THE ROLE OF NATURAL KILLER CELLS IN PREVENTING HIV-1 ACQUISITION AND CONTROLLING DISEASE PROGRESSION

Presented by

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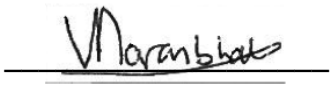
AUTHORS DECLARATION

I, the undersigned, hereby declare that the work contained in this document is of my own original work and that I have not previously, in its entirety or in part, submitted it at any university for a degree. The experiments in this thesis constitute work carried out by the candidate and comply with the stipulations set out for the degree of Doctor of Philosophy by research papers.



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ETHICS DECLARATION

The studies described in this thesis were approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal (B015/09 and B073/10).

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LIST OF FIGURES

LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cell-mediated Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
cART	Combination Antiretroviral Therapy
CD	Cluster of Differentiation
CMV	Cytomegalovirus
EBV	Epstein Barr Virus
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HSV-2	Herpes Simplex Virus Type 2
HTLV-III	Human T-cell Lymphotropic Virus III
IFN-γ	Interferon gamma
I-FABP	Intestinal fatty-acid binding protein
IL	Interleukin
KIR	Killer-cell Immunoglobulin-like Receptor
LAV	Lymphadenopathy associated virus
LILR	Leukocyte immunoglobulin-like receptors
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MICA	MHC polypeptide-related sequence A
MICA	MHC polypeptide-related sequence B
NCR	Natural cytotoxicity receptor
NK cell	Natural Killer cell
NKR	Natural Killer cell Receptor
NRTI	Nucleoside analogue Reverse Transcriptase Inhibitor
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PrEP	Pre Exposure Prophylaxis
SHIV-1	Simian/Human Immunodeficiency Virus

SIV	Simian Immunodeficiency Virus
TNF-α	Tumour Necrosis Factor alpha
TRAIL	Tumour necrosis factor-related apoptosis inducing ligand
ULBP	UL16 binding protein
VZV	Varicella Zoster Virus

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ABSTRACT

In sub-Saharan Africa, women carry a disproportionate burden of the Human Immunodeficiency Virus Type 1 (HIV-1) pandemic. The high risk of HIV acquisition in these women and the variability in their disease progression is not fully understood. Natural Killer (NK) cells, which are innate immune antiviral lymphocytes, present systemically and at mucosal surfaces, may play a role in preventing HIV acquisition and/or altering disease progression, as they are key early mediators of the response to viral infections and are equipped to kill infected cells.

The purpose of this study was to evaluate the role of NK cells in HIV-1 acquisition and following acquisition, in disease progression.

The study participants were selected women who were participating in a randomized controlled trial assessing the effectiveness of 1% Tenofovir gel in preventing HIV-1 (CAPRISA 004 trial). The study design was a case-control study nested within the cohorts followed up in the CAPRISA 004 trial. In this trial, 889 sexually-active women aged 18-40 years were randomized to receive Tenofovir or placebo gel and prospectively followed. Assessment of HIV infection was performed monthly by rapid HIV-1 antibody tests, supplemented by HIV-1 RNA polymerase chain reaction (PCR), p24 Western blotting and/or ELISA. Samples obtained prior to the first positive rapid antibody test were retrospectively tested by HIV specific PCR to identify window period infections. The date of infection in this study was estimated as the midpoint between the last negative and first positive antibody test, or 14 days prior to the first HIV-1 RNA-PCR positive result. Multi-parametric flow cytometry techniques developed and validated in healthy blood donors were used to asses the bidirectional relationship between NK cells and HIV-1. To simulate *in vivo* interaction between NK cells and autologous HIV infected cells, an *in vitro* infection and coculture assay was used in addition to conventional assays of NK cell recognition of HLA-deficient cell lines. These were supplemented with measurement of plasma cytokines by Luminex and microbial products by ELISA. In this study, 44 cases who acquired HIV-1 were

sampled prior to infection and 39 controls who remained HIV-1 negative despite high behavioural exposure at the timepoint when their preceding sexual activity was highest. To understand how HIV infection affected NK cells during early HIV-1 infection, the first sample obtained after acquisition was studied and compared to preinfection samples from the same participant. The case and control groups were broadly similar in the proportions using tenofovir gel, proportions infected with HSV-2 and number of sexual partners but tended to be marginally older than cases (27.6 vs 23.3 years). By design control women had higher sexual activity than cases (mean 11 vs. 5.7 sex acts per month).

The frequency of IFN-γ secreting NK cells from women who acquired HIV infection were significantly lower than from women who remained uninfected in response to 721 cells-an EBV transformed B cell line (background-adjusted median 13.7% vs. 21.6%; p=0.03) and to autologous HIV infected T-cells (background-adjusted median 0.53% vs. 2.09%; p=0.007). NK cells from HIV acquirers displayed impaired proliferation but enhanced spontaneous degranulation compared with non-acquirers after co-culture with HIV uninfected or infected autologous T-cell blasts. Adjusting for age, gel arm, HSV-2 infection status and levels of NK cell activation, IFN-γ+ NK cell responses to autologous HIV infected cells were associated with reduced odds of HIV acquisition (OR 0.582; 95% CI 0.35-0.98; p=0.04). In addition, even in the absence of *ex vivo* stimulation, HIV acquirers had higher levels of generalised innate immune activation measured by systemic cytokine concentrations (TNF-α, IL2, IL-7 and IL12p40), peripheral blood platelet concentrations (p=0.038), and non-specific *ex vivo* NK cell activation (p<0.001). Generalised NK cell activation measured directly *ex vivo* without stimulation was associated with acquisition. Further, if innate immune activation was assembled as a principal component in an unsupervised fashion but taking into account all the measures made, it was significantly associated with HIV acquisition (OR adjusted for age, tenofovir gel use, and HSV-2 status for PC with innate immune factor loadings 11.27; 95% CI 1.84-69.09; p=0.009). The causes of preinfection innate immune activation could not be established in this study but the degree of activation could not be

explained by microbial translocation as both HIV acquirers and non-acquirers had similar levels of plasma lipopolysaccharide (LPS), soluble CD14 (sCD14) and intestinal fatty-acid binding protein (I-FABP). Similarly, both HIV acquirers and non-acquirers had similar NK cell and cytokine responses to Toll-like Receptor (TLR)-2, 3 or 7/8 agonists 11. During early HIV-infection, NK cells demonstrated significantly higher activation ($p=0.03$), expression of Killer-cell immunoglobulin-like Receptors (KIR) ($p=0.006$) and expression of chemokine receptor 7 (CCR7, $p<0.0001$) compared with prior to acquisition. Although NK cells had reduced cytolytic potential following HIV acquisition, antiviral IFN-γ secretion appeared to be preserved. NK cell responses were not different between tenofovir and placebo gel recipients, but women who acquired HIV whilst using tenofovir gel had higher gag-specific IFN-γ CD4+ T-cell responses during early infection.

Overall, the findings suggest that the frequency of NK cells producing IFN-γ specifically after co-culture with HIV-1 infected target cells was associated with protection from HIV-1 acquisition but, generalised, non-specific activation of NK cells and other innate immune components enhanced HIV acquisition. Since neither microbial translocation nor TLR responsiveness were associated with pre-existing immune activation further studies will be required to identify the drivers of generalised innate immune activation. Methods to dampen generalised innate immune activation and/or augment specific NK cell antiviral responses in women at risk for HIV-1 may reduce HIV-1 acquisition.

During primary HIV-1 infection, NK cells underwent impairment of cytolytic function but not IFN-γ secretory function; this may affect their ability to affect disease progression. Although Tenofovir gel did not alter innate immune responses in women with breakthrough infection, it preserved HIV-specific T-cell immune responses, the consequences of which need further exploration. Understanding how Tenofovir gel mediated preservation of adaptive immune responses may lead to interventions that will reinforce protective host responses.

In conclusion, innate immune responses by NK cells have been shown to impact HIV acquisition; HIV-specific IFN-γ responses by NK cells were protective while generalised NK activation was detrimental. The causes of innate immune activation are not known but these effects were independent of the impact of Tenofovir gel. Future prevention strategies targeting mucosal transmission of HIV should assess their impact on NK cell responses, to avoid general innate immune activation and enhance their ability to protect against HIV acquisition.

CHAPTER ONE: INTRODUCTION, AIMS AND OBJECTIVES

1. 1 Introduction, Aims and Objectives

Heterosexual transmission is the primary mode of spread of HIV-1 in high-burden countries. However, the correlates of acquisition remain inadequately understood. Whilst some genetic, immunologic and behavioural factors have been identified (Miyazawa et al., 2009, Horton et al., 2010) to be associated with acquisition or protection, there are limited data on host-related biological correlates that are important for the development of targeted prophylactic interventions such as microbicides or vaccines. Likewise, whilst events during early infection have been shown to affect rates of progression to the Acquired Immunodeficiency Syndrome (AIDS), the reasons for the heterogeneity of HIV-1 control during early infection are inadequately understood. Moreover, most studies of HIV-1 acquisition and control have focused on adaptive immune responses, which are slow to develop, require antigen priming, are impaired by the targeting of adaptive immune cells by HIV-1, and generally appear to correlate with control of HIV-1 but not acquisition. In contrast, NK cells are the earliest antiviral cells to respond to HIV-1 and may have a role in HIV-1 prevention and disease progression.

The overall aim of this study was to determine whether, and how, NK cells may be involved in heterosexual acquisition and control of HIV-1 in high-risk women in KwaZulu-Natal, South Africa. The objectives were:

1. To develop and assess multiparametric flow cytometric and cell preparation methods for quantifying NK cell activation, chemokine receptor expression and function as well as high throughput genotyping for KIR and HLA typing.
2. To assess whether NK cells have primed anti-HIV-1 responses, and, if so, whether these have any impact on HIV-1 acquisition.
3. To assess the extent of non-specific NK cell activation and whether it has any role in HIV-1 acquisition.

4. To investigate whether microbial translocation or TLR responsiveness play a role in pre-infection innate immune activation.
5. To assess the effect of primary HIV-1 infection on NK cell activation and function.
6. To determine whether exposure to Tenofovir gel has any impact on innate and adaptive immune responses during early HIV-1 infection.

1.2 Thesis Framework

Chapter one (this chapter) describes the rationale of study, the aims, objectives and the structure of the thesis.

Chapter two is a literature review, which describes HIV-1, the burden and the problem, risk factors, course of infection and opportunities for protection/control. This is followed by an overview of NK cells, their functions, mechanisms of recognition of target cells and the pertinent literature regarding their involvement in HIV-1. A brief overview of the methods used to study NK cells and their limitations is provided, followed by a description of the role of immune activation in HIV-1 pathogenesis. This study is nested in a recent Tenofovir gel study (Abdool Karim et al., 2010), therefore the literature review encompasses background to the clincal study, and the literature indicating a possible immunomodulatory effect of Tenofovir.

Chapter three deals with the experimental methods established to measure key parameters of NK cell function, namely activation, ability to respond to chemokines by virture of appropriate receptor expression, antiviral cytokine secretion and degranulation. These experiments validated the methods used in the subsequent chapters and, importantly, established their limitations.

Chapter four documents the development a new method for genotyping two genes families that are important to NK cell function: Killer-cell Immunoglobulin-like Receptor (KIR) and Human Leukocyte Antigen (HLA). Although, the cohorts studies here were not powered to interrogate KIR/HLA

associations in HIV, these methods may prove useful for future studies of how KIR and HLA interactions are involved in HIV acquisition.

Chapter five describes an investigation into the role of antiviral NK cell reponses in women exposed to HIV-1, some of whom later acquired HIV-1. Previous studies have suggested a role for NK cells in preventing HIV acquisition but were limited by cohort, sample timing and use of models not directly translatable to HIV-1. Using a series of different assays, NK cell function was assayed prior to HIV acquisition and in women who did not acquire HIV. This chapter presents evidence for primed NK cell anti-HIV-1 responses being protective against HIV-1 acquisition and explores the implications of this finding for vaccine development.

Chapter six presented, evidence that non-specific innate immune activation is antecedent to and strongly associated with HIV-1 acquisition. In order to identify correlates of HIV acquisition, soluble and cellular mediators of immunity were examined prior to HIV acqusiition. The implications of this finding for development of the next generation of microbicide candidates is discussed.

Chapter seven reports results from studies to determine whether microbial translocation or differences in TLR responsivness may have acocunted for the innate immune activation reported in chapter six. Immune activation is a familiar pathophysiological feature of established HIV-1 infection. Therefore it was plausible that similar factors to those that drive activation during established infection may be operational in women at risk for, but not infected with HIV-1. Chapter six summarises findings that exclude microbial translocation and TLR responsiveness as major players in innate immune activation preceding HIV-1 acquisition.

Chapter eight details the complex impact of HIV on NK cells and T-cells. I compared NK cell (and some T-cell parameters) prior to and following HIV-1 acquisition. Although HIV is known to impair NK cell responses, these studies suggest that during early infection the impact is more nuanced than broad

scale impairment as some NK cell functions are lost whilst others relatively preserved.

Chapter nine reports findings of studies of women who acquired infection whilst using Tenofovir gel compared to those who had used placebo gel. Although no major effect of Tenofovir gel was noted in women at-risk for HIV-1 (mentioned in chapters five and six), in women who acquired HIV-1, Tenofovir gel use appeared to be associated with preservation of specific adaptive immune responses but had no effect on the innate responses measured.

Chapter ten presents, as the final chapter, the conclusions of this study, its limitations and implications for future research.

CHAPTER TWO: REVIEW OF THE LITERATURE

2.1 HIV-1

AIDS was first recognised as an immunodeficiency syndrome by the United States Centre for Disease Control and Prevention (US CDC) following reports of *Pneumocystis jiroveci* pneumonia and Kaposi's sarcoma amongst homosexual men and intravenous drug users (C.D.C., 1981a, C.D.C., 1981b). Although initially referred to as gay-related immunodeficiency disease (GRID) reports of cases in the general population resulted in the name change to AIDS in 1982 (Kher, 2003).

Human Immunodeficiency Virus Type 1 (HIV-1), a single-stranded, positive-sense, enveloped retrovirus of the lentivirus family, was initially identified independently by Barre-Sinoussi and colleagues (Barre-Sinoussi et al., 1983) and Gallo and colleagues (Gallo et al., 1983), as LAV (Lymphadenopathy associated virus) and HTLV-III (Human T-lymphotropic Virus III) respectively. By 1986 LAV and HTLV-III were renamed HIV-1 as it became clear that these were the same viruses and the cause of AIDS. Later, a second strain of HIV was isolated from a patient from West Africa (Clavel et al., 1986). The latter came to be known as HIV-2 and has been shown to have a less virulent course of infection in humans (Fauci and Clifford-Lane, 2012). Subsequent genetic and phylogenetic studies demonstrate that HIV-1 and HIV-2 are both zoonoses that originated from distinct simian immunodeficiency viruses (SIV), common amongst other primates. HIV-1 arose from a cross species transmission of an SIV virus (SIVcpz) present amongst *Pan troglodytes troglodytes,* the common African chimpanzee, through three independent trans-specific transmission events that led to strains HIV-1 M, N and O (Gao et al., 1999). Similarly, HIV-2 originated from an SIV virus (SIVsmm) present in *Cercocebus atys,* the African sooty mangabey (Hirsch et al., 1989). Although HIV-1 and HIV-2 share distant relatedness as shown in figure 1 below, the former is the major cause of the HIV-1 pandemic worldwide and HIV-2 is therefore not further discussed in this work.

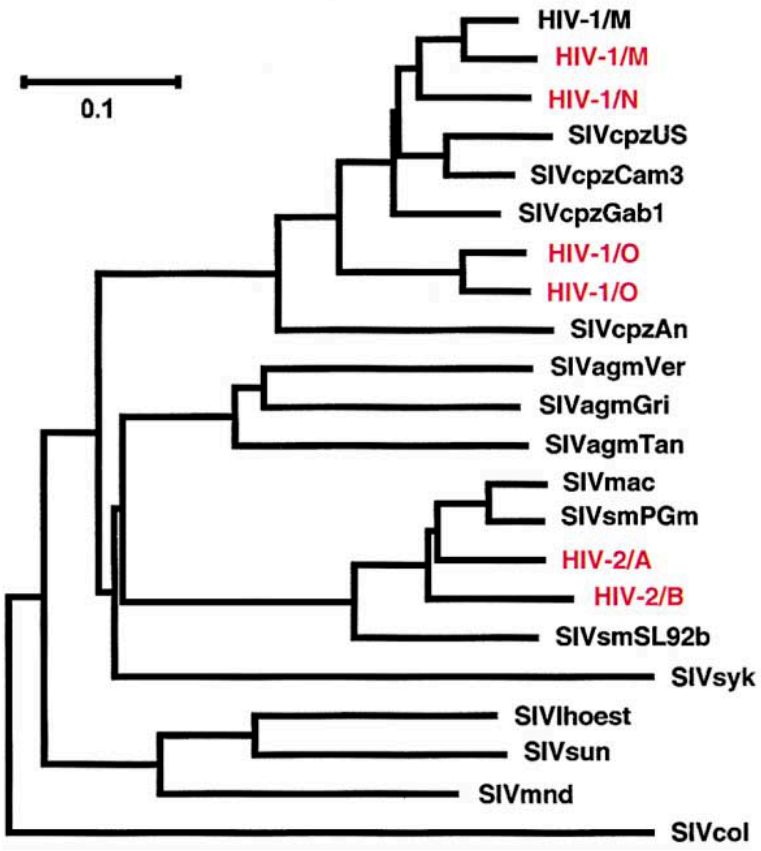

Figure 1 *The distinct but distantly shared origins of HIV-1 and HIV-2. Phylogenetic tree showing the distinct origins of HIV-1 and HIV-1-2 from SIVcpz and SIV smm respectively, as based on pol sequences from HIV-1, HIV-1-2 or SIV (strain origin denoted by suffix). Reproduced with permission from Elsevier (License no. 3036490007234) (Sharp, 2002).*

HIV-1 is transmitted through sexual contact and via bloodborne transmission between intravenous drug-users or recipients of contaminated blood-products. HIV-1 infects Langerhans cells, macrophages, dendritic cells and CD4+ cells, however, the primary target for reproduction appears to be CD4+ T-cells (Boggiano and Littman, 2007, Hladik et al., 2007). The immunopathology related to HIV-1 infection is: (i) the result of direct and indirect killing of immune cells (Alimonti et al., 2003); (ii) distortion of immune tissue architecture (van Grevenynghe et al., 2008); and (iii) the induction of dysfunctional immune responses. Classically, patients with established HIV-1 disease present with features of viral infection related to lymphoid activation-

such as lymphadenopathy, splenomegaly, and polyclonal gammopathy, and features of immunodeficiency namely opportunistic infections (Fauci and Clifford-Lane, 2012). Recently, the greater risk of metabolic (Norbiato, 2012), cardiovascular (Hsue et al., 2012) and malignant disease amongst patients with HIV-1 has been recognized and partially linked to aberrant immune activation (Hunt, 2012).

2.1.1 HIV-1, the burden and the problem

Infection with HIV-1 is the defining public health problem of our times. Nearly 60 million people have been infected - the majority -80%- by sexual acquisition, >25 million have died from HIV-1 and related diseases (UNAIDS, 2010). By the end of 2011 about 34 million adults and children (31.4-35.9 million) were living with HIV-1 globally (UNAIDS, 2012). Sixty-nine percent of people living with HIV-1/AIDS are residents of sub-Saharan Africa and 58% of them are women. Thus women in sub-Saharan Africa are disproportionately affected by the generalized epidemic. Epidemic trends are dynamic and while incidence rates overall have declined by 25% between 2001 and 2011 in sub-Saharan Africa, rates have increased in North Africa, Eastern Europe, central Asia and the Middle East as the contained epidemic becomes generalized in these regions (De Cock et al., 2012). There is therefore a continued need for prevention and treatment in both current high-burden countries and in regions with increasing incidence rates.

Understanding of the biology and correlates of heterosexual HIV-1 acquisition and control is key to the development of effective prophylactic interventions.

2.1.2 Risk factors for sexual HIV-1 acquisition and targeted prevention interventions

The risk of acquisition of HIV-1 is dependent on three primary factors: the probability and quantity of exposure to replication-competent virus, the frequency and type of exposure and host factors. Several parameters that

correlate with the probability of exposure have been demonstrated as epidemiologic risk factors for acquisition including the population prevalence of infection and the viral load and amount of virus shed in the transmitting partner (Quinn et al., 2000, Operskalski et al., 1997). Different frequencies and types of exposure are associated with distinct probabilities of transmission per-exposure with risk probabilities generally declining from receptive-anal > receptive-vaginal > insertive-anal/insertive-vaginal > receptive-oral intercourse (Boily et al., 2009). The overall per-act risk of transmission from a positive individual is estimated at about 0.124% (95% CI 0.078-0.199%) but rates are highly heterogenous across different demographic groups and study methodologies. Finally, a range of host factors have been reported be associated with acquisition risk and include the circumcision status of the male at-risk partner (Siegfried et al., 2005), presence of a sexually transmitted infection in the at-risk individual (especially HSV-2 and other genital ulcer diseases) and host genetic factors, in particular a CCR5Δ32 mutation that reduces CCR5 expression and reduces acquisition risk-the only widely accepted host genetic correlate for acquisition(Liu et al., 1996a). More than two-dozen additional (but unvalidated) factors have been suggested to modulate host risk for HIV-1 acquisition but these findings are often hampered by their discovery through comparison of HIV-1 exposed seronegative (HESN) individuals to individuals already infected or not at risk for HIV-1, limiting their generalizability (Miyazawa et al., 2009, Horton et al., 2010).

HIV acquisition prevention or intervention studies have been designed based on a wide variety of known HIV risk factors (Padian et al., 2011). These include interventions that tested behaviour change, financial incentives to enable prevention, treatment of STI's, use of female diaphragms, medical male-circumcision, antiretroviral treatment of positive partners, antiretroviral and non-antiretroviral microbicides, oral pre-exposure prophylaxis (PrEP) and vaccines. Overall, these intervention classes vary in effectiveness with discrepant results within each subclass of intervention. Nine of forty-five randomised trials of interventions with incidence outcomes had yielded evidence of effectiveness (Auvert et al., 2005, Bailey et al., 2007, Baird et al.,

2012, Cohen et al., 2011, Gray et al., 2007, Rerks-Ngarm et al., 2009, Grant et al., 2010, Grosskurth et al., 1995, Abdool Karim et al., 2010). Further delineation of validated risk factors will allow development of novel interventions.

2.1.3 The natural history of HIV-1 infection in humans and opportunities for protection/control

Animal and *in vitro* studies, indicate that HIV-1 traverses the mucosa via various mechanisms, and this is followed by local replication and expansion of a genetically restricted viral population in a small founder population of infected cells (Haase, 2010, Li et al., 2005). At this stage eradication of virus appears still be possible. Within about one week after infection the virus spreads to the draining lymph node and then to the broader circulation seeding lymphoid tissues richly endowed with HIV-1 target cells, causing massive CD4+ T-cell depletion and generating reservoirs of latently infected cells (Chun and Fauci, 2012). The detectable viraemia peaks within two to three weeks of acquisition as viral replication in lymphoid tissue explodes before it declines to a plateau known as the viral setpoint, a level that correlates with the rate of HIV-1 progression to AIDS (Gupta et al., 1995, Mellors et al., 1995). The median period between HIV-1 acquisition and progression to clinical features of AIDS is estimated at about 8-10 years in untreated adults (Fauci and Clifford-Lane, 2012). During this period patients are at substantially higher risk of opportunistic infection and AIDS-related malignancies. Onset of AIDS is heralded by further CD4+ T-cell lymphopaenia, increases in the level of HIV-1 viraemia and further increases in susceptibility to opportunistic infections and eventual death (Fauci and Clifford-Lane, 2012). These events are summarised in Figure 2.

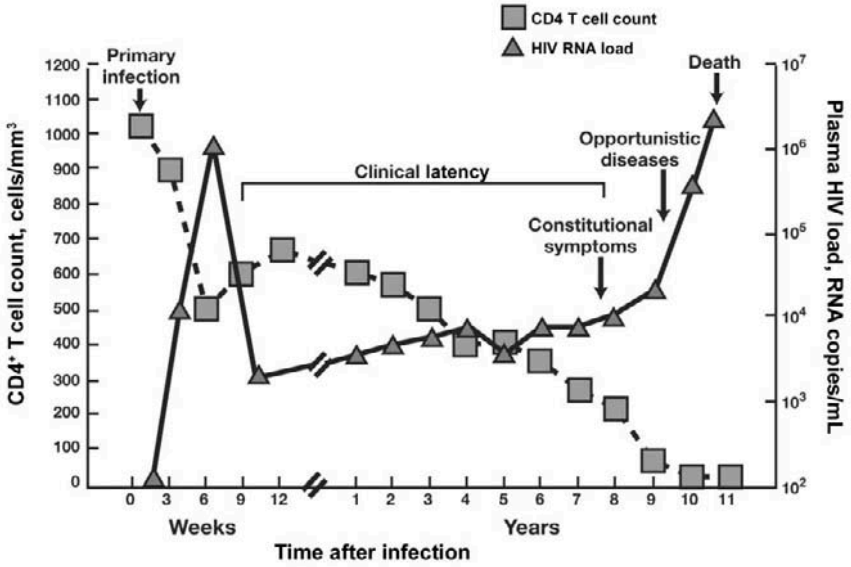

Figure 2: *The course of untreated HIV-1 infection in humans. The figure shows the CD4+ T-cell count (squares) and plasma HIV-1 RNA concentration (triangles) in relation to time in a typical individual. Reproduced with permission from Oxford University Press (License no. 3036520625885), (Fauci, 2007).*

Combination antiretroviral therapy (cART), a combination of at least three antiviral drugs that directly inhibit replication of HIV-1, has revolutionised the treatment of HIV-1 even in resource-limited settings (Ivers et al., 2005). Survival of patients with HIV-1 on cART now approaches that of the normal population (van Sighem et al., 2010, Walensky et al., 2006). Nevertheless, because of continued morbidity despite cART (Deeks, 2011), and the economic burden and side-effects of cART, methods to prevent HIV-1 and limit the need for cART are desirable. The critical periods for halting the establishment of infection or changing the course of infection are therefore during the first few days of infection or during early infection respectively(Haase, 2010).

Similar to other viral infections (Biron, 1999), HIV-1 infection leads to a characteristic pattern of immune responses with early increases in type-1 interferons and IL-15 followed by expansion of NK cells and the generation of Th1 cytokines that facilitate the development of the adaptive T-cell response

(Figure 3). The quality and quantity of antiviral cytotoxic (CD8+) and helper (CD4+) T-cell responses during early infection have been shown to correlate with the degree of viral control during infection (McMichael et al., 2010, Walker and Burton, 2008). Hence the primary approach to developing prophylactic interventions has been attempting to augment protective immune responses (McMichael et al., 2010). Whereas both CD8+ and CD4+ T-cell responses are acquired responses that take days to generate, the earliest antiviral cellular immune response is mediated by NK cells, innate immune cells equipped to kill virus infected cells (Alter and Altfeld, 2009).

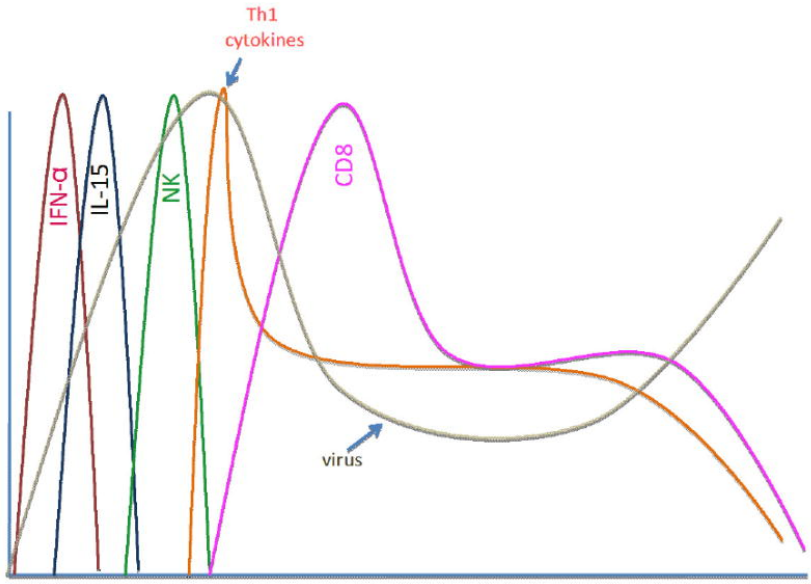

Figure 3 *Kinetics of the immune response to viral infections. Reproduced with permission from John Wiley and sons (License no. 3036530611015) (Alter and Altfeld, 2009).*

2.2 Natural Killer cells and HIV-1

2.2.1 Natural Killer cell function

Natural Killer cells were discovered by Jondal, Kiessling, Klein, Pross and Wigzell in mice and humans as a unique class of lymphocytes that displayed ‘natural killing’ of tumour cells without previous sensitisation to them (Kiessling et al., 1975, Pross and Jondal, 1975). By 1979, Timonen and colleagues had

demonstrated that the natural killing activity could be attributed to a class of large granular lymphocytes (Timonen et al., 1979). NK cells can be identified by the absence of T and B-cell markers on their surface (such as the cluster of differentiation (CD) markers CD3, CD4 and/or CD19), and the surface expression of the FcγRIIIa (CD16), Neural Cell Adhesion Molecule (NCAM, CD56), CD7 and CD335 (NKp46). They make up about 5-10% of circulating lymphocytes in uninfected individuals (Trinchieri, 1989) but contribute substantially to mucosal lymphocyte populations - up to 45% of duodenal epithelial lymphocytes (Eiras et al., 1998) and up to 75% of uterine decidual lymphocytes during pregnancy (Moffett and Loke, 2006). NK cells are part of the first line of defence against dysplasia and viral or other intracellular pathogens in humans and other jawed vertebrates. Although NK cells were classically thought not to undergo priming, recall responses mediated by NK cells have been demonstrated in mice (O'Leary et al., 2006, Sun et al., 2009) raising the possibility that similar 'NK cell memory' responses may be present in humans (Paust and von Andrian, 2011).

The role of NK cells in viral infection was highlighted in patients with NK cell deficiency who presented with increased susceptibility and severity of human herpesvirus infections(Biron et al., 1989, Orange and Ballas, 2006) including Epstein-barr virus (EBV) (Fleisher et al., 1982), Human papilloma virus (HPV) (Ballas et al., 1990), Varicella Zoster virus (VZV) (Etzioni et al., 2005) and Cytomegalovirus (CMV) ((Orange, 2006).

Unlike T-cells or B-cells which undergo genetic recombination of receptor encoding genes to enable antigen specificity, NK cells have germline-encoded receptors and are able to respond to viral infections within the first days of infection (Lodoen and Lanier, 2006). The antiviral effects of NK cells are mediated by direct killing (via granzyme or perforin mediated cytolysis) or by secretion of cytokines with antiviral properties (including IFN-γ, Tumour Necrosis Factor alpha (TNF-α), CCL3 and CCL5). The cytokine secretion also plays an important role in recruitment of additional immune effectors. In addition, NK cells induce apoptosis of target cells through expression of Fas ligand (FasL) and tumour necrosis factor-related apoptosis inducing ligand

(TRAIL) (Zamai et al., 1998). The cytolytic and cytokine secretory roles are performed by different NK cell subsets that partially overlap in function and appear to be different maturation stages: a cytolytic subset (CD56dim) and a cytokine secreting less mature subset (CD56hi)(Di Santo, 2006). NK cell function is enhanced by interferon-alpha (IFN-α), interferon beta (IFN-β), interleukin 2 (IL-2), IL-12 and IL-15 (Cooper et al., 2001).

2.2.2 Mechanisms of NK cell recognition of viral infected cells

NK cells constantly circulate and survey for possible target cells therefore a mechanism of differentiating healthy cells from dysplastic or virus-infected targets is critical. In 1986 Ljunggren and Kärre demonstrated that NK cell killing of tumour cell targets required the loss of Human Leukocyte Antigens (HLA) from the target cell surface (Ljunggren and Karre, 1985, Karre et al., 1986). This loss of HLA abrogated inhibition of NK cell responses allowing recognition of 'the missing-self' cell and activation of NK cells. This missing-self model for NK cell function holds that, activation and consequent antiviral activity occurs, when abrogation of inhibitory and promotion of activating signals transduced by NK cell receptors (NKR) occurs (Karre, 2008).

2.2.2.1 The role of NK cell receptors in virus-infected cell recognition and in HIV-1 infection

Natural Killer-cell Receptors (NKRs) that mediate signalling include the killer-cell immunoglobulin-like receptors (KIR), Leukocyte immunoglobulin-like receptors (LILR), the C-type lectin receptors-NKG2A-F, and the natural cytotoxic receptors (NCRs) -NKp30, NKp44, NKp46 (Lanier, 1998). These receptors have differing ligands (Figure 4). Recognition of infected cells by NK cells can therefore occur through modification of the receptor-ligand interactions. For example, the activating C-type lectin receptor NKG2D is the receptor for the stress-induced ligands UL16 binding protein (ULBP), MHC polypeptide-related sequence A (MICA) and MICB. Therefore NK cells may recognise cells undergoing stress by their expression of ULBP, MICA or MICB. Likewise NKp30, NKp44 and NKp46 recognise different heparin

sulphate/heparin sequences (Hecht et al., 2009) and NKp46 has been shown to directly recognize cells infected with influenza or parainfluenza virus (Mandelboim et al., 2001). The dominant regulators of NK cell recognition of virus-infected cells are thought to be KIR because these are the natural receptors for HLA (Rajalingam, 2012). In the course of autoimmune, malignant and viral disease, loss of HLA expression has been widely reported (Klein and Sato, 2000). Moreover, some viruses have evolved specific mechanisms to reduce HLA expression as a means of evading adaptive immune responses. For example, HIV-1 Nef protein induces endocytosis of HLA as a mechanism for host evasion (Schwartz et al., 1996).

The lectin-like receptors and the natural cytotoxic receptors occur in all humans with little or no sequence variation, but the KIR family of receptors is encoded for by a diverse region on human chromosome 19q13.4 (Parham, 2005). There is tremendous haplotypic, allelic and protein expression variation at KIR loci with more than 1000 alleles described for 16 KIR genes and giving rise to many different specific haplotypes. Humans inherit KIR genes in one of two broad haplotypes (haplotype A or B) that vary in the relative gene content and specificity of activating/inhibitory KIR. The folded KIR protein consist of either two or three immunoglobulin-like extracellular (EC) domains (3D or 2D respectively), a transmembrane region, and either a long (denoted L) or short (denoted S) intra-cytoplasmic tail that signals via immune-tyrosine activating or inhibitory motifs (ITAM or ITIM's respectively) and additional adaptor molecules. As shown in Figure 4, KIRs have distinct specificity for HLA ligands.

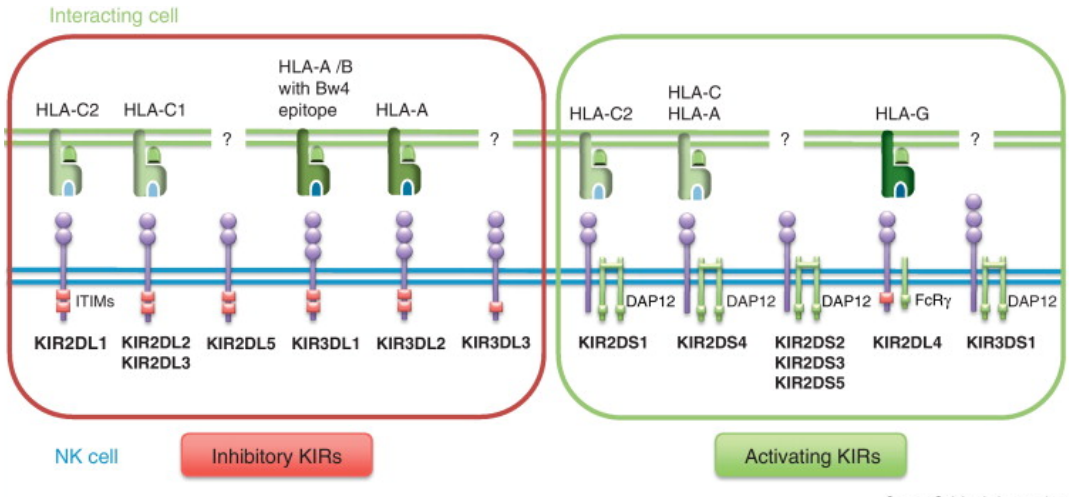

Figure 4. *Inhibitory (left) and activating (right) KIR on NK cells interact with specific HLA ligands on a potential target cell (top). Above each receptor, the adaptor molecule involved in signalling is mentioned. ITIM immunotyrosine inhibitory motif, ITAM immunotyrosine activating motif. Reproduced with permission from Elsevier (Licence no. 3036640145821) (Thielens et al., 2012).*

HLA ligands for some KIR receptors have been described, but the precise ligands for many remain unknown (Khakoo and Carrington, 2006). Studies have also indicated a strong role for peptides in modulating KIR/HLA recognition (Hansasuta et al., 2004, Stewart et al., 2005, Stewart-Jones et al., 2005). Further, the exact way in which the variety of KIR expressed on NK cells interact with the high density of HLA molecules presenting host or pathogen-derived peptides remains to be fully elucidated. Given the significant variation in NK cell receptors, particular KIR, and the unique biology NK cell receptors have been actively studied for their possible involvement in acquisition and course of human disease.

Specific KIR-HLA relationships have been described in a variety of viral, autoimmune and malignant diseases (Jamil and Khakoo, 2011, Khakoo and Carrington, 2006). In the context of HIV-1, Martin et al., (Martin et al., 2002) demonstrated in a large population immunogenetic study in Caucasians infected with HIV-1, that individuals who carry genes to encode for KIR3DS1 and its ligand (HLA-Bw4 80Ile) have a markedly slower progression to

AIDS/death. Qi *et al.* further studied *in vivo* effects of this relationship demonstrating early containment of HIV-1 viral load and protection against late opportunistic infection in KIR3DS1/HLA-Bw4 80Ile encoding patients (Qi et al., 2006). It is noteworthy that the frequency of KIR3DS1, an activating KIR, is very low in Africa (Norman et al., 2007). Other studies have demonstrated a number of immunogenetic, and functional correlates of control in relation to KIR3DS1 (Alter et al., 2007a, Alter et al., 2009), however other KIRs may be operational. The strongest evidence for this comes from work by Alter and colleagues (Alter et al., 2011) in which they demonstrated KIR-associated amino-acid polymorphisms in the sequence of HIV-1 from chronically infected individuals. These results indicate that KIR expressing cells exert immunological pressure on viruses forcing evolution and selection of virus subpopulations with escape mutations. KIRs are expressed on some T-cell subsets as well as NK cells. But their dominant role is presumed to be on NK cells. Figure 5 summarises how NK cells may recognise HIV-1 infected cells through change in interactions between NK cell receptors and their ligands, and the consequences this may have on enabling NK cell cytolytic or cytokine responses.

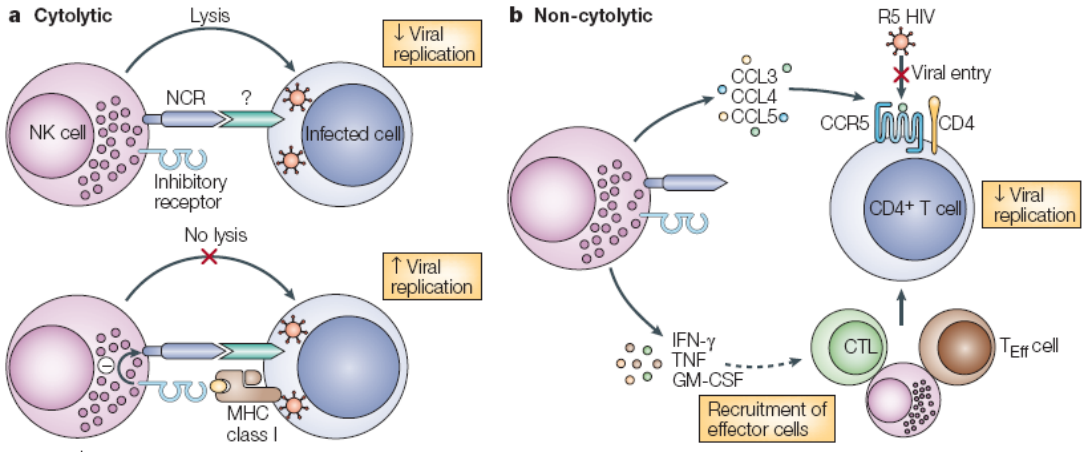

Figure 5 *Consequences of NK cells recognition virus-infected cells through receptor-ligand interactions for HIV-1 replication. Loss of inhibitory receptor binding to cognate HLA ligands (left lower panel) allow NK cells become activated. Activated NK cells may reduce viral replication through three mechanisms: lysis of target cells (top left), production of cytokines that inhibit viral entry (top right) or recruitment of additional effector cells (bottom right). CCR5, CC-chemokine receptor 5; CTL, cytotoxic T lymphocyte; NCR, natural cytotoxicity receptor; R5 HIV-1, CCR5-tropic HIV-1; TEff cell, effector T cell. Reproduced with permission form Nature Publishing Group (License no. 500735895). (Fauci et al., 2005)*

NK cells may also recognise virus-infected cells indirectly. Since NK cells are equipped to recognise Immunoglublin G (IgG) through expression of receptors for the Fc region ($Fc_{\gamma}RIIIa$), they are able to bind to cells that may have been recognised by IgG directed against viral antigens. This process, known as antibody-dependent cell-mediated cytotoxicity (ADCC) is primarily mediated by NK cells. Moreover, it has been shown that ADCC responses may direct peptide-specific NK cell activities (Chung et al., 2011, Stratov et al., 2008). Other groups have demonstrated that peptide-specific NK cell responses may be of clinical value as they are associated with lower viral loads, higher CD4+ T-cell counts and stronger CD4+ T-cell anti-HIV-1 responses (Tiemessen et al., 2010, Thobakgale et al., 2012). Activating KIR and HLA-C1 alleles have been associated with NK cell anti-viral peptide-specific HIV-1 responses (Tiemessen et al., 2011).

