HLA-I, NATURAL KILLER CELLS AND T LYMPHOCYTES IN PAEDIATRIC HIV FUNCTIONAL CURE

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To my parents, Bruce and Vida, my grandmother, Judy, my sister, Emma, and my fiancée, Kelly — thank you all for your unwavering support throughout my academic journey.

ABSTRACT

Achieving a functional cure for HIV-1 remains one of the greatest challenges in medicine. Post-treatment control (PTC), where individuals sustain virological suppression after discontinuing antiretroviral therapy (ART), represents a particularly promising strategy. Infants appear to have enhanced PTC potential due to early ART initiation, immune tolerance, and the infection of less fit viruses compared to adults, making this group particularly informative for studying mechanisms of PTC. This thesis investigates the immunogenetic and immunological determinants of PTC in early ART-treated children living with HIV, to inform cure-directed interventions and identify paediatric candidates for analytical treatment interruption (ATI) trials. Chapter 3 reveals that children carrying low-expression HLA-A alleles exhibit reduced HIV-1 DNA levels, suggesting smaller reservoirs and enhanced cure potential, which were linked with NK cell phenotype and function. In contrast, females with protective HLA-B alleles displayed larger reservoirs associated with Th17-skewed immune profiles and therefore reduced cure potential. Chapter 4 demonstrates the potential for HIV-1 to alter population-level HLA-I frequencies in KwaZulu-Natal and that the introduction of ART has halted this selective pressure. Additionally, infants born in the ART era were shown to have reduced frequencies of high-expression HLA-A alleles compared to the pre-ART era, favouring PTC. Chapter 5 explores immunological differences between HLA-B*58:01 and HLA-B*58:02. HLA-B*58:01 was linked to heightened immune activation relative to HLA-B*58:02 in both healthy adults and early-ART treated children, suggesting that, despite its 'protective' classification in ART-naïve settings, HLA-B*58:01 may be less compatible with functional cure in early ART-treated children compared to HLA-B*58:02. Together, these findings highlight factors that enhance paediatric functional cure, including low HLA-A expression, heightened NK cell functionality, 'disease-susceptible' HLA-B alleles, and high ART coverage. This work helps advance our understanding of factors that enhance paediatric HIV-1 functional cure and supports the long-term vision of relieving children of lifelong ART.

CONTRIBUTIONS TO THIS THESIS

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Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious.

Stephen Hawking

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GLOSSARY OF TERMS

ACRONYMS AND ABBREVIATIONS

3TC	Lamivudine
ABC	Abacavir
AIDS	acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ATI	analytical treatment interruption
AZT	Zidovudine
BFA	Brefeldin A
CM	central memory
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DMSO	dimethyl sulfoxide
EC	elite controller
EM	effector memory
Env	envelope
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FRESH	Females rising through education, safety and health
Gag	group-specific antigen
GSEA	gene set enrichment analysis
GWAS	genome-wide association study

HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HPP	HIV-1 Pathogenesis Program
ICS	intracellular cytokine staining
IFN-I	type 1 interferon
IFNg	interferon gamma
IL	interleukin
ISG	interferon stimulated genes
KIR	killer-cell immunoglobulin-like receptor
KZN	KwaZulu-Natal
LILR	inhibitory leukocyte immunoglobulin-like receptors
LOD	limit of detection
LVP	Lopinavir
LWH	living with HIV
MHC	Major Histocompatibility Complex
Nef	negative regulator factor
NGS	next-generation sequencing
NK	Natural Killer
NVP	Nevirapine
OR	odds ratio
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction

PEHSS	paediatric early HAART and strategic treatment interruption study
PIC	post-intervention control
PLWH	people living with HIV
Pol	polymerase
PTC	post-treatment control
pVL	plasma viral load
RA	rheumatoid arthritis
RNA	ribonucleic acid
scRNA-seq	single cell RNA sequencing
Seb	Staphylococcal Enterotoxin B
TEMRA	terminal effector memory RA
Tfh	T follicular helper
TM	terminal memory
TNFa	tumour necrosis factor alpha
UKZN	University of KwaZulu-Natal
UNAIDS	Joint United Nations Programme on HIV/AIDS
VRC	viral replicative capacity
WB	whole blood

AMINO ACID ABBREVIATIONS

M	Methionine
T	Threonine

CHAPTER 1: INTRODUCTION

Despite four decades of research and biomedical advances since the first confirmed case of acquired immunodeficiency syndrome (AIDS) (Gottlieb et al. 1981), HIV-1 remains one of the most significant global public health challenges. Antiretroviral therapy (ART) has transformed HIV-1 from a fatal infection into a manageable chronic condition, allowing people living with HIV-1 (PLWH) to achieve near-normal life expectancy (Samji et al. 2013). However, ART is not a cure, where in most patients rapid rebound in viremia is observed within weeks of ART interruption (Li et al. 2022; Sneller et al. 2020). ART must be taken lifelong, carries potential side effects and toxicities, incurs substantial financial costs, and requires sustained access to healthcare, an ongoing challenge in many low-resource settings (UNAIDS 2023). Most critically, ART cannot eliminate the persistent latent viral reservoir, the main barrier to cure, as it seeds viral rebound upon treatment interruption (Pitman et al. 2018). For these reasons, the development of an HIV-1 cure remains a central goal of the field.

Broadly, two curative strategies have been proposed (**Fig 1.1**): a sterilising cure, which entails complete elimination of the virus from the body, and a functional cure, in which viral replication is durably controlled in the absence of ART, even if some virus remains (Deeks, Lewin, and Havlir 2013). A sterilising cure has only been observed in a small number of exceptional cases (Dickter et al. 2024; Gupta et al. 2019; Hsu et al. 2023; Hütter et al. 2009; Jensen et al. 2023; Sáez-Cirión et al. 2024), typically involving high-risk and high-cost interventions such as allogeneic stem cell transplantation. In contrast, a functional cure, while also rare, is a more realistic and scalable target for the wider population living with HIV, as the interventions are less risky and are more cost-efficient.

Three phenotypes of functional cure exist: elite control (EC), post-treatment control (PTC), and post-intervention controllers (PIC). Briefly, ECs maintain viral control without ART while PTCs achieve viral control following ART discontinuation (reviewed in Deng et al. 2025). PICs are similar to PTCs; however, these individuals receive experimental medical interventions, such as broadly neutralising antibodies, and then experience sustained periods of viral control following treatment interruption. The underlying mechanisms of PIC remain poorly understood due to the limited number of studies conducted; consequently, the PIC phenotype will not be the focus of this introduction.

Although both ECs and PTCs achieve viral suppression without continuous ART, the mechanisms are strikingly different. Immune features contributing to EC include 'protective' Human leukocyte antigen (HLA) class I alleles and potent HIV-specific CD8+ T cell responses (Fellay et al. 2007; Mothe et al. 2012; O'Connell, Bailey, and Blankson 2009; Pereyra et al. 2008; The Major Genetic Determinants of HIV-1 Control Affect HLA Class I Peptide Presentation 2010a). On the other hand, PTCs appear to achieve control through a combination of early ART initiation, reduced HIV-1 reservoir size, low levels of immune activation, and the notable absence of a strong HIV-specific CD8+ T cell response (Etemad et al. 2023; Sáez-Cirión et al. 2013). The number of PTCs ranges between 5 and 15% in early ART-treated studies (Goujard et al. 2012; Hocqueloux et al. 2010; Lodi et al. 2012), while ECs represent a smaller subset of PLWH (<1%) (Grabar et al. 2009; Okulicz et al. 2009; Salgado et al. 2024). This highlights PTC as a more promising and scalable pathway to achieve a functional cure compared to EC (5-15% prevalence vs <1%, respectively).

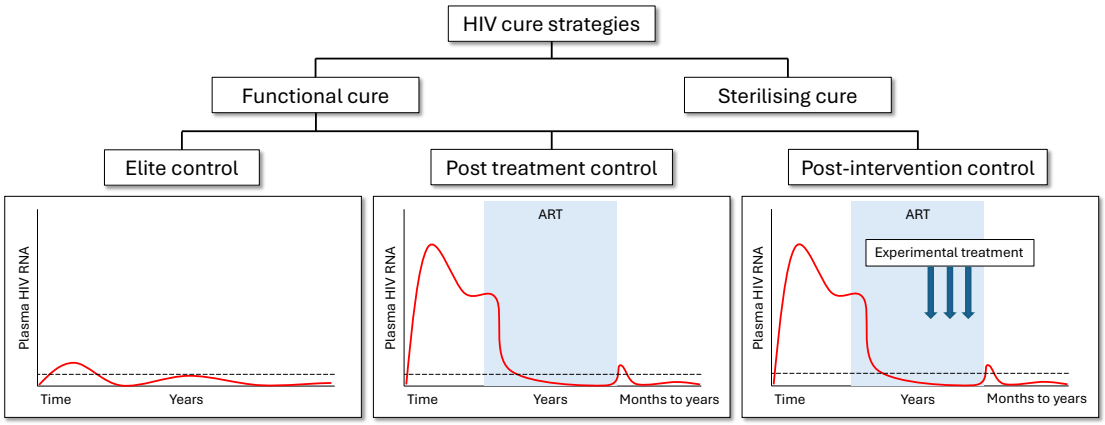

Figure 1.1. Schematic of HIV-1 cure strategies, adapted from Ole Søgaard's plenary talk at CROI 2025.

Interestingly, it has been proposed that PTC is more easily attainable in children compared to adults (Goulder, Lewin, and Leitman 2016). Although the unique features of early-life immunity typically result in rapid HIV-1 disease progression in ART-naive infection, these same factors favour infants in achieving PTC (Herbert and Goulder 2023). First, we will examine the HIV-1 reservoir, the primary barrier to cure, and the key immune cells responsible for clearing infected cells. We will then discuss why PTC may be more readily achieved in children than in adults, providing the context for this thesis.

THE HIV-1 VIRAL RESERVOIR

Following receptor-mediated entry (CD4 and CCR5 or CXCR4) of HIV-1 into the target cell, the viral RNA genome is reverse-transcribed into double-stranded DNA and integrated into the host genome as a provirus (reviewed in Deeks et al. 2015) (**Fig 1.2A**). Once integrated, the provirus hijacks host transcriptional and translational machinery to produce viral proteins and assemble new virions. The formation of integrated proviruses underlies the HIV-1 viral reservoir, the principal barrier to cure. Shortly after acute infection, HIV-1 seeds the reservoir by integrating into long-lived host cells; when these proviruses become transcriptionally

silent (latent), infected cells escape (i) ART, which targets active replication, and (ii) immune recognition, because the absence of antigen expression and location of infected cells prevents the detection by host effector cells, largely the major antiviral immune cells Natural Killer (NK) cells and CD8+ T cells (Deeks et al. 2015; Finzi et al. 1997). Reservoirs persist within multiple cell types and across multiple anatomical sites (**Fig 1.2BC**) but are concentrated in resting memory CD4+ T cells and T follicular helper (Tfh) cells in germinal centres of secondary lymphoid tissues; cell types that are long-lived and relatively inaccessible to circulating effector cells (Perreau et al. 2013). Clonal expansion of infected cells contributes to reservoir maintenance and can amplify integrated proviruses without new rounds of infection (Bui et al. 2017; Murray et al. 2016). The reservoir's slow decay (half-lives measured in years during ART) explains why stopping therapy typically causes rapid viral rebound (Crooks et al. 2015; Siliciano et al. 2003).

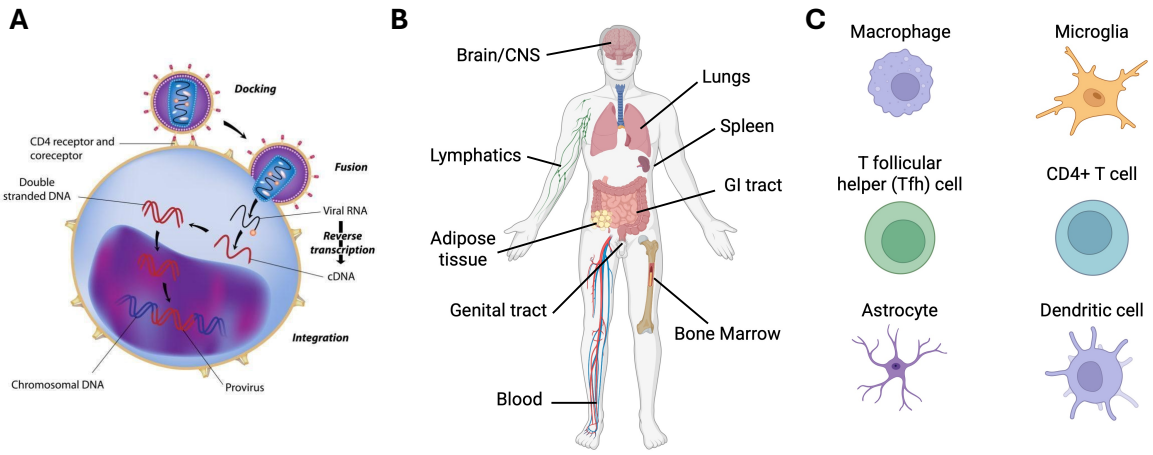

Figure 1.2. The HIV-1 reservoir. A. Establishment of the HIV-1 provirus within CD4+ target cells, adapted from Interactive Biology, 2025. B. Anatomical compartments of the HIV-1 reservoir. C. Cell types that are infected by HIV-1. B and C are adapted from (Henderson et al. 2020) using BioRender.

HOW CD8+ T CELLS REDUCE THE VIRAL RESERVOIR

CD8+ Cytotoxic T Lymphocytes (CTLs) are essential adaptive immune effector cells that eliminate virally infected cells by recognising viral peptide presented on HLA class I molecules and subsequently delivering cytolytic granules (perforin, granzymes) or inducing apoptosis via the Fas/FasL pathway (McMichael and Rowland-Jones 2001). This mechanism allows CTLs to eliminate cells that transiently express viral antigen following spontaneous low-level transcription or pharmacological latency reversal, thereby limiting the pool of replication-competent proviruses. Multiple lines of evidence support the importance of CD8+ T cells in shaping the reservoir size: Elite controllers, as mentioned previously, display vigorous, polyfunctional HIV-specific CD8+ T cell responses (Migueles and Connors 2015; Salgado et al. 2024), and in vivo depletion experiments in SIV-infected macaques result in rapid viral rebound, demonstrating active reservoir containment (Schmitz et al. 1999). Furthermore, therapeutic strategies to enhance CD8+ T cell responses have shown promise in preclinical models (Borducchi et al. 2016). However, CTL-mediated clearance is significantly limited by several barriers: many reservoir cells reside in anatomically restricted sites like germinal centres where CD8+ T cells are scarce or functionally constrained (He et al. 2016); chronic HIV infection induces CD8+ T cell exhaustion, characterized by inhibitory receptor expression (PD-1, TIM-3) and reduced killing capacity (Wherry and Kurachi 2015); and viral escape mutations can impair CTL recognition (Goulder and Watkins 2008). Critically, the primary limitation is that the majority of proviruses in the stable reservoir are deeply latent and do not express sufficient antigen for CTL recognition.

HOW NK CELLS REDUCE THE VIRAL RESERVOIR

NK cells are critical innate lymphocytes that function to eliminate virally infected or transformed cells through both direct cytotoxicity and antibody-dependent cellular

cytotoxicity (ADCC) (Vivier et al. 2011). Unlike CD8+ T cells, NK cells recognise targets independently of peptide-HLA class I presentation, instead integrating signals from a diverse repertoire of activating and inhibitory receptors to determine the killing response. The inhibitory signal, typically delivered by inhibitory KIR receptors binding to HLA class I epitopes or NKG2A to HLA-E, is overridden when the target cell is stressed, downregulates HLA class I molecules, or is coated with antibodies, leading to higher levels of activating receptor binding, tipping the balance in favour of activation over inhibition, thereby leading to the release of cytolytic granules. ADCC is mediated by the CD16 (FcγRIIIa) receptor, linking NK cells to antibody-opsonised, infected cells.

NK cells are classically divided into functional subsets based on CD56 expression. In healthy individuals, peripheral blood NK cells are dominated by $CD56^{dim}$ cells (~90%) and a smaller population of $CD56^{bright}$ cells (~10%), each with distinct functional specialisations (Cooper, Fehniger, and Caligiuri 2001). $CD56^{bright}$ NK cells localise preferentially to secondary lymphoid tissues and are potent cytokine producers, secreting interferon-gamma (IFN-γ), TNF-α, IL-10, and GM-CSF in response to IL-12, IL-15, and IL-18. They have relatively low cytotoxicity but play essential roles in immune regulation, shaping dendritic cell maturation and T-cell priming (Martín-Fontecha et al. 2004). Conversely, $CD56^{dim}$ NK cells are highly cytotoxic, expressing high levels of perforin, granzymes, and CD16 (FcγRIIIa), enabling potent ADCC (Mavilio et al. 2005). A third subset, $CD56^{neg}$ NK cells, expands significantly during chronic HIV-1 infection and other inflammatory states (Del Zotto et al. 2017). These cells exhibit profound functional defects, including reduced cytotoxicity, impaired cytokine production, and diminished responsiveness to activating signals (Del Zotto et al. 2017). Phenotypically, they carry markers of chronic activation and exhaustion, resembling dysfunctional NK cells seen in lymphoid tissue fibrosis or chronic viral stimulation.

Importantly, the expansion of $CD56^{neg}$ NK cells in HIV-1 correlates with higher viral load, immune dysfunction, and impaired NK-mediated control of viral replication (Brunetta, Hudspeth, and Mavilio 2010).

Mechanistic and experimental data strongly implicate NK cells in the control of the HIV-1 reservoir. In vitro, activated NK cells efficiently kill latently infected CD4+ T cells following pharmacological latency reversal (Garrido et al. 2018). In vivo, combinatorial strategies pairing latency reversal agents with adoptively transferred NK cells or IL-15 superagonists have been shown to reduce the rebounding reservoir and delay viral rebound in humanised mice (Kim et al. 2022a). Furthermore, human cohort studies have consistently linked superior NK cell function with reduced viral persistence. For instance, the inducibility of activating receptors NKp30 and NKp46, as well as IFN-γ production, correlates inversely with HIV-1 DNA in both controllers and progressors (Marras et al., J Virol, 2017), while the total proportion of circulating mature NK cells has been shown to predict reservoir size (Blazkova et al. 2024; Olesen et al. 2015). In a paediatric ART-naïve HIV-1 cohort, NKG2A and NKp46 expression on $CD56^{dim}$ NK cells, as well as low HLA-A expression and the presence of Bw4 epitopes, were linked to lower levels of HIV-1 DNA (Vieira et al. 2021). More recently, mass cytometry analyses of ART-suppressed individuals have identified specific receptor-ligand combinations, notably the expression of KIR2DL1/L3 and HLA-Bw6, that are robustly associated with lower measures of HIV-1 DNA and RNA persistence (Ivison et al. 2022). In addition, antibody-mediated NK cell activation was found to be associated with delayed viral rebound in an ATI cohort study (Bartsch et al. 2021). Lastly, a study on PTC's identified NK cell activation and function in restricting cell-associated RNA before treatment interruption and suppressing viral rebound during early ATI (Etemad et al. 2023).

Collectively, these findings suggest that specific NK phenotypes and favourable KIR/HLA genotypes are critical determinants of reservoir size.

INFANT VERSUS ADULT IMMUNITY

The difference between early life and adult immune polarisation depends fundamentally on the cytokines produced by innate immune cells in response to pathogen exposure (**Fig 1.3**). The Th1-supporting cytokines IFN-γ and IL-12 facilitate the strong HIV-specific T-cell responses seen in adults that play a major part in immune control of HIV-1, whereas in children these particular responses are not well supported and are consequently relatively weak (Goulder and Watkins 2008; Hsieh et al. 1993; Kollmann et al. 2009, 2012; Leitman et al. 2017; Saravia, Chapman, and Chi 2019). However, the early life immune system is designed to serve other functions, such as a more regulated and tolerogenic immune environment with a relatively high level of regulatory T-cell activity, and also to promote other specific responses such as antibody production via Tfh cell supporting cytokines, IL-6 and IL-21 (Nurieva et al. 2007). Thus, the early life immune system is not ‘immature’ or systematically ‘weaker’ than the adult immune system but is designed differently to achieve optimal outcomes for the unique challenges faced in early life.

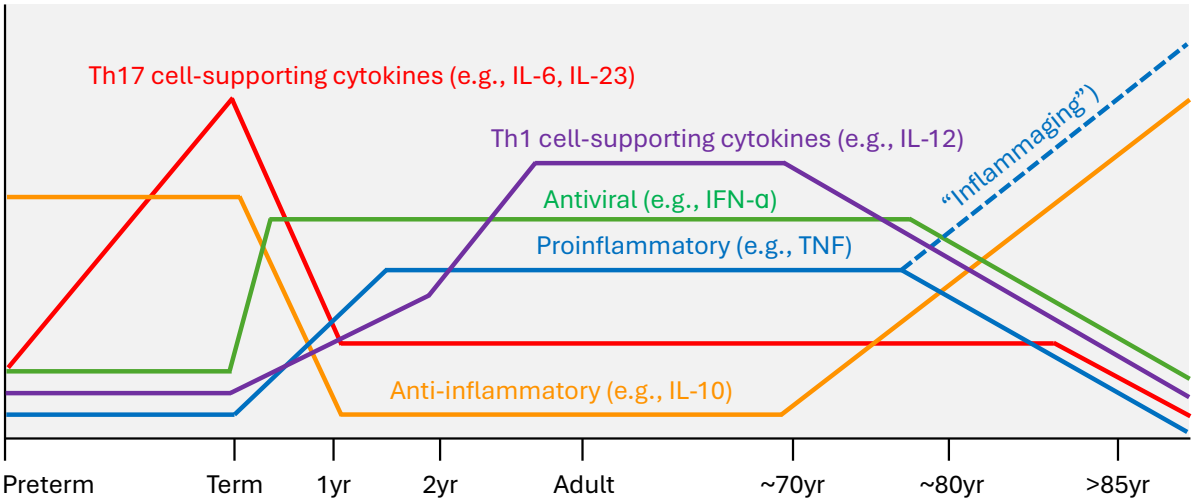

Figure 1.3. Age-dependent changes in cytokine production by immune cells in response to Toll-like receptor agonists, adapted from Kollmann et al., 2012.

Indeed, in certain instances, compared to adults, early life immunity can generate superior immune responses, such as high-frequency and highly potent broadly neutralising antibodies against HIV-1 (Goo et al. 2014; Muenchhoff et al. 2016), and antibody responses to non-HIV-1 and HIV-1 vaccines (Fouda et al. 2015; Martinez, Permar, and Fouda 2016; Ota et al. 2004). The early-life immune response can also achieve superior disease outcomes. A recent example is SARS-CoV-2 infection, where mortality in children aged 7 years, for example, was 25-fold lower than in adults aged 30 years (COVID-19 Forecasting Team 2022). Chickenpox is a second example, where mortality in adults ≥20 years old is 21-fold higher than in children under 14 years of age (Lopez, Harrington, and Marin 2021). By contrast, in HIV-1 infection, compared to adults, the early life immune system is less well adapted in the absence of ART to achieve successful disease outcomes. However, following early ART initiation, PTC may be more easily attained in children than in adults. The reason for this is that the immune responses that most readily achieve immune control of HIV-1 and minimise disease in natural, ART-naïve infection – rapid activation of the immune system, and generation of a broad HIV-specific CD8+ T-cell response – are, in broad terms, the opposite of what is successful in achieving PTC.

It is important to note that paediatric and adult HIV-1 infection differ in one key respect in addition to the stage of immune ontogeny at which each becomes infected: the child is infected by the mother, whereas the adult is infected by an unrelated donor. This means that the virus that is transmitted from mother to child may be pre-adapted to the child's immune system as a result, for example, of HLA I-associated CTL escape in the mother involving HLA alleles shared with the child (Carlson et al. 2016; Goulder et al. 2001). As discussed, this may have implications for children living with HIV (LWH), both for HIV-1 pathogenesis in natural infection in the absence of ART, and for HIV-1 cure potential following early ART initiation.

CD8+ T CELLS AND HIV-1 DISEASE IN ART-NAÏVE INFECTION IN CHILDREN VERSUS ADULTS

The natural course of paediatric HIV-1 infection is characterised by faster disease progression to AIDS and shorter time to death compared to adults. More than 50% of ART-naïve children LWH have died by 2 years of age (Newell et al. 2004), whereas in ART-naïve adults the median survival time is 11 years (Collaborative Group on AIDS Incubation and HIV-1 Survival including the CASCADE EU Concerted Action 2000). In adult infection, slow disease progression and immune control of HIV-1 are associated with polyfunctional, broad HIV-specific CD8+ T-cell responses (Betts and Harari 2008; Kiepiela et al. 2007; Migueles et al. 2002; Migueles and Connors 2015), especially those targeting the highly conserved and abundant Gag capsid protein. The HLA-I molecules that are strongly linked with disease protection are, in Caucasian populations, HLA-B*27 and HLA-B*57 (Kaslow et al. 1996; The International HIV-1 Controllers Study 2010), and, in African populations, HLA-B*57, HLA-B*58:01 and HLA-B*81:01 (Kiepiela et al. 2004; Leslie et al. 2010). In each case,

these HLA-I molecules present immunodominant CTL epitopes that are located within the capsid protein. Although escape mutations to evade these responses can be selected by the virus, in each case they come at a considerable cost to viral replicative capacity (Crawford et al. 2007; Goepfert et al. 2008; Leslie et al. 2004; Prince et al. 2012; Schneidewind et al. 2007; Tsai et al. 2016; Wright et al. 2012). Thus, immune control mediated by these HLA-I molecules results either from successful containment of viral replication via CTL-mediated killing, or via significant reduction in viral replicative capacity after the selection of immune escape.

In paediatric infection, as explained above, the HIV-specific T-cell response is not strongly supported by early life immune polarisation. The low-frequency, narrowly based HIV-specific CD8+ T-cells in the first two years of life do little to contain viral replication, and it is during this time period that the majority of children LWH succumb to AIDS. In contrast to the protective part played by HLA-I molecules such as HLA-B*27/57/58:01/81:01 in slowing disease progression in adults LWH, in children these HLA-I have little impact (Adland et al. 2015; Vieira et al. 2021). In part, this is because the virus transmitted from mother-to-child may have escape mutants that abrogate the beneficial effects of CTL responses restricted by these protective alleles, but principally it is because the HIV-specific CD8+ T-cell responses are so weak in early life.

NK CELLS AND HIV-1 DISEASE IN ART-NAÏVE INFECTION IN CHILDREN VERSUS ADULTS

NK cell activity in adult infection can protect against HIV-1 disease progression, as shown by immunogenetic studies demonstrating that the combination of Bw4-80I alleles such as HLA-B*57 with high-expressing KIR3DL1 allotypes enhance the effects of HLA-B*57 alone in

slowing disease progression and improving control of viral load (Martin et al. 2007). In addition, functional studies have demonstrated the ability of NK cells to exert immune control on HIV-1 sufficient to drive the selection of escape variants capable of evading these antiviral NK responses (Alter et al. 2007, 2011; Hölzemer et al. 2015; Ziegler et al. 2021). However, these studies also indicate that, in adults, the dominant impact of protective HLA-I such as HLA-B*27/57 is independent of these NK cell interactions. By contrast, in ART-naïve paediatric infection, NK responses have a greater impact on disease progression than HIV-specific CD8+ T-cell responses (Vieira et al. 2021). As stated above, HIV-specific CD8+ T-cell responses in the first few years of life are relatively weak and their impact on immune control becomes greater as children transition into adolescence. Consistent with this is the observation that HLA-B*58:02, which is disease-susceptible in adult infection, and is Bw4-expressing and therefore serves as a ligand for KIR3DL1, does not carry any disease-susceptible effect in children until >10 years of age; by contrast, the other two principal disease-susceptible HLA-I in African populations, HLA-B*18 and HLA-B*45, which are both Bw6 expressing, have a substantial detrimental impact in the first 10 years of life as well (Vieira et al. 2021).

IMPACT OF IMMUNE SEX DIFFERENCES ON HIV-1 INFECTION SUSCEPTIBILITY AND DISEASE

The very wide impact of immune sex differences on outcomes from infections and immunisations, cancers, inflammatory conditions, and autoimmune disorders is becoming increasingly evident (Klein and Flanagan 2016). However, with respect to HIV-1, the very significant sex differences in outcomes have taken a long time to appreciate because studies in resource-rich countries have focused mainly on males and in resource-poor countries on females. Thus, it was not until 2017 that it became apparent that, among adults, females

achieve elite control 5x more frequently than males (Yang et al. 2017). Among children, elite control is 10x less common than in adults (Vieira et al. 2019), principally for the reasons described above related to the weakness of the HIV-specific CD8+ T-cell responses in early life. However, among the few paediatric elite controllers described, females outnumber males by 9:1 (Vieira et al. 2019). In adult and paediatric infection, viral setpoints are lower in females compared to males, in adults by 0.5 log (Gandhi et al. 2002). In children, the superior immune control of HIV-1 among females becomes apparent from approximately 2 years of age; below that age, males have lower viral loads (European Collaborative Study 2002; Ruel et al. 2011). These age- and sex-specific differences in immune control of HIV-1 relate to the fine balance that exists between the beneficial impact of early immune activation of the innate immune system, and subsequently of adaptive immunity (Ndhlovu et al. 2015), and the negative effects of increased immune activation which fuels the fire of viral replication and, in chronic infection, causes immune dysfunction and accelerates HIV-1 disease progression (Deeks et al. 2004; Hunt et al. 2014; Meditz et al. 2011; Utay and Hunt 2016). The higher level of immune activation in females compared to males (Meier et al. 2009; Santinelli et al. 2020; Ziegler and Altfeld 2016) that is present from intrauterine exists onwards (Adland et al. 2020) and is associated with increased female susceptibility to *in utero* infection (Adland et al. 2020; European Collaborative Study 2004; Marinda et al. 2007; Maswabi et al. 2021; Taha et al. 2005; Violari et al. 2018; Virological response to very early ART in neonates with in utero HIV: IMPAACT P1115 2019) and, as stated above, higher viral loads in females in the first two years of life. Beyond this age, the benefits of a stronger adaptive immune response, and more specifically the anti-HIV-1 CD8+ T-cell response, are increasingly brought to bear on the virus.

The explanation for these observations lies in the fact that the female immune response is typically more activated and responds to pathogen exposure more rapidly and robustly. At the heart of this is the higher production of type I interferons (IFN-I) by plasmacytoid dendritic cells in females compared with males (Berghöfer et al. 2006; Meier et al. 2009; Sampson et al. 2022; Webb et al. 2019). In addition to the direct antiviral effects of interferon-stimulated gene products (ISGs), such as the HIV-1 restriction factors tetherin, SAMHD1, TRIM5a, APOBEC3, and MX2 (Kane et al. 2016), IFN-I activates and accelerates antiviral NK cell activity and also the HIV-specific adaptive immune response. The counterpoint to the more activated and aggressive immune response observed in females compared to males is increased immunopathology, with more rapid CD4 decline in chronic HIV-1 infection in females than males for a given HIV-1 viral load (Gandhi et al. 2002; Meier et al. 2009) and more autoimmune diseases such as systemic lupus erythematosus (Souyris et al. 2019) and rheumatic diseases such as ankylosing spondylitis and rheumatoid arthritis (KVIEN et al. 2006). **Fig 1.4** summarises the favourable and unfavourable immune factors associated with viral control in the absence of therapy discussed in the preceding sub-chapters.

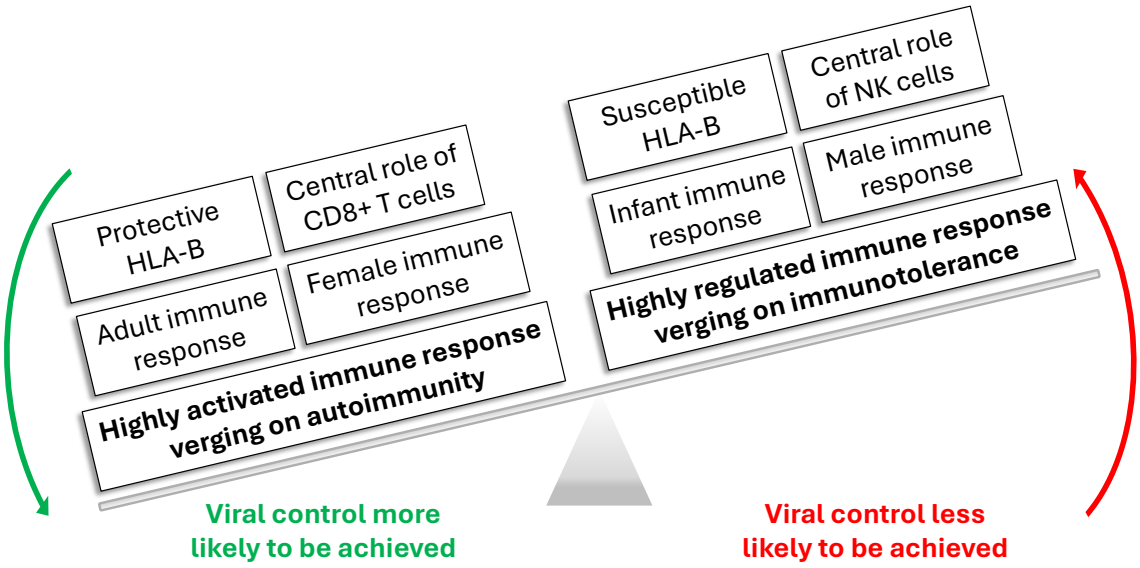

Figure 1.4. Factors that favour, and do not favour, viral control in the absence of therapy. Adapted from (Goulder and Deeks, 2018).

ART-NAÏVE IMMUNE CONTROL VERSUS POST-TREATMENT CONTROL OF HIV-1

Studies of the mechanisms underlying PTC in adult infection have unexpectedly pointed to striking differences with those that underpin immune control in ART-naïve infection. First, whereas protective HLA-I are strongly associated with the latter, the HLA-I associated with PTC are those that are disease-susceptible in ART-naïve infection (Martin et al. 2017; Ramsuran et al. 2018; Sáez-Cirión et al. 2013). In Caucasian populations, this is HLA-B*35 (especially subtypes HLA-B*35:02 and B*35:03) (Carrington et al. 1999; Gao et al. 2001), and among African cohorts these are HLA-B*18, B*45 and B*58:02 (Kiepiela et al. 2004; Leslie et al. 2010). These disease-susceptible HLA-I molecules typically present a small number of HIV-specific epitopes, especially of Gag-specific epitopes. In addition, they tend to bind with strong avidity to the leucocyte immunoglobulin-like receptors LILRB1 and LILRB2, that has the effect of dampening down the initiation of virus-specific CD8+ T-cell responses (Bashirova et al. 2014). Second, consistent with the HLA-I associations, HIV-specific CD8+ T-cell responses among PTC are unremarkable (Etemad et al. 2023; Sáez-Cirión et al. 2013). Third, low levels of immune activation are associated with PTC (Etemad et al. 2023; Sáez-Cirión et al. 2013), whereas immune control of ART-naive infection correlates with the speed and extent of immune activation (Ndhlovu et al. 2015). It is no surprise therefore that, while adult elite controllers may exhibit the high levels of immune activation needed to control HIV-1 infection, they also suffer an increased risk of coronary atherosclerosis (Pereyra et al. 2012). Furthermore, the HLA-I molecules associated with high immune activation and elite control of HIV-1 infection are in many cases also linked with autoimmune diseases (Bowness 2015; Chen et al. 2012; Mallal et al. 2008), the best

characterised example being certain HLA-B*27 subtypes with ankylosing spondylitis (Bowness 2015).

The fourth feature of PTC is the central role played by NK responses. While NK cell activity contributes to immune control in ART-naïve infection in adults (Alter et al. 2007, 2011; Hölzemer et al. 2015; Martin et al. 2007; Ziegler et al. 2021) and plays a major part in preventing disease progression among ART-naïve children LWH (Vieira et al. 2021), NK responses appear to play a central role in PTC (Etemad et al. 2023; Garcia-Broncano et al. 2019; Kim et al. 2022; McKinnon et al. 2014; Saez-Cirion 2019). In particular, it has been proposed that PTC is associated with an HLA-I signature that combines disease-susceptible HLA-B molecules with HLA genotypes that mediate a KIR-biased education of NK cells (Saez-Cirion 2019). The features contributing to such a genotype are low-expressing HLA-A molecules (Ramsuran et al. 2018), HLA-B molecules expressing the Bw4 motif, HLA-B molecules expressing Threonine at residue -21 in the signal peptide, and HLA-C molecules within the C2 group. Low-expressing HLA-A molecules reduce the expression of HLA-E, which is the ligand for the NKG2A/CD94 heterodimer (Braud et al. 1998), as less signal peptide from HLA-A molecules is available to be loaded onto HLA-E. With regards to HLA-B, the leader sequence in a portion of HLA-B molecules has threonine at residue -21, which does not bind to HLA-E, whilst those with methionine do (Lee et al. 1998; Lin et al. 2023) as shown in **Fig 1.5 (left panel)**. Thus, threonine at residue -21 in the HLA-B leader sequence results in the reduction of HLA-E expression, favouring KIR-education of NK cells. HLA-Bw4 is the ligand for KIR3DL1, and the C2 group of HLA-C molecules are ligands for the KIR2DL1 receptor, which have a stronger binding affinity than does C1 group and KIR2DL2/L3. These genotypes are in strong linkage disequilibrium, for example 11/12 HLA-B molecules that have Bw4 epitopes also have threonine at residue -21 of the signal

peptide, leading to haplotypes that generally favour either KIR-education or NKG2A-education of NK cells (Horowitz et al. 2016). The fact that these HLA-I haplotypes have been brought together over the course of evolution across many different populations is strong evidence for their importance in the immune response.

