Fc-functional antibody immunity to HIV: the role of neutrophils and IgA

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Abstract

A safe and effective prophylactic vaccine against HIV-1 is an essential component to limit the HIV-1 epidemic. The RV144 HIV vaccine efficacy trial has highlighted the importance of generating Fc functional antibodies to prevent the further spread of HIV infection. Fc functional antibody responses have also been shown to correlate with delayed HIV disease progression. Despite the intensification of interest in Fc-mediated responses to HIV infection, there has been limited research focused on the Fc functional capacity of neutrophils, which are a key innate immune cell at mucosal surfaces and in the blood. The majority of Fc-effector studies in HIV focus upon examining NK cells and/or monocytes responses, while other effector cells such as neutrophils remain understudied. NK cells lack the FcαR and cannot mediate any IgA-dependent Fc-mediated effector responses therefore, other immune cells like neutrophil are necessary for IgA to be studied.

Neutrophils are highly functional innate effector cells with the potential to induce both antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis. In chapter 2, methods were optimized to evaluate antibody-dependent neutrophil phagocytosis (ADNP) and neutrophil-mediated rapid fluorometric antibody-dependent cellular cytotoxicity (RFADCC) effector responses, using freshly isolated primary human neutrophils from blood. *In vitro*, neutrophil-mediated RFADCC responses peaked at 4 hours, which was faster than primary NK cells or monocyte mediated responses. There was a large spectrum of responses of both ADNP and neutrophil-mediated RFADCC responses across a cohort of 41 viremic antiretroviral-therapy naïve HIV positive subjects. ADNP and RFADCC responses correlated well with each other, suggesting that they measure overlapping functions. The viral load of the patients inversely correlated with the ADNP responses, suggesting that these antibody-mediated neutrophil-based assays could prove useful in dissecting HIV-specific immunity.

The role that IgA plays in active HIV infection remains controversial, with some reports of HIV-specific IgA being able to inhibit HIV infection and potentially being protective. Chapter 3 investigated if HIV progression was influenced by HIV-specific ADNP and neutrophil-mediated RFADCC responses and the effects of IgA on these responses. It was shown that, although neutrophil-mediated RFADCC responses were higher in the plasma of subjects who controlled their viremia levels (viremic controllers), IgA from both viremic controllers and viremic non-controllers inhibited both ADNP and neutrophil-mediated RFADCC responses

similarly. The IgG mediated ADNP responses from both viremic controller and viremic non-controllers were broadly inhibited by both autologous HIV positive IgA and HIV negative pooled purified IgA. The IgA inhibition was able to be blocked by pretreating neutrophils with an Fcα receptor (FcαR) blocking antibody. This suggests that IgA inhibition of ADNP responses can be mediated by 2 mechanisms; 1) antigen dependent/FcαR independent and 2) antigen independent/FcαR dependent.

The RV144 vaccine trial has generated interest in Fc functional antibodies and in the role that HIV-specific IgA can play during HIV vaccination strategies and in HIV infection. The RV144 vaccine induced IgG antibodies that were able to mediate ADCC responses. However, the vaccine efficacy was reduced in the presence of high concentrations of HIV-specific IgA. Monoclonal IgA that was isolated from the plasma of the RV144 vaccinees was able to block the potentially protective IgG antibodies from binding similar epitopes, thus preventing ADCC responses with NK cells. This indicates there was epitope competition between IgA and IgG antibodies in the RV144 vaccine trial. NK cells lack the FcαR and cannot mediate any IgA-dependent Fc-mediated effector responses. Chapter 4 assessed plasma samples from the RV144 vaccine trial for their ability to induce neutrophil-mediated responses and if IgA was able to inhibit these responses. IgG from the RV144 vaccinees was able to induce modest HIV-specific ADNP and neutrophil-mediated RFADCC responses. Plasma IgA from the vaccinees was able to inhibit ADNP responses but not neutrophil-mediated RFADCC responses. Using pooled IgG from the vaccinees, it was shown that pooled purified IgA from vaccinees, pooled purified IgA from HIV positive donors and pooled purified HIV negative IgA were able to inhibit the IgG mediated ADNP responses.

Overall, this thesis shows that neutrophils can mediate HIV-specific antibody-dependent phagocytosis and neutrophil-mediated RFADCC responses. HIV-specific IgG mediated neutrophil responses, induced by either infection or vaccination, can be inhibited by plasma IgA in an antigen dependent mechanism and an antigen independent mechanism that is a FcαR dependent mechanism. The inhibitory effects of IgA may assist in understanding HIV pathogenesis and improving future HIV vaccine designs.

Declaration

This is to certify that:

I. This thesis comprises my original work towards the PhD except where indicated in the Preface

II. Due acknowledgement has been made in the text to all other material used

III. This thesis is less than 100,000 words in length, exclusive of tables, figures, references and appendices

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Matthew Worley

Preface

Work for this thesis was performed in the Kent laboratory, Department of Microbiology and Immunology, the University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria, Australia. This was conducted under the supervision of Prof Stephen Kent and Dr Amy Chung.

Chapter 2 of this thesis is included as an unchanged pdf of the published manuscript:

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Author contributions: MJW designed the study, performed experiments, analysed data, wrote and edited the manuscript; KF performed HL-60 experiments and wrote the HL-60 portion of manuscript; AJL performed fluorescent microscopy; ADK provided the HIV positive plasma samples and the CD4 and viral loads of subjects; SJK designed the study, edited the manuscript, AWC designed the study, performed and analysed RFADCC time course experiments and ADNP fluorescent microscopy, edited the manuscript.

Chapter 3: Professor Anthony Kelleher (Kirby institute) provided the plasma samples for the viremic controllers and the HIV non-controllers with the CD4 count and viral loads.

Chapter 4: Ester Lopez (Peter Doherty Institute) performed the surface plasmon resonance experiments and analysed that data

Other publications during candidature (see Appendix for published manuscripts):

Selva KJ, Juno JA, **Worley MJ**, Chung AW, Kent SJ and Parsons MS
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I would like to thank the member of the Kent lab that were present throughout my PhD for making my time here enjoyable. Firstly, I would like to thank all the post-doctoral fellows Matthew Parsons, Adam Wheatley, Jennifer Juno, Rob De Rose, Sinthujan Jegaskanda, Hyon-Xhi Tan, Vijaya Madhavi and Wendy Winnall for all their support. I am grateful to all the students Josh, Sarah, Fernanda, Wen Shi, Kevin, Hillary, Yi, Julius, Hannah, Timon and Kuangyu for all the good times and late-night giggles. The lab is only kept running because of the amazing research assistants that work tirelessly to keep everything in order, thank you to all of you Thakshila, Vinca, Anne, Sriveni, Ester, Robyn and Kathleen it wouldn't have been possible without you. A special thanks to Thakshila, Vinca and Hannah for dealing with my constant requests for blood and being so accommodating with my persistent short notice requests.

Finally, I would like to thank my friends and family for the continuously support and encouragement throughout this time in my life. I have experienced so much during my PhD but I have also missed seeing you all. I hope to be able to make more time to spend with you in the years ahead. I wouldn't have been able to finish my PhD without the support of my close friends and family that were always there with encouragement when I needed it.

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Abbreviations

Ad26	Adenovirus type 26
Ad5	Adenovirus type 5
ADCC	Antibody-dependent cellular cytotoxicity
ADCML	Antibody-dependent complement-mediated lysis
ADCP	Antibody-dependent cellular phagocytosis
ADCVI	Antibody-dependent cell-mediated viral inhibition
ADNP	Antibody-dependent neutrophil phagocytosis
ADP	Antibody-dependent phagocytosis
AIDS	Acquired immunodeficiency syndrome
AMP	Antibody-Mediated Prevention
APC	Antigen presenting cells
ART	Antiretroviral therapy
bNAbs	Broadly neutralising antibody
BSA	Bovine serum albumin
CCL	C-C chemokine ligand
CCR	C-C chemokine coreceptor
CD	Cluster of differentiation
CDC	Centre for Disease control and prevention
CFSE	Carboxyfluorescein succinimidyl ester
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
dIgA	Dimeric IgA
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EC	Elite controllers
EC50	Effective concentration 50
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme-linked immune absorbent spot
Env	Envelope protein

ER	Endoplasmic reticulum
Fab	Fragment antigen binding
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisable region
FcR	Fc Receptor
FCS	Foetal calf serum
FcαR	Fc alpha receptor
FcγR	Fc gamma receptor
G-CSF	Granulocyte-colony stimulating factor
G-MDSC	Granulocytic myeloid-derived suppressor cells
gag	Group specific antigen protein
GALT	Gut associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
HCDR3	Heavy-chain complementarity-determining region 3
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HESN	Highly exposed seronegative
HIV	Human immunodeficiency virus
HIVIG	HIV immune globulin
HIVIGA	HIV immune globulin A
HLA	Human leukocyte antigen
HLE	Human leukocyte elastase
HNP	Human neutrophil peptides
HRP	Horse radish peroxidase
HVTN	HIV Vaccine Trials Network
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITAMi	Inhibitory ITAM
LDG	Low density granulocytes
LTNP	Long-term non-progressors

LTR	Long terminal repeats
mAb	Monoclonal antibody
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MHRP	Military HIV Research Program
MPER	Membrane proximal external region
MPO	Myeloperoxidase
MSM	Men who have sex with men
MVA	Modified vaccinia Ankara
NADPH	Nicotinamide adenine dinucleotide phosphate
Nef	Negative regulatory factor
NET	Neutrophil extracellular traps
NHP	Non-human primate
NIH	National Institute of Health
NK	Natural killer cell
NKG2D	Natural-killer group 2, member D
NLR	NOD-like receptors
nm	Nanometers
OD	Optical density
OLFM4	Olfactomedin 4
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PCP	*Pneumocystis carinii* pneumonia
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
pDC	Plasmacytoid dendritic cell.
pNPP	p-nitrophenyl-phosphate
PR3	Proteinase 3
PrEP	Pre-exposure prophylaxis
PRR	Pattern recognition receptors
RBC	Red blood cells

Rev	Regulator of expression of virion proteins
RFADCC	Rapid fluorometric ADCC assay
RIG	Retinoic acid-inducible gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park memorial institute
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
SHIV	Simian human immunodeficiency virus
sIgA	Secretory IgA
SIV	Simian immunodeficiency virus
SPR	Surface plasmon resonance
ssRNA	Single stranded ribonucleic acid
ssDNA	Single stranded deoxyribonucleic acid
TasP	Treatment-as-prevention
Tat	Trans-Activator of Transcription
TCR	T cell receptor
Tfh	T follicular helper
Th17	T helper 17
TLR	Toll like receptors
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumor necrosis factor
UNAIDS	United Nations Programme on HIV/AIDS
V1V2	Variable regions 1 and 2
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VC	Viremic controllers

Chapter 1

General introduction

1.1. The HIV epidemic

Human immunodeficiency virus 1 (HIV-1) was first isolated as the causative agent of acquired immunodeficiency syndrome (AIDS) in 1983 [1, 2]. The HIV/AIDS pandemic has claimed an estimated 32 million (23.6 million- 43.8 million) lives to date with another 37.9 million (32.7 million–44.0 million) people living with HIV in 2018 [3]. UNAIDS estimates that the spread of the epidemic reached its peak in 1996, when the number of new infections reached its highest at 3.4 million. Despite this, there was an estimated 1.7 million new infections and 770,000 (570,000–1.1 million) succumbed to AIDS related illnesses in 2018 [3]. Sub-Saharan Africa continues to be the worst affected region in the world, accounting for some 25.6 million infections (68% of all HIV infections) [3]. There are nearly 5000 new infections every day and 64% of these occur in Sub-Saharan Africa. The Asia Pacific region is the next most affected region, accounting for some 5.9 million total HIV infections. In Australia, there is an estimated 27,545 people living with HIV with an estimated nearly 3000 unaware of their HIV status [4].

The introduction of antiretroviral therapy (ART) has vastly improved the lives of people living with HIV, but treatment remains life long and these treatments still have limited availability in many regions including Sub-Saharan Africa [5, 6]. Despite the many advances in antiretroviral treatment strategies, in 2018 only 62% of people living with HIV were accessing ART worldwide [3]. A safe and effective prophylactic vaccine against HIV-1 is widely viewed as an essential component to further limiting the HIV-1 epidemic [7, 8].

In the absence of a HIV vaccine, a number of other technologies and strategies have been implemented to assist in the prevention of HIV acquisition. The risk of transmitting or acquiring HIV is reduced by consistent male and female condom use, reductions in concurrent and/or sequential sexual and needle-sharing partners, male circumcision, pre-exposure prophylaxis (PrEP) and treatment-as-prevention (TasP) with antiretroviral medications [9]. Trials of PrEP have provided efficacy ranges from 40% to well above 90% [10-14]. The efficacy ranges vary due to a number of factors including gender, social/environmental impacts and most importantly adherence to medical regimens [15]. TasP has also been shown to reduce the number of HIV transmissions in virally undetectable HIV-discordant couples, although this has yet to be translated to population level benefits [16]. If the HIV positive partner has achieved viral suppression a number of studies have shown no HIV transmission to the HIV

negative partner [17-19]. These technologies require a high level of commitment and adherence which may be more difficult to sustain compared to vaccination.

1.2. HIV-1 origins

In 1981 the United States Centre for Disease Control and Prevention (CDC) reported 5 cases of a rare lung infection, *Pneumocystis carinii* pneumonia (PCP), in young, previously healthy gay men in Los Angeles. All 5 men suffered from other rare and unusual infections that indicated that they were all suffering from a compromised immune system. By the end of 1981 there were 270 reported cases of severe immune deficiency in homosexual men, with 121 succumbing to this illness. In the following years 2 separate groups, the first led by Robert Gallo and the second led by Luc Montagnier with Françoise Barré-Sinoussi, independently isolated a retrovirus as the cause of AIDS and this would later be named HIV [1, 2]. It is now known that there are two main strains of HIV (HIV-1 and HIV-2) that cause AIDS in humans, with HIV-1 being the focus of this thesis.

HIV-1 has become widely spread throughout the world, while HIV-2 is less prevalent and found principally in Western Africa. HIV-1 and HIV-2 both have zoonotic origins in the simian immunodeficiency virus from non-human primates. HIV-2 has close genetic heritage to simian immunodeficiency virus (SIV) strains which have been isolated from the sooty mangabey [20]. The closest SIV genetic relatives to HIV-1 have been found in Gorillas and Chimpanzees living in Western Africa [21, 22]. In extensive phylogenetic studies it has been shown that four lineages of HIV-1 have arisen independently [23-29] reviewed in [30]. Through historically documented sequences and evolutionary modelling (molecular clock analysis), the origin of the spread of HIV-1 into human populations dates back to the early twentieth century [31, 32].

1.3. HIV-1 structure and replication cycle

HIV-1 is a spherical lentivirus with a diameter of ~145nm and is part of the Retroviridae family (Schematic representation in Figure 1.1) [33]. The HIV-1 genome is composed of two copies of positive single-stranded RNA (ssRNA), that contains 9 viral genes (Table 1.1) with overlapping reading frames (Figure 1.2) and making 19 proteins [34]. The ssRNA is reverse transcribed in to DNA that integrates into the host cell's DNA, creating a latent infection. The ssRNA viral genome is bound to nucleocapsid proteins (Vif, Vpr, Nef and p7) and several

important viral enzymes such as protease, integrase, reverse transcriptase (RT) and retroviral ribonuclease H. The core of the virus is enclosed by a conical capsid composed of approximately 1500-2000 copies of p24 [35, 36]. The conical capsid is then further surrounded by a matrix protein p17, which assists in the structural integrity of the virus. Directly surrounding the viral matrix is a host-cell derived lipid membrane. The lipid membrane is made up of phospholipid bilayer embedded with ~14 envelope protein spikes (Env) that are essential for viral entry [37]. The functional envelope protein spike has a trimeric structure of heterodimers composed of 3 gp120 (surface) subunits and 3 gp41 (transmembrane) subunits and is essential for cell entry.

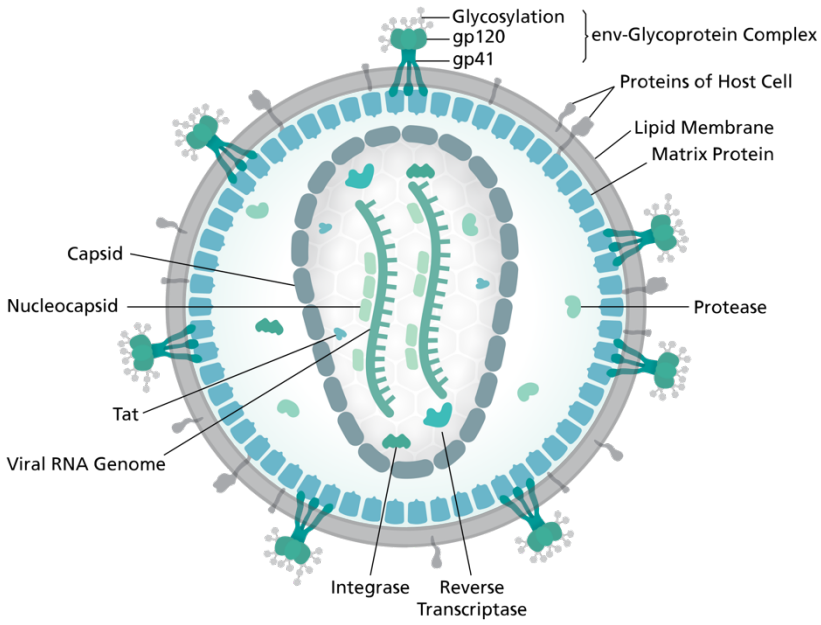

Figure 1.1: A schematic representation of the HIV virus. Image produced by Thomas Splettstoesser, use permitted by Creative Commons CC BY-SA 3.0 license

Table 1.1: The genes, major proteins and functions of the HIV-1 particle (reviewed in [38])

Class	Genes	Proteins/ enzymes	Function
Structural proteins	*gag*	Matrix protein (p17) Capsid protein (p24) Nucelocapsid (p7) P6	Forms the inner membrane layer Forms the conical capsid nucleoprotein/RNA complex involved in viral particle release
	env	gp120 gp41	Attachment of virus to cell anchorage of gp120, fusion of viral and cell membranes
Enzymatic Proteins	*pol*	Protease (p10) Reverse transcriptase (p51) Integrase (p32) RNase H (p15)	Protein cleavage Transcription of HIV RNA in proviral DNA Integration of proviral DNA into host DNA Degradation of viral RNA
Essential regulatory elements	*tat*	Tat	Activator of transcription from the LTR
	rev	Rev	Regulates nuclear export of viral mRNA
Accessory regulatory protein	*nef*	Nef	Down-regulates CD4 Down-regulates MHC-1 Increase viral infectivity
	vif	Vif	Promotes infection
	vpr	Vpr	Involved in nuclear import of DNA Cell growth arrest Transactivation of cellular genes
	vpu	Vpu	Degradation of CD4 in ER and Promotes virion release Modulates intracellular trafficking

The HIV replication cycle is depicted in Figure 1.3 and is reviewed in detail in [39][39][39][39-43]. Very briefly, HIV infects cells firstly by attachment where the gp120 binds to the CD4 cell surface molecule on T cells, leading to a conformational change of the envelope protein [44]. This conformational change allows for the gp120 to bind to the co-receptor (CCR5 or

CXCR4) which results in a second conformational change, pushing the gp41 subunit up and exposing a fusion peptide of the gp41 subunit for viral entry (reviewed in [45] and [40]). The virion is then endocytosed with the fusion peptide, puncturing the membrane of the cell and allowing fusion between the cell and virion membranes. This fusion of the membranes allows for the viral capsid to be released into the cytoplasm. Blocking viral entry through antibodies (i.e. neutralising antibodies) to the Env is a key goal in many vaccine approaches.

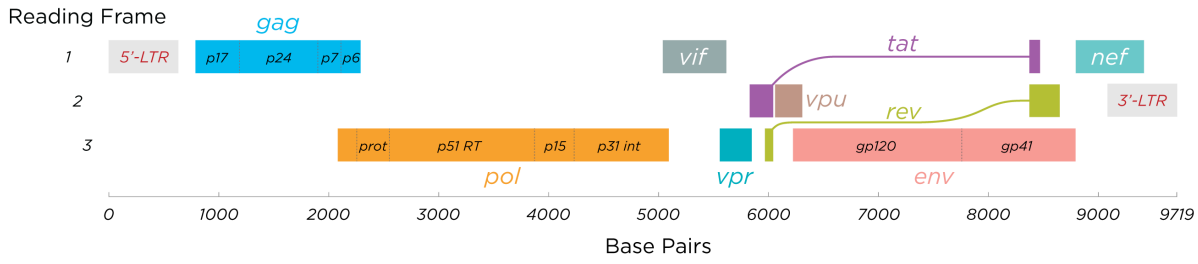

Figure 1.2: Organisation of the HIV RNA genome. The genome consists 9 genes with overlapping reading frames that code for 19 proteins *Image produced by Thomas Splettstoesser, use permitted by Creative Commons CC BY-SA 3.0 license*

After entry, capsid is taken up by endosome and processed removing the capsid, ultimately releasing the capsid contents into the cytoplasm. The HIV reverse transcriptase then transcribes the single-stranded HIV RNA genome into ssDNA then into dsDNA (proviral DNA). The proviral DNA complexed with integrase is then transported through the nucleopores into the nucleus and integrates into the host cell DNA. The long terminal repeats (LTR) of the now integrated provirus can act as attachment sites for host transcriptional machinery to bind to synthesise the viral RNA. This is followed by the splicing of Tat and Rev which regulate the synthesis and generation of new virus. The gp160 trimer precursors go to the endoplasmic reticulum (ER) where they undergo folding and formation of trimers and ultimately post-translational modifications (reviewed in [46]). The trimeric gp160 envelope proteins are then modified by the Golgi complex and cleaved into the gp120 and gp41 complexes which are non-covalently bound. The Env protein is transported by the Golgi complex to the surface of the cells and the gp41 binds in the plasma membrane. The Env protein is a target for both neutralising antibodies and non-neutralising antibodies with fragment crystallisable region (Fc) mediated functions that likely play a role in controlling and preventing HIV infection, which is a key focus of this thesis and will be discussed in greater

detail below. The gag, pol, viral enzymes and nucleocapsid associate with the viral RNA and the p24 capsid is formed around this complex, then the p17 matrix forms an outer capsid. The gag and pol genes are relatively conserved internal proteins and are the common targets of T cell immunity. This outer capsid assembles with the envelope protein on the cell surface. The immature virus is attached to the inner wall of the cell membrane which allows for the budding of the virus and release from the host cell.

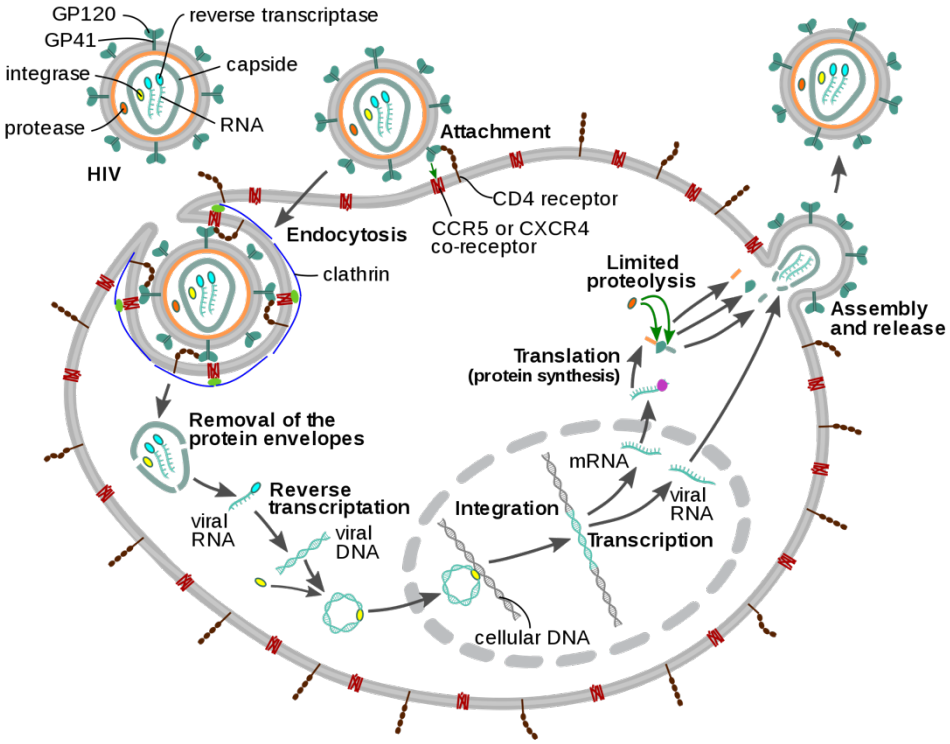

Figure 1.3: HIV replication cycle. Image produced by Thomas Splettstoesser, use permitted by Creative Commons CC BY-SA 3.0 license

1.4. HIV pathogenesis and disease progression

The majority, ~80% HIV-infected heterosexuals [47-49] and ~60-75% of HIV-infected men who have sex with men (MSM) [50-52], of HIV transmission is the result of productive infection by a single founder virus (or virally infected cell). The transmission of HIV primarily (>80%) occurs from virus exposure at the mucosal surfaces of the genital or rectal tract [53, 54]. In rectal transmission, there is a single layer of columnar epithelium that is frequently disturbed during intercourse [55]. This results in free virions or virally infected cells to come in close contact with target CD4+ T cells that can readily be infected. In vaginal transmission,

macaque models have shown that CD4+ T cells can also be observed to be directly infected by SIV at the mucosa [56]. However, some studies have indicated that dendritic cells (e.g. Langerhans cells) bind to the envelope gp120 of HIV through the unique C-type lectin langerin receptor and ultimately internalise the virus [57]. The virus internalised by dendritic cells is then transmitted to vulnerable CD4+ T cells in the sub-mucosal tissue or draining lymphoid tissue leading to a productive infection [58]. The establishment of infection with a founder virus (usually a single virus strain) occurs within hours of exposure resulting in an established founder population which expands locally for a few days [59]. Free virus released from this early founder population continues to disseminate in the lymphoid organs, including gut-associated lymphoid tissue (GALT), which is a primary site of CD4+ T cell depletion [60, 61]. Without treatment there is continuous replication of HIV, and rapid turnover and ultimately depletion of CD4+ T cells. Although T cells, macrophages and dendritic cells (DCs) are primarily in the submucosal compartment, neutrophils are also able to access the mucosal lumen [62-64]. This is important as they may be able to mediate anti-HIV effector functions prior to CD4+ T cells being targeted by HIV, this is discussed in greater detail below.

In the absence of treatment, HIV infection can be characterized into 4 stages of infection (Figure 1.4) [65]. The primary infection phase is an asymptomatic incubation that includes the early stages of HIV infection and replication. This is followed by the acute infection phase which includes the wide dissemination of HIV in lymphoid tissue and establishment of the latent reservoir. During the acute phase many individuals will develop an influenza-like or a mononucleosis-like illness, typically 2–4 weeks after infection [66]. This is followed by the clinical latency phase, which is generally asymptomatic and lasts for up to 20 years or more with an average clinical latency of 6-10 years [67]. Potent cellular and humoral immune responses help partially control HIV viremia during this phase, although immune escape is well described [68-70] and progressive disease eventually ensues in almost all infected subjects [66]. The last stage is defined by the development of AIDS which results in CD4+ T cells counts <200/μl of blood with the development of opportunistic infections, cancers and constitutional symptoms like weight loss, diarrhea and fever [71]. Without ART intervention the patient is vulnerable to multiple opportunistic infections commonly leading to death. The early diagnosis of HIV infection by detection of antibodies and instigation of ART regimens greatly improves clinical outcomes.

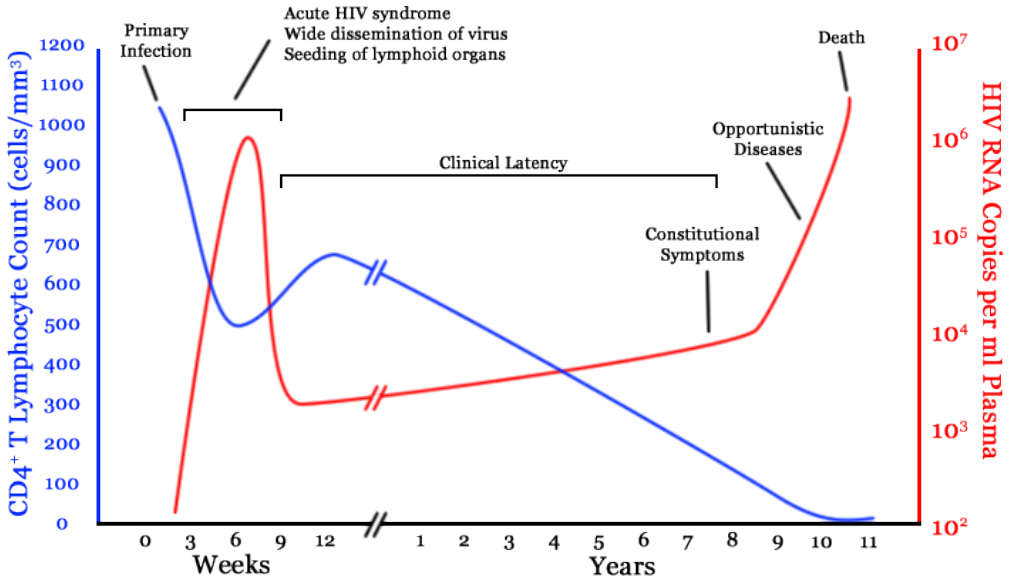

Figure 1.4: Disease progression of untreated HIV (adapted from [66])

1.5. Immune response to HIV

1.5.1. Innate immunity responses

An understanding of immunity to HIV infection is essential to rationally design and dissect vaccine-induced immunity. The innate immune response is the first line of defense against invading pathogens and does not require prior exposure for their anti-microbial activity. The innate immune system involves a number of immune cells including macrophages, monocytes, neutrophils, dendritic cells and natural killer cells (NK) [72]. The macrophages, monocyte and neutrophils are all able to phagocytose free virus or virally infected cells and clear the pathogens. Neutrophils are a focus of this thesis and they are discussed in further detail in section 1.7. NK cells are able to recognize and kill virally infected cells. DCs are able to capture the pathogens and present viral antigens to induce adaptive T and B cell responses.

DCs are able to recognize HIV pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) which include toll like receptors (TLRs), RIG-1 like receptors and potentially NOD-like receptors (NLRs). DCs expressing intracellular TLR7/8 can recognise HIV RNA which can activate the DCs to secrete a TNF-α and type 1 interferons [73]. DCs can also interact directly with HIV gp120 protein with DC-SIGN to secret IL-10 which can inhibit other innate immune cells [74]. It should also be noted that DCs are depleted during HIV infection which limits their ability to prime the adaptive immune response later during chronic infection [75]. Plasmacytoid dendritic cells (pDCs) that are exposed to HIV are

known to secrete inflammatory cytokines and IFN-α, however, they also induce differentiation of CD4+ T cells into regulatory T cells, which are known to suppress HIV-specific immune responses [76]. It is evident that DCs can play a beneficial role in the course of HIV infection, but they can also inhibit the immune responses, potentially to protect from over activation of the immune system.

NK cells are able to recognize virally infected cells by different mechanisms and lyse HIV infected target cells. HIV infected cells express a range of stress induced ligands that can bind and activate the NKG2D receptor that is expressed on NK cells, thus mediating the killing of HIV infected cells [77, 78]. However, HIV limits the expression of these stress induced ligands through accessory proteins like Nef [79]. NK cells also express FcγRIII (CD16), which can recognize and be activated by the Fc portion of the IgG antibodies that can opsonize HIV infected cells, which are eliminated by an Antibody-Dependent Cellular Cytotoxicity (ADCC) response [80, 81]. NK cell mediated ADCC responses have been implicated in providing protection from HIV infection in the RV144 vaccine trial (discussed in detail below in section 1.9.4) [82]. NK cell phenotypes have been associated with delayed HIV disease progression through direct cell lysis and the secretion of β-chemokines CCL3, CCL4 and CCL5. The β-chemokines can compete for engagement of the same receptors HIV uses to infect target cells [83, 84]. The expression of FcγRIIc has also been associated with delayed HIV progression, however, FcγRIIc is only expressed by ~20% of the population [85]. Interestingly, enhanced NK cell functions have also been detected in HIV exposed uninfected individuals, emphasizing the importance of innate immunity in protection against infection [86]. Neutrophils are also a key innate immune cell that can respond to HIV and are discussed in greater detail below in section 1.7.

1.5.2. Cellular immune responses

Early in HIV infection, cytotoxic CD8+ T cell (CTL) responses are generated and are able to reduce the initial viral spike [87]. The CTL responses occur prior to the appearance of detectable levels of antibodies in blood plasma [88]. CTLs are able to exert pressure on HIV by secreting cytokines and chemokines that can prevent HIV from infecting new CD4+ T cells or by directly killing infected cells through the TCR recognition of HIV peptides on major histocompatibility complex class I (MHC) [89]. The early control of HIV viral load does not last indefinitely due to a strong selective pressure for escape mutants which ultimately broadens

the target epitopes [90, 91]. During chronic HIV infection, the plasma HIV RNA (viral load) is not usually completely suppressed and the persistent activation of T cells can lead to immune exhaustion and further inhibition of the immune response to HIV (reviewed in [92]). CTLs that become functionally exhausted in chronic infection exhibit impaired cytokine production, impaired proliferation and upregulation of inhibitory receptors like PD1 [93]. Of note, there has been a number of human leukocyte antigen (HLA) alleles that have been associated with delayed progression to AIDS, including HLA B57 and B27, with B57 associated with HIV elite controllers (EC) [94-97]. ECs are HIV positive people that without ART remain with undetectable viral loads (<50 RNA copies/ml) [98]. Conversely, HLA B35 and CW04 have been associated with a more rapid progression to AIDS [99-101]. CTL responses have been the target for HIV vaccines due to their capacity to control early HIV infection (HIV vaccines are discussed in greater detail below in section 1.9).

CD4+ helper T cells can recognize peptides displayed on the MHCII by antigen presenting cells (APC) and are vital in generating and maintaining CTL and humoral responses to HIV [102]. CD4+ T cells are the primary target for HIV to infect and this ultimately will lead to impaired T cell help [103]. During HIV infection, HIV-specific CD4+ T cells are activated and recruited to the sites of viral replication which can result in further infection and depletion of these specific cells [104]. In some cases, a strong CD4+ T cell response against Gag epitopes has been associated with control of the virus in the absence of ART [105, 106]. T follicular helper (Tfh) cells are a subset of CD4+ T cells that reside in the germinal centers of lymph nodes and provide help to B cells, which drives somatic hypermutation and affinity maturation of antibody responses [107]. It has been shown that HIV can cause dysregulation of Tfh cell functions (reviewed in [108]). However, the frequency of Tfh subsets and early preservation of these cells has been correlated with the development of broadly neutralising antibodies (bNAbs) during infection [109]. The early initiation of ART has been shown to have positive outcomes for maintaining Tfh cell populations and preventing dysregulation of functions, but was less beneficial at later stages [110, 111].

1.5.3. Humoral immune responses

The first B cell responses to HIV develop approximately 1 week following detectable plasma viremia and this is initially as immune complexes on B cells [88]. The first circulating antibodies following infection occur a few days later and are directed toward gp41. The

development of anti-gp120 antibodies occurs several weeks later [88]. Antibodies that can mediate a number of Fc-mediated effector responses (including ADCP, ADCC, complement deposition, and cellular trogocytosis are generated early on infection [112]. Studies have found ADCC antibodies are present early in infection, often coinciding with the detection of early CTL responses [113]. Non-neutralising antibody responses have been associated with decreased viremia during acute infection [113]. Within 12 months of infection, most subjects develop neutralising antibodies directed towards the initial infecting autologous strain, however, these antibodies are usually unable to neutralise divergent virus isolates [114]. The neutralising antibodies provide selective immune pressure, such that the virus rapidly mutates to evade these immune responses [115, 116]. Following years of persistent infection, bNAbs develop in a subset of infected subjects that can neutralise a broad range of divergent virus isolates [117-119]. A number of these neutralising antibodies have been isolated and monoclonal antibodies generated and profiled for their neutralising potential. These broadly neutralising monoclonal antibodies protect macaques from SIV-HIV chimeric viruses [120-123]. One such monoclonal antibody, VRC01, is currently being assessed in an efficacy study for the prevention of HIV transmission in humans (discussed further below in section 1.9.7) [124]. Although neutralising antibodies are a critical immune mechanism to control and prevent HIV, the induction of bNAbs by vaccination is at present difficult, although it is the subject of intense research [125]. This thesis focusses primarily on non-neutralising antibodies with Fc-mediated functions.

1.5.4. Fc-mediated functions

HIV-specific antibodies can mediate effector responses via the Fc portion of the antibody interacting with Fc-receptors (FcR) on innate effector cells like monocytes, macrophages, NK cells and neutrophils. Fc-mediated responses form a bridge between adaptive and innate immunity, providing the specificity of the adaptive immune responses to HIV target antigens via the fragment antigen binding (Fab) region of the antibody, while activating a range of potent effector functions of innate immune cells. These cells are able to mediate effector responses including ADCC, antibody-dependent cell-mediated viral inhibition (ADCVI) and antibody-dependent phagocytosis (ADP) via different FcR (reviewed in [126]). In contrast to neutralising antibodies, antibodies that can mediate Fc-dependent effector responses arise earlier in HIV infection [88]. Innate immune cells express a range of Fc receptors that mediate a variety of functions (Table 1.2). Most of the focus of Fc-mediated responses to HIV has been

on NK cells mediating ADCC, primarily through IgG binding to FcγRIIIa, and monocyte/macrophages mediating ADCP, primarily through IgG binding to FcγRIIa [127, 128]. There has been less focus on the role of neutrophils which express a range of Fcγ receptors and also express FcαRI that can bind IgA, a focus of this thesis discussed further below in sections 1.6 and 1.7.

Neutrophils and monocytes can mediate both ADCC responses and ADP responses via FcγRs (discussed further below in 1.7.3) (Figure 1.5) (reviewed in [129] and [130]). Monocytes and NK cells are able to mediate ADCC responses to HIV via the FcγRIIIa (CD16) which ultimately leads to lysis of the infected target cell by the release of perforin and granzyme B [131-133]. Along with cytotoxicity, the crosslinking of activating FcγRs can also lead to the release of cytokines and chemokines with some of these being able to directly inhibit HIV infection by blocking CCR5 which is termed as ADCVI [113, 134, 135]. ADP responses can be directed to both free circulating HIV virions and to whole infected cells and can be mediated by monocytes, macrophages, DCs and neutrophils [136-139]. In addition, HIV-specific antibodies can also activate complement responses via the classical pathway and kill infected target cells by a process termed antibody-dependent complement-mediated lysis (ADCML) [140, 141]. There is increasing interest in HIV-specific antibody-mediated trogocytosis [142, 143]. Trogocytosis has been shown in monocytes to occur rapidly and reduce the viability of target cells [143]. Despite the growing interest in trogocytosis it has not been investigated in HIV with neutrophils.