2.2.3 The bi-directional relationship between NK cells and HIV-1

Natural Killer cells are equipped to directly recognise and kill virus-infected cells and guide the development of adaptive immunity by interacting with dendritic cells and macrophages in the regional lymph node, and at mucosal sites (Colonna, 2009). Given that NK cells are amongst the earliest cells to respond in HIV-1 infection, and that they are the dominant cytolytic force in acute infection- before the development of acquired T-cell/B-cell immunity three hypotheses of how NK cells impact on HIV-1 have been widely evaluated: i) NK cells may be important in preventing HIV-1 infection; ii) NK cells may be important in the early control of HIV-1 infection (mentioned above); and iii) NK cells may be important in modifying disease progression. However, HIV-1 infection is known to cause systemic immunopathology, and data on whether NK cells can be infected by HIV-1 are controversial (Milush et al., 2009, Valentin et al., 2002). It is plausible that NK cells, like other lymphocytes they may succumb to bystander activation, senescence or apoptosis. Therefore, the impact of HIV-1 on NK cells has also been of considerable interest to the field (Fauci et al., 2005).

2.2.3.1 The role of NK cells in acquisition of HIV-1 in adults

Studies of resistance to infection have historically been limited by an immunogenetic approach, small sample sizes and limited measures of specific anti-HIV-1 NK cell function, but have given strong rationale for studying the functional changes of NK cells directly *ex-vivo*. Scott-Algara and colleagues (Scott-Algara et al., 2003) reported that 27 Vietnamese intravenous-drug users (IVDU) who were exposed to HIV-1, but appeared to resist infection, expressed a repertoire of predominantly activating NKR. The same group in 2007 (Ravet et al., 2007) observed a greater ratio of activating NKR to inhibitory NKR transcripts (*KIR3DL1*: *KIR3DS1* and *NKG2C*: *NKG2A*) amongst 25 exposed uninfected intravenous drug users compared with IVDU and/or healthy controls. In exploring the functional consequence of this observation, they found enhanced constitutive degranulation activity. The

immunogenetic data first described by Scott-Algara are consistent with later findings in other populations (Boulet et al., 2008, Jennes et al., 2006b). Jennes and colleagues found that the frequency of activating KIR was significantly greater amongst 41 HIV-1 exposed uninfected versus HIV-1 infected African sex workers. Boulet et al. (2008) studied 80 HIV-1 exposed uninfected Canadian adults and found that the frequency of *KIR3DS1* homozygous individuals was greater amongst these individual than a comparison group of participants infected with HIV-1. Montoya and colleagues (Montoya et al., 2006) demonstrated increased Interferon-γ secretory function of NK cells following non-specific activation from 30 sexually exposed uninfected Colombian adults but were unable to define what role activating NKR repertoire had in this and whether this affected specific anti-HIV-1 activity. *In vitro* studies of NK cells derived from HIV-1 negative individuals that expressed activating KIR (*KIR3DS1*) in the presence of HIV-1 infected target cells expressing cognate HLA ligand (HLA Bw4 80Ile) were shown to have enhanced NK cell mediated killing of HIV-1-infected target cells (Alter et al., 2007a). In addition, specific KIR-HLA combinations have been suggested to mediate protection from mother-to-child transmission of HIV-1 (Paximadis et al., 2011), but whether similar mechanisms may operate in adults are not well defined.

While the above studies have offered insight into the role of specific activating receptors, they are limited in that they did not assess HIV-1-specific NK cell function nor is the ideal comparison group included (individuals who acquire HIV-1). Thus, the correlates of protection on NK cells and their mechanisms of infected-cell recognition and eradication remain unclear. Therefore, the systematic characterization of NK cells (frequency, receptor expression, activation, and specific anti-HIV-1 activity) from women exposed to HIV-1 but who remained uninfected or later acquired HIV-1 could reveal important insight into the role of the NK cells in preventing HIV-1 acquisition.

2.2.3.2 The role of NK cells in early viral control

Few studies exploring the role of NK cells in early viral containment have been reported. Alter and colleagues (Alter et al., 2007b) described the changes in NK cell populations in 29 acutely infected individuals. They found that expansion of NK cell populations preceded the development of CD8+ T-cell immunity, and showed that the expansion correlated with viral replication, was limited by HAART-induced suppression of viral replication and rebounded on cessation of HAART. Given the strong immunogenetic data supporting activating NKR activity in chronic HIV-1 infection, and the evidence supporting their role in resistance to infection, it is plausible that enhanced NK cell activation in acute infection modulated by NKR may occur before the development of adaptive responses and can lead to early viral control and lower viral load set points. Initial viral control is known to predict the course of HIV-1 disease.

In non-human primate models of acute/early SIV infection, NK cell activity in sooty mangabeys and rhesus macaques correlates with lower viral set points (Pereira et al., 2008) and participate in an early innate response to infection (Giavedoni et al., 2000).

2.2.3.3 The deleterious effect of HIV-1 on NK cells

The interplay between NK cells and HIV-1 is bidirectional in that while NK cells likely affect HIV-1 disease, HIV-1 has been shown to result in expansion of anergic NK cells, incomplete activation and reduced cytolytic function (Fogli et al., 2004, Mavilio et al., 2006, Mavilio et al., 2005). Transient expansion of NK cell subsets occurs during acute infection, followed by declines in cytotoxic subsets and expansion of anergic subsets, a pattern that can be partially reversed by suppressing viral replication (Alter et al., 2005, Alter et al., 2009). Some functions of NK cells are preserved through chronic infection, though whether their specific anti-HIV-1 activity is preserved is less clear (Fogli et al., 2008). Taken together, this suggests that expansion of anergic

NK cells arise during HIV-1 infection and are linked to impairments of NK cell function. Understanding the kinetics of NK cell dysfunction in HIV-1 and relationship to other parts of the immune system is limited.

2.2.3.5 Natural Killer cells are a component of a broader immune system

Natural Killer cells play a key role in the cooperation of the broader immunological system. Through direct cell-to-cell contact and via secreted soluble factors NK cells mediate the maturation and function of dendritic cells (Fernandez et al., 1999) and thereby also affect the development of antigen presentation by DCs and the consequent adaptive immune response (Della Chiesa et al., 2005). Moreover, as producers of cytokines NK cells mediate recruitment and activation of other innate and adaptive immune cells including monocytes and CD4+ and CD8+ T-cells. Natural Killer cells play a central role in initiating, supporting or quelling immune responses therefore their activity in relation to other cells in individuals at risk for HIV-1 is of crucial importance. HIV-1 infection has been showed to partially impair this crosstalk (Mavilio et al., 2006).

2. 3 Methods for the study of Natural Killer cells

2.3.1 Blood processing

The study of NK cells in humans requires robust methods to identify and where necessary isolate them from blood. Flow cytometric methods developed by Fulwyler and Herzenberg (Fulwyler, 1965, Julius et al., 1972), including the use of fluorophore-conjugated monoclonal antibodies allows identification of NK cells based on their expression of surface markers such as CD7, CD16, CD56, CD335. Although these methods can be applied to whole blood, because of the relative ease of cryopreservation of peripheral blood mononuclear cell, the latter are the preferred starting material in clinical studies of lymphocytes. A widely-used method for isolating peripheral blood mononuclear cells (PBMC) is by the depletion of erythrocytes and granulocytes by the centrifugation of whole blood mixed with a sodium

diatrizoate/polysaccharide mixture with a density of 1.77g/ml to create a gradient that causes roleaux formation thus increasing the density of erythrocytes (Thorsby and Bratlie, 1970, Boyum, 1968a, Boyum, 1968c, Boyum, 1968b). Density gradient centrifugation has been reported to markedly affect T-cell, monocyte and dendritic cell measures (Berhanu et al., 2003, Ekong et al., 1993, Tamul et al., 1995), but its effects on NK cells is uncertain. An alternative approach to applying density gradient centrifugation techniques to isolate PBMC for cryopreservation is to use fresh whole blood. But in the context of a clinical trial, delays in processing blood may also have introduced artefacts of processing as has been reported for other cell types (Betensky et al., 2000, McKenna et al., 2009, Meier et al., 2008, Nicholson et al., 1984). Isolation methods therefore required validation in order to understand the limitations of the studies reported here.

2.3.2 Functional measurement

Since their description as cells able to kill tumour cells, the gold standard for assessing cytolytic NK cell function has been the Cr_{51} release assay (Pross and Jondal, 1975). In this method, target cells are labelled with Cr_{51} and co-incubated with NK cells. Killing is inferred from measurement of radioactive Cr_{51} in the supernatant of the co-culture. The technical demands of the assay, in particular the need for radioactive isotopes and detection equipment, limit its application more widely, and for this reason several flow cytometric assays to infer degranulation capacity were developed between 1986 and 1994 (Chang et al., 1993, Hatam et al., 1994, Kane et al., 1996, Zarcone et al., 1986). Since the degranulation event results in externalisation of granule contents including perforin, granzyme A and granzyme B, direct intracellular staining of granule contents continues to be an alternative method (Li, 2010). More recently, Alter and colleagues demonstrated that surface expression of lysosomal-associated membrane protein 1 (LAMP-1 or CD107a) was a reliable surrogate marker for degranulation of NK cells (Alter et al., 2004, Li, 2010).

The target cell used in the original and more recently developed assays though warrant attention. In the classic chromium release assays, the preferred target cells were P815 mouse mastocytoma cells and later K562 cells-a human cell line developed from an adult female with chronic myelogenous leukaemia (Lozzio and Lozzio, 1975, Lozzio and Lozzio, 1979), or 721.221 cells-a human EBV transformed lymphoblastoid cell line that was originally reported to be deficient in HLA class I (Kavathas et al., 1980). Although NK cells recognise and respond vigorously to these various target cells, each assay has drawbacks. For example, K562 cells are HLA expressing cells that present peptides derived from the mutated bcr-abl protein that is partly responsible for its phenotype (Clark et al., 2001), and 721 cells retain some class I HLA expression. Further, NK cell responsiveness to cells deficient in HLA may plausibly differ from response to autologous virus-infected cells which may have more subtle alteration of HLA expression.

For these reasons the work described here used flow cytometric detection of CD107a on NK cells as a surrogate marker of NK cell degranulation. To simulate *in vivo* interactions between various cell populations and autologous virus-infected cells an assay using mixed peripheral blood mononuclear cells cultured together with autologous virus-infected cells was developed for the work described here and used alongside classical responses to 721.221 cells.

2.3.3 Immunogenetic approaches

Both the *KIR* and *HLA* coding regions are highly polymorphic. HLA typing is a well-defined clinical assay that originated with serological assays and can also be conducted by sequence-specific oligonucleotide polymorphism polymerase chain reaction (SSOP-PCR), sequence-specific primer PCR (SSP-PCR), sequence based typing (SBT) or newer high throughput sequencing methods (Hurley, 2008). Similarly SSP-PCR, SSOP-PCR and SBT assays are used in KIR genotyping (Lebedeva et al., 2007, Nong et al., 2007, Gomez-Lozano and Vilches, 2002, Martin and Carrington, 2008, Single et al., 2008). These assays have drawbacks such as cost, labour and the need for large agarose gel electrophoresis equipment. Although higher throughput assays have been

reported (Sun et al., 2004, Houtchens et al., 2007, Kulkarni et al., 2010), large-scale genotyping of KIR and HLA pose a significant challenge. Further higher throughput assays are therefore desirable.

2.4 The role of immune activation in HIV-1 pathogenesis

Activation of the innate immune system accompanies recognition of pathogen or other threats and is necessary for the development of adaptive immune responses to viruses. Activation is mediated by a variety of pattern recognition receptors (PRRs) such as the Toll-like Receptor (TLR) family of receptors, TLR1-13 (Kawai and Akira, 2011). But non-specific chronic activation or excessively broad inflammatory responses come at the cost of immunopathology and exhaustion of adaptive immune cells (Figure 6) (Deeks, 2011). Non-specific activation of CD8+ T-cells has been demonstrated in many settings as a significant correlate of subsequent HIV disease course in adults (Giorgi et al., 1994, Giorgi et al., 1993, Liu et al., 1996b, Yagi et al., 1992, Hazenberg et al., 2003) and a driver of immunosenescence and premature age-related cardiovascular, liver, kidney, bone, and metabolic disease (Deeks, 2011). Further, in a cohort of women similar to that studied here, higher proinflammatory cytokine concentrations in the genital tract were associated with higher viral load setpoints (Roberts et al., 2012), lower CD4+ T-cell counts (Bebell et al., 2008), and accelerated disease progression (Roberts et al., 2010). The causes of immune activation during HIV-1 infection include the HIV-1 viral load, CMV viral load (Hunt, 2012) and loss of thymic regulatory function (Deeks, 2011), (Figure 6).

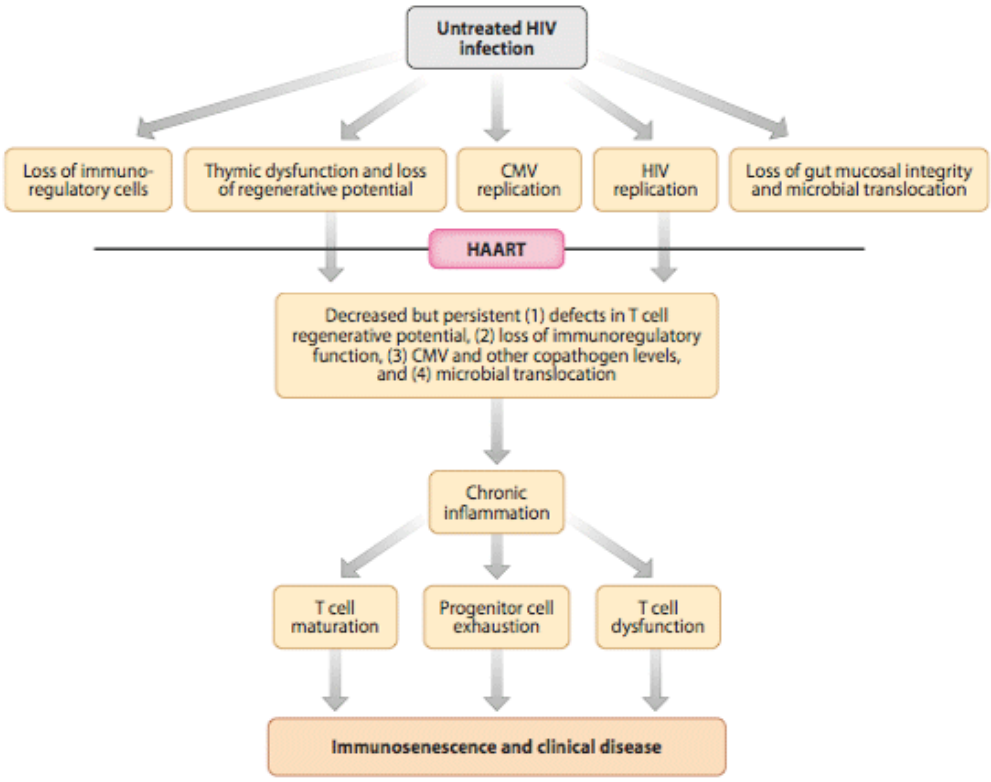

Figure 6 *The effects of untreated HIV-1 and subsequent HAART lead to immunosenescence and clinical disease. Reproduced from Annual Reviews. (Deeks, 2011).*

Studies have suggested that the translocation of enteric microbial products is a further driver of systemic immune activation following HIV-1 infection (Brenchley et al., 2006, Sandler and Douek, 2012). Whether these factors also mediate NK cell activation is less clear. A study of NK cells in patients with inflammatory bowel disease suggested that NK cells are not further activated *in vitro* by microbial products (Gregson et al., 2009). But due to the underlying genetic and immunologic basis for inflammatory bowel disease these results are not generalisable to NK cells in individuals at risk or infected with HIV-1. As a key part of the innate immune response, the role that NK cells play in immune activation is of relevance to the study of activation in the context of HIV-1 acquisition. Data are conflicting in this regard with some studies arguing that activation is protective and others suggesting that

quiescence is protective (Begaud et al., 2006, Jennes et al., 2006a, McLaren et al., 2010, Pancino et al., 2010) cf (Biasin et al., 2000, Jennes et al., 2003, Suy et al., 2007, Tomescu et al., 2011, Tran et al., 2006). These studies relied on comparisons between exposed uninfected individuals and either unexposed individuals or those with established HIV-1 infection. Hence, a key reason for lack of clarity in this respect is the difficulty in studying pre-infection samples from individuals who acquire HIV-1.

2.5 Tenofovir gel

2.5.1 Tenofovir gel for the prevention of HIV-1 acquisition in women

Tenofovir is a nucleotide-analogue reverse transcriptase inhibitor (NRTI) widely used for the treatment of HIV-1. Tenofovir was formulated as a gel for evaluation as a microbicide for prophylaxis from HIV-1 infection because of its favourable safety, tolerability and pharmacokinetic profile (Rohan et al., 2010, Tsai et al., 1995, Parikh et al., 2009), and encouraging preclinical and animal studies of the active metabolite (Mayer et al., 2006). The phase IIb double-blind randomised controlled clinical trial of tenofovir microbicide gel, from which samples used in this study were obtained, tested the safety and efficacy of 1% Tenofovir gel delivered intravaginally prior to and after coitus in woman at high risk of HIV-1 infection In South Africa (Abdool Karim et al., 2010). The trial demonstrated that 1% Tenofovir gel was both safe and effective at preventing HIV-1 acquisition, reducing incidence rates by 39% overall. Although encouraging, even amongst women with >80% adherence to gel use, effectiveness was only 54% indicating a need to identify alternative biologic mechanisms of infection for further development. Although the CAPRISA004 study demonstrated evidence of protection, the finding has not yet been replicated in other studies. The VOICE (Vaginal and oral interventions to control the epidemic) study was a placebo-controlled phase IIb study of daily vaginal tenofovir gel, oral tenofovir or truvada to prevent HIV that was conducted in South Africa, Uganda and Zimbabwe. The study was stopped for futility, plausibly because of low adherence to the study regimen (Marrazzo J., 2013). A larger efficacy trial of the vaginal gel, the Follow-on

African Consortium for Tenofovir Studies (FACTS) study is underway in South Africa. The CAPRISA004 study prospectively collected peripheral blood mononuclear cells, plasma and vaginal specimens and enrolled women who acquired HIV into continued follow-up. Therefore, the CAPRISA004 study in its design made this study possible.

2.5.2 Indirect and direct mechanisms of immunomodulation by Tenofovir gel

Tenofovir gel may plausibly limit viral replication in the mucosa. By modulating events during the mucosal replication stage of viral replication, a privileged situation may arise in which NK cell exposure to HIV-1 may occur in the absence of productive infection. Therefore, an opportunity arises for host mechanisms to clear infection before the virus can spread beyond the regional lymph nodes (Haase, 2010). Evidence for the ability of Tenofovir to create privileged exposures to non-productive HIV-1 by aborting infection at a mucosal replication stage is inferred from animal studies in which oral Tenofovir facilitated the induction of CD8+ T-cell immune responses despite lack of productive HIV-1 infection following mucosal exposure to Simian/Human Immunodeficiency Virus (SHIV-1) (Kersh et al., 2011). Therefore, it can be hypothesised that viral exposure, albeit transient may have been sufficient to prime immune responses, or to alter the kinetics of such responses.

In addition, *in vitro* and *in vivo* studies suggest that Tenofovir may have immunomodulatory activity on NK cells (Calio et al., 1994). These findings were not replicated, however, in a SIV-infection macaque model (Van Rompay et al., 2004).

CHAPTER THREE:

Impact of blood processing variations on Natural Killer cell frequency, activation status, chemokine receptor expression and function

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Research paper

Impact of blood processing variations on natural killer cell frequency, activation, chemokine receptor expression and function

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ABSTRACT

Understanding the role of natural killer (NK) cells in human disease pathogenesis is crucial and necessitates study of patient samples directly *ex vivo*. Manipulation of whole blood by density gradient centrifugation or delays in sample processing due to shipping, however, may lead to artifactual changes in immune response measures. Here, we assessed the impact of density gradient centrifugation and delayed processing of both whole blood and peripheral blood mononuclear cells (PBMC) at multiple timepoints (2–24 h) on flow cytometric measures of NK cell frequency, activation status, chemokine receptor expression, and effector functions. We found that density gradient centrifugation activated the NK cells and modified the chemokine receptor expression. Delays in processing beyond 8 h activated NK cells in PBMC but not in whole blood. Likewise, processing delays decreased chemokine receptor (CCR4 and CCR7) expression in both PBMC and whole blood. Finally, delays in processing PBMC were associated with a decreased ability of NK cells to degranulate (as measured by CD107a expression) or secrete cytokines (IFN-γ and TNF-α). In summary, our findings suggest that density gradient centrifugation and delayed processing of PBMC can alter measures of clinically relevant NK cell characteristics including effector functions; and therefore should be taken into account in designing clinical research studies.

1. Introduction

Quantitative measures of natural killer (NK) cell frequency, activation, trafficking potential and effector functions are important to consider in clinical research because these measures are associated with reduced disease pathogenesis of viral and neoplastic diseases (Orange and Ballas, 2006). Nonetheless, the study of human NK cells from diseased patients directly *ex vivo* has two important challenges. Firstly, in multicenter trials and developing country settings the time from venipuncture to sample processing may be prolonged due to long transport times to a central research laboratory. Secondly, there is little consensus on whether peripheral blood mononuclear cells (PBMC) and whole blood are equivalent sample types for studies of NK cells. These are important considerations in designing clinical studies because it is often not feasible to isolate PBMCs from whole blood in a timely manner. Thus, delays in processing and the type of starting sample may affect measurements of NK cell characteristics; however, their impact has not been well characterized.

Previous studies have shown that PBMC prepared by density gradient centrifugation may not be equivalent to

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whole blood for measuring some leukocyte parameters. Specifically, density gradient centrifugation altered the proportion of T-cells expressing cytokine receptors (varying by specific receptor), and increased the proportion of T-cells expressing adhesion molecules (Renzi and Ginns, 1987; Tamul et al., 1995; Lin et al., 2002; Berhanu et al., 2003). Likewise, delays in the time from venipuncture to sample processing altered the phenotype or functional responses of leukocytes. Ekong and colleagues found that delays in processing PBMC reduced T-cell frequencies (Ekong et al., 1993) and others have reported impaired T-cell responses as measured by cytokine secretion or proliferation (Betensky et al., 2000; Bull et al., 2007; Kierstead et al., 2007; McKenna et al., 2009; Weinberg et al., 2009). As a potential mechanism to explain these findings, McKenna and colleagues (McKenna et al., 2009) recently demonstrated that delayed processing of blood increased the frequency of activated CD11bpos and CD15pos granulocytes, and that these leukocytes inhibited T-cell responses. Furthermore, the induction of changes in activation and functional responses is not confined to T-cells. Delayed processing also reduced monocyte and dendritic cell responses to Toll like receptor ligands (Meier et al., 2008). Thus, density gradient centrifugation and delayed processing of blood or PBMC can affect important leukocyte parameters; however, their effects on NK cells have not been well described.

Here we report the impact of density gradient centrifugation and delayed processing on NK cell frequency, activation, chemokine receptor expression (as markers of trafficking potential) and effector function in whole blood and PBMC at multiple timepoints (2–24 h.). Our results suggest that while delayed sample processing does not affect NK cell frequencies, both delayed processing and density gradient centrifugation alter chemokine receptor expression and *in vitro* effector functions. It is therefore crucial to take these factors into account in designing clinical studies that measure innate immune responses.

2. Materials and methods

2.1. Study subjects

Blood samples from a total of 11 adult (20–31 years) females enrolled in an observational cohort of healthy women at Edendale Hospital (a district level hospital in Pietermaritzburg, KwaZulu Natal, South Africa) were included in these studies. Participants gave informed consent. The University of KwaZulu Natal Biomedical Research Ethics Committee (E118/06), the Edendale Hospital ethics committee and the Massachusetts General Hospital Internal Review Board approved this study.

2.2. Assessment of NK cell frequency, activation and chemokine receptor expression in whole blood and PBMC

A total of 34 ml of blood was drawn into four acid citrate dextrose (ACD) tubes (BD Biosciences), transported by vehicle at atmospheric temperature (23–25 °C) to the research laboratory in Durban, South Africa, and kept at ambient room temperature (20–23 °C) until being processed. All donors were bled within 15 min of each other and all samples reached the laboratory within two-hours of venipuncture. At 2, 8, 16 and 24 h after venipuncture, PBMC were prepared by density gradient centrifugation using Histopaque 1077 (Sigma, St Louis, MO) as per manufacturers protocol. Whole blood (processed at 2 and 24 h after venipuncture) and PBMC (processed at 2, 8,16 and 24 h after venipuncture) were stained using separate panels of antibodies to measure NK cell frequency, activation and chemokine receptor expression by multiparametric flow cytometry on an LSRII flow cytometer (BD Biosciences).

For whole blood staining, 300 μl whole blood was stained with three separate antibody cocktails for 20 min at 4 °C in the dark. Subsequently, red blood cells were lysed with 1 ml Versalyse Lysing Solution (Beckman Coulter, France) and the cells were concomitantly fixed with IOTest3 (Beckman Coulter, France), per manufacturers protocol for the concomitant 'Fix and Lyse' procedure.

For PBMC staining, two million PBMC per staining condition were washed and resuspended in 50 μl of calcium and magnesium free Dulbecco's phosphate buffered saline (DPBS) (Invitrogen). The cells were then stained for 20 min with LIVE/DEAD fixable aqua dead cell stain (Invitrogen) per manufacturers instructions. Next, the samples were washed, resuspended in DPBS, and stained with each of the three separate antibody cocktails for 20 min at 4 °C in the dark. The cells were then washed twice to remove excess antibody and fixed with 1% paraformaldehyde (Sigma, St Louis, MO) before being acquired on the LSRII.

2.2.1. Antibody cocktails for staining panels

All antibodies were purchased from BD Biosciences unless otherwise indicated. The antibody cocktails consisted of anti-CD3 Qdot-605 (Invitrogen) or anti-CD4 Qdot-605 (Invitrogen), anti-CD8 Qdot-655 (Invitrogen), anti-CD14 Pacific Orange (Invitrogen), anti-CD19 Pacific Orange (Invitrogen), anti-CD16 Pacific Blue (Invitrogen), anti-CD38 PE-Cy7 or anti-CCR7 PE-Cy7, anti-CD56 PE-Cy5, anti-CD69 FITC or anti-CCR4 FITC or anti-NKp46, anti-HLA-DR APC-H7, anti-CD69 APC or anti-CCR5 APC, anti-CD45 AlexaFluor 700 (Invitrogen)—for whole blood staining only or anti-CD3 AlexaFluor 700 (Invitrogen) conjugated antibodies. All antibodies were titrated to determine optimal saturating concentrations for staining.

2.3. Stimulation of PBMC for the assessment of NK cell degranulation and intracellular cytokine expression

One million PBMC were cultured in 1 ml of RPMI 1640 (supplemented with 10% fetal calf serum and 1% Penicillin/streptomycin), 0.5 μl Golgistop (BD Biosciences), 5 μg/ml brefaldin A (SIgma, St Louis, MO), and simultaneously stained with 10 μl anti-CD107a PE-Cy5 (BD Pharmingen). After 4 h, Phorbol-12-myristate-13-acetate (PMA) (Sigma, St Louis, MO) plus Ionomycin (Sigma, St Louis, MO) were added at 1 μg/ml and 0.5 μg/ml, respectively, and incubation continued further for 2 h at 37 °C, 5% CO_2. After a total of 6 h of incubation, cells were washed twice with DPBS and stained for 20 min with LIVE/DEAD fixable aqua dead cell stain (Invitrogen) per manufacturers instructions. After being washed twice, cells were surface stained for 20 min at 4 °C in the dark with anti-CD3 AlexaFluor 700 (Invitrogen), anti-CD14 Pacific Orange (Invitrogen), anti-CD16 Pacific Blue (Invitrogen) and anti-CD56 PE (BD Biosciences) conjugated antibodies. Next, the cells were washed with DPBS and fixed with 100 μl Fix&Perm Reagent A (Invitrogen) for 20 min at room temperature. Following

fixation, cells were washed and incubated in 100 μl Fix&Perm Reagent B (Invitrogen) containing anti-Interferon-γ PE-Cy7 and anti-tumour necrosis factor-α FITC conjugated antibodies, for 20 min further at 4 °C in the dark. Finally, cells were washed, resuspended in DPBS and 500,000–1,000,000 events were acquired on the LSRII. In addition, eight-peak mid-range beads (BD Biosciences) were used to standardize instrument settings on the LSRII between each run.

2.4. Analysis of flow cytometric data

The proportions of activated, chemokine receptor-expressing, degranulating, or cytokine secreting NK cells were analyzed using Flowjo (v9.0.1, Treestar). Phenotypically, NK cells were defined as aqua viability dye negative (live), CD3neg, CD14neg, CD19neg, CD16pos and/or CD56pos. In the gating strategy, gates were standardized using fluorescence minus one (FMO) controls. Time and singlet gates were included. We assessed both proportions of NK cells and levels of marker expression as measured by mean fluorescence intensity (MFI). All measures of frequency and activation were done in duplicate using independent flow cytometric panels. Means for duplicate measures have been indicated.

Data were summarized and statistical tests were performed in Graphpad Prism (v5, Graphpad Prism Inc.). Paired t-tests were applied to assess the statistical significance of differences between different timepoints, or between different sample-types from an individual. A repeated measures ANOVA was used to assess statistical significance of differences between several (>2) timepoints. To test for linear trend, a test for trend was calculated, and where relevant we report the p value testing for nonlinear variation after correcting for a linear trend. P-values were not corrected for comparisons that we defined *a priori*.

3. Results

Previous studies have shown that delays in blood sample processing or the process of isolating PBMC may alter phenotypes or functions of leukocytes (Renzi and Ginns, 1987; Tamul et al., 1995; Lin et al., 2002; Berhanu et al., 2003) (Betensky et al., 2000; Bull et al., 2007; Kierstead et al., 2007; McKenna et al., 2009; Weinberg et al., 2009). To quantify the impact of density gradient centrifugation and delayed processing on NK cells, we evaluated whole blood and PBMC samples from five healthy adults processed at different timepoints after venipuncture (Fig. 1A). These timepoints corresponded to the processing of the samples immediately on receipt (2 h), at the end of a routine working day (8 h), after an overnight rest (16 h), or one full day later (24 h). Using multiparameter flow cytometry, NK cells were defined based on the absence of T-cell, monocyte, or B-cell markers (CD3, CD14, and CD19, respectively), and the presence of CD16 and/or CD56 (Fig. 1B). In addition, non-viable cells and cell doublets were excluded from the analysis.

3.1. Frequencies of NK cells are not significantly affected by delays in blood sample processing

Following delays in the preparation of PBMC from whole blood at 2, 8, 16, and 24 h post-venipuncture, the frequencies of NK cells remained unchanged; however, there was a weak trend towards reduced frequencies of NK cells with time (repeated measure ANOVA $p = 0.147$, post test for trend $p = 0.031$, $r^2 = 0.1$) (Fig. 2A). Likewise, the frequencies of NK cells, as a proportion of all CD45pos cells in whole blood, were similar in blood processed at 2 h and 24 h post venipuncture (means of 10.3% and 9.1% respectively), (Fig. 2B). Whole blood was not assessed at 8 or 16 h post venipuncture due to limited sample availability. Overall, we found no change in NK cell frequencies over time in both whole blood and PBMC. Based on previous reports of lymphocyte alterations attributed to sample processing (Renzi and Ginns, 1987; Tamul et al., 1995; Lin et al., 2002; Berhanu et al., 2003), we next wanted to assess whether density gradient centrifugation affected the quality of the NK cells.

3.2. Density gradient centrifugation alters NK cell activation and the expression of chemokine receptors

Using multi-parametric flow cytometry, we measured the activation status of NK cells *ex vivo* and the expression of selected chemokine receptors in the samples processed from the same five donors used previously (Fig. 3). First, to assess NK cell activation we quantified the expression of CD38, CD69, HLA-DR and NKp46 on NK cells in whole blood vs. PBMC prepared 2 h after venipuncture (Fig. 3A). We chose these markers for two reasons. Firstly, on NK cells, the levels of CD69 and HLA-DR expression have previously been used as surrogates for acute and chronic activation, respectively (Ziegler et al., 1993; Fogli et al., 2004; Ravet et al., 2007). Secondly, we and other investigators have reported a loss of NKp46 expression on activated NK cells (Wong et al., 2010; Fogli et al., 2008); thus, the level of NKp46 expression may also serve as a surrogate measure of NK cell activation.

The proportions of NK cells expressing CD69, a marker of acute activation, roughly doubled from 0.36% in whole blood to 0.85%, following density gradient centrifugation ($p = 0.034$) (Fig. 3A). There was, however, no change in the proportions of NK cells expressing HLA-DR. This increase in CD69 expression was not associated with a significant decrease in the proportion of NKp46 expressing NK cells (Fig. 3A). In addition, we observed a modest, but statistically significant decrease in the proportion of NK cells expressing CD38 following density gradient centrifugation (69.78% in whole blood vs. 65.5% in PBMC, $p = 0.026$). These results suggest that NK cells may be acutely activated by density gradient centrifugation; however, the degree of this effect was small.

Unlike some activation markers, chemokine receptors undergo rapid internalization and endocytic recycling, and therefore, have high rates of turnover at the cell surface (Neel et al., 2005). We therefore predicted that the cell surface expression of chemokine receptors might be more susceptible to the effects of density gradient centrifugation than activation markers. To test this hypothesis we quantified the expression of two chemokine receptors on NK cells in whole blood vs. PBMC (Fig. 3B). We chose to specifically assess CCR4 and CCR7 since both are expressed on NK cells and have ligands that are critical for trafficking to the skin and lymph nodes, respectively (Berahovich et al., 2006). With density gradient centrifugation we found a substantial increase in the average proportion of CCR4-expressing NK cells (10.5% in whole blood vs. 50.9% in

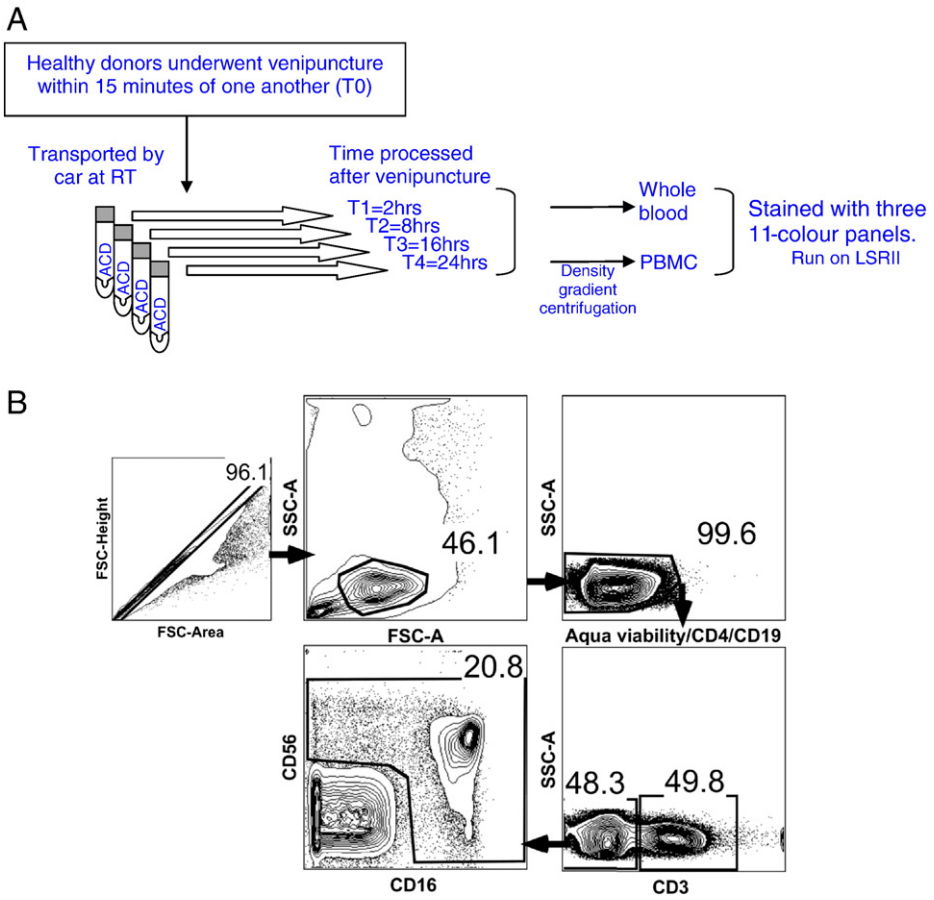

Fig. 1. Study workflow. (A) Sample acquisition and processing time delays: A total of 34 ml of blood per individual was collected from five healthy adult female volunteers. The blood samples were collected into ACD tubes and subsequently processed at 2, 8, 16 or 24 h after venipuncture. Peripheral blood mononuclear cells (PBMC) prepared by density gradient centrifugation and whole blood (WB) samples were surface stained with monoclonal antibodies, fixed and analyzed on an LSRII multiparameter flow-cytometer at the DDMRI, Durban, South Africa. (B) Gating strategy to define NK cell populations in PBMC (time, singlet, Live/CD14neg/CD19neg, CD3neg, CD56pos and/or CD16pos). For the analysis of whole blood, CD45 staining was used to identify lymphocytes (CD45pos).

PBMC, $p<0.001$) (Fig. 3B). However, we observed a tendency for proportionally fewer NK cells expressing CCR7 (37.5% in whole blood vs. 21.8% in PBMC) following density gradient centrifugation, but this difference was not statistically significant ($p=0.13$). Consistent with our findings of increased proportions of CCR4-expressing NK cells, we also observed a tendency towards increased surface expression of CCR4 following density gradient centrifugation (2.7 $\log_{10}$ MFI in whole blood vs. 3.24 $\log_{10}$ MFI in PBMC, $p=0.077$). Likewise, we observed a statistically significant reduction of CCR7 expression on NK cells following density gradient centrifugation (3.1 $\log_{10}$ MFI in whole blood vs. 2.61 $\log_{10}$ MFI in PBMC, $p=0.014$) (Supplementary Table 1). Taken together, these data suggest that density gradient centrifugation marginally alters the activation status of NK cells, but dramatically alters their expression of chemokine receptors such as CCR4.

3.3. Processing of blood beyond 8 h after venipuncture results in activation of NK cells and alteration of chemokine receptor expression

Based on our findings of changes in activation and chemokine receptor expression profiles following density gradient centrifugation, we next wanted to know how delays in processing, affected these parameters. Hence, we measured these parameters on NK cells in PBMC at 2, 8, 16 and 24 h after venipuncture and in whole blood at 2 and 24 h after venipuncture (Fig. 4).

Similar to our previous findings, we observed changes in the expression of activation markers and chemokine receptors. We found no significant change in the proportions of NK cells in PBMC expressing either CD38 or HLA-DR with delays in processing (Fig. 4A). However, we observed greater proportions of NK cells expressing CD69 ($p=0.027$) and lower proportions of NK cells expressing NKp46 in PBMC processed more than 8 h after venipuncture ($p<0.001$) (Fig. 4A). The magnitude of these effects was greater than the comparison between whole blood and PBMC. Consistent with these changes, we also noted an increase in the MFI of CD69 and a decrease in the MFI of NKp46 (Supplementary Table 1). Thus, the delays in processing activated NK cells in PBMC substantially reduced the proportions of NK cells expressing either CCR4 ($p<0.0001$) or CCR7 ($p=0.0008$) (Fig. 4B). However, these decreases were only noted in PBMC processed more than 8 h after venipuncture. Consistent with these findings, over time we also noted lower MFIs for both

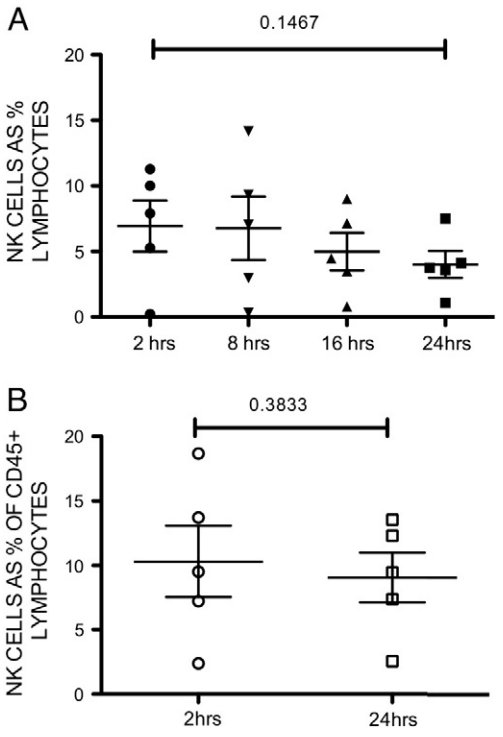

Fig. 2. Frequencies of NK cells are not affected by delays in blood sample processing (n = 5). A shows the frequency of NK cells as a proportion of lymphocytes in PBMC processed at 2, 8, 16 or 24 h after venipuncture. The indicated p-value represents the result of a paired t-test. B shows the frequency of NK cells as a proportion of CD45pos lymphocytes in whole blood processed at 2 or 24 h after venipuncture. The indicated p-value represents the result of repeated measures analysis of variance (ANOVA). A post-test for linear trend gives p = 0.031, r = 0.312. Data shown represent means of duplicate measures for each participant (n = 5). Open shapes (○▽△□) represent means of duplicate measures from whole blood, closed shapes represent means of duplicate measures from PBMC (■▼▲●); circles (● or ○), inverted triangles (▼ or ▽), upright triangles (▲ or △) and squares (■ or □) represent measures of NK cell frequency at 2, 8, 16 and 24 h respectively, and as indicated. Each data point represents one individual.

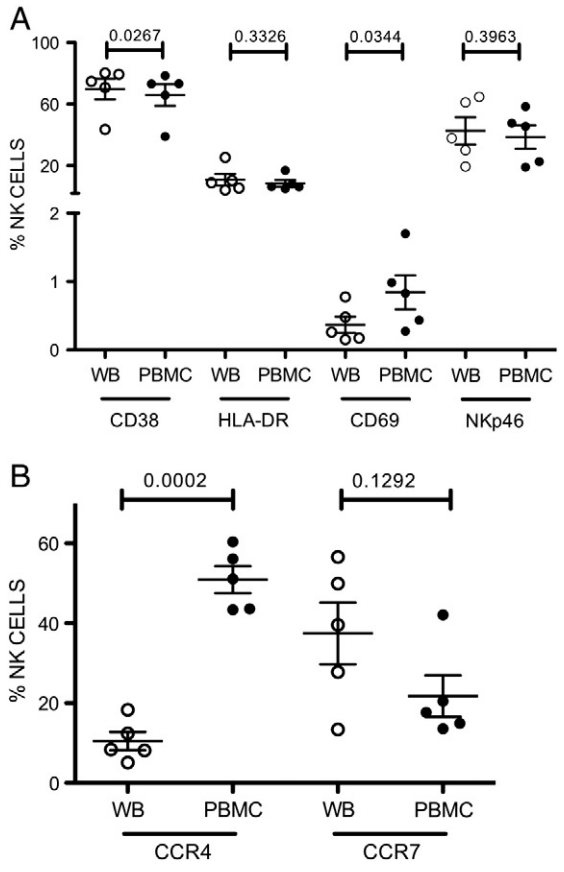

Fig. 3. Density gradient centrifugation alters NK cell activation and the expression of chemokine receptors (n = 5). A shows the proportion of NK cells expressing CD38, HLA-DR, CD69 or NKp46 in whole blood (WB) and PBMC processed 2 h after venipuncture. Fig. 1B shows the proportion of NK cells expressing CCR4 or CCR7 in WB and PBMC processed 2 h after venipuncture. The indicated p-values represent the results of paired t-tests. Data shown in A represent means of duplicate measures for each participant (n = 5). Open shapes (○▽△□) represent means of duplicate measures from whole blood, closed shapes represent means of duplicate measures from PBMC (■▼▲●); circles (● or ○), inverted triangles (▼ or ▽), upright triangles (▲ or △) and squares (■ or □) represent measures of NK cell frequency at 2, 8, 16 and 24 h respectively, and as indicated. Each data point represents one individual.

chemokine receptors (Supplementary Table 1). Finally, to address whether these effects were limited to NK cells we further analyzed measures of activation and chemokine receptor expression on T-cells in the same samples. Similar to our findings from NK cells, we observed a trend of increased activation for both CD4 and CD8 T-cells as measured by CD38 (Supplementary Fig. 1), We also observed proportionally fewer CCR4, CCR5 and CCR7-expressing T-cells over time (Supplementary Fig. 1). Thus the changes we observed were consistent for both T-cells and NK cells.

Likewise, in whole blood stained at 2 and 24 h after venipuncture, we observed no change in the proportions of NK cells expressing CD38, HLA-DR or CD69 (Fig. 4C). In contrast, we found a decrease in the proportion of NKp46-expressing NK cells (42.6% at 2 h vs. 28.06% at 24 h, p = 0.009) (Fig. 4C). These findings were consistent with decreased surface densities of these receptors as measured by MFI (Supplementary Table 1). Furthermore, we discovered a dramatic loss of CCR7 staining on NK cells in whole blood stained at 24 h after venipuncture (37.5% at 2 h vs. 0.25% at 24 h, p = 0.008), which was substantiated by a concomitant decline in the levels of CCR7 expression (Supplementary Table 1). In contrast, we found no difference in the proportions of CCR4-expressing NK cells (10.5% at 2 h vs. 6.8% at 24 h, p = 0.11) (Fig. 4D). Similarly, MFIs for CCR4 were only marginally lower at 24 h (2.70 MFI (log10) vs. 2.61 at 2 h. and 24 h., respectively, p = 0.04; Supplementary Table 1). Taken together, these data suggest that delayed processing of whole blood minimally impacted NK cell activation status, but profoundly diminished expression of some chemokine receptors. Notably, these effects were more apparent with PBMC than whole blood.

3.4. Delayed processing of blood decreases functional responses of NK cells following in vitro stimulation

The significant loss of NKp46 expression in PBMC over time suggested that delayed processing could alter functional

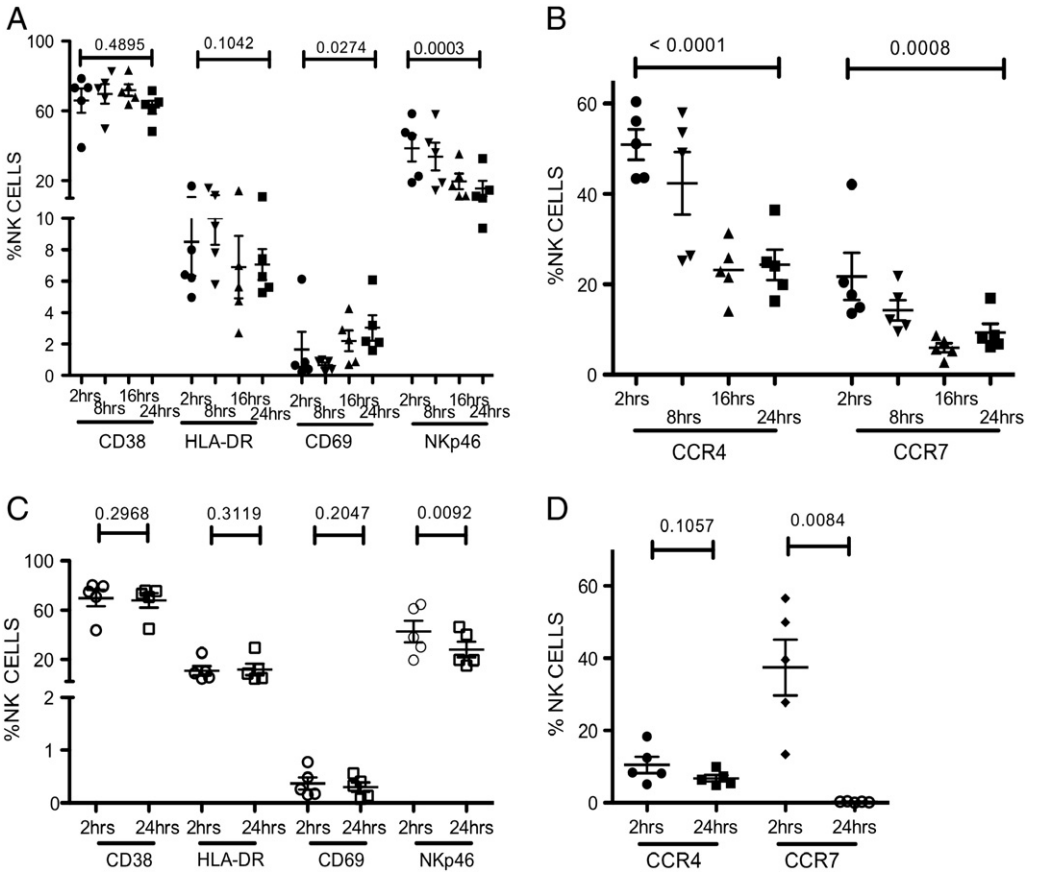

Fig. 4. Delayed processing of blood alters NK cell activation and the expression of chemokine receptors (n = 5). A shows the proportion of NK cells expressing CD38, HLA-DR, CD69 or NKp46 in PBMC processed after 2, 8, 16 or 24 h after venipuncture. B shows the proportion of NK cells expressing CCR4 or CCR7 in PBMC processed after 2, 8, 16 or 24 h after venipuncture. The indicted p-values in A and B represent the results of repeated measures analysis of variance (ANOVA). C shows the proportion of NK cells expressing CD38, HLA-DR, CD69 or NKp46 in whole blood (WB) at 2 or 24 h after venipuncture. D shows the proportion of NK cells expressing CCR4 or CCR7 in WB at 2 or 24 h after venipuncture. The indicated p-values in C and D represent the results of a paired t-test. Data shown in A and C represent means of duplicate measures for each participant (n = 5).Open shapes (○▽△□) represent means of duplicate measures from whole blood, closed shapes represent means of duplicate measures from PBMC (■▼▲●); circles (● or ○), inverted triangles (▼ or ▽), upright triangles (▲ or △) and squares (■ or □) represent measures of NK cell frequency at 2, 8, 16 and 24 h respectively, and as indicated. Each data point represents one individual.

responses. Previously, De Maria and colleagues demonstrated diminished functional responses by NK cells with lower NKp46 expression (De Maria et al., 2003). To assess whether the changes we observed were accompanied by alterations in NK cell effector functions, we measured the impact of delayed sample processing on degranulation and cytokine secretion following *in vitro* stimulation (Fig. 5). For these assays we selected 6 additional healthy donors. We used PMA plus Ionomycin to stimulate NK cells in PBMC prepared 2, 16 and 24 h after venipuncture (stimulation of PBMC prepared at 8 h was not performed due to insufficient sample availability). We quantified functional responses using flow cytometry by gating on NK cells expressing CD107a, a surrogate for degranulation (Alter et al., 2004), and interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α).

Following *in vitro* stimulation we observed a significant reduction in both NK cell degranulation and cytokine secretion with delays in processing that was most marked with delays of greater than 16 h (Fig. 5). The proportion of NK cells expressing CD107a correlated with the proportion expressing IFN-γ ($p = 0.002$, $r^2 = 0.59$), but not with the proportion expressing TNF-α. However, we noted a positive correlation between proportions of NK cells expressing IFN-γ and TNF-α ($p = 0.008$, $r^2 = 0.37$). These findings suggest that delays in the processing of PBMCs significantly impair NK cell cytokine secretion and degranulation.

4. Discussion

In clinical studies there are few standardized methods for assessing NK cell parameters, including their frequency, activation, chemokine receptor expression, or effector functions. Some investigators have reported marked differences in antiviral responses mediated by NK cells using fresh whole blood vs. PBMC prepared by density gradient centrifugation (Tiemessen et al., 2009). This finding along with previous reports of leukocyte alterations with density gradient centrifugation(Renzi and Ginns, 1987; Tamul et al., 1995; Lin et al., 2002; Berhanu et al., 2003) suggests that these factors are important to consider in designing studies using blood samples. Our work further extends these findings by quantifying the impact of sample type and processing delays

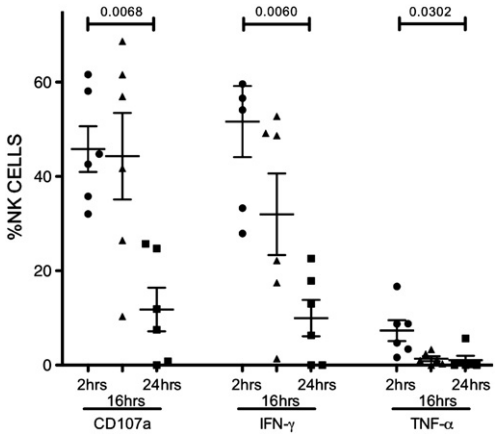

Fig. 5. Delayed processing of blood decreases functional responses by NK cells as measured by CD107a expression and cytokine secretion following *in vitro* stimulation (n = 6).Fig. 5 shows the proportion of NK cells expressing CD107a (a surrogate for degranulation), interferon-γ (IFN-γ) or Tumour Necrosis Factor-α (TNF-α) in response to PMA/Ionomycin stimulation ofPBMC processed at 2, 16 or 24 h after venipuncture. The indicted p-value represents the results of repeated measures analysis of variance (ANOVA) (n = 6). Circles, triangles and squares (■▲●) represent the NK cell parameter as denoted on the x-axis label, at 2, 16, and 24 h respectively, and as indicated. Each data point represents one individual.

on NK cells. For the first time we demonstrate that delays in processing alter chemokine receptor expression on NK cells and decrease NK cell functional responses.

Our overall aim was to assess the impact of density gradient centrifugation and delayed processing on NK cell frequency, activation, chemokine receptor expression and effector function. We found that NK cell frequencies in whole blood and PBMC were unaffected by the delays in the processing up to 24 h. This is not unexpected since NK cells have a half-life of about 7–10 days *in vivo* (Zhang et al., 2007) so they would not be predicted to die or proliferate dramatically over a 24-hour period *ex vivo*. We also found that density gradient centrifugation acutely activated NK cells; however the magnitude of this change was relatively small and most likely biologically insignificant. In comparison, much greater alterations in acute activation of NK cells occur following vaccination (Horowitz et al., 2010). Similarly, we found slight differences in CD38 expression due to sample processing although the function of CD38 on NK cells remains undetermined. By using consistent sample processing methods these differences may be taken into account.

In contrast, the effects of density gradient centrifugation on chemokine receptor expression are dramatic, particularly for CCR4. Our results suggest that density gradient enhances the detection of CCR4 on NK cells. One possible explanation for this finding is that the reagents used in the density gradient centrifugation may stabilize the cell-surface expression of highly labile chemokine receptors, Similar reported effects of enhanced chemokine receptor expression on T-cells further support this hypothesis (Berhanu et al., 2003). Thus, our results suggest that chemokine receptor expression is not comparable between NK cells in whole blood and PBMC. Using different sample types for comparisons may yield inconsistent results.

In addition, delays in processing for more than 8 h dramatically affect chemokine receptor expression on NK cells. This finding is consistent with similar reported effects on T-cells (Nicholson et al., 1984; Ekong et al., 1993; Betensky et al., 2000; Lin et al., 2002; Berhanu et al., 2003; Bull et al., 2007; Meier et al., 2008; McKenna et al., 2009). With NK cells, the expression of both CCR4 and CCR7 diminishes over time. This effect on CCR4 expression is most notable in PBMC; whereas, the effect on CCR7 expression is most notable in whole blood. Taken together, our findings imply that clinical samples must be analyzed within 8 h of collection to reliably assess chemokine receptor expression on NK cells. Such guidelines are useful considerations for the design and implementation of clinical studies that quantify NK cell subsets.

Similarly, we found that delays of more than 16 h significantly impaired degranulation and cytokine secretion by NK cells. These findings are consistent with previously described changes in functional responses by T-cells, dendritic cells and monocytes (McKenna et al., 2009; Meier et al., 2008). Notably delays of more than 8 h severely impair dendritic cell and monocyte cytokine secretion following *in vitro* stimulation (Meier et al., 2008). Thus, 8 h is a general benchmark for quality control assessment in deciding the usability of blood samples for measuring NK cell functional responses. While the mechanisms underlying dysfunction remain unknown, we speculate that, as previously shown for T-cells, the expansion of activated CD11bpos and CD15pos granulocytes may dampen NK cell responses through changes in the cytokine milieu (McKenna et al., 2009). Alternatively, intracellular signaling components required for robust responses may become depleted over time *ex vivo*. To understand the basis of this impairment further investigations are warranted; however, it is apparent that the time from sample collection to assay implementation is a critical consideration in obtaining valid results. This study extends the work of previous studies by providing an estimate of time for the assessment of NK cell parameters *ex vivo*. Samples that are analyzed greater than 16 h after collection may yield deficient NK cell responses. Since we did not assess time-points between 2 and 16 h we cannot provide a more accurate estimate of a suitable limit on sample transport to permit functional analyses but based on the findings reported here, and those of others (McKenna et al., 2009; Meier et al., 2008), we believe 8 h is a reasonable benchmark.

A few limitations of our study warrant mention. Firstly, our conclusions are limited to blood samples from adults. Lin and colleagues have reported different effects of sample processing variation using blood samples from young children and infants (Lin et al., 2002).Secondly, for these assays we used blood drawn on ACD treated tubes. It is plausible that blood drawn into ethylenediamine tetra-acetic acid (EDTA) tubes may yield different results, particularly for measures of activation. EDTA chelates divalent cations that are crucial for activation processes and thus may reduce the effects of *ex vivo* activation. Finally, since our cohort was small, there is risk of a type II error in the interpretation of the data; hence findings of no effect should be interpreted with caution, and attention to possible trends should be made.

5. Conclusion

Based on these findings, interpretation of studies using NK cells processed after delays of greater than 8 h or after density

gradient centrifugation warrants careful consideration. In particular, variations in sample processing time and/or methods have the greatest impact on NK cell chemokine receptor expression and *in vitro* functional responses. Thus, where possible, delays in processing should be avoided, and careful attention should be made in the selection of NK cell parameters that are least affected by these variables in the assessment of clinical samples.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jim.2011.01.001.

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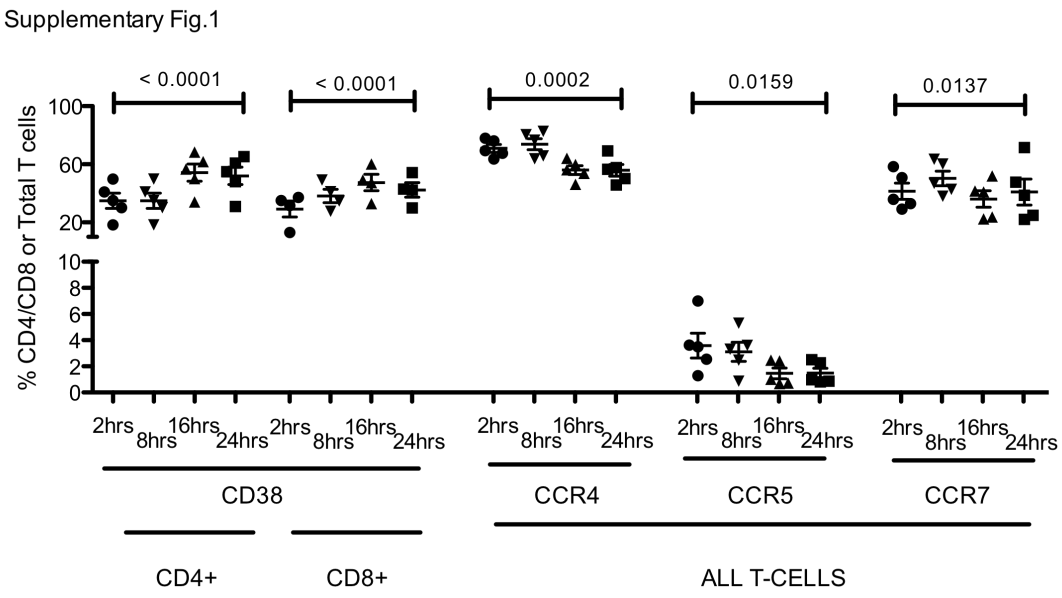
LIST OF TABLES

NK cell readout	Parameter	Measure	Comparison Group 1	Comparison Group 2	Comparison Group 3	Comparison Group 4	Statis compa *
			WB	**PBMC**			
Activation	CD38	**% of NK**	**69,78**	**65,95**			**0,0**
		MFI (log_{10})	3,34	2,99			0,1
	CD69	**% of NK**	**0,37**	**0,85**			**0,0**
		MFI (log_{10})	-0,54	1,64			0,0
	HLA-DR	% of NK	10,82	8,51			0,3
		MFI (log_{10})	**2,47**	**2,38**			**0,0**
	NKp46	% of NK	42,64	38,64			0,4
		MFI (log_{10})	**2,62**	**3,08**			**0,0**
Chemokine receptor	CCR4	**% of NK**	**10,47**	**50,92**			**0,00**
		MFI (log_{10})	2,70	3,24			0,0
	CCR7	% of NK	37,46	21,76			0,1
		MFI (log_{10})	**3,10**	**2,56**			**0,0**
			WB 2hr	**WB 24hr**			
Frequency of NK		NK as % CD45+ lymphocytes	10,30	9,10			0,3
Activation	CD38	% of NK	69,78	67,92			0,3
		MFI (log_{10})	3,35	3,18			0,0
	CD69	% of NK	0,37	0,30			0,2
		MFI (log_{10})	2,49	2,50			0,5
	HLA-DR	% of NK	10,82	11,86			0,3
		MFI (log_{10})	-0,21	-1,80			0,2
	NKp46	**% of NK**	**42,64**	**28,06**			**0,0**
		MFI (log_{10})	**2,62**	**2,39**			**0,0**
Chemokine receptor	CCR4	% of NK	10,47	6,77			0,1
		MFI (log_{10})	**2,70**	**2,61**			**0,0**
	CCR7	**% of NK**	**37,46**	**0,26**			**0,0**
		MFI (log_{10})	**3,10**	**1,60**			**<0,0**
			PBMC 2hr	**PBMC 8hr**	**PBMC 16hr**	**PBMC 24hr**	
Frequency of NK		NK as % Lymphocytes	6,94	6,76	4,99	4,00	0,1
Activation	CD38	% of NK	65,95	69,66	71,89	62,03	0,4
		MFI (log_{10})	**2,96**	**3,45**	**3,16**	**2,92**	**0,0**
	CD69	**% of NK**	**1,66**	**0,63**	**2,21**	**3,03**	**0,0**
		MFI (log_{10})	**2,38**	**2,49**	**2,58**	**2,57**	**0,00**
	HLA-DR	% of NK	8,51	10,00	6,90	7,06	0,1
		MFI (log_{10})	**1,67**	**2,02**	**-1,27**	**-1,65**	**<0,0**
	NKp46	**% of NK**	**38,64**	**33,82**	**19,60**	**15,54**	**0,0**
		MFI (log_{10})	**3,08**	**2,99**	**2,71**	**2,60**	**<0,0**
Chemokine receptor	CCR4	**% of NK**	**50,92**	**42,36**	**23,18**	**24,34**	**<0,0**
		MFI (log_{10})	**3,24**	**3,00**	**2,67**	**2,69**	**0,0**
	CCR7	**% of NK**	**13,60**	**9,54**	**2,76**	**6,14**	**0,00**
		MFI (log_{10})	**2,56**	**2,41**	**2,14**	**2,20**	**<0,0**

Two-tailed t-tests or repeated measures ANOVA

Supplementary Fig 1.**Delayed processing PBMC alters T-cell activation and chemokine receptor expression.**

Supplementary Fig 1 shows the proportion of CD4pos, CD8pos or bulk T-cells expressing CD38, or chemokine receptors, CCR4, CCR5 or CCR7 (n=5). Open shapes (○▽△□) represent means of duplicate measures from whole blood, closed shapes represent means of duplicate measures from PBMC (■▼▲●); circles (● or ○), inverted triangles (▼ or ▽), upright triangles (▲ or △) and squares (■ or □) represent measures of NK cell frequency at 2, 8, 16 and 24 hours respectively, and as indicated. Each data point represents one individual.



CHAPTER FOUR:

Killer-cell immunoglobulin-like receptor genotyping and HLA killer-cell immunoglobulin-like receptor-ligand identification by real-time polymerase chain reaction

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Killer-cell immunoglobulin-like receptor genotyping and HLA killer-cell immunoglobulin-like receptor-ligand identification by real-time polymerase chain reaction

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Key words
genotyping; human leukocyte antigen killer-cell immunoglobulin-like receptor-ligand; killer-cell immunoglobulin-like receptor; real-time polymerase chain reaction

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Abstract

The effector function of natural killer (NK) cells is modulated by surface expression of a range of killer-cell immunoglobulin-like receptors (KIRs) that interact with human leukocyte antigen (HLA) class I ligands. We describe the use of real-time polymerase chain reaction (PCR) assays that allow easy and quick detection of 16 *KIR* genes and the presence/absence of KIR-ligands based on allelic discrimination at codon 80 in the *HLA-A/B Bw4* and *HLA-C C1/C2* genes. These methods overcome the tedious and expensive nature of conventional *KIR* genotyping and HLA class I typing using sequence-specific primer (SSP) PCR, sequence-specific oligonucleotide (SSO) hybridization or sequence-based typing (SBT). Using these two cost-effective assays, we measured the frequencies of KIRs, KIR-ligands and KIR/KIR-ligand pairs in a cohort of Black women recruited in South Africa.

Introduction

Natural killer (NK) cells comprise approximately 10%–15% of peripheral blood lymphocytes, and their primary function is the detection and destruction of malignant and virus-infected cells (1, 2). A number of cell surface receptors that control the effector function of NK cells have been characterized, such as the killer-cell immunoglobulin-like receptors (KIRs), C-type lectins and natural cytotoxicity receptors (NCRs) (3, 4).

KIR proteins are encoded in the leukocyte receptor complex (LRC) located on chromosome 19, and are characterized by the presence of two-dimensional (2D) or three-dimensional (3D) extracellular immunoglobulin-like domains that determine ligand specificity (3, 5). In addition, KIRs fall into two broad groups with different cytoplasmic tail lengths. Receptors with long tails (L) suppress NK cell activation, whereas short-tail (S) receptors are stimulatory, inducing the secretion of interferon-γ (IFN-γ) and the release of perforin and granzyme (6).

Sixteen *KIR* genes have been identified; 7 are inhibitory (*KIR2DL1-3*, *KIR2DL5*, *KIR3DL1-3*), 6 are activating (*KIR 2DS1-5* and *KIR3DS1*), 2 are pseudogenes (*KIR2DP1* and *KIR3DP1*) (5, 7) and 1 has both inhibitory and activating potential (*KIR2DL4*) (8). Certain KIRs, upon engagement with specific human leukocyte antigen (HLA) class I ligands, may either inhibit or stimulate NK cell activity (6, 9). *HLA* and *KIR* genes are highly polymorphic and map to chromosomes 6 and 19, respectively, and thus assort independently. On the basis of *KIR* gene combinations, two haplotype groups have been identified; haplotype A, with a fixed content of nine *KIR* genes (*KIR2DL1/3/4*, *KIR3DL1-3, KIR2DS4, KIR2DP1* and *KIR3DP1*) and haplotype B, with variable gene content. Collectively, this allows for a large range of KIR/HLA diversity (10).

KIR-HLA ligands (KIR-ligands) for KIR2DL1-3, KIR2DS1 and KIR2DS4 are HLA-C molecules. KIR2DL2 and KIR2DL3 recognize HLA-C group 1 (C1) allotypes, which have an asparagine at position 80 (C1:N^{80}), whereas KIR2DL1 and KIR2DS1 bind to the HLA-C group 2 (C2) allotypes, which have a lysine at position 80 (C2:K^{80}) (11). Whilst KIR2DS4 can bind to both subsets of HLA-C C1 and C2 allotypes, it has also been found to bind to two HLA-A alleles (HLA-A*11:01 and A*11:02) (12). For KIR3DL1 and putatively KIR3DS1, the ligands are HLA-B allotypes with the Bw4 motif designated by five variable amino acids spanning positions 77–83, with either isoleucine (Bw4:I^{80}) or threonine (Bw4:T^{80}) at position 80 (13, 14). In addition, HLA-B Bw4:T^{80} variants can vary with alanine (Bw4:$T^{80}A^{81}$) or leucine (Bw4:$T^{80}L^{81}$) at position 81 (13–15). Some HLA-A allotypes also have the Bw4 motif (Bw4:I^{80}) and bind KIR3DL1 and putatively KIR3DS1, whereas HLA-A allotypes lacking the Bw4 motif (non-Bw4:T^{80}) and HLA-B Bw6 allotypes (Bw6:N^{80}) are not known to bind any KIRs.

Specific KIR and KIR-ligand combinations have been associated with numerous infectious/autoimmune disease outcomes as well as in pregnancy and transplantation complications (10, 16). The presence of specific KIRs with or without their corresponding ligands can influence susceptibility to disease and/or improved response to therapy (10, 17–19). Conventional *KIR* genotyping is done by sequence-specific primer polymerase chain reaction (SSP-PCR), and HLA class I typing is done by SSP-PCR, sequence-specific oligonucleotide (SSO) hybridization or sequence-based typing (SBT) assays. These methods are often cumbersome and expensive and require large quantities of high-quality genomic DNA. Amplified SSP products are visualized by agarose gel electrophoresis, which is time-consuming as well as hazardous if using ethidium bromide staining (16).

We have optimized and validated two real-time PCR assays for *KIR* genotyping and identification of KIR-ligands using samples previously characterized by SSP-PCR and SBT. The *KIR* genotyping assay is able to determine the presence/absence of 16 *KIR* genes, while the KIR-ligand assay identifies the presence/absence of KIR-ligands (HLA-A/B Bw4, and HLA-C C1/C2). We then applied these new methods to identify *KIR* genotype, KIR-ligand frequencies and KIR/KIR-ligand pairs in a small South African cohort.

Methodology

Validation samples

Stored genomic DNA was available for 230 samples previously genotyped for *KIR* or *HLA* class I using commercial assays. The real-time PCR *KIR* genotyping assay was validated against 50 samples, consisting of 24 South African Black individuals and 26 Caucasians that were *KIR* genotyped by SSP-PCR. Importantly, all 16 *KIR* genes were represented in these samples. The real-time PCR KIR-ligand assay was validated against 220 samples that had been *HLA* typed by SBT, in which all variants at position 80 were represented. To accommodate HLA class I allelic variation, we tested samples from South African Black individuals ($n = 107$) and Caucasians ($n = 83$), as well as published HLA-A, -B and -C reference samples ($n = 30$) obtained from the International Histocompatibility Working Group (http://www.ihwg.org).

Study samples

Whole-blood ethylenediaminetetraacetic acid (EDTA) samples were collected from consenting Black women ($n = 81$) recruited from the Carletonville mining area, South Africa. A salting-out method (20) was used to extract genomic DNA from leukocytes isolated from 1 ml of whole blood. DNA concentration was measured on a nanodrop spectrophotometer (Thermo Scientific, Wilmington, MA). The study was approved by the Human Research Ethics Committee (Medical) at the University of the Witwatersrand and informed written consent was obtained from all participants.

KIR PCR primers and real-time PCR

To date, real-time PCR-based KIR genotyping and HLA KIR-ligand identification assays have been described by Alves et al. (21) and Koehler et al. (22). In our system, we wished to avoid the use of fluorescent probe chemistry and nested PCR methods used by Koehler et al. (22) in favor of SYBR® green chemistry. For our real-time KIR genotyping assay, we selected previously published KIR primer sequences (16, 21, 23), which we mapped to updated *KIR* gene alignments in the Immuno Polymorphism Database (IPD) database (24). A combination of primers was used to improve detection of KIR allelic variants, with the exception of KIR2DL3 and KIR3DP1, where a single primer set was used (Table 1). The KIR2DL3 primer set detects all known alleles; however, the pseudogene KIR3DP1 primer set does not detect KIR3DP1*004 and KIR3DP1*009 alleles. Furthermore, an internal control gene galactosylceramidase (*GALC*) (21) was included.

For real-time PCR amplification, each 5-μl reaction contained 2× SYBR® Green master mix (Roche, Mannheim, Germany), 0.2 μM KIR-specific primers, 0.2 μM GALC-specific primers and 5 ng of genomic DNA. PCR cycling was performed in a 96-well format on an ABI PRISM® 7500 real-time instrument (Applied Biosystems, Foster City, CA) as follows: 10 min at 95°C, 30 cycles of 15 s at 95°C and 1 min at 60°C. Melt curve analysis was performed after cycling (Figure 1A) and could discriminate between GALC (T_m = 74.89°C) and the KIR amplicons (T_m = 78.5.5°C–85.9°C), thereby making it simple to identify the presence/absence of specific *KIR* genes. The presence of an internal control amplicon ensured that the absence of a KIR amplicon was a true result.

KIR-ligand primers and real-time PCR

We designed our KIR-ligand primers from HLA class I alignments available at the International Immunogenetics Project (IMGT)/HLA database (25) to discriminate Bw4 from Bw6 allotypes at codon 80. Primers were synthesized with 3′ locked nucleic acid (LNA) bases (Exiqon, Vedbaek, Denmark) in order to increase template specificity (Table 2). Each PCR included an internal positive control gene albumin (*ALB*) (26).

For real-time PCR amplification, each 5-μl reaction contained 2× SYBR® Green master mix (Roche), 0.2 μM KIR-specific primers, 0.2 μM ALB-specific primers and 5 ng of genomic DNA. PCR cycling was performed in a 96-well format on an ABI PRISM® 7500 real-time instrument (Applied Biosystems) as follows: 10 min at 95°C, 30 cycles of 15 s at 95°C, 5 s at 60°C and 1 min at 72°C. Melt curve analysis was performed after cycling (Figure 1B), and similar to the KIR assay, differences in melting temperature could discriminate between ALB (T_m = 75.62°C) and the KIR-ligand amplicons (T_m = 87.3°C–88.3°C).

High-resolution HLA typing

High-resolution (four-digit) HLA typing was performed on validation samples as follows: DNA was extracted from either peripheral blood mononuclear cells or granulocytes using the Pel-Freez DNA isolation kit (Dynal Biotech, Ullernchausseen, Norway). HLA-A, -B and -C typing was performed by DNA sequencing of exons 2, 3 and 4 using SBTexcellerator kits (Qiagen, Dusseldorf, Germany) on an ABI3100 Genetic Analyzer (Applied Biosystems) and ASSIGN-SBT v3.5 software (Conexio Genomics, Fremantle, Western Australia). Any ambiguities resulting from either polymorphisms outside the sequenced exons or identical heterozygote combinations were resolved using statistical methods (27).

SSP-PCR KIR genotyping

KIR genotyping was performed on validation samples using either a commercially available kit, sequence-specific primer *KIR* PCR genotyping kit (Olerup, Loewengasse, Austria) or a published protocol (16). These methods both detect the presence/absence of the following 16 *KIR* genes: *KIR2DL1-5, KIR2DS1-5*, *KIR3DL1-3*, *KIR3DS1*, *KIR2DP1* and *KIR3DP1*.

On the basis of individual *KIR* gene combinations, two genotype groups were stratified, the AA genotype with nine genes (*KIR2DL1/3/4*, *KIR3DL1-3, KIR2DS4, KIR2DP1* and *KIR3DP1*) and the Bx genotype, which can be a mixture of AB or BB haplotypes. In addition, *KIR* Bx genotype numbers were assigned according to published nomenclature on the Allele Frequencies database (28).

Data analysis

For both sets of data (SSP-PCR, SBT and real-time PCR), carrier frequencies for the observed KIR and KIR-ligands were determined by direct counting (individuals positive for the gene divided by the individuals tested per population × 100). Real-time PCR KIR and KIR-ligand assays were validated by direct comparison with SBT- and SSP-PCR-based typing results of the same samples.

Results

Validation of real-time PCR KIR assay

Using samples that represented the full spectrum of the *KIR* genes, we tested real-time PCR KIR assay on the KIR validation samples (n = 50) and compared our results to the gel-based (SSP-PCR) *KIR* genotyping kit (Olerup) and published methods (16). We found the real-time PCR KIR assay was 100% concordant with conventional SSP-PCR methods (Table 3).

Validation of real-time PCR KIR-ligand assay

In comparison with the commercial high-resolution SBT method, the real-time KIR-ligand assay correctly genotyped

Table 1 PCR primer sets used for real-time *KIR* genotyping assay

KIR gene	PCR well no.	Primers	Sequences (5′–3′)	Primer binding site	Product T_m (°C)	Alleles not detected
2DL1	1	F1[a]	GTTGGTCAGATGTCATGTTTGAA	Exon 4	80.36	—
		R1[a]	GGTCCCTGCCAGGTCTTGCG	Exon 4		
	2	F1[a]	TGGACCAAGAGTCTGCAGGA	Exon 8	82.12	KIR2DL1*005
		R1[a]	TGTTGTCTCCCTAGAAGACG	3′UTR		
2DL2	3	F1[a]	CTGGCCCACCCAGGTCG	Exon 4	80.68	KIR2DL2*004, *00601, *00602, *00303, *004
		R1[a]	GGACCGATGGAGAAGTTGGCT	Exon 4		
	4	F1[b]	AAACCTTCTCTCTCAGCCCA	Exon 5	83.30	KIR2DL2*009
		R1[b]	GCCCTGCAGAGAACCTACA	Exon 5		
2DL3	5	F[b]	AGACCCTCAGGAGGTGA	Exon 9	79.47	—
		R[b]	CAGGAGACAACTTTGGATCA	Exon 9		
2DL4	6	F1[a]	CAGGACAAGCCCTTCTGC	Exon 3	78.08	—
		R1[a]	CTGGGTGCCGACCACT	Exon 3		
	7	F1[a]	ACCTTCGCTTACAGCCCG	Exon 5	84.21	—
		R1[a]	CCTCACCTGTGACAGAAACAG	Exon 5		
2DL5	8	F1[a]	GCGCTGTGGTGCCTCG	Exon 3	82.19	—
		R1[a]	GACCACTCAATGGGGGAGC	Exon 3		
	9	F1[a]	TGCAGCTCCAGGAGCTCA	Exon 5	83.09	—
		R1[a]	GGGTCTGACCACTCATAGGGT	Exon 5		
2DS1	10	F1[b]	TCTCCATCAGTCGCATGAG	Exon 4	79.34	—
		F2[b]	TCTCCATCAGTCGCATGAA	Exon 4		
		R[b]	GGTCACTGGGAGCTGAC	Exon 4		
2DS2	11	F1[a]	TTCTGCACAGAGAGGGGAAGTA	Exon 4	80.55	—
		R1[a]	GGGTCACTGGGAGCTGACAA	Exon 4		
	12	F1[a]	CGGGCCCCACGGTTT	Exon 5	83.47	KIR2DS2*00104
		R1[a]	GGTCACTCGAGTTTGACCACTCA	Exon 5		
2DS3	13	F1[c]	AAACCTTCTCTCTCAGCCCA	Exon 5	84.07	—
		R1[b]	GCATCTGTAGGTTCCTCCT	Exon 5		
	14	F1[a]	CTATGACATGTACCATCTATCCAC	Exon 5	83.07	—
		R1[a]	AAGCAGTGGGTCACTTGAC	Exon 5		
2DS4	15	F1[a]	CTGGCCCTCCCAGGTCA	Exon 4	80.83	—
		R1[a]	TCTGTAGGTTCCTGCAAGGACAG	Exon 4		
	16	F1[a]	GTTCAGGCAGGAGAGAAT	Exon 5	84.35	—
		R1[a]	GTTTGACCACTCGTAGGGAGC	Exon 5		
2DS5	17	F1[a]	TGATGGGGTCTCCAAGGG	Exon 4	81.54	KIR2DS5*003
		R1[a]	TCCAGAGGGTCACTGGGC	Exon 4		
	18	F1[a]	ACAGAGAGGGGACGTTTAACC	Exon 4	82.18	—
		R1[a]	ATGTCCAGAGGGTCACTGGG	Exon 4		
2DP1	19	F1[a]	GTCTGCCTGGCCCAGCT	Exon 3	80.67	—
		R1[a]	GTGTGAACCCCGACATCTGTAC	Exon 3		
	20	F1[a]	CCATCGGTCCCATGATGG	Exon 4	79.28	—
		R1[a]	CACTGGGAGCTGACAACTGATG	Exon 4		
3DL1	21	F1[a]	CGCTGTGGTGCCTCGA	Exon 3	79.62	KIR3DL1*009, *042, *057
		R1[a]	GGTGTGAACCCCGACATG	Exon 3		
	22	F1[b]	CCATCGGTCCCATGATGCT	Exon 4	79.13	—
		F2[b]	CCATT GGTCCCATGATGCT	Exon 4		
		F3[b]	TCCATCGGTCCCATGATGTT	Exon 4		
		R[b]	CCACGATGTCCAGGGGA	Exon 4		
3DL2	23	F1[a]	CAAACCCTTCCTGTCTGCCC	Exon 3	82.94	KIR3DL2*013, *014
		R1[a]	GTGCCGACCACCCAGTGA	Exon 3		
	24	F1a	CCCATGAACGTAGGCTCCG	Exon 5	83.70	KIR3DL2*018
		R1a	CACACGCAGGGCAGGG	Exon 5		
3DL3	25	F1[a]	GTCAGGACAAGCCCTTCCTC	Exon 3	82.32	—
		R1[a]	GAGTGTGGGTGTGAACTGCA	Exon 3		
	26	F1[a]	TTCTGCACAGAGAGGGGATCA	Exon 4	82.47	—
		R1[a]	GAGCCGACAACTCATAGGGTA	Exon 4		

Table 1 *Continued*

KIR gene	PCR well no.	Primers	Sequences (5′–3′)	Primer binding site	Product T_m (°C)	Alleles not detected
3DS1	27	F1[a]	AGCCTGCAGGGAACAGAAG	Exon 8	81.41	—
		R1[a]	GCCTGACTGTGGTGCTCG	3′UTR		
	28	F1[b]	CATCGGTTCCATGATGCG	Exon 4	80.38	—
		F2[b]	CATCAGTTCCATGATGCG	Exon 4		
		R[b]	CCACGATGTCCAGGGGA	Exon 4		
3DP1	29	F[b]	GTACGTCACCCTCCCATGATGTA	5′UTR	85.97	KIR3DP1*004, *009002
		R[b]	GAAAACGGTGTTTCGGAATAC	Exon 3		
GALC (control)	30	F[c]	TTACCCAGAGCCCTATCGTTCT		74.89	—
		R[c]	GTCTGCCCATCACCACCTATT			

GALC, galactosylceramidase; KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction; UTR, untranslated region.
[a]Primers designed by Martin and Carrington (16).
[b]Primers designed by Vilches et al. (23).
[c]Primers designed by Alves et al. (21).

