# Vd1 T Cells as a Potential Candidate for an HIV Cure

**Brittany Haas**

Thesis submitted to the University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science in Microbiology and Immunology

Department of Biochemistry, Microbiology, and Immunology

Faculty of Medicine

University of Ottawa

**Abstract**

Modified autologous lymphocytes as treatment for disease has become the focus of increasing interest. In the context of HIV, this approach is costly, time-consuming, and, to date, has not been particularly effective. The pathology of HIV is incredibly complex, and achieving a functional cure would involve clearing the infection from the mucosal tissues where there is poor drug penetration for those on antiretrovirals. Delta One T (DOT) cells, an expanded population of gamma delta 1 (Vd1) T cells, may represent a novel immunotherapy for targeting the HIV reservoir within the mucosa. Vd1 T cells preferentially reside in the mucosa, which is typically where they perform their anti-viral/anti-tumoral functions. Previous studies have demonstrated their cytotoxicity towards target cells *in vitro* and *in vivo* through a variety of cytotoxic receptors including natural killer (NK) receptors such as NKG2D and NKp30. While there is no distinct marker of cells latently infected with HIV, there is some evidence to suggest that they express ligands for these NK receptors. Furthermore, the lack of major histocompatibility complex (MHC) restriction on Vd1 T cells means that these cells can be given to multiple donors without the risk of inducing graft-versus-host-disease (GvHD), making this a potentially scalable treatment.

**Acknowledgements**

I would like to offer my sincerest thanks to my supervisor Dr. Jonathan Angel for his guidance throughout my project and for allowing me the freedom to decide what directions to take. I am a better scientist thanks to my time in his lab.

I would also like to thank the members of the Angel lab for their advice and expertise with a special thanks to Bengisu for ranting with me over lemon loaves and coffee.

Thank you to my partner Max for sitting through every presentation even though the science made him bleary eyed and sleepy.

My thanks to my TAC members Dr. Zakia Djaoud and Dr. Subash Sad for their advice and expertise regarding my project's design and execution.

Finally, I would like to thank all of the blood donors and the nurses within the Clinical Investigations Unit who drew the blood which allowed for the primary cell work in this thesis to be made possible.

**List of abbreviations**

| | |
|---|---|
| ANOVA | Analysis of variance |
| αβ T cells | Alpha beta T cells |
| BSA | Bovine serum albumin |
| cART | Combination antiretroviral therapy |
| CCR5 | C-C chemokine receptor type 5 |
| CD2 | Cluster of differentiation 2 |
| CD4 | Cluster of differentiation 4 |
| CD96 | Cluster of differentiation 96 |
| CD150 | Cluster of differentiation 150 |
| CD155 | Cluster of differentiation 155 |
| CXCL10 | C-X-C chemokine motif ligand 10 |
| CXCL11 | C-X-C chemokine motif ligand 11 |
| CXCR3 | C-X-C chemokine receptor type 3 |
| CXCR4 | C-X-C chemokine receptor type 4 |
| DOT Cell | Delta one T cell |
| DNAM-1 | DNAX accessory molecule-1 |
| ELISA | Enzyme-linked immunosorbent assay |
| GALT | Gut-associated lymphoid tissue |
| γδ T cell | Gamma delta T cell |
| GMP | Good manufacturing process |
| HI-FBS | Heat-inactivated fetal bovine serum |
| HIV | Human immunodeficiency virus |

| | |
|---|---|
| HSA | Heat stable antigen |
| IFNg | Interferon gamma |
| IMDM | Iscove's modified dulbecco's medium |
| LRA | Latency reversing agent |
| MDM | Monocyte-derived macrophage |
| MHC | Major histocompatibility complex |
| NK cell | Natural killer cell |
| NKG2D | Natural killer group 2D |
| NKp30 | Natural killer protein 30 |
| NKp44 | Natural killer protein 44 |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PE | Phycoerythrin |
| PE-Cy7 | Phycoerythrin-cyanine 7 |
| PFA | Paraformaldehyde |
| PVR | Polio virus receptor |
| RPMI-1640 | Roswell Park memorial institute 1640 medium |
| RT | Room temperature |
| SFM | Serum free medium |
| TCR | T cell receptor |
| TNFa | Tumor necrosis factor alpha |
| Vd1 T cell | Variable delta 1 T cell |
| Vd2 T cell | Variable delta 2 T cell |

**List of figures**

**List of tables**

**Table of Contents**

# Chapter 1: Introduction

## 1.1 Human immunodeficiency virus

### 1.1.1 HIV and the need for a cure

Approximately 1.3 million people were newly infected with human immunodeficiency virus (HIV) in 2022, with an estimated number of HIV-related deaths totalling 630,000.[1] Currently, combination antiretroviral therapy (cART) is the standard for treating HIV, but it is not a cure. cART targets different steps in the viral replication cycle to prevent the virus from reproducing, but cessation of this treatment results in the resurgence of virus production.[2] There are many barriers in place that may prevent people from accessing cART regularly.[3–5] Aside from patient adherence to cART, drug-drug interactions may also effect cART efficacy, which is becoming increasingly worrisome as the HIV populace ages and develops co-morbidities that require medication.[4] When discussing an HIV cure, there are different definitions as to what a cure may look like. One definition, referred to as HIV remission, is a long-term, medication-free, suppression of the virus.[6] A study from people living with HIV (PLWH) in the Netherlands found that the most desired outcome of an HIV cure is the reduction of societal stigma, potentially lessening future health risks, and no longer having to worry about viral transmission.[6] An HIV cure therefore does not necessarily need to be a "sterilizing" cure where all of the virus is removed from the body, potentially allowing for more leeway in the field of HIV cure research.[6]

### 1.1.2 The life cycle of HIV

CD4+ T cells are the primary target of HIV, but other CD4 expressing cells such as those from a monocyte/macrophage lineage can become infected.[7,8] The HIV envelope protein gp120 will first bind to the primary CD4 receptor, and this binding causes a conformational change that

exposes the chemokine co-receptors C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4), which can also be bound by gp120.[2] Binding of the co-receptors allows gp41, a transmembrane protein on HIV, to contact the target cell and facilitate fusion to the cell membrane.[2] Fusion of the virion with the cell membrane allows the virion to release its RNA into the cell where it can be reverse-transcribed into DNA.[2] This DNA can then integrate into the host genome where it will stay in the target cell for the remainder of the cells lifespan.[2] These infected cells can become latent, or can use the host machinery to transcribe and translate the viral DNA.[2] The production of viral particles is done by a combination of viral and host transcription.[9] Viral proteins get transported to the cell membrane where they get assembled prior to release via the vesicular sorting pathway.[9] Since virion transmission from the cell occurs using a naturally occurring pathway rather than lysing the cell, one infected cell can produce a few thousand virions before dying, allowing the infectious cycle to continue.[9,10]

### 1.1.3 The HIV viral reservoir

Cells harboring HIV DNA become latent when a patient begins cART, which is why cessation of cART often results in a rebound of viremia.[2] These latently infected cells contribute to what is called a "viral reservoir" and are extremely difficult to target as they can occur in different cell types and tissue sources throughout the body.[11] In people living with HIV (PLWH), the gut-associated lymphoid tissue (GALT) represents one of the major reservoirs, potentially harboring 80-95% of all HIV-infected cells in the human body.[12] Part of this has to do with poor penetration of cART drugs into tissues, creating these viral sanctuaries.[12] Aside from harboring the virus, mucosal tissues also play a fundamental role in HIV transmission.[13] Therefore, a major

effort in HIV cure research strategies has been to design a treatment that is able to penetrate into mucosal tissues to access and eliminate these infected cells.

## 1.2 Gamma delta T cells

### 1.2.1 Characteristics of gamma delta T cells

Gamma delta (γδ) T cells are a distinct subset of T cells that present glycoprotein gamma and delta chains that comprise their T cell receptor (TCR).[14] This unique subset of T cells only accounts for 0.5-5% of all T cells, with alpha beta (αβ) T cells making up the vast majority of the remaining population.[14] γδ T cells are often referred to as non-conventional or innate-like T cells, owing to their ability to act as mediators between the innate and adaptive immune system.[15] They can act directly on their target through degranulation and cytokine production, or they can act indirectly on other effector cells.[16,17] There are multiple subtypes of γδ T cells, but the two most common ones express the variable delta chains 1 or 2, and are thus denoted as Vd1 or Vd2 T cells.[18] Interestingly, these two subtypes preferentially localize to different areas of the body. Vd1 T cells are most predominant in the thymus and peripheral tissues while Vd2 T cells can comprise up to 90% of the γδ T cells found in blood.[18,19]

### 1.2.2 Gamma delta T cells during HIV infection

Both the Vd1 and Vd2 T cell populations are impacted during HIV infection.[20] Vd1 T cells expand while the Vd2 T cell population gets dramatically reduced.[20] These changes are referred to as an inversion of the Vd1:Vd2 ratio, where the Vd1 T cell population outnumbers the Vd2 T cell population in the periphery.[21] The alpha 4 beta 7 integrin on Vd2 T cells appears to function as a co-receptor with CCR5 for HIV binding and subsequent infection which would

explain their reduction.[22] However, the explanation behind the expansion of the Vd1 T cell population is less clear. It has been hypothesized that Vd1 T cells may play an antiviral role,[23] or they may be expanding indirectly as a result of the leaky gut that occurs during HIV infection.[24] If the Vd1 T cells do expand in direct response to HIV infection, they are unable to retain their effector function during chronic infection and appear to become exhausted.[25] It is therefore possible that revitalization of these cells may help to boost anti-viral immunity.[26]

### 1.3 Adoptive transfer for immunotherapies

#### 1.3.1 Adoptive transfer of alpha beta T cells

Unlike γδ T cells αβ T cell effector function is restricted by major histocompatibility complex (MHC) recognition on the target cell.[14,27] Administration of αβ T cells to a recipient therefore needs to be autologous as using allogeneic cells can lead to a phenomenon known as graft-versus-host disease (GvHD).[28] GvHD can have significant impacts on patient morbidity and mortality, and is typically avoided by harvesting and re-infusing alpha beta T cells from the same donor.[28] The process of collecting αβ T cells for modification prior to re-infusion has a plethora of associated costs due to the amount of time and manipulation required.[28] The efficacy of αβ T cell immunotherapy is also questionable due to the high rate of patient relapse (up to 60%).[29] Therefore, using a γδ T cell therapy which does not require patient matching is ideal to avoid GvHD and may also prove to be more effective overall.

1.3.2 Adoptive transfer of gamma delta T cells

Studies into the clinical potential of adoptively transferred γδ T cells is a relatively recent concept. Mice do not possess γδ T cell receptors homologous to humans, so pre-clinical studies have had to use immunodeficient or humanized mice.[30] There are downsides to both of these models since immunodeficient mice cannot fully represent the immune landscape during disease, and humanized mice frequently experience GvHD which can limit the relevance of immunotherapies.[31] Multiple studies of adoptive transfer of Vd2 T cells have been done in both of these mouse models to successfully treat different types of cancers, providing enough of a basis to bring this treatment into the clinic.[32,33] In 2021, a phase I clinical trial adoptively transferred allogeneic Vd2 T cells for the treatment of lung and liver cancer with promising results regarding its safety and efficacy.[34] Since Vd1 T cells are fairly similar to Vd2 T cells with respect to their lack of MHC restriction and functionality, it is likely that adoptive transfer of Vd1 T cells would be just as safe. However, the lack of appropriate expansion methods to generate a robust population of Vd1 T cells in addition to the limited number of *in vivo* studies has proved to be a hindrance in translating this treatment into clinical research.

## 1.4 Delta One T cells as a potential immunotherapy

1.4.1 Delta One T (DOT) cells

There have been multiple attempts to expand Vd1 T cells from the blood, however this has proved to be extremely difficult due to the extremely small size of the circulating Vd1 T cell population. A study in 2012 first tried to expand Vd1 T cells from peripheral blood using phytohemagglutinin (PHA) and interleukin-2 (IL-2).[35] Another study in 2015 followed a similar direction and used PHA and interleukin-7 (IL-7) for expansion.[36] These studies were not

clinically relevant, however, only generating a few hundred thousand cells. Even if the number of cells generated had been enough for adoptive transfer, mitogenic plant lectins such as PHA are not good manufacturing process (GMP) approved, meaning that there may be limited clinical adaptability for these expansion methods.[37,38] A method that can expand vast amounts of Vd1 T cells while using GMP approved reagents is therefore a necessity for the progression to clinical trials. This rationale led to the development of the Delta One T (DOT) cell protocol by the Silva-Santos lab in 2016.[38] This protocol involves harvesting γδ T cells from the peripheral blood before using a GMP approved cytokine cocktail for a period of 16-20 days. However, there was still the problem of cell number with this method since the total γδ T cell population in the peripheral blood is less than 5%, meaning that the starting number of cells is incredibly small.[14] In 2022, the DOT cell method was improved by the Silva-Santos lab by swapping the γδ T cell isolation with an αβ T cell depletion.[39] This alteration removed the largest competitors for the cytokine cocktail while keeping in cells such as monocytes that can provide co-stimulation for the Vd1 T cells.[39,40] This method results in hundreds of millions of cells that have achieved a final Vd1 T cell population of 60% or higher. While this method is the best that has been developed so far, it has not achieved complete purity and may not expand cells from all donors tested.[41] To increase purity and final Vd1 T cell population size, a lab in 2022 found that adding in an additional CD56 depletion prior to expansion helps to remove natural killer (NK) cells and further decrease cytokine competition.[41]

### 1.4.2 Functionality of DOT cells

Since this expansion protocol was recently developed, the research into the potential applications of this cellular product has been limited to its killing of lymphocytic and myelocytic

leukemia.[38,39,42] DOT cells were found to be highly efficient at killing leukemic cells, and significantly decreased tumor burdens in mouse models.[39,42] The primary mechanism behind DOT cell killing of leukemic cells was determined to be engagement of the natural killer protein 30 (NKp30) receptor on the DOT cells leading to subsequent degranulation and expression of perforin.[38,42] Engagement of the Vd1 T cell receptor has also found to be involved, but this effect is less pronounced and seems to vary between donors.[38,42] It is possible that NK receptors such as NKp30 have a transient high-affinity interaction with the target cell that allows for the formation of a stable low affinity TCR-ligand complex to form.[43] The ligands recognized by the NKp30 receptor and Vd1 TCR have been poorly characterized, thus making it difficult to determine what other tumoral or virally infected cells would be susceptible to DOT cell therapy.[37,44]

### 1.4.3 DOT cells and HIV

Although NKp30 has been the primary receptor implicated in the clearance of leukemic cells, there are an abundance of other cytotoxic receptors expressed on DOT cells that could play a role in anti-viral immunity.[38] NK receptors such as natural killer group 2D (NKG2D) and natural killer protein 44 (NKp44) are upregulated on DOT cells, and the ligands recognized by these receptors also appear to become upregulated during HIV infection.[45] Furthermore, DOT cells express receptors that may be crucial for controlling HIV infection, such as CD96, CD150, DNAM-1, etc.[46–48] The problem, however, is that it is unclear whether cells that are latently infected with HIV continue to express the ligands that would be recognizable to the DOT cells. In fact, many researchers have employed the use of latency reversing agents (LRAs) to force infected cells to produce viral particles and express ligands that will allow them to be recognized and killed by immune cells.[49,50] However, LRAs have failed to decrease the size of the latent

reservoir, thus suggesting that increasing the expression of these ligands is not enough to generate the required immune response on its own.[51] The exhaustion of immune cells that occurs during HIV infection may explain this limited response, in addition to the diversity of tissues that make up the HIV reservoir.[52,53] An immunotherapy of activated cells such as DOT cells which are able to penetrate mucosal tissues and reach the latent reservoir may therefore be a novel solution. The lack of MHC restriction of these cells also allows for an off-the-shelf therapy that will be widely accessible. There is also the possibility that this immunotherapy could be combined with other treatments currently being researched, such as LRAs.

### 1.5 Research hypothesis and objectives

The anti-HIV potential of DOT cells requires further investigation. It is hypothesized that DOT cells will kill HIV-infected cells from immunologically relevant models of infection, including cell lines and primary cells.

In order to assess the anti-HIV potential of DOT cells, there are two main objectives:

1. Optimize and characterize cells expanded using the DOT cell protocol.
2. Determine DOT cell killing of HIV-uninfected and HIV-infected target cells
    a. Determine if HIV infection alters DOT cell killing
    b. Determine the mechanism of DOT cell killing

## Chapter 2: Materials and methods

### 2.1 Reagents

#### 2.1.1 Media

Gibco® Roswell Park Memorial Institute 1640 medium (RPMI-1640) with phenol red indicator (denoted as R10 when supplemented), Gibco® Dulbecco's Modified Eagle's medium (DMEM), Gibco® phosphate buffered saline (PBS), pH 7.4, Gibco® Iscove's Modified Dulbecco's medium (IMDM), and Gibco® CTS™ OpTmizer™ T cell expansion serum free medium (SFM) without phenol red, were purchased from ThermoFisher Scientific (Ottawa, ON).

#### 2.1.2 Virus Strains

$HIV_{BAL}$ (NL4.3 BAL-IRES-HSA) a CCR5 (R5) tropic virus was obtained from Dr. Michel J Tremblay at the Université de Laval.

