Impact of Duffy Antigen Receptor for Chemokines (DARC)-null linked Neutropenia on Neutrophil and Natural Killer cell Function in HIV-1 Infection

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University of KwaZulu-Natal, Durban.

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PREFACE

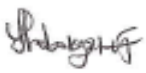
This project represents original work done by the author and where others have made contributions it has been acknowledged in the text. This work has not been submitted in any form for any degree or diploma at another tertiary institution.

The experimental work described in this thesis was performed in the HIV Pathogenesis Programme Laboratory at the Doris Duke Medical Research Institute at the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal in Durban, South Africa. The work was supervised by Dr Christina F. Thobakgale-Tshabalala and Professor Thumbi Ndung'u.

Kewreshini K. Naidoo

Signed:  Date: 04 December 2018

Christina F. Thobakgale-Tshabalala

Signed:  Date: 04 December 2018

As the candidate's supervisor I agree to the submission of this thesis.

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DECLARATION

I, Kewreshini K. Naidoo, declare that

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PUBLICATIONS AND PRESENTATIONS

Peer-reviewed Publications

Thobakgale, C., **Naidoo, K.**, Mckinnon, L. R., Werner, L., Samsunder, N., Karim, S. A., Ndung'u, T., Altfeld, M. & Naidoo, K. 2017. Interleukin 1-Beta (IL-1beta) Production by Innate Cells Following TLR Stimulation Correlates With TB Recurrence in ART-Treated HIV-Infected Patients. Journal of Acquired Immune Deficiency Syndromes, 74, 213-220.

Zulu, M. Z., **Naidoo, K. K.**, Mncube, Z., Jaggernath, M., Goulder, P. J. R., Ndung'u, T., Altfeld, M. & Thobakgale, C. F. 2017. Reduced Expression of Siglec-7, NKG2A, and CD57 on Terminally Differentiated CD56(-)CD16(+) Natural Killer Cell Subset Is Associated with Natural Killer Cell Dysfunction in Chronic HIV-1 Clade C Infection. AIDS Res Hum Retroviruses, 33, 1205-1213 **(shared first author).**

Naidoo, K.K., Ngubane, A., Gaza, P., Moodley, A., Ndung'u, T., Thobakgale, C.F. Neutrophil Effector Functions are not Impaired in Duffy Antigen Receptor for Chemokines (DARC)-null Black South Africans. Frontiers in Immunology (*in press*).

Presentations

Naidoo, K.K., Mbhele, N., Ngubane, A., Dong, K.L., Ndung'u, T. and Thobakgale-Tshabalala, C.F. NK Cell Responses in a DARC-null Zulu/Xhosa African HIV-1 Infected Population. Keystone Symposia on Molecular and Cellular Biology: HIV Vaccines, 20-24 March 2016, Olympic Valley, California, USA. Abstract X8 3047 (poster presentation).

Naidoo, K.K., Mbhele, N., Rashid, T., Ngubane, A., Dong, K.L., Ndung'u, T. and Thobakgale-Tshabalala, C.F. NK Cell Responses in a DARC-null Zulu/Xhosa African HIV-1 Infected Population. University of KwaZulu-Natal College of Health Sciences Research Symposium, 8-9 September 2016, Durban, KwaZulu-Natal, South Africa (oral presentation).

Naidoo, K.K., Ngubane, A., Gaza, P., Moodley, A., Ndung'u, T., Thobakgale, C.F. Neutrophil Effector Functions are not Impaired in Duffy Antigen Receptor for Chemokines (DARC)-null Black South Africans. European Society for Clinical Investigation (ESCI) Congress, 22-24 May 2019, Coimbra, Portugal (poster presentation).

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ABSTRACT

Sub-Saharan Africa carries a disproportionate burden of the HIV-1 epidemic. The Duffy Antigen Receptor for Chemokines (DARC)-null polymorphism is a predictor of ethnic neutropenia which commonly occurs in persons of African ethnicity and is thought to account for up to 11% of HIV-1 infections on the African continent. Neutrophils are recognised for their killing mechanisms and have been noted for their regulatory mechanisms in recent years. For example, a role for neutrophils in natural killer (NK) cell priming in the periphery has been suggested, and neutrophil deficiency has been implicated in contributing to NK cell immaturity and dysfunction. While the DARC-null genotype is well associated with lower absolute neutrophil counts (ANCs), studies that assess the effects of the polymorphism on neutrophil functionality are lacking and the impact of DARC-null linked neutropenia on HIV disease progression is debatable. Furthermore, the influence of DARC-null neutropenia on the NK cell compartment is unknown. In this cross sectional pilot study, we assessed the impact of the DARC-null trait on neutrophil effector functions and also characterised NK cell profiles in Zulu/Xhosa African individuals from a high incidence HIV setting in Durban, South Africa. We hypothesised that in the context of the DARC-null genotype and lower ANCs in our cohort participants, neutrophils would have impaired functionality and would be unable to efficiently prime NK cells; thus affecting NK cell maturation and function, and altering NK cell homeostatic activities such as survival and proliferation. We further hypothesised that the impaired cellular responses in DARC-null individuals would be more prominent in HIV-1 infected individuals compared to HIV negative individuals.

Neutrophil killing mechanisms were measured in HIV-1 chronically infected (n=22) individuals and HIV negative (uninfected) controls (n=20). For assessment of key neutrophil effector functions, isolated neutrophils were evaluated for Fc receptor-mediated phagocytosis following uptake of IgG opsonised beads using flow cytometry; reactive oxygen species (ROS) emission was measured by chemi-luminesce after activation of neutrophils with phorbol 12-myristate 13-acetate (PMA). Activated neutrophils were also visualised by fluorescent microscopy for neutrophil extracellular trap (NET) quantification. Assessment of the NK cell compartment in chronically HIV-1 infected (n=18) and uninfected (n=20) individuals using multi-parametric flow cytometry determined NK cell subsets, maturation profiles, cytokine production and degranulation. Annexin V and propidium iodide assays were used to determine NK cell survival, whilst CFSE staining was used to examine cytokine-activated NK cell

proliferation. Study subjects were genotyped for the DARC trait using TaqMan allelic discrimination assays and ANCs were measured by full blood count.

Our findings confirmed a high prevalence of the DARC-null allele in the African population and the polymorphism was significantly associated with lower ANCs. Neutrophil functional analysis detected rapid and higher phagocytic activity in the absence of DARC at 10 minutes ($p=0.05$ and $p=0.009$) and 60 minutes ($p=0.05$ and $p=0.07$) in HIV negative and HIV-1 infected subjects respectively. ROS and NET production in neutrophils were mostly unaffected by DARC negativity irrespective of HIV status. The only exception to this was a reduction in NET production in neutrophils from DARC-null HIV infected subjects ($p=0.04$) following prolonged *in vitro* stimulation. In the NK cell compartment, individuals showed similar NK cell counts irrespective of HIV status. In HIV negative individuals, a marked reduction of total NK cell counts was noted in the absence of DARC ($p=0.006$) and this correlated with lower ANCs ($p=0.002$) and a weak trend towards higher CD56 bright subset proportions was noted in DARC-null individuals ($p=0.08$). HIV negative DARC-null subjects also displayed a less mature NK cell phenotype with higher proportions of hypo-responsive KIR-NKG2A- NK cells ($p=0.06$) and lower frequencies of terminally differentiated CD57 ($p=0.02$) expressing NK cells. However, this immature phenotype did not translate to differences in expression of NK cell activation markers CD69 or HLA-DR and exhaustion marker PD-1 by DARC state. Furthermore, no differences in relation to NK cell degranulation and cytokine production were detected in the absence of DARC in HIV negative subjects. In contrast to HIV negative individuals, HIV-1 infected subjects displayed NK cell subset redistribution marked by higher CD56 negative NK cells, marginally higher frequencies of less mature NK cells, accompanied by higher expression of activation and exhaustion markers and lower cytolytic potential. However, these observed phenotypic and functional differences were lost upon DARC stratification in HIV-1 infected persons. Lastly, examination of NK cell survival capacity demonstrated only marginal differences during HIV infection in the absence of DARC ($p=0.09$); no changes were detected in NK cell proliferation by DARC state in HIV negative or infected individuals.

Together our data suggests that the DARC-null polymorphism and lower ANCs does not adversely affect neutrophil activity irrespective of HIV status. We also show that while HIV negative individuals with the DARC-null genotype displayed reduced NK cell counts with a less mature phenotype, the condition did not compromise NK cell functionality or homeostatic activities. Furthermore, no significant differences were exhibited in the context of DARC

during HIV infection, suggesting that any advantage that the DARC-positive trait may offer pre-infection is lost in chronic infection. DARC-null associated neutropenia is considered a mild condition and thus our findings support reports that the effect of ethnic neutropenia is not as pronounced as exhibited in severe neutropenia. Overall the data presented here provides mechanistic evidence behind the asymptomatic clinical characteristics associated with benign ethnic neutropenia.

ETHICS

The study was granted ethical approval by the Biomedical Research Ethics Council (BREC) of the University of KwaZulu-Natal, Ref BE229/15.

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ABBREVIATIONS

α, alpha

β, beta

µg, microgram

µl, microlitre

µm, micrometre

µM, micromolar

°C, degrees Celsius

ADCC, antibody-dependent cellular cytotoxicity

AIDS, Acquired Immune Deficiency Syndrome

AIN, autoimmune neutropenia

ANC, absolute neutrophil count

APC, Allophycocyanin

ARV, antiretroviral

BD, Becton Dickinson

BREC, Biomedical Research Ethical Council

BSA, bovine albumin serum

BV, Brilliant Violet

CA, capsid

CC, DARC-null

CCR5, C-C chemokine receptor 5

CD, Cluster of Differentiation

CFSE, carboxyfluorescein succinimidyl ester

CO_2, carbon dioxide

CXCR4, CXC chemokine receptor 4

DARC, Duffy antigen receptor for chemokines

DC, dendritic cell

ddH_2O, double distilled water

DMSO, Dimethyl Sulfoxide

DNA, Deoxyribo Nucleic Acid

dPBS, Dulbecco's phosphate-buffered saline

EDTA, Ethylenediaminetetraacetic acid

Env, Envelope glycoprotein

ESCRT, endosomal sorting complex required for transport

FBS, foetal bovine serum

FcɣR, fragment crystallisable gamma receptor

FcR, fragment crystallisable receptor

FITC, Fluorescein isothiocyanate

FRESH, Females Rising through Education, Support and Health

FMO, fluorescence minus one

FSC-A, forward scatter area

G-CSF, granulocyte-colony stimulating factor

Gag, Group specific antigen

GM-CSF, granulocyte-macrophage-colony stimulating factor

g, gravitational force

gp, glycoprotein

HAART, highly active antiretroviral therapy

HIV, Human Immunodeficiency Virus

HIV-1, Human Immunodeficiency Virus Type 1

HLA, human leukocyte antigen

HPP, HIV Pathogenesis Programme

HSA, human serum albumin

HSC, hematopoietic stem cell

ICAM, intercellular adhesion molecule

ICS, intracellular staining

Ig, Immunoglobulin

IL, Interleukin

IFN, interferon

IN, integrase

IQR, interquartile range

ITAM, immunoreceptor tyrosine-based activation motif

ITIM, immunoreceptor tyrosine-based inhibitory motif

KIR, killer cell immunoglobulin-like receptor

KZN, KwaZulu-Natal

LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry

LDN, Low density neutrophils

LTR, long terminal repeat

M, molar

MA, matrix

MHC, major histocompatibility complex

MIP, macrophage inflammatory protein

mg, milligram

ml, millilitre

mm, millimetre

mRNA, messenger RNA

MSC, mesenchymal stem cell

na, not applicable

Nab, neutralising antibody

NADPH, nicotinamide adenine dinucleotide phosphate

NB, nucleic-acid binding

NC, nucleocapsid

NCAM, neural cell adhesion molecule

NCR, natural cytotoxicity receptor

NDN, normal density neutrophils

Nef, negative regulation factor

NET, neutrophil extracellular trap

ng, nanogram

NK, natural killer

NKR, natural killer receptor

nM, nanomolar

NP, neutrophil phagocytosis

ns, not significant

PAMP, pathogen-associated molecular pattern

PBMC, peripheral blood mononuclear cells

PBS, phosphate-buffered saline

PCR, polymerase chain reaction

PE, Phycoerythrin

PI, propidium iodide

PIC, pre-integration complex

PMA, phorbol 12-myristate 13-acetate

PMN, polymorphonuclear neutrophil

Pol, Polymerase

PR, protease

r, Spearman rho

RBC, red blood cell

Rev, regulator of virion protein

RNA, Ribo Nucleic Acid

ROS, reactive oxygen species

RPMI, Roswell Park Memorial Institute

RT, reverse transcriptase

RTC, reverse transcription complex

RT-PCR, real time polymerase chain reaction

SCN, severe congenital neutropenia

SIV, Simian Immunodeficiency Virus

SLAN+ DC, 6-sulfo $Lac^{+}NAc^{+}$ dendritic cell

SNP, single-nucleotide polymorphism

SSC-A, side scatter area

SU, surface subunit glycoprotein

Tat, transactivator of transcription factor

TC/TT, DARC-positive

TCR, T cell receptor

Th, two helper

TLR, Toll-like receptor

TM, transmembrane protein

TNF, tumour necrosis factor

UKZN, University of KwaZulu-Natal

USA, United States of America

Vif, viral infectivity factor

Vpr, viral protein R

Vpu, viral protein U

CHAPTER 1: LITERATURE REVIEW

1.1 HUMAN IMMUNODEFICIENCY VIRUS (HIV)-1

Since its identification over thirty years ago, Human Immunodeficiency Virus (HIV)-1 has become the defining public health concern of our time. HIV-1 targets cluster of differentiation (CD)4+ T cells of the immune system and infection leads to progressive immune dysfunction and deterioration. This allows opportunistic infections and cancers to thrive, resulting in Acquired Immunodeficiency Syndrome (AIDS) (Fauci and Lane, 2005).

1.1.1 HIV-1 Epidemiology

According to UNAIDS an estimated 36.9 million people were living with HIV in 2017 (Figure 1.1), with 1.8 million new infections in that year alone. Sub-Saharan Africa remains the worst affected region, accounting for almost 70% of infected people worldwide. South Africa is one of the top ranking countries in the world in terms of HIV prevalence, estimated at 18.8% in 2017 (UNAIDS, 2017b). Of the 7.9 million people infected with HIV in South Africa, the number of infections is disproportionately skewed towards the black South African population with an HIV prevalence of 16.6%, compared to other racial groups of 1.1%, 5.3% and 0.8% in the Caucasian, Coloured and Indian/Asian ethnic groups respectively (HRSC, 2017).

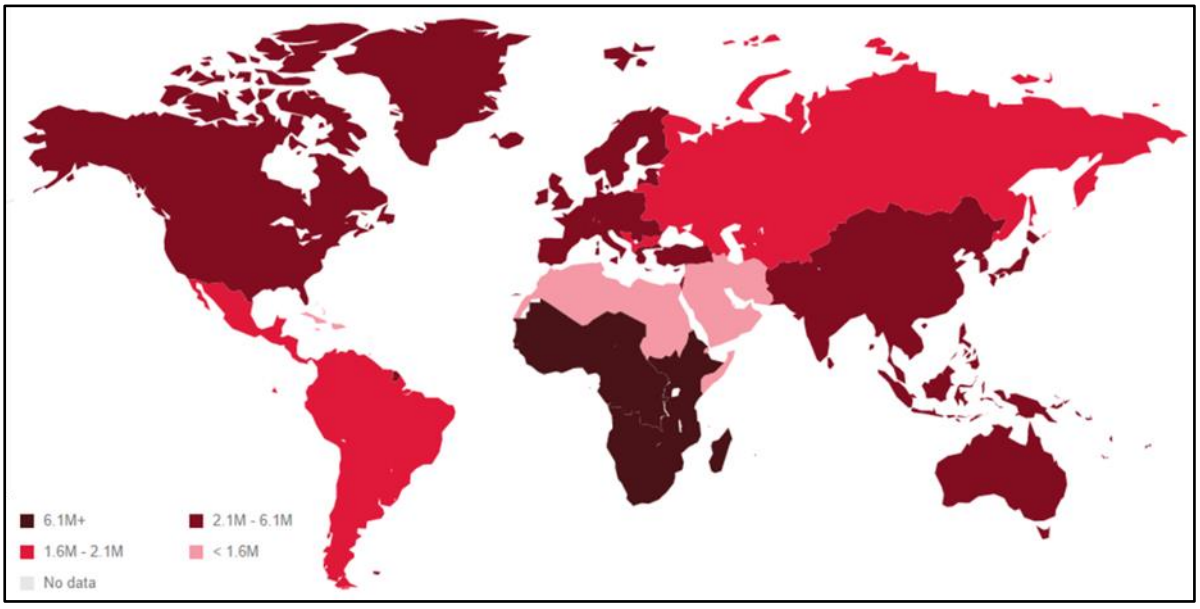


Figure 1.1 Estimated Number of People Living with HIV-1 in 2017 (UNAIDS, 2017a).

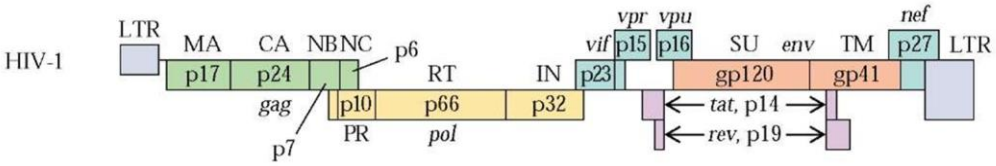
1.1.2 HIV-1 Genome Organisation and Structure

HIV-1 is a single-stranded, positive-sense, enveloped retrovirus that belongs to the *Lentivirus* genus of the *Retroviridae* family (Fauci and Lane, 2005). The HIV-1 retroviral genome, coded by an approximately 9kb Ribo Nucleic Acid (RNA), comprises nine overlapping genes that are flanked by identical long terminal repeat (LTR) regions at the 5' and 3' ends of the genome. These nine genes, namely group specific antigen (*gag*), polymerase (*pol*), envelope glycoprotein (*env*), viral infectivity factor (*vif*), viral protein R (*vpr*), viral protein U (*vpu*), negative regulation factor (*nef*), transactivator of transcription factor (*tat*) and regulator of virion protein (*rev*) encode for structural, accessory and gene regulatory proteins as depicted in Figure 1.2a (Freed, 2001, Fanales-Belasio et al., 2010).

Mature HIV-1 virions are encapsulated by a lipoprotein membrane derived from host plasma membrane. The viral Env components glycoprotein (gp) 41 and gp120 are inserted into this lipid membrane. Bound to the lipid layer are matrix (MA) molecules composed of viral protein p17 which surrounds the conical capsid (CA) and protects the inner molecules of the virus particle. Enclosed within the CA are nucleocapsid (NC) molecules that are in tight conjunction with the single-stranded viral RNA, reverse transcriptase (RT), protease (PR) and integrase (IN) molecules (Fanales-Belasio et al., 2010). The structure of the HIV-1 virion is illustrated in Figure 1.2.b.

The three structural proteins Gag, Env and Pol, which are common to all retroviruses, are initially synthesised as polyproteins and later cleaved by either viral PR or host cellular enzymes during or after the budding phase of the replicative cycle (described in section 1.1.3) into mature structural and enzymatic proteins. The accessory proteins Vif, Vpr, Vpu and Nef are thought to impede host defences and enhance viral pathogenicity, while the regulatory proteins Tat and Rev direct viral gene expression and nuclear export (Frankel and Young, 1998, Freed, 2001).

A



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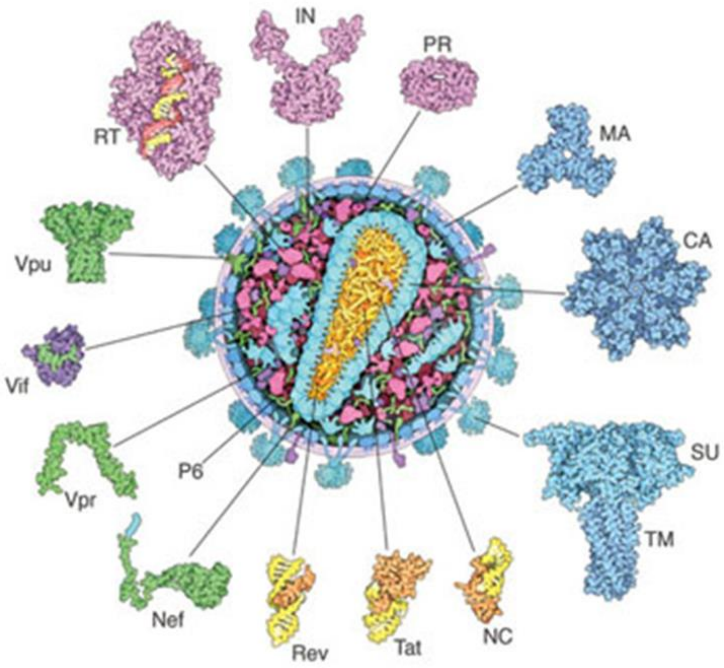


Figure 1.2 HIV-1 Genome Organisation and Virion Structure

(A) The HIV-1 genome is flanked by two long terminal repeat (LTR) regions. The structural genes *gag*, *pol*, and *env* encode for the proteins: matrix (MA), capsid (CA), nucleic-acid binding (NB), nucleocapsid core proteins (NC), protease (PR), reverse transcriptase (RT), integrase (IN) surface subunit glycoprotein (SU, gp120), and a smaller transmembrane protein (TM, gp41). *Tat*, *rev*, *vif*, *nef*, *vpr* and *vpu* encode for regulatory and accessory proteins. **(B)** Structure of the HIV-1 virion with identified proteins (Goodsell, 2011).

1.1.3 HIV-1 Replication Cycle

The replicative cycle of HIV-1 bears considerable similarity to that of other retroviruses, although there are select aspects that are exclusive to HIV-1 replication (Freed, 2001). The cycle can be described in several sequential steps and are referred to as 1) binding and entry; 2) uncoating; 3) reverse transcription of RNA to Deoxyribo Nucleic Acid (DNA); 4) nuclear import; 5) provirus integration; 6) viral protein synthesis and assembly; 7) budding and cell exit and 8) maturation (Freed, 2001, Fanales-Belasio et al., 2010) as depicted in Figure 1.3.

Virus particle entry into the target cell is facilitated by interaction of the viral Env with various host cellular membrane factors. Attachment to any of these factors brings Env into close proximity and enables adhesion with the primary HIV host receptor CD4 (Ugolini et al., 1999, Wilen et al., 2012). This high affinity interaction promotes the rearrangement of the variable loops V1/V2 and subsequently the V3 variable loop of gp120. In addition, a "bridging sheet" made up of four-stranded β sheets is formed (Kwong et al., 1998, Chen et al., 2005). Both these events are thought to be critical for co-receptor engagement with either the C-C chemokine receptor 5 (CCR5) or the CXC chemokine receptor 4 (CXCR4) proteins (Wilen et al., 2012).

Co-receptor binding results in the formation of a ternary complex composed of gp120, CD4 and the co-receptor, which in turn prompts the exposure of the highly hydrophobic N-terminus of gp41, known as the "fusion peptide" that inserts directly into the target membrane. Tethering of the viral and target membranes permits folding of the fusion peptide of each gp41 creating a six-helix bundle structure which aligns viral and host membranes and instigates the formation of the fusion pore and delivery of the virus into the host cell cytoplasm (Freed, 2001, Melikyan, 2008, Wilen et al., 2012).

Once the viral particle gains access to the cytoplasm, several events, collectively described as "uncoating" occur and seem essential to the success of the proceeding step of reverse transcription. Here, the CA destabilises and is lost, while some of the structural and enzymatic components, including MA, NC, RT, IN and Vpr remain intact as part of a high molecular weight reverse transcription complex (Freed, 1998, Freed, 2001).

Next the viral genome undergoes reverse transcription where the single-stranded viral RNA is converted to a linear double stranded DNA (Hu and Hughes, 2012). Although the mechanism by which the viral complex is transported to the nucleus is debatable, it is widely believed that

Vpr, and the DNA "flap" synthesised during reverse transcription, are required for nuclear import (Zennou et al., 2000, Freed, 2001).

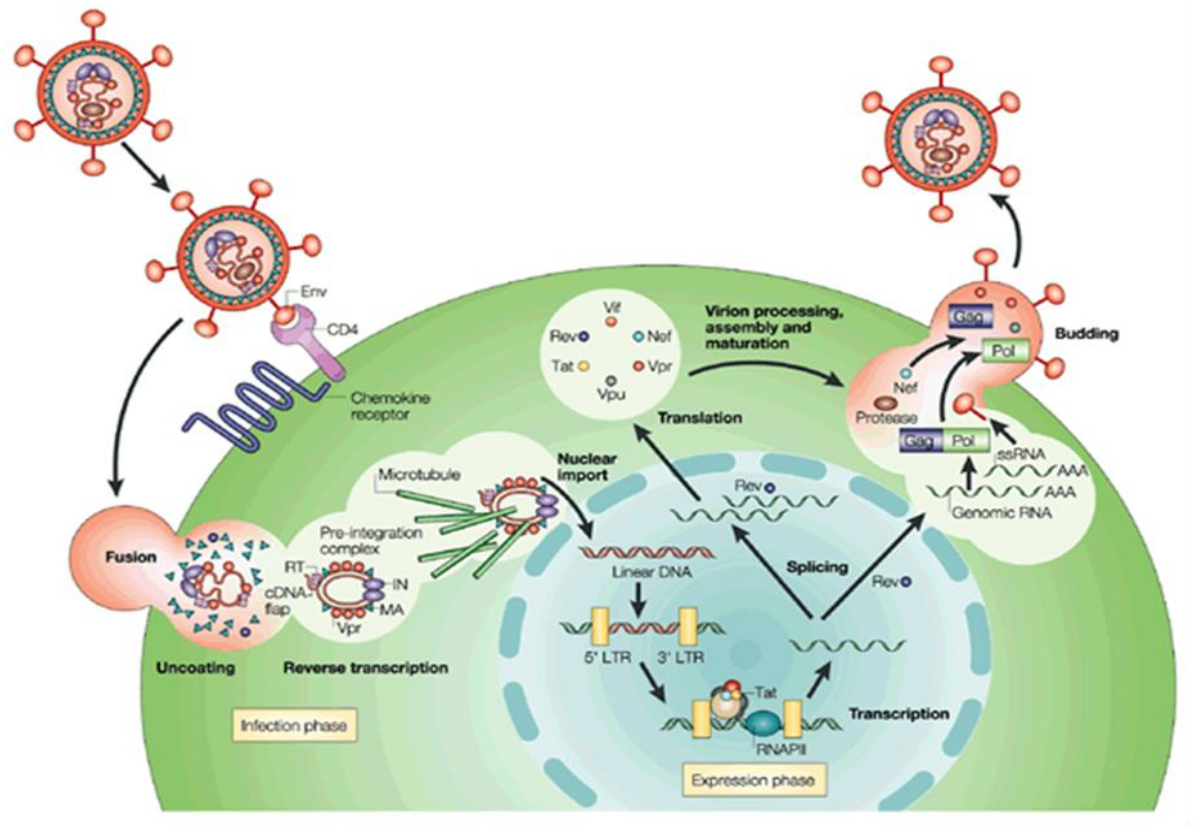


Figure 1.3 HIV-1 Replication Cycle

The diagram highlights the major sequential steps of the HIV-1 replication cycle, namely: binding and entry; uncoating; reverse transcription; nuclear import; provirus integration; viral protein synthesis and assembly; budding and maturation (Peterlin and Trono, 2003).

Insertion of the linear, double-stranded viral DNA into the host cell chromosome is catalysed by the virus-encoded IN protein. The integrated viral DNA termed the "provirus" serves as the transcriptional template for viral RNA synthesis (Freed, 2001, Craigie and Bushman, 2012). The viral LTR region comprises various DNA regulatory elements and is the site of transcriptional initiation (Lee et al., 2009). Soon after infection only short, fully spliced

messenger (m) RNAs which encode for Tat and Rev are produced. Tat triggers the transcription and synthesis of lengthened transcripts, so that as infection progresses, there is accelerated transcription of partially sliced mRNAs that encode Env, Vif, Vpr and Vpu; and full-length transcripts which serve as the mRNA for the Gag-Pol polyproteins (Fanales-Belasio et al., 2010, Karn and Stoltzfus, 2012).

Most viral mRNAs are fully sliced before export to the cytoplasm. However transport of certain longer transcripts, such as those that encode Gag, Pol and Env, are facilitated by Rev (Fanales-Belasio et al., 2010). Following export from the nucleus to the cytoplasm, RNA translation occurs on either free ribosomes or ribosomes attached to the endoplasmic reticulum to produce Gag and GagPol polyprotein precursors and Env glycoproteins (Freed, 2015).

Virion assembly occurs in dedicated micro-domains, the formation of which is induced by Gag at the cell plasma membrane. Single-stranded genomic viral RNA directs RNA "packaging signal" which interacts with the NC domain of Gag and "captures" the viral RNA into virus particles (Sundquist and Krausslich, 2012, Freed, 2015). The central Gag domain, CA, facilitates protein-protein interactions that are required for virion assembly. Gag molecules are aligned and radially packed, MA domain binds to the inner surface of the viral membrane and the C terminus forms a conical shell layer that is positioned towards the particle core. MA trimers and the gp41 cytoplasmic tail of Env are central to the recruitment and incorporation of the Env glycoproteins into the forming virions, although the mechanism is not clearly understood (Freed, 2015).

In events that remain unclear, the virus interacts with cellular machinery, thus catalysing membrane scission and virion particle release or budding (Freed, 2015). During or soon after budding, viral PR cleaves Gag and GagPol polyprotein precursors, generating mature Gag and Pol proteins and sets into motion a series of morphological rearrangements that lead to virus maturation. The maturation period is viewed as a phase when the immature virion particle transforms into virus particle that can enter and replicate in a new target cell (Sundquist and Krausslich, 2012, Freed, 2015).

1.2 HIV-1 PATHOGENESIS

The main routes of HIV-1 transmission occurs through exposure of a mucosal surface to the virus, although other modes include intravenous drug use, by blood transfusion and mother-to-child transmission. The initial phase before the detection of viral RNA is termed the eclipse phase (McMichael et al., 2010, Mogensen et al., 2010). Data collected from Simian Immunodeficiency Virus (SIV) infected rhesus macaques and acutely infected subjects alike suggest that resident memory CD4+ CCR5+ T cells are the first mucosal cell type to be infected (Li et al., 2005, Salazar-Gonzalez et al., 2009), and that productive infection emerges from a specific infectious founder virus. Local replication and expansion of the genetically restricted viral population is supported by signalling mucosal epithelial cells that recruit cytokine secreting dendritic cells, which subsequently attract additional activated CD4+ T cells that are susceptible to infection (Haase, 2010, Sagar, 2010), Figure 1.4.

Primary or acute HIV-1 infection is distinguished as the period of infection when HIV-1 RNA first becomes detectable in the blood plasma until the formation of HIV-1-specific antibodies that are produced 3-4 weeks following infection (McMichael et al., 2010, Mogensen et al., 2010). Within a week of infection, cell-free virus and infected cells disseminate and are able to infect HIV-specific CD4+ T cells in the draining lymph node. It is during this time that HIV-1 RNA first becomes detectable in the blood plasma (10-17 days post-infection), followed by detection of viral p24 antigen (17-22 days post-infection) (Fiebig et al., 2003, Lee et al., 2009).

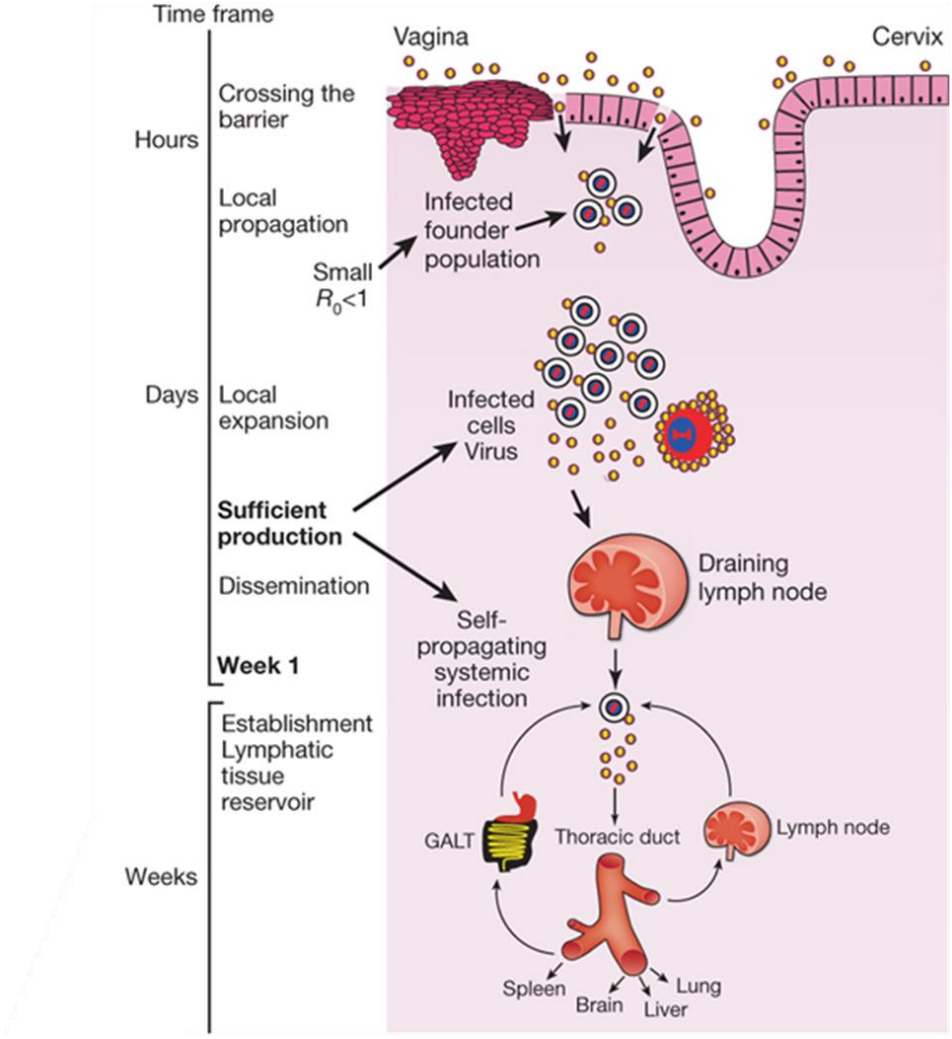


Figure 1.4 HIV-1 Transmission and the Early Events of HIV-1 Infection

Key interactions between virus and host in the early stages of HIV-1 infection, from crossing the cervicovaginal mucosal barrier and infection of 'resting' CD4+ T cells in the lamina propria, through to expansion in the draining lymph nodes and subsequently the lymphoid tissues within weeks of infection (Haase, 2010).

Consequently, entry by the virus into the general circulation and secondary lymph nodes allows efficient infection of the main target cell of choice i.e. CD4+ T cells, which are found in abundance at these sites (Haase, 2010, McMichael et al., 2010). Documented studies in SIV models and HIV-infected individuals provide evidence that acute infection is characterised by substantial memory CD4+ T cell depletion predominantly in mucosal tissues, particularly the gut associated lymphoid tissue (GALT) (Veazey et al., 1998, Brenchley et al., 2004). Interestingly, it has been documented that SIV infects up to 60% of the infected organism's memory CD4+ T cells and that the majority of these cells undergo apoptosis within days (Mattapallil et al., 2005). Importantly, acute infection does not efficiently target CCR5 devoid

T cell subsets, including naïve and resting central memory T cells, thus allowing the regenerative potential of these subsets to remain intact (Grossman et al., 2006).

Peak plasma viremia (>1 million HIV-1 RNA copies/ml) is reached 21-28 days post-infection and is accompanied by diminished peripheral CD4+ T cell counts. While circulating T cell numbers subsequently rebound close to pre-infection levels, CD4+ T cell levels in the GALT remain severely suppressed. It is at this stage that host HIV-specific, albeit non-neutralising, antibodies first become measureable (Fiebig et al., 2003, Lee et al., 2009, Mogensen et al., 2010), Figure 1.5. Establishment of viral reservoirs of primarily latently infected resting memory CD4+ T cells occurs during this acute stage of infection and cannot be eliminated by highly active antiretroviral therapy (HAART) (Dinoso et al., 2009). At this stage of infection, individuals may present with acute retroviral syndrome symptoms that include influenza-like signs with fever, sore throat and lymphadenopathy (Kahn and Walker, 1998, McMichael et al., 2010). Eventually, at approximately 25-31 days post-infection, plasma viremia starts to decrease over 12-20 weeks to reach a stable plateau termed viral set point which is viewed as a predictor of rate of disease progression (Fiebig et al., 2003, Lee et al., 2009). Adaptive immune responses in the form of cytotoxic CD8+ T cells emerge as viral loads peak and it is widely recognised that these cells are predominantly responsible for the consequent decline in viremia (Borrow et al., 1994).

Chronic infection ensues and is characterised by a gradual decline in circulating CD4+ T cells, rapid cell turnover and immune dysfunction. Central to HIV-1 pathogenesis is the heightened immune activation which is evidenced by the increased expression of immune activation markers including CD38 which is considered a reliable marker for immune activation, progression to AIDS and death (Liu et al., 1997). Prolonged activation is disadvantageous to the host and leads to immune exhaustion. Several factors have been implicated in chronic immune activation; these include increased activation of T cells, translocation of bacterial products across the damaged mucosal barrier and subsequent heightened pro-inflammatory cytokine production leading to the activation of latently infected cells (Ko et al., 2010, Mogensen et al., 2010).

Progressive activation results in the loss of cytotoxic and cytokine-secreting abilities of immune cells, leading to their dysfunction. Thus, the capacity to control HIV-1 replication is further compromised and CD4+ T cell decline continues. At this time the host becomes highly susceptible to opportunistic infections and certain cancers, and an infected individual with

presentation of these clinical features is considered to have AIDS. The period between HIV-1 acquisition and progression to AIDS is 8-10 years without antiretroviral treatment, although this can vary substantially amongst individuals where several factors such as immunological factors, genetic factors, infecting strain virulence and co-infections may influence disease progression (Fauci and Lane, 2005).

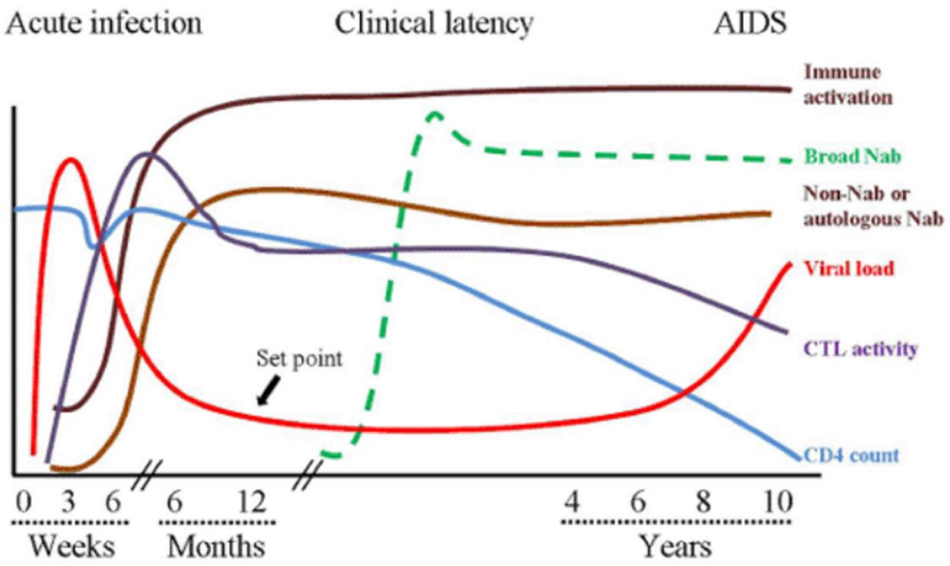


Figure 1.5 Representation of the Clinical Course of HIV-1 Infection

Schematic showing the markers of disease progression (CD4 count and viral load) and well as immune responses in the acute, chronic and AIDS phases of HIV-1 infection (The dashed line indicates that broad Nab have been found in only a portion of HIV-1 infected persons.) Abbreviations: Nab, neutralising antibody (Chen, 2015).

1.3 THE HOST IMMUNE RESPONSE TO HIV-1

The human immune system is multifaceted; consisting of innate and adaptive cell-mediated responses, humoral components and host restriction factors that provide protection against foreign, and often harmful microorganisms. Even from the earliest stages of HIV-1 infection, the host elicits a strong, although only partially successful, response to eradicate the virus.

Innate immune cells, including dendritic cells (DCs), macrophages and natural killer (NK) cells, are the first line of defence which the HIV-1 virus encounters upon crossing the mucosal barrier. Epidermal DCs are probably the first immune cells to encounter the virus, and are activated via cytokines secreted by mucosal epithelial cells or through direct Toll-like receptor-mediated recognition of viral RNA (Gonzalez et al., 2010). Activated DCs produce type 1 interferons which trigger various antiviral pathways (Chang and Altfeld, 2010) and upregulate host restriction factors including APOBEC3G, TRIM5α and tetherin (Chakrabarti and Simon, 2010). Follicular DCs are principally involved in the presentation of antigen to T cell lymphocytes and also provide signals for the activation of B cell lymphocytes. Macrophages also participate in cytokine secretion and antigen presentation which result in the activation of innate and adaptive immune responses (Collman et al., 2003).

Natural killer (NK) cells proliferate in response to type 1 interferon production by DCs. Stimulated NK cells produce cytokines and chemokines that activate T cell lymphocyte proliferation; NK cell IFN-γ secretion also inhibits viral replication. Further to this, NK cells directly target HIV-1 infected cells that exhibit diminished expression of major histocompatibility complex (MHC) class I through cytolytic activity. Since MHC class I expression is crucial for peptide presentation to T cells, NK cells are essential when HIV escapes T cell responses (Fauci et al., 2005). The relationship between NK cells and HIV-1 is further explored in section 1.3.2.3.

The adaptive immune system consists of T and B cell lymphocytes and humoral factors. MHC class I on infected CD4+ T cells display intracellularly degraded HIV peptide fragments for recognition by CD8+ T lymphocytes (Paranjape, 2005). CD8+ T cells are able to lyse these infected cells and secrete cytokines, i.e. IFN-γ, TNF-α, and chemokines, i.e. MIP-1 α, MIP β and RANTES, that inhibit virus replication and block viral entry into CD4+ T cells (Benito et al., 2004). The humoral immune response occurs later in infection. Non-neutralising antibodies against HIV-1 structural proteins appear approximately 20 days post infection and generally do not persist. Several months post infection, neutralising antibodies that are specific to viral

proteins involved in target cell entry are generated. Potent neutralizing antibodies have been shown to play a major role in HIV-1 controllers (Alter and Moody, 2010).

There is mounting evidence that suggests a pivotal role for the earlier and quicker responses of innate immunity in HIV-1 infection, long before the induction of adaptive immune responses. The sections below describe two innate immune cell types that are of interest in this study, namely neutrophils and NK cells.

1.3.1 Neutrophils

Polymorphonuclear neutrophils (PMNs) are innate immune cells that are typically recognised as part of the first line of defence against invading pathogens through direct antimicrobial activity. They are formed in the bone marrow and continuously differentiate from myeloid precursor cells through regulation by hematopoietic cytokines, mainly granulocyte colony stimulating factor (G-CSF) and enter the circulation as terminally mature cells (Richards et al., 2003, Summers et al., 2010). Daily neutrophil turnover is exceedingly rapid with approximately 10^9 cells per kilogram of body weight exiting the bone marrow in healthy individuals, making them the most abundant white blood cells in the periphery, constituting 50-70% of circulating leukocytes (von Vietinghoff and Ley, 2008, Summers et al., 2010).

Neutrophils are considered short-lived cells with a half-life of approximately 6-8 hours in circulation under basal conditions, although recent findings challenge this long perceived notion of a shortened lifespan. Experimental data from an in vivo study using 2H2O labelling now estimates an extended lifespan of 5.4 days under homeostatic conditions in humans (Pillay et al., 2010). During inflammation, neutrophil lifespan is increased several fold through activation by microbial products, endogenous cytokines and other pro-inflammatory mediators which results in the inhibition of cellular apoptosis. Prolonged lifespan during chronic inflammation often contributes to deteriorating disease prognosis. (Summers et al., 2010, Kolaczkowska and Kubes, 2013, Silvestre-Roig et al., 2016). Aged circulatory neutrophils exhibit increased expression of CXCR4 and CCR5; and reduced expression of CXCR2 and CD62L (Rankin, 2010). The altered surface marker profile ensures homing of senescent neutrophils to the bone marrow where they either die spontaneously by necrosis or apoptosis or are destroyed through phagocytosis by bone marrow macrophages (Kolaczkowska and Kubes, 2013, Hong, 2017).

Mature neutrophils are characterised by a lobulated or segmented nucleus and are enriched by cytoplasmic granules and secretory vesicles. The proposition of heterogeneous neutrophil phenotypes is a fairly recent concept. Although there is compelling evidence that suggests the presence of distinct neutrophil subsets, whether these subsets develop from a single precursor cell and represent different stages of maturity or reflect distinct neutrophil lineages remains unclear (Borregaard, 2010, Kolaczkowska and Kubes, 2013). Classical neutrophils or normal density neutrophils (NDNs) are primarily terminally differentiated and are thought to be in a resting state phase. Throughout systemic inflammation, 'emergency granulopoiesis' leads to enhanced production and mobilisation of neutrophils into the circulation. A portion these of mobilised neutrophils are immature; without the development of granules which are typically acquired during differentiation. These neutrophils are less granular and as such termed low density neutrophils (LDNs) (Hong, 2017).

Different LDN subsets have been suggested, namely immunosuppressive and pro-inflammatory LDNs. The pro-inflammatory LDN subset is highly exhibited in various autoimmune diseases and displays intensified type I interferon production and diminished phagocytic activity. However, since they share similar phenotypic and functional characteristics to that of activated NDNs, it is probable that these cells represent pre-exhausted activated NDNs rather than a separate LDN subset. Immunosuppressive LDNs have been found in individuals with haematological malignancies, sepsis and HIV-1 infection. Contrary to NDNs, these cells are linked to suppression of T cell responses including proliferation and IFN-ɣ production. Phenotypic characterisation of these cells, however, remains to be determined (Silvestre-Roig et al., 2016, Hong, 2017).

During steady state conditions, neutrophils transverse the circulation as quiescent cells, continuously and randomly probing the endothelium. Recruitment of neutrophils into inflamed tissues is initiated by surface changes of local endothelial cells. Through several sequential steps, neutrophils are recruited from the vasculature to the tissue. Once in the interstitial space, neutrophil behaviour is dictated by chemotactic gradients toward invading pathogens. Chemo-attractants also bind membrane receptors, initiating signalling pathways (Amulic et al., 2012, Kolaczkowska and Kubes, 2013).

1.3.1.1 Neutrophil Effector Mechanisms

The most well-known neutrophil effector processes include phagocytosis, degranulation, and production of reactive oxygen species (ROS). In subsequent years, an active form of cell death resulting in the release of neutrophil extracellular traps (NETs) has been described (Brinkmann et al., 2004), Figure 1.6.

