

HIV-1-SPECIFIC FC-GAMMA-RECEPTOR-MEDIATED EFFECTOR FUNCTIONS: THE ROLE OF HOST FACTORS

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, August 2020

DECLARATION

I, Ntando Ghwenneth Phaahla, declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

18th day of August 2020 in Johannesburg

ABSTRACT

Antibodies recruit potent innate immune effector functions through engagement of their Fc portion with Fc-gamma-receptors (FcγRs) expressed on the surface of effector cells. FcγR-mediated effector functions such as the antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) are mediated by binding antibodies, with immunoglobulin G (IgG) as the most predominant isotype. IgG occurs as four subclasses (IgG1-IgG4) that have distinct affinities for FcγRs and differentially regulate HIV-1-specific FcγR-mediated effector functions. Effector cells further modulate FcγR-mediated effector functions through varied cellular distribution and expression of FcγRs. Although there is a consensus on the importance of Fc-mediated antibody function in protective immunity to HIV-1, less is known about the exact underlying mechanisms of HIV-1 prevention or replication control. This is due to the complex interaction of various host factors and the impact of chronic HIV-1 infection on these mechanisms. Therefore, we sought to characterize FcγR-mediated effector functions in HIV-1 infected Black South Africans (located in the region that is hardest hit by the AIDS epidemic), by assessing the modulatory effects of some host factors on these effector functions in the context of chronic HIV-1 infection and HIV-1 control.

We investigated the cellular distribution of FcγRIIIa on cytotoxic lymphocytes – natural killer cells and CD8⁺ T cells – and the effect of the FcγRIIIa-F158V variant on ADCC capacity in HIV-1-infected individuals (n = 23) and healthy controls (n = 23) using the GranToxiLux ADCC assay (Chapter 2). Study participants were matched for F158V genotypes, carried two copies of the *FCGR3A* gene and were negative for expression of the inhibitory FcγRIIb on NK cells. We found that the distribution of CD56^{dim}FcγRIIIa^{bright} and CD56^{neg}FcγRIIIa^{bright} NK cell subsets, but not FcγRIIIa surface expression, differed significantly between HIV-1 negative and HIV-1 positive donors. Intriguingly, healthy donors bearing at least one 158V allele had higher ADCC responses compared to those homozygous for the 158F allele, whereas the opposite was observed for the HIV-1-infected group, although the difference was not statistically significant. Furthermore, FcγRIIIa⁺CD8^{bright} and FcγRIIIa⁺CD8^{dim} T cell subsets were observed in both HIV-1 negative and HIV-1 positive donors, with median proportions that were significantly higher in HIV-1 positive donors compared to healthy controls. We demonstrate that CD8⁺ T cells acquire innate functions and can mediate HIV-1-specific ADCC through the delivery of granzyme B, which was overall lower compared to that of autologous NK cells.

We next sought to investigate whether hypergammaglobulinaemia affects HIV-1-specific ADCC responses in individuals with different HIV-1 disease progression phenotypes (Chapter 3). Here, HIV-1 gp120-specific ADCC responses were compared and correlated with levels of total IgG and gp120-specific antibody isotypes and subclasses (IgM, IgA [IgA1-2] and IgG [IgG1-4]) in plasma obtained from 22 elite controllers, 35 viraemic controllers and 73 progressors. HIV-1-specific ADCC responses at low plasma dilutions, high concentrations of purified total IgG and high HIV-1-specific IgG were significantly reduced compared to higher dilutions of these antibody sources, thus exhibiting a prozone effect in the presence and in the absence of plasma constituents. Within this prozone window, ADCC responses were significantly higher in elite controllers compared to viraemic controllers and progressors. This is not attributable to higher HIV-1-specific IgG levels in elite controllers, since this group had significantly reduced total plasma IgG levels and gp120-specific IgG, IgG1, IgG2, IgG4, IgA1 and IgA2 compared to viraemic controllers and progressors. Conversely, elite controller ADCC responses at low plasma dilutions were inversely correlated with total plasma IgG levels, which suggests a regulatory effect of hypergammaglobulinaemia on this Fc effector function. In addition, CD4/CD8 ratios were directly correlated to ADCC responses in a subset of 20 controllers, with higher range CD4/CD8 ratio (>1) controllers mediating enhanced ADCC responses compared to those with lower range CD4/CD8 ratios (<1).

The capacity of IgG to mediate HIV-1-specific phagocytosis and the role of antibody levels were subsequently determined for the same controller and progressor cohort (Chapter 4). Using the THP-1 phagocytic gp120-coated bead-uptake method in the presence of low (1 µg/ml) and high (50 µg/ml) concentrations of purified IgG, we observed that the median ADCP activity in elite controllers was significantly reduced compared to progressors at 1 µg/ml IgG, while there was no significant difference at 50 µg/ml IgG. ADCP responses at 1 µg/ml IgG, but not 50 µg/ml IgG, positively correlated with total plasma IgG levels. A modulatory effect of hypergammaglobulinemia may, thus, partly explain the ADCP differences observed between elite controllers and progressors at 1 µg/ml IgG, which is confounded by higher IgG concentrations in the assay used. Envelope gp120-specific IgG1 normalized for bulk plasma IgG was significantly elevated in elite controllers compared to progressors, while normalized gp120-specific IgG2 and IgG4 subclass levels were elevated in progressors compared to controllers. Furthermore, only the normalized gp120-specific IgG1 and IgG4 subclasses significantly positively associated with ADCP responses at 1 µg/ml and 50 µg/ml in the complete HIV-1 infected cohort. Although viral load and CD4⁺ T cell count

did not associate with ADCP responses, CD4/CD8 ratios were inversely correlated to ADCP responses at 1 µg/ml in a subset of 20 controllers, where those with <1 CD4/CD8 ratios mediating higher ADCP responses compared to controllers with >1 CD4/CD8 ratios. Therefore, Chapter 3 and 4 findings collectively suggest that immune hyper-activation that results in elevated antibody levels positively associates with ADCP responses as the disease progresses and yet the same phenomenon associates with a loss of ADCC activity in HIV-1 progressors. A comprehensive understanding of how these two possibly interdependent FcγR-mediated effector functions are modulated during the course of an HIV-1 infection may contribute to the development of an effective HIV-1 vaccine or functional cure.

Overall, this study underscores the need to characterize antibody levels and composition (Chapter 3 and 4), as well as FcγR expression, cellular distribution and functional consequences of FcγR genetic variants (Chapter 2) within a specific environment or disease state.

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TABLE OF CONTENTS

DECLARATION.....	ii
ABSTRACT.....	iii
PUBLICATIONS AND PRESENTATIONS.....	vi
ACKNOWLEDGEMENTS.....	vii
TABLE OF CONTENTS.....	viii
LIST OF FIGURES.....	xiii
LIST OF TABLES.....	xiv
ABBREVIATIONS.....	xv

Chapter 1: Introduction	1
1.1 Human Immunodeficiency Virus.....	2
1.1.1 HIV-1 epidemiology.....	2
1.1.2 HIV-1 structure.....	3
1.1.3 Transmission.....	4
1.1.4 HIV-1 life cycle	5
1.1.5 Clinical course of HIV infection in humans	6
Acute phase.....	6
Chronic phase (asymptomatic)	7
The symptomatic phase	7
1.1.6 Variable disease progression phenotypes	8
HIV-1 controllers.....	8
HIV-1 progressors	9
1.2 HIV-1-specific antibody responses.....	10
1.2.1 Fc γ R-mediated effector functions	12
ADCC	12
ADCP.....	14
1.3 Modulators of Fc-mediated effector functions	15

1.3.1	Antibody isotypes and subclasses.....	15
	IgM	17
	IgA	17
	IgG	19
	IgG1	19
	IgG2	20
	IgG3	21
	IgG4	22
1.3.2	Fc-gamma-Receptors	23
	Fc-FcγR interactions.....	23
	FcγRIIIa as a mediator of ADCC	24
	FcγRIIa as a mediator of ADCP	25
1.3.3	NK cells	26
	NK cell subsets	27
	NK cell responses and HIV-1 infection.....	27
1.3.4	CD8 ⁺ T cells	28
	HIV-1-specific CD8 ⁺ T cell responses	28
	Nonconventional CD8 ⁺ T cells	29
	FcγRIIIa ⁺ CD8 ⁺ T cells.....	29
1.3.5	Phagocytes	30
	Phagocytes in HIV-1 infection	30
1.4	Study Aims and Objectives.....	31
Chapter 2: Chronic HIV-1 Infection Alters the Cellular Distribution of Fc-Gamma-Receptor IIIa and the Functional Consequence of the Fc-Gamma-Receptor IIIa-F158V Variant		
2.1	Introduction.....	34
2.2	Materials and Methods.....	35

2.2.1	Cohort	35
2.2.2	<i>FCGR</i> variant genotyping.....	36
2.2.3	Monoclonal antibodies and flow cytometric analysis	37
2.2.4	Isolation of specific cell populations	37
2.2.5	Antibodies.....	38
2.2.6	CEM.NK ^R .CCR5 as targets	38
2.2.7	ADCC	39
2.2.8	Statistics.....	40
2.3	Results.....	40
2.3.1	Study population.....	40
2.3.2	FcγRIIIa expression on NK cell subsets.....	42
2.3.3	NK cell-mediated ADCC responses	43
2.3.4	Expression of FcγRIIIa on CD8 ⁺ T cells	45
2.3.5	CD8 ⁺ T cells mediate ADCC.....	46
2.4	Discussion	48
Chapter 3: Low Levels of Hypergammaglobulinaemia Correlate with Enhanced HIV-1-Specific ADCC Responses in HIV-1 Elite Controllers		52
3.1	Introduction.....	53
3.2	Methods.....	54
3.2.1	Study participants	54
3.2.2	Peripheral blood mononuclear cells (PBMCs) as effector cells	55
3.2.3	Total IgG purification	55
3.2.4	HIVIG	55
3.2.5	ADCC assay	56
3.2.6	Quantitating total IgG	56
3.2.7	Quantitating HIV-1-specific IgG subclasses	57
3.2.8	Statistical Analysis	58

3.3	Results.....	58
3.3.1	ADCC responses are inhibited at low plasma dilutions and high purified IgG concentrations.....	58
3.3.2	At low plasma dilutions, HIV-1 elite controllers have higher ADCC responses compared to viraemic controllers and chronic progressors.....	59
3.3.3	Elite controllers have low antibody levels that inversely correlate with ADCC responses	61
3.3.4	Strong negative correlations between ADCC at the lowest plasma dilution and HIV-1 gp120-specific total IgG and IgG1 in elite controllers	62
3.3.5	Reduced gp120-specific serum IgA subclass levels in elite controllers that negatively correlate with ADCC responses	65
3.3.6	Significant negative correlation between gp120-specific IgM levels and ADCC responses in Elite controllers.....	66
3.3.7	CD4/CD8 ratios directly associated with ADCC responses at the low plasma dilution	67
3.4	Discussion	70
Chapter 4: Progressive HIV-1 Infection and Hypergammaglobulinaemia Associate with Enhanced Env-specific Phagocytic Responses.....		75
4.1	Introduction.....	76
4.2	Materials and Methods.....	77
4.2.1	Study cohort.....	77
4.2.2	Antibodies.....	77
4.2.3	HIV-1 gp120.....	77
4.2.4	ADCP.....	78
4.2.5	HIV-specific IgG subclass detection	78
4.2.6	Statistics.....	79
4.3	Results.....	79
4.3.1	IgG concentration modulates the association between ADCP and natural control of HIV-1 infection	79

4.3.2	Phagocytic responses directly correlate with hypergammaglobulinaemia.....	80
4.3.3	Plasma levels of HIV-1 gp120-specific IgG1 and IgG4 directly correlate with phagocytic capacity	81
4.3.4	CD4/CD8 ratios inversely correlated with ADCP responses at the low IgG concentration in a subset of controllers.....	84
4.4	Discussion.....	85
Chapter 5:	Summarizing Discussions and Conclusions	89
Chapter 6:	References	99
Chapter 7:	Appendices	131
Appendix 1:	Supplementary figures.....	132
Appendix 2:	Plagiarism Declaration.....	137
Appendix 3:	Ethical clearance.....	138
Appendix 4:	Thesis title change.....	139

LIST OF FIGURES

Figure 1.1. HIV-1 genome and virion structure.	4
Figure 1.2. HIV-1 life cycle.....	6
Figure 1.3. Typical clinical course of an HIV-1 infection.....	8
Figure 1.4. Variable HIV-1 disease progression phenotypes.	10
Figure 1.5. HIV-1-specific antibodies that arise in early HIV-1 infection.....	11
Figure 1.6. FcγR-mediated effector functions.	13
Figure 1.7. Antibody isotypes and subclasses.	16
Figure 1.8. Human Fcγ receptors (FcγRs).	24
Figure 2.1. NK cell mediated ADCC responses at different effector-to-target (E:T) cell ratios.	40
Figure 2.2. Study participant selection.	42
Figure 2.3. FcγRIIIa expression on NK cell subsets in HIV-1 uninfected and HIV-1 infected individuals matched for <i>FCGR</i> genetic variants.....	44
Figure 2.4. FcγRIIIa expression on CD8 ⁺ T cells in whole blood obtained from HIV-1 negative and HIV-1 positive donors.	45
Figure 2.5. FcγRIIIa expression on CD8 ⁺ T cells in HIV-1 uninfected and HIV-1 infected individuals matched for <i>FCGR</i> genetic variants.....	47
Figure 3.1. Low plasma dilutions and high antibody concentrations inhibit ADCC responses.	59
Figure 3.2. Higher ADCC responses in HIV-1 elite controllers compared to viraemic controllers and progressors at the low plasma dilution.	60
Figure 3.3. Total plasma IgG levels in controllers compared to progressors and correlated to ADCC responses.....	62
Figure 3.4. Association between ADCC responses (1:50 plasma dilution) and HIV-1-specific antibodies.....	64
Figure 3.5. IgA subclass levels, IgG/IgA ratios and associations between ADCC responses at the lowest plasma dilution.	66

Figure 3.6. ADCC responses at low plasma dilutions and correlation with gp120-specific IgM levels.....	67
Figure 3.7. Correlation of ADCC at the low plasma dilution (1:50) with markers of disease progression.....	69
Figure 4.1. Comparison of ADCP responses at low and high IgG concentrations.	80
Figure 4.2. Phagocytic responses and antibody levels.	81
Figure 4.3. HIV-1 gp120-specific IgG and IgG1-IgG4 subclass levels normalized for total plasma IgG and correlated with phagocytic scores.	83
Figure 4.4. Correlation of ADCP at the low IgG concentration with markers of disease progression.....	85

SUPPLEMENTARY FIGURES

Figure S1. ADCC mediated by purified NK cells and CD8 T cells.....	132
Figure S2. Comparison of granzyme B activity by antibodies from HIV-1 infected and uninfected individuals.....	133
Figure S3. Correlation of ADCC responses at 1:50 dilution with markers of disease progression	134
Figure S4. Antibody-dependent cellular phagocytosis gating strategy and comparison of activity	135
Figure S5. Correlation of ADCP responses with markers of disease progression.....	136

LIST OF TABLES

Table 2.1. Clinical and demographic characteristics of study cohort.....	36
Table 3.1 Clinical and demographic characteristics of HIV-1 disease phenotype groups.	54

ABBREVIATIONS

ADCCDC	antibody-dependent complement-dependent cytotoxicity
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
ADCVI	antibody-dependent cellular virus inhibition
ADE	antibody-dependent enhancement
AIDS	acquired immunodeficiency syndrome
ART	anti-retroviral therapy
BAMA	binding antibody multiplex assay
BCA	bicinchoninic acid
BCR	B cell receptor
CA	capsid
CCR5	C-C chemokine receptor type 5
CD	cluster of differentiation
CD4BS	CD4-binding site
CH	constant heavy
CNR	copy number variable region
CNR1	gene copy number variable region 1
CNV	copy number variability
ConC	Consensus C
CRF	circulating recombinant forms
CXCR4	C-X-C Motif Chemokine Receptor 4
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC	elite controller
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Env	Envelope
E:T	effector:target
Fab	antigen binding fragment
Fc	crystallisable fragment
<i>FCGR</i>	Fc gamma receptor gene
FcR	Fc receptor
FcRn	neonatal FcR
FCS	foetal calf serum
Fc α Rs	Fc-alpha receptors
Fc γ R	Fc gamma receptor
FSC	forward scatter
FMO	fluorescence minus one
GPI	glycosylphosphatidylinositol
GTL	GranToxiLux
GzB	granzyme B
HAART	highly active antiretroviral treatment
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HIVIG	HIV immune globulin
HLA	human leukocyte antigen
HRP	horse reddish peroxide
HVL LTNP	high-viral-load long-term non-progressor
Ig	immunoglobulin
IgG	immunoglobulin G
IN	integrase
IQR	interquartile range
ITAM	immunoreceptor tyrosine-based activation motif

ITIM	immunoreceptor tyrosine-based inhibitory motif
Kb	kilobases
kDa	kilodaltons
LTNP	long-term non-progressor
MA	matrix
MACS	magnet-activated cell separation
MHC	major histocompatibility complex
MLPA	multiplex ligation-dependent probe amplification
MPER	membrane-proximal external region
MTCT	mother-to-child transmission
NC	nucleocapsid
NHP	non-human primate
NK	natural killer cells
nm	nanometre
NPC	nuclear pore complex
ORF	open reading frame
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed death-1
PIC	pre-integration complex
PR	protease
r	Spearman and Pearson correlation coefficient
Rev	regulator of virion
RNA	ribonucleic acid
RT	reverse transcriptase
RTRNA	reverse transcriptase ribonucleic acid
SD	standard deviation
SIV	simian immunodeficiency virus
SIVcpz	simian immunodeficiency virus of Central African Chimpanzee
SIVsm	simian immunodeficiency virus of West African sooty mangabeys
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SSC	side scatter
Tat	trans-activator of transcription
TCR	T cell receptor
TE	Tris/ethylenediaminetetraacetic acid
TMB	tetramethylbenzidine
URF	unique recombinant forms
VC	viraemic controller
VE	vaccine efficacy
VH	variable domain
Vif	Viral infectivity factor
VL	viral load
Vpr	viral protein r
Vpu	viral protein u

Chapter 1: Introduction

1.1 Human Immunodeficiency Virus

1.1.1 HIV-1 epidemiology

HIV is an enveloped, positive-sense single stranded RNA virus that is grouped under the Lentivirus genus, within the Orthoretrovirinae subfamily and Retroviridae family. The virus is differentiated into HIV type 1 (HIV-1) and type 2 (HIV-2) based on genetic characteristics and unique viral antigens. Available data suggest that HIV-1 evolved from simian immunodeficiency virus (SIV) of Central African Chimpanzee (SIVcpz) and HIV-2 from SIV of West African sooty mangabeys (SIVsm) (Gao et al., 1999, Sharp and Hahn, 2011, Faria et al., 2014). Although the pandemic history of HIV-1 remains unclear, phylogenetic and epidemiologic data trace the first introduction of the virus into the human population between 1920 and 1940 from central Africa in Kinshasa, the capital city of the Democratic Republic of Congo (Korber et al., 2000, Salemi et al., 2001, Worobey et al., 2008, Rodgers et al., 2017).

HIV-1 is the predominant type circulating worldwide and has a highly complex molecular epidemiology. The virus type is divided into four groups known as major (M), outlier (O), non-major/ non-outlier (N) and putative (P), with O, P and N groups primarily circulating in a small fraction of Cameroonian and Gabonese HIV-1 infected individuals (Plantier et al., 2009, Vallari et al., 2010, Vallari et al., 2011). The most predominant M group comprises 9 subtypes: A, B, C, D, F, G, H, J and K. Subtypes A, B, and C are the most abundant, with subtype C accounting for half of all circulating HIV-1 strains in the world. Subtype C is predominant in southern Africa, the horn of Africa and some Asian countries. Further HIV-1 diversity occurs through sub-subtype divisions such as A1, A2, A3, and A4, as well as recombination events between different subtypes in co-infected individuals giving rise to unique recombinant forms and circulating recombinant forms (URFs and CRFs, respectively) (Foley et al., 2018). This high HIV-1 variation has contributed to the complexity of developing effective cure and treatment strategies.

Current HIV treatment and prevention programmes have shown an overall positive trajectory in the battle against the virus (UNAIDS and Statistics, 2019). Since 2010, AIDS-related deaths have dropped by an estimated 33% and new infections by 16% (UNAIDS and Statistics, 2019). By the end of 2018, approximately 76% of all individuals living with HIV were aware of their status, nearly 78% of people with an HIV diagnosis were on anti-retroviral therapy (ART), and 86% of these individuals were virally suppressed. Despite these successes, there are factors that reinforce the HIV epidemic in developing countries such as shortage of health-care workers, lack of resources, and HIV-associated stigma and

discrimination (UNAIDS, 2017). These factors are threatening to slow down the global objective to end the acquired immunodeficiency syndrome (AIDS) epidemic by 2030, starting with the 90-90-90 targets that aim to have 90% of all infected individuals knowing their status; 90% of these individuals put on treatment; and 90% being virally suppressed, and all three aims being achieved by the year 2020 (HIV/AIDS, 2014).

Developing countries within sub-Saharan Africa have the greatest HIV-1-related morbidity and mortality and the highest incidence in young adults, especially young women. Southern and eastern Africa, in particular, account for 53% of all individuals living with HIV-1 in 2017, with South Africa contributing 33% of new infections in this region (UNAIDS, 2017). Despite the wide distribution and accessibility of ART in South Africa, treatment associates with negative factors including chronic inflammation, drug resistance and the requirement of daily medication. Thus, there remains a need to develop new approaches to treat and prevent HIV-1 infection.

1.1.2 HIV-1 structure

HIV-1 is an enveloped virus with a cylindrical capsid that ranges from 100 nm to 150 nm in diameter. The genome of the provirus is approximately 9.8 kb in length (Muesing et al., 1985). Each virion consists of two positive-sense single-stranded RNA copies that code for 15 proteins, which are divided into three groups (Fig 1.1). The first group comprises structural proteins that include an assembled trimer of heterodimers of gp120 and gp41 subunits that form the envelope glycoprotein (Env) and the Gag glycoprotein containing the capsid (CA, p24), matrix (MA, p17) and the nucleocapsid (NC) of the virus (Levy, 2007, Sauter et al., 2012). The second group comprises viral enzymes that include reverse transcriptase (RT, p51), integrase (IN, p32), RNase H (p15) or RT plus RNase H (together p66), and protease (PR). The structural proteins and enzymes are synthesized as polyproteins encoded by three genes (*gag*, *pol* and *env*) (Fig 1.1A) (Levy, 2007, Sauter et al., 2012). The *gag* gene encodes proteins of the outer core membrane, the *pol* gene encodes viral enzymes, and the *env* gene encodes envelope associated proteins (reviewed in (German-Advisory-Committee-Blood, 2016)). The third group of proteins comprises accessory proteins: trans-activator of transcription (Tat), negative regulatory factor (Nef), viral infectivity factor (Vif), regulator of virion (Rev), viral protein r (Vpr), viral protein u (Vpu), and P6. Tat and Rev are important for initiating viral replication, while Nef, Vif, Vpr and Vpu are necessary for viral replication, virus budding and pathogenesis (Levy, 2007, Sauter et al., 2012).

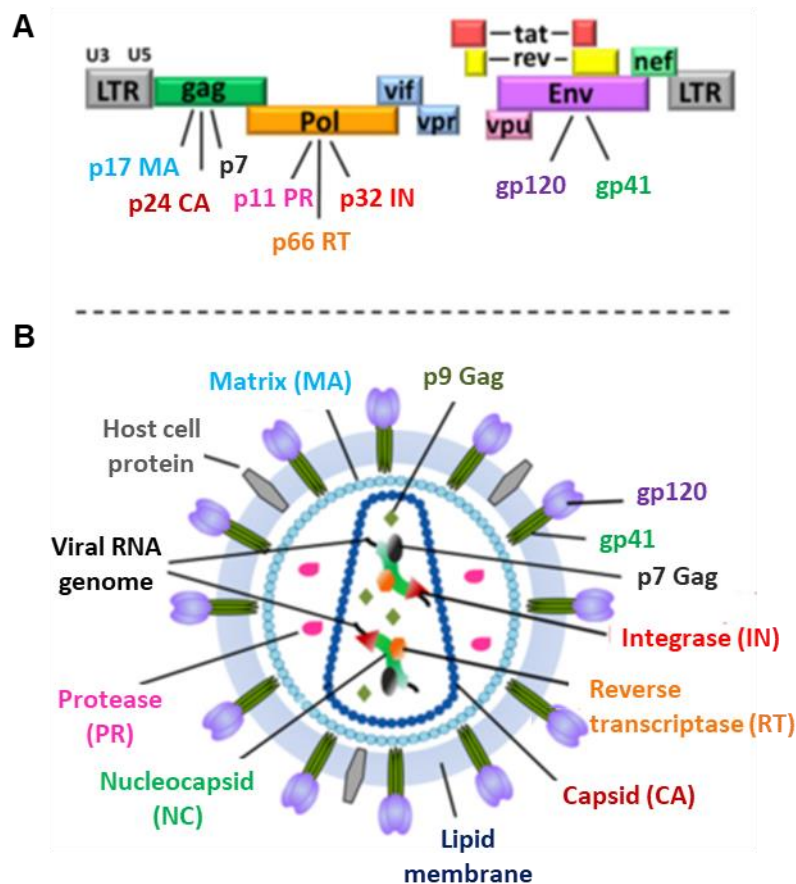


Figure 1.1. HIV-1 genome and virion structure.

A) Illustration of genes within the HIV-1 genome. Structural components of the virion including the glycoprotein 120 (gp120) and gp41 proteins, capsid (CA), matrix (MA), and nucleocapsid (NC) are encoded by *gag* and *env* genes. The *pol* gene encodes three viral enzymes, reverse transcriptase (RT), protease (PR), and integrase (IN). **B)** Illustration of the HIV-1 virion and viral proteins. Reproduced from (Musumeci et al., 2015).

1.1.3 Transmission

Most HIV-1 infections occur through sexual exposure where the virus penetrates the genital or rectal mucosa. Heterosexual transmissions account for 70% of HIV-1 infections globally; the remaining 30% is shared between men who have sex with men, mother-to-child transmission, blood transfusions and injecting drug-use (Shaw and Hunter, 2012). Although the exact mechanism by which the virus crosses the genital mucosal epithelium is not clear, it has been postulated to involve direct contact with dendrites of intraepithelial dendritic cells or transcytosis (Cunningham et al., 2013, Gupta et al., 2013). Under normal conditions, crossing of the genital mucosal epithelium is rare; however, the process may be aided by genital inflammation or damaged genital mucosa through physical trauma or sexually transmitted infections (Galvin and Cohen, 2004, Haaland et al., 2009, McKinnon and Kaul, 2012, Kaul et al., 2015, Masson et al., 2015, Kriek et al., 2016, Passmore et al., 2016). Other important

contributing factors to risk of virus transmission include the stage of HIV-1 disease, route of exposure, and male circumcision (Powers et al., 2008, Boily et al., 2009, Baeten et al., 2011).

1.1.4 HIV-1 life cycle

The HIV-1 life cycle begins when the cell-free virus binds to target cell receptors via its trimeric Env protein spikes on the surface of the virion. Recognition sites for the primary receptor, CD4, and co-receptors, C-C chemokine receptor type 5 (CCR5) and C-X-C Chemokine Receptor 4 (CXCR4), are found on the gp120 glycoprotein (Kwong et al., 1998). Through gp41 mediation, viral contents enter the host cell cytoplasm (Wilén et al., 2012, West et al., 2014, Harrison, 2015). Discharge of the viral contents into the cytoplasm is followed by reverse transcription of the viral RNA genome into DNA, which is mediated by the viral reverse transcriptase (Fig 1.2). A pre-integration complex is generated, which consists of the newly formed viral DNA, viral integrase, capsid proteins, and other cellular proteins (Lusic and Siliciano, 2017). The pre-integration complex is transported to the nucleus where it enters through the nuclear pore complex in non-dividing cells. Through the activity of viral integrase, viral DNA is integrated into the host cellular DNA (Lusic and Siliciano, 2017). The preferred location for the process of viral integration is one with a high gene density and transcriptional activity, although it can occur at different locations in the host cell genome (Schroder et al., 2002, Han et al., 2004, Mitchell et al., 2004, Barr et al., 2006, Lewinski et al., 2006, Sherrill-Mix et al., 2013). The integrated viral DNA, also known as a provirus, can be transcriptionally silenced in resting CD4⁺ T cells, which may lead to the formation of a latent viral reservoir that is a major obstacle in eradication of HIV-1. However, when infected CD4⁺ T cells are activated, the provirus becomes a template for transcription and translation of viral proteins such as Env, Gag and Pol polyproteins which are transported to the plasma membrane. At this site, budding of progeny virus from cells occurs and immature virions are released and their maturation is carried out by virion-encoded protease through proteolysis (Lusic and Siliciano, 2017).

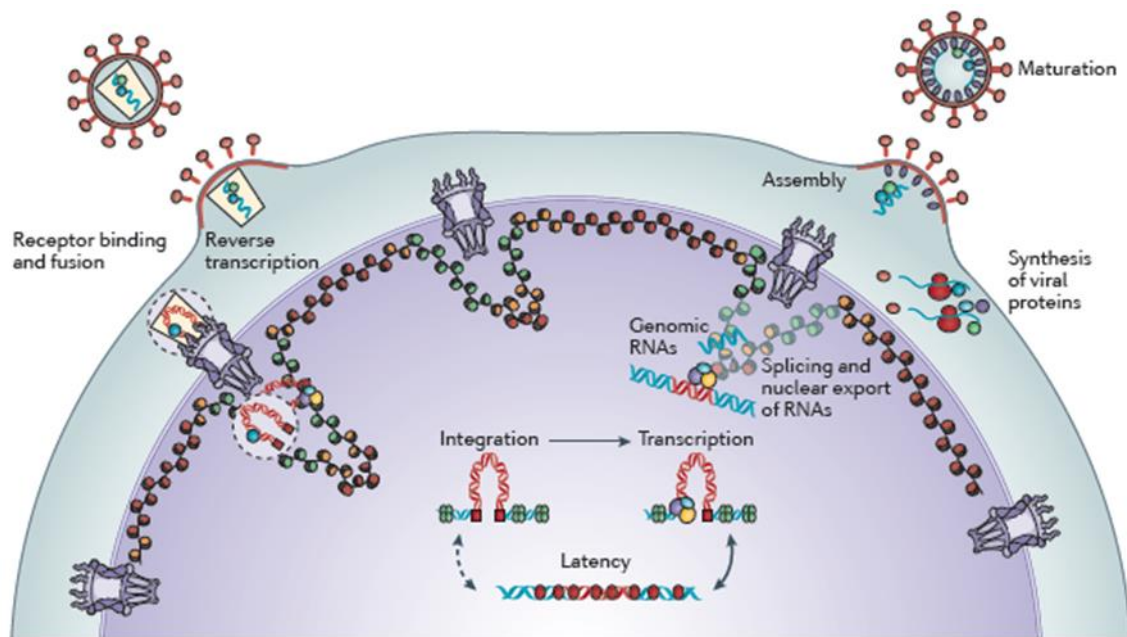


Figure 1.2. HIV-1 life cycle.

Following the fusion of HIV-1 virion with plasma membrane receptors, reverse transcription of viral genomic RNA into DNA occurs. Subsequently, the viral capsid disassembles, viral DNA enters into the nucleus where it integrates into cellular chromatin. In productive infection, transcribed viral RNA is spliced and nuclear exported, and new virus particles are produced as they assemble for budding from the plasma membrane. Mature virus particles become infectious. Alternatively, proviral latency may result from silencing of integrated viral DNA through mechanisms that allow silent viral reservoirs to form. Illustration adapted from (Lusic and Siliciano, 2017).

1.1.5 Clinical course of HIV infection in humans

The clinical course of an HIV-1 infection progresses through three phases: the acute, chronic (asymptomatic) and symptomatic phases (Fig 1.3).

Acute phase

The acute phase comprises six stages, known as Fiebig stages I-VI of acute viraemia and seroconversion characterised by different viral and host immune markers (Fiebig et al., 2003). Stage I: HIV RNA positive, Stage II: HIV RNA and p24-antigen positive, Stage III: antibody positive/Western blot negative, Stage IV: antibody positive/Western blot indeterminate, Stage V: Western blot positive but missing p31, and lastly, stage VI: Western blot positive and including p31 (Fiebig et al., 2003). Following their sharp decline in the acute phase, CD4⁺ T cells return to near normal levels as the acute phase ends and the chronic phase begins (Haase, 1999).

Chronic phase (asymptomatic)

Following acute infection, HIV-1-specific adaptive immune responses establish a viral set point, that is lower than the peak acute viraemia approximately 12 weeks post infection and may remain steady for 5 to 15 years. During the chronic phase, primarily cytotoxic CD8⁺ T cells and the activity of antibodies control viral replication (Koup et al., 1994, Goonetilleke et al., 2009). Autologous neutralizing antibodies emerge 12 weeks post-infection in most HIV-1 infected individuals, whilst the more potent broadly neutralizing antibodies usually arise between 1 and 3 years post-infection in few HIV-1 infected individuals (Moore et al., 2008, Moore et al., 2012). Despite the presence of these and other antibodies, a slow decline in CD4⁺ T cells continues over the years, in association with exhaustion of cytotoxic CD8⁺ T cell responses, leading to overall inability of the adaptive immune response to maintain the steady viral load set point (Catalfamo et al., 2011, Hoffmann et al., 2016).

The symptomatic phase

This phase is mainly characterised by an increase of plasma viraemia and decline of CD4⁺ T cell count due to exhausted and significantly reduced HIV-1-specific T cell responses. It triggers the appearance of opportunistic infections and eventual collapse of the host immune system, as evidenced by the development of AIDS-related symptoms that is followed by death in the absence of ART (An and Winkler, 2010). The rate of disease progression in the absence of ART varies between individuals and is influenced by various factors such as pre-existing clinical conditions, host genetics, environmental conditions (e.g. malnutrition and absence of treatment) and virus virulence. The advent of ART has significantly altered the clinical course of HIV-1 infection, and as a result, the disease has become a manageable chronic disease for many infected individuals across the globe.

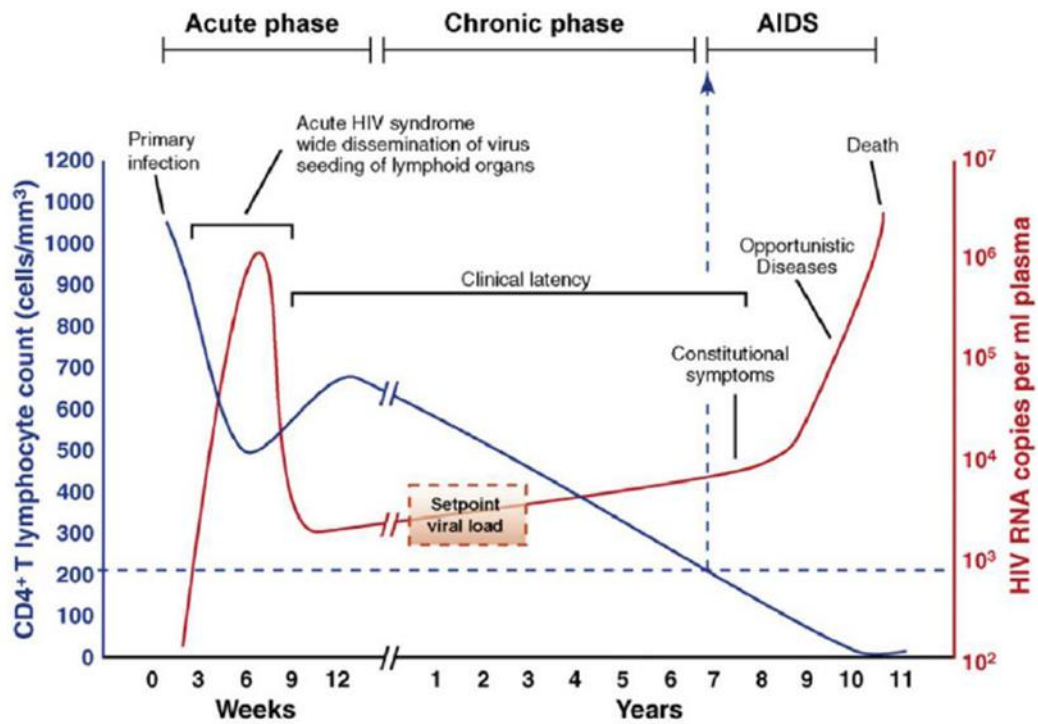


Figure 1.3. Typical clinical course of an HIV-1 infection.

The acute phase begins with a rapid rise in RNA viral load and a decline of CD4⁺ T cell count which returns to normal levels shortly afterwards. The chronic phase starts after the decline of viral load and maintains a viral load setpoint for years (clinical latency), followed by a gradual increase of plasma viraemia and decline of CD4⁺ T cell count that results in the symptomatic phase defined by the development of opportunistic diseases, and ultimate death without treatment. Reproduced from (O'Brien and Hendrickson, 2013).

