Prevention of HIV-1 acquisition and determinants of disease progression

by

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the

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Preface

The study described in this thesis was carried out at the Africa Health Research Institute (AHRI) and at the HIV Pathogenesis Programme (HPP), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal in Durban in South Africa between June 2016, and February 2021 under the supervision of Dr Jaclyn K Mann, Dr Alex Sigal and Prof Thumbi Ndung'u.

The study described in this thesis is original work done and reported by the author. The study has not been used in any form, by any person or submitted to any tertiary institution for award of a degree or diploma. Some of the work has been published in accredited journals in line with the thesis guidelines of UKZN. Due acknowledgements have been accorded where other people's work has been used in the text.

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Publications

The publications (published, in print and/or submitted) that constitute this thesis are presented here.

Publication 1

Moyano A, Lustig G, Rodel HE, Antal T, Sigal A (2020) Interference with HIV infection of the first cell is essential for viral clearance at sub-optimal levels of drug inhibition. PLoS Comput Biol 16(2): e1007482. https://doi.org/10.1371/journal.pcbi.1007482

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Moyano A, Lustig G, Rodel HE, Antal T, Sigal A. Interference with HIV infection of the first cell is essential for viral clearance at sub-optimal levels of drug inhibition. PLoS Comput Biol. 2020 Feb 4;16(2):e1007482. doi: 10.1371/journal.pcbi.1007482. PMID: 32017770; PMCID: PMC7039526.

List of acronyms

HIV-1	Human Immunodeficiency Virus type 1
ARVs	Antiretroviral regimens
PrEP	Pre-Exposure Prophylaxis
PEP	Post-Exposure Prophylaxis
AIDS	Acquire Immunodeficiency Syndrome
RT	Reverse Transcriptase
LTR	Long Terminal Repeats
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
RTIs	Reverse Transcriptase Inhibitors
PIs	Protease Inhibitors
ART	Antiretroviral Therapy
CTLs	Cytotoxic T lymphocytes
RP	Rapid Progressors
NP	Non-progressors
VL	Viral Load
HLA	Human Leukocyte Antigen
HLA-I	Human Leukocyte Antigen class I
TFV	Tenofovir
ATV	Atazanavir
NK	Natural Killer
SIV	Simian Immunodeficiency Virus
N_0	Initial number of infected cells
R_0	Basic reproductive ratio
RevCEM	Rev dependent indicator CEM T cell line
GFP	Green Fluorescence Protein
P_c	Probability of clearance
PBMCs	Peripheral Blood Mononuclear Cells
P_{lat}	Probability of latency
LTNP	Long-term Non-progressors
LTS	Long-term Survivors
ES	Elite Suppressors
HIC	HIV-1 Controllers

EC	Elite Controllers
VC	Viraemic Controllers
NC	Non-controllers
CP	Chronic Progressors
KIRs	Killer Inhibitory Receptors
CDK	Cyclin Dependent Kinase
IFN-γ	Interferon gamma
DCs	Dendritic Cells
TLR	Toll Like Receptors
ADCC	Antibody-dependent Cellular Cytotoxicity
IL	Interleukin
ISG	Interferon-stimulated genes
VC+	Viraemic Controllers with protective HLA-I alleles
VC-	Viraemic Controllers without protective HLA-I alleles
MIP	Macrophage Inflammatory Protein
TNF-α	Tumour Necrosis Factor Alpha
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
FRESH	Females Rising with Education, Support and Health
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
ELISpot	Enzyme-Linked Immunosorbent Spot assay
OLPs	Overlapping peptides
PBS	Phosphate Buffered Saline
FBS	Foetal Bovine Serum
PHA	Phytohemagglutinin
SFU	Spot Forming Unit
RT	Room Temperature
NCR	Natural Cytotoxicity Receptors
ICS	Intracellular Cytokine Stimulation
UI	Uninfected Individuals
APCs	Antigen Presenting Cells
FACS	Fluorescence-activated Cell Sorting

ABSTRACT

Introduction

HIV-1 infection can be managed using multiple strategies, including preventative approaches and therapeutic approaches. Current preventative and treatment strategies are suboptimal and there is a need to develop an effective prophylactic or therapeutic vaccine and to improve the public health approaches against the virus. This requires more detailed understanding of the infection, from prevention to natural disease progression. We performed several studies that cover a range of infection attributes, from understanding the mechanism of action of pre-exposure prophylaxis (PrEP) and determining the effectiveness of different compounds in blocking initial infection, to gaining further insight into potential mechanisms of natural control of HIV-1 disease progression in viraemic controllers (VC) with (VC+) and without (VC-) protective class I human leukocyte antigen (HLA-I) alleles. In order to cover this range of infection attributes we investigated two hypotheses: (i) initial low dose infection can be cleared with sub-optimal drug inhibition, which allows ongoing viral replication, as long as the drug mechanism acts before the first cell is infected; and (ii) individuals without protective HLA-I alleles have CD8+ T cell-independent mechanisms of control.

Methods

To understand the mechanism of action of PrEP, the probability of extinction of new infections in the presence of two drug mechanisms (interference of initial infection with tenofovir (TFV), or reduction of burst size with atazanavir (ATV)), or with no drug, was modelled as a function of initial infected cells and viral replication ratio. The fraction of extinguished infections was experimentally determined with low viral input in the presence of either drug, or with no drug, in an *in vitro* model of PrEP.

To gain insight into potential mechanisms of control, we studied immune cells in 12 VC+ and 9 VC- and, compared these 21 controllers with 5 rapid progressors (RP). Measurements included the magnitude and breadth of CTL responses using the ELISpot assay, as well as flow cytometry-based characterization of NK cell and T cell populations, which included the measurement of surface markers for activation, maturation, and exhaustion on these populations. Further, NK cell function was measured by intracellular cytokine staining following stimulation of these cells.

Results

Our study showed that TFV dramatically increased clearance while ATV did not, both for our mathematical model and our experimental study.

We observed that both VC, in particular VC-, had a higher contribution of Gag CTL responses to the total CTL response than RP (p=0.04), however there was no significant difference in the magnitude and breadth of CTL responses between VC+ and VC-. In addition, VC- NK cells had higher levels of the activation markers HLA-DR (p=0.007) and co-expression of CD38 and HLA-DR (p=0.03) when compared to VC+ and uninfected individuals (UI), and lower cytokine expression (MIP-1β and TNF-α) than VC+ NK cells (p=0.05 and p=0.04, respectively). We found a negative correlation between the expression of MIP-1β and the co-expression of CD38 and HLA-DR (r =-0.45, p=0.05). Furthermore, VC- T cells had higher levels of CD38 and HLA-DR co-expression (p=0.05), and a trend of higher HLA-DR (p=0.07) as well as CD57 expression (p=0.09) when compared to VC+.

Conclusions

The ability of drugs to clear initial but not established infection depends only on the ability to target initial infection. This implies that in diseases which involve transmission of low pathogen numbers upon exposure, but have robust replication when established, such as HIV-1, a possibility to clear infection should exist even with relatively weak inhibition as long as the drug has the mechanism of targeting the initial infection. This finding is particularly relevant in scenarios of variable adherence that result in sub-optimal drug levels or possible future PrEP strategies with drugs that have long half-lives yet do not completely suppress viral replication.

VC have a more Gag focused CTL response than RP, however this feature did not distinguish VC+ from VC-. NK and T cell profiles differ between VC+ and VC-. VC- have a more activated NK cell profile with lower cytokine expression, and a more active and terminally differentiated T cell profile than VC+. A possible explanation for our results is that the increased CD38+HLA-DR+ NK cells in VC- may represent NK cells acting as antigen presenting cells (APCs), which may then directly interact with a more activated and terminally differentiated population of T cells observed in VC-. Further work to test this hypothesis is necessary to better understand the mechanisms underlying control in these two groups of VC patients. It is also suggested that transcriptomic studies may contribute further to understanding the distinct NK and T cell profiles observed between VC+ and VC- and how these may result in differing mechanisms of control.

CHAPTER 1

Introduction and literature review

1.1 Problem statement

HIV-1 remains a global epidemic with approximately 39.6 million people living with HIV-1 worldwide (1). There are multiple strategies employed to manage the infection, including preventative approaches, which involve, for example, sexual education, circumcision programs, and clean needle programs (preventing parenteral transmission). Alternatively, antiretroviral regimens (ARVs) may be used to prevent establishment of infection in the form of pre-exposure prophylaxis (PrEP – for preventing transmission during contact with an HIV-1 infected partner), post-exposure prophylaxis (PEP – after a high-risk encounter between an uninfected individual and an HIV-1 positive individual), or treatment of infected mothers (preventing vertical transmission). ARVs may also be used to control disease progression in individuals with established infection. Despite having multiple strategies for preventing infection or controlling disease progression, we are faced with several limitations, including implementation struggles for prevention strategies (barriers accessing ARVs or related services) and difficulties with adherence to ARVs (for example, stigma, side effects, treatment fatigue, distance to clinics, appointment waiting times, and scarce supplies of ARVs) (2). The ideal goal is to develop a prophylactic or therapeutic vaccine, which will reduce side effects of ARVs, allow independence of access to treatment (or prevention possibilities), and be discrete enough to reduce stigma, thus changing the global HIV-1 landscape. Currently two vaccine efficacy trials are underway in southern Africa to test the safety and efficacy of different approaches. In parallel, the Antibody Mediated Prevention programme is testing passive immunisation for prevention and the Chinese Centre for Disease Control and Prevention team are also testing efficacy of different vaccine products. Despite these promising immunisation candidates, there are multiple challenges in late-stage development (such as manufacturing scale-up, regulatory requirements or determination of public health benefit against the cost) that will need to be addressed (3). Our project attempts to contribute to these areas by understanding the mechanisms of action of PrEP and factors influencing PrEP efficacy as well as the natural mechanisms of control of HIV-1 disease progression, as this knowledge will be important for developing more effective strategies to combat the HIV-1 epidemic.

1.2 Background

1.2.1 Structure, genome, life cycle

The Human Immunodeficiency Virus type 1 (HIV-1) is a retrovirus that causes acquired immunodeficiency syndrome (AIDS). Each virion is composed of two positive sense single stranded RNAs, protected by a conical capsid formed by 1200-2500 copies of the p24 viral protein (Figure 1). Enclosed within this capsid are the enzymes required for the development of a new virion, such as reverse transcriptase (RT), proteases

and integrases. The capsid is surrounded by the viral envelope, that consists of an Env-glycoprotein complex including the gp160 spike (gp41 and gp120) (4).

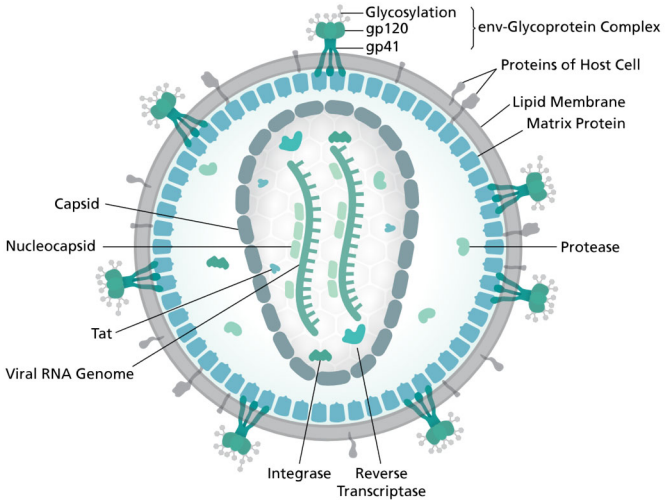

Fig. 1. HIV-1 virion structure.
HIV-1 structural components (outer envelope, matrix, capsid and nucleocapsid in association with the HIV-1 RNA genome) as well as the viral enzymes (integrase, reverse transcriptase, and protease) are shown. The figure was taken from https://commons.wikimedia.org/wiki/File:HI-virion-structure_en.svg.

The HIV-1 genome encodes for nine genes: *gag, pol, env, tat, rev, vpu, nef, vpr* and *vif* (Figure 2). It is flanked by long terminal repeats (LTR). The *gag, pol* and *env* genes encode for the main structural proteins; In particular, *gag* encodes for the matrix, the capsid (p24), the nucleocapsid and p6; *pol* encodes for the viral protease, RT and integrase; and *env* encodes for the glycoprotein gp160, a precursor of gp120 and gp41. The *tat, rev, vpu, nef, vpr* and *vif* genes are regulatory and accessory genes that code proteins which control the ability of HIV-1 to infect a cell, produce new copies of the virus, or induce pathogenesis (4).

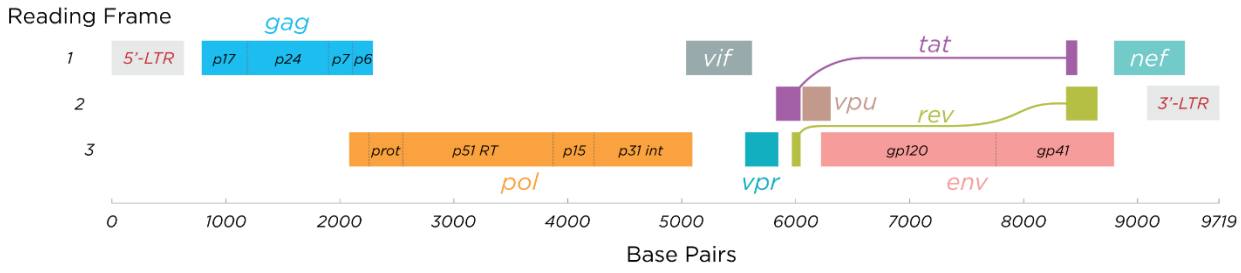

Fig. 2. HIV-1 genome structure.
HIV-1 genome consists of nine genes (*gag, pol, env, tat, rev, vpu, nef, vpr* and *vif*). It is flanked by long terminal repeats (LTR). The *gag, pol* and *env* genes encode for the main structural proteins; while *tat, rev, vpu, nef, vpr* and *vif* genes are regulatory and accessory genes. The figure was taken from https://commons.wikimedia.org/wiki/File:HIV-genome.png.

The HIV-1 replication cycle starts with the entry of HIV-1 into the cell. This viral entry is characterized by the binding of HIV-1 envelope gp120 to the CD4 receptor on the target cell membrane (the primary target for HIV-1 are CD4+ T cells). This interaction induces a conformational change in the envelope protein that allows the additional binding of the virion to chemokine co-receptors on the surface of the host cell, which determines the tropism of the virus (4). Macrophage tropic (M-tropic) strains of HIV-1, called R5 viruses, use the CCR5 chemokine co-receptor, whereas T cell tropic (T tropic) strains of HIV-1, called X4 viruses, bind to CXCR4. R5 viruses are the most common strain sexually transmitted, while X4 viruses are normally found during late stages of disease (5). Additionally, HIV-1 can enter the host cell via a pH-independent endocytosis (Figure 3) (6).

Once the virion is stably bind to the host membrane, HIV-1 gp41 then mediates the fusion with the cellular membrane, which allows uncoating of the viral core, releasing the viral RNA genome and the viral proteins into the host cell. Once the viral genome is inside the host cell, a process of reverse transcription takes place. The RT contains RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity and RNase H activity. RT reverse transcribes a single positive viral RNA genome into a double stranded DNA copy. The RNase H is used to remove the original RNA viral template from the first DNA strand (7). This DNA is transported into the nucleus with the integrase and Vpr, where it integrates into the host DNA. The integrated viral copy DNA, known as proviral DNA, can lead to transcriptionally active or latent forms of infection, which is going to depend on the level of activation of the host cell. Whether the proviral DNA leads to a transcriptionally active or a latent form is going to depend on host factors, mainly NF-κβ, a family of host proteins that regulate the transcription of cellular genes involved in immune recognition and activation (4). If the host cell is activated, following integration, the cellular machinery will initiate transcription. Some of the transcribed RNA created undergoes splicing to produce messenger RNA (mRNA). The regulatory protein Rev will facilitate the export of unspliced and incompletely spliced transcribed viral RNA from the nucleus to the cytoplasm (8). The mRNA is transported into the cytoplasm where is translated into proteins. Some of the full-length transcribed viral RNAs function as new copies of the viral genome, while multiply spliced transcripts will encode for Nef, Tat and Rev. Other singly spliced or unspliced viral transcripts encode multiple polyproteins, such as gp160, Gag and Gag-Pol polyproteins (9). The cleavage products of gp16, gp120 and gp41, are transported to the plasma membrane of the infected cell. The Gag and Gag-Pol polyproteins also become associated with the plasma membrane of the infected cell, and together with the HIV-1 transcribed RNA and Vpr form the immature virion that begins the budding process (Figure 3). The immature virion needs further cleave of the Gag and Gag-Pol polyproteins, this cleavage is mediated by the HIV-1 protease. Cleavage of Gag results in the capsid (p24) and

nucleocapsid, while cleavage of the Gag-Pol precursor results in the production of protease, integrase, and RT (4).

On the other hand, if the host cell is not active, the proviral DNA will enter a so-called latent state. Viral latency is a state of reversibly non-productive infection, where there is a stable integration of reverse transcribed viral cDNA into the host cell genome. This state allows HIV-1 to persist in the host cell while being transcriptionally silent for long periods of time (10). This latent state of HIV-1 infection results in the most significant barrier to eradicate HIV-1 infection, since the immune system, and ARVs, are unable to eliminate this long-lived, latently infected cells (11). These cells can be re-activated and re-establish infection at any time and proceed with the transcription and finish the viral replication cycle.

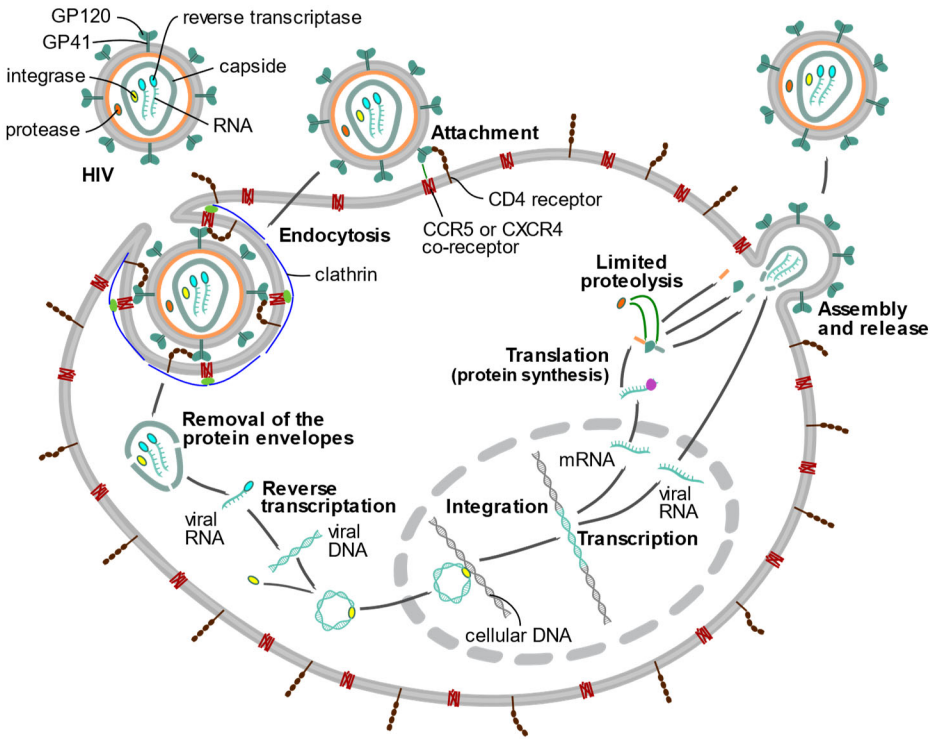

Fig. 3. HIV-1 life cycle.
HIV-1 enters a cell through either endocytosis or binding of Env to host receptors and subsequent membrane fusion. The HIV-1 capsid is released into the cell, viral RNA is reverse transcribed into viral DNA. Viral DNA is integrated into the host genome and following transcription, new proteins and viral RNA are produced and assembled into new virions, that exit the cell via budding. The figure was taken from https://commons.wikimedia.org/wiki/File:HIV-replication-cycle-en.svg.

1.2.2 Treatment and prevention

1.2.2.1 Antiretroviral drugs

ARVs interact with the HIV-1 replication cycle at different stages, ultimately blocking HIV-1 from infecting new cells. There are six classes based on their molecular mechanism and resistance profiles: 1) nucleoside-analogue reverse transcriptase inhibitors (NRTIs), 2) non-nucleoside reverse transcriptase inhibitors (NNRTIs), 3) integrase inhibitors, 4) protease inhibitors (PIs), 5) fusion inhibitors, and 6) co-receptor antagonist. NRTIs and NNRTIs target the RT of the virus, both affect the DNA polymerization activity of the enzyme and block the generation of full-length viral DNA. Integrase inhibitors block the next step on the HIV-1 life cycle, specifically they inhibit strand transfer and block integration of HIV-1 DNA into the cellular DNA. PIs block proteolysis of the viral polyprotein, which is required for virion maturation; PIs are amongst the most potent agents developed to date but given their large size they require the coadministration of a "boosting" agent to inhibit their metabolism and enhance drug levels, the only boosting agent available is ritonavir. And lastly, fusion inhibitors and co-receptor antagonists target the first step of the viral cycle, the attachment and fusion of the viral membrane with the host membrane (Figure 4) (12).

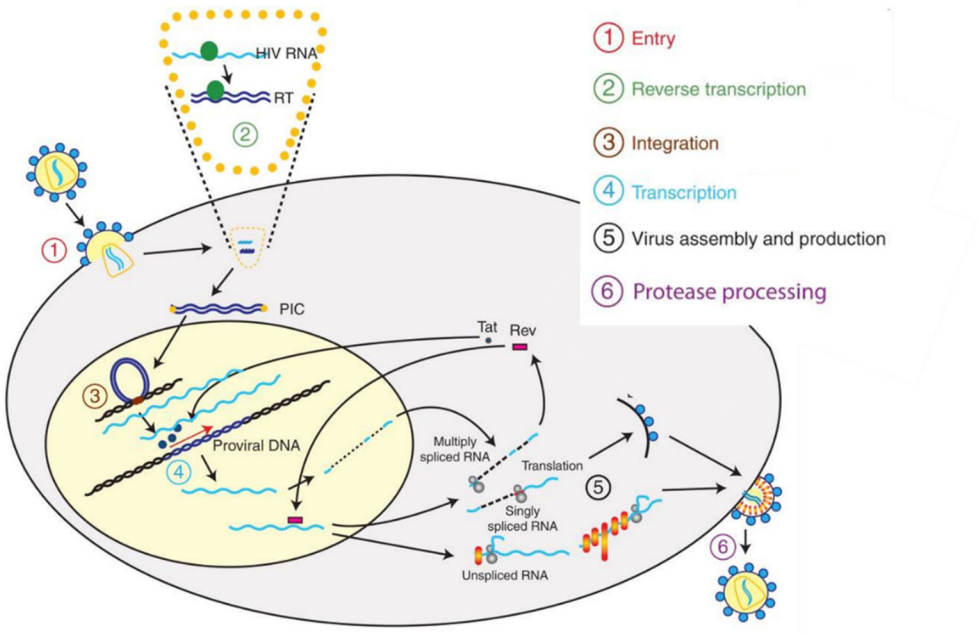

Fig. 4. HIV-1 life cycle and targets of antiretroviral drugs.
Schematic of the HIV-1 life cycle showing the six steps of the viral cycle. Fusin inhibitors and co-receptor antagonist interact with step 1, viral entry; RTIs block step 2 of the HIV-1 cycle, reverse transcription; integrase inhibitors block step 3 of the cycle and PIs act in the last step of the cycle, protease processing. The figure was modified from *HIV-1 Antiretroviral Drug Therapy (12)*.

The HIV-1 replication cycle can be broadly divided into two stages: pre-integration and post-integration. ARVs such as entry inhibitors (co-receptor or receptor antagonists (CD4 binding, CCR5 binding) and fusion inhibitors) and reverse transcriptase inhibitors (NRTIs/NNRTIs) act before the viral RNA is integrated into the host genome (pre-integration). On the other hand, integrase inhibitors, transcriptase inhibitors and protease inhibitors act once the viral genome is already integrated into the host genome (post-integration) (4, 12).

It must be noted that despite the diverse range of ARVs discovered, not all of them have been approved for use in humans. Current approved ART regimes for adults include: a first-line ART regime compose of two NRTIs plus a NNRTI or an integrase inhibitor; second-line ART regime consisting of two NRTIs plus a ritonavir-boosted PI; and third-line regimens should include new drugs with minimal risk of cross-resistance to previously used regimens, such as integrase inhibitors and second generation NNRTIs and PIs (13).

1.2.2.2 Prevention methods

Prevention of HIV-1 acquisition is promoted by a combination of interventions that modifies sexual behaviours, in addition to biomedical interventions. These include the use of condoms, antiretroviral therapy (ART) in infected individuals to reduce the viral load to untransmissible levels, voluntary medical male circumcision, access to clean needles, treatment of infected mothers to prevent vertical transmission, PrEP, and PEP (2, 14, 15).

PrEP is a prevention tool, based on the administration of ARVs prior to the exposure to HIV-1. Clinical trials have investigated if the administration of ARVs, present in the standard regimens used to treat HIV-1 infected individuals, to uninfected individuals prior to HIV-1 exposure will prevent the acquisition of infection (16-30). These trials have tested different combinations of RTIs (tenofovir, emtricitabine, dapivirine), different mechanisms of delivery (oral pills, preventive vaccine, vaginal gel, vaginal rings) and different study populations (men who have sex with men, transgender women, people who inject drugs, sero-discordant couples, heterosexual men, and women), in order to assess all the possible range of side effects, interaction with other drugs, and adherence, amongst other factors. Results of these trials show that administration of PrEP prevents HIV-1 acquisition in up to 86% of cases. By reducing the ARVs employed in PrEP to only one or two compounds the side effects and potential drug interaction is reduced, making it more amenable for use in uninfected individuals.

Current guidelines stablish that PrEP may be taken orally, using an ARV containing Tenofovir and Emtricitabine, or topically as a vaginal gel containing Tenofovir (31). One key limitation of this approach

is the delivery of the ARVs, and the need of taking a pill every day in order to prevent HIV-1 infection is a major issue, especially in settings where accessing to clinics is complicated and stigma around HIV-1 and ARVs is present. A way around this issue is the development of an effective vaccine. The challenges of developing an effective vaccine against HIV-1 are numerous. The vaccine must be able to elicit an immune response effective in clearing the infection within the first few days before the virus establishes a latent reservoir. In addition, it needs to be effective against the wide diversity of HIV-1 strains (32). Currently two vaccine efficacy trials are underway in southern Africa (HVTN705/706 and PrEPVacc), testing the safety and efficacy of different approaches. In parallel the Antibody Mediated Prevention programme is testing passive infusion of antibodies (HVTN703/HPTN081-HVTN704/HPTN085), where the goal is to use broad and potent antibodies, alone or in combination, as a prevention method. In addition, the Chinese Centre for Disease Control and Prevention team are testing efficacy of different vaccine products (ChiaCTRPRC-10001287; NCT01705223; ChiCTR1900021442). Despite these promising vaccine candidates, multiple challenges regarding the roll-out will need to be addressed once we find an effective and safe vaccine, such as public health benefit-costs, manufacturing scale-up, regulatory requirements, amongst others (3).

1.2.3 Disease progression

1.2.3.1 Natural disease course

The typical course of infection is illustrated in Figure 5 (grey lines) and reviewed in Munier et al. (2007) and Gatell et al. (2015) (4, 33). Briefly, following HIV-1 infection, there is a massive viral replication with the virus typically peaking at $>10^6$ RNA copies/ml in the blood followed by a reduction in the number of CD4+ T cell lymphocytes. This is known as the acute phase of infection. This initial replication allows a quick viral dissemination throughout the body to different organs and tissues. At approximately 2-3 weeks post-infection, a strong cytotoxic T cell response (which precedes the development of HIV-1-specific antibodies) is associated with a pronounced drop in viral RNA levels (to a level known as the viral load set point) and a partial reestablishment in CD4+ T cell counts. This is the beginning of the asymptomatic phase, that will last until the development of AIDS (4). During the asymptomatic phase, viral replication is ongoing, but the individual maintains an immune response that partially controls the infection. Despite this control, CD4+ T-cell lymphocytes decrease gradually over time at an approximate rate of 25-60 cells/µl per year eventually triggering the development of AIDS, typically in approximately 8-10 years. During the phase of AIDS, the disease is characterized by the emergence of clinical symptoms, CD4+ T-cell lymphocytes <300cells/µl, reduction of the HIV-specific CD8+ cytotoxic T lymphocytes (CTLs), and an increase in the levels of viral RNA in plasma (4, 33).

1.2.3.2 Heterogeneity in disease progression rate

In 80-85% of HIV-1-infected individuals the asymptomatic phase lasts between 8-10 years, however the course of infection varies greatly between individuals, with rapid progressors (RPs) and non-progressors (NPs) at the extremes of the spectrum (Figure 5) (34).

Individuals that display a faster decrease in CD4+ T-cell lymphocytes with development of AIDS at 2-5 years post-infection, as well as a high viral load set point following acute infection are classified as RPs, who constitute approximately 10% of infected individuals (Figure 5, blue lines). RPs are described as patients with (i) two or more CD4+ T cell measurements <350/mm^3 within 3 years after seroconversion, with no value ≥350/ mm^3 thereafter, in the absence of ART, and/or (ii) ART initiated within 3 years of seroconversion and at least one preceding CD4 <350/ mm^3, and/or (iii) AIDS or AIDS-related death within 3 years of seroconversion and at least one preceding CD4 <350/ mm^3 (34-36).

On the other side of the spectrum, NPs are a subset of 5-10% of infected individuals, in whom levels of CD4+ T-cell lymphocytes are maintained over time (longer than 10 years) and viral load set point is low in the absence of ARVs (34) (Figure 5, green lines). NPs can be further classified according to clinical and/or diagnostic criteria, years of follow-up and viral load (VL) quantification (see Chapter 3).

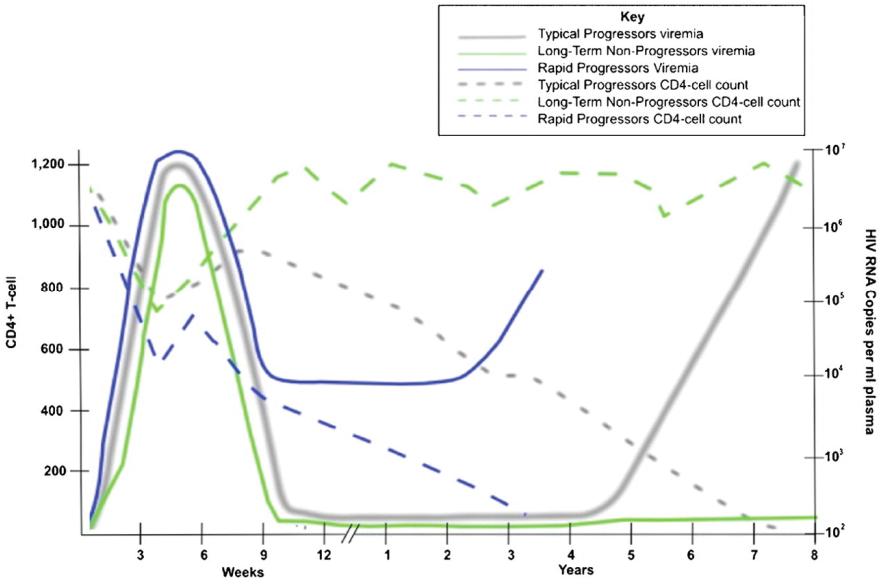

Fig. 5. Disease progression in HIV-1 infected individuals.
Schematic of HIV-1 disease progression in typical progressors (grey), long-term non-progressors (green) and rapid progressors (blue). Levels of viremia are represented with a normal line, and CD4+ T-cell counts with a dashed line. The figure was taken from *Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression* (37).

1.2.3.3 Overview of factors influencing disease progression rate

The difference in HIV-1 disease progression rate between individuals may be explained by a complex relationship between virologic, immunologic and genetic factors. Infection with an attenuated virus may explain control in a minority of controllers – some NP are infected with viruses that have attenuated Env function or mutations in Nef, Vpr, Vif or Rev (37, 38). Host genetics may also play a role. The most well-known host genetic factors associated with altered HIV-1 disease progression are mutations in the chemokine receptor gene CCR5 and mutations in the CXCR4 receptor's main ligand (SDF-1), used by HIV-1 as co-receptors to enter the cell (37, 39). For example, the best characterized co-receptor mutation influencing disease progression is a deletion of 32 base pairs in the CCR5 coreceptor gene (CCR5-Δ32) – individuals that are homozygous for this mutation are resistant to infection by virus with R5 tropism (37).

HLA class I (HLA-I) is the most significant host genetic determinant of clinical outcome in HIV-1 infection (40). The expression of "protective" HLA-I alleles, such as HLA-B*27, HLA-B*57, HLA-B*58:01, HLA-B*81:01, HLA-A*74, is associated with low viral loads and slower progression to AIDS (40-48). HLA-I molecules present viral peptides to HIV-specific CD8+ CTLs, and a major mechanism by which these individuals control infection is thought to be through CTL activity (34, 37, 49, 50). Furthermore, specific Gag CD8+ T cell responses are associated with the control of viral replication (51-53). However, a study done in viraemic controllers with and without protective HLA-I alleles showed that escape mutations within the Gag epitope, or loss of breadth in Gag CTL responses with or without associated escape, can lead to loss of viraemic control by individuals possessing these protective alleles (54). This indicates that control in individuals with protective alleles is associated with the ability of their CD8+ T cells to control viral replication and loss of control is often precipitated by decreased breadth in Gag CTL responses (54). Despite the known mechanisms of control through CTL responses in individuals with protective HLA-I alleles, the individuals without these alleles were able to maintain control and were characterised by CTLs with poor ability to suppress HIV-1 replication *ex vivo*. The mechanisms of control that are independent of CD8+ T cell response in these individuals are not yet fully understood (54).

The mechanisms involved in influencing disease progression are still under investigation, although a complex interaction between virologic, immunologic and genetic factors is thought to hold the key for the controller phenotype. This topic is reviewed in detail in Chapter 3.

1.3 Rationale for the present study

Over 690 000 people died of AIDS-related illness in 2019, and over 38 million people are currently infected with HIV-1 worldwide, and South Africa is classified as one the highest HIV-1 burden countries globally.

Despite having multiple strategies for preventing infection or controlling disease progression, the ideal goal is to develop a prophylactic or therapeutic vaccine, which will reduce side effects of ARVs, allow independence of access to treatment (or prevention possibilities), and be discrete enough to reduce stigma. In order to do this, we need to further understand many aspects of the infection, from prevention to natural disease progression.

Despite clinical trial data showing the effectiveness of PrEP, there has not yet been data accurately describing the mechanism of action of PrEP, and why certain drug mechanisms (RTIs) are effective in reducing HIV-1 acquisition. Understanding the mechanism of action of PrEP and determining the effectiveness of different compounds in blocking infection (including drug classes other than RTIs), could lead to improvements in PrEP, such as new ARVs that require less doses (helping with adherence issues), or new delivery strategies (reducing stigma, or issues accessing treatment).