### 2.2 Cell culture

#### 2.2.1 Cell lines

Jurkat (ATCC #: TIB-152) and HL60 (ATCC #: CCL-240) cell lines were obtained from American Type Culture Collection (ATCC, Manassas VA). Jurkats are a model of acute T-cell leukemia, while HL60s are a model of acute promyelocytic leukemia. The J1.1 cell line (NIH # ARP–1340) is derived from the parental Jurkat cell line and is a model of latent HIV infection in T cells. The OM10.1 cell line (NIH # ARP–1319) is derived from the parental HL60 cell line and is a model of latent HIV infection in myeloid cells. HIV-infected cell lines were obtained through the NIH AIDS Reagents Program, OM10.1 cells courtesy of Dr. Salvatore Butera[54] and J1.1 cells from Dr. Thomas Folks.[55] Jurkats, J1.1s, and OM10.1s were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 2mM L – Glutamine

(ThermoFisher Scientific) penicillin (100units(U)/mL) and streptomycin (10µg/mL) (PenStrep). HL60s were cultured in IMDM supplemented with 20% HI-FBS, 2mM L-glutamine, and 100U/mL PenStrep. All cell lines were maintained with a 1:5 passage schedule occurring every 2 days.

2.2.2 Generation of Delta One T (DOT) cells

Peripheral blood was drawn from a healthy donor into sterile 60 ml syringes, containing 100U/ml filter-sterilized Heparin Sodium (LEO Pharma Inc.). Within 3 hours of collection, whole blood was spun down at 400 x g for 10 minutes and the plasma was collected. The plasma was then heat-inactivated at 56°C for 30 minutes, after which it was spun down at 1400 x g (ST Plus Series, Sorvall) and the supernatant was collected for media supplementation. The volume of plasma that had been removed from the blood was then replaced with PBS, and the blood was further diluted 2X in PBS. 30 mL of the diluted blood was then layered over 15 ml of Lymphoprep$^{TM}$ density gradient medium (Stemcell Technologies) and was centrifuged at 400 x g for 30 minutes (Megafuge 1.0, Heraeus Instruments) without braking. The buffy coats were then collected into 50 ml Falcon tubes and topped up with PBS to 50 ml. Cells were pelleted by centrifugation (300 x g for 20 min). Cell pellets from individual donors were then pooled into one 50ml Falcon tube, and cells were washed twice with PBS (400 x g for 10min). Following the final wash, peripheral blood mononuclear cells (PBMC) were counted by trypan blue exclusion and re-suspended in sort buffer (2% HI-FBS, 1mM EDTA in PBS) at a concentration of $50x10^6$ cells/mL.

The Delta One T (DOT) cell protocol was first described by Almeida *et al.,* 2016, and an updated protocol from the same lab was published in 2022 (Sánchez Martínez *et al.,* 2022). Thus,

the updated protocol was utilized for this study. An αβ T cell depletion was performed on the isolated PBMCs using the EasySep™ Human TCR Alpha/Beta Depletion Kit (Stemcell Technologies) according to the manufacturer's instructions. A further depletion was then performed using the EasySep™ Human CD56 Positive Selection Kit II (Stemcell Technologies). The remaining cells were then re-suspended at a concentration of $2x10^6$ cells/mL in OpTmizer™ (ThermoFisher) supplemented with 2.5% autologous heat-inactivated plasma, 2mM L-glutamine (ThermoFisher), and 50U/mL PenStrep (ThermoFisher) and cultured in a T175 (Sarstedt) at 37°C, 5% $CO_2$ for 16-20 days. On day 0, the following stimuli were added (all animal-free human cytokines from PeproTech); rIL-4 (100ng/mL), rIFNγ (70ng/mL), rIL-21 (7ng/mL), rIL-1β (15ng/mL) and 140ng/mL of a soluble mAB anti-CD3 (Biolegend) (Table 1). On day 7, the cells were spun down and re-suspended at a concentration of $2x10^6$ cells/mL in fresh complete OpTmizer™ supplemented with 2µg/mL anti-CD3 (OKT3), rIL-21 (13ng/mL), and rIL-15 (70ng/mL). On day 11, the cells were again spun down and re-suspended at $2x10^6$ cells/mL in fresh complete OpTmizer™ supplemented with anti-CD3 (1µg/mL) and rIL-15 (100ng/mL). On day 15, the cells were given the same treatment they received on day 11. DOT cells were harvested on day 17. Verification of expansion was done by characterizing the Vd1 TCR+ cell population via flow cytometry. Expanded cells were cryopreserved in HI-FBS (Life Technologies) containing 10% DMSO (Sigma) and stored in liquid nitrogen until use (See Figure 1).

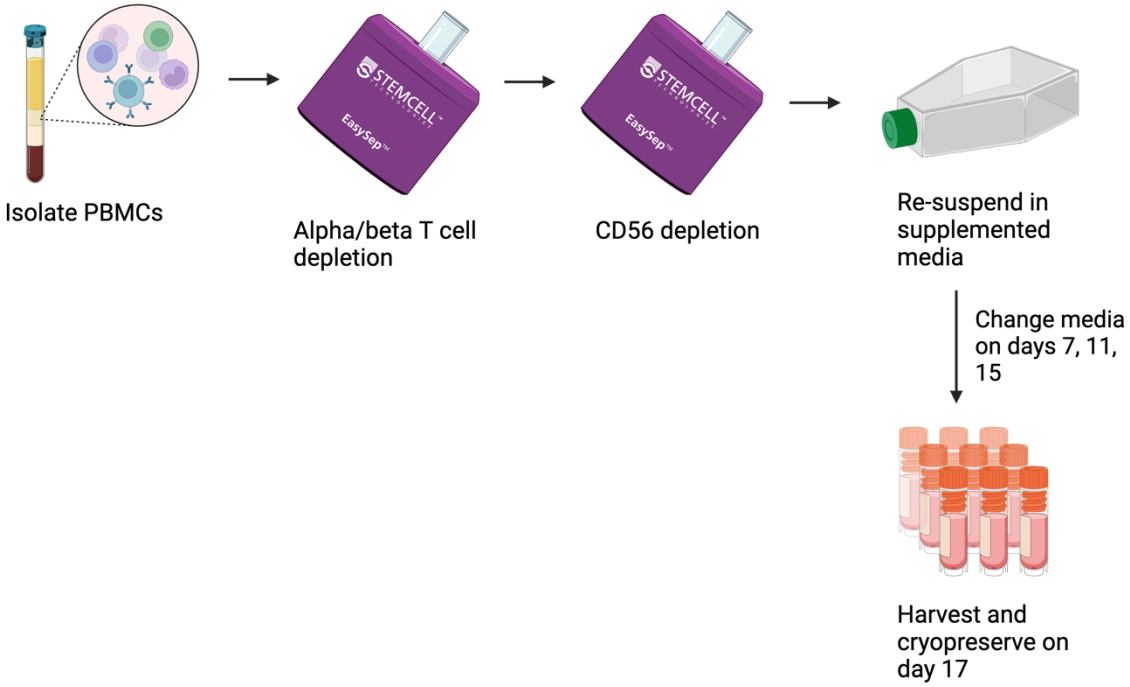

Isolate PBMCs
Alpha/beta T cell depletion
CD56 depletion
Re-suspend in supplemented media
Change media on days 7, 11, 15
Harvest and cryopreserve on day 17

**Figure 1. The DOT cell expansion protocol.**
Schematic representation depicting the selective expansion of Vd1 T cells from PBMCs using the DOT cell protocol. Image created with BioRender.com.

**Table 1. Stimuli used in DOT cell expansion**

| Name | Species | Product number | Company |
|---|---|---|---|
| Anti-human CD3 mAb | Mouse (Clone OKT-3) | BE0001-2 | Bio X cell |
| IL-1β (Interleukin-1beta) | Human | AF-200-01B | Peprotech |
| IFN-γ (Interferon-gamma) | Human | AF-300-02 | Peprotech |
| IL-4 (Interleukin-4) | Human | AF-200-04 | Peprotech |
| IL-21 (Interleukin-21) | Human | AF-200-21 | Peprotech |
| IL-15 (Interleukin-15) | Human | AF-200-15 | Peprotech |

2.2.3 Generation of monocyte-derived macrophages (MDMs)

Peripheral blood was drawn from a healthy donor into sterile 60 ml syringes, containing 100U/ml filter-sterilized Heparin Sodium (LEO Pharma Inc., Thornhill, ON). Within 3 hours of collection, 30 ml of whole blood was layered over 15 ml of Lymphoprep$^{TM}$ density gradient medium (Stemcell Technologies, Vancouver, BC) and was centrifuged at 400 x g for 30 minutes (Megafuge 1.0, Heraeus Instruments, Germany) without braking. The buffy coats were then collected into 50 ml Falcon tubes and topped up with PBS to 50 ml. Cells were pelleted by centrifugation (300 x g for 20 min). Cell pellets from individual donors were then pooled into one 50ml Falcon tube, and cells were washed twice with PBS (400 x g for 10min). Following the final wash, peripheral blood mononuclear cells (PBMC) were counted by trypan blue exclusion and re-suspended in the appropriate media for further experiments. Healthy blood donors provided written informed consent to partake in the study. This was approved by The Ottawa Health Science Network Research Ethics Board.

Following PBMC isolation, the PBMCs were re-suspended in serum-free RPMI-1640 supplemented with 100U/mL PenStrep and 2mM L-glutamine at a concentration of $6.25x10^6$ cells/mL. $6.25x10^7$ cells were then plated in 100 $cm^2$ suspension polystyrene tissue culture dishes (Sarstedt) and left to adhere at 37°C for 2 hours. Following this incubation, the plates were washed 3 times with warm endotoxin-free PBS (Sigma-Aldrich) to remove the non-adherent lymphocytes. 10mL of RPMI-1640, supplemented with 100U/mL PenStrep, 25U/mL macrophage colony stimulating factor (M-CSF) (BioLegend # 574802), 2mM L-glutamine, and 10% heat-inactivated human AB serum (MP Biomedical) was added to the plates. Plates were then incubated at 37°C, 5% $CO_2$ for 7 days. On day 3 post-plating, adherent cells were washed twice with warm endotoxin-free PBS, and 10 mL of RPMI-1640 supplemented with 10% heat-inactivated human AB serum, 100U/mL PenStrep, and 2mM L-glutamine (MDM media) was added to the plates. MDMs were ready to be infected with HIV on day 8.

2.2.4 DOT cell characterization

To determine if the DOT cell expansion had achieved a final Vd1 T cell population of >60%, 100,000 cells were collected from the expansion and washed with 1% HI-FBS in PBS at 400 x g for 5 minutes. The cells were then re-suspended in 100ul of 1% HI-FBS in PBS with anti-Vd1 TCR PE (Table 2). Cells were incubated at 4°C for 20 minutes prior to being washed with 1% HI-FBS in PBS at 400 x g for 5 minutes. The cells were then re-suspended in 100ul 4% paraformaldehyde (PFA) (ThermoFisher) and incubated at 4°C for 20 minutes prior to being washed with 1% HI-FBS in PBS at 400 x g for 5 minutes. The cells were re-suspended in 300ul PBS and were then analyzed by flow cytometry using the CytoFLEX Flow Cytometer (Beckman

Coulter, Brea, CA, USA) with the CytExpert Software (Beckman Coulter). The results were further analyzed using FlowJo Software (BD Biosciences, Mississauga, ON, Canada).

To evaluate the Vd1 TCR negative population, cryopreserved DOT cells were thawed, and 100,000 cells were washed with 1% HI-FBS in PBS at 300 x g for 5 minutes. The cells were then re-suspended in 100ul 1% HI-FBS in PBS with the following antibodies (Table 2); anti-Vd1 TCR PE-Cy7 (Thermofisher), anti-CD3 FITC (Biolegend), anti-γδ TCR PE (Biolegend), and anti-CD56 APC (Biolegend) for 20 minutes at 4°C. Following this incubation, the cells were washed with 1% HI-FBS in PBS at 300 x g for 5 minutes. The cells were then re-suspended in 100ul 4% PFA (ThermoFisher) and incubated at 4°C for 20 minutes. Following this incubation, the cells were washed with 1% HI-FBS in PBS at 300 x g for 5 minutes. The cells were re-suspended in 300ul PBS and were then analyzed using the CytoFLEX Flow Cytometer (Beckman Coulter) with the CytExpert Software (Beckman Coulter). The results were further analyzed using FlowJo Software (BD Biosciences).

### 2.3 Infection and quantification of infection

#### 2.3.1 Infecting MDMs with $HIV_{BAL}$

Monocyte-derived macrophages were generated as described in section 2.2.3. On day 8 following PBMC isolation, the generated MDMs were washed twice with warm endotoxin-free PBS (Sigma-Aldrich), and 5mL accutase (Sigma-Aldrich) was added to the 100 $cm^2$ suspension dishes and left to incubate at 37°C, 5% $CO_2$ for 30-45 minutes. Following this incubation with accutase, the MDMs were pipetted off the plates and spun down at 300 x g for 5 mins. The supernatant was discarded, and the cell pellet was re-suspended in 10mL MDM media for counting via trypan blue exclusion. The macrophages were then re-suspended at 200,000

cells/mL in MDM media, and 1mL was added to each well of a 24 well suspension plate (Sarstedt). The MDMs were then allowed to adhere overnight at 37°C, 5% $CO_2$. The following day (day 9), the media was removed, and the adhered MDMs were washed once with warm endotoxin-free PBS. $HIV_{BAL}$ was added to each well at a final concentration of 20ng p24 in 500ul of MDM media. The following day (day 10), the wells were topped up in 500ul R10. On day 12, a half-media change was performed by removing 500ul from each well and replacing it with 500ul of warm MDM media. On day 15, supernatant was collected for verification of infection by p24 ELISA prior to co-culture.

2.3.2 Enzyme-linked immunosorbent assay (ELISA) for p24 antigen (p24)

An ELISA was used to determine the capsid protein concentration of HIV-1 (p24) from supernatants of monocyte-derived macrophages to quantify infection and viral replication. This assay and its reagents were provided by the AIDS and Cancer Virus Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research (Frederick, MD, USA), supported with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. Supernatants were incubated for 1 hour at 37°C with 1% Triton X-100 to lyse any remaining cells and inactivate remaining viral particles. The ELISA was then performed according to the manufacturer's protocol. Samples were run in duplicate. Absorbance was read at a 450nm wavelength with a reference wavelength of 650nm using the Multiskan Ascent 96 Plate Reader (ThermoFisher Scientific).

## 2.4 DOT cell co-culture experiments

### 2.4.1 DOT cell and cell line co-culture

Cryopreserved DOT cells were thawed and rested overnight in R10 at a concentration of $4x10^6$ cells/mL at 37°C, 5% $CO_2$. The following day CellTrace™ Carboxyfluorescein succinimidyl ester (CFSE) Cell Proliferation assay (Life Technologies) was used to tag the cell line of interest and allow them to be distinguished from DOT cells. 5mM stock concentration of CFSE was prepared in DMSO. The target cells were stained with 5μM CFSE as indicated by the manufacturer's instructions. Following CFSE staining, the target cells and rested DOT cells were combined in a 5mL polypropylene tube (ThermoFisher) at the following ratios of DOT:Target; 1:1, 2:1, 5:1, and 10:1. The co-culture was then incubated for either 3 hours (lymphocytic) or 24 hours (myelocytic) at 37°C, 5% $CO_2$. Lower ratios of DOT:Target (1:16, 1:8, 1:4, 1:2) were also tested for 24 hours with Jurkats and HL60s to determine if DOT cell function was maintained over time when outnumbered. For cytokine analysis, the 10:1 cell co-cultures were spun down following a 24-hour incubation at 400 x g for 5 minutes and the supernatant was collected for analysis by IFNg ELISA. For analysis of target cell apoptosis, 500,000 cells were washed with 1% HI-FBS in PBS at 300 x g for 5 minutes. Cells were then re-suspended in 100ul of annexin V binding buffer (Biolegend) with annexin V PE (Table 2) and incubated at room temperature (RT) for 15 minutes. Following this incubation, 200ul of annexin V binding buffer and 100ul 4% paraformaldehyde (PFA) (ThermoFisher) was added. Cells were then incubated for another 15 minutes at RT prior to reading on the CytoFLEX Flow Cytometer (Beckman Coulter) with the CytExpert Software (Beckman Coulter). The results were further analyzed using FlowJo Software (BD Biosciences).

2.4.1.1 IFNg detection in co-culture supernatant

Cryopreserved DOT cells were thawed and rested overnight in R10 at a concentration of $4x10^6$ cells/mL at 37°C, 5% $CO_2$. The following day, DOT cells were co-cultured with Jurkats, J1.1s, HL60s, and OM10.1s at a 10:1 effector:target (E:T) ratio for 24 hours at 37°C, 5% $CO_2$. Following co-culture, the cells were spun down at 400 x g for 5 minutes after which the supernatant was collected and frozen at -80°C until ready to be used. To quantify IFNg levels in the supernatant, an ELISA MAX™ Deluxe Set IFNg kit (Biolegend) was used according to manufacturer's instructions. Supernatants were lysed with 1% Triton X-100 for 1 hour at 37°C to lyse any remaining cells and to inactivate any viral particles. Samples were run in triplicate. Absorbance was read at 450nm wavelength using the Multiskan Ascent 96 Plate Reader (ThermoFisher Scientific).

2.4.1.2 DOT cell receptor blocking

Cryopreserved DOT cells were thawed and rested overnight at $4x10^6$ cells/mL in R10 (37°C, 5% $CO_2$). Prior to co-culture with the lymphocytic cell lines, $1x10^6$ DOT cells in 1mL of R10 were incubated with 5ug of various antibodies for 1 hour. The antibodies used for blocking (Table 2) were human anti-Vd1 TCR clone TS8.2 (Fisher Scientific), human anti-Nkp30 clone P30-15 (Biolegend), human anti-Nkp44 clone P44-8 (Biolegend), human anti-CD2 clone RPA-2.10 (Biolegend), and mouse IgG1k clone MOPC-21 (Biolegend). Following this incubation, 100,000 target cells (either Jurkats or J1.1s stained with CFSE as described in section 2.4.1) were added to the blocked DOT cells for a final E:T ratio of 10:1. The cells were then incubated for 3 hours after which 500,000 cells were washed with 1% HI-FBS in PBS prior to re-suspending in 100ul annexin V binding buffer (Biolegend) with annexin V PE (Biolegend). Cells were

incubated at RT for 15 minutes. Following this incubation 200ul annexin V binding buffer and 4% PFA (ThermoFisher) were added to the cells. The cells were then incubated for an additional 15 minutes at RT prior to reading on the CytoFLEX Flow Cytometer (Beckman Coulter) with the CytExpert Software (Beckman Coulter). The results were further analyzed using FlowJo Software (BD Biosciences).