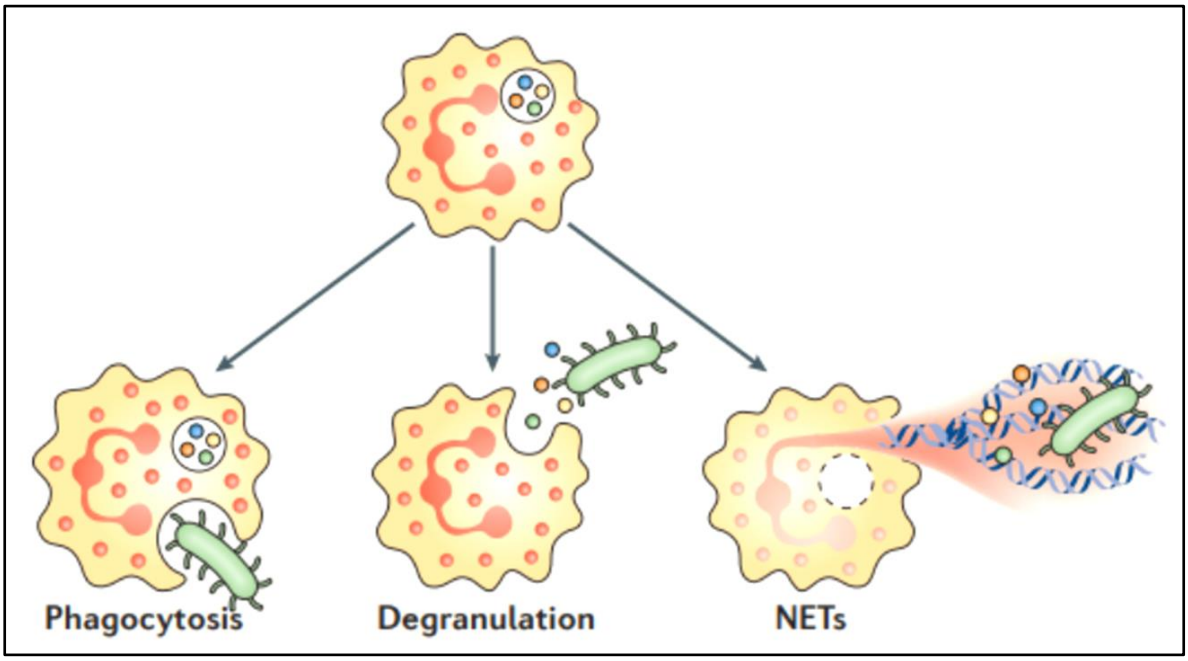


Figure 1.6 Neutrophil Killing Mechanisms

Neutrophils eliminate pathogens by multiple means, both intra- and extracellular. The most well-known mechanisms, phagocytosis, degranulation and production of neutrophil extracellular traps (NETs) are represented in the diagram (Kolaczkowska and Kubes, 2013).

Phagocytosis is the principal process to clear pathogens and cell debris. It is an active, receptor-mediated mechanism where foreign particles or microbes are internalised by the neutrophil cellular membrane into a vacuole known as a phagosome (Amulic et al., 2012). Internalisation is dependent on the interaction between the neutrophil and the pathogen and can be directly through recognition of pathogen-associated molecular patterns (PAMPs) or may be opsonin mediated. The well-characterised opsonin mediated phagocytosis can occur via two mechanisms, either fragment crystallisable gamma receptor (FcγR)-mediated or complement receptor-mediated phagocytosis, the former of which requires the formation of membrane

extensions for the engulfment of Immunoglobulin (Ig)G opsonised particles (Underhill and Ozinsky, 2002, Lee et al., 2003).

Engulfment alone is insufficient for pathogen elimination. Neutrophil cytoplasmic granules are unique organelles that store a plethora of antimicrobial proteins, metalloproteases and plasma-derived proteins. Upon complete cell activation, granules fuse with the phagosomal membrane and release their contents into the vacuole lumen, thus creating an antimicrobial environment that is lethal to invading pathogens (Amulic et al., 2012). However, certain pathogens have adapted strategies to evade engulfment, modify phagosome maturation and create hospitable phagosomal conditions (Staali et al., 2006, McCaffrey et al., 2010).

In parallel to fusion of granules with the phagosome, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase assembles on the phagosome membrane and the reactive oxygen cascade commences by reducing oxygen to superoxide, which readily forms several reactive oxygen intermediates, and this collectively creates an uninhabitable environment to most pathogens. Despite the continuing fusion of acidic granules to the phagosome, NADPH oxidase activity sustains the maintenance of an alkaline pH within the phagosomal lumen, which is essential to the activation of serine proteases (Amulic et al., 2012).

First described by Brinkmann and colleagues (2004), NETosis is an active form of cell death which results in the release of fibrous structures referred to as NETs into the extracellular space in response to surplus chemical or biological stimulus. The released NETs comprise decondensed chromatin, to which histones and antimicrobial proteins are attached and are thought to directly eliminate microbes through exposure to high concentrations of antimicrobials (Brinkmann et al., 2004, Papayannopoulos and Zychlinsky, 2009). The mechanisms involved in NET formation are not completely known, although formation is dependent on NADPH, myeloperoxidase and neutrophil elastase (Fuchs et al., 2007, Patel et al., 2010, Metzler et al., 2011).

1.3.1.2 Neutrophil Regulatory Mechanisms

Historically, neutrophils were viewed as no more than “professional killers”. However, recent research has highlighted their role in forming the foundation of the inflammatory response. As one of the earliest immune cellular components to arrive at the site of inflammation, neutrophils not only secrete cytokines and chemokines for the recruitment of other cell types, but they are

also responsible for creating adequate micro-milieu conditions to promote adaptive immune responses.

In comparison to other cells types, neutrophils characteristically produce reduced volumes of cytokines per cell, but since they are abundant in frequency, their contribution to overall cytokine levels at the site of inflammation is quite substantial (Nathan, 2006). Initially cytokine release is principally Interleukin (IL)-8 and is directed toward the recruitment of cellular support, in particular other neutrophils (Scapini et al., 2000). Additionally, neutrophil-derived IL-1β and Tumour Necrosis Factor (TNF)-α promote the secretion of neutrophil chemo-attractants by other cell types (Sica et al., 1990, Kasama et al., 2005).

Neutrophils also shape the immune response by communicating with other cell types including monocytes/macrophages, dendritic cells (DCs), NK cells and T and B cell lymphocytes through cell-cell contact (Figure 1.7) (Mantovani et al., 2011). Neutrophils can induce monocyte recruitment either directly through production of conventional monocyte chemo-attractants or indirectly through the upregulation of adhesion molecules on the endothelium and encourage monocyte extravasation through secretion of granule proteins. Moreover, neutrophils can directly moderate monocyte/macrophage cytokine production, strengthening their effector activities (Soehnlein et al., 2008a, Soehnlein et al., 2008b, Amulic et al., 2012). Neutrophils also contribute to the recruitment, activation and programming of DCs. Neutrophils have been found to modulate maturation of DCs via contact-dependent communications. In contrast, neutrophil-derived elastase can diminish the allo-stimulatory capacity of monocyte-derived DCs and neutrophil secreted ectosomes have been shown to inhibit monocyte-derived DC maturation (Mantovani et al., 2011).

Neutrophils also crosstalk with T and B cell lymphocytes. The production of various chemokines by neutrophils can attract two helper (Th)1 and Th17 cells to the site of inflammation through chemotaxis (Scapini et al., 2000, Pelletier et al., 2010). Neutrophils can also directly modulate T cell function by secreting IL-12 which is essential for Th1 cell differentiation, and are also known to express B cell development and maturation factors (Huard et al., 2008, Scapini et al., 2008). Recently it has been demonstrated that antigen-pulsed neutrophils can cross-present exogenous antigens and promote the differentiation of cytotoxic CD8+ T cells (Beauvillain et al., 2007). The regulatory interactions between neutrophils and NK cells are of particular interest in this study and are described in detail in section 1.3.3.

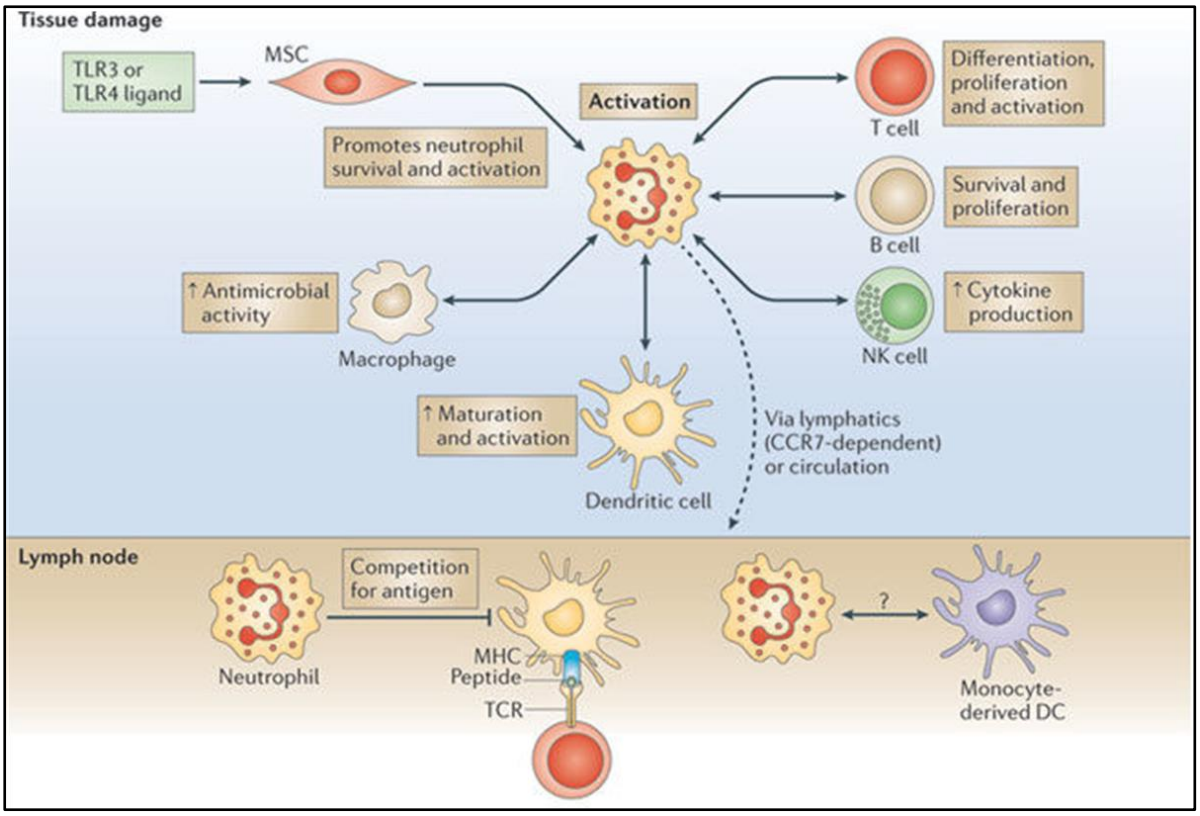


Figure 1.7 Neutrophils interact with immune and non-immune cells in tissues and lymph nodes

Circulating neutrophils are activated by systemic pathogens and crosstalk with platelets and endothelial cells. Activated neutrophils leave the circulation and crosstalk with resident and recruited immune cells, including macrophages, dendritic cells (DCs), natural killer (NK) cells and B and T cells. Neutrophils can also migrate to the lymph nodes and interact with DCs (Mantovani et al., 2011).

1.3.1.3 Neutrophils and HIV-1 Infection

Various studies have demonstrated that neutrophils and their associated factors are linked to increased HIV acquisition. Toll-like receptor and HIV-1 stimulated neutrophils from highly exposed seronegative (HESN) individuals were found to have reduced cytokine production compared to HIV-1 infected patients (Hernandez et al., 2015). Interestingly, high levels of human neutrophil peptides and cathelicidin from cervicovaginal samples in Kenyan sex workers pre-infection were associated with subsequent HIV acquisition (Levinson et al., 2009), and neutrophil pro-inflammatory cytokine IL-8 was increased in the cervicovaginal lavages (CVLs) of women that seroconverted compared to women that remained uninfected in the CAPRISA 004 microbicide trial in South Africa (Masson et al., 2015). Importantly, further analysis of the CAPRISA 004 cohort revealed increased CVL neutrophil proteases positively associated with IL-17 expression. This neutrophil/Th17 axis could represent an increased capacity of neutrophils to preferentially recruit Th17 target cells to the female genital tract

which could result in higher risk of HIV acquisition (Arnold et al., 2016, Hensley-McBain and Klatt, 2018).

Various studies have suggested that neutrophils have an important role in controlling viral infections (Bastian and Schafer, 2001, Fujisawa, 2001, Yasin et al., 2004). The majority of studies investigating the role of neutrophils in HIV-1 infection have focused on the susceptibility of infected individuals to opportunistic bacterial and fungal co-infections (Casulli and Elbim, 2014). It has now become clear that the association between neutrophils and HIV-1 is far more complex.

Despite neutrophil-derived mediators such as ROS, TNF-α and IL-8 having been implicated in augmenting HIV-1 replication (Ho et al., 1995, Yoshida et al., 2007), recent literature suggests an active role of granule-derived proteins in controlling replication. Numerous studies have reported the inhibitory effect of α-defensins on several steps of the HIV-1 replicative cycle. It has been demonstrated that α-defensins can broadly block viral infectious particles at an early stage by specifically binding both CD4 on target cells and viral gp120 envelope glycoprotein, thereby inhibiting fusion of gp120 to CD4 and preventing viral entry regardless of co-receptor specificity or genetic subtype (Chang et al., 2005, Furci et al., 2007, Demirkhanyan et al., 2012).

Defensins have also been shown to downregulate both CD4 and CXCR4 expression and inhibit replication at the nuclear import, reverse transcription and integration phase (Furci et al., 2007, Seidel et al., 2010, Demirkhanyan et al., 2012). Defensins may also act indirectly by upregulating CC-chemokine expression and secretion (Macrophage Inflammatory Protein (MIP)-1α and MIP-1β) which in turn inhibits HIV-1 infection of macrophages (Guo et al., 2004) or encourage HIV-specific lymphocyte proliferation responses and amplify interferon (IFN)-ɣ and perforin production by CD4+ and CD8+ T cells (Mohan et al., 2013).

In comparison to negative controls, HIV-infected individuals were shown to have increased α-defensin expression and production which was unaffected by antiretroviral therapy and may be associated with chronic immune activation (D'Agostino et al., 2009). Interestingly, in breastfed infants it was found that milk concentration of α-defensins significantly associated with decreased intrapartum and postnatal HIV transmission risk (Kuhn et al., 2005). Other granule stored antimicrobial peptides, specifically indolicidin (Robinson et al., 1998) and lactoferrin (Carthagena et al., 2011) also exhibit effective inhibitory actions against HIV-1.

Neutrophils are able to detect HIV-1 through surface expressed Toll-like receptor (TLR)-7 and TLR-8 (Kawai and Akira, 2008). Engagement of viral nuclei acids with TLRs triggers ROS production and promotes α-defensin and myeloperoxidase action (Klebanoff and Coombs, 1992, Moguilevsky et al., 1992). In a study conducted by Saitoh et al. (2012) it was shown that via NET formation, neutrophils can capture HIV-1 and through α-defensin and myeloperoxidase action were able to promote HIV-1 elimination. However, in response, HIV-1 was able to induce DC-derived IL-10 production which consequently suppresses TLR-mediated NET release (Saitoh et al., 2012).

HIV-1 does not actively establish infection in neutrophils but rather leads to weakened effector responses and an escalated rate of apoptosis. Despite a handful of contradicting reports (Bandres et al., 1993, Schwartz et al., 2010), the general consensus is that neutrophils from HIV-1 individuals, particularly those in the advanced stage of disease, display variable functional irregularities resulting in defective bacterial and fungal killing. HIV-1 alters phagocytosis of extracellular pathogens (Michailidis et al., 2012) presumably by downregulation of FcγR (Kedzierska et al., 2002) and also modifies ROS production by disturbing the distribution of the NADPH oxidase complex within the neutrophil (Salmen et al., 2012), although the mechanism remains undefined. Continued exposure of neutrophils to HIV-1 or its products may reduce chemotactic receptor expression and thereby negatively impacting chemotaxis and the recruitment of the neutrophils (Casulli and Elbim, 2014). Additionally, a changed plasma cytokine environment, as seen in HIV-infected persons, may play a part in neutrophil dysfunction (Casulli and Elbim, 2014). Administration of IL-15 has been shown to augment neutrophil function and induce production of monocytic IL-8, which is the main neutrophil chemo-attractant (Mastroianni et al., 2000, d'Ettorre et al., 2002). It has also been established that resting neutrophils from treatment naïve HIV-infected individuals displayed elevated basal activation as measured by CD11b and L-selectin expression (Elbim et al., 1994).

Neutrophil apoptosis accelerates considerably from the very early stages of HIV-1 infection and this shortened survival contributes to the observed neutropenia observed in advanced stages of infection (Casulli and Elbim, 2014). Several factors may be responsible for the increased rate of cell death including heightened basal ROS production (Salmen et al., 2007), increased activity of calpains, which are non-caspase cysteine proteases involved in apoptosis (Lichtner et al., 2006) or a decline of pro-inflammatory cytokines such as G-CSF and

granulocyte-macrophage-colony stimulating factor (GM-CSF) that are critical to neutrophil survival (Casulli and Elbim, 2014).

1.3.2 Natural Killer (NK) Cells

NK cells are an integral component of the innate immune system. They are a unique class of large, granular lymphocytes with the capacity to directly recognise and rapidly respond to non-self, damaged, tumour and virally-infected cells without prior sensitisation (Lodoen and Lanier, 2006, Ward and Barker, 2008). Derived primarily from the bone marrow micro-environment, they differentiate and mature from common lymphoid progenitors that are also known to generate T and B lymphocytes. In the periphery, NK cells range from 2 to 18% of total circulating lymphocytes with a predicted turnover of approximately 2 weeks (Lanier, 2005, Vivier et al., 2008).

NK cells are distinguished from their lymphoid counterparts by the absence of T and B cell receptors such as CD3, T-cell receptor (TCR) or surface Ig B cell receptors and were thus initially termed "null" cells (Strauss-Albee and Blish, 2016). Presently they are defined as cells that lack CD3 and express neural cell adhesion molecule (NCAM, CD56), with the majority also expressing CD16. NK cells can be phenotypically classified according to the cell density of surface CD56 into two subsets specifically CD56 bright (expressing high levels of CD56) and CD56 dim (expressing low levels of CD56) cells (Cooper et al., 2001). There is sufficient evidence that these subsets have unique functional characteristics implying their distinct assigned roles in immunity. Constituting about 90% of NK cells in the periphery, CD56 dim cells are fully mature NK cells that primarily mediate cytotoxic responses. In contrast, CD56 bright cells, representing approximately 10% of NK cells in circulation, express little or no CD16 and it is highly debatable as to whether these cells symbolise an immature, precursor subset of CD56 dim NK cells. They are responsible for abundant cytokine production and are limited in their cytolytic activity (Cooper et al., 2001).

1.3.2.1 Natural Killer Cell Recognition and Activation

NK cells are dissimilar to T and B cells in that they lack the ability to undergo somatic recombination, and thus cannot generate antigen specific receptors. Rather they respond via a multitude of sophisticated germline-encoded receptors that regulate their activation,

proliferation and effector mechanisms through a contemporary modification of a process referred to as the "missing-self hypothesis" (Karre et al., 1986, Ljunggren and Karre, 1990, Karre, 2002, Lanier, 2005) as illustrated in Figure 1.8.

Proposed by Kärre and colleagues (Karre et al., 1986, Ljunggren and Karre, 1990), the missing-self hypothesis works on the concept that NK cells are repressed from responding to self-cells that actively express major histocompatibility complex (MHC) class I molecules through interaction with NK inhibitory receptors. In the absence or downregulation of MHC class I expression on hematopoietic cells, as is the circumstance with cellular transformation and certain viral infections, there is dampening of this inhibitory effect, rendering the cell susceptible to NK cytotoxicity (Karre et al., 1986, Ljunggren and Karre, 1990, Karre, 2002).

To a large extent the presumptions of this concept still holds true, although subsequent research has revealed the complexity of NK cell activation. Upon adhesion to a potential target cell, interactions between NK cell receptors and target cell ligands determines whether the NK cell detaches and moves to the next potential target cell or remains bound and responds. It is now widely recognised that the outcome of NK cell activity relies on a counterbalance of signals from both activating and inhibitory receptors, such that unengaged inhibitory receptors results in unsuppressed activating signals from interaction with stress induced ligands and subsequent response initiation.

Three major natural killer receptor (NKR) "superfamilies" have been described; the killer cell immunoglobulin-like receptors (KIRs) superfamily, the C-type lectin superfamily and the natural cytotoxicity receptors (NCRs). Receptor signalling is mediated either by conserved sequences within the cytoplasmic domains of these receptors or by their associated adaptor proteins. Cytoplasmic domains of all well-defined inhibitory receptors contain copies of the consensus sequence immunoreceptor tyrosine-based inhibitory motif (ITIM), whereas numerous activating receptors signal through adaptor proteins that possess the immunoreceptor tyrosine-based activation motif (ITAM) (Lanier, 2005). In the instance of inhibitory receptors binding to target cell ligand, the ITIM is phosphorylated and there is recruitment of phosphatases which work at the membrane to either dampen or completely arrest NK cell effector functions. Whereas in the case of activating receptors, tyrosine phosphorylation of ITAMs results in calcium influx, degranulation, and cytokine and chemokine gene transcription (Lanier, 2005).

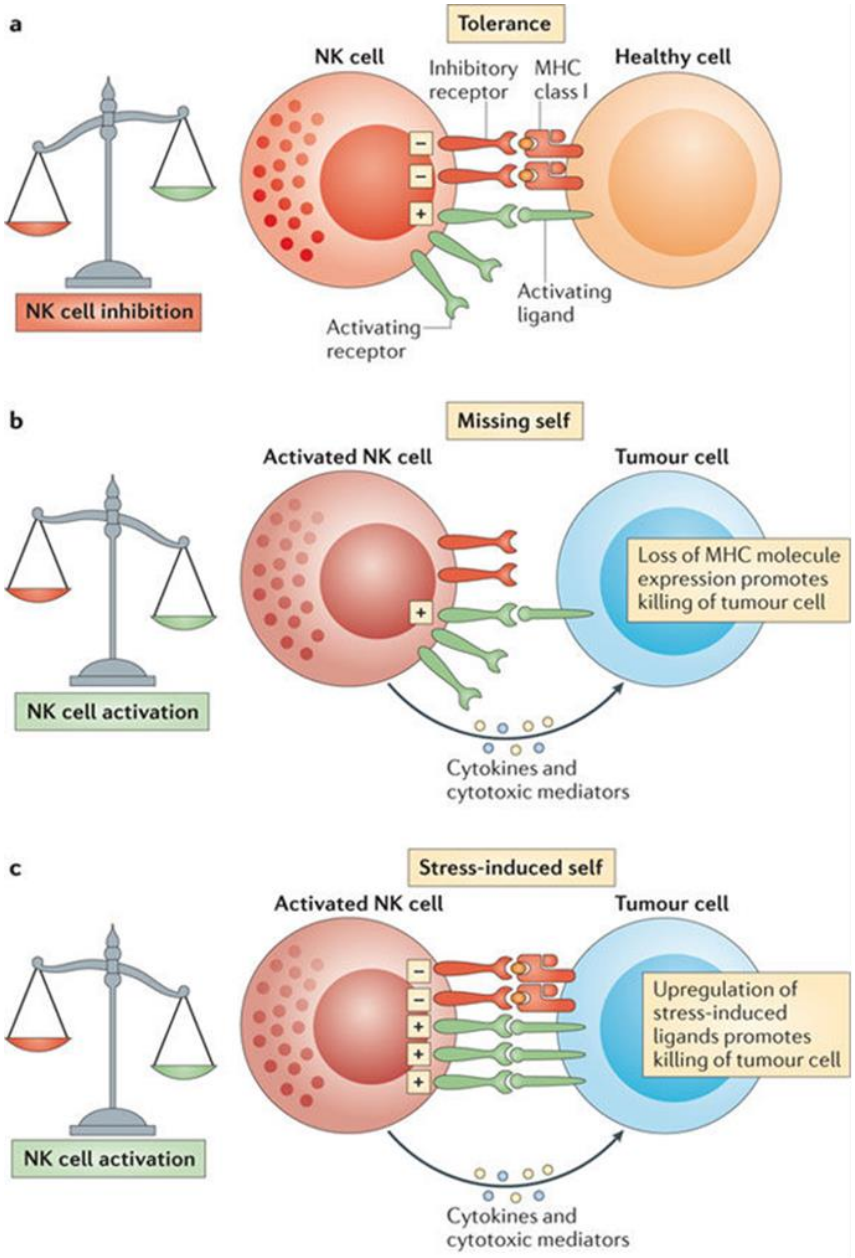


Figure 1.8 Natural killer cell Recognition and Activation

(a) In the case of healthy cells, when the strength of the activating signals are dampened by the engagement of inhibitory receptors, NK cells are tolerant to host cells. (b) Tumour/transformed cells may lose expression of MHC class I molecules, termed as 'missing-self'. NK cells become activated and are no longer held in check by the inhibitory signal delivered by MHC class I molecule engagement. (c) Additionally, NK cells are selectively activated by 'stressed' cells, which upregulate activating ligands for NK cells and thereby overcome the inhibitory signalling delivered by MHC class I molecules (Vivier et al., 2012).

1.3.2.2 Natural Killer Cell Functions

NK cells are predominantly cytolytic and there are a number of mechanisms employed to mediate this activity. These include the release of cytoplasmic granules such as perforin and granzymes (degranulation) which are released when the NK cell is in close proximity to the cell targeted for killing. Additionally, in a process termed antibody-dependent cellular cytotoxicity (ADCC), NK cells can actively lyse target cells expressing surface antigen that are bound by specific antibodies produced as part of the humoral immune response. NK cells are equipped to recognise IgG-opsonised infected cells through expression of CD16, and upon interaction commences NK cell activation and release of cytotoxic granules (Cooper et al., 2001, Caligiuri, 2008).

Apart from their ability to mediate spontaneous cytotoxicity, NK cells are also a major source of several pro-inflammatory cytokines and chemokines including IFN-ɣ, TNF-α and GM-CSF, and are thus viewed as immune-regulatory cells able to "bridge" innate and adaptive immunity. They are also capable of directly modulating immune responses through interactions with macrophages, DCs and T cell lymphocytes. NK cells, in combination with cell receptors and cytokines, are able to enhance macrophage and DC maturation and activation (Vivier et al., 2008). Furthermore, NK cells can directly influence adaptive immune responses by acting on T cells. NK cell-derived IFN-ɣ can promote CD4+ Th1 priming (Martin-Fontecha et al., 2004, Morandi et al., 2006). NK cells are also capable, in the case of insufficient expression of MHC class I molecules, of killing activated T cells by dampening CD94-NKG2A inhibitory signalling in NK cells (Lu et al., 2007).

1.3.2.3 Natural Killer Cells and HIV-1 Infection

The role of NK cells in viral infection has been repeatedly highlighted in individuals with NK cell deficiencies (Orange, 2006, Orange and Ballas, 2006). There is presently a wide consensus that in addition to adaptive immune responses, NK cells may be critical to early HIV-1 containment well before the development of T and B cell responses, and may influence disease progression as discussed below.

Several studies have suggested a significant role of NK cells in resistance to HIV-1 infection. In a group of Vietnamese intravenous drug users who were continually exposed to HIV-1 but remained uninfected, it was found that their apparent resistance to infection coincided with a repertoire of predominantly activating NKRs that endowed these individuals with higher

cytokine production and enhanced degranulation activity when compared to HIV-1 infected and healthy uninfected individuals (Scott-Algara et al., 2003, Ravet et al., 2007). This data has been repeatedly confirmed in other highly exposed, uninfected populations including Canadian adults (Boulet et al., 2008), exposed uninfected Colombian adults (Montoya et al., 2006) and African sex workers (Jennes et al., 2006).

There is mounting evidence that NK cells have a direct role in the containment of HIV-1 infection and therefore influences disease progression. This has been attributed to specific KIR genotypes in combination with their MHC class I ligand genotypes in certain individuals. Most convincing was a study conducted by Martin and colleagues (2002) which suggested a pivotal role for NK cells in controlling viral replication. The presence of KIR3DS1 in combination with the ligand human leukocyte antigen (HLA)-Bw4 was strongly associated with slower progression to advanced disease (Martin et al., 2002). Further study of this KIR3DS1/HLA-Bw4 relationship demonstrated early containment of HIV-1 viremia and protection against opportunistic infections (Qi et al., 2006). In addition, in an *in vitro* study, Alter et al. (2007) demonstrated that KIR3DS1 expressing NK cells derived from healthy donors displayed enhanced NK cell-mediated killing in the presence of HIV-1 infected target cells expressing HLA Bw4 80Ile (Alter et al., 2007).

NK cells have been implicated in directly mediating antiviral immune pressure and thereby contributing to viral evolution. In examining HIV-1 subtype B chronically infected individuals, Alter and colleagues (2011) provided evidence that KIR-associated polymorphisms of the HIV-1 sequence allowed for improved binding of NK cell inhibitory KIRs to HIV-1 infected CD4+ T cells, resulting in reduced antiviral activity of these KIR+ NK cells (Alter et al., 2011). Further to this, the presence of specific KIR/HLA combinations associated with p24 Gag sequence mutations were identified in a HIV-1 subtype C South African cohort, suggestive of viral evasion from NK cell activity through selection of HLA-presented epitope mutations (Holzemer et al., 2015).

Primary HIV-1 infection results in activation and expansion of NK cells before the development of antibody responses. However, similar to other cell types, persistent HIV-1 replication during chronic infection is associated with NK impairment, and this is seen most notably in an early loss of CD56 bright cells, secondary loss of CD56 dim cells and in the accumulation of a NK CD56 low cell type that are unable to respond to stimulation (Alter and Altfeld, 2006). Conflicting data surrounds NK cell activity in HIV-1 infection. While earlier

studies documented reduced NK cell activity in terms of declined IL-2, defective IFN-α secretion and impaired cytolysis (Alter and Altfeld, 2006), more recent reports have suggested elevated NK cell activity with raised cytokine production in viremic individuals (Ironson et al., 2001, Parato et al., 2002, Alter et al., 2005). Infection also leads to altered NK cell receptor profiles, particularly an increase of inhibitory KIR expression. Expression of CD94 has been shown to fluctuate throughout infection, and even more striking is the diminished expression of NKG2A in chronic infection, both of which are likely due to the stepwise loss of CD56 bright cells during infection (Alter and Altfeld, 2006, Altfeld et al., 2011, Jost and Altfeld, 2012).

1.3.3 Neutrophil / NK cell Interaction

As previously mentioned, neutrophils have emerged as important regulators, capable of interacting with both the innate and adaptive arms of the immune system, including NK cells. The bi-directional interactions between neutrophils and NK cells have gained considerable appreciation in recent years and a number of studies have examined the interplay between these two cell types as described below.

Early studies investigating possible regulatory mechanisms reported that NK responses could be modulated by neutrophil-derived ROS and granule proteins. Although initial studies were conducted before the identification of NK cells as a specific cell type, subsequent reports using NK-enriched lymphocytes and purified NK cell populations confirm that neutrophils were able to inhibit NK cell cytotoxicity and accelerate NK cell apoptosis in a ROS-dependent manner (Mellqvist et al., 2000, Bellner et al., 2005, Costantini and Cassatella, 2011). Specifically, it was established that modulation of NK cells was subset specific, where natural cytotoxicity receptor NKp46, important for inducing lysis of tumour cells, was down-regulated on NK CD56 low cells (Romero et al., 2006) which were highly sensitive to cell death (Thoren et al., 2007, Harlin et al., 2007).

Additional studies have revealed the modulatory effects of antimicrobial and signalling molecules contained within neutrophil granules. Arginase-1 depletes the micro-milieu of the amino acid arginine which consequently results in diminished NK proliferation and supressed IL-12/IL-18-stimulated production of IFN-ɣ by NK cells (Oberlies et al., 2009). In contrast, neutrophil elastase (Aoki et al., 1982) and lactoferrin (Shau et al., 1992) have been shown to increase NK cell-mediated cytotoxic activity. Azurophil granule proteins have also been

reported to amplify NK cell killing activity (Lala et al., 1992, Lindemann et al., 1994, Yamazaki and Aoki, 1998).

More recently, neutrophils have been shown to directly, or in cooperation with other cell types, regulate NK cell development and function (Sporri et al., 2008, Costantini et al., 2011a, Costantini et al., 2011b, Jaeger et al., 2012). Neutrophils were found to be critical in the resolution of *Legionella pneumophila* infection in mice. In the case of depleted neutrophils, NK cells were unable to produce sufficient IFN-ɣ and clear infection. The authors proposed that together with dendritic cell derived IL-12, neutrophil activation of caspase-1 and IL-18 production were essential in the activation of NK cells (Sporri et al., 2008).

Similarly, the interactive network between human neutrophils, NK cells and 6-sulfo $Lac^{+}NAc^{+}$ dendritic cells (SLAN+ DCs) was described *in vitro* and is illustrated in Figure 1.9. Stimulated neutrophils were found to directly induce IFN-γ production by NK cells through cell-cell interaction via intercellular adhesion molecule 3 (ICAM-3) and the CD18/CD11d complex expressed on neutrophils and NK cells respectively. Neutrophils could likewise, through cellular contact via ICAM-1 and the SLAN+ DC CD18/CD11a complex, promote the release of IL-12p70 by SLAN+ DCs, which in turn potentiated the production of IFN-γ by NK cells. By a positive feedback loop, the release of IFN-γ by NK cells subsequently augmented the cross talk between neutrophils and SLAN+ DCs and the release of IL-12p70 (Costantini et al., 2011a, Costantini et al., 2011b), Figure 1.9.

Using an *N*-ethyl *N*-nitrosourea mutagenesis strategy, Jaeger and colleagues (2012) identified the mouse pedigree, *Genista*, with diminished NK cell reactivity. Further examination of this mutant phenotype revealed that mature neutrophils were essential in both the bone marrow and in circulation to allow for proper NK cell maturation and function. Neutrophil-deficient mice had NK cells that were impeded at an immature stage of development and were hypo-responsive. In addition, their NK cells exhibited hyper-proliferation and reduced survival. These findings, demonstrating the fundamental role of neutrophils as mediators of NK cell function, were verified in individuals with established severe congenital neutropenia and autoimmune neutropenia (Jaeger et al., 2012).

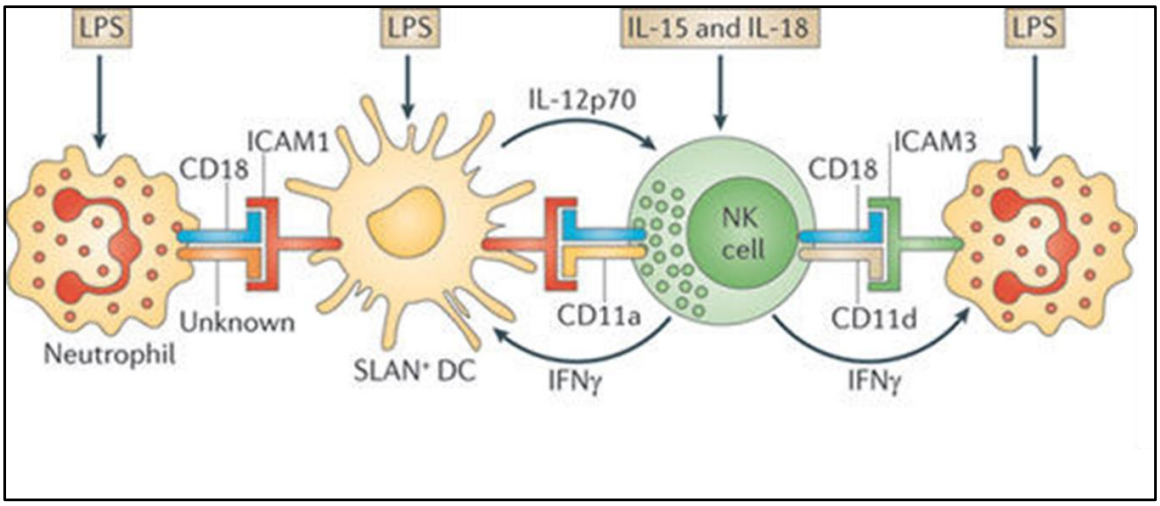


Figure 1.9 Crosstalk between neutrophils, NK cells and SLAN+ DCs

During inflammation cell–cell interactions between activated neutrophils and SLAN+ DCs (mediated by CD18 and ICAM1) induces release of IL-12p70 by SLAN+ DCs, which in turn enhances the production IFN-γ by activated NK cells. Activated neutrophils can also directly stimulate the production of IFN-γ by NK cells through engagement of CD11d–CD18 on NK cells by ICAM3 (Mantovani et al., 2011).

Historically, the extent to which NK cells influence neutrophils has received little consideration. Activated NK cells produce various CC chemokines including CCL3, CCL4, CCL5 and CCL8 (Robertson, 2002). The release of CCL8, an effective neutrophil chemoattractant, may affect neutrophil migration to the site of infection, whilst CCL3, CCL4 and CCL5 could activate neutrophils and thus modulate effector mechanisms including respiratory burst and microbial killing under certain inflammatory conditions (Hartl et al., 2008).

Researchers have recently established that neutrophil activation and survival are moderated by NK cells. The release of soluble factors such as IFN-γ, TNF-α and GM-CSF by cytokine-activated NK cells facilitated the activation and the prolonged survival of neutrophils under co-culture conditions. Depending on the cytokines to which the NK cells were exposed, NK cells were further able to enhance the expression patterns of certain neutrophil activation markers including CD11b and CD64. These neutrophils also exhibited sustained phagocytic functionality and preserved capacity to produce ROS (Bhatnagar et al., 2010, Costantini et al., 2010).

1.4 THE DUFFY ANTIGEN SYSTEM AND HIV-1 IN SUB-SAHARAN AFRICA

As previously mentioned, sub-Saharan Africa carries a disproportionate burden of the HIV-1 epidemic. Though sub-Saharan Africa is home to about 12% of the global population, 70% of HIV-1 infected people live in this region. Several factors have been implicated to explain the alarmingly high HIV-1 incidence rate in this region. Of these, high risk sexual behavioural patterns has been perceived as the major factor contributing to the spread of infection (Velayati et al., 2007, Kenyon et al., 2013), although other factors including political factors, poor healthcare and access to antiretroviral therapy, to name a few, have been cited.

However, the impact of various biological factors cannot be dismissed. There is substantial evidence linking sexually transmitted infections (STIs) and HIV transmission, although the varying influence on HIV susceptibility is microorganism dependent. Reviewed data of 22 studies indicate herpes simplex virus type 2 (HSV-2) sero-positivity as the highest STI risk factor (Braunstein et al., 2009, Ward and Ronn, 2010). Furthermore, bacterial vaginosis which is highly prevalent amongst African women has been associated with increased risk of STI acquisition, including HIV infection. The increased presence of strict anaerobic species in bacterial vaginosis causes disruption of the mucosal epithelial barrier through production of pro-inflammatory metabolites and enzymes (Bayigga et al., 2019). Of recent interest is a variation of the Duffy antigen receptor for chemokines (DARC) that influences HIV-1 infection as discussed in the following section.

1.4.1 Duffy Antigen System

The first description of the Duffy Antigen System was reported by Cutbush and colleagues (1950) in *Nature* following the detection of agglutinin in the serum of a haemophilic patient who had received multiple blood transfusions (Cutbush et al., 1950). To date six Duffy antigens have been described, although only three, specifically Fy^a, Fy^b and Fy3, are considered to be clinically relevant. The Duffy antigen receptor for chemokines (DARC), also referred to as Fy glycoprotein (FY), is a glycosylated cell membrane receptor that in humans is encoded by the DARC gene located on the long arm of chromosome 1 (q21-q25) (Donahue et al., 1968). Two main, codominant alleles exist namely FYA and FYB which are differentiated by a single-nucleotide polymorphism (SNP) (125G→A) with the corresponding antigens, Fy^a and Fy^b, differing by a single amino acid (Gly42Asp) (Langhi and Bordin, 2006). Genotypes give rise to four phenotypes: Fy(a+b+), Fy(a+b-), Fy(a-b+) and Fy(a-b-), the last of which results from

a point mutation in the FYB allele with a single T to C substitution in the gene promoter region at nucleotide position -33. This prevents transcription, causing a disruption of Duffy antigen expression and results in the "erythrocyte silent" (ES) phenotype (Howes et al., 2011)

Distribution of these phenotypes vary globally according to racial heredity, with the Fy^a and Fy^b antigens relatively common in Caucasians and Asians. Frequencies of these antigens are far less in individuals of African descent and the Fy(a-b-) phenotype prevails in these populations (Reid and Lomas-Francis, 2004), Figure 1.10.

The receptor is expressed primarily on the surface of erythrocytes, and to some extent is also located on endothelial cells of blood vessels, epithelial cells of lung alveoli, collecting ducts of kidneys and Purkinje cells of the cerebellum. Functionally, the receptor is multi-specific and binds several circulating chemokines including CXR and CC chemokines, as well as IL-8 and CCL5, thus acting as a chemokine sink and reservoir. However, no pathway signalling has been attributed to this receptor (Mohandas and Narla, 2005). Interestingly, point mutation in the FYB allele as exhibited in the Fy(a-b-) or DARC-null phenotype, only results in disruption of Duffy antigen expression on the surface of RBCs, while expression on non-erythroid cells is preserved (Duchene et al., 2017) This disruption may contribute to lower absolute neutrophil counts as discussed below.

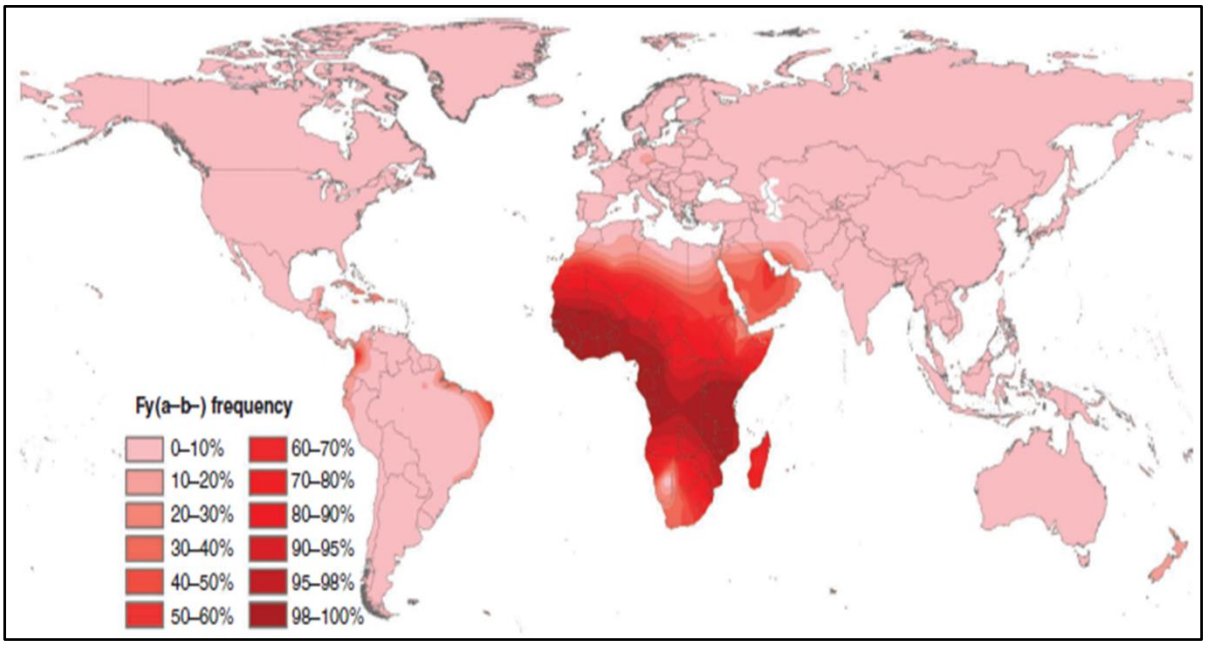


Figure 1.10 Distribution of the Duffy negativity phenotype

Global prevalence of the Duffy negative phenotype (Howes et al., 2011).

Clinically, the presence of Duffy antigen on RBCs has been implicated in the acquisition of malarial parasite infection. Experimental data has demonstrated the dependency of *Plasmodium vivax* on the Duffy antigen system to establish erythrocyte infection (Howes et al., 2011). The absence of these antigens on erythrocytes, as is common in populations in sub-Saharan Africa, has long been thought to confer relative resistance to certain malarial parasites in these individuals. In fact the variation in phenotype distribution among different racial groups seems to be the result of positive selection pressure in regions where *Plasmodium vivax* has historically been endemic, and may explain the relatively low prevalence of *P. vivax* malaria in the region (Howes et al., 2011).

Recent literature identifies the DARC-null variant as the principal determinant of benign ethnic neutropenia. Using admixture mapping of African Americans and European Americans, a polymorphism within the Duffy gene on chromosome 1 was found to have a strong association with lower white blood cell (WBC) counts, specifically neutrophils in persons of African ancestry (Reich et al., 2009). It has been postulated that the DARC-null trait could account for up to 20% of the variation in WBCs between African Americans and European Americans (Reich et al., 2009). However, the mechanisms involved in this association are not fully understood. As mentioned earlier, DARC is a multi-specific receptor and may regulate peripheral chemokine levels that consequently affect neutrophil chemotaxis, migration and localisation. It is plausible that through DARC antigen disruption on RBCs and subsequent changes to circulatory chemokines, neutrophil recruitment from the bone marrow is diminished thus leading to lower circulating neutrophils (Paz et al., 2011).

1.4.2 Benign Ethnic Neutropenia

Ethnic neutropenia is a condition characterised by significantly reduced numbers of circulating neutrophils and has been shown to be exceedingly prevalent in individuals of African descent (25-50%) (Haddy et al., 1999, Grann et al., 2008). High prevalence of lower neutrophil counts has been repeatedly confirmed, not only in persons of African descent (Bain et al., 1984, Kourtis et al., 2005, Wells et al., 2006), but also in a few ethnic groups of Middle Eastern origin (Weingarten et al., 1993, Denic et al., 2009) and has been termed benign ethnic neutropenia (Haddy et al., 1999). Although the ailment was considered to have seemingly no adverse

clinical consequences, recent reports have associated DARC-linked neutropenia to HIV-1 acquisition.

1.4.3 DARC-null Genotype, Ethnic Neutropenia and HIV-1 Infection

Interestingly, even before the connection between the DARC-null variant and ethnic neutropenia was established, He et al. (2008) suggested that the DARC-null genotype played a role in HIV-1 acquisition and disease progression. It was reported that in African Americans, individuals in possession of the null genotype had a 40% increased odds in HIV-1 acquisition and may even account for up to 11% of the epidemic in Africa (He et al., 2008). Conversely, the authors suggested that absence of the DARC trait associated with delayed development to AIDS related dementia, delayed loss of CD4+ T cells and prolonged survival (He et al., 2008).

A few mechanisms were proposed to explain these findings. Firstly, before onset of infection, HIV-suppressive chemokines that are associated with DARC-expressing red blood cells (RBCs) may act as a protective shield, preventing HIV attachment to RBCs and averting subsequent transfer of HIV-1 to target cells. However, following HIV-1 acquisition, HIV-1 binds to DARC on the surface of RBCs and transfers virus to target cells, indicating that DARC-expressing RBCs might act as carriers of infectious HIV-1 particles to susceptible cells (such as CD4+ T lymphocytes). Additionally, DARC-positive individuals may be predisposed to a more pro-inflammatory state that promotes HIV replication and spread (He et al., 2008, Kulkarni et al., 2009).

However, it is important to note the considerable controversy generated by the He et al. (2008) paper. Subsequent data generated by four independent groups demonstrated contrasting outcomes. Two groups found no association between the DARC-null variant and HIV acquisition, whilst all four groups found unaltered disease progression in the absence of DARC. (Horne et al., 2009, Julg et al., 2009, Walley et al., 2009, Winkler et al., 2009).