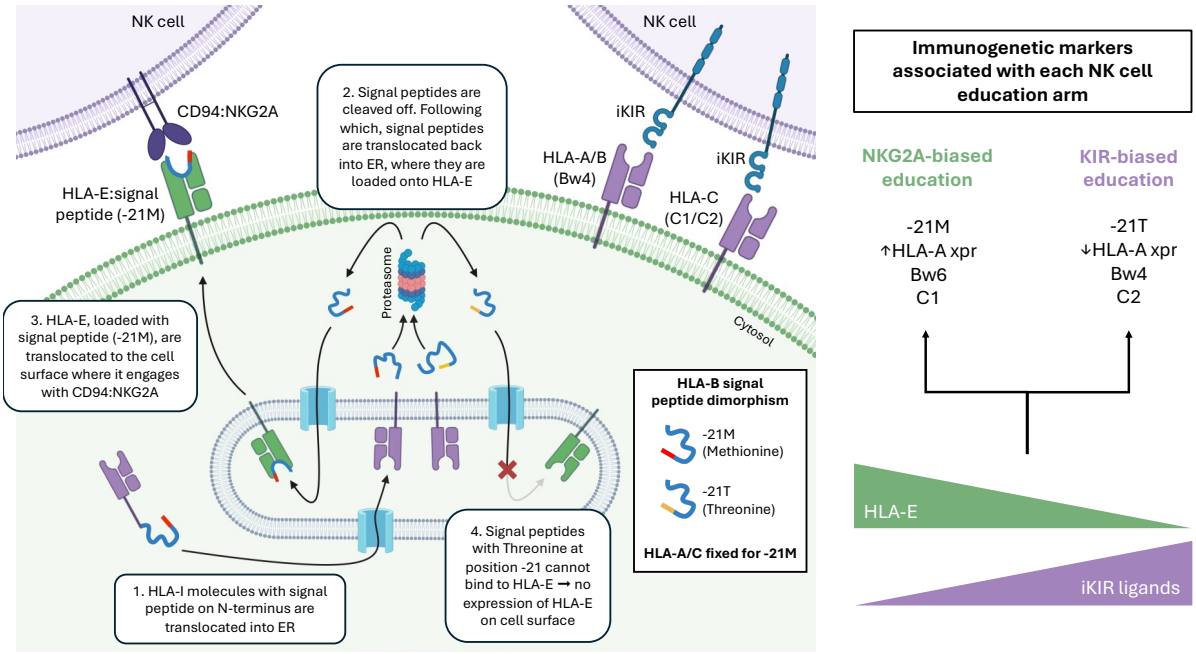

Figure 1.5. Natural Killer cell education. A schematic outlining the process of how HLA class I molecules' signal peptides impact HLA-E surface expression, the ligand binding partner of NKG2A, and the ligand binding partners of inhibitory KIR receptors. HLA-B allele signal peptides contain either a methionine (−21M) or threonine (−21T) residue at position -21. The -21M peptides strongly bind to MHC-E molecules, leading to its expression on the cell surface (point 3). Through the conserved CD94:NKG2A receptors, the HLA-E complexes educate NK cells through NKG2A. In contrast, -21T leader peptides do not interact with MHC-E molecules. Therefore, the presence of HLA-B alleles with -21T signal peptides leads to decreased surface level of MHC-E expression, favouring KIR-educated NK cells (point 4). HLA-A molecules are fixed with -21M but differ in their expression levels. High expressing HLA-A alleles result higher levels of signal peptide available to be loaded onto HLA-E and subsequently resulting in more HLA-E expression on the surface of the cell; favouring NKG2A educated NK cells. Low expression HLA-A alleles on the other hand leads to lower levels of HLA-E on the cell surface; favouring KIR-educated NK cells. C2 groups on HLA-C molecules have a stronger affinity for inhibitory KIRs than do C1 groups; favouring KIR educated NK cells. Bw4 is the ligand binding partner of KIR3DL1, an inhibitory KIR receptor. The ligand binding partner of Bw6 is unknown, however is not NKG2A or an inhibitory KIR; therefore favouring NKG2A-educated NK cells. Adapted from (Bruijnesteijn, de Groot, and Bontrop 2020). The far-right panel summarised the immunogenetic markers of each NK cell education arm.

The education of NK cells, and therefore their response to infection, are guided by these interactions between NK cell inhibitory receptors (inhibitory KIR receptors and NKG2A) and their HLA class I ligands. These engagements enable NK cells to distinguish diseased cells, which have perturbed expression of HLA class I molecules, from normal healthy cells, known as NK cell education. As mentioned, there are two schools of NK cell education (**Fig 1.5, right panel**): either KIR-educated NK cells, where more KIR-HLA class I interactions occur, or NKG2A-educated NK cells, where more NKG2A-HLA-E interactions occur (Horowitz et al. 2016). The two schools of education lead to NK cells that are phenotypically and functionally distinct (Horowitz et al. 2016). Education through HLA-E, and therefore NKG2A-education of NK cells, produces a more phenotypically diverse population of NK cells that are more effective at mediating ADCC, whilst those from individuals educated through KIR show more immature, less phenotypically diverse NK cells. Interestingly, KIR-educated $CD56^{dim}$ NK cells, identified by -21TT genotype, can respond more robustly to autologous HIV-infected target cells with degranulation and secretion of the cytokines IFN-γ and macrophage inflammatory protein 1β (MIP-1β) than NK cells that are NKG2A-educated, identified by -21MM genotype (Lisovsky et al. 2015).

These observations suggest that KIR-educated NK cells could be more effective in responding to some types of infection, like HIV-1, than NKG2A-educated NK cells. Indeed, HLA-I genotypes that educate NK cells through KIR are associated with reduced susceptibility to HIV-1 infection in adults (Merino et al. 2012) as well as with more effective NK cell-mediated control of HIV-1 both in adults (Merino et al. 2013; Ramsuran et al. 2018) and in children LWH (Vieira et al. 2021).

Consideration of the differences between the factors that contribute to immune control of ART-naïve and PTC control among adults highlights the fact that the early-life immune system is especially poorly designed to achieve ART-naïve immune control of HIV-1. However, it also prompts the hypothesis that the early-life immune system is especially well-designed to achieve PTC, summarised in **Fig 1.6**. All four features of PTC identified above are supported by early-life immunity in children LWH. Indeed, children LWH are enriched for disease-susceptible HLA-I because mother-to-child transmission is strongly associated with high viraemia - and therefore with disease-susceptible HLA-I in the mother (Kiepiela et al. 2004). As described above, the early life immune polarisation does not support virus-specific CD8+ T-cell responses, but does promote a highly regulated, tolerogenic immune environment with low levels of immune activation. By contrast with CTL in early life, NK cell numbers are at their highest at birth, and NK responses are active and capable of effective HIV-1 disease prevention from birth and potentially even *in utero* (Guilmot et al. 2011; Murphy et al. 2023; Strauss-Albee et al. 2017; Vieira et al. 2021).

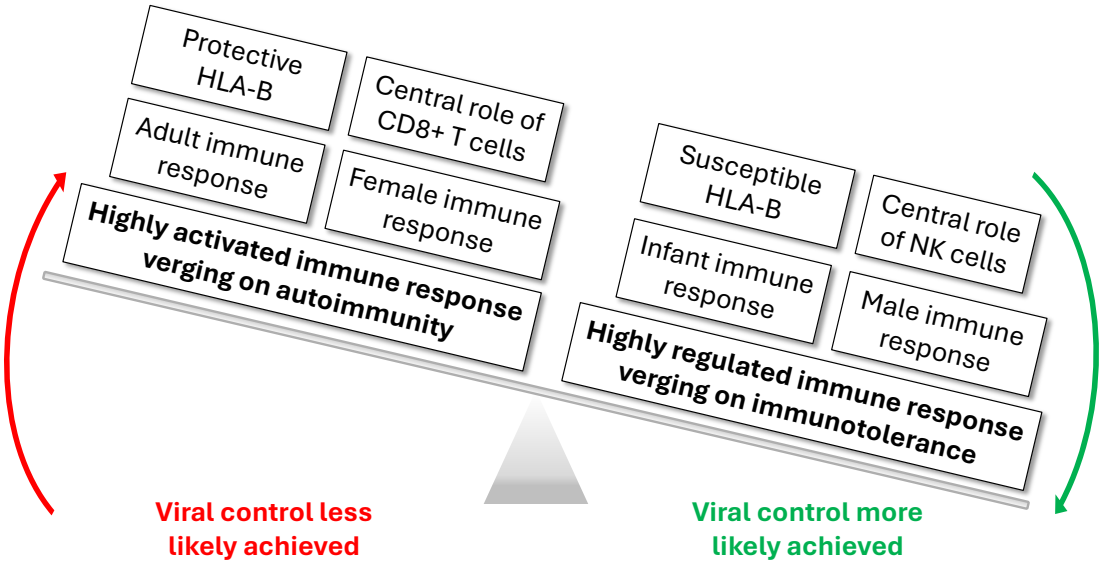

Figure 1.6. Factors that favour, and do not favour, post-treatment control. Adapted from (Goulder and Deeks, 2018).

An additional fifth feature of PTC is early ART initiation. Infants can receive ART immediately after birth and therefore sooner after infection compared to adults (Rouzioux et al. 1995). It has been shown both in non-human primate and human studies that early ART initiation, followed by an analytical treatment interruption (ATI), results in lower viral setpoints than arise in natural infection (Jacobson et al. 2006; Okoye et al. 2018, 2021). Early ART initiation suppresses the high levels of immune activation that rapidly drive immune dysfunction and also blocks the rapid selection of CTL escape viruses that occur in natural infection, thus allowing a broad and effective antiviral immune response to develop during the initial period on ART. Following treatment interruption, the lower virus setpoint is in part due to virus-specific CD8+ T-cell activity, as demonstrated by CD8+ T-cell depletion studies in NHP (Okoye et al. 2021) using the anti-CD8β monoclonal antibody, which does not deplete NK cells. However, as stated above, antiviral NK cell responses are a stronger correlate of PTC than virus-specific CD8+ T-cell responses, and in early life, the dominant antiviral impact of NK cells may again place children LWH in a stronger position than adults to achieve functional cure following early ART initiation.

VIRAL FACTORS IN PAEDIATRIC INFECTION

This introduction has focused on immune-related factors, especially HLA-I, CD8+ T cells, NK cells and sex differences, that impact HIV-1 disease progression in children. An additional important factor is the impact of the virus transmitted to children compared with that transmitted to adults. The replicative capacity of the virus transmitted from mother-to-child is lower than that of the virus quasispecies circulating in the mother (Naidoo et al. 2017a), whereas the replicative capacity of the virus transmitted from adult-to-adult is similar or somewhat higher in the recipient compared to that of the donor (Deymier et al. 2015; Iyer et al. 2017). Transmission of a low replication virus is important because HIV-1 disease

progression is slower, and immune activation and proviral DNA load are lower in the recipient (Claiborne et al. 2015). In mother-to-child transmission, lower replicative capacity viruses are transmitted to females (Adland et al. 2020), which may be related to the higher levels of immune activation observed in females versus males *in utero*, and also the selection of IFN-I-resistant viruses in mother-to-female fetal transmission. In the same way that CD8+ T-cell escape mutants often carry a cost to replicative capacity (Crawford et al. 2007; Goepfert et al. 2008; Leslie et al. 2004; Prince et al. 2012; Schneidewind et al. 2007; Tsai et al. 2016; Wright et al. 2012), innate immune escape variants may have similar effects on virus replication efficiency.

The consequence of lower 'fitness', IFN-I resistant viruses being transmitted to female fetuses would, in ART-naïve infection, likely result in superior outcomes among female children LWH. The higher frequencies of paediatric elite controllers who are female, the lower viral loads in female children above 2 years of age, and the female preponderance of paediatric non-progressors (Mori et al. 2015) – healthy children with normal-for-age CD4 T-cell counts and low levels of immune activation despite persistently high viraemia (Muenchhoff et al. 2016) – are all consistent with this notion. However, the transmission of IFN-I-resistant viruses predominantly to females may undermine the ability of innate immunity to control viral rebound following treatment interruption, since the rebounding virus following ATI is highly IFN-I-resistant (Gondim et al. 2021).

Children LWH exhibit very different HIV-1 disease outcomes compared with adults. In natural, ART-naïve infection, early-life immunity and features linked with mother-to-child transmission mitigate against effective antiviral CD8+ T-cell responses and precipitous disease progression is usually the outcome. By contrast, in adults, rapid CD8+ T-cell

activation and suppression of viraemia as early as possible in acute infection is associated with much longer periods of disease-free infection. However, as observed in elite controllers, immune control may come at the cost of high immune activation which itself brings well-documented, significant pathologies in chronic infection.

The situation following early ART initiation is almost diametrically opposed to that in ART-naïve infection. Recent studies have highlighted the key features associated with PTC. Early ART initiation facilitates PTC, but the salient immunological features that have been identified are low immune activation; the notable absence of an association with strong HIV-specific CD8+ T-cell activity or with 'protective' HLA-I; but a central role for antiviral NK responses. The immunogenetic link with PTC is rather with 'disease susceptible' HLA-I, potentially through reduced immune activation via strong LILR-B2 binding affinities, and with HLA genotypes that favour KIR education of NK cells and more effective antiviral NK responses in HIV-1 infection. In these respects, early-life immunity appears better-positioned than the adult immunity to achieve PTC following early ART initiation.

SCOPE AND AIMS OF THIS THESIS

Motivated by this, this thesis aims to investigate factors associated with paediatric PTC, as infants appear particularly informative for studying mechanisms underlying PTC. Although cases of paediatric PTC are rare (Bengu et al. 2024; Frange et al. 2016; Luzuriaga et al. 2015; Persaud et al. 2013; Violari et al. 2019), likely due to the risk of conducting ATI's on infants, they have provided important evidence that functional cure or remission may be achievable in this setting. The findings presented in this thesis may help researchers better understand the determinants of PTC potential in infants LWH and potentially aid in identifying children with high PTC potential who could be considered for ATI studies.

- In my first results chapter (**Chapter 3**), I investigated immunogenetic and immunological factors associated with low total HIV-1 DNA, a proxy for HIV-1 reservoir size and therefore a biomarker for PTC potential, in children who remained virally suppressed from a cohort of early-ART treated children, a subset of children who may be considered for ATI studies. We further investigated 5 male infants who achieved periods of PTC in this cohort.

- In **Chapter 4**, I investigated the potential for HIV-1 to cause natural selection on disease-associated HLA-I alleles in KwaZulu-Natal, South Africa, a high-burden population, since the start of the pandemic and the impact of ART on this process. This study is valuable for two reasons. Firstly, it provides a contemporary study investigating the potential for an infectious disease to drive HLA-I frequency changes in a population, since, although widely accepted, there are few studies demonstrating this. Secondly, this study will provide us with clarity of the overall gene pool of children currently LWH and how it has changed since ART have been introduced, and what this may mean for paediatric PTC in the current ART era.

- In **Chapter 5**, I investigated the immunological implications of subtle polymorphisms in the peptide-binding region of HLA-B by comparing two closely related alleles: HLA-B*58:01 and HLA-B*58:02, a 'protective' and 'disease-susceptible' HLA-B allele, respectively. This chapter investigated this in the unique setting of healthy adults and early ART-treated children. This chapter highlights how even minimal genetic differences in HLA class I alleles can shape the immune landscape in ways that influence immune activation, and ultimately, cure potential. Additionally, it

provides supporting evidence for ‘disease-susceptible’ HLA-B alleles being favourable to achieve PTC, which this study extends to paediatric PTC.

By integrating host genetics, innate and adaptive immunity, transcriptomics, proteomics and viral reservoir dynamics, this thesis aims to deepen our understanding of what factors are associated with durable HIV-1 remission in children. This work adds to a small but growing body of evidence that functional cure is not only biologically plausible but may be systematically predicted, as has been previously shown for male sex (Bengu et al. 2024). As such, it provides important insights to guide future paediatric HIV-1 cure strategies, including the design of ATI studies, and sheds light on which children may be most likely to achieve remission.

In a field where scalable, long-term treatment strategies remain urgently needed, such as PTC, the most promising mechanism of HIV-1 cure, findings presented here contribute both mechanistic understanding and translational relevance.

CHAPTER 2: METHODS

STUDY POPULATIONS

Chapters 3, 4, and 5 utilised samples from the **Ucwaningo Lwabantwana cohort**.

- The Ucwaningo Lwabantwana cohort (meaning 'Learning from Children', otherwise known as the Baby Cure cohort) is a cohort of >330 in utero–infected children enrolled and followed in KwaZulu-Natal, South Africa, from 2015 to the present date (Adland et al. 2020; Bengu et al. 2024; Millar et al. 2020). All infants were tested at birth via the SoC dried blood spot total nucleic acid PCR (COBAS AmpliPrep/COBAS TaqMan HIV-1 Qualitative PCR v2, Roche Molecular Diagnostics) that was run in a central laboratory. If the result of this test was positive or indeterminate, a confirmatory or repeat test, respectively, was undertaken at approximately 7 days of age. All children born to mothers LWH received ART in the delivery room within minutes of birth (either NVP alone or AZT plus NVP, according to local guidelines). Infants of mothers at high risk of in utero HIV-1 transmission were also tested for HIV-1 as soon as possible after birth using PoC testing (in addition to the SoC testing) to detect total nucleic acid via PCR on whole blood (GeneXpert Qualitative HIV-1 PCR, Cepheid). Baseline data were collected at a median of 1.0 (IQR 0.9–1.8) day of age and 11 (IQR 9–14) days of age from the PoC-diagnosed and SoC-diagnosed infants, respectively. Initial ART for infants with confirmed HIV-1 infection comprised twice-daily NVP, AZT and lamivudine (3TC) as per local guidelines. This regimen was switched to RTV-boosted LPV, 3TC and abacavir (ABC) at 42 weeks corrected gestational age or at 1 month of age. Mother and infant follow-up occurred monthly for 6 months and then every 3 months. At

each visit, blood was drawn for CD4+ T cell quantification, pVL (HIV-1 RNA PCR, NucliSens) and storage of PBMCs and plasma. The Ucwaningo Lwabantwana cohort is an ongoing study which has been approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BF450/14) and the Oxfordshire Research Ethics Committee (06/Q1604/12). Written informed consent for the infant's and mother's participation in the study was obtained from the mother or the infant's legal guardian.

Chapter 4 utilised HLA class I typing data from the **PEHSS cohort**.

- The PEHSS cohort (Paediatric Early HAART and Structured Treatment Interruption Study) is a historical cohort of early-treated infants living with HIV-1 followed in 2002–2005 in Durban, KwaZulu-Natal, South Africa (Mphatswe et al. 2007). Antenatal mothers were recruited from October 2002, and paediatric study subjects were enrolled between July 2003 and September 2005. At this time, single-dose nevirapine (sd-NVP) was the only intervention available for prevention of mother-to-child transmission. Mothers were given sd-NVP (200 mg) at the onset of labour and infants were given sd-NVP (2mg/kg) within 72h of birth. Plasma viral loads were determined from 662 mothers and 71 infants using Roche Amplicor assay version 1.5 (Roche Molecular Systems, Branchburg, New Jersey, USA). This cohort included non-transmitting mothers. The PEHSS cohort was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, Durban and the Institutional Review Board of the Massachusetts General Hospital, Boston, Massachusetts, USA.

Chapter 4 utilised HLA class I typing data from a **healthy adult cohort from Durban, South Africa (2000)**.

- The HIV-uninfected control cohort from the pre-ART era was a cohort of 110 blood donor adults enrolled by the Natal Blood Transfusion Service, Pinetown, Durban, KwaZulu-Natal, South Africa in the year 2000.

Chapters 4 and 5 utilised HLA class I typing data and PBMCs from the **FRESH cohort**.

- The FRESH cohort (Females Rising through Education, Support and Health) is a prospective study that recruits women who are HIV-1 negative, aged 18–24 years and are tested for HIV-1 RNA in plasma twice weekly for 1 year (Dong et al. 2018). Women are recruited from the Umlazi district, KwaZulu-Natal, South Africa, a high HIV-1 prevalence area. Each time the women come to the study centre, they participate in peer-support groups and receive a stipend. In addition to semi-weekly virus testing by PCR with reverse transcription, whole blood is collected four times (including during enrollment) throughout the year from participants. If a plasma test comes back positive, the participant is asked to come back to the clinic that day to collect a blood sample. Samples are then collected weekly through the first 6 weeks of infection and regularly afterwards as long as the participant continues to return to the study centre. Participants who test positive for viral RNA are initiated on ART when they are called back into the study centre for their first post-infection sample collection. The FRESH study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE699/18), where all study participants provided written informed consent.

Chapter 4 utilised HLA class I typing data from the **Ucwaningo Lwamawele cohort**.

• The Ucwaningo Lwamawele cohort (meaning 'Learning from Twins') is an ongoing study which recruits mothers and their twin children from Durban, KwaZulu-Natal, both mothers living with and without HIV. Chapter 4 utilised HLA class I typing (two digits) from 32 uninfected mothers. The Ucwaningo Lwamawele cohort is an ongoing study which has been approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BF450/14) and the Oxfordshire Research Ethics Committee (06/Q1604/12). Written informed consent for the infant's and mother's participation in the study was obtained from the mother or the infant's legal guardian.

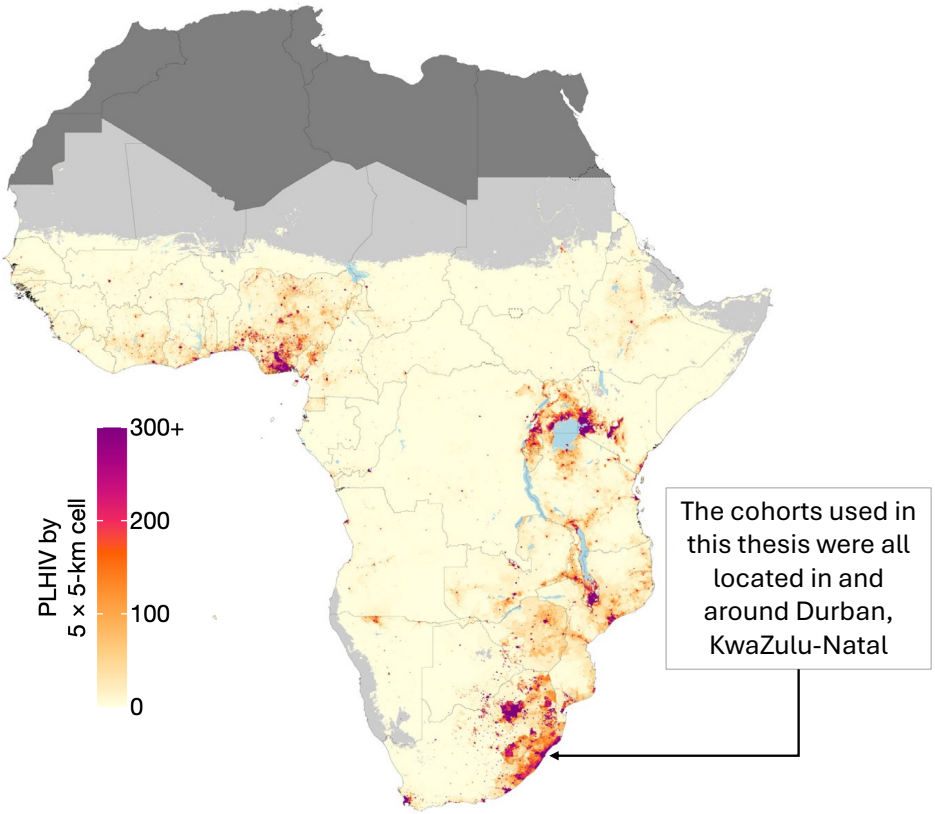

Figure 2.1. Number of people living with HIV-1 for adults aged 15–49 on the African continent in 2017. Figure adapted from Dwyer-Lindgren, Nature, 2019.

BLOOD PROCESSING AND CRYOPRESERVATION

Chapters 3 and 5 utilised plasma and PBMC samples from the Ucwaningo Lwabantwana cohort. Blood samples from the Ucwaningo Lwabantwana cohort were taken at their respective clinics located in KwaZulu-Natal and processed at the Human Pathogenesis Laboratory (HPP) based at the Nelson Mandela School of Medicine in Durban city centre. The clinics are in Edendale, Stanger, Empangeni and Umhlanga. 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 off. Plasma samples were separated after the whole blood was spun and stored at -80^{o}C. The PBMC 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 into cryovials. PBMCs were initially stored at -80^{o}C in a freezing container (Nalgene) for slow cooling at 1^{o}C/min. The following day, samples were transferred into a liquid nitrogen tank.

HLA TYPING

Samples from the PEHSS cohort were HLA typed using a targeted next-generation sequencing (NGS) method. Briefly, locus-specific primers are used to amplify HLA-A and B (exons 1 to 4) and C (exons 1 to 5) genes with Fluidigm Access Array (Fluidigm Singapore PTE Ltd, Singapore). The Fluidigm polymerase chain reaction (PCR) amplicons are pooled and subjected to sequencing on an Illumina MiSeq sequencer (Illumina, San Diego, CA

92122 USA). HLA alleles and genotypes are called using the Omixon HLA Explore (version 2.0.0) software (Omixon, Budapest, Hungary). For the HIV-negative KZN adults from 2000, genomic DNA samples were initially typed to an oligo-allelic level using Dynal RELITM reverse Sequencing Specific Oligonucleotide (SSO) kits for the HLA-A, -B, -C loci (Dynal Biotech). Refining the genotype to the allele level was performed using the Dynal Biotech sequence-specific priming kits in conjunction with the previous SSO type. Where alleles were still not defined to the allele level, bespoke sequence-specific priming primer mixes were utilised. All HLA class I alleles in the IMGTallele release 2.4.0 were considered for typing. For samples from the FRESH cohort, Ucwaningo Lwamawele cohort and Ucwaningo Lwabantwana cohort, HLA typing was done using in house locus-specific primers, which were used to amplify HLA-A/B and C (exons 2 and 3) genes. The products were purified and sequenced using in-house designed primers on an AB3730 DNA analyser. Traces were analysed using bespoke software and BioEdit.

TOTAL HIV-DNA QUANTIFICATION

Total HIV-1 DNA levels in the paediatric cohort were quantified using droplet-digital PCR (ddPCR, Bio-Rad) starting from 1×10^6 PBMCs. Samples were screened with three different primer/probe sets, two annealing to the 5′LTR and gag conserved regions of HIV-1 genome (Morón-López et al. 2017) and an additional degenerated primer/probe set adapted to clade C (iSCA-int Forward Degenerate TTTGGAAAGGACCAGCMAA, iSCA-int Reverse CCTGCCATCTGTTTTCCA, iSCA-int Probe AAAGGTGAAGGGGCAGTAGTAATACA) targeting integrase gene, to overcome HIV-1 sequence variability. Input cell number was verified by quantifying the amount of genomic RPP30 gene, in a parallel ddPCR assay, and this value was used to infer the limit of detection for each sample.

IMMUNOPHENOTYPING OF PERIPHERAL NK CELLS

NK cells were phenotyped from virally suppressed children (n=28) from the Ucwaningo Lwabantwana cohort in chapter 3. PBMC were thawed, counted and 1 million cells were rested in R10 medium for 3 hours at 37˚C in 5% CO2. Cells were washed in PBS and stained with Live/Dead near-IR stain (Invitrogen) according to the manufacturer's instructions in room temperature for 30 min. PBMC were washed again in FACS Buffer and incubated for another 30 min at 4˚C in a suspension with their respective set of the fluorochrome-conjugated surface antibodies: CD14 (APC-Cy7, HCD14, Biolegend), CD19 (APC-Cy7, HIB19, Biolegend), CD3 (APC-Cy7, UCHT1, Biolegend), CD56 (BV650, 5.1H11, Biolegend), NKG2A (PE-Vio770, GL183, Miltenyi), and a cocktail of mainly inhibitory KIRs (iKIRs) on APC (KIR2DL1 143211 R&D Systems, KIR2DL2/L3/S2 GL183 Beckman Coulter, KIR3DL1 DX9 Miltenyi, KIR3DL2 539304 R&D Systems), CD16 (SB600, 3G8, Life Technologies), NKG2D (PerCP-Cy5.5, DX27, Miltenyi), NKp30 (PE-Vio615, REA823, Miltenyi), NKp46 (V450, 9E2, BD Bioscience) and DNAM-1 and (BV510, 11A8, Biolegend). Cells were washed, fixed in 2% PFA, and acquired on a LSR II (BD Bioscience). Data was analysed using FlowJo software v10.10.10 (TreeStar Inc.).

IMMUNOPHENOTYPING OF PERIPHERAL T CELLS

T cells were phenotyped from virally suppressed children (n=27) from the Ucwaningo Lwabantwana cohort in chapter 5 and from the FRESH cohort (n=35, HIV-1 uninfected) in chapter 5. PBMC were thawed, counted and 1 million cells/mL were resuspended in R10 medium. Cells were washed in PBS and stained with Live/Dead near-IR stain (Invitrogen) according to the manufacturer's instructions at room temperature for 30 min. PBMC were

washed again in FACS Buffer and incubated for another 30 min at 4˚C in a suspension with their respective set of the fluorochrome-conjugated surface antibodies: CD3 (BV605, UCHT1, Biolegend), CD4 (BV650, RPA-T4, Biolegend), CD8a (BV570, RPA-T8, Biolegend), CD45RA (FITC, H100, Biolegend), CCR7 (Pacific Blue, G043H7, Biolegend), CD39 (APC, A1, eBioscience), PD-1 (PE-eFluor610, J105, eBioscience), CD27 (BV510, M-T271, Biolegend), CCR5 (PE-Cy7, HM-CCR5, Biolegend), HLA-DR (APC-R700, G46.6, BD), CD38 (PerCP-Cy5.5, HIT2, Biolegend), CD73 (PE, AD-2, Biolegend). Cells were washed and acquired on a LSR II (BD Bioscience), and data was analysed using FlowJo software v10.10.10 (TreeStar Inc.).

NK CELL FUNCTIONAL ASSAYS

NK cell functional assays were conducted on PBMCs from the Ucwaningo Lwabantwana cohort in chapter 3, including K562 stimulation (n=21), RAJI/Rtxb stimulation (n=22), and IL-12/IL18 stimulation (n=21) assays, summarised in **Fig 2.2**. Half to one million thawed PBMC per condition were rested in R10 medium for 5 hours for cytokine stimulation and overnight for K562 and RAJI cells assays at 37˚C in 5% CO2. To assess cytokine stimulation, PBMC were either left unstimulated or incubated with 10 ng/mL of rhIL-12 (R&D Systems) and 100 ng/mL of rhIL18 (R&D Systems) for 18 hours at 37˚C. Cells were incubated with CD107a (APC, H4A3, BD Bioscience) at the start of stimulation and Brefeldin A (BFA, 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 ratio). For ADCC study, RAJI cells (1 million cells/mL) were coated with 2.5 µg/ml of anti-CD20 (R&D Systems) for 30 min, washed and incubated with PBMC at a 10:1 effector to target ratio for 6 hours at 37˚C. For both K562 and RAJI assays,

cells were incubated with CD107a (APC, H4A3, BD Bioscience) at the start of stimulation and BFA/Monensin were added in the last 5 hours. At the end of incubation, cells were washed in PBS and stained with Live/Dead near-IR stain (Invitrogen) in room temperature for 30 min, followed by surface staining for another 30 min at 4˚C with CD14 (APC-Cy7, HCD14, Biolegend), CD19 (APC-Cy7, HIB19, Biolegend), CD3 (APC-Cy7, UCHT1, Biolegend), CD56 (BV650, 5.1H11, Biolegend), CD16 (SB600, 3G8, Life Technologies), NKG2A (PE-Vio770, GL183, Miltenyi), NKG2C (PE, 134591, R&D Systems), CD57 (SPRD (Cy5), NK-1, Southern Biotech), a cocktail of inhibitory KIRs (iKIRs) on Alexa700 (KIR2DL1 143211, KIR2DL2/L3/S2/S4 180704, KIR3DL1 177407, KIR3DL2 539304, R&D Systems). Cells were then washed in FACS buffer (1% FBS in PBS) and then fixed and permeabilized (Foxp3/Transcription Factor Staining Kit, Thermo Fisher) as per manufacturer's instructions. Cells were then stained for 45 min at 4˚C with TNFa (FITC, Mab11, BD Bioscience), PLZF (PE-CF594, R17-809, BD Bioscience) and IFN-γ (BV421, 4S.B3, BD Bioscience) for 30 min at 4˚C. Cells were washed and acquired on a BD LSR II, and data were analysed using FlowJo software v10.10.10 (TreeStar Inc.). The frequency of CD107a+, IFNg+ and TNFa+ cells are shown in figures after subtracting the background. Boolean gating was used to analyse their frequency in $CD56^{dim}$ NK cells expressing NKG2A and iKIRs, as well as CD57 and NKG2C.

Here, I measured three complementary NK cell functions to capture the breadth of their antiviral capacity: (i) recognition and killing of "missing self" targets (K562 stimulation), (ii) antibody-dependent cellular cytotoxicity (ADCC, RAJI/Rituximab stimulation), and (iii) responsiveness to pro-inflammatory cytokines (IL-12/IL-18 stimulation). These functional capacities reflect the major pathways through which NK cells can contribute to the elimination of HIV-infected cells. First, I examined NK cell ADCC activity using the RAJI–

Rituximab assay. In this model, RAJI cells, a continuous, non-adherent human B-lymphoblastoid cell line derived from a Burkitt lymphoma patient, are pre-coated with a monoclonal anti-CD19 antibody (Rituximab). Antibody opsonisation enables engagement of the CD16 (FcγRIIIa) receptor on NK cells, triggering ADCC. Upon encountering antibody-coated RAJI cells, NK cells degranulate, a process which I quantified by surface expression of CD107a. This assay specifically measures the ability of NK cells to mediate Fc-dependent killing, a critical mechanism in HIV-1 infection where antibody-opsonised infected cells or viral particles can be targeted. Second, I assessed missing-self recognition using K562 cells. K562 is an MHC class I deficient erythroleukaemia cell line classically used to probe NK activation in the absence of inhibitory HLA–KIR signalling. Because HIV-1-infected $CD4^+$ T cells often downregulate MHC I molecules such as HLA-A and HLA-B to evade $CD8^+$ T cell recognition, the K562 assay models how efficiently NK cells respond when inhibitory signals are reduced. Degranulation or cytokine production following K562 stimulation, therefore, reflects the cytotoxic readiness of NK cells to detect and eliminate stressed or HLA-deficient targets. Finally, I measured NK cell responsiveness to innate immune cytokines by stimulating cells with IL-12 and IL-18. These cytokines synergistically induce robust IFN-γ production and enhance cytotoxic granule release. IL-12/IL-18 stimulation tests the capacity of NK cells to integrate inflammatory cues, a major pathway driving NK activation in early viral infection and an important determinant of antiviral function.

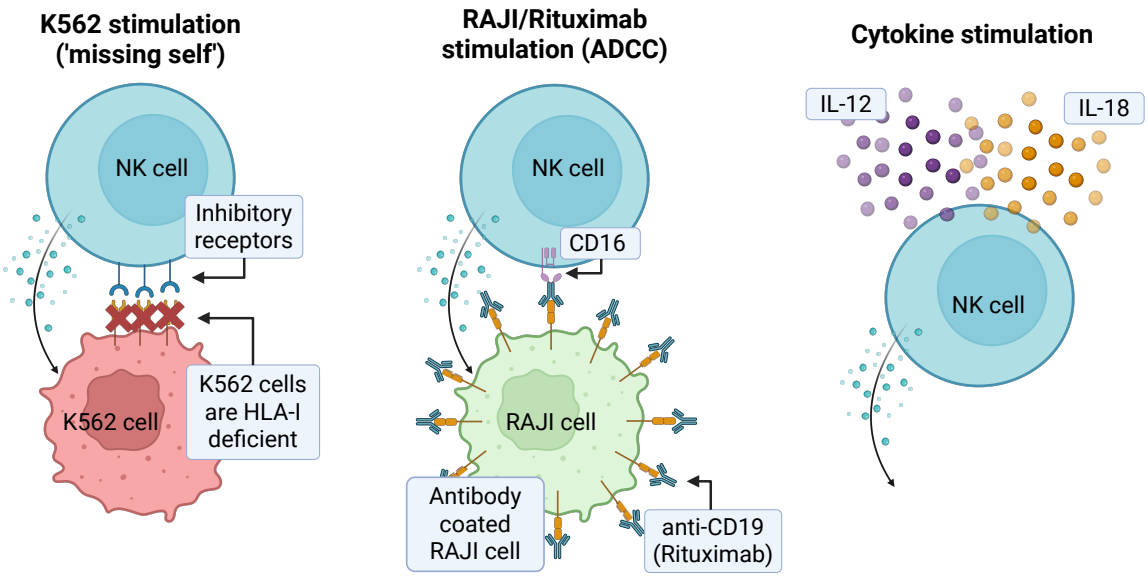

Figure 2.2. NK cell functional assays. Figure made with Biorender.

WHOLE BLOOD HIV-1 PEPTIDE STIMULATION AND INTRACELLULAR CYTOKINE STAINING

In chapter 5, whole blood HIV-1 peptide stimulations were conducted to access HIV-1 specific T cell responses from the Ucwaningo Lwabantwana cohort at HPP in Durban, South Africa. Within 2 hours of the blood samples being taken, 500ul of blood was added to each condition [unstimulated, Gag, Pol, Nef, Env, TRVVV, positive control (SEB)] along with co-stimulant antibody cocktail (BD: 347690) and their respective HIV-1 peptide pools (2ug/mL) or SEB (2ug/mL). Samples were incubated at 37°C in a water bath. After 5-6 hours, BFA was added (5ug/mL) and incubated for 5 hours in a timed water bath. The next morning, no more than 10 hours after water bath was switched off, 2mM EDTA was added per condition for 15 min. Following this, each sample was added to FACS Lysing solution (BD: 349202) for 10 mins at room temperature in the dark. Samples were then spun and washed in PBS and then resuspended in freezing media [FCS containing 10% Dimethyl sulfoxide (DMSO, Sigma)]

into individual cryovials. Samples were initially stored at -80ºC in a freezing container (Nalgene) for slow cooling at 1ºC/min. The following day, samples were transferred into a liquid nitrogen tank.