A number of studies have demonstrated a vital role of Fc-mediated functions in delaying HIV disease progression. In macaques, a passive transfer of a neutralising antibody called b12 with a mutated Fc portion which abrogated complement activation and Fc receptor binding ability, had reduced protective efficacy from simian human immunodeficiency virus (SHIV), with only 5 out of 9 macaques protected [144]. In contrast, 8 out of 9 macaques passively transferred with the standard neutralising antibody were protected [144]. More recent studies, using a more potent neutralising antibody, PGT121, showed that Fc functions were less important in the efficacy of that antibody [145]. Macaque passive transfer studies using non-neutralising antibodies showed a reduction in the number of SHIV founder viruses that the macaques acquired [144, 146, 147].

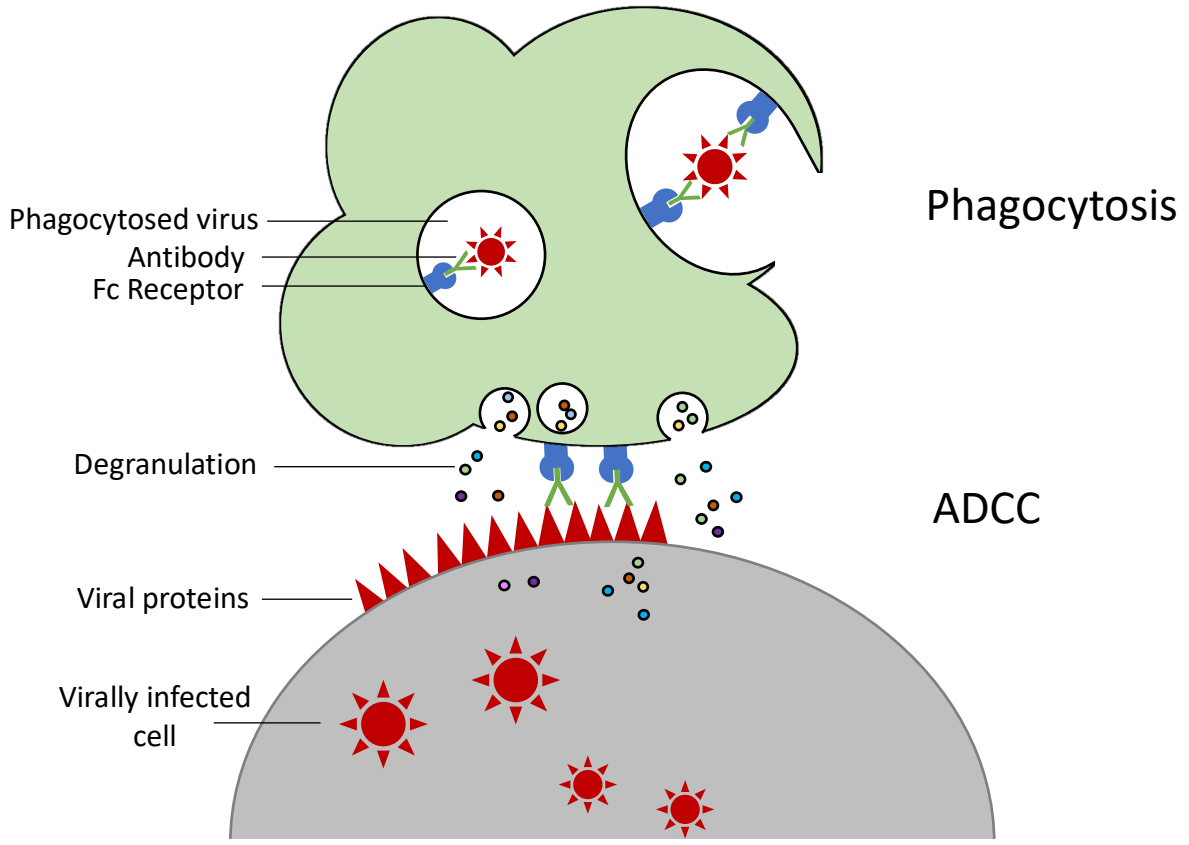

Figure 1.5: Schematic representation of Fc receptor mediated antibody-dependent phagocytosis and antibody-dependent cellular cytotoxicity again viral pathogen.

The importance of ADCC mediating antibody responses has also been highlighted by linking it with delayed HIV disease progression in humans and viral escape from these immune responses [148-152]. Other Fc-mediated effector functions including ADCVI [113] and ADCP [134, 153] have been associated with delayed disease progression. The range of Fc-mediated responses to HIV, the innate effector cells involved and the influence they have on diseases progression is still to be explored. Understanding ADCP and ADCC responses, mediated by neutrophils, to HIV is a key focus of my thesis.

Table 1.2: Fc Receptor expression on leukocytes (reviewed in [142, 154-156])

	Constitutively expressed	**Induced expression**	**Effect following binding to antibody**
FcγRI		Macrophages Monocytes Neutrophils Eosinophils Mast cells	Phagocytosis, oxidative burst cytotoxicity, complement activation
FcγRIIA	Macrophages Monocytes Neutrophils Eosinophils Mast cells Basophils Mast cells		Phagocytosis, oxidative burst, cytokine production, degranulation and complement activation
FcγRIIB	Macrophages Monocytes Neutrophils Basophils B cells Mast cells		Inhibition of cell activity Antigen presentation
FcγRIIIA	Natural killer cells Macrophages Monocytes	Dendritic cells	ADCC and Induction of cytokine release
FcγRIIIB	Neutrophil	Eosinophils	phagocytosis of immune complexes
FcαRI	Macrophages Monocytes Neutrophils Eosinophils		phagocytosis of immune complexes and ADCC responses
FcεRI	Monocytes Eosinophils Mast cells Basophils		Phagocytosis and degranulation

1.6. IgA antibodies

IgA is the most abundant antibody at the mucosal surfaces which is the primary site of HIV transmission and the second most abundant antibody in the blood behind IgG [157]. The IgA antibodies are able to interact with FcαRI (CD89) which is present on neutrophils, monocytes, macrophages, DCs and eosinophils [158]. Human IgA antibodies can be divided into two subclasses, IgA1 and IgA2 (Figure 1.6). IgA1 antibodies have a hinge region consisting of 19 amino acids with a number of O-linked oligosaccharides, while IgA2 antibodies contain just 6 amino acids and lack O-linked glycosylation [159-161]. These structural differences have been shown to have different biological functions and susceptibilities to proteases [162]. In human serum ~90% of IgA present is of the IgA1 subclass and with the remaining ~10% being IgA2, while at the genital and rectal mucosal sites ~60-90% are IgA2 subclass [163, 164]. In human serum, IgA antibodies are primarily monomeric (>80%), however, in the mucosal fluid IgA is predominately dimeric (dIgA). In dIgA, the Fc regions of the two monomeric IgA are bound together by a single disulphide bond to a J-chain [165]. In addition, mucosal IgA contains a secretory component added during passage of the epithelial layer resulting in secreted IgA (sIgA) [163]. The local distribution is important as the monomeric IgA and dimeric IgA bind to FcαRI with different avidity. Monomeric IgA and dIgA binding to the FcαRI is similar, however, dIgA and IgA immunocomplexes disassociate at a much slower rate than monomeric IgA [166]. Monomeric IgA1 and IgA2 subclasses bind FcαRI with similar affinity. IgA crosslinking of the FcαRI has been shown to be able to induce phagocytosis [167, 168], ADCC [169], degranulation [170], reactive oxygen release [171] and the release of neutrophil extracellular traps [172] to a number of pathogens and is seen in autoimmune diseases.

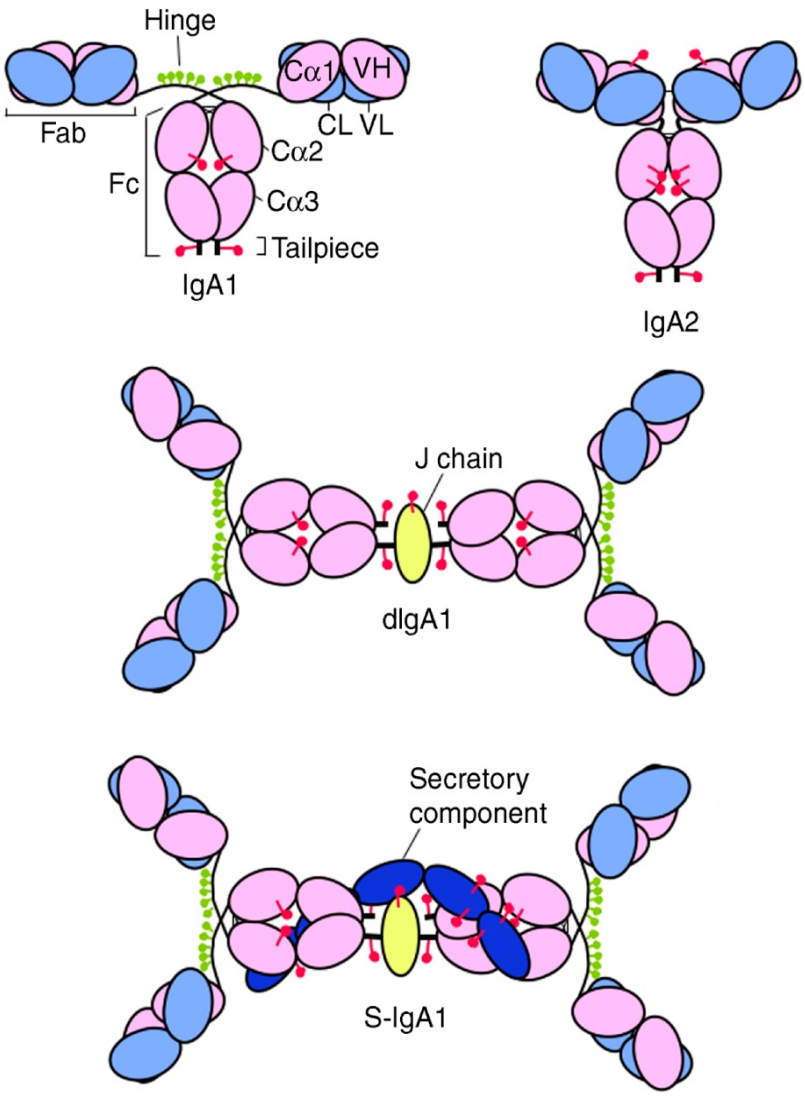

Figure 1.6: Human IgA structure. Schematic diagrams of IgA1, IgA2, dimeric IgA1 and secretory IgA1. Adapted from [163]

The role that IgA plays during HIV infection is likely to be complex. In non-human primate (NHP) studies, neutralising monoclonal dimeric IgA administered to the rectal lumen was shown to prevent SHIV (Chimeric simian/human immunodeficiency virus) challenge. [173] This study also demonstrated in the mucosal surface that dimeric IgA1 was better at protecting macaques than IgA2 or IgG antibodies [173]. The larger more flexible hinge region of the IgA1 may have facilitated more viral binding and therefore better efficacy in preventing infection. In NHP following vaccination with gp41 subunit virosome (utilising intramuscular

and mucosal delivery routes) HIV-specific IgA and IgG antibodies were generated [174]. Anti-gp41 IgA was induced in both serum and vaginal fluids and correlated with protection from vaginal challenge by blocking transcytosis of the virus [174]. Interestingly, there was epitope specificity differences in the mucosal and systemic antibody responses [174]. It should be noted that there is significant differences in studying IgA in macaques compared to humans as macaques only have one IgA subclass which is similar to IgA2 in humans [175]. This complicates the interpretation of IgA in NHP model systems and more studies on IgA and HIV infection in humans are warranted.

In humans, the total concentration IgA antibodies present in the serum increases as HIV disease progresses, conversely, mucosal IgA concentrations decreases as HIV infection progresses [176-178]. The role that IgA plays during HIV infection is complicated by monocytes and neutrophils also exhibiting decreased FcαRI expression as the diseases progresses [179]. HIV-exposed, persistently seronegative people have been shown to have mucosal and plasma IgA that inhibits transcytosis of HIV across epithelial cells [180]. The IgA from these people has also been shown to prevent HIV infection of peripheral blood mononuclear cells *in vitro*. IgA was shown to inhibit IgG mediated ADCC responses with peripheral blood mononuclear cell (PBMCs) in subjects with primary HIV infection, but no inhibition was observed in elite controllers [181]. This suggests that Env specific IgA is capable of negatively modulating ADCC responses to HIV but the particular innate immune cells involved have not been well studied.

IgA was shown to bind conserved regions of the membrane proximal external region (MPER) of the gp41 envelope protein in HIV-exposed, persistently seronegative people and has been shown to be able to neutralise HIV [182, 183]. In addition, HIV-1 Env specific mucosal IgA antibodies in breast milk have been shown to reduce mother to child transmission of HIV-1 [184], highlighting the potential protective capabilities of IgA. In contrast, two separate studies in 1995 showed that IgA resulted in modest enhancement of HIV replication *in vitro* [185, 186]. These studies suggest that there is an FcαRI-mediated enhancement of HIV replication in the presence of serum IgA but the relevance to mucosal IgA remains uncertain. The mechanism for the enhanced HIV replication has not been investigated thoroughly. Interestingly in other infections, IgA has been shown to inhibit IgG responses in an antigen-dependent manner to both *Candida albicans* and Epstein-Barr virus [187, 188]. Thus, IgA may

be both beneficial and inhibitory during infections and the role it plays during HIV infection is still emerging.

1.7. Neutrophils

Neutrophils are the most abundant white blood cell accounting for 40-70% of circulating leukocytes and have a short half-life of ~6 hours without stimulation [189]. Following activation and migration into the tissues, however, neutrophils can survive for several days [190]. Neutrophils are first responder immune cells and are regarded as an essential effector cell of the innate immune response to many pathogens [191]. Neutrophils can undergo rapid chemotaxis and migrate into the tissue to the site of infection and can recruit a number of other immune cells to the site [192]. Neutrophils are abundant in the mucosal surfaces, particularly in vaginal tissue, which is a key site of HIV transmission [193]. They have long been associated with the protection and clearance of bacteria and fungi, while the role that they play in viral infections is a field of increasing interest [194].

1.7.1. Plasticity of neutrophil subsets

There is currently much debate into the heterogeneity and plasticity of neutrophils. Strategies utilised to elucidate neutrophil subsets have included distinct cell surface markers, cell maturity, function and residency under both homeostatic and pathological conditions [195]. The surface expression of OLFM4 or CD177 and PR3 has been used to define subsets of neutrophils associated with autoimmune diseases [196, 197], however, the definition of these subsets remains controversial. This is in part due to the substantial individual variation of CD177 expression and the fact that it is not expressed on neutrophils by all individuals [198]. In addition, a number of subsets of neutrophils have been reported in autoimmunity [199], cancer [200, 201], cardiovascular disease [202, 203], pregnancy [204] and during infections [205-207]. Despite the advances in identification of new neutrophil subsets it remains to be verified if they are distinct subsets that derive from separate lineages or represent different activation or polarization of a common neutrophil precursor.

The effects of HIV infection on neutrophils is an emerging area of interest and the role of neutrophil subsets is of importance. A subset of neutrophils known as a granulocytic myeloid-derived suppressor cells (G-MDSCs) have been identified with a distinct phenotype (CD15+ CD11b+ CD66b+ CD33int CD14-) [208]. G-MDSCs have the ability to suppress T cell

functions (including proliferation and cytokine production) by multiple mechanisms including the release of arginase-1, resulting in a depletion of arginine and downregulation of TCR ζ chain, production of reactive oxygen species (ROS), production of regulatory cytokines and induction of regulatory T cells [205, 209]. Subjects with HIV infection have significantly higher levels of G-MDSCs in the peripheral blood [210]. It has also been shown neutrophils in the blood of HIV-1-infected individuals express high levels of PD-L1 which correlated with PD-1 expression on CD4+ and CD8+ T cells [211]. This in turn correlated with the frequency of low-density neutrophils expressing the G-MDSC phenotype [211]. Low-density granulocytes (LDG), that are phenotypically G-MDSC, are a major source of peripheral arginase and the frequency of LDGs inversely correlated with the CD4+ T cell count and positively correlated with plasma HIV RNA copies/ml [212]. These studies indicate the neutrophil subsets may play a role in HIV infection including potentially suppressing the adaptive immune system. It is important to note that LDG are still being evaluated for their source, with some speculating that they are immature neutrophil while others contend that they are activated and highly degranulated neutrophils, resulting in the lower density [195]. Despite this, LDG have other distinctive features including the increased synthesis of type 1 IFNs [213], decreased phagocytosis of bacteria [199] and an enhanced capacity to produce neutrophil extracellular traps (NETs)[214, 215].

1.7.2. Neutrophils in HIV infection

Despite their essential nature in protection and control of infections, neutrophils can also contribute to tissue damage through their release of reactive oxygen species (ROS) and other granule contents [216]. Neutrophils are implicated in several autoimmune diseases and may also contribute to pathological processes in a number of infections [171, 217, 218]. It is not known if neutrophils contribute to the observable pathology in HIV infections. However, the role of neutrophils in atherosclerotic cardiovascular diseases during HIV infection is currently being investigated by several groups [219, 220]. Neutrophils have remained understudied in viral infections, and HIV is no exception to this, despite their potential to prevent infection. This is at least in part due to the difficulty in studying them due to their greatly reduced viability after cryopreservation, necessitating the study of fresh isolates [221, 222]. Despite these limitations it is known that neutrophils have impaired antimicrobial functional capacity (including chemotaxis, phagocytosis, bactericidal activity, and oxidative burst abilities) during HIV infection [223-227].

It is common for HIV infected individuals to experience a decrease in peripheral blood neutrophils when compared to uninfected individuals. Neutropenia (neutrophil count <2000μl) has been found to affect approximately 10-50% of HIV infected individuals [228-230]. In the largest study on neutropenia in HIV infected women, it was found that during 7.5 years of follow-up 79% of women presented at least one time with neutropenia [228]. It should be noted that neutropenia in these studies has been associated with more advanced HIV disease progressing i.e. lower CD4+ T cell counts and with higher HIV-1 RNA levels [231]. The instigation of ART can assist in the prevention of the development of neutropenia [228]. The use of different antiretrovirals has different implications for neutrophils; azidothymidine has been shown to increase the probability of developing neutropenia, however, it is no longer commonly used in treatment [229]. Neutropenia in HIV infection can be treated with G-CSF or GM-CSF which increases neutrophil production and effector functionality [231-235].

The cause of the neutropenia in HIV infection remains unknown, but several factors have been suggested (reviewed in [236]). HIV has been shown to destroy multipotent hematopoietic stem cells through direct infection, Fas-dependent apoptosis and suppression of proliferation granulomonocytic progenitor cells [237-240]. HIV infection leads to changes in the bone marrow microenvironment which decreases the frequency of progenitor cells and there is a decrease in granulocyte development factors like G-CSF [241, 242]. It has also been shown that during HIV infection there is impaired production of neutrophil supporting cytokines by T cells and other mononuclear cells [243, 244]. It has also been proposed that the relationship between HIV-1 RNA and neutrophil induced cytotoxicity contributes to neutropenia [228]. In HIV infected subjects that have progressed to AIDS their neutrophils have been shown to have increased apoptosis and decreased life span [245]. It has also been speculated that the peripheral neutropenia that is frequently observed is a result of increased trafficking to effector site like key mucosal sites [246].

The neutrophils of HIV infected individuals have impaired phagocytosis [247], oxidative burst [248], chemotaxis [249] and bactericidal activity [224] in both untreated infection and following ART [250, 251]. This impaired neutrophil functionality has been associated with an increased risk of a range of secondary infections, including aspergillosis and bacteremia [225, 252]. The treatment with G-CSF has been shown to reduce the bacterial and fungal infections

in progressive HIV infection [253, 254]. The functionality of neutrophils has not been well studied following the intervention with ART, however, dideoxynucleoside antiretroviral drugs have been demonstrated to enhance neutrophil killing of *Candida albicans* and maintained antibacterial killing and chemotaxis [255]. In contrast, HIV protease inhibitors have been shown to severely impair a range of neutrophil functions including chemotaxis, phagocytosis, ROS production and apoptosis [256]. The influence of different ART strategies complicates the study of neutrophils from HIV infected individuals.

A definitive role for neutrophils in the prevention of HIV is not clear, however there is supporting clinical evidence to their potentially protective nature. There is an association of low peripheral neutrophil counts and the HIV infection susceptibility [257]. In addition, mothers with low neutrophil counts also had increased intrauterine perinatal transmission of HIV [258]. Female sex workers with neutropenia have also been shown to have increased risk of HIV transmission [257]. In males, it was shown that prior to HIV seroconversion there is high IL-8 concentration in the foreskin that attracts neutrophils to the site and there is a higher CD4+ T cell density in the foreskin [259]. This aligns with data on the protective effect of circumcision on HIV infection [260-262] and reviewed in [263]. HIV infected individuals have abundant neutrophils in inflamed oral, genital, and gut mucosae and these cells have also been observed in draining lymph nodes, contributing to the pathology [264]. Overall, these studies highlight that neutrophils play a multifaceted role in HIV being both beneficial and potentially protective, but they can be the source of detrimental pathology.

1.7.3. Neutrophil responses to HIV infection

Neutrophils mediate a range of effector responses to Fc receptor signalling including phagocytosis, ADCC, degranulation and NET formation. Degranulation of neutrophils results in a release of important antimicrobial proteins that are contained in the granules [190, 265]. Human neutrophil peptides (HNPs), also known as alpha-defensins, has potent antiviral activity and has been shown to be crucial in a number of viral infections including HIV (reviewed in [266]), influenza [267], HSV1 [268]. HNP1, HNP2 and HNP3 can all negatively affect HIV infection through a range of mechanisms including directly binding and inactivating HIV [269], bind the gp120 blocking viral entry [270, 271] and can block nuclear import and transcription [272]. HNPs has been shown to be elevated in the foreskin of men in a population of highly exposed seronegative (HESN) individuals, indicating a potential role in protection

[273]. Degranulation of neutrophils results in the release of myeloperoxidase (MPO) and ROS which produces hypochlorous acid, which is in turn able to directly kill HIV and denature viral proteins [274]. The MPO responses is broad spectrum and highly potent and is thought to be the primary functioning antimicrobial compound of neutrophils [275].

Neutrophils can utilise a novel mechanism called neutrophil extracellular traps (NETs) which can capture and kill pathogens [276]. The primary constituents of NETs are DNA and histones with a number of granule-derived antimicrobial peptide and enzymes including MPO, elastase, HNPs and several others [276, 277]. In has been shown *in vitro*, that NETs are able to bind to HIV and irreversibly inactive it, preventing infection of CD4+ T cells [278]. This inactivation of HIV is thought to be mediated by the presence of MPO and HNPs being present in the NETs [278]. NETs have been shown to be induced by HIV virions and *in vitro* can occur rapidly within 5 minutes of exposure [278]. Recently, it was shown that neutrophils isolated from the female genital tract were able to mediate rapid NET responses to HIV and irreversibly inactive HIV [279]. NET responses could yield a unique opportunity in preventing HIV prior to establishment of a HIV reservoir but this has to be tightly regulated due to the potential tissue damage that it can cause.

A key immune effector mechanism of neutrophils is phagocytosis. Neutrophils can phagocytose immune complexes of HIV or HIV infected cells [138, 280]. Neutrophils utilise NADPH (nicotinamide adenine dinucleotide phosphate) which is an oxygenase-dependent mechanism to generate ROS, which are then released into the phagosome to kill the pathogen [281]. In addition, the phagosome binds to azurophilic granules releasing a number of anti-microbial enzymes including HNP, MPO, human leukocyte elastase (HLE), cathepsins, lactoferrin and LL-37 that can all directly kill pathogens including HIV [282]. It has recently been shown that neutrophils are able to mediate phagocytosis using purified IgG from HIV infected subjects to gp120 [138]. It was found that there was no difference in antibody-dependent neutrophil-mediated phagocytosis between ECs or viremic controller (VC) subjects on anti-retroviral therapy or subjects not taking anti-retroviral therapy [138]. The ability of these clinical groups to mediate neutrophil ADCC responses had not been evaluated to date and this is one aspect that is studied in Chapter 3.

Neutrophils are traditionally thought of as a phagocytic cell, primarily targeting bacteria [168, 283]. There is however increasing interest in the capacity of neutrophils to mediate antiviral capabilities using FcRs [129, 284, 285]. Neutrophils were shown to reduce influenza virus infection in cell culture by ADCC mechanisms [286]. Neutrophils can also mediate ADCC responses using IgG and IgA antibodies to cancers [287, 288]. In addition, neutrophils from healthy donors and HIV-infected subjects have long been shown to have the capacity to mediate HIV-specific ADCC to infected cells *in vitro* [289, 290]. ADCC responses in NK cells and monocytes have been well studied and mechanisms of target cell death is well established [291-294]. Despite this, the mechanisms that neutrophils utilize to mediate ADCC remains poorly studied, as they lack both perforin and granzyme which are utilised by NK cells and monocytes to mediate ADCC responses [295-297]. Despite this, neutrophils are well positioned anatomically for anti-HIV functions such as ADP and ADCC but remain relatively understudied in comparison to other innate cells such as NK cells and monocyte/macrophages.

1.8. Viral controllers of HIV

A small subset of individuals infected with HIV are able to control the infection in the absence of ART, they are described as ECs and VCs. ECs are able to control the viral load maintaining <50 HIV RNA copies/ml blood while VCs maintain viral load of <2000 HIV RNA copies/ml blood over time without antiretroviral treatment [298]. HIV controllers are also known to have potent HIV-specific CD8+ T cell response compared to HIV progressors and the CD8+ T cell responses in these subjects is often restricted by HLA alleles B57 and B27 [98, 299]. There is also a subset of controllers that are likely a result of mutated viral strains, such as viruses with Nef/LTR deletions [300].

In addition, controllers have been shown to have significantly higher Fc-mediated antibody responses including ADCC [152]. Seminal studies by Lambotte et al observed that despite lacking neutralising antibody responses, HIV VCs had significantly higher ADCC responses compared to viremic individuals [152]. Multiple other studies have confirmed these observations, where enhanced ADCC responses are present in HIV long-term non-progressors (LTNP) and can alter the course of infection [150, 301]. Higher ADCC responses are detected in HIV controllers that lacked the HLA B57 allele [302, 303]. However, ECs are highly enriched for the protective HLA alleles including B57, therefore, it may be more useful to study VCs for antibody mediated protection. More recently, polyfunctional Fc-mediated responses

(including ADCC, ADCP, ADCVI and complement) were also associated with HIV controllers [138]. The combinations of Fc-mediated responses during acute infection (including ADCVI, and IgG subclass profiles) were able to predict HIV control from HIV progressors [304].

In comparison to Fc responses mediated by IgG, IgA immunity in HIV controllers is understudied. A recent study by Ruiz et al showed that total serum with IgA removed from primary and chronic HIV infection subjects showed enhanced ADCC responses [181]. This suggests that serum IgA inhibits IgG Fc-mediated functions. Surprisingly, IgA depletion from a small cohort of ECs (n=4) had no effect on the functional capacity of IgG antibodies, suggesting that the IgA from controllers may have different biophysical and functional capacities compared to that of progressors [181]. The authors of this study hypothesize that blocking of IgG by IgA of the same epitope may have caused the functional inhibition within the progressor patient, however, no follow up experiments were conducted to confirm this [181]. Exploring the differences between functional IgG antibodies and IgA inhibition in controllers versus progressors will be a key focus of Chapter 3 of my thesis.

1.9. HIV vaccines

The development of an effective prophylactic vaccine for HIV faces a number of challenges that need to be overcome. A prophylactic vaccine has to prevent the establishment of the viral reservoir shortly after the initial infection. The generation of bNAbs from vaccination has been prevented by the HIV Env protein having high diversity and a complex structure with limited accessible conserved regions. The recruitment of CD4+ T cells following vaccination should be considered as they are required for full development of an immune response but can also serve as kindling for greater HIV infection in high risk populations. There are challenges with conventional types of immunity to HIV and alternative approaches like antibody-mediated neutrophil functions and the analysis of IgA antibodies need to be pursued. Despite the number of challenges in generating an effective prophylactic vaccine, some candidate vaccines have successfully prevented SIV and SHIV infections in NHP models. These vaccines have given hope that a successful vaccine is possible, and a number of these vaccines have entered efficacy trials in humans.

1.9.1. The STEP trial

A recombinant Adenovirus type 5 (Ad5) vaccine expressing HIV-1 Gag/Pol/Nef, was tested in the STEP (HVTN 502) and Phambili (HVTN 503) trials. The STEP trial was conducted in MSM and high-risk women in the Americas, Caribbean, and Australia, however, the trial was stopped prematurely due to futility by a lack of efficacy [305]. The vaccine showed no vaccine-induced reduction in the rate of HIV-1 infections in the study participants and no reduction in viral load of infected vaccinees. There was an increase in HIV infection rates in the vaccinees compared to placebo in some analyses, although the precise cause or mechanism is unknown. Vaccinees who were uncircumcised and had higher serum titres of Ad5 antibodies prior to vaccination showed an increased rate of HIV infection, [305] suggesting the possibility that local anti-Ad5 immunity could have played a role in enhancing infection [306]. Despite this, in further sieve analysis it was suggested that there was a weak vaccine induced pressure on the founder viruses [307]. The recombinant Ad5 Gag/Pol/Nef vaccine elicited cell mediated immunity in most subjects yet the breadth, strength, and/or focus of these responses was insufficient to prevent infection or reduce viral load [308]. A similar regimen to the STEP trial that was later conducted in Indian rhesus macaque recapitulated similar results, showing no protection to an intrarectal SIVE660 challenge [309] or penile SIVmac251 challenges [310].

1.9.2. AD26 vaccines

The HVTN705 HIV vaccine efficacy trial is currently being conducted in Sub-Saharan Africa and enrolling 1300 vaccinees and 1300 placebo recipients. The vaccine is testing four recombinant Adenovirus type 26 (Ad26) vectored mosaic sequences given at 0, 12, 24 and 52 weeks, followed by recombinant trimeric gp140 Env booster given concurrently with the Ad26 vaccine at 24 and 52 weeks. The results of this trial are not expected for some years, however, a smaller phase 1/2a trial utilising Ad26 mosaic vector vaccines has been completed known as the APPROACH trial.

The APPROACH trial was conducted in East Africa, South Africa, Thailand, and the USA. It enrolled 393 participants to receive the vaccine, all of whom were considered to be at low risk of HIV infection [311]. The vaccinees were primed at 0 and 12 weeks, with Ad26.Mosaics.HIV (Env/Gag/Pol) and booster vaccines given at week 24 and 48 with either Ad26.Mosaics.HIV (Env/Gag/Pol) or modified vaccinia Ankara (MVA) with or without a clade C Env gp140 protein. It was found that the vaccines were well tolerated with limited

adverse side effects. The mosaic Ad26/Ad26 with the high dose gp140 HIV booster vaccine was shown to be the most immunogenic, inducing Env-specific binding antibody responses (100% of vaccinees) and ADCP responses with THP-1 cells (80% of vaccinees) at week 52, and T-cell responses by ELISpot at week 50 (83% of vaccinees) [311]. Similar results were shown in rhesus macaque with Ad26/Ad26 plus gp140 boost inducing similar magnitude, durability, and phenotype of immune responses as compared with humans and afforded 67% protection against a series of six repetitive, heterologous, intrarectal SHIV challenges [311].

Interestingly, the vaccines tested did not induce broadly neutralising antibody responses, indicating that other functional antibody responses, like ADCP, and T cell responses were responsible for the protection provided in the macaque trial. Correlates of protection analysis in the macaque trial suggested a combination of Env-specific binding antibodies and T cell responses may have facilitated protective immunity. In prior Ad26 HIV vaccines in Rhesus monkeys it has been shown that protection correlated with Env-specific binding and functional antiviral antibody responses, including ADCP [312]. As noted above, the vaccine concept utilised in the APRROACH trial is currently being evaluated in a phase 2b clinical efficacy study in Sub-Saharan Africa (HVTN705, ClinicalTrials.gov NCT03060629).

1.9.3. AIDSVAX gp120 protein based vaccines

The early HIV vaccine efficacy trials utilised recombinant gp120 protein to vaccinate, which had proved to be efficacious in NHP models. In Phase I and II trials these vaccines were shown to be safe and capable of inducing antibody responses. With these promising results two large scale phase III trials were conducted using the AIDSVAX vaccine composed of a recombinant gp120 protein. The first trial, VAX004, was completed in North America and the Netherlands enrolling 5403 (5095 men and 308 women) at high risk of HIV transmission with subtype B gp120 protein [313]. The second trial, VAX003, was completed in Thailand enrolling 2546 individuals at high risk of HIV transmission as injection drug users, with the AIDSVAX formulation based on the B and E subtype viruses [314]. Despite some early success in chimpanzee models, neither vaccine trialled displayed significant efficacy in the prevention of HIV acquisition or any delayed HIV disease progression upon infection during the initial analysis [315, 316]. There is, however, some evidence that the trials induced ADCVI responses that correlated with reduced rates of infection and this was thought to be mediated by ADCC

responses [317]. Although the trials were negative, they suggest that an Fc-mediated type immunity may be useful in protective immunity.

1.9.4.RV144

Despite the absence of vaccine efficacy in VAX003/004, the AIDSVAX was used as a booster protein vaccination component to the ALVAC vaccine which was utilised in the phase III Thai RV144 trial. The ALVAC [vCP1521] vaccine is a nonreplicating canarypox virus encoding the HIV Env/Gag/Pol. The ALVAC vaccine had previously been evaluated in a phase I/II trial and was shown to induce limited (<25%) CD8+ T cell responses [318]. The Thai RV144 trial enrolled 16,402 volunteers with 8197 receiving the vaccine and 8198 receiving a placebo. The vaccinees received 4 priming injections with ALVAC at weeks 0, 4,12, and 24, and boosted with the AIDSVAX B/E gp120 proteins at weeks 12 and 24 [319]. Despite the limited successes of the individual vaccines, when combined in the Thai RV144 trial it conferred the first, and to date, only significant but modest vaccine efficacy of 31.2% ($p = 0.04$) [319]. Interestingly, in post-hoc analyses the vaccine generated a vaccine efficacy of 60% at 12 months and waned over time, consistent with the waning levels of antibodies over time [320]. The vaccinees that later became infected with HIV displayed no significant reduction in viral load or changes in CD4+ T cell counts compared to the placebo recipients who became infected [321].

The initial immune correlates analysis of the RV144 trial identified that the binding of IgG antibodies to the V1V2 region of the Env protein correlated inversely with the rate of HIV-1 infection [82]. In contrast, high levels of plasma IgA antibodies to the Env decreased the vaccine efficacy [82]. Sieve analysis of break through virus in vaccinees showed, mutations in Env around the sites that the V1V2 specific antibodies bind to likely resulting in reduced efficacy and further supporting the correlation between V1V2 antibodies and protection [322]. The same V2 epitopes identified in the sieve analysis have been identified in HIV positive subjects and they can mediate broad ADCC activity [323]. The RV144 induced very little CTL response and limited neutralising antibodies (Tier 1 but no tier 2 neutralisation) were induced by the vaccines, suggesting these responses played no role in the modest protection observed [82, 324].

In secondary post-hoc correlation analysis, it was revealed that reduced risk of infection correlated with IgG antibodies mediating ADCC responses to the Env protein but only in the presence of low plasma titres of anti-Env IgA [82]. The IgG antibodies mediating this effect were subsequently attributed to the IgG3 subclass of antibodies mediating Fc-functional responses to the V1V2 region of Env protein [325, 326]. The IgG3 subclass has been shown to be important in Fc-functional responses and in bNab responses and have correlated with delayed HIV progression [138, 304, 327, 328]. In contrast, Env-specific IgG2, IgG4, and serum IgA titres did not correlate with reduced risk of infection [325, 326]. Correlates of risk analyses showed that the vaccine recipients who contracted HIV had a higher Env-specific plasma IgA:IgG ratio compared to the uninfected vaccine recipients [82]. It was shown that an IgA mAb generated from the RV144 vaccinees was able to inhibit the plasma IgG mediated ADCC responses [329]. This indicated that IgA is inhibiting IgG mediated ADCC responses by epitope competition [329]. In the presence of low plasma anti-Env IgA antibody levels, it was shown that there were inverse correlations with the risk of infection with IgG avidity, ADCC, neutralising antibodies, and CD4+ T cells that were of borderline significance [82]. In further analysis of the CD4+ T cells responses elicited by the vaccine, it was shown that two polyfunctional subsets (firstly co-expressed CD40 ligand, IL-2, IL- 4, IFN-γ, and TNF-α; secondly CD40 ligand, IL- 2, and IL- 4) were recruited and correlated with reduced risk of HIV infection [330]. The expression of IL-4 by CD4+ T cells is associated with decreased or no expression of CCR5 which could be contributing to the efficacy of the vaccine. This is consistent with the ALVAC vaccine recruiting CD4+ T cells that are relatively resistant to HIV infection [331]. There is ongoing evaluation of subsets of the RV144 vaccinees including the RV305 (discussed below in section 1.9.5) and a trial of a similar vaccine in South Africa known as the HVTN100 trial (discussed below in section 1.9.6).

1.9.5. Late boosting of the RV144 regimen: the RV305 study

the RV305 trial was a follow-on trial which re-immunized vaccinees from the RV144 trial, who had remained uninfected, to boost their B cell repertoires. HIV uninfected RV144 vaccine recipients (162 subjects) were re-immunized 6-8 years later with AIDSVAX B/E gp120 alone, ALVAC-HIV alone, or a combination of ALVAC-HIV and AIDSVAX B/E gp120 [332]. The vaccinations increased the V1V2 specific IgG antibodies, but the increased IgG titres were short lived [332]. The boosting of the RV144 vaccinees in the RV305 increased the somatic mutation rate of the Env-specific memory B cell (from 2.9% post RV144 to 6.7% post RV305)

[333]. There was no increase in broadly neutralising antibody activity following these booster vaccinations. However, there was an expanded population of CD4 binding site reactive memory B cells which had long third heavy chain complementarity determining regions (HCDR3) [333]. Two of these antibodies were found to be able to recognise the open confirmation of the near native gp140 trimer. The authors hypothesised that vaccination with stably closed trimers may enhance the antibodies to recognise the closed confirmation of the Env trimer and ultimately, at least modestly, improve their neutralisation potential [333].

1.9.6. HVTN100 and HVTN702

The HVTN100 trial was conducted as a follow on from the RV144 utilising a similar vaccination strategy and vaccines adapted to the circulating HIV clades present in South Africa. The HVTN100 trial enrolled 210 participants to receive the vaccine and 42 for the placebo [334]. The vaccinees received ALVAC-HIV vector (vCP2438, similar to the ALVAC vector used in RV144 but with clade C HIV gene inserts) at 0, 1, 3, 6, and 12 month plus bivalent subtype C gp120 protein in a MF59 adjuvant at 3, 6, and 12 months. 56% of vaccinees induced a CD4+ T cell response which was elevated compared to the RV144 vaccine [334]. 100% of the vaccinees induced IgG antibody responses specific for the gp120 Env with significantly higher levels compared to the RV144 regimen [334]. Despite this, the IgG antibody titres to the V1V2 region were lower compared to the RV144 trial which was largely attributed to the protection provided in the RV144 [334]. The IgA responses following the vaccinations have not been reported despite the interest in such responses following on from the importance in the RV144 trial. A precursor to the HVTN100 was the HVTN97 that used a similar vaccine schedule and compared the immune responses to the RV144 [335]. The HVTN97 induced higher magnitudes of IgG responses to the gp120 Env protein [335]. There were also high levels of ADCC induced antibody responses than the RV144 vaccinees (72.6% to 58.5% respectively) [335]. In addition, 100% of the vaccinees were able to mediate an ADCP response with monocytes [335].

The HVTN702 efficacy trial was a follow on to the HVTN100 trial that showed high antibody responses [336]. Unfortunately, the HVTN702 trial was stopped early for lack of efficacy. The trial utilised a similar vaccine strategy to the RV144 but adapted to the HIV clade C and the Env protein booster used an alternative adjuvant. In addition, there was the incorporation of later stage booster vaccinations to enhance the longevity of the immune responses generated.

There were 129 HIV infections in the vaccinated group and 123 in those who received the placebo. The subjects that received vaccinations are continued to be followed but no further vaccinations are planned to be given.

1.9.7. Passive transfer of HIV antibodies: the AMP efficacy trials

An alternative approach to the traditional vaccination with HIV antigens is to repeatedly passively transfer known bNAbs to people that are at high risk of HIV infection and to maintain levels that would be protective in the event of an exposure. This strategy comes with some challenges including i) the high cost of antibody manufacture, ii) the repetition of transfusions to maintain protective antibody titres, iii) the requirement for trained personnel to administer, and iv) the temporary level of protection generated compared to the potential of a lifelong protection from a prophylactic vaccine. This strategy circumvents the difficulties in generating neutralising antibodies via vaccination and provides a proof-of-principle approach showing that antibodies can mediate effective immunity in humans. Macaque studies have long suggested that passive infusion of neutralising antibodies can protect against SHIV infection if the strain is recognised by the neutralising antibody [120-123, 337-341].

Despite the difficulties associated with passively transferred recombinant antibodies, there is currently only one clinically approved monoclonal antibody (mAb), known as Palivizumab, for use against a viral pathogen. Palivizumab is used to prevent respiratory syncytial virus (RSV) infections in infants at high-risk [342]. It has also been demonstrated that mAbs to Ebola were able to clear the virus from infected primates [343-345] and very recently anti-Ebola antibodies appear to be effective in human Ebola outbreaks ([346],Press report [347]). In NHP models of HIV infection, a number of mAbs have been evaluated targeting different regions of the HIV envelope protein (including: CD4bs, V1V2, V3-glycan and the MPER region) for their protective potential [120-123]. In these studies, the mAbs have shown varying levels of protection from challenge likely due to the plasma antibody titres, challenge virus and the epitope target. In addition, the mechanisms of protection provided by these mAbs can be from both neutralisation and FcR cell mediated effector functions and this might be contributing to the varying levels of protection [147].

As noted in an earlier section, the importance of the FcγR in protective immunity to HIV has been shown in NHPs using the broadly neutralising b12 mAb compared to a b12 mAb LALA variant which has two mutations in the Fc portion of the antibody preventing it from binding to FcγRs. It was shown that the b12 LALA antibody has decreased ability to protect the NHPs from SHIV challenge compared to the native b12 mAb [144]. It was also demonstrated using a b12 variant, KA (K322A), that removed complement activation, that it was driven by FcγR and not by complement [144]. This result was mimicked in an ongoing low-dose viral challenge with SHIV delivered to the mucosal within macaques [341]. It was shown that the b12 variant was more than twice as effective as the LALA variant in reducing the infection risk across each of the repeated challenges [341]. These two studies highlight that FcR binding can enhance the protective abilities of passively transferred antibodies. It has also been shown that not all bNAbs have the same potential to mediate Fc-mediated effector functions. The balance between protection mediated by neutralising capacity and Fc-mediated functions was recently challenged by Parsons et al, when macaques passively infused with PGT121 broadly neutralising antibodies with abrogated Fc binding (LALA variant) were also protected from infection, with identical efficacy to those transfused with standard PGT121 bNAbs [145]. There is the potential to re-engineer Fc regions of some bNAbs to improve Fc-mediated responses and enhance protection provided in humans [348].

Recently, two large phase IIb clinical trials (HVTN 703 and HVTN 704, clinicaltrials.gov NCT02568215 and NCT02716675, respectively) have commenced, testing the efficacy of VRC01 in preventing HIV transmission in high risk populations. The VRC01 antibody is a broadly neutralising recombinant antibody that binds to the CD4 binding site of the HIV envelope protein and neutralises 90% of circulating strains [349]. The VRC01 antibody has previously been shown to be safe and well tolerated in adults in two small clinical trials, and to reduce HIV viral load in infected subjects [350, 351]. The HVTN 703 trial is enrolling 1500 participants and the HVTN 704 is enrolling 2700 participants. These trials are known as the Antibody-Mediated Prevention (AMP) studies and are the first to study the ability of bNAbs to prevent HIV-1 infection in humans. The AMP studies will both use 20 or 30 mg/kg of VRC01 transfused 10 times at 8-week intervals for a period of 80 weeks [352]. The goals of the AMP trials are to determine the efficacy of the passive transfer of the VRC01 antibody and also to set a benchmark for plasma antibody titres to provide protection of an at-risk population. The AMP trials are ongoing, and results are expected in 2020.

1.10. Rationale and aims of this thesis

There is increasing interest in the Fc-mediated responses and IgA antibodies in HIV infection and vaccine development. Fc-mediated responses have been shown to correlate with delayed HIV disease progression [148-152]. The importance of Fc functional antibodies was also highlighted by the RV144 HIV vaccine trial showing modest efficacy, which was attributed to ADCC mediating IgG antibodies [319] [82]. Despite the rise in interest in Fc-mediated responses in HIV, limited research has been conducted on neutrophils which are key innate immune cells at mucosal surfaces and in the blood. Methods were developed to evaluate antibody-dependent neutrophil-mediated effector responses to HIV (Chapter 2). The RV144 vaccine trial generated interest into HIV-specific plasma IgA with HIV-specific IgA responses in the vaccine trial being associated with reduced vaccine efficacy [82]. The HIV-specific plasma IgA from the RV144 was shown to block protective IgG mediated antibodies binding and preventing ADCC responses with NK cells [353]. The role that IgA plays in active HIV infection remains controversial, with other reports of HIV-specific IgA inhibiting HIV infection and being potentially protective [180, 182, 183]. IgG and IgA responses from VCs and ART naïve HIV positive subjects were investigated for their ability to induce neutrophil-mediated responses and assessed if IgA was able to inhibit these responses (Chapter 3). The RV144 vaccine trial has previously been evaluated for a range of antibody mediated responses, including Fc-mediated responses with NK cells and monocytes, but has not been investigated previously with neutrophils. IgG and IgA responses from the plasma samples from the RV144 vaccine trial were investigated for their ability to induce neutrophil-mediated responses and if IgA was able to inhibit these responses (Chapter 4). Overall this thesis aimed to investigate the role of antibody-dependent neutrophil responses in HIV infection and in HIV vaccinations, and the effects of IgA on these responses.

Aim 1: To develop and optimise methods to evaluate HIV-specific antibody- dependent neutrophil effector functions

Aim 2: Evaluate the effect of IgA on IgG antibody-dependent neutrophil functions from HIV-1 viremic controllers and HIV non-controller cohort

Aim 3: Evaluate the effect of IgA on IgG antibody-dependent neutrophil functions from RV144 vaccinees

Chapter 2

Neutrophils mediate HIV-specific antibody-dependent phagocytosis and ADCC

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Research paper

Neutrophils mediate HIV-specific antibody-dependent phagocytosis and ADCC

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ABSTRACT

There is growing evidence to support the role of Fc-mediated effector functions, such as Antibody-Dependent Cellular cytotoxicity (ADCC) and Antibody-Dependent Phagocytosis (ADP) in the protection and control of HIV. The RV144 trial and other recent HIV vaccine studies have highlighted the importance of ADCC responses in protection against HIV. The role of neutrophils, the most abundant leukocyte in the blood, has not been thoroughly evaluated for Fc-mediated effector functions to HIV. We optimized HIV-specific neutrophil ADCC and Antibody-Dependent Neutrophil Phagocytosis (ADNP) assays using freshly isolated primary human neutrophils from blood. We also developed methods to study ADP using the neutrophil-like HL-60 cell line. We found that neutrophils mediate both HIV-specific ADP and ADCC responses. In vitro, neutrophil-mediated ADCC responses peaked at 4 h, much faster than primary NK cell or monocyte-mediated responses. We detected a wide range of responses in the ADNP, HL-60 mediated ADP and ADCC across a cohort of 41 viremic antiretroviral therapy naïve HIV positive subjects. HL-60 and Neutrophil-mediated ADP and ADCC responses correlated well with each other, suggesting that they measure overlapping functions. The ADNP and HL-60 ADP inversely correlated with HIV viral load, suggesting that these antibody-mediated neutrophil-based assays should prove useful in dissecting HIV-specific immunity.

1. Introduction

HIV-specific antibodies with Fc-mediated functions may play an important role in protection from HIV. Recent human and non-human primate HIV vaccine studies have identified Fc receptor (FcR) mediated responses as potential correlates of protective immunity (Haynes et al., 2012; Bradley et al., 2017; Chung et al., 2015; Barouch et al., 2013, 2015). In non-human primate vaccine studies ADP and ADCC responses have correlated with protection (Bradley et al., 2017; Barouch et al., 2012, 2013). The importance of ADCC and ADP mediating antibodies during HIV infection has also been highlighted by numerous studies correlating ADCC and ADP with decreased disease progression (Lambotte et al., 2009; Baum et al., 1996; Wren et al., 2013; Chung et al., 2011a; Vaine et al., 2010; Ana-Sosa-Batiz et al., 2014; Ackerman et al., 2013). In addition, the moderately efficacious RV144 human Phase III vaccine trial demonstrated the potentially protective capabilities of FcR mediated responses, with ADCC responses correlating with reduced risk of infection in the absence of IgA (Haynes et al., 2012). Multiple different effector cells, including plasmacytoid dendritic cells (pDC) (Tjiam et al., 2015), NK cells (Chung et al., 2011a), monocytes/macrophages (Kramski et al., 2012a), other dendritic cell subsets (Altfeld et al., 2011) and neutrophils (Smalls-Mantey et al., 2013), have the capacity to mediate potent anti-viral Fc-effector responses against HIV. Recent research suggests that polyfunctional Fc-effector responses (i.e. the capacity to engage and recruit multiple different effector cells and functions) may be associated with protection

Abbreviations: HIV-1, human immunodeficiency virus 1; ADP, antibody-dependent phagocytosis; ADNP, antibody dependent neutrophil phagocytosis; ADCC, antibody dependent cellular cytotoxicity; RFADCC, rapid-fluorometric Antibody dependent cellular cytotoxicity; CFSE, carboxyfluorescein succinimidyl ester; FcR, Fc receptor; DMSO, dimethyl sulfoxide; ART, antiretroviral therapy; SHIP, specific hybridisation internalization probe

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and control of HIV infection (Ackerman et al., 2016, Chung et al., 2014). However, the majority of Fc-effector studies in HIV focus upon examining NK cells and/or monocytes responses, while other effector cells such as neutrophils remain understudied.

Neutrophils are the most abundant circulating leukocyte in the blood (30–70%) and can rapidly migrate to sites of infection and can mediate a range of effector responses (Kolaczkowska and Kubes, 2013; Mantovani et al., 2011; Palmer et al., 2006). Although neutrophil functions have long been associated with the killing and control of bacterial and fungal infections, there is growing interest in their role in control of viral infections (Mocsai, 2013; Galani and Andreakos, 2015; Naumenko et al., 2018). In the context of HIV infection, which is predominantly a mucosally acquired infection, it is important to note that neutrophils are abundantly present at mucosal surfaces, especially vaginal tissues and their presence is upregulated during HIV infection (Somsouk et al., 2015; Sips et al., 2016). Neutrophils express FcγRI (induced by cytokines) (Bovolenta et al., 1998), FcγRII and FcγRIII and can mediate a range of antibody-dependent effector functions, including ADP and phagocytosis-independent ADCC responses, however little is known of their importance in HIV infection (Bradley et al., 2017; Ackerman et al., 2016; Sips et al., 2016). Furthermore, while neutrophils have previously been reported to mediate ADCC killing of HIV infected cells, it remains unclear if this was in part mediated by phagocytosis (Baldwin et al., 1989; Jenkins et al., 1993).

NK cells have been extensively studied for ADCC responses (Isitman et al., 2012; Seidel et al., 2013), and mediate target cell lysis through the release of perforin and granzymes (Bryceson et al., 2006). In contrast, the mechanisms that neutrophils utilize to mediate ADCC remains controversial, as they lack perforin and granzyme (Grossman and Ley, 2004; Metkar and Froelich, 2004). Neutrophils release reactive oxygen intermediates following crosslinking of FcR which have been associated with ADCC responses (Horner et al., 2007). However, neutrophils isolated from chronic granulomatous disease patients lack reactive oxygen intermediates but are still able to mediate ADCC responses (Roberts et al., 1993). This indicates that reactive oxygen intermediates, contribute to, but are not the sole mechanism of neutrophil ADCC responses.

The rapid-fluorometric ADCC (RFADCC) has been used extensively to evaluate ADCC responses with monocytes, NK cells and PBMCs (Vaine et al., 2010; Gomez-Roman et al., 2006; Chung et al., 2009, 2011b; Ruiz et al., 2016; Lai et al., 2014). The short half-life of fresh primary neutrophils (6–8 h) means that it would be useful to develop cell-line based neutrophil assays (Summers et al., 2010). The human promyelocytic leukemia HL-60 cell line has the capacity to be differentiated into neutrophil-like CD11b+ cells after culture with dimethyl sulfoxide (DMSO) (Chang et al., 2006; Collins et al., 1978), and can be used to evaluate neutrophil effector functions (Yaseen et al., 2017; Fleck et al., 2005; Kim et al., 2015).

In this study, we optimized and validated HIV-specific neutrophil-mediated RFADCC assays and ADP assays and developed a neutrophil-like cell-line based HL-60 ADP assay. IgG purified from plasma of 41 viremic antiretroviral therapy (ART) naïve HIV positive subjects were readily able to mediate ADNP, HL-60 ADP and neutrophil RFADCC responses. Furthermore, these Fc-mediated neutrophil responses inversely correlated with viral load, suggesting that these optimized assays should prove useful in the evaluation of immune responses, where functional antibodies and neutrophils may play an important role.

2. Material and methods

2.1. Study subjects/plasma samples

Plasma was collected from HIV positive ($n = 41$) subjects, previously described (Wren et al., 2013; Chung et al., 2011a), and HIV negative healthy donors ($n = 13$). All HIV positive subjects were antiretroviral therapy (ART) naive. HIV positive subjects had a median CD4 T cell count of 520 cells/μl (range 296–1156 cells/μl) and a median plasma HIV-1 RNA level of 26,700 copies/ml, (range 399–339,000 copies/ml) to reflect the spectrum of HIV disease states (Table 1). All subjects provided written informed consent and the studies were approved by the relevant institutional ethics committees.

Table 1
Clinical characteristics of study cohort.

	HIV positive cohort
Number of subjects	41
Median CD4 count entry, cells/μl (range)	520 (296–1156)
Median plasma HIV-1 RNA copies/ml (range)	26,700 (399–339,000)

2.2. Primary cell isolation and culture

Neutrophils were isolated by adapting previously published methods (Bowers et al., 2014; Nauseef, 2007). Briefly, fresh heparinized blood was obtained from HIV negative donor leukocytes, separated using Ficoll density centrifugation. The granulocytes were enriched for by using 3% dextran sedimentation for 25 min at room temperature. The remaining cells were washed in and suspended in RPMI 1640 media supplemented with 10% FCS and penicillin (100 U/ml)/Streptomycin/(100 μg/ml) and L-Glutamine (2 nM) (Gibco, 10378-16) (RF10). The enriched cells were then collected and treated with a hypotonoic lysis reagent to remove any remaining red blood cells. The purity of the isolated cells was evaluated by staining with anti-CD16 BV605, CD3 PerCP (Biolegend), CD32 FITC, CD89 APC, CD64 BV510, CD66 BV421, CD14 APC-H7 and CD56 PE (BD biosciences). Monocytes and NK cells were isolated with RosetteSep kits (Stem Cell Technologies) as per manufacturer's instructions.

2.3. HL-60 cells maintenance and differentiation

HL-60 cells (ATCC) were cultured in Iscove's Modified Dulbecco's Medium (Sigma) with 20% heat-inactivated FCS and penicillin (100 U/ml)/Streptomycin/(100 μg/ml) and L-Glutamine (2 nM). To differentiate the HL-60 cell into a neutrophil-like subset, sterile DMSO (Sigma) was added into media at a final concentration of 1.3%, culturing for five days, as previously described (Collins et al., 1978; Martin et al., 1990; Birnie, 1988). The generation of neutrophil-like cells was assessed by staining with CD11b (Chang et al., 2006; Collins et al., 1978), a marker that has previously been identified as essential for neutrophil Fc receptor-mediated cytotoxicity (van Spriel et al., 2001).

2.4. IgG antibody purification

Total IgG was purified using Melon gel resin (Thermo Scientific) following the manufacturer's instructions. Briefly, purification columns (Pierce/Thermo Scientific) were prepared by loading 500 μl of Melon gel resin and washed with Melon gel purification buffer (Thermo Scientific). Plasma samples were diluted 1:5 in purification buffer, added to columns and incubated at room temperature on an orbital rotator for 5 min. The flow-through was collected then placed back into the column and incubate at room temperature for a further 5 min on the orbital rotator. The columns were centrifuged for 1 min at 3000*g* to elute the purified IgG.

The purified antibodies were quantified using an anti-IgG ELISA kit (Mabtech) following the manufacturer's instructions. Briefly, Maxisorb 96 wells plates (Nunc) were coated with the MT145 (2 μg/ml) capture antibody overnight at 4 °C. The plate was washed with PBST (PBS with 0.05% Tween 20) and blocked with 1% BSA/PBST for 2 h. The plate was washed and purified IgG antibodies diluted 1:20000 and 1:50,000 in 1% BSA/PBST were added for 2 h, alongside the IgG standard. The plate was washed and the MT78-ALP secondary added to each well and

incubated for 1 h at room temperature. The plate was washed five times and developed using of *p*-nitrophenyl-phosphate (pNPP) and detected for optical density at 405 nm on a Thermo Fisher Multiskan Ascent plate reader.

2.5. Rapid fluorometric antibody-dependent cellular cytotoxicity (RFADCC)

The RFADCC was performed using similar methods to previously published methods (Kramski et al., 2012b) with different effector cells. Briefly, 1×10^6 CEM.NKr-CCR5 cells in 50 µl of RF10 medium were coated in 3 µg of HIV-1_{BAL} envelope protein gp120 (NIH AIDS reagent program) by incubation at room temperature for 1 h. Uncoated CEM.NKr-CCR5 cells were treated identically but without the addition of the $gp120_{BAL}$. The cell membrane of coated and uncoated CEM.NKr-CCR5 cells were initially stained with 7.5×10^{-7} M of PKH26 (Sigma) diluted in diluent-C for 5 min, stopped with 500 µl of FCS and washed twice with PBS. The cytosols of coated and uncoated CEM.NKr-CCR5 cells were then stained with 1×10^{-6} M CFSE (Sigma) for 3 min, stopped with 500 µl of FCS and washed twice with PBS, then suspended in RF10 media. 2×10^4 PKH26+ CFSE+ coated and uncoated CEM.NKr-CCR5 cells per well were incubated with the purified IgG for 15 min at room temperature to allow for opsonization of target cells. 2×10^5 effector cells (freshly isolated neutrophils, monocytes, NK cells or PBMCs) were then added to the target cells and incubated for a range of times between 0 and 5-5 h at 37 °C with CO_2. Post incubation, the cells were immediately placed on ice and then fixed with a final concentration of 2% formaldehyde and the proportion of PKH26+ cells that had lost intracellular CFSE staining (lysed target cells) was determined using flow cytometry on a BD LSR Fortessa with high-throughput sampler.

2.6. Antibody-dependent neutrophil phagocytosis (ADNP)

HIV-1_{BAL} gp120 proteins were biotinylated using the EZ-Link Sulfo-NHS-LC biotinylation kit (Thermo scientific) using a 50 mmol excess biotin according to manufacturer's instructions. After the reaction, the free biotin was removed by buffer exchange using Amicon 30 kDa centrifugal filters (EMD millipore). The biotinylated $gp120_{BAL}$ was used to coat the binding sites of 1 µm fluorescent NeutrAvidin Fluosphere (Invitrogen) overnight at 4 °C. The excess antigen was removed by washing the beads with 2% BSA/PBS and diluted 1:100 in 2% BSA/PBS. Then 10 µl of the diluted beads were incubated with the purified IgG for 2 h at 37 °C. The purified neutrophils were added to the bead/antibody mix and incubated for a range of times between 0.5 and 5 h at 37 °C with CO_2 to allow for phagocytic uptake. The cells were then fixed with a final concentration of 2% formaldehyde and cells were acquired by flow cytometry on a BD LSR Fortessa with a high-throughput sampler attachment. The data was analyzed using FlowJo version 9.8.5 and the phagocytic score (% bead positive cells × mean fluorescent intensity)/10^4 was calculated as previously described (Darrah et al., 2007) and was used to compare between conditions.

2.7. HL-60 antibody-dependent phagocytosis

The HL-60 ADP was performed using a similar method to the ADNP. $gp120_{BAL}$ coated NeutrAvidin Fluospheres were prepared in the same manner as the ADNP. The diluted beads were then incubated with the purified IgG for 2 h at 37 °C. The differentiated HL-60 were washed and resuspended in fresh media then 1×10^5 HL-60 cells were added to the beads/IgG mix and incubated for 20 h overnight. The HL-60's were then stained with CD11b BV785 (1:100) and fixed with a final concentration of 2% formaldehyde and acquired by flow cytometry on a BD LSR Fortessa.

2.8. HL-60 ADP-SHIP (specific hybridisation internalization probe) assay

To confirm that the fluorescent beads were completely phagocytosed, the HL-60 ADP-SHIP (specific hybridization internalization probe) assay was performed as previously described for THP-1 cells (Ana-Sosa-Batiz et al., 2014). Briefly, 3 µg biotinylated $gp120_{BAL}$ (NIH AIDS Reagent Program) were incubated with 1 µl NeutrAvidin Fluosphere 1 µm beads (Invitrogen) and 1 µl of 150 mM biotin- and Cy5-labelled florescent internalization probe (FIPCy5) (5′ Cy5-TCAGTTCA GGACCCTCGGCT-N3 3′, Integrated DNA Technologies) overnight at 4°C. The gp120-coated beads were washed twice with sterile 2% PBS-BSA and diluted in 100 µl 2% PBS-BSA. Then 10 µl of the diluted beads were incubated with the purified IgG for 2 h at 37 °C. Opsonized beads were incubated with 1×10^5 DMSO-stimulated HL-60 cells in a total volume of 100 µl in fresh media. After incubation for a range of times between 1 and 20 h at 37°C, cells were stained with CD11b BV785. Surface-bound beads were quenched by adding 1 µg/ml^{-1} of the complementary quenching probe (QPC) (5′ -AGCCGAGGGTCCTGAAC TGA-BHQ2- 3′ Integrated DNA Technologies) for 10 min at 4°C and were subsequently washed with PBS and fixed in 2% formaldehyde. Cells were acquired on a Fortessa LSRII (BD Bioscience) and analyzed using Flowjo.

2.9. Confocal imaging

Samples used for microscopy were treated the same way as described for the neutrophil RFADCC and the ADNP assay with the exception that a membrane staining step was performed before fixing. Following the neutrophil RFADCC for 4 h, the cells were stained with CD66b APC, CD89 APC and CD11b APC. While the ADNP samples were stained with CD16 APC and CD32 APC. The samples were washed and fixed with 2% formaldehyde, then loaded on to poly-L-lysine (Sigma) coated slides and cover slips mounted with Ultramount no.4 (Fronine). The Cells were visualized on Zeiss LSM710 laser scanning confocal microscope and images were analyzed using ImageJ.

2.10. Statistical analysis

Statistical analyses were completed using Prism GraphPad version 7.0a (GraphPad Software, San Diego, CA). A Kruskal-Wallis multiple comparisons analysis was used to compare the responses of PBMCs, neutrophils, monocytes and NK cells at each time point. A Mann-Whitney *U* test was used to determine the difference between the HIV positive IgG responses and the HIV negative IgG (healthy donors) for the neutrophil RFADCC, ADNP and the HL-60 ADP. Nonparametric Spearman correlation analyses were used to test for correlations. A *p*-value of < 0.05 was considered to indicate a significant difference.

3. Results

3.1. Purified primary blood neutrophils express a range of Fc receptors

Antibody-mediated neutrophil functions are of interest in HIV immunity since neutrophils can be present at mucosal surfaces in high frequency (Sips et al., 2016) and antibody-mediated functions can contribute to control and protective immunity to HIV (Wren et al., 2013; Chung et al., 2011a; Rerks-Ngarm et al., 2009). To evaluate models of neutrophil-mediated antibody functions, primary neutrophils were isolated from freshly collected heparinized blood by density gradient and dextran sedimentation from normal healthy subjects. Neutrophils were defined as small, highly granular, FSC low, SSC high, CD66b+, CD32+ and were isolated with a mean purity of 99% (Fig. 1A). The Fc Receptor expression on freshly isolated neutrophils was evaluated, with the median MFI for FcαRI (3471), FcγRI (7882), FcγRII (7079) and FcγRIII (34992) determined (Fig. 1B, C). Neutrophils were isolated from 6 different healthy donors had only slightly varying

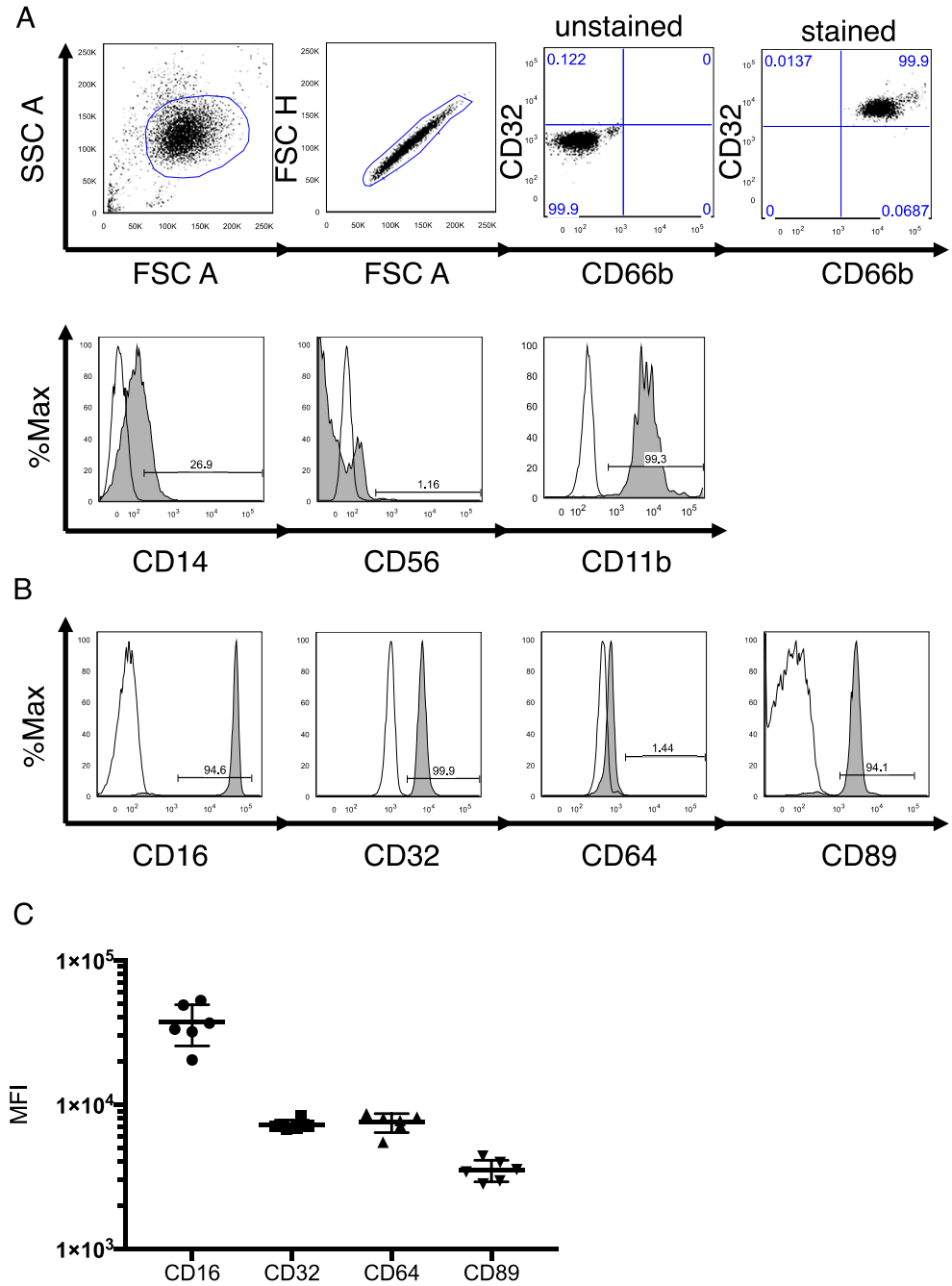


Fig. 1. Purity of isolated neutrophils and Fc receptor expression. A. Representative flow cytometry plots of staining for neutrophil purity for CD66b+ and CD32+. The isolated cells were confirmed to have typical neutrophil phenotypic markers including CD11b+ and CD56- with low levels of CD14. B. Representative flow cytometry plots of isolated neutrophils for the Fc receptors: CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI), CD89 (FcαRI) expression. C. The MFI for Fc receptor surface expression of CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI) and CD89 (FcαRI) on the isolated neutrophils for 6 donors.

levels of Fc receptors present on the surface (Fig. 1C).

3.2. Neutrophil-mediated ADCC as measured by the RFADCC assay

Neutrophils have previously been shown to mediate ADCC in response to antibody opsonized target cells (van der et al., 2002; Peipp et al., 2008) and cancer/tumor cells (Schneider-Merck et al., 2010; Albanesi et al., 2013; Keler et al., 1997), but their ability to mediate ADCC responses against HIV expressing targets has been rarely investigated (Baldwin et al., 1989). To set up a model of neutrophil-dependent HIV-specific ADCC activity, fresh neutrophils were evaluated for their ability to mediate ADCC responses using the rapid fluorometric ADCC (RFADCC) assay (Gomez-Roman et al., 2006). Neutrophils were cultured in the presence of HIV antibodies and HIV-1 envelope protein gp120 $_{BAL}$ coated PKH and CFSE labelled CEM.NKr-CCR5 target cells. Lysis of target cells were defined as PKH+ and CFSE− cells, with a

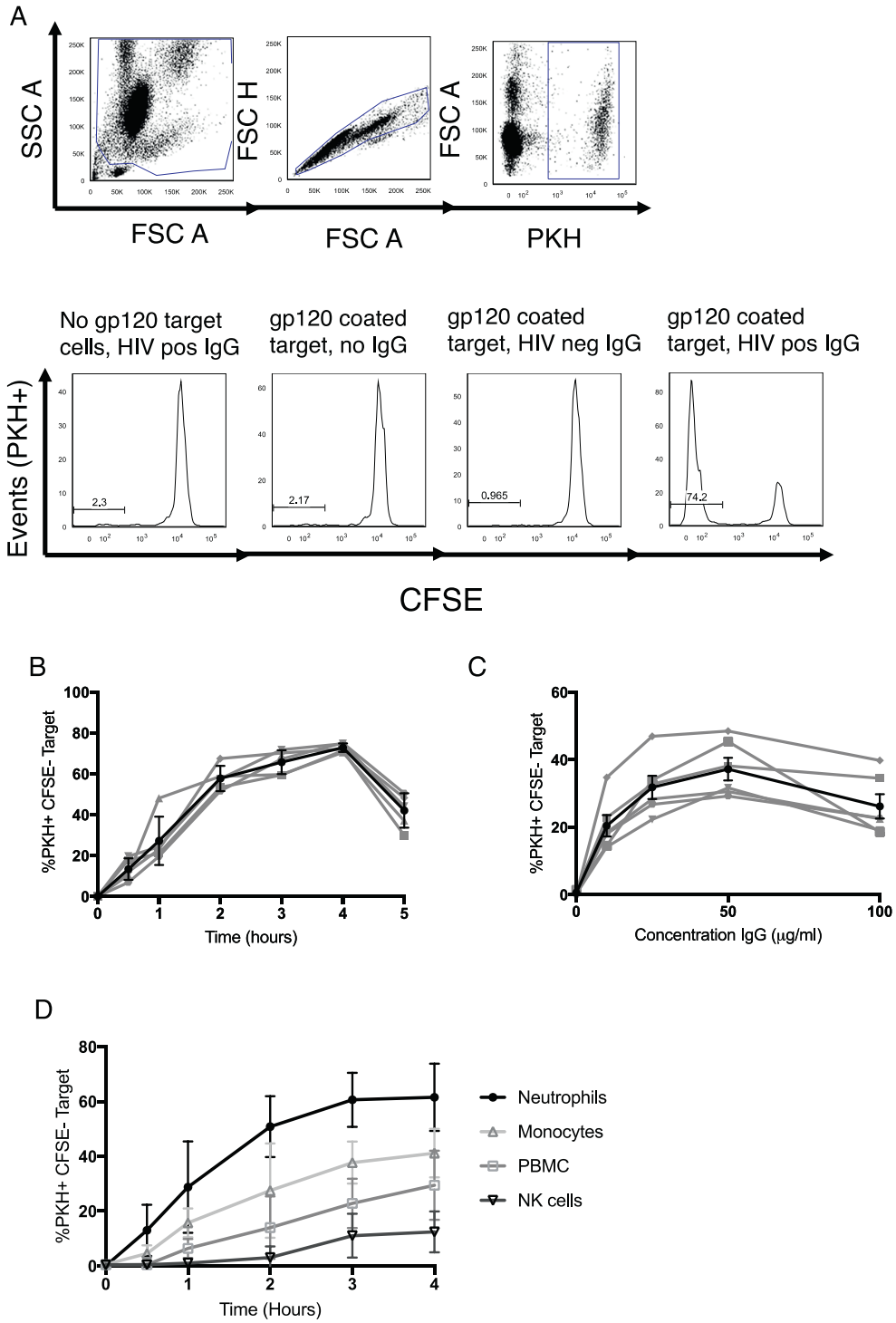


Fig. 2. Neutrophil-mediated RFADCC assay. A. Representative gating strategy for the neutrophil RFADCC. The target cells were stained with PKH membrane dye, CFSE cytoplasmic dye and coated with HIV-1_{BAL} gp120 and incubated with isolated neutrophils. Target cell lysis was identified as cells that were PKH+ and CFSE- with responses only observed for the HIV-1_{BAL} gp120 coated target cells in the presence of HIV positive IgG. B. The %PKH + CFSE- cells at time points 0, 0.5, 1, 2, 3,4 and 5 h utilising 25 μg/ml of a pooled purified IgG from HIV positive donors with 6 neutrophil donors, grey lines each represent different donors, while black line represents the mean response. C. The %PKH + CFSE− cells at different concentrations (100, 50, 25 10 μg/ml) of pooled purified IgG from HIV positive donors with 6 neutrophil donors, grey lines represent different donors, while black line represents the mean response. D. The difference in RFADCC %PKH + CFSE− cell responses with different effectors cells utilising neutrophils, monocytes, NK cells and PBMCs. A Kruskal-Wallis multiple comparisons analysis was performed and showed neutrophils killed more target cells at 0.5 h than NK cells (p = .0008) and PBMCs (p = .0001), at 1 h NK cells ($p \leq .0001$), PBMC (p = .0032), 2 h ($p \leq .0001$), PBMC (p = .0032), 3 h NK cells ($p \leq .0001$), PBMC (p = .0003), 4 h NK cells ($p \leq .0001$), PBMC (p = .0041). The monocytes responded higher than the NK cells at 0.5, 1, 2, 3, 4 h (P = .0018, 0.023, 0.018, 0.023 respectively).

representative gating strategy shown in Fig. 2A. To optimize the assay, we evaluated 5 different healthy neutrophil donors. We found that neutrophils across all donors could readily mediate HIV-specific ADCC and optimal ADCC responses were observed at 4 h, with a median lysis of 72.2% (Fig. 2B).

The neutrophil RFADCC assay was further evaluated to determine the optimal concentration of purified plasma IgG antibody. Purified IgG from 11 different HIV positive donors were evaluated at 4 different concentrations (100, 50, 25 and 10 μg/ml) and an EC50 was determined. A spearman nonparametric correlation (r value) compared the 1/EC50 to the percent lysis for each IgG concentration. For the different concentrations of antibody we found the following results: 100 μg/ml $r = 0.2091$ and $p = .5393$; 50 μg/ml $r = 0.5818$ and $p = .0656$; 25 μg/ml $r = 0.8182$ and $p = .0033$ and 10 μg/ml $r = 0.900$ and $p = .0004$ (Fig. S1). We used 25 μg/ml of IgG in future experiments with the HIV positive cohort as this level had the maximum dynamic range of responses. The neutrophil variability (donors $n = 6$) at different IgG concentrations were evaluated with maximal responses occurring at 50 μg/ml and decreasing at 100 μg/ml showing a prozone effect (Fig. 2C).

A potential advantage of neutrophils as ADCC effectors compared to other immune cells is their ability to rapidly mediate effector functions and thereby rapidly limiting viral spread. Isolated neutrophils were evaluated against other isolated immune cells for their potency in mediating lysis of the target cells over 4 h in the RFADCC assay (Fig. 2D). We found in vitro that neutrophils exhibited higher levels of ADCC at all time points compared to NK cells ($p \leq .001$) and PBMC ($p \leq .005$), while the monocytes responded higher than the NK cells at all time points ($p \leq .05$). Neutrophils also induced higher activity than monocytes, however the responses were not significant. This demonstrates the rapid ability of neutrophils to kill HIV-1 gp120 coated target cells.

3.3. Multiple mechanisms of target cell cytotoxicity are measured by the RFADCC assay

The RFADCC assay has been used widely to evaluate HIV-specific lysis of target cells. Interestingly, Kramski et al. showed that the RFADCC also measures phagocytosis of the labelled lysed target cells by monocytes (Kramski et al., 2012b). We hypothesized such a phenomenon may also occur with neutrophils. The neutrophil-mediated RFADCC assay was therefore adapted to include the CD66b granulocyte marker surface staining to determine if the target cell lysis was mediated by ADCC and/or phagocytosis of the target cells using gating strategy in Fig. 3A. The neutrophil-mediated RFADCC at early time-points of 0.5 h observed high levels of PKH+ CFSE+ CD66b+ populations (47.4%), suggesting the potential early phagocytosis of un-lysed PKH+ CFSE+ target cells by the CD66b+ neutrophils. While at the same time-point low levels of PKH+ CFSE− CD66b+ were observed (10.1%) indicating the rapid lysis of phagocytosed target cells or potentially uptake of lysed target cell membranes. This PKH+ CFSE− CD66b+ population rapidly increased to 48.8% at the 1 hour time point, coinciding with the decrease of PKH+ CFSE+ CD66b+ populations (3.74%) (Fig. 3B). Collectively this early association of the neutrophil and target cell (CD66b+ PKH+ CFSE+) changing to a neutrophil and lysed target cell (CD66b+ PKH+ CFSE−) may indicate that the target cell has been phagocytosed.