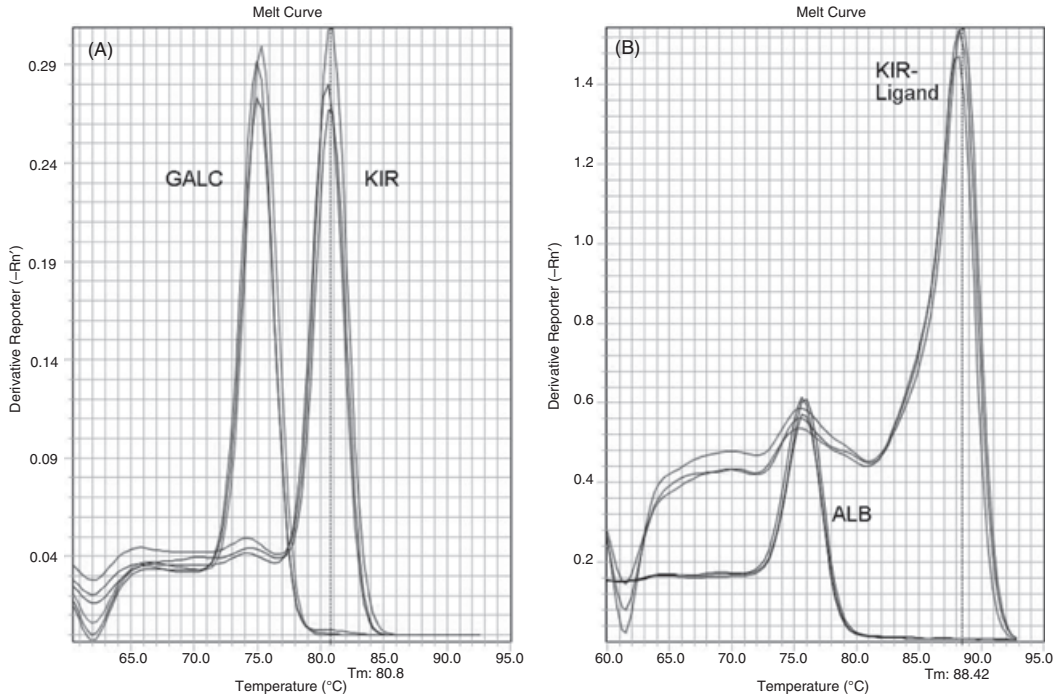

Figure 1 (A) Melt curve analysis of killer immunoglobulin-like receptor (KIR)-polymerase chain reaction (PCR) products melting to the right of the internal control galactosylceramidase (GALC) (74.89°C) and (B) KIR-ligand PCR amplicons melting to the right of the internal control albumin (ALB) (75.62°C).

99.1% (218/220) of individuals tested. All KIR-ligand primer sets, except HLA-B Bw4:$T^{80}A^{81}$, were 100% concordant with SBT results (Table 4). Our assay failed to correctly assign HLA-B*38:02 to the Bw4:$T^{80}A^{81}$ allotype for two DNA samples obtained from the IHWG database. The sample was retyped by SBT and confirmed to be correctly assigned based on exon 2, 3 and 4 sequences. In addition, the genomic sequences for HLA-B*38:02:01, B*38:02:02 and B*38:02:03 were available to confirm that the reverse primer binding site had no changes. SBT typing does not include the 5′ untranslated region (5′UTR); however, genomic sequences were available for HLA-B*38:01:01, B*38:02:01 and B*38:14 and confirmed that the forward primer was conserved except for a −7 mismatch. It is unlikely that this was the cause of a failed PCR amplification as the forward primer was found to successfully amplify other HLA-B alleles with the same mismatch. It is therefore possible that these two samples are undescribed B*38:02 alleles with changes in the 5′UTR. Nevertheless, the HLA-B*38:02 allele is prevalent at a low frequency in people of Taiwanese/Chinese descent (0.11% and 0.08%, respectively) and has not been reported within the African continent (28).

Table 2 PCR primer sets used for real-time PCR KIR-ligand genotyping assay

Gene		Primers	Sequences (5′–3′)[a]	Product *T*m (°C)	Reference
HLA-A	Bw4:I^{80}	F	CCATTGGGTGTCGGGTTTC[C]	87.39	This study
		R[b]	CTCTGGTTGTAGTAGCGGAGCGCG[A]		
	Non-Bw4:T^{80}	F	AATCAGTGTCGTCGCGGTC[G]	87.65	
		R	TGTAGTAGCCGCGCAGG[G]		
HLA-B	Bw6:N^{80}	F	TCAGCGTCGCCGGGGTCCC[A]	87.73	This study
		R	TGTAGTAGCCGCGCAGG[T]		
	Bw4:I^{80}	F	ACCCGGACTCAGAATCTCC[T]	87.96	
		R[b]	CTCTGGTTGTAGTAGCGGAGCGCG[A]		
	Bw4:$T^{80}A^{81}$	F	ACCCGGACTCAGAATCTCC[T]	88.08	
		R	TGTAGTAGCGGAGCGCG[G]		
	Bw4:$T^{80}L^{81}$	F	ACCCGGACTCAGAATCTCC[T]	87.73	
		R	CTCTGGTTGTAGTAGCGGAGCAGG[G]		
HLA-C	C1:N^{80}	F	AGCCAATCAGCGTCTCCGC[A]	88.34	This study
		R	GCTCTGGTTGTAGTAGCCGCGCAG[G]		
	C2:K^{80}	F	CCATTGGGTGTCGGGTTCT[A]	88.06	
		R	GCTCTGGTTGTAGTAGCCGCGCAG[T]		
ALB (control)		F	TCGATGAGAAAACGCCAGTAA	75.62	(26)
		R	ATGGTCGCCTGTTCACCAA		

ALB, albumin; HLA, human leukocyte antigen; KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction.
[a]3′ Locked nucleic acid bases are depicted between square brackets. Melting temperature (T_m) for each PCR product was determined automatically following melt curve analysis.
[b]The same reverse primer is used for detection of HLA-A and -B Bw4:I^{80} alleles.

Table 3 Validation of real-time PCR *KIR* genotyping assay ($n = 50$)[a]

	Carriers/noncarriers		
KIR genes	SSP KIR PCR	Real-time KIR assay	Concordant (%)
2DL1	48/2	48/2	100
2DL2	27/23	27/23	100
2DL3	41/9	41/9	100
2DL4	50/0	50/0	100
2DL5	26/24	26/24	100
2DS1	15/35	15/35	100
2DS2	27/23	27/23	100
2DS3	10/40	10/40	100
2DS4	47/7	47/7	100
2DS5	21/29	21/29	100
2DP1	48/2	48/2	100
3DL1	47/3	47/3	100
3DL2	50/0	50/0	100
3DL3	50/0	50/0	100
3DS1	13/37	13/37	100
3DP1	50/0	50/0	100

KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction; SSP, sequence-specific primer.
[a]Concordance between assays was established by directly comparing the number of individuals who did/did not carry the KIR gene divided by the KIR genes present/absent x 100.

KIR genotyping in a South African population group

KIR genotyping using the real-time PCR KIR assay showed similar gene frequencies to other South African population groups studied (29, 30). We identified 20 different *KIR* genotype profiles (Table 5), 2 of which have not been previously reported (*Bx46* and *Bx70*) and 1 undescribed *KIR* Bx genotype with all *KIR* genes present with the exception of *KIR2DL2*, *KIR2DL3*, *KIR2DS1*, *KIR2DS2* and *KIR3DS1*.

Table 4 Validation of real-time PCR KIR-ligand genotyping assay ($n = 220$)[a]

		Carriers/noncarriers		
HLA	KIR-ligand	SBT HLA sequence	Real-time KIR-ligand assay	Concordant (%)
HLA-A	Bw4:I^{80}	62/158	62/158	100
	Non-Bw4:T^{80}	215/5	215/5	100
HLA-B	Bw6:N^{80}	180/40	180/40	100
	Bw4:I^{80}	93/127	93/127	100
	Bw4:$T^{80}A^{81}$	49/171	47/171	95.9
	Bw4:$T^{80}L^{81}$	11/209	11/209	100
HLA-C	C1:N^{80}	173/47	173/47	100
	C2:K^{80}	161/59	161/59	100

HLA, human leukocyte antigen; KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction; SBT, sequence-based typing; SSP, sequence-specific primer.
[a]Concordance between assays was established by directly comparing the number of individuals who did/did not carry the *KIR*-ligand gene divided by the *KIR*-ligand genes present/absent × 100.

KIR-ligand genotyping in a South African population group

Using the real-time PCR KIR-ligand assay, we determined the absence/presence of the HLA-A/B Bw4 and HLA-C C1/C2 motifs. At the HLA-A locus, 2.5% of individuals were homozygous for Bw4:I^{80} (ligands for KIR3DL1/S1), 69.1% were homozygous for non-Bw4:T^{80}

Table 5 Real-time PCR *KIR* genotyping in a South African Cohort ($n = 81$)[a]

KIR genotype	*KIR* genes																No. of *KIR* genes	Genotype frequency *N* (%)
	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1		
AA1	■	□	■	■	□	□	□	□	■	□	■	■	■	■	□	■	9	20 (24.7)
Bx21	■	■	■	■	■	□	■	□	■	■	■	■	■	■	□	■	13	11 (13.6)
Bx5	■	■	■	■	■	□	■	■	■	□	■	■	■	■	□	■	13	7 (8.6)
Bx10	■	■	■	■	□	□	■	□	■	□	■	■	■	■	□	■	11	6 (7.4)
Bx20	■	■	■	■	■	□	□	□	■	■	■	■	■	■	□	■	12	6 (7.4)
Bx112	■	■	□	■	■	□	■	■	■	■	■	■	■	■	□	■	13	5 (6.2)
Bx73	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	15	5 (6.2)
Bx92	■	■	□	■	■	■	■	□	■	■	■	■	■	■	□	■	13	4 (4.9)
Bx228	■	■	□	■	■	□	■	□	■	■	■	■	■	■	□	■	12	3 (3.7)
Bx6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	16	2 (2.5)
Bx9	■	■	■	■	■	■	■	□	■	■	■	■	■	■	□	■	14	2 (2.5)
Bx91	■	■	□	■	■	■	■	■	■	■	■	■	■	■	□	■	14	2 (2.5)
Bx118	■	■	□	■	■	■	■	□	■	■	■	■	■	■	■	■	14	1 (1.2)
Bx27	■	□	■	■	■	□	□	■	■	□	■	■	■	■	■	■	12	1 (1.2)
Bx32	■	□	■	■	■	□	□	□	■	■	■	■	■	■	□	■	11	1 (1.2)
Bx35	■	□	■	■	■	■	□	□	■	■	■	■	■	■	□	■	12	1 (1.2)
Bx4	■	■	■	■	□	□	■	□	■	□	■	■	■	■	□	■	11	1 (1.2)
Bx46	■	■	■	■	■	■	□	□	■	■	■	■	■	■	□	■	13	1 (1.2)
Bx70	■	■	■	■	■	■	■	■	□	■	■	□	■	■	■	■	14	1 (1.2)
Bx*	■	□	□	■	■	□	□	■	■	■	■	■	■	■	□	■	11	1 (1.2)
Gene frequency *N* (%)	81 (100)	56 (69)	60 (74)	81 (100)	54 (67)	19 (23)	50 (62)	24 (30)	80 (99)	46 (57)	81 (100)	80 (99)	81 (100)	81 (100)	10 (12)	81 (100)		

KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction.

[a]Frequency of each *KIR* gene is expressed as a percentage and is defined as the number of individuals having the gene divided by the number of individuals in the cohort ($n = 81$). Genotype frequency is defined by the number of individuals having a particular genotype divided by the number of individuals within the cohort. The black boxes indicate the presence of the gene, whereas the open boxes indicate the absence of the gene. Bx* is a new KIR genotype that has not been reported.

and the remaining 28.4% were heterozygotes (Figure 2). At the HLA-B locus, 40.7% of individuals were Bw6:N^{80} homozygous. Of the remainder who carried a Bw4 allele (ligands for KIR3DL1/S1), 48.1% were heterozygous for Bw6:N^{80}/Bw4:I^{80}, 3.7% were Bw4:I^{80}/$T^{80}A^{81}$ heterozygotes and 7% were Bw4:I^{80} homozygotes. The Bw4:I^{80}/$T^{80}L^{81}$ was detected in 1.2% of individuals, while no Bw4:$T^{80}L^{81}$ homozygotes or Bw4:$T^{80}L^{81}$/Bw6:N^{80} heterozygotes were detected in this population. At the HLA-C locus, 16% of individuals were homozygous for C1:N^{80}, 38% were homozygous for C2:K^{80} and the remaining 46% were heterozygotes.

KIR/KIR-ligand pairs in a South African population group

KIR and KIR-ligand data, when taken together, allow receptor-ligand combinations to be determined (Table 6). Within this relatively small South African cohort ($n = 81$), the frequency of activating KIR/KIR-ligand pairs was lower than that of the inhibitory KIR/KIR-ligand pairs. Activating *KIR2DS1* paired with the HLA-C C2 ligand was found at a frequency of 16% and *KIR3DS1* paired with the putative HLA-B Bw4 ligand was present in 6.2% of individuals. Inhibitory *KIR* genes (*KIR2DL1/2/3* and *KIR3DL1*) and their ligands HLA-C C2, HLA-C C1, HLA-C C1 and HLA-A/B Bw4 were present at frequencies of 84%, 44%, 44% and 58%, respectively.

Discussion

We have developed a fast and effective tool to determine KIR and KIR-ligand profiles using real-time PCR and SYBR® Green chemistry. Our KIR and KIR-ligand assays were validated against commercial and published methods and

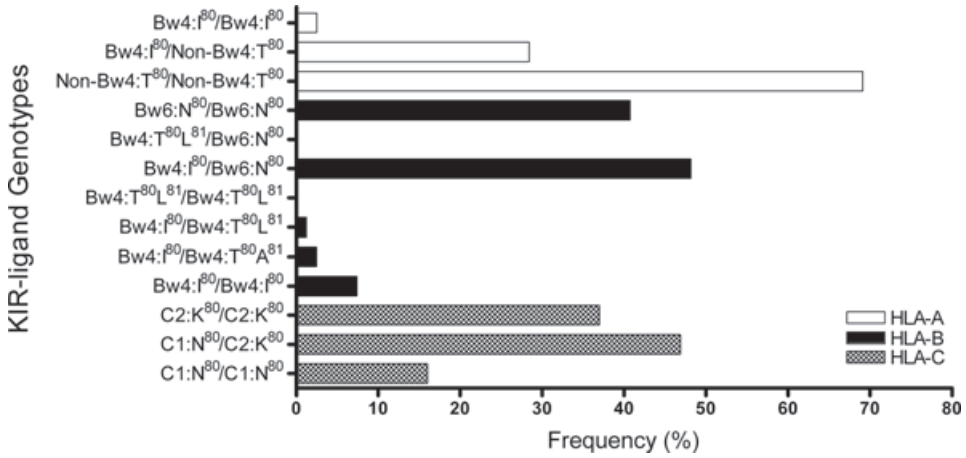

Figure 2 Frequency of killer immunoglobulin-like receptor (KIR)-ligand allele combinations in a South African Cohort ($n = 81$). On the basis of allelic discrimination at codon 80, the KIR-ligand assay can identify the presence/absence of KIR-ligands human leukocyte antigen (HLA)-A (Bw4:I^{80} and non-Bw4:T^{80}) and HLA-B (Bw4:I^{80}, Bw4:$T^{80}A^{81}$, Bw4:$T^{80}L^{81}$ and Bw6:N^{80}) and HLA-C (C1 and C2).

Table 6 KIR/KIR-ligand pairs and frequencies in a South African cohort ($n = 81$)[a]

	KIR gene	*KIR* gene *N* (%)	KIR-ligand	KIR-ligand *N* (%)	KIR + KIR-ligand frequency *N* (%)
Inhibitory	2DL1	81 (100)	C2	68 (84)	68 (84.0)
	2DL2	56 (69)	C1	51 (63)	36 (44.4)
	2DL3	60 (74)	C1	51 (63)	36 (44.4)
	3DL1	80 (99)	Bw4	58 (72)	47 (58.0)
Activating	2DS1	19 (23)	C2	68 (84)	13 (16.0)
	3DS1	10 (12)	Bw4[b]	58 (72)	5 (6.2)

KIR, killer-cell immunoglobulin-like receptor.

[a]Frequency of each KIR/KIR-ligand combination is expressed as a percentage and defined as the number of individuals having the paired KIR/KIR-ligand (*N*) divided by the number of individuals within the cohort ($n = 81$).

[b]Putative KIR-ligand for KIR3DS1 based on homology to KIR3DL1.

were found to be 100% and 99.1% concordant, respectively. The advantage of our assays compared to the commercial gel-based KIR SSP-PCR and HLA SBT methods, as well as published KIR/KIR-ligand typing methods, includes its low input DNA concentration, simple technique and ease of interpretation of results. To enhance detection of *KIR* genes that may contain sequence variation in the primer binding sites, multiple primers were used for all except three genes (Table 1). In addition, each of the assays uses a single internal positive control gene with a melting peak significantly lower than the KIR and KIR-ligand amplicon melting peaks. This enables proper discrimination between the absence of a target gene and a failed PCR. Most importantly, the KIR and KIR-ligand assays are very cost-effective, costing as little as 15%–20% of the price of commercial KIR and SBT typing kits, provided the users have access to a real-time PCR instrument. However, with respect to the HLA SBT typing one must keep in mind the level of allele-specific detail that sequencing provides as opposed to the real-time KIR-ligand allotype designations (*HLA-A/B Bw4* and *HLA-C C1/C2*). Our assay is a single-round detection using SYBR® Green chemistry and melting curve analysis, which is cheaper and simpler than other real-time PCR methods that make use of fluorescent probes and multiple amplification steps (22).

Limitations of the KIR and KIR-ligand assay designs are that due to the large number of *KIR* and *HLA* genes described and allelic variation within these, certain alleles can be either undetected or misclassified. The KIR primers used detect all known allelic variants, except the pseudogene KIR3DP1 primer set which does not detect KIR3DP1*004 and KIR3DP1*00902. The KIR-ligand assay failed to detect HLA-B*38:02 as a Bw4:$T^{80}A^{81}$ allotype, but was not likely due to a primer mismatch. Furthermore, the KIR-ligand primers were designed to discriminate Bw4 from Bw6 at codon 80. By definition, amino-acid residues at positions 77 and 80-83 are important in defining the Bw4 (NXXIALR, NXXTALR, SXXIALR, DXXTLLR and SXXTLLR) and Bw6 (SXXNLRG and GXXNLRG) amino-acid motifs (14, 31). Therefore, a limitation of our approach is that unclassified HLA-B alleles such as HLA-B*44:11, B*51:50, B*57:08 and HLA-A*24:61 that contain amino-acid changes within the Bw4 motifs will be classified as Bw4. Likewise, unclassified HLA-B alleles such as HLA-B*07:11, B*07:57, B*08:17, B*35:56, B*39:20, B*40:37, B*55:12, B*78:06, B*95:35, B*08:29, B*27:42, B*037:05, B*25:52, B*37:14, B*47:03, B*40:76 and B*41:05 that have amino-acid changes that are divergent from the Bw6 motifs will be classified as Bw6. Lastly, a number of HLA-B alleles encode the C1 amino-acid motif (B*07:13, B*07:15, B*08:15, B*15:57, B*18:06,

B*35:74, B*39:27, B*40:73, B*46:01-18, B*55:03, B*67:02 and B*73:01) and will be misclassified as Bw6 allotypes by our assay. Therefore, complimentary HLA typing would be necessary in the geographic areas where these alleles have high frequencies, such as HLA-B*46 in Asia. Likewise for KIR2DS4 ligand identification, which consists of HLA-A*11:01/02 and a subset of HLA-C alleles, further HLA typing would be necessary.

When applied to a small South African sample set, we found similar *KIR* gene frequencies to other published studies (29, 30), and identified an unreported *KIR* genotype profile (with all *KIR* genes present with the exception of *KIR2DL2*, *KIR2DL3*, *KIR2DS1*, *KIR2DS2* and *KIR3DS1*). In addition, paired KIR and KIR-ligand combinations could be determined showing a dominance of inhibitory compared to stimulatory KIR receptor pairings. Specific combinations of HLA and KIRs have been associated with higher risk for autoimmune disease (32, 33) and differential risk for certain infectious diseases (17, 19), thus it remains to be seen how the representation of activating/inhibitory *KIR* genes and their ligand pairs might impact on disease susceptibility/resistance in our population.

Conventional *KIR* and *HLA* genotyping methodologies involve gel-based SSP-PCR and SBT methods, respectively. These methods can be expensive, labor intensive and time-consuming. We describe an optimized and validated real-time PCR-based KIR and KIR-ligand assays that show high concordance with commercial KIR and HLA typing methods. Melt curve analysis offers straightforward determination of the presence/absence of KIRs or known HLA ligands for KIRs, thereby generating results much faster than conventional methods. In addition, the assay is inexpensive and allows for high-throughput data generation. Together, these two assays have relevance to understanding NK cell function in disease as well as in complications with pregnancy or bone marrow transplantation.

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Conflict of interest

We disclose that there are no conflicts of interest or any commercial links pertinent to this article.

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CHAPTER FIVE:

Natural Killer cell function in women at high risk for HIV-1 acquisition: Insights from the CAPRISA 004 microbicide trial

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Natural killer cell function in women at high risk for HIV acquisition: Insights from the CAPRISA004 microbicide trial

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Objective: To assess the role of natural killer(NK) cells in HIV acquisition.

Design: We conducted a nested case-controlsub-study to the CAPRISA004 microbicide trial.

Methods: Thirty women who acquired HIV infection(cases) and thirty women with high-risk sexual activity who remained HIV negative(controls) were selected. Proliferation, degranulation and interferon-gamma(IFN-γ) secretion was measured by multiparametric flow cytometry after culture of recombinant human interleukin-2(rhIL-2)-activated PBMC with 721.221 cells or *in vitro* HIV-infected, autologous CD4+T-cell blasts. Relationships between NK-cell responses and HIV acquisition were modeled with logistic regression models.

Results: Natural Killer cells from cases had lower IFN-γ responses to HLA-deficient 721.221cells(median %IFN-γ_{pos}NK-cells: 13.7% vs. 21.6%, $p = 0.03$). rhIL-2 activated NK cells from cases had responses to autologous HIV-infected target cells distinct from controls: cases had fewer proliferating and more frequent degranulating NK cells. NK cells from caseshad significantly lower IFN-γ responses to *in vitro* HIV-infected autologous T-cells even after adjusting for responses to uninfected blasts(median %IFN-γ_{pos}NK-cells: 0.53% vs. 2.09%, $p = 0.007$). Responses to *in vitro* HIV-infected autologous T-cells were significantly lower in HSV-2 infected women ($p = 0.003$). IFN-γ NK cell responses to autologous HIV-infected cells were associated with lower risk of HIV acquisition (OR adjusted for age, gel arm, HSV-2 and immune activation:0.582, 95% CI 0.347–0.977, $p = 0.04$).

Conclusions: At the time of exposure to HIV, women with impaired NK-cell IFN-γ responses were more likely to acquireHIV infection. NK cells,as early responders to viral exposure, were associated withlower risk ofHIV acquisition, independent of the intercalated effect of HSV-2 infection suppressing NK cell responses.

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Keywords: correlates of protection, herpes simplex virus, HIV infection, interferon-gamma, natural killer cells, tenofovir gel

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Introduction

In 2010, 2.6 million adults were newly infected with HIV globally [1]. In some regions, more than half of all young women having their first child are already infected with HIV [2]. Within these high-risk communities, there are individuals who are at heightened risk due to their sexual behavior but only some of them acquire HIV. Understanding the reasons for why some high-risk women do not acquire HIV may yield new ways to prevent HIV in high-incidence settings.

Few studies have identified reliable immunological correlates of protection [3–7], yet this is crucial for the development of a preventive HIV-1 vaccine. Previous studies have focused primarily on adaptive immune responses. These studies have found that adaptive responses are slow to develop [8], do not consistently correlate with protection [9] and are severely impaired by HIV infection [10]. Nevertheless, adaptive responses are associated with control of HIV in chronically infected individuals [11,12]. In contrast, Natural Killer (NK) cells are the earliest antiviral cells to respond to HIV [8]. These innate immune cells make up a significant proportion (10–15%) of circulating lymphocytes and are potently armed to recognize virally infected cells [13]. However a limitation of previous studies has been the characterization of HIV exposure; individuals defined as exposed based on risk factors alone have been compared with individuals at low or unknown HIV-risk, or HIV infected individuals [14–20]. Few studies have evaluated functional immune responses prior to HIV acquisition. Moreover, in prior studies that measured NK cell function in HIV-exposed uninfected individuals [17,19], NK cell activity following stimulation with non-HIV-specific stimulants was measured, which might not correlate with responses to HIV.

The purpose of this study was to determine the relationship betweenNK cell function andsusceptibility to HIV infection by quantifying NK cell responses to HIV-infected cells and Human Leukocyte Antigen (HLA)-deficient cell lines among women who did or did not subsequently acquire HIV.

Methods

Study population

This study was nested in the CAPRISA 004 Tenofovir gel trial [21]. The CAPRISA 004 trial was a double-blind, randomized controlled trial and was conducted to assess the effectiveness and safety of 1% Tenofovir gel for preventing HIV in women. The trial characteristics and primary outcomes have been published elsewhere [22].

For the case-control analysis presented here, 30 HIV acquirers (cases) were studied at the last pre-infection visit at which cryopreserved peripheral-blood mononuclear cell (PBMC) samples were available (mean of 127 days before the estimated date of infection, range 15–404 days). As a control group for this analysis samples from 30 women who reported the highest sexual exposure but remained uninfected during the trial (HIV non-acquirers) were studied at the visit at which they reported their highest coital frequency in the preceding month [21]. We confirmed sexual activity by counts of returned, used gel applicators. The sample size was similar to previous studies of NK cell function in HIV protection [14,16–20]. Previous studies have implicated specific KIR repertoires with NK cell function [18] and protection from HIV [14,16], but this study focused on NK cell function and like previous studies, was not adequately powered to draw conclusion on KIR involvement in HIV acquisition.

On average, controls had about double the frequency of sexual acts and similar self-reported condom use to HIV acquirers, but were older. In the overall clinical trial cohort, we have previously reported that younger age and HSV-2 infection was associated with HIV acquisition, and accordingly these covariates are adjusted for in further analyses. Ethics approval for this study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE073/010). Participants gave written informed consent for their samples to be collected and stored for these studies after the nature and possible consequences of the studies were explained.

Study procedure

In brief, HIV serostatus, safety, sexual behavior, and gel and condom use were assessed at monthly follow-up visits for 30 months as described [21]. Standard haematology analyses were conducted at 3, 12, 24 months; when clinically indicated and at study exit. In addition, plasma and peripheral blood mononuclear cells (PBMC) were obtained and cryopreserved at 3,12 and 24 months following enrolment. HSV-2 infection was assessed serologically using the Kalon Enzyme Immunoassay (Kalon, Biologicals, Ashgate, UK).

Diagnosis of HIV endpoint

Two HIV rapid antibody tests (Determine HIV1/2-Abott Laboratories, Chicago, Illinois) and Uni-Gold Recombigen (Trinity Biotech, Wicklow, Ireland) were used at each monthly visit to screen for incident HIV and samples stored from prior visits were assessed by RNA PCR Roche CobasTaqMan HIV-1 Monitor v1.0 (Roche Diagnostics, Branchburg, New Jersey) to exclude window period infection in the last stored pre-infection sample. The date of infection was estimated as the midpoint, in days, between the last negative and first

positive HIV test result, or 14 days prior to the first HIV RNA positive result.

Generation and infection of autologous CD4+ T-cell blasts

To generate autologous CD4+ T-cell blasts, cryopreserved PBMC were cultured with 0.5 ug/ml of a bispecific antibody directed against both CD3 and CD8 (Harvard Centre for AIDS Research) in R10/100 medium (RPMI1640 (Invitrogen) and 10% foetal calf serum supplemented with 100 IU/ml rhIL-2 (Invitrogen)) for 96 hours (day 4) at 37°C, 5% CO_2. By 96 h, the population was >90% pure for CD4+ T-cells for all donors. The CD4+ T-cell blasts were infected with a VSV-g pseudotyped HIV-1 laboratory strain (JRCSF) by spinoculation [23]. In brief, the virus was co-incubated with 1×10^6 cells in each well of a 48 well plate, and centrifuged for 90 min at 1500 rpm at 21°C. Cells were washed twice and incubated for a further 48 h (day 6) at 37°C, 5% CO_2. The mean infection rate (based on intracellular detection of p24 by flow cytometry) was 48% (range 15.4–74%) by 48 hours after infection.

NK-cell functional response

NK cell functional responses were measured using cryopreserved PBMCs in batches. Cryopreserved PBMC were rapidly thawed in warm media, washed and pre-activated for 12 h in R10/100 media (containing rhIL-2). Cells were washed and re-suspended in R10/100 medium containing 0.5 μlGolgistop (BD Biosciences), 5 μg/mlbrefaldin A (SIgma, St Louis, MO), and an antibody directed against CD107a; cell culture was continued for a further 6 h.

For PMA/Ionomycin stimulation of cells, Phorbol-12-myristate-13-acetate (PMA) (Sigma, St Louis, MO) plus Ionomycin (Sigma, St Louis, MO) was added at 1 μg/ml and 0.5 μg/ml, respectively. For experiments in which PBMC were cocultured with autologous (HIV infected or uninfected) CD4+ T-cell blasts, or with 721 cells, a 1:1 ratio of PBMC to target cells was used. Sample viability and cell counts were determined by staining with Viacount (Millipore) and run on a Guava PCA (Millipore). The cells were stained with Yellow Viability Dye (Invitrogen), anti-CD14 and -CD20 (to exclude nonviable cells, monocytes and B-cells), surface stained for with anti-CD3, -CD8, -CD56, -CD69 (BD biosciences) and -CD7 (Beckman Coulter) antibodies, then washed, fixed and permeabilised before staining intracellular with anti-Ki-67 and -IFN-γ antibodies (BD Biosciences). Because IL-2 causes loss of CD16 on NK cells [24], CD7 expression was used to classify viable, $CD14_{neg}$, $CD20_{neg}CD3_{neg}$ cells as NK cells. Stained cells were run on a BD LSRII and at least 250,000 cells were collected for analysis. The data were analyzed using Flowjo (Treestar). Fluorescent minus one (FMO) gates were used to determine the proportion of cells expressing CD107a, IFN-γ or Ki-67. A representative example of NK cell responses to the various stimuli assessed is shown in Supplementary Figure 1, http://links.lww.com/QAD/A238.

Statistical methods

Sample assay were conducted blinded to whether the sample was from a case or control and all were conducted prior to study product allocation in the CAPRISA 004 trial being unblinded. Comparisons were made using a non-parametric Mann-Whitney test in GraphPad Prism v5 (GraphPad Software). All other statistical analyses were conducted in SAS v9.2 (SAS Institute, Cary, North Carolina). A logistic regression model was performed using the "Proc logistic" procedure in SAS. The "/stepwise" option implemented in "Proc logistic" was used for stepwise regression models.

Results

Natural Killer cells from HIV acquirers (cases) had diminished IFN-γ responses toHLA-deficient 721.221 cells

To determine whether NK cells from cases (HIV acquirers) differed from controls (HIV non-acquirers) in their ability to recognize non-self targets, NK cell effector functions were measured after 6 h of co-culture with 721.221 cells, an HLA deficient B-cell line. This assay has been routinely used to quantify NK cell functional capacity [25]. In these experiments, cases demonstrated a lower proportion of IFN-γ secreting NK cells than controls (median %NK cells secreting IFN-γ after adjusting for background in cases vs. controls: 13.7% vs. 21.6%, $p = 0.03$, Fig. 1a). In contrast, a similar proportion of NK cells from cases and controls secreted IFN-γ after stimulation with PMA/Ionomycin (median %NK cells secreting IFN-γ in cases vs. controls: 56.6% vs. 40.7%, $p = 0.17$, Fig. 1b). There was no difference in NK cell functional responses *ex vivo*-in the presence of only IL-2-enriched media (median %NK cells secreting IFN-γ in cases vs. controls: 2.6% vs. 3.5%, $p = 0.77$, Fig. 1c). The NK cell response to 721.221 cells or to PMA/Ionomycin was not associated with HIV protection in adjusted logistic regression models (data not shown).

rhIL-2 activated Natural Killer cells from HIV acquirers (cases) and non-acquirers (controls) had distinct responses to autologous in-vitro infected CD4+ T-cells

Next, to mimic *in vivo* interactions between NK cells and HIV-infected target cells, rhIL-2 activated PBMC from cases and controls were cultured with autologous CD4+ T-cell blasts infected *in vitro* with a VSV-Gpseudotyped-HIV-1 (JRCSF) virus and functional responses assessed. A VSV-g pseudotyped virus was used in order to achieve high infection rates, and maximize interaction between NK cells and autologous CD4 T-cell blasts infected with

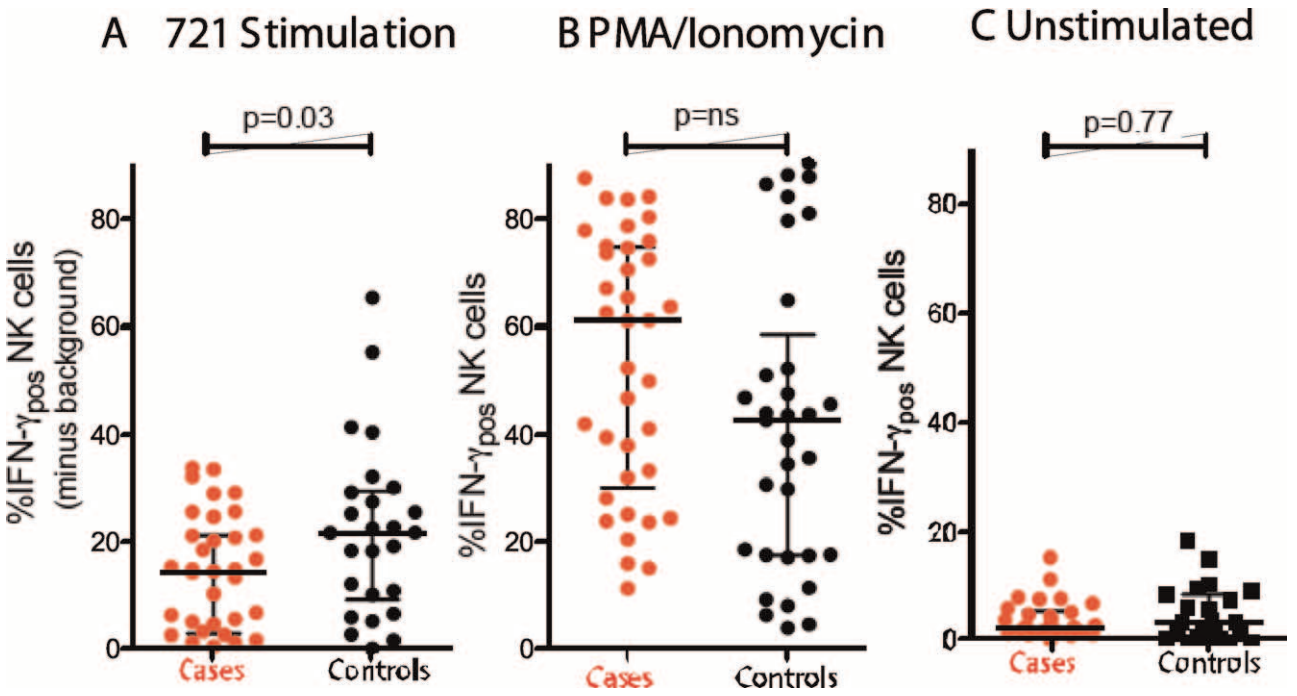

Fig. 1. The frequencies of IFN-γ secreting Natural Killer cells in cases (HIV-acquirers) compared with controls (HIV non-acquirers) were (a) greater following co-culture with 721.221 cells, (b) equivalent following PMA/Ionomycin stimulation, and (c) equivalent directly *ex vivo*. ● denotes cases, ■ denotes controls.

HIV. Uninfected autologous CD4+ T-cell blasts were included for comparison.

Overall, NK cell proliferative, degranulation and IFN-γ responses differed between cases and controls and showed divergent patterns of response after culture with HIV uninfected and HIV infected autologous T-cells (Fig. 2). Cases had fewer recently divided (Ki-67_{pos}) NK cells than controls after culture with uninfected or infected blasts (median %Ki-67_{pos} NK cells in cases vs. controls: after culture with uninfected T-cell targets: 8.55% vs. 38.05%, $p < 0.0001$, after culture with HIV infected T-cell blast targets: 8.47% vs. 29%, $p < 0.0001$, Fig. 2a). However, after subtracting responses to uninfected T-cell targets, cases had greater proliferating NK cells following culture with infected T-cell targets (median %Ki-67_{pos} NK cells in cases vs. controls: −0.5% vs. −7.6%, $p = 0.018$, Fig. 2b). Cases had greater proportions of degranulating ($CD107a_{pos}$) NK cells than controls (median %$CD107a_{pos}$ NK cells in cases vs. controls: after culture with uninfected target cells: 13% vs. 7.5%, $p = 0.003$, after culture with HIV infected target cells: 17.8% vs. 11.2%, $p = 0.018$, Fig. 2c). There was no difference between cases and controls in the proportion of degranulating NK cells after the data were adjusted by subtracting responses to uninfected T-cells (median %$CD107a_{pos}$ NK cells in cases vs. controls, 2.6% vs. 3.2%, $p = 0.61$, Fig. 2d). Among cases, the proportion of recently divided NK cells was correlated inversely with the proportion of degranulating NK cells ($r = -0.54$, $p = 0.0017$ after culture with uninfected target cells, $r = -0.51$, $p = 0.0023$ after culture with infected target cells) whereas in controls there was no significant correlation between these two effector functions. The pattern of IFN-γ secretory response after culture with uninfected T-cell blasts was similar in cases and controls (Fig. 2e), but cases had significantly lower proportions of IFN-γ-secreting NK cells after culture with infected T-cell blasts than controls (median %NK cells secreting IFN-γ in cases vs. controls: 5.93% vs. 8.44%, $p = 0.028$, Fig. 2e). After subtracting IFN-γ responses after culture with uninfected blast targets from those to infected blast targets, cases had a significantly lower IFN-γ responses to *in vitro* HIV infected autologous blasts than controls (median %NK cells secreting IFN-γ in cases vs. controls: 0.53% vs. 2.09%, $p = 0.007$, Fig. 2f).

Functional responses of NK cells to autologous HIV infected T-cell blasts are altered by HSV-2 infection

Since NK-cell responses to HIV may plausibly be altered by host factors and pre-existing infections, we sought to determine their contribution to NK cell responses to HIV-infected cells. Neither the degree of pre-existing concurrent NK cell activation (measured by CD38 or HLA-DR expression on unstimulated cells), nor age, nor gel arm were significant modifiers of IFN-γ responses to HIV infected T-cells. HSV-2 infection, however, significantly skewed responses after culture with HIV infected T-cell blasts and subtraction of response to uninfected blasts (median %NK cells secreting IFN-γin HSV-2 sero-negative vs. -positive cases and controls:

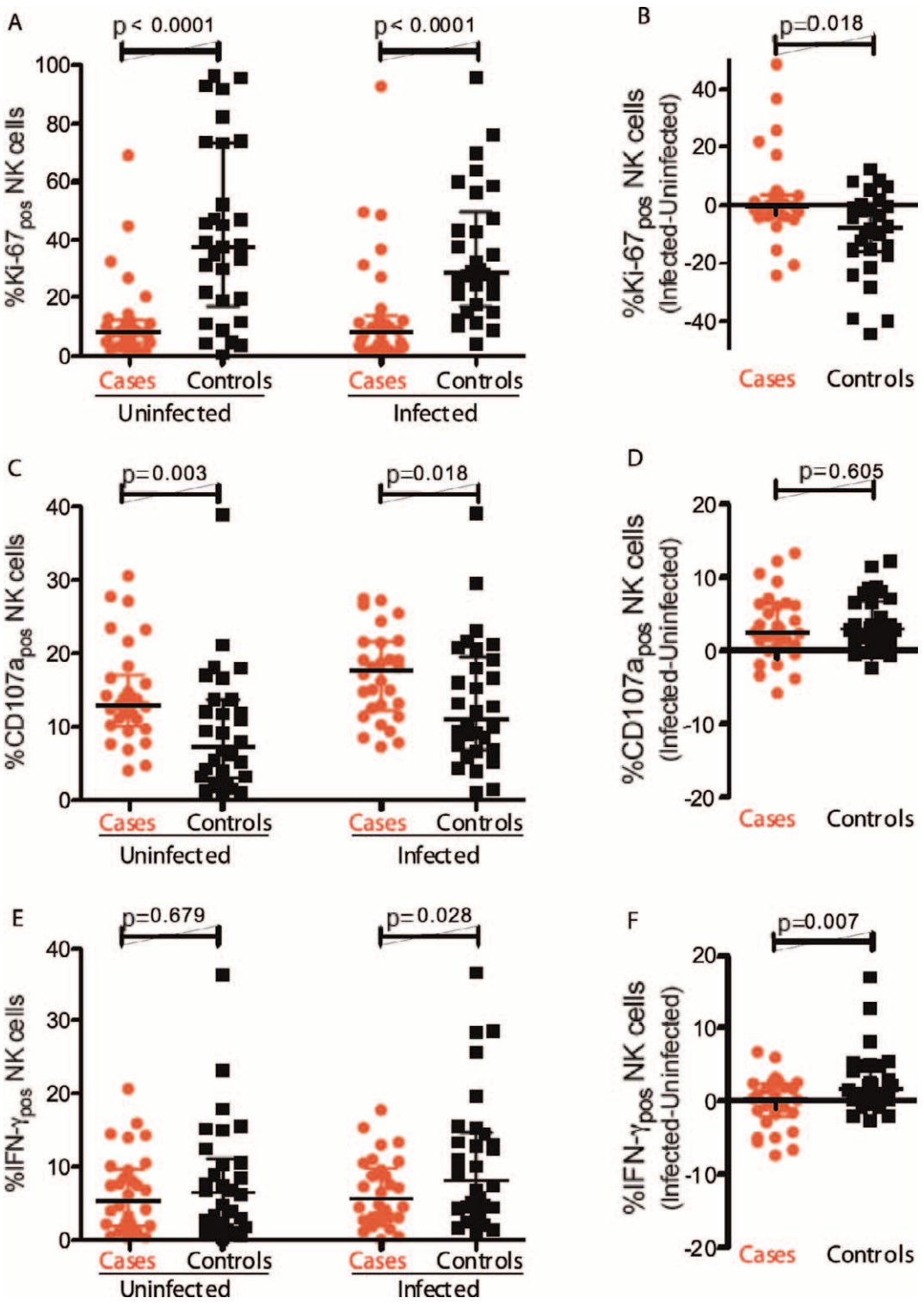

Fig. 2. Natural Killer cells from cases (HIV-acquirers) and controls (HIV non-acquirers) show different patterns of proliferation, degranulation and IFN-γ secretion following culture with HIV-infected and uninfected autologous CD4+ T-cell blasts. (a & b) Show the proportion of NK cells from cases and controls expressing Ki-67, a marker of recent cell division, (a) after cultured with HIV uninfected or infected autologous CD4+ T-cell blasts, or (b) when the response to uninfected T-cell blasts is subtracted from those of infected T-cell blasts. The proportion of NK cells from cases and controls expressing CD107a (a surrogate marker of degranulation) are shown in c & d and the proportion secreting IFN-γ are shown in e & f. In (d) and (f), asin (b), theresponses to HIV infected blasts minus responses to uninfected blasts are shown. ● denotes cases, ■ denotes controls.

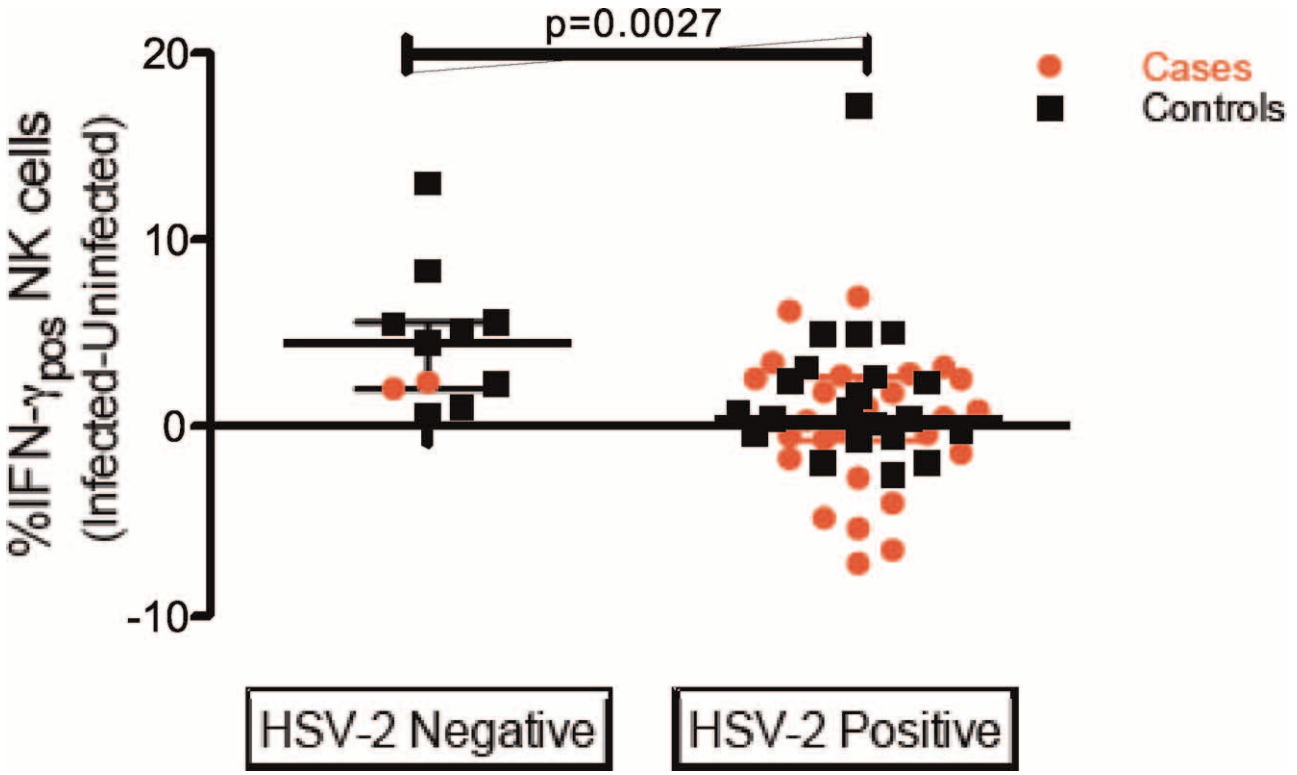

Fig. 3. Natural Killer cells from women with HSV-2 infection have significantly lower responses to *in vitro* HIV infected, autologous T-cell blasts. Herethe proportion of IFN-γ secreting NK cells after culture with HIV uninfected blasts is subtracted from the response to HIV infected blasts. ● denotes cases, ■ denotes controls.

4.6% vs. 0.6%, p = 0.003, Fig. 3). The frequency of HSV-2 infection was higher amongst cases (93%, 26/28-two individuals had weakly positive serology and were categorized as indeterminate and regarded as HSV-2 negative for this analysis) compared with controls (70%, 21/30, p = 0.03).

Natural Killer cell responses to autologous HIV infected blasts are associated with reduced HIV acquisition

To test whether NK cell functional responses to autologous T-cell blasts were of predictive value, over and above previously described risk factors for HIV acquisition, their relationship with HIV acquisition was modeled. In addition to NK cell functional responses to HIV infected cells (measured by Ki-67, CD107a or IFN-γ response to HIV-infected minus uninfected autologous T-cell blasts), the following covariates were included: age, gel arm, HSV-2 serostatus,and NK-cell CD38 expression-as we recently found reduced levels to be a robust marker of innate immune activation linked to enhanced HIV acquisition risk [26]. In both univariate and multivariate logistic regression models the quantitative trait of NK cell IFN-γ secretion following stimulation with autologous HIV infected cells, was associated with reduced HIV acquisition (Odds Ratio for HIV acquisition: 0.582 95% CI 0.347–0.977, p = 0.04, Table 1).

Although the association between NK cell IFN-γ response and HIV acquisition was expectedly weakened when HSV-2 serostatus was added to the model (OR with HSV-2 serostatus as a covariate:0.656 vs. OR without HSV-2 serostatus as covariate:0.546), the association was significant with or without HSV-2 serostatus in the model (Table 1 footnote). Finally, a stepwise logistic regression approach found that women who were in the tenofovir gel arm, of increased age, had increased NK cell CD38 expression(correlated with lower immune activation) or had greater proportion of IFN-γ_{pos} NK cells that responded to HIV infected autologous cells by secreting IFN-γ, had reduced odds of HIV acquisition. In summary, IFN-γ NK cell responses to HIV infected autologous cells were associated with protection from HIV acquisition independently of other covariates and consistently across the multivariate models.

Discussion

In this study of women at high-risk for HIV acquisition, those who acquired HIV infection had NK cells withlower IFN-γ responses to 721.221 cells and to autologous HIV-1 infected CD4+ T-cell blasts, prior to infection. Impaired NK cell IFN-γ responses to autologous HIV-1 infected CD4+ T-cell blasts were

Table 1. Logistic regression models of HIV acquisition.

	Univariate Regression Unadjusted OR (95% CI)	*P* value	Multivariate Regression Adjusted OR (95% CI)	*P* value	Stepwise Regression OR (95% CI)	*P* value
Age (per year increase)	**0.875 (0.783–0.976)**	**0.017**	**0.722 (0.567–0.920)**	**0.008**	**0.713 (0.566–0.897)**	**0.004**
Tenofovir vs. Placebo	0.381 (0.111–1.163)	0.09	**0.055 (0.004–0.636)**	**0.0202**	**0.04 (0.004–0.412)**	**0.007**
HSV-2 positive vs. negative)	5.155 (0.998–26.316)	0.050	1.851 (0.101–33.333)	0.678	–	–
$CD38_{pos}$ NK cells	**1.062 (1.022–1.101)**	**0.02**	**1.111 (1.036–1.192)**	**0.003**	**1.116 (1.044–1.193)**	**0.001**
IFN-γ_{pos} NK cells	**0.769 (0.632–0.935)**	**0.008**	**0.582 (0.347–0.977)***	**0.040**	**0.656 (0.457–0.940)**	**0.022**
Ki-67_{pos} NK cells	1.034 (0.998–1.070)	0.065	1.041 (0.982–1.104)	0.177	–	–
$CD107a_{pos}$ NK cells	0.967 (0.854–1.095)	0.593	1.156 (0.851–1.572)	0.353	–	–

*If HSV-2 is omitted from the multivariate model, then the adjusted OR for HIV acquisition is 0.546 (95% CI: 0.350-0.879), p = 0.01.

associated with HIV acquisition even after adjusting for known risk factors for HIV including HSV-2 infection. Although HSV-2 infection markedly reduced NK cell IFN-γ responses to autologous HIV-1 infected CD4+ T-cells, the association between NK cell responses and HIV infection was observed even after adjusting for the presence or absence of HSV-2 infection. The proliferative and degranulation response of NK cells to autologous T-cell blasts that were uninfected or infected *in vitro* with HIV was lower and higher, respectively,in cases compared with controls. These results are consistent with a model in which NK cells, as early responders to viral exposure, play an important role in protection from HIV acquisition.

This study prospectively measured NK cell proliferative, degranulation and IFN-γ responses in the absence of stimulation, against 721.221 cells and against autologous HIV infected cells. This approach allowed a more comprehensive assessment of NK cell function against HIV in a model that simulates *in vivo* interactions between NK cells and targets during HIV exposure. Further, the controls in this study were women at high risk for HIV, who did not acquire HIV and were drawn from a well-characterized clinical trial population [22], thereby avoiding the possible bias of comparing at-risk to not-at-risk individuals,as encountered in previous studies [14,16–20]. Enhanced NK cell IFN-γ responses have been reported from cross-sectional studies [17,19] in HIV exposed intravenous drug users and sexually-exposed adults in response to K562 cells or PMA/Ionomycin respectively, in comparison with low-risk seronegative or HIV infected individuals. Further, NK cell IFN-γ responses to specific HIV-derived peptides were associated with protection against mother-to-child transmission of HIV [27]. Our findings suggestthat NK cell responses play a role in preventing sexual transmission of HIV, asdiminished IFN-γ responses increase the risk of acquisition of HIV in women.Although the differences in NK-cell IFN-γ responses that we discovered were small, similar low responses to autologous HIV infected cells have been reported previously [28]. We speculate that only a low frequency of NK cells respond to HIV infected cells; however these cells likely activate other cells of the innate immune system to provide protective immunity. Recent findings of enhanced NK cell and dendritic cell cross-talk in HIV exposed, uninfected individuals provide further support for such a model [29].

Although we used a novel assay to mimic NK cell function in vivo in HIV exposed individuals, the response to 721.221 cells largely mirrored responses to autologous HIV-infected cells. Thus, functional assays using HLA-null cells could be potential alternative to the demanding assay described here, but these assays will not capture the role of activating KIR in HIV responses as NK cell responses to HLA-null cells are through abrogation of inhibitory KIR signaling. Moreover, the assay as described here may be a proxy for in vitro inhibition assays and useful for mechanistically probing responses targeted against HIV-infected cells, by for example, selective receptor blockade.

The analyses presented here took into account possible confounders, including HSV-2. HSV-2 has been shown to increase the number of NK cells and their activation [30] while diminishing NK cell cytotoxicity via a secreted peptide [31]. Our findings suggest that HSV-2 infection may also impact NK cell responses to autologous HIV infected cells. Further research is required to establish whether enhanced HIV acquisition and disease progression [32,33] is mediated by diminished NK cell responsesor whether HSV-2 acquisition and impaired NK cell function are caused by a proximal factor.

Some limitations of this study warrant attention. Firstly, this case-control study cannot demonstrate causal relationships between NK cell responses and HIV acquisition. The HIV status of the women's partners and the exact date on which they were exposed to HIV was unknown. The assays of NK cell function described here were however performed within a few months prior to prospectivelydeterminedHIV acquisition. From the high background prevalence of HIV (25% in the population screened for the CAPRISA004 trial) and the similar HIV-risk profiles of the cases and controls, it can be inferred that both cases and controls were at similarly high risk of sexual exposure to HIV. A larger control population may have given better estimates of NK

cell function in HIV exposed but uninfected controls. Secondly, our data do not directly answer whether the HIV-specific response against autologous HIV-infected T-cells measured here were directed against HIV-derived peptides, stress-induced peptides or may have constituted NK cell memory [34,35] selectively induced in controls. This will require further mechanistic studies. Thirdly, because only a single timepoint for cases and controls was studied here, it will be important for future studies to examine specimens from earlier timepoints to establish whether the reduced proliferative and enhanced IFN-γ secretory response in cases is due to transient or sustained reduction in cases' NK cell function.

Finally, although IFN-γ responses can be plausibly linked to anti-HIV activity [36] and are thought to be protective in other models of HIV risk [37], their mechanism of protection remains elusive. The extent to which these systemically measured responses are present at the mucosal sites of HIV exposure [38], and whether other cell types [39] are involved in the mechanism of protection in heterosexual women requires further investigation.

In summary, our data showed thatimpaired HIV specific NK cell IFN-γ responseswere associated with HIV acquisition in high-risk women. Methods to enhance HIV-specific NK cell responses may be a new way to reduce the spread of HIV in the millions of young women at high risk for infection.

Acknowledgements

We pay tribute to the participants of CAPRISA 004; women whose dedication and commitment to improving their and their peers' health and kindly donating samples during the conduct of the trial make this research possible. We gratefully acknowledge Lise Werner for advice on the statistical analysis. We thank the Africa Center for Health and Population Studies at the University of KwaZulu-Natal for the use of their BSL2+ bio-containment facility.

Author's contributions V.N. designed the case control study and experiments, performed the data collection and analysis and co-wrote the manuscript. MA, TN and WHC supervised the data collection and co-wrote the manuscript. SSAK and Q.K designed, conducted and analysed the parent CAPRISA 004 trial, co-designed the case control study and co-wrote the manuscript. S.S.A.K. supervised statistical analyses.

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Competing Interests: SSAK and QAK were the co-Principal Investigators of the CAPRISA 004 trial of tenofovir gel. QAK is co-Principal Investigator of the HIV Prevention Trials Network, which undertook the HPTN 052 trial of treatment for prevention. SSAK is an executive committee member of the Microbicide Trials Network, which is undertaking the VOICE trial of oral and topical PrEP.SSAK and QAK are also co inventors of two pending patents (61/354.050 and 61/357,892) of tenofovir gel against HSV-1 and HSV-2 with scientists from Gilead Sciences.Gilead Sciences did not have any role in the experiments or analyses presented here.

Clinical trials registration number of parent trial: NCT00441298.

Conflicts of interest

None declared.

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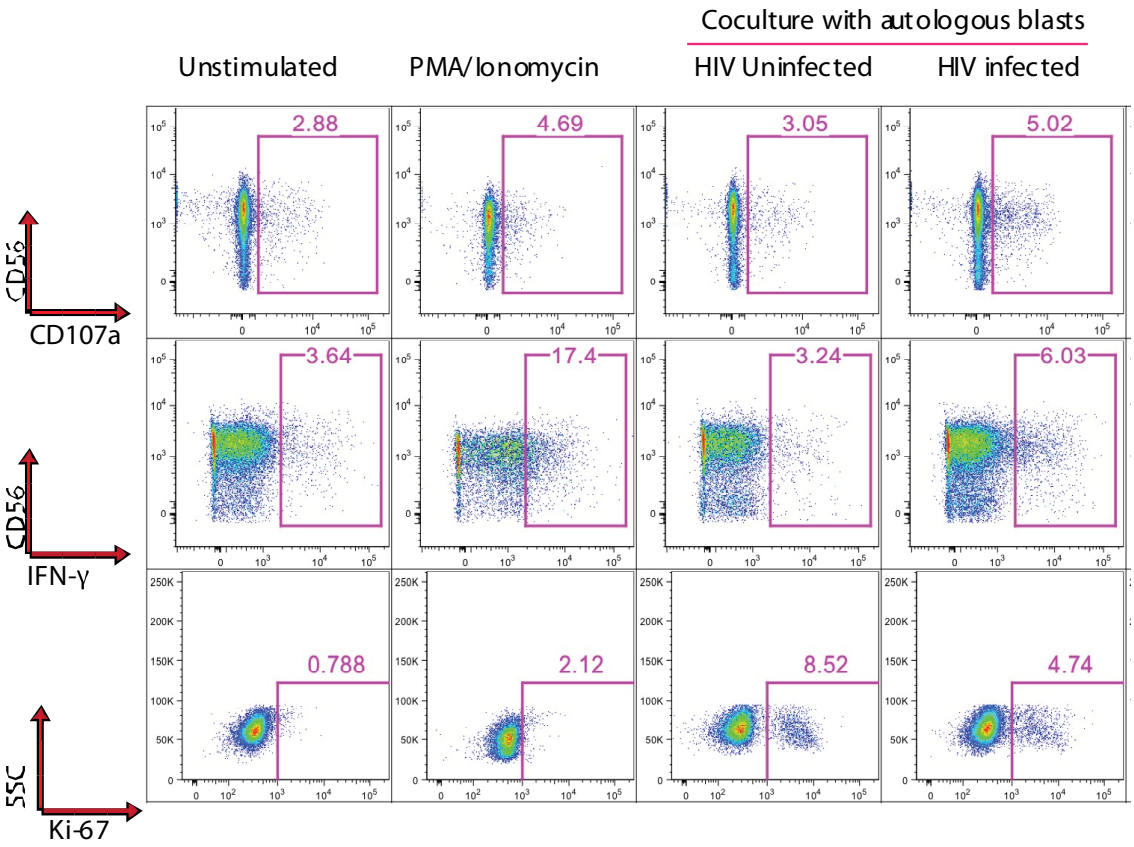
List of supplementary material:

1. Supplementary Figure 1: Representative results of flow cytometric analyses of Natural Killer cells from one representative donor showing each response measure (indicated along y-axis) under each stimulation condition (indicated along x-axis).

Supplementary Figure 1



CHAPTER SIX:

Innate Immune activation enhances HIV-1 acquisition in women, diminishing the effectiveness of tenofovir microbicide gel

J Infect Dis. 2012 Oct 1;206 (7):993-1001. Epub 2012 Jul 24.

MAJOR ARTICLE

Innate Immune Activation Enhances HIV Acquisition in Women, Diminishing the Effectiveness of Tenofovir Microbicide Gel

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The antiretroviral agent, tenofovir, formulated as a vaginal microbicide gel, reduces human immunodeficiency virus (HIV) acquisition by 39% in women. This study assessed the role of preexisting immune activation in HIV acquisition in women from the CAPRISA 004 trial, to identify potential strategies to increase the effectiveness of tenofovir gel. Systemic cytokine and cellular immune mediators (platelets and natural killer [NK] cells) were assessed in women at high risk for HIV assigned to either tenofovir or placebo gel in the CAPRISA 004 trial. Notwithstanding tenofovir gel use, women who acquired HIV had significantly higher systemic innate immune activation prior to infection than women who remained uninfected. Activation of both soluble (cytokine) and cellular (NK cells) immune mediators were associated with HIV acquisition, individually or in combination. Hence, an innate immune activation suppressant could be added to tenofovir gel as a potential combination gel strategy in developing the next generation of higher efficacy antiretroviral microbicides.

Women comprise just over half of the 33.3 million people living with human immunodeficiency virus (HIV) globally [1]. Each year there are 2.6 million new infections [1]. The highest prevalence is in sub-Saharan Africa, where young women (15–24 years) bear the brunt of this epidemic [2]. Reducing HIV infection rates in young women is key to altering current epidemic trajectories and is the impetus for developing prevention modalities for women [3]. Results from preexposure prophylaxis (PrEP) studies of both oral and topical formulations of antiretroviral drugs signal new hope for preventing sexual transmission in young women [4]. Notwithstanding the 2 oral PrEP trials [5, 6] that have been stopped for futility, the 3 positive trials involving women [7, 8] demonstrate partial protection that ranges from 39% to 73%. New technologies that further enhance the efficacy of antiretroviral agents are therefore needed.

The CAPRISA 004 study was a phase IIb, randomized, placebo-controlled clinical trial to assess the safety and effectiveness of 1% tenofovir gel in preventing HIV infection in women [9, 10]. The trial showed a 39% reduction in HIV infection. Even in the most adherent of women, protection was no higher than 54%. Thus, the occurrence of infections even in women using tenofovir gel highlights the need to identify strategies to enhance the effectiveness of tenofovir gel.

Some recent studies have suggested that higher levels of systemic immune activation are associated with the risk of HIV acquisition and concluded that a "quiescent

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immune" phenotype is protective [11–14], but others conclude that activation is protective [15–19]. To resolve this question we investigated the role of immune activation in HIV acquisition in the CAPRISA 004 trial.

METHODS

Subjects

This case–control study was nested in the CAPRISA004 Tenofovir gel trial [9, 10] (see supplementary methods). Samples from 44 HIV acquirers (cases) were studied at the last preinfection visit for which cryopreserved plasma and peripheral blood mononuclear cells (PBMCs) were available (mean, 12.1 months post–trial enrollment). Samples from 37 women were selected as controls if they reported the highest sexual exposure but remained uninfected during the trial (HIV nonacquirers), at the visit at which they reported the greatest frequency of sex in the preceding month (mean, 7.8 months post–trial enrollment). This study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE073/010). Participants gave informed consent for their samples to be used for these studies.

Study Procedure

HIV serostatus, safety, sexual behavior, and gel and condom use were assessed at monthly follow-up visits for up to 30 months postenrollment In addition, plasma and PBMCs were obtained and cryopreserved at 3, 12, and 24 months postenrollment.

Plasma Cytokine Quantitation

Plasma samples from cases and controls were assessed using a high-sensitivity human cytokine premixed 13-plex kit (Millipore, Billerica, Massachusetts) for 13 cytokines: GMCSF, interferon gamma (IFN-γ), interleukin (IL)-1β, IL-2 ,-4, -5, -6, -7, -8, -10, -12(p70), -13, and tumor necrosis factor α (TNF-α) per the manufacturer's protocol. Plasma was assayed in duplicate or triplicate after a single thaw, without dilution. Cytokine measures beneath the detection limit of the assay were given a value of the midpoint between zero and the lower detection limit of the assay and were included in the analysis.

Phenotypic Analysis

Natural killer (NK) cell and T-cell activation was measured in cryopreserved PBMCs in batch analyses using optimized flow cytometry methods [20]. Cryopreserved PBMCs were rapidly thawed in warm media, washed, and rested for 2 hours. Sample viability and cell counts were determined by staining with Viacount (Millipore) and run on a Guava PCA (Millipore). PBMCs were stained with yellow viability dye (Invitrogen), anti-CD14 and -CD20 antibodies (to exclude nonviable cells, monocytes, and B cells, respectively). In addition, the cells were stained with anti-CD3, -CD16, -CD38, -CD56, -CD69, -CD158a, -CD158b (BD Biosciences) and-CD158e1/e2 (Beckman Coulter) antibodies. Stained cells were run on a BD LSRII, and the data were analyzed using Flowjo (Treestar). NK cells were defined as viable, $CD14^{neg}$, $CD20^{neg}$, $CD3^{neg}$, and $CD16/56^{pos}$ lymphocytes. The frequency of NK cells that were $CD69^{pos}$, $HLA\text{-}DR^{pos}$, or $CD38^{pos}$ was determined using fluorescent minus one (FMO) gates. The frequency of T cells, defined as viable, $CD14^{neg}$, $CD20^{neg}$, $CD3^{pos}$ cells, expressing CD69 or both CD38 and HLA-DR was similarly determined.

NK-cell and T-cell effector functions were measured using cryopreserved PBMCs in batched analyses. Each of the following 4 NK-cell effector functions were quantified in the absence of exogenous stimulation, excepting IL-2 in the culture media: degranulation (using CD107a expression as a surrogate [21]), cytokine secretion (IFN-γ), activation (CD69), and recent cell division (using Ki-67 as a surrogate [22]). Cryopreserved PBMC were rapidly thawed in warm media, washed, and rested for 12 hours in media containing 50 U/mL IL-2. For experiments in which cells were stimulated with K562 cells (Supplementary Figure 1), NK cells from HIV-uninfected healthy donors were cultured with equal numbers of K562 cells. Cells were washed, resuspended in medium containing an antibody directed against CD107a, and cultured for a further 6 hours. Samples were processed for flow cytometry as detailed above with the following modification. Cells were surface stained with anti-CD3, -CD8, -CD56, -CD69 (BD Biosciences), and -CD7 (Beckman Coulter) antibodies, then washed, fixed, and permeabilized before staining with anti-Ki-67 and -IFN- γ (BD Biosciences) antibodies. To account for NK cells that have lost CD16 expression due to activation [23], CD7 expression was used to classify viable, $CD14^{neg}$, $CD20^{neg}$, and $CD3^{neg}$ cells as NK cells [24]. FMO gates were used to determine the proportion of cells responding with ≥1 of the 4 effector functions measured (CD107a, CD69, IFN-γ, or Ki-67). Boolean gates were used to enumerate the proportions of NK cells or CD8 T cells expressing any particular combination of the 4 effector functions. Spice (v5.2) and Pestle (v1.6.2) software (kindly provided by M. Roederer) were used to analyze these data.

Statistical Methods

Comparisons Between Cases and Controls

Assays were conducted blinded. All except the cytokine assays were conducted prior to the unblinding of the CAPRISA004 trial arm allocation. Comparisons were made using a nonparametric Mann–Whitney *U* test in GraphPad Prism v5 (GraphPad). The cytokine analysis was corrected for multiple comparisons using a false-discovery step-down procedure. The heatmap presented in Figure 1 was assembled in GeneCluster and visualized in TreeStar (Michael Eisen, Stanford University). All other statistical analyses were conducted in SAS v9.2 (SAS Institute, Cary, North Carolina). Logistic regression analyses were implemented in SAS v9.2 with the "Proc logistic" procedure.

Principal Component Analysis

Principal component analysis (PCA) was run on all variables that differed between cases and controls. Orthogonally rotated (varimax) components that had an eigenvalue of >1 (see Supplementary Figure 2 for the scree plot) and had at least 3 factors loading on it each with a loading score of >0.4, were included. The principal components (PCs), their eigenvalues, variance, and the important variables that composed a PC are shown in Supplementary Table 1.

RESULTS

Behavioral and Biological Risk Factors for HIV Acquisition in the CAPRISA 004 Trial

In the CAPRISA 004 trial population of 889 high-risk women (445 randomized to 1% tenofovir gel, 444 randomized to placebo gel), neither coital frequency (protected or unprotected by condoms), nor the number of sexual partners, nor symptomatic sexually transmitted infections (STIs) were strong predictors of HIV acquisition (data not shown). In contrast, younger age and prevalent herpes simplex virus type 2 (HSV-2) infection were predictive of HIV acquisition (data not shown). But taken together, these risk factors did not entirely explain HIV acquisition risk in this high-risk cohort of young women.

Nested Case-Control Analysis of Immunological Risk Factors for HIV Acquisition

To identify immunological risk factors for HIV acquisition in high-risk women from the CAPRISA 004 trial, we selected 44 cases who acquired HIV (cases), and 37 controls who, despite being at the highest sexual risk in the trial, did not acquire HIV. Baseline demographic characteristics, sexual behaviors during the trial and HSV-2 serostatus by group are detailed in Table 1. Controls had a higher average number of self-reported sex acts per month and a congruently higher average number of used vaginal gel applicators returned per month than cases. Cases were, on average, younger than controls. In total, 33 of 44 cases and 22 of 37 controls were in the Tenofovir treatment arm (p-0.41).

Cases Had a Proinflammatory Signature of Plasma Cytokine Expression

Cases had a distinctive pattern of plasma inflammatory cytokines prior to infection compared with controls (Figure 1). The cytokine profile (regardless of treatment arm assignment in the trial) of cases was marked mainly by higher concentrations of the proinflammatory and T-cell homeostatic cytokines TNF-α, IL-2, IL-7, and IL-12p70 (Table 2). In an unsupervised hierarchical cluster analysis, cases were clustered separately from controls (data not shown). To probe for the source or functional consequences of these cytokine differences, the cellular profiles of peripheral blood collected at the same timepoints were studied.

Cases Had Higher Numbers of Platelets Just Prior to HIV Acquisition

Consistent with a proinflammatory cytokine profile in plasma, cases had significantly higher platelet counts than did controls ($P = .04$, Table 2). Cases and controls had similar neutrophil and lymphocyte counts (Table 2). In contrast, there was no difference in the platelet count between cases and controls at study enrollment (data not shown). Next, we explored whether the differences in platelet counts extended to other components of the innate immune system.

Cases Had Higher Levels of Systemic Innate Immune Activation

Cases had significantly higher proportions of activated NK cells than did controls. The proportions of NK cells that

Table 1. Baseline Demographic Characteristics, Sexual Behavior, and Herpes Simplex Type 2 (HSV-2) Serostatus

Baseline	Cases (n = 44 women)	Controls (n = 37 women)	*P*
Demographic characteristics			
Mean age, years	23.3	27.6	<.001
Mean days preinfection at sample collection (range)	127 (15–404)		
Sexual behavior			
Mean no. of sex acts/month	5.7	11	<.001
Mean no. of returned used applicators/month	6	10.7	<.001
Mean no. of partners	0.9	1.2	.059
Overall mean self-reported condom use, %	87.1	84.6	.650
HSV-2 serostatus, no. (%)			
HSV-2 positive at baseline	28 (63.6)	21 (56.8)	.788
HSV-2 positive at exit	33 (75.0)	25 (67.6)	.693
Gel arm comparison group, no. (%)			
Tenofovir arm	33 (75)	22 (60)	.417

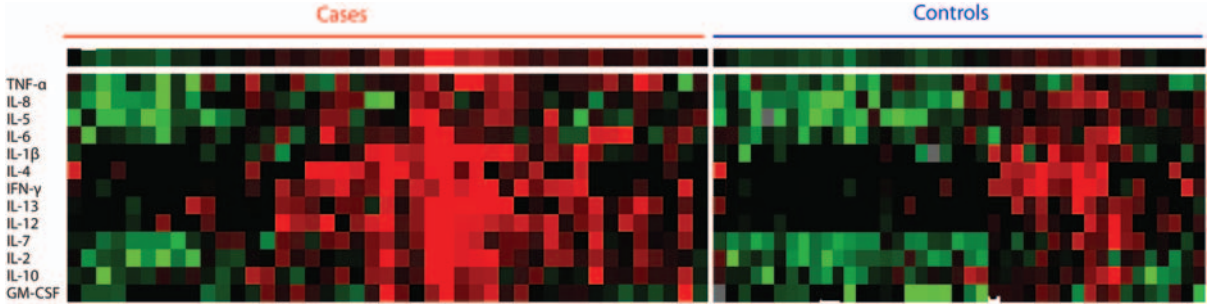

Figure 1. Heat map representation of 13 plasma cytokines measured in cases and controls. Color changes in the spectrum green to red denote increasing levels of cytokines relative to the median for all participants.

Table 2. Plasma Cytokine Levels, NK-Cell and T-Cell Activation, and Peripheral White Blood Cell Counts

Variable	Cases (n = 44 women)	Controls (n = 37 women)	*P*
Median plasma cytokine, pmol/L (IQR)			
IL-10	7.82 (4.50–18.93)	5.00 (2.46–9.18)	.101[a]
IFN-γ	0.36 (0.15–2.84)	0.15 (0.15–0.95)	.101[a]
TNF-α	2.16 (1.26–3.17)	1.21 (1.01–1.98)	.017[a]
IL-2	0.30 (0.15–1.07)	0.10 (0.06–0.28)	.003[a]
IL-4	0.07 (0.07–2.02)	0.07 (0.07–1.36)	.213[a]
IL-6	0.67 (0.28–1.51)	0.48 (0.24–1.12)	.338[a]
IL-7	0.55 (0.21–1.09)	0.12 (0.06–0.34)	.003[a]
IL-1β	0.03 (0.03–0.11)	0.03 (0.03–0.05)	.084[a]
IL-5	0.08 (0.03–0.19)	0.08 (0.08–0.14)	.445[a]
IL-12p70	0.68 (0.06–3.19)	0.06 (0.06–0.11)	.007[a]
IL-13	0.53 (0.24–1.52)	0.24 (0.24–0.24)	.053[a]
IL-8	0.60 (0.26–0.99)	0.59 (0.21–1.15)	.873[a]
GMCSF	0.23 (0.15–0.49)	0.23 (0.23–0.25)	.062[a]
Median peripheral blood cell counts (IQR)			
Platelets ($\times 10^9$/L)	345 (277–383)	289 (257–355)	.038
WBC	6.76 (4.79–8.01)	5.89 (4.99–7.41)	.763
Neutrophil	3.50 (2.39–4.57)	3.08 (2.31–4.36)	.647
Lymphocyte	2.11 (1.91–2.65)	2.32 (1.6–2.78)	.289
Median NK-cell activation (IQR)			
%HLA-DR^{pos}	7.43 (4.81–11.10)	4.08 (2.37–5.61)	.0001
%CD38^{pos}	46.40 (35.00–55.90)	67.0 (49.8–76–95)	<.0001
%CD69^{pos}	0.07 (0.4–0.15)	0.04 (0.01–0.09)	.049
Median (total) T-cell activation (IQR)			
%CD38^{pos} and HLA-DR^{pos}	0.93 (0.66–1.14)	1.08 (0.77–1.56)	.370
%CD69^{pos}	0.17 (0.10–0.31)	0.15 (0.7–0.30)	.350

Abbreviations: GMCSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IQR, interquartile range; NK, natural killer; TNF, tumor necrosis factor; WBC, white blood cell.

[a] Adjusted for multiple comparisons using a false discovery rate step-down procedure

expressed HLA-DR, a marker of chronic activation and CD69, a marker of recent activation were raised in cases relative to controls ($P = .0001$ and $P = .05$, respectively, Figure 2*A* and Table 2). In addition, cases had markedly lower proportions of CD38 expressing NK cells than controls ($P < .0001$, Figure 2*B* and Table 2). In an independent analysis, we observed CD38 down-regulation following in vitro stimulation with K562 cells (Supplementary Figure 1). Thus, lower CD38 expression on NK cells is consistent with higher activation, unlike T cells.

To validate these findings as of functional relevance, the effector function of NK cells in cases and controls was studied. In the absence of in vitro stimulation excepting for IL-2 in the culture medium, cases had a greater proportion of degranulating NK cells than controls (median % CD107a^{pos} NK cells in cases: 8.5%, interquartile range [IQR], 6.08%–11.58% vs controls: 4.8%, IQR 2.52%–7.48%: $P = .0004$). Cases also had fewer recently divided NK cells than controls (median, %Ki-67^{pos} NK cells in cases: 8.94%, IQR 3.99%–18.85% vs controls: 17.1%, IQR 10.0%–24.03%: $P = .003$), but the proportion of unstimulated NK cells secreting IFN-γ was similar (median, % IFN- γ^{pos} NK cells in cases: 2.43%; IQR, 1.65%–4.95% vs controls: 3.32%; IQR, 0.83%–8.04%, $P = .93$).

To account for the observed differences in NK-cell activation between cases and controls, NK-cell maturation status and killer immunoglobulin–like receptor (KIR) genotypes and phenotypes were examined. There was no difference in the proportion of NK cells expressing CD57, a marker of lymphocyte differentiation (mean %CD57+ NK cells, 56.7% vs 50.7% for cases and controls, respectively; $P = .16$) Furthermore, there were no differences in KIR genotypes between cases and controls in this study. In this study only 1 donor possessed the *KIR3DS1* gene, which has been previously implicated in HIV disease pathogenesis [25, 26]. Finally, there was no difference between cases and controls in the proportion of NK cells expressing KIRs for which commercial antibodies for flow cytometric staining were available (data not shown). Although others have shown that HSV-2 infection is associated with

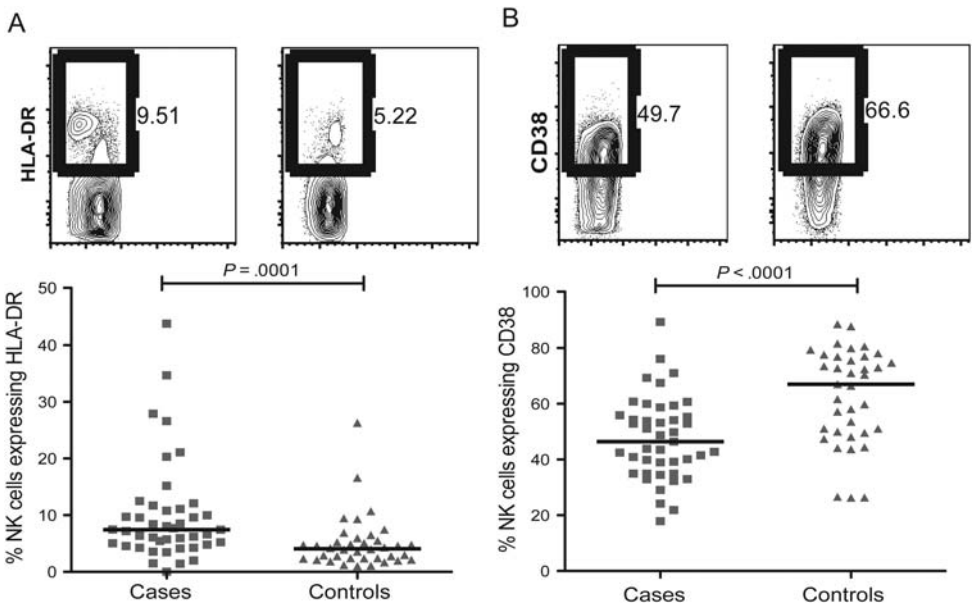

Figure 2. Cases had higher levels of natural killer (NK) cell activation in blood. *A–B*, Proportion of NK cells expressing (*A*) HLA-DR or (*B*) CD38 shown as representative flow cytometry plots from a case (*left*) in comparison to a control (*right*) and summary graphs of the same (*below*). Squares, cases; triangles, controls.

elevated white blood cell counts and increased NK cell degranulation in HIV-coinfected individuals [27], there was no difference in NK cell activation between HSV-2 seropositive and seronegative individuals in this study (data not shown).

Cases Had Higher Levels of Spontaneous Cytotoxic T-Cell Degranulation

The degree of bulk T-cell activation using conventional markers of activation (coexpression of CD38 and HLA-DR) was similar between cases and controls ($P = .37$, Table 2). We did not measure activation of T-cell subsets. In contrast, the proportion of degranulating cytotoxic (CD8) T cells in the absence of in vitro stimulation (excepting for IL-2 in the culture medium) was significantly higher among cases compared to controls ($P = .0026$, Figure 3).

In a Principal Component Analysis of Immunological Drivers of HIV Acquisition, Innate Immune Activation Was Predictive of Acquisition

To conduct a more comprehensive analysis, the putative factors related to acquisition were subjected to a principal components analysis. Four PCs with eigenvalues >1 were extracted (Supplementary Figure 2) and collectively explained almost 70% of the variability in activation measures. Each PC measured a qualitatively different immune response, which were labeled accordingly (Supplementary Table 1): PC1-T-cell homeostatic cytokines, PC2-innate immune activation, PC3-innate immune quiescence, and PC4-innate immune activation (independent of $CD8^{pos}$ T-cell degranulation). The PCs were included in an adjusted logistic regression model of HIV acquisition.

In this logistic regression analysis, adjusted for treatment arm, age and HSV-2 serostatus, PC2-innate immune activation was a significant risk factor for HIV acquisition (OR, 11.27; 95% CI: 1.84–69.09, $P = .009$, Table 3). Conversely and in congruence, PC3-innate immune quiescence was a significant independent protective factor (OR, 0.06; 95% CI: .013–.33, $P = .001$, Table 3).

Finally, to exclude tenofovir treatment as a confounding effect, and to determine whether immune activation enhances HIV acquisition over and above the protective effect of tenofovir gel, the logistic regression was repeated focusing on the 2 measures of activation most divergent between cases and controls in the stratum of women who were using tenofovir gel. In this analysis, which was also adjusted for age, HSV-2 infection, and HLA-DR expressing NK cells, a reduced proportion of CD38-expressing NK cells was independently associated with HIV acquisition among women who were using tenofovir gel (OR, 0.90; 95% CI: .82–.97, $P = .005$, Table 4). In summary, after adjusting for multiple potential confounders, systemic innate immune activation measured collectively in a principal component or by a reduced proportion of CD38 expressing NK cells in blood was associated with HIV acquisition.

DISCUSSION

In this study systemic innate immune activation was associated with HIV acquisition, even among women using tenofovir

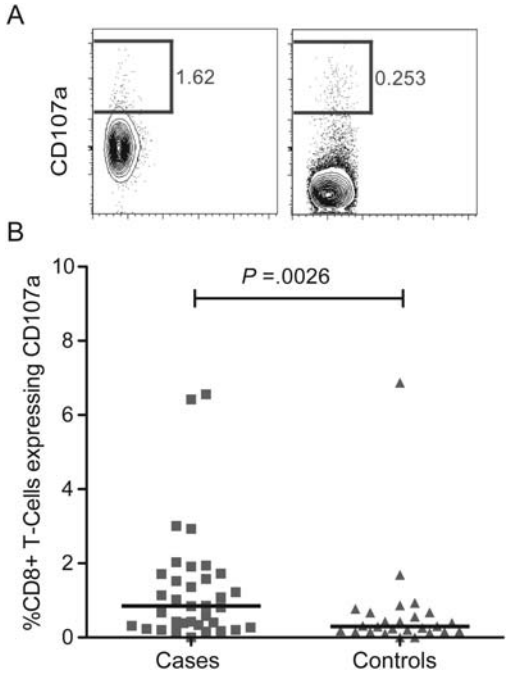

Figure 3. Cases had higher levels of spontaneous CD8 T-cell degranulation. *A–B*, Proportion of $CD8^{pos}$ T cells expressing CD107a shown as (*A*) representative flow cytometry plots from a case (*left*) in comparison to a control (*right*), and (*B*) a summary graph of the same. Squares, cases; triangles, controls.

gel. Relative to women at high HIV risk who remained uninfected, women who acquired HIV had higher levels of proinflammatory cytokines, platelets, activated NK cells, and spontaneous cytotoxic T-cell degranulation in blood. Many of these various measures of immune activation were predictors of HIV acquisition independent of other risk factors but when these factors were assembled into principal components, innate immune activation was a strong predictor of HIV acquisition. We infer from these collective data that systemic innate immune activation enhances HIV acquisition, and thus modulating activation may provide a new strategy for improving the efficacy of antiretroviral agents in preventing HIV acquisition.

Table 3. Logistic Regression Model for HIV Acquisition in Case–Control Analysis Including Principal Components

Component	Adjusted Odds Ratio (95% CI) for HIV Acquisition	*P*
Placebo vs tenofovir	7.15 (1.02–50.28)	.048
$HSV\text{-}2^{pos}$ vs $HSV\text{-}2^{neg}$ serostatus	21.85 (1.31–364.45)	.031
Age (per year increase)	0.72 (.58–.88)	.001
PC 1: "T-cell homeostatic cytokines"	9.36 (.35–247.90)	.181
PC 2: "Innate immune activation"	11.27 (1.84–69.09)	.009
PC 3: "Innate immune quiescence"	0.06 (.013–.33)	.001
PC 4: "NK-cell activation independent of $CD8^{+}$ T-cell degranulation"	1.65 (.69–3.95)	.260

Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; HSV, herpes simplex virus; NK, natural killer; PC, principal component.

Table 4. Logistic Regression Model for HIV Acquisition: Tenofovir Gel Arm Only Including Individual Measures of NK-Cell Activation

Component	Adjusted Odds Ratio (95% CI) for HIV Acquisition	*P*
$HSV\text{-}2^{pos}$ vs $HSV\text{-}2^{neg}$	77.33 (.90–999)	.056
Age (per year increase)	0.67 (.48–.91)	.011
%$CD38^{pos}$ NK cells (per 1% decrease)	1.11 (1.03–1.22)	.005
%$HLA\text{-}DR^{pos}$ NK cells (per 1% increase)	1.38 (.99–1.92)	.058

Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; HSV, herpes simplex virus; NK, natural killer.

The search for risk factors for HIV has yielded an array of genetic, innate, humoral, and cellular immune response factors that differentiate highly exposed seronegative (HESN) individuals from healthy controls [28]. However, many promising correlates have not been reproducible: some studies conclude that a quiescent immune phenotype is protective [11–14], whereas others conclude that activation is protective [15–19]. These divergent findings may be because of differences in comparison groups (HESN to healthy controls—women with low/no sexual risk for HIV—or HIV-infected patients) and the definition of exposure used. We propose that subsequent HIV acquisition is a more accurate measure of HIV exposure. In the CAPRISA 004 trial of tenofovir gel, conventional risk factors for HIV acquisition [29] did not fully account for overall HIV risk. This confirmed the relative homogeneity in the risk profiles of the trial participants and the underlying population [2]. Further, it validated the search for additional immunological risk factors by comparing preinfection samples from women who acquired HIV (cases) to samples from those who did not (controls).

Women who acquired HIV had higher levels of systemic innate immune activation than those who remained HIV negative despite high risk exposure. Platelets, which are closely intertwined with the innate immune system [30], were also more abundant in the blood of cases than controls, consistent with our data from a similar population in a previous study [31]. NK cells are the first line of defense against a variety of intracellular viral pathogens and are potently equipped to kill HIV-infected cells or to recruit other cells [32]. Thus it was

reasoned that their basal state of activation broadly portrays the extent of innate immune activation and thereby may correlate with HIV endpoints. Using several different measures of activation, NK cells from cases were more activated and more avidly degranulated than those from controls. An activated NK-cell phenotype associated with higher levels of degranulation and lower proliferation is reminiscent of a more differentiated or exhausted NK cell. But the finding that a similar proportion of NK cells in cases and controls expressed CD57, a marker of differentiation [33], suggests that this does not account for differences in NK-cell activation and function in this study.

Our data suggest reduced CD38 expression on NK cells is an activation phenotype associated with HIV. Ligation of CD38 on NK cells results in proliferation, cytokine production, and HLA-DR expression [34–36] but because CD38 colocalizes with CD16 [37, 38] and CD16 is rapidly downregulated on NK-cell activation [23], we conclude that in this context, lower frequencies of CD38 expressing NK cells are consistent with prior NK-cell activation. But the role of CD38 in HIV-infection is complex [39].

Although this study had a similar sample size to other studies that have found specific KIR or KIR-HLA compound genotypes associated with protection from HIV acquisition [40–42], we did not find evidence to support this. High genetic diversity of KIR genes has been described among sub-Saharan Africans [43]. Thus, power to detect genetic associations in this study was limited.

Two limitations of this study warrant attention: its small size and the paucity of longitudinal measures of activation. Despite the increased Type II error because of a small sample size, we found marked differences that remain statistically significant after correcting for multiple comparisons. These differences withstood rigorous testing in adjusted regression models. Second, because of the rarity of stored samples (particularly PBMCs) in the CAPRISA 004 trial, the availability of and access to longitudinal samples was limited. Nevertheless, for women who did not acquire HIV, NK-cell and T-cell activation was similar at the highest sex visit (reported here) compared to the last visit in the trial for controls (data not shown). Hence, it could be inferred that the single timepoint measurement accurately portrayed underlying activation.

A particular strength of this analysis is that it compares women who acquire HIV to women who do not acquire HIV followed prospectively. The participants were drawn from a clinical trial population conducted in a community with high background HIV incidence rates. Jennes and colleagues have reported an immune quiescence phenomenon in highly exposed seronegative African women in comparison to healthy women [14]. Our data are consistent with these observations and suggest that prior to infection, women have a potentially modifiable set of immunological parameters that are strongly associated with their risk of HIV acquisition. The temporal relationship between the presence of inflammation prior to infection and HIV acquisition and the high degree of consistency across multiple measures of systemic immune activation increase the likelihood that systemic immune activation is causally related to HIV acquisition. But what biological plausibility is there for immune activation as a driver of acquisition?

There are at least 2 potential mechanisms by which innate immune activation may drive acquisition. First, activated T cells are more susceptible to HIV infection and support higher rates of viral replication [44]. We did not observe evidence of bulk T-cell activation, but importantly we did not measure activation on T-cell subsets ($CD4_{pos}$ or $CD8_{pos}$ T cells) in peripheral blood, nor at the site of HIV exposure (genital tract). But an increased frequency of innate lymphocytes that activate these cells at the genital tract mucosa or regional lymph node level may (1) recruit potential target cells to the site of infection [45] or (2) increase the likelihood that the initial viral reproductive rate R_0 will exceed 1 ($R_0 > 1$), thereby establishing productive infection [46]. In additional concordant studies done in this study population, reported higher cervicovaginal cytokine concentrations in women who acquired HIV [47]. Further work to measure T-cell subset activation and the crosstalk between genital and systemic activation is warranted. Second, in the presence of generalized systemic immune activation, the ability to generate a protective immune response may be compromised. Immune activation is associated with greater rates of immunosenescence, disruption of anatomic niches, and excessive bystander apoptosis [48]. Consequently, these effects may impair defense against HIV, aside from their effects on defense against other pathogens.

Perhaps the most promising finding from this study is the identification of a potentially modifiable risk factor for HIV. If methods to dampen immune activation were available, the efficacy of tenofovir gel may be improved. One approach is to identify and target the upstream drivers of immune activation. The finding of systemic activation suggests that a multipotent factor may be driving activation, perhaps a common pathogen. In chronic HIV infection, there is evidence that herpes viruses, such as human cytomegalovirus (CMV), may drive immune activation, and reducing CMV replication with valganciclovir reduces activation [49]. In our study population, almost all adults are infected with CMV (A Kharsany, personal communication) so is unlikely to be distributed unequally in cases and controls so as to account for differences in activation. But a search for other pathogens is nonetheless warranted.

An alternative approach to targeted treatment of specific pathogens is to develop and test methods that directly dampen inflammation. There are recent data to suggest that reducing inflammation may prevent HIV. In a monkey model of HIV acquisition, the modulation of Toll-like receptor (TLR)

signaling events at the genital tract mucosa protects from infection [50].

Future studies will seek to identify pathogens that drive activation prior to HIV and to determine whether innate immune activation in women who acquire HIV has an underlying host genetic component. Incorporating an intervention that dampens innate immune activation among the existing HIV prevention methods may meanwhile provide additional protection for young African women, even in the tenofovir gel era. More research is needed before the results can be confidently translated into clinical applications.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. Salim Abdool Karim and Quarraisha Abdool Karim were the co-Principal Investigators of the CAPRISA 004 trial of tenofovir gel. Q. A. K. is co-Principal Investigator of the HIV Prevention Trials Network, which is undertaking HPTN 052 trial of treatment for prevention. S. S. A. K. is an executive committee member of the Microbicide Trials Network, which is undertaking the VOICE trial of oral and topical PrEP. S. S. A. K. and Q. A. K. are also coinventors of 2 pending patents (61/354.050 and 61/357.892) of tenofovir gel against HSV-1 and HSV-2 with scientists from Gilead Sciences.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Supplementary Methods

The CAPRISA004 Tenofovir gel trial was a Phase IIb randomized, placebo-controlled trial of coitally-related use of 1% Tenofovir gel (or placebo).This trial enrolled women 18-40 years of age, resident in a high-prevalence community who were sexually active (defined as >2 sex acts in preceding 30 days). The overall HIV incidence in this trial was 7.3 per 100 women-years. Controls were eligible for selection if they reported an average of more than two sex-acts per week throughout the trial, returned a corresponding number of used gel applicators, had PBMC samples available and had given informed consent for future research. We excluded 3 control participants initially selected due to low viability of thawed PBMC. Experimental procedures were conducted blind to whether the sample was from a case or a control. Although participants were tested for sexually transmitted infections and bacterial vaginosis if clinically indicated, the prevalence of STI's was similar amongst cases and controls(11.4%(5/44) cases vs. 8.35%(3/36) controls).

Study procedure

HIV serostatus, safety, sexual behavior, and gel and condom use were assessed at monthly follow-up visits for up to 30 months post-enrollment In addition, plasma and peripheral blood mononuclear cells(PBMC) were obtained and cryopreserved at 3, 12 and 24 months post-enrollment.In brief the HIV testing was performed at each visit with two HIV-1 rapid antibody tests from two different manufacturers (Determine HIV1/2 from Abbott Laboratories, Chicago, Illinois and the UnigoldRecombigen test (Trinity Biotech, Wicklow Ireland). To avoid the window period and to estimate date of HIV acquisition, if either or both tests were reactive, previously stored and freshly obtained specimens were tested for HIV RNA.

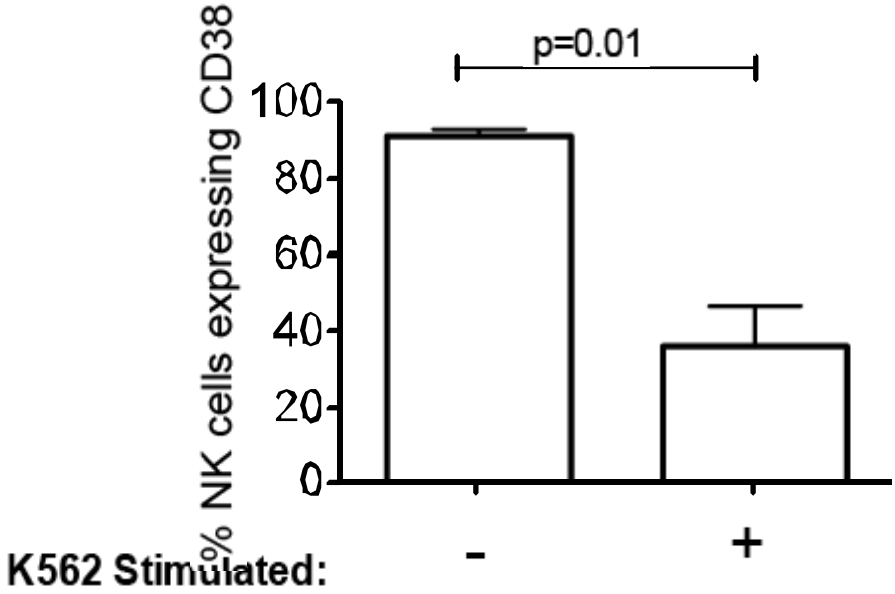
Supplementary Table 1. Principal component (PC) 1, PC2, PC3 and PC4 composition. Principal component analysis of measures of immune activation resulted in 4 major principal components (PC), which explained 68.68% of the total variation in the data set. For each PC, the major contributing variables are listed. Influential variables composing a principal component, with rotated factor based scores >0.4 or <-0.4 are shown in bold.

	Principal Component					
	1	2	3	4		
	"T-cell homeostatic cytokines"	"Innate immune activation"	"Innate immune quiescence"	"NK cell activation independent to CD8+ T-cell degranulation"		
Eigenvalue	2.15	1.72	1.17	1.13		
% Variance	23.97	19.14	13.04	12.53		
					Communality estimate	Items
	0.15	**0.89***	-0.03	-0.03	0.81	TNF-α
	0.88*	-0.01	-0.08	-0.08	0.78	IL-2
	0.58*	0.17	0.10	0.06	0.38	IL-7
	0.98*	-0.04	-0.02	0	0.96	IL-12p70
	-0.05	0.18	0.08	**0.79***	0.66	%CD69^{pos} NK cells
	-0.10	0.21	**0.85***	0.09	0.78	%CD38^{pos} NK cells
	0.02	**0.54***	**-0.56***	0.24	0.66	%HLA-DR^{pos} NK cells
	-0.15	0.31	**-0.58***	-0.06	0.46	Platelets (X 10^9/l)
	-0.07	**0.47***	0.04	**-0.67***	0.68	%CD107a^{pos} CD8 T-cells

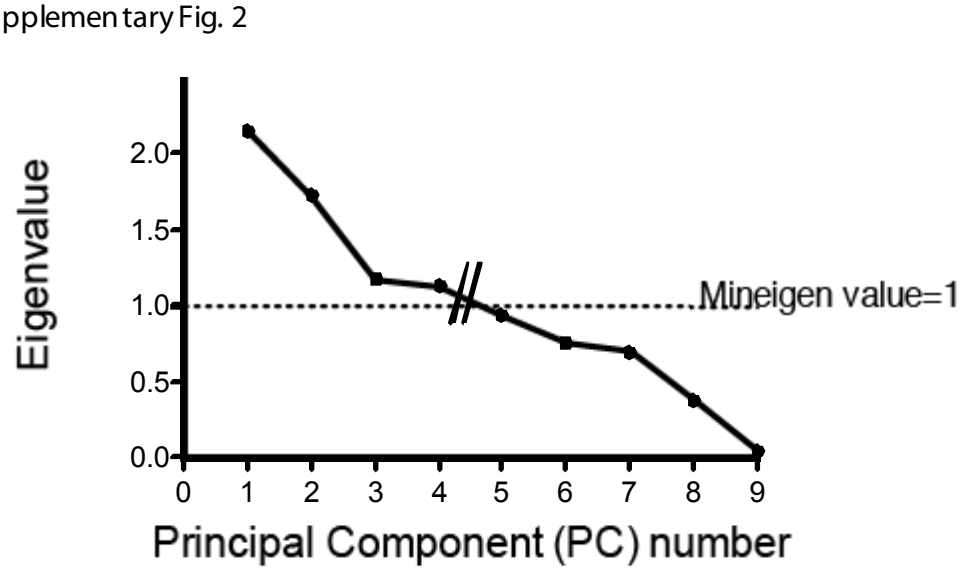
Supplementary Figure Legends

Supplementary Figure 1. Natural Killer cells from HIV uninfected healthy donors (n=3) reduce CD38 cell-surface expression when stimulated with K562 cells.

Supplementary Fig. 1



Supplementary Figure 2. Scree plot of principal component analysis: all generated components (x-axis) versus total Eigen value (y-axis).



CHAPTER SEVEN:

Neither microbial translocation nor TLR responsiveness are likely explanations for pre-existing immune activation in women who subsequently acquired HIV-1 in CAPRISA004

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Neither Microbial Translocation Nor TLR Responsiveness Are Likely Explanations for Preexisting Immune Activation in Women Who Subsequently Acquired HIV in CAPRISA 004

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Abstract: Innate immune activation was a strong predictor of HIV acquisition in women at risk for HIV in CAPRISA 004. Identifying the cause(s) of activation could enable targeted prevention interventions. In this study, plasma concentrations of lipopolysaccharide, soluble CD14, and intestinal fatty acid–binding protein did not differ between subjects who did or did not subsequently acquire HIV nor were these levels correlated with plasma cytokines or natural killer cell activation. There was no difference between HIV acquirers and non-acquirers in the chemokine and cytokine responses of peripheral blood mononuclear cells stimulated with TLR2, 4, or 7/8 agonists. Further studies are required.

Key Words: HIV, microbial translocation, LPS, sCD14, I-FABP, TLR2, TLR4, TLR7/8, immune activation

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INTRODUCTION

Nonspecific activation of the immune system is a pathological feature of HIV infection.[1] The degree of activation of $CD8^+$ T cells is associated with the rate of HIV disease progression.[2] But our recent studies[3] validate previous studies[4–6] in suggesting that innate immune activation is also a pathological determinant of HIV acquisition. In CAPRISA 004, a randomized placebo-controlled trial of 1% Tenofovir gel, women who

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V. N., N. S., and N. G. S. contributed equally to the work.

S. S. A. K. and Q. A. K. were the co-principal investigators of the CAPRISA 004 trial of tenofovir gel. S. S. A. K. and Q. A. K. are also co-inventors of 2 pending patents (61/354.050 and 61/357,892) of tenofovir gel against HSV-1 and HSV-2 with scientists from Gilead Sciences. Gilead Sciences did not have any role in the experiments or analyses presented here.

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Author contributions: All the authors contributed to the design of the sub-study. V. N. designed the study. V. N., N. S. and N. G. S. designed and executed the experiments, performed the data collection and analysis. T. N., M. A., D. C. D. and S. S. A. K. supervised the data collection. S. S. A. K. and Q. A. K. designed, conducted, and analyzed the parent CAPRISA 004 trial. All authors reviewed and approved the final manuscript.

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acquired HIV had significantly higher plasma levels of interleukin (IL)-2, IL-7, IL12p70, and tumor necrosis factor alpha (TNF-α), and more frequent activation and degranulation of natural killer (NK) cells in the blood than the women who remained uninfected.[3] Even after adjusting for potential confounders, innate immune activation was associated with HIV acquisition. The identification of determinants of activation would allow for the design and testing of targeted interventions to reduce activation as a method to reduce HIV acquisition.

During established HIV infection, increased microbial translocation[1] and chronic cytomegalovirus infection[7] are associated with greater immune activation. There has been some speculation that a higher pathogen burden in settings with poorer sanitation may result in higher levels of microbial translocation, even among HIV-uninfected individuals.[8] Others have suggested that differences in toll-like receptor (TLR) responsiveness exist between women who are exposed to HIV but remain uninfected, and HIV-uninfected controls.[9] We previously reported that the prevalence of cytomegalovirus infection in this cohort is almost universal[3] so this is not likely to drive the differences. Although HSV-2 was associated with HIV acquisition and suppressed NK cell antiviral response,[10] it did not weaken the association between innate immune activation and HIV acquisition.[3] Here, we report on further studies that aimed to determine whether differences in microbial translocation or TLR responsiveness could explain differences in innate immune activation in women who were exposed to HIV in CAPRISA 004.

METHODS

Study Population and Sampling

For this study, specimens from women who acquired HIV and women who remained uninfected were used. Peripheral blood mononuclear cells (PBMCs) and plasma were collected from participants of CAPRISA 004, a randomized controlled trial of 1% Tenofovir gel conducted in eThekweni and Vulindlela (KwaZulu-Natal, South Africa), as previously described.[11] The participants in this study matched those used in previous studies of immune activation in this trial.[3] Briefly, samples from HIV acquirers were from the last trial visit before HIV acquisition. Samples from HIV non-acquirers were from the trial visit at which sexual activity in the preceding month was each participant's personal maximum. HIV non-acquirers were eligible for selection if they reported an average of more than 2 sex acts per week throughout the trial, returned a corresponding number of used gel applicators (as a surrogate measure of actual sexual activity), had PBMC samples available, and had given informed consent for future research. HIV-uninfected status was verified by polymerase chain reactions, Western blot, and rapid antibody assays. Due to limited cell numbers, a different number of samples were used for each TLR agonist studied (16 acquirers and 13 non-acquirers for aldrithiol-inactivated HIV [AT-2]; 14 and 9, respectively, for heat-killed *Listeria monocytogenes* [HKLM]; and 10 and 6 for lipopolysaccharide [LPS]). The baseline demographic characteristics, sexual history, and study procedures have been previously described.[3] Receipt of Tenofovir gel did not affect any of the measures presented[3,12] so is not further described in this report.

This study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE073/010). Participants gave informed consent for their samples to be used for these studies.

Measurement of LPS, Soluble CD14, and Intestinal Fatty Acid–Binding Protein

LPS, soluble CD14 (sCD14; a marker of monocyte response to LPS), and intestinal fatty acid–binding protein (I-FABP; a marker of enterocyte damage) were measured in duplicate in cryopreserved plasma from participants. Plasma had not been thawed more than once previously. LPS levels were measured using the Limulus Amebocyte Assay (Lonza, Walkersville, MD), as previously described.[13] A commercially available enzyme-linked immunosorbent assay was used for sCD14 (R&D Systems, Minneapolis, MN) on plasma diluted to 0.5% according to the manufacturer's instructions. I-FABP was measured using an adaptation of FABP2 kit on plasma diluted to 10% in 50% fetal calf serum in phosphate buffered saline (R&D Systems).

NK Cell Phenotypic Characterization

NK cell activation was measured in cryopreserved PBMCs in batch analyses using optimized procedures and flow cytometry methods as previously described.[3]

Characterization of Cytokine and Chemokine Response of PBMC to TLR Agonists

Cryopreserved PBMCs were thawed in warm R10 culture media: RPMI 1640 (Gibco; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen), 2 mmol/L of L-glutamine and 1% penicillin/streptomycin. After 2 hours of incubation at 37°C, cells were stained with Viacount (Millipore, Billerica, MA) and counted with a Guava PCA (Millipore). PBMC were resuspended at 1×10^6/mL in R10 culture media. TLR agonists or R10 media alone were added to 1×10^6 PBMC in 4 mL FACS Tubes (Becton Dickinson, Franklin Lakes, NJ) at the following concentrations 0.7 μg/mL AT-2 HIV, 2 μL/mL HKLM, or 0.1 μg/mL LPS. Cells were cultured overnight at 37°C in a sterile incubator maintained at 5% CO_2. Supernatant was harvested by centrifugation and immediately cryopreserved at −80°C.

The concentrations of 13 cytokines: GMCSF, interferon (IFN)-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, and TNF-α were assessed using a high-sensitivity human cytokine premixed 13-plex kit (Millipore) as per the manufacturer's instructions. Samples were assayed in duplicate after a single thaw, without dilution. Samples from HIV acquirers and non-acquirers were equally spread across assay plates to minimize plate-to-plate variability as a confounder. Cytokine measures beneath the detection limit of the assay were given a value of the midpoint between 0 and the lower detection limit of the assay and were included in the analysis.

Statistical Analysis

Assays were conducted blinded to whether the sample was from an HIV acquirer or non-acquirer. The study had 80% power to detect a difference of approximately 4 pg/mL in LPS, 0.17×10^6 pg/mL in sCD14, or 208 pg/mL in I-FABP levels between the 2 groups.

Comparisons were made using a nonparametric Mann–Whitney test in GraphPad Prism v5 (GraphPad Software). To account for multiple comparisons, the unadjusted *P* value is reported and the relevant Bonferroni adjusted *P* value is given in the text or figure caption.

RESULTS

HIV-exposed women who acquired HIV while enrolled in CAPRISA 004 were previously reported to have higher levels of circulating IL-2, IL-7, IL12p70, and TNF-α in the blood, and higher levels of activated NK cells relative to women who did not acquire HIV.[3] To explore possible explanations for the differences in activation, samples from the same 44 women who acquired HIV and 37 who remained uninfected and were previously studied, were evaluated for evidence of microbial translocation or differential response to microbial products. The baseline characteristics of this study population have been previously described.[3]

No Appreciable Difference in Microbial Translocation between HIV Acquirers and Non-acquirers

In primary or chronic HIV infection, plasma levels of LPS are a marker of gut permeability and microbial translocation.[1] Both LPS and its ligand sCD14 are associated with immune activation in HIV infection. We measured LPS, sCD14, and I-FABP—a marker of enterocyte damage.[14] There was no significant difference in plasma levels of LPS, sCD14, or I-FABP in HIV acquirers and non-acquirers, although surprisingly, some of the clinically well participants had high levels of LPS or I-FABP consistent with significant translocation or intestinal epithelial cell necrosis (Fig. 1).

Levels of LPS, sCD14, and I-FABP were not associated with NK cell activation, regardless of which marker of NK cell activation was used (HLA-DR, CD38, or CD69), as shown in Figure S1 (see **Supplemental Digital Content**, http://links.lww.com/QAI/A414). Similarly, levels of LPS, sCD14, or I-FABP were not significantly associated with any of the plasma cytokines measured in previous studies after correcting for multiple comparisons (the Bonferroni corrected alpha for significance was $P < 0.0013$).

TLR Responsiveness

Some previous studies have suggested that differences in the ability to respond to microbial products may lead to differential levels of TLR-mediated activation.[9] To determine whether there were any differences in TLR responsiveness between HIV acquirers (n = 21) and non-acquirers (n = 18), cytokine and chemokine release was quantified after overnight in vitro culture of PBMC with agonists for TLR2 (HKLM), TLR4 (LPS), or TLR7/8 (AT-2 HIV).

Neither the absolute increase in concentration nor the fold change increase in concentration relative to media alone of any of the measured cytokines differed between PBMC from HIV acquirers and non-acquirers stimulated with a TLR2, TLR4, or TLR7/8 agonist (Fig. 2). IFN-γ concentrations were higher in the supernatant of PBMC from HIV acquirers cultured with AT-2 HIV ($P = 0.05$), but after adjusting for the 39 comparisons made, the difference was not significant.

Chronic stimulation by LPS is thought to reduce further responsiveness to stimulation of TLR4 by LPS ("LPS tolerance").[15] In this study, there was no evidence that circulating LPS levels were associated with in vitro cytokine or chemokine

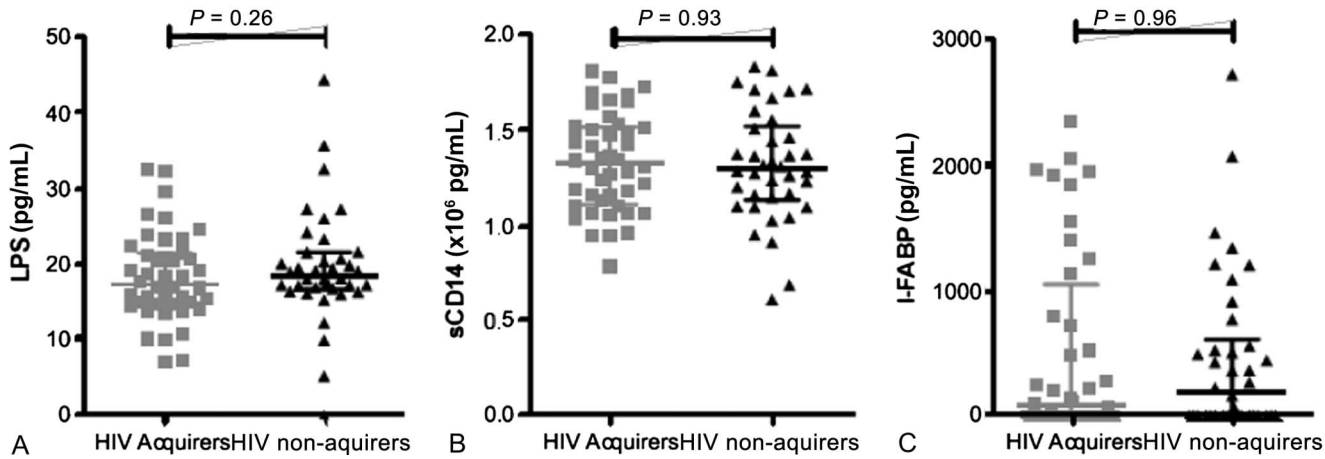

FIGURE 1. Plasma levels of LPS (A), sCD14 (B), and I-FABP (C) do not differ between women who acquired HIV samples pre-infection (gray squares) and women who remained HIV uninfected (black triangles). Lines demarcate the median and interquartile range. *P* value from Mann–Whitney test.

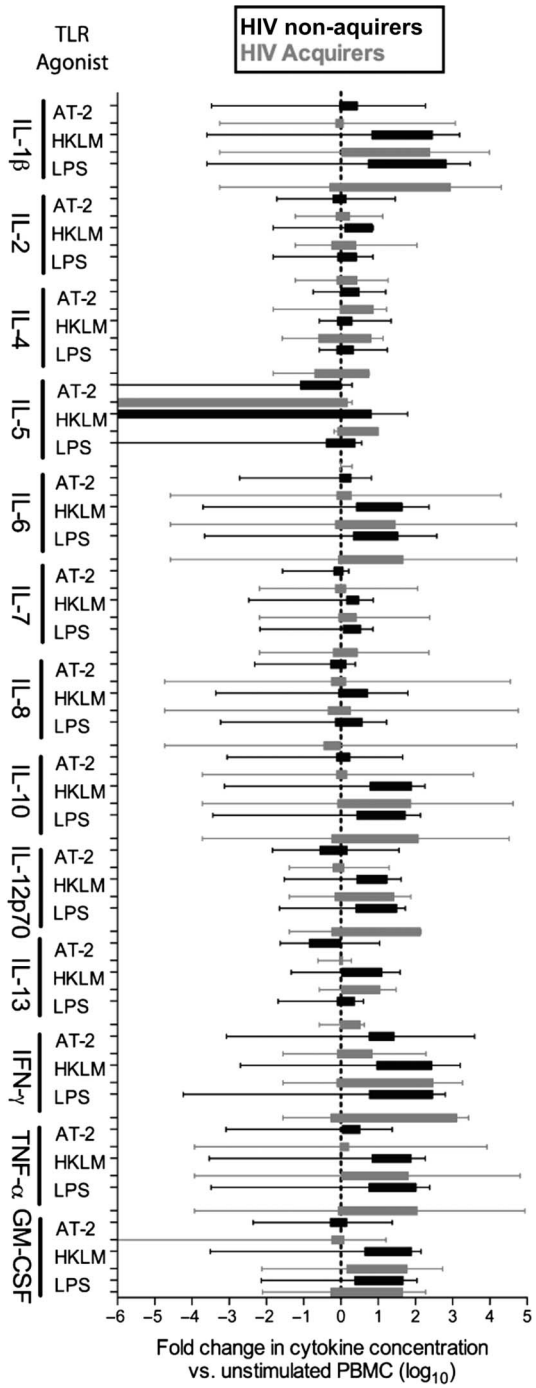

FIGURE 2. PBMC from HIV acquirers (black) and non-acquirers (gray) exhibit a similar fold change (log_{10}) in the secretion of chemokines and cytokines after stimulation with a TLR7/8 agonist (AT-2 HIV), TLR2 agonist HKLM, or a TLR4 agonist LPS relative to media alone. PBMC were stimulated overnight and secreted cytokines/chemokines measured by Luminex in duplicate. Data are shown as box and whiskers to 5th and 95th percentile. Adjusted *P* values are not shown as none achieved significance, although the nominal *P* value for the comparison of IFN secretion after AT-2 stimulation in HIV acquirers and non-acquirers was $P = 0.05$ (Bonferroni adjusted $P < 0.0013$ for significance).

responses of PBMC stimulated with LPS (see **Table S1**, **Supplemental Digital Content**, http://links.lww.com/QAI/A414).

DISCUSSION

Immune activation was a potent predictor of HIV acquisition in CAPRISA 004.[3] In chronic HIV infection, immune activation is associated with circulating levels of microbial products,[1] which predict disease course.[1,13] To our knowledge, there have not been any previous studies of microbial translocation in women who are exposed to HIV. Here, we found that microbial translocation was similar among HIV acquirers and non-acquirers. Microbial translocation was, furthermore, not associated with NK cell activation or any of the cytokines measured. We speculate that the levels of microbial translocation measured in HIV acquirers and non-acquirers may represent homeostatic levels and an alternative more potent mediator of activation may remain undiscovered among HIV acquirers. We tested an alternative hypothesis that TLR responsiveness may differ among HIV acquirers and non-acquirers. In this study, there was no significant difference in PBMC response to TLR agonists, whereas a previous report found greater TLR responsiveness in HIV-exposed uninfected women compared with healthy blood donors.[9] The difference may be due to the comparison group in our study being women who actually acquired infection.

We observed that PBMC from HIV acquirers produced more of the antiviral cytokine, IFN-γ, after stimulation with AT-2 HIV than HIV non-acquirers. Although this finding is likely due to chance, as it did not reach statistical significance after controlling for the multiple tests performed, the finding points to the possibility that women who later acquired HIV may have had sustained exposure to HIV and consequently developed an HIV-specific immune response in the absence of infection. As reported elsewhere,[16] we did not detect sustained or robust HIV-specific T-cell responses by Enzyme-Linked Immunosorbent SPOT in these participants. Therefore HIV-specific responses, if present in these women, are weak. Assessments of HIV-specific immune responses in future studies of HIV acquisition continue to be warranted.

Our findings are limited by the sample size; we are unable to rule out a difference smaller than what the study was designed to detect. Moreover, we studied bulk PBMC and plasma. Studies in established HIV infection have demonstrated that TLR responsiveness is cell type, disease stage, and agonist specific.[17] Moreover, measurement of these parameters in the periphery may be inadequate to reveal what actually happens in the female genital tract, the site of infection, and therefore a study of the mucosa would perhaps be appropriate in future work.[18]

These data collectively suggest that neither microbial translocation nor TLR responsiveness of PBMC are sufficient to explain the higher levels of innate immune activation observed in women who subsequently acquired HIV in CAPRISA 004. Further studies to explore host genetic factors or differences in the enteric or genital tract microbiome may be required to identify the upstream causes of preexisting immune activation in women who subsequently acquired HIV in CAPRISA 004.

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CHAPTER EIGHT:

Changes in Natural Killer cell activation and function during primary HIV-1 infection

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Changes in Natural Killer Cell Activation and Function during Primary HIV-1 Infection

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Abstract

Background: Recent reports suggest that Natural Killer (NK) cells may modulate pathogenesis of primary HIV-1 infection. However, HIV dysregulates NK-cell responses. We dissected this bi-directional relationship to understand how HIV impacts NK-cell responses during primary HIV-1 infection.

Methodology/Principal Findings: Paired samples from 41 high-risk, initially HIV-uninfected CAPRISA004 participants were analysed prior to HIV acquisition, and during viraemic primary HIV-1 infection. At the time of sampling post-infection five women were seronegative, 11 women were serodiscordant, and 25 women were seropositive by HIV-1 rapid immunoassay. Flow cytometry was used to measure NK and T-cell activation, NK-cell receptor expression, cytotoxic and cytokine-secretory functions, and trafficking marker expression (CCR7, $\alpha_4\beta_7$). Non-parametric statistical tests were used. Both NK cells and T-cells were significantly activated following HIV acquisition ($p = 0.03$ and $p < 0.0001$, respectively), but correlation between NK-cell and T-cell activation was uncoupled following infection (pre-infection $r = 0.68; p < 0.0001$; post-infection, during primary infection $r = 0.074; p = 0.09$). Nonetheless, during primary infection NK-cell and T-cell activation correlated with HIV viral load ($r = 0.32'p = 0.04$ and $r = 0.35; p = 0.02$, respectively). The frequency of Killer Immunoglobulin-like Receptor-expressing (KIR_{pos}) NK cells increased following HIV acquisition ($p = 0.006$), and KIR_{pos} NK cells were less activated than KIR_{neg} NK cells amongst individuals sampled while seronegative or serodiscordant ($p = 0.001; p < 0.0001$ respectively). During HIV-1 infection, cytotoxic NK cell responses evaluated after IL-2 stimulation alone, or after co-culture with 721 cells, were impaired ($p = 0.006$ and $p = 0.002$, respectively). However, NK-cell IFN-y secretory function was not significantly altered. The frequency of CCR7+ NK cells was elevated during primary infection, particularly at early time-points ($p < 0.0001$).

Conclusions/Significance: Analyses of immune cells before and after HIV infection revealed an increase in both NK-cell activation and KIR expression, but reduced cytotoxicity during acute infection. The increase in frequency of NK cells able to traffic to lymph nodes following HIV infection suggests that these cells may play a role in events in secondary lymphoid tissue.

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Competing Interests: The authors have read the journal's policy and have the following conflicts: Salim Abdool Karim and Quarraisha Abdool Karim were the co-Principal Investigators of the CAPRISA 004 trial of tenofovir gel. QAK is co-Principal Investigator of the HIV Prevention Trials Network, which is undertaking HPTN 052 trial of treatment for prevention. SSAK is an executive committee member of the Microbicide Trials Network, which is undertaking the VOICE trial of oral and topical PrEP. Salim and Quarraisha Abdool Karim are also co-inventors of two pending patents (61/354.050 and 61/357,892) of tenofovir gel against HSV-1 and HSV-2 with scientists from Gilead Sciences. Gilead Sciences did not fund this project, however the drug used in the clinical trial from which samples were used for this study belongs to Gilead Sciences. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Understanding immunological responses that modulate HIV-1 pathogenesis is important for vaccine and immunotherapy development. Events that occur during the earliest period of HIV-1 infection disproportionately influence the outcome and course of disease. In particular, generalized activation of CD8 T-cells is associated with faster disease progression [1], whilst HIV-specific CD8+ and CD4+ T-cell responses during primary infection are associated with slower disease progression and lower set point viral load [2,3,4]. Although the effects of HIV-1 infection on adaptive immune cells, particularly T-cells have been well described, the impact on innate immune responses are less well understood. Natural Killer (NK) cells, are part of the innate immune defense against viral infections and modulate subsequent adaptive immune responses [5]. *In vitro* and animal studies suggest a possible role of NK cells in controlling viral replication during primary HIV-1 infection [6]. NK cells can limit HIV replication through direct killing of infected cells as well as the secretion of anti-viral cytokines. However, HIV can also impair immune responses by NK cells [7,8,9]. By examining the responses of NK cells prior to infection and at the earliest time points following infection, we may better unravel cause-effect relationships of HIV impact on immune responses and vice-versa.

During chronic HIV-1 infection NK-cell cytotoxicity and cytokine secretion are impaired, but these deficiencies likely start earlier in the course of disease [10]. The impairments are associated with expansion of an "anergic" NK cell subset that expresses CD16 and relatively low levels of CD56 ($CD56_{neg}CD16_{pos}$). Some investigators have also proposed that this subset functionally impairs the total NK cell population [11]. *In vitro* models of HIV infection suggest that HIV viraemia contributes to this impairment [9]. Viraemia peaks early following acquisition, during primary HIV infection. Thus, NK cell dysregulation *in vivo* likely begins during primary infection. This hypothesis is supported by the findings of Alter and colleagues [7]. In a study of 10 acutely infected individuals, they reported quantitative expansion of NK cell populations during the seronegative phase of primary HIV-1 infection that rapidly returned to baseline, but remained qualitatively different from 14 healthy HIV-uninfected individuals, or 45 individuals with chronic HIV. They observed NK cell expansion prior to the development of adaptive (CD8+ T-cell) responses. They also found that NK-cell degranulation and interferon-gamma (IFN-γ) secretion were elevated in the seronegative phase of infection but declined subsequently independent of antiretroviral treatment status. However the lack of blood samples from the same individuals prior to HIV acquisition limited the conclusions from this study [12].

Few studies have quantified immune responses before and after HIV infection among the same individuals. Most previous studies compare immune responses in HIV acutely infected individuals to uninfected individuals [13]. Here we extend the findings of previous studies by characterizing innate immune responses in the same individuals before and after HIV acquisition. To better understand the kinetics of NK-cell responses in primary HIV-1 infection we compared innate immune responses in paired blood specimens collected prior to and during primary infection among women who acquired HIV whilst enrolled in a randomized, controlled clinical trial of 1% Tenofovir microbicide gel. Our findings provide new insights into the effects of HIV on NK-cell responses.

Methods

Study Subjects

This prospective cohort study was nested in the CAPRISA 004 Tenofovir gel trial of coitally-related use of 1% Tenofovir gel [14]. The trial enrolled 889 sexually active (defined as >2 sex acts in preceding 30 days) women 18–40 years of age, who were resident in high-prevalence rural and urban communities. For this study, 41 consenting adult women, who acquired HIV whilst in the trial, were selected based on sample availability. Incident HIV infection was established by two rapid HIV antibody tests and confirmed by negative qualitative HIV PCR testing of pre-infection samples. In cases of diagnostic uncertainty, Western blots and/or an HIV-1 ELISA were performed. To characterize changes in NK cells in women who acquired HIV-1, and whether these were associated with changes in T-cell activation or function, we examined NK cell and T-cell responses at the last sampling time point before infection and during primary HIV-1 infection.

Participants gave informed consent for their samples to be stored and used for this study. This study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (#BE073/010). The clinical trials registration number of parent trial was NCT00441298.

Natural Killer cell and T-cell activation

Activation, CD107a expression and intracellular cytokine production were measured in cryopreserved peripheral blood mononuclear cells (PBMC) in batch analyses using optimized flow cytometry methods as previously described [15]. Cryopreserved PBMC were rapidly thawed in warm media (RPMI 1640; Gibco), washed and rested for two hours. Sample viability and cell counts were determined by staining with Viacount (Millipore) and run on a Guava PCA (Millipore). PBMC were stained with Yellow Viability Dye (Invitrogen), anti-CD14 (clone Tuk4, Invitrogen) and -CD20 (clone HI47, Invitrogen) antibodies (to exclude nonviable cells, monocytes and B-cells, respectively). In addition, the cells were stained with anti-CD3 (clone SP34-2, BD Biosciences),-CD16 (clone 3G8, BD Biosciences),-CD38 (clone HIT2, BD Biosciences),-CD56 (clone B159, BD Biosciences),-CD69 (clone FN50, BD Biosciences),-CD158a (cone HP-3E4, BD Biosciences),-CD158b (clone CH-L, BD Biosciences), -HLA-DR (clone L243, BD Biosciences) and anti-CD158e1/e2 (clone Z27, Beckman Coulter) antibodies. Flow cytometry data were collected on a BD LSRII and analyzed using FlowJo v9.3.1 (Treestar). NK cells were defined as viable, $CD14_{neg}$, $CD20_{neg}$, $CD3_{neg}$, $CD16/56_{pos}$ lymphocytes. The frequency of NK cells that were $HLA\text{-}DR_{pos}$ was determined using fluorescence minus one (FMO) gates. The frequency of T-cells, defined as viable, $CD14_{neg}$, $CD20_{neg}$ $CD3_{pos}$ cells, expressing both CD38 and HLA-DR, was similarly determined. The gating strategy is shown in Figure S1.

NK-cell effector functions

Using cryopreserved PBMCs in batched analyses each of the following three NK-cell effector functions were quantified following IL-2 stimulation (100 U/ml R+D Systems) alone, IL-2 plus 721 cells or PMA/Ionomycin: degranulation (using CD107a expression as a surrogate [16]), cytokine secretion (IFN-γ), and recent cell division (using Ki-67 as a surrogate [17]). Cryopreserved PBMC were rapidly thawed in warm media, washed and rested for 12 hours in IL-2 enriched culture medium (RPMI 1640 media containing 50 U/ml IL-2 and 10% fetal calf serum). For experiments in which cells were stimulated with 721 cells, the NK cells were cultured with equal numbers of 721 cells. Cells were washed, resuspended in culture medium containing an antibody

directed against CD107a, and cultured for a further 6 hours. Samples were processed for flow cytometry as detailed above with the following modification. Cells were surface stained with anti-CD3 (clone SP34-2, BD Biosciences), -CD8 (clone 3B5, Invitrogen), -CD56 (clone B159, BD Biosciences), -CD69 (clone FN50, BD Biosciences) and anti-CD7 (clone 8H8.2, Beckman Coulter) antibodies, then washed, fixed and permeabilised before staining with anti-Ki-67 (clone B56, BD Biosciences) and -IFN-γ (clone 4S.B3, BD Biosciences) antibodies, respectively. To account for NK cells that have lost CD16 expression due to activation and to exclude monocyte/DC-like cells, anti-CD7 antibody was used as described [18]. FMO gates were used to determine the proportion of cells responding with one or more effector functions (i.e., CD107a, IFN-γ or Ki-67). The gating strategy is shown in Figure S2.

Statistical methods

Assays were conducted blinded to time point of sampling. For comparisons between paired specimens at pre- and post-infection time points from the same individual, a non-parametric Wilcoxon signed rank test was performed. For comparisons between different time points post-infection, a Kruskal-Wallis test was performed. Statistical analyses were conducted in GraphPad Prism v5 (GraphPad).

Results

To characterize the earliest changes in innate and adaptive immune responses induced by HIV-1, we examined NK-cell and T-cell responses, respectively, prior to HIV acquisition and during primary infection. Unlike most prior studies these responses were measured in the same individuals over time.

Study cohort

A total of 41 recently infected women were included in this study, (mean age 23.3 years). Thirty-one were in the tenofovir arm and eight in the placebo arm. To take into account differences between post-infection sampling time points in this cohort, the results were stratified by stage of HIV-1 infection at the time of sample collection. Blood samples collected at the last visit prior to HIV acquisition (hereafter referred to as pre-infection) were collected at a median of 119 days prior to HIV acquisition, IQR 67.25–169.25). The samples collected from these women at the first visit following HIV infection were designated as post-infection samples. HIV serostatus (seronegative versus seropositive) of the samples was only used in reference to samples collected after HIV acquisition (i.e., post-infection). Five participants were sampled during the seronegative stage of primary infection based on two HIV rapid tests (*seronegative* group), 11 whilst having indeterminate or discordant antibody responses (*discordant* group) and 25 during the seropositive stage of infection (seropositive group). Due to limited cell numbers, we were unable to assess NK-cell degranulation, proliferation or cytokine secretion in two seronegative, two discordant, and three seropositive individuals.

Based on estimates of the date of infection confirmed by BEAST analysis of viral sequences, seronegative, discordant and seropositive individuals were sampled at a median (IQR) of 14 (13.5–14), 15 (13–25) and 131 (105.5–157) days post-infection respectively. The median viral load (log_{10} copies/ml) (IQR) was 4.95 (4.39–5.29), 4.63 (4.19–5.34) and 3.86 (3.38–4.80) for seronegative, discordant and seropositive groups respectively. The corresponding probable Fiebig staging was based on a combination of the estimated time post-infection at sampling and ancillary HIV PCR, Western blot and/or ELISA results, where available, were I/II/early III; III/IV, and V/VI for seronegative, discordant and seropositive groups, respectively.

Consistent with a previous report on this cohort by Mureithi et al. [19] we did not observe any significant differences in any of the measured parameters between women who acquire HIV whilst receiving 1% Tenofovir gel compared with women on placebo gel. For this reason the groups were combined in further analyses.

NK-cell and T-cell activation were uncoupled by primary HIV-1 infection, but both were positively correlated with HIV viral load

The proportions of activated T-cells and NK cells were significantly increased during primary HIV-1 infection ($p<0.0001$ and $p = 0.03$, Figures 1A and 1B, respectively). Prior to HIV acquisition, the proportions of activated NK cells and T-cells were positively correlated ($r = 0.68$, $p<0.0001$, Figure 1C), whereas following infection, they were uncoupled ($r = 0.074$, $p = 0.09$, Figure 1D). Nevertheless, the proportions of activated T-cells and NK cells correlated independently with HIV viral load ($r = 0.32$, $p = 0.04$ and $r = 0.35$, $p = 0.02$, respectively; Figures 1E and 1F) but not with CD4+ T-cell counts (Figure 1G and H).

As a proportion of lymphocytes, NK cells were not significantly expanded during primary infection (Figure S3A). To take into account differences between post-infection sampling time points in this cohort the results were stratified by stage of HIV-1 infection when samples were collected. We observed no significant difference in frequencies of NK cells during primary infection between seronegative, serodiscordant and seropositive individuals ($p = 0.41$; Figure S3B) although a weak trend to greater frequencies in seronegative individuals was present. Similarly, there was no difference in the frequencies of recently divided NK cells as measured by expression of Ki-67 (Figure S3C and 3D). Although the difference in proportions of activated NK cells was not statistically significant, there was a tendency for greater activation during later stages of primary infection (Figure S3E). The frequencies of the $CD56_{neg}$ (anergic), $CD56_{dim}$ and $CD56^{hi}$ NK cell subsets did not differ between pre-infection and post-infection time points, nor amongst the stages of primary infection (Figure S4A and 4B). In contrast, the proportion of recently divided CD8+ T-cells increased following HIV-1 infection ($p<0.0001$, Figure S5A).

The frequency of Killer Immunoglobulin-like Receptor expressing NK cells (KIR_{pos}) increased following HIV-1 infection with KIR_{pos} NK cells being less activated than KIR_{neg} NK cells

Next, to explore whether KIRs contributed to the observed changes in activation profiles of NK cells, KIR expression was evaluated by flow cytometry. For this analysis commercial antibodies, which detected the most commonly expressed KIRs (KIR2DL1, KIR2DS1, KIR2DL2, KIR2DL3, KIR3DL1 and KIR3DS1), were used. A significant expansion in the proportion of KIR_{pos} NK cells was evident following HIV-1 infection ($p = 0.006$, Figure 2A) and appeared higher in individuals with seronegative primary HIV-1 infection but this was not significant (Figure 2B). Since KIR modulate NK cell activation, activation on KIR_{pos} and KIR_{neg} cells was compared in each category of samples. Prior to HIV infection, the proportion of KIR_{pos} NK cells that were activated was significantly lower than KIR_{neg} NK cells ($p<0.0001$, Figure 2C). During primary HIV-1 infection, KIR_{pos} NK cells were similarly less activated than KIR_{neg} NK cells amongst those with discordant or seropositive primary HIV-1 infection ($p = 0.001$, $p<0.0001$ respectively, Figure 2C). This did

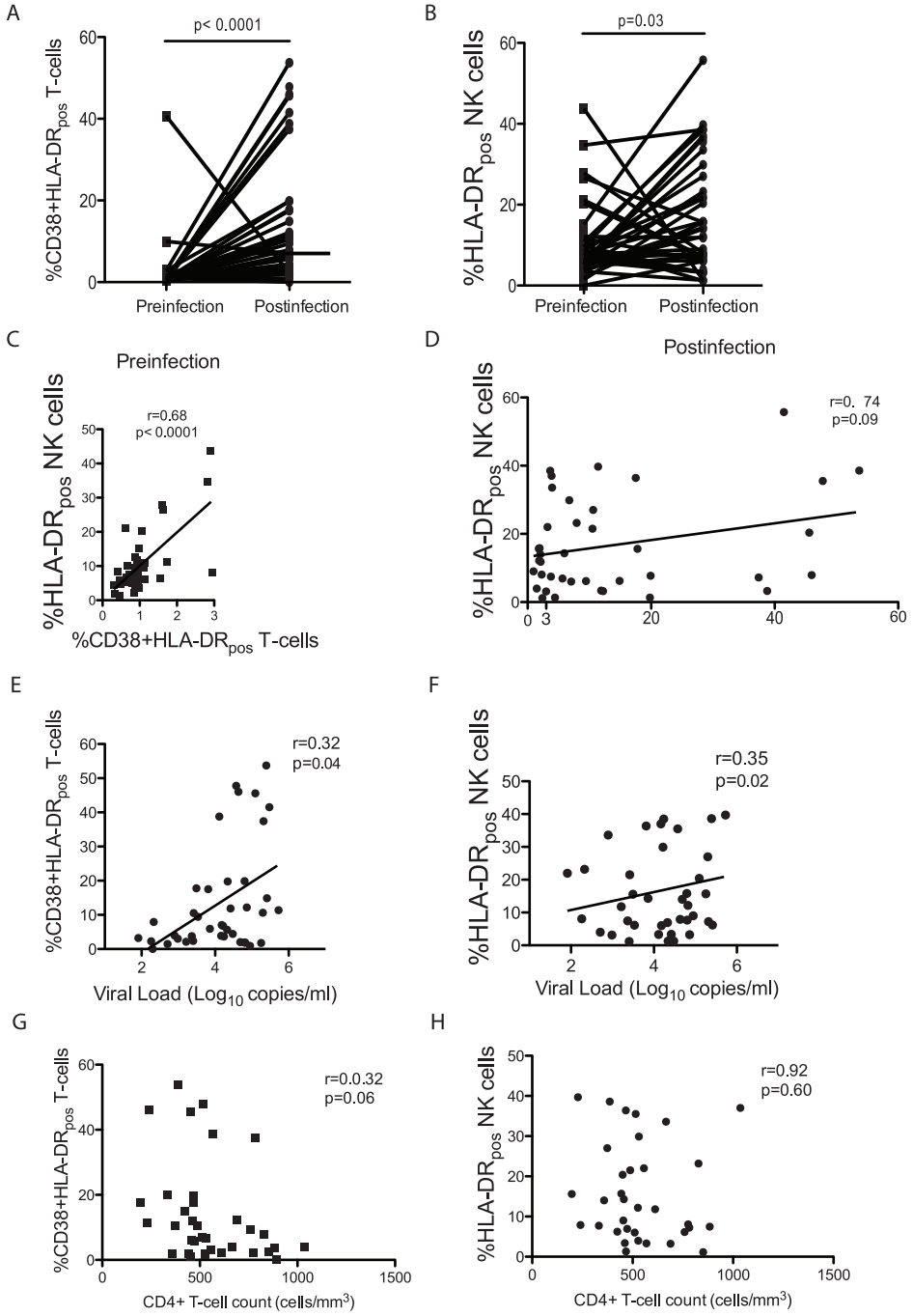

Figure 1. Primary HIV-1 infection was associated with differential T-cell and NK-cell activation. T-cells (A) and NK cells (B) were activated following HIV infection. The proportion of activated T-cells and NK cells was significantly correlated before (C), but not following HIV-1 infection (D). In contrast, during primary infection, both T-cell (E) and NK-cell (F) activation were positively correlated with HIV viral load (log_{10} copies/ml) but not CD4+ T-cell counts (G and H respectively).
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not achieve statistical significance in the smaller group with seronegative primary HIV-1 infection (p = 0.13, Figure 2C).

NK cell function was impaired during primary HIV-1 infection

To quantify the effects of HIV on NK-cell function, we assessed NK cell degranulation and interferon-gamma (IFN-γ) secretion in paired blood samples collected prior to HIV acquisition and after infection, during the early stages of primary infection. The samples were analyzed after *ex vivo* culture in media containing Interleukin-2 (IL-2) alone, or after exposure to 721 cells (a B-cell line deficient in HLA class 1 expression) and IL-2, or PMA/Ionomycin and IL-2. For this assay NK cells were defined as $CD3_{neg}$ cells expressing CD7, as done previously by Milush and colleagues [18].

Following *ex vivo* culture in media containing IL-2 stimulation alone, the proportion of degranulating NK cells was elevated

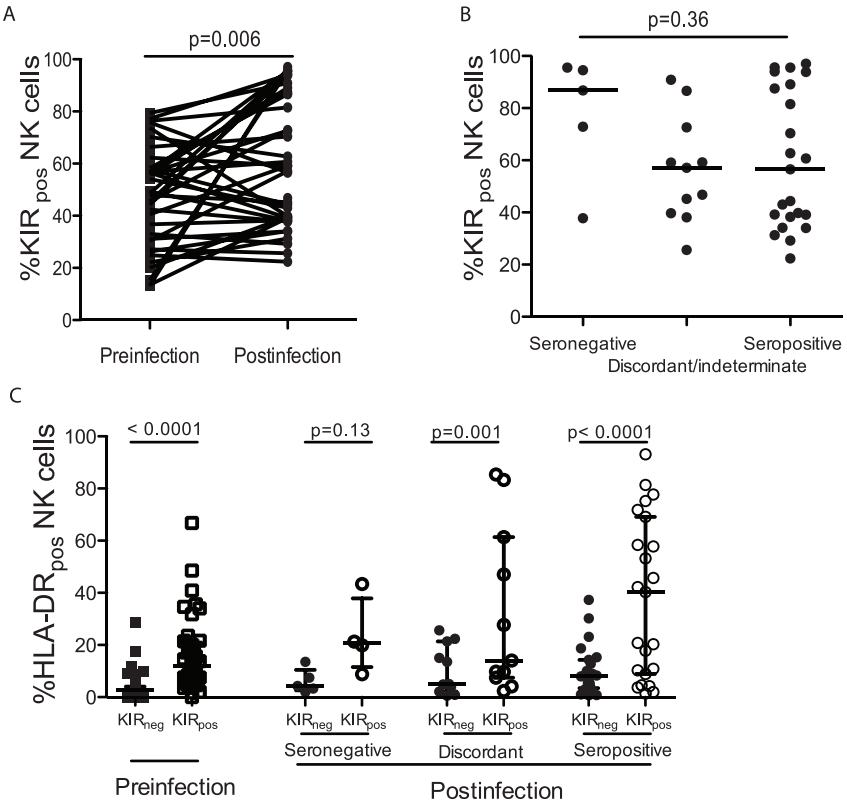

Figure 2. Following infection, an increased proportion of activated NK cells in blood expressed Killer Immunoglobulin-like Receptors (KIR). The proportion of KIR_{pos} NK cells in blood increased following HIV infection (A). The proportion of KIR_{pos} NK cells did not differ between stages of primary HIV infection (seronegative, sero-discordant or seropositive stages) (B). KIR_{pos} NK cells were more activated than KIR_{neg} NK cells (C).
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during primary HIV-1 infection compared to prior to HIV acquisition, ($p = 0.006$, Figure 3A). In contrast, the degranulation response of NK cells to 721 cells, (a model of "missing self") [20], was significantly reduced during primary HIV-1 infection ($p = 0.002$, Figure 3A). Similarly, stimulation by PMA/Ionomycin showed the same tendency, but this difference did not reach statistical significance ($p = 0.06$, Figure 3A). There was no difference in the proportion of NK cells secreting IFN-γ following *ex vivo* IL-2 stimulation alone (Figure 3B). Following stimulation with 721 cells there was a tendency of fewer NK cells secreting IFN-γ after HIV acquisition ($p = 0.09$, Figure 3B). Likewise, the proportion of NK cells secreting IFN-γ following stimulation with PMA/Ionomycin was significantly lower after infection ($p = 0.03$). In contrast to NK cells, the proportion of degranulating CD8+ T was increased following HIV-1 infection ($p = 0.02$, Figure S5B) and there was no change in the proportion of IFN- γ_{pos} CD8+ T-cells (Figure S5C).

The frequency of NK cells expressing markers for homing to lymphoid tissues is increased during primary HIV-1 infection

Finally, to measure the impact of HIV acquisition on NK cell trafficking we quantified the expression of receptors for lymphoid and gastrointestinal tissue homing. We quantified the frequency of NK cells expressing either CCR7, which enables trafficking to lymphoid tissues [21], or α_4 and β_7 integrins, which enable homing to the gut [22]. After infection the frequency of CCR7+ cells increased markedly ($p = <0.0001$, Figure 4A). The proportion of CCR7+ NK cells was decreased with later category of primary HIV-1 infection but did not return to baseline ($p<0.0001$, Figure 4A). In contrast, the proportion of NK cells expressing both the α_4 and β_7 integrin subunits remained unchanged (Figure 4B).

Discussion

Primary HIV-1 infection is associated with differential NK cell and T-cell activation, expansion of NK cells expressing KIR, impairment of cytotoxic NK cell function in the absence of significant increase in anergic subsets, and NK cells equipped to home to secondary lymphoid tissues. These data offer insight into the impact of primary HIV-1 infection on NK cells.

Unlike prior studies that cross-sectionally analysed acutely infected and uninfected individuals [7,12], this study was unique in following immune responses over-time before and after HIV acquisition. The relatively small sample size of samples collected during the seronegative phase of infection limited our ability to compare responses during the seonegative versus seropositive stages of acute infection. Nevertheless, our finding that both NK cells and T-cells were activated early following HIV infection was consistent with previous findings by Alter and colleagues [12]. However, during primary infection we did not observe a significant expansion in the frequency of NK cells amongst total lymphocytes that has been described previously [12]. Alter and colleagues noted an expansion of NK cells among HIV-infected, seronegative individuals compared to uninfected individuals. Since most of the post-infection samples used in this study were collected during the seropositive phase of primary infection, we speculate

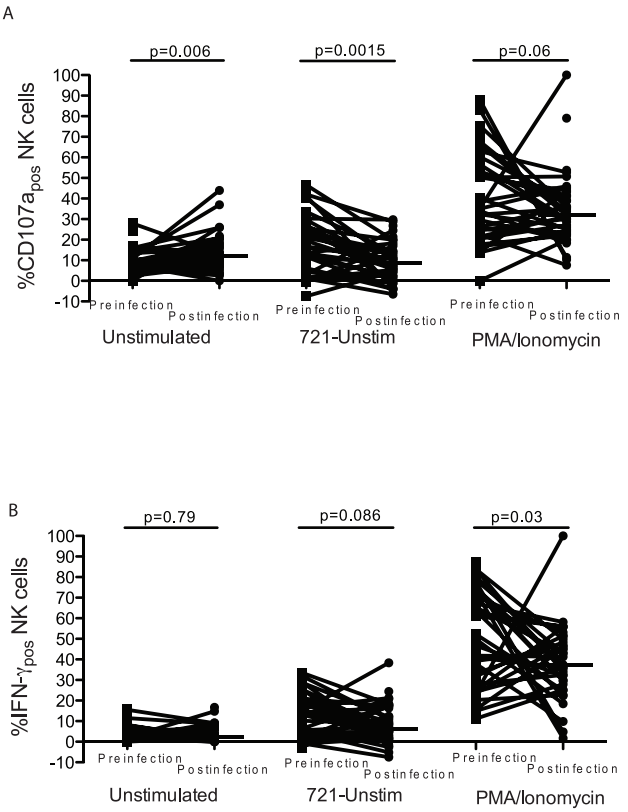

Figure 3. Following HIV infection NK-cell responses to stimulation were diminished. Natural Killer cell degranulation (A) and IFN-γ secretion (B) responses after stimulation with IL-2 alone or with IL-2 and 721 cells (adjusted for background) or with PMA/Ionomycin. Data are adjusted for background responses to media alone.
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that NK-cell expansion may have occurred prior to our sampling time points. In addition, the relatively low number of seronegative individuals limited our statistical power to detect these differences. Therefore, our results are consistent with other reports that suggest that NK cell population expansion occurs early in primary infection but may be transient [7,12].

In this study NK cell and T-cell activation were uncoupled by primary HIV-1 infection illustrating the differential effects of HIV on NK cells and T-cells. This finding appears to be mostly explained by the relatively greater increase in T-cell activation. Alter and colleagues have previously shown that T-cell responses occur after NK-cell responses and are inversely correlated with the magnitude of NK cell activity during primary infection [23]. Primary HIV-1 infection was associated with expansion of KIR_{pos} NK cells, consistent with a previous study, but was not associated with enhanced KIR_{neg} NK cell activation. We speculate that this may be due to either expansion of NK cells able to detect HIV-1-infected cells that down-regulate HLA expression, or a regulatory response that diminishes NK cell reactivity. Fogli and colleagues have previously demonstrated that KIR_{pos} NK cells are incompletely activated in individuals with chronic HIV infection [8], which implies that this may be a regulatory mechanism. In this study we were not able to delineate which specific KIR (inhibitory versus activating) were expressed on the surface of NK cells. Further studies using newer reagents that can distinguish specific KIR are needed to understand the regulation of KIR expression in NK cells during primary infection.

Consistent with previous studies, NK cell function following stimulation with 721 cells or PMA/Ionomycin was impaired as early as primary HIV-1 infection even in the absence of significant expansion of anergic NK cells ($CD56_{neg}CD16_{pos}$) [7]. After infection, a higher proportion of NK cells degranulated in the presence of only IL-2 than before infection. This suggests that HIV may prime responses or that NK cells are degranulating *in vivo* in response to HIV. However, their response to culture with 721 cells was diminished. Similarly, their responses to non-specific stimulation with PMA/Ionomycin were reduced. These results suggest that NK cell functional deficiencies during primary infection might be attributed to impaired or incomplete activation rather than the accumulation of anergic NK cells.

Finally, we observed an increase in the frequency of blood NK cells expressing CCR7, a receptor for homing to lymphoid tissues. We did not observe a similar expansion of NK cells expressing $\alpha_4\beta_7$ integrins for homing to the gut. In contrast to our findings, Luteijn and colleagues did not observe a higher frequency of CCR7+ NK cells in blood from HIV-infected compared to uninfected individuals in their cross-sectional analysis [24]. One difference between these studies is that the participants in the study by Luteijn et al. were beyond the acute phase, but within the first year of HIV infection. Thus, the changes in CCR7 expression that we observed may be transient and limited to the acute phase of infection. In a previous study we found that chemokine receptor expression on NK cells changes rapidly [15]. In addition, the wide variation between individuals in the magnitude of change may also explain differences in the outcomes. In this study we noted a relatively large increase in the proportion of CCR7+ NK cells among some individuals and a much more subtle increase among others. Longitudinal assessment of changes in the frequency of CCR7+ NK cells within individuals would take some of this variability into account, and thus enable the detection of differences not apparent in a cross-sectional analysis. We propose that the increase in the frequency of KIR_{pos} NK cells in blood may also increase the proportion of NK cells that are able to traffic to lymphoid tissues. In support of this model, Marcenaro and colleagues previously showed that KIR-ligand mismatch was required for the *ex vivo* up-regulation of CCR7 expression by human NK cells interacting with either monocyte-derived dendritic cells or Epstein Barr Virus (EBV)-transformed B-cell lines [25]. It has been well-described that HIV decreases the expression of selected HLAs, which are ligands for KIRs [26]. Based on these observations, we speculate that the expansion of KIR-expressing NK cells concurrent with relative reduction in HLA-ligand expression simulates KIR-ligand mismatch and may facilitate NK cell CCR7 up-regulation and homing to lymphoid tissues. But Luteijin and colleagues observed relatively few KIR_{pos} NK cells in the lymph nodes of HIV-infected individuals [24] suggesting that these events may be transitory. Additional studies are need to determine if changes in NK-cell compartmentalization occur *in vivo* and whether it influences the viral load set point in the acute phase of HIV infection. Studies may also need to track NK cell homing longitudinally in order to determine whether, for example, the observation of lack of $\alpha_4\beta_{7pos}$ NK cell expansion is because NK cells home to the gut earlier than we were able to observe.

Several factors limit our conclusions. Firstly, we previously found that women in this cohort, who acquired HIV-1, had higher activation and lower NK cell responses prior to infection than women who remained uninfected [27,28]. Hence, these results may be generalizable to South African women who subsequently acquire HIV heterosexually, but they may not be generalizable to other populations. Future studies should consider whether pre-

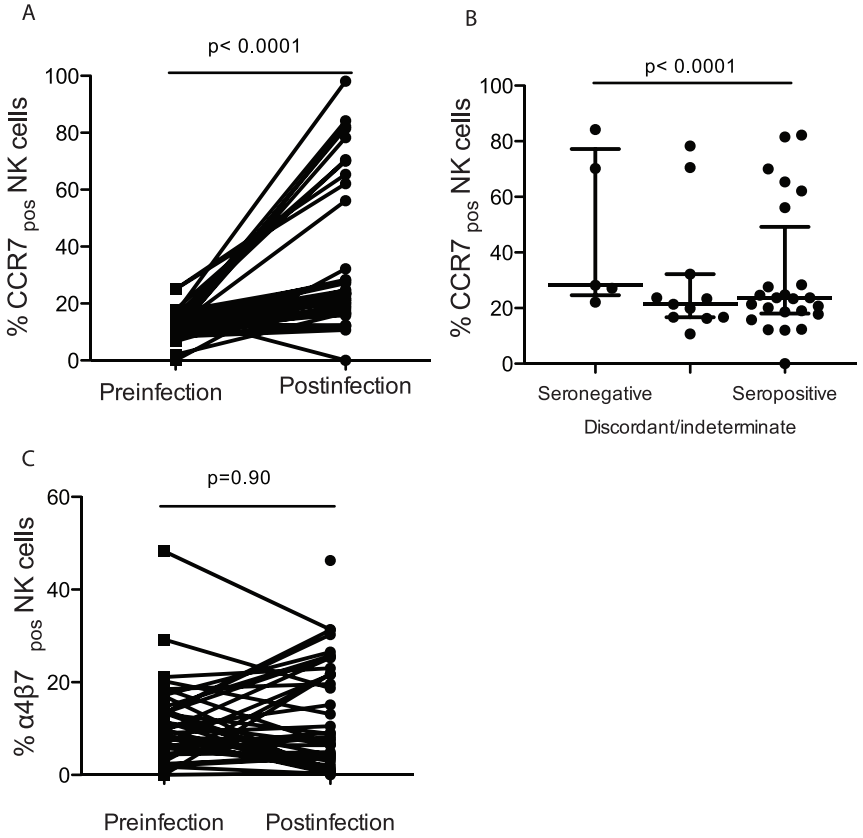

Figure 4. Early after HIV infection blood NK cells increased their expression of lymphoid, but not gut homing receptors. The proportion of NK cells expressing CCR7 was increased following HIV infection (A). The proportion of NK cells expressing CCR7 was reduced during later stages of primary infection (B). The proportion of NK cells that expressed α4β7, was not changed during primary HIV-1 infection (C).
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infection immune responses confound inferences about immune responses in the early post-infection period. Secondly, most women in this study were studied after the earliest phase of primary HIV-1 infection, as most had either discordant or positive serology at the time of first sampling. Thus transient events that occur very early infection may have been missed.

Overall, we found that primary HIV-1 infection was associated with activation of NK cells, potentially modulated by KIR expression. We observed functional impairment of NK cells early after HIV acquisition in the absence of anergic NK cell expansion. Further, during primary infection NK cells acquired the ability to home to secondary lymphoid tissues. This implies that they may play a role in early events outside the peripheral blood compartment. This study of matched blood specimens obtained before HIV-1 acquisition and during the stages of primary infection has helped delineate directionality in the relationship between NK cells and HIV during primary HIV-1 infection.

Supporting Information

Figure S1 The gating strategy for defining NK cells that expressed HLA-DR, a marker of activation, in matched samples obtained pre-infection (A) or post-infection (B), included (clockwise from top left for A and B) the gating of singlets, with forward (FSC) and side-scatter (SSC) properties consistent with lymphocytes, that were not monocytes ($CD14_{neg}$), B-cells ($CD20_{neg}$) or dead cells (Viability dye_{neg}) and that were CD3 negative and expressed CD16/CD56. Figure shows a single donor at each timepoint.
(EPS)

Figure S2 The gating strategy for defining NK cells expressing CD107a or IFN- γ and obtained pre-infection (A) or post-infection (B) were evaluated by multiparametric flow cytometry. NK cells were defined (shown clockwise for A and B) after gating on singlets, lymphocytes, non monocytes ($CD14_{neg}$), non B-cells ($CD20_{neg}$) or dead cells (Viability dye_{neg}) and that were CD3 negative but expressed CD7 (as described [18]). For each donor, both pre-infection and post-infection responses were evaluated following stimulation with media containing rhIL-2 alone, or with 721 cells or PMA/Ionomycin.
(EPS)

Figure S3 The frequency of NK cells (A), and the frequency of recently divided NK cells ($Ki\text{-}67_{pos}$, C) was not altered by HIV infection (B) nor during the stages of primary HIV infection (D). The proportion of activated NK cells was not altered during primary infection (E).
(EPS)

Figure S4 The proportion of anergic ($CD56_{neg}$), cytotoxic ($CD56_{dim}$) or cytokine-secreting ($CD56_{hi}$) NK cells was not significantly altered by HIV infection (A) nor by stage of primary infection (B).
(EPS)

Figure S5 The proportion of recently divided CD8+ T-cells (A), and degranulating CD8+ T-cells (B) was increased in primary HIV infection but the proportion of $CD8_{pos}$ T-cells secreting IFN-γ was not significantly altered (C).
(EPS)

Acknowledgments

We pay tribute to the participants of CAPRISA 004; women whose dedication and commitment to improving their and their peers' health and kindly donating samples during the conduct of the trial make this research possible. We gratefully acknowledge Lise Werner for advice on the statistical analysis and Galit Alter for advice on design and conduct of experiments.

Author Contributions

Conceived and designed the experiments: VN MA WHC. Performed the experiments: VN. Analyzed the data: VN MA QAK TN SSAK WHC. Contributed reagents/materials/analysis tools: VN MA QAK TN SSAK WHC. Wrote the paper: VN MA QAK TN SSAK WHC.

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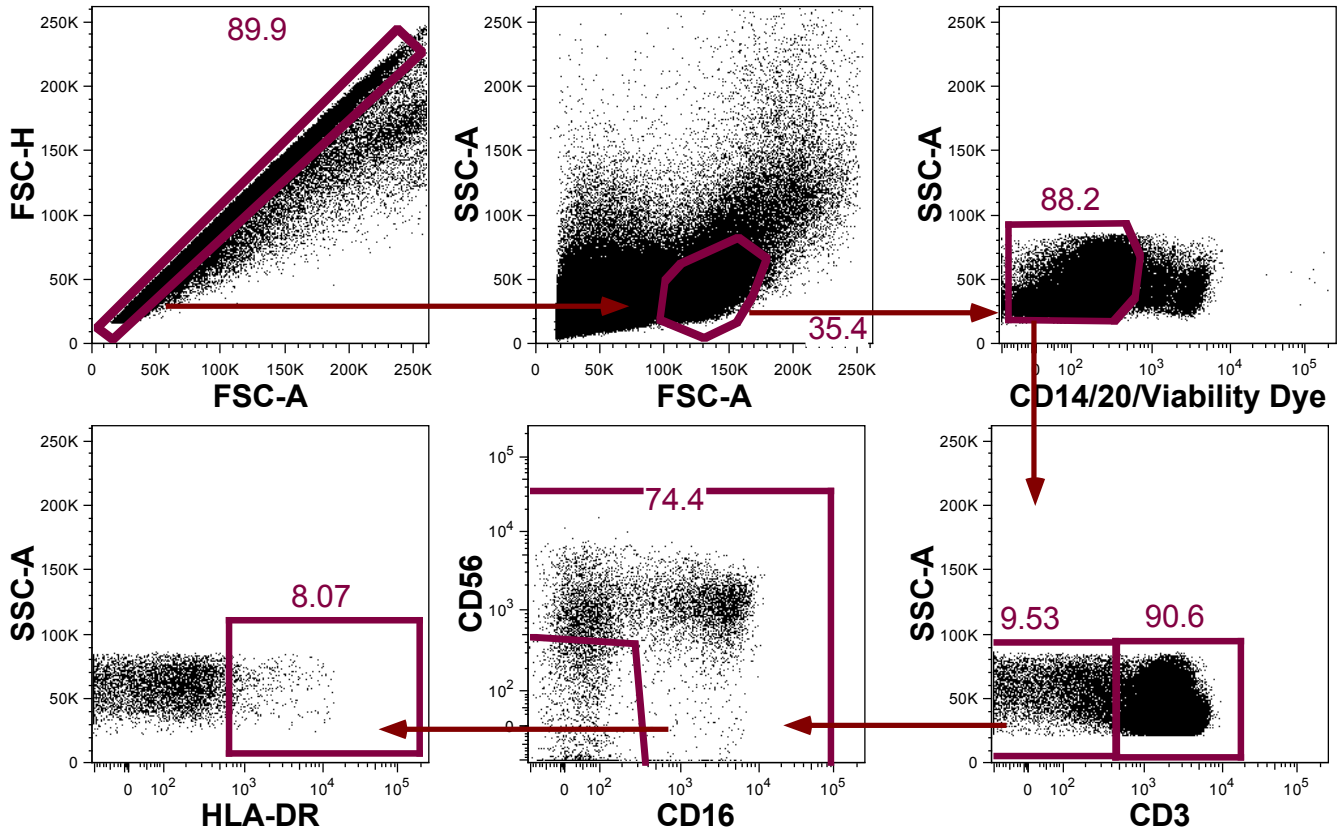
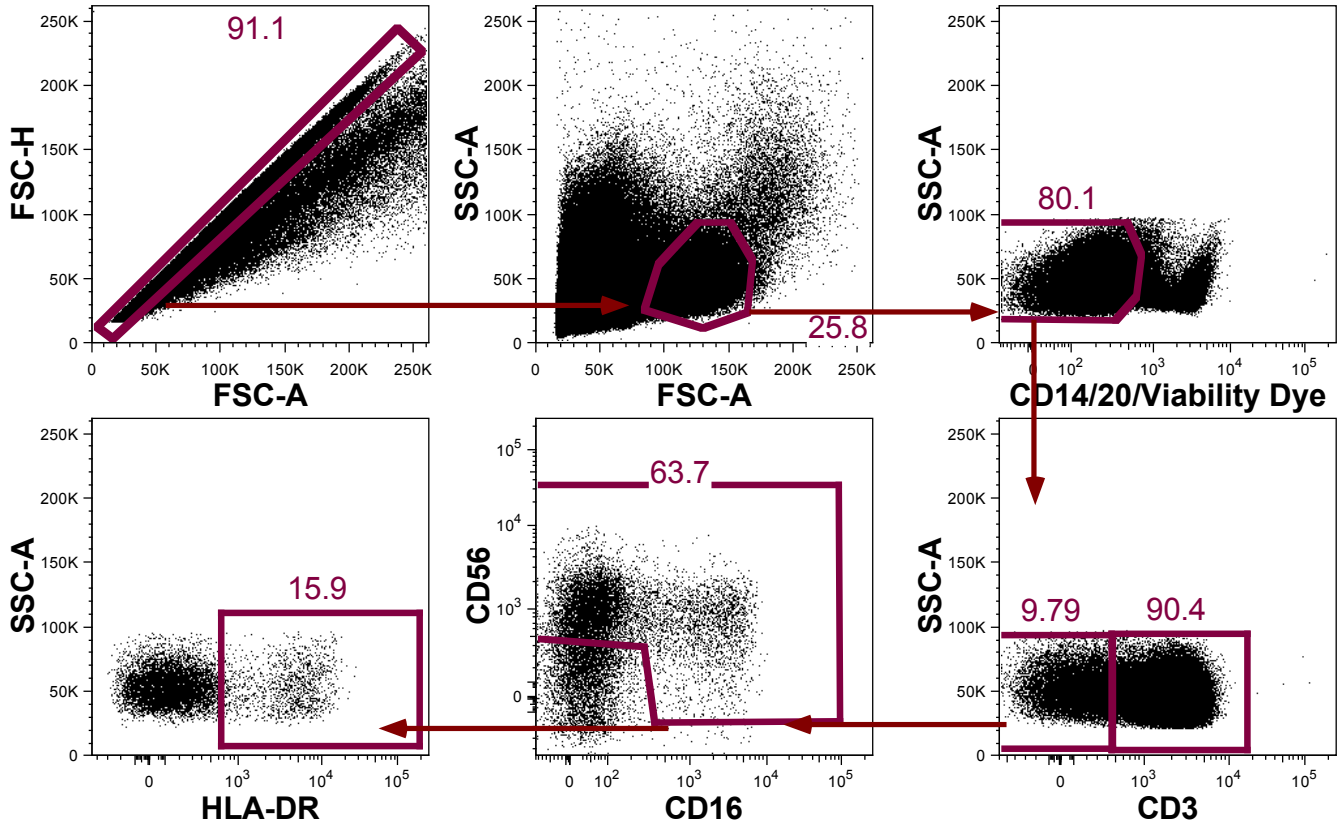
Supplementary Figure 1

A Pre-infection

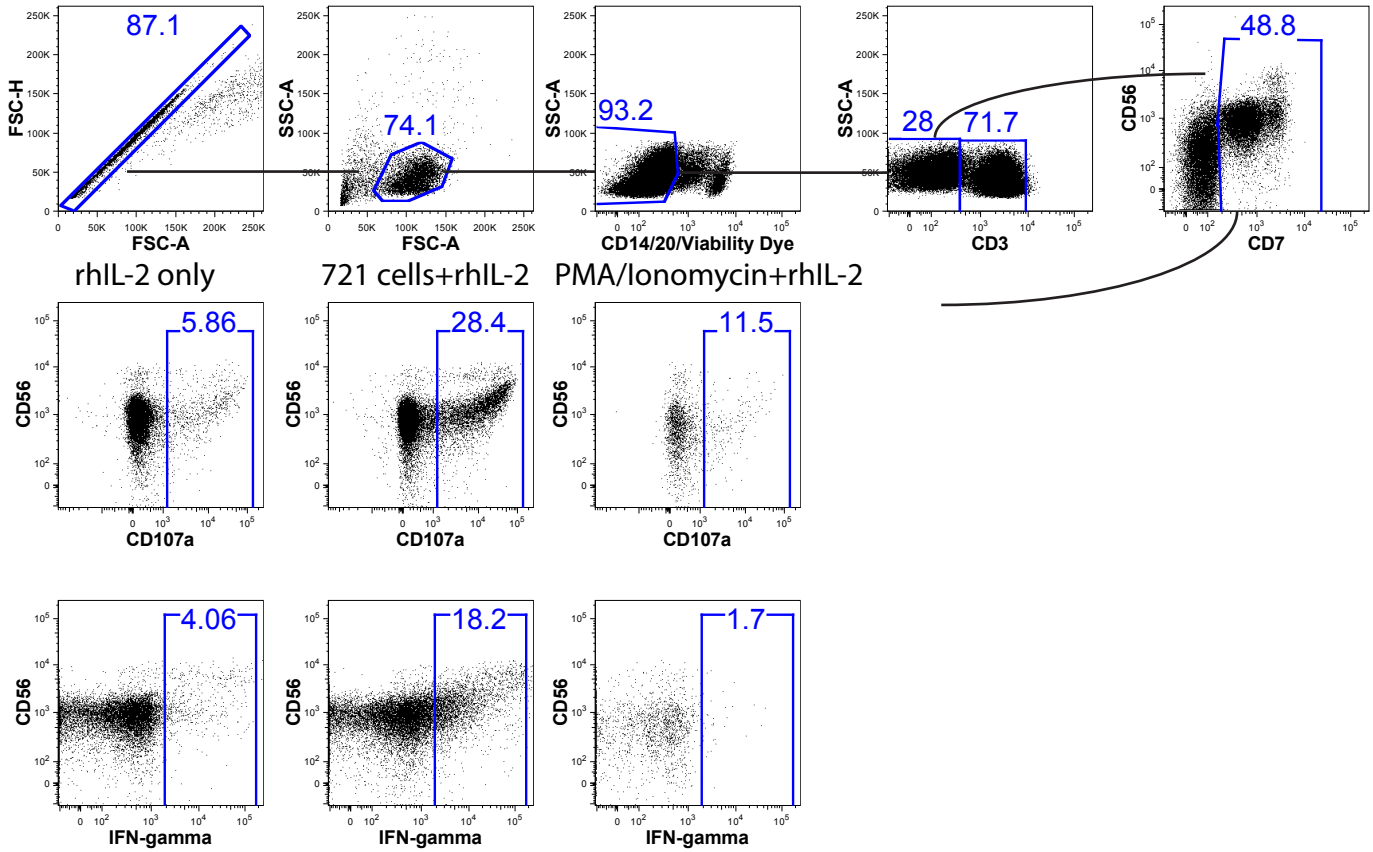


B Post-infection

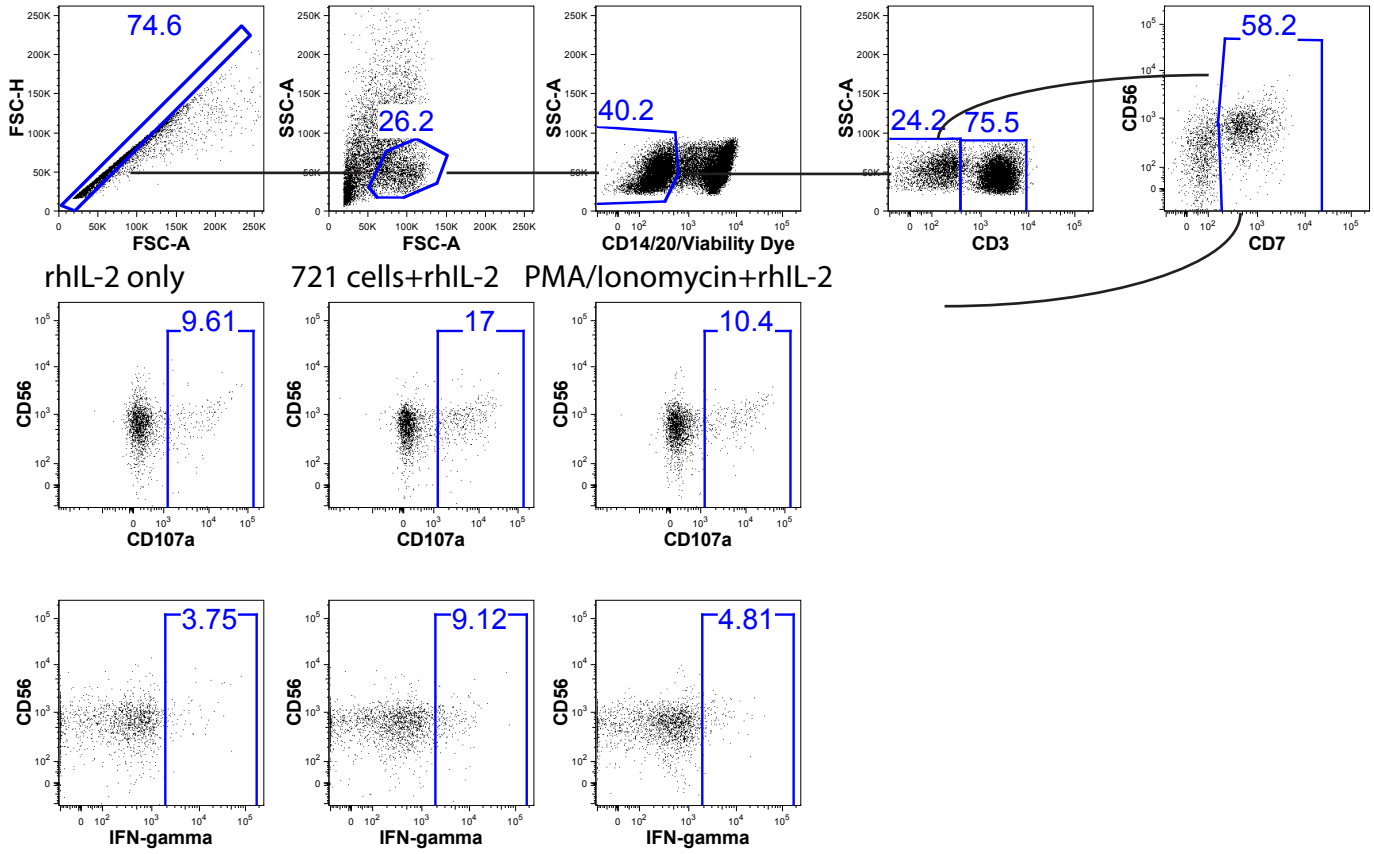


Supplementary Figure 2

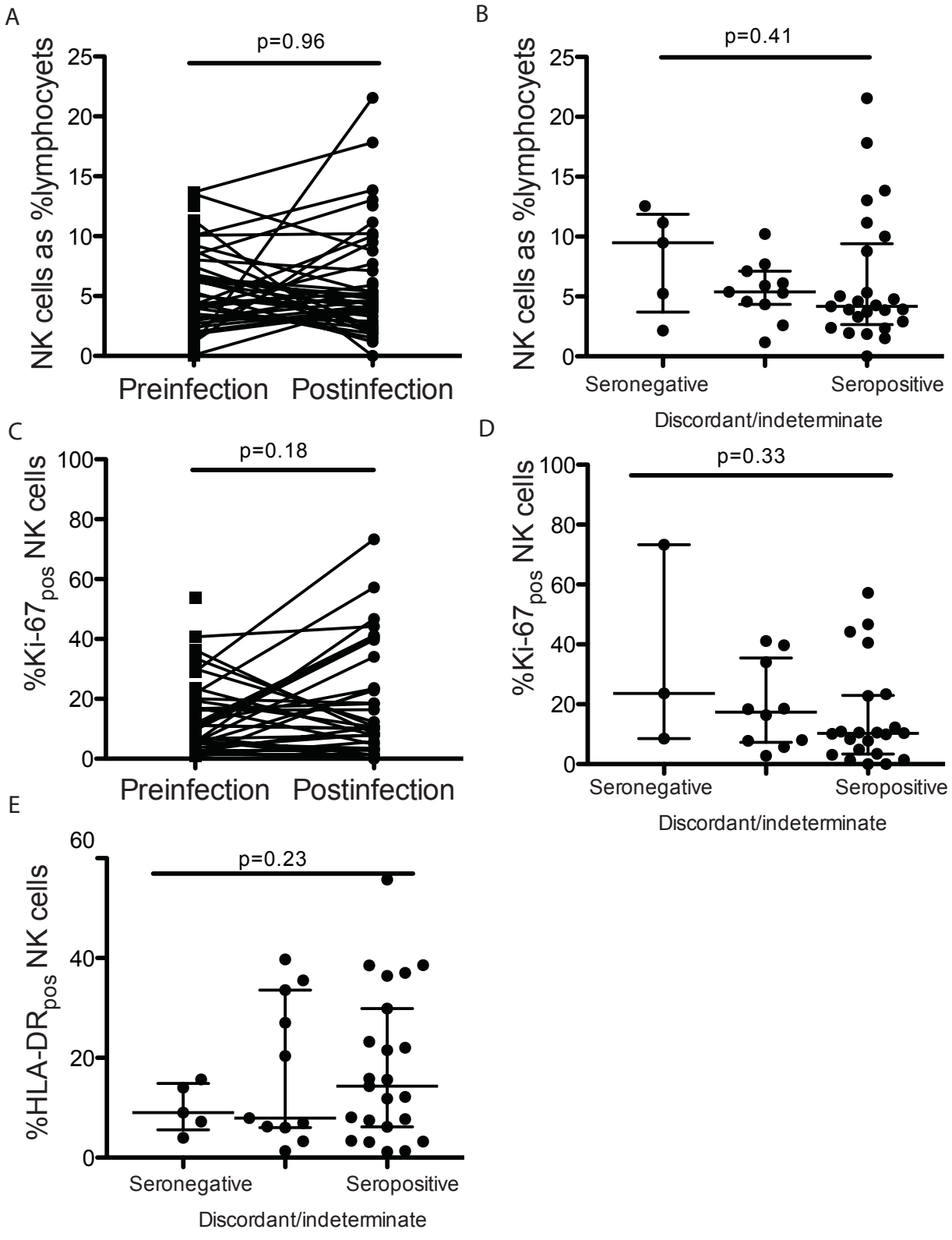
A Pre-infection



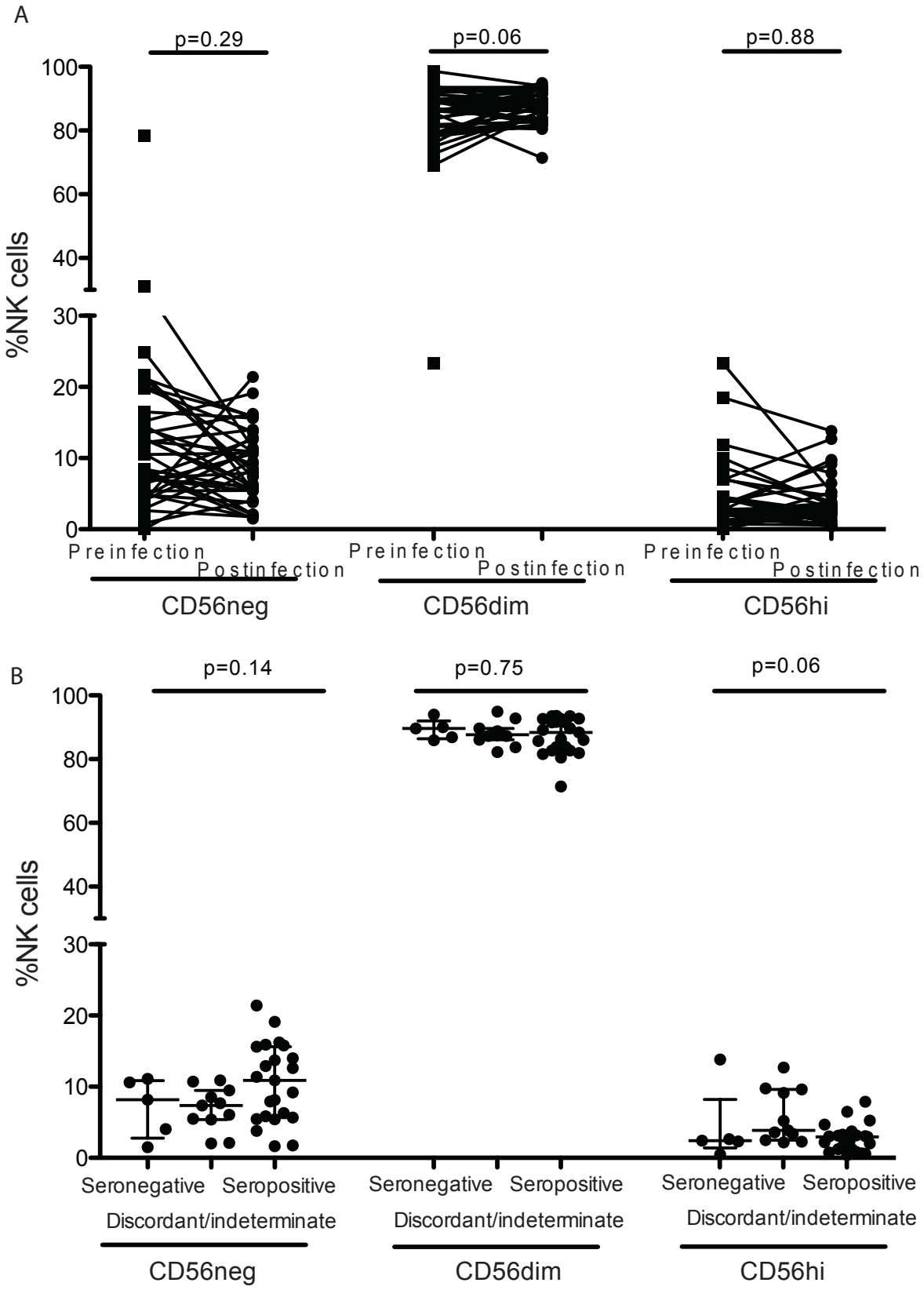
B Post-infection



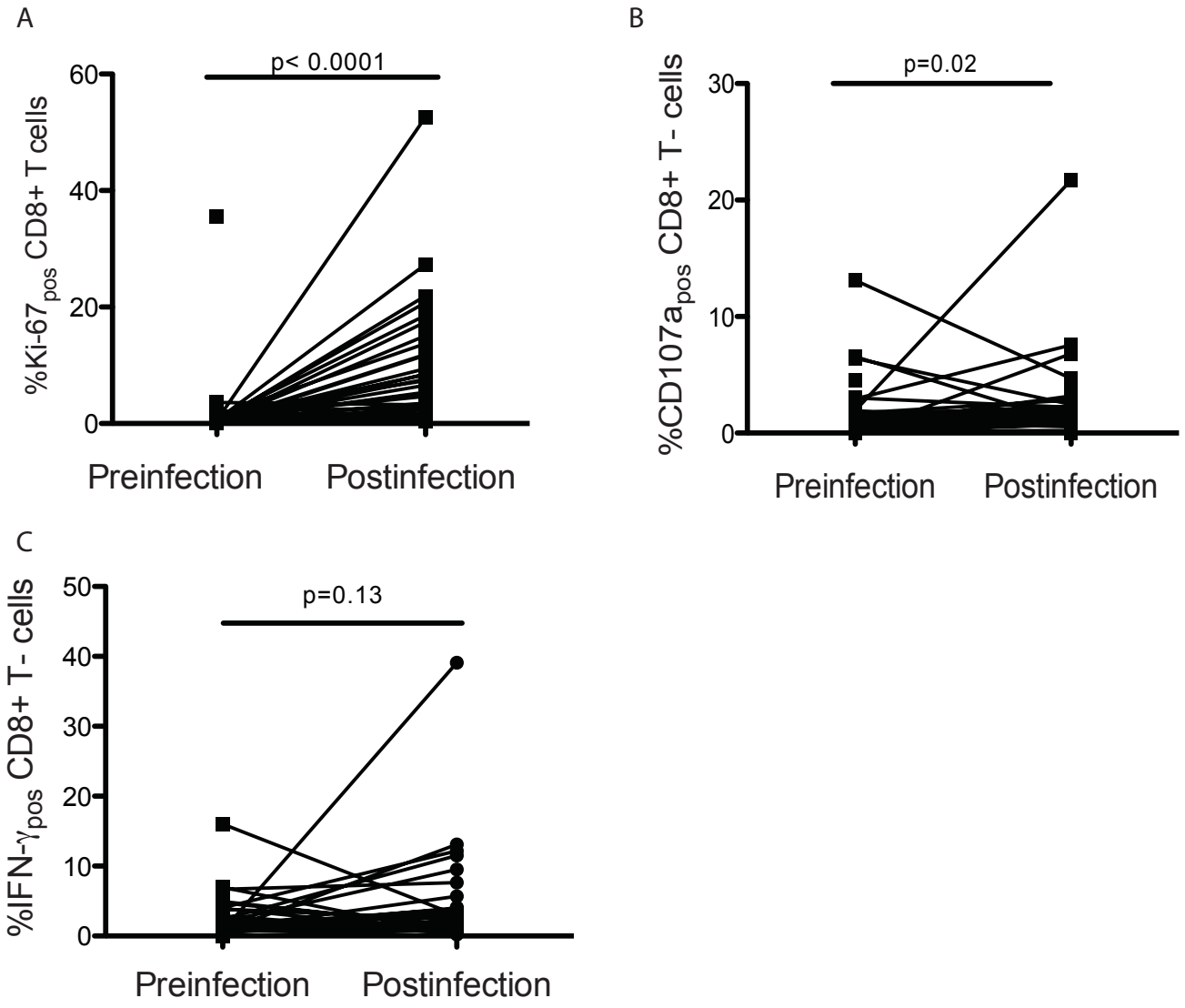
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



CHAPTER NINE:

Preservation HIV-1-specific CD4+ T IFNg cell responses in intercurrent infections following exposure to Tenofovir Gel – CAPRISA 004 microbicide trial

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Preservation HIV-1–Specific IFNγ+ CD4+ T-Cell Responses in Breakthrough Infections After Exposure to Tenofovir Gel in the CAPRISA 004 Microbicide Trial

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Abstract: The Centre for the AIDS Program of Research in South Africa 004 trial demonstrated reduction of sexual HIV-1 acquisition in women using a vaginal microbicide containing tenofovir. A better understanding of the consequences of antiretroviral-containing microbicides for immune responses in individuals with intercurrent HIV-1 infection is needed for future trials combining the use of microbicides with HIV-1 vaccines. Investigation of immune responses in women who acquired HIV-1 although using tenofovir gel showed significantly higher (P = 0.01) Gag-specific IFNγ+ CD4+ T-cell responses. The use of tenofovir-containing gel around the time of infection can modulate HIV-1 immunity, and these immunological changes need to be considered in future trials combining vaccines and microbicides.

Key Words: HIV-1, vaginal microbicide, tenofovir, HIV-1–specific CD4+ T-cell help

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The results from this study were presented in an oral presentation at the AIDS Vaccine meeting, 2011, Bangkok, Thailand.

The authors Q.A.K. and S.S.A.K. are coinventors with investigators from Gilead Sciences on two pending patents on tenofovir gel. The authors S.S.A.K. once received $2500 for serving on an Advisory Panel on PrEP for Merck. M.A. once received $1500 as a CME Honorarium from Gilead Science. M.W.M. was supported by the National Institutes of Health Office of the Director, Fogarty International Center, through the International Clinical Research Fellows Program at Vanderbilt (R24 TW007988).

The authors have no other conflicts of interest to disclose.

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INTRODUCTION

A recent randomized controlled trial undertaken by the Centre for the AIDS Program of Research in South Africa (CAPRISA) reported that a vaginal microbicide gel containing 1% tenofovir reduced the risk of HIV-1 infection in women by 39%.[1] Although confirmation of these findings in additional studies are needed and similar studies are currently underway, these results will have significant implication for the design of future prevention trials, including vaccine trials in which both vaccinees and placebo recipients might receive prophylactic antiretroviral drugs in gel or oral formulation.[2] A better understanding of the consequences of antiretroviral-containing microbicides for immune responses in individuals with intercurrent HIV-1 infection is therefore critical to take these immunological changes into consideration in the design of future trials combining the use of microbicides with HIV-1 vaccines.[2] In the present study, we investigated innate and adaptive immune responses during primary HIV-1 infection in women who acquired HIV-1 although using either tenofovir gel or placebo in the CAPRISA004 trial.

MATERIALS AND METHODS

Study Population

Cryopreserved peripheral blood mononuclear cell (PBMCs) were collected from sexually active HIV-1 clade C–infected 18-year-old to 40-year-old women in urban and rural KwaZulu-Natal, South Africa, enrolled in CAPRISA 004.[1] The eligibility and exclusion criteria for the parent trial have been previously reported.[1] Participants who

acquired HIV stopped using study gel on confirmation of HIV infection, as per the trial protocol.[1] From the total of 98 intercurrent HIV infections that occurred, 36 randomly selected HIV-1–infected female adults exposed either to tenofovir gel (n = 17) or placebo (n = 19) were selected for this substudy. The study was approved by the University of KwaZulu-Natal and Massachusetts General Hospital Biomedical Research Ethics Committee, Family Health International Protection of Human Subjects Committee, and the South African Medicines Control Council with each subject giving informed consent for participation.

Characterization of Phenotype and Function of Innate and Adaptive Immune Cells Using Multiparameter Flow Cytometry

The phenotypic characteristics of natural killer (NK) cells and myeloid dendritic cells (mDCs) were assessed by multiparameter flow cytometry using cyropreserved PBMC collected within 3 months of HIV-1 infection. After gating on lineage negative (CD3neg, CD19neg, CD56neg) lymphocytes, anti-CD11c was used to identify mDCs, as described.[3,4] Antibodies directed against Human Leukocyte Antigen (HLA)-DR, CD83, and CD86 were used to study the activation and maturation stage of mDCs directly ex-vivo. NK cells were defined as CD3negCD56/CD16+ cells, and antibodies directed against HLA-DR, CD38, and CD69 were used to study the activation status of NK cells, as described.[5]

Intracellular measurement of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin 2 (IL-2) production by CD4+ and CD8+ T cells was undertaken as described.[6,7] Cryopreserved PBMCs were stimulated for 6 hours at 37°C; 5% CO_2 with Gag and Nef peptide pools spanning the HIV-1 clade C consensus sequence (final concentration 2μg/ml per peptide) in the presence of brefeldin A. Negative controls with PBMCs and media alone and positive controls stimulated with phytohemaggultinin were included in the assays. Between 500,000–1,000,000 events were acquired on a LSRII flow cytometer, and data analysis was performed using FlowJo version 8.8.2 (TreeStar, Inc). Boolean gating was used to create a full array of the 8 possible response patterns when testing 3 functions, and data were further analyzed using SPICE 5 and PESTLE software programs (kindly provided by Drs Roederer and Nozzi, National Institute of Allergy and Infectious Diseases, National Institutes of Health).

Statistical Analyses

Statistical analyses and graphical presentation was done using Graphpad Prism 5 (Graphpad). Results are given as averages with standard deviations. Paired 2-tailed Student *t* tests were used to test statistical significance. Differences after comparisons were considered statistically significant if $P < 0.05$.

RESULTS

Characteristics of Study Subjects

Cryopreserved PBMCs collected from 36 sexually active HIV-1–infected women in urban and rural KwaZulu-Natal enrolled into the CAPRISA 004 trial[1] were randomly selected and studied. Investigators were blinded regarding the study group for the immunological analysis. Seventeen of these women were part of the tenofovir gel arm and 19 of the placebo arm. The demographic, immunological, and virological characteristics of the study cohort are presented in Table 1. Women in the 2 arms did not differ in the days since infection, and HIV-1 viral loads and CD4+ T-cell counts at presentation or analysis, and did also not differ in the distribution of low, intermediate, and high adherers.

TABLE 1. Characteristics of Study Subjects

	Tenofovir	Placebo	*P*
n	17	19	—
Age	24 (19–37)	23 (19–31)	0.69
Days post infection	85 (20–345)	64 (15–481)	0.30
Initial CD4+ T-cell count	454 (182–955)	556 (240–1036)	0.17
CD4+ T-cell count post infection	464 (197–955)	510 (240–1036)	0.11
Initial viral log (log)	4.66 (2.60–6.53)	4.42 (2.60–5.85)	0.72
Viral load post infection (log)	4.96 (2.60–5.86)	4.51 (2.60–5.51)	0.58

Average and range is shown.

Lack of Differences in Innate Immune Cells Between the Study Arms at 3 Months Post Infection

We initially examined the frequencies and activation status of NK cells and mDCs between women in the tenofovir or placebo arms. The average frequencies of NK cells did not differ between the 2 arms (average NK cells 4.7% ± 3.8% vs. 5.5% ± 4.6%). Furthermore, the average percentage of NK cells expressing HLA-DR (12% ± 6.3% vs. 12.9% ± 9.2%), CD38 (68.9% ± 25.3% vs. 67.1% ± 28.6%) or CD69 (10.3% ± 10.9% vs. 10.7% ± 7.1%) did not differ between the women who received tenofovir compared with those who received placebo ($P > 0.05$ for all comparisons). Similarly, the average percentage of mDCs (5.7% ± 3.2% vs. 5.9% ± 1.9%) and their activation status (HLA-DR: 19.8% ± 19.5% vs. 17.5% ± 11.3%; CD83: 6.8% ± 9.5% vs. 6.7% ± 4.2%, and CD86: 19.3% ± 21.4% vs. 11.5% ± 11.7%; $p > 0.05$ for all comparisons) did not differ between tenofovir and placebo gel recipients. Taken together, these data demonstrate that frequencies and activation status of cells of the innate immune system were not significantly impacted by the use of tenofovir gel in women with breakthrough HIV-1 infection approximately 3 months after infection.

Preservation of HIV-1–Specific IFNγ CD4+ T Cells During Primary Infection

Previous studies have shown that HIV-1 Gag and Nef are targeted by HIV-1–specific T cells during primary infection, and that virus-specific CD4+ T-cell responses are rapidly lost after infections.[8] We compared HIV-1 clade C Gag-specific and Nef-specific CD4+ and CD8+ T-cell activity

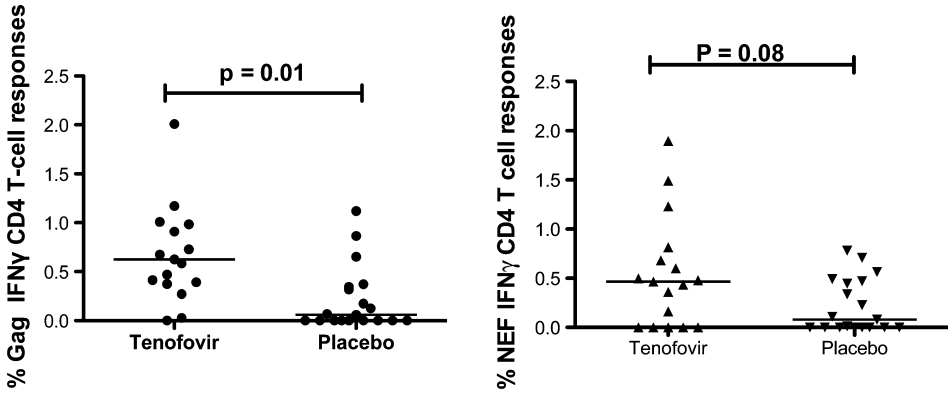

FIGURE 1. Higher HIV-1 Gag-specific and Nef-specific CD4+ T-cell responses in tenofovir recipients. The dot plot represents the median % IFNγ+ CD4+ T cells in response to HIV-1 Gag (left panel) and Nef (right panel) in women enrolled in the tenofovir arm (n = 17) compared with women who received placebo (n = 19).

between women who received tenofovir gel or placebo recipients. As shown in Figure 1, Gag-specific IFN-γ+ CD4+ T-cell responses were significantly higher in the women in the tenofovir gel arm compared with placebo ($P = 0.01$). No correlation was observed between days postinfection and Gag-specific IFN-γ+ CD4+ T-cell responses ($R = -0.2$; $P = 0.3$). HIV-1 Nef-specific CD4+ T-cell responses also trended to be higher in women in the tenofovir gel arm compared with placebo recipients ($P = 0.08$, Fig. 1). No significant differences were observed in the percentage of Gag-specific and Nef-specific CD4+ T-cells producing TNF-α or IL-2 between the 2 groups (data not shown).

Gag-specific and Nef-specific IFN-γ+ CD8+ T-cell responses were also higher in the tenofovir arm compared with placebo though those differences did not reach statistical significance (Gag: average 1.1% IFN-γ+ CD8+ T cells ± 1.0% in tenofovir arm vs. 0.7% ± 0.7% in placebo, $P = 0.33$; Nef: average 1.1% IFN-γ+ CD4+ T cells ± 1.4% in tenofovir arm vs. 0.35% ± 0.5% in placebo, $P = 0.35$). Furthermore, Gag and Nef-specific CD8+ T cells IL-2 and TNF-α production did not differ significantly between the 2 groups (data not shown). We also assessed the polyfunctionality of the CD4+ and CD8+ T cells by the simultaneous quantification of three functions (IL-2, IFN-γ, and TNF-α production) in response to Gag and Nef stimulation and observed no significant differences in the polyfunctionality of the CD4+ and CD8+ T cells in the tenofovir arm compared with placebo ($P > 0.05$). Overall, a vast majority of HIV-1–specific T cells were monofunctional (>90%) in both the tenofovir gel and placebo group. Taken together, these data demonstrate that the HIV-1–specific CD8+ T-cell responses in women with breakthrough infection during tenofovir microbicide use did not significantly differ from those that used placebo, but that Gag-specific IFNγ+ CD4+ T cells responses were significantly higher in HIV-1–infected women randomized to the tenofovir gel arm.

DISCUSSION

The use of a tenofovir-containing vaginal microbicide gel for the prevention of HIV-1 infection in sexually active, HIV-1–uninfected adult women in KwaZulu-Natal, South Africa, showed a significant reduction of HIV-1 infection rates by 39% in the primary intent-to-treat analysis.[1] Here, we examined the innate and adaptive immune responses in women with breakthrough HIV-1 infection that either used tenofovir microbicide gel or were part of the placebo arm. The frequencies and activation status of principal effector cells of the innate immune response, including NK cells and mDCs, assessed within 3 months of HIV-1 infection were not affected by the use of the tenofovir gel. Previous studies have demonstrated a significant expansion of NK cells in infected individuals during the early phase of HIV-1 infection and a subsequent contraction of NK cell and mDC populations.[9–11] We can therefore not exclude that NK cell and mDC frequencies in the initial 3 months of infection might have been affected by the use of the tenofovir gel, and that these changes in innate effector cells might have subsequently contributed to the observed functional differences in HIV-1–specific CD4+ T cells.[12]

HIV-1–specific T cells are considered critical for the control of HIV-1 replication and disease progression.[8,13–15] Although HIV-1–specific CD8+ T-cell responses to both Gag and Nef peptides did not significantly differ between the groups, HIV-1 Gag-specific IFN-γ+ CD4+ T-cell responses were significantly higher in women in the tenofovir gel arm compared with the placebo arm. HIV-1–specific CD4 + T cell responses are rapidly lost after acute infection in the presence of continuing HIV-1 replication, and this is in contrast to HIV-1–specific CD8+ T-cell frequencies which tend to increase over at least the first year of HIV-1 infection with continues exposure to antigen.[16–20] Previous studies have shown that CD4+ T cells and in particular gut-associated CD4+ T cells are severely depleted within the first few days/weeks of infection[21] and that HIV-1 preferentially infects HIV-1–specific CD4+ T cells.[22] The presence of an antiretroviral agent, such as tenofovir, during this crucial period might protect CD4 + T cells from deletion, allowing for a preservation of HIV-1-specific IFN-γ CD4+ T cells. The observation that women receiving tenofovir gel maintained higher Gag-specific CD4+ T-cell responses despite the reported lack of differences in viral load set-point between tenofovir gel and placebo recipients[1] was however unexpected, and the long-term clinical benefit of this preservation, and its consequences for HIV-1-specific immune function, will require further investigation.

Taken together, our studies in a subset of women who experienced breakthrough HIV-1 infection despite randomization to tenofovir gel usage demonstrate no significant alteration in the frequencies and activation status of key innate immune cells and HIV-1–specific CD8+ T-cell responses 3 months after infection. However, tenofovir gel applied vaginally around the time of HIV-1 transmission might protect HIV-1–specific CD4+ T-cell responses in infected individuals. This study demonstrates for the first time that the use of vaginal microbicides containing antiretroviral drugs can modulate HIV-1–specific immunity in individuals with breakthrough infection. These consequences of microbicide use for immune responses in individuals that acquire HIV-1 have to be considered in the design of future trials that will combine microbicides with HIV-1 vaccines.

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CHAPTER TEN: DISCUSSION AND CONCLUSIONS

10.1 Study conclusions

The overall aim of this study was to determine whether NK cells alter the events by which HIV-1 infects human cells and thereafter establish a productive HIV-1 infection process. As a result, this study assessed whether NK cells are involved in heterosexual HIV-1 acquisition and control and whether HIV-1 infection and Tenofovir gel impact NK cell responses. Notwithstanding the limitations of these studies, the following outcomes and conclusions from this series of studies can be drawn:

a. **NK cell assay development:**
   Multiparametric flow-cytometry using PBMC prepared within 8 hours can be used, with caveats, to study NK cells. Further, a novel method for high throughput KIR and HLA genotyping was developed.
b. **HIV-specific NK cell responses were associated with reduced HIV risk:**
   NK cell HIV-1 specific antiviral responses may play a role in protection against HIV-1 acquisition
c. **Generalised innate immune activation was associated with increased HIV risk:**
   The cause of the activation has yet to be established but microbial translocation and TLR responsiveness is unlikely to be a cause,
d. **HIV-1 infection impacts NK cell activity:**
   HIV-1 infection caused incomplete activation of NK cells, impaired cytolytic potential and preservation of antiviral responses starting during primary infection.
e. **Tenofovir gel altered T-cell but not innate responses:**
   Tenofovir gel did not alter innate immune responses in women with breakthrough infection but may have preserved adaptive immune responses.

10.1.1 **NK cell assay development**

Validation of multiparametric flow cytometric and cell preparation methods for quantifying NK cell activation, chemokine receptor expression and function

Ex vivo study of lymphocytes has become the standard approach for investigating their role in health and disease in humans, even though specific subset of lymphocytes respond differently to *ex vivo* manipulations. Studies of NK cells reported here demonstrate that delayed processing beyond eight hours introduces artefacts of increased activation, reduced chemokine receptor expression and reduced measures of NK cell cytolytic and cytokine-secretory function. Activated NK cells that have been through density gradient centrifugation show a distortion in their expression of chemokine receptors differentially for CCR4 (increased) and CCR7 (reduced). Hence, for studies in which inference about *in vivo* NK cell function is desired, sample processing should occur at least within eight hours after venepuncture. Ideally, whole blood assays should be considered, but where this is not feasible as in the studies presented in Chapters 4-9, the caveats attendant on using PBMC should be acknowledged.

Development of a novel method for high throughput genotyping for KIR and HLA

KIR and HLA have complex interactions that give rise to associations with disease. The development or a real-time PCR based typing method to simultaneously determine KIR gene content and HLA ligands allows further large-scale testing of KIR/HLA associations with human disease. When this method was used in the studies here, preliminary results (see Conference presentation 1- page xi), suggested that specific KIR genotypes were associated with lower viral loads during the first two years of HIV-1 infection. This methodology allows future studies to validate this preliminary finding by genotyping larger sample sets.

10.1.2 **HIV-specific NK cell responses were associated with reduced HIV risk**

HIV-1 specific responses were hypothesized to be protective. To probe the specific response of NK cells to HIV-1 infected cells, an assay that recapitulates *in vivo* conditions was designed and used. Using this assay, women who remained uninfected despite higher sexual risk than the women who became infected, had significantly higher proportions of NK cells that produced IFN-γ after co-culture with autologous HIV-1 infected T-cell blasts. Infection with HSV-2 was associated with impairment in NK cell IFN-γ responses. Although NK cell IFN-γ responses to 721.221 cells were higher in non-acquirers than acquirers, only the antiviral cytokine response to HIV-1 infected cells was associated with protection from acquisition. This association remained significant even after adjusting for other acquisition risk factors, including innate immune activation. Therefore, these data indicate that specific IFN-γ NK cell responses may be protective against HIV-1.

10.1.3 **Generalised innate immune activation was associated with increased HIV risk**

Current studies of the role of immune activation in HIV-1 acquisition are divergent in their conclusions. Some suggest that greater activation is universally protective, others suggested immune quiescence is protective. To date, studies of immune activation have used healthy unexposed individuals or chronic HIV-infected patients as controls; this is likely to have contributed to the varying propositions of immune activation and also immune quiescence as mechanisms of HIV protection. In contrast, the results here demonstrate that women who later acquired HIV-1 had significantly higher *ex vivo* levels of innate immune activation, as measured by higher levels of pro-inflammatory cytokines including IL-2, IL-7, IL-12p70 and TNF-α, platelets, NK cell activation, and constitutive CD8+ T-cell degranulation. That this finding withstood adjustment for potential confounders, different regression

analyses and was evident even in an unbiased principal components analysis points to the finding being robust. Hence we conclude that innate immune activation may plausibly have enhanced HIV-1 acquisition in women who participated in the Tenofovir gel trial.

During primary HIV-1 infection translocation of microbial products is thought to be a major cause of immune activation. Although women exposed to HIV-1 would not be expected to have the gut immunopathology characteristic of primary infection, there may be alternative causes of increased microbial burden in these individuals. Despite marked variability in levels of LPS, sCD14 or I-FABP, we found no evidence for microbial translocation as a correlate of immune activation in women exposed to HIV-1, but not infected. Differential response to pathogen-derived TLR ligands has been suggested as an alternative mechanism for enhanced activation in women exposed to HIV-1. Therefore, in a pursuant line of inquiry, cytokine responses of PBMC to HKLM (TLR2 ligand), LPS (TLR4 ligand) or AT-2 HIV (TLR7/8 ligand) were measured. Responses to TLR ligands were not associated with cellular activation. Therefore, neither microbial translocation nor TLR responsiveness are probable upstream causes of the activation profiles observed. Alternative contributors to immune activation may be alterations in the microbiome and/or chronic intercurrent infections perhaps by herpes viridae as HSV-2 infection was observed to alter NK cell function in this study and the herpesviridae are known modulators of systemic immune responses.. Future studies are required to clarify the causes of immune activation in women at risk for HIV.

10.1.4 **HIV-1 infection impacts NK cell activity**

Analyses of immune cells before and during primary HIV-1 infection revealed an increase frequency in activated and KIR-expressing NK cells, but reduced cytolytic potential during primary infection. The increase in frequency of NK cells able to traffic to lymph nodes following HIV-1 infection suggests that these cells may localise to the site of early viral replication. What role they may play within lymph nodes will require additional study.

10.1.5 **Tenofovir gel altered T-cell but not innate responses**

Breakthrough infection whilst using tenofovir gel may have facilitated protected exposure to HIV-1. Women who acquired HIV-1 whilst using Tenofovir gel had similar NK cell and dendritic cell (DC) activation profiles but had relative preservation of *gag*-specific IFN-γ+ CD4+ T-cell responses. Because of the small number of participants in this part of the study, firm conclusions cannot be drawn but these results do suggest that tenofovir gel may modulate some post-infection adaptive immune parameters in humans.

10.2 Limitations

The series of studies described here has several limitations:
Firstly, the case-control nature of this study limits the extent to which causality can be inferred for generalised innate immune activation as enhancing or HIV-specific NK cell antiviral response as protecting from HIV acquisition. Although statistical adjustment was performed for known differences, such as age, between cases and controls, it is still possible that unmeasured covariates may partially account for some of the differences observed between cases and controls. Prospective human cohort or experimental animal studies are both feasible next steps for strengthening causal inference.

Secondly, the studies reported here were limited to measures of systemic NK cell responses using cryopreserved specimens. Given the distribution of NK cells in mucosal tissue, and their recently reported ability to secrete IL-22, an important cytokine for mucosal immunity (Malmberg and Ljunggren, 2009), it is plausible that NK cells may have different roles at the mucosa than those described here. In collaboration with other investigators (Roberts L., 2011), it was demonstrated that immune activation in the genital tract correlates with HIV-1 acquisition, recapitulating the systemic observations. Further studies are required to delineate the relationship between systemic and mucosal immune activation. As observed in chapter three, sample processing may

markedly affect *ex vivo* measures of immune cell function. The use of cryopreserved cells was unfortunately, not addressed by these studies and although widely practiced in clinical trials, use of cryopreserved specimens may not extrapolate to *in vivo* events. More generally, the *ex vivo* measures here are mere correlates of events *in vivo* so may include implicit biases.

Thirdly, the sample size in these studies, though comparable to similar studies, was modest. Therefore the risk of Type II errors-failing to reject a false null hypothesis- was substantial. Larger studies may have allowed delineation of NK cell related factors that have smaller effects on HIV-1 protection or control.

Fourthly, the post-infection timepoints studied were usually after acute HIV-1 infection. Hence events that may have occurred during very early HIV-1 and may correlate with disease course may have been missed.

Finally, this study was limited to women participating in a microbicide trial in South Africa. Although the Tenofovir study included women similar to those at moderate to high risk of HIV in these settings, the findings may not be generalizable to settings other than clinical trials, women of different ages and in different geographical areas. Although Tenofovir gel did not appear to confound the results observed, further studies are required to determine whether the findings described here apply to risk groups in other settings. For example, it would be useful to assess this in the VOICE study (Marrazzo J., 2013) in which tenofovir gel was made available to the study participants but adherence to using gel every day, was low.

The major strength of this analysis is the study of clinically well defined high-risk women at timepoints prior to infection. This offers strength to causal inference as temporality is observed. A further strength is the wide array of measures assessed and in particular, the measurement of NK cell responses to autologous *in vitro* HIV infected cells. This assay models the likely events during early infection when NK cells first encounter infected cells. Lastly, the

setting of this study simulates those where the highest burden of infection is seen.

10.3 Implications for Future Research

This work has two major implications for the development of prevention interventions. Since antiviral NK cell responses targeted against HIV appear to be involved in preventing infection, work to define whether these responses constitute NK cell memory and whether they could be primed by a novel vaccination strategy may be fruitful. Likewise, consistent with previous evidence, non-specific immune activation increased the risk HIV infection by HIV-1 (Shapira-Nahor et al., 1998), it appears likely that systemic innate immune activation is in the causal pathway of HIV-1 acquisition. Monitoring of generalised and local immune activation during interventions to prevent HIV may therefore be helpful in predicting unexpected outcomes such as those observed in the STEP vaccine trials (McElrath et al., 2008, Buchbinder et al., 2008). Reducing generalised innate immune activation may be a new target for prophylaxis of HIV-1 acquisition. Further elucidation of this potential causal pathway and identification of the points where intervention may be possible should be pursued. In collaboration with colleagues at CAPRISA and University of Cape Town, exploratory studies on how the systemic findings observed here link to genital tract events are continuing in conjunction with an assessment of whether genital microbiome changes may be associated with altered genital mucosal immune activation.

The observation that NK cell function is impaired early in HIV infection supports the idea that immunomodulatory interventions to elicit therapeutic responses will need to take into account early impairment of NK cell cytolytic function.

Finally, the findings that CD4+ T-cell responses may be selectively preserved during early infection following breakthrough infection whilst on Tenofovir gel suggest that even failed antiviral prophylaxis may have some associated

benefits. Replication of these data in larger numbers of individuals, and in additional studies is necessary.

Future studies would benefit by addressing weaknesses inherent to the design of these studies. First, a larger cohort in which complete ascertainment of exposure variables of interest such as immune activation and NK cell IFN- responses could be made would be advantageous. Use of freshly obtained specimens with several complementary approaches such as the autologous HIV-infected assay used here as well as heterologous killing assays may avoid confounding by specimen preservation or by assay-specific bias. Second, future studies should aim to sample both the genital tract and systemic compartments (ideally including lymph nodes). Finally, definitive demonstration of the role of innate immune activation, and of NK cells responding to HIV may require adoptive transfer of these cells into a suitable model (perhaps humanised mice) for challenge with heterologous virus. Similarly, putative causes of activation could be formally tested with mechanistic models complementary to the observations in humans.

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