Intracellular staining was then conducted on these samples at HHP in Durban, South Africa. Briefly, cells were thawed and resuspended in R10 medium. Cells were washed with PBS and then transferred into 96 well plates. Cells were again washed and then resuspended in surface staining antibody cocktail [CD3 (BUV295, UCTH, BD), CD4 (BUV496, OKT4, BD), CD8a (V500, RPA-T8, BD)] for 30 min at 4ºC in the dark. Cells were then washed in FACS buffer (1% FBS in PBS) and then fixed and permeabilised (Foxp3/Transcription Factor Staining Kit, Thermo Fisher) as per the manufacturer's instructions. Cells were then stained for 45 min at 4˚C with TNFa (AF700) and IFNg (PE-Cy7) for 30 min at 4˚C. Cells were washed, fixed in 2% PFA, and acquired on a BD Fortessa. Data were then analysed using FlowJo software v10.10.10 (TreeStar Inc.).

OLINK TARGETED PROTEOMICS

Plasma samples (n=34) from children in the Ucwaningo Lwabantwana cohort were sent to Probiomics S.r.l, Rome, Italy, to conduct proteomic assays, which contributed to chapter 5. Here, serum immune patterns were characterised using Olink's Multiplex Proximity Extension Assay (PEA technology) on the Inflammation and the Immune Response panels. A total of 180 proteins were quantified on plasma samples at 9 and 24 months as previously described (Morrocchi et al. 2022). Briefly, after thawing and randomisation, 1ul of plasma for each sample was transferred into a 96-well plate with U-bottom (Cat.#CLS3799) for the overnight incubation at 4°C with an Incubation mix, as described by the manufacturer in the

user manual (Olink Target96). The day after, the extension of the coupled oligos and the amplification steps were carried on as recommended by Olink. The Fluidigm BioMark platform was used for the Real-Time PCR quantification. Proteomics data were reported with the arbitrary log2 scale unit Normalised Protein eXpression (NPX), wherein a higher NPX correlates with higher protein expression. The data were preprocessed using the NPX Manager Software and OlinkAnalyze R package (version 3.4.1). Proteins with more than 80% of values below the limit of detection were discarded. Experimental and computational analysis were executed by Probiomics S.r.l. (Rome, Italy).

SINGLE-CELL RNA SEQUENCING

Cryopreserved PBMCs (n=8) were thawed rapidly in a 37 °C water bath, then slowly diluted in pre-warmed R10, centrifuged and resuspended in PBS. To obtain single-cell RNA-seq data, 10x Genomics Chromium Next GEM Single Cell 3' Kit v3.1 was used according to the manufacturer's instructions. Briefly, PBMCs were diluted in PBS, washed, and then incubated with Cell multiplexing oligos according to the manufacturer's instructions. After washing and prior to 10X chip loading, cells were counted and showed high viability (>80%). Subsequently, cell-plexed cells were mixed (2 samples mixed), counted again and then loaded on a Chromium Next GEM chip G (10X). Post GEM-RT clean up, cDNA amplification and library construction were performed following the manufacturer's instructions. Libraries quality was determined through TapeStation D5000 ScreenTape (Agilent Technologies). Libraries were quantified by Qubit 2.0 (ThermoFisher). Samples were sequenced using the Illumina NovaSeq 6000. The single-cell transcriptome data were aligned and quantified by Cell Ranger v3.1 using GRCh38 (Ensembl 93). Pooled donor samples were deconvolved using Souporcell101, which yielded a genotype variant that

allowed donor identity to be matched across samples. Cells with the following features were filtered out: more than 20% mitochondrial gene content, more than 7500 counts per cell, fewer than 500 genes, or more than 5000 genes. Additionally, genes expressed in fewer than three cells were removed. Downstream analysis was performed using the *Seurat R package*.

STATISTICS

For scatterplots, median values, interquartile ranges, and biological replicates are indicated. For categorical variables, comparisons were performed using Fisher's exact tests. Spearman's test was used to show correlation, and a simple linear regression model for the best-fit line and 95% confidence interval. All comparisons were non-parametric. When comparing three or more groups (unpaired); Kruskal-Wallis tests followed by Dunn's test for multiple comparisons were conducted. When comparing two groups (unpaired); Mann Whitney U-tests were conducted. When comparing three or more groups that were paired, Friedman tests were conducted. The heatmap analysis was done using the *pheatmap R package* after standardising each parameter's frequency data to z-score and calculating the mean per group; the markers were clustered using the Euclidean method. All p-values were two-sided, and a p-value less than 0.05 was considered significant throughout this thesis. All calculations performed and graphs generated using GraphPad Prism v10.4.1 (GraphPad Software), except for the single-cell RNA seq figures and heatmaps, which were produced in RStudio.

CHAPTER 3: LOW HLA-A EXPRESSION LINKED TO NK CELL PHENOTYPE AND FUNCTIONALITY IS ASSOCIATED WITH LOW TOTAL HIV-1 DNA AND CURE/REMISSION IN VERY EARLY ART-TREATED CHILDREN

INTRODUCTION

Post-treatment control (PTC) following early ART initiation has been proposed to be more easily attainable in children than adults living with HIV-1 (LWH) for three main reasons (Goulder et al. 2016). First, infants can receive antiretroviral therapy (ART) immediately after birth and therefore sooner after infection compared to adults (Rouzioux et al. 1995), leading to a small viral reservoir with low viral diversity (Bitnun et al. 2014; Garcia-Broncano et al. 2019). The size of the viral reservoir is a major predictor of time to viral rebound following ART interruption (Assoumou et al. 2015; Li et al. 2015; Williams et al. 2014). Studies in humans and nonhuman primates show clearly that early ART initiation results in lower viral setpoints following subsequent treatment interruption (Jacobson et al. 2006; Okoye et al. 2018, 2021). Secondly, the replicative capacity of the virus transmitted from mother to child is lower than that of the virus circulating the mother (Bengu et al. 2024; Naidoo et al. 2017b). The replicative capacity of the transmitted virus has a profound impact on immune activation, proviral DNA load, and outcome in the recipient (Claiborne et al. 2015). Thirdly, the tolerogenic early life immune environment results in higher levels of immune regulation and lower levels of immune activation than those found in adults

(Kollmann et al. 2009; Levy 2007; Michaëlsson et al. 2006). Low levels of immune activation has been identified as a key feature of PTC in adults (Etemad et al. 2023; Sáez-Cirión et al. 2013). Together, these findings suggest PTC may be more easily attainable in children compared to adults LWH.

Furthermore, whereas immune control in ART-naïve adult HIV-1 infection is associated with effective HIV-specific CD8+ T-cell activity, and the expression of 'protective' HLA-I molecules such as HLA-B*57, among PTC, HIV-specific CD8+ T-cell responses are reportedly unremarkable (Etemad et al. 2023; Sáez-Cirión et al. 2013) and there is a striking underrepresentation of 'protective' HLA-B alleles (Martin et al. 2017; Sáez-Cirión et al. 2013). Although NK cell activity contributes to immune control in ART-naive infection in adults (Alter et al. 2007, 2011; Hölzemer et al. 2015; Martin et al. 2007; Ziegler et al. 2021), its impact is less than that of HIV-specific CD8+ T-cells (Martin et al. 2007). However, NK responses reportedly play a central role in PTC (Essat et al. 2025; Etemad et al. 2023; Kim et al. 2022b; McKinnon et al. 2014). There is therefore a stark contrast between the immune responses that lead to viral control in the absence of therapy compared to those that are associated with PTC (reviewed in Herbert and Goulder 2023). Children appear to be better adapted to achieve PTC than ART-naïve control of HIV-1, which is extremely rare following vertical transmission (Vieira et al. 2019). Whereas effective HIV-specific CD8+ T-cell responses are not supported by early-life immunity (Kollmann et al. 2009; Leitman et al. 2017), NK responses play a major part in viral control and preventing disease progression in ART-naïve children (Vieira et al. 2021). NK cell numbers are at their highest at birth, and NK responses are active and capable of effective HIV-1 disease prevention from birth and potentially even in utero (Guilmot et al. 2011; Murphy et al. 2023; Strauss-Albee et al. 2017;

Vieira et al. 2021). These factors may contribute to a greater potential for PTC to be achieved in paediatric versus adult infection.

It has been proposed that PTC in adults LWH is associated with an HLA-I signature that combines disease-susceptible HLA-B molecules with HLA genotypes that mediate a KIR-biased education of NK cells (Saez-Cirion et al 2013, Essat et al. 2025). Further studies have also shown that HLA-I genotypes that favour KIR-education of NK cells are associated with favourable outcomes in HIV-1 infection. Threonine at -21 of HLA-B signal peptides has been associated with reduced susceptibility to HIV-1 infection in adults (Merino et al. 2012) as well as with more effective NK cell-mediated control of HIV-1 both in adults (Merino et al. 2013). Furthermore, high HLA-A expression has been associated with impaired viral control in ART-naïve adults through the inhibition of NKG2A+ NK cells (Ramsuran et al. 2018). Lastly, and importantly for our study, both low HLA-A expression and the presence of Bw4 epitopes have been associated with immunological and viral control in ART-naïve children LWH (Vieira et al. 2021).

To evaluate the role of NK responses in paediatric PTC, we investigated the genetic and immunological factors associated with low total HIV-1 DNA, a proxy for HIV-1 reservoir size, in children who remained virally suppressed within an early-ART treated paediatric cohort from KwaZulu-Natal, South Africa. In this study, we additionally examined the impact of this HLA-I signature on NK cell phenotype and functionality to gain mechanistic insights into how this HLA-I profile modulates NK cell responses. Finally, we examined the unique NK cell profiles of five male participants from this cohort who exhibited persistent ART-free aviraemia after unscheduled treatment interruption (Bengu et al. 2024).

RESULTS

Birth total HIV-1 DNA is associated with cure/remission outcome in paediatric infection

To evaluate mechanisms of PTC in children LWH, we prospectively studied 274 very-early-ART-treated children from KwaZulu-Natal, South Africa. We initially sought to identify factors associated with low total HIV-1 DNA loads since low DNA load is linked to HIV-1 cure/remission potential (Assoumou et al. 2015; Li et al. 2015; Williams et al. 2014). Whilst the size of the viral reservoir is not directly accessible, being mainly located in lymphoid tissue, a surrogate measure of this is the total HIV-1 DNA copies per million PBMCs (Eriksson et al. 2013). We quantified total HIV-1 DNA from baseline PBMC samples collected from the cohort (**Fig 3.1A**) and observed that birth total HIV-1 DNA load was strongly associated with time to reach undetectable HIV-1 DNA levels (**Fig 3.1BC,** $p<0.0001$). 50% of children with baseline total HIV-1 DNA loads of <200cpm PBMC (in the 4^{th} quartile) had achieved undetectable DNA loads by 6 months, whereas virtually no children whose baseline total HIV-1 DNA loads are >2451cpm PBMC (in the 1^{st} quartile) reach undetectable total HIV-1 DNA loads at any stage.

Furthermore, as previously reported (Bengu et al. 2024), we unexpectedly identified 5 children who maintained ART-free aviraemia for at least 3-17 months following unscheduled treatment interruption. Bearing in mind that in adult studies, the average time to viral rebound following analytical treatment interruption is 2-3 weeks, and only 4% of individuals maintain aviraemia for 3 months (Gunst et al. 2025), these 5 children are highly unusual in having achieved this degree of remission. Importantly, low baseline HIV-1 DNA loads were associated with the achievement of cure/remission in these 5 children (**Fig 3.1D**).

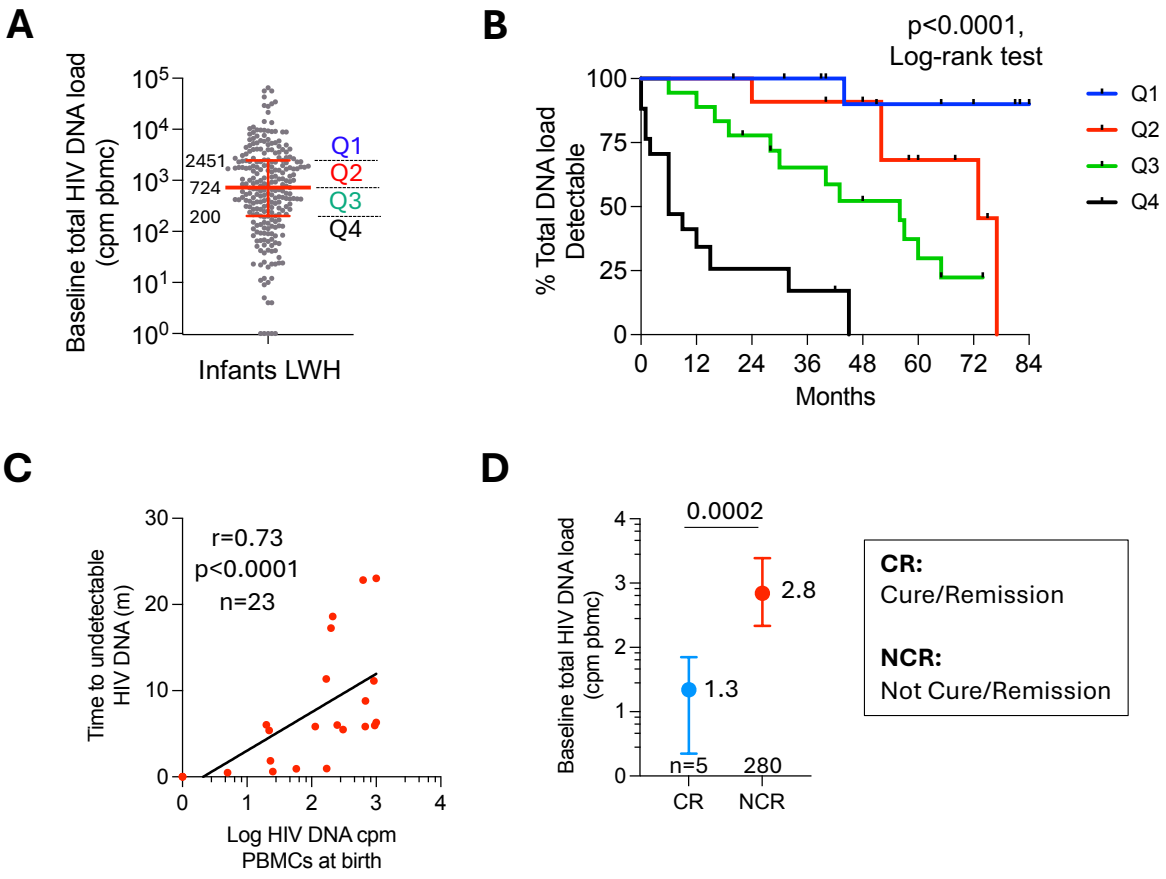

Figure 3.1. Magnitude of total HIV-1 DNA load at birth associates with time to undetectable total HIV-1 DNA load and is notably low in 'atypical' males. A. Total HIV-1 DNA load at birth for cohort (n=274). B. By splitting children into groups based on the magnitude of total HIV-1 DNA load at birth by interquartile ranges, the time to undetectable total HIV-1 DNA load was shown for each group using a Kaplan-Meier curve. A log-rank test was performed. C. Correlation between total HIV-1 DNA at birth (excluding samples with LoD<30 cpm PBMCs due to low DNA recovery) and time to achieve an undetectable total HIV-1 DNA load. A Spearman Rank test was conducted and included a simple linear best-fit line. D. Comparing total HIV-1 DNA load at birth for 'atypical' males, who experienced periods of ART-free viral control, and the rest of the cohort. Mann-Whitney U tests were conducted.

Together, these findings highlight the critical importance of low total HIV-1 DNA at baseline as a predictor both of time to achieving undetectable HIV-1 DNA load and of achieving HIV-1 cure/remission.

Low HLA-A expression is associated with low total HIV-1 DNA load at baseline

We next investigated factors that might drive low baseline total HIV-1 DNA loads. Consistent with previous adult and paediatric studies showing an association between high viral loads and high-expressing HLA-A molecules (Ramsuran et al. 2018; Vieira et al. 2021), we observed an association between total HIV-1 DNA load at birth with HLA-A expression (**Fig 3.2A**, r=0.14, p=0.01). Previously defined allele-specific HLA-A expression values (Ramsuran et al. 2018) were used to calculate a total HLA-A expression score for each participant. For each individual, the expression scores of their two HLA-A alleles were summed to give a total HLA-A expression z-score, as previously done (Ramsuran et al. 2018; Vieira et al. 2021). Low-expressed HLA-A molecules such as HLA-A*74 were associated with low total HIV-1 DNA load and high-expressed HLA-A molecules such as HLA-A*01 were associated with high total HIV-1 DNA load, unadjusted for multiple comparisons (**Fig 3.2B**). Additionally, those children with low HLA-A expression (z-score below-0.5) were associated with a faster time to achieve undetectable HIV-1 DNA, although this did not reach statistical significance (p=0.08, **Fig 3.2C**). Further support for the notion that KIR-educated NK cells are linked with lower HIV-1 viral loads (Ramsuran et al. 2018; Vieira et al. 2021) is indicated by the observation that increasing copies of Bw4 epitopes, which are KIR3DL1 ligands, are associated with decreasing HIV-1 DNA, although this did not reach statistical significance here (**Fig 3.2D**).

We next investigated the immunogenetic features of children aged 9 months who had maintained viral suppression on ART, focusing on this group to ensure that viremia or transient viral rebounds did not confound HIV-1 DNA measurements. As expected, total HIV-1 DNA loads at 9 months correlated with those at baseline/birth (**Fig 3.2E**) and also correlated with HLA-A expression (**Fig 3.2F**). Of note, those children who had achieved

undetectable total HIV-1 DNA by 9 months had significantly lower HLA-A expression levels (p=0.004, **Fig 3.2G**).

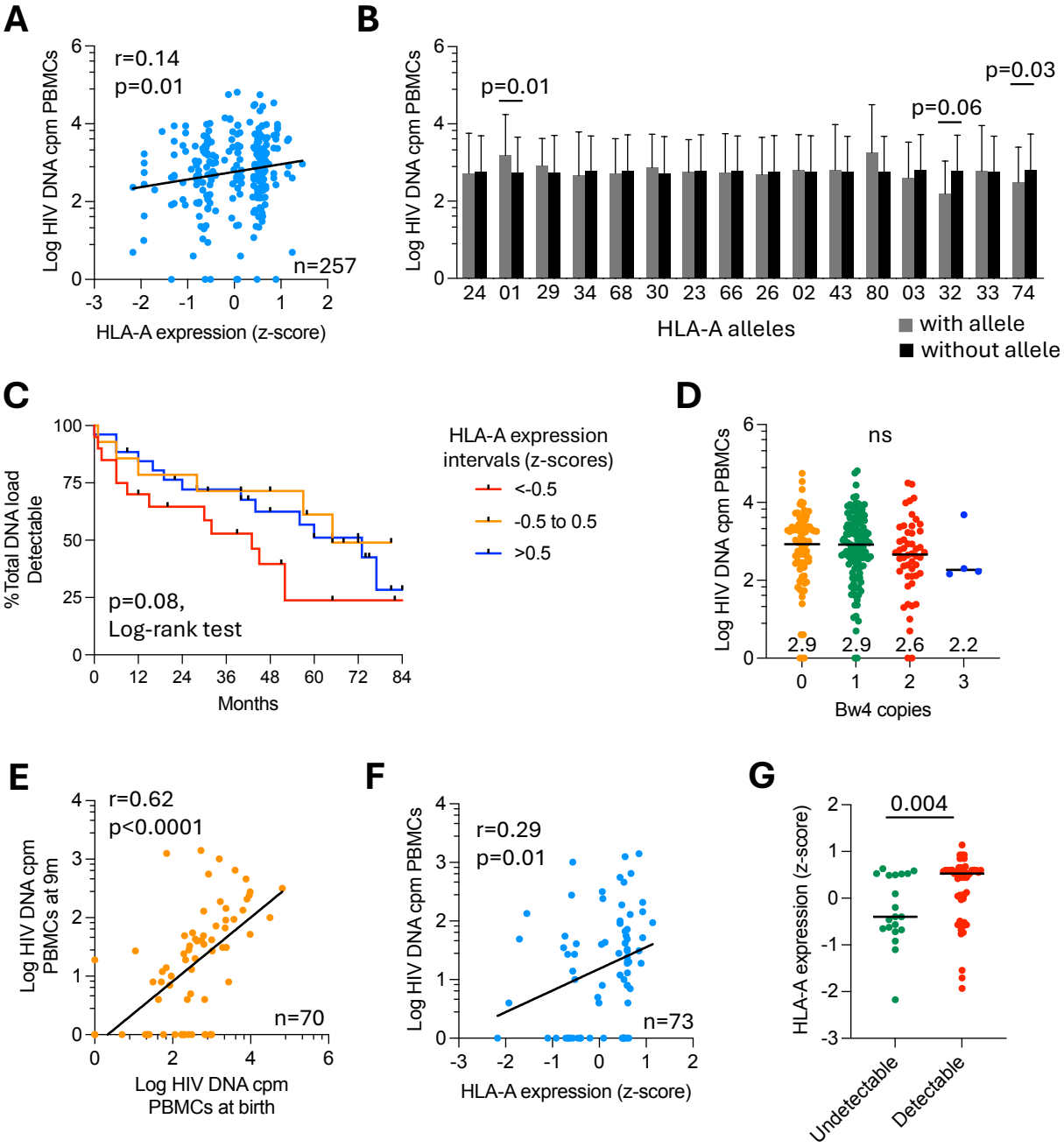

Figure 3.2. Low HLA-A expression associates with low total HIV-1 DNA load. A. Correlation between HLA-A expression levels (z-score) with total HIV-1 DNA load at birth. A Spearman Rank test was conducted, including a simple linear best-fit line. B. Comparisons between total HIV-1 DNA load at birth in individuals with the specified HLA-A allele (grey bars) compared to those without the allele (black bars). Mann-Whitney U tests were

conducted for each HLA-A allele, uncorrected for multiple comparisons. C. By splitting children into groups based on the HLA-A expression intervals, the time to undetectable total HIV-1 DNA load was shown for each group using a Kaplan-Meier curve. D. Comparing total HIV-1 DNA load at birth from individuals with differing numbers of Bw4 epitopes (from both HLA-A and HLA-B alleles). A Kruskal-Wallis test followed by Dunn's test for multiple comparisons was conducted. E. Correlation between total HIV-1 DNA load at birth and at median 9 months old. A Spearman Rank test was conducted and included a simple linear best-fit line. F. Correlation between total HIV-1 DNA load and median 9 months old and HLA-A expression levels (z-score). A Spearman Rank test was conducted and included a simple linear best-fit line. G. Comparison of HLA-A expression levels (z-score) between those that had undetectable total HIV-1 DNA load at median 9 months old and those with detectable total HIV-1 DNA load. A Mann-Whitney U test was conducted.

We also investigated other immunogenetic markers associated with HLA-A, HLA-B or HLA-C that might be related to total HIV-1 DNA at birth (**Fig 3.3**) and in virally suppressed children at 9 months old (**Fig 3.4**) but observed no significant associations.

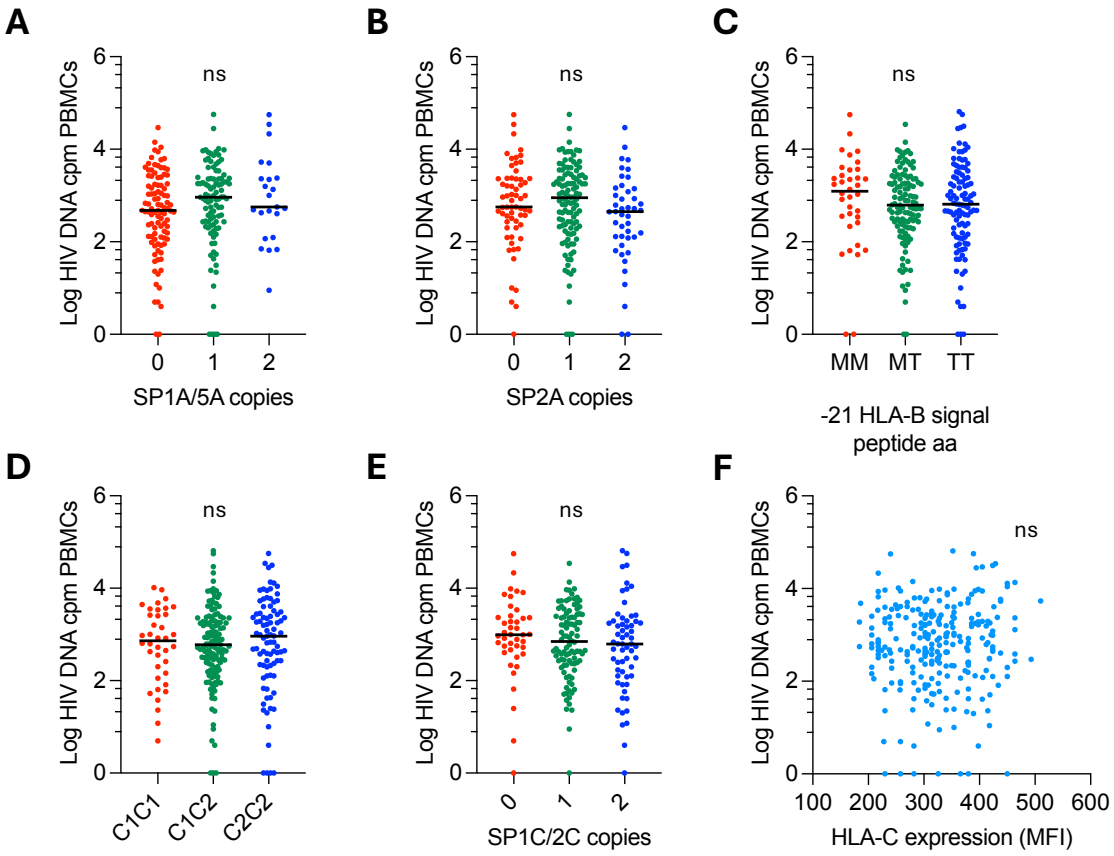

Figure 3.3. Total HIV-1 DNA load associations with HLA-A, HLA-B, and HLA-C at birth. A-B. Total HIV-1 DNA load between individuals with different SP1A/5A copy number and SP2A copy number. C. Total HIV-1 DNA load between individuals with different -21M/T

genotypes. D. Total HIV-1 DNA load between individuals with different C1/C2 genotypes. E. Total HIV-1 DNA load between individuals with different SP1C/SP2C copy number. F. Correlation between total HIV-1 DNA load and HLA-C expression (MFI), a Spearman Rank test was conducted. A Kruskal-Wallis test followed by Dunn's test for multiple comparisons was conducted for all analyses unless stated otherwise.

Of note, however, we observed a pattern of decreasing copies of SP2A signal peptides, favouring KIR-educated NK cells, was associated with lower total HIV-1 DNA in virally suppressed children at median 9 months old, whilst 2 copies of SP1A/5A signal peptides, which favour NKG2A-educated NK cells, had higher total HIV-1 DNA than zero copies (Lin et al. 2023) (**Fig 3.4A-B**).

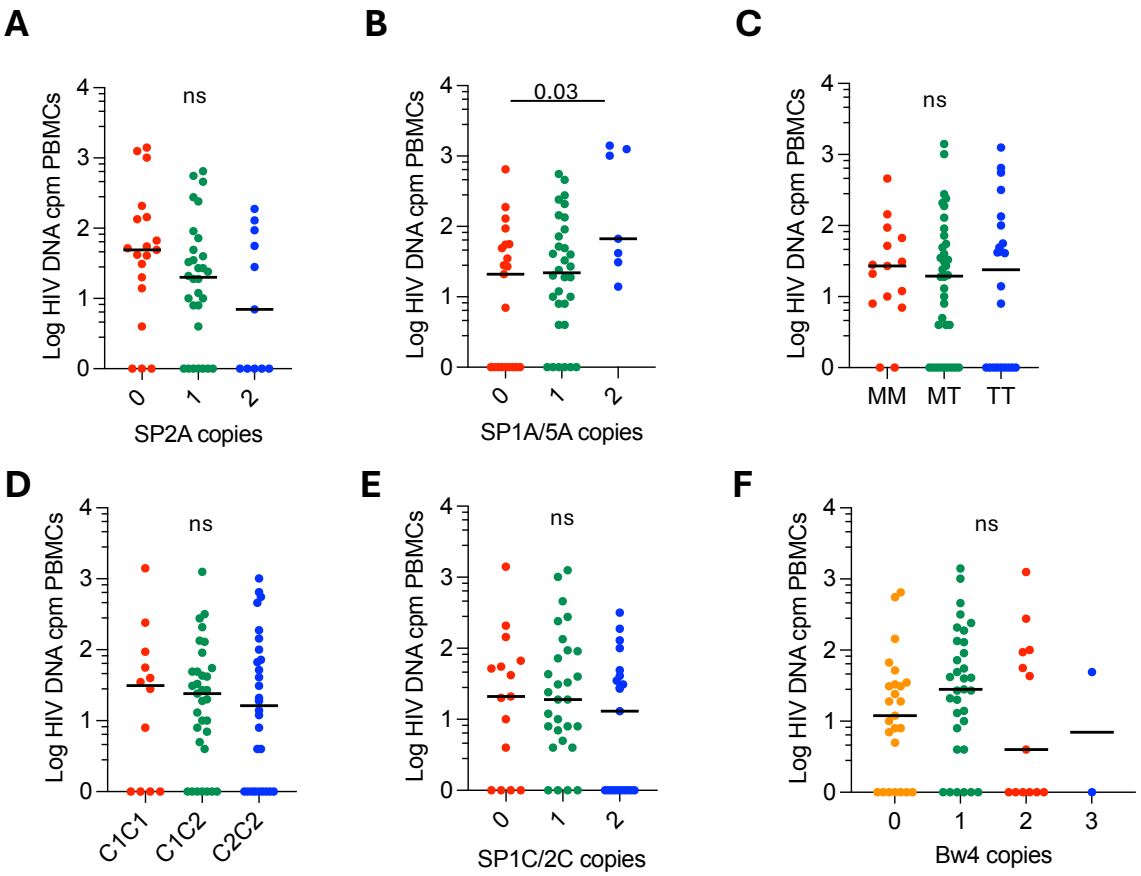

Figure 3.4. Total HIV-1 DNA load associations with HLA-A, HLA-B and HLA-C in virally suppressed children at median 9 months old. A. Total HIV-1 DNA load between individuals with different SP2A copy numbers. B. Total HIV-1 DNA load between individuals with differing SP1A/5A copy numbers. C. Total HIV-1 DNA load between individuals with different -21M/T genotypes. D. Total HIV-1 DNA load between individuals with different -HLA-C1/2 genotypes. E. Total HIV-1 DNA load between individuals with different SP1C/2C

genotypes. C. Total HIV-1 DNA load between individuals with different Bw4 copy numbers. A Kruskal-Wallis test followed by Dunn's test for multiple comparisons was conducted for all analyses.

Together, these data show low HLA-A expression as a predictor of low initial levels of total HIV-1 DNA in children LWH, and no other HLA-related factors investigated here, increasing the likelihood of achieving undetectable levels of total HIV-1 DNA. Since low HLA-A expression is associated with KIR-educated NK cells (Horowitz et al. 2016; Ramsuran et al. 2018), this suggests that certain NK cell functionality may be modulating total HIV-1 DNA in early life and therefore influencing PTC potential.

High NKG2D-expressing CD56^{dim} NK cells associated with low HLA-A expression levels and with low total HIV-1 DNA load

To identify NK cell immunological factors associated with low HLA-A expression and low total HIV-1 DNA, we next evaluated NK cell immunophenotype in age-matched children maintaining viral suppression on ART. We investigated activation and inhibitory markers on CD56^{bright}, CD56^{dim} and CD56^{neg} NK cell subsets, as well as CD56^{dim} NK cell subsets that expressed NKG2A and/or iKIR, in order to further understand those subsets involved with NK cell education at various HLA-A expression levels (**Fig 3.5A**).

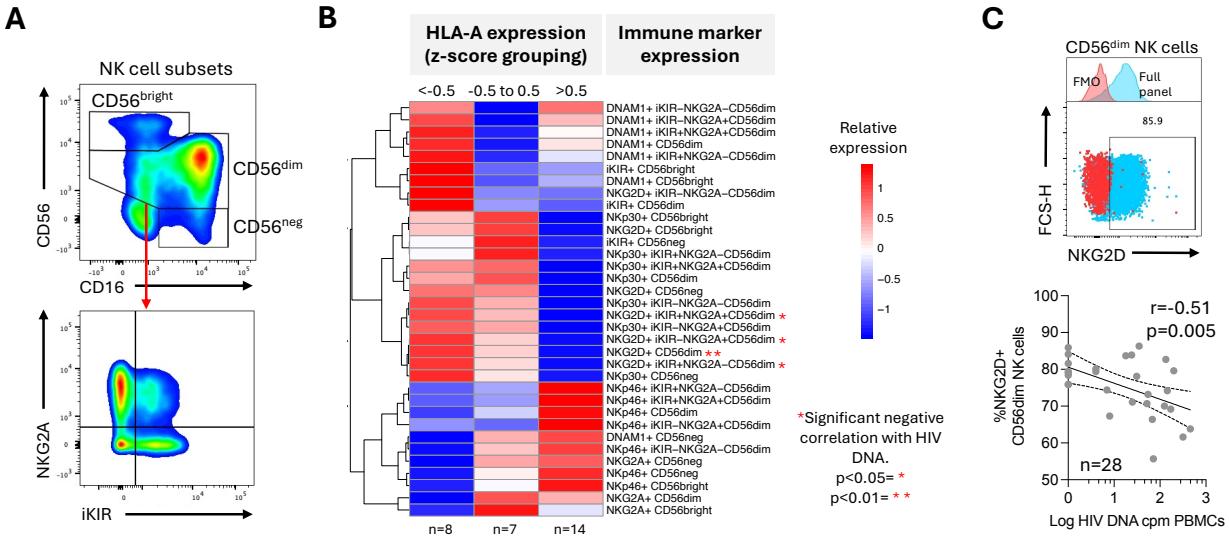

Figure 3.5. NK cell phenotypes compared between infants with differing HLA-A expression levels at median 9 months old. A. NK cell gating for different NK cell subsets, including further subsetting CD56^{dim} NK cells into those that expressed a cocktail of inhibitory KIRs and/or NKG2A using Boolean gating. B. Heatmap showing median z-score based on the frequency of the positive population for each surface marker split between different HLA-A expression intervals using hierarchical clustering. Those with red stars show markers that negatively correlate with total HIV-1 DNA. C. Gating for NKG2D+ CD56^{dim} NK cells and a correlation between the frequency of positive NKG2D CD56^{dim} NK cells and total HIV-1 DNA. A Spearman Rank test was conducted, including a best-fit line with 95% confidence intervals.

We observed that children with low HLA-A expression levels (z-score<-0.5) had relatively higher DNAM1, NKp30 and NKG2D expression and relatively low NKp46 expression across subsets compared to those with high HLA-A expression levels (z-score>0.5, **Fig 3.5B**). We observed that NKG2D expression on CD56^{dim} NK cells, and subsets expressing iKIR and/or NKG2A, negatively correlated with total HIV-1 DNA load (**Fig 3.5C**, r=-0.51, p=0.005). Together, high NKG2D-expressing CD56^{dim} NK cells are associated with both low HLA-A expression levels and low total HIV-1 DNA loads.

High frequency of CD107a+ CD56^{dim} NK cells associated with low HLA-A expression levels and with low total HIV-1 DNA load

We next investigated NK cell functionality in relation to HLA-A expression and total HIV-1 DNA load in children maintaining viral suppression on ART. Low HLA-A expression levels were associated with relatively higher expression of the degranulation marker CD107a in the absence of in vitro stimulation across all NK cell subsets, with the exception of adaptive NK cells (CD57+NKG2C+PLZF^{low}CD56^{dim}) (**Fig 3.6A**). Here, we observed CD107a expression on several CD56^{dim} NK cell subsets that negatively correlated with total HIV-1 DNA, such as CD107a expression by NKG2A+ CD56^{dim} NK cells (p=0.008) (**Fig 3.6B**). Following in vitro NK cell stimulation with IL-12/IL-18 (**Fig 3.6C**), NKG2A+ CD56^{dim} NK cells expressing IFNg were associated with decreased total HIV-1 DNA loads (p=0.01, **Fig 3.6D**), but these cells were infrequent in individuals with low-expressing HLA-A molecules.