To further interrogate the mechanism behind the PKH+ CFSE− target cell lysis, confocal microscopy imaging was used to evaluate the neutrophil-mediated RFADCC assay. Fig. 3C presents PKH+ membrane (red) of target cells internalized in the neutrophil (blue) in the presence (Fig. 3C ii) and absence (Fig. 3C iii) of the CFSE (green) showing phagocytosis of a lysed target cell. There is also evidence that traditional ADCC responses are present, with PKH+CFSE− target cells in close proximity to neutrophils (Fig. 3Ci). In addition, different levels of PKH+ membrane were observed within neutrophils which may indicate the neutrophils are phagocytosing PKH+ membranes of dead cells killed by traditional ADCC responses (Fig. 3Cvi).

3.4. HIV-specific ADNP assay

The ADNP has been previously published (Ackerman et al., 2016) but has not been directly compared to neutrophil RFADCC assays. We employed a modification of the previously reported gp120-coated bead-based phagocytosis assays to study ADNP by flow cytometry using the gating strategy shown in Fig. 4A. ADNP was evaluated using a pool of HIV-specific polyclonal antibodies (HIVIG) at different concentrations measuring phagocytosis with different neutrophil donors ($n = 6$) (Fig. 4B). There was little difference between the 50 and 100 μg/ml concentrations over the course of 5 h. However, when ADNP variability (donor $n = 6$) testing for different IgG concentrations were evaluated at 4 h incubation, similar to the RFADCC assay we saw a plateauing of responses at concentrations of 50 μg/ml or higher (Fig. 4C). To determine that the beads were phagocytosed and not associated with the surface cell membrane, bead internalization was confirmed by confocal microscopy. Fig. 4D illustrates bead (green) surround by membrane (blue) showing the beads are phagocytosed. *Z*-stack movie confirmed complete phagocytosis (Fig. S2). There was no uptake of the beads observed in the absence of HIV positive IgG.

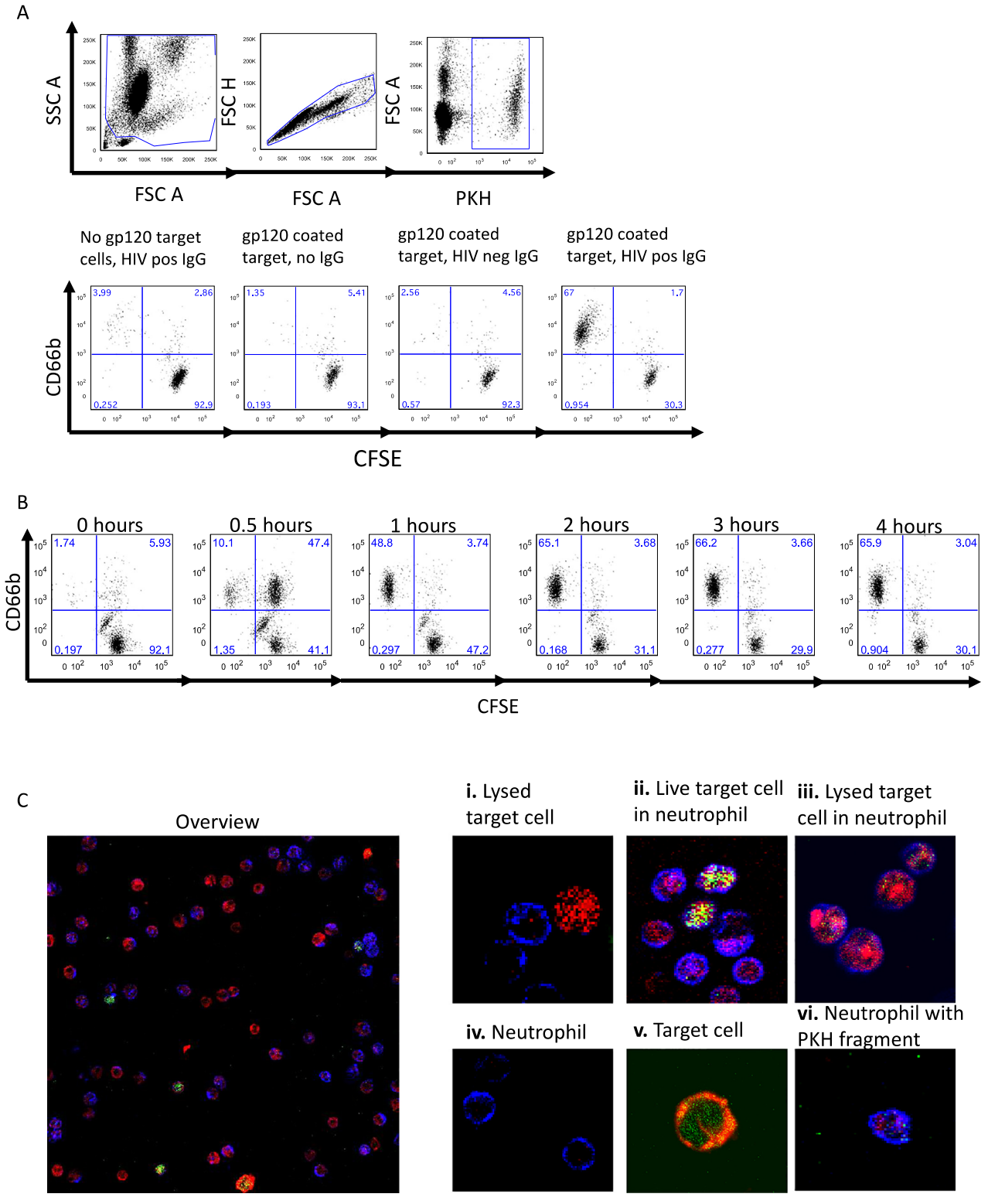
3.5. HIV-specific HL-60 ADP assay

Neutrophils have a short half-life upon isolation, thus requiring constant fresh leukocyte sources for studies. In contrast, the HL-60 neutrophil-like cell line can be continuously grown in culture and differentiated with DMSO when required. We developed a HL-60 ADP assay as a proxy for the evaluation of antibody-dependent primary neutrophil phagocytosis. The HL-60 cells were differentiated with 1.3% DMSO, confirmed by the upregulation of CD11b, a marker that has previously been identified as essential for neutrophil Fc receptor-mediated cytotoxicity and is commonly used as a marker for HL-60 neutrophil differentiation (Birnie, 1988; van Spriel et al., 2001). Furthermore, we confirmed the upregulation of CD14, CD16, CD32, CD89 surface marker expression compared to HL-60 cells prior to differentiation (Fig. 5A). Prior to differentiation, HL-60 cells expressed CD89 (MFI 2178) but had low/no expression of CD11b, CD14, CD16, CD32 and CD64. Following 5 days of differentiation with DMSO, they CD11b (MFI 753) CD89 (MFI 2914), CD14 (MFI 392), CD16 (MFI 641), CD32 (MFI 2204) but with no expression of CD64.

The HL-60 ADP assay gating strategy relies on gating for CD11b positive cells (differentiated HL-60 cells) and then gating on the fluorescent beads (Fig. 5B), while the CD11b negative cells (undifferentiated HL-60 cells) show only low levels of phagocytosed beads. To determine the optimal incubation time of the HL-60 ADP assay, a time-course was completed and showed that maximal responses were reached at 20 h (Fig. 5C). To validate that the beads were internalized, the HL-60 ADP was modified to include a hybridization internalization probe (SHIP) present on the bead (Ana-Sosa-Batiz et al., 2014). Increasing levels of phagocytosis (7.56% at 1 h and 36% at 3 h) were observed over time with 98.6% internalization of the beads observed following a 12 hour period (Fig. 5D).

3.6. HIV positive IgG cohort ADNP, HL-60 ADP and neutrophil-mediated RFADCC

Having established three separate neutrophil-mediated HIV-specific antibody assays, we asked how these assays performed across a cohort of 41 HIV positive subjects who were viremic and not on ART, compared to 13 healthy donors. All three assays clearly discriminated between HIV positive and HIV negative subjects (Fig. 6A). RFADCC results were analyzed, gating on both total PKH+ CFSE− cells and CD66b+ PKH+ CFSE− cells (a measure of Neutrophil uptake of target cells and



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target cell membranes). There were only small marginal differences between the responses with these 2 gating strategies (Fig. 6A).

The ADNP and HL-60 ADP both had a large dynamic range of responses, however, the HL-60 ADP had somewhat greater non-specific uptake of the gp120-coated beads using HIV negative IgG. None-the-less, the ADNP and HL-60 ADP assay exhibited a strong positive

Fig. 3. Neutrophil- mediated RFADCC is a measure of both extracellular cytotoxicity and phagocytosis. A. Alternative gating strategy for the neutrophil RFADCC. Gating on PKH+ CD66b+ and CFSE− shows that CD66b+ neutrophils acquire target cell PKH and this occurs only in the presence of HIV-1_{BAL} gp120 coated target cells and HIV positive IgG. B. Gating on PKH+ cells and then CD66b and CFSE over 0, 0.5, 1, 2, 3 and 4 h utilising 25 μg/ml of a pooled purified IgG from HIV positive donors. C. Confocal microscopy imaging of the neutrophil RFADCC with the neutrophils stained with CD66b APC, CD89 APC and CD11b APC (PKH: red, CFSE: green, Neutrophil markers: blue). Inset panels show i) Dead target cell (PKH+ CFSE−) next to neutrophils, ii) live target cell inside neutrophil, iii) Dead target cell inside of neutrophil, iv) Neutrophil alone, v) CEM.NKr-CCR5 (target) cell alone, vi) small amount of target cell membrane inside of neutrophil. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

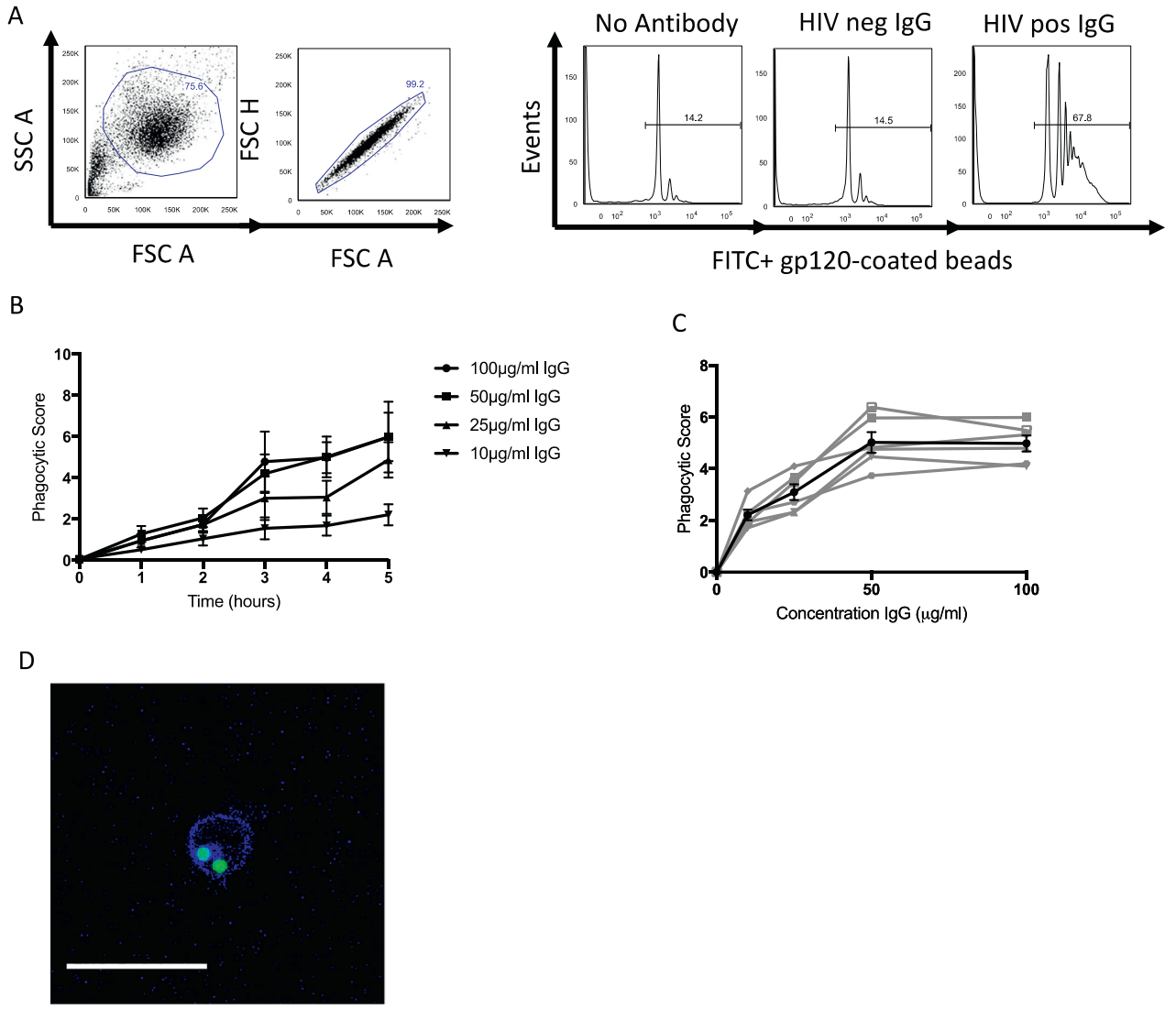

Fig. 4. ADNP responses to HIV positive IgG. A. Representative gating strategy for the ADNP assay. The panels 3 and 4 show minimum uptake of the FITC+ beads in the absence of antibody and in the presence of HIV negative IgG. Panel 5 shows that beads coated with HIV-1_{BAL} gp120 in the presence HIV positive IgG can be taken up by neutrophils. Phagocytic score = %beads positive cells × mean fluorescence intensity/10^4. B. ADNP at time points 0, 0.5, 1, 2, 3, 4 and 5 h utilising different concentrations (100, 50, 25 10 μg/ml) of pooled purified IgG from HIV positive donors with 6 neutrophil donors. C. ADNP responses at 4 h using different concentrations (100, 50, 25 10 μg/ml) of pooled purified IgG from HIV positive donors with 6 neutrophil donors; grey lines represent each donor and black line is mean. D. Confocal microscopy showing the internationalisation of 2 FITC+ beads following a 4 h incubation (neutrophils stained with CD16 APC and CD32 APC), scale bar = 20 μm.

correlation ($r = 0.7718$, $p \leq .0001$, Fig. 6B, right panel). Interestingly, we observed that the neutrophil RFADCC with CD66b gating also showed a significant correlation with both the ADNP ($r = 0.5692$, $p \leq .0001$) and the HL-60 ADP ($r = 0.4524$, $p = .0030$) (Fig. 6B). In addition, the neutrophil RFADCC without CD66b gating also showed a significant correlation with both the ADNP ($r = 0.5655$ $p \leq .0001$) and the HL-60 ADP ($r = 0.4551$, $p = .0028$) (Fig. S3). The same 5 (out of 41) HIV positive donors responded very weakly in all 3 assays.

Since all 41 subjects were ART naive and had a range of HIV viral levels, we were able to evaluated the neutrophil RFADCC, ADNP and HL-60 ADP assays for clinical relevance. We found that there was a significant negative correlation with vial load and ADNP ($r = -0.32$,

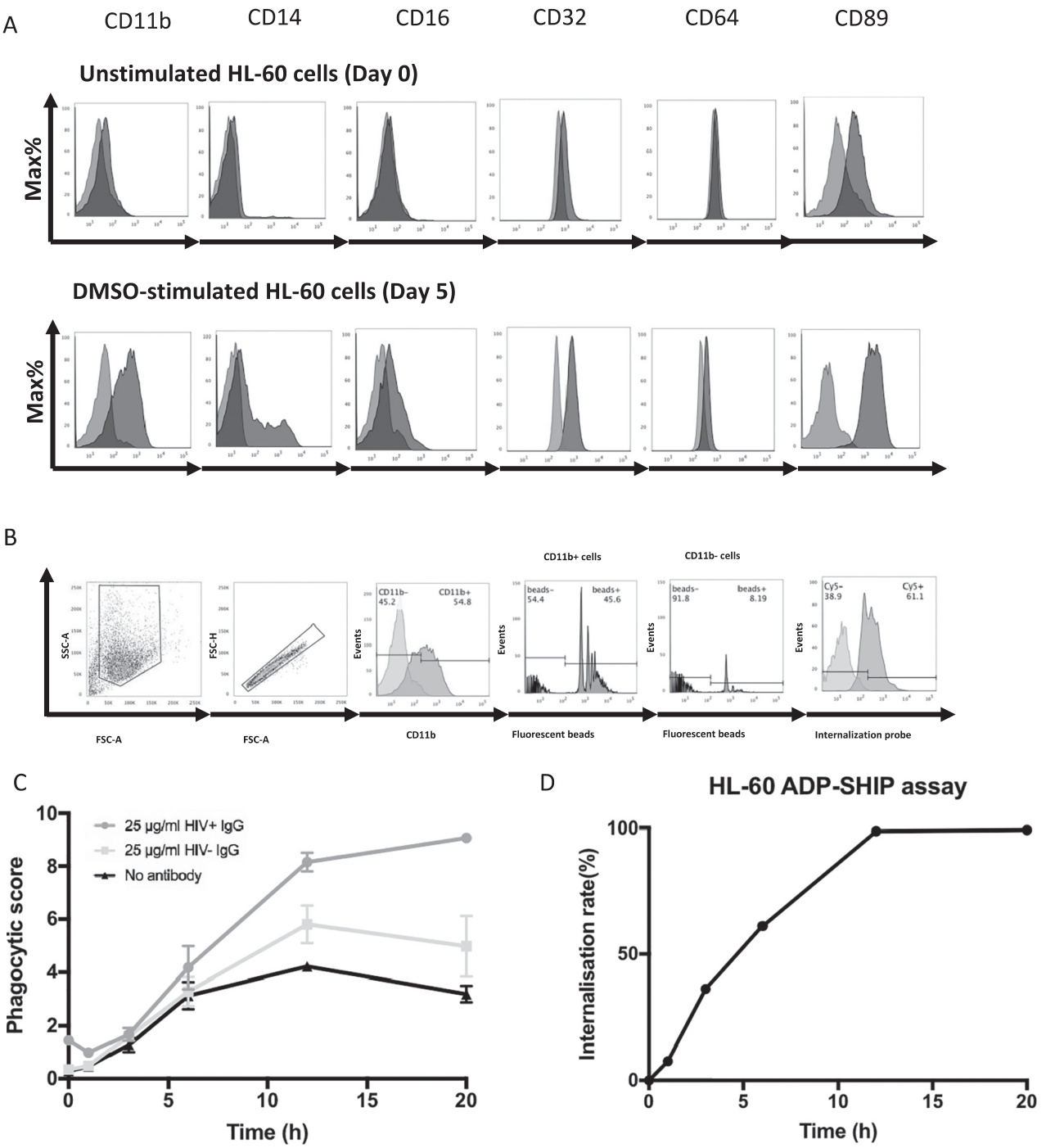

Fig. 5. Differentiated CD11b+ HL-60 cell as a model for ADNP. A. Representative flow cytometry plot for cell markers and Fc receptors profiles in HL-60 cells. D0 were unstimulated naïve HL-60 cells, and D5 were cells stimulated by 1.3% DMSO for five days. Lighter grey populations were unstained control and darker grey populations were stained cells. B. Gating strategy for HL-60 ADP assay and HL-60 ADP-SHIP assay. The first 3 panels show gating strategy on CD11b+ cells. The fourth panel shows the bead (FITC+) phagocytosis activity of CD11b positive HL-60s when cultured with HIV positive IgG and gp120 coated fluorescent beads. The fifth panel shows the bead phagocytosis activity of CD11b negative HL-60s. The sixth panel shows gating on FITC+ cells, identifying internalized beads by gating for Cy5+ internalization probes. FITC+/Cy5+ events were identified as internalized beads and FITC+/Cy5- events were identified as surface-associated beads. C. Time-course plot of HL-60 ADP assay. The phagocytic activity was measured using HIV positive IgG and IgG purified from HIV negative subjects. Phagocytic score = %beads positive cells × mean fluorescence intensity/10^4. The error bars represent mean ± s.e.m of three replicates. D. Time-course plot of beads internalization rate in HL-60 ADP-SHIP assay. Internalization rate% = FITC+Cy5+ cells/ FITC+ cells * 100%.

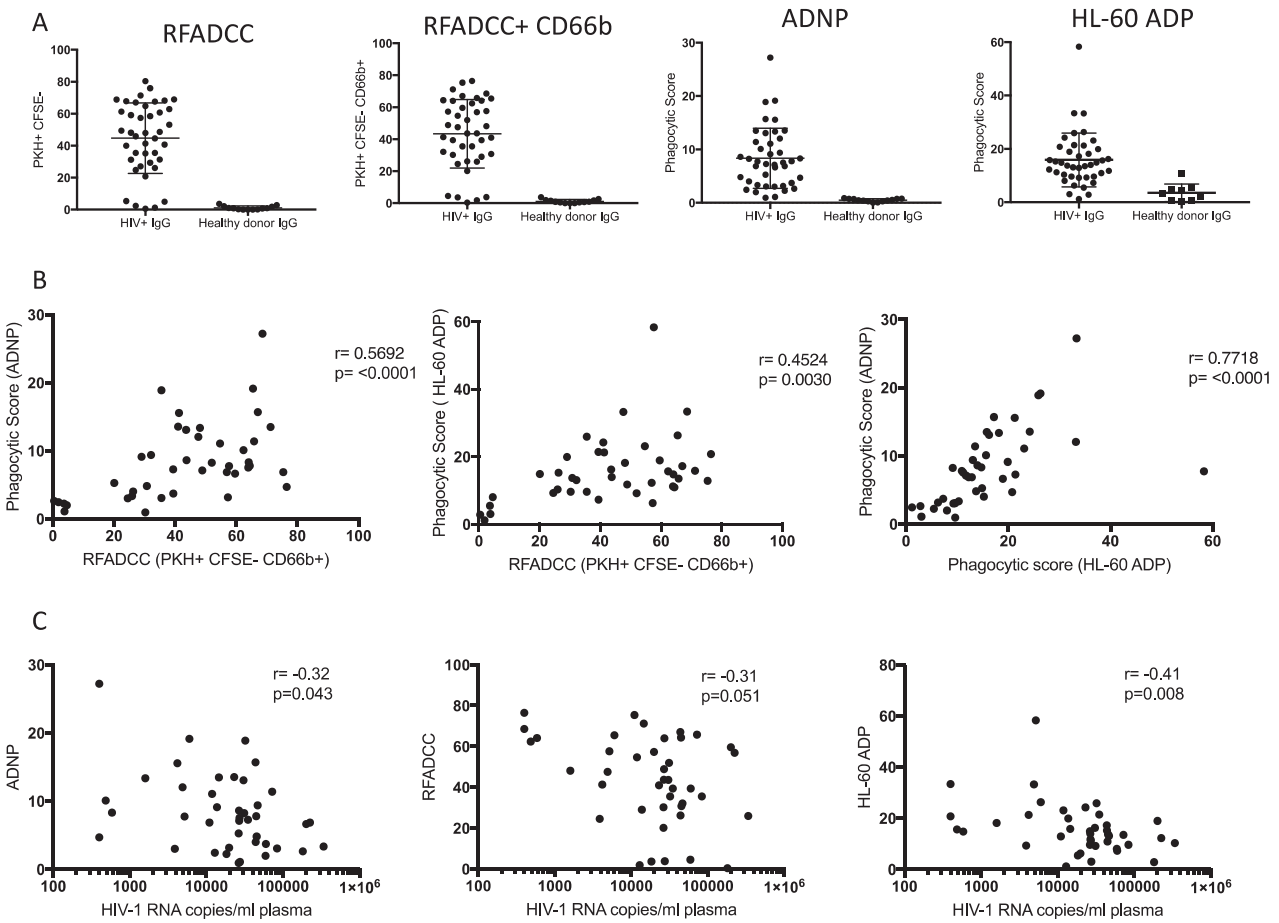

Fig. 6. Neutrophil Fc-Effector responses of HIV positive cohort. A. 41 HIV positive plasma samples and 13 HIV negative plasma samples were purified for IgG and were then assess for responses in the neutrophil RFADCC, neutrophil RFADCC with CD66b gating, ADNP and the HL-60 ADP. B. The HIV positive IgG cohort responses were correlated (spearman) between the 3 assays showing strong significant correlations between the assays. C. The HIV positive IgG cohort responses were correlated (spearman) with viral load (HIV-1 RNA copies/ml plasma) and showed significant negative correlations with the ADNP and HL-ADP and approaching significance with the neutrophil RFADCC with the CD66b gating.

$p = .043$) and the HL-60 ADP ($r = -0.41$, $p = .008$), with the RFADCC approaching significance ($r = -0.31$, $p = .051$) (Fig. 6C). This may indicate that neutrophils play a role in controlling HIV viral load in some patients.

4. Discussion

There is a growing interest in the role of Fc-mediated effector functions, such as ADCC and ADP in the protection and control of HIV. However, there has been limited investigation into the role of neutrophil-mediated Fc-responses in HIV infections. We show that neutrophils are efficient Fc-effector cells and they respond faster than monocytes or NK cells when specifically measured by the RFADCC assay. These differences may be due to the different effector mechanism involved, as neutrophils express a range of FcγRs and can mediate both phagocytosis and ADCC responses, whereas NK cells only express FcγRIIIa and mediate only ADCC responses (Sips et al., 2016; Selvaraj et al., 1988). Since the neutrophil RFADCC is measuring in part phagocytosis responses, it is unsurprising that these responses correlate with the primary neutrophil ADNP assay and neutrophil-like cell line HL-60 ADP assay. The HL-60 neutrophil cell line assay correlated strongly with the primary neutrophil ADNP assay and provides a more tractable model for primary neutrophils in the ADP assays. Importantly, the neutrophil RFADCC, ADNP and HL-60-ADP assays inversely correlated with the viral load of the HIV positive IgG donors at the time of plasma collections, suggesting the possibility that these assays are of biological relevance.

The RFADCC assay has been widely used to assess FcγR mediated responses of PBMCs (Vaine et al., 2010; Gomez-Roman et al., 2006; Chung et al., 2009). While NK cells have been shown to mediate ADCC responses, monocyte responses have largely been attributed to trogocytosis and phagocytosis of the target cells (Kramski et al., 2012b). Our data indicates that the neutrophil RFADCC assay more represents phagocytosis of whole target cells, phagocytosis of killed target cell debris, or trogocytosis. When comparing HIV positive versus negative sample responses, there is an increase in the population of the PKH+ CFSE+ CD66b+ cells (ie neutrophils internalizing live target cells) at early time points (0.5 h) that are higher on the FSC and SSC, which may indicate the target cells have been phagocytosed (Fig. S4). While at the same early time point, there is also a small population of PKH+ CFSE− CD66b+ cells (i.e. neutrophils that have internalized target cell membrane only) which have smaller FSC vs SSC profiles, similar to HIV negative samples, suggesting that the neutrophils have ingested smaller fragments of dead target cells killed by more traditional extracellular ADCC mechanisms. We also observe more PKH+ cells when testing HIV positive IgG samples compared to HIV negative IgG samples,

despite the same number of target cells being added to each well (Fig. S5), which may be caused by neutrophils mediating traditional extracellular ADCC target cell lysis prior to the neutrophil phagocytosing fragments of dead target cells. Alternatively, multiple neutrophils may interact with the same target cell and compete to phagocytose resulting in the sharing of target cell membrane between effector cells.

The neutrophil RFADCC, HL-60-ADP and ADNP responses inversely correlated with the viral load of the patients. ADNP responses have recently been shown not to differ between elite controllers, viremic controllers or patients on antiretroviral therapy (Ackerman et al., 2016). It will be of interest to evaluate the differences in clinical populations in the antibody-mediated neutrophil assays in future studies. In addition, neutrophil phagocytic activity is progressively impaired during HIV infection despite antiretroviral therapy (Tsachouridou et al., 2017). In future studies it will be of interest to investigate neutrophils isolated from different clinical populations of HIV positive donors for their ability to mediated antibody-dependent functions, although these studies are technically demanding due to the need for fresh neutrophils. Our and other studies (Bradley et al., 2017; Smalls-Mantey et al., 2013; Ackerman et al., 2016) have utilized circulating neutrophils isolated from blood, however, it is known that neutrophils residing in tissues and mucosal surfaces can have altered FcγR expression profiles, which could potentially alter the Fc-effector potentials of neutrophils at different sites (Sips et al., 2016) and should be taken into consideration when evaluating FcγR responses. While this study focused exclusively on exploring neutrophil mediated Fc-effector responses, multiple other FcγR bearing innate immune effector cells including pDC, NK cells and monocytes/macrophages may also participate in antiviral control and their respective contributions also deserve further evaluation.

The strong correlation between the ADP and HL-60 ADP assay suggest that HL-60 cells can represent a useful tool in the evaluation of other neutrophil effector functions in the future. Due to the difficulty of working with primary isolated neutrophils that have short half lives and require fresh blood for assays, as neutrophils cannot be reliably cryopreserved without severely effecting functionality (Boonlayangoor et al., 1980), usage of neutrophil-differentiated HL-60 cell lines may allow for easier, more robust, high throughput evaluation of Fc-mediated effector responses. However, HL-60 have several differences compared to primary neutrophils including differences in gene expression and the lack of several neutrophil proteins including the CD66b surface marker (Ozeki and Shively, 2008). In addition, HL-60 neutrophils lack the development of the secondary and tertiary granules which are hall marks of neutrophils, required for some effector functions upon cell activation (Gaines et al., 2005). HL-60 neutrophils have been shown to mediate lower levels of antimicrobial activity and lower reactive oxygen production compared to primary blood derived neutrophils (Yaseen et al., 2017; Watson et al., 1997). These differences should be taken into consideration when utilising HL-60 cells to evaluate alternative Fc-effector functions.

In summary, the neutrophil RFADCC, ADNP or HL-60 ADP were validated, evaluated and compared for neutrophil effector functions. As neutrophil Fc-mediated responses were associated with reduced viral loads in HIV ART naïve subjects, this suggests that neutrophil-mediated effector responses should be investigated in future HIV vaccine trials for their potential to control viremia, especially due to their rapid response compared to other FcγR innate immune cells and their abundance at key sites of HIV transmission. The assays described should help form the foundation for future neutrophil Fc-effector studies in HIV infection and vaccination.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.03.007.

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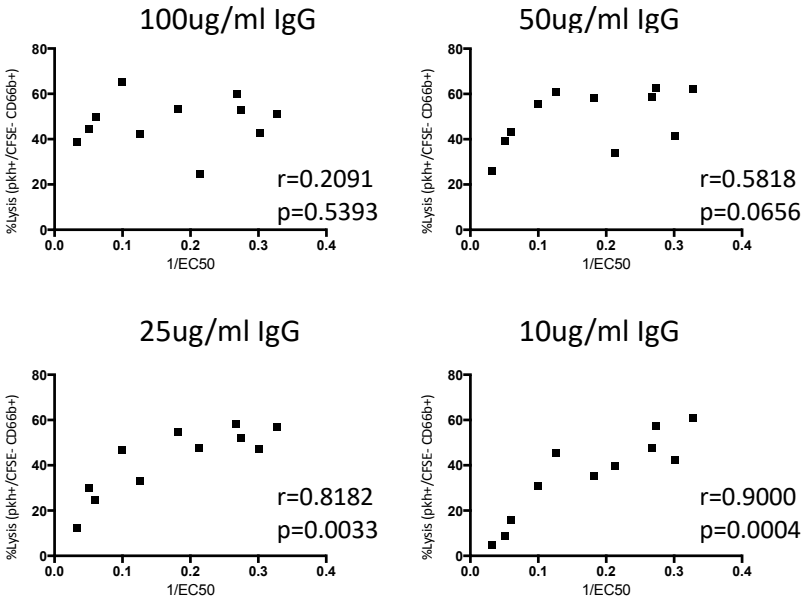
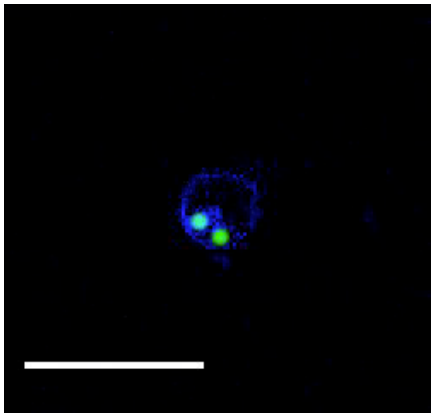
Supplementary Figures

Fig.S1



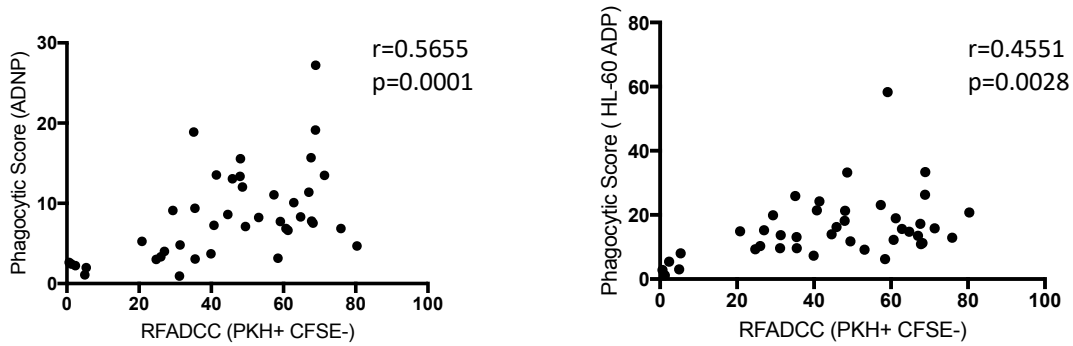
Supplementary 1: EC50 correlations for the RFADCC

Fig.S2



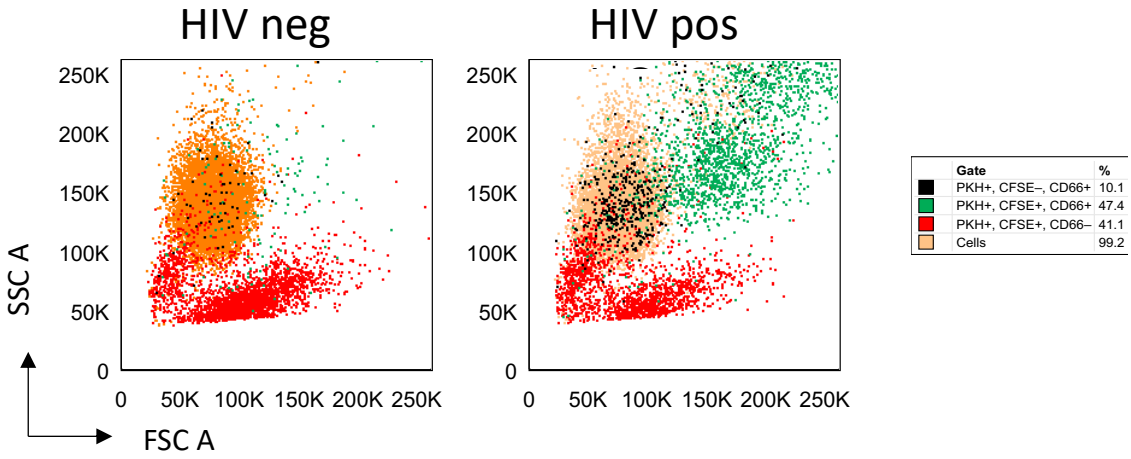
Supplementary 2: Confocal microscopy of the ADNP. Z-stack showing the internationalisation of 2 FITC+ beads following a 4 hour incubation (neutrophil stained with CD16 APC and CD32 APC), scale bar=20 nm

Fig.S3



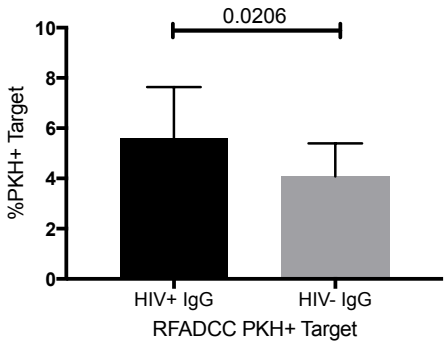
Supplementary 3: RFADCC without gating on CD66b correlates well with the ADNP and the HL-60 ADP

Fig.S4



Supplementary 4: RFADCC with HIV positive IgG creates a higher FSC SSC population that is PKH+ CFSE- CD66b+ that is absent in the HIV negative IgG RFADCC. HIV pos IgG sample FSC vs SSC plot taken at time 0.5 hours (same sample shown in Figure 3B). 47% of CD66+ neutrophils (green) appear to have internalized PKH+CFSE+ (ie live) target cells which have larger FCS vs SSC profiles. In contrast, the 10% of PKH+CFSE+ CD66b+ cells (ie neutrophils that have internalized dead target cells- shown as black), have much smaller FSC vs SCC profiles which suggests that either the neutrophils have rapidly phagocytosed live target cells and then digested them to the point that the FSC vs SSC significantly is reduced, or they have ingested fragments of dead target cells killed by more traditional extracellular ADCC (as illustrated in confocal imaging Figure 3C vi)

Fig.S5



Supplementary 5: There is an increase ($p = 0.0206$) in PKH+ cells in the RFADCC with HIV positive IgG compared to HIV negative IgG.

Chapter 3

Viremic controllers and viremic non-controllers IgG mediated neutrophil phagocytosis is inhibited by non-epitope specific plasma IgA

3.1. Introduction

Despite advances in antiretroviral treatment for HIV, there is still an urgent need for the development of an effective prophylactic vaccine. Recent human and non-human primate (NHP) HIV vaccine studies have identified Fc-mediated responses as potential correlates of protective immunity [82, 312, 354, 355]. Antibody-dependent phagocytosis (ADP) and Antibody-dependent cellular cytotoxicity (ADCC) responses have correlated with protection in NHP vaccine studies [312, 354, 356]. Multiple studies have correlated ADCC and ADP responses with delayed HIV disease progression in infected humans [137, 148, 150-152, 357, 358]. The importance of functional antibodies was highlighted by the RV144 HIV vaccine trial, demonstrating a correlation between ADCC mediated IgG antibodies with reduced risk of infection in the absence of IgA [82, 319]. The binding of plasma IgA antibodies to Env correlated directly with the rate of HIV infection [82, 319]. The RV144 trial also found that a higher ratio of plasma IgA:IgG showed decreased vaccine efficacy [353]. Monoclonal IgA derived from RV144 recipients was shown to block purified IgG from the vaccinees from mediating ADCC responses with NK cells via epitope competition [353]. These surprising results have focussed attention on the role of IgA in HIV infection. Whether epitope competition is the only mechanism whereby IgA inhibits Fc-mediated effects is unclear.

IgA is the most abundant antibody at the mucosal surfaces and the second most abundant antibody in the blood behind IgG [157]. There are two subclasses of IgA in the humans, IgA1 and IgA2, that have different local distributions. In human serum ~90% of IgA present is of the IgA1 subclass, while at the genital and rectal mucosal sites ~60-90% are the IgA2 subclass [163, 164]. The role of IgA in HIV infection is complex with only a limited number of studies performed to date. HIV-specific IgA passively transferred into NHP or induced by vaccination has been showed to protect at the mucosal surfaces from SHIV challenges [173, 174]. HIV-exposed, persistently seronegative people have been shown to have mucosal and plasma IgA that inhibit transcytosis of HIV across epithelial cells and have the ability to neutralise HIV [180] [359, 360]. HIV-1 envelope-specific mucosal IgA antibodies in breast milk have been shown to reduce mother to child transmission of HIV-1 [184]. In contrast, it has also been shown that high levels of IgA can result in a modest enhancement of HIV replication [185, 186]. More recently, IgA was shown to inhibit IgG mediated ADCC responses with PBMC's in subjects with primary HIV infection, but no inhibition was observed in elite controllers [181]. It is evident that there is a complicated relationship of HIV infection and IgA responses

that requires further investigation in different clinical settings. This complicated relationship of HIV infection and IgA responses requires further investigation. Importantly, despite evidence linking both FcR engagement and IgA in protection and control of HIV, there has been limited investigation of the role of IgA interactions with FcαR in HIV infection.

Furthermore, despite the strong interest in both IgA and Fc-mediated responses to HIV there have been few investigations into responses mediated by neutrophils. Neutrophil are the most abundant leukocyte in the blood (30-70% of all leukocytes) and they can rapidly migrate to sites of infection. They are abundant in the mucosal surfaces, which are key sites of HIV transmission [193]. During HIV infection neutrophils are upregulated at a number of mucosal surfaces including the foreskin [259]. Neutrophils express FcγRI (induced by cytokines) [361], FcγRII and FcγRIII (table 1.2) and can mediate a range of antibody-dependent effector functions, including ADP and phagocytosis-independent ADCC responses, however little is known of their importance in HIV infection [138, 193, 354]. Importantly, neutrophils also express FcαR, unlike NK cells, making them a suitable cell to study Fc-mediated responses initiated by IgA. It is unknown what role that neutrophils may play in the control of HIV in particular in the control or progression of the infection.

To probe the utility of IgA-mediated neutrophil responses in HIV infection, plasma samples from subjects who naturally control HIV (viremic controllers) and subjects that are viremic non-controllers were studied. Purified IgG from a cohort viremic controllers and viremic non-controllers was evaluated for its ability to mediate ADNP and neutrophil RFADCC responses. Plasma and IgA-depleted plasma were evaluated for ADNP and neutrophil RFADCC responses to determine the role that IgA may play in these responses. A subset cohort of viremic controllers and viremic non-controllers was evaluated for the ability of IgA from different sources (including autologous IgA, HIV negative pooled purified IgA, HIV positive pooled purified IgA, HIV negative IgA1 and HIV negative IgA2) to inhibit IgG mediated ADNP responses. The inhibitory effects of IgA on IgG mediated ADNP were investigated for inhibition mediated by FcαR dependent mechanisms by using an FcαR blocking antibody.

3.2. Materials and Methods

3.2.1. Study subjects and samples

All HIV positive plasma samples were kindly provided by Professor Anthony Kelleher at the Kirby Institute who maintains the Sydney long-term non-progressor cohort [150, 362, 363]. Plasma was obtained from 33 HIV positive subjects that were defined as viremic controllers (VCs) as having a plasma HIV-1 RNA level of less than 2200 (RNA copies/ml) plasma HIV-1 RNA level. Plasma was collected from 41 HIV positive subjects not on ART, that were defined as viremic non-controllers, as having a plasma HIV-1 RNA level of more than 25,000 (RNA copies/ml). Clinical data for the study cohorts are summarised in table 3.1 and as expected the viral load and CD4 T cell count are significantly different (p=<0.0001 and 0=0.0053 respectively) between clinical groups (Figure 3.1A and 3.1B). All subject samples utilised were anti- retroviral therapy (ART) naïve. Plasma samples for 13 healthy HIV negative subjects were collected and utilised as controls for this study. All subjects provided written informed consent and the studies were approved by the relevant institutional ethics committees.

Table 3.1: Clinical characteristics of ART-naïve study cohort.

	Viremic controllers	Viremic non-controllers	P value
Number of samples	33	41	
Age, median (Range)	45 (28-76)	42 (28-68)	ns
CD4 count entry, cells/µl, median (Range)	703 (273-1190)	581 (84-874)	0.0053
Viral load at entry copies/ml, Median (Range)	757 (80-2200)	63,000 (28,500-750,000)	<0.0001

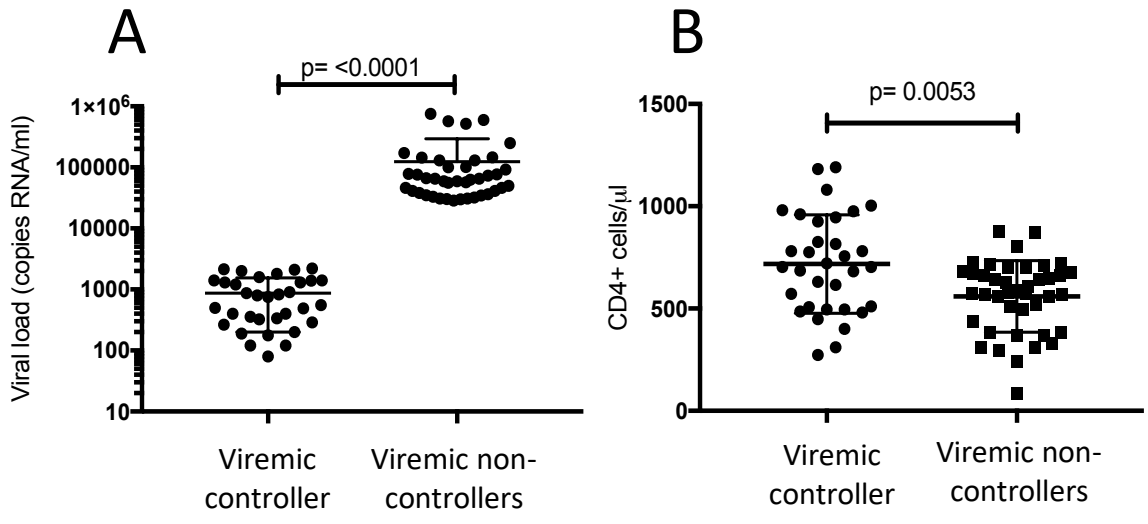


Figure 3.1: **The clinical characteristics of the viremic controllers and viremic non-controllers cohorts**, **A)** Viral load (copies RNA/ml of blood) were significantly (Mann-Whitney U test) different between viremic controllers and viremic non-controllers p=<0.0001. **B)** CD4+ T cell count (cells/µl) were significantly (Mann-Whitney U test) different between viremic controllers and viremic non-controllers p=0.0053

3.2.2. Other IgG and IgA antibodies:

A number of antibodies were utilised in addition to the clinical samples to evaluate IgA responses. A pooled HIV positive IgG (HIVIG) was supplied by the NIH AIDS reagent program and was used as a known positive sample. In addition, serum from 11 HIV+ subjects on ART (provided by Dr Stephen Kent through the Melbourne Sexual Health Centre) was used to purify IgA and create a pool of IgA from HIV positive subjects. A pooled purified HIV negative IgA was created using the same 13 HIV negative healthy donors used as controls. The purified HIV negative IgA1 and IgA2 were both supplied by Athens Research and Technology (cat: 090701-1M and 090701-2M).

3.2.3. Primary Neutrophil isolation

Neutrophils were isolated by adapting previously published methods [211, 364]. Briefly, fresh heparinized blood was obtained from HIV negative healthy donor leukocytes, separated using Ficoll density centrifugation. The granulocytes were enriched by using 3% dextran sedimentation for 25 minutes at room temperature. The remaining cells were washed in and suspended in RPMI 1640 media supplemented with 10% FCS and penicillin (100 U/ml)/Streptomycin/(100 µg/ml) and L-Glutamine (2 nM) (Gibco, 10378-16) (RF10). The enriched cells were then collected and treated with a hypotonic lysis reagent to remove any remaining red blood cells. The purified neutrophils were then counted and utilized as effector cells in HIV antibody assays. Alternatively, the neutrophils were pre-treated with 60µg/ml of MIP8a antibody (Invitrogen Cat:MA5-16857) a FcαR blocking antibody for 20 minutes on ice followed by washing with RF10 to remove excess antibody.

3.2.4. IgA antibody purification

Plasma IgA purification was performed using peptide M agarose resin (InvivoGen) following the manufacturer's instructions with minor modifications. Briefly, purification columns were prepared by loading 300µl of peptide M agarose into 0.5ml Pierce™ spin columns (Thermo Fisher) and washed with PBS. Plasma samples were added to columns and incubated at room

temperature on an orbital rotator for 45 minutes. After the incubation, the IgA depleted flow-through was collected for later evaluation in ADNP and ADCC assays and used for IgG purification. The columns were washed with PBS 6 times and the IgA eluted off the columns using IgG elution buffer (Thermo Fisher) and immediately neutralised with Tris 1M pH 8.0 (Life Technologies). The eluted fractions were concentrated using 50k Amicon ultra 0.5ml filters. The purified IgA was then processed in the protein G multitrap columns (GE Healthcare) at room temperature for 15 minutes to remove any contaminating IgG. The purified IgA samples were concentrated again through 50k Amicon ultra 0.5ml filters and then evaluated for both IgA and IgG concentrations.

3.2.5. IgA antibody concentration quantification

The total plasma IgA and the purified IgA antibodies were quantified using an anti-IgA ELISA kit (Mabtech) following the manufacturer's instructions. Briefly, Maxisorb 96 well plates (Nunc) were coated with the MT57 (2 μg/ml) capture antibody overnight at 4 °C. The plate was washed with PBST (PBS with 0.05% Tween 20) and blocked with 1% BSA/PBST for 2 hours. The plate was washed and purified IgA antibodies diluted 1:10,000 and 1:50,000 in 1% BSA/PBST were added for 2 hours, alongside the IgA standard. The plate was washed and the MT20-ALP secondary added to each well and incubated for 1 hour at room temperature. The plate was washed five times and developed using p-nitrophenyl-phosphate (pNPP) and detected for optical density at 405 nm on a Thermo Fisher Multiskan Ascent plate reader or on the BMG Labtech CLARIOstar plate reader.

3.2.6. IgG antibody concentration quantification

The total plasma IgG, the IgG in the IgA-depleted plasma and the purified IgG antibodies were quantified using an anti-IgG ELISA kit (Mabtech) following the manufacturer's instructions. Briefly, Maxisorb 96 well plates (Nunc) were coated with the MT145 (2 μg/ml) capture antibody overnight at 4 °C. The plate was washed with PBST (PBS with 0.05% Tween 20) and blocked with 1% BSA/PBST for 2 hours. The plate was washed, and purified IgG antibodies diluted 1:20,000 and 1:150,000 in 1% BSA/PBST were added for 2 hours, alongside the IgG standard. The plate was washed, and the MT78-ALP secondary added to each well and incubated for 1 hours at room temperature. The plate was washed five times and developed using pNPP and detected for optical density at 405 nm on a Thermo Fisher Multiskan Ascent plate reader or on the BMG Labtech CLARIOstar plate reader.

3.2.7. Antibody-dependent neutrophil phagocytosis (ADNP)

HIV-1_{BAL} gp120 proteins were biotinylated using the EZ-Link Sulfo- NHS-LC biotinylation kit (Thermo Scientific) using a 50 mmol excess biotin according to manufacturer's instructions. After the reaction, the free biotin was removed by buffer exchange using Amicon 30kDa centrifugal filters (EMD Millipore). The biotinylated HIV-1_{BAL} gp120 (NIH AIDS reagent program) was used to coat the binding sites of 1 μm fluorescent NeutrAvidin Fluosphere (Invitrogen) overnight at 4°C. The excess antigen was removed by washing the beads with 2% BSA/PBS and diluted 1:100 in 2% BSA/PBS. Then 10 μl of the diluted beads were incubated with the purified 25 μg/ml of IgG for 2 hours at 37 °C. The purified neutrophils were added to the bead/antibody mix and incubated for 3 hours at 37 °C with CO_2 to allow for phagocytic uptake. The cells were then fixed with a final concentration of 2% formaldehyde and cells were acquired by flow cytometry on a BD LSR Fortessa with a high-throughput sampler attachment. The data were analyzed using FlowJo version 9.8.5 and the phagocytic score (% bead positive cells × geometric mean fluorescent intensity)/ 10^4 was calculated as previously described [365, 366] and was used to compare between conditions.

3.2.8. Rapid fluorometric antibody-dependent cellular cytotoxicity (RFADCC)

The RFADCC was performed using similar methods to previously published methods [367, 368] with different effector cells. Briefly, 1×10^6 CEM.NKr-CCR5 cells in 50μl of RF10 medium were coated in 3 μg of HIV-1_{BAL} gp120 (NIH AIDS reagent program) by incubation at room temperature for 1 hour. Uncoated CEM.NKr-CCR5 cells were treated identically but without the addition of $gp120_{BAL}$. The cell membrane of coated and uncoated CEM.NKr- CCR5 cells were initially stained with 7.5×10^{-7} M of PKH26 (Sigma) diluted in diluent-C for 5 minutes, stopped with 500 μl of FCS and washed twice with PBS. The cytosols of coated and uncoated CEM.NKr-CCR5 cells were then stained with 1×10^{-6} M CFSE (Sigma) for 3 minutes, stopped with 500 μl of FCS and washed twice with PBS, then suspended in RF10 media. 2×10^4 PKH26+ CFSE+ coated and uncoated CEM.NKr-CCR5 cells per well were incubated with 25 μg/ml of purified IgG for 15 minutes at room temperature to allow for opsonization of target cells. 2×10^5 effector cells (freshly isolated neutrophils) were then added to the target cells and incubated for 3 hours at 37 °C with CO_2. Post incubation, the cells were immediately placed on ice and then fixed with a final concentration of 2% formaldehyde and

the proportion of PKH26+ cells that had lost intracellular CFSE staining (lysed target cells) was determined using flow cytometry on a BD LSR Fortessa with high- throughput sampler.

3.2.9. Statistical analysis

Statistical analyses were completed using Prism GraphPad version 7.0a (GraphPad Software, San Diego, CA). A Mann-Whitney U test was used to determine the differences in viral load and the CD4+ T cells between the viremic controllers and the viremic non-controllers. A Kruskal-Wallis multiple comparisons analysis with Dunn's multiple comparison's test was used to determine the difference in the ADNP and the neutrophil RFADCC responses. A Mann-Whitney U test was used to determine the differences between the IgA-depleted plasma and the whole plasma for both the ADNP and neutrophil RFADCC. A p- value of < 0.05 was considered to indicate a significant difference. Except where stated, experiments were repeated at least two times.

3.3. Results

3.3.1. Viremic controllers induce higher neutrophil RFADCC responses than the viremic non-controllers

Many studies have shown a strong association between Fc-mediated responses with delayed HIV disease progression [150-152, 301]. The RV144 HIV vaccine trial also highlighted the importance of Fc-mediated responses in providing protection against HIV infection [82]. These studies have utilized monocytes and NK cells but have rarely evaluated neutrophils for their Fc-mediated response in clinical cohorts. The methods previously developed in this thesis were utilized to study neutrophil-mediated phagocytosis and killing (ADNP and RFADCC assays in Chapter 2 [139]) in two large cohorts of HIV infected subjects. Purified IgG antibodies (25μg/ml) from a cohort of 33 Viremic controllers, 41 viremic non-controllers and 13 HIV negative subject samples were evaluated for ADNP and neutrophil RFADCC responses. The viremic controllers and viremic non-controllers both induced detectable ADNP responses above the HIV negative IgG subject samples (Figure 3.2A). There was no significant difference between the viremic controllers and the viremic non-controllers for the ADNP responses (Figure 3.2A). The viremic controllers and viremic non-controllers also both induced neutrophil RFADCC responses higher than the HIV negative IgG subject samples (Figure 3.2B). The viremic controllers induced significantly higher responses (median 1.2-fold increase, p=0.0094) than the viremic non-controllers (Figure 3.2B).

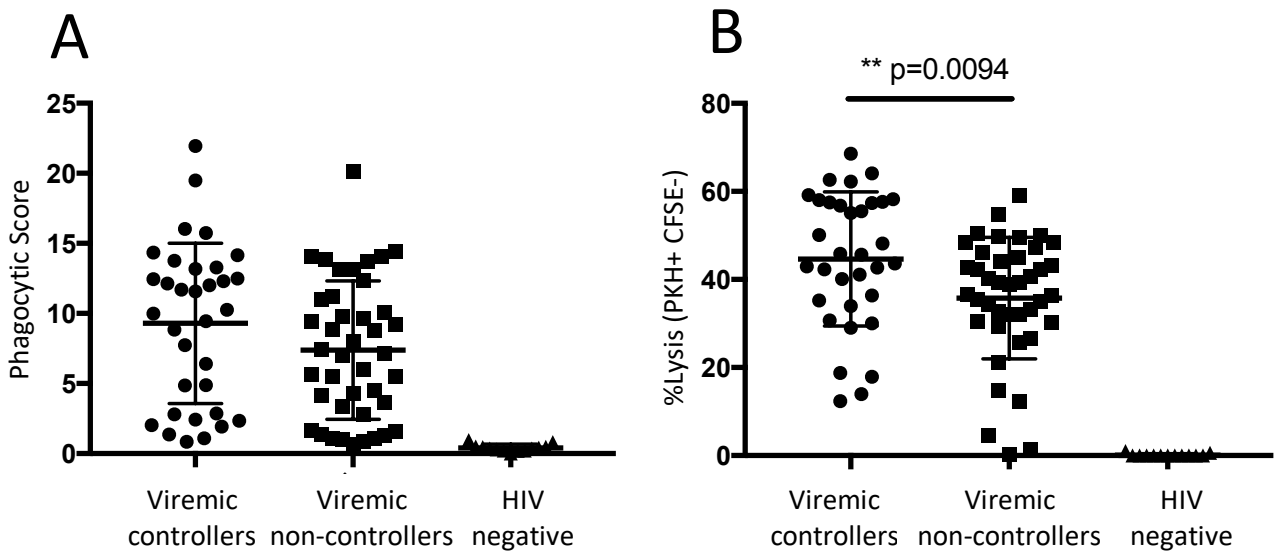


Figure 3.2: **Viremic controllers induce higher levels of neutrophil RFADCC responses than the viremic non controllers but not ADNP responses**. Purified plasma IgG from 33 HIV viremic controllers, 41 HIV viremic non-controllers and 13 HIV negative subjects were evaluated for their ability to mediate **A)** ADNP and **B)** neutrophil RFADCC responses.

3.3.2. IgA-depleted plasma induced higher neutrophil RFADCC responses compared to whole plasma in both viremic controllers and viremic non-controllers

Post hoc analyses of samples from the RV144 HIV vaccine trial showed IgA has the potential to block potentially protective ADCC mediating IgG antibodies from binding to the Env protein. This is thought to have contributed to the reduced vaccine efficacy observed in vaccinees with high titers of IgA to the Env protein. The role that IgA plays in clinical HIV infection has not been well investigated. A consistent concentration of IgG (25µg/ml) for all cohort samples was used to compare the neutrophil RFADCC responses of plasma and IgA-depleted plasma for a cohort of viremic controllers and viremic non-controllers. The IgA-depleted plasma from the viremic controllers induced a significantly higher level of neutrophil ADCC responses than the plasma (1.4 fold median increase, p=<0.0001, Figure 3.3A). The IgA-depleted plasma from the viremic non-controllers also induced a significantly higher level of neutrophil ADCC responses than the plasma (1.5-fold median increase, p=<0.0001, Figure 3.3B). This indicates that IgA is negatively affecting the IgG mediated neutrophil ADCC responses in the plasma samples.

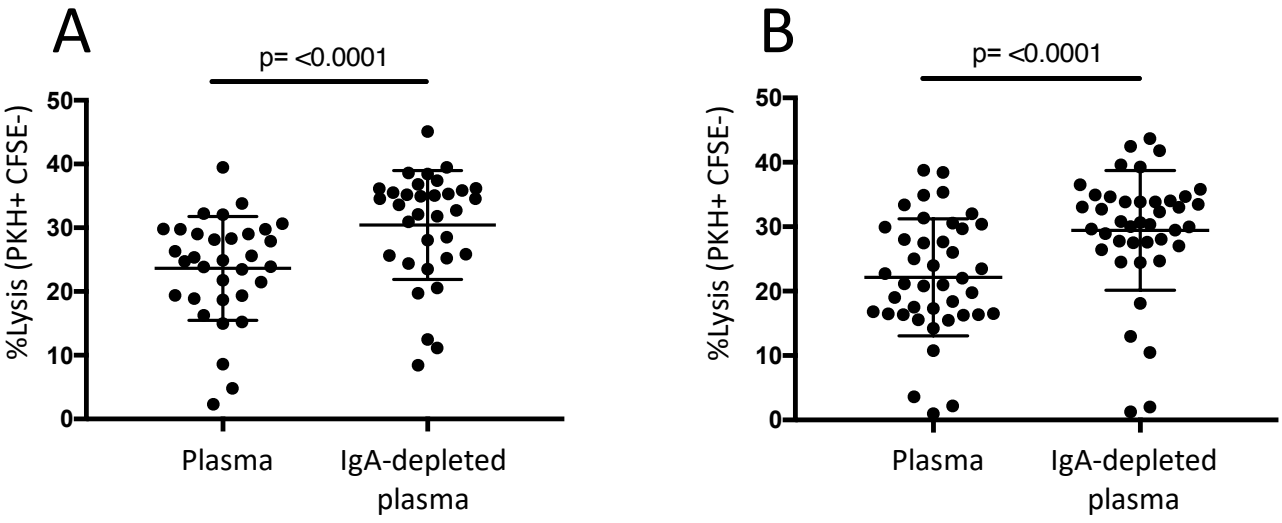


Figure 3.3: **IgA-depleted plasma induced higher neutrophil RFADCC responses than whole plasma for both HIV viremic controllers and HIV viremic non-controllers**. Using 25µg/ml of IgG from IgA-depleted plasma and whole plasma from **A)** 33 HIV viremic controllers and **B)** 41 HIV viremic non-controllers were evaluated for their ability to mediate neutrophil RFADCC responses.

3.3.3. IgA-depleted plasma induced higher ADNP responses compared to whole plasma in both viremic controllers and viremic non-controllers

The role that IgA plays in HIV is controversial with certain studies observing that IgA can provide protection via virus neutralisation [369-371]. In contrast, another study suggests that IgA can potentially block Fc-mediating IgG antibodies via epitope competition [353]. Neutrophils express the IgA-binding FcαR however the effect of IgA on neutrophil-mediated phagocytosis had not been evaluated in HIV infection. A consistent concentration of total IgG (25μg/ml) was used to compare the ADNP responses of plasma and IgA-depleted plasma for a cohort of viremic controllers and viremic non-controllers. The IgA-depleted plasma from the viremic controllers induced a significantly higher level of ADNP responses than the plasma (1.7-fold median increase, p=<0.0001, Figure 3.4A). The IgA-depleted plasma from the viremic non-controllers induced a significantly higher level of ADNP responses than the plasma (1.5-fold median increase, p=<0.0001, Figure 3.4B). This indicates that IgA is negatively affecting the IgG mediated ADNP responses in the plasma samples.

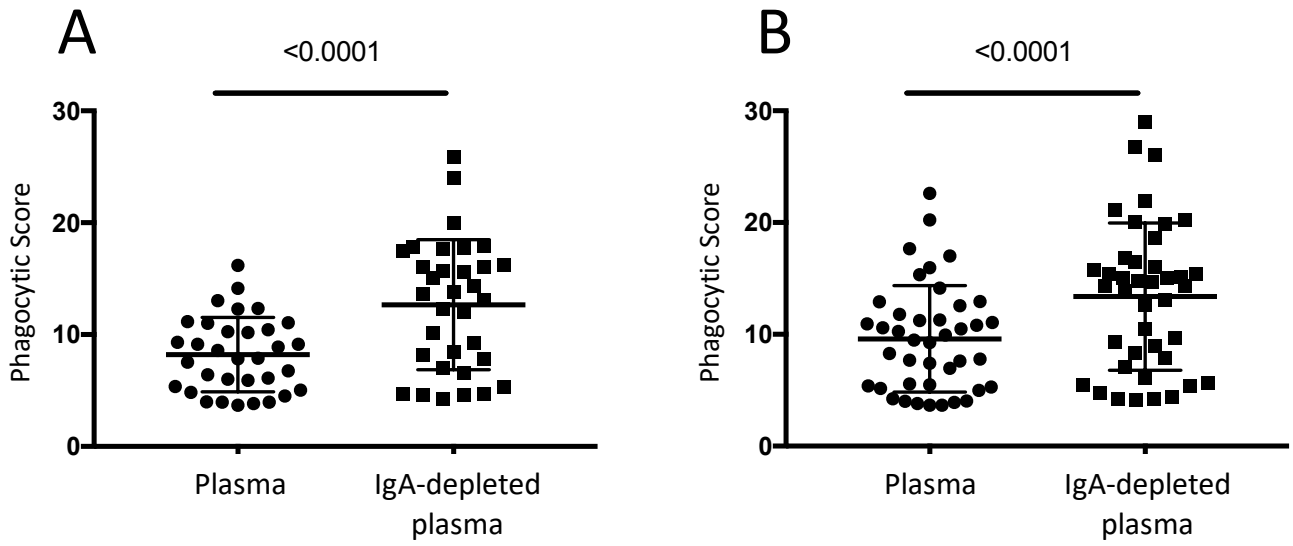
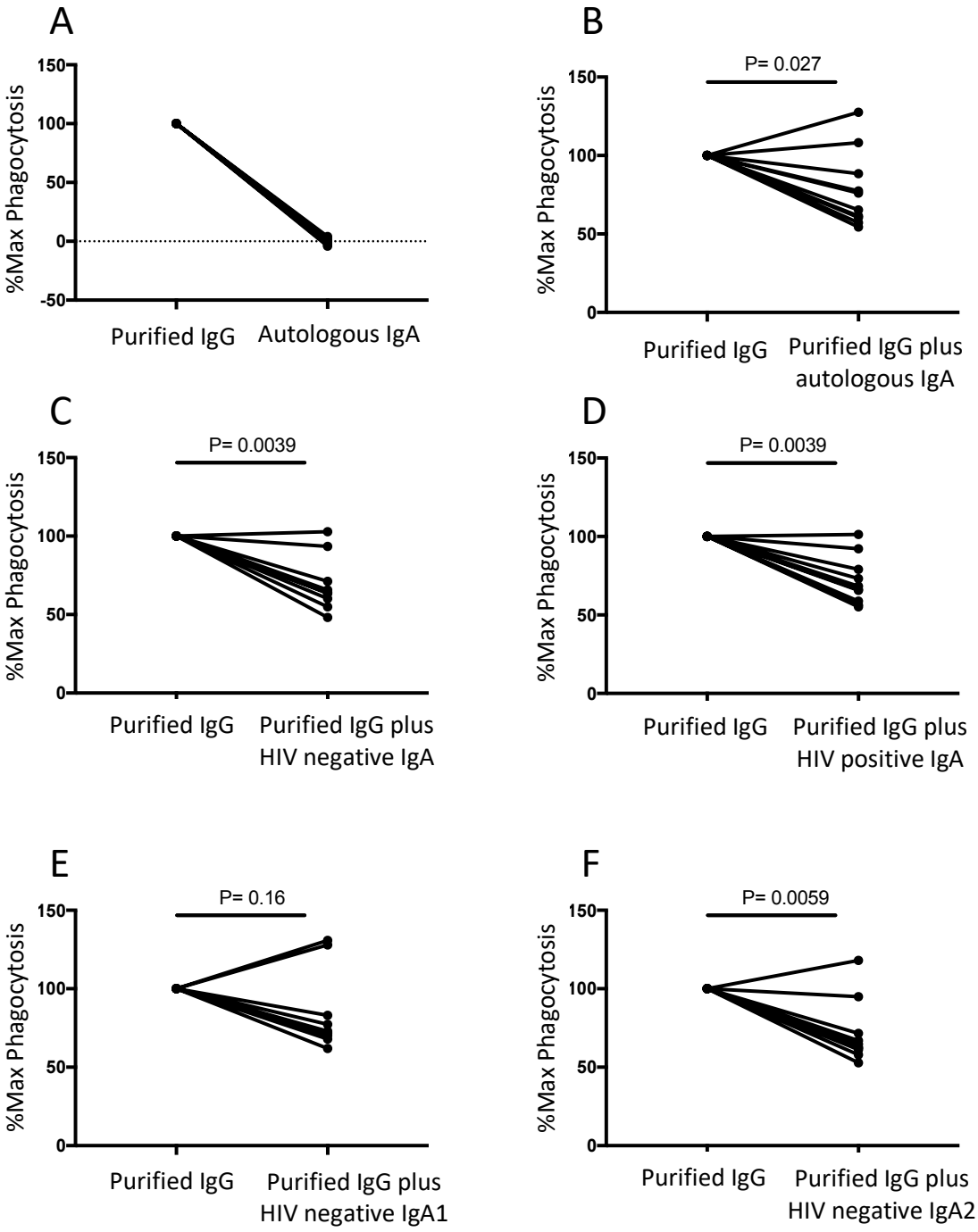


Figure 3.4: **IgA-depleted plasma induced higher ADNP responses than whole plasma for both HIV viremic controllers and HIV viremic non-controllers**. Using 25μg/ml of IgG from IgA-depleted plasma and whole plasma from **A)** 33 HIV viremic controllers and **B)** 41 HIV viremic non-controllers were evaluated for their ability to mediate ADNP responses.

3.3.4. IgA can inhibit the IgG from viremic controllers from mediating ADNP

IgA has previously been shown to be able to inhibit HIV-specific NK cell mediated ADCC responses via epitope competition [353] but has not been investigated for FcαR dependent inhibition. There are structural differences between IgA1 and IgA2 that influence the binding kinetics to the FcαR that may influence neutrophil inhibition. To elucidate the role of IgA in the viremic controllers, a subgroup of the cohort was studied in more depth. A cohort of 10 viremic controllers that were known to have higher IgG mediated ADNP responses that also had sufficient amounts of available IgA were selected for further evaluation. This cohort was evaluated for the ability of IgA from different sources (including autologous IgA, HIV negative pooled purified IgA, HIV positive pooled purified IgA, HIV negative IgA1 and HIV negative IgA2) to inhibit IgG mediated ADNP responses. The autologous IgA antibodies from all 10 viremic controllers were unable to mediate any ADNP (Figure 3.5A). When the IgG from the 10 viremic controller samples were mixed with the autologous IgA, the ADNP responses decreased with a median of 70.71% ($p=0.027$) of the maximal phagocytose response and range of 54.4-127.6% (Figure 3.5B). The HIV negative pooled purified IgA when added to the viremic controllers IgG the ADNP responses decreased with a median of 64.08% of the maximal phagocytose responses ($p=0.0039$) and a range of 48.2-102.8% maximal phagocytosis (Figure 3.5C). The HIV positive pool purified IgA when added to the viremic controllers IgG the ADNP responses decreased with a median of 67.29% ($p=0.0039$) maximal phagocytose response and a range of 55.2-101.3% maximal phagocytosis (Figure 3.5D). The HIV negative purified IgA1 when added to the viremic controllers IgG did not significantly change the IgG mediated ADNP responses (Figure 3.5E). The HIV negative purified IgA2 when added to the viremic controllers IgG reduced the ADNP responses with a median of 63.7% ($p=0.0059$) maximal phagocytose response and a range of 52.8-118.1% maximal phagocytosis (Figure 3.5F). Overall, it was found that IgA inhibited IgG-mediated ADNP responses. Importantly, the IgA inhibition of IgG-mediated ADNP responses was similarly mediated by both HIV specific and non-HIV specific IgA antibodies. A Kruskal-Wallis ANOVA of the phagocytic score shows the same inhibition and that IgA was negatively impacting the IgG mediated ADNP responses ($p<0.0001$) (Figure 3.5G). This highlighted the viremic controller IgA was not able to mediate any HIV specific ADNP responses.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Purified IgG
Purified IgG plus HIV negative IgA2
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Phagocytic score
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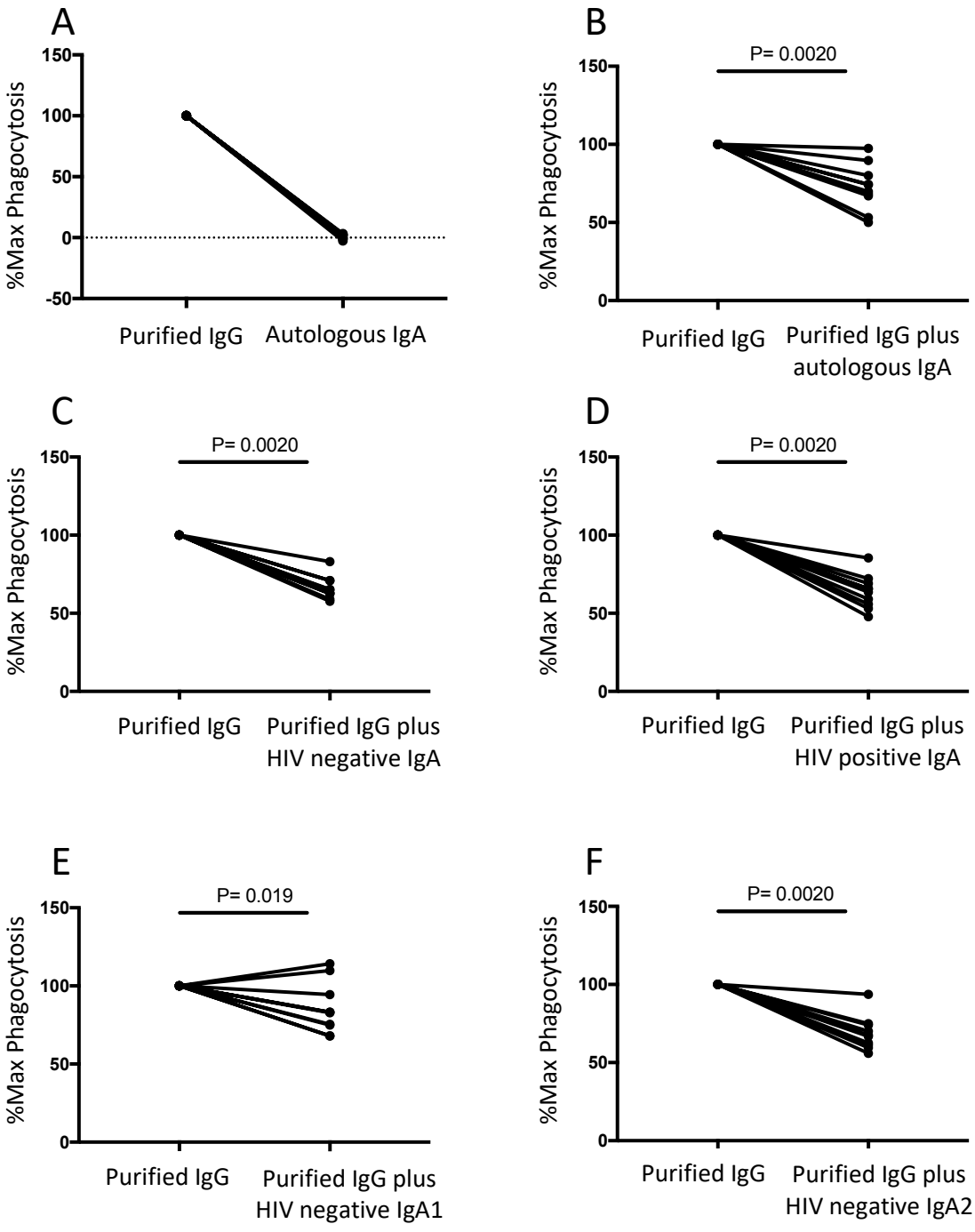
15
10
5
0
Purified IgG
Autologous IgA
Autologous IgA
HIV Negative IgA
HIV Positive IgA
IgA1 HIV negative
IgA2 HIV negative
IgA:IgG 3:1

Figure 3.5: **IgA inhibits the IgG of viremic controllers from mediating ADNP.** A cohort of 10 viremic controllers were evaluated for the ability of their purified IgG and IgA to induce ADNP responses and compared as a percent maximal response of the respective viremic controllers **(A)**. The inhibitory effects of IgA was compared by mixing the viremic controller IgG with different IgAs at a 1:3 (IgG:IgA) ratio including **B)** autologous IgA, **C)** HIV negative pool purified IgA, **D)** HIV positive pooled purified IgA, **E)** HIV negative IgA1 and **F)** HIV negative IgA2. **G**) The phagocytic scores of viremic controllers shows that IgG is inhibited by IgA Kruskal-Wallis test with Dunn's multiple comparison's test ($p<0.0001$)

3.3.5. IgA can inhibit the IgG from viremic non-controllers from mediating ADNP

Similar to section 3.3.4, a subgroup (n=10) of the cohort viremic non-controllers was studied to elucidate the role of IgA in this clinical setting. The ability of IgA to inhibit IgG mediated ADNP responses of viremic non-controllers was investigated using a 3:1 ratio of IgA:IgG. The autologous IgA antibodies from all 10 viremic non-controllers were unable to mediate any ADNP (Figure 3.6A). The purified IgG responses were compared with the addition of the IgA. When the IgG from the 10 viremic non-controller samples were mixed with the autologous IgA the ADNP responses decreased with a median of 71.99% ($p=0.0020$) of the maximal phagocytose response and a range of 50-97.4% maximal phagocytosis (Figure 3.6B). The HIV negative pool purified IgA when added to the viremic non-controllers IgG decreased the ADNP responses by a median of 63.95% ($p=0.0020$) of the maximal phagocytose response and a range of 57.66-83.03% maximal phagocytosis (Figure 3.6C). The HIV positive pool purified IgA when added to the viremic non-controllers IgG decreased the ADNP responses with a median of 64.65% ($p=0.0020$) of the maximal phagocytose response and a range of 47.8-85.45% maximal phagocytosis (Figure 3.6D). The HIV negative purified IgA1 when added to the viremic non-controllers IgG decreased ADNP responses by a median 82.95% ($p=0.019$) of the maximal phagocytose response and a range of 68-114.1% maximal phagocytosis (Figure 3.6E). The HIV negative purified IgA2 when added to the viremic non-controllers IgG decreased the ADNP responses by a median of 67.2% ($p=0.0020$) of the maximal phagocytose response and a range of 56.95-93.65% maximal phagocytosis (Figure 3.5F). These results recapitulate the results of the viremic controllers in Figure 5 showing that IgA can inhibit the responses in an antigen intendent manner. The viremic non-controllers showed similar levels of inhibition of IgG responses compared to the viremic controllers. A Kruskal-Wallis ANOVA of the phagocytic score shows the same inhibition and that IgA was negatively impacting the IgG mediated ADNP responses ($p<0.0001$) (Figure 3.6G). The IgA along from viremic was not able to mediate any ADNP responses.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autologous IgA
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Purified IgG
Purified IgG plus
HIV negative IgA
D
P= 0.0020
%Max Phagocytosis
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100
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Purified IgG plus
HIV positive IgA
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P= 0.019
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Purified IgG plus
HIV negative IgA1
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P= 0.0020
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HIV negative IgA2
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Purified IgG
Autologous IgA
Autologous IgA
HIV Negative IgA
HIV Positive IgA
IgA1 HIV negative
IgA2 HIV negative
IgA:IgG 3:1

Figure 3.6: **IgA inhibits the IgG of viremic non-controllers from mediating ADNP**. A cohort of 10 viremic non-controllers were evaluated for the ability of their purified IgG and IgA to induce ADNP responses and compared as a percent maximal response of the respective viremic controllers **(A)**. The inhibitory effects of IgA was compared by mixing the viremic non-controllers IgG with different IgAs at a 1:3 (IgG:IgA) ratio including **B)** autologous IgA, **C)** HIV negative pool purified IgA, **D)** HIV positive pooled purified IgA, **E)** HIV negative IgA1 and **F)** HIV negative IgA2. **G**) The phagocytic scores of viremic non-controllers shows that IgG is inhibited by IgA Kruskal-Wallis test with Dunn's multiple comparison's test ($p<0.0001$)

3.3.6. ADNP can be inhibited by IgA in an antigenic-specific and antigen-independent manner

The above experiments found IgA inhibited IgG-mediated HIV-specific ADNP responses for both the viremic controllers and viremic non-controller cohorts. To further elucidate the potency of IgA on ADNP mediated IgG responses a series of concentrations of different IgA sources (HIV negative pool purified IgA, HIV positive pooled purified IgA, HIV negative IgA1, HIV negative IgA2) were compared for their ability to inhibit ADNP responses. A fixed pool of HIV positive purified IgG at the same concentration (25μg/ml) and titrated in different amounts of IgA (8.33μg/ml [1:3 ratio], 25μg/ml [1:1] and 75μg/ml [3:1]) was used to evaluate the effect of their inhibition. An IgA dose dependent inhibition of IgG mediated ADNP responses for the HIV negative pool purified IgA, HIV positive pooled purified IgA and HIV negative IgA2, with greater than 50% inhibition at the 3:1 ratio for all 3 IgA preparations (Figure 3.7). The level of inhibition observed in the HIV negative IgA1 was less compared to the HIV negative pool purified IgA and HIV positive pooled purified IgA.

The inhibition of the IgG-mediated ADNP responses by non-HIV specific IgA suggests that the IgA is mediating the inhibition through binding FcαR rather than by epitope competition alone. The inhibition was validated to be through FcαR, by the addition of the MIP8a antibody that binds to and blocks FcαR signaling [372]. There was a substantial (23.6% and 30.3%) resurrection of HIV-specific IgG mediated ADNP responses by the HIV negative pooled purified IgA and the HIV negative IgA2. This is consistent with a FcαR dependent inhibition of IgG mediated responses that is independent of antigen. There was no resurrection (2.1%) of the ADNP responses with the HIV negative IgA1 antibody when the FcαR on the neutrophils was blocked. There was a more partial resurrection of the HIV-specific IgG mediated ADNP responses by the HIV positive pooled purified IgA (10.7%) compared to the HIV negative

pooled purified IgA and the HIV negative IgA2. This suggests that there may be both antigen dependent inhibition (epitope competition) and an antigen-independent inhibition (through FcαR) by the HIV-specific IgA. Overall, these results show that IgA can be inhibitory to neutrophil mediated ADNP independent of the IgA antigen specificity.

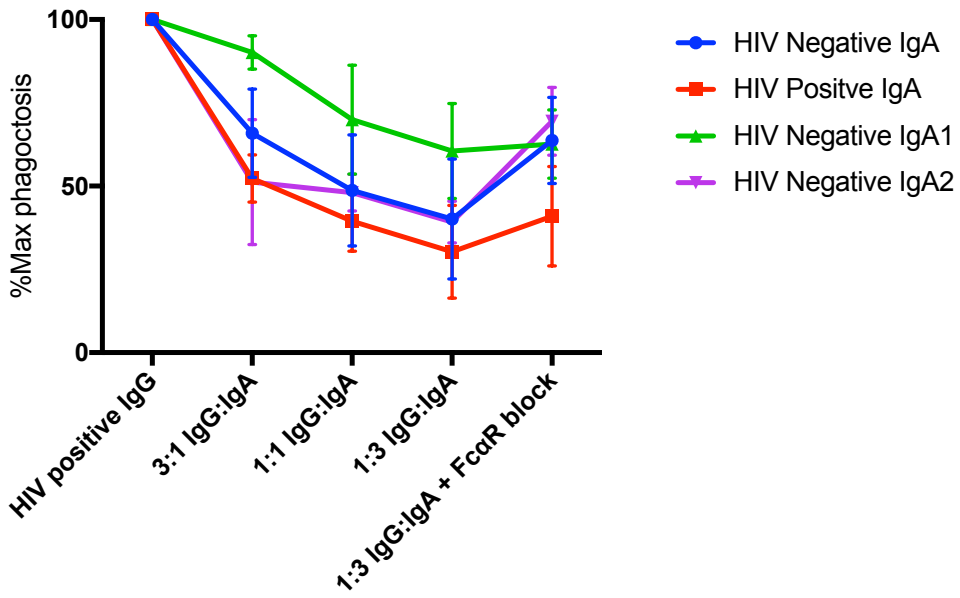

Figure 3.7: **ADNP can be inhibited by IgA in an antigenic-specific and antigen-independent manner.** Using a fixed pool of HIV positive IgG at 25μg/ml and titrating in different amounts of IgA (8.33μg/ml [1:3 ratio], 25μg/ml [1:1] and 75μg/ml [3:1]) to evaluate the effect of their inhibition. To identify whether the blocking of FcαRI reduces IgA-mediated inhibition of phagocytosis, neutrophils were pre-treated with an anti-FcαRI antibody to block signalling before the addition of the IgA and IgG antibodies at a 3:1 ratio. Results from two independent experiments are shown.

3.4. Discussion

In this thesis, plasma samples of two large viremic HIV positive cohorts were investigated to probe the role of antibody-dependent neutrophil function on disease progression. Purified IgG samples from both viremic controller and the viremic non-controller cohorts showed detectable ADNP and neutrophil ADCC responses, although the neutrophil ADCC responses were weaker in the cohort of viremic non-controllers. The comparison of the plasma samples and the IgA-depleted plasma samples indicated both the viremic controller and viremic non-controllers IgG were equally inhibited by IgA, for both the ADNP and neutrophil ADCC responses. HIV-specific IgG ADNP responses mediated by plasma from both viremic controllers and viremic non-controllers were inhibited by their autologous IgA, HIV negative pool purified IgA, HIV positive pooled purified IgA, HIV negative IgA2.

The mechanism of IgA inhibition of IgG was evaluated for an FcαR dependent mechanism of inhibition opposed to just an epitope competition. IgA was shown to inhibit IgG-mediated neutrophil responses in a concentration dependent manner and that the inhibition of antigen independent IgA antibodies was able to be blocked by pretreating neutrophils with an FcαR blocking antibody. However, the HIV specific IgA antibodies only had partial resurrection of responses following pretreatment of neutrophils with an FcαR blocking antibody. This suggests that the HIV specific IgA antibodies are blocking HIV specific IgG with an antigen specific mechanism in addition to a FcαR dependent mechanism also being present. Overall, the data are consistent with 2 mechanisms that IgA is able to inhibit ADNP responses: 1) antigen dependent/FcαR independent and 2) antigen independent/FcαR dependent.

It was recently shown that IgA negatively influenced PBMCs RFADCC responses in HIV infected individuals within 12 months of infection [181]. Interestingly, the same study showed PBMC mediated RFADCC responses in elite controllers were unaffected by IgA [181]. Our results using primary neutrophil based assays indicate that, irrespective of clinical progression (viremic non-controllers or viremic controllers), IgA is able to inhibit both ADNP and neutrophil ADCC responses. This difference may be a product of the difference in cells being evaluated. It would be of interest to investigate if IgA from elite controllers also effect IgG mediated neutrophil functions as Ruiz et al previously observed with PBMCs [181]. To further elucidate the role of IgA on ADCC responses in HIV infected subjects, a larger number of

clinical groups including elite controllers studied longitudinally should be evaluated across a range of effector cells. There is a need to investigate the differences in IgA1 and IgA2 from HIV positive plasma to evaluate any potential Fc-mediated responses and any inhibitory effects it can have on IgG mediated responses.

Our work on polyclonal HIV antibody responses in sera is complemented by recent work using HIV-specific monoclonal antibodies engineered to be IgA or IgG. The 2F5 IgA gp41 specific monoclonal antibodies was shown to induce ADCC responses using monocytes as effector cells [373]. The ADCC responses of the 2F5 IgA when combined with 2F5-IgG or with 10E8-IgG antibodies show cooperation and results in enhanced target cell lysis [373]. Evaluating HIV specific monoclonal IgA and IgG antibodies with neutrophils would be an area of interest to evaluate if these results are recapitulated. It has been suggested that IgA monoclonal antibodies may be used to protect at risk populations from HIV [374, 375]. The differences observed between using monoclonal antibodies and patient derived antibodies should be evaluated further using a range of effector cells and Fc-blocking reagents which could allude to a mechanism of action and lead to improved vaccine designs.

In our studies, the IgA antibodies were generally found to inhibit IgG mediated ADNP responses. Interestingly, a subset of 2 viremic controllers (Figure 3.5B) IgG antibodies showed similar or slight increases in ADNP responses when combined with their autologous IgA. This was unexpected as the autologous IgA alone is unable to mediate ADNP responses. The same two viremic controllers IgG also showed an increase in ADNP responses when combined with the HIV negative IgA1 (Figure 3.5E). Two of the viremic non-controllers IgG antibodies showed similar or slight increases in ADNP responses with the HIV negative IgA1 (Figure 3.6E). This suggest the possibility that there is a complex role that IgA can play with IgG whereby IgA does not negatively affect all polyclonal HIV-specific IgG mediated ADNP responses equally. As the IgA remains consistent between IgG samples (with the exception of the autologous IgA) it suggests that there are differences in IgG that drives the possibility of a synergistic relationship. This may be driven by a number of different factors including IgG subclass presence which may also affect the FcγR affinity or which FcγR that is bound to. In addition, the IgG antibody glycosylation can play a role in FcγR binding and mediation of ADCC responses and may influence if IgG is able to overcome IgA mediated inhibition of neutrophils [376, 377].

The neutrophils of HIV infected individuals have been shown to have impaired phagocytosis [378], oxidative burst [379], chemotaxis [380] and bactericidal activity [381] in both untreated infection and following ART. It is also known that FcαRI expression is downregulated on the neutrophil surfaces as HIV disease progresses [382, 383]. The experiments performed here have all been completed with neutrophil isolated from HIV uninfected healthy donors. The effects that HIV has on IgA's ability to inhibit IgG mediated responses using primary neutrophils from HIV-infected subjects should be investigated. The ability of neutrophils from HIV infected individuals to mediate ADNP and ADCC responses is critical to understanding the role of IgA in HIV infected subjects.

Our experiments were completed on isolated circulating neutrophils. This is a common practice as it is a convenient source of neutrophils [132, 138, 354]. There is a need to investigate the differences of neutrophils isolated from tissues, particularly mucosal tissues where HIV is first encountered. It had been previously shown that neutrophils isolated from different tissues express different FcR frequencies which could influence the effector responses [193]. The different FcR profiles could also influence whether neutrophils are inhibited by HIV negative IgA due to different IgA exposure during the life of the cell.

In summary, antiretroviral naïve viremic non-controllers and viremic controllers are both able to mediate HIV-specific ADNP and neutrophil ADCC responses and these responses can be inhibited in the presence of IgA. HIV-specific IgA and non-specific IgA was able to inhibit HIV specific IgG mediated ADNP responses. These results suggest that there could be 2 mechanisms that IgA is able to inhibit ADNP responses, 1) antigen dependent/FcαR independent and 2) antigen independent/FcαR dependent. The non-specific IgA2 was more effective at inhibiting HIV-specific IgG mediated ADNP responses. The mechanism of IgA inhibition of neutrophils should be investigated further, and this could lead to improved therapeutics and vaccine development in HIV and may contribute to our understanding of HIV pathogenesis.

Chapter 4

RV144 vaccine-induced neutrophil phagocytosis is inhibited by IgA

4.1. Introduction

The phase III RV144 ALVAC/AIDSVAX B/E HIV-1 vaccine trial in Thailand demonstrated a vaccine of efficacy 31.2% at 3.5 years after the first vaccination [319]. The vaccine had an estimated vaccine efficacy of 60.5% at the 1 year time point, but waned over time in association with declining vaccine-induced antibody responses [320] . The initial immune correlates analysis indicated that plasma IgG antibody responses to Env were associated with a lower risk of infection in the vaccinees [82]. Plasma IgG levels to the V1V2 region of the envelope protein correlated with reduced risk of infection [82]. However, high levels of IgA antibodies to the envelope protein were associated with decreased vaccine efficacy [82]. In the presence of low levels of IgA antibodies to the envelope protein, ADCC responses inversely correlated with risk of infection, while in the presence of high levels IgA antibodies to the envelope protein there was no correlation with ADCC with risk of infection [82].

Antibody effector functions rely partially on their ability to engage to Fc receptors on innate immune effector cells. Various effector cells express different FcRs, which bind with differing capacities to antibody isotypes and subclasses. There are two human IgA antibody subclasses, IgA1 and IgA2 [159-161]. In human serum ~90% of IgA present is the IgA1 subclass and ~10% being IgA2, while in the mucosa of the genitorectal tract it is ~60-90% IgA2 [163, 164]. IgA1 and IgA2 have different binding kinetics and have altered structure, allowing for different antigen binding kinetics [166]. Monoclonal IgA1 was shown to protect macaques against SHIV challenge more effectively than IgA2 or IgG1 [173]. The location a pathogen is encountered will influence the immune responses generated by IgA antibodies.

RV144 vaccination induced IgG antibodies with the capacity to activate NK cells to mediate ADCC responses [82]. However, monoclonal Env-specific IgA antibodies isolated from vaccinees were able to block plasma IgG mediated ADCC responses of NK cells [353]. This suggested that IgA may have inhibited IgG mediated ADCC responses from NK cells by epitope competition, although how important this is in the context of total polyclonal responses in plasma is unclear. NK cells express FcγRIIIa however, they do not express the IgA-binding FcαRI (CD89) which is expressed by other FcγR expressing leukocytes including monocytes/macrophages and neutrophils [384]. Whether vaccine-induced IgA can inhibit IgG-mediated Fc effector functions in cells that also express FcαRI, such as neutrophils, has not previously been studied.

Neutrophils are the most abundant circulating leukocyte in the blood (30–70%), can rapidly migrate to sites of infection and can initiate a range of Fc-mediated effector responses [189-191]. Neutrophils are abundantly present at mucosal surfaces where HIV is commonly acquired, including vaginal and rectal tissues [193] [385]. Neutrophils have been shown to be able to mediate HIV-specific antibody-dependent phagocytosis (ADP) and antibody-dependent cellular cytotoxicity (ADCC) in the context of HIV infection [132, 138, 139]. Neutrophils have remained an understudied area in the context of vaccination against HIV.

This chapter investigates polyclonal IgG and IgA isolated from the serum of RV144 vaccinees for their ability to mediate antibody-dependent neutrophil responses. Samples from the RV144 vaccinees were investigated for Env-specific IgG that mediated ADNP and neutrophil RFADCC. The RV144 vaccinees plasma and IgA-depleted plasma was investigated for ADNP and RFADCC responses. The IgG mediated ADNP responses of the RV144 vaccinees were evaluated to for their potential to be inhibited by pooled purified IgA from RV144, pooled purified IgA from HIV positive donors, pooled purified IgA from HIV negative donors, HIV negative IgA1 or HIV negative IgA2. These findings support the finding in Chapter 3 and provide new leads on the influence of plasma IgA on HIV vaccine induced antibody immunity.

4.2. Materials and Methods

4.2.1. Study subjects/plasma samples

Plasma samples from the RV144 vaccine trial (80 vaccinees and 20 placebo) were kindly provided by Military HIV Research Program (MHRP). Additionally, plasma samples from 10 baseline samples prior to vaccination were obtained from the MHRP. The vaccine and placebo plasma samples were all collected and stored at the 6 month time point following initial vaccination, 2 weeks after the completion of the vaccination and at a time of maximal antibody response. All RV144 trial participants gave written informed consent [319]. The subjects utilised in this study did not become infected with HIV during the course of the vaccine trial.

4.2.2. Other IgG and IgA antibodies:

A number of antibodies were utilised in addition to the clinical samples to evaluate IgA responses. A pooled HIV positive IgG (HIVIG) was supplied by the NIH AIDS reagent program and was used as a known positive sample. In addition, serum from 11 HIV+ subjects (provided by Dr Stephen Kent through the Melbourne Sexual Health Centre with written

informed consent) was used to purify IgA and create a pool of IgA from HIV+ subjects (HIVIGA). A pooled purified HIV negative IgA was created using 13 HIV negative healthy donors. The purified HIV negative IgA1 and IgA2 were both supplied by Athens Research and technology and was sourced from myeloma patients (cat: 090701-1M and 090701-2M).

4.2.3. Primary Neutrophil isolation

Neutrophils were isolated by adapting previously published methods [139, 211, 364] and previously described in section 3.2.3. Briefly, freshly collected heparinized blood was obtained and leukocytes were removed after Ficoll density centrifugation. The granulocytes were enriched by using dextran sedimentation for 15-20 minutes at room temperature. The remaining RBC in the granulocytes was treated with a hypotonic lysis reagent. The remaining neutrophils were washed in and suspended in RF10.

4.2.4. IgA antibody purification

Plasma IgA was performed using peptide M/agarose resin (InvivoGen) following the manufacturer's instructions with minor modifications as detailed in section 3.2.4. Briefly, purification columns were prepared by loading 300µl of peptide M agarose into spin columns and washed with PBS. Plasma samples were added to columns and incubated at room temperature on an orbital rotator for 45 minutes. The columns were washed with PBS 6 times and the IgA eluted off the columns using IgG elution buffer and immediately neutralised with Tris 1M pH 8.0. The purified IgA was then processed in protein G multitrap columns to remove any contaminating IgG and then evaluated for both IgA and IgG concentrations.

4.2.5. IgA antibody concentration quantification

The purified antibodies (IgG, IgA) and the plasma samples (IgA-depleted plasma and whole plasma) were quantified using an anti-IgA ELISA kit (Mabtech) following the manufacturer's instructions and how is detailed in section 3.2.5.

4.2.6. Plasma IgA1 antibody concentration quantification

The concentration of IgA1 was evaluated for all plasma samples from RV144 vaccinees and placebos. Maxisorb 96 wells plates (Nunc) were coated with 100µl of the Mabtech MT57 (2 µg/ml) capture antibody overnight at 4 °C. The plate was washed 5 times with PBST (PBS with 0.05% Tween 20) and blocked with 200µl of 1% BSA/PBST for 1 hour at room

temperature. The plate was washed as before and plasma was diluted to 1:10,000, 1:20,000 or 1:30,000 in 1% BSA/PBST were added for 2 hours at room temperature, alongside an IgA1 standard (Athens Research and Technology cat# 16-16-090701-1M). The plate was washed as before and 100 µl/well of mouse Anti-Human IgA1-HRP (Southern Biotech, cat 9130-05) diluted 1:1000 in incubation buffer and incubated for 2 hours at room temperature. The plate was washed as before and 100 µl/well of 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma, Cat T0440) was added to develop and stopped with 100µl sulfuric acid (0.16mol). The plate was then measured for optical density at 450 nm on a Thermo Fisher Multiskan Ascent plate reader or on the BMG Labtech CLARIOstar plate reader.

4.2.7. Plasma IgA2 antibody concentration quantification

The concentration of IgA2 was evaluated for all plasma samples from RV144 vaccinees and placebos. Maxisorb 96 wells plates (Nunc) were coated with 100µl of the Mabtech MT57 (2 µg/ml) capture antibody overnight at 4 °C. The plate was washed 5 times with PBST (PBS with 0.05% Tween 20) and blocked with 200µl of 1% BSA/PBST for 1 hour at room temperature. The plate was washed as before and plasma was diluted to 1:1,000, 1:5,000 or 1:10,000 in 1% BSA/PBST were added for 2 hours at room temperature, alongside an IgA2 standard (Athens Research and technology cat# 16-16-090701-2M). The plate was washed as before and 100 µl/well of mouse Anti-Human IgA2-HRP (southern biotech, cat 9140-05) diluted 1:4000 in incubation buffer and incubated for 2 hours at room temperature. The plate was washed as before and 100 µl/well of 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma, Cat T0440) was added to develop and stopped with 100µl sulfuric acid (0.16mol). The plate was then measured for optical density at 450 nm on a Thermo Fisher Multiskan Ascent plate reader or on the BMG Labtech CLARIOstar plate reader.

4.2.8. IgG antibody concentration quantification

The purified antibodies (IgG, IgA) and the plasma samples (IgA-depleted plasma and whole plasma) were quantified using an anti-IgG ELISA kit (Mabtech) following the manufacturer's instructions and detailed in section 3.2.6. The purified antibodies (IgG, IgA) and the plasma samples (IgA-depleted plasma and whole plasma) were diluted to 1:20,000 and 1:250,000 to ensure they were well placed in the standard curves.

4.2.9. Antibody-dependent neutrophil phagocytosis (ADNP)

See Chapter 2 for detailed methods including gating strategy and optimization of this assay [139]. The assays here were completed with minor modifications as follows. Briefly, the gp120 protein was either Clade AE(A244) or Clade B (MN) from Immune Technologies. 300μg/ml purified of IgG (from purified IgG, Plasma IgG or IgA-depleted plasma) or a combination of purified IgG with 300μg/ml of purified IgA (pooled purified IgA from RV144, pooled purified IgA from HIV positive donors, pooled purified IgA from HIV negative donors, HIV negative IgA1 or HIV negative IgA2), was incubated with the protein coated beads.

4.2.10. Rapid fluorometric antibody-dependent cellular cytotoxicity (RFADCC)

See Chapter 2 for detailed methods including gating strategy and optimization of this assay [139]. The assays here were completed with minor modifications as follows. The CEM.NKr-CCR5 cells were coated with in 3 μg of gp120 (MN or A244) (Immune Technologies). The coated CEM.NKr-CCR5 cells were incubated with 300μg/ml of IgG (from purified IgG, Plasma IgG or IgA-depleted plasma) for 15 minutes at room temperature to allow for opsonization of target cells.

4.2.11. Fcα-receptor surface plasmon resonance analysis

Surface plasmon resonance (SPR) experiments were run on a Biacore 3000 to measure IgA to Fcα receptor (FcαR) binding activity. The Fcα receptor was biotinylated using the EZ-Link Sulfo- NHS-LC biotinylation kit (Thermo Scientific) using a 5 mmol excess of biotin according to manufacturer's instructions. Biotinylated FcαR was coated onto a sensor Chip SA (BR100032) at a density of 200RU. 6.25μg/ml of purified IgA antibodies from the RV144 vaccinees and placebo were passed over sensor chips at 25 ul/minute for 60 seconds. The binding to the FcαR was assessed using Bia Evaluation software and quantified as the relative response units of signal at maximal binding.

4.2.12. Statistical analysis

Statistical analyses were completed using Prism GraphPad version 7.0a (GraphPad Software, San Diego, CA). A Mann- Whitney U test was used to determine the differences between the vaccinees and placebo samples. A Kruskal-Wallis multiple comparisons analysis was used to determine the difference between the IgA-depleted plasma and the whole plasma for vaccinees and placebo for both the ADNP and neutrophil RFADCC. Nonparametric Spearman

correlation analyses were used to test for correlations. A p-value of < 0.05 was considered to indicate a significant difference. Except where stated, experiments were repeated at least two times.

4.3. Results

4.3.1. Antibody concentration of plasma IgG, plasma total IgA, plasma IgA1 and plasma IgA2

As total antibody titres within plasma can contribute to differences in Fc functions across samples, the total IgG and IgA concentration levels from both vaccine and placebo plasma samples was evaluated. The total plasma IgG concentrations were similar between the vaccinees (median 9.8mg/ml and range of 4.48-17.45 mg/ml) and placebo (median 10.29 mg/ml and range of 5.91-14.11 mg/ml, Figure 4.1A). The plasma IgA concentrations also showed no differences between the vaccinees (median 1.5mg/ml and range of 0.63-4.0mg/ml) and placebo subjects (median 1.65mg/ml and range of 0.56-3.96mg/ml, Figure 4.1B). There was no difference between the vaccine (median 1.87mg/ml and range of 0.71-4.85mg/ml) and placebos (median 1.91mg/ml and range of 1.02-3.39mg/ml) for both IgA1 (Figure 4.1C) and IgA2 (vaccine median 0.10mg/ml and range of 0.013-0.31mg/ml; placebo median 0.10mg/ml and range of 0.018-0.35mg/ml, Figure 4.1D).

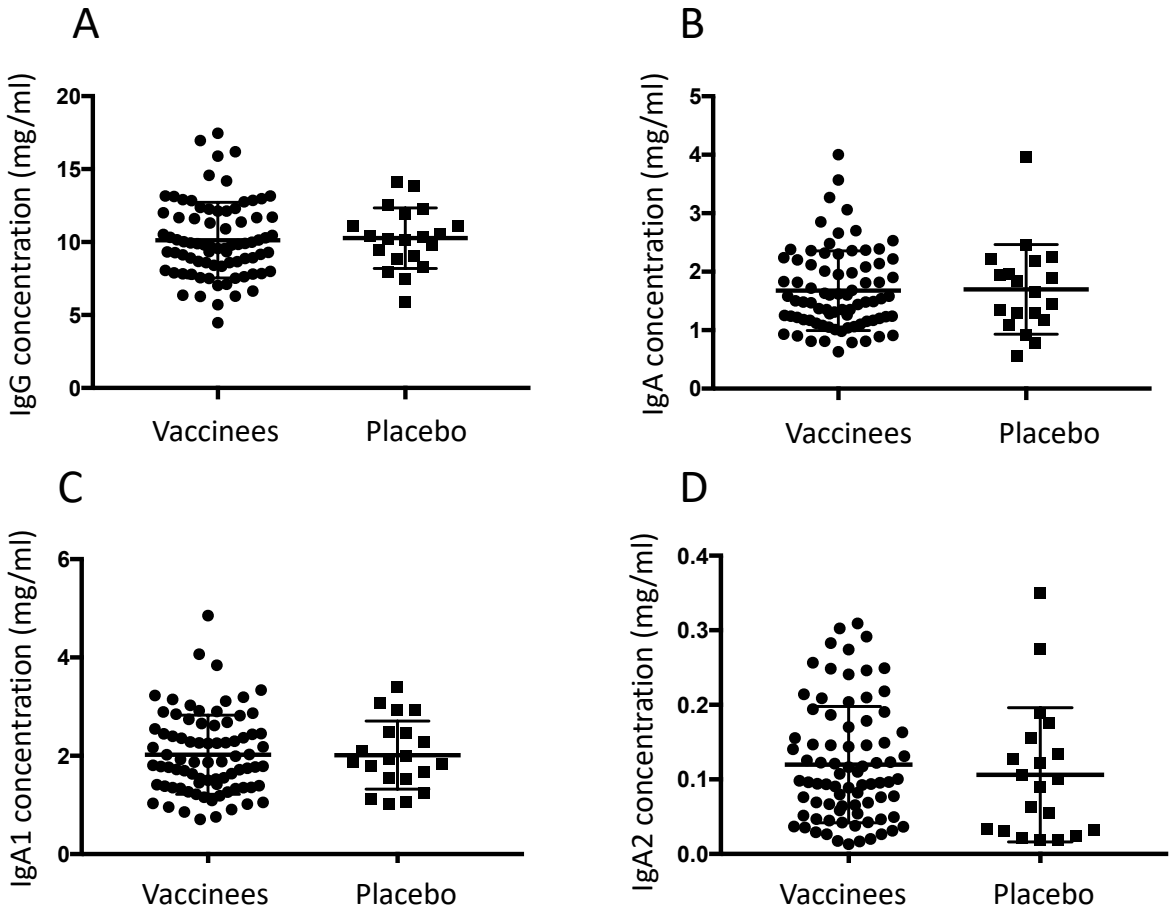

Figure 4.1: **Antibody concentration of plasma IgG, plasma total IgA, plasma IgA1 and plasma IgA2 as measured by ELISA**. There were no differences in plasma IgG **(A)**, plasma total IgA **(B)**, plasma IgA1 **(C)** and plasma IgA2 **(D)** antibody concentrations between the RV144 vaccinees and the placebo subjects.

4.3.2. Antibody concentrations of IgA-depleted plasma, whole plasma and purified IgG

Small levels of contaminating IgG within the IgA samples could potentially confound functional antibody studies. The IgA and IgG antibody concentrations were therefore evaluated in the purified IgG samples, purified IgA and the IgA-depleted plasma samples. The purified IgG was confirmed to be free of contaminating IgA (Figure 4.2A) with similar levels of IgG purified for both vaccinees (median 2.3mg/ml and range 1.42-4.01mg/ml) and placebo recipients (median 2.2mg/ml and range 1.43-3.01mg/ml). The purified IgA was also confirmed to be free of any contaminating IgG (Figure 4.2B) with similar levels of IgA purified for both vaccinees (median 0.85mg/ml and range 0.26-1.56mg/ml) and placebo recipients (median 0.71mg/ml and range 0.02-1.14mg/ml). The IgA-depleted plasma was verified to have been depleted of the IgA (Figure 4.2C) with no IgA detected via ELISA and again similar levels of IgG between the vaccinees (median 4.99mg/ml and range 2.31-8.96mg/ml) and placebo recipients (median 5.78mg/ml and range 3.76-8.08mg/ml).

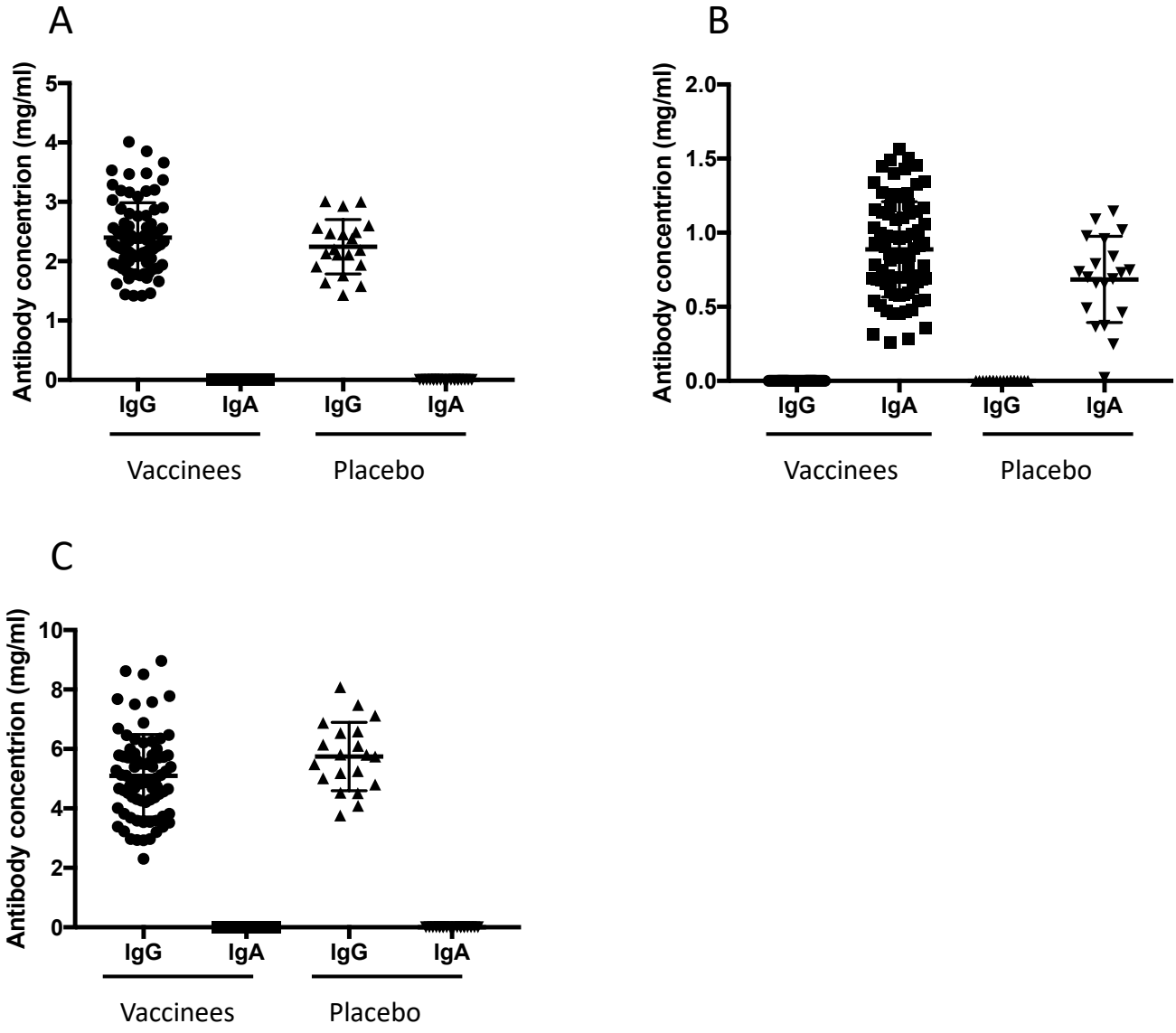

Figure 4.2: **Antibody concentrations of purified IgG, purified IgA and the IgA-depleted plasma.** The purified IgG was free of IgA and no difference in antibody concentrations were observed between the RV144 vaccinees and placebo subjects (**A**). The purified IgA was free of contaminating IgG and with no observed differences in antibody concentrations between the RV144 vaccinees and placebo subjects **(B)**. The IgA-depleted plasma was free of IgA and no difference in antibody concentrations were observed between the RV144 vaccinees and placebo subjects **(C)**.

4.3.3. Correlations between the EC50 and the ADNP phagocytic scores

It was hypothesised that the RV144 vaccinees would have lower levels of HIV-specific IgG antibodies than HIV infected subjects. Therefore, the optimal concentration of purified plasma IgG antibody to use in functional assays was determined. The ADNP assay was employed to

study different concentrations from plasma samples of 20 randomly selected RV144 vaccinees. Purified IgG samples were evaluated at 300, 200, 100, 50, 25 and 10μg/ml, the phagocytic score was calculated and EC50 determined using prism non-linear regression curve fit for each sample. A Spearman nonparametric correlation was utilized to compare the EC50 with the phagocytic score for each IgG concentration 300μg/ml r=0.8917 and p=<0.0001 (Figure 4.3A); 200μg/ml r=0.7865 and p=<0.0001 (Figure 4.3B); 100μg/ml r=0.7895 and p=<0.0001 (Figure 4.3C); 50μg/ml r=0.4602 and p=0.0412 (Figure 4.3D); 25μg/ml r=0.2286 and p=0.3324 (Figure 4.3E), and 10μg/ml r=0.02406 and p=0.9198 (Figure 4.3F). The antibody concentrations tested did not reach a plateau in responses, indicating the maximum response had not been reached. However, the correlations were strong at 300μg/ml and the limited volume of samples precluded testing higher concentrations (Figure 4.3G). The strongest correlation of responses was the 300 μg/ml of IgG and this was utilised for subsequent assays in this chapter.

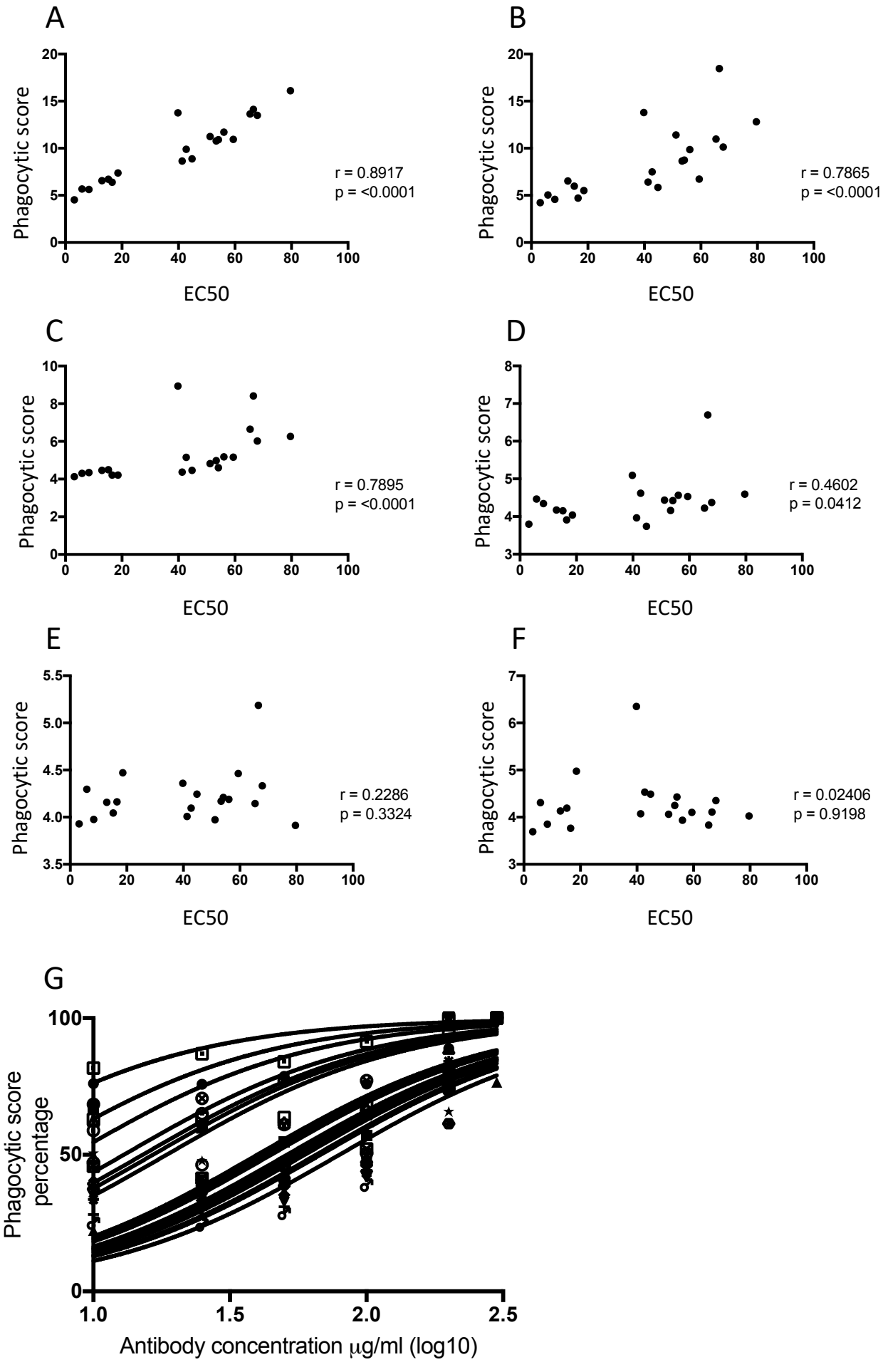
A
Phagocytic score
EC50
r = 0.8917
p = <0.0001
B
Phagocytic score
EC50
r = 0.7865
p = <0.0001
C
Phagocytic score
EC50
r = 0.7895
p = <0.0001
D
Phagocytic score
EC50
r = 0.4602
p = 0.0412
E
Phagocytic score
EC50
r = 0.2286
p = 0.3324
F
Phagocytic score
EC50
r = 0.02406
p = 0.9198
G
Phagocytic score percentage
Antibody concentration μg/ml (log10)

Figure 4.3: Correlations between the EC50 and the ADNP phagocytic scores. Purified IgG from 20 RV144 vaccinees were used to determine the optimal concentration for the ADNP assay. A Spearman nonparametric correlation was utilized to compare the EC50 with the ADNP phagocytic score for each IgG concentration of 300µg/ml r=0.8917 and p=<0.0001 (**A**); 200µg/ml r=0.7865 and p=<0.0001 (**B**); 100µg/ml r=0.7895 and p=<0.0001 (**C**); 50µg/ml r=0.4602 and p=0.0412 (**D**); 25µg/ml r=0.2286 and p=0.3324 (**E**), and 10µg/ml r=0.02406 and p=0.9198 (**F**). The IgG antibodies concentrations did not reach the maximum responses (**G**)

4.3.4. Antibody-dependent neutrophil phagocytosis is inhibited by IgA

Plasma from RV144 vaccinees has been shown to mediate ADCP responses with monocytes and this response is largely driven by IgG3 [326]. ADNP responses of purified IgG from RV144 samples using Env-coated beads and fresh healthy donor blood neutrophils were assessed. There was a large range (0-26.12 phagocytic score) of responses for purified IgG mediated ADNP responses with a median phagocytic score of 2.02 (Figure 4.4A). Although the responses were modest, the majority, 62 of the 80 samples, had phagocytic score above 2 standard deviation (2 standard deviation is 0.255) of the mean of the placebo responses (mean 0.046 phagocytic score).