1.1.6 Variable disease progression phenotypes

HIV-1 controllers

Before viral load testing became available in the mid 1990's, the term “long-term non-progressors (LTNPs)” was used to describe a small percentage of individuals who maintained stable CD4⁺ T cell counts, spontaneously controlled viral replication for extended periods, and had delayed development of AIDS symptoms in the absence of ART (Sheppard et al., 1993, Cao et al., 1995, Pantaleo et al., 1995). The definition of this HIV-1 infected group became complicated with viral load testing. Significant viral load differences were observed within this group of individuals, although they shared the common trait of maintaining stable CD4⁺ T cell counts (Deeks and Walker, 2007). Currently, these individuals are collectively known as HIV-1 controllers, which comprise 5-15% of all HIV-1 infected people and are divided into sub-groups based on viral load. These include elite controllers (ECs), defined as a rare group (<1% of infected population) that spontaneously maintains undetectable HIV RNA (<50 copies per ml) (Fig 1.4) (reviewed in (Lopez-Galindez et al., 2019), as well as viraemic controllers (VCs) that maintain viral RNA between 50 to 2000 copies per ml, and the rare

high-viral-load long-term non-progressors (HVL LTNPs) with HIV RNA above 100000 copies per ml for over 3 years without CD4⁺ T cell count decline (Sáez-Cirión et al., 2012, Telenti, 2014, Ryu, 2016). However, there is lack of a clear consensus in how these groups are defined across studies (reviewed in (Gurdasani et al., 2014)).

Both viral and host factors affect HIV-1 control. Host factors that impact HIV-1 control include aspects of innate, cellular and humoral immunity (reviewed in (Li et al., 2018)). Several protective human leukocyte antigen (HLA) and CCR5 alleles have been identified in HIV-1 controllers. For instance, the protective HLA-B*57 observed across multiple ethnicities (Migueles et al., 2000, Carrington and Walker, 2012, McLaren et al., 2012, Leszczyszyn-Pynka et al., 2015, Dominguez-Molina et al., 2017) as well as protective HLA B*58:01 and B*81:01 in South African patients (Kiepiela et al., 2004). The CCR5delta32 variant prevalent in Caucasians, results in a loss of this HIV-1 co-receptor on the surface of target cells, where homozygosity for this allele associates with prevention of HIV-1 infection with CCR5-utilizing strains (Huang et al., 1996, Samson et al., 1996, Hutter et al., 2009) and heterozygosity associates with a slower disease progression course (Dean et al., 1996, Borinskaya et al., 2012). Some HIV-1 controllers have no known protective HLA or CCR5 alleles, thus pointing to additional mechanisms underlying HIV-1 control. These may include superior HIV-1-specific CD8⁺ T cell cytotoxicity (although some are related to protective HLA) (Migueles et al., 2008, Hersperger et al., 2011), which is responsible for inhibiting or preventing replication of HIV-1 in autologous CD4⁺ T cells *in vitro* (Sáez-Cirión et al., 2007). Elite controllers, in particular, maintain strong, polyfunctional CD8⁺ T cell responses (Owen et al., 2010).

HIV-1 progressors

Compared to HIV-1 controllers, HIV-1 progressors have higher viral load set points that predict the faster disease progression observed for this group in the absence of ART (Fig 1.4) (Pantaleo and Fauci, 1996, Langford et al., 2007). Typical progressors develop AIDS within 3–7 years and rapid progressors in less than 3 years (Lyles et al., 2000). Some of the associated deleterious genetic factors include HLA alleles, such as HLA-B*08 (Carrington and O'Brien, 2003), HLA-B*35Px (Gao et al., 2010), HLA-B*58:02 (Kiepiela et al., 2004, Carlson et al., 2012), HLA-A*36:01, HLA-B*51:01 (Carlson et al., 2012), HLA-A*24 (Kaslow et al., 1990) and a promoter variant in the HLA-C gene (SNP rs9264942 -35T), which reduces its surface expression (Fellay et al., 2009, Thomas et al., 2009, Kulpa and Collins, 2011). T cell exhaustion, marked by the upregulation of inhibitory programmed

death-1 receptor on HIV-1-specific CD8⁺ T cells, further contributes to loss of HIV-1 viral replication control (Zhang et al., 2007, Youngblood et al., 2013, Hoffmann et al., 2016). The impact of antibodies and Fc-mediated effector functions on HIV-1 disease progression and control is discussed in more detail below.

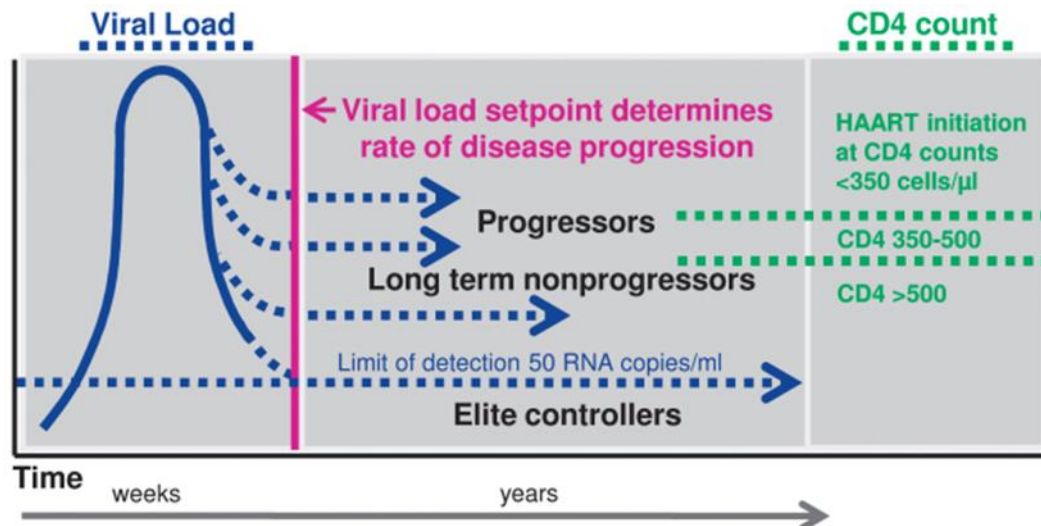


Figure 1.4. Variable HIV-1 disease progression phenotypes.

Schematic illustration of the different categories of HIV-infected individuals with various disease progression rates, based on markers of disease progression, namely, CD4⁺ T cell count (cells/μl) and/or viral load (RNA copies/ml) reproduced from (Tiemessen and Martinson, 2012). The viral load setpoint determines the rate of disease progression, which is rapid in progressors, moderate in long-term non-progressors, and slow in elite controllers who maintain viral RNA copies/ml below the limit of detection for years without treatment.

1.2 HIV-1-specific antibody responses

Non-neutralizing antibodies are the first to appear in the early stages of an HIV-1 infection (Fig 1.5). The earliest detection of antibodies occurs 8 days after the first detectable viraemia in a form of immune complexes, which comprise 22% of total circulating virus in plasma at that stage (Liu et al., 2011). As infection progresses to the chronic phase, immune complexes may comprise 17-67% of circulating plasma viraemia (Liu et al., 2011). Thirteen days following detection of plasma viral RNA, HIV-1-specific binding antibodies are detected in free form as gp41-specific-IgM with subsequent class switching to IgG and IgA (Tomaras et al., 2008, Mikell et al., 2011). These antibodies do not contribute significantly to control of HIV-1 replication. Eighteen days following detectable plasma viral RNA, Gag-specific antibodies begin to appear as p24-specific IgG and p55-specific IgG, with subsequent detection of p17-specific IgG about 15 days later (Tomaras and Haynes, 2009).

Approximately one month after viraemia is detected in the blood, the first gp120-specific IgG antibodies appear in a form of non-neutralizing antibodies that target the V3 epitope (Cooper et al., 1987, Tomaras et al., 2008, Tomaras and Haynes, 2009, McMichael et al., 2010), with the subsequent appearance of CD4-binding site antibodies (Lynch et al., 2012). These early antibodies lack neutralization breadth; however, they have the capacity to mediate effector functions both *in vivo* (Horwitz et al., 2017) and *in vitro* (Holl et al., 2006, Kramski et al., 2013, Lewis, 2013, Bruel et al., 2017).

Excessive production of immunoglobulin antibodies in circulation due to non-specific polyclonal B cell activation, known as hypergammaglobulinaemia, accompanies HIV-1 infection (Lane et al., 1983). It is characterized by the impairment of antigen-specific humoral immunity in HIV-1 infection that may be the result of improper B cell differentiation that yields low-specificity antibodies (reviewed in (Hu et al., 2015)). This B cell dysfunction and affected antigen-specific immunity in HIV-1 infected individuals (Moir et al., 2001, De Milito et al., 2004, Moir and Fauci, 2008), may therefore contribute to an increased risk of accelerated HIV-1 disease progression.

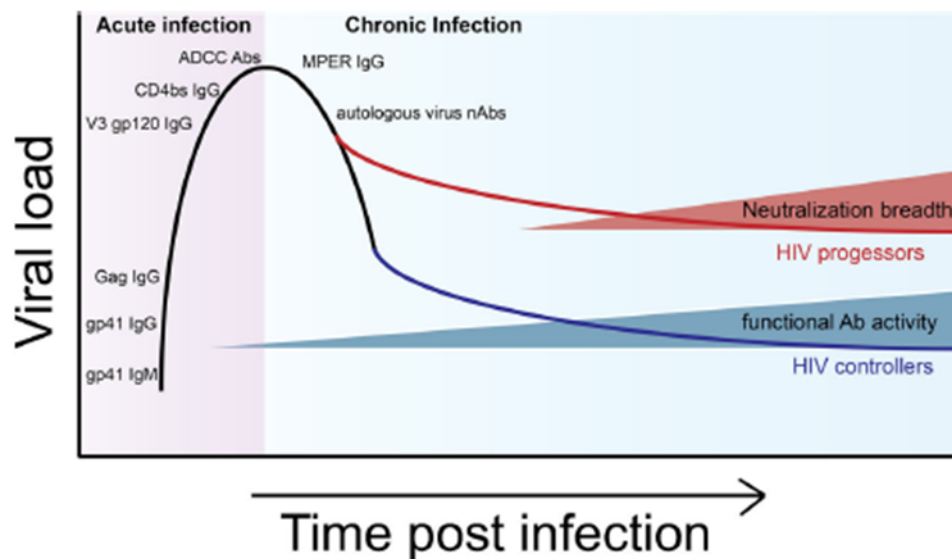


Figure 1.5. HIV-1-specific antibodies that arise in early HIV-1 infection.

In the early weeks of the acute phase of HIV-1 infection, HIV-1 envelope-specific IgM and IgG antibodies appear sequentially, and gradually broaden epitope specificity to include gp120, gp41, CD4-binding site (CD4BS), V3 loop and membrane-proximal external region (MPER). These are non-neutralizing antibodies that have the capacity to induce Fc-mediated effector functions including the antibody-dependent cellular cytotoxicity (ADCC). Months after HIV-1 infection, the first neutralizing antibodies appear, followed by the development of broadly neutralizing antibodies years later in some infected individuals (reproduced from (Butler et al., 2019)).

1.2.1 FcγR-mediated effector functions

Fc-gamma-receptor (FcγR)-mediated effector functions form a direct bridge between the adaptive and innate immune systems that enable effective immune responses against invading pathogens (Fig 1.6). When the Fc domain of antigen-bound antibodies engages FcγRs expressed on the surface of innate immune cells, it triggers a variety of antibody-mediated responses that ultimately leads to the clearing of opsonised pathogens, infected cells or immune complexes from the body. These mechanisms include the antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent complement-dependent cytotoxicity (ADCDC) (Fig 1.6).

ADCC

NK-cell-mediated ADCC is an FcγR-mediated effector function mediated through the release of cytotoxic granules consisting of perforins and granzymes by effector cells to initiate cytotoxic killing of infected cells. Initiation of ADCC occurs mainly through signal transduction by cross-linked FcγRIIIa on NK cells, with IgG1 or IgG3 as potent mediators (Odin et al., 1991, Benhamou et al., 1993, Kiener et al., 1993, Rankin et al., 1993, Kimura et al., 1996). This cytotoxic mechanism has been demonstrated to play a role in elimination or control of various intracellular pathogens including malaria parasites (Jafarshad et al., 2007, Tiendrebeogo et al., 2015, Lu et al., 2016b), influenza A virus (Wu et al., 2015, He et al., 2016) and *Chlamydia trachomatis* (Moore et al., 2002). Based on the growing list of pathogens cleared or controlled through ADCC as well as the capacity for killing abnormal cells, it is no coincidence that this mechanism has been the focus of developing effective monoclonal therapeutics, particularly in cancer research (reviewed in (Weiner, 2015)). Numerous studies have looked into enhancing ADCC activity for more effective therapeutics directed at various autoimmune and infectious diseases, with more attention directed at IgG Fc domain modifications (Umana et al., 1999, Shields et al., 2002, Zeitlin et al., 2011, Jefferis, 2012).

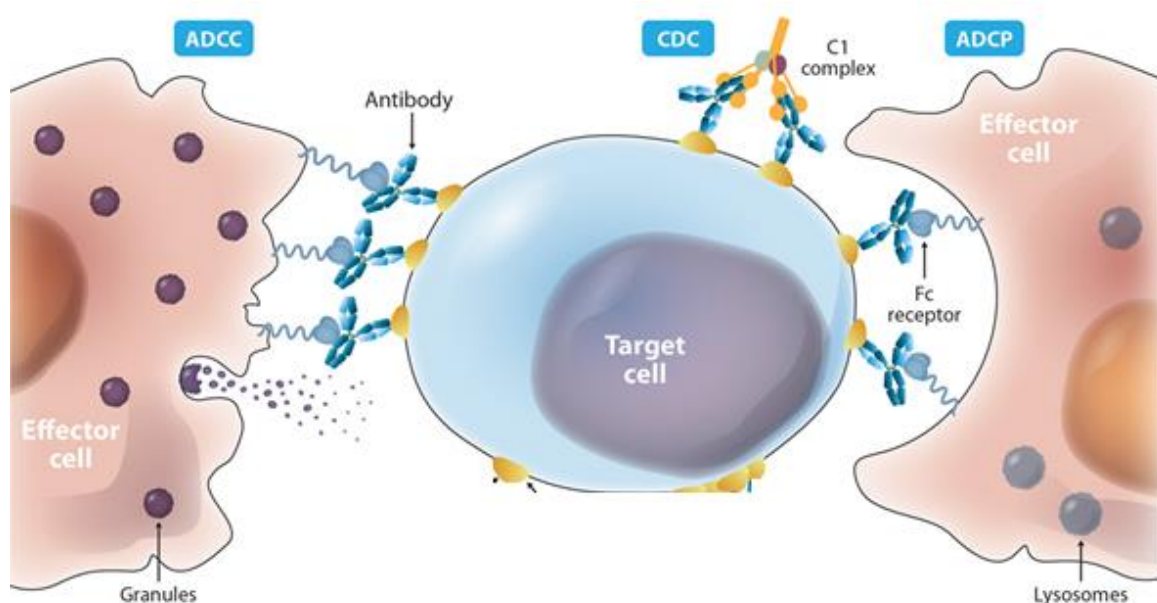


Figure 1.6. FcγR-mediated effector functions.

Crosslinking of Fc receptors expressed on effector cells to the Fc portion of antigen-bound antibodies transmits activation signals that result in Fc-mediated effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP) and antibody-dependent complement-mediated cytotoxicity (ADCDC). Illustration reproduced from Almagro et al. (Almagro et al., 2018).

ADCC in HIV-1 infection. The potential of ADCC to prevent or control HIV-1 has been explored in both humans and non-human primates (NHPs) (reviewed in (Lewis et al., 2017, Lee and Kent, 2018)). Several studies associate ADCC with control of HIV-1 viraemia in humans (Baum et al., 1996, Ahmad et al., 2001, Forthal et al., 2001, Lambotte et al., 2009, Lambotte et al., 2013, Wren et al., 2013), as well as in NHPs (Banks et al., 2002, Gomez-Roman et al., 2005, Xiao et al., 2010). In particular, antibodies or plasma from HIV-1 controllers mediated higher ADCC compared to viraemic individuals using assays such as the chromium release ADCC assay (Baum et al., 1996, Lambotte et al., 2009) and the GranToxiLux (GTL) ADCC assay (Lambotte et al., 2013). In the Thai RV144 vaccine trial, the first to demonstrate a modest efficacy against HIV-1 acquisition, ADCC responses associated with lower risk of HIV-1 infection in the absence of high HIV-1 gp120-specific IgA antibody levels (Haynes et al., 2012, Tomaras et al., 2013, Yates et al., 2014). Similarly, the RV144 vaccine regimen tested in the South African population (HVTN 097 trial) induced enhanced gp120-specific ADCC and Env-specific ADCP responses, together with higher or comparable magnitude and prevalence of gp120-specific IgG3 and V1V2-specific IgG3 antibody responses compared to that observed for the Thai RV144 trial (Gray et al., 2019).

Despite these promising associations, it still remains unclear how ADCC provides protection or controls virus replication (reviewed in (Lee and Kent, 2018)).

ADCP

While most FcγRs expressed on phagocytes mediate the internalization of antibody-opsonized targets, phagocytic capacity is primarily determined by FcγRIIa expression levels (Dugast et al., 2011, Ackerman et al., 2013b), the type of myeloid phagocyte, and stage of cell differentiation (reviewed in (Lu et al., 2018)). FcγR-expressing phagocytes include monocytes, macrophages, dendritic cells, neutrophils, eosinophils, basophils and mast cells, which are all capable of mediating ADCP of opsonized infected cells, extracellular pathogens such as virions and bacteria, as well as their derivatives (Dale et al., 2008). Pathogen clearance that occurs through ADCP is often accompanied by signal activation for antigen processing and presentation, regulation of T cell responses, and the secretion/release of cytokines, antimicrobial peptides, lipid mediators, metalloproteinases, defensins, reactive nitrogen species and reactive oxygen species (Hoving et al., 2014, van Egmond et al., 2015, Bournazos et al., 2016).

ADCP in HIV-1 infection. ADCP responses are generally impaired in HIV-1 infected individuals, with the loss of function correlating with increased HIV-1 disease progression (Biggs et al., 1995, Kedzierska et al., 2000, Kedzierska et al., 2002). This may be attributable to a reduction of FcγRIIa expression on professional phagocytes that accompanies progressive HIV-1 infection (Dugast et al., 2011). Antibodies from viraemic individuals have poorer overall Fc effector function capacity, whilst antibodies purified from elite controllers mediate a more polyfunctional humoral immune response that incorporate ADCP, ADCC and ADCDC (Ackerman et al., 2016). In addition, associations of ADCP-mediating antibodies with prevention of HIV-1 acquisition have been observed in both human and NHP studies. These include the polyfunctional gp120-specific V1V2 IgG3 antibody in the RV144 vaccine trial post analysis (Chung et al., 2014b), as well as protection against intrarectal and vaginal SHIV challenges in NHP vaccine studies (Barouch et al., 2013, Moog et al., 2014). Despite this evidence, more data are required on the direct role of ADCP in both control and prevention of HIV-1 infection.

1.3 Modulators of Fc-mediated effector functions

The collective of multiple viral and host factors determines the ability of the host to control HIV-1 infection. The inordinate complexity thereof makes it difficult to fully comprehend the mechanisms that underlie effective HIV-1-specific immunity. This complexity remains even when isolated to features that impact on a specific axis such as antibodies and their cognate Fc receptors. Factors that modulate Fc γ R-mediated effector functions during HIV-1 infection include: i) antibody binding affinity (Bruhns et al., 2009), ii) the distribution of Fc γ Rs on effector cells (Ackerman et al., 2013b, Bournazos et al., 2016), iii) effector cell proportions and distribution (Guilliams et al., 2014, Bournazos et al., 2016), iv) degree of Fc glycosylation on antibodies (Jefferis et al., 1998, Chung et al., 2014a), and v) distribution of Ig isotypes and IgG subclasses (Jefferis et al., 1994). The following sections will focus on antibody isotypes and subclasses, effector cells and Fc γ Rs as it pertains to this thesis.

1.3.1 Antibody isotypes and subclasses

The secreted antibody is a heterodimeric immunoglobulin glycoprotein structure of four polypeptide chains composed of 82-96% proteins and 4-18% carbohydrates that make up two identical 50 kDa heavy chains and two identical 25 kDa light chains joined together by interchain disulfide bonds (Fig 1.7). Each heavy chain consists of 3 constant domains (CH1, CH2 and CH3) with a hinge region between CH1 and CH2, and an N-terminal variable domain (VH) (Wu et al., 1993). In contrast, the two identical light chains each contain a single constant domain and an N-terminal variable domain. The two types of light chains known as lambda (λ) and kappa (κ) have a 1:2 ratio in humans that may vary in immunoglobulin isotypes and subclasses, as well as from one species to the next. The variable fragment antigen binding (Fab) domain is formed by the association of the VH and CH1 domains with the light chain, with an amino acid terminal region composed of highly variable amino acids that are involved in antigen binding (Kabat et al., 1978, Potter, 1983). The lower hinge region, as well as CH2 and CH3, together form the fragment crystalline domain. The heavy and light chains of the Fc domain consist of highly conserved amino acid sequences that play important roles in assembling proteins (complement activation), placental transport

and mediating Fc effector functions (reviewed in (Rispen and Vidarsson, 2013)).

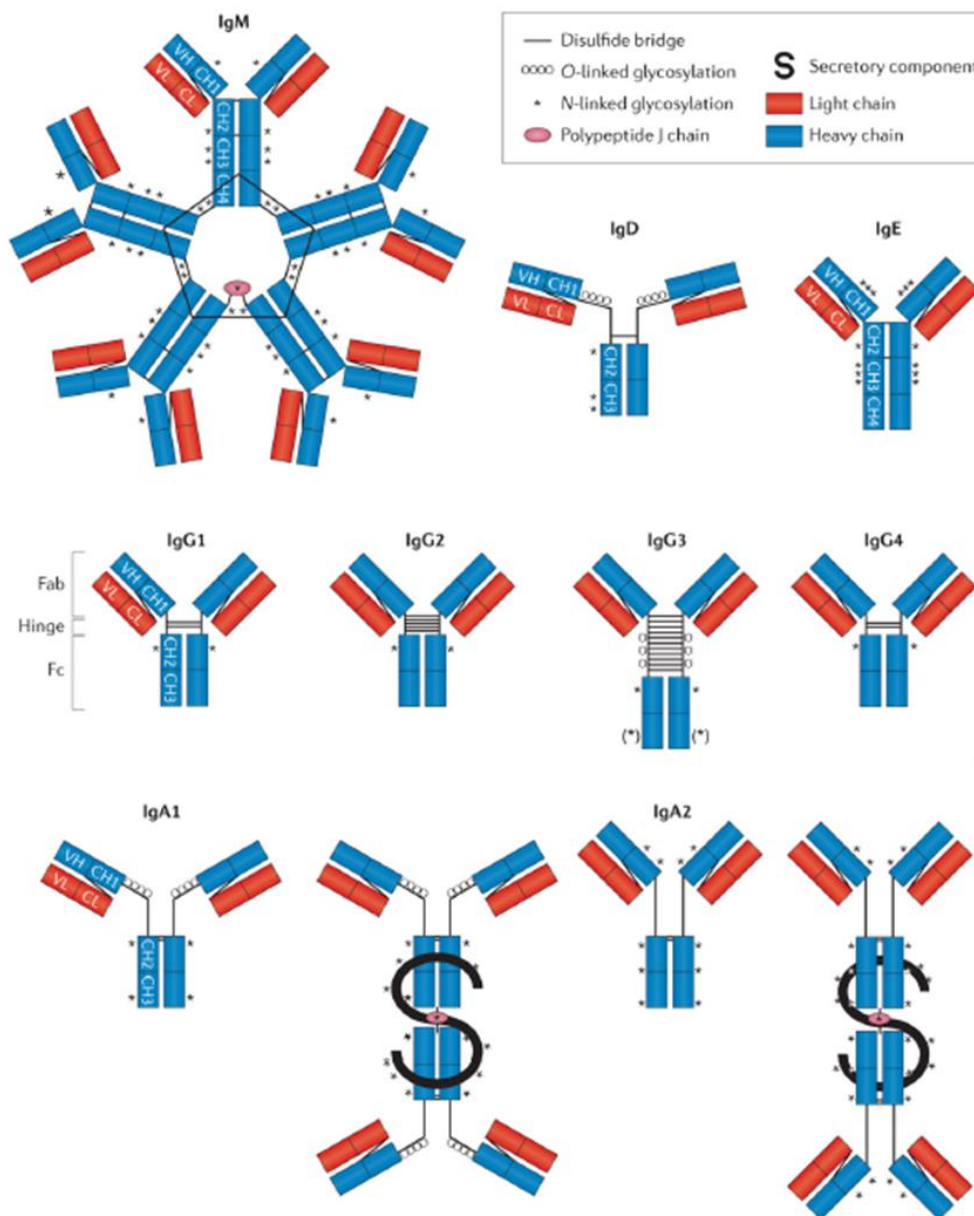


Figure 1.7. Antibody isotypes and subclasses.

The basic antibody structure is composed of two domains: the crystallisable fragment (Fc) domain that interacts with FcRs expressed on the surface of effector cells, and the fragment (Fab) domain that engages antigens of the invading pathogen. The Fc and Fab domains are connected by a hinge region, and are formed by four chains that are divided into two identical light chains (red) and two identical heavy chains (blue). These are further divided into constant (CH) and variable (VH) domains that make up the Fc and Fab domains. Upon encountering an antigen, antibody diversity occurs through class switching and post-translational modification, resulting in different antibody isotypes and subclasses. In humans, there are five isotypes (IgM, IgE, IgD, IgG, and IgA) and six subclasses (IgG1-IgG4, and IgA1-IgA2), that occur as monomeric and/or polymeric structures that are joined by disulphide bridges, polypeptide J chains or the secretory component. Structural differences between the antibodies affect their conformation and/or flexibility, which impact their interaction with immune receptors and

by default, their capacity to mediate different FcR effector functions (Illustration reproduced from (Lu et al., 2018)).

IgM

IgM is the first antibody class produced by naïve B cells. In contrast to typical antibody structures of one Fc region per antibody class, IgM is secreted in a pentameric form, with five Fc regions connected at the centre by disulfide bonds and one J chain (Czajkowsky and Shao, 2009). The pentameric IgM structure plays an important role in activating the complement system (Czajkowsky and Shao, 2009). As antigens of a pathogen bind to the IgM antibody, more than one C1 complex can bind to Fc regions of the same antibody, thereby connecting the complexes to initiate the complement cascade. In addition to activating the complement cascade, the pentameric IgM structure has increased avidity to antigens (Onoue et al., 1968). IgM antibodies are involved in neutralizing virus infectivity, making them suitable antibodies for defense of the host against viral or bacterial infections, particularly in early infection, where they have the capacity to mediate pathogen clearance through phagocytosis. However, these antibodies have a short half-life of one day.

In the context of HIV-1 vaccine development, intrarectal passive transfer of recombinant monoclonal IgM produced from class-switching of the neutralizing monoclonal IgG1 antibody 33C6-IgG1, demonstrated protection from infection following SHIV challenge of rhesus macaques (Gong et al., 2018). This study implicated efficient virus capture through cross-linking of the pentameric IgM. In the context of HIV-1 infection, as the first antibody isotype to emerge during the acute phase, HIV-1-specific IgM plays various roles. These include inhibition of chemokine or HIV-1 virion binding through direct interaction with CCR5 and CXCR4 co-receptors as well as a reduction of T cell activation and lowered generation of some pro-inflammatory cytokines (Lobo et al., 2008a, Lobo et al., 2008b, Lobo et al., 2012). Men infected with HIV-1 have elevated semen IgM levels compared to uninfected individuals (Pillay et al., 2018), and total IgM levels are increased in intestinal secretions of HIV-1 infected individuals (Hel et al., 2017). The role of IgM in Fc-mediated effector functions, however, is not well established.

IgA

IgA is the most abundant antibody in the human body and the second most abundant in the blood. It is predominantly located in the mucosal surfaces that include the digestive, respiratory and reproductive tracts (Kerr, 1990). IgA-producing B cells appear approximately

three months after birth in humans (Cooper et al., 1974) and IgA is also secreted into breastmilk where it aids in protecting the intestinal mucosa of infants. In contrast to IgG, IgA occurs in numerous forms that include monomeric, dimeric and secretory. In humans, there are two types of IgA subclasses termed serum IgA (IgA1) and secretory IgA (IgA2) that share high sequence similarity but differ in hinge length, antigen-binding capacity, primary function and distribution (reviewed in (Zhou and Ruprecht, 2014)).

Serum IgA comprises 85% IgA1, which is predominantly monomeric with a longer hinge composed of a 16-amino-acid sequence. The longer hinge region increases IgA1 flexibility and antigen-binding capacity, but also makes IgA1 highly susceptible to bacterial proteases, compared to IgA2 (reviewed in (Kerr, 1990)). In contrast to IgA1, the secretory IgA (IgA2) predominates in mucosal secretions in humans (reviewed in (Woof and Mestecky, 2005)), which may be due to its shorter hinge region that confers greater resistance to bacterial proteolysis. The dimeric structure of IgA2 resembles two IgG molecules joined at the Fc region tails, resulting in four Fab regions. This structure is complexed to a secretory component, which is a highly glycosylated polypeptide chain (Macpherson et al., 2008). The dimeric structure of secretory IgA is well suited for its primary function of guarding/protecting mucosal surfaces against pathogen invasion, as it allows transport of secretory IgA through the intestinal wall into the intestine and resists degradation or erosion from the acidic environment and enzymes found in the intestine (reviewed in (Kerr, 1990, Macpherson et al., 2008)). Both IgA1 and IgA2 are highly polyfunctional by way of neutralizing pathogenic molecules and microbes as well as mediating a spectrum of innate immune responses such as ADCC and ADCP by monocytes and polymorphonuclear leukocytes via Fc α Rs (Monteiro and van de Winkel, 2003). IgA2 is a more potent ADCC mediator compared to IgA1 (Dechant et al., 2002, Dechant et al., 2007), which may explain the choice of IgA2 in development of anticancer therapeutics (Lohse et al., 2018).

It has long been determined that monomeric IgA1 occur at significantly higher proportions in serum compared to IgA2 in HIV-1 infection (Kozlowski and Jackson, 1992). Some studies have reported elevated levels of total serum IgA in HIV-1 infected individuals in connection to hypergammaglobulinaemia (Yarchoan et al., 1986, Shirai et al., 1992, Moir and Fauci, 2009). Functionally, HIV-1-specific IgA1 in elite controllers reacted with significantly more HIV-1 antigens compared to that in viraemic controllers and progressors (Nabi et al., 2017).

The contribution of HIV-1-specific IgA in effector functions is unclear. In the RV144 vaccine trial, ADCC-mediating gp120-specific IgG antibodies correlated with lower infection risk in

the presence of low gp120-specific IgA antibody levels, which suggested competition between the two isotypes (Tomaras et al., 2013). The inhibitory effect of IgA on ADCC activity was further demonstrated through depletion of HIV-1-specific IgA from plasma samples of HIV-1 infected individuals and a consequent elevation in ADCC activity (Ruiz et al., 2016). It should be noted that studies of RV144 vaccinees did not measure the ADCC-mediating capacity of vaccine-induced IgA antibodies directly due to the absence of Fc α RI/CD89 expression on effector cells used in the assays. However, a more recent study demonstrated that HIV-1 Env gp41-targeting IgA antibodies, through Fc α Rs expressed on monocytes and neutrophils, may cooperate with IgG-mediated ADCC rather than compete against it (Duchemin et al., 2018).

IgG

IgG is the most abundant antibody in the blood, making up 75% of all immunoglobulins (Lefranc and Lefranc, 2001). It has several unique features that include: a half-life of roughly three weeks as a result of recycling by FcRn receptors (Mankarious et al., 1988, Akilesh et al., 2007, Kim et al., 2007), the ability to travel through the placenta from a mother to her unborn child, and has one Fc domain linked by a hinge region to two Fab domains. There are four IgG subclasses that are named in order of decreasing composition in plasma: IgG1 (60–70%), IgG2 (20–30%), IgG3 (5–8%), and IgG4 (1–3%) (Schur, 1988, Lefranc and Lefranc, 2001). Several structural properties distinguish these subclasses and collectively influence their functionality. These include differences in the content of their CH2 amino acids, the ratio of the λ and κ light chains, as well as the length and flexibility of their hinge regions (Bruhns et al., 2009). The hinge flexibility decreases in the order IgG3 > IgG1 > IgG4 > IgG2 (Redpath et al., 1998, Carrasco et al., 2001) based on the number of amino acid residues and interchain disulfide bonds. The Fc domain contains a binding site for Fc γ Rs expressed on innate immune cells, located on the residues most proximal to the hinge region in the CH2 domain, which facilitates Fc-mediated effector functions. The general Fc-mediated effector function activating capacity order of the four IgG subclasses is IgG3 > IgG1 >> IgG2 > IgG4 (Vidarsson et al., 2014).

IgG1

IgG1 is the predominant subclass (60-75%) in serum and dominates the early antibody responses to membrane proteins and soluble protein antigens (Schur, 1988, Ferrante, 1990, Rieben et al., 1991, Tomaras and Haynes, 2009). The IgG1 hinge has 15 amino acid residues and is freely flexible so that Fab regions can rotate about their axes of symmetry and travel

within a sphere positioned centrally at the first of two inter-chain disulfide bridges. It is a pro-inflammatory IgG subclass that has been positively associated with increased effector function capacity (Redpath et al., 1998).

IgG1, primarily specific to HIV-1 Env, is the most predominant antibody subclass during the acute and chronic phases of HIV-1 infection (Klasse and Blomberg, 1987, Mergener et al., 1987). IgG1 maintains high levels during the acute and chronic phases of HIV-1 infection, but begins to decline at the symptomatic phase in HIV-1 progressors (Klasse and Blomberg, 1987, Broliden et al., 1989, Yates et al., 2011). With its high affinity for FcγRs and cell activation capacity, IgG1 plays an important role in HIV-1-specific Fc-mediated effector functions. In particular, several studies of natural HIV-1 infection showed that HIV-1-specific IgG1 and IgG3 subclass levels correlated with reduced disease progression and potent ADCC in HIV-1 controllers (reviewed in (Moris et al., 2019)). In contrast to few studies associating gp120-specific IgG1 levels with HIV-1 control (Banerjee et al., 2010, French et al., 2013), there has been a strong focus on Gag-specific IgG1 (especially p24-specific IgG1) in the context of HIV-1 control (Banerjee et al., 2010, Johansson et al., 2011, French et al., 2013, Tjiam et al., 2015, Tjiam et al., 2016, Ahmad et al., 2018, Alter et al., 2018, Chung et al., 2018).

IgG2

The hinge region of IgG2 is 12 amino acid residues, similar to IgG4. However, IgG2 has an additional cysteine deletion at the lower hinge region, which makes it the shortest of all IgG subclasses (Pumphrey, 1986, Redpath et al., 1998). The presence of a poly-proline helix in the hinge that is stabilized by a few extra inter-heavy chain disulfide bridges, further restricts movement of the IgG2 Fab arms. IgG2 has the capacity to activate the complement system and plays a role in ADCP activity through its engagement with FcγRIIa, whereas it has low binding for other FcγRs (Rabinovitch, 1995, Ravetch and Bolland, 2001, Williams et al., 2014, Bruhns and Jönsson, 2015). IgG2 is the primary IgG subclass to respond to bacterial capsular polysaccharide antigens (Siber et al., 1980, Barrett and Ayoub, 1986, Schauer et al., 2003). Its importance in anti-glycan responses is demonstrated in IgG2 deficient individuals who lack anti-carbohydrate IgG and an associated increased risk of bacterial infection (Hammarström and Smith, 1983, Kuijpers et al., 1992), despite the ability of IgG1 and IgG3 to respond to protein-conjugated glycans and other carbohydrates (Hammarström et al., 1987, Vidarsson et al., 1998). The HIV-1 Env spike is heavily shielded by glycans which assist in evading antibody neutralization and effector functions (Wei et al., 2003). HIV-1-specific

IgG2 binding the glycan shield, such as the broadly neutralizing antibody 2G12 (Sanders et al., 2002, Murin et al., 2014), may therefore be important in HIV-1 protective immunity (Stanfield et al., 2015).

HIV-1 Env-specific IgG2 is detected throughout various stages of HIV-1 infection (Chiodi et al., 1989, Lambotte et al., 2009), but often at lower levels than other subclasses in the periphery (Klasse and Blomberg, 1987, McDougal et al., 1987, Khalife et al., 1988). Although it is established that Env-specific IgG2 contributes to Fc γ RIIa-mediated effector functions (French et al., 2010), there is no clear consensus regarding the role of this subclass with HIV-1 disease progression. A more recent study reported Env-specific IgG2 levels to vary through the course of disease progression, with higher levels observed at early time-points in HIV-1 controllers and gradually increasing levels over time in progressors (Sadanand et al., 2018). In agreement, gp120-specific IgG2 (alongside IgG4) antibodies have been found to be prevalent in viraemic individuals and to inversely associate with Fc-mediated effector functions in HIV-1 progressors (Ackerman et al., 2016) and in RV144 vaccinees (Chung et al., 2014b). Others consider Env-specific IgG2 as a marker of immune responses correlated to HIV-1 control (Ngo-Giang-Huong et al., 2001, Martinez et al., 2005, Lai et al., 2014, Tjiam et al., 2015), with one study observing that lack of this antibody correlated with progressive HIV-1 infection (Lal et al., 1991). These observations warrant further investigation.