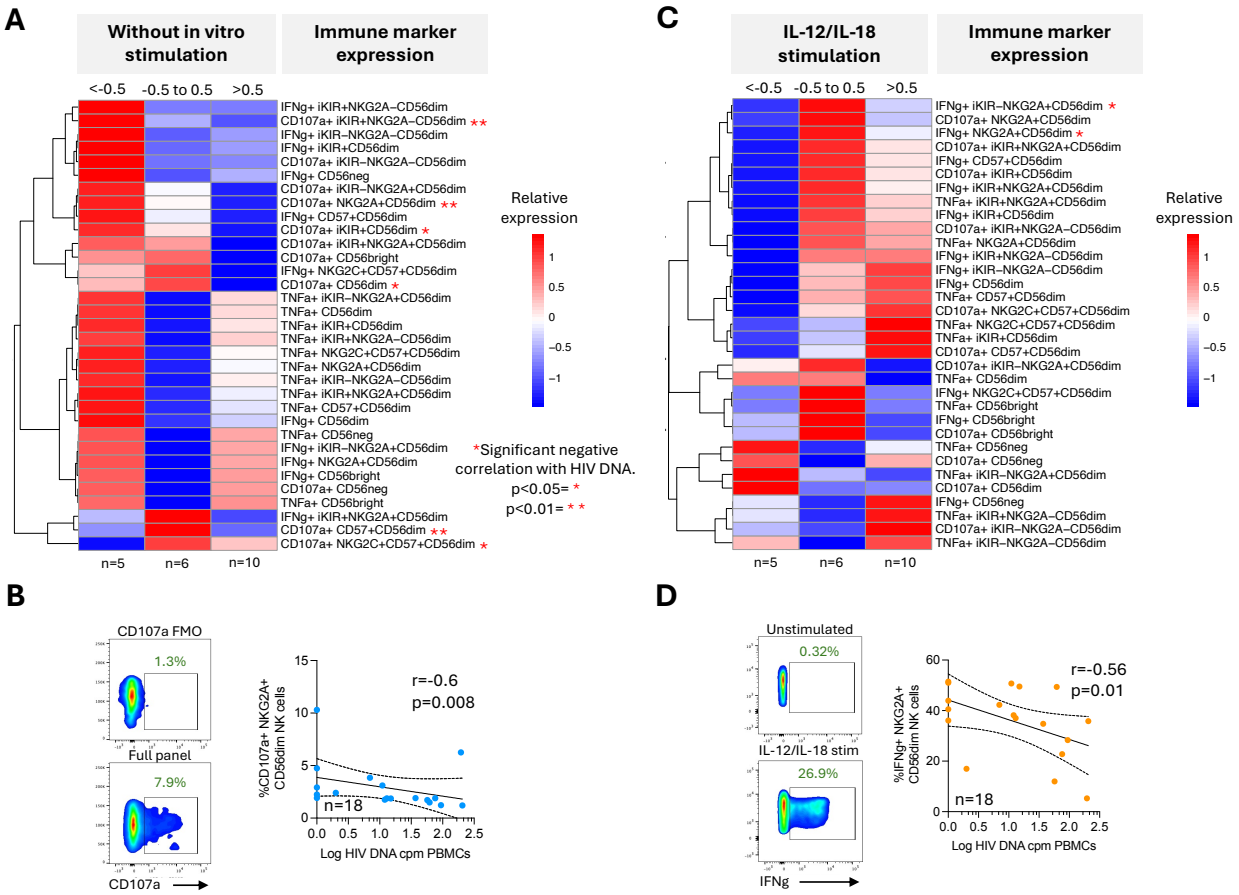

Figure 3.6. NK cell phenotypic and functional comparisons between the different HLA-A expression intervals using age-matched samples. A. Heatmap showing relative expression of CD107a, IFNg and TNFa on NK cell populations between differing HLA-A expression intervals without in vitro stimulation. B. Correlation between CD107a-expressing NKG2A+ $CD56^{dim}$ NK cells and total HIV-1 DNA. A Spearman Rank test was conducted, including a best-fit line with 95% confidence intervals. C. Heatmap showing relative expression of functional markers on NK cell populations between differing HLA-A expression intervals following IL-12/IL-18 stimulation. D. A correlation between IFNg expressing NKG2A+ $CD56^{dim}$ NK cells and total HIV-1 DNA. A Spearman Rank test was conducted, including a best-fit line with 95% confidence intervals. CD107a, IFNg and TNFa expression levels were applied after subtracting background expression for C. Heatmaps used hierarchical clustering.

Following in vitro NK cell stimulation with K562 cells or Rituximab-coated Raji cells, differential functionalities were observed in relation to HLA-A expression, but these responses did not significantly impact HIV-1 DNA load (**Fig 3.7**).

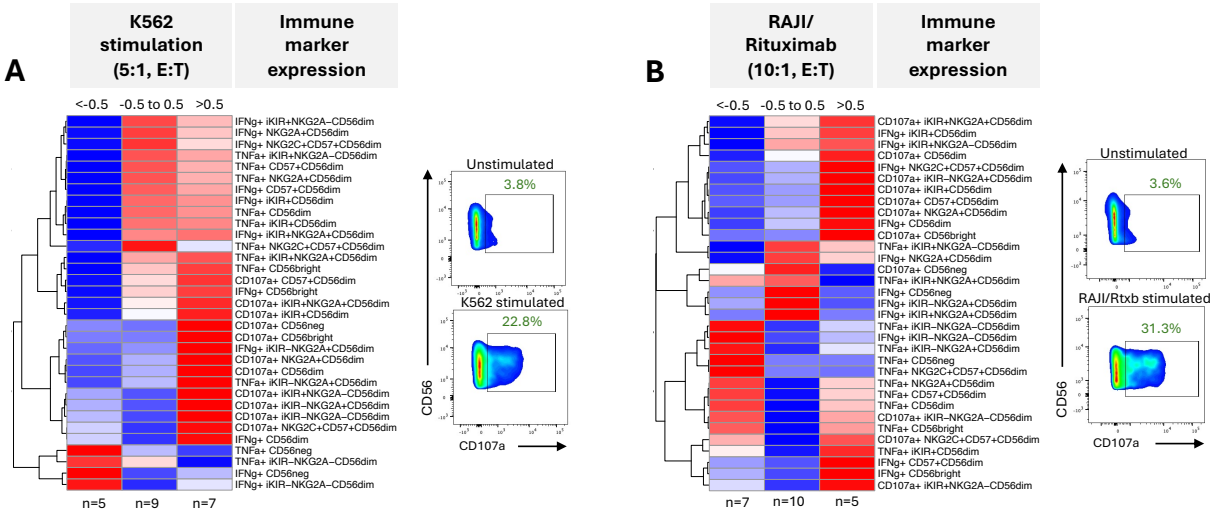

Figure 3.7. Heatmaps showing relative expression of functional markers on NK cell populations between differing HLA-A expression intervals following (A) K562 stimulation (5:1, E:T ratio) and (B) RAJI/Rituximab stimulation (10:1, E:T ratio). Relative CD107a, IFNg and TNFa are shown after subtracting background expression. Heatmaps used hierarchical clustering.

In summary, the frequency of CD107a+ CD56^{dim} NK cells, including those that express iKIR and/or NKG2A, negatively correlated with HIV-1 DNA loads and were enriched in children with low HLA-A expression levels. Together, these data suggest that increased NK cell cytotoxic functionality, reflected by CD107a expression, and an enhanced ability to kill stressed or activated cells expressing NKG2D ligands, are features of children LWH who carry low-expressed HLA-A alleles, and are associated with low HIV-1 DNA loads and a relatively high potential for HIV-1 cure/remission.

'Atypical' males display relatively higher resting CD56^{dim} NK cell functional capacity

We previously reported 5 'atypical' male children who achieved prolonged periods of viral suppression following unscheduled ART interruption (Bengu et al. 2024). We compared NK cell phenotypic features and functional characteristics in these 'atypical' males with age-matched 'typical' males and females (in whom viral suppression is ART-dependent).

NKG2D expression levels were somewhat higher in the 'atypical' males in all NK cell subsets compared to the other two groups although with these small numbers of study subjects this did not reach statistical significance (**Fig 3.8A**).

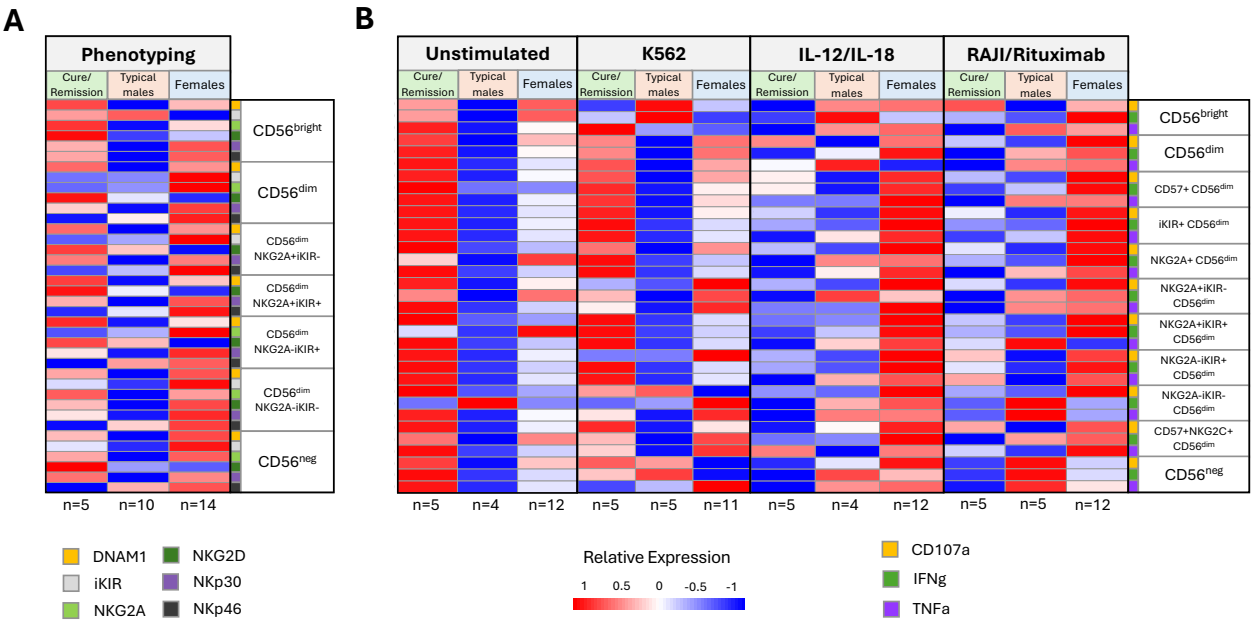

Figure 3.8. NK cell phenotypic and functional comparisons of five 'atypical males' (Cure/Remission) who achieved sustained viral suppression in the absence of ART to age-matched typical males and females. A. Heatmap showing relative expression of phenotypic markers on NK cell populations between the atypical males, typical males and typical females. B. Heatmap showing relative expression of functional markers on NK cell populations in the following conditions: unstimulated, after K562 stimulation (5:1, E:T ratio), after IL-12/IL18 stimulation, and after RAJI/Rituximab stimulation (10:1, E:T ratio). Relative CD107a, IFNg and TNFa are shown for the stimulated conditions after subtracting background expression. Heatmaps used hierarchical clustering.

Analysis of NK cell functionality in the 'atypical' males in the absence of in vitro stimulation again showed a distinct pattern from that observed in 'typical' males and females, with relatively higher CD107a, IFNg and TNFa expression across all NK cell subsets except for IFNg on NKG2A+, NKG2A+iKIR+ and NKG2A-iKIR- $CD56^{dim}$ NK cells (**Fig 3.8B**). A similar pattern was observed following K562 stimulation. As shown previously (Cheng et al. 2023), NK responses to stimulation by K562 cells, by cytokines, and by Rituximab-coated

Raji cells were generally stronger in females, reaching statistical significance in the case of CD107a expressing CD56^{dim} NK cell stimulation by Rituximab-coated Raji cells versus typical males (p=0.02) **(Fig 3.9A-C)**. Overall, NK cell phenotype and NK functionality of the 'atypical' males in the absence of in vitro stimulation and following K562 stimulation was distinct from 'typical' males and somewhat similar to females. However, numbers of 'atypical' males were low (n=5), and these differences did not reach statistical significance.

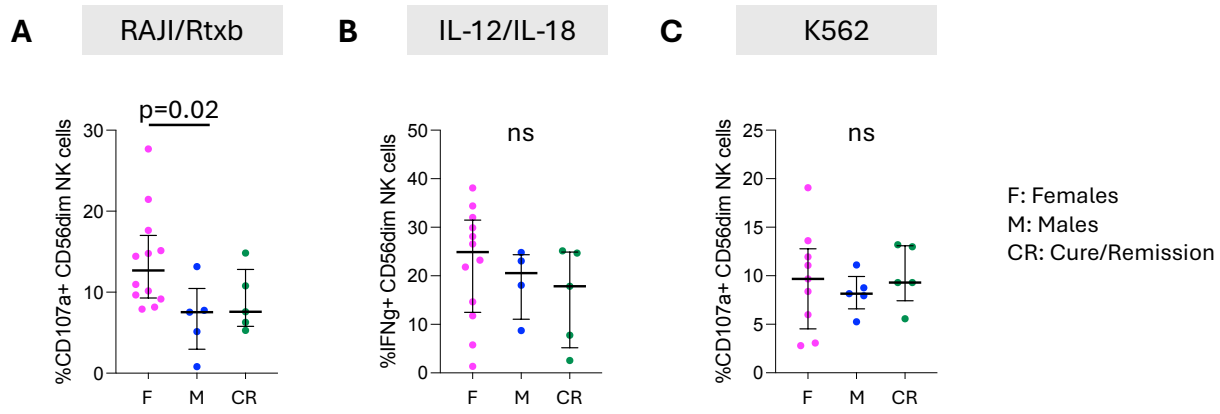

Figure 3.9. Age-matched comparison of responses of typical females, typical males and 'atypical males' (Cure/Remission) following (A) RAJI/Rituximab stimulation (10:1, E:T ratio), (B) IL-12/IL-18 stimulation, and (C) K562 stimulation (5:1, E:T ratio). CD107a and IFNg expression are compared after subtracting background expression. A Kruskal-Wallis test followed by Dunn's test for multiple comparisons was conducted for all analyses.

Females with protective HLA-B have elevated total HIV-1 DNA loads at birth and upregulation of the IL-17 cytokine signaling pathway

As mentioned previously, adult PTC studies have shown an under-representation of individuals expressing protective HLA-B alleles (Sáez-Cirión et al. 2013). We noted that children with protective HLA-B (HLA-B*57/58:01/81:01) had total HIV-1 DNA loads at birth approximately 0.3 $\log_{10}$ higher than those without, although this did not reach statistical significance (**Fig 3.10A**). However, females with protective HLA-B had baseline total HIV-1 DNA loads 0.7 $\log_{10}$ higher than males with protective HLA-B, and 0.6 $\log_{10}$ higher than

males or females without protective HLA-B (p=0.01, 0.007 and 0.01, respectively, **Fig 3.10B**).

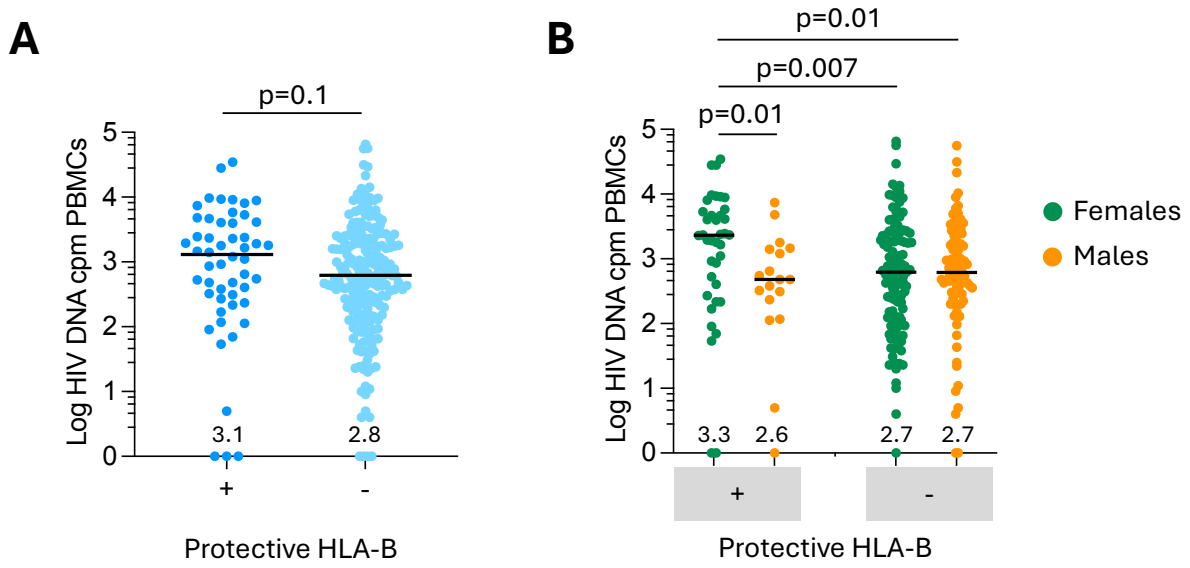

Figure 3.10. Total HIV DNA and disease-associated HLA-B at birth. A. Comparison of total HIV-1 DNA load at birth between those with protective HLA-B alleles (HLA-B*57, -B*58:01, -B*81:01) and those without. B. Comparison of total HIV-1 DNA load at birth between those with protective HLA-B alleles and those without, split by sex. A Mann-Whitney U test was conducted for both analyses.

Given the observation that NK responses are stronger in females (Cheng et al. 2023) and that, in several studies, including the current one, certain NK responses are linked with lower total HIV-1 DNA loads (Vieira et al. 2021), the finding of higher HIV-1 DNA loads in females, particularly in the setting of protective HLA-B molecules, might appear counter-intuitive. To explore the immune mechanism that might underlie this finding, we generated proteomic data from 34 children who had remained virally suppressed, simultaneously running two Olink panels, the 'Inflammatory' and 'Immune Response' panels, which we analysed using the online Olink Statistical Analysis software.

First, we compared protein concentration levels between those children with protective HLA-B compared to those without (**Fig 3.11A**). We observed in those children with protective HLA-B significantly higher concentrations of chemokines such as CXCL5, CXCL1, CCL28. Next, comparing sexes, males were observed to have significantly higher IL-10, a key anti-inflammatory cytokine, compared to females, which was confirmed in both panels (**Fig 3.11B**). Next, females with protective HLA-B were compared with all other children (**Fig 3.11C**). Those proteins that were at significantly higher concentrations in females with protective HLA-B across both panels (namely, MMP-1, GALNT3, CCL28, CXCL1, CXCL5, CDCP1, beta-NGF, IL-33 and NRTN) were subjected to the Enrichr pathway enrichment analysis utilizing the 'KEGG 2021 Human' and 'GO Biological Processes 2021' databases (**Fig 3.11D**).

Interestingly, we found that females with protective HLA-B were enriched for the IL-17 signaling pathway ($q=8.2x10^{-5}$), a highly pro-inflammatory pathway. Indeed, IL-6 and IL17A were upregulated in females with protective HLA-B (red circles, **Fig 3.11C**), although these increased levels did not reach statistical significance. Also, the Rheumatoid Arthritis (RA) pathway was enriched in females with protective HLA-B, which may be expected as IL-17 signaling is implicated in RA disease pathogenesis (Gaffen 2009). By comparing key proteins associated with the IL-17 signaling pathway, we show these are elevated in females with protective HLA-B (**Fig 3.11E**).

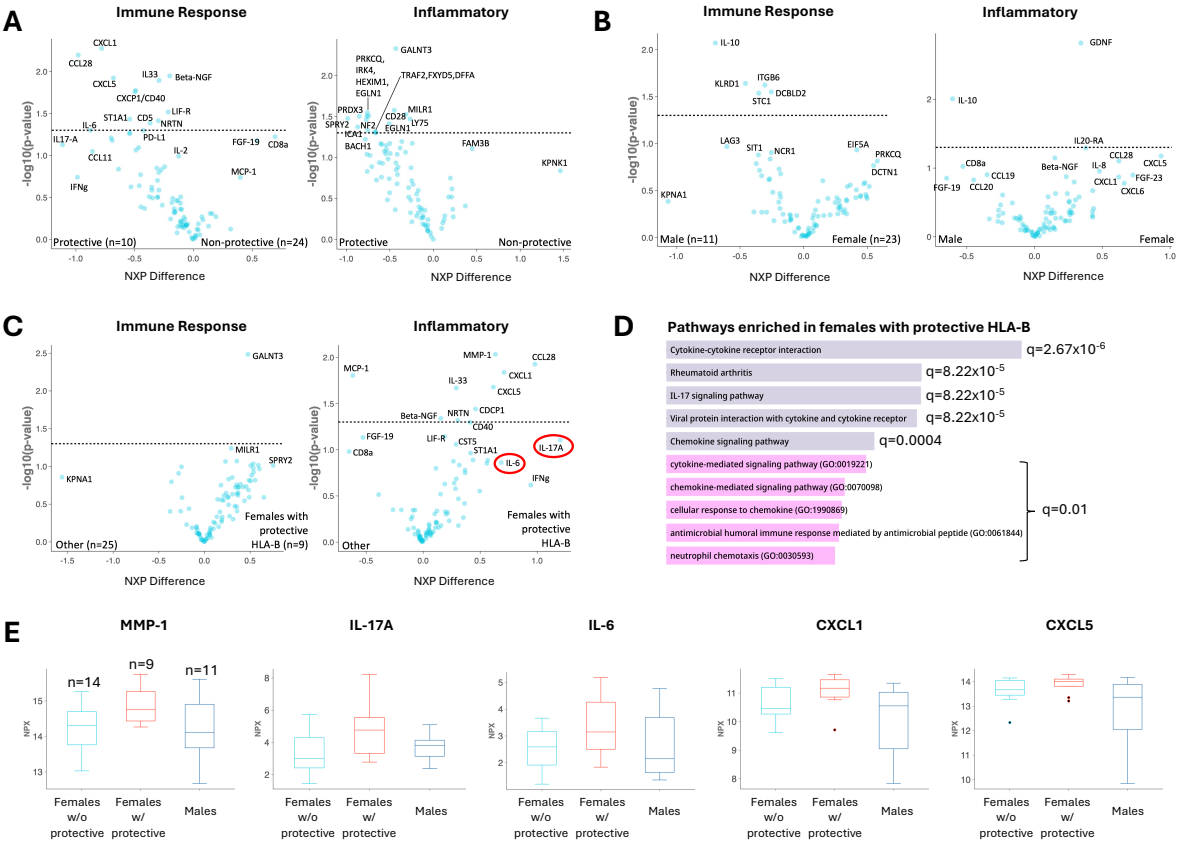

Figure 3.11. Comparison of plasma proteomic profile in age-matched samples by sex, presence of protective HLA-B alleles (HLA-B*57, -B*58:01, -B*81:01), and females with protective HLA-B. A. Volcano plots of plasma protein concentrations between those with protective HLA-B compared to those without, from both the 'Immune Response' and 'Inflammatory' Olink panels. B. Volcano plots of plasma protein concentrations compared between males and females, from both the 'Immune Response' and 'Inflammatory' Olink panels. C. Volcano plots of plasma protein concentrations between females with protective HLA-B compared to all others, from both the 'Immune Response' and 'Inflammatory' Olink panels. D. Proteins that were significantly differentially expressed in C (MMP-1, GALNT3, CCL28, CXCL1, CXCL5, CDCP1, beta-NGF, IL-33 and NRTN) were subjected to the Enrichr pathway enrichment analysis utilising the 'KEGG 2021 Human' and 'GO Biological Processes 2021' databases. These identified pathways that were significantly enriched by these proteins. E. A comparison of protein concentrations that are enriched in the IL-17 signaling pathway was made between females without protective HLA-B, females with protective HLA-B, and males. All analyses here were conducted using online Olink Statistical Analysis software, except for the Enrichr pathway enrichment analysis. NXP Difference is equivalent to log 2 fold change.

In summary, we show that females with protective HLA-B have a uniquely upregulated IL-17 pathway. This suggests that the CD4+ T cell compartment is skewed towards a Th17 phenotype. Th17 cells are highly susceptible to HIV-1 infection (Gosselin et al. 2010; Kim et al. 2013) and HIV-1 replicates preferentially within Th17 cells (El Hed et al. 2010). These

findings are consistent therefore with the observation that female newborns with protective HLA-B have higher levels of total HIV-1 DNA at birth.

DISCUSSION

This study sought to identify genetic and immune factors associated with low total HIV-1 DNA load in very-early ART-treated children who had remained virally suppressed on ART. We show that the initial 'seeding' or baseline total HIV-1 DNA load strongly influences time to achieve undetectable total HIV-1 DNA and is associated with achievement of sustained ART-free aviraemia in children following unscheduled treatment interruption. We demonstrate that low HLA-A expression, favouring KIR-educated NK cells, is linked both to this lower initial 'seeding' of HIV-1 DNA at birth and time to total HIV-1 DNA load following very-early ART; and that, in $CD56^{dim}$ NK cells, high NKG2D expression and high CD107a expression is associated with low HIV-1 DNA loads and with low HLA-A expression. Together, these data are consistent with the notion that NK cells play an important role in HIV-1 cure/remission in very-early ART-treated children. In addition, we showed that, although females have stronger NK cell functionality, upregulation of the pro-inflammatory IL-17 pathway in females expressing the 'protective' HLA-B molecules, HLA-B*57/58:01/81:01, may contribute to the higher baseline total HIV-1 DNA loads observed in females and enhanced potential for HIV-1 cure/remission observed in male children (Bengu et al. 2024).

The role of NK cells in HIV-1 cure/remission or PTC has been highlighted in previous adult and paediatric studies. PTC has been associated with an HLA-I signature that combines disease-susceptible HLA-B molecules with HLA genotypes that mediates a KIR-biased

education of NK cells (Essat et al. 2025), the genotype being: low HLA-A expression, threonine at p-21 of the HLA-B signal peptide, the presence of Bw4 epitopes and HLA-C molecules in the C2 group. In the current study, we observed that low HLA-A expression was associated with lower total HIV-1 DNA loads, at birth and in virally suppressed children at median 9 months old, consistent with similar findings of low HLA-A expression linked with enhanced immune control of viral replication both in ART-naïve adults (Ramsuran et al. 2018) and ART-naïve children (Vieira et al. 2021). Similar observations were also made in the very-early ART-treated paediatric cohort in Botswana, noting a trend for a stronger decline of intact proviruses in carriers of HLA-A alleles known to be expressed at lower levels (Hartana et al. 2022). This study therefore supports the notion that an HLA-I signature that favours KIR-educated NK cells is associated with paediatric PTC, observed through low HLA-A expression.

We also show resting CD107a+ NKG2A+ $CD56^{dim}$ NK cells (**Fig 3.6B**) and IFNg production by NKG2A+ $CD56^{dim}$ NK cells following IL-12/IL-18 stimulation negatively correlated with total HIV-1 DNA (**Fig 3.6D**). Previous studies have shown that NKG2A-expressing NK cells play a favourable role in HIV-infection (Lisovsky et al. 2015; Merino et al. 2013) and in preventing transmission of HIV-1 (Merino et al. 2012). Previously, NKG2A+ $CD56^{dim}$ NK cells from -21TT individuals were shown to be more effective in immune control of HIV-1 (Lisovsky et al. 2015). A previous study by our group of ART-naïve children LWH, NKG2A expression on $CD56^{dim}$ NK cells negatively correlated with total HIV-1 DNA and plasma HIV-1 RNA viral load (Vieira et al. 2021). These previous studies are consistent with the data presented here, indicating that NKG2A+ $CD56^{dim}$ NK cells play a favourable role in controlling HIV-1 in the setting of an HLA-I signature favouring KIR-educated NK cells (Lisovsky et al. 2015; Vieira et al. 2021).

The observation here, that NKG2D expression on $CD56^{dim}$ NK cells negatively correlated with total HIV-1 DNA, is noteworthy. The NKG2D receptor has an important role in NK cell cytotoxicity through its binding to stress-inducible homologues of HLA class I molecules (Bauer et al. 1999). HIV-infected cells induce the expression of NKG2D ligands ULBP-1, -2 and -3 (Ward et al. 2007), triggering NK cells killing in the absence of cytokine or antibodies (Ward et al. 2007). HIV-1 Vpr upregulates the expression of NKG2D ligands on infected CD4+ T- cells, promoting NK cell-mediated killing (Richard et al. 2010). The ability of NKG2D-expressing NK cells to facilitate control of HIV-1 infection via recognition of CD4+ T-cells expressing ULBP-2 or MICB was recently also demonstrated via *in vitro* studies (Pi et al. 2025; Zhao et al. 2024). These studies, therefore are consistent with the findings here that an enhanced ability of NK cells to kill stressed or activated cells expressing NKG2D ligands is associated with lower total HIV-1 DNA loads and therefore an increased likelihood of achieving cure/remission.

The 'atypical' male children who were able to maintain undetectable plasma viraemia following unscheduled ART interruptions lasting >3 months (Bengu et al. 2024) had an NK cell phenotypic profile with relatively higher activation and inhibitory receptor expression levels compared to 'typical' males (**Fig 3.8A**), and relatively higher resting functional activity and K562 responses across NK cell subsets (**Fig 3.8B**). The resting CD107a expression levels of $CD56^{dim}$ NK cells correlated negatively with total HIV-1 DNA load (**Fig 3.6A**), consistent with the notion that these NK cell responses may be contributing to the ability of these 'atypical' males to achieve ART-free viral control. However, the numbers of these 'atypical' males are low, limiting definitive conclusions here.

One of the more striking findings from the original VISCONTI study (Sáez-Cirión et al. 2013) and supported in other reports (Martin et al. 2017) was the paradoxical low frequency in PTCs of 'protective' HLA-I such as HLA-B*57, and high frequency of disease-susceptible HLA-I, such as HLA-B*35 in B clade infection. By contrast, in adults who initiated ART in chronic infection who subsequently underwent ATI following no immunological interventions, 'protective' HLA-B were associated with delayed time to viral rebound (Park et al. 2017) and one early-ART-treated individual in the placebo arm of the RV405 trial, who had HLA-B*57, time to rebound was 6 months (Colby et al. 2018). More recently, in the FRESH cohort, most of whom initiated ART during Fiebig I, following intervention with bnAbs and a TLR7 agonist, longer time to rebound and lower viral setpoints during ATI were associated with protective HLA. Thus, the impact of protective and disease susceptible HLA on outcome in PTC studies may depend upon many factors, including timing of ART initiation, the involvement or not of an immunological intervention, the sex of study participants (all FRESH study participants were female, and all RV405 participants were male, for example), clade of virus, and, here, paediatric versus adult infection.

Here we observed higher total HIV-1 DNA loads at baseline in children expressing 'protective' HLA-I, and, particularly this was the case in females (**Fig 3.10B**). Since females have higher levels of immune activation (Flanagan et al. 2017; Klein and Flanagan 2016; Meier et al. 2009) even at birth (Adland et al. 2020) and that certain protective HLA-B alleles have been associated with autoimmune diseases, (Brewerton et al. 1973; Chen et al. 2012; Schlosstein et al. 1973), we initially hypothesised that a combination of being females and having 'protective' HLA-B would lead to even higher levels of immune activation through an additive effect, thus 'fuelling the fire of HIV' replication. Proteomic analyses here showed that the IL-17 signaling pathway is upregulated in females with protective HLA-B, with

proteins such as CXCL5 and CXCL1 highly expressed both in children with protective HLA-B (**Fig 3.11A**) *and* also in females (**Fig 3.11B**). Th17 CD4+ T-cells are highly susceptible to HIV-1 infection (Gosselin et al. 2010; El Hed et al. 2010; Kim et al. 2013) and are maintained in individuals with undetectable plasma viral loads (Ndhlovu et al. 2008). Thus, despite females typically having superior NK cell functionality compared to males (Cheng et al. 2023), upregulation of the pro-inflammatory IL-17 pathway in females with protective HLA-B molecules may predispose to increased viral replication and the observed elevated levels of HIV-1 DNA among females in early life.

It is important to highlight the caveats of this study. Firstly, in this study the total HIV-1 DNA levels have been investigated, but not other valuable measures of HIV-1 persistence such as cell-associated HIV-1 RNA, intact proviruses or replication-competent reservoir. Despite the real added value of these alternative approaches, as proviral DNA overestimates the true size of the reservoir due to the high proportion of defective proviruses, this was the only measurement that allowed us to compare viral dynamics at scale across the entire cohort and at longitudinal timepoints. Nevertheless, previous studies have demonstrated a strong correlation between total and intact proviral DNA (Bailón et al. 2025; Gálvez et al. 2022; Puertas et al. 2021), supporting the biological relevance and interpretability of our findings. In our study, we did have a number of timepoints that showed undetectable total HIV-1 DNA, which importantly still tells us that no intact provirus/replication competent HIV-1 DNA was detectable at those timepoints. Secondly, it is important to fully understand the direction of causation. While this study identified several correlations, the causal relationships remain difficult to define. Associations between NK cell functional markers or phenotypes and total HIV-1 DNA should be interpreted cautiously, as they do not necessarily imply causation. However, in the case of HLA-A expression, there is greater confidence in a

causal link, as genetic variation precedes and likely influences total HIV-1 DNA levels, as has also been shown in previous studies (Hartana et al. 2022; Ramsuran et al. 2018; Vieira et al. 2021).

In conclusion, very-early ART-treated children who have low HLA-A expression levels typically have lower HIV-1 DNA loads that are associated with a distinctive NK cell phenotype and functionality, with high NKG2D expression and CD107a expression. The enhanced potential for HIV-1 cure/remission that has been reported in very-early ART-treated males may be related in part to upregulation of the proinflammatory IL-17 pathway in females, particularly if they express protective HLA-B molecules. Further evaluation of the impact on immune responses of 'protective' and 'disease-susceptible' HLA-I, in addition to that of sex differences, is warranted in adults as well as children, given their importance to HIV-1 cure/remission outcomes, as demonstrated from this and other previous studies.

CHAPTER 4: ANTIRETROVIRAL THERAPY WEAKENS NATURAL SELECTION ON PROTECTIVE AND DISEASE-SUSCEPTIBLE HLA-B ALLELES IN HIV-1 INFECTION

INTRODUCTION

The HLA class I region is the most polymorphic in the human genome (Robinson et al. 2011). The association of particular HLA types with outcome from a range of human diseases, especially infectious and autoimmune diseases, but also other inflammatory conditions and cancers (Trowsdale and Knight 2013), is consistent with the notion that HLA-associated human disease drives HLA polymorphism. However, specific examples of selection pressure mediated by human pathogens operating to alter gene frequencies are rare.

The HIV-1 epidemic provides a unique opportunity to investigate the potential impact of a human infection in driving altered HLA frequencies, since HLA-I associations with differential HIV-1 disease progression are well-documented (Carrington and Walker 2012; Goulder and Walker 2012; Goulder and Watkins 2008), and with just over 80 million people infected with HIV-1 since the initial cases were described in 1981 (UNAIDS 2024), the numbers affected could be sufficient to observe population-level impact on HLA-I frequencies. In particular, this applies to the countries in sub-Saharan Africa that have been the worst-affected, accounting for two-thirds of people living with HIV-1 (PLWH). South Africa alone accounts for >20% of PLWH worldwide, and, within this country, KwaZulu-Natal has been the most severely affected province, with antenatal seroprevalence rates maintained at a relatively stable level of ~40% since antiretroviral therapy roll-out 20 years

ago (Tendesayi Kufa-Chakezha et al. 2024). For these reasons, the studies described below focus on cohorts we have studied both pre-ART (1998-2005) and subsequent to ART roll-out (2015-2025) in KwaZulu-Natal, South Africa.

Studies in KwaZulu-Natal have shown that, in ART-naïve infection, the HLA-I molecules most strongly associated with low plasma viral loads, high absolute CD4 counts and therefore slow progression to AIDS are HLA-B*57, HLA-B*58:01 and HLA-B*81:01 (Kiepiela et al. 2004; Leslie et al. 2010). The HLA-I molecules associated with high plasma viral loads, low absolute CD4 counts and rapid progression to AIDS are HLA-B*18, HLA-B*45:01 and HLA-B*58:02. The mechanism underlying these HLA-I associations with HIV-1 disease outcome has been related to the ability of HLA class I molecules such as HLA-B*57 to present multiple epitopes within the abundant and highly conserved Gag capsid protein that enable HIV-specific CD8+ T-cells to recognise and kill virus-infected target cells effectively (Julg et al. 2010; Kawada et al. 2008; Kiepiela et al. 2007; Sacha et al. 2007, 2008, 2009; Schneidewind et al. 2007; Streeck et al. 2007). At the same time, the selection of viral escape mutations is severely limited by the cost to viral replicative capacity ('fitness') of variation within the highly conserved regions of the HIV-1 proteome (Crawford et al. 2007; Friedrich et al. 2004; Leslie et al. 2004; Martinez-Picado et al. 2006; Miura et al. 2009; Schneidewind et al. 2007, 2008). By contrast, 'disease-susceptible' HLA such as HLA-B*58:02 present no Gag-specific CD8+ T-cell epitopes (Kiepiela et al. 2007; Ngumbela et al. 2008) and therefore exert little or no selection pressure on this critical region of the virus (Matthews et al. 2008).

Thus, time to AIDS and death in the pre-ART era was strongly associated with the specific HLA-I molecules expressed. In addition, the risk of adult HIV-1 transmission is strongly related to viral load (Broyles et al. 2023; Cohen et al. 2013; Quinn et al. 2000), and in

children born to mothers LWH in the pre-ART era, the risk of vertical transmission was also linked with maternal plasma viral load (Garcia et al. 1999). We therefore hypothesised that, in the pre-ART era, mothers expressing 'disease-susceptible' HLA would have higher plasma viral loads and therefore would be more likely to transmit vertically than mothers expressing 'protective' HLA-I. In the pre-ART era, the prognosis for children following vertical transmission was especially poor, with a 60% 2-year mortality (Newell et al. 2004). The prognosis for even HIV-exposed, uninfected children whose mothers had AIDS was also poor (Newell et al. 2004). Thus, we hypothesised that the ability of mothers expressing 'disease-susceptible' HLA-I to have children who themselves would survive to reproductive age would be substantially reduced in the pre-ART era, both because of reduced survival of the mothers and of their children.