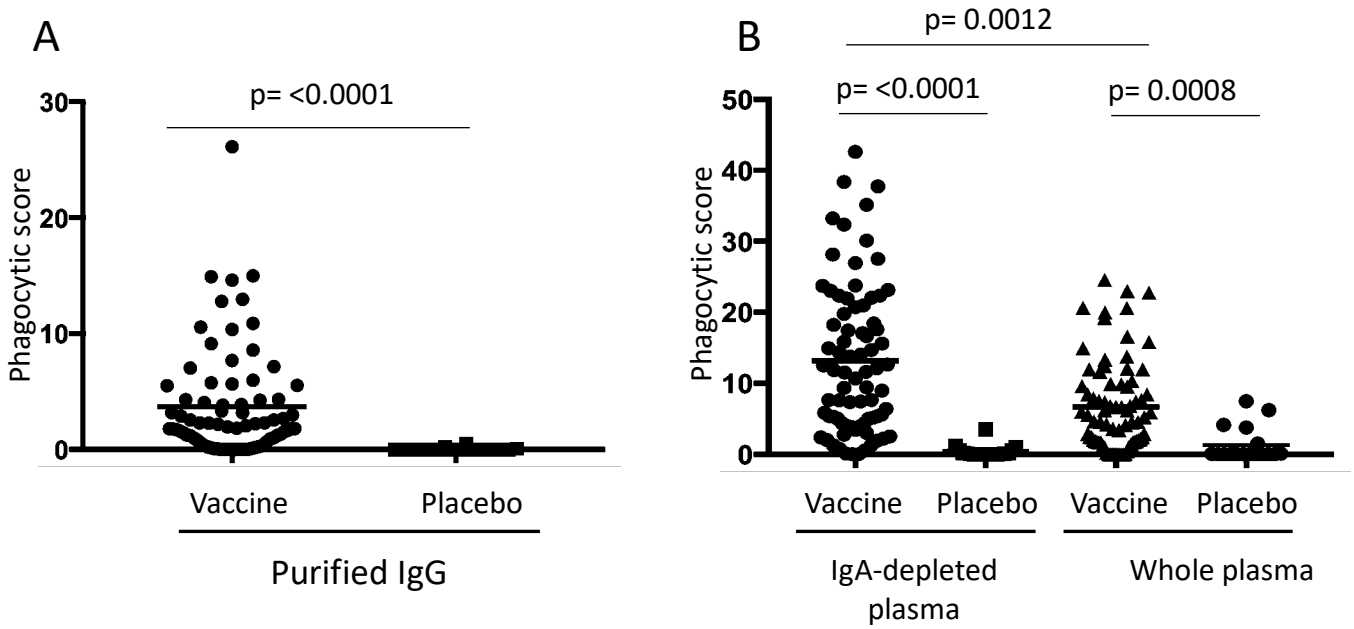

Figure 4.4: **ADNP is enhanced when IgA is depleted from plasma.** Purified IgG from 80 RV144 vaccinees were able to mediate higher ADNP responses compared to 20 placebo subjects p=<0.0001 (**A**). The ADNP responses increased when the IgA was depleted from whole plasma (p=0.0012) from the vaccinees (**B**). The vaccinees induced higher ADNP response with the IgA-depleted plasma p=<0.0001 and the whole plasma p=0.0008 compared to the placebo subjects.

IgA antibody responses to the Env protein in the RV144 vaccine trial has been associated with decreased vaccine efficacy and were shown to block potentially protective IgG antibodies [82, 353]. IgA-depleted plasma and whole plasma were evaluated for ADNP responses to determine if IgA was able to inhibit ADNP responses. The IgA-depleted plasma (median 11.56; range 4.40-42.61 phagocytic score) from the vaccinees mediated higher ADNP responses than the whole plasma (median 4.92; range 1.76-24.57 phagocytic score) samples (Figure 4.4B, p=0.0012). The ADNP responses for the vaccinees with IgA-depleted plasma and whole plasma were higher than their respective placebo recipients (p=<0.0001 and p=0.0008). These results suggest that IgG mediated ADNP responses are inhibited by autologous plasma IgA antibodies.

4.3.5. Neutrophil-mediated RFADCC is not inhibited by IgA

The RV144 vaccine trial showed that ADCC responses inversely correlated with the efficacy in the presence of low IgA levels. NK cells have been shown to mediate the Env-specific ADCC responses with antibodies induced by the RV144 vaccine [82]. However, other immune cells are also able to mediate HIV-specific ADCC responses including neutrophils. RFADCC responses using neutrophils as effector cells were investigated, with 300μg/ml of IgG (from purified IgG, whole plasma and IgA-depleted plasma) from 80 vaccinees and 20 placebo recipients after vaccination and 10 additional samples prior to vaccination. The mean RFADCC response in baseline samples prior to vaccination were subtracted from all samples to allow for comparison between the different sample types. There was a large range (median 0.78% and range of 0-22.51% lysis) of RFADCC responses in RV144 vaccinees purified IgG samples. Twenty-two of the 80 samples had a RFADCC response 2 standard deviations (2.1%) above the mean placebo responses (mean 0.39% lysis) (Figure 4.5A). The IgG from placebo recipients had negligible levels of neutrophil-mediated RFADCC (median 0% lysis, range 0-4.42% p= 0.0002 vs vaccinees).

Post-hoc analyses of samples from the RV144 HIV vaccine trial suggested that monoclonal IgA antibodies isolated from the vaccinees were able to block potentially protective ADCC mediating IgG antibodies. The epitope competition of IgA and IgG is thought to have contributed to the reduced vaccine efficacy [353]. This previous study utilized NK cells which do not express the FcαRI. Therefore, the implications of IgA engagement with FcαR by

utilizing neutrophils as effector cell (that express FcαRI) was investigated. There was no difference in the HIV Env-specific RFADCC response between the vaccinees IgA-depleted plasma (median 1.51%; range 0-8.76% lysis, 54 responders above 2 standard deviation (0.71) above the mean placebo (0.225) and the whole plasma (median 1.98%; range 0-11.93%, 59 responders above 2 standard deviation (0.587) above the mean placebo (0.285) RFADCC IgG mediated responses (Figure 4.5B). The RFADCC responses of the vaccinees for both IgA-depleted plasma and whole plasma were higher than their respective placebo recipients (p=<0.0001 and p=<0.0001). This suggests that blood IgG mediated neutrophil RFADCC responses induced by the RV144 regimen are not significantly inhibited by plasma IgA antibodies.

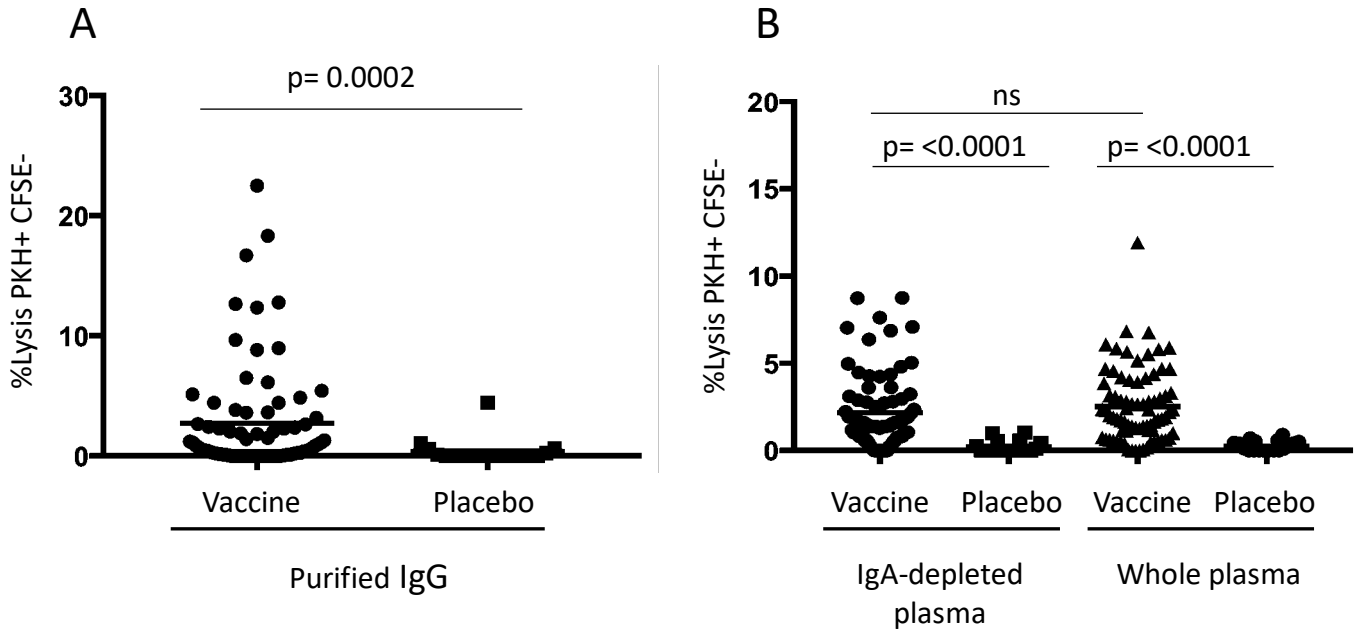

Figure 4.5: **Neutrophil-mediated RFADCC is not inhibited by IgA.** Purified IgG from 80 RV144 vaccinees were able to mediate higher neutrophil RFADCC responses compared to 20 placebo subjects p=0.0002 (**A**). The vaccinees induced higher neutrophil RFADCC response with the IgA-depleted plasma (p=<0.0001) and the whole plasma (p=<0.0001) compared to the placebo subjects (**B**). Similar levels of neutrophil RFADCC responses were observed between the whole plasma and IgA-depleted plasma.

4.3.6. IgA from RV144 vaccinees have higher binding to FcαRI than placebo samples

Total polyclonal IgA present in the RV144 vaccinee plasma was found to inhibit HIV-specific IgG mediated ADNP responses. To begin to investigate the mechanism of IgA inhibition, the binding of purified IgA from the vaccinees and the placebo samples to FcαRI via SPR was determined. Purified IgA was passed across the FcαRI coated sensor chip and the binding was

assessed. The SPR data was kindly supplied by Ms Ester Lopez. Samples from the vaccinees (median 34.99RU; range 16.26-48.8RU) had an enhanced binding to the FcαRI compared to the placebo samples (median 22.27RU; range 11.01-41.3RU) (p=<0.0001, Figure 4.6A). It was hypothesised that the reduced ADNP activity in the plasma compared to the IgA-depleted plasma was a result of IgA binding to FcαRI similar to the results observed in Chapter 3. The IgA binding to FcαRI was compared to the change in ADNP responses that was defined as the IgA-depleted plasma ADNP phagocytic score responses minus the whole plasma ADNP phagocytic score responses from the vaccinees. Binding of IgA to the FcαRI correlated with the change in ADNP responses (r=0.34, P=0.002, Figure 4.6B). This indicates that the inhibition of the ADNP responses may be FcαRI dependent.

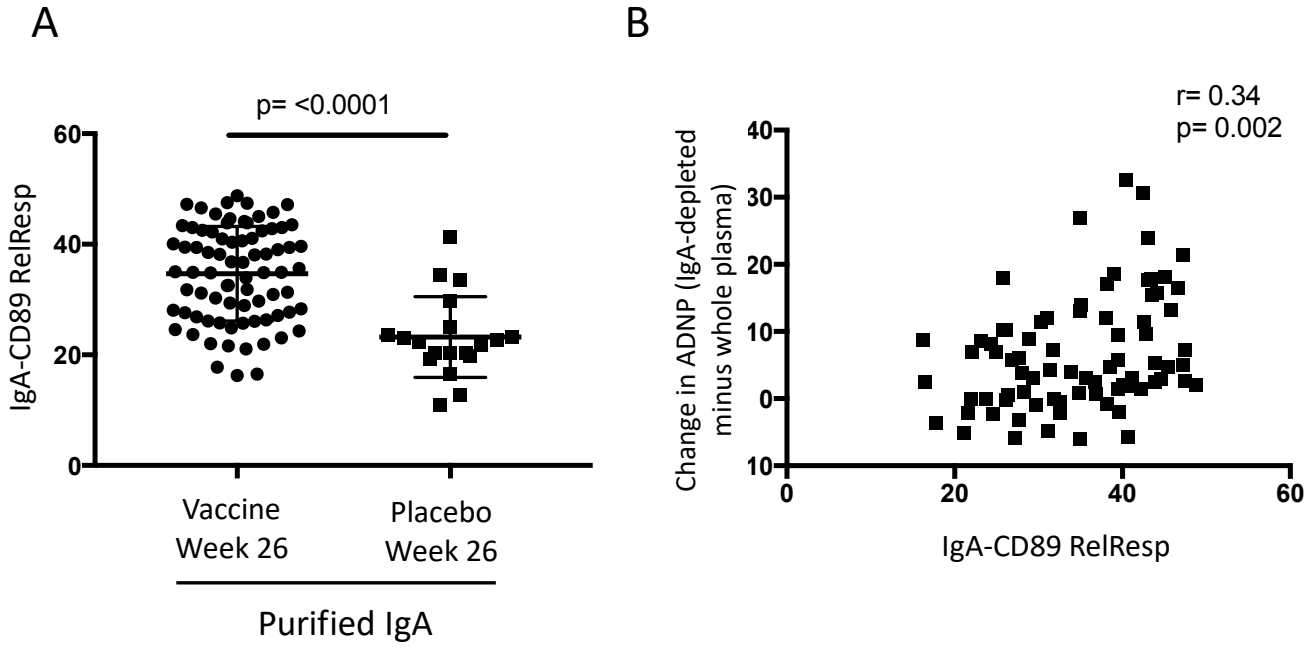

Figure 4.6: **RV144 vaccinee plasma IgA has higher binding to CD89 (FcαRI) than placebo.** (**A**) Purified IgA from RV144 vaccinees were shown to have higher relative binding to the FcαRI than placebo using surface plasmon resonance (p=<0.0001). (**B**) The vaccinees IgA -CD89 RelResp was correlated with the change in ADNP responses from the IgA-depleted plasma to the whole plasma ADNP response (r= 0.34, p= 0.002)

4.3.7. RV144 IgA inhibition of ADNP responses

The above experiments found depletion of IgA enhanced the IgG-mediated HIV-specific ADNP responses in the RV144 vaccinees. To more specifically elucidate the role of IgA on the vaccinees' IgG mediated ADNP responses, purified IgA from the vaccinees was evaluated for their ability to inhibit the RV144 IgG mediated response. To probe the contribution of

HIV-specific vs non-specific IgA, different IgA preparations (including pooled purified IgA from HIV positive donors, pooled purified HIV negative IgA, HIV negative IgA1 and HIV negative IgA2) were evaluated for their ability to inhibit the vaccinees' IgG mediated ADNP responses. Due to the limited antibody availability of the RV144 vaccine samples and variability in the ADNP responses among the vaccinees, a subset of RV144 vaccinees were studied. Thirteen samples from the 36 highest ADNP responders were selected that had sufficient IgG and IgA purified to allow a 1:1 (300µg/ml) ratio of IgG to IgA to be co-cultured with the ADNP assay. Owing to the limited volume of samples, the vaccinees IgA samples were pooled and the assay was completed in triplicate. Initially the IgA antibody pools and preparations were evaluated for ADNP responses alone, including the RV144 pooled purified IgA, pool purified IgA from HIV positive donors, HIV negative IgA1 and HIV negative IgA2 (Figure 4.7A). The pooled IgA from RV144 vaccinees mediated no HIV-specific ADNP responses similarly to all the IgA preparations including the pool purified IgA from HIV positive and pool purified IgA from HIV negative donors. The vaccinees pooled IgG was then mixed at a 1:1 ratio with either autologous IgA or the other different IgA preparations and ADNP studied. The percentage of maximum phagocytosis of the IgG mediated responses was calculated and used to compare the inhibition responses of the IgA. It was found that all preparations of IgA decreased the vaccinees IgG mediated ADNP responses, however, significant inhibition was observed from the pooled purified HIV positive IgA (median inhibition 57.11%; range 61.81-7.96%; p= 0.0198), pooled purified HIV negative IgA (median inhibition 41.39%; range 57.69-30.5%; p= 0.05) and purified HIV negative IgA2 (median inhibition 55.7%; range 70.53-40.14%; p= 0.0021, Figure 4.7B).

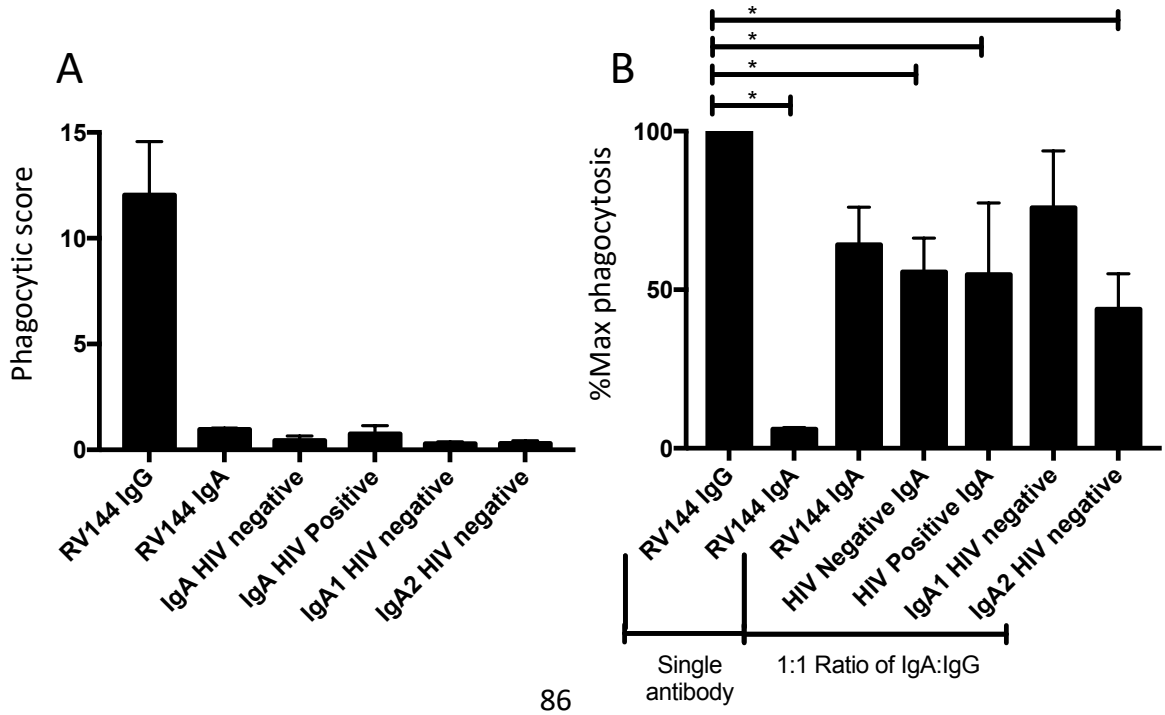

Figure 4.7: IgA inhibits the IgG from RV144 vaccinees from mediating ADNP responses. A cohort of 13 vaccinees IgG were pooled together and IgA evaluated for its ability to inhibit the IgG mediated ADNP responses. (**A**) The RV144 vaccinees pooled IgG was compared to IgA alone to ensure the IgA induced low nonspecific ADNP. (**B**) The RV144 vaccinees pooled IgG in the first column was compared to the RV144 vaccinees pooled IgA in the second column as single antibodies. The RV144 vaccinees pooled IgG was inhibited by pooled purified HIV negative IgA and HIV negative IgA2 but was not significant against the pooled RV144 vaccinees IgA, pooled purified HIV positive IgA or the HIV negative IgA1. The samples were compared with a Kruskal-Wallis test and considered significant with a p-value below 0.05.

4.4. Discussion

There have been six human HIV vaccine efficacy studies conducted, however, to date, only the RV144 trial provided significant, albeit modest, efficacy [319]. High levels of IgA antibodies to the envelope protein were associated with decreased vaccine efficacy [82] and it was later shown that IgA from vaccinees could block potentially protective IgG from binding to the envelope protein [353]. The RV144 vaccine trial highlighted the importance of inducing FcγR mediated effector responses in providing protection. It has also raised questions about the role that IgA may play in HIV vaccination. To date there has been no studies on the role of neutrophils in mediating Fc-dependent responses in the RV144 trial. IgG antibodies from the RV144 vaccines were able to induce both ADNP responses and neutrophil RFADCC responses. Depletion of IgA from plasma resulted in an increase in ADNP responses compared to the whole plasma samples. A similar enhancement of neutrophil RFADCC responses for the IgA-depleted plasma compared to whole plasma was not observed. Using pooled IgG from the vaccinees, it was found that pooled purified matched vaccinee IgA, pooled purified HIV negative IgA, pooled purified IgA from HIV positive donors and HIV negative IgA2, but not HIV negative IgA1, were able to inhibit ADNP responses. This inhibition of vaccine-induced Env-specific IgG mediated responses by the different sources of IgA is consistent with the IgA inhibition observed in HIV infection (Chapter 3) with HIV negative IgA2 providing the greatest inhibition of the IgG mediated responses.

The role that IgA plays in HIV remains controversial with NHP and humanized mouse models indicating that IgA can provide some protection and much of this data are based on mucosal IgA not plasma [386, 387]. IgA isolated from highly HIV exposed persistently seronegative

subjects has been shown to neutralise HIV and inhibit transcytosis across epithelial cells [180, 370]. Mucosal IgA antibodies in breast milk have also been shown to reduce mother to child transmission of HIV-1 [184]. In the RV144 vaccine trial it was shown that monoclonal-IgA antibodies derived from the vaccinees were able to block IgG mediated ADCC responses by NK cells, by epitope competition [353]. Our results indicate that IgA purified from HIV positive individuals can inhibit ADNP responses. In addition, ADNP responses were also inhibited by IgA not specific for HIV. It can be hypothesised, that the HIV negative IgA inhibits the IgG responses by an FcαR dependent mechanism. FcαR when binding monomeric IgA is also known to be able to mediate an inhibitory ITAM (ITAMi) signaling cascade that reduces the effector potential of IgG mediated responses [388]. This is thought to be a regulatory mechanism for FcαRI expressing cells to prevent over activation and resulting inflammation [389]. Neutrophils, macrophages, monocytes and eosinophils are all thought to be regulated by FcαR ITAMi signaling and could potentially be affected by plasma IgA inhibiting other effector mechanisms like degranulation [390-392].

There was only a weak correlation between the FcαR binding with IgA and the change in ADNP responses when comparing the plasma ADNP responses and IgA-depleted plasma responses. There are a number of confounding factors that may influence the comparison between plasma and IgA depleted plasma especially in the correlation of change in ADNP responses and FcαR binding. One being that the change in ANDP responses may also be affected by other factors (like cytokines) present in the plasma that may also be affected by the removal of IgA antibodies. The ratio of IgA1 and IgA2 present in the plasma may also have affected the difference in inhibition between the samples. This is of particular importance as IgA2 is inhibitory to IgG mediated ADNP while IgA1 is not. In addition, a similar occurrence to what is see in chapter 3 where there can be an increase in ADNP responses may be present in some subjects affecting this correlation.

Plasma IgA is mainly composed of monomeric IgA1, while, mucosal IgA is primarily dimeric IgA2 that also contains the secretory component. There are different binding kinetics for FcαR binding to dimeric IgA compared to monomeric IgA, with monomeric IgA disassociating more rapidly [166]. The IgA responses investigated here were all isolated from plasma samples, as the RV144 trial did not collect mucosal samples [319]. Since HIV is primarily transmitted at the genitorectal tract, where the majority of IgA antibodies are dimeric IgA, it can be

hypothesized that the inhibitory responses may not be observed at this site. It would be of interested to investigate the potentially inhibitory role of IgA with neutrophil responses utilizing IgA isolated from mucosal surfaces, especially from key sites of HIV transmission.

The neutrophil RFADCC responses were similar between the IgA-depleted plasma and whole plasma when comparing the same concentration of IgG mediated responses. This is contradictory to what was shown in the ADNP responses with the IgA-depleted plasma inducing elevated responses. The neutrophil RFADCC responses are also contrary to the viremic controller and HIV non-controller responses presented in Chapter 3, which also showed elevated responses in the IgA-depleted plasma compared to the whole plasma IgG mediated responses. It is not clear why there is this difference in regulation of IgA between the neutrophil RFADCC in the vaccinees and the HIV positive plasma. A potential explanation could be that it is the result of differences in HIV-specific IgA antibodies' concentration and subclasses being present relative to the target epitopes. IgA antibody glycosylation patterns may also play a role in the level of inhibition and could be investigated further. In addition, the neutrophil-mediated RFADCC responses, while detectably different from placebo recipients, were very modest. The ADNP assay may present a more sensitive assay for detecting IgA mediated inhibition than the neutrophil RFADCC assay. This could also explain the difference between the inhibition of ADNP with whole plasma, which was not observed when using the neutrophil RFADCC assay with the RV144 vaccinee samples.

The IgG purified from RV144 vaccinees induced both ADNP and neutrophil RFADCC responses, albeit lower than HIV positive plasma samples from Chapter 3. The ADNP IgG mediated responses were inhibited by the plasma IgA. The RV144 vaccinees IgG mediated ADNP responses were also inhibited by pooled purified HIV negative IgA, pooled purified IgA from HIV positive donors and HIV negative IgA2, but not by the HIV negative IgA1. Understanding the role of IgA in plasma and how it contributes or inhibits vaccine efficacy is vitally important to future HIV vaccine trials. There is a need for collection of mucosal samples in future HIV vaccine trials to determine the effects of IgA at the sites of HIV transmission.

Chapter 5

General discussion

5.1. Discussion

5.1.1. General summary of findings

There is increasing interest in Fc-mediated responses and IgA antibodies in HIV infection and vaccine development. Several studies have highlighted the importance of Fc effector functions in delaying HIV disease progression and preventing infection [134, 148-153, 311, 312]. The importance of Fc functional antibodies was also highlighted by the RV144 HIV vaccine trial's modest efficacy which was partly attributed to antibody-dependent cellular cytotoxicity (ADCC) [82, 319, 325, 393]. Despite the rise in interest in Fc-mediated responses in HIV, limited research has been conducted on neutrophils, that are key innate immune cells at both mucosal surfaces and in the blood. Neutrophils are highly functional effector cells with the potential to induce both ADCC and antibody-dependent phagocytosis but their involvement in HIV prevention and disease progression remains understudied. Chapter 2 outlines developed methods to evaluate antibody-dependent neutrophil phagocytosis (ADNP) and neutrophil-mediated ADCC effector responses to HIV and validated these methods with confocal microscopy. The ADNP and neutrophil ADCC responses were found to inversely correlate with the viral load demonstrating the potential importance of these neutrophil-mediated Fc effector functions for control of HIV viremia. This has consequently opened additional avenues of research that will be discussed in greater detail below.

The RV144 vaccine trial has generated interest in the role that HIV-specific IgA can play during HIV vaccination strategies and in HIV infection, with IgA being shown in the trial to be associated with reduced vaccine efficacy [82]. The role that IgA plays in active HIV infection remains controversial, with some reports of HIV-specific IgA being able to inhibit HIV infection and potentially be protective [180, 182, 183]. Chapter 3 investigated if HIV progression was influenced by HIV-specific ADNP and neutrophil ADCC responses and the effects of IgA on these responses. This thesis showed that, although neutrophil ADCC responses were higher in the plasma of subjects who controlled their viremia levels (viremic controllers), IgA from both viremic controllers and viremic non-controllers inhibited both ADNP and neutrophil ADCC responses similarly. The ADNP responses were broadly inhibited by both autologous IgA and HIV negative pooled purified IgA. Importantly, the addition of an FcαR blocking antibody was able to prevent the IgA mediated inhibition of the ADNP responses. This suggests that IgA inhibition of ADNP responses can be mediated by 2

mechanisms that were identified as 1) antigen dependent/FcαR independent, and 2) antigen independent/FcαR dependent.

HIV-specific plasma IgA from the RV144 vaccine trial was shown to block protective IgG mediated antibodies binding to the Env and prevent ADCC responses with NK cells, indicating epitope competition between the antibodies [353]. Chapter 4 investigated the plasma samples from the RV144 vaccine trial for their ability to induce neutrophil-mediated responses and if IgA was able to inhibit these responses. It was found that IgG from the vaccinees was able to induce modest HIV-specific ADNP and neutrophil ADCC responses. IgA from the vaccinees was able to inhibit the ADNP responses but not the neutrophil ADCC responses. Using pooled IgG from the vaccinees, it was shown that pooled purified IgA from vaccinees, pooled purified IgA from HIV positive donors and pooled purified HIV negative IgA were all able to inhibit the ADNP responses. Overall, this thesis shows that neutrophils can mediate antibody-dependent effector responses directed to HIV, and that both HIV-specific IgA and IgA from HIV negative subjects can inhibit ADNP responses.

This final discussion will focus on several research questions that arise from my research and suggest future experimental work to further clarify the role of antibody-dependent neutrophil responses in HIV infection. In particular, this discussion will focus on (i) the role that IgA plays in HIV infection, (ii) mechanisms that neutrophils can use to protect from HIV infection, (iii) the risk of neutrophils increasing the rate of HIV infection, (iv) the differences between neutrophils in the mucosal tissue and circulating neutrophils and (v) the mechanisms of how neutrophils can mediate ADCC responses.

5.1.2. In HIV, is IgA a hero or a villain?

This thesis showed that plasma IgA was able to inhibit IgG mediated responses in an antigen specific and an antigen independent manner. The IgA inhibition of IgG mediated responses was dependent upon FcαR engagement. This was a consistent association both in samples from HIV infected subjects (viremic controllers and non-controllers, Chapter 3), as well as in the setting of vaccination (RV144 samples, Chapter 4). Mucosal IgA has been associated with protection of highly exposed seronegative (HESN) subjects [394]. IgA isolated from the plasma and mucosal surfaces from HESN subjects has been shown to inhibit transcytosis of HIV that assists in preventing infection [369, 371, 395, 396]. IgA from HESN subjects has

been shown to bind to the MPER region of the gp41 protein and can neutralise a broad range of HIV isolates [182, 183, 371]. In addition, HIV neutralising IgA antibodies at the foreskin have been associated with protection from HIV infection [397]. HIV-specific mucosal IgA in breast milk has been associated with reduced mother to child transmission but the mechanism remains unknown [184]. Despite these observations, HIV-specific mucosal IgA has also been reported as absent in a number of HESN studies [398-401].

The discrepancy between the findings, especially regarding the mucosal IgA, may be the result of a number of confounding factors including the varying use of protease inhibitors in collected mucosal fluids, varying assay sensitivities and the use of a jacalin-based IgA isolation. Jacalin resins preferentially bind to IgA1 [402, 403], which would be preferentially degraded in the absence of protease inhibitors. This is important as in the blood 90% of IgA present is of the IgA1 subclass, while in the genitorectal tract ~60-90% is the IgA2 subclass [163, 164]. It is also notable that previous studies in mucosal IgA have largely neglected to define the differences between IgA1 and IgA2 for their ability to mediate protection to HIV. This thesis showed that IgG mediated ADNP responses are inhibited IgA2 but not with IgA1. The inhibition of IgA2 was FcαR dependent and could be blocked by pre-treating neutrophils with the FcαR blocking antibody. This difference in the antibody inhibition may alter neutrophil dependent effector responses based on their exposure to IgA antibodies at mucosal sites versus blood. However, it is important to acknowledge that for the studies in Chapters 3 and 4, high concentrations of IgA2 were used that are not normally observed in human plasma. Future studies are needed to understand the relative contribution of IgA2 and IgA1 at physiological conditions and the mechanisms by which the two IgA subclasses may contribute to FcαR dependent inhibition.

The RV144 vaccine trial showed that IgA can potentially block protective IgG via epitope competition [353]. The IgA antibodies generated were shown to not mediate ADCC responses with NK cells. Wills et al isolated two monoclonal IgA antibodies from the RV144 vaccinees and they were both found to be able to mediate phagocytosis by monocytes [374]. One of the isolated monoclonal IgA antibodies blocked HIV Env binding to galactosylceramide which is an alternative HIV-1 receptor [374]. Recently, a 2F5-IgA monoclonal antibody that is specific to gp41, was shown to mediate FcαRI dependent ADCC responses with monocytes [373]. The 2F5-IgA is also able to cooperate with the 2F5-IgG to enhance monocyte ADCC responses to

kill Env-expressing target cells [373]. This cooperation of 2F5-IgA also exists between the broadly neutralising 10E8-IgG [373]. This is in contrast to the results in this thesis that showed no HIV-specific IgA mediated neutrophil responses and that the HIV-specific IgG mediated responses were inhibited by IgA. This difference may be the result of using monoclonal antibodies as opposed to polyclonal antibodies isolated from HIV positive subjects. Repeating the 2F5-IgA experiments with non-specific IgA may yield results more similar to the results observed in this thesis. It would be of interest to investigate if the 2F5-IgA blocks 2F5-IgG with NK cells similar to the results published in the RV144 vaccine trial. Investigating if this cooperation of IgA and IgG is limited to monocytes or if it is extends to neutrophils may influence future studies in antibody-dependent neutrophil responses.

In contrast to the number of studies that link IgA with protection, IgA has also been associated with a number of undesirable responses with HIV. In two separate studies IgA was demonstrated to modestly enhance HIV replication *in vitro* [185, 186]. This suggests there is an FcαRI-mediated enhancement of HIV replication in the presence of serum IgA but the relevance to mucosal IgA remains uncertain. More recently, plasma IgA was shown to inhibit IgG mediated RFADCC responses with PBMCs from subjects with primary and chronic HIV infection [181]. Moreover, IgA from a small cohort (n=4) of ECs was shown to have no effect on IgG mediated RFADCC with PBMCs [181]. This suggests that IgA is able to inhibit IgG mediated Fc functions but IgA acts differently from different clinical groups. More studies are needed to evaluate the differences between mucosal IgA and plasma IgA for mediating protection and for their ability to neutralise HIV and/or mediate Fc responses. Understanding the dynamic that IgA can play in HIV infection may also provide insights into other infectious diseases, with IgA being shown to inhibit IgG responses in an antigen-dependent manner to both *Candida albicans* and Epstein-Barr virus infection [187, 188].

Another small cohort of ECs (n = 10) were shown to have higher anti-gp41 IgA antibody responses compared to viremic non-controllers [404]. The avidity of the anti-gp41 IgA was also higher in the ECs than the viremic non-controllers. The authors suggest that the IgA from elite controllers have greater affinity maturation than that from the viremic non-controllers [404]. Despite the concentration differences in IgA and IgG in the ECs they demonstrated similar avidity suggesting they could compete for antigens. In HIV+ serodiscordant couples, there were higher ADCC responses compared to HIV positive subjects that were known to

have transmitted HIV [405]. Additionally, HIV+ serodiscordant couples had higher HIV Env-specific IgA antibodies and a higher IgG/IgA ratio of total antibodies. Interestingly, the higher IgG/IgA antibody ratio correlated with ADCC responses in the HIV+ serodiscordant couples' but not in the HIV transmitters'. In this thesis it was shown that viremic controllers and viremic non-controllers increased ADCC responses with the removal of IgA from plasma. Similarly, the HIV+ serodiscordant couples and HIV transmitters ADCC responses increased following the removal of IgA from plasma [405]. This indicates that IgA may be inhibiting ADCC responses and the concentrations of IgA in HIV positive subjects plasma may alter how infectious they are. This needs to be investigated further in a larger clinical cohort and to elucidate potential mechanisms.

In this thesis it was shown that IgA was able to inhibit ADNP and neutrophil ADCC responses. The ADNP responses were also shown to be inhibited by purified IgA from HIV positive subjects and IgA purified from HIV negative subjects. The inhibitory effects of the IgA from HIV negative subjects were abrogated by blocking the FcαR. This indicates that there is an FcαR dependent mechanism through which IgA can inhibit the neutrophil-mediated phagocytosis. It can be hypothesised that the inhibition of neutrophils with plasma IgA from HIV negative donors is a result of monomeric IgA binding to the FcαR and inducing an inhibitory immunoreceptor tyrosine-based activation motif (ITAMi) signalling cascade. There are two proposed mechanisms by which IgA can inhibit IgG mediated phagocytosis; 1) epitope competition which is antigen dependent and FcαR independent and, 2) ITAMi signalling that is antigen independent and FcαR dependent (Figure 5.1). ITAMi signalling is a result of monomeric IgA binding to the FcαR with a failure to cross link the FcαR [392]. This binding induces the partial phosphorylation of the FcR γ-chain of the FcαR/FcR γ-chain complex and downregulates other activating FcRs (Figure 5.2 schematic of the ITAMi signalling) [392]. This inhibitory signalling involves the recruitment of SHP-1 (Src homology region 2 domain-containing phosphatase-1) and the formation of what is commonly termed the 'inhibisome' [391]. The inhibisome clusters impair the phosphorylation of Syk, LAT, and ERK and thereby impair the function of other activating Fc receptors (including FcεRI or FcγRs), TNF receptors, CCR2 and a number of TLRs [406]. On the molecular level recruitment of SHP-1 is known to be involved in the FcγRII inhibitory signalling and inhibition of TCR signalling [406]. If the FcαR ITAMi signalling is being induced by IgA in HIV infection or following vaccination, this is of importance and needs to be investigated further. The FcαR ITAMi signalling is

thought to be a regulatory mechanism to prevent innate immune cells from over activating and causing excessive inflammation and tissue damage [407]. Understanding how the FcαR can induce ITAMi signalling could lead to new therapeutics, which could allow for the innate immune cells to be reactivated. It would also be of interest to know if the FcαR ITAMi signalling is present in tissue residing neutrophils, or if this is exclusively in circulating neutrophils. In addition, since the IgA used in this thesis was primarily monomeric IgA as it was isolated from plasma, it would be of interest to obtain mucosal IgA containing a higher frequency of dimeric IgA and secretory IgA, to evaluate if there is still inhibition of IgG mediated neutrophil responses.

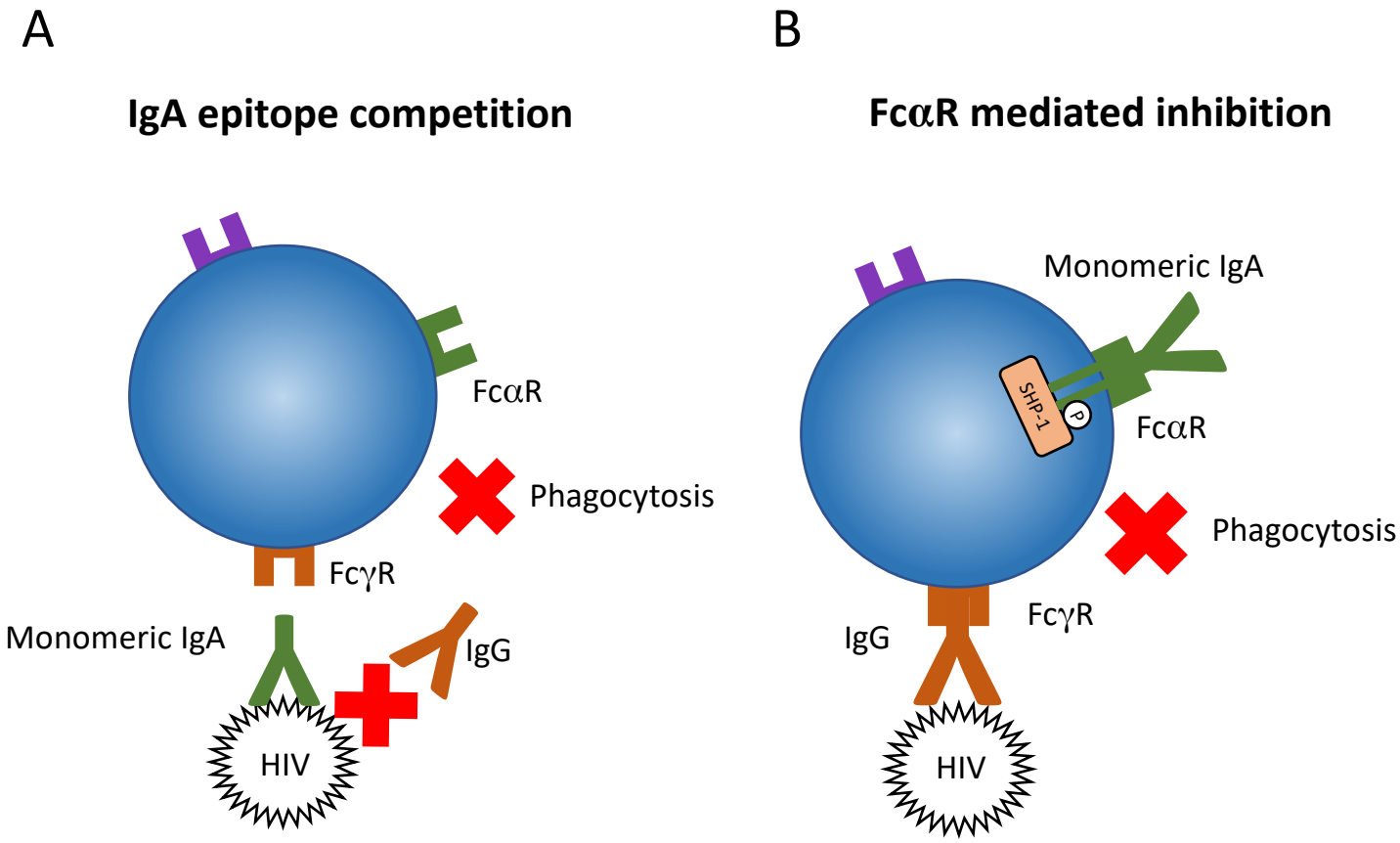

Figure 5.1: Hypothesized mechanisms of IgA mediated inhibition of IgG ADNP responses. A) epitope competition relies on the IgA binding to the HIV virion and blocking IgG from accessing antigens, therefore preventing IgG mediated responses. B) FcαR mediated inhibition by inhibitory ITAM signalling relies on monomeric IgA binding to the FcαR and recruiting SHP-1 to the ITAM region, creating an inhibisome which results in reduced IgG mediated effector responses being induced.

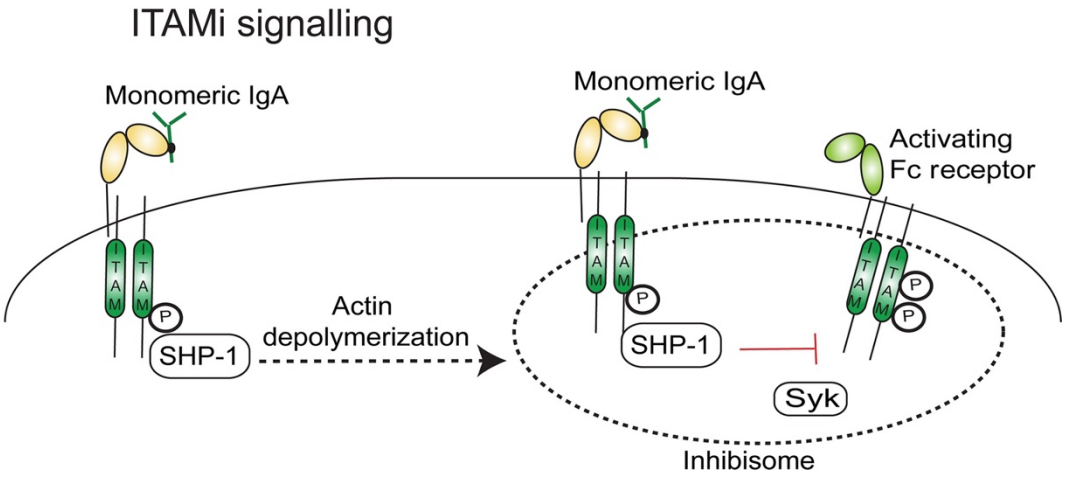

Figure 5.2: Inhibitory ITAM signaling pathway following FcαR binding to monomeric IgA. In the absence of FcαRI crosslinking it leads to partial phosphorylation of ITAMs and the recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP-1) to the FcαR. This forms inhibisome clusters that impair phosphorylation of Syk, LAT and ERK. Adapted from [408].

5.1.3. How do neutrophils protect from HIV infection?

A definitive role for neutrophils in the prevention or treatment of HIV is not clear, however, there is supporting clinical evidence for their potential. There has been a demonstrated association between low circulating peripheral neutrophil counts and the increased risk of HIV infection, which has implicated neutrophils for their potentially protective role [257, 258]. Women at high risk of HIV infection who also have a genetic basis of ethnic neutropenia and circulating neutrophil counts <2500 cells/μl had a ~3-fold increased risk of HIV infection compared to those with higher neutrophil counts [257]. The mechanism for the associated protection has not been evaluated but this may in part be a result of neutrophils directly mediating anti-HIV effector responses.

Neutrophils can mediate a range of effector responses to Fc-receptor signalling including phagocytosis, ADCC, degranulation and neutrophil extracellular trap (NET) formation [409]. Neutrophil degranulation releases granules that contain a range of antimicrobial proteins that can inhibit HIV infection [190, 265]. α-defensins (HNP1-3) are released from the azurophil granules and are able to directly bind and inactivate HIV [269], bind the gp120 blocking viral entry [270, 271] and can block nuclear import and transcription [272]. Neutrophil azurophil granules contain the cathelicidin LL-37 peptide which can inhibit HIV replication [410, 411].

Myeloperoxidase (MPO) and reactive oxygen species (ROS) released from the azurophil granule produce hypochlorous acid, which is in turn able to directly kill HIV and denature viral proteins [274]. Neutrophils can release several other antimicrobial peptides (e.g. azurocidin, cathepsin G, elastase, lactoferrin, neutrophil gelatinase-associated lipocalin) that have been shown to kill other microbes but have not been evaluated in HIV [412].

Neutrophil activation can also trigger a novel anti-microbial mechanism called neutrophil extracellular traps (NETs). NETs involve the release of a chromatin net-like structure that is decorated in a range of antimicrobial peptides including MPO, elastase, HNPs and histones among others. Mature neutrophils are able to release NETs following activation by a number of mechanisms including TLR stimulation, IL-8, platelet-induced activation and from Fc receptor activation [276, 277]. NETs have been shown to induce and promote a number of autoimmune diseases including atherosclerosis [413], rheumatoid arthritis [414] and systemic lupus erythematosus [415], psoriasis [416] and gout [417]. Despite the pathologic potential of NET formation, they have the potential to prevent HIV infection and contribute to controlling HIV disease progression. Saitoh et al showed that neutrophils can recognize HIV-1 by TLR7/8 and this can induce ROS ultimately triggering NET formation [278]. The NET formation was shown to be able to capture HIV and immobilise and irreversibly inactive HIV, preventing infection of CD4 cells [278]. The NET formations were also shown to be inhibited by IL-10 that dendritic cells release after directed exposure to HIV [278]. Neutrophils isolated directly from the female genital tract are able to release more NETs upon exposure to HIV than peripheral circulating neutrophils [279]. This difference in neutrophil NET potential could be investigated further for the impact this could have on HIV transmission at the mucosal surfaces and in blood. HIV Env was also shown to be a trigger for NET formation giving support to neutrophils being able to recognise and kill HIV in the absence of other immune factors [279]. Neither of these studies evaluated the capacity of antibodies to induce NET release to HIV and if these antibodies would enhance or inhibit HIV Env induced NET release. While, IgA antibodies opposed to IgG have been shown to induce NET release in higher frequencies in rheumatoid arthritis [171, 172]. The death of neutrophils upon NET release may also contribute to the neutropenia that occurs during HIV infection. Understanding the role that NETs play in HIV infection may provide insight into how they can be regulated for improved immune outcomes in HIV infected subjects.

The data presented in this thesis (Chapters 3-4), corroborates the work of Ackerman et al who showed that neutrophils can mediate HIV-specific antibody-dependent phagocytosis in the setting of HIV infection [138]. Neutrophil-mediated phagocytosis was one of the polyfunctional effector responses that were shown to be induced by ECs [138]. Neutrophils have also been previously shown to mediate ADCC responses to HIV [132, 289, 290]. Neutrophils have, however, not been evaluated for ADCC responses as part of an EC cohort or subjects on ART. A potential issue in studying ADNP and neutrophil-mediated ADCC responses in human HIV-infected cohorts is that the precise infecting strain and time of infection are rarely known. There has been no work on ADNP or neutrophil-mediated ADCC responses in NHP models, where the strain and timing of SIV or chimeric SHIV infection can be carefully controlled. The role that ADNP and ADCC responses play in predicting progression would benefit from evaluating their potential for mediating protection in NHP models, to better understand their role in providing protection from HIV and how they can be utilised in HIV vaccines.

5.1.4. How do neutrophils mediate ADCC responses?

There has been substantial research linking ADCC responses with the delayed HIV disease progression and viral escape [148-152]. The majority of these studies have utilised NK cells or PBMCs as the effector cells. The ADCC responses in NK cells and monocytes have been extensively studied and the mechanism of target cell death is well established to be mediated by perforin and granzymes [291-294]. Despite this, the mechanisms that neutrophils utilize to mediate ADCC remains contentious and poorly understood. The expression of granzymes and perforin by neutrophils was contentiously illustrated by two groups [418, 419] by intracellular flow cytometry. It was however, previously shown by Sayer et al that neutrophils lacked these proteins, by a range of techniques including intracellular flow cytometry, western blots and ELISA [420]. Grossman et al demonstrated that neutrophils lacked granzymes and perforin utilising improved mAbs and stringent flow cytometry techniques [295]. Metkar et al demonstrated that neutrophils lack granzymes and perforin with a combination of flow cytometry, western blots and a granzyme enzymatic activity assay (Ile-glu-Thr-Asp-pNitroaniline assay) [296]. It has been speculated that the expression of granzyme and perforin detected by Wagner et al and Hochegger et al was a result of failure to adequately block Fc receptors and impure neutrophil populations [295, 296]. In a small study with limited patient samples (n=7), it was shown that human neutrophils from healthy donors and donors with

diverse diseases were shown to lack granzyme and perforin production which recapitulated the results shown in mice [421].

From the above studies, it appears unlikely that neutrophils mediate ADCC via granzyme or by perforin. Neutrophils are known to release ROS intermediates following crosslinking of FcR which have been associated with ADCC responses [422]. However, neutrophils isolated from chronic granulomatous disease patients, which lack reactive oxygen intermediates, are still able to mediate ADCC responses [423]. In a model of cancer, it has been noted that there are ROS dependent and ROS independent mechanisms for neutrophil ADCC responses [424]. This suggests that reactive oxygen intermediates, contribute to, but are not the sole mechanism of neutrophil ADCC responses. Neutrophil ADCC responses may also be mediated by the release of NETs that can kill bystander cells [425], or by the secretion of CD63+ azurphilic granules [426]. Azurphilic granules are known to contain HNP1-3 and LL37 which have both been shown *in vitro* to mediate cell death [427]. This may indicate that neutrophil ADCC responses are a result of many neutrophil derived proteins working in different intracellular pathways to perform ADCC.

A new mechanism of neutrophil-mediated ADCC was recently described in cancer [428]. The process relies on a trogocytosis-induced lysis of tumour cells, which the authors termed trogoptosis. The authors found that neutrophils endocytosed cytoplasmic fragments of target cells in an antibody-dependent manner that induces cancer cell death. This type of ADCC was also shown to release danger-associated molecular patterns (DAMPs) that recruit other immune cells to the site for further clearance of target cells. Trogoptosis was also shown to be enhanced by CD47- SIRPα checkpoint inhibition, suggesting that this mechanism may be able to be modulated to increase ADCC responses [142, 143]. This appears to be a different mechanism than the monocyte mediated trogocytosis observed in HIV which does not appear to directly kill the target cell but reduce viability [143]. . It remains uncertain if neutrophils can mediate either trogoptosis or trogocytosis directed towards HIV.

There is no consensus on the mechanism that neutrophils employ to mediate an ADCC response. It is most likely that neutrophil ADCC responses are comprised of multiple mechanisms that all work to kill the target cell. In the RFADCC assays shown in Chapter 2, it is clear that there is both ADCC and phagocytosis events that occur during the assay [139].

The neutrophil RFADCC assay may serve as a measure of multiple cytotoxic mechanisms. Furthermore, the results of the neutrophil RFADCC assays may be confounded by the different mechanisms which gives scope for this assay to be further developed and simultaneously identify the different mechanisms. Understanding how neutrophils mediate ADCC responses may yield improved methods for measuring the different mechanisms of neutrophil-mediated cytotoxicity.

5.1.5. Origin of neutrophils and how they respond

In HIV infection neutrophils are known to have impaired functionality (including chemotaxis, phagocytosis, bactericidal activity, and oxidative burst abilities) [223-227, 247-249, 378]. This impaired neutrophil functionality is present in untreated subjects and is partially corrected with the instigation of ART [250, 251, 378]. Understanding how neutrophil responses are impaired during HIV infection could lead to improved therapeutics. In this thesis, for practical reasons, healthy donor neutrophils for all studies were utilised. It would be of interest to investigate if neutrophils, from HIV positive subjects that are viremic controllers and viremic non-controllers of HIV, mediated different responses, in particular the effect of IgA on ADNP and neutrophil-ADCC responses. These studies would be technically demanding due to the requirement of freshly supplied blood because of the short life of neutrophils.

The majority of HIV transmissions occur from virus exposure at the mucosal surfaces of the genital or rectal mucosae [53, 54]. Despite this, the majority of studies on neutrophils have focused on the effects of circulating neutrophils, including the experiments in this thesis [132, 138, 354]. Neutrophils isolated from tissues and mucosal surfaces can have altered FcR expression profiles. These differences may alter the Fc-effector potentials of neutrophils at different crucial sites of HIV transmission, including the genital or rectal mucosae. Cervical and colonic neutrophils express high levels of both FcγRIII and FcαRI but only low levels of FcγRI and FcγRII [193]. Interestingly, FcγRII was only expressed on 14% of colon residing neutrophils and in 9% of cervical neutrophils [193]. It has previously been reported that neutrophils constitutively express FcγRII [429]. Neutrophils were also shown to be present in rectal, colonic mucosa and in the upper female genital tract including the endocervix and uterus [193]. Isolated colonic neutrophils outperformed colonic macrophages in phagocytic activity against HIV highlighting their potential in protecting from HIV transmission [193].

There are a number of difficulties in obtaining neutrophils from fresh tissue samples and this is further complicated by neutrophils having a short half-life. Cheeseman et al found there were significantly more neutrophils in the blood compared to penile and cervical tissues [430]. However, viable neutrophils were found infrequently in the mucosal tissues they isolated. This is likely a result of their short half-life, preventing an analysis of Fc receptor expression profiles of their isolated neutrophils [189, 430]. Despite this, neutrophils have been isolated from the ectocervix, endocervix and endometrium and shown to mediate anti-HIV responses [279]. It is vital to know the types of responses that neutrophils can mediate and by which Fc receptors at different sites of HIV transmission, to allow for the evaluation of future HIV vaccines. This may require the investigation of neutrophils in NHP models of HIV infection to establish improved techniques for their evaluation.

5.1.6. Neutrophils could potentially increase HIV acquisition

Neutrophils isolated from the blood of HESN subjects expressed lower levels of pattern-recognition receptors and cytokine mRNAs *ex vivo [431]*. The HESN neutrophils had lower cytokine production compared to neutrophils from HIV positive subjects following TLR stimulation and HIV stimulation [431]. This suggests that reduced neutrophil activation is associated with protection from HIV infection. A caveat of this, is that it is unknown if the neutrophils from HIV positive subjects are more responsive to stimulation than that of uninfected controls. This may complicate comparisons between that of HESN and HIV positive subjects. High levels of LL-37 and HNPs in cervicovaginal secretions have been associated with subsequent HIV infection [432]. Despite this, LL-37 and HNPs have both previously been shown to neutralise HIV, preventing infection [269, 270, 272, 410, 411, 433]. It is unclear if the presence of LL-37 and HNPs in the secretions are directly contributing to the risk of HIV acquisition or are a predicting factor.

Similar to the presence of HNPs in the cervicovaginal lavages, higher levels of HNPs in the foreskin of men has been associated with the acquisition of HIV infection. Higher levels of IL-8 in the penile coronal sulcus have been associated with the risk of HIV transmission [259]. IL-8 is a potent neutrophil chemoattractant that has been shown to recruit neutrophils to the site of infection or tissue damage [434-436]. This is thought to be due to neutrophils being recruited to the site and neutrophils recruiting Th17 and Th1 cells to the foreskin prior to infection [259]. While a number of neutrophil innate peptides have anti-HIV properties, many

act as proinflammatory signalling molecules that can promote epithelial cell remodelling and inflammation which may overshadow their protective capabilities [437, 438].

Neutrophils and Th17 cells participate in reciprocal recruitment through the production of various cytokines and chemokines. This relationship is commonly referred to as the Th17/neutrophil axis and is well studied in a number of bacterial, parasitic and viral infections [439-443]. This relationship may be exposing Th17 cells to greater risk of HIV infection. The presence of IL-8 in cervicovaginal secretions have been associated with later HIV infection, compared to women that remained uninfected in the CAPRISA 004 microbicide trial [444]. The IL-8 observed in the cervicovaginal secretions is thought to have recruited neutrophils to the female genital tract. In a follow up to the CAPRISA 004 trial, the cervicovaginal secretions contained increased levels of neutrophil proteases that correlated with increased inflammatory cytokines (including GM-CSF, IL-1β, MIP-3α, IL-17, and IL-8), an altered cytoskeleton, and increased endocervical CD4+ T cells prior to HIV infection [445]. The correlation of neutrophil proteases and IL-17 suggests there would be an increase of Th17 cells present in the female genital tract. This relationship could represent a role of activated neutrophils in increasing the rate of HIV acquisition as they increase the frequency of target cells. It has been demonstrated previously that Th17 cells are preferentially infected in the female genital tract [446]. Taken together, this may suggest that recruitment of neutrophils for the prevention of HIV may have an unintended effects, that may result in a higher rate of infection and this needs to be investigated thoroughly for the potential of improved vaccine design.

5.1.7. Conclusions

The body of work in this thesis has shown that neutrophils were able to mediate HIV-specific antibody-dependent phagocytosis and neutrophil ADCC responses with IgG from HIV viremic controllers, viremic non-controllers and the RV144 vaccinees. The IgG from HIV viremic controllers and viremic non-controllers mediated ADNP responses. These ADNP responses are able to be inhibited by IgA purified from HIV positive and HIV negative subjects. Similarly, the RV144 IgG mediated ADNP responses are able to be inhibited by IgA from the RV144 vaccinees, HIV positive and HIV negative subjects. There are two mechanisms that IgA can inhibit IgG mediated ADNP responses; 1) antigen dependent/FcαR independent, and 2) antigen independent/FcαR dependent mechanisms. There is a requirement for future work to evaluate if the IgA mediated inhibition is restricted to plasma IgA or if IgA from the mucosal

surfaces are also able to mediate FcαR dependent neutrophil inhibition. The effects of IgA on cell mediated effector responses, including neutrophils, needs to be considered during the development and evaluation of future HIV vaccines. The potentially protective role of neutrophils should be evaluated further for different effector responses and how these responses might affect vaccine efficacy and HIV disease progression. The FcαR is expressed by other innate immune cells that could be inhibited by IgA in a similar mechanism and requires further investigation. It is still an open question if neutrophils can be a correlate for protection in vaccine studies and the impact that neutrophils play in slowing HIV progression.

Overall, it is clear that many challenges remain in the development of an effective prophylactic HIV vaccine. There is great interest in the role of Fc-mediated effector responses in protection against HIV. Further understanding of the role that neutrophils and IgA play in prevention of HIV infection could lead to improved HIV vaccine design and the analysis of correlates of protection.

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Appendix

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Short Communication: Effect of Seminal Plasma on Functions of Monocytes and Granulocytes

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Abstract

Most HIV-1 transmissions occur at mucosae and involve exposure to semen. Semen contains immunomodulatory factors, which inhibit anti-HIV-1 natural killer cell and T cell responses. We demonstrate high concentrations (1:2 dilution) of seminal plasma (SP) inhibit monocyte phagocytosis and anti-HIV-1 Fc-dependent functions of both neutrophils and monocytes. In addition, slightly lower SP concentrations (1:2–1:10 dilutions) inhibit granulocyte phagocytosis and oxidative burst of both monocytes and granulocytes. These observations may have implications for HIV-1 infectivity after mucosal exposure.

Keywords: HIV-1, semen, antibodies, Fc receptors, granulocytes, monocytes

There is interest in utilizing anti-HIV-1 antibody Fc-dependent effector cell functions to protect against HIV-1 infection. The RV144 vaccine trial showed partial protection from HIV-1 infection correlated with non-neutralizing immunoglobulin G (IgG) directed to envelope.[1] Secondary analyses revealed that the ability of anti-HIV-1 IgG to trigger antibody-dependent cellular cytotoxicity (ADCC), an Fc-dependent function, correlated with protection in individuals carrying low levels of anti-HIV-1 immunoglobulin A.[1] Similarly, Fc-dependent functions of anti-viral IgG have been implicated in protection of infants from HIV-1 infection through breastfeeding[2] and protection of macaques immunized with live-attenuated simian immunodeficiency virus.[3] Much research regarding anti-HIV-1 Fc-dependent functions has focused on natural killer cell-mediated ADCC. It is important to note, however, Fc-dependent functions are also mediated by neutrophils and monocytes,[4,5] which might be present at sites of mucosal exposure to HIV-1.

Most new HIV-1 infections are established after sexual exposure at mucosal membranes, often involving semen. Semen is a complex solution containing factors that can drive or inhibit HIV-1 infection.[6] Semen contains proinflammatory components, which can serve to recruit leukocytes, including macrophages, to the female reproductive tract postcoitus.[7] Semen also contains immunosuppressive factors that inhibit immune responses *in vitro*.[8–10] We previously showed T cell and NK cell anti-HIV-1 functions are inhibited by seminal plasma (SP),[9] but have not analyzed the effect of SP on neutrophils and monocytes.

We first tested the effect of SP on phagocytosis of opsonized heat-inactivated fluorescein isothiocyanate-labeled *Escherichia coli* (PHAGOTEST™ kit; Glycotope Biotechnology). Whole blood (50 μL) was mixed with 50 μL of an HIV-1-uninfected SP pool (BioIVT) diluted in RF10 or RF10 alone. *E. coli* (20 μL of 2×10^9/mL) was added and incubated for 10 min at 37°C. Samples were then placed on ice and quenched to prevent detection of surface-bound bacteria. Finally, samples were treated with 500 μL of ACCUTASE (StemCell Technologies) for 10 min to promote detachment before analysis by flow cytometry, using forward and side scatter to identify monocytes and granulocytes. Robust phagocytosis of opsonized bacteria was detected in the absence of SP by both leukocyte populations, with between 83.0%–94.0% monocytes and 68.9%–96.5% granulocytes containing FITC-labeled bacteria (Fig. 1A). SP inhibited phagocytosis activity of both leukocyte populations in a dose-dependent manner. Significant decreases in granulocyte-mediated phagocytosis were noted in the

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presence of 1:2, 1:5, and 1:10 SP dilutions. A significant decrease in monocyte-mediated phagocytosis was only observed in the presence of a 1:2 SP dilution.

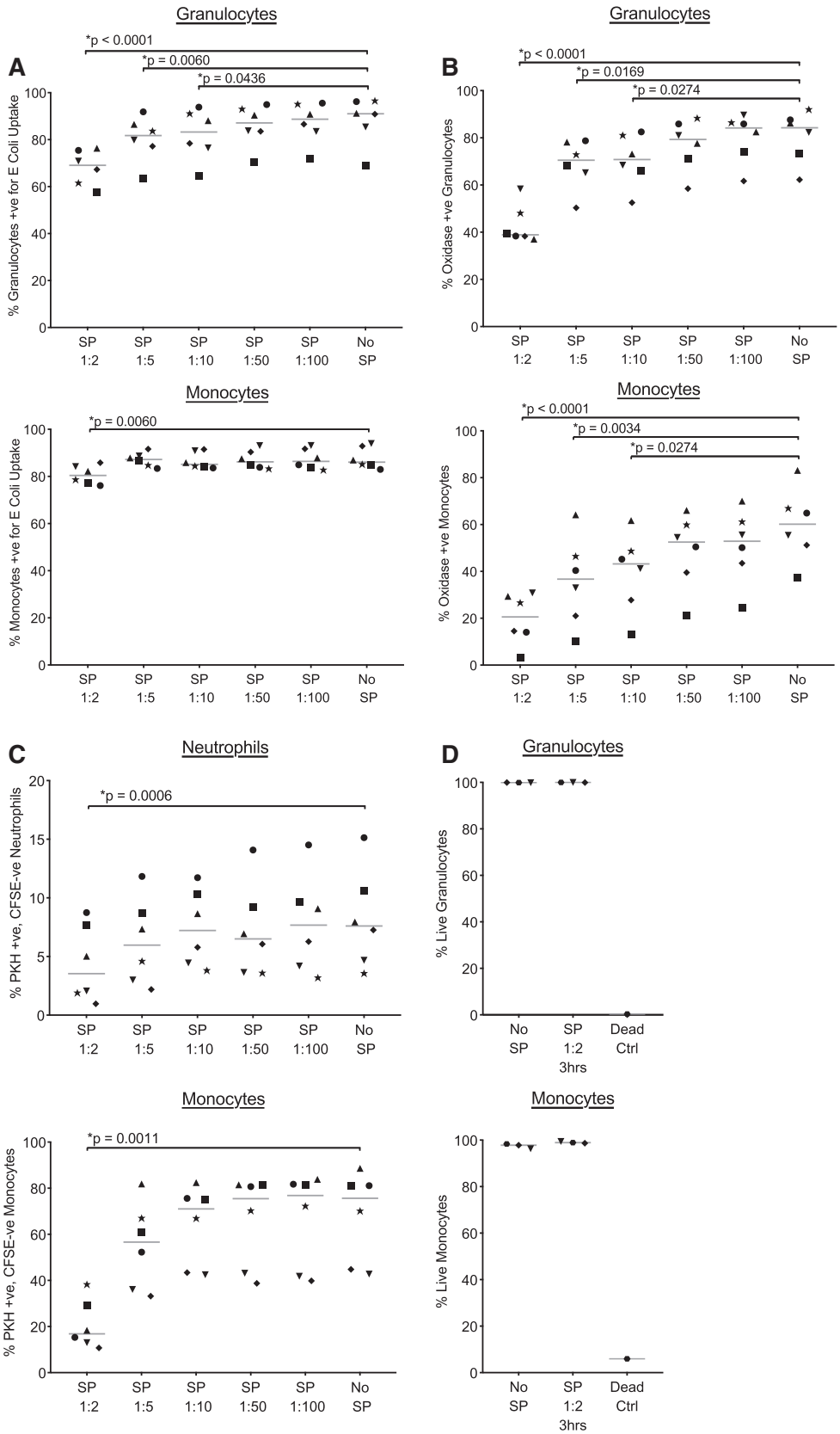
Oxidative burst functions of phagocytes are critical to the killing of engulfed pathogens by granulocytes and monocytes. We next tested the effect of SP on oxidative burst, as measured by conversion of a fluorogenic substrate dihydrorhodamine 123 to rhodamine 123, of granulocytes and monocytes after phagocytosis of *E. coli* (PHAGOBURST™ kit; Glycotope Biotechnology). Samples were prepared as previously, except that after the 10 min incubation at 37°C, 20 μL of substrate was added to tubes, which were then incubated for another 10 min at 37°C. Oxidative burst activity was detected in 37.4%–83.1% monocytes and 62.2%–92.0% granulocytes in the absence of SP (Fig. 1B). The addition of 1:2–1:10 dilutions of SP significantly inhibited oxidative burst activity for both granulocytes and monocytes.

Anti-HIV-1 antibody-mediated functions of phagocytic cells may be important effector mechanisms of HIV-1 prevention,[11] but the effect of SP on these functions has not been studied. The rapid fluorescent ADCC (RFADCC) assay detects anti-HIV-1 antibody-dependent functions of effector cells using a labeled HIV-1 envelope-coated $CD4^+$ T cell line as targets.[4,5] Although envelope-coated target cells do not recapture the trimeric closed conformation of envelope observed on HIV-1-infected cells, antibodies targeting CD4-bound envelope are common in plasma IgG from HIV-1-infected donors.[12] As such, utilization of gp120-coated target cells allows detection of robust anti-HIV-1 antibody-dependent responses using plasma IgG. Peripheral blood mononuclear cells or enriched neutrophils (EasySep™ Direct Human Neutrophil Isolation Kit; StemCell Technologies) were coincubated for 1 h with CFSE and PKH26 labeled HIV-1_{BaL} gp120-coated CEM.NKr-CCR5 cells at a 5:1 effector to target cell ratio. Incubations were conducted in the presence of 100 μg/mL of pooled HIV-1 immunoglobulin (HIVIG), as the source of anti-HIV-1 antibody, and in the presence or absence of SP. The HIV-1 specificity of monocyte and neutrophil RFADCC responses has been previously confirmed.[4,5] Purified neutrophils, or monocytes within PBMC, that took up $PKH26^+$ CEM.NKr-CCR5 membrane were quantified by flow cytometry. SP had a modest negative impact on anti-HIV-1 RFADCC, significantly inhibiting the response at a 1:2 dilution only (Fig. 1C).

High concentrations of SP were employed to assess modulation of leukocyte responses. Importantly, SP is known to have cytotoxic effects after cell culture.[13] To determine if cytotoxicity explained inhibition of leukocytes, we incubated 50 μL of blood from three donors with a 1:2 dilution of SP for 3 h. Next, blood was washed twice with phosphate buffered saline (PBS), to remove serum proteins, and stained with LIVE/DEAD™ far red stain (Invitrogen). Lastly, samples were treated with lysis buffer (BD Biosciences), washed in PBS and analyzed by flow cytometry. As shown in Figure 1D, granulocytes and monocytes within blood treated with SP exhibited similar viability as those not exposed to SP. To confirm the viability dye identified dead cells, blood from one donor was fixed with 1% formaldehyde for 10 min before washing and staining. Cell death of monocytes and granulocytes were detected in this sample (Fig. 1D).

The capacity of SP to influence HIV-1 susceptibility is likely multifactorial, involving both induction of mucosal inflammation and alterations in immune function. We previously showed SP at very low concentrations (i.e., 1:100–1:1,000 dilutions) significantly inhibited NK cell and T cell functions.[9] In this study, we show SP inhibits monocyte phagocytosis and monocyte and neutrophil RFADCC responses, but relatively high concentrations (i.e., 1:2 dilution) of SP are required for this effect. In the absence of SP exposure to underlying innate cells through microtears of the mucosa, this concentration may not be achieved. Interestingly, intermediate concentrations of SP (i.e., 1:5 and 1:10) were sufficient to inhibit granulocyte phagocytosis and monocyte and granulocyte oxidative burst responses. These data are consistent with previous literature demonstrating SP to inhibit monocyte and neutrophil/granulocyte responses.[8,10] The heightened sensitivity of

FIG. 1. Effects of SP on the functions of phagocytes. **(A)** The capacity of granulocytes and monocytes within heparinized whole blood from six donors to ingest FITC-labeled *Escherichia coli* was assessed using the PHAGOTEST™ kit. Experiments were conducted in the absence of SP, or in the presence of a range of SP dilutions (1:2. 1:5, 1:10, 1:50, and 1:100). The graphs depict the percentage of granulocytes (*top*) or monocytes (*bottom*) positive for *E. coli* uptake in each experimental condition. **(B)** The capacity of granulocytes and monocytes within heparinized whole blood from six donors to mediate oxidative burst after *E. coli* exposure was measured using the PHAGOBURST™ kit. Experiments were conducted in the absence of SP, or in the presence of a range of SP dilutions (1:2. 1:5, 1:10, 1:50, and 1:100). The graphs depict the percentage of granulocytes (*top*) or monocytes (*bottom*) positive for oxidative burst in each experimental condition. **(C)** The capacity of enriched neutrophils and monocytes within PBMC from six donors to absorb the $PKH26^+$ membrane of PKH26- and CFSE-labeled HIV-1_{BaL} gp120-coated CEM.NKr-CCR5 target cells, in an anti-HIV-1 antibody-dependent manner, was assessed using the RFADCC assay. Experiments were conducted in the absence of SP, or in the presence of a range of SP dilutions (1:2. 1:5, 1:10, 1:50, and 1:100). The graphs depict the percentage of $PKH26^+$ neutrophils (*top*) or monocytes (*bottom*). **(D)** The effect of SP on granulocyte and monocyte viability was assessed by incubating whole blood alone or in the presence of SP (1:2 dilution) for 3 h before washing blood with phosphate buffered saline and staining with a viability dye. As a control for detecting dead cells, a whole blood sample was fixed with 1% formaldehyde before washing and staining with viability dye. The graphs depict the percentage of live granulocytes (*top*) or monocytes (*bottom*), gated by forward and side scatter, in each experimental condition. For assays depicted in **(A, B, C)**, statistical differences between experimental conditions were compared using the Friedman test. The condition conducted in the absence of SP was compared with each condition containing a dilution of SP using Dunn's multiple comparisons *post hoc* tests. Statistical differences between the conditions are represented by the *p* values at the *top* of each graph. The horizontal lines within graphs refer to the median of each experimental condition. The symbols within each graph refer to individual effector cell donors. ADCC, antibody-dependent cellular cytotoxicity; FITC, fluorescein isothiocyanate; SP, seminal plasma; PBMC, peripheral blood mononuclear cells; RFADCC, rapid fluorescent ADCC.


A
Granulocytes
*p < 0.0001
*p = 0.0060
*p = 0.0436
% Granulocytes +ve for E Coli Uptake
B
Granulocytes
*p < 0.0001
*p = 0.0169
*p = 0.0274
% Oxidase +ve Granulocytes
Monocytes
*p = 0.0060
% Monocytes +ve for E Coli Uptake
Monocytes
*p < 0.0001
*p = 0.0034
*p = 0.0274
% Oxidase +ve Monocytes
SP 1:2
SP 1:5
SP 1:10
SP 1:50
SP 1:100
No SP
C
Neutrophils
*p = 0.0006
% PKH +ve, CFSE-ve Neutrophils
D
Granulocytes
% Live Granulocytes
No SP
SP 1:2 3hrs
Dead Ctrl
Monocytes
*p = 0.0011
% PKH +ve, CFSE-ve Monocytes
Monocytes
% Live Monocytes

the PHAGOBURST response to inhibition by SP led us to speculate that inhibition of oxidative burst could create a situation whereby SP-exposed phagocytic cells engulf but fail to destroy viral particles. This could increase the likelihood of noninfected virus-harboring cells infecting new target cells through infectious synapses. Future research is required to confirm that SP-mediated inhibition of monocyte and granulocyte function occurs under physiologically relevant conditions. Several studies have identified macrophages and neutrophils in cervicovaginal fluid,[14,15] which would represent mucosal immune cells exposed to the highest concentration of SP. The contribution of these cells to immune function, however, is difficult to determine. Once released from tissue and shed into the vaginal lumen, these cells are likely transitory and not long retained in the reproductive tract. In addition to determining the effects of SP on leukocytes within vaginal fluid, the impact of vaginal fluid itself on SP-mediated immunomodulation should be assessed. Furthermore, determining the degree that SP is diluted by vaginal fluid postcoitus will establish the physiological relevance of SP dilutions that suppress leukocyte function *in vitro*. Additional work in tissue explants and animal models should evaluate how immunomodulation by SP interacts with other features of semen, such as the capacity to recruit cells to mucosal tissues[7,16] and the presence of amyloid fibrils reported to enhance HIV-1 infectivity,[13,17,18] to shape susceptibility to HIV-1 infection.

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Author Disclosure Statement

The authors have no conflicts of interest to declare.

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