IgG3

IgG3 is the most functional and potent effector function mediator due to its long hinge region and superior binding affinity to Fc γ Rs (Ferrante, 1990, Redpath et al., 1998, Niwa et al., 2005). It has the longest hinge region of all IgG subclasses, containing up to 62 amino acids (Pumphrey, 1986) with 11 disulfide bridges in the polyproline double helix (Redpath et al., 1998, Vidarsson et al., 2014). The highly elongated hinge region places the Fab domains further from the Fc domain, enabling greater rotational capacity that reinforces high flexibility and accessibility to antigens (Roux et al., 1997, Carrasco et al., 2001). This may aid in overcoming steric interference and contribute to better binding activating Fc γ Rs and ultimately the enhanced effector function mediating capacity of IgG3 (Roux et al., 1997, Bruhns et al., 2009, Vidarsson et al., 2014),

IgG3 is found in the initial stages of an antibody response to a wide range of invading pathogens such as bacteria, viruses and parasites where it interacts with Fc γ Rs expressed on

effector cells including NK cells, monocytes, neutrophils and macrophages (Chung et al., 2011b, Lu et al., 2016b, McLean et al., 2017, Wang et al., 2017). In particular, IgG3 is the first IgG subclass to appear in a viral infection (Ferrante, 1990) and plays an important role in controlling or preventing infectious diseases. IgG3's function is regulated by a short half-life (~7 days) and low levels within circulating IgG.

Env-specific IgG3 is present in both HIV-1 controllers and progressors at early time-points, but levels decline over time in progressors (Sadanand et al., 2018). However, high total IgG3 levels in HIV-1 infected patients have been reported with concurrent high B cell dysfunction that associates with hypergammaglobulinaemia (Béniguel et al., 2004). This may suggest that declining HIV-1-specific IgG3 levels coincide with elevation of dysfunctional non-specific IgG3 levels in HIV-1 progressors, resulting in higher total IgG3 levels. This may partially explain the gradual decline of Fc-mediated effector functions that are linked to loss of HIV-1-specific IgG3 responses and increased immune activation over time (Dugast et al., 2014).

Conversely, the potent effector function capacity of HIV-1-specific IgG3 appears to be advantageous in HIV-1 vaccine protection. Post-RV144 analysis showed that HIV-1 gp120 V1V2-specific IgG3 was the only IgG subclass that was directly linked to the moderate success of the RV144 vaccine trial (Yates et al., 2014). These antibodies declined shortly after vaccination. However, their high effector functionality was demonstrated through depletion of IgG3 from RV144 vaccinees plasma samples that resulted in loss of Fc effector polyfunctionality and significantly lower Fc effector functions including ADCC and ADCP when compared with non-depleted samples *in vitro* (Chung et al., 2014b).

IgG4

IgG4 antibodies are generally not associated with a primary response to antigen but arise following repeat exposure to antigen. This antibody subclass may become dominant upon induction by an allergen or in response to therapeutic proteins (Aalberse et al., 1983a, Aalberse et al., 1983b, Nouri-Aria et al., 2004, van Helden et al., 2008). IgG4's shorter hinge region and limited flexibility (Roux et al., 1997) may impact its lower effector function capacity that, in conjunction with its high antigen-binding affinity, has led to the hypothesis that chronic antigen exposure leads to IgG4 stimulation in order to limit the humoral immune response (reviewed in (Koneczny, 2018)). IgG4 was initially considered to lack binding capacity to human receptors and several IgG4-based therapeutic antibodies were manufactured based on this notion. However, a study by Bruhns *et al.* demonstrated its

capacity to bind human FcγRI, FcγRIIa, FcγRIIb, FcγRIIc and FcγRIIIa with varying affinities (Bruhns et al., 2009). IgG4, alongside IgG2, is less efficient at activating Fc-mediated effector functions due to its lower binding affinity to FcγRs (Bruhns et al., 2009).

Not all HIV-1 infected individuals have detectable HIV-1-specific IgG4. If detected, it is at low levels and may be present in all phases of HIV-1 infection (McDougal et al., 1987, Ljunggren et al., 1988). However, it is more prevalent during the chronic phase with a slight increase in AIDS patients (Ljunggren et al., 1988, Mathiesen et al., 1989, Ackerman et al., 2016), where it was also inversely correlated with Fc-mediated effector functions (Ackerman et al., 2016).

1.3.2 Fc-gamma-Receptors

FcγRs are a family of glycoproteins that are widely expressed throughout the haematopoietic system and their cellular expression pattern differs. There are ten FcγRs expressed on human cells that have been identified (Fig 1.8): six classical FcγRs known as FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb; as well as neonatal FcR (FcRn), cytosolic tripartite motif-containing protein 21 (TRIM21) (Yan et al., 1999), and the two FcR-like receptors; FcRL4 (CD307d) and FcRL5 (CD307e) (Wilson et al., 2012, Franco et al., 2013). Classical FcγRs have an extracellular domain that consists of two Ig domains (three for FcγRI) connected by a hinge region that makes them structurally and functionally related to the Ig receptor superfamily (Nimmerjahn and Ravetch, 2006).

Fc-FcγR interactions

The IgG-FcγR complex forms a 1:1 stoichiometric complex between the membrane-proximal Ig-like domain of FcγR and the horseshoe Fc portion at the hinge-proximal region of CH2 (Maxwell et al., 1999, Sonderrmann et al., 2000, Ramsland et al., 2011). In humans, activating FcγRs comprise the FcγRI (CD64), FcγRIIa/c (CD32a/c) and FcγRIIIa (CD16a). FcγRI and FcγRIIIa depend on the association of the FcγR γ-chain signalling protein for expression, assembly to the membrane and signal transduction to occur (Wirthmueller et al., 1992, Duchemin et al., 1994, Indik et al., 1994). Conversely, FcγRIIa and FcγRIIc contain an intracellular immunoreceptor tyrosine-based activating motif (ITAM) domain enabling signal transduction without accessory activation proteins (Ghazizadeh et al., 1994, Wang et al., 1994). Most FcγRs have a low binding affinity that prevents non-specific activation of pro-inflammatory responses by circulating monomeric IgG (Nimmerjahn and Ravetch, 2008). Consequently, successful signal transduction requires crosslinking of multiple FcγRs on the

cell surface (Bruhns et al., 2009). The only known inhibitory and most broadly expressed classical FcγR is FcγRIIb, which contains the intrinsic immunoreceptor tyrosine based inhibition motif (ITIM) in its cytoplasmic region (Daëron et al., 1995, Daëron, 1997, Nimmerjahn and Ravetch, 2006). FcγRIIb-mediated signal transduction reduces cellular activation and effector responses and thereby maintains a cell activation threshold (Daëron and Lesourne, 2006).

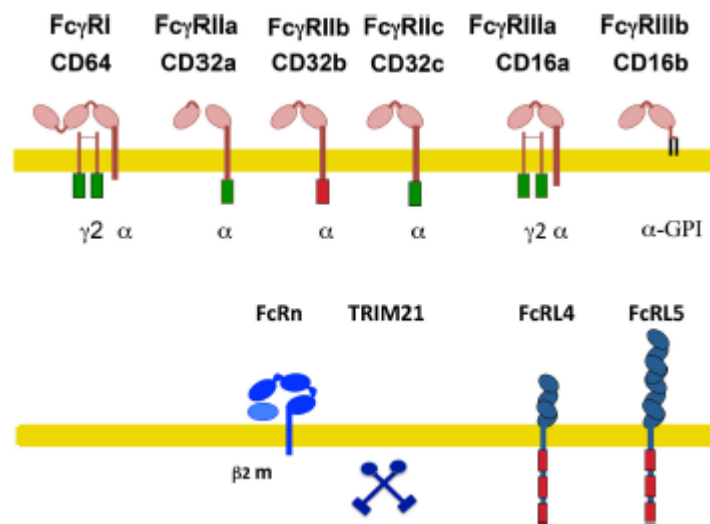


Figure 1.8. Human Fcγ receptors (FcγRs).

Human FcγRs for IgG schematically illustrated with their respective signaling subunits across the cell membrane represented by a yellow line. The upper panel depicts classical FcγRs, while the lower panel consists of non-classical FcγRs. FcR gamma subunit dimer (γ2); beta-2 microglobulin (β2m); immunoreceptor tyrosine-based activation motif (ITAM), green rectangle; immunoreceptor tyrosine-based inhibition motif (ITIM), red rectangle; neonatal Fc receptor (FcRn). TRIM21 is a cytosolic receptor. FcRL4 and FcRL5 are inhibitory FcR-like receptors that are only expressed on B cells. Schematic illustration reproduced from Rosales (Rosales, 2017).

FcγRIIIa as a mediator of ADCC

FcγRIIIa (CD16a) is an activating receptor that is widely expressed on NK cells, macrophages, a subset of monocytes, and γδ T cell receptor (TCR) T lymphocytes (Nimmerjahn and Ravetch, 2008). This receptor also occurs on pre-B lymphocytes and small subsets of CD8⁺ T and CD4⁺ T lymphocytes (Gessner et al., 1995, de Andres et al., 1999, Clemenceau et al., 2008, Chauhan and Moore, 2012). FcγRIIIa has a low-binding affinity for monomeric IgG and preferentially binds to immune complexes. It has a broad IgG subclass specificity (Nimmerjahn and Ravetch, 2010) and the capacity to mediate ADCC, ADCP, and cytokine release depending on the cell type (Anderson et al., 1990, Wu et al., 1997). The inhibitory FcγRIIb, when co-expressed on NK cells due to a segmental deletion in *FCGR2C*,

inhibits ADCC responses (Niederer et al., 2010, van der Heijden et al., 2012, Mueller et al., 2013).

FCGR3A, the gene that encodes for FcγRIIIa, is polymorphic. It is subject to gene copy number variation and bears a well-characterized functional nonsynonymous single nucleotide variant that modulates antibody binding affinity. A T to G variation at nucleotide 559 alters a valine (V) to phenylalanine (F) at amino position 158 (F158V, rs396991) (Gough et al., 2003). The 158V allele confers higher binding affinity for IgG1, IgG2, IgG3 and IgG4 compared to the 158F variant (Ravetch and Perussia, 1989, Bruhns et al., 2009, Bruhns, 2012). This increased binding leads to enhanced activation of FcγRIIIa-bearing cells and consequently enhanced effector functions (Vance et al., 1993, Wu et al., 1997). Additionally, FcγRIIIa surface expression directly correlates with the number of gene copies and associates with differences in FcγRIIIa-mediated effector function capacity (Breunis et al., 2009). In particular, lower ADCC responses are observed in individuals bearing a single copy of *FCGR3A* and associated reduced FcγRIIIa surface expression (Breunis et al., 2009).

Studies that assessed the effect of FcγRIIIa polymorphisms on HIV-1 acquisition, mother-to-child transmission (MTCT), and disease progression have shown inconsistent findings (reviewed in (Geraghty et al., 2019)). For instance, despite an association of reduced risk of MTCT with the maternal FcγRIIIa V allele in a South African cohort (Lassaunière et al., 2016) and associations of this allele with HIV-1 control in Caucasian Americans (Deepe et al., 2012), the African American cohort from Baltimore contrastingly associated the FcγRIIIa V allele with HIV-1 infection risk and increased disease progression (Poonia et al., 2010). Therefore, demographics may account for variable results.

FcγRIIa as a mediator of ADCP

FcγRIIa (CD32a) is a functionally diverse receptor that is expressed on all myeloid cells including neutrophils, eosinophils, basophils, mast cells, platelets megakaryocytes, monocytes, macrophages, and dendritic cells (reviewed in (Gillis et al., 2014)). Interestingly, this receptor is exclusively expressed in humans and has not been found in other species (reviewed in (Bruhns and Jönsson, 2015)). The wide expression of FcγRIIa on myeloid cells corresponds to its functional diversity, with immunological functions such as ADCC, cytokine production, DC maturation, generating reactive oxygen species (van de Winkel et al., 1989, Anderson et al., 1990, Löfgren et al., 1999, Boruchov et al., 2005, Antczak et al., 2011), and mainly ADCP (van de Winkel and Capel, 1993, Bruhns, 2012). Similar to

FCGR3A, *FCGR2A* contains a functional polymorphism that alters antibody binding affinity. An A to G point mutation at nucleotide 519 alters a histidine (H) to arginine (R) at position 131 (H131R, rs1801274). The 131H variant has higher binding affinity for IgG1 and IgG2 (Warmerdam et al., 1991, Salmon et al., 1992), with a consequent enhancement of effector function capacity compared to 131R (Warmerdam et al., 1990, Bruhns et al., 2009, Bruhns, 2012). FcγRIIa H131R polymorphism has been linked to autoimmune and infectious diseases such as systemic lupus erythematosus and bacterial infections, respectively (Zegers et al., 1994, Haseley et al., 1997).

In the context of HIV-1-specific ADCP responses, the role of FcγRIIa genetics has been implicated in the association of the heterozygous FcγRIIa 131HR allele and the homozygous high affinity FcγRIIa 131HH allele with slowing down HIV-1 disease progression, compared to the FcγRIIa 131RR allele associated with accelerated disease progression (Forthal et al., 2007). It was further demonstrated that the presence of at least one H allele is required for binding to IgG2, and binding to IgG1 and IgG3 is higher compared to binding to receptors with the R allele (Bruhns et al., 2009), thus suggesting higher ADCP responses in individuals bearing at least one H allele. However, other studies have not seen an association between FcγRIIa polymorphisms and HIV-1 disease progression, as observed in Kenyan female sex workers (Weis et al., 2015). More studies are required to define the roles of FcγR SNPs in HIV-1 control and disease progression.

1.3.3 NK cells

NK cells are large lymphocytes that form an essential part of the innate immune system. They can interface with the adaptive immune system through interaction with dendritic cells and T cells (Cooper et al., 2001, Moretta et al., 2002a, Caligiuri, 2008). Following their development and differentiation in the bone marrow, they enter into circulation where they constitute up to only 15% within total blood lymphocytes (Lanier et al., 1986). Despite their small population in circulation, NK cells play an essential role as effectors of the innate immune system (Trinchieri, 1989, Pollara et al., 2011). A complex network of surface activating and inhibiting receptors that recognize MHC class I molecules regulate the functionality of NK cells (Lanier et al., 1998, Moretta et al., 2002a, Moretta et al., 2002b, Lanier, 2005). In addition to these, NK cells express co-activating accessory receptors that regulate their response to targets that do not have MHC class I molecules. This plethora of receptors contribute to the overall capacity of NK cells to serve as effector cells in antiviral immune responses such as ADCC. Increased susceptibility to viral attack has been associated

with NK cell deficiency (Fleisher et al., 1982, Biron et al., 1989). The NK cell-mediated ADCC mechanism can be modified by a number of factors, including disease progression, NK cell maturation, FcγR genotypic variants and NK cell subset distribution (Parsons et al., 2014, Gooneratne et al., 2015).

NK cell subsets

NK cells can be differentiated into several subsets based on their relative CD56 and FcγRIIIa expression (Cooper et al., 2001). The predominant CD56^{dim}FcγRIIIa^{bright} NK cell subset constitutes about 90% of total circulating NK cells in healthy individuals. This NK subset expresses higher levels of FcγRIIIa, secretes high levels of perforin and granzyme, and low levels of cytokines, and is the most cytotoxic NK cell subset (Moretta, 2010). Within the total CD56^{dim} population, a less characterised minor CD56^{dim}FcγRIIIa^{dim/neg} population has been observed (Moretta, 2010). Other minor populations include the CD56^{bright}FcγRIIIa^{dim/neg} subset with limited cytotoxic potential but higher cytokine production as well as the CD56^{neg}FcγRIIIa^{bright} NK subset that is rarely present in healthy individuals and expands during chronic HIV-1 infection (Mavilio et al., 2005, Brunetta et al., 2010). The CD56^{neg}FcγRIIIa^{bright} NK subset is labelled dysfunctional or pathologic due to its significantly reduced cytotoxic activity that adversely impacts the overall ADCC-mediating capacity of the entire NK cell population in virus infected individuals (Cooper et al., 2001).

NK cell responses and HIV-1 infection

Epidemiological and functional evidence suggest a crucial role for NK cells in the control of HIV-1 replication (Martin et al., 2002, Alter et al., 2007a, Martin et al., 2007). Around the third day of the acute phase, NK cells proliferate and expand before plasma viraemia reaches peak levels *ex vivo* (Alter et al., 2007b, Clay et al., 2007, Borrow and Bhardwaj, 2008, Alter et al., 2009). These cells mediate control of viral replication through cytolysis of infected target cells and production of chemokines and cytokines. Following establishment of the adaptive immune response at the acute and early chronic phase of HIV-1 infection, NK cells interact with HIV-1-specific antibodies to mediate ADCC responses (Chung et al., 2011a, Chen et al., 2018) which are associated with lower viral set point in primary HIV-1 infection (Chen et al., 2018).

As HIV-1 infection progresses, the virus evolves mechanisms to evade NK cell recognition. These include reduced expression of NK cell receptors ligands on infected cells (Ward et al., 2007, Goonetilleke et al., 2009, Carrington and Alter, 2012, Ploquin et al., 2012) as well as

inducing functional and phenotypic abnormalities of NK cells that lead to NK cell decline and dysfunctionality (Mavilio et al., 2003, Fauci et al., 2005, Iannello et al., 2008, Hong et al., 2010). Consequently, during chronic HIV-1 infection, the proportion of cytotoxic CD56^{dim}FCγRIIIa^{bright} NK subset declines and is replaced by an expansion of the dysfunctional CD56^{neg}FCγRIIIa^{bright} subset (Mavilio et al., 2005, Brunetta et al., 2010, Hong et al., 2010). The latter lacks overall NK cell effector functions and causes impairment of total NK cell immune responses (Brunetta et al., 2010). It is unclear whether the impaired NK cell effector function might cause induction of FCγRIIIa expression on other cytotoxic cell populations, such as CD8⁺ T cells, to compensate for loss of NK cell activity, as implicated to occur during the aging process (reviewed in (Pereira and Akbar, 2016)).

1.3.4 CD8⁺ T cells

CD8⁺ T cells typically mediate lysis of infected and abnormal cells through recognition of antigen presented on major histocompatibility complex (MHC) molecules expressed on cell surfaces. These classical cytotoxic functions occur primarily along three pathways, namely, the Fas-Fas ligand pathway, granule exocytosis (perforin and granzymes), and release of cytotoxic cytokines (tumor necrosis factor and lymphotoxin) (Hsieh, 2014).

HIV-1-specific CD8⁺ T cell responses

Studies on CD8⁺ T cell responses in the course of an HIV-1 infection are largely based on the MHC-restricted mechanisms (reviewed in (Walker and McMichael, 2012)). These CD8⁺ T cell responses occur in the early acute phases of HIV-1 infection, before the first antibodies are detected (Koup et al., 1994, Pantaleo et al., 1994, Wilson et al., 2000), and peak two weeks later where they play an important role in viral replication control (Koup et al., 1994, Goonetilleke et al., 2009). CD8⁺ T cells suppress viral replication and/or eliminate virus-infected cells through their polyfunctionality, which involves secretion of soluble antiviral factors and via their lytic cell killing functions (independent of antibodies) (Yang et al., 1997, Purbhoo et al., 2001, Stinchcombe et al., 2001, Faroudi et al., 2003, Zloza et al., 2009). Following viral load decline, viral sequences mutate in response to ongoing immune pressure and sequences are changed drastically from the original founder virus (Bernardin et al., 2005, Goonetilleke et al., 2009). Founder virus epitopes are then eliminated from plasma, followed by a rapid decline in the respective epitope-specific CD8⁺ T cell responses.

During the chronic phase of HIV-1 infection, continuous CD8⁺ T cell activation eventually leads to exhaustion, with effects of declining functionality. As a consequence, plasma

viraemia rises and HIV-1 disease progression is no longer controlled through CD8⁺ T cell responses (Khaitan and Unutmaz, 2011).

Nonconventional CD8⁺ T cells

Although the majority of T lymphocytes utilize the MHC system, discovery of non-conventional T cells that are non-MHC-restricted are increasingly appreciated in the protection against invading pathogens (reviewed in (Godfrey et al., 2015)). These cell types join features of the innate and adaptive immune systems, as seen with the CD8⁺ T cells with NK-like properties (reviewed in (Arosa, 2017)). NK-like CD8⁺ T cells are multifunctional lymphocytes largely found in peripheral tissues and organs (Smolders et al., 2013, Thome and Farber, 2015). Their diverse function includes protection against pathogen invasion, regeneration of tissues and healing of wounds (Ramirez et al., 2015, Vasanthakumar and Kallies, 2015). Although emergence of this cell population warrants further investigation, their expansion has been associated with aging (reviewed in (Michel et al., 2016, Pita-López et al., 2016), and virus infections (Bjorkstrom et al., 2008, Clemenceau et al., 2008). Of particular interest to the current study are cytotoxic FcγRIIIa⁺ CD8⁺ T cells based on their capacity to mediate FcγR-mediated effector functions.

FcγRIIIa⁺ CD8⁺ T cells

Although CD8⁺ T cells are crucial lymphocytes that play an important role in eliminating infected and abnormal cells, their ADCC-mediating capacity was not always recognized. This is despite the ability of αβ T cells and γδ T cells to express FcγRIIIa and thus mediate cytotoxicity through granzyme B and perforin release being demonstrated since the 1980's (Lanier et al., 1985, Braakman et al., 1992, Drugarin et al., 1996, Clemenceau et al., 2008). Fc-mediated effector function capacity of CD8⁺ T cells was linked with the presence of chronic virus infections, including hepatitis C virus and Epstein Barr virus, which trigger induction of FcγRIIIa expression on these cells (Bjorkstrom et al., 2008, Clemenceau et al., 2008). This FcγRIIIa⁺CD8⁺ T cell subset displays NK cell-like functional properties, including ADCC activity and the release of pore forming perforin and serine protease granzyme B (Bjorkstrom et al., 2008, Clemenceau et al., 2008). Furthermore, FcγRIIIa⁺ CD8⁺ T cells were also demonstrated to be present at higher proportions in virus infected compared to uninfected individuals (Bjorkstrom et al., 2008, Clemenceau et al., 2008, Clemenceau et al., 2011). However, the presence and role of this CD8⁺ T cell subset in HIV-1 infection is not well studied. Nayiluma et al. very recently described HIV-1-specific ADCC mediating capacity of FcγRIIIa⁺ CD8⁺ T cells comparable to that of NK cells, as well as the expansion

of this population in chronically HIV-1 infected individuals who are not on ART (Naluyima et al., 2019). The aforementioned study succeeded and cited the original work described for this cell type in the present thesis (presented in Chapter 2 and published (Phaahla et al., 2019)).

1.3.5 Phagocytes

The word phagocytes, first coined in 1884 (Metchnikoff, 1884), describes cells that protect the body by engulfing pathogens, harmful foreign molecules, and dead/dying cells through an antibody dependent or independent mechanism of phagocytosis, ultimately resulting in the death of invaders and elimination of waste and debris (Metchnikoff, 1884). Phagocytes play important effector and immunomodulatory roles in both the innate and adaptive arms of immune responses, which is largely a consequence of the wide range of FcγRs and complement receptors they express (Dale et al., 2008). Distinct effector functions may occur from antibody-opsonised target and phagocyte interactions on the basis of the type of phagocyte within a particular tissue location and period following infection (Guilliams et al., 2014). Monocytes are one of the well characterised phagocytes known to have diverse activation when their FcγRs are engaged to antibody-opsonised targets. The resulting immune responses include ADCP, ADCC, antigen presentation, and immunomodulation. In addition, monocytes are mononuclear phagocytes that retain their capacity to proliferate and are thus precursors for other phagocytes: macrophages and dendritic cells (Szabolcs et al., 1996, Zhou and Tedder, 1996, Palucka et al., 1998).

Phagocytes in HIV-1 infection

Although phagocytes that mediate ADCP in HIV-1 infection are not well studied compared to cells that mediate HIV-1-specific ADCC, the reduction of FcγRIIa expression on phagocytes such as monocytes and dendritic cells in chronic HIV-1 infection has been implicated in impaired antibody mediated phagocytosis (Dugast et al., 2011). The study that recently compared ADCP mediated by various phagocytes using a method adapted from Ackerman et al. (Ackerman et al., 2011) found that the ADCP profile of the THP-1 cell line differed to that of neutrophils, and that primary monocytes showed high donor-to-donor variation that hindered data analysis (Powell et al., 2019). Therefore, despite its drawbacks, the THP-1 mediated bead-uptake method (Ackerman et al., 2011) remains one of the more commonly used for antibody activity assessment because of the THP-1 cell line consistency (Ackerman et al., 2013b, Ana-Sosa-Batiz et al., 2014, Ackerman et al., 2016, Gach et al., 2017). However, this leaves *in vivo* HIV-1-specific ADCP by different autologous phagocytes

largely unexplored and little known. For instance, in contrast to being associated with protective immune responses, ADCP is also known to associate with a worse disease outcome in a number of disease settings including HIV-1 infection in a process called antibody-dependent enhancement (ADE) (Reviewed in (Tay et al., 2019)). Although the exact mechanism is not well understood, it is postulated to involve the fact that phagocytes such as macrophages and monocytes are targets for HIV-1 infection and have been reported to be reservoirs of the virus (Crowe et al., 1987, Carter and Ehrlich, 2008, Coleman and Wu, 2009). Such observations warrant further investigation in the context of HIV-1 control or disease progression and ADCP capacity.

1.4 Study Aims and Objectives

The overall aim of the current study was to characterize host factors that may modulate HIV-1-specific FcγR-mediated effector functions (ADCC and ADCP) in the Black South African population in a quantitative cohort study that is experimental in nature.

Objective 1. To define the proportion and distribution of FcγRIIIa-expressing lymphocytes (NK cells and CD8⁺ T cells) and characterize HIV-1-specific ADCC responses according to FcγRIIIa F158V functional variants in HIV-1 viraemic individuals and uninfected controls.

Hypothesis: We hypothesize that FcγR expression, cellular distribution and functional consequences of FcγRIIIa genetic variants are altered in the presence of an HIV-1 infection which affects the capacity of cytotoxic lymphocytes to mediate ADCC.

Objective 2. To characterize the role of bulk IgG levels as well as HIV-1 gp120-specific antibody levels, according to isotype and subclass, in HIV-1-specific ADCC responses in HIV-1 elite controllers, viraemic controllers and progressors.

Hypothesis: We hypothesize that hypergammaglobulinaemia modulates HIV-1-specific effector functions and can, in part, explain differences in ADCC capacity observed between individuals with variable disease progression profiles.

Objective 3. To investigate the modulation of HIV-1-specific ADCP responses by bulk IgG and gp120-specific IgG isotype and IgG1-IgG4 subclass levels in HIV-1 infected controllers compared to progressors.

Hypothesis: We hypothesize that hypergammaglobulinaemia as well as levels and distribution of HIV-1-specific IgG subclasses modulate ADCP responses.

The methods used will all be discussed in each results chapter.

Chapter 2: Chronic HIV-1 Infection Alters the Cellular Distribution of Fc-Gamma-Receptor IIIa and the Functional Consequence of the Fc-Gamma-Receptor IIIa-F158V Variant

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2.1 Introduction

Receptors for the Fc domain of immunoglobulin G (IgG), so called Fc-gamma-receptors (FcγRs), link the specificity of IgG with potent effector functions of the innate immune system. FcγRs comprise a family of activating (FcγRI, FcγRIIa, and FcγRIIIa) and inhibiting (FcγRIIb) receptors that are differentially expressed on innate immune cells such as natural killer (NK) cells, monocytes, dendritic cells, neutrophils and granulocytes (Nimmerjahn and Ravetch, 2008, Bournazos and Ravetch, 2017b, Rosales, 2017). During an infection, these receptors play an important role in activating IgG-induced protective inflammatory processes and regulating immune responses (Ravetch and Bolland, 2001, Nimmerjahn and Ravetch, 2005, Ackerman et al., 2012, Wren et al., 2013).

Increasing evidence support an important role for FcγR-mediated effector functions, in particular antibody-dependent cellular cytotoxicity (ADCC), in HIV-1-specific immunity (Ackerman et al., 2012, Lewis, 2014). In adults, ADCC has been associated with a reduced risk of HIV-1 acquisition in the RV144 vaccine trial, and in infants born to HIV-1 infected mothers, passively acquired ADCC activity associated with reduced mortality (Milligan et al., 2015). Similarly, ADCC responses associate with slower disease progression in HIV-1 infected adults (Ahmad et al., 2001, Gomez-Roman et al., 2005, Hessel et al., 2007, Lambotte et al., 2009).

In the periphery, CD56^{dim}FcγRIIIa^{bright} NK cells are the primary effectors of ADCC responses (Pollara et al., 2011). During a chronic HIV-1 infection, however, shedding of FcγRIIIa from the surface of cytotoxic CD56^{dim}FcγRIIIa^{bright} NK cells together with an increase in the CD56^{neg}FcγRIIIa^{bright} NK cell subset leads to a significant reduction in NK cell function (Mavilio et al., 2005, Brunetta et al., 2010). It is unknown whether this loss in NK cell-mediated ADCC capacity is compensated for by other cytotoxic cells, where FcγR expression is induced upon immune cell activation. For chronic hepatitis C virus or Epstein Barr virus infections, for instance, it has been shown that FcγRIIIa expression is induced on an effector memory CD8⁺ T cell subset (Bjorkstrom et al., 2008, Clemenceau et al., 2008). This FcγRIIIa⁺CD8⁺ T cell subset acquires NK cell-like functional properties, including ADCC activity accompanied by the release of pore forming perforin and serine protease granzyme B (Bjorkstrom et al., 2008, Clemenceau et al., 2008). The presence and role of this CD8⁺ T cell subset in HIV-1 infection is currently undefined.

In addition to the effect of an actively replicating virus on FcγRIIIa expression, host genetics also contribute to variability of FcγRIIIa expression and/or ADCC responses. *FCGR3A* copy number variation directly correlates with the surface density of FcγRIIIa, with individuals

bearing a single *FCGR3A* copy and correspondingly lower FcγRIIIa surface densities, having reduced ADCC responses compared to individuals with two or more gene copies (Breunis et al., 2009). In addition, a phenylalanine (F) to valine (V) substitution at amino acid 158 in the proximal Ig-like domain of FcγRIIIa confers increased binding for IgG1, IgG3 and IgG4, which has been associated with higher NK cell activation and ADCC responses (Koene et al., 1997, Wu et al., 1997, Bruhns et al., 2009). Unlike *FCGR3A* copy number and the FcγRIIIa-F158V variant, a deletion of a copy number variable region (CNR) encompassing *FCGR3B* and *FCGR2C* – known as CNR1 – does not affect FcγRIIIa directly (Niederer et al., 2010). However, it juxtaposes the 5'-regulatory sequences of *FCGR2C* with the open reading frame of *FCGR2B*, creating a chimeric *FCGR2B'* gene (Mueller et al., 2013). This results in the expression of the inhibitory FcγRIIb on NK cells where it regulates FcγRIIIa-mediated ADCC responses (van der Heijden et al., 2012, Mueller et al., 2013).

FCGR variants are rarely adjusted for in studies that compare NK cell-mediated ADCC capacity between HIV-positive and HIV-negative individuals. Moreover, it is unclear if the altered immune milieu accompanying an HIV-1 infection modulates the functional consequences of the aforementioned variants. In this study, we sought to characterize FcγRIIIa expression on cytotoxic lymphocytes – NK cells and CD8⁺ T cells – and associated ADCC responses in healthy donors and viraemic HIV-1 individuals matched for *FCGR* genetic variants.

2.2 Materials and Methods

2.2.1 Cohort

All study participants were black South Africans recruited from the city of Johannesburg, Gauteng province, South Africa (Table 2.1). Self-reported HIV-1 uninfected individuals who did not have an acute or chronic illness at the time of sample collection were prospectively recruited from the National Institute for Communicable Diseases as healthy controls. Viraemic, treatment naïve HIV-1 infected individuals were identified from an existing cohort recruited from hospitals in Johannesburg and Soweto. Samples used for this cohort were collected and stored between 2015 and 2017. In 2015, the South African national guidelines for antiretroviral treatment initiated the prioritization of patients with CD4 counts below 500 cells/μl or with severe HIV disease irrespective of CD4 count. The average CD4 count at the time of sample collection was >500 cells/μl for participants (508 and 562 cells/μl for 158FF and 158FV/VV individuals, respectively) (Table 2.1). This study was carried out in accordance with the recommendations of the National Health Research Ethics Council

(NHREC) of the South African Department of Health. The protocol was approved by the University of the Witwatersrand Ethics Committee (Ethics clearance certificate no. M1511102). All participants provided written informed consent in accordance with the Declaration of Helsinki.

Table 2.1. Clinical and demographic characteristics of study cohort.

	HIV-1 negative	HIV-1 positive	P-value
<i>N</i>	23	23	
Age [mean (SD)]	36.3 [8.5]	40.2 (8.8)	0.143*
Gender [% Females]	69.6	82.6	0.491*
HIV-1 Viral load [median (IQR)]			
158FF	-	6931 (3598-15090)	0.563†
158FV/VV	-	4732 (1134-11430)	
CD4⁺ T cell count [mean (SD)]			
158FF	-	508 (267)	0.629†
158FV/VV	-	562 (261)	
Other diseases or infections			
<i>Mycobacterium tuberculosis</i>	0 (0%)	2 (8.7%)	0.489*

*Comparison between HIV-1 negative and HIV-1 positive groups.

†Comparison between genotypes.

2.2.2 FCGR variant genotyping

Genomic DNA was isolated from ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood. Study participants were genotyped for *FCGR* variants using the *FCGR*-specific multiplex ligation-dependent probe amplification (MLPA) assay (MRC Holland, Amsterdam, The Netherlands) as previously described (Schouten et al., 2002, Breunis et al., 2008). In the negative control sample, DNA was replaced with TE (10 mM Tris-HCl pH 8.2 + 0.1 mM EDTA) buffer. This assay detects the genomic copy number of *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A*, and *FCGR3B* and functional allelic variants that include FcγRIIa-H131R (alias H166R, c.497A>G, rs1801274), FcγRIIb-I232T (c.695T>C, rs1050501), FcγRIIIa-F158V (alias F176V, c.634T>G, rs396991), FcγRIIIb-HNA1a/b/c, *FCGR2C* gene expression variants c.169T>C (X57Q) and c.798+1A>G (rs76277413), and the *FCGR2B/C* promoter variants c.-386G>C (rs3219018) and c.-120T>A (rs34701572) in two multiplex reactions.

Capillary electrophoresis was used to separate the MLPA assay amplicons on an ABI Genetic Analyzer 3500. Data were analyzed with Coffalyser.NET software created by the MLPA assay manufacturer, MRC Holland.

2.2.3 Monoclonal antibodies and flow cytometric analysis

Frozen PBMCs were thawed and rested overnight in R10 media [10% FCS (Gibco) + 0.1% Gentamicin (Sigma) in RPMI 1640 (Gibco)] at 37°C, 5% CO₂. On the following day, 5 x 10⁶ PBMCs resuspended in 1ml phosphate-buffered saline (PBS) were stained with the BD Horizon™ Fixable Viability Stain 510 (1:1000 dilution), which was compensated with the ArC amine reactive compensation bead kit (Thermo Scientific) according to manufacturer's instructions. Subsequently, multicolor staining was performed with antibody concentrations determined from prior titrations. The following monoclonal antibodies were added to 100 µl final volume of 1 x 10⁶ cells/ml PBMCs in a FACS tube: 5 µl CD3-PerCP (SK7), 2.5 µl CD56-AF647 (B159), 1 µl CD8-APC-H7 (SK1), and 0.25 µl CD16-PE (3G8) for determination of CD16 (FcγRIIIa) expression on NK cells and CD8 T cells; as well as 5 µl CD3 PerCP (SK7), 2.5 µl CD56-AF647 (B159), 5 µl CD16-FITC (NKP15), and 2 µl CD32-PE (FLI8.26) (all antibodies obtained from BD Biosciences) to identify CD32 (FcγRIIa) expression on NK cells in a separate FACS tube. FACS tubes were gently vortexed and then incubated for 15 minutes in the dark at room temperature, followed by washing with 3 ml FACS flow and subsequent centrifugation at 2000 rpm for 5 minutes. After resuspension in 200 µl of 1% paraformaldehyde, samples were stored at 4°C until acquisition on a BD Fortessa X20 (BD Biosciences) and analyzed on FlowJo version 9.8.1 software (Tree Star, San Carlos, CA, USA). Each monoclonal antibody was compensated with BD™ CompBeads. Fluorescence-minus-one (FMO) controls were used to set the appropriate gates for analyses.

