Immunoglobulin allotypes, antibody-dependent neutrophil phagocytosis: impact on HIV and influenza

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Abstract

Antibodies (Abs) are highly functional immune proteins. Abs can deactivate pathogens through neutralization and mediating various Fc receptor (FcR) functions. Two common FcR-mediated functions are Ab-dependent cellular cytotoxicity (ADCC) and Ab-dependent (cellular) phagocytosis (ADCP or ADP). These functions are triggered by binding of the Fc region of Abs to FcRs on innate immune cell surface. Studies including analyses of the moderately protective human phase III RV144 HIV vaccine trial suggest that antibodies with the ability to mediate FcR functions may be important in HIV protection and control. IgG, the most abundant Ab isotype, has different subclasses including IgG1, IgG2, IgG3, and IgG4. The IgG1 and IgG3 subclasses when complexed with antigens, bind efficiently to all Fcγ receptors (FcγRs), thus initiating FcR-mediated functions (the FcR-mediated functions discussed in this thesis are all FcγR-mediated functions).

There are several allelic variations (termed allotypes) of IgG1 in the constant region including G1m1 and G1m3. The prevalence of allotypes differs among ethnic groups. There is growing evidence that IgG1 allotypes differentially influence IgG subclass levels, FcR binding, and FcR-mediated functions. However, the understanding of the influence of IgG1 allotype on FcR-mediated functions is still limited at present. This thesis focuses primarily on the investigation of the impact of IgG1 allotypes on vaccine-induced immune responses.

The first chapter provides the literature review of the whole study, ranging from the general humoral immunity to specific FcR-mediated functions. It also introduces IgG1 allotypes and the importance of FcR functions in infectious diseases including Human Immunodeficiency Virus (HIV) and Influenza. The

second chapter focuses on the material and methods utilized in these studies. Information on the subjects who provided samples are also described in chapter two.

The third chapter addresses two approaches used to determine the IgG1 allotypes. Normally the IgG1 allotypes are determined by sequencing as the DNA sequence difference is the intrinsic difference among different IgG1 allotype carriers. This study optimized another ELISA-based approach of IgG1 allotyping on protein levels utilizing only purified IgG or plasma to identify allotypes. This approach makes it possible to allotyp subjects when genomic DNA is unavailable. Furthermore, this study validates that sequencing allotyping and ELISA allotyping for the G1m1 and G1m3 allotypes are consistent with each other.

In the fourth chapter, a neutrophil-like HL-60 cell line was developed as a model to study neutrophil responses to Fc-functional HIV-specific antibodies. Neutrophils are innate immune cells that express a range of FcRs and can trigger various FcR-mediated functions including Ab-dependent cellular phagocytosis (ADCP). However, the role of neutrophil FcR-mediated functions in HIV control is not well explored, largely due to the difficulty in working with primary neutrophils as they die rapidly (half-life 6-8 hours) *ex vivo*. In this chapter we cultured HL-60 cells for 5 days with DMSO such that they expressed a range of neutrophil-like cell markers. We then successfully used these neutrophil-like HL-60 cells to measure neutrophil FcR-mediated ADCP against HIV.

The fifth chapter explored the influence of IgG1 allotypes in response to Influenza vaccination. IgG subclass levels, IgG-FcR binding, ADCC and ADCP (using the HL-60 method generated above) were all evaluated. We found that IgG1 allotypes are associated with antigen-specific IgG subclass levels upon Influenza vaccination. However, no functional assay difference regarding ADCC

and ADCP was found, implying substantial complexity in allotypic effects on FcR-mediated functions. The final chapter covers the overall discussion and conclusion of the study.

In summary, this project developed useful methods for IgG1 allotyping and Fc-functional assays of a neutrophil-like cell line. We utilized these methodologies to provide insight into the role of IgG1 allotypes upon vaccine-induced immune responses. This work could ultimately have implications for the development of global vaccinations against infectious diseases including HIV, where antigen-specific IgG subclasses contribute to protection.

Declaration

This is to certify that:

1. The thesis comprises only my original work towards the Master of Research program except what was indicated in text or preface.
2. Due acknowledgement has been made in the text to all other material used
3. The thesis is less than 100,000 words in length, exclusive of tables, figures, references.

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Kuangyu Fei

July 16, 2018

Preface

As required by thesis regulation of University of Melbourne, I acknowledge these works presented in the thesis were collaborative:

Chapter 2 and Chapter 5: Matthew Worley and Fernanda Ana-Sosa-Batiz performed IgG purification for the HIV-positive antiretroviral therapy (ART) naïve cohort and the Influenza vaccine cohort, respectively**.** Amy Chung performed the multiplex assay to determine IgG subclass levels. Ester Lopez performed the SPR assay on Biacore to determine the kinetics of IgG-FcR binding. Ester Lopez and Timon Damelang purified DNA from Fluvax003 cohorts. Anne B. Kristensen performed the Fc receptor dimer-binding assay for HIV-positive samples and the plate-bound natural killer (NK) cell activation assay to determine the binding of antibodies with Fc receptor and the capacity of IgG to trigger ADCC.

The thesis contains published materials from:

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Author contributions: Kuangyu Fei: Performed experiments, study design, data analysis and writing manuscripts that relate to HL-60 cell line.

This published article is provided as an appendix to the thesis.

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Thanks to my parents, relatives, and friends back in China for their support, help over these two years. It would be impossible for me to make it through without their assistance! Thanks again to all these nice people!

Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
ADCP or ADP	Antibody-dependent (cellular) phagocytosis
ADE	Antibody-dependent enhancement
ADNP	Antibody-dependent neutrophil phagocytosis
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
bp	Base pair
BSA	Bovine serum albumin
CCR5	C-C chemokine receptor type 5
CD	Cluster differentiation
CDC	Complement-dependent cytotoxicity
CXCR4	C-X-C chemokine receptor type 4
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding
Fc	Fragment crystallisable
FcR	Fc receptor
FcγR	Fc gamma receptor
FcRn	Neonatal Fc receptor
FIP	Fluorescent internalization probe
Gm	Genetic marker
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
HA	Hemagglutinin
HIV	Human immunodeficiency virus
HIVIG	Anti-HIV immune globulin
HRP	Horseradish peroxidase
Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
IVIG	Intravenous immunoglobulin
MFI	Mean fluorescent intensity (for flow cytometry) Median fluorescent intensity (for multiplex)

MHC	Major histocompatibility complex
mRNA	Messenger RNA
NA	Neuraminidase
nAb	Neutralizing Ab
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
NK	Natural killer
NO	Nitric Oxide
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
pNPP	p-nitrophenyl-phosphate
ROS	Reactive oxygen species
SHIP	Sensor hybridization internalization probe
SHIV	SIV/HIV
SIV	Simian immunodeficiency virus
SPR	Surface plasmon resonance
TAE	Tris base, acetic acid and EDTA
TIV	Trivalent inactivated influenza vaccine
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TPA	12-O-tetradecanoylphorbol-13-acetate
Vax	vaccination
WHO	World health organisation

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Chapter 1 Introduction

1.1 Human immune system-a brief overview

The immune system contains the entirety of molecules, cells, tissues, and organs that mediate resistance to infectious diseases [1][1]. Functionally, the human immune defence system is composed of innate immunity, which is responsible for the early and initial protection against pathogens, and adaptive immunity which mediates later, stronger and more specific immunity against pathogens [2]. Innate immunity is the first line of defence and is ready to eliminate infectious microorganisms at any time even if there is no stimulation by microbes [3]. The primary components of innate immunity include phagocytes, dendritic cells, and nature killer cells (NK cells), which are able to recognize various microbial components including bacterial cell wall constituents by germline-encoded pattern-recognition receptors (PRRs) [4]. The cells of the innate immune system develop defects with advanced age, including reduced cell numbers and functions [5]. Adaptive immunity must be stimulated by invading pathogens or by vaccination, which responds more effectively against a specific type of microbe. The process of adaptive immunity normally involves the following stages: antigen recognized by lymphocytes, proliferation and differentiation of lymphocytes upon activation into effector and memory cells, eradication of the pathogens, decline of the immune response with a long-term immunological memory [1][2].

Adaptive immunity can be further divided into cell-mediated immunity which is mediated by T lymphocyte and mainly fights against intracellular pathogens, and humoral immunity which is mediated by antibodies that primarily bind to extracellular microbes and toxins [3].

[1] Refer to page 3, 18 in reference [1].

[2] Refer to page 35 in reference [1].

1.2 The importance of non-neutralizing antibodies

Naïve B cells can differentiate into effector B cells (plasma cells) with the help of antigen stimulation and helper T cells ($CD4^+$ T cells). Antibodies bind to extracellular pathogens, toxins or antigens on the cell surface, which leads to neutralization or elimination by NK cells, phagocytes and complement system [1][1]. Based on diverse functions, antibodies can be classified into two categories, neutralizing antibodies and non-neutralizing antibodies. Neutralizing antibodies are able to bind directly to microbes and toxins, preventing the microbes from attaching to host cells and neutralizing the pathogenicity of toxins. However, it is difficult for antibodies to enter the cytoplasm and neutralize intracellular pathogens [6]. Effective clearance of these pathogens may require another antibody function mechanism, which is the role of antibody-dependent Fc-mediated functions.

Non-neutralizing antibodies can coat the pathogens or the antigens on the cell surface and can be recognized by phagocytes by binding to Fc receptors (FcR) on phagocytes, and then stimulating the phagocytosis activities of phagocytes. Non-neutralizing antibodies coated on microbes or infected cells can also be recognized by antibody-dependent cellular cytotoxicity (ADCC) effector cells such as NK cells through FcR, and thereby causing the lysis of target cells. It is worth noticing that not only non-neutralizing antibodies but neutralizing Abs may also activate FcR-mediated effector functions [7]. IgE, one specific Ab isotype, is capable of activating mast cells and eosinophils, which plays a critical role in the immunity against parasites and allergic reactions. Non-neutralizing antibodies can also activate the complement activation cascade, leading to phagocytosis or cell lysis of the pathogen and causing inflammation [8]. Non-neutralizing antibodies have been shown to play a role in various infectious diseases including influenza and HIV [9-12].

[1] Refer to page 410 in reference [1].

1.3 Human Fc receptors

1.3.1 Fc receptors present on innate immune cells

A group of innate immune cells bears a family of cell surface receptors called Fc receptors, which specifically bind the Fc portion of antibodies. Different Fc receptors are distributed in different types of cells including B cells, dendritic cells, NK cells, macrophages/monocytes, neutrophils, eosinophils, basophils and mast cells, which upon engagement induce different types of functions [1][1].

According to the isotypes of the antibody they recognize, Fc receptors can be divided into several types. Fc gamma receptors (FcγR) bind to the most common antibody isotype, immunoglobulin G (IgG), and Fc alpha receptors (FcαR) bind to the critical antibody isotype in mucous membrane, immunoglobulin A (IgA). Fc epsilon receptors (FcεR) bind to the parasite defending antibody isotype, immunoglobulin E (IgE) [1][1].

Two types of Fc receptors interact with IgE, FcεRI and FcεRII [13]. Mast cells and basophils bear FcεRI which binds IgE with high affinity and induces the secretion of inflammatory cytokines and thus recruiting more innate immune cells to eliminate pathogens [14]. FcεRII is expressed on monocytes, eosinophils and B cells, which binds IgE with low affinity and is found to be involved in the pathogenesis of inflammatory diseases, allergic reaction and cancer [15, 16].

FcαRI (CD89) is the only Fc receptor in Fc-alpha receptor group, which is expressed on monocytes/macrophages, neutrophils, eosinophils and some dendritic cells [17]. IgA is known as the critical antibody at mucosal membrane which protects the mucosal surface from invading microbes [18]. The multimeric FcαRI crosslinking triggers the phagocytosis of effector cells, antibody-

[1] Refer to page 418 in reference [1].

dependent cellular cytotoxicity, reactive oxygen production and cytokine release [19].

The most important subgroup of Fc receptors for inducing phagocytosis or cell lysis of opsonized pathogens are the Fc-gamma (Fcγ) receptors [13]. This subgroup includes FcγRI (CD64), FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIc (CD32c), FcγRIIIa (CD16a), FcγRIIIb (CD16b), which vary in their antibody-Fc receptors binding affinity, IgG subclass specificity, signalling pathway and effector functions [20, 21]. In humans, these Fcγ receptors are commonly expressed on various innate immune cells including NK cells, macrophages/monocytes, neutrophils, eosinophils, basophils and dendritic cells [21]. Polymorphisms of Fc receptor have been previously described: In humans, the FcγRIIA exhibits two variants differing at position 131 (H131 and R131), and the FcγRIIIA displays two variants differing at position 158 (V158 and F158) [22].

1.3.2 Signalling pathway of Fcγ receptors

The Fc region of antibodies can bind the Fcγ receptors and activate the cell which expresses these Fcγ receptors [23]. Based on downstream effects, Fcγ receptors can be divided into two broad types: activation receptors and inhibition receptors [24]. Crosslinking of activation receptors such as FcγRIIa transduces signals through immunoreceptor tyrosine-based activation motif (ITAM) sequences. A signalling cascade is activated once the tyrosine residue of the ITAM is phosphorylated by tyrosine kinases, which ultimately induces phagocytosis in phagocytes [24, 25].

Inhibitory receptors such as FcγRIIb possess immunoreceptor tyrosine-based inhibitory motif (ITIM) sequence to transduce signals. A signalling cascade is initiated upon the phosphorylation of ITIM, leading to the termination of ITAM-initiated signalling cascade [26]. The negative signalling pathway helps to suppress overactivated effector cells, down-regulating the Fc receptor-mediated

functions and immune complex-induced inflammation [27]. It is also crucial for the regulation of activated B cells [28].

Table 1.1 Characteristics of human Fcγ receptors [29-32]

Name	FcγRI (CD64)	FcγRIIa (CD32a)	FcγRIIb (CD32b)	FcγRIIIa (CD16a)	FcγRIIIb (CD16b)
Cells	Dendritic cells Macrophages monocytes Neutrophils Mast cells	Macrophages monocytes Langerhans cells Neutrophils Eosinophils Basophils	Plasma cells Macrophages monocytes Langerhans cells Neutrophils Eosinophils Basophils Mast cells	Macrophages monocytes NK cells Mast cells	Neutrophils Eosinophils (stimulated)
Function	Effector cell phagocytosis	Effector cell phagocytosis Degranulation ADCC	Inhibition of effector cells	Effector cell ADCC phagocytosis	Trigger calcium influx
Effect on effector cell	Activation	Activation	Inhibition	Activation	Activation
Binding affinity for IgG subclasses	IgG3>IgG1> IgG4>>IgG2	Variant H131 IgG3>IgG1 >IgG2>>IgG4 Variant R131 IgG3>IgG1 >>IgG2, IgG4	IgG3>IgG1, IgG4>>IgG2	Variant V158 IgG3>IgG1 >>IgG2,IgG4 Variant F158 IgG3>>IgG1 >>IgG2,IgG4	IgG3>IgG1>> IgG2= IgG4

1.3.3 Fc receptor-mediated functions

The crosslinking of Fcγ receptors initiates a signalling cascade and eventually induce specific Fc receptor-mediated functions depending on the type of Fcγ receptors and the type of effector cells. Two most common Fcγ receptor-mediated functions triggered by the interaction of Antibodies and Fcγ receptors is Ab-dependent cellular cytotoxicity (ADCC) and Ab-dependent cellular phagocytosis (ADCP) [25].

NK cells, monocytes, macrophages and mast cells, they express an Fcγ receptor called FcγRIIIa (CD16a), which can bind to arrays of IgG (preferentially IgG1 and IgG 3) attached to the infected cells [30, 33]. The binding then triggers downstream signalling pathway, which results in effector cells secreting granules which contain cytotoxic substances like perforin and granzyme that will enter the target cells and trigger apoptosis. This process is called antibody-dependent cellular cytotoxicity (ADCC), which is broadly involved in antimicrobial and antitumor immunity [33, 34].

Phagocytes such as monocytes, macrophages, dendritic cells, neutrophils and eosinophils, bear other Fc receptors called FcγRI (CD64) and FcγRIIa (CD32) which also bind preferentially to IgG1 and IgG3 opsonizing microbes. The interaction induces the phagocytes to extend its cell membrane and phagocytose the microbes by triggering numerous biochemical signalling pathways in phagocytes. Phagocytes can produce large amounts of reactive oxygen, nitric oxide (NO) and enzymes to kill and lyse ingested pathogens. This process is called Ab-dependent cellular phagocytosis (ADCP or ADP), which is an important mechanism to protect the body from encapsulated bacteria and probably some viruses such as HIV [1, 35].

In addition to ADCP and ADCC which is commonly mediated through macrophages, neutrophils and NK cells, Fc receptors also play a key role in the recruitment and activation of inflammatory cells [36]. Mast cells, basophils and eosinophils express FcεRI which specifically bind IgE. Bound IgE activates mast cells and basophils, giving rise to degranulation of these cells, secreting

inflammatory cytokines and thus recruiting more leukocytes to destroy pathogens [3].

This thesis primarily focuses on ADCC and ADCP, which is are key Fc receptor effector mechanisms. There is growing evidence that Fc receptor-mediated functions are an effective way to control pathogens, such as HIV and influenza, that commonly evade neutralizing antibody responses [37-39].

1.4 Neutrophils and HL-60 cell lines

1.4.1 Neutrophils

Neutrophils are the most abundant type of circulating granulocytes and even the most abundant circulating leukocyte (30%-70%) in human blood. Their abundance and diverse functions make them an indispensable part of the innate immune system [40, 41]. Neutrophils are continuously generated in the bone marrow from multipotential hematopoietic stem cells to maintain large numbers in the circulation. Mature neutrophils are released into the blood and can be captured by the endothelial cells where there is a signal of infection. Once migrated to the site of infection through chemotaxis, neutrophils are able to mediate a variety of effector functions to kill the microbes [42].

Apart from recruiting and activating other members of the innate and adaptive immune system, neutrophils have three primary means to directly eliminate microbes: phagocytosis, degranulation and generation of neutrophil extracellular traps (NETs) [40]. Neutrophils can release granules which contain reactive oxygen species and antimicrobial proteins such as myeloperoxidase, NADPH oxidase into the extracellular milieu to combat microbes [42, 43]. Highly activated neutrophils can release a net-like structure of DNA element called a neutrophil extracellular trap, which is composed of a net of chromatins and enzymes. NETs help to trap microbes and prevent them from spreading and

damaging, and subsequently kill them with antimicrobials or allow for phagocytosis [44, 45]. Another killing mechanism is phagocytosis, in which neutrophils phagocytose microbes or particles. This process forms a phagosome to capture the microbes where reactive oxygen species and antimicrobial proteins (defensins, lactoferrin, lysozyme, etc.) are secreted [40].

Neutrophils express a range of Fc receptors including FcγRI, FcγRII and FcγRIII and can mediate Fc receptor-mediated functions including ADCP and ADCC [29, 46-48], which are considered as important mechanisms of neutrophil phagocytosis and degranulation. In neutrophil-mediated ADCC, reactive oxygen intermediates are released to kill target microbes [48]. In neutrophil-mediated ADCP, neutrophils are capable of recognizing and ingesting microbes or particles that have been opsonized by antibodies. Moreover, neutrophils have previously been reported to mediate ADCC and ADCP towards target cells in HIV infection [49, 50]. Neutrophils can be found at common sites of HIV acquisition[51]. Hence, there is growing interest in their functions in HIV infection.

The β2-integrin CD11b/CD18 plays a role in the rapid immobilisation and migration of neutrophils [52], and is also found to be essential for Fc receptor-mediated neutrophil cytotoxicity and phagocytosis [53, 54]. It is noteworthy that the upregulation of expression of CD11b is normally recognized as a sign of neutrophil activation [55, 56].

1.4.2 HL-60 cell lines

The HL-60 cell line is a human leukemia cell line that was derived from a female patient with acute myeloid leukemia [57]. The HL-60 cells grow continuously in culture with a doubling time of 20 to 45 hours [58]. The majority of HL-60 cells show neutrophilic promyelocytic morphology under normal cell culture circumstance [57]. Notably, HL-60 cells can be induced to differentiate into mature granulocytes-like cells by certain reagents, including dimethyl sulfoxide (DMSO) and retinoic acid. HL-60 cells can also be induced to differentiate into

eosinophil-like, monocyte-like and macrophage-like by granulocyte-macrophage colony-stimulating factor (GM-CSF), 1, 25-dihydroxyvitamin D3 and 12-O-tetradecanoylphorbol-13-acetate (TPA), respectively [57, 59-61]. This differentiation process with DMSO usually takes 5-8 days, which results in a considerable increase in the proportion of the cells with neutrophil-like characteristics. The terminally differentiated HL-60 cells lose immortality and subsequently die through apoptosis [62].

The HL-60 cell line provides a useful tool to study proliferation, differentiation and gene expression of human myeloid cell lineage cells [58]. They were also used to study cell apoptosis, transepithelial cell migration and evaluate neutrophil effector functions [62-64]. Although HL-60 cells are a potentially useful model of primary neutrophils, they have not been studied for HIV-specific ADP which is the primary goal of chapter four of this thesis.

1.5 Antibody subclasses and allotypes

1.5.1 Antibody subclasses

Antibodies form a large portion of serum proteins and are made of five isotypes called IgG, IgM, IgD, IgA, and IgE. Different isotypes vary in terms of heavy chain structure and effector functions. IgG is an abundant and useful subclass, which can be divided into four subclasses: IgG1, IgG2, IgG3 and IgG4. IgG subclasses share over 90% same amino acid sequence, but differ both in molecular structure and physiological functions, including Fc receptor-mediated responses. [1, 65, 66]

IgG1 is the most abundant subclasses and is effective for neutralization, complement activation and Fc receptor responses. IgG3 is another effective subclass to trigger the complement pathway and Fc receptor function with low abundance and short half-life which may prevent over-reaction of inflammation [66]. The second most abundant subclass, IgG2 is characterized as the effective responder to polysaccharide antigens [67]. Moreover, IgG2 is able to trigger

ADCP [68]. The rarest subclass IgG4 is usually induced by allergy under long-term exposure to allergens [69]. This thesis primarily focuses on IgG1 and IgG3, since they are two primary inducers of Fc receptor-mediated functions such as ADCP.

Table 1.2 Characteristics of human IgG subclasses [66, 70]

	IgG1	**IgG2**	**IgG3**	**IgG4**
Amino acids in hinge region	15	12	62 (differs according to allotypes)	12
Relative abundance (%)	60	32	4	4
Antibody response to proteins	++	+/-	++	++
Antibody response to polysaccharides	+	+++	+/-	+/-
Antibody response to allergens	+	-	-	++
Activation of Fc receptors	Strong activation to FcγRI, FcγRIIa, FcγRIIIa	Weak activation to FcγRIIa (H131 variant) FcγRIIIa (V158 variant)	Strong activation to FcγRI, FcγRIIa , FcγRIIIa	Weak activation to FcγRI, FcγRIIa FcγRIIIa (V158 variant)

1.5.2 Antibody allotypes

Antibodies are formed of four polypeptides, two heavy chains and two light chains connected by disulfide bonds. Among each chain, there are the variable region and the constant region. The amino acid difference in the constant region due to genetic polymorphisms is called immunoglobulin allotypes[71]. The allotypes identified on the constant region of the human immunoglobulin heavy gamma chains of the IgG1 are designated as Gamma one marker (G1m) [72], which is the primary focus of the thesis.

For IgG1, G1m1 was the first discovered allotype by Grubb when he studied the agglutination of human erythrocytes coated with anti-Rh sera [73, 74]. Now it is well known that the G1m1 allotype comprises aspartate 356 and leucine 358 in the CH3 domain, while non-G1m1 (nG1m1) expresses glutamate 356 and methionine 358. G1m1 and nG1m1 are two genetically exclusive allotypes on the CH3 domain [75]. The G1m2 allotype was also identified on the CH3 domain of IgG1, which associates to glycine at position 431. The IgG1 without this allotype expresses an alanine at the same position [76]. G1m3 and G1m17, two mutually exclusive allotypes, however, is found to locate on the CH1 domain. The G1m3 allotype expresses an arginine at position 214 and G1m17 contains a lysine at the same position [77].

Table 1.3 Specific amino acid positions for G1m allotypes

	CH1 domain	**CH3 domain**	
G1m1		Asp 356 Leu 358	
nG1m1		Glu 356 Met 358	
G1m2			Gly 431
G1m3	Arg 214		
G1m17	Lys 214		

(Positions are based on Eu numbering, http://www.imgt.org)

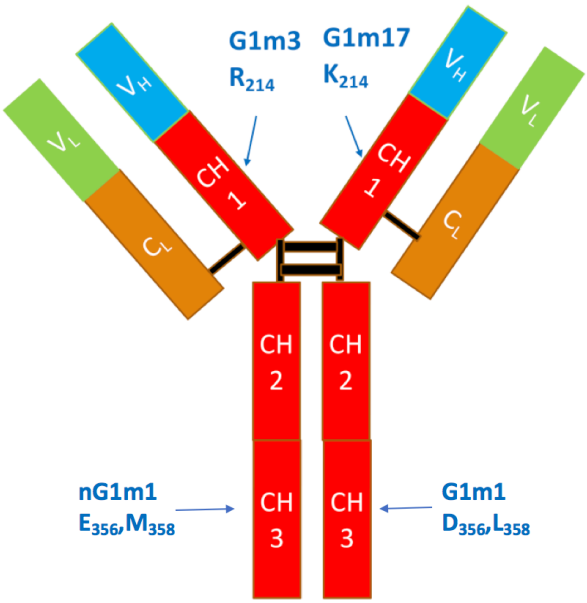


Figure 1.1 Localization of IgG1 allotypes on IgG1 molecule. The constant region of heavy chains can be divided into three domains: CH1, CH2 and CH3. G1m3 correlates to an arginine at position 214 in CH1 domain, while G1m17 correlates to a lysine. G1m1 correlates to an aspartate 356 and a leucine 358, while nG1m1 correlates to a glutamate and methionine at the same position. V_H: variable region on heavy chains. V_L : variable region on light chains. C_L: constant region on light chains.

For other IgG subclasses, IgG2 has two allotypes. A more extensive and complex allotype pool is found in IgG3, while IgG4, in contrast, have no actual allotypic determinants determined as yet [66, 72].

G1m haplotype means the sets of all IgG1 allotypes that are inherited. Interestingly, the prevalence of different G1m haplotypes widely varies among different ethnicities according to some population genetic studies [78]. It is worth noticing that G1m17 allotype almost always links to G1m1 allotype, while G1m3 allotype can exist either with or without G1m1 allotype [79, 80].

Table 1.4 Common G1m haplotypes in each ethnicity [78]

Ethnicity	G1m haplotypes
Caucasian	(G1m1, G1m17) , (nG1m1, G1m3)
African	(G1m1, G1m17)
Asian	(G1m1, G1m17), (G1m1, G1m3)

(Only prevalent haplotypes are shown. Rare haplotypes can be present in specific regions and races.)

Allotypes have potentially important clinical implications beyond serological findings. Some research suggests that certain allotypes of monoclonal antibodies

can affect the outcome of antibody-based cancer therapy, because it can alter ADCC, complement-dependent cytotoxicity (CDC) activity and viral immunoevasion [81]. For instance, it is shown that G3m21 IgG3 binds better than G3m5 IgG3 to C1q, which is an important component in the complement system [82]. It is also found that allotypes may affect the immune response of certain infections [83-85]. One pharmaceutical study showed that G1m1,3 monoclonal antibodies bind more effectively to the neonatal FcR (FcRn) [86], and one HIV vaccine study observed that G1m1 allele carriers are more likely to obtain elevated HIV-specific IgG1/IgG2 ratios compared to G1m3 allele carriers [79, 80]. All these researches suggest that IgG1 allotypes might have an impact on the antibody effector functions [87]. However, there has not been a deep and thorough exploration of the influence of IgG1 allotypes on functional antibody responses against HIV and influenza, including FcR-Ab binding and IgG subclass levels. This thesis offers a further investigation of the influence of IgG1 allotypes on the functionality of HIV or influenza-specific IgG.

1.6 Human Immunodeficiency Virus (HIV) infection

1.6.1 Basic introduction

The most serious acquired immunodeficiency is caused by a retrovirus called Human Immunodeficiency Virus (HIV). Discovered for more than 30 years, this global pandemic has become one of the most significant infectious diseases in human history by influencing negatively on economy, culture and people's livelihood in almost every country on the planet. Although the anti-retroviral therapy (ART) halts disease progression and prolongs the survival time[88, 89], the complete eradication of the virus is still largely unachievable. HIV primarily infects $CD4^+$ T lymphocytes which are at the core of adaptive immunity [90].HIV infection makes the $CD4^+$ T lymphocytes decline to lower level, leading to the loss of cell-mediated immunity. The loss of adaptive immunity results in the

increased susceptibility to opportunistic infections and cancers and, if untreated, eventual death [91]. HIV can be transmitted through the contact or the transfer of body fluids such as blood, semen and vaginal fluids [92]. In 2017, it was estimated that 1.8 million people were newly infected with HIV and 0.94 million people died of HIV infection [93]. Furthermore, the ART is an expensive treatment and can trigger the emergence of drug resistance. Therefore, a prophylactic vaccine is considered to be an essential component to combat the HIV pandemic.

1.6.2 HIV classification

Being a member of genus Lentivirus, family retroviridae [94], two types of HIV have been discovered: HIV-1 and HIV-2. These two types of HIV differ in viral genomics, geographical prevalence, virulence and infectivity: HIV-1 is a more virulent and contagious type with a shorter latency and higher morbidity than HIV-2, and HIV-1 is prevalent in every continent while HIV-2 is mostly confined to Western Africa [95-97]. This thesis primarily studies the infection by HIV-1.

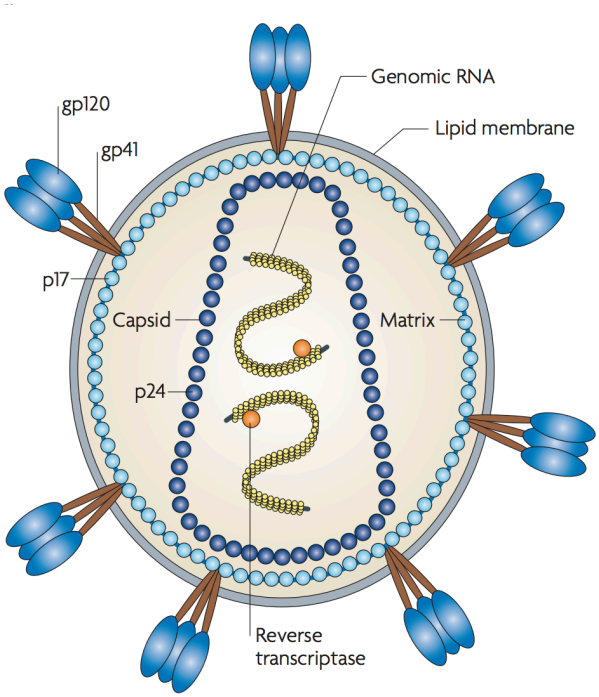
HIV-1 can be further classified into three genetic groups: group M, group O and group N. Group M are responsible for most HIV-1 infections around the planet, and can be divided into nine subtypes (clades) based on genetic composition: A, B, C, D, F, G, H, J, K. They are differentially distributed on the planet [98].

1.6.3 HIV-1 structure

The HIV-1 virus is approximately 100 nm in diameter [99]. The viral protein p24 constitutes the viral capsid that encloses the core region. The viral core contains two copies of the genomic RNA which is approximately 9749 nucleotides long and the viral enzymes such as reverse transcriptase and integrase [100-102]. Another viral protein p17 makes up the matrix between the envelop and the core

region [103]. The viral envelop consists of the lipid bilayer and the embedded glycoprotein (gp). The envelope protein contains the trimeric gp120-gp41 complex, and the cytoplasmic tail of the gp41 anchors into the matrix protein p17 [99, 104, 105]. The envelope protein gp120 and gp41 are the only protein on the virus surface coded by viral genome, and are the primary target of the humoral immunity against HIV infection [106, 107].

Figure 1.2 The structure of HIV-1 virus



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1.6.4 HIV-1 replication cycle

HIV infects human immune cells such as $CD4^+$ T lymphocytes in several steps: attachment and entry, reverse transcription of viral DNA and its integration into host DNA, expression of viral genes, assembly and exocytosis of viral particle [1][1]. The envelope glycoprotein of HIV gp120 plays a key role in the viral entry of host cells. Trimeric gp120 binds to both CD4 and a chemokine co-receptor (CXCR4 or CCR5). The binding makes it possible for the virus to attach host cells, fuse the viral membrane with the cell membrane and enter the cell to release the viral proteins and genome [108]. When the viral capsid enters the

[1] Refer to page 547 in Reference [1].

infected cell, the single-stranded RNA is freed and copied into a complementary DNA using reverse transcriptase [109]. The high error rate of reverse transcriptase creates large amounts of mutations that lead to virus evasion from the immune system and drug resistance [110, 111]. Once a double-stranded viral DNA is formed, the viral DNA can be integrated into the genome of the infected cell using integrase, which may then become dormant or activated during viral replication [112-114]. Activated viral DNA is transcribed and translated, producing viral genome and proteins. Finally, new HIV virions undergo assembly and release from the host cell, and become ready to find another victim [115, 116].

1.6.5 Immune response against HIV

The pathogen-associated molecular patterns (PAMPs) of HIV can be recognized by the pattern-recognition receptors (PRRs) of several types of innate immune cells, including NK cells, neutrophils, macrophages and dendritic cells [117]. This recognition can activate these innate immune cells and initiate many mechanisms of the antiviral immunity, including releasing cytokines and chemokines [118, 119]. $CD8^+$ T lymphocytes are also a critical component of HIV immune control. HIV infection can be latent as viral gene integrated into $CD4^+$ T lymphocytes genome, which is out the reach of antibodies. When viral proteins are expressed intracellularly and presented by MHCI molecules, the infected cells can be recognized and lysed by HIV-specific $CD8^+$ T lymphocytes response [120]. A large number of highly functional HIV-specific $CD8^+$ T lymphocytes are found in rarer HIV patients who control virus replication so well that they do not develop immunodeficiency for many years to decades even in the absence of drug therapy [121, 122].

In addition to the innate immunity and cellular immunity, humoral immunity is also a key aspect of natural HIV control and prevention [107]. Neutralizing Abs primarily bind to HIV envelope glycoproteins, gp120 and gp41 which is

necessary for viral entry. Therefore, Abs can interfere with viral entry by blocking CD4 binding or co-receptor binding. Most people are capable of producing moderate titres of broadly neutralizing antibodies, and nearly 50% of individuals develop neutralizing antibodies that can neutralize more than 50% of viruses [123]. A limitation of neutralizing antibodies (and indeed other anti-HIV immune response) is that the rapid replication rate and high error rate of the reverse transcriptase of HIV mean that resistant variants are produced that can be selected for in the face of immune pressure [124]. Serial escape from the neutralizing antibody response occurs in subjects with HIV infection. Recent passive transfer experiments in human showed that it was very difficult to eliminate all virus with monoclonal Ab [125, 126].

Fc receptor-mediated functions including ADCC and ADCP are also important for HIV immunity and pathogenesis supported by numerous studies [21, 127], which can be achieved by neutralizing or non-neutralizing Abs [7]. Anti-envelope antibodies such as anti-gp120 Env antibodies that mediate ADCC are normally produced during the early stage of infection [128]. Anti-envelope antibodies can inhibit HIV replication process by ADCC, which cause the direct killing of infected $CD4^+$ T lymphocytes by NK cells, neutrophils or macrophages [129, 130]. The importance of ADCC-mediating antibodies have been demonstrated by a wealth of data correlating clinical outcome and ADCC titres, including human vaccine trial, nonhuman primate studies and passive transfer studies [21, 127]. The most well-known one would be the partially protective human vaccine trial (an estimated 31.2% efficacy), the RV144 trial, which showed that anti-V1/V2 antibodies that mediate ADCC correlated with reduced infection risk [9]. IgG3 has a high affinity towards FcγRI, FcγRIIa, FcγRIII, which makes it capable of mediating ADCC and ADCP. HIV-1 V1/V2 IgG3, in particular, was found to correlate with reduced risk of infection in the RV144 trial [131]. Immune cells that mediate ADCP are also capable of killing infected $CD4^+$ T lymphocytes, serving as another approach to clear HIV infection [21, 38, 51]. Non-neutralizing

ADCP responses were discovered to correlate with vaccine protection in a monkey SHIV study [35]. However, the role of ADCP-mediating antibodies in human HIV infection is supported by limited evidence compared to ADCC-mediating antibodies. This thesis focus on the role of ADCP mediated by neutrophils during HIV infection.

1.7 Influenza Virus infection

1.7.1 Basic introduction

Influenza virus, which causes an infectious disease called influenza, belongs to the family of the *orthomyxoviruses* [132]. Unlike HIV which is a newly discovered virus, influenza viruses have caused epidemic and pandemic outbreaks for more than a thousand years, negatively impacting the population and economy of human society. Influenza virus typically infects the epithelial cells in the nose, throat, trachea and lung of human, resulting in a range of symptoms including high fever, rhinitis, coughing, headache, muscle pain[133, 134]. Severe influenza infection can cause complications such as viral pneumonia, co-infection bacterial pneumonia and multisystem failure which can be deadly. It is estimated by the United States Centers for Disease Control and Prevention (CDC) that influenza has caused between 9.2 million and 35.6 million cases, between 12000 and 56000 mortalities each year. To prevent influenza infection, trivalent inactivated vaccine (TIV) or live attenuated influenza vaccine (LAIV) is recommended. However, these seasonal vaccines are required annually and did not have ideal effectiveness [135, 136]. A more broadly protective influenza vaccine is in urgent need.

1.7.2 Influenza virus classification

Four types of seasonal influenza viruses have been identified so far based on their antigenic distinct viral proteins: Type A, B, C and D. Most seasonal influenza epidemics are caused by influenza A, but influenza B infection is also common. Influenza A viruses are the most virulent type, and can be further divided into subtypes according to the antigenic characteristics of 18 hemagglutinin (HA) and 11 neuraminidase (NA) [137]. Influenza B virus is less common and less virulent than influenza A virus, and they lack antigenic diversity [138]. Influenza C only causes mild symptoms in children and influenza D primarily infects cattle, thus they are not a severe problem for public health [139, 140]. This thesis only concerns immune response against influenza A virus.

1.7.3 Influenza virus structure

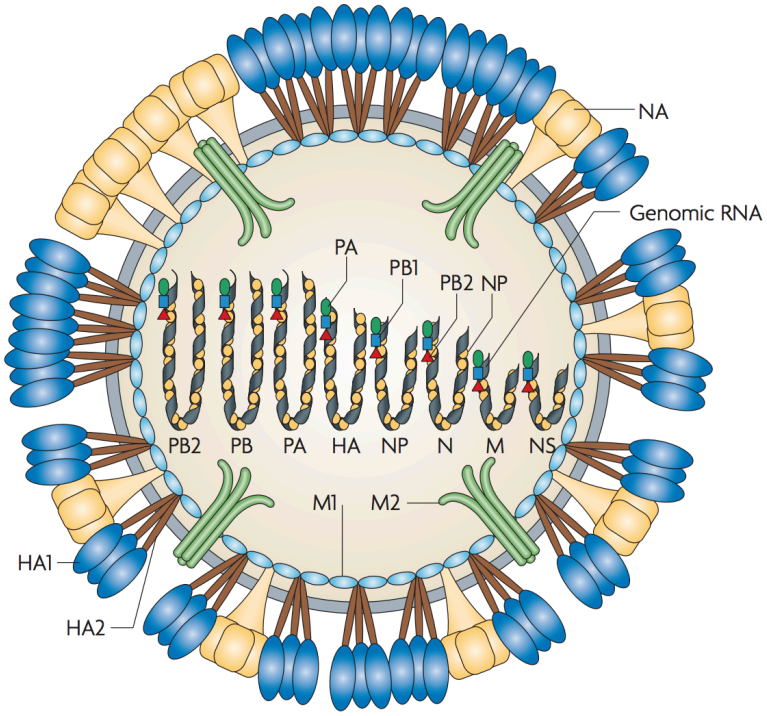
The influenza A virus is approximately 100 nm in diameter [99]. The viral envelope contains three types of viral proteins: trimeric hemagglutinin (HA), tetrameric neuraminidase (NA) and tetrameric M2 (figure 1.3). In the core region of the virus lies its RNA genome, which includes seven or eight segmented single-stranded RNA that encodes for 11 viral proteins [141, 142]. HA and NA are two important envelope glycoproteins which both recognize sialic acid on host cells. HA is a lectin that binds to sialic acid-containing receptors on the host cell and mediates the entry of the virus into the host cell, while NA is involved in the viral release and spreading to other cells by cleaving sialic acids from cellular receptors and extracellular inhibitors [143]. There are 18 different HA and 11 NA types found in influenza A virus [137]. HA and NA are primary targets for antiviral drug and influenza vaccine and humoral immunity [144, 145].

1.7.4 Immune response against influenza virus

The innate immunity is the first line of defence when the influenza virus infects the respiratory tract. The innate immune cells such as NK cells, neutrophils,

macrophages and dendritic cells can recognize the PAMPs of influenza virus by PRRs like Toll-like receptors (TLRs). Activation of PRRs can initiate various innate immune cells responses, leading to the activation of antiviral defence genes and promoting disease tolerance [146]. Cellular immunity is also crucial for the clearance of influenza virus [147, 148]. Internal viral antigens such as NP and Matrix can be expressed and presented by MHC class I molecules on the surface of the infected cells. The $CD8^+$ T lymphocytes can recognize presented antigens and cause cell lysis of the infected cell [149, 150].

Figure 1.3 The structure of influenza A virus



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Humoral immunity adds another important mechanism for influenza prevention and control. Naïve influenza antigen-specific B lymphocytes can activate and differentiate into plasma cells or memory B cells once stimulated by infection or vaccination [151]. The neutralizing antibodies mainly target at HA

and NA, two major envelope proteins [152, 153]. HA-specific antibodies can interfere with the viral attachment and entry to the host cell, while NA-specific antibodies can inhibit viral release from the host cell. Neutralizing antibodies correlate with the protection against viral infection [154, 155] and are primarily induced by current vaccine technologies [156].

Some influenza-specific antibodies can trigger Fc receptor-mediated functions including ADCC and ADCP [37, 157, 158]. NK cells, neutrophils or macrophages can kill the infected cells by ADCC or ADCP, inhibiting the viral replication and spread. It is demonstrated by a range of evidence including human vaccine trials and animal studies that ADCC-mediating antibodies might play a role in influenza protection [10, 39, 159]. Fc receptor-mediated phagocytosis is found to contribute to influenza virus clearance in mice [160]. However, the role of influenza-specific ADCP-mediating antibodies and the factors that affect Fc receptor-mediated function in human influenza infection are still not well investigated.

To induce adaptive immune responses against influenza virus infections, annual vaccination of trivalent inactivated vaccine (TIV) or live attenuated influenza vaccine (LAIV) is recommended [161]. These vaccines contain representative strains from influenza A H1N1, A H3N2 and B viruses, which is prevalent in annual epidemics [162]. The vaccine confers protection by inducing HA or NA-specific antibodies, which correlates with the protection against influenza infections [163]. With the role of non-neutralizing antibodies in influenza infection increasingly studied, it is also found that TIV vaccine can induce ADCC or ADCP-antibodies [164, 165]. In the influenza vaccine cohort on which the thesis focuses, an enhanced Fc receptor-mediated function was observed in both HIV-positive and HIV-negative subjects[159]. Since IgG polymorphisms might impact on Fc receptor-mediated functions, it would be interesting to know if the IgG allotype would affect the enhancement of Fc receptor-mediated functions induced by TIV, which is primarily discussed in

chapter five. HIV infection status should be taken into consideration because HIV is a risk factor for severe influenza [166].

1.8 Summary, Hypothesis and Aims

1.8.1 Summary

Humoral immunity is mediated by antibodies, of which IgG is an abundant and effective isotype. IgG1, the most abundant subclass of IgG, exhibits allelic variations in the IgG1 constant region named allotypes, including G1m1 (CH3 domain), G1m3 and G1m17 (CH1 domain). Fc receptor-mediated functions which are triggered by binding of Fc portion of Antibodies and Fc receptor on the cell surface is an essential function of IgG1. It is shown by many studies that Fc receptor-mediated functions play a part in HIV and influenza protection and control. There is growing evidence that IgG1 allotypes potentially modulate Fc receptor-mediated functions, such as Ab-dependent cellular cytotoxicity (ADCC) and Ab-dependent cellular phagocytosis (ADCP).

Neutrophils are innate immune cells that express a range of Fc receptors and can mediate various Fc receptor-mediated functions such as ADCP and ADCC. However, the role of neutrophils in HIV and influenza infection is underexplored due to the difficulty in working with primary neutrophils. A high throughput method using neutrophil cell lines to evaluate neutrophil effector functions is needed.

1.8.2 Overall hypothesis

IgG1 allotypes impact IgG subclass levels and Fc receptor-mediated functions including ADCC and ADCP in HIV and influenza infection.

1.8.3 Aims

1) Validate the serological and genetical approach to determine IgG1 allotypes
2) Develop an HL-60 cell line-based method to evaluate neutrophil-mediated ADCP
3) Evaluate the influence of IgG1 allotypes on HIV and influenza-specific IgG subclass levels, Fc receptor-antibody binding, ADCP, and ADCC.

Chapter 2 Materials and Methods

2.1 Study Cohorts.

Influenza vaccine cohort. This cohort was previously described in an influenza vaccine study[159] . 30 healthy and 26 HIV-positive subjects were recruited from the Peter Doherty Institute and the Melbourne Sexual Health Centre. The components of the 2015 vaccine were 15 μg of hemagglutinin of A/California/7/2009 (pH1N1), B/Phuket/3073/2013 and A/South Australia/55/2014 (an H3N2 strain) which is a very similar strain to A/Switzerland/9715293/2013. Plasma samples were obtained before and with a mean of four weeks after vaccination. IgG antibody was then purified from plasma samples using protein G purification. All subjects provided written informed consent and the study was approved by the respective institutional ethics committees**.**

Table 2.1: Characteristics of Influenza vaccine cohort

Characteristic	**HIV-negative (n=30)**	**HIV-positive (n=26)**
Age (years)	Mean: 40.4 Range: 22-55	Mean: 41.4 Range: 26-54
% of male gender	43.4	96.3
Duration of HIV infection (years)	N/A	Mean: 8.1 Range: 1-20.8
CD4+ T cell count (cells/μl)	N/A	Mean: 729 Range: 257-1843
Days between Fluvax vaccine and 2nd blood sampling	Mean: 27 Range: 22-29	Mean: 29 Range: 27.5-31.5
Recruitment location	Melbourne, Australia	

Fluvax003 cohorts. 23 healthy adults from Peter Doherty Institute, University of Melbourne, Parkville, Australia was recruited. All subjects provided written informed consent and the study was approved by the respective institutional ethics committees**.** Plasma samples were collected at baseline and four weeks after the administration of the seasonal quadrivalent vaccine. Each 0.5 ml dose contains 15 μg hemagglutinin antigens of the following type: A/Singapore/GP1908/2015, A/Hong Kong/4801/2014, B/Phuket/3073/2013, B/Brisbane/46/2015. Genomic DNA was extracted and purified from blood before vaccination using QIAamp DNA mini kit (Qiagen).

Table 2.2: Characteristics of Fluvax003 cohorts

Numbers of subjects	23
Age (years)	23-53
% of male gender	52.2%
Recruitment location	Melbourne, Australia
Days between vaccine and 2nd blood sampling	28 days

CSLCT-WHO-08-51 Cohort This cohort was previously described in an influenza study [164]. 50 healthy younger subjects (age from 18 to 59 years old) and 41 healthy older subjects (age from 60 to 79 years) were recruited. Participants were vaccinated with the southern hemisphere 2008 seasonal TIV (CSL Behring, Australia) with 15 μg of H1N1 A/Solomon Islands/3/2006, H3N2 A/Brisbane10/2007 and B/Florida/4/2006. Blood samples were collected at baseline and three weeks after vaccination. IgG antibody was then purified from plasma samples using protein G purification. All subjects provided written informed consent and the study was approved by the WHO human ethics committee**.**

Table 2.3: Characteristics of CSLCT-WHO-08-51 Cohort

	Younger adults	**Older adults**
Numbers of subjects	50	41
Age (years)	18-59	60-79
Days between vaccine and 2nd blood sampling	21 ± 4 days	
Recruitment location	Melbourne, Australia	

HIV-positive antiretroviral therapy (ART) naïve cohort. This cohort of 41 subjects was previously described in several HIV studies[50, 167]. Plasma samples were collected from 41 HIV-positive subjects who had not received antiretroviral therapy. IgG was purified from plasma using IgG Melon Gel purification. All subjects provided written informed consent and the study was approved by the respective institutional ethics committees. To increase the number of availbale for allotyping analysis, an additional 17 were only included for allotype analysis in chapter 3 but excluded in chapter 4. We did not have CD4 and HIV RNA data ,so not included in chapter four.

Table 2.4: Characteristics of HIV-positive ART naive cohort

Numbers of subjects	41	17
Recruitment location	Melbourne, Australia	Melbourne, Australia
CD4 count entry, cells/μl	Mean: 520 Range: 296-1156	Not available
Plasma HIV-1 RNA, copies/ml	Mean: 26700 Range: 399-339000	<400
Used in chapter	Chapter 3 & 4	Chapter 3

Normal range of CD4 count: 400-1600 cells/μl

2.2 Allotyping Sequencing

2.2.1 DNA purification

Genomic DNA was purified from human blood peripheral blood mononuclear cell (PBMC) using QIAamp DNA Mini Kit (Qiagen). Briefly, blood cells were lysed using protease and transferred to the QIAamp Mini spin columns. Then the solution was washed and centrifuged to remove other components. The purified DNA was obtained with elution buffer and centrifugation. If only human plasma was available, the heparin in the plasma should be degraded by heparinase I from Flavobacterium Heparinum (Sigma-Aldrich) to prevent the heparin from interfering with DNA amplification. Dissolve the enzyme at 1 mg/ml in 20 mM Tris-HCl (Sigma-Aldrich), 50 nM NaCl, 4 mM $CaCl_2$, pH 7.5 and 0.01% bovine serum albumin (BSA, Sigma-Aldrich). Incubate 200 µl plasma with 2 µl heparinase solution for 2 hours at room temperature. Genomic DNA was purified from heparin-free plasma with QIAamp DNA Mini Kit (Qiagen).

2.2.2 Polymerase chain reaction (PCR)

For CH1 domain (allotype G1m3 and G1m17): Primers 5'-CCCCTGGCACCCTCCTCCAA-3' and 3'-TACGTCGGGGTCAGGTCCCG-5' (Sigma-Aldrich) were used for amplification of a 364-bp DNA fragment from IgG1 gene[168]. PCR was performed in GeneAmp PCR system 9700 using 50 ng DNA template and 0.5 µl Phusion polymerase (New England Biolabs) in a 50 µl reaction solution containing both primers at a final concentration of 0.5 pm/µl. Conditions for PCR amplification were as follows: 7 min at 96°C, and 30-50 cycles of 1 min at 96°C, 30 s at 70°C and 1 min at 72°C. The final elongation step at 72°C lasts 5-8 minutes. The amplification products should be stored at 4°C.

For CH3 domain (allotype G1m1 and nG1m1): Forward primers 5'-GAGCCCAAATCTTGTGACAA-3' and reverse primer 5'-GGCGATGTCGCTGGGA-3' (Sigma-Aldrich) were used for amplification of an approximately 700bp DNA fragment from IgG1 gene[169]. PCR was performed in GeneAmp PCR system 9700 using 50 ng DNA template and 0.5 µl Phusion polymerase (New England Biolabs) in a 50 µl reaction solution containing both primers at a final concentration of 0.5 pm/µl. Conditions for PCR amplification were as follows: 7 min at 96°C, and 30-50 cycles of 1 min at 96°C, 25 s at 57°C and 50 s at 72°C. The final elongation step at 72°C lasts 5-8 minutes. The amplification products should be stored at 4°C.

Table 2.5: Component of PCR reaction solution for IgG sequencing

Component	**50 µl reaction**	**Initial concentration**	**Final concentration**
Nuclease-free water (Promega)	To 50 µl		
Phusion HF buffer (New England Biolabs)	10 µl	5X	1X
dNTPs (Bio Line)	1 µl	10 mM	200 µM
Forward Primer (Sigma-Aldrich)	2.5 µl	10 µM	0.5 µM
Reverse Primer (Sigma-Aldrich)	2.5 µl	10 µM	0.5 µM
DMSO (New England Biolabs)	1.5 µl	100%	3%
Phusion DNA polymerase (New England Biolabs)	0.5 µl	2 unit/µl	1 unit/50 µl
Template DNA	Variable	Variable	< 5 ng/ µl

2.2.3 Amplification products purification

Amplification products were separated by electrophoresis in 2% agarose gels. DNA agarose gel was prepared by dissolving 2 g agarose (Life Technologies) in 100 ml TAE buffer (Promega) with 10 µl SyBR Safe DNA dye (Life Technologies) added. Transfer the gel into gel electrophoresis machine full of TAE buffer and add PCR samples to slots of the gel, then run electrophoresis on 110 volts for 30 minutes. Scan the gel with Gel reader (doc) machine and identify the position of PCR products. Cut a small piece of gel that contains amplification products with surgical blades. Amplification products were purified from gel using QIAquick Gel Extraction Kit (Qiagen).

2.2.4 Sequencing

Plates for sequencing were prepared by the requirements of AGRF (Australian Genome Research Facility). Briefly, each well of the sequencing plate contains 2 µl of amplification products, 1 µl of 10 pmol primers and 9 µl of nuclease-free water. Then the plate was completely sealed with a silver lid and sent for sequencing. Sequencing was performed by AGRF company using Sanger Sequencing purified DNA (PD) procedures. Sequence analysis was performed using Geneious Software.

2.3 IgG antibody purification and quantification

2.3.1 IgG Melon Gel purification

Melon Gel IgG Purification Kits (Thermo Scientific) was used for the purification of antibodies from plasma samples according to the manufacturer's instructions. Melon Gel Resin can bind and remove serum proteins, thereby allowing pure IgG to be collected in the flow-through fraction (ThermoFisher product overview). Briefly, add 500 µl Melon gel resin to the purification column and wash the

column with Melon gel purification buffer. Plasma diluted in purification buffer were added to the columns and incubate at room temperature for 10 minutes on a rotator. Collect the flow-through and incubate in the column for another 10 minutes on the rotator. The final purified IgG was obtained by centrifuging for 1 minute at 3000 g.

2.3.2 Protein G IgG purification

IgG was purified from plasma samples utilizing the protein G HP Multitrap and the Antibody Buffer Kit (GE Healthcare, Buckinghamshire, UK) based on manufacturer's instructions. Briefly, prepare two collection plates with 15 µl neutralizing buffer (1 M Tris-HCl) per well. Place the MultiTrap plate on a collection plate after shaking it gently, and remove the storage solution by centrifuging for 1 min at 100 g. Then add 300 µl binding buffer (0.02 M sodium phosphate) and centrifuge for 30 s to equilibrate. Add 300 µl antibody sample and incubate for 4 minutes while mixing. Wash twice using 300 µl binding buffer. Replace the collection plate with a new one and add 200 µl elution buffer (0.1 M glycine-HCl). Following elution which was repeated two times pooled elutes were washed two times with PBS utilizing Amicon 30kDa centrifugal filters (EMD Millipore).

2.3.3 IgG quantification

The concentration of purified IgG antibody was determined by Human IgG ELISA kit (Mabtech). Briefly, coat the MT145 antibody onto Maxisorb 96 wells plate (Nunc) overnight. Then wash the plate with 0.05% PBS-Tween(U-CyTech) for 5 times. The plate was blocked with 1 % PBS-BSA for 2 hours. Then wash the plate with 0.05% PBS-Tween for 2 times. Add purified IgG antibody diluted 1: 100000 in 1% PBS-BSA and incubated for 2 hours. Then wash the plate with 0.05% PBS-Tween for 5 times. Add MT78-ALP as secondary antibody and incubate for one hour. Then wash the plate with 0.05% PBS-Tween for 5 times.

Finally, add p-nitrophenyl-phosphate (pNPP) and develop for an hour. Optical density at 405nm was read using plate reader (Thermo Fisher Multiskan Ascent plate reader). Raw data were analyzed on the website elisaanalysis.com which produce directly the final concentration of each sample.

2.4 IgG1 Allotyping ELISA

2.4.1 First version of allotyping ELISA

The original version of ELISA procedure for IgG1 allotyping was adapted from a previous HIV vaccine study [79, 80], which can be used to identify G1m1 and G1m3 allotypes in IgG samples. Allotype-specific detection antibody for G1m3 IgG1 is monoclonal Anti-Human IgG1 (Fab specific) [G1m(f)] antibody produced in mouse-clone SG-16 (Sigma) and detector Ab for G1m1 IgG1 is Mouse Anti-Human IgG1 Hinge-BIOT (Southern Biotech). Briefly, coat the mouse anti-human G1m1 or G1m3 IgG1 detection antibodies onto Maxisorb 96 wells plate (Nunc) overnight. Then wash the plate with 0.05% PBS-Tween for 5 times. Following blocking by PBS-BSA and washing, add the samples (25 μg/ml human IgG or serum diluted 1:400 in PBS) and incubate for 2 hours. HIVIG （NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Catalog#3957）was used as positive control and no antibody well was set as negative control. Then wash the plate with 0.05% PBS-Tween for 5 times. Add mouse anti-human IgG Ab-biotin (Thermo Fisher Scientific) followed by streptavidin-HRP (Thermo Fisher Scientific) after washing and incubate for one hour each. Then wash the plate with 0.05% PBS-Tween for 5 times. Finally, add TMB substrate (Thermo Fisher Scientific) and 1M sulphuric acid after developing for 10 minutes. Optical density at 450nm was read using plate reader (Thermo Fisher Multiskan Ascent plate reader).

2.4.2 Optimized version of allotyping ELISA

The optimized version of allotyping ELISA is adapted from the first version. The most prominent difference is that IgG samples were firstly coated onto the plate overnight instead of G1m1 or G1m3 detection antibodies. Briefly, coat the samples (25 μg/ml human IgG or serum diluted 1:400 in PBS) onto Maxisorb 96 wells plate (Nunc) overnight. HIVIG was used as positive control and no antibody well was set as negative control. Then wash the plate with 0.05% PBS-Tween for 5 times. Following blocking by PBS-BSA and washing, add allotype-specific antibody and incubate for 2 hours. Then wash the plate with 0.05% PBS-Tween for 5 times. Then add Rat Anti-mouse IgG1-HRP (eBioscience, diluted 1:1000 in incubation buffer) as secondary antibody and incubate for one hour. Then wash the plate with 0.05% PBS-Tween for 5 times. Finally, add TMB substrate (ThermoFisher) and 1M sulphuric acid after developing for 10 minutes. Optical density at 450nm was read using plate reader (Thermo Fisher Multiskan Ascent plate reader).

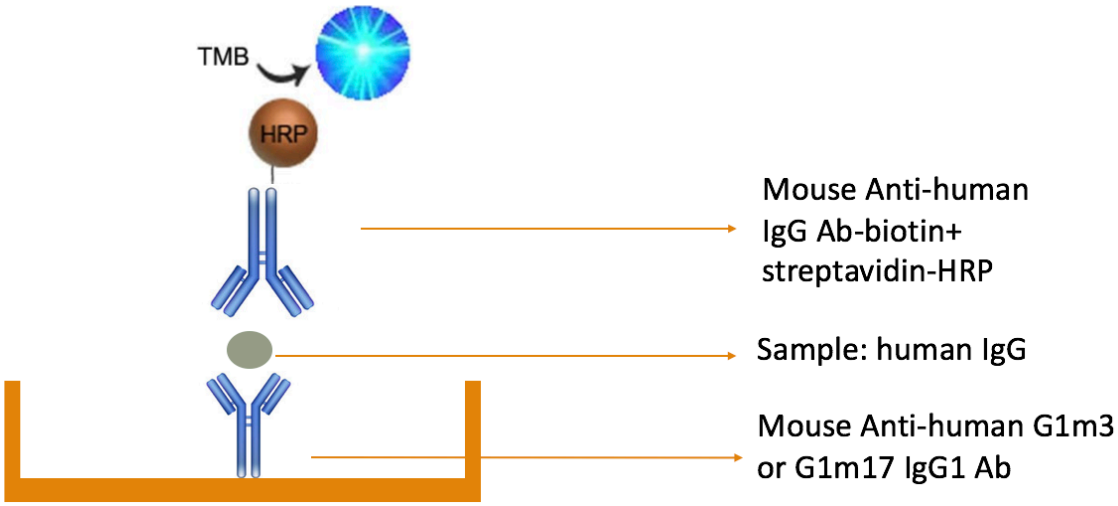


Figure 2.1 Diagram of allotyping ELISA 2.4.1

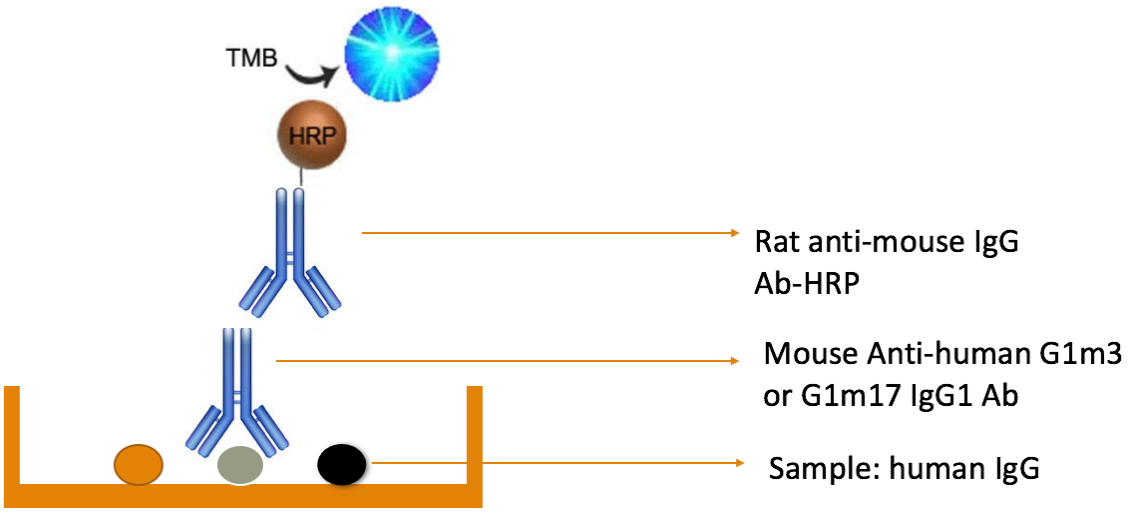


Figure 2.2 Diagram of allotyping ELISA 2.4.2

2.5 HL-60 cells maintenance, differentiation and staining

The maintenance and differentiation of HL-60 cells (ATCC) were previously described[62, 63]. HL-60 cells were cultured in Iscove's Modified Dulbecco's Medium (Sigma) with 20% heat-inactivated FCS and penicillin (100U/ml)-streptomycin (100 µg/ml)-glutamine (2nM). To differentiate the HL-60 cell into a neutrophil-like subset, sterile DMSO (Sigma-Aldrich) was added into media at a final concentration of 1.3% for five days. The generation of neutrophil-like cells was assessed by staining with CD11b BV785 (BioLegend, 1:100), a marker that was crucial in neutrophil Fc receptor-mediated functions [53].

DMSO-induced HL-60 cells were stained with the BV785 anti-human CD11b antibody (BioLegend, 1:100) for 30 minutes at room temperature. After washed by PBS, the HL-60 cells were stained with a cocktail solution of APC-H7 anti-human CD14 antibody (BD Biosciences,1:50), AF-700 anti-human CD16 antibody(BD Biosciences,1:333), FITC anti-human CD32 antibody(BD Biosciences, 1:50), BV510 anti-human CD64 antibody (BD

Biosciences,1:50)and APC anti-human CD89 antibody(BioLegend,1:100). The stained cells were washed by PBS after staining for half an hour at room temperature and fixed with formaldehyde. Cell marker positive frequency and mean fluorescence intensity were measured by BD LSRII Fortessa and analyzed by Flowjo 10.3 software.

2.6 HL-60 Antibody-dependent phagocytosis (ADP) assay

Phagocytosis assay is designed to measure the capacity of IgG samples to trigger antibody-dependent phagocytosis of HL-60 cells. Briefly, HIV-1 Bal gp120 antigens (NIH AIDS Reagent Program) were biotinylated with Sulfo-NHS-LC Biotin (Thermo Fisher Scientific). HIV-1 Bal is a clade B strain, and thus it is suitable for use in samples from Australian cohort where clade B HIV-1 is prevalent. The excessive biotin was removed using Amicon 30kDa centrifugal filters (EMD Millipore). Biotinylated gp120 antigens were incubated with 1 μm fluorescent neutravidin beads (Invitrogen) at 4°C overnight with a mess ratio of 3:1. Then the free antigens were removed by washing twice with PBS-BSA (2%) and beads were diluted 1:100 in PBS-BSA (2%). Then 10 μl Antigen-coated beads were then incubated with 25 μg/ml IgG samples for 2 hours at 37°C. A concentration of 25 μg/ml was chosen based on experiments in Chapter 4. Antigen-coated beads were also incubated with HIVIG (NIH AIDS reagent), IVIG (Privigen®, CSL Behring) or no Abs to be used as positive or negative controls. The differentiated HL-60 cells were washed and resuspended in fresh media to remove DMSO. HL-60 cells (1×10^5 cells) were added and incubated at 37°C with 5% CO2 in air for 20 hours. Cells were stained with CD11b BV785 (BioLegend, 1:100) and then fixed in 2% formaldehyde. Beads uptake was determined by BD LSRII Fortessa and Flowjo analysis software version 10.3.

The phagocytic score was calculated by the formula: beads-positive frequency of CD11b-positive HL-60 cells× mean fluorescent intensity (MFI)/10,000.

2.7 Antibody-dependent neutrophil phagocytosis (ADNP) assay

This Phagocytosis assay is previously described to measure the capacity of IgG samples to trigger antibody-dependent phagocytosis of neutrophils[50]. Briefly, HIV-1 Bal gp120 antigens (NIH AIDS Reagent Program) were biotinylated with Sulfo-NHS-LC Biotin (Thermo Fisher Scientific) using a 50 mmol excess biotin. The excessive biotin was removed using Amicon 30kDa centrifugal filters (EMD Millipore). Biotinylated gp120 antigens were incubated with 1 μm fluorescent neutravidin beads (Invitrogen) at 4°C overnight. Then the free antigens were removed by washing twice with PBS-BSA (2%) and beads were diluted 1:100 in PBS-BSA (2%). Then 10 μl Antigen-coated beads were then incubated with purified 25 μg/ml IgG samples for 2 hours at 37°C. Antigen-coated beads were also incubated with HIVIG, IVIG (Privigen®, CSL Behring) or no Abs to be used as positive or negative controls. Primary neutrophils were purified from fresh heparinised blood of healthy subjects via density gradient and dextran sedimentation. The purified fresh neutrophils were added to the beads/IgG solution and incubated at 37°C with 5% CO_2 in air for 0.5 to 5 hours. Cells were then fixed in 2% formaldehyde. Beads uptake was determined by BD LSRII Fortessa and Flowjo analysis software version 9.8.5. The phagocytic score was calculated by the formula: beads-positive frequency × MFI/10,000.

2.8 HL-60 ADP assay using a specific hybridization internalization probe

To confirm that the fluorescent beads were completely phagocytosed, the HL-60 ADP-SHIP assay and was performed as previously described for THP-1 cells[38]. Briefly, 3 μl biotinylated HIV-1 Bal gp120 (NIH AIDS Reagent Program) were incubated with 1 μl FITC-labeled NeutrAvidin Fluosphere 1 μm beads (Invitrogen) and 1μl of 150mM biotin- and Cy5-labelled florescent internalisation probe (FIPCy5) (5' Cy5-TCAGTTCAGGACCCTCGGCT-N3 3', Integrated DNA Technologies, Coralville, IA, USA) overnight at 4°C. gp120-coated Beads were washed with sterile 2% PBS-BSA and diluted in 100 μl 2% PBS-BSA. Diluted beads were incubated with 25 μg/ml purified IgG for 2 hours at 37°C. Opsonized beads were incubated with 1x 10^5 DMSO-stimulated HL-60s in a total volume of 100 μl. After incubating at 37°C for 20 hours, cells were stained with BV785 mouse anti-human CD11b (Biolegend) for 30 minutes. Surface-bound beads were quenched by adding 1 μg/ml of the complementary quenching probe (QPC) (5' -AGCCGAGGGTCCTGAACTGA-BHQ2-3' Integrated DNA Technologies) for 10 min at 4°C and were subsequently washed with PBS and fixed in 2% formaldehyde. Fixed cells were acquired on a Fortessa LSRII (BD Bioscience) and analyzed using Flowjo analysis software version 10.3.

2.9 Multiplex assay for IgG subclasses

This assay was designed to measure the HA-specific IgG subclass levels of plasma samples and was previously described[170, 171]. Briefly, a panel of various hemagglutinin proteins with different fluorescence was covalently conjugated to individual microspheres[170]. Then Incubate the protein conjugated beads with influenza vaccine cohort plasma from baseline and post-vaccination with 1:100 plasma dilution on a plate shaker, overnight at 4°C. The beads were incubated on a plate shaker at room temperature for two hours after washing utilizing a Bio-Rad magnetic plate-washer (Bioplex Pro wash station).

Subsequently, the beads were incubated with PE-conjugated anti-human antibodies that recognized total IgG, IgG1, IgG2, IgG3 or IgG4 (Southern Biotech) for one hour. Finally, a Bio-Plex reader (Bio-Plex MAGPIX, Bio-Plex Manager 5.0, Bio-Rad) was used to detect the microspheres and evaluate the binding of PE fluorescence to calculate a median fluorescence intensity (MFI). The average MFI for each microsphere set that incubated with the PE-conjugated detection reagent without Ab sample was defined as background, and was subtracted from MFI for all the samples.

2.10 FcγR dimer-binding assay

2.10.1 For influenza or HIV-specific IgG

This method was previously described to measure the binding of Fc receptors with antibody[159]. Recombinant soluble homodimers of FcγRIIa (H131) or FcγRIIIa (V158) were constructed as single, biotin-tagged polypeptides. Briefly, influenza HA or SIV gp120 (Sino biological), HIV-1 Bal gp120 (NIH) at 50 ng per well diluted in PBS were coated on 96-well flat-bottom MaxiSorp plates (Nalgene Nunc, Rochester, NY). HIVIG or IVIG was coated on the plate to normalize FcR-IgG binding across different plates. Following overnight incubation at 4˚C, the ELISA plates were washed with PBS containing 0.05% Tween20 (U-CyTech) and blocked for one hour at 37°C with 100 μl PBS containing 1% BSA (Sigma-Aldrich) and 1 mM EDTA per well. Plates were washed and 1:20 diluted plasma or 100 μg/ml purified IgG in 1% PBS-BSA was added and incubated for one hour （two hours for purified IgG）at 37°C. After incubation and washing, incubate with 0.1 μg/ml of FcγRIIIa dimer or 0.2 μg/ml of FcγRIIa dimer diluted in PBS with 1 mM EDTA and 1% BSA for one hour at 37°C. Then 100 ng/ml of HRP-conjugated streptavidin (Thermo Fisher Scientific) was added diluted in PBS-EDTA-BSA for one hour at 37°C. Finally, add TMB

substrate (Sigma-Aldrich) and stop solution (1 M hydrochloric acid) after developing for 10 minutes. OD450 of wells was read using spectrophotometer as final data after normalizing using the data from control wells containing 5μg/ml human intravenous immunoglobulin (IVIG, Sandoglobublin) or HIVIG (NIH AIDS reagent).

2.10.2 For total IgG

This method was adapted from method 2.10.1 to measure the binding of Fc receptors with the whole pool of IgG instead of influenza or HIV-specific IgG. Briefly, 100 μg/ml purified IgG (50 μl per well) were coated on 96-well flat-bottom MaxiSorp plates (Nalgene Nunc, Rochester, NY). HIVIG or IVIG was coated on the plate to normalize FcR-IgG binding across different plates. Following overnight incubation at 4˚C, the ELISA plates were washed with PBS containing 0.05% Tween20 (U-CyTech) and blocked for one hour at 37°C with 100 μl PBS containing 1% BSA (Sigma-Aldrich) per well. After incubation and washing, incubate with 0.1 μg/ml of FcγRIIIa dimer or 0.2 μg/ml of FcγRIIa dimer diluted in PBS -1% BSA for one hour at 37°C. Then 100 ng/ml of HRP-conjugated streptavidin (Thermo Fisher Scientific) was added diluted in PBS- -BSA for one hour at 37°C. Finally, add TMB substrate (Sigma-Aldrich) and stop solution (1 M hydrochloric acid) after developing for a 10 minutes. OD450 of wells was read using spectrophotometer as final data after normalizing using the data from control wells containing 5μg/ml human intravenous immunoglobulin (IVIG, Sandoglobublin) or HIVIG (NIH AIDS reagent).

2.11 Plate-bound natural killer (NK) cell activation assay

This method was previously described to measure the capacity of HA-specific antibodies to induce NK-92 cell expression of CD107a[159]. Briefly, 600 ng/ well influenza HA or SIV gp120 (Sino biological) diluted in PBS were coated on

96-well flat-bottom MaxiSorp plates (Nalgene Nunc, Rochester, NY) overnight at 4˚C. Then the plates were washed and blocked in 5% PBS-BSA, and incubated with heat-inactivated plasma samples with 1:40 dilution for two hours at 37°C. NK-92 cells (2×10^5 cells) were added and incubated at 37°C with 5% CO2 in air for 5 hours. Cells were stained with CD107a-allophycocyanin (Clone H4A3, BD Biosciences) and 1 mM EDTA for 30 mins at room temperature. NK-92 cells were subsequently washed twice with PBS and fixed with 1% formaldehyde. Fixed cells were acquired on a Fortessa LSRII (BD Bioscience) and analyzed using Flowjo analysis software version 10.0.7.

2.12 Surface plasmon resonance analysis

A BIACORE 3000 (GE Healthcare) was used to determine the binding kinetics of subjects purified IgG to both the low and the high-affinity variants of FcRIIa (H131 or R131) and FcRIIIa (V158 or F158). Biotinylated FcRs (Sino Biological) were immobilized on a streptavidin sensor chip (GE Healthcare) at a flow rate of 5 µl/ min, with flow cell 1 left blank to serve as a reference surface. Each FcR was immobilized at the following ligand densities FcRIIAH131: 270 RU, FcRIIAR131:917 RU, FcRIIIa F176: 798 RU, FcRIIIAV176: 660 RU. To collect kinetic binding data, each analyte (purified IgG) in 10 mM HEPES, 150 mM NaCl, 0.005% P20 (polysorbate 20 surfactant), 0.1% T20 (Tween 20 surfactant) pH 7.4, was injected over flow cells at concentrations of 333.3, 166.7, 83.3, 41.7 and 20.8 nM at a flow rate of 30 µl/min and at a temperature of 25°C. For the FcRIIIa ligands, the complex was allowed to associate and dissociate for 120 and 360 s respectively, and the surfaces were regenerated with a 2 x 5ul injection of 10 mM Glycine HCl pH 2.25. For the FcRIIa ligands, the complex was allowed to associate and dissociate for 120 and 360 s respectively, and the surfaces were regenerated with a 3 x 5ul injections of 10 mM NaoH. Data were analyzed and

globally fitted to a 1:1 interaction model with Scrubber version 2 software (BioLogic Software, Campbell, Australia).

2.13 Statistical analysis

Statistical analysis was performed on Prism GraphPad version 7.0b (GraphPad Software, San Diego, CA). The non-parametric Spearman correlation analysis was performed to test the correlation between HL-60 ADP assay, ADNP assay, FcγR dimer-binding assay and viral load. The non-parametric Mann-Whitney U test and multiple comparisons of Kruskal-Wallis test were performed to compare the difference between G1m1 IgG1 subjects, G1m3 IgG1 subjects and G1m1/G1m3 IgG1 subjects for IgG subclasses levels, FcγR dimer-binding assay, surface plasmon resonance analysis, plate-bound NK cell activation assay and HL-60 ADP assay. A p value of less than 0.05 indicates a significant difference (*$p<0.05$, **$p<0.01$, ***$p<0.001$,****$p<0.0001$).

The half-maximal effective concentration (Ec50) of IgG for the phagocytosis assay was determined at 25 μg/ml by the nonlinear curve fit using a standard process from Prism Graphpad 7.0b. Briefly, concentrations of all samples were transformed by logarithm and phagocytic scores were normalized (highest score set 100% and lowest score set at 0%). Then a nonlinear curve fit was done based on integrated data and the concentration that trigger 50% normalized phagocytic score was set as Ec50.

Chapter 3 ELISA Allotyping

3.1 Introduction

Allotypes are genetic polymorphisms in the constant region of antibodies. IgG1 is the most abundant immunoglobulin G subclass and commonly performs important neutralization and Fc receptor-mediated functions. Two common allotypes for IgG1 in the CH1 domain are G1m3 which presents CH1 arginine at position 214 and G1m17 which codes CH1 lysine at the same position. Two common allotypes for IgG1 in CH3 domain are G1m1 allotype which comprises aspartate at position 356 and leucine at position 358, and nG1m1 allotype which expresses glutamic acid at position 356 and methionine at position 358 (see introduction section 1.5.2 for further details). The amino acid sequence is the primary structure of antibodies, which may influence the tertiary and quaternary structure, and thus changing the binding affinity of antibodies with Fc receptors and ultimately affecting the antibodies effector function. It is possible that allotypes have an impact on Fc receptor-mediated functions such as Antibody-dependent cell-mediated phagocytosis (ADCP) and Antibody-dependent cell-mediated cytotoxicity (ADCC).

There are two primary approaches for the IgG allotyping. One is the genetic-based test, i.e. PCR, which is more time-consuming and requires human cells to extract DNA or mRNA. It normally takes 1-2 days to run the allotyping process in this way. The other is the serology-based test, i.e. ELISA, which is much more convenient if adequately validated. It can be done using serum or purified IgG and only takes several hours to finish the test.

In this project, an ELISA-based allotyping assay was optimized and validated. This ELISA allotyping assay was used to determine the G1m1 and G1m3

allotypes for the purified IgG or plasma samples from HIV-positive antiretroviral therapy (ART) naïve cohort and influenza vaccine cohort. We found that this ELISA allotyping is consistent with sequencing results, indicating that this assay should be useful to determine the IgG1 allotypes of purified IgG or plasma samples where cells are not available.

3.2 Results

3.2.1 Determination of IgG1 allotypes from PBMC by PCR and Sanger sequencing

PCR and sequencing methods for determining IgG1 allotypes have been developed and validated [168, 169]. These published methods were adapted to PCR and sequence IgG1 genes from PBMC stored from 23 donors of FLUVAX003 cohort. We used this cohort initially since PBMCs were readily available. Amplification products of all samples showed a band of expected size in electrophoresis (figure 3.1A, 3.1C), and were successfully purified and submitted for Sanger sequencing. It was noted that the CH3 domain PCR did generate various side products (figure 3.1C), but these by-products did not affect the purification and sequencing of the target segment products. In the analysis of chromatographs, the homozygous allotype samples manifest themselves as a single peak while the heterozygous allotype samples manifest themselves as overlapping peaks because of two different nucleotides encoded by different G1m alleles in the same subjects (figure 3.1B, 3.1D). Among the 23 donors, six subjects were found to be homozygous G1m3, and four subjects were found to be homozygous G1m17 with the remaining 13 subjects were found to carry both

allotype alleles. For CH3 domain sequencing, 10 subjects were homozygous G1m1, and three subjects were homozygous nG1m1, plus 10 subjects which were heterozygous G1m1/nG1m1.

3.2.2 Determination of IgG1 allotypes from plasma by PCR and Sanger sequencing

Given the PCR and sequencing procedures were validated in the Fluvax003 cohort, all the participants from a larger cohort, the influenza vaccine cohort, were evaluated utilizing the same method. PBMC was not available in this cohort but only plasma. It is difficult to extract and amplify the tiny amount of genomic DNA in the plasma, thus large volume (approximately 400 μl) of plasma was used to purify genomic DNA for each sample. Since the heparin in the plasma hindered PCR, all the plasma samples were treated with heparinase to remove the heparin. The PCR method (section 2.2.2) was adapted for the plasma-derived DNA: the cycle number increased to 50 cycles, and the annealing temperature was lowered 2-3°C. All amplification products were eventually obtained after rounds of attempts. The analysis of the sequencing results indicated that 23 donors were determined to be G1m3/nG1m1 homozygotes, eight donors were found to be G1m1/G1m17 homozygotes, and 25 donors carried both G1m1 and G1m3 allotype alleles. All these results were compared with ELISA allotyping results as below.

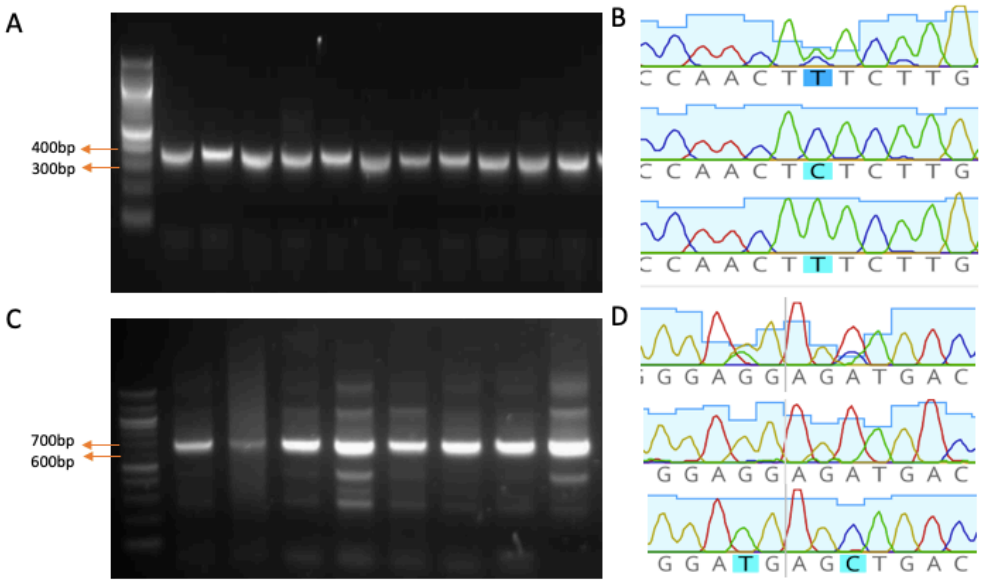


Figure 3.1 Determination of IgG1 allotypes via PCR and sequencing (A) Representative electrophoresis image of amplification products in CH1 domain. The ladder was in the first lane, and products showed an expected length of 364 bps. (B) Representative sequence chromatographs corresponding to G1m3/G1m17 heterozygotes (top), G1m3 homozygotes (middle) and G1m17 homozygotes (bottom) samples. The highlighted position is the polymorphic nucleotide locus. (C) Representative electrophoresis image of amplification products in CH3 domain. First lane stands the ladder, and amplification products showed an expected length of approximately 700 bps. (D) Representative sequence chromatographs corresponding to G1m1/nG1m1 heterozygotes (top), nG1m1 homozygotes (middle) and G1m1 homozygotes (bottom) samples. The highlighted position is the polymorphic nucleotide locus.

3.2.3 Development and optimization of the allotyping ELISA on extracted IgG and plasma

Neither PBMC or plasma was available for the HIV-positive ART naïve cohort, only extracted IgG. A serological approach to determine the IgG1 allotypes that only uses purified IgG samples was therefore developed for this application. The first version of the ELISA-based allotyping assay was adapted from a previous study using the same G1m1 or G1m3-specific detection antibody to distinguish

IgG1 allotypes [79, 80]. 53 samples from the HIV-positive ART naïve cohort were analysed by the methods outlined in section 2.4.1 above for IgG1 allotypes. The G1m3 allotyping results showed adequate discrimination difference of positive and negative results with low background signal and decent positive OD 450 nm (figure 3.2A). However, the G1m1 allotyping results encountered a difficulty distinguishing positive and negative subjects because of the high background and continuous OD450 distribution (Figure 3.2A). To address this problem, we designed a novel allotyping ELISA (method 2.4.2). In the first version of allotyping ELISA (method 2.4.1, figure 2.1), the G1m1 or G1m3-specific detection antibody was coated on the plate as the capture antibody, and then the samples were added. In this new version of the ELISA-based assay (method 2.4.2, figure 2.2), the samples (purified IgG) were coated on the plate overnight as the capture antibody, and the G1m1 or G1m3-specific detection antibody was added to be captured. 58 samples from the HIV-positive ART naïve cohort were analyzed by new versions of allotyping ELISA for IgG1 allotypes (figure 3.2B). Using this ELISA-based approach, both G1m3 and G1m1 groups showed low background and clear discrimination difference between positive and negative samples after optimization. Within this cohort, 20 subjects were determined to be G1m3/nG1m1 homozygotes, eight subjects were found to be G1m1/G1m17 homozygotes, and 30 subjects carried both G1m1 and G1m3 allotype alleles.

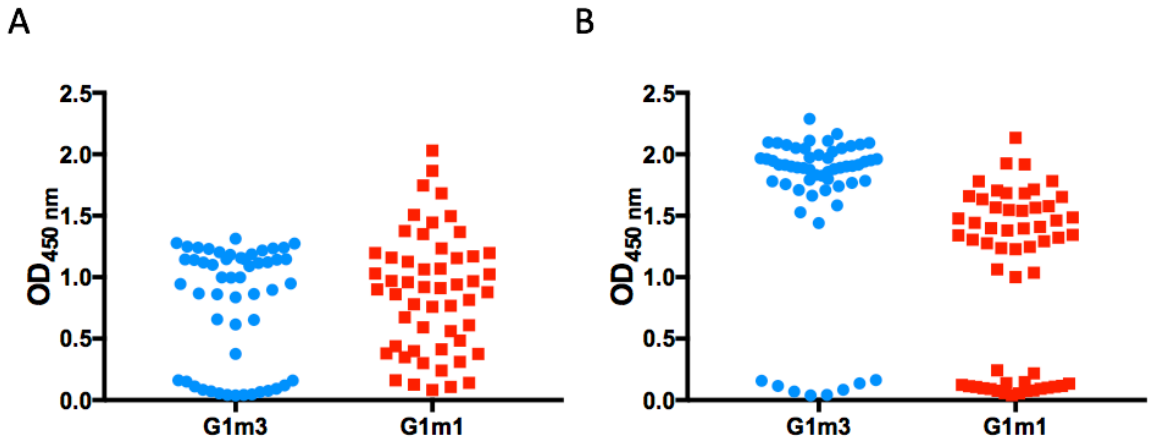


Figure 3.2 Distribution of OD 450 nm of HIV-positive ART naïve cohort in the first version of ELISA allotyping (method 2.4.1, A) and in the optimized version of ELISA allotyping (method 2.4.2, B). The results of the ELISA allotyping were further divided into two groups. In the G1m3 group (each blue dot represents a subject in the cohort), anti-G1m3 IgG1 detector antibody was used. In the G1m1 group (each red dot represents a subject in the cohort), anti-G1m1 IgG1 detector antibody was used. Samples with OD 450 nm>0.5 were identified to be positive for G1m3 or G1m1. Note that the G1m1 allotyping had poor discrimination of positive and negative samples in the first method (red samples in A) but this is improved with the second method (red samples in B).

A minimum volume of 50 µl plasma samples is commonly required to purify IgG. It would spare much plasma if coating with raw plasma (less than 1 µl) also efficiently discriminates allotypes. Eight subjects whose allotypes have been identified by sequencing were compared via allotyping ELISA using diluted plasma and purified IgG (Figure 3.3). Purified IgG and plasma samples provided the same result: Those who only carries G1m3 allotype allele exhibited positive signals for G1m3 allotyping ELISA and negative signals for G1m1 allotyping ELISA, and those who only carry G1m1 allotype allele showed positive signals for G1m1 allotyping ELISA and negative signals for G1m3 allotyping ELISA, and heterozygotes displayed positive signals for both allotypes. This result demonstrated that both plasma and purified IgG samples can be directly used for

the ELISA protocol. The plasma diluted 1:800 was as efficient as 25 μg/ml purified IgG in ELISA allotyping.

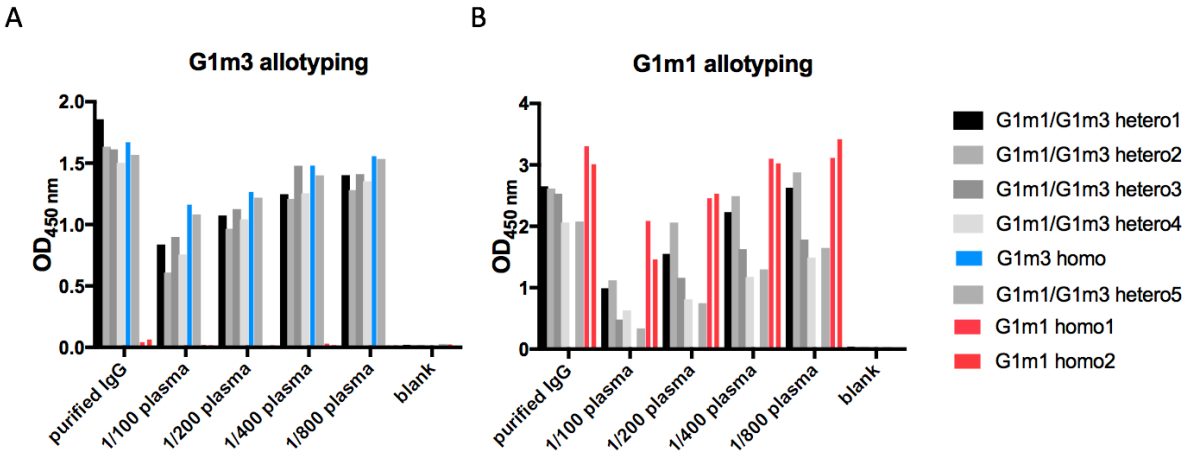


Figure 3.3 Evaluation of G1m1 and G1m3 ELISA allotyping with plasma in selected subjects of Fluvax003 cohort. Each bar shows the OD 450 nm value for purified IgG or diluted plasma from a specific participant. G1m1/G1m3 hetero: the subject is found to be G1m1/G1m3 heterozygote by sequencing. G1m3 (G1m1) homo: the subject is found to be G1m3 (G1m1) homozygote by sequencing. (A) ELISA allotyping for G1m3 allotype. Subjects whose allotypes were determined by sequencing were tested with purified IgG and plasma of different sets of dilution. (B) ELISA allotyping for G1m1 allotype. The blue bar of G1m3 homozygotes is not clear because it is very close to zero.

Likewise, the allotyping ELISA was also performed with samples of the adult and elderly group in CSLCT-WHO-08-51 cohort, the whole Fluvax003 cohort and the influenza vaccine cohort as it is optimized (figure 3.4). We observed low background signal and clear discrimination difference between positive and negative samples in all cohorts as expected. The distribution of different allotypes in all the cohorts in the thesis was acquired after analysis (table 3.1).

Unfortunately, the ethnicity of each participant is only available in the Fluvax003 cohort. Population genetic analysis of IgG1 allotypes covered approximately 20 samples (table 3.2).

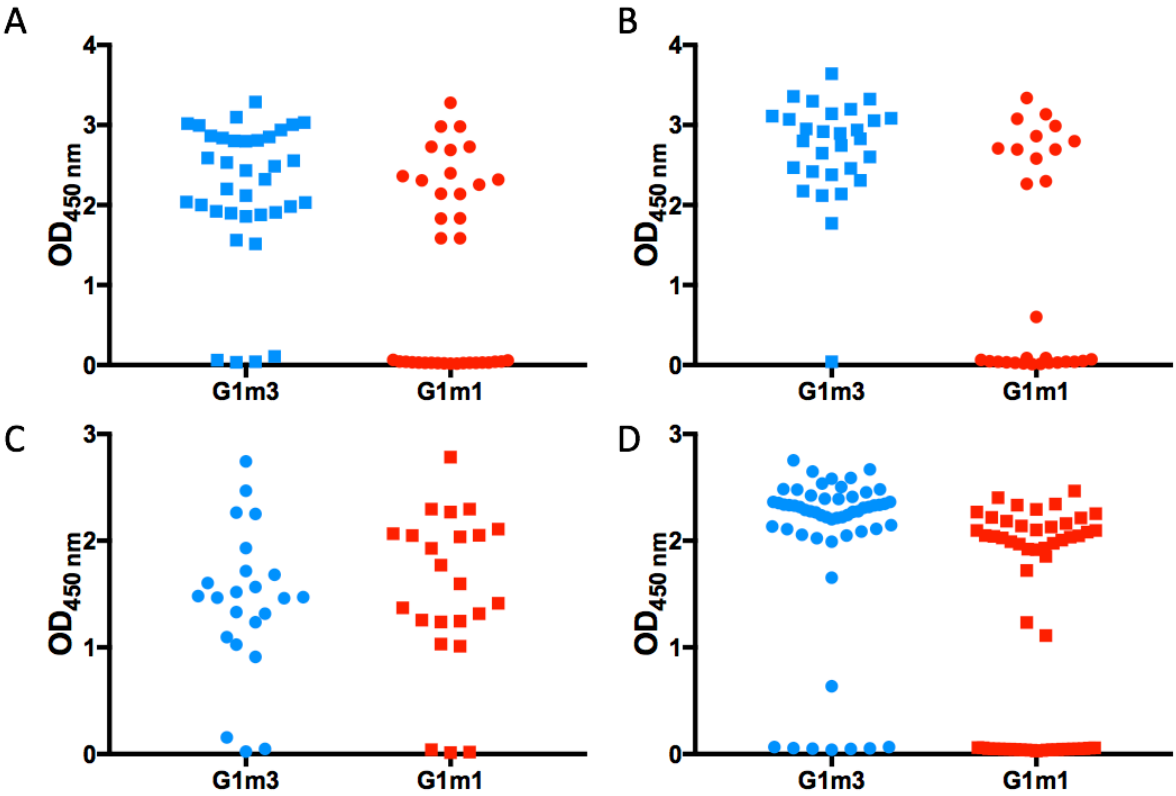


Figure 3.4 Distribution of OD 450 nm of optimized ELISA allotyping in various cohorts. The results of the ELISA allotyping are further divided into two groups. In the G1m3 group (each blue dot represents a subject in the cohort), anti-G1m3 IgG1 detector antibody was used. In the G1m1 group (each red dot represents a subject in the cohort), anti-G1m1 IgG1 detector antibody was used. Samples with OD 450 nm>0.5 were identified to be positive for G1m3 or G1m1.(A) Purified IgG samples of adult subjects in CSLCT-WHO-08-51 cohort (B) Purified IgG samples of elderly subjects in CSLCT-WHO-08-51 cohort (C) Diluted plasma samples in Fluvax003 cohort (D) Purified IgG samples in Influenza vaccine cohort

3.2.4 Concordance of the allotyping ELISA and allotype sequencing

To further validate the utility of the ELISA allotyping, a correlation of allotyping ELISA and sequencing results was implemented in the complete Fluvax003 cohort and influenza vaccine cohort (table 3.3). The comparison of ELISA-based and sequencing-based data showed that the PCR and sequencing approach was consistent with the ELISA approach, thus confirming the reliability of the

allotyping ELISA protocol. This cross-validation means the allotyping ELISA can replace sequencing for G1m1 and G1m3 discrimination in certain scenarios.

Table 3.1 the distribution of different allotypes in four cohorts

Cohort	HIV-positive ART naïve cohort	CSLCT-WHO-08-51 cohort adults	CSLCT-WHO-08-51 elderly cohort	Fluvax003 cohort	Influenza vaccine cohort
G1m3 homozygotes numbers (%)	20 (34.5%)	14 (41.2%)	17 (58.6%)	3 (13.0%)	23 (41.1%)
G1m1 homozygotes numbers (%)	8 (13.8%)	3 (8.8%)	1 (3.4%)	4 (17.4%)	8 (14.3%)
G1m1/G1m3 heterozygotes numbers (%)	30 (51.7%)	17 (50%)	11 (37.9%)	16 (69.6%)	25 (44.6%)
Total number	58	34	29	23	56

Table 3.2 The IgG1 haplotypes present in each ethnicity of Fluvax003 cohort

Ethnicity	IgG1 haplotypes	Allele number	Percentage
Caucasians	(G1m1,G1m17)	11	42.3%
	(G1m1,G1m3)	0	0%
	(nG1m1, G1m3)	15	57.7%
Asians	(G1m1,G1m17)	5	35.7%
	(G1m1,G1m3)	9	64.3%
	(nG1m1, G1m3)	0	0%
Caucasian-Asian Hybrid	(G1m1,G1m17)	5	83.3%
	(G1m1,G1m3)	0	0%
	(nG1m1, G1m3)	1	16.7%

Table 3.3 The correlation of sequencing allotyping and ELISA allotyping in Fluvax003 cohort and influenza vaccine cohort

Sequencing allotyping		ELISA allotyping		
IgG1 Allotypes	**Number**	**IgG1 Allotypes**	**Number**	**Discordant results**
G1m1,3,17 (has nG1m1)	29	G1m1,3	29	0
G1m1,3	5	G1m1,3	5	0
G1m1,3 (has nG1m1)	2	G1m1,3	2	0
G1m1,3,17	5	G1m1,3	5	0
G1m1,17	12	G1m1	12	0
G1m3	26	G1m3	26	0

3.3 Discussion

This chapter developed and cross-validated several potential methods to determine G1m1 and G1m3 allotypes by both PCR and ELISA that should prove useful in future studies. The first version of the ELISA-based allotyping had quite obvious background signals and did not have clear discrimination difference in the G1m1 ELISA despite several attempts of optimization. Since the G1m3 ELISA exhibited reliable discrimination, it suggested that the G1m1 ELISA is not a problem with the assay plate, the buffer, the samples, the detection antibody, the enzyme conjugate or the substrate, but something of the G1m1-specific capture antibody (Method section 2.4.1). One possible explanation is that the anti-IgG-Hinge antibody coated on the plate might generate some unexpected steric effects, thus interfering with its interactions with G1m1-IgG1.

To avoid the problem of coating with G1m1-specific antibody, a novel allotyping method was developed and optimized in which the purified IgG or whole plasma samples were coated as the capture antibody. Although the IgG is a major component of plasma samples, the plasma is a complicated mixture which has other proteins such as albumins. Despite the complex composition of the plasma, this second allotyping ELISA method produced quite low background signals and obvious discrimination differences, suggesting the high sensitivity and specificity of both G1m1 and G1m3-specific antibodies.

The validation of allotyping ELISA provides an alternative approach to determine the IgG1 allotypes when PBMCs or large volume of plasma is not available. Furthermore, the ELISA method is a simpler process and takes less time to perform. Admittedly, the sequencing allotyping offers more accurate information across a wider range of IgG polymorphisms which may be critical to broader analyses. For instance, the G1m(1,3) allele carriers determined by ELISA-based allotyping can be G1m(1,3), G1m(1,3,17), G1m(1,n1,3) and

G1m(1,n1,3,17) genotypes determined by sequencing. The greater detail provided by sequencing means more precise comparison can be done. In conclusion, the sequencing and ELISA methods have their respective advantages and can play a role in specific situations (table 3.4).

Table 3.4 The comparison of the ELISA and sequencing allotyping

	ELISA allotyping	Sequencing allotyping
Time required	Several hours	1-2 days
Financial cost	Lower	Higher
Information provided	Existence of G1m1 or G1m3 allotypes	G1m1, nG1m1, G1m3 and G1m17
Materials required	Purified IgG or whole plasma	Cells or >200 μl plasma

IgG1 allotypes are differentially distributed among various ethnicities. Some population genetic studies showed that G1m3/nG1m1 haplotype was common in Caucasians but rare in Asians or Africans. On the contrary, the G1m3/G1m1 haplotype was present in Asians but rarely found in Caucasians or Africans [72, 78]. In the ethnicity analysis of Fluvax003 cohort, all the haplotypes of G1m1, nG1m1, G1m3 and G1m17 identified in the subjects corresponded to the previous population study [78], in which the Caucasians were found to carry G1m3/nG1m1 haplotype while the G1m3/G1m1 haplotype was found to present in Asians. Despite the shortage of samples, G1m3/nG1m1 haplotype was predominant in Caucasian subjects which also corresponded to the previous statistical investigation [78]. Unfortunately, ethnicity data for other cohorts are not available but further exploration on this would be of interest in the future. However, it is noticed that the number of G1m1 homozygotes in all the cohorts was lower than other genotypes. It accorded with the fact that all participants

were recruited in Melbourne where the majority of the population were Caucasians whose predominant haplotype are G1m3/nG1m1.

Chapter 4 Development of the HL-60 cell line to study neutrophil-mediated ADP

4.1 Introduction

HL-60 is a human leukemic cell line which came from the peripheral blood of a patient with acute promyelocytic leukemia. Under normal cell culture (method section 2.5, HL-60 maintenance part), the majority of cells are close to promyelocytes, while a small percentage of cells show properties of myelocytes, metamyelocytes, and neutrophils[57]. Interestingly, this cell line holds the capacity of differentiating into granulocyte-like or monocyte-like cells based on inducing agents. The polar compound such as dimethyl sulphoxide (DMSO) and other compounds like retinoid acid can induce HL-60 cells to granulocyte-like[59]. Numerous morphological, histochemical and genetic changes can be observed when induced to differentiation, and subsequently followed by death via apoptosis[62]. All these characteristics of the HL-60 cell line make it an appropriate model to study myeloid cell differentiation, proliferation and gene expression in vitro.

Neutrophils play an important role in Fc receptor-mediated functions such as ADCP and ADCC[51, 172]. HIV infection is primarily acquired via mucosal surfaces where neutrophils are abundantly distributed [51]. Neutrophils express a range of Fc receptors [25, 36], thus having the potential of mediating Fc functions against HIV. However, studies that examined neutrophil Fc functions in HIV and other infectious diseases are still limited, largely due to the difficulty of studying neutrophils. Neutrophils have a short half-life of 6-8 hours and die rapidly ex vivo[173]. Therefore, fresh cells from blood are required when doing Fc receptor-mediated functional assays with primary neutrophils and even then,

since some assays require several hours of incubation, a high proportion of the primary neutrophils can be dead or dying during assays.

It would be convenient to develop a cell line-based method to complement the study of antibody-mediated neutrophil functions. HL-60 cells have previously been used to study neutrophil effector functions, in which they were utilized to measure antimicrobial activity, production of ROS and the release of NETs [174, 175]. Furthermore, HL-60 cells have previously been applied to measure antibody-dependent phagocytosis activity towards bacteria [64]. Our aim is to study neutrophil ADCP responses using HL-60 cells. If the correlation of ADCP function profile of neutrophil differentiated HL-60 cells and that of primary neutrophils is strong, it is possible to substitute neutrophils with neutrophil differentiated HL-60 cells in Fc receptor-mediated functional assays.

In this thesis, an HL-60 cell line-based HIV-antibody specific phagocytosis assay was optimized and validated (method 2.6) to quantify the phagocytic activity of antibodies purified from clinical samples. This assay was adapted from previous studies which employed antigen-coated fluorescent beads to simulate microbes [176], but CD11b-positive DMSO-stimulated HL-60 cells were used for phagocytosis instead of THP-1 cells. The HL-60 ADP assay and neutrophil phagocytosis assay were used to evaluate the purified IgG from HIV-positive antiretroviral therapy (ART) naïve cohort. The neutrophil phagocytosis assay (method 2.7) supported that neutrophils can mediate HIV-specific ADCP in HIV-positive subjects [50]. We found that HL-60 ADP assay correlated strongly with primary neutrophils assay, indicating that this assay should be practical to evaluate neutrophil effector functions. Some results from this chapter have been published in: Worley, M. J., Fei K., et al. (2018). "Neutrophils mediate HIV-specific antibody-dependent phagocytosis and ADCC." J Immunol Methods 457: 41-52.

4.2 Results

4.2.1 The differentiation of HL-60 cells into neutrophil-like cells

DMSO has been previously used for differentiating HL-60 cells [177]. However, the concentration of DMSO and duration of stimulation for differentiating HL-60 cells into ADCP-mediating neutrophil-like cells has not been finely analysed. To achieve this aim, the appropriate cell stimulation condition to induce the differentiation of HL-60 cells needed to be determined. HL-60 cells were stimulated with a concentration gradient of DMSO, 1.0%, 1.3%, 1.6% and 1.9%. In the cell count result (figure 4.1A), HL-60 cells ceased growing and died quickly in the group of 1.6% and 1.9% DMSO. More morphological changes were found in these two groups, indicating the cytotoxicity of DMSO (>1.6%) might inhibit the cellular activity and trigger apoptosis of HL-60 cells. HL-60 cells stimulated by 1.0% DMSO maintained a rapidly growing phenotype, while HL-60 cells in the 1.3% DMSO group grew slowly compared to cells cultured in normal media.

The expression level of neutrophilic activation markers and Fc receptors on HL-60 cells were then measured to determine which day and which concentration is most suitable for developing neutrophil-like cells for an ADP assay. We first measured CD11b, CD16 and CD32 staining on DMSO-stimulated HL-60 cells (figure 4.1B-D). CD11b is recognized as an activation marker of neutrophils [55]. CD16 (FcγRIII) and CD32 (FcγRII) are primary Fc receptors that mediate ADCC and ADCP, respectively. The CD11b, CD16, and CD32 were all upregulated in 1.3%, 1.6% and 1.9% DMSO-stimulated groups, especially on the fifth day after stimulation. Moreover, a preliminary phagocytosis assay using gp120-coated beads and HIV+ IgG was evaluated in four DMSO-stimulated groups (figure 4.1E). It is observed that the phagocytosis activity reached a peak at day 5 in the 1.3% DMSO group. A five-day stimulation of 1.3% DMSO to induce HL-60 cells

to differentiate into neutrophil-like is also recommended by several previous studies [178-180]. Based on the results of the CD11b and Fc receptor staining, cell counts, product instructions by the manufacturer, pre-experiment of the phagocytosis assay, and previous literature, we chose 1.3% DMSO to stimulate and on the fifth day after stimulation to perform the phagocytosis assay.

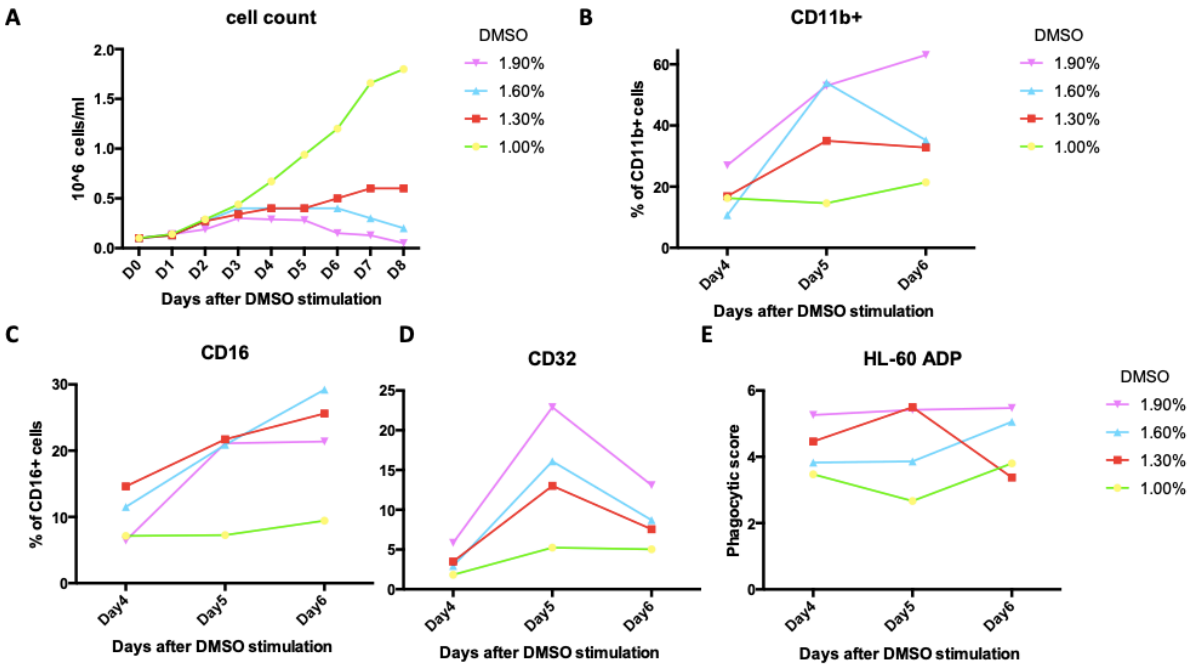


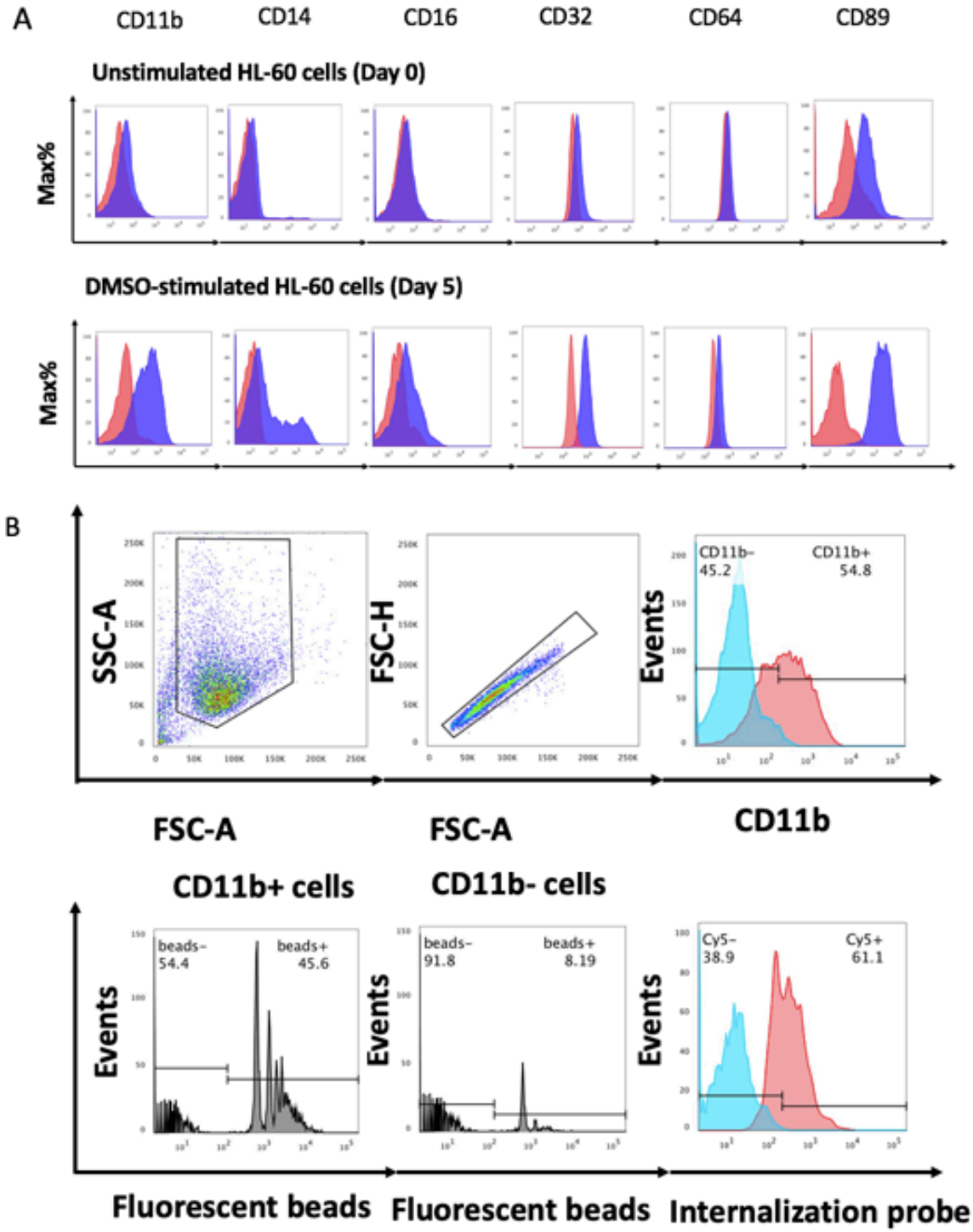
Figure 4.1. HL-60 cells respond to DMSO stimulation. (A) Cell counts after the stimulation of HL-60 cells with various concentrations of DMSO Cells are seeded at a concentration of 0.1*10^6 cells/ml at day 0 and incubated under various concentration of DMSO. Cells are counted manually by hemocytometer. Viability was counted by trypan blue dye exclusions. **(B-D) The Activation marker and Fc receptors staining of DMSO-activated HL-60 cells** The Y axis means the frequency of marker-positive cells of all cells. **(E) The phagocytosis assay of DMSO-activated HL-60 cells** Phagocytic score= %beads positive cells × mean fluorescence intensity/10^4.

4.2.2 Validation of HIV-specific HL-60 ADP assay

The HL-60 cells were differentiated into neutrophil-like using 1.3% DMSO for five days, as determined above. The expression of a broad range of surface markers (CD11b and CD14) and Fc receptors including CD16, CD32, CD64(FcγRI) and CD89 (FcαRI) of neutrophil-differentiated HL-60 cells was assessed by immunofluorescent staining with flow cytometric analysis (figure 4.2A). CD11b, a marker for neutrophil Fc receptor-mediated cytotoxicity, was upregulated upon DMSO stimulation, indicating the neutrophilic differentiation of HL-60 cells. Unstimulated HL-60 cells expressed low/no level of CD 11b, CD14, CD16, CD32, and CD64 but expressed CD89 (MFI 2178). After stimulation with 1.3% DMSO for five days, CD11b (MFI 753), CD14 (MFI 392), CD16 (MFI 641), CD32 (MFI 2204), and CD89 (MFI 2914) were all upregulated. The only exception is CD64, with barely any expression whether at day 0 or day 5.

The gating strategy of the HL-60 ADP assay first gates on CD11b+ HL-60 cells (figure 4.2B a-c) and subsequently gates on the gp120-coated fluorescent beads (figure 4.2B d). CD11b+ HL-60 cells exhibited a high level of beads phagocytosis while CD11b- HL-60 cells had a low level of phagocytosis activity (figure 4.2B e), suggesting that CD11b is a proper marker for measuring Fc receptor-mediated phagocytosis in HL-60 cells. To identify the appropriate incubation time of the assay, a time-course HL-60 ADP assay was performed (figure 4.2C). It is observed that the maximal phagocytic score reached at the 20-hour time point before cells began to die rapidly. Meanwhile, there is a discriminative difference between positive controls and negative controls at 20-hour incubation time point. To confirm that the beads were internalized, an ADP-SHIP assay developed by Fernanda Ana-Sosa-Batiz [38] which used FITC and Cy5 (fluorescent internalization probe, FIPcy5) labelled beads were applied to HL-60 cells (figure 4.2D, method 2.8). This assay can distinguish surface-bound

and internalized beads after the addition of a quenching DNA sequence. This quenching DNA segment can only bind to FIP_{cy5} of the beads on cell surface and lead to quenching of the Cy5 fluorescence, but unable to penetrate into the cytomembrane. Surface-bound beads are identified by flow cytometry as FITC+ Cy5- cells and internalized beads are identified as FITC+ Cy5+ cells (figure 4.2B f). The time-course HL-60 ADP-SHIP assay showed that levels of internalization increased with time (7.56% at 1 hour, 36% at 3 hour) and eventually reached approximately 100% internalization (98.6% at 12 hour) (figure 4.2D). The beads uptake with HIV-positive IgG control was significantly higher than HIV-negative IgG or in the absence of IgG, suggesting the beads were phagocytosed in an antibody-dependent manner (figure 4.2E).


A
CD11b
CD14
CD16
CD32
CD64
CD89
Unstimulated HL-60 cells (Day 0)
Max%
DMSO-stimulated HL-60 cells (Day 5)
Max%
B
SSC-A
FSC-A
CD11b+ cells
FSC-H
FSC-A
CD11b- cells
Events
CD11b-
45.2
CD11b+
54.8
CD11b
Events
beads-
54.4
beads+
45.6
Fluorescent beads
Events
beads-
91.8
beads+
8.19
Fluorescent beads
Events
Cy5-
38.9
Cy5+
61.1
Internalization probe

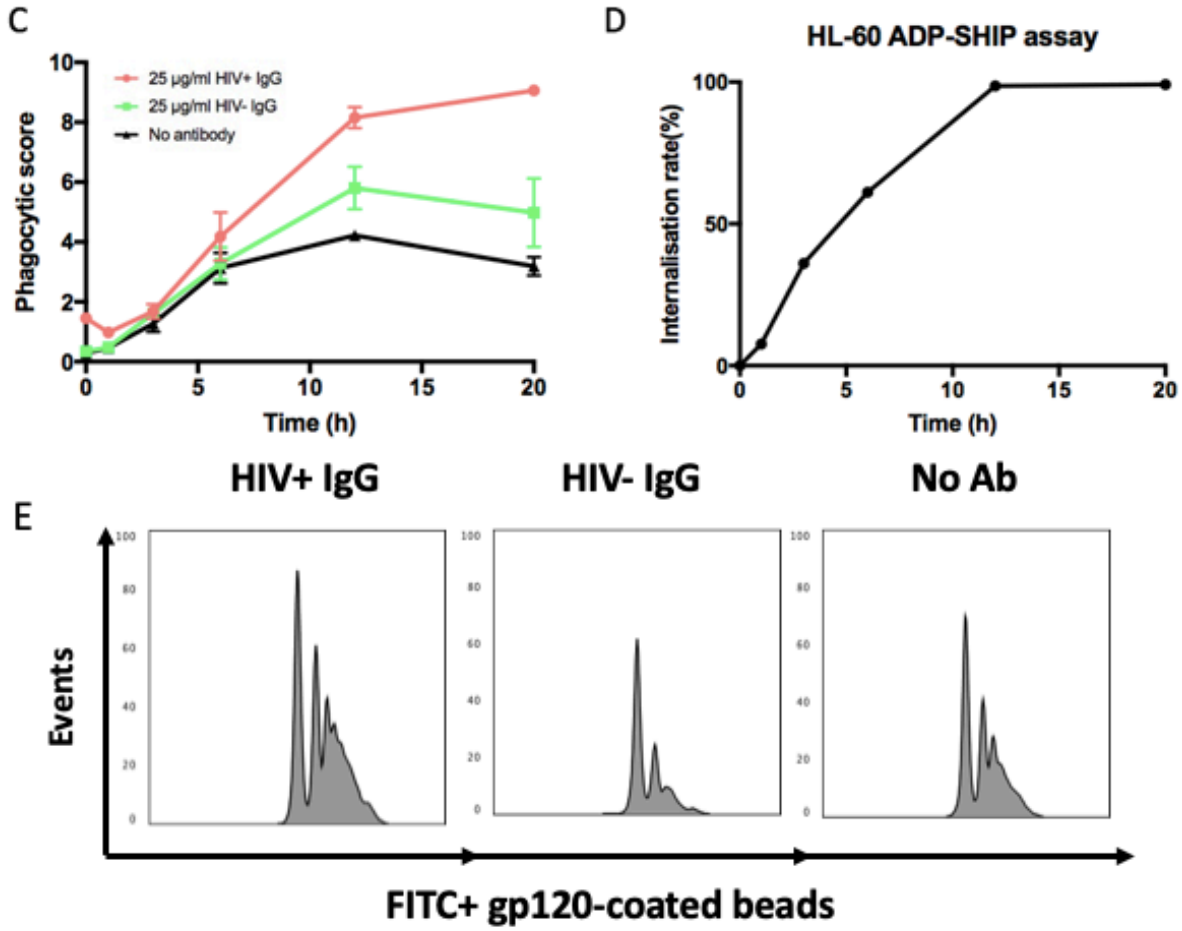

C
Phagocytic score
Time (h)
25 µg/ml HIV+ IgG
25 µg/ml HIV- IgG
No antibody
D
HL-60 ADP-SHIP assay
Internalisation rate(%)
Time (h)
E
HIV+ IgG
HIV- IgG
No Ab
Events
FITC+ gp120-coated beads

Figure 4.2 Differentiated CD11b+ HL-60 cell as a model of ADNP A. Representative flow cytometry plot for cell markers and Fc receptors profiles in HL-60 cells. D0 were unstimulated naïve HL-60 cells, and D5 were cells stimulated by 1.3% DMSO for five days. Red populations were unstained control and blue populations were stained cells. **B. Gating strategy for HL-60 ADP assay and HL-60 ADP-SHIP assay.** The first 3 panels show gating strategy on CD11b+ cells. The panel d shows the bead (FITC+) phagocytosis activity of CD11b positive HL-60s when cultured with HIVIG and gp120 coated fluorescent beads. The panel e shows the beads phagocytosis activity of CD11b negative HL-60s. The panel f shows gating on internalised beads by gating on red population which is Cy5 unstained controls. FITC+/Cy5+ events were identified as internalised beads and FITC+/Cy5- events were identified as surface-associated beads. **C. Time-course plot of HL-60 ADP assay.** The phagocytic activity was measured over time using IgG purified from HIV+ subjects (HIV+ IgG sample, 2 replicates) and from HIV- subjects (HIV- IgG sample, 3 replicates). Phagocytic score= %beads positive cells $\times$ mean fluorescence intensity/10^4. The error bars represent mean $\pm$ S. E. M. **D. Time-course plot of beads internalisation rate in HL-60 ADP-SHIP assay.** Internalisation rate%= FITC+Cy5+ cells/ FITC+ cells*100%. **E. Representative flow cytometry plots for ADP responses to HIV-positive IgG, HIV- negative IgG and in the absence of antibody.** The panels 2 and 3 showed minimum uptake of the beads without antibody or with HIV-negative IgG. Panel 1 showed beads uptake with HIV-positive IgG by HL-60 cells. Note: Panels A, B, C, D were published in Worley, **Fei** et al., *J Immunol Methods* 2018 (appendix 2).

To find the appropriate IgG concentration to do the phagocytosis assay, purified IgG from ten plasma samples from the HIV-positive ART naïve cohort were titrated in an HL-60 ADP assay with a large range of concentration (figure 4.3A). The phagocytosis activity of HL-60 cells (represented by phagocytic score) increased when the concentration of purified IgG became higher. Half-maximal effective concentration (Ec50) of IgG for the phagocytosis assay was determined at 25 μg/ml by the nonlinear curve fit using a standard process from Prism Graphpad 7.0b (figure 4.3B). Using 25 μg/ml purified IgG samples as the trigger of HL-60 ADP, a novel HL-60 cell-based antibody-dependent phagocytosis assay was developed and optimized (method 2.6).

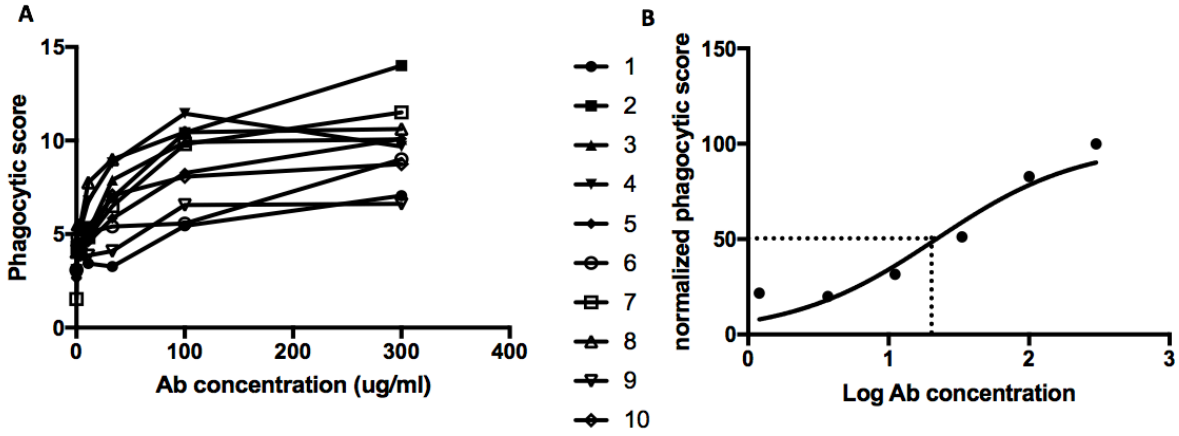


Figure 4.3. Determine Ec50 for the antibody usage in HL-60 phagocytosis assay. Antibodies (from the HIV-positive ART naïve cohort) were titrated from 300 μg/ml to 1.2 μg/ml in 10 samples, concentration was transformed by logarithm and phagocytic score was normalized (highest score set 100% and lowest score set at 0%). **(A) Titration of ten purified IgG samples in HL-60 ADP assay** The Y axis represents the phagocytosis activity and the X axis shows the concentration of the IgG samples used **(B) Nonlinear fit curve for the IgG titration** The Y axis represents the integrated normalized phagocytic score of the ten samples and the X axis shows the logarithm of the concentration of the IgG.

4.2.3 The analysis of HL-60 ADP assay in plasma from an HIV-positive cohort

With the HL-60 ADP assay developed and optimized, the assay was performed on 41 HIV-positive subjects from the HIV-positive ART naïve cohort and nine healthy donors (figure 4.4A). The ADP assay (method 2.7) using primary neutrophil was also performed on the same subjects, and ADNP data analysis can be found in the appendix (figure 6C). HL-60 ADP responses of all subjects were broadly distributed with a large dynamic range. HL-60 cells had a higher level of non-specific phagocytosis response using HIV-negative IgG from healthy donors compared to neutrophils. However, it still showed reasonable discriminative differences between HIV-positive IgG and HIV-negative IgG samples. To further validate the involvement of Fc receptor activation, the HL-60 ADP assay was correlated with a CD32 dimer binding assay (figure 4.4C). This ELISA-based assay was developed to quantify the binding of antigen-specific antibodies to the

recombinant soluble dimeric ectodomains of FcγRs, which simulates cell surface-expressed FcγIIa [181], the primary Fc receptor for ADP responses. The FcγR dimer-binding assay can reflect Fc receptor-mediated functions against HIV proteins [171]. The two assays exhibited a reasonable correlation with a spearman r of 0.6522 ($p<0.0001$).

To determine whether HL-60 cells can be a suitable model to study primary neutrophil phagocytosis functions, the ADNP and HL-60 ADP assay were compared (figure 4.4B). The two assays showed a robust positive correlation with a spearman r of 0.7718 ($p<0.0001$).

Several Fc receptor-mediated functions correlate with the control of viremia in HIV infection [182], but this has not been studied extensively using neutrophils or neutrophil-like cells. We therefore analysed the clinical implication of the HL-60 ADP assay, which can be assessed since all subjects did not receive antiretroviral therapy and all viral loads are available (figure 4.4D). The HL-60 assay exhibited a significant negative correlation with viral load with a spearman r of -0.41 ($p=0.008$), suggesting that neutrophil ADP might play a role in controlling HIV viral load. Additionally, the primary neutrophil ADP response also showed a significant inverse correlation with viral load, albeit with a slightly lower spearman correlation and higher p-value ($r =-0.32$, $p=0.043$).

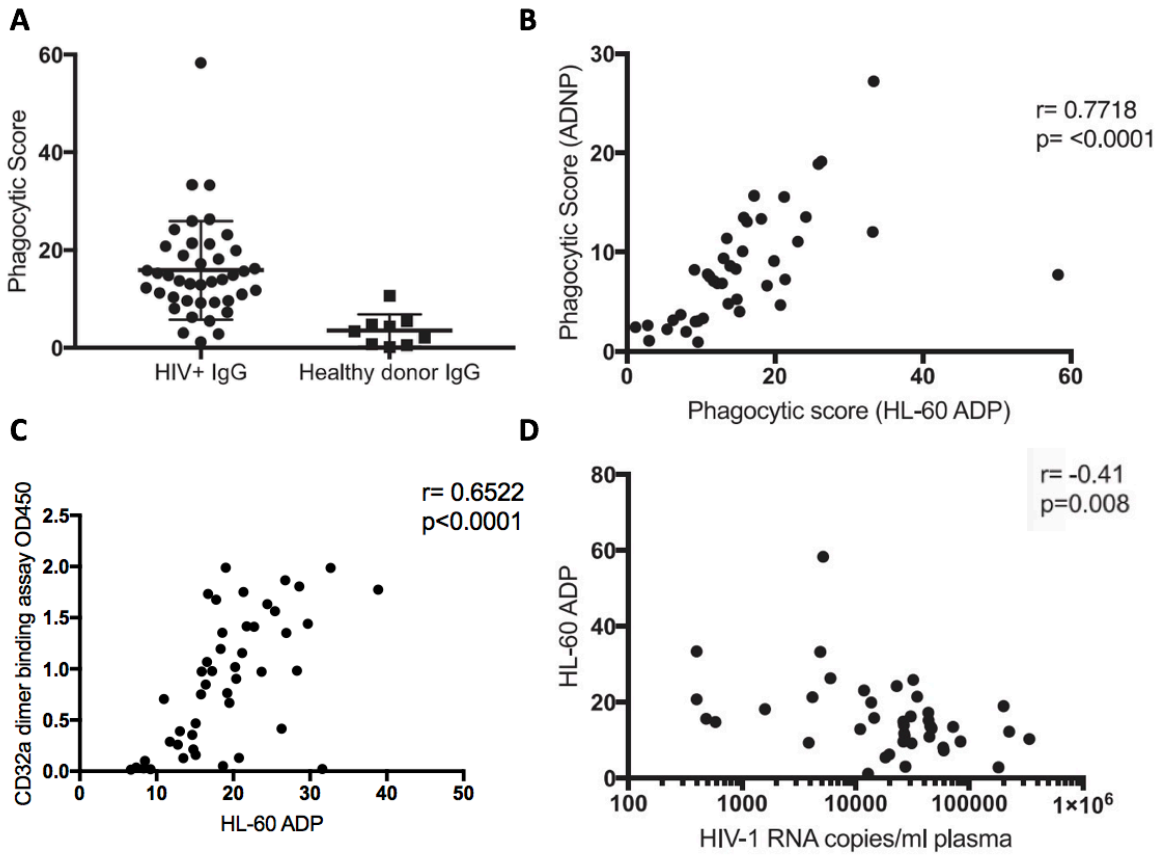


Figure 4.4. Analysis of HL-60 cells ADCP responses of the HIV-positive ART naïve cohort (A) 41 HIV-positive subjects from and 9 HIV-negative healthy donors were evaluated for ADCP responses in the HL-60 ADP assay. **(B) Correlation of HL-60 cells phagocytosis with neutrophils.** HL-60 ADP assay and neutrophil ADNP assay were performed in the HIV-positive ART naïve cohort. The two assay showed a strong significant correlation with a spearman R of 0.7718 ($p<0.0001$). **(C) Correlation of HL-60 cells phagocytosis with CD32a dimer-binding assay.** HL-60 ADP assay and CD32a dimer-binding assay were performed in the HIV-positive ART naïve cohort. The two assay showed a strong significant correlation with a spearman R of 0.6522 ($p<0.0001$). **(D) Correlation of HL-60 cells phagocytosis with viral load** The HL-60 ADCP response with correlated with viral load (HIV-1 RNA copies/ml plasma) and showed a significant negative correlation with a spearman R of -0.41 ($p=0.008$) Note: Panels A, B, C, D were published in Worley, **Fei** et al., *J Immunol Methods* 2018 (appendix 2).

4.3 Discussion

Neutrophils are crucial innate immune cells that manifest multiple functions against microbes. However, the primary neutrophils have a short half-life and die rapidly outside human blood. Since the cryopreservation would impair the functions of neutrophils [183], they must be freshly isolated for studies. Therefore, a neutrophil-like cell line provides a potentially simpler tool for neutrophil investigations if it adequately captures neutrophil-mediated functions. The HL-60 cell line has been used in some bacterial studies to assess antimicrobial functions of neutrophils [174, 175]. Here a novel high throughput HL-60 ADP assay was developed to evaluate neutrophil antibody-mediated effector functions in HIV infection. The HL-60 ADP assay exhibited a robust correlation with the primary neutrophil ADNP assay. Furthermore, the HL-60 ADP assay showed a strong correlation with CD32a dimer binding assay, indicating the HL-60 ADP works in an Fc receptor-mediated manner. These correlations suggest that HL-60 ADP assay can be a useful mean to evaluate neutrophil Fc receptor-mediated phagocytosis functions. Moreover, neutrophils can mediate ADCC and DMSO-stimulated HL-60 cells have been shown to express CD16, the primary Fc receptor to mediate ADCC. Hence, it deserves further investigation to develop an HL-60 ADCC assay to evaluate neutrophil ADCC functions.

However, there are several differences between HL-60 cells and primary neutrophils or other innate immune cells that need to be taken into account. The HL-60 cell line is a human leukaemia cell line which has distinctions in gene expression compared to neutrophils. Furthermore, it is observed that the antimicrobial activities of HL-60 cells were distinctly lower than primary neutrophils, including the production of reactive oxygen species and formation of NETs [175]. HL-60 cells have the ability to differentiate into neutrophil-like, monocyte-like, macrophage-like or eosinophil-like cells when induced by various reagents [184]. DMSO is known to induce HL-60 cells to differentiate into

neutrophil-like cells [184]. The cell subtypes are evaluated by various cell surface receptors, antigens or enzymes [184]. Admittedly, this thesis did not probe further into the differentiation of other cell subtypes or use additional surface markers. Further work is needed to study the diversity of granulocytes and monocyte/macrophages in the HL-60 model and their capacity to mediate ADCP.

Increasing evidence points out that Fc receptor-mediated functions including ADCC and ADCP contribute to HIV immunity [21, 127]. However, the role of neutrophil-mediated Fc receptor functions in HIV infection has not been fully understood. The neutrophil ADCP response in HIV-positive patients is found to decline over time even with ART, indicating the impairment of neutrophil immunity in HIV infection [185]. Additionally, the ADNP activity has been showed not to differ between elite controllers, viremic controllers, infected subjects on ART therapy and infected subjects off ART therapy [182]. Here we observed that HL-60 ADP negatively correlated with the viral load, indicating that the Fc receptor-mediated responses of neutrophils might play a part in HIV control and is worth further investigation in the future.

Similarly, there is growing evidence that Fc receptor-mediated functions play a part in influenza prevention and control. However, the role of neutrophil Fc receptor-mediated effector functions has not been well investigated due to the difficulty of using primary neutrophils. The antimicrobial functions of neutrophils have been shown to be impaired during influenza infections [186, 187]. Neutrophils are found to participate in antibody-mediated viral clearance of influenza A virus [188]. The HL-60 ADP assay can be applied into influenza research to evaluate the role of neutrophil Fc receptor-mediated functions in influenza infection, which is discussed in chapter five.

Chapter 5 Influence of allotypes on Fc-mediated antibody responses to influenza

5.1 Introduction

Antibodies are highly functional immune proteins that can mediate various Fc receptor functions, including Ab-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC). These functions are triggered by the binding of the Fc region of antibodies to Fc receptors on innate immune cells. Numerous studies suggest that antibodies that mediate Fc receptors effector functions may be important in HIV and influenza protection and control [7, 21, 51, 160, 164].

The IgG1 and IgG3 subclasses when complexed with antigens, bind avidly to all Fc receptors, thus initiating strong Fc receptor-mediated functions. There are several allelic variations (allotypes) of IgG1 in the constant region including G1m1 and G1m3 which differ among various ethnic groups. There is growing evidence that IgG1 allotypes differentially impact IgG subclass distribution, Ab-Fc receptor binding, and functions. This chapter focuses on the investigation of the influence of IgG1 allotypes on influenza vaccine-induced immune responses.

The results of this chapter were based on an influenza vaccine cohort. IgG was purified from plasma, and genomic DNA was also extracted from plasma samples, which were collected from 30 HIV-negative and 26 HIV-positive subjects at baseline and four weeks after seasonal influenza vaccination. IgG1 allotypes were determined by PCR and Sanger sequencing in table 3.1.

The IgG1 allotype analysis provided a means for regrouping the influenza vaccine cohort. The HIV-negative and HIV-positive subjects can be further divided into G1m1 homozygous subjects, G1m3 homozygous subjects, and

G1m1/G1m3 heterozygotes. Therefore, the influence of IgG1 allotypes on antibody-dependent functions can be analyzed by comparing the antibody-dependent immune responses between different allotype groups. Here we analyzed the IgG subclass levels, Fc receptor binding, ADCC and ADCP responses data (table 5.1). The IgG subclass level was assessed by the multiplex assay to influenza-specific antibodies. The Fc receptors dimer binding ELISA assay and the HL-60 ADP assay (developed in chapter four) were used to determine the capacity of influenza-specific IgG to bind FcγRIIa and FcγRIIIa and trigger Fc receptor-mediated phagocytosis. The plate-bound natural killer (NK) cell activation assay was performed to assess the ability of IgG samples to activate NK cells which reflects the ADCC activity. Our data imply that IgG1 allotypes significantly affect IgG subclass levels and influence IgG-Fc receptors interactions upon vaccination. However, ADCC and ADCP responses did not differ significantly across different allotype groups.

Table 5.1 Summary of all assays performed in chapter five

Subjects immunized with TIV	Antigen-specific IgG tested	Assays and methods
HIV-negative N=30 G1m1=4 G1m3=15 G1m1/G1m3=11	1. HA (A/Switzerland/9715293/2013) 2. HA (B/Phuket/3073/2013)	Multiplex assay for IgG subclasses (method 2.9)
	1. HA (A/Switzerland/9715293/2013) 2. Total IgG	FcγR dimer-binding assay (method 2.10)
	Total IgG	Surface plasmon resonance analysis (method 2.12)
	HA (A/Switzerland/9715293/2013)	Plate-bound natural killer (NK) cell activation assay (method 2.11)
	HA (A/Switzerland/9715293/2013)	HL-60 Antibody-dependent phagocytosis (ADP) assay (method 2.6)
HIV-positive N=26 G1m1=4 G1m3=8 G1m1/G1m3=14	1. HA (A/Switzerland/9715293/2013) 2. Gp120 (Bal)	Multiplex assay for IgG subclasses (method 2.9)
	1. HA (A/Switzerland/9715293/2013) 2. Total IgG	FcγR dimer-binding assay (method 2.10)
	Total IgG	Surface plasmon resonance analysis (method 2.12)
	HA (A/Switzerland/9715293/2013)	Plate-bound natural killer (NK) cell activation assay (method 2.11)
	HA (A/Switzerland/9715293/2013)	HL-60 Antibody-dependent phagocytosis (ADP) assay (method 2.6)

5.2 Results

5.2.1 Association of IgG1 allotypes with antigen-specific IgG subclasses levels

The sequence allotyping and ELISA allotyping results in the third chapter showed that in the influenza vaccine cohort, 23 (41.1%) participants are G1m3 homozygotes, 8 (14.3%) participants are G1m1 homozygotes, and 25 (44.6%) subjects are G1m1/G1m3 heterozygous, carrying both allotype alleles. IgG1 allotyping data allowed for the analysis of the impact of IgG1 allotypes on IgG subclass profiles of these 56 individuals. To evaluate the influence of IgG1 allotypes on IgG subclass distribution profiles, a multiplex assay was performed to determine influenza or HIV-specific IgG subclass profiles in the cohort.

For the HIV-negative subjects, the HA-specific IgG subclasses for 2 of the antigens within the administered trivalent inactivated influenza vaccine, HA (A/Switzerland/9715293/2013) and HA (B/Phuket/3073/2013), were assessed. The non-parametric multiple comparisons of Kruskal-Wallis test and Mann-Whitney test was used to compare three allotypic groups. Regarding HA (A/Switzerland)-specific IgG subclass responses (figure 5.1), we found that influenza vaccination of G1m1 homozygotes/heterozygotes induced elevated levels of HA-specific IgG1 in comparison to G1m3 homozygotes (figure 5.1A-B). In contrast, G1m3 homozygotes/heterozygotes possessed elevated levels of influenza-specific IgG3 in comparison to G1m1 homozygotes (figure 5.1G-H). Moreover, we found that G1m3 allele carriers might have the potential to increase levels of HA-specific IgG4 compared to homozygous G1m1 subjects (figure 5.1J-K). No significant differences in HA-specific IgG2 responses were observed (Figure5.1D-E).

For HA (B/Phuket)-specific IgG (figure 5.2), similarly, the data exhibited that G1m1 allele carriers had higher HA-specific IgG1 levels when compared to G1m3 homozygotes (figure 5.2A-B). Participants homozygous or heterozygous for G1m3 had a trend of inducing increased HA-specific IgG3 levels in comparison to homozygous G1m1 carriers (figure 5.2G-H). It is also observed that G1m3-carriers seemed to induce elevated HA-specific IgG4 levels compared to G1m1 homozygotes (figure5.2K). HA-specific IgG2 levels were found not to differ between different allotypes (figure5.2E-F). Moreover, G1m1/G1m3 allotypes were not found to significantly influence the IgG subclass increase in response to the influenza vaccination (figure 5.1,2C, F, I, L). All the p values of the statistical comparisons (non-parametric Mann-Whitney test) between different allotype carriers are summarized in the appendix table 7.1. Numbers of participants of the influenza vaccine cohort were not sufficient to exhibit a significant difference between G1m1 (n=8) and G1m3 (n=23) allele carriers in some situations. Therefore, all subjects that were G1m1 (or G1m3) homozygotes

or G1m1/G1m3 heterozygotes were combined as one group and compared with G1m3 (or G1m1) homozygotes (appendix table 7.1).

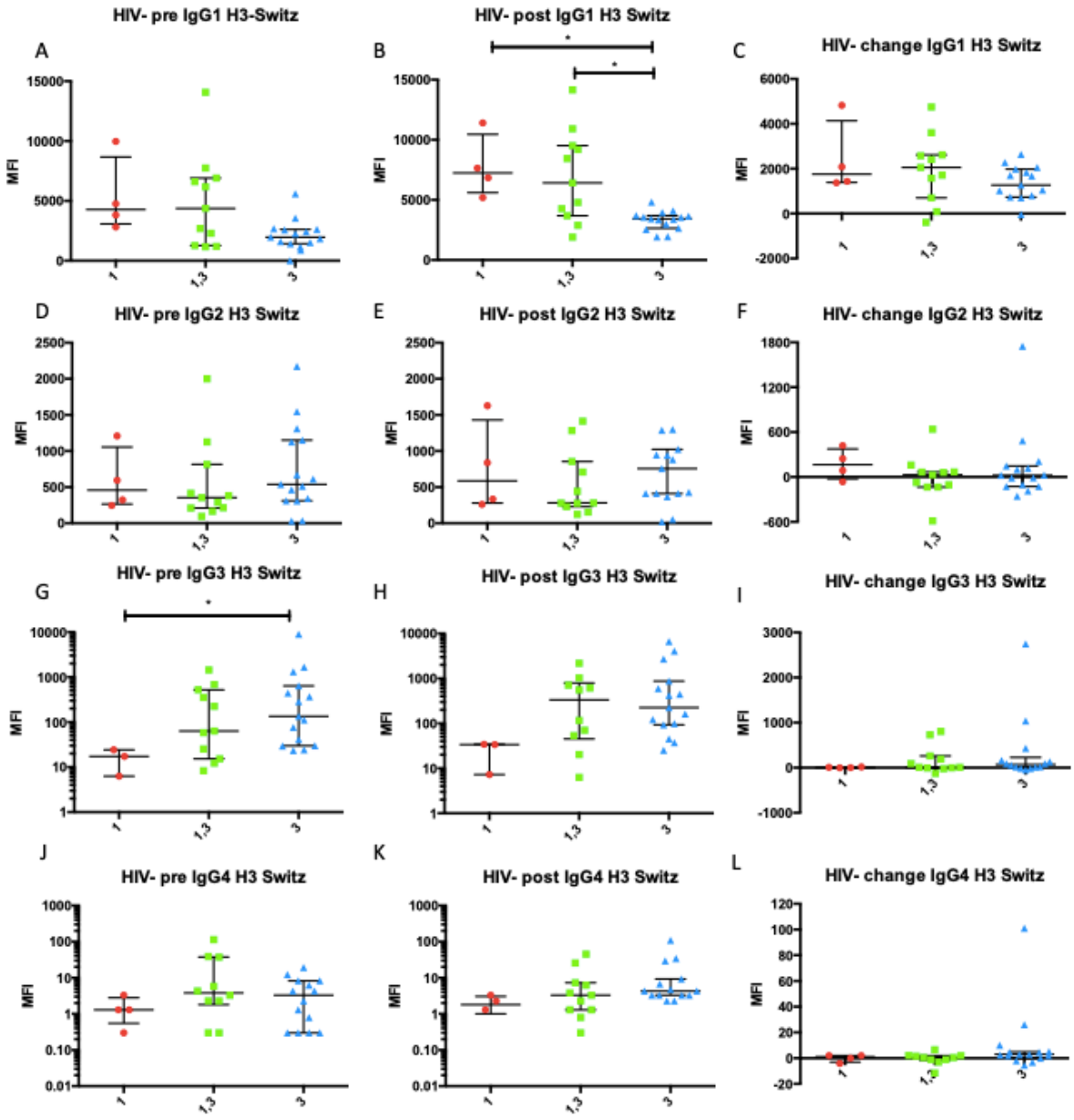


Figure 5.1 Association of HA-specific IgG subclass levels and IgG1 allotypes in HIV-negative subjects. HA (A/Switzerland/9715293/2013) specific IgG levels were measured. MFI, median fluorescence intensity of the multiplex beads assay. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Error bar: median with interquartile range. **(A-B)** Differences in HA-specific IgG1 levels at pre-vaccination and post-vaccination. **(C)** Differences in HA-specific IgG1 level changes in response to vaccination. **(D-E)** Differences in HA-specific IgG2 levels at pre-vaccination and post-vaccination. **(F)** Differences in HA-specific IgG2 level changes in response to vaccination. **(G-H)** Differences in HA-specific IgG3 levels at pre-vaccination and post-vaccination. **(I)** Differences in HA-specific IgG3 level changes in response to vaccination. **(J-K)** Differences in HA-specific IgG4 levels at pre-vaccination and post-vaccination. **(L)** Differences in HA-specific IgG4 level changes in response to vaccination. The non-parametric multiple comparisons of Kruskal-Wallis test (*p<0.05).

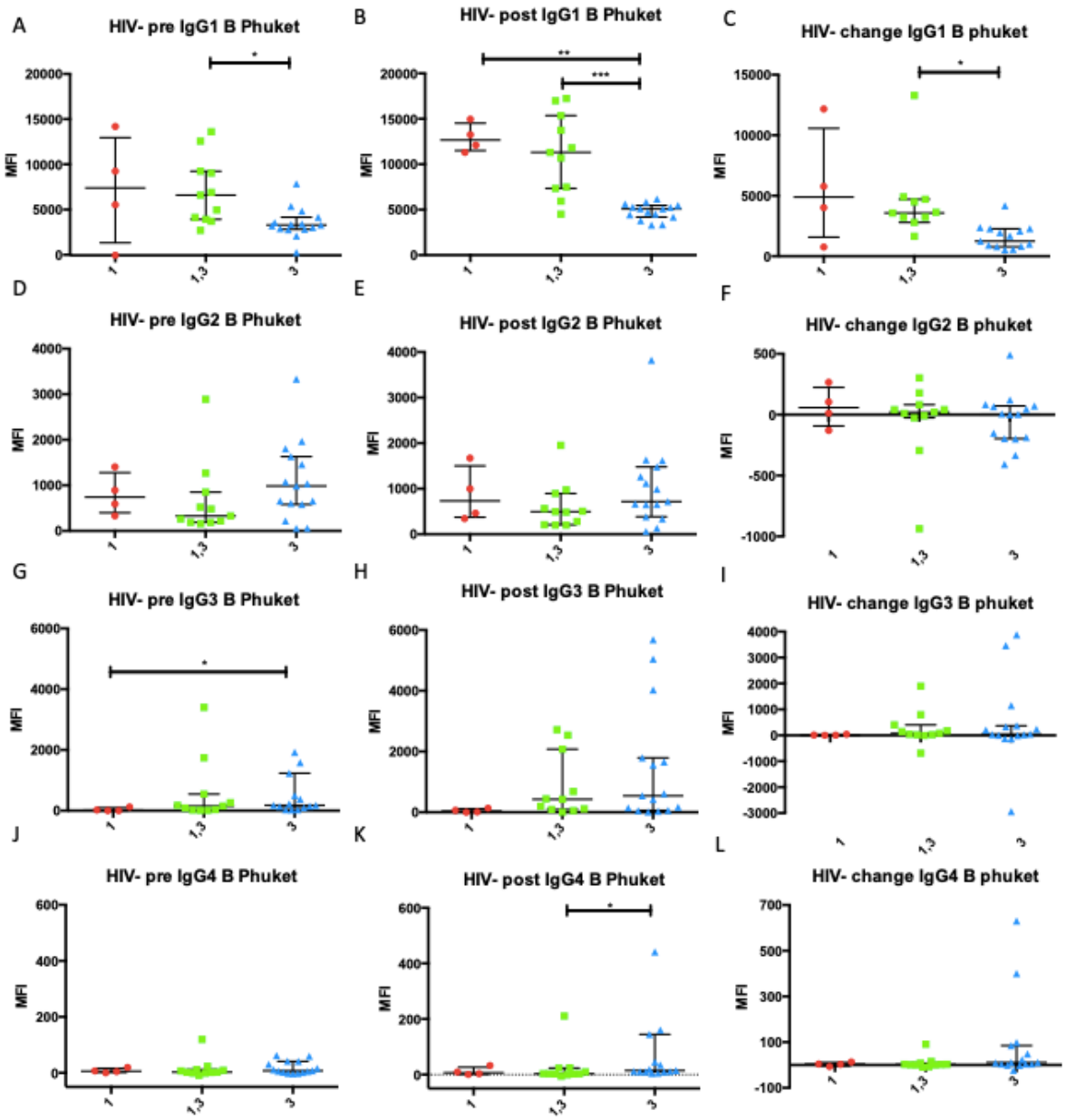


Figure 5.2 Association of influenza B-specific IgG subclass levels and IgG1 allotypes in HIV-negative subjects. HA (B/Phuket/3073/2013) specific IgG levels were measured. MFI, median fluorescence intensity of the multiplex beads assay. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Error bar: median with interquartile range. **(A-B)** Differences in influenza B-specific IgG1 levels at pre-vaccination and post-vaccination. **(C)** Differences in influenza B-specific IgG1 level changes in response to vaccination. **(D-E)** Differences in influenza B-specific IgG2 levels at pre-vaccination and post-vaccination. **(F)** Differences in influenza B-specific IgG2 level changes in response to vaccination. **(G-H)** Differences in influenza B-specific IgG3 levels at pre-vaccination and post-vaccination. **(I)** Differences in influenza B-specific IgG3 level changes in response to vaccination. **(J-K)** Differences in influenza B-specific IgG4 levels at pre-vaccination and post-vaccination. **(L)** Differences in influenza B-specific IgG4 level changes in response to vaccination. The non-parametric multiple comparisons of Kruskal-Wallis test (*p<0.05, **p<0.01, ***p<0.001).

For the HIV-positive subjects, the HA (A/Switzerland/9715293/2013)-specific IgG subclass profiles were assessed by the non-parametric multiple comparisons of the Kruskal-Wallis test. Likewise, G1m1 homozygotes/heterozygotes had increased levels of HA-specific IgG1 in comparison to G1m3 homozygotes (figure 5.3A-B). However, the HA-specific IgG3 level lost significant difference with HIV infection (figure 5.3G-H). Furthermore, no identified difference in HA-specific IgG2 or IgG4 levels was shown (figure 5.3D, E, J, K). IgG subclass level changes due to vaccination showed no significant difference with regard to different allotypes in HIV-positive subjects (figure 5.3 C, F, I, L).

To determine the influence of IgG1 allotypes in HIV infection, the HIV-1 gp120-specific IgG subclass profiles were evaluated. Interestingly, it was found that G1m3 allele carriers had elevated levels of gp120-specific IgG3 compared to G1m1 homozygotes (figure5.4E-F), while no significant difference was discovered in gp120-specific IgG1 or IgG2 levels (figure 5.4A-D). Additionally, G1m3 allele carriers seemed to induce higher levels of gp120-specific IgG4 against HIV infection in comparison to G1m1 homozygotes (figure 5.4G-H). It is noted that the gp120-specific antibody subclass responses were reproducible and did not change significantly after influenza vaccination. All the p values of the statistical comparisons (non-parametric Mann-Whitney test) between HIV-positive different allotype carriers were summarized in the appendix table 7.2.

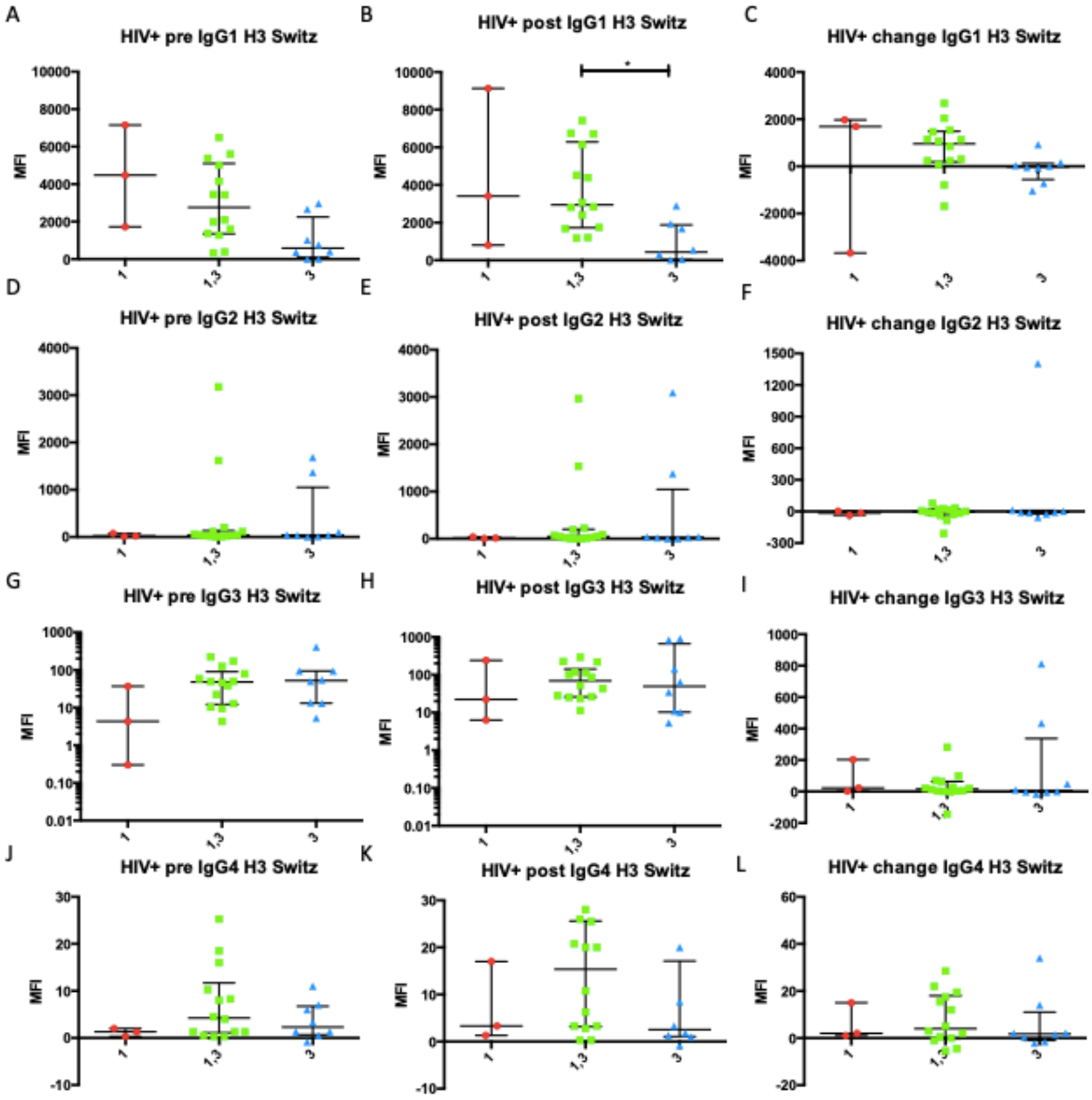


Figure 5.3 Association of HA-specific IgG subclass levels and IgG1 allotypes in HIV-positive subjects. HA (A/Switzerland/9715293/2013) specific IgG levels were measured. MFI, median fluorescence intensity of the multiplex beads assay. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Error bar: median with interquartile range. Some extremely high or low data points are off axis limit. **(A-B)** Differences in HA-specific IgG1 levels at pre-vaccination and post-vaccination. **(C)** Differences in HA-specific IgG1 level changes in response to vaccination. **(D-E)** Differences in HA-specific IgG2 levels at pre-vaccination and post-vaccination. **(F)** Differences in HA-specific IgG2 level changes in response to vaccination. **(G-H)** Differences in HA-specific IgG3 levels subjects at pre-vaccination and post-vaccination. **(I)** Differences in HA-specific IgG3 level changes in response to vaccination. **(J-K)** Differences in HA-specific IgG4 levels at pre-vaccination and post-vaccination. **(L)** Differences in HA-specific IgG4 level changes in response to vaccination. The non-parametric multiple comparisons of Kruskal-Wallis test (*p<0.05)

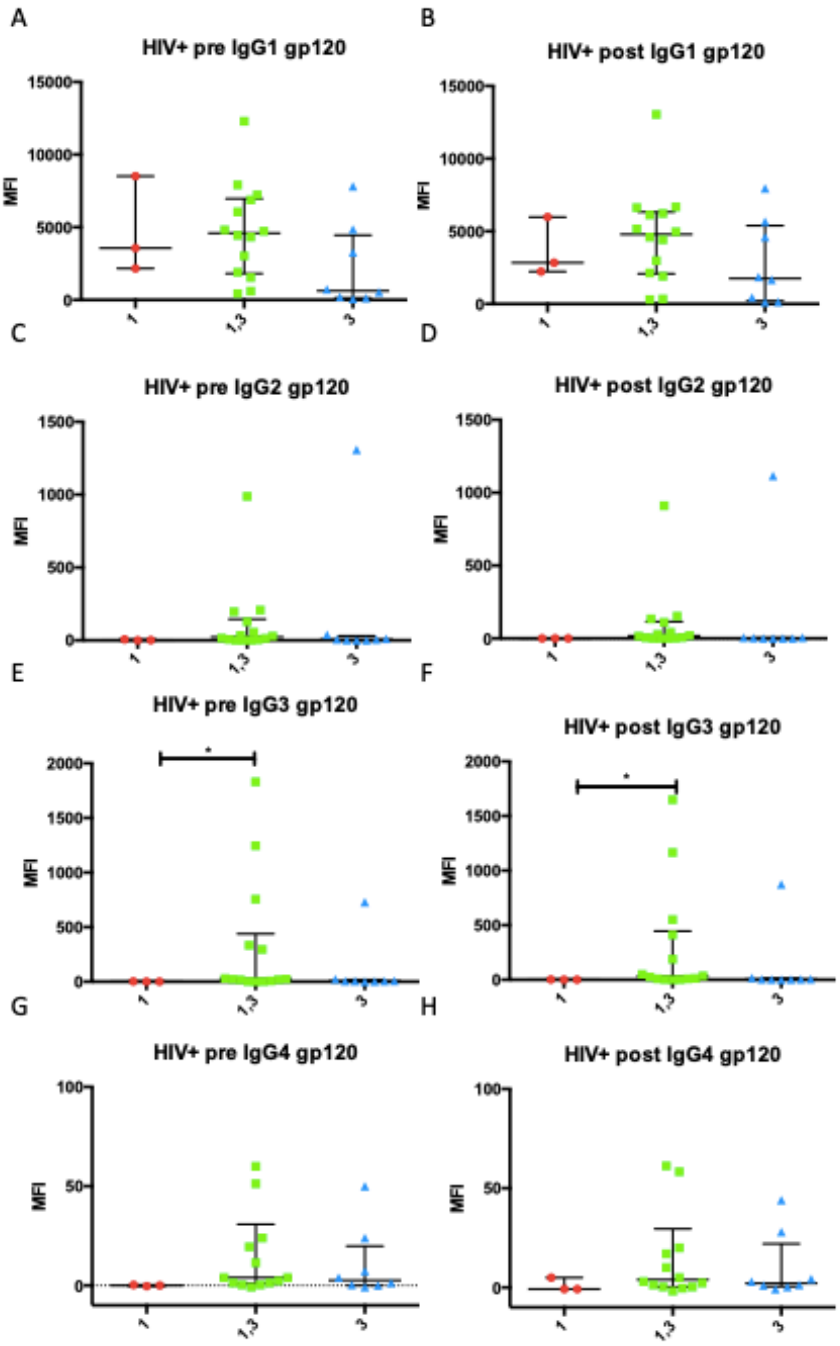


Figure 5.4 Association of HIV-specific IgG subclass levels and IgG1 allotypes in HIV-positive subjects. HIV gp120-specific IgG levels were measured. MFI, median fluorescence intensity of the multiplex beads assay. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Error bar: median with interquartile range. Some extremely high or low data points are off axis limit. **(A)** Differences in HIV-specific IgG1 levels before vaccination. **(B)** Differences in HIV-specific IgG1 levels post-vaccination. **(C)** Differences in HIV-specific IgG2 levels before vaccination. **(D)** Differences in HIV-specific IgG2 levels post-vaccination. **(E)** Differences in HIV-specific IgG3 levels before vaccination. **(F)** Differences in HIV-specific IgG3 levels post-vaccination. **(G)** Differences in HIV-specific IgG4 levels before vaccination. **(H)** Differences in HIV-specific IgG4 levels post-vaccination. The non-parametric multiple comparisons of Kruskal-Wallis test (*$p<0.05$).

5.2.2 Association of HA-specific or total IgG-Fc receptor binding with IgG1 allotypes

Due to our interest in Fc-mediated functions in HIV or influenza infection, an FcγR dimer-binding assay was applied to evaluate the impact of IgG1 allotypes on the FcγR-IgG binding. This novel ELISA-based assay was developed to quantify the binding of antigen-specific antibodies to the recombinant soluble dimeric ectodomains of FcγRs, which mimic cell surface-expressed FcγRs [181]. The FcγR dimer-binding assay can reflect Fc receptor-mediated functions against influenza proteins [159]. FcγRIIIa (CD16a), the primary Fc receptor to mediate ADCC responses, was evaluated for its binding with HA-specific or total IgG in the influenza vaccine cohort (figure 5.5). There was no significant difference in CD16a dimer binding with HA-specific IgG, whether in HIV-negative subjects or HIV-positive subjects. Interestingly, total IgG-CD16a binding was observed to be slightly higher in G1m1 homozygotes than G1m3 homozygotes (figure 5.5D). However, G1m3 homozygotes had a trend of inducing stronger binding of CD16a with total IgG in response to the vaccination (figure 5.5F), thus reaching the same binding levels with G1m1 homozygotes post-vaccination (figure 5.5E). All the p values of the statistical comparisons (non-parametric Mann-Whitney test) between different allotype carriers were summarized in the appendix table 7.3.

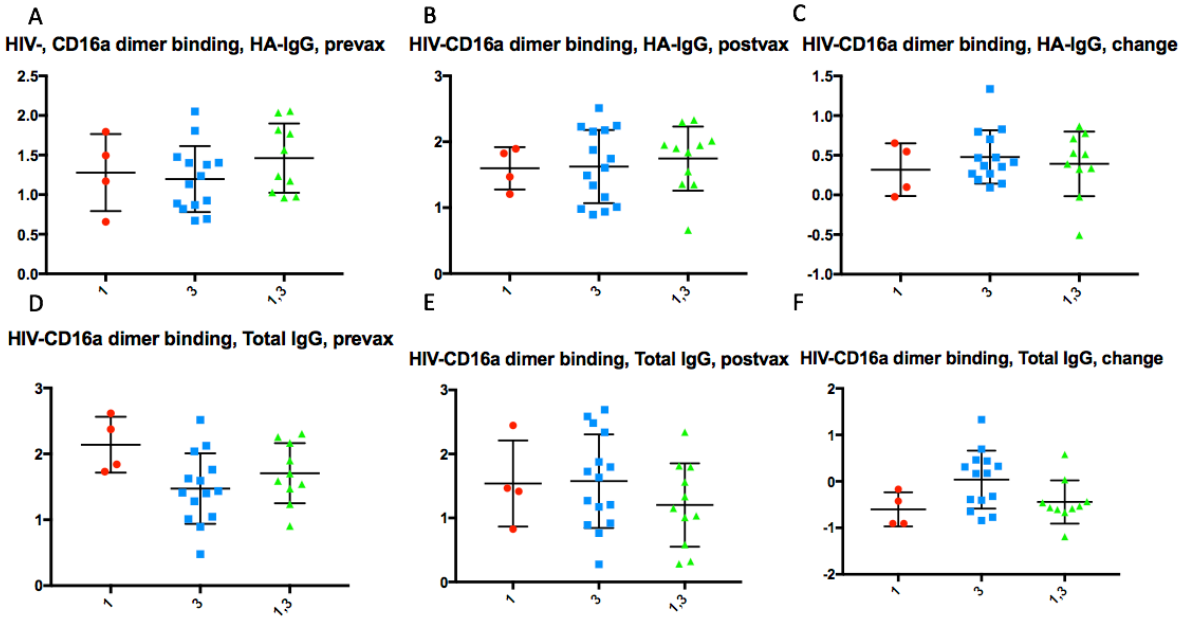


Figure 5.5 Association of HA-specific or total IgG-CD16a binding and IgG1 allotypes in HIV-negative subjects. The binding of CD16a with HA (A/Switzerland/9715293/2013)-specific IgG was measured. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Error bar: SEM. Change: post-vaccination level minus pre-vaccination level **(A)** Differences in the CD16a binding with HA-specific IgG before vaccination. **(B)** Differences in the CD16a binding with HA-specific IgG post-vaccination. **(C)** Differences in the CD16a binding change with HA-specific IgG in response to the influenza vaccine. **(D)** Differences in the CD16a binding with total IgG before vaccination. (E) Differences in the CD16a binding with total IgG post-vaccination. (F) Differences in the CD16a binding change with total IgG in response to the influenza vaccine. The non-parametric multiple comparisons of Kruskal-Wallis test.

On the other hand, FcγRIIa (CD32a), the major Fc receptor known to mediate ADCP responses, was also assessed for its binding with HA-specific or total IgG in the influenza vaccine cohort (figure 5.6). Similarly, no significant difference was identified to CD32a dimer binding with HA-specific IgG for the whole cohort. In the same way, total IgG-CD32a binding trends to be lower in G1m3 homozygotes than G1m1 allele carriers (figure 5.6D, $p<0.1$). Nevertheless, G1m3 homozygotes potentially induced higher binding of CD32a with total IgG in response to the vaccination (figure 5.6F). Therefore, G1m1 or G1m3 homozygotes had approximately the same binding levels post-vaccination (figure 5.6E). All the p values of the statistical comparisons (non-parametric Mann-

Whitney test) between different allotype carriers were summarized in the appendix table 7.4.

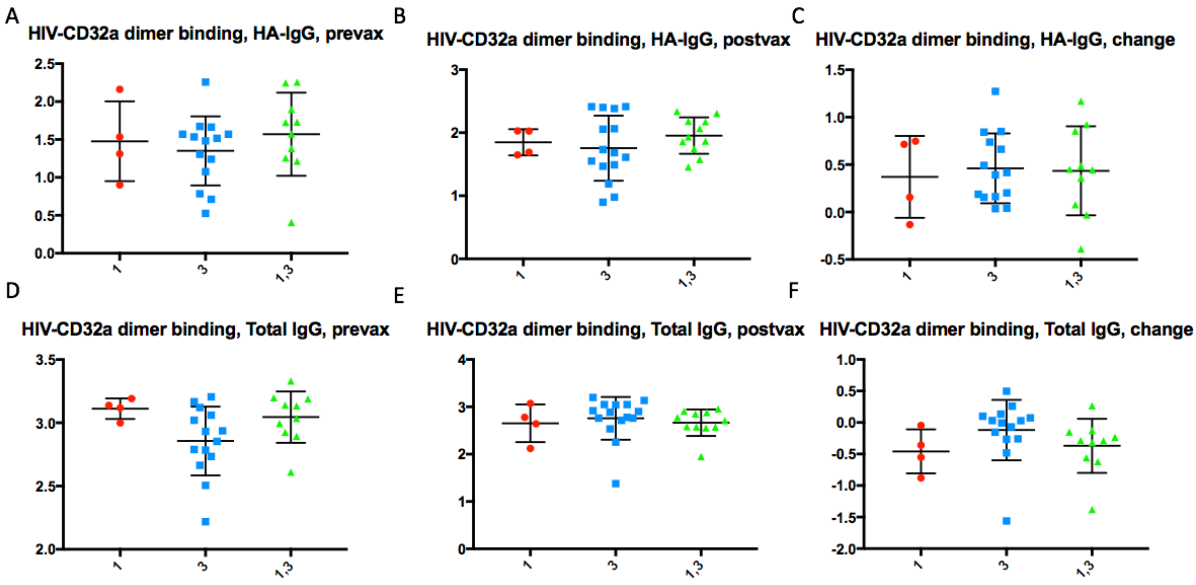


Figure 5.6 Association of HA-specific or total IgG-CD32a binding and IgG1 allotypes in HIV-negative subjects. The binding of CD32a with HA (A/Switzerland/9715293/2013)-specific IgG was measured. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Error bar: SEM. Change: post-vaccination level minus pre-vaccination level **(A)** Differences in the CD32a binding with HA-specific IgG before vaccination. **(B)** Differences in the CD32a binding with HA-specific IgG post-vaccination. **(C)** Differences in the CD32a binding change with HA-specific IgG in response to the influenza vaccine. **(D)** Differences in the CD32a binding with total IgG before vaccination. **(E)** Differences in the CD32a binding with total IgG post-vaccination. **(F)** Differences in the CD32a binding change with total IgG in response to the influenza vaccine. The non-parametric multiple comparisons of Kruskal-Wallis test.

The limitation of the Fc receptor dimer binding assay is that there is a gap between primary binding affinity and optical density of the ELISA-based assay. Higher OD does not necessarily mean higher binding affinity. Besides, the ELISA-based assay cannot provide information concerning the association and dissociation process. To obtain and compare the binding kinetics of total IgG with CD16a and CD32a, a surface plasmon resonance (SPR) assay was performed with selected samples from the influenza vaccine cohort. The following kinetic variables were determined and compared:

- Ka, the association rate constant, measures the rate of free IgG to associate with fixed CD16a or CD32a on the chips.
- Kd, the dissociation rate constant, measures the rate of bound IgG to dissociate with fixed CD16a or CD32a on the chips.
- KD, the equilibrium dissociation constant, measures the propensity of IgG to dissociate reversibly from fixed CD16a or CD32a on the chips.

Regarding the total IgG binding with CD16a in HIV-negative subjects, we found that G1m3 homozygotes showed a higher Kd compared to G1m1 homozygotes (figure 5.7C-D). However, there was no identifiable difference in Ka and KD between different allotype carriers (figure 5.7A-B, E-F). Similarly, no significant variance of Ka and KD was observed in IgG-CD32a binding in HIV-negative subjects (figure 5.8A-B, E-F). Surprisingly, G1m3 homozygotes did not been found to have a higher Kd binding to CD32a, which was observed to IgG-CD16a binding (figure 5.8C-D). Within HIV-positive subjects, no significant difference was found in any kinetic variables between different allotype groups (figure 5.9).

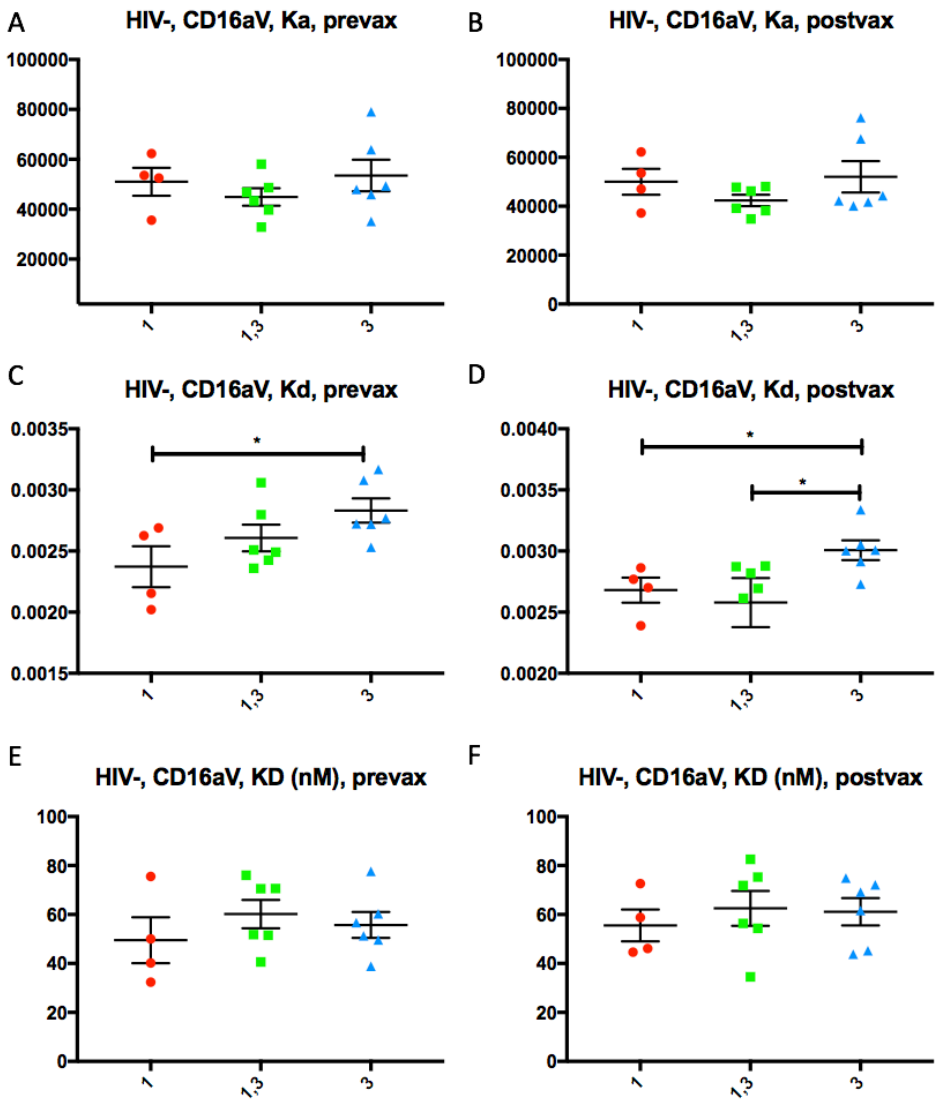


Figure 5.7 Association of total IgG-CD16a binding kinetics and IgG1 allotypes in HIV-negative subjects. The binding of CD16aV with total IgG was measured by the SPR assay. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Ka, the association rate constant. Error bar: SEM. Kd, the dissociation rate constant. KD, the equilibrium dissociation constant. **(A)** Differences in the CD16aV binding Ka with total IgG before vaccination. **(B)** Differences in the CD16aV binding Ka with total IgG post-vaccination. **(C)** Differences in the CD16aV binding Kd with total IgG before vaccination. **(D)** Differences in the CD16aV binding Kd with total IgG post-vaccination. **(E)** Differences in the CD16aV binding KD with total IgG before vaccination. **(F)** Differences in the CD16aV binding KD with total IgG post-vaccination. Non-parametric Mann-Whitney test (*$p<0.05$).

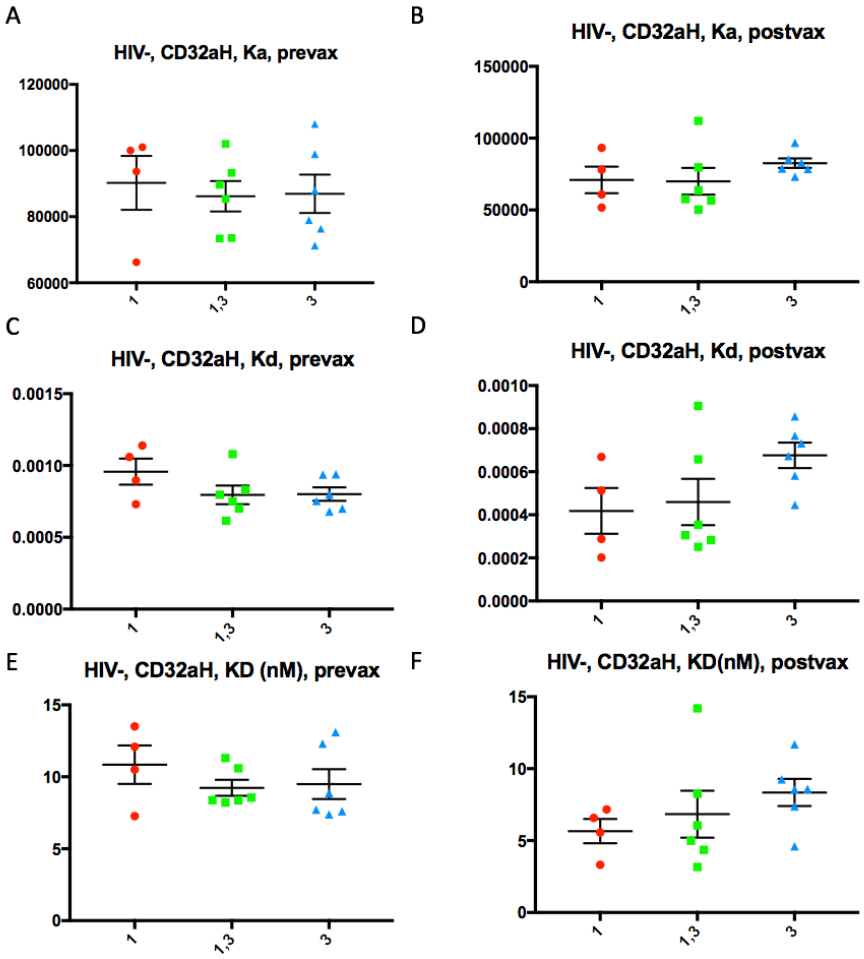


Figure 5.8 Association of total IgG-CD32a binding kinetics and IgG1 allotypes in HIV-negative subjects. The binding of CD32aH with total IgG was measured by the SPR assay. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Error bar: SEM. Ka, the association rate constant. Kd, the dissociation rate constant. KD, the equilibrium dissociation constant. **(A)** Differences in the CD32aH binding Ka with total IgG before vaccination. **(B)** Differences in the CD32aH binding Ka with total IgG post-vaccination. **(C)** Differences in the CD32aH binding Kd with total IgG before vaccination. **(D)** Differences in the CD32aH binding Kd with total IgG post-vaccination. **(E)** Differences in the CD32aH binding KD with total IgG before vaccination. **(F)** Differences in the CD32aH binding KD with total IgG post-vaccination. Non-parametric Mann-Whitney test.

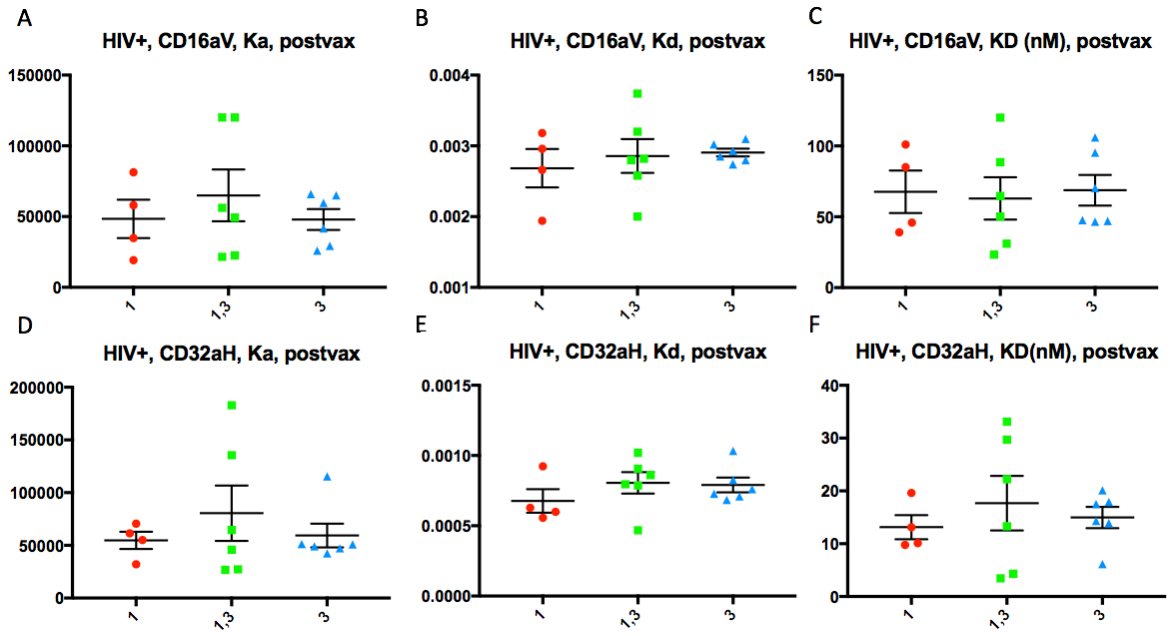


Figure 5.9 Association of total IgG-Fc receptor binding kinetics and IgG1 allotypes in HIV-positive subjects. The binding of CD16aV and CD32aH with total IgG was measured by the SPR assay. Error bar: SEM. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Ka, the association rate constant. Kd, the dissociation rate constant. KD, the equilibrium dissociation constant. **(A)** Differences in the CD16aV binding Ka with total IgG post-vaccination. **(B)** Differences in the CD16aV binding Kd with total IgG post-vaccination. **(C)** Differences in the CD16aV binding KD with total IgG post-vaccination. **(D)** Differences in the CD32aH binding Ka with total IgG post-vaccination. **(E)** Differences in the CD32aH binding Kd with total IgG post-vaccination. **(F)** Differences in the CD32aH binding KD with total IgG post-vaccination. Non-parametric Mann-Whitney test.

5.2.3 Association of NK cell activation and HL-60 ADCP response with IgG1 allotypes

Since IgG1 allotypes might affect the antigen-specific IgG subclass levels and IgG-Fc receptor binding, it is worth investigating their influence on influenza-specific ADCC and ADCP responses. An influenza-specific plate-bound natural killer (NK) cell activation assay (figure 5.10A-F) and HL-60 ADP assay (figure 5.10H-M) was applied to assess the capacity of HA-specific IgG to trigger NK cell activation and HL-60 cell phagocytosis. Disappointingly, NK cell activation and HL-60 ADCP response to vaccination were shown not to differ between G1m1 homozygotes, G1m3 homozygotes and G1m1/G1m3 alleles carriers in HIV-positive or -negative participants.

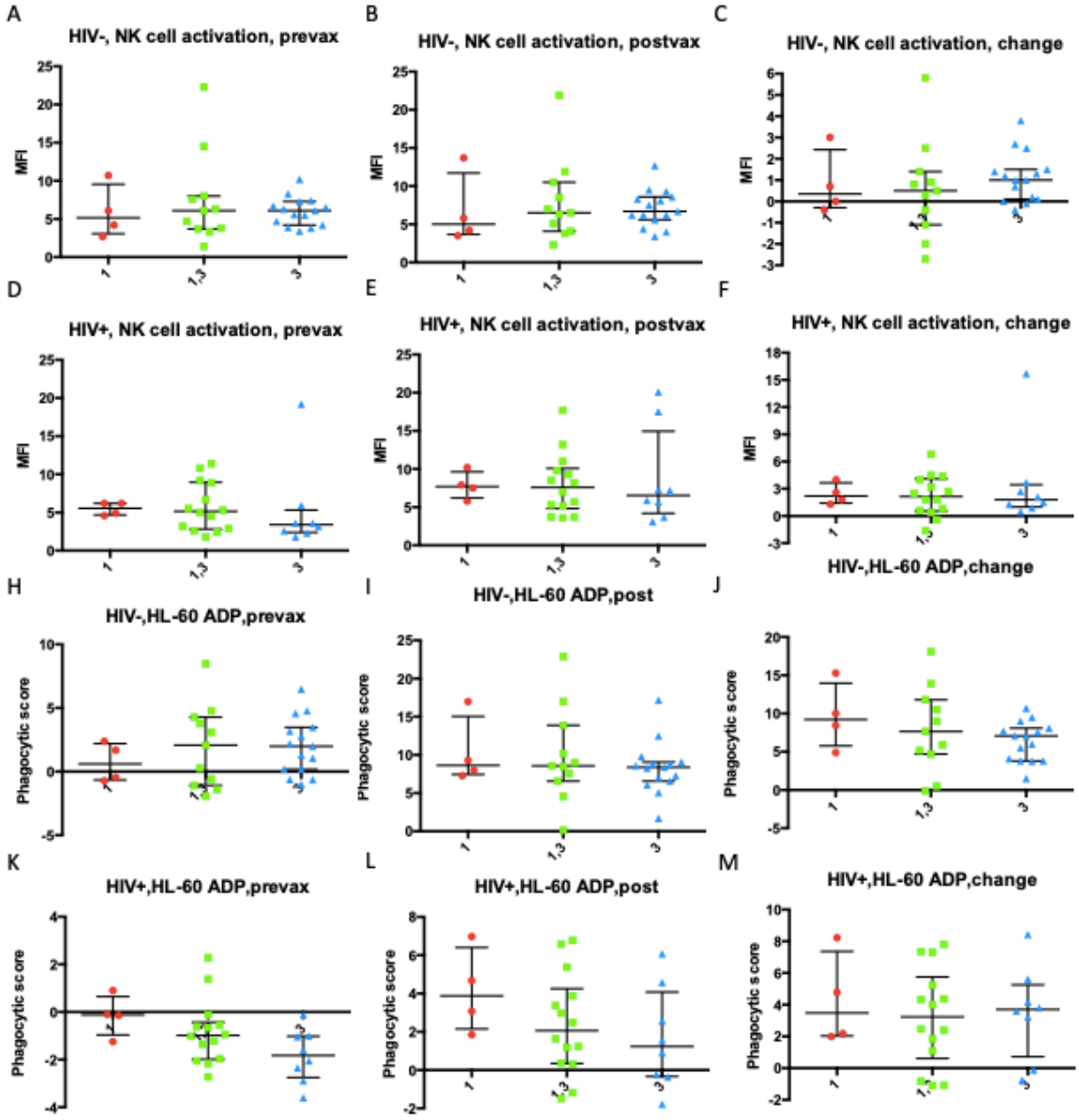


Figure 5.10 Association of NK cell activation, ADCP response and IgG1 allotypes. HA (A/Switzerland/9715293/2013) specific IgG were selected for NK cell and HL-60 cell activation. 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers. Error bar: median with interquartile range. Change: post-vaccination level minus pre-vaccination level. **(A-B)** Differences in NK cell activation in HIV-negative subjects at pre-vaccination and post-vaccination. **(C)** Differences in NK cell activation changes in HIV-negative subjects in response to vaccination. **(D-E)** Differences in NK cell activation in HIV-positive subjects at pre-vaccination and post-vaccination. **(F)** Differences in NK cell activation changes in HIV-positive subjects in response to vaccination. **(H-I)** Differences in HL-60 ADP response in HIV-negative subjects at pre-vaccination and post-vaccination. **(J)** Differences in HL-60 ADP response changes in HIV-negative subjects in response to vaccination. **(K-L)** Differences in HL-60 ADP response in HIV-positive subjects at pre-vaccination and post-vaccination. **(M)** Differences in HL-60 ADP response changes in HIV-positive subjects in response to vaccination. Non-parametric Mann-Whitney test.

5.3 Discussion

There is growing evidence that IgG allotypes are involved in immune response against certain infections. The sequencing and ELISA allotyping methods provide the opportunity to analyze the influence of IgG1 allotypes including G1m1 and G1m3 on antigen-specific IgG subclass levels, IgG-FcR binding, and Fc receptor-mediated effector functions. Concerning the relations of IgG subclass levels and IgG allotypes, previous studies have shown that: (a) Specific combinations of IgG1, IgG2, and IgG3 allotypes could result in significantly lower levels of total IgG2 or total IgG3 [189]. (b) Certain IgG2 allotypes are associated with a lower level of IgG2 and IgG4 [190, 191]. (c) The G1m1 allele was associated with higher HIV-specific IgG1 levels and decreased IgG2 levels compared to G1m3 carriers [79, 80]. Our data indicated that IgG1 homozygotes developed increased HA-specific IgG1 levels and decreased HA-specific IgG3 levels. On the contrary, G1m3 homozygotes generated higher HA-specific IgG3 levels and lower HA-specific IgG1 levels. Furthermore, G1m3 allele carriers had a trend of elevated HA-specific IgG4 levels in comparison to G1m1 homozygotes. All these conclusions were not contradictory to the previous studies but an important further supplement in the influenza-specific field and across both HIV-negative and HIV-positive subjects. Different IgG subclasses possess distinct functional profiles such as activation of Fc receptors. Therefore, the influence of IgG1 allotypes on IgG subclass levels have the potential to further affect other immune effector functions.

Although polymorphisms of Fc receptors have been shown to affect the binding affinity with IgG [30], whether the polymorphism of IgG can contribute to the binding remains underexplored. One pharmacokinetic study found that G1m1/G1m17 monoclonal antibodies bind more efficiently to FcRn compared to nG1m1/G1m3 monoclonal antibodies [86]. This thesis evaluated the binding kinetics of total IgG with CD16a and CD32a. In CD16a binding, G1m1 allele

carriers tend to have a lower Kd compared to G1m3 homozygotes. Since the IgG-Fc receptor binding was shown to be mediated by antigen-specific IgG1 levels [79, 80], these data presumably mean that G1m1 IgG1 dissociate more slowly than G1m3 IgG1when bound to CD16a. However, the same difference was not observed in CD32a binding or in HIV-positive subjects. Further investigation is required to determine the reliability of the observation in CD16a binding. KD, the equilibrium dissociation constant, was found not to differ between G1m1 and G1m3 allele carriers. Hence, the role of G1m1 and G1m3 allotype might not be decisive in IgG binding with CD16a and CD32a. CD16b which is expressed on neutrophils can exhibit different binding affinity with IgG compared with CD16a [192]. The CD16b dimer reagent was currently unavailable, but CD16b dimer binding analysis are potential future directions.

Similarly, polymorphisms of Fc receptors are known to affect the ADCC response [193], and increasing studies have shown that IgG1 allotypes might play a role in Fc receptor-mediated functions: (a) Some cancer studies found that G1m3 allotype IgG1 inhibited ADCC response of NK cells against cancer cells [194, 195]. (b) G1m3 homozygotes exhibited higher NK cell activation against Herpes Simplex Virus Type 1 compared to G1m17 homozygotes [85].

However, no significant impact was identified in NK cell activation or HL-60 phagocytosis. It is unsurprising since we did not observe convincing evidence showing that the binding affinity is different between G1m1 and G1m3 allotype carriers. Furthermore, NK cells or HL-60 cells express a broad range of Fc receptors beyond CD32a (including CD16a, CD16b, CD32b). This complexity may obscure influences of specific IgG1 allotypes. Additionally, it is known that both IgG1 and IgG3 can effectively mediate Fc receptor-mediated functions [66]. G1m1 homozygotes induce higher IgG1 levels while G1m3 homozygotes produce elevated IgG3 levels, resulting in an equalized Fc receptor-mediated functions.

Admittedly, the lack of G1m1 homozygotes could be one of the reasons why we did not observe significant difference in FcR-IgG binding and ADCC, ADCP responses. Further investigations that involve adequate numbers of G1m1 homozygous subjects are required.

Another noticeable phenomenon is that the influence of allotypes diminished in HIV-positive subjects compared to HIV-negative subjects, which is reflected in the increased p-value of the same comparison condition. The IgG glycosylation is known to regulate the biological activity of IgG, including Fc receptor-mediated functions [196]. The antibody glycosylation is greatly altered in the inflammatory diseases such as HIV infection [197]. The IgG glycosylation can dramatically alter the IgG functional profiles, which may mask the comparatively weak influence of IgG1 allotypes. Moreover, a direct comparison of the levels of HA-specific antibodies elicited in HIV-positive and HIV-negative subjects with different allotypes would be interesting, which can be important future analyses to conduct. It reveals the impact of HIV infection on the IgG subclass response to influenza. The analysis is not included here since it separates from the focus of this thesis which is the influence of IgG1 allotypes on IgG subclass levels.

In conclusion, we found that IgG1 allotypes G1m1 and G1m3 significantly affected HA-specific IgG subclass levels in response to influenza vaccination. G1m1 allele carriers tend to trigger higher HA-specific IgG1 responses, while G1m3 allele carriers can induce elevated levels of HA-specific IgG3. However, no convincing functional assay difference regarding ADCC and ADCP was found, as well as FcR-IgG binding, indicating the complexity of allotypic influence on Fc receptor-mediated functions.

Chapter 6 General discussion

This master thesis primarily investigates two related projects: (1) A novel HL-60 cell line-based assay to measure HIV or influenza-specific neutrophil ADCP response was developed and optimized. The neutrophil ADCP responses in an HIV-positive cohort was evaluated using this assay. (2) The development of an ELISA-based assay to determine IgG1 allotypes. The influence of IgG1 allotypes on Fc receptor-mediated functions (including the HL-60 neutrophil ADCP) was assessed in an influenza vaccine cohort based on allotype results. The general discussions will examine my findings and integrate them with the previous studies on the subject and suggest future directions for research in this field.

6.1 Methods in IgG polymorphism detection: ELISA and Sequencing

Although newer and more elaborate methods for identifying antibody allotypes are continuously developed, the two most common methods for routine use in laboratories are still the ELISA and the PCR, sequencing procedure. The benefits of these two methods are apparent: (1) They are straightforward to perform even with new users. (2) They are accessible to most labs even in resource-poor regions. (3) They have good sensitivity and specificity, and are highly repeatable in a standard manner. (4) They are both high-throughput methods that can screen large numbers of samples in a 96 well plate format.

The production of monoclonal antibodies against specific IgG allotypes by mouse hybridomas has made it possible to utilize ELISA for a determination of IgG allotypes [198, 199], and most previous studies on IgG allotypes applied

ELISA techniques [200-202]. With the wider accessibility of the sequencing technologies, the allotype genotyping became lower-cost and time-saving. To obtain more accurate information across a wider range of allotypes, the field is now more inclined to use sequencing to determine IgG allotypes [85, 86, 169]. Besides, some rare allotypic mutations can only be determined by sequencing. However, the advantages of the serological-based ELISA should not be neglected. For an inexperienced user in the developing world, the ELISA is a simple process which costs less time and less money. Moreover, the ELISA is the only eligible option when stored cells or DNA are unavailable.

The ELISA and PCR techniques for G1m1 and G1m3 allotypes were validated in this thesis. Nonetheless, the ELISA or the sequencing method that this thesis used is only capable of identifying one single allotype. A multiplex-based method is worth developing in the future which is beneficial when large numbers of samples need to be examined for multiple allotypes [203]. In the multiplex assay, allotype-specific antibodies are coated onto fluorescent microspheres. The results can be measured by flow cytometry by the fluorescent signature of these beads. The different allotypes can be determined by the number of different fluorescent colors [204, 205]. This multiplex immunoassay provides a powerful and effective mean for wider IgG allotype determinations. However, the simpler ELISA might still be useful in low-resource clinical settings.

6.2 Role of ADCP in HIV infection

Antibody responses against HIV are generated early after infection [124]. There is growing evidence illustrating the importance of humoral immunity against HIV. Neutralizing antibodies can protect nonhuman primates from SHIV challenge [206, 207], and is thought to partially protect human from HIV-1 infection [208] and suppress viral load in HIV-infected subjects [209]. Though developing a vaccine to trigger a broadly neutralizing antibody response is believed to be one

means for HIV prevention, eliciting potent and broadly neutralizing antibodies has yet to be achieved [210, 211].

Non-neutralizing antibody-mediated immune functions including ADCC and ADCP offers another approach to prevent and control HIV infection [21]. The role of ADCC responses has already been supported by numerous studies where ADCC activities correlated with clinical outcome in human or nonhuman studies [21, 128, 167, 212, 213]. Comparatively, little has been done on the role of ADCP response in combatting HIV infection. In a monkey vaccine trial against SHIV-SF162P3 challenge, ADCP responses were found to correlate with vaccine protection [35], leading to the recent initiation of an HIV human efficacy trial [214]. Enhanced ADCP activities are also observed early in HIV infection, although whether ADCP responses increased in the RV144 trial is still controversial [38, 215].

Our study showed that ADCP-antibodies against HIV envelope proteins were present in HIV-positive subjects and are able to mediate ADCP responses via a neutrophil-like cell line (HL-60 cells matured with DMSO). Importantly, we found that neutrophil-like HL-60 cells mediated HIV-specific ADCP responses which correlated with reduced HIV viral loads in a cohort of 41 subjects not on ART. This provides insight into the role of ADCP activities in HIV infection and future HIV vaccine development. New vaccine strategies and regimens that can trigger ADCP responses should be considered due to their potential to control viral load. The probable mechanism of viral load control is that ADCP can eradicate latently infected cells, since macrophages and neutrophil are more abundantly distributed in gut and lymph nodes where the HIV reservoir exists [51, 216]. Moreover, neutrophils are present in the rectum and the female genital tract which are potential sites of transmission, therefore ADCP might potentially prevent HIV transmission [51].

Admittedly, there are limitations of Fc receptor-mediated functions against viral infections. Antibody-dependent enhancement (ADE) can enhance virus

infection of host cells where the interaction of virus-antibody complex and surface Fc receptors facilitates the viral entry [217]. This particular phenomenon has also been observed in HIV infection [218, 219].

6.3 Role of neutrophils in viral infection

As the most abundant circulating leukocyte population, neutrophils are considered as essential effector cells in the innate immune system [41]. The most well-known weapons to eliminate microbes wielded by neutrophils are degranulation in which neutrophils release antimicrobial molecules from secretory granules, and production of Reactive Oxygen Species (ROS) in the respiratory burst [220]. Another antimicrobial mechanism of neutrophils has been discovered recently in which neutrophils released decondensed chromatin decorated with defensins and proteases called NETs to trap microorganisms [45]. NETs formation is induced during HIV infection and can facilitate HIV elimination [221]. However, NETs might not be important for immunity against influenza infection [222]. Overall, the role of NETs in antiviral responses are not clearly understood and require further attention.

Neutrophils express FcγRI, FcγRII, and FcγRIII, which makes Fc receptor-mediated function as another potential mechanism against viral infection [51]. Little is shown so far on the role of neutrophil Fc receptor-mediated functions in HIV or influenza infection. Our study showed that neutrophils could mediate ADCC and ADCP responses against HIV infection. Moreover, these neutrophil-mediated effector functions might contribute to viremia control. A neutrophil-like HL-60 cell line is used to evaluate the neutrophil-mediated ADCP response in a high-throughput manner. HL-60 cell line can potentially be used to evaluate ADCC responses and these functions in other viral infection in the future. Our study provided important information and useful tool in the exploration of the role of neutrophil in viral infection.

However, neutrophils are not always beneficial against viral infection. For instance, NETs release chromatin and neutrophil component proteins to induce autoantibodies, which results in autoimmune diseases such as systemic lupus erythematosus [223, 224]. Furthermore, neutrophils communicate with other immune cells by releasing cytokines, which sometimes cause inhibition[220]. Some studies suggest that neutrophils expressing high levels of PD-L1 in chronic HIV-1 individuals suppress T cell function [225]. We speculate that dysregulated activation of neutrophils may also have potentially detrimental effects. Further research studying antibody-mediated functions of neutrophils and neutrophil activation status during HIV infection is warranted.

6.4 Influence of IgG profiles on Fc receptor functions

Since the importance of Fc receptor-mediated functions has been explored, there is growing interest in what factors affect the magnitude of these effector functions. On the one hand, numerous genetic variations are present in Fc receptors [22]. The Fc receptor polymorphism is shown to influence Fc receptor-mediated functions like ADCC [194, 226, 227]. On the other hand, the IgG Fc fragment also exhibits variations including glycosylation variations and genetic polymorphisms. The N297-glycans attached to the Fc fragment varies between antibodies and is known to modulate Fc receptor-mediated functions [228, 229]. There are numerous IgG allotypes in different IgG subclasses, but the evidence of their role on ADCC and ADCP is limited. Some studies found IgG allotypes can alter ADCC responses against cancer cells and HSV infection [85, 194, 195]. One pharmacokinetic study showed that G1m1,17 monoclonal antibodies bind more efficiently to FcRn than G1m3,-1 monoclonal antibodies [86]. Neutrophils express a range of Fc receptors, including FcγRIIa and FcRn which are involved in neutrophil ADCP activity [230]. Therefore, IgG1 allotypes potentially affect neutrophil phagocytosis. However, we observed no significant difference in HL-60 ADP responses between different allotype carriers, which requires further

validation. Multiple allotype combinations impact the susceptibility to certain infections such as malaria [231, 232], suggesting it may be important to explore other allotypes apart from G1m1 and G1m3. However, few have been looking into the role of IgG allotypes in influenza infection.

Our study has shown that G1m1 allele carriers induced higher levels of influenza-specific IgG1 while G1m3 allele carriers had elevated levels of influenza-specific IgG3. However, we found no convincing evidence that G1m1/G1m3 allotypes modulated Fc-IgG binding, ADCC or ADCP responses. The possible reasons are: (1) IgG1 and IgG3 are both effective in mediating ADCC or ADCP responses. Higher IgG1 levels or higher IgG3 levels can produce similar FcR-mediated activity. (2) The lack of numbers of subjects weakens the statistical comparison, especially G1m1 homozygous subjects. (3) Other factors such as IgG glycosylation conceal the influence of allotypes.

There are several directions for further work: (1) Investigate the influence of allotypes in more subjects, preferably a more ethnically diverse population. (2) Investigate the influence of additional allotypes. (3) Find out if this is an influenza-specific phenomenon or more widely applicable to other viral infections such as HIV.

The influence of IgG profiles provides implications for vaccine development and antibody engineering. IgG allotypes are differentially distributed among various ethnic groups. Africans generally do not carry G1m3 alleles, while both G1m1 and G1m3 are common amongst Asians and Caucasians [72, 78]. Various subclasses play a different role in immunity against viral infection. The partially protective HIV RV144 vaccine trial was conducted in Asia and correlated HIV-specific IgG3 responses with vaccine efficacy [131]. This might result from a higher HIV-specific IgG3 level induced by G1m3 allele carriers. Speculatively, the vaccine might not be effective in Africa where G1m1 homozygotes are prevalent who cannot induce adequate HIV-specific IgG3. This should be taken into consideration when developing an HIV vaccine for Africans. Similarly, Fc-

engineered therapeutic antibodies should consider the type of glycosylation and allotypes that provides better immune responses.

6.5 Concluding remarks

HIV and influenza infection are two important threats to public health and economy. Many studies highlight the role of FcR-mediated functions in viral protection, which is investigated in the thesis. A robust cell line-based assay was developed and validated to measure neutrophil-mediated ADCP responses in HIV infection. Here we show that neutrophils potentially mediate ADCP during HIV infection and contribute to viral load control. Furthermore, this study highlights the importance of IgG allotypes in the response to Influenza vaccination, in which they related to antigen-specific IgG subclass levels. An ELISA-based method to determine IgG1 allotypes was also developed and validated. Overall, this thesis has future implications for the understanding of immune responses and the development of global vaccinations against infectious diseases including HIV and influenza.

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Appendix

1. Summary table of Statistics in chapter five

Table 7.1 Statistical summary of the difference in antigen-specific IgG subclass distribution between different IgG1 allotypes subjects in the HIV-negative group

P value (Mann-Whitney test)	1 vs 3	1 vs 1,3	1,3 vs 3	1+1,3 vs 3	1 vs 1,3+3
HIV- pre IgG1 H3 Switz	0.0062 Median 1: 4287 Median 3: 1974	ns	ns	0.0075 Median 1+1,3: 4363 Median 3: 1974	ns
HIV- pre IgG1 B Phuket	ns	ns	0.0045 Median 1,3: 6596 Median 3: 3298	0.0043 Median 1+1,3:6596 Median 3: 3298	ns
HIV- pre IgG2 H3 Switz	ns	ns	ns	ns	ns
HIV- pre IgG2 B Phuket	ns	ns	ns	ns	ns
HIV- pre IgG3 H3 Switz	0.0013 Median 1: 11.8 Median 3: 134.3	0.0396 Mean 1: 11.8 Mean 1,3: 63.3	ns	ns	0.0029 Median 1:11.8 Median 1,3+3: 123.1
HIV- pre IgG3 B Phuket	0.0062 Median 1: 13 Median 3: 178.5	ns	ns	ns	0.0088 Median 1:13 Median 1,3+3: 178
HIV- pre IgG4 H3 Switz	ns	ns	ns	ns	ns
HIV- pre IgG4 B Phuket	ns	ns	ns	ns	ns
HIV- post IgG1 H3 Switz	0.0005 Median 1: 7243 Median 3: 3436	ns	0.0077 Median 1,3: 6421 Median 3: 3436	0.0005 Median 1+1,3: 6842 Median 3: 3436	0.0315 Median 1:7243 Median 1,3+3: 3661
HIV- post IgG1 B Phuket	0.0005 Median 1: 12682 Median 3: 5089	ns	<0.0001 Median 1,3: 11303 Median 3: 5089	<0.0001 Median 1+1,3: 11801 Median 3: 5089	0.0262 Median 1:12682 Median 1,3+3: 5556
HIV- post IgG2 H3 Switz	ns	ns	ns	ns	ns
HIV- post IgG2 B Phuket	ns	ns	ns	ns	ns
HIV- post IgG3 H3 Switz	0.0021 Median 1: 20.55 Median 3: 224.3	ns	ns	ns	0.0088 Median 1:20.55 Median 1,3+3: 193.1
HIV- post IgG3 B Phuket	0.0206 Median 1: 37.5 Median 3: 544.5	ns	ns	ns	0.0181 Median 1:37.5 Median 1,3+3: 433
HIV- post IgG4 H3 Switz	0.0307 Median 1: 1.8 Median 3: 4.3	ns	ns	ns	ns
HIV- post IgG4 B Phuket	ns	ns	0.0103 Median 1,3: 2.6 Median 3: 14.6	0.0041 Median 1+1,3: 3.6 Median 3: 14.6	ns

- Antigens-specific IgG: HA of A/Switzerland/9715293/2013 and B/Phuket/3073/2013
- 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers
- Pre: pre-vaccination, post: post-vaccination
- Non-parametric Mann-Whitney test. Ns, not significant ($p>0.05$)

Table 7.2 Statistical summary of the difference in antigen-specific IgG subclass distribution between different IgG1 allotypes subjects in the HIV-positive group

P value (Mann-Whitney test)	1 vs 3	1 vs 1,3	1,3 vs 3	1+1,3 vs 3	1 vs 1,3+3
HIV+ pre IgG1 H3 Switz	0.0485 Median 1: 4482 Median 3: 584.7	ns	0.0159 Median 1,3: 2765 Median 3: 584.7	0.0074 Median 1+1,3: 3429 Median 3: 584.7	ns
HIV+ pre IgG1 gp120	ns	ns	ns	0.0495 Median 1+1,3: 4445 Median 3: 636.5	ns
HIV+ pre IgG2 H3 Switz	ns	ns	ns	ns	ns
HIV+ pre IgG2 gp120	ns	ns	ns	ns	ns
HIV+ pre IgG3 H3 Switz	ns	0.05 Median 1: 4.3 Median 1,3: 47.8	ns	ns	0.0374 Median 1:4.3 Median 1,3+3: 49.65
HIV+ pre IgG3 gp120	ns	0.0309 Median 1: 0 Median 1,3: 23.45	ns	ns	0.0257 Median 1:0 Median 1,3+3: 18.2
HIV+ pre IgG4 H3 Switz	ns	ns	ns	ns	ns
HIV+ pre IgG4 gp120	ns	0.0294 Median 1: 0 Median 1,3: 4	ns	ns	0.0426 Median 1:0 Median 1,3+3: 4
HIV+ post IgG1 H3 Switz	ns	ns	0.0029 Median 1,3: 2948 Median 3: 437	0.0019 Median 1+1,3: 3060 Median 3: 437	ns
HIV+ post IgG1 gp120	ns	ns	ns	ns	ns
HIV+ post IgG2 H3 Switz	ns	ns	ns	ns	ns
HIV+ post IgG2 gp120	ns	ns	ns	ns	ns
HIV+ post IgG3 H3 Switz	ns	ns	ns	ns	ns
HIV+ post IgG3 gp120	ns	0.0309 Median 1: 0 Median 1,3: 30.75	ns	ns	0.03 Median 1: 0 Median 1,3+3: 11
HIV+ post IgG4 H3 Switz	ns	ns	ns	ns	ns
HIV+ post IgG4 gp120	ns	ns	ns	ns	ns

- Antigens-specific IgG: HA of A/Switzerland/9715293/2013 and $gp120_{Bal}$ of HIV-1
- Pre: pre-vaccination, post: post-vaccination
- 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers
- Non-parametric Mann-Whitney test. Ns, not significant ($p>0.05$)

Table 7.3 Statistical summary of the difference in CD16a binding with antigen-specific or total IgG between different IgG1 allotypes subjects

P value (Mann-Whitney test)	1 vs 3	1 vs 1,3	1,3 vs 3	1+1,3 vs 3	1 vs 1,3+3
HIV-, HA-specific IgG-CD16A dimer binding pre	ns	ns	ns	ns	ns
HIV-, HA-specific IgG-CD16A dimer binding post	ns	ns	ns	ns	ns
HIV-, HA-specific IgG-CD16A dimer binding change	ns	ns	ns	ns	ns
HIV+, HA-specific IgG-CD16A dimer binding pre	ns	ns	ns	ns	ns
HIV+, HA-specific IgG-CD16A dimer binding post	ns	ns	ns	ns	ns
HIV+, HA-specific IgG-CD16A dimer binding change	ns	ns	ns	ns	ns
HIV-, total IgG-CD16A dimer binding pre	0.0346 Median 1: 2.107 Median 3: 1.424	ns	ns	ns	0.0351 Median 1: 2.107 Median 1,3+3: 1.564
HIV-, total IgG-CD16A dimer binding post	ns	ns	ns	ns	ns
HIV-, total IgG-CD16A dimer binding change	0.0464 Median 1: -0.665 Median 3: 0.172	ns	ns	0.0186 Median 1+1,3: -0.244 Median 3: 0.172	ns

Table 7.4 Statistical summary of the difference in CD32a binding with antigen-specific or total IgG between different IgG1 allotypes subjects

P value (Mann-Whitney test)	1 vs 3	1 vs 1,3	1,3 vs 3	1+1,3 vs 3	1 vs 1,3+3
HIV-, HA-specific IgG-CD32A dimer binding pre	ns	ns	ns	ns	ns
HIV-, HA-specific IgG-CD32A dimer binding post	ns	ns	ns	ns	ns
HIV-, HA-specific IgG-CD32A dimer binding change	ns	ns	ns	ns	ns
HIV+, HA-specific IgG-CD32A dimer binding pre	ns	ns	ns	ns	ns
HIV+, HA-specific IgG-CD32A dimer binding post	ns	ns	ns	ns	ns
HIV+, HA-specific IgG-CD32A dimer binding change	ns	ns	ns	ns	ns
HIV-, total IgG-CD32A dimer binding pre	ns	ns	ns	0.035 Median 1+1,3:3.127 Median 3: 2.893	ns
HIV-, total IgG-CD32A dimer binding post	ns	ns	ns	ns	ns
HIV-, total IgG-CD32A dimer binding change	ns	ns	0.0484 Median 1,3: -0.288 Median 3: 0.0115	0.0162 Median 1+1,3:-0.305 Median 3: 0.0115	ns

- Antigens-specific IgG: HA of A/Switzerland/9715293/2013
- HIV-: HIV-negative subjects, HIV+: HIV-positive subjects
- 1: G1m1 homozygotes. 3: G1m3 homozygotes. 1,3: G1m1/G1m3 carriers
- Pre: pre-vaccination, post: post-vaccination, change: post-pre
- Non-parametric Mann-Whitney test. Ns, not significant ($p>0.05$)

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Research paper

Neutrophils mediate HIV-specific antibody-dependent phagocytosis and ADCC

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Antibody

ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

2. Material and methods

2.1. Study subjects/plasma samples

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

Table 1
Clinical characteristics of study cohort.

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

2.2. Primary cell isolation and culture

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

2.3. HL-60 cells maintenance and differentiation

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

2.4. IgG antibody purification

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

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

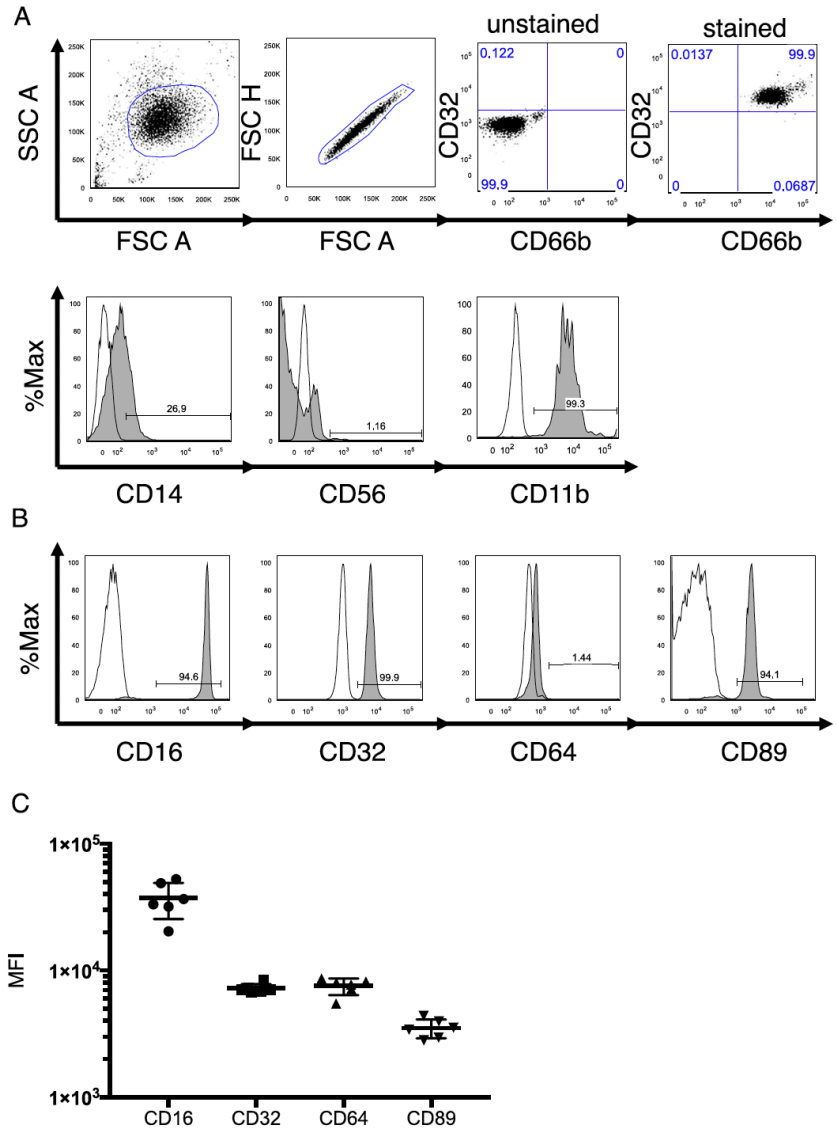


Fig. 1. Purity of isolated neutrophils and Fc receptor expression. A. Representative flow cytometry plots of staining for neutrophil purity for CD66b+ and CD32+. The isolated cells were confirmed to have typical neutrophil phenotypic markers including CD11b+ and CD56- with low levels of CD14. B. Representative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

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

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

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

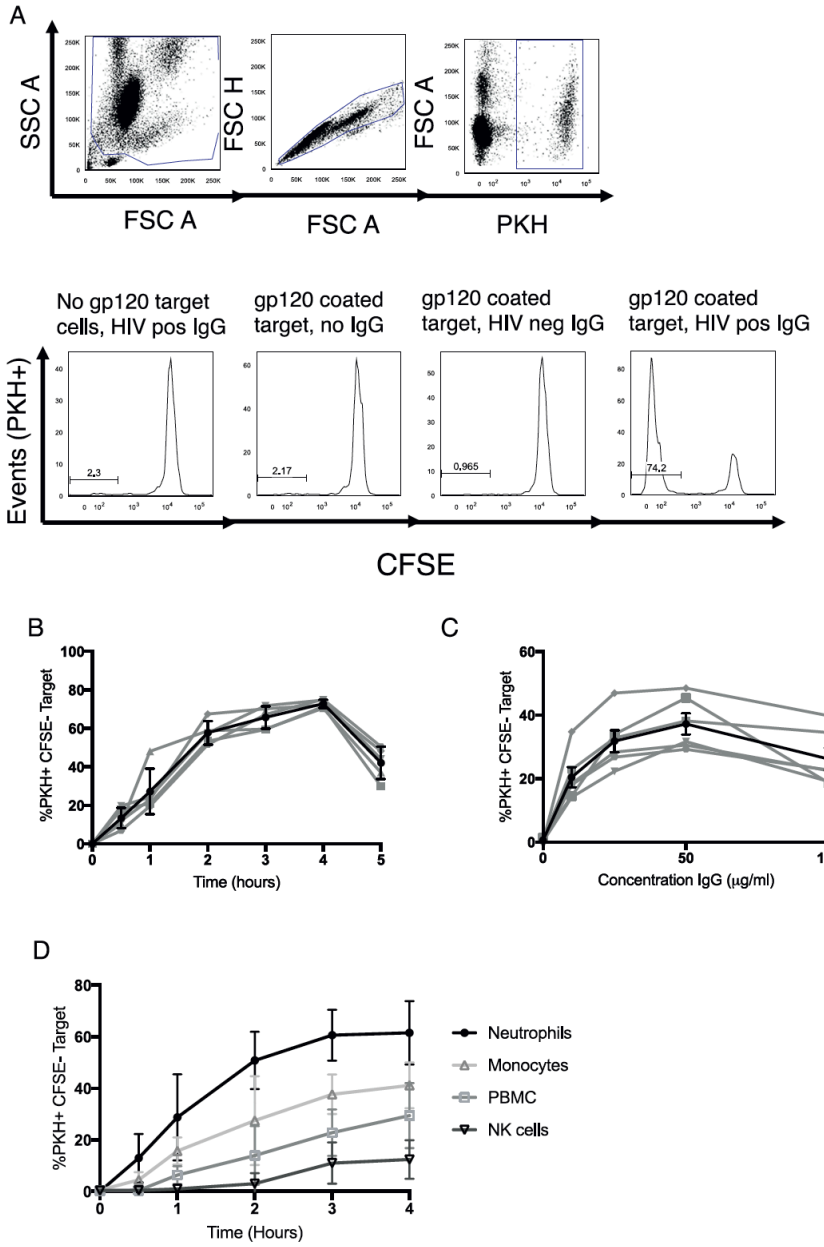


Fig. 2. Neutrophil-mediated RFADCC assay. A. Representative gating strategy for the neutrophil RFADCC. The target cells were stained with PKH membrane dye, CFSE cytoplasmic dye and coated with HIV-1_{BAL} gp120 and incubated with isolated neutrophils. Target cell lysis was identified as cells that were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

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

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

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

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

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

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

3.4. HIV-specific ADNP assay

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

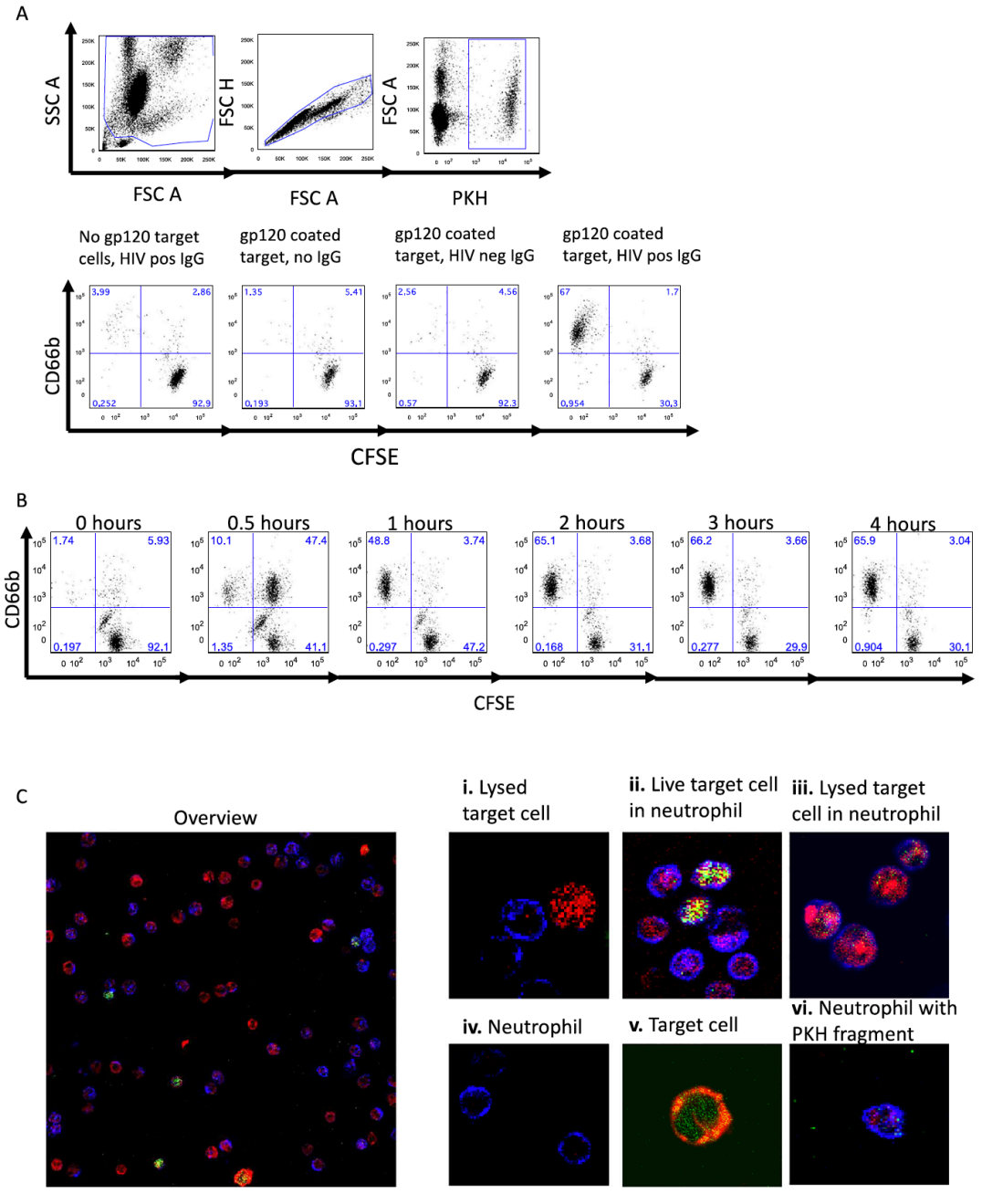
3.5. HIV-specific HL-60 ADP assay

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

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

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

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



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

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

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

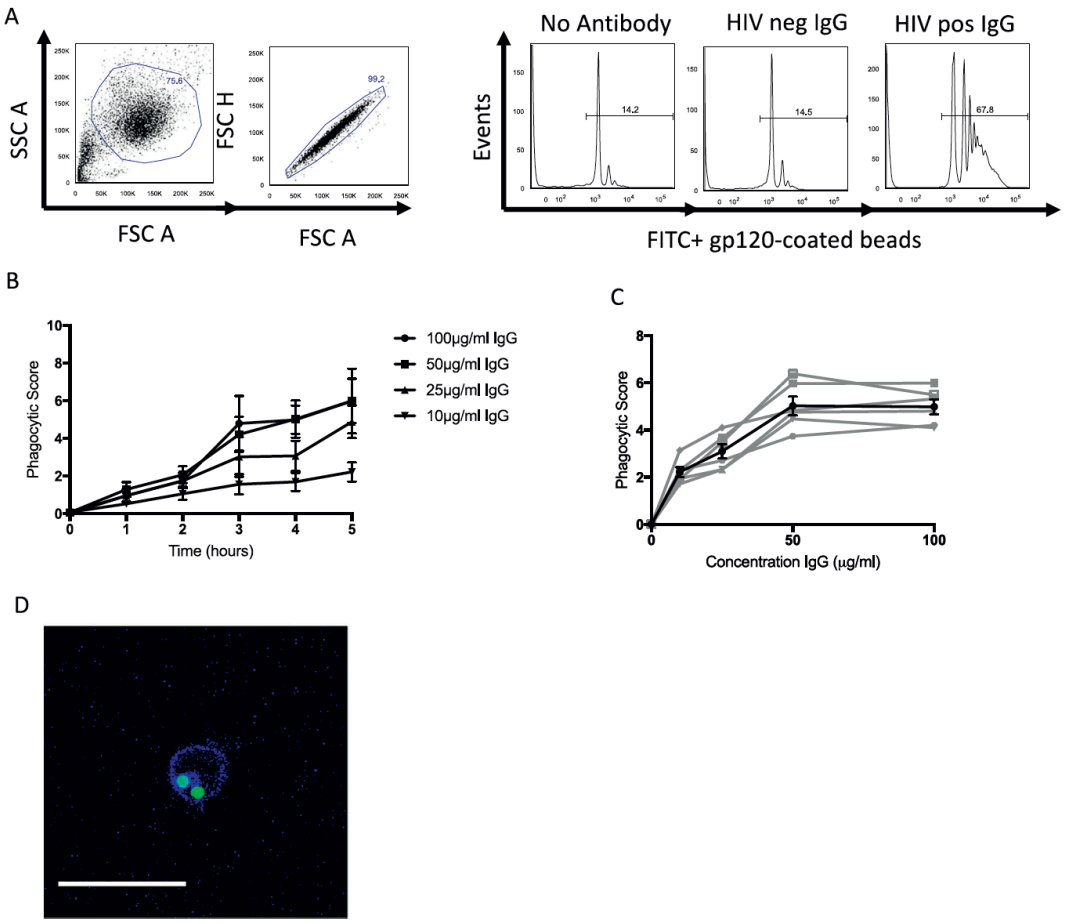

Fig. 4. ADNP responses to HIV positive IgG. A. Representative gating strategy for the ADNP assay. The panels 3 and 4 show minimum uptake of the FITC+ beads in the absence of antibody and in the presence of HIV negative IgG. Panel 5 shows that beads coated with HIV-1_{BAL} gp120 in the presence HIV positive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

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

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

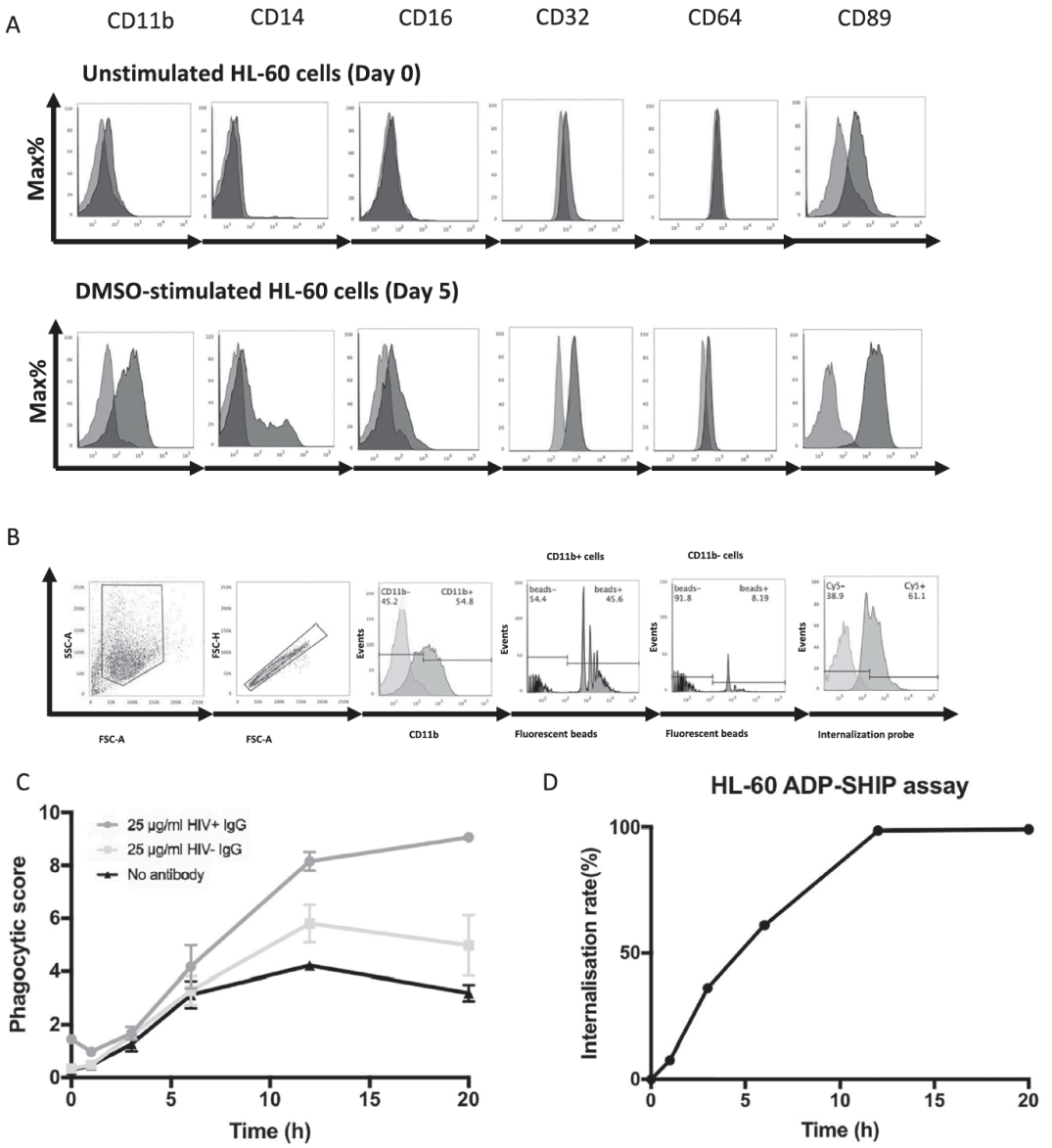

Fig. 5. Differentiated CD11b+ HL-60 cell as a model for ADNP. A. Representative flow cytometry plot for cell markers and Fc receptors profiles in HL-60 cells. D0 were unstimulated naïve HL-60 cells, and D5 were cells stimulated by 1.3% DMSO for five days. Lighter grey populations were unstained control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

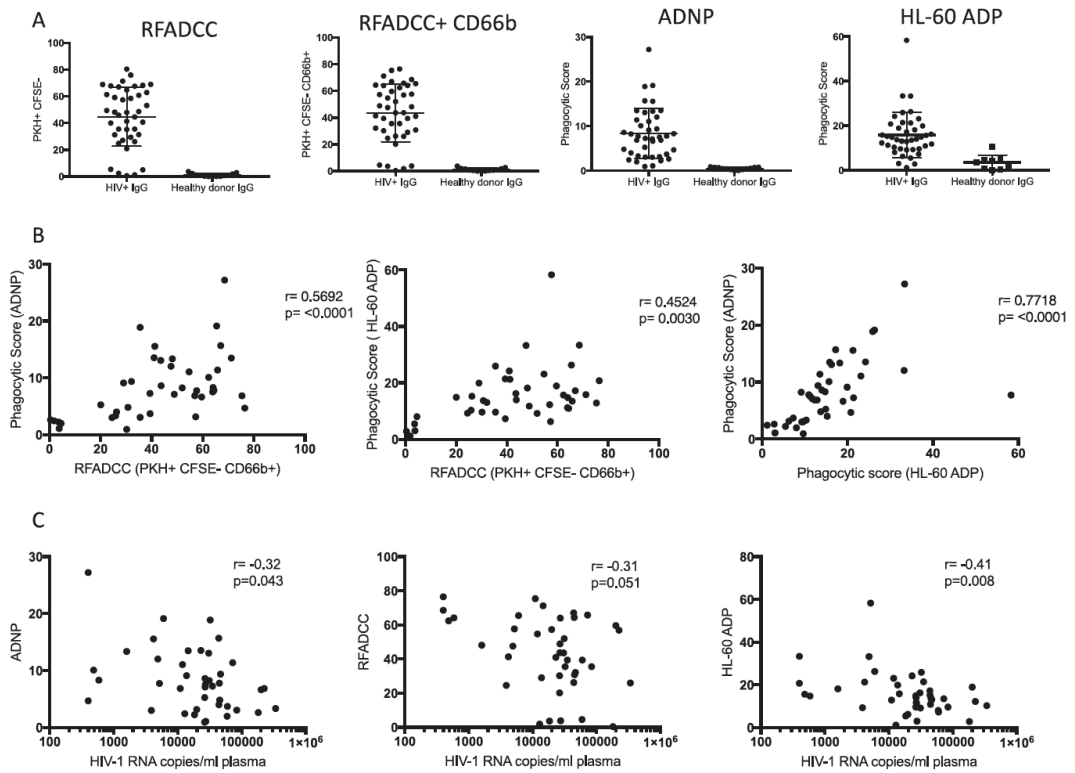

Fig. 6. Neutrophil Fc-Effector responses of HIV positive cohort. A. 41 HIV positive plasma samples and 13 HIV negative plasma samples were purified for IgG and were then assess for responses in the neutrophil RFADCC, neutrophil RFADCC with CD66b gating, ADNP and the HL-60 ADP. B. The HIV positive IgG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

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

4. Discussion

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

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

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

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

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

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

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Appendix A. Supplementary data

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