In correspondence to the He et al. (2008), Winkler and colleagues considered population substructure. It was stated that since DARC −46C is a marker for African ancestry, population substructure is a major confounding factor. Their analysis using ancestry-informative markers revealed minimal population substructure stratification between HIV negative and HIV positive subjects in a cohort of injecting drug users in Baltimore, USA. After taking these

considerations into account, the results indicated no association between the DARC-null genotype and HIV acquisition. Further to this, there were no significant associations between DARC-null and CD4 decline or progression to AIDS. Overall, the authors concluded that the infection signal observed in the He et al. (2008) study was the result of inadequate correction of population substructure between cases and control which led to false-positive associations (Winkler et al., 2009).

Similarly, no associations between the DARC-null polymorphism and HIV acquisition and progression were found in a report by Walley et al. (2009). Using logistic regression models, the study found no effect of the DARC-null genotype on risk of HIV acquisition in African Americans. Furthermore, the authors noted no correlation between DARC and viral load set point, CD4+ T cell decline or progression to AIDS, thus indicating no associations with faster disease progression (Walley et al., 2009). Two studies further reiterated that the DARC-null polymorphism was not linked to HIV disease progression in African Americans (Horne et al., 2009) or black South Africans (Julg et al., 2009) when assessed for clinical outcomes including rate of CD4+ T cell decline, plasma viral load burden and progression to AIDS and death.

In correspondence that followed, He and colleagues highlighted several inconsistencies between their study and the other groups. These included subject selection and controls, sample size, route of HIV infection and end-point analysis (He et al., 2009). Overall, it should be noted that direct comparison between the He et al. (2008) and these latter reports is problematic due to the differences in cohort designs and study end-points.

A subsequent study investigating high risk South African women identified the DARC-null trait as a predictor of low neutrophil counts with increased risk of HIV-1 infection. It was demonstrated that women with a pre-seroconversion neutrophil count of less than 2500 cells/mm^3 had an approximate 3 fold greater risk of HIV infection acquisition (Ramsuran et al., 2011). Consistent with this finding, it has also been established that low neutrophil counts, independent of lymphocyte and monocyte counts, are associated with an increased risk of mother-to-child HIV transmission in an African cohort (Kourtis et al., 2012).

1.5 STUDY RATIONALE, HYPOTHESIS AND OBJECTIVES

Taken together, the current literature suggests a role for DARC-null linked neutropenia in HIV-1 acquisition, while the impact of the polymorphism on HIV disease progression remains unclear. Thobakgale and Ndung'u (2014) hypothesised the manner in which DARC-null linked neutropenia in African populations may be associated with increased susceptibility or inability to control HIV-1 infection. They reasoned that in the context of ethnic neutropenia, neutrophils may be unable to clear initial infection due to either reduced circulating numbers or altered functional capacity. In addition, neutrophils were incapable of priming innate and adaptive immune cells, thus leading to an increased risk of infection and pathogen spread (Figure 1.11) (Thobakgale and Ndung'u, 2014).

The influence of DARC-null linked neutropenia on neutrophil effector functions and the effect of the condition on downstream interaction with NK cells are unknown. In a previous study conducted in the highly HIV prevalent Umlazi region in Durban, South Africa, it was determined that of the 247 participants enrolled, 64.8% individuals were homozygous for the DARC-null allele, 31.2% were heterozygous and 3.9% were homozygous wildtype (Julg et al., 2009), making this community setting ideal to assess the influence of the DARC-null trait in HIV-1 infection.

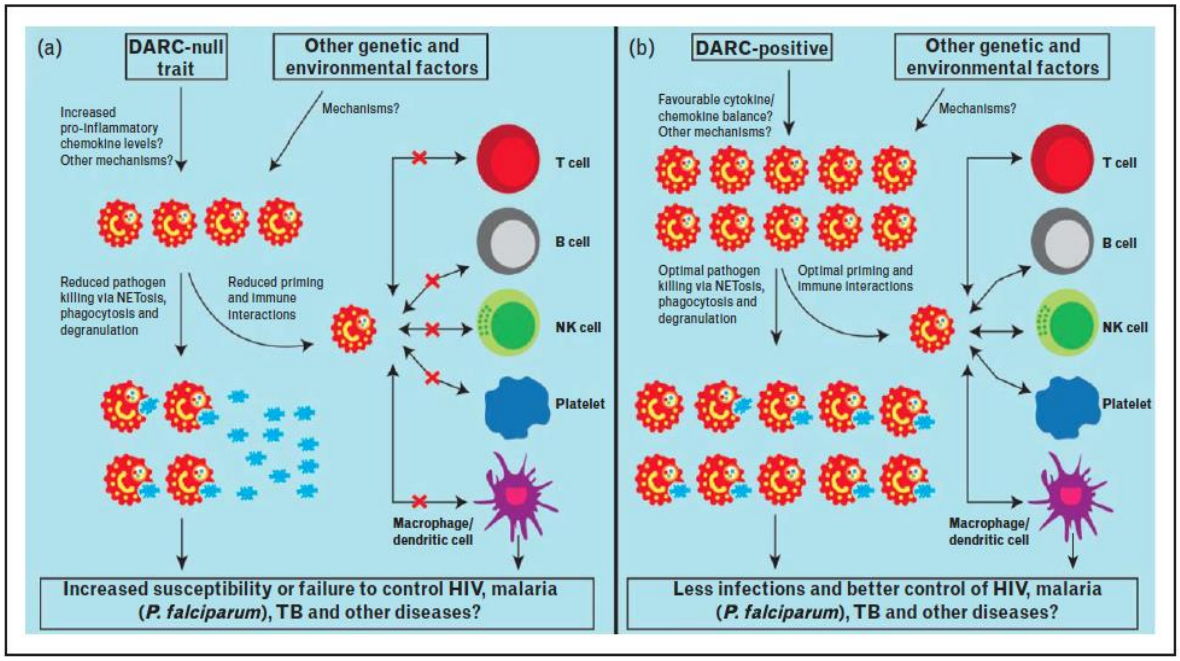


Figure 1.11 Association of DARC-null Genotype with Disease Susceptibility and Progression

Proposed mechanism of how DARC-null linked neutropenia may affect susceptibility or ability to control diseases. (a) In the context of low ANCs in DARC-null individuals, neutrophils are unable to clear infection and there may be an inability to prime immune cells, leading to increased risk of infection and pathogen spread. (b) When neutrophils counts and functions are optimal there is enhanced pathogen clearance, and neutrophil priming and cross-talk with other immune cells results in desired clinical outcome. (Thobakgale and Ndung'u, 2014).

This study aimed to establish the consequences of DARC-null linked neutropenia in HIV negative and HIV-1 chronically infected individuals. We hypothesised that in the context of DARC-null and lower ANCs in our cohort participants, neutrophils would be impaired in their key antimicrobial functions and would be unable to efficiently prime NK cells, affecting NK cell maturation and function, and altering NK cell homeostatic activities such as survival and proliferation. We further hypothesised that the impaired cellular responses would be more prominent in HIV-1 infected individuals as compared to HIV negative individuals.

In this cross sectional pilot study, we recruited HIV negative and HIV-1 chronically infected individuals from DARC-null prevalent Zulu/Xhosa African individuals from a high incidence HIV-1 setting in Durban, South Africa to address the following objectives:

Objective 1: To assess the impact of the DARC-null trait on key neutrophil effector functions, specifically Fc-mediated phagocytosis, ROS and NET production.

Objective 2: To assess the role of DARC-null trait on NK phenotypic profiles and functionality.

Objective 3: To assess HIV-specific NK cell mediated activation in individuals with and without ethnic neutropenia.

Objective 4: To assess the impact of the DARC-null trait on the NK cell homeostatic activities (survival and proliferation).

The knowledge generated from this study will be beneficial in establishing the biological consequences of DARC-null associated ethnic neutropenia in HIV-1 infection for improved biomedical interventions in African ancestry populations.

CHAPTER 2: DIFFERENTIAL NEUTROPHIL FUNCTION IN THE ABSENCE OF DUFFY CHEMOKINE RECEPTOR FOR CHEMOKINES (DARC) TRAIT

(This chapter has been accepted in part for publication in Frontiers in Immunology; Manuscript ID: 440706, see Appendix 1)

2.1 INTRODUCTION

Neutrophils constitute the earliest and most dominant innate immune response to invading pathogens. Their role in microbial clearance such as fungal and extracellular bacterial infections is well documented (Mocsai, 2013, Nauseef and Borregaard, 2014), and there is evidence of the key role of neutrophils in viral immunity (Bastian and Schafer, 2001, Fujisawa, 2001, Yasin et al., 2004). Investigations of neutrophil responses in Human Immunodeficiency Virus (HIV)-1 infection have largely focused on susceptibility to bacterial and fungal co-infections. Only recently have the direct interactions between neutrophils and HIV-1 been fully appreciated (Casulli and Elbim, 2014).

Disorders of neutrophil counts (neutropenia) and function are usually associated with recurrent infection complications, particularly in severe cases where the absolute neutrophil count (ANC) drops to below 500 cells/mm^3 (Hsieh et al., 2010, Thobakgale and Ndung'u, 2014). Congenital disorders driven by diminished neutrophil production and release from the bone marrow exhibit a higher risk of microbial infections in comparison to peripheral acquired neutropenia (Boxer, 2012). Ethnic neutropenia is the most frequent form of congenital neutropenia globally, commonest in persons of African descent. Clinically, the condition is considered moderate and is not associated with obvious increased oral and systemic infection incidence as observed in other forms of neutropenia (Hsieh et al., 2010). It is unclear whether ethnic neutropenia is associated with any mechanistic defects in neutrophil functions.

Absence of the Duffy Antigen Receptor for Chemokines (DARC), a glycosylated cell membrane receptor encoded by the DARC gene located on chromosome 1, has been identified as the principal genetic determinant of ethnic neutropenia (Reich et al., 2009). A single nucleotide polymorphism (-46T>C) in the DARC promoter region abolishes gene expression and results in selective loss of DARC expression on erythrocytes (Howes et al., 2011). However, the mechanism through which the DARC-null polymorphism induces neutropenia remains poorly understood. Previous studies have established an association between lower circulating neutrophils exhibited in the absence of DARC and increased risk of HIV acquisition and mother-to-child transmission (He et al., 2008, Ramsuran et al., 2011, Kourtis et al., 2012). Following HIV infection, it has been suggested that the DARC-null state is accompanied with slower disease progression and that the polymorphism may impart a survival advantage in leukopenic HIV infected individuals (He et al., 2008, Kulkarni et al., 2009). Conversely, other reports contesting these findings demonstrated that DARC status did not impact the rate of

disease progression based on viral load set points, CD4+ T cell decline or progression to AIDS (Horne et al., 2009, Julg et al., 2009, Walley et al., 2009).

Overall, the evidence appears stronger for an association between DARC-null-linked ethnic neutropenia and HIV acquisition than it does for its role in HIV disease progression. However, it is plausible that the influence of the DARC-null trait is complex, with many of its underlying unknown components contributing to HIV pathogenesis. While the absence of DARC is well associated with low circulating neutrophils, the effect of the polymorphism on neutrophil effector functions has not been investigated. Here we aimed to determine the influence of the DARC genotype on neutrophil effector functions in HIV-1 negative versus HIV-1 chronically infected Zulu/Xhosa black African individuals in Durban, South Africa. We hypothesised that neutrophil effector mechanisms including phagocytosis, production of reactive oxygen species (ROS) or formation of neutrophil extracellular traps (NETs) would be impaired in the absence of DARC and that this dysfunction would be more distinct in HIV infected persons compared to uninfected individuals.

2.2 METHODS

2.2.1 Participant Recruitment

HIV negative participants were recruited from the Females Rising through Education, Support and Health (FRESH) cohort, an acute HIV infection monitoring cohort that targets high risk women, whilst HIV-1 subtype C chronically infected treatment naïve participants were recruited from the HIV Pathogenesis Programme (HPP) Acute Infection Cohort, an acute-chronic HIV infection cohort. Both of these cohorts are located in the Umlazi region in Durban, KwaZulu-Natal (KZN), South Africa. Ethical approval for the maintenance of the cohorts have been attained from the University of KwaZulu-Natal (UKZN) Biomedical Research Ethical Council (BREC) (FRESH BREC Reference Number: BF131/11 and HPP Acute Infection Cohort BREC Reference Number: E036/06). All participants gave written informed consent to voluntarily participate in these cohorts. This particular study obtained further ethical clearance as a subsidiary of the above studies (BREC Reference Number: BE229/15). A total of 42 individuals sub-divided into HIV negative donors (n=20) and HIV-1 chronically infected individuals (n=22) were assessed. These individuals were further stratified by DARC status.

2.2.2 ANCs, CD4 Counts and Viral Load

Blood for full blood counts, CD4+ T cell count measurement and viral load quantification was collected in Ethylenediaminetetraacetic acid (EDTA) anticoagulated vacutainer tubes (Becton Dickinson (BD), Franklin Lakes, New Jersey, USA). ANCs were enumerated by full blood count using the automated XN 1000 Haematology Analyser (Sysmex, Kobe, Hyōgo, Japan). CD4 counts were measured using BD Trucount and analysed on a four-parameter FACS Calibur flow cytometer (BD). Viral loads were determined using the NucliSENS EasyQ HIV-1 v2.0 kit with a detection limit of 20 copies/ml (BioMérieux, Marcy-l'Étoile, France).

2.2.3 Quantification of Antiretroviral (ARV) Drugs in Plasma

This study aimed to recruit HIV-1 chronically infected, treatment naïve individuals. ARV therapy usage in chronically infected patients was self-reported. Certain subjects maintained viral loads below 1000 RNA copies/ml at the time of assessment. To rule out ARV drug use, plasma samples were collected from these study participants and screened for ARV drugs using a quantitative liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method. The method screened for nine ARVs, namely Emtracitabine, Tenofovir, Lopinavir,

Ritonavir, Nevirapine, Abacavir, Lamivudine, Zidovudine and Efavirenz. A plasma sample volume of 50 µl was processed using a protein precipitation method, ARV drug analytes were chromatographically separated on a Agilent Zorbax Eclipse Plus C18 (2.1 x 50mm, 3.5 µm) HPLC column (Agilent Technologies, Santa Clara, California, USA), detected using an AB Sciex 5500 triple quadrupole mass spectrometer (Sciex, Framingham, Massachusetts, USA) and quantitated using Analyst® 1.6.2 software (Sciex).

2.2.4 DARC Genotyping

DARC -46T→C (rs2814778) single-nucleotide polymorphism (SNP) genotyping was performed by TaqMan allelic discrimination assays which has been previously verified by direct sequence analysis by Julg et al. (2009). Briefly, genomic DNA was isolated from stored buffy coats using the QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. DNA concentration was standardised at 50ng/µl with polymerase chain reaction (PCR) grade water. A cocktail containing TaqMan Genotyping master mix (Life Technologies, Carlsbad, California, USA) and predesigned probes for the DARC gene (SNP ID: rs2814778, Applied Biosystems, Foster City, California, USA) was used to amplify target sequence in 50ng genomic DNA by real time PCR (RT-PCR) in the Light Cycler 480 (Roche, Basel, Switzerland) according to the manufacturer's protocol.

2.2.5 Neutrophil Isolation

Blood was collected in sodium heparin BD Vacutainers (BD) from each participant and was processed within 4 hours of collection. Granulocyte fraction was prepared by layering an equal volume of whole blood over a Histopaque-1119 cushion (Sigma-Aldrich, St. Louis, Missouri, USA) and centrifugation at 800 x g for 20 minutes at room temperature, low brakes as previously described (Brinkmann et al., 2010). Centrifugation with the Histopaque-1119 poly-sucrose solution allows for the rapid separation of cellular components based on density. Erythrocytes aggregate and sediment resulting in pellet formation. Polymorphonuclear leukocytes (PMNs) form a band in the histopaque-1119 solution above the pelleted erythrocytes, whereas peripheral blood mononuclear cells (PBMCs), which are lower in density form a band higher in the histopaque-1119 solution between the plasma layer and the PMN band (Figure 2.1).

The PMN band was collected, washed with Dulbecco's phosphate-buffered saline (dPBS) and centrifuged at 300 x g for 10 minutes at room temperature, followed by a second wash with chilled wash buffer (Dulbecco's phosphate-buffered saline (dPBS) containing 0.5% bovine albumin serum (BSA)) and centrifugation at 300 x g for 10 minutes at 4°C. Pelleted cells were then incubated with magnetically labelled CD15 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at 4°C. Cells were again washed with chilled wash buffer and centrifuged at 300 x g for 10 minutes at 4°C. Pelleted cells were resuspended in 500µl chilled wash buffer and loaded into a MACS column placed in the magnetic field of a MACS Separator. Chilled wash buffer was added to the column 3 times, allowing the unlabelled cells to run through the column. The magnetically labelled CD15+ cells were retained within the column. The column was then removed from the magnetic field and the retained cells eluted as the CD15+ selected cell fraction, washed once in wash buffer and resuspended at 1million cells/ml in Roswell Park Memorial Institute (RPMI) 1640 Medium without phenol red indicator and containing 0.05% human serum albumin (HSA). Post purification cell counts were determined by 1:5 dilution with Trypan Blue Stain (Gibco) using a haemocytometer under a light microscope. The median neutrophil count was 15.2 million cells (IQR: 10-22 million cells). The median neutrophil viability following isolation was 98% (IQR: 98 – 100%).

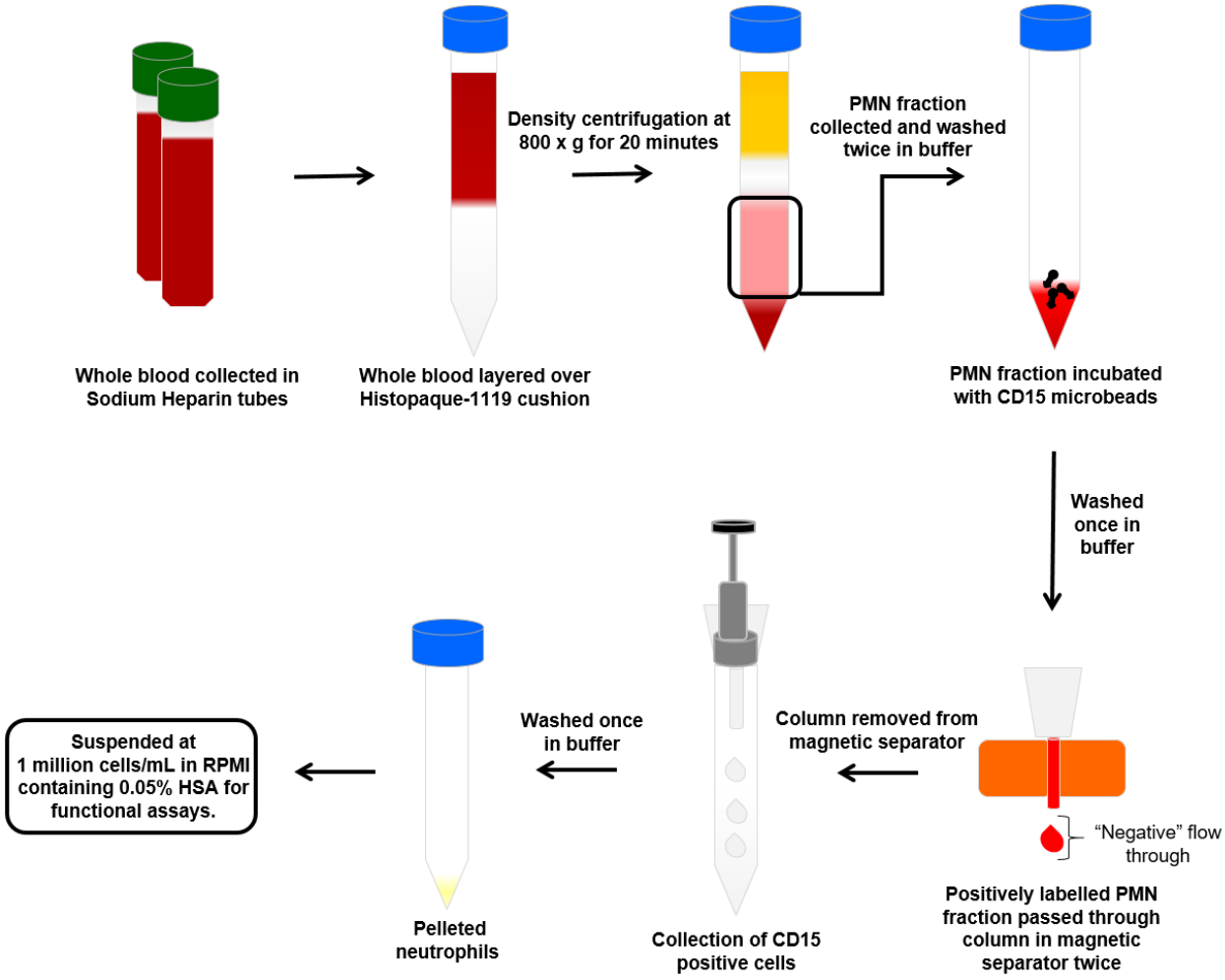


Figure 2.1 Schematic of Neutrophil Isolation

Whole blood layered over equal volumes of Histopaque-1119. Following density centrifugation, the PMN layer was removed, washed twice and incubated with CD15 microbeads. Cell suspension was passed through a column within a magnetic field. Positively labelled cells were retained within the column whilst unlabelled cells ran through the column as the "negative" flow through and was discarded. Removal of the column from the magnetic separator allowed for the positively labelled cells to be eluted as the CD15+ cell fraction.

2.2.6 Neutrophil Purity

Neutrophil purity was measured by assessing the expression of CD66b and CD15 surface markers on the isolated cell suspension. CD66b is a glycosylphosphatidylinositol (GPI)-linked protein that is exclusively expressed on two granulocyte populations; i.e. neutrophil and eosinophil cells (Lakschevitz et al., 2016). CD15 is a carbohydrate adhesion molecule that is expressed on glycoproteins, glycolipids and proteoglycans on the surface of all myeloid lineage cells (granulocytes and monocytes), except basophils (Gadhoum and Sackstein, 2008). By assessing the expression of CD66b and CD15 in the isolated cell suspension, the level of neutrophil purity can be estimated (neutrophils will express both markers), with only minimal eosinophil contamination. Any eosinophil contamination is considered negligible since neutrophils comprise over 90% of circulating granulocytes, whilst eosinophils contribute to less than 5% of the population (Geering et al., 2013).

To determine the purity of the isolated cell suspension, half a million cells were centrifuged at 13000 x g for 1 minute, supernatant removed and the pelleted cells stained with monoclonal antibodies anti-CD66b Fluorescein isothiocyanate (FITC, BD Biosciences) and anti-CD15 Brilliant Violet (BV) 605 (Biolegend, San Diego, California, USA) for 20 minutes at room temperature in the dark. Cells were then washed with dPBS, centrifuged at 13000 x g for 1 minute and supernatant removed before addition of fixation Medium A (Invitrogen, Carlsbad, California, USA) to ensure that morphological characteristics of the cells remained intact. Fluorescence minus one (FMOs) for CD66b and CD15 were prepared for each experiment to exclude background fluorescence in the gating strategies. Samples were acquired on an LSR II flow cytometer (BD) and at least 10000 events per sample were recorded. FlowJo Software Version 9 (TreeStar, Inc., Ashland, Oregon, USA) was used for sample analysis. Only neutrophils with a purity of at least 90% were used for functional assays (Figure 2.2).

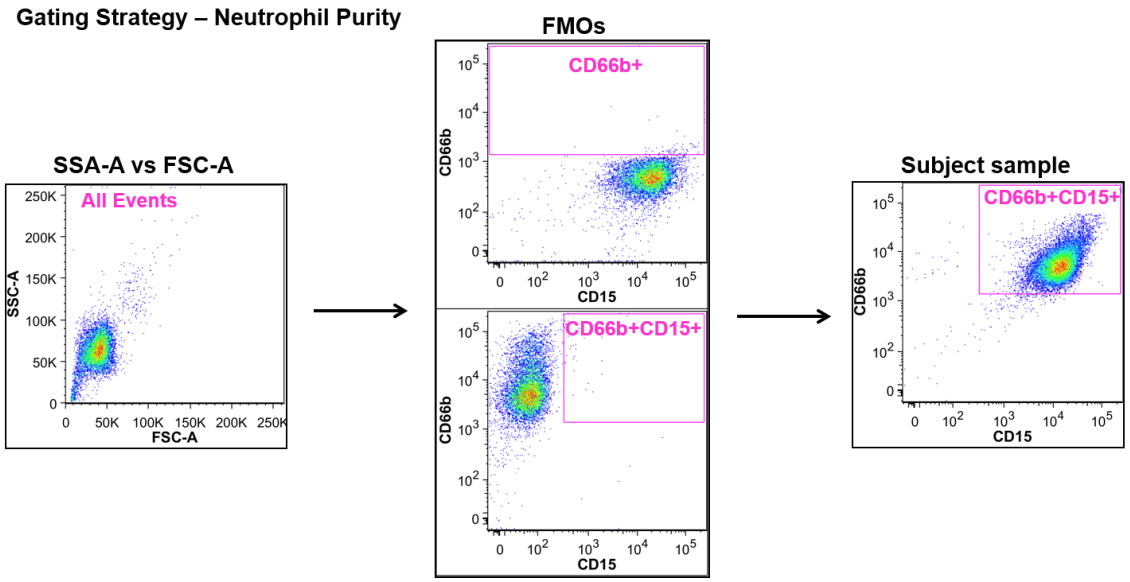


Figure 2.2 Representative gating strategy for neutrophil purity

All events from the side scatter area (SSC-A) vs forward scatter area (FSC-A) plot were included to determine neutrophil purity of each subject sample using flow cytometry. Fluorescence minus one (FMOs) for CD66b and CD15 were used to exclude cells that did not stain positive for CD66b and/or CD15. Neutrophils were identified as CD66b+CD15+.

2.2.7 Phagosome Maturation

Phagosome maturation refers to a series of sequential events that occur within the phagosome following internalisation. Delivery of antimicrobial molecules through secretory vesicle and granule fusion is central to this dynamic process and confers the phagosome the ability to degrade internalised pathogens (Lee et al., 2003). To evaluate the phagocytic capacity of neutrophils we employed a strategy developed by Podinovskaia et al. (2013) which measures the extent of bulk proteolysis that occurs within the phagosome. Immunoglobulin G (IgG) opsonised particles are modified to carry a reporter fluorochrome and DQ Green-BSA complex. Binding of IgG to the neutrophil Fc receptor (FcR) mediates the extension of pseudopods and engulfment of the opsonised particle. Thereafter, delivery of antimicrobial molecules to the phagosome results in degradation of the Green-BSA complex and emission of fluorescence that is used as an indicator of phagosome progression.

DQ Green-BSA reporter beads were prepared as previously described (Podinovskaia et al., 2013). Briefly, 5mg of 3.0µm carboxylate-modified silica particles (Kisker Biotech GmbH & Co., Steinfurt, Germany) were washed three times with 1ml of dPBS and centrifuged at 2000 x g for 60 seconds at room temperature. Particles were resuspended in 1ml PBS containing

20mg cyanamide (Sigma-Aldrich) and incubated on a shaker for 20 minutes at room temperature. Particles were then washed three times in dPBS before resuspending in 1ml coupling buffer (0.1M boric acid in double distilled water (ddH_20), adjusted to pH 8.0 with 10M sodium hydroxide) containing 3mg DQ Green-BSA (Life Technologies, Carlsbad, California, USA) and 100µg IgG (Sigma-Aldrich) and incubated on a shaker overnight at room temperature. Particles were washed twice in coupling buffer and once in dPBS before resuspending in 500µl dPBS containing 0.02% sodium azide (Sigma-Aldrich). Particles were stored at -20°C in aliquots protected from light.

One million isolated neutrophils in RPMI-1640 medium without phenol red indicator and containing 0.05% HSA was used to measure phagosome maturation after addition of 5µl prepared DQ Green-BSA reporter beads and incubation at 37°C for either 10 minutes, 60 minutes or 120 minutes. Samples were fixed with paraformaldehyde (1% final concentration, Sigma-Aldrich) and acquired on an LSRII Flow Cytometer (BD), recording at least 50000 events. For each experiment, a tube containing neutrophils incubated for 120 minutes without DQ Green BSA was acquired for gating strategies and a tube containing DQ Green-BSA reporter beads alone was acquired to determine background fluorescence. Flow Cytometry Standard files were analysed using FlowJo Software Version 9 (TreeStar, Inc., Ashland, Oregon, USA).

2.2.8 ROS production

In parallel to phagosome maturation, assemblage of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex on the phagosome membrane results in the production of ROS intermediates. Accumulation of ROS within the phagosome creates a toxic environment to most microbes. ROS production by neutrophils was measured by chemi-luminescence as previously described (Fuchs et al., 2007).

Briefly, $1x10^5$ isolated neutrophils in RPMI-1640 medium without phenol red indicator (0.05% HSA) and containing 50µM luminol (Sigma-Aldrich) and Horseradish peroxidase (Sigma-Aldrich) at a final concentration of 1.2 units/ml were seeded per well in a 96 well plate and allowed to rest at 37°C 5% CO_2 for 30 minutes. Thereafter, cells were cultured with either RPMI-1640 medium alone as a negative control or with 10nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) in triplicate and luminescence was read every 2 minutes for a period of 120 minutes in a Glomax Multi Detection System (Promega, Madison, Wisconsin, USA).

Triplicate readings for unstimulated and PMA stimulated conditions were averaged. Unstimulated readings were used to subtract background luminescence from PMA stimulated readings.

2.2.9 NET Quantification

A form of programmed cell death that is distinct from apoptosis and necrosis, leads to the generation of NETs (Brinkmann et al., 2004). The process involves the release of nuclear material studded with antimicrobial factors from the cell. These extracellular fibres are able to bind pathogens and thus aid to eliminate invading microbes. Here we adapted a fluorescent microscopy based technique to quantify NET production following neutrophil activation with PMA stimulus as previously described (Brinkmann et al., 2010).

Fifty thousand isolated neutrophils in 400µl RPMI supplemented with 0.05% HSA were seeded on 14mm coverslips placed in 24 well plates. Neutrophils were either left untreated as a negative control (100µl RPMI) or activated with 50nM PMA and incubated at 37°C 5% CO_2 for 10 minutes, 60 minutes or 120 minutes before addition of paraformaldehyde fixative solution (2% final concentration) to halt further NET formation. Plates were stored at 4°C overnight until coverslip staining.

Coverslips with the attached cells were removed from the 24 well plate with fine forceps and washed three times for 5 minutes by floating coverslips with the fixed cells facing downwards on dPBS drops placed on hydrophobic parafilm. Cells were permeabilised with 0.5% Triton X-100 (Sigma-Aldrich) in dPBS for 5 minutes, and thereafter blocked in blocking buffer (3% donkey serum, 3% fish gelatine, 0.5% Tween (all from Sigma-Aldrich), 1% BSA (Biowest, Nuaillé, France) in dPBS) for 30 minutes at room temperature. Thereafter solutions containing antibody directed against the subnucleosomal complex of Histone 2A, Histone 2B, and chromatin (mouse anti-PL2-3, Zychlinsky Lab, Max Planck Institute for Infection Biology, Berlin, Germany) and rabbit anti-neutrophil elastase (Calbiochem, San Diego, California, USA) diluted in blocking buffer were applied to the coverslips and placed in a humid chamber for 1 hour in the dark. After washing with dPBS three times for 5 minutes, solution containing Hoechst 33342, dye-conjugated goat anti-mouse Alexa Flour 568 and goat anti-rabbit Alexa Flour 488 (all from Life Technologies) diluted in blocking buffer was applied to the coverslips and placed in a humid chamber for 45 minutes in the dark. Coverslips were then washed twice

with dPBS and once with distilled water before mounting with mowiol solution (Calbiochem) to glass slides, allowed to dry overnight and subsequently stored at 4°C.

To quantify NET production, images were taken from five randomly selected regions of each coverslip using the 10× lens on a Zeiss upright fluorescence microscope equipped with an AxioCam MRC microscope camera (Oberkochen, Germany). The exposure times for each channel were kept constant over the course of an experiment after using a bright representative region for calibration. Images were loaded separately for each channel onto ImageJ/FIJI software. The Hoechst 33342 fluorescent channel image was used to collect data of total cell number in a selected region. The image was converted to an 8-bit image and binarised using Bernsen's thresholding method with the parameter 1 contrast threshold value set at 15. Particles were analysed with the size set to a minimum of 20 pixels. The PL2-3 fluorescent channel image was used to collect data of NET production in the selected region. The threshold was applied manually to an image fixed with cells that were activated for 10 minutes with PMA. The threshold was adjusted to the minimum value that allowed only objects larger than 75 pixels (spontaneous NETs > 75 pixels) to be visible. This minimum threshold value was then applied to coverslip regions stimulated for different time intervals for the same specimen. The summarised output was exported to a spreadsheet programme for further processing. NET production was calculated as follows:

NET-rate (%) = 100 × Objects counted (PL2-3 channel) / Objects counted (Hoechst channel).

The NET production on each coverslip was calculated as an average of the five randomly selected regions. RGB-merged images of the three channels Hoechst, PL2-3 and neutrophil elastase were compiled and used as control images to manually assess and exclude non-neutrophil cells and artefacts from the results.

2.2.10 Statistical Analysis

GraphPad Prism Version 5 software (GraphPad software Inc., La Jolla, California, USA) was used for statistical data analysis. Differences between two studied groups was examined by Mann-Whitney U test. Differences between more than two groups was examined by 1 way ANOVA followed by Bonferonni's Multiple Comparison Test. Differences with a p value <0.05 were considered statistically significant. For ROS production, area under curve was calculated with the baseline y value = 0 and all values above the baseline were taken into consideration.

2.3 RESULTS

To assess the possible influence of the DARC-null trait and ethnic neutropenia on neutrophil effector functions, 42 participants of Zulu/Xhosa ethnicity were enrolled into our study cohort. The cohort comprised 20 HIV negative donors and 22 HIV infected subjects that were therapy naïve. The majority of the subjects (40 out of 42) were female with a median age of 20, interquartile range (IQR) (19-22) and 22 years, IQR (20-25) in HIV negative and HIV infected individuals respectively.

Assessment of our participant cohort by HIV status indicated that CD4 counts were significantly lower in HIV infected persons compared to HIV negative individuals (median CD4 count of 653 and 892 cells/mm^3 respectively, p=0.002, data not shown). Further analysis by DARC status showed no significant differences in CD4 counts in HIV negative subjects (DARC-null median CD4 count of 824 cells/mm^3; DARC-positive median CD4 count of 1198 cells/mm^3, p=0.08) or infected individuals (DARC-null median CD4 count of 582 cells/mm^3; DARC-positive median CD4 count of 618 cells/mm^3, p=0.53, Table 2.1). Furthermore viral loads were not statistically different by DARC genotype in HIV-infected individuals (DARC-null median viral load of 4150 RNA copies/ml; DARC-positive median viral load of 12250 RNA copies/ml, p=0.48 Table 2.1).

Table 2.1 Clinical Characteristics of Study Participants used to assess Neutrophil Function

	HIV Negative			HIV Positive		
	DARC CC (n=13)	DARC TC/TT (n=7)	p value	DARC CC (n=16)	DARC TC/TT (n=6)	p value
Absolute Neutrophil Count, 10^3 cells/mm^3	**2.64** (1.95-3.17)	**5.07** (4.68-7.08)	0.0007	**2.22** (1.78-2.66)	**4.08** (3.13-4.78)	0.03
CD4 count, cells/mm^3	**824** (469-1561)*	**1198** (672-1583)*	0.08	**582** (341-927)*	**618** (466-940)*	0.53
Viral Load, RNA copies/ml	na	na	na	**4150** (1175-30750)	**12250** (3750-24500)	0.48

Data is represented as median (IQR). * Full range CD4 counts are indicated in brackets instead of IQR. Abbreviations: DARC, Duffy Antigen Receptor for Chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute Neutrophil Count; IQR, Interquartile range; n, number; na, not applicable. Female participants: n=40, Male participants: n=2.

2.3.1 High Prevalence of the DARC-null trait in the Zulu/Xhosa Population

The DARC-null trait is exceedingly prevalent on the African continent (Howes et al., 2011). High prevalence of this polymorphism has previously been reported in persons of Zulu/Xhosa ethnicity where 64.8% of HIV-infected individuals and 64.7% of high risk South African women displayed the DARC-null allele (Julg et al., 2009, Ramsuran et al., 2011). Here we used allelic discrimination assays for SNP genotyping in order to distinguish DARC-null and DARC-positive individuals.

Twenty nine (69%) of the participants were negative for the DARC trait irrespective of HIV status. In HIV negative individuals, 13 of 20 participants (65%) were DARC-null, whilst 7 of 20 (35%) were DARC-positive. In HIV-infected subjects, the presence of the polymorphism was even higher, 16 of 22 participants (73%) were DARC-null and 6 of 22 subjects (27%) were DARC-positive (Table 2.1).

Thus, despite the comparatively smaller sample size used here, our data is in agreement with past studies (Julg et al., 2009, Ramsuran et al., 2011) that indicate high prevalence of the DARC-null allele in populations within this region.

2.3.2 DARC-null trait is associated with reduced ANCs

Neutrophils constitute 50-70% of circulating leukocytes with typical counts ranging from $2.0x10^3$ to $7.5x10^3$ cells/mm^3 of whole blood. Previous reports have indicated a strong association of the DARC variant with ANCs. The DARC-null polymorphism is accompanied with persistently low concentrations of circulating neutrophils and is a predictor of ethnic neutropenia where ANCs are repeatedly less than 1.5x10 cells/mm^3 (Hsieh et al., 2010). To ascertain whether our studied cohort displayed similar associations, we compared ANCs according to DARC trait in both HIV negative and HIV-1 infected individuals.

Among our study participants we found no differences in ANCs by HIV status where median ANCs were $3.17x10^3$ and $2.56x10^3$ cells/mm^3 in HIV negative and HIV-infected individuals respectively (data not shown). We did, however, observe that median ANCs were significantly lower in DARC-null compared to DARC-positive individuals in both HIV negative ($p=0.0007$) and HIV infected ($p=0.03$) persons (median ANC of $2.64x10^3$ cells/mm^3 in HIV negative and $2.22x10^3$ cells/mm^3 in infected DARC-null compared to $5.07x10^3$ cell/mm^3 in HIV negative and $4.08x10^3$ cells/mm^3 in infected DARC-positive subjects, Table 2.1). Significantly reduced ANCs were observed when comparing DARC-null and DARC-positive individuals,

irrespective of HIV status, although this finding was more prominent in HIV negative individuals (p=0.0007 in HIV negative individuals, Figure 2.3A and p=0.03 in HIV infection, Figure 2.3B). As an indication of ethnic neutropenia in our cohort of African individuals, only 13.8% (4 of 29) of our recruited DARC-null subjects had ANCs of $1.5x10^3$ cells/mm^3 or less at the time of sampling (data not shown).

In line with current literature (Reich et al., 2009), our data suggested that DARC-null individuals exhibited significantly lower circulating neutrophil counts, although only a minority had a count low enough to meet the clinical definition of neutropenia.

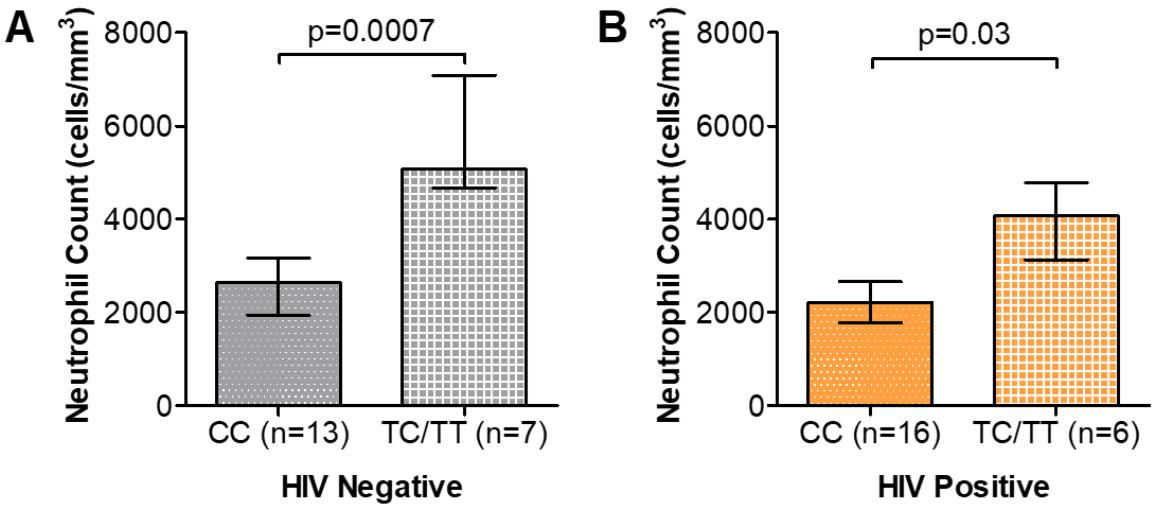


Figure 2.3 ANCs stratified by DARC variant

Comparison of ANCs measured by full blood count according to DARC genotype in HIV negative **(A)** and HIV positive **(B)** individuals. Bar graphs indicate medians extended to interquartile range (IQR) with whiskers. The p-values refer to differences in ANC between DARC-null and DARC-positive participants at the time of sampling. Abbreviations: ANC, Absolute Neutrophil Count; DARC, Duffy Antigen Receptor for Chemokines; CC, DARC-null; TC/TT, DARC-positive; IQR, interquartile range.

2.3.3 Phagocytic activity enhanced in the absence of DARC

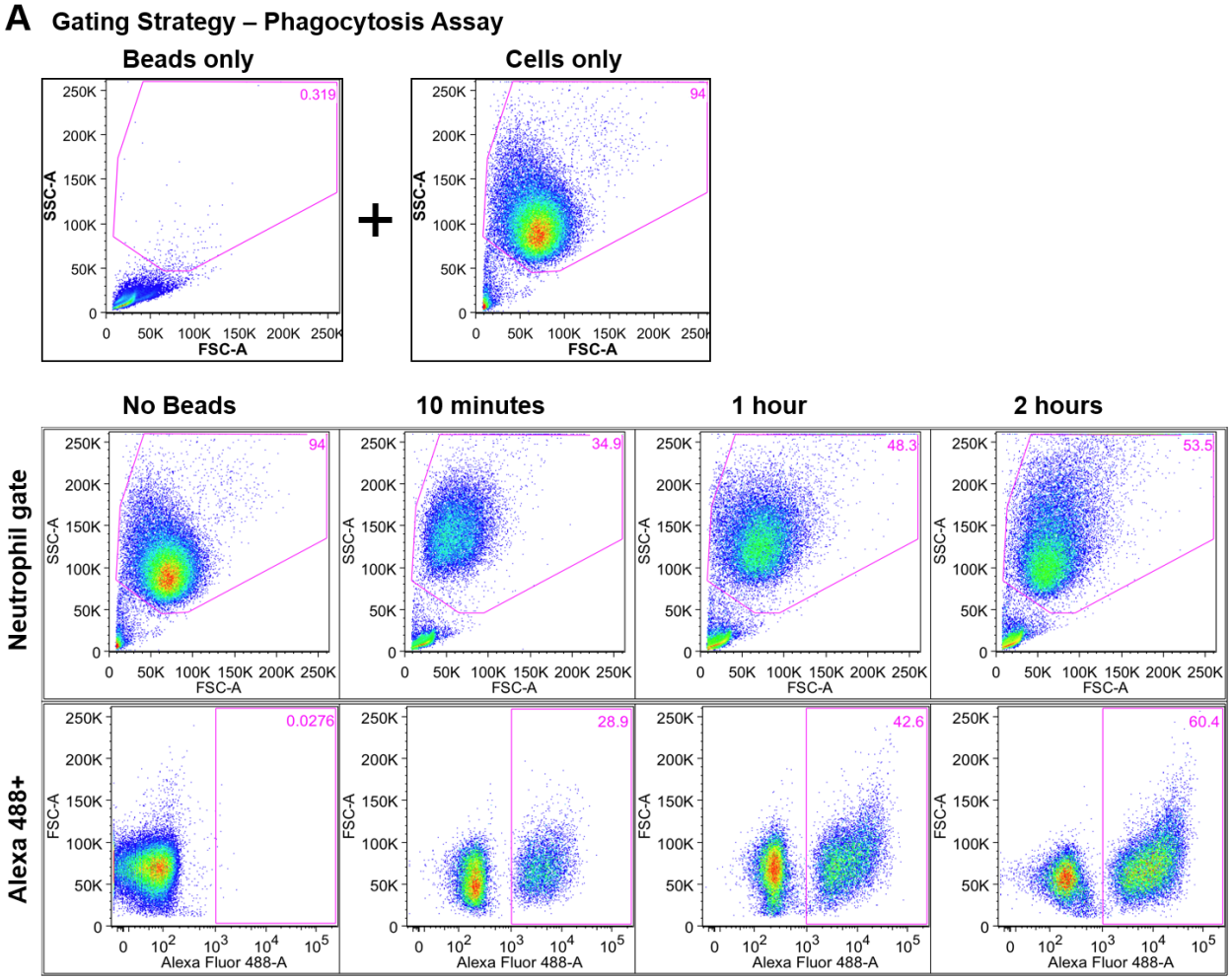
Recognition, internalisation and phagosome maturation are the hallmarks of the neutrophil response to effectively clear invading pathogens (Mayadas et al., 2014). We thus sought to measure the phagocytic capacity of neutrophils in DARC-null individuals and whether this phagocytic ability is altered by DARC status or ANCs in HIV negative and HIV-1 infected individuals. Phagocytosis is routinely quantified by measurement of bacterial or opsonised bead internalisation. Here we measured phagosome maturation by flow cytometry after 10 minutes, 60 minutes and 120 minutes of introducing IgG opsonised beads to isolated neutrophils (Figure 2.4A). Phagosome maturation exhibited a steady increase over all time intervals in the 120 minute period in all studied subjects ($p<0.0001$, Figure 2.4B), and this increase was irrespective HIV status (Figure 2.4C and Figure 2.4D).

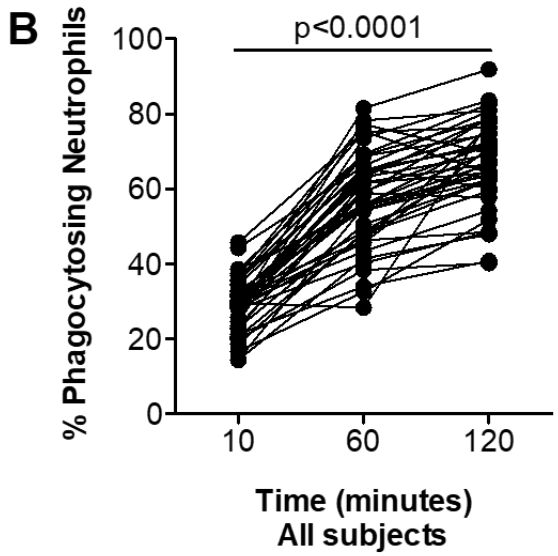
HIV infection reduces neutrophil phagocytosis capacity, although the mechanisms underlying this altered activity are not fully understood (Casulli and Elbim, 2014). Here a trend of lower phagocytic ability in HIV chronically infected compared to HIV negative persons was observed at all measured time intervals over a 120 minute period (Figure 2.4E, $p=0.08$ at 10 minutes; $p=0.24$ at 60 minutes; $p=0.06$ at 120 minutes). Subsequent analysis by DARC status revealed that DARC-null individuals (irrespective of HIV status) had a higher proportion of neutrophils able to phagocytose opsonised beads at 10 minutes and 60 minutes (Figure 2.4F, $p=0.05$ at 10 minutes and $p=0.05$ at 60 minutes in HIV negative individuals; and Figure 2.4G, $p=0.009$ at 10 minutes and $p=0.07$ at 60 minutes in HIV infected individuals). The median frequency of phagocytosing neutrophils was still higher in DARC-null compared to DARC-positive individuals at 120 minutes post bead incubation (74.5% versus 64.3% in HIV negative and 67.9% versus 55.4% in HIV infection in DARC-null and DARC-positive individuals respectively). However, overall no statistical differences were observed in the phagocytic potential between DARC-null and DARC-positive individuals in HIV negative or HIV infected persons at 120 minutes post bead incubation (Figures 2.4F and 2.4G).

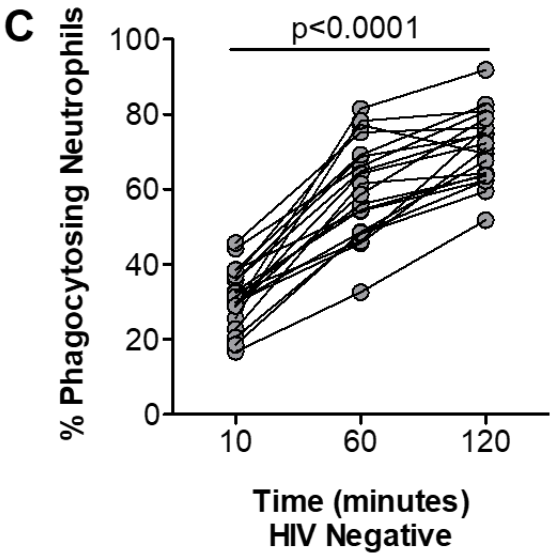
In HIV negative individuals, the frequency of phagocytosing neutrophils did not correlate with ANCs, whilst, in contrast, the number of phagocytosing neutrophils inversely correlated with ANCs in chronically infected individuals at 10 minutes (a trend was observed) and 60 minutes following bead incubation (Figure 2.4H).

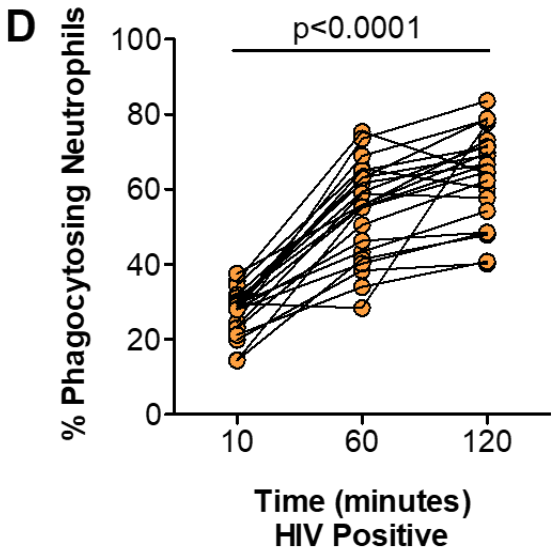
Taken together our data suggests that the DARC genotype may play a role in the phagocytosing potential of neutrophils irrespective of HIV status. In the absence of the DARC phenotype a

higher proportion of neutrophils are able to phagocytose in an FcR dependent manner soon after bead incubation (10 minutes and 60 minutes).




B
p<0.0001
% Phagocytosing Neutrophils
100
80
60
40
20
0
10
60
120
Time (minutes)
All subjects


C
p<0.0001
% Phagocytosing Neutrophils
100
80
60
40
20
0
10
60
120
Time (minutes)
HIV Negative


D
p<0.0001
% Phagocytosing Neutrophils
100
80
60
40
20
0
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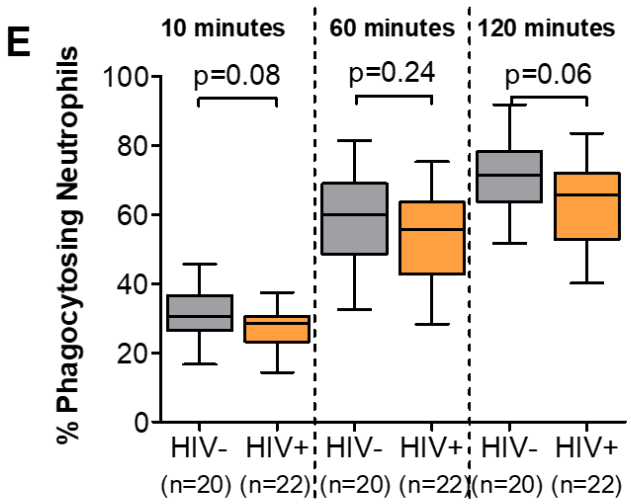
60
120
Time (minutes)
HIV Positive


E
10 minutes
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p=0.08
p=0.24
p=0.06
% Phagocytosing Neutrophils
100
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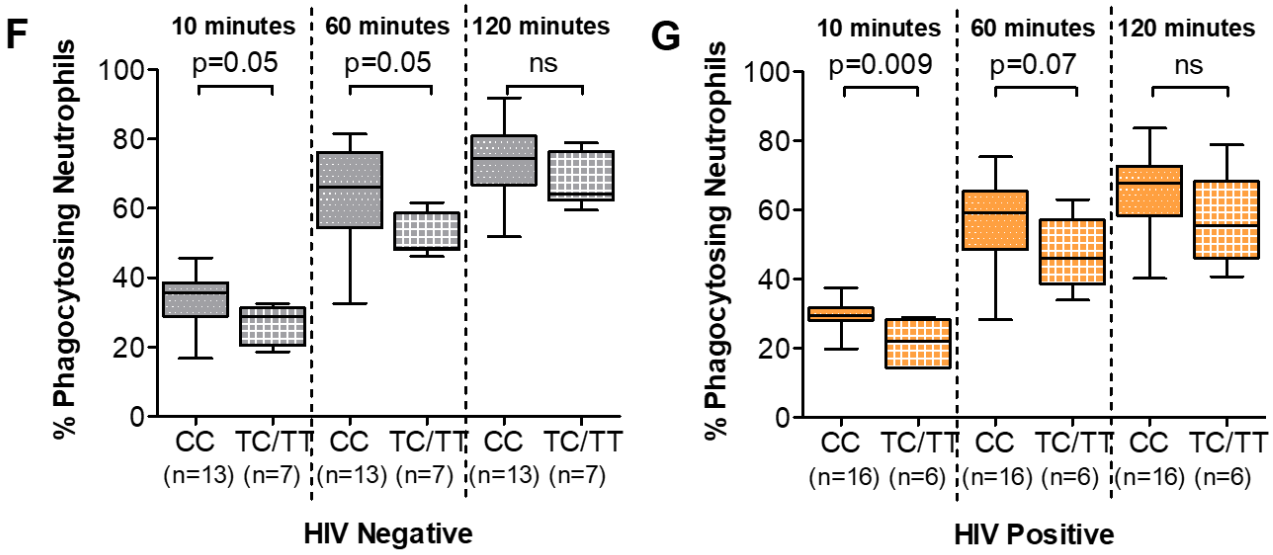
HIV-
HIV+
(n=20)
(n=22)


F
10 minutes
60 minutes
120 minutes
p=0.05
p=0.05
ns
% Phagocytosing Neutrophils
CC
TC/TT
(n=13)
(n=7)
HIV Negative
G
p=0.009
p=0.07
(n=16)
(n=6)
HIV Positive

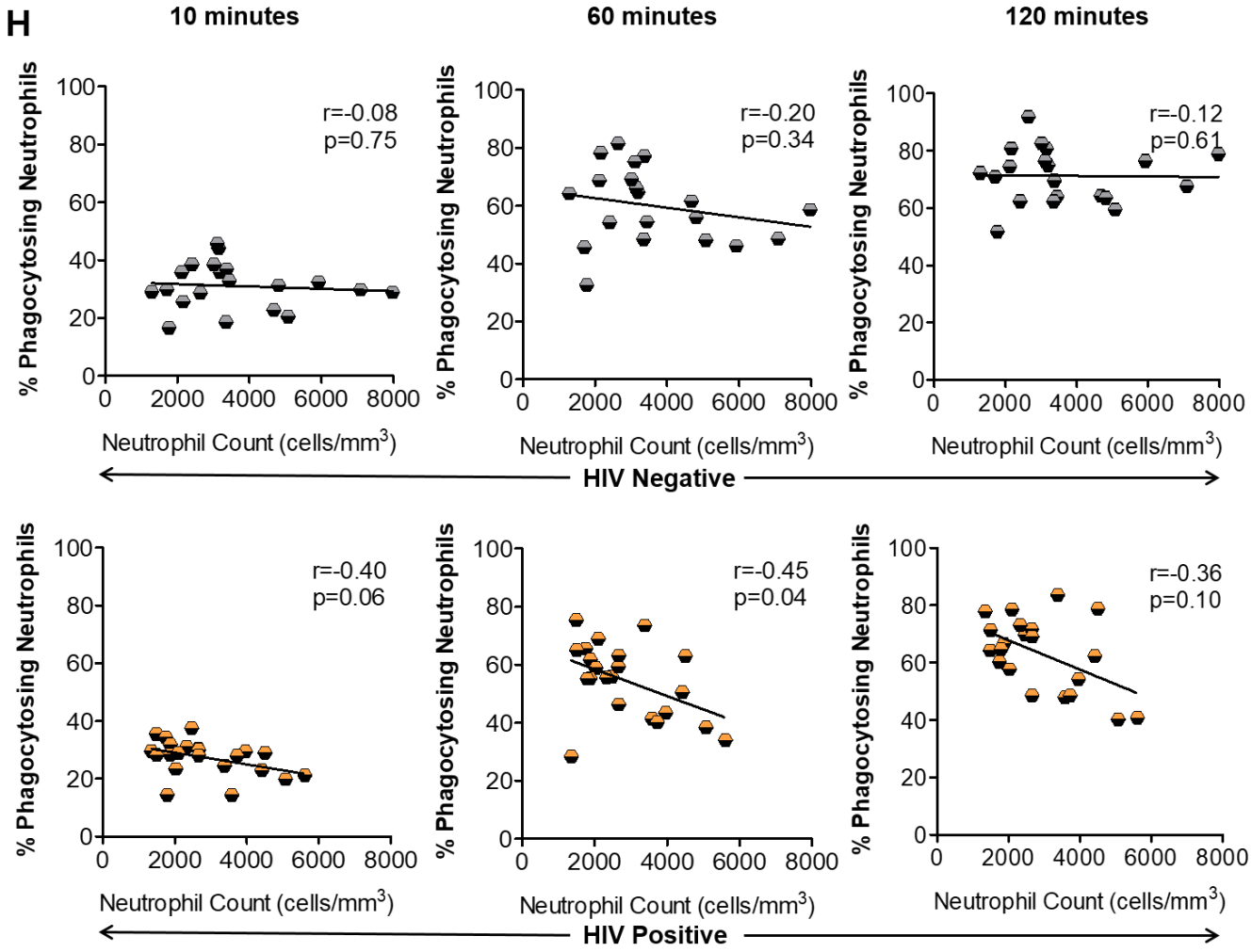


Figure 2.4 Neutrophil Phagocytosis (NP) in HIV negative and HIV infected individuals

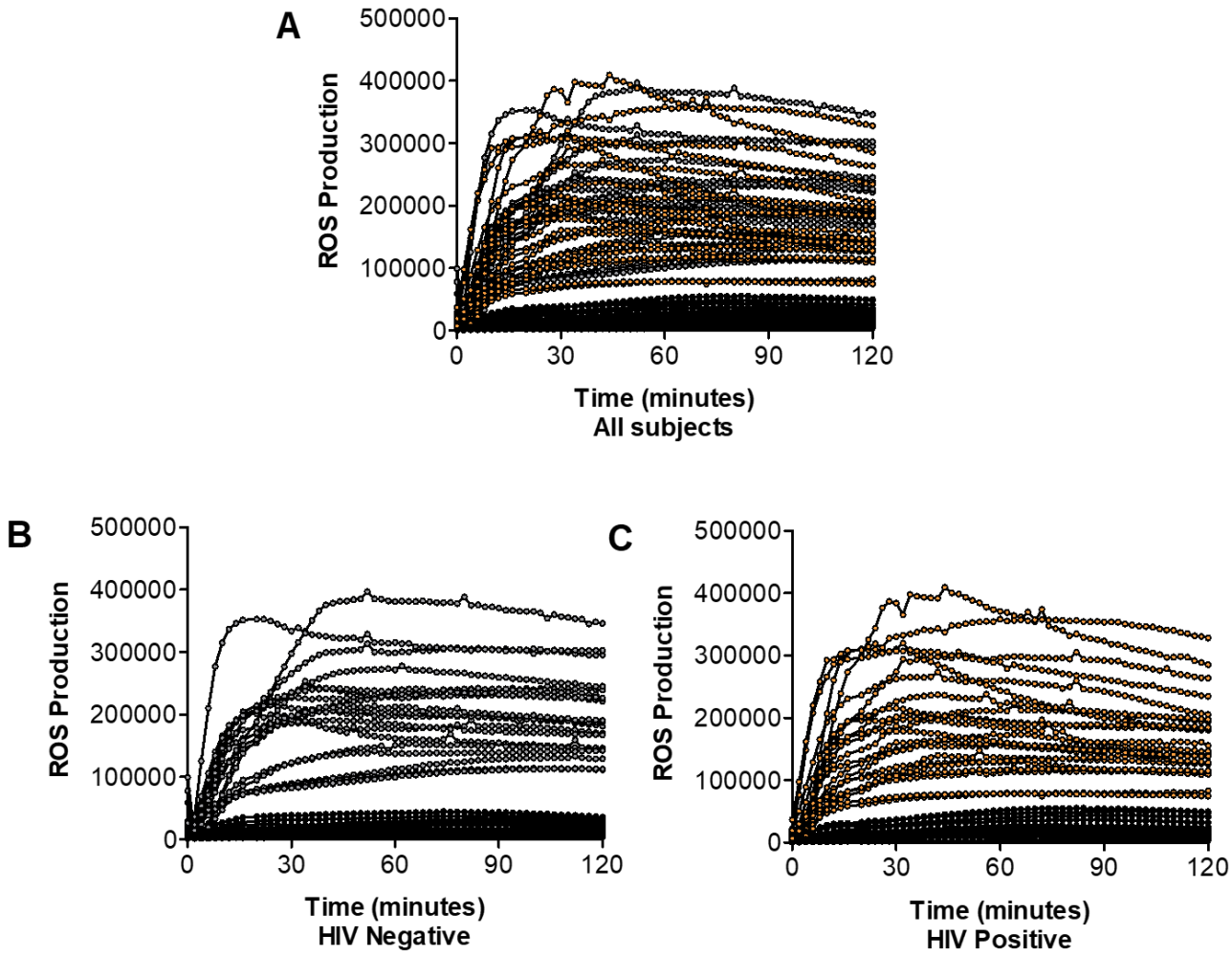
(A) Representative gating strategy employed for measuring phagosome maturation by flow cytometry. Background fluorescence was determined by acquisition of DQ Green-Bovine Serum Albumin (BSA) reporter beads alone. The gating strategy for neutrophils was determined by acquisition of neutrophils without DQ Green-BSA reporter beads. The gating strategy was applied to neutrophil samples incubated with DQ Green-BSA reporter beads for 10 minutes, 60 minutes and 120 minutes. Neutrophil phagocytosis (NP) was measured as DQ Green-BSA (Alexa Flour 488-A) positive. NP is shown for all **(B)**, HIV negative **(C)** and HIV-infected **(D)** subjects over all timed intervals. Dots indicate data points for each participant joined by lines over the 120 minute time interval. 1 way ANOVA was used to examine differences in neutrophil phagocytosis (NP) over time. The p-values refer to the differences in median NP across all timed intervals (i.e. 10, 60 and 120 minutes). **(E)** Comparison of NP by HIV status. The p-values refer to differences in NP between HIV negative and HIV-infected subjects. NP was further assessed by DARC status in HIV negative **(F)** and HIV-infected **(G)** individuals respectively. The p-values indicate differences in NP between DARC-null and DARC positive participants. Boxes extend from the 25th to 75th percentile and medians are indicated by the horizontal line. Whiskers indicate minimum and maximum values. **(H)** Correlations between NP and ANCs in HIV negative and HIV-infected individuals at 10 minutes, 60 minutes and 120 minutes. Data was examined by Spearman correlation analysis. Lines of best fit are indicated in correlation graphs. Dots indicate individual data points. Abbreviations: NP, Neutrophil Phagocytosis; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute Neutrophil Count; ns, p-value not significant; r, Spearman rho.

2.3.4 ROS Production is unaffected by DARC status

During the phagocytic process several enzymatic mechanisms are involved in microbial killing. Superoxide burst leads to the release of toxic compounds within the phagosome and is crucial to these processes. We next assessed the superoxide producing potential of neutrophils from study participants following in vitro PMA stimulation. Luminol oxidation measured by chemi-luminescence was used as an indicator of ROS production. Similar to other effector mechanisms, the ability of neutrophils to generate ROS has been shown to be compromised during HIV infection (Casulli and Elbim, 2014). Here, ROS release was observed within 10 minutes of PMA stimulation and reached peak secretion within 60 minutes irrespective of HIV status (Figures 2.5A, 2.5B and 2.5C, see Supplementary Figure 1 for averaged data). ROS production was higher in HIV negative compared to infected persons at 60 and 120 minutes (median luminol oxidation was measured at 9.04×10^6 at 60 minutes and 1.91×10^7 Relative Light Units (RLU) at 120 minutes compared to 8.59×10^6 at 60 minutes and 1.70×10^7 RLU at 120 minutes in HIV negative and infected individuals respectively). However statistical analysis showed no significant differences between HIV uninfected and infected individuals at 10, 60 or 120 minutes following stimulation (Figure 2.5D).

ROS production was further assessed in DARC-null compared to DARC-positive participants in HIV negative and HIV positive individuals respectively. In HIV uninfected individuals, median luminol oxidation was measured at 9.53×10^6 and 1.92×10^7 RLU in DARC-null compared to 8.74×10^6 and 1.91×10^7 RLU in DARC-positive persons at 60 and 120 minutes respectively. In infected individuals, median luminol oxidation was measured at 8.62×10^6 and 1.84×10^7 RLU in DARC-null compared to 7.26×10^6 and 1.50×10^7 RLU in DARC-positive persons at 60 and 120 minutes respectively. Statistical analysis displayed no significant disparities in overall superoxide release according to DARC status (Figures 2.5E and 2.5F) or by ANCs (Figure 2.5G) in uninfected or infected persons.

Taken together our data suggests that the potential of neutrophils to produce ROS is unaffected by HIV infection in this study population. This was unexpected as it was in contrast to previous reports of diminished ROS production in HIV infected individuals. Furthermore our results indicate that neither the absence of DARC nor lower ANCs (when neutrophil numbers are standardised) impact neutrophil ROS release.


A
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ROS Production
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60
90
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Time (minutes)
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400000
300000
200000
100000
0
ROS Production
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30
60
90
120
Time (minutes)
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ROS Production
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90
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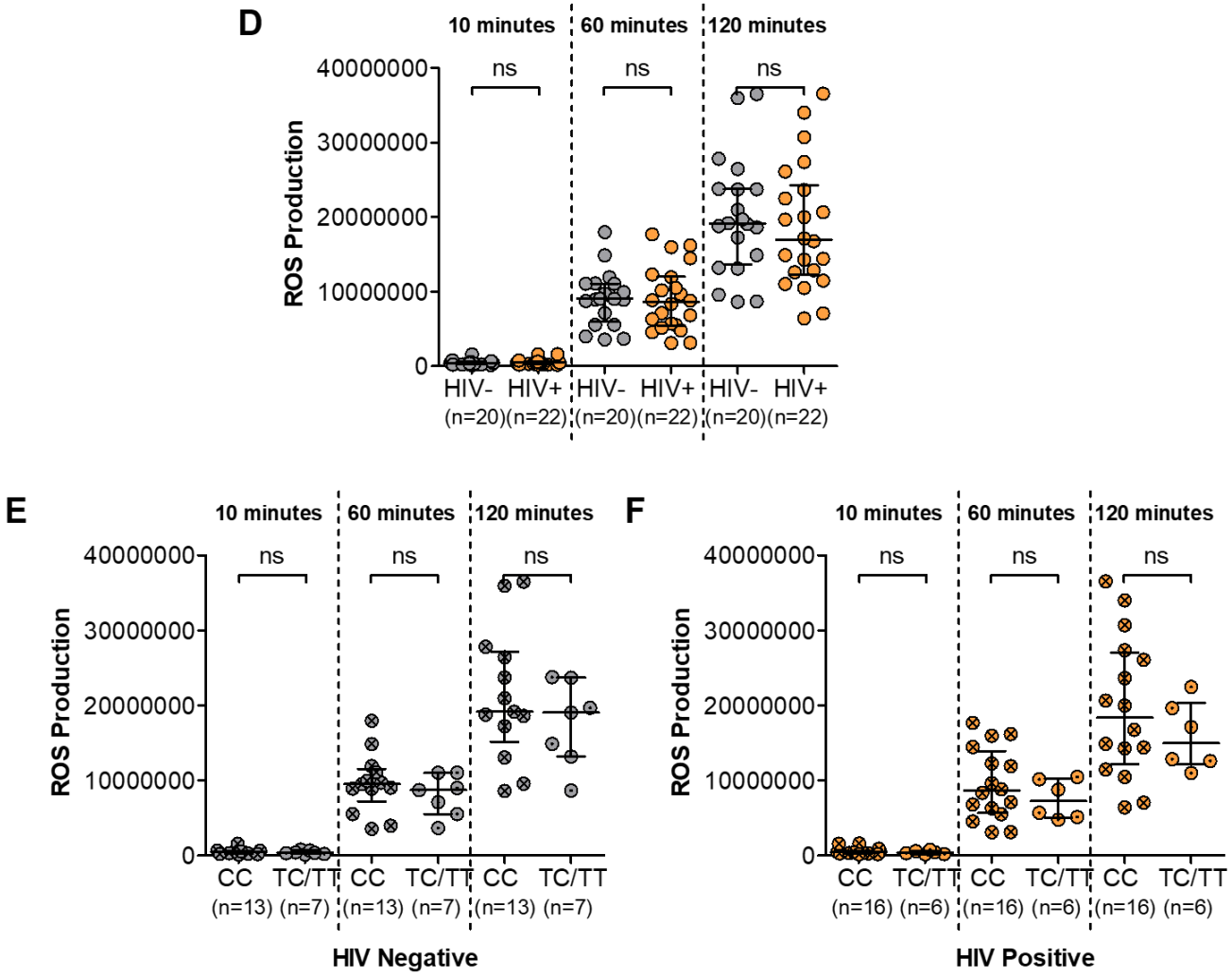
Time (minutes)
HIV Positive


D
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ROS Production
40000000
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HIV+
(n=20)
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HIV Negative
HIV Positive

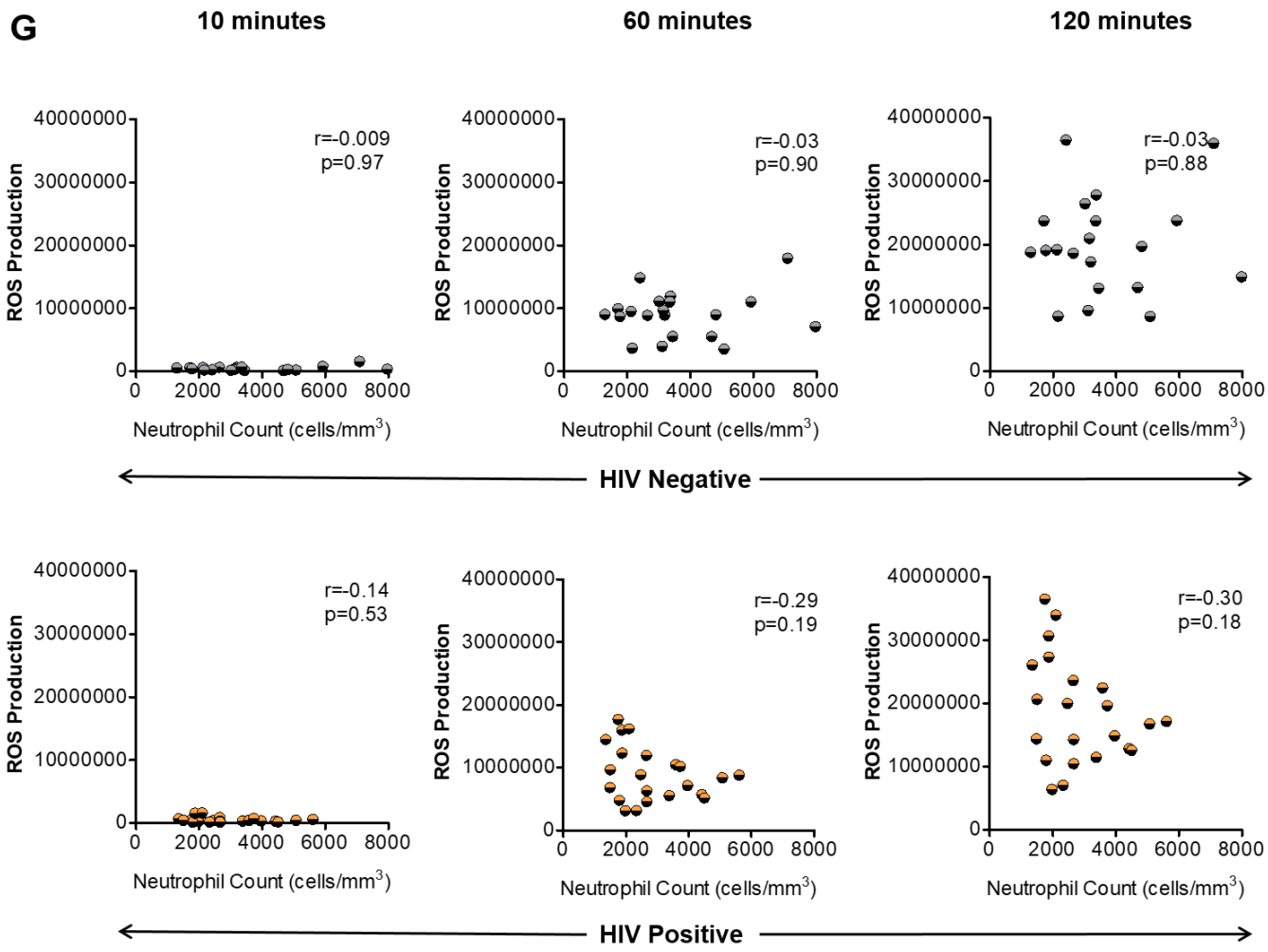


Figure 2.5 Neutrophil reactive oxygen species (ROS) release in HIV negative and HIV infected individuals

Neutrophil reactive oxygen species (ROS) production in all **(A)**, HIV negative **(B)** and infected **(C)** subjects as measured by chemi-luminescence every 2 minutes over a 120 minute time interval. Dots indicate data points for each participant joined by lines over the 120 minute time interval. Black dots indicate unstimulated samples. Stimulated samples are indicated by grey (HIV negative) and orange (HIV positive) dots. **(D)** Comparison of Neutrophil ROS production by HIV status. Neutrophil ROS production was further assessed by DARC status in HIV negative **(E)** and infected **(F)** individuals respectively. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences by HIV or DARC status. **(G)** Correlations between Neutrophil ROS production and ANCs in HIV negative and infected individuals at 10 minutes, 60 minutes and 120 minutes. Dots indicate individual data points. Abbreviations: ROS, reactive oxygen species; DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute Neutrophil Count; IQR, interquartile range; ns, p-value not significant; r, Spearman rho.

2.3.5 Prolonged exposure to stimulus leads to lower NET formation in DARC-null persons during HIV infection

The ability of neutrophils to form NETs in a process called NETosis is a relatively recent discovery (Brinkmann et al., 2004). It involves the release of genomic material together with antimicrobial factors in an effort to counter larger pathogens that evade phagocytosis (Branzk et al., 2014). Here we measured NETosis following neutrophil stimulation with PMA over a 120 minute period using fluorescent microscopy (Figure 2.6A). Data demonstrating NET quantification during HIV infection is lacking and also it is unknown whether DARC state impacts NET production. To address this gap, we hypothesised that NET production upon neutrophil activation would be altered in the absence of DARC and that this impairment would be more marked during HIV infection.

No significant differences in frequency of NET producing neutrophils were observed by HIV status in our study (Figure 2.6B). The median frequency of NET producing neutrophils was 27% at 60 minutes and 76% at 120 minutes in HIV negative individuals compared to 18% at 60 minutes and 71% at 120 minutes in HIV infected persons (Figure 2.6B).

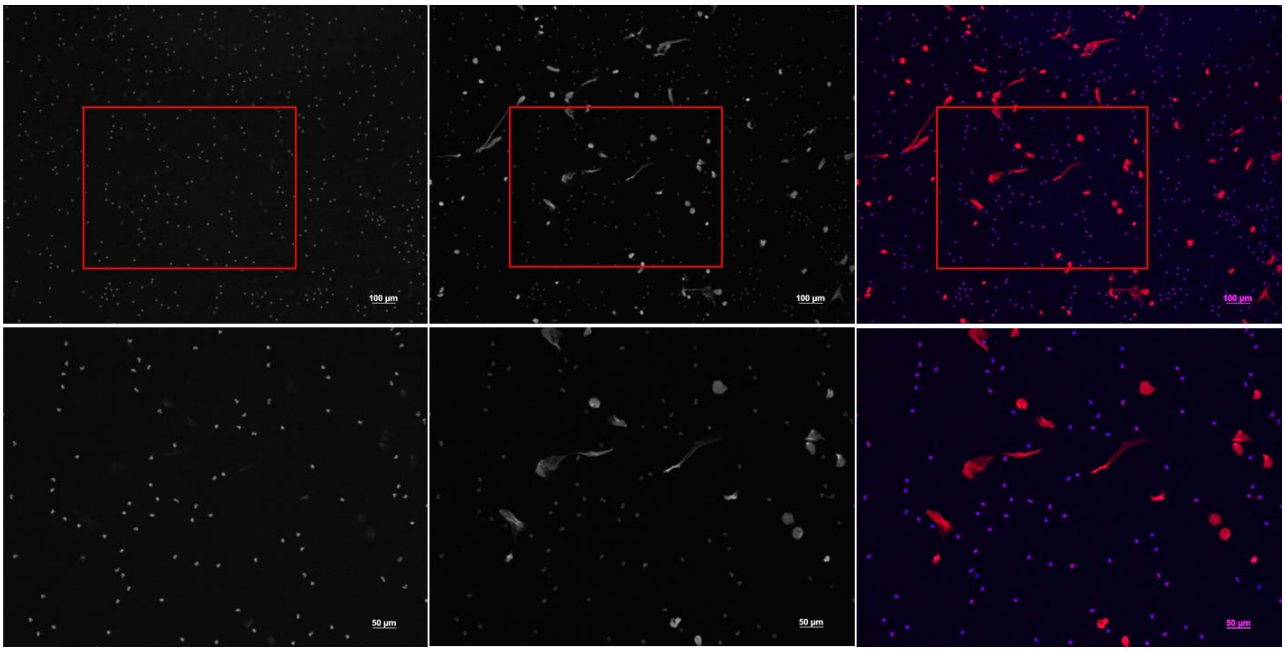
Further assessment by DARC genotype showed that the median frequency of NET producing neutrophils was 0.3% and 42% in DARC-null compared to 0.2% and 7% in DARC-positive persons at 10 minutes and 60 minutes post stimulation in HIV negative individuals respectively. In chronically infected persons, the median frequency of NET producing neutrophils was 1% and 20% in DARC-null compared to 0.03% and 11% in DARC-positive subjects at 10 minutes and 60 minutes post stimulation respectively. However, statistical analysis did not show any significant differences in NET production between DARC-null and DARC-positive individuals at 10 minutes or 60 minutes post stimulation irrespective of HIV status (Figures 2.6C and 2.6D).

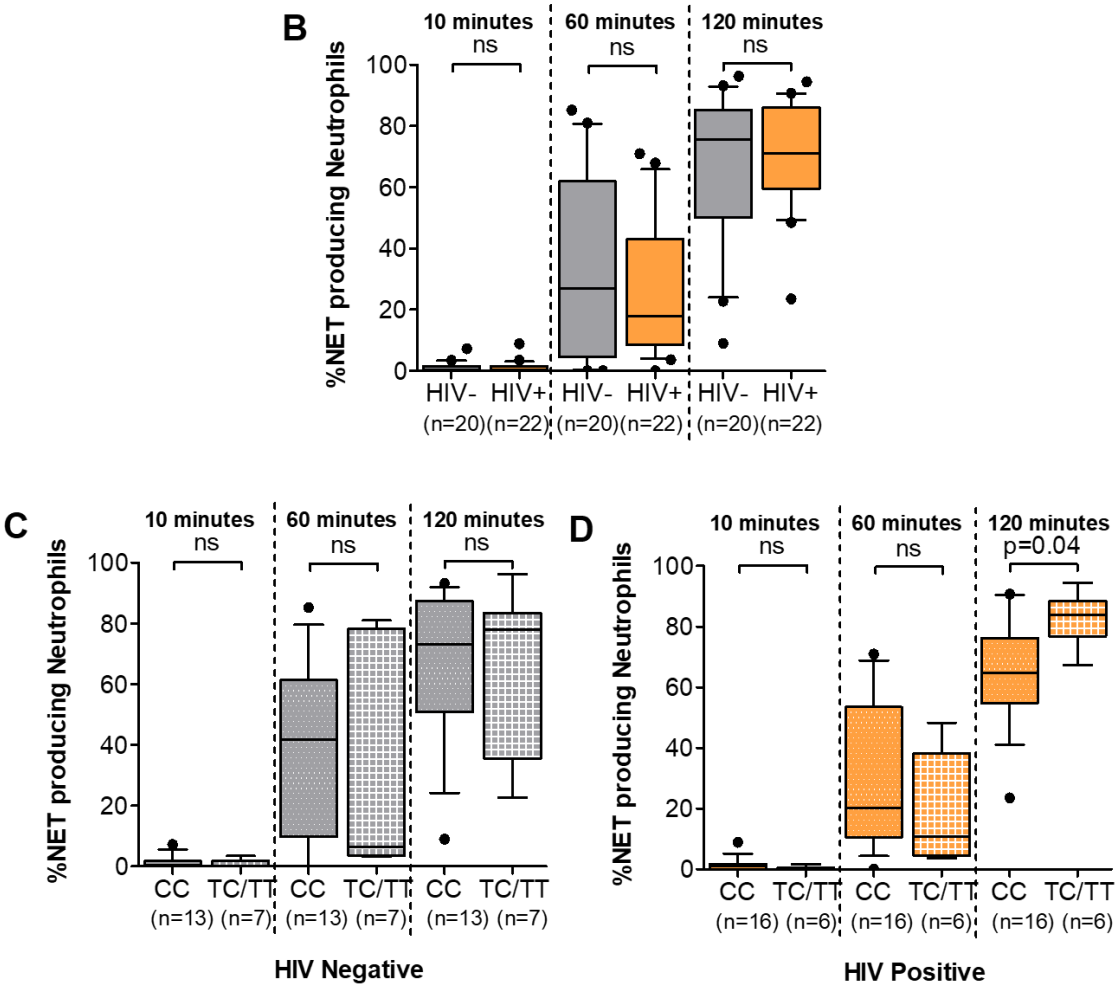
In contrast, following prolonged exposure to stimulus (120 minutes), DARC-null individuals had lower NET production medians compared to DARC-positive persons. Median frequencies of NET producing neutrophils was 73% in DARC-null compared to 78% in DARC-positive subjects in HIV negative individuals. During HIV infection, median NET production was 65% in DARC-null compared to 84% in DARC-positive individuals (Figures 2.6C and 2.6D). Moreover, overall examination by DARC status revealed significantly lower NET release in DARC-null chronically infected persons compared to their DARC-positive counterparts

(Figure 2.6D, p=0.04). Further analyses revealed no association between the frequency of NET producing neutrophils and ANCs regardless of HIV or DARC status (Figure 2.6E).

Taken together this data suggests that neutrophils are not significantly impaired in their ability to produce NETs during HIV infection. Neutrophils from DARC-null individuals are rapidly activated within the first hour and produce NETs in response to stimulus. However, during chronic HIV infection, prolonged activation of neutrophils may lead to impairment of NETosis activity in DARC-null individuals.

A

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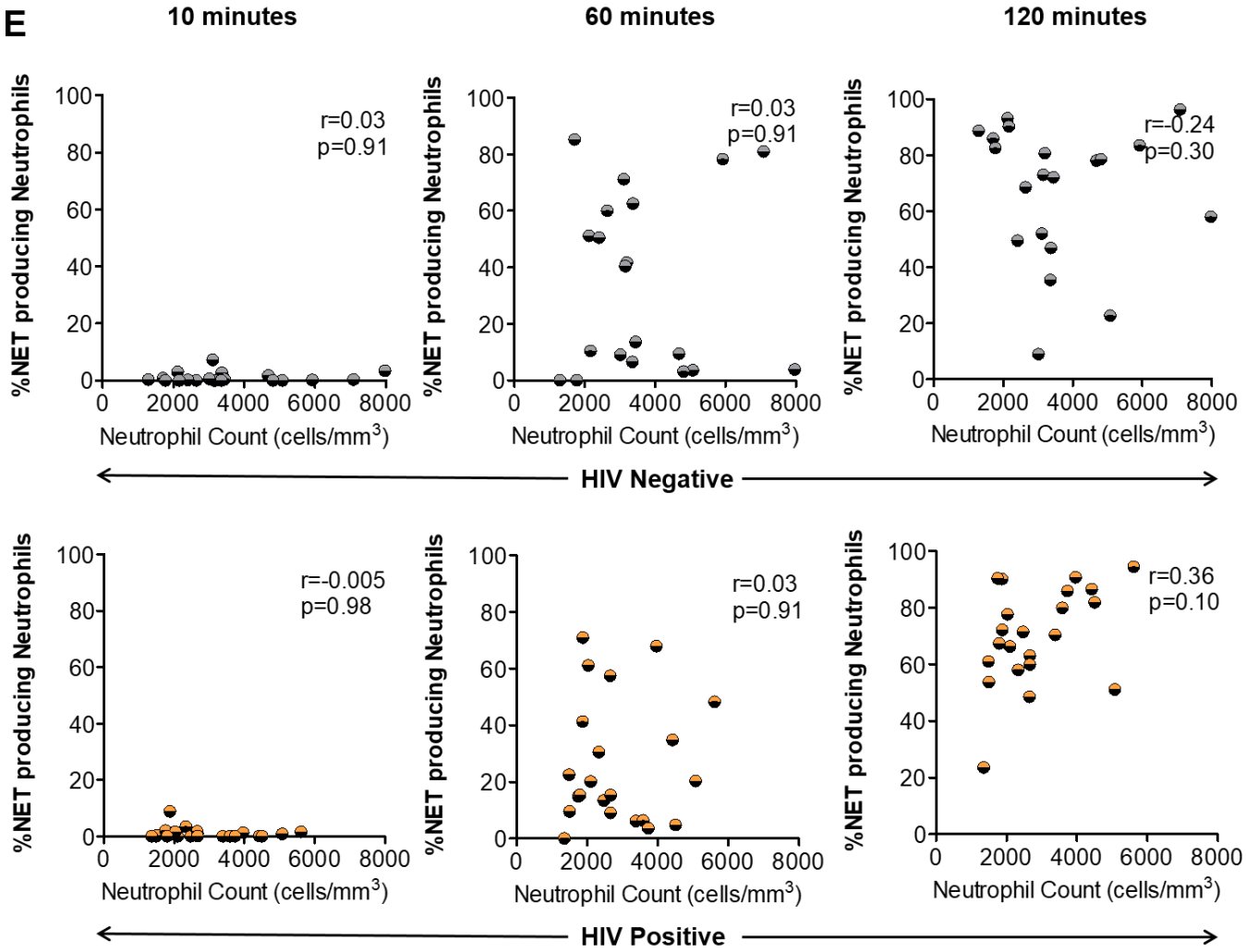


Figure 2.6 Neutrophil extracellular trap (NET) production in HIV negative and HIV infected individuals

(A) Representative microscopy images showing Hoechst (blue) and PL2-3 (red) staining at 10x and 20x magnification after 60 minutes post PMA activation. **(B)** Comparison of NET production by HIV status. The p-values refer to differences in NET production between HIV negative and HIV-infected subjects. Net production was further assessed by DARC status in HIV negative **(C)** and HIV-infected **(D)** individuals respectively. The p-values refer to differences in NET production between DARC-null and DARC-positive subjects. Boxes indicate the 10-90% data spread and whiskers extend to the interquartile range (IQR). Medians are indicated by the horizontal line inside the box. Dots indicate outliers. **(E)** Correlations between NET production and ANCs in HIV negative and infected individuals at 10 minutes, 60 minutes and 120 minutes. Abbreviations: NET, Neutrophil extracellular trap; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute Neutrophil Count; IQR, interquartile range; ns, p-value not significant; r, Spearman rho.

2.4 DISCUSSION

DARC-null linked neutropenia is associated with increased susceptibility to HIV acquisition. Interestingly, subsequent to infection, reports suggest a survival advantage imparted on leukopenic DARC-null persons in comparison to non-leukopenic DARC-null and DARC-positive individuals. However, the mechanisms involved in these associations are not fully elucidated. Here we investigated the influence of DARC on neutrophil functionality in HIV negative and therapy naïve HIV infected individuals in a DARC-null prevalent population from Durban, KwaZulu-Natal, South Africa.

The DARC-null polymorphism was highly prevalent in our studied cohort and we observed that ANCs were reduced in individuals with the DARC-null trait regardless of the HIV status. The functional capacity of neutrophils as assessed by three key effector functions, i.e. the ability to phagocytose, produce ROS and NETs demonstrated differential results. A trend of lower phagocytic capacity was observed in HIV infected individuals compared to HIV negative subjects; however, neither production of ROS or NETs were altered in HIV infection. Assessment of the neutrophil functions by DARC status, revealed higher phagocytosis activity in DARC-null compared to DARC-positive individuals, which was more pronounced in HIV infected than in HIV negative subjects. In contrast, a reduction in NET production was noted in HIV infected individuals with the DARC-null trait following 2 hours of *in vitro* stimulation.

The DARC-null trait is predominantly displayed in persons of African ancestry, and the absence of DARC has been associated with lower neutrophil counts in circulation (He et al., 2008, Reich et al., 2009). Assessment of HIV-1 chronically infected African individuals based in Durban indicated that 64.8% (247 of 381) of the patients were homozygous for the DARC-null allele (Julg et al., 2009), whilst a longitudinal study of high risk South African woman described that 64.7% (92 of 142) of their study subjects possessed the DARC-null trait (Ramsuran et al., 2011). We here observed comparable frequencies of the DARC-null variant (69%) within a similar population group of Zulu/Xhosa individuals from the Durban area. Furthermore, in accordance with previous reports, the DARC-null genotype was significantly associated with reduced neutrophil counts in studied individuals irrespective of HIV status, although this finding was substantially less prominent in chronic HIV infection. Accelerated neutrophil apoptosis is generally observed in HIV infection (Casulli and Elbim, 2014). Whether the effect of the DARC-null variant on neutrophil count is overshadowed by this accelerated apoptosis is unclear and requires further investigation.

There are conflicting reports of neutrophil responses in HIV-1 disease. While few studies have described an increase of key responses such as phagocytosis, oxidative burst and intracellular bacterial killing in HIV-1 infected patients compared to their HIV negative counterparts (Bandres et al., 1993, Schwartz et al., 2010), the general consensus suggests that infection leads to defective neutrophil responses and a higher rate of neutrophil apoptosis (Casulli and Elbim, 2014). Past studies have reported significantly impaired neutrophil phagocytosis and reduced superoxide burst in HIV-1 infected individuals (Casulli and Elbim, 2014). In addition, previous investigations have demonstrated the adverse effects of HIV infection on neutrophil phagocytosis of extracellular bacteria (Pos et al., 1992, Michailidis et al., 2012). Ingestion of *Staphylococcus aureus* was compromised in AIDS patients with CD4 counts below 200 cells/mm^3 compared to controls following 60 minutes incubation with opsonised bacteria (Pos et al., 1992). Furthermore, superior neutrophil functional responses were detected in patients with better immunological status and lower viral loads (Michailidis et al., 2012). While the mechanism for this altered activity remains unclear, reports of monocyte function suggest that HIV proteins impede phagocytosis through downregulation of the gamma signalling chain of the FcR (Kedzierska et al., 2002) and inhibit phagosome formation in a Nef-dependent manner (Mazzolini et al., 2010). In agreement with these reports, we observed lower FcR-mediated phagocytosis of opsonised beads in HIV infected persons compared to HIV negative subjects, although this difference was not significant ($p=0.08$ at 10 minutes and $p=0.06$ at 120 minutes).

We also found that HIV-infected individuals studied here did not display lower ROS production in comparison to uninfected individuals. Plasma RNA levels have been shown to be associated with reduced ROS production. Individuals with less than 1000 viral copies/ml have been shown to display no differences in oxidative metabolism to HIV negative persons. Reduced ROS release was detected in patients with more than 1000 viral copies/ml, while extreme ROS dysfunction was observed in patients with more than 10,000 viral copies/ml (Diz Dios et al., 2000). Interestingly, neutrophils from HIV negative subjects exhibited supressed release of ROS following priming with synthetic HIV-1 envelope peptide, signifying the direct role of HIV virions in the alteration of neutrophil functional responses (Munoz et al., 1999). Moreover, no significant modification of neutrophil ROS production was described in HIV-1 asymptomatic individuals with a CD4 count above 200 cells/mm^3 following neutrophil activation with PMA (Munoz et al., 1999).

Importantly, these previous reports noted that the severity of neutrophil impairment is dependent on the stage of HIV disease. Neutrophil dysfunction is more evident in the later

stages of infection in patients with high HIV-1 plasma concentrations and lower CD4 lymphocyte counts (Campbell, 1990, Mastroianni et al., 1999). Moreover, the prevalence of coinfection with opportunistic pathogens during advanced disease contributes to diminished neutrophil response (Shi et al., 2014). Chronically infected subjects that were studied here presented with low viral loads at the time of sample collection, 59% (13 of 22) had a viral load lower than 10,000 copies/ml and all HIV infected persons studied had a CD4 count above 300 cells/mm^3. Clinically, these data indicate that none of these individuals were in the later stages of disease, and could partly explain our observation of no significant differences in FcR-mediated phagocytic capacity or levels of ROS production between HIV negative and infected individuals.

Furthermore, our data suggests that NET release following PMA exposure was unaffected in HIV infection; it is plausible that variations in NET production due to HIV may not have been detected due to lower viral loads in our studied cohort. In contrast to other neutrophil functions, data on NET formation is lacking with regard to HIV infection. The role mediated by NET formation in preventing HIV transmission has been described (Jenne et al., 2013, Barr et al., 2018), however, whether this mechanism is modified following infection remains unclear.

Various genetic mutations have been associated with neutrophil disorders either in count and/or function including phagocytosis and superoxide release (Lakshman and Finn, 2001). It is well established that the absence of the DARC phenotype is associated with lower circulating ANCs characteristic of ethnic neutropenia. Yet the influence of this polymorphism on neutrophil function has been virtually unexplored. A recent study reported similar neutrophil gene expression in subjects with and without ethnic neutropenia, suggesting intact neutrophil function in individuals with low DARC expression (Charles et al., 2018).

We thus assessed the impact of the DARC-null polymorphism on neutrophil effector functions in our study cohort. We noted enhanced FcR-mediated phagocytosis in neutrophils from DARC-null individuals and this activity was not affected by HIV infection. Higher phagocytic activity was exhibited within the first hour following incubation with beads and thereafter no differences in bead uptake was exhibited between DARC-null and DARC-positive persons. Neutrophil phagocytosis through FcR engagement is characteristically very rapid. Uptake of IgG opsonised particles has been measured within minutes, and the rate at which neutrophils can efficiently phagocytose is a clear advantage in terms of host immunity (Herant et al., 2006, Mayadas et al., 2014). A recent report indicated that DARC knockout murine models exhibited

phenotypically distinct neutrophils. Notably, neutrophils from DARC-deficient mice displayed a higher expression of FcγR and CD45, which is known to enhance FcγR function (Duchene et al., 2017). Similarly, neutrophils characterised from HIV negative DARC-negative individuals were found to selectively upregulate FcγRIIIb (CD16) and CD45 expression in comparison to DARC-positive individuals (Duchene et al., 2017). Here we show higher FcR-mediated phagocytosing potential in neutrophils from DARC-null individuals, specifically at the earlier timed intervals. This is a probable consequence of augmented FcR expression and may be beneficial in the faster clearance of pathogens in DARC-null individuals.

Furthermore, we observed higher (although not significant) frequencies of neutrophils able to produce ROS and NETs in DARC-null individuals within the first hour following stimulation regardless of HIV status. Neutrophil activation progresses through multiple steps, and neutrophils are considered to be fully activated when they are able to fully function (i.e. induce phagocytosis, produce ROS and release NETs). Responsiveness to stimuli priming allows for rapid and efficient neutrophil activation (Mayadas et al., 2014). Higher frequencies of ROS and NET production in DARC-null individuals within the first hour could indicate a more rapid response to stimulus in DARC-null persons. The data may suggest that neutrophil activation is more easily induced in DARC-null persons, resulting in a higher frequency of cells with earlier responses to stimulus compared to DARC-positive individuals. Interestingly, in the absence of DARC, murine models displayed modified hematopoietic stem cell (HSC) differentiation and exhibited higher proportions of committed myeloid progenitor cells in the bone marrow (Duchene et al., 2017). Furthermore, genome expression indicated marginally activated neutrophil profiles in DARC-null individuals with benign ethnic neutropenia (Charles et al., 2018). Whilst leukocyte migration and HSC mobilisation pathways were most affected (Charles et al., 2018), this slightly activated state of neutrophils from DARC-null persons could contribute to rapid induction of neutrophil defence mechanisms.

While earlier functional responses (within the first hour) to stimulus were seemingly slower in neutrophils from DARC-positive individuals, prolonged stimulation (2 hours) resulted in neutrophil activity that was comparable to neutrophils from DARC-null neutrophils. An exception to this observation was a more robust NET response following prolonged stimulation in neutrophils from DARC-positive HIV infected individuals compared to their DARC-negative counterparts. Interestingly, this variation in NET release was not exhibited in HIV negative individuals by DARC state, suggesting that chronic inflammation during HIV infection may contribute to lower NET production in DARC-null HIV infected persons. It is

possible that the slightly activated neutrophil profile exhibited in the absence of DARC coupled with continued inflammatory signals displayed in HIV infection could induce faster neutrophil cell exhaustion and death in DARC-null persons. While diminished NET production would be unfavourable in clearing pathogens, it has been noted that disproportionate NET formation occurring in chronic activation elicits antibody-mediated autoimmune activity and organ damage (Chen and Nunez, 2010, Barnado et al., 2016). Thus lower NET formation in DARC-null chronically infected persons may be beneficial in averting excessive tissue damage in these individuals.

While the data presented here provides evidence of variation in neutrophil function in the absence of the DARC allele, we were limited by numerous factors. Neutrophils are a fragile cell subset and the four hour period between blood collection and processing was longer than the ideal conditions when assessing neutrophil activity; however, this time period was consistent for all samples. Furthermore, positive selection separation for the enrichment of neutrophils using the CD15+ MACS beads was not ideal and could have resulted in activation and altered phenotypic characteristics of the isolated neutrophils. However, this technique provided the best neutrophil purity levels compared to other approaches that were tested. The limitation was taken into account and all assays included a control sample to subtract background from possible activation during the isolation procedure. Other limitations included a small sample size and chronically infected patients with relatively lower viral loads than that typical of untreated HIV infected patients. Our observations may therefore not be representative of typical disease progression in the absence of antiretroviral therapy. Furthermore, many of our DARC-null subjects were not neutropenic as defined by the ANC threshold of <1500 cells/mm^3, and since these patients were not followed longitudinally we cannot determine which patients possessed neutropenic characteristics. Additional sample collection from chronically infected treatment naïve individuals became challenging during the study due to the global implementation of the HIV test and treat policy. Despite these limitations, we were able to identify variation in the neutrophil response, particularly with regard to phagocytosis. We noted differences in neutrophil phagocytosis as either an effect of HIV infection or lack of DARC. Although participants with identified ethnic neutropenia were scarce, we were able to provide evidence of the influence of the DARC-null polymorphism rather than lower ANCs on neutrophil effector functions irrespective of HIV status.

In summary, we addressed the impact of the DARC-null allele on three key neutrophil responses in persons recruited from an HIV-1 prevalent population. We focused on these three

functions as they are primary to the killing efficiency of neutrophils as a first line of host immunity. Beyond these effector functions, neutrophils have emerged as immunomodulatory cells that contribute to ongoing immune responses through either soluble protein secretion or direct interaction with other immune cells (Mayadas et al., 2014), and these mechanisms in the context of DARC require future attention.

To our knowledge this is the first study to assess the influence of the DARC-null polymorphism on neutrophil function in an HIV-1 setting. Our findings opposed our hypothesis of impaired neutrophil responses in the absence of DARC. Neutrophils from DARC-null individuals with lower ANCs were not functionally impaired. Interestingly, our data suggested that neutrophils displayed higher phagocytic activity in the absence of DARC which could suggest a compensatory mechanism for lower ANCs in these individuals. Higher neutrophil activity could explain the asymptomatic characteristics associated with ethnic neutropenia as demonstrated by higher neutrophil activity in individuals with DARC null trait regardless of HIV status. Furthermore, higher neutrophil phagocytosis detected in DARC-null individuals may serve as an advantage in chronic HIV infection, where a higher proportion of neutrophils able to rapidly phagocytose foreign pathogens could benefit DARC-null individuals in immune defence against opportunistic infection in stages of advanced disease. Overall, our data provide evidence that the DARC-null allele is not deleterious to neutrophil function in people of African descent.

CHAPTER 3: NATURAL KILLER (NK) CELL PROFILES IN HIV-1 INFECTED INDIVIDUALS WITH DUFFY ANTIGEN RECEPTOR FOR CHEMOKINES (DARC)-NULL LINKED GENOTYPE

3.1 INTRODUCTION

Natural killer (NK) cells represent a distinct class of innate lymphoid cells that are able to rapidly respond to tumour and virally infected cells without prior sensitisation (Lanier, 2005). NK cell responses are governed by counterbalance of signals from a diverse array of germline encoded activating and inhibitory receptors. Activation of NK cells leads to the initiation of effector mechanisms that include direct cytotoxicity through release of cytoplasmic granules, antibody dependent cellular cytotoxicity (ADCC) and production of soluble proteins that are involved in immune response modulation (Lanier, 2005, Vivier et al., 2008).

Substantial evidence suggests the imperative role of NK cells in HIV-1 protection (Lederman et al., 2010, Hens et al., 2016), and previous studies have highlighted the significance of these cells in the early containment and control of HIV-1 infection subsequent to transmission (Carrington and Alter, 2012). Furthermore, the impact of NK cells in HIV infection is supported by the evolution of viral strategies to evade NK cell mechanisms (Lisnic et al., 2010, Jost and Altfeld, 2012). Prolonged HIV-1 infection without antiretroviral therapy intervention is associated with several NK cell defects and impaired immune responses. Together these factors can influence the course of HIV-1 infection and disease outcome.

Neutrophils have emerged as immune-regulatory cells in recent years; amongst their functions is their ability to either directly or in cooperation with other cell types ensure terminal maturation and attainment of complete effector functionality in NK cells (Costantini et al., 2011a, Costantini et al., 2011b, Jaeger et al., 2012). Specifically, one such study demonstrated that neutrophil-deficient mice had NK cells that were impeded at an immature stage of development and were hypo-responsive. This key role of neutrophils as mediators of NK cell function was subsequently verified in severe congenital neutropenia (SCN) and autoimmune neutropenia (AIN) patients (Jaeger et al., 2012) where NK cell maturation and function were impaired in neutropenic patients.

Disorders that result in low neutrophil counts (below 500 cells/mm^3) are usually rare and contribute to severe immune complications (Hsieh et al., 2010). Contrary to this, a type of neutropenia – termed benign ethnic neutropenia – is common in the African population, occurring in 25-50% of persons of African ethnicity (Thobakgale and Ndung'u, 2014). The condition is characterised by repeatedly low absolute neutrophils counts (ANCs) below 1500 cells/mm^3, but is not associated with increased oral and systemic infections (Hsieh et al., 2010). The Duffy antigen receptor for chemokines (DARC)-null polymorphism is the principal

genetic determinant of benign ethnic neutropenia (Thobakgale and Ndung'u, 2014). The ailment was initially considered to have no adverse clinical consequences (Haddy et al., 1999). However, recent data has implicated the DARC-null trait and its linked neutropenia to several health issues including asthma, rheumatoid arthritis, cancer and transplant rejection (Ulvmar et al., 2011).

Individuals in possession of the DARC-null genotype and its associated neutropenia are reported to have an increased HIV-1 acquisition risk (He et al., 2008, Ramsuran et al., 2011). Furthermore, low neutrophil counts, independent of lymphocyte and monocyte counts, have been associated with an increased risk of mother-to-child HIV transmission (Kourtis et al., 2012). The effect of the DARC-null state on disease progression is less certain. Suggestions that the absence of DARC may predispose individuals to a survival advantage (Kulkarni et al., 2009) is debatable. Contrasting studies in HIV-1 chronically infected persons of African descent showed no association between the DARC-null allele and markers of HIV disease progression (Horne et al., 2009, Hesslein et al., 2011, Sun and Lanier, 2011, Julg et al., 2009).

Thus, while the implications of DARC-null linked neutropenia in HIV-1 acquisition and possibly disease progression have been described, the underlying mechanisms for this association remain unclear. Different mechanisms have been proposed; the low circulatory levels of neutrophils present in DARC-null neutropenic individuals could impact both the priming and functional capacity of NK cells, thus leading to an impaired ability of NK cells to mediate an effective response against HIV-1 resulting in adverse disease outcomes (Thobakgale and Ndung'u, 2014).

In this chapter we hypothesised altered neutrophil priming of NK cell in the presence of the DARC-null polymorphism that would result in modified NK cell phenotypic patterns and impaired NK cell functionality. We assessed the impact of DARC-null genotype and lower ANCs on NK cell profiles from HIV negative and HIV-1 chronically infected treatment naïve black South African individuals from Durban, KwaZulu-Natal in South Africa. In particular, we assessed NK cell maturation/differentiation characteristics and functionality in whole blood and cryopreserved peripheral mononuclear cells (PBMCs) from these individuals.

Overall we found that in HIV negative individuals, absence of DARC associated with lower NK cell numbers and higher frequencies of less differentiated NK cells. However, this immature phenotype did not amount to functional differences by DARC genotype. In contrast HIV-1 infected individuals displayed NK cell subset redistribution and higher activation which

was accompanied by lower cytolytic potential. However, these observed phenotypic and functional differences were lost upon stratification by DARC genotype.

3.2 METHODS

3.2.1 Participant Recruitment

HIV negative participants were recruited from the Females Rising through Education, Support and Health (FRESH) cohort, an acute HIV infection monitoring cohort that targets high risk women. HIV-1 subtype C chronically infected treatment naïve subjects were recruited from the HIV Pathogenesis Programme (HPP) Acute Infection Cohort, an acute-chronic HIV infection cohort. Both of these cohorts are located in the Umlazi region in Durban, KwaZulu-Natal (KZN), South Africa. Both studies attained ethical approval from the University of KwaZulu-Natal (UKZN) Biomedical Research Ethical Committee (BREC) (FRESH BREC Reference Number: BF131/11 and HPP Acute Infection Cohort BREC Reference Number: E036/06). All participants gave written informed consent to voluntarily participate in these studies. This particular study obtained further ethical clearance as a subsidiary of the above mentioned studies (BREC Reference Number: BE229/15). A total of 39 individuals sub-divided into HIV negative donors (n=20) and HIV-1 chronically infected individuals (n=19) were assessed. These individuals were further stratified by DARC status as described below and summarized in Table 3.1.

3.2.2 ANCs, CD4 Counts and Viral Load

Blood for full blood counts, CD4+ T cell count measurement and viral load quantification was collected in Ethylenediaminetetraacetic acid (EDTA) anticoagulated vacutainer tubes (Becton Dickinson (BD), Franklin Lakes, New Jersey, USA). ANCs were enumerated by full blood count using the automated XN 1000 Haematology Analyser (Sysmex, Kobe, Hyōgo, Japan). CD4 counts were measured using BD Trucount and analysed on a four-parameter FACS Calibur flow cytometer (BD). Viral loads were determined using the NucliSENS EasyQ HIV-1 v2.0 kit with a detection limit of 20 copies/ml (BioMérieux, Marcy-l'Étoile, France).

3.2.3 Quantification of Antiretroviral (ARV) Drug in Plasma

This study aimed to recruit HIV-1 chronically infected, treatment naïve individuals. ARV therapy usage in chronically infected patients was self-reported. Certain subjects maintained viral loads below 1000 RNA copies/ml at the time of assessment. To rule out ARV drug use, plasma samples were collected from these study participants and screened for ARV drugs using a quantitative liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

method. The method screened for nine ARVs, namely Emtracitabine, Tenofovir, Lopinavir, Ritonavir, Nevirapine, Abacavir, Lamivudine, Zidovudine and Efavirenz. A plasma sample volume of 50 µl was processed using a protein precipitation method, ARV drug analytes were chromatographically separated on a Agilent Zorbax Eclipse Plus C18 (2.1 x 50mm, 3.5 µm) HPLC column (Agilent Technologies, Santa Clara, California, USA), detected using an AB Sciex 5500 triple quadrupole mass spectrometer (Sciex, Framingham, Massachusetts, USA) and quantitated using Analyst® 1.6.2 software (Sciex).

3.2.4 DARC Genotyping

DARC -46T→C (rs2814778) single-nucleotide polymorphism (SNP) genotyping was performed by TaqMan allelic discrimination assays which has been previously verified by direct sequence analysis by Julg et al. (2009). Briefly, genomic DNA was isolated from stored buffy coats using the QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. DNA concentration was standardised at 50ng/µl with polymerase chain reaction (PCR) grade water. A cocktail containing Taqman Genotyping master mix (Life Technologies, Carlsbad, California, USA) and predesigned probes for the DARC gene (SNP ID: rs2814778, Applied Biosystems, Foster City, California, USA) was used to amplify target sequence in 50ng genomic DNA by real time PCR (RT-PCR) in the LightCycler 480 (Roche, Basel, Switzerland) according to the manufacturer's protocol.

3.2.5 PBMC Isolation, Cryopreservation and Thawing

Blood collected in sodium heparin BD Vacutainers (BD) from each participant was processed within 6 hours of collection. One millilitre (1ml) of blood was reserved for the whole blood assays, and the remainder was used for lymphocyte fraction preparation by density centrifugation on a Histopaque-1119 cushion (Sigma-Aldrich, St. Louis, Missouri, USA) at 800 x g for 20 minutes, low brakes as previously described (Brinkmann et al., 2010).

Cell counts were determined by 1:5 dilution with Trypan Blue Stain (Gibco) counted using a haemocytometer under a light microscope. Cells were prepared for cryopreservation by refrigerated centrifugation and re-suspended at a final concentration of 10 million cells/ml in ice cold freezing solution containing 1:10 Dimethyl Sulfoxide (DMSO) (Merck Millipore, Billerica, Massachusetts, USA) and Foetal Bovine Serum (FBS). The frozen samples were stored in 2ml cryogenic vials in a liquid nitrogen Biorack ultralow freezer for long term storage

and subsequently batched for phenotypic and intracellular staining (ICS) assays. Cell viability was not statistically different by HIV or DARC status. Median viability was 92% in HIV negative and 88% in HIV infected individuals (p=0.12). By DARC status, median viability in HIV negative individuals was 91% and 94% in DARC-null and DARC-positive individuals respectively (p=0.82), and in HIV infected individuals was 88% and 84% in DARC-null and DARC-positive individuals respectively (p=0.59).

Cryopreserved cells were rapidly thawed, washed twice in pre-warmed R10 medium (RPMI Medium 1640 (Gibco) containing 10% gamma irradiated, heat inactivated FBS (Gibco), 1% L-Glutamine, 1% Penicillin Streptomycin and 1% Hepes buffer 1M (all from Lonza, Basel, Basel-Stadt, Switzerland) at 500 x g for 8 minutes at room temperature. Cells were rested in R10 medium in a 37°C, 5% CO_2 incubator for at least 2 hours. Sample viability and cell counts were determined by trypan blue exclusion, and cells were re-suspended at 10 million cells/mL in R10 medium for subsequent assays.

3.2.6 PBMC Ex vivo phenotyping and in vitro ICS staining

All PBMC staining was performed in 96-well plates. Each staining / fixation incubation was for 20 minutes at room temperature in the dark, followed by washing with Dulbecco's phosphate-buffered saline (dPBS) and centrifuged at 850 x g for 6 minutes. For phenotype staining, one million cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen, Carlsbad, California, USA) to exclude dead cells, followed by cell surface staining with the following monoclonal antibodies: anti-CD3 PE-CF594 (clone:UCHT1), anti-CD14 V500 (clone:M5E2), anti-CD19 V500 (clone:HIB19) (to exclude of T-cells, monocytes and B-cells respectively), and NK cell markers Alexa-Fluor-700 conjugated anti-CD56 (clone:B159) and anti-CD16 APC-Cy7 (clone:3G8, all from BD Biosciences). In addition, to assess activation and differentiation, cells were stained with anti-CD69 fluorescein isothiocyanate (FITC (clone:FN50, BD Biosciences), anti-CD38 Phycoerythrin (PE), (clone:HB7 BD Biosciences) anti-HLA-DR Brilliant Violet (BV) 711 (clone:L243 Biolegend, San Diego, California, USA), anti-CD27 BV605 (clone:O323 Biolegend), anti-CD11b PE-Cy5 (clone:ICRF44), anti-PD-1 BV421 (clone:EH12.2H7 Biolegend) and anti-CD57 APC (clone:NK-1, BD Biosciences), before addition of fixation Medium A (Invitrogen, Carlsbad, California, USA) which ensures that the morphological characteristics of the cells remain intact.

For *in vitro* functional assays: 1 million cells were assessed for cytokine production and degranulation capacity in the presence of degranulation marker anti-CD107a PE-Cy5 (clone:H4A3, BD Biosciences), BD GolgiStop protein transport inhibitor (GolgiStop, BD Biosciences) and Brefaldin A (Sigma-Aldrich) at a final concentration of 10µg/ml with either R10 medium alone, Recombinant Human Interleukin (IL)-2, Recombinant Human IL-15 (both from R&D Systems, Minneapolis, Minnesota, USA), and Recombinant Human IL-18 (Medical & Biological Laboratories, Japan) all at a final concentration of 10ng/ml alone, K562 cell line alone (effector:target ratio of 10:1), or both for 18 hours at 37°C, 5% CO_2.

Following overnight culturing, samples were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain followed by surface staining with anti-CD3 PE-CF594, anti-CD14 BV650 (clone:M5E2), anti-CD19 BV650 (clone:HIB19, both from Biolegend), Alexa-Fluor-700 conjugated anti-CD56 and anti-CD16 APC-Cy7 as described above and anti-CD158a PE (clone: HP-3E4), anti-CD158b PE (clone:CH-L, both from BD Biosciences), anti-CD158e1/e2 PE (clone:) and anti-NKG2A APC (clone: Z199, both from Beckman Coulter), followed by fixation with Medium A and ICS staining with antibody cocktail containing cytokine markers anti-Tumor necrosis factor (TNF)-α PerCP-Cy5.5 (clone:Mab11) and anti-Interferon gamma (IFN-ɣ) PE-Cy7 (clone:B27, both from Biolegend) in the presence of permeabilisation buffer Medium B (Invitrogen).

3.2.7 Whole Blood Cytokine and Degranulation Assay

Simultaneous to PBMC isolation, the frequency of CD107a positive and IFN-ɣ producing NK cells in whole blood was determined following HIV-1 peptide stimulation as previously described (Thobakgale et al., 2012). The whole blood assay was used to assess antibody mediated NK cell activity following stimulation with HIV peptides and therefore requires the presence of plasma which would be absent in PMBCs. In brief, 200µl of whole blood was stimulated with either gp120 or gp41 HIV-1 peptides at a final concentration of 2µg/ml, in the presence of 1µg co-stimulatory anti-human CD28/CD49 antibodies (BD Biosciences, San Jose, California, USA). A negative control with whole blood alone and a positive control with whole blood stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin at a final concentration of 1µg/ml and 0.5µg/ml respectively were included for each participant sample. Brefaldin A at a final concentration of 10µg/ml, 1µl of a 1:10 dilution of GolgiStop to R10 and degranulation marker anti-CD107a PE-Cy5 were added to each sample before incubation at

37°C, 5% CO_2 for 6 hours. After incubation, 20µl of EDTA was added to arrest activation and remove adherent cells, before lysis of erythrocytes with BD FACS lysing solution and permeabilisation of leucocytes was done with BD FACS permeabilising solution (all from BD BioSciences). Cells were washed and stained with an antibody cocktail containing anti-CD3 PECy5.5 (clone:7D6), anti-CD14 Pacific Blue (clone:TuK4), anti-CD19 Pacific Blue (clone:SJ25-C1) (all from Invitrogen), anti-CD56 Alexa-Fluor-700, anti-CD16 APC-Cy7 and anti-IFN-ɣ PE-Cy7 for 1 hour and fixed with Medium A before data acquisition.

3.2.8 Description of NK cell markers

CD56 or Neural-Cell Adhesion Molecule (NCAM) is a homophilic binding glycoprotein. Although CD56 is typically used as an NK cell marker, the function of CD56 on NK cells is still unknown (Van Acker et al., 2017).

CD16 or FcγRIII, is found on the surface of several leukocytes and participates in signal transduction. The CD16 molecule on NK cells is involved in triggering antibody-dependent cellular cytotoxicity (ADCC) (Vivier et al., 2008).

NK cell subsets are defined by the relative expression of CD56. The fully mature CD56 dim subset (CD56+CD16+) is potently cytotoxic and generally comprises about 90% of total circulatory NK cells in healthy individuals. The CD56 bright cells (CD56+CD16-) are considered as an immature subset with cytokine secreting potential that account for <10% of the NK cell population (Cooper et al., 2001, Lanier, 2005). Chronic HIV infection is associated with an accumulation of the aberrant CD56 negative subset (CD56-CD16+) (Jost and Altfeld, 2012).

CD11b is an integrin family member that is expressed on the surface of many leukocytes. Functionally, CD11b mediates inflammatory responses through regulation of leukocyte adhesion and migration (Rosetti and Mayadas, 2016).

CD27 is a costimulatory receptor belonging to the TNF family and is constitutively expressed on naive T and B cells and on NK cells (Grant et al., 2017).

CD11b/CD27 NK cell maturation as proposed by Fu and colleagues (2011) suggests that four populations defined by CD11b and CD27 expression (CD11b-CD27-; CD11b-CD27+; CD11b+CD27+ and CD11b+CD27-) represents distinct development stages of NK cells with

varying functional capabilities. At the least mature stage, NK cells exhibits a CD11b-CD27- phenotype. Through stepwise acquisition of CD27 and CD11b, NK cells progress through intermediate development stages which are defined as CD11b-CD27+ and CD11b+CD27+. Finally loss of CD27 demarcates full maturation to the CD11b+CD27- phenotype. Development stages based on CD11b and CD27 expression have been suggested to functionally correspond to CD56 bright and CD56 dim subsets, where CD11b-CD27+ and CD11b+CD27+ intermediate phenotypes correspond to the cytokine producing CD56 bright subset and the fully mature CD11b+CD27- phenotype corresponds to the potently cytolytic CD56 dim subset (Fu et al., 2011).

CD158a, CD158b and CD158e1/e2 are inhibitory killer-cell immunoglobulin-like receptors (KIRs) with long cytoplasmic domains. Recognition and binding of KIRs to HLA class I molecules on self-cells triggers inhibitory signals via immune tyrosine-based inhibitory motifs (ITIMs) which results in suppression of NK cell activity (Lanier, 2005).

NKG2A belongs to the CD94/NKG2 family of C-type lectin receptors. NKG2A is an inhibitory receptor with long cytoplasmic domains. Recognition and binding of NKG2A to HLA-E on self-cells triggers inhibitory signals via ITIM which results in suppression of NK cell activity (Lanier, 2008).

The **KIR/NKG2A model** described by Beziat et al. (2010) commences with the CD56 bright subset that highly express NKG2A and few KIRs, transitioning through intermediate phases to the terminally differentiated KIR+NKG2A- subset. Based on a stepwise loss of NKG2A and the correlated acquisition of KIRs, the model thus forms a basis to predict the functional fate of NK cells. Each phenotypic stage of differentiation is characterised by unique functional attributes where KIR-NKG2A+ cells respond by cytokine secretion, KIR+NKG2A+ and KIR+NKG2A- cells are dominantly cytotoxic, whilst KIR-NKG2A- are regarded as hypo-responsive cells (Beziat et al., 2010).

CD57 is expressed as a carbohydrate epitope on the surface of NK cells. Increased CD57 expression defines terminal differentiation of peripheral NK cell maturation and is an indicator of potently cytotoxic NK cells with impaired sensitivity to cytokine stimulus and diminished replicative capacity (Nielsen et al., 2013).

CD69 is a human transmembrane C-Type lectin protein that acts as an immune regulator. It is induced shortly after NK cell activation with a potential role in triggering cell cytotoxicity (Fogel et al., 2013).

HLA-DR is an MHC class II cell surface receptor. HLA-DR presents peptide antigens to immune cells. Increased **HLA-DR** expression is a marker of prolonged activation typically seen in chronic infection (Fogli et al., 2004).

Upregulation of **PD-1** antigen expression is commonly associated with NK cell dysfunction and is routinely used as a marker of cell exhaustion (Beldi-Ferchiou et al., 2016).

CD107a is expressed primarily across lysosomal membranes. Cell surface expression of CD107 has been identified as a marker for granule release (i.e. degranulation) on NK cells (Alter et al., 2004a).

IFN-ɣ is a dimerised soluble immune-stimulatory cytokine. It is produced predominantly by NK and cytolytic T cells and is an important activator of macrophages and inducer of class II MHC molecule expression. IFN-ɣ is also able to inhibit viral replication directly (Schoenborn and Wilson, 2007).

TNF-α is a cell signalling cytokine produced by various immune cells and is involved in systemic inflammation. TNF-α is able to induce fever and cellular apoptosis; and inhibit tumorigenesis and viral replication (Pfeffer, 2003).

3.2.9 Sample Acquisition and Statistical Analysis

Samples were acquired on an LSR Fortessa flow cytometer (BD). Daily routine instrument QC was performed by the flow cytometry administrator using Cytometer Setup and Tracking beads (BD Biosciences). Compensation was performed for each experiment using the Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (BD Biosciences). A compensation tube for each panel antibody was prepared as follows; one drop of anti-mouse Ig, κ and one drop of negative control beads were mixed in a 5ml round bottom polypropylene tube and stained with the antibody of interest for 20 minutes. Compensation tubes were stored in the dark at 4°C until acquisition. Compensation was calculated using FACS Diva Software (BD) on the experiment template used to acquire experimental samples. At least 250 000 events per sample were recorded. Fluorescence minus one (FMOs) were used in each experiment to exclude

background fluorescence in the gating strategies for each activation/differentiation marker. FMOs were prepared by staining a PBMC sample with a mastermix containing all the antibodies of the phenotype panel except the activation/differentiation marker of interest. For ICS assays, degranulation and cytokine frequencies were examined after background subtraction from unstimulated control. FlowJo Software Version 9 (TreeStar, Inc., Ashland, Oregon, USA) was used for sample analysis. Differences between studied groups was examined by Mann-Whitney U test using GraphPad Prism Version 5 software (GraphPad software Inc., La Jolla, California, USA). Differences with a p value <0.05 were considered statistically significant.

3.3 RESULTS

To determine whether the DARC-null trait and the associated ethnic neutropenia modifies NK cell phenotypic characteristics and functional responses, we examined frequencies, maturation and differentiation profiles, cytolytic activity and cytokine production of NK cells in Zulu/Xhosa individuals. A total of 39 individuals were assessed, of which 20 individuals were HIV-uninfected (DARC-null=12 and DARC-positive=8), and 19 individuals were HIV-1 chronically infected (DARC-null=10 and DARC-positive=9) (Table: 3.1).

All individuals but one were female, with a median age of 21, interquartile range (IQR) (19-22) and 23 IQR (20-24) years in HIV negative and HIV infected patients respectively. Thirty nine individuals were assessed in this chapter, of which 22 (56%) were found to display the DARC-null trait. ANCs by HIV status did not differ significantly ($p=0.08$, data not shown). ANCs were significantly lower in DARC-null compared to DARC-positive individuals in both HIV negative ($p=0.0004$) and HIV-1 infected ($p<0.0001$) subjects. CD4 counts were significantly lower in HIV infected persons compared to HIV negative individuals ($p=0.003$, data not shown). Assessment by DARC status showed no differences in CD4 counts in HIV negative subjects (DARC-null = median CD4 count of 883 cells/mm^3; DARC-positive = median CD4 count of 919 cells/mm^3) or infected individuals (DARC-null = median CD4 count of 608 cells/mm^3; DARC-positive = median CD4 count of 709 cells/mm^3, Table 1). Further to this, no significant differences were observed in viral loads by DARC genotype in infected individuals (DARC-null = median viral load of 9300 RNA copies/ml; DARC-positive = median viral load of 7200 RNA copies/ml, Table 3.1).

Table 3.1 Clinical Characteristics of Study Participants used to assess NK cell Phenotype and Function

	HIV Negative			HIV Positive		
	DARC CC (n=12)	DARC TC/TT (n=8)	p value	DARC CC (n=10)	DARC TC/TT (n=9)	p value
Absolute Neutrophil Count, 10^3 cells/mm^3	**2.59** (2.13-3.39)	**4.99** (4.48-5.73)	0.0004	**1.75** (1.59-2.21)	**4.34*** (3.27-5.45)*	<0.0001
CD4 count, cells/mm^3	**883** (720-981)	**919** (693-1066)	0.7	**608** (487-759)	**709** (533-845)	0.44
Viral Load, RNA copies/ml	na	na	na	**9300** (3275-23250)	**7200** (750-70500)	0.9

Data is represented as median (IQR). *ANC median and IQR was calculated from 8 participants (ANC was not available for 1 participant). Abbreviations: DARC, Duffy Antigen Receptor for Chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute Neutrophil Count; IQR, Interquartile range; n, number; na, not applicable. Female participants: n=38, Male participants: n=1. Recruitment of HIV negative participants was from an all-female cohort, thus we had no male HIV negative participants. While both males and females were recruited in our HIV positive cohort, almost 83% of patients enrolled were females (183 of 221 patients). Samples were collected from individuals that were recruited / presented at the clinic during sample collection and met the criteria of not having initiated antiretroviral treatment. Thus while recruitment of HIV participants was unbiased, we did have unequal gender distribution in this group.

3.3.1 Marked reduction of total NK cell counts in DARC-null HIV negative individuals

To gain insight of the circulating NK cell population in the context of DARC-null persons, NK cell frequency and subset composition were assessed. NK cells were quantified based on the absence of CD3, CD14 and CD19 and the presence of CD56 and/or CD16 surface markers in total viable lymphocytes. NK cells were further defined by the relative expression of CD56 and CD16 into three subsets, namely CD56+CD16- (CD56 brights), CD56+CD16+ (CD56 dims) and CD56-CD16+ (CD56 negatives) subsets (Figure 3.1A).

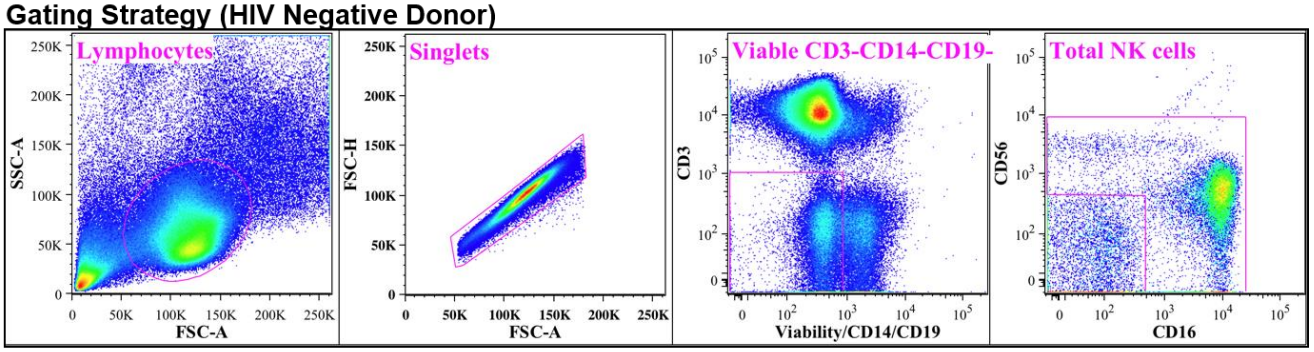
NK cells typically comprise 2-18% of total circulating lymphocytes (Vivier et al., 2008). As a proportion of lymphocytes, total NK cell frequencies observed here were comparable between HIV negative and HIV-1 chronically infected persons (median frequency of 4.76% and 3.39% respectively, Figure 3.1B). Subsequent analysis by DARC status indicated strikingly lower total NK cell frequencies in DARC-null HIV negative individuals compared to DARC-positive HIV negative individuals ($p=0.006$, Figure 3.1C). Total NK cell frequencies also strongly correlated with ANCs in HIV negative individuals ($p=0.002$, Figure 3.1E). Assessment of HIV-infected individuals showed no differences in total NK cell frequencies when grouped by DARC status or ANCs (Figure 3.1D and Figure 3.1F).

NK cells are phenotypically and functionally diverse and can be characterised as CD56 dim (CD56+CD16+), CD56 bright (CD56+CD16-) or CD56 negative cells (CD56-CD16+) (Caligiuri, 2008). In accordance with other studies our data indicated higher frequencies of the CD56 dim population in HIV negative individuals compared to HIV-infected donors ($p=0.01$, Figure 3.1G), and elevated CD56 negative NK cell subset counts were observed in the HIV-infected group ($p=0.005$, Figure 3.1G). Although NK cell subset frequencies were not significantly different when stratified by DARC genotype irrespective of HIV status (Figure 3.1H and Figure 3.1I), a weak trend was noted towards higher CD56 bright frequencies in DARC-null compared to DARC-positive HIV negative donors ($p=0.08$, Fig 3.1H). In addition, a negative correlation was observed between CD56 bright frequencies and ANCs in HIV negative individuals ($p=0.05$, data no shown).

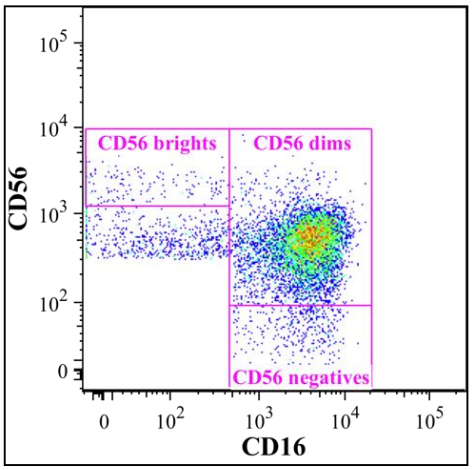
Taken together, these data indicate that the DARC-null trait and lower ANCs are associated with reduced peripheral NK cell counts in HIV negative persons. Furthermore, higher CD56 bright frequencies in HIV negative donors suggest that the NK cell subset composition in these DARC-null individuals may be skewed toward an immature profile. In contrast, the results

suggest that DARC-null neutropenia may have no effect on NK cell counts or subset distribution during chronic HIV-1 infection.

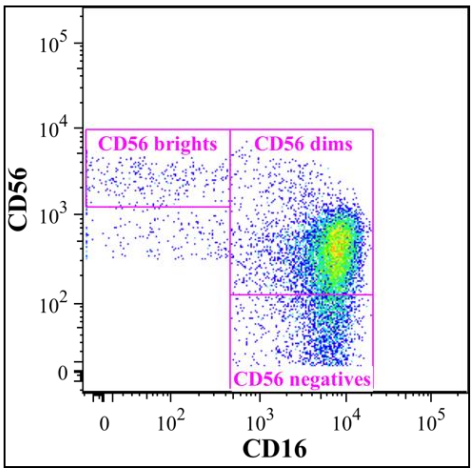
A **Gating Strategy (HIV Negative Donor)**

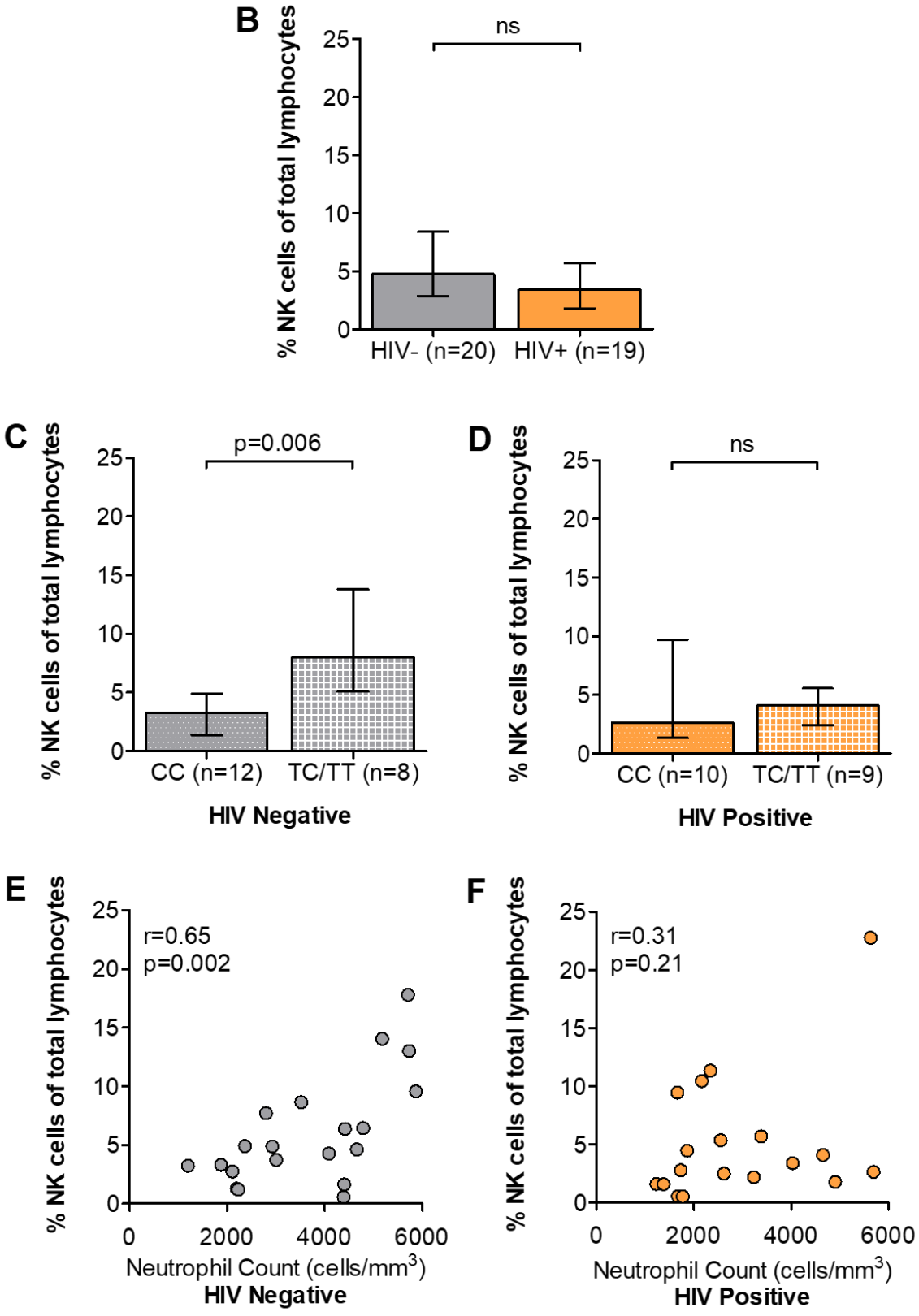


NK cell subsets (HIV Negative)



NK cell subsets (HIV Positive)




B
ns
% NK cells of total lymphocytes
25
20
15
10
5
0
HIV- (n=20)
HIV+ (n=19)
C
p=0.006
% NK cells of total lymphocytes
CC (n=12)
TC/TT (n=8)
HIV Negative
D
ns
% NK cells of total lymphocytes
CC (n=10)
TC/TT (n=9)
HIV Positive
E
r=0.65
p=0.002
% NK cells of total lymphocytes
0
2000
4000
6000
Neutrophil Count (cells/mm3)
HIV Negative
F
r=0.31
p=0.21
% NK cells of total lymphocytes
Neutrophil Count (cells/mm3)
HIV Positive

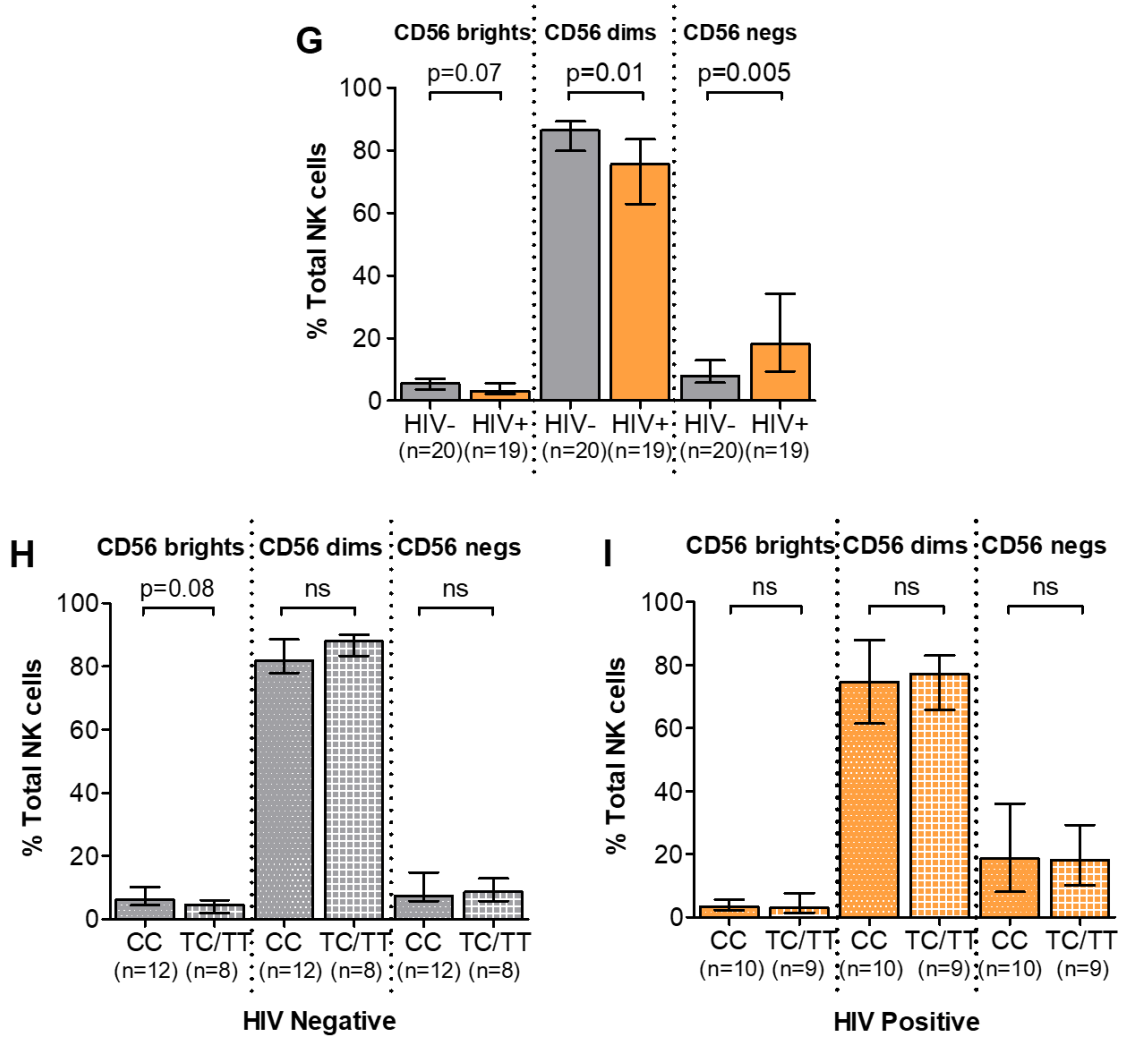


Figure 3.1 Total NK cell and subset frequencies in HIV negative and HIV infected individuals

(A) Representative gating strategy employed for the identification of total NK cells from lymphocytes using multi-parametric flow cytometry. Gating was on side scatter area (SSC-A) vs forward scatter area (FSC-A) to identify lymphocytes followed by exclusion of doublets by forward scatter height (FSC-H) vs FSC-A, and exclusion of non-viable cells, T-cells, B-cells and monocytes by use of viability dye and anti-CD3, anti-CD19 and anti-CD14 monoclonal antibodies. NK cells were identified as CD56 positive and/or CD16 positive cells. NK cells subsets were further identified from total NK cells as CD56+CD16- (CD56 brights), CD56+CD16+ (CD56 dims) or CD56-CD16+ (CD56 negatives). **(B)** NK cell frequency as a percentage of total viable lymphocytes in HIV negative and HIV infected individuals. NK cell frequency as a percentage of total viable lymphocytes in HIV negative **(C)** and HIV infected **(D)** individuals as grouped by DARC genotype. Bar graphs indicate medians extended to interquartile range (IQR) with whiskers. Correlations between NK cells as a percentage of total lymphocytes and absolute neutrophil counts (ANCs) are shown in HIV negative **(E)** and HIV infected **(F)** participants. **(G)** CD56 brights, CD56 dims and CD56 negatives subset frequencies as a percentage of total NK cells in HIV negative and HIV infected subjects. NK cell subset frequencies were further stratified by DARC genotype in HIV negative **(H)** and HIV-infected **(I)** donors. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute neutrophil count; IQR, interquartile range; ns, p-value not significant; r, Spearman rho.

3.3.2 Absence of DARC is associated with a less differentiated NK cell profile in HIV negative individuals

NK cells are a heterogeneous population which proceed through several stages of development (Cooper et al., 2001). Our earlier finding showed that there was a trend of higher frequencies of the CD56 bright NK cell subset in HIV negative individuals with reduced ANCs. This result suggested a less mature NK cell phenotype in these individuals compared to persons with typical ANCs. We thus assessed the maturation/differentiation status of NK cells in these participants to determine whether distinct NK cell phenotypes were evident in HIV infection, and whether these varied by DARC genotype and ANCs in HIV negative and HIV-1 infected individuals. Two groups have proposed maturation/differentiation models for NK cells in previous reports (Beziat et al., 2010, Fu et al., 2011), while CD57 expression has been described as a marker for terminal differentiation (Nielsen et al., 2013).

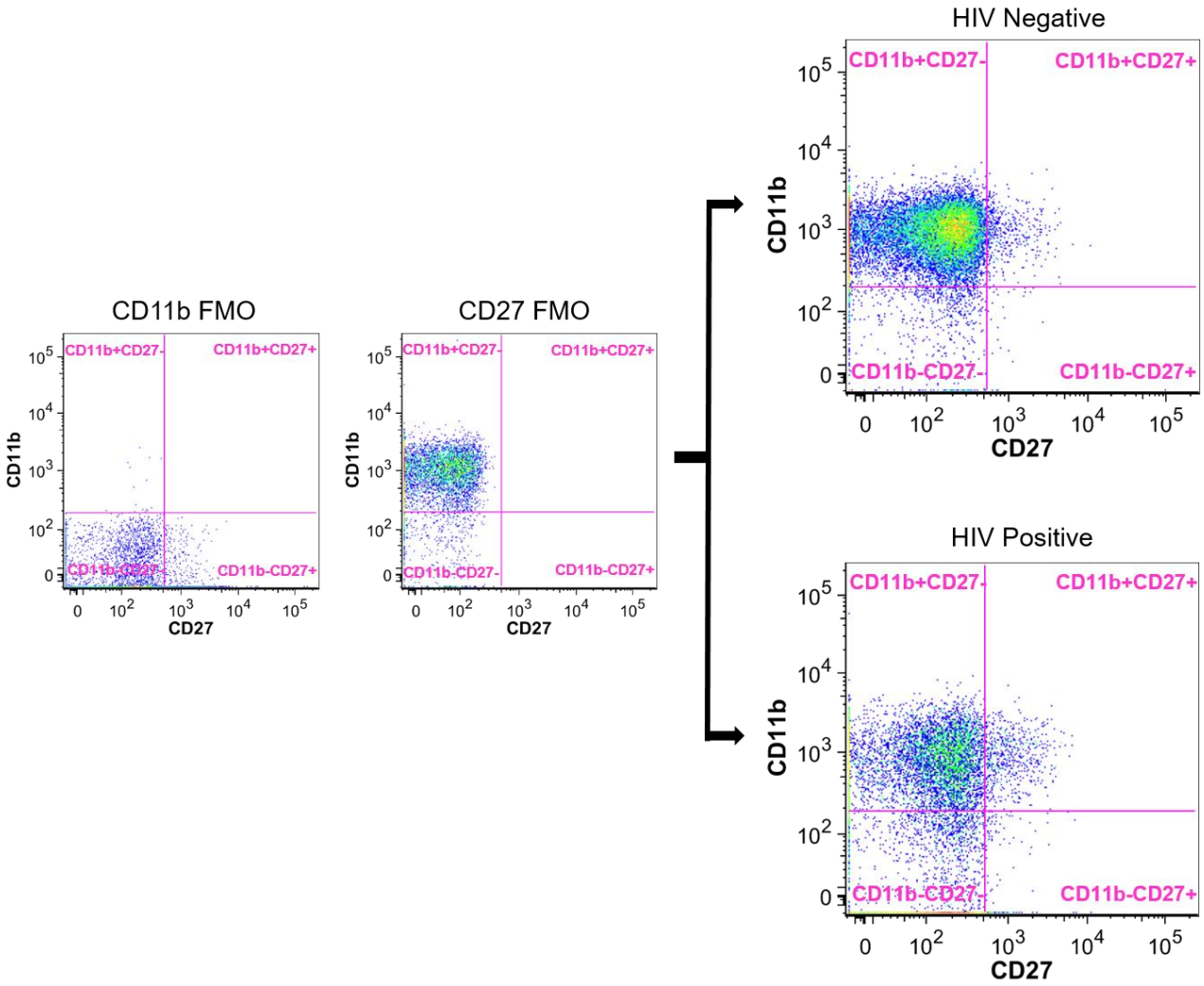
NK cells were assessed by the maturation model proposed by Fu and colleagues by measuring CD11b and CD27 expression (gating strategy depicted in Figure 3.2A) Assessment of HIV negative and HIV positive participants demonstrated comparable frequencies in three of the four subsets. The majority of NK cells were mature CD11b+CD27- irrespective of HIV status (median frequencies of 83.5% and 78.2% in HIV negative and infected individuals respectively, Figure 3.2B). HIV infected subjects demonstrated higher frequencies of the intermediate CD11b-CD27+ subset, although this subset comprised only a minor proportion of NK cells (median frequencies of 0.33% and 0.65% in HIV negative and HIV infected individuals respectively, Figure 3.2B).

In HIV negative individuals, DARC-null individuals had lower (although not significant) frequencies of the mature CD11b+CD27- subset. Furthermore significantly higher proportions of intermediate CD11b+CD27+ and CD11b-CD27+ subsets ($p=0.01$ and $p=0.05$, respectively, Figure 3.2C) were observed in DARC-null individuals. These observations were not apparent in the HIV infected group where CD11b/CD27 subset frequencies were unaffected by DARC genotype or ANC (Figure 3.2D).

Using both the relative expression of CD56 and the CD11b/CD27 model offers a more thorough assessment of NK cells in terms of maturation subsets. The data suggests that CD11/CD27 maturation subsets are not significantly altered during HIV infection. Assessment of CD11b/CD27 subsets by DARC genotype confirmed our previous result of higher frequencies of the immature CD56 bright NK cells in DARC-null HIV negative individuals.

The model used here also indicates higher levels of a less mature NK cell phenotype in the absence of DARC and may suggest that the DARC trait may contribute to full maturation of NK cells in HIV negative study subjects. Conversely, the data indicates that lack of DARC does not modify CD11b/CD27 NK cell subsets in chronic HIV infection.

A **Gating Strategy – CD11b/CD27 Subsets**



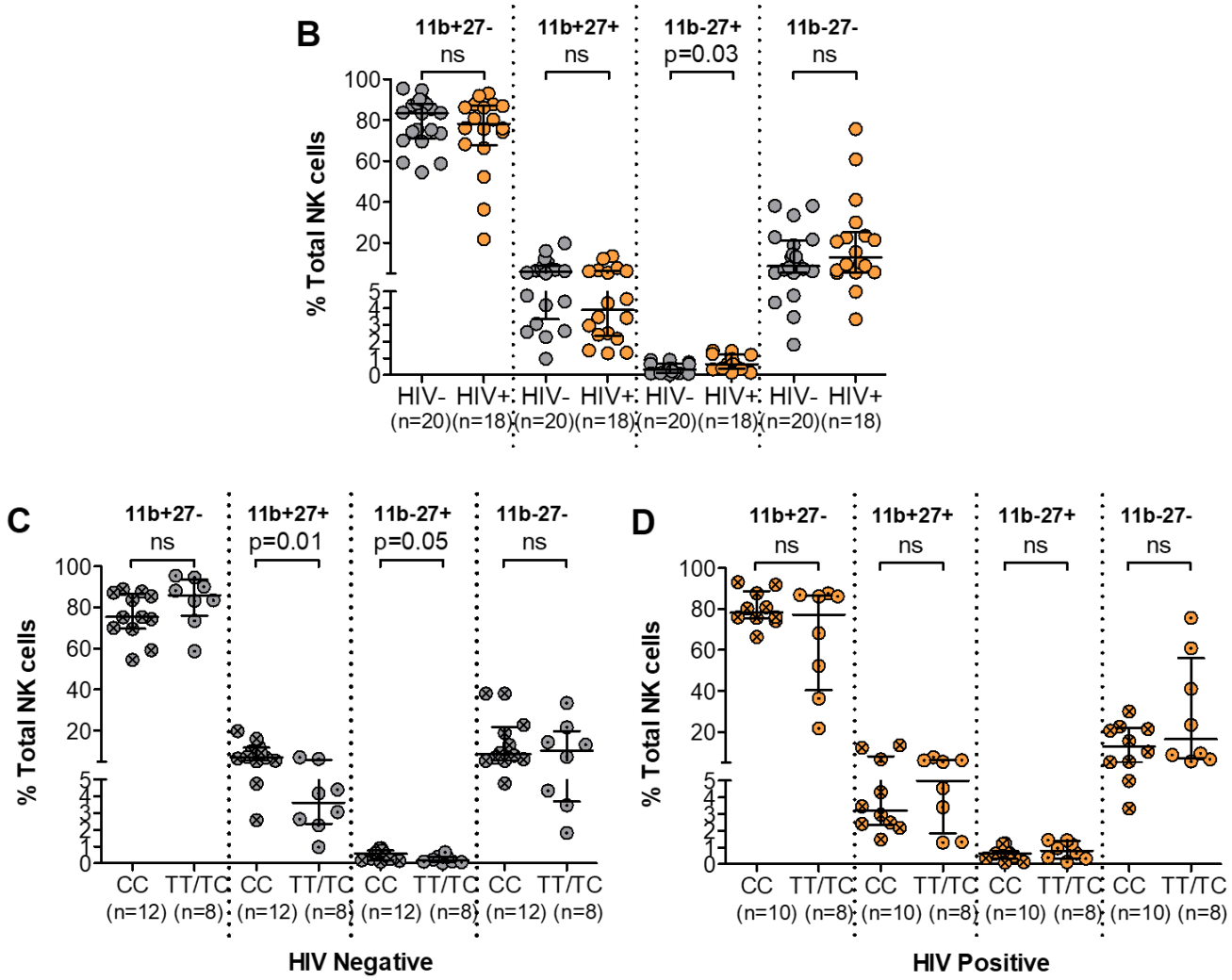


Figure 3.2 Frequency of CD11b/CD27 subsets in total NK cells

(A) Representative gating strategy used to identify CD11b/CD27 subsets from total NK cells (as described in Figure 1A) using multi-parametric flow cytometry. FMO samples for CD11b and CD27 were employed on total NK cells to adjust the quadrant gates to identify subpopulation combinations CD11b+CD27-; CD11b+CD27+; CD11b-CD27+ and CD11b-CD27- respectively. Here an example for both an HIV negative and HIV positive sample are shown after adjustment of the quadrant gating. **(B)** CD11b/CD27 subsets as a percentage of total NK cells in HIV negative and HIV infected participants. CD11b/CD27 subsets were further stratified by DARC genotype in HIV negative **(C)** and HIV infected **(D)** individuals. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute neutrophil count; 11b+/27-, CD11b+CD27- NK cell subset; 11b+/27+, CD11b+CD27+ NK cell subset; 11b-/27+, CD11b-CD27+ NK cell subset; 11b-/27-, CD11b-CD27- NK cell subset; IQR, interquartile range; ns, p-value not significant.

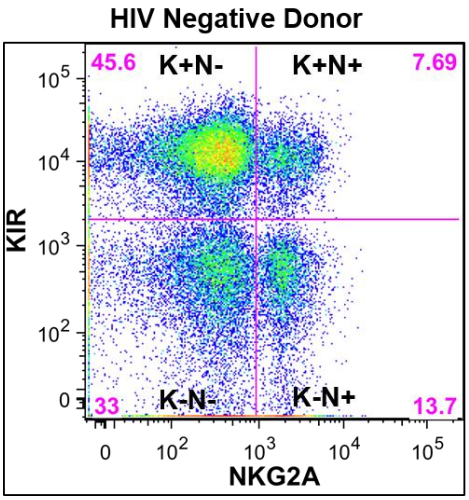
Next to assess terminal differentiation, NK cells were evaluated using the KIR and NKG2A model described by Beziat et al. (2010). Frequency gating for KIR/NKG2A is shown in Figure 3.3A. Lower frequencies of the cytolytic KIR+NKG2A+ subset were detected in HIV infected persons compared to uninfected individuals (p=0.05, Figure 3.3B). Moreover higher (although not significant) median frequencies of the hypo-responsive KIR-NKG2A- subset were noted in HIV infection (median frequencies of 13.3% and 36.8% in HIV negative and infected participants respectively, Figure 3.3B).

HIV negative donors with the DARC-null trait displayed lower (although not significant) median frequencies of the cytotoxic KIR+NKG2A- subset (median frequencies 38.9% and 48.3% in DARC-null and DARC-positive persons respectively, Figure 3.3C). Furthermore, a trend of higher frequencies of the hypo-responsive KIR-NKG2A- subset was observed in DARC-null individuals compared to DARC-positive individuals (p=0.06, Figure 3.3C) and ANCs were negatively associated with this KIR-NKG2A- subset (p=0.03, data not shown). No differences were indicated in any of the KIR/NKG2A subset frequencies in the HIV infected group when assessed by DARC genotype or ANC (Figure 3.3D).

Taken together our data suggested a slightly less differentiated NK cell phenotype during HIV infection. In the context of DARC, absence of DARC was associated with higher proportions of less differentiated NK cells in HIV negative individuals, whilst this difference in NK cell phenotype by DARC genotype was lost in HIV infection.

In summary, the two models used to assess NK cell phenotype together provide insight of the impact of HIV and DARC on NK cell maturation/differentiation. Both models confirm higher proportions of a less mature NK cell phenotype in HIV infected individuals. Assessment of HIV negative individuals indicated a less mature NK cell phenotype in those that lacked the DARC allele. In contrast, both models revealed no alteration in maturation/differentiation status of NK cells by DARC trait in HIV infected subjects.

A Gating Strategy – KIR/NKG2A Subsets



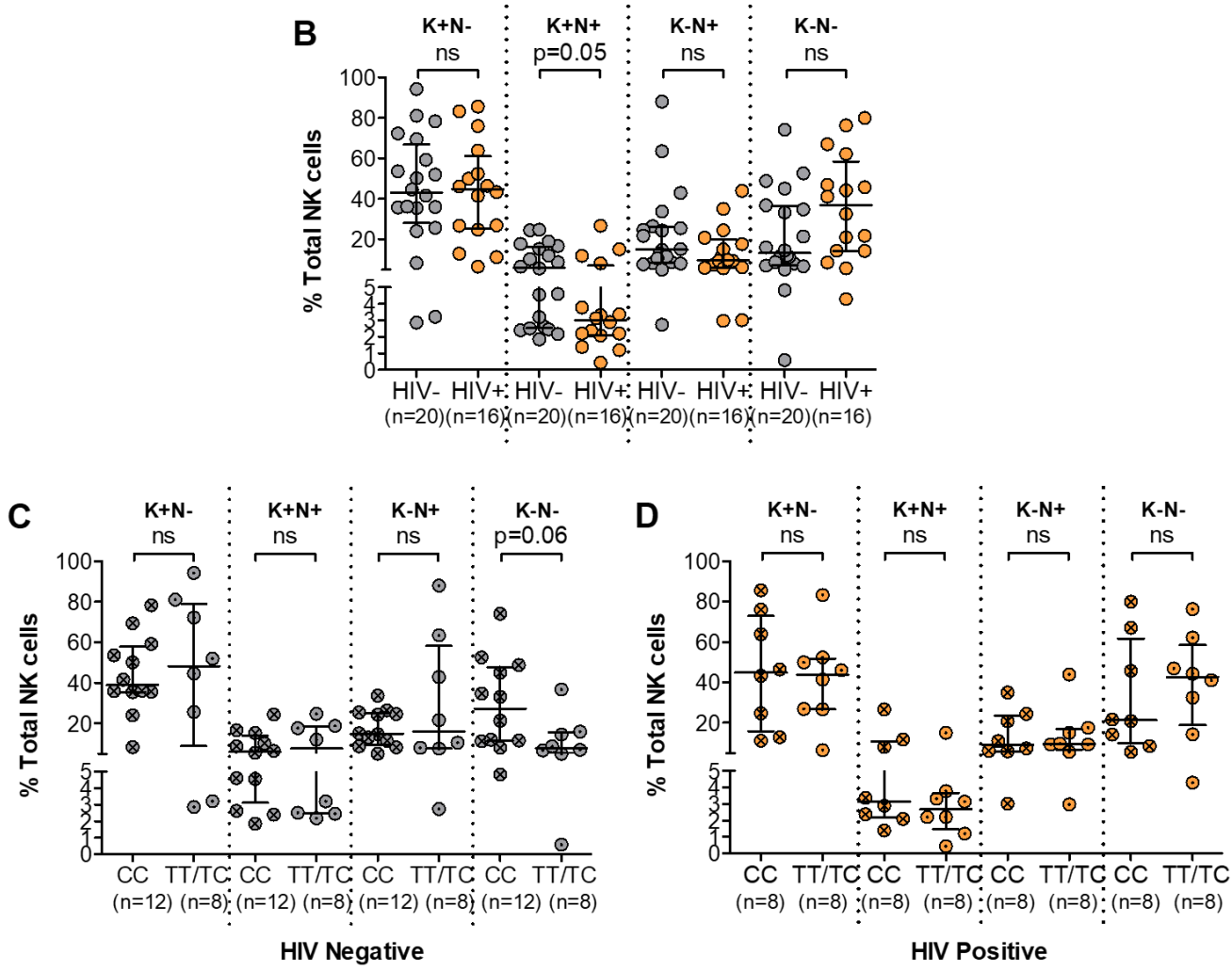


Figure 3.3 Expression of KIR/NKG2A subsets in total NK cells

(A) Representative strategy employed for the identification of KIR and/or NKG2A expressing NK cells following ex vivo phenotype antibody staining using multi-parametric flow cytometry. Here an example HIV negative sample is shown after adjustment of the quadrant gating. **(B)** KIR/NKG2A subsets as a percentage of total NK cells in HIV negative and HIV infected individuals. KIR/NKG2A subsets further stratified by DARC genotype in HIV negative **(C)** and HIV infected **(D)** individuals. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute neutrophil count; K+N-, KIR+NKG2A- NK cell subset; K+N+, KIR+NKG2A+ NK cell subset; K-N+, KIR-NKG2A+ NK cell subset; K-N-, KIR-NKG2A- NK cell subset; IQR, interquartile range; ns, p-value not significant.

To further characterise differentiation status of NK cells, we quantified terminal differentiation using CD57 expression in these participants. HIV negative and HIV infected persons demonstrated comparable CD57 expression (Figure 3.4A). In HIV negative participants, CD57 expression was considerably lower in DARC-null compared to DARC-positive individuals ($p=0.02$, Figure 3.4B), and CD57 expression strongly correlated with ANC ($p=0.009$, Figure 3.4D). No differences in CD57 expression were observed in HIV-infected individuals based on DARC genotype or ANC (Figure 3.4C and Figure 3.4E).

In summary, this data suggests similar frequencies of fully differentiated NK cells in HIV negative and infected persons. The results indicated lower proportions of fully differentiated NK cells in DARC-null compared to DARC-positive subjects that is mostly noted in HIV negative subjects and lost in chronic HIV-infection. Interestingly, these findings support our earlier maturation and differentiation data, showing that the DARC genotype may influence NK cell progression to a fully differentiated profile.

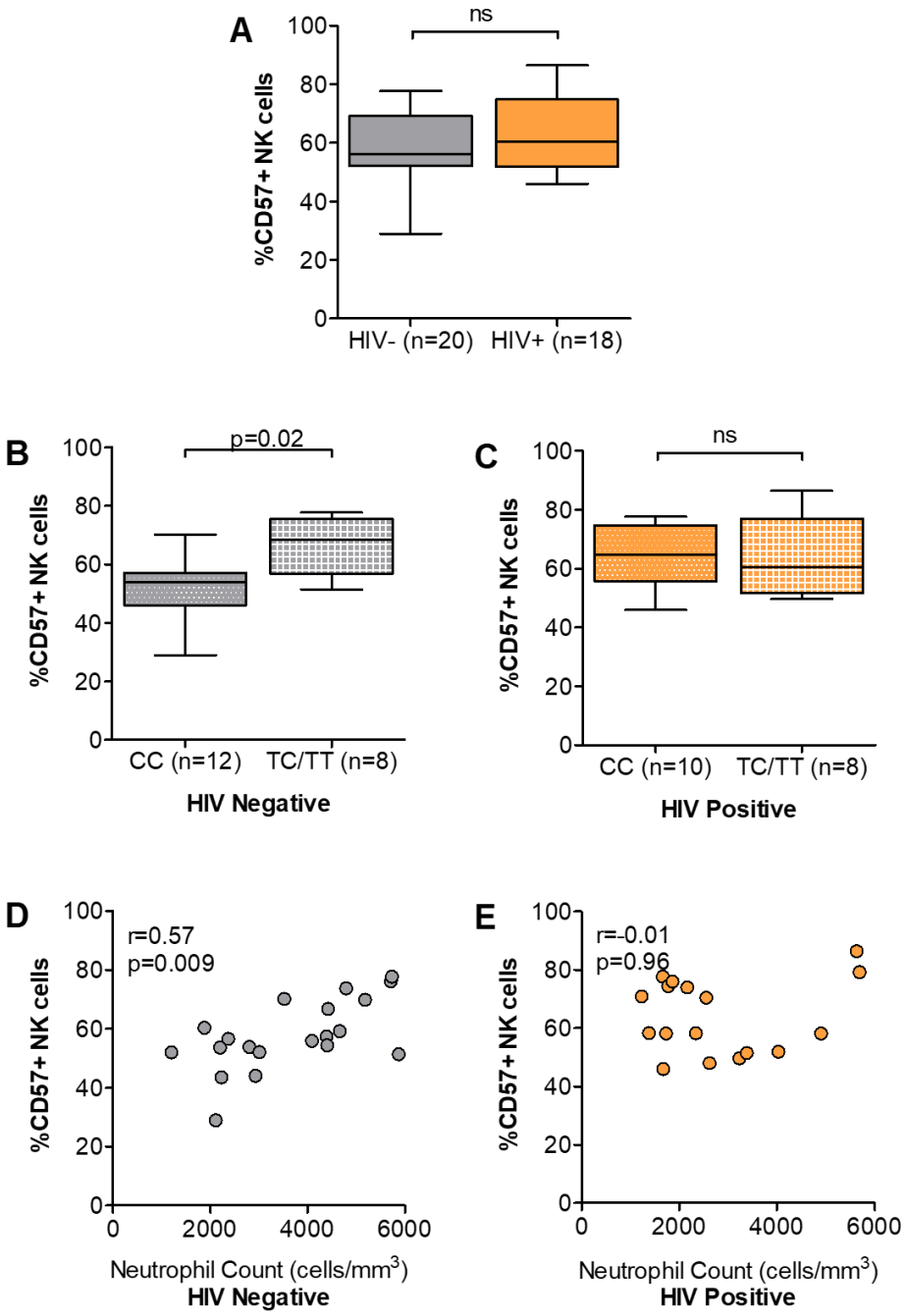


Figure 3.4 Expression of CD57 in total NK cells

(A) CD57 expressing NK cells as a percentage of total NK cell count in HIV negative and HIV infected individuals. CD57 expressing NK cells as a percentage of total NK cells in HIV negative **(B)** and HIV infected **(C)** individuals grouped by DARC genotype. Boxes extend from the 25th to 75th percentile and medians are indicated by the horizontal line. Whiskers indicate minimum and maximum values. Correlations between CD57 expressing NK cells and absolute neutrophil counts (ANCs) are shown in HIV negative **(D)** and HIV infected **(E)** participants. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute neutrophil count; IQR, interquartile range; ns, p-value not significant.

3.3.3 DARC-null trait has no impact on NK cell activation status and functionality

Next, we aimed to determine whether NK cell activation ex vivo differed in patients by HIV status and DARC genotype. We hypothesised that the absence of DARC would contribute to modified NK cell phenotype, specifically we expected higher NK cell activation status in DARC-null individuals independent of HIV status. To address this question, previously described markers of acute and chronic activation, CD69 and HLA-DR respectively, were quantified on NK cells. In addition, we examined cell exhaustion as quantified by the PD-1 antigen which is commonly associated with NK cell dysfunction (Beldi-Ferchiou et al., 2016).

HIV negative donors were found to have higher frequencies of CD69 expressing NK cells compared to HIV infected individuals ($p=0.008$, Figure 3.5A). Consistent with a previous report (Fogli et al., 2004), chronic NK cell activation as measured by HLA-DR was evident in HIV infected persons compared to HIV negative controls ($p=0.02$, Figure 3.5A). HIV-infected persons also showed a trend of higher expression of exhaustion marker PD-1 compared to uninfected individuals ($p=0.09$, Figure 3.5A). DARC-null HIV negative individuals displayed lower median frequencies of CD69 expressing NK cells compared to DARC-positive individuals (median frequencies of 10.8% and 17.78% respectively, Figure 3.5B). However, these differences in CD69 expression were not significant. Moreover, NK cell activation as measured by HLA-DR and PD-1 exhaustion marker was not affected by DARC genotype or ANCs in HIV negative persons (Figure 3.5B and Supplementary Figures 2A, 2C and 2E). Likewise, no differences in NK cell activation or exhaustion were displayed in HIV infected individuals by DARC status or ANCs (Figure 3.5C and Supplementary Figures 2B, 2D and 2F).

Taken together this data indicated variation in NK cell activation profiles during HIV infection. Furthermore, NK cells from HIV infected individuals exhibited a more exhausted phenotype in comparison to HIV negative individuals. Contrary to our hypothesis our data revealed that neither DARC genotype nor ANC had an impact on activation and exhaustion status of NK cells.

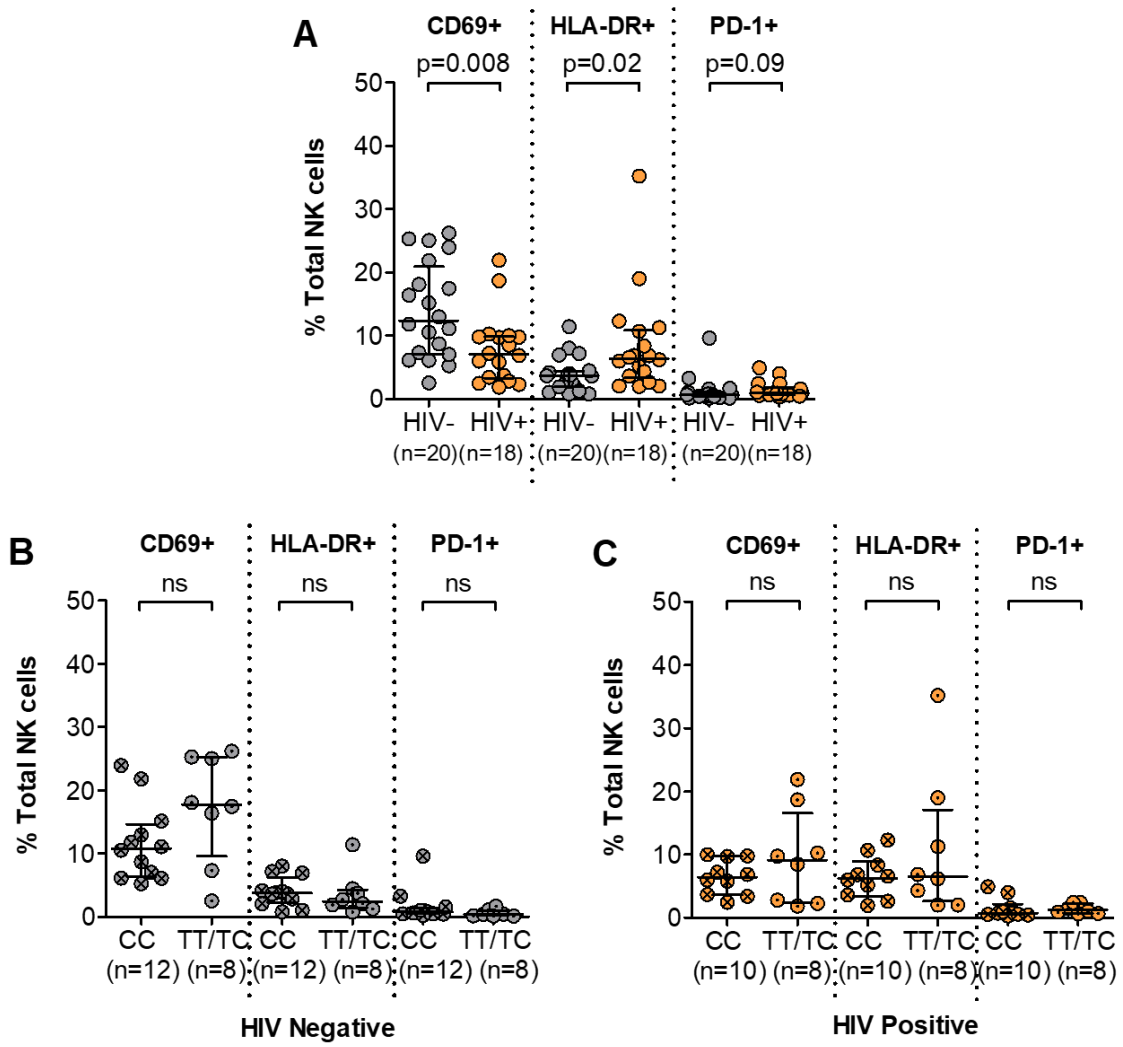


Figure 3.5 Expression of CD69, HLA-DR and PD-1 in total NK cells

(A) Expression of CD69, HLA-DR and PD-1 on total NK cells HIV negative and HIV infected individuals. Expression of CD69, HLA-DR and PD-1 on total NK cells in HIV negative **(B)** and HIV infected **(C)** individuals grouped by DARC genotype. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; IQR, interquartile range; ns, p-value not significant.

The functional activity of NK cells is predominantly characterised by direct killing of infected/transformed cells through release of cytoplasmic granules. Cell surface expression of CD107 has been previously identified as a marker for granule release (degranulation) (Alter et al., 2004a). NK cells also produce various pro-inflammatory cytokines and chemokines that shape the adaptive immune response (Vivier et al., 2008). To investigate the impact of DARC genotype and ANC on NK cell functionality we assessed degranulation (CD107a) and cytokine production (TNF-α and IFN-ɣ) in PBMCs following overnight stimulation with MHC-devoid K562 target cell line and a combination of cytokines (IL-2, IL-15, IL-18).

Our results indicated higher CD107a expression in total NK cells from HIV negative compared to HIV infected individuals ($p=0.05$, Figure 3.7A). Moreover, no differences in the proportion of TNF-α and IFN-ɣ secreting NK cells were evident in HIV infection (data not shown). Further assessment detected no differences in degranulation (Figures 3.7B-3.7D) or cytokine production (data not shown) when stratified by DARC trait or ANC irrespective of HIV status.

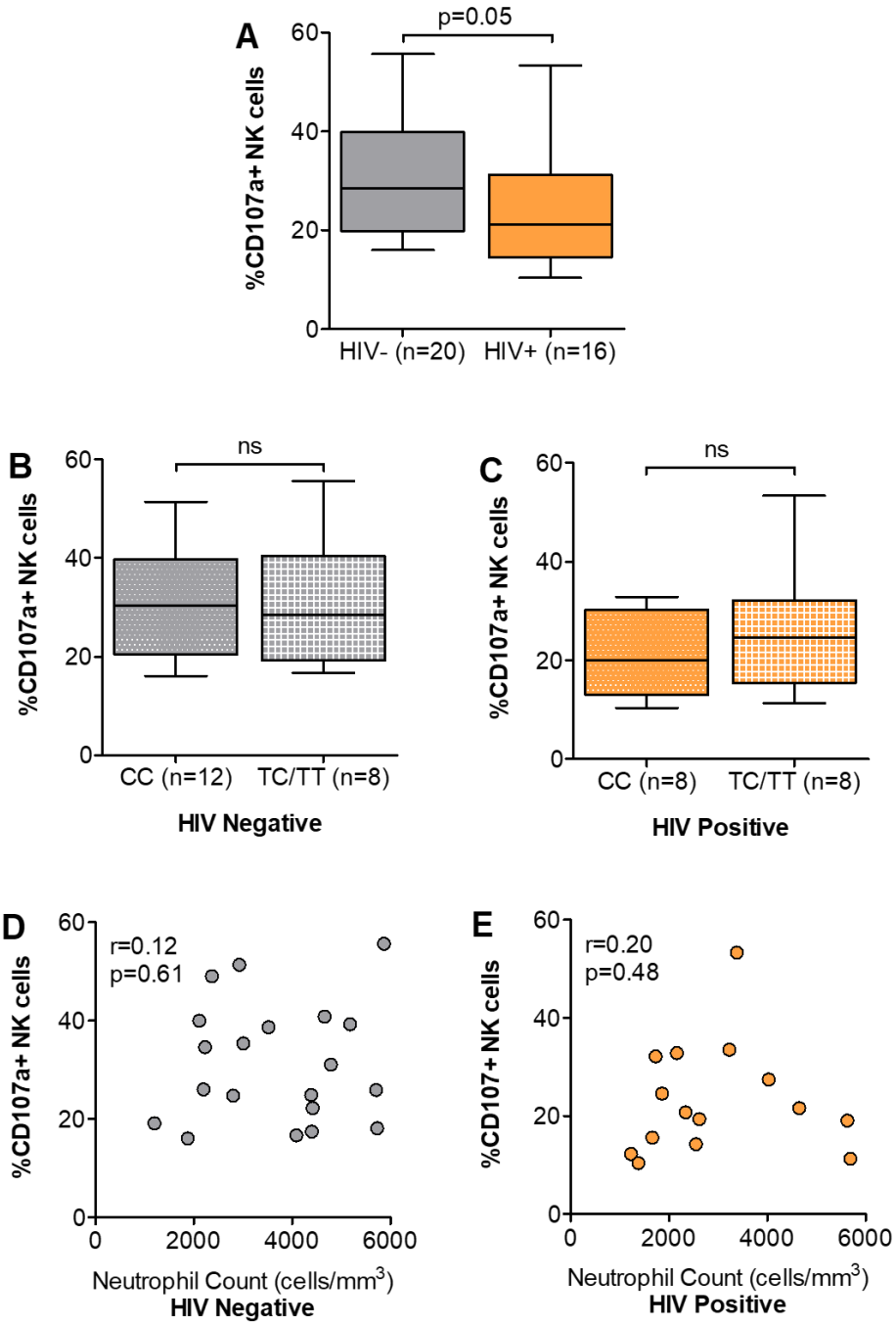


Figure 3. 6 Expression of CD107a in NK cells

(A) CD107a expressing NK cells as a percentage of total NK cell count in HIV negative and HIV infected individuals. CD107a expressing NK cells as a percentage of total NK cells in HIV negative **(B)** and HIV infected **(C)** individuals grouped by DARC genotype. Boxes extend from the 25th to 75th percentile and medians are indicated by the horizontal line. Whiskers indicate minimum and maximum values. Correlations between percentage of NK cells expressing CD107a and absolute neutrophil count (ANC) in HIV negative **(D)** and HIV infected **(E)** individuals. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute neutrophil count; IQR, interquartile range; ns, p-value not significant; r, Spearman rho.

Frequent and strong antibody mediated NK cell responses have been detected in chronic HIV-1 infection following stimulation with HIV-1 peptides. These responses targeted Env antigens more than other HIV-1 proteins and were shown to be mediated by plasma (Thobakgale et al., 2012). We thus sought to assess this HIV-specific NK cell activity in whole blood in our cohort following stimulation with HIV-1 gp120 and gp41 peptides, for gating strategies see Supplementary Figure 3.

In line with previous findings we detected little or no response to HIV-1 antigens in uninfected individuals, whilst some (but not all) HIV-1 chronically infected individuals displayed measurable expression of CD107a and IFN-ɣ, particularly in response to gp120 (Figure 3.8A and Figure 3.9A). However, comparison of CD107a expression and IFN-ɣ production in HIV negative or HIV-1 chronically infected persons by DARC genotype showed no disparities (Figures 3.8B-3.8C and Figures 3.9B-3.9C), and further analyses exhibited no relationship between ANCs and CD107a (Supplementary Figure 4) or IFN-ɣ expression (Supplementary Figure 5) in response to gp120 or gp41 respectively.

In summary, our data indicated lower degranulation activity in response to MHC devoid cell line during HIV infection. Plasma mediated NK cell degranulation was detected, particularly in response to HIV Env gp120 antigen in HIV infected individuals and this is consistent with previous data (Thobakgale et al., 2012). Investigation of NK cell activity in PBMCs and whole blood assays suggested that while the absence of DARC in HIV negative individuals may contribute to a less differentiated NK cell profile, this may not translate to measurable functional impairment of these cells. Similarly, DARC-null trait in HIV-1 infection did not affect functional activity.

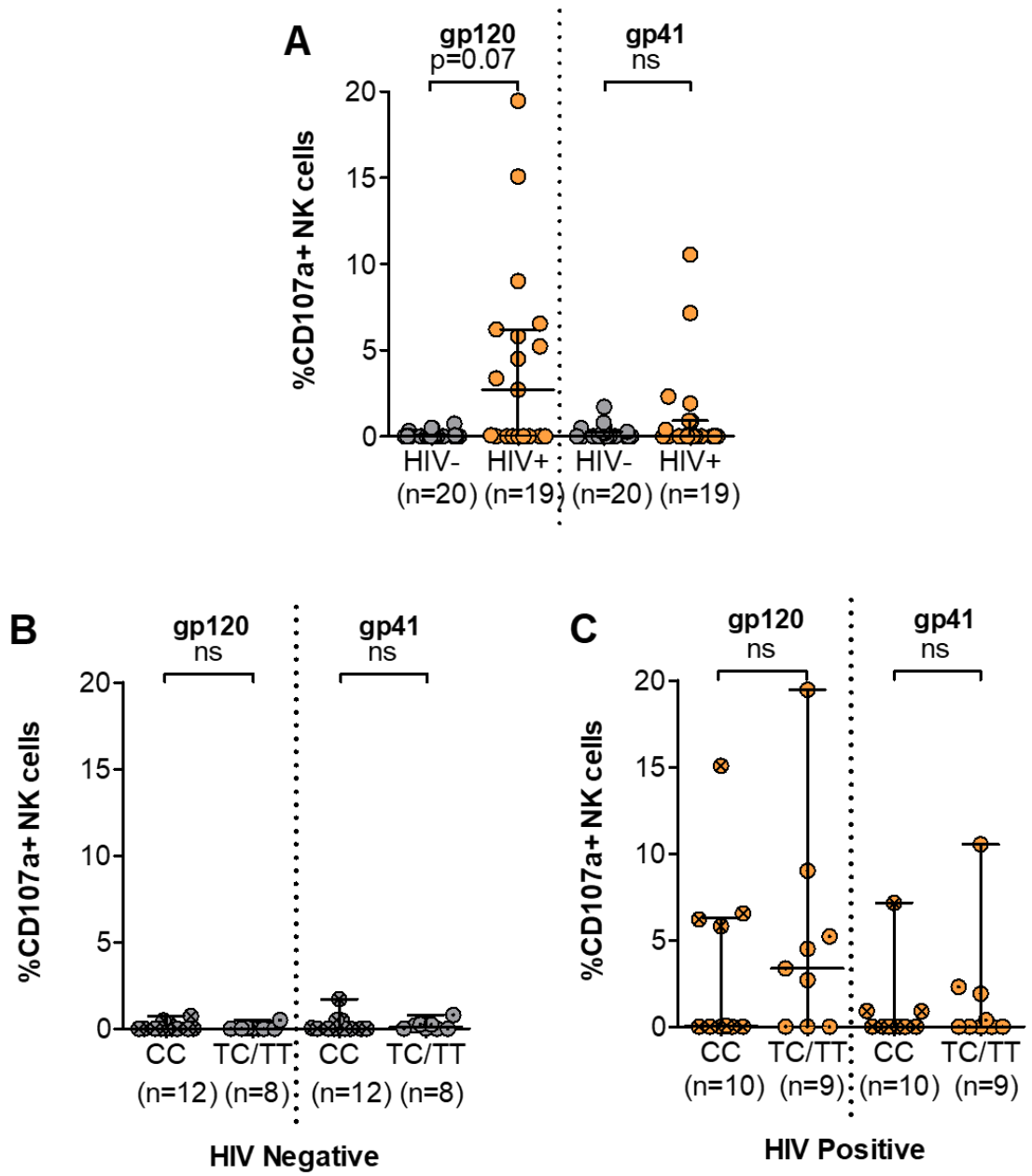


Figure 3. 7 CD107a expression following whole blood stimulation with HIV-1 peptides gp120 or gp41

(A) Percentage of CD107a expressing NK cells in HIV negative and HIV infected individuals. Percentage of CD107a expressing NK cells further grouped by DARC genotype in HIV negative **(B)** and HIV infected **(C)** individuals. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; IQR, interquartile range; ns, p-value not significant.

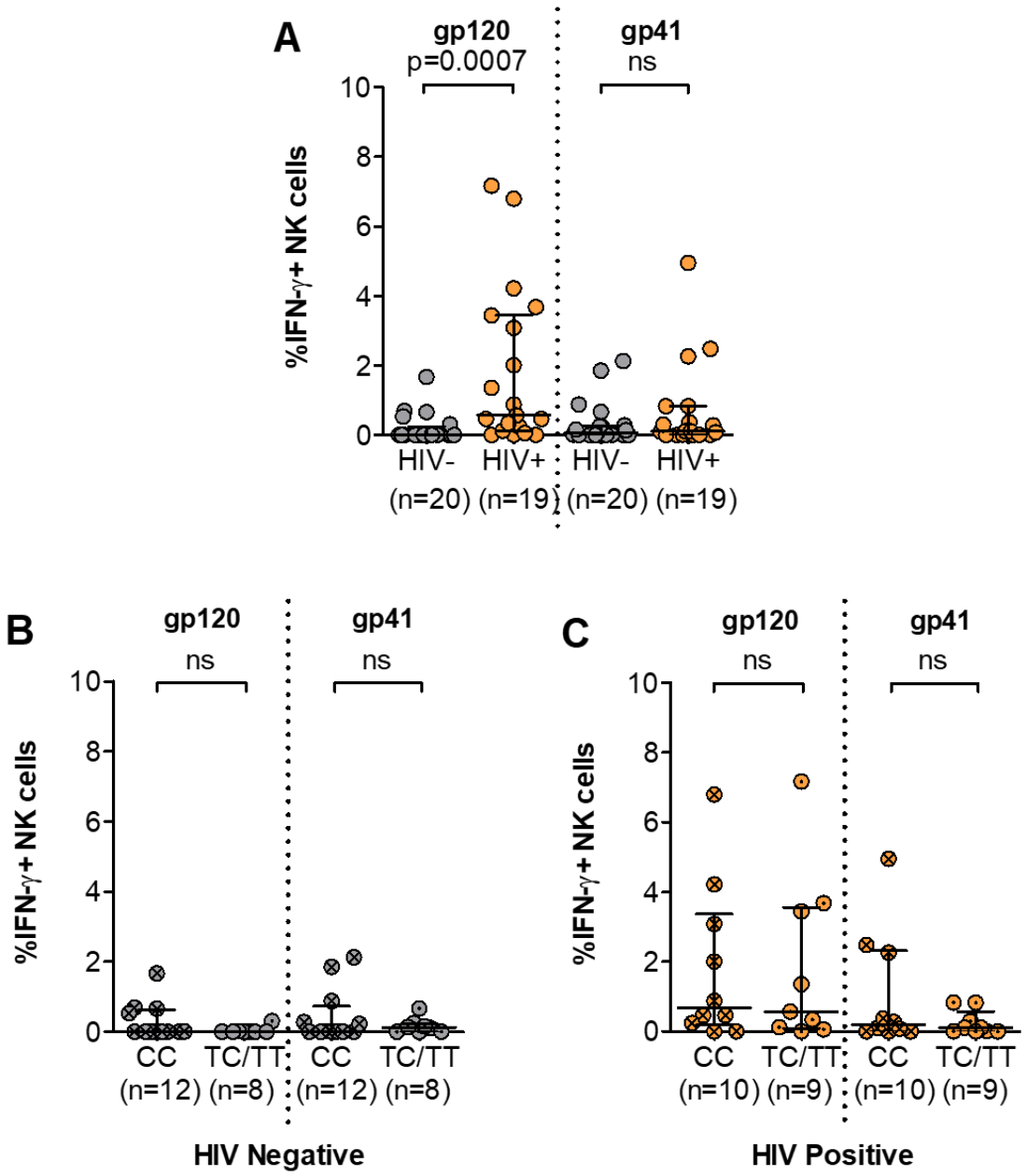


Figure 3. 8 IFN-ɣ expression following whole blood stimulation with HIV-1 peptides gp120 or gp41

(A) Percentage of IFN-ɣ expressing NK cells in HIV negative and HIV infected individuals. Percentage of IFN-ɣ expressing NK cells further grouped by DARC genotype in HIV negative **(B)** and HIV infected **(C)** individuals. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; IQR, interquartile range; ns, p-value not significant;

3.4 DISCUSSION

DARC-null linked neutropenia is associated with increased HIV-1 infection risk in sub-Saharan Africa, however its impact on HIV pathogenesis is unclear. Notably, NK cell priming and effector functionality are dependent on continual interactions with mature neutrophils in the bone marrow and peripheral compartments. Here we assessed possible modifications introduced by the absence of the DARC trait and low circulating neutrophils on peripheral NK cell differentiation, maturation and effector profiles in HIV negative and HIV-1 infected Zulu/Xhosa individuals from the Umlazi region in Durban, South Africa.

The data presented here showed that 56% (22 out of 39) of our subjects possessed the DARC-null polymorphism and these individuals exhibited reduced absolute ANCs independent of HIV status. Examination of NK cell dynamics in these individuals showed similar NK cell counts irrespective of HIV status. We also found that in HIV negative individuals, the DARC-null trait was associated with reduced NK cell counts but elevated CD56 bright subset frequencies when compared to DARC-positive individuals. Determination of NK cell maturation and differentiation by CD11b/CD27, KIR/NKG2A and CD57 quantification revealed an immature phenotype with lower frequencies of terminally differentiated NK cells in DARC-null compared to DARC-positive HIV negative individuals. In contrast HIV-1 infected individuals displayed NK cell subset redistribution marked by higher CD56 negative cells, marginally higher proportions of less mature NK cells, higher expression of markers of activation and exhaustion accompanied by lower cytolytic potential. However, these observed phenotypic and functional differences were lost upon stratification by DARC genotype.

Numerous studies allude to the alteration of NK cell profiles associated with HIV infection, and there is general consensus that high viremic infection leads to reduction in total NK cell counts and CD56 subset redistribution (Alter et al., 2004b, Milush et al., 2013, Mikulak et al., 2017). Here we did not detect a difference in NK cell counts between HIV negative and infected individuals. Many of our infected subjects had relatively low viral loads to those typically observed in chronic infection and could explain our observation of moderate reduction of NK cell counts. Nonetheless, in line with literature (Fauci et al., 2005, Mikulak et al., 2017), we observed diminished CD56 bright and CD56 dim subset proportions and substantially higher frequencies of the irregular CD56 negative subset in HIV infected individuals.

NK cell differentiation is evaluated using receptor expression profiling models either alone or in combination (Bozzano et al., 2017). To gain a comprehensive understanding of the NK cell

maturation/differentiation profile in our study cohort, we assessed NK cells using two previously proposed NK cell models (CD11b/CD27 and KIR/NKG2A subset profiles) and CD57 expression. Previous reports suggest NK cell profile pattern variance in terms of differentiation status during HIV infection. HIV-infected individuals have been shown to exhibit a more mature/differentiated NK cell repertoire. Specifically, higher frequencies of terminally differentiated NK cells as measured by CD57 expression (Hong et al., 2010, Kaczmarek et al., 2017) and lower frequencies of NKG2A and CD27 expression (Kaczmarek et al., 2017), which are receptors associated with a less mature phenotype (Beziat et al., 2010, Fu et al., 2011). Overall, our data demonstrated comparable frequencies of fully differentiated NK cells in HIV negative and HIV infected subjects. This was indicated by similar proportions of CD57 positive NK cells irrespective of HIV status. Furthermore, assessment by maturation/differentiation models showed no differences in the frequencies of the fully mature CD11b+CD27+ and KIR+NKG2A- NK cell subsets. Although we did observe higher frequencies of the intermediate CD11b-CD27+ NK cell subset in HIV infected individuals, this CD11b/CD27 subset was only a minor subset in all participants (>5%) while the remaining CD11b/CD27 subsets showed no differences by HIV status. The only other difference noted during HIV infection was a reduction of the intermediate KIR+NKG2A+ NK cell subset. This observation of lower proportions of the KIR+NKG2A+ subset during HIV infection has been previously described (Peppa et al., 2018), but in contrast to a shift toward a mature or terminally differentiated NK cell phenotype (Peppa et al., 2018), we noted here that KIR+NKG2A+ reduction was accompanied with higher (although not significant) proportions of the hypo-responsive KIR-NKG2A- NK cell subset. Dissimilarities between our and past observations could be due to variations in NK cell analysis. Most studies have reported on CD56 positive NK cells whilst our analyses comprised total NK cells including the dysfunctional CD56 negative subset. We have previously described the phenotypic characteristics of the CD56 negative subset, and we noted reduced CD57 expression on NK cells of this subset in chronically infected individuals (Zulu et al., 2017). Thus phenotypic alterations within this subset could account for differences observed between our observations in this cohort compared to previous reports.

Consistent with previous reports (Kuri-Cervantes et al., 2014, Beldi-Ferchiou et al., 2016), we show here higher NK cell exhaustion and activation (HLA-DR expression) in chronic HIV-1 infected subjects. Elevated frequencies of activated and exhausted NK cells have been associated with disease progression to AIDS (Kuri-Cervantes et al., 2014, Mikulak et al., 2017).

Although HIV infection is associated with NK cell activation, there are contrasting reports on the effect of HIV viremia on NK cell functional responses and these mechanisms seemingly depend on disease state (Mikulak et al., 2017). Previous studies have reported that NK cells exhibit higher levels of cytolytic potential (as measured by degranulation marker CD107a) and elevated secretion of antiviral cytokines during chronic disease (Alter et al., 2004b, Wong et al., 2010). Following in vitro culturing with MHC devoid cell line, NK cell degranulation tended to correlate with patient viral load (Wong et al., 2010). Inconsistent with these reports, chronic HIV-1 infection in our study setting was associated with lower degranulation capacity, and production of cytokines (TNF-α and IFN-ɣ) were not different between HIV negative and HIV-infected individuals. Possible reasons including lower viral loads in our study group and an accumulation of the irregular and dysfunctional CD56 negative subset in HIV infected individuals could drive inferior cytolytic NK cell activity (Fauci et al., 2005, Zulu et al., 2017) in this group compared to HIV negative donors.

Various populations have been assessed for frequency of the DARC-null polymorphism (Grann et al., 2008, He et al., 2008, Hodgson et al., 2014). Noteworthy, are the reports of high risk South African woman and HIV-1 chronically infected individuals based in Durban where 64.7% (92 of 142) and 64.8% (247 of 381) of patients were described to be homozygous for the DARC-null allele respectively (Julg et al., 2009, Ramsuran et al., 2011). Despite our small sample size in this pilot study, we also demonstrated a high DARC-null distribution of over 50% in our African cohort. In addition, we also observed here that the DARC-null genotype significantly associated with reduced neutrophil counts in individuals regardless of HIV status, consistent with previous literature that reported the DARC-null polymorphism as the genetic determinant of ethnic neutropenia in persons of African descent (Reich et al., 2009).

There are limited reports on the effect of neutrophil deficiency on NK cell phenotype and effector function (Sporri et al., 2008, Jaeger et al., 2012, Ueda et al., 2016) and to our knowledge this is the first to explore this mechanism in African individuals in a high HIV-1 incidence setting. Interestingly, most of the observations made in the context of HIV infection above were not associated with absence of the DARC trait. Despite observations of lower NK cell frequencies with a less differentiated phenotype in DARC-null HIV negative individuals, the DARC-null trait was not associated with other NK cell profile modifications.

Jaeger et al. (2012) previously described comparable peripheral NK cell counts in controls and patients with neutropenia, such as severe congenital neutropenia (SCN) and autoimmune

neutropenia (AIN). It was observed by the authors in parallel neutrophil deficient mouse models that increased cell death sensitivity was counterbalanced with hyper-proliferative capacity, resulting in unaltered NK cell frequencies (Jaeger et al., 2012). In contrast to this report, we observed reduced NK cell counts in HIV negative DARC-null individuals with lower ANCs. Our findings may suggest modified peripheral NK cell homeostasis in DARC-null HIV negative individuals. Thus the important question of whether NK survival and/or proliferation is affected by the DARC-null allele is addressed in detail in Chapter 4.

Furthermore, it has been shown that neutropenic individuals exhibit higher frequencies of less mature CD56 bright cells coupled with impaired cell reactivity in terms of IFN-ɣ release in response to the K562 cell line (Jaeger et al., 2012). Similarly, we observed that the DARC-null trait was associated with higher proportions of the CD56 bright subset and a less mature NK cell phenotype as measured by CD11b/CD27 profiling in HIV negative individuals. DARC Further, quantification of KIR/NKG2A and CD57 expression were indicative of fewer terminally differentiated NK cells in HIV negative DARC-null individuals with lower ANCs compared to DARC-positive subjects. These findings of a less matured/differentiated NK cell profile were suggestive of hypo-responsive NK cells in HIV negative DARC-null individuals.

Surprisingly, despite our findings of a less differentiated NK cell profile in DARC-null HIV negative individuals, our functional data demonstrated that cytolytic potential and cytokine production were unaffected by the DARC-null trait and no differences were detected by activation and exhaustion status ex vivo. Hypo-responsive KIR-NKG2A- cells can be re-educated and become fully functional following specific cytokine stimulation (Beziat et al., 2010). This supports the notion that NK cells are not halted at a less differentiated state, and following appropriate stimulus are able to further differentiate and gain functional capabilities. While HIV negative DARC-null individuals exhibited a less differentiated NK cell phenotype ex vivo, it is possible that in vitro stimulation allowed for further differentiation and increased cytolytic activity in these cells, and may explain our observation of no differences in NK cell cytotoxic mechanisms by DARC status.

NK cell variations that were detected between HIV negative DARC-null and DARC-positive individuals were not observed in HIV-1 infected individuals, implying that DARC-null trait does not influence NK cell responses in chronic infection. It is plausible that any advantage that DARC-positive individuals are predisposed to is masked by the general leukopenia commonly detected in chronic disease (De Santis et al., 2011). Alternatively, these advantages

could be lost in untreated chronic infection. We were unable to determine the time of infection in these individuals, and without longitudinal samples pre and post infection, we could not determine the stage during infection when such advantages are lost. Thus the current cohort could not fully assess the impact of DARC-null genotype on NK cells and whether this trait is beneficial or detrimental in chronic HIV-1 infection.

Since this was intended to be a pilot study, limitations include a small sample size. Also, although testing negative for antiretroviral drugs, some of our chronically infected patients had relatively lower viral loads (<1000 RNA copies/ml) than previously described chronic patients. Many of our DARC-null subjects were not neutropenic as defined by the ANC threshold of <1500 cells/mm^3 at the time of sampling, and since these patients were not followed longitudinally we cannot conclude which patients were neutropenic. Moreover, the HIV universal test and treat policy that was implemented during our study, made additional sample collection from chronically infected treatment naïve subjects increasingly challenging.

To our knowledge no previous studies have assessed the impact of DARC-null polymorphism and low neutrophil counts on NK phenotypic characteristics and functionality in an HIV-1 setting. Taken together, our findings suggest that in HIV negative individuals, DARC-null neutropenia results in reduced NK cell frequencies. Reduced cell counts may result in dampened NK cell responses and may indicate a greater risk to viral infections in these individuals (Orange, 2006, Orange and Ballas, 2006). Although NK cells in these individuals are seemingly less mature, it is encouraging that NK cells are not functionally impaired as seen in patients with severe neutropenia. The DARC-null trait and lower ANCs is considered a milder condition compared to other neutrophil deficiencies, and thus our findings support reports that the effect of DARC-null genotype is not as pronounced as that seen in severe neutropenia (Paz et al., 2011).

In conclusion the mechanism proposed by Thobakgale and Ndung'u (2014) of differential NK cell priming and function in DARC-null populations does not fully elucidate the association of DARC-null neutropenia with HIV-1 risk or disease progression. Only disparities in NK cell count and priming was noted in HIV negative donors, whilst no NK cell functional impairment was observed by DARC status independent of HIV status, indicating that NK cell priming is not altered in the presence of the DARC-null polymorphism and lower ANCs. Nevertheless, the mechanism proposed by Thobakgale and Ndung'u includes impairment of several cell types that are known to interact with neutrophils (Thobakgale and Ndung'u, 2014). Here we only

assessed this model with respect to NK cell phenotype and function, thus evaluation of other cell types in similar settings is warranted.

CHAPTER 4: NK CELL SURVIVAL AND PROLIFERATION IN DUFFY ANTIGEN RECEPTOR FOR CHEMOKINES (DARC)-NULL INDIVIDUALS

4.1 INTRODUCTION

Natural Killer (NK) cells are bone marrow-derived leukocytes that differentiate from lymphoid progenitors. Cell development occurs primarily in the bone marrow, although NK cell maturation has been reported in secondary lymphoid organs, before eventual migration to the peripheral tissues (Andrews and Smyth, 2010, Bernardini et al., 2013). NK cells are critical to the early detection of infection, transformation or stress through continuous surveillance of host tissue well before the development of adaptive immune responses.

The role of NK cells during infectious diseases is most notable in their defence against viral pathogens, particularly those viruses that are elusive to T-cell mediated activity. Interestingly, the impact of NK cells in such instances is highlighted by the evolution of certain viruses to employ strategies that evade specific NK cell mechanisms (Lisnic et al., 2010, Jost and Altfeld, 2012). The importance of NK cells in health and disease is further emphasised in individuals that are lacking in NK cell number and/or function. Deficiency of peripheral NK cells are implicated in susceptibility to viral infections that result in severe clinical outcomes (Orange, 2006, Orange, 2013).

Alterations to NK cell homeostasis is key to understanding NK cell deficiency and hypo-responsiveness. NK cells typically constitute 2-18% of the total circulating lymphocyte pool with a turnover of approximately 2 weeks. Homeostatic maintenance of circulating NK cell numbers is influenced by the generation of new cells within the bone marrow and the survival and proliferation of existing cells in circulation. Several factors tightly regulate these processes including cell-cell interactions and cytokine signals that are transmitted through cell surface receptors (Ranson et al., 2003, Sun and Lanier, 2011). Jaeger et al. (2012) described a regulatory role for neutrophils that ensures NK cell terminal maturation. Dissection of a neutrophil deficient mouse phenotype suggested impaired NK cell homeostasis, as displayed by poor survival and hyper-proliferation, which was associated with hypo-responsiveness in these cells. The essential role of neutrophils in NK cell dynamics are seemingly conserved across species (Jaeger et al., 2012) and thus necessitates the examination of NK cell biology in neutropenia-associated conditions in humans.

Most human neutrophil disorders in cell count and function are rare (Hsieh et al., 2010). Dissimilarly, ethnic neutropenia is extremely common on the African continent. A single nucleotide polymorphism (SNP) in the Duffy Antigen Receptor for Chemokines (DARC) promoter region results in selective loss of DARC on erythrocytes. While this DARC-null

polymorphism coincides with a phenotype that serves as a resistant factor to invasion of certain malarial parasites, it is also the causative genetic factor of ethnic neutropenia (Reich et al., 2009, Howes et al., 2011). DARC-null linked neutropenia overlaps strikingly with global HIV prevalence and has been associated with a 3-fold higher risk of HIV acquisition (Ramsuran et al., 2011).

The implications of DARC-null neutropenia on the NK cell compartment have been hypothesised (Thobakgale and Ndung'u, 2014), where low neutrophils are proposed to have a detrimental effect on NK cell biological processes important in optimal clearance of pathogens. Here we examined NK cell homeostasis in DARC-null individuals in a population with high HIV prevalence. Specifically we assessed NK cell survival and proliferation in cryopreserved peripheral mononuclear cells (PBMCs) from these individuals. We hypothesised that NK survival and proliferation would be impaired in the presence of the DARC-null trait and lower absolute neutrophil counts (ANCs).

4.2 METHODS

4.2.1 Participant Recruitment

HIV negative participants were recruited from the Females Rising through Education, Support and Health (FRESH) cohort and HIV-1 subtype C chronically infected treatment naïve participants were recruited from the HIV Pathogenesis Programme (HPP) Acute Infection Cohort as previously described in Chapter 2. In this analysis, a total of 36 individuals sub-divided into HIV negative donors (n=19) and HIV-1 chronically infected individuals (n=17) were assessed. These individuals were further stratified by DARC status.

4.2.2 ANCs, CD4 Counts and Viral Load

Blood for full blood counts, CD4+ T cell count measurement and viral load quantification was collected in Ethylenediaminetetraacetic acid (EDTA) anticoagulated vacutainer tubes (Becton Dickinson (BD), Franklin Lakes, New Jersey, USA). ANCs were enumerated by full blood count using the automated XN 1000 Haematology Analyser (Sysmex, Kobe, Hyōgo, Japan). CD4 counts were measured using BD Trucount and analysed on a four-parameter FACS Calibur flow cytometer (BD). Viral loads were determined using the NucliSENS EasyQ HIV-1 v2.0 kit with a detection limit of 20 copies/ml (BioMérieux, Marcy-l'Étoile, France).

4.2.3 Quantification of Antiretroviral (ARV) Drug in Plasma

This study aimed to recruit HIV-1 chronically infected, treatment naïve individuals. ARV therapy usage in chronically infected patients was self-reported. Certain subjects maintained viral loads below 1000 RNA copies/ml at the time of assessment. To rule out ARV drug use, plasma samples were collected from these study participants and screened for ARV drugs using a quantitative liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method. The method screened for nine ARVs, namely Emtracitabine, Tenofovir, Lopinavir, Ritonavir, Nevirapine, Abacavir, Lamivudine, Zidovudine and Efavirenz. A plasma sample volume of 50 µl was processed using a protein precipitation method, ARV drug analytes were chromatographically separated on a Agilent Zorbax Eclipse Plus C18 (2.1 x 50mm, 3.5 µm) HPLC column (Agilent Technologies, Santa Clara, California, USA), detected using an AB Sciex 5500 triple quadrupole mass spectrometer (Sciex, Framingham, Massachusetts, USA) and quantitated using Analyst® 1.6.2 software (Sciex).

4.2.4 DARC Genotyping

DARC -46T → C (rs2814778) SNP genotyping was performed by TaqMan allelic discrimination assays which has been previously verified by direct sequence analysis (Julg et al., 2009). Briefly, genomic DNA was isolated from stored buffy coats using the QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. DNA concentration was standardised at 50ng/µl with polymerase chain reaction (PCR) grade water. A cocktail containing Taqman Genotyping master mix (Life Technologies, Carlsbad, California, USA) and predesigned probes for the DARC gene (SNP ID: rs2814778, Applied Biosystems, Foster City, California, USA) was used to amplify target sequence in 50ng genomic DNA by real time PCR (RT-PCR) in the LightCycler 480 (Roche, Basel, Switzerland) according to the manufacturer's protocol.

4.2.5 PBMC Isolation, Cryopreservation and Thawing

Blood collected in sodium heparin BD Vacutainers (BD) from each participant was processed within 4 hours of collection. Whole blood was used for lymphocyte fraction preparation by density centrifugation on a Histopaque-1119 cushion (Sigma-Aldrich, St. Louis, Missouri, USA) at 800 x g for 20 minutes, low brakes as previously described (Brinkmann et al., 2010).

Cell counts were determined by 1:5 dilution with Trypan Blue Stain (Gibco) counted using a haemocytometer under a light microscope. Cells were prepared for cryopreservation by refrigerated centrifugation and re-suspended at a final concentration of 10 million cells/ml in ice cold freezing solution containing 1:10 Dimethyl Sulfoxide (DMSO) (Merck Millipore, Billerica, Massachusetts, USA) and heated inactivated Foetal Bovine Serum (FBS, Gibco). The frozen samples were stored in 2ml cryogenic vials in a liquid nitrogen Biorack ultralow freezer for long term storage and subsequently batched for NK cell survival and proliferation assays.

Cryopreserved cells were rapidly thawed, washed twice in pre-warmed R10 medium (RPMI Medium 1640 (Gibco) containing 10% gamma irradiated, heat inactivated FBS, 1% L-Glutamine, 1% Penicillin Streptomycin and 1% Hepes buffer 1M (all from Lonza, Basel, Basel-Stadt, Switzerland) at 500 x g for 8 minutes at room temperature. Cells were rested in R10 medium in a 37°C, 5% CO_2 incubator for at least 2 hours. Sample viability and cell counts were determined by trypan blue exclusion, and cells were re-suspended at 10 million cells/mL in R10 medium for subsequent assays.

4.2.6 NK cell Survival

NK cells were assessed for survival by plating one million PBMCs in 96-well plates with either R10 medium alone or in the presence K562 cell line (effector:target ratio of 10:1) and 10ng each of Recombinant Human Interleukin (IL)-2, Recombinant Human IL-15 (both from R&D Systems, Minneapolis, Minnesota, USA), and Recombinant Human IL-18 (Medical & Biological Laboratories, Japan) and incubated for 18 hours at 37°C, 5% CO_2.

Following overnight culture, cells were stained for surface markers with antibody cocktail containing anti-CD3 Allophycocyanin (APC, clone: UCHT1), anti-CD56 Alexa-Fluor-700 (clone: B159), anti-CD16 APC-Cy7 (clone: 3G8), anti-CD14 V500 (clone: M5E2) and anti-CD19 V500 (clone: HIB19, all from BD Biosciences). Fluorescein isothiocyanate (FITC) conjugated Annexin V and propidium iodide (PI) staining solution were included in the antibody cocktail in the presence Annexin V binding buffer (all from BD Biosciences) to detect for cell death via apoptosis and necrosis respectively. Cells were stained for 20 minutes at room temperature in the dark, washed in 2% FCS in Dulbecco's phosphate-buffered saline (dPBS) and centrifuged at 850 x g for 6 minutes. The supernatant was discarded and fixation Medium A (Invitrogen, Carlsbad, California, USA) added which ensured that the morphological characteristics of the cells remained intact. Cells were stored at 4°C until sample acquisition.

4.2.7 NK cell Proliferation

To assess for the replicative capacity of NK cells, PBMCs were washed once with DPBS to remove traces of FSC and centrifuged at 500 x g for 6 minutes. Pelleted PMBCs were stained with carboxyfluorescein succinimidyl ester (CFSE) dye (Invitrogen) for 7 minutes at 37°C with 5% CO_2. Cells were immediately washed with ice cold FSC to stop the reaction and centrifuged at 500 x g for 6 minutes. Cells were then washed once with R10 medium, centrifuged at 500 x g for 6 minutes and resuspended at 10 million cells/ml in R10 medium. One million PBMCs were plated in 96-well plates with either R10 medium alone or in the presence of cytokine mixture containing 10ng each of Recombinant Human Interleukin (IL)-2, Recombinant Human IL-15 (both from R&D Systems, Minneapolis, Minnesota, USA), and Recombinant Human IL-18 (Medical & Biological Laboratories, Japan) and incubated for 5 days at 37°C with 5% CO_2. R10 medium was changed on day 3 by removing 100µl supernatant from each well without disturbing the cell pellet and replaced with fresh R10 medium.

On day 5 cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen) to exclude dead cells, followed by cell surface staining with monoclonal antibodies: anti-CD3 APC (clone:UCHT1), Alexa-Fluor-700 conjugated anti-CD56 (clone:B159) and anti-CD16 APC-Cy7 (clone:3G8), anti-CD14 BV650 (clone: M5E2, Biolegend), anti-CD19 BV650 (clone: HIB19, Biolegend) before fixing with Medium A. Staining / fixation incubations were for 20 minutes at room temperature in the dark, followed by washing with Dulbecco's phosphate-buffered saline (dPBS) and centrifugation at 850 x g for 6 minutes. Cells were stored at 4°C until sample acquisition.

4.2.8 Sample Acquisition and Statistical Analysis

Samples were acquired on an LSR II flow cytometer, recording at least 250 000 events per sample. FlowJo Software Version 9 (TreeStar, Inc., Ashland, Oregon, USA) was used for sample analysis. For survival assays, fluorescence minus one (FMOs) for Annexin V and PI were prepared with each experiment to exclude background fluorescence in the gating strategies. For proliferation assays, unstimulated cells were used to subtract background from the stimulated cells for each patient sample. Differences between studied groups was examined by Mann-Whitney U test using GraphPad Prism Version 5 software (GraphPad software Inc., La Jolla, California, USA). Differences with a p value <0.05 were considered statistically significant.

4.3 RESULTS

To gain insight into the possible impact of the DARC-null trait on NK cell homeostasis, we examined the survival and proliferation of NK cells within our participant cohort. A total of 36 individuals were assessed, of which 19 individuals were HIV-uninfected (DARC-null=11 and DARC-positive=8), and 17 subjects were HIV-1 chronically infected (DARC-null=9 and DARC-positive=8) (Table 4.1).

Thirty five of thirty six subjects were female with a median age of 21 (IQR 19-22) and 23 (IQR 20-24) years in HIV negative and HIV infected patients respectively. Twenty participants (55.6%) were negative for the DARC allele. DARC-null individuals had significantly lower absolute neutrophil counts (ANCs) compared to DARC-positive individuals in both HIV negative and HIV-1 infected ($p=0.0006$ and $p=0.0003$ respectively, data not shown) subjects. CD4 counts were significantly lower in HIV infected persons compared to HIV negative individuals (median CD4 count of 653 and 892 cells/mm^3 respectively, $p=0.003$).

Assessment by DARC status, showed no differences in CD4 counts in HIV negative subjects (DARC-null = median CD4 count of 892 cells/mm^3; DARC-positive = median CD4 count of 919 cells/mm^3) or infected individuals (DARC-null = median CD4 count of 563 cells/mm^3; DARC-positive = median CD4 count of 743 cells/mm^3, Table 4.1). Further to this, no significant differences were observed in viral loads by DARC genotype in infected individuals (DARC-null = median viral load of 9500 RNA copies/ml; DARC-positive = median viral load of 11500 RNA copies/ml, Table 4.1).

Table 4.1 Clinical Characteristics of Study Participants used to assess NK cell Survival and Proliferation

	HIV Negative			HIV Positive		
	DARC CC (n=11)	DARC TC/TT (n=8)	p value	DARC CC (n=9)	DARC TC/TT (n=8)	p value
Absolute Neutrophil Count, 10^3 cells/mm^3	**2.80** (2.11-3.52)	**4.99** (4.48-5.73)	0.0006	**1.77** (1.52-2.25)	**4.03*** (3.23-4.90)*	p=0.0003
CD4 count, cells/mm^3	**892** (770-984)	**919** (693-1066)	0.8	**563** (487-729)	**743** (607-848)	0.14
Viral Load, RNA copies/ml	na	na	na	**9500** (2650-30500)	**11150** (700-95250)	0.96

Data is represented as median (IQR). *ANC median and IQR was calculated from 7 participants (ANC was not available for 1 participant). Abbreviations: DARC, Duffy Antigen Receptor for Chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute Neutrophil Count; IQR, Interquartile range; n, number; na, not applicable. Female participants: n=35, Male participants: n=1.

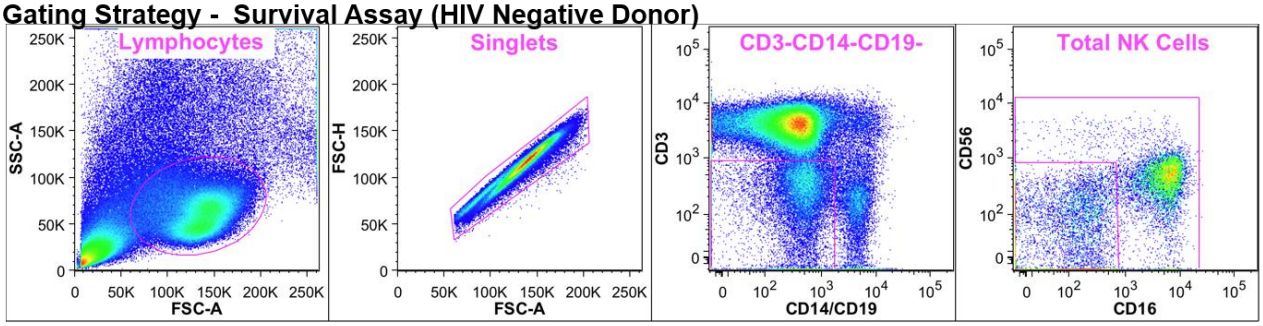
4.3.1 Reduced survival of activated NK cells in HIV infected individuals with DARC-null genotype

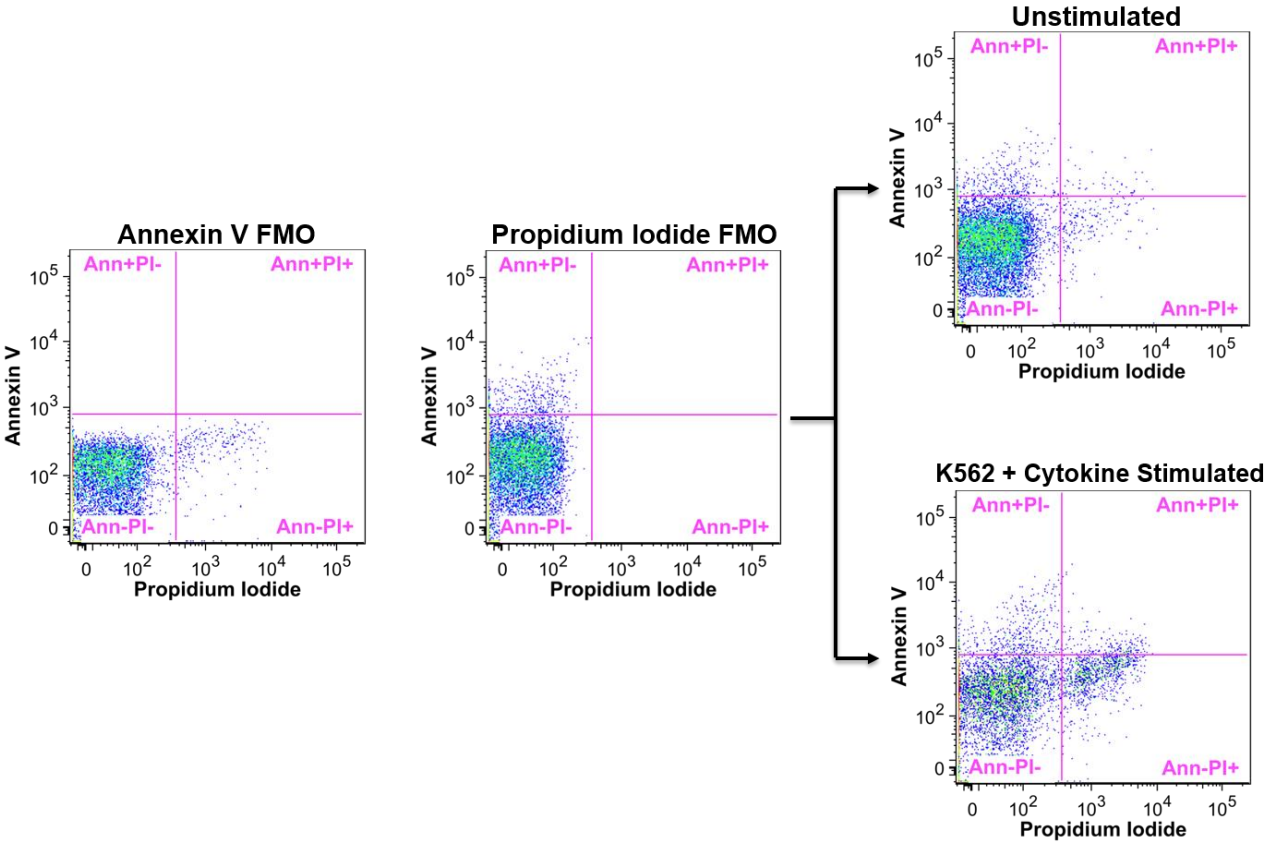
Cell death can proceed either through the apoptotic or necrotic pathways. Cellular activation can increase cell sensitivity to apoptosis (programmed cell death) and activation-induced cell death (Childs and Berg, 2013). We used a combination of Annexin V and propidium iodide staining to detect NK cells undergoing either apoptosis or necrosis following activation with K562 cell line and cytokines. NK cells that survived overnight stimulation were negative for Annexin V and propidium iodide staining (Figure 4.1A).

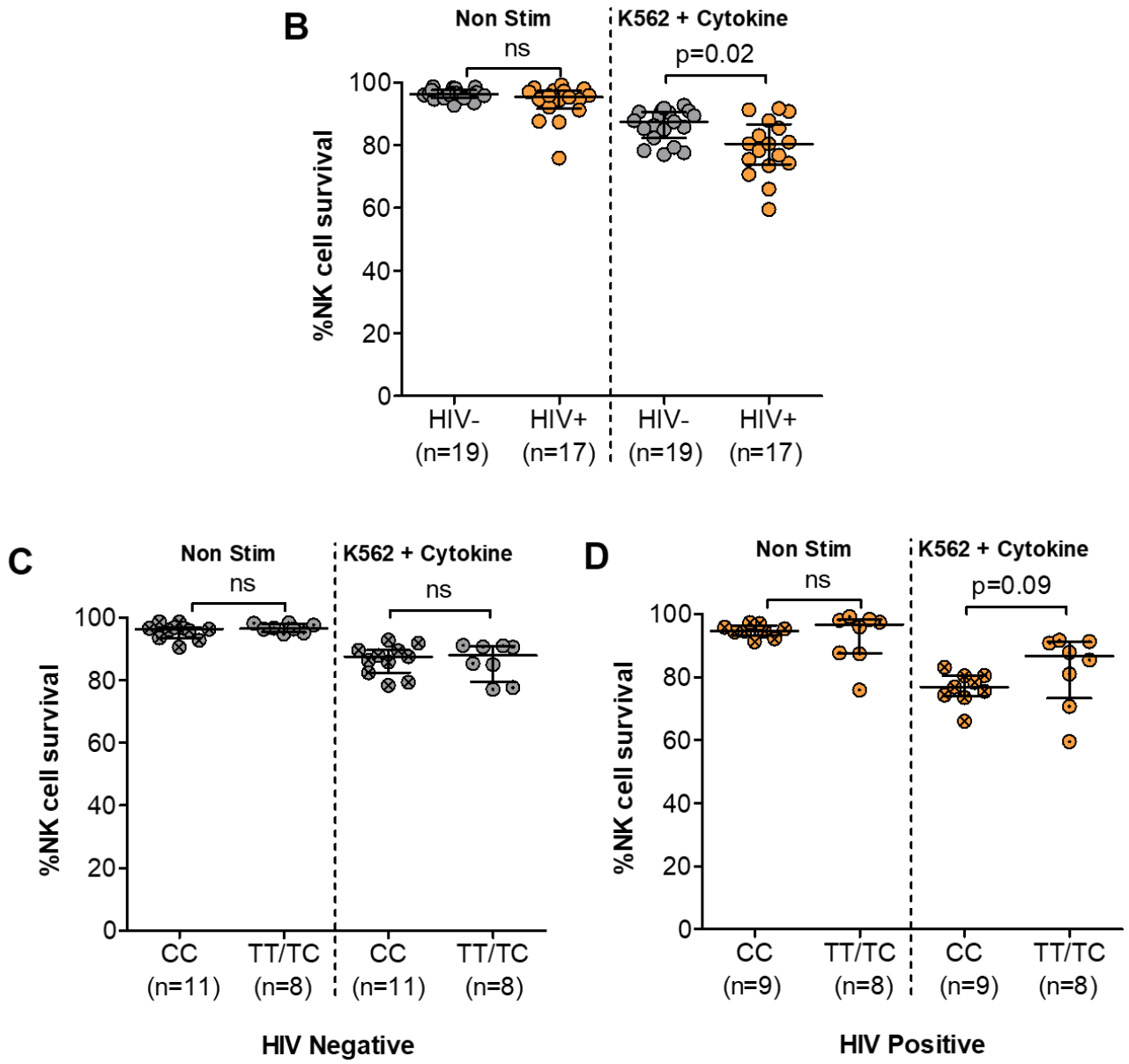
We observed similar median frequencies of NK cell survival of 96% in HIV negative and 95% in HIV-infected individuals when cells were left untreated (data not shown). Median frequencies of total NK cell survival were significantly higher in HIV negative (87%) compared to HIV infected individuals (80%) following cellular activation with K562 cell line and cytokines ($p=0.02$, Figure 4.1B). HIV negative individuals grouped by DARC trait showed no differences in the frequency of NK cell survival under untreated or stimulated conditions (Figure 4.1C). Ex vivo assessment of NK cell survival showed no differences by DARC status in HIV chronically infected persons. However, a trend of lower NK cell survival was noted in activated NK cells from DARC-null individuals after stimulation with K562 cell line and cytokines compared to DARC-positive participants (Figure 4.1D; $p=0.09$). Further examination showed no association between NK cell survival and ANCs or DARC genotype in either HIV negative or HIV infected individuals (Figure 4.1E).

Together the data suggests that NK cell survival is unaffected by DARC status in HIV negative individuals. However, survival of activated NK cells is reduced during HIV infection, and this is more evident in DARC-null individuals where there tended to be lower NK cell survival compared to DARC-positive persons.

A Gating Strategy - Survival Assay (HIV Negative Donor)






B
Non Stim
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TT/TC
(n=8)
CC
(n=11)
TT/TC
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HIV Negative
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K562 + Cytokine
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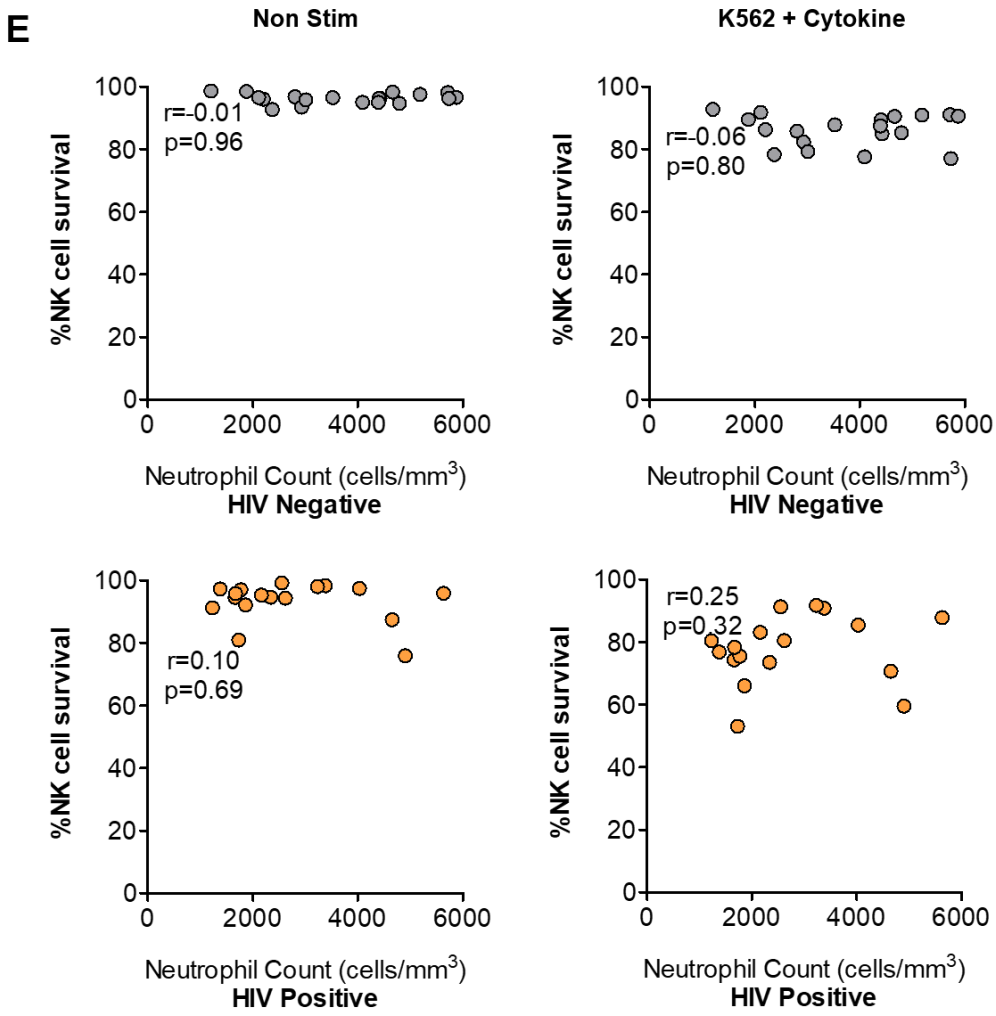


Figure 4.1 Total NK cell survival in HIV negative and HIV infected donors

Representative gating strategy employed to assess NK cell survival using multi-parametric flow cytometry. Initial gating was on side scatter area (SSC-A) vs forward scatter area (FSC-A) to identify lymphocytes followed by exclusion of doublets by forward scatter height (FSC-H) vs FSC-A, and exclusion of T-cells, B-cells and monocytes using anti-CD3, anti-CD19 and anti-CD14 monoclonal antibodies. Total NK cells were identified as CD56 positive and/or CD16 positive cells. Survival was assessed by staining with Annexin V (Ann) and Propidium Iodide (PI). FMO samples for Ann and PI were used to adjust the quadrant gates to identify staining combinations Ann+PI-; Ann+PI+; Ann-PI+ and Ann-PI- from total NK cells. The gating strategy was applied to unstimulated and K562+cytokine stimulated samples for each participant. NK cells that survived were identified as Ann-PI-. **(B)** Percentage of NK cell survival for unstimulated and K562+cytokine stimulated cells in HIV negative and HIV infected individuals. Participants were further stratified to show percentage of NK survival for unstimulated and K562+cytokine stimulated cells in HIV negative **(C)** and HIV infected **(D)** individuals by DARC trait. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences by HIV or DARC status. **(E)** Correlations between absolute neutrophil counts (ANCs) and NK survival for unstimulated and K562+cytokine stimulated cells in HIV negative and HIV infected individuals. Dots indicate individual data points. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute Neutrophil Count; Ann, Annexin V; PI, Propidium Iodide; IQR, interquartile range; ns, p-value not significant; r, Spearman rho.

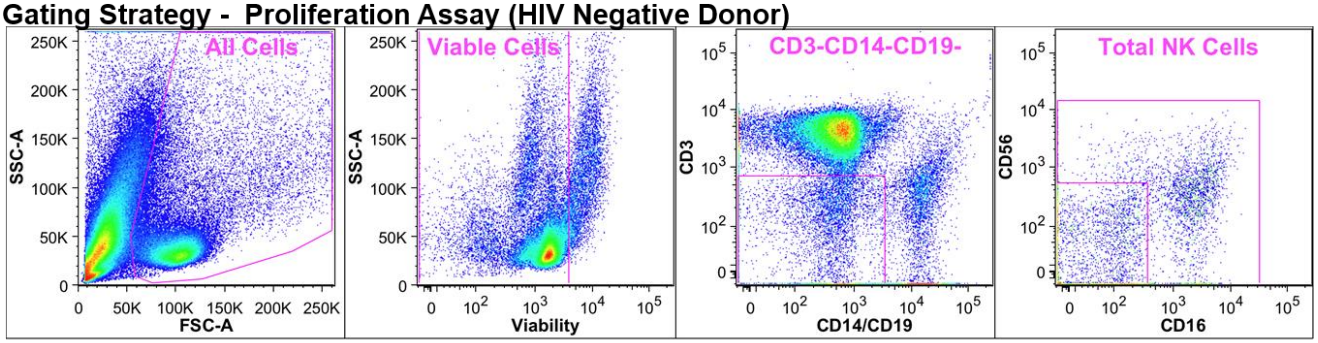
4.3.2 No differences in NK cell proliferation by HIV or DARC status in studied individuals

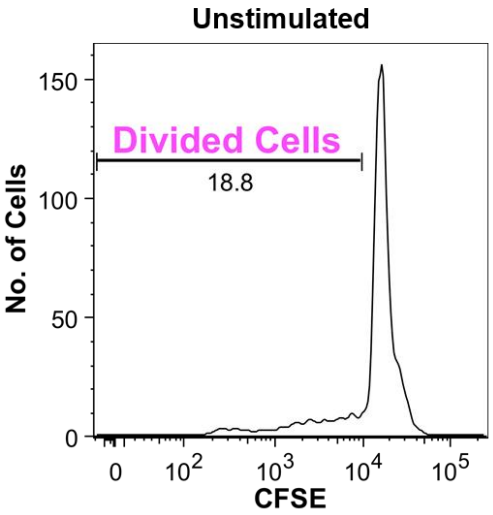
Peripheral NK cell proliferation levels are relatively low at physiological state. Upregulation of pro-proliferative cytokines that utilise the common gamma chain drives NK cell expansion. To investigate NK cell proliferation we employed CFSE labelling of PMBCs to track NK cell divisions in response to cytokines that are known facilitate NK cell activation and generation. Antigen-induced cellular proliferation is initiated during HIV infection. We thus hypothesised that HIV infected individuals in our cohort would have higher levels of dividing NK cells.

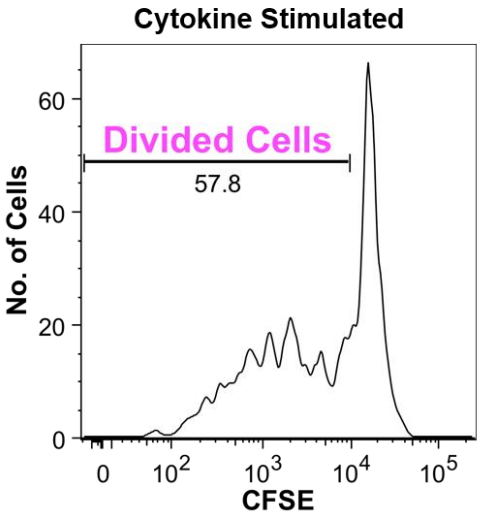
NK cell proliferation due to activation was measured by subtracting CFSE divisions in unstimulated from CFSE divisions in cytokine stimulated NK cells (Figure 4.2A). In contrast to our hypothesis, we observed similar levels of NK cell expansion following cytokine induction in HIV negative and HIV infected subjects where median NK cell proliferation frequencies were 50% and 45% respectively (Figure 4.2B). As verification of our data we assessed NK cell CFSE divisions between HIV negative and HIV infected individuals in unstimulated cultures alone and stimulated cultures without subtraction of unstimulated divisions. Consistent with our initial observation, we did not detect differences between HIV negative and HIV infected individuals in either case. Median NK cell proliferation frequencies were 16% and 14% in HIV negative and HIV infected individuals for unstimulated cultures, and 69% and 58% in HIV negative and HIV infected individuals in stimulated cultures (data not shown). Furthermore, we detected no differences in NK cell proliferation by DARC trait or ANCs in both HIV negative (Figure 4.2C and Figure 4.2E) and chronically infected subjects (Figure 4.2D and Figure 4.2F).

Together our results shows no difference in NK cell proliferation by HIV status. Furthermore the data indicates that DARC status and ANC levels do not impact on NK cell proliferation in HIV negative and infected individuals.

A Gating Strategy - Proliferation Assay (HIV Negative Donor)







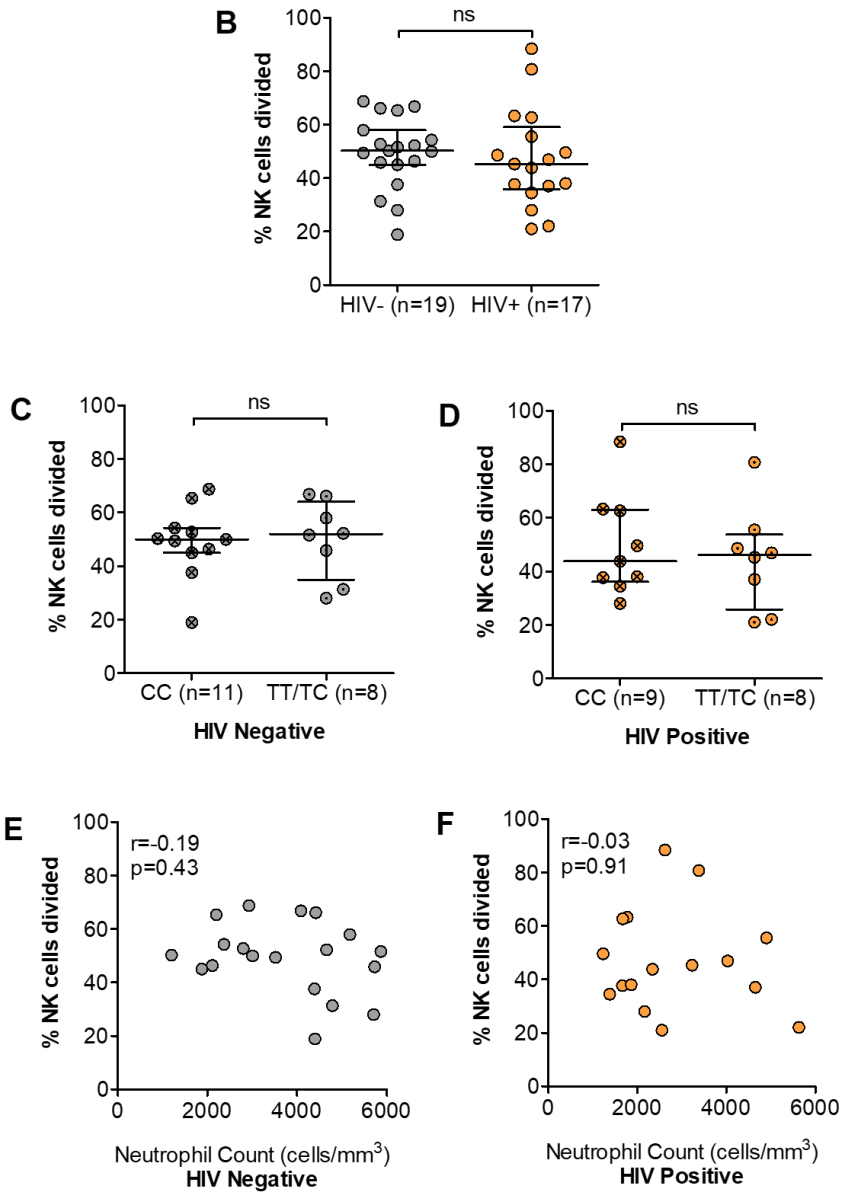


Figure 4.2 Total NK cell proliferation in HIV negative and HIV infected donors

Representative gating strategy employed to assess NK cell proliferation using multi-parametric flow cytometry. Gating on side scatter area (SSC-A) vs forward scatter area (FSC-A) to identify all cells was followed by exclusion of non-viable cells using viability dye. T-cells, B-cells and monocytes were excluded and total NK cells identified as described in Figure 1 above. Proliferation was assessed on CFSE stained NK cells where divided cells are identified as cells with lower CFSE fluorescence compared to undivided cells. Divided cells in unstimulated samples were subtracted from divided cells in K562+cytokine stimulated samples for each participant. **(B)** Percentage of NK cells divisions in HIV negative and HIV infected individuals. Participants were further stratified to show percentage of NK cells divided in HIV negative **(C)** and HIV infected **(D)** individuals by DARC trait. Dots indicate individual data points. Medians are indicated and extended to interquartile range (IQR) with whiskers. The p-values as indicated by ns refer to no significant differences by HIV or DARC status. **(E)** Correlations between absolute neutrophil counts (ANCs) and NK cells divisions in HIV negative and HIV infected individuals. Dots indicate individual data points. Abbreviations: DARC, Duffy antigen receptor for chemokines; CC, DARC-null; TC/TT, DARC-positive; ANC, Absolute Neutrophil Count; CFSE, carboxyfluorescein succinimidyl ester; IQR, interquartile range; ns, p-value not significant; r, Spearman rho.

4.4 DISCUSSION

Our previous data as part of the current investigation showed that the DARC-null polymorphism and lower ANCs were associated with lower peripheral NK cell numbers in HIV negative individuals (Chapter 3). Reduced NK cell count and/or dysfunction can arise from modulation of NK cell homeostatic activities. In an effort to explain our previous findings, we here investigated the possible influence of the DARC-null trait on NK cell survival and proliferation in HIV negative and HIV-1 infected persons in a DARC prevalent population.

We observed no differences in spontaneous (unstimulated) NK cell survival by HIV status. Conversely, activated NK cells from HIV infected subjects exhibited lower survival compared to NK cells from HIV negative individuals. Moreover, examination by DARC genotype indicated that absence of DARC may be a contributing factor in reduced NK cell survival following activation in HIV infection but not in HIV negative individuals. Further assessment of this cohort showed that NK cell proliferation did not differ by HIV status or DARC trait.

The pathological changes in NK cell homeostatic activities during chronic HIV infection have been previously described (Mikulak et al., 2017). Past studies have indicated progressive loss of NK cells through programmed cell death during HIV infection (Douglas et al., 2001, Meier et al., 2005, Kottilil et al., 2007) where NK cell death susceptibility has been attributed to the upregulation of genes that promote apoptosis as a consequence of HIV envelope protein exposure (Kottilil et al., 2006, Kottilil et al., 2007). In vitro assays revealed that activation-induced apoptosis contributes mostly to this accelerated cell death in viremic individuals, whereas spontaneous NK cell death accounted for less than 5% *ex vivo* irrespective of HIV status (Kottilil et al., 2007). Here we report similar findings in our participant cohort. We observed minimal spontaneous cell death in HIV negative and chronically infected individuals. Furthermore, upon NK cell activation with MHC devoid cell line and cytokines, NK cells from HIV infected individuals exhibited reduced survival in comparison to NK cells from HIV negative subjects.

NK cell proliferation rates are dependent on opposing factors during HIV infection. Upregulation of IL-15 serum levels during retroviral infection are known to drive rapid NK cell expansion and result in higher NK cell numbers (Zhao and French, 2012, Littwitz-Salomon et al., 2016). On the other hand, soluble protein modulation and various cell interactions in viremic individuals have been shown to reduce proliferation of NK cells (Mavilio et al., 2006,

Gasteiger et al., 2013). Furthermore HIV encoded proteins have been demonstrated to directly decrease NK cell proliferation. Experiments mimicking the HIV envelope concentrations detected in AIDS patients demonstrated diminished NK cell proliferation responses through upregulation of genes associated with suppression of cellular expansion (Kottilil et al., 2006). In comparison, we did not observe different levels of cytokine-induced NK cell proliferation between HIV negative and infected subjects. Since HIV viremia is a contributing factor to either supressing or inducing NK cell expansion, the especially low viral loads measured in the cohort participants could explain the absence of differences in proliferation levels by HIV status.

The impact of low peripheral neutrophils on NK cell homeostasis in humans is lacking. In experiments conducted by Jaeger and colleagues (2012), mice that were deficient of peripheral neutrophils were shown to have impaired NK cell homeostasis. This was exhibited by higher NK cell proliferation rates that were counterbalanced by increased sensitivity to cell death. Interestingly, these changes in cellular homeostasis were limited to NK cells, whereas T cell and B cell compartments were unaffected. Though these NK cell homeostatic alterations were demonstrated in mouse models, the authors indicated a conserved role for neutrophils in NK cell biology across species that was independent of the genetic aetiology responsible for the neutropenic phenotype (Jaeger et al., 2012). Thus we hypothesised that low circulating neutrophils due to the DARC-null polymorphism would confer altered NK cell survival and proliferation levels. However, surprisingly, we detected no changes in most homeostatic activities in the absence of DARC. The only exception was that activated NK cells from DARC-null HIV infected individuals exhibited reduced survival than NK cells from their DARC-positive counterparts. While only two of our DARC-null participants had neutrophil counts that were indicative of ethnic neutropenia (<1500 cells/mm^3), no association was made between absolute neutrophil counts and NK cell survival or proliferation in either HIV negative or HIV infected subjects. It has been suggested that deficiency of neutrophils in both bone marrow and circulation are required to observe severe modifications within the NK cell compartment (Jaeger et al., 2012). The relatively higher neutrophil counts in our cohort compared to individuals with other types of neutropenia could explain the unaltered observations in most NK cell homeostatic activities measured in our study.

Here we were limited by a small sample size and that many of the chronically infected patients studied had moderately lower viral loads (<1000 RNA copies/ml) than previously described chronic patients. Experiments executed in this chapter were planned according to findings in

the previous chapter. In order to maintain the same sampling cohort, we were restricted by the availability of cryopreserved cells from each participant.

In summary we deduce that lower absolute neutrophil counts associated with the DARC-null polymorphism have marginal effect on the survival of NK cells. Further we attest that the capacity of activation-induced NK cell proliferation is unaffected in DARC-null persons. We conclude that any NK cell homeostatic defects associated with neutropenia are either mild or not apparent in African persons with the DARC-null trait. Overall these results indicate that the reduced NK cell counts observed in HIV negative DARC-null individuals (Chapter 3) was not due to impaired cell survival or proliferation. This work focused on the impact of low circulating neutrophils on NK cell survival and proliferation. Beyond the scope of this study, other factors that drive lower peripheral NK cell counts may be at play including diminished NK cell release from the bone marrow or altered trafficking of NK cells between tissues and circulation (Sun and Lanier, 2011). Nonetheless, the observations presented here are in line with our earlier results which suggested that the DARC-null trait does not have a detrimental effect on NK cell functionality. Altered NK cell homeostasis has been associated with NK cell hypo-responsiveness. Together with our Chapter 3 results we demonstrate that there is no evidence for either NK cell functional or homeostatic impairments due to the DARC-null polymorphism or lower ANCs in persons of African ethnicity. The findings presented here support previous literature that suggest that ethnic neutropenia does not confer clinical disadvantage (Haddy et al., 1999, Grann et al., 2008, Hsieh et al., 2010), indicating that individuals with the DARC-null polymorphism are able to mount an appropriate NK cell immune response to infection.

CHAPTER 5: GENERAL DISCUSSION, LIMITATIONS, FUTURE DIRECTIONS AND CONCLUSION

5.1 GENERAL DISCUSSION

The DARC-null allele is the principal genetic factor contributing to benign ethnic neutropenia which is common in 25-50% persons of African ethnicity (Reich et al., 2009, Haddy et al., 1999). Whilst the condition does not confer a clinical disadvantage (Hsieh et al., 2010), literature advocates that the DARC-null polymorphism and the associated neutropenia predisposes individuals to increased HIV susceptibility (He et al., 2008, Ramsuran et al., 2011) and mother-to-child transmission (Kourtis et al., 2012). Conversely, the effect of the DARC-null trait in relation to HIV disease progression is controversial. Few studies suggest slower HIV disease progression in the absence of DARC (He et al., 2008, Kulkarni et al., 2009). This is highly debated by reports of no association between DARC-null polymorphism and rate of disease progression (Horne et al., 2009, Julg et al., 2009, Walley et al., 2009).

Several proposed mechanisms have attempted to clarify the possible associations between the DARC genotype and progression to AIDS. Among these, Thobakgale and Ndung'u (2014) suggested a role for DARC-null linked ethnic neutropenia in HIV disease progression. While lower ANCs in DARC-null individuals may plausibly confer inadequate pathogen killing, it was further postulated that absence of the DARC allele may further compromise other unknown mechanisms. Additionally, it was hypothesised that lower circulating neutrophils would result in fewer immuno-regulatory interactions and inefficient priming of other cellular compartments. In the context of HIV infection, these immune deficiencies would lead to suboptimal pathogen clearance and failure to control pathogen spread, thus adversely contributing to disease progression (Thobakgale and Ndung'u, 2014).

This study aimed to explore the question of whether DARC-null genotype compromises immune responses in African individuals with and without HIV infection; in particular we investigated the impact of the DARC-null genotype and its associated low ANCs on key neutrophil effector functions. We further assessed the role of DARC-null on NK cell priming by measuring NK cell phenotypic maturation / differentiation markers and functional activity. Lastly, we examined the capacity of NK cells to survive and proliferate in DARC-null individuals.

Our results demonstrated the following main findings; firstly, enhanced neutrophil phagocytosis was observed in DARC-null individuals with and without HIV infection demonstrating that this function was a common characteristic in individuals with the DARC-null allele irrespective of infection. Other neutrophil functions such as ROS and NET release

did not differ significantly by DARC state, with the exception that we detected lower NET formation in DARC-null HIV infected persons following sustained stimulus exposure (Chapter 2). Secondly, HIV negative individuals with the DARC-null trait displayed lower NK cell frequencies which strongly associated with lower ANCs. Furthermore, lack of the DARC phenotype in HIV negative subjects associated with a less differentiated NK cell profile. However, examination of NK cell functionality in these DARC-null HIV negative individuals showed no measurable impact on NK cell degranulation or cytokine expression. The differences observed in HIV negative persons by DARC state were undetectable in HIV infected individuals where no differences in NK cell frequency, differentiation or function were observed (Chapter 3). To determine the mechanistic link between DARC-null and lower NK cell counts in HIV negative persons, we lastly demonstrated that NK cell survival was lower in DARC-null individuals, although this outcome was only observed in individuals with HIV infection. Moreover, we found no differences in proliferation of NK cells by DARC state in individuals with and without HIV infection (Chapter 4). The findings from this study are discussed in detail below with reference to literature:

5.1.1 DARC-null polymorphism imparts differential neutrophil functionality

Neutrophils are recognized for their killing mechanisms and disorders of neutrophil count and function are associated with recurrent pathogenic infections (Hsieh et al., 2010, Mocsai, 2013, Nauseef and Borregaard, 2014). While the absence of the DARC genotype is associated with lower circulating ANCs that is characteristic of ethnic neutropenia (Reich et al., 2009), studies exploring the influence of this polymorphism on neutrophil function are lacking. Thus, in an analysis of 20 HIV negative and 22 HIV-1 infected individuals, we set out to determine the possible impact of the DARC-null trait and ethnic neutropenia on key neutrophil effector mechanisms namely, Fc receptor mediated phagocytosis, ROS and NET production.

Impairment of neutrophil responses manifest during late stages of HIV infection when HIV-1 plasma concentrations escalate and CD4 lymphocyte counts are significantly reduced (Michailidis et al., 2012, Casulli and Elbim, 2014). Evaluation of participants in this study indicated a trend for lower phagocytic activity in HIV infected individuals; however, neither ROS nor NET production were altered in HIV infection. Clinically, the individuals studied here exhibited low viral loads and moderate CD4 counts which are not reflective of advanced

disease, and this could partly explain our observation of no significant differences in neutrophil phagocytosis or levels of ROS production in HIV infection.

Unlike other neutrophil functions, data on NET formation with respect to HIV infection is lacking and whether this mechanism is altered following infection remains unclear. No differences in NET release were detected here between HIV negative and infected subjects. It is worth mentioning that individuals in the HIV infected group presented with lower viral loads than that typical of chronic infection while it has been reported that higher viral load concentration is a major factor in relation to neutrophil deterioration (Mastroianni et al., 1999). Thus, our results need to be interpreted with caution as the effect of HIV infection on NET formation may be under estimated in this cohort and requires further investigation.

Stratification of our study cohort by DARC trait indicated high occurrence of the DARC-null polymorphism in the Zulu/Xhosa African population which is in agreement with past studies (Julg et al., 2009, Ramsuran et al., 2011). We further assessed neutrophil functionality by DARC state, hypothesising that neutrophil responses would be impaired in the absence of the DARC genotype. Conversely, we demonstrated rapid and enhanced Fc receptor mediated phagocytosis in DARC-null individuals. Interestingly, this activity was more prominent in HIV infected individuals suggesting that this heightened function was a common characteristic in individuals with the DARC-null polymorphism. Fc receptor upregulation in DARC-null neutrophils from HIV negative individuals has been previously described (Duchene et al., 2017) and may explain the heightened phagocytic activity demonstrated in DARC-null Africans in our study. Overall this superior response may be advantageous by promoting faster pathogen clearance in DARC-null individuals.

Examination of ROS and NET formation indicated higher median activity in response to stimulus in the presence of the DARC-null polymorphism in individuals with and without HIV infection. This result suggested that neutrophil activation was more easily induced in neutrophils from DARC-null persons, an observation that is supported by previous data that indicated marginally activated neutrophil profiles in DARC-null individuals with benign ethnic neutropenia (Charles et al., 2018). However, statistical analysis of ROS and NET production generally displayed no significant differences by DARC trait irrespective of HIV status. An exception to this observation was the lower NET production exhibited after prolonged activation in DARC-null neutrophils from HIV infected individuals. This finding suggests that persistent inflammation during HIV infection may induce accelerated neutrophil cell

exhaustion and/or death in DARC-null individuals. Though lower NET production in these individuals would reduce pathogen killing, diminished NET formation could minimise tissue damage by NETs in these individuals (Chen and Nunez, 2010, Barnado et al., 2016).

5.1.2 DARC-null polymorphism does not adversely affect NK cell priming

NK cells are innate lymphoid cells that are crucial for cytolytic killing and bridging innate and adaptive immunity (Lanier, 2005, Vivier et al., 2008). Recent data suggests that NK cell priming is dependent on immune-regulatory interactions with mature neutrophils (Jaeger et al., 2012). We thus investigated the possible impact of DARC-null associated neutropenia on NK cell profiles, hypothesising that low circulating neutrophils would adversely affect NK responses and homeostasis.

Assessment of NK cell dynamics in our study participants showed similar NK cell counts irrespective of HIV status, although HIV-1 infected individuals displayed NK cell subset redistribution marked by higher CD56 negative cells. Past studies including from our group of African individuals have described diminished CD56 bright NK cells and an accumulation of the aberrant CD56 negative subset in HIV infection (Alter et al., 2005, Jost and Altfeld, 2012, Zulu et al., 2017). To determine NK cell maturity during HIV infection, we profiled NK cells comprehensively using three NK cell maturation / differentiation models. Past studies have described higher proportions of mature NK cells in HIV infected individuals in relation to CD57 expression (Hong et al., 2010, Kaczmarek et al., 2017) and lower proportions of NKG2A and CD27 expressing NK cells (Beziat et al., 2010, Kaczmarek et al., 2017). In contrast, we associated HIV infection with marginally higher frequencies of less mature NK cells. We reasoned that dissimilarities in our and past reports was owing to our different profiling approaches. Most studies report on CD56 positive NK cell repertoires whilst our analyses incorporated total NK cells including the dysfunctional CD56 negative subset. We previously noted reduced CD57 expression on the CD56 negative subset of NK cells from chronically infected persons (Zulu et al., 2017). These phenotypic alterations within this CD56 negative subset could account for the differences observed between past reports and our observations in this cohort.

Consistent with previous studies (Kuri-Cervantes et al., 2014, Beldi-Ferchiou et al., 2016), we demonstrated higher expression of markers of activation (HLA-DR) and exhaustion (PD-1) on NK cells from HIV infected subjects. However, in contrast to literature (Alter et al., 2004b,

Wong et al., 2010), the phenotypic alterations observed in NK cells during HIV infection was accompanied by lower cytolytic potential (measured by degranulation marker CD107a) and no differences in cytokine production (IFN-ɣ and TNF-α) of these cells. Chronic HIV infection is characteristically associated with higher cytolytic potential and cytokine secretion which tend to correlate with plasma viremia (Mikulak et al., 2017). Unusually low viral loads exhibited by chronically infected individuals and higher proportions of the dysfunctional CD56 negative NK cell subset in our cohort probably contributed to our contradictory findings.

Limited reports describe the effect of neutrophil deficiency on NK cell dynamics (Sporri et al., 2008, Jaeger et al., 2012, Ueda et al., 2016), and to our knowledge, none have explored this mechanism in DARC-null African individuals in the context of HIV infection. Jaeger et al. (2012) previously examined NK cell profiles in patients diagnosed with severe congenital neutropenia (SCN) and autoimmune neutropenia (AIN). Though comparable NK cell counts were observed in controls and those with neutrophil deficiency, it was revealed that neutropenia associated with higher proportions of less mature CD56 bright subset coupled to impaired cell cytotoxicity (Jaeger et al., 2012). In contrast, we demonstrated lower NK cell counts in HIV negative DARC-null individuals with lower ANCs. Similar to SCN and AIN patients (Jaeger et al., 2012), DARC-null linked neutropenia associated with elevated frequencies of the CD56 bright subset and a less mature / differentiated NK cell phenotypic profile in HIV negative individuals that was indicative of hypo-responsive NK cells.

Surprisingly, despite our findings of a less differentiated NK cell profile in DARC-null HIV negative individuals, we detected no differences by activation and exhaustion status *ex vivo* in NK cells from these individuals compared to their DARC-positive counterparts. Furthermore, we demonstrated that cytolytic potential, cytokine production and antibody dependent cytotoxicity were unaffected by the DARC-null trait in HIV negative subjects. Interestingly, it has been reported that hypo-responsive NK cells can be re-educated and become fully functional following cytokine stimulation (Beziat et al., 2010). While HIV negative DARC-null individuals here exhibited a less differentiated NK cell repertoire *ex vivo*, it is plausible that *in vitro* cytokine stimulation culminated in fully differentiated NK cells with optimal effector activities, and may account for comparable NK cell cytotoxicity and cytokine secretion by DARC state.

Interestingly, most of the observations made in the context of HIV infection above were not associated with absence of the DARC trait. The observed phenotypic and functional differences

associated with HIV infection were lost upon stratification by DARC genotype and NK cell frequencies, differentiation and functionality were comparable in DARC-null and DARC-positive HIV infected individuals.

Jaeger et al. (2012) previously reported that neutrophil deficient mouse models demonstrated increased cell death sensitivity which was counterbalanced with hyper-proliferative capacity, resulting in unaltered NK cell frequencies (Jaeger et al., 2012). As earlier mentioned, analysis of HIV negative individuals here showed reduced NK cell counts associated with lower ANCs in DARC-null HIV negative subjects, suggesting modified peripheral NK cell homeostasis in DARC-null HIV negative individuals. Thus in an effort to explain our previous findings, we here investigated the possible influence of the DARC-null trait on NK cell survival and proliferation in HIV negative and HIV-1 infected.

Past studies have shown that NK cells are more susceptible to cell death with progressive loss of these cells in HIV infection (Douglas et al., 2001, Meier et al., 2005). Furthermore, modified cytokine-induced NK cell proliferation has been noted in chronically infected individuals (Mavilio et al., 2006, Zhao and French, 2012). Here we report lower NK cell survival upon activation amongst infected subjects. However, in contrast to literature, we observed no differences in cytokine induced NK cell expansion by HIV status. HIV viremia contributes to altered NK cell expansion (Kottilil et al., 2006) and thus our unexpected observation was probably a consequence of the low plasma viremia exhibited by infected individuals in our cohort.

Further analysis of NK cell survival and proliferation by DARC trait indicated a trend of lower NK cell survival in HIV infected DARC-null persons, however, this difference was not measured by DARC state in HIV negative subjects. Thus, contradictory to our hypothesis, we concluded that absence of DARC had no apparent or mild effects on NK survival. Lastly, we found that NK cell expansion was not altered by DARC-null neutropenia irrespective of HIV status. Deficiency of neutrophils in both the bone marrow and circulation are required to detect severe modifications within the NK cell compartment (Jaeger et al., 2012). The relatively higher neutrophil counts in our cohort compared with other types of neutropenia could account for the intact NK cell homeostatic activities (survival and proliferation) measured in our study. Furthermore, other factors that drive lower peripheral NK cell counts may play a role including diminished NK cell release from the bone marrow or altered trafficking of NK cells between tissues and circulation (Sun and Lanier, 2011).

5.2 STUDY LIMITATIONS

While the data presented here provides evidence of immune variation in the absence of the DARC allele, particularly in relation to neutrophil function, our study was limited by numerous factors. Neutrophils are a fragile immune cell subset and research on these cells has been previously hindered due to their experimentally inflexible nature (Amulic et al., 2012). The time interval between blood collection and processing was longer than the ideal conditions when assessing neutrophil activity; however, this time period was consistent for all samples. Many of the limitations of this study were cohort driven. Since this was intended as a cross sectional, pilot study, our cohort size was small and with respect to our NK cell experiments, we were restricted to cryopreserved samples. We were further restricted by the availability of HIV chronically infected samples from antiretroviral therapy naïve individuals, and the time of infection was unestablished in these infected individuals. Since many changes to immune cell dynamics occur during early infection, it is plausible that advantages, specifically in terms NK cell frequency and maturation that were found in DARC-positive HIV negative individuals were lost during chronic infection. However, without longitudinal data, we were unable to fully appreciate the effect of DARC-null neutropenia on NK cells from infected persons and whether these effects contributed to HIV disease progression. We also noted that our infected cohort consisted of individuals with relatively lower viral loads than those previously described in HIV chronically infected patients. Thus our observations in this HIV infected group may not be representative of typical disease progression in the absence of antiretroviral therapy. Further sample collection from treatment naïve individuals became increasingly challenging during the study due to the implementation of the HIV test and treat policy. The DARC-null polymorphism is a predictor of ethnic neutropenia, however many of our DARC-null subjects were not neutropenic as defined by ANC measurements of <1500 cells/mm^3. Furthermore, these patients were not followed longitudinally, and since repeated ANC measurements are lacking, we cannot determine which patients possessed neutropenic characteristics. Despite these limitations, our study was able to detect and, for the most part, confirm past observations pertaining HIV-1 infection. Further, we were able to detect neutrophil response variation in the absence of DARC, and NK cell phenotypic differences that associated with the DARC-null genotype and lower ANCs.

5.3 FUTURE DIRECTIONS

Neutrophils are primarily identified for their killing mechanisms that contribute to first line immunity. Our study focused on three key neutrophil killing responses (phagocytosis, ROS and NET production), although other mechanisms such as degranulation and soluble protein production should be assessed in the context of the DARC-null trait in futures studies. Our study suggested no detrimental impact of the DARC-null genotype on the above mentioned neutrophil functions. In fact, phagocytic data suggest that this activity is enhanced in the presence of the DARC-null polymorphism.

Recent HIV vaccine studies have highlighted the importance of Antibody-Dependent Cellular Cytotoxicity (ADCC) responses in HIV protection. Interestingly, neutrophils have been found to mediate both HIV-specific Antibody-Dependent Phagocytosis (ADP) and ADCC responses in vitro; and these responses were exhibited much faster than monocyte or NK cell-mediated responses (Worley et al., 2018). Furthermore, passive immunotherapy administration in mouse models revealed that neutrophils, while limited in controlling viral propagation, were crucial inducers of a potent host humoral antiviral response (Naranjo-Gomez et al., 2018). Together, these studies offer insight into the key role of neutrophils in protective immunity induction and suggests the importance of neutrophil activity in an effective HIV vaccine or immunotherapy. Ultimately, preservation of neutrophil functions and counts may be significant in successful protective immunity strategies.

Here we presented favourable neutrophil functionality in the absence of DARC in HIV uninfected and infected individuals. While these findings offers promise that vaccines or immunotherapy centred on neutrophil-mediated induction of protective immune responses may still be plausible in the African setting, the issue of DARC-null neutropenia still requires extensive consideration. Lower circulatory neutrophil counts may prove ineffective in these circumstances as these interventions may not offer the anticipated protection against HIV. Indeed, the mechanisms behind DARC-null neutropenia requires better understanding. But even then, therapies aimed to overcome neutropenia such as enhancing neutrophil mobilisation and migration from the bone marrow be prove unfeasible.

Besides their effector mechanisms, neutrophils are more recently recognised as important immune regulators (Nathan, 2006). Here we focused on neutrophil / NK cell dynamics by investigating the impact of the DARC-null allele and lower ANCs on NK cell phenotype and function. Neutrophils interact with numerous immune cells including macrophages, dendritic

cells, T cells and B cells (Nathan, 2006, Mantovani et al., 2011, Amulic et al., 2012). It may be worth profiling these cell types in the context of DARC-null and ethnic neutropenia.

An interesting observation in our cohort was the association of lower NK cell counts with lower ANCs in HIV negative DARC-null individuals. In an attempt to ascertain the mechanism behind this lower NK cell count, we assessed for possible changes in NK cell survival and expansion. However, our proposed hypothesis that lower ANCs in DARC-null individuals could modify NK cell survival and / or proliferation proved incorrect. NK cells are important cytolytic cells and are also critical to the development of adaptive immune response. Thus recent HIV prophylactic and therapeutic vaccine strategies have focused on enhancing NK cell activity (Alter and Altfeld, 2009, Florez-Alvarez et al., 2018). Such interventions could prove less efficient in the context of lower NK cells in ethnic neutropenic populations. Other factors including reduced NK cell release from the bone marrow or modified NK cell trafficking between tissues and circulation may contribute to lower peripheral NK cell counts (Sun and Lanier, 2011), these and other possible mechanisms should be further investigated.

Time of infection in our HIV positive group was unknown, thus we were unable to fully appreciate the effect of DARC-null genotype on neutrophils or NK cells from infected persons in terms of HIV pathogenesis. Longitudinal data from HIV infected persons may better define the immune responses examined here in relation to time of infection and disease progression. However, because of the newly implemented policy of initiating antiretroviral therapy on diagnosis, this may not be possible.

5.4 CONCLUSION

DARC-null associated ethnic neutropenic populations are at higher risk to HIV-1 acquisition. However, the role of lower neutrophil counts in the absence of the DARC-null trait on HIV disease progression is unclear. Thus, in this series of studies we examined neutrophil and NK cell responses in DARC-null individuals, hypothesising that the absence of DARC would impair neutrophil and NK cell responses and contribute adversely to HIV pathogenesis.

Conversely, we demonstrate to our knowledge for the first time the advantageous effect of the DARC-null polymorphism in conferring superior neutrophil phagocytic activity irrespective of HIV status. Furthermore, we attest that the DARC-null trait does not affect ROS production, and only marginally reduces NET formation in HIV infected individuals. The data suggests that the DARC-null genotype does not adversely affect neutrophil functionality irrespective of HIV state. From the data presented here, it is probable that any disadvantage DARC-null individuals may display pre or post infection is likely due to reduced neutrophil counts rather than impaired neutrophil functions.

While reduced NK cells frequencies with a less differentiated phenotype were observed in HIV negative DARC-null subjects, overall NK cell functional responses were not impaired. Differences observed by DARC state in HIV negative individuals were absent in HIV infection, suggesting that any advantage that DARC-positive HIV negative persons may exhibit in relation to NK cells are lost during infection. The condition does not impede downstream NK cell functionally, suggesting that NK cell priming is not hindered by lower ANCs. Lastly, NK cell survival and proliferation were not hampered in either HIV negative or chronically infected individuals by DARC state, suggesting that the DARC-null polymorphism does not impair these homeostatic activities.

Overall, the data presented here suggests that the DARC-null trait and the associated lower neutrophil counts does not adversely affect neutrophil function or compromise NK cell dynamics. This is encouraging as it suggests that the absence of DARC does not diminish neutrophil or NK cell responses and does not contribute adversely to HIV pathogenesis in this manner. We provide mechanistic evidence in support of the asymptomatic clinical characteristics commonly associated with benign ethnic neutropenia.

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APPENDIX 1

Dear Dr Thobakgale,

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Journal: Frontiers in Immunology, section Molecular Innate Immunity
Article type: Original Research
Authors: Kewreshini Kasturi Naidoo, Ayanda Ngubane, Pedzisai Gaza, Amber Moodley, Thumbi Ndung'u, Christina Fanesa Thobakgale
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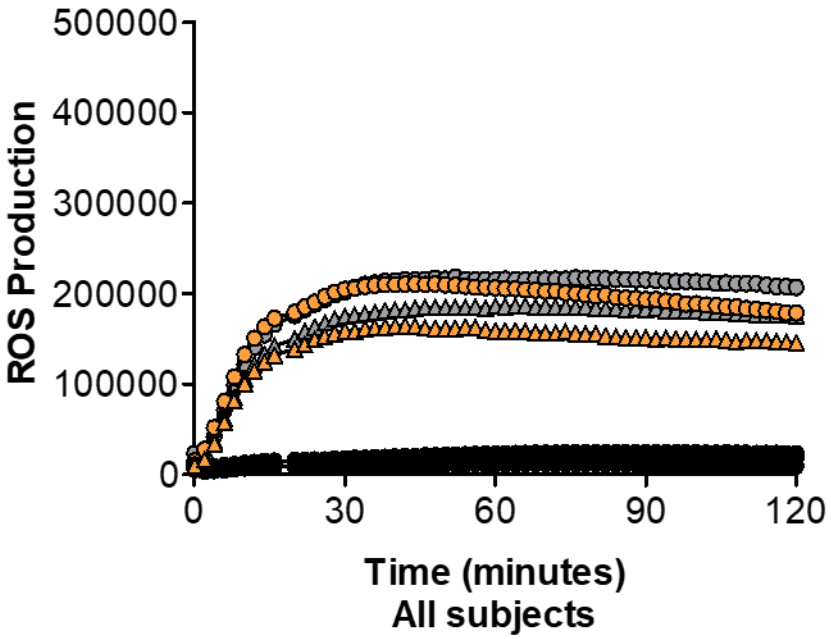
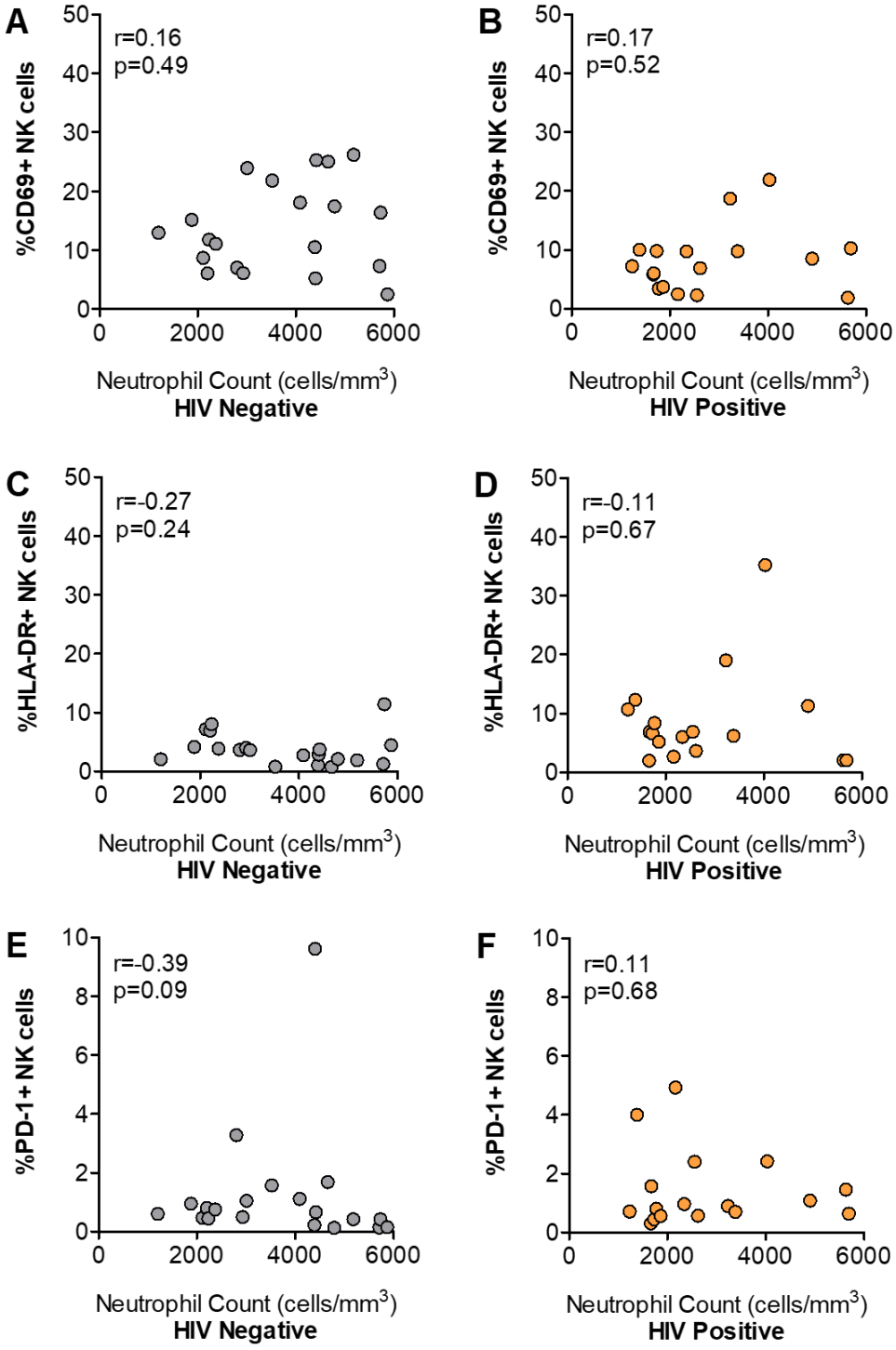
APPENDIX 2



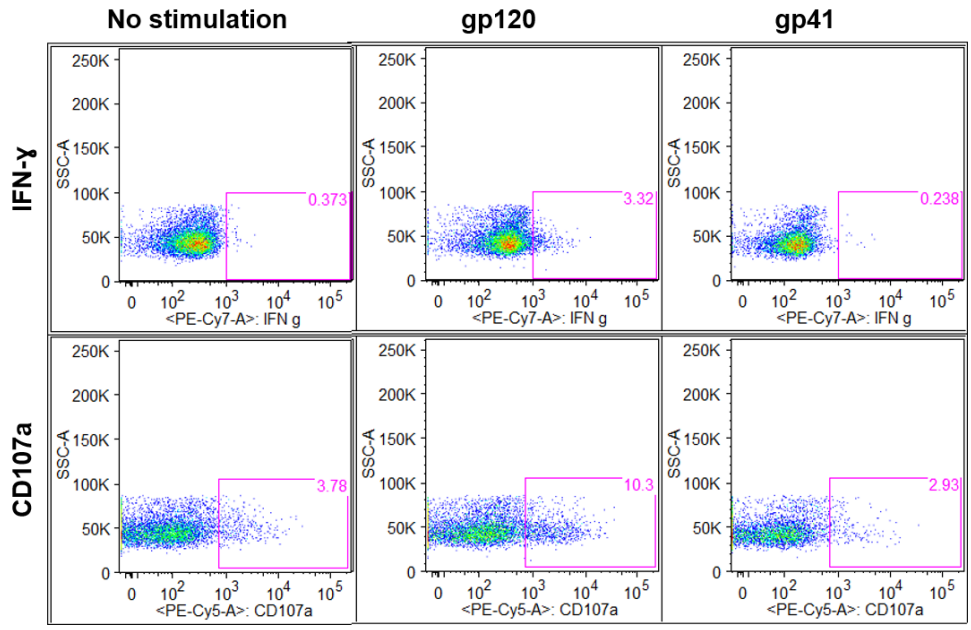
Supplementary Figure 1 Neutrophil Reactive Oxygen Species (ROS) production

Summary lines for ROS production over time is shown for the studied groups. Unstimulated controls for all groups are represented as black circles; PMA stimulated samples for HIV negative individuals are represented as grey circles (DARC-null) or triangles (DARC-positive); PMA stimulated samples for HIV positive individuals are represented as orange circles (DARC-null) or triangles (DARC-positive). Summary lines were calculated as the average of unstimulated or PMA stimulated samples of all participants within each studied group. Abbreviations: ROS, reactive oxygen species; DARC, Duffy Antigen Receptor for Chemokines.



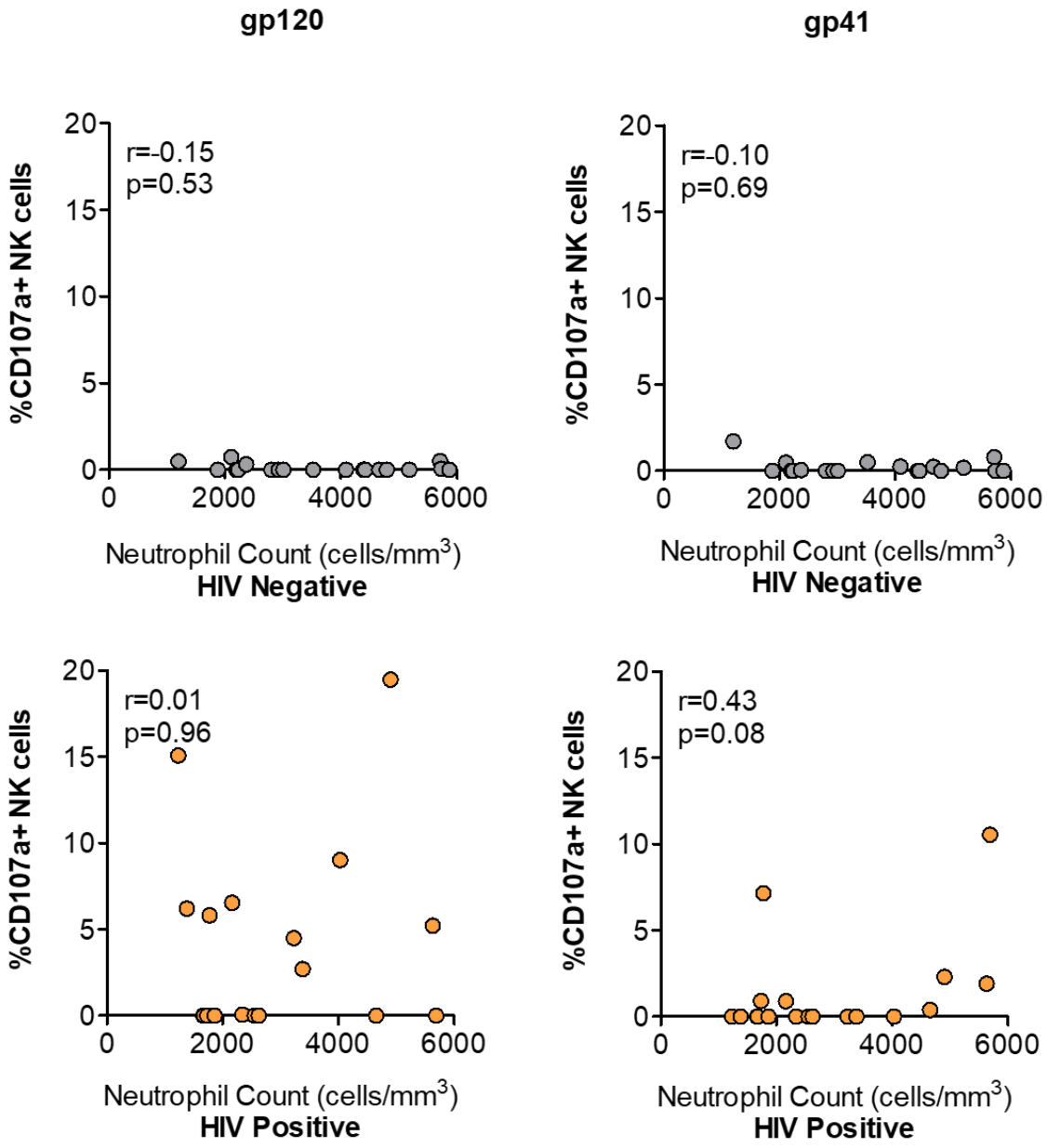
Supplementary Figure 2 Correlation between CD69, HLA-DR or PD-1 expression on total NK cells with ANC

Correlations between ANC and percentage of NK cells expressing either CD69 in HIV negative **(A)** and HIV infected **(B)** persons; HLA-DR in HIV negative **(C)** and HIV infected **(D)** persons; or PD-1 in HIV negative **(E)** or HIV infected **(F)** persons. Dots indicate individual data points. Abbreviations: ANC, Absolute neutrophil count; r, Spearman rho.



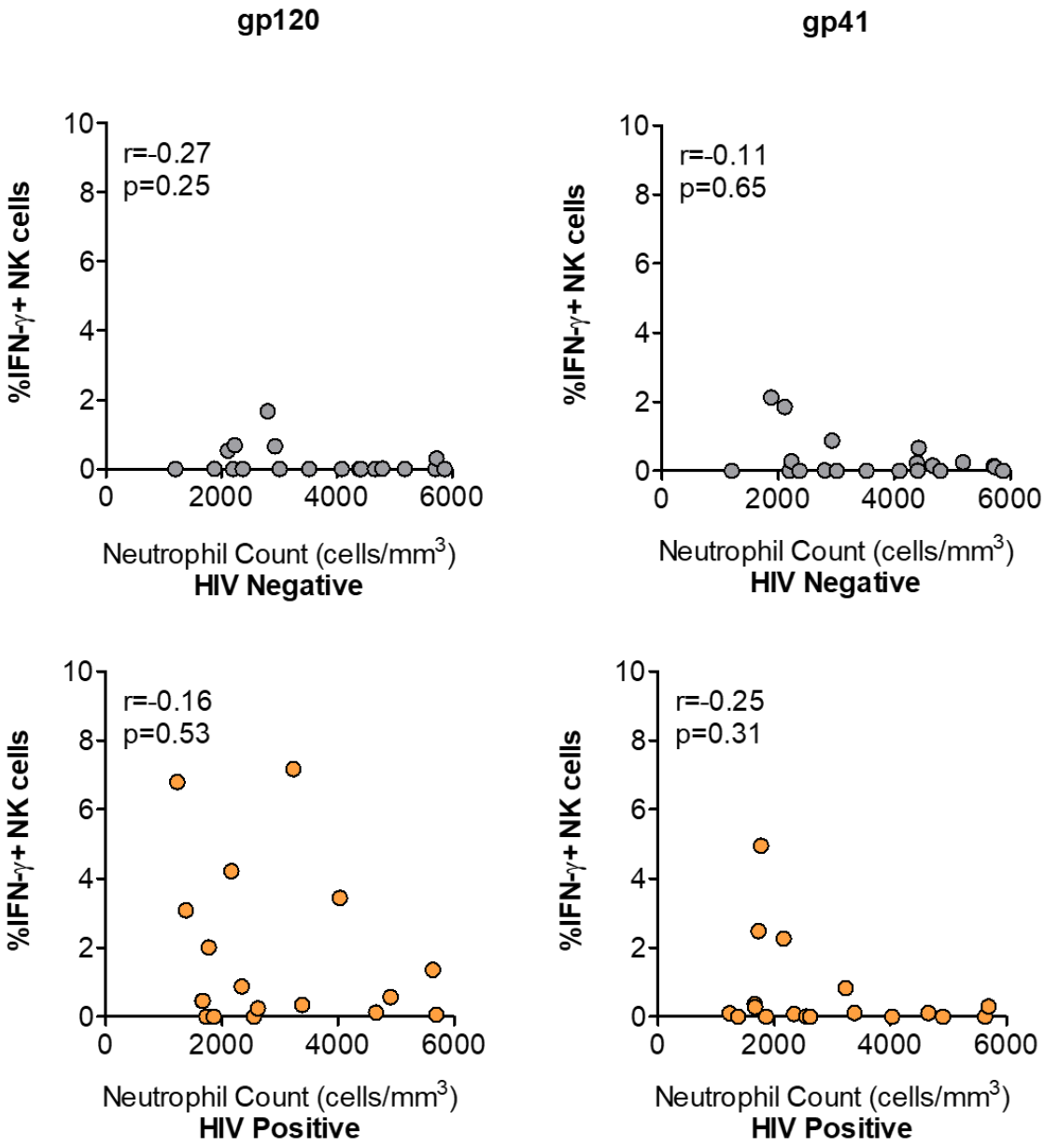
Supplementary Figure 3 IFN-ɣ and CD107a expression in NK cells following whole blood stimulation with HIV-1 peptides gp120 and gp41

Representative gating strategy employed for the analysis of IFN-ɣ and CD107a expression in NK cells after stimulation with gp120 and gp41. Frequencies of no stimulation samples were used to subtract background from stimulated gp120 and gp41 samples.



Supplementary Figure 4 Expression of CD107a relative to absolute neutrophil count (ANC) following whole blood stimulation with HIV-1 peptides gp120 or gp41

Correlations between percentage of NK cells expressing CD107a and absolute neutrophil count (ANC) in HIV negative and HIV infected individuals after stimulation with gp120 or gp41. Dots indicate individual data points. The p-values as indicated by ns refer to no significant differences. Abbreviations: ANC, Absolute neutrophil count; r, Spearman rho.



Supplementary Figure 5 Expression of IFN-ɣ relative to absolute neutrophil count (ANC) following whole blood stimulation with HIV-1 peptides gp120 or gp41

Correlations between percentage of NK cells expressing IFN-ɣ and absolute neutrophil count (ANC) in HIV negative and HIV infected individuals after stimulation with gp120 or gp41. Dots indicate individual data points. Abbreviations: ANC, Absolute neutrophil count; r, Spearman rho.

Supplementary Table 1 Flow Cytometry Antibodies

Panel	Marker	Fluorochrome	Species	Isotype	Clone	Manufacturer	Volume (µl)
Phenotype/ICS	CD3	PE-CF594	Mouse	BALB/c IgG1, κ	UCHT1	BD Biosciences	2
Whole Blood	CD3	PE-Cy5.5	Mouse	IgG2a, κ	7D6	Invitrogen	2
Phenotype	CD14	V500	Mouse	IgG2a, κ	M5E2	BD Biosciences	2
ICS	CD14	BV650	Mouse	IgG2a, κ	M5E2	Biolegend	2
Whole Blood	CD14	Pacific Blue	Mouse	IgG2a, κ	TuK4	Invitrogen	2
Phenotype	CD19	V500	Mouse	IgG1, κ	HIB19	BD Biosciences	2
ICS	CD19	BV650	Mouse	IgG1, κ	HIB19	Biolegend	2
Whole Blood	CD19	Pacific Blue	Mouse	IgG2a, κ	SJ25-C1	Invitrogen	2
All panels	CD16	APC-Cy7	Mouse	BALB/c x DBA/2	3G8	BD Biosciences	1.5
All panels	CD56	Alexa-Fluor-700	Mouse	IgG1, κ	B159	BD Biosciences	1
Phenotype	CD69	FITC	Mouse	IgG1, κ	FN50	BD Biosciences	5
Phenotype	CD38	PE	Mouse	IgG1, κ	HB7	BD Biosciences	4
Phenotype	HLA-DR	BV711	Mouse	IgG2a, κ	L243	Biolegend	2
Phenotype	CD27	BV605	Mouse	IgG1, κ	O323	Biolegend	2.5
Phenotype	CD11b/Mac-1	PE-Cy5	Mouse	IgG1, κ	ICRF44	BD Biosciences	10
Phenotype	PD-1	BV421	Mouse	IgG1, κ	EH12.2H7	Biolegend	2
Phenotype	CD57	APC	Mouse	IgM, κ	NK-1	BD Biosciences	2.5
ICS	CD158a	PE	Mouse	BALB/c IgM	HP-3E4	BD Biosciences	5
ICS	CD158b	PE	Mouse	BALB/c IgG2b, κ	CH-L	BD Biosciences	5
ICS	CD158e1/e2	PE	Mouse	IgG1, κ	Z27.3.7	Beckman Coulter	5
ICS	NKG2A	APC	Mouse	IgG2b, κ	Z199.1	Beckman Coulter	5
ICS/Whole Blood	CD107a	PE-Cy7	Mouse	BALB/c IgG1, κ	H4A3	BD Biosciences	3.5
ICS	TNF-α	PerCP-Cy5.5	Mouse	IgG1, κ	Mab11	Biolegend	5
ICS/Whole Blood	IFN-ɣ	PE-Cy7	Mouse	IgG1, κ	B27	Biolegend	5

The table indicates the monoclonal antibodies used for NK cell phenotype, intracellular staining (ICS) or whole blood staining for assessment by flow cytometry. The species, isotype and clone for each antibody is specified. Details of manufacturer and the final titrated volumes used for staining is supplied. Abbreviations: µl, microliter; ICS, intracellular staining.