2.2.4 Isolation of specific cell populations

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated whole blood using Ficoll-Paque™ PLUS density gradient centrifugation (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and stored at -80°C. NK cells and CD8⁺ T cells were positively selected from overnight rested PBMCs using MACS® magnetic cell separation technology (Miltenyl Biotec, Bergisch Gladbach, Germany). To limit the presence of NK cells in the enriched CD8⁺ T cell preparation, NK cells were first isolated from PBMCs using CD56⁺ beads prior to isolation of CD8⁺ T cells with CD8⁺ beads. Viability and number of each participant's NK and CD8⁺ T cells were determined by trypan blue exclusion through direct cell counting on a haemocytometer. Cells with >80% viability were used for subsequent

tests. The purity of enriched cell fractions was determined by flow cytometric phenotyping assays using the following monoclonal antibodies: CD3-PerCP (SK7), CD56-AF647 (B159), and CD8-APC-H7 (SK1), as well as the BD Horizon™ Fixable Viability Stain 510. For the purity of CD8 T cells from individuals that were selected for ADCC analysis, the proportion of CD3⁺CD8⁺ cells in the live cell population was considered pure with a median purity of 95.6 %. Whereas for NK cells, the proportion of CD3⁻CD56⁺ cells in the live cell population was considered pure for all individuals.

2.2.5 Antibodies

ADCC capacity was evaluated using pooled IgG isolated from the HIV-1 infected individuals included in the study. IgG was isolated from plasma using the Melon Gel IgG Purification kit according to the manufacturer's instructions (Thermo Scientific). In brief, 500 µl of resuspended Purification Support (gel slurry) was dispensed into a spin column placed in a microcentrifuge tube. The uncapped column was centrifuged at 6000 × g (all centrifugation steps performed at this speed) for 1 minute followed by washing with 300 µl Purification Buffer twice and pulse centrifugation for 10 seconds. After discarding flow-through 100 µl of 1:10 diluted plasma sample was added to the column. The capped column was incubated for 5 minutes at room temperature with end-over-end mixing. Following a 1 minute spin, purified IgG was collected and detected with the Bicinchoninic acid (BCA) assay (Sigma-Aldrich, St. Louis, MO, USA) on a VersaMax microplate reader (Molecular Devices, Silicon Valley, CA, USA) at 562 nm. HIV immune globulin (HIVIG; NIH AIDS reagent program) was used as a positive control.

2.2.6 CEM.NK^R.CCR5 as targets

The human T-lymphoblastoid cell line, CEM.NK^RCCR5 (NIH AIDS Research and Reference Reagent Program, NAIAD, NIH), was used as a target in HIV-1-specific ADCC assays and the cells were maintained in R10 media [10% FCS (Gibco) + 0.1% Gentamicin (Sigma) in RPMI 1640 (Gibco)] at densities below 1 × 10⁶ cells per ml and kept at 37°C, 5% CO₂ in an incubator. Following three days of incubation, cultured cells were centrifuged at 400 × g for 5 minutes, and resuspended in fresh 10 ml R10 media after discarding supernatant for cell maintenance. For the ADCC assay, HIV-1-specific Consensus C (ConC) gp120 (a gift from Prof L Morris, Virology, NICD) was added to a final concentration of 2.5 µg/ml to 1 × 10⁶ CEM.NK^RCCR5 target cells re-suspended in 1 ml R10 media, followed by incubation at 37°C for 75 minutes with gentle flicking every 30 minutes (Pollara et al., 2011).

2.2.7 ADCC

NK cells and CD8⁺ T cells both utilize the pore forming perforin and serine protease granzyme B during cytotoxic responses. It is therefore possible to evaluate ADCC responses of both NK cells and CD8⁺ T cells with the HIV-specific GranToxiLux assay (Pollara et al., 2011). Since concentrations of isolated IgG used in the ADCC assay varies notably across studies, the optimal concentration of isolated IgG (30 µg/ml) was determined empirically. In this assay, 1 ml of HIV-1 gp120 coated CEM.NK^RCCR5 cells (1×10^6 cells/ml) were combined with 1 µl fluorescent target cell marker (TFL4, OncoImmunin, Gaithersburg, MD, USA) and 1 µl viability marker (NFL1; OncoImmunin) and incubated for 15 minutes at 37°C. Following incubation, 25 µl gp120-coated target cells and 25 µl effector cells (NK cells or CD8⁺ T cells) (E:T ratio of 10:1) were co-cultured with 25 µl purified IgG sample from each HIV-1 infected and uninfected individual and 75 µl granzyme B substrate that fluoresces when cleaved by granzyme B. Following a 45 minute incubation, cells were washed, resuspended in 200 µl Wash Buffer, and stored at 4°C until acquisition on the BD Fortessa X20 (BD biosciences, San Jose, CA, USA) and data were analyzed using FlowJo version 7.6.1 software (Tree Star, San Carlos, CA, USA). ADCC responses were reported as the proportion of granzyme B-positive (%GzB) live target cells (Flow plots shown in Appendix 1, Fig S1). Granzyme B activity in the absence of any antibody (background killing) was determined for each subject – NK cells and CD8⁺ T cells – and subtracted from granzyme B activity in the presence of antibody. To minimize inter-assay variation between groups, participants in the different groups were proportionally represented in each ADCC assay performed. The inter assay coefficient of variation, as calculated from a HIVIG-specific ADCC response measured for a single donor included in every run, was <10%. The HIV status of HIV-1 uninfected individuals was indirectly confirmed through data analysis of Granzyme B activity of their antibodies, which is expected to be below a certain baseline across a series of dilutions. Granzyme B activity above the said baseline was considered an outlier, and therefore excluded from the data because it indicated possibility of an HIV-1 infection. All the self-reported HIV negative controls met this criteria.

To assess CD8⁺ T cell mediated granzyme B responses, HIV-1 positive donors with <5% NK cell contamination in their enriched CD8⁺ T cell fractions were identified and selected for further analysis. This proportion of NK cells equates to a <0.5:1 NK-to-target ratio in a 10:1 CD8-to-target preparation. In our experience, the NK cell-mediated granzyme B activity at 0.5:1 is on average 14.1% (standard deviation [SD]: 2.8%) of that observed for NK cells at a ratio of 10:1 (Fig 2.1). Using these data, a positivity threshold for CD8⁺ T cell-mediated

granzyme B responses was calculated relative to autologous NK cell-mediated granzyme B responses whereby the mean granzyme B activity for autologous NK cells (E:T = 10:1) was multiplied with 22.4% (14.1% + 3×SD:2.8%).

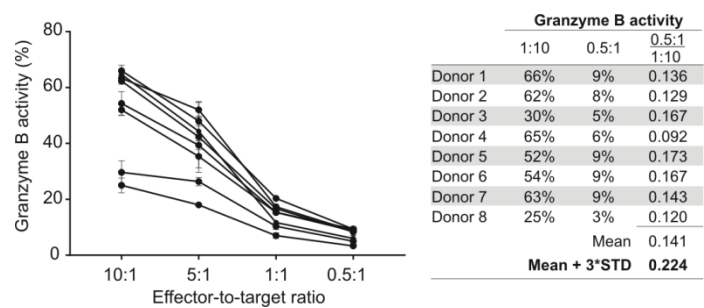


Figure 2.1. NK cell mediated ADCC responses at different effector-to-target (E:T) cell ratios. NK cells isolated from eight HIV-1 negative donors were tested at different E:T ratios in an HIV-specific GranToxiLux assay using an ADCC-mediating monoclonal antibody (A32) at 2.5 µg/ml. The mean fraction of Granzyme B activity observed at an E:T of 0.5:1 relative to 10:1 was used to calculate a 99% confidence level cut-off value for NK cell-mediated ADCC responses at an E:T of 0.5:1. Data points represent the mean of triplicate measures and the standard deviation indicated by error bars.

2.2.8 Statistics

All statistical analyses were performed using GraphPad Prism 7 software version 7.04 (GraphPad Software). P-values from 2-tailed tests less than 0.05 were considered statistically significant. The significance of differences between unpaired data sets were analysed with the Mann-Whitney U tests and paired data sets with the Wilcoxon matched-pairs signed rank test. The significance of differences between more than two data sets were analysed using Kruskal-Wallis tests. Correlation analyses of data between two groups were assessed using the nonparametric Spearman rank correlation coefficient. To determine the role of FcγRIIIa-F158V alleles in ADCC responses, the V allele was studied under a dominant model due to the low prevalence of VV homozygotes (Lassaunière and Tiemessen, 2015).

2.3 Results

2.3.1 Study population

Thirty-seven chronic HIV-1 infected, viraemic, and antiretroviral naïve individuals with sufficient sample available were identified from an existing cohort of HIV-1 positive individuals (Fig 2.2). Following *FCGR* genotyping, seven individuals were excluded due to the possession of an *FCGR3A* gene duplication (n = 1), *FCGR3A* gene deletion (n = 2), or CNR1 deletion that results in the expression of the inhibitory FcγRIIb on NK cells (n = 4). To ensure that individuals carrying an undetectable CNR1 deletion – possess a duplication of this

region on a single chromosome and a deletion on the other – were also excluded, expression of FcγRIIb on NK cells was monitored using flow cytometry. During the eligibility screening of individuals, such an individual was indeed identified and excluded from the study; thus, five HIV-1 positive individuals in total expressed FcγRIIb on their NK cells and were excluded.

Within the HIV-1 positive group, FcγRIIIa-158FF donors were paired with FcγRIIIa-158FV donors according to their HIV-1 RNA plasma viral load. Poor matching of HIV-1 RNA plasma viral load for six individuals with different genotypes resulted in them being excluded from further analysis. Twenty-three HIV-1 positive individuals were eligible for further analysis, with the FcγRIIIa-F158V genotype distribution closely resembling that observed in the general black population from the same region in South Africa (Lassaunière and Tiemessen, 2015). As a comparative group, thirty-three HIV-1 negative individuals were genotyped for *FCGR* variants. Six individuals were excluded due to the possession of an *FCGR3A* duplication (n = 1) or *CNR1* deletion (n = 5). Twenty-three eligible HIV-1 negative individuals were subsequently paired with HIV-1 positive individuals based on the FcγRIIIa-F158V genotype. None of the study participants expressed the activating FcγRIIc on their NK cells, as determined by the *FCGR2C* c.798+1A>G splice-site variant, or carried the *FCGR3A* intragenic haplotype previously associated with increased FcγRIIIa surface density (van der Heijden et al., 2012, Lassauniere et al., 2013).

Age and gender did not differ significantly between HIV-1 negative and positive donors (Table 2.1). Two HIV-1 positive individuals had tuberculosis, of which one was an FcγRIIIa-158FF donor and the other an FcγRIIIa-158FV donor. Their TB infection status was diagnosed from sputum samples within the three year follow-up period. However, the treatment status is unknown. No other infections were noted for these patients. Human cytomegalovirus (HCMV) infection status was not determined for study participants. However, the prevalence is likely 100% for the HIV-1 positive individuals and >85% for HIV-1 negative individuals as observed in other cohorts in rural and urban South Africa ((Schaftenaar et al., 2014) and Tiemessen, unpublished data). Other sexually transmitted infections were not tested for in the study cohort.

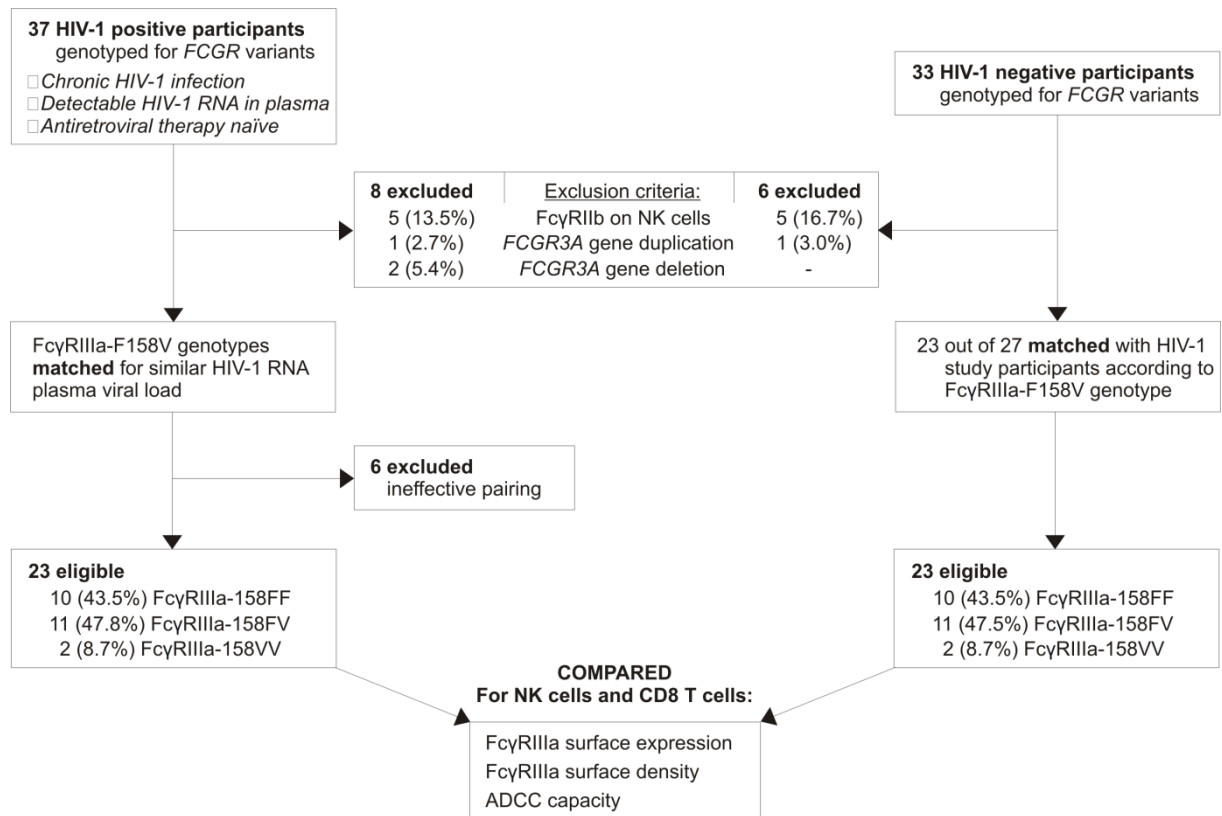


Figure 2.2. Study participant selection.

2.3.2 FcγRIIIa expression on NK cell subsets

NK cell subsets were defined based on the relative surface expression of CD56 and FcγRIIIa. Four subsets were identified that include CD56^{bright}FcγRIIIa^{dim/neg}, CD56^{dim}FcγRIIIa^{bright}, CD56^{neg}FcγRIIIa^{bright}, and CD56^{dim}FcγRIIIa^{dim/neg} (Fig 2.3A). The distribution of these subsets within the NK cell population differed significantly between HIV-1 positive and HIV-1 negative donors. Compared to the HIV-1 negative group, the HIV-1 positive group had a smaller median proportion of CD56^{dim}FcγRIIIa^{bright} cells (60.2% vs 77.0%, $P = 0.0002$, Fig 2.3B) that was offset by a larger median proportion of CD56^{neg}FcγRIIIa^{bright} cells (14.9% vs 2.1%, $P < 0.0001$, Fig 2.3B). The inverse relationship between the NK cell subsets in HIV-1 positive individuals, but not HIV-1 negative individuals, was further observed in a correlation analysis ($r = -0.633$, $P = 0.001$, Fig 2.3F). The relative proportions of CD56^{bright}FcγRIIIa^{dim/neg} and CD56^{dim}FcγRIIIa^{dim/neg} did not differ significantly between HIV-1 positive and HIV-1 negative donors. In contrast to the observed differences in NK cell subsets, FcγRIIIa surface density on neither cytotoxic CD56^{dim}FcγRIIIa^{bright} nor CD56^{neg}FcγRIIIa^{bright} NK cells differed between the two groups ($P = 0.948$ and $P = 0.486$, respectively; Fig 2.3C).

2.3.3 NK cell-mediated ADCC responses

The capacity of CD56⁺ NK cells to mediate ADCC was tested in an HIV-1-specific granzyme B assay in the presence of pooled IgG isolated from HIV-1-infected South Africans. ADCC responses, measured as granzyme B activity in target cells, were reduced in the HIV-1 positive group compared to the HIV-1 negative group, although not statistically significantly different (31.0% vs 43.3%, $P = 0.184$, Fig 2.3D). In both groups, the ADCC responses were affected by the FcγRIIIa-F158V variant (Fig 2.3E). Healthy donors bearing at least one V allele had a higher median NK cell-mediated granzyme B activity compared to those homozygous for the F allele (48.1% vs 34.1%, $P = 0.284$). This trend was, however, not observed in the HIV-1 positive group. In contrast, HIV-1 positive donors bearing at least one V allele had reduced granzyme B activity compared to those homozygous for the F allele (26.4% vs 34.6%, $P = 0.522$), although not statistically significant.

Functional differences between the various NK subsets warrant investigation of their individual correlation with ADCC responses. ADCC responses of both HIV-1 negative and HIV-1 positive donors negatively correlated with the proportion of immunoregulatory CD56^{bright}FcγRIIIa^{dim/neg} cells ($r = -0.486$, $P = 0.019$; and $r = -0.454$, $P = 0.030$, respectively; Fig 2.3F). Furthermore, ADCC responses of HIV-1 negative donors, but not HIV-1 positive donors, positively correlated with the proportion of CD56^{dim}FcγRIIIa^{bright} cells ($r = 0.665$, $P = 0.0005$; and $r = 0.233$, $P = 0.284$, respectively; Fig 2.3F). FcγRIIIa expression levels on the cytotoxic CD56^{dim}FcγRIIIa^{bright} cell subset did not correlate with ADCC responses in either HIV-1 negative or HIV-1 positive group. P-values for correlations were reported unadjusted for multiple comparisons.

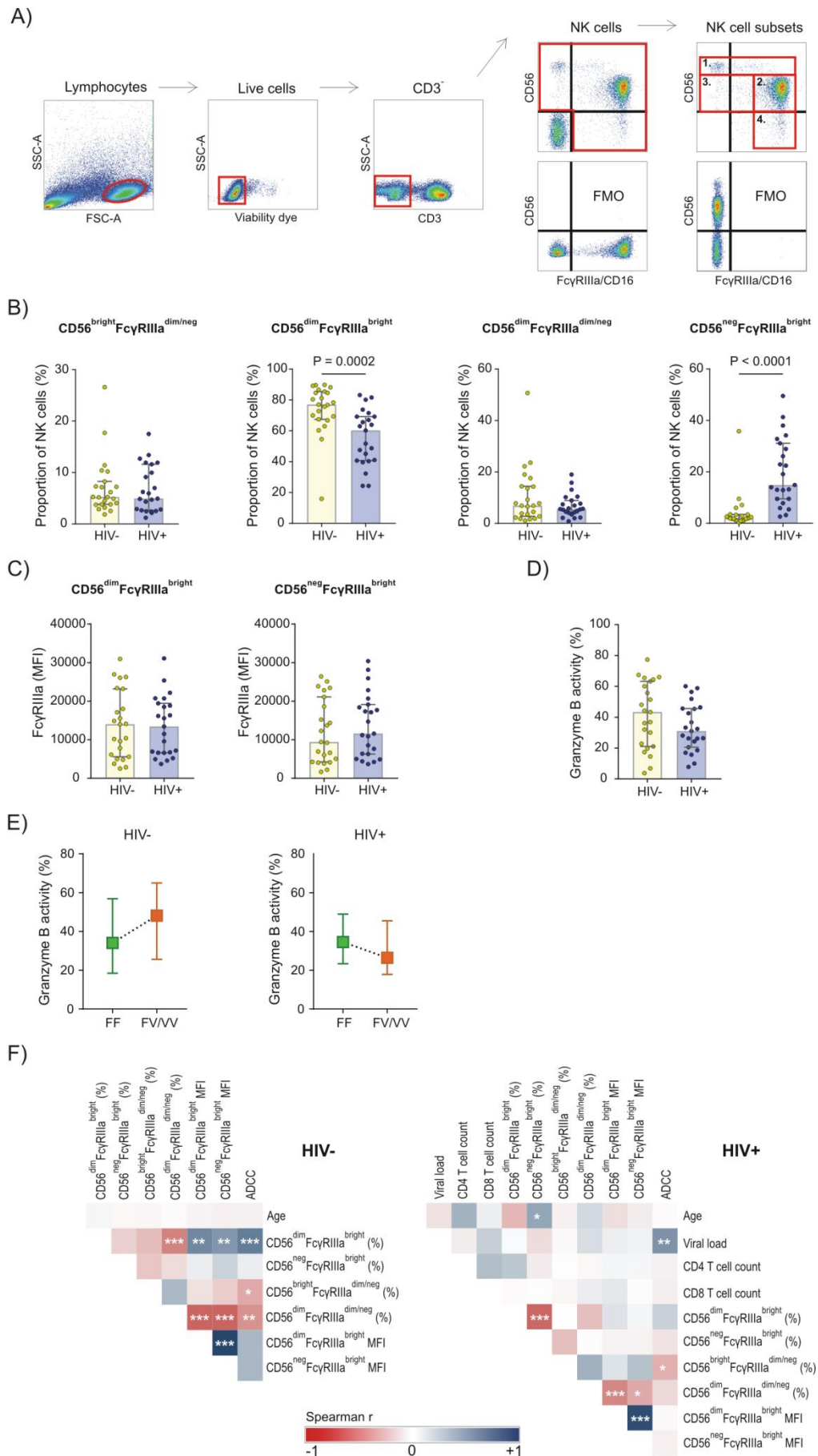


Figure 2.3. FcγRIIIa expression on NK cell subsets in HIV-1 uninfected and HIV-1 infected individuals matched for *FCGR* genetic variants.

A) Gating strategy for defining four NK cell subsets: 1. CD56^{bright}FcγRIIIa^{dim/neg}, 2. CD56^{dim}FcγRIIIa^{bright}, 3. CD56^{dim}FcγRIIIa^{dim/neg}, and 4. CD56^{neg}FcγRIIIa^{bright}. B) Comparison of NK cell subsets between HIV-1 uninfected and infected individuals. C) FcγRIIIa surface density on FcγRIIIa bright NK cell subsets. D) ADCC activity of NK cells at a effector-to-target cell ratio of 10:1 with isolated IgG pooled from HIV-1 study participants. E) Median ADCC responses for individuals homozygous for the FcγRIIIa-158F allele and individuals bearing at least one FcγRIIIa-158V allele. F) Correlation analysis between demographic, clinical, phenotypic and functional variables in HIV-1 uninfected and HIV-1 infected individuals. The colour represents the strength and direction of the correlation. Red = negative correlation, white = no correlation, and blue = positive correlation (*** P < 0.001; ** P < 0.01; * P < 0.05).

2.3.4 Expression of FcγRIIIa on CD8⁺ T cells

During the course of characterizing FcγRIIIa expression on peripheral leukocytes in whole blood obtained from a preliminary cohort of HIV-1 negative and HIV-1 positive donors, we observed a CD8^{bright} T cell subset expressing FcγRIIIa in both groups (Fig 2.4A). The proportion of FcγRIIIa⁺CD8^{bright} T cells within the CD8^{bright} T cell population varied extensively, ranging from 4.4% to 45.9%, with the median proportion significantly higher in the HIV-1 infected group compared to the healthy control group (17.8% vs 9.8%, P = 0.002, Fig 2.4B).

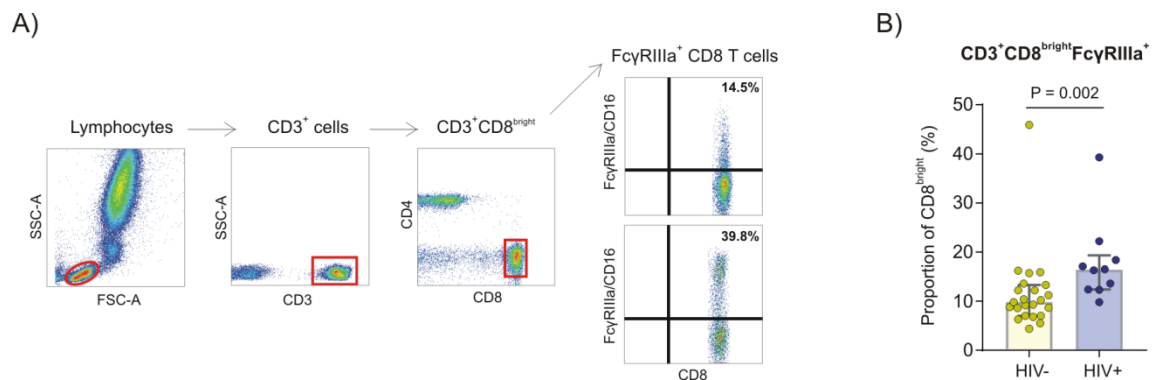


Figure 2.4. FcγRIIIa expression on CD8⁺ T cells in whole blood obtained from HIV-1 negative and HIV-1 positive donors.

A) Gating strategy showing two representative individuals with low and high proportions of FcγRIIIa⁺CD8^{bright} T cell subsets. B) Frequencies of FcγRIIIa⁺CD8^{bright} T cells in whole blood isolated from a separate preliminary cohort of ‘before’ HIV-negative healthy controls (n = 23) and HIV-infected individuals (n = 10).

In the present validation study, two CD8⁺ T cell subsets are identified, designated CD8^{bright} and CD8^{dim} (also referred to as CD8^{high} and CD8^{low}), according to the relative expression of CD8 on CD3⁺ T cells (Fig 2.5). The CD8^{dim} subset accounted for 13.9% and 13.4% of the total CD8⁺ T cell population in HIV-1 negative and HIV-1 positive donors, respectively (P = 0.978). In agreement with our observations in whole blood, 2-44.8% of CD8^{bright} cells and 2-68.4% of CD8^{dim} cells expressed FcγRIIIa. Compared to its FcγRIIIa⁺CD8^{bright} counterpart, the FcγRIIIa⁺CD8^{dim} subset expressed significantly higher levels of FcγRIIIa in both the HIV-

1 positive and negative group (471 vs 1610, $P < 0.001$; and 377 vs 740, $P < 0.001$, respectively; data not shown).

Compared to the HIV-1 negative group, the HIV-1 positive group had a significantly higher median proportion of Fc γ RIIIa⁺CD8^{bright} and Fc γ RIIIa⁺CD8^{dim} T cell subsets (15.7 vs 8.3%, $P = 0.016$ and 18.2 vs 14.1%, $P = 0.038$, respectively) and correspondingly higher median Fc γ RIIIa surface densities on both Fc γ RIIIa⁺CD8⁺ T cell subsets (471 vs 377, $P = 0.031$; and 1610 vs 740, $P = 0.021$, respectively) (Fig 2.5B). The proportion of Fc γ RIIIa⁺CD8^{bright} and Fc γ RIIIa⁺CD8^{dim} cells positively correlated in the HIV-1 negative group ($r = 0.671$, $P = 0.001$; Fig 2.5C). However, this relationship was not observed for the HIV-1 positive group. In the latter group, neither the presence nor levels of Fc γ RIIIa correlated with CD4⁺ T cell count or HIV-1 plasma viral load (Fig 2.5C). However, the proportion of Fc γ RIIIa on CD8^{bright} T cells negatively correlated with age in both groups, although this correlation was only statistically significant for the HIV-1 positive group ($r = -0.510$, $P = 0.013$; Fig 2.5C).

2.3.5 CD8⁺ T cells mediate ADCC

To assess CD8⁺ T cell-mediated ADCC responses, CD8⁺ T cells were positively selected from PBMCs after depletion of NK cells. The proportion of potential contaminating NK cells was determined using flow cytometry and six HIV-1 positive donors were identified with less than 5% NK cells in their enriched CD8⁺ T cell fractions (mean: 1.3%; range: 0.3-2.9%). A threshold for positive CD8⁺ T cell-mediated granzyme B responses (5.6%) was calculated based on the corresponding NK cell granzyme B responses at an NK-to-target ratio of 10:1 (described in section 2.2.7). Five out of six donors had positive CD8⁺ T cell-mediated granzyme B responses that ranged from 7.5% to 25.6% (mean: 14.5%; Fig 2.5D). Overall, the CD8⁺ T cell-mediated granzyme B activity was comparatively lower than that observed for their NK cell counterparts ($P = 0.031$, Fig 2.5D).

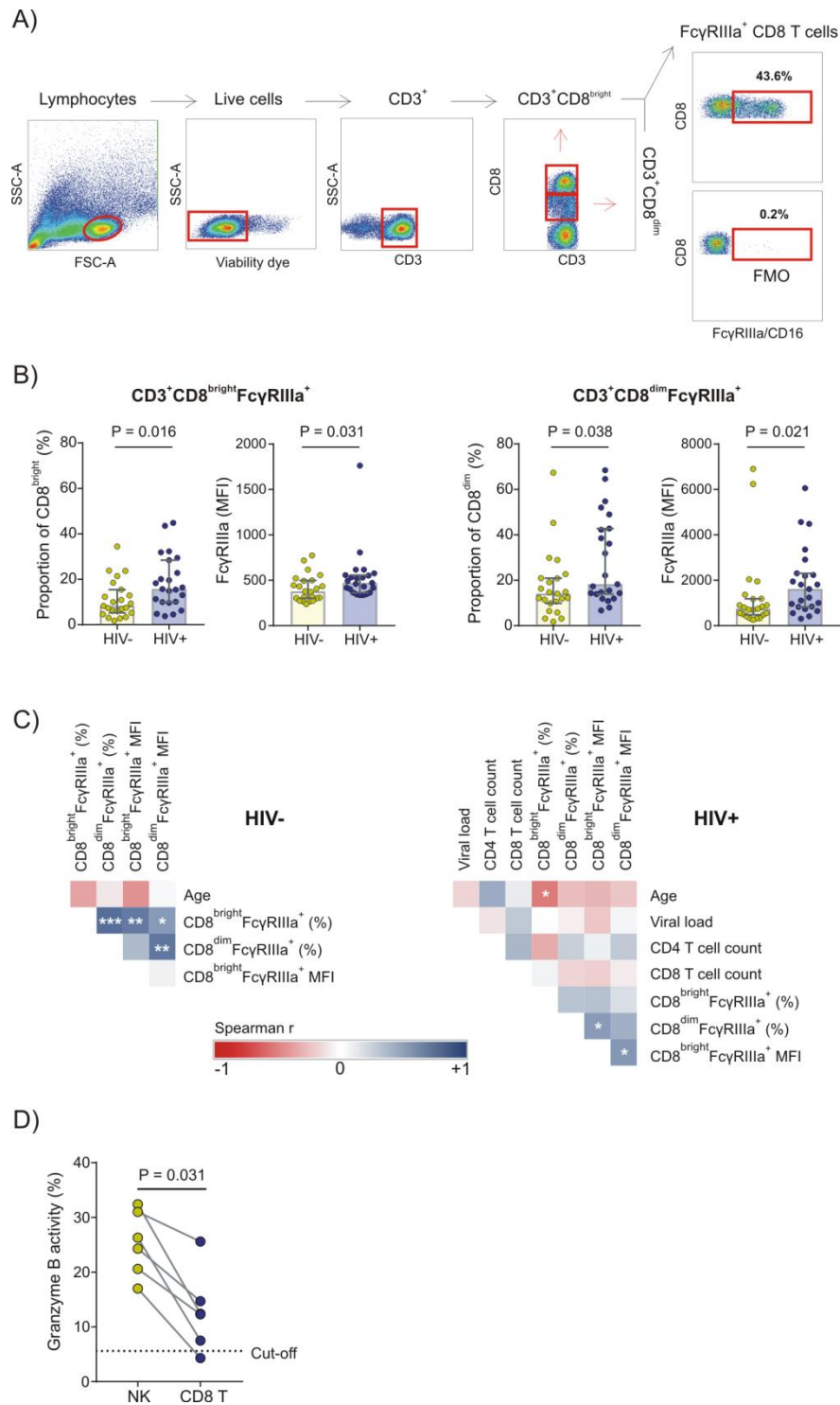


Figure 2.5. FcγRIIIa expression on CD8⁺ T cells in HIV-1 uninfected and HIV-1 infected individuals matched for FCGR genetic variants.

A) Gating strategy. B) The proportion of FcγRIIIa⁺CD8^{bright} and FcγRIIIa⁺CD8^{dim} T cells and corresponding median fluorescence intensity (MFI) of FcγRIIIa on these FcγRIIIa⁺CD8⁺ T cell subsets in a cohort of HIV-1 uninfected and infected individuals. C) Correlation analysis between demographic, clinical, phenotypic and functional variables in HIV-1 uninfected and HIV-1 infected individuals. D) CD8⁺ T cell-mediated ADCC responses of HIV-1 positive donors relative to autologous NK cell-mediated ADCC responses at the same effector-to-target cell ratio (*** P < 0.001; ** P < 0.01; * P < 0.05).

2.4 Discussion

This study set out to comprehensively characterize FcγRIIIa variability and its effect on ADCC responses in HIV-1 positive and HIV-1 negative South African individuals, the population that bears the largest HIV-1 epidemic. Accurate assessment of FcγRIIIa variability requires careful selection of study participants to exclude confounding genetic variables that modify FcγRIIIa expression and FcγRIIIa-mediated cell activation. Study participants within the comparative groups were further matched for the FcγRIIIa-F158V variant that not only alters ADCC responses, but also affects measurements of FcγRIIIa surface expression with the most commonly used anti-FcγRIIIa antibody, clone 3G8 (Congy-Jolivet et al., 2008).

In comparing a cohort of Black South African HIV-1 negative and HIV-1 positive donors matched for genotypic variants, we confirmed previously described significant differences in the distribution of NK cell subsets, while differences in FcγRIIIa surface density on cytotoxic NK cells were not observed. NK cell-mediated ADCC responses of HIV-1 positive donors were both reduced and differentially affected by the FcγRIIIa-F158V variant compared to HIV-1 negative donors. In addition, FcγRIIIa expression was identified on a subset of cytotoxic CD8⁺ T cells where it potentially contributes to HIV-1-specific ADCC responses in a granzyme B-dependent manner.

In healthy individuals, NK cells typically comprise a dominant CD56^{dim}FcγRIIIa^{bright} population and minor populations that include CD56^{bright}FcγRIIIa^{dim/neg}, CD56^{dim}FcγRIIIa^{dim/neg} and CD56^{neg}FcγRIIIa^{bright}. Perturbation of NK cell subsets in the presence of an HIV-1 infection has been extensively described (Fauci et al., 2005, Mavilio et al., 2005, Brunetta et al., 2010). In HIV-1 infected individuals, the cytotoxic CD56^{dim}FcγRIIIa^{bright} subset contracts with an associated expansion of the CD56^{neg}FcγRIIIa^{bright} subset. The latter is characterized by higher levels of inhibitory NK cell receptors, lower levels of natural cytotoxicity receptors, and reduced secretion of cytokines compared to the CD56^{dim}FcγRIIIa^{bright} subset (Mavilio et al., 2005, Brunetta et al., 2010). This hyporesponsive NK cell subset was similarly increased in the South African HIV-1 positive cohort; however, it did not associate with HIV-1 viral load as observed by Mavilio et al. (Mavilio et al., 2003, Mavilio et al., 2005). The lack of an association with HIV-1 viral load may be explained by an approximate 5- to 6-fold lower median HIV-1 plasma viral load of the South African cohort compared to the other cohorts. Moreover, in the present study, NK cell subsets were studied for overnight rested PBMCs as opposed to freshly isolated negatively-selected NK cells.

The dysregulation of NK cell subsets is typically associated with reduced ADCC responses in HIV-1 infected individuals (Ahmad and Menezes, 1996, Scott-Algara and Paul, 2002,

Mavilio et al., 2005, Brunetta et al., 2010). In the present study, lower ADCC responses were similarly observed for HIV-1 infected individuals. Since ADCC capacity was studied for CD56⁺ NK cells it precluded an analysis of the association between the CD56^{neg}FcγRIIIa^{bright} subset and ADCC responses, but not for the CD56^{dim}FcγRIIIa^{bright} and CD56^{bright}FcγRIIIa^{dim/neg} subsets. A negative correlation observed between the CD56^{bright}FcγRIIIa^{dim/neg} subset and ADCC responses for both HIV-1 negative and positive donors would suggest that ADCC capacity is similarly affected by the immunoregulatory CD56^{bright}FcγRIIIa^{dim/neg} subset in both groups. However, as demonstrated by a positive correlation between the cytotoxic CD56^{dim}FcγRIIIa^{bright} subset and ADCC responses for HIV-1 negative donors, but not HIV-1 positive donors, not all factors modulating ADCC capacity may be shared between healthy individuals and HIV-1 infected individuals.

