Mechanisms of slow progression and viraemic control in vertical HIV infection



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"I know just about nothing. But I am suspicious of many things."
Guimarães Rosa, Grande Sertão: Veredas

Abstract

HIV infection in children progresses faster than in adults due to an immune system well-adapted to allow adequate development and protection against threatening extracellular pathogens but left vulnerable to intracellular pathogens like HIV. Early life immunity against HIV contrasts with the aggressive response in adults, where viraemic control is driven by a strong adaptive response. Although viraemic control is rare, 10% of antiretroviral therapy (ART)-naïve children reach late childhood with normal-for-age CD4+ T-cells numbers and function by adopting a strategy like the African monkeys in SIV infection of low immune activation and CCR5 expression on long-lived CD4+ T-cell subsets, thereby confining viral replication to short-lived cells.

Here, I explore the mechanisms contributing to immunological non-progression and viraemic control in children living with HIV. In Chapter 3, I describe an inherited signature that favours a KIR-education program, associated with an NK cell population with more plasticity in individuals with higher CD4+ T-cell count and better viral control. This role of NK cells contrasts with the more central role of HLA-B molecules and CD8+ T-cell-mediated immune control in adults. In Chapter 4, I show, in a cohort of early treated infants who underwent analytical treatment interruption, that PD-1 expression on CD8+ T-cells predicted progression and anticipated cardinal features of the slow progression phenotype. Moreover, the CD8+ T-cells of slow progressors expressed a stem-like phenotype with high proliferative capacity and potential to acquire antiviral functions. In Chapter 5, I describe a rare population of paediatric elite controllers. This group is strongly biased towards the female sex, and their T-cell compartment is populated with a polyfunctional HIV-specific response. In Chapter 6, I describe two mechanisms by which viral control is lost in another rare group of children who achieve aviraemia but, unlike the elite controllers, cannot maintain it.

This work strengthens our understanding of HIV infection in children. The opportunity to start ART after birth, the tolerogenic immunity and the transmission of virus with a lower replicative capacity increase the chance of restricting the size and diversity of the viral reservoir, thereby to achieve remission in children. ART alone, however early, is proven not enough, and this work contributes to identifying immune signatures and routes that can be exploited to boost the antiviral response when ART can be stopped, and viral rebound curbed by the child's immune system.

Contributions to this Thesis

Above all, I want to thank the participants in the studies that are part of this thesis and their caregivers. Without their consent to be part of the studies and donate their time and blood, the work presented here would not exist. I also want to express my gratitude to the studies' team, responsible for participant recruitment, clinical follow-up, and blood processing.

I am also immensely thankful to the following individuals for their contributions with their expertise over these years.

Clinical samples

The work presented in this thesis comprises several cohorts. I want to name a few people whose determination contributed immensely to this thesis: **Pieter Jooste** from Kimberley Hospital (South Africa) set up the cohort of paediatric slow progressors at the local HIV out-patient clinic; **Maximilliam Muenchhoff** and **Julia Roider** from the Ludwig-Maximilians-University Munich (Germany), and **Jane Millar** from the University of Oxford (UK) set up and managed the PSP/pART cohort in Durban (South Africa) before my arrival; **Ayanda Ngubane**, **Pedzisai Gaza** and everyone at Prince Mshiyeni Memorial Hospital (South Africa) who brilliantly manage the cohorts in Durban; **Gareth Tudor-Williams** from St Mary's Hospital (UK), and **Alasdair Bamford** from Great Ormond Street Institute of Child Health (UK) enrolled and provided samples of the paediatric elite controllers followed in the clinics. **Christian Brander** from the IrsiCaixa AIDS Research Institute (Spain) and **Claudia Fortuny** from Sant Joan de Déu Hospital (Spain) identified and provided samples of the two paediatric elite controllers followed in

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Chapter 3

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Ellen Leitman from the University of Oxford (UK) compiled the PEHSS cohort clinical data. **Christina Thobakgale** provided the PEHSS ELISPOT raw data for analysis. **Henrik Kløverpris** and **Alasdair Leslie** from the Africa Health Research Institute (South Africa) conceptualised the cell sorting and RNAseq study, while **Alveera Singh** assisted with the experiment. **Jennifer Giandhari** from the Kwazulu-Natal Research Innovation and Sequencing Platform (South Africa) assisted

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But you can go on thinking and imagining forever
further and stop at no decisions to pick up a bag
for the thinkings. Turn your thinking into your
work, your thoughts a book, in sieges.
Jack Kerouac, On the Road

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Glossary of Terms

Units of Measurements

AU	Arbitrary unit
bp	base pair
mm^3	cubic millimetre
^oC	degree Celsius
kb	kilobyte
MFI	median fluorescence intensity
µg	microgram
µL	microlitre
mL	millilitre
mM	millimolar
min	minute
M	molar (mol per litre)
MOI	multiplicity of infection
ng	nanogram
%	percent
rpm	revolutions per minute

Acronyms and abbreviations

AIDS	Acquired Immunodeficiency Syndrome
aNKR	activating Natural Killer cells receptor
AVNP	adult viraemic non-progressor
AGM	African green monkey
ATI	analytical treatment interruption
ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
BM	bone marrow
bnAb	broadly neutralising antibody
CCR/CXCR	C-C motif or C-X-C motif chemokine receptors
CM	central memory

ART	combination antiretroviral therapy
CI	confidence interval
CMV	cytomegalovirus
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DGE	differential gene expression
DMSO	dimethyl sulfoxide
dsDNA	double stranded DNA
EM	effector memory
EC	elite controller
Env	envelope
EBV	Epstein-Barr virus
Tex	exhausted T-cells
FCS	fetal calf serum
FMO	fluorescence Minus One
GI	gastrointestinal
GFP	green fluorescent protein
Gag	group-specific antigen
HR	hazard ratio
HEU	HIV exposed uninfected
HIV	human immunodeficiency virus 1
HLA	human leucocyte antigen
ISG	IFN stimulated gene
iNKR	inhibitory Natural Killer cells receptor
IFN	interferon
IL	interleukin
IP	intermediate progressor
IQR	interquartile ranges
ICS	intracellular cytokine staining
UNAIDS	Joint United Nations Programme on HIV/AIDS
KIR	killer cell immunoglobulin-like receptor
KLRG	killer cell lectin like receptor G
LASSO	least absolute shrinkage and selection operator
LILRB-1	leukocyte immunoglobulin-like receptor–1
LPS	lipopolysaccharide
LN	lymph node

MIP	macrophage inflammatory protein
MTCT	mother-to-child-transmission
NCR	natural cytotoxicity receptor
NK	Natural Killer
Nef	negative regulator factor
NVP	nevirapine
NGS	next-generation sequencing
NHP	non-human primates
OR	odds ratio
PEHSS	paediatric early HAART and strategic treatment interruption study
PEC	paediatric elite controller
PP	paediatric progressor
PSP	paediatric slow-progressor
PFA	paraformaldehyde
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PHA	phytohemagglutinin
Pol	polymerase
PCR	polymerase chain reaction
PTC	post-treatment controller
PC	principal component
PCA	principal component analysis
PD-1	programmed death 1
RP	rapid progressor
Rev	regulator of viral expression
RM	resident memory
RT	room temperature
SA	South Africa
SIV	simian immunodeficiency virus
SPICE	simplified presentation of incredibly complex evaluations
SNP	single-nucleotide polymorphism
SP	slow progressor
SM	Sooty mangabey
SFC	spot-forming cell
SEB	Staphylococcal Enterotoxin B

SSA	Sub-Saharan Africa
Th	T helper
Treg	T regulatory cells
TCF-1	T-cell factor 1
TCR	T-cell receptor
TEMRA	terminal effector memory
TEC	thymic epithelial cell
TLR	toll-like receptor
Tat	trans-activator of transcripton
TA	transient aviraemic
TM	transitional memory
TGF	tumour growth factor
TNF	tumour necrosis factor
UK	United Kingdom
UKZN	University of KwaZulu-Natal
VA	viraemic adult
VC	viraemic controller
VNC	viraemic non-controller
Vpr	viral protein R
Vpu	viral protein U
VRC	viral replicative capacity
Vif	virion infectivity factor
WT	wild type

Amino acid abbreviations

G	Glycine
M	Methionine
P	Proline
Q	Glutamine
T	Threonine

1

Introduction

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1.5. Overview and aims of this thesis

1.1. Epidemiology

1.1.1. Emergence of the HIV epidemic in Sub-Saharan Africa

The first case report of Acquired Immunodeficiency Syndrome (AIDS) was published in 1981 (Gottlieb et al. 1981), and only two years later the presence of the disease in the African continent was published (Feremans et al. 1983). At that time, the human immunodeficiency virus 1 (HIV-1, hereafter referred as HIV) had been circulating in Africa for decades after its introduction in humans via the adaptation of the simian immunodeficiency virus (SIV) from non-human primates (NHP) (Korber et al. 2000). The first epidemiological data published in 1989 showed a striking prevalence among lorry drivers of 35.2% in east Africa (Carswell, Lloyd, and Howells 1989). The stigmatization and discrimination allied with socio-political conflicts and poverty led to the denial of the epidemic for years and to an immense burden of new infections and HIV-related mortality in the continent (Figure 1.1). By 2000, the prevalence of HIV infection in sub-Saharan Africa (SSA) was nearly 8% of the adult population with more than 1 million new infections each year (UNAIDS 2020).

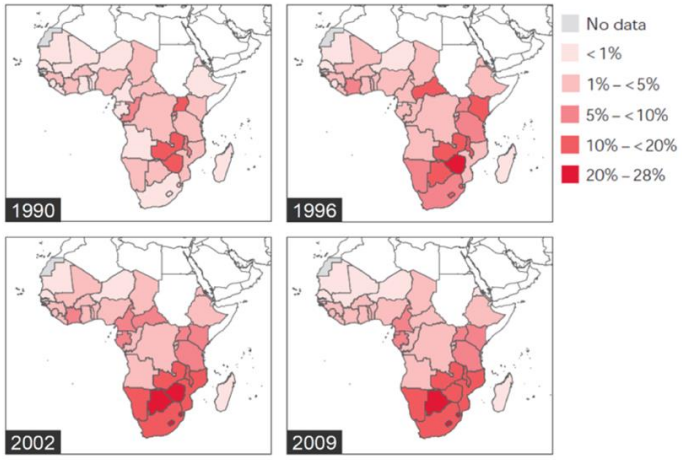


Figure 1.1 Longitudinal HIV-prevalence in adults in sub-Saharan Africa.
Figure reproduced with permission from UNAIDS 2010 Global Report.

1.1.2. A historical perspective of mother-to-child transmission

It was early in the epidemic when the first case series of AIDS in infants became public, and soon it was evident that mother-to-child-transmission (MTCT) of HIV was arising (CDC 1982; Scott et al. 1984). In the absence of any intervention, the risk of MTCT can reach 40% (5-10% intrauterine, 10-20% intrapartum and 10-20% via breastfeeding) (De Cock et al. 2000). By the start of the year 2000, more than 400,000 infants were infected every year in SSA (UNAIDS 2020). The direction of the paediatric epidemic only started to change when the HIVNET 012 study showed that a single dose of nevirapine (NVP) at labour onset effectively reduced intrapartum MTCT by approximately 70% (Guay et al. 1999).

The combination of local socio-political awareness and a coordinated international effort scaled-up the resources to fight AIDS with a special focus on prevention. After the significant impact of combination antiretroviral therapy (ART) in reducing overall transmission shown by HPTN 052 (Cohen et al. 2011), the Joint United Nations Programme on HIV/AIDS (UNAIDS) changed their policy in 2014 and started to advocate ART regardless of CD4+ T-cell count, including for pregnant women (WHO 2014). To eliminate transmission, UNAIDS set up the 90-90-90 target, aiming to achieve 90% of people living with HIV to know their status, 90% of diagnosed people starting ART, and 90% of treated people to be virologically suppressed (UNAIDS 2014). ART taken during pregnancy can bring down MTCT to 1-2% (Siegfried et al. 2011), and substantial progress has been made towards curbing vertical transmission with a significant decrease in new paediatric infections (Figure 1.2). However, due to the patchy coverage of pre-natal programs and because of ongoing maternal seroconversion during pregnancy, 126,000 new paediatric infections

were reported in SSA in 2019 and 1.8 million children are living with HIV worldwide (UNAIDS 2020).

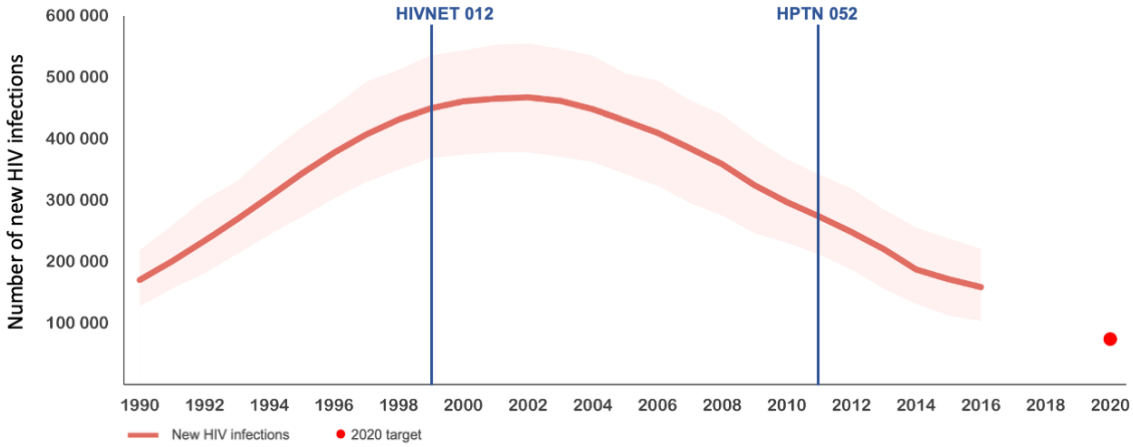


Figure 1.2 Number of new HIV infections in children (1990 – 2016).
Public release of HIVNET 012 and HPTN 052 trials are shown in blue lines. Figure adapted with permission from UNAIDS 2017 Global Report.

1.1.3. Natural history in paediatric infection

Children living with HIV progress faster to AIDS than adults. While HIV disease progression in adults takes a median of 11 years (Babiker A 2000), a sharp CD4+ T-cell decline, faltering growth and recurrent infections are frequently observed in the first year of life in paediatric infection, and up to half of the children living with HIV die by the age of two years (Newell et al. 2004). Nonetheless, the progression rate follows a bimodal curve and 25% would be alive by the age of ten (Marston et al. 2011). In absence of ART, plasma viraemia peaks after three months at approximately 6 $\log_{10}$ HIV-RNA copies/mL, but a setpoint is only achieved after five years (Rouet et al. 2003; Muenchhoff et al. 2016). This contrast with adults where a set point of 30,000 HIV-RNA copies/mL is achieved within months of infection as a result of a partially effective adaptive immune response (Dong et al. 2018). Regardless of the natural history, the current guideline recommends therapy independently of the

clinical-stage or immunological progression for all children living with HIV (WHO 2014).

1.2. HIV pathogenesis and immune response in early life

1.2.1. HIV replication cycle

HIV is part of the lentiviruses genus and belongs to the group of pathogenic human retroviruses (Reitz and Gallo 2015). They reverse transcribe the viral RNA into double-stranded DNA (dsDNA) to further integrate it into the host genome. The HIV proviral DNA has a length of 9.7 kb spanned by the long terminal repeats and encompassing of the *gag, pol* and *env* genes. The *gag* gene encodes the Group-specific antigen (Gag) precursor of the virus core (p24, p6 and p7) and matrix (p17) proteins. The Polymerase (Pol) enzymes Protease, Reverse Transcriptase and Integrase are encoded by the *pol* gene. The *env* gene encodes the Envelope (Env) glycoproteins gp120 and gp41. Several accessory proteins are transcribed within the code of the primary genes: Vif (Virion infectivity factor), Vpr (Viral protein R), Tat (Trans-activator of transcription), Rev (Regulator of viral expression), Vpu (Viral Protein U), and Nef (Negative regulator factor) (Foley B 2018) (Figure 1.3.A).

The HIV cycle starts when the Env-gp120 binds to the CD4 receptor and establishes the initial interaction between the virus and the host-cell. CD4 molecules are expressed mainly on T helper (Th) cells, but also monocytes, macrophages and dendritic cells (DCs). The gp120-CD4 binding leads to a conformational change in gp120 that allows its interaction with the C-C motif or C-X-C motif chemokine receptors 5 or 4 (CCR5 or CXCR4, respectively). CCR5-tropic viruses are usually the transmitted virus, while CXCR4-tropic viruses are frequently identified in later stages

of infection (Kahn and Walker 1998). Upon gp120-CCR5/CXCR4 interaction, the Env-gp41 is free to anchor and fuse to the cell membrane, leading to viral entry. The p24-capsid is disassembled using the host-cell machinery, and viral dsDNA is synthesized. The pre-integration complex, which includes Gag-p7/Vpr/Pol-Integrase, transfers the dsDNA to the nucleus, where it is integrated into the host genome. The proviral DNA uses the host-cell machinery for the synthesis of RNA. The processing of viral genomic RNA and the translation of viral messenger RNA are complex. Initially, the translation favours the production of the regulatory proteins Tat, Rev and Nef, but the progressive increase in Rev concentration changes the splicing, and favours the late phase Gag, Pol, Env, Vpu, Vpr and Vif proteins, as well genomic RNA. Subsequently, Gag-p7 binds to Gag-p24 and genomic RNA at the host-cell membrane's lipid rafts, where the matrix Gag-p17 interacts with the transmembrane Env-gp41. At the outer membrane, Env-gp41 can then interact with Env-gp120, ending the cycle with the virion assembling, budding, and releasing of the viral particle [reviewed in (Freed 2015; Reitz and Gallo 2015)] (Figure 1.3.B-C).

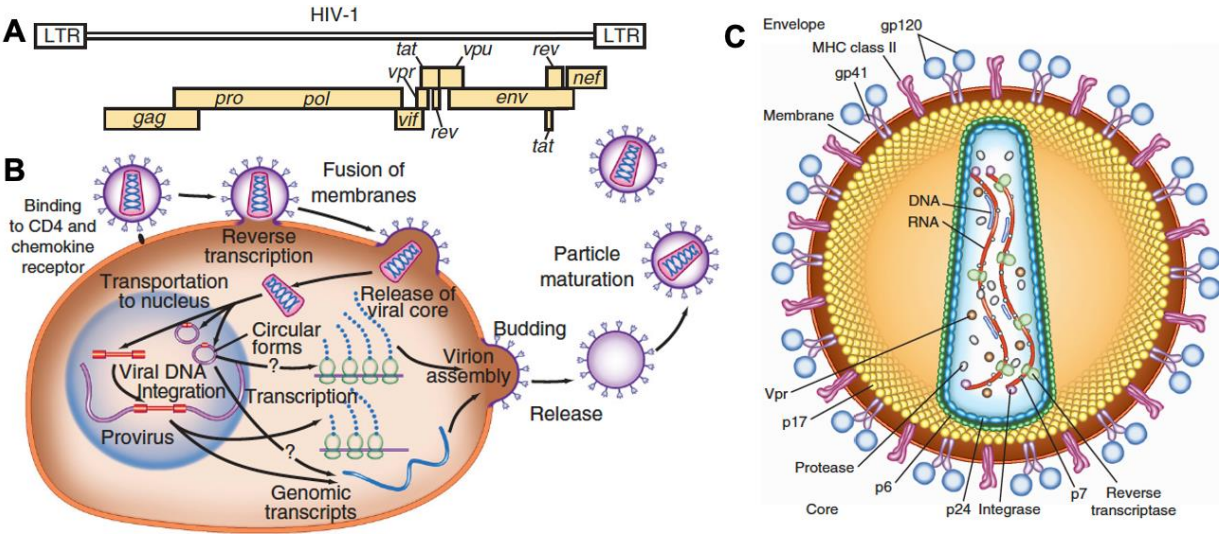


Figure 1.3 HIV structure and life cycle.
Genomic structure of HIV (A), the life cycle (B) and HIV-1 virion (C). Figure reproduced with permission from Reitz and Gallo 2015.

1.2.2. The route from transmission to disease progression

Regardless the transmission route, HIV infection is usually established by a single transmitted-founder virus that has a consensus-like sequence and a CCR5 tropism (Shaw and Hunter 2012; Naidoo et al. 2017). After either breaching the mucosa barrier or the bloodstream, DCs recognise Env-gp120, leading to their activation and migration to lymph nodes (LNs) (Cunningham, Harman, and Donaghy 2007). The role of DCs as antigen presenting cells (APCs) is a double-edged sword in HIV pathogenesis by priming lymphocytes to initiate the adaptive immune response against the virus, but also activating resting CD4+ T-cells. HIV exploits DCs as a transporter to regions fully populated with target cells.

The direct link between HIV replication in CD4+ T-cells seems to be the obvious cause of their progressive depletion that leads to AIDS. However, the vast majority of cell death occurs due to abortive infections that trigger a highly inflammatory pathway called "pyroptosis" (Doitsh et al. 2014). The inflammatory milieu attracts more healthy cells and ignites a vicious cycle (Figure 1.4.A). Chronic inflammation is now accepted as the leading cause of progression to AIDS. Indeed, CD38 and Human Leucocyte Antigen (HLA)-DR, well-known markers of T-cell activation, are a stronger predictor of HIV disease progression than plasma viral load (Giorgi et al. 1999).

The cause of chronic inflammation is multifactorial, and it is conceivable that different mechanisms have different weights according to the disease stage (Figure 1.4.B) [reviewed in (Paiardini and Müller-Trutwin 2013)]. Different cell types are activated in the course of infection and are the source of pro-inflammatory molecules that help maintain the cycle. The reactivation of latent infections such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) can also stimulate cells and

contribute to chronic inflammation (Doisne et al. 2004). Nonetheless, the major contributor is the disruption of the gastrointestinal (GI) barrier and microbial translocation, leading to the release of bacterial and fungal by-products that induce the secretion of pro-inflammatory molecules. Lipopolysaccharide (LPS) and bacterial genetic materials are increased in the circulation during HIV infection (Abad-Fernández et al. 2013). Moreover, HIV preferentially infects the Th17 subset that populates the GI tract and plays a vital role in maintaining mucosal integrity (Brenchley et al. 2008). Bacterial translocations are possibly one of the drivers of faster progression in children due to the immature and more permeable gut epithelium seen in infants (Sherman 2010).

HIV also preferentially targets the long lived, self-renewal and tissue resident central memory (CM) CD4+ T-cells (Okoye et al. 2007; Brenchley et al. 2012). Their activation and death stimulate homeostatic proliferation, especially in LNs, which help maintain viral replication and the inflammatory state.

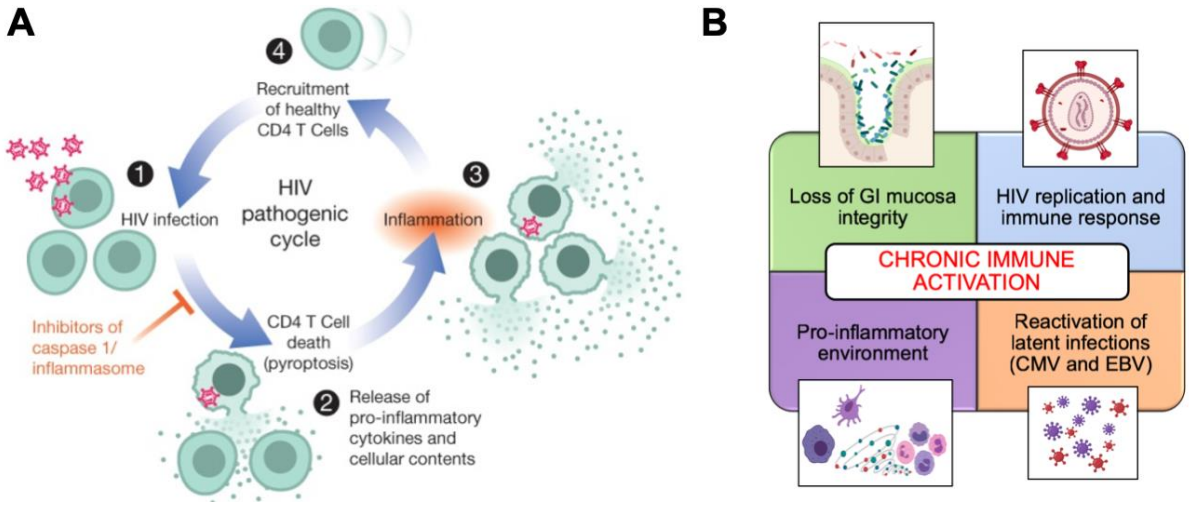


Figure 1.4 The pathway towards chronic immune activation.
A. The vicious cycle of CD4+ T-cell "pyroptosis" death. Figure reproduced with permission from Doitsh et al. 2014. B. The multifactorial causes of chronic immune activation in HIV pathogenesis. Figure done on Biorender.

1.2.3. The immune response in early life

The apparently immature infants' immune response in fact is a well-adapted system to face the obstacles of the intense intrauterine maternal-fetal interplay and the immediate post-birth environment of an almost naïve immune system exposed to life-threatening extracellular pathogens (Figure 1.5). However, it is widely exploited by intracellular pathogens like HIV.

Monocytes and DCs in infants can effectively sense HIV and prime lymphocytes to initiate the adaptive immune response. HIV is primarily recognised by receptors that bind to viral genetic substrates, such as Toll-like receptor (TLR)-7 and -9 (Rustagi and Gale 2014). Upon recognition, pro-inflammatory pathways are activated, including the interferon (IFN) type I pathway, leading to the expression of IFN-stimulated genes (ISGs) that attenuate viral entry and replication and promote the recruitment of cytotoxic cells (Stetson and Medzhitov 2006). Although efficient in their APC function, DCs in infants secret cytokines that prime the CD4+ T-cell towards Th2 and Th17 responses, such as Interleukin (IL)-10, IL-6 and IL-23, to the detriment of a Th1 response that requires secretion of IL-12 and IFN-γ (Kollmann et al. 2009). The immune strategy adopted in early life avoids the harmful effect of a Th1 response and protects the infants against life-threatening extracellular bacteria and fungi (Levy 2007), but makes them more susceptible to HIV.

The Th1 response is fundamental for the development and maintenance of antigen specific CD8+ T-cells. Cytotoxic T-lymphocytes (CTLs) are known to be the key controllers of viral replication in adults (Koup et al. 1994; Borrow et al. 1994; Pereyra et al. 2010), but in vertical transmission they encounter important obstacles in addition to the Th2 bias in children. Infants inherit a virus that can be already pre-

adapted to half of their HLA class I (HLA-I) alleles and, therefore, unable to effectively target the transmitted virus (Goulder et al. 2001). In addition, although HIV-specific CD8+ T-cells can be detected after birth, they usually target non-Gag regions, have lower magnitude and are less polyfunctional (Scott et al. 2001; Lohman et al. 2005; Huang et al. 2008; Leitman et al. 2017).

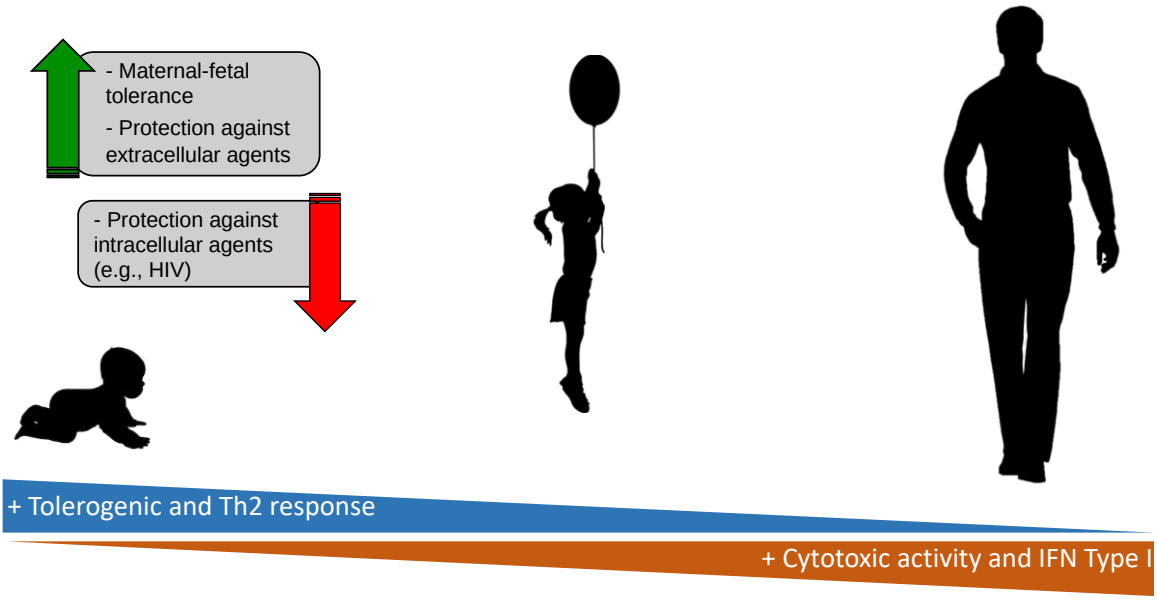


Figure 1.5 The contrasting immune system in children and in adults.

Infants also have an increased frequency of T regulatory (Treg) cells. Tregs downregulate the function of effector cells and make them less responsive, which enable adequate intrauterine interplay between fetus and their mother (Michaëlsson et al. 2006), but do not contribute to the generation of a strong HIV-specific response. Although the number of Tregs falls towards the adult levels after birth, intrauterine-primed cells persist through childhood and promote suppressive functions (Mold et al. 2008).

Overall, the faster progression to AIDS in children is a consequence of a more tolerogenic immune system. It effectively avoids inflammation in a period of rapid

development, and also protects children against life-threatening extracellular pathogens but it creates a scenario where HIV cannot be effectively controlled.

1.2.4. Slow progression and the non-pathogenic model

Even though children progress faster to AIDS than adults, around 10% of ART-naïve children reach late childhood with normal-for-age CD4+ T-cell count and no signal of clinical disease, referred to as paediatric slow progressors (PSPs) (Mphatswe et al. 2007; Ananworanich et al. 2010). PSPs are usually viraemic and maintain tolerance to viral replication without AIDS-progression, similar to the much less frequent adult viraemic non-progressors (AVNPs) (Rotger et al. 2011) and to the African monkeys natural hosts of SIV [reviewed in (Klatt, Silvestri, and Hirsch 2012)].

The lack of AIDS-progression in African monkeys is the result of a coevolution with SIV that dates back more than 30,000 years (Worobey et al. 2010). SIV is host-specific and non-African monkeys like the Rhesus macaque develop a disease course similar to AIDS when infected. Most non-pathogenic models come from studies in sooty mangabeys (SMs), African green monkeys (AGMs) and mandrills (Figure 1.6). After acute infection, a plasma viral setpoint is achieved similar to the pathogenic infection, but a normal peripheral CD4+ T-cell frequency is maintained despite ongoing viral replication, and the development of immunodeficiency is extremely rare (Pandrea et al. 2006). Viraemic control is not commonly seen and the SIV-specific T-cell response is lower when compared to the HIV response in humans (Dunham et al. 2006). Furthermore, CD8+ T-cell depletion has minimal effect on SIV plasma viraemia (Barry et al. 2007).

The innate response is robust during acute infection and ISGs are found equally upregulated in AGMs and in Rhesus macaques (Bosinger et al. 2009; Jacquelin et al. 2009). However, regulatory pathways come into play and rapidly downregulate ISGs to avoid chronic inflammation in the non-pathogenic model (Jacquelin et al. 2009). The mechanisms responsible to curb inflammation after acute infection are still a matter of debate and include rapid upregulation of programmed death 1 (PD-1), Treg activity, containment of apoptosis-inducing genes (e.g., TRAIL) and low TLR signalling (Kim et al. 2007; Estes et al. 2008; Mandl et al. 2008).

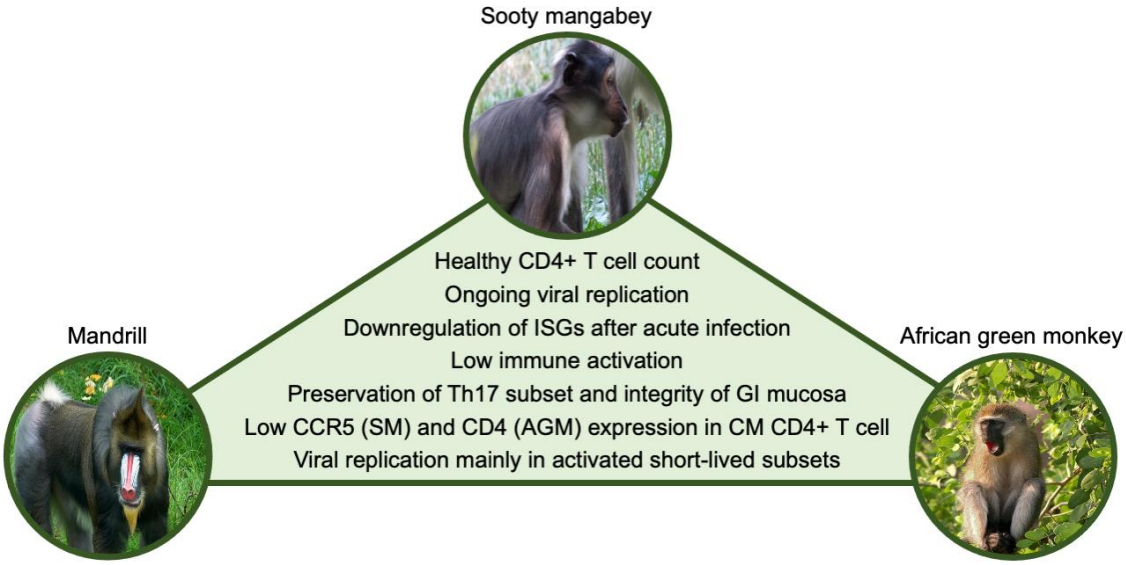


Figure 1.6 The non-pathogenic SIV model.
Reproduction was authorized by copyright holders: SM - Giulio Russo; Mandrill -Sanjay Acharya; AGM - Whit Welles.

The lack of systemic immune activation during the chronic phase is the major feature of the non-pathogenic model. Although mucosal CD4+ T-cells are depleted during acute infection in natural hosts, the Th17 subset is preserved, and the GI barrier is not damaged. In addition, the vicious cycle of activation and homeostatic proliferation of long-lived subsets seen in HIV-pathogenesis is absent in natural hosts. CM CD4+ T-cells are somehow protected due to low CD4 and CCR5 expression on AGM and SM, respectively (Pandrea et al. 2007; Beaumier et al. 2009).

PSPs and AVNPs share profound similarities to the natural hosts of SIV (Rotger et al. 2011; Klatt et al. 2014; Muenchhoff et al. 2016). African monkeys coevolved with SIV towards a non-aggressive and indulgent model that allows viral replication without systemic inflammation and progression to AIDS. The more tolerogenic immune system observed in early life may explain the higher frequency of slow-progressors in the paediatric group. PSPs have lower levels of systemic inflammation and T-cell activation (HLA-DR+CD38+) and exhaustion (PD-1+) when compared to paediatric progressors (PPs) (Muenchhoff et al. 2016). CCR5 expression on CD4+ T-cells is also lower in the CM subset of PSPs, confining viral replication in the short-lived population of effector memory (EM) cells (Muenchhoff et al. 2016). However, the mechanisms leading to this phenotype has not yet been explored in humans.

1.2.5. Viraemic controllers

Individuals with spontaneous control of plasma viraemia, also known as Elite Controllers (ECs), are a relatively rare group. The most accepted definition is of an ART-naïve individual with at least three consecutive plasma viral load below the limit of detection (conventionally, 50 plasma HIV-RNA copies/mL) spanning at least a year (Deeks and Walker 2007). Their prevalence ranges between 0.2 to 0.5% (Okulicz and Lambotte 2011; Olson et al. 2014; Yang et al. 2017) but it can vary according to the criteria used and the cohort design. Truly, only a minority of adult ECs fully control plasma viraemia and maintain <1 copy/mL, and fluctuations in residual viraemia are relatively common (Pereyra et al. 2009; Hatano et al. 2009; Noel et al. 2015).

The clinical follow-up of ECs reveals a heterogenous group with different levels of viral suppresion (Figure 1.7) [reviewed in (Navarrete-Muñoz et al. 2020)]. A study of

seroconverters indicated that viraemic control is typically achieved within the first 12 months of infection (Madec et al. 2013). Overall, loss of viral suppression occurs in a low number varying between 1.22 to 4.22% per year (Okulicz and Lambotte 2011; Yang et al. 2017). However, studies done using public database and historical cohorts largely overestimate its duration by losing the early stages of infection. Elite control can be detected in up to 6% of the adults living with HIV in the phase after seroconversion, and it is transitory for the vast majority (Madec et al. 2013). Several factors have been associated with loss of EC status, including residual plasma viraemia, viral blips, HIV-DNA levels, change in viral tropism and female sex (Madec et al. 2013; Yang et al. 2017; Rosás-Umbert et al. 2019).

ECs usually have normal CD4+ T-cell counts, and HIV disease progression and CD4+ T-cell decline are usually preceded by the loss of viral suppression (Rosás-Umbert et al. 2019). Nonetheless, ECs in general have higher systemic inflammation than healthy individuals and a higher risk of developing non-AIDS defining conditions, including cardiovascular events (Hunt et al. 2008). Therefore, it is currently advised to offer ART to this group.

The natural history of ECs can give some clues of the mechanisms that lead to viral control, including viral and host factors. Some defects in accessory genes such as *nef*, *vif* and *vpu* have been reported in ECs, which could favour host restriction factors and the priming of the adaptive immune response (Lum et al. 2003; Mwimanzi et al. 2013; Chen et al. 2015). However, the presence of detectable plasma viraemia during acute infection and the relatively common rate of blips and viral rebound exclude viral factors as the main mechanism. Virus isolated from some ECs generally has a similar replication capacity and genetic evolution compared to non-controllers (Blankson et al. 2007). Moreover, the same fully competent transmitted founder virus

was isolated from a non-controller and an EC (Buckheit et al. 2012), consistent with host factors determining elite control.

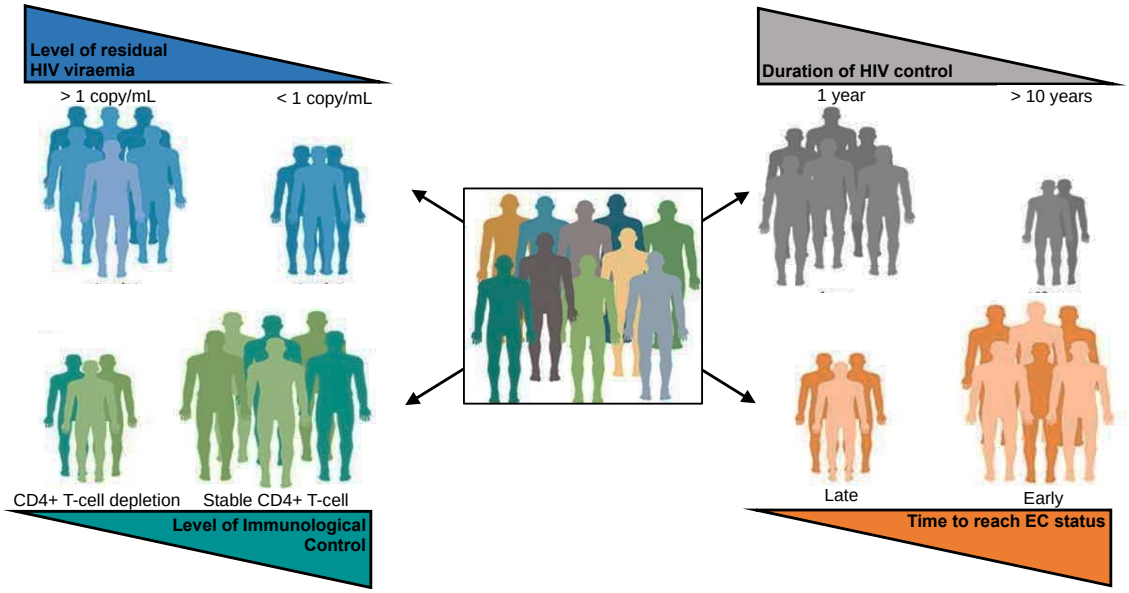


Figure 1.7 Elite Controllers' natural history in adults.
Figure reproduced with permission from Navarrete-Muñoz *et al.* (2020).

The appearance of the HIV-specific T-cell immunity is temporally associated with reaching spontaneous viraemic control. The breadth and magnitude of HIV-specific CD8+ T-cell responses, particularly those targeting Gag, have been associated with lower viral setpoint and slower progression in adults (Edwards et al. 2002). The two most extensive genome-wide association studies looking at single-nucleotide polymorphisms (SNPs) consistently pointed out the HLA-I genes as the key regions associated with elite control (Fellay et al. 2007; Pereyra et al. 2010). Adult ECs are enriched with particular HLA-B molecules, such as HLA-B*27, *57, *58:01 and *81:01 (Pereyra et al. 2008; Leslie et al. 2010; Carlson et al. 2012). As a shared feature, these alleles can bind and present peptides from conserved and fitness valuable regions of the virus, such as Gag and Pol. ECs are enriched with Gag-specific CD8+ T-cells, while non-controllers frequently target regions with a lower fitness barrier, such as Env and Nef (Kiepiela et al. 2007; Berger et al. 2011; Turk et al. 2013; Sunshine et

al. 2014). However, not all individuals expressing these protective HLA-B alleles become ECs. The SNPs identified only explain around 20% of viraemic control (Pereyra et al. 2010). Additionally, since EC prevalence is approximately 0.3% (Yang et al. 2017) and HLA-B*57 prevalence in most populations is approximately 7%, <5% of adults living with HIV and expressing HLA-B*57 are ECs.

Other functional aspects of the CD8+ T-cell response have also been associated with viral control. ECs' CTLs have a greater cytolytic capacity and release more granzymes and perforins upon encountering HIV-infected cells (Migueles et al. 2002; Hersperger et al. 2010). They also secrete more antiviral and pro-inflammatory cytokines and chemokines, such as IFN-γ, Tumour necrosis factor (TNF)-α and Macrophage inflammatory protein-1β (MIP-1β) (Sáez-Cirión et al. 2007; Almeida et al. 2007), and they are also generally more polyfunctional (Betts et al. 2006; Almeida et al. 2007). Furthermore, the HIV-specific CD8+ T-cell population is highly enriched with resting CM cells with a self-renewal and rapid expansion capacity (López et al. 2011; Ndhlovu, Stampouloglou, et al. 2015; Rutishauser et al. 2021).

Although the CD4+ T-cells of ECs are susceptible to HIV-infection, some studies have identified a higher capacity of MIP secretion by these cells, making them more resistant to CCR5-tropic virus (Walker et al. 2015). Additionally, ECs have lower amount of integrated HIV-DNA and more nuclear non-integrated circular HIV-DNA (Graf et al. 2011; Walker et al. 2015). HIV-specific CD4+ T-cells may also contribute to viral suppression, and they are associated with a lower viral set point (Rosenberg et al. 1997; Soghoian et al. 2012). Th1 responses characterized by high IFN-γ and IL-2 secretion in Gag-restricted CD4+ T-cells are higher among ECs (Emu et al. 2005; Zimmerli et al. 2005; Chevalier et al. 2011). But the true impact of effector HIV-

specific CD4+ T-cell response is still a matter of debate, and it might be a consequence rather than a cause of viral control and immunological non-progression.

ECs in the paediatric population has only been sporadically described as case reports (Ananworanich et al. 2004; Tang et al. 2010). Overall, a more pro-inflammatory and aggressive HIV-specific CD8+ T-cell and Th1 responses seem to be more effective in suppressing viraemia, opposing to the tolerogenic and Th2 biased responses observed in children.

1.3. Different impact of acute and chronic infections in the CD8+ T-cell development

1.3.1. CD8+ T-cell ontogenesis

Hematopoietic stem cells residing in the bone marrow (BM) migrate to the thymus, where the T-cell development occurs. Progenitor T-cells interact with thymic epithelial cells (TECs), circulating outward and inward the cortical and medullar regions. Progenitor cells differentiate towards the T-cell lineage by acquiring CD45RA, CD7 and CD1a during their proliferation and maturation process. They start as double negative for CD4 and CD8, followed by a single-positive CD4+CD8- stage and a late double-positive CD4+CD8αβ+ stage. Meanwhile, the T-cell receptor (TCR) undergoes a complex and stepwise rearrangement up to the expression of the αβ-TCR. Double positive T-cells with weak to intermediate TCR affinity to self-peptides presented by HLA-I and -II on cortical TECs are positively selected and become CD8+ and CD4+ single-positive T-cells, respectively. Self-tolerance and negative selection continue via interaction with TECs and DCs at the medullar region. After receiving survival signals, fully mature naïve T-cells emerge from the

thymus to populate lymphoid organs [reviewed in (Kumar, Connors, and Farber 2018)].

1.3.2. CD8+ T-cell differentiation in acute infection

In acute infections and after vaccination, antigen-specific naïve CD8+ T-cells are primed by APCs and activated (Henrickson et al. 2008). This initiates a process of rapid expansion of memory precursors T-cells, differentiation, and migration. Most cells achieve the stage of terminally differentiated effector memory (TEMRA) CD8+ T-cells capable of eliminating infected cells via secretion of cytokines and cytolytic granules. Upon pathogen clearance, TEMRA CD8+ T-cells undergo intense contraction but around 5-10% of pathogen-specific CD8+ T-cells survive to become the various subsets of memory cells (Figure 1.8.A) (Hammarlund et al. 2003). They represent a broad continuum comprising different populations with heterogeneous functions and survival capacity (Rubinstein et al. 2008). The literature so far indicates that the differentiation into memory subsets is branched upon naïve T-cell activation. However, effector cells derived from IL-7Rα^{high} memory precursors T-cells have a certain ability to transit back and forth into transitional memory (TM) and CM T-cells, while those derived from IL-7Rα^{low} die in the contraction phase [reviewed in (Cui and Kaech 2010)].

Memory T-cell subsets have different levels of expansion and effector capacity (Figure 1.8.B). The CM population is long-lived with extensive self-renewal ability. These cells express IL-7Rα at high levels and secrete IL-2 but lack prompt effector function (Kaech and Wherry 2007). Co-stimulatory signalling coming from CD27/CD28 (Williams and Bevan 2007) and IL-2 secretion from CD4+ Th cells (Shedlock and Shen 2003) are essential for memory acquisition. CM CD8+ T-cells express CCR7

and are mainly localised in LNs, while some subsets express other homing molecules (e.g. CD69) and become specialised tissue-resident memory (RM) CD8+ T-cells [reviewed in (Kumar, Connors, and Farber 2018)]. Upon activation, CM CD8+ T-cells can expand and acquire cytotoxic and cytolytic functions and respond to secondary infections.

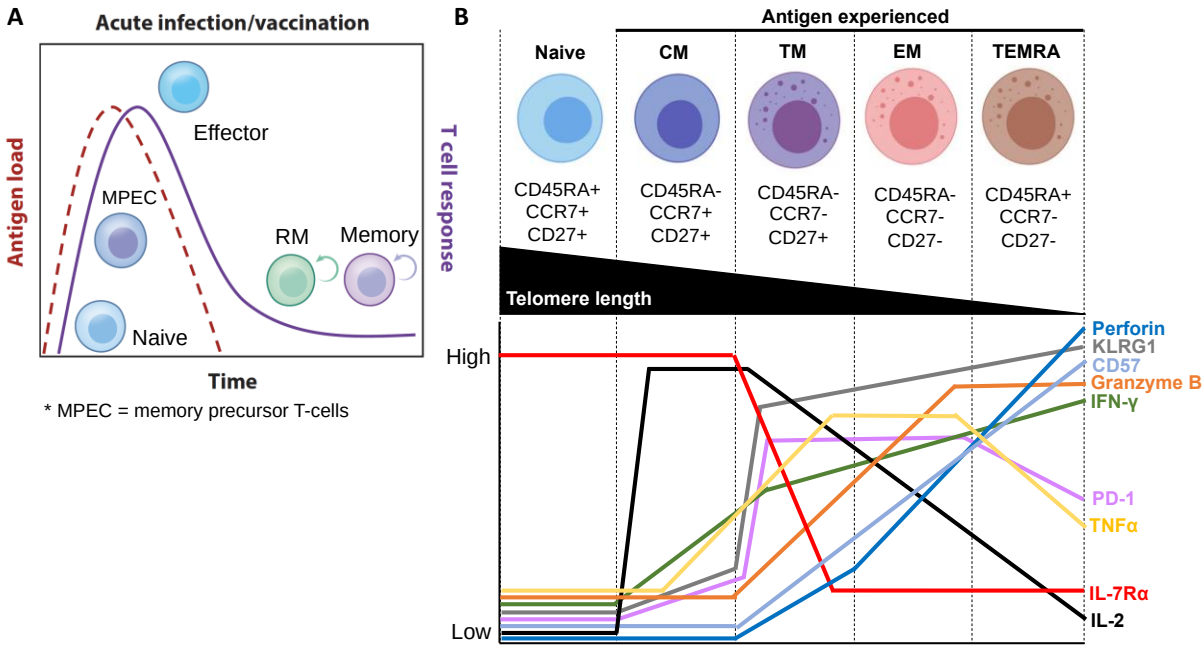


Figure 1.8 CD8+ T-cell in acute infection and its subsets markers.
A. CD8+ T-cell differentiation in acute infection and vaccination. Figure reproduced with permission from McLane LM et al. 2019. B. Phenotypic markers associated with the differentiation state and the effector function of CD8+ T-cell subsets. Modified with permission from Appay V et al (2008) using Biorender.

EM CD8+ T-cells have a self-limited expansion potential [reviewed in (Cui and Kaech 2010)] but respond faster to secondary infections with prompt IFN-γ and cytolytic granules release. Pro-inflammatory cytokines, IL-12 and TCR signalling are crucial for EM CD8+ T-cell development (Curtsinger, Lins, and Mescher 2003; Pearce and Shen 2007). The EM population is more frequent in the periphery and progressively downregulates CCR7, CD28/CD27 and IL-7Rα (Appay et al. 2008).

1.3.3. CD8+ T-cell differentiation in chronic infection

The continuous antigen load in chronic infections and cancer dramatically modifies the CD8+ T-cell differentiation process described above, disturbing memory development through the emergence of exhausted CD8+ T-cell (Tex) (Figure 1.9.A) [reviewed in (McLane, Abdel-Hakeem, and Wherry 2019)]. The term is misleading, however, and the Tex population are not CD8+ T-cells lacking effector functions but rather a heterogenous population with a hierarchical loss of functional capacity driven by profound epigenetic and transcriptional adaptations. The repeated TCR signalling found in chronic infections and cancer promotes the survival of early Tex population, also known as stem-like Tex, who, like CM CD8+ T-cells, maintain their expansion potential but lack prompt effector function (Shin et al. 2007). Upon the acquisition of effector roles, the persistent TCR signalling leads to accumulation of inhibitory receptors and a progressive decrease in IL-2, TNF-α and ultimately in IFN-γ and granules release in terminally Tex (Wherry et al. 2003).

The pillars of CD8+ T-cell activation in acute infection are the TCR signalling, the co-stimulation and the presence of pro-inflammatory cytokines. However, during chronic infection, this process is disturbed by the persistent signalling coming from the TCR engagement in a chronic inflammatory environment, while the costimulatory pathways are abrogated by the accumulation of inhibitory receptors (Figure 1.9.B). Some cytokines up-regulated in this context contribute to exhaustion, including IL-10 and Tumour growth factor (TGF)-β, while the homeostatic cytokines IL-2 and IL-21 are usually absent (Yi, Du, and Zajac 2009; Said et al. 2010). Similarly, some soluble molecules like prostaglandin E2 and adenosine also favours exhaustion (Kalinski 2012). CD39, an enzyme responsible for converting extracellular ATP/ADP

to adenosine, is upregulated in these conditions and is a marker of terminally Tex (Gupta et al. 2015).

PD-1 is one of the primary inhibitory receptors expressed in Tex. It is up-regulated in the first hours after CD8+ T-cell activation and works as a negative feedback to avoid uncontrolled immune activation and tissue damage in acute infection. Upon antigen cessation, PD-1 is downregulated and contributes to the development of memory CD8+ T-cells (Ahn et al. 2018). In chronic infections, where antigen stimulation is continuous, PD-1 is persistently expressed on the CD8+ T-cell surface, followed by other inhibitory receptors (e.g. Tim-3, Lag-3, TIGIT and CTLA-4) (Blackburn et al. 2009). The PD-1 ligands are PD-L1 (B7.H1), expressed by various cells, including non-immune cells, and PD-L2 (B7.DC), expressed by DCs, macrophages and some B-cells. Two intracytoplasmic tyrosine residues (ITIM and ITSM) recruits SHP-2, leading to the inhibition of the TCR- and CD28-signalling pathways (Figure 1.9.C). The downstream effect of its persistent expression is the change in the T-cell metabolism and cell cycle, decreasing the secretion of IL-2 and IFN-γ and the proliferation rate [reviewed in (McLane, Abdel-Hakeem, and Wherry 2019)].

Tex are phenotypically and functionally different from senescent T-cells. Tex cells progenitors come from a pool of IL-7Rα+ memory precursors and not from the terminal senescent Killer Cell Lectin Like Receptor G (KLRG)-1+ TEMRA CD8+ T-cells (Angelosanto et al. 2012). The stem-like Tex population expresses intermediate levels of PD-1, but not other inhibitory receptors, and maintains a large capacity of self-renewal and of generating the larger population of effector and terminally Tex. Stem-like Tex subset expresses high levels of the transcription factor T-cell factor 1 (TCF-1), which is also highly expressed in naïve cells and involved in

the generation of memory T-cells after acute infections. TCF-1 maintains the IL-2/15Rβ pathway and inhibits the effects of IFN-I (Im et al. 2016; Wu et al. 2016). TCF-1 also induces the expression of Eomes and Bcl-2, both associated with memory maintenance, and inhibit the expression of T-bet and effector molecules such as Granzyme B (McLane, Abdel-Hakeem, and Wherry 2019). By inducing Eomes and Bcl-2, TCF-1 helps to maintain the pool of CD8+ Tex population. Tex differentiation is driven by a decrease in TCF-1 expression, a progressive increase in PD-1 and gain of effector functions up to terminally Tex population, where the accumulation of other inhibitory receptors leads to functional exhaustion (Paley et al. 2012) (Figure 1.9.D). Thymocyte selection-associated high mobility group box protein (Tox) is a critical regulator of T-cell exhaustion by playing a fundamental role in the differentiation and maintenance of Tex. Tox calibrates the transcriptional and epigenetic modifications in the context of chronic stimulation, acting in the differentiation from stem-like exhausted TCF-1^{high} Tex population to TCF-1^{low}PD-1^{high} terminally Tex (Alfei et al. 2019).

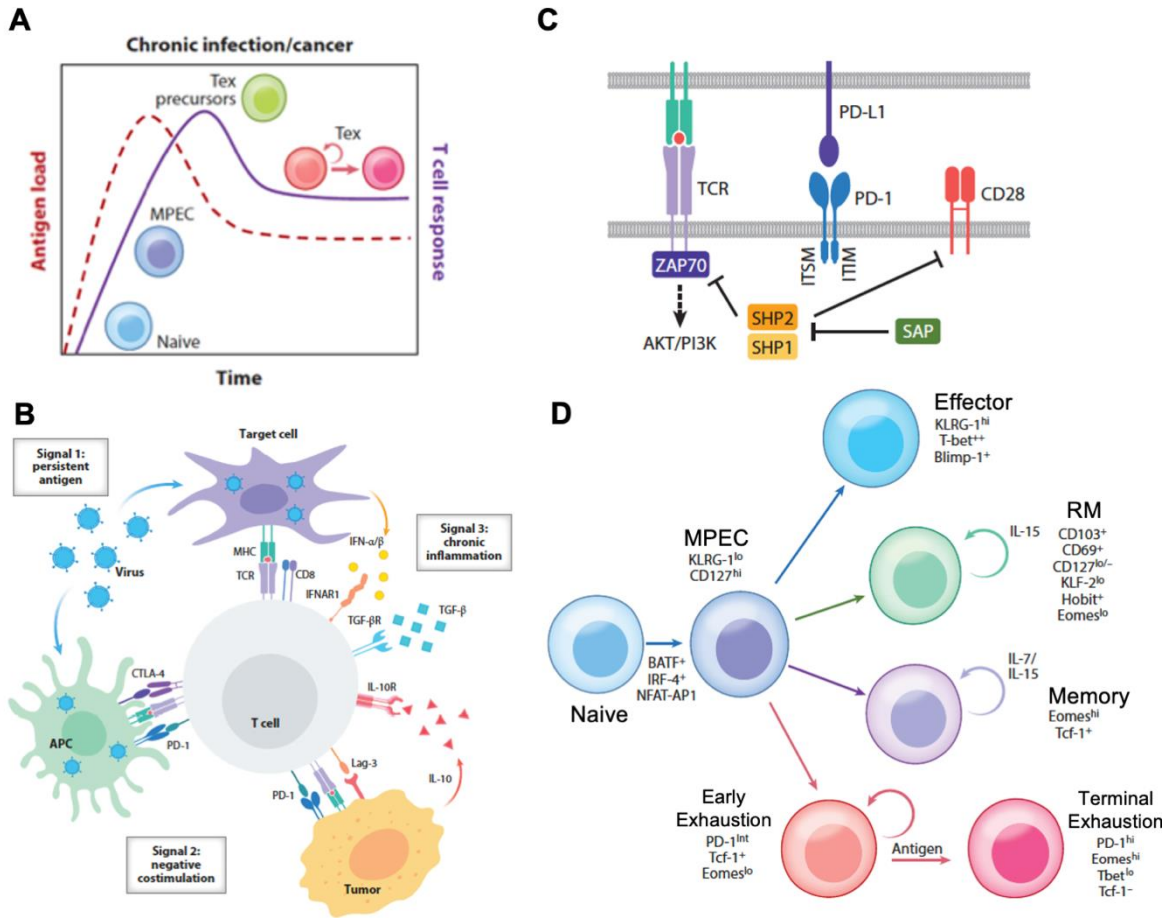


Figure 1.9 CD8+ T-cell in chronic infection.
A. CD8+ T-cell differentiation in the context of chronic infection and cancer. B. Signals present during chronic TCR-stimulation. C. Inhibitory pathway promoted by PD-1 engagement. D. CD8+ T-cell development in the context of transient and continuous antigen presence. Figures reproduced with permission from McLane LM et al. 2019.

1.3.4. PD-1 in HIV infection

PD-1 expression has been constantly associated with poor outcome and HIV disease progression, predicting faster viral rebound after analytical treatment interruption (ATI) in individuals treated as early as a few months after seroconversion (Trautmann et al. 2006; Day et al. 2006; Hurst et al. 2015; Rutishauser et al. 2017). PD-1 expressing HIV-specific CD8+ T-cells commonly have the phenotype of terminally Tex including co-expression of other inhibitory receptors and impaired effector functions that are only partially reversed with ART (Rutishauser et al. 2017).

In vitro PD-1 blockade has shown to revigorated the effector function and increase the capacity to kill HIV-infected target cells (Trautmann et al. 2006; Day et al. 2006). Similarly, *in vivo* PD-1 blockade in NHP models led to expansion and functional recover of SIV-specific CD8+ T-cells and lower plasma viral load (Velu et al. 2009). However, the field still lacks *in vivo* human data, as individuals living with HIV are usually excluded in immunotherapy studies due to safety precautions. A trial recently reported a mild effect of PD-1 and CTLA-4 dual blockade in latency reversal in individuals on ART, but no data exploring the impact on HIV-specific T-cell effector function has been published (Rasmussen et al. 2021).

Despite the safety concerns and the lack of robust effects observed so far in HIV infection, immune checkpoint blockade is a rapidly evolving field that can bring immense benefits to the HIV therapeutical arsenal. The heterogeneity within the PD-1+ CD8+ T-cell population has not been fully explored in HIV. The proliferative and differentiation potential of the stem-like Tex population has been associated with better response to immunotherapy in cancer, while the terminally Tex population responds poorly to PD-1 blockade (Siddiqui et al. 2019a; Miller et al. 2019a). TCF-1+ virus-specific CD8+ T-cells are increased in viraemic controllers in HIV and SIV infection and their frequency is positively correlated with *in vitro* expansion and memory capacity (Rutishauser et al. 2021). The tolerogenic and non-inflammatory environment observed in infants, especially in PSPs, may favour the development of a stem-like Tex population with higher expansion potential not yet explored in HIV infection.

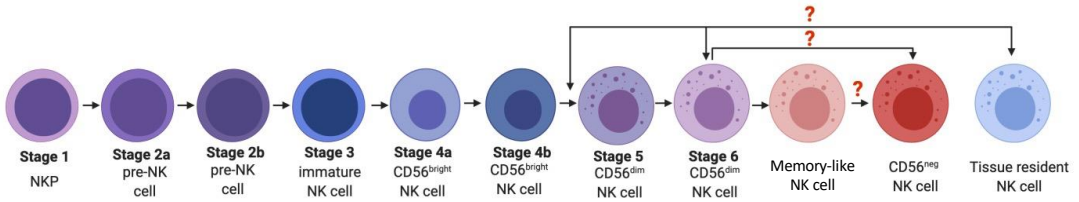
1.4. NK cells in HIV infection

1.4.1. NK cell development

Natural Killer (NK) cells represent 5-15% of peripheral lymphocytes in humans and employ essential functions, including immune-surveillance and -regulation, and effector activity against tumours, infected and non-allogenic cells. They are traditionally defined as a CD3- (non-T), CD19- (non-B) lymphocyte capable of secreting IFN-γ upon stimulation. NK cells share some phenotypic similarities with the T-cells, especially CD8+ T-cells, but they develop and function differently, leading to complementary roles in immunity [reviewed in (Freud et al. 2017; Di Vito, Mikulak, and Mavilio 2019)].

NK cell development primarily occurs in the BM, but all developmental stages have been identified in different sites, such as secondary lymphoid tissues, peripheral blood, liver and uterus. The commitment of the CD45RA+ hematopoietic stem cells to the NK lineage occurs in two subsequential steps: the loss of CD34 and the gain of CD122 (IL-2/IL-15R β subunit) and IL-1βR, which eliminate their potential to differentiate into T-cells and DCs; followed by the gain of activating NK receptors (aNKRs), leading to the rise of immature NK cells and preventing them from differentiating in other innate lymphocytes. It is followed by the sequential acquisition of CD56 and CD94. The fully mature $CD56^{bright}$ NK cells are characterised by the expression of the aNKRs NKG2A, NKG2D, NKp30 and NKp46. The progressive gain of CD16, Killer cell immunoglobulin-like receptors (KIRs) and granzyme granules marks the transition to the $CD56^{dim}$ population. The differentiation process further continues with the loss of NKG2A and the gradual expression of CD57. Although described as a sequential stage, NK cells development has some plasticity, and not all $CD56^{bright}$

are fated to become CD56^{dim} (Figure 1.10) [(Björkström et al. 2010), reviewed in (Freud et al. 2017; Di Vito, Mikulak, and Mavilio 2019)].



	Stage 1 NKP	Stage 2a pre-NK cell	Stage 2b pre-NK cell	Stage 3 immature NK cell	Stage 4a CD56bright NK cell	Stage 4b CD56bright NK cell	Stage 5 CD56dim NK cell	Stage 6 CD56dim NK cell	Memory-like NK cell	CD56neg NK cell	Tissue resident NK cell
CD34	+	+	+	-	-	-	-	-	-	-	
CD10	+	+/-	+/-	-	-	-	-	-	-	-	
HLA-DR	+	+	+	-	-	-	-	-	+	+	
CD117	-	+	+	+	+/low	low/-	-	-	-	-	
CD127	+	+	+	+	-	-	-	-	-	+	
CD45RA	+	+	+	+	+/-	+/-	-	-	-	-	
IL-1β	-	-	+	+	+/low	low/-	low/-	low/-	low/-	-	
CD122	-	-	+	+	+	+	+	+	+	+	
CD161	-	-/low	-/low	-/+	+	+	+	+	low/-	+	
CD56	-	-	-/low	-/low	++	++	+	+	+	-	
CD94	-	-	-	-	+	+	+/-	+/-	+	+	
NKG2A	-	-	-	-	+	+	low/-	low/-	low/-	low/-	
NKG2D	-	-	-	-/low	+	+	+	+	+	+	
NKp30	-	-	-	-/low	+	+	+	+	low/-	low	
NKp46	-	-	-	-/low	++	++	+	+	low	low	
NKp80	-	-	-	-	-	+	+	+	+	+	
NKG2C	-	-	-	-	low/-	low/n	+	+	++	+/low	
CD16	-	-	-	-	-	-	+	+	+	+	
KIRs	-	-	-	-	-	-	+	+	+	+	
CD57	-	-	-	-	-	-	-	+	+	+	

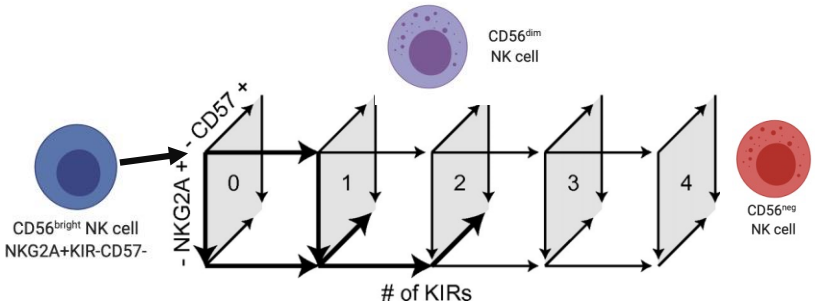


Figure 1.10 NK cell ontogenesis and surface markers.
Figures adapted with permission from Di Vito et al. (2019) and Björkström et al. (2010) using Biorender.

Several transcription factors are expressed in different stages of NK cell development. Although most are not specific for NK cell lineage, E4BP4 is singular to NK cell commitment and its deficiency leads to the lack of only NK cells in mice (Male et al. 2014). Ets-1 and PU.1 are induced in the early stages and are essential in the transition from hematopoietic stem cells to NK cell precursors (Taveirne et al. 2020). As mentioned, the gain of CD122 is fundamental in NK cell maturation, and Runx3

regulates its expression. CD122 leads to JAK3, JAK1, and STAT5 activation and downstream modifications on NK cell machinery (Miller et al. 2019b). Tox plays an essential role in NK cell differentiation from hematopoietic stem cells and also induces T-bet in later stages (Aliahmad, de la Torre, and Kaye 2010). Both T-bet and Eomes are necessary to promote the effector functions of NK cells in the mature subsets, including the expression of cytolytic proteins and IFN-γ. Blimp-1 and Zeb2 are T-bet dependent and act in the later stages of NK cell maturation and their release from the BM [reviewed (Bi and Wang 2020)].

The $CD56^{bright}$ NK cells, while representing less than 10% of the peripheral NK cells, are the majority in tissues and secondary lymphoid organs. They are highly responsive to cytokine, secreting IFN-γ and immunomodulatory cytokines and chemokines upon stimulation. Almost 90% of NK cells identified in the peripheral blood are the $CD56^{dim}$ subset. They are recognised by the high levels of granules containing granzymes and perforins, CD16 expression and late cytokine releasing, which invest in them the cytolytic effector role. Among this group, there is a population of mature CD57+ cells with a memory-like signature, primarily identified in the context of CMV infection. These memory-like NK cells usually express the activating heterodimer CD94/NKG2C, bear an "adaptiveness" epigenetic signature resembling CTLs, can expand when re-exposed to CMV and exert efficient antibody-dependent cellular cytotoxicity (ADCC) killing. A population of $CD56^{negative}$ NK cells emerges in the context of chronic viral infection and expands with HIV disease progression. They are considered terminally differentiated (CD57+KIR+NKG2A-) NK cells and functionally exhausted. Finally, tissue resident NK cells are $CD56^{bright}$, highly specialised and distinct from NK cells in the peripheral blood, particularly the populations identified in LNs, tonsils, uterus and liver [reviewed in (Del Zotto et al. 2017; Di Vito, Mikulak, and Mavilio 2019)].

1.4.2. NK cell receptors

In contrast to the T-cells, the NK cells are regulated by a group of germline non-rearranged activating and inhibitory receptors with close interaction with HLA-I alleles (Figure 1.11). The regulatory role employed by the HLA-I molecules occurs through a process called "education" and allows educated cells to lyse target cells lacking HLA-I due to the abrogation of the inhibitory signal. The HLA-I and NK cell synergism are regulated by polymorphic and non-polymorphic receptor-ligand interactions [reviewed in (Freud et al. 2017; Bastidas-Legarda and Khakoo 2019)].

KIRs are encoded by polymorphic genes and bind to HLA-A, -B and -C. They have two or three extracellular domains (2D, 3D) and can contain a long inhibitory (L) or a short activating (S) cytoplasmic tail. *KIR* genes are in strong linkage disequilibrium (LD), and two KIR haplotypes (A and B) have been described, with high frequencies of the inhibitory KIRs (iKIRs) in both and a more variable amount of the activating KIRs (aKIRs). Allelism also increases KIRs diversity and alter specificity and levels of expression. Interestingly, because they are inherited independently, an individual can express a KIR allele but not its HLA-I ligand [reviewed in (Freud et al. 2017; Bastidas-Legarda and Khakoo 2019)].

The non-polymorphic interaction consists mainly of the heterodimer CD94/NKG2A, which binds to the conserved HLA-E and provides an inhibitory signal to the NK cells. Another non-polymorphic inhibitory interaction occurs with the Leukocyte immunoglobulin-like receptor–1 (LILRB1/CD85j) and HLA-I [reviewed in (Freud et al. 2017; Bastidas-Legarda and Khakoo 2019)].

Upon the loss of the inhibitory signals, effector functions are triggered by aNKRs and their binding with a vast number of ligands usually upregulated in malignancy transformation, inflammation and pathogen presence. NKp46, NKp44 and NKp30, jointly named as Natural Cytotoxicity Receptors (NCRs), are constitutively expressed in mature NK cells (except for NKp40, only expressed in activated cells) and recognize different ligands, including Env-gp41. NKG2D is another extensively expressed receptor that binds the transcripts from MICA, MICB (HLA-I polypeptide–related sequence A and B) and ULBP (UL16 binding protein), which are commonly upregulated in "stress" conditions. Other surface molecules work as coreceptors, including NKp80, 2B4, NTBA, DNAM-1 and CD59. NK cell activation is also possibly induced by the expression of TLRs. Finally, fully differentiated $CD56^{dim}$ NK cells can express CD16, an Fcγ receptor that triggers ADCC [reviewed in (Lanier 2015; Barrow, Martin, and Colonna 2019)].

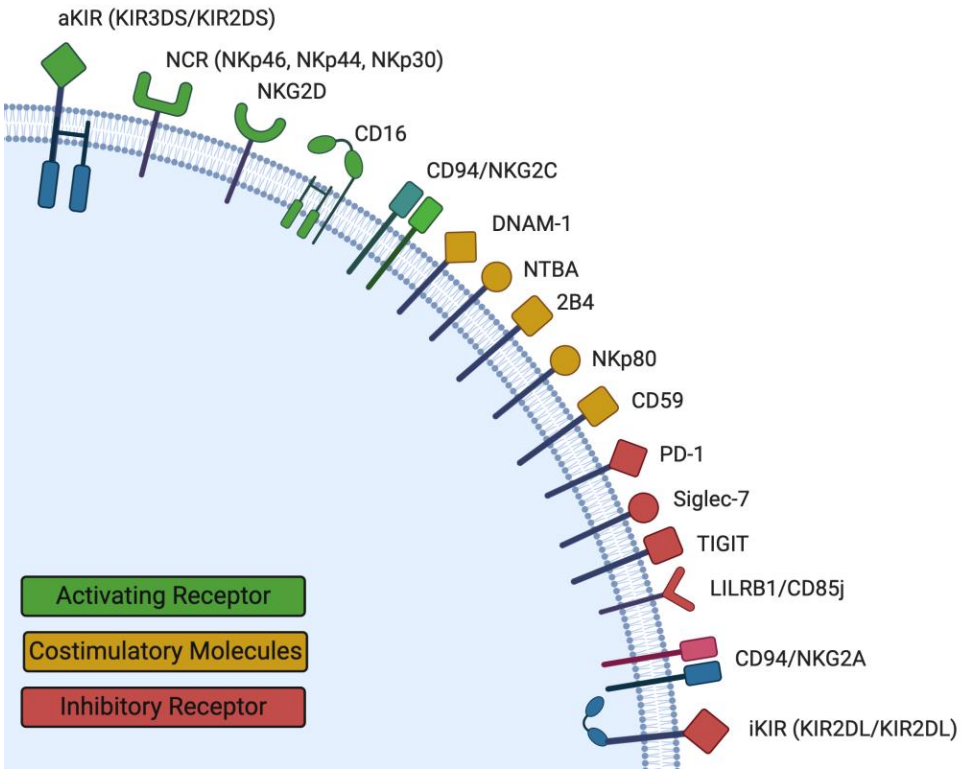


Figure 1.11 Inhibitory and activating NK cell receptors.
Figure made with Biorender.

1.4.3. NK cell education

The ability to sense and target abnormal cells is governed by the interaction of the inhibitory NK receptors (iNKRs) with HLA-I molecules in the process called "education". The diversity of the iNKRs and the HLA-I molecules and the stochastic nature of iNKRs expression build up a range of different education programs between and within individuals that are also parallel and independent of the NK cell differentiation (Björkström et al. 2010). Educated NK cells are suppressed in normal conditions due to the constant inhibitory signal coming from the iNKRs interaction with their ligands. Upon the abrogation of the negative net signal, they become highly responsive. In comparison, uneducated NK cells express less iNKRs but are less responsive, requiring a much stronger positive net signal beyond the aNKR interaction to become reactive, such as cytokines stimulation and CD16 engagement (Figure 1.12) [(Boudreau and Hsu 2018)].

Several models have been offered to explain the role of education on NK cell function. The licensing model proposes that the iNKRs engagement with their ligands empowers NK cells with a signalling program that makes them more responsive when the negative signal is abrogated. The disarming model proposes that all NK cells are initially reactive, but with the absence of inhibition, they become anergic. The encounter of iNKRs with their ligands would shield the NK cells from the constant activating stimuli and save them from anergy. The rheostat model proposes that the strongest the inhibition capacity, the stronger the effector function. In that model, the more possible interactions between iNKRs and their cognate ligand, the higher is the NK cell reactivity. Finally, the tuning model proposes that NK cell education is dynamic, and it can be tuned according to the environmental conditions. In general,

one model does not exclude the other, and they possibly coexist [reviewed in (Boudreau and Hsu 2018)].

NK cell education happens based on the capacity of KIRs and CD94/NKG2A to recognise their ligands. The KIR binding site is located within the HLA's polymorphic regions next to the peptide-binding groove over the α1 and α2 helices and the C-terminal region of the peptide. While HLA-A and HLA-B evolved in the context of T-cell immunity, HLA-C evolution is closely linked to NK cell immunity. All HLA-C molecules bind to a KIR and the dimorphism in the residues 77 and 80 of the α1 helix differentiates the HLA-C molecules into C1 and C2 groups, promoting their binding with KIR2DL2/L3 and KIR2DL1, respectively. C2 alleles provide stronger KIR binding than C1 alleles [reviewed in (Saunders et al. 2015)].

The determinant of HLA-A and -B binding to KIRs is found on the Bw4 motif, from residues 77 to 83 present in the α1 helix region. The Bw4 motif, present in around 20% of the HLA-A and one-third of the HLA-B allotypes, allows their interaction with KIR3DL1. Its counterpart, the Bw6 motif, has a Glycine at position 83, eliminating a salt-bridge that provides the KIR3DL1 recognition and does not bind to a KIR. Furthermore, the presence of Isoleucine at position 80 (Ile80) of the Bw4 motif enhances the binding and inhibitory signal of KIR3DL1. Polymorphisms on the KIR3DL1 side also influence its expression and binding strength. For instance, KIR3DL1*015 and *001 have a stronger binding interaction with Bw4-Ile80 allotypes, while KIR3DL1*005 does not show that preference [reviewed in (Saunders et al. 2015)].

An older arm of NK cell education is based on the interaction between the heterodimer CD94/NKG2 and the non-polymorphic HLA-E. CD94 provides the

primary binding site to HLA-E, while the NKG2 subunit provides the cell signalling. The affinity to HLA-E is more than five times stronger with the inhibitory NKG2A than with activating NKG2C. HLA-E surface expression is more stable when bound to peptides with Methionine at position 2, mainly provided by the leader sequence of classical HLA-I molecules. All HLA-A and HLA-C allotypes have a Methionine at position -21 (-21M) and provide an excellent anchor to HLA-E, while most of the HLA-B molecules has a Threonine at that position (-21T), with poor binding to the HLA-E. Thus, an individual homozygous for HLA-B -21M alleles has higher expression of HLA-E in their cells surface, which inflicts functional changes on the NK cell population. [(Horowitz et al. 2016), reviewed in (Saunders et al. 2015)].

The number of HLA-E molecules available on the cell surface will depend on the amount of the leader sequence peptide being expressed and, therefore on the *HLA-A, -B* and *-C* gene expression. The transcriptional level of different *HLA-B* alleles within and between individuals is relatively stable, and its impact on HLA-E surface expression will be mainly dependent on whether an individual expresses -21T or -21M alleles. On the other hand, HLA-A and HLA-C molecules will always provide a good anchor to HLA-E, but their transcriptional levels vary considerably according to the allotype. *HLA-A* is around 2-fold more polymorphic and its expression is up to 18-fold higher than *HLA-C* (Apps et al. 2015). Hence, the surface level of HLA-E is mainly modulated by the HLA-B allele -21 residue (-21M or -21T) and by the expression level of the *HLA-A* allele [(Ramsuran et al. 2018), reviewed in (Saunders et al. 2015)].

The complexity of these interactions is uncovered by the segregation of HLA-I haplotypes in two distinct groups with a direct consequence on the NK cell education program (Horowitz et al. 2016). Eleven of 12 HLA-B Bw4 alleles are also -21T and

therefore do not provide the peptide for HLA-E. Also, more than 92% of the HLA-C2 haplotypes are in LD with -21T HLA-B alleles. The haplotypes encoding -21M HLA-B are Bw6 and C1, providing good expression of HLA-E but not strong and variable KIR binding, favouring NK cell inhibition via NKG2A. Due to linkage disequilibrium, -21M HLA-B haplotypes have not only a low diversity of C1 alleles, but they are enriched with those with a low level of expression. This group is highly dominated by HLA-C*07, which is affected by microRNA-148a downregulation (Kulkarni et al. 2011), leading to poor KIR binding. On the other hand, haplotypes encoding -21T HLA-B can offer both HLA-C1 and -C2 alleles, and also the Bw4 motif, increasing the amount of KIR ligands and decreasing the supply of peptides to HLA-E. This clear segregation creates two distinct education programs that affect the diversity of NK cells and their ability to sense abnormalities, as well their effector function and response to diseases (Horowitz et al. 2016).

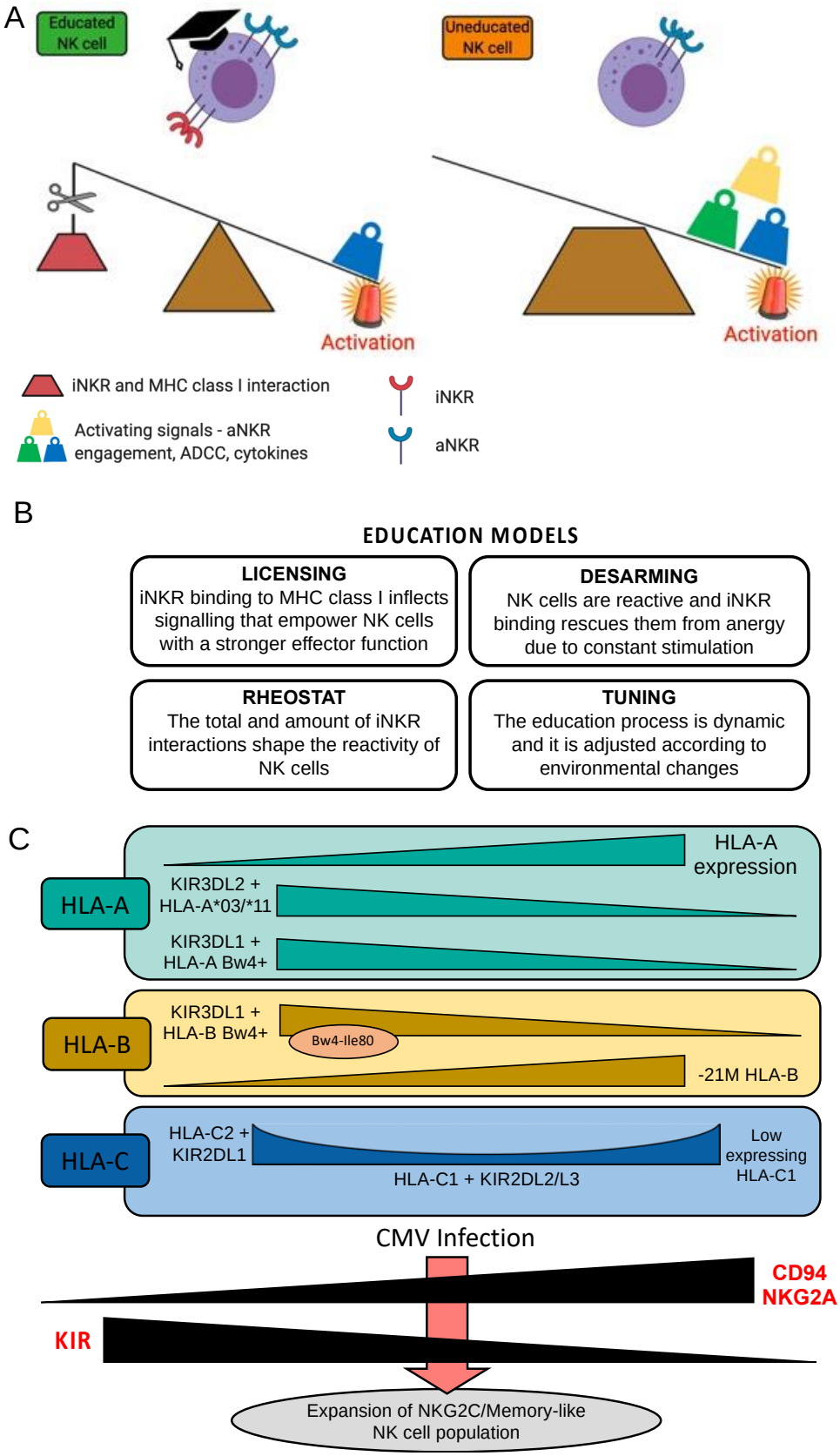


Figure 1.12 NK cell education.
Functional outcome (A), models (B) and immunogenetic factors (C) of NK cell education. Figure made with Biorender.

1.4.4. NK cells in HIV pathogenesis

In acute HIV infection, while the adaptive arm of the immune response requires antigen sensitization to mount its response, NK cells are first-line defenders. They expand during acute infection (Alter, Teigen, et al. 2007) and potentially influence viral setpoint (Lifson et al. 1997). The accumulated epidemiologic, genetic, phenotypic, and functional data demonstrate the critical role NK cells have in HIV pathogenesis.

The capacity of HIV to mutate and evade the adaptive response is widely known [reviewed in (Goulder and Watkins 2008)]. The KIR-HLA interaction also include the peptide region, and viral mutations can also modulate and evade NK cell response, as initially shown by the enrichment of specific polymorphisms in Vpu/Env, Gag and Nef regions that increases the affinity to KIR2DL2 and, consequently, NK cell inhibition (Alter et al. 2011). Further studies reinforced that NK cells can impose immune pressure and promote HIV evasion (Hölzemer et al. 2015; Ziegler et al. 2021).

Several populational studies associated the presence of certain KIRs and their cognate ligand with HIV disease course. First, HLA-Bw4 homozygosity was associated with slower progression to AIDS (Flores-Villanueva et al. 2001) and later the combination KIR3DS1 and HLA-Bw4^{I80} was associated with disease protection (Martin et al. 2002; Qi et al. 2006). This impact on HIV disease course was further confirmed in other populations (Jiang et al. 2013) and combined with functional data showing an enhanced cytotoxic and HIV-killing profile of KIR3DS1+ compared to KIR3DS1- NK cells (Alter, Martin, et al. 2007; Song et al. 2014). Unfortunately, the KIR3DS1 binding with Bw4 alleles is not completely uncovered and possibly peptide dependent (O'Connor et al. 2007; O'Connor et al. 2015), and the effect initially observed was

later attributed to the presence of KIR3DL1 (Pelak et al. 2011). KIR3DL1, in particular those high expressing allotypes (KIR3DL1*h), combined with HLA-Bw4^{I80} was associated with slow HIV disease progression and viral control (López-Vázquez et al. 2005; Martin et al. 2007; Tomescu et al. 2012). The combination KIR3DL1*h-HLA-B57 is significantly enriched in adult ECs (Martin et al. 2018). The presence of high expressing inhibitory KIR3DL1 with their cognate ligand allows the NK cells to be licensed with a potent cytotoxicity profile upon downregulation of HLA-Bw4 molecules (Boudreau et al. 2016). This licensing effect is also observed in KIR2DL, when bound to their HLA-C cognate, with data showing expansion and higher polyfunctionality of KIR2DL2+ population in HIV infection (Körner et al. 2014).

The HLA-E and CD94/NKG2A arm of NK education is also known to influence HIV disease course. NKG2A+ NK cells from M/T and T/T individuals living with HIV showed increased cytotoxic and killing capacity compared to those from M/M donors, highly inhibited by the HLA-E interaction (Merino et al. 2013). In a Zambian study involving serodiscordant couples, the presence of -21T HLA-B was linked to protection against transmission (Merino et al. 2012). However, this relation is complex and another study showed a protective effect in women homozygous for the highly expressed HLA-E^{G} allele (Lajoie et al. 2006). Surface expression of HLA-E is also influenced by the *HLA-A* allele and its expression levels. High expressing *HLA-A* alleles favour NK cell inhibition via NKG2A and positively correlate with plasma viral load and negatively with CD4+ T-cell count. This effect is additive when looked in combination with -21M/M HLA-B individuals (Ramsuran et al. 2018).

Overall, the protection against HIV disease progression and viraemic control has been consistently associated with a KIR-educated program that decreases the amount of NK cell inhibition via NKG2A in individuals having Bw4, -21T HLA-B and low

expressing *HLA-A* alleles. The lower expression of HLA-E on the cell surface translates into a larger NKG2A+ NK cell population (Horowitz et al. 2016). NKG2A+ NK cells, particularly from -21T HLA-Bw4 donors, have been linked with higher polyfunctionality and degranulation via missing-self assays and higher killing capacity against autologous HIV infected-CD4+ T-cell targets (Merino et al. 2013; Lisovsky et al. 2015). Interestingly, this effect was only observed in HLA-Bw4 donors (Lisovsky et al. 2015). HIV disease progression has also been associated with the expansion of the terminally differentiated and hypofunctional $CD56^{neg}CD57^{pos}KIR^{neg}$ subset, which is only partially reverted in suppressed ART-treated individuals (Mavilio et al. 2003; Brunetta, Hudspeth, and Mavilio 2010).

NK cells in paediatric HIV-infection are less well-studied. Children living with HIV have lower frequency of circulating NK cells than uninfected children and show a more terminally differentiated phenotype, (Azzoni et al. 2005; Slyker et al. 2012; Mahapatra et al. 2019) with impaired missing-self effector function and ability to employ ADCC (Ziegner et al. 1999; Ballan et al. 2007). Besides the lack of studies investigating their role in different HIV disease courses, in a large cross-sectional study, KIR3DL1-HLA-Bw4 was associated with lower plasma viral load and higher CD4+ T-cell counts in all ages (Singh et al. 2016).

In children, NK cells achieve their full effector potential by the age of five months (Yabuhara, Kawai, and Komiyama 1990), therefore several years before the CTL response has become fully effective. The effector potential of NK cells in early life combined with specific genotypes previously linked with HIV disease protection allow us to hypothesize that certain inherited imprints may contribute to the AIDS-resistant phenotype and viraemic control observed in a group of children living with HIV that have yet to be explored.

1.5. Overview and aims of this thesis

Post-treatment control of HIV may be easier to achieve in children compared with adults (Figure 1.13) (Goulder, Lewin, and Leitman 2016) due to: (i) the possibility to initiate ART immediately after birth in all children born to mothers living with HIV, shortening the window between transmission and suppression as most intrauterine infections arise late in pregnancy (Rouzioux et al. 1995); (ii) the tolerogenic immune response in early life, with high levels of immune regulation and low activation (Michaëlsson et al. 2006; Levy 2007; Kollmann et al. 2009); (iii) the virus transmitted from mother-to-child is overall of lower replicative capacity than the viruses circulating in the mother (Adland et al. 2015; Claiborne et al. 2015). All these add barriers to viral seeding and, after years of viral ART-suppression, the size and diversity of the viral reservoir achieved can be very low. Several anecdotal cases of children living with HIV who received early ART and subsequently became post treatment controllers (PTCs) have been reported (Persaud et al. 2013; Frange et al. 2016; Violari et al. 2019). However, starting ART even immediately after birth will evidently not be sufficient to scale up the numbers of paediatric PTCs to a populational level (Millar et al. 2020; Kuhn et al. 2020) and additional therapeutical strategies need to be explored.

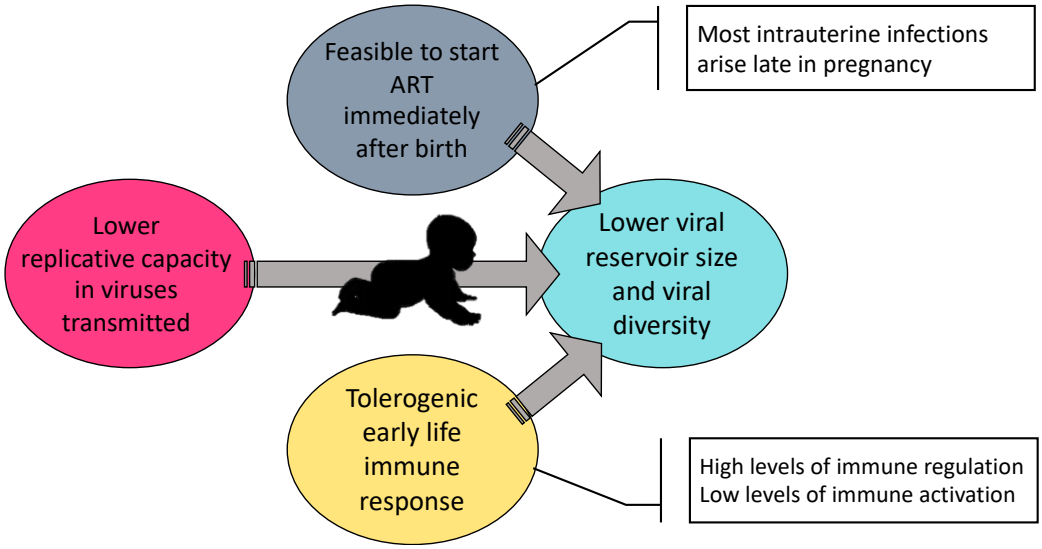


Figure 1.13 Potential to achieve post-treatment control is hypothesized to be higher in children.

This thesis investigates mechanisms that can help us understand how post treatment control can be achieved in the paediatric population by studying models of slow-clinical progression and viraemic control in children living with HIV. The first results chapter (Chapter 3) examines the contribution of inherited factors, known to influence NK cells education, on HIV disease progression and investigates the immunophenotype and function of the NK cell compartment in different paediatric groups of children living with HIV. Chapter 4 identifies early T-cell markers associated with immunological non-progression in a group of early treated infants who underwent ATI after one year on ART, building up an HIV-specific CD8+ T-cell compartment populated with non-terminally exhausted cells with high potential of expansion. Chapter 5 uncovers the rare group of paediatric ECs (PECs), including the role of the HIV-specific T-cell response in viral control, and how these populations contrast with the adult ECs. Chapter 6 investigates two different mechanisms of viral rebound in the rare group of paediatric transient aviraemic (TA) individuals.

Detailed reasoning and hypotheses are specified in each chapter.

2

Methods

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2.1. Study subjects

The following definitions are used throughout this thesis to define different groups of children living with HIV:

- *Paediatric Slow Progressors (PSPs):* ART-naïve children who reached the age of 5 years with an absolute CD4+ T-cell count $\geq$350 cells/mm^3 and a CD4 percentage of $\geq$20%, irrespective of plasma viral load.
- *Paediatric Progressors (PPs):* ART-naïve children with an absolute CD4+ T-cell count <350 cells/mm^3 and/or CD4 percentage <20%. In Chapter 3, PPs are defined as those who reached this definition before the age of 5 years and PSP-PPs as those with CD4+ T-cell progressive decline and reaching the above definition between 5 and 10 years of age.
- *HIV-exposed uninfected (HEU):* HIV-uninfected children born to a mother who was living with HIV during pregnancy. Due to the lack of information on maternal viral suppression and ART during pregnancy, we did not have information in terms of the level of HIV exposure. I acknowledge that HEUs are immunologically different from HIV-unexposed uninfected children, but they are still distinct from children living with HIV and are helpful as a reference group. They are also likely to match the HIV-infected groups better socio-economically.

Groups unique to a particular study are detailed in their respective chapter.

All participants or their caregivers provided informed consent at enrolment.

Participants from the following cohorts were studied:

(i) PSP/pART Durban cohort (Muenchhoff et al. 2016; Muenchhoff et al. 2019): children and adolescents were recruited at the Ithembalabantu Clinic and at Prince Mshiyeni Memorial Hospital, Durban, SA. The PSP cohort consisted of ART-naïve children living with HIV aged between 1 to 5 years who have a relative CD4+ T-cell count of >25% and an absolute count of 500 cells/mm^3 or greater, or aged 5-18 years with an absolute count >350 cells/mm^3. PSPs who started ART due to progression or because of new universal treatment guideline were transferred to pART. pART also included ART-naïve children living with HIV about to start ART at study entry independent of their immunological status. Both cohorts recruited HIV negative siblings, and some were included as HEUs (however, no HIV-negative and HIV-positive siblings were included in the same study). Participants were seen twice a year for clinical follow-up and blood sample collection. Siblings were seen once a year and always tested for HIV. All HEUs and part of PSPs and PPs studied in Chapter 3 to 6 were recruited through this cohort. PEC-4 studied in Chapter 5 and TA-1 studied in Chapter 6 are part of this cohort. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (UKZN), SA), and the University of Oxford, United Kingdom (UK).

(ii) Kimberley cohort (Adland et al. 2015; Muenchhoff et al. 2016; Adland et al. 2018): PSPs and PPs followed at Kimberley Hospital in Kimberley, SA, are part of a cohort established in collaboration with Dr Pieter Jooste. They comprise most of the HLA-I and KIR-typed children in Chapter 3. PEC-6 in Chapter 4 and some TAs described in Chapter 6 are also part of the Kimberley cohort. Ethical approval was given by the University of the Free State Ethics Committee, Bloemfontein, SA, and the University of Oxford, UK.

(iii) Treatment interruption cohort (Mphatswe et al. 2007; Prendergast et al. 2008): the participants were part of an ART feasibility study named "Paediatric Early HAART and Strategic Treatment Interruption Study (PEHSS)" that enrolled 75 newborns with confirmed HIV-infection at St. Mary's and Prince Mshiyeni Hospitals in KwaZulu-Natal, SA, between 2003 and 2005. Women living with HIV were recruited in the third trimester of pregnancy, and NVP was given at delivery and to the infant immediately after birth. This was the only available MTCT prevention treatment in SA at the time. When HIV transmission was confirmed, infants were randomised into one of the three arms. The infants studied in Chapter 4 were allocated to immediate uninterrupted ART for 12 months, followed by ATI. After ATI, ART was restarted according to the guideline at the time for treatment initiation: paediatric stage III or advanced stage II disease or relative CD4+ T-cell count <20% or <15% if below or above 18 months of age, respectively (National Department of Health South Africa 2004). The study was approved by the Biomedical Research Ethics Committee of the UKZN, SA, the Institutional Review Boards of the Massachusetts General Hospital, USA, and the University of Oxford, UK.

(iv) London cohort (Muenchhoff et al. 2016)**:** children fulfilling the criteria of PSPs were recruited from the HIV clinics in London, UK, at the St Mary's Hospital in collaboration with Dr Gareth Tudor-Williams, and at the Great Ormond Street Hospital for Children NHS Trust in collaboration with Dr Alasdair Bamford. The PSPs from this cohort included in this thesis are originally from SSA countries and emigrated to the UK at a later stage. Some of these children were HLA-I and KIR typed and are studied in Chapter 3. PEC-1 and TA-2 also belong to this cohort. The study was approved by the London Stanmore Research Ethics Committee, UK, and the University of Oxford, UK.

(v) Paediatric Elite Controllers (Vieira et al. 2019): PECs and TAs were identified in several out-patient clinics around the globe, and they are described in detail in Chapter 5 and 6. Blood samples were collected and studied here for PEC-1 to PEC-4. PEC-1 and PEC-4 are part of the Durban and London cohorts, respectively. PEC-2 and PEC-3 were originally from SSA countries but recruited at Sant Joan de Déu Children's Hospital in Barcelona, Spain, in collaboration with Dr Claudia Guash and Prof Christian Brander.

(vi) Durban Adult cohort (Kiepiela et al. 2004; Leslie et al. 2004; Kiepiela et al. 2007): adults living with HIV being ART-naïve at the time of enrolment were recruited from out-patient follow-up clinics in Durban, SA. All the HLA and KIR-typed adults studied in Chapter 3 belong to this cohort. The study was approved by the Biomedical Research Ethics Committee of the UKZN, SA, the Institutional Review Boards of the Massachusetts General Hospital, USA, and the University of Oxford, UK.

(vii) Thames Valley Cohort (Payne et al. 2010): ART-naïve adults living with HIV were recruited from the Royal Berkshire Hospital (Reading, UK), Northampton General Hospital (Northampton, UK), Churchill Hospital (Oxford, UK) and Wycombe Hospital (High Wycombe, UK). Some viraemic adults of SSA origin and infected with clade C virus were selected from this cohort and studied in Chapter 4. The study was approved by the corresponding Institutional Review Boards and the University of Oxford, UK.

2.2. Blood processing and cryopreservation

Samples were processed at their respective cohort site location except for the cohorts located in the UK, where blood processing was done in our laboratory at Peter Medawar Building for Pathogen Research. Plasma samples were separated after the whole blood was spun, and peripheral blood mononuclear cells (PBMCs) obtained using the density gradient separation method. Briefly, whole blood was added to a 50 mL Falcon tube containing Lymphoprep (Stemcell technologies) and spun for 25 min at 2000 rpm with brakes offs. PBMCs layer was collected with a Pasteur pipette and transferred to a new 50 mL Falcon Tube and washed three times with RPMI-1640 (Sigma) and resuspended in R10 medium [RPMI-1640, 10% heat-inactivated fetal calf serum (FCS, Sigma), 1% L-glutamine (Sigma) and 1% penicillin-streptomycin (Sigma)]. After counting, PBMCs were pelleted and resuspended slowly into cold freezing media [FCS containing 10% Dimethyl sulfoxide (DMSO, Sigma)] and aliquoted in cryovials.

Plasma samples were stored at -80^{o}C. PBMCs were initially stored at -80^{o}C placed into a freezing container (Nalgene) for slow cooling at a 1^{o}C/min rate and transferred the following day to a liquid nitrogen tank.

PBMCs were thawed by transferring the cryovials to a water bath at 37^{o}C and cautiously diluting into 20 mL of pre-warmed R10. Cells were two times spun and resuspended in R10 to wash off the DMSO, and then counted and resuspended in R10.

2.3. Flow Cytometry

Full descriptions of antibody panels are shown in the Appendices.

2.3.1. Surface staining

Thawed PBMCs were rested for 5 hours or overnight according to the assay in R10 at 37ºC in 5% CO_2. Then, cells were spun and resuspended to have 0.5 to 1 million PBMCs in 200 µL of R10 and transferred to a 96-well V-bottom plate. R10 was washed off by spinning the plates at 2000 rpm for 3 min and resuspended in 200 µL of Phosphate-buffered saline (PBS, Gibco) two times. PBMCs were resuspended in 50 µL of PBS containing diluted Live/Dead near-IR stain (Invitrogen) and incubated for 30 min at room temperature (RT) and in the dark according to the manufacturer instructions. Cells were washed twice with FACS Buffer (PBS, 0,5% FCS and 2mM EDTA) and resuspended in 50 µL of a solution containing all surface antibodies diluted in FACS Buffer. PBMCs were incubated for another 30 min at 4ºC and protected from light. Finally, cells were washed twice and fixed in 2% paraformaldehyde (PFA) and kept at 4ºC in the dark until acquisition.

2.3.2. Intracellular and intranuclear staining

When intracellular markers were present in the panel, after 30 min of incubation with antibodies targeting surface markers, cells were fixed and permeabilized with Foxp3 Fixation/Permeabilization solution (eBioscience) for 45 min at 4°C. Then, cells were washed twice with 200 µL of Permeabilization Buffer (eBioscience) and stained with antibodies targeting intracellular and/or intranuclear molecules for 30-45 min at 4°C

protected from light. Cells were washed two times and resuspended in PBS before acquisition.

2.3.3. NK cells functional assays

This protocol was standardized in collaboration with Dr David Malone (University of Oxford, UK) and performed in the study described in Chapter 3.

Three assays were done to investigate different NK cell functions: (i) cytokine stimulation with IL-12/IL-18; (ii) missing-self activation using the K562 cell line, which lacks expression of HLA-I molecules; (iii) ADCC function via anti-CD20 coated RAJI cells (Figure 2.1). Half to one million thawed PBMCs per condition were rested in R10 for 5 hours for cytokine stimulation and overnight for K562 and RAJI cells assays at 37°C in 5% CO_2. To assess cytokine stimulation, PBMCs were either left unstimulated or incubated with 10 ng/mL of rhIL-12 (R&D Systems) and 100 ng/mL of rhIL-18 (R&D Systems) for 18 hours at 37°C. Brefeldin A (Biolegend) and Monensin (Biolegend) were added in the last 5 hours. For K562 cells condition, PBMCs were incubated for 6 hours at 37°C with and without the target cell (5:1 effector to target cell ratio). For ADCC assay, RAJI cells (1 million cells/mL) were coated with 2.5 µg/mL of either murine IgG or anti-CD20 (R&D Systems) for 30 min, washed twice in R10 and incubated with PBMCs at a 10:1 effector to target ratio for 6 hours at 37°C. CD107a (APC, H4A3, BD Bioscience) was add from incubation outset. For both K562 and RAJI assays, Brefeldin A and Monensin were added in the last 5 hours. At the end of incubation, cells were washed in PBS and surface and intracellular stained as previously described.

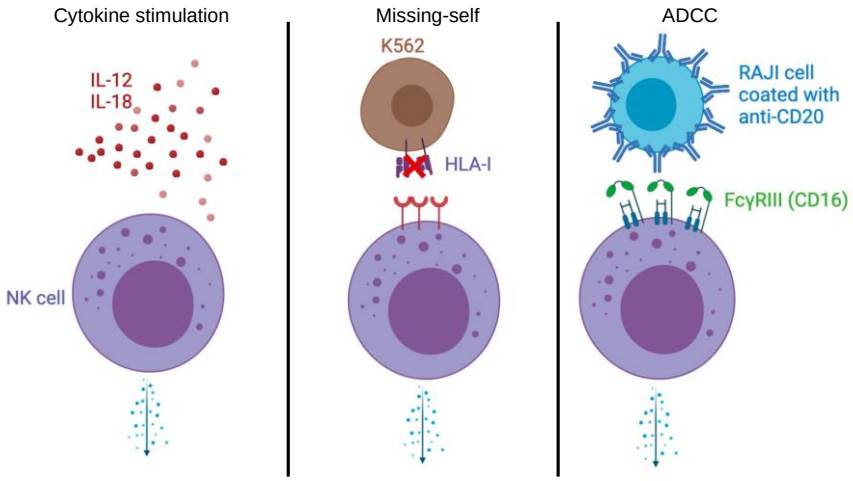


Figure 2.1 NK cell functional assays.
The three NK cell effector functions assessed in Chapter 3 are illustrated. Figure made with Biorender.

2.3.4. Intracellular cytokine staining (ICS) to detect HIV-specific T-cell responses

This protocol was standardized in collaboration with Dr Priscilla Ramos (University of Sao Paulo, Brazil) and performed in the studies described in Chapter 5 and 6.

Thawed PBMCs were rested in R10 overnight at 37°C in 5% CO_2. Cells were stimulated with Gag, Env and/or Nef pools of overlapping HIV-1 clade C consensus peptides (NIH AIDS Reagent Program) at a final concentration of 2 µg/mL for each peptide in the presence of anti-CD28/anti-CD49d at 1 µg/mL (BD) and anti-CD107a (PE-Cy7, H4A3, Biolegend). PMA/Ionomycin cocktail (Biolegend) and 0.2% DMSO were used as a positive and a negative control, respectively. Cells were incubated for 6 hours and Brefeldin A and Monensin added after 1-hour of stimulation outset. At the end of incubation, cells were washed in PBS and surface and intracellular staining performed as described.

2.3.5. Tetramer staining of virus-specific CD8+ T-cells

To identify virus-specific CD8+ T-cells in Chapter 4, four sets of peptide-HLA tetramers conjugated with BV421 were obtained from ImmunAware ApS (Denmark): HLA-B*42:01/HIV-Gag-TL9 (TPQDLNTML), HLA-B*42:01/CMV-pp65-RV10 (RPHERNGFTV), HLA-B*81:01/HIV-Gag-TL9 (TPQDLNTML), and HLA-B*81:01/CMV-pp65-GL8 (GPISGHVL). Two million cryopreserved PBMCs were thawed, and surface stained according to the manufacture instructions before viability staining. The subsequent staining steps followed as described.

2.3.6. Proliferation assay combined with cytokine stimulation and PD-1 blockade

This protocol was standardized in collaboration with Dr Ane Ogbe (University of Oxford, UK), Dr Julieta Carabelli and Miguel Marín López (Irsi Caixa, Spain).

The proliferation assay using CellTrace Violet (CTV, Life Technologies) labelled PBMCs associated with ICS and PD-1 blockade was performed in Chapter 4 and briefly summarized in Figure 2.2. Thawed PBMCs were rested overnight in R10 at 37°C in 5% CO_2. Cells were washed twice with PBS and stained with CTV for 10 min at RT. Cold FCS was used to quench the reaction for 5 min at 37°C, and labelled PBMCs were washed twice and resuspended in RAB10 [R10, 10% human AB serum, (Sigma)]. 250,000 cells were plated in duplicate in a 96-well round-bottom plate in the presence of anti-CD28/anti-CD49d at 1 µg/mL (BD), HIV-Gag clade C or CMV-pp65 pools of overlapping consensus peptides (NIH AIDS Reagent Program) at a final concentration of 2 µg/mL for each peptide. Negative and positive control included DMSO in the same concentration of the peptide pools and Staphylococcal Enterotoxin

B (SEB, Sigma), respectively. PD-1 blockade was performed by adding anti-PD-1 (NA/LE Mouse anti-Human CD279, BD) or its Isotype at 10 µg/mL. Cells were incubated for 7 days at 37°C in 5% CO_2 with a 20% top-up of RAB10 on Day 4. Twelve hours before the end of incubation on Day 6, the stimulation was boosted with the same concentration of the respective stimulus, and Brefeldin A and Monensin were added to allow ICS in the proliferated CD8+ T-cells. Surface and intracellular staining followed as described.

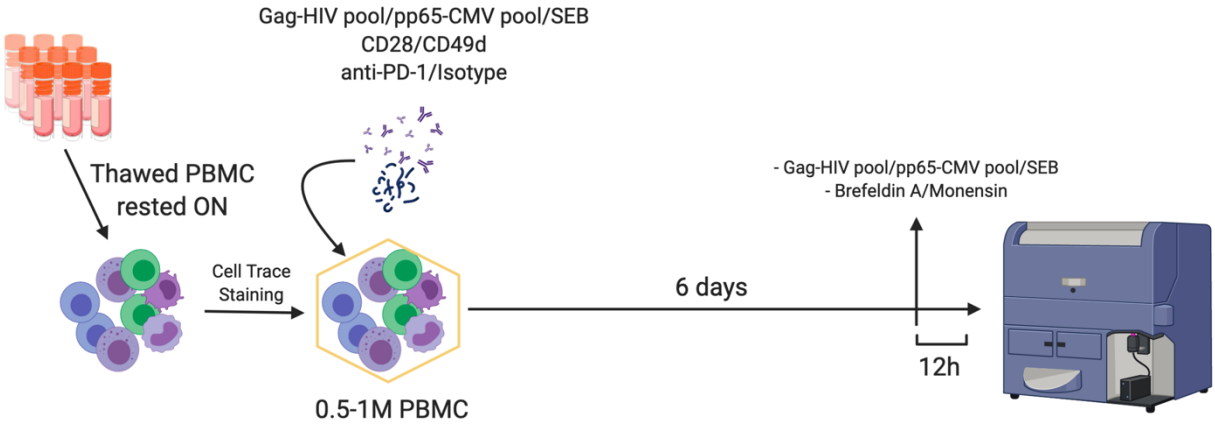


Figure 2.2 Summary of the proliferation and cytokine stimulation assay.
Figure made with Biorender.

2.3.7. Sample acquisition and data analysis

Samples were acquired on a BD LSR II and the data analysed in FlowJo v10.6.2 (Tree Star LLC). The gating strategies are detailed in their respective chapter. Positive gates were selected using Fluorescence Minus One (FMO). Memory subsets and co-expression of different markers and cytokines were obtained using Boolean Gates. For functional assays, positive responses were gated according to the negative control, and the background was subtracted later. For ICS, positive responses were considered if at least three times above the negative control.

2.4. ELISPOT

A screening assay was initially performed using a panel of 410 overlapping 18mer peptides covering the entire HIV-1 proteome based on C clade consensus sequence mixed in 72 pools with 9-12 peptides with each peptide represented in 2 different pools, as previously described (Kiepiela et al. 2004; Kiepiela et al. 2007; Thobakgale et al. 2007). A confirmatory assay was run to confirm an individual set of peptides when a positive response was identified in the two pools containing the particular peptides. In Chapter 6, the titration curves to evaluate peptide affinity was done by serial dilution of peptides (from 10^{-4} to 10^{-10} M) done in triplicates, as previously described (Almeida et al. 2007).

Briefly, 96-well plates with a Polyvinylidene Fluoride membrane (Milipore) were coated with anti-human monoclonal antibody (Mabtech) in PBS and incubated at 4°C overnight. Before starting the assay, the plate was washed with blocking buffer (PBS and 1% FCS). 50,000 or 100,000 PBMCs and the peptides at a final concentration of 2 μg/mL were added to the wells. Negative and positive controls included DMSO in the same concentration of the peptide pools and phytohemagglutinin (PHA, Sigma). After 14-16-hour incubation at 37°C in 5% CO_2, IFN-γ-producing cells were revealed after subsequent washes with PBS and incubations with biotinylated anti-IFN-γ antibody (Mabtech), streptavidin-alkaline phosphatase conjugate antibody (Mabtech), and alkaline phosphatase substrate reagents (Bio-Rad). The last reaction was stopped washing the plates with tap water and left to air-dry overnight. The number of spots per well was quantified using an automated ELISPOT plate reader (AID ELISPOT reader system; Autoimmun Diagnostika GmbH, Germany) and manually checked. A response was defined as

positive if ≥50 spots-forming cells (SFC)/million PBMCs after background subtraction (mean of the negative controls + 3 times the standard deviation).

2.5. HLA typing

HLA-A, HLA-B and HLA-C were determined by Polymerase chain reaction (PCR) amplifying Exons 2 and 3 for each locus using sequence-specific primers (Cano et al. 2007). The HLA-I typing was performed along the previous years in collaboration with different groups: Prof Mary Carrington (Frederick National Laboratory for Cancer Research, USA), Prof William Hildebrand (University of Oklahoma Health Sciences Center, USA) and Timothy Rostron (University of Oxford, UK).

2.6 KIR typing

KIRs were typed in collaboration with Mary Carrington's laboratory and performed by Maureen P. Martin (Frederick National Laboratory for Cancer Research, USA). The genotyping for the presence or absence of each KIR gene was conducted by PCR with sequence-specific priming as described previously (Martin and Carrington 2008), with some modifications. Each PCR was conducted in a volume of 5 μL using 5 ng genomic DNA and SYBR Green PCR Master Mix with Platinum Taq (Invitrogen). The presence and absence of specific PCR products were detected by melting curve analysis on the 7900 Real-Time PCR System (Applied Biosystems). An algorithm developed in-house assigned KIR genotypes based on melting curve data. Additional 2DS4 subtyping to detect the null variant, which has a 22 bp deletion in exon 5, was performed by size discrimination of the 2DS4 PCR products using the LabChip GX instrument (Caliper).

2.7. Total HIV-DNA quantification

Total HIV-DNA quantification was done in collaboration with Prof Javier Martinez-Picado's laboratory and performed by Maria C. Garcia-Guerrero and Mari C. Puertas (Irsi Caixa, Spain). After thawing, at least 2 million total PBMCs were incubated overnight in lysis buffer and frozen at -80ºC. Samples were sent to Barcelona where total HIV-DNA were measured by using droplet digital PCR (BioRad) with *5'LTR* or *gag* primers and probes, as previously described (Morón-López et al. 2017). Briefly, DNA copy numbers were calculated using the QuantaSoftTM (BioRad) software and normalised to the number of input cells per reaction using quantification of the RPP30 single-copy gene to express the result as copies per million PBMC. The limit of detection for each sample was estimated according to the total input cell number. Total HIV-DNA copies were further adjusted to 1.0 million CD4+ T-cell according to the frequency obtained in the same sample, as analysed by flow cytometry.

2.8. Statistical analysis

Clinical and demographic characteristics were described using absolute numbers and percentages for categorical variables and medians and interquartile ranges (IQR) for numerical variables. All statistical tests are clearly stated in the corresponded figure's legend. Overall, comparisons for categorical variables were made using Chi-square or Fisher's exact test followed by Bonferroni-Holm adjustment for pairwise comparisons as appropriate. Comparisons of continuous variables between two groups were performed using Student's t-test (parametric) or Mann-Whitney U test (non-parametric) according to sample size. Similarly, when there were three or more groups, comparisons were made using ANOVA (parametric) or Kruskal-Wallis (non-parametric) followed by pairwise comparisons with adjusted p-values using Tukey's

or Dunn's tests, respectively. Associations were done using the non-parametric Spearman's test and a simple linear regression model for best-fit line and 95% confidence interval (CI) is shown in the graphs. For all analysis, a two-sided alpha error of 0.05 was assumed. Graphs and statistical analysis were performed with Prism v9.0.1 (GraphPad Software) unless otherwise stated.

Specific statistical analysis and graphs are described below:

(i) Correlation Matrix: the correlation matrixes shown in Chapter 3 to 5 were built with the `corrplot` package in R. Positive and negative correlations are displayed in blue and red shades, respectively. Both colour intensity and size of the squares are proportional to the correlation coefficients. Only correlations with p-values <0.05 are shown.

(ii) Cumulative incidence analysis: the cumulative ART-free analysis done in Chapter 3 are shown in a Kaplan-Meier plot, and curves were compared with log-rank (Mantel-Cox) test for p-value and hazard ratio (HR). We used the time to start ART as a result of meeting the prevailing CD4+ T-cell count criteria for ART initiation (absolute and/or relative CD4+ T-cell count $<$ 350 cells/mm^3 and/or $<$ 20%) as the primary outcome. Data was censored if ART was started in the absence of immunological progression. Subsequently, a Cox proportional-hazard model was built including several co-variates using the `coxph` function in R (The R Core Team) and the forest plot using `ggforest`.

(iii) Least Absolute Shrinkage and Selection Operator (LASSO) model (Tibshirani 1996; Friedman, Hastie, and Tibshirani 2010): in Chapters 3 and 4, when multiple covariates were identified in the correlation matrix, a penalised

multivariate linear regression model was run to select the relevant predicting covariates. The package `glmnet` was used to obtain the optimal penalty via 10-fold cross-validation. The code for this analysis was written and cross-validated in collaboration with Prof Andreas Groll (Department of Statistics, TU Dortmund University, Germany).

(iv) Logistic Regression Model: the models shown in Chapter 3 were done using the `glm` function in R to identify the impact of different covariates on categorical outcomes. The `confit` function was used to obtain the CIs.

(v) Linear Regression Model (Bates et al. 2015): a linear mixed-effect regression model was used for the longitudinal analysis of CD4+ T-cell count and plasma HIV-RNA copies in Chapter 3 using the `lme4` package in R, with the `lmer` function for continuous outcomes. The model accounted for the random effect attributable to the age (in years) at which each donor had data available and a fixed effect estimate by the presence of HLA-Bw4 alleles and *HLA-A* gene expression. Graphs were generated using `ggplot2`, and the smoothed lines are shown using LOESS. The code for this analysis was written and cross-validated in collaboration with Dr Azim Ansari (University of Oxford, UK).

(vi) Heatmap: the heatmap graphs shown in Chapter 3 and 5 were built using the `pheatmap` package in R, after standardizing each parameter's frequency data to z-scores and calculating the median z-score per group.

(vii) Simplified Presentation of Incredibly Complex Evaluations (SPICE v6) (Roederer, Nozzi, and Nason 2011): co-expression of a group of markers or evaluation of T-cell polyfunctionality were done with Boolean Gates on FlowJo.

Bar and pie charts and comparisons were made using the permutation and Student's t-test in SPICE, as recommended.

(viii) Principal Component Analysis (PCA): the PCA analysis shown in Chapter 5 was performed using the function `prcomp` with the parameters detailed in the chapter and the biplot graph built using the `ggbiplot` package in R. The graph shows the two principal components (PCs) that most explained the total variation in the dataset (PC1 and PC2).

3

An HLA-I signature favouring KIR-educated Natural Killer cells mediates immune control of HIV in children

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3.1 Background

In adult HIV infection, the major immunogenetic factors contributing to disease outcome in African populations are the 'protective' HLA-B molecules 57/58:01/81:01 and the 'disease-susceptible' HLA-B molecules 18:01/45:01/58:02 (Pereyra et al. 2008; Leslie et al. 2010; Carlson et al. 2012). HLA-C expression levels also affect HIV disease outcome via CD8+ T-cell activity (Apps et al. 2013). In paediatric HIV infection, these molecules have a much more modest effect (Adland et al. 2015) and are not apparently enriched in PECs (Chapter 5). Although HIV-specific CD8+ T-cell responses are detectable from birth (Luzuriaga et al. 1995; Thobakgale et al. 2007), they generally impose weak selection pressure on the virus and lack sufficient breadth to have a substantial impact on viraemia (Leitman et al. 2017).

HLA-I alleles also influence NK cell effector function and allows educated cells to lyse target cells lacking HLA-I due to the abrogation of the inhibitory signal provide by the ligand interaction [reviewed in (Boudreau and Hsu 2018)]. HLA-A and HLA-B molecules expressing the Bw4 motif in combination with its ligand KIR3DL1 licence NK cells to kill (Boudreau et al. 2016) and are associated with slower progression in adult and paediatric HIV-infection (Flores-Villanueva et al. 2001; Martin et al. 2007; Kamya et al. 2011; Tomescu et al. 2012; Jiang et al. 2013; Singh et al. 2016; Martin et al. 2018). Low *HLA-A* expression alleles has also been associated with slower progression and better viraemic control in adults (Ramsuran et al. 2018) by modulating HLA-E surface expression, the ligand for the inhibitory CD94/NKG2A receptor. HLA-E expression on the cell surface is highly dependent on *HLA-A* gene expression or whether HLA-B expresses Threonine or Methionine at position -21 (position 2 in the signal peptide). The -21M variant binds to HLA-E (Lee et al. 1998) and it is present in all HLA-A and -C alleles and in the minority of HLA-B alleles.

The combination of high expressing *HLA-A* alleles and -21M HLA-B alleles synergizes and mediates NK cell inhibition via NKG2A and impairs HIV control in adults (Ramsuran et al. 2018).

NK cell antiviral functions are similar in infants and adults (Yabuhara, Kawai, and Komiyama 1990; Jenkins, Mills, and Kohl 1993), but the part played by NK cells in immune control of HIV in early life and the potential for NK responses to facilitate HIV remission in paediatric infection have yet to be explored. In the VISCONTI cohort of adult PTCs, the HIV-specific CD8+ T-cell responses were no more potent than those of non-controllers, and the cohort was enriched for disease-susceptible HLA-I, suggesting a mechanism independent of strong CTL activity (Sáez-Cirión et al. 2013). Subsequent studies in this cohort have suggested that an immunogenetic signature reflecting KIR educated NK cells and a corresponding NK cell phenotype and functionality may mediate HIV remission in these subjects (Scott-Algara et al. 2015). Studies in AGM, natural hosts of SIV, support the possible role of NK cells in purging lymphoid tissue sanctuary sites of virus-infected cells that are inaccessible to CD8+ T-cells (Huot et al. 2017; Bunders and Altfeld 2017; Huot et al. 2021). A study of early-ART-treated human infants living with HIV demonstrated correlations between NK cell responses and intact proviral reservoir at only 12 weeks of age (Garcia-Broncano et al. 2019). Together, these findings suggest the possibility that early life NK responses may have potential to help control viral replication, and therefore to contribute also to functional cure in early-ART-treated children undergoing ATI.

We studied a historical cohort of >300 children living with HIV from SSA that we have followed for up to two decades to determine the major immunogenetic factors influencing disease outcome in children compared with an adult cohort in Durban,

SA. We then investigated the functional aspects of the NK cell responses in a representative subset of these children.

3.2. Chapter-specific population and methods

3.2.1. Study population and comparison groups

We included 310 ART-naïve children living with HIV after MTCT of SSA origin from four different cohorts: Kimberley (SA), Durban (SA), London (UK), and Barcelona (Spain). The children were categorised as either PPs (n=73) or PSPs (n=237) as described in Chapter 2. The median time to start ART due to immunological progression (absolute or relative CD4+ T-cell < 350 cells/mm^3 or < 20%) in the PP group was 0.5 years (IQR 0.3 – 0.8), while 49% of PSPs started ART due to progression at a median age of 11.2 years (IQR 8.3 – 13.0).

The PSP group was further subdivided into delayed progressors (PSP-PP, n=57) if they met the criteria to initiate ART due to immunological progression before the age of 10 years; in case they did not progress by the age of 10 years, they were classified as viraemic non-controllers (PSP-VNC, n=112) or viraemic controllers (PSP-VC, n=68) according to whether their median plasma HIV-RNA load during follow-up was above or below 10,000 copies/mL, respectively (Figure 3.1). PSP-PP had a median plasma HIV-RNA load of 71,095 copies/mL (IQR 28,000 – 170,623) and a median absolute and relative CD4+ T-cell count of 446 cells/mm^3 (IQR 383 – 543) and 16% (IQR 14 – 18) at the timepoint of immunological progression. PSP-VNC had a median plasma HIV-RNA load of 45,049 copies/mL (IQR 27,000 – 106,229) and a median absolute and relative CD4+ T-cell count of 619 cells/mm^3 (IQR 506 – 910) and 26.5% (IQR 23.3 – 30.9). Finally, PSP-VC had a median plasma

HIV-RNA load of 2,614 copies/mL (IQR 584 – 5,762) and a median absolute and relative CD4+ T-cell count of 779 cells/mm^3 (IQR 568 – 985) and 30.9% (IQR 26.1 – 33.3).

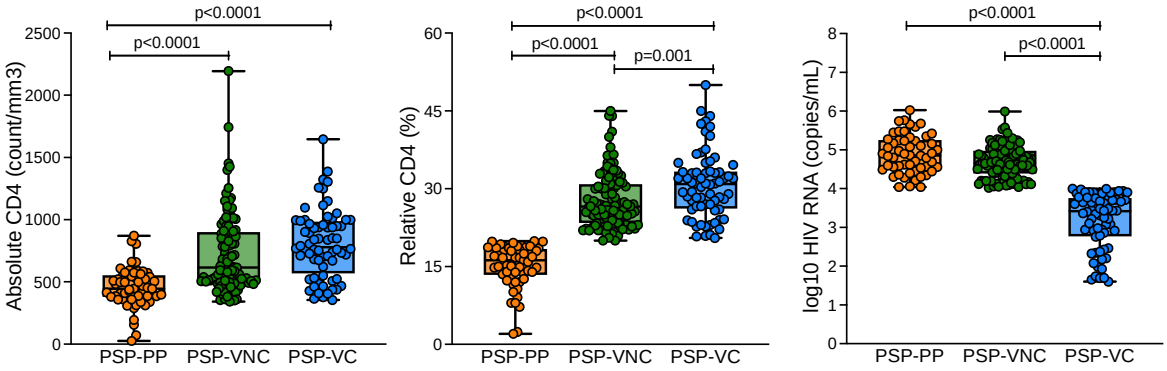


Figure 3.1 Clinical data of PSPs.
Comparison of absolute and relative CD4+ T-cell count and plasma HIV-RNA load between PSP-PP, PSP-VNC and PSP-VC. Statistical comparison between the three groups were based on ANOVA followed by Tukey's test for multiple comparisons.

We also investigated 998 ART-naïve adults living with HIV in the chronic stage of infection from a previously described cohort in SA (Kiepiela et al. 2004; Kiepiela et al. 2007; Leslie et al. 2010). Clinical data, plasma HIV-RNA load and CD4+ T-cell counts were collected as part of their regular follow-up. As AVNPs with normal CD4+ T-cell counts are very rarely seen (Rotger et al. 2011; Klatt et al. 2014), we classified the adult population according to their immunological progression and viral control in adult progressors (Adult-P, n=289), defined as having a sustained absolute CD4+ T-cell count of <200 cells/mm^3, viraemic non-controllers (Adult-VNC: absolute CD4+ T-cell count >200 cells/mm^3, and plasma HIV-RNA load >10,000 copies/mL, n=449); and viraemic controllers (Adult-VC: absolute CD4+ T-cell >200 cells/mm^3, and plasma HIV-RNA load <10,000 copies/mL, n=260), similar to previously published studies (Martin et al. 2002; Martin et al. 2007). Adult-P had a median plasma HIV-RNA load of 143,500 copies/mL (IQR 50,300 – 450,000) and a median absolute CD4+ T-cell count of 161 cells/mm^3 (IQR 99 – 192), while Adult-VNC had

a median plasma HIV-RNA load of 68,250 copies/mL (IQR 26,675 – 132,500) and a median absolute CD4+ T-cell count of 360 cells/mm^3 (IQR 303 – 496). Adult-VC had a median plasma HIV-RNA load of 2,805 copies/mL (IQR 673 – 4854) and a median absolute CD4+ T-cell count of 492 cells/mm^3 (IQR 438 – 696).

For subjects where NK cell immunophenotype was evaluated, CMV IgG levels were measured with ARCHITECT CMVG assay (Abbott Diagnostics, IL, USA) from frozen plasma samples and considered positive if >15 AU/mL following the recommendations by the manufacturer.

3.3. Results

3.3.1. HLA-Bw4 and low expressing HLA-A alleles are associated with slow-progression in children

To evaluate the impact of HLA-I and KIRs on disease outcome in paediatric HIV infection, we studied 310 children living with HIV from SSA. Using time to start ART as a result of meeting the prevailing CD4 criteria for ART initiation at the time of follow-up (absolute and relative CD4+ T-cell count < 350 cells/mm3 and/or < 20%) as the primary outcome and censoring the data at the last timepoint before ART if therapy was started in absence of immunological progression (change of guideline to universal ART), the presence of protective HLA-I alleles (B*57/58:01/81:01) in HIV-children living with HIV trended towards slower progression without reaching significance (HR=0.76, p=0.1; Figure 3.2A and B), while disease-susceptible HLA-I alleles (B*18:01/45:01/58:02) were significantly associated with faster progression (HR=1.44, p=0.02; Figure 3.2C and D). The effect of HLA-B*18:01 and HLA-B*45:01 was most marked in the first 5-6 years of life, while the effect of HLA-B*58:02 was

only observed in late childhood. Thus, based on these univariate analyses, both protective and disease-susceptible HLA-I appear to have some impact on HIV disease progression in paediatric infection that is consistent with the HLA-I effects observed in adult infection.

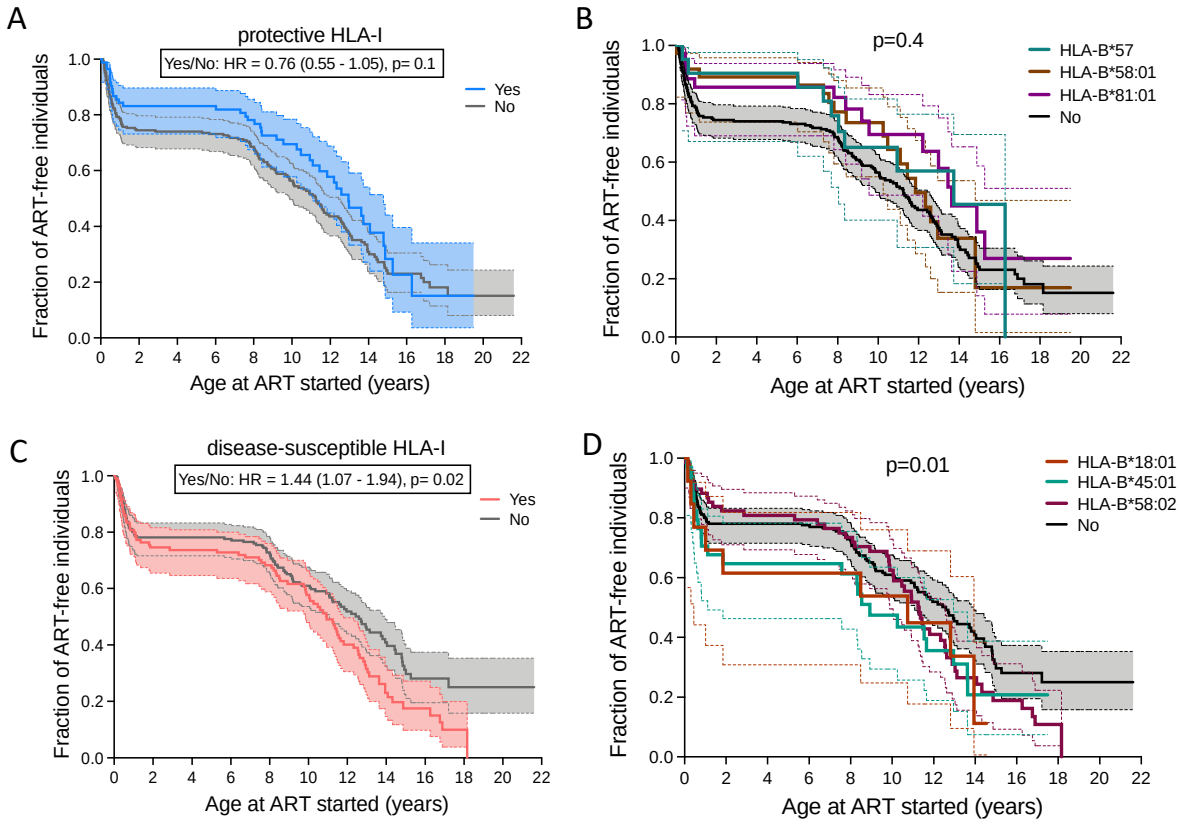


Figure 3.2 Adult defined disease-protective and disease susceptible HLA-I in paediatric infection.

A and B. Effect of combined (A) and individual protective HLA-B alleles (57/58:01/81:01) on time to start ART due to progression (absolute and relative CD4+ T-cell count <350 cells/mm^3 and/or < 20%). Data was censored at the timepoint before therapy if ART initiated without immunological progression. Hazard ratio (HR) and p-values are based on log-rank comparisons between the presence or not of the listed HLA-I alleles. C and D. Same for combined (C) and individual (D) disease-susceptible HLA-B alleles (18:01/45:01/58:02).

The combination of HLA-Bw4 and KIR3DL1 has been associated with slow progression in HIV infection in both adult (Martin et al. 2007; Kamya et al. 2011; Tomescu et al. 2012; Wang et al. 2016; Martin et al. 2018) and paediatric (Singh et al. 2016) studies. In our paediatric cohort, individuals with at least one HLA-Bw4

allele progressed significantly slower than HLA-Bw6/Bw6 individuals (HR=0.63, p=0.01, Figure 3.3A). Since almost all individuals in the cohort express KIR3DL1, the presence or absence of KIR3DL1 did not affect the analyses. Of note, the disease-susceptible HLA-B*58:02 is a Bw4 allele and, as noted, its detrimental effect in accelerating progression is only seen after the age of 10 years in children (Figure 3.2D). Mixed-effects regression analyses of longitudinally followed children aged >5 years who had not met the prevailing criteria to initiate ART showed a tendency to lower plasma HIV-RNA load in children expressing alleles with the Bw4 motif (Figure 3.3B). The effect was not clear-cut for absolute CD4+ T-cells (Figure 3.3C).

To evaluate the role of *HLA-A* allele expression on HIV disease progression in the paediatric cohort, we used previously published data to estimate for each study subject in the paediatric cohort the gene expression levels of different alleles (Ramsuran et al. 2018). We observed an association between low-expressing *HLA-A* and slow progression to meet criteria to initiate ART (Figure 3.3D, p=0.01). The mixed-effects regression analyses showed a lower plasma HIV-RNA load in children with the lowest HLA-A z-scores (p=0.03, Figure 3.3E) when compared to the group with highest z-scores. We observed similar but less clear benefits of low *HLA-A* expression on absolute CD4+ T-cell count (Figure 3.3F). Due to the low frequency of -21M homozygous, we were not able to see any additive effect in our analyses (data not shown), as previously has been demonstrated in adults (Ramsuran et al. 2018).

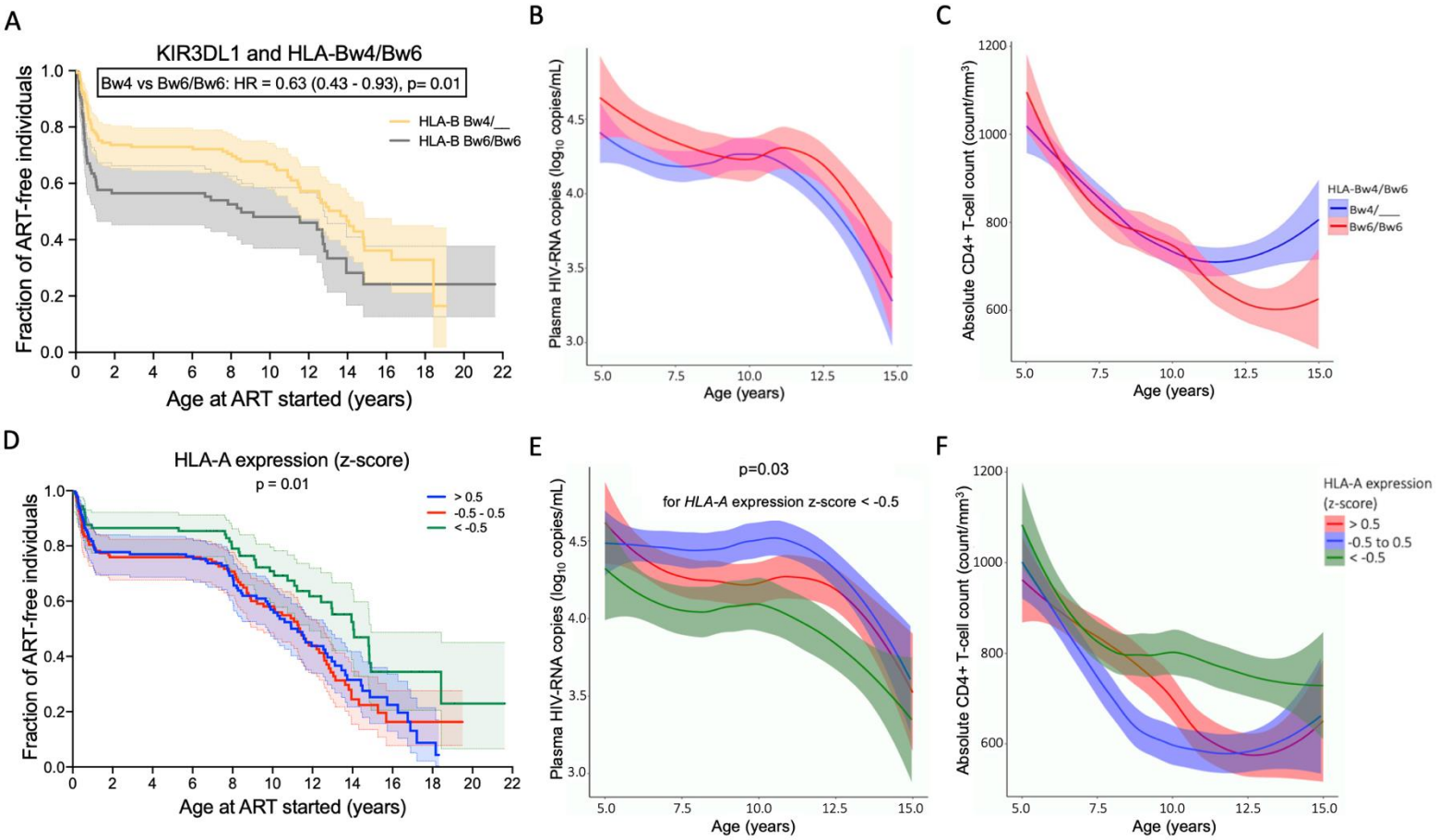


Figure 3.3 HLA-Bw4 and HLA-A allele expression in paediatric infection.

A. Effect of HLA-Bw4 on time to start ART due to progression. Data was censored if ART initiated without immunological progression. HR and p-values are based on log-rank comparisons between the presence or not of HLA-Bw4 alleles. B and C. Impact of Bw4 on longitudinal viral load (B) and absolute CD4+ T-cell count (C) in children older than five years old. The best fit line and the 95% confidence interval are shown for each category D to F. Same but for *HLA-A* expression z-score. p-value was obtained from the linear mixed-effects modelling comparing the groups.

Next, we categorised the paediatric cohorts into PP and PSP, subsequently subdividing the PSP group into PSP-PP, PSP-VNC and PSP-VC, as described in the methods section. Table 3.1 describes the distribution of HLA-I factors and KIR genotypes among the groups. Neither protective nor disease-susceptible HLA-I were significantly enriched in any of these groupings (Figure 3.4A), apart from HLA-B*57 when compared PP with PSP and PSP-VC (p=0.05 and p=0.03, respectively; Figure 3.4B and C). However, HLA-Bw4 alleles were more frequent in the PSP and PSP-VC groups (p=0.008 and p=0.002) compared to PP, and *HLA-A* expression was also lower among PSP-VC compared to PP (p=0.05, Figure 3.4D).

We did not observe any clear effect of HLA-C1 and C2 alleles (Figure 3.5A). Again, due to the low frequency of -21M homozygous in our cohort, we were not able to investigate its impact on the HIV disease outcome. Interestingly, the PSP and PSP-VC tend to have more KIR and their cognate HLA-I ligand than PP and the PSP-VNC (Figure 3.5B), allowing more KIR-HLA interactions.

To compare these immunogenetic effects of HLA-I and KIR in the paediatric cohort with those observed in adults, we conducted similar analyses in the group of ART-naïve, HLA-I typed adults from SA, of whom 405 were also KIR typed. Although the protective and disease-susceptible HLA-I were very strongly associated with immunological and virological status (Figure 3.6A), the impact of HLA-Bw4 alleles (Figure 3.6A) and *HLA-A* expression levels (Figure 3.6B) appeared substantially more modest; the opposite to what was observed in the paediatric cohort.

Table 3.1 Frequency of HLA-I and KIR genotypes.

HLA-I	PP (n=73)	PSP (n=237)	*p*	PSP-PP (n=57)	PSP-VNC (n=112)	PSP-VC (n=68)
pHLA[1]	19% (14)	29% (69)	*ns*	23% (13)	29% (32)	35% (24)
HLA-B*57	3% (2)	10% (24)	0.05	9% (5)	9% (10)	13% (9)
HLA-B*58:01	10% (7)	11% (26)	*ns*	10% (6)	12% (13)	10% (7)
HLA-B*81:01	7% (5)	11% (26)	*ns*	7% (4)	9% (10)	18% (12)
sHLA[2]	41% (30)	35% (84)	*ns*	40% (23)	33% (37)	35% (24)
HLA-B*18:01	8% (6)	4% (10)	*ns*	2% (1)	5% (6)	4% (3)
HLA-B*45:01	16% (12)	9% (22)	*ns*	9% (5)	10% (11)	9% (6)
HLA-B*58:02	18% (13)	23% (55)	*ns*	30% (17)	19% (21)	25% (17)
Bw4	49% (36)	67% (158)	0.009	68% (39)	64% (72)	69% (47)
HLA-C1	63% (46)	57% (136)	*ns*	54% (31)	58% (65)	59% (40)
HLA-C2	81% (59)	72% (171)	*ns*	63% (36)	72% (81)	79% (54)

KIR	PP (n=73)	PSP (n=126)	*p*	PSP-PP (n=13)	PSP-VNC (n=70)	PSP-VC (n=43)
KIR2DL1	100% (73)	99% (125)	*ns*	100% (13)	99% (69)	100% (43)
KIR2DL2	71% (52)	66% (83)	*ns*	69% (9)	71% (50)	56% (24)
KIR2DL3	79% (58)	71% (89)	*ns*	54% (7)	73% (51)	72% (31)
KIR2DL4	100% (73)	99% (125)	*ns*	100% (13)	99% (69)	100% (43)
KIR2DL5	70% (51)	60% (76)	*ns*	77% (10)	63% (44)	51% (22)
KIR2DS1	25% (18)	17% (22)	*ns*	31% (4)	16% (11)	16% (7)
KIR2DS2	68% (50)	65% (82)	*ns*	69% (9)	70% (49)	56% (24)
KIR2DS3	23% (17)	26% (33)	*ns*	8% (1)	30% (21)	26% (11)
KIR2DS4[3]	86% (63)	87% (110)	*ns*	54% (7)	90% (63)	93% (40)
KIR2DS5	58% (42)	47% (59)	*ns*	69% (9)	47% (33)	40% (17)
KIR3DL1	100% (73)	98% (124)	*ns*	100% (13)	99% (69)	98% (42)
KIR3DS1	11% (8)	10% (12)	*ns*	8% (1)	9% (6)	12% (5)
KIR3DL2	100% (73)	99% (125)	*ns*	100% (13)	99% (69)	100% (43)
KIR3DL3	100% (73)	99% (125)	*ns*	100% (13)	99% (69)	100% (43)

[1]p-HLA: disease-protective HLA (HLA-B*57/58:01/81:01)

[2]s-HLA: disease-susceptible HLA (HLA-B*18:01/45:01/58:02)

[3]full-length gene

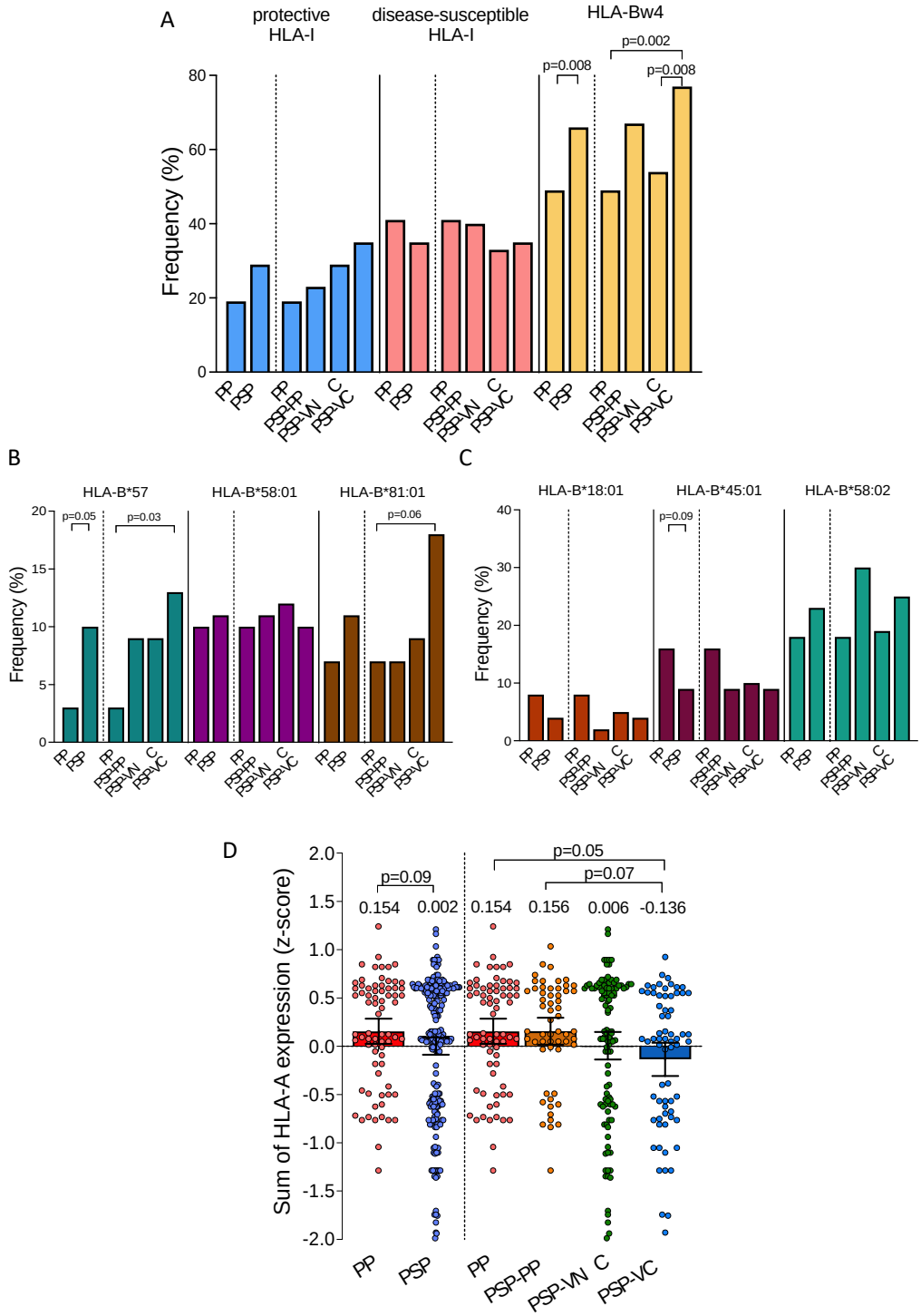


Figure 3.4 HLA-I in each paediatric categorical group of HIV infection.
A. Frequency of the disease-protective and the disease-susceptible HLA-I and HLA-Bw4 alleles in each group. Comparisons were based on Chi-square test and corrected with Bonferroni-Holm due to multiple-comparison tests. B and C. Frequency of individual disease-protective (B) and disease-susceptible (C) HLA-B alleles. D. *HLA-A* expression levels z-score of each individual is plotted. Statistical comparison between two groups were based on unpaired t-test and between the four groups on ANOVA followed by Tukey's test for multiple comparisons.

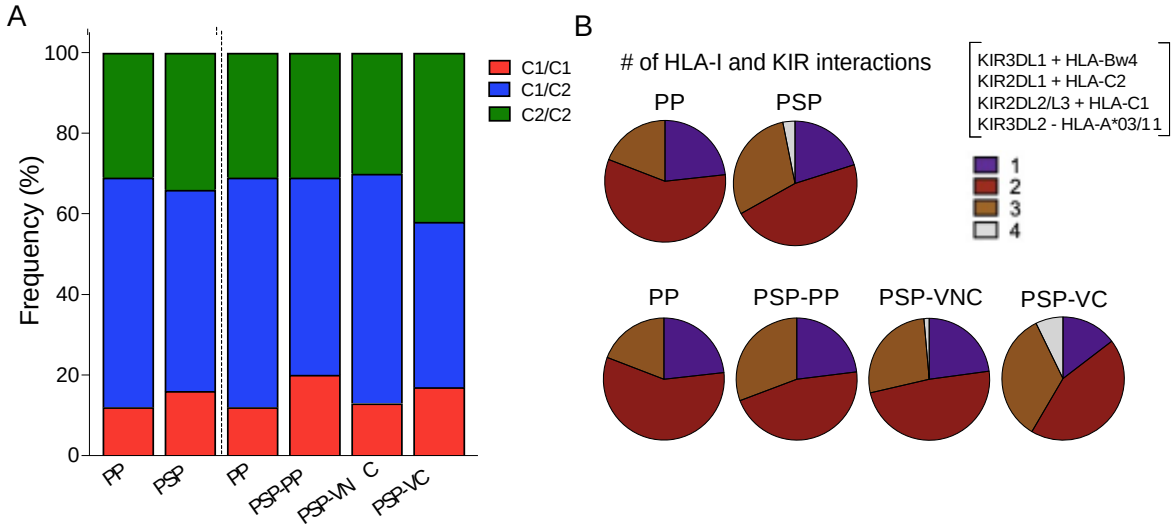


Figure 3.5 HLA-C and KIR-HLA interactions.

A. Proportion of HLA-C C1 and C2 alleles in each group. B. Proportion of individuals grouped according to the number of KIR-HLA interactions each individual can have according to the presence of a KIR and their cognate HLA-I ligand in each paediatric group.

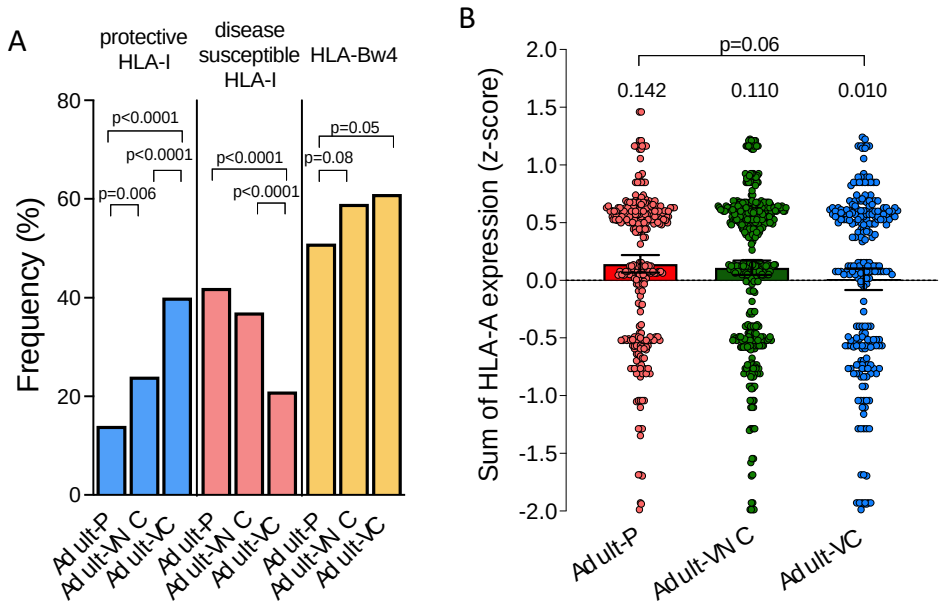


Figure 3.6 HLA-I in each categorical group of HIV infection.

A. Frequency of the disease-protective and the disease-susceptible HLA-I and HLA-Bw4 alleles in each adult group. Comparisons were based on Chi-square test and corrected with Bonferroni-Holm due to multiple-comparison tests. A. *HLA-A* expression levels z-score of each individual is plotted. Statistical comparison between two groups were based on unpaired t-test and between the four groups on ANOVA followed by Tukey's test for multiple comparisons.

3.3.2. Regression analyses indicate stronger influence of HLA-A and Bw4 alleles than HLA-B on HIV outcome in children

To estimate the relative impact of each of the immunogenetic factors studied on HIV disease outcome in children and adults, we first used a Cox proportional-hazard model to determine hazard ratios of these parameters for protection against progression to meet CD4+ T-cell criteria for ART initiation in children (Figure 3.6). Whilst there was no significant independent effect of protective or disease-susceptible HLA-B alleles on outcome, the presence of HLA-Bw4 and low *HLA-A* expression levels were independently associated with a slower HIV disease progression in children.

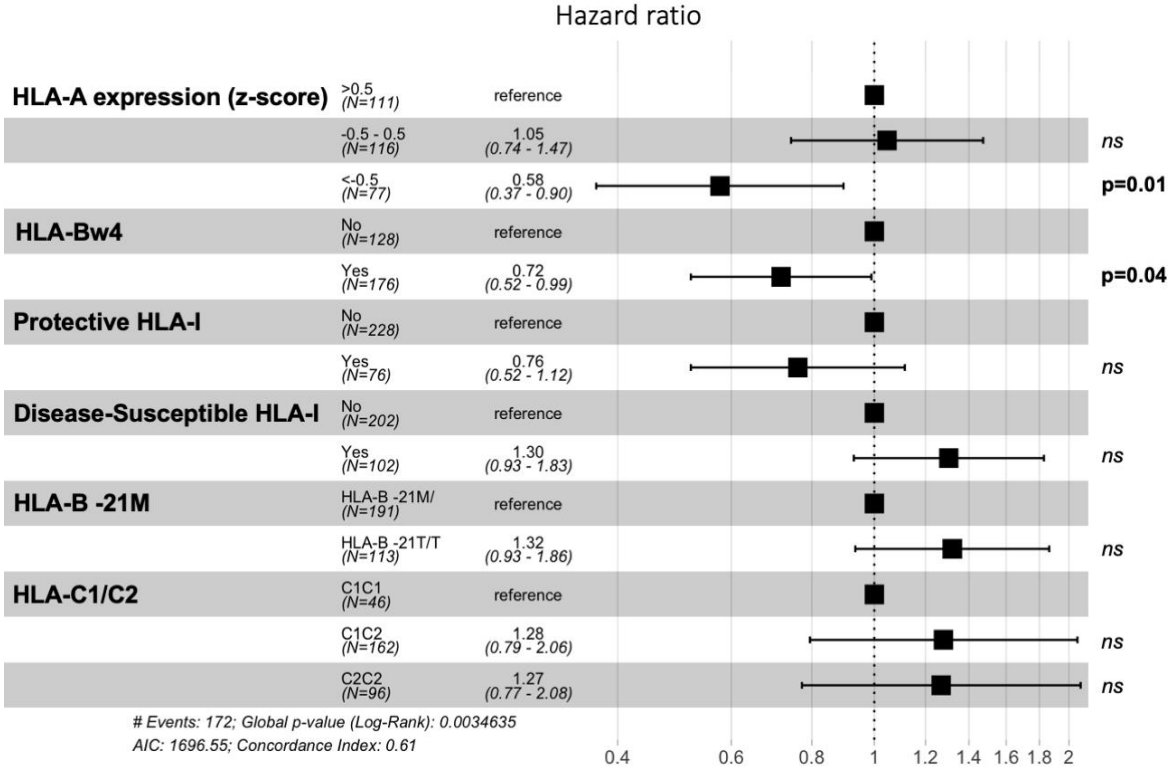


Figure 3.7 Evaluation of immunogenetic factors on time to start ART.
Cox-proportional hazard model evaluating the effect of protective and disease susceptible HLA-I alleles, HLA-Bw4, HLA-B -21T/M, HLA-C1/C2 and *HLA-A* expression level on time to start ART due to progression (absolute and relative CD4+ T-cell count <350 cells/mm3 and/or <20%). Data was censored at the timepoint before therapy if ART initiated without immunological progression.

We next ran three logistic models including all the co-variates, having as outcomes for the paediatric groups in the first model the categorical variables PP and PSP, and, in the second, PP, PSP-PP, PSP-VC and PSP-VNC. For the adult cohort, a third model used as outcomes: Adult-P, Adult-VNC and Adult-VC (Table 3.2). In both paediatric models, HLA-Bw4 and *HLA-A* expression levels were significantly associated with outcome. Of note, Bw4 remained significant even after adjusting for protective HLA-I alleles. The HLA-A effect was even more marked in the second model that included viraemic control among the outcomes. In neither paediatric model the adult defined protective and disease-susceptible HLA-B have a significant impact on outcome. By contrast, in the adult model, protective and disease-susceptible HLA-B had the strongest effects. HLA-Bw4 and *HLA-A* expression were significant albeit lower impact in adults. Similar findings were obtained when individual protective and disease-susceptible HLA-I alleles were considered (Table 3.3).

Therefore, the combination seen here of Bw4 alleles and low expressing *HLA-A* alleles decreases the inhibition via NKG2A and favours the education via KIRs of NK cells, these being the immunogenetic factors most strongly associated with immune control in the paediatric cohort.

Table 3.2 Logistic regression models in the paediatric and adult cohorts.

Model 1. Outcome: PP=1; PSP=0

Predictor	Coefficient	Standard error	p-value	OR[1]	95% CI of OR	
					Lower	Upper
Intercept	0.19266	0.10975	0.08035	1.21	0.98	1.5
pHLA-I	-0.06371	0.06079	0.29557	0.94	0.93	1.06
sHLA-I	0.0818	0.05669	0.15017	1.08	0.97	1.21
HLA-Bw4	-0.17295	0.05742	**0.00285**	**0.84**	**0.75**	**0.94**
HLA-B -21T	0.08933	0.10645	0.40214	1.1	0.89	1.35
HLA-C C1C1	*Reference*					
C1C2	0.07499	0.07783	0.33619	1.08	0.92	1.25
C2C2	0.0603	0.07783	0.33619	1.06	0.9	1.25
HLA-A (z-score)	0.08134	0.03966	**0.04130**	**1.08**	**1.01**	**1.17**

Model 2. Outcome: PP=3, PSP-PP=2, PSP-VNC=1, PSP-VC=0

Predictor	Coefficient	Standard error	p-value	OR[1]	95% CI of OR	
					Lower	Upper
Intercept	1.36496	0.27711	<0.0001	3.92	2.27	6.74
pHLA-I[2]	-0.2735	0.15349	0.07594	0.76	0.56	1.02
sHLA-I[3]	0.13246	0.14312	0.35555	1.14	0.86	1.51
HLA-Bw4	-0.4274	0.14498	**0.00349**	**0.65**	**0.49**	**0.86**
HLA-B -21T	0.23293	0.26877	0.38693	1.26	0.74	2.14
HLA-C C1C1	*Reference*					
C1C2	0.15877	0.19652	0.41989	1.17	0.8	1.72
C2C2	0.07129	0.21078	0.73546	1.07	0.71	1.62
HLA-A (z-score)	0.30516	0.10015	**0.00255**	**1.35**	**1.12**	**1.65**

Model 3. Outcome: Adult-P=2, Adult-VNC=1, Adult-VC=0

Predictor	Coefficient	Standard error	p-value	OR[1]	95% CI of OR	
					Lower	Upper
Intercept	1.068053	0.85302	<0.0001	2.91	2.46	3.44
pHLA-I	-0.270221	0.055879	**<0.0001**	**0.76**	**0.68**	**0.85**
sHLA-I	0.249808	0.053009	**<0.0001**	**1.28**	**1.16**	**1.42**
HLA-Bw4	-0.142084	0.054087	**0.00875**	**0.87**	**0.78**	**0.96**
HLA-B -21T	0.004181	0.080804	0.95875	1	0.86	1.18
HLA-C C1C1	*Reference*					
C1C2	0.020518	0.063699	0.74744	1.02	0.9	1.16
C2C2	0.023111	0.068771	0.7369	1.02	0.89	1.17
HLA-A (z-score)	0.078734	0.033122	**0.01764**	**1.08**	**1.01**	**1.15**

[1] OR: odds ratio

[2] pHLA-I: disease-protective HLA-I (B*57/58:01/81:01)

[3] sHLA-I: disease-susceptible HLA-I (B*18:01/45:01/58:02)

Table 3.3 Logistic regression models in the paediatric and adult cohorts considering individual protective and disease-susceptible HLA-I alleles.

Model 1. Outcome: PP=1; PSP=0

Predictor	Coefficient	Standard error	p-value	OR[1]	95% CI of OR	
					Lower	Upper

Intercept	-1.42550	0.63540	0.025	0.24	0.06	0.79
HLA-B*57	-0.42900	0.82140	0.601	0.65	0.09	2.76
HLA-B*58:01	0.38110	0.58550	0.515	1.46	0.43	4.42
HLA-B*81	-1.05680	0.56550	0.062	0.35	0.10	0.98
HLA-B*18:01	0.64630	0.57980	0.265	1.91	0.60	5.97
HLA-B*45	0.32170	0.44200	0.467	1.38	0.57	3.24
HLA-B*58:02	0.30090	0.45430	0.508	1.35	0.55	3.29
HLA-Bw4	-1.10800	0.41140	**0.007**	**0.33**	**0.14**	**0.73**
HLA-B -21T	0.30290	0.60120	0.614	1.35	0.43	4.77
HLA-C C1C1	*Reference*					
C1C2	0.55810	0.47100	0.236	1.75	0.72	4.64
C2C2	0.69660	0.52680	0.186	2.01	0.73	5.89
HLA-A (z-score)	0.63240	0.25620	**0.014**	**1.88**	**1.16**	**3.18**

Model 2. Outcome: PP=3, PSP-PP=2, PSP-VNC=1, PSP-VC=0

Predictor	Coefficient	Standard error	p-value	OR[1]	95% CI of OR	
					Lower	Upper
Intercept	1.43455	0.27989	<0.001	4.20	2.43	7.27
HLA-B*57	-0.40597	0.28743	0.159	0.67	0.38	1.17
HLA-B*58:01	0.05056	0.24089	0.834	1.05	0.66	1.69
HLA-B*81	-0.61485	0.22609	**0.007**	**0.54**	**0.35**	**0.84**
HLA-B*18:01	0.16412	0.28724	0.568	1.18	0.67	2.07
HLA-B*45	0.16588	0.21434	0.440	1.18	0.78	1.80
HLA-B*58:02	-0.07274	0.19547	0.710	0.93	0.63	1.36
HLA-Bw4	-0.42616	0.17675	**0.017**	**0.65**	**0.46**	**0.92**
HLA-B -21T	0.13436	0.27388	0.624	1.14	0.67	1.96
HLA-C C1C1	*Reference*					
C1C2	0.20382	0.1971	0.302	1.23	0.83	1.80
C2C2	0.21345	0.22207	0.337	1.24	0.80	1.91
HLA-A (z-score)	0.34711	0.10176	**0.001**	**1.41**	**1.16**	**1.73**

Model 3. Outcome: Adult-P=2, Adult-VNC=1, Adult-VC=0

Predictor	Coefficient	Standard error	p-value	OR[1]	95% CI of OR	
					Lower	Upper
Intercept	1.079563	0.084901	<0.001	2.94	2.49	3.48
HLA-B*57	-0.300951	0.099949	**0.003**	**0.74**	**0.61**	**0.90**
HLA-B*58:01	-0.126502	0.08845	0.153	0.88	0.74	1.05

HLA-B*81	-0.297718	0.079431	**<0.001**	**0.74**	**0.64**	**0.87**
HLA-B*18:01	0.336877	0.095352	**<0.001**	**1.40**	**1.16**	**1.69**
HLA-B*45	0.067484	0.089174	0.449	1.07	0.90	1.27
HLA-B*58:02	0.310816	0.069619	**<0.001**	**1.36**	**1.19**	**1.56**
HLA-Bw4	-0.19336	0.06544	**0.003**	**0.82**	**0.72**	**0.94**
HLA-B -21T	0.005349	0.082449	0.948	1.01	0.86	1.18
HLA-C C1C1	*Reference*					
C1C2	0.024693	0.065404	0.706	1.03	0.90	1.17
C2C2	0.015861	0.073897	0.830	1.02	0.88	1.17
HLA-A (z-score)	0.083442	0.033594	**0.013**	**1.09**	**1.02**	**1.16**

[1] OR: odds ratio

3.3.3. A NK cell signature characterised by high NKG2A expression is strongly associated with favourable paediatric outcome

The differentiation and functional activity of the NK cell population are influenced by different factors including the immunogenetics and the impact of immunogenetics on the education program, but also age and infections (e.g., HIV and CMV). To investigate the NK cell immunophenotype of the distinct clinical groupings of children living with HIV defined above, we first compared age-matched individuals who fulfilled the criteria for the PSP-PP (n=10), PSP-VNC (n=8), and PSP-VC (n=10) groups (Table 3.4). PP were excluded from this analysis due to the very-early stage of progression and ART-initiation. As a reference group, we included HEU (n=9). As expected at this age in children from SA, all individuals with plasma available were CMV seropositive. Although there was no difference in total NK cell frequency, HIV disease progression was associated with a tendency to lower frequency of the $CD56^{bright}$ subset and an expanded $CD56^{neg}$ population (Figure 3.8A and B; Appendix I, Table 1 for antibody panel), as previously described in adult studies (Mavilio et al. 2003; Brunetta, Hudspeth, and Mavilio 2010). The PSP-VC had a similar subset distribution to the HEU.

Table 3.4 Individual clinical data for samples used in the NK cell immunophenotype.

PID	Sex	Age (years)	HLA-A		HLA-B		HLA-C		Viral load (copies/mm ^{3})	Absolute CD4	% CD4	CD4:CD8	CMV IgG (AU/mL)
PSP-PP-1	Female	10.47	30:01	66:01	39:10	42:01	12:03	17:01	19,000	297	12	0.18	>250
PSP-PP-2	Male	9.73	*Not available*						210,000	55	3	0.12	126.1
PSP-PP-3	Male	9.25	*Not available*						820,000	218	3		244.7
PSP-PP-4	Male	8.22	02:05	66:01	14:01	58:02	06:02	08:04	44,000	164	7	0.16	65.9
PSP-PP-5	Female	9.55	24:02	68:02	07:02	15:10	07:02	08:04	470,000	86	7	0.10	137.7
PSP-PP-6	Male	7.46	02:05	74:01	15:03	58:01		07:01	54,000	251	9	0.28	>250
PSP-PP-7	Female	10.24	02:02	30:02	08:01	57:03	07:01	07:01	3,700,000	112	2		126.5
PSP-PP-8	Female	8.65	02:01	30:02	45:01	53:01	04:01	16:01	740,000	242	9	0.12	>250
PSP-PP-9	Female	9.91	30:01	33:03	07:02	42:01	07:02	17:01	590,000	41	3	0.08	231.1
PSP-PP-10	Male	8.47	02:01	03:01	58:01	58:02	03:02	06:02	57,000	321	8	0.36	109.2
PSP-VNC-1	Male	10.12	30:02	68:02	15:10	42:01	03:04	17:01	57,000	933	45	1.69	198.2
PSP-VNC-2	Female	11.88	02:05	29:00	42:01	44:03	02:02	17:01	110,000	629	38	1.24	>250
PSP-VNC-3	Male	11.88	02:01	26:01	08:01	41:01	07:01	17:01	23,000	721	28	0.81	173
PSP-VNC-4	Male	12.72	03:01	23:01	13:03	44:03	03:03	06:02	58,000	1345	25	0.53	68
PSP-VNC-5	Female	13.69	02:01	34:02	44:03	45:01	04:01	16:01	24,000	1051	37	0.90	12.2
PSP-VNC-6	Female	10.19	29:02	30:02	14:02	57:03	08:02	18:02	140,000	954	34	0.89	66.7
PSP-VNC-7	Female	10.50	02:14	03:01	44:03	58:02	04:01	06:02	18,000	715	25	0.44	>250
PSP-VNC-8	Male	11.34	29:02	74:01	15:03	44:03	02:10	07:01	27,000	375	18	0.36	-
PSP-VC-1	Female	12.75	03:01	68:02	14:02	58:02	06:02	08:02	50	700	35	1.13	186.4
PSP-VC-2	Female	11.77	03:01	30:02	14:01	15:10	03:04	08:02	25	1258	37	1.00	72.5
PSP-VC-3	Female	13.58	03:01	33:03	35:01	53:01	04:01	16:01	26	1316	39	2.01	93.3
PSP-VC-4	Female	12.00	02:01	74:01	27:03	42:01	02:02	07:01	35	768	32	0.88	242.3
PSP-VC-5	Female	11.54	30:09	68:01	58:02	81:01	04:01	06:02	1,800	902	39	0.86	105.7
PSP-VC-6	Female	11.90	39:02	66:01	39:10	44:03	07:01	12:03	430	1007	38	1.18	30.6
PSP-VC-7	Male	12.86	02:05	68:02	14:01	15:10	03:04	08:04	1,700	982	33	1.63	>250
PSP-VC-8	Male	13.50	02:05	30:01	58:01	81:03	07:01	08:04	20	1007	34	1.37	>250
PSP-VC-9	Female	12.61	*Not available*						1,000	819	38	1.67	-
PSP-VC-10	Male	12.68	*Not available*						1,200	665	34	0.88	32.8
HEU-1	Male	12.33	*Not available*						-	-	-	-	47.2
HEU-2	Female	11.72	*Not available*						-	-	-	-	167.7
HEU-3	Male	12.11	*Not available*						-	-	-	-	>250
HEU-4	Female	13.12	*Not available*						-	-	-	-	226.6
HEU-5	Male	10.27	*Not available*						-	-	-	-	95.5
HEU-6	Female	11.55	*Not available*						-	-	-	-	72.2
HEU-7	Female	11.14	*Not available*						-	-	-	-	>250
HEU-8	Female	10.61	*Not available*						-	-	-	-	109.6
HEU-9	Male	13.23	*Not available*						-	-	-	-	138.8

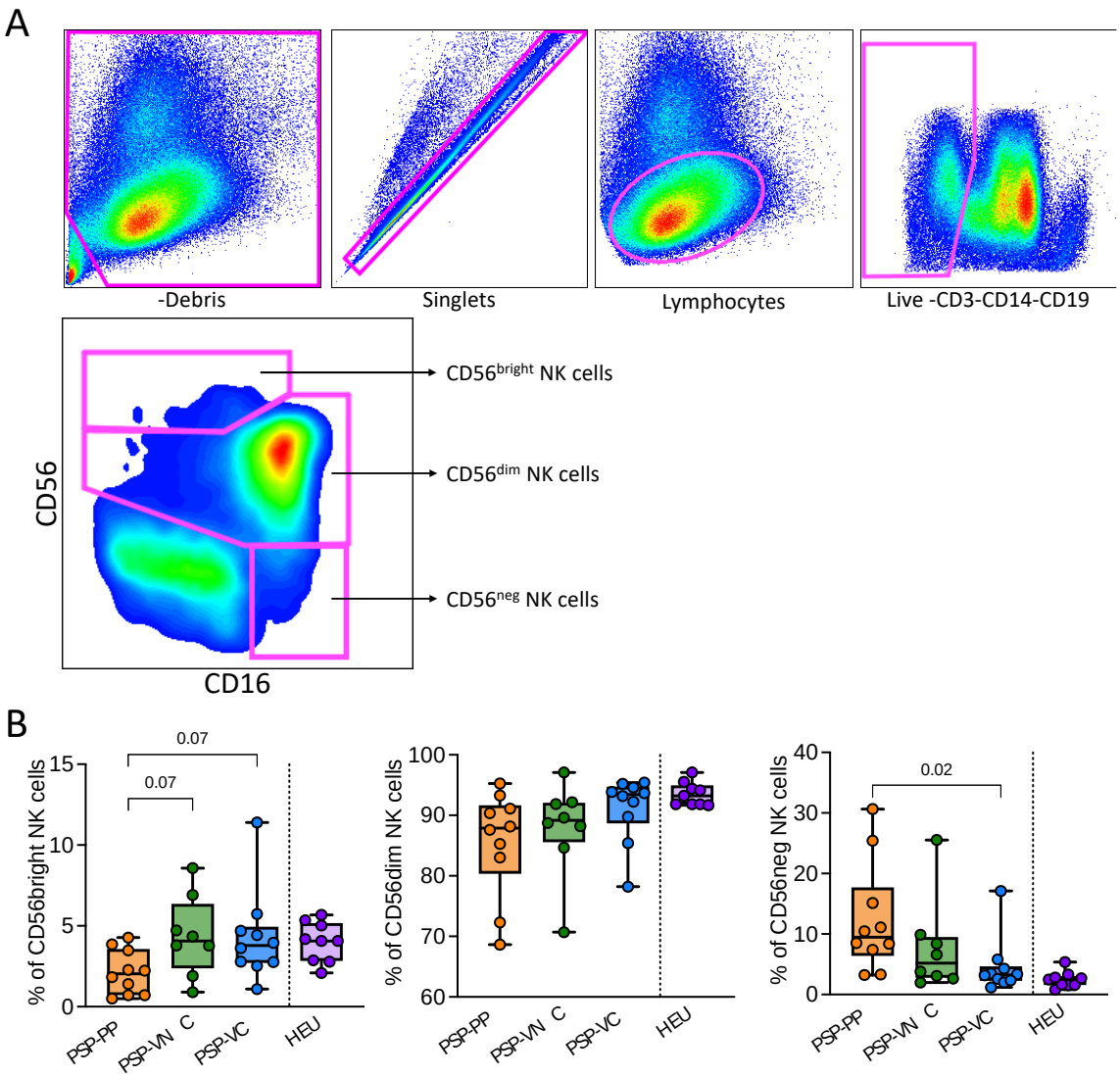


Figure 3.8 Subset distribution of NK cell population.

A. Strategy analyses to gate on NK cell subsets. The gate position for the other markers was based on the FMO control. B. Frequency of CD56^{bright}, CD56^{dim} and CD56^{neg}CD16+ NK cell subsets, respectively, among PSP-PP, PSP-VNC, PSP-VC and HEU. Statistical comparison between the three groups was based on Kruskal-Wallis test followed by Dunn's test for multiple comparisons.

Assessing the surface expression of several markers on the CD56^{dim} subset, PSP-VC had a higher frequency of NCRs on their surface than PSP-PP, being statistically significant for NKp46 (p=0.007) (Figure 3.9A and B). We did not observe any difference in the other activating markers, such as DNAM-1, NKG2D, NKG2C and CD16. In the heatmap, NCRs expression clustered together with NKG2A in the HEU and PSP-VC groups. NKG2A and NKp46 were highly co-expressed in the PSP-VC

compared to PSP-PP and PSP-VNC (p=0.002 and p=0.07, respectively, Figure 3.9C). By contrast, CD38, CD85j, NKG2C were clustered together with iKIRs and tend to be more expressed in the PSP-PP.

The frequency of NKG2A+ cells was higher in the PSP-VC than in the PSP-PP (p=0.003, Figure 3.10A). The NKG2A+ $CD56^{dim}$ NK cell population, either positive or negative for iKIRs, was distinctly expanded in the PSP-VC. This enrichment of the NKG2A+ population with higher expression of NCRs in PSP-VC shows a population skewed towards a less mature phenotype. Co-expression patterns showed that NKG2A expression alone or in combination with iKIRS, CD85j and CD57 was significantly higher in the PSP-VC, suggesting different stages of NK cell differentiation, whereas no difference was observed in the terminally differentiated iKIRs+CD57+ cells (Figure 3.10B).

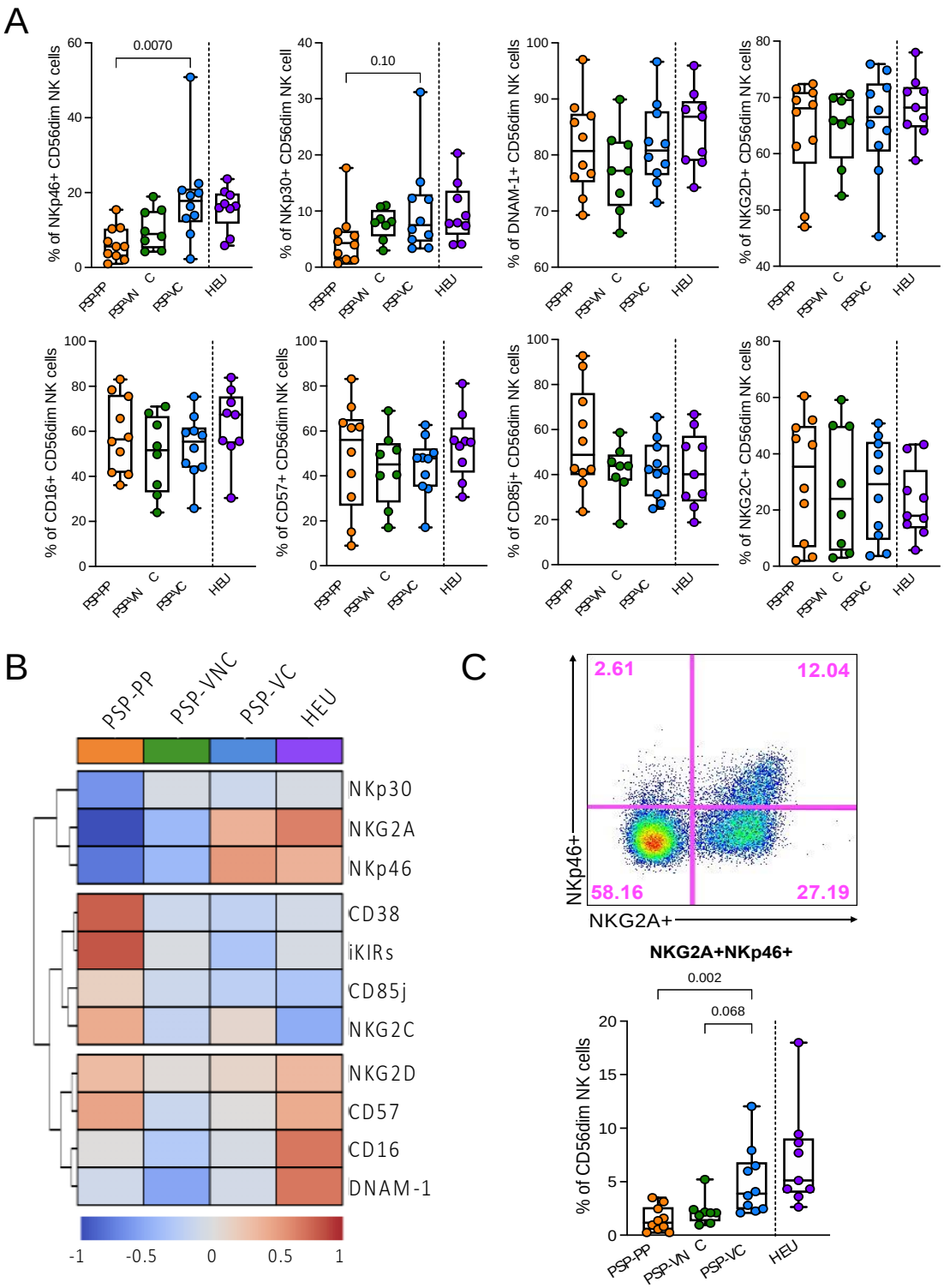


Figure 3.9 Expression of receptors and coreceptors on CD56^{dim} NK cells.
A. Frequency of difference surface markers shown for CD56^{dim} NK cell in the four groups. B. Heatmap showing the median z-score base on the frequency of the positive population for each surface marker using the average linkage method for clustering. C. Frequency of NKG2A and NKp46 co-expression difference on CD56dim NK cell in the four groups. Statistical comparison between the three groups was based on Kruskal-Wallis test followed by Dunn's test for multiple comparisons.

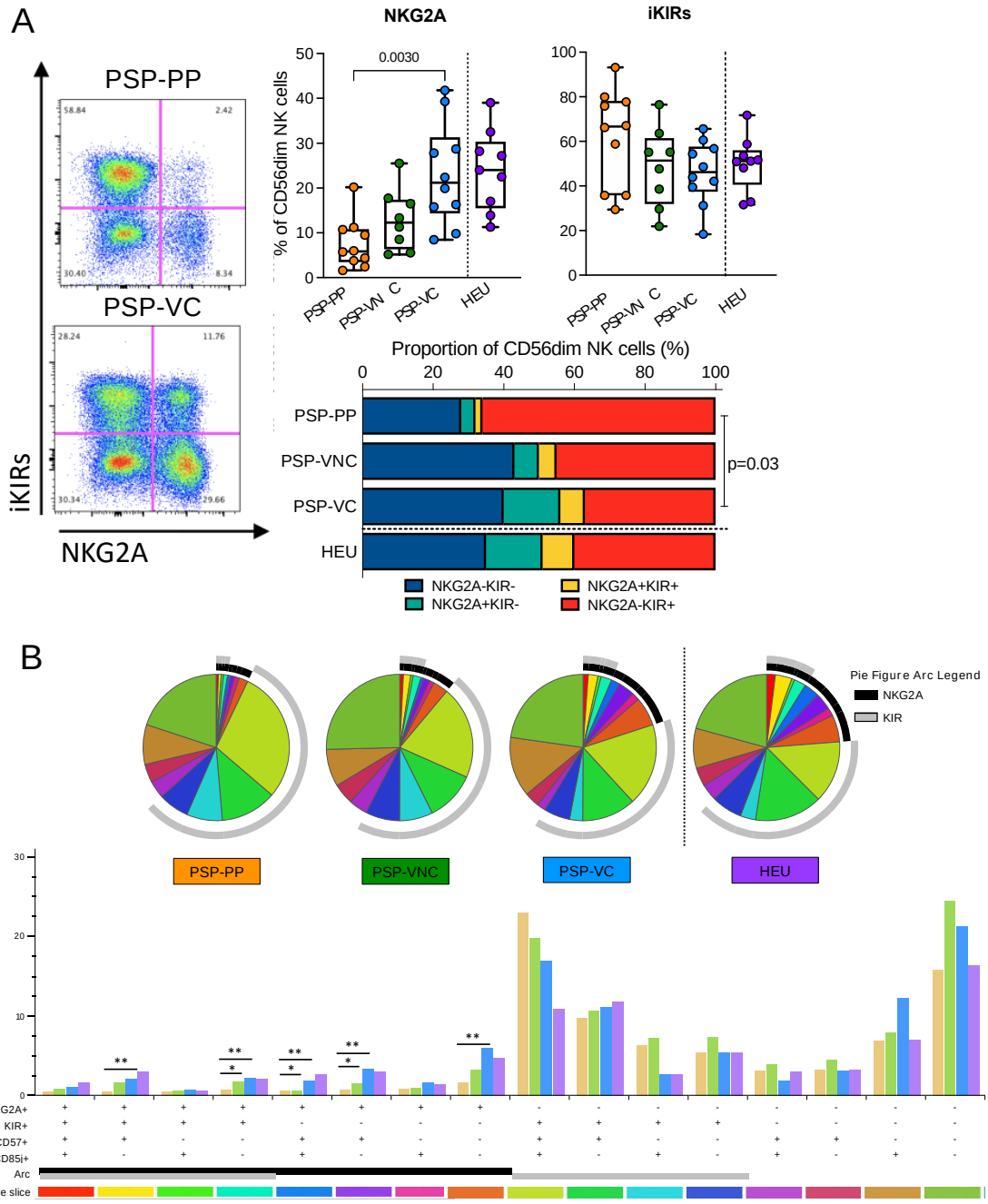


Figure 3.10 Frequency of maturations markers on CD56^{dim} NK cell.
A. Typical FACS plot obtained with NKG2A and iKIRs staining followed by the frequency of these markers in each group. Statistical comparison between the three groups was based on Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Combination of NKG2A and iKIRs fractions in CD56^{dim} NK cell standardized to the total from the median of each subset per group. Statistical comparison based on the permutation test. B. Pie and bar charts analysis performed by SPICE looking the combination of NKG2A, iKIRs, CD85j and CD57. Each pie slice corresponds to a different combination in the bar chart. The arcs represent the populations who NKG2A+ (black) and iKIRs+ (grey). Student t-test was performed by SPICE to compare the groups; *p<0.05, **p<0.005, ***p<0.001.

Despite the small sample size studied here, we considered whether the immunogenetic signature found in the larger cohort correlated with the NK cell immunophenotype. Indeed, we observed a trend towards a higher frequency of NKG2A+ cells in the participants with low-expressing *HLA-A* and HLA-Bw4 alleles (Figure 3.11A-C). We did not find any effect of -21M/T HLA-B on NKG2A expression, probably as a consequence of the low representation of -21M homozygous individuals (Figure 3.11D) and the impact of CMV seropositivity in our study (Cubero et al. 2020). Together these findings further support some influence of the inherited genetic factors on the NK cell population's immunophenotype.

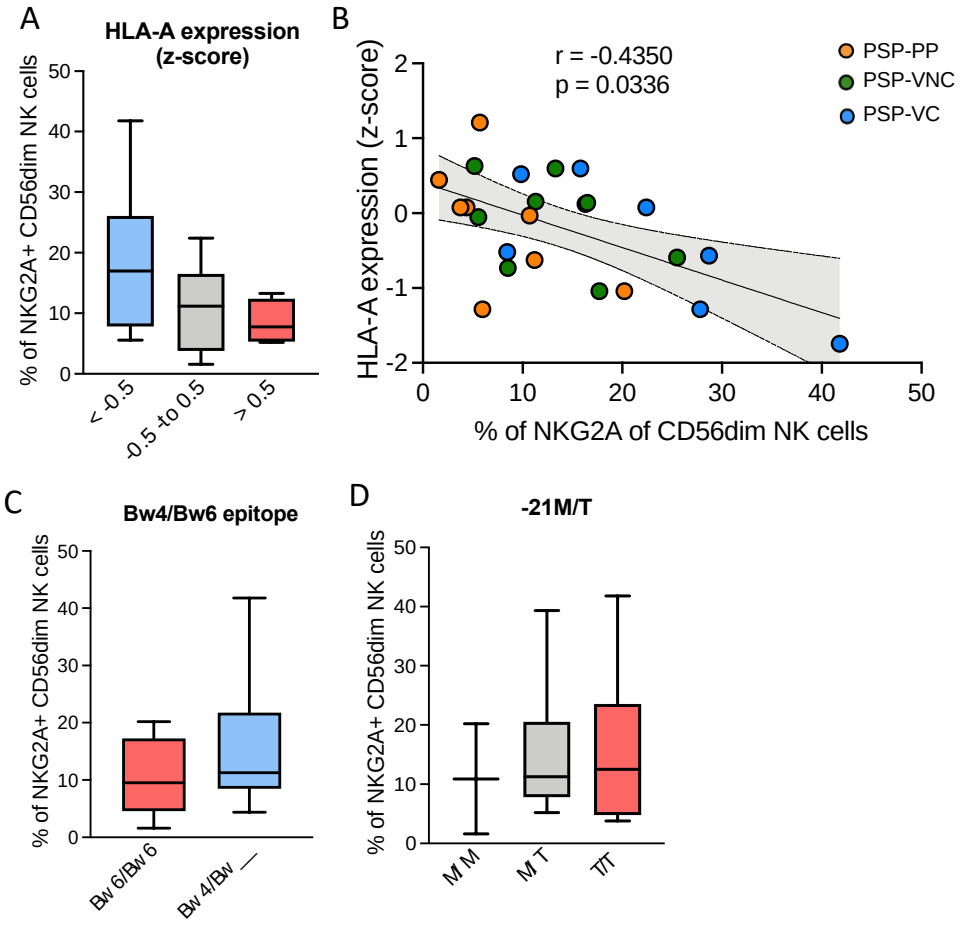
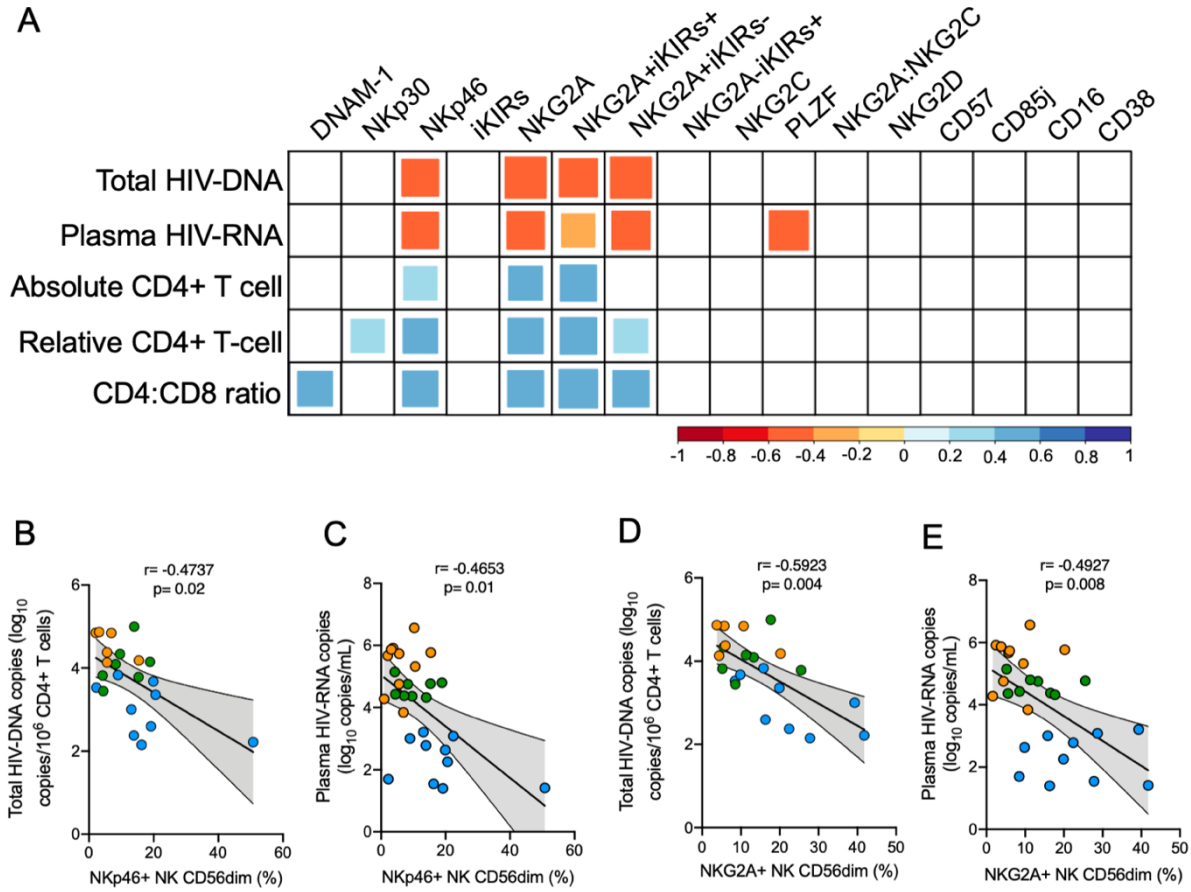


Figure 3.11 Effect of immunogenetics on NKG2A expression.
Effect of *HLA-A* expression z-score (A and B), HLA-Bw4 (C) and HLA-B -21M/T (D) on the frequency of NKG2A+ $CD56^{dim}$ NK cells. Spearman rank test was performed, and a simple linear regression best-fit line and 95% CI is shown within the grey area.

Next, we investigated whether expression of these immunophenotypic parameters on the CD56^{dim} NK cell subset correlated with markers of immunological HIV disease progression and viraemic control. NKp46 and NKG2A expression both correlated negatively with total HIV-DNA and plasma HIV-RNA load, and positively with absolute and relative CD4+ T-cell count, and CD4:CD8 ratio (Figure 3.12A to E). These associations of NKG2A+ cells were independent of iKIRs. Next, the LASSO principle was used to identify which were the major predicting covariates that contributed to these outcomes (Figure 3.12F). NKG2A+ or NKG2A+iKIRs- were selected in all models and had the highest impact in the outcome investigated, with the single exception of CD4:CD8 ratio, for which DNAM-1 expression had a similar impact. These findings reinforce the strong relation between the NKG2A+ NK cell population and the favourable immunological and virological outcomes in the paediatric group.

In contrast to the PSP-VNC and PSP-VC groups, we observed in the PSP-PP a higher proportion of a more mature and memory-like CD56^{dim}. Although PSP-PP did not show a clear expansion NKG2C+ cells (Figure 3.8A), we found a lower NKG2A:NKG2C ratio and an increase of the NKG2A-NKG2C+ population in this group (Figure 3.13A and B). Moreover, PSP-PP clearly displayed a downregulation of PLZF and FcεRI-γ in the CD56^{dim} subset (Figure 3.13C and D), a recognised signature of the memory-like NK cell subset. We did not observe any correlation between CMV IgG levels and NGK2A:NKG2C ratio or PLZF expression (Figure 3.13E), as has previously been described in adults (Peppa et al. 2018).



F

LASSO β-coefficient	NKp46	NKG2A	NKG2A+ iKIRs-	PLZF	CD16	DNAM1	CD38
Total HIV-DNA copies (log copies/million CD4+ T-cell)			**-0.1673**		-0.0007		
Plasma HIV-RNA copies (log copies/mL)	-0.0155		**-0.2403**	-0.0004			
Relative CD4+ T-cell count (%)	0.0866	**0.1977**					-0.0466
CD4:CD8 ratio	0.0082	0.0110				**0.0152**	

Figure 3.12 Correlation between different markers on CD56dim NK cells and disease severity.

A. Matrix showing only significant correlation between each different marker and total HIV-DNA load (log_{10} copies/10^6 CD4+ T-cell), plasma HIV-RNA load (log_{10} copies/mL), absolute CD4+ T-cell count (count/mm^3), relative CD4+ T-cell count (%) and CD4:CD8 ratio. B to E. Plots showing NKp46+ and NKG2A+ $CD56^{dim}$ NK cell subsets correlations. Spearman rank test was performed for correlations and simple linear regression best-fit line and 95% CI are shown within the grey area. PSP-PP, PSP-VNC and PSP-VC are shown in orange, green and blue dots, respectively. F. Covariates selected by the LASSO model and their β-coefficients. All variables that were significant correlated to the outcome in Figure 6A were included in the final model.

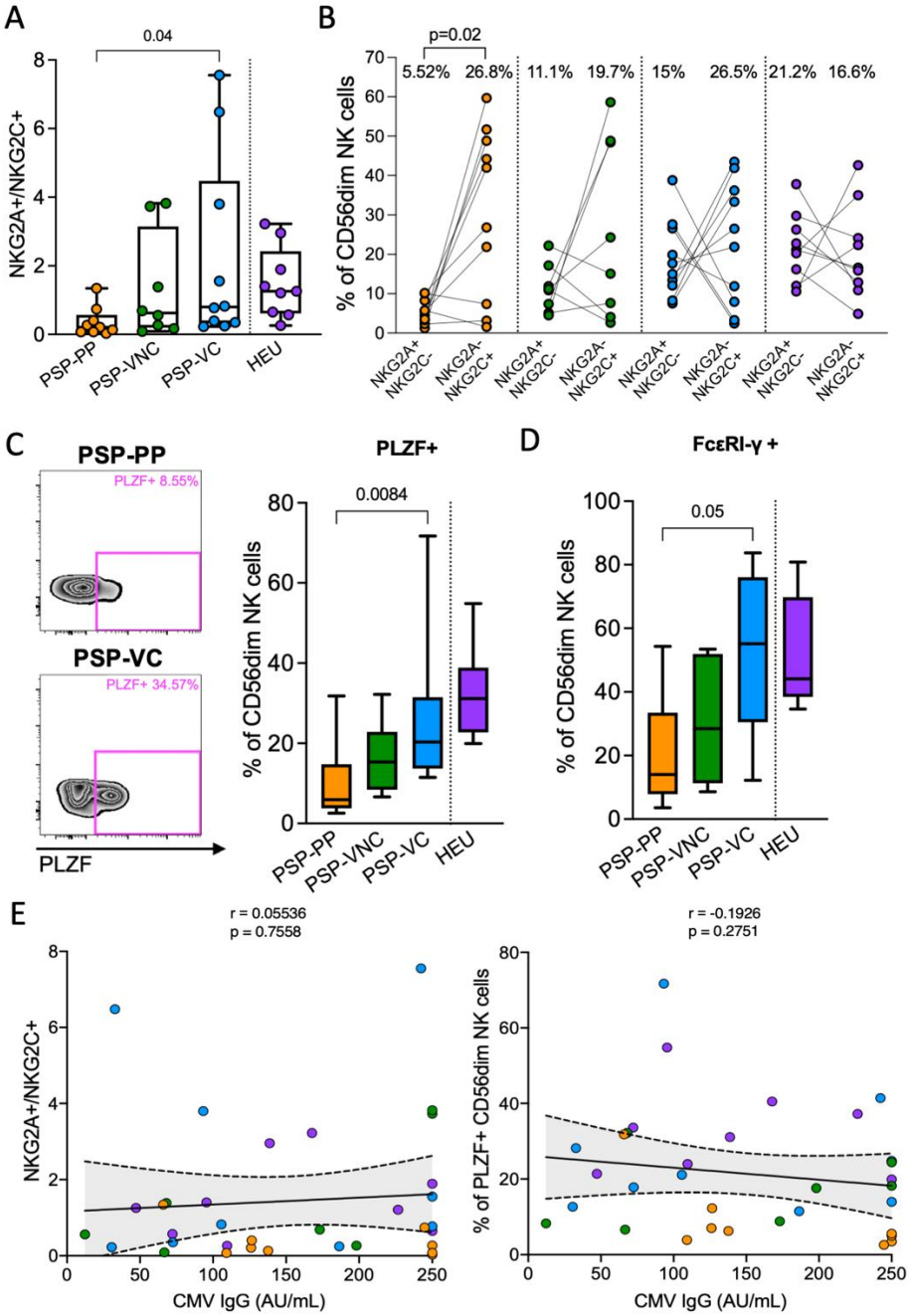


Figure 3.13 Expansion of memory-like NK cells in PSP-PP.

A. NKG2A/NKG2C ratio in each of the four groups. Statistical comparison was based on Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Pairwise comparison of NKG2A+NGK2C- and NKG2A-NKG2C+ subsets in each group. Values shown correspond to the median. Wilcoxon matched-pairs test was performed to compare the two populations. B. Typical FACS plot and frequency of PLZF+ and FcεRI-γ+ in $CD56^{dim}$ NK cell subset in each group. Statistical comparison was based on Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Correlation of CMV IgG levels with NKG2A/NKG2C ratio and PLZF. Spearman rank test are shown for correlations. Simple linear regression best-fit line and 95% CI are shown within the grey area.

3.3.4. Enhanced cytokine responsiveness of CD56^{dim} NK cells in viraemic controllers

To assess the impact this less mature phenotype has on CD56^{dim} NK cell effector function, we evaluated the ability of these cells in the different paediatric groups to secrete IFN-γ and degranulate via different pathways. IL-12 and IL-18 stimulation induced strong IFN-γ secretion in the PSP-VC compared to PSP-PP (p=0.02, Figure 3.14A; Appendix I, Table 2 for antibody panel). As expected, a higher proportion of IFN-γ+ cells were NKG2A+ and PLZF+ in the PSP-VC. Similarly, we observed higher levels of degranulation (CD107a) in the PSP-VC compared to PSP-PP (p=0.03) and PSP-VNC (p<0.001). The frequency of IFN-γ positive cells correlated positively with the frequency of NKG2A+ CD56dim NK cells, and CD4+ T-cell percentage, while negatively with plasma viral load and total HIV-DNA (Figure 3.14B).

When incubated with K562, a cell line lacking HLA-I, we did not observe the same difference in the IFN-γ expression. However, a tendency towards higher degranulation (CD107a+) was present among the PNP-VC (Figure 3.14C). Similarly, PSP-VC tended to have a higher proportion of CD107a+ cells expressing NKG2A.

Finally, the ability to perform ADCC was assessed in response to RAJI cells coated with anti-CD20. The median percentage of IFN-γ and CD107a was similar between the groups but tended to be higher in the PSP-PP group, which was expected due to the more mature and memory-like phenotype of their NK cell population (Figure 3.14D).

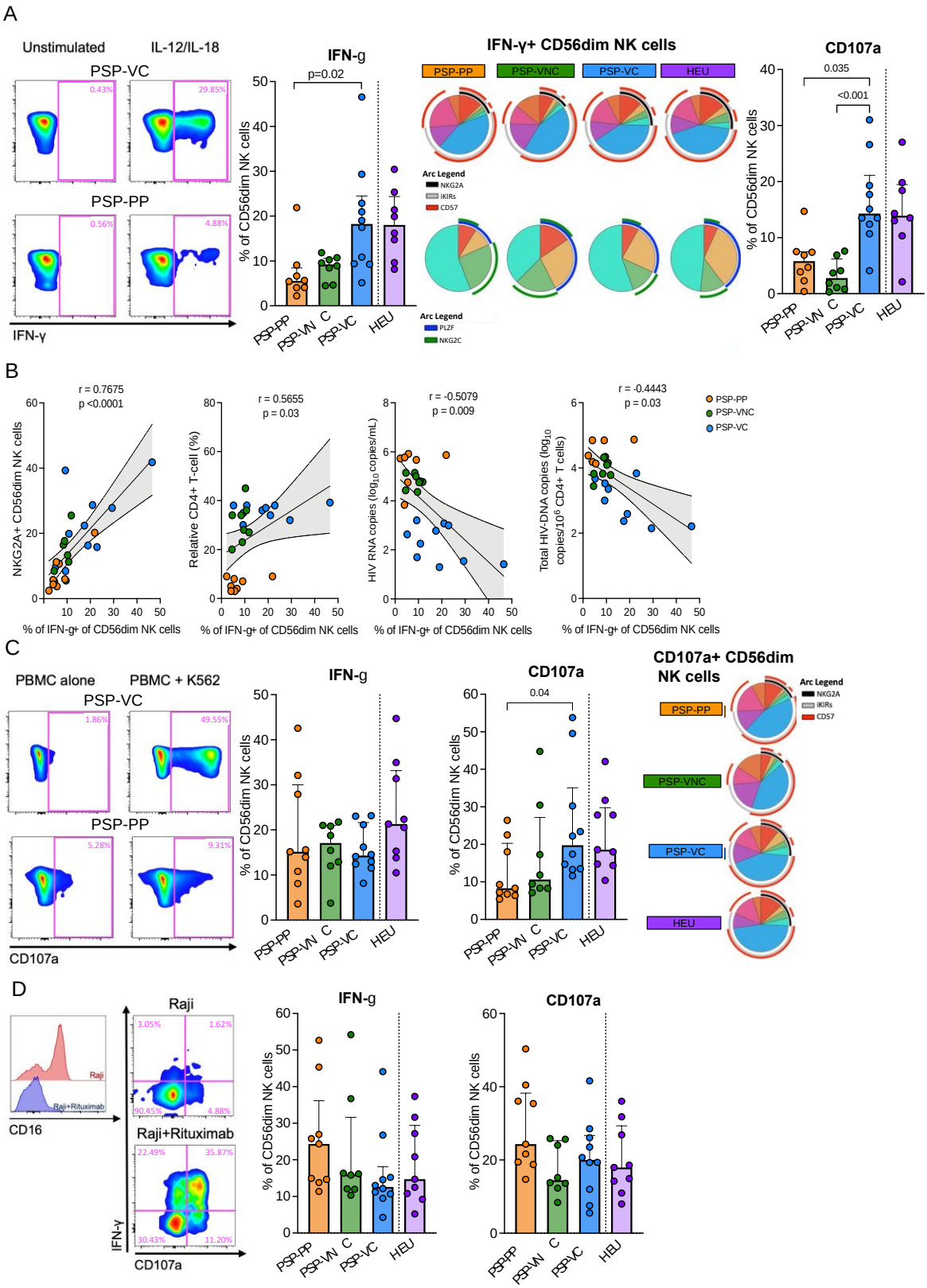

A
Unstimulated
IL-12/IL-18
PSP-VC
PSP-PP
IFN-γ
IFN-g
p=0.02
% of CD56dim NK cells
PSP-PP
PSP-VN
C
PSP-VC
HEU
IFN-γ+ CD56dim NK cells
PSP-PP
PSP-VNC
PSP-VC
HEU
Arc Legend
NKG2A
iKIRs
CD57
Arc Legend
PLZF
NKG2C
CD107a
0.035
<0.001
B
r = 0.7675
p <0.0001
NKG2A+ CD56dim NK cells
% of IFN-g+ of CD56dim NK cells
r = 0.5655
p = 0.03
Relative CD4+ T-cell (%)
r = -0.5079
p = 0.009
HIV RNA copies (log10 copies/mL)
r = -0.4443
p = 0.03
Total HIV-DNA copies (log10 copies/10^6 CD4+ T cells)
PSP-PP
PSP-VNC
PSP-VC
C
PBMC alone
PBMC + K562
PSP-VC
PSP-PP
CD107a
IFN-g
% of CD56dim NK cells
CD107a
0.04
CD107a+ CD56dim NK cells
Arc Legend
NKG2A
iKIRs
CD57
PSP-PP
PSP-VNC
PSP-VC
HEU
D
Raji
Raji+Rituximab
CD16
IFN-γ
CD107a
IFN-g
CD107a
% of CD56dim NK cells

Figure 3.14 Effector function of CD56^{dim} NK cells.
A. Summary of IL-12/IL-18 cytokine stimulation showing typical FACS plots and the frequency of IFN-γ+ of total CD56^{dim} NK cells. Pie charts performed by SPICE looking the combination of NKG2A, iKIRs, and CD57 (top), and NKG2C and PLZF (bottom) on IFN-γ+ CD56^{dim} NK cells. Frequency of cells expressing CD107a is shown on the top right. B. Correlation of IFN-γ+ upon IL-12/IL-18 stimulation with NKG2A+ CD56^{dim} NK cells, relative CD4+ T-cell, plasma HIV-RNA and total HIV-DNA loads. Spearman rank test are shown for correlations. Simple linear regression best-fit line and 95% CI are shown within the grey area. C. Summary of K562 cell line as target cells showing typical FACS plots and the frequency of CD107a+ and IFN-γ+ of total CD56^{dim} NK cells. Pie charts performed by SPICE looking the combination of NKG2A, iKIRs, and CD57 on CD107a+ CD56^{dim} NK cells. D. Summary of ADCC assay showing engagement of CD16 receptor, typical FACS plots and frequency of CD107a+ and IFN-γ+ of total CD56^{dim} NK cells. Statistical comparison between the three groups was based on Kruskal-Wallis test followed by Dunn's test for multiple comparisons.

3.4 Discussion

In this chapter, we evaluated HLA-I factors influencing HIV disease outcome in children. In an ART-naïve historical cohort of >300 children living with HIV from SSA, we showed that maintenance of CD4+ T-cell counts and also lower plasma viraemia were significantly associated with low-expressing *HLA-A* alleles and/or with expression of the Bw4 motif. However, the HLA-B molecules that in adults confer strong protection against progression, or that are associated with more rapid disease progression, did not significantly contribute to outcome in paediatric infection. This immunogenetic signature associated with better disease outcome in children favours a KIR-education program of the NK cell compartment. The immunophenotypic and functional analyses of NK cells indicated a less differentiated population with more plasticity characterised by high NKG2A/NKp46 expression on CD56^{dim} NK cells in viraemic controllers that correlated with low total HIV-DNA copies, while NK cells in progressors showed a terminally differentiated phenotype. Finally, the major functional feature distinguishing CD56^{dim} NK cells in slow progressor/low viraemic

children is their enhanced ability to produce cytokines and degranulate in response to IL-12/IL-18 stimulation as opposed to ADCC-mediated function.

These findings contrast with the predominant immunogenetic mechanisms of immune control of adult HIV infection. Although *HLA-A* and *HLA-C* expression levels, and HLA-Bw4 in association with KIR3DL1, have important influences on adult HIV disease outcome, these do not have as great impact as differences in HLA-B type. The dominant immunogenetic impact of HLA-B on adult HIV outcome is highlighted also by the data shown in the current study. This HLA-B effect is believed to be principally, but not solely, related to the broad, immunodominant Gag-specific CD8+ T-cell responses restricted by protective HLA-B molecules such as HLA-B*57/58:01/81:01 (Klein et al. 1995; Edwards et al. 2002; Zuñiga et al. 2006; Kiepiela et al. 2007; Streeck et al. 2007). Gag is an important target since it is both highly abundant and conserved in sequence. As a result, Gag escape mutants often inflict a cost on viral replicative capacity (Leslie et al. 2004; Kiepiela et al. 2007; Schneidewind et al. 2007). Other factors contributing to the dominant effect of HLA-B include the ability of HLA-B to bind LILRB2, and thereby influence the innate immune response (Bashirova et al. 2014), and the tapasin dependence of HLA molecules, which strongly influences epitope breadth (Bashirova et al. 2020).

These data support previous studies showing that differences in HLA-B do not significantly affect plasma viral load in children living with HIV and that even HIV-specific CD8+ T cell responses restricted by protective HLA-B do not successfully control viraemia in children (Adland et al. 2015). As in adults, Gag- and Pol-specific CD8+ T-cell responses in early life contribute somewhat to improved control of viraemia, but a median breadth of only one Gag-specific response over the first five years of life in children living with HIV (Leitman et al. 2017) explains the modest

impact on viraemia compared with broad Gag-specific responses generated within weeks of infection observed in adult controllers (Kiepiela et al. 2007). Several factors might contribute to the narrow response observed in children, notably the low levels of Th1-cell polarising cytokines produced by early-life innate immune cells (Kollmann et al. 2012; Prendergast, Klenerman, and Goulder 2012) and the vertical transmission of viruses carrying escape mutants pre-selected by the HLA-I molecules shared by the mother (Goulder et al. 2001; Thobakgale et al. 2007; Carlson et al. 2016).

NK responses in infants have been less well-studied than in adults but NK cell numbers and antiviral functions are reportedly similar in neonates and adults, and indeed non-cytolytic inhibition of CCR5-tropic HIV replication by neonatal NK cells through the production of MIP-1β is deemed to be superior (Yabuhara, Kawai, and Komiyama 1990; Jenkins, Mills, and Kohl 1993; Nakazawa, Agematsu, and Yabuhara 1997; Bernstein et al. 2004). Previous studies have demonstrated that HLA-Bw4 in combination with KIR3DL1 is associated with higher CD4+ T-cell counts in children living with HIV, and HLA-Bw4^{I80} in combination with KIR3DS1 with higher plasma viral loads (Singh et al. 2016). Preliminary data from an early-ART-treated cohort of infants in Botswana demonstrated an association between CD56^{dim} NK cells expressing NKp30 and low intact proviral reservoir size within three months of birth (Garcia-Broncano et al. 2019). Our own data showing a strong presence of Bw4 alleles and of low-expressing *HLA-A* alleles that create an immunogenetic signature favouring NK cell education via KIRs, along with the presence of a less terminally differentiated NK cell phenotype dominated by expression of NKG2A, are associated with better immune and viral control in children. Adding to these findings, the adult disease-susceptible effect of the Bw4 allele HLA-B*58:02 was only observed in children older than 10 years, reinforcing the lower impact of T-cell activity and the protective effect of the Bw4 motif in HIV disease progression in early life. By contrast the

disease-susceptible Bw6 alleles HLA-B*45 and HLA-B*18:01 had a marked detrimental impact during the first 10 years of life.

Although these findings are consistent with NK responses in children having a stronger impact on disease outcome than HIV-specific CTL responses, nonetheless there is some contribution of the HLA-B alleles that are disease-protective and those that are disease-susceptible to outcome in paediatric infection. In particular, HLA-B*81 is unusual if not unique among protective HLA-B alleles in expressing the Bw6 motif, and HLA-B*81 appears to have some independent protective effect in children. So, while the NK responses appear to have stronger effects in control of viraemia in early life, the HIV-specific CTL responses mediated by protective HLA-B alleles also make some contribution.

The fact that the immunogenetics underpin the correlations favouring a KIR-education program leading to better paediatric HIV disease outcome suggests that the NKG2A-expressing NK cell signature can be at least partially a consequence of the immunogenetics (Horowitz et al. 2016) and has a causal link with immune control of HIV in children, as opposed to being an effect of it. Indeed, individuals with the immunogenetics favouring lower HLA-E expression and a KIR-education program result in higher expression of NKG2A on the cell surface (Horowitz et al. 2016). Our findings are consistent with previous work showing that NKG2A-expressing NK cells are superior in cytotoxic and non-cytotoxic activities in HIV-infection (Lisovsky et al. 2015; Merino et al. 2013) and in preventing transmission of HIV (Merino et al. 2012). A NK signature expressing NKp46/NKp30 has been associated with better control of viral reservoir and IFN-γ secretion upon cytokine stimulation in HIV infection (Marras et al. 2017). The less mature phenotype observed in slow progressors and viraemic controllers favours an NK cell population with higher plasticity and

responsiveness to cytokines, while a more terminally mature phenotype and fixed population was observed in HIV disease progressors. Of note, the expression pattern of NKG2A and KIRs observed here in association with better HIV disease outcome in children can also be at a certain level uncoupled and parallel to the process of NK cell education outlined by the immunogenetics (Björkström et al. 2010). For example, the perturbed homeostasis and altered cytokine environment that may arise in progressors would preferentially drive the terminal differentiation of NK cells and/or immune exhaustion. Thus, whilst the immunophenotypic and functional NK cell pattern associated with immune control of HIV in paediatric infection is consistent with the immunogenetic signature favouring KIR education observed here, factors additional to the genetics can also contribute to disease outcome.

We observed a negative correlation between *HLA-A* expression on paediatric HIV outcome with the less mature NKG2A+NKp46+ $CD56^{dim}$ NK cells, but no effect of the polymorphism at residue -21 of the signal peptide of HLA-B molecules. -21M variants bind HLA-E and therefore individuals with HLA-B molecules that express the -21M variants could synergise with high-expressing *HLA-A* alleles in facilitating education via NKG2A, leading to more NK cell inhibition and less killing of the infected targets (Ramsuran et al. 2018). In adult studies this synergy was observed in -21M homozygous and the effect of heterozygous -21M/T is somewhat lower in HIV disease outcome (Ramsuran et al. 2018). Indeed, the effect of -21M on HLA-E surface expression is only evident in homozygous (Horowitz et al. 2016) and, due to our sample size, the number of children expressing -21M/M was low.

Strong linkage disequilibrium exists segregating HLA-I allotypes that support either a KIR- or a NKG2A-education (Horowitz et al. 2016). The vast majority of HLA-B Bw4 alleles are also -21T and are usually inherited with both HLA-C2 and -C1 alleles,

which provides KIR-binding with KIR2DL1 and KIR2DL2/L3, respectively. Indeed, we observed a higher proportion of inherited KIRs and their cognate HLA-I ligands in slow progressors/viraemic controllers than in the other groups. In contrast, -21M haplotypes are relatively frequent in HLA-B Bw6 alleles, which do not bind KIRs and are highly dominated by the HLA-C*07, an HLA-C1 allele with less strong KIR interaction and affected by microRNA-148a mediated downregulation (Kulkarni et al. 2011). Although we easily detected an enrichment of Bw4 alleles, we did not find a higher proportion of HLA-C2 and -21T-HLA-B in slow progressors/viraemic controllers. Interestingly, the HLA-C2 segregation with -21T and Bw4 alleles is not as clear in African populations and may help explain our findings (Horowitz et al. 2016).

The dichotomous effect of -21M/T is also less clear in CMV positive populations due to the effect of CMV infection in reshaping the NK cell compartment (Cubero et al. 2020). Our data show an expansion of a memory-like subset in paediatric progressors. Interestingly, albeit children were CMV positive, we did not observe any near correlation between IgG levels and the frequency of memory-like NK cells, as in adults (Peppa et al. 2018). Our findings might reflect a role of HIV viraemia/inflammatory environment to imprint the expansion of this phenotype independent of CMV. Memory-like NK cells are expanded in HIV-infected adults correlated with systemic immune activation (Gumá et al. 2006). However, it seems likely that CMV intermittently reactivates in association with AIDS-progression independently of IgG levels. This population has specific anti-HIV/SIV activity (Reeves et al. 2015) and was shown to be expanded in secondary lymphoid tissues in non-pathogenic SIV-infection (Huot et al. 2021). Although lower in frequency, we cannot exclude their potential activity in paediatric viraemic control outside the periphery.

There are limitations to our study. First, due to faster progression in children and the rarity of viraemic controllers, we do not have a large sample size to detect the possible effect of less frequent genotypes, such as -21M/M. However, we were able to detect a stronger association of KIR-HLA-I interaction with HIV disease progression and viral control than in adults. Due to the cross-sectional nature of functional aspects of this study, further concerns might be raised about the causality between the NKG2A+ phenotype and slow progression. However, our identification of inherited factors not modifiable by disease course and previously known to influence the distribution and function of the NK cell population supports our findings. We have not demonstrated through functional analyses a specifically effective anti-HIV response in this study. This was not possible owing to unavailability of adequate samples for these assays. It is important to underline that that these analyses used cryopreserved samples from a historical cohort because now WHO guidelines have recommended that all children living with HIV are treated with ART irrespective of CD4+ T-cell count.

Here, we have shown genetic and phenotype signatures associated with slower progression and viraemic control in children living with HIV after MTCT. The reduced role of the CTLs response in paediatric HIV disease progression and the full effector potential of NK cells in early life have important implications in cure strategies for children living with HIV. In an era of early suppressed children with low levels of the viral reservoir, we can identify potential immunogenetic signatures or adjuvant therapies that exploit the NK cell response to help achieve remission upon ART interruption, when you expected the child immune system would have to act to control viral reactivation.

4

Early PD-1 expression on CD8+ T-cells and the development of a stem-like memory phenotype are associated with HIV slow progression in children

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4.1 Background

PSPs are more prevalent than the adult counterpart AVNPs. Both PSPs and AVNPs maintain normal CD4+ T-cell count in the absence of ART by adopting a strategy similar to the natural SIV hosts (Paiardini et al. 2011; Klatt et al. 2014; Muenchhoff et al. 2016). The mechanisms leading to this slow clinical progression phenotype are not fully elucidated in humans. African monkeys control the inflammation and immune activation observed during acute SIV infection through immunoregulatory mechanisms including upregulation of the inhibitory receptor PD-1 on CD8+ T-cells located in LNs (Estes et al. 2008; Jacquelin et al. 2009).

The low level of immune activation despite ongoing viral replication is a key feature of the slow progression phenotype and appears to be more compatible with the tolerogenic and Th2-bias immune response observed in early life than with the aggressive Th1-driven immune response observed in adults (Muenchhoff, Prendergast, and Goulder 2014). A highly potent CTL response seen in adults can lead to better viral control but also is associated with higher levels of immune activation (Hunt et al. 2008; Hatano et al. 2013; Noel et al. 2015). In chronic infections, prolonged antigen exposure culminates in T-cell terminal exhaustion with impaired effector functions (Day et al. 2006; Gupta et al. 2015; McLane, Abdel-Hakeem, and Wherry 2019). Despite this, adult ECs maintain viral and immune control for decades by mounting an effective and polyfunctional HIV-specific CTL response characterised by its high proliferative capacity (Migueles et al. 2002) and populated with CM (López et al. 2011; Ndhlovu, Stampouloglou, et al. 2015) and stem-like Tex (Rutishauser et al. 2021) subsets that support a sustained and non-exhausted antiviral activity. PD-1+ CD8+ T-cells expressing the transcription factor TCF-1 have been recognized by their memory and proliferative potential as capable of generating effector cells in the

context of chronic antigen load in murine (Wu et al. 2016; Im et al. 2016; Chen et al. 2019; Siddiqui et al. 2019b) and human (Wieland et al. 2017; Rutishauser et al. 2021) studies. The ability to induce stem-like Tex capable of proliferating and sustaining the burst of cytotoxic effector cell activity required for immune control has been proposed as a target for T-cell based immunotherapies in HIV infection (Rutishauser et al. 2021).

Here, we sought to investigate early T-cell markers associated with slow progression in a group of early treated infants living with HIV after MTCT who underwent ATI after one year on ART. As observed in the NHPs, early PD-1 expression on CD8+ T-cells was the key predictor of slow HIV disease progression, leading us to explore further the phenotype and function of PD-1 expressing cells in the PSP population.

4.2. Chapter-specific population and methods

4.2.1. Study population and comparison groups

The PEHSS study was a feasibility study to investigate early ART initiation and ATI in infants living with HIV that was undertaken in 2002-2005, at a time before universal ART was implemented. Detailed protocol and study results have been previously published (Thobakgale et al. 2007; Prendergast et al. 2008). We selected participants randomised to immediate ART given for one year followed by ATI. ART was restarted after ATI according to the current guidelines prevailing at the time (National Department of Health South Africa 2004): clinical paediatric stage III or advanced stage II; or a CD4+ T-cell percentage ≤ 20% at <18 months of age or <15% at >18 months of age. To investigate T-cell markers associated with immunological progression, we investigated 13 infants who were suppressed and had

cryopreserved PBMCs available up to three months before ATI. The infants were further categorised into rapid progressors (RP) if they restarted ART before one year after ATI, intermediate progressors (IP) if therapy was restarted between one and four years after ATI, and slow progressors (SP) if ART restarted more than four years after ATI. Multiple timepoints from two subjects PS-021-C and PS-114-C were obtained for the additional longitudinal study.

In addition, we investigated the immunophenotype of PD-1 expressing CD8+ T-cells in older ART-naïve PSPs, PPs and HEUs and ART-naïve viraemic adults (VAs) with chronic infection and of SSA origin, as described in Chapter 2. In the immunophenotype study done after ART-initiation, the participants were suppressed for one to two years.

4.2.2. Cell sorting, library preparation and RNA-Sequencing

Cell sorting and RNA-sequencing library preparation were done in collaboration with Dr Henrik Kløverpris and Dr Alveera Singh from Africa Health Research Institute, SA. We selected three SPs and three RPs with cryopreserved PBMCs at the timepoint before ATI. After thawing and staining, one hundred PD-1^{high} CD8+ T-cells were sorted in triplicates using a FACSAria Fusion (BD) directly into RLT buffer (Qiagen) containing 1% β-mercaptoethanol and stored at -80 °C. RNA extraction, cDNA conversion and whole-transcriptome-amplification were done using Smart-seq2 as previously described (Kløverpris et al. 2016). Quality and concentration of the material amplified were confirmed with BioAnalyzer (Aligent Technologies) and Qubit assay kit (ThermoFisher Scientific). The process was followed by tagmentation and amplification of diluted samples and barcoded using Nextera XT DNA Library prep kit (Illumina). Sample was cleaned using AMPure XP SPRI beads (Beckman

Coulter) and sequenced on a HiSeq system using HiSeq SBS Kit v4 (Illumina) to an average depth of 10×10^6 reads.

The sequenced data were analysed by Nicholas Lim (University of Oxford, UK). Briefly, the sequences were demultiplexed using bcl2fastq snapshot 5 (mask_short_adapter_reads 22, minimum_trimmed_read_length 35) before alignment. FASTQs were aligned using the smartseq2 snapshot 8 workflows using "GRCh38_ens93filt" as the reference genome. Both protocols were adopted from the Cumulus workflow. The cell by gene matrix was subsequently loaded and analysed using DESeq2 (v 1.2.8.1) in R. Samples with fewer than 9 million total reads and genes not found in more than 3 samples, or greater than a sum of 5 counts were excluded from further analysis. Differential gene expression (DGE) was conducted using the Wald test. Gene set enrichment analysis was performed on genes pre-ranked based on the Wald statistic and analysed via GSEA version 4.0.3 using the curated Hallmark gene set. Significant gene sets were filtered based on p-adjusted value less than 0.05.

4.3. Results

4.3.1. Early PD-1 expression on CD8+ T-cells before ATI predicts disease progression

Thirteen infants living with HIV initiated on ART following HIV diagnosis at a median age of 27 days (IQR 22 – 42 days) and underwent ATI after 12 months on ART. All participants were suppressed, and CD4+ T-cell counts were within the normal range for age at ATI (Table 4.1). Viral rebound occurred within a median of 15 days (IQR 14.1 – 24.9 days) of treatment interruption in all participants (Figure

4.1A), but the time to reach the criteria to restart ART varied extensively, and participants were categorised in RP, IP and SP (Figure 4.1B and C).

To identify parameters that predict immunological progression during ATI, we analysed cryopreserved PBMCs at the timepoint immediately before ATI for the expression of activation and exhaustion markers on the T-cell compartment (Appendix I, Table 3 for antibody panel). HLA-DR expression on CM and TEMRA CD4+ T-cells negatively correlated with time to restart therapy, while, unexpectedly, PD-1 expression on all CD8+ T-cell subsets was positively correlated (Figure 4.2). In the LASSO model, where the duration of viral suppression during their first year, age at ART initiation, plasma viral load and CD4+ T-cell count were also included, PD-1 expression on EM CD8+ T-cells was the primary variable selected and associated with slow progression.

Higher PD-1 expression, especially on EM CD8+ T-cells, was associated with lower CCR5+ and HLA-DR+ on total and CM CD4+ T-cells before ATI, the two cardinal features previously identified in PSP (Figure 4.3) (Muenchhoff et al. 2016). High PD-1 expression on EM CD8+ T-cells was also associated with lower PD-1 expression on total CD4+ T-cells, with a higher CD4 and CD8 ratio and with higher absolute CD4+ T-cell count immediately after ATI and viral rebound.

Table 4.1 Clinical progression in infants after ATI.

			Before ATI			At viral rebound after ATI		
PID	Sex	Time off ART after ATI (years)	Viral load (copies/mL)	Absolute CD4+ T cell (cells/mm^3)	Relative CD4+ T-cell (%)	Viral load (copies/mL)	Absolute CD4+ T cell (cells/mm^3)	Relative CD4+ T-cell (%)
Rapid progressors (RP) - ART restart ≤ 1 year after ATI								
PS-344-C	Male	0.2	<50	896	21	7,040,000	575	13
PS-435-C	Female	0.1	<50	1919	27	596,000	972	18
PS-590-C	Male	0.3	<50	947	25	>750,000	1124	23
PS-675-C	Female	0.2	<50	2132	39	>750,000	2775	34
PS-732-C	Male	0.3	<50	1372	37	>750,000	1392	30
Intermediate progressors (IP) - ART restart between 1-4 years after ATI								
PS-001-C	Female	2.1	<50	2073	53	3,340,000	4039	26
PS-149-C	Female	1	<50	4450	45	19,140,000	991	29
PS-222-C	Female	1.1	<50	795	30	56,900	1075	31
PS-586-C	Male	2.9	<50	1948	35	>750,000	2949	32
Slow progressors (SP) - ART restart after 4 years of ATI								
PS-021-C	Female	10.1	<50	1926	42	673,000	2178	34
PS-114-C	Female	6.2	<50	2410	49	521,000	2129	39
PS-380-C	Male	6.4	<50	1622	36	1,354,000	1134	26
PS-559-C	Male	4.6	<50	2137	47	40,100	2277	50

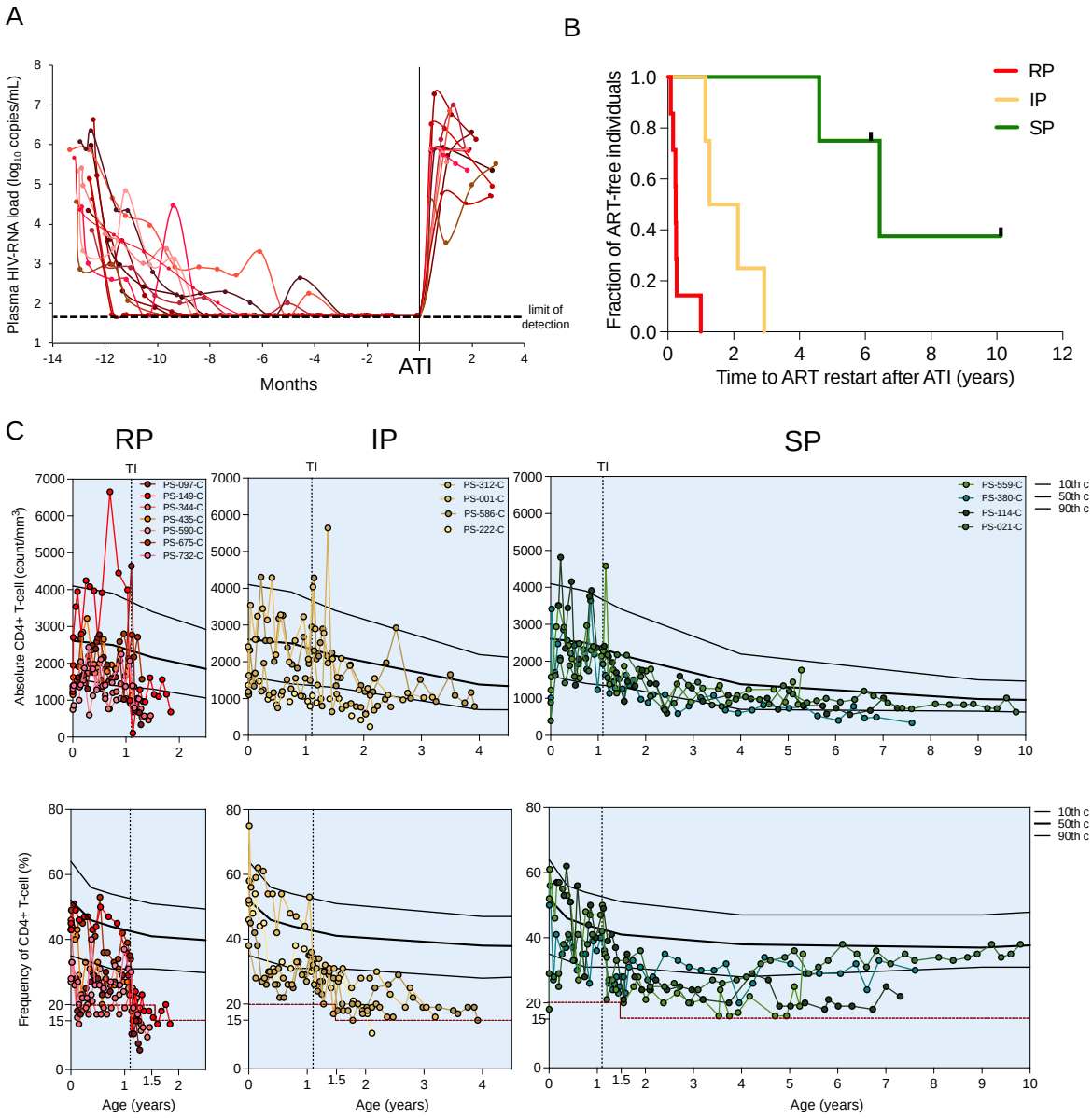
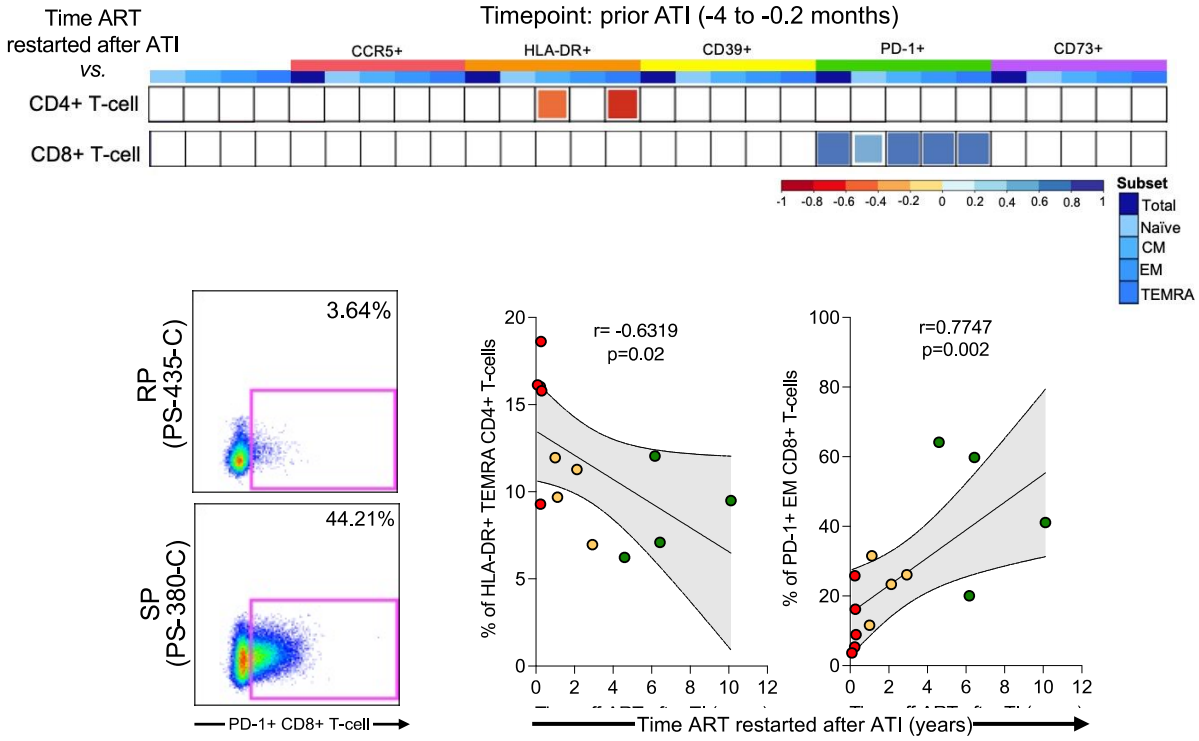


Figure 4.1 Longitudinal follow-up after ATI.

A. Longitudinal plasma HIV-RNA load for all participants who underwent ATI after 1 year on ART. The horizontal dashed line represents the limit of detection and the vertical line the time of ATI. B. Time to restart ART after ATI for each participant according to their group. C. Individual longitudinal absolute and relative CD4+ T-cell count in each group. The 10th, 50th and 90th percentiles for HIV-uninfected children are represented by the three black lines. Hashed red line represents the threshold to restart ART according to the guideline at the time ($\leq$ 20% if younger than 18 months or 15% if older than 18 months of age).



Time ART restarted after ATI (years)	LASSO β-coefficient
PD-1+ EM CD8+ T-cell	1.0899
HLA-DR+ TEMRA CD4+ T-cell	-0.5518

Figure 4.2 Early expression of PD-1 on CD8+ T-cell before ATI is associated with slow progression.

Markers significantly associated with time to restart ART after ATI are shown in the correlation matrix. FACS plot showing PD-1 expression on CD8+ T-cell in a RP and a SP. HLA-DR on TEMRA CD4+ T-cells and PD-1+ on EM CD8+ T-cells were selected by the LASSO model that best associated with time to restart ART after ATI. The best-fit line and 95% confidence bands are shown. Red, yellow and green dots represent RP, IP and SP, respectively.

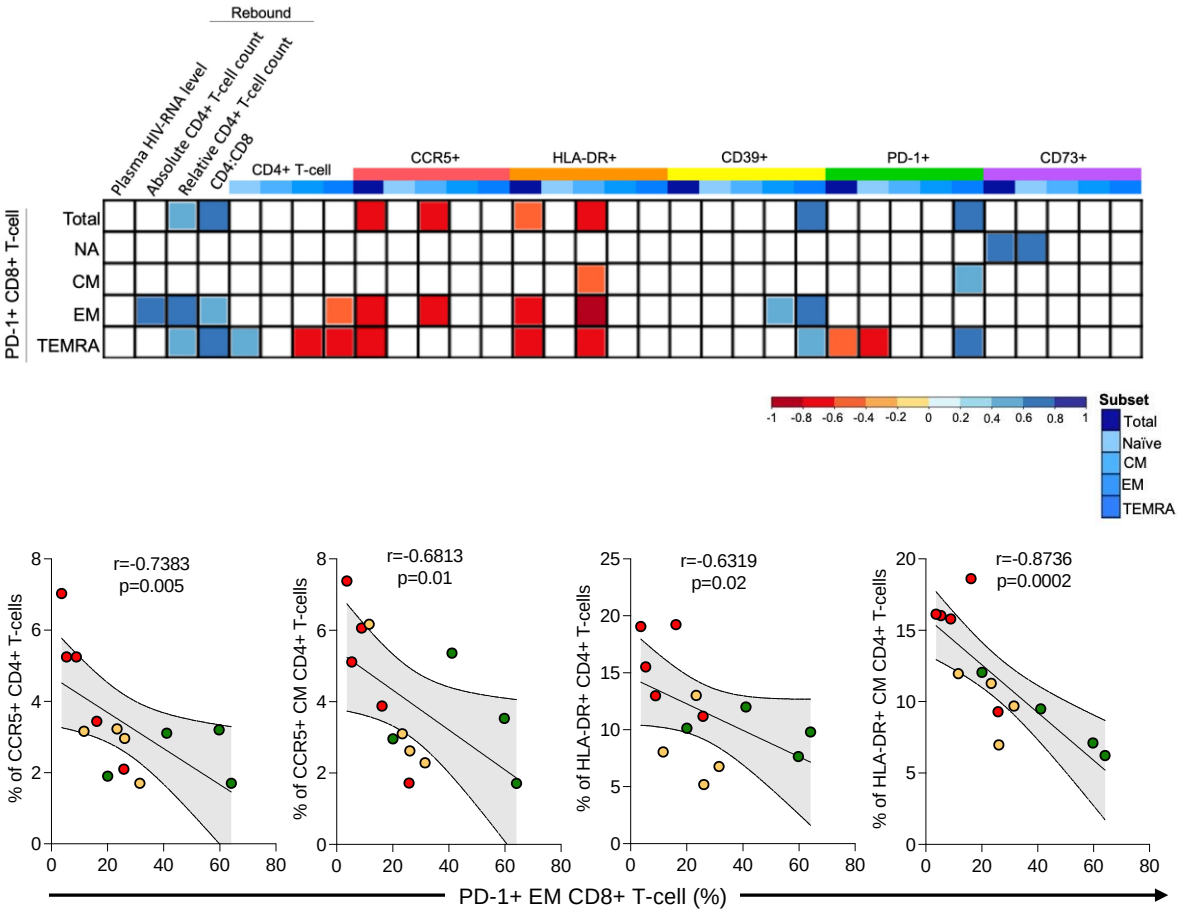


Figure 4.3 PD-1 on CD8+ T-cell before ATI is associated with immunological markers of slow progression.
Markers on CD4+ T-cells significantly associated with PD-1 expression on CD8+ T-cell subsets. Spearman rank tests were used for correlations. The best-fit line and 95% confidence bands are shown. Red, yellow and green dots represent RP, IP and SP, respectively

4.3.2. Early upregulation of PD-1+ on CD8+ T-cells predicts stronger HIV-specific response in infants after ATI

We hypothesized that the early upregulation of PD-1 would act as 'damage control' and avoid the hyperactivation and terminal exhaustion of the CD8+ T-cell compartment that is commonly seen with HIV disease progression, while preserving the effector potential of this population. In order to compare the genes expressed by PD-1+ CD8+ T-cells in RP versus SP, we undertook transcriptomic analysis of populations sorted by PD-1 expression on CD8+ T-cells in 3 RPs and 3 SPs (sample

availability limited the numbers that could be studied). Although the transcriptomic analysis in sorted PD-1+ CD8+ T-cells from the timepoint immediately before ATI showed that a large proportion of differentially expressed genes appeared to be largely driven by individual heterogeneity (Figure 4.4A), the RPs showed higher levels of the inhibitory receptor Tim-3 (*HAVCR2*), a marker of terminally exhausted PD-1+ CD8+ T-cells (Figure 4.4B), while SPs showed a clear upregulation of pathways directly related to antiviral function, cell proliferation and sustained effector activity (Figure 4.4C).

To investigate if PD-1 expression on CD8+ T-cells prior to the ATI predicted a stronger and preserved antiviral activity after viral rebound, we examined the HIV-specific responses via ELISPOT assays of samples three months after ART interruption. This timepoint was chosen because beyond this time the RP group had all met criteria to restart ART. Despite the small numbers, SPs tended to have higher magnitude and breadth of HIV-specific CD8+ T-cell response when compared to RPs (Figure 4.5A). Total magnitude of HIV-specific CD8+ T-cell responses (Gag, Pol, Env and Nef) 3 months post-ATI positively correlated with pre-ATI PD-1 expression on CD8+ T-cells ($p=0.03$) and negatively correlated with pre-ATI HLA-DR+ expression on CD4+ T-cells ($p=0.06$) (Figure 4.5B). Similar trends were observed for the Gag-specific response and total breadth. These data are consistent with the notion that, in these infants, PD-1 expression on EM CD8+ T-cells prior to ATI is associated with a less exhausted CD8+ T-cell compartment and predicts stronger antiviral response during ATI.

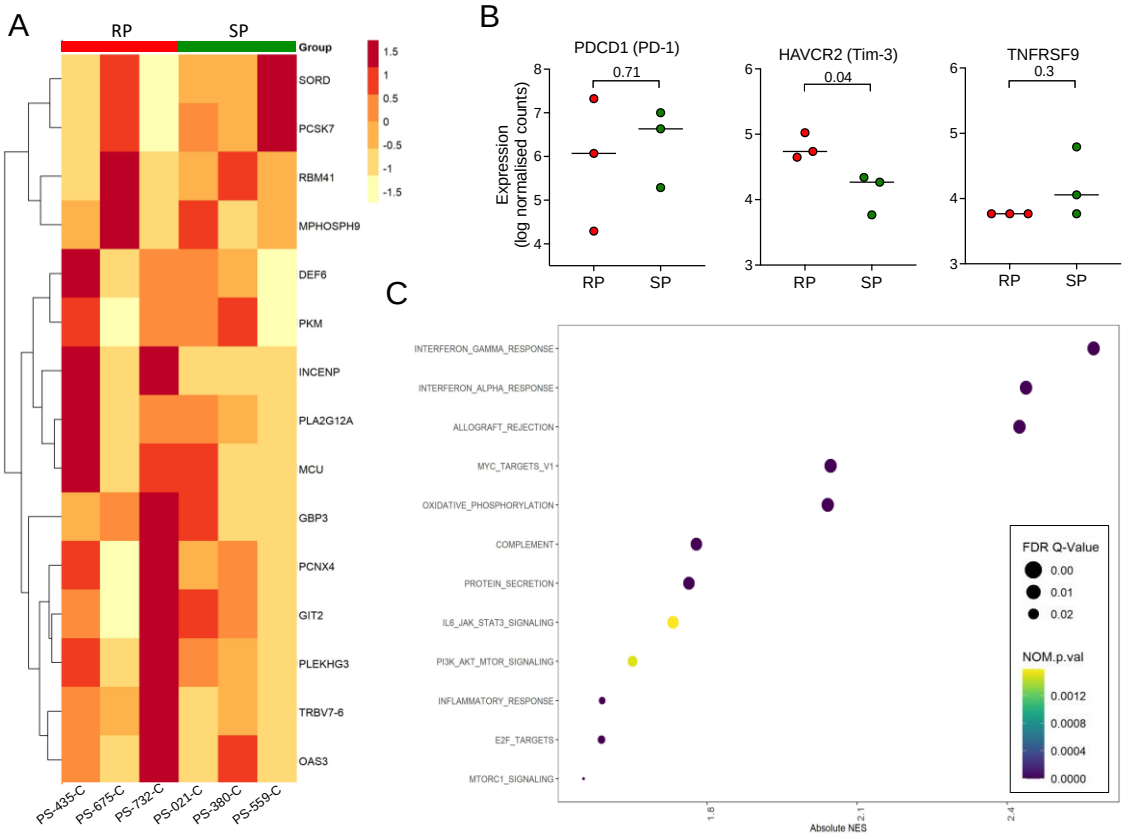


Figure 4.4 PD-1 expression on CD8+ T-cell is associated with lower Tim-3 (*HAVCR2*) gene expression and preserved effector function on SPs.

A. Heatmap is showing the DGEs in sorted PD-1+ CD8+ T-cell population of three SPs and RPs. B. Scaled log-normalized expression values of genes of interest. C. Pathway enrichment for genes significantly associated with SPs.

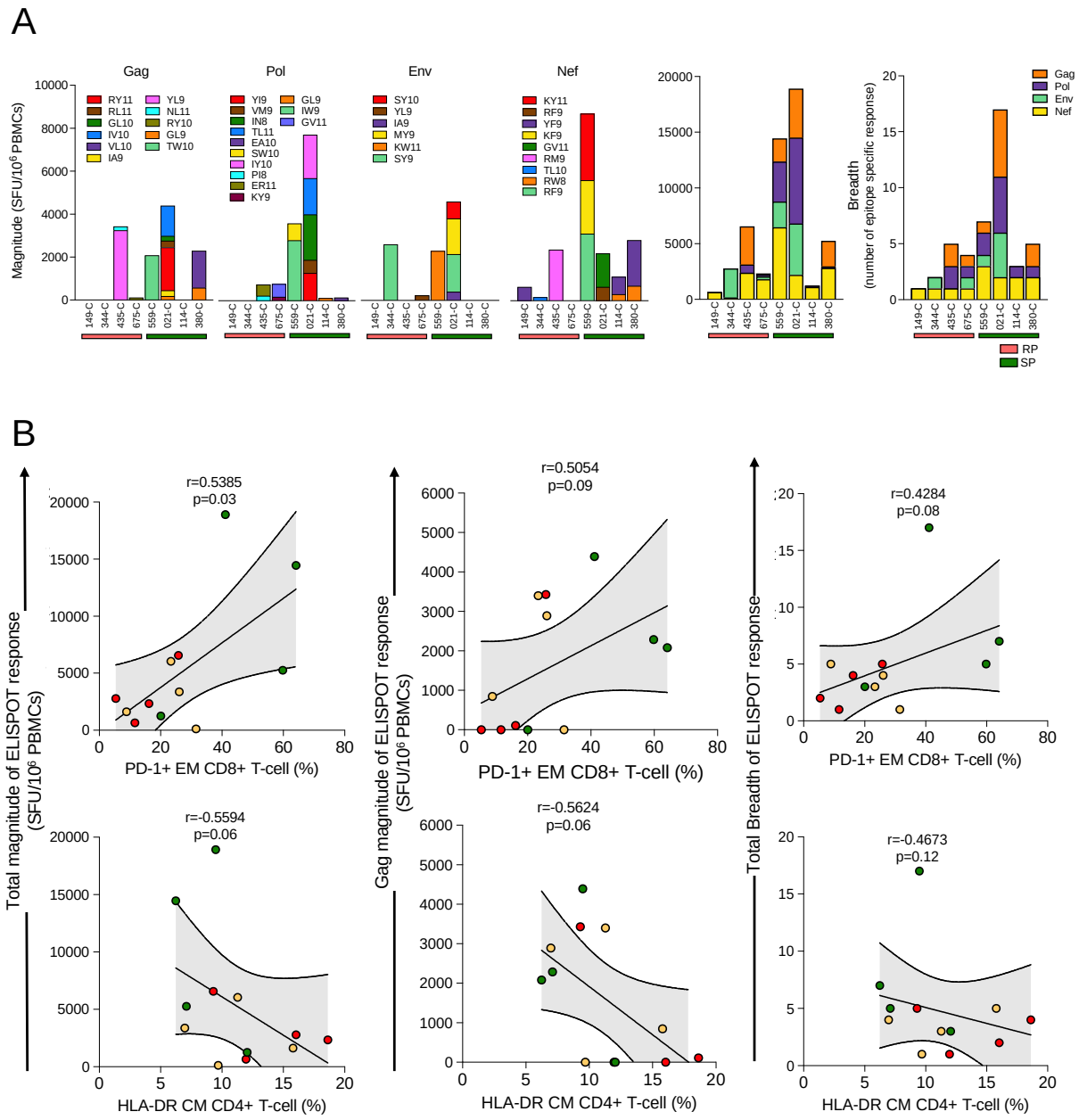


Figure 4.5 PD-1 expression on CD8+ T-cells is associated with stronger HIV-specific response.

A. Gag, Pol, Env, Nef and total magnitude and total breadth ELISPOT response in three RPs (red) and three SPs (green) three months after ATI. B. Total and Gag magnitude and total breadth of ELISPOT response correlated with PD-1+ EM CD8+ T-cell and HLA-DR+ CM CD4+ T-cell before ATI. Spearman rank tests were used for correlations. The best-fit line and 95% confidence bands are shown. Red, yellow and green dots represent RP, IP and SP, respectively.

4.3.3. CD8+ T-cells from PSPs have a stem-like PD-1+ phenotype

In adults living with HIV, PD-1 expression is typically associated with T-cell exhaustion, impaired effector function and is a signal of HIV disease progression (Day et al. 2006; Sauce et al. 2011). When we evaluated the expression of PD-1 on CD8+ T cells in a group of 182 ART-naïve children combining current data and data generated previously by Dr Emily Adland, PD-1 was only positively associated with immune activation on CD4+ T-cells in children older than five years, but not in the younger group (Figure 4.6), suggesting a different role of PD-1 or different populations of PD-1 expressing CD8+ T-cells in early life compared to later childhood and adults.

To investigate how PD-1 expression and associated markers on T-cells alter through childhood, we further studied two of the four SPs from the ATI study across 7-10 years of follow-up, neither of whom met the prevailing WHO criteria to restart ART, and who had similarly stable plasma HIV-RNA load (median 73,806 and 85,892 copies/mL) but contrasting CD4+ T-cell trajectories (Figure 4.7). Both of these SPs met the criteria for PSPs in that they maintained absolute CD4+ T-cell counts of >350 cells/mm^3 in the absence of ART at age of 5 years or above. In PS-021-C, whose relative CD4+ T-cell count increased from a nadir of 20% to a peak of 40% at 9.8yrs, PD-1 expression on CD8+ T-cells was maintained stable at a moderate level in association with high expression of CD73, a marker associated with memory maintenance (Bono et al. 2015; Flores-Santibáñez et al. 2015). By contrast, PS-114-C, who was close to the criteria for restarting ART throughout the follow up period, experienced an upregulation of PD-1 and HLA-DR and downregulation of CD73 on CD8+ T-cells.

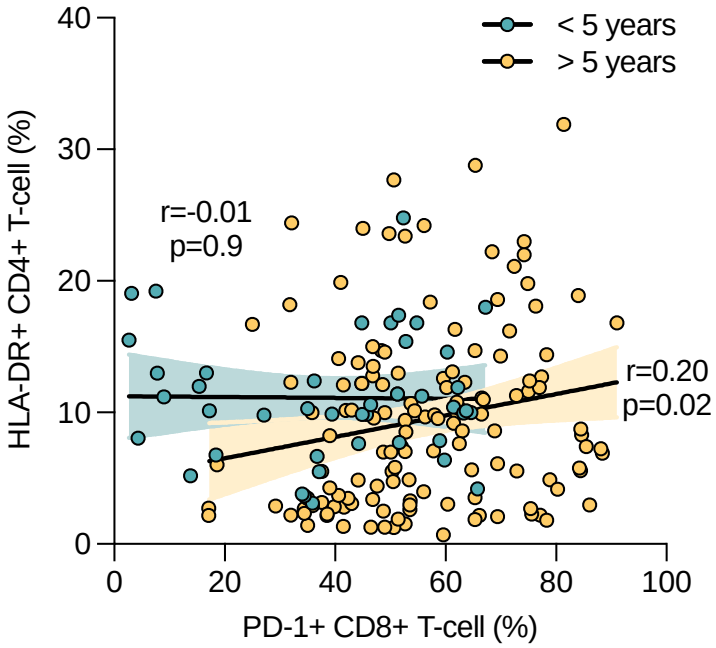


Figure 4.6 PD-1 expression on CD8+ T-cells and immune activation on CD4+ T-cells during childhood.

PD-1+ CD8+ T-cell correlation with immune activation (HLA-DR+) on CD4+ T-cell in children living with HIV before and after 5 years of age. Spearman rank tests were used for correlations. The best-fit line and 95% confidence bands are shown.

Next, we investigated the phenotype of PD-1 expressing CD8+ T-cells in a group of older ART-naïve PSPs (n=18, median 12.1 years, IQR 10.9 – 15.2) and PPs (n=18, median 13.4 years, IQR 11.9 – 16.8), matched by age and sex with a group of HEUs (n=16, median 13.8 years, IQR 8.3 – 16.9). A fourth group of horizontally infected VAs (n=18, median 29.0 years, IQR 26.0 – 35.5) was included for comparison (Table 4.2; Appendix I, Table 4 for antibody panel). As reported in previous studies, the frequency of PD-1+ CD8+ T-cells was higher in the HIV-infected groups (Figure 4.8A) and at greater levels in TM and EM subsets (Figure 4.8B). PD-1 expressing CD8+ T-cells were significantly lower in frequency in PSPs when compared with PPs (p=0.002) and VAs (p=0.05). The absolute levels of PD-1 expression measured by the median fluorescence intensity (MFI) were also higher in PPs and VAs when compared to HEUs (Figure 4.8C).

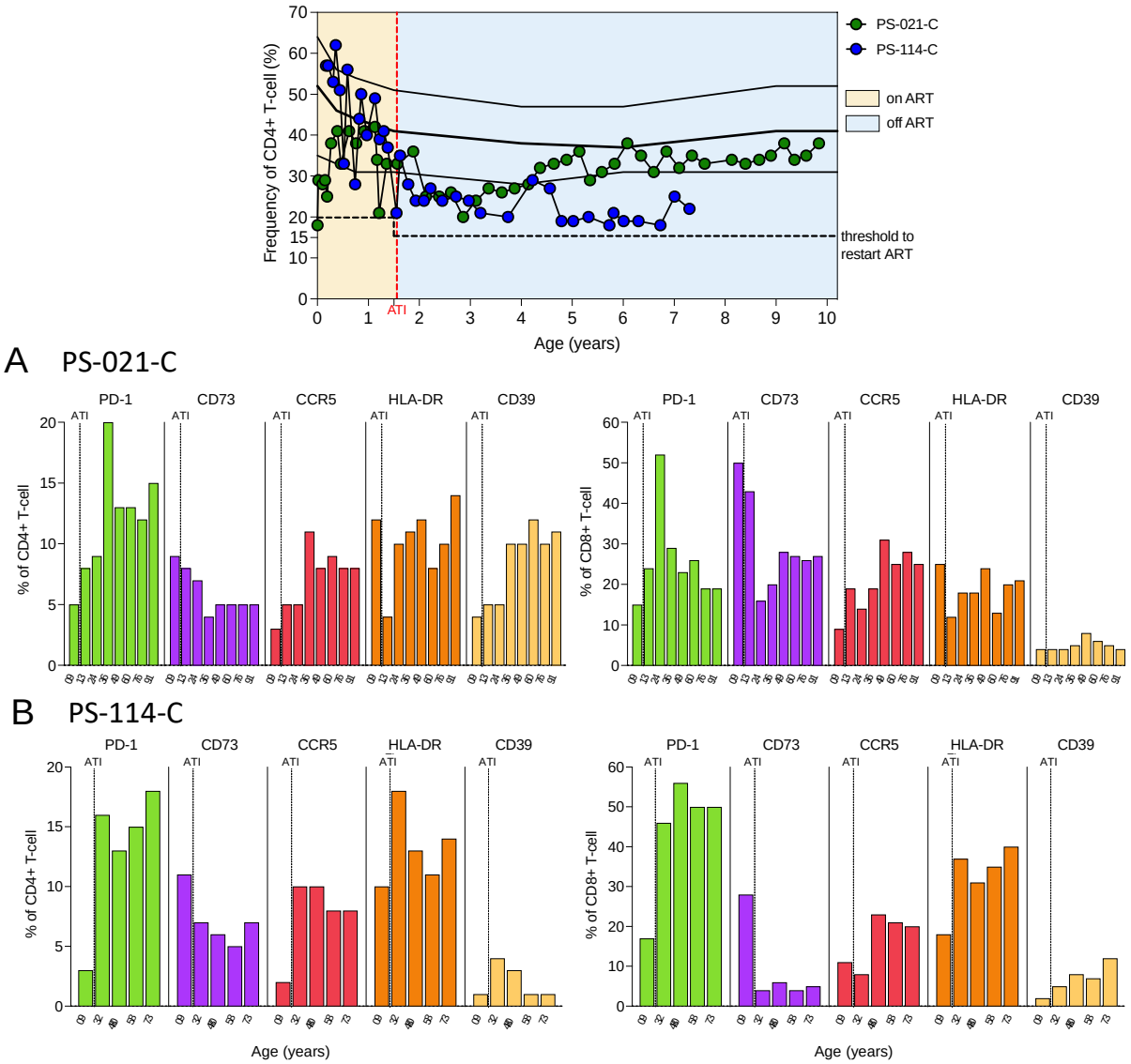


Figure 4.7 Longitudinal T-cell immunophenotype of two SPs.
PS-021-C (green dots) maintained CD4+ T-cells within the normal range while PS-114-C (blue dots) experienced a decline of CD4+ T-cells after ATI and near the threshold to re-start ART (black dotted line). The 10th, 50th and 90th percentiles for HIV-uninfected children are represented by the three black lines. The expression of each marker on CD4+ and CD8+ T-cell are shown before and after ATI for PS-021-C (A) and PS-114-C (B).

Table 4.2 Clinical data of the paediatric and the adult groups.

	HEU (n=16)	PSP (n=18)	PP (n=18)	VA (n=18)
Age (years)	13.8 [8.3 - 16.9]	12.1 [10.9 - 15.2]	13.35 [11.9 - 16.8]	29 [26 - 35.5]
Sex	9M:7F	8M:10F	9M:9F	9M:9F
Plasma HIV RNA (copies/mL)	-	18,000 [980 - 120,000]	165,000 [43,500 - 590,000]	24,883 [17,128 - 196,563]
Absolute CD4+ T-cell ($cell/mm^3$)	-	750 [665 - 917]	246 [102 - 306]	411 [388 - 515]
CD4+ T-cell percentage (%)	-	31 [25 - 34]	9 [3 - 16.5]	26 [19 - 37]
CD4:CD8 ratio	-	0.71 [0.44 - 1.10]	0.19 [0.10 - 0.36]	0.4 [0.27 - 0.73]

PD-1 is not necessarily a marker of terminally exhausted T-cells (McLane, Abdel-Hakeem, and Wherry 2019) but when co-expressed with CD39, an NTPDase classically upregulated in activated cells, PD-1 expression characterizes a CD8+ T-cell population with impaired effector function and increased expression of other inhibitory receptors (Gupta et al. 2015). Indeed, CD39+PD-1+ on CD8+ T-cells were increased in PPs (p=0.009) and VAs (p=0.009), but not on PSPs when compared to HEUs (Figure 4.8D). By contrast, when associated with TCF-1, PD-1 expressing T-cells are characterized by a stem-like phenotype with increased proliferative capacity (Chen et al. 2019). We found a higher frequency of TCF-1+PD-1+ on CD8+ T-cells in the paediatric groups with a p-value <0.0001 when VAs were compared with HEUs or with PSPs (Figure 4.8E). The small TCF-1+ population in VAs also showed the lowest MFI levels (Figure 4.8F), and a TCF-1^{high} population was only apparent in HEUs and PSPs (Figure 4.8G). Moreover, PD-1+TCF-1+ was highly associated with the expression of CD127, the IL7Rα required in the generation of long-term memory cells. Although TCF-1+PD-1+ CD8+ T-cells have been associated with the

expression of CXCR5 in murine models, we did not observe this correlation in the paediatric group (discussed further below).

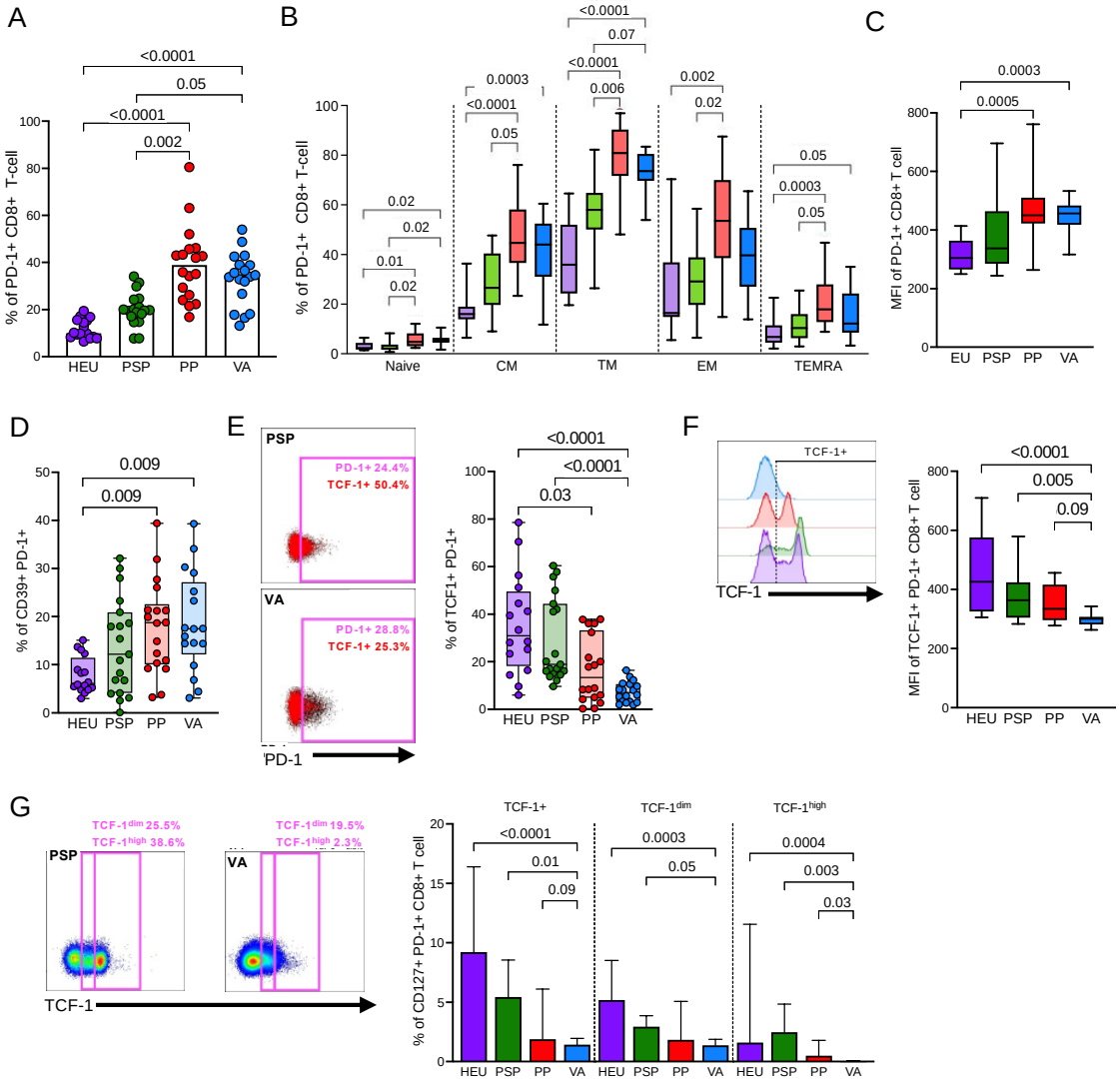


Figure 4.8 PD-1 expressing CD8+ T-cells are characterized by a stem-like TCF-1+ phenotype in PSPs.

A to C. Frequency of PD-1+ on total (A) and on memory (B) CD8+ T-cell and absolute levels of PD-1 (C) measured by the MFI among HEUs, PSPs, PPs and VAs. D. Frequency of CD39+ on PD-1+CD8+ T-cells as a marker of terminal exhaustion. E. Typical FACS plot showing PD-1+ population (pink gate) and TCF-1+ population (red dots) on CD8+ T-cells in PSPs and VAs. Frequency of TCF-1+PD-1+ CD8+ T-cell. F. Typical histogram and MFI levels of TCF-1+ in PD-1+ CD8+ T-cells. G. Typical FACS plot showing typical frequency of TCF-1+ CD8+ T-cell in PSPs and VAs. Frequency of CD127+PD-1+ CD8+ T-cell according to TCF-1 expression levels (TCF-1+, TCF-1^{dim} and TCF-1^{high}). Statistical comparison between four groups was done using Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons.

Consistent with these findings and with adult data (Trautmann et al. 2006; Day et al. 2006), the PD-1+ population shrank after ART initiation in the paediatric groups, mainly driven by the contraction of the short-lived terminally exhausted CD39+ subset, while the long-lived TCF-1+ population relatively expanded (Figure 4.9).

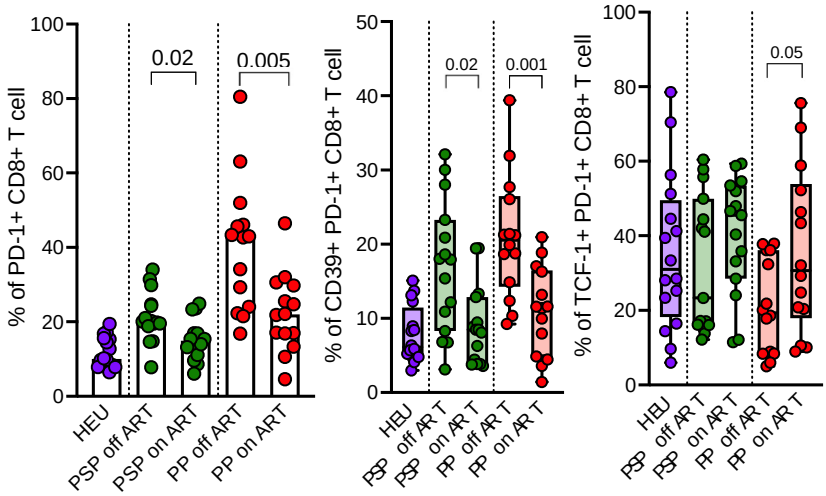


Figure 4.9 PD-1+ CD8+ T-cell before and after ART-initiation.
Pairwise comparison of different PD-1+ populations on CD8+ T-cells before and after 1-2 years suppression on ART. Wilcoxon matched-pairs test was performed for pairwise comparisons.

To determine whether the findings observed in bulk CD8+ T-cells were also present in HIV-specific CD8+ T-cells, we generated HLA-B*42:01- and HLA-B*81:01-restricted TL9-Gag-specific tetramers to characterize HIV-specific CD8+ T-cells in selected individuals in the PSP, PP and VA groups (Figure 4.10A; Appendix I, Table 5 for antibody panel). We observed similar frequencies of PD-1+ and T-bet+ on HIV-specific cells in the three groups. However, Granzyme B was significantly higher, and TCF-1 expression was lower in VAs than PSPs (p=0.009 and p=0.04, respectively) on HIV-specific CD8+ T-cells. There was also a tendency for higher levels of CD39+ on antigen-specific CD8+ T-cells in the VAs. As expected, the CD8+ T-cell compartment in VAs was markedly populated with more differentiated subsets than in PSPs and PPs (p=0.009 and p=0.03, respectively, Figure 4.10B). These findings

indicate an HIV-specific CTL population enriched with a stem-like phenotype in the PSP group.

To test if the constant TCR stimulation in HIV viraemic individuals affects the PD-1+TCF-1+ population, we stained the same individuals with tetramers to identify CMV-pp65 specific CD8+ T-cells. The frequency of PD-1+, CD39+ and TCF-1+ population was significantly lower in the CMV-specific CD8+ T-cells than in the HIV-specific CD8+ T-cells and no differences were observed among the three groups (Figure 4.10C), highlighting the importance of antigen load for the survival of this PD-1+ antigen-experienced CTLs.

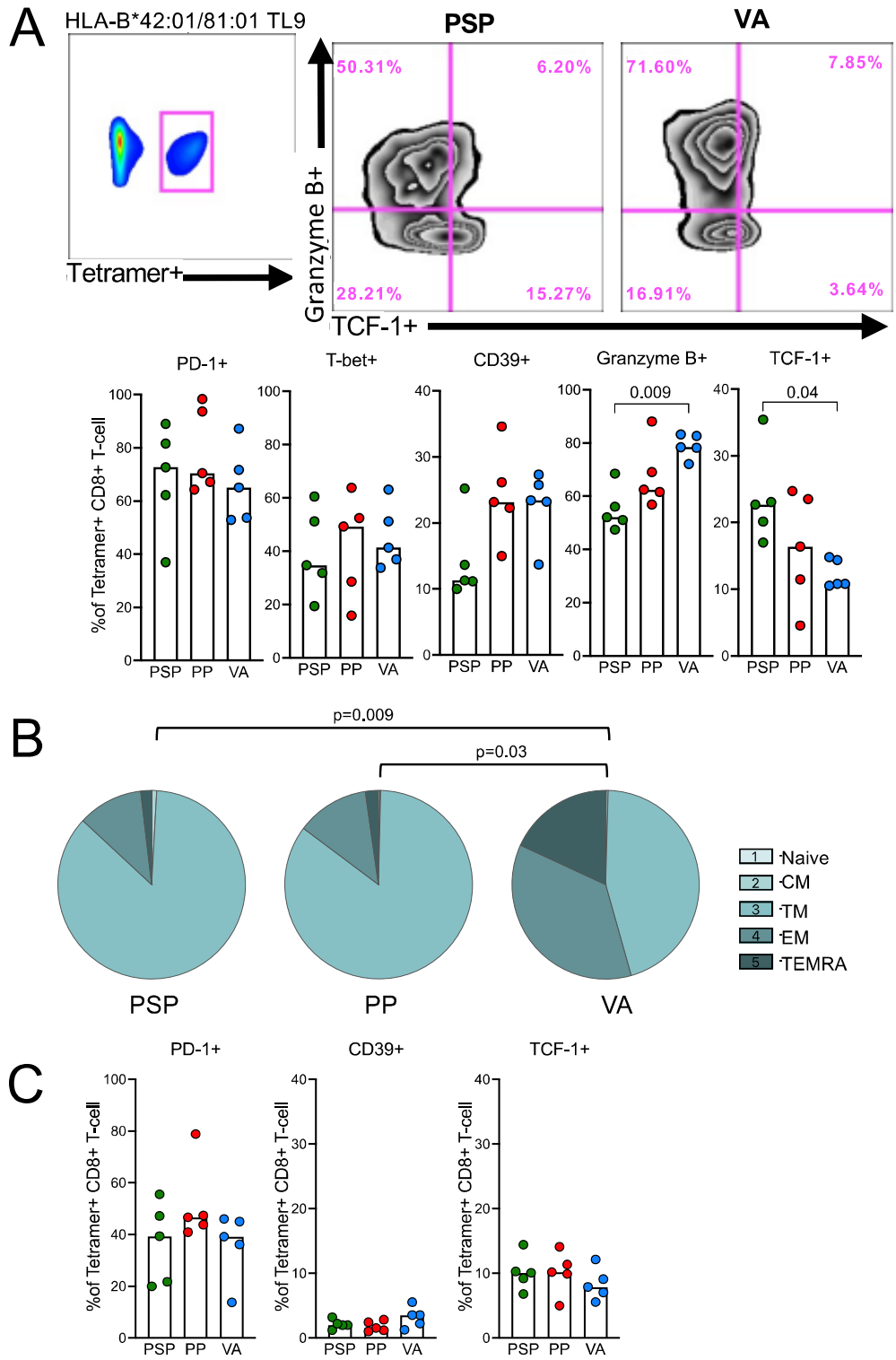


Figure 4.10 Immunophenotype of HIV-Gag and CMV-pp65+ CD8+ T-cells.
A. Typical FACS plot of Gag-specific CD8+ T-cell showing Granzyme B and TCF-1 gating in a PSP and VA. Frequency of PD-1+, T-bet+, CD39+, Granzyme B+ and TCF-1+ on Gag-TL9+ CD8+ T-cell for each group. B. Memory subset distribution on Gag-TL9+ CD8+ T-cell. C. Frequency of PD-1+, CD39+, and TCF-1+ on pp65-specific CD8+ T-cell for each group. Statistical comparison between three groups was done using Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons.

4.3.4. HIV-specific CD8+ T-cells have stronger proliferative capacity in PSPs

To investigate if the higher frequency of antiviral-specific TCF-1+PD-1+ CD8+ T-cells population in PSPs translates to higher proliferative capacity, CTV labelled PBMCs were stimulated for seven days with HIV-Gag or CMV-pp65 pool (Appendix I, Table 6 for antibody panel). Although not statistically significant, we observed a tendency to a higher frequency of proliferating CD8+ T-cells in PSPs than in PPs and VAs in PBMCs stimulated with Gag pool (Figure 4.11A). In the final 12 hours of incubation, we boosted the stimulation with the same Gag and pp65 pools to measure the ability of these proliferated CD8+ T-cells to secrete cytokines. Again, we observed a tendency to higher IFN-γ+ and TNF-α+ proliferated CD8+ T-cells in PSPs (Figure 4.11B and C). The frequency of TCF-1+PD-1+ CD8+ T-cells prior to stimulation positively correlated with the frequency of total proliferating CD8+ T-cells and with IFN-γ+ and TNF-α+ CD8+ T-cells (Figure 4.11D). As anticipated by the immunophenotype, when we looked at the population stimulated with the pp65-CMV pool, we observed an overall lower proliferation rate and IFN-γ+ and TNF-α+ CD8+ T-cells compared to HIV-Gag stimulated PBMCs, and again with no difference between the three groups (Figure 4.11E).

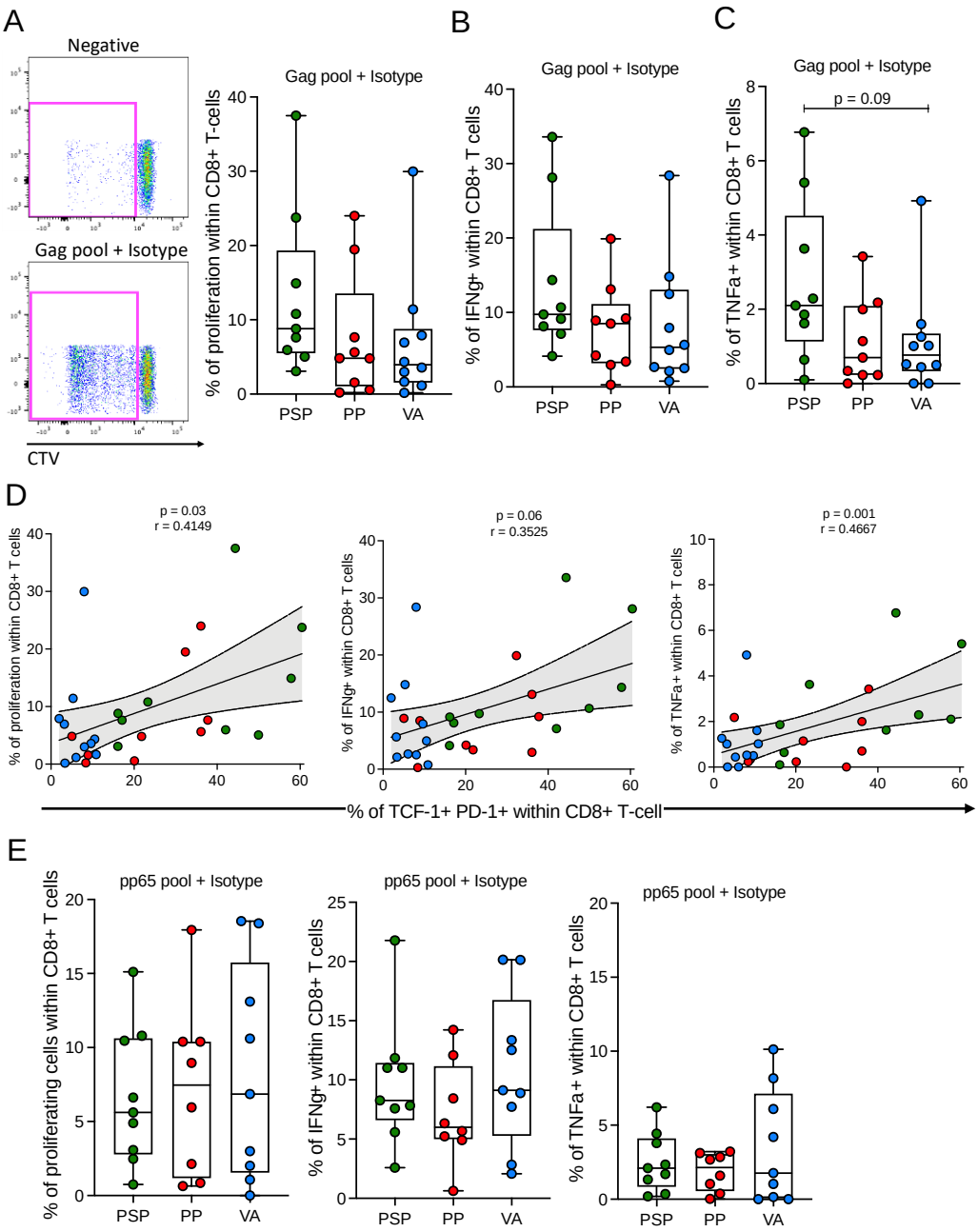


Figure 4.11 Stem-like PD-1+ CD8+ T-cell positively correlate with proliferated Gag-stimulated CD8+ T-cells.

A. Typical FACS plot of the proliferation rate of unstimulated and Gag-stimulated CTV-labelled CD8+ T-cells. Frequency of proliferated CD8+ T-cell after seven days of stimulation with Gag-pool. B and C. IFN-γ+ (B) and TNF-α+ (C) on proliferated CD8+ T-cells. D. Frequency of total, IFN-γ+ and TNF-α+ proliferated CD8+ T-cell correlated with TCF-1+PD-1+ CD8+ T-cell. E. Frequency of total, IFN-γ+ and TNF-α+ proliferated CD8+ T-cell after seven days of stimulation with pp65-pool. Statistical comparison between three groups was done using Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons. Spearman rank tests were used for correlations. The best-fit line and 95% confidence bands are shown. Green, red and blue dots represent PSPs, PPs and VAs, respectively.

4.3.5. Distinct effects of PD-1 blockade in children and adults

The boost on CTL effector functions after PD-1/PD-L1 blockade has been associated with the presence of the stem-like TCF-1+PD-1+ population identified here and its capacity to expand and differentiate into effector populations (Wu et al. 2016; Im et al. 2016; Siddiqui et al. 2019b). We stimulated CTV labelled PMBCs with Gag pool for seven days in the presence of anti-PD-1 or its isotype. As expected, PD-1 blockade increased the proliferation rate and consistently improved the CTL effector functions in adults (Figure 4.12). Unexpectedly and in contrast to adults, PD-1 blockade did not boost CTL function in children and leaned towards a decrease in the proliferation capacity of HIV-specific CD8+ T-cells, particularly in the PSP group ($p=0.06$, Figure 4.12A). The frequency of IFN-γ+ expression was also decreased after PD-1 blockade ($p=0.05$, Figure 4.12B), and a similar trend was observed for TNF-α (Figure 4.12C).

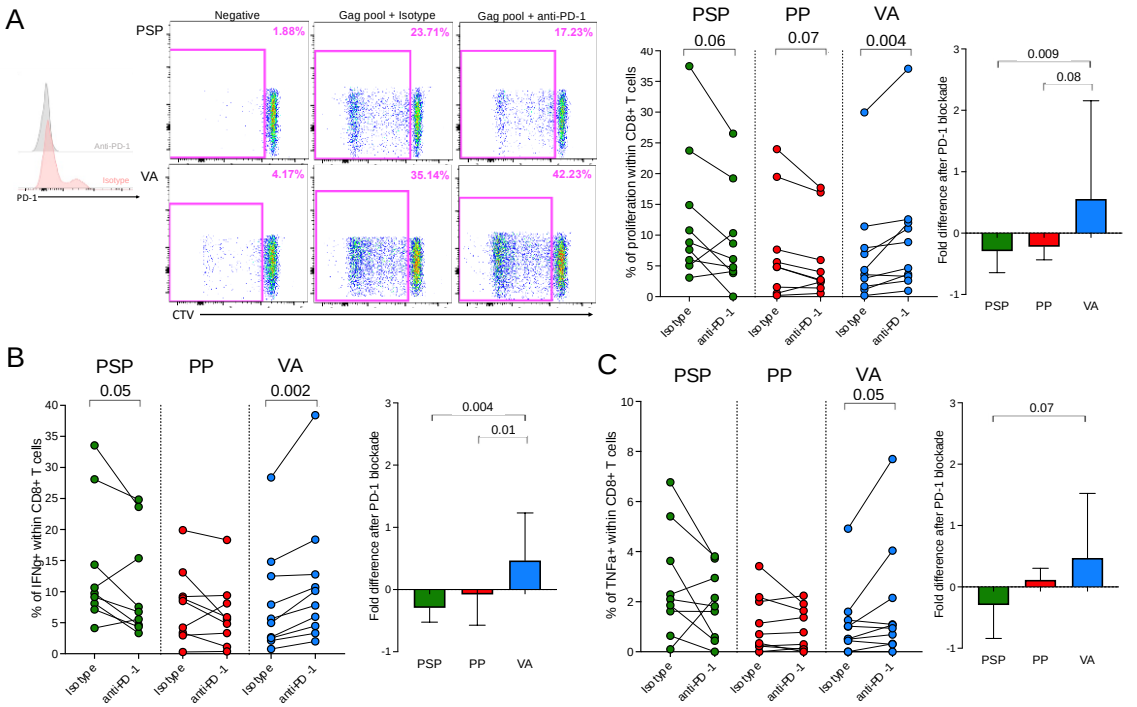


Figure 4.12 Distinct effects of PD-1 blockade in children and adults living with HIV.

A. Histogram is showing PD-1 blockade and typical proliferation graphs with and without anti-PD-1 antibody for a PSP and VA individual. Pairwise comparison of proliferation frequency of Gag-stimulated CD8+ T-cell between isotype and anti-PD-1. Fold-difference after PD-1 blockade for each group. B and C. IFN-γ+ (B) and TNF-α+ (C) frequency on CD8+ T-cell with and without anti-PD-1 antibody followed by fold-difference after PD-1 blockade. Wilcoxon matched-pairs test was performed for pairwise comparisons. Statistical comparison between three groups was done using Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons.

4.4. Discussion

To increase the numbers of PTCs, it is important to identify mechanisms that promote potent and sustained antiviral responses and facilitate immediate viral control after ART cessation. Within a group of early-treated infants that underwent ATI after one year on ART, we show here that early expression of PD-1 on the CD8+ T-cells strongly predicts slower HIV disease progression during subsequent ATI and appears to drive the cardinal features previously observed in ART-naïve paediatric long-term non-progressors. This PD-1+ CD8+ T-cell population, representing a non-

terminally exhausted T-cell phenotype with preserved effector potential, predicts a stronger HIV-specific CTL response during ATI. When we investigated the PD-1+ CD8+ T-cells in ART-naïve adolescent PSPs, we identified an enrichment of the TCF-1+ population with a higher proliferative capacity capable of generating effector functions upon stimulation. In contrast, ART-naïve progressors and viraemic adults were enriched with a more terminally exhausted subset.

PSPs and the natural hosts of SIV appear to share the same fundamental immunological adaptations that contribute to maintaining normal CD4+ T-cell counts despite ongoing viral replication, namely including low immune activation and low surface expression of CCR5 on the long-lived CD4+ T-cell populations (Paiardini et al. 2011; Klatt et al. 2014; Muenchhoff et al. 2016). The mechanism leading to this phenotype is not elucidated in humans. Still, in experimental studies in NHPs, both pathogenic (Rhesus monkeys) and non-pathogenic (AGM and SM) models upregulate genes associated with immune activation in CD4+ T-cells during SIV acute infection (Jacquelin et al. 2009). However, this is followed by a natural downregulation of these genes in non-pathogenic SIV infection, leading to the tolerogenic state of low immune activation and viral replication in the chronic phase. Rapid upregulation of PD-1 on CD8+ T-cells located in the LNs has been temporally associated with resolution of immune activation in SM and proposed as an important mechanism to control overactivation of the cytotoxic response and avoid tissue damage. In the pathogenic model, this early upregulation of PD-1 is not seen, and PD-1 is upregulated during the progression phase (Estes et al. 2008). Similarly, in the ART-treated infants who underwent subsequent ATI described here, PD-1 expression on CD8+ T-cells predicted slower HIV disease progression and negatively correlated with immune activation and CCR5 expression on the CD4+ T-cells. PD-1 was only positively associated with higher immune activation in older ART-naïve children. Interestingly,

in a recent anecdotal case of PTC in a vertically infected child, the PD-1 level, but not HLA-DR and CCR5, was elevated on CD4+ and CD8+ T-cells (Violari et al. 2019).

The PD-1+ CD8+ T-cells before in the SP group evaluated prior to ATI did not express the classical terminally exhausted phenotype at the transcriptional level but demonstrated lower Tim-3 gene expression and the upregulation of pathways associated with sustained effector functions when compared to RPs. PD-1 expression on CD8+ T-cell pre-ATI also positively correlated with the magnitude of virus-specific CD8+ T-cell responses three months later post-ATI. However, in the first 2 years of life, these HIV-specific CD8+ T-cell responses have much more modest antiviral effect compared to those generated in adulthood (Thobakgale et al. 2007; Leitman et al. 2017), for reasons related to the more tolerogenic and Th2 polarised immune response in young children (Kollmann et al. 2009; Kollmann et al. 2012; Prendergast, Klenerman, and Goulder 2012). Nonetheless, the observed phenotype contributes to avoid hyperstimulation of the CD8+ T-cell compartment and to preserve both CD4+ T-cell numbers and function, as well as antigen-specific CD8+ T-cells that are not terminally exhausted and ready to be explored as the child matures in age.

The HIV-specific CD8+ T-cell population expressing PD-1 and TCF-1 represents a promising target for T-cell therapies because of their long-term survival and their ability to expand. Better viraemia control in adult ECs is associated with both stronger proliferative capacity of HIV-specific CTLs (Migueles et al. 2002; Rutishauser et al. 2021) and with the presence of long-lived memory populations (Ndhlovu, Stampouloglou, et al. 2015; López et al. 2011), including the TCF-1+ stem-like Tex subset (Rutishauser et al. 2021). Reinforcing their recall ability in chronic

infections, TCF-1+PD-1+ CD8+ T-cells led to the expansion of virus-specific CTLs upon stimulation even after months of antigen cessation in patients with hepatitis C virus infection (Wieland et al. 2017). However, expanded TCF-1+ cells need to downregulate TCF-1 to allow themselves further differentiation into effector CTLs. Although we did not sort TCF-1+ cells to observe its downregulation in proliferated cells, we observe a positive correlation between the proportion of TCF-1+ CD8+ T-cell prior stimulation and the frequency of antiviral function by the IFN-γ expression in proliferated Gag-specific CTL after seven days of stimulation.

In addition to the acquisition of effector functions, TCF-1+ CTLs also need to migrate to lymphoid tissues to target the viral reservoir. Interestingly, in mouse models, virus-specific stem-like TCF-1+PD-1+ CD8+ T-cells have been associated with CXCR5 expression (Im et al. 2016). Although our data and a previous study did not find such correlation in peripheral blood in humans, PD-1+CXCR5+ CD8+ T-cell frequency in peripheral blood was recently associated with better effector function and higher CD4+ T-cell counts in adult HIV-infection (Jiao et al. 2017).

The window of opportunity to start ART immediately after birth following intrauterine infection, thereby quickly suppressing antigen load, allows the seeding of the viral reservoir to be blocked rapidly, protecting the T-cell compartment from chronic antigen stimulation (Garcia-Broncano et al. 2019). In adults, immune activation and exhaustion can only be partially reverted with ART even when started during primary infection (Hurst et al. 2015). By contrast, the T-cells in early-treated infants can be preserved by long-term suppression up to the time children can generate a more potent Th1 polarised effector response. The presence of a preserved T-cell compartment that is not terminally exhausted can be explored in immunotherapeutic approaches that generate and boost HIV-specific responses in

older children. Although rare, children controlling viral replication exist, as described in Chapter 5. PECs only achieve viraemic control at an older stage by maintaining a very low level of immune activation and exhaustion while mounting a Gag-specific response that is highly polyfunctional, usually in the absence of the well-known protective HLA-B alleles.

PD-1 expression imposes an inhibition in downstream TCR-stimulation, and *in vitro* and *in vivo* blockade of its pathway improves cytotoxic function of CD8+ T-cells, including in HIV-infection (Trautmann et al. 2006; Day et al. 2006; Petrovas et al. 2006). PD-1 blockade is the front-runner in the immunotherapy revolution in medicine, and it is used now with several levels of success in the cancer field (Wolchok et al. 2017). Moreover, in murine models, the boost of the effector function is directly associated with the presence of the stem-like PD-1+ CD8+ T-cell population, while the terminally exhausted PD-1+ CTLs do not respond well to blockade (Im et al. 2016; Wu et al. 2016). In recent human trials, the clinical response to the anti-PD-1 immunotherapy has found similar results (Sade-Feldman et al. 2018; Miller et al. 2019b). Albeit we observed the same positive effect of PD-1 blockade in the VAs, it was not seen in children and had a surprisingly opposite effect in the paediatric groups. The mechanism behind these findings is not yet clear and requires subsequent studies. It might be associated with a more immune regulatory function of PD-1 in children. Nonetheless, in a recent study, *in vitro* PD-1 blockade reduced the effector function of CXCR5+PD-1+ CD8+ T-cell in HIV-infected adults, while the expected effect was observed in the CXCR5-PD-1+ subset (Jiao et al. 2017). PD-1 blockade has not been widely explored in the paediatric HIV field but failed to improve the function of CD4+ T-cells in one study (Foldi et al. 2017).

The study presented in this Chapter has some limitations. Sample availability from this historical cohort prevented transcriptomic evaluations in more than three individuals in each group. Also, in this analysis, PD-1^{high} cells were sorted, which decreased our capacity to capture differences between the SP and RP groups, since PD-1 is expressed at a moderate level in the stem-like CD8+ T-cell subset. Considerations of sample availability from the historical PEHSS cohort also prevented further analyses of the function and phenotype of the PD-1+ population that arise early in infection and after ATI. Epigenetics plays an essential role in modulating *PD-1* gene expression and can be a critical factor in identifying mechanisms leading to early PD-1 upregulation and HIV slow progression. Finally, it would be optimal to explore the tissue-resident cells and their role in HIV pathogenesis instead of extrapolating findings obtained with peripheral blood cells, which we were not able to do in this study. In particular, studies of the CXCR5+ population of TCF-1+ CD8+ T-cell in murine models support the need to evaluate human lymphoid tissue to assess the immense potential of stem-like CD8+ T-cells in future T-cell therapeutic strategies for HIV cure/remission.

The findings here make a novel contribution to the mechanisms leading to HIV slow progression in children and add to the previously described its similarities with the mechanisms underlying SIV non-progression in the African monkeys. Moreover, the phenotype of stem-like CD8+ T-cell is associated with a population of HIV-specific CTLs with high proliferative capacity that can be exploited in future adjuvant therapies in the pursuit of HIV remission. The data presented here also strengthen the notion that, owing to the immune differences that exist in early life compared to adulthood, remission may be more easily achievable in the children. In future work, it will be important to identify therapeutic candidates that can harness the high proliferative capacity and long-term survival of PD-1+TCF-1+ virus-specific CTLs

while at the same time exploiting their cytotoxic potential in individuals living with HIV.

5

Paediatric elite controllers are strongly biased towards the female sex and marked by polyfunctional HIV-specific T-cell response

Contents

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5.1 Background

In the paediatric population, immune control is usually not followed by control of HIV viral replication (Blanche et al. 1997; Paul et al. 2005; Mphatswe et al. 2007; Muenchhoff et al. 2016) and PSPs are not enriched with the protective HLA-I alleles (HLA-B*27, *57, *58:01 and *81:01) that in adults are associated with lower viral setpoint and elite control (Pereyra et al. 2008; Pereyra et al. 2010; Leslie et al. 2010; Carlson et al. 2012) . On the other hand, viraemic non-progressors are rare in adults (Rotger et al. 2011; Klatt et al. 2014), and immune control is usually in association with plasma viraemic control.

Definitions of ECs have varied but one that reached acceptance is: three or more consecutive plasma HIV-RNA load below 50 copies/mL spanning at least one year in an ART-naïve individual (Okulicz et al. 2009; Olson et al. 2014; Yang et al. 2017). PECs have scarcely been described in the literature (Ananworanich et al. 2004; Tang et al. 2010), while the estimated frequency of ECs in adult infection range between 0.18 and 0.56% (Grabar et al. 2009; Okulicz and Lambotte 2011; Olson et al. 2014; Yang et al. 2017). The immune features responsible for viraemic control remain incompletely understood, although contributing factors include the spectrum of HLA-I and KIR alleles expressed, as well as specificity and functionality of the HIV-specific CD8+ T-cell response (Migueles et al. 2002; Betts et al. 2006; Fellay et al. 2007; Pereyra et al. 2008; Sáez-Cirión et al. 2009; Pereyra et al. 2010; Mothe et al. 2012).

Here, we first describe a group of 12 ART-naive children living with HIV after MTCT who fulfil the criteria of EC. Next, we investigated the activation and exhaustion marker expression on T-cell memory subsets, and the HIV-specific T-cell response in four PECs compared to PSPs, PPs and HEUs adolescents.

5.2. Chapter-specific population and methods

5.2.1. Study population and comparison groups

To identify PECs, we investigated cohorts of children living with HIV in SA, the country with the largest number of paediatric HIV infections, Brazil and Thailand, also countries with substantial number of infections, and smaller cohorts of children being followed in Europe, which nonetheless are largely constituted of children immigrated from SSA countries. PEC was defined as an ART-naive child living with HIV after MTCT with three or more consecutive plasma HIV-RNA load over a year or more, that were below the limit of detection (Okulicz et al. 2009; Olson et al. 2014; Yang et al. 2017). The limit of detection varied according to the centre and the historical period when the assays were done and ranged between less than 150 and less than 20 copies/mL. 'Blips' higher than 1000 copies/mL were not allowed. Medical records were reviewed for clinical data, plasma viral loads and CD4+ and CD8+ T-cell counts.

The study involved nine out-patient clinics caring for children living with HIV around the world: Kimberley Hospital (Kimberley, SA), Ithembalabantu Clinic (Durban, SA), the Family Clinical Research Unit in Tygerberg Academic Hospital (Cape Town, SA), Instituto Emílio Ribas (Sao Paulo, Brazil), Universidade Federal de Minas Gerais (Belo Horizonte, Brazil), Great Ormond Street Hospital (London, UK), St Mary's Hospital (London, UK), Sant Joan de Déu Children's Hospital (Barcelona, Spain), and The HIV Netherlands Australia Thailand Research Collaboration, Thai Red Cross AIDS Research Centre (Bangkok, Thailand).

To investigate the T-cell compartment and HIV-specific response, cryopreserved PBMCs were obtained from four PECs during elite control. For comparison, we matched by age a group of PSPs (n=13), PPs (n=10), and HEUs (n=9) from SA. No data were available on plasma viraemia and drug suppression of HEU's mothers. PPs and PSPs were defined as described in Chapter 2. All participants were diagnosed before the age of 10 years and also, in every case where the maternal HIV status could be ascertained, were born to a mother with HIV-infection, consistent with vertical transmission.

5.3. Results

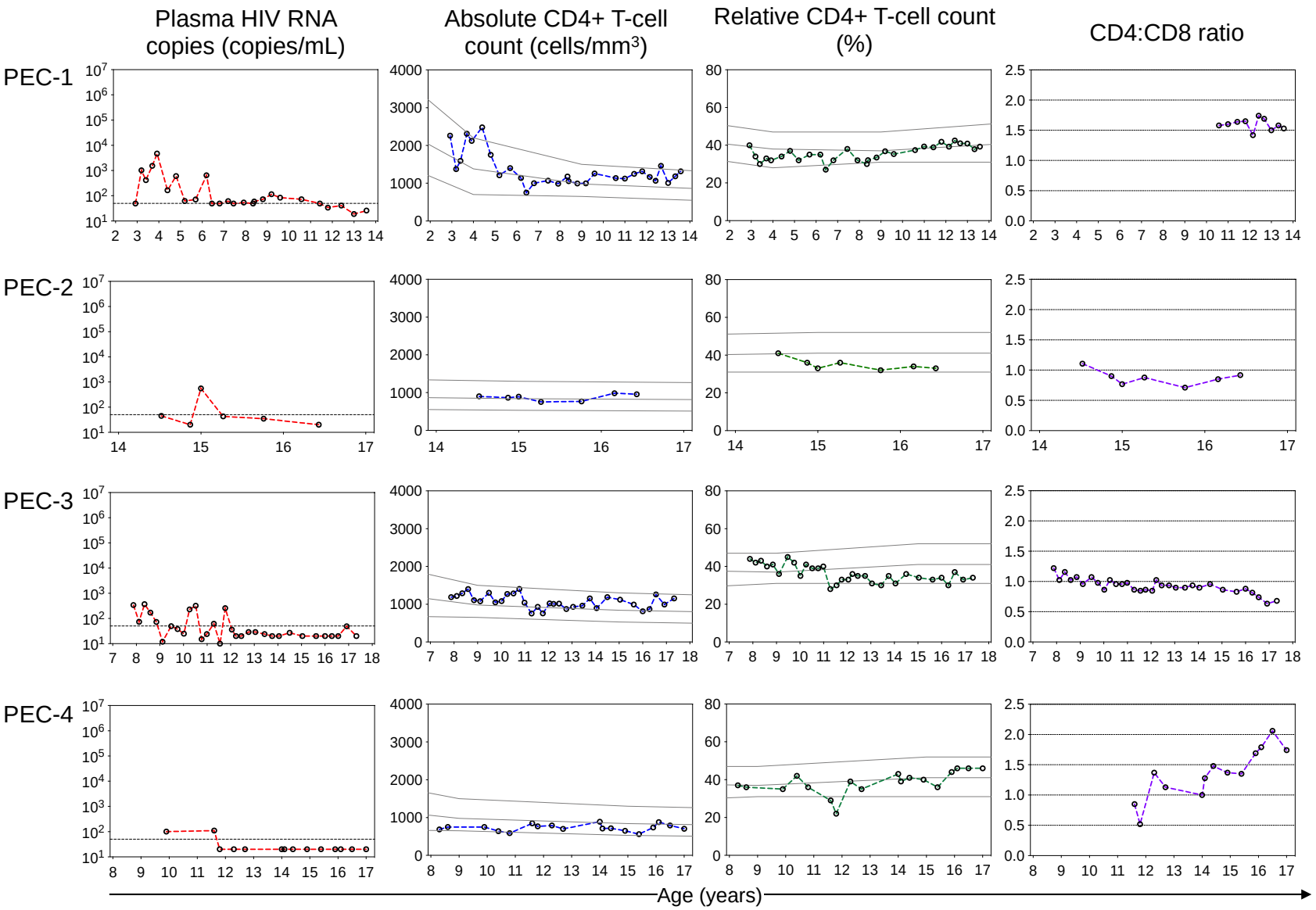
5.3.1. PECs are strongly biased towards the female sex and viraemic control is short lived

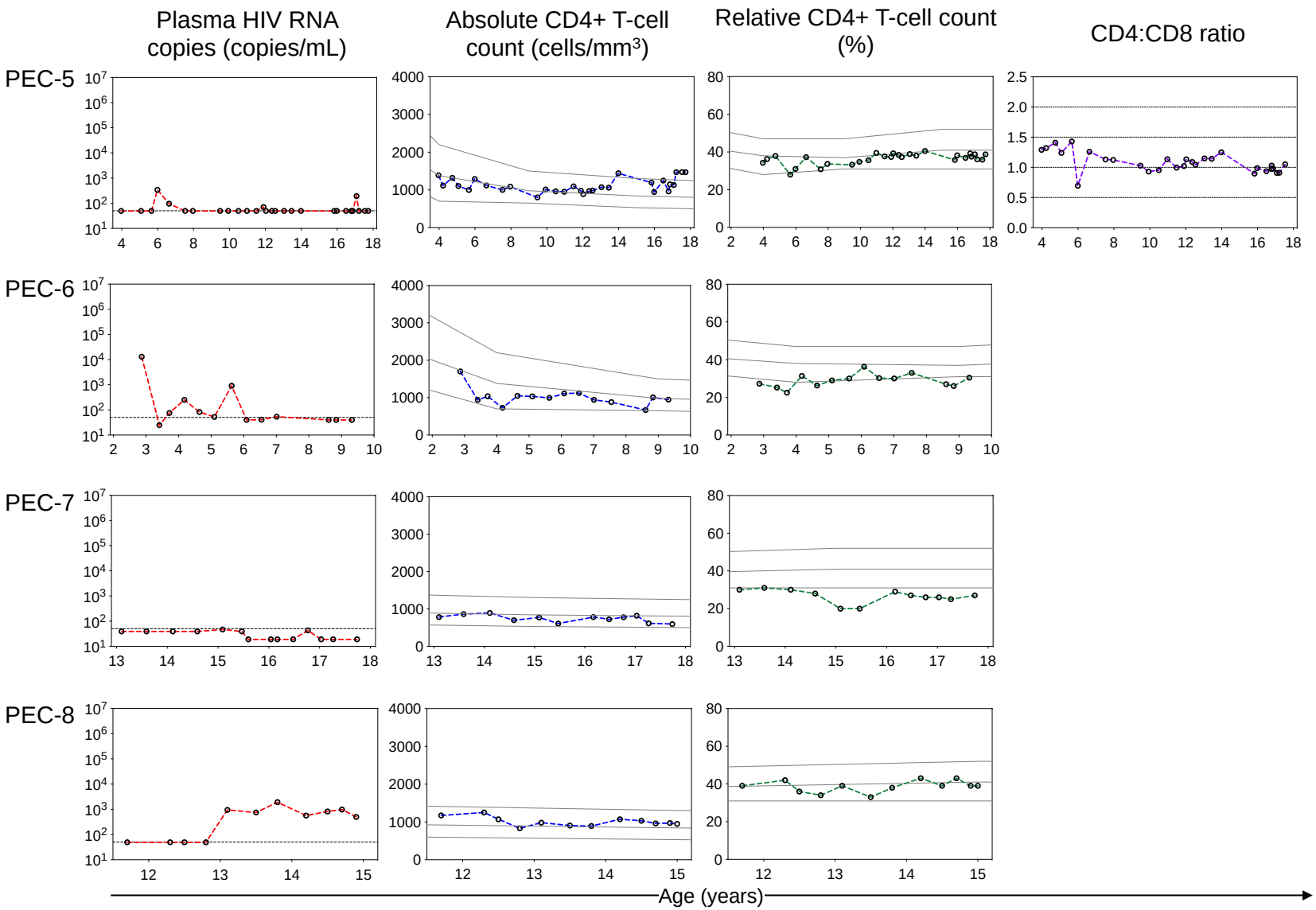
Twelve PECs were identified according to the criteria described above (Table 5.1 and Figure 5.1). The group included nine (75%) patients born in African countries, two (17%) in Latin America, and one (8%) in Asia. Eleven (92%) were females. The median age at enrolment was 6.9 years (IQR 2.9–11.1).

Table 5.1 Characteristics of the 12 PECs and their cohorts.

PID	Country of origin	Sex	Clinic of origin	Number in cohort	Year of birth	Age at enrolment (years)
PEC-1	Ghana	Female	London, UK[1]	400	2004	2.9
PEC-2	Nigeria	Female	Barcelona, Spain[1]	100	2003	14.5
PEC-3	Ethiopia	Female	Barcelona, Spain[1]	100	2002	7.9
PEC-4	SA	Female	Durban, SA[1]	200	2002	8.3
PEC-5	Brazil	Female	Belo Horizonte, Brazil[1]	644	1993	4.0
PEC-6	SA	Male	Kimberley, SA	2501	2007	2.9
PEC-7	SA	Female	Stellenbosch. SA	9038	1999	13.8
PEC-8	Uganda	Female	London, UK[1]	278	2001	12.0
PEC-9	Zimbabwe	Female	London, UK[1]	278	2000	7.1
PEC-10	SA	Female	Stellenbosch. SA	9038	2010	2.9
PEC-11	Brazil	Female	São Paulo, Brazil[1]	500	1999	2.0
PEC-12	Thailand	Female	Bangkok, Thailand[1]	382	1996	6.7

[1] Local clinics – the PECs number and cohort size cannot represent the paediatric population living with HIV in that region.


Plasma HIV RNA copies (copies/mL)
Absolute CD4+ T-cell count (cells/mm3)
Relative CD4+ T-cell count (%)
CD4:CD8 ratio
PEC-1
PEC-2
PEC-3
PEC-4
Age (years)


Plasma HIV RNA copies (copies/mL)
Absolute CD4+ T-cell count (cells/mm3)
Relative CD4+ T-cell count (%)
CD4:CD8 ratio
PEC-5
PEC-6
PEC-7
PEC-8
Age (years)

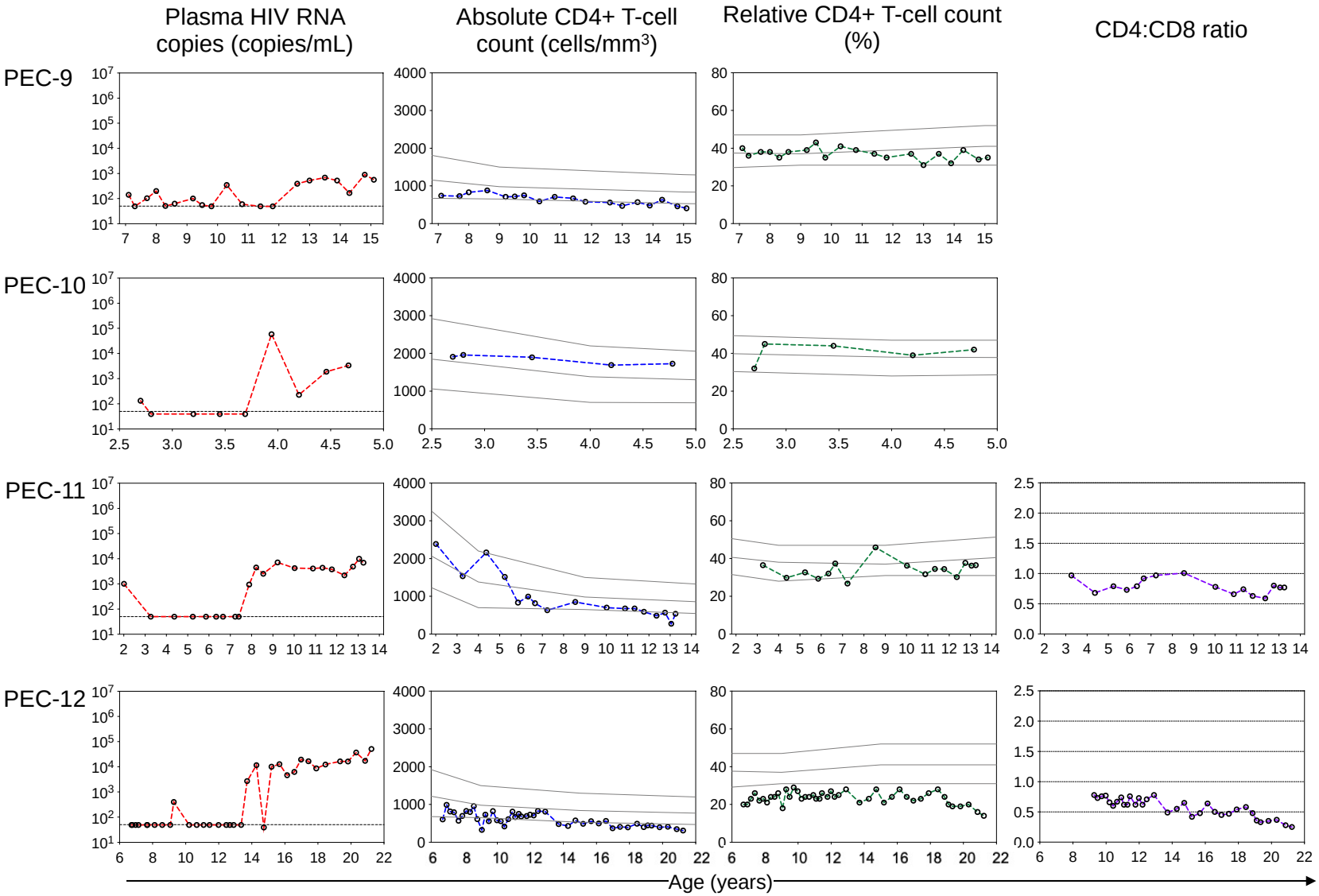

Plasma HIV RNA copies (copies/mL)
Absolute CD4+ T-cell count (cells/mm3)
Relative CD4+ T-cell count (%)
CD4:CD8 ratio
PEC-9
PEC-10
PEC-11
PEC-12
Age (years)

Figure 5.1 Longitudinal clinical follow-up.
Plasma HIV-RNA load, absolute and relative CD4+ T-cell count and CD4:CD8 ratio are shown for the 12 PECs. The horizontal dashed line for plasma HIV-RNA copies represents the limit of 50 copies/mL. The 10th, 50th and 90th percentiles for absolute and relative CD4+ T-cell count for HIV-uninfected children are represented by the three grey lines.

The median absolute and relative CD4+ T-cell count in the 12 patients at enrolment were 1170 cells/mm^3 (IQR 726–1808) and 34% (IQR 31–38), respectively. All patients had an absolute and relative CD4+ T-cell count within the normal range for age during the period of elite control apart from PEC-12. Five were viraemic when enrolled but became aviraemic subsequently, whereas the other seven participants were already aviraemic when clinical follow-up started. The median age at which viraemic control was first achieved among the five initially viraemic was 5.2 years (IQR 3.3–8.6). For only three participants (PEC-5, PEC-10 and PEC-11) we could ascertain that viraemic control was clearly achieved before 5 years of age.

Loss of elite control was observed in five of the 12 children (42%) during follow-up. The viral rebound arose at a median age of 12.6 years (IQR 7.9–13.1). In a linear mixed model analysis, absolute CD4+ T-cell count was significantly higher in PECs who maintained control of viraemia than those who subsequently lost it (Figure 5.2, $p=0.03$). Relative CD4+ T-cell count was also higher among those maintaining viraemic control, although this did not reach statistical significance.

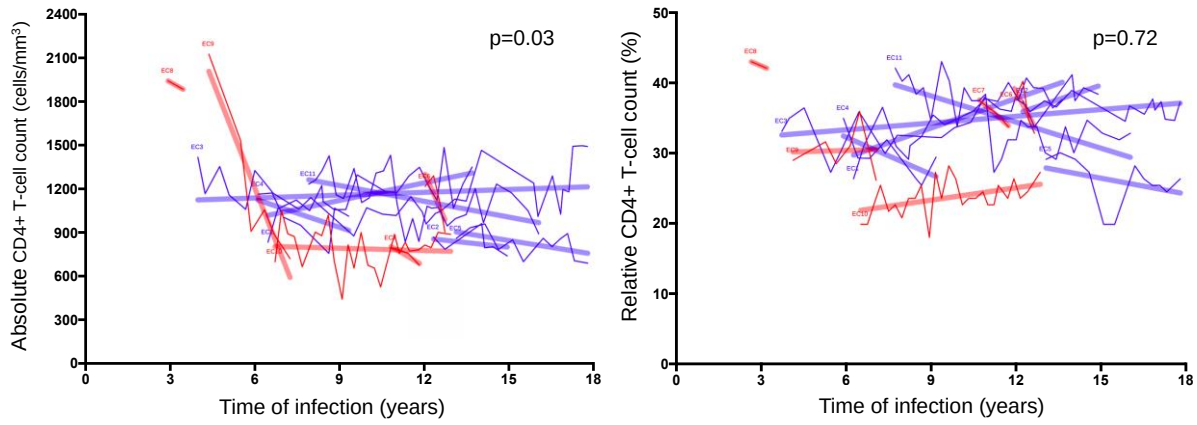


Figure 5.2 Longitudinal effect of CD4+ T-cell count in paediatric elite control. Mixed linear model of absolute and relative CD4+ T-cell count for those with persistent EC status (blue) and transient EC status (red).

5.3.2. CD4+ and CD8+ T-cells of PECs express low levels of immune activation and exhaustion

To evaluate the T-cell compartment in PECs, we investigated PEC-1 to PEC-4 (median age 14.6 years, range 12.7 to 16.6 years of age) during their period of elite control and compared with age-matched PSPs (median 13.6 years, IQR 12.2-15.7), PPs (median 13.0 years, IQR 11.3-15.0), and HEUs (median 14.5 years, IQR 12.0-16.6). The clinical characteristics of each individual are summarized in Table 5.2. As expected, PECs tended to have a lower total proviral HIV-DNA load (median 286 total HIV-DNA copies/10^6 CD4+ T-cells) when compared to the PSPs (median 7244, p=0.12) and the PPs (median 22,574, p=0.003) (Figure 5.3A). When combining the three groups, total proviral HIV-DNA copies correlated negatively with plasma HIV-RNA copies and positively with absolute and relative CD4+ T-cell count and CD4:CD8 ratio (Figure 5.3B).

Table 5.2 Individual clinical data at the time point studied.

Group	Sex	Age (years)	Plasma HIV RNA (copies/mL)	Total HIV DNA (copies/10^6 CD4+ T-cells)	Absolute CD4+ T-cell count (cells/mm^3)	Relative CD4+ T-cell (%)	CD4 CD8 ratio
PEC-1	Female	13.6	26	172	1316	39	1.5
PEC-2	Female	15.7	35	399	768	32	0.71
PEC-3	Female	16.6	<20	146	1258	37	0.74
PEC-4	Female	12.7	<20	3500	700	35	1.13
PSP-1	Male	16.4	1,800	2,267	779	34	0.42
PSP-2	Female	16.9	20	4,885	621	34	1.75
PSP-3	Male	14.1	1,700	647	982	33	1.63
PSP-4	Female	17.9	1,000	238	1007	34	1.37
PSP-5	Male	11.3	1,500	7,293	819	38	1.67
PSP-6	Female	10.1	1,800	11,118	933	45	1.69
PSP-7	Female	11.1	140,000	15,445	629	38	1.24
PSP-8	Male	12.7	8,000	11,781	463	31	0.96
PSP-9	Female	13	570	12,981	547	22	0.52
PSP-10	Male	14.2	370	40,435	550	28	0.45
PSP-11	Male	11.9	23,000	22,370	721	28	0.81
PSP-12	Female	15.2	59,000	6,274	1345	25	0.53
PSP-13	Male	10.4	23,000	7,120	954	34	0.89
PP-1	Female	13.2	470,000	11,576	387	18	0.43
PP-2	Male	14	44,000	86,633	164	7	0.16
PP-3	Female	12.7	19,000	79,728	86	7	0.1
PP-4	Male	17.3	210,000	142,913	476	9	0.09
PP-5	Male	18.1	8,200,000	6,260	375	18	0.36
PP-6	Male	15.2	5,400,000	558	235	21	0.34
PP-7	Female	11.5	7,400,000	25,594	251	9	0.28
PP-8	Female	10.7	5,900,000	80,950	242	9	0.12
PP-9	Male	14.9	57,000	19,554	41	3	0.08
PP-10	Female	12.5	3,700,000	14,386	321	8	0.36
HEU-1	Male	12.3	-	-	-	-	-
HEU-2	Female	16.7	-	-	-	-	-
HEU-3	Male	15	-	-	-	-	-
HEU-4	Female	12	-	-	-	-	-
HEU-5	Male	14	-	-	-	-	-
HEU-6	Female	10.2	-	-	-	-	-
HEU-7	Female	14.5	-	-	-	-	-
HEU-8	Female	16.1	-	-	-	-	-
HEU-9	Male	18.9	-	-	-	-	-

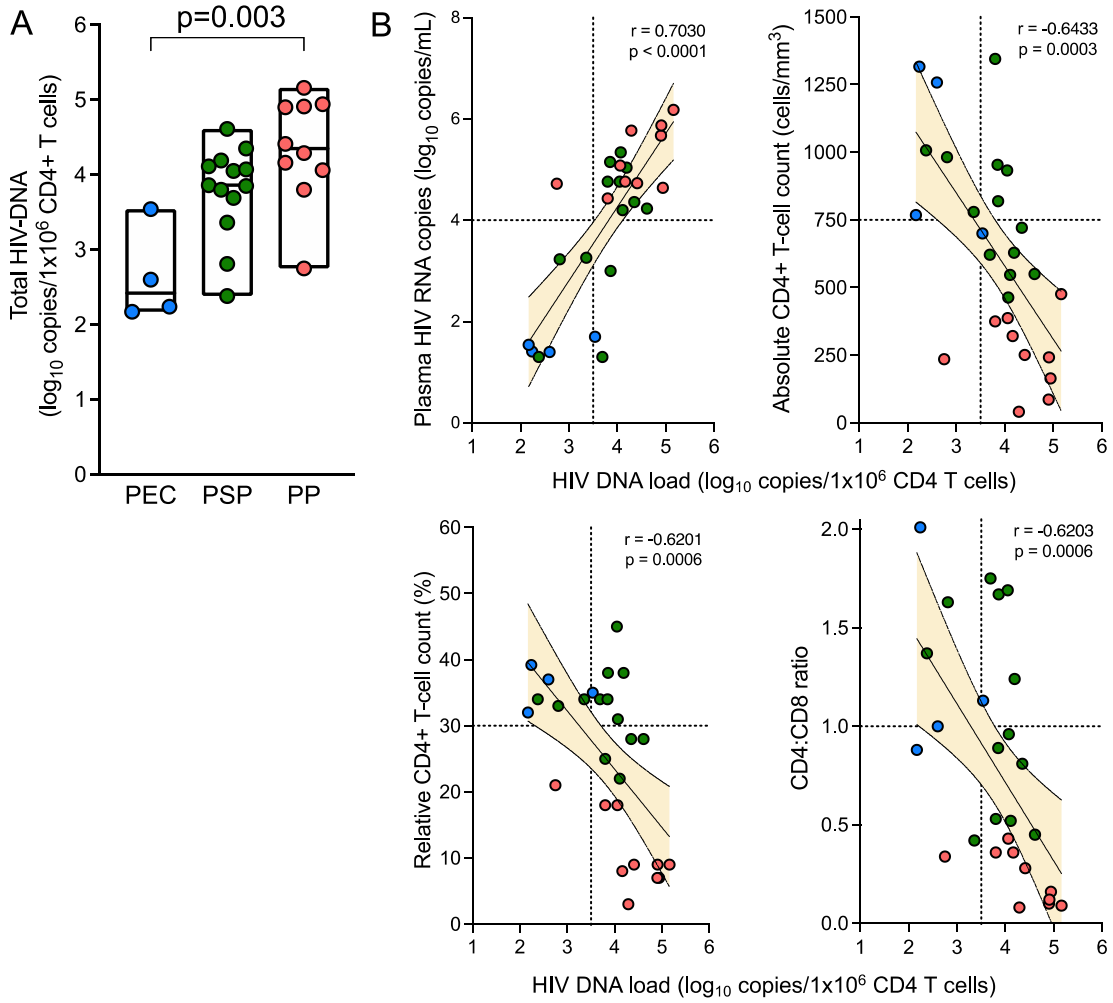


Figure 5.3 Total proviral HIV-DNA levels distinguish PECs from PPs.
A. Total HIV-DNA copies adjusted to CD4+ T-cell count in PECs (blue), PSPs (green), PPs (red). Boxplots represent median values and minimum and maximum values. B. Total proviral HIV-DNA load correlated with plasma HIV-RNA copies, absolute and relative CD4+ T-cell count, and CD4:CD8 ratio. Statistical comparison between groups was done with Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Spearman rank tests were used for correlations. Best-fit line and 95% confidence bands are shown.

The CD4+ T-cell compartment in PECs was distributed towards a higher proportion of naïve cells, similar to those observed in the HEUs and the PSPs, while the EM population was expanded in the PPs (Figure 5.4 and Figure 5.5A; Appendix I, Table 7 for antibody panel). Consistent with this pattern, the levels of immune activation (HLA-DR+CD38+) on CM and TM were significantly lower in PECs and comparable

to HEUs (Figure 5.5B and Table 5.3). PD-1 and CD39 were also more highly expressed on CD4+ T-cells of PPs when compared to HEUs.

By contrast to the less terminally differentiated phenotype of CD4+ T-cells in the PECs, the CD8+ T-cell compartment in all HIV-infected groups exhibited a pattern typical of chronic viral infection. There was a progressive decrease of the naïve population and a corresponding expansion of the effector subsets of TM, EM and TEMRA in these groups, markedly so in the PPs (Figure 5.5C). Interestingly, both PSPs and PPs, but not PECs, had a higher frequency of activated and PD-1+ CD8+ T-cells than HEUs (Figure 5.5D and Table 5.4). Again, all individuals living with HIV, but not PECs, expressed higher levels of CD39 and lower levels of CD73 compared to HEUs.

To further visualise immune differences between the four groups, a PCA was undertaken using memory subset distribution, activation and exhaustion markers (Figure 5.6). Undeniable confounders such as total proviral HIV-DNA and plasma HIV-RNA copies, and CD4+ T cell count were not included in the PCA. The PPs clustered in a distinct and a broader group, while the PECs grouped closer to the HEUs and PSPs. The main variables and their loading coefficients that explain the variations observed in PC1 were HLA-DR+CD38+ (0.8523), PD-1+ (0.8014), and CD39+ (0.7683) in CD4+ T-cells, and CD73+ (-0.8392), PD-1+ (0.7603) and naïve cell markers (-0.8245) in CD8+ T-cells.

The expression of activation and exhaustion markers on CD4+ and CD8+ T-cells also positively correlated with total proviral HIV-DNA and plasma HIV-RNA copies, and inversely correlated with absolute and relative CD4+ T-cell count, and CD4:CD8

ratio. Lower total HIV-DNA was associated with CD73 expression on both CD4+ and CD8+ T-cell populations (Figure 5.7A and B).

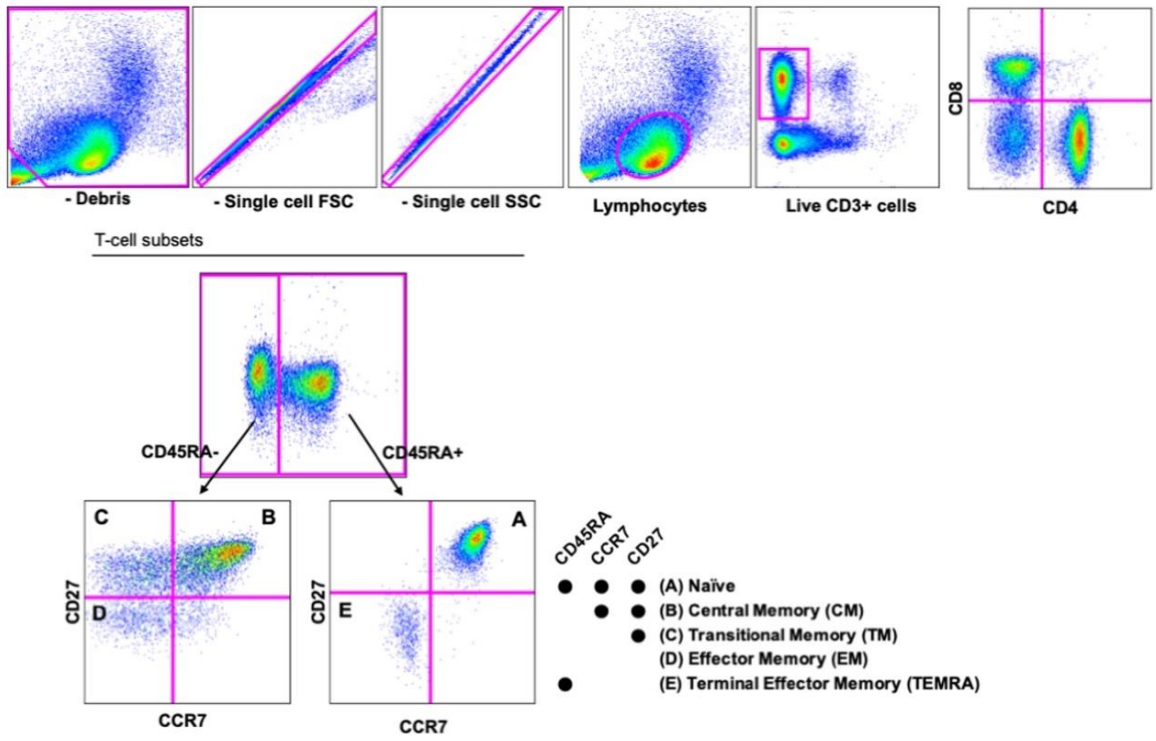


Figure 5.4 Gating strategy for T-cell immunophenotype.
Scheme to gate the naïve and memory subsets of live CD8+ and CD4+ T-cells. The gate position for the other markers was based on the FMO control.

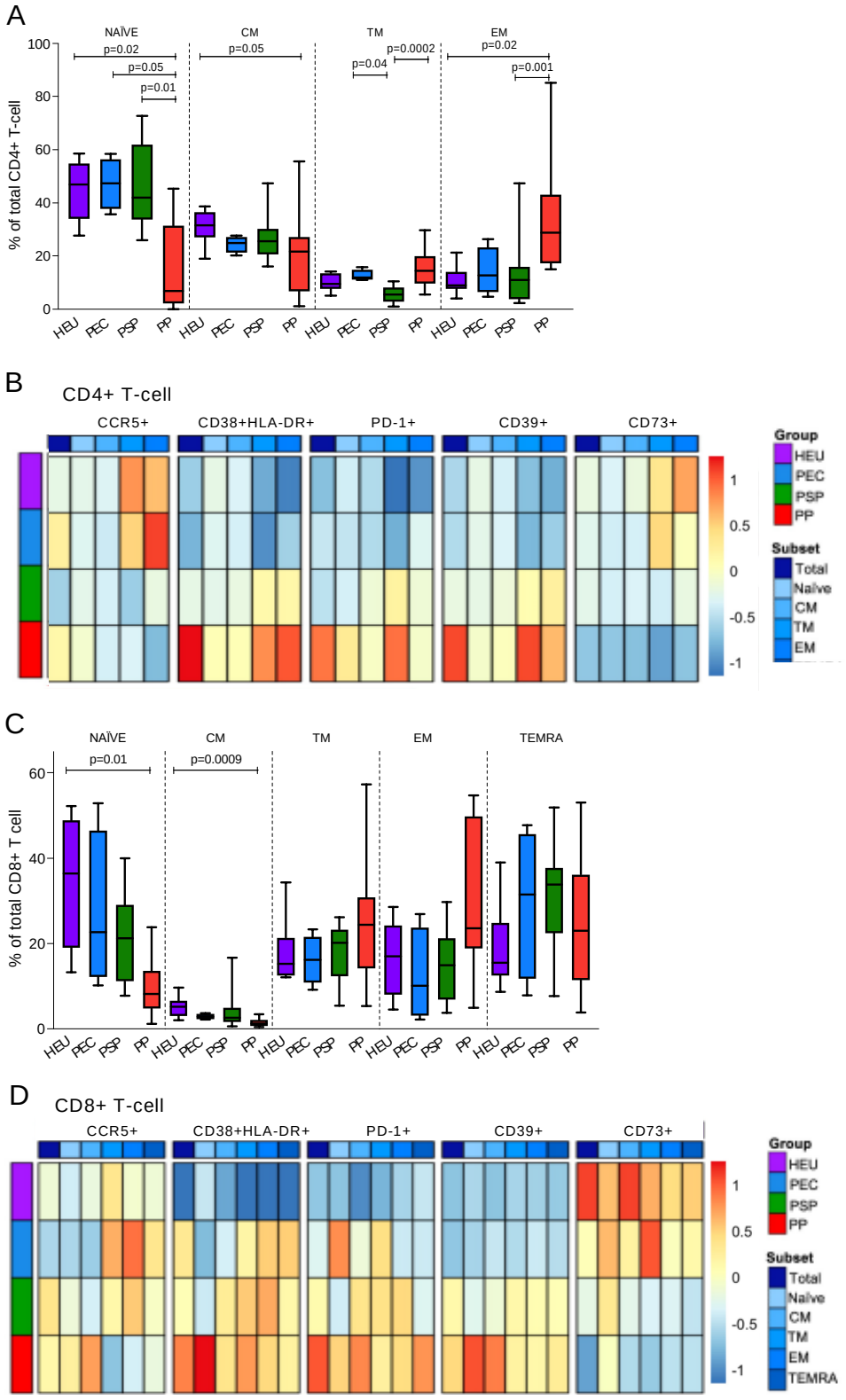


Figure 5.5 T-cell memory subsets and activation markers.

A. Frequency of CD4+ T-cell subsets defined in HEUs (purple), PECs (blue), PSPs (green) and PPs (red). Comparisons were done with Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons. B. Heatmap showing the median z-score of the frequency of CCR5+, HLA-DR+CD38+, PD-1+, CD39+ and CD73+ for each CD4+ T-cell subset. C and D. Same as A and B, respectively, but for CD8+ T-cells.

Table 5.3 Statistical comparison for markers on CD4+ T-cells.

CD4+ T-cell	*HEUs vs. PECs*	*HEUs vs. PSPs*	*HEUs vs. PPs*	*PECs vs. PSPs*	*PECs vs. PPs*	*PSPs vs. PPs*
Total						
CCR5+	-	-	-	-	-	-
HLA-DR+CD38+	-	-	0.0007	-	-	-
PD-1+	-	-	0.03	-	-	-
CD39+	-	-	0.01	-	-	-
CD73+	-	-	-	-	-	-
Naïve						
CCR5+	-	-	-	-	-	-
HLA-DR+CD38+	-	-	-	-	-	-
PD-1+	-	-	-	-	-	-
CD39+	-	-	-	-	-	-
CD73+	-	-	0.007	-	-	-
CM						
CCR5+	-	-	-	-	-	-
HLA-DR+CD38+	-	0.04	0.0003	-	0.003	-
PD-1+	-	-	0.002	-	-	-
CD39+	-	-	0.004	-	-	-
CD73+	-	-	-	-	-	-
TM						
CCR5+	-	-	0.001	-	0.02	-
HLA-DR+CD38+	-	-	0.003	-	0.002	-
PD-1+	-	-	0.002	-	-	0.03
CD39+	-	-	0.04	-	0.01	-
CD73+	-	-	-	-	-	-
EM						
CCR5+	-	-	-	-	-	-
HLA-DR+CD38+	-	0.03	0.0008	-	-	-
PD-1+	-	-	-	-	-	-
CD39+	-	-	-	-	-	-
CD73+	-	-	-	-	-	-

* Comparison was done with Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons.

Table 5.4 Statistical comparison for markers on CD8+ T-cells.

CD8+ T-cell	*HEUs vs. PECs*	*HEUs vs. PSPs*	*HEUs vs. PPs*	*PECs vs. PSPs*	*PECs vs. PPs*	*PSPs vs. PPs*
Total						
CCR5+	-	-	-	-	-	-
HLA-DR+CD38+	-	0.03	0.001	-	0.05	-
PD-1+	-	-	0.0002	-	-	-
CD39+	-	0.02	0.0004	-	-	-
CD73+	-	-	0.002	-	-	0.03
Naïve						
CCR5+	-	-	-	-	-	-
HLA-DR+CD38+	-	-	0.01	-	-	-
PD-1+	-	-	-	-	-	-
CD39+	-	-	0.0002	-	0.008	-
CD73+	-	-	-	-	-	-
CM						
CCR5+	-	-	-	-	-	-
HLA-DR+CD38+	-	-	0.0005	-	-	-
PD-1+	-	0.01	0.002	-	-	-
CD39+	-	-	0.001	-	-	-
CD73+	-	-	-	-	-	-
TM						
CCR5+	-	-	0.01	-	-	-
HLA-DR+CD38+	-	0.04	0.006	-	-	-
PD-1+	-	-	0.0003	-	0.004	-
CD39+	-	0.01	0.004	-	-	-
CD73+	-	-	0.005	-	-	-
EM						
CCR5+	-	-	-	-	-	-
HLA-DR+CD38+	-	0.005	0.01	-	-	-
PD-1+	-	-	-	-	-	-
CD39+	0.02	0.002	0.02	-	-	-
CD73+	-	-	-	-	-	-
TEMRA						
CCR5+	-	-	-	-	-	-
HLA-DR+CD38+	-	0.004	0.0007	-	-	-
PD-1+	-	-	-	-	-	-
CD39+	0.002	0.04	0.03	-	-	-
CD73+	-	-	-	-	-	-

* Comparison was done with Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons.

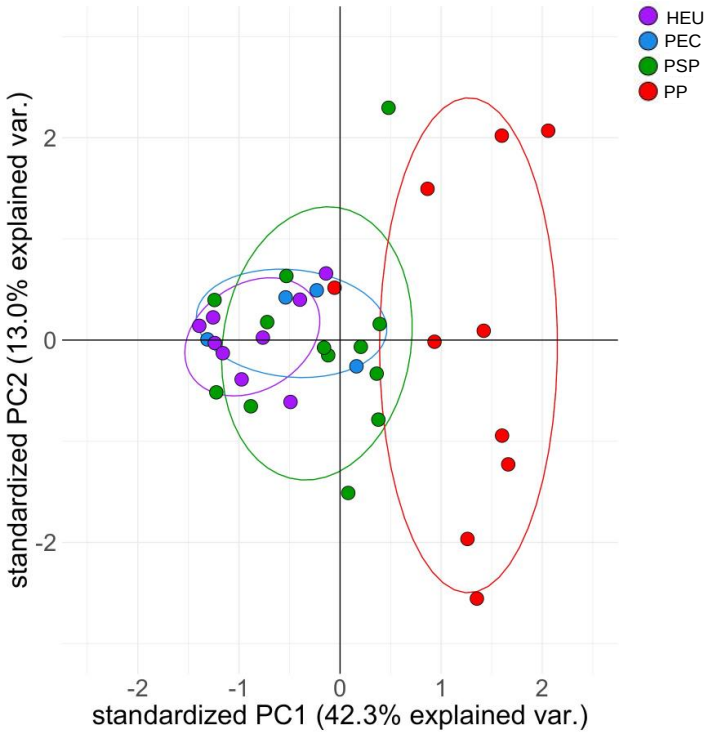


Figure 5.6 Multivariate analysis of T-cell exhaustion, activation and memory differentiation markers in the four groups.
The two PCA axes selected explain most of the variations found.

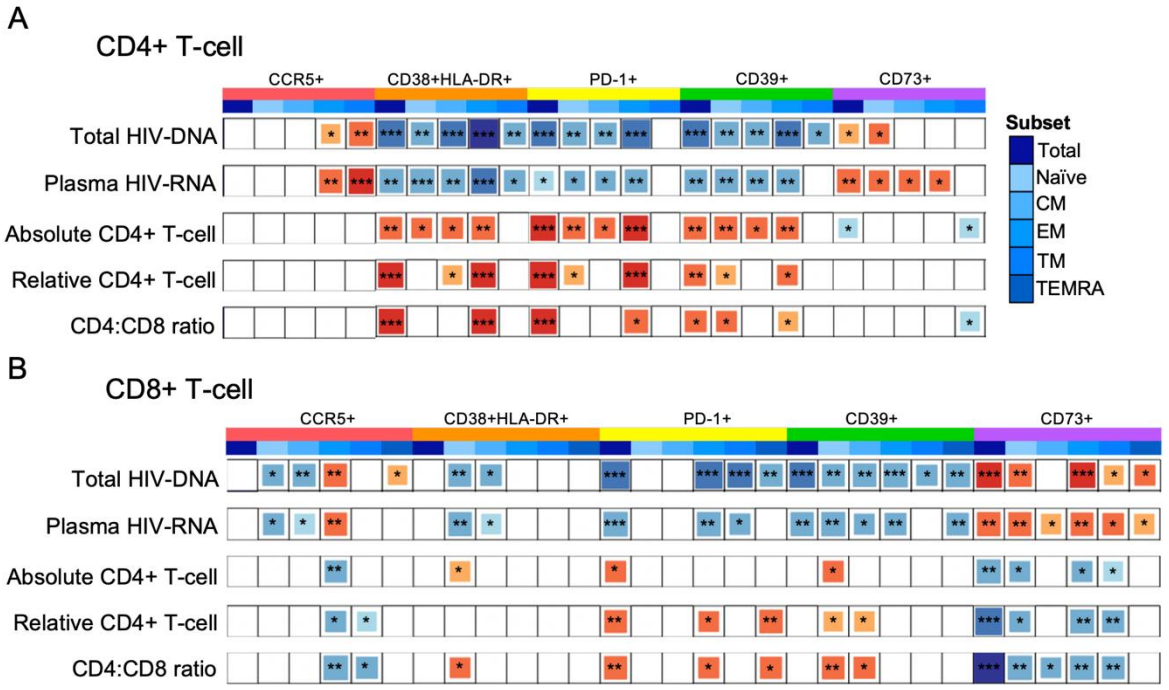


Figure 5.7 Correlations of exhaustion and activation markers on T-cells.
Total HIV-DNA and plasma HIV-RNA copies, absolute and relative CD4+ T-cell counts and CD4:CD8 ratio with different markers and subsets of CD4+ (A) and CD8+ (B) T-cell are shown. Layers of red indicate a negative correlation and layers of blue indicate a positive correlation. (*, $p<0.05$; ** $p<0.01$; ***, $p<0.001$).

5.3.3. Robust and polyfunctional HIV-specific T-cell response in PECs

Next, we investigated the role of the HIV-specific T-cell response in control of viraemia among PECs. Interestingly, only 2 of the 7 PECs who have been HLA-I typed expressed one of HLA-B*27/57/58:01/81:01 described as protective in adult EC (Table 5.5).

Table 5.5 HLA- I alleles for seven PECs.

HLA-I	A1*	A2*	B1*	B2*	C1*	C2*
PEC-1	03:01	33:03	35:01	53:01	04:01	16:01
PEC-2	03:01	30:02	14:01	15:10	03:04	08:02
PEC-3	02:01	74:01	27:03	42:01	02:02	07:01
PEC-4	03:01	68:02	14:02	58:02	06:02	08:02
PEC-8	23:01	68:02	08:01	14:01	03:04	08:02
PEC-9	03:01	30:02	18:01	57:03	07:01	07:04
PEC-11	23:01	74:01	15:16	53:01	04:01	14:02

* In green is highlighted the HLA-B alleles associated with viraemic control and in red those associated with rapid disease progression in adult cohorts.

Robust IFN-γ and degranulation (CD107a+) CD8+ T-cell responses were observed in PECs upon stimulation with Gag pool, but neither these markers nor IL-2, TNF-α and MIP-1β differed in magnitude from the Gag-specific responses observed in PSPs and PPs (Figure 5.8 and Figure 5.9; Appendix I, Table 8 for antibody panel). Of note, PPs had significantly higher magnitude Nef-specific CD8+ T-cell responses in line with the described high magnitudes in paediatric and adult progressors (Kiepiela et al. 2007; Sunshine et al. 2014; Leitman et al. 2017). The expression of cytolytic granules such as Granzyme B and Perforin were also not different among the groups (Figure 5.10A). However, the Gag-specific CD8+ T-cells were markedly more polyfunctional in PECs, expressing more 2, 3 or 4 functions simultaneously when compared with PSPs (p=0.009) and PPs (p=0.01) (Figure 5.10B).

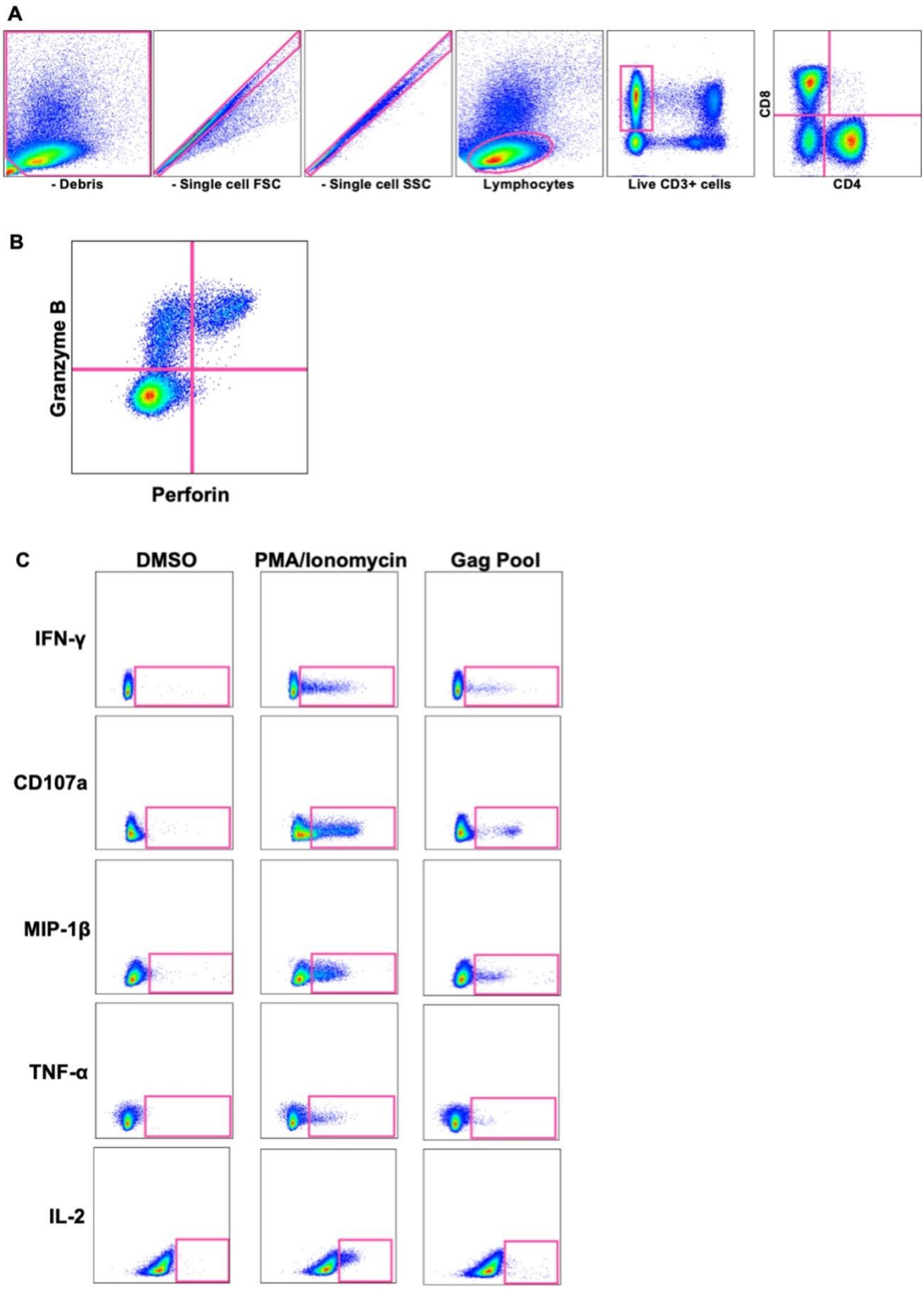


Figure 5.8 Gating strategy for ICS of HIV-specific T-cells.
A. Scheme to gate CD4+ and CD8+ T-cells. B. Typical plot for Granzyme B and Perforin. C. Typical DMSO, PMA/Ionomycin and Gag pool plots for IFN-γ, CD107a, MIP-1β, TNF-α and IL-2.

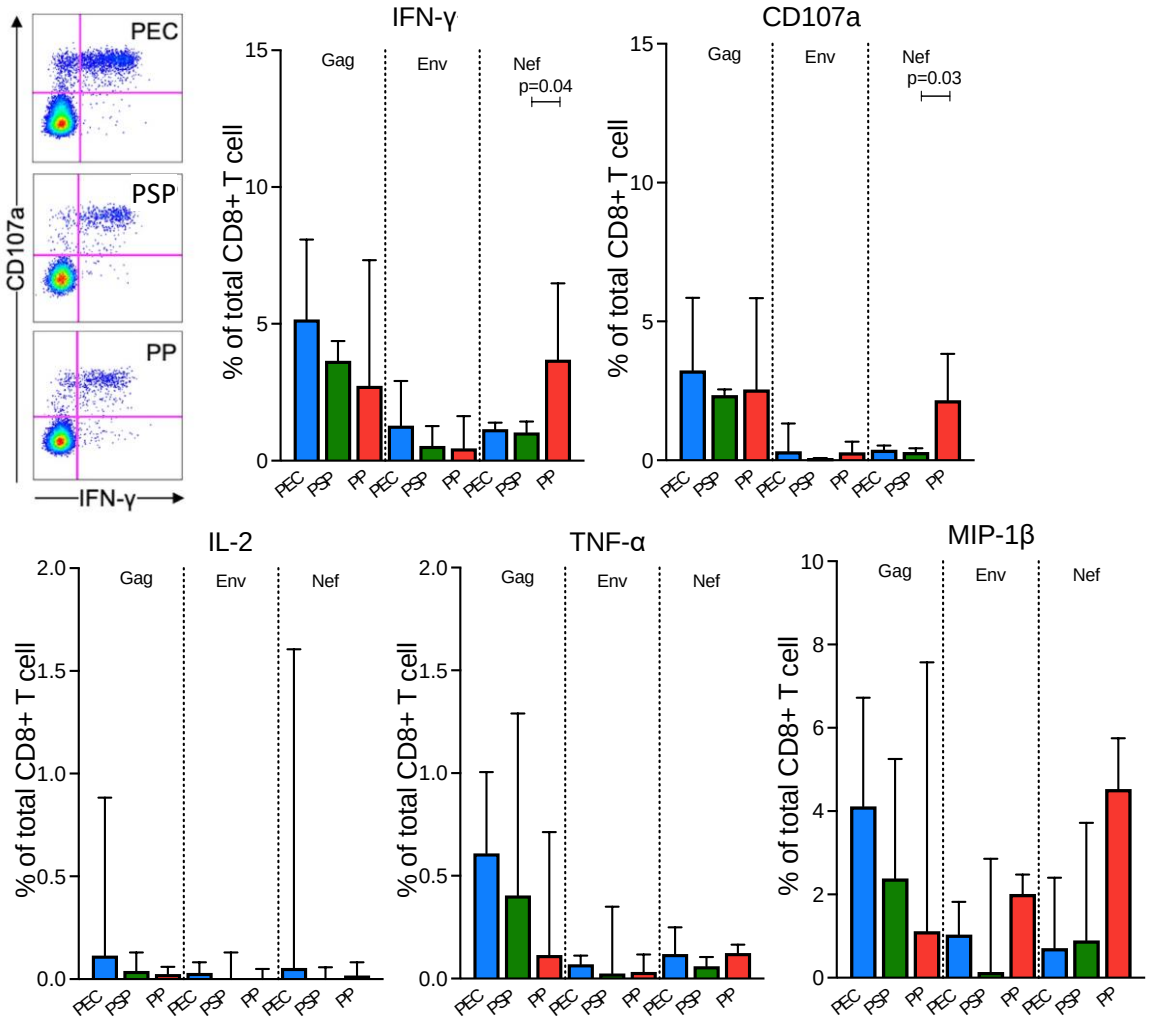


Figure 5.9 HIV-specific CD8+ T-cell response.

Representative FACS plot of CD107a+ and IFN-γ+ CD8+ T-cell upon stimulation with Gag in PECs, PSPs and PPs followed by frequency of total CD8+ T-cell expressing IFN-γ, CD107a, IL-2, TNF-α and MIP-1β upon stimulation with Gag, Env and Nef pools. Comparisons were done with Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons.

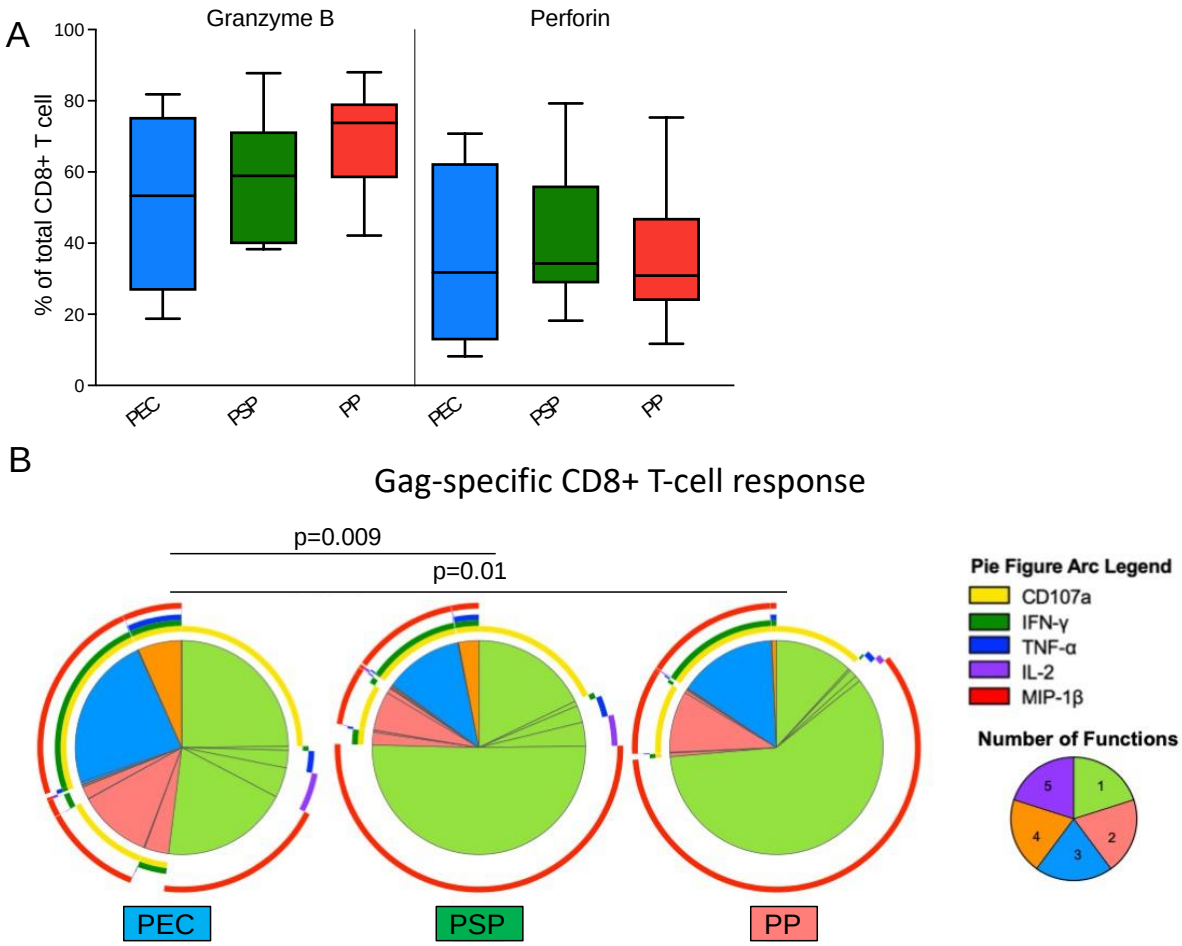


Figure 5.10 Cytolytic function and polyfunctionality of CD8+ T-cells.
A. Frequency of Granzyme B and Perforin in total CD8+ T-cells. Comparisons were done with Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons. B. Functional profile of Gag-specific CD8+ T-cells. Each slice of the pie chart represents the average relative proportions of each possible combination of the functions measured. Slices are coloured according to the number of simultaneous functions. Comparisons were done using the permutation test in SPICE 6.0.

Gag-specific CD4+ T-cell responses differed considerably in magnitude and quality from the CD8+ T-cell responses. These cells expressed more IFN-γ upon Gag stimulation in PECs (Figure 5.11A), and the frequency of IFN-γ+ in CD4+ T-cells negatively correlated with plasma HIV-RNA copies (Figure 5.11B), consistent with previous data in children (Prendergast et al. 2011). Although the frequency for the other markers were not different among the groups (data not shown), the CD4+ T-

cells that responded to Gag pool were again more polyfunctional in the PECs when compared to PPs (p=0.001) (Figure 5.11C).

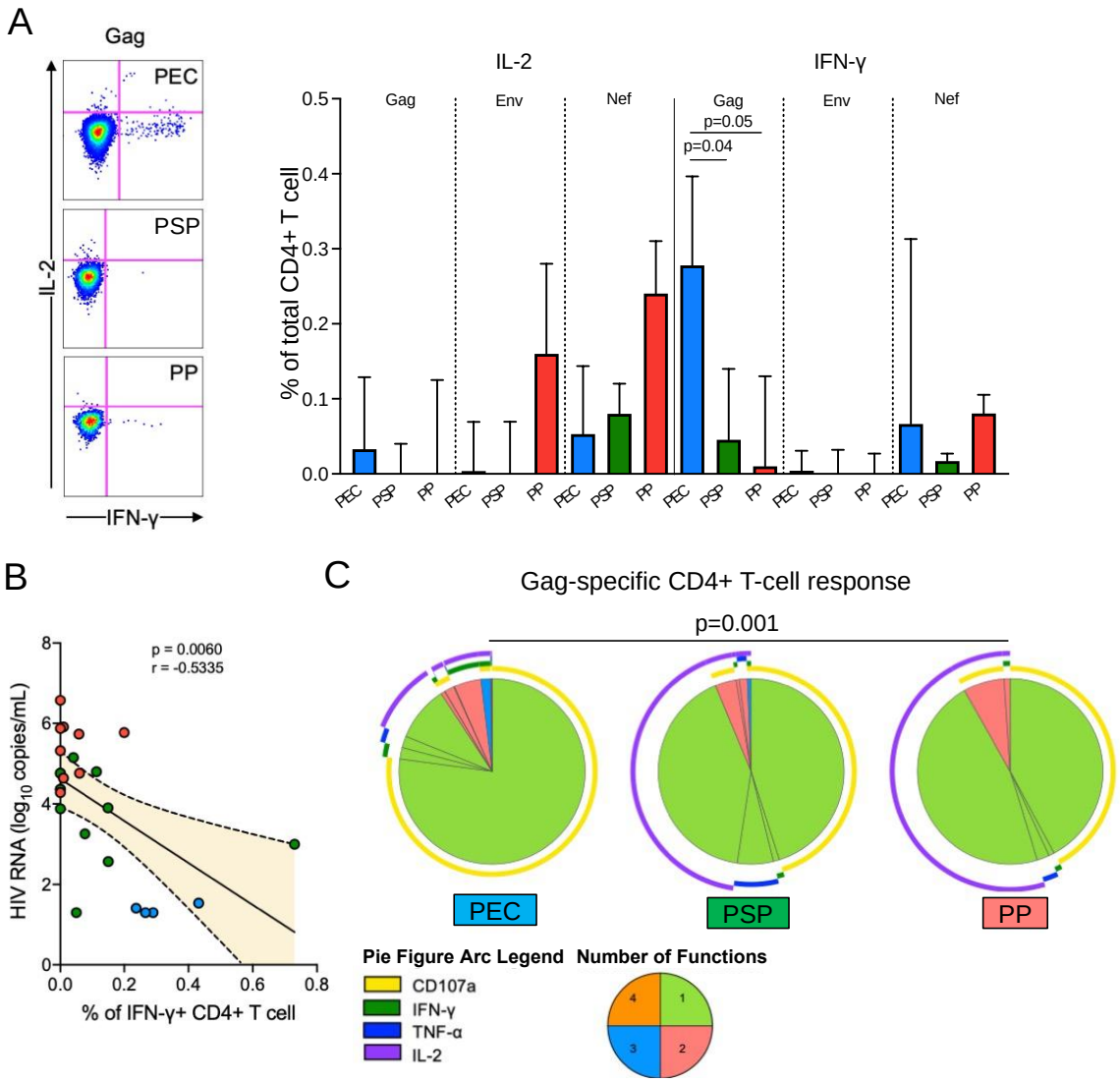


Figure 5.11 HIV-specific CD4+ T-cell response.

A. Representative plot and frequency of IL-2+ and IFN-γ+ CD4+ T-cells upon stimulation with Gag pool. Comparisons were done with Kruskal-Wallis' test followed by Dunn's test to correct for multiple comparisons. B. Correlation between plasma HIV-RNA load and percentage of CD4+ T-cells secreting IFN-γ upon Gag pool stimulation. Spearman correlation and best-fit line are shown. C. Functional profile of Gag-specific CD4+ T-cells. Each slice of the pie chart represents the average relative proportions of each possible combination of the functions measured. Slices are coloured according to the number of simultaneous functions. The arcs illustrate the specific cytokine response. Comparisons were done using the permutation test in SPICE 6.0.

5.4 Discussion

To identify PECs, nine cohorts of children living with HIV were studied from South America (Brazil), Europe (the UK and Spain), Africa (SA) and Asia (Thailand). Within these cohorts, we identified 12 children qualifying as ECs. Although five of the PECs were identified within European cohorts, all originated from SSA. This group, thus likely represents a broad range of populations and subclades of HIV according to the global distribution: subtype B (Brazil), subtype C (Southern Africa and Ethiopia), subtype D (Uganda) and subtype CRF01_AE (Thailand) (Hemelaar et al. 2011). Despite only a small number of PECs subjects available to study and the cautious we need to have when interpreting statistical differences here, it was possible to identify features in the T-cell compartment and in the antiviral response that distinguish PECs from ART-naïve children/adolescents living with HIV who also maintain normal-for-age CD4+ T-cell counts but do not control viraemia (PSPs). The principal differences are in a more polyfunctional Gag-specific CD8+ T-cell responses combined with higher frequencies of Gag-specific CD4+ T-cell IFN-g responses in PECs compared to PSPs. These data provide some clues as to how HIV-specific T-cell responses in PECs are distinct from those in paediatric viraemic non-progressors

The question of the prevalence of ECs among children versus adults is difficult to address when analysing this study's data, given that the paediatric cohorts from which these 12 children have been identified have not been followed from birth. In particular, the European cohorts, which are largely constituted of children born in SSA, are clearly selected for those who survived long enough to have emigrated. The fact that the average age at enrolment of these children is 6.9 years illustrates the point that many healthy children are likely to remain undiagnosed, especially in less

well-resourced countries with high adult seroprevalence where the priority is to treat adults and children with known infection and prevent disease progression. The most accurate estimates of PECs are from Kimberley and Stellenbosch, SA, in which the structure of paediatric care is such that a high proportion of children living with HIV have been followed. In these two provinces of 11,539 children living with HIV, there were three PECs. These data would yield an estimated prevalence of 0.03% (95% CI 0–0.06). This may represent an underestimate as healthy EC children are less likely to be identified in such settings. Furthermore, as it appears that maintenance of elite control status can be quite short-lived in paediatric infection, some PECs have likely lost control of viraemia by the time that they present to clinicians. Conversely, other children who are future ECs have not achieved control of viraemia when first presenting and also might have started ART before viraemic control onset. The other seven cohorts studied, as described above, are relatively selected, and therefore, would represent an overestimate of prevalence. Overall, the nine cohorts provide a PEC prevalence estimate of 0.08% (95% CI 0.04–0.1%). Given the prevalence of ECs among adult cohorts of ~0.5%, and even after considering the imperfections of prevalence estimate here of PECs, these data suggest that ECs prevalence is 5–10-fold lower among children than adults.

Several features of PECs are apparent even from this small group. The high proportion of females (92%) is consistent with the observation that adult females are five-fold more likely than males to be ECs (Yang et al. 2017). Although there appears to be an increased susceptibility among females specifically to in utero MTCT (Taha et al. 2005; Piwoz et al. 2006; Biggar et al. 2006; Marinda et al. 2007; Adland et al. 2020), this does not appear to apply to intrapartum or postpartum infections (Taha et al. 2005; Marinda et al. 2007), so overall, the numbers of females infected via MTCT do not exceed those of males by more than approximately 10% (Marinda et

al. 2007). In adult infection, the initial viral setpoint in females is lower than in males (Anastos et al. 2000; Napravnik et al. 2002; Collazos, Asensi, and Cartón 2007), in association with a more vigorous type I IFN response in acute infection in females (Meier et al. 2009; Chang et al. 2013). In some paediatric studies, female children also have been shown to have lower plasma viral loads (Study 2002; Ruel et al. 2011), although this sex difference was not observed in one large study of greater than 2000 ART-naive children (Mori et al. 2015). In the same study, among 282 PSPs, 59% were female, indicating that in female children, absolute CD4+ T-cell count declines more slowly than males. Nonetheless, the number of females in the group of children identified here is significantly higher than that of this PSP cohort, suggesting that female children control viraemia better than males, as well as maintaining higher absolute CD4+ T-cell counts.

Despite the limitations, many of the other features of PECs appear to contrast with observations in adult ECs. The time to achieve viraemic control among the PECs who were viraemic at enrolment, and we can assure elite control onset, was 5.2 years, whereas adults ECs are usually recognised within the first year of infection (Madec et al. 2013). Similarly, in ART-naïve children, the viral set point is a gradual and usually incomplete process that takes several years (Muenchhoff et al. 2016), which in adults is completed within weeks (Mellors et al. 1997; Robb et al. 2016). Again, it should be noted that this estimate of the time to achieve elite control among children does not take into account the possibility that some PECs who achieved elite control at an earlier age were not recognised as ECs as a result of having lost viraemic control by the time they presented; indeed, several PECs here experienced periods of undetectable plasma viral loads before fulfilling the criteria of EC.

EC status appears to be more short-lived in children than in adults, in whom the median time to viral rebound among adult women is greater than 30 years (Yang et al. 2017), although these studies usually miss individuals who control viraemia for a short period after acute infection (Madec et al. 2013). We show in Chapter 6 that children achieving undetectable plasma viral loads only transiently are relatively more common, and it prompts the hypothesis that the developing immune response over childhood helps to bring plasma viraemia down over a period of years, but once sufficient selection pressure is brought to bear on the virus, in most cases it can escape. By contrast, in adults, the critical battles between the immune system and the virus are waged chiefly, although not exclusively, in the first weeks of infection (Koup et al. 1994; Goulder and Watkins 2008; Ndhlovu, Kamya, et al. 2015). The frequency of blips of less than 1000 copies/mL among the PECs suggests that control of viral replication is more profound among adult ECs. The average plasma viral loads among adult ECs are reportedly 1–5 RNA copies/mL (Maldarelli et al. 2007; Dinoso et al. 2008; Hatano et al. 2009; Pereyra et al. 2009).

The immune strategies by which elite control is reached in children and adults can represent distinct strategies and reflects the contrasting developmental stages of the immune system. In adults, the speed and extent of activation of CD8+ T-cells following infection predict the degree to which viraemia will be suppressed (Ndhlovu, Kamya, et al. 2015). Rapid immune control of viraemia requires high levels of immune activation of T-cells, but in early life, these high levels of immune activation result in accelerated CD4+ T-cell decline (Muenchhoff et al. 2016). Like PSPs, PECs have an immunophenotype resembling that of the natural hosts of SIV, the hallmark being a low degree of immune activation, lack of exhaustion of the CD4+ T-cell population and the low expression of CCR5 in long-lived memory cells (Silvestri et al. 2003;

Muenchhoff et al. 2016). As seen here, this phenotype of low immune activation and exhaustion is even more marked in the four PECs than in PSPs.

As children transition towards an adult-like immune system, the T-cell response progressively shifts towards a more aggressive antiviral response that is eventually associated with the acquisition of viral control in PECs (Muenchhoff, Prendergast, and Goulder 2014). Whereas in adults, 60% of Caucasian ECs express one of HLA-B*57 or HLA-B*27 (Pereyra et al. 2008), and in African ECs, 60% express one or more of HLA-B*57, HLA-B*58:01 or HLA-B*81:01 (Pereyra et al. 2008; Pereyra et al. 2010; Leslie et al. 2010; Carlson et al. 2012), only 2 out of 7 HLA-I typed PECs expressed a protective allele. However, genetic associations explain only 20% of host control in adults (Pereyra et al. 2010), and other features also play a role in the antiviral response. The polyfunctionality of the HIV-specific CD8+ T-cell response is enhanced in adults ECs compared to normal progressors (Betts et al. 2006; Almeida et al. 2007), however the rarity of AVNPs has made it difficult to determine whether CD8+ T-cell polyfunctionality is a cause or consequence of maintained CD4+ T-cell counts among adult ECs. The existence of PSPs who have maintained normal-for-age CD4+ T-cell counts and yet have markedly reduced HIV-specific CD8+ T-cell polyfunctionality compared to PECs suggests that CD8+ T-cell polyfunctionality is an important driver of PECs.

A further distinguishing feature of PECs was the expression of immune activation and exhaustion markers. We noted a distinct upregulation of CD39 and downregulation of CD73 in PPs and PSPs, but not in PECs, when compared to HEUs that was especially marked in the CD8+ T-cells. In adults, CD39 is positively correlated with HIV disease progression, while CD73 has the opposite effect (Tóth et al. 2013; Gaiha et al. 2014). CD39 is upregulated in activated cells during

inflammation (Allard et al. 2017). By contrast, CD73, which functions to convert adenosine monophosphate into adenosine, is downregulated in CD8+ T-cells upon activation and in terminally differentiated cells (Allard et al. 2017). Indeed, the adenosine produced by CD73 is important in the generation and maintenance of memory cells (Bono et al. 2015). This phenotype, together with low expression of CD38, HLA-DR and PD-1 in the CD8+ T-cell compartment in PECs maintains a more effective and sustained effector function.

We also observed an increased IFN-γ frequency on Gag-specific CD4+ T-cell in PECs associated with a more polyfunctional phenotype compared to PPs. Gag-specific CD4+ T-cell responses have been associated with a lower viral set point in adults and children, although not always in association with high expression of IFN-γ (Prendergast et al. 2011; Muenchhoff et al. 2016; Adland et al. 2018). As noted in previous studies of both adult and paediatric infection (Kiepiela et al. 2007; Muenchhoff et al. 2016; Leitman et al. 2017), we observed here again the association of Nef-specific CD8+ T-cell responses with ineffective control of viraemia. Together, these data support the notion that polyfunctional Gag-specific CD4+ and CD8+ T-cell activity contribute increasingly through childhood to control of viraemia and may play a progressively more important part in antiviral immunity as part of strategies to achieve functional cure in children from mid-childhood onwards.

As observed in AVNPs (Garbuglia et al. 1995; Lambotte et al. 2005) and adult ECs (Graf et al. 2011; de Masson et al. 2014; García et al. 2017), the total proviral HIV-DNA in ART-naïve children was lower in the PECs than in the paediatric viraemic groups. Their ability to control the viral reservoir could be explained by their ability to control viral replication or to resist viral infection. The CD4+ T-cell compartment of PECs is mainly populated with naïve and CM cells with the lowest immune

activation and exhaustion levels among individuals living with HIV. In previous studies in PSPs, total HIV-DNA were mainly integrated in the short-lived EM CD4+ T cells, in contrast to higher copy numbers in the long-lived CM in future adult progressors (Muenchhoff et al. 2016). In adult studies, low levels of HIV-DNA viral reservoir have also been related to lower immune activation in both CD4+ and CD8+ T-cells (Yukl et al. 2010; Cockerham et al. 2014; Khoury et al. 2017). However, in adults, achieving low plasma viral loads is related to the ability of the HIV-specific CD8+ T-cell response in acute infection to become rapidly and fully activated (Ndhlovu, Kamya, et al. 2015). The low immune activation observed in association with chronic elite control in adults, therefore, is the consequence of the aggressive response in acute infection. In PECs, by contrast, where viraemic control is usually only achieved after years of infection, low immune activation presumably precedes immune control of viraemia.

It is important to acknowledge limitations to this study, that include its cross-sectional nature and the low number of PECs that could be assessed. The small sample size also does not allow us to exclude a contributing role for protective HLAs in paediatric elite control. The rarity of the elite control phenotype, combined with the introduction of the universal treatment guidelines, make the generated data unique, even if somewhat anecdotal. Despite the low numbers, we identified clear-cut differences between PECs and other paediatric phenotypes, shedding light on the immune strategies adopted to achieve viral suppression in the context of vertical transmission. In addition, we have not subtyped the participants prior to the HIV-specific T-cell assays but our group has previously determined that 100% of more than 600 HIV-infected individuals from Durban (SA) are C clade infected (Kawashima et al. 2009). Therefore, it is reasonable to assume that the PSPs and PPs and PEC-4, all from Durban, were also C clade infected. However, because

plasma viral loads were so low in the PEC individuals, we were unable to determine the clade of infection in these PECs. It is possible that some of the PECs were infected by non-C clade viruses, in particular PEC-1 and PEC-2, whose country of origin were Ghana and Nigeria, respectively. We also only measured total proviral HIV-1 DNA, which limits our capacity to interpret the viral reservoir in these populations further. Finally, innate immunity and B-cells were not assessed in this study, and these cells are likely also to contribute to viraemic control. Previous studies have demonstrated a correlation between plasma viral load and the neutralization breadth of anti-HIV antibodies (Goo et al. 2014; Muenchhoff et al. 2016). Similarly, NK cells exert important effector functions, and the role of these cells was the focus of Chapter 3.

This study provides the first initial description of a group of longitudinally tracked PECs and the differences that exist between these and adult ECs. We have demonstrated that elite control in children is strongly biased towards the female sex and takes longer to be achieved. Furthermore, the T-cell compartment in PECs has very low levels of immune activation and exhaustion like the natural host of SIV, while maintaining a polyfunctional HIV-specific T-cell response, similar to the adult ECs. This dual phenotype can provide important clues to remission strategies in PTCs, where viraemic suppression is also typically achieved in absence of protective HLA-I molecules (Sáez-Cirión et al. 2013).

6

Two distinct mechanisms leading to loss of virological control in the rare group of antiretroviral therapy-naïve, transiently aviraemic children

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6.1. Background

HIV-specific CD8+ T-cell responses play a central part in immune control of HIV in adults (Pereyra et al. 2009; Mothe et al. 2012; Koup et al. 1994; Borrow et al. 1994). In children living with HIV, although HIV-specific CTL responses are detectable from birth (Luzuriaga et al. 1995; Thobakgale et al. 2007), due to the non-Th1 polarising nature of early life immunity (Kollmann et al. 2009; Prendergast, Klenerman, and Goulder 2012), these are of lower breadth and do not impose strong selection pressure on the virus until mid-childhood (Adland et al. 2015). In paediatric HIV, a viral setpoint is only observed after the age of five years in those who survive without ART (Muenchhoff et al. 2016). Although as many as 10% of ART-naïve children living with HIV are PSPs (Blanche et al. 1997; Paul et al. 2005; Ananworanich et al. 2010), having maintained normal-for-age CD4+ T-cell counts at 5 years, viraemic controllers are rare. PSPs are usually viraemic, but those with better viral control are marked by a CD8+ T-cell response directed to the conserved Gag/Pol regions rather than to Env, Nef or the other accessory proteins (Tat, Rev, Vif, Vpr, Vpu), as observed previously (Leitman et al. 2017) and reinforced by the data in Chapter 5.

The effect of HLA-B molecules in immune control of HIV is substantially stronger in adults than in paediatric infection (Adland et al. 2015). However, even in adults, HLA-I polymorphisms only explain 20% of differences in plasma viral load (Pereyra et al. 2010). Although 40-50% of ECs express HLA-B*57 (Pereyra et al. 2008), since EC prevalence is approximately 0.5% (Yang et al. 2017) and HLA-B*57 prevalence in most populations is approximately 7%, <10% of adults living with HIV and expressing HLA-B*57 are ECs. Other factors influencing CD8+ T-cell function clearly also play a role in immune control. Notably, HIV-specific CD8+ T-cells in ECs have higher proliferative capacity and are more polyfunctional than in non-controllers in

both adults (Migueles et al. 2002; Migueles et al. 2015; Sáez-Cirión et al. 2007; Almeida et al. 2007; Betts et al. 2006) and in children (Chapter 5).

In the cohorts described in the previous chapter, we also identified ART-naïve children with transient periods of aviraemia but who did not maintain viral suppression sufficiently long-term to meet the criteria for PEC. The vast majority rebounded to a plasma viral load >10,000 copies/mL after a short-period of viral control. To further assess the mechanisms linked with failure to maintain aviraemia in these children, we characterised the HIV-specific T-cell responses and longitudinally performed deep sequencing of the virus in three transiently aviraemic children (TAs), each of whom expressed HLA-B*81:01, an allele strongly associated with HIV immune control in the adult African populations (Kiepiela et al. 2004; Pereyra et al. 2010; Leslie et al. 2010).

Of note, the assays described in this Chapter is all my own except where stated otherwise in the text. The analyses and interpretation of the raw data are also my own work.

6.2. Chapter-specific population and methods

6.2.1. Study population

We identified a total of 21 TAs looking at the clinical follow up of ART-naïve children in the same cohorts described in Chapter 5, except for TA-3, who is followed at the out-patient clinic in Karolinska University Hospital (Stockholm, Sweden). They were defined as those with spontaneous plasma HIV-RNA below the limit of detection (<50 copies/mL) on at least one occasion, but without fulfilling the criteria of a PEC.

The three TAs described in detail are originally from SA (Kimberley and Durban) and were selected based on the availability of longitudinal plasma and PBMCs samples and the expression of the disease-protective HLA-B*81:01.

6.2.2. Virus sequencing and epitope haplotype calling

Sanger sequencing was performed by Dr Emily Adland (University of Oxford, UK). Sequencing was generated from genomic DNA extracted from whole blood and amplified by nested PCR. Sequencing was undertaken using the Big Dye ready reaction terminator mix (V3) (Department of Zoology, University of Oxford, UK). Sequence data were analysed using Sequencher v.4.8 (Gene Codes Corporation). Nucleotides for each gene were aligned manually in Se-Al v.2.0a11.

Next-generation sequencing (NGS) was performed by Dr David Bonsall's research group (Big Data Institute, University of Oxford, UK) and previously described elsewhere in detail (Bonsall et al. 2020). Briefly, plasma samples were thawed at RT and total RNA was extracted using the NucliSens easyMAG system (bioMerieux) and concentrated with Agencourt RNAClean XP (Beckman Coulter). Libraries were prepared using the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara Bio). The RNA was denatured at 72°C with tagged random hexamers, followed by cDNA synthesis using in-house sets indexed primers (Lamble et al. 2013). 5 µL of each amplified library were pooled and shorter libraries eliminated with a lower ratio than recommended of Agencourt AMPure XP. High Sensitivity D1000 ScreenTape assay on a TapeStation system (Agilent) and a Qubit dsDNA HS Assay (Thermo Fisher Scientific) were used to measure size distribution and concentration. A total of 500 ng of pooled libraries was hybridized (SeqCap EZ Reagent kit, Roche) to a mixture of custom HIV-specific biotinylated 120-mer

oligonucleotides 46 (xGen Lockdown Probes, Integrated DNA Technologies), then captured by streptavidin-conjugated beads. Unbound DNA was washed off (SeqCap EZ Hybridization and Wash kit, Roche), and the remaining libraries were then PCR amplified for sequencing using a MiSeq instrument (Illumina) resulting in read length up to 300 nucleotide paired-end.

Epitope haplotype calling was performed with the assistance of Dr Nicholas Grayson (University of Oxford, UK). To obtain the epitope ratios from NGS reads, initially the software trimmomatic (Bolger, Lohse, and Usadel 2014) was used to trim Illumina adaptors and low quality data from the raw reads. Where possible reads were then merged using a minimum overlap of 10 nucleotides. The processed reads were then blasted against Los Alamos Sequence Database (http://www.hiv.lanl.gov/) HIV-1 "Web Alignments 2017" to obtain the HXB2 alignment positions for each read. Reads that entirely contained the epitope were used to find the epitope ratios at each position. The panel used of characterized epitopes of the full HIV-1 clade C proteome restricted by HLA-I alleles was previously compiled (Leitman et al. 2017).

6.2.3. Viral mutagenesis

Viral mutagenesis was performed by Dr Emily Adland and Dr Ming-Han Chloe Tsai (University of Oxford, UK). Single mutations were introduced into the Gag region of HIV-1 using the B clade NL4-3 plasmid and the patient derived C clade Gag-protease sequence, SK-254 (M), which had been modified to consensus C. Glutamine (G) was substituted to proline (P), glycine (G) or threonine (T) at the 182nd position in B clade and 179th position in C clade. This was done using Quickchange Lightning site-directed mutagenesis kit (Agilent technologies) using custom primers from Eurofins genomics. The forward primers, with mutations, are: (NL4-3 Q182T) 5'-GCA TTA

TCA GAA GGA GCC ACC CCA ACG GAT TTA AAT ACC ATG CTA AAC ACA-3'; (Q182G) 5' -GCA TTA TCA GAA GGA GCC ACC CCA GGA GAT TTA AAT ACC ATG-3'; (Q182P) 5'-AA GGA GCC ACC CCA CCA GAT TTA AAT ACC ATG C-3'; (SK-254(M) Q179T) 5'-GCA TTA TCA GAA GGA GCC ACC CCA ACG GAT TTA AAC ACC ATG TTA AAT ACA G-3'; (Q179G) 5'-GCA TTA TCA GAA GGA GCC ACC CCA GGA GAT TTA AAC ACC ATG-3'; (Q179P) 5'-GAA GGA GCC ACC CCA CCA GAT TTA AAC ACC ATG T-3'. All plasmids were maxipreped following the manufacturer's instructions (HiSpeed plasmid Maxi Kit, Qiagen, Hilden, Germany). The mutation was confirmed by sequencing.

6.2.4. Replication Capacity Assay

Viral replication capacity (VRC) was performed by Dr Emily Adland and Dr Ming-Han Chloe Tsai. A deleted version of pNL4-3 was constructed that lacks the entire Gag and Protease region (Stratagene Quick-Change XL kit) replacing this region with a BstE II (New England Biolabs) restriction site at the 5' end of Gag and the 3' end of protease. To generate recombinant viruses, 10 µg of BstEII-linearized plasmid plus 50 µL of the second-round amplicon (approximately 2.5 µg) were mixed with $2x10^6$ cells of a Tat-driven green fluorescent protein (GFP) reporter T cell line (GXR 25 cells) in 800 µL of R10 medium and transfected by electroporation using a Bio-Rad GenePulser II instrument. Following transfection, cells were rested for 45 min at RT, transferred to T25 culture flasks in 5 mL warm R10 and fed with 5 mL R10 on day 4. GFP expression was monitored by flow cytometry, and once GFP expression reached >30% among viable cells supernatants containing the recombinant viruses were harvested and aliquots stored at -80°C.

The replication capacity of each chimera is determined by infection of GXR cells at a low multiplicity of infection (MOI) of 0.003. The mean slope of exponential growth from day 2 to day 7 was calculated using the semi log method in Excel. This was divided by the slope of growth of the wild type (WT) NL4-3 control included in each assay to generate a normalized measure of replication capacity. Replication assays were performed in triplicate, and results were averaged. These VRC determinations were undertaken entirely blinded to the identity of the study subject.

6.3. Results

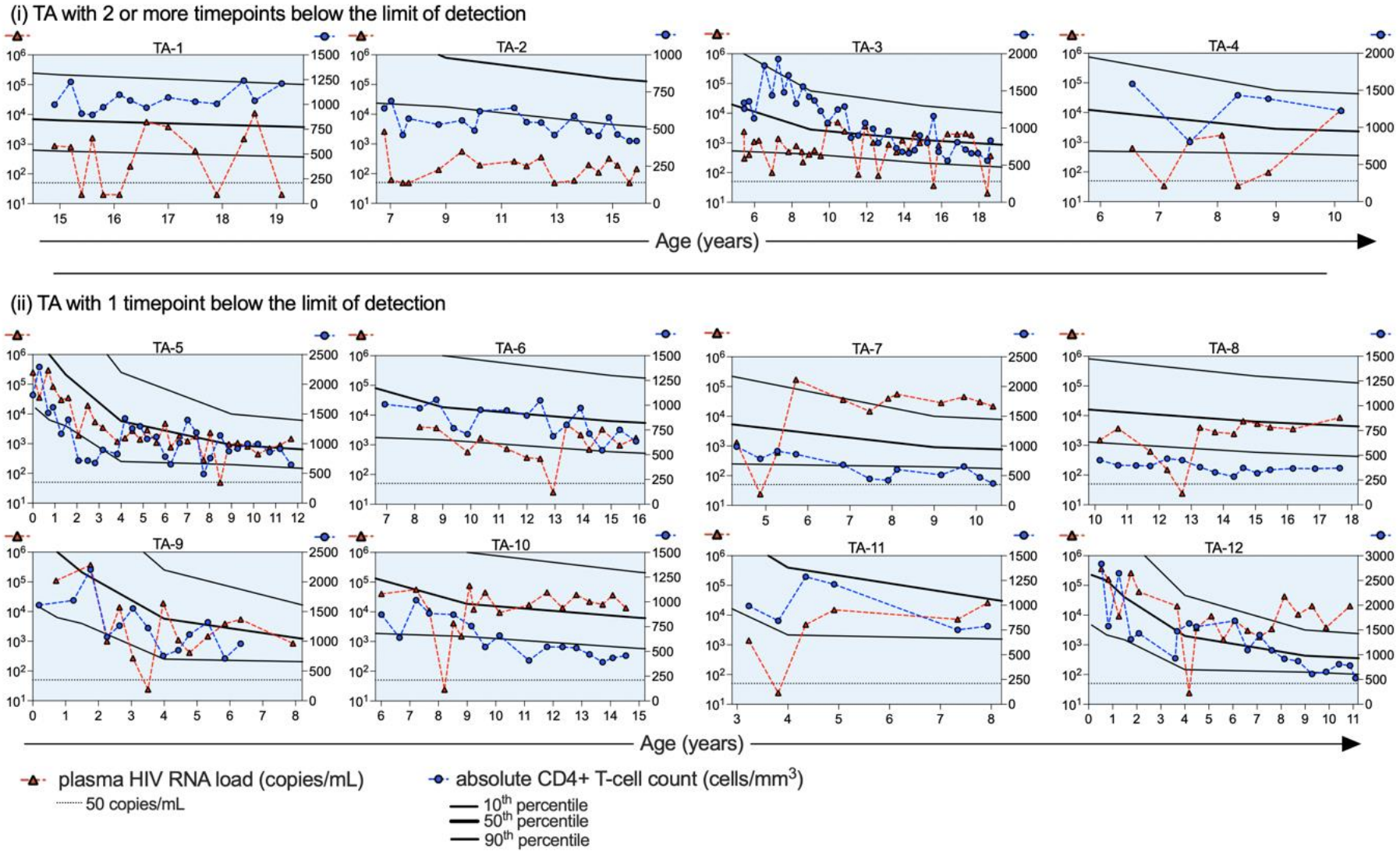
6.3.1. Longitudinal follow-up of Transient Aviraemics

We identified twenty-one children/adolescents who had at least one timepoint with viraemia below the limit of detection, however, without fulfilling the criteria for elite control (Table 6.1 and Figure 6.1).

In contrast to PECs, TAs were more evenly distributed among female and male sex (13F:8M) and the age at aviraemia widely varied from 1.8 to 15.1 years (median 7.4, IQR 4.6 – 12.4). Although 81% had an absolute CD4+ T-cell count above the 10^{th} percentile, five individuals experienced a sharp CD4+ T-cell decline and progressed after viral rebound. Four had transiently suppressed the virus for more than one timepoint (TA-1 to TA-4), but these were not consecutive or spanned less than one year. In TAs, the plasma viral load was frequently below 10,000 copies/mL.

Table 6.1 Characteristics of the TAs.

PID	Country of origin	Sex	Clinic of origin	Age at aviraemia (years)
TA-1	South Africa	Male	Durban, AS	15.4
TA-2	Zimbabwe	Female	London, UK	7.5
TA-3	Somalia	Female	Stockholm, Sweden	6.9
TA-4	South Africa	Male	Kimberley, SA	7.1
TA-5	Brazil	Male	São Paulo, Brazil	8.5
TA-6	South Africa	Female	Kimberley, SA	12.9
TA-7	South Africa	Female	Kimberley, SA	4.9
TA-8	South Africa	Female	Kimberley, SA	12.7
TA-9	South Africa	Male	Kimberley, SA	3.5
TA-10	South Africa	Female	Kimberley, SA	8.2
TA-11	South Africa	Male	Kimberley, SA	3.8
TA-12	South Africa	Female	Kimberley, SA	4.2
TA-13	South Africa	Male	Kimberley, SA	7.0
TA-14	South Africa	Female	Kimberley, SA	10.9
TA-15	South Africa	Female	Kimberley, SA	12.1
TA-16	South Africa	Female	Kimberley, SA	6.1
TA-17	South Africa	Female	Kimberley, SA	15.1
TA-18	South Africa	Female	Kimberley, SA	1.8
TA-19	South Africa	Male	Kimberley, SA	4.4
TA-20	South Africa	Female	Kimberley, SA	7.4
TA-21	South Africa	Female	Durban, SA	16.9


(i) TA with 2 or more timepoints below the limit of detection
TA-1
TA-2
TA-3
TA-4
Age (years)
(ii) TA with 1 timepoint below the limit of detection
TA-5
TA-6
TA-7
TA-8
TA-9
TA-10
TA-11
TA-12
Age (years)
plasma HIV RNA load (copies/mL)
50 copies/mL
absolute CD4+ T-cell count (cells/mm3)
10th percentile
50th percentile
90th percentile

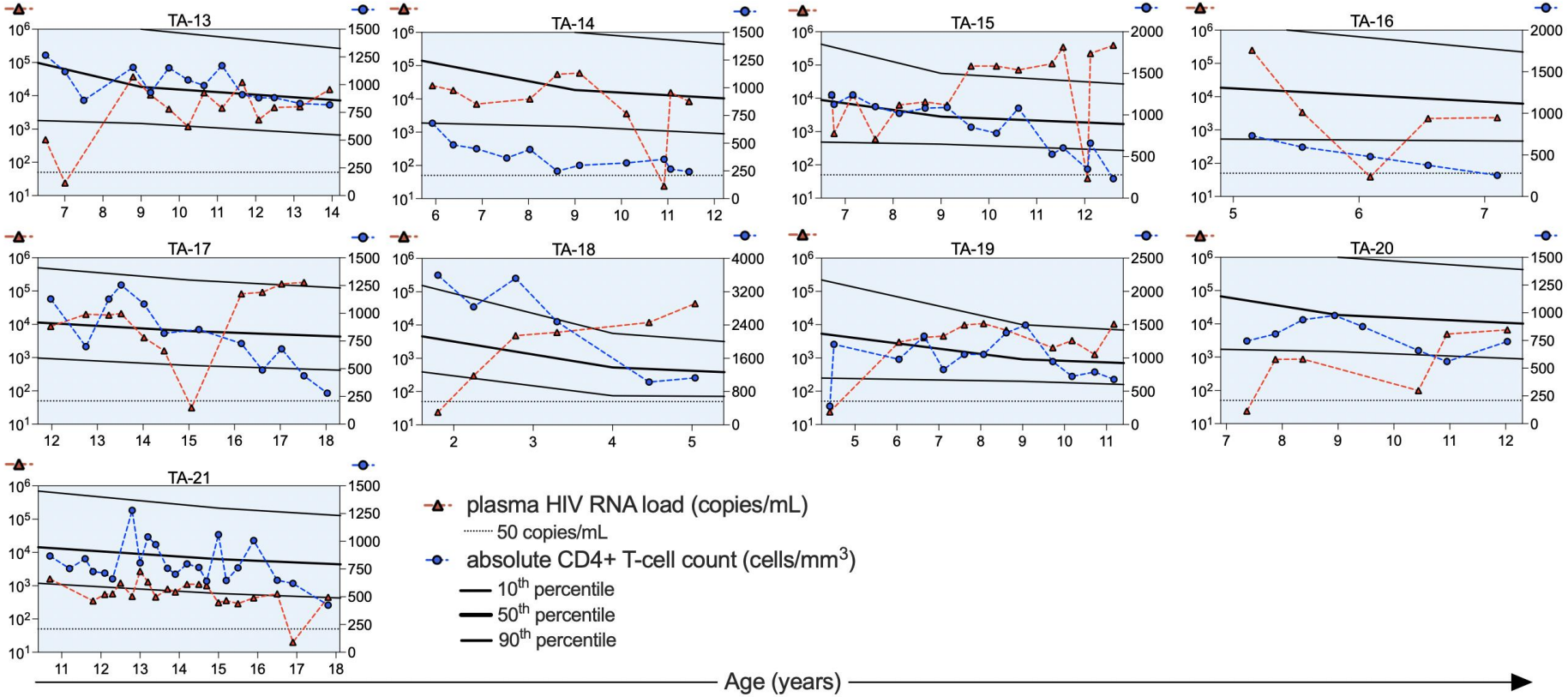


Figure 6.1 Longitudinal clinical data.

Plasma HIV-RNA load (red triangle) and absolute CD4+ T-cell count (blue circle) are shown for the 21 TAs. The horizontal dashed line for plasma HIV-RNA copies represents the limit of 50 copies/mL. The 10th, 50th and 90th percentiles for absolute CD4+ T-cell count for HIV-uninfected children are represented by the three grey lines.

6.3.2. Loss of virological control associated with selection of an escape variant

To look at the mechanism leading to viral rebound, we selected three individuals (TA-1, TA-10 and TA-12) with longitudinally available samples expressing the HLA-B*81:01 allele, a well-known adult defined disease-protective HLA-I (Pereyra et al. 2008; Pereyra et al. 2010; Leslie et al. 2010; Carlson et al. 2012).

TA-10 and TA-12 were both female ART-naive children from Kimberley, SA. In both these cases, we observed transient aviraemia followed by viral rebound and CD4+ T-cell decline. TA-10 reached aviraemia at the age of 8.2 years, within 3 months rebounding to a plasma viral load of 4,100 copies/mL (Figure 6.2A). Within 9 months, her viral load had returned to the pre-existing setpoint (median of 17,294 copies/mL), which was now followed by a progressive decline in her CD4+ T-cell count. TA-12 was followed from birth (Figure 6.2B). Similar to TA-10, but at a younger age, TA-12 reached undetectable viraemia at the age of 4.2 years, within 3 months rebounding to 3,100 copies/mL. Within 9 months, a new viral setpoint was maintained at 9,100 copies/mL, and as in TA-10, this was followed by a decline in the CD4+ T-cell count. TA-1, an older male from Durban, SA, followed from 14.8yrs, reached undetectable viraemia four times across 4.5 years of follow-up, but without achieving the criteria for EC (Figure 6.2C). His median plasma viraemia was 700 copies/mL and he maintains a healthy CD4+ T-cell count to date, most recently 1200 CD4+ T cells/mm^3. Whereas TA-10 and TA-12 have both now started ART, TA-1 has, despite medical advice, opted to remain ART-naïve.

We had previously reported the results of population sequencing of the virus encoding the immunodominant HLA-B*81:01-restricted Gag epitope TPQDLNTML (Gag 180-188, 'TL9') from two timepoints available in TA-10 and TA-12 (Leitman et al. 2017). To achieve better resolution of the kinetics of viral selection pressure in parallel with HIV-specific CD8+ T-cell response, we here deep-sequencing a further five and seven timepoints, respectively, in TA-10 and TA-12, and 4 timepoints for TA-1. In TA-10, following the initial timepoint studied at 8.3yrs, the viral sequence became 100% dominated by the Q182G variant (Figure 6.2D). In TA-12, where follow up extended over more than 11 years from infancy, the Q182G variant also reached 100% fixation, but in this case via Q182T and subsequently Q182S (Figure 6.2E). By contrast, TA-1 deep sequencing of the virus demonstrated no evidence of any selection of escape mutants in Gag-TL9, despite these multiple viral rebounds (Figure 6.2F). Both TA-10 and TA-12 children also selected escape mutants within the HLA-B*81:01-restricted Pol epitope SPIETVPVKL (Pol 158-167, 'SL10') and Vpr epitope FPRIWLHGL (Vpr 34-42, 'FL9). Escape mutants also arise in Pol epitopes SPAIFQSSM (Pol 311-319, 'SM9') for TA-10 and TPVNIIGRNLL (Pol 136-146, 'TL11') for TA-12 (Figure 6.3A and B). Similarly, we did not find any evidence in TA-1 of escape mutations in any of the known HLA*81:01 restricted epitopes, except for the G41S variant in the Vpr FL9 (Figure 6.3C)

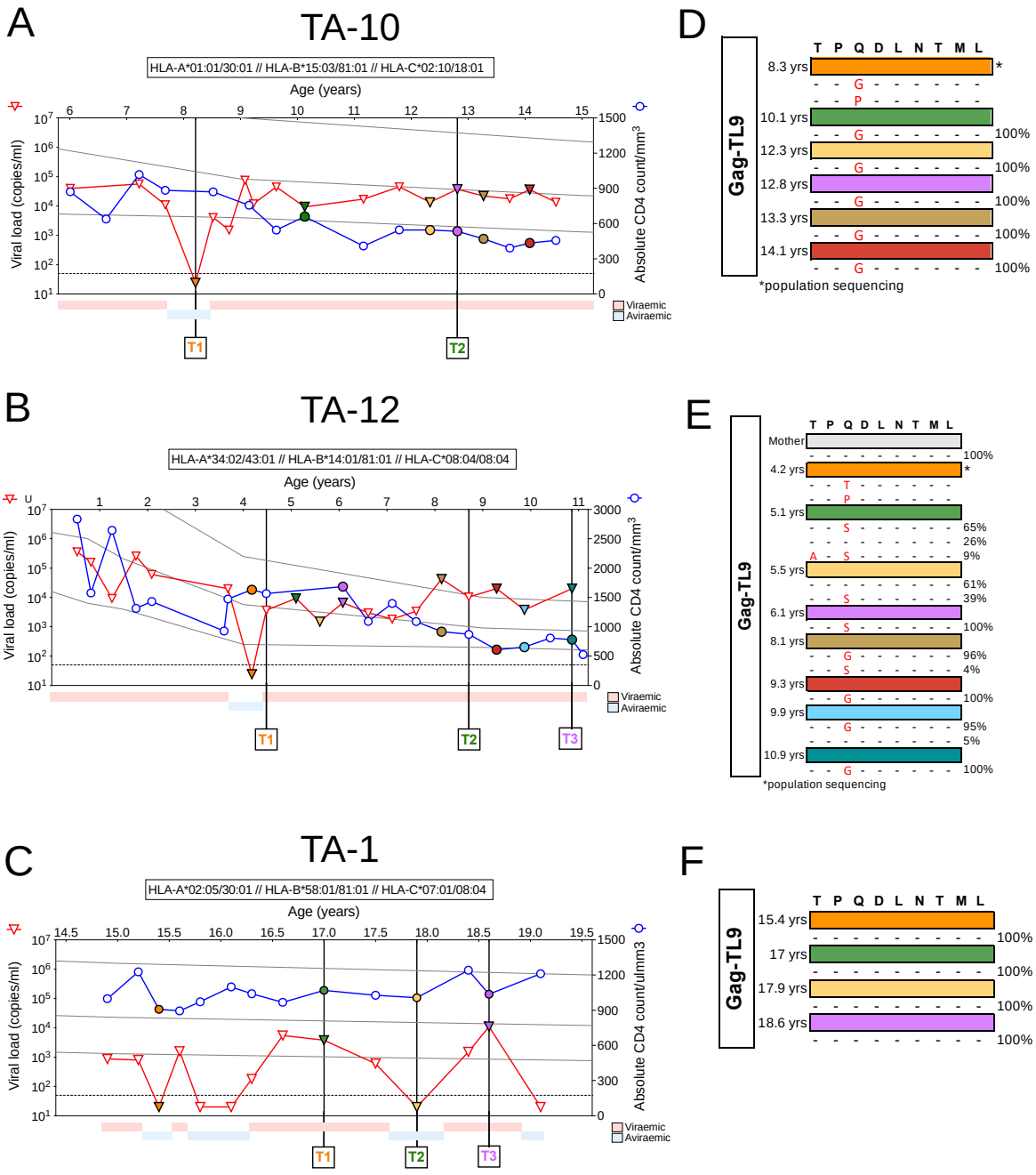
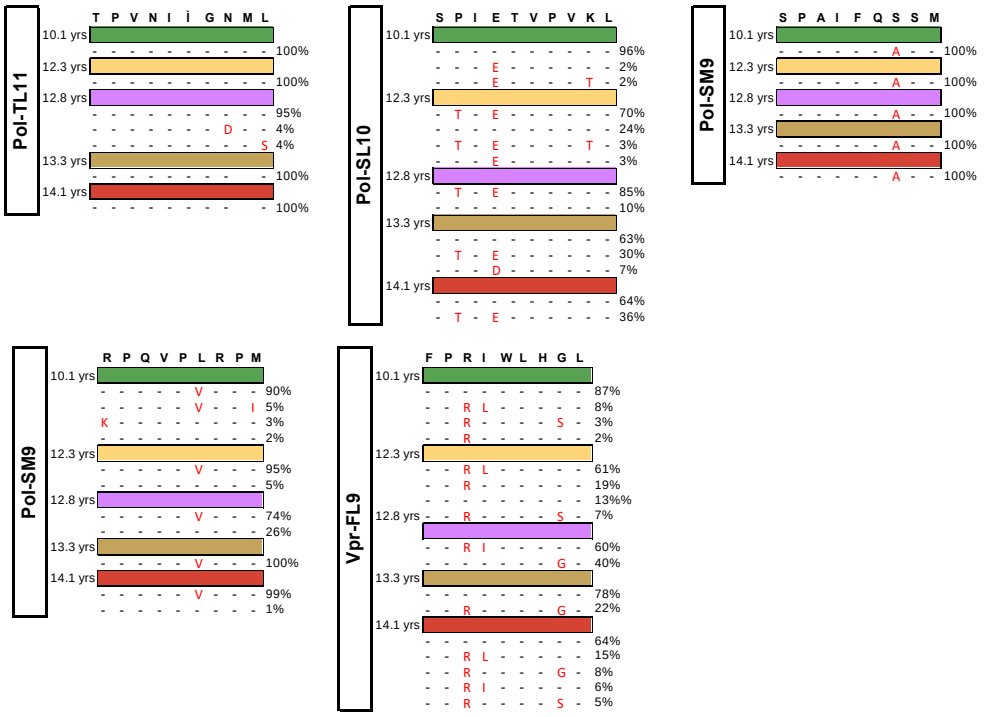


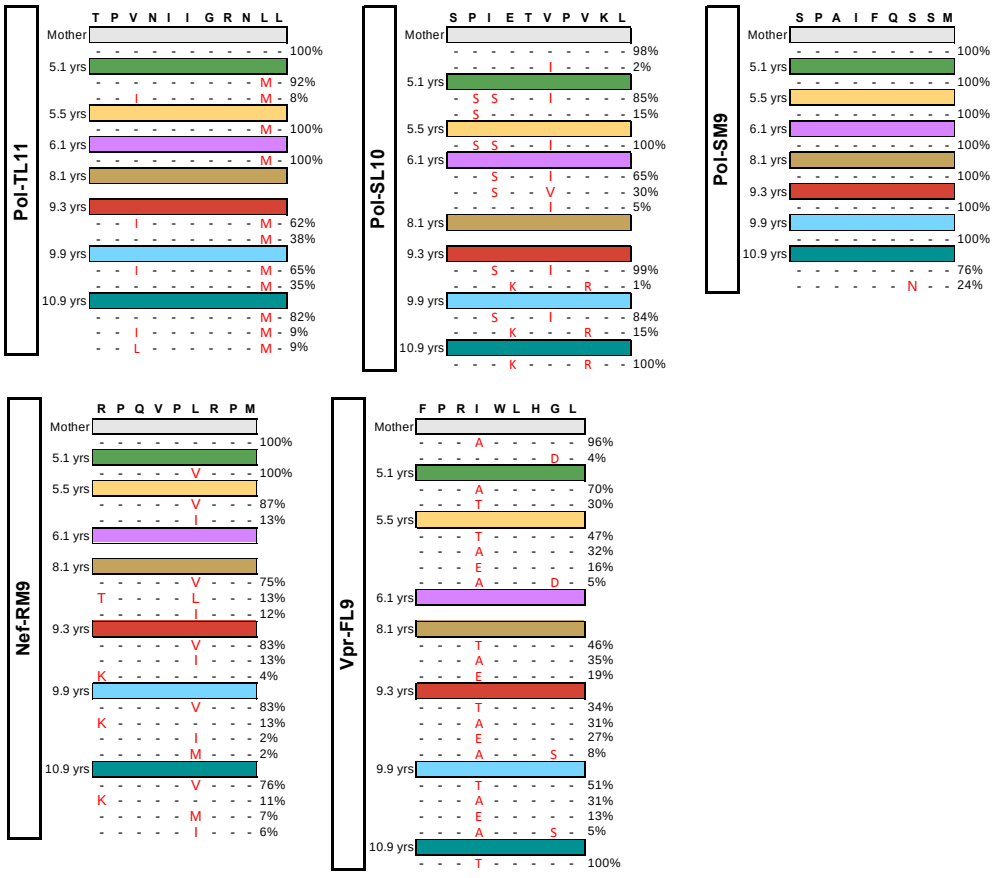
Figure 6.2 Longitudinal clinical data and escape in the dominant TL9-Gag epitopes after transient aviraemia.

Plasma HIV-RNA load (red triangle) and absolute CD4+ T-cell count (blue circle) are shown for the TA-10 (A), TA-12 (B) and TA-1 (C). The horizontal dashed line for plasma HIV-RNA copies represents the limit of 50 copies/mL. The 10th, 50th and 90th percentiles for absolute CD4+ T-cell count for HIV-uninfected children are represented by the three grey lines. D to F. Population or deep sequencing are shown for the TL9-Gag epitope. Timepoints are matched by colour. T1 to T3 are the timepoints selected for the avidity assay in Figures 6.5 and 6.6.

A TA-10


Pol-TL11
T P V N I İ G N M L
10.1 yrs 100%
12.3 yrs 100%
12.8 yrs 95%
D 4%
S 4%
13.3 yrs 100%
14.1 yrs 100%
Pol-SL10
S P I E T V P V K L
10.1 yrs 96%
E 2%
E T 2%
12.3 yrs 70%
24%
T E T 3%
E 3%
12.8 yrs 85%
10%
13.3 yrs 63%
T E 30%
D 7%
14.1 yrs 64%
T E 36%
Pol-SM9
S P A I F Q S S M
10.1 yrs A 100%
12.3 yrs A 100%
12.8 yrs A 100%
13.3 yrs A 100%
14.1 yrs A 100%
Pol-SM9
R P Q V P L R P M
10.1 yrs V 90%
V I 5%
K 3%
2%
12.3 yrs V 95%
5%
12.8 yrs V 74%
26%
13.3 yrs V 100%
14.1 yrs V 99%
1%
Vpr-FL9
F P R I W L H G L
10.1 yrs 87%
R L 8%
R S 3%
R 2%
12.3 yrs R L 61%
R 19%
13%%
12.8 yrs R S 7%
R I 60%
G 40%
13.3 yrs 78%
R G 22%
14.1 yrs 64%
R L 15%
R G 8%
R I 6%
R S 5%

B TA-12


Pol-TL11
T P V N I I G R N L L
Mother 100%
5.1 yrs M 92%
I M 8%
5.5 yrs M 100%
6.1 yrs M 100%
8.1 yrs
9.3 yrs I M 62%
M 38%
9.9 yrs I M 65%
M 35%
10.9 yrs M 82%
I M 9%
L M 9%
Pol-SL10
S P I E T V P V K L
Mother 98%
I 2%
5.1 yrs S S I 85%
S 15%
5.5 yrs S S I 100%
6.1 yrs S I 65%
S V 30%
I 5%
8.1 yrs
9.3 yrs S I 99%
K R 1%
9.9 yrs S I 84%
K R 15%
10.9 yrs K R 100%
Pol-SM9
S P A I F Q S S M
Mother 100%
5.1 yrs 100%
5.5 yrs 100%
6.1 yrs 100%
8.1 yrs 100%
9.3 yrs 100%
9.9 yrs 100%
10.9 yrs 76%
N 24%
Nef-RM9
R P Q V P L R P M
Mother 100%
5.1 yrs V 100%
5.5 yrs V 87%
I 13%
6.1 yrs
8.1 yrs V 75%
T L 13%
I 12%
9.3 yrs V 83%
I 13%
K 4%
9.9 yrs V 83%
K 13%
I 2%
M 2%
10.9 yrs V 76%
K 11%
M 7%
I 6%
Vpr-FL9
F P R I W L H G L
Mother A 96%
D 4%
5.1 yrs A 70%
T 30%
5.5 yrs T 47%
A 32%
E 16%
A D 5%
6.1 yrs
8.1 yrs T 46%
A 35%
E 19%
9.3 yrs T 34%
A 31%
E 27%
A S 8%
9.9 yrs T 51%
A 31%
E 13%
A S 5%
10.9 yrs T 100%

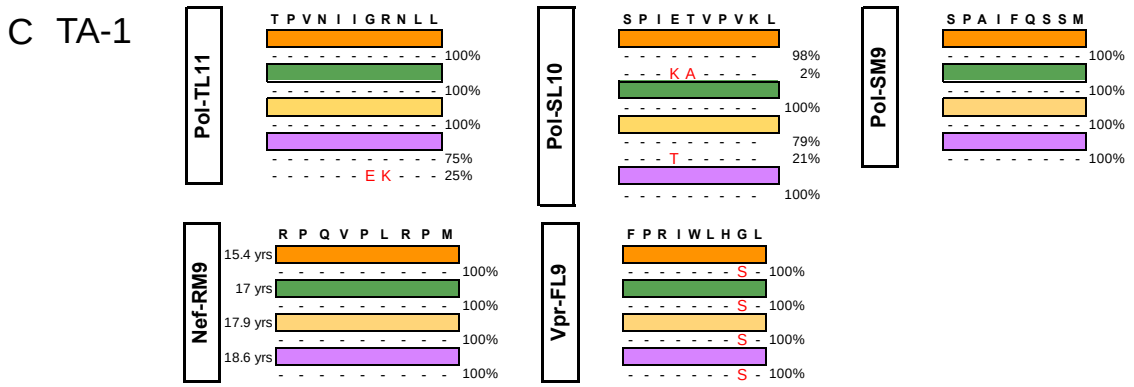


Figure 6.3 Deep sequencing for HLA-B*81:01 targeted epitopes.
Deep sequencing data are shown for well-known HLA-B*81:01 targeted epitopes for TA-1 (A), TA-2 (B), and TA-1 (C). Timepoints are matched by Figure 1 colours.

To investigate why these particular TL9 escape mutants arose in TA-10 and TA-12 and, in the case of Q182G, were maintained, we evaluated the impact of the TL9 escape variants on virus replication, comparing variant viral growth against either NL4-3 or a chimeric consensus C clade Gag-Pro/NL4-3 virus (Figure 6.4A and B). The Q182P variant was detected as a low-frequency variant at the aviraemic timepoint in both TA-10 and TA-12 via population proviral DNA sequencing, but because plasma viral RNA at these aviraemic timepoints could not be amplified, no RNA sequence was available. The Q182P variant reduced viral replication capacity to beneath detectable limits, explaining its temporary appearance only. The more common TL9 escape variants decreased VRC but less dramatically, without crippling the virus, in both B and C clade Gag backbones, but the VRC reductions were broadly similar for all of these more common variants. Hence the selection of Q182G rather than Q182T or Q182S is likely the result of the relative benefit of the escape mutant in evading the CTL response.

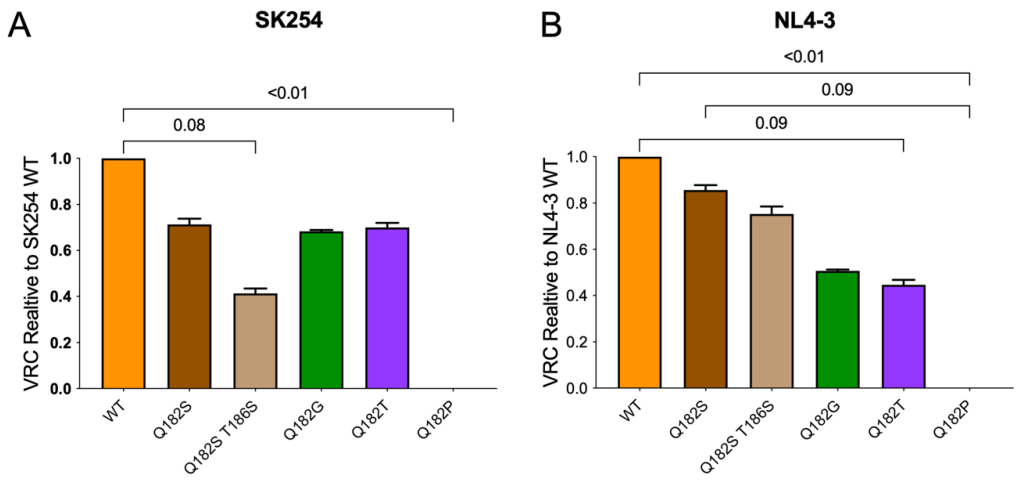


Figure 6.4 Lower VRC in TL9-escape variants.
Viruses containing a single Q182X mutation or a Q182S/T186S were constructed, and VRC measured compared to the growth of a WT virus in C clade (A) and B clade (B) infection. Triplicates were compared was done using Friedman test to obtain the exact p-value.

To investigate this further, we compared the functional avidity of CTL responses to the WT TL9 at early timepoints with that of the Q182G-specific response at later timepoints. In both TA-10 and TA-12, the functional avidity of the Q182G-specific response was substantially lower, with an SD50 1.03 and 2.62 log10 higher than that of WT during viraemic control (Figure 6.5A and B). This lower functional avidity of the Q182G-specific CTL compared with WT TL9-specific CTL is therefore consistent with the failure to control viraemia and to prevent CD4+ T-cell decline in these two TAs, despite initially cornering the virus via the variant-specific CTL response. These findings are also consistent with studies of HLA-B*81:01-positive adults showing emergence of both Q182S and Q182G variants in association with HIV disease progression (Ntale et al. 2012).

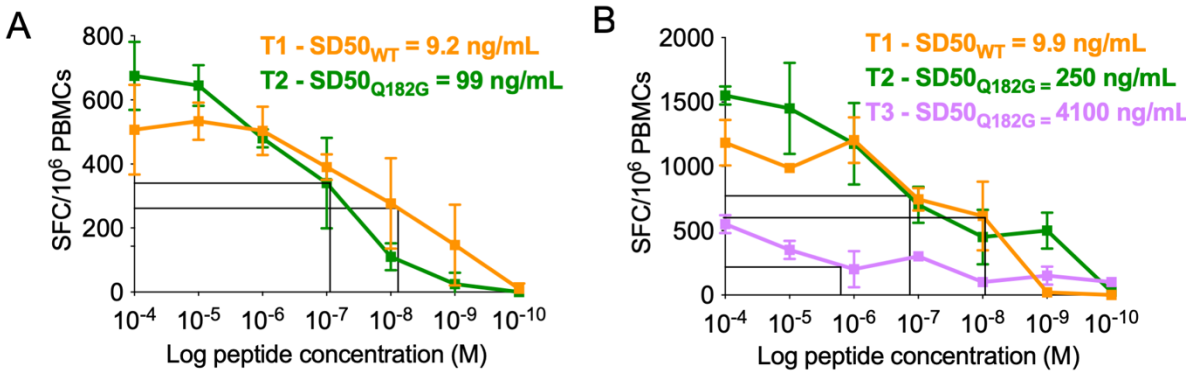


Figure 6.5 Lower avidity in the Q182G-specific CD8+ T-cell response.
Avidity curves of the dominant TL9 CD8+ T-cell response determined by IFN-γ ELISPOT assay are shown for the wild-type (WT) and selected escape Q182G for TA-10 (A) and TA-12 (B) in different timepoints.

6.3.3. Loss of virological control associated with fluctuations in the quality of HIV-specific CD8+ T-cell response

To identify the mechanism of viral rebound in TA-1, we investigated whether functional differences in HIV-specific CD8+ T-cell responses were present at aviraemic timepoints compared with subsequent timepoints when viral rebound had occurred. Viral rebound was associated with a >3-fold decrease in the magnitude of the TL9-specific IFN-γ secreting CD8+ T-cell ELISPOT response and >2-fold decrease in the ICS response magnitude. Functional avidity of the TL9 response was also somewhat decreased at viraemic timepoints (Figure 6.6). Degranulation (CD107a), IFN-γ and MIP-1β expression were all decreased during the periods of viral rebound, and responses were less polyfunctional (defined as 2 or more functions) during the viraemic timepoints (Figure 6.7A). As previously observed in paediatric non-progressors (Muenchhoff et al. 2016), despite changes in plasma viral load, levels of immune activation (HLA-DR+CD38+) were unaltered, and exhaustion (PD-1+ and CD39+) markers likewise were unaffected. High levels of CD73 were also unchanged by fluctuations in viraemia. When looking the viraemic period, TA-10 and TA-12 also showed a lower proportion of Gag-specific CD8+ T-cells performing more

than 2 functions, a higher frequency of TEMRA cells and higher levels of CD38+HLA-DR+ expression on CD8+ T-cells (Figures 6.7B and C) when compared to TA-1.

In contrast to TA-10 and TA-12, where attainment of aviraemia had occurred once only, and was followed by CTL escape, persistent viraemia and CD4+ T-cell decline, TA-1 transitioned from total and partial control of viraemia in association with fluctuations in the quality of the CD8+ T-cell response, no selection of CTL escape, and maintenance of highly stable and normal-for-age absolute CD4+ T-cell counts.

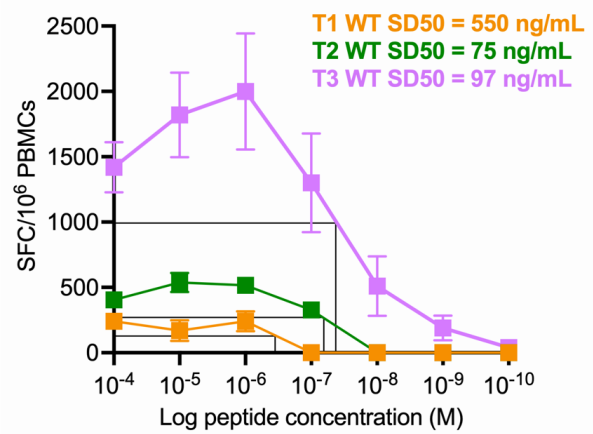


Figure 6.6 Functional avidity of the TL9-specific CD8+ T-cell response.
Avidity curves of the dominant TL9-specific CD8+ T-cell response determined by IFN-γ ELISPOT assay are shown for the WT for TA-1 in different timepoints.

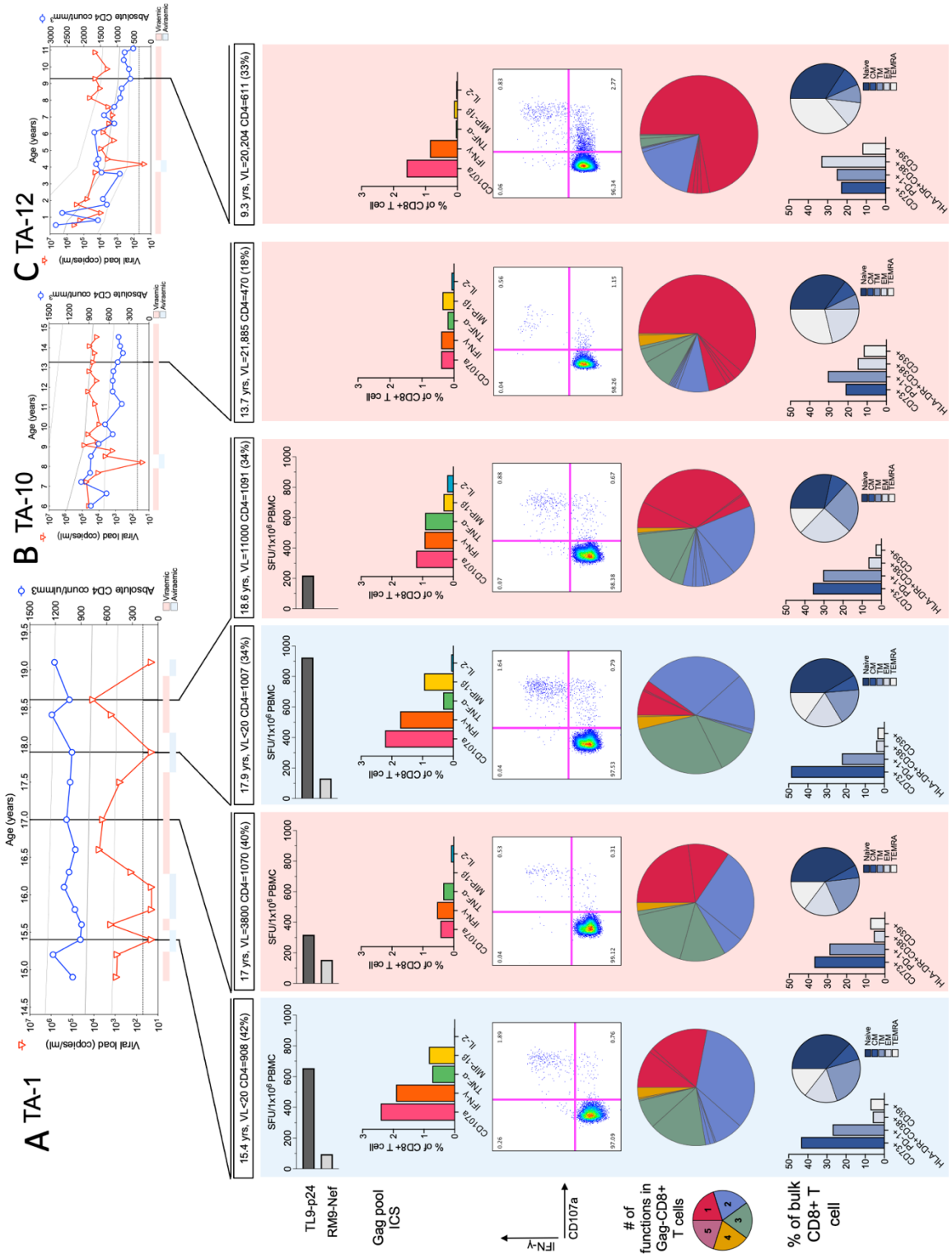

A TA-1
B TA-10
C TA-12
Age (years)
Viral load (copies/ml)
Absolute CD4 count/ulmm3
Viraemic
Aviraemic
15.4 yrs, VL<20 CD4=908 (42%)
17 yrs, VL=3800 CD4=1070 (40%)
17.9 yrs, VL<20 CD4=1007 (34%)
18.6 yrs, VL=11000 CD4=1091 (34%)
13.7 yrs, VL=21,885 CD4=470 (18%)
9.3 yrs, VL=20,204 CD4=611 (33%)
SFU/1x10^6 PBMC
TL9-p24
RM9-Nef
Gag pool ICS
% of CD8+ T cell
CD107a
IFN-γ
TNF-α
MIP-1β
IL-2
# of functions in Gag-CD8+ T cells
% of bulk CD8+ T cell
Naive
CM
TM
EM
TEMRA
CD27+
PD-1+
HLA-DR+CD38+
CD39+

Figure 6.7 Fluctuations on the magnitude and quality of CD8+ T-cell response determine viral control during transient aviraemia in TA-1.
A to C. Longitudinal data showing plasma HIV-RNA load (red triangle) and absolute CD4+ T-cell count (blue circle) for TA-1 (A), TA-10 (B) and TA-12 (C). Magnitude of TL9 CD8+ T-cell response determined by IFN-γ ELISPOT assay are shown for TA-1, followed by intracellular cytokine staining of CD107a, IFN-γ, TNF-α, MIP-1β and IL-2; polyfunctionality showing co-expression of markers on CD8+ T-cells after Gag-stimulated PMBCs; memory subset distribution; and expression of activation/exhaustion markers (CD73, PD-1, CD38, HLA-DR and CD39).

6.4. Discussion

In this study, we investigated PSPs who transiently controlled viraemia to undetectable plasma viral load. Although the vast majority rebounded after a single timepoint, four individuals were aviraemic during multiple timepoints without fulfilling the criteria of an EC. Looking at the same cohorts described in Chapter 5, we found that TAs are more prevalent than PECs and more evenly distributed between sex. We then investigated three TAs expressing the disease-protective HLA-B*81:01 and identified two distinct mechanisms linked to loss of viral control: (i) the selection of an escape variant that partially evaded the CD8+ T-cell response leading to viral rebound (TA-10 and TA-12), and (ii) fluctuations in the magnitude and quality of the CD8+ T-cell response influencing the levels of viral control (TA-1).

Even in an era prior universal treatment, the observation of TAs in ART-naive children has been rarely noted in paediatric infection. A possible reason is the low frequency and regular monitoring of plasma viraemia at dedicated outpatient follow-up facilities in the more poorly resourced SSA countries where greater than 90% of children living with HIV are based. The mechanisms underlying TA remain unknown. It is possible that a proportion of these children would eventually become PECs, but in the vast majority, an undetectable plasma viral load is followed by a viral rebound above 10,000 copies/mL. However, it is of interest that among these paediatric TAs,

eight of 21 (38%) are boys, a proportion similar to that among PSPs (41%) (Mori et al. 2015) and higher than among PECs (Chapter 5). Numbers are too small to be definitive, but these suggest the possibility that male children might be more susceptible to viral rebound once aviraemia has been achieved compared with female children.

Through childhood, the HIV-specific CD8+ T-cell response becomes increasingly more effective at killing infected targets and progressively strengthens pressure for the selection of CTL escape mutants (Leitman et al. 2017). Previously, our group demonstrated that PSPs with immune control of HIV had lower magnitude and breadth of HIV-specific CD8+ T-cell responses when compared to viraemic non-controllers, but effectively targeted conserved epitopes in Gag and Pol, adopting a cornering-strategy by mounting a new response to the selected escaped variants (Leitman et al. 2017). This strategy drives the virus down an evolutionary cul-de-sac with progressive loss of viral fitness that constantly need to escape further to avoid variant-specific CTL responses. TA-10 and TA-12 are clear examples of the cornering strategy seen in children, who mounted a response against the selected variants of Gag-TL9 epitope and induced the fixation of the Q182S and Q182G mutants with lower replicative capacity. However, in these children the new responses failed to control viraemia completely and led to a progressive decline in the CD4+ T-cell count. In neither of these two cases was the more frequently-observed escape mutant at Gag-186, T186S or, less often, T186M (Carlson et al. 2012; Huang et al. 2008; Kløverpris et al. 2015) selected, that reduce VRC substantially more than the common variants at Gag-182 (Tsai et al. 2016; Leitman et al. 2017). This may account for their short period of viraemic control in these two children and subsequent CD4+ T-cell decline. In these two cases the escape mutant selected did not damage viral replication excessively and the variant-specific responses had lower functional avidity compared

with the WT-specific responses had been. In an HLA-B*81:01-positive child described previously (Leitman et al. 2017), where cornering of the virus had been described in association with maintained normal-for-age CD4+ T-cell counts, T186M had been selected, crippling viral replication, and at the same time the variant-specific response had the same functional avidity as the WT-specific response.

The mechanism identified in TA-1 seems to be more sustained in the long-term. In this case the child was enrolled at the age of 14 years and had reached adolescence with a healthy CD4+ T-cell count and a median plasma viral load below 1000 copies/mL. The periods of detectable viraemia were marked by a decrease in both the magnitude and quality of the specific CD8+ T-cell response rather than by the selection of an escape variant. The mechanism seen here is reminiscent of that described in PECs, where viral control is mediated by a highly effective polyfunctional Gag-specific CD8+ T-cell response while maintaining low immune activation and exhaustion levels (Chapter 5). This finding is consistent with previous work in adults showing improved viral control in association with superior qualitative aspects of the immune response (Migueles et al. 2003; Migueles et al. 2002; Sáez-Cirión et al. 2007; Almeida et al. 2007; Betts et al. 2006). Indeed, most adults living with HIV expressing the protective HLA-B*57 allele progress to AIDS despite having access to same HLA-B*57-restricted epitopes as HLA-B*57-positive ECs (Pereyra et al. 2008). The polyfunctionality of the HIV-specific response is a common feature of adult ECs, irrespective of the HLA-I molecules expressed (Migueles et al. 2015).

It is important to note the limitations of the current study. First, the cases described are necessarily anecdotal, given the rarity of elite control and even transient aviraemia in paediatric infection. Second, because of sample availability, we have had to focus on the immunodominant HLA-B*81:01-restricted TL9 response rather than

evaluating other virus-specific responses. Despite this, the deep sequencing approach allowed us to observe differences between TA-10/TA-12 and TA-1 in terms of escape mutants across all the HLA-B*81:01-restricted CTL epitopes. Finally, the altered magnitude and quality of the CTL responses in TA-1 in association with fluctuations in viraemia could be the cause or consequence of viraemic changes. Nonetheless, the immunogenetic data showing that HLA-B*81:01 protects against HIV disease progression support the hypothesis that the HLA-B*81:01-restricted TL9 responses strongly influence immune control of HIV (Goulder and Walker 2012).

Even if anecdotal, the cases described add new insights into the mechanisms of viraemic control in children (Leitman et al. 2017; Adland et al. 2015; Muenchhoff et al. 2016). The cornering approach is more commonly observed in children than in adults (Leitman et al. 2017) and has been previously modelled as a vaccine strategy (Ferguson et al. 2013) by leading the virus down an evolutionary cul-de-sac to a variant of low replicative capacity that is still well-recognised by a variant-specific CTL response. However, if the variant is sufficiently 'fit', and the variant-specific response is less efficacious, HIV disease progression will result. A robust HIV-specific T-cell response of high quality that is capable of efficiently controlling viral replication would appear to be more sustainable in the long-term. Although in TA-1 the common escape variants are less well-seen than the WT, it may be that when the CTL response is most efficacious, replication is suppressed and variants are not generated, and when the CTL response is less potent, the small selective advantage of the escape mutant is outweighed by the high cost to viral replicative capacity. These findings support the development of therapeutic models that prioritise induction of high-quality, robust, and polyfunctional HIV-specific T-cell responses that target immunodominant and conserved epitopes.

7

Conclusions

The year of this thesis completion – 2021 – marks the 40 years of the first case report of HIV/AIDS in 1981 by the CDC (Gottlieb et al. 1981) and the start of a pandemic that devastated entire populations and has killed more than 32 million people globally (UNAIDS 2020). Although we now have the tools to end HIV/AIDS pandemic and interrupt MTCT, nearly 700,000 people die anually from AIDS and 150,000 children were newly diagnosed with HIV in 2019 (UNAIDS 2020).

This year also marks one year of Timothy Brown's death (from a relapse of his leukaemia), also known as the "Berlin patient" and the first individual considered cured of HIV back in 2008 (Hütter et al. 2009). To date, only Timothy Brown and, more recently, the "London patient" (Gupta et al. 2019) have been considered cured, and no replicant competent virus has been found after treatment interruption. Both patients underwent bone-marrow transplant from a donor with a homozygous CCR5 delta-32 mutation after chemotherapy due to associated haematological malignancy. Albeit an undeniable accomplishment, the complex road to achieving cure in these two cases seems unlikely to be implemented on a populational scale.

Meanwhile, reports of viral remission solely achieved by the early start of ART both in the adult (Lisziewicz et al. 1999; Sáez-Cirión et al. 2013; Namazi et al. 2018) and

the paediatric (Persaud et al. 2013; Frange et al. 2016; Violari et al. 2019) populations were received with great enthusiasm by the scientific community. In contrast to "true" cure, remission means undetectable plasma HIV-RNA by the standard assays in the absence of therapy, while HIV-DNA remains at low levels in latently infected cells. After the "Mississippi baby" report (Persaud et al. 2013), clinical studies were designed to replicate this case by diagnosing HIV infection soon after birth and starting therapy in a very early stage of the disease course (Millar et al. 2020; Kuhn et al. 2020; Garcia-Broncano et al. 2019). Part of the scientific community believes remission might be easier to achieve in the context of intrauterine transmission. First, most infections arise late in pregnancy (Rouzioux et al. 1995), and ART can be initiated immediately after birth, hindering viral seeding and diversity at an early stage. The tolerogenic immune system in early life (Kollmann et al. 2009; Levy 2007; Michaëlsson et al. 2006) associated with early ART can protect the immune system from inflammation, also contributing to restrain viral reservoir. Moreover, the virus transmitted to the newborn is generally of lower replicative capacity (Adland et al. 2015; Naidoo et al. 2017) and possibly easier to control after ATI. Unfortunately, most studies showed that starting ART in the neonatal period does not result in post-treatment control for the vast majority (Viganò et al. 2006; Cotton et al. 2013; Prendergast et al. 2008), even when started within the first hours after birth (Millar et al. 2020; Kuhn et al. 2020).

Although, with rare exceptions, remission will not be largely achieved by ART alone, children early suppressed for years will have a very limited size and diversity of viral reservoir by the time other immune interventions can be implemented and advantage can be taken of a more mature immune system. The ideal age to implement a new therapy and stop ART is uncertain, but optimally it should happen before late childhood and adolescence, when the struggle with adherence hit its hardest.

Interestingly, this exact period between 4 and 12 years is called the "honeymoon period of infectious diseases" by immunologists (Ahmed, Oldstone, and Palese 2007), during which outcome from many infections is superior to that observed in younger and older age groups. Although being equally susceptible to infection, children at this stage have lower mortality and morbidity from several other infections including tuberculosis, influenza, mumps, measles, and Varicella-Zoster (Langford 2002; Ahmed, Oldstone, and Palese 2007). Partly for this reason, it has not been surprising that children have been overall undamaged by the SARS-CoV-2 pandemic (Havers et al. 2021; Dong et al. 2020; Rankin et al. 2021). Although not yet completely elucidated, this period of life comprises the perfect balance between developing an immune response and the harmful damage it may provoke. The innate response and the priming of the adaptive response are likely more efficient and better regulated at this age than in adults.

The existence of PTCs shows clear evidence that remission can be achieved, and they provide a model to identify potential interventions necessary to scale up to the population level. Through cohorts of PTCs, it is clear that they are not future ECs who started ART, but a subset of individuals who, for reasons that remain unclear, controlled viral replication after treatment interruption (Namazi et al. 2018; Sáez-Cirión et al. 2013).

In this thesis, I had access to a unique population of children living with HIV representing various disease phenotypes that delineate different aspects of host and pathogen interaction leading up to a range of disease outcomes that can help us understand how remission can be achieved in children. Using a large cohort of PSPs, we identified an inherited signature associated with slower HIV disease progression and better viraemic control, directly shedding light on the NK cells' potential in

future therapeutic interventions (Chapter 3). Next, using unique samples of early-treated children who underwent ATI, we could identify aspects of the T-cell development that led to slow clinical progression in children and that is associated with the emergence of an HIV-specific CTL population with stem-like properties and enormous potential to be exploited in T-cell boosting strategies (Chapter 4). Finally, albeit anecdotally, we could for the first time delve into the mechanisms behind the rare group of children who spontaneously control viraemia and how they differ from adult ECs, including detailed analyses of the T-cell compartment of four PECs (Chapter 5) and the mechanism of viral rebound in TAs (Chapter 6).

NK cells play an essential role in antiviral immunity, including immunological control of HIV (Scully and Alter 2016), but it had been scarcely explored in the paediatric population. In Chapter 3, we made a detailed study of the immunogenetic markers regulating NK cells and the stage of maturation and function of the NK cell population in the context of different paediatric HIV disease outcomes. We found a clear signature favouring a KIR-education program associated with slow progression and viraemic control in children while reinforced the dwarfed effect of the adult protective HLA-B molecules linked with CTL activity. This inherited signature can be further explored in cohorts of long-term suppressed children to identify those with higher potential to become PTCs. Unpublished data from the VISCONTI cohort shows a potential role of NK cells in PTCs (Scott-Algara et al. 2015). Previously, NK cells expressing NCRs were correlated with the size of intact proviral HIV-DNA in a cohort of very early treated babies (Garcia-Broncano et al. 2019). Our findings also reinforce the detrimental role of HLA-A expression in HIV control via NKG2A mediated-inhibition of NK cells (Ramsuran et al. 2018). NK2GA blockade is now being explored in cancer and can potentially be a tool in the HIV field to unleash NK cells and boost their antiviral activity (van Hall et al. 2019). Other therapeutic

strategies being explored include ADCC-mediated function promoted by broadly neutralising antibodies (bnAbs) and the use of cytokine superagonists to stimulate NK cells survival, proliferation, and maturation (Halper-Stromberg and Nussenzweig 2016; Seay et al. 2015). In a recent study of SHIV infected NHP, remission was achieved in a group of ART suppressed monkeys after infusion of PGT121 bnAb associated with a TLR-7 agonist, and part of the antiviral effect after ATI was attributed to NK cells (Borducchi et al. 2018). Although we have not shown a direct increase in the ADCC function in viraemic controllers, we observed an NK cell population with higher plasticity and responsiveness to cytokines that renders a higher potential to be exploited in therapeutic interventions, and that can be potentially replicated by damping inflammation with early suppression of infants on ART.

In Chapter 4, we could identify regulatory mechanisms behind the emergence of the slow progression phenotype. Previously, our group showed the similarity between PSPs and the non-pathogenic model of SIV infection in African Monkeys. Both confine viral replication to the population of short-lived CD4+ T-cells while shielding long-lived subsets via low levels of inflammation and CCR5 expression (Muenchhoff et al. 2016; Paiardini et al. 2011). We identified a positive correlation between early expression of PD-1 on CD8+ T-cells and these cardinal features of slow progression. The PD-1 expressing population was the main predictor of slower immunological progression after ATI. Similar to the observed in NHP models (Estes et al. 2008), the regulatory effect of PD-1 in controlling CD8+ T-cell activation and exhaustion acts as a damage control and, likely the proposed in the “honeymoon period”, protects the immune system from an unrestrained cycle of inflammation and, consequently, HIV/SIV disease progression. The inhibitory effect of PD-1 contributes to avoiding hyperactivation and terminal differentiation of the CTL population, supporting the

generation of a CD8+ T-cell compartment non-terminally exhausted and with high proliferative potential and capable of acquiring effector functions.

The complete role of TCF-1 in the ontogenesis of CD8+ T-cell remains unknown as well if eliciting such population will contribute to long-lasting and potent antiviral/antitumour function. However, PD-1+TCF-1+ CD8+ T-cell population carries an enormous potential that can be explored in immunotherapies targeting the T-cell compartment (McLane, Abdel-Hakeem, and Wherry 2019). The inherent "less combative" immune system in early life associated with the opportunity to start ART immediately after birth creates an ideal scenario that protects the T-cell compartment of terminal exhaustion up to the time the optimal window to elicit an antiviral response that is long-lasting and capable of eliminating reactivated cells is reached, and ART can be suspended.

Our findings in Chapter 4 also highlight the importance of conducting specific studies in the paediatric population rather than extrapolating conclusions from the adults. The stem-like Tex population has been highly explored in the cancer field as the source of the effective response to PD-1 blockade, promoting a boost of antitumor CTL response (Wolchok et al. 2017). Similar results have been proposed in HIV *in vitro* models and SIV studies in NHP (Trautmann et al. 2006; Velu et al. 2009; Grabmeier-Pfistershammer et al. 2017). We also detected this effect in our adults' assays, but no effect in the paediatric samples. Although preliminary, PD-1 blockade did not increase the proliferation and effector capacity of HIV-specific CD8+ T-cells in both PSPs and PPs. Similar results were obtained by a previous study looking at the CD4+ T-cell in children living with HIV (Foldi et al. 2017). PD-1 might have different functional roles along life and, according to the immunological context, exert

an important function in immune tolerance and regulation that has not been completely uncovered and needs to be further explored.

In this thesis, we also had the opportunity to report for the first time on a group of children living with HIV and capable of spontaneously controlling viraemia. PECs as a group have never been described in the literature and, despite the small sample size, we could identify distinct characteristics from their adult counterparts. Of all these features, the time to start controlling viraemia and the strong bias towards the female sex are highlighted. Distinct from adults, in which viraemic control is usually achieved after acute infection (Madec et al. 2013), to start controlling viral replication after years of infection in children means that the immune system needs to adopt a strategy similar to the viraemic slow progressors up to the point the immune system is mature enough to generate a highly effective antiviral response. The striking proportion of females also reinforces the importance to study sex difference in the immune response.

In several aspects, PECs resemble more PTCs than adult ECs. As shown in Chapter 3 and reinforced by the data in Chapter 5, the effect of disease-protective HLA-B alleles linked with CTL activity is lower in the paediatric population and likely to the observed in PTCs (Sáez-Cirión et al. 2013). Similar to PSPs (Muenchhoff et al. 2016), the viral reservoir in PTCs is highly confined to short-lived CD4+ T-cell subsets and considerably low in naïve and CM subsets (Sáez-Cirión et al. 2013; Chéret et al. 2015). All four PECs and the TA-1 presented in Chapter 6 had a CD4+ T-cell compartment with very low levels of immune activation and a CD8+ T-cell compartment less prone to exhaustion. PTCs reports also show lower levels of immune activation when compared to the aggressive immune response generated by adult ECs (Sáez-Cirión et al. 2013; Chéret et al. 2015; Frange et al. 2016; Violari et al. 2019), which leads to higher levels of systemic inflammation and increases non-AIDS related morbidity. In

Chapter 4 to 6, we also described different functional aspects of the CTL response, including proliferation potential and polyfunctionality, that goes beyond the rationale of responses restricted by specific HLA-I alleles as the major drivers for viral control that can also be explored in future studies in PTCs.

Working with these projects allowed me to explore broad aspects of the immune system in the paediatric population and the complex interactions between host and HIV. The work described in this thesis adds to our understanding of how some outstanding individuals have developed successful strategies to survive HIV infection pacifistically despite the persistent high levels of virus, or have gone even further and achieved success by engaging in an aggressive battle with HIV. From inherited genetic factors, and from the first defence barrier provided by NK cells to the development of an adaptive T-cell compartment that is more resilient and effective against HIV, this work has sought to open new roads that will hopefully assist in our way towards HIV remission. Our findings now can be explored in cohorts of very early treated infants – some of them fully suppressed for years with undetectable proviral HIV-DNA and who are therefore well-placed to achieve remission. We need now to further explore these findings and investigate the existence of anti-HIV specific response in this group, the impact of very early ART on the T-cell compartment development or the presence of inherent genetic markers contributing to control viral reservoir in this population of very early suppressed children, and combine them with clinical trials investigating new therapeutic strategies (e.g., bnAbs, vaccines).

Ultimately, we ought not forget that the tools to end HIV and stop new infections are already here. Although it is undeniable the importance to pursuit an HIV cure/remission and the burden a lifelong ART can bring, our focus also needs to be directed to regions where HIV is still an acute calamity and poverty, gender equality,

access to healthcare and HIV treatment and prevention are the main barrier to end the HIV/AIDS pandemic. Beyond that, a world without HIV will require an effort to understand the role of people's surroundings, social interaction, and cultural and religious beliefs in HIV transmission and treatment adherence.

"Adianta querer saber muita coisa? O senhor sabia, lá para cima - me disseram. Mas, de repente chegou neste sertão, viu tudo diverso diferente, o que nunca tinha visto. Sabença aprendida não adiantou para nada... Serviu algum?"
Guimarães Rosa

Appendices

Appendix I: Antibody panels used in Flow Cytometry assays.

Table 1. Antibodies used for NK cell immunophenotype (Chapter 3).

Antibody	Fluorochrome	Clone	Company	Dilution
CD14	APC-Cy7	HCD14	Biolegend	1:50
CD19	APC-Cy7	HIB19	Biolegend	1:50
CD3	APC-Cy7	UCHT1	Biolegend	1:100
CD56	BV650	5.1H11	Biolegend	1:50
CD16	Alexa700	3G8	Biolegend	1:50
CD16	SB600	3G8	Life Technologies	1:100
NKG2A	PE-Vio770	GL183	Miltenyi	1:50
NKG2D	PerCP-Cy5.5	DX27	Miltenyi	1:50
NKG2C	Biotin*	REA205	Miltenyi	1:50
CD57	BV421	NK-1	BD	1:50
KIR2DL1	APC	143211	R&D Systems	1:50
KIR2DL2/L3/S2	APC	GL183	Beckman Coulter	1:50
KIR3DL1	APC	DX9	Miltenyi	1:50
KIR3DL2	APC	539304	R&D Systems	1:50
NKp30	PE-Vi0615	REA823	Miltenyi	1:50
NKp46	V450	9E2	BD	1:50
DNAM-1	BV510	11A8	Biolegend	1:100
CD38	PE-Cy5	HIT2	Biolegend	1:50
PLZF	PE-CF594	R17-809	BD	1:50
FcεRI-γ	FITC	polyclonal	Sigma-Aldrich	1:100
CD85j	PE	GHI/75	Biolegend	1:50
Viability	near-IR	N/A	Invitrogen	1:50 (of 1:200 stock)

*Conjugated with Streptavidin BV570 (Biolegend)

Table 2. Antibodies used for NK cell functional assays (Chapter 3).

Antibody	Fluorochrome	Clone	Company	Dilution
CD14	APC-Cy7	HCD14	Biolegend	1:50
CD19	APC-Cy7	HIB19	Biolegend	1:50
CD3	APC-Cy7	UCHT1	Biolegend	1:100
CD56	BV650	5.1H11	Biolegend	1:50
CD16	SB600	3G8	Life Technologies	1:100
NKG2A	PE-Vio770	GL183	Miltenyi	1:50
NKG2C	PE	1345591	R&D Systems	1:50
CD57	BV421	NK-1	BD	1:50
KIR2DL1	Alexa700	143211	R&D Systems	1:50
KIR2DL2/L3/S2	Alexa700	180704	R&D Systems	1:50
KIR3DL1	Alexa700	177407	R&D Systems	1:50
KIR3DL2	Alexa700	539304	R&D Systems	1:50
PLZF	PE-CF594	R17-809	BD	1:50
IFN-γ	BV421	4S.B3	BD	1:50
TNF-α	FITC	MAb11	BD	1:100
Viability	near-IR	N/A	Invitrogen	1:50 (of 1:200 stock)

Table 3. Antibodies used for T-cell immunophenotype in the ATI study (Chapter 4).

Antibody	Fluorochrome	Clone	Company	Dilution
CD3	BV605	UCHT1	Biolegend	1:50
CD4	BV650	RPA-T4	Biolegend	1:50
CD8α	BV570	RPA-T8	Biolegend	1:50
CD45RA	AlexaFluor700	H100	Biolegend	1:50
CCR7	Pacific Blue	G043H7	Biolegend	1:50
CCR5	PE-Cy7	HM-CCR5	Biolegend	1:100
HLA-DR	APC-R700	G46-6	BD	1:100
CD39	APC	A1	eBioscience	1:50
CD73	PE	AD-2	Biolegend	1:200
PD-1	PE-eFluor610	J105	eBioscience	1:50
Viability	near-IR	N/A	Invitrogen	1:50 (of 1:200 stock)

Table 4. Antibodies used for PD-1+ CD8+ T-cell immunophenotype (Chapter 4).

Antibody	Fluorochrome	Clone	Company	Dilution
CD3	PE-Cy5	UCHT1	Biolegend	1:50
CD4	BV650	RPA-T4	Biolegend	1:50
CD8α	PE-Cy7	RPA-T8	Biolegend	1:50
CD45RA	BV605	H100	Biolegend	1:50
CCR7	Pacific Blue	G043H7	Biolegend	1:50
CD27	BV510	M-T271	Biolegend	1:100
CXCR5	AlexaFluor700	J252D4	Biolegend	1:50
CD127	AlexaFluor647	HIL-7R-M21	BD	1:10
CD39	APC	A1	eBioscience	1:50
TCF-1	PE	7F11A10	Biolegend	1:50
PD-1	PE-eFluor610	J105	eBioscience	1:50
Viability	near-IR	N/A	Invitrogen	1:50 (of 1:200 stock)

Table 5. Antibodies used for Tetramer+ CD8+ T-cell immunophenotype (Chapter 4).

Antibody	Fluorochrome	Clone	Company	Dilution
CD3	BV711	UCHT1	Biolegend	1:50
CD4	BV650	RPA-T4	Biolegend	1:50
CD8α	BV570	RPA-T8	Biolegend	1:50
CD45RA	AlexaFluor700	H100	Biolegend	1:50
CCR7	PerCP-Cy5.5	G043H7	Biolegend	1:50
CD27	BV510	M-T271	Biolegend	1:100
Tetramers	Pacific Blue		ImmunAware	1:2.5
CD127	BV605	A019D5	Biolegend	1:25
CD39	APC	A1	eBioscience	1:50
TCF-1	PE	7F11A10	Biolegend	1:50
PD-1	PE-eFluor610	J105	eBioscience	1:50
T-bet	PE-Cy7	4B10	Biolegend	1:25
Granzyme B	FITC	GB11	Biolegend	1:50
Viability	near-IR	N/A	Invitrogen	1:50 (of 1:200 stock)

Table 6. Antibodies used for proliferation assay (Chapter 4).

Antibody	Fluorochrome	Clone	Company	Dilution
CD3	PE-Cy5	UCHT1	Biolegend	1:50
CD4	BV605	RPA-T4	Biolegend	1:50
CD8α	BV570	RPA-T8	Biolegend	1:50
IFN-γ	AlexaFluor700	B27	Biolegend	1:50
TNF- α	FITC	MAb11	Biolegend	1:50
CD107a	BV650	H4A3	Biolegend	1:25
CTV	Pacific Blue	N/A	Life Technologies	1:2.5
CD127	PE-Cy7	HIL-7R-M21	BD	1:25
CD39	APC	A1	eBioscience	1:50
TCF-1	PE	7F11A10	Biolegend	1:50
PD-1	PE-eFluor610	J105	eBioscience	1:50
Viability	near-IR	N/A	Invitrogen	1:50 (of 1:200 stock)

Table 7. Antibodies used for T-cell immunophenotype (Chapters 5 and 6).

Antibody	Fluorochrome	Clone	Company	Dilution
CD3	BV605	UCHT1	Biolegend	1:50
CD4	BV650	RPA-T4	Biolegend	1:50
CD8α	BV570	RPA-T8	Biolegend	1:50
CD45RA	AlexaFluor700	H100	Biolegend	1:50
CCR7	Pacific Blue	G043H7	Biolegend	1:50
CD27	BV510	M-T271	Biolegend	1:100
CCR5	PE-Cy7	HM-CCR5	Biolegend	1:100
HLA-DR	APC-R700	G46-6	BD	1:100
CD38	PerCP-Cy5.5	HIT2	Biolegend	1:100
CD39	APC-R700	A1	eBioscience	1:50
CD73	PE	AD-2	Biolegend	1:200
PD-1	PE-eFluor610	J105	eBioscience	1:50
Viability	near-IR	N/A	Invitrogen	1:50 (of 1:200 stock)

Table 8. Antibodies used for ICS of HIV-specific T-cell response (Chapters 5 and 6).

Antibody	Fluorochrome	Clone	Company	Dilution
CD3	BV605	UCHT1	Biolegend	1:50
CD4	BV650	RPA-T4	Biolegend	1:50
CD8α	BV570	RPA-T8	Biolegend	1:50
CD107a	PE-Cy7	H4A3	Biolegend	1:100
TNF-α	AlexaFluor700	MAb11	Biolegend	1:50
IFN-γ	PE-Dazzle594	B27	Biolegend	1:100
IL-2	BV510	MQ1-17H12	Biolegend	1:50
MIP-1β	PE	D21-1351	BD	1:50
Perforin	FITC	B-D48	Biolegend	1:50
Granzyme B	APC	GB11	Invitrogen	1:50
Viability	near-IR	N/A	Invitrogen	1:50 (of 1:200 stock)

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