To address these hypotheses, we investigated two antenatal cohorts in KwaZulu-Natal, one prior to the ART era (Mphatswe et al. 2007) (1998-2005), and one subsequent to it (Bengu et al. 2024; Millar et al. 2020) (2015-2025). To simulate the impact of HIV-1 on frequencies of 'disease-susceptible' and 'protective' HLA-I in this population, we developed an evolutionary-epidemiological model of HLA-B frequencies based on KwaZulu-Natal. We fitted this model to observations from both the pre-ART and post-ART era, to estimate the changes in HLA-B frequencies that could have occurred in KZN throughout this period. We also conducted a hypothetical counterfactual simulation where ART was never rolled out.

RESULTS

Natural selection on protective and disease-susceptible HLA-B alleles in the pre-ART era

Antiretroviral therapy (ART) was introduced in South Africa in 2004, but access was limited initially, with less than 1% of the 5.3 million people living with HIV-1 in South Africa in 2004 receiving treatment in that year (Larsen et al. 2019; Steinbrook 2004). We studied antenatal cohorts (Kiepiela et al. 2004; Mphatswe et al. 2007) in KwaZulu-Natal, South Africa, in the pre-ART era (1998-2005), at a time when HIV-1 prevalence was increasing from 35% to plateau at a relatively stable ~40% (**Fig 4.1A**). Mothers testing seropositive for HIV-1 infection were enrolled during pregnancy and, as expected (Garcia et al. 1999), plasma viral loads (pVL) were higher in vertically transmitting mothers compared to non-transmitting mothers (87,400 median versus 20,100, $p<0.0001$, **Fig 4.1B**). We had previously shown in the Zulu-Xhosa population in KwaZulu-Natal (Kiepiela et al. 2004; Leslie et al. 2010) that HLA-B*57, HLA-B*58:01 and HLA-B*81:01 were 'protective' against HIV-1 disease progression, being associated with low pVL and high absolute CD4 counts, and that HLA-B*18, HLA-B*45:01 and HLA-B*58:02 are 'disease-susceptible', being associated with high pVL and low absolute CD4 counts. Not unexpectedly, therefore, these same associations were observed in the cohort of mothers enrolled antenatally here (**Fig 4.1CD, FG**).

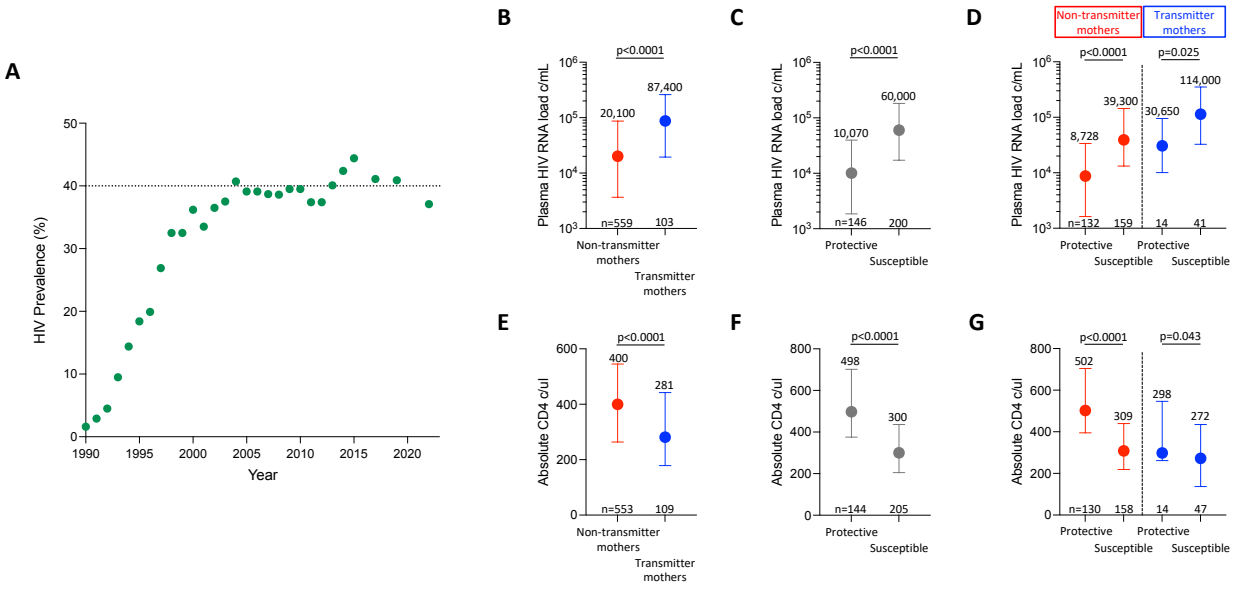

Figure 4.1. The HIV-1 epidemic curve among antenatal woman (1990-2022) and pVL and CD4 counts from pre-ART mothers (1998-2005). A. HIV-1 epidemic curve among antenatal woman in KwaZulu Natal (1990-2022). This graph was adapted from the Antenatal HIV-1 Sentinel Survey (Tendesayi Kufa-Chakezha et al. 2024). These data points were used to fit our model in Fig 4. B. Viral loads at pregnancy of non-transmitting and transmitting mothers. C. Viral loads at pregnancy compared between mothers who had one 'protective' (HLA-B*57/58:01/81:01) or one 'disease-susceptible' (HLA-B*18/45:01/58:02) HLA-B allele. D. Viral loads at pregnancy compared between mothers with either one 'protective or 'disease-susceptible' HLA-B alleles in non-transmitting and transmitting mothers. E. CD4 counts at pregnancy of non-transmitting and transmitting mothers. F. CD4 counts at pregnancy compared between mothers with either one 'protective' or 'disease-susceptible' HLA-B alleles. G. CD4 counts at pregnancy compared between mothers with either one 'protective or 'disease-susceptible' HLA-B alleles in non-transmitting and transmitting mothers. One-tailed Mann-Whitney tests were used to determine the p values shown. All p values <0.025 shown remained significant after Bonferroni-Holm correction for multiple tests (Table S1). Those mothers with both protective and disease-susceptible HLA-B alleles were excluded. Numbers correspond to median values and error bars indicate IQR.

Furthermore, as we had hypothesised, the frequencies of disease-susceptible HLA-I were significantly higher in the transmitting mothers and their infants than in the non-transmitting mothers (p=0.01 (odds ratio 1.6) and p=0.006 (odds ratio 1.7)), respectively; these were also higher than in a cohort of HIV-uninfected adults in KwaZulu-Natal (p=0.003 and p=0.003, respectively, **Fig 4.2A**). Similarly, the frequencies of protective HLA-I were significantly lower in the transmitting mothers and their infants than in the non-transmitting mothers (p=0.002 (odds ratio 0.47) and p=0.004 (odds ratio 0.5), **Fig 4.2B**) and somewhat lower than

in the control cohort of uninfected adults, although this latter difference did not reach statistical significance. Thus, in ART-naïve infection, 'protective' HLA-B are associated with slow HIV-1 disease progression to AIDS and death, whereas 'disease-susceptible' HLA-B alleles are associated with rapid progression to AIDS and death in the mothers, as well as an increased risk of vertical transmission and subsequently reduced infant survival. This process of natural selection, if continued without intervention, would therefore tend to increase the frequencies of 'protective' HLA-B and reduce the population frequencies of the disease-susceptible HLA-B alleles.

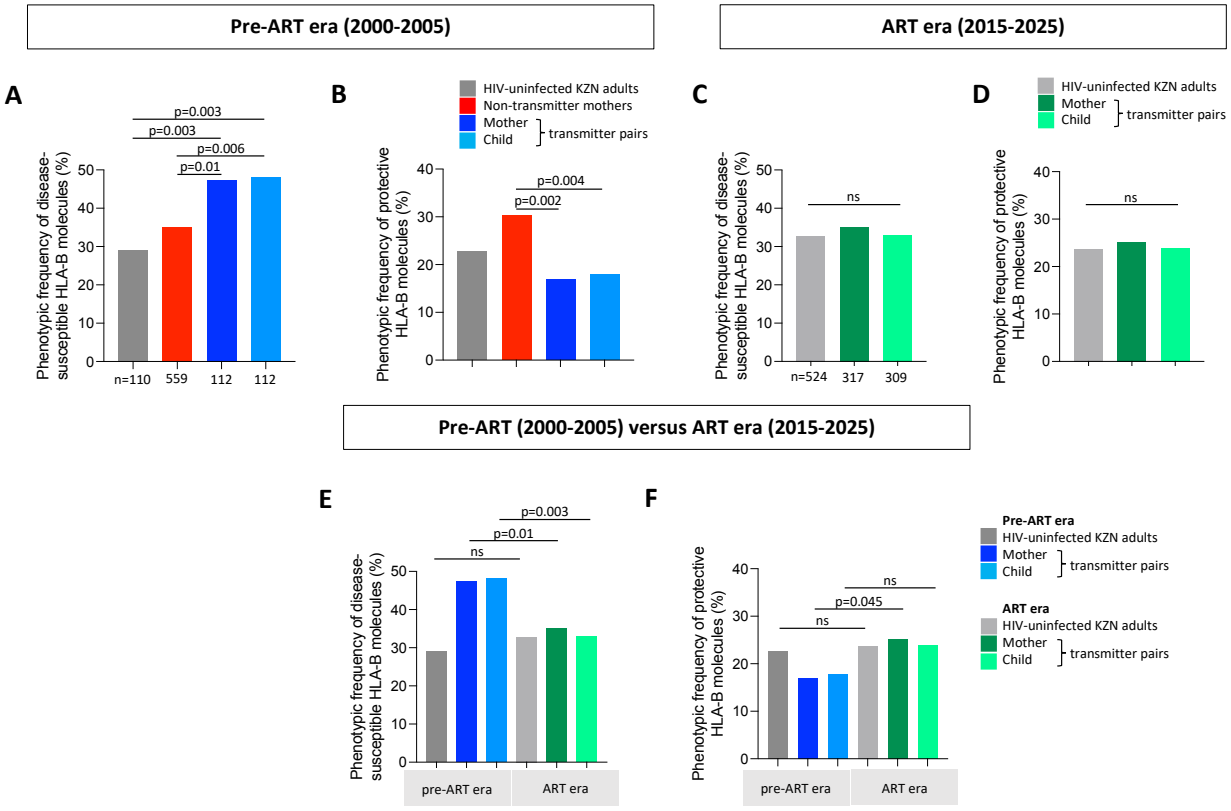

Figure 4.2. Disease-associated HLA-B phenotypic frequencies in mother-child pairs in KwaZulu-Natal, South Africa from the pre-ART era (1998-2005) and ART era (2015-2023). A. Phenotypic frequencies of disease-susceptible HLA-B alleles from the pre-ART era. B. Phenotypic frequencies of protective HLA-B alleles from the pre-ART era. C. Phenotypic frequencies of disease-susceptible HLA-B alleles from the ART era. D. Phenotypic frequencies of protective HLA-B alleles from the ART era. E. Phenotypic frequencies of disease-susceptible HLA-B alleles from the pre- and ART era. F. Phenotypic frequencies of protective HLA-B alleles from the pre- and ART era. One-sided Fisher's exact tests were used to

determine the p values shown. All p values <0.025 shown remained significant after Bonferroni-Holm correction for multiple tests (Table S1) . Dark grey; HIV-1 negative adults from pre-ART era. Red; non-transmitting mothers from pre-ART era. Dark blue; transmitting mothers from pre-ART era. Light blue; children from pre-ART era. Light grey; HIV-1 negative mothers from ART era. Dark green; transmitting mothers from ART era. Light green; children from ART era.

Weakened natural selection on HLA-B alleles in the ART era

By 2023, an estimated 5.9 million (77%) of the 7.7 million PLWH in South Africa were receiving ART (UNAIDS 2025). Long-term survival is now the expected outcome in PLWH receiving ART. Similarly, ART has dramatically reduced HIV-1 transmission rates, since HIV-1 transmission is effectively completely prevented when viraemia is suppressed by ART (Cohen et al. 2016). Accordingly, the intra-uterine vertical transmission rate is 0.4% in the current ART era (Millar et al. 2020), compared to the vertical transmission rate for *in utero* infection of 7.5% in the pre-ART era (Goulder et al. 2016; Mphatswe et al. 2007). Most of the vertical transmissions in the current era arise when, for social reasons, the mothers have not been able to access ART, or have not been aware of their HIV-1 status, either having become infected themselves during pregnancy or testing seropositive for the first time during the pregnancy (Millar et al. 2020). Thus, we hypothesised that, in the current ART era, both long-term survival in PLWH and vertical transmission are no longer related to HLA-I-mediated immune control of HIV-1 viraemia, but are now more strongly affected by HLA-independent factors that determine whether ART is accessed.

To test this hypothesis with respect to vertical transmission, we studied a second mother-child cohort enrolled in KwaZulu-Natal during the ART era (2015-2025) (Millar et al. 2020). We compared the frequencies of protective and disease-susceptible HLA-B molecules in transmitter mother-child pairs with those of a contemporaneous HIV-uninfected cohort of

age-matched women in KwaZulu-Natal (Dong et al. 2018; Ndung'u et al. 2018) and observed that there are no significant differences between the frequencies of either of these HLA-B alleles between HIV-uninfected women and transmitter mothers and their infants (**Fig 4.2C-D**). The numbers of study participants studied in the ART era were sufficient to provide >98% power (<2% type II error rate) to detect a significant difference between the groups had the difference between transmitter mothers and HIV-ve adults in the ART era been the same as that which we observed in the pre-ART era. Comparing HLA-B frequencies in the transmitter pairs in the pre-ART era versus the transmitter pairs in current ART era, as hypothesised, we observed that the frequencies of 'disease-susceptible' HLA-B alleles were significantly lower in the ART era compared to the pre-ART era, whereas these do not differ between the respective HIV-uninfected cohorts (**Fig 4.2E**, Table S1). As anticipated, 'protective' HLA-B alleles were increased in frequency in the transmitter mother-child pairs in the ART era compared to the pre-ART era, significantly so for the transmitter mothers ($p=0.045$, **Fig 4.2F**).

These analyses were also undertaken individually for the three 'disease-susceptible' and for the three 'protective' HLA-B alleles (**Fig 4.3A-F**). The most prevalent of these six alleles is the disease-susceptible HLA-B*58:02. This allele was enriched in the transmitter mother-child pairs in the pre-ART era (phenotypic frequency ~33%) but not so in the current ART era (phenotypic frequency ~22%) (**Fig 4.3C**).

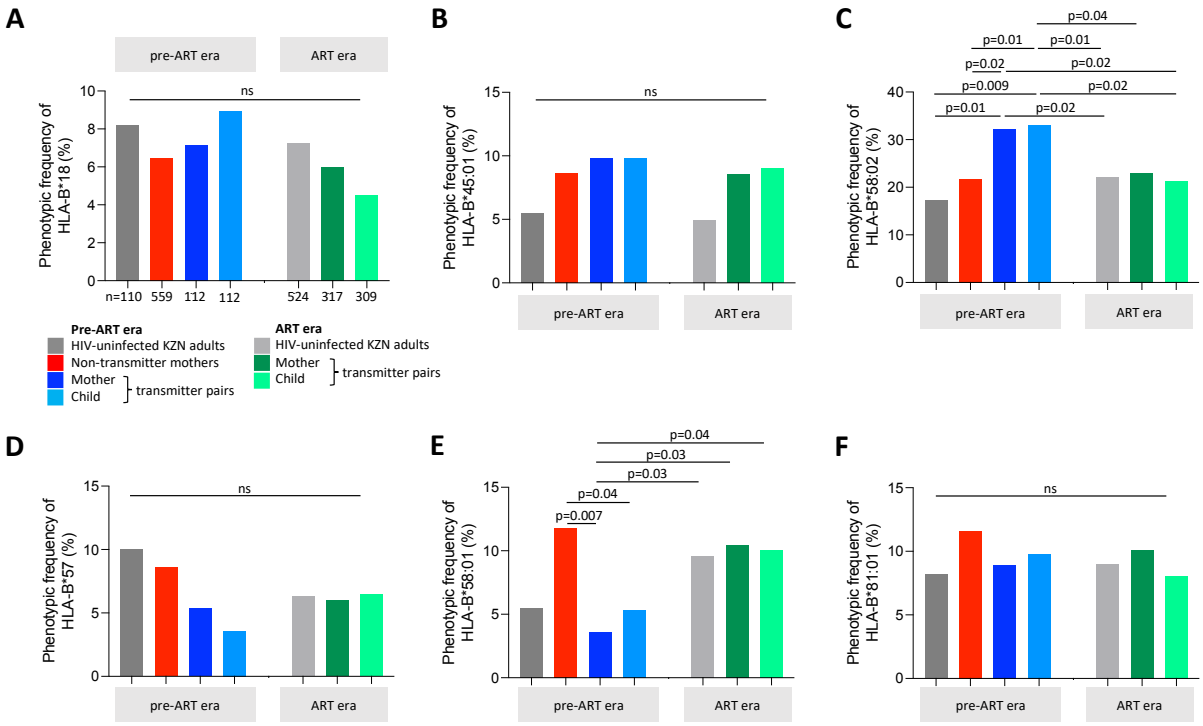

Figure 4.3. Phenotypic frequencies of individual protective and disease-susceptible HLA-B alleles across cohorts. A-C. Phenotypic frequencies of disease-susceptible HLA-B alleles; HLA-B*18, 45:01 and 58:02, respectively. D-F. Phenotypic frequencies of protective HLA-B alleles; HLA-B*57, 58:01 and 81:01, respectively. The p values shown are from Fisher's exact tests uncorrected for multiple comparisons.

With respect to the relationship between HLA-B expression and survival in adult LWH in the current ART era, as described above, in the antenatal clinic in KZN we studied, 90% of mothers LWH have undetectable plasma viral loads at delivery and 95.7% had plasma viral loads of <1000 c/ml (**Fig 4.4A**). These findings are in close agreement with published data for all antenatal clinics in KZN, reporting >80% of mothers with undetectable plasma viral loads, and 94.1% of mothers with plasma viral loads <1000 c/ml (Tendesayi Kufa-Chakezha et al. 2024). In the 0.4% of mothers LWH in KZN who transmit the virus in the ART era, there is no significant effect of HLA-B on plasma viral load (**Fig 4.4B**). However, it is noteworthy that, even though absolute CD4 counts are relatively high among transmitter mothers in the ART era (mean 487 versus 320 cells/ul in the pre-ART era), an HLA-B effect on absolute CD4 counts and CD4% remains (**Fig 4.4CD**). Thus, although ART is weakening

natural selection on HLA-B alleles, the fact that in adults ART is not initiated immediately following acquisition of HIV, and in a subset of people, illustrated by transmitter mothers, ART adherence is suboptimal, demonstrates that the selection pressure imposed by HIV-1 on HLA-B has not completely halted even in the ART era.

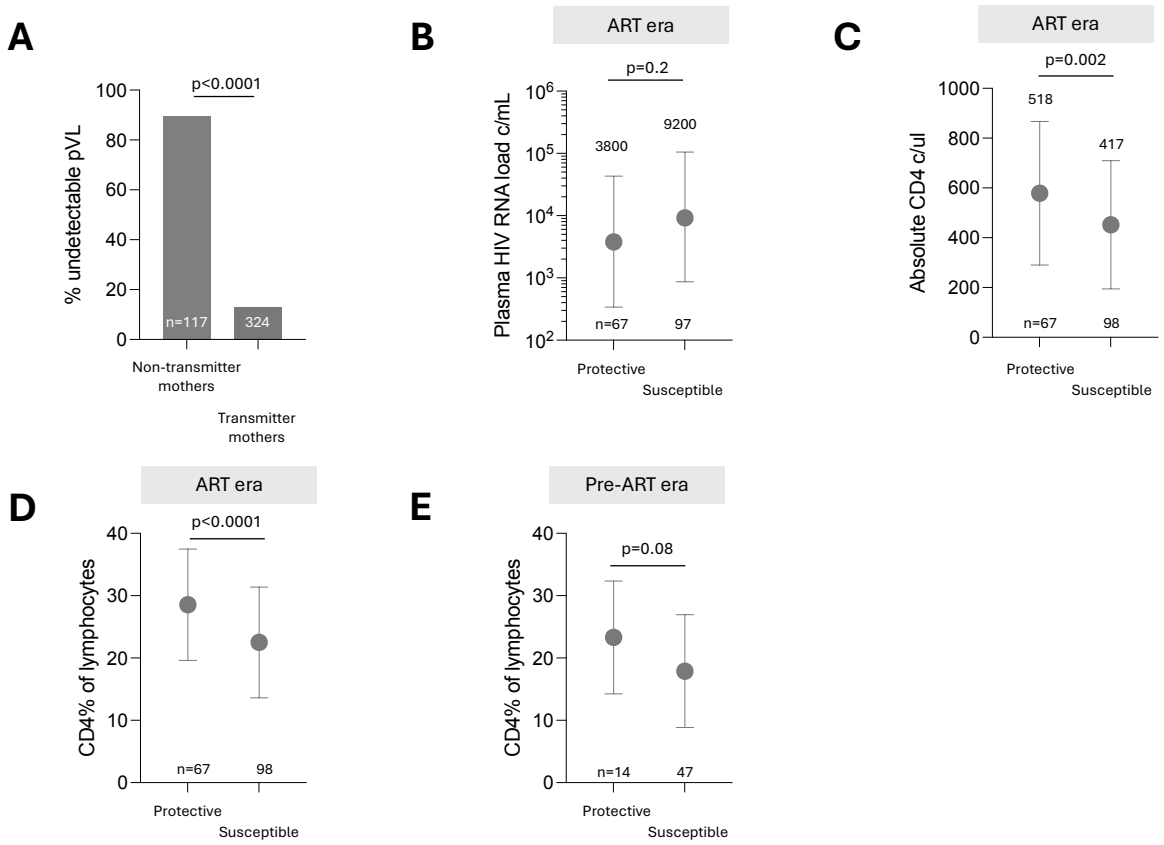

Figure 4.4. pVL and CD4 counts of ART era (2015-2025) and pre-ART era (2000-2005) mothers at birth. A. Percentage of mothers with undetectable pVL in ART era compared between non-transmitter and transmitter mothers. Fisher's exact tests were used as a statistical comparison. B. Comparison of pVL of ART era transmitter mothers with either protective (HLA-B*57/58:01/81:01) or disease-susceptible (HLA-B*18/45:01/58:02) HLA-B alleles at birth. C. Comparison of absolute CD4 counts of ART era transmitter mothers with either protective or disease-susceptible HLA-B alleles at birth. D. Comparison of CD4% of lymphocytes of ART era transmitter mothers with either protective or disease-susceptible HLA-B alleles at birth. E. Comparison of CD4% of lymphocytes of pre-ART era transmitter mothers with either protective or disease-susceptible HLA-B alleles at birth. The p values shown were determined via two-tailed Mann-Whitney tests. Numbers correspond to median values and error bars indicate IQR. Those with both protective and disease-susceptible HLA-B alleles were excluded.

ART weakens selection of disease-susceptible, high-expressing HLA-A alleles during vertical transmission

Having observed evidence of the HIV-1 epidemic driving changes in HLA-B frequencies, we next considered the possibility that the same selection pressures might be operating on the HLA-A locus. Although differences between HLA-A molecules have substantially less impact on HIV-1 pVL in ART-naïve individuals than differences in HLA-B molecules (Kiepiela et al. 2004; Leslie et al. 2010), high HLA-A expressing alleles are associated with high pVL and low absolute CD4 counts in ART-naïve adult and paediatric infection, mediated through inhibition of NKG2A+ NK cell responses by high-expressing HLA-A alleles (Ramsuran et al. 2018; Vieira et al. 2021). In the pre-ART and ART era cohorts studied here, surprisingly, we observed no difference overall in HLA-A expression levels between the different groups (**Fig 4.5A**). However, individuals with disease-susceptible, high-expressing HLA-A alleles (z-score >0.5) were significantly increased in frequency amongst the transmitter mother-child pairs in the pre-ART era compared to the ART era ($p<0.0001$, **Fig 4.5B**). Similarly, children with protective, low-expressing HLA-A alleles (z-score <-0.5) were somewhat more frequent among the transmitter mother-child pairs in the ART era compared to the pre-ART era, significantly so for the children ($p=0.007$, **Fig 4.5C**). In analyses of individual HLA-A alleles, the lowest-expressed and therefore most protective HLA-A allele is HLA-A*74:01, and children expressing HLA-A*74:01 were present at a higher frequency in the ART era versus the pre-ART era ($p=0.005$ pre-Bonferroni-Holm correction for multiple tests; $p=0.085$ post-correction, **Fig 4.5D**). Taking into account the fact that HLA-A*74:01 is in linkage disequilibrium with the strongly protective HLA-B*57:03, we reanalysed these data removing individuals expressing HLA-B*57:03. There remained a somewhat increased frequency of the protective HLA-A*74:01 in the ART era children versus the pre-ART era children ($p=0.017$, uncorrected for multiple tests).

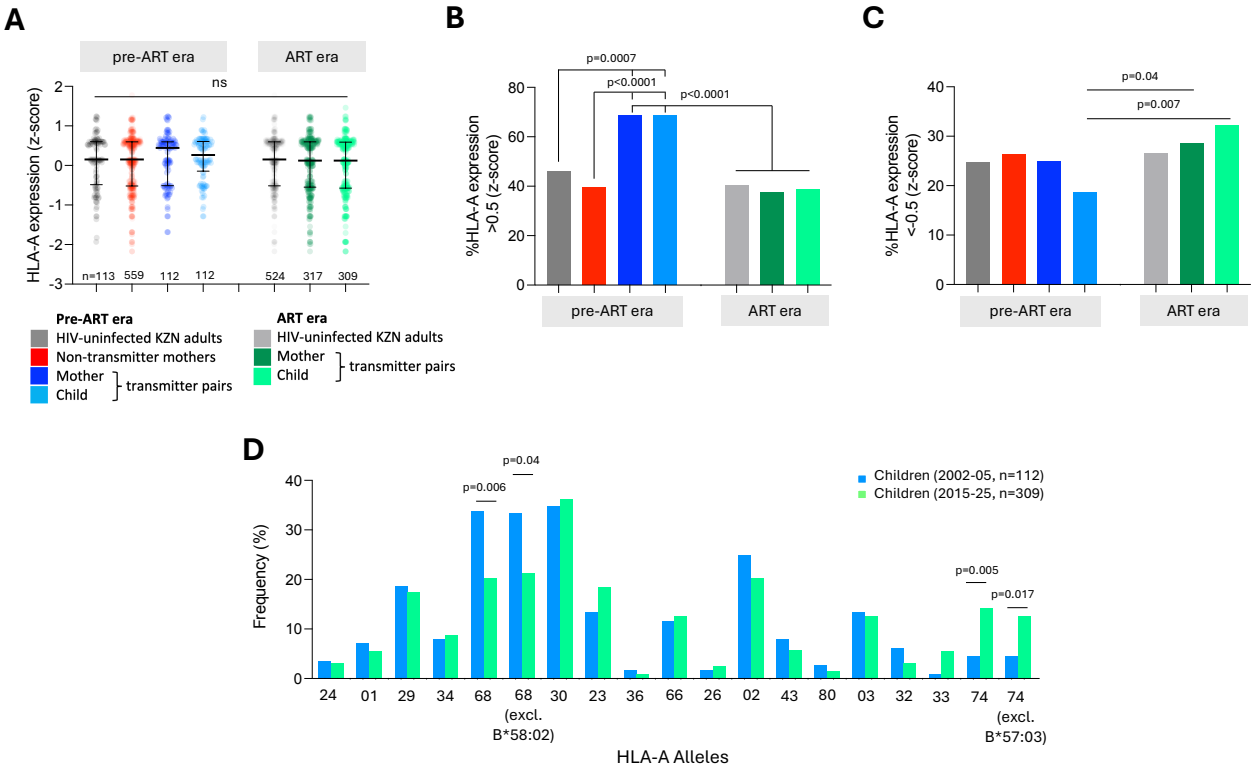

Figure 4.5. High HLA-A expression enriched in transmitter pairs in the pre-ART era. A. HLA-A expression (z-score) compared between cohorts (sum of both alleles). Median and interquartile range are shown. P values shown were determined using two-tailed Mann-Whitney tests. Medians are included and error bars indicate IQR. B. Frequency of individuals with HLA-A expression (z-scores) above 0.5. C. Frequency of individuals with HLA-A expression (z-scores) below -0.5. D. Frequency of HLA-A alleles compared between the pre- (light blue) and ART era children (light green). The p-values shown, determined using Fisher's exact tests, were uncorrected for multiple comparisons.

Simulating the KZN HIV-1 epidemic: ART radically slows allele frequency changes

To evaluate the impact of ART on HLA-B population genetics in the KwaZulu-Natal HIV-1 epidemic, we developed an evolutionary-epidemiological model of HIV-1 transmission and treatment (**Fig 4.6**) in a population containing 3 possible types of HLA-B allele: protective, disease-susceptible and indifferent. We began our simulation in the year 1990, using allele frequencies inferred from the HIV-negative HLA-typed dataset from the year 2000, since this was the earliest time point for which we had genetic data. We assumed that genotype

frequencies were at Hardy Weinberg equilibrium, and started our simulation with an HIV-1 prevalence of 0.016 (the proportion of HIV-infected women in KZN reported in the National HIV-1 Surveillance Programme 1990 antenatal survey (Küstner, Swanevelder, and Van Middelkoop 1994)). We assumed, based on behavioural observations (Johnson et al. 2012), that the parameter determining the horizontal transmissibility of HIV-1 could take 3 different values in 3 different time periods (1990-1998; 1999-2008 and 2009 onwards), and that ART treatment rates, when non-zero, took 2 different values (2004-2016 and 2017 onwards) (see Supporting Information). We obtained suitable values of these transmission and treatment parameters by fitting the model to antenatal HIV-1 survey data reported for KZN province (Küstner et al. 1994; Küstner, Swanevelder, and van Middelkoop 1998; Tendesayi Kufa-Chakezha et al. 2024; US Global HV/AIDS Surveillance Database 2024), and to ART treatment data from Conan *et al* (Conan et al. 2022) and the Sentinel Survey (Tendesayi Kufa-Chakezha et al. 2024), using Bayesian MCMC (full details in Supporting Information). The datapoints used for fitting are visualised in **figure 4.6A**.

Amornkul *et al* (Amornkul et al. 2013) report times to AIDS for HIV-1 C (the clade of virus that predominates in Southern Africa) and also reports the impact of having protective or disease susceptible HLA-B alleles on times to AIDS in sub Saharan African populations. Our pre-ART data allows us to estimate the odds ratios for disease susceptible or protected HLA-B genotypes vertically transmitting HIV-1 relative to genotypes containing neither such allele (Table S2). We brought together this information to generate three different HLA-B evolutionary scenarios: (i) a best estimate scenario, in which HLA-B specific times to AIDS and risks of vertical transmission are taken from the relevant hazard ratio or odds ratio calculated directly from the aforementioned data sources; (ii) a conservative scenario, in which the impact of HLA-B on times to AIDS and risks of vertical transmission are the

minimal values implied by the data (e.g. the lower bound of the 95% confidence interval of a hazard ratio or odds ratio if the ratio is greater than 1 or the upper bound of that interval if the ratio is less than 1), and (iii) an extreme scenario, in which the impact of HLA-B on times to AIDS and risks of vertical transmission are the maximum values implied by the data (e.g. the upper bound of the 95% confidence interval of a hazard ratio or odds ratio if the ratio is greater than 1 or the lower bound of that interval if the ratio is less than 1). Full details of these scenarios are provided in the Appendix and Table S3.

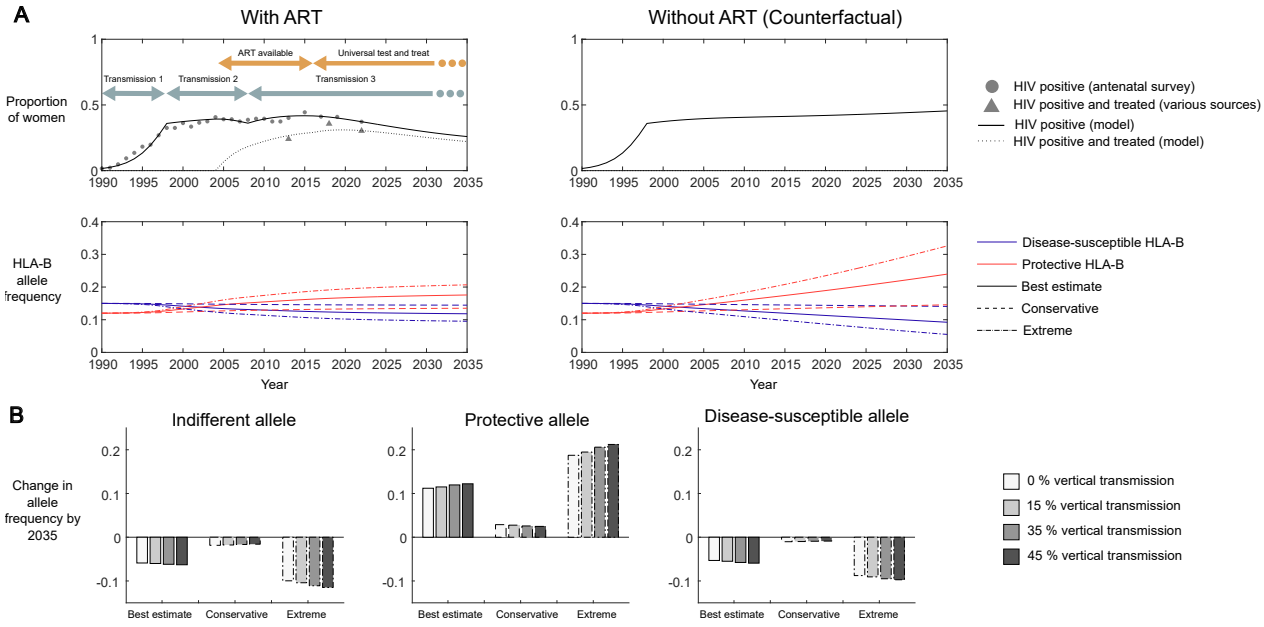

Figure 4.6. Simulating a KZN-like HIV-1 epidemic and its consequences for HLA-B allele frequencies. A. A time series of our evolutionary epidemiological model from 1990 to 2035. The upper row of panel A displays HIV-1 prevalence in women, for our best estimate HLA-B properties scenario (see figure S6 for the equivalent figures for the conservative and extreme scenarios). The lower row displays frequencies of HLA-B alleles deemed disease-susceptible (blue) or protective (red). The allele trajectories associated with our best estimate, conservative and extreme HLA-B scenarios are indicated by different line styles, with the best estimate scenario a solid line, the conservative scenario a dashed line and the extreme scenario a dotted-dashed line. In the panels on the left, we have fitted the model to antenatal survey data on HIV-1 prevalence (squares) and ART uptake (triangles). We assumed, based on behavioural observations, that the parameter determining the transmissibility of HIV-1 could take 3 different values in 3 different time periods (1990-1998; 1999-2008 and 2009 onwards), and that treatment rates could take 2 different values (2004-2016 and 2017 onwards). Each of these transmission rates and treatment rates were fitted to the data. In addition to fitting the model to data between 1990 and 2022 we extrapolate to 2035. Note that the trajectory of this extrapolation is a continuation of the trend seen in the final few years of

data, and any interpretation of the extrapolation depends on the strength of our belief in this trend. Panels on the right illustrate a counterfactual simulation. Both left- and right-hand panels are identical up until the year 2004. In the counterfactual simulation, ART is not rolled out, and we take the transmission parameter that has been fitted for the period 1999-2008 and allow transmission at that rate to continue in the absence of ART. The consequences for HIV-1 transmission and HLA-B allele frequencies are shown. In all of panel A, vertical transmission of HIV-1 occurs at a probability of 35% for mothers who are homozygous for the indifferent HLA-B allele (and is increased or decreased from this baseline depending on maternal genotype, see Supporting Information for further details). A vertical transmission probability of 35% is within the range of probabilities of vertical transmission for HIV-1 positive breastfeeding mothers estimated by de Cock et al (78) (whose maximum estimate is 45% in mothers breastfeeding for 2 years). In panel B. we explore how changing the baseline level of vertical transmission impacts HLA-B allele frequencies, in the absence of ART, to understand the potential changes in HLA-B allele frequencies that have been averted by ART. Each bar chart displays allele frequency changes (from the starting conditions in 1990) that we predict could have occurred by 2035, for the indicated HLA-B properties scenario and for the probability of vertical transmission in homozygotes for the indifferent allele given on the x axis.

Despite the devastating impact of the HIV-1 epidemic on KZN throughout the 1990s and early 2000s, frequencies of protective and disease-susceptible alleles only shifted from 0.12 to 0.14 (protective) and 0.15 to 0.14 (susceptible) between 1990 and 2004 in our best-estimate HLA-B properties scenario (**Fig 4.6**). This equates to 28% of the population possessing any susceptible allele in 1990 decreasing to 25% of the population possessing any susceptible allele in 2004 (and 23% of the population possessing a protective allele in 1990 increasing to 27% of the population possessing a protective allele in 2004). Within 15 years of ART becoming available, allele frequencies start to stabilise, with the protective allele at a frequency of 0.17 and the susceptible allele at a frequency of 0.12 in 2019 in our best estimate scenario. These frequencies only shift slightly more by 2035 (0.18 protective; 0.12 susceptible), resulting in the 2035 population having 22% with any susceptible allele and 32% with any protective allele. The extreme HLA properties scenario and the conservative HLA properties scenario result in greater and smaller overall allele frequency changes respectively but show the same stabilising effect of ART (**Fig 4.6A**).

In the absence of ART, in our best estimate scenario, the susceptible allele is at a frequency of 0.092 in 2035 (a decline of 38% compared to its initial allele frequency in 1990), and the protective allele has reached an allele frequency of 0.24 (double its allele frequency in 1990). This equates to just 18% of the population possessing any susceptible allele and 42% of the population possessing any protective allele in 2035, had there been no introduction of ART.

Figure 4.6B displays the allele frequency changes that occur by 2035 in the absence of ART, with four different values for the baseline probability of vertical transmission. The baseline probability of vertical transmission is the probability of vertical transmission of HIV-1 from infected mothers who are homozygous for indifferent HLA-B alleles. The protective or disease susceptibility effects of other genotypes are included in the model by changing their risks of vertical transmission relative to this baseline. When the baseline probability is 0%, the only mechanism driving HLA-B frequency changes is the impact of HIV-1 on lifespan. As the baseline probability of vertical transmission is increased, we can see that vertical transmission does have an effect on allele frequencies, but that this effect is small compared to that driven by the impact of HIV-1 on lifespan.

In our best-estimate and extreme HLA-B evolutionary scenarios, protective and disease susceptible HLA-B genotypes affect both times to AIDS and vertical transmission of HIV, and the greater the level of vertical transmission, the greater the increase in protective allele (or decrease in susceptible allele) frequencies by 2035 (**Fig 4.6B**). In our conservative scenario, HLA-B has a relatively small effect on times to AIDS and does not affect the probability of vertical transmission (see Supporting Information and tables S2 and S3). **Figure 4.6B** thus illustrates the counter intuitive fact that if HLA-B genotype affects time to AIDS but not vertical transmission, higher rates of vertical transmission of HIV-1 reduce the

rate of allele frequency change. If vertical transmission is experienced to the same extent by all HLA-B genotypes, the relative advantage of having a longer time to AIDS is lessened.

DISCUSSION

We have shown here, within a maternal cohort in KwaZulu-Natal, South Africa in the pre-ART era (1998-2005), HIV-1 survival and vertical transmission are both HLA-B dependent. AIDS-free survival and non-transmission are favoured by the expression of 'protective' HLA-B (HLA-B*57/58:01/81:01) whereas rapid progression to AIDS and vertical transmission are favoured by the expression of 'disease-susceptible' HLA-B (HLA-B*18/45:01/58:02). Using data from this cohort and other published studies to describe this process of natural selection, we developed a model to estimate the impact of HIV-1 on HLA-B frequencies over time in KZN. Our best estimate of the interaction between HIV-1 and HLA-B suggests that, in the absence of ART, HIV-1 selection could have doubled the frequencies of protective HLA-B alleles and reduced the frequencies of disease-susceptible HLA-B allele frequencies by 38% over a 45-year period. Even more extreme allele frequency changes are within the bounds of possibility (**Figure 4.6A**). Evaluation of a KwaZulu-Natal cohort in the ART era (2015-2025) showed that the HLA-B-dependent effects on HIV-1 survival and vertical transmission are substantially weakened by ART, and our model suggests this weakening is sufficient to radically slow HLA-B allele frequency change.

These studies are consistent with previous work on chimpanzees, proposing that, 2-3 million years ago, a selective sweep of chimpanzee MHC-I occurred as a result of widespread HIV-1/SIVcpz infection, removing animals expressing MHC-I associated with HIV-1/SIVcpz-associated disease (de Groot et al. 2002). The chimpanzees surviving today have a relatively

small repertoire of MHC-I molecules that are, however, associated with resistance to HIV-1/SIVcpz AIDS (Balla-Jhagjhoorsingh 1999; de Groot et al. 2010; de Groot and Bontrop 2013). Bonobos appear to have an even more diminished MHC class I repertoire compared to chimpanzees, which suggests that the selective sweep may have predated the speciation of common chimpanzees and bonobos (de Groot et al. 2017). It is striking that the HIV-1-specific epitopes targeted by CD8+ T-cells restricted by these protective chimpanzee MHC-I molecules, (Patr-03:01, Patr-B*01:01/03:01/05:01) are virtually the identical Gag-specific epitopes that are presented by protective HLA-B (HLA-B*27/57/58:01) molecules in humans (Goulder and Walker 2012; de Groot et al. 2010). Similarly, in rhesus macaques (*Macaca mulatta*) and pig-tailed macaques (*Macaca nemstrina*), that are not naturally infected with HIV-1 or SIV, immune control of experimental SIV infection is observed in association with protective MHC-I molecules (Mamu-A*01, Mamu-B*08, Mamu-B*17, Mamu-A1*065:01 (90-120-Ia), Mane-A*10) that target virtually the identical epitopes presented by protective HLA in humans, HLA-B*27/57/58:01/81:01 (Allen et al. 2001; Goulder and Walker 2012; Maness et al. 2008; Mothé et al. 2002; Nomura and Matano 2012; Smith et al. 2005). These findings support the hypothesis that immune control of immunodeficiency virus infection is mediated by MHC-I molecules that can present Gag epitopes for recognition by virus-specific CD8+ T-cells. These epitopes are highly abundant and conserved, with the result that virus-infected target cells are recognised and killed early in the viral life cycle (Sacha et al. 2007), and immune escape is limited by purifying selection against low fitness variants (Friedrich et al. 2004; Leslie et al. 2004).

The impact of HIV-1 described here in driving increases in protective HLA-B allele frequencies and decreases in disease-susceptible HLA-B allele frequencies over the course of the epidemic in the absence of ART is also broadly consistent with previous studies that have

estimated changes in gene frequencies as a result of HIV-1-mediated natural selection (Cromer, Wolinsky, and McLean 2010; Schliekelman, Garner, and Slatkin 2001). The analysis by Cromer *et al*, based on a hypothetical population where the HIV-1 prevalence plateaus at 30%, focused on the disease-susceptible HLA-B allele combination, HLA-B*35-Px/B53, in people living with HIV-1 B clade infection, the clade of virus that predominates in the Western world. In this study, the authors estimate a 50% decrease in frequency of this 'frail' HLA-B allele combination over 50 years. These estimates are similar to the 38% reduction over 45 years of the disease-susceptible HLA-B allele combination HLA-B*18/45:01/58:02 calculated here for the KwaZulu-Natal population affected by C clade HIV-1.

What is distinct about the current study is, first, that it is focused on a population in Sub-Saharan Africa, where two-thirds of the global HIV-1 pandemic is centred; second, the HIV-1 seroprevalence figures here of 30-40% corresponds to the specific situation for antenatal populations in KZN (**Fig 4.1A**); likewise, survival times in people living with C clade infections are derived from data in people living in Sub-Saharan Africa, and these differ from the survival figures in the PLWH in North America (the average time to AIDS when infected with clade C is 4.5 years (Amornkul et al. 2013), as opposed to the average time to AIDS of ~10 years seen in North America (Bacchetti and Moss 1989)); third, we have taken into account the impact of HLA on vertical transmission and child survival, again using actual data; fourth, we have included analysis of 'protective' HLA-B, where the increase over time (doubling over 45yrs in our best estimate scenario) is faster than the decrease in disease-susceptible HLA-B; and, finally, we have incorporated the impact of ART roll-out in the current analysis.

The current study is also broadly consistent with a study of the impact of CCR5 variants (Schliekelman et al. 2001), the coreceptor that facilitates entry of HIV-1 into target CD4+ T-cells via binding to CCR5 in addition to CD4. That HLA has the strongest genetic effect on HIV-1 outcome is abundantly clear from several genome-wide association studies (Carrington and Walker 2012; McLaren et al. 2013, 2015; The International HIV-1 Controllers Study 2010; Vergara et al. 2023). The HIV-resistant CCR5 variants most prevalent in Africa populations affect HIV-1 survival by approximately 2yrs (Gonzalez et al. 1999), and these effects are therefore weaker than those of protective HLA-B (see Table S3). Correspondingly, the estimates are that the CCR5 variants affecting HIV-1 survival would alter more slowly than HLA-B alleles affecting HIV-1 survival, with HIV-resistant CCR5 variants increasing by 33% over 100 years and HIV-susceptible variants decreasing by 50% over 100 years.

Although the major HLA effects on HIV-1 outcome are through HLA-B (Carrington and Walker 2012; McLaren et al. 2013, 2015; The International HIV-1 Controllers Study 2010; Vergara et al. 2023), differences in HLA-A genes also affect HIV-1 disease outcome, high-expressed HLA-A being associated with high viraemia and rapid progression to AIDS (Ramsuran et al. 2018; Vieira et al. 2021). We did not observe evidence of an impact of HLA-A on AIDS progression and HIV-1 survival in the adult cohorts we studied here. However, we did observe a striking significant enrichment of individuals with high expressing HLA-A alleles (z-score >0.5) in the transmitter mother-child pairs during the pre-ART era compared to non-transmitters and seronegative individuals ($p<0.0001$). This enrichment had disappeared in the ART era transmitter pairs (**Figure 4.5B**). These data are therefore consistent with the HLA-B data in showing that disease-susceptible HLA-I alleles were enriched in transmitter pairs in the pre-ART era, and that following the advent of ART,

they are no longer significantly different from uninfected cohorts. Overall, however, the part played by HLA-A in affecting survival of children born to mothers living with HIV-1 is quite small relative to the impact of HLA-B on adult survival, and, given that any HLA-A effect on adult survival is only evident after removal of the HLA-B effect, it is unlikely that significant changes in HLA-A frequencies would result over the course of an ART-free HIV-1 epidemic.

It is important to highlight limitations of this study. One limitation is the size of our cohorts, and the time points at which populations were sampled. We do not show a change in frequency of protective and disease-susceptible HLA-I resulting from the differential impact of HIV-1 on survival and reproductive rate described above. The reason is, first, that HLA data from the same Zulu ethnic group being studied here are not available pre-1990. Second, HLA typing at this time depended on serological methods (TERASAKI and McCLELLAND 1964) and HLA types were 2-digit only. As a result, detailed, large cohort (>1000 individuals) studies during this period were unable to distinguish, for example, between HLA-B*58:01 (protective) and HLA-B*58:02 (disease-susceptible), and HLA-B*81 did not feature at all, being difficult to distinguish from HLA-B*07 or HLA-B*42. Up to 10% of individuals were not successfully typed owing to limitations in the class I antisera available. Third, although HIV-1 seroprevalence in antenatal mothers increases exponentially during the period 1990-2000, >80% of antenatal mothers were seronegative for most of this decade, and at the population level HIV-1 seroprevalence would be lower still, especially among males who account for <40% of infections (Karim et al. 2011). Thus, changes in HLA frequencies resulting from the differential impact of HIV-1 on disease outcomes would take some time to become evident at the population level. This is reflected in the data generated by the model here and is consistent also with the model of Cromer et al (Cromer et al. 2010).

We do observe the phenotypic frequency of protective HLA-B alleles increasing marginally from 22.7% in the uninfected pre-ART cohort from the year 2000 to 23.6% in the uninfected ART era cohort from 2015-2025 (**Fig 4.2F**); but this difference is not statistically significant. Nevertheless, the magnitude of the change is consistent with the behaviour of the model (specifically, between 2000 and 2020 in the model, the proportion of the population with any protective allele at all changes from 25% to 30%). We do not observe an equivalent change in the phenotypic frequency of disease-susceptible HLA-B alleles between the two uninfected cohorts (**Fig 4.2F**).

It is important also to note that, whereas in the analyses of HLA frequencies in the pre-ART era, a cohort of non-transmitter mothers were available to compare against the transmitter mother-child pairs, such a cohort was not available in the ART era. In the pre-ART era HIV-uninfected adults served as an additional comparator group, and while the HLA frequencies in this group did not differ significantly from those in the non-transmitter mothers, the disease-susceptible HLA-B frequency in HIV-uninfected adults was significantly lower than in the transmitter mother-child pairs. In the ART era, ART coverage is high and, specifically, ~95% of antenatal mothers have low or undetectable viral loads (<1000 c/ml; (Tendesayi Kufa-Chakezha et al. 2024) & our data, **Fig 4.4**). With these levels of viral suppression, it is not surprising that birth transmission rates in the ART era are <1% (Millar et al. 2020). Non-transmitter mothers in the ART era therefore represent >99% of mothers LWH. Given this strong evidence of very effective control of HIV-1 among non-transmitter mothers in the ART era, it is reasonable to argue that HIV-uninfected adults in the ART era represent an appropriate contrast transmitting mother-child pairs. Of note, the risk of adults (or mothers) themselves becoming HIV-1 infected is independent of their HLA-I type. This has been shown both in GWAS studies (Lane et al. 2013; Shevchenko et al. 2021) and in studies that

have focused specifically on HLA-I susceptibility to infection (Gao et al. 2010; Vince et al. 2014).

A further point to note in relation to the finding of no significant difference in disease-susceptible HLA-B frequencies in the ART era between HIV-uninfected adults and transmitter mother-child pairs is the possibility that sample sizes were insufficient to detect the difference of the magnitude that was observed in the pre-ART era. The sample sizes of n=524 in the HIV-ve adults and n=317 in the transmitter mothers in the ART era provide >98% power (<2% type II error rate) to detect a significant difference of this magnitude between these two groups in the ART era.

Whilst the analyses presented here indicate that the process of natural selection driven by HIV-1 in the pre-ART era has been substantially slowed by ART, it is not possible to say that this process has been halted altogether. The reason for this is that ART coverage is not 100% from the time of acquisition of infection, and, as highlighted in the paper, disease progression to AIDS in Sub-Saharan Africa for people who express disease-susceptible HLA-B may only take 3-4 years (see supporting information and Table S3).This is well illustrated by the analysis of the 0.4% of mothers LWH who are transmitters: although viral load is rapidly suppressed by ART, the differential impact of HIV-1 on CD4 counts according to HLA-B type shows that this process of natural selection is not completely halted (**Fig 4.4**). Indeed, were ART to be interrupted for any sustained period at the population level for whatever reason, it is clear that the HLA-dependent effects observed in the pre-ART era would be activated very rapidly once again.

An important limitation of our modelling analysis is the relative lack of information about how different HLA-B alleles combine to impact HIV-1 progression. The classification of HLA-B alleles as being relatively protective or risky for HIV-1 is well-supported by multiple lines of evidence, but the existence and completeness of any dominance effects is less clear. Furthermore, relatively few data are available for HIV-1 subtype C, the type which predominates in KZN. We used the average time-to-AIDS data reported by Amornkul *et al* (Amornkul et al. 2013) for subtype C in our model. We used Amornkul's hazard ratios for the presence/absence of B*57 and B*45 to adjust this time to AIDS up or down for "protective" and "risky" genotypes, since these are the only available estimates of the impact of HLA-B on time to AIDS that are specific to sub Saharan African populations. However, Amornkul *et al*'s HLA-B results are based on their entire dataset (which is not exclusively subtype C). We had to treat results for the presence/absence of B*57 and B*45 as indicative of the difference between indifferent genotypes and all protective and disease susceptible allele containing genotypes for the purposes of our model, which was an extrapolation. We further assumed that a compound heterozygote for a protective and a disease susceptible HLA-B allele would experience the population average time to AIDS (i.e. we assumed the protective and risky effects cancelled each other out) – see Table S3. Such heterozygotes are relatively rare in the model, but it would still have been far better if estimates of the relative risk of HIV-1 C progression for all possible genotypic combinations were available to parametrise the model.

We made the simplifying assumption that the onset of AIDS meant an individual no longer contributed to HIV-1 transmission and would no longer successfully reproduce. Fertility rates and rates of sexual contact are much lower after the onset of AIDS in women (Ross et al. 2004), but it would have been more realistic to model a continuous decline according to

WHO disease stages, especially if it were possible to stratify the rate of progression between these stages by HLA genotype.

In addition to the HIV/AIDS specific limitations detailed above, our model makes multiple simplifying assumptions in representing a population within an evolutionary-epidemiological framework (which attempts to account for rapid infectious disease dynamics and long-term population genetic trends within the same model). We assumed a constant birth rate over time, and assumed there was no difference in the fertility of women LWH versus women without HIV. We modelled only the female population, implicitly assuming that the impact of HIV-1 on women and their HLA frequencies would be mirrored in the male population. In fact, HIV-1 rates in KZN show sex specific patterns, with incidence rates peaking at lower ages in women than in men, and with shifts in this pattern over time (Akullian et al. 2021); to account for this would require extensive data not only on those age specific patterns but also on how sexual contact between different age groups has changed, or not, over time. Despite these challenges, it would be extremely interesting to incorporate greater population stratification in future models and explore which mechanisms accelerate or decelerate HLA allele frequency changes.

As noted in the Introduction, it is widely accepted that MHC/HLA polymorphism is driven by natural selection from infectious diseases. Superficially, our results appear to contradict this, since the model would indicate that selection from HIV-1 reduces HLA diversity (indeed, the long-term outcome of our model is that disadvantageous alleles are driven to extinction within a few hundred years in the extreme and best-estimate scenarios, and are at extremely low levels within 600 years even in the conservative scenario, **Fig S1**). However, we do not predict this would be the long-term outcome in any population, even in the absence

of ART. Two processes not captured by our model are (i) co-evolution, whereby the pathogen evolves alongside the host, as previously described for HIV-1 in relation to protective HLA-B (Payne et al. 2014); and (ii) selection from multiple antigenically diverse pathogens. We have shown the impact of a single pathogen over a short time. Over evolutionary time, processes similar to those shown here would play out repeatedly, driven by different pathogens and favouring different MHC/HLA types. Even if lost from a sub-population, MHC/HLA types can be re-introduced via migration or even introgression between species. Selection from a HIV/SIV like pathogen may have skewed MHC frequencies in the ancestors of chimpanzees and bonobos as proposed by de Groot *et al* (de Groot et al. 2002, 2017), but apes, like humans, also have to contend with completely different pathogens such as *Laverania* malaria parasites, whose evolutionary impact on bonobo MHC frequencies has also recently been demonstrated (Wroblewski et al. 2023). It would not be realistic to expect any primate to have solely adapted to HIV/SIV.

In summary, here we demonstrate that HLA-B genotype impacts not only HIV-1 survival rates but also the vertical transmission of HIV-1, in the absence of ART. We also demonstrate the potential for an infectious disease to rapidly alter HLA frequencies. The HIV-1 pandemic in KZN, if left untreated, could have doubled protective HLA-B allele frequencies and decreased disease susceptible HLA-B alleles by 38% within 45 years. The introduction of ART, together with high levels of adherence and successful suppression of viraemia, is substantially weakening this process of natural selection.

CHAPTER 5: DISTINCT IMMUNE DIFFERENCES BETWEEN HLA-B*58:01 AND HLA-B*58:02

INTRODUCTION

HLA class I molecules bind peptides derived from intracellularly processed host or pathogen proteins (Abbas, Lichtman, and Pillai 2019). These peptide-HLA complexes are assembled in the endoplasmic reticulum and transported to the cell surface, where they are surveyed by CD8+ T cells. During thymic development, T cells that recognise self-peptides presented by HLA class I are eliminated via negative selection, allowing mature T cells to selectively respond to non-self peptides and thereby mount immune responses against intracellular pathogens.

Among the HLA class I loci, HLA-B has consistently shown the strongest association with HIV-1 disease outcome. In clade C infection, certain HLA-B alleles, notably HLA-B*57, -B*58:01, and -B*81:01, are associated with lower plasma viral loads, higher $CD4^+$ T-cell counts, and delayed progression to AIDS in the absence of antiretroviral therapy and have therefore been termed ‘protective’ HLA-B alleles (Kiepiela et al. 2004; Leslie et al. 2010). These alleles present immunodominant cytotoxic T lymphocyte (CTL) epitopes derived from the viral capsid protein, Gag, which elicit potent $CD8^+$ T-cell responses. Although HIV-1 can escape these responses through mutations, such mutations frequently carry a high fitness cost (Crawford et al. 2007; Goepfert et al. 2008; Leslie et al. 2004; Prince et al. 2012; Schneidewind et al. 2007; Tsai et al. 2016; Wright et al. 2012). Thus, immune control in

these cases arise either through efficient viral clearance from the immune response or from reduced viral replicative capacity following immune escape.

In contrast, HLA-B alleles such as HLA-B*18, -B*45, and -B*58:02 present a narrower range of HIV-1 epitopes in clade C infection, particularly few or no Gag-derived epitopes (Kiepiela et al. 2004; Leslie et al. 2010). Additionally, these molecules tend to bind with high affinity to inhibitory leukocyte immunoglobulin-like receptors (LILRB1 and LILRB2), which can suppress the initiation of effective $CD8^+$ T-cell responses (Bashirova et al. 2014). As a result, individuals with these alleles tend to exhibit higher viral setpoints and more rapid disease progression when untreated (Kiepiela et al. 2007; Ngumbela et al. 2008) which has led to these HLA-B alleles being termed disease-susceptible.

Genome-wide association studies (GWAS) have pinpointed specific amino acid residues within the HLA-B peptide-binding groove that modulate viral control (de Bakker et al. 2006; The Major Genetic Determinants of HIV-1 Control Affect HLA Class I Peptide Presentation 2010b). The most significant of these is position 97, located in the C pocket of the peptide-binding cleft. This residue plays a critical role in peptide anchoring and in shaping the three-dimensional structure of the HLA-peptide complex, which influences both stability and T-cell receptor recognition (Blanco-Gelaz et al. 2006; Fagerberg, Cerottini, and Michielin 2006). Notably, variation at position 97 is strongly associated with divergent clinical outcomes in ART naïve HIV-1 infection: for example, the protective HLA-B*58:01 and the disease-susceptible HLA-B*58:02 differ only at positions 94, 95, and 97 (Marsh SGE, Parham P, and Barber LD 2000), highlighting the clinical relevance of small sequence changes within the peptide-binding cleft.

In this study, we investigated the immunological differences between individuals carrying either HLA-B*58:01 or HLA-B*58:02, two closely related alleles that are respectively associated with HIV-1 disease protection and susceptibility, respectively. While previous studies have primarily focused on ART-naïve individuals to assess HIV-specific CD8+ T cell responses and disease progression by these alleles (Kiepiela et al. 2004; Leslie et al. 2010), this study is distinct in examining these differences in both healthy adults and early ART-treated children, the latter of whom we hypothesise to have weak CD8+ T cell responses, providing insight into how subtle molecular variations influence immune function across age and disease contexts.

RESULTS

Differential transcriptional pathway activity distinguishes HLA-B*58:01 and HLA-B*58:02 across immune cell subsets in healthy adult females

To explore this, we first utilised samples from the FRESH cohort (Females Rising through Education, Support and Health), which is a prospective study that recruits women from the Umlazi district, KwaZulu-Natal, South Africa who are HIV-negative, aged 18–24 years and are of African descent (Dong et al. 2018). We chose to study this cohort to control for biological sex, age and ethnicity. We undertook single-cell RNA sequencing (scRNA-seq) using PBMC samples from n=4 uninfected donors with HLA-B*58:01 and n=4 uninfected donors with HLA-B*58:02 (**Fig 5.1A**). Donors were selected to match for HLA-B type where possible. Full HLA class I types can be found in **Table S8**.

In total, 15 cell populations were identified and manually annotated based on canonical PBMC markers (**Fig. 5.1B**). Differential gene expression analysis was then performed between cells expressing HLA-B*58:01 and HLA-B*58:02 within each subset, namely CD4+

T cells, CD8+ T cells, a granulocytic NK/CD8+ T-cell population, NK cells, B cells, and myeloid cells (**Fig. 5.1C-H**).

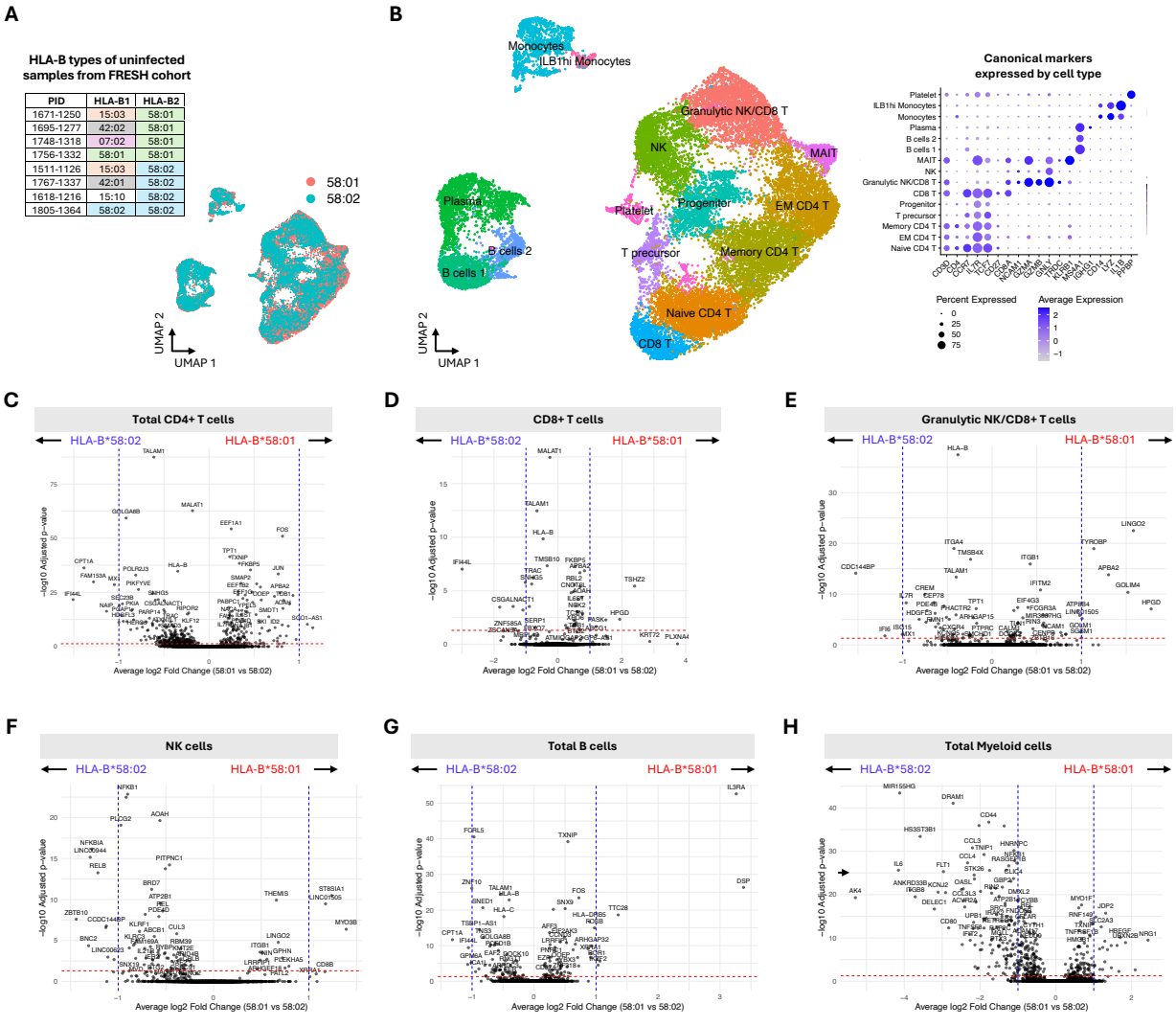

Figure 5.1. Single cell RNA seq comparison of healthy women with HLA-B*58:01 and HLA-B*58:02. A. HLA-B types are shown for each individual whose PBMCs were included in the single cell RNA seq assay as well as a UMAP showing cells that were either HLA-B type. B. UMAP with annotated cell populations included a dot plot with canonical markers used to identify each cluster. C-H. Volcano plots showing differentially expressed genes between HLA-B subtypes within each major cell population.

Using these differential gene expression results, gene set enrichment analysis (GSEA) was carried out to identify pathways that differ between each HLA-B type within each immune

cell subset (**Fig. 5.2A-F**). Dot sizes of 1.3 or more indicate statistically significant pathways, indicated by an asterisk.

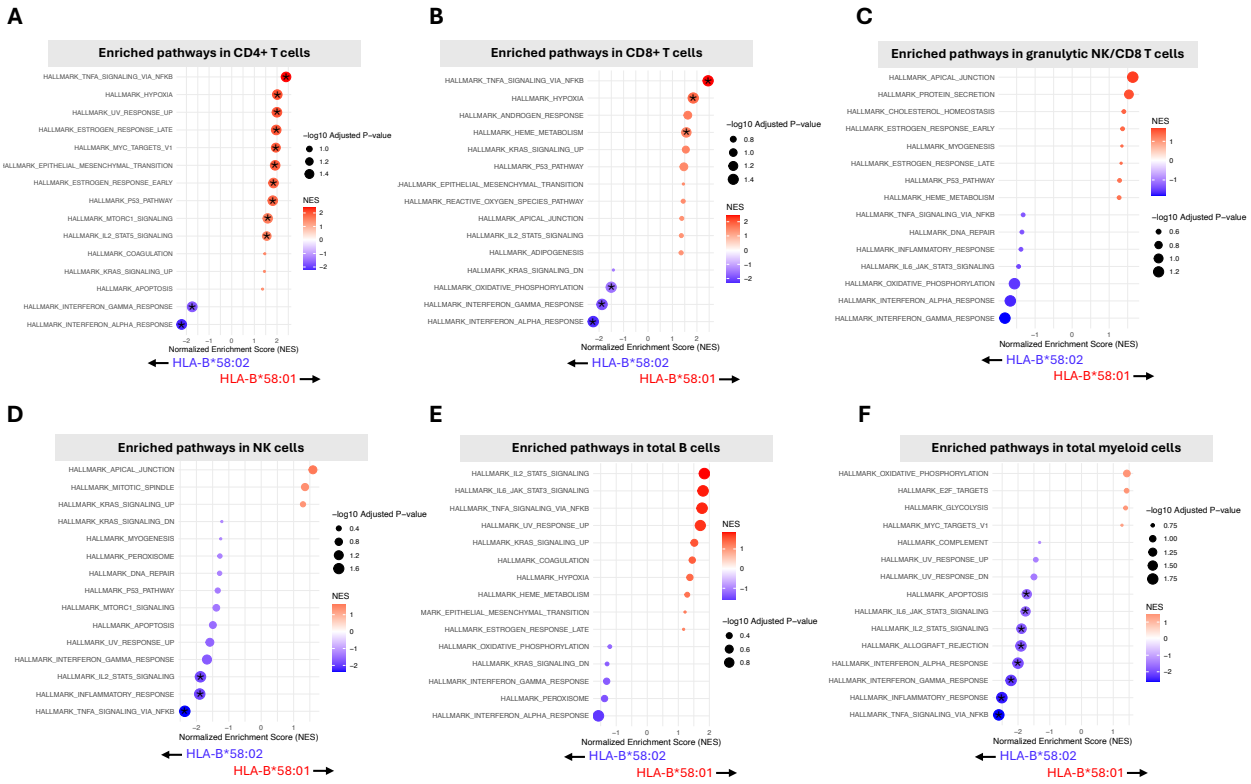

Figure 5.2. Gene set enrichment analysis data between the HLA-B types, within each cell population. Cell populations included CD4+ T cells (A), CD8+ T cells (B), granulytic NK/CD8+ T cells (C), NK cells (D), B cells (a combination of 'B cells 1', 'B cells 2' and 'Plasma' cells, E), and myeloid cells (a combination of 'Monocytes' and 'IL1Bhi Monocytes', F). -10 adjusted p values of 1.3 and above are significant, shown by an asterisk ($p<0.05$).

In CD4+ T cells (**Fig 5.2A**), individuals with HLA-B*58:01 showed significant upregulation of inflammatory pathways, such as TNFa signalling via NF-κB and IL2-STAT5 signalling, and proliferative pathways, such as hypoxia signalling, c-Myc target signalling, mTORC1 signalling. Conversely, HLA-B*58:02 was significantly associated with elevated IFNa and IFNg responses. Similarly, in CD8+ T cells, individuals with HLA-B*58:01 showed significant upregulation of inflammatory pathways, such as TNFa signalling via NF-κB, and proliferative pathways, such as hypoxia signalling and heme metabolism. While individuals

with HLA-B*58:02 were significantly enriched with elevated IFNa and IFNg signalling. Interestingly, in the granulocytic NK/CD8+ T cell subset (**Fig 5.2C**), no pathways were significantly enriched, although, HLA-B*58:02 was still enriched for IFNa and IFNg response pathways.

In NK cells (**Fig 5.2D**), HLA-B*58:01 drove enrichment in proliferative pathways, such as mitotic spindle formation and the inflammatory atypical junction, although these did not reach statistical significance. While HLA-B*58:02 showed significant enrichment for inflammatory response, IL2-STAT5 signalling, and TNFa signalling. In B cells (**Fig 5.2E**), no pathways reach statistical significance. However, of note, HLA-B*58:01 was again enriched for TNFa signalling while HLA-B*58:02 again showed enrichment for IFNa and IFNg signalling. Finally, in myeloid cells (**Fig 5.2F**), no pathways were significantly enriched in individuals with HLA-B*58:01. However, HLA-B*58:02 showed a significant skew toward inflammatory responses, such as TNFa signalling, IFNa and IFNg responses, IL6-JAK/STAT3 signalling and IL2/STAT5 signalling.

These findings show that HLA-B*58:01 is consistently associated with proliferative transcriptional programs and TNFa signalling across T and B cells. In contrast, HLA-B*58:02 is characterised by the upregulation of interferon-driven antiviral pathways across T cells, B cells and myeloid cells. These distinct immune profiles potentially reflect allele-specific differences. In the context of T cells, IFN signalling pathways function to clear viral infections (Goodbourn, Didcock, and Randall 2000), while TNFa signalling via NF-kB promotes inflammation, survival and activation (Aggarwal 2003). With this in mind, we next sought to confirm whether these differentially enriched transcriptional programs observed by single-cell RNA seq could be confirmed by flow cytometry with regards to T cell activation.

HLA-B*58:01 display more activated T cells compared to HLA-B*58:02 by flow cytometry in healthy adult females

To confirm our findings, we phenotyped healthy peripheral T cells from PBMCs from n=4 with HLA-B*58:01 and n=21 with HLA-B*58:02 with T cell activation markers from the same cohort (**Fig 5.3A**). The same PBMC samples utilised in the single-cell RNA seq assay were all included in the phenotyping assay here. We observed that those with HLA-B*58:01 had a significantly higher frequency of CD38+ CD4+ T cells (p=0.04), but not HLA-DR or HLA-DR+CD38+ CD4+ T cells (**Fig 5.3B**). For CD8+ T cells, we show no difference in activation markers across groups. However, we did show that those with HLA-B*58:02 had lower PD1 expression on effector memory CD8+ T cells and in transitional memory CD8 T cells compared to the other groups, significantly so for effector memory CD8 T cells (p=0.03) (**Fig 5.3C**). A previous study has shown that PD1 plays a regulatory role during naive-to-effector CD8+ T cell differentiation and suggests that PD1 likely acts as a negative feedback system for T cell activation to optimise T cell effector and memory responses (Ahn et al. 2018). Therefore, we hypothesise that lower levels of PD1 expression on effector and transitional memory CD8+ T cells in those with HLA-B*58:02 may reflect lower levels of general immune activation compared to those with HLA-B*58:01.

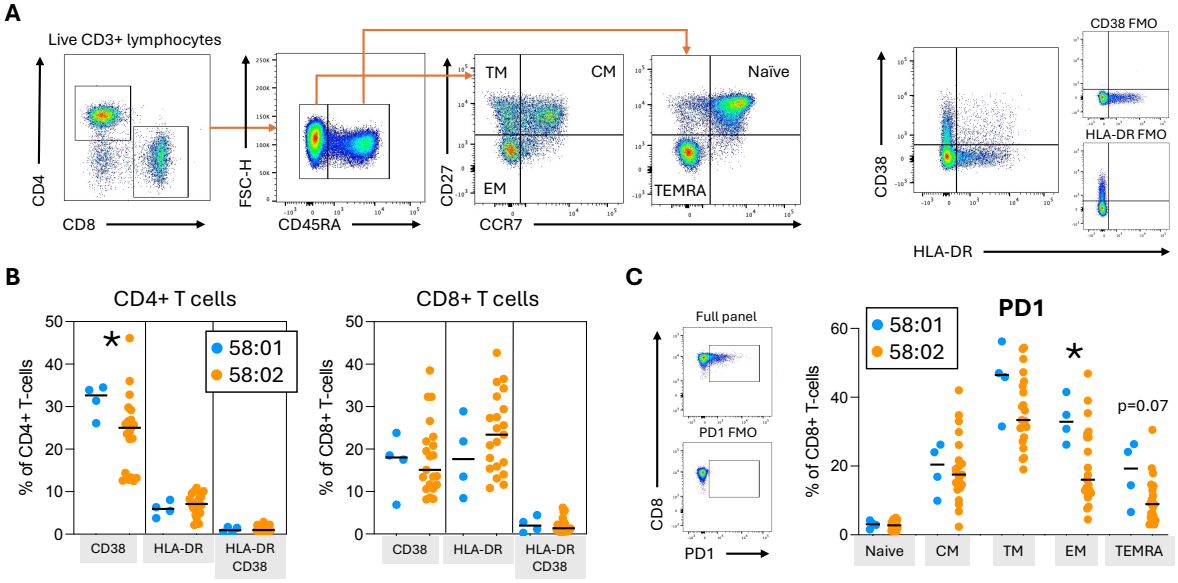

Figure 5.3. Comparison of T cell activation markers between uninfected woman from the FRESH cohort with HLA-B*58:01 and HLA-B*58:02. A. Gating strategy for T cell subsets and gating strategy for HLA-DR/CD38 double positive cells. B. Comparison of activation markers between those with HLA-B*58:01 and HLA-B*58:02, for both CD4+ T cells and CD8+ T cells. C. Frequency PD1 expressing CD8+ T cell subpopulations. Mann-Whitney U-tests were used as statistical comparisons uncorrected for multiple comparisons.

These data suggest that T cells in individuals with HLA-B*58:01 were more activated compared to individuals with HLA-B*58:02, observed through CD38+ CD4+ T cells and PD1 expression on effector memory CD8+ T cells. This difference may be due T cells in individuals with HLA-B*58:01 being enriched with inflammatory pathways, such as TNFa signaling and IL2/STAT5 signaling, and proliferative pathway, such as hypoxia, compared to individuals with HLA-B*58:02.

Virally suppressed, early ART-treated children with HLA-B*58:01 display higher levels of T cell activation and CCR5 expression compared to those with HLA-B*58:02

Next, we investigated whether the distinct phenotypic differences observed between HLA-B*58:01 and HLA-B*58:02 in healthy adult females could be confirmed in virally suppressed, early ART-treated children, at median 30 months old. Here, we phenotyped T cells from age-matched children with different HLA-B phenotypes: HLA-B*58:01, HLA-

B*58:02, HLA-B*57:03, and HLA-B*81:01, the latter two being additional ‘protective’ HLA-B alleles included for comparison. We also included seven individuals expressing ‘neutral’ HLA-B alleles to serve as a reference group. Phenotypic profiles across total CD4+ T cells and CD4+ T cell subsets (naïve, central memory, effector memory, transitional memory, and TEMRA) were summarised in a heatmap, displaying relative expression of markers between HLA-B types as z-scores (**Fig 5.4A**).

By using the heatmap to guide analyses, we identified the top three differentially expressed markers for each CD4+ T cell subset and total CD4+ T cells (**Fig 5.4B**). We observed that HLA-DR is consistently higher in individuals with HLA-B*58:01, significantly so for total CD4+ T cells (p=0.01), effector memory (p=0.007) and transitional memory CD4+ T cells (p=0.007). Additionally, we observe that CCR5 expression in total CD4+ T cells and central memory CD4+ T cells is higher in those with HLA-B*58:01, significantly so for total CD4+ T cells (p=0.03).

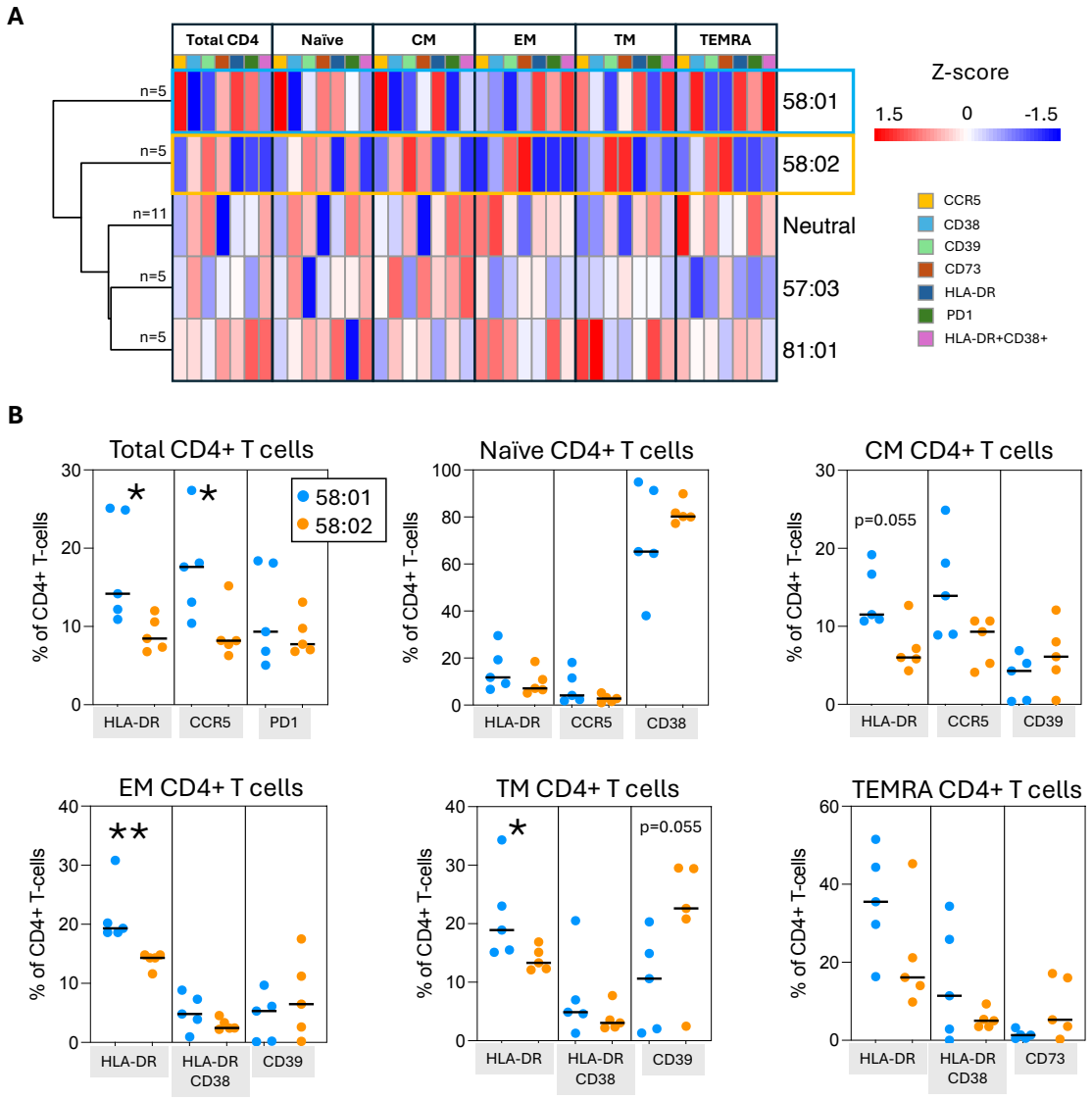

Figure 5.4. Phenotypic comparison of CD4+ T cell markers across selected HLA-B types in early ART-treated children. A. Heatmap showing relative marker expression between HLA-B types (z-score). B. A comparison of the top 3 differentially expressed markers between HLA-B types per subset. Mann-Whitney U-tests were used as statistical comparisons uncorrected for multiple comparisons. * represent p-values <0.05, and ** represent p-values <0.01.

Next, by phenotyping CD8+ T cells (**Fig 5.5A**), we similarly show a general enrichment of HLA-DR expression across subsets in those with HLA-B*58:01, significantly so for effector memory CD8+ T cells (p=0.03) (**Fig 5.5B**). Together, these data firstly highlight the impact HLA-B type has on T cell phenotype, as shown in the heatmaps across HLA-B types as well as direct comparisons between children with either HLA-B*58:01 or HLA-B*58:02. We

observe a general increase in HLA-DR expression in individuals with HLA-B*58:01 compared to HLA-B*58:02 across the majority of T cell subsets. We also observe an enrichment of CCR5 expression on total CD4+ T cells in individuals with HLA-B*58:01 compared to HLA-B*58:02.

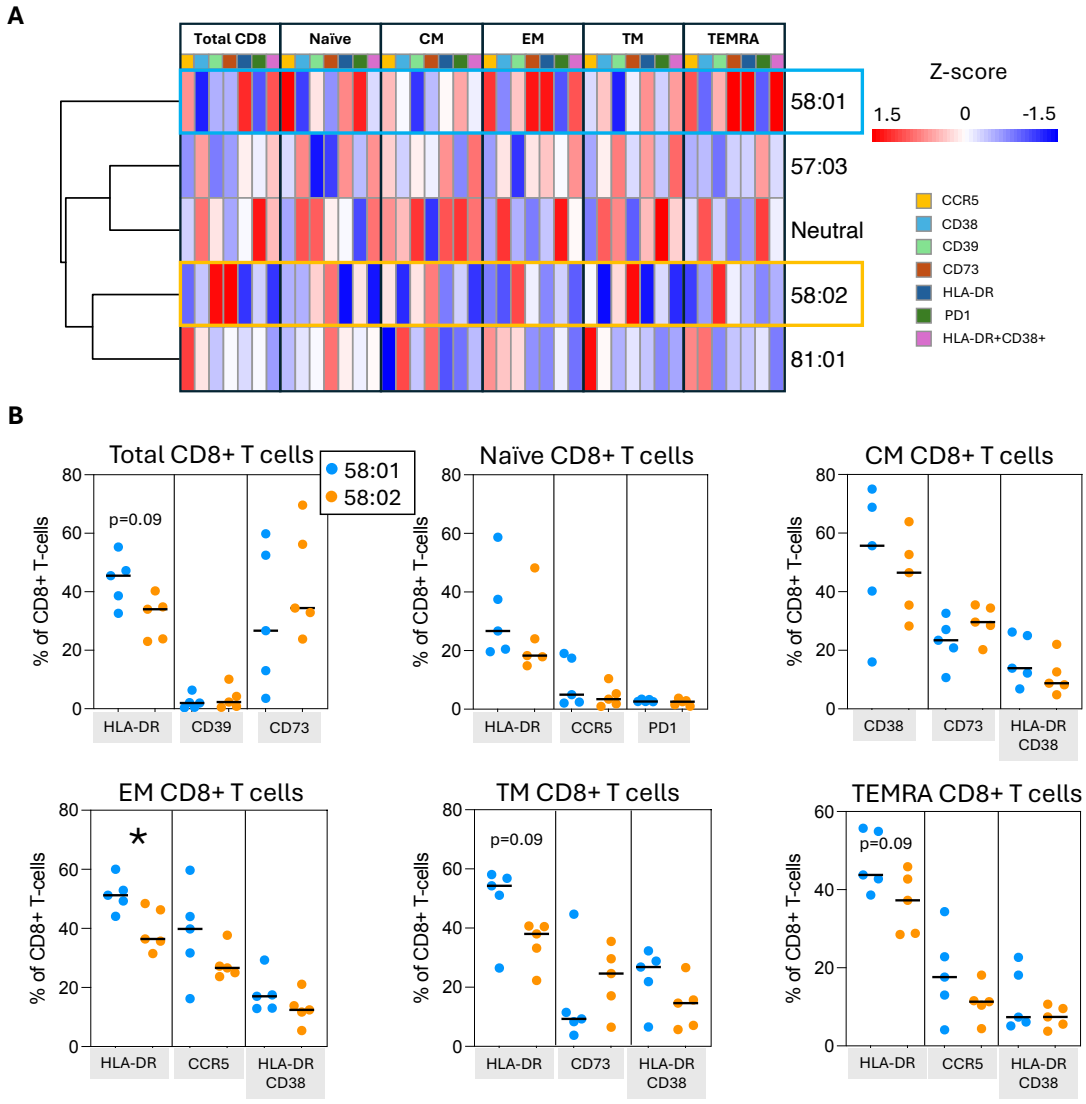

Figure 5.5. Phenotypic comparison of CD8+ T cell markers across selected HLA-B types in early ART-treated children. A. Heatmap showing relative marker expression between HLA-B types (z-score). B. A comparison of the top 3 differentially expressed markers between HLA-B types per subset. Mann-Whitney U-tests were used as statistical comparisons uncorrected for multiple comparisons. * represent p-values <0.05.

Together, we have shown that T cells from individuals with HLA-B*58:01 are generally more activated than those with HLA-B*58:02, observed here in both healthy adult women and early-ART treated children.

Weak HIV-specific immune response in virally suppressed children

We next investigated whether children who had remained virally suppressed exhibited substantial HIV-specific immune responses, as we hypothesised that this subset of children may not have been exposed to enough virus to develop any strong HIV-specific immunity. We did so by stimulating whole blood (WB) with HIV-1 peptide pools (2ug/mL for peptide pools and SEB, 10-hour incubation) and then conducted intracellular cytokine staining for IFNg (**Fig 5.6**). By stimulating WB from a child who had experienced prolonged periods of viremia, we show a relatively strong HIV-specific CD8+ T cell response, particularly to Gag, Pol and Env peptide pools (**Fig 5.6A**). However, when we looked at children who have remained consistently virally suppressed (**Fig 5.6B**) or have experienced a brief period of viremia but were suppressed at the time of the WB ICS assay (**Fig 5.6C-D**), we show that they had relatively weak CD8+ T cell responses compared to the child who had experienced prolonged periods of viremia. Here, although using pilot data, we demonstrate that those children who have remained virally suppressed have weak HIV-specific immune responses, which has been widely documented (Lohman et al. 2005; Sandberg et al. 2003; Thobakgale et al. 2007). We therefore did not conduct further HIV-specific T cell assays between virally suppressed children with HLA-B*58:01 and HLA-B*58:02, as this data shows that they would not likely have any significant responses to compare. Further pilot data from our group has confirmed that this group of early-ART treated children who have remained virally suppressed lack comparable HIV-specific T cell responses (not shown).

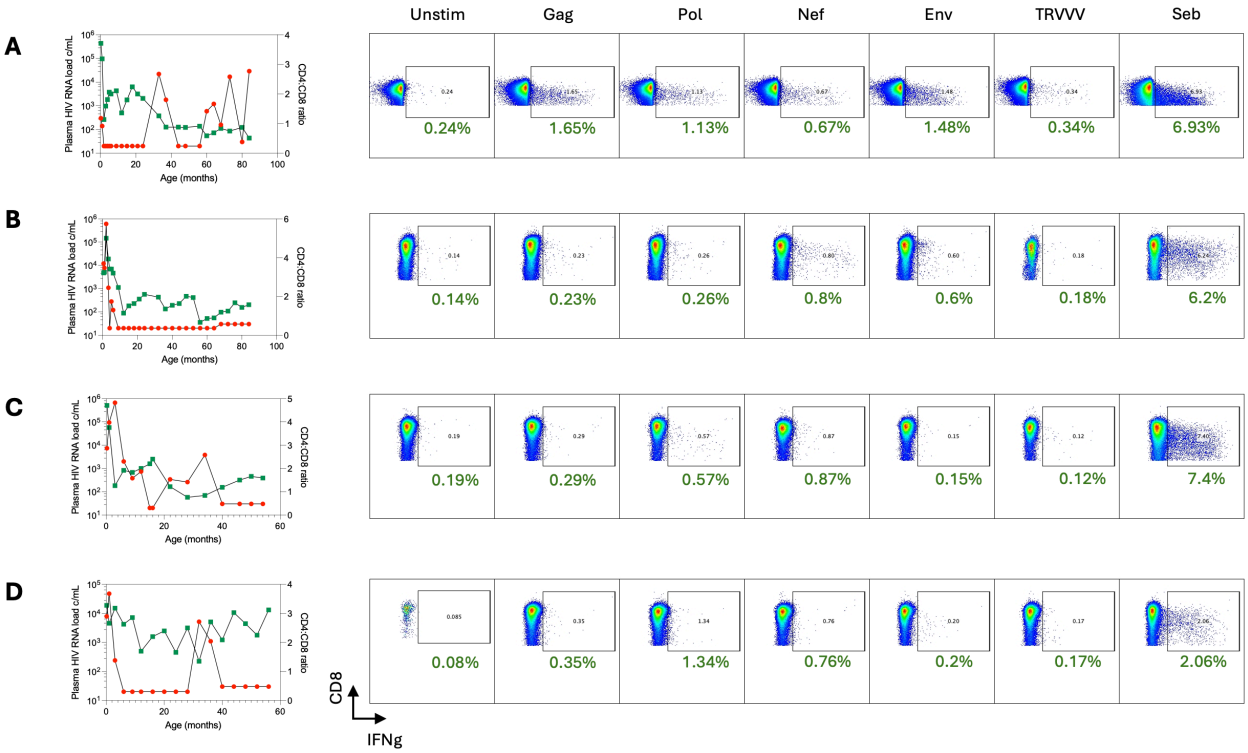

Figure 5.6. Comparison of HIV-1 specific CD8+ T cell responses to HIV-1 peptides by IFNg between a viraemic child (A) and virally suppressed children (B-D).

The difference in transcriptional activity and T cell phenotype between individuals with HLA-B*58:01 and HLA-B*58:02, observed here from both healthy women and early-ART-treated children, suggests that these differences may have disease associations considering the differences observed in this study related to immune activation. We investigated this first in the context of horizontal HIV-1 acquisition in adults from the same uninfected, adult cohort used in this study (FRESH cohort), and secondly, the viral characteristics of the same cohort of early ART-treated children and their mothers at birth.

Adult females with HLA-B*58:01 associated with a higher risk of horizontal HIV-1 acquisition and higher viral fitness compared to those with HLA-B*58:02

Women in the FRESH cohort were recruited HIV-negative and followed longitudinally for 9 months, where they were tested for HIV-1 RNA in plasma twice weekly (Dong et al. 2018).

Since n=159 women who participated in the study were HLA class I typed, we compared the proportion of women who became infected during the 9-month study by their HLA-B allele phenotype (**Fig 5.7A**). Interestingly, HLA-B*58:01 and HLA-B*58:02 showed significantly different proportions of women who become infected during the study, where HLA-B*58:01 had a 3-fold higher proportion of women who became infected compared to HLA-B*58:02 (75% vs 25.8%, respectively, p=0.001, OR 8.6). By comparing CCR5 expression on CD4+ T cells from the uninfected samples previously phenotyped, there was no significant difference between women with HLA-B*58:01 versus HLA-B*58:02 (**Fig 5.7A**).

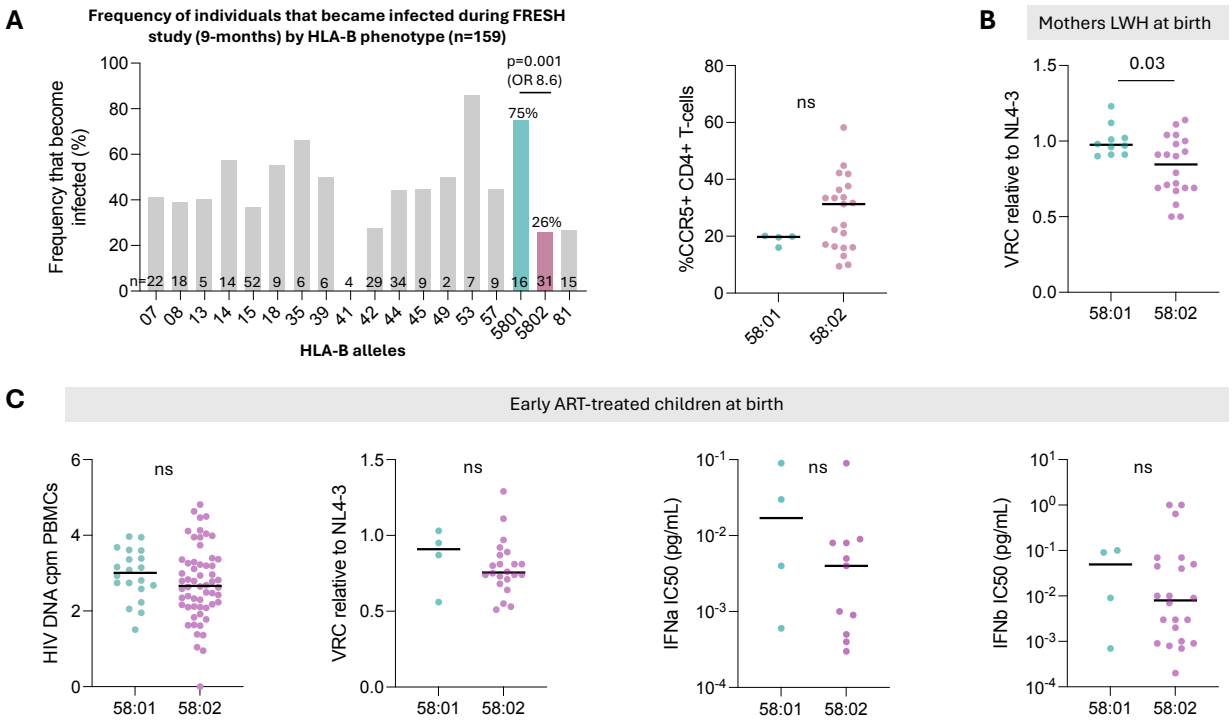

Figure 5.7. Contrasting risk of HIV-1 acquisition, VRC in mothers LWH, and viral characteristics in early-ART treated children. A. Phenotypic frequency of individuals with each HLA-B type that acquired HIV-1 during 9-month within the FRESH study. Fishers Exact test uncorrected for multiple comparison was conducted. Comparison of CCR5 expressing CD4+ T cells between HLA-B groups from the FRESH cohort using uninfected samples. B. Comparison of viral replicative capacity (VRC) between mothers with HLA-B*58:01 and HLA-B*58:02 at pregnancy from the Baby Cure cohort. C. Comparison of total HIV-1 DNA, viral replicative capacity (VRC), IFNa IC50, and IFNb IC50 between children with HLA-B*58:01 and HLA-B*58:02 from the Baby Cure cohort at birth. Mann-Whitney U-tests were used as statistical comparisons unless stated otherwise.

Next, to understand the consequences of having either HLA-B*58:01 or HLA-B*58:02 in the context of HIV infection, we next investigated early ART treated mothers and children at birth. In the mothers, there was no significant difference in plasma VLs between the two HLA-B types (not shown). However, when we look at viral characteristics, we show that those mothers with HLA-B*58:01 had a higher VRC virus compared to mothers with HLA-B*58:02 (p=0.03) (**Fig 5.7B**) but observed no difference in IFNa and IFNb resistance (not shown).

We next sought to investigate whether the same HLA-B dependent patterns in the mothers are also observed in the children LWH at birth. We show that children with HLA-B*58:01 have higher total HIV-1 DNA, VRC, IFNa and IFNb resistance (**Fig 5.7C**). Although none of these comparisons reached statistical significance, the trends are consistent across assays, which were all conducted independently, suggesting that children with HLA-B*58:01 may harbour a higher-fitness virus than those with HLA-B*58:02. While additional biological replicates are required to confirm these findings, the data imply that early ART treated children with HLA-B*58:01 may have a lower functional cure potential compared to those with HLA-B*58:02 as they may harbour a higher fitness virus following in utero infection.

DISCUSSION

This study provides insights into the immunological differences conferred by two closely related HLA-B alleles, HLA-B*58:01 and HLA-B*58:02, that differ by only three amino acids within the peptide binding cleft (Marsh SGE et al. 2000). This study investigated these differences in the novel setting of healthy adult females and early ART-treated children, compared to previous studies investigating HIV-specific immune responses in ART-naïve

settings between the two alleles (Kiepiela et al. 2004; Leslie et al. 2010). Our study suggests that, in both healthy adult females and early ART treated children, those with HLA-B*58:01 have higher levels of T cell immune activation compared to those with HLA-B*58:02. By single cell RNA seq, we show that T cells from the healthy adult females with HLA-B*58:01 were significantly enriched with the pro-inflammatory TNFa signaling pathway via NF-kB while those with HLA-B*58:02 were significantly enriched for the antiviral interferon signaling pathways. It is important to note that these samples from the healthy adult females were not stimulated or primed prior to the flow cytometry or the single-cell RNA seq protocol, and therefore our data show basal differences in T cell activity potentially driven by differences in HLA-B*58 subtype. While in early ART-treated children, although virally suppressed, the PBMCs from these children could be seen are pre-stimulated by the child's own virus prior to the assays. None-the-less, we observe T cells more activated in children with HLA-B*58:01 compared to HLA-B*58:02, observed through HLA-DR expression, consistent with our findings from the healthy adult females, with whom we observed these differences through CD38 and PD1 expression.

HLA-I molecules associated with protection of HIV-1 infection are in many cases also linked with autoimmune diseases (Bowness 2015; Chen et al. 2012; Mallal et al. 2008), the best characterised example being certain HLA-B*27 subtypes with ankylosing spondylitis (Bowness 2015). Autoimmune diseases are associated with heightened inflammation and immune activation (Xiang et al. 2023) and our study therefore supports the notion that 'protective' HLA-B alleles in HIV-1 infection, such as HLA-B*58:01, are associated with increased risk of autoimmune diseases. Indeed, HLA-B*58:01 is associated with severe adverse reactions to allopurinol, a gout and hyperuricaemia therapy (Pham et al. 2025). While more studies are required to confirm these findings, our study does shed light on why HIV-1

protective HLA-B alleles are associated with autoimmune diseases, at least in the context of HLA-B*58:01 compared to HLA-B*58:02, as those with HLA-B*58:01 are linked with heightened immune activation, potentially driven by amplified basal TNFa signaling compared to those with HLA-B*58:02. Indeed, TNFa signaling plays a central role in many inflammatory diseases (Smulski 2024).

Disease-susceptible HLA-B alleles have been found to be enriched among adult PTCs who are characterised by low levels of immune activation (Sáez-Cirión et al. 2013). Indeed, our study demonstrates that the 'disease-susceptible' HLA-B allele HLA-B*58:02 has lower levels of immune activation compared to the protective HLA-B*58:01, observed in both healthy adults and early ART-treated children. This therefore raises the possibility that children carrying HLA-B*58:01 may be at a disadvantage in achieving PTC compared to those with HLA-B*58:02 in the context of early ART initiation and ATI studies. In contrast, the more quiescent immune profile observed among early ART treated children with HLA-B*58:02 may be more favourable to achieve PTC. In support of this notion, we observed that those children with HLA-B*58:02 appear to harbour a less fit viruses compared to children HLA-B*58:01. Further studies are required to show statistical differences between the viral characteristics and total HIV-1 DNA load between the HLA-B*58 subtypes.

Of note, healthy adult women with HLA-B*58:02 were enriched for the antiviral interferon pathways, both type I and type II interferons, across T cells and myeloid cells, compared to HLA-B*58:01. From our cohort of mothers LWH, we observed that mothers with HLA-B*58:02 harboured a virus with lower VRC compared to HLA-B*58:01. Since both the healthy adult females and mothers LWH are of comparable age and same ethnicity, we may hypothesis that the mothers LWH with HLA-B*58:02 have a stronger interferon response

compared to those with HLA-B*58:01, leading to the observed difference in VRC due to selective pressure between the two HLA-B*58 subtypes. However, further studies will need to confirm the enrichment of immune pathways in mothers LWH.

While we observed that these two closely related HLA-B subtypes exhibit distinctly different immune-pathway enrichment and T-cell activation, the precise mechanisms underlying these differences remain unclear. Residue 97 is known to play a critical role in peptide anchoring and in shaping the three-dimensional structure of the HLA-peptide complex, thereby influencing complex stability and T-cell receptor recognition (Blanco-Gelaz et al. 2006). Supporting this, a recent study demonstrated that micropolymorphisms among the closely related alleles HLA-B*57:03, B*57:02 and B*58:01 lead to differences in peptide abundance, HLA-peptide stability, and the conformation of the surface-exposed peptide-HLA complex (Illing et al. 2018). In addition, structural studies have shown that even minimal differences between HLA-B*57:02 and HLA-B*57:03 result in measurable changes in TCR binding kinetics and downstream cellular activation (Stewart-Jones et al. 2012). Taken together, differences in peptide-binding conformation, peptide-HLA complex stability, peptide repertoire, or TCR engagement offer plausible mechanisms by which HLA-B micropolymorphisms shape T-cell immunity and may underlie the distinct immune activation and pathway enrichment patterns observed between the HLA-B*58 subtypes in our study.

Another potential mechanism involves differential binding affinities of the leukocyte immunoglobulin-like receptors 1 and 2 (LILRB1/2) for the HLA-B*58 subtypes. LILRB2 is predominantly expressed on myeloid cells whereas LILRB1 can also be expressed by lymphoid populations. Prior work demonstrated that these receptors interact with HLA class I molecules with varying affinities depending on the specific HLA allele (Bashirova et al.

2014). Engagement of LILRB1 or LILRB2 delivers inhibitory signals that can modulate immune activation levels, and allele-specific differences in this interaction have been linked to variation in plasma viral loads. In the Bashirova et al. study, LILRB1/2 binding was assessed for HLA-B*58:01 but not for HLA-B*58:02. It is therefore plausible that differences in LILRB1/2 binding affinities between these closely related alleles contribute to the distinct pathway enrichment patterns and T-cell activation phenotypes observed in the present study. Specifically, if HLA-B*58:01 engages LILRB1/2 with weaker affinity than HLA-B*58:02, this could lead to reduced inhibitory signalling and consequently higher T-cell immune activation, consistent with our findings in healthy adult females and virally suppressed children carrying HLA-B*58:01 compared with those carrying HLA-B*58:02.

Another interesting finding was observed from the longitudinal data from the FRESH cohort, where we found that adult women with HLA-B*58:01 were significantly more likely to acquire HIV-1 over a 9-month follow-up period compared to those with HLA-B*58:02, by 3-fold (p=0.001, **Fig 5.7A**). This increased acquisition risk could not be explained by differences in CCR5 expression in the pre-infection samples, suggesting that other factors may be involved. A previous study in the FRESH cohort has shown that significantly higher levels of genital inflammation led to an increased risk of HIV-1 acquisition (Masson et al. 2015). Since CD4+ and CD8+ T cells activation in blood has been shown to significantly predict CD4+ and CD8+ T cell activation in the cervical compartment (by HLA-DR and CD38 expression) (Jaspan et al. 2011), our findings that CD4+ T cells in peripheral blood have higher levels of immune activation in those with HLA-B*58:01 compared to HLA-B*58:02 may partially explain why those with HLA-B*58:01 have a 3 fold increased risk of HIV-1 acquisition compared to those with HLA-B*58:02.

Collectively, our findings highlight the impact of small sequence changes within the HLA-B peptide-binding groove on immune activation and pathway enrichment. Although HLA-B*58:01 and HLA-B*58:02 differ at only three amino acid positions, their divergent immune transcriptional signatures, T cell phenotypes, and associations with viral acquisition and fitness underscore the fine-tuned relationship between HLA polymorphism and immunity. In conclusion, this study supports the hypothesis that protective HLA-B molecules are unfavourable and that disease-susceptible HLA-B alleles are favourable in achieving PTC and may be extended to paediatric PTC. Our study uniquely showed that those with HLA-B*58:02 had lower levels of immune activation in the context of healthy adults, potentially reducing the risk of HIV-1 acquisition, and in early ART-treated children, potentially leading to a lower fitness virus. These insights improve our understanding of host genetic factors that may either promote or hinder the achievement of PTC.

CHAPTER 6: CONCLUSION

This year, 2025, marks 44 years since the first case report of HIV-1 infection in 1981 by the Centers for Disease Control and Prevention (Gottlieb et al. 1981). Since the start of the pandemic, HIV-1 has been estimated to have killed 44.1 million people from HIV-related causes, with 91.4 million people having been infected (World Health Organisation 2024). At the end of 2024, around 40.8 million people were living with HIV-1 globally. Although we now have the tools to end the HIV-1 epidemic and interrupt mother-to-child transmission, nearly 700 000 people die annually from HIV-related causes, and around 150 000 children were newly diagnosed with HIV-1 in 2024 (World Health Organisation 2024).

Achieving an HIV-1 cure remains one of the most ambitious and urgent goals in the field, yet it continues to pose substantial challenges. While sterilising cure has only been observed in a small number of exceptional cases (Dickter et al. 2024; Gupta et al. 2019; Hsu et al. 2023; Hütter et al. 2009; Jensen et al. 2023; Sáez-Cirión et al. 2024), typically involving high-risk and high-cost interventions such as allogeneic stem cell transplantation, it is not viable to scale to the wider population LWH. In contrast, a functional cure such as PTC, while also rare, is a more realistic and scalable option for the wider population LWH.

Among the potential strategies being explored, PTC, where individuals maintain virological suppression in the absence of ongoing ART, represents the most promising path toward achieving a widely applicable functional cure as covered in Chapter 1. Previous reports of PTC achieved by early ART initiation have been shown in adults (Lisziewicz et al. 1999; Namazi et al. 2018; Sáez-Cirión et al. 2013) and in children (Bengu et al. 2024; Frange et al. 2016; Persaud et al. 2013; Violari et al. 2019), demonstrating its possibility. After the "Mississippi baby" report (Persaud et al. 2013), clinical studies were designed to replicate

this case by diagnosing HIV-1 infection soon after birth and starting therapy in the very early stage of the disease course (Garcia-Broncano et al. 2019; Kuhn et al. 2020), including our own study, the Baby Cure cohort, in 2015 (Bengu et al. 2024; Millar et al. 2020). Notably, infants appear to have a uniquely elevated capacity to achieve PTC, likely due to a combination of immunological tolerance, early ART initiation, and the infection of lower fitness viruses (Goulder et al. 2016). Although most studies in early-ART treated children have not resulted in PTC (Cotton et al. 2013; Kuhn et al. 2020; Prendergast et al. 2008; Viganò et al. 2006), our study on the Baby Cure cohort has uniquely shown 5 male infants who have experienced periods of sustained ART-free viral control and therefore periods of PTC (Bengu et al. 2024). Here we postulated that a small subset of early ART-treated children exists that may achieve PTC, and previously predicted that the factors that associate with ART-free viral control include male sex and the transmission of low-replicative-capacity, IFN-I-sensitive virus (Bengu et al. 2024). This thesis adds to this, showing that (1) low HLA-A expression, (2) enhanced NK cell responses (which were enhanced in these five male infants), (3) disease-susceptible HLA-B alleles such as B*58:02, and (4) high frequency ART coverage enough to halt HLA-B dependent risk of vertical transmission are further factors that this thesis shows favour paediatric PTC.

However, with rare exceptions, PTC is thought not to be achieved by ART alone and may require additional therapeutic interventions such as broadly neutralising antibodies (Goulder et al. 2016). The ideal age to initiate a therapeutic intervention and interrupt treatment is uncertain, but optimally it should happen before late childhood and adolescence, when the risk of non-adherence is at its highest. Interestingly, the period between 4 and 12 years old is called the ‘honeymoon period of infectious diseases’ (Ahmed, Oldstone, and Palese 2007; Langford 2002), during which the 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 (Ahmed et al. 2007; Langford 2002). Although not yet completely understood, this period of life comprises the perfect balance between developing an immune response and the harmful damage it may provoke and may provide a unique window to utilise therapeutic interventions and conduct ATIs in early ART-treated children (Goulder et al. 2016).

The existence of PTCs shows clear evidence that functional cure/remission can be achieved, even though they are rare, and they provide a model to identify potential interventions necessary to scale up to the population level. Motivated by this, this thesis focused on elucidating the immunogenetic and immunological determinants of PTC potential in children LWH. Here, I had access to a unique population of early ART-treated children LWH from the Baby Cure cohort (Bengu et al. 2024; Millar et al. 2020), including access to samples from five children within the cohort who have experienced periods of PTC. A deeper understanding of the factors that contribute to PTC is critical for informing future curative strategies and identifying paediatric candidates for ATI trials. Across three data chapters, this thesis presents novel insights into factors that favour paediatric PTC.

In **Chapter 3**, we showed that early ART-treated children who possessed low-expression HLA-A alleles had significantly lower levels of HIV-1 DNA, a proxy for the size of the latent viral reservoir. This builds on existing ART naïve adult and paediatric studies that low HLA-A expression is favourable in the context of HIV-1 infection and likely acts via enhanced NK cell-mediated immunity. In the early-ART-treated paediatric setting, we extend this understanding by showing that low HLA-A expression may similarly favour smaller reservoir seeding. We also characterised five atypical male infants who sustained ART-free viral suppression and found that they exhibited enhanced resting NK cell function relative to

typical infants on ART. Although the sample size was small, this observation suggests that elevated resting NK cell activity may partially contribute to the natural control observed in these unique cases. Unpublished data from our group has shown that these 'atypical' males have weak HIV-specific responses, as do other early-ART treated children who have remained virally suppressed, such as those presented in Fig 5.5B-D. This possibly gives more significance to the identification that they have enhanced NK functionality relative to typical infants. Additionally, females with protective HLA-B alleles exhibited higher levels of HIV-1 DNA at birth, positioning them less favourably for functional cure. Mechanistically, we observed an enrichment of IL-17 signalling in this group, consistent with a Th17-skewed CD4+ T cell compartment. As Th17 cells are more susceptible to HIV-1 infection and contribute to the establishment of the latent reservoir, this may explain the paradoxically higher reservoir sizes observed in these females. Taken together, this chapter highlights the protective role of NK cells in paediatric PTC, identifies low HLA-A expression as a genetic correlate of reduced reservoir size and enhanced cure potential, and the complex interaction of biological sex and HLA-I on immune activation.

Chapter 4 explored the long-term evolutionary consequences of HIV-1 on host HLA genetics at the population level in KwaZulu-Natal, a region severely impacted by the HIV-1 epidemic and where all the studies of this thesis are based. Prior to the introduction of widespread ART, natural selection may have favoured the accumulation of protective HLA-B alleles, those associated with reduced vertical transmission and slower disease progression, while eradicating disease-susceptible alleles. However, our study suggests that this evolutionary pressure has been weakened by the introduction of ART. Interestingly, we also observed a shift in the HLA-A allele pool, whereby children born during the ART era have lower frequencies of high-expression HLA-A alleles. When taken together with the findings of Chapter 3, this suggests that the current generation of infants acquiring HIV-1 possess a

more favourable genetic landscape for achieving PTC or functional cure, due to the enrichment of low-expression HLA-A alleles associated with smaller reservoirs.

Chapter 5 examined how subtle polymorphisms within the HLA-B peptide-binding region influence immunity by comparing the closely related alleles HLA-B*58:01 and HLA-B*58:02 in the unique setting of healthy adults and early ART-treated children. Given the link between low immune activation and PTC achievement, our findings suggest that early ART treated children who carry HLA-B*58:01 may be less likely to achieve PTC than those with HLA-B*58:02. The more quiescent immune profile and less fit viruses observed in HLA-B*58:02 carriers may instead favour durable remission. Indeed, in Chapter 3, our proteomic data shows that those early-ART treated children with protective HLA-B are enriched with proteins linked with the inflammatory IL-17 pathway compared to those without. Our results are consistent with prior evidence that disease-susceptible HLA-B alleles are associated with adult PTCs, which we show may be extended to paediatric PTC. These insights may support the identification of early ART-treated children most likely to achieve remission in future ATI studies.

This thesis provides a multifaceted view of PTC potential in children living with HIV, drawing on immunogenetics, immunology, virology, and population-level analyses. By identifying favourable biomarkers, such as low HLA-A expression, disease-susceptible HLA-B alleles, and enhanced NK cell function, this research may help inform future strategies for selecting paediatric candidates for ATI trials. It is also encouraging that the current ART era has a gene pool of children with a lower frequency of high expressing HLA-A alleles, theoretically improving the chances of children achieving PTC/functional cure and highlights the importance of high ART coverage for PTC potential. A hopeful outcome is that we may one day be able to accurately predict which children have high cure potential, allowing study

resources to be more efficiently used by identifying those children with the highest potential. Additionally, and importantly, this work will hopefully help realise the vision of relieving children of a lifetime on ART and help to reduce the stigma still associated with LWH.

Some important future work could advance the projects presented in this thesis. The Baby Cure study is currently implementing treatment interruption trials, creating an opportunity for future work to identify immunogenetic and immunological correlates of time to viral rebound, a stronger clinical measurement of PTC potential than total HIV DNA levels. In addition, rather than inferring HLA-A expression from previously published allele-specific values, future studies could quantify HLA-A expression directly using flow cytometry to obtain more accurate, participant-specific measurements. In parallel, NK cell functional assays could be further optimised, particularly through stimulation with autologous HIV-1–infected PBMCs, to provide a more detailed understanding of NK-mediated antiviral responses in early-treated children. Lastly, chapter 5 could be built upon by verifying whether other cohorts have observed differences in the risk of acquiring HIV-1 between those with HLA-B*58:01 versus HLA-B*58:02.

Ultimately, we ought not to forget that the tools to end the HIV-1 pandemic and stop new infections are already here, including ART and pre-exposure prophylaxis. Although pursuing an HIV-1 cure remains critically important, greater focus must be directed to regions where HIV-1 continues to be a major public health concern. In these settings, poverty, gender inequality, stigma, limited awareness, and restricted access to treatment and prevention remain key barriers, compounded by fragile health systems, treatment adherence challenges, and the disproportionate burden among certain key populations (such as young woman in South Africa), all of which are likely true for the cohorts studied in this thesis. A world

without HIV-1 will require a holistic approach that encompasses understanding all of these important aspects, including the factors associated with paediatric PTC.

Overall, nobody has a better or worse set of compatibility genes: there's no hierarchy in the system. The fact that we differ is what's important; the way our species has evolved to survive disease requires us to be different.

Daniel Davis, The Compatibility Gene (2013)

CHAPTER 7: REFERENCES

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CHAPTER 8: APPENDIX

Supporting information for Chapter 4:

The evolutionary-epidemiological model

To investigate the potential impact of HIV-1 on HLA frequencies in KZN, with or without ART, we constructed an evolutionary epidemiological model. The code for the model can be found at: https://github.com/BSPenmanBiol/HIV_HLA. We classified HLA-B alleles as being disease-susceptible (allele type 1, associated with shorter times to AIDS), indifferent (allele type 2, associated with the population average time to AIDS) or protective (allele type 3, associated with longer times to AIDS (Kiepiela et al. 2004)). There were thus 6 possible HLA-B genotypes in the model (see Table S3). The model consisted of linked ordinary differential equations representing susceptible (S), infected (I), or treated (T) hosts of HLA-B genotype *I* and age class *k* (0= reproductively immature, 1= reproductively active).

The model equations are as follows:

$$\frac{dS_{i0}}{dt} = b_i - (g + \mu)S_{i0} \qquad \textit{Equation 1}$$

$$\frac{dS_{i1}}{dt} = gS_{i0} - (\lambda + \mu)S_{i1} \qquad \textit{Equation 2}$$

$$\frac{dI_{i1}}{dt} = \lambda S_{i1} - (\alpha_i + \sigma + \mu)I_{i1} \qquad \textit{Equation 3}$$

$$\frac{dT_{i1}}{dt} = \sigma I_{i1} - \mu T_{i1} \qquad \textit{Equation 4}$$

The force of infection is calculated as follows:

$$\lambda = \beta\left(\frac{\sum_{i=1}^{i=6}(I_{i1})}{\sum_{i=1}^{i=6}(S_{i1} + I_{i1} + T_{i1})}\right) \qquad \textit{Equation 5}$$

β determines how the rate of becoming infected relates to the proportion of individuals in the population who are already infected. B is one of the parameters that we fit to the data (using Bayesian methods, described later in this document). Our model concerns the proportion of infected women, hence parameter β acts as a proxy for the entire process by which women become infected as a result of other women being infected. One way to think of this is to imagine the scenario in which an HIV-1 positive woman (woman 1) infects a man, who then infects another woman (woman 2). Parameter β captures the overall pathway of transmission from woman 1 to woman 2 without explicitly simulating the proportion of infected men. A higher value of β could indicate higher rates of heterosexual partner change and/or lower rates of condom usage.

b_i is the total number of births of genotype *I* per model time step. b_i is calculated as follows:

$$b_i = rNp_i \qquad \textit{Equation 6}$$

Where N = the total population size (i.e. the sum of all categories in the model); r = the birth rate and p_i = the proportion of genotype *I* among offspring who do *not* receive HIV-1 by vertical transmission from an infected mother.

To calculate p_i , first we calculate the potential reproductive contributions of males and females separately. Male contributions ($f_{i,\ males}$) are assumed to be equal to the frequency of each genotype in the adult population:

$$f_{i,males} = \frac{S_{i1} + I_{i1} + T_{i1}}{\sum_{i=1}^{i=6}(S_{i1} + I_{i1} + T_{i1})} \qquad \textit{Equation 7}$$

Note that our model is only of the female population and hence we are making the simplifying assumption that HLA frequencies are equivalent in males and females.

Female contributions must be adjusted to account for reproductive failure due to vertical transmission of HIV. We calculate an adjusted female contribution from each genotype, accounting for the maternal genotype specific probability of a mother transmitting HIV-1 to her baby (γv_i, see tables S3 and S4):

$$f_{i,females} = \frac{S_{i1}+(1-\min(\gamma v_i,1))I_{i1}+T_{i1}}{\sum_{i=1}^{i=6}(S_{i1}+I_{i1}+T_{i1})} \qquad \textit{Equation 8}$$

We multiply a column vector of the values of $f_{i,males}$ by a row vector of the values $f_{j,females}$ and obtain a 6x6 matrix **M** in which $(\mathbf{M})_{a,b}$ represents the proportion of offspring which we expect to come from males of genotype *a* reproducing with females of genotype *b*. We finally obtain p_i as follows:

$$p_i = \sum_{a,b=1}^{a,b=6}((\boldsymbol{M})_{ab}(\boldsymbol{Z_i})_{ab}) \qquad \textit{Equation 9}$$

Where $\boldsymbol{Z_i}$ is 6x6 matrix where $(\boldsymbol{Z_i})_{ab}$ corresponds to the proportion of offspring from the pairing of parental genotypes *a* and *b* which will have genotype *i*. For example, $(\boldsymbol{Z_1})_{11} = 1$, indicating that the offspring from a pairing between genotype 1 and genotype 1 (both homozygous for a disease-susceptible allele, see table S3) will all be homozygous for a disease-susceptible allele. $(\boldsymbol{Z_1})_{12} = 0$, indicating that none of the offspring from a pairing between genotype 1 and genotype 2 (one homozygous disease-susceptible, the other homozygous indifferent) will be homozygous for a disease-susceptible allele. $(\boldsymbol{Z_1})_{14} = 0.5$, indicating that half of the offspring from a pairing between genotype 1 and genotype 4 (one homozygous disease-susceptible, the other heterozygous disease-susceptible /indifferent) will be homozygous for a disease-susceptible allele.

All other parameters of the model are explained further in Table S4, which also gives the values used in the simulations.

Starting conditions

The starting conditions of the model were: a population in which 48% of the population are reproductively immature and the remainder are reproductively mature (equilibrium proportions associated with the birth rate and aging parameters in Table S4, in the absence of HIV). Since HIV-1 transmission is frequency dependent, the overall population size does not affect the behaviour of the model, so the results are applicable regardless of the starting population size.

The starting allele frequencies were: 0.15 disease-susceptible HLA-B allele; 0.12 protective HLA-B allele; 0.73 indifferent HLA-B allele. These proportions were based on the HLA frequencies we observed in a HIV-1 negative cohort from the year 2000 (this was the earliest

dataset we had available – see table S7). The population was assumed to be at Hardy Weinberg equilibrium at the beginning of the simulation.

Zero individuals were in the treated category at time=0. The starting proportion of the population infected with HIV-1 was assumed to be 0.016, reflecting the proportion of HIV-infected women reported in the National HIV-1 Surveillance Programme 1990 antenatal survey (Küstner et al. 1994).

Model fitting

The transmission parameter (β) is likely to vary with time due to behavioural factors (e.g. condom use). There is evidence to suggest a substantial increase in condom use between 1998 and 2000 in South Africa (Jonnson *et al* (Johnson et al. 2012), figure 1; 1998 is also the year that HIV-1 life skills programmes began in SA schools). Condom usage appears to peak between 2008 and 2012 (Simbayi *et al* (Simbayi LC et al. n.d.), see figure 3.12 and table 3.49). We therefore defined three broad time periods in which we allowed different values of β to apply: 1990-1998, 1999-2008 and 2009-2024. Parameter σ (the rate of starting ART) will also vary with time. Σ takes a nonzero value from 2004 onwards following the introduction of free ART in SA. There is likely to have been a change in σ around 2016, the year in which universal test and treat was introduced in SA. We thus fitted two different nonzero values of σ: 2004-2016 and 2017-2024.

We used Bayesian Markov Chain Monte Carlo (MCMC) sampling with a binomial likelihood function to estimate the posterior distribution of each of the 5 parameters ($\beta_{1990\text{-}1998}$, $\beta_{1999\text{-}2008}$, $\beta_{2009\text{-}2024}$, $\sigma_{2004\text{-}2016}$ and $\sigma_{2017\text{-}2024}$). We used a binomial likelihood because the available data was in the form of proportions (proportions of women infected and proportions of women

virally supressed – data sources are described below and in Table S6). We chose uninformative uninform priors for each function: a uniform distribution between 0.01 and 2 for each of the transmission parameters and a uniform distribution between 0.01 and 1 for each of the treatment parameters. To implement this method, we used the Slice Sampling algorithm provided in Matlab (Neal 2003), and we used Tracer (Rambaut et al. 2018) to calculate parameter estimates, 95% HPD intervals and estimated sample sizes (ESS). We ran each MCMC for long enough that ESS >200 for every parameter. We investigated 3 different HLA-B scenarios (a conservative scenario, a best estimate scenario and an extreme scenario, described in detail below). Since these involved different HIV-1 mortality rates, we performed MCMC fitting separately for each scenario. The fitted parameters for each of these scenarios are provided in table S5 and the joint posterior distributions of the fitted parameters are shown in figure S2.

We fitted the model to antenatal HIV-1 survey data reported for KZN province (Küstner et al. 1994, 1998; Tendesayi Kufa-Chakezha et al. 2024). Wherever possible, we used the raw data in the form of the numbers HIV-1 positive and the total number tested in our likelihood function, most of which we obtained directly from records in the US global HIV/AIDS surveillance database (US Global HV/AIDS Surveillance Database 2024). For some years (1990, 1992, 1998-2002), raw numbers were not available in either publications or the HIV/AIDS surveillance database, but proportions were available. For 1990, based on visual inspection of figure 1 of (Küstner et al. 1994), and the described study methods, we took 500 as a conservative estimate for the total sample size upon which the KZN proportion was based. For 1992, from the same figure in the same study, we used 1000 as a conservative estimate of the KZN sample size. For the years 1998-2002, no indications of sample size were available, but considering that every national antenatal screening programme KZN

sample recorded in the HIV/AIDs surveillance database from 1991 onwards has a sample size of >1000 (see Table S5), we again took 1000 as a conservative estimate of the sample size in those years.

To capture the impact of ART, we used estimates of the proportion of women virally suppressed from a community based study carried out by Conan *et al* (Conan et al. 2022): 60% viral suppression amongst women in 2013 based on a sample size of 1064 and 87.2% viral suppression in 2018 based on a sample size of 638. We also used the Sentinel Antenatal Survey 2022 report (Tendesayi Kufa-Chakezha et al. 2024) to add an additional datapoint of 81% viral suppression for KZN in 2022, based on an assumed sample size of 7821. To calculate this sample size, we took the overall 2022 sample size reported for KZN (9201) and multiplied it by the proportion of women in KZN reported as having had a viral load test (85%). For completeness we note that Conan *et al* used a cut off of <1000 copies/mL to define viral suppression and the Sentinel survey used <50 copies/mL to define viral suppression.

HLA-B evolutionary scenarios

Key genotype-specific parameters in the model are: α_i, the rate at which infected individuals of genotype *I* are removed from the population and v_i , which controls any genotype-specific increase or decrease in the baseline risk of vertical transmission of HIV-1 from genotype *i*.

We used rates of progression to AIDS, rather than time to death, to define α_i. This assumes that once an individual develops AIDS they are unlikely to successfully reproduce, and their contribution to infecting other individuals is also likely to be small. We took Amornkul *et al*'s (Amornkul et al. 2013) estimate of the median time to clinical AIDS for subtype-C (4.5

years) as the best available estimate of the mean time to AIDS in the KZN population, giving us an average rate of progression to AIDS of ¼.5 $years^{-1}$. In the absence of further genotype-specific information, we made the simplifying assumption that homozygotes for indifferent alleles would progress to AIDS at this average rate. Amornkul *et al* also provide hazard ratios and confidence intervals for the increase or decrease in the rate of progression to clinical AIDS for those with the protective allele HLA-B*57 versus those without, or those with the disease-susceptible allele HLA-B*45 versus those without, in sub Saharan African populations. It would have been ideal if these estimates were available for exclusively subtype C infections, and gave hazard ratios directly comparing different diploid genotypes, but this was not possible. The population in which these estimates were made had subtype C as the dominant HIV-1 type present. We assumed that genotypes containing protective alleles would have an increase in time to AIDS relative to the indifferent genotype equivalent to that which Amornkul reports for genotypes containing HLA-B*57 versus those without HLA-B*57. We further assumed that genotypes containing disease-susceptible alleles would have a decrease in time to AIDS relative to the indifferent genotype equivalent to that which Amornkul reports for genotypes containing HLA-B*45 versus those without HLA-B*45. Finally we assumed that genotypes containing both susceptible and protective alleles would have the population average time to AIDS for HIV-1 subtype-C (4.5 years). We used Amornkul's hazard ratios and their confidence intervals to generate a "best estimate" HLA-B evolutionary scenario (using the estimated hazard ratios directly); an "extreme" HLA-B evolutionary scenario (giving protective genotypes the longest possible time to AIDS implied by the confidence interval of the relevant hazard ratio, and giving genotypes with disease susceptible alleles the shortest possible time to AIDS implied by the confidence interval of the relevant hazard ratio), and a "conservative" scenario (giving protected genotypes the smallest increase in time to AIDS implied by the confidence interval of the relevant hazard

ratio and assigning disease susceptible genotypes no change in time to AIDS, because that side of the relevant confidence interval for the hazard ratio encompasses 1 in Amornkul's data). The resulting genotype-specific values of α_i for each of the HLA-B evolutionary scenarios are given in Table S3.

We took a similar approach to genotype-specific risks of vertical transmission. For this, we used our own data (Table S2). Since the sample sizes for some genotypes were small, we combined groups containing susceptible or protective alleles together to generate Ors to parameterise the model (Table S2b). We observed that the protective/disease-susceptible heterozygote seemed to be relatively protected against vertical transmission (Table S2a), and hence we assumed that for vertical transmission, the impact of the protective allele dominates over that of the disease-susceptible allele. For the "best estimate" scenario we used genotype-specific vertical transmission risks based directly on our calculated odds ratios for vertical transmission. For the "extreme" scenario we took the upper bound of the OR for the disease susceptible genotypes and the lower bound of the OR for the protected genotypes. For the "conservative" scenario we assigned all genotypes the same risk of vertical transmission. The resulting values of v_i for all three of our HLA-B evolutionary scenarios are given in Table S3.

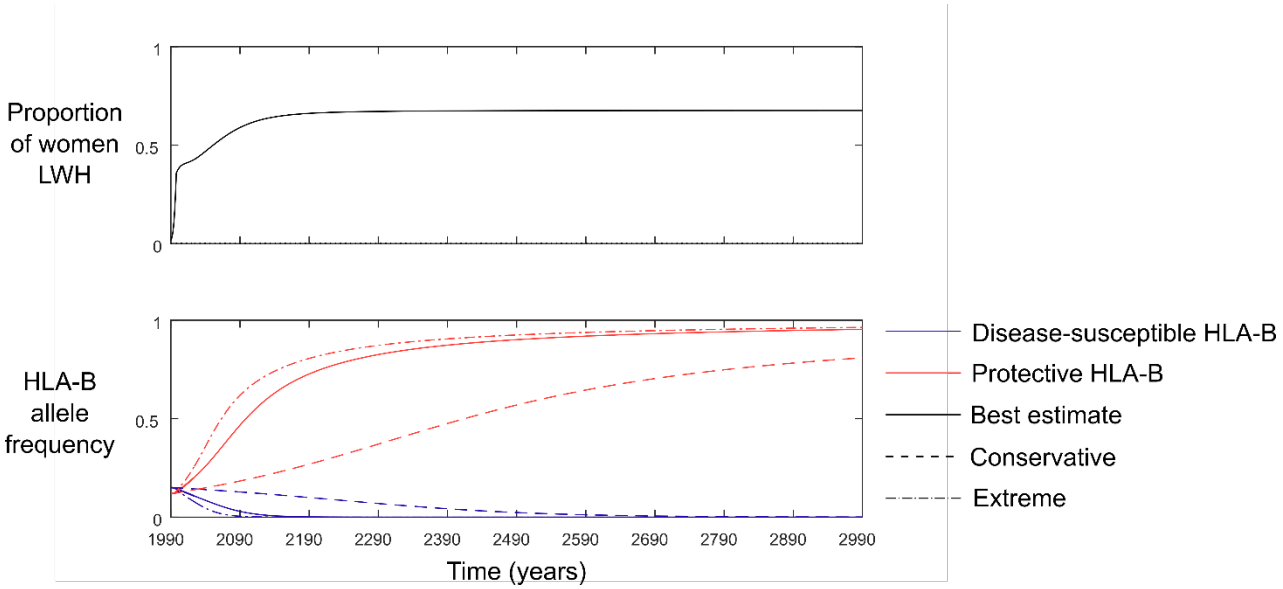

Figure S1. Extending the counterfactual scenario for 1000 years. This figure shows the behaviour of the model, under counterfactual conditions (no ART), for a 1000 year period between 1990 and 2990. Model parameters and starting conditions are exactly as described in the methods; this shows the longer-term outcomes of the behaviour illustrated in the right hand panel of figure 4.4A. Note that as the disease susceptible HLA-B allele is lost from the population, the proportion of women infected with HIV-1 increases. This reflects the fact that in the absence of the individuals who have the quickest time to AIDS (those with the disease susceptible HLA-B), on average HIV-1 infected people are living longer (which both means that they stay in the population for longer and can cause more secondary infections). The protective HLA-B allele is still increasing in the population after 1000 years, but at a slower rate. This reflects the fact that at the late stage of the simulation, there are relatively few homozygotes for the indifferent allele in the population, so most people in the population now have the longest possible times to AIDS.

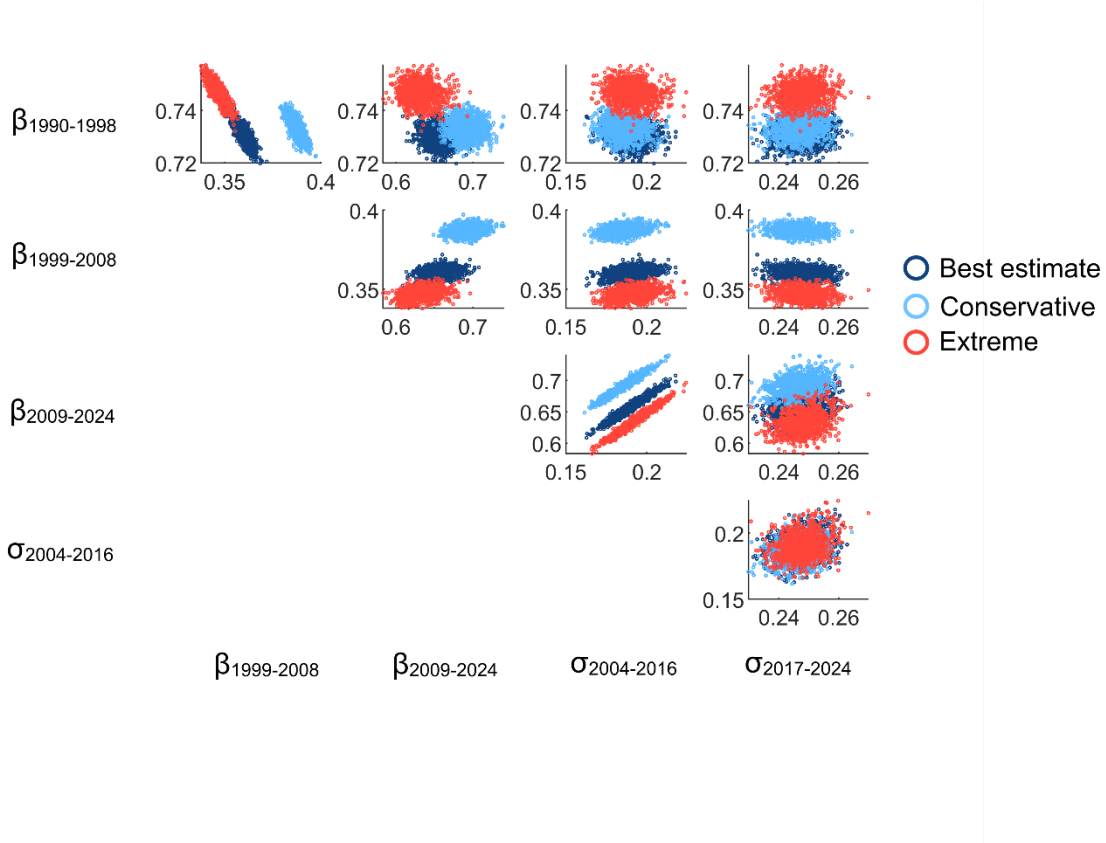

Figure S2. Joint posterior distributions of the fitted parameters. Each panel shows 1000 values sampled from the joint posterior distribution of the parameters indicated on the x and y axes. We performed model fitting separately for our 3 different HLA-B scenarios (best estimate, conservative and extreme). This resulted in slightly different posterior distributions, illustrated by the three different colours of marker (see key).

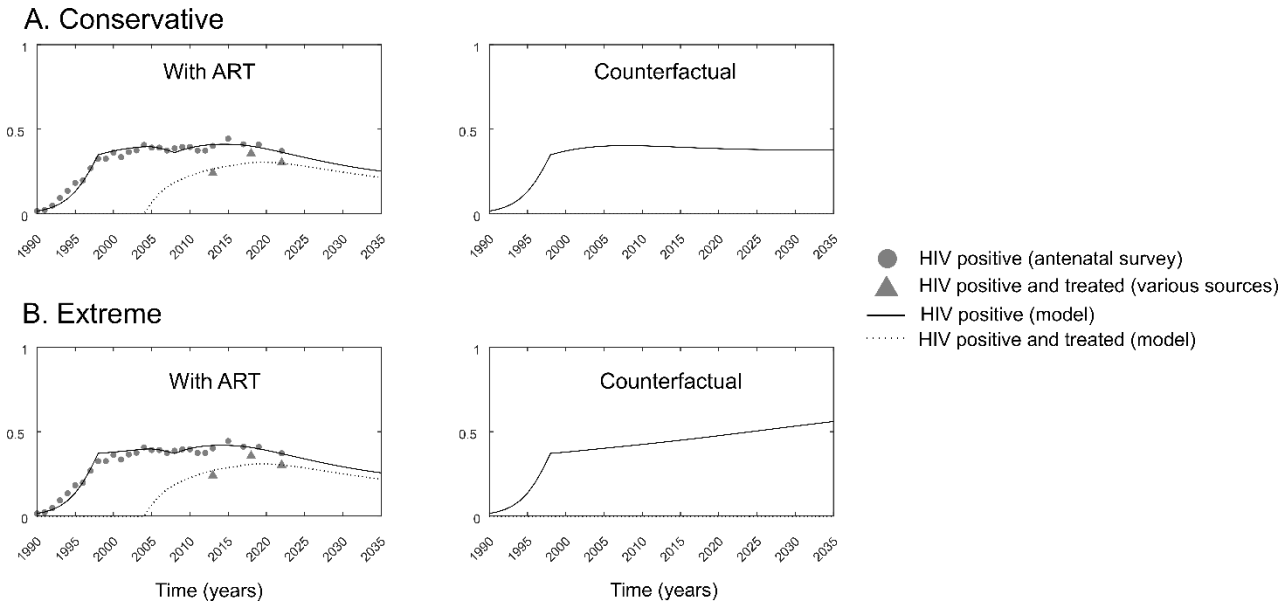

Figure S3: Fitted epidemiological behaviours for the conservative and extreme HLA-B property scenarios. The top row of figure 4.4A in the main text illustrates our simulated time series for the proportion of the population who are LWH, and those on ART, using the transmission and treatment parameter values for our best-estimate HLA-B properties scenario. Here we show the equivalent figures using the fitted values for our conservative (A) and extreme (B) HLA-B properties scenarios (see Table S4 for all fitted parameter values). Very similar fits are achieved to the data for all three HLA-B scenarios (see ART scenario on the left-hand side), but the different values of the $\beta_{1999\text{-}2008}$ parameter (which gets applied indefinitely in the counterfactual simulation) result in different longer-term dynamics in the counterfactual scenarios.

Table S1. Table describing statistical tests used in Figures 1 and 2.

	Tests	Question	Test	p value	p value
Fig 4.1B	Test 1	Was pVL higher in Transmitter Mothers vs Non-transmitter Mothers in the pre-ART era	1-sided MW	p<0.0001	n/a
Fig 4.1C	Test 1	Was pVL higher in Mothers expressing DS vs Protective HLA in the pre-ART era	1-sided MW	p<0.0001	n/a
Fig 4.1D	Test 1	Was pVL higher in Non-Transmitter Mothers expressing DS vs Protective HLA in the pre-ART era	1-sided MW	p<0.0001	n/a
	Test 1	Was pVL higher in Transmitter Mothers expressing DS vs Protective HLA in the pre-ART era	1-sided MW	p=0.025	n/a
Fig 4.1E	Test 1	Was CD4 higher in Non-Transmitter Mothers vs Transmitter Mothers in the pre-ART era	1-sided MW	p<0.0001	n/a
Fig 4.1F	Test 1	Was CD4 higher in Mothers expressing Protective vs DS HLA in the pre-ART era	1-sided MW	p<0.0001	n/a
Fig 4.1G	Test 1	Was CD4 higher in Non-Transmitter Mothers expressing Protective vs DS HLA in the pre-ART era	1-sided MW	p<0.0001	n/a
	Test 1	Was CD4 higher in Transmitter Mothers expressing Protective vs DS HLA in the pre-ART era	1-sided MW	p=0.043	n/a
Fig 4.2A	Test 1	Is DS HLA frequency lower in HIV-ve adults vs Transmitter Mothers & Children?	1-sided FET	p=0.003 (mothers)	p=0.003 (children)
	Test 2	Is DS HLA frequency lower in Non-transmitter Mothers vs Transmitter Mothers & Children?	1-sided FET	p=0.01 (mothers)	p-0.006 (children)
Fig 4.2B	Test 1	Is Protective HLA frequency lower in HIV-ve adults vs Transmitter Mothers & Children	1-sided FET	p=ns (mothers)	p=ns (children)
	Test 2	Is Protective HLA frequency lower in Non-transmitter Mothers vs Transmitter Mothers & Children	1-sided FET	p=0.002 (mothers)	p=0.004 (children)
Fig 4.2C	Test 1	Is DS HLA frequency lower in HIV-ve adults vs Transmitter Mothers & Children	1-sided FET	p=ns (mothers)	p=ns (children)
Fig 4.2D	Test 1	Is Protective HLA frequency lower in HIV-ve adults vs Transmitter Mothers & Children	1-sided FET	p=ns (mothers)	p=ns (children)
Fig 4.2E	Test 1	Is DS HLA frequency higher in Transmitter Mothers/Children Pre-ART era vs Transmitter Mothers/Children ART era	1-sided FET	p=0.01 (mothers)	p=0.003 (children)
	Test 2	Is DS HLA frequency higher in HIV-uninfected adults pre-ART vs ART era	1-sided FET	p=ns	
Fig 4.2F	Test 1	Is Prot HLA frequency lower in Transmitter Mothers/Children Pre-ART era vs Transmitter Mothers/Children ART era	1-sided FET	p=0.045 (mothers)	p=ns (children)
	Test 2	Is Prot HLA frequency lower in HIV-uninfected Pre-ART era vs HIV-uninfected ART era	1-sided FET	p=ns	

MW: Mann-Whitney test. FET: Fisher's Exact test. Prot: Protective. DS: Disease-susceptible.

Table S2. Odds ratios for risk of vertical transmission in the absence of ART for different genotypes from the PEHSS cohort (2000-2005). In panel A we show all 6 possible genotypes and their Ors for vertical transmission relative to the homozygous indifferent genotype. In panels B and C we group all genotypes with any protective allele together, and all genotypes with any susceptible allele (but no protective allele) together. Panel B provides Ors relative to the homozygous indifferent genotype. These are the estimates that we use directly in the model. Panel C provides Ors relative to the susceptible group of genotypes, to demonstrate the difference between protected and susceptible genotypes.

A.

Genotype	**Transmitter**	**Non-transmitter**	**OR**	**CI**
Homozygous for a disease-susceptible HLA-B allele	5	21	1.22	(0.43-3.41)
Homozygous for an indifferent HLA-B allele	45	231	1	
Homozygous for a protective HLA-B allele	1	14	0.37	(0.05-2.86)
Heterozygous for disease-susceptible and indifferent HLA-B alleles	43	138	1.60	(1.00-2.55)
Heterozygous for disease-protective and indifferent HLA-B alleles	13	118	0.57	(0.29-1.09)
Heterozygous for disease-susceptible and protective HLA-B alleles	5	37	0.69	(0.26-1.86)

B.

Genotype	**Transmitter**	**Non-transmitter**	**OR**	**CI**
Homozygous for a disease-susceptible HLA-B allele or Heterozygous for disease-susceptible and indifferent HLA-B alleles	48	159	1.55	(0.98,2.44)
Homozygous for an indifferent HLA-B allele	45	231	1	
Homozygous for a protective HLA-B allele, Heterozygous for disease-	19	169	0.58	(0.32,1.02)

protective and indifferent HLA-B alleles or Heterozygous for disease-susceptible and protective HLA-B alleles				

C.

Genotype	**Transmitter**	**Non-transmitter**	**OR**	**CI**
Homozygous for a disease-susceptible HLA-B allele or Heterozygous for disease-susceptible and indifferent HLA-B alleles	48	159	1	
Homozygous for an indifferent HLA-B allele	45	231	0.65	(0.41,1.02)
Homozygous for a protective HLA-B allele, Heterozygous for disease-protective and indifferent HLA-B alleles or Heterozygous for disease-susceptible and protective HLA-B alleles	19	169	0.37	(0.21,0.66)

Table S3. Genotype specific parameters used in the simulations. Note that the genotype specific risk of vertical transmission given in this table, v_i , is multiplied by a baseline probability of vertical transmission to give an overall probability of vertical transmission (see equation 11 and Table S5).Values for the rates of progression to AIDS are based on data reported in (Amornkul et al. 2013), and values for the risks of vertical transmission are based on data in Table S2. See the Supplementary Methods for more detail.

i	**Genotype identity**	**Rate of progression to AIDS (α_i), years^{-1}**			**Genotype specific risks of vertical transmission of HIV-1 (v_i)**		
		Conservative	**Best estimate**	**Extreme**	**Conservative**	**Best estimate**	**Extreme**
1	Homozygous for a disease-susceptible HLA-B allele	¼.5	1/3.4	½.3	1	1.55	2.44
2	Homozygous for an indifferent HLA-B allele	¼.5	¼.5	¼.5	1	1	1
3	Homozygous for a protective HLA-B allele	1/6.5	1/15	1/34.6	1	0.58	0.32
4	Heterozygous for disease-susceptible and indifferent HLA-B alleles	¼.5	1/3.4	½.3	1	1.55	2.44
5	Heterozygous for protective	1/6.5	1/15	1/34.6	1	0.58	0.32

	and indifferent HLA-B alleles						
6	Heterozygous for disease-susceptible and protective HLA-B alleles	¼.5	¼.5	¼.5	1	0.58	0.32

Table S4: Model parameters used in the simulations (excluding genotype specific parameters)

Parameter	Description	Value used	Notes
β	Transmission parameter	Fitted as part of model fitting process	See Table S5
σ	Rate at which HIV+ women start ART	Fitted as part of model fitting process.	See Table S5
μ	Rate of leaving the modelled population	1/45 $years^{-1}$	Setting this parameter to be 1/45 $years^{-1}$ means that the average "lifetime" of a host within the model is 45 years. We chose this value of the parameter based on the simplifying assumption that individuals over the age of 45 are not reproductively active.
G	Aging rates	g = 1/13 $years^{-1}$	This value means the mean time spent in the reproductively immature (i.e. child) age category is 13 years.
R	Birth rate	0.07	This value means that the number of new births entering the population each year is equal to 7% of the total size of the modelled population.

			The modelled population only represents ages 0-45, which is likely to be ~85% of the population for KZN in the 1990s, meaning that 0.07 in the model would translate to a crude birth rate of 0.0595, or 59.5 births per 1000 of the population. This is higher than the 1900s values reported for South Africa by the World Bank (20-31 per 1000 people). Of African countries, Angola, Niger, Chad, Uganda and South Sudan had crude birth rates>50 per 1000 people in 1991 according to World Bank estimates. We chose a value this high so that HIV-1 did not already start to cause a population decline before 2004, and we speculate that the crude birth rate may be higher in KZN than in South Africa as a whole.
γ	Baseline probability of vertical transmission of	A value of 0.35 used during model fitting and	This value is modified by the parameter v_i to account for genotype specific differences in the risk of vertical transmission (see Table S3).

	HIV-1 from an infected mother to her baby	for the main simulation. Values between 0 and 0.75 tested for panel B of the main text figure.	If the product of v_i and γ is ≥1 then vertical transmission occurs 100% of the time for maternal genotype *I* (see equation 8). The value of 0.35 is within the range of probabilities of vertical transmission of HIV-1 for breastfeeding mothers estimated by de Cock *et al (De Cock et al. 2000)*, whose highest estimate is 0.45 for mothers breastfeeding for up to 2 years.

Table S5: Fitted parameters used in the simulations

Parameter	**Fitted value (95% HPD)**		
	Conservative HLA scenario	**Best estimate HLA scenario**	**Extreme HLA scenario**
$\beta_{1990\text{-}1998}$	0.73 (0.73, 0.74)	0.73 (0.72,0.74)	0.75 (0.73, 0.76)
$\beta_{1999\text{-}2008}$	0.39 (0.38, 0.39)	0.36 (0.35, 0.37)	0.35 (0.33,0.36)
$\beta_{2009\text{-}2024}$	0.69 (0.66, 0.72)	0.66 (0.63, 0.69)	0.63 (0.60,0.67)
$\sigma_{2004\text{-}2016}$	0.19 (0.17, 0.20)	0.19 (0.17, 0.21)	0.19 (0.17, 0.21)
$\sigma_{2017\text{-}2024}$	0.25 (0.24, 0.26)	0.25 (0.24, 0.26)	0.25 (0.24, 0.26)

Table S6: Antenatal survey HIV-1 prevalence data for KZN used to fit the model, sourced from (Küstner et al. 1994, 1998; Tendesayi Kufa-Chakezha et al. 2024) and the US global HIV/AIDS surveillance database (US Global HV/AIDS Surveillance Database 2024). As described in the Supplementary Methods, the datapoints for 1990, 1992 and 1998-2002 (given in italics) are based on reported proportions and conservative estimates of the original sample size, in the absence of definitive information on sample size.

Year	**Number HIV-1 positive**	**Total number tested**
1990	*8*	*500*
1991	65	2957
1992	*48*	*1000*
1993	125	1338
1994	235	1738
1995	283	1552
1996	316	1601
1997	497	1846
1998	*325*	*1000*
1999	*325*	*1000*
2000	*362*	*1000*
2001	*335*	*1000*
2002	365	1000
2003	1277	3406
2004	1433	3522
2005	1369	3500
2006	2664	6814
2007	2588	6920
2008	2703	6985
2009	2665	6744
2010	2720	6887
2011	2511	6714
2012	2614	6990
2013	2783	6940
2015	3028	6819
2017	3387	8242
2019	3448	8430
2022	3423	9201

Table S7: HLA typing, plasma viral load, and CD4 count data per cohort.

	Number of biological replicates (n)		
	HLA typed	pVL	CD4 count (cells/ul)
Antenatal cohort from pre-ART era (2000-2005)			
Nontransmitter mothers	559	559	553
Transmitter mothers	112	103	109
Uninfected cohort from 2000	110		
Antenatal cohort from ART era (2015-2025)			
Nontransmitter mothers		117	
Transmitter mothers	317	324	324
Uninfected cohort from 2015-2025	524		

Supporting information for Chapter 5:

Table S8: HLA class I types of FRESH cohort single-cell RNA seq samples.

PID	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2
1671-1250	02:01	30:01	15:03	58:01	03:02	18
1695-1277	29:02	66:01	42:02	58:01	03:02	17:01
1748-1318	02:05	30:02	07:02	58:01	04:01	07:02
1756-1332	02:05	02:05	58:01	58:01	07:01	07:01
1511-1126	30:01	43:01	15:03	58:02	02:10	06:02
1767-1337	30:01	30:04	42:01	58:02	06:02	17:01
1618-1216	02:05	43:01	15:10	58:02	06:02	08:04
1805-1364	66:01	66:01	58:02	58:02	06:02	06:02