Given the association of ADCC-mediating antibody responses with HIV-1 protective immunity, it could be hypothesized that genetic determinants of NK cell-mediated ADCC capacity, in particular the FcγRIIIa-F158V variant, may associate with HIV-1 acquisition risk or disease progression. The FcγRIIIa-158V isoform has greater avidity for complexes comprising IgG1, IgG3, and IgG4 than the FcγRIIIa-158F isoform and confers increased NK cell activation and ADCC responses in healthy individuals (Koene et al., 1997, Wu et al., 1997, Bruhns et al., 2009). Despite its potential contribution to ADCC responses, the FcγRIIIa-158V isoform is yet to be positively associated with HIV-1 acquisition and disease progression. On the contrary, the FcγRIIIa-158V isoform has been associated with an increased risk of HIV-1 infection (Poonia et al., 2010), disease progression (Poonia et al., 2010), and HIV-1-associated Kaposi's sarcoma (KS) and Cryptococcal disease (Foster et al., 2000, Rohatgi et al., 2013). Furthermore, homozygosity for the FcγRIIIa-158V allele associated with a higher rate of HIV-1 infection among vaccinated men in the VAX004 trial (Forthal et al., 2012), while homozygosity for the FcγRIIIa-158F allele associated with greater protection from HIV-1 disease progression in male participants in the RV144 vaccine trial (Kijak et al., 2012). Taken together, these findings are more indicative of FcγRIIIa-158V-mediated antibody-dependent enhancement of infection rather than improved ADCC responses to the benefit of the individual. Alternatively, it is possible that the functional consequence of the FcγRIIIa-F158V isoforms may be different in the presence of an HIV-1 infection and that a different mechanism(s) may underlie the aforementioned associations.

FcγRIIa/FcγRIIb polymorphic variants, for example, show distinct differences in oxidative burst responses of resting neutrophils; however, once neutrophils were pre-activated with IFNγ and G-CSF these differences were no longer observed (van der Heijden et al., 2014).

Other non-FcγR genetic variants have also been shown to differentially affect gene expression or cytokine production of activated and resting immune cells (Jonkers and Wijmenga, 2017). In the present study, we show a similar trend for the FcγRIIIa-F158V variant, higher ADCC responses for HIV-1 negative donors bearing the FcγRIIIa-158V allele compared to those homozygous for the FcγRIIIa-158F allele, whereas in HIV-1 positive donors this trend was lost or even slightly reversed. The effect of the variant on ADCC responses was, however, not significant in either group. It is possible that the independent effect size of the FcγRIIIa-F158V variant is too small to detect with the current sample size. Larger cohort studies that adjust for other NK cell activation and inhibitory receptors are required to further define the role of this variant in HIV-1 infection. Nonetheless, these findings may partially explain the inconclusive role of FcγRIIIa genetic variants in HIV-1-specific immunity (reviewed by (Cocklin and Schmitz, 2014)).

In addition to modulating the function of an allelic variant, infection can lead to the induction of FcγRIIIa on other cytotoxic cells, including CD8⁺ T cells. An FcγRIIIa⁺CD8⁺ T cell population was first described in the 1980's and has since been characterized in the context of hepatitis C virus and Epstein Barr virus infections (Lanier et al., 1985, Bjorkstrom et al., 2008, Clemenceau et al., 2008). These terminally differentiated CD8⁺ T cells belong to the T effector memory CD45RA⁺ lymphocyte subset, are perforin positive, directly mediate ADCC ex vivo, and increase in vivo during hyperlymphocytosis (Bjorkstrom et al., 2008, Clemenceau et al., 2008). In addition to FcγRIIIa, this cell subset also has increased expression of other NK-like receptors including NKG2A, NKG2D, KIR2DL2/L3 and KIR2DL1/S1 when compared to FcγRIIIa⁻CD8⁺ T cells (Clemenceau et al., 2011). The present study validates the expression of FcγRIIIa on cytotoxic CD8⁺ T lymphocytes and is in agreement with other studies that have consistently showed an increase in the proportion of FcγRIIIa⁺CD8⁺ T cells in the presence of a virus infection (Bjorkstrom et al., 2008, Clemenceau et al., 2008, Clemenceau et al., 2011). Compared to CD8^{bright} cells, the surface density of FcγRIIIa was significantly higher on CD8^{dim} cells, a subset characterized by higher activation levels, increased cytotoxicity and increased cytokine production (Trautmann et al., 2003, Kienzle et al., 2004, Falanga et al., 2017). The higher proportions of FcγRIIIa⁺CD8⁺ T cells in HIV-1-infected individuals suggests that HIV-1 in its own right is a driver of these cell expansions, whereas increasing age associated with reduced proportions of FcγRIIIa-expressing CD8⁺ T cells. The increase in FcγRIIIa expression on CD8⁺ T cells in HIV infection contrasts with decreased proportions of FcγRIIIa expressing NK cells. This suggests

the development of an ADCC capacity by CD8⁺ T cells that could compensate to some extent for the reduction in NK cell ADCC function.

Attributing the otherwise innate cell function of ADCC to CD8⁺ T cells – mediated through expression of FcγRIIIa and engagement of HIV-1-specific antibodies – is reminiscent of other known examples of innate-like unconventional T cell populations. Among these are invariant NKT cells, mucosal-associated invariant T cells, and γδT cells, that recognize foreign/self-lipid presented by non-classical MHC molecules (Lanier, 2013, Godfrey et al., 2015).

Another interesting unconventional CD8⁺ T cell subset is one with a prominent innate/memory phenotype identified by co-expression of eomesodermin (Eomes) and KIR/NKG2A (Jacomet et al., 2017). The current study highlights the addition of another unconventional CD8⁺ T cell population, capable of ADCC function, that warrants further investigation.

In conclusion, our findings underscore the importance of expanding studies of HIV-specific antibodies to include the influence of different host cell types that share expression of FcγRs (constitutive or induced), the respective functional cellular capabilities, as well as host genotypes, in the context of the presiding immune milieu which is altered as a consequence of chronic HIV infection. Continuing investigations are warranted to further define effector functions, cytokine production and activation status of these different FcγRIIIa⁺ NK and CD8⁺ T cell subsets in similarly selected individuals.

Author Contributions

Phaahla N. G. recruited HIV-1 negative donors, performed the majority of the experiments, analyzed the data and wrote the manuscript. Dr. Lassauniere, R. designed the study, contributed to the data analysis, and writing of the manuscript. Dr. Da Costa Dias, B. contributed to the flow cytometry experiments. Dr. Waja, Z. and Dr. Martinson, N. recruited HIV-1 positive donors, Prof. Tiemessen, C. T. in her capacity as head of the laboratory, allocated funds toward the study, supervised the research, and provided the necessary infrastructure to perform the work.

**Chapter 3: Low Levels of
Hypergammaglobulinaemia Correlate with
Enhanced HIV-1-Specific ADCC Responses in
HIV-1 Elite Controllers**

3.1 Introduction

Increasing evidence support a beneficial role for HIV-1-specific ADCC in preventing HIV-1 infection and slowing disease progression. Several studies have indirectly associated ADCC with blocking HIV/SIV acquisition (reviewed in (Lewis, 2014, Lewis et al., 2017)). In addition, passively acquired ADCC activity from HIV-1 infected mothers to their infants associated with reduced infant mortality (Milligan et al., 2015). Lastly, ADCC has been implicated in post-infection control of viraemia in human (Baum et al., 1996, Forthal et al., 1999, Ahmad et al., 2001, Forthal et al., 2001, Chung et al., 2011b, Brombin et al., 2013, Wren et al., 2013) and non-human primate studies (Banks et al., 2002, Gomez-Roman et al., 2005, Xiao et al., 2010).

Total circulating antibody levels and HIV-1-specific antibody isotypes and subclasses are important regulators of Fc-mediated effector functions and have been associated with HIV-1 control as well as prevention of HIV-1 acquisition (reviewed in (Mayr et al., 2017b, Lee and Kent, 2018, Su et al., 2019)). A recent review (Moris et al., 2019) briefly outlined several studies in natural HIV-1 infection that correlated levels of HIV-1-specific IgG1 and IgG3 with reduced disease progression in HIV-1 controllers (Lambotte et al., 2009, Banerjee et al., 2010, Ackerman et al., 2016). In contrast, gp120-specific IgG2 and IgG4 responses were absent or low in HIV-1 controllers, prevalent in chronic progressors and were inversely correlated to functional activity (Ackerman et al., 2016), and reduced infection risk in the RV144 vaccine trial (Chung et al., 2014b). In the same vaccine trial, gp120-specific V1V2 IgG3 antibodies and ADCC responses in the presence of low gp120-specific IgA levels were associated with reduced risk of infection (Yates et al., 2014), and the gp120-specific V1V2 IgG3 responses were higher when the same vaccine regimen was tested in South African participants (Gray et al., 2019). Despite these associations, little is known about the actual mechanisms of HIV-1 prevention or control.

We hypothesize that the level of non-specific activation of B cells and excessive antibody production (hypergammaglobulinaemia) that accompanies HIV-1 infection, modulates HIV-1-specific ADCC responses in the context of disease progression. We therefore set out to further define the role for ADCC in HIV-1 control by investigating the collective effect of levels and composition of antibodies in HIV-1 elite controllers and viraemic controllers, compared to progressors. An in-depth study of factors that modulate FcγR-mediated effector functions is required to define their role in HIV-1 disease progression and control of HIV-1

replication. This may provide insight into HIV-1 protective immunity to facilitate rational vaccine design or cure efforts.

3.2 Methods

3.2.1 Study participants

All study participants (male and female over the age of 18 years) were Black South Africans recruited from the Johannesburg metropolitan region in South Africa. Participants provided written informed consent and the University of the Witwatersrand Ethics Committee approved the study (Ethics clearance certificate no. M1511102). Self-reported HIV-1 uninfected individuals (n = 18) were recruited from the National Institute for Communicable Diseases (NICD) as healthy controls. The study assessed HIV-1-specific ADCC responses in antiretroviral treatment-naïve HIV-1 controllers (n = 57) and HIV-1 chronic progressors (n = 73) recruited from hospitals and clinics in Johannesburg and Soweto, South Africa (Table 3.1). The HIV-1 controllers comprised 22 elite controllers (ECs) and 35 HIV-1 viraemic controllers (VCs). Elite controllers were defined as HIV-1-infected individuals with viral loads <50 HIV-1 RNA copies/ml for at least two consecutive clinic visits 6-12 months apart. Viraemic controllers had viral loads <2 000 RNA copies/ml. HIV-1 infected patients with progressive HIV-1 disease were selected from an existing cohort of HIV-1 infected individuals based on a CD4⁺ T cell decline to at least <300 cells/μl and had viral loads >10 000 RNA copies/ml prior to starting ART.

Table 3.1 Clinical and demographic characteristics of HIV-1 disease phenotype groups.

	Elite controllers	Viraemic controllers	Chronic progressors
N	22	35	73
Age (years) at enrolment	44.6 (9.6)	41.3 (8.5)	46.5 (7.3)
Gender (% Females)	77.3	94.3	83.6
CD4⁺ T cell count (cells/μl)	730.2 (233.3)	737.8 (234.7)	182.7 (72.3)
Viral load (RNA copies/ml)	<20	366.0 (253-860)	39322 (17941-105195)
Time since diagnosis (years)	10 (4-12)	3 (2-11)	6 (1-7)

Mean with standard deviation (SD) is reported for 'Age' and 'CD4⁺ T cell count'; median with interquartile range is reported for 'Viral load' and 'Time since diagnosis'. The CD4⁺ T cell count and viral load at study enrolment were used for HIV-1 controllers, while for HIV-1 chronic progressors, the CD4⁺ T cell count and viral load before initiation of antiretroviral therapy was utilized.

3.2.2 Peripheral blood mononuclear cells (PBMCs) as effector cells

Effector cells were obtained from a single individual to minimize assay associated variability of relative plasma antibody HIV-specific ADCC. Since a single donor was used for comparison of plasma ADCC activity between HIV-1 infected groups, absolute numbers of effector cells was not determined since it will not have confounded the findings. Effector cells were PBMCs isolated from a single HIV-1 uninfected donor homozygous for the FcγRIIIa-158V allele. Within the PBMC preparation, NK cells are the primary ADCC effector cells killing through granzyme B activity. PBMCs were separated from EDTA whole blood on Ficoll-Paque™ PLUS (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) density gradient media, washed with phosphate buffered saline (PBS), re-suspended in freezing medium (90% foetal calf serum [FCS] and 10% dimethyl sulfoxide [DMSO]) and stored at -80°C overnight, followed by storage for several months in liquid nitrogen. Before performing an ADCC assay, stored PBMCs were thawed and rested overnight in R10 media [10% FCS (Gibco) + 0.1% Gentamicin (Sigma) in RPMI 1640 (Gibco)] at 37°C, 5% CO₂ in an incubator.

3.2.3 Total IgG purification

Total IgG of any specificity, including HIV-1 and non-HIV-1 IgG, was isolated from plasma samples using the Melon Gel IgG Spin Purification Kit (Thermo Scientific) as detailed in Chapter 2, section 2.2.5.

3.2.4 HIVIG

HIV immunoglobulin obtained from HIVIG, NIH AIDS Reagent Program served as positive control for ADCC assays. HIVIG is prepared from pooled plasma of asymptomatic, HIV antibody positive individuals with CD4⁺ T cell counts >400 per µl. The donors were selected for high titre p24 antibody. IgG was purified by QAE Sephadex gel, an ion exchange resin that yields monomeric, unfragmented, and undenatured IgG. Following additional purification and inactivation steps, the final antibody preparation is a protein solution (5%) in NaCl (0.9%) without preservatives, stabilizing agents or buffers. At least 95% of the protein content has the electrophoretic mobility of gamma globulin with >90% being monomeric. The end product is generated as a sterile, non-pyrogenic solution appropriate for intravenous injection (NIH-AIDS-Reagent-program, 2018).

3.2.5 ADCC assay

HIV-1-specific ADCC antibody responses were determined using a slightly modified GranToxiLux assay described in Chapter 2, section 2.2.6 and 2.2.7. In brief, 25 µl gp120-coated target cells (CEM.NK^RCCR5) at 2.5 µg/ml gp120 and 25 µl effector cells (healthy donor PBMCs) (E:T ratio of 30:1) were co-cultured with 25 µl of diluted plasma or purified IgG sample from each HIV-1 infected individual and 75 µl granzyme B substrate. Following acquisition on the BD Fortessa X20 (BD biosciences), data were analyzed using FlowJo version 7.6.1 software (Tree Star). ADCC responses were reported as the proportion of granzyme B-positive (%GzB) live target cells (Flow plots shown in Appendix 1, Fig S2). Background ADCC activity was determined from a duplicate no-antibody control and was subtracted from study subjects' sera %GzB activity. A cut-off value for positive responses was determined using %GzB activity data from 18 HIV-1 sero-negative donors and calculated as the mean + 3 standard deviations. %GzB activity at a serum dilution of 1:50 was performed in triplicate for each patient and the mean used in analyses. To minimize the risk of inter-run variability affecting comparisons between groups, each ADCC assay performed on a given day comprised patients from each group at a ratio that closely reflected that of the study cohort, which is 1:2:4 for elite controller: viraemic controller: progressor. The coefficient of variation for all ADCC assays was 7.95%, as calculated from a duplicate HIV immunoglobulin positive control (HIVIG) included in each assay.

3.2.6 Quantitating total IgG

From whole plasma samples of participants, total IgG concentrations were determined using the IgG Human SimpleStep ELISA (Enzyme-Linked Immunosorbent Assay) kit (Abcam, Burlingame, CA, USA) according to manufacturer's instructions. Briefly, 50 µl of serially diluted plasma sample ($1:32 \times 10^6$ final dilution) was added to a well coated with anti-tag antibody, followed by addition of 50 µl antibody cocktail that comprises an affinity tag labelled capture antibody and a reporter conjugated detector antibody. The entire complex was immobilized to the well by incubating at room temperature for 40 minutes on a plate shaker. After incubation, the well was washed 3 times with 350 µl wash buffer to remove unbound material, followed by adding 100 µl 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate and incubation for 3 minutes in the dark on a plate shaker. Following catalysis by horse reddish peroxidase (HRP), the reaction generated a blue colour that was subsequently added with 100 µl Stop Solution at a defined endpoint to stop the reaction, which turns the contents of the wells from blue to yellow after shaking for 1 minute. A signal was generated

according to the concentration of IgG in each plasma sample and measured at 450 nm on a VersaMax microplate reader.

3.2.7 Quantitating HIV-1-specific IgG subclasses

IgG subclasses were measured using a customized binding antibody multiplex assay (BAMA). ConC gp120 was covalently coupled at 6.25 µg to 1.25×10^6 carboxylated magnetic beads (Bio-Rad, Hercules, CA, USA) using a standard two-step carbodiimide coupling procedure developed by Luminex xMAP technology (Dunbar and Hoffmeyer, 2013). Briefly, beads were washed and suspended in activation buffer (0.1 M NaH₂PO₄, pH 6.2) then 50 mg/ml N hydroxysulfosuccinimide (sulfo-NHS, Thermo Scientific) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Thermo Scientific) were added. Activated beads were washed twice with coupling buffer (50 mM MES, pH 5.0) and incubated with 6.25 µg ConC gp120 for 2 hours in the dark at room temperature on a rotational shaker. Coupled beads were washed twice with storage/blocking buffer consisting of 0.1% BSA, 0.02% Tween-20 and 0.05% Sodium azide diluted in PBS (PBS/TBN), pH 7.4, and counted with a haemocytometer followed by storage in the dark at 4°C.

A mixture of the HIV antigen-coupled beads was prepared by diluting the appropriate volume of beads in assay buffer (1% BSA/PBS). Fifty µl of the bead mixture, 40 µl assay buffer and 25 µl of patient plasma (1:75 dilution) were added to each well of a 96-well flat bottom plate (Greiner, Friedrich-Schiedel-Straße, Nußbach, Austria). Following a 2 hour incubation at room temperature with shaking, the plate was washed twice with wash buffer (PBS, 0.1% BSA, 0.05% Tween-20) and a final wash with assay buffer. HIV-1-specific antibody isotypes were measured by adding 2 µg/ml R-phycoerythrin (PE)-conjugated mouse anti-human IgG1 to IgG4 (Southern Biotech, Birmingham, AL, USA), mouse anti-human IgM (Southern Biotech), mouse anti-human IgA1 (Southern Biotech) or mouse anti-human IgA2 (Southern Biotech) at 50 µl/well. After 2 hour incubation at room temperature with shaking, the beads were washed three times with wash buffer and resuspended in 125 µl sheath fluid. Beads were analysed using the Bio-Plex 200 instrument (Bio-Rad) by measuring the mean fluorescence signal for 50 beads/analyte. The average MFI for the bead set when incubated with PE detection antibody in the absence of patient plasma was used as the background signal, which was subtracted from the MFI of each sample. The cut-off for sero-positivity was calculated as the mean MFI value from the pooled plasma from HIV-1 uninfected individuals plus 3 standard deviations. HIVIG served as a positive control on each plate and the MFI values were tracked with a Levey-Jennings plot and the one point

outside of 3SD (13S) control rule was followed, namely the positive control and therefore the plate run was rejected if it exceeded the mean plus/minus 3SD. HIV-1-specific antibody detection was performed by Dr. Diana Schramm.

3.2.8 Statistical Analysis

Continuous variables were tested for normality using the Shapiro Wilk test. Continuous data between unrelated groups were compared using the nonparametric Kruskal-Wallis test for non-normally distributed data followed by Dunn's multiple comparison tests, where multiple comparisons tests were applicable. The adjusted P-value (designated $P_{\text{Dunn's}}$) is reported. For normally distributed data, continuous variables were compared using one way ANOVA with Tukey multiple comparisons test. Matched continuous data were compared using the Wilcoxon matched-pairs signed rank test. Correlation analysis of non-normally distributed data sets was assessed using the nonparametric Spearman rank correlation coefficient, while Pearson's was used to correlate normally distributed data sets. All statistical analysis were performed in GraphPad Prism 8 software version 8.3.0 (GraphPad Software, La Jolla, CA, USA). P-values less than 0.05 were considered statistically significant (2-tailed tests).

3.3 Results

3.3.1 ADCC responses are inhibited at low plasma dilutions and high purified IgG concentrations

HIV-1-specific ADCC activity measured by various assays is characterized by a prozone phenomenon, where ADCC responses are inhibited at low plasma dilutions (Fig 3.1A). In a cohort of 130 HIV-1 infected South African individuals with variable disease progression profiles, the percentage granzyme B (%GzB) activities were overall significantly reduced at plasma dilutions of 1:50 compared to those observed for 1:1250 (median: 13.3% vs 48.0%, $P < 0.0001$, Fig 3.1B). Given the heterogeneity of plasma, it is unclear whether the observed inhibition is due to high levels of antibodies or other plasma constituents. To assess the effect of IgG levels in the absence of other plasma factors, total IgG was isolated from plasma and tested at 4-fold serial diluted concentrations ranging from 167 $\mu\text{g/ml}$ to 2.6 $\mu\text{g/ml}$. A prozone effect was also observed for high concentrations of isolated total IgG (Fig. 3.1C).

To determine whether the presence of non-HIV-1 antibodies contribute to the observed inhibition, we next tested a polyclonal HIV-1 specific antibody preparation. A similar, albeit less drastic, reduction in %GzB activity was also observed for HIV-specific immunoglobulin

(HIVIG) isolated from pooled plasma of asymptomatic, HIV antibody positive donors. Using effector cells from different donors, HIVIG concentrations of 16.7 $\mu\text{g/ml}$ had significantly lower median %GzB activity compared to 3.3 $\mu\text{g/ml}$ or 0.67 $\mu\text{g/ml}$ (28.9% vs 36.6% or 34.0%, respectively; $P < 0.0001$ for both comparisons; Fig 3.1D). It is unlikely that diluent constituents could account for this inhibition since the antibody preparation contains no preservatives, buffers or stabilizing agents and is suitable for intravenous injection. Taken together, these data demonstrate that not only do high levels of total IgG, but also high levels of HIV-specific IgG, inhibit HIV-specific ADCC responses.

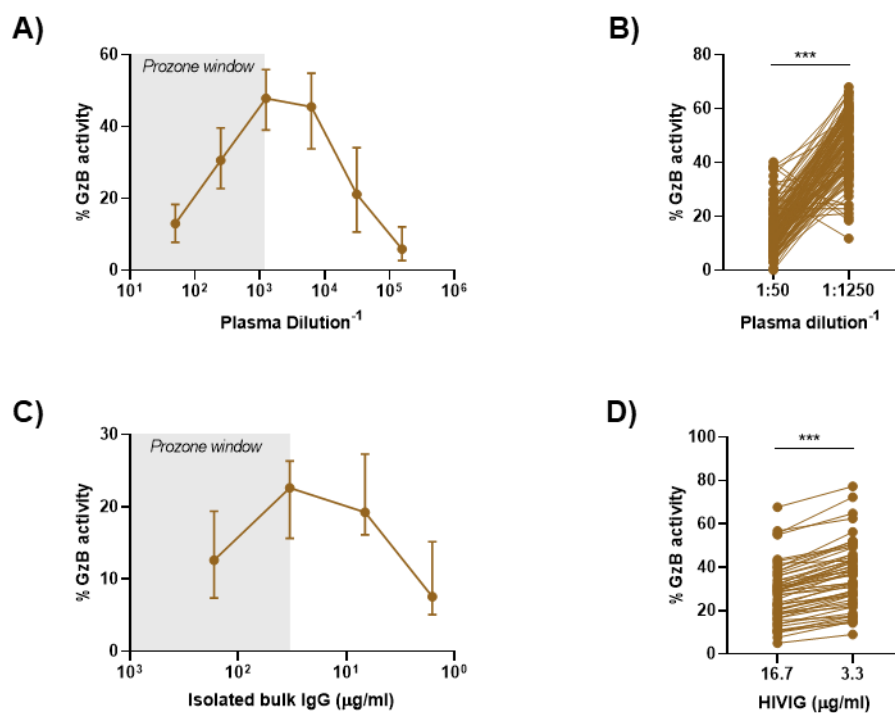


Figure 3.1. Low plasma dilutions and high antibody concentrations inhibit ADCC responses.

A) Median %GzB activity with interquartile ranges observed for a 5-fold titration curve using plasma obtained from 130 HIV-1 infected individuals. The prozone phenomenon where ADCC responses are inhibited at low plasma dilutions is indicated with a grey box. B) Comparison between ADCC responses at a 1:50 vs 1:1250 plasma dilution using Wilcoxon matched-pairs signed rank test. C) Titration curve for ADCC responses using total IgG isolated from plasma of Danish HIV-1 infected individuals. D) Using Wilcoxon matched-pairs signed rank test to compare ADCC responses between two different concentrations (16.7 $\mu\text{g/ml}$ vs. 3.3 $\mu\text{g/ml}$) of HIV-1 specific IgG (HIVIG) (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

3.3.2 At low plasma dilutions, HIV-1 elite controllers have higher ADCC responses compared to viraemic controllers and chronic progressors

We next sought to establish whether the inhibition at low plasma dilutions differs between HIV-1 controllers and chronic progressors. The median titration curve obtained for elite controllers ($n = 23$), viraemic controllers ($n = 35$) and chronic progressors ($n = 73$), indicated that at plasma dilutions within the prozone 'window' – 1:50 and 1:250 – elite controllers had higher median %GzB activity compared to their viraemic counterparts (Fig 3.2A). Subsequent

triplicate measurements of ADCC responses at a 1:50 plasma dilution confirmed this observation, with elite controllers having a median %GzB activity of 27.3% compared to 18.5% for viraemic controllers ($P = 0.010$) and 17.0% for chronic progressors ($P < 0.001$) (Fig 3.2B). In contrast, other measures of ADCC responses – area under the curve (AUC), titre, and maximum %GzB activity – showed higher median %GzB activity in progressors compared to controller groups. In particular, the median %GzB activity determined from the AUC were significantly higher in progressors compared to elite controllers (32.0% vs. 20.0%, $P_{\text{Dunn's}} = 0.03$, Fig 3.2C). In addition, median titre and median maximum %GzB activity were higher in progressors compared to controller groups, although the difference was not statistically significant (Fig. 3.2C).

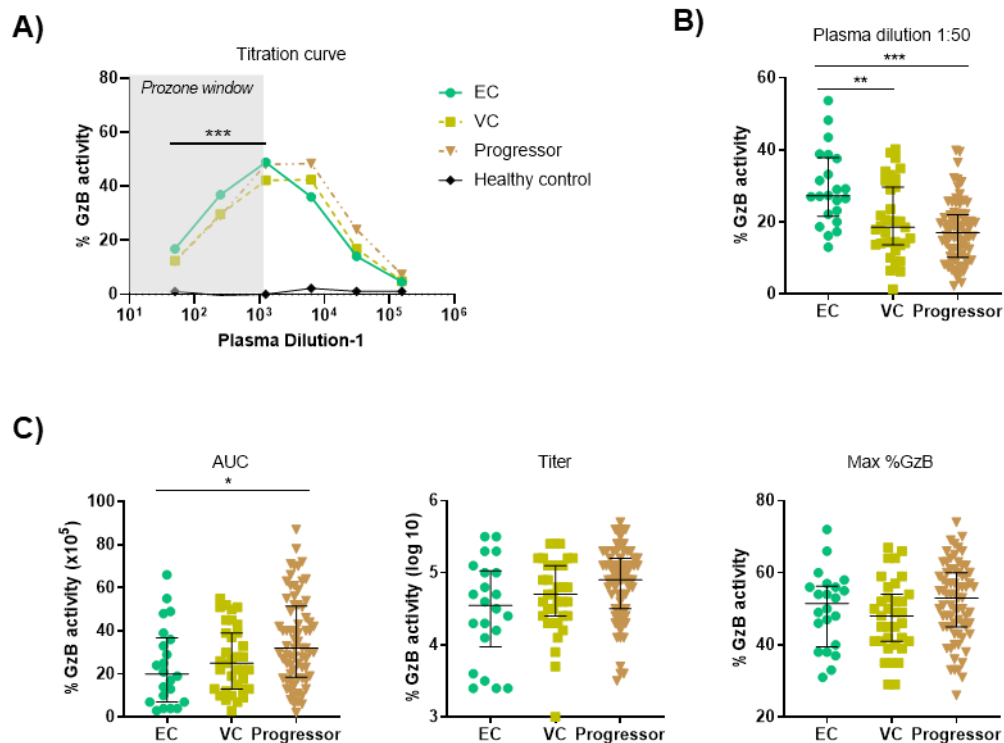


Figure 3.2. Higher ADCC responses in HIV-1 elite controllers compared to viraemic controllers and progressors at the low plasma dilution.

A) ADCC responses of 6 serial dilutions of plasma starting from 1:50 up to 1:156250 using median %GzB activity in a 5-fold titration curve to represent HIV-1 infected 22 elite controllers, 35 viraemic controllers, 73 chronic progressors and 18 healthy controls. ADCC responses of 22 elite controllers compared to 35 viraemic controllers and 73 chronic progressors using B) 1:50 plasma dilution, and C) area under the curve (AUC), titre and maximum granzyme B activity as measurements of activity compared between groups by Kruskal Wallis or One-way Anova using Dunn's or Tukey's tests, respectively, for multiple comparisons (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$). The horizontal black line represents the median with the interquartile range as error bars.

3.3.3 Elite controllers have low antibody levels that inversely correlate with ADCC responses

To investigate the association between hypergammaglobulinaemia and ADCC responses in HIV-1 disease progression, we determined the total plasma IgG levels for HIV-1 infected groups. In HIV-1 infected individuals, hypergammaglobulinemia is defined as having a total IgG level exceeding 16 mg/ml (Morris et al., 1998). Elite controllers had significantly lower median IgG levels (12.6 mg/ml) compared to viraemic controllers (33.2 mg/ml; $P_{\text{Dunn's}} = 0.004$) and chronic progressors (39.7 mg/ml; $P_{\text{Dunn's}} < 0.001$) (Fig 3.3A). In the complete cohort, ADCC responses were inversely correlated with plasma IgG levels ($r = -0.333$, $P < 0.001$) (Fig 3.3B). However, this negative correlation was primarily attributable to the HIV-1 controller group ($r = -0.512$, $P < 0.001$) and was not observed for the HIV-1 progressor group ($r = 0.015$, $P = 0.902$) (Fig 3.3B).

When ADCC responses at a plasma dilution of 1:50 are normalized for the levels of plasma IgG, the original observation of increased ADCC responses in elite controllers compared to viraemic controllers and progressors remained (2.3 vs. 0.5, $P_{\text{Dunn's}} = 0.001$, and 2.3 vs. 0.4, $P_{\text{Dunn's}} < 0.001$; respectively, Fig 3.3C). Progressors and viraemic controllers did not differ significantly (0.4 vs. 0.5, $P_{\text{Dunn's}} = 0.052$) (Fig 3.3C).

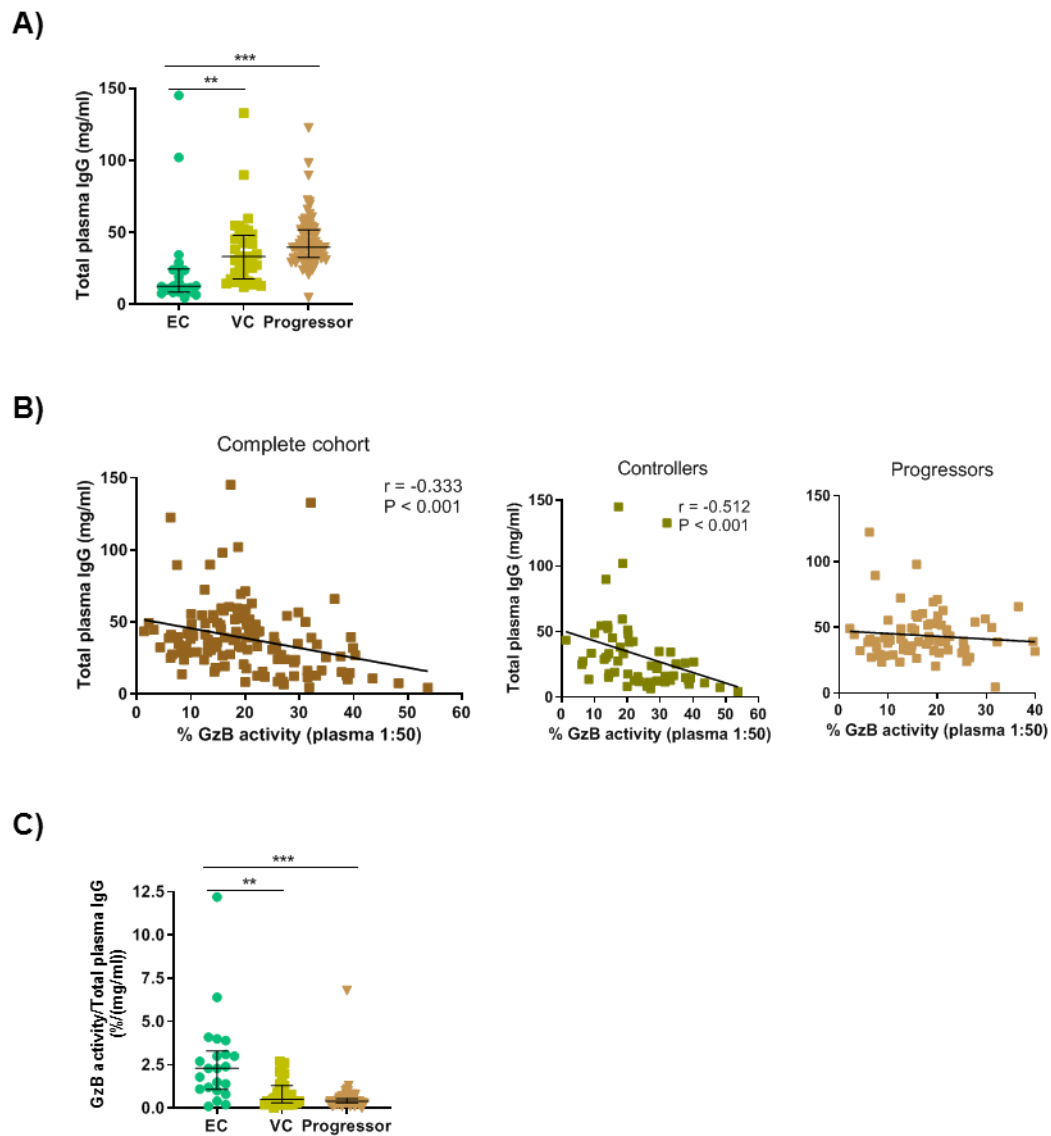


Figure 3.3. Total plasma IgG levels in controllers compared to progressors and correlated to ADCC responses.

A) Comparison of total IgG levels between 22 elite controllers, 35 viraemic controllers and 73 progressors. B) Correlation of total plasma IgG levels with ADCC responses at the lowest plasma dilution in the complete cohort and in separate controller and progressor groups using the nonparametric Spearman rank correlation coefficient and linear regression analysis. C) ADCC responses at the low plasma dilution normalized for total plasma IgG in 22 elite controllers, 35 viraemic controllers and 73 progressors. All comparisons tested by Kruskal-Wallis using Dunn's tests for multiple comparisons (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$). The median is represented by a horizontal black line and error bars represent the interquartile range.

3.3.4 Strong negative correlations between ADCC at the lowest plasma dilution and HIV-1 gp120-specific total IgG and IgG1 in elite controllers

We next sought to determine the role of autologous HIV-1-specific IgG subclasses in ADCC responses and HIV-1 control. In this cohort gp120-specific IgG plasma levels directly correlated with bulk plasma IgG ($r = 0.457$, $P < 0.001$, Fig 3.4A). It is known that IgG1 dominates the HIV-1 IgG response (Tomaras and Haynes, 2009) and likely explains the

comparable measurements observed for total gp120-specific IgG and IgG1. Although other subclasses were comparably lower than IgG1, they all (except IgG3) showed a similar trend of significantly enhanced levels in progressors compared to elite controllers (all $P < 0.001$, Fig 3.4B).

We next evaluated the extent to which autologous HIV-1-specific IgG and subclass levels associate with ADCC responses at low plasma dilutions. ADCC responses had stronger negative associations with total gp120-specific IgG and IgG1 ($r = -0.504$, $P < 0.001$ and $r = -0.570$, $P < 0.001$, respectively; Fig 3.4B), and weaker associations with gp120-specific IgG2 and IgG4 ($r = -0.193$, $P = 0.001$ and $r = -0.353$, $P < 0.001$, respectively; Fig 3.4B), whereas no significant correlation was observed for gp120-specific IgG3. Further analysis in separate HIV-1 infected groups showed that HIV-1 gp120-specific IgG negatively correlated with ADCC responses in elite controllers, viraemic controllers and progressors ($r = -0.817$, $P < 0.001$; $r = -0.528$, $P = 0.001$; and $r = -0.385$, $P < 0.001$, respectively; Fig 3.4C). Similar correlations were observed between ADCC and gp120-specific IgG1 in elite controllers, viraemic controllers and progressors ($r = -0.869$, $P < 0.001$; $r = -0.502$, $P = 0.002$; and $r = -0.407$, $P < 0.001$, respectively; Fig 3.4C). In both cases, elite controllers had stronger correlations. Notably, gp120-specific IgG4 weakly correlated to ADCC in elite and viraemic controllers ($r = -0.425$, $P = 0.043$ and $r = -0.474$, $P = 0.004$, respectively; Fig 3.4C). In contrast, levels of gp120-specific IgG2 and IgG3 did not significantly correlate to ADCC responses in neither HIV-1 controllers nor progressors (Fig 3.4C), which may be due to low circulating levels in plasma (Fig 3.4B) (Yates et al., 2011).