In addition to understanding prevention of infection through PrEP, understanding the mechanisms leading to the heterogeneity in rate of disease progression in HIV-1 infected individuals, especially the mechanisms that allow for control in the absence of ART in certain individuals, could reveal important insights that could be exploited for development of a therapeutic/preventative vaccine.

In this study we focussed on two aspects of HIV-1 infection: prevention and disease progression. On one hand we studied the basic science behind PrEP, through testing the ability of two different antiretrovirals (tenofovir - TFV and atazanavir - ATV) to extinguish new infection in both a reporter cell line and primary cells. On the other hand, we investigated natural determinants of HIV-1 disease progression through studying viraemic controllers with and without protective HLA-I alleles to gain insight into differing mechanisms of control in these two groups of individuals. Rapid progressors or uninfected individuals were included as control groups for comparison to controllers where appropriate.

1.4 Aims and objectives

1.4.1 Research Aims

1. Understand the mechanism of action of PrEP and determining the effectiveness of different ARVs in blocking initial infection.
2. Investigate the natural mechanisms of control in non-progressor individuals with and without protective HLA-I alleles.

1.4.2 Research Objectives

1. Determination of the mechanism of action of PrEP.

1.1. Selection of two antiretrovirals with different mechanisms of action - tenofovir (TFV) which is a reverse transcriptase inhibitor and atazanavir (ATV) which is a protease inhibitor - and calibration of both ARVs to allow the same level of ongoing replication (calculation of the viral replication ratio).

1.2. Calibration of the viral infectious units. In order to replicate initial infection, we calibrated the amount of viral copies to add to obtain ≈3 infected cells (initial infected cells).

1.3. Examination of the fraction of extinguished infections with low viral input in the presence of either TFV, ATV or no drug in an *in vitro* model of PrEP.

1.4. Modelling the probability of extinction of new infections in the presence of either drug mechanism, or with no drug, as a function of initial infected cells and viral replication ratio.

2. Determination of natural mechanisms of control and lack of control of HIV-1 disease progression.

2.1. Identify patients that fulfil the criteria of viraemic controllers (viral load <2000copies/ml maintained for at least 14 months) with and without protective HLA-I alleles (HLA-B*27, HLA-B*57, HLA-B*58:01, HLA-B*81:01, HLA-A*74), and RP (two or more CD4+ T cell measurements <350/mm^3 within 3 years after seroconversion, with no value ≥350/ mm^3 afterwards in the absence of ART, and/or ART initiated within 3 years of seroconversion, and at least one preceding CD4 <350/ mm^3, and/or AIDS or AIDS-related death within 3 years of seroconversion, and at least one preceding CD4 <350/ mm^3 (25-27)).

2.2. Determine the magnitude and breadth of CTL responses to Gag to test the hypotheses that viraemic control in individuals with protective HLA-I alleles versus non-protective HLA-I alleles is associated with greater breadth of Gag CTL responses.

2.3. Perform a phenotypic characterization of CD56CD16 cells (NK cells) and CD3+ cells (T cells) using cell surface markers and intracellular cytokine staining to explore differences between viraemic controllers with and without protective HLA-I alleles that could explain their ability to control HIV-1 infection in the absence of ARVs.

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CHAPTER 2

Determination of the mechanisms of action of PrEP

The following chapter has been published by PLOS Computational Biology and it is attached as appendix 1.

Moyano A, Lustig G, Rodel HE, Antal T, Sigal A. Interference with HIV infection of the first cell is essential for viral clearance at sub-optimal levels of drug inhibition. PLoS Comput Biol. 2020 Feb 4;16(2):e1007482. doi: 10.1371/journal.pcbi.1007482. PMID: 32017770; PMCID: PMC7039526.

Abstract

HIV-1 infection can be cleared with antiretroviral drugs if they are administered before exposure, where exposure occurs at low viral doses which infect one or few cells. However, infection clearance does not happen once infection is established, and this may be because of the very early formation of a reservoir of latently infected cells. Here we investigated whether initial low dose infection could be cleared with sub-optimal drug inhibition which allows ongoing viral replication, and hence does not require latency for viral persistence. We derived a model for infection clearance with inputs being drug effects on ongoing viral replication and initial number of infected cells. We experimentally tested the model by inhibiting low dose infection with the drug tenofovir, which interferes with initial infection, and atazanavir, which reduces the cellular virion burst size and hence inhibits replication only after initial infection.

Drugs were used at concentrations which allowed infection to expand. Under these conditions, tenofovir dramatically increased clearance while atazanavir did not. Addition of latency to the model resulted in a minor decrease in clearance probability if the drug inhibited initial infection. If not, latency strongly decreased clearance even at low latent cell frequencies. Therefore, the ability of drugs to clear initial but not established infection can be recapitulated without latency and depends only on the ability to target initial infection. The presence of latency can dramatically decrease infection clearance, but only if the drug is unable to interfere with infection of the first cells.

2.1 Introduction

HIV-1 can be suppressed with antiretroviral therapy (ART) to clinically undetectable levels in the blood. However, established HIV-1 infection cannot be cleared with ART, and generally rebounds several weeks after ART interruption. This persistence is driven by a reservoir of infected cells which decays minimally in the face of ART (1,2). There is extensive evidence that a key component of the HIV-1 reservoir is a population of latently infected cells: cells where functional proviral HIV-1 DNA is integrated into the cellular genome but is not expressed (3-6). Such cells may start producing virus when they are activated (7, 8) and due to stochastic fluctuations in HIV-1 Tat protein production, initiating a positive feedback loop in HIV-1 gene expression (9, 10).

The exception to the failure of ART to clear infection occurs when ART is present during or immediately after an infection attempt. An approach termed pre-exposure prophylaxis (PrEP) aims to administer ART to uninfected, at risk individuals to take advantage of this fact. The majority of clinical studies have shown that PrEP is effective in a variety of populations, transmission modes, and drug delivery modalities (11-19).

The shift from an infection which can be cleared with ART to one which cannot, is generally attributed to the formation of the latent reservoir. The early formation of a reservoir of infected cells in the face of ART has been demonstrated in a non-human primate model (20) and latency has been proposed to be a key driver in the initial establishment of HIV-1 infection (21). While this mechanism is consistent with the very early transition to irreversible infection, it relies on the assumption that ART regimens completely inhibit viral replication in the mucosal tissues of the genital and rectal tracts, the initial HIV-1 infection sites, and that the infection becomes irreversible if the latent reservoir is established before this complete inhibition takes place.

It may be important to consider whether a mechanism which does not rely on the assumption of complete suppression of viral replication in the mucosa with ART, and therefore the rapid formation of a latent reservoir, can lead to this observed behaviour of HIV-1 infection. There are several reasons to consider such an alternate: 1) While there is strong evidence that ART levels as measured in the blood are more than sufficient to completely suppress HIV-1 replication (22), drug penetration may be lower in the mucosa. Therefore, whether inhibition is complete in this compartment is less clear (23); 2) a challenge in PrEP is to maintain adherence to the treatment, as it is administered to uninfected individuals (11-14, 24-26). If adherence to PrEP is variable, sub-optimal ART concentrations should occur in at least a subset of treated individuals. PrEP was shown to be effective in a non-human primate model of low dose infection even when dosing was intermittent (27), suggesting it may still be effective under conditions of sub-optimal drug; 3) incomplete suppression of viral replication may be relevant to future PrEP approaches (28) which may use agents that have advantages such as long half-lives but do not completely inhibit HIV-1 replication; 4) it may be relevant to understanding basic principles of initial viral infection by using the well characterized HIV-1 infection system which has as a toolkit antiretroviral drugs with different mechanisms of action.

An alternative mechanism would need to explain why, if infection can expand, ART can nevertheless inhibit infection if administered very early after exposure. The alternative hypothesis we propose is that if the initial number of infected cells is small (~1), it is possible to clear initial infection at sub-optimal inhibitor levels, where such sub-optimal levels would allow infection to expand if the number of initial infected cells was larger. The key conditions are a low initial number of infected cells and an inhibitor which acts before the first cell is infected. The basic reasoning is that under these conditions, the first infected cell is either present or absent. If the inhibitor succeeds in eliminating that infected cell, the infection is cleared regardless of the fact that the infected cell could initiate an expanding infection.

The evidence that a low number of initial infected cells is in fact the physiological condition in vivo is that the probability for an individual exposed to HIV-1 by sexual contact to become infected does not exceed 0.02 per sexual act under any set of conditions and is usually much lower (29, 30). Moreover, infection is established most often with a single viral founder clone (31, 32), and experimental infection with SIV in non-human primates shows the existence of an infection bottleneck at initial infection (33, 34). These observations indicate that initial transmission is at a low viral dose, sufficient to infect at most one or few cells. This may also be consistent with initial HIV-1 transmission occurring by cell-free HIV-1 infection, where cell-free virions rely on diffusion to reach an infectable cell and therefore have a low probability to infect (35-51). In contrast, an infected cell is likely to deliver considerable numbers of virions (10^3 to 10^4 virions are produced per cell (52, 53)) if it is at close range.

To test whether it is necessary to inhibit before the first infected cells for sub-optimal inhibition to be effective, it is possible to use antiretroviral drugs with different mechanisms of action. HIV-1 reverse transcriptase inhibitors (RTI) such as tenofovir (TFV) prevent the initial infection of the cell but do not interfere with viral production from an already infected cell. That is, they decrease infection frequency. Protease inhibitors (PI) such as atazanavir (ATV) do not interfere with cellular infection but reduce the number of viable mature virions an infected cell produces—the burst size per cell of viable virions. The effect of decreasing infection frequency or viral burst size should be symmetrical at a high viral dose: The number of successful infections will be decreased if fewer virions successfully infect cells or if the ability of infected cells to produce viable virions is reduced (Figure 1, left panel). However, these effects may not be symmetrical at an initial low viral dose (Figure 1, right panel). Since PIs act with a delay—protease mediated cleavage occurs in the virion during budding from an already infected cell—they can be used to study the effects of the delay on the probability of infection clearance with drug when the initial viral dose is only sufficient to infect one or few cells.

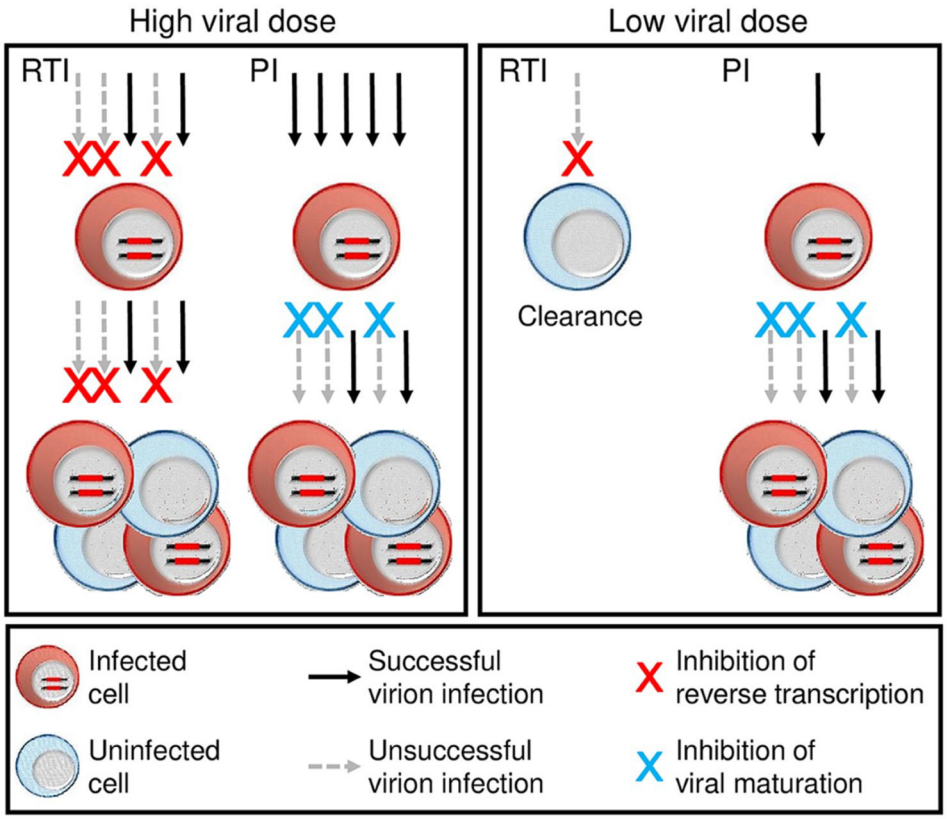

Fig.1. Low dose HIV-1 transmission is vulnerable to clearance before infection of the first cells. Illustrated is partial inhibition of infection ($R_0 \sim 2$ with drug) with drugs such as a reverse transcriptase inhibitor (RTI), which acts before HIV-1 integrates into the cellular genome, and a protease inhibitor (PI), which acts after the infection of the first cells by interfering with HIV-1 maturation. Left panel shows high dose transmission between individuals, inhibited by drugs at levels where infection is still able to replicate. Here, the effects of the RTI and PI are symmetrical and neither clears infection. Right panel shows low dose transmission between individuals inhibited at the same drug levels. While the RTI may not clear every infection attempt, it may be successful at clearing infection if the number of infection attempts are few. In contrast, once the first cells are infected, as would occur with the PI, this advantage is lost. In the event $R_0 \lessapprox 1$ with drug, both drug mechanisms can clear infection.

Here we tested the hypothesis that the probability of HIV-1 infection clearance with drug levels which allow for viral replication in established infection depends on preventing initial infection of the first one or few cells. We modelled HIV-1 infection as a function of the measurable initial number of infected cells (N_0) and the basic reproductive ratio (R_0)—the number of cells infected on average by one infected cell when infectable cells are not limiting. We then performed experiments with low N_0 and two types of inhibition: reduction of infection frequency by TFV and reduction of viral burst size per cell by ATV. With both drugs, we used drug concentrations where $R_0 > 1$ in the presence of drug. That is, infection could expand. We observed that while both drugs reduced R_0 to a similar extent at the concentrations used, only TFV, which prevented successful infection of the first set of cells, was effective at clearing infection.

2.2 Materials and methods

2.2.1 Ethics statement

The study protocol for blood collection from healthy donors was approved by the University of KwaZulu-Natal Institutional Review Board (approval BE083/18). Blood was obtained with informed written consent from each donor.

2.2.2 Inhibitors, viruses, and cells

The following reagents were obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health: the antiretroviral drugs ATV and TFV; RevCEM cells from Y. Wu and J. Marsh; HIV-1 NL4-3 CCR5 tropic infectious molecular clone (pNL (AD8)) from E. Freed; pBABE.CCR5, from N. Landau. Cell-free virus was produced by transfection of HEK293 cells with pNL (AD8) using TransIT-LT1 (Mirus) transfection reagent. Supernatant containing released virus was harvested two days post-transfection and filtered through a 0.45-micron filter (GVS). The number of HIV-1 RNA genomes in viral stocks was determined using the RealTime HIV-1 viral load test (Abbott Diagnostics). The RevCEM HIV-1 infection GFP indicator cell line was modified as follows for experiments with the CCR5 tropic virus: The E7 clone was generated from RevCEM cells as described in (36). Briefly, the RevCEM cell line was sub-cloned by limiting dilution. Clones derived from single cells were expanded into duplicate 96-well plates, one optical and one standard tissue culture for continued growth. The optical plate was infected with HIV-1 strain NL4-3 virus and optical wells were scanned by microscopy to select clones with highest infection percentage by GFP expression. The clone E7 was selected based on greater than 70% GFP positive cells upon infection, expanded from the uninfected replicate plate and frozen. To generate the CCR5 expressing B8 reporter clone, RevCEM-E7 cells were infected with the pBABE. CCR5 retroviral vector which stably expressed CCR5 under the LTR promoter. Cells were sub-cloned by limiting dilution. Clones derived from single cells were expanded into duplicate 96-well plates, one optical and one standard tissue culture for continued growth. The optical plate was infected with HIV-1 strain NL(AD8) CCR5 tropic HIV-1 and wells were scanned by microscopy to select clones which maintained similar GFP expression to the parental RevCEM-E7 clonal cell line. The clone RevCEM-B8 was selected based on greater than 70% GFP positive cells upon infection, expanded from the uninfected replicate plate, and frozen. Cell culture medium was complete RPMI 1640 supplemented with L-Glutamine, sodium pyruvate, HEPES, non-essential amino acids (Lonza), and 10% heat-inactivated FBS (Hyclone).

2.2.3 Infection and flow cytometry

For determination of drug effect on R_0 and N_0, cells were infected with 2.5×10^7 viral RNA copies in 2ml of cell culture containing 5×10^5 cells/ml. The number of infected cells was acquired every 2 days with a FACSCalibur machine (BD Biosciences) using the 488nm laser line. Flow rate on the machine was measured at each time-point, and acquisition time was multiplied by the inverse of the flow rate to obtain the number of infected cells per millilitre.

For experiments measuring P_c^{drug}, 200 µl of cells at a density of 5×10^5 cells/ml were infected with 6.3×10^3 viral RNA copies. Results were analysed using FlowJo 10.0.8 software. The background frequency of positive cells was determined by acquiring uninfected samples (n = 17 from 4 independent experiments). A sample was scored as infected if the number of GFP positive cells was greater than that in the highest background samples (0.01% positive cells).

2.2.4 Passaging of infected cell cultures

For determination of drug effect on R_0 and N_0, the uninfected and drug treated cell cultures were passaged at a split ratio of 1:2 every 2 days, where half the cell culture was removed and fresh media with drug (for TFV and ATV) or without drug (for uninfected cells) was added. Proliferation of uninfected cells was sufficient to maintain uninfected cell numbers, and infection was below 5% for both drug conditions at all time-points, ensuring target cells were not limiting. For the no drug condition, the infection expanded much more rapidly. Therefore, the infected cell culture was passaged by diluting the infected cells 1:100 every 2 days into uninfected cells. Hence, 20 µl infected cells were added to 2 ml of fresh, uninfected cells at 5×10^5 cells/ml. The removed fraction of cells was used to detect infection by flow cytometry.

For experiments measuring P_c^{drug}, cell cultures with either no infection or containing ATV or TFV, passaging conditions were the same as for the experiments used to determine drug effect on R_0 except that no culture was removed. Instead, new media with drug was added for the TFV and ATV conditions, and new media with no drug was added for the uninfected condition. The infection volume therefore doubled every 2 days, and the cell culture was transferred to larger volume wells to preserve a constant surface to volume ratio. After 8 days (4 passages), cells were spun down, washed once in medium with no drug, and resuspended at 5×10^5 cells/ml in fresh medium with no drug. Cells were then further passaged in the absence of drug for 6 days (3 passages) using a 1:2 dilution every 2 days to amplify any infection in the culture. For infection in the absence of drug, cells were passaged for 6 days (3 passages) using a 1:2 dilution every 2 days with fresh medium without removing any of the cell culture. The number of infected cells was

acquired at the end of the experiment (14 days post-infection for the uninfected, TFV, and ATV conditions, and 6 days for the no drug infection condition) with a FACSCalibur machine as above.

2.2.5 Measurement of infected cell half-life

For determination of the half-life of infected cells in the presence of ATV, RevCEM-B8 cells were pre-incubated with 16nM ATV for 48h. 10^6 cells/ml were then infected with NL(AD8) in the presence of ATV to obtain saturating infection (approximately 70% GFP positive resulting from 10^9 viral RNA copies) so that the population of uninfected cells was small and reduction in infected cell number due to cell death could be tracked without the confounding effect of new infections. The cells were maintained with ATV and the number of live infected cells was tracked 2-, 4- and 6-days post-infection by pulsing cells with 4 μg/ml of the death detection dye propidium iodide (Sigma-Aldrich) and acquiring for 1 minute with a FACSCalibur machine.

2.2.6 Measurement of infection clearance in peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich) and cultured at 2×10^6 cells/ml in complete RPMI 1640 medium supplemented with L-glutamine, sodium pyruvate, HEPES, and non-essential amino acids (Lonza), 10% heat-inactivated FBS (GE Healthcare), and IL-2 at 5 ng/ml (PeproTech). Phytohemagglutinin at 12 μg/ml (Sigma-Aldrich) was added for 1 day to activate cells. For cell-free infection, PBMCs were pre-treated with either TFV, ATV or no drug for 48 hours after activation and before infection. Cells were then infected with 2×10^8 viral RNA copies of NL(AD8) in 1 ml of culture. 2 days post-infection, the number of infected cells was determined by fixing and permeabilizing PBMCs using the BD Cytofix/Cytoperm Fixation/ Permeabilization kit (BD Biosciences) according to the manufacturer's instructions. Cells were then stained with anti-HIV p24 FITC conjugated antibody (KC57-FITC, Beckman Coulter, Brea, CA) to detect the presence of intracellular HIV-1 Gag protein. For coculture infection, PBMCs were activated as above. After activation, cells were split into two fractions: donor cells infected with cell-free virus, and target cells to be infected by the addition of the infected donor cells. Donor cells were infected in the absence of drug with 2×10^9 viral RNA copies of NL (AD8) in 2 ml of culture. Target cells were incubated with TFV or ATV. 1-day post-donor cell infection, TFV or ATV was added to donor cells. 2 days post-donor cell infection, infected donor cells were stained with carboxyfluorescein succinimidyl ester at 1.5 μM (CFSE, Thermo Fisher Scientific) vital stain to differentiate them from target cells and added to target cells at 1:300 p24-positive infected donor to uninfected target cell ratio. 2 days post target infection, the number of HIV-1 infected, CFSE-negative target cells was quantified by fixing and permeabilizing as for cell-free infection

and staining with anti-HIV p24 PE conjugated antibody (KC57-PE, Beckman Coulter, Brea, CA). For determination of clearance probability, 0.5 ml of PBMCs at 10^6 cells/ml were activated as above and pre-incubated with drug for 48h, then infected with 2.5×10^5 viral RNA copies of cell-free NL(AD8). After 2 days, cells were spun down and resuspended into new growth media with drug. After 4 days, cells were washed in 2 ml growth media, then resuspended in 0.5 ml media without drug and added to 1.5 ml RevCEM-B8 cells at 0.7×10^6 cells/ml to amplify infection. 4 days after addition of PBMCs to RevCEM-B8 cells, the number of infected, GFP positive RevCEM-B8 cells was acquired with a FACSCalibur machine. A sample was scored as infected if the number of GFP positive cells was greater than that in the highest background samples (0.01% positive cells). To approximate N_0 in PBMCs with 2.5×10^5 viral RNA copies, 0.5 ml of PBMC cultured at 10^6 cells/ml was infected at four virus stock dilutions in triplicate: 1.3×10^7, 6.3×10^6, 3.2×10^6, 1.6×10^6 RNA copies. The number of infected PBMCs was measured after 2 days by flow cytometry using anti-HIV p24 FITC conjugated antibody staining. Infected cell numbers at the viral stock dilutions above were (mean ± std): $1.4 \pm 0.08\times10^3$, $6.6 \pm 1.9\times10^2$, $4.7 \pm 0.2\times10^2$, $2.2 \pm 0.9\times10^2$. Data was fit using linear regression to determine N_0, calculated to be approximately 29 infected cells.

2.3 Results

2.3.1 A model for infection clearance

We first set out to model the effect of drugs on the probability to clear infection (P_c). Let N_i be the number of infected cells in the i-th transmission step within the newly infected host. The sequence N_i, $i = 0, 1, 2, \ldots$ is a Markov chain, or, more specifically, a branching process (54) with the random number N_{i+1} of infected cells at the $(i + 1)$-st infection step determined from the number N_i of infected cells in the previous infection step by the formula

$$N_{i+1} = \sum_{c=1}^{N_i} I_c \qquad (1)$$

Here I_c are independent identically distributed random variables denoting the number of new cells infected by each infected cell in step i. We note that in the case where host cells are not limiting, as occurs in the initial stages of infection, infection chains originating from individual infected cells are independent of each other. The infection is cleared if the number of infected cells N_i becomes zero at any point.

Infection starts with a number of infected cells N_0 as a result of exposure to HIV-1 from an infected individual, where $N_0 \geq 0$. N_0 is expected to depend on several factors, among which is the transmitted viral dose during exposure and the cellular infection frequency per virion. N_i, where $i \geq 1$, would then depend on N_0 and the basic reproductive ratio (R_0), the number of cells infected on average by one infected cell in the

initial stages of HIV-1 infection, where host cells are not limiting. R_0 depends on both the viral replication rate and the half-life of the infected cells (55, 56) and is approximately 10 *in vivo* (55). Eventual infection clearance is certain for $R_0 \leq 1$. For $R_0 > 1$, infection may still be cleared if, at any point in the infection chain, the number of infected cells is zero.

To infect new cells, an infected cell produces a burst of κ virions, where κ is on the order of 10^3 to 10^4 (52, 53), and each virion can infect a cell independently with probability r. The number of cells infected by a single infected cell in one transmission step has a binomial distribution with mean $R_0 = r\kappa$ (56). In the biologically relevant case where κ is large and r is small, with $R_0 = r\kappa$ finite, this binomial distribution can be replaced by the simpler Poisson distribution with mean R_0 (57). That is, the probability that a single infected cell infects m cells (progenies) in one step is $P_m = R_0^m e^{-R_0}/m!$.

We denote by q the probability that an infection starting from exactly one infected cell is cleared. In this case, it is required that all m identical progenies originating from the original infected cell are cleared. Since each progeny is cleared with the same probability q, all progenies are cleared with probability qm, assuming independence of progenies. Therefore:

$$q = \sum_{m\geq 0} q^m P_m. \tag{2}$$

Note that the right-hand side of Eq (2) is called the generating function, in this case, of the number of progenies of a single infected cell (54).

Replacing P_m with the Poisson distribution with mean R_0 as described above and using the Taylor series of the exponential function $\sum_{m\geq 0} x^m/m! = e^x$, we find:

$$q = \frac{\sum_{m\geq 0} q^m R_0^m e^{-R_0}}{m!} = e^{R_0(q-1)}. \tag{3}$$

The (smallest non-negative (57)) solution of the above equation gives the probability of clearing the infection for a single initial infected cell:

$$q = -R_0^{-1} W(-R_0 e^{-R_0}). \tag{4}$$

Here, W is the Lambert W–function (58), the inverse of the function $x \to xe^x$. The relationship between q and R_0 is graphed in Supplementary Figure 1, which shows that $q = 1$ for $R_0 \leq 1$ and $q \to 0$ at $R_0 >> 1$.

Eq (4) derived the probability of infection clearance for exactly one infected cell. The initial number of infected cells may not be one but may be described as a random variable (21). We choose it to be a Poisson

random variable with mean N_0 which is a biologically relevant distribution in viral infection. Therefore, the probability that the initial number of infected cells is n has probability $\emptyset_n = N_0^n e^{-N_0}/n!$. For a fixed number n of initial infected cells the infection is cleared with probability q_n, assuming infections originating in individual infected cells are independent. To find the probability of infection clearance P_c for a random number of initial infected cells, we take the average over n:

$$P_c = \sum_{n\geq 0} q^n \emptyset_n = \sum_{n\geq 0} \frac{q^n N_0^n e^{-N_0}}{n!} = e^{-N_0(q-1)} . \quad (5)$$

This is the probability that an infection starting from a Poisson distributed random number of infected cell is cleared, where q is given by Eq (4).

We now consider the effect of the antiretroviral drug mechanism on N_0 and q. We note that antiretroviral drugs reduce either infection frequency r or burst size κ. For drugs which reduce infection frequency, $r \rightarrow d_1 r$, and for drugs which reduce viral burst size, $k \rightarrow d_2 k$, where $0 \leq d_1, d_2 \leq 1$. The no drug case is recovered for $d_1 = d_2 = 1$. Given $R_0 = r\kappa$ and therefore $R_0 \rightarrow R_0\, d_1 d_2$, the effects of the drug mechanisms are symmetrical on q

$$q_{drug} = -(R_0 d_1 d_2)^{-1}\, W\left(-R_0 d_1 d_2 e^{-R_0 d_1 d_2}\right). \quad (6)$$

Hence, if the drugs decrease R_0 to a similar extent, their effect on q will also be similar.

However, given an initial transmission with cell-free virus, only the drug mechanism that decreases infection frequency will reduce the mean initial number of infected cells N_0. The mechanism which reduces burst size will only affect the success of the next transmission cycle. Therefore, the probability to clear infection with drugs becomes

$$P_c^{drug} = e^{N_0 d_1 (q_{drug}-1)}. \quad (7)$$

Here q_{drug} is determined by Eq (6). The limits for Eq (7) for $R_0 \leq 1$ and $R_0 >> 1$ with drug are 1 and $e^{-N_0 d_1}$. At the upper limit for R_0, infection clearance is simply determined by the probability of obtaining $n = 0$ initial infected cells, where the probability to obtain n infected cells is a random number from a Poisson distribution with mean $N_0 d_1$. What constitutes a high value for R_0, at which P_c^{drug} only depends on $N_0 d_1$, is discussed below.

To visualize the effects of decreasing R_0 versus N_0, we plotted Eq (5) for a range of parameter values (Figure 2A). It can be observed that for $R_0 \leq 1$, infection terminates. At $R_0 > 1.5$, infection is not strongly sensitive

to the exact R_0 value provided $N_0 \gtrapprox 3$. However, at all $R_0 > 1$ values, the probability of infection clearance is very sensitive to N_0, provided N_0 is small. This sensitivity is greatly reduced when $N_0 \gtrapprox 3$.

To examine the effects of drug mechanism, we plotted infection clearance according to Eq (7) at two conditions of R_0 and N_0 relative to d_1 and d_2 (Figure 2B). In the first condition, R_0 was sufficiently small to be decreased below 1 by the drugs in the inhibition range used, while N_0 was large (Figure 2B, top panel). In the second condition, R_0 was large while N_0 was small (Figure 2B, bottom panel). In the first condition, both drug mechanisms had a similar effect on infection clearance, and $P_c = 1$ when the effect of either drug reduced R_0 below 1. In the second condition, only d_1, which decreased infection frequency, substantially increased P_c. d_2, which acted on burst size, had a minimal effect. We note that based on observations of $R_0 \approx 10$ *in vivo* (55) and a probability of infection of at most 0.02 per exposure in the absence of PrEP (29, 30), the second condition likely reflects the physiological situation.

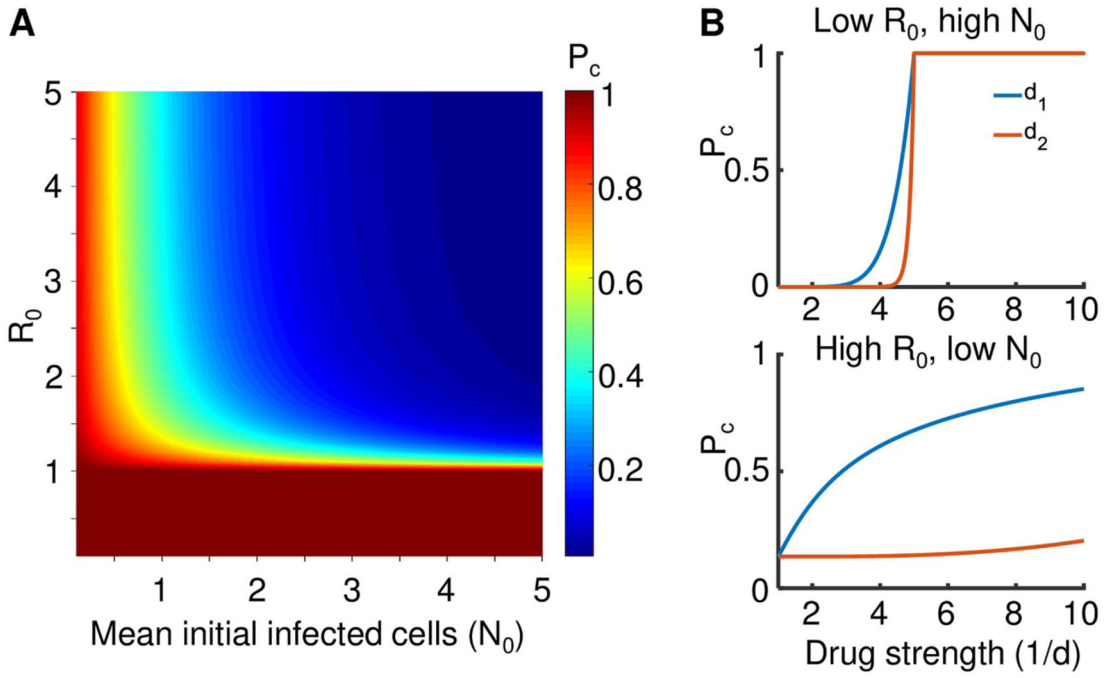

Fig. 2. Effects of the initial infected cell number N_0 and R_0 on the probability of infection clearance P_c. (A) P_c according to Eq (5) at different parameter values for N_0 and R_0. (B) P_c according to Eq (7) when a drug attenuating infection frequency (d_1, blue line) or burst size (d_2, orange line) acts on N_0 and R_0. Top panel shows the case where $R_0 = 5$, $N_0 = 20$, while bottom panel shows the case where $R_0 = 20$, $N_0 = 2$. X-axis is drug strength as 1/d, y-axis is P_c.

2.3.2 Experimental determination of the probability of infection clearance with drug

We examined experimentally whether Eq (7) predicts P_c for different drug mechanisms after infection with a low HIV-1 dose, the likely *in vivo* condition for transmission. We used the antiretroviral drugs TFV and

ATV to inhibit infection initiating as cell-free HIV-1. We measured the effect of each drug on the initial number of infected cells N_0 resulting from the initial input of cell-free HIV-1 virions. After this initial cycle of infection, the initial number of infected cells was cultured with uninfected target cells (coculture infection). We define established infection as infection where infected cells are present and can infect new cells using both the cell-free infection route and by cell-to-cell spread (59). R_0 was measured during this phase of infection.

For virus, we used HIV-1 NL(AD8), an HIV-1 strain with a CCR5 tropic envelope protein. CCR5 tropism has been shown to be the predominant transmitted form between individuals (31). As target cells for infection, we used a clone of the RevCEM infection indicator cell line (60) which we first subcloned to increase detection efficiency (36) then modified to express the CCR5 receptor (Materials and methods). Detection of infected cells was done by quantifying the number of GFP positive cells using flow cytometry.

We titrated TFV and ATV to obtain a similar effect on ongoing coculture infection. This occurred at 60 μM TFV and 16 nM ATV. To maintain nutrients for cell growth and prevent uninfected cell depletion, we passaged cells every two days (Materials and methods). Such passaging is necessary to maintain conditions where uninfected cells are not limiting in an expanding infection over multiple cell division and viral replication cycles (35).

Despite the use of the same HIV-1 cell-free input dose, there were pronounced differences at day 2 between TFV and ATV (Figure 3A). This time-point reflects the results of the initial cell free infection given an approximately 2-day viral cycle (61). Cell-free infection was strongly inhibited by TFV relative to no drug. As expected, the effect of ATV on cell-free infection was much weaker since cell-free virus produced in a cell not exposed to a protease inhibitor is already mature. After the day 2 time-point, infection expanded with similar dynamics for both drug conditions, and much more rapidly when no drug was present.

We plotted the total number of infected cells, corrected for cells removed during passaging, versus time (Figure 3B). We then calculated the effect of drug on R_0 over a two-day cycle (Table 1). R_0 values showed that infection expanded at a similar rate for the TFV and ATV conditions. We then measured the effect of the drugs on N_0 after the first cycle of infection (day 0 to day 2) and compared the results to infection in the absence of drug. N_0 in the presence of drug divided by N_0 for the no drug condition (N_0^{norm}) was 0.027 ± 0.014 for TFV and 0.88 ± 0.16 for ATV, (Figure 3C, Table 1). The decrease in N_0 for TFV versus ATV was significant ($p = 6\times10^{-14}$, t-test).

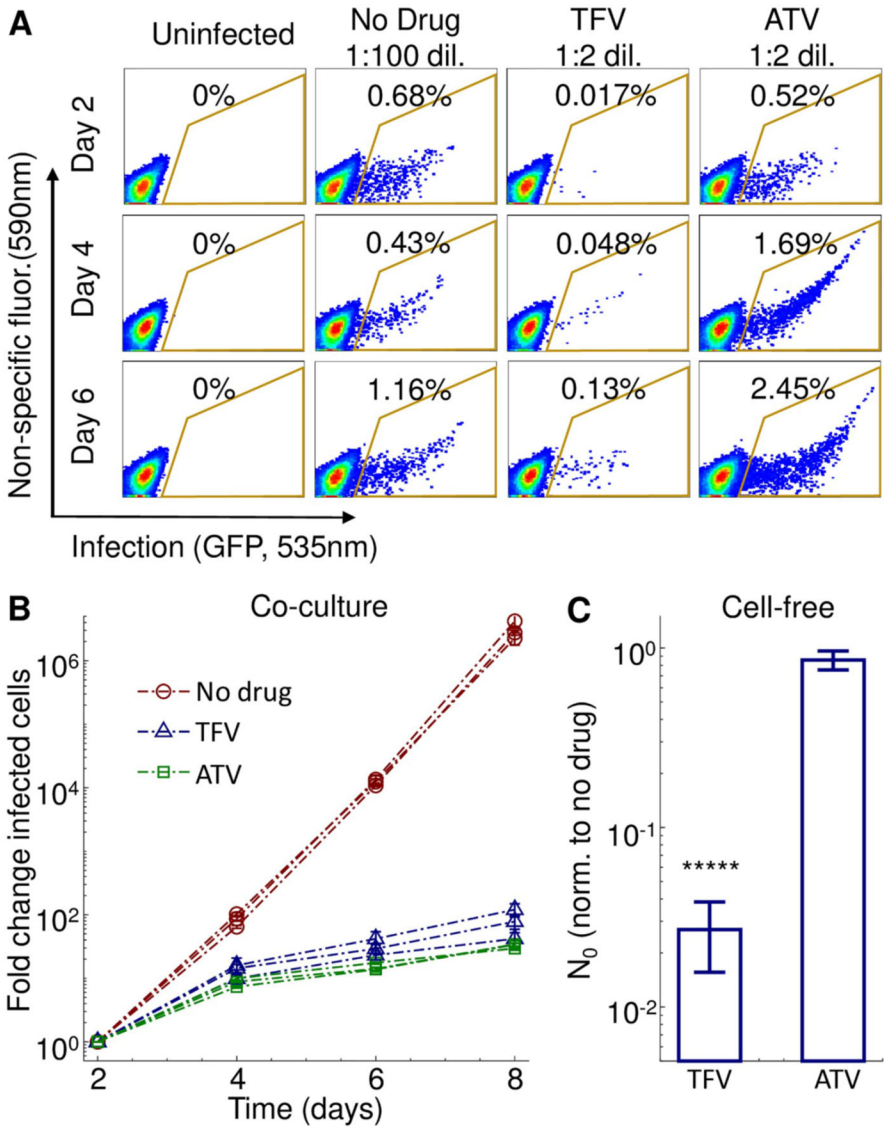

Fig. 3. Experimental measurement of drug effect on R_0 and the initial number of infected cells N_0. (A) Flow cytometry plots of the fraction of infected cells at different days post infection in the absence of drug or presence of 60 μM TFV or 16 nM ATV. Day 2 is the first time-point after the initial cell-free infection, corresponding to approximately one viral cycle. X-axis is GFP fluorescence, y-axis is autofluorescence, with the fraction of infected cells corresponding to the cells within the area outlined in yellow. Infected cell cultures in the presence of either drug were diluted 1:2 every 2 days. Infected cultures in the absence of drug were diluted 1:100 into uninfected cells every 2 days. (B) Measurement of R_0 in the absence and presence of drug. The number of infected cells at each time-point is normalized by the number of infected cells at day 2 and corrected for the dilution factor used in each infection cycle. 3 independent experiment were performed, with each point denoting the mean ± std of 3 experimental replicates per experiment. Infection in the absence of drug is shown as red circles, TFV as blue triangles, and ATV as green squares. (C) Effect of drug on N_0. For each drug condition N_0 was measured 2 days after cell-free HIV-1 infection and normalized by N_0 for no drug. Mean ± std of 3 independent experiments, where normalization was with N_0 in the absence of drug as measured in the same experiment. Raw numbers of infected cells averaged over all experiments were $1.3\times10^4 \pm 1.5\times10^3$ for no drug infection, $3.4\times10^2 \pm 1.3\times10^2$ for TFV and $1.1\times10^4 \pm 7.4\times10^3$ for ATV (mean ± std). The difference between TFV and ATV was significant ($p = 6\times10^{-14}$ by t-test).

Table 1. Measured parameter values.

Treatment	N_0^{norm} *	R_0
No drug	1	143 ± 15
60 µM TFV	0.027 ± 0.014	4.2 ± 0.73
16 nM ATV	0.88 ± 0.16	3.2 ± 0.088

* N_0 normalized by N_0 no drug.

We then set out to investigate whether TFV and ATV could increase the probability of clearance of low dose infection, corresponding to in vivo exposure. We used 6.3×10^3 viral copies (Materials and methods), predicted to result in approximately 3 initial infected cells based on a regression of the number of infected cells versus input viral load (Figure 4A). Infection was initiated with the same cell-free viral dose for all conditions, and infected cells were cultured for 8 days in the presence of drugs. Any infection present was then amplified for detection by culturing cells in the absence of drug. After amplification, infection was either clearly visible or absent (Figure 4B).

We did not experimentally observe clearance of infection in the absence of drug. In the presence of TFV, clearance rose dramatically, with approximately three quarters of infections extinguished. In contrast, only a minor increase of infection clearance was observed with ATV (Figure 4C, red bars). Clearance with TFV was significantly higher relative to no drug and ATV ($p=9\times10^{-8}$ and $p=5\times10^{-9}$ by Fisher's exact test, respectively), while ATV was not significantly different from no drug. Calculation of P_c^{drug} based on Eq (7) using the measured values for N_0 and R_0 for each drug condition replicated an essential feature of the experimental results: treatment with TFV was predicted to result in a much higher clearance probability relative to treatment with ATV (Figure 4C, grey bars). If no effect of drug on N_0 was included in the model, TFV and ATV were predicted to have similar, and small, effects on P_c^{drug} (Figure 4C, yellow bars). Hence, Eq (7) was able to predict the relative effectiveness of each drug to terminate infection.

One explanation for the difference between TFV and ATV clearance frequencies is that the initially infected cells in the presence of ATV were still present at the end of drug treatment due to lack of cell death and gave rise to the infected cell population when ATV was removed. We therefore measured the half-life of cells in the presence of ATV. We observed a half-life of approximately 1 day (Supplementary Figure 2). Hence, less than one-tenth of the initially infected cells are expected to survive to the end of drug treatment, making infection persistence with ATV due to a long half-life unlikely.

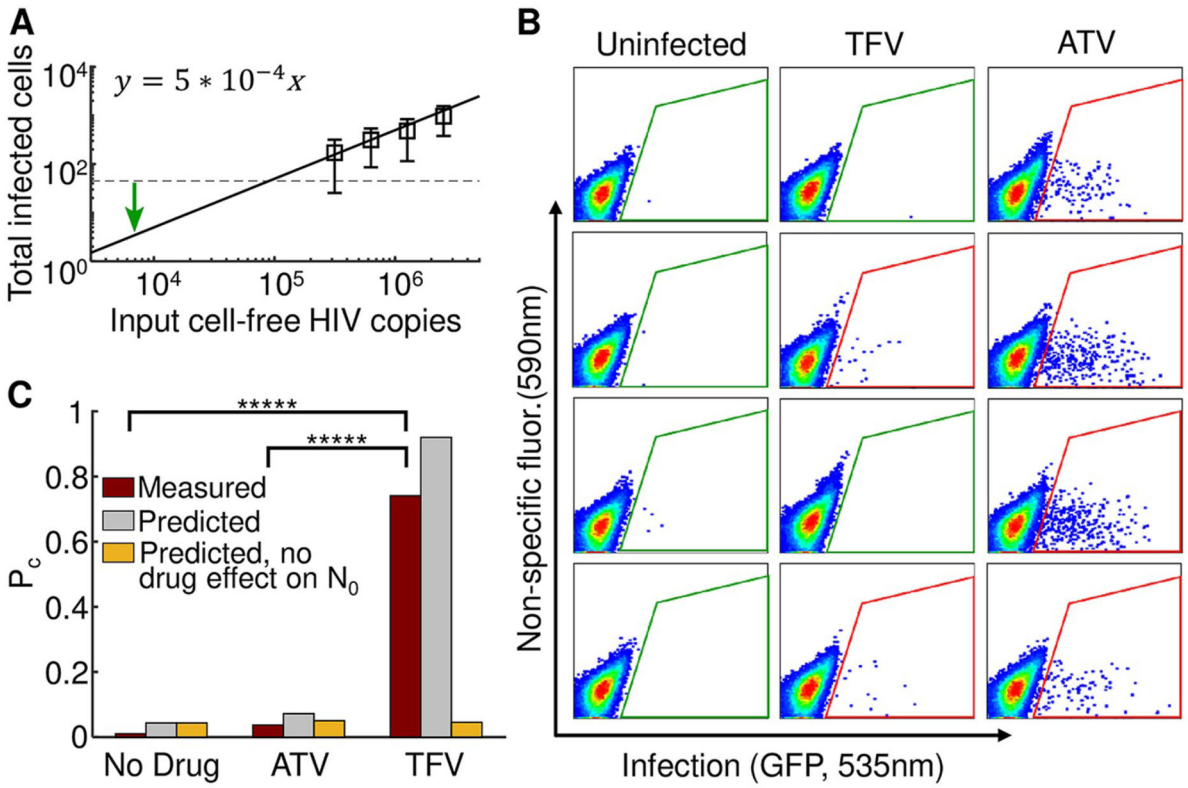

Fig. 4. Probability of infection clearance depends on drug mechanism.
(A) Determination of N_0. The number of infected cells was measured using flow cytometry as a function of cell-free HIV-1 RNA copies for four virus stock dilutions after one infection cycle (2 days). Data was fit using linear regression to determine the input viral dose for 3 infected cells. Mean ± std of 5 independent experiments. Dashed line is limit of detection. Green arrow marks number of HIV-1 RNA copies used in the experiments. (B) Representative flow cytometry plots after 8 days of infection with the input cell-free virus in the presence of TFV or ATV and further 6 days amplification in the absence of drug. Each plot represents one independently cultured replicate of the experiment. Uninfected samples are shown in the left column, and infection in the presence of TFV or ATV is shown in the middle and right columns, respectively. X-axis is GFP fluorescence, y-axis is autofluorescence. The fraction of infected cells corresponds to the cells within the area outlined in green or red, with green indicating background GFP signal level as determined using the uninfected samples, and red indicating above background signal. (C) P_c^{drug} as experimentally measured (red bars), and as predicted by Eq (7) (grey bars) based on the measured drug effects on R_0 and N_0. Presence of infection was assayed in 26 (no drug) or 27 (TFV and ATV) cell-free virus infections from 4 independent experiments. Observed P_c^{TFV} was significantly higher than P_c^{ATV} and P_c^{Nodrug} ($p=9\times10^{-8}$ and $p=5\times10^{-9}$ by Fisher's exact test, respectively). P_c^{ATV} and P_c^{Nodrug} were not significantly different. Yellow bars show predicted P_c^{drug} if both drugs act on R_0 only. That is, $P_c^{drug} = e^{N_0(q_{drug}-1)}$.

To examine whether the qualitative pattern of the results obtained for the cell line would also be obtained in primary cells, we repeated the experiment in peripheral blood mononuclear cells (PBMCs) from an HIV-1 uninfected blood donor. PBMCs were infected with a low dose of NL(AD8) strain HIV-1 in the presence of TFV and ATV (Materials and methods). Drug concentrations used were 40 μM for TFV and 24 nM for ATV. At these drug concentrations, both drugs reduced infection by approximately one order of magnitude

when infection was by coculture of infected with uninfected cells (Supplementary Figure 3A–3C), as occurs in established infection. When the infection source was cell-free virus, TFV reduced infection by two orders of magnitude while ATV reduced infection 3-fold (Supplementary Figure 3D). The reduction with ATV of cell-free infection is consistent with a previous report showing some effect of protease inhibitors on cell-free infection (62), while the greater effect of TFV on cell-free versus coculture infection is consistent with multiple previous studies (35-37, 39, 41, 43, 46, 51). When infection was with low dose cell-free virus, TFV led to almost complete infection clearance, with no clearance detected for the ATV and no drug conditions (Supplementary Figure 3E). These results validate the observed behaviour of the cell-line infection in primary human cells.

2.3.3 Effects of latency on the probability of infection clearance

The results above showed that at sub-optimal drug concentrations where HIV-1 infection can replicate, infection can still be cleared if the initial number of infected cells is low and the drug decreases infection frequency before the first cells are infected. This effect does not presuppose the existence of latency. However, given the strong evidence for latency, we investigated the expected effect of latency on infection clearance.

We introduce a probability of a cell to become latent P_{lat} (21). Estimates for P_{lat} vary between approximately 0.5 in *in vitro* infections and modelling (10, 63, 64), to 10^{-4} *in vivo*, based on the frequency of intact HIV-1 DNA in the face of ART in CD4+ T cells in the peripheral blood compartment (65, 66), and 10^{-3}, based on total HIV-1 DNA copies in rectal CD4+ T cells of individuals on ART (67). The latter values do not measure the ability of the HIV-1 DNA to produce infectious virus, and therefore the frequency of latent cells containing inducible infectious virus may be lower. However, the value of P_{lat} at initial infection is difficult to determine, and therefore values in the upper part of the range cannot be ruled out.

Once a latent cell is produced, the infection may no longer be cleared, since latently infected cells may maintain the reservoir by homeostatic latent cell proliferation as opposed to new rounds of infection (68). Therefore, infection can persist even if $R_0 \leq 1$, provided a latent cell is present.

Infection originating in exactly one initial infected cell clears if it both stays non-latent with probability $1 - P_{lat}$ and independently if all of its progenies clear, which occurs for each of them, again independently, with probability q:

$$q = (1 - P_{lat}) \sum_{m \geq 0} q^m P_m = (1 - P_{lat}) e^{R_0(q-1)}. \quad (8)$$

The solution for q of Eq (8) is:

$$q_{lat} = -R_0 W(-R_0(1 - P_{lat})e^{-R_0}). \quad (9)$$

To account for latency, we simply use q_{lat} instead of q in Eq (7) to calculate P_c.

We visualize Eq (9) as the probability to clear infection in the face of increasing drug strength under conditions where the initial number of infected cells is small, while R_0 is within the in vivo range for initial infection ($N_0 = 2$, $R_0 = 10$, (55)). Therefore, at drug level $1/d > 10$, $R_0^{drug} < 1$ (Figure 5A, horizontal green lines in each graph). We examined clearance with P_{lat} ranging from 0 to 0.5 (Figure 5A). We compared the effects on clearance of drug mechanism d_2 which decreases viral burst size, versus d_1 which decreases infection frequency. In the case where $P_{lat} = 0$, $P_c = 1$ at $R_0^{drug} \leq 1$. As previously described, clearance was lower with mechanism d_2 relative to d_1 at drug levels where $R_0^{drug} > 1$ and the difference decreased as $R_0^{drug} \rightarrow 1$.

Even at a relatively low frequency of latent cells ($P_{lat} = 0.01$), latency had a visible effect on clearance probability with drug mechanism d_2 (Figure 5A, orange lines). This effect became more pronounced as the frequency of latent cells increased. At $P_{lat} = 0.5$, less than a quarter of infection attempts were cleared at the d_2 drug strength where $R_0^{drug} = 1$. In comparison, all infection attempts are cleared at this drug strength without latency. Interestingly, increasing d_2 further increased clearance. This reflects the fact that at higher drug strength, the number of transmission events between cells becomes smaller before the infection terminates. Therefore, the probability of forming a latent cell and hence making infection unclearable becomes lower.

We next examined the sensitivity of drug mechanism d_1 to latency. Unlike with drug d_2, it was difficult to discern the effect of latency on probability of clearance with drug d_1 (Figure 5A, blue lines). We therefore calculated the drug strength necessary to clear 50% or 75% of infection attempts (Figure 5A, dashed lines). Drug strength of drug d_1 required for clearance of 50% of infection attempts was almost unchanged across the range of latent cell frequencies, while drug strength required for 75% clearance increased slightly (Figure 5B). Therefore, in contrast to d_2, drug mechanism d_1 was far less sensitive to the presence of latency, even at the highest frequency of latent cells.

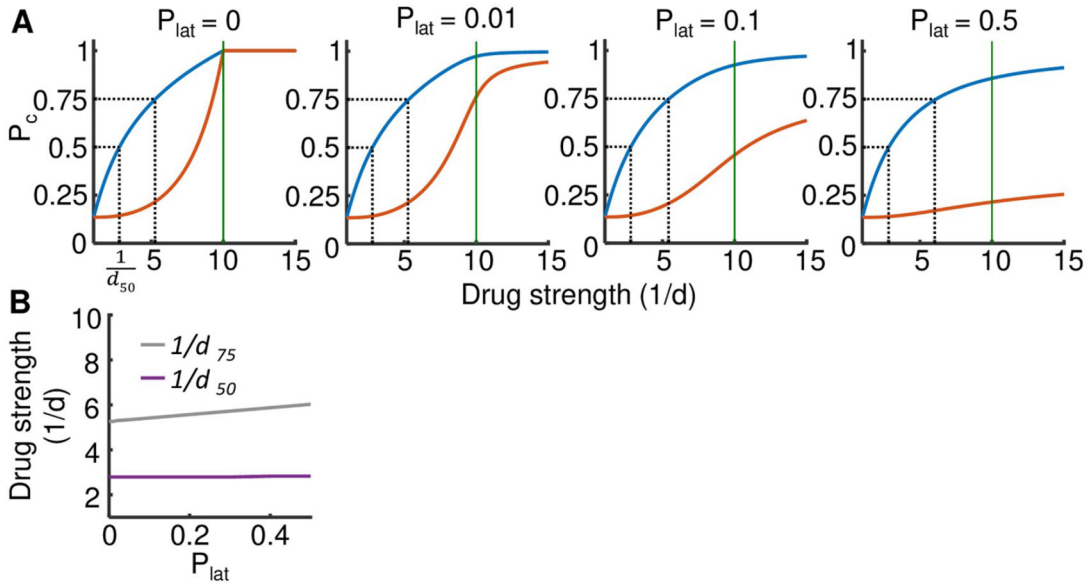

Fig. 5. Effect of latency on the probability of infection clearance.
(A) P_c was calculated as a function of increasing drug strength (1/d) with $N_0 = 2$ and $R_0 = 10$. Therefore, at 1/d > 10 (denoted by green line), $R_0^{drug} < 1$. Drug d_1 (blue line) decreases infection frequency and d_2 (orange line) decreases burst size from an already infected cell. The probability of an infected cell to become latent was P_{lat}, and the graphs show calculated P_c at the different P_{lat} values indicated above each panel. Drug strength for drug d_1 required to clear 50% and 75% of infection attempts are shown by dashed lines. $1/d_{50}$ is indicated in the first panel. (B) Drug strength for drug d_1 required to clear 50% (purple line) and 75% (grey line) of infection attempts as a function of P_{lat}.

2.4 Discussion

In this study we modelled and experimentally measured the clearance probability of HIV-1 infection as a function of the effect of drug on the basic reproductive ratio of infection R_0 and the number of initial cells N_0 infected by the viral input dose. We chose drug concentrations where HIV-1 infection was able to expand to investigate the effect of sub-optimal HIV-1 inhibition.

The reasons to consider sub-optimal drug concentrations are that ART penetration may be lower in the mucosa where the infection takes place, that it is challenging to maintain adherence in healthy individuals on PrEP, and that it is useful to future approaches to understand the basic principles of initial viral infection.

We have shown analytically and experimentally that, under conditions where drugs do not completely inhibit expansion of established infection, it is still possible to clear initial infection provided the number of initial infected cells per infection attempt is low. We derive the clearance probability in Eq (7) and show that clearance is dependent on using a drug which is able to decrease infection frequency and therefore act before the generation of the first infected cells. The intuition is that if R_0 of infection is relatively large despite the drug, termination of infection originating in an initially infected cell becomes unlikely.

However, either an initially infected cell is present, or it is not, and the probability of this depends on N_0. If N_0 is low, a drug which can decrease it further will have a strong effect on the probability of infection clearance regardless of its effect downstream of the first infection.

The model output using the measured values for N_0 and R_0 resulted in predicted probabilities of infection clearance which were higher than the experimentally observed clearance frequencies for all conditions. We speculate that this is due to an underestimation of the input number of infected cells N_0. We measured N_0 one viral cycle after cell-free infection. If GFP expression in an infected cell was below threshold of detection at that time, the infected cell would not be detected, yet still amplify infection. Despite this, the relative effectiveness of each drug mechanism was clearly predicted by the model.

Factors in vivo which may lead to deviations from model predictions include transmission by cell-to-cell spread (35-51). Cell-to-cell spread of HIV-1 should reduce the effectiveness of PrEP since the drugs would only act on R_0 and not on N_0. If the initial exposure is indeed to cell-free virus, the higher efficiency of cell-to-cell spread which results in lower drug sensitivity would make sub-optimal levels of ARVs even less likely to be able to clear infection once initial cellular infection has taken place.

In our analysis we assumed that once the first cells are infected, infection proceeds without further bottlenecks and essentially depends on the value of R_0 in the presence of drug. We further considered that a small number of initially infected cells is the physiological situation. Intravaginal SIV infection of rhesus macaques supports the view that the major bottleneck to the establishment of infection is infection of the initial cells. It was observed that even with exposure to a large dose of virus, most of the inoculum was lost at the initial infection stage, and the rest gave rise to few infected cells (33). Other bottlenecks to systemic infection spread may exist, and establishment of infection may be a two-step process (21), where resting CD4+ T cells are initially infected in the mucosa (33, 69). HIV-1 is then transmitted with a delay from the mucosa to lymph nodes, a process which may involve transmission of virions on dendritic cells homing to the lymph nodes to present antigen (34, 70). Therefore, a relatively large number of initially infected cells in the mucosa may decay to one or few infected cells which initiate systemic infection (21). In this case, it has been shown that the probability to establish infection is$\sim N_0 P_{estab}$, where P_{estab} is the probability for one initially infected cell to establish infection (21). Hence, even in a two-step infection process, the sensitivity to N_0 still holds.

HIV-1 has been observed to rapidly seed a latent reservoir of infected cells (20). We therefore examined the effect of latency on the probability to clear infection as a function of drug strength. Interestingly, for a drug which could target initial infection, clearance probability was similar regardless of whether latency

was present or absent. In contrast, latency had a far stronger effect on the probability of infection clearance if the inhibitor used could not interfere with initial infection, even when $R_0 < 1$. The latter observation is consistent with a critical role for latency in infection establishment under unfavourable conditions for viral replication (21).

The current study shows that sub-optimal drug inhibition can clear HIV-1 infection before it is established, provided the number of initial HIV-1 infected cells is low, and the drug is able to target initial infection. In this situation, the presence or absence of latency has a weak impact on the outcome. More generally, it indicates that in diseases which involve transmission of low pathogen numbers upon exposure, but have robust replication when established, a possibility to clear infection should exist even with relatively weak inhibition if initial infection is targeted.

2.5 Conclusion

We investigated why initial HIV-1 infection can be cleared with inhibitors before it is established but not after. We modelled infection with a branching process and used *in vitro* experimentation to test the model. We examined two drug mechanisms: inhibition of infection frequency, and reduction of the burst size of viable virions from an already infected cell. We found that the small difference in timing between the two mechanisms is critical in clearing of low dose HIV-1 transmission. Despite similar effects of both drug mechanisms on HIV-1 replication, only the drug mechanism reducing infection frequency, which could act before the first cells were infected, was able to clear infection. We conclude that the difference may not require the presence of a latent reservoir, but is rather a numbers game: while an imperfect drug may not clear every infection attempt, it may be successful at clearing infection if the number of cellular infection attempts are few.

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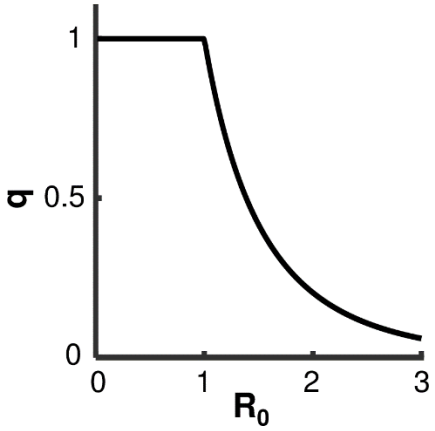
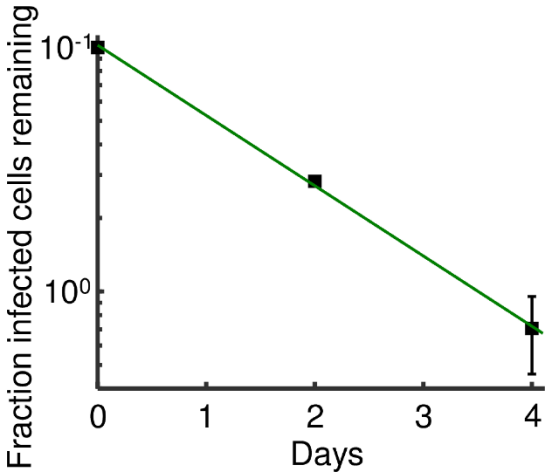
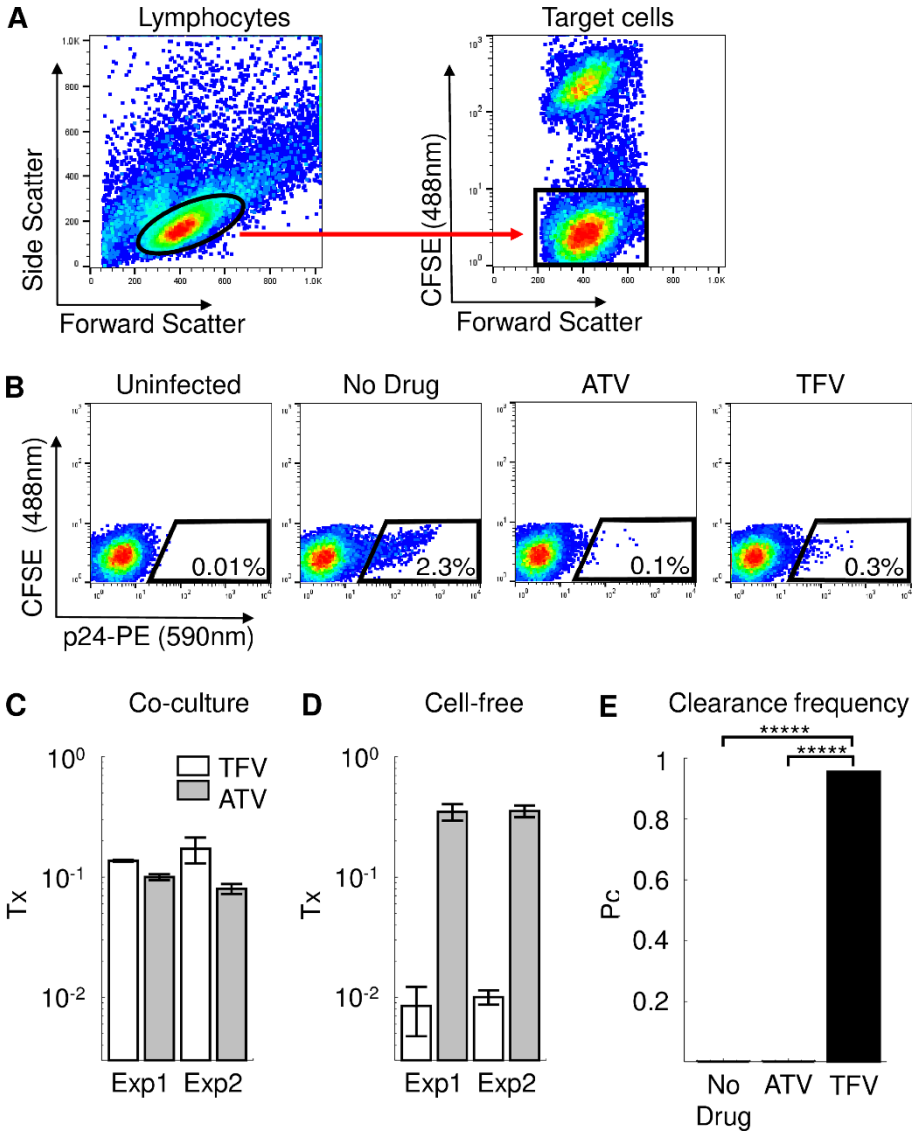
Supplementary figures



Supplementary Figure 1: *q* as a function of R_0.



Supplementary Figure 2: Estimation of the half-life of infected cells in the presence of ATV.
Half-life of infected cells was estimated using the fraction of live infected cells over time in the presence of ATV after saturating infection. Shown are the means and standard deviations of the number of live infected cells normalized by the number at the first time-point measured. Line is the fit to $y = e^{rt+b}$, with r = -0.66/day. Half-life was 1.05 days.



Supplementary Figure 3: Infection clearance with drugs in primary cells.

(A) Gating strategy to detect the number of infected cells in coculture infection. Cells were first infected with cell-free HIV-1 and used as the infecting (donor) cells for coculture infection. Donor cells were labelled with CFSE and added to uninfected target cells (Materials and methods). To quantify the number of infected target cells, the lymphocyte population was selected using forward scatter (FSC) and side scatter (SSC) and donor cells were gated out by selecting the CFSE negative population. (B) Fraction of infected target cells in coculture infection. X-axis shows infection as detected using a stain for intracellular HIV-1 Gag protein, y-axis is CFSE fluorescence. First plot shows uninfected cells, second plot shows infection in the absence of drug, third plot shows infection with 24 nM ATV, and forth plot is infection with 40 μM TFV. (C) Decrease in coculture infected target cells with drug relative to no drug with 40 μM TFV or 24 nM ATV. Tx = (number infected cells with drug)/(number infected cells without drug). Mean and standard deviation of 3 replicates from two independent experiments. (D) Decrease in the number of cell-free infected cells with drug relative to no drug (Tx, equivalent here to N_0^{norm}) with 40 μM TFV or 24 nM ATV. (E) Probability of infection clearance with 40 μM TFV or 24 nM ATV. Pooled data from 5 independent experiments, n = 45 samples each for no drug, TFV, and ATV. None of the infection attempts with no drug or ATV were cleared, while all but 2 of the infection attempts were cleared with TFV. Difference between TFV and the other two conditions was significant ($p=2 \times 10^{-23}$ by Fisher's exact test).

CHAPTER 3:

Determinants of HIV-1 control

Abstract

HIV-1 infection usually progresses to AIDS within 10 years in untreated individuals, but there is a group of infected individuals, known as HIV-1 controllers, who show no disease progression and are able to maintain low levels of HIV-1 RNA in plasma as well as normal CD4+ T cell counts. This group of individuals is highly heterogeneous because of the different mechanisms involved in HIV-1 control. In this review we highlight the viral and host factors, both immunological and genetic, that have been associated with HIV-1 controller status.

3.1 Introduction

HIV-1 infection remains a global epidemic with approximately 39.6 million people living with HIV-1 worldwide (1). The natural course of HIV-1 infection, in the absence of ARVs, is divided into three phases (reviewed in (2, 3)). Briefly, the initial phase is known as acute infection, where following HIV-1 infection, there is a massive viral replication with the virus typically peaking at $>10^6$ RNA copies/ml in the blood followed by a reduction in the number of CD4+ T cell lymphocytes. This initial replication allows a quick viral dissemination throughout the body to different organs and tissues. At approximately 2-3 weeks post-infection the second phase starts, the asymptomatic phase, where a strong cytotoxic T cell response (which precedes the development of HIV-1-specific antibodies) is associated with a pronounced drop in viral RNA levels (to a level known as the viral load set point) and a partial reestablishment in CD4+ T cell counts. During the asymptomatic phase, viral replication is ongoing, but the individual maintains an immune response that partially controls the infection. Despite this partial control, CD4+ T-cell lymphocytes decrease gradually over time at an approximate rate of 25-60 cells/µl per year eventually triggering the development of AIDS, typically at 8-10 years post-infection. During the phase of AIDS, the disease is characterized by the emergence of clinical symptoms, CD4+ T-cell lymphocytes <300cells/µl, reduction of the HIV-specific CD8+ cytotoxic T lymphocytes (CTLs), and an increase in the levels of viral RNA in plasma. However, the course of infection varies greatly between individuals where a combination of host genetic, host immunological and viral factors contribute to differences in HIV-1 disease progression patterns (Figure 1, panel A and B). Multiple studies have sought to determine these factors and understand their impact on disease progression, with the hope of harnessing these factors to control progression even in the absence of antiretroviral drugs (ARVs) (4-9).

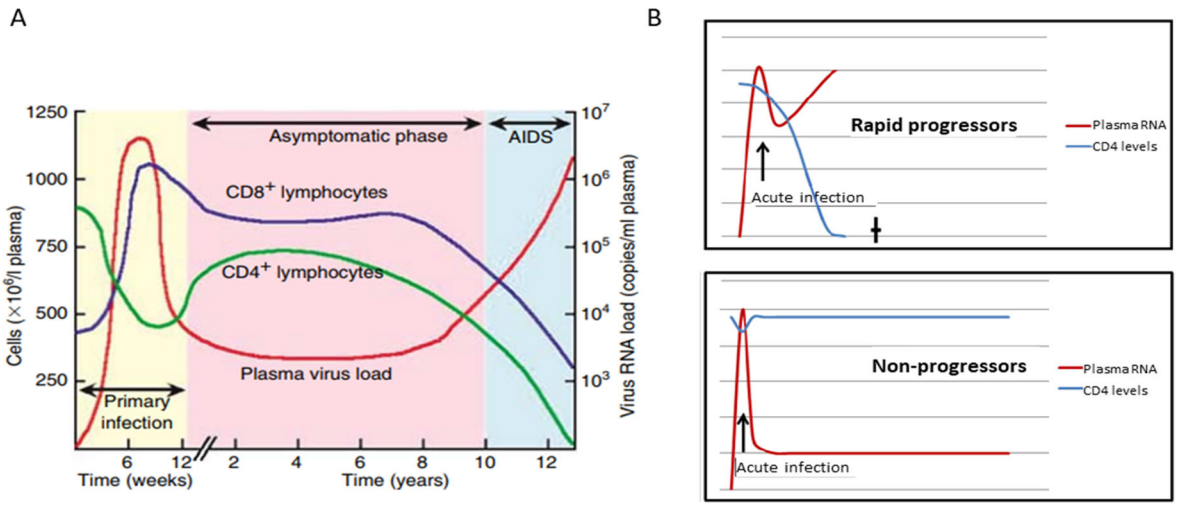


Fig. 1. Schematic of typical HIV-1 infection and different rates of disease progression.
Schematic of typical course of HIV-1 infection showing changes in CD4+ and CD8+ T-cell counts in peripheral blood and viral load (VL) (Modified from Munier and Kelleher, 2007(2)) (A); Clinical course of HIV-1 infection in rapid progressors (top panel) and in non-progressors (bottom panel), showing changes in VL and CD4+ T-cell counts (Modified from Casado lab) (B).

Various types of disease classifications have been used, based on clinical and/or diagnostic criteria, years of follow-up and viral load quantification (3-6). The classifications include non-progressor (NP), long-term non-progressors (LTNPs), long-term survivors (LTS), elite suppressors (ES), HIV-1 controllers (HIC), elite controllers (EC), viraemic controllers (VC), non-controllers (NC), chronic progressors (CP) and rapid progressors (RP). Table 1 describes the criteria used to define these different terms and simplifies the classification of disease progression phenotypes (10, 11), with the goal of clarifying the terminology used to describe disease progression.

The mechanisms underlying natural HIV-1 control are not fully understood. Multiple factors are at play in different individuals; the achievement of control cannot be explained by one single factor (5, 7, 12) (Figure 2). The purpose of this review is to give a concise overview of the different viral and host factors associated with differences in HIV-1 disease progression rate to date. While these factors are discussed separately, it is important to note that they are closely interlinked: the majority of host genetic factors linked to altered disease progression mediate their effect through influencing host immune responses to HIV-1; similarly, most viral genetic factors associated with slower or faster rates of disease progression are themselves consequences of host immune responses and/or affect pathogenesis through altering the effectiveness of host immune responses.

Table 1: HIV-1 disease progression classifications and criteria.

Name	CD4+ cell counts (cells/ul)	Years of follow-up	Viral load (copies/ml) (Plasma HIV-1 RNA)	ART	Symptomatic infection
Non-progressor (NP)	Includes all patients with lack of disease progression				
Elite controller (EC)	>350/>400-500	*/>10/ From 6 months to 16 years	<50	No	No
Viraemic controller (VC)	>350	*/>10	50-2000	No	No
Long-term non-progressor (LTNP)	>350	>7/>10	>2000	No	No
Long-term survivor (LTS)	500	10	*	No	No symptoms/AIDS-free
Elite suppressor (ES)	Normal levels	Several years	<50	No	No
HIV-1 controller (HIC)	*	10/1	400/2000	No	*
Viraemic non-controller (NC)	*	>10	>2000	No	No
Chronic progressor (CP)	*	*	>2000	No	Yes
Rapid progressor (RP)	▪ ≥2 CD4 T cell measurements <350 within 3 years after seroconversion, with no value >350 afterwards in the absence of antiretroviral therapy (ART). ▪ And/or, ART initiated within 3 years after seroconversion, and at least one preceding CD4 <350. ▪ And/or, AIDS or AIDS-related Death within 3 years after seroconversion and at least one preceding CD4 <350.	3 (time to end-point)	*	No	Death, AIDS, or ART initiation used as endpoints.

*Parameter not used in this definition.

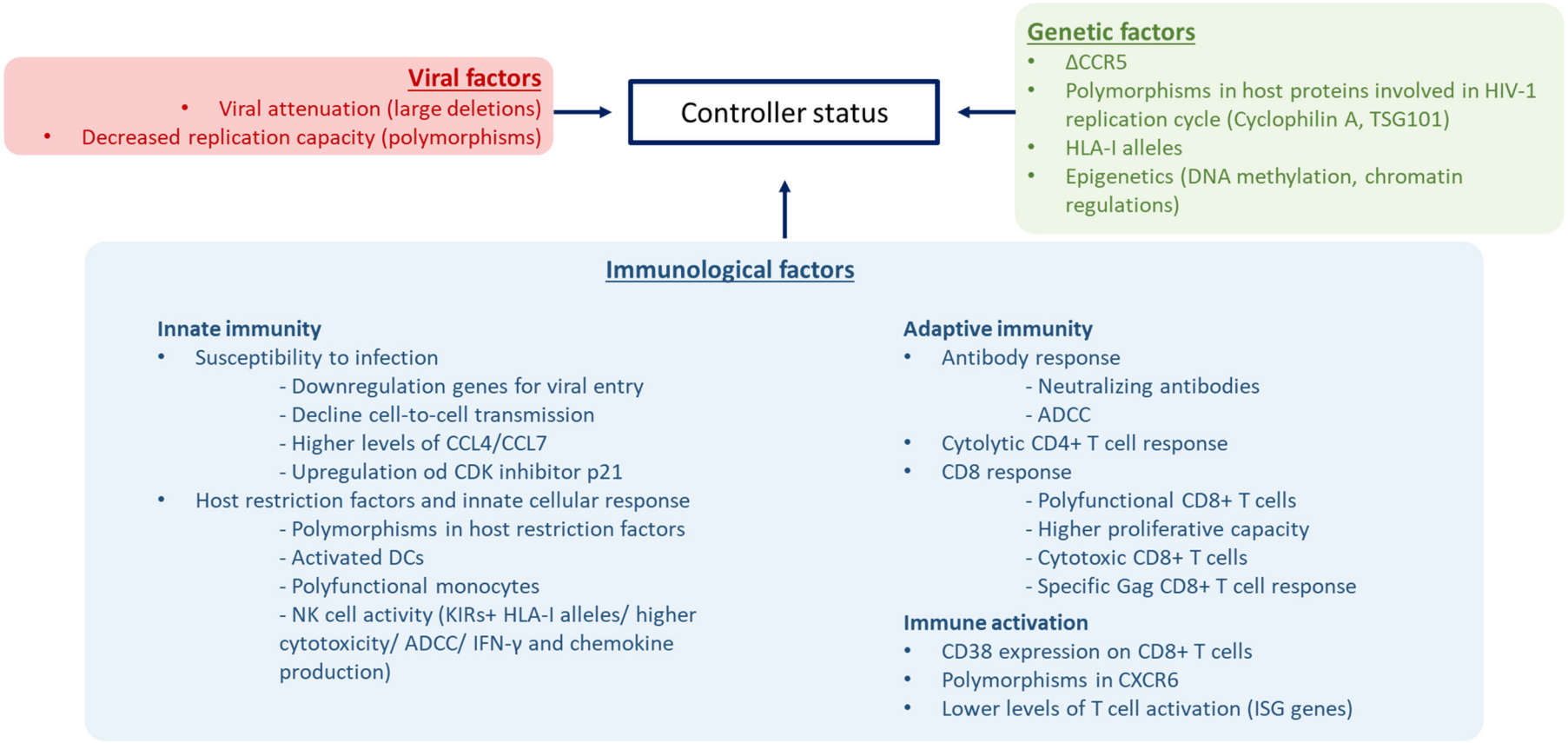


Fig.2. Factors involved on the control of HIV-1 disease progression. Certain factors have been associated with the presence of controller status to date, including viral (red), genetic (green) or immunological (blue) factors. It should be noted that factors have been distributed into the different categories for easy visualization, and there may be some overlap between genetic and immunological factors (genetic traits may have a direct impact on immune responses, e.g. single nucleotide polymorphisms may impact on expression levels of immunological mediators).

3.2 Viral factors

The impact of the virus strain on HIV-1 disease progression is clearly demonstrated by non-progression to disease in individuals infected with strains containing large deletions in the *nef* gene (13-15). Control in these cases may be explained by significant attenuation of the virus replication due to the deletions, as well as the absence of the many antagonistic effects of Nef, such as CD8+ cytotoxic T cell (CTL) evasion through Nef-mediated down-regulation of HLA class I (HLA-I) in infected cells (15). However, large viral deletions or gross sequence defects of the transmitted virus account for the minority of control cases (16). Nevertheless, transmission of viruses with decreased replication capacity due to single nucleotide polymorphisms rather than gross defects, has also been shown to result in benefit to the host. For example, transmission of strains with attenuating CTL escape mutations in *gag* to HLA mismatched hosts has been shown to result in lowered viral load set point or slower CD4 decline in the host (17-21), and this may even facilitate development of controller status in some cases (22). However, an attenuated virus alone is not sufficient for control, as is evidenced by loss of viral control when effective CTL responses are lacking despite virus attenuation (23-25). While numerous studies have shown an overall tendency for attenuated function of various proteins isolated from the plasma of EC during chronic infection (9, 22, 26-29), it is likely that this is due to the immune responses of the EC attenuating the plasma virus, while replication competent virus is archived in the proviral DNA, rather than the attenuated virus being the cause of the control (30-36). Furthermore, the isolation from some EC of replication competent viruses with replication/pathogenic potential equivalent to that of laboratory strains or viruses isolated from CP (33, 34, 37), as well as transmission of replication competent viruses from EC to others who become progressors, illustrates that the development of controller status is likely to depend more on host factors than virus factors.

3.3 Host factors

3.3.1 Host genetics

Polymorphisms in host proteins that are involved in the replication cycle of HIV-1, such as CCR5 (a co-receptor for virus entry), cyclophilin A (promotes HIV-1 infectivity by facilitating viral uncoating) and Tsg101 (participates in HIV-1 budding by interacting with viral protein), have been associated with differences in susceptibility to HIV-1 infection or in the rate of progression to AIDS (38-40). CCR5 is the most well-known example here, where individuals who are homozygous for a 32-base pair deletion in the CCR5 gene show almost complete protection against CCR5-tropic HIV-1 acquisition (5, 40, 41) and bone marrow transplantation from donors homozygous for the CCR5 deletion mutation has led to the only 2

known cases of complete cure of HIV-1 - the “Berlin patient” and the “London patient” (42, 43). In addition, those who are heterozygous for the CCR5 deletion mutation show delayed progression to AIDS (44-46). Interestingly, lower levels of CCR5 gene DNA methylation have also been associated with viral control (47), indicating that epigenetics (modifications, determined by DNA methylation or chromatin regulations, that regulate gene transcription and expression without changing the DNA sequence) could also play a role in clinical course of HIV-1 (4, 48, 49).

Besides polymorphisms in host proteins involved in virus replication, polymorphisms in host proteins key in the immune response against HIV-1 are associated with differences in disease progression rate. Indeed, the most significant genetic determinant of clinical outcome in HIV-1 infection is the HLA-I profile of the host (6, 50). HLA-I molecules present viral peptides to HIV-specific CD8+ cytotoxic T lymphocytes (CTLs), allowing for recognition and elimination of infected cells. “Protective” HLA-I alleles, such as HLA-B*27, HLA-B*57, HLA-B*58:01, HLA-B*81:01, HLA-A*74, have been associated with low viral loads and slower progression to AIDS (35, 50-57), while “risk” HLA-I alleles, such as HLA-B*35, HLA-B*08, HLA-B*58:02, HLA-B*18, have been associated with a susceptibility to rapid disease progression (51, 56). The amino acid variants at positions in the peptide binding groove appear to distinguish these “protective” and “risk” HLA-I alleles (50). Protective HLA-I alleles in conjunction with specific natural killer (NK) receptors, known as killer inhibitory receptors (KIRs), have also been shown to increase the likelihood of achieving controller status (58, 59). For example, KIR3DS1 and KIR3DL1, when interacting with HLA-B alleles, are associated with delayed disease progression in cohorts of HIV-1-positive individuals with spontaneous control of viral load (60). Additionally, HLA-B*57 expressed in combination with KIR3DL1*h/*y (61), as well as a higher KIR3DS1/L1 ratio (corresponding to a lower threshold for NK activation) (62), is more prevalent in exposed seronegative individuals, suggesting that these characteristics may contribute to HIV-1 resistance. The underlying basis for the particularly strong association between HLA-I alleles and HIV-1 disease progression (and/or resistance to HIV-1 infection) is not fully understood but appears to involve the specificity and quality of the CTL response, the interaction between HLA-I alleles and NK cells, as well as the relationship between HLA-I alleles and immune activation status as further discussed below.

3.3.2 Host immune response

Consistent with the strong association between different HLA-I alleles and differences in clinical course, the CD8+ T cell response, which is determined in part by HLA-I alleles, is the dominant feature of immune defence in EC (63). However, there is considerable heterogeneity between controllers, and additional factors may act together with, or independently of CTLs, to achieve virus control (64). In addition, a subset

of EC may eventually lose control while others maintain durable control (8, 65). More recently, transcriptome studies have identified genes that are differentially expressed in CP and controllers, thereby contributing to the understanding of pathogenesis as well as potential mechanisms involved in control of disease progression and these studies are highlighted below. A discussion on various immune responses and their role in determining rate of disease progression, as well as durability of virus control, follows.

3.3.2.1 Innate response

3.3.2.1.1 Susceptibility to infection

Data suggests that EC have a reduced susceptibility of target cells to support HIV-1 infection. Zhang et al. (2018) performed transcriptome analysis and observed that CXCR6 and SIGLEC1 genes were downregulated in EC, suggesting that a mechanism for increased control in EC is decreased susceptibility of T lymphocytes to HIV-1 entry and declined cell-to-cell transmission mediated by myeloid cells (66-68). They also describe higher levels of CCL4 and CCL7 in EC than CP; CCL4 and CCL7 are chemokines that bind to CCR5, one of the coreceptors used by HIV-1 to enter the cell (48). Multiple studies show that CD4+ T cells from EC are resistant to HIV-1 infection in culture, and some have associated this phenotype with increased levels of cyclin dependent kinase (CDK) inhibitor p21. It has been suggested that p21 may indirectly block HIV-1 reverse transcription by inhibiting CDK2-dependant phosphorylation (69-71).

3.3.2.1.2 Host restriction factors and innate cellular response

Host restriction factors constitute a first line of defence; they block steps in the viral replication cycle, and some can also act as sensors that trigger innate responses against infections. Polymorphisms in the IFN-α receptor as well as restriction genes upregulated by IFN-α, namely APOBEC3G, SAMHD1, tetherin, and TRIM5a have been linked to differences in disease progression (72-75). However, it appears that polymorphisms in identified restriction factors are not the cause of viral control in the majority of EC (76).

Innate cells, including dendritic cells, monocytes and NK cells may play a role in determining the rapidity of disease progression. HIV-1 activates dendritic cells (DCs) via toll like receptors (TLR) and induces the secretion of cytokines, such as type 1 IFN. Studies show an increase in the antigen-presenting properties of myeloid DCs of EC, while their TLR- dependent secretion of proinflammatory cytokines is reduced (77). Multiple studies have shown that EC have higher levels of plasmacytoid DCs than CP, and similar levels to uninfected individuals, with preserved functionality that translates into sustained secretion of type 1 IFN and induction of T cell apoptosis, thereby reducing viral production (76, 78-80). Superior monocyte function is also indicated in controllers; specifically, transcriptomic studies suggest that monocytes may

contribute to the phenotype of viral control. In monocytes from LTNPs, compared with CP, there is an upregulation of interrelated pathways of TLR signalling (with down-stream expression of antiviral cytokines), cytokine-cytokine receptor interactions, cell-cycle, apoptosis and trans-endothelial migration, which indicates superiority in the innate immune response in monocytes from LTNP compared to CP (81). Furthermore, a longitudinal single cell transcriptomic analysis suggests that monocytes, as well as NK cells, acting alongside T cells could play a role in the development of the controller phenotype (82). In that study, the hyper-acute phase was characterized by proinflammatory T cell differentiation, prolonged monocyte MHC II upregulation and persistent NK cell cytolytic killing. During the first weeks of infection in two individuals who became VC, they identified polyfunctional monocytes, as well as a subset of cytotoxic, proliferating NK cells and suggest that the proliferating NK cells may function alongside CTLs early in infection, mitigating CTL antigenic load and subsequent exhaustion.

Various other studies have also linked better NK functionality with viraemic control (62, 83, 84). As described in the host genetics section of this review, specific NK receptors in conjunction with protective HLA-I alleles have been shown to increase the likelihood of achieving controller status (58, 59). These receptor-HLA combinations may associate with better NK functionality. For example, HIV-1 controllers expressing HLA-Bw4*801 on target cells and KIR3DL1 on NK cells displayed a stronger target cell-induced NK cytotoxicity compared with CD8+ T cells of the same individuals (85). A study evaluating the phenotypic and functional properties of CD56/CD16 NK cells, found higher IFN-γ expression and cytolytic activity in the CD3-CD56+ NK subset in LTNP and controllers than in CP (86). This subset of NK cells usually diminishes with HIV-1 infection (6, 86). Further, increased IFN-γ and chemokine production (CCL3, CCL4 and CCL5; natural ligands of CCR5) of NK cells has been associated with resistance to HIV-1 infection and delayed disease progression (83).

3.3.2.2 Adaptive immunity

3.3.2.2.1 Antibody response

Several studies have shown that EC have lower titers of broadly neutralizing antibodies and similar levels of autologous neutralizing antibodies when compared with CP (87-89), suggesting that neutralizing antibody responses are not a main determinant of elite control of HIV-1 replication. Data suggests that sufficient antigenic stimulation is generally required to develop broadly neutralizing antibody activity (89), however, there is considerable heterogeneity in controllers, and although less common, broadly neutralizing antibodies have been detected in EC (90). Interestingly, neutralizing antibodies to a conserved gp41 epitope

were reported to be more common in LTNP (24%) than CP (<5%) and hypothesized to contribute to long-term control in these individuals (91).

There is some evidence that non-neutralizing antibody activity may play a role in viral control. NK cells can mediate antibody-dependent cellular cytotoxicity (ADCC), linking innate and adaptive immunity, and these responses were reported to be stronger in HIV-1 controllers (87, 92). ADCC against Envelope and Vpu proteins, which is mediated largely by NK cells, is also associated with EC (5, 93). However, the causal link between ADCC and elite control is not determined, particular since, compared with EC, equally potent ADCC activity was shown in some acutely infected individuals and individuals on ART, which may suggest that persistent viremia is responsible for a loss in ADCC activity (92).

3.3.2.2.2 CD4 responses

HIV-specific CD4+ T cell responses of EC and LTNPs have a higher cytolytic response and proliferative potential than those of CP, and also result in the secretion of multiple cytokines, including IL-2, upon stimulation, while CD4+ T cells from CP mostly secrete IFN-γ (75, 76, 94-96). Further, there are preserved central memory and activated effector memory CD4+ T cell subsets in HIV-1 controllers (97, 98). The preservation of a strong CD4+ T cell response in HIV-1 controllers may be important for CD8+ T cell-mediated control of virus replication, but whether or not it is crucial is unknown (8, 99, 100). However, a study has shown that IL-21-secreting CD4+ T cells (preserved in EC) may contribute to viral control through enhancing CD8+ T cell function (101). It is also unclear whether preserved CD4+ T cell responses in controllers are a cause or consequence of low viremia and there is conflicting data in this regard (76, 94, 99, 102). It is clear at least that the proliferative capacity of HIV-specific CD4+ T cells can be restored by ART to levels observed in LTNPs, suggesting that this characteristic is influenced by the level of viremia (94, 102, 103).

3.3.2.2.3 CD8 responses

Most of the immunological studies focus on CD8+ T cells as there is a consensus that they are the main immunological driver of control. As with HIV-specific CD4+ T cell responses, there are qualitative differences in HIV-specific CD8+ T cell responses between EC or VC and CP. HIV-specific CD8+ T cells from EC and/or LTNPs are more polyfunctional (can secrete multiple cytokines) (104), have a higher proliferative capacity when stimulated (105), are more efficient at lytic granule loading, and have a higher per-cell killing capacity (106). Interestingly, some studies have found restoration of CD8+ T cell polyfunctionality by ART, suggesting that polyfunctionality might be a consequence rather than cause of low viremia (107). It is argued that polyfunctionality is not likely to be an important determinant of immune

control as polyfunctional cells form a small subset of the total HIV-specific CD8+ T cell response (108). However, proliferative and cytotoxic capacities of CD8+ T cells were superior in LTNPs when compared with patients on ART and these characteristics may contribute to immune control of HIV-1 (7, 76).

Several studies show that Gag-specific CD8+ T cell responses are associated with better suppression of viral replication (7, 109-111). In particular, controllers with protective HLA-I alleles have CD8+ T cell responses focused on key Gag epitopes which have limited tolerance to sequence variation due to structural and functional constraints, thereby allowing them to maintain immune pressure on the virus (112). Furthermore, CD8+ T cells from HIV-1 controllers present a higher capacity to suppress viral infection *ex-vivo* (8, 64, 113), which is suggested to be the primary mechanism of control in controllers with protective HLA-I alleles, but not in those without protective alleles (64). Also, CD8+ T cells restricted by the protective HLA-I alleles are not suppressed by T regulatory cells, in contrast with those restricted by non-protective alleles (114). Specific TCR clonotypes that interact with the peptide-HLA-I allele complex, together with protective HLA-I alleles, may also determine the antiviral efficacy (115, 116).

While some individuals are able to maintain control for long periods of time, a proportion of controllers eventually lose control (8). Loss of control in HIV-1 controllers has been associated with CD8+ T cell activity. In controllers, prior to loss of control, a decrease in antiviral *in vitro* capacity of CD8+ T cells, together with an increase in expression of T cell activation and exhaustion markers (high levels of PD-1 expressing CD8+ T cells), is a predictor of failing immune control (65). CTL escape mutations were not, however, significantly correlated with loss of control in that study (65). A longitudinal study of EC identified the characteristics of those individuals that eventually lose control, termed as “transient EC”, showing that these individuals present lower Gag-specific T cell polyfunctionality, a higher viral diversity and a profile of higher proinflammatory cytokine levels before loss of control, when compared to persistent EC (8). Interestingly, a decrease on CD8+ T cell breadth has been associated with a loss of control in HIV-1 controllers with protective HLA-I alleles, while individuals without HLA-I protective alleles exhibit durable control which appears to be independent of CD8+ T cell responses (64).

It is worth noting that most studies have focused on studying CD8+ T cells responses in blood, however a recent study has associated elite control with distinct functional and transcriptional signatures of CD8+ T cells in lymphoid tissue (117). That study showed higher levels of memory and follicle-homing HIV-specific CD8+ T cells in lymph nodes of EC when compared to CP. These cells suppressed viral replication without demonstrable cytolytic activity and presented a down-regulation of inhibitory receptors and cytolytic molecules as well as an up-regulation of multiple cytokines. This suggests that the CTL-mediated

mechanisms of action may differ somewhat between blood and tissues, and more studies of cells in tissues is warranted.

3.3.2.3 Immune activation

There is much evidence supporting that immune activation plays a role in HIV-1 disease progression. The expression of CD38 (a marker of activation) on CD8+ T cells can predict progression to AIDS to a similar degree as HIV-1 viral load in early infection and is the strongest predictor in later infection (118). In addition, polymorphisms in the CXCR6 receptor (a mediator of inflammation) are strongly associated with long-term non-progression (119), and polymorphisms in genes encoding pro-inflammatory (e.g. tumour necrosis factor-α) and anti-inflammatory cytokines (e.g. IL-10) have been associated with altered rates of disease progression (39). EC have lower levels of HIV-specific CD8+ and CD4+ T cell activation (120), and have immune activation restricted to the T cell effector compartment and not a generalised pattern of immune activation (97). T cell transcriptome analysis shows a role of reduced interferon-stimulated genes (ISGs) associated with non-progressor status in LTNP and EC, and the reduction of ISG genes expression translates in a reduction of the immune system activation (4). Whole blood transcriptome studies describe a novel ISG gene (LY6E), which restrains the hyperactivation of monocytes during HIV-1 infection, which was upregulated in CP (121). Activation markers, such as the above mentioned CD38, as well as LAG-3 (coinhibitory molecule) were also downregulated in NP (4). Recently, a novel mechanism of HLA-I mediated protection was described for certain HLA alleles; namely the reduction of microbial translocation and consequently reduction in immune activation during acute HIV-1 infection (122). Collectively, these studies highlight that restriction of immune activation is a key feature in NP.

3.4 Conclusions

The existence of individuals able to control HIV-1 infection in the absence of antiretroviral therapy provides evidence that control of disease progression is possible. Despite the broad heterogeneity within HIC, there is compelling evidence that CTL responses act as the main driver of control in the majority of these individuals, especially in those with protective HLA-I alleles. However not all controllers rely on protective HLA-I alleles and CD8+T cells as mechanisms of control. Further investigation of controllers without protective HLA-I alleles is required as it seems that this subset of controllers exhibit more durable control of HIV-1 disease progression. Understanding the immune defence mechanisms in these individuals perhaps also provides more hope for harnessing a response in the general population, either for protective or therapeutic vaccines or to achieve a functional cure in infected individuals, that does not rely on the expression of protective HLA-I alleles.

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CHAPTER 4:

NK and T cell characterization in viraemic controllers with and without protective HLA-I alleles

Abstract

Within HIV-1 infected individuals a group known as viraemic controllers (VC) are able to durably maintain viral loads below 2000 copies/ml in the absence of antiretroviral therapy. Different studies have linked HIV-1 control to several virologic, immunologic or genetic factors. The aim of the present study was to gain further insight into potential mechanisms of control in VC with and without protective HLA-I alleles, particular since control is reported to be more durable in VC-. We studied 12 controllers with protective HLA-I alleles (VC+) and 9 controllers without protective HLA-I alleles (VC-) and also compared these 21 controllers with 5 rapid progressors (RP) and 4 healthy uninfected individuals (UI). Measurements included cytotoxic T lymphocyte (CTL) responses by the ELISpot assay, as well as flow cytometry-based characterization of NK cell and T cell populations, specifically analysing surface markers for activation, maturation, and exhaustion on these populations as well as cytokine secretion from stimulated NK cells. We found that both VC groups, in particular VC-, had a higher contribution of Gag CTL responses to the total CTL response than RP ($p=0.04$), however there was no significant difference in the magnitude and breadth of CTL responses between VC+ and VC-. In addition, VC- NK cells had higher levels of activation markers (HLA-DR alone ($p=0.007$) and co-expression of CD38 and HLA-DR ($p=0.03$)) and lower cytokine expression (MIP-1β and TNF-α) ($p=0.05$ and $p=0.04$, respectively) than VC+ NK cells. We found a negative correlation between the expression of MIP-1β and the co-expression of CD38 and HLA-DR ($r = -0.45$, $p=0.05$). Furthermore, VC- T cells had higher levels of CD38 and HLA-DR co-expression ($p=0.05$), and a trend of higher HLA-DR+ ($p=0.07$) as well as the senescence/terminal differentiation marker CD57 ($p=0.09$) when compared to VC+. Altogether these results suggest that VC- have a more activated NK cell profile with lower cytokine expression, and a more terminally differentiated and activated T cell profile than VC+. Further studies are required to understand how these distinct NK and T cell profiles may contribute to differing mechanisms of control in VC+ and VC-.

4.1 Introduction

HIV-1 infected individuals have different rates of disease progression in the absence of antiretroviral therapy (ART), with a subset who rapidly progress to AIDS and, at the other end of the spectrum, a subset who is able to control infection. Multiple studies have linked HIV-1 control, or lack of it, to several virologic, immunologic and genetic factors (1-6).

HLA class I (HLA-I) is the most significant genetic determinant of clinical outcome in HIV-1 infection (7). The expression of "protective" HLA-I alleles, such as HLA-B*27, HLA-B*57, HLA-B*58:01, HLA-B*81:01, and HLA-A*74, is associated with low viral loads and slower progression to AIDS (7-15); while

the expression of "risk" HLA-I alleles, such as HLA-B*35, HLA-B*08, HLA-B*58:02, and HLA-B*18, has been associated with a susceptibility to rapid disease progression (8, 13). HLA-I molecules present viral peptides to HIV-specific CD8+ cytotoxic T lymphocytes (CTLs), and a major mechanism by which individuals with protective HLA-I alleles control infection is thought to be through CTL activity (1, 4, 16-18). Furthermore, Gag-specific CTL responses are associated with the control of viral replication (19-21). However, escape mutations within Gag epitopes, or loss of breadth in Gag CTL responses with or without associated escape, can lead to loss of viraemic control by individuals possessing these protective HLA-I alleles (22). This indicates that control in individuals with protective HLA-I alleles is associated with the ability of their CD8+ T cells to control viral replication and loss of control may be precipitated by decreased breadth in Gag CTL responses (22). Despite the known mechanisms of control through CTL responses in individuals with protective HLA-I alleles, there are individuals without these alleles who maintain control and are characterised by CTLs with poor ability to suppress HIV-1 replication *ex vivo*. The mechanisms of control independent of the CTL response in these individuals are not yet fully understood (22).

Natural killer (NK) cells represent a subset of peripheral lymphocytes that play a critical role in the innate immune response to virus-infected and tumour transformed cells (23). NK cells have multiple direct antiviral functions and also act as immune regulators (24, 25), through production of several cytokines and chemokines (26). NK cells can be subdivided into different subsets: $CD56^{dim}$ (CD56+CD16+) NK cells are cytotoxic and produce perforin and granzyme B predominantly; $CD56^{bright}$ (CD56+CD16-) NK cells are immune-regulatory and secrete cytokines including IFN-γ, TNF-α, IL-10, and IL-13; and $CD56^{neg}$ (CD56-CD16+) NK cells are thought to represent a dysfunctional subset (27, 28). This dysfunctional subset expresses low levels of the natural cytotoxicity receptors NKp30 and NKp46, has low expression of IFN-γ, and exhibits impaired cytotoxicity (based on the expression of Siglec-7) (29). During HIV-1 infection there is a redistribution of NK cell subpopulations: in the acute phase there is an early depletion of the immune-regulatory subset ($CD56^{bright}$), then ongoing viral replication is followed by a reduction of the cytotoxic subset ($CD56^{dim}$) with a parallel increase in the dysfunctional subset ($CD56^{neg}$) (27, 30).

Different studies have linked NK cell receptors with differences in HIV-1 disease progression (2, 5, 26-28, 31, 32). Specific NK receptors in conjunction with protective HLA-I alleles have been shown to increase the likelihood of achieving controller status (33, 34). NK cells and a minority of T cells express a family of type I transmembrane glycoproteins known as killer inhibitory receptors (KIRs), which interact with HLA-I molecules to regulate their killing function (35). According to Genovese et al. (2013), KIR3DS1 and KIR3DL1, when interacting with HLA-B alleles, are associated with delayed disease progression in cohorts of HIV-1-positive individuals with spontaneous control of viral load (36). The HIV-1 controllers expressing HLA-Bw4*801 on target cells and KIR3DL1 on NK cells displayed a stronger target cell-induced NK

cytotoxicity compared with CD8+ T cells of the same individuals (36). Additionally, a higher KIR3DS1/L1 ratio has been observed in HIV-1 Exposed Seronegative (ESN) cohorts, which corresponds to the presence of more "reactive" NK cells with a lower activation threshold, suggesting that this phenotype may contribute to HIV-1 resistance (27).

Some studies report higher functionality of NK cells in HIV-1 controllers (5, 37). NK-mediated antibody-dependent cellular cytotoxicity (ADCC) responses were reported to be stronger in HIV-1 controllers (38). ADCC against Env and Vpu proteins, which is mediated largely by NK cells, is also associated with elite control (5, 39). Another study comparing controllers and long-term non-progressors (LTNP) evaluated the phenotypic and functional properties of NK cells, and suggested that LTNP could have a phenotypic and functional intermediate state between HIV-1 progressors and HIV-1 controllers. IFN-γ expression was higher in the CD3-CD56+ NK subset in LTNP and controllers than in progressors and healthy donors, and this subset also had greater cytolytic activity in the non-progressor groups (37). In addition, IFN-γ and chemokine production (MIP-α, MIP-β and RANTES) by NK cells has been associated with delayed disease progression (26). While there is data linking specific NK receptors (in combination with specific HLA alleles), NK-mediated ADCC and NK-mediated production of cytokines and chemokines with HIV-1 control, it remains unknown if NK cell function differs between viraemic controllers with and without protective HLA-I alleles.

The goal of the present study was to gain further insight into potential mechanisms of control in viraemic controllers (VC) with and without protective HLA-I alleles. We studied 12 controllers with protective HLA-I alleles (VC+) and 9 controllers without protective HLA-I alleles (VC-) and also compared these 21 controllers with 5 rapid progressors (RP) and 4 healthy uninfected individuals (UI). Measurements included the breadth and magnitude of CTL responses, as well as frequencies of NK cell and T cell populations, surface markers for activation, maturation, and exhaustion profiling on these cellular populations, and cytokine secretion from stimulated NK cells (as a measure of their function).

4.2 Methodology

4.2.1 Determination of natural mechanisms of control of HIV-1 disease progression

4.2.1.1 Study participants

Patients meeting the study criteria were from several cohorts based in Durban; namely the Sinikithemba cohort (Mechanisms of HLA-associated control and lack of control of HIV infection, BREC reference E028/99) (11), the Acute infection cohort (Characterisation of the evolution of adaptive and innate immune

responses in acute HIV clade C virus infection, BREC reference E036/06) (40, 41), the Females Rising with Education, Support and Health (FRESH) Study (Establishment and long-term follow-up of a cohort of HIV negative women in Umlazi, SA, BREC reference BF131/11) (42), and the Elite controllers cohort (Immunology and Virology of HIV Controllers, BREC reference BE102/14).

Viraemic controllers (VC) were patients who maintained a viral load of <2000 copies/ml for at least 14 months. VC studied included 12 VC with protective HLA-I alleles (VC+), where protective alleles were HLA-B*27, HLA-B*57, HLA-B*58:01, HLA-B*81:01, and HLA-A*74 (7-9, 11, 13, 14, 22), and 9 VC without protective HLA-I alleles (VC-). We also included a group of rapid progressors (RP), described as patients with (i) two or more CD4+ T cell measurements <350/mm^3 within 3 years after seroconversion, with no value ≥350/mm^3 thereafter, in the absence of antiretroviral therapy (ART), and/or (ii) ART initiated within 3 years of seroconversion and at least one preceding CD4 <350/mm^3, and/or (iii) AIDS or AIDS-related death within 3 years of seroconversion and at least one preceding CD4 <350/mm^3 (4, 43, 44). A group of healthy uninfected individuals (UI) (n=4) was included for the cellular phenotypic characterization.

Participants were ART naïve at all time points analysed. HLA-I data as well as longitudinal viral load (VL) and CD4 count data were available for all participants. There was no significant difference in VL or CD4 counts between VC+ and VC- at the timepoints analysed (Mann-Whitney, $p>0.05$; data not shown). All samples selected for analysis were based on availability of PBMCs and plasma samples, and a minimum follow-up time of 12 months of viral control for VC. For the timepoints studied, plasma was tested by the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method for the quantification of antiretrovirals (ARVs) as described previously (45) to confirm that participants were not taking ARVs. The LC-MS/MS method is a highly sensitive technique for accurate quantification of ARVs in plasma (46, 47). Participant characteristics are summarized in Table 1.

All study subjects provided written informed consent and this study was approved by the University of KwaZulu-Natal's Biomedical Research Ethics Committee.

Table 1: Study participant characteristics.

Group [a]	Patient identifier	HLA A	HLA A	HLA B	HLA B	HLA C	HLA C	Months follow-up [b]	VL [c]	CD4 count [d]	Months follow-up [e]	VL [f]	CD4 count [g]
VC+	SK-235	01:01	66:01	81:01	39/67	12:03	18	26	No VL	434	13	No VL	322
VC+	SK-354	30:01	74	35	81	4	4	34	No VL	213			
VC+	SK-362	03	74	15:03	15:10	02:02	04:01	44	No VL	406	58	310	413
VC+	SK-282/206-30-0020-0	02	34:02	44	58:01	04	07	157	<20	630	134	<20	743
VC+	111-30-0005-0	30:01	68:01	42:01	81:01	04:01	17:01	28	<20	1133	28	<20	1133
VC+	111-30-0015-0	30:04	74:01	35:01	44:03	04:01	04:01	28	<20	903	28	<20	903
VC+	SK-469/206-30-0011-0	29:02	30:02	57:03	81:01	18	18	46	550	1414	46	550	1414
VC+	AS-30-0018	02:02	02:05	57:03	58:01	06:02	07:01	85	<20	772	85	<20	772
VC+	SK-481/206-30-0007-0	01:01	74:01	15:03	81:01	02:10	18	21	65	859	21	65	858
VC+	SK-490/206-30-0012-0	30:01	68:02	39:10	58:01	03:02	15:05	21	<20	950	21	<20	950
VC+	127-33-0397-268	01:01	66:01	39:10	81:01	18:01	12:03	22	<20	672	25	260	574
VC+	SK-453	74:01	74:01	15:10	57:03	03:04	07:01	23	No VL	834	23	No VL	834
VC-	SK-209	03:01	30:01	08:01	42:01	07:02	17:01	32	No VL	416	60	1140	325
VC-	SK-275	03	30:01	08	39:10	07	12	20	No VL	550	62	0	634
VC-	SK-317	23:01	33	42:01	44	03	17:01	29	No VL	592	57	1504	675
VC-	111-30-0041-0	23:01	29:11	13:02	14:02	06:02	08:02	28	1300	587	28	1300	587
VC-	SK-452/206-30-0004-0	02:05	68:02	15:10	15:10	03:04	08:04	92	<20	391	92	<20	391
VC-	SK-470/206-30-0005-0	30:01	33:03	42:01	42:01	17:01	17:01	44	23	596	44	23	596
VC-	206-30-0024-0	33:03	43:01	14:01	53:01	04:01	08:04	14	<20	788	14	<20	788
VC-	SK-475/206-30-0002-0	30:01	66:01	15:03	58:02	02:10	06:02	22	160	1086	22	160	1086
VC-	127-33-0035-039	2:05	66:01	14:01	39:10	08:04	12:03	33	<20	465	33	<20	465
RP	127-33-0108-093	02:01	30:01	15:10	42:02	08:04	17	8	41000	216			
RP	127-33-0251-186	03:01	74	15:03	58:02	02:10	06:02	8	230000	340			
RP	AS02-0973	30:01	43:01	42:01	42:01	17:00	17:00	1	159059	208			
RP	AS02-0110	02:14	29:02	44:03	44:03	04:01	07:01	3	466000	290			
RP	AS33-0182	29:02	30:02	07:02	15:03	02:10	07:01	1	300817	147			

[a] VC+, Viraemic controller with protective HLA-I alleles; VC-, Viraemic controllers without protective HLA-I alleles; RP, Rapid Progressor.
[b] Months of follow-up time at the time point used for the ELISpot analysis.
[c] Viral load (copies/ml) at the time point used for the ELISpot analysis.
[d] CD4 counts measured as cells/μL at the timepoint used for the ELISpot analysis.
[e] Months of follow-up time at the time point used for the T cell and NK cell characterization.
[f] Viral load (copies/ml) at the time point used for the T cell and NK cell characterization.
[g] CD4 counts measured as cells/μL at the timepoint used for the T cell and NK cell characterization.

4.2.1.2 Determination of the magnitude and breadth of CTL responses

HIV-1 CTL responses were enumerated from frozen whole peripheral blood mononuclear cells (PBMCs) by the gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assay as previously described (11, 48). PBMCs were stimulated with 410 consensus clade C 18-mer overlapping peptides (OLPs) covering the entire HIV-1 proteome using a matrix system of 11-12 peptides per pool. This was followed by a separate confirmation ELISpot assay to confirm which individual peptides were recognised within a reactive pool.

Briefly, Millipore 96-well plates were coated with 100 µl of anti-IFN-γ-antibody solution (5 µl of antibody into 10 ml phosphate buffered saline (PBS)) (2 mg/well, Microsep) and stored at 4ºC, for up to two weeks, until use. PBMCs were thawed in 10 ml of R10 media [RPMI 1640 (Lonza) supplemented with 10% gamma irradiated, heat-inactivated foetal bovine serum (FBS) (Biocom), 100 U/ml penicillin/streptomycin (Lonza), 2 mM L-glutamine (Lonza) and 10 mM HEPES buffer (Lonza)], washed twice, resuspended at $1x10^6$ cells/ml, and incubated for 2 hours at 37ºC with 5% CO_2. Coated plates were washed 6 times with 100 µl of blocking buffer (PBS and 1% FBS), followed by the addition of 50 µl of R10 per well, 10 µl of the positive control (Phytohemagglutinin - PHA) to the specific control wells, 10 µl of the OLPs, or the confirmation peptides (at 33 µg/ml stock concentration and a final concentration of 2 µg/ml), and 100 µl of the cells (at a final concentration of $0.1x10^6$/well). The plate was incubated overnight at 37ºC with 5% CO_2. The following day the plate was washed 6 times with 100 µl of PBS, 100 µl biotinylated IFN-γ-antibody solution (5 µl of antibody into 10ml PBS) (2 mg/well, Microsep) was added and the plate was incubated for 90 minutes in the dark. Following PBS washes, 100 µl of streptavidin alkaline-phosphatase conjugate solution (5 µl of conjugate into 10 ml PBS) (2 mg/well, Microsep) was added and the plate was incubated for a further 45 minutes in the dark. The plate was washed with PBS and then developed using the AP Conjugate Kit (Bio-Rad) as indicated in the user manual.

The IFN- γ-secreting cells were counted using an AID ELISpot reader (AID-Diagnostika). The spot forming unit (SFU) value was multiplied by 10 (to express as per $1x10^6$ PBMC) and the background (the mean of the negative controls plus 3 times the standard deviation of the negative controls) was subtracted to calculate the SFU/well, which was then expressed as SFU per $1x10^6$ PBMC. Responses ≥100 SFU/$1x10^6$ PBMC were considered to be positive.

4.2.1.3 NK cell and T cell phenotypic and intracellular staining characterization

To perform cell phenotypic characterization, the surface markers used included those selecting for viable cells and specific cell populations (CD3 to select T cells, CD14 to exclude monocytes, CD19 to exclude B

cells, and CD56 and CD16 to select NK cells), activation markers (CD69, CD38, HLA-DR and NKG2C), maturation markers (NKG2A and CD57), the exhaustion marker PD-1 and the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46. Further to this, PBMCs were stimulated for analysis of NK cell degranulation (CD107a) and intracellular cytokine (IFN-γ, TNF-α and MIP-1β) expression was determined.

Cryopreserved PBMCs were thawed, resuspended at $1x10^6$ cells/ml in R10 media [supplemented with FCS (Biocom) instead of FBS], and rested for 2 hours at 37°C, 5% CO_2. Cells were resuspended at $10x10^6$/ml and plated in a 96 well-plate at $1x10^6$ cells/well. To phenotype the NK and T cells, the cells were centrifuged at 1800 rpm for 8 minutes, supernatant was discarded and cells were stained for 20 minutes at room temperature (RT) in the dark using LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen) and specific monoclonal antibodies: CD3-BV785, CD14-BV650, CD19-BV650, CD56-Alexa Flour 700, CD16-APC-Cy7, CD69-PerCPCy5.5, CD38-BV711, HLA-DR-PE-CF594, NKG2C-PE, NKG2A-APC, CD57-FITC, PD-1-BV421, NKp30-PE-Cy7, NKp44-PE-Cy7, and NKp46-PE-Cy7 (Supplementary Table 1). Cells were then washed with PBS, fixed with Fix/Perm Medium A (Caltag), and resuspended in 200 µl PBS until acquisition.

To assess NK cell function, PBMCs were stimulated followed by measurement of intracellular cytokines and CD107a, a degranulation marker. The stimulation was performed by plating the PBMCs in R10 in a 96 well-plate at $1x10^6$ cells/well together with K562 cells, a tumour cell line, that lacks HLA expression (ATCC, Manassas, Virginia, USA; ATCC® CCL-243™), in a target: effector PBMC ratio of 1:10. The K562 cell line lacks expression of HLA-I, resulting in activation of NK cells as there is a lack of HLA-I to provide an inhibitory signal to the NK cells (49). An unstimulated sample (PBMCs alone) was included as a baseline control. Brefeldin A (5 µg/ml, Sigma) and Golgi stop (1:10 diluted in R10) (BD Biosciences) were included in all wells to enhance the detection of intracellular cytokines and CD107a-PE-Cy5 (Supplementary Table 1) was included for detection of degranulation marker CD107a. Cells were cultured for 18 hours at 37°C and 5% CO_2. Following stimulation, PBMCs were washed with PBS and stained for 20 minutes at RT in the dark with LIVE/DEAD™ Fixable Aqua Dead Cell Stain and specific monoclonal antibodies: CD3-BV785, CD14-BV650, CD19-BV650, CD56-Alexa Flour 700, CD16-APC-Cy7. Cells were washed with PBS, fixed with Fix/Perm Medium A for 20 minutes at RT in the dark. Cells were washed again, permeabilized (Fix/Perm B, Caltag) and stained for intracellular expression of IFN-γ-PECy7, TNF-α-BV605 and MIP-1β-PE (Supplementary Table 1) for 20 minutes at RT in the dark, washed and resuspended in PBS until acquisition.

Acquisition of cells was done on an LSR Fortessa flow cytometer (BD Biosciences). At least 200,000 events were acquired per sample when possible and analysed using FlowJo software (version 10.6.1).

Compensation was calculated on the DIVA software using stained Anti-Mouse Ig, κ/Negative Control Compensation Particles (BD Biosciences). Fluorescence minus one (FMOs) were used to exclude background fluorescence in the gating strategies for each activation/differentiation marker. The gating strategies are shown in Figure 1 (cell subsets) and 2 (cell surface markers and intracellular cytokines). Expression of differentiation/activation markers were compared between patient groups (VC+, VC- UI). NK cell expression of intracellular cytokines and the CD107a degranulation marker in response to stimulation was calculated by subtracting the unstimulated condition from the K562-stimulated condition, and also compared between patient groups.

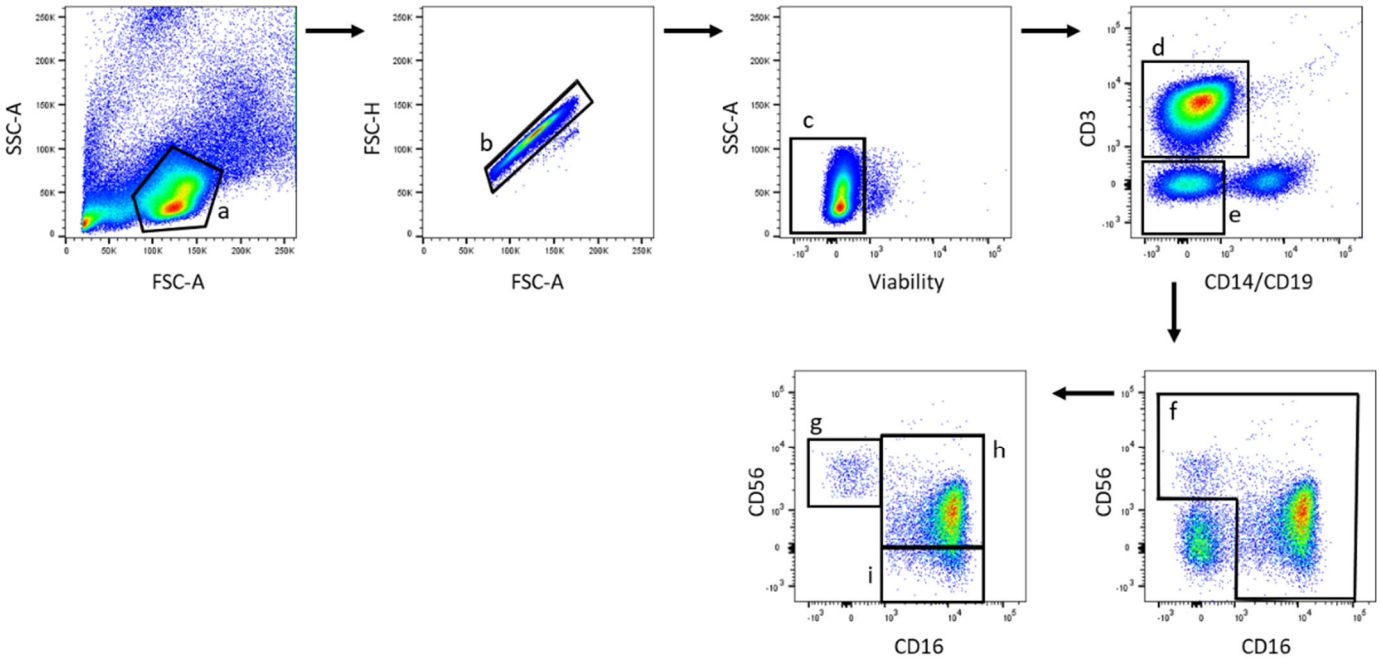

Fig. 1. Gating strategy for NK cell subsets and T cells.
Determination of the proportion of lymphocytes (a), single cells (b), viable cells (c), $CD3^{+}CD14^{-}CD19^{-}$ cells (T cells) (d), $CD3^{-}CD14^{-}CD19^{-}$ cells (e), total NK cells (f), $CD56^{bright}$ (g), $CD56^{dim}$ (h) and $CD56^{neg}$ (i).

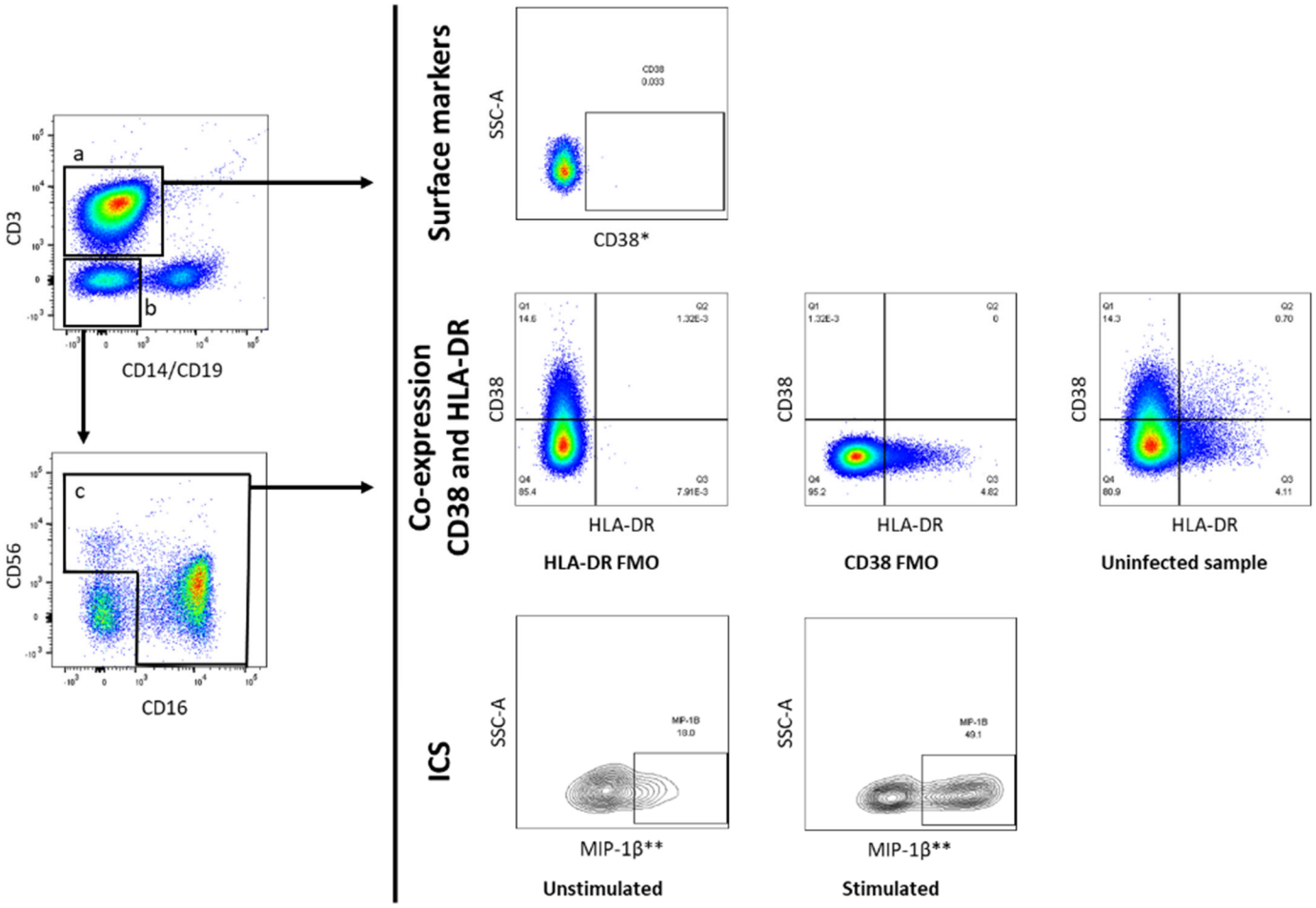

Fig. 2. Gating strategy for cell surface markers in NK cells and T cells and intracellular cytokine staining (ICS) in NK cells.

Flow cytometry representation of the gating strategy for surface markers (top panel), co-expression of CD38 and HLA-DR (middle panels) and ICS (bottom panels). CD38* is used as a representation, same strategy was used for CD69, HLA-DR, NKG2C, NKG2A, CD57, PD-1, NKp30, NKp44 and NKp46. The quadrants for co-expression were drawn using the Fluorescence minus one (FMO) sample. For ICS the gates on both unstimulated and stimulated condition were done and then values were calculated by subtracting the unstimulated condition background; MIP-1β** is used as a representation, same strategy was used for IFN-γ, TNF-α and CD107a. Gate "a" represents the CD3+ population (T cells), gate "b" represents CD3- population and gate "c" represents the NK cell population.

4.2.1.4 Data analysis

GraphPad Prism version 7 was used to construct graphs and perform statistical analyses. A significance cut off of $p \leq 0.05$ was used. One-way ANOVA with Tukey post-hoc tests were used to assess if there were significant differences in breadth/magnitude of CD8+ T cell responses, cell populations, expression of surface markers or cytokine expression between the different patient groups where more than 2 groups were compared. The Mann-Whitney test (unpaired, non-parametric t-test) was used to compare parameters when

only 2 patient groups were compared. Pearson or Spearman tests were used for correlation analysis depending on whether or not the data was normally distributed.

4.3 Results

4.3.1 VC have a more Gag-focussed CTL response than RP

A previous study from our group on VC with and without protective HLA-I alleles, suggested that control in individuals with protective HLA-I alleles may be driven by Gag CTL responses with potent viral inhibitory capacity, while control among individuals without protective alleles may be more durable and mediated by CTL-independent mechanisms (22). We therefore first sought to compare the breadth and magnitude of CTL responses in an extended group of VCs (where only 3 VC+ and 3 VC- study subjects were also in the previous study), and then perform more detailed characterisation of the NK cell and T cell (CD3+ cells) populations to further explore potential CTL-independent mechanisms of control in these VC groups.

The breadth and magnitude of CTL responses overall and to each individual protein are shown in Supplementary Tables 2 and 3. There was no significant overall difference in total breadth and magnitude between the different patient groups (VC+, VC- and RP) by ANOVA (p=0.45 and p=0.37, respectively) (Figure 3A and 3B). Similar results were obtained when results were analysed separately for each protein.

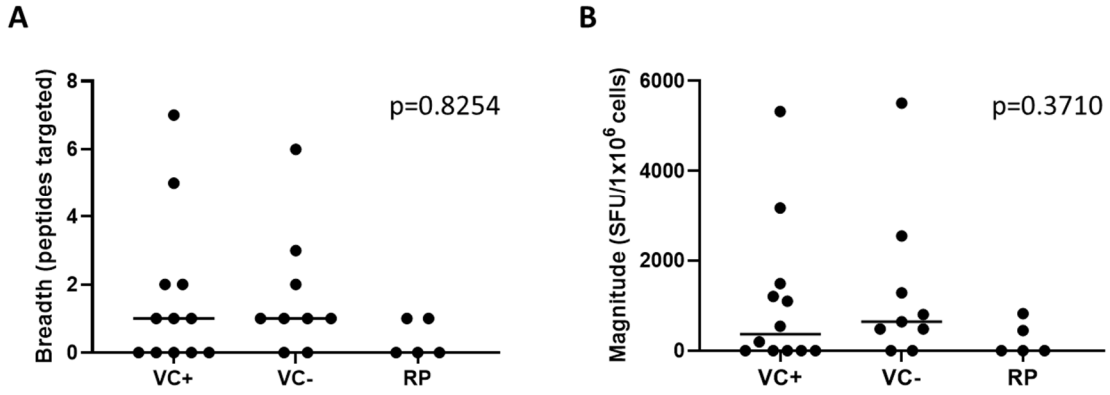

Fig. 3. Total breadth and magnitude of CD8+ T cell (CTL) responses.
Total breadth (A) and magnitude (B) of CTL responses measured by the ELISpot assay were compared between viraemic controllers with protective alleles (VC+), viraemic controllers without protective alleles (VC-) and rapid progressors (RP) using ANOVA (p value shown). The magnitude of CTL responses to individual peptides was measured in spot forming units (SFU) per million cells. Bars represent the median.

Previous reports have shown a difference in the magnitude and breadth of CTL responses between those individuals with protective alleles and those without when focussing only on epitopes in the most conserved

region of Gag (amino acids 1-56, 57-96 and 97-135) (50). Therefore, the analysis was narrowed down to overlapping peptides from those conserved regions only, however no significant difference in CTL breadth and magnitude to the overlapping peptides in those regions was observed between the patient groups overall (ANOVA, p=0.15 and p=0.19, respectively; Supplementary Figure 1).

The large majority of the CTL responses were to Gag peptides; there was a strong correlation between total breadth of response and Gag breadth (Spearman's test, r =0.87 and p<0.0001) (Figure 4A). Nearly all of the Gag responses were to the p24 region, as indicated by a very tight correlation between Gag breadth and Gag p24 breadth (Spearman's test, r =0.99 and p<0.0001) (Figure 4B).

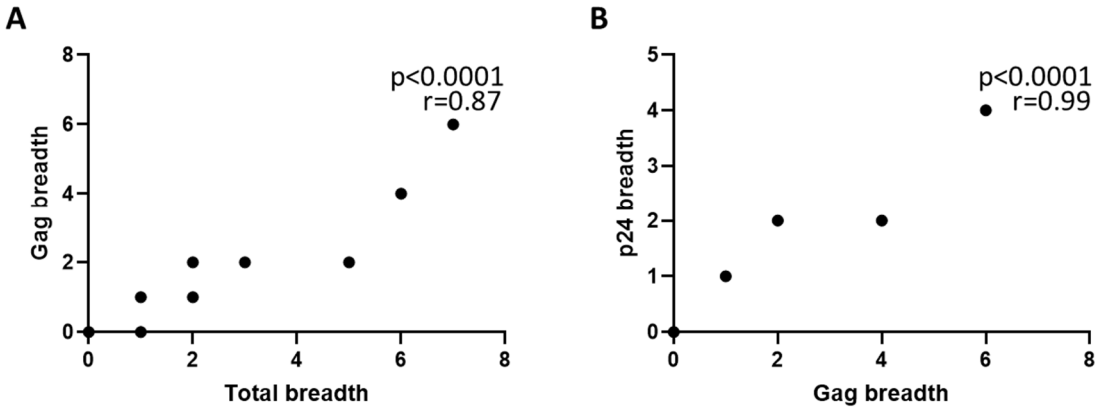

Fig. 4. Correlation of total breadth, Gag breadth and p24 breadth.
Graphs show the Gag breadth and total breadth correlation (A) as well as p24 breadth and Gag breadth correlation (B), as measured by the ELISpot assay for all groups. Spearman's correlation test was used (p value and r value shown).

Gag-focussed CTL responses as opposed to Env-focussed CTL responses have been associated with lower viral loads (19), and CTL responses to certain epitopes within the highly conserved p24 region have previously been associated with slower disease progression (20). Therefore, the contribution of the Gag CTL response to the total CTL response in an individual was calculated and compared between the patient groups. Here a significant difference was observed overall between the groups (ANOVA, p=0.04) (Figure 5), with Tukey post-hoc tests showing a significant difference between the VC- and RP groups specifically (p<0.05), and no significant difference between the VC+ and VC- groups.

In summary, there was no difference between patient groups in the breadth or magnitude of CTL responses as a whole or to specific viral proteins/regions. VC, and more especially VC-, had a greater contribution of Gag CTL responses to the total CTL response than RP, however this feature of a Gag-focussed CTL response did not distinguish the VC+ and VC- groups (Mann-Whitney, p=0.21).

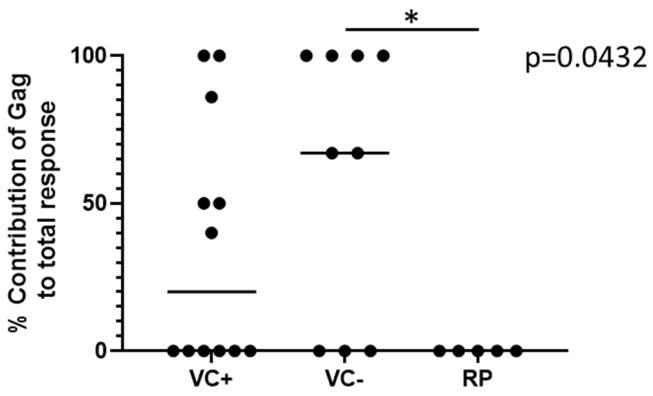

Fig. 5. Analysis of Gag-specific CD8+ T cell (CTL) responses.
Contribution of Gag CTL responses to the total CTL response (A) measured by the ELISpot assay were compared between viraemic controllers with (VC+) and without (VC-) protective HLA alleles and rapid progressors (RP) using ANOVA (p value shown) with Tukey post-hoc tests (* represents $p<0.05$). Bars represent the median.

4.3.3 NK and T cell populations: cell surface marker expression and intracellular cytokine expression

HIV-1 infection is characterized by the depletion of CD4+ T cells and the main driver of control of infection is known to be CTL responses mediated by CD8+ T cells (18). Recent studies suggest that NK cells might also play a significant role in control, nevertheless most papers focus on groups such as elite controllers (EC), LTNP, VC, viraemic non-controllers or acutely infected patients (26-28, 31, 32). Since our hypothesis is based on previous data suggesting a CD8+ T cell mediated mechanism for control in VC+ versus a CD8+ T cell-independent mechanism for control in VC- (22), and this was not fully supported by the CTL determination data, we decided to explore other immune mechanisms that might explain the different mechanisms of control between these groups. A phenotypic characterization of NK cells and T cells using cell surface markers was performed and the expression of certain intracellular cytokines in NK cells was measured.

4.3.4 NK cells from VC- have higher levels of activation markers than those from VC+ ex vivo

We observed no significant differences in NK cell subset frequencies as distinguished by expression of CD56 and CD16 markers ($CD56^{bright}$, $CD56^{dim}$ and $CD56^{neg}$) between VC+ and VC- (Mann-Whitney, $p \geq 0.25$; Figure 6), and therefore we continued further analyses on the whole NK cell population.

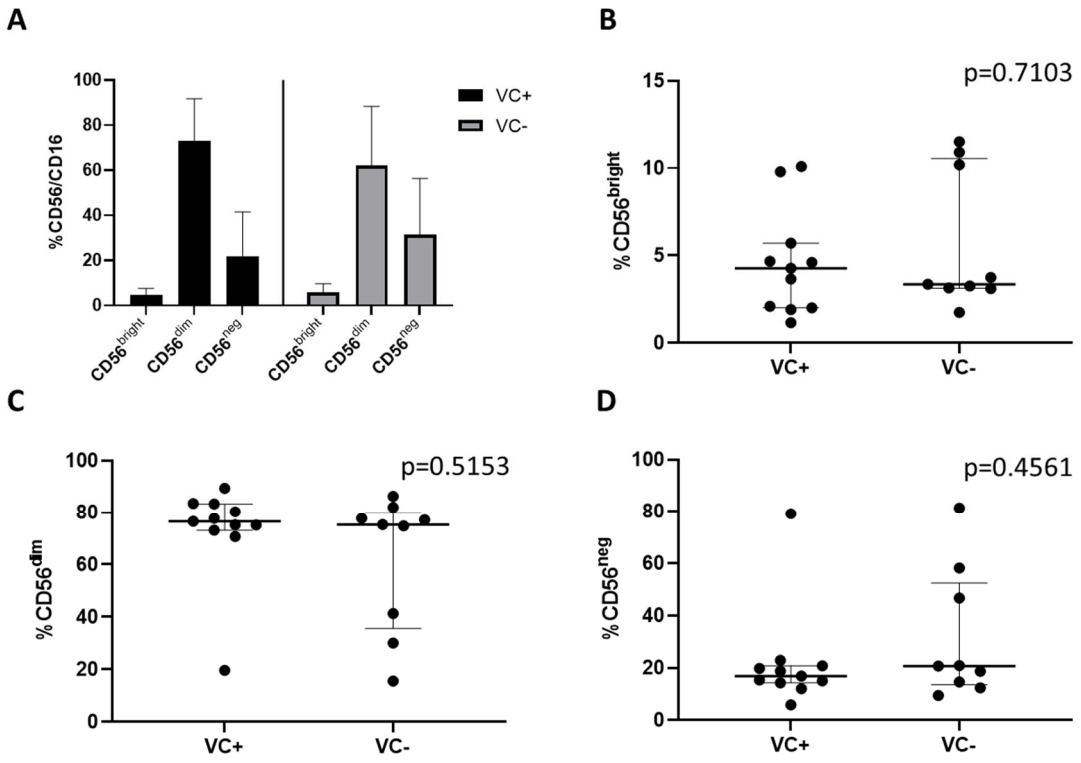

Fig. 6. Flow cytometry analysis of the NK cell subpopulations.
Frequency of the different NK cell subpopulations, as measured by expression of CD56 and CD16 surface markers, was compared between viraemic controllers with protective alleles (VC+) and viraemic controllers without protective alleles (VC-). Graphs show the frequency of the three NK cell subpopulation per VC group (VC+ and VC-) (A), and a comparison of the frequency of the CD56^{bright} subpopulation (B), the CD56^{dim} subpopulation (C) and the CD56^{neg} subpopulation (D) between VC+ and VC-. Mann-Whitney test was used (p values are shown). Lines represent the median and bars show the interquartile range.

Similarly, there were no significant differences between VC+ and VC- in NCR, maturation markers or exhaustion markers (Supplementary Table 4). However, there was a tendency of higher expression of the activation marker HLA-DR, but not other activation markers, in VC- compared to VC+ (p=0.06) (Figure 7A). To provide further insight we compared the expression of HLA-DR in VC+ and VC- to that in healthy uninfected controls (UI). Significant differences in HLA-DR expression were found between VC+, VC- and UI (ANOVA, p=0.007), where VC- had higher HLA-DR expression than UI (p<0.01) (Figure 7B). This shows that, although there is a trend of higher activation in VC- than in VC+, both VC groups have somewhat higher activation than UI (only significant for VC-), which is to be expected given that the VC in this study are chronically infected individuals.

Since most studies measure HLA-DR co-expressed with CD38 as a marker of immune activation (28), we compared the frequency of CD38+HLA-DR+ NK cells between patient groups. Similar to HLA-DR+ cells,

there was a trend of higher co-expression of CD38 and HLA-DR in VC- compared to VC+ (p=0.07) (Figure 7C). Further, CD38 and HLA-DR co-expression was significantly different between VC+, VC- and UI overall (ANOVA, p=0.03) (Figure 7D). Although, post-hoc tests were not significant (Figure 7D), VC+ were more similar to UI, while VC- showed the highest CD38 and HLA-DR co-expression overall.

Taken together, these results support higher NK cell activation in VC compared to UI, but more especially higher NK cell activation in VC-, whereas no differences in the frequency of the different NK cell subsets or other phenotypes were found between VC+ and VC-.

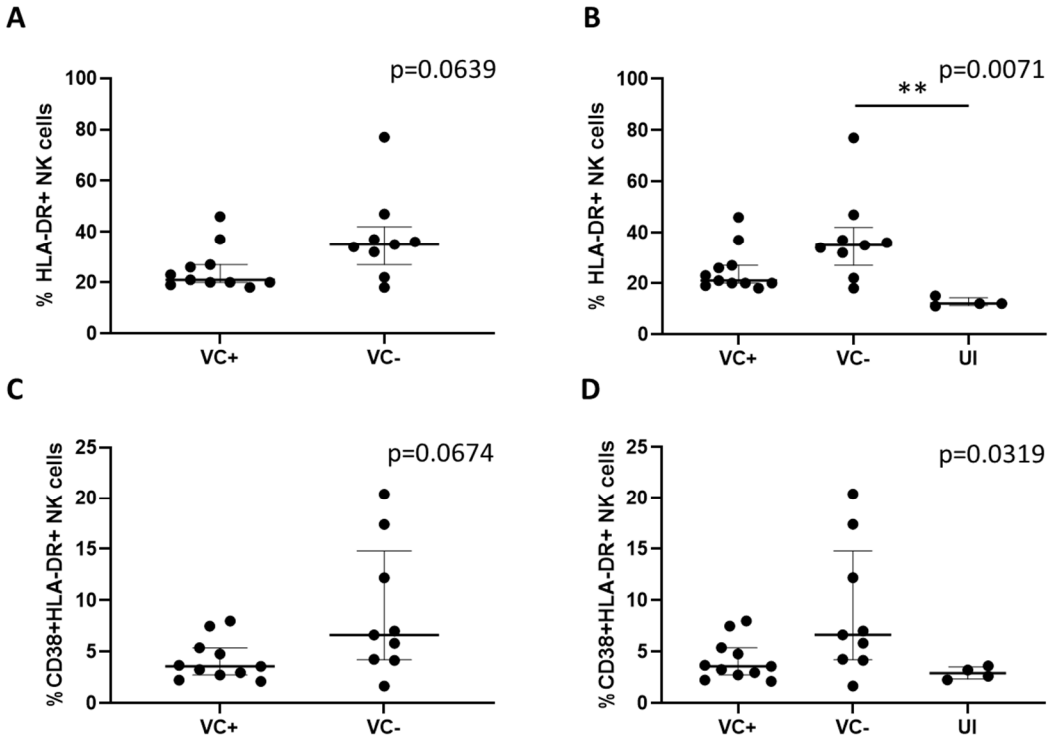

Fig. 7. Flow cytometry analysis of the activation marker HLA-DR and the co-expression of the markers CD38 and HLA-DR within the NK cell population.
Expression of the activation marker HLA-DR within the NK cell population was compared between viraemic controllers with protective alleles (VC+) and viraemic controllers without protective alleles (VC-) (A), and VC+, VC- and uninfected individuals (UI) (B). Similarly, co-expression of CD38 and HLA-DR within the NK cell population was compared between VC+ and VC- (C), and VC+, VC- and UI (D). Mann-Whitney test and One-way ANOVA with the Tukey post-hoc test were used (p values are shown). Lines represent the median and bars show the interquartile range. ** represents $p<0.01$.

4.3.5 NK cells from VC+ express higher levels of intracellular cytokines in response to stimulation than those from VC-

To investigate the functionality of NK cells in response to stimulation, we incubated the PBMCs from VC with a tumour cell line and then measured NK cells for degranulation and intracellular cytokine expression. Degranulation (CD107a) and intracellular cytokines (IFN-γ, TNF-α and MIP-1β) in the unstimulated condition was subtracted from the stimulated condition. Although stimulation induced a slightly higher median CD107a expression in VC+ than VC- (19.9% vs 14.7% of NK cells), this was not statistically significant (Mann-Whitney, p=0.23; data not shown). However, VC+ expressed significantly higher MIP-1β and TNF-α (p=0.05 and 0.04, respectively) than VC- in response to stimulation (Figure 8A and 8B). To further interrogate the results showing a significant difference in expression of MIP-1β and TNF-α between the VC groups, we compared the expression of these cytokines in the VC groups to UI. VC+, VC- and UI have a significantly different expression of MIP-1β (ANOVA, p=0.02), were VC have lower median expression than UI although only VC- have significantly lower MIP-1β expression than UI (Figure 8C). However, TNF-α expression in both VC- and VC+ following stimulation was not significantly different from UI (ANOVA, p=0.25) (Figure 8D). Overall, NK cells from VC+ show greater expression of MIP-1β and TNF-α upon stimulation than NK cells from VC-. Further, NK cells from VC+ were more similar to UI in terms of MIP-1β expression.

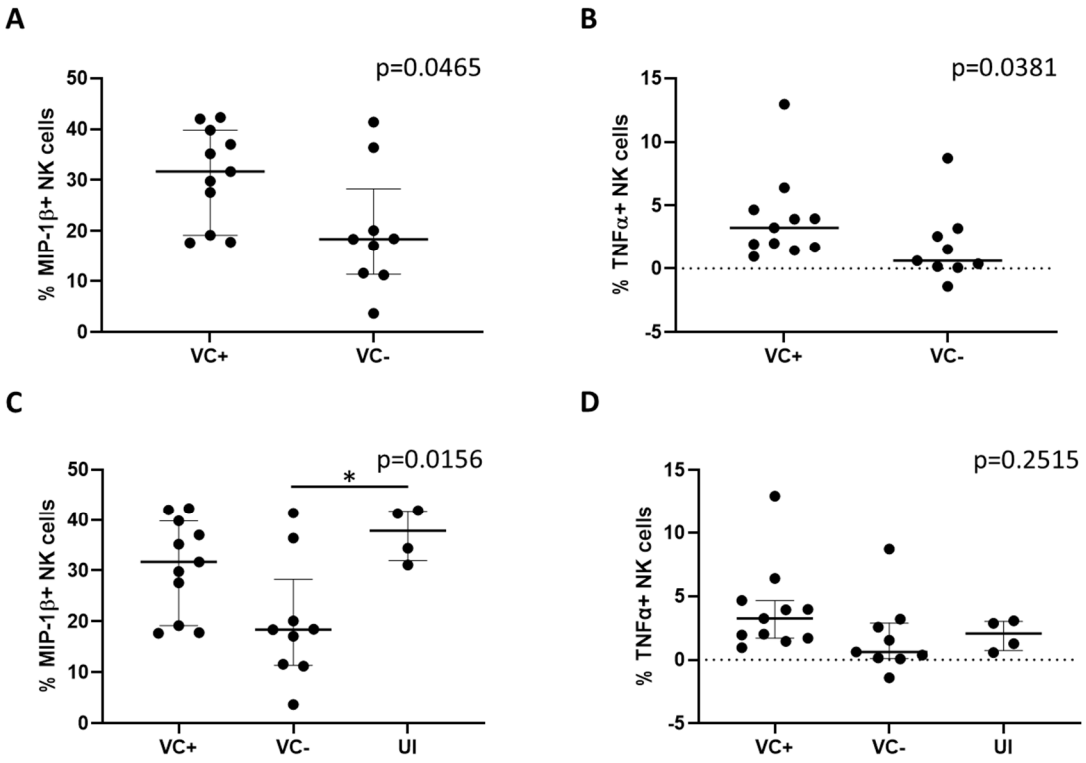

Fig. 8. Flow cytometry analysis of the expression of the intracellular cytokines MIP-1β and TNF-α within the NK cell population.

Frequency of expression of the intracellular cytokines MIP-1β (A) and TNF-α (B) within the NK cell population was compared between viraemic controllers with protective alleles (VC+) and viraemic controllers without protective alleles (VC-). Similarly, expression of MIP-1β was compared between VC+, VC- and UI (C); and expression of TNF-α was compared between VC+, VC- and UI (D). Mann-Whitney test and One-way ANOVA with Tukey post-hoc test were used (p values are shown). Lines represent the median and bars show the interquartile range. * represents $p<0.05$.

Since VC- showed a higher NK activation than VC+ (as measured by CD38 and HLA-DR expression), yet VC- showed lower cytokine expression (MIP-1β and TNF-α) upon stimulation than VC+, we analysed if there was a correlation between the co-expression of CD38 and HLA-DR *ex vivo* and the induction of the cytokines MIP-1β and TNF-α in stimulated cells (Figure 9A and 9B, respectively). There was a significant inverse correlation between MIP-1β expression and the co-expression of CD38 and HLA-DR in the total NK population from VC (Pearson's Test, r=-0.45 and p=0.05), but no significant relationship between TNF-α and co-expression of CD38 and HLA-DR, although these parameters were negatively associated (Pearson's Test, r=-0.29 and p=0.21).

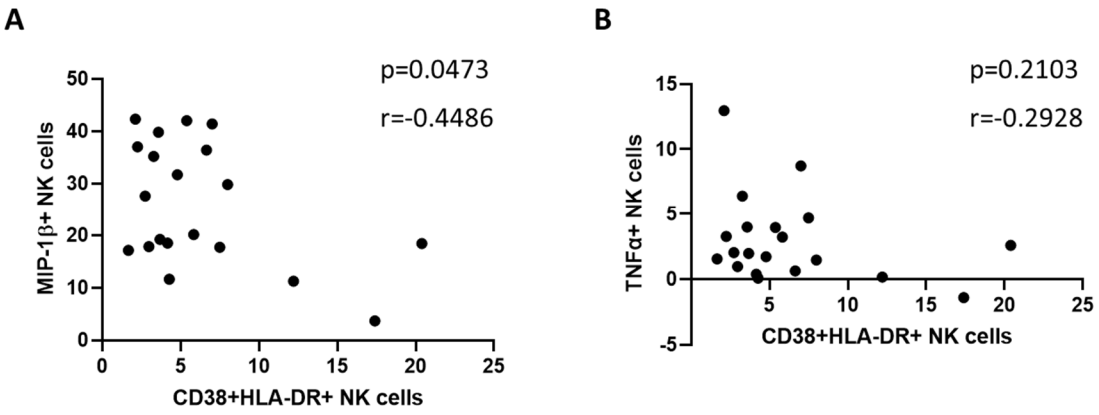

Fig. 9. Correlation analysis of the expression of the intracellular cytokines MIP-1β and TNF-α with co-expression of CD38 and HLA-DR within the NK cell population.
Graphs show correlation of the expression of MIP-1β (A) and TNF-α (B) with the co-expression of CD38 and HLA-DR markers within the NK cell population for all viraemic controllers, with protective alleles and without protective alleles, using Pearson's correlation analysis (p values and r values are shown).

4.3.6 VC- T cells have a more activated and terminally differentiated profile than VC+ T cells

Since T cells have been known to act as immune regulators and interact with NK cells, we measured the total frequency of CD3+ populations (T cells) and the expression of the activation markers CD69, CD38 and HLA-DR, as well as the senescence/terminal differentiation marker CD57 and the exhaustion marker PD-1, on T cells (Supplementary Table 5). There were no significant differences between VC+ and VC- for any of the markers, although there was a tendency of higher expression of the activation marker HLA-DR ($p=0.07$) in T cells from VC- when compared to VC+ (Figure 10A). To provide further insight we compared the expression of HLA-DR in VC+ and VC- to that in UI. Significant differences in HLA-DR expression were found between VC+, VC- and UI overall (ANOVA, $p=0.003$), where VC had higher expression than UI and VC- had significantly higher HLA-DR expression than UI ($p<0.01$, Figure 10B). This shows that, similar to NK cells, although there is a trend of higher activation in VC- than in VC+, both VC groups have higher overall activation than UI, which is to be expected given that the VC in this study are chronically infected individuals.

Since HLA-DR co-expressed with CD38 in T cells is known to be a marker of immune activation (28, 51-53), we compared the frequency of CD38+HLA-DR+ T cells between patient groups. Similar to HLA-DR+ cells, there was significantly higher co-expression of CD38 and HLA-DR in VC- compared to VC+ (Mann-Whitney, $p<0.05$) (Figure 10C). However, VC+, VC- and UI did not differ significantly in CD38 and HLA-

DR co-expression, although VC+ were more similar to UI and VC- showed the highest expression overall (ANOVA, p=0.23) (Figure 10D).

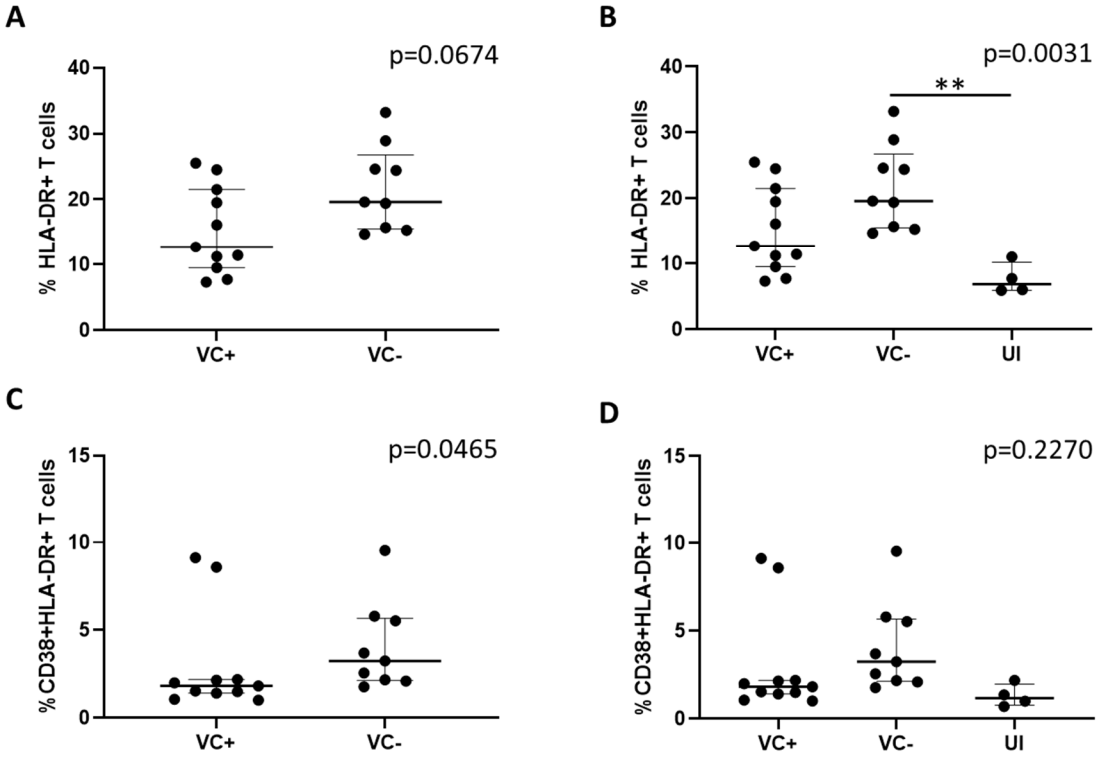

Fig. 10. Flow cytometry analysis of the activation marker HLA-DR and the co-expression of the markers CD38 and HLA-DR within the T cell population.
Expression of the activation marker HLA-DR within the T cell population between viraemic controllers with protective alleles (VC+) and viraemic controllers without protective alleles (VC-) (A), and VC+, VC- and UI (B); and co-expression of CD38HLA-DR within the T cell population between VC+ and VC- (C), and VC+, VC- and UI (D). Mann-Whitney test and One-way ANOVA with Tukey post-hoc test were used (p values are shown). Lines represent the median and bars show the interquartile range. ** represents $p<0.01$.

In addition, we found a trend of higher expression of CD57 in VC- compared to VC+ (p=0.09) (Figure 11A), were VC- have a higher proportion of CD57+ T cells. We compared the expression of CD57 in VC+, VC- and UI and, although VC+ were again more similar to UI and VC- had the highest CD57 expression, overall there was no significant difference in CD57 expression between our groups (Figure 11B).

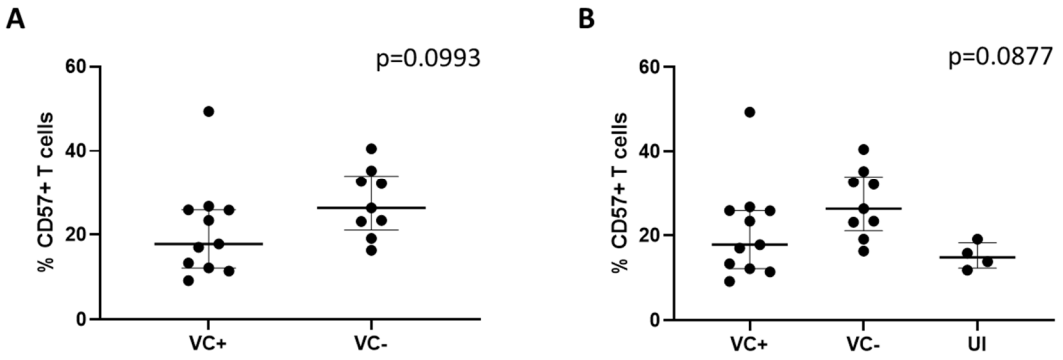

Fig. 11. Flow cytometry analysis of the senescence/terminal differentiation marker CD57 within the T cell population.

Expression of the senescence/terminal differentiation marker CD57 within the T cell population between viraemic controllers with protective alleles (VC+) and viraemic controllers without protective alleles (VC-) (A), and VC+, VC- and UI (B). Mann-Whitney test and One-way ANOVA were used (p values are shown). Lines represent the median and bars show the interquartile range.

Altogether, these results suggest that VC- have HLA-DR expression, and CD38/HLA-DR co-expression, higher than VC+, who have values more similar to UI individuals, suggesting the CD3+ cells in VC- show a more activated profile when compared to UI and VC+ individuals. VC- also have a trend of higher CD57 expression than VC+ who again have more similar CD57 levels to UI individuals, suggesting the CD3+ population in VC- might show a higher terminally differentiated profile when compared to UI and VC+ individuals.

4.4 Discussion

In this study we examined the differences in immunological responses between VC+ and VC-, by measuring the CTL breadth and magnitude, and performing a phenotypic characterization of total NK cells and T cell populations, to gain further insight into potential differences in mechanisms of control between VC+ and VC-.

While we observed a significantly greater contribution of Gag CTL responses to the total CTL response in VC, especially in VC-, compared to RP, consistent with previous work (54, 55), we did not observe differences in the breadth or magnitude of CTL responses measured by ELISpot between VC+ and VC-. The lack of significant differences between VC+ and VC- could be due to various reasons. Since both groups are matched for clinical parameters (VL, CD4 counts, years of HIV-1 control), and the only difference is the presence of HLA-I protective alleles, we may expect the difference between both groups to be very subtle. Previous reports suggest that HIV-specific T cell responses cannot be adequately differentiated by ELISpot assays, and a follow-up with an *in vitro* HIV-1 suppression assay is recommended, as different studies have shown it is likely the most informative assay in the functional

evaluation of CD8+ T cell responses (18, 56). As reported by another study from our group, there was no difference in total breadth of CTL responses or breadth of Gag CTL responses as measured by ELISpot assay between VC+ and VC- but there was a significant difference in *ex vivo* virus inhibition capacity, where VC+ had CD8+ T cell responses with significantly more potent viral inhibitory capacity than VC-. In that study, the suppression assays showed a clear difference in the mechanism of control between VC+ and VC- while the ELISpot analysis did not, supporting that HIV-1 suppression assays are preferable to assess HIV-specific CTL responses especially when comparing groups with similar disease progression profiles (22).

We next explored the possibility that innate immune responses, and in particular NK cells, may differentiate VC+ and VC- mechanisms of control. Our results showed no significant difference in the frequency of NK cell subsets, NCR, maturation markers or exhaustion markers between VC+ and VC-. It should be noted that despite seeing no differences between VC+ and VC- in total % of expression of NCRs markers, our panel did not allow for the distinction of different NCRs (since they are all stained for the same fluorochrome), and it cannot be excluded that there may be differences in specific NCRs between VC+ and VC-.We, however, observed a tendency of higher expression for the activation marker HLA-DR, and co-expression of CD38 and HLA-DR in NK cells from VC- when compared to VC+, suggesting that VC- have a more activated NK cell profile than VC+. CD38 has been described as a marker of disease progression, where the expression of CD38 on NK cells is associated with soluble and immunological factors found in advanced HIV-1 disease progression (28). In our study, there was no significant difference in the expression of CD38 alone between VC+ and VC-, whereas we did observe higher expression of HLA-DR alone or in combination with CD38 in VC-, rendering it unclear based on the previous study (28) if this feature of VC- NK cells is unfavourable or not. Even though VC- had higher levels of activation markers, VC+ NK cells were more responsive upon stimulation than VC- in that they expressed higher levels of MIP-1β and TNF-α, and this was negatively correlated with the co-expression of CD38 and HLA-DR, which was higher in VC-. One possibility is that the higher HLA-DR in VC- NK cells could indicate that NK cells in VC- may be acting as antigen presenting cells (APCs) to a greater degree than in VC+: HLA-DR expressing NK cells combine phenotypic characteristics of both NK cells and dendritic cells, and have recently been proposed as professional APCs (57).

While we speculate that there may be differences in NK-mediated APC activity between VC+ and VC- based on the HLA-DR expression differences, our data does not indicate differences in cytolytic activity between the groups. We did not observe differences between NK cells from VC+ and VC- in expression of CD57, which has been associated with increasing prevalence of KIR+ and granzyme B+ cells (32), which

suggests that the level of KIRs or granzyme B expressing cells is unlikely to be a distinguishing mechanism of control of the infection between the VC groups. Similarly, our results on CD107a expression, used as a functional marker for the identification of NK cell activity that correlates with TNF-α and IFN-γ secretion, and NK cell-mediated lysis of target cells (58), showed no difference between VC+ and VC-.

We further explored whether phenotypic characteristics of T cells might reveal additional potential different mechanisms of control between VC+ and VC-. Firstly, a major limitation in our study is the lack of markers to differentiate between CD4+ and CD8+ T cells, as a result of focusing on phenotypic characterization of NK cells and prioritizing of markers. Thus, our conclusions apply to the whole T cell population and further analysis is needed to distinguish between the CD4+ and CD8+ subsets. Our results show a significant difference in T cell phenotype between VC+ and VC-, where VC- show a higher T cell activation (higher frequency of CD38/HLA-DR co-expression on T cells). Similar to NK cells, we observed a trend of higher expression of HLA-DR in VC- when compared to VC+, with both groups of VCs having significantly higher HLA-DR expression than UI. This suggests that, similar to NK cells, VC have a more activated T cell profile than UI and particularly more so in VC- than VC+. Previous studies have shown that CD38 and HLA-DR co-expression on CD8+ T cells is the strongest predictor for HIV-1 disease progression and higher expression of this profile is associated with adverse outcomes (28, 51-53). In addition, the Ki-67 marker of cell cycling (59-61) correlates positively with HLA-DR expression but not with CD38 expression (59), indicating that chronically infected VC who have higher HLA-DR expression than UI may have a higher turnover of T cells than UI. Indeed, studies have found that in both CD4+ and CD8+ subsets the percentage of cycling cells is ~3 fold higher in HIV-positive individuals than in healthy controls (59, 60). Our results showing a significantly higher HLA-DR expression in VC when compared to UI, could be explained by the chronic infection with HIV-1 and thus the need of a higher T cell turnover (62), while the trend of higher HLA-DR expression on T cells in VC- compared to VC+ might suggest that VC- compensate for lower CD8+ T cell inhibitory capacity with a higher turnover of T cells.

VC- also had slightly higher CD57 (senescence/terminal differentiation) expression on T cells than VC+, who were more similar to UI in terms of CD57 expression, suggesting that VC- have a slightly more terminally differentiated T cell profile. CD57 has been described as a marker of replicative senescence (63), and an increase of its expression on T cells and NK cells has been described as a general marker of proliferative inability and a history of more cell divisions. It is suggested that an increase in CD57+ T cells results from chronic antigen stimulation in HIV-1 infection (64, 65). On the other hand, CD57 expression on CD4+ T cells identifies cytolytic cells, and its expression correlates with cytolytic granules, granzyme B and perforin expression (66). The trend of differences observed in CD57 expression between VC+ and VC-, while not significant, might therefore indicate a slightly more cytolytic CD4+ T cell population in our

VC-, however this cannot be definitely concluded, since the higher CD57 expression could be on CD8+ T cells and not CD4+ T cells. Of interest, CD8+ CD57+ T cells are associated with antibody neutralization breadth against HIV-1 in viraemic controllers, although the frequencies of CD8+CD57+, in their study, did not differ between VC+ and VC- (67). Further analysis on antibody neutralization from our patients would be interesting to address the differences between our groups in this regard.

Altogether, our results identify different NK and T cell profiles between VC+ and VC-. VC- have a more activated and terminally differentiated T cell population than VC+ and UI (higher CD38 and HLA-DR co-expression, as well as higher HLA-DR and CD57 expression), and a more activated NK cell population than VC+ (higher HLA-DR expression and higher CD38 and HLA-DR co-expression), while VC+ have a higher cytokine expression in NK cells (higher production of MIP-1β and TNF-α). In addition to this, previous CTL data has shown that VC- might have a CD8+ T cell independent mechanism of control. A possible explanation for our results is that the increase we observed in HLA-DR+ NK cells from VC- suggests that these NK cells act as APCs (57) in VC-, and this NK APCs would then directly interact with a more activated and terminally differentiated population of T cells observed in VC-. Further work to test this hypothesis is necessary to better understand the mechanisms underlying control in these two groups of VC patients.

4.5 Conclusions

VC have a more Gag focused CTL response than RP, however this feature did not distinguish VC+ from VC. Yet NK and T cell profiles differ between VC+ and VC-. VC- have a more activated NK cell profile with lower cytokine expression, and a more activated and terminally differentiated T cell profile than VC+. A possible explanation for our results is that the increased CD38+HLA-DR+ NK cells in VC- may represent NK cells more efficient as APCs, which may then imply better antiviral activity as a consequence of interaction with activated and terminally differentiated population of T cells observed in VC-. Further work to test this hypothesis is necessary to better understand the mechanisms underlying control in these two groups of VC patients.

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Supplementary Table 1: Antibodies used for cell phenotypic and intracellular staining characterization.

Markers	Fluorochrome	Manufacturer	Clone	Volume (µl)
CD3	Brilliant Violet (BV)785	BioLegend	OKT3	2.5
CD14	BV 650	BioLegend	M5E2	2.5
CD19	BV 650	BioLegend	HIB19	2.5
CD56	Alexa Fluor 700	BD Biosciences	B159	2
CD16	Allophycocyanin (APC)/Cy7	BD Biosciences	3G8	1.5
CD69	Peridinin Chlorophyll Protein (PerCP)/Cy5.5	BioLegend	FN50	2.5
CD57	Fluorescein isothiocyanate (FITC)	BD Biosciences	NK-1	5
PD-1	BV 421	BioLegend	EH12.2H7	2.5
NKG2A	APC	Beckman Coulter	Z199	5
CD38	BV 711	BioLegend	HIT2	2.5
HLA-DR	Phycoerythrin (PE)/CF594	BD Biosciences	G46-6	1
NKG2C	PE	R&D Systems	134591	5
NKp30	PE/Cy7	BioLegend	P30-15	5
NKp44	PE/Cy7	BioLegend	P44-8	3
NKp46	PE/Cy7	BioLegend	9E2	5
CD107a	PE/Cy5	BD Biosciences	H4A3	3.5
TNF-α	BV 605	BioLegend	MAb11	2.5
MIP-1β	PE	BD Biosciences	D21-1351	2.5
IFN-γ	PE/Cy7	BioLegend	B27	0.5

Supplementary Table 2: Breadth of total HIV-specific CTL responses and by individual HIV proteins.

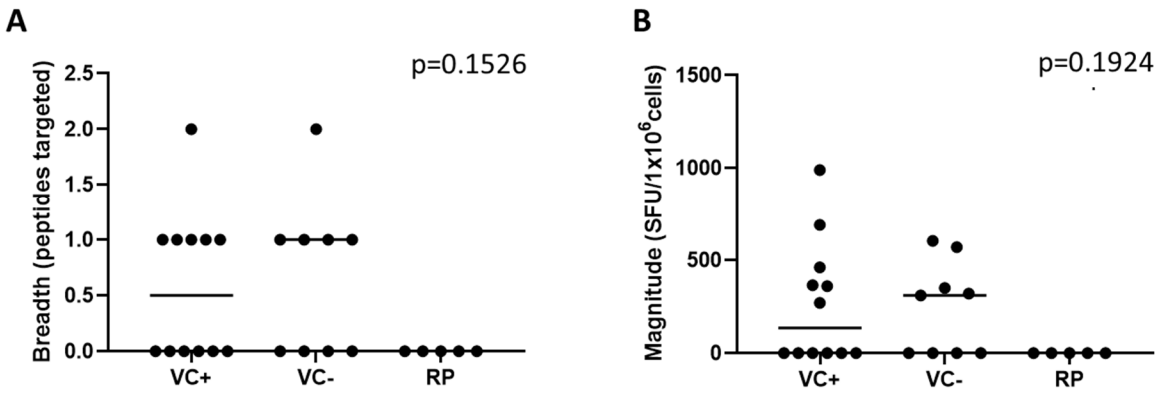
		Number of peptides targeted						
Group [a]	**Patient identifier**	**Total**	**Gag**	**p24**	**Nef**	**Protease**	**RT**	**Env**
VC+	SK-235	1	1	1	0	0	0	0
VC+	SK-362	0	0	0	0	0	0	0
VC+	SK-354	0	0	0	0	0	0	0
VC+	SK-282/206-30-0020-0	1	0	0	1	0	0	0
VC+	111-30-0005-0	1	1	1	0	0	0	0
VC+	111-30-0015-0	0	0	0	0	0	0	0
VC+	SK-469/206-30-0011-0	2	1	1	0	1	0	0
VC+	AS-30-0018	7	6	4	1	0	0	0
VC+	SK-481/206-30-0007-0	2	1	1	0	0	1	0
VC+	SK-490/206-30-0012-0	0	0	0	0	0	0	0
VC+	SK-453	0	0	0	0	0	0	0
VC+	127-33-0397-268	5	2	2	0	0	3	0
VC-	SK-209	2	2	2	0	0	0	0
VC-	SK-275	0	0	0	0	0	0	0
VC-	SK-317	1	1	1	0	0	0	0
VC-	111-30-0041-0	1	0	0	1	0	0	0
VC-	SK-452/206-30-0004-0	6	4	2	1	0	0	1
VC-	SK-470/206-30-0005-0	1	1	1	0	0	0	0
VC-	206-30-0024	0	0	0	0	0	0	0
VC-	SK-475/206-30-0002-0	1	1	1	0	0	0	0
VC-	127-33-0035-039	3	2	2	0	0	1	0
RP	127-33-0251-186	1	0	0	0	0	0	1
RP	AS02-0973	0	0	0	0	0	0	0
RP	AS02-0110	0	0	0	0	0	0	0
RP	AS33-0182	1	0	0	1	0	0	0
RP	127-33-0108-093	0	0	0	0	0	0	0

[a] VC+, Viraemic controller with protective HLA-I alleles; VC-, Viraemic controllers without protective HLA-I alleles; RP, Rapid Progressor.

Supplementary Table 3: Magnitude of total HIV-specific CTL responses and by individual proteins.

		Magnitude (spot forming units/million cells)						
Group [a]	**Patient identifier**	**Total**	**Gag**	**p24**	**Nef**	**Protease**	**RT**	**Env**
VC+	SK-235	365	365	365	0	0	0	0
VC+	SK-362	0	0	0	0	0	0	0
VC+	SK-354	0	0	0	0	0	0	0
VC+	SK-282/206-30-0020-0	600	0	0	600	0	0	0
VC+	111-30-0005-0	270	270	270	0	0	0	0
VC+	111-30-0015-0	0	0	0	0	0	0	0
VC+	SK-469/206-30-0011-0	743	462	462	0	281	0	0
VC+	AS-30-0018	2330	2020	1720	310	0	0	0
VC+	SK-481/206-30-0007-0	549	361	361	0	0	188	0
VC+	SK-490/206-30-0012-0	0	0	0	0	0	0	0
VC+	SK-453	0	0	0	0	0	0	0
VC+	127-33-0397-268	1510	820	820	0	0	690	0
VC-	SK-209	570	570	570	0	0	0	0
VC-	SK-275	0	0	0	0	0	0	0
VC-	SK-317	350	350	350	0	0	0	0
VC-	111-30-0041-0	150	0	0	150	0	0	0
VC-	SK-452/206-30-0004-0	2753	1544	864	518	0	0	691
VC-	SK-470/206-30-0005-0	320	320	320	0	0	0	0
VC-	206-30-0024	0	0	0	0	0	0	0
VC-	SK-475/206-30-0002-0	242	242	242	0	0	0	0
VC-	127-33-0035-039	1272	938	938	0	0	334	0
RP	127-33-0251-186	185	0	0	0	0	0	185
RP	AS02-0973	0	0	0	0	0	0	0
RP	AS02-0110	0	0	0	0	0	0	0
RP	AS33-0182	145	0	0	145	0	0	0
RP	127-33-0108-093	0	0	0	0	0	0	0

[a] VC+, Viraemic controller with protective HLA-I alleles; VC-, Viraemic controllers without protective HLA-I alleles; RP, Rapid Progressor.



Supplementary Fig. 1. Total breadth and magnitude of CD8+ T cell (CTL) responses to epitopes in the most conserved region of Gag.

Total breadth (A) and magnitude (B) of CTL responses focussing only on epitopes in the most conserved region of Gag (amino acids 1-56, 57-96 and 97-135), measured by the ELISpot assay were compared between viraemic controllers with protective alleles (VC+), viraemic controllers without protective alleles (VC-) and rapid progressors (RP) using ANOVA (p value shown). The magnitude of CTL responses to individual peptides was measured in spot forming units (SFU) per million cells. Bars represent the median.

Supplementary Table 4: Percentage of expression of different surface markers and intracellular cytokines within the total NK cell population.

			% expression												
Patient identifier	**Sex**	**Group[a]**	**CD38+**	**HLA-DR+**	**CD57+**	**CD69+**	**HLA-DR+ CD38+**	**NKG2A+**	**NKG2C+**	**NKp30 /p44/p46+**	**PD-1+**	**CD107a+**	**IFN-γ+**	**MIP-1β+**	**TNF-α+**
127-33-1457-1080	F	UI	68.0	14.7	63.7	11.3	2.6	15.5	68.7	85.1	1.4	17.2	5.7	31.1	0.6
127-33-1782-1347	F	UI	70.9	11.2	73.0	16.7	2.3	6.0	76.7	64.8	1.5	13.1	3.0	34.4	1.3
127-33-1896-1440	F	UI	82.8	12.5	81.8	10.6	3.6	12.8	63.9	54.5	1.8	17.9	7.1	41.3	2.9
127-33-1854-1412	F	UI	72.9	11.6	68.4	19.1	3.2	37.5	47.3	73.4	7.5	22.0	3.6	41.9	3.1
AS30-0018	F	VC+	56.8	18.7	55.1	15.7	2.1	32.5	10.7	54.8	0.8	38.0	21.7	42.3	13.0
127-33-0397-268	F	VC+	75.7	20.1	64.4	39.7	5.4	29.0	60.9	66.6	0.9	14.3	12.4	42.0	3.9
SK-453	M	VC+	30.4	20.4	83.3	19.2	2.2	22.4	18.9	40.8	0.7	2.9	4.3	37.0	3.3
SK-235	M	VC+	71.5	21.0	39.3	28.1	3.6	38.5	32.0	69.9	1.8	26.1	10.2	39.8	4.0
206-30-0011-0	F	VC+	52.0	37.0	85.3	32.1	7.5	40.4	3.8	41.8	0.5	20.0	6.9	17.7	4.7
206-30-0020-0	M	VC+	50.6	27.1	63.4	26.3	3.7	13.7	56.1	42.4	0.6	17.3	5.2	19.2	2.0
206-30-0012-0	F	VC+	40.5	46.1	56.3	19.5	2.7	35.1	2.4	36.0	5.4	21.2	7.4	27.6	2.0
111-30-0005-0	F	VC+	73.0	25.7	56.0	18.6	4.8	33.0	40.9	56.4	1.8	19.4	2.5	31.7	1.7
206-30-0007-0	F	VC+	63.6	23.3	21.7	36.4	3.3	53.2	4.7	73.3	3.8	36.0	15.1	35.2	6.4
SK-362	F	VC+	85.4	17.5	55.1	30.9	8.0	43.6	35.1	60.9	6.7	26.7	9.2	29.8	1.5
111-30-0015-0	F	VC+	42.9	19.9	62.4	28.2	3.0	27.9	54.5	66.4	2.3	8.2	2.0	17.8	1.0
SK-317	F	VC-	33.6	47.0	45.2	14.3	7.0	35.2	53.5	32.1	0.5	21.0	17.7	41.4	8.7
206-30-0002	F	VC-	35.1	31.7	69.6	24.8	4.2	13.7	66.7	52.8	7.5	4.9	2.3	18.5	0.4
111-30-0041-0	F	VC-	55.7	21.8	58.1	42.6	4.3	21.3	61.4	51.7	2.0	1.6	0.0	11.6	0.1
206-30-0005-0	F	VC-	55.8	36.5	11.8	22.5	1.7	47.1	10.0	75.6	2.0	25.5	7.3	17.1	1.5
206-30-0004-0	F	VC-	54.1	33.8	58.7	32.6	6.6	15.1	7.3	32.7	1.8	14.7	4.2	36.4	0.6
SK-275	F	VC-	96.7	36.9	63.6	21.3	20.4	26.5	58.8	56.8	1.1	28.2	12.2	18.4	2.6
127-33-0035-039	F	VC-	85.5	18.5	28.9	27.6	5.8	44.8	27.7	65.9	1.4	26.0	7.7	20.1	3.2
206-30-0024-0	F	VC-	37.2	77.0	54.9	28.4	12.2	9.8	38.5	44.4	1.9	0.7	0.4	11.2	0.2
SK-209	F	VC-	79.5	34.7	43.6	12.8	17.4	56.1	43.6	64.3	4.3	0.3	0.4	3.7	-1.4

[a] UI, Healthy Uninfected controls; VC+, Viraemic controller with protective HLA-I alleles; VC-, Viraemic controllers without protective HLA-I alleles.

Supplementary Table 5: Percentage of expression of different surface markers and intracellular cytokines within the CD3+ population.

			% expression									
PID	**Sex**	**Group[a]**	**CD38+**	**CD57+**	**CD69+**	**HLA-DR+**	**PD-1+**	**HLA-DR+ CD38+**	**CD107a+**	**IFN-γ+**	**MIP-1β+**	**TNF-α+**
127-33-1457-1080	F	UI	38.0	19.1	4.5	6.0	19.1	0.7	-1.2	0.1	0.9	0.0
127-33-1782-1347	F	UI	37.4	15.8	14.5	11.0	14.7	2.2	-2.6	0.0	1.1	0.0
127-33-1896-1440	F	UI	56.7	13.8	5.8	5.9	19.0	1.0	-9.6	0.1	0.7	0.1
127-33-1854-1412	F	UI	37.1	11.8	9.3	7.7	8.9	1.4	-0.6	0.0	0.3	0.0
AS30-0018	F	VC+	18.3	23.4	16.5	11.4	25.6	1.5	0.0	1.1	7.2	0.9
127-33-0397-268	F	VC+	47.1	17.8	29.1	21.5	17.2	9.1	-3.5	0.0	-1.4	0.0
SK-453	M	VC+	26.9	25.8	8.6	11.2	34.8	1.4	5.5	-0.3	-1.4	-0.1
SK-235	M	VC+	31.5	17.0	15.2	12.7	17.0	2.2	-1.9	0.3	-0.7	0.1
206-30-0011-0	F	VC+	28.1	12.2	29.6	16.1	14.0	1.8	-0.7	0.1	-0.8	0.0
206-30-0020-0	M	VC+	6.8	49.4	19.5	19.5	18.3	1.1	-5.3	0.0	0.3	0.0
206-30-0012-0	F	VC+	19.6	9.2	24.7	7.7	24.1	1.5	-5.6	0.2	2.5	0.1
111-30-0005-0	F	VC+	33.0	13.3	19.8	9.5	24.1	2.0	-4.9	0.1	0.4	0.1
206-30-0007-0	F	VC+	16.0	26.7	15.4	24.5	13.1	2.1	-8.4	0.0	0.9	0.0
SK-362	F	VC+	44.4	25.9	28.6	25.5	43.2	8.6	0.2	0.0	1.4	0.0
111-30-0015-0	F	VC+	20.6	11.4	12.8	7.3	13.7	1.0	7.0	-0.1	-2.0	0.0
SK-317	F	VC-	25.0	40.5	12.0	24.4	23.7	3.2	-0.7	0.1	3.6	0.1
206-30-0002	F	VC-	18.4	19.1	17.8	19.6	23.1	1.8	-2.6	-0.1	0.6	0.0
111-30-0041-0	F	VC-	20.7	32.6	17.8	15.3	19.4	2.2	0.0	-0.1	0.1	0.0
206-30-0005-0	F	VC-	22.2	32.1	15.9	14.7	17.3	2.1	-0.4	0.0	1.1	0.0
206-30-0004-0	F	VC-	28.3	26.3	35.2	28.9	20.4	5.8	0.2	-0.1	0.4	0.0
SK-275	F	VC-	42.9	23.1	20.6	15.7	19.8	5.5	6.0	0.0	-0.1	0.0
127-33-0035-039	F	VC-	27.1	35.3	23.8	19.4	26.9	3.7	-4.2	-0.1	5.4	0.0
206-30-0024-0	F	VC-	24.7	16.3	17.2	24.6	22.5	2.6	-13.7	0.0	2.1	0.0
SK-209	F	VC-	32.0	23.4	11.9	33.2	17.6	9.5	0.9	0.0	0.0	0.0

[a] UI, Healthy Uninfected controls; VC+, Viraemic controller with protective HLA-I alleles; VC-, Viraemic controllers without protective HLA-I alleles.

CHAPTER 5:

Final discussion, conclusions, and future research

5.1 Final discussion and conclusions

HIV-1 remains a global pandemic despite access to preventive and therapeutic measures to control acquisition of infection or disease progression (1). These include non-pharmaceutical interventions to promote safer sex or access to clean needles and pharmaceutical interventions such as antiretrovirals (ARVs) for prevention (Pre-Exposure Prophylaxis [PrEP] or Post-Exposure Prophylaxis [PEP]), or treatment of infected individuals (2, 3). Access and adherence to ARVs, treatment stigma, treatment side effects, amongst others, still exist despite having multiple measures for prevention or control of disease progression (4). The ideal goal is to develop a prophylactic or therapeutic vaccine, which will reduce side effects of ARVs, allow independence of access to treatment (or prevention possibilities), and be discrete enough to reduce stigma, thus changing the global HIV-1 landscape.

Our project contributed to these areas by understanding the mechanisms of action of PrEP and factors influencing PrEP efficacy, as well as giving a deeper understanding on the natural mechanisms of control of HIV-1 disease progression within the group known as viraemic controllers (VC). This information could be important for developing more effective strategies to combat the HIV-1 epidemic.

In Chapter 2 we showed the basic mechanism of action of PrEP. In our study we modelled and experimentally measured the probability of clearing HIV-1 infection as a function of the effect of drug on the basic reproductive ratio of infection (R_0) and the number of initial cells (N_0) infected by the viral input dose. We chose drug concentrations where HIV-1 infection was able to expand to investigate the effect of sub-optimal HIV-1 inhibition. The reasons to consider sub-optimal drug concentrations were that ARVs penetration may be lower in the mucosa where the initial infection takes place, the possible challenge to maintain adherence in healthy individuals on PrEP, and its usefulness for future approaches to understand the basic principles of initial viral infection. We have shown analytically and experimentally that, under conditions where drugs do not completely inhibit expansion of established infection, it is still possible to clear initial infection provided the number of initial infected cells per infection attempt is low and the drug used is able to decrease infection frequency, and therefore act before the generation of the first infected cells. We also show that the presence or absence of latency has a weak impact on the outcome of the probability to clear initial infection as long as the drug targets initial infection. It should be noted that these results apply to the specific conditions used in the models, and thus a different modality of initial infection, such as conditions where higher viral load is transmitted (via intravenous drug use for example), could have an impact on preventing initial infection if the sub-optimal concentrations are not able to prevent initial infection. In addition, these models do not take into consideration PEP, where the administration of ARVs

occurs after the initial exposure, and thus further studies should be done to understand this mechanism of prevention.

Chapters 3 and 4 provide a better understanding of natural HIV-1 disease progression and control, or lack of control, in the absence of antiretroviral therapy (ART). In Chapter 3 we delivered an up-to-date review of the literature, highlighting the viral and host factors (genetic and immunological) that have been associated with HIV-1 control or lack of control in individuals without ART. In Chapter 4 we focussed our study on a group of individuals able to durably control infection in the absence of ART, known as VC, both with (VC+) and without (VC-) HLA-I protective alleles, and compared them with a group of individuals that rapidly progress to AIDS, known as rapid progressors (RP), and healthy uninfected individuals (UI). We studied the differences in immunological responses between VC+ and VC- by measuring the breadth and magnitude of CTL responses and performing a phenotypic characterization of total NK cell and T cell populations, with the aim of offering insights into potential mechanisms of control. While our CTL results did not show any significant difference between VC+ and VC-, we do see a significantly difference in the contribution of Gag to the total breadth/magnitude for VC compared to RP, in particular VC-. As discussed in Chapter 4 the lack of significant difference between VC+ and VC- could be due to limitations in our approach of using ELISpots assays, and the need of following up with an *in-vitro* HIV-1 suppression assay to functionally evaluate CD8+T cell responses (5, 6). Further, previous reports using suppression assays showed a difference in the mechanism of control between VC+ and VC-: VC+ seemed to have a more effective CD8+ T cell response with failure in control associated with a reduction in breadth of Gag CD8+ T cell responses, while VC- seemed to have a more robust mechanism of control independent of CD8+ T cell responses (7). Lastly, our phenotypic characterization of total NK cells and T cells from VC+ and VC- highlighted the presence of slightly different cell profiles between our groups. It is important to take into account that our experiments were done in a small subset of patients, due to availability of samples; this could confine our statistical analysis and as thus they should be taken into account only as a proof of concept, to guide further analysis in these populations. A second limitation in our phenotypic characterization of these cell types would be the lack of surface markers to differentiate between CD3+ cells (CD4+ and CD8+), the reason being the prioritization of cell surface markers specific of NK cells, following previous data from our group suggesting the already existing differences between CD8+ T cells as mentioned above (7). We acknowledge that further characterization of CD4+ T cells and their subsets is warranted to explore alternative mechanisms of viral control in these individuals. It should be noted that we lacked enough samples to deeply interrogate all potential mechanisms and cell types that may contribute to the differences in viral control. NK cells were not purified from PBMCs before NK functional assays and this is a limitation as other cell types may influence cell activity

and introduce inaccuracy in cell numbers, although it also best reflects the situation in vivo where cells interact to define a specific immunological outcome. Moreover, we believe that performing these assays without purifying cells may better reflect the scenario in vivo, where different cell types interact to achieve and immunological outcome. Furthermore, it should be noted that a limitation of our study was limited PBMC numbers, which precluded us from investigating all potential mechanisms of viral control involving different cell subsets. Despite these limitations, our results showed that VC- have a more activated and terminally differentiated T cell population than VC+ and UI (higher CD38 and HLA-DR co-expression, and higher HLA-DR and CD57 expression), and a more activated NK cell population than VC+ (higher HLA-DR expression and higher CD38 and HLA-DR co-expression), while VC+ have a higher cytokine expression in NK cells (higher expression of MIP-1β and TNF-α) upon stimulation. A possible hypothesis for our results is that the increase we observed in HLA-DR+ NK cells suggests this population is more activated and therefore more efficient in acting as antigen presenting cells (APCs) (8) in VC-, which would then imply superior antiviral activity as a consequence of a more functional interaction with activated and terminally differentiated population of T cells. Further analysis on this hypothesis is necessary to better understand the mechanisms underlying control in this group of patients.

Altogether our study adds new insights into both prevention and disease progression of HIV-1 infection. Our study of PrEP was able to define the basic mechanism of action of this approach and suggests the possibility to focus on new ARVs that might be able to block initial infection at lower concentrations or be administered less frequently (resulting in what is considered sub-optimal levels), as long as they act before the integration of HIV-1 into the host genome. In addition to this, our study on the mechanisms of control of disease progression in VC in the absence of ART broadens our understanding on the immunological mechanisms of control. This understanding is necessary in order to design an effective preventative vaccine or to design therapies to help boost immune responses in HIV-1 infected individuals.

5.2 Future Research

Key future studies which will help understand possible mechanisms of control, or lack of it, in HIV-1 infected individuals in the absence of ART are suggested:

Although neutralizing antibodies have been ruled out as a main determinant of control of HIV-1 replication, there is data associating broadly neutralizing antibodies with elite controllers and long-term non-progressors (9, 10). While our study described in Chapter 4 focussed on the differences in immunological responses between VC+ and VC-, we did not measure the presence of neutralizing antibodies within these

individuals, and whether or not they may significantly contribute to control, particularly in VC-. We propose this measurement would add value and be a key future study. We suggest performing neutralization assays using heterologous HIV-1 single-round competent pseudoviruses incubated with TZM-bl cells against the plasma from the individuals; followed by a measurement of the neutralization fingerprint, as described in Ndlovu et al 2020 (11).

We have hypothesized that NK cells from VC- might be acting as APCs, presenting antigens to a more active and terminally differentiated population of T cells. This is based on recent studies suggesting the possibility of NK cells to act as APCs (8, 12, 13). To what degree NK cells are regarded as APCs, or what would induce this switch from "ordinary" NK cell activity to presenting antigens is currently unknown and further evidence is needed in order to answer these questions. NK cells are also known to mediate antibody-dependent cellular cytotoxicity (ADCC), and some studies have reported higher functionality of this cells in HIV-1 controllers (14-16). Another possibility for the differences in activation of NK cells we observed between VC- and VC+ might be related to their ability to mediate ADCC, and further studies could clarify this.

Previous reports have used integrated single-cell analysis of multicellular dynamics during hyperacute HIV-1 infection (17) to describe the cellular responses during the first year of HIV-1 infection. We suggest that using this approach to measure the subtle differences between VC+ and VC-, and to compare them with RP (matched to VC for viral load in acute infection) as well as uninfected individuals, would be an adequate strategy to get baseline data for generating further testable hypotheses on possible mechanisms of control or lack of control. This approach could also bring some clarity to the differences we observed in Chapter 4 between VC+ and VC- NK cells and T cells, and the pathways associated with the cytokine expression we see in VC+ NK cells. Further, this approach would allow us to determine the transcriptome profile of NK cells and determine if they act as APCs (12), as well as the ADCC profiles between groups or the differences in expression of surface markers associated with disease progression (such as CD38) (18).

Lastly, although the impact of viral factors in disease progression are not regarded as a dominant factor in achieving control, there are multiple studies associating viral attenuation through immune escape variants or viral defects with control of disease progression (19-31). The study of viral sequences in our patients would provide a more complete picture of the factors contributing to control of disease progression in our patients.

Figure 1 provides a summary of the differences in immune cells that we observed between VC+ and VC- and shows the possible future research directions to build on these findings and to more completely understand different natural mechanisms of control in VC+ and VC-.

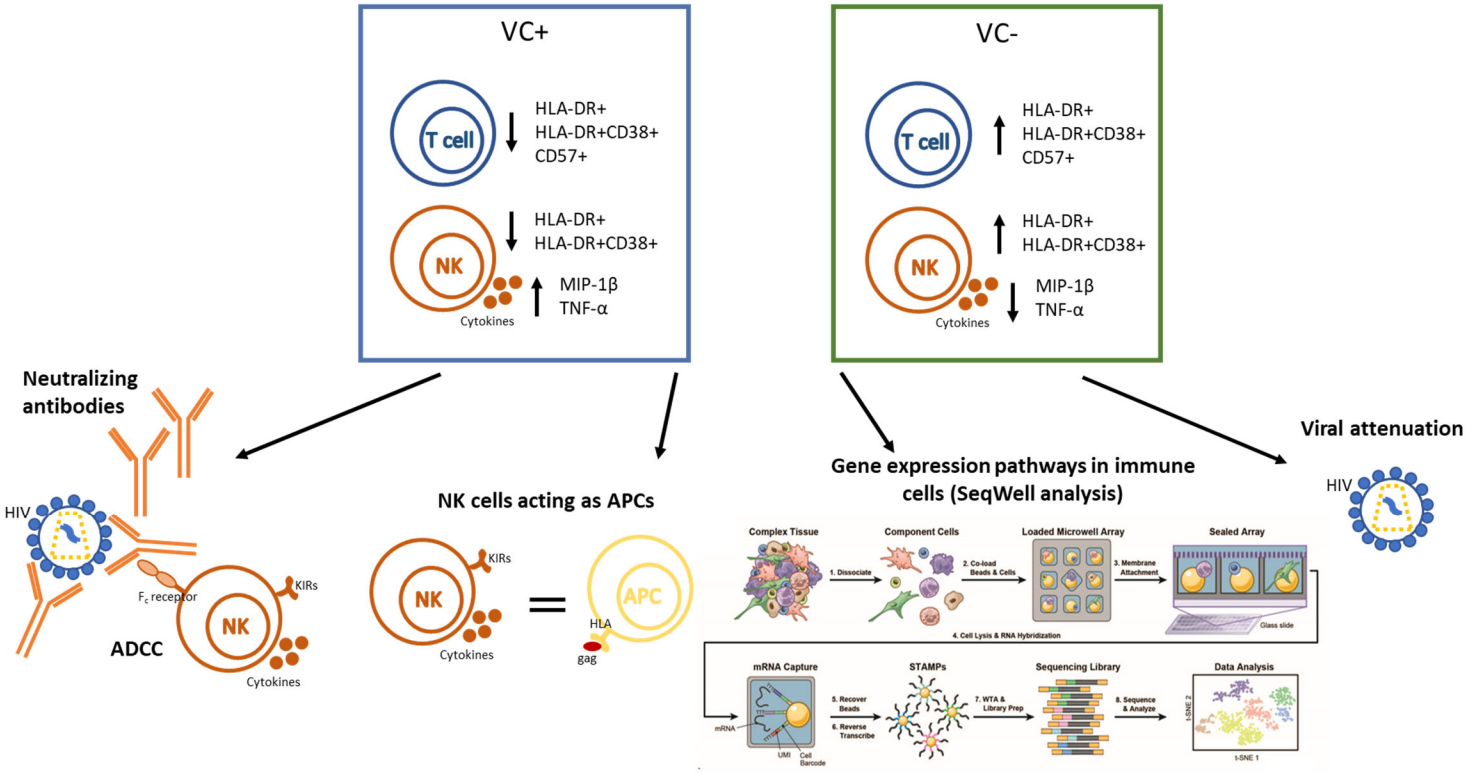


Fig.1. Illustration of differences found between viraemic controllers with (VC+) and without (VC-) protective HLA-I alleles and future research proposed.

Top panels show the results obtained after cell phenotypic characterization of T cells and natural killer (NK) cells and intracellular cytokine expression in NK cells. Bottom panels show the multiple hypotheses of different mechanisms of control present in VC+ and VC- which may form the basis of future research.

ADCC refers to antibody-dependent cellular cytotoxicity; APCs refer to antigen presenting cells; SeqWell analysis refers to integrated single-cell analysis of multicellular dynamics.

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APPENDIX 1:

Paper published.

The following is a paper on the basic mechanism of action of PrEP. It is in the form of a publication and has been published by PLOS Computational Biology:

PLOS COMPUTATIONAL BIOLOGY

RESEARCH ARTICLE

Interference with HIV infection of the first cell is essential for viral clearance at sub-optimal levels of drug inhibition

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Abstract

HIV infection can be cleared with antiretroviral drugs if they are administered before exposure, where exposure occurs at low viral doses which infect one or few cells. However, infection clearance does not happen once infection is established, and this may be because of the very early formation of a reservoir of latently infected cells. Here we investigated whether initial low dose infection could be cleared with sub-optimal drug inhibition which allows ongoing viral replication, and hence does not require latency for viral persistence. We derived a model for infection clearance with inputs being drug effects on ongoing viral replication and initial number of infected cells. We experimentally tested the model by inhibiting low dose infection with the drug tenofovir, which interferes with initial infection, and atazanavir, which reduces the cellular virion burst size and hence inhibits replication only after initial infection. Drugs were used at concentrations which allowed infection to expand. Under these conditions, tenofovir dramatically increased clearance while atazanavir did not. Addition of latency to the model resulted in a minor decrease in clearance probability if the drug inhibited initial infection. If not, latency strongly decreased clearance even at low latent cell frequencies. Therefore, the ability of drugs to clear initial but not established infection can be recapitulated without latency and depends only on the ability to target initial infection. The presence of latency can dramatically decrease infection clearance, but only if the drug is unable to interfere with infection of the first cells.

Author summary

A feature of viral infections such as HIV is that successful transmission occurs with low probability and is preventable by administration of drugs before exposure to the virus. Yet, once established, the infection is difficult or impossible to eradicate within its host. In the case of HIV, this may be explained by the establishment of a latent reservoir of infected cells insensitive to antiretroviral drugs. Here we use a combined modelling and experimental approach to determine whether low dose HIV infection can be cleared at drug concentrations which allow the expansion of HIV infection once established. We show

that such sub-optimal drug levels are effective at clearing infection, provided they target the virus before it infects the first set of cells. The difference in the effect of drugs before and after the initial cells are infected does not require the establishment of viral latency. Rather, it is a quantitative effect, where the low infection dose can be cleared before amplifying viral numbers by infecting the first cells.

Introduction

HIV can be suppressed with antiretroviral therapy (ART) to clinically undetectable levels in the blood. However, established HIV infection cannot be cleared with ART, and generally rebounds several week after ART interruption. This persistence is driven by a reservoir of infected cells which decays minimally in the face of ART [1, 2]. There is extensive evidence that a key component of the HIV reservoir is a population of latently infected cells: cells where functional proviral HIV DNA is integrated into the cellular genome but is not expressed [3–6]. Such cells may start producing virus when they are activated [7, 8] and due to stochastic fluctuations in HIV Tat protein production, initiating a positive feedback loop in HIV gene expression [9, 10].

The exception to the failure of ART to clear infection occurs when ART is present during or immediately after an infection attempt. An approach termed pre-exposure prophylaxis (PrEP) aims to administer ART to uninfected, at risk individuals to take advantage of this fact. The majority of clinical studies have shown that PrEP is effective in a variety of populations, transmission modes, and drug delivery modalities [11–19].

The shift from an infection which can be cleared with ART to one which cannot is generally attributed to the formation of the latent reservoir. The early formation of a reservoir of infected cells in the face of ART has been demonstrated in a non-human primate model [20] and latency has been proposed to be a key driver in the initial establishment of HIV infection [21]. While this mechanism is consistent with the very early transition to irreversible infection, it relies on the assumption that ART regimens completely inhibit viral replication in the mucosal tissues of the genital and rectal tracts, the initial HIV infection sites, and that the infection becomes irreversible if the latent reservoir is established before this complete inhibition takes place.

It may be important to consider whether a mechanism which does not rely on the assumption of complete suppression of viral replication in the mucosa with ART, and therefore the rapid formation of a latent reservoir, can lead to this observed behavior of HIV infection. There are several reasons to consider such an alternate: 1) While there is strong evidence that ART levels as measured in the blood are more than sufficient to completely suppress HIV replication [22], drug penetration may be lower in the mucosa. Therefore, whether inhibition is complete in this compartment is less clear [23]; 2) a challenge in PrEP is to maintain adherence to the treatment, as it is administered to uninfected individuals [11–14, 24–26]. If adherence to PrEP is variable, sub-optimal ART concentrations should occur in at least a subset of treated individuals. PrEP was shown to be effective in a non-human primate model of low dose infection even when dosing was intermittent [27], suggesting it may still be effective under conditions of sub-optimal drug; 3) incomplete suppression of viral replication may be relevant to future PrEP approaches [28] which may use agents that have advantages such as long half-lives but do not completely inhibit HIV replication; 4) it may be relevant to understanding basic principles of initial viral infection by using the well characterized HIV

infection system which has as a toolkit antiretroviral drugs with different mechanisms of action.

An alternative mechanism would need to explain why, if infection can expand, ART can nevertheless inhibit infection if administered very early after exposure. The alternative hypothesis we propose is that if the initial number of infected cells is small (~ 1), it is possible to clear initial infection at sub-optimal inhibitor levels, where such sub-optimal levels would allow infection to expand if the number of initial infected cells was larger. The key conditions are a low initial number of infected cells and an inhibitor which acts before the first cell is infected. The basic reasoning is that under these conditions, the first infected cell is either present or absent. If the inhibitor succeeds in eliminating that infected cell, the infection is cleared regardless of the fact that the infected cell could initiate an expanding infection.

The evidence that a low number of initial infected cells is in fact the physiological condition *in vivo* is that the probability for an individual exposed to HIV by sexual contact to become infected does not exceed 0.02 per sexual act under any set of conditions and is usually much lower [29, 30]. Moreover, infection is established most often with a single viral founder clone [31, 32], and experimental infection with SIV in non-human primates shows the existence of an infection bottleneck at initial infection [33, 34]. These observations indicate that initial transmission is at a low viral dose, sufficient to infect at most one or few cells. This may also be consistent with initial HIV transmission occurring by cell-free HIV infection, where cell-free virions rely on diffusion to reach an infectable cell and therefore have a low probability to infect [35–51]. In contrast, an infected cell is likely to deliver considerable numbers of virions (10^3 to 10^4 virions are produced per cell [52, 53]) if it is at close range.

To test whether it is necessary to inhibit before the first infected cells for sub-optimal inhibition to be effective, it is possible to use antiretroviral drugs with different mechanisms of action. HIV reverse transcriptase inhibitors (RTI) such as tenofovir (TFV) prevent the initial infection of the cell but do not interfere with viral production from an already infected cell. That is, they decrease infection frequency. Protease inhibitors (PI) such as atazanavir (ATV) do not interfere with cellular infection but reduce the number of viable mature virions an infected cell produces—the burst size per cell of viable virions. The effect of decreasing infection frequency or viral burst size should be symmetrical at a high viral dose: The number of successful infections will be decreased if fewer virions successfully infect cells or if the ability of infected cells to produce viable virions is reduced (Fig 1, left panel). However, these effects may not be symmetrical at an initial low viral dose (Fig 1, right panel). Since PIs act with a delay—protease mediated cleavage occurs in the virion during budding from an already infected cell—they can be used to study the effects of the delay on the probability of infection clearance with drug when the initial viral dose is only sufficient to infect one or few cells.

Here we tested the hypothesis that the probability of HIV infection clearance with drug levels which allow for viral replication in established infection depends on preventing initial infection of the first one or few cells. We modeled HIV infection as a function of the measurable initial number of infected cells (N_0) and the basic reproductive ratio (R_0)—the number of cells infected on average by one infected cell when infectable cells are not limiting. We then performed experiments with low N_0 and two types of inhibition: reduction of infection frequency by TFV and reduction of viral burst size per cell by ATV. With both drugs, we used a drug concentrations where $R_0 > 1$ in the presence of drug. That is, infection could expand. We observed that while both drugs reduced R_0 to a similar extent at the concentrations used, only TFV, which prevented successful infection of the first set of cells, was effective at clearing infection.

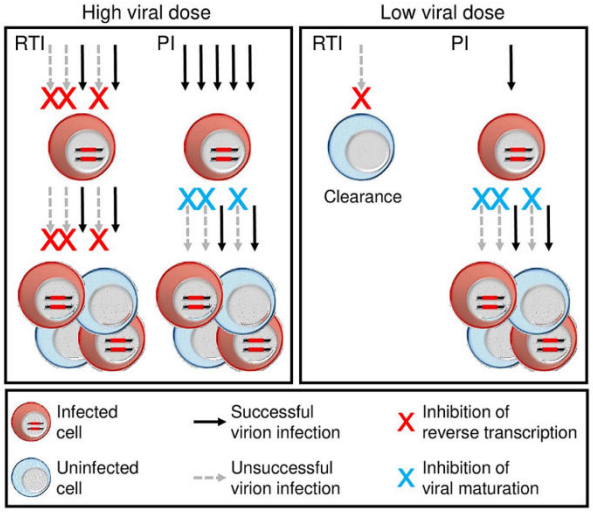

Fig 1. Low dose HIV transmission is vulnerable to clearance before infection of the first cells. Illustrated is partial inhibition of infection ($R_0 \sim 2$ with drug) with drugs such as a reverse transcriptase inhibitor (RTI), which acts before HIV integrates into the cellular genome, and a protease inhibitor (PI), which acts after the infection of the first cells by interfering with HIV maturation. Left panel shows high dose transmission between individuals, inhibited by drugs at levels where infection is still able to replicate. Here, the effects of the RTI and PI are symmetrical and neither clears infection. Right panel shows low dose transmission between individuals inhibited at the same drug levels. While the RTI may not clear every infection attempt, it may be successful at clearing infection if the number of infection attempts are few. In contrast, once the first cells are infected, as would occur with the PI, this advantage is lost. In the event $R_0 \lesssim 1$ with drug, both drug mechanisms can clear infection.

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Materials and methods

Ethics statement

The study protocol for blood collection from healthy donors was approved by the University of KwaZulu-Natal Institutional Review Board (approval BE083/18). Blood was obtained with informed written consent from each donor.

Inhibitors, viruses and cells

The following reagents were obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health: the antiretroviral drugs ATV and TFV; RevCEM cells from Y. Wu and J. Marsh; HIV-1 NL4-3 CCR5 tropic infectious molecular clone (pNL(AD8)) from E. Freed; pBABE.CCR5, from N. Landau. Cell-free virus was produced by transfection of HEK293 cells with pNL(AD8) using TransIT-LT1 (Mirus) transfection reagent. Supernatant containing released virus was harvested two days post-transfection and filtered through a 0.45 micron filter (GVS). The number of HIV RNA genomes in viral stocks was determined using the RealTime HIV-1 viral load test (Abbott Diagnostics). The RevCEM HIV infection GFP indicator cell line was modified as follows for experiments with the CCR5 tropic virus: The E7 clone was generated from RevCEM cells as described in [36]. Briefly, the RevCEM cell line was sub-cloned by limiting dilution.

Clones derived from single cells were expanded into duplicate 96-well plates, one optical and one standard tissue culture for continued growth. The optical plate was infected with HIV strain NL4-3 virus and optical wells were scanned by microscopy to select clones with highest infection percentage by GFP expression. The clone E7 was selected based on greater than 70 percent GFP positive cells upon infection, expanded from the uninfected replicate plate and frozen. To generate the CCR5 expressing B8 reporter clone, RevCEM-E7 cells were infected with the pBABE.CCR5 retroviral vector which stably expressed CCR5 under the LTR promoter. Cells were sub-cloned by limiting dilution. Clones derived from single cells were expanded into duplicate 96-well plates, one optical and one standard tissue culture for continued growth. The optical plate was infected with HIV strain NL(AD8) CCR5 tropic HIV and wells were scanned by microscopy to select clones which maintained similar GFP expression to the parental RevCEM-E7 clonal cell line. The clone RevCEM-B8 was selected based on greater than 70 percent GFP positive cells upon infection, expanded from the uninfected replicate plate, and frozen. Cell culture medium was complete RPMI 1640 supplemented with L-Glutamine, sodium pyruvate, HEPES, non-essential amino acids (Lonza), and 10 percent heat-inactivated FBS (Hyclone).

Infection and flow cytometry

For determination of drug effect on R_0 and N_0, cells were infected with 2.5×10^7 viral RNA copies in 2ml of cell culture containing 5×10^5 cells/ml. The number of infected cells was acquired every 2 days with a FACSCalibur machine (BD Biosciences) using the 488nm laser line. Flow rate on the machine was measured at each time-point, and acquisition time was multiplied by the inverse of the flow rate to obtain the number of infected cells per milliliter. For experiments measuring P_c^{drug}, 200 μl of cells at a density of 5×10^5 cells/ml were infected with 6.3×10^3 viral RNA copies. Results were analyzed using FlowJo 10.0.8 software. The background frequency of positive cells was determined by acquiring uninfected samples (n = 17 from 4 independent experiments). A sample was scored as infected if the number of GFP positive cells was greater than that in the highest background samples (0.01% positive cells).

Passaging of infected cell cultures

For determination of drug effect on R_0 and N_0, the uninfected and drug treated cell cultures were passaged at a split ratio of 1:2 every 2 days, where half the cell culture was removed and fresh media with drug (for TFV and ATV) or without drug (for uninfected cells) was added. Proliferation of uninfected cells was sufficient to maintain uninfected cell numbers, and infection was below 5 percent for both drug conditions at all time-points, ensuring target cells were not limiting. For the no drug condition, the infection expanded much more rapidly. Therefore, the infected cell culture was passaged by diluting the infected cells 1:100 every 2 days into uninfected cells. Hence, 20μl infected cells were added to 2ml of fresh, uninfected cells at 5×10^5 cells/ml. The removed fraction of cells was used to detect infection by flow cytometry. For experiments measuring P_c^{drug}, cell cultures with either no infection or containing ATV or TFV, passaging conditions were the same as for the experiments used to determine drug effect on R_0 except that no culture was removed. Instead, new media with drug was added for the TFV and ATV conditions, and new media with no drug was added for the uninfected condition. The infection volume therefore doubled every 2 days, and the cell culture was transferred to larger volume wells to preserve a constant surface to volume ratio. After 8 days (4 passages), cells were spun down, washed once in medium with no drug, and resuspended at 5×10^5 cells/ml in fresh medium with no drug. Cells were then further passaged in the absence of drug for 6 days (3 passages) using a 1:2 dilution every 2 days to amplify any infection in the culture. For

infection in the absence of drug, cells were passaged for 6 days (3 passages) using a 1:2 dilution every 2 days with fresh medium without removing any of the cell culture. The number of infected cells was acquired at the end of the experiment (14 days post-infection for the uninfected, TFV, and ATV conditions, and 6 days for the no drug infection condition) with a FACSCalibur machine as above.

Measurement of infected cell half-life

For determination of the half-life of infected cells in the presence of ATV, RevCEM-B8 cells were pre-incubated with 16 *n*M ATV for 48h. 10^6 cells/ml were then infected with NL(AD8) in the presence of ATV to obtain saturating infection (approximately 70 percent GFP positive resulting from 10^9 viral RNA copies) so that the population of uninfected cells was small and reduction in infected cell number due to cell death could be tracked without the confounding effect of new infections. The cells were maintained with ATV and the number of live infected cells was tracked 2, 4 and 6 days post-infection by pulsing cells with 4 μg/ml of the death detection dye propidium iodide (Sigma-Aldrich) and acquiring for 1 minute with a FACSCalibur machine.

Measurement of infection clearance in peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich) and cultured at 2×10^6 cells/ml in complete RPMI 1640 medium supplemented with L-glutamine, sodium pyruvate, HEPES, and non-essential amino acids (Lonza), 10 percent heat-inactivated FBS (GE Healthcare), and IL-2 at 5 *n*g/ml (PeproTech). Phytohemagglutinin at 12 μg/ml (Sigma-Aldrich) was added for 1 day to activate cells. For cell-free infection, PBMCs were pretreated with either TFV, ATV or no drug for 48 hours after activation and before infection. Cells were then infected with 2×10^8 viral RNA copies of NL(AD8) in 1ml of culture. 2 days post-infection, the number of infected cells was determined by fixing and permeabilizing PBMCs using the BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) according to the manufacturer's instructions. Cells were then stained with anti-HIV p24 FITC conjugated antibody (KC57-FITC, Beckman Coulter, Brea, CA) to detect the presence of intracellular HIV Gag protein. For coculture infection, PBMCs were activated as above. After activation, cells were split into two fractions: donor cells infected with cell-free virus, and target cells to be infected by the addition of the infected donor cells. Donor cells were infected in the absence of drug with 2×10^9 viral RNA copies of NL(AD8) in 2ml of culture. Target cells were incubated with TFV or ATV. 1 day post-donor cell infection, TFV or ATV was added to donor cells. 2 days post-donor cell infection, infected donor cells were stained with carboxyfluorescein succinimidyl ester at 1.5μM (CFSE, Thermo Fisher Scientific) vital stain to differentiate them from target cells, and added to target cells at 1:300 p24-positive infected donor to uninfected target cell ratio. 2 days post target infection, the number of HIV infected, CFSE-negative target cells was quantified by fixing and permeabilizing as for cell-free infection and staining with anti-HIV p24 PE conjugated antibody (KC57-PE, Beckman Coulter, Brea, CA). For determination of clearance probability, 0.5ml of PBMCs at 10^6 cells/ml were activated as above and pre-incubated with drug for 48h, then infected with 2.5×10^5 viral RNA copies of cell-free NL(AD8). After 2 days, cells were spun down and resuspended into new growth media with drug. After 4 days, cells were washed in 2ml growth media, then resuspended in 0.5 ml media without drug and added to 1.5ml RevCEM-B8 cells at 0.7×10^6 cells/ml to amplify infection. 4 days after addition of PBMCs to RevCEM-B8 cells, the number of infected, GFP positive RevCEM-B8 cells was acquired with a FACSCalibur machine. A sample was scored as infected if the number of GFP positive cells was

greater than that in the highest background samples (0.01% positive cells). To approximate N_0 in PBMCs with 2.5×10^5 viral RNA copies, 0.5 ml of PBMC cultured at 10^6 cells/ml was infected at four virus stock dilutions in triplicate: 1.3×10^7, 6.3×10^6, 3.2×10^6, 1.6×10^6 RNA copies. The number of infected PBMCs was measured after 2 days by flow cytometry using anti-HIV p24 FITC conjugated antibody staining. Infected cell numbers at the viral stock dilutions above were (mean±std): $1.4 \pm 0.08 \times 10^3$, $6.6 \pm 1.9 \times 10^2$, $4.7 \pm 0.2 \times 10^2$, $2.2 \pm 0.9 \times 10^2$. Data was fit using linear regression to determine N_0, calculated to be approximately 29 infected cells.

Results

A model for infection clearance

We first set out to model the effect of drugs on the probability to clear infection (P_c). Let N_i be the number of infected cells in the i-th transmission step within the newly infected host. The sequence N_i, $i = 0, 1, 2, \ldots$ is a Markov chain, or, more specifically, a branching process [54] with the random number N_{i+1} of infected cells at the $(i + 1)$-st infection step determined from the number N_i of infected cells in the previous infection step by the formula

$$N_{i+1} = \sum_{c=1}^{N_i} I_c. \tag{1}$$

Here I_c are independent identically distributed random variables denoting the number of new cells infected by each infected cell in step i. We note that in the case where host cells are not limiting, as occurs in the initial stages of infection, infection chains originating from individual infected cells are independent of each other. The infection is cleared if the number of infected cells N_i becomes zero at any point.

Infection starts with a number of infected cells N_0 as a result of exposure to HIV from an infected individual, where $N_0 \geq 0$. N_0 is expected to depend on several factors, among which is the transmitted viral dose during exposure and the cellular infection frequency per virion. N_i, where $i \geq 1$, would then depend on N_0 and the basic reproductive ratio (R_0), the number of cells infected on average by one infected cell in the initial stages of HIV infection, where host cells are not limiting. R_0 depends on both the viral replication rate and the half-life of the infected cells [55, 56] and is approximately 10 *in vivo* [55]. Eventual infection clearance is certain for $R_0 \leq 1$. For $R_0 > 1$, infection may still be cleared if, at any point in the infection chain, the number of infected cells is zero.

To infect new cells, an infected cell produces a burst of κ virions, where κ is on the order of 10^3 to 10^4 [52, 53], and each virion can infect a cell independently with probability r. The number of cells infected by a single infected cell in one transmission step has a binomial distribution with mean $R_0 = r\kappa$ [56]. In the biologically relevant case where κ is large and r is small, with $R_0 = r\kappa$ finite, this binomial distribution can be replaced by the simpler Poisson distribution with mean R_0 [57]. That is, the probability that a single infected cell infects m cells (progenies) in one step is $P_m = R_0^m e^{-R_0}/m!$.

We denote by q the probability that an infection starting from exactly one infected cell is cleared. In this case, it is required that all m identical progenies originating from the original infected cell are cleared. Since each progeny is cleared with the same probability q, all progenies are cleared with probability q^m, assuming independence of progenies. Therefore:

$$q = \sum_{m \geq 0} q^m P_m. \tag{2}$$

Note that the right hand side of Eq (2) is called the generating function, in this case, of the number of progenies of a single infected cell [54].

Replacing P_m with the Poisson distribution with mean R_0 as described above and using the Taylor series of the exponential function $\Sigma_{m\geq 0}\, x^m/m! = e^x$, we find:

$$q = \sum_{m\geq 0} \frac{q^m R_0^m e^{-R_0}}{m!} = e^{R_0(q-1)}. \tag{3}$$

The (smallest non-negative [57]) solution of the above equation gives the probability of clearing the infection for a single initial infected cell:

$$q = -R_0^{-1} W(-R_0 e^{-R_0}). \tag{4}$$

Here, W is the Lambert W–function [58], the inverse of the function $x \mapsto xe^x$. The relationship between q and R_0 is graphed in S1 Fig, which shows that $q = 1$ for $R_0 \leq 1$ and $q \to 0$ at $R_0 \gg 1$.

Eq (4) derived the probability of infection clearance for exactly one infected cell. The initial number of infected cells may not be one, but may be described as a random variable [21]. We choose it to be a Poisson random variable with mean N_0 which is a biologically relevant distribution in viral infection. Therefore, the probability that the initial number of infected cells is n has probability $\phi_n = N_0^n e^{-N_0}/n!$. For a fixed number n of initial infected cells the infection is cleared with probability q^n, assuming infections originating in individual infected cells are independent. To find the probability of infection clearance P_c for a random number of initial infected cells, we take the average over n:

$$P_c = \sum_{n\geq 0} q^n \phi_n = \sum_{n\geq 0} \frac{q^n N_0^n e^{-N_0}}{n!} = e^{N_0(q-1)}. \tag{5}$$

This is the probability that an infection starting from a Poisson distributed random number of infected cell is cleared, where q is given by Eq (4).

We now consider the effect of the antiretroviral drug mechanism on N_0 and q. We note that antiretroviral drugs reduce either infection frequency r or burst size κ. For drugs which reduce infection frequency, $r \to d_1 r$, and for drugs which reduce viral burst size, $\kappa \to d_2\kappa$, where $0 \leq d_1, d_2 \leq 1$. The no drug case is recovered for $d_1 = d_2 = 1$. Given $R_0 = r\kappa$ and therefore $R_0 \to R_0 d_1 d_2$, the effects of the drug mechanisms are symmetrical on q

$$q_{\text{drug}} = -(R_0 d_1 d_2)^{-1} W(-R_0 d_1 d_2 e^{-R_0 d_1 d_2}). \tag{6}$$

Hence, if the drugs decrease R_0 to a similar extent, their effect on q will also be similar. However, given an initial transmission with cell-free virus, only the drug mechanism that decreases infection frequency will reduce the mean initial number of infected cells N_0. The mechanism which reduces burst size will only affect the success of the next transmission cycle. Therefore, the probability to clear infection with drugs becomes

$$P_c^{\text{drug}} = e^{N_0 d_1 (q_{\text{drug}}-1)}. \tag{7}$$

Here q_{drug} is determined by Eq (6). The limits for Eq (7) for $R_0 \leq 1$ and $R_0 \gg 1$ with drug are 1 and $e^{-N_0 d_1}$. At the upper limit for R_0, infection clearance is simply determined by the probability of obtaining $n = 0$ initial infected cells, where the probability to obtain n infected cells is a random number from a Poisson distribution with mean $N_0 d_1$. What constitutes a high value for R_0, at which P_c^{drug} only depends on $N_0 d_1$, is discussed below.

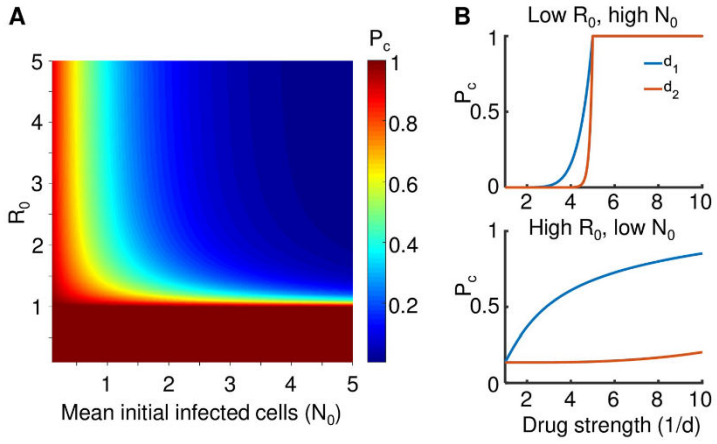

Fig 2. Effects of the initial infected cell number N_0 and R_0 on the probability of infection clearance P_c. (A) P_c according to Eq (5) at different parameter values for N_0 and R_0. (B) P_c according to Eq (7) when a drug attenuating infection frequency (d_1, blue line) or burst size (d_2, orange line) acts on N_0 and R_0. Top panel shows the case where $R_0 = 5$, $N_0 = 20$, while bottom panel shows the case where $R_0 = 20$, $N_0 = 2$. X-axis is drug strength as $1/d$, y-axis is P_c.

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To visualize the effects of decreasing R_0 versus N_0, we plotted Eq (5) for a range of parameter values (Fig 2A). It can be observed that for $R_0 \leq 1$, infection terminates. At $R_0 > 1.5$, infection is not strongly sensitive to the exact R_0 value provided $N_0 \gtrapprox 3$. However at all $R_0 > 1$ values, the probability of infection clearance is very sensitive to N_0, provided N_0 is small. This sensitivity is greatly reduced when $N_0 \gtrapprox 3$.

To examine the effects of drug mechanism, we plotted infection clearance according to Eq (7) at two conditions of R_0 and N_0 relative to d_1 and d_2 (Fig 2B). In the first condition, R_0 was sufficiently small to be decreased below 1 by the drugs in the inhibition range used, while N_0 was large (Fig 2B, top panel). In the second condition, R_0 was large while N_0 was small (Fig 2B, bottom panel). In the first condition, both drug mechanisms had a similar effect on infection clearance, and $P_c = 1$ when the effect of either drug reduced R_0 below 1. In the second condition, only d_1, which decreased infection frequency, substantially increased P_c. d_2, which acted on burst size, had a minimal effect. We note that based on observations of $R_0 \approx 10$ *in vivo* [55] and a probability of infection of at most 0.02 per exposure in the absence of PrEP [29, 30], the second condition likely reflects the physiological situation.

Experimental determination of the probability of infection clearance with drug

We examined experimentally whether Eq (7) predicts P_c for different drug mechanisms after infection with a low HIV dose, the likely *in vivo* condition for transmission. We used the antiretroviral drugs TFV and ATV to inhibit infection initiating as cell-free HIV. We measured the effect of each drug on the initial number of infected cells N_0 resulting from the initial input of cell-free HIV virions. After this initial cycle of infection, the initial number of infected cells was cultured with uninfected target cells (coculture infection). We define established infection as infection where infected cells are present and can infect new cells using both the cell-free infection route and by cell-to-cell spread [59]. R_0 was measured during this phase of infection.

For virus, we used HIV NL(AD8), an HIV strain with a CCR5 tropic envelope protein. CCR5 tropism has been shown to be the predominant transmitted form between individuals [31]. As target cells for infection, we used a clone of the RevCEM infection indicator cell line [60] which we first subcloned to increase detection efficiency [36] then modified to express the CCR5 receptor (Materials and methods). Detection of infected cells was done by quantifying the number of GFP positive cells using flow cytometry.

We titrated TFV and ATV to obtain a similar effect on ongoing coculture infection. This occurred at 60μM TFV and 16nM ATV. To maintain nutrients for cell growth and prevent uninfected cell depletion, we passaged cells every two days (Materials and methods). Such passaging is necessary to maintain conditions where uninfected cells are not limiting in an expanding infection over multiple cell division and viral replication cycles [35].

Despite the use of the same HIV cell-free input dose, there were pronounced differences at day 2 between TFV and ATV (Fig 3A). This time-point reflects the results of the initial cell-free infection given an approximately 2 day viral cycle [61]. Cell-free infection was strongly inhibited by TFV relative to no drug. As expected, the effect of ATV on cell-free infection was much weaker since cell-free virus produced in a cell not exposed to a protease inhibitor is already mature. After the day 2 time-point, infection expanded with similar dynamics for both drug conditions, and much more rapidly when no drug was present.

We plotted the total number of infected cells, corrected for cells removed during passaging, versus time (Fig 3B). We then calculated the effect of drug on R_0 over a two day cycle (Table 1). R_0 values showed that infection expanded at a similar rate for the TFV and ATV conditions. We then measured the effect of the drugs on N_0 after the first cycle of infection (day 0 to day 2), and compared the results to infection in the absence of drug. N_0 in the presence of drug divided by N_0 for the no drug condition (N_0^{norm}) was 0.027 ± 0.014 for TFV and 0.88 ± 0.16 for ATV, (Fig 3C, Table 1). The decrease in N_0 for TFV versus ATV was significant ($p = 6 \times 10^{-14}$, t-test).

We then set out to investigate whether TFV and ATV could increase the probability of clearance of low dose infection, corresponding to *in vivo* exposure. We used 6.3×10^3 viral copies (Materials and methods), predicted to result in approximately 3 initial infected cells based on a regression of the number of infected cells versus input viral load (Fig 4A). Infection was initiated with the same cell-free viral dose for all conditions, and infected cells were cultured for 8 days in the presence of drugs. Any infection present was then amplified for detection by culturing cells in the absence of drug. After amplification, infection was either clearly visible or absent (Fig 4B).

We did not experimentally observe clearance of infection in the absence of drug. In the presence of TFV, clearance rose dramatically, with approximately three quarters of infections extinguished. In contrast, only a minor increase of infection clearance was observed with ATV (Fig 4C, red bars). Clearance with TFV was significantly higher relative to no drug and ATV ($p = 9 \times 10^{-8}$ and $p = 5 \times 10^{-9}$ by Fisher's exact test, respectively), while ATV was not significantly different from no drug. Calculation of P_c^{drug} based on Eq (7) using the measured values for N_0 and R_0 for each drug condition replicated an essential feature of the experimental results: treatment with TFV was predicted to result in a much higher clearance probability relative to treatment with ATV (Fig 4C, grey bars). If no effect of drug on N_0 was included in the model, TFV and ATV were predicted to have similar, and small, effects on P_c^{drug} (Fig 4C, yellow bars). Hence, Eq (7) was able to predict the relative effectiveness of each drug to terminate infection.

One explanation for the difference between TFV and ATV clearance frequencies is that the initially infected cells in the presence of ATV were still present at the end of drug treatment

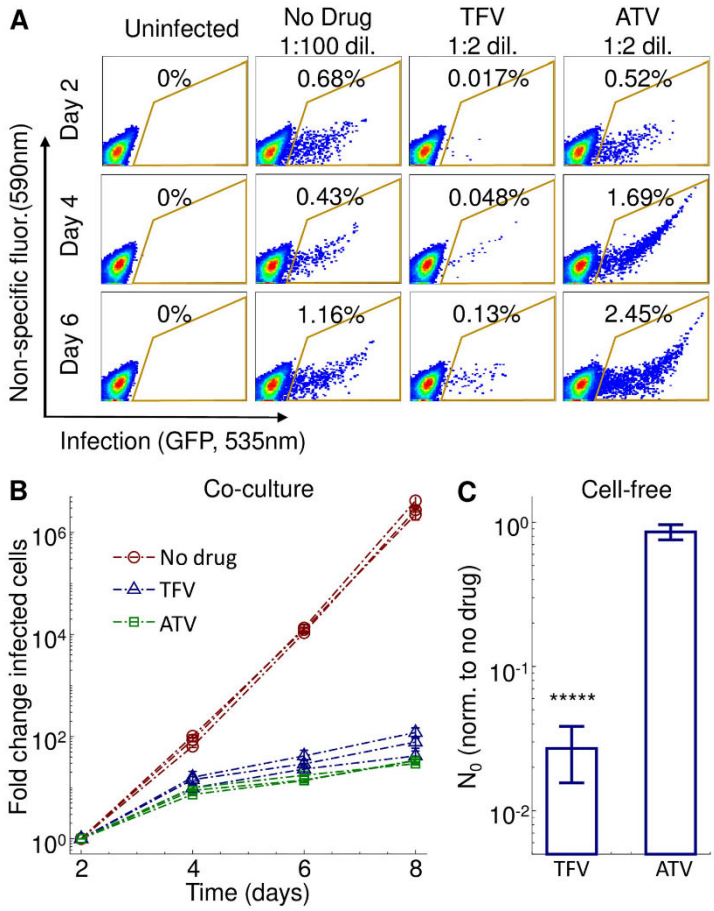

Fig 3. Experimental measurement of drug effect on R_0 and the initial number of infected cells N_0. (A) Flow cytometry plots of the fraction of infected cells at different days post infection in the absence of drug or presence of 60 μM TFV or 16 nM ATV. Day 2 is the first time-point after the initial cell-free infection, corresponding to approximately one viral cycle. X-axis is GFP fluorescence, y-axis is autofluorescence, with the fraction of infected cells corresponding to the cells within the area outlined in yellow. Infected cell cultures in the presence of either drug were diluted 1:2 every 2 days. Infected cultures in the absence of drug were diluted 1:100 into uninfected cells every 2 days. (B) Measurement of R_0 in the absence and presence of drug. The number of infected cells at each time-point is normalized by the number of infected cells at day 2 and corrected for the dilution factor used in each infection cycle. 3 independent experiment were performed, with each point denoting the mean ± std of 3 experimental replicates per experiment. Infection in the absence of drug is shown as red circles, TFV as blue triangles, and ATV as green squares. (C) Effect of drug on N_0. For each drug condition N_0 was measured 2 days after cell-free HIV infection and normalized by N_0 for no drug. Mean + std of 3 independent experiments, where normalization was with N_0 in the absence of drug as measured in the same experiment. Raw numbers of infected cells averaged over all experiments were $1.3 \times 10^4 \pm 1.5 \times 10^3$ for no drug infection, $3.4 \times 10^2 + 1.3 \times 10^2$ for TFV and $1.1 \times 10^4 + 7.4 \times 10^3$ for ATV (mean + std). The difference between TFV and ATV was significant ($p = 6 \times 10^{-14}$ by t-test).

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Table 1. Measured parameter values.

Treatment	N_0^{norm*}	R_0
No drug	1	143 ± 15
$60\mu M$ TFV	0.027 + 0.014	4.2 + 0.73
$16nM$ ATV	0.88 ± 0.16	3.2 ± 0.088

* N_0 normalized by N_0 no drug.

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due to lack of cell death and gave rise to the infected cell population when ATV was removed. We therefore measured the half-life of cells in the presence of ATV. We observed a half-life of approximately 1 day (S2 Fig). Hence, less than one-tenth of the initially infected cells are expected to survive to the end of drug treatment, making infection persistence with ATV due to a long half-life unlikely.

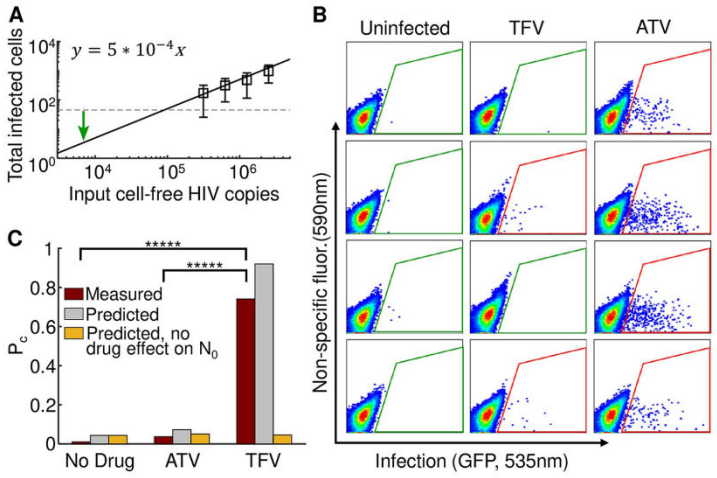

Fig 4. Probability of infection clearance depends on drug mechanism. (A) Determination of N_0. The number of infected cells was measured using flow cytometry as a function of cell-free HIV RNA copies for four virus stock dilutions after one infection cycle (2 days). Data was fit using linear regression to determine the input viral dose for 3 infected cells. Mean ± std of 5 independent experiments. Dashed line is limit of detection. Green arrow marks number of HIV RNA copies used in the experiments. (B) Representative flow cytometry plots after 8 days of infection with the input cell-free virus in the presence of TFV or ATV and further 6 days amplification in the absence of drug. Each plot represents one independently cultured replicate of the experiment. Uninfected samples are shown in the left column, and infection in the presence of TFV or ATV is shown in the middle and right columns respectively. X-axis is GFP fluorescence, y-axis is autofluorescence. The fraction of infected cells corresponds to the cells within the area outlined in green or red, with green indicating background GFP signal level as determined using the uninfected samples, and red indicating above background signal. (C) P_c^{drug} as experimentally measured (red bars), and as predicted by Eq (7) (gray bars) based on the measured drug effects on R_0 and N_0. Presence of infection was assayed in 26 (no drug) or 27 (TFV and ATV) cell-free virus infections from 4 independent experiments. Observed P_c^{TFV} was significantly higher than P_c^{ATV} and P_c^{Nodrug} ($p = 9 \times 10^{-8}$ and $p = 5 \times 10^{-9}$ by Fisher's exact test, respectively). P_c^{ATV} and P_c^{Nodrug} were not significantly different. Yellow bars show predicted P_c^{drug} if both drugs act on R_0 only. That is, $P_c^{drug} = e^{N_0(\frac{1}{R_{drug}} - 1)}$.

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To examine whether the qualitative pattern of the results obtained for the cell line would also be obtained in primary cells, we repeated the experiment in peripheral blood mononuclear cells (PBMCs) from an HIV uninfected blood donor. PBMCs were infected with a low dose of NL(AD8) strain HIV in the presence of TFV and ATV (Materials and methods). Drug concentrations used were 40 μM for TFV and 24 nM for ATV. At these drug concentrations, both drugs reduced infection by approximately one order of magnitude when infection was by coculture of infected with uninfected cells (S3A–S3C Fig), as occurs in established infection. When the infection source was cell-free virus, TFV reduced infection by two orders of magnitude while ATV reduced infection 3-fold (S3D Fig). The reduction with ATV of cell-free infection is consistent with a previous report showing some effect of protease inhibitors on cell-free infection [62], while the greater effect of TFV on cell-free versus coculture infection is consistent with multiple previous studies [35–37, 39, 40, 43, 46, 51]. When infection was with low dose cell-free virus, TFV led to almost complete infection clearance, with no clearance detected for the ATV and no drug conditions (S2E Fig). These results validate the observed behavior of the cell-line infection in primary human cells.

Effects of latency on the probability of infection clearance

The results above showed that at sub-optimal drug concentrations where HIV infection can replicate, infection can still be cleared if the initial number of infected cells is low and the drug decreases infection frequency before the first cells are infected. This effect does not presuppose the existence of latency. However, given the strong evidence for latency, we investigated the expected effect of latency on infection clearance.

We introduce a probability of a cell to become latent P_{lat} [21]. Estimates for P_{lat} vary between approximately 0.5 in *in vitro* infections and modelling [10, 63, 64], to 10^{-4} *in vivo*, based on the frequency of intact HIV DNA in the face of ART in CD4+ T cells in the peripheral blood compartment [65, 66], and 10^{-3}, based on total HIV DNA copies in rectal CD4+ T cells of individuals on ART [67]. The latter values do not measure the ability of the HIV DNA to produce infectious virus, and therefore the frequency of latent cells containing inducible infectious virus may be lower. However, the value of P_{lat} at initial infection is difficult to determine, and therefore values in the upper part of the range cannot be ruled out.

Once a latent cell is produced, the infection may no longer be cleared, since latently infected cells may maintain the reservoir by homeostatic latent cell proliferation as opposed to new rounds of infection [68]. Therefore, infection can persist even if $R_0 \leq 1$, provided a latent cell is present.

Infection originating in exactly one initial infected cell clears if it both stays non-latent with probability $1 - P_{\text{lat}}$ and independently if all of its progenies clear, which occurs for each of them, again independently, with probability q:

$$q = (1 - P_{\text{lat}}) \sum_{m \geq 0} q^m P_m = (1 - P_{\text{lat}}) e^{R_0(q-1)}. \tag{8}$$

The solution for q of Eq (8) is:

$$q_{\text{lat}} = -R_0^{-1} W(-R_0(1 - P_{\text{lat}})e^{-R_0}). \tag{9}$$

To account for latency, we simply use q_{lat} instead of q in Eq (7) to calculate P_c.

We visualize Eq (9) as the probability to clear infection in the face of increasing drug strength under conditions where the initial number of infected cells is small, while R_0 is within the *in vivo* range for initial infection ($N_0 = 2$, $R_0 = 10$, [55]). Therefore, at drug level $1/d > 10$, $R_0^{\text{drug}} < 1$ (Fig 5A, horizontal green lines in each graph). We examined clearance with P_{lat}

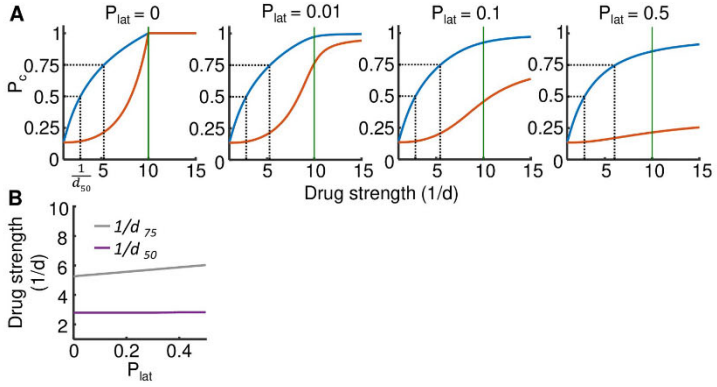

Fig 5. Effect of latency on the probability of infection clearance. (A) P_c was calculated as a function of increasing drug strength ($1/d$) with $N_0 = 2$ and $R_0 = 10$. Therefore, at $1/d > 10$ (denoted by green line), $R_0^{drug} < 1$. Drug d_1 (blue line) decreases infection frequency and d_2 (orange line) decreases burst size from an already infected cell. The probability of an infected cell to become latent was P_{lat}, and the graphs show calculated P_c at the different P_{lat} values indicated above each panel. Drug strength for drug d_1 required to clear 50% and 75% of infection attempts are shown by dashed lines. $1/d_{50}$ is indicated in the first panel. (B) Drug strength for drug d_1 required to clear 50% (purple line) and 75% (gray line) of infection attempts as a function of P_{lat}.

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ranging from 0 to 0.5 (Fig 5A). We compared the effects on clearance of drug mechanism d_2 which decreases viral burst size, versus d_1 which decreases infection frequency. In the case where $P_{lat} = 0$, $P_c = 1$ at $R_0^{drug} \leq 1$. As previously described, clearance was lower with mechanism d_2 relative to d_1 at drug levels where $R_0^{drug} > 1$ and the difference decreased as $R_0^{drug} \rightarrow 1$.

Even at a relatively low frequency of latent cells ($P_{lat} = 0.01$), latency had a visible effect on clearance probability with drug mechanism d_2 (Fig 5A, orange lines). This effect became more pronounced as the frequency of latent cells increased. At $P_{lat} = 0.5$, less than a quarter of infection attempts were cleared at the d_2 drug strength where $R_0^{drug} = 1$. In comparison, all infection attempts are cleared at this drug strength without latency. Interestingly, increasing d_2 further increased clearance. This reflects the fact that at higher drug strength, the number of transmission events between cells becomes smaller before the infection terminates. Therefore, the probability of forming a latent cell and hence making infection unclearable becomes lower.

We next examined the sensitivity of drug mechanism d_1 to latency. Unlike with drug d_2, it was difficult to discern the effect of latency on probability of clearance with drug d_1 (Fig 5A, blue lines). We therefore calculated the drug strength necessary to clear 50% or 75% of infection attempts (Fig 5A, dashed lines). Drug strength of drug d_1 required for clearance of 50% of infection attempts was almost unchanged across the range of latent cell frequencies, while drug strength required for 75% clearance increased slightly (Fig 5B). Therefore, in contrast to d_2, drug mechanism d_1 was far less sensitive to the presence of latency, even at the highest frequency of latent cells.

Discussion

In this study we modeled and experimentally measured the clearance probability of HIV infection as a function of the effect of drug on the basic reproductive ratio of infection R_0 and the number of initial cells N_0 infected by the viral input dose. We chose drug concentrations

where HIV infection was able to expand to investigate the effect of sub-optimal HIV inhibition. The reasons to consider sub-optimal drug concentrations are that ART penetration may be lower in the mucosa where the infection takes place, that it is challenging to maintain adherence in healthy individuals on PrEP, and that it is useful to future approaches to understand the basic principles of initial viral infection.

We have shown analytically and experimentally that, under conditions where drugs do not completely inhibit expansion of established infection, it is still possible to clear initial infection provided the number of initial infected cells per infection attempt is low. We derive the clearance probability in Eq (7) and show that clearance is dependent on using a drug which is able to decrease infection frequency and therefore act before the generation of the first infected cells. The intuition is that if R_0 of infection is relatively large despite the drug, termination of infection originating in an initially infected cell becomes unlikely. However, either an initially infected cell is present, or it is not, and the probability of this depends on N_0. If N_0 is low, a drug which can decrease it further will have a strong effect on the probability of infection clearance regardless of its effect downstream of the first infection.

The model output using the measured values for N_0 and R_0 resulted in predicted probabilities of infection clearance which were higher than the experimentally observed clearance frequencies for all conditions. We speculate that this is due to an underestimation of the input number of infected cells N_0. We measured N_0 one viral cycle after cell-free infection. If GFP expression in an infected cell was below threshold of detection at that time, the infected cell would not be detected, yet still amplify infection. Despite this, the relative effectiveness of each drug mechanism was clearly predicted by the model.

Factors *in vivo* which may lead to deviations from model predictions include transmission by cell-to-cell spread [35–51]. Cell-to-cell spread of HIV should reduce the effectiveness of PrEP since the drugs would only act on R_0 and not on N_0. If the initial exposure is indeed to cell-free virus, the higher efficiency of cell-to-cell spread which results in lower drug sensitivity would make sub-optimal levels of ARVs even less likely to be able to clear infection once initial cellular infection has taken place.

In our analysis we assumed that once the first cells are infected, infection proceeds without further bottlenecks and essentially depends on the value of R_0 in the presence of drug. We further considered that a small number of initially infected cells is the physiological situation. Intravaginal SIV infection of rhesus macaques supports the view that the major bottleneck to the establishment of infection is infection of the initial cells. It was observed that even with exposure to a large dose of virus, most of the inoculum was lost at the initial infection stage, and the rest gave rise to few infected cells [33]. Other bottlenecks to systemic infection spread may exist, and establishment of infection may be a two-step process [21], where resting CD4+ T cells are initially infected in the mucosa [33, 69]. HIV is then transmitted with a delay from the mucosa to lymph nodes, a process which may involve transmission of virions on dendritic cells homing to the lymph nodes to present antigen [34, 70]. Therefore, a relatively large number of initially infected cells in the mucosa may decay to one or few infected cells which initiate systemic infection [21]. In this case, it has been shown that the probability to establish infection is $\sim N_0 P_{estab}$, where P_{estab} is the probability for one initially infected cell to establish infection [21]. Hence, even in a two-step infection process, the sensitivity to N_0 still holds.

HIV has been observed to rapidly seed a latent reservoir of infected cells [20]. We therefore examined the effect of latency on the probability to clear infection as a function of drug strength. Interestingly, for a drug which could target initial infection, clearance probability was similar regardless of whether latency was present or absent. In contrast, latency had a far stronger effect on the probability of infection clearance if the inhibitor used could not interfere with

initial infection, even when $R_0 < 1$. The latter observation is consistent with a critical role for latency in infection establishment under unfavourable conditions for viral replication [21].

The current study shows that sub-optimal drug inhibition can clear HIV infection before it is established, provided the number of initial HIV infected cells is low, and the drug is able to target initial infection. In this situation, the presence or absence of latency has a weak impact on the outcome. More generally, it indicates that in diseases which involve transmission of low pathogen numbers upon exposure, but have robust replication when established, a possibility to clear infection should exist even with relatively weak inhibition if initial infection is targeted.

Conclusion

We investigated why initial HIV infection can be cleared with inhibitors before it is established but not after. We modelled infection with a branching process and used *in vitro* experimentation to test the model. We examined two drug mechanisms: inhibition of infection frequency, and reduction of the burst size of viable virions from an already infected cell. We found that the small difference in timing between the two mechanisms is critical in clearing of low dose HIV transmission. Despite similar effects of both drug mechanisms on HIV replication, only the drug mechanism reducing infection frequency, which could act before the first cells were infected, was able to clear infection. We conclude that the difference may not require the presence of a latent reservoir, but is rather a numbers game: while an imperfect drug may not clear every infection attempt, it may be successful at clearing infection if the number of cellular infection attempts are few.

Supporting information

S1 Fig. q as a function of R_0.
(TIF)

S2 Fig. Estimation of the half-life of infected cells in the presence of ATV. Half-life of infected cells was estimated using the fraction of live infected cells over time in the presence of ATV after saturating infection. Shown are the means and standard deviations of the number of live infected cells normalized by the number at the first time-point measured. Line is the fit to $y = e^{rt+b}$, with r = -0.66/day. Half-life was 1.05 days.
(TIF)

S3 Fig. Infection clearance with drugs in primary cells. (A) Gating strategy to detect the number of infected cells in coculture infection. Cells were first infected with cell-free HIV and used as the infecting (donor) cells for coculture infection. Donor cells were labelled with CFSE and added to uninfected target cells (Materials and methods). To quantify the number of infected target cells, the lymphocyte population was selected using forward scatter (FSC) and side scatter (SSC) and donor cells were gated out by selecting the CFSE negative population. (B) Fraction of infected target cells in coculture infection. X-axis shows infection as detected using a stain for intracellular HIV Gag protein, y-axis is CFSE fluorescence. First plot shows uninfected cells, second plot shows infection in the absence of drug, third plot shows infection with 24 nM ATV, and forth plot is infection with 40 μM TFV. (C) Decrease in coculture infected target cells with drug relative to no drug with 40 μM TFV or 24 nM ATV. Tx = (number infected cells with drug)/(number infected cells without drug). Mean and standard deviation of 3 replicates from two independent experiments. (D) Decrease in the number of cell-free infected cells with drug relative to no drug (Tx, equivalent here to N_0^{norm}) with 40 μM TFV or 24 nM ATV. (E) Probability of infection clearance with 40 μM TFV or 24 nM ATV. Pooled

data from 5 independent experiments, $n = 45$ samples each for no drug, TFV, and ATV. None of the infection attempts with no drug or ATV were cleared, while all but 2 of the infection attempts were cleared with TFV. Difference between TFV and the other two conditions was significant ($p = 2 \times 10^{-23}$ by Fisher's exact test).
(TIF)

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