**Table 2. Monoclonal antibodies used in flow cytometry and blocking experiments**

| Target | Reactivity | Host Species | Product number | Company | Clone | Conjugated fluorophore | ug per test |
|---|---|---|---|---|---|---|---|
| Vd1 TCR | Human | Human | 130-120-440 | Miltenyi Biotech | REA173 | PE | 0.1ug |
| Vd1 TCR | Human, Non-human primate | Mouse | 25-5679-41 | Thermofisher | TS8.2 | PE-Cy7 | 0.075ug |
| γδ TCR | Human, Cynomolgus, Rhesus | Mouse | 331209 | Biolegend | B1 | PE | 1.2ug |
| CD3 | Human | Mouse | 317305 | Biolegend | OKT3 | FITC | 0.8ug |
| CD56 | Human, African green, Baboon, Cynomolgus, Rhesus | Mouse | 318309 | Biolegend | HCD56 | APC | 0.15ug |
| Phosphatidyl serine (PS) | Human, Mouse, Rat | Escherichia coli | 640908 | Biolegend | Annexin V | PE | 0.006ug MDM, HL60, Jurkat, J1.1 & 0.012 ug OM10.1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Heat stable antigen (HSA) | Mouse | Rat | 138505 | Biolegend | 30-F1 | APC | 0.4ug |
| Vd1 TCR | Human, Non-human primate, Rhesus monkey | Mouse | TCR1730 | Thermofisher | TS8.2 | - | 5ug |
| CD2 | Human, Cynomolgus, Rhesus | Mouse | 300202 | Biolegend | RPA-2.10 | - | 5ug |
| NKp30 | Human | Mouse | 325202 | Biolegend | P30-15 | - | 5ug |
| NKp44 | Human | Mouse | 325102 | Biolegend | P44-8 | - | 5ug |
| IgG1k isotype | - | Mouse | 400165 | Biolegend | MOPC-21 | - | 5ug |

2.4.2 DOT cell and MDM co-culture

Cryopreserved DOT cells were thawed and rested overnight in R10 at a concentration of $4x10^6$ cells/mL at 37°C, 5% $CO_2$. The following day, the media from wells containing HIV-infected or mock-infected MDMs was removed (with some saved for analysis by p24 ELISA) and DOT cells were then added to the wells at a 1:1 E:T ratio. Following a 24-hour incubation at 37°C, 5% $CO_2$, the supernatant was removed from the wells (with some being saved for analysis by p24 ELISA) and the MDMs were washed once with warm endotoxin free PBS (Sigma-Aldrich). 1mL of warm accutase (Sigma-Aldrich) was added to each well and the cells were left to detach at 37°C, 5% $CO_2$ for 1 hour. The cells were then pipetted off the plate and transferred to a 5mL polypropylene tube (ThermoFisher) where they were subsequently spun down at 300 x g for 5 minutes. The cells were then ready to be stained for flow cytometry.

2.4.2.1 Flow cytometry analysis of HIV-infected monocyte-derived macrophages

Detached MDMs were washed with 1mL PBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich) at 300 x g for 5 minutes. Following this wash, the cells were re-suspended in 100ul of PBS containing 10% human AB serum (MP Biomedical) and 20% normal goat serum (Life Technologies). The cells were then incubated at 4°C for 20 minutes prior to the addition of 100ul of PBS with anti-HSA APC (Table 2). Cells were vortexed and then incubated for an additional 15 minutes at 4°C. Cells were then washed with 1mL of PBS at 300 x g for 5 minutes. Cells were then re-suspended in 100ul of annexin V binding buffer (Biolegend) with annexin V PE (Table 2). Cells were incubated at RT for 15 minutes. Following this incubation 200ul of annexin V binding buffer and 100ul of 4% PFA were added. Cells were incubated for an additional 15 minutes at RT prior to reading on the CytoFLEX Flow Cytometer (Beckman Coulter) with the CytExpert Software (Beckman Coulter). The results were further analyzed using FlowJo Software (BD Biosciences).

2.4.2.2 DOT cell migration assay

Cryopreserved DOT cells were thawed and rested overnight in R10 at a concentration of $4x10^6$ cells/mL at 37°C, 5% $CO_2$. 900ul of fresh MDM media was added to the MDM wells that had either been mock-infected or HIV-infected. An 8um Millicell Hanging Cell Culture Insert (ThermoFisher) was placed above the wells and 200ul of DOT cells from donor 262 were added to the filter at different E:T ratios (1:1, 2:1, 5:1, and 10:1). The transwell assay was then incubated for 24 hours at 37°C. The following day the filters were carefully removed and the supernatant containing suspension cells was collected. The cells were then counted using trypan blue exclusion, excluding cells of obvious macrophage morphology.

## 2.5 Statistics

Statistics were performed using GraphPad Prism 10 software (San Diego, CA). Linear regression, one-way ANOVA, and two-way ANOVA were used. P values $<0.05$ were considered significant.

## Chapter 3: Results

### 3.1 DOT cell characterization

#### 3.1.1 CD56 depletion improves DOT cell expansion

During initial testing of the DOT cell generation method described in Sánchez Martínez *et al* 2022, there was limited success. Only 2 out of 8 donors (25%) were successfully expanded using this method (Table 3). An additional CD56 depletion prior to expansion as described in Ferry *et al* 2022 was found to greatly improve DOT cell expansion, as 4 out of 6 donors (66%) were successfully expanded, over twice as successful as the original method (Table 3). Overall, 6 donors successfully reached a Vd1 T cell population size >60% with donors 262 and 289 being the only donors successfully expanded without CD56 depletion (Figure 2).

**Table 3. Vd1 T cell expansion of healthy donors using the DOT cell expansion method**

| Donor ID | Traditional | +CD56 depletion |
|---|---|---|
| 014 | <60% | <60% |
| 211 | <60% | <60% |
| 253 | <60% | NA |
| 262 | >60% | NA |
| 281 | <60% | NA |
| 282 | <60% | NA |
| 289 | >60% | NA |
| 300 | NA | >60% |
| 304 | NA | >60% |
| 305 | NA | >60% |
| 309 | <60% | NA |
| 312 | NA | >60% |

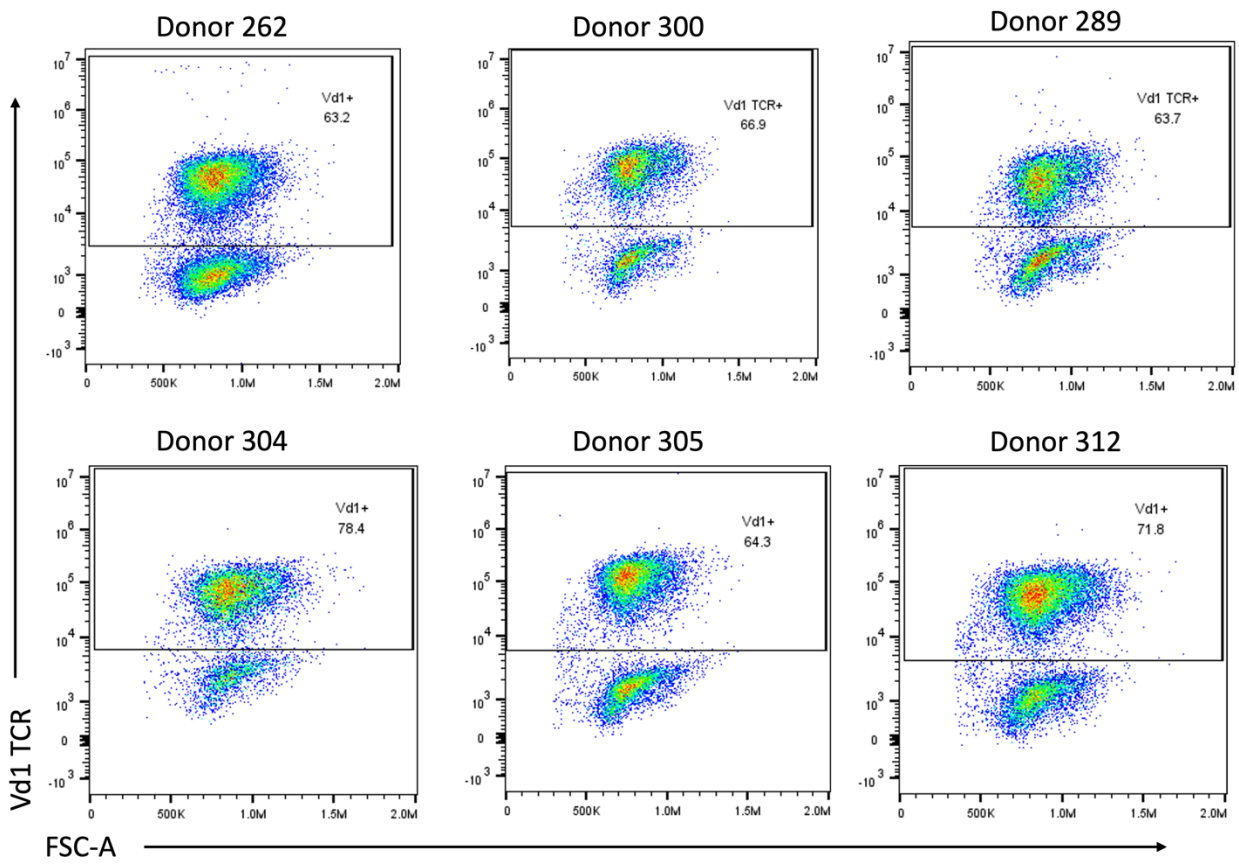

Donor 262
Vd1+
63.2
Donor 300
Vd1 TCR+
66.9
Donor 289
Vd1 TCR+
63.7
Donor 304
Vd1+
78.4
Donor 305
Vd1+
64.3
Donor 312
Vd1+
71.8
Vd1 TCR
FSC-A

**Figure 2. Vd1 T cell population of 6 healthy donors following DOT cell expansion.**
DOT cells were collected on day 17 from 6 different donors and stained with anti-Vd1 TCR prior to being analyzed by flow cytometry.

3.1.2 The majority of Vd1 TCR negative cells are other gamma delta T cells

Since a successful DOT cell expansion is deemed to have a final Vd1 T cell population >60%, there was a question of what other cell type comprised the remaining cells. Analysis by flow cytometry revealed that the majority of the Vd1 TCR negative fraction is other γδ T cells (Figure 3). However, within the Vd1 TCR negative fraction there was a small population of cells that were CD3 and γδ TCR negative. Further analysis showed that the majority of these cells were CD56 positive (Supplementary Figure 2). This population is therefore likely to be residual NK cells.

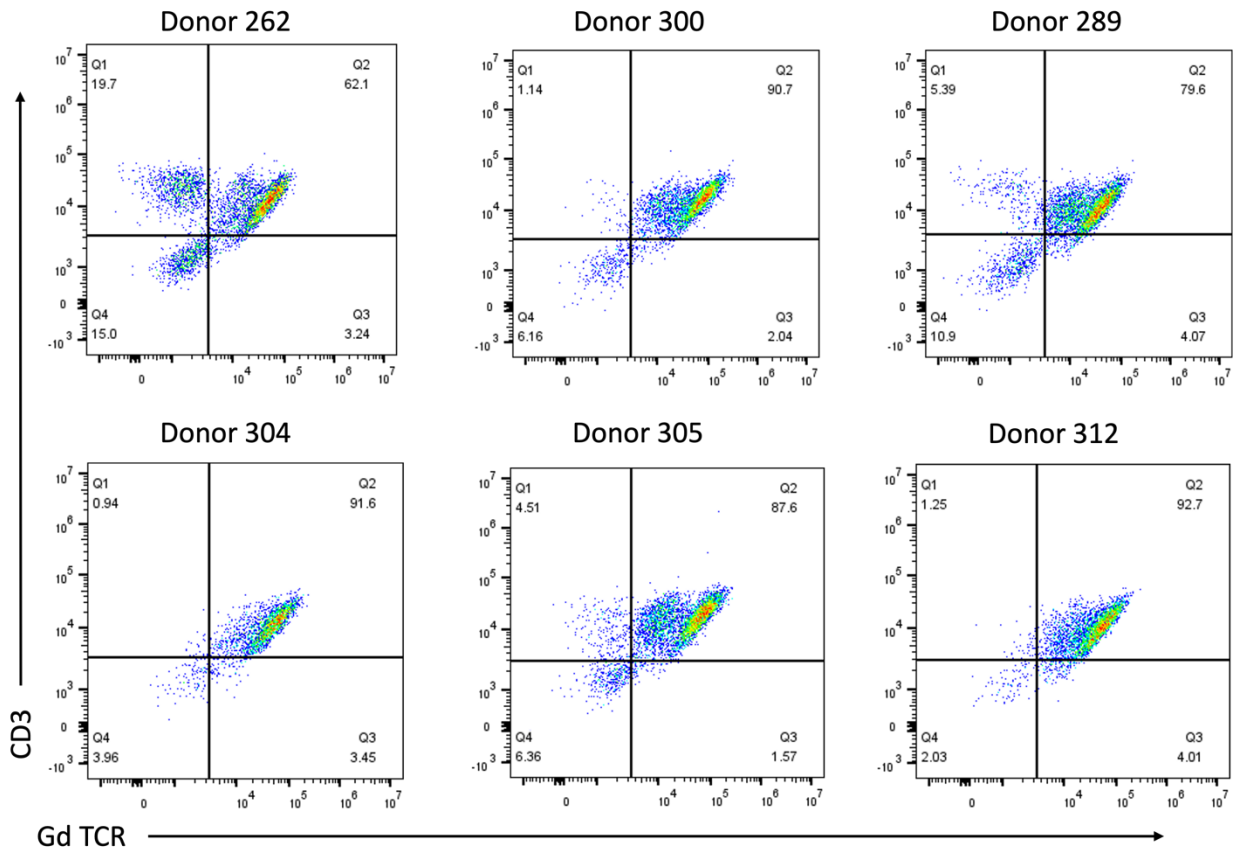

Donor 262
Q1 19.7
Q2 62.1
Q4 15.0
Q3 3.24
Donor 300
Q1 1.14
Q2 90.7
Q4 6.16
Q3 2.04
Donor 289
Q1 5.39
Q2 79.6
Q4 10.9
Q3 4.07
Donor 304
Q1 0.94
Q2 91.6
Q4 3.96
Q3 3.45
Donor 305
Q1 4.51
Q2 87.6
Q4 6.36
Q3 1.57
Donor 312
Q1 1.25
Q2 92.7
Q4 2.03
Q3 4.01
CD3
Gd TCR

**Figure 3. Gamma delta T cells are the primary cell type comprising DOT cells.**
DOT cells were collected on day 17 from 6 different donors and cryopreserved. Cells were thawed prior to being stained with anti-Vd1 TCR, anti-CD3, anti-γδ TCR, and anti-CD56 and were analyzed by flow cytometry.

### 3.1.3 Expanded DOT cells kill different leukemic target cells

To determine the functionality of generated DOT cells, they were co-cultured with two different cell lines of leukemic origin. Jurkats were selected as a model of lymphocytic cells (CD4+ T cells) while HL60s are a model of myelocytic cells (monocytes/macrophages). DOT cells have been shown to kill leukemic cell lines of lymphocytic and myelocytic origin in previous studies.[38,42] DOT cells were co-cultured with the Jurkats for 3 hours while the HL60s required the co-culture to be extended to 24 hours to observe a similar dose-dependent increase in target cell killing (Figure 4). The following E:T ratios were selected; 1:1, 2:1, 5:1 and 10:1. DOT cells were highly effective at killing both cell models even at the lowest E:T ratio.

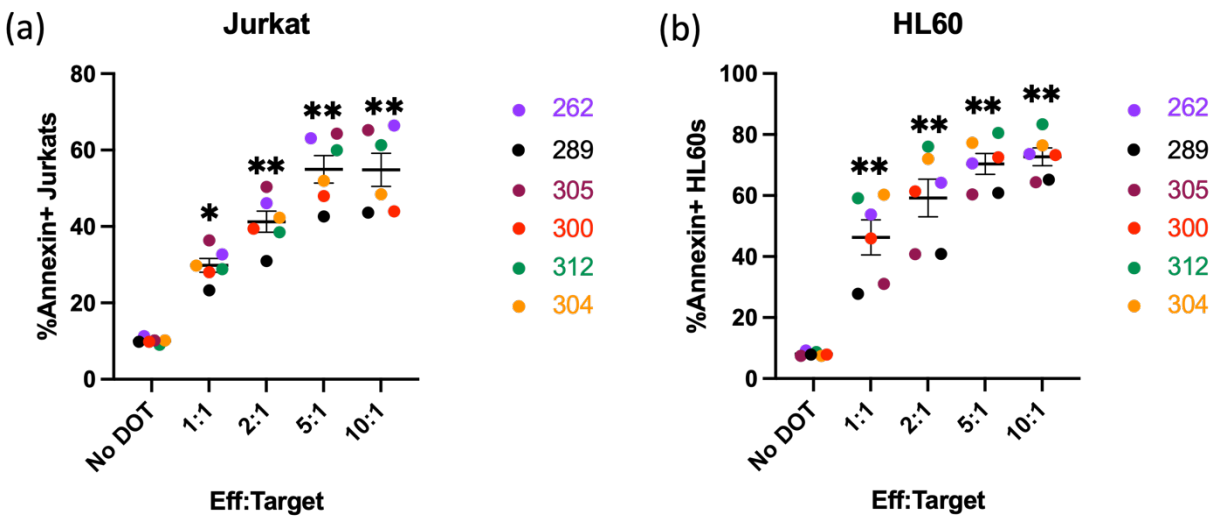

(a)
Jurkat
%Annexin+ Jurkats
80
60
40
20
0
No DOT
1:1
2:1
5:1
10:1
Eff:Target
*
**
**
**
262
289
305
300
312
304
(b)
HL60
%Annexin+ HL60s
100
80
60
40
20
0
No DOT
1:1
2:1
5:1
10:1
Eff:Target
**
**
**
**
262
289
305
300
312
304

**Figure 4. DOT cells kill lymphocytic and myelocytic leukemic cell lines.**
DOT cells were incubated with the lymphocytic Jurkat cell line for 3 hours (a) or the myelocytic HL60 cell line for 24 hours (b). Following co-culture, cells were stained with annexin V and analyzed via flow cytometry. $p<0.0001$ by ordinary one-way ANOVA. * = $p<0.001$, ** = $p<0.0001$ relative to no DOT control by Dunnett's post-test. Data represents the mean +/- the SEM of three technical replicates (n=6).

### 3.1.4 DOT cells effectively kill lymphocytic and myelocytic target cells at low effector: target ratios

Lower E:T ratios were next selected to determine if there was a threshold at which the DOT cells would no longer kill the target cells. DOT cells were co-cultured with the lymphocytic Jurkats or the myelocytic HL60s for 24 hours at the following E:T ratios 1:16, 1:8, 1:4, and 1:2 (Figure 5). While not significant, the lowest effector: target ratio of 1:16 still exhibited a slight increase in target cell killing when compared to the no DOT control. Overall, DOT cells appeared to be very potent.

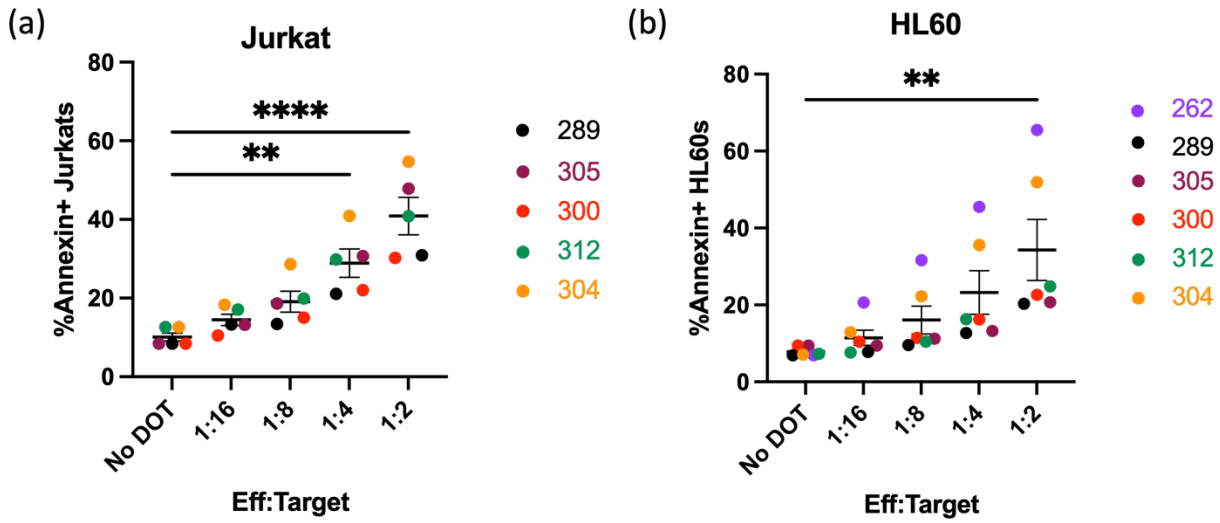

(a)
Jurkat
%Annexin+ Jurkats
80
60
40
20
0
****
**
No DOT
1:16
1:8
1:4
1:2
Eff:Target
289
305
300
312
304
(b)
HL60
%Annexin+ HL60s
**
262
289
305
300
312
304

**Figure 5. DOT cells remain cytotoxic at low effector: target ratios.**
DOT cells were co-cultured with Jurkats (a) or HL60s (b) for 24 hours after which annexin V expression on the target cells was analyzed via flow cytometry. $p<0.0001$ for Jurkats and $p=0.0047$ for HL60s by ordinary one-way ANOVA. ** = $p\leq0.001$, **** = $p<0.0001$ relative to no DOT control by Dunnett's post-test. Data represents the mean +/- SEM (n=5 for Jurkat, n=6 for HL60).

### 3.2 DOT cell killing of HIV-infected cell lines

3.2.1 DOT cell killing of latently HIV-infected cells is dependent on cell type

It was hypothesized that HIV infection in the target cells would either increase DOT cell killing or that there would be no change due to the potential of DOT cells to recognize stress ligands that may be upregulated during HIV infection. Therefore, once DOT cells had had their effector function towards the two cell line models (Jurkat – lymphocytic and HL60 – myelocytic) established, they were then tested with the latently HIV-infected daughter cells of these two cell lines (J1.1s and OM10.1s, respectively). The co-cultures with the HIV-infected daughter cells were performed in the exact same way as had been done with the uninfected parental cell lines i.e., J1.1s were co-cultured with DOT cells for 3 hours and OM10.1s were co-cultured with DOT cells for 24 hours at the same E:T ratios (1:1, 2:1, 5:1, and 10:1). These co-cultures revealed that there were significant differences in the ability of DOT cells to kill the latently HIV-infected J1.1s in comparison to the parental Jurkats (Figure 6a). Specifically, DOT cells were unable to kill the HIV-infected J1.1s as well as the uninfected parental Jurkats. This suggests that HIV infection in the lymphocytic model significantly impaired DOT cell killing. However, there was no significant difference in the killing of the latently HIV-infected OM10.1s in comparison to the parental HL60s (Figure 6b). This suggests that DOT cells are employing a different mechanism of killing between the lymphocytic and myelocytic cell lines, and they appear to be better suited for targeting the myelocytic cells in the context of HIV infection.

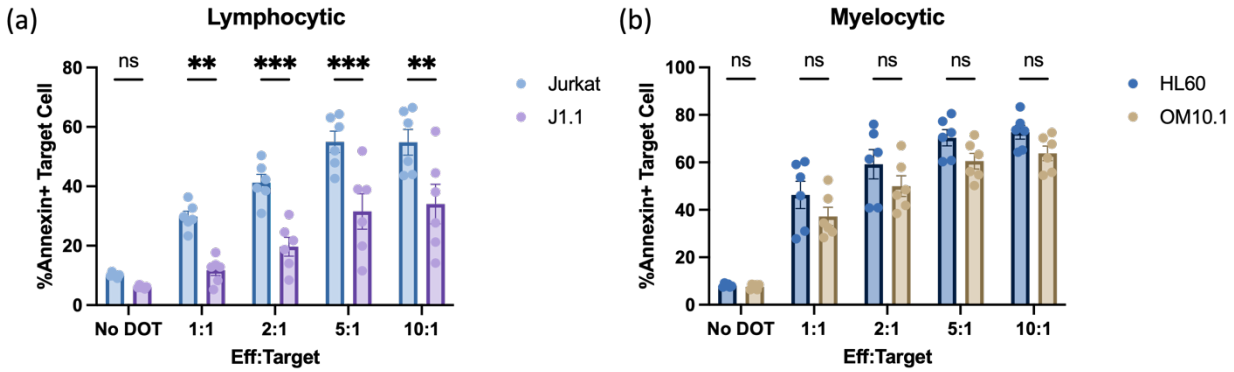

(a)
Lymphocytic
%Annexin+ Target Cell
ns
**
***
***
**
No DOT
1:1
2:1
5:1
10:1
Eff:Target
Jurkat
J1.1
(b)
Myelocytic
%Annexin+ Target Cell
ns
ns
ns
ns
ns
No DOT
1:1
2:1
5:1
10:1
Eff:Target
HL60
OM10.1

**Figure 6. DOT cells kill HIV-infected target cells differently depending on target cell type.** DOT cells were co-cultured with either Jurkats or the latently HIV-infected J1.1s for 3 hours after which annexin V expression was measured on the target cell by flow cytometry (a). DOT cells were co-cultured with either HL60s or the latently HIV-infected OM10.1s for 24 hours after which annexin V expression was measured on the target cell by flow cytometry (b). $p=0.0753$ for lymphocytes and $p=0.7175$ by ordinary two-way ANOVA. ns = not significant, ** = $p \leq 0.001$, *** = $p < 0.0001$ by Šidák's post-test. Data represents mean +/- SEM (n=6).

3.2.2 Vd1 T cell population size in DOT cells may have a correlation with target cell killing

Since some donors had differences in the Vd1 T cell population size after DOT cell expansion, it is possible that this may contribute to the difference in killing observed between the lymphocytic and myelocytic cell lines. To determine this, Vd1 T cell population size was plotted against the killing of the four target cell types (Figure 7). Although not significant, the lymphocytic cell lines appeared to have a slightly negative correlation with Vd1 T cell population size and killing. In particular, the latently HIV-infected J1.1s seemed to have more of a negative correlation than the uninfected parental Jurkats. In contrast, the myelocytic cell lines appeared to have a positive correlation with Vd1 T cell population size, however, only the latently HIV-infected OM10.1s had a significant positive correlation. A similar trend was observed when the Vd1 TCR negative γδ TCR positive population was compared to the extent of killing (data not shown). This suggests a difference in how the cells are being killed, and that myelocytic killing is dependent on the size of the γδ T cell population.

To determine if DOT cell killing matched the expansion of Vd1 T cells, donor 304 DOT cells were collected at 4 different timepoints during the expansion period (Day 0, 7, 11, and 17). These cells were then co-cultured with Jurkats for 3 hours, or HL60s for 24 hours at a 10:1 E:T ratio. The killing of HL60s seemed to be dependent on the presence of Vd1 T cells within the DOT cell population, as there was a linear increase in killing which matched the increasing Vd1 T cell population size (Figure 8a). However, the killing of Jurkats did not appear to be dependent on the size of the Vd1 T cell population (Figure 8b). This was not surprising due to the slight negative correlation observed between killing and Vd1 T cell population size previously (Figure 7a).

Since it was clear at this point that the killing of the lymphocytic cell lines was independent of the size of the γδ T cell population, the NK cell population was plotted against the killing of the target cells (Figure 9). Surprisingly, there was still no correlation between NK cell population size and killing of Jurkats (Figure 9a). However, there was a significant relationship between NK cell population size and the killing of the latently HIV-infected J1.1s (Figure 9b). This suggested that Jurkats are likely killed by multiple cell types/mechanisms whereas J1.1s are primarily killed by NK cells within the DOT cell population. The NK cell population was also negatively correlated with the killing of the cells of myeloid origin, which further supports the previous results that suggested a complete dependence on Vd1 T cells to facilitate killing.

Since OM10.1s were found to be the only cell line that had a significant relationship between Vd1 T cell population size and killing, OM10.1s were then tested with DOT cell expansions that did not meet the >60% Vd1 T cell population threshold (deemed pseudo-DOTs). It was a possibility that the cells that out-competed the Vd1 T cells could contribute to the killing of target cells, and therefore cause the correlation to disappear. Pseudo-DOTs and OM10.1s were co-cultured together for 24 hours at a 1:1 E:T ratio. The pseudo-DOTs were found to be less efficient at killing OM10.1s (Figure 10a), and the correlation between Vd1 T cell population size and killing of OM10.1s remained strong (Figure 10b). This showed that it was unlikely that other cells in the DOT cell population contributed to OM10.1 killing at all, and that this process is primarily mediated by the Vd1 T cells.

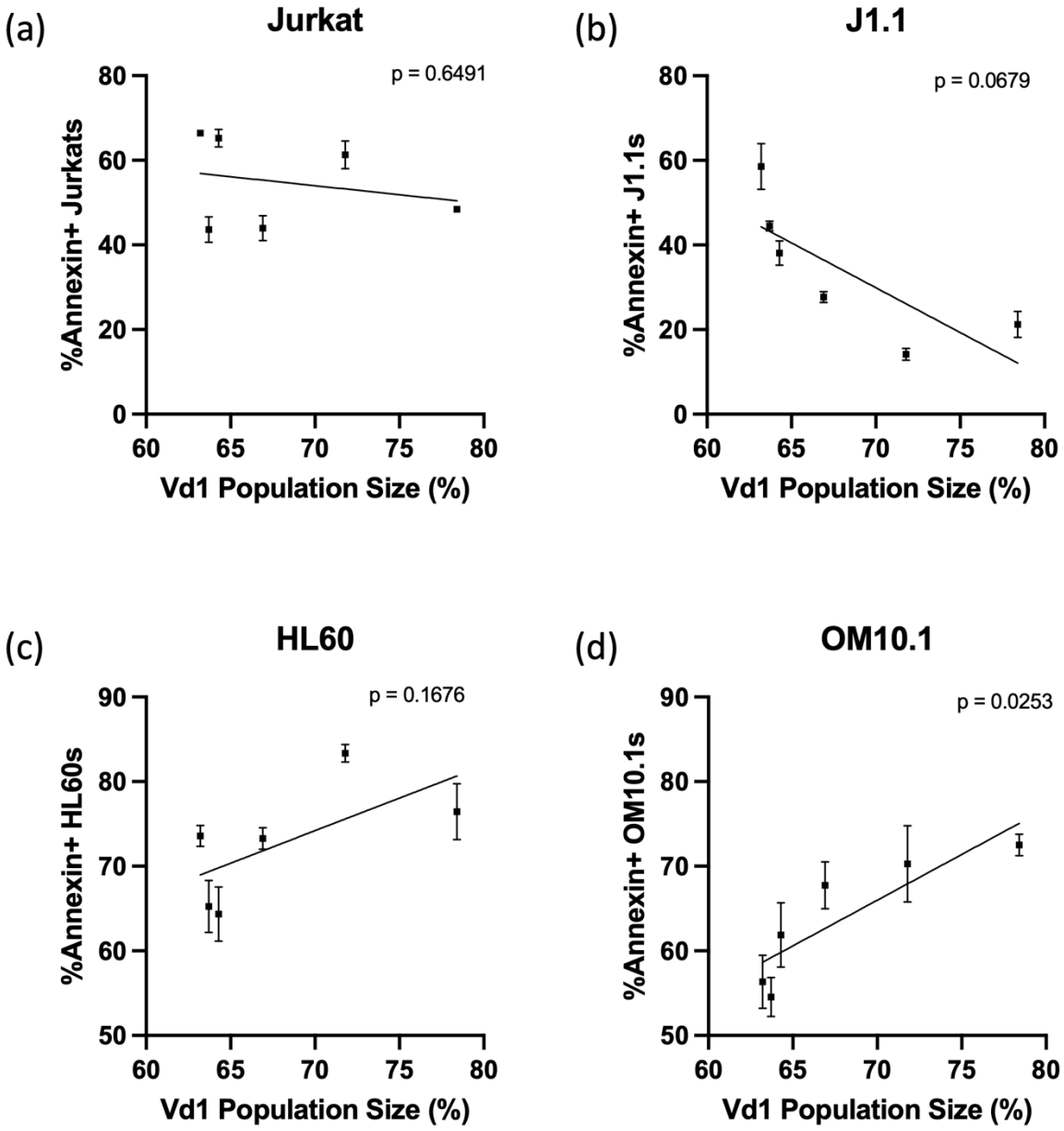
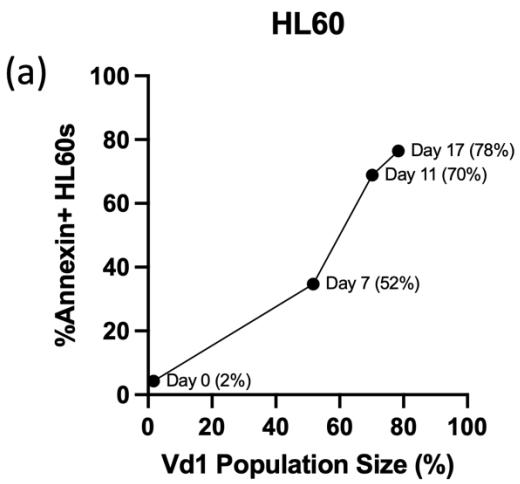

(a)
Jurkat
p = 0.6491
%Annexin+ Jurkats
Vd1 Population Size (%)
(b)
J1.1
p = 0.0679
%Annexin+ J1.1s
Vd1 Population Size (%)
(c)
HL60
p = 0.1676
%Annexin+ HL60s
Vd1 Population Size (%)
(d)
OM10.1
p = 0.0253
%Annexin+ OM10.1s
Vd1 Population Size (%)

**Figure 7. Vd1 T cell population in DOT cells is correlated with OM10.1 killing but not that of other target cells.**
Vd1 T cell population size of the 6 different donors was plotted against the killing of lymphocytic uninfected Jurkats (a), lymphocytic latently HIV-infected J1.1s (b), myelocytic uninfected HL60s (c), and myelocytic latently HIV-infected OM10.1s (d). Linear regression performed. Data represents the mean of three technical replicates +/- SEM of 10:1 E:T ratio (n=6).


HL60
(a)
%Annexin+ HL60s
Vd1 Population Size (%)
Day 0 (2%)
Day 7 (52%)
Day 11 (70%)
Day 17 (78%)

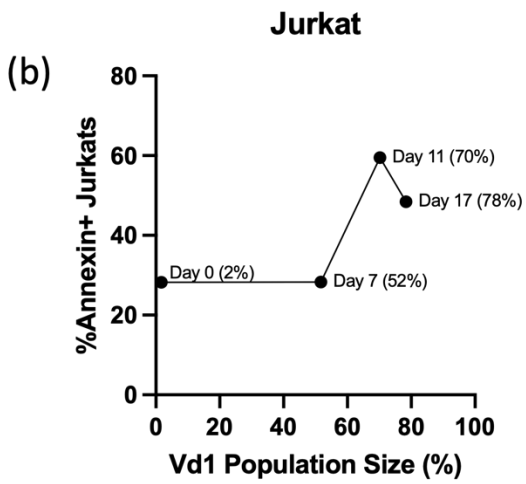

Jurkat
(b)
%Annexin+ Jurkats
Vd1 Population Size (%)
Day 0 (2%)
Day 7 (52%)
Day 11 (70%)
Day 17 (78%)

**Figure 8. DOT cell killing of HL60s but not Jurkats may be dependent on Vd1 T cell population.**
DOT cells from donor 304 were collected at different timepoints during the DOT cell expansion period and co-cultured for 24 hours at a 10:1 E:T ratio with HL60s (a) or for 3 hours at a 10:1 E:T ratio with Jurkats (b). Annexin expression measured on the target cells via flow cytometry (n=1).

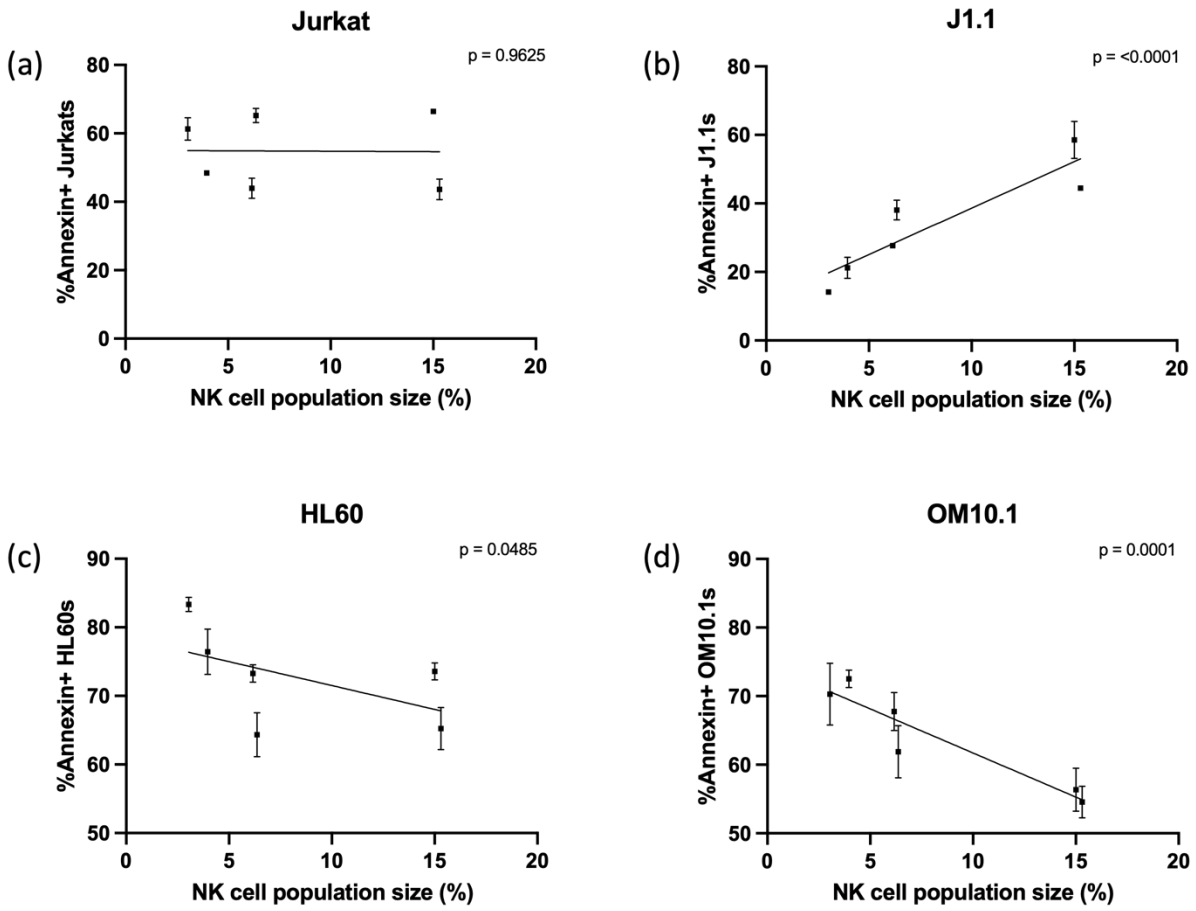

(a)
Jurkat
p = 0.9625
%Annexin+ Jurkats
NK cell population size (%)
(b)
J1.1
p = <0.0001
%Annexin+ J1.1s
NK cell population size (%)
(c)
HL60
p = 0.0485
%Annexin+ HL60s
NK cell population size (%)
(d)
OM10.1
p = 0.0001
%Annexin+ OM10.1s
NK cell population size (%)

**Figure 9. NK cell population in DOT cells is required to kill HIV-infected lymphocytes.** NK cell population size of the 6 different donors was plotted against the killing of lymphocytic uninfected Jurkats (a), lymphocytic latently HIV-infected J1.1s (b), myelocytic uninfected HL60s (c), and myelocytic latently HIV-infected OM10.1s (d). Linear regression performed. Data represents the mean of three technical replicates +/- SEM of 10:1 E:T ratio (n=6).

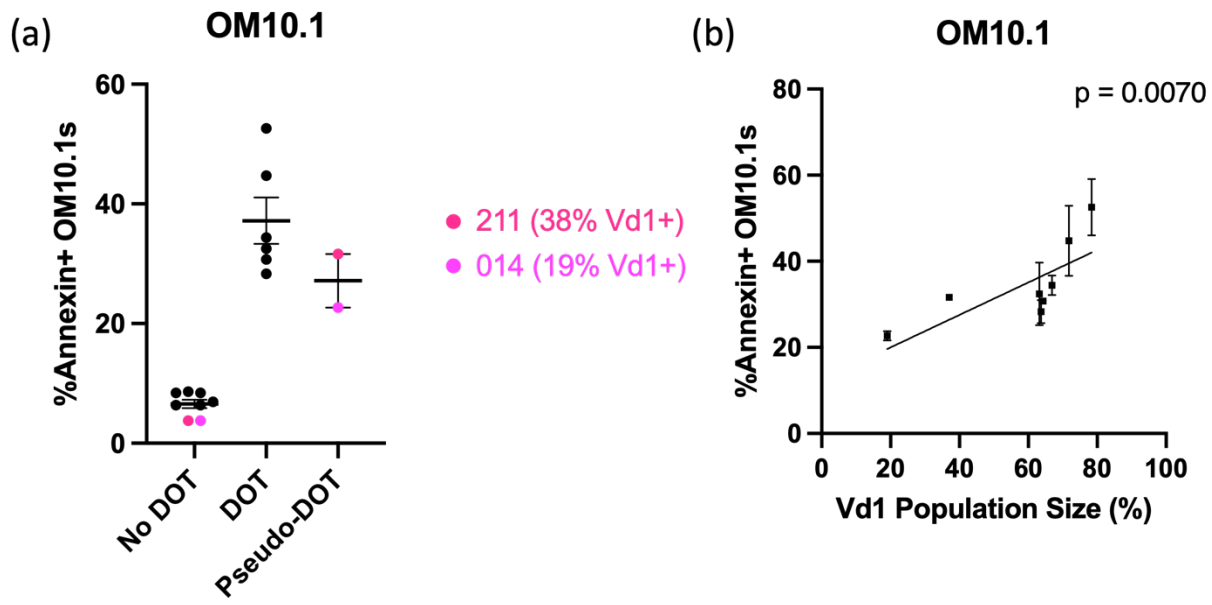

(a)
OM10.1
%Annexin+ OM10.1s
60
40
20
0
No DOT
DOT
Pseudo-DOT
211 (38% Vd1+)
014 (19% Vd1+)
(b)
OM10.1
p = 0.0070
%Annexin+ OM10.1s
80
60
40
20
0
0
20
40
60
80
100
Vd1 Population Size (%)

**Figure 10. Unsuccessful DOT cell expansions ("Pseudo-DOTs") are less cytotoxic towards OM10.1s.**
Pseudo-DOTs were co-cultured with OM10.1s for 24 hours at a 1:1 E:T ratio after which annexin V expression was measured on the OM10.1s via flow cytometry. Annexin V expression following pseudo-DOT co-culture is markedly less compared to DOT cell co-culture (a). Correlation analysis shows a linear relationship between Vd1 T cell population size and killing of OM10.1s (b). Data represents the mean of 2 technical replicates for pseudo-DOTs and 3 technical replicates for DOT cells +/- SEM. n=2 for panel a, n=8 for panel b. Linear regression used for analysis.

### 3.2.3 DOT cell killing of lymphocytic cell lines is dependent on NK receptors

To elucidate the mechanism of killing that the DOT cells employed against the lymphocytic cell lines, various receptors were blocked on the DOT cells prior to co-culture. Compared to the isotype control, there was a slight non-significant decrease in killing when the Vd1 TCR was blocked prior to co-culture with Jurkats, however this effect was not seen in the latently HIV-infected J1.1s (Figure 11). Both Jurkats and J1.1s had the biggest decrease in cell death compared to the no DOT control when the NKp30 and NKp44 receptors were blocked on the DOT cells. These results support the hypothesis that the uninfected Jurkats are killed by a combination of cells/receptors while J1.1s can only be killed through NK receptors which are likely present on residual NK cells in the population.

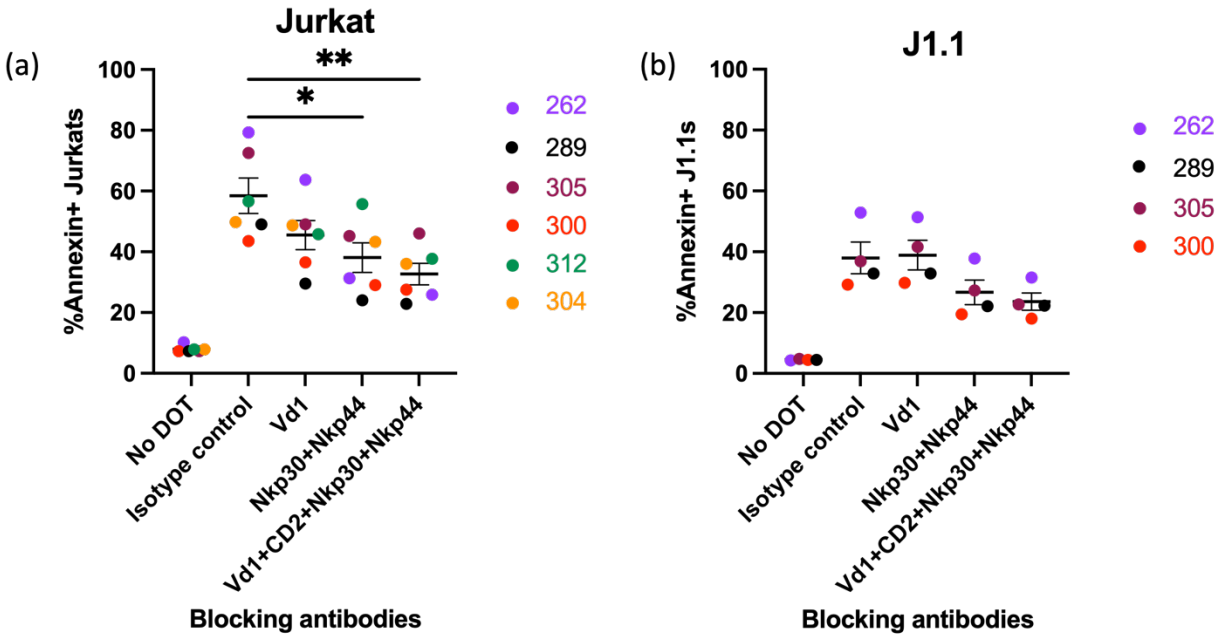

(a)
Jurkat
**
*
%Annexin+ Jurkats
100
80
60
40
20
0
No DOT
Isotype control
Vd1
Nkp30+Nkp44
Vd1+CD2+Nkp30+Nkp44
Blocking antibodies
262
289
305
300
312
304
(b)
J1.1
%Annexin+ J1.1s
100
80
60
40
20
0
No DOT
Isotype control
Vd1
Nkp30+Nkp44
Vd1+CD2+Nkp30+Nkp44
Blocking antibodies
262
289
305
300

**Figure 11. DOT cell killing of lymphocytic cell lines is dependent on NK receptors.**
DOT cells were incubated with different antibodies for 1 hour prior to a 3-hour co-culture at a 10:1 E:T ratio with uninfected Jurkats (a) or latently HIV-infected J1.1s (b). $p=0.0034$ for Jurkats and $p<0.0001$ for J1.1s by ordinary one-way ANOVA. * = $p<0.05$, ** = $p<0.001$ relative to isotype control by Tukey's post-test. Data represents the mean +/- SEM (n=6 for Jurkat, n=4 for J1.1).

3.2.4 DOT cells do not produce IFNg after a 24-hour co-culture with target cell lines

Vd1 T cells and specifically DOT cells, have been shown to produce tumor-antagonistic cytokines like IFNg following exposure to tumor cells.[38,56] However, these studies looked at IFNg production *in vivo*, so the speed of IFNg production following effector-target interaction was unclear. γδ T cells generally have been shown to provide an early source of IFNg for tumor immunity, so it was hypothesized that Vd1 T cells would rapidly produce IFNg during a co-culture with tumoral target cells.[57] No IFNg was detected in any supernatants from 24-hour co-cultures between DOT cells and Jurkats, J1.1s, HL60s, or OM10.1s at a 10:1 E:T ratio (data not shown). A 48-hour or 72-hour co-culture may have resulted in IFNg production, but this was not tested. Furthermore, a lower E:T ratio may also have facilitated more of an IFNg response since there would have been more receptor engagement on the DOT cells. Overall, these results suggest that when DOT cells are cultured with relatively few target cells for a period of 24 hours, they are not induced to produce an inflammatory response.

**3.3 DOT cell killing of HIV-infected MDMs**

3.3.1 DOT cells may selectively kill HIV-infected monocyte-derived macrophages

Since DOT cells had been shown to be very effective at killing HIV-infected cell lines of myeloid origin, they were next tested on primary monocyte-derived macrophages. Macrophages are one of the cell types that make up the viral reservoir, and their propensity to reside in tissues makes them difficult to target.[58] It was hypothesized that DOT cells would specifically target the HIV-infected macrophages while bypassing the uninfected bystander cells. Uninfected bystander cell death is a key element of HIV pathogenesis, and a selective therapy to avoid this would be ideal.[59] To test the specificity of DOT cell killing, DOT cells from donor 262 were co-cultured

for 24 hours with allogeneic MDMs that had been either mock-infected or HIV-infected. Overall cell death was measured on MDMs following this co-culture (Supplementary Figure 4) as well as annexin expression on HIV-infected and uninfected fractions of the co-culture (Figure 12a). To determine the HIV-specific response, the HSA negative annexin expression was subtracted from the HSA positive fraction (Figure 12b). These results suggest a potential for DOT cells to specifically target HIV-infected MDMs. The results depicted in Figure 12a show that HIV-infected MDMs treated with DOT cells had increased cell death of the HIV-infected population, while the uninfected bystander cells did not exhibit this increase. When the results were further distinguished to look specifically at the difference in cell death exhibited by the HIV-infected cells when treated with DOT cells as shown in Figure 12b, there was a clear increase in cell death of the DOT cell treated HIV-infected MDMs compared to the untreated no DOT cell control. While this difference in cell death between the treated and untreated groups may seem small at approximately 10%, these results are actually quite promising since the HIV-infected population in the MDM model tends to be around 5-10%. However, since only one donor was tested, the observation of HIV-specific cytotoxicity of DOT cells remains preliminary.

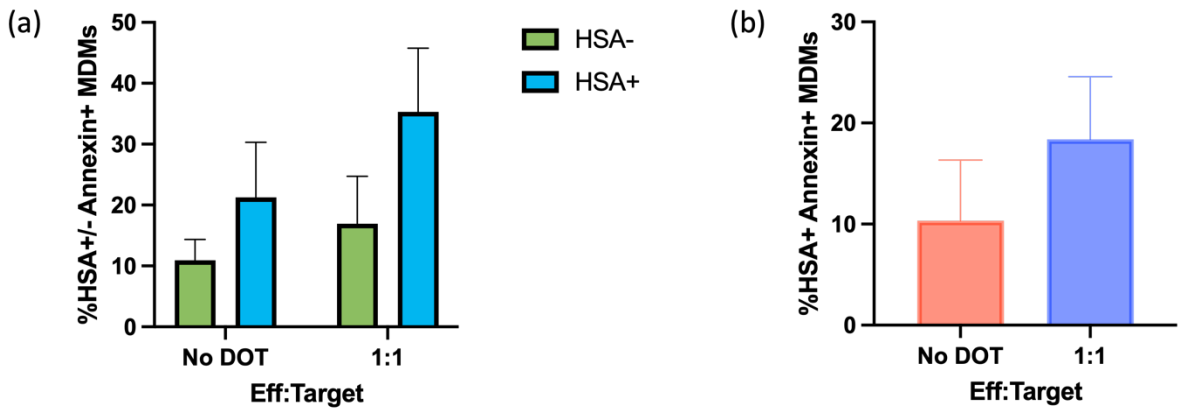

(a)
%HSA+/- Annexin+ MDMs
50
40
30
20
10
0
No DOT
1:1
Eff:Target
HSA-
HSA+
(b)
%HSA+ Annexin+ MDMs
30
20
10
0
No DOT
1:1
Eff:Target

**Figure 12. DOT cells may exhibit HIV-specific killing of monocyte-derived macrophages.** DOT cells from donor 262 were co-cultured with allogeneic monocyte-derived macrophages for 24 hours at a 1:1 E:T ratio after which HIV infection and subsequent death was measured by staining for the HSA reporter protein and analyzing the annexin expression in the infected and uninfected cell populations (a). Background HSA negative annexin expression was subtracted from the HSA positive population to show the overall HIV-specific death (b). Data shows the mean of 4 technical replicates +/- SEM (n=1).

### 3.3.2 HIV infection in monocyte-derived macrophages may not selectively induce DOT cell migration in a transwell model

It was hypothesized that DOT cells may be induced to migrate towards HIV infected cells due to their expression of various chemokine receptors. To test this, DOT cells from donor 262 were separated from HIV-infected or mock-infected MDMs by an 8um filter for 24 hours, after which migrated cells in the supernatant were counted by light microscopy. There appeared to be no specific induction of migration of the DOT cells towards HIV-infected MDMs regardless of whether an allogeneic or autologous model was used (Figure 13). It is possible that the experimental design was not optimized to accurately depict the migration of DOT cells, as well as there only being an n of 1.

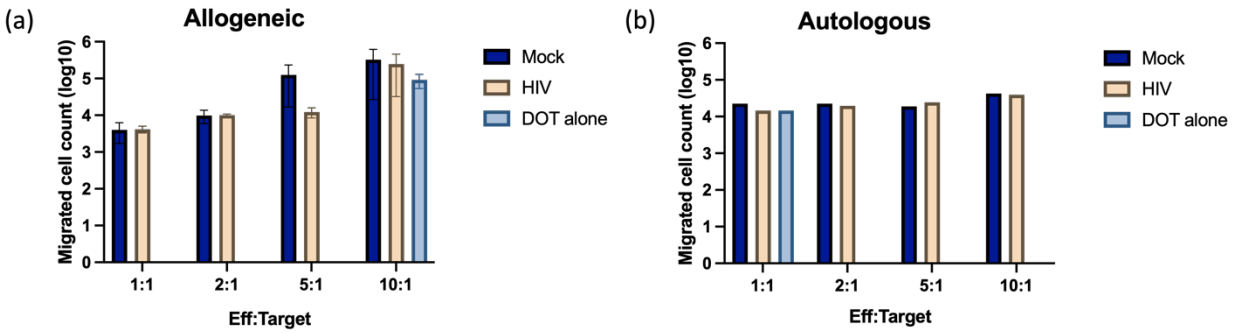

(a)
Allogeneic
Migrated cell count (log10)
0
1
2
3
4
5
6
1:1
2:1
5:1
10:1
Eff:Target
Mock
HIV
DOT alone
(b)
Autologous
Migrated cell count (log10)
0
1
2
3
4
5
6
1:1
2:1
5:1
10:1
Eff:Target
Mock
HIV
DOT alone

**Figure 13. DOT cells do not appear to preferentially traffic to monocyte-derived macrophages infected with HIV.**
DOT cells were separated from monocyte-derived macrophages that had been mock-infected or HIV-infected via an 8um filter for 24 hours. Migrated cells were collected from the supernatant and counted by light microscopy. DOT cells were used from allogeneic donors (a) or from an autologous donor (b). Data from panel a represents the mean +/- SEM of 4 technical replicates (n=1).

## Chapter 4: Discussion

To summarize the findings of this project, the DOT cell expansion method was further optimized by adding in a CD56 depletion. This depletion increased the expansion of Vd1 T cells by removing NK cells that competed for the cytokine cocktail. When the DOT cell populations were characterized, they were found to be enriched in γδ T cells, with the expansions that had a CD56 depletion prior to expansion having a smaller proportion of residual NK cells.

DOT cells effectively killed lymphocytic (Jurkats) and myelocytic (HL60s) target cell lines at low effector:target ratios. The hypothesis of this study was that DOT cells would kill latently HIV-infected target cells at rates higher than or similar to their uninfected parental target cells. However, DOT cell killing of J1.1s, the latently HIV-infected daughter cell of Jurkats, was significantly less, while DOT cell killing of OM10.1s, the latently HIV-infected daughter cells of HL60s did not experience this decrease. An independent mechanism of killing may explain these differences, with myelocytic killing relying completely on the Vd1 T cell population whereas lymphocytic killing is mediated by NK receptors that may be present on residual NK cells within the DOT cell population. DOT cell killing of target cell lines was also not associated with the secretion of IFNg. The length of the co-culture (24 hours) and the effector:target ratio used (10:1) may not have provided enough engagement of the DOT cells to induce the production of inflammatory cytokines.

With respect to primary cells, DOT cells may have an HIV-specific response towards HIV-infected monocyte-derived macrophages, however this data is preliminary, and more work needs to be done to confirm this specificity with different donors. Lastly, DOT cells may not preferentially migrate towards HIV-infected monocyte-derived macrophages in a transwell assay. However, this data is also preliminary and there may be donor differences in

migratory response. Furthermore, it is unknown what would induce DOT cell migration so the lack of a positive control for this assay is a large limitation.

### 4.1 DOT cells are an enriched population of gamma delta T cells

The threshold for a successful DOT cell expansion is a Vd1 T cell population >60%. This leaves the question of what the remaining ~40% of cells are comprised of since the Vd1 TCR negative population of DOT cells has been poorly characterized in the past. This study has shown that the majority of Vd1 TCR negative cells are other subtypes of γδ T cells, meaning that DOT cells are a highly enriched population of γδ T cells. There are at least 8 subtypes of γδ T cells, so the Vd1 TCR negative population could comprise any number of Vd2-Vd8 T cells.[60] Unfortunately, a further characterization into the exact proportions of each subtype is difficult since no commercial antibodies exist outside of the ones available for Vd1 and Vd2 T cells.

DOT cell donors that had been CD56 depleted prior to expansion had an increased purity of γδ T cells and also had a far smaller proportion of residual NK cells in the population. Therefore, adding in an additional CD56 depletion prior to expansion may not only improve overall Vd1 T cell yield but decrease the number of contaminant cells.

### 4.2 DOT cell killing of lymphocytic but not myelocytic cells is altered by HIV infection

Interestingly, the killing of lymphocytic cell lines that were infected with HIV (J1.1 cells) was significantly less than that observed in their uninfected parental cell line (Jurkat cells). However, this difference was not observed in the myelocytic cell lines, where the HIV-infected cell line (OM10.1 cells) had only slightly less killing compared to the uninfected parental cell

line (HL60 cells). Correlation analyses indicated that the killing of the myeloid cells was positively correlated with the population size of the Vd1 T cells within the DOT cell population, however only OM10.1s had a significant relationship. The lymphoid cells had no correlation (Jurkat cells) or negative correlation (J1.1 cells) when the Vd1 T cell population size was plotted against killing, however neither was significant. The first explanation that seems most likely based on these findings is that different cell types within the DOT cell product are responsible for killing of the lymphoid vs the myeloid cells. Correlation of the NK cell population with killing of the latently HIV-infected J1.1s revealed a positive correlation. It therefore appears that J1.1s are primarily killed by NK cells while the myelocytic cell lines seem to be primarily killed by Vd1 T cells. The uninfected Jurkats seemed to be killed by a combination of cells and couldn't be linked to any specific cell type. A previous study has shown that NK cells are capable of killing J1.1s, and this killing can be increased by upregulating NKG2D ligands.[61] Having a multicellular DOT cell product may therefore be beneficial for targeting the different cell types that make up the latent HIV reservoir.

The next likely explanation is that another receptor may be involved in the killing of myelocytic cells. DNAM-1 is an NK receptor that synergizes with other activating receptors to help mediate cytotoxicity and happens to be upregulated on DOT cells.[62] A recent publication supports the interaction between DNAM-1 on DOT cells and the ligand polio virus receptor (PVR)/CD155 on acute leukemic myeloid cells.[63,64] Nectin-2, a ligand recognized by DNAM-1, was found to be unchanged on monocytes from PLWH compared to healthy donors, so it is possible that this killing could be translated to HIV-infected cells.[65]

Another possibility for differences in cell type killing may be the downregulation of immune ligands during HIV infection.[45] As shown in this study, the lymphocytic cell lines were primarily killed by the NK receptors NKp30 and NKp44. This is supported by other work that has shown the role of NKp30 in the killing of lymphocytic and myelocytic leukemias.[38,42,66] Both Jurkats and HL60s express high levels of B7 homolog 6 (B7-H6), one of the ligands known to bind to the NKp30 receptor.[67] In fact, over-expression of B7-H6 is thought to contribute to the pathogenesis of leukemia *in vivo* by leading to chronic engagement of the NKp30 receptor.[67,68] HIV infection is known to change the expression of ligands for NKp30 on T cells, but its expression on monocytes/macrophages following HIV infection is unclear.[45] It is therefore possible that NKp30L may be a triggering ligand on both cell types, but only gets downregulated on the HIV-infected lymphocytes.

A final possibility to explain the difference in killing of different HIV-infected cells by DOT cells is that there may be a difference in the anti-apoptotic mechanisms of each cell type. Other studies have found J1.1s to generally be more resistant to cell death than their parental uninfected cell line.[69,70] Some of this difference can be explained by a decreased activation of apoptosis proteins called caspases in the latently HIV-infected J1.1s compared to the uninfected Jurkats.[69] In our lab, we have also observed a greater difference in apoptosis between Jurkats and J1.1s in comparison with HL60s and OM10.1s following treatment with apoptosis inducers like staurosporine and campothecin. It is therefore possible that any treatment that results in apoptosis will see this difference between the lymphocytic and myelocytic cell lines.

### 4.3 DOT cells do not secrete IFNg during a 24-hour co-culture with target cells

IFNg is an important inflammatory cytokine that is associated with a Th1 (pro-inflammatory) immune response.[71] Furthermore, the secretion of IFNg has also been shown to help boost the motility and cytotoxicity of T cells.[72] This study found that IFNg was not produced after a 24-hour co-culture of DOT cells with any of the target cell lines, regardless of HIV infection. However, DOT cells were strongly cytotoxic towards all of the cell lines tested. This suggests that DOT cells can kill their target cells rapidly and will only secrete cytokines after repeated receptor-ligand engagement over an extended period of time. Previous studies have shown that when adoptively transferred, DOT cells were shown to produce both TNFa and IFNg months after the initial transfer.[38] It is therefore likely that 24 hours is an insufficient period of time to capture this immune response, and DOT cells are still most likely to be Th1 effectors.[73]

### 4.4 Potential for DOT cell migration

DOT cells highly express C-X-C chemokine receptor type 3 (CXCR3) which should allow them to follow gradients of interferon inducible ligands.[74] A previous study from 2005 using CD4+ T cells and monocyte-derived macrophages determined that HIV infection resulted in the production of C-X-C motif chemokine ligand 10 (CXCL10) and C-X-C motif chemokine ligand 11 (CXCL11), both of which acted on the CXCR3 receptor of CD4+ T cells to induce migration.[75] Based on this 2005 study, it seems as though DOT cells should also have been induced to migrate. There are many factors that could explain this difference, one being that in the 2005 study peak CXCL10 production and subsequent peak CD4+ T cell migration occurred 3

days post-infection.[75] In our study, the migration analysis was performed 8 days post-infection, and it is therefore possible that CXCL10 production had been lost. The kinetics of migration may also differ in CD4+ T cells than in Vd1 T cells, so it is impossible to know if Vd1 T cells would migrate if the experimental design had been performed in the exact same manner. In PLWH, CXCL10 is elevated in the plasma in nearly all patients regardless of treatment status.[76] Therefore, the transitory CXCL10 production seen in an *in vitro* model is not necessarily relevant to the immune landscape *in vivo* and DOT cell migration should not be ruled out until it can be properly tested in an *in vivo* model.

### 4.5 Limitations and future directions

In this study, only 6 donors were used to generate DOT cells, meaning that there was a relatively small sample size. Using CD56 depleted DOT cells in combination with CD56 undepleted DOT cells may have contributed to donor variability, especially with respect to the killing of the HIV-infected lymphocytes.

This study primarily focused on the ability of DOT cells to kill cell lines. It is important to consider that the origin of these cell lines was leukemia, which DOT cells have been shown to kill in previous studies.[38] Since DOT cells kill these cells regardless of HIV-infection, the specificity of DOT cells towards HIV-infected cells needs further investigation. It does appear as though there may be HIV-specific killing when DOT cells were co-cultured with HIV-infected monocyte-derived macrophages, however only an n of 1 was tested and more DOT cell donors need to be tested to make any conclusions. Furthermore, a true model of HIV latency in primary cells was not used in this study, which would also have been beneficial in determining DOT cell

specificity.

The models included in this study were all performed *in vitro,* so the ability of DOT cells to efficiently target the HIV reservoir is still unknown. The complexities of the immune landscape during HIV infection would play a role in how DOT cells would function following adoptive transfer.[77] DOT cells have been cleared by the FDA for treatment for acute myeloid leukemia under the name GDX012 and is currently in phase 1 and 2 clinical trials.[78] Should the preliminary safety studies go well, the transition to other clinical studies should be relatively simple and using DOT cells to treat HIV could be a real possibility in the future.

**4.6 Summary and significance**

In summation, this project has contributed to the characterization of the DOT cell population and indicated specific cell types within this population with the killing of different types of target cells. DOT cells can kill cells latently infected with HIV, but target cell killing was not increased by HIV infection, and the killing of lymphocytic cells was significantly decreased by HIV infection. Killing of the HIV-infected lymphocytes appeared to be dependent on residual NK cells within the DOT cell population, while the killing of the myelocytes appeared to be dependent on the Vd1 T cell population. Therefore, having a multicellular DOT cell product may be beneficial for targeting the different cell types comprising the HIV reservoir.

This project has significant implications into the subject of HIV cure research and may pose a novel method for targeting and eliminating the HIV viral reservoir. PLWH remain a vulnerable population, not only through societal stigma, but they are disproportionately at risk for other infections, as displayed by the COVID-19 pandemic.[79] DOT cells may provide a path

towards achieving a functional cure by exploiting the anti-viral capabilities of Vd1 T cells to control HIV infection.

**References**


1. https://www.unaids.org/sites/default/files/media_asset/UNAIDS_FactSheet_en.pdf UNAIDS. UNAIDS Fact Sheet 2024. Preprint.
2. Kirchhoff, F. (2013). HIV Life Cycle: Overview. In Encyclopedia of AIDS (Springer New York), pp. 1–9. https://doi.org/10.1007/978-1-4614-9610-6_60-1.
3. Kalichman, S.C., Ramachandran, B., and Catz, S. (1999). Adherence to combination antiretroviral therapies in HIV patients of low health literacy. J Gen Intern Med *14*, 267–273. https://doi.org/10.1046/j.1525-1497.1999.00334.x.
4. Tseng, A., Seet, J., and Phillips, E.J. (2015). The evolution of three decades of antiretroviral therapy: Challenges, triumphs and the promise of the future. Br J Clin Pharmacol *79*, 182–194. https://doi.org/10.1111/bcp.12403.
5. Wester, C.W., Bussmann, H., Koethe, J., Moffat, C., Vermund, S., Essex, M., and Marlink, R.G. (2009). Adult combination antiretroviral therapy in sub-Saharan Africa: lessons from Botswana and future challenges.
6. Power, J., Dowsett, G.W., Westle, A., Tucker, J.D., Hill, S., Sugarman, J., Lewin, S.R., Brown, G., and Lucke, J. (2020). The significance and expectations of HIV cure research among people living with HIV in Australia. PLoS One *15*. https://doi.org/10.1371/journal.pone.0229733.
7. Dalgeish, A.G., Beverley, P.C.L., Clapham, P.R., Crawford, D.H., Greaves, M.F., and Weiss, R.A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus.
8. Kruize, Z., and Kootstra, N.A. (2019). The Role of Macrophages in HIV-1 Persistence and Pathogenesis. Front Microbiol *10*, 2828. https://doi.org/10.3389/fmicb.2019.02828.
9. Simon, V., Ho, D.D., and Abdool Karim, Q. (2006). HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Preprint at Elsevier B.V., https://doi.org/10.1016/S0140-6736(06)69157-5 https://doi.org/10.1016/S0140-6736(06)69157-5.
10. de Boer, R.J., Ribeiro, R.M., and Perelson, A.S. (2010). Current estimates for HIV-1 production imply rapid viral clearance in lymphoid tissues. PLoS Comput Biol *6*. https://doi.org/10.1371/journal.pcbi.1000906.
11. Hong, F.F., and Mellors, J.W. (2015). Changes in HIV reservoirs during long-term antiretroviral therapy. Preprint at Lippincott Williams and Wilkins, https://doi.org/10.1097/COH.0000000000000119.
12. Moretti, S., Schietroma, I., Sberna, G., Maggiorella, M.T., Sernicola, L., Farcomeni, S., Giovanetti, M., Ciccozzi, M., and Borsetti, A. (2023). HIV-1–Host Interaction in Gut-Associated Lymphoid Tissue (GALT): Effects on Local Environment and Comorbidities. Preprint at Multidisciplinary Digital Publishing Institute (MDPI), https://doi.org/10.3390/ijms241512193 https://doi.org/10.3390/ijms241512193.
13. Belyakov, I.M., and Berzofsky, J.A. (2004). Immunobiology of Mucosal HIV Infection and the Basis for Development of a New Generation of Mucosal AIDS Vaccines.
14. Zhao, Y., Niu, C., and Cui, J. (2018). Gamma-delta (γδ) T Cells: Friend or Foe in Cancer Development. Preprint at BioMed Central Ltd., https://doi.org/10.1186/s12967-017-1378-2 https://doi.org/10.1186/s12967-017-1378-2.

15. Bonneville, M., O'Brien, R.L., and Born, W.K. (2010). γ δ T cell effector functions: A blend of innate programming and acquired plasticity. Preprint, https://doi.org/10.1038/nri2781 https://doi.org/10.1038/nri2781.
16. Bonneville, M., O'Brien, R.L., and Born, W.K. (2010). γδ T cell effector functions: a blend of innate programming and acquired plasticity. Nat Rev Immunol *10*, 467–478.
17. Caron, J., Ridgley, L.A., and Bodman-Smith, M. (2021). How to Train Your Dragon: Harnessing Gamma Delta T Cells Antiviral Functions and Trained Immunity in a Pandemic Era. Preprint at Frontiers Media S.A., https://doi.org/10.3389/fimmu.2021.666983 https://doi.org/10.3389/fimmu.2021.666983.
18. Vantourout, P., and Hayday, A. (2013). Six-of-the-best: Unique contributions of γδ T cells to immunology. Preprint, https://doi.org/10.1038/nri3384 https://doi.org/10.1038/nri3384.
19. Fonseca, S., Pereira, V., Lau, C., Teixeira, M.D.A., Bini-Antunes, M., and Lima, M. (2020). Human Peripheral Blood Gamma Delta T Cells: Report on a Series of Healthy Caucasian Portuguese Adults and Comprehensive Review of the Literature. Preprint at NLM (Medline), https://doi.org/10.3390/cells9030729 https://doi.org/10.3390/cells9030729.
20. David Pauza, C., Poonia, B., Li, H., Cairo, C., and Chaudhry, S. (2015). γδ T cells in HIV disease: Past, present, and future. Preprint at Frontiers Media S.A., https://doi.org/10.3389/fimmu.2014.00687 https://doi.org/10.3389/fimmu.2014.00687.
21. De Paoli, P., Gennari, D., Martelli, P., Basaglia, G., Crovatto, M., and Battistin, S. (1991). A subset of γδ lymphocytes is increased during HIV-1 infection.
22. Li, H., and David Pauza, C. (2011). HIV envelope-mediated, CCR5/47-dependent killing of CD4-negative T cells which are lost during progression to AIDS. https://doi.org/10.1182/blood-2011.
23. Hudspeth, K., Fogli, M., Correia, D. V, Mikulak, J., Roberto, A., Bella, S. Della, Silva-Santos, B., and Mavilio, D. (2012). Engagement of NKp30 on Vδ1 T cells induces the production of CCL3, CCL4, and CCL5 and suppresses HIV-1 replication. https://doi.org/10.1182/blood-2011.
24. Harris, L.D., Klatt, N.R., Vinton, C., Briant, J.A., Tabb, B., Ladell, K., Lifson, J., Estes, J.D., Price, D.A., Hirsch, V.M., et al. (2010). Mechanisms underlying γδ T-cell subset perturbations in SIV-infected Asian rhesus macaques. Blood *116*, 4148–4157. https://doi.org/10.1182/blood-2010-05-283549.
25. Cimini, E., Agrati, C., D'Offizi, G., Vlassi, C., Casetti, R., Sacchi, A., Lionetti, R., Bordoni, V., Tumino, N., Scognamiglio, P., et al. (2015). Primary and chronic HIV infection differently modulates mucosal Vδ1 and Vδ2 T-cells differentiation profile and effector functions. PLoS One *10*. https://doi.org/10.1371/journal.pone.0129771.
26. Wherry, E.J., and Kurachi, M. (2015). Molecular and cellular insights into T cell exhaustion. Preprint at Nature Publishing Group, https://doi.org/10.1038/nri3862 https://doi.org/10.1038/nri3862.
27. Van Laethem, F., Tikhonova, A.N., and Singer, A. (2012). MHC restriction is imposed on a diverse T cell receptor repertoire by CD4 and CD8 co-receptors during thymic selection. Trends Immunol *33*, 437–441. https://doi.org/10.1016/j.it.2012.05.006.
28. Aladağ, E., Kelkitli, E., and Göker, H. (2020). Acute Graft-Versus-Host Disease: A Brief Review. Turkish Journal of Hematology *37*. https://doi.org/10.4274/tjh.galenos.2019.2019.0157.

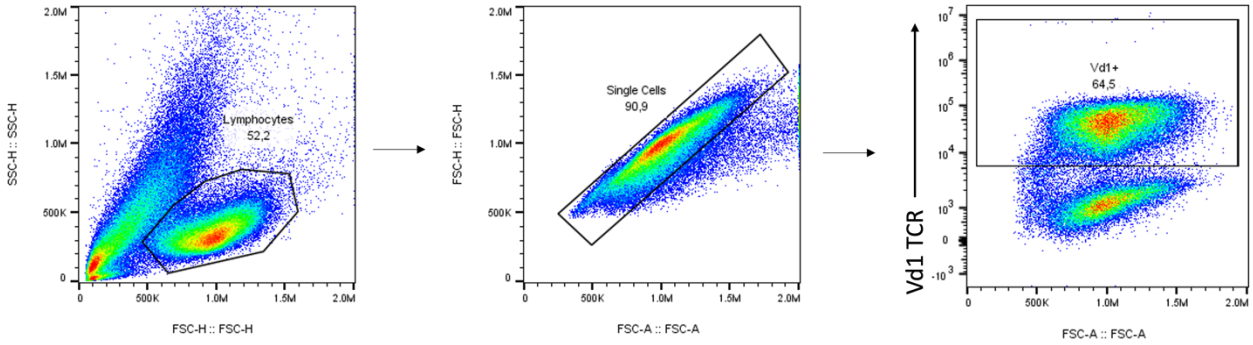
29. Chen, Y.-J., Abila, B., and Mostafa Kamel, Y. (2023). CAR-T: What Is Next? Cancers (Basel) *15*, 663. https://doi.org/10.3390/cancers15030663.
30. Kabelitz, D., Serrano, R., Kouakanou, L., Peters, C., and Kalyan, S. (2020). Cancer immunotherapy with γδ T cells: many paths ahead of us. Preprint at Springer Nature, https://doi.org/10.1038/s41423-020-0504-x https://doi.org/10.1038/s41423-020-0504-x.
31. Joalland, N., and Scotet, E. (2020). Emerging Challenges of Preclinical Models of Anti-tumor Immunotherapeutic Strategies Utilizing Vγ9Vδ2 T Cells. Preprint at Frontiers Media S.A., https://doi.org/10.3389/fimmu.2020.00992 https://doi.org/10.3389/fimmu.2020.00992.
32. Sun, L., Li, Y., Jiang, Z., Zhang, J., Li, H., Li, B., and Ye, Z. (2016). Vγ9Vδ2 T cells and zoledronate mediate antitumor activity in an orthotopic mouse model of human chondrosarcoma. Tumor Biology *37*, 7333–7344. https://doi.org/10.1007/s13277-015-4615-4.
33. Beck, B.H., Kim, H.-G., Kim, H., Samuel, S., Liu, Z., Shrestha, R., Haines, H., Zinn, K., and Lopez, R.D. (2010). Adoptively transferred ex vivo expanded γδ-T cells mediate in vivo antitumor activity in preclinical mouse models of breast cancer. Breast Cancer Res Treat *122*, 135–144. https://doi.org/10.1007/s10549-009-0527-6.
34. Xu, Y., Xiang, Z., Alnaggar, M., Kouakanou, L., Li, J., He, J., Yang, J., Hu, Y., Chen, Y., Lin, L., et al. (2021). Allogeneic Vγ9Vδ2 T-cell immunotherapy exhibits promising clinical safety and prolongs the survival of patients with late-stage lung or liver cancer. Cell Mol Immunol *18*, 427–439. https://doi.org/10.1038/s41423-020-0515-7.
35. Knight, A., Mackinnon, S., and Lowdell, M.W. (2012). Human Vdelta1 gamma-delta T cells exert potent specific cytotoxicity against primary multiple myeloma cells. Cytotherapy *14*, 1110–1118. https://doi.org/10.3109/14653249.2012.700766.
36. Wu, D., Wu, P., Wu, X., Ye, J., Wang, Z., Zhao, S., Ni, C., Hu, G., Xu, J., Han, Y., et al. (2015). *Ex vivo* expanded human circulating Vδ1 γδT cells exhibit favorable therapeutic potential for colon cancer. Oncoimmunology *4*, e992749. https://doi.org/10.4161/2162402X.2014.992749.
37. Siegers, G.M., and Lamb, L.S. (2014). Cytotoxic and regulatory properties of circulating Vδ1+ γδ t cells: A new player on the cell therapy field? Preprint at Nature Publishing Group, https://doi.org/10.1038/mt.2014.104 https://doi.org/10.1038/mt.2014.104.
38. Almeida, A.R., Correia, D. V., Fernandes-Platzgummer, A., Da Silva, C.L., Da Silva, M.G., Anjos, D.R., and Silva-Santos, B. (2016). Delta one T cells for immunotherapy of chronic lymphocytic leukemia: Clinical-grade expansion/differentiation and preclinical proof of concept. Clinical Cancer Research *22*, 5795–5804. https://doi.org/10.1158/1078-0432.CCR-16-0597.
39. Martínez, D.S., Tirado, N., Mensurado, S., Martínez-Moreno, A., Romecín, P., Agüera, F.G., Correia, D. V., Silva-Santos, B., and Menéndez, P. (2022). Generation and proof-of-concept for allogeneic CD123 CAR-Delta One T (DOT) cells in acute myeloid leukemia. J Immunother Cancer *10*. https://doi.org/10.1136/jitc-2022-005400.
40. Serrano, R., Coch, C., Peters, C., Hartmann, G., Wesch, D., and Kabelitz, D. (2021). Monocyte-dependent co-stimulation of cytokine induction in human γδ T cells by TLR8 RNA ligands. Sci Rep *11*. https://doi.org/10.1038/s41598-021-94428-6.
41. Ferry, G.M., Agbuduwe, C., Forrester, M., Dunlop, S., Chester, K., Fisher, J., Anderson, J., and Barisa, M. (2022). A Simple and Robust Single-Step Method for CAR-Vδ1 γδT Cell

Expansion and Transduction for Cancer Immunotherapy. Front Immunol *13*. https://doi.org/10.3389/fimmu.2022.863155.
42. Di Lorenzo, B., Simões, A.E., Caiado, F., Tieppo, P., Correia, D. V., Carvalho, T., Da Silva, M.G., Déchanet-Merville, J., Schumacher, T.N., Prinz, I., et al. (2019). Broad cytotoxic targeting of acute myeloid leukemia by polyclonal delta one T cells. Cancer Immunol Res *7*, 552–558. https://doi.org/10.1158/2326-6066.CIR-18-0647.
43. Legut, M., Cole, D.K., and Sewell, A.K. (2015). The promise of γδT cells and the γδT cell receptor for cancer immunotherapy. Cell Mol Immunol *12*, 656–658. https://doi.org/10.1038/cmi.2015.28.
44. von Strandmann, E.P., Shatnyeva, O., and Hansen, H.P. (2015). NKp30 and its ligands: Emerging players in tumor immune evasion from natural killer cells. Preprint at AME Publishing Company, https://doi.org/10.3978/j.issn.2305-5839.2015.09.08 https://doi.org/10.3978/j.issn.2305-5839.2015.09.08.
45. Ward, J., Bonaparte, M., Sacks, J., Guterman, J., Fogli, M., Mavilio, D., and Barker, E. (2007). HIV modulates the expression of ligands important in triggering natural killer cell cytotoxic responses on infected primary T-cell blasts. Blood *110*, 1207–1214. https://doi.org/10.1182/blood-2006-06-028175.
46. Eriksson, E.M., Keh, C.E., Deeks, S.G., Martin, J.N., Hecht, F.M., and Nixon, D.F. (2012). Differential Expression of CD96 Surface Molecule Represents CD8+ T Cells with Dissimilar Effector Function during HIV-1 Infection. PLoS One *7*. https://doi.org/10.1371/journal.pone.0051696.
47. Sidorenko, S.P., and Clark, E.A. (2003). The dual-function CD150 receptor subfamily: the viral attraction.
48. Davis, Z.B., Sowrirajan, B., Cogswell, A., Ward, J.P., Planelles, V., and Barker, E. (2017). CD155 on HIV-Infected Cells Is Not Modulated by HIV-1 Vpu and Nef but Synergizes with NKG2D Ligands to Trigger NK Cell Lysis of Autologous Primary HIV-Infected Cells. AIDS Res Hum Retroviruses *33*, 93–100. https://doi.org/10.1089/aid.2015.0375.
49. Desimio, M.G., Covino, D.A., and Doria, M. (2019). Potential of the NKG2D/NKG2DL axis in NK cell-mediated clearance of the HIV-1 reservoir. Preprint at MDPI AG, https://doi.org/10.3390/ijms20184490 https://doi.org/10.3390/ijms20184490.
50. Garrido, C., Spivak, A.M., Soriano-Sarabia, N., Checkley, M.A., Barker, E., Karn, J., Planelles, V., and Margolis, D.M. (2016). HIV latency-reversing agents have diverse effects on natural killer cell function. Front Immunol *7*. https://doi.org/10.3389/fimmu.2016.00356.
51. Grau-Expósito, J., Luque-Ballesteros, L., Navarro, J., Curran, A., Burgos, J., Ribera, E., Torrella, A., Planas, B., Badía, R., Martin-Castillo, M., et al. (2019). Latency reversal agents affect differently the latent reservoir present in distinct CD4+ t subpopulations. PLoS Pathog *15*. https://doi.org/10.1371/journal.ppat.1007991.
52. Fenwick, C., Joo, V., Jacquier, P., Noto, A., Banga, R., Perreau, M., and Pantaleo, G. (2019). T-cell exhaustion in HIV infection. Preprint at Blackwell Publishing Ltd, https://doi.org/10.1111/imr.12823 https://doi.org/10.1111/imr.12823.
53. Chen, J., Zhou, T., Zhang, Y., Luo, S., Chen, H., Chen, D., Li, C., and Li, W. (2022). The reservoir of latent HIV. Preprint at Frontiers Media S.A.,

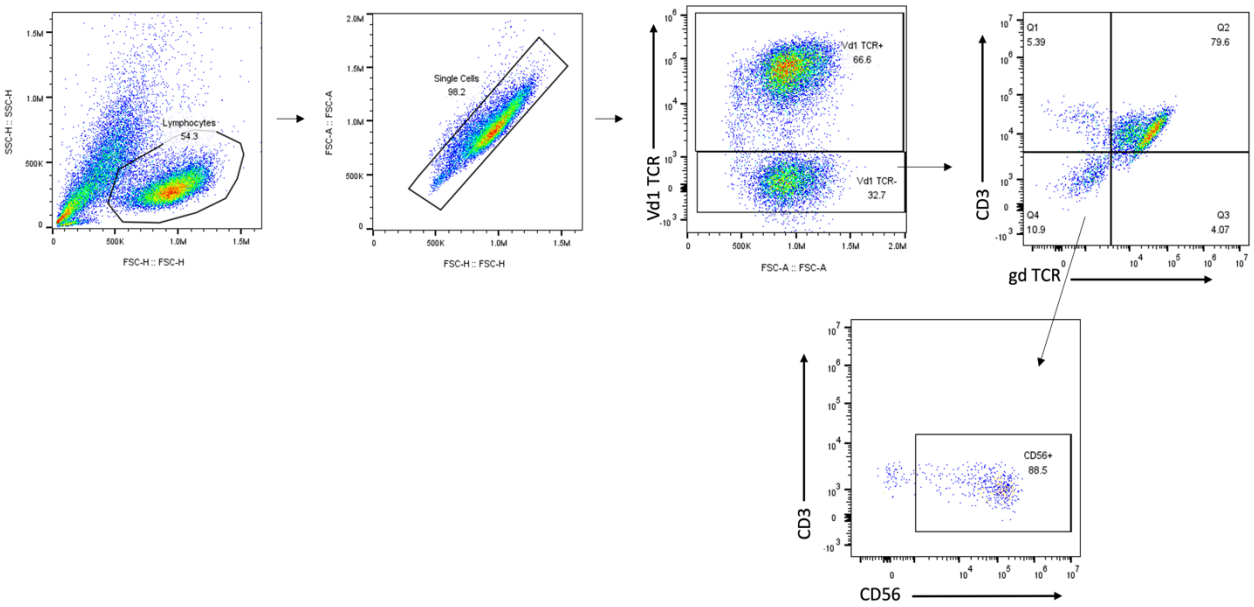
https://doi.org/10.3389/fcimb.2022.945956 https://doi.org/10.3389/fcimb.2022.945956.
54. Butera, S.T., Perez, V.L., Besansky, N.J., Folks, T.M., Chan, W.C., Wu, B.-Y., and Nabel, G.J. (1991). Extrachromosomal human immunodeficiency virus type-1 DNA can initiate a spreading infection of HL-60 cells. J Cell Biochem *45*, 366–373. https://doi.org/10.1002/jcb.240450410.
55. Perez, V.L., Rowe, T., Justement, J.S., Butera, S.T., June, C.H., and Folks, T.M. (1991). An HIV-1-infected T cell clone defective in IL-2 production and Ca2+ mobilization after CD3 stimulation. J Immunol *147*, 3145–3148.
56. Wu, Y., Biswas, D., Usaite, I., Angelova, M., Boeing, S., Karasaki, T., Veeriah, S., Czyzewska-Khan, J., Morton, C., Joseph, M., et al. (2022). A local human Vδ1 T cell population is associated with survival in nonsmall-cell lung cancer. Nat Cancer *3*, 696–709. https://doi.org/10.1038/s43018-022-00376-z.
57. Gao, Y., Yang, W., Pan, M., Scully, E., Girardi, M., Augenlicht, L.H., Craft, J., and Yin, Z. (2003). Gamma delta T cells provide an early source of interferon gamma in tumor immunity. J Exp Med *198*, 433–442. https://doi.org/10.1084/jem.20030584.
58. Woottum, M., Yan, S., Sayettat, S., Grinberg, S., Cathelin, D., Bekaddour, N., Herbeuval, J.-P., and Benichou, S. (2024). Macrophages: Key Cellular Players in HIV Infection and Pathogenesis. Viruses *16*, 288. https://doi.org/10.3390/v16020288.
59. Garg, H., Mohl, J., and Joshi, A. (2012). HIV-1 Induced Bystander Apoptosis. Viruses *4*, 3020–3043. https://doi.org/10.3390/v4113020.
60. Wu, D., Wu, P., Qiu, F., Wei, Q., and Huang, J. (2017). Human γδT-cell subsets and their involvement in tumor immunity. Preprint at Chinese Soc Immunology, https://doi.org/10.1038/cmi.2016.55 https://doi.org/10.1038/cmi.2016.55.
61. Desimio, M.G., Giuliani, E., and Doria, M. (2017). The histone deacetylase inhibitor SAHA simultaneously reactivates HIV-1 from latency and up-regulates NKG2D ligands sensitizing for natural killer cell cytotoxicity. Virology *510*, 9–21. https://doi.org/10.1016/j.virol.2017.06.033.
62. Huntington, N.D., Martinet, L., and Smyth, M.J. (2015). DNAM-1: would the real natural killer cell please stand up! Oncotarget *6*, 28537–28538.
63. Kabelitz, D. (2024). Delta One T cells recognize AML via DNAM-1. Blood *143*, 1434–1436. https://doi.org/10.1182/blood.2024024120.
64. Mensurado, S., Condeço, C., Sánchez-Martínez, D., Shirley, S., Coelho, R.M.L., Tirado, N., Vinyoles, M., Blanco-Domínguez, R., Barros, L., Galvão, B., et al. (2024). CD155/PVR determines acute myeloid leukemia targeting by Delta One T cells. Blood *143*, 1488–1495. https://doi.org/10.1182/blood.2023022992.
65. Bisio, F., Bozzano, F., Marras, F., Di Biagio, A., Moretta, L., and De Maria, A. (2013). Successfully treated HIV-infected patients have differential expression of NK cell receptors (NKp46 and NKp30) according to AIDS status at presentation. Immunol Lett *152*, 16–24. https://doi.org/10.1016/j.imlet.2013.03.003.
66. Correia, D. V., Fogli, M., Hudspeth, K., Gomes Da Silva, M., Mavilio, D., and Silva-Santos, B. (2011). Differentiation of human peripheral blood Vδ1+ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells. Blood *118*, 992–1001. https://doi.org/10.1182/blood-2011-02-339135.
67. Baragaño Raneros, A., Rodriguez, R.M., Bernardo Flórez, A., Palomo, P., Colado, E., Minguela, A., Suárez Álvarez, B., and López-Larrea, C. (2021). Bromodomain protein

BRD4 is an epigenetic activator of B7-H6 expression in acute myeloid leukemia. Oncoimmunology *10*. https://doi.org/10.1080/2162402X.2021.1897294.
68. Yang, S., Yuan, L., Wang, Y., Zhu, M., Wang, J., and Ke, X. (2020). B7-H6 promotes cell proliferation, migration and invasion of non-hodgkin lymphoma via ras/MEK/ERK pathway based on quantitative phosphoproteomics data. Onco Targets Ther *13*, 5795–5805. https://doi.org/10.2147/OTT.S257512.
69. Fernández Larrosa, P.N., Croci, D.O., Riva, D.A., Bibini, M., Luzzi, R., Saracco, M., Mersich, S.E., Rabinovich, G.A., and Peralta, L. (2008). Apoptosis resistance in HIV-1 persistently-infected cells is independent of active viral replication and involves modulation of the apoptotic mitochondrial pathway. Retrovirology *5*. https://doi.org/10.1186/1742-4690-5-19.
70. Delgado-Figarella, R., Rosario, G., Roche, M., Del Llano, A.M., and Lavergne, J.A. (2001). Cyclin levels during TNF-alpha-induced apoptosis in HIV-infected cells. Cell Mol Biol (Noisy-le-grand) *47*, 1129–1142.
71. Swanson, M.A., Lee, W.T., and Sanders, V.M. (2001). IFN-γ Production by Th1 Cells Generated from Naive CD4+ T Cells Exposed to Norepinephrine. The Journal of Immunology *166*, 232–240. https://doi.org/10.4049/jimmunol.166.1.232.
72. Bhat, P., Leggatt, G., Waterhouse, N., and Frazer, I.H. (2017). Interferon-γ derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. Cell Death Dis *8*, e2836–e2836. https://doi.org/10.1038/cddis.2017.67.
73. Donia, M., Ellebaek, E., Andersen, M.H., Straten, P.T., and Svane, I.M. (2012). Analysis of Vδ1 T cells in clinical grade melanoma-infiltrating lymphocytes. Oncoimmunology *1*, 1297–1304. https://doi.org/10.4161/onci.21659.
74. Kohli, K., Pillarisetty, V.G., and Kim, T.S. (2022). Key chemokines direct migration of immune cells in solid tumors. Preprint at Springer Nature, https://doi.org/10.1038/s41417-021-00303-x https://doi.org/10.1038/s41417-021-00303-x.
75. Foley, J.F., Yu, C.-R., Solow, R., Yacobucci, M., Peden, K.W.C., and Farber, J.M. (2005). Roles for CXC Chemokine Ligands 10 and 11 in Recruiting CD4+ T Cells to HIV-1-Infected Monocyte-Derived Macrophages, Dendritic Cells, and Lymph Nodes. The Journal of Immunology *174*, 4892–4900. https://doi.org/10.4049/jimmunol.174.8.4892.
76. Simmons, R.P., Scully, E.P., Groden, E.E., Arnold, K.B., Chang, J.J., Lane, K., Lifson, J., Rosenberg, E., Lauffenburger, D.A., and Altfeld, M. (2013). HIV-1 infection induces strong production of IP-10 through TLR7/9-dependent pathways. AIDS *27*, 2505–2517. https://doi.org/10.1097/01.aids.0000432455.06476.bc.
77. Koay, W.L.A., Siems, L. V., and Persaud, D. (2018). The microbiome and HIV persistence: Implications for viral remission and cure. Preprint at Lippincott Williams and Wilkins, https://doi.org/10.1097/COH.0000000000000434 https://doi.org/10.1097/COH.0000000000000434.
78. Takeda A Study of GDX012 in Adults With Relapsed or Refractory Acute Myeloid Leukemia. https://classic.clinicaltrials.gov/ct2/show/NCT05886491.
79. Patterson, W., Rosenthal, M., and Rajulu, D.T. (2023). Brief Report: Trends in HIV Mortality in the Context of the COVID-19 Pandemic. JAIDS Journal of Acquired Immune Deficiency Syndromes *94*, 91–94. https://doi.org/10.1097/QAI.0000000000003228.

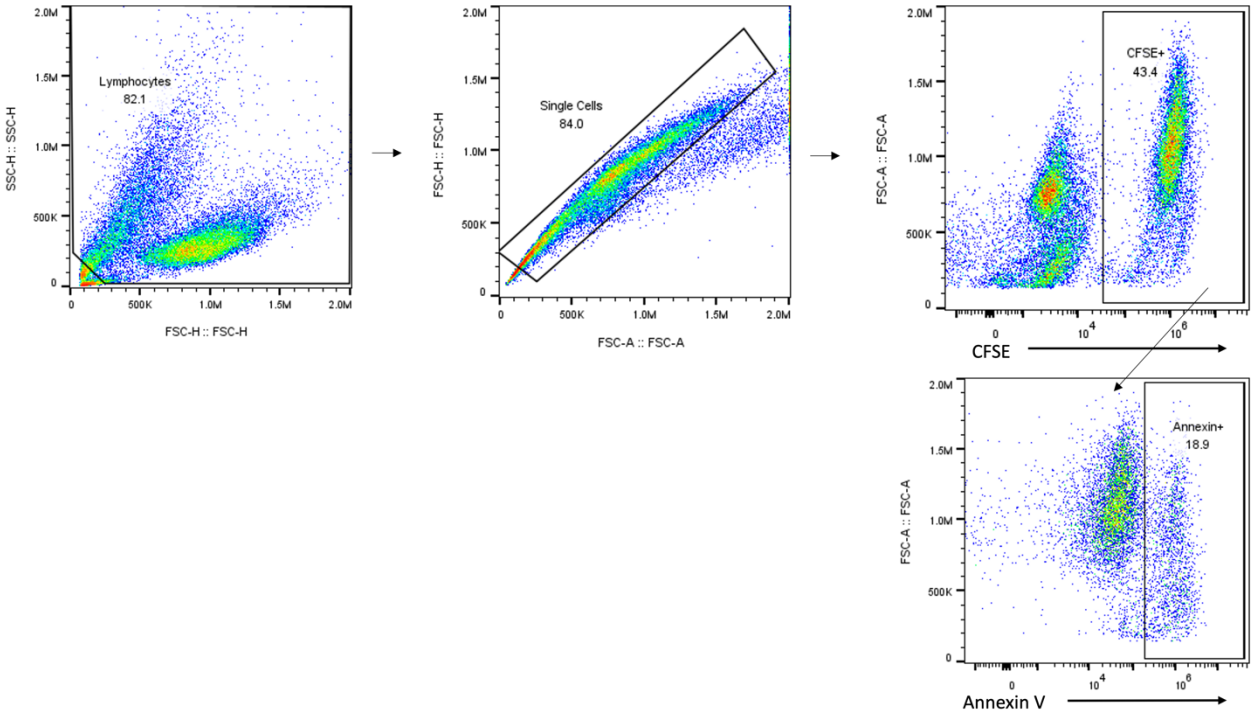
## Supplementary data



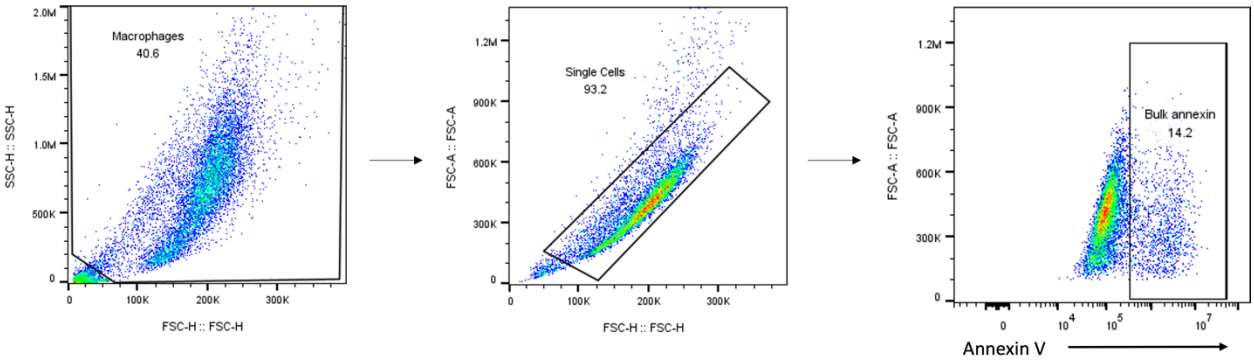

**Supplementary Figure 1. Gating strategy for verification of Vd1 T cell population size following DOT cell expansion.**
Lymphocytes were first gated on prior to doublet exclusion. Within the single cell population, the proportion of cells positive for Vd1 T cell receptor was observed. Donor 262 is depicted in this example gating strategy.



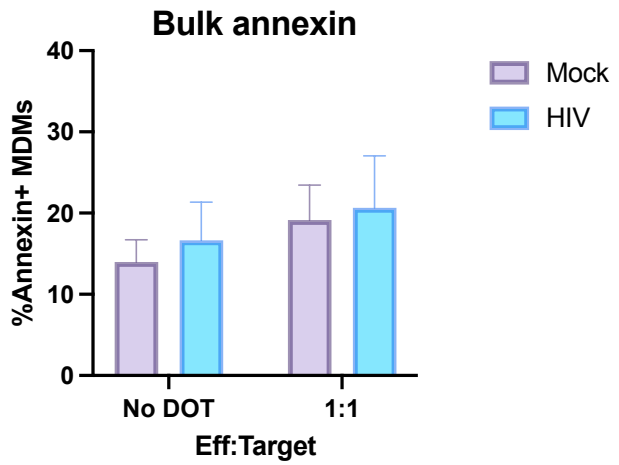

**Supplementary Figure 2. Gating strategy for characterization of the Vd1 TCR negative population of DOT cells.**
Lymphocytes were gated on prior to exclusion of doublets. Within the single cell population, the proportion of cells negative for Vd1 T cell receptor was further characterized by gating on CD3 and gamma delta T cell receptor expression. Within the CD3 negative gamma delta T cell receptor negative population the expression of CD56 was further gated on. Donor 289 is depicted in this example gating strategy.



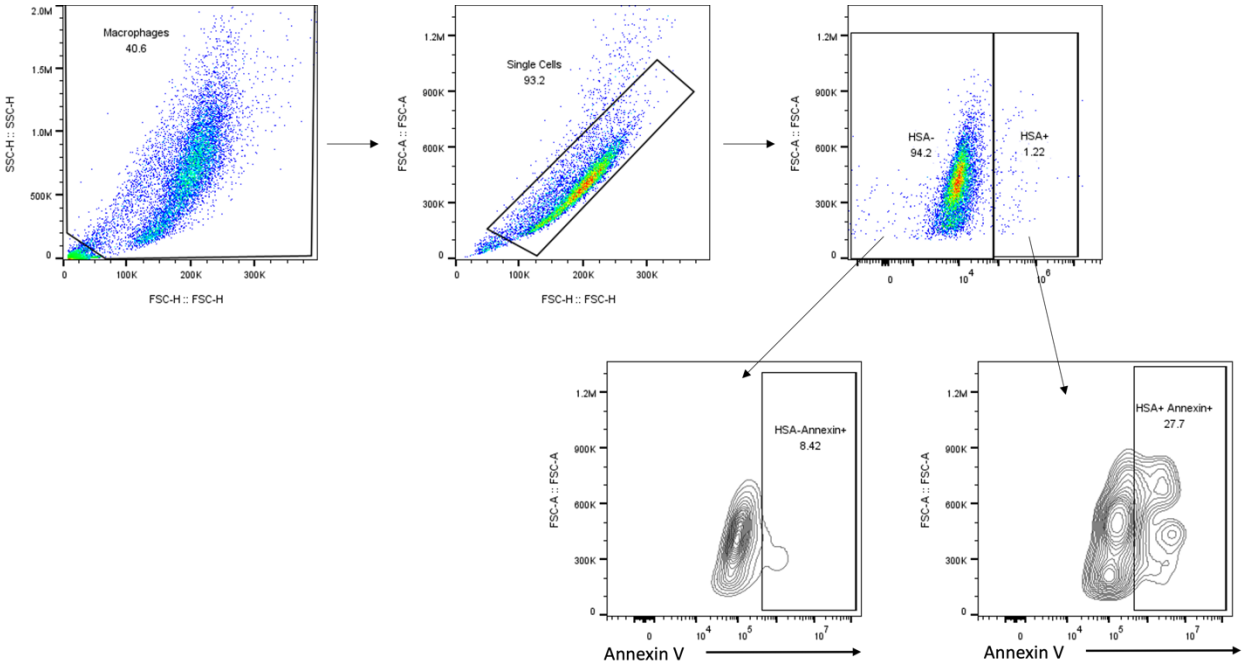

**Supplementary Figure 3. Gating strategy for analysis of annexin expression of target cell lines following DOT cell co-culture.**
Debris was excluded prior to excluding doublets. Within the single cell population, cells expressing CFSE were gated on and annexin expression was determined within this population. Jurkats co-cultured with DOT cells from donor 289 at a 1:1 E:T ratio for 3 hours is depicted in this gating strategy,




**Supplementary Figure 4. Gating strategy for analysis of annexin expression on monocyte-derived macrophages following DOT cell co-culture.**
Debris was excluded prior to excluding doublets. Within the single cell population, annexin expression was determined. HIV-infected MDMs at 1:1 E:T ratio is depicted in this example gating strategy.




**Supplementary Figure 5. DOT cells do not result in non-specific death of uninfected bystander cells.**
Annexin V was measured on bulk monocyte-derived macrophages following a 24-hour co-culture with donor 262 DOT cells by flow cytometry. (n=1). Data represents the mean +/- the SEM of four technical replicates.




**Supplementary Figure 6. Gating strategy for analysis of annexin expression within HIV-infected and uninfected populations.**
Debris was excluded prior to excluding doublets. Within the single cell population, expression of the HSA reporter protein was further gated on, and annexin expression in the HSA positive and HSA negative populations were determined. HIV-infected MDMs at 1:1 E:T ratio is depicted in this example gating strategy.