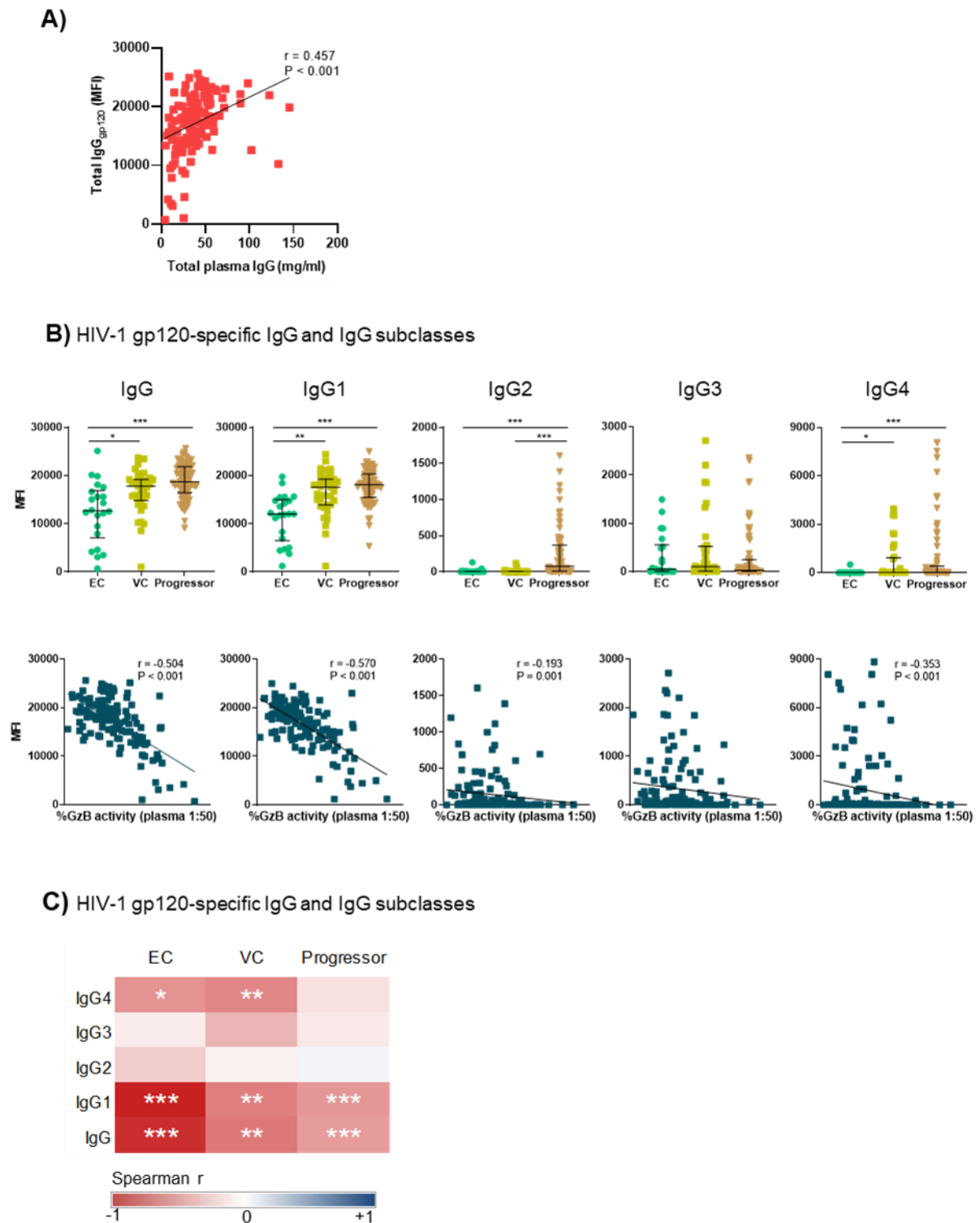


Figure 3.4. Association between ADCC responses (1:50 plasma dilution) and HIV-1-specific antibodies.
A) Total HIV-1 gp120-specific IgG levels associated with total IgG levels purified from 130 HIV-1 infected individuals. B) HIV-1 gp120-specific IgG and gp120-specific IgG1-IgG4 subclass levels compared between HIV-1 infected groups (the horizontal black line represents the median with the interquartile range as error bars) and correlated to ADCC responses in 130 HIV-1 infected individuals combined and in C) separate HIV-1 infected 22 elite controllers, 35 viraemic controllers and 73 progressors using the Spearman's or Pearson's coefficient correlation after normality tests. The colour represents the strength and direction of the correlation. Red = negative correlation, white = no correlation, and blue = positive correlation (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

3.3.5 Reduced gp120-specific serum IgA subclass levels in elite controllers that negatively correlate with ADCC responses

We next sought to evaluate the role of IgA levels in the ADCC responses measured for the different HIV-1 controller and progressor groups. Compared to IgA1, IgA2 occurs at lower levels in plasma/serum (reviewed in (Lopez et al., 2018)). Despite differences in levels, the two subclasses showed similar distributions among the HIV-1 infected groups. The median serum gp120-specific IgA1 levels were significantly higher in progressors compared to elite controllers and viraemic controllers (MFI 224.0 vs.179.0, $P_{\text{Dunn's}} = 0.03$ and 224.0 vs. 168.0, $P_{\text{Dunn's}} = 0.007$, respectively; Fig 3.5A). Similarly, progressors had higher median gp120-specific IgA2 levels compared to elite and viraemic controllers (MFI 45.0 vs. 11.0, $P_{\text{Dunn's}} < 0.001$ and 45.0 vs. 19.6, $P_{\text{Dunn's}} = 0.006$, respectively; Fig 3.5A). These data suggest an association between elevated IgA levels and more progressive disease.

Although controversial, competitive behavior of HIV-1-specific IgA towards IgG in gp120-specific binding has been previously illustrated to reduce ADCC responses in post-vaccination studies (Tomaras et al., 2013) and in natural HIV-1 infection (Ruiz et al., 2014, Ruiz et al., 2016). Even though we did not perform competitive binding assays, we observed an association between elevated IgA levels and reduced ADCC activity. ADCC responses in elite controllers and progressors were negatively correlated to gp120-specific IgA1 levels ($r = -0.728$, $P = 0.0001$ and $r = -0.328$, $P = 0.005$, respectively; Fig. 3.5B) and gp120-specific IgA2 levels ($r = -0.774$, $P < 0.0001$, $r = -0.415$, $P = 0.0004$, respectively; Fig 3.5C), as well as to gp120-specific IgA2 levels in viraemic controllers ($r = -0.366$, $P = 0.031$, Fig 3.5C). Furthermore, majority of IgG/IgA ratios positively correlated to ADCC responses, and statistically significant correlations included the IgG/IgA1 ratio in progressors ($r = 0.253$, $P = 0.031$, Fig 3.5B), as well as IgG/IgA2 and IgG1/IgA2 ratios in progressors ($r = 0.466$, $P < 0.0001$, and $r = 0.414$, $P = 0.0003$, respectively, Fig 3.5C). Contrastingly, viraemic controllers showed negative correlations between ADCC responses and gp120-specific IgA1 ratios, with the IgG4/IgA1 ratio being statistically significant ($r = -0.487$, $P = 0.004$, Fig 3.5B).

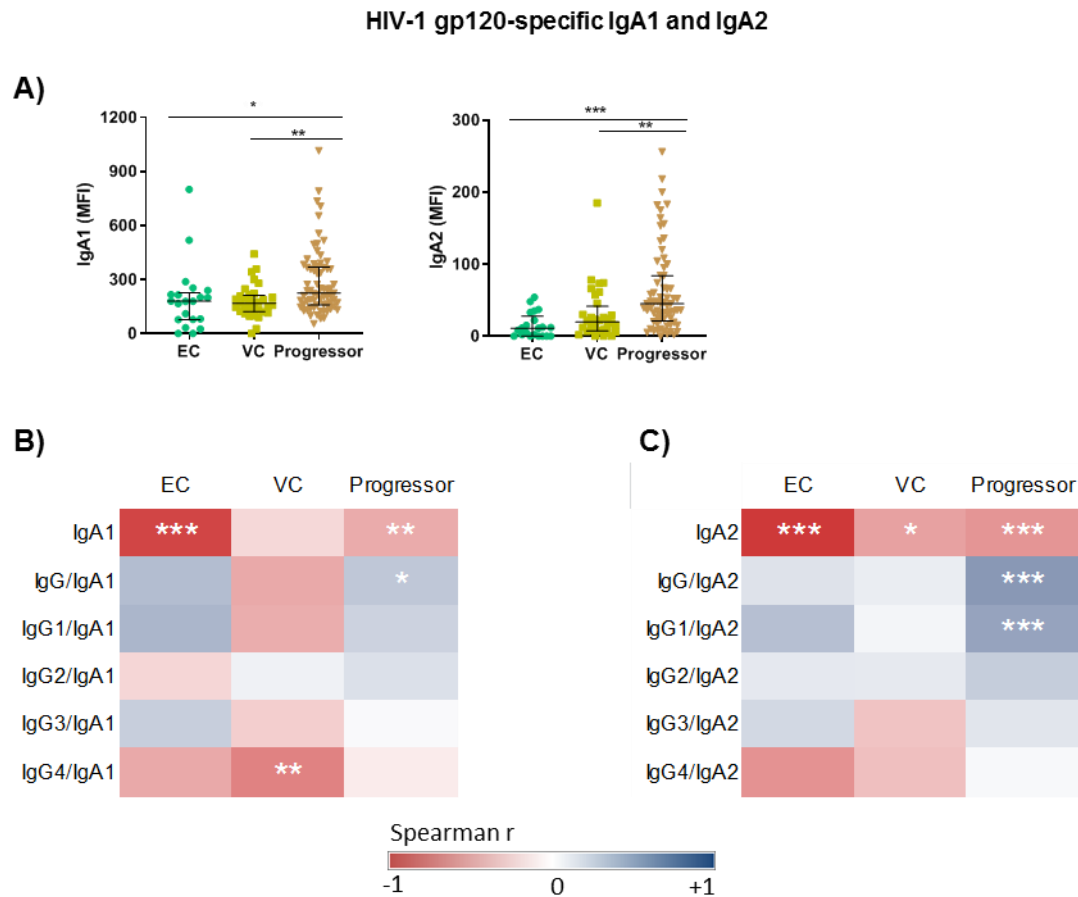


Figure 3.5. IgA subclass levels, IgG/IgA ratios and associations between ADCC responses at the lowest plasma dilution.

A) Levels of circulating gp120-specific IgA1 and IgA2 compared between 22 HIV-1 elite controllers, 35 viraemic controllers and 73 progressors by Kruskal-Wallis using Dunn's tests for multiple comparisons. The horizontal black line represents the median with the interquartile range as error bars. Correlations between ADCC (1:50 plasma dilution) and HIV-1 gp120-specific B) IgA1, IgG/IgA1, IgG1/IgA1, IgG2/IgA1, IgG3/IgA1, and IgG4/IgA1 as well as C) IgA2, IgG/IgA2, IgG1/IgA2, IgG2/IgA2, IgG3/IgA2, and IgG4/IgA2 in HIV-1 infected groups using the non-parametric Spearman rank or Pearson's correlation coefficient depending on the outcome of Shapiro Wilk's normality test. The colour represents the strength and direction of the correlation. Red = negative correlation, white = no correlation, and blue = positive correlation (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

3.3.6 Significant negative correlation between gp120-specific IgM levels and ADCC responses in Elite controllers

When we measured gp120-specific IgM levels in HIV-1 infected controllers and progressors we observed higher gp120-specific IgM levels in viraemic groups compared to elite controllers, although not statistically significant (Fig 3.6A). In addition, ADCC responses at the low plasma dilution correlated to gp120-specific IgM levels, with significantly inverse correlations in elite controllers ($r = -0.643$, $P = 0.005$, Fig 3.6B).

HIV-1 gp120-specific IgM

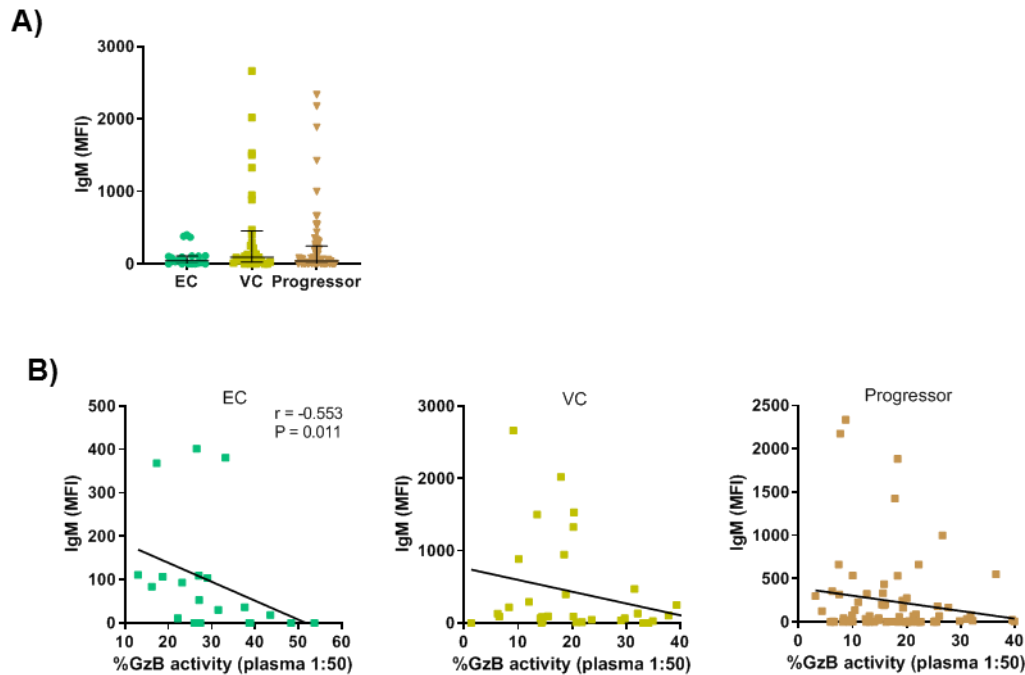


Figure 3.6. ADCC responses at low plasma dilutions and correlation with gp120-specific IgM levels.

A) HIV-1 gp120-specific IgM levels compared between elite controllers ($n = 22$), viraemic controllers ($n = 35$) and progressors ($n = 73$) using Kruskal Wallis with Dunn's test for multiple comparisons. The horizontal black line represents the median with the interquartile range as error bars. B) ADCC activity correlated with gp120-specific IgM levels in elite controllers, viraemic controllers and progressors (outliers removed). The non-parametric Spearman rank coefficient was used for assessments after groups failed normality tests.

3.3.7 CD4/CD8 ratios directly associated with ADCC responses at the low plasma dilution

Further analysis of host factors that may influence effector functions entailed investigating the association between markers of disease progression and ADCC responses. We did not find significant correlations between $CD4^+$ T cell count and ADCC responses at 1:50 plasma dilution in individual groups of elite controllers ($n = 22$), viraemic controllers ($n = 35$) and progressors ($n = 73$), whereas viral load negatively associated with ADCC responses in elite controllers and viraemic controllers ($r = -0.483$, $P = 0.02$ and $r = -0.364$, $P = 0.032$, respectively, Appendix 1, Fig S3). $CD8^+$ T cell counts were recorded for 20 controllers (12 elite controllers and 8 viraemic controllers), thus enabling analysis of ADCC responses relative to the CD4/CD8 ratio, an established marker of HIV-1 clinical outcome and immunopathogenesis (reviewed in (Lu et al., 2015)). We found a positive correlation between ADCC responses and CD4/CD8 ratios ($r = 0.701$, $P < 0.001$, Fig 3.7A).

Since progressive HIV-1 infection has been linked to CD4/CD8 ratios below 1 and hypergammaglobulinaemia (Balter, 1997), we categorized CD4/CD8 ratios according to values above and below 1. Controllers with CD4/CD8 ratio >1 had a higher median ADCC response compared to controllers with CD4/CD8 ratios <1 (36.4% vs. 21.1%, $P < 0.001$, Fig 3.7B). This difference was primarily attributed to the elite controller group (38.9% vs. 26.0%, $P = 0.001$, Fig 3.7B), with viraemic controllers not showing a significant difference between higher and lower range CD4/CD8 ratios. Furthermore, gp120-specific IgG subclass levels may affect this difference, with reduced mean IgG1 (and total gp120-specific IgG, not shown) levels in the higher compared to lower range CD4/CD8 ratios (MFI 9017 vs. 16458, $P = 0.009$, Fig 3.3C), and the contrary for mean IgG2 and IgG4 levels on higher compared to lower range CD4/CD8 ratios (MFI 4.10 vs. 0.833, $P = 0.039$ and 1710 vs. 1337, $P = 0.026$; respectively, Fig 3.3C), whilst IgG3 levels did not differ significantly between the two ratios (Fig. 3.3.C). Similar to gp120-specific IgG1 levels, gp120-specific IgA2 and IgM levels were significantly reduced in individuals with higher compared to lower range CD4/CD8 ratios (MFI, 1 vs. 12, $P = 0.027$ and 0.0001 vs. 68.5, $P = 0.021$; respectively), while IgA1 levels did not differ significantly between the two ratios (100.5 vs. 182.8, $P = 0.073$) (Fig 3.7D).

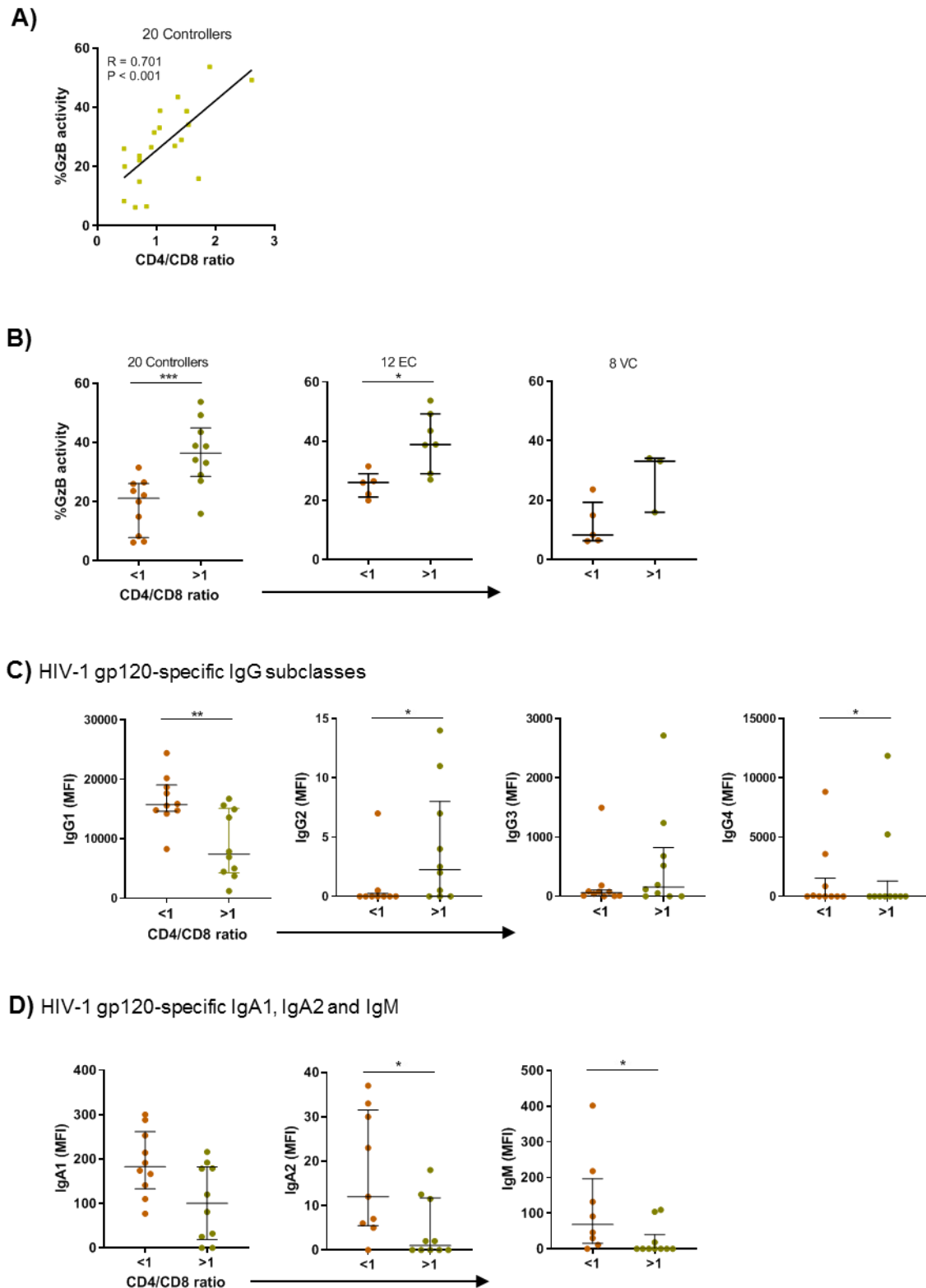


Figure 3.7. Correlation of ADCC at the low plasma dilution (1:50) with markers of disease progression. CD8⁺ T cell counts were recorded at time points selected for testing ADCC responses only for a subset of 20 controllers (12 elite controllers and 8 viraemic controllers) and used to determine the CD4/CD8 ratios which were A) correlated to ADCC responses at 1:50 plasma dilution. B) ADCC responses of the 20 controllers at 1:50 plasma dilution were divided into lower range (<1) and higher range (>1) CD4/CD8 ratios for comparison, and further divided into 12 elite and 8 viraemic controller groups. C) HIV-1 gp120-specific IgG1-IgG4 and D) gp120-specific IgA1, IgA2 and IgM levels were compared between the lower and higher range CD4/CD8 ratios. The horizontal black line represents the median with the interquartile range as error bars. All comparisons were performed with the Mann-Whitney test. (***) P < 0.001; ** P < 0.01; * P < 0.05).

3.4 Discussion

To define the role of antibody levels as regulators of HIV-1-specific effector functions, we measured ADCC responses for serially diluted plasma from 130 HIV-1 infected South Africans against HIV-1 gp120 coated CEM.NK^R.CCR5 cells. At low plasma dilutions, ADCC responses from HIV-1 infected individuals were reduced, known as the prozone phenomenon. It is unlikely that this inhibitory effect is solely determined by non-IgG plasma constituents, since ADCC responses remained low at high concentrations of isolated IgG. Moreover, high levels of IgG only targeting HIV-1 appears to have the same effect. It is plausible that high total IgG levels, whether exclusively HIV-1-specific or a combination of specific and non-specific IgG, may compete for FcγR or gp120 binding via their Fc or Fab domains, respectively, thus effectively lowering ADCC responses independently of other non-antibody plasma factors. A similar concept was illustrated in cancer therapeutics, wherein total serum IgG is thought to compete with therapeutic IgG1 trastuzumab (Herceptin[®]) for binding to FcγRs (Preithner et al., 2006).

It has been recognized since the early 1980's that HIV-1 infection is accompanied by hypergammaglobulinaemia (Lane et al., 1983), a condition characterized by polyclonal B cell activation that results in excessive production of immunoglobulin antibodies in circulation, IgG being the most predominant. Whether effects of hypergammaglobulinaemia may have important consequences for HIV-1-specific antibody effector function implications in untreated HIV-1 infected individuals with different clinical phenotypes (elite and viraemic controllers, progressors) is less explored.

Associations between ADCC responses and HIV-1 control are currently controversial, primarily due to conflicting findings that arise from variations within assays (reviewed in (Forthal and Finzi, 2018, Lewis et al., 2019)). Differences within assays include the use of different effector cell types (primary cells or cell lines), targets (antigen coated or virus infected), and purified IgG or plasma (Black et al., 1996, Tomaras et al., 2013, Duchemin et al., 2018). In addition, various end-point measures of ADCC activity further complicate data analysis. We demonstrate in the current study that ADCC measured as the percentage granzyme B (%GzB) activity at a plasma dilution of 1:50 displayed higher responses in elite controllers compared to viraemic controllers and progressors, while ADCC responses expressed as AUC, titre and maximum %GzB activity were conversely higher in progressors compared to elite and viraemic controllers. Although AUC is likely a more accurate analytical

measurement as it considers all points in the dilution series, we opted for ADCC at the low plasma dilution (1:50) for further data analysis since it more closely resembles physiological conditions where plasma is not highly diluted. Since enhanced ADCC associates with HIV-1 elite control at a plasma dilution of 1:50, we sought to investigate the potential role of hypergammaglobulinaemia in these correlations.

It has been established that HIV-1 viraemic groups develop hypergammaglobulinaemia, while HIV-1 controllers or long-term non-progressors (LTNPs) have lower antibody levels compared to viraemic individuals (Keating et al., 2017), as seen in the current study. In agreement, a South African study inversely correlated total serum IgG levels (including adenosine deaminase and lipopolysaccharide-binding protein) with CD4⁺ T cell count in untreated HIV-1 infected individuals (Ipp et al., 2013). Hypergammaglobulinaemia has been implicated in impairment of antigen-specific humoral immunity in HIV-1 infection that may occur as a result of improper B cell differentiation that produces low-specificity antibodies (reviewed in (Hu et al., 2015)). Several studies postulated that B cell hyperactivation and dysfunction may reduce antigen-specific antibody responses in viraemic individuals (Lane et al., 1983, Bowers et al., 2014, Aggarwal et al., 2017). On the contrary, studies by Titanji et al. demonstrated an inverse correlation between increasing disease progression (declining CD4⁺ T cell count) and hypergammaglobulinaemia, however, the authors do agree that hypergammaglobulinaemia is accompanied by a gradual loss of memory B cells that is severe in chronic progressors compared to long-term non-progressors (LTNPs) (Titanji et al., 2005, Titanji et al., 2006). It may therefore be this loss of antibody specificity during increased hypergammaglobulinaemia that contributes to impaired ADCC responses as observed for progressors, but not elite controllers who had significantly lower hypergammaglobulinaemia.

IgG1 and IgG3 are potent mediators of ADCC due to their relatively strong binding to FcγRs (Bruhns et al., 2009). In the context of HIV-1, IgG1 and IgG3 subclasses have been associated with increased HIV-1-specific ADCC responses and spontaneous control of viraemia (Banerjee et al., 2010, Ackerman et al., 2016, Madhavi et al., 2017). It is therefore unexpected that, in the present study, higher levels of gp120-specific IgG1 associated with reduced ADCC responses. Therefore, despite high affinity for FcγRs and abundance of gp120-specific IgG1, these antibodies fail to recruit ADCC responses. Conversely, the most potent mediator of ADCC, IgG3, did not associate with the magnitude of ADCC responses. The mechanism(s) underlying these observations is unclear. However, it may pertain to consequences of hypergammaglobulinaemia such as alterations in global and HIV-1-specific

IgG Fc glycosylation that affects Fc-FcγR interactions (Ackerman et al., 2013a, Chung et al., 2014a), potential competition for FcγR binding in the presence of excessive IgG (Preithner et al., 2006), or uneven distribution of antibodies that recognize potent ADCC-specific epitopes in HIV-1 infected individuals, which may account for differences in ADCC-mediating capacity (reviewed in (Butler et al., 2019)).

Immune-virological features in HIV-1 controllers have been shown to develop in antiretroviral therapy (ART)-treated HIV-1 infected individuals, particularly in early and prolonged treatments (Cellerai et al., 2011). A recent review (French et al., 2017) discussed effects of ART that included the lowering of viral load which occurs in concert with reduction of the pool of B lymphocytes that generate anti-HIV-1 IgG, thereby reversing effects of hypergammaglobulinaemia and resulting in lower total IgG (Morris et al., 1998, Aggarwal et al., 2017) and lower gp120-specific IgG1 and IgG3 (Jensen et al., 2015) in treated compared to untreated HIV-1 infected individuals. Despite improvement of NK cell function, ADCC-mediating capacity of antibodies decreases in patients receiving ART (Madhavi et al., 2014, Jensen et al., 2015), contrary to ADCC capacity in controllers. Therefore, understanding the maintenance of ADCC potency in elite controllers may assist improvement of ART or development of an HIV-1 functional cure.

The current observation of elevated gp120-specific IgA1 and IgA2 levels in progressors when compared to elite and viraemic controllers likely reflects the nonspecific polyclonal B cell activation associated with hypergammaglobulinaemia, similar to suggestions made by others (Amadori and Chieco-Bianchi, 1990, Moir and Fauci, 2009). However, the exact mechanisms behind enhanced IgA production during HIV-1 infection have not been defined. Also, the association of IgA with ADCC responses is not well understood due to data that presents IgA as either an inhibitor (Mestecky et al., 2004, Tomaras et al., 2013, Ruiz et al., 2016, Ruiz et al., 2017, Tuen et al., 2019) or promoter (Ackerman et al., 2018, Duchemin et al., 2018) of ADCC responses. Despite circulating gp120-specific IgG levels being approximately 100-fold higher than gp120-specific IgA levels in HIV-1 infection as observed in the current and previous study (Yates et al., 2013), our findings point to the inhibitory role of IgA based on the observation of high gp120-specific IgA subclass levels in progressors who present with reduced ADCC responses, and inverse correlations of both gp120-specific IgA subclasses with ADCC responses.

Similar to gp120-specific IgA observations, progressors had elevated gp120-specific IgM levels that may be attributed to effects of hypergammaglobulinaemia in the current study.

Declining CD4⁺ T cell counts in progressors may have negatively impacted on effective class switching of IgM to IgG and to IgA, resulting in elevated IgM levels and lower ADCC responses due to the IgM/IgG ratio being affected. This concept has been demonstrated in the mucosa, as reviewed by Kulkarni and Ruprecht (Kulkarni and Ruprecht, 2017). It warrants further investigation in systemic circulation, particularly in the course of an HIV-1 infection.

The importance of high CD4⁺ T cell count (often found in controllers) in conjunction with low CD8⁺ T cell count to impacting potent effector functions, is evident in that CD4/CD8 ratios, that were determined for a subset of controllers (12 elite controllers + 8 viraemic controllers), were directly correlated to increasing ADCC responses. Moreover, higher range CD4/CD8 ratios mediated enhanced ADCC responses compared to lower range CD4/CD8 ratios, which was attributed to the elite controller group. The reason for these associations may be partly explained by findings from a recent study (Tuen et al., 2019) that showed higher CD4/CD8 ratios and HIV-1 control being linked to enhanced IgG antibody binding to native Env. The authors speculate that the observation of slower disease progression being predicted by the antibody binding against wild-type Env (Study time point 1) and two V1V2 (Study time point 2) antigens may indicate that these responses are markers at specific stages of the disease, for other, protective functional responses, which may or may not be related. However, in the same study, CD4/CD8 ratios were directly correlated to Env-specific IgG levels (Tuen et al., 2019), in contrast to the current reduction of gp120-specific IgG in controllers with higher range CD4/CD8 ratios. Differences may be influenced by the small sample size of 4 Cameroonian individuals studied longitudinally in the Tuen et al. study. Nonetheless, current results on CD4/CD8 ratios support the notion of similarities between HIV-1 controllers and ART-treated patients. After ART, higher range CD4/CD8 ratios were associated with CD4⁺ T cell recovery, viral suppression (Mussini et al., 2015) and reduced gp120-specific IgG1 levels as a sign of reversal of hypergammaglobulinaemia (Jensen et al., 2015), which is comparable to controllers suppressing virus replication, maintaining stable CD4⁺ T cell count and low hypergammaglobulinaemia. In addition, viral load in controllers was observed to inversely correlate with ADCC responses.

Our data suggests that there may be a connection between the constant immune activation that accompanies HIV-1 disease progression in the absence of ART and the reduction of some FcγR-mediated effector functions such as ADCC. How the extent of immune activation that prevails during ART might impact on these same functions also remains to be determined. Although the exact mechanisms of ADCC inhibition by elevated antibody levels are not

clearly defined, therapeutic interventions seeking to harness ADCC in already HIV-1 infected individuals may need to address causes and mechanisms underlying hypergammaglobulinaemia in order to facilitate an antibody environment that is conducive to generating robust ADCC responses.

Chapter 4: Progressive HIV-1 Infection and Hypergammaglobulinaemia Associate with Enhanced Env-specific Phagocytic Responses

4.1 Introduction

Antibody-dependent cellular phagocytosis (ADCP) mediates protective immunity by eliminating immune complexes, opsonized pathogens, and infected cells. This effector function is initiated when the Fc portion of antigen-bound antibodies engage Fc-receptors (FcRs) expressed on phagocytes that include macrophages, monocytes, neutrophils, dendritic cells, and mast cells (Rabinovitch, 1995, Ravetch and Bolland, 2001, Garcia-Garcia and Rosales, 2002, Dale et al., 2008, Guillems et al., 2014, Bruhns and Jönsson, 2015). While best known for its extracellular anti-bacterial and anti-fungal functions, phagocytosis also plays a role in an effective defence against viral invasions (reviewed in (Tay et al., 2019)). ADCP and its contribution to protective immunity has been implicated in *in vivo* studies for influenza virus (Huber et al., 2001, He et al., 2017), West Nile virus (Chung et al., 2007), and SARS coronavirus (SARS-CoV) (Yasui et al., 2014).

A protective function for ADCP may also extend to HIV-1 (reviewed in (Lewis, 2014)). Several lines of evidence suggest a role for phagocytosis in protection against HIV-1 and SHIV/SIV infection following vaccination in humans and non-human primates, respectively (Barouch et al., 2013, Chung et al., 2014b, Barouch et al., 2015, Chung et al., 2015, Bradley et al., 2017, Ackerman et al., 2018). In addition, macrophages and neutrophils from the colon and cervix have been shown to mediate robust phagocytic capacity for HIV-1 antibody immune complexes and likely contribute to protection against virus entry at these vulnerable sites (Sips et al., 2016).

Establishing a clear role for ADCP in HIV-1 disease progression has been difficult. This may be due to the altered immune milieu present during chronic HIV-1 infection that severely affects critical components in the mechanism. For instance, impaired ADCP in chronic viraemic HIV-1 infected individuals was linked to reduced FcγRIIa expression on their phagocytes (Dugast et al., 2011). Furthermore, FcγR polymorphisms that affect antibody binding may further modulate phagocytic capacity. In a cohort of predominantly Caucasian men, individuals homozygous for an arginine at amino acid 131 in FcγRIIa, which results in reduced IgG2 binding, had a faster rate of CD4⁺ T cell decline compared to those bearing at least one histidine in this position, thereby associating the FcγRIIa 131RR allele with accelerated disease progression (Forthal et al., 2007).

In addition to variability within the cellular component of ADCP, different antibody properties may further alter ADCP responses. Antibody isotypes and subclasses are important regulators of Fc-mediated effector functions due to their unique binding affinities and

specificities for FcRs expressed on various effector cells (reviewed in (Vidarsson et al., 2014, Lu et al., 2018)). Several antibody characteristics have been linked to ADCP responses in HIV-1 disease progression. These include antibody polyfunctionality (Ackerman et al., 2016), Fc glycosylation (Ackerman et al., 2013a, Chung et al., 2014a) and the IgG2 subclass (Ngo-Giang-Huong et al., 2001). In particular, Env- and Gag-specific IgG2 levels were linked to HIV-1 control, whilst Env- and Pol-specific IgG1 levels correlated with HIV replication (Ngo-Giang-Huong et al., 2001). In addition, recombinant gp120 (rgp120) vaccine-elicited IgG2 had an inhibitory effect on internalization of viral-like particles by healthy donor monocytes, and deglycosylated rgp120 bound poorly to IgG2 and IgG4, but better to IgG1 and IgG3 (Forthal et al., 2011). Lack of direct associations indicates that the role of ADCP in the context of HIV-1 disease progression and natural control of infection requires further investigation.

There is currently a paucity of data on HIV-1-specific ADCP responses for South Africans, the population with one of the greatest HIV-1 epidemics. To address this, we describe ADCP responses in South African HIV-1 infected elite controllers, viraemic controllers compared to progressors relative to IgG isotype and subclass levels and clinical markers of disease severity.

4.2 Materials and Methods

4.2.1 Study cohort

The HIV-1 infected cohort consists of 22 elite controllers, 35 viraemic controllers and 73 progressors as described in Chapter 3, section 3.2.1 and table 3.1. Only 6 of the 18 HIV-1 uninfected individuals were included in the current study objective as healthy controls.

4.2.2 Antibodies

IgG was purified from plasma samples using the Melon Gel IgG Purification kit (Thermo Scientific, Rockford, Ill, USA) as described in Chapter 2, section 2.2.5.

4.2.3 HIV-1 gp120

An HIV-1 gp120 protein derived from the consensus sequence of HIV-1 subtype C viruses (HIV-1 ConC gp120, a gift from Lynn Morris, Virology, NICD) was selected as the target antigen in the phagocytosis assay. The protein was biotinylated using the Sulfo-NHS LC

biotin reagent (Thermo Scientific) according to manufacturer's instructions. HIV-1 gp120 (3 µg) was incubated with 12 µl yellow-green 1.0 µm FluoroSpheres™ NeutrAvidin™-Labeled Microspheres (Thermo Scientific) overnight at 4°C in the dark. Coated beads were washed and resuspended in 2% BSA/PBS (1-part beads to 100 parts diluent). Saturation of beads was determined empirically by incubation with differing amounts of HIV-1 gp120. The bead coating concentration that gave the maximal phagocytic score was determined at 90 µg/ml biotinylated gp120 by using HIVIG as a control polyclonal antibody at a concentration range from 1 µg/ml - 1000 µg/ml.

4.2.4 ADCP

HIV-1-specific antibody-mediated phagocytosis was measured as previously described (Ackerman et al., 2011). Briefly, HIV-1 gp120-coated fluorescent beads were opsonised with purified IgG at 1 µg/ml or 50 µg/ml for 2 hours at 37°C, 5% CO₂. Antibody-independent bead uptake was measured in parallel duplicate reactions containing HIV-1 gp120-coated fluorescent beads without antibodies. Following incubation, 1×10^5 THP-1 monocytic cells (NIH AIDS Research and Reference Reagent Program) reported to be homozygous for the H allele associated with superior IgG2 binding (Tebo et al., 2002), were added to each assay well and incubated for 16 hours at 37°C, 5% CO₂. Cells were subsequently fixed with 4% paraformaldehyde, acquired on a BD Fortessa X20 and analysed on FlowJo version 9.8.1 software (Tree Star, San Carlos, CA). Flow plots illustrating the gating strategy are included in Appendix 1, Fig S4. A phagocytic score was calculated by multiplying the proportion of bead-positive THP-1 cells with the mean fluorescence intensity (MFI) of the bead-positive population. The phagocytic score is widely used for this assay and in the present study it was representative of other output measurements, including bead-positive THP-1 cells (Spearman $r = 0.993$, 95% CI: 0.990-0.995; $P < 0.0001$), MFI of the bead-positive population (Spearman $r = 0.956$, 95% CI: 0.9396-0.9675; $P < 0.0001$), and the proportion of bead-positive THP-1 cells in the presence of antibody minus that of the antibody negative control (Spearman $r = 0.993$, 95% CI: 0.990-0.995; $P < 0.0001$). THP-1 cells were checked for total FcγRIIa expression by staining with the anti-CD32-AF642 monoclonal antibody (BD Biosciences).

4.2.5 HIV-specific IgG subclass detection

HIV-1 gp120-specific IgG1-IgG4 antibody levels were quantitated according to the description in Chapter 3, section 3.2.7.

4.2.6 Statistics

All statistics were performed as described in Chapter 3, section 3.2.8.

4.3 Results

4.3.1 IgG concentration modulates the association between ADCP and natural control of HIV-1 infection

An international consensus for the optimal concentration of isolated IgG to use in the HIV-1 gp120-specific fluorescent bead-uptake assay (Ackerman et al., 2011) has not been established. Study-dependent optimal concentrations have primarily been determined empirically from phagocytosis dose response curves (Ackerman et al., 2011, McAndrew et al., 2011, Ackerman et al., 2013b, Chung et al., 2014b). However, these IgG concentrations varied from 0.05-5 µg/ml (McAndrew et al., 2011), 1 µg/ml (Ana-Sosa-Batiz et al., 2014), to 6.25 µg/ml (Lai et al., 2014), whereas others reported an antibody titre calculated as half-maximal phagocytic activity observed in an IgG titration curve (Ackerman et al., 2013b).

In the present study, phagocytosis dose response curves were used to select isolated IgG concentrations (Fig 4.1A). To detect possible ADCP differences that may be confounded by high antibody levels, we selected a low IgG concentration of 1 µg/ml. Conversely, to assess functionality at higher IgG concentrations, we selected the highest possible IgG concentration for all patients that is 50 µg/ml. While circulating bulk IgG concentrations in healthy human serum range between 7-16 mg/ml (Dati et al., 1996) and are generally higher (> 16 mg/ml) in hypergammaglobulinemic HIV-1 infected individuals (Morris et al., 1998), a concentration of 50 µg/ml may be more representative of physiological IgG levels than 1 µg/ml.

Phagocytic responses were overall lower at 1 µg/ml compared to 50 µg/ml IgG (all $P < 0.001$, Fig 4.1B). Intriguingly, differences between the HIV-1 infected groups were only observed at low IgG concentrations (Fig 4.1C). At 1 µg/ml IgG, median phagocytic responses were significantly lower in elite controllers compared to chronic progressors (5.1 vs. 6.2, $P = 0.010$, Fig 4.1C). Conversely, at 50 µg/ml isolated IgG, the median phagocytic response of elite controllers was equivalent to that of chronic progressors (Fig 4.1C). The latter observation is likely not due to an increase in non-specific ADCP activity, since phagocytic activity from HIV-1 negative individuals at 50 µg/ml remained low.

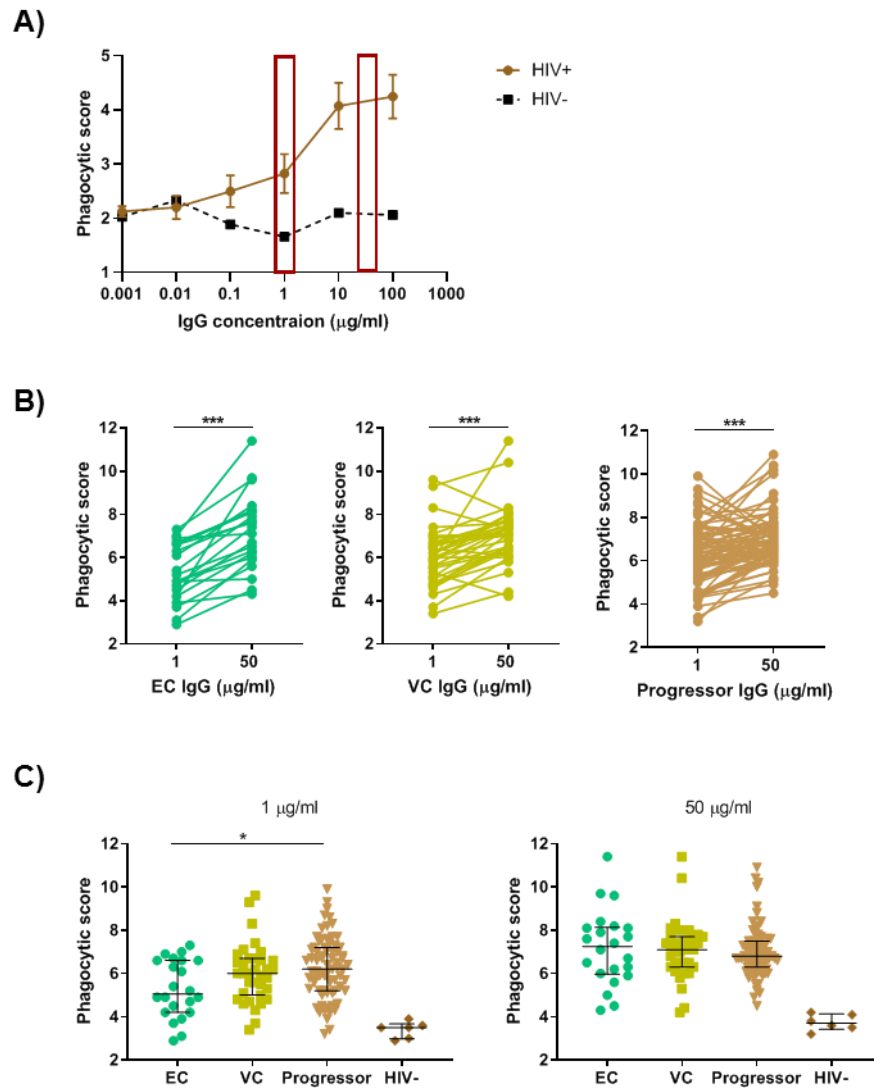


Figure 4.1. Comparison of ADCP responses at low and high IgG concentrations.

A) Median phagocytic scores of HIV-1 infected individuals (n = 4) and a healthy control in a phagocytosis dose-response curve with a concentration range from 0.001 µg/ml to 100 µg/ml. ADCP responses of HIV-1 infected elite controllers (n = 22), viraemic controllers (n = 35) and progressors (n = 73) compared between B) 1 µg/ml and 50 µg/ml using Wilcoxon matched pairs signed rank test, and C) using Mann-Whitney U to compare phagocytic scores of HIV-1 infected groups with each other (*** P < 0.001; ** P < 0.01; * P < 0.05). A horizontal black line represents the median with the interquartile range as error bars. Phagocytic scores of HIV-1 uninfected individuals (n = 6) were significantly lower than all HIV-1 infected groups, separately and combined (All P < 0.001 not illustrated on the figures).

4.3.2 Phagocytic responses directly correlate with hypergammaglobulinaemia

To determine whether non-specific B cell activation and excessive antibody production (hypergammaglobulinaemia) modulate HIV-1-specific IgG phagocytic capacity, we studied HIV-1-specific ADCP responses in relation to bulk IgG levels in plasma (Fig 4.2). As shown in Chapter 3, elite controllers had significantly lower median IgG levels (12.6 mg/ml) compared to viraemic controllers (33.2 mg/ml; $P_{\text{Dunn's}} = 0.004$) and chronic progressors (39.7 mg/ml; $P_{\text{Dunn's}} < 0.001$) (Fig 4.2A). In contrast to what we observed for ADCC responses,

ADCP responses at 1 $\mu\text{g/ml}$ directly correlated with plasma levels of IgG in the complete cohort ($r = 0.241$, $P = 0.006$, Fig 4.2B), but not in individual groups (data not shown). On the other hand, there was no significant correlation at 50 $\mu\text{g/ml}$ in the complete cohort (Fig 4.2C) and in individual groups (data not shown).

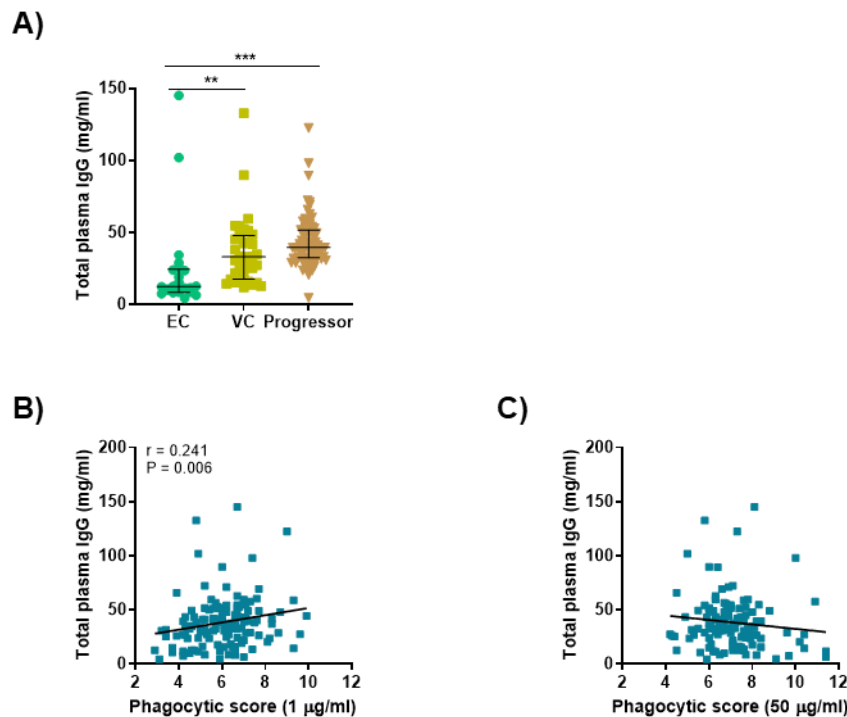


Figure 4.2. Phagocytic responses and antibody levels.

A) Kruskal-Wallis with Dunn's tests for multiple comparisons was used to compare total plasma IgG (mg/ml) levels between elite controllers ($n = 22$), viraemic controllers ($n = 35$) and progressors ($n = 73$) (median represented by a horizontal black line and error bars represent the interquartile range), which were correlated to ADCP responses in the complete cohort at B) 1 $\mu\text{g/ml}$ and C) 50 $\mu\text{g/ml}$ isolated IgG using nonparametric Spearman rank correlation coefficient and linear regression analysis. (***) $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

4.3.3 Plasma levels of HIV-1 gp120-specific IgG1 and IgG4 directly correlate with phagocytic capacity

To determine whether the differences observed for phagocytic scores at 1 $\mu\text{g/ml}$ isolated IgG may be determined by the availability of HIV-1 gp120-specific IgG in bulk IgG, we measured plasma gp120-specific IgG levels relative to bulk plasma IgG. The latter had higher median levels in elite controllers compared to viraemic controllers and progressors, although not statistically significant (Fig 4.3A). Despite the differences in normalized gp120-specific IgG levels between the groups, they all displayed a positive correlation with phagocytic responses as a collective at 1 $\mu\text{g/ml}$ ($r = 0.199$, $P = 0.020$, Fig 4.3B) and a stronger correlation at 50

µg/ml ($r = 0.395$, $P < 0.001$, Fig 4.3C), as well as in individual groups, with statistical significance at 1 µg/ml in viraemic controllers ($r = 0.354$, $P = 0.04$, Fig 4.3D) and at 50 µg/ml in viraemic controllers and progressors ($r = 0.445$, $P = 0.007$ and $r = 0.323$, $P = 0.005$, respectively, Fig 4.3D).

In the different gp120-specific IgG subclasses, the predominant IgG1 relative to bulk plasma IgG followed a similar pattern to gp120-specific IgG of higher levels in elite controllers compared to viraemic controllers (not significant) and progressors (MFI/(mg/ml), 678.8 vs 437.5, $P = 0.03$, Fig 4.3A), while all groups correlated significantly to ADCP responses at 50 µg/ml combined ($r = 0.371$, $P < 0.001$, Fig 4.3C) and in individual elite controllers, viraemic controllers and progressors ($r = 0.451$, $P = 0.04$; $r = 0.378$, $P = 0.03$ and $r = 0.288$, $P = 0.01$, respectively; Fig 4.3D). Conversely, gp120-specific IgG2 and IgG4 levels relative to bulk plasma IgG were significantly higher in progressors compared to elite controllers (MFI/(mg/ml), 3.0 vs. 0.005, $P < 0.001$ and 0.650 vs. 0.020, $P < 0.001$) and viraemic controllers for normalized IgG2 (3.0 vs. 0.020, $P < 0.001$) (Fig 4.3D). However, normalized IgG4 levels were significantly positively associated with ADCP responses at 1 µg/ml ($r = 0.320$, $P < 0.001$, Fig 4.3B) and 50 µg/ml ($r = 0.221$, $P = 0.01$, Fig 4.3C) in all groups combined, while failing to correlate significantly in individual groups. Although significant, correlations in figures 4.3B and 4.3C appear to be not significant due to majority of IgG4/total IgG values being zero or less than 1 as a result of IgG4 MFI values that are generally lower compared to other subclasses, whilst few individuals had high IgG4 MFI values.

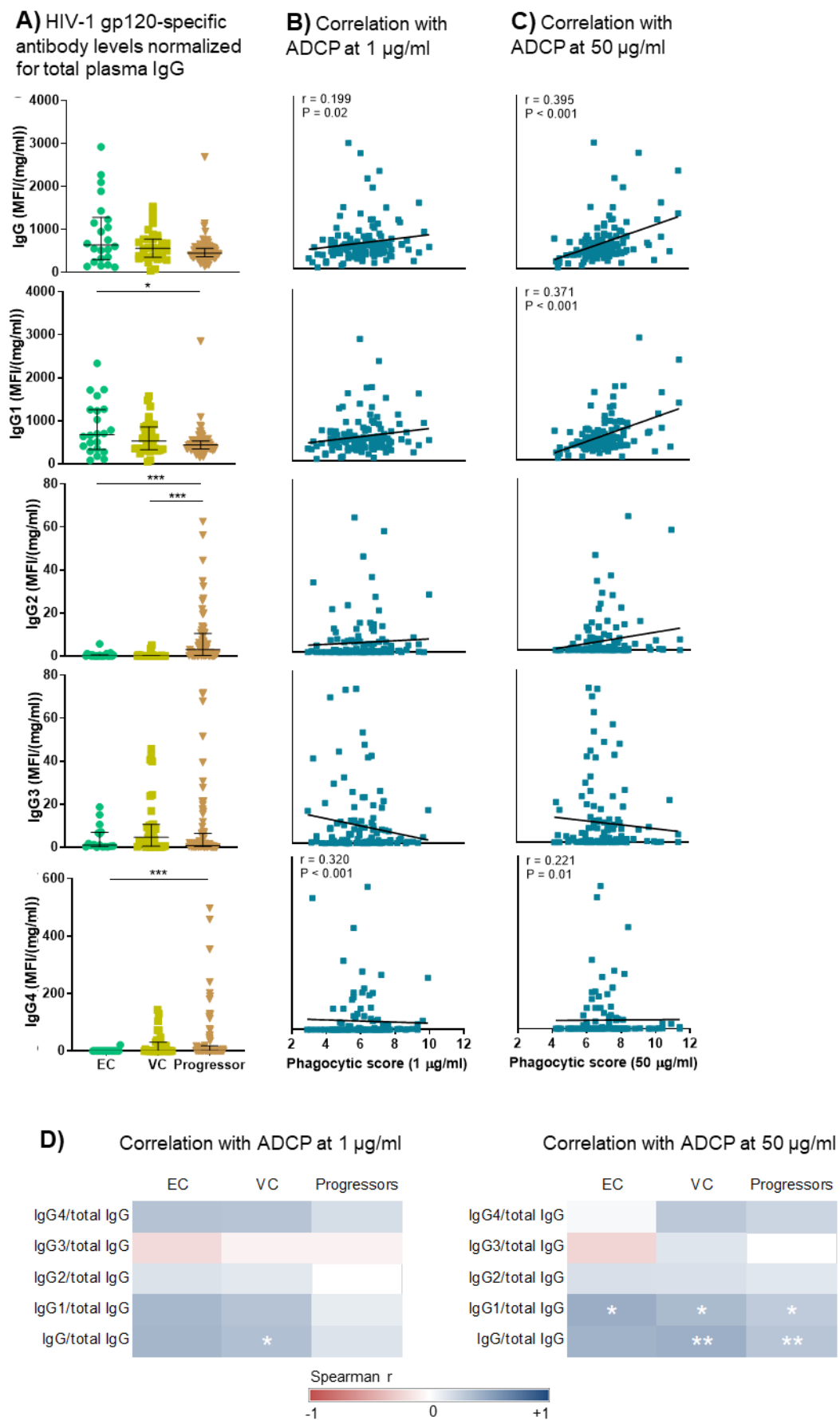


Figure 4.3. HIV-1 gp120-specific IgG and IgG1-IgG4 subclass levels normalized for total plasma IgG and correlated with phagocytic scores.

A) Comparing gp120-specific antibody levels/total IgG (MFI/(mg/ml)) between elite controllers (n = 22), viraemic controllers (n = 35) and HIV-1 progressors (n = 73) using Kruskal-Wallis with Dunn's multiple comparison. A horizontal black line represents the median with the interquartile range as error bars (*** P < 0.001; ** P < 0.01; * P < 0.05). Normalized gp120-specific antibody levels were correlated with phagocytic scores at B) 1 µg/ml and C) 50 µg/ml in all HIV-1 infected groups (n = 130) combined and in D) separate elite controllers, viraemic controllers and progressors using Spearman or Pearson correlation tests following Shapiro-Wilk normality tests.

4.3.4 CD4/CD8 ratios inversely correlated with ADCP responses at the low IgG concentration in a subset of controllers

Further investigation of host factors that may influence phagocytic responses entailed determining whether phagocytic scores had any associations with markers of disease progression (viral load and CD4⁺ T cell count), which were found to be absent at phagocytic responses mediated by the high purified IgG concentration (50 µg/ml) (Appendix 1, Fig S5). Although viral load and CD4⁺ T cell count did not correlate significantly to ADCP responses mediated by 1 µg/ml purified IgG in controllers or progressors analysed separately (Appendix 1, Fig S5), an increase in viral load weakly correlated positively with phagocytic scores in the complete cohort (r = 0.196, P = 0.026, Fig 4.4A). With reports of CD8⁺ T cell expansion during HIV-1 infection (Cao et al., 2009, Cao et al., 2016, Younes et al., 2016), and the acknowledgement of CD4/CD8 ratios as reliable markers of HIV-1 clinical outcome and HIV-1 related immune dysfunction (Fahey et al., 1990, Lu et al., 2015), we analysed possible associations between CD4/CD8 ratios and phagocytic responses in the current study. Although CD8⁺ T cell counts were not recorded for progressors and some controllers at time-points used to measure phagocytosis, we correlated CD4/CD8 ratios to phagocytic scores at 1 µg/ml for 20 controllers that comprised 12 elite controllers and 8 viraemic controllers. We found that phagocytic scores were inversely correlated to CD4/CD8 ratios (r = -0.506, P = 0.02, Fig 4.4B). Furthermore, we observed significantly enhanced median phagocytic scores in controllers that had CD4/CD8 ratios below 1 (6.07 vs. 4.53, P = 0.04, Fig. 4.4C), but they were not significantly different in separate elite controllers and viraemic controllers (Fig 4.4C).

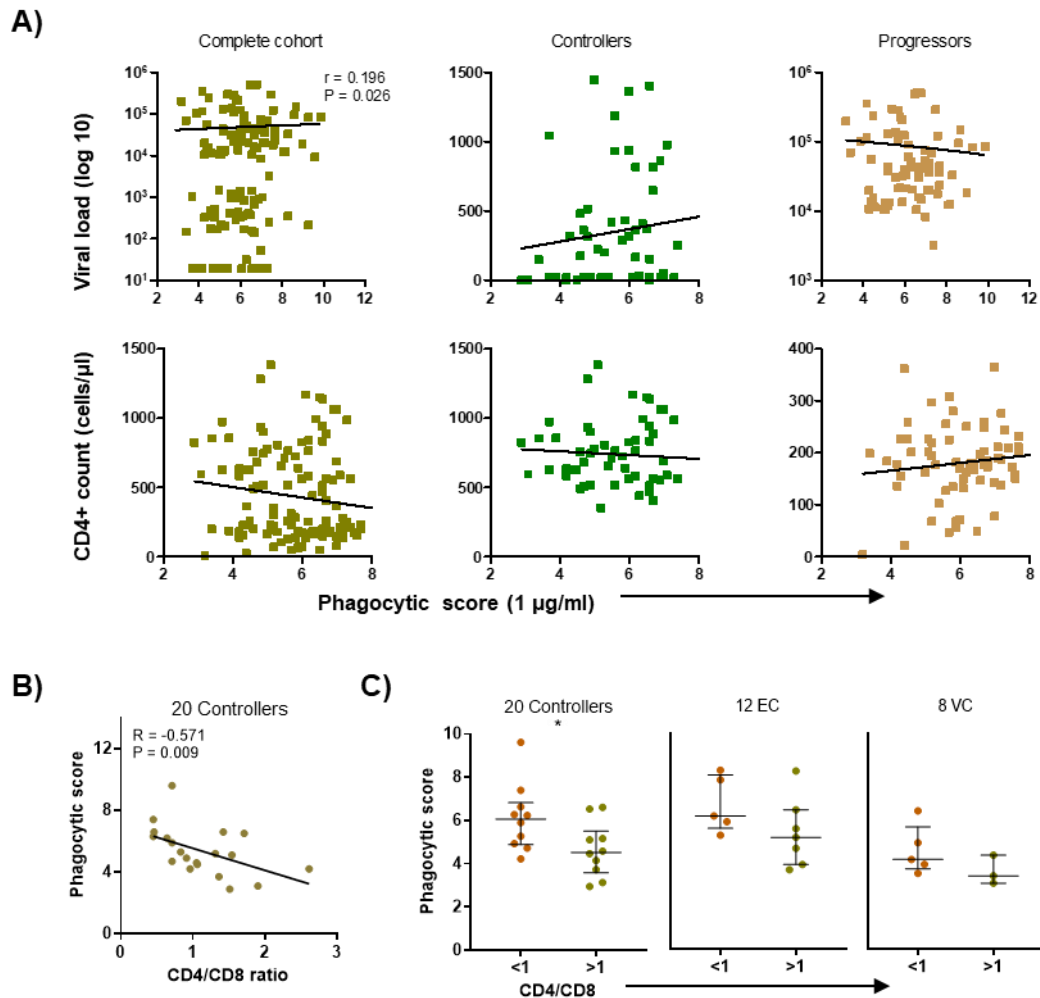


Figure 4.4. Correlation of ADCP at the low IgG concentration with markers of disease progression.

A) Viral load and CD4⁺ T cell count were correlated with ADCP at 1 μg/ml in the complete HIV-1 positive cohort (n = 130), and in separate controllers (n = 57) and progressors (n = 73) using the Spearman's rank coefficient correlation. CD4⁺ T cell counts were recorded at time points selected for testing phagocytic responses only for a subset of 20 controllers (12 elite controllers and 8 viraemic controllers) and used to determine the CD4/CD8 ratios which were B) correlated to ADCP responses at 1 μg/ml. C) ADCP responses at 1 μg/ml were divided into lower range (<1) and higher range (>1) CD4/CD8 ratios and compared in combined controllers (n = 20) and in separate elite controllers (n = 12) and viraemic controllers (n = 8). The median is represented by a horizontal black line and black bars represent the interquartile range (***) $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

4.4 Discussion

The role of circulating antibody isotype and subclass proportions in modulating FcγR-mediated effector functions in the course of an HIV-1 infection is becoming increasingly recognized. However, this is primarily studied in the context of HIV-1-specific ADCC responses. Less is known about ADCP and HIV-1 control, especially in the South African population that bears the brunt of the HIV-1 pandemic. Therefore, we investigated the effect of circulating HIV-1-specific IgG and IgG subclass levels on ADCP responses in a South African cohort of HIV-1 controllers and progressors.

We demonstrate that the concentration of isolated IgG used to assess and compare phagocytic responses of patient groups affects the outcome of associations. In particular, elite controllers had significantly lower ADCP responses compared to untreated chronic progressors when measured for 1 µg/ml isolated IgG; however, these differences disappear when using 50 µg/ml isolated IgG. The latter is consistent with the findings of Ackerman et al., who did not observe differences between elite controllers and chronically infected viraemic patients after using the concentration of antibody required for half-maximal phagocytosis (PC₅₀) from a dose-dependent curve of 0.01-100 µg/ml IgG concentration range in the THP-1 phagocytosis assay similar to the currently used (Ackerman et al., 2013b). One study that measured ADCP responses at 1 µg/ml had only evaluated these responses in primary and early chronic HIV-1 infection, and found that ADCP activity increased over a 2 year period in 96.8 % of tested individuals (Ana-Sosa-Batiz et al., 2014). This may imply continual increase in ADCP responses as HIV-1 disease progresses, thus partly supporting current findings. However, Ana-Sosa-Batiz and collaborators had not looked into the role of HIV-1-specific antibody levels in ADCP responses.

HIV-1-specific antibody levels may modulate ADCP responses, as illustrated by positive associations between gp120-specific IgG levels adjusted for bulk plasma IgG and ADCP responses in all HIV-1-infected groups, with associations that are stronger when ADCP is mediated with 50 µg/ml and weaker with 1 µg/ml purified IgG. Furthermore, associations at 50 µg/ml ADCP are significant in viraemic groups (viraemic controllers and progressors), which may implicate the role of hypergammaglobulinaemia, a condition characterized by excessive production of Ig antibodies in circulation due to polyclonal B cell hyperactivation (Lane et al., 1983). The direct relationship between HIV-1 replication and production of HIV-1-specific IgG, reviewed recently (French et al., 2017), is evident in treated patients reversing the effects of hypergammaglobulinaemia through reduction of viral load accompanied by a decrease in the pool of B lymphocytes that produce anti-HIV-1 IgG, resulting in lower total IgG levels than in untreated HIV-1 infected individuals (Morris et al., 1998, Aggarwal et al., 2017). In addition, Ackerman et al. observed significantly enhanced ADCP responses by IgG purified from chronically infected viraemic individuals and controllers compared to those on ART (Ackerman et al., 2013b).

While bulk plasma IgG was significantly higher in progressors compared to controllers in the current study, gp120-specific IgG that was adjusted for total plasma IgG was reduced in progressors compared to controllers, although the difference was not significant. Based on the

association of hypergammaglobulinaemia with dysfunctional B cells that produce high levels of non-specific antibodies (reviewed in (Hu et al., 2015)), it is plausible that continuous HIV-1 replication in conjunction with elevated hypergammaglobulinaemia (of both specific and non-specific antibodies) in untreated progressors provides the antigen-specific antibody:bulk plasma antibody ratio required to enhance some HIV-1-specific antibody responses such as ADCP in an attempt to control virus replication. However, this exercise seems futile *in vivo*, confronted with defects in immune responses that arise from progressive HIV-1 disease including impaired ADCP (Biggs et al., 1995, Kedzierska et al., 2000, Kedzierska et al., 2002).

HIV-1-specific IgG subclass levels add another layer of regulating ADCP responses in the course of an HIV-1 infection. The gp120-specific IgG1 adjusted for bulk plasma IgG positively associated with ADCP responses at 50 µg/ml in all HIV-1 infected individuals collectively, and in individual groups. Lai et al. demonstrated positive associations between gp120-specific IgG1 levels and ADCP responses mediated at the low 6.25 µg/ml using the same assay in a cohort of HIV-1 elite controllers only, and suggested that gp120-specific IgG1 levels impacted effector function mediating capacity (Lai et al., 2014). The demonstration of higher gp120-specific IgG1 adjusted for bulk plasma IgG levels in elite controllers compared to progressors is consistent with studies that demonstrated higher gp120-specific- IgG1 levels in controllers compared to progressors (Banerjee et al., 2010, Ackerman et al., 2016). Furthermore, the positive association of this antibody subclass with ADCP responses in both controllers and progressors may be contributed by the fact that it is one of the highly potent Fc-effector function mediating antibody subclasses (reviewed in (Moris et al., 2019)) and the most abundant (Banerjee et al., 2010).

We further demonstrated that gp120-specific IgG2 and IgG4 levels after adjusting for bulk plasma IgG were expectedly significantly elevated in progressors compared to controllers. Recent findings support increasing levels of Env-specific IgG2 antibodies in chronic progressors (Sadanand et al., 2018). Furthermore, Ackerman et al. found gp120-specific IgG2 and IgG4 responses to be prevalent amongst HIV-1 chronically infected individuals (treated and untreated), and to be absent or rare in elite controllers (Ackerman et al., 2016). IgG2 and IgG4 are also known as less functional subclasses compared to IgG1 and IgG3 on the basis of their lower binding affinity (Bruhns et al., 2009). Ackerman et al. negatively associated gp120-specific IgG2 and IgG4 with antibody-mediated effector functions including ADCP in an HIV-1 infected American cohort (Ackerman et al., 2016). We observed a lack of association of normalized IgG2 levels with ADCP responses, while normalized IgG4 levels

weakly positively associated with ADCP responses at both 1 µg/ml and 50 µg/ml in the complete cohort. Whether gp120-specific IgG4 levels may play a role in enhanced phagocytic responses observed in progressors warrants further investigation.

Although HIV-1-specific IgG subclass levels appeared to play a role in phagocytic responses, markers of disease progression did not, with the exception of the CD4/CD8 ratios that were only determined in 12 elite controllers and 8 viraemic controllers. We found that phagocytic scores were inversely correlated to CD4/CD8 ratios, and that controller individuals with the lower range CD4/CD8 ratios had enhanced ADCP responses compared to those with the higher range ratios. Since CD4/CD8 ratio is an established marker of immune activation, disease progression and clinical outcome (Taylor et al., 1989, Serrano-Villar et al., 2013, Serrano-Villar et al., 2014), it can be postulated that the expansion of CD8⁺ T cells indicative of enhanced immune activation and inflammation that results in the lower range CD4/CD8 ratios (reviewed in (Lu et al., 2015)), may associate with enhanced ADCP responses as HIV-1 disease progresses. However, a future study of these ratios and ADCP responses in HIV-1 progressors is required to validate this hypothesis.

Antibody levels may play an important role in HIV-1-specific ADCP in the course of an HIV-1 infection. Our findings suggest that hypergammaglobulinaemia alters antibody characteristics that favour ADCP functionality. The hyperactive immune conditions found in progressors may be conducive to efficient antibody-mediated phagocytic responses *in vitro*. Although not entirely reflective of *in vivo* conditions, such findings may potentially lead to better understanding of the role of ADCP in HIV-1 infection that can be translated to developing an effective treatment or functional cure.

Chapter 5: Summarizing Discussions and Conclusions

The goal to develop an effective HIV-1 vaccine continues to be explored primarily through antibody functions (reviewed in (Lu et al., 2018)). The polyfunctionality of antibodies, along with their ability to target a plethora of pathogens at multiple stages of infection and at multiple sites (antigens and epitopes), and the ultimate antibody-mediated pathogen clearance from circulation or tissues by joining the adaptive and innate arms of the immune system, places antibodies firmly in focus for rational vaccine design (reviewed in (Bournazos and Ravetch, 2017b)). Following the RV144 vaccine trial, more attention has been directed towards non-neutralizing antibody-mediated effector functions including ADCC, ADCP and ADCDC that can be modulated by a variety of host factors that comprise the antibody, effector cell and target cell components (reviewed in (Bournazos and Ravetch, 2017a)). Although these mechanisms and their plethora of host factors are usually assessed independently in *in vitro* HIV-1-specific immune responses, their interdependence in the course of an HIV-1 infection is crucial but less understood. In addition, demographic host factors that are sometimes unaccounted for - such as ethnicity, race, gender, age and genetics - further complicate interpretation and comparison of findings across studies. This contributes to a general lack of understanding of the relationship between Fc-mediated effector functions and their role in disease control/progression. The lack is more pronounced in the African context as such studies are often conducted with American or European cohorts that are also primarily infected with HIV-1 subtype B. Therefore, an intense study of populations who are predominantly infected with subtype C virus that is widely spread in southern African countries is necessary to help close the gap in knowledge pertaining to HIV-1 infection and Fc-mediated effector functions in this region, which could lead to the formulation of effective HIV vaccine, cure or treatment strategies.

FcγRs modulate Fc effector functions through levels of expression on effector cells as well as FcγR genetic variants (reviewed in (Bournazos et al., 2016, Rosales, 2017)). There is evidence of variation at the *FCGR* locus in healthy individuals and in different disease settings as impacted by demographic differences such as countries and ethnicities (Niederer et al., 2010, Machado et al., 2012, Lassaunière and Tiemessen, 2015, Nagelkerke et al., 2019b), which has implications for effector function capabilities. Given the influence of FcγR SNPs on antibody binding affinity, and copy number variation on FcγR expression levels and distribution (reviewed in (Hargreaves et al., 2015)), studies investigating HIV-1 infection in the context of FcγR genotypes and expression levels are warranted across the globe. But such studies are often complicated by the high sequence homology between the *FCGR* genes. The MLPA assay, first developed almost 2 decades ago (Schouten et al., 2002), remains the

golden standard for analyzing the highly similar sequences of the distinct *FCGR* genes that function differently and have unique regulation mechanisms. Although recent efforts to move to genome wide testing for larger sample sizes were conducted, they were met with challenges such as the inability to directly genotype the *FCGR3A* SNP (rs396991), thus resulting in unreliable imputation (Geraghty et al., 2019). Therefore, the MLPA assay has been generating reliable data that propels efforts to elucidate the *FCGR* genes and FcγRs in association with effector functions and diseases (Breunis et al., 2008, Lassaunière et al., 2016). Nonetheless, adjusting for factors that are known to influence ADCC responses such as copy numbers of the *FCGR3A* gene and the expression of inhibitory FcγRIIb on NK cells (Breunis et al., 2009, Niederer et al., 2010, van der Heijden et al., 2012, Mueller et al., 2013) that may invariably affect HIV-1 acquisition, MTCT, and disease progression is essential for analysis and interpretation of data. Therefore, following use of the MLPA assay, we found that when Black South African study participants were matched for F158V genotypes, carried two copies of the *FCGR3A* gene and were negative for FcγRIIb expression on NK cells, healthy donors bearing at least one 158V allele had higher NK-cell mediated ADCC responses compared to those homozygous for the 158F allele, whereas the opposite was observed for the HIV-infected group. However, the difference was not statistically significant, which may have been affected by having few study participants, which is a limitation of the current study. Therefore, a study of *FCGR* genes in a larger cohort with different disease states compared to healthy controls, bearing in mind demographic differences, may lead to prospects of developing a more personalized medicine approach (reviewed in (Nagelkerke et al., 2019a)).

In addition to *FCGR* genes, less is known about FcγR expression levels on some critical HIV-1-specific cell populations that are less appreciated for their capacity to mediate ADCC. These include the innate-like CD8⁺ T cells, and the influence of FcγRIIIa expression on their cytotoxic activity during HIV-1 disease progression, a process known to alter cellular distribution and function. According to our knowledge, our study is the first published study to demonstrate the upregulation of FcγRIIIa on CD8⁺ T cells of HIV-1 infected individuals when compared to uninfected individuals, as well as the capacity of FcγRIIIa⁺ CD8⁺ T cells to mediate ADCC (Phaahla et al., 2019), which has been previously described in disease settings other than HIV-1 (Bjorkstrom et al., 2008, Clemenceau et al., 2008). Our study was recently cited and validated by Naluyima et al. who associated chronic, untreated, HIV-1 infection with the expansion of an FcγRIIIa⁺ CD8⁺ T cell population that has the capacity to mediate HIV-1-specific ADCC (Naluyima et al., 2019). Their study further characterized this cell

population to be late-stage differentiated and to express high levels of receptors such as NKp80 that were recently associated with the development and maturation of fully functional NK cells (Freud et al., 2016), thus displaying a ‘hybrid’ CD8⁺ T cell and NK cell. A recent study described a continuous ‘innateness gradient’ that has been developed from transcriptional and functional data from human lymphocyte populations, with adaptive cells (T lymphocytes) on one end of the spectrum, innate-like T cells in the middle (i.e Mucosal associated invariant T cells, iNKT, $\gamma\delta$ T cells), and innate effector cells (i.e NK cells) on the other end (Gutierrez-Arcelus et al., 2019). Although their work excluded characterization of the Fc γ RIIIa⁺ CD8⁺ T cell population in their innate-like T cell group, they described common transcriptional and functional traits within the group that are likely applicable to Fc γ RIIIa⁺ CD8⁺ T cells as supported by Naluyima et al. (Naluyima et al., 2019). One of the illuminating findings from Gutierrez-Arcelus et al. is that T lymphocytes prioritize transcription of cellular translational machinery and promote proliferation, innate cells primarily focus on transcribing genes that encode effector functions, and the hybrid innate-like T cells harbour traits that promote both proliferation and rapid effector function (Gutierrez-Arcelus et al., 2019). Harbours both traits puts innate-like T cells such as Fc γ RIIIa⁺ CD8⁺ T cells at an advantage when it comes to developing effective cure or treatment strategies against HIV-1. Therefore, future studies would benefit from full characterization of this cell population which could potentially contribute to the evolution of therapeutic strategies that include Fc γ R-mediated effector functions for elimination of HIV-1 reservoirs (Bruehl et al., 2016, Lu et al., 2016a, Schoofs et al., 2016).

It can be postulated that the ‘hybrid’ Fc γ RIIIa⁺ CD8⁺ T cell population develops from an HIV-1-specific CD8⁺ T cell population that is no longer effective in eliminating the virus due to viral mutations that occur in the chronic phase of HIV-1 infection (Bernardin et al., 2005, Goonetilleke et al., 2009, Khaitan and Unutmaz, 2011). Therefore, developing Fc γ RIIIa⁺ CD8⁺ T cells use a different mechanism (ADCC) of virus replication control that deviates from the normal CD8⁺ T cell MHC-dependent cytotoxic killing of infected cells that dominates the acute phase of HIV-1 infection (Koup et al., 1994, Goonetilleke et al., 2009). It is plausible that the emergence of this cell population is meant to compensate for the loss of innate-like effector functions occurring in chronic HIV-1 infection as the cytotoxic CD56^{dim}Fc γ RIIIa^{bright} NK cell subset shrinks and the dysfunctional CD56^{neg}Fc γ RIIIa^{bright} NK cell subset expands. This may not be the only means of compensation that occurs in chronic HIV-1 infection. Based on their findings, Dugast et al. suggested that following acute HIV-1 infection, Fc- and Fab-related antibody functions are modulated in a distinct manner (Dugast

et al., 2014). Perhaps this notion extends to distinct modulation of different Fc effector functions in the course of an HIV-1 infection, leading to the possibility that the decline of some Fc effector functions are compensated by the increased potency of others during progression of HIV-1 disease.

We observed a loss of ADCC activity that may have been compensated by a gain in potent ADCP activity by antibodies purified from HIV-1 progressors. The reverse was observed in HIV-1 elite controllers. Although causes for these differences are uncertain, antibody levels appeared to play an important modulatory role in these effector functions. Indeed, we observed an inhibitory effect on ADCC responses at low plasma dilutions (and high purified IgG concentrations) in all HIV-1 infected individuals. This effect was more pronounced in HIV-1 progressors who showed significantly lower plasma-mediated ADCC activity in the presence of elevated hypergammaglobulinaemia compared to HIV-1 controllers who displayed enhanced plasma-mediated ADCC responses at low hypergammaglobulinaemia. In addition, the composition of antibodies was also implicated to play a role, as hypergammaglobulinaemia has been associated with dysfunctional B cells that produce high levels of non-specific antibodies, particularly in chronically HIV-1 infected individuals (reviewed in (Hu et al., 2015)). Therefore, reduced ADCC responses in chronic progressors may possibly be a culmination of having high total IgG levels composed of high levels of non-specific IgG to compete with HIV-1-specific IgG for binding to gp120 or Fc γ Rs. The impact of antibody:antigen ratios and antigen-specific antibody:total antibody ratios on effector function capacity has been primarily elucidated in disease settings other than HIV-1 infection, wherein antibody deficiency or surplus was shown to have adverse effects on antibody mediated responses (Taborda et al., 2003, Preithner et al., 2006). On the other hand, requirement for non-specific IgG antibodies within total IgG to mediate enhanced effector functions compared to antibodies that are exclusively antigen-specific has also been previously demonstrated. In one example, a glycoengineered (GE) IgG with enhanced Fc γ RIIIa binding affinity (GA101 anti-CD20 monoclonal antibody) mediated higher ADCP and ADCC activities when tested in the presence of non-specific, endogenous IgG antibodies versus when tested on its own (Herter et al., 2014). It appears that the collaborate effect of HIV-1-specific and non-specific IgG mimic physiologic conditions and mediate enhanced phagocytic responses, particularly at higher concentrations in circulation. This illustrates the requirement for not only optimal circulating total antibody concentrations, but also the correct composition of antibodies within the total for the formation of immune complexes that can activate maximal effector function responses. This is supported by enhanced normalized

gp120-specific IgG2 and IgG4 in progressors compared to controllers in the current study, with Env-specific IgG2 being known to contribute to FcγRIIa-mediated effector functions (French et al., 2010). Therefore, it is plausible that the amount of gp120-specific and non-specific antibodies making up total IgG in progressors is optimal for mediating enhanced ADCP responses, but competitive for ADCC responses based on the mechanistic differences between the two effector functions. In addition, the difference between ADCC and ADCP responses in these groups of HIV-1 infected individuals may be contributed by that we tested ADCP responses with purified IgG without plasma constituents to affect activity.

The mediation of ADCC responses using plasma dilutions opens the possibility of interference by plasma constituents other than antibodies. Although both low plasma dilutions and high IgG concentrations had an inhibitory effect on ADCC responses, the prozone effect observed in HIV-1 infected individuals became more exacerbated when plasma instead of purified IgG was used, thus implicating an inhibitory role for factors such as IgA. In particular, our data showed higher plasma gp120-specific IgA subclass levels in progressors who mediated reduced ADCC responses at low plasma dilutions compared to controllers. This supports earlier reports of low plasma dilutions having an inhibitory effect on ADCC, while high plasma dilutions showed less ADCC inhibition using the chromium release assay (Baum et al., 1996, Battle-Miller et al., 2002). This was postulated to result from IgA being diluted out as an inhibitory factor. Furthermore, IgA in plasma has been shown to bind FcαRs expressed on cell types that are present in PBMCs, such as monocytes, without activating them, but causing interference with FcγR-mediated responses such as ADCP (Wilton, 1978, Nikolova and Russell, 1995, Pasquier et al., 2005). More recently, plasma IgA1 binding affinity to FcαRs was observed to be high in HIV-1 progressors and low in controllers, and that progressors had higher plasma IgA1 concentrations compared to controllers (Chung, 2018). The same study went on to speculate that the weaker binding affinity observed in controllers may be responsible for higher effector function mediating capacity in these individuals. Therefore, in addition to IgA subclass levels, differential IgA binding affinity in controllers and progressors may be important in Fc effector functions. However, the contribution of this isotype is uncertain due to various findings that have either demonstrated IgA to have an inhibitory effect (Tomaras et al., 2013, Ruiz et al., 2016) or a supportive role (Duchemin et al., 2018) on ADCC. Protective effects of IgA have been demonstrated in some NHP vaccine studies for both mucosal and serum IgA (Bomsel et al., 2011, Ackerman et al., 2018). The complexity of the IgA-FcαR interaction lies in the dual functionality, where it has the capacity to be activating or inhibitory on the same γ chain (Pasquier et al., 2005).

Therefore, elucidation of the IgA role in effector functions has a potential to increase understanding of disease progression as well as HIV-1 control.

Despite the uncertainty surrounding the causal factors of HIV-1 control, elite controllers remain one of the most intensely studied groups of HIV-1 infected individuals, with hopes that they may shed light on mechanisms of HIV-1 control and inform HIV cure or vaccine strategies. Based on the overlap between correlates of protection and HIV-1 control such as gp120-specific V1V2 antibodies (reviewed in (Zolla-Pazner et al., 2019)), it is hoped that the information provided by HIV-1 control studies can potentially guide the design of effective vaccines (reviewed in (Li et al., 2018)). In particular, more evidence is pointing towards the significance of ADCC-mediating antibodies in the elite controller phenotype. This includes the detection of these antibodies in the early stages of an HIV-1 infection, their enrichment in HIV-1 controllers, or association with HIV-1 replication control (Baum et al., 1996, Ahmad et al., 2001, Forthal et al., 2001, Lambotte et al., 2009). However, the causal relationship between the elite controller phenotype and antibodies is not well understood, particularly with some speculating whether these antibodies are merely a marker for other protective responses (reviewed in (Duerr and Gorny, 2019)).

In the current study, elite controllers maintained low total and gp120-specific IgG levels compared to progressors due to minimum immune activation associated with low hypergammaglobulinaemia (Keating et al., 2017). It is well established that reduced total IgG levels are among the shared characteristics between elite controllers and ART-treated patients. Indeed, treated patients reverse hypergammaglobulinaemia through reduction of viral load and recovery of CD4⁺ T cell count that occurs in parallel with a decrease in the pool of B lymphocytes that produce anti-HIV-1 IgG, with consequences of lower total IgG levels than in untreated HIV-1 infected individuals (Morris et al., 1998, Aggarwal et al., 2017). Restoration of CD4⁺ T cell counts impacts recovery of CD4/CD8 ratios from <1 to >1 or >0.8 in some treated individuals (Mussini et al., 2015, Lee et al., 2017, Petoumenos et al., 2017, Yue et al., 2017). The CD4/CD8 ratio is an established marker of immune activation, disease progression and clinical outcome (Taylor et al., 1989, Serrano-Villar et al., 2013, Serrano-Villar et al., 2014). Lower range CD4/CD8 ratios (<1), caused by CD4⁺ T cell depletion and CD8⁺ T cell expansion as the disease progresses, have been linked to a high extent of immune activation that involves enhanced proliferation of HIV-1-infected CD4⁺ T cells and consequential persistence of the DNA reservoir (Chomont et al., 2009, Barouch and Deeks, 2014). Conversely, higher range CD4/CD8 ratios (>1) were associated with CD4⁺ T cell recovery and viral suppression after ART (Mussini et al., 2015). Hence, in the current study it

was not surprising to find that controllers with higher range CD4/CD8 ratios had reduced gp120-specific antibody levels, including IgG, IgG1, IgG4, IgA1, IgA2 and IgM levels (except IgG2 and IgG3), lower phagocytosis and higher ADCC responses compared to those with the lower range CD4/CD8 ratio. Therefore, ADCC associates with reduced hypergammaglobulinaemia and HIV-1 control, while ADCP is linked to elevated hypergammaglobulinaemia and disease progression. Despite proven similarities between elite controllers and ART-treated individuals, there are important effector function capacity differences. In particular, IgG purified from ART-treated individuals has been shown to mediate lower ADCC (Madhavi et al., 2014, Jensen et al., 2015), and lower ADCP responses (Ackerman et al., 2013b) compared to untreated HIV-1 infected individuals. Therefore, apart from reduced hypergammaglobulinaemia in elite controllers, there are other features of their ADCC-mediating antibodies that may contribute to higher activity. Uncovering and understanding these features may be applied in the context of ART to improve functional cure studies.

Recent studies have investigated antibodies from elite controllers in the emerging field of systems serology, which provides an unbiased, thorough and wide-ranging approach to methodically survey humoral immune responses. Their findings suggest that beyond antibody titres, overall antibody features (Fc glycosylation, hinge length etc.) and polyfunctionality (including ADCC, ADCP, ADCDC and ADCVI) may contribute to the less understood mechanisms of HIV-1 control and protection from infection (Chung et al., 2015, Ackerman et al., 2016, Ackerman et al., 2017, Chung and Alter, 2017). In particular, Fc glycosylation is well established as a factor that influences Fc-mediated effector functions (Raju, 2008, Quast et al., 2015). For example, Ackerman and collaborators observed that while potent Fc effector function of antibodies from HIV-1 elite controllers was not linked to titre or IgG subclass, elite controllers had a higher IgG glycan skewing, with significantly increased levels of agalactosylated glycoforms on HIV-1-specific antibodies compared to HIV-1 progressors. Intriguingly, these antibodies were shown to have higher binding capacity to FcγRIIIa, which may result in enhanced ADCC/ADCVI activity (Ackerman et al., 2013a). In addition, Chung et al. found that agalactosylated monoclonal antibodies reduced ADCP activity while having no influence on ADCC activity (which was affected by galactose arm linkages that impacted FcγRIIIa binding) (Chung et al., 2014a). Collectively, these data provide an additional explanation for enhanced ADCC in elite controllers, as well as differences in ADCC and ADCP mediating capacity of antibodies from elite controllers and progressors in the current

study. Therefore, a caveat of the current study is lack of full antibody characterization, which should be undertaken in future studies.

Nonetheless, systems serology is limited by the fact that findings from different studies are usually not directly comparable due to variation between assays. It is well described how different types of ADCC assays may be partly responsible for some of the contrasting findings in HIV-1-specific ADCC responses reported across the literature (reviewed in (Lewis et al., 2019)). These assays include: the chromium release assay (Blumberg et al., 1987), rapid fluorometric ADCC assay (Gomez-Roman et al., 2006), GranToxiLux ADCC assay (Pollara et al., 2011), ADCC measured by target cell derived luciferase activity (Alpert et al., 2012), bound virion ADCC (Guan et al., 2013), flow-cytometry-based ADCC assay (Richard et al., 2014) and infected cell elimination (Mayr et al., 2017a). The different ADCC assays are not all directly comparable. Even variations within one particular assay such as the widely used, high-throughput GranToxiLux ADCC assay (Pollara et al., 2011), can affect comparability between studies. Differences within assays include the use of different effector cell types (primary cells or cell lines), targets (antigen coated or virus infected), and purified IgG or plasma (Black et al., 1996, Tomaras et al., 2013, Duchemin et al., 2018). Additional studies measuring HIV-1-specific ADCC responses by the different assays, where the aforementioned inter-assay differences are well defined, are necessary to allow for a meta-analysis of studies to better define the role of ADCC in the context of natural HIV-1 control. In addition, end-point measurement of cytotoxicity during data analysis is an often overlooked factor that has great implications in interpretation of data. Given the various outcome measure of cytotoxicity, most studies select one method, thus neglecting others and the implications that may arise from unexplored options. For instance, in the current study, had we opted to use AUC or titre as the only means of ADCC measurements, as typically done, we would have seen higher ADCC by HIV-1 progressors compared to elite controllers. However, taking into account the importance of specifically evaluating ADCC at the lowest plasma dilution (or highest IgG concentration) which is more reflective of physiological conditions, enabled us to see opposite results of higher ADCC mediating capacity by elite controllers compared to progressors. On the other hand, there is a lack of consensus on the measure of ADCP responses. This may be a result of study-specific dose-dependent curves being used to select the optimal concentration for further data analysis (Ackerman et al., 2011, McAndrew et al., 2011, Ackerman et al., 2013b, Chung et al., 2014b). This results in various studies having unique optimal concentrations, which makes direct comparison between studies impossible. Future studies can evaluate a broader range of antibody

concentrations (including full antibody characterization) as well as their Fc-mediated effector function capacity beyond ADCC and ADCP, to unveil the complex interdependent relationships between antiviral effector functions and develop strategies for effective vaccines. Indeed, the design of effective therapeutic monoclonal antibodies has already begun in other disease settings, particularly in cancer and autoimmune diseases (reviewed in (Sondermann, 2016, Sondermann and Szymkowski, 2016)). Therefore, the same is possible in HIV-1 infection.

In addition to the antibody component, the effector cell itself is a very important component of the equation. Aside from expression of receptors that bind antibody, which are modulated by HIV-1 infection (Nimmerjahn and Ravetch, 2008, Rosales and Uribe-Querol, 2013), HIV-1 infection also impacts on cell numbers as well cell functions beyond merely those mediated by specific antibody binding to cognate receptors. Inflammatory environments are also associated with cell anergy and exhaustion (Catalfamo et al., 2011, Hoffmann et al., 2016). In Fc effector functions, while an individual may have an effective antibody response as measured in an *in vitro* assay, *in vivo* the particular effector cell may be incapable of mounting the appropriate response. This was illustrated by associations of impaired ADCP with reduced FcγRIIa expression on phagocytes of individuals with chronic HIV-1 infection (Dugast et al., 2011). Furthermore, while NK cells are important ADCC effector cells, FcRs are expressed by all innate immune cells (Nimmerjahn and Ravetch, 2008). These include cell populations that can mediate FcγR-mediated effector functions such as monocytes/macrophages (Biburger et al., 2011, Kramski et al., 2012, Kramski et al., 2013, Alrubayyi et al., 2018) and neutrophils (Smalls-Mantey et al., 2013, Worley et al., 2018, Powell et al., 2019). However, the ADCC-mediating capacity of these cell types remains understudied relative to NK cells. Therefore, a simultaneous and full characterization of FcγR expression levels and FcγR genetic variants on monocytes, neutrophils, NK cells, and T lymphocytes in the context of HIV-1-specific immunity, as well as their cellular distribution (and subset distribution in some cases) and impact on effector function capacity across different continents, countries, races and ethnicities could be of benefit in the development of effective vaccine or cure strategies.

Overall, this work emphasizes the need to elucidate the collective interaction of many parts and players of the antiviral humoral immune response and their modulation of Fc effector functions within a specific environment or at different stages of an infection.

Chapter 6: References

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Chapter 7: Appendices

Appendix 1: Supplementary figures

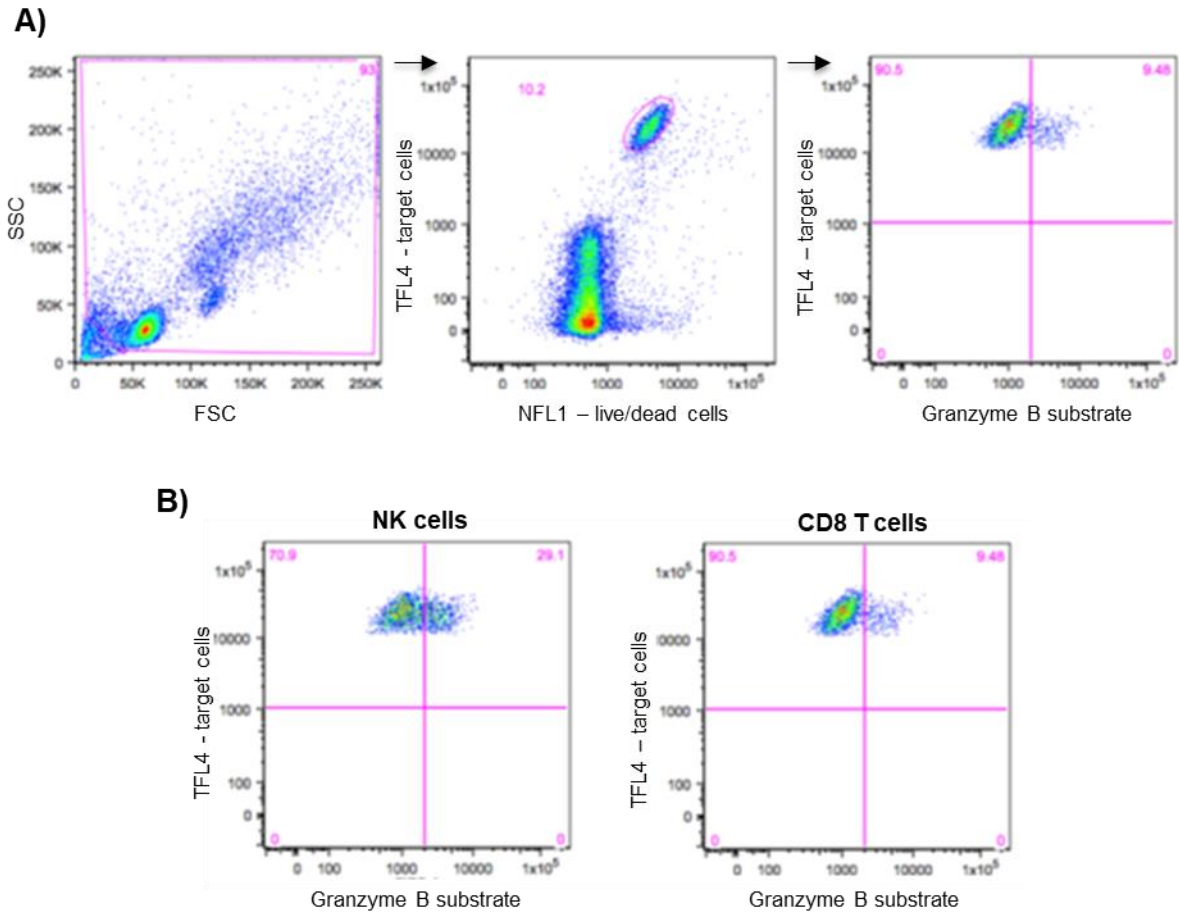


Figure S1: ADCC mediated by purified NK cells and CD8 T cells

A) The gating strategy for ADCC mediated by NK cells or CD8 T cells is similar to the previously described (Pollara et al., 2011). Briefly, the forward scatter/side scatter (FSC/SSC) channel was used to gate the entire cell population, the TFL4 marker identifies target cells, the NFL1 marker eliminates target cells that died before initiating the GranToxiLux ADCC, and granzyme B-positive cells are identified as cells killed through ADCC. B) At 30 μ g/ml HIV+ pooled IgG, NK cell-ADCC generally produced higher granzyme B activity compared to CD8 T cell-ADCC.

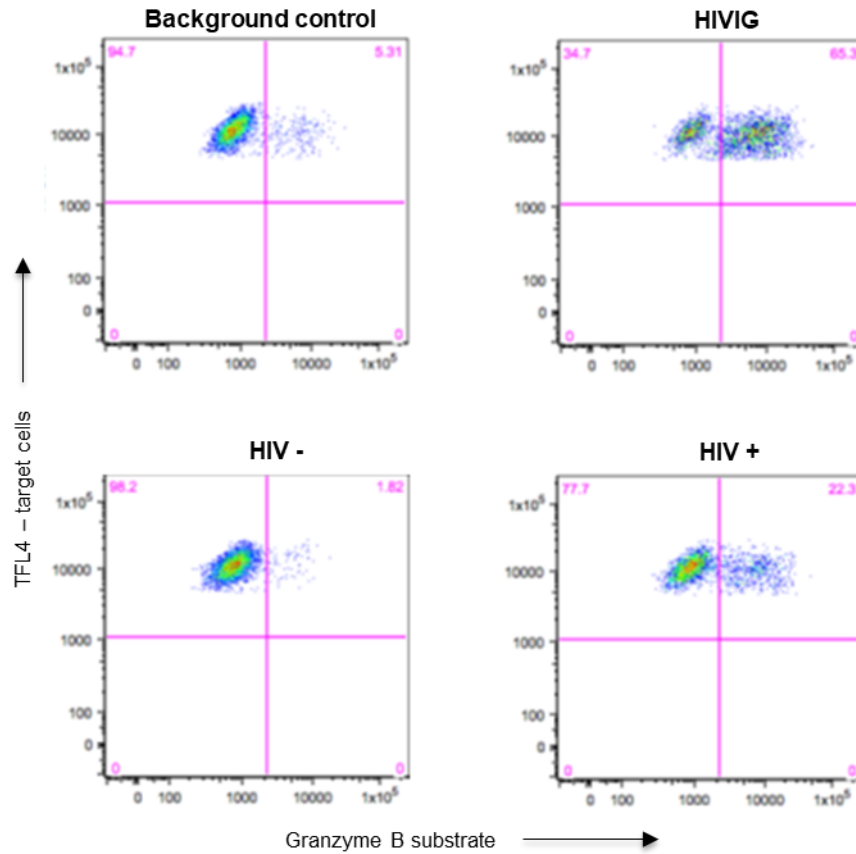


Figure S2: Comparison of granzyme B activity by antibodies from HIV-1 infected and uninfected individuals.

The background control (without antibody) was subtracted from test samples. HIVIG was used as a positive control. At 1:50 plasma dilution, HIV-1 uninfected individuals had reduced granzyme B activity compared to HIV-1 infected individuals. The granzyme B activity was below a determined baseline in order to confirm the status of self-reported HIV-1 uninfected individuals.

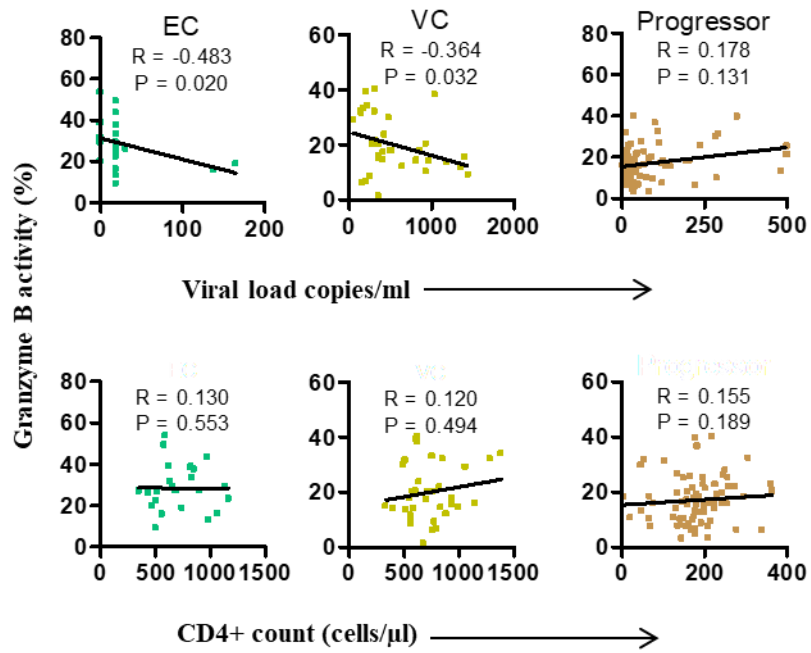


Figure S3: Correlation of ADCC responses at 1:50 dilution with markers of disease progression.

Viral load and CD4⁺ T cell count were correlated with ADCC at 1:50 plasma dilution in elite controllers (n = 23), viraemic controllers (n = 35), and progressors (n = 73) using the Spearman's rank coefficient correlation. (*** P < 0.001; ** P < 0.01; * P < 0.05). Viral load for progressors was divided by 1000 for ease of presentation.

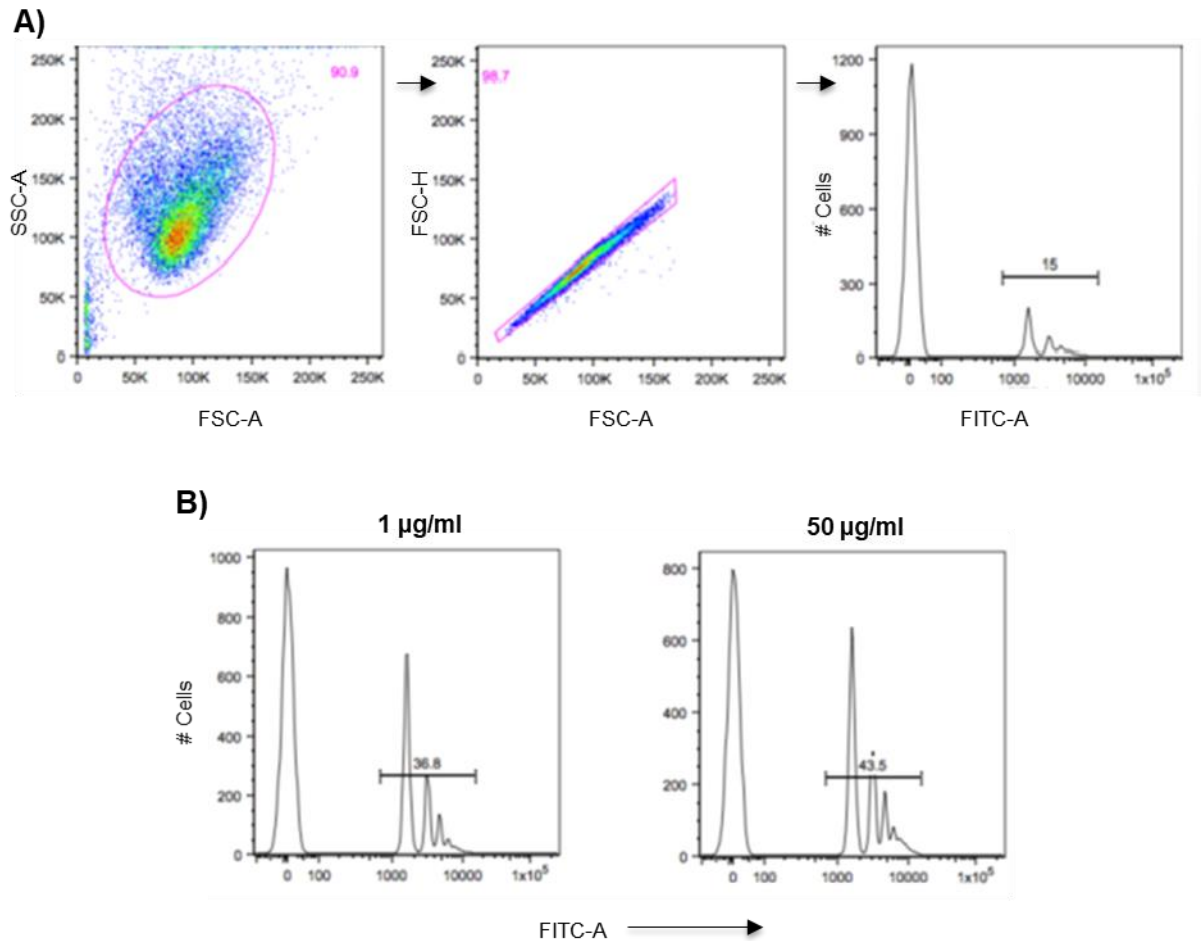


Figure S4: Antibody-dependent cellular phagocytosis gating strategy and comparison of activity.

A) In the gating strategy, the FSC-A/SSC-A channel was used to gate for THP-1 cells, singlets were gated with the FSC-H/FSC-A channel, and the FITC-A channel identified %bead-positive cells in the negative control sample (without IgG). B) HIV+ IgG tested at 1 µg/ml generally had reduced ADCP compared to ADCP at 50 µg/ml. The background control was subtracted from these values and the median fluorescence intensity (MFI) was used to calculate the phagocytic score.

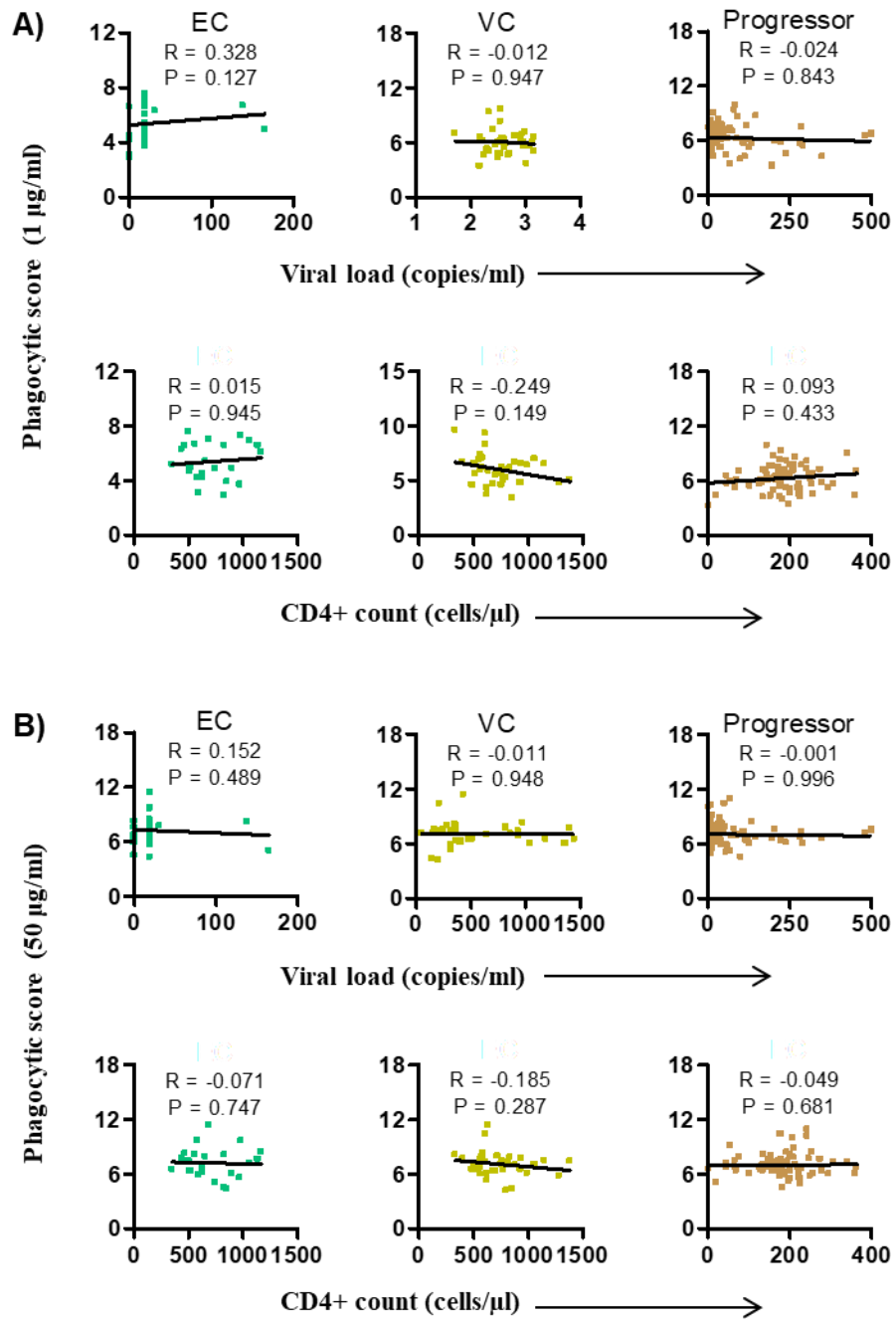


Figure S5: Correlation of ADCP responses with markers of disease progression.

Viral load and CD4⁺ T cell count were correlated with ADCP at A) 1 µg/ml and B) 50 µg/ml in elite controllers (n = 23), viraemic controllers (n = 35), and progressors (n = 73) using the Spearman's rank coefficient correlation. (***) P < 0.001; (**) P < 0.01; (*) P < 0.05). Viral load for all progressors were divided by 1000.

Appendix 2: Plagiarism declaration

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG



FACULTY OF
HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Ntando Ghwenneth Phaahla (Student number: 699653) am a student registered for the degree of PhD (Virology) in the academic year 2020.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: 

Date: 18/08/2020

Appendix 3: Ethical clearance



R14/49 Ms Ntando Ghwenneth Mnisi

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1511102

NAME: Ms Ntando Ghwenneth Mnisi
(Principal Investigator)

DEPARTMENT: Virology
Centre for HIV and STI's (CHIVSTI), National Institute
for Communicable Diseases (NICD)
National Health Laboratory Services (NHLS),
Johannesburg, South Africa

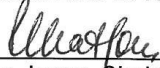
PROJECT TITLE: Fc Gamma Receptor-Mediated Effector Functions in the
Context of Differential Control of HIV-1 and of Anti-HIV-1
Vaccination

DATE CONSIDERED: Adhoc

DECISION: Approved unconditionally

CONDITIONS: Sub-Study

SUPERVISOR: Prof Caroline Tiemessen and Dr Ria Lassauniere

APPROVED BY: 
Professor P Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 09/12/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature _____

Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix 4: Thesis title change



Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Mrs Sandra Benn
E-mail: sandra.benn@wits.ac.za

10 December 2019
Person No: 699653
TAA

Mrs NG Phaahla
422 Kaalfontein
Ext 1 Fantail Street
Midrand
1682
South Africa

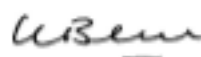
Dear Mrs Ntando Phaahla

Doctor of Philosophy: Change of title of research

I am pleased to inform you that the following change in the title of your Thesis for the degree of Doctor of Philosophy has been approved:

From: Fc-gamma-receptor-mediated effector functions in the context of differential control of HIV-1 and of anti-HIV-1 vaccination
To: HIV-1-specific Fc-gamma-receptor-mediated effector functions: The role of host factors

Yours sincerely



Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences