Defining Fc-mediated Functions in People Living with HIV during Respiratory Viral Infection and Vaccination

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

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Declaration

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

Boitumelo Madika Motsoeneng

2nd day of April 2024 in Johannesburg

To my greatest support,

My parents, Molupi D. and Ntombizodwa D. Motsoeneng, for your patience, putting my education first, and providing endless love, support, and encouragement.

My forever, Sibusiso Mbandazayo, who has journeyed with me from undergrad to here. Thanks for being my pillar of strength.

To even bigger dreams.

Presentations

Talks

Simone I. Richardson, Nelia Manamela, Thandeka Moyo, Tandile Hermanus, Prudence Kgagudi, Frances Ayres, Zanele Molaudzi, **Boitumelo M. Motsoeneng**, Zelda van der Walt, Talita de Villiers, Wesley van Hougenhouck-Tulleken, Veronica Ueckermann, Lynn Morris, Theresa Rossouw, Michael T. Boswell, Penny L. Moore. Distinct humoral trajectories associated with both HIV co-infection and COVID-19 survival in a hospitalized South African SARS-CoV-2 cohort. Virtual VIIIth Conference of the South African Immunology Society (SAIS). September 2021.

Boitumelo M. Motsoeneng, Nisha Dhar, Marta C. Nunes, Florian Krammer, Lynn Morris, Shabir A. Madhi, Penny L. Moore, Simone I. Richardson. Differential hemagglutinin stalk-directed Fc-mediated antibody functions in HIV-infected individuals in response to influenza vaccination. Virtual VIIIth Conference of the South African Immunology Society (SAIS). September 2021.

Boitumelo M. Motsoeneng, Nisha Dhar, Marta C. Nunes, Florian Krammer, Lynn Morris, Shabir A. Madhi, Penny L. Moore, Simone I. Richardson. Differential hemagglutinin stalk-directed Fc-mediated antibody functions in HIV-infected individuals in response to influenza vaccination. 10th Infectious Diseases in Africa (IDA) Symposium. October 2021.

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Simone I. Richardson, **Boitumelo M. Motsoeneng**, Nelia P. Manamela, Haajira Kaldine, Prudence Kgagudi, Tandile Hermanus, Frances Ayres, Zanele Molaudzi, Thandeka Moyo-Gwete, Mieke A. van der Mescht, Alane Izu, Fareed Abdullah, Michael T. Boswell, Veronica Ueckermann, Theresa M. Rossouw, Shabir A. Madhi and Penny L. Moore. People living with HIV achieve equivalent but delayed antibody function after SARS-CoV-2 infection and vaccination. Human Vaccine Trial Network, Sub-Saharan regional meeting. February 2023. Johannesburg, South Africa.

Boitumelo M. Motsoeneng, Nelia P. Manamela, Haajira Kaldine, Prudence Kgagudi, Tandile Hermanus, Frances Ayres, Zanele Molaudzi, Thandeka Moyo-Gwete, Mieke A. van der Mescht, Alane Izu, Fareed Abdullah, Michael T. Boswell, Veronica Ueckermann, Theresa M. Rossouw, Shabir A. Madhi, Penny L. Moore and Simone I. Richardson. People living with HIV have differential humoral response kinetics in SARS-CoV-2 infection and vaccination. NICD scientific meeting. February 2023. Johannesburg, South Africa.

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Shabir A. Madhi, Penny L. Moore and Simone I. Richardson. Despite delayed kinetics, people living with HIV achieve equivalent antibody function after SARS-CoV-2 infection or vaccination. The 11th SA AIDS conference and symposium. Invited talk. June 2023. Durban, South Africa.

Boitumelo M. Motsoeneng, Nisha Dhar, Marta C. Nunes, Florian Krammer, Shabir A. Madhi, Penny L. Moore, Simone I. Richardson. Hemagglutinin stalk-specific Fc effector functions are associated with protection after vaccination with trivalent inactivated influenza vaccine. The Africa Network for Influenza Surveillance and Epidemiology (ANISE) 7th Meeting. September 2023. Johannesburg, South Africa.

Posters

Boitumelo M. Motsoeneng, Nisha Dhar, Marta C. Nunes, Shabir A. Madhi, Lynn Morris, Simone I. Richardson, Penny L. Moore. Differential Hemagglutinin Stalk Antibody-Dependent Phagocytosis in HIV-Infected and -Uninfected Pregnant Women Pre- and Post-Influenza Vaccination. University of the Witwatersrand, Faculty of Health Sciences Virtual Research Day. October 2020.

Simone I. Richardson, Nelia Manamela, Thandeka Moyo, Tandile Hermanus, Prudence Kgagudi, Frances Ayres, Zanele Molaudzi, **Boitumelo M. Motsoeneng**, Zelda van der Walt, Talita de Villiers, Wesley van Hougenhouck-Tulleken, Veronica Ueckermann, Lynn Morris, Theresa Rossouw, Michael T. Boswell, Penny L. Moore. Distinct humoral trajectories associated with both HIV co-infection and COVID-19 survival in a hospitalized South African SARS-CoV-2 cohort. HIV R4P Virtual Conference. January 2021.

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Boitumelo M. Motsoeneng, Nisha Dhar, Marta C. Nunes, Florian Krammer, Shabir A. Madhi, Penny L. Moore and Simone I. Richardson. Differential hemagglutinin stalk-specific Fc-mediated functions in individuals living with or without HIV in response to influenza vaccination. Gordon Research Conference (GRC) Immunochemistry and Immunobiology. June 2022. Castelldefels, Spain.

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Boitumelo M. Motsoeneng, Nisha Dhar, Marta C. Nunes, Florian Krammer, Shabir A. Madhi, Penny L. Moore, Simone I. Richardson. Hemagglutinin stalk-specific Fc effector functions are associated with protection after vaccination with trivalent inactivated influenza vaccine. 18th International Congress of Immunology (IUIS). November 2023. Cape Town, South Africa.

Publications

From this Thesis

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Boitumelo M. Motsoeneng, Nisha Dhar, Marta C. Nunes, Florian Krammer, Shabir A. Madhi, Penny L. Moore and Simone I. Richardson. Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women. Under review, Journal of Infectious Diseases, February 2024.

Boitumelo M. Motsoeneng, Nelia P. Manamela, Haajira Kaldine, Prudence Kgagudi, Tandile Hermanus, Frances Ayres, Zanele Molaudzi, Thandeka Moyo-Gwete, Mieke A. van der Mescht, Fareed Abdullah, Michael T. Boswell, Veronica Ueckermann, Theresa M. Rossouw, Shabir A. Madhi, Penny L. Moore and Simone I. Richardson. Despite delayed kinetics, people living with HIV achieve equivalent antibody function after SARS-CoV-2 infection and vaccination. Frontiers in Immunology, 2023, doi.org/10.3389/fimmu.2023.1231276

Other

Simone I. Richardson, Nelia P. Manamela, **Boitumelo M. Motsoeneng**, Haajira Kaldine, Frances Ayres, Zanele Makhado, Mathilda Mennen, Sango Skelem, Noleen Williams, Nancy J. Sullivan, John Misasi, Glenda G. Gray, Linda-Gail Bekker, Veronica Ueckermann, Theresa M. Rossouw, Michael T. Boswell, Ntobeko A.B. Ntusi, Wendy A. Burgers, Penny L. Moore. SARS-CoV-2 Beta and Delta variants trigger Fc effector function with increased cross-reactivity. Cell Reports Medicine, 2022, doi: 10.1016/j.xcrm.2022.100510.

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Abstract

There are approximately 39 million people living with human immunodeficiency virus (HIV) (PLWH) worldwide. Furthering our understanding of humoral immune responses to respiratory viral infection and vaccination in PLWH is essential for reducing the burden of these diseases, in high HIV prevalence settings, and informing vaccine implementation in this population.

Influenza virus hemagglutinin (HA) stalk-specific antibodies have been associated with protection and shown to mediate Fc-mediated functions. This thesis describes HA stalk-specific antibody-dependent cellular phagocytosis (ADCP), cellular cytotoxicity (ADCC) and complement deposition (ADCD) between PLWH and people without HIV (PWOH) following immunization with a seasonal trivalent inactivated influenza vaccine (TIV). Irrespective of HIV status, ADCD was boosted while ADCC was not. ADCP was only enhanced in PWOH. The coordination of these functions differed by HIV status. Additionally, differences in the regulation of these HA stalk Fc responses by HIV infection was reported. Furthermore, ADCC was not associated with protection in this study. Pre-existing ADCP reduced the risk of influenza virus infection while TIV-induced ADCD provided protection against influenza-illness. Overall, PLWH have unique responses to TIV and HA stalk-specific ADCD correlated with protection following TIV.

For SARS-CoV-2, antiretroviral treatment (ART)-naïve PLWH had reduced humoral responses to respiratory infection. The infecting variants D614G and Beta differentially triggered ADCC, ADCD and antibody-dependent cellular trogocytosis (ADCT). Regarding the kinetics, PLWH infected with D614G had delayed neutralization and ADCP while Beta infection delayed ADCT, regardless of HIV status. PLWH showed improved coordination between immune responses following respiratory infection. ChAdOx-1 nCoV-19 vaccination differed from infection in that PLWH had delayed IgG binding while neutralization and ADCP were not delayed, and ADCC was substantially enhanced than in PWOH. In conclusion, despite the delayed and differential kinetics, PLWH on ART developed equivalent responses to PWOH, supporting the rapid rollout of ART and SARS-CoV-2 vaccines to PLWH.

This thesis highlights the need to include high-risk groups with different responses in future vaccination trials and also supports the assessment of novel correlates of protection for future vaccines. Overall, this thesis provided insights into the mechanisms required for protection against severe respiratory diseases and improved our understanding of vaccine-induced immunity in PLWH.

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Part 1: Integrated Narrative

Abbreviations

AIDS	acquired immunodeficiency syndrome
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
ADCT	antibody-dependent cellular trogocytosis
ADCD	antibody-dependent complement deposition
ART	antiretroviral treatment
B-cells	B lymphocytes (bursa derived)
BCRs	B-cell receptors
cHAs	chimeric HAs
CD4	clusters of differentiation 4
CSR	class switch recombination
COBRA	computationally-optimized broadly reactive antigens
CH and CL	constant heavy and constant light
COVID-19	coronavirus disease 2019
DCs	dendritic cells
ELISA	enzyme linked immunosorbent assay
Fab	fragment of antigen binding
Fc	fragment crystallisable
FcγR	Fc gamma receptors
GISRS	Global Influenza Surveillance and Response System
HA	hemagglutinin
HAI	hemagglutination inhibition
hACE2	human angiotensin-converting enzyme 2
HIV	human immunodeficiency virus
Ig	immunoglobulins
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
IIV	inactivated influenza vaccines
LAIV	live attenuated influenza vaccines
MERS-CoV	Middle East respiratory syndrome coronavirus
mRNA	messenger RNA
NK	natural killer cells
NTD	N-terminal domain

NA	neuraminidase
NAI	neuraminidase inhibition
PLWH	people living with HIV
PWOH	people without HIV
QIV	quadrivalent inactivated influenza vaccine
RBD	receptor binding domain
rHA	recombinant hemagglutinin
RNA	ribonucleic acid
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
S	spike
T-cells	T lymphocytes (thymus derived)
TIV	trivalent inactivated influenza vaccine
VH and VL	variable heavy and variable light
VOCs	variants of concern
WHO	World Health Organization

Symbols and Units

%	percentage
~	approximately
α	alpha
+	positive
>	greater than
<	less than
ul	microliter
µg	microgram
nm	nanometer
kb	kilobases
kDa	kilo Daltons

Part 1: Integrated Narrative

For ease of reading, studies emanating from this thesis, and contained in Part 2 are marked with an *

1. People Living with HIV

Human immunodeficiency virus (HIV) remains a major public health concern, having caused 85.6 million infections worldwide, over the past 40 years (1,2). In 2022, there were an estimated 39 million people living with HIV (PLWH) globally (1). Sub-Saharan Africa is the most heavily impacted by HIV, accounting for two thirds of PLWH and approximately 500,000 new infections in the past year (3). Although these new infections have declined by 57% since 2010, women and girls of all ages are still disproportionately affected (3,4). Furthermore, the reduction in HIV incidence differs distinctly between countries in this region (3). Eastern and southern Africa have achieved 92% of PLWH knowing their status, 83% on antiretroviral treatment (ART) and 77% virally suppressed, making progress towards the Joint United Nations Programme on HIV/acquired immunodeficiency syndrome (AIDS) 95-95-95 targets (95% of PLWH diagnosed, 95% on ART and 95% virally suppressed) (3). However, South Africa with over 8.5 million PLWH (**Figure 1**), has only 75% of PLWH on ART, leaving more than 2 million not virally suppressed (5–7).

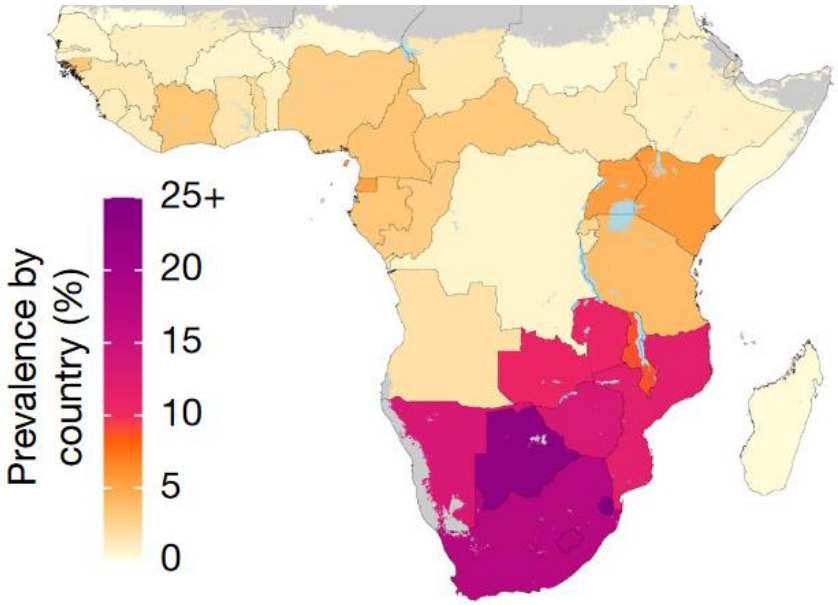


Figure 1: Prevalence of HIV in sub-Saharan Africa.
Map showing the prevalence of HIV in sub-Saharan Africa by country with the darker colours indicating higher prevalence (%). The countries with the highest HIV prevalence (>20%) are Botswana, Swaziland, South Africa and Lesotho. Source: Dwyer-Lindgren *et al.,* 2019 (7).

HIV infection is characterized by decreasing $CD4^+$ T-cell counts, cellular immune dysfunction, dysregulated B-cell responses, resulting in polyclonal activation, reduced specific antibody production or hypergammaglobulinemia and can progress to AIDS (8,9). Access to ART has resulted in a decrease of AIDS-related deaths such that HIV infection can be a manageable chronic condition. PLWH on ART have a life expectancy nearing that of people without HIV (PWOH) (10,11).

Despite the use of ART, PLWH still have an increased risk of severe respiratory viral disease and mortality, as reported for respiratory illnesses caused by influenza and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viruses (12–20). This could be a consequence of incomplete immune restoration by ART and ongoing inflammation associated with HIV (21–23). As a result, generating immunity against other infections and vaccinations in PLWH should be a priority. Furthermore, the absence of ART heightens the risk of respiratory viral infections in children and adults with HIV by up to 19-fold in comparison with PWOH (24,25). Therefore, understanding immune responses to respiratory viral infection and vaccination in PLWH is crucial for reducing the burden of these diseases and informing vaccine implementation in this population.

2. The Burden of Respiratory Viral Infections in People Living with HIV

2.1 Influenza

Seasonal influenza is an acute respiratory infection caused by influenza viruses, which cause substantial annual morbidity and mortality globally. These annual epidemics are estimated to result in 3-5 million cases of severe illness worldwide, and the occurrence of approximately 290,000-650,000 respiratory deaths (26,27). Low-middle income countries in sub-Saharan Africa are reported to have the highest influenza-associated mortality rates (27). In South Africa, seasonal influenza epidemics lead to more than 56,000 hospitalizations and over 11,000 fatalities each year (12).

In South Africa, the public health and economic burden of severe influenza-illness remains significant (28,29). High-risk groups of severe respiratory illnesses include, pregnant women, children under 5 years, the elderly, individuals with tuberculosis and PLWH, who together account for the majority of influenza-associated severe (86.8%) and fatal (94.5%) illnesses

(26,29–31). Of the fatal illnesses, 44.9% are among PLWH (29). PLWH therefore require effective vaccination strategies in order to reduce the impact of severe influenza-illnesses (32). Furthermore, while HIV infection is not associated with influenza virus transmission, long-term influenza viral shedding has been associated with low $CD4^+$ T-cell counts in PLWH, which may contribute to influenza viral evolution (33,34).

In 2016, the National Advisory Group on Immunization in South Africa recommended the prioritization of pregnant women and PLWH in receiving annual influenza vaccination (35). More specifically, influenza vaccination for pregnant women living with HIV would reduce the burden of influenza in this country and be cost-effective (32,36). While studies support these recommendations and vaccine efficacy has been observed in PLWH, the immune responses to influenza vaccines is typically lower than in PWOH (37–42). In maternal influenza vaccination trials moderate (54.4%) and high (70.6%) vaccine efficacy was reported for PWOH and PLWH, respectively. However, hemagglutination inhibition (HAI) titers did not fully explain the high efficacy in PLWH (41). Hemagglutinin (HA) stalk antibody were then measured and an association with protection against influenza-illness was reported (43). Consequently, it is necessary to gain a deeper understanding of the immune responses and their mechanisms of protection in PLWH following seasonal influenza vaccination.

2.2 SARS-CoV-2

Since December 2019, the novel coronavirus disease 2019 (COVID-19) pandemic, caused by the SARS-CoV-2 virus, has resulted in more than 770 million infections. As of November 2023, approximately 7 million deaths have occurred worldwide. South Africa accounts for the majority of known SARS-CoV-2 cases in Africa with over 4 million infections and 100,000 deaths (44).

Risk factors for severe COVID-19 include old age (>65 years), male sex assigned at birth, hypertension, diabetes and HIV infection (45). In particular, low $CD4^+$ T-cell counts in PLWH were associated with a greater likelihood of testing positive for SARS-CoV-2 (46). There was also evidence of an increased risk of SARS-CoV-2 hospitalizations and mortality in PLWH, heightened in those with high HIV viral loads and who were not on ART (14–19,47–53). Furthermore, studies have shown that in both uncontrolled HIV infection and ART-treated PLWH there may be higher SARS-CoV-2 viral loads upon infection in comparison to PWOH, along with prolonged virus shedding and the emergence of mutations that were concurrently detected in the SARS-CoV-2 variants of concern (VOCs) (54–59). These studies highlighted

that PLWH should be prioritized for SARS-CoV-2 clinical management and vaccination programmes.

Several vaccines have been developed to control the COVID-19 pandemic (60–68). Since PLWH have poor immune responses to several vaccines, such as influenza and hepatitis B, there has been an urgent need to evaluate the immunogenicity of SARS-CoV-2 vaccines in PLWH (40,41,69,70). Furthermore, as the SARS-CoV-2 virus continues to evolve, understanding immune responses to infection and vaccination in PLWH is important for reducing the public health impact in a high HIV prevalence setting.

3. Respiratory Viral Pathogens

The respiratory viruses discussed here are both of zoonotic origin; avian or swine to humans for influenza viruses, and potentially bats to an intermediate host to humans for SARS-CoV-2 viruses (71–74). Human-to-human transmission of infectious virus particles is through inhalation of respiratory droplets and aerosols from an infected individual to a new host, and through direct contact with contaminated surfaces (75,76). The incubation period for influenza viruses is 1-2 days, while it is 2-14 days for SARS-CoV-2 viruses (77). For both influenza and SARS-CoV-2 infections, infected individuals may be asymptomatic or display mild illness characterized by symptoms such as fever, cough, sore throat, headache, runny nose, muscle pain and general malaise or they may develop severe respiratory disease, which may lead to fatalities (78).

3.1 Influenza

3.1.1 Classification and Structure

Influenza viruses belong to the family of *Orthomyxoviridae* and are divided into 4 types influenza A, B, C and D viruses. Influenza A and B viruses are the most clinically relevant types, causing severe respiratory disease and mortality in humans (26). Detected less frequently are influenza C viruses, which typically cause mild illness in humans. The distantly related influenza D viruses primarily infect cattle and can infect other animals including pigs (79,80).

Influenza viruses have a host-derived lipid envelope and are approximately 80-120 nm in diameter (**Figure 2**). The negative-sense ribonucleic acid (RNA) genome of ~13.6 kb is

composed of eight single-stranded segments (81). Three segments encode the RNA-dependent RNA polymerases (PB1, PB2 and PA) that are involved in RNA synthesis and virus replication. Surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which mediate host cell entry and egress, are encoded by two segments. The RNA genome is bound by the viral nucleoprotein (NP). The remaining segments encode for matrix protein (M1) that aids in giving the virus particle structure; membrane protein (M2) a proton ion channel; non-structural protein (NS1) a virulence factor and nuclear export protein (NEP/NS2) that regulates the trafficking of viral RNA segments (81,82). The influenza A viral surface envelope proteins consist of the HA, NA and the less abundant M2 proteins, while influenza B consist of HA, NA and instead of M2, NB and BM2 (81).

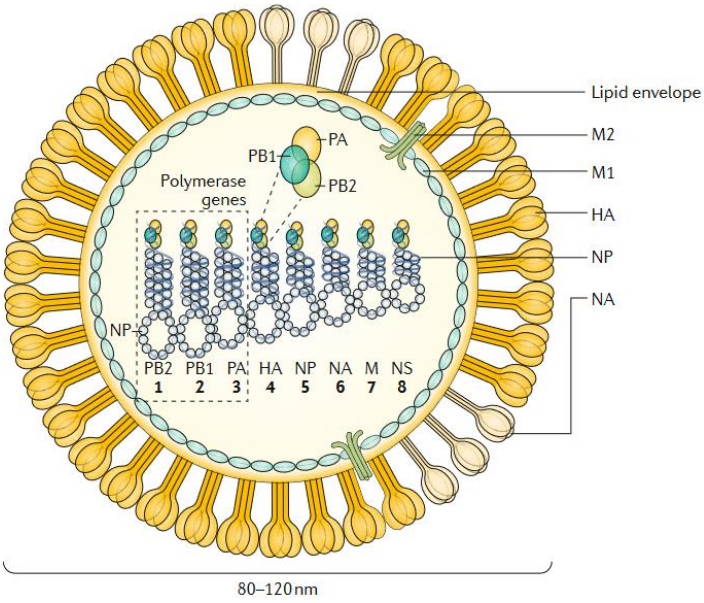


Figure 2: Influenza A and B virus structure.
Schematic diagram of influenza virus particle with a segmented, enveloped, negative-sense RNA genome. The genome encodes RNA-dependent RNA polymerases (PB1, PB2 and PA), glycoproteins (HA and NA). The viral NP is encoded by RNA segment 5. RNA segments 7 and 8 encode more than one protein, namely, the matrix protein (M1) and membrane protein (M2) or NB and BM2 in the case of influenza B and the NS1 and NEP proteins (not shown). Source: Krammer *et al.,* 2018 (81).

3.1.2 Influenza Virus Hemagglutinin

Hemagglutinin (HA) is the most abundant influenza virus surface glycoprotein (83). HA is a homotrimeric structure and is composed of an antigenically variable globular head (HA head) and a highly conserved stalk (HA stalk) domain (**Figure 3A**) (84,85). The HA head contains

the receptor binding site, whilst the HA stalk mediates membrane fusion and cell entry. Human influenza viruses have HA receptor binding specificity for the α2,6-linked sialic acid receptor (83). Influenza A viruses are divided into subtypes that carry distinct combinations of 18 HA proteins (H1-H18), split into phylogenetic groups 1 and 2 (**Figure 3B**), and 11 NA proteins. The influenza virus B/Lee is the ancestral strain from which influenza B viruses have diverged into B/Yamagata and B/Victoria lineages (**Figure 3B**). Seasonal influenza epidemics are caused by influenza virus types A/H1N1 (group 1), A/H3N2 (group 2) and both influenza B lineages (84). However, since the start of the COVID-19 pandemic the B/Yamagata lineage has not been detected due to the lack of sustained transmission (86).

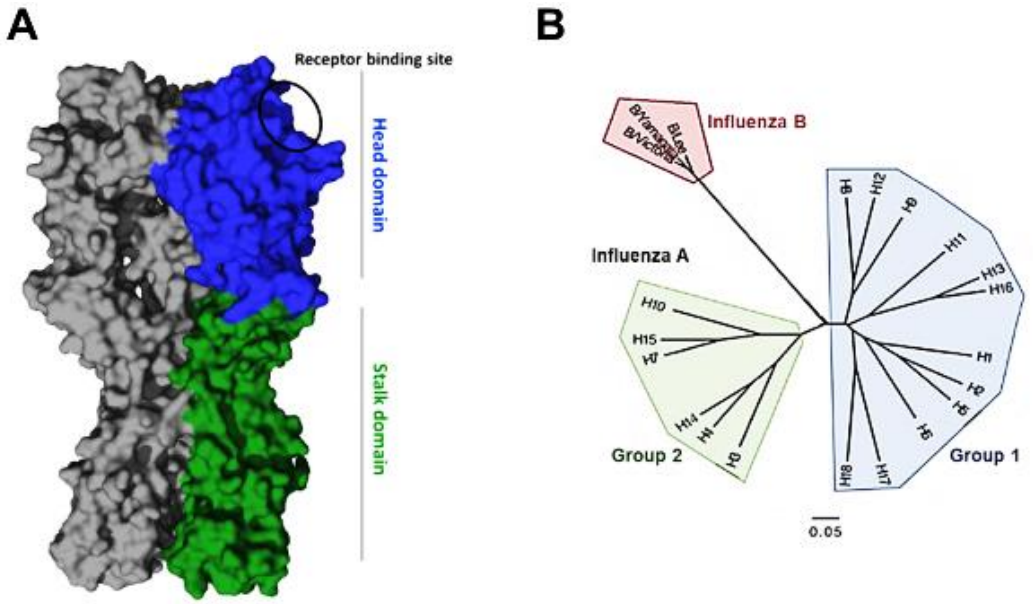


Figure 3: Structure and phylogenetic tree of influenza hemagglutinins.
(A) HA homotrimeric structure, with a monomer of the head domain coloured in blue, a monomer of the stalk domain coloured in green and the receptor binding site circled in black. (B) Phylogenetic tree of influenza A and B hemagglutinins. Blue shading shows influenza A group 1 HAs, green shows influenza A group 2 HAs and red denotes influenza B HAs. Figure adapted from: Kirkpatrick *et al.,* 2018 (84).

3.1.3 Viral Evolution and Pandemics

Influenza viruses are constantly evolving within animal and human hosts. This is due to antigenic changes that occur in the surface glycoproteins HA and NA. Viral evolution through the continuous accumulation of point mutations is known as antigenic drift (81). The error-prone activity and low proofreading capabilities of influenza viruses' RNA-dependent RNA polymerase causes the point mutations that allow for influenza viruses to escape pre-existing immunity from previous infections and vaccination (87). The evolutionary rate is faster for the HA head domain than the conserved HA stalk domain and other influenza viral proteins, resulting in high levels of mutations (84). Additionally, influenza A viruses evolve more rapidly than influenza B viruses (88). Antigenic drift is responsible for generating closely related, unique influenza virus clades that result in annual seasonal epidemics.

Influenza A viruses can also undergo a process referred to as antigenic shift (81). This process involves the genetic re-assortment of the HA (and to a lesser extent NA) genome segments across two or more strains. While it is sporadic, zoonotic influenza viruses can re-assort with a human influenza virus and gain the ability to infect humans, which can lead to novel pandemic influenza strains (87). Since the 1918 pandemic, antigenic shift has led to the emergence of three influenza A virus pandemics in 1957, 1968 and 2009 (**Figure 4**) (85).

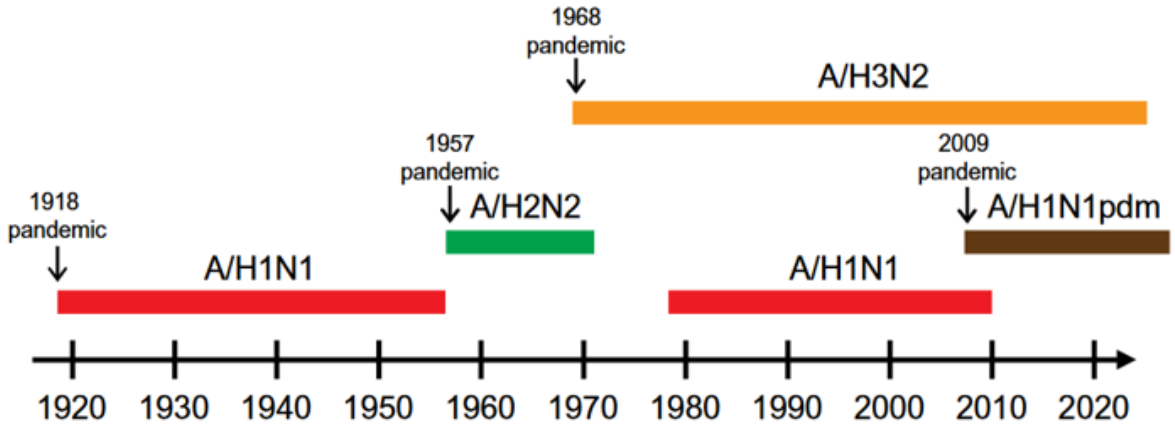


Figure 4: Timeline of human influenza pandemics.
As indicated, the 1918 pandemic was caused by the H1N1 virus, which circulated in humans until the 1957 H2N2 pandemic. The circulating H2N2 virus was then replaced by the 1968 H3N2 pandemic virus. H3N2 has been co-circulating with H1N1, since it re-emerged in 1977. Then in 2009 the pandemic was caused by the H1N1pdm virus. Figure adapted from: Wang *et al.,* 2022 (85).

The 1918 influenza pandemic, known as the “Spanish Flu”, had the most significant public health impact with approximately 500 million infections and over 50 million deaths (72). The 1918 H1N1 virus displayed high pathogenicity and mortality in young adults (20-45 years of age), likely due to the lack of pre-existing immunity (89,90). Relative to the 1918 pandemic, the subsequent pandemics showed decreased virulence, with relatively lower mortality. In 1957, “Asian Flu” caused by an H2N2 strain led to between 1–4 million deaths worldwide (91). Then in 1968, the “Hong Kong Flu” or H3N2 strain first emerged and completely replaced the H2N2 strain, leading to about 1 million deaths worldwide (91). The most recent influenza A pandemic, “Swine Flu”, was caused by an antigenically distinct H1N1 influenza A virus in 2009, resulting in an estimated 280,000 deaths (92). This was reported to be a triple re-assortment of genes from human, swine and avian influenza A viruses (93,94).

Prior to the emergence of SARS-CoV-2, it was predicted that the next pandemic would be caused by a novel influenza strain (95).

3.2 SARS-CoV-2

3.2.1 Classification and Structure

SARS-CoV-2 forms part of the family *Coronaviridae,* which is classified into four genera namely, *Alpha, Beta, Gamma* and *Deltacoronaviruses* (96). SARS-CoV-2 viruses belong to the same genus (*Betacoronaviruses*) as SARS-CoV-1 and the Middle East respiratory syndrome coronavirus (MERS-CoV) which are highly pathogenic in humans (97,98). In contrast, endemic human coronaviruses include *Alphacoronaviruses* (229E and NL63) and *Betacoronaviruses* (HKU1 and OC43) that usually cause mild symptoms, such as the common cold (74).

These enveloped viruses contain a positive-sense, single-stranded RNA genome of 26-32 kb, which is amongst the largest genomes for RNA viruses (99). Two-thirds of the genome encodes for sixteen non-structural proteins involved in virus replication and transcription (100). The remaining genome encodes for structural proteins known as the spike (S), nucleocapsid (N), membrane (M) and envelope (E) proteins (**Figure 5**). The spike glycoprotein, with its crown-like (corona) appearance, mediates viral attachment and fusion to host cells (101). Unlike other *Betacoronaviruses,* SARS-CoV-2 lacks the hemagglutinin-esterase glycoprotein, generally used in conjunction with the spike glycoprotein for viral attachment (102,103). The membrane protein shapes the virus and plays a role in viral

assembly (104). The envelope protein has ion channel activity that is a determinant of virus pathogenesis and it aids in viral assembly and release (105). Lastly, the nucleocapsid protein allows for binding of viral RNA, packaging it into a helical ribonucleoprotein complex (106). Additionally, the genome encodes nine accessory proteins, which are involved in various functions that impact virus release, apoptosis, pathogenesis and host immune escape (99).

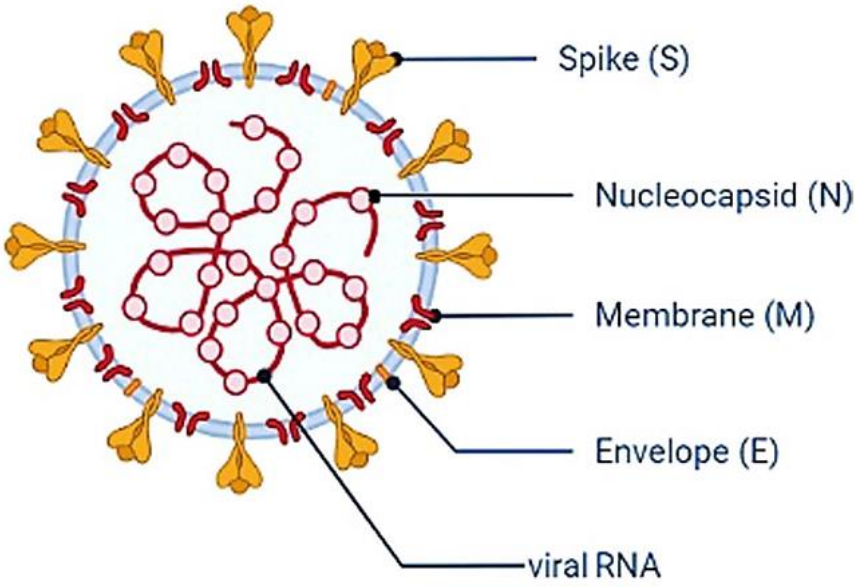


Figure 5: SARS-CoV-2 structure.
Schematic diagram of SARS-CoV-2 virus particle with spike glycoprotein (S), membrane glycoprotein (M) and envelope (E) embedded in the host-derived lipid bilayer encapsulating the helical nucleocapsid (N) comprising of viral RNA. Source: Saberiyan *et al.,* 2022 (107).

3.2.2 SARS-CoV-2 Spike Glycoprotein

The spike glycoprotein is a heavily glycosylated homotrimeric structure, with each monomer consisting of S1 and S2 subunits (**Figure 6**) (108,109). The S1 subunit contains two immunodominant regions known as the receptor binding domain (RBD) and N-terminal domain (NTD) (110). The S1 RBD facilitates the recognition of the receptor, human angiotensin-converting enzyme 2 (hACE2), which is expressed not only on cells of the respiratory tract but also the kidneys, intestines, pancreas, heart and other tissues (111,112). SARS-CoV-2 infection can therefore lead to multi-organ injury (111). The function of the highly variable NTD is unclear but it has been shown to allosterically mediate membrane fusion and cell entry, and mutations occurring in the NTD result in immune escape (113–115). After hACE2 binding and viral attachment, the S2 subunit proceeds with membrane fusion and viral entry via endocytosis (110). Fusion is triggered by the low pH of endosomes

and proteolytic cleavage by transmembrane protease serine 2 (TMPRSS2) at the polybasic furin cleavage site (116,117). This process has a critical role in infectivity and virulence, as it facilitates the conformational changes required for high affinity hACE2 receptor binding leading to entry into the host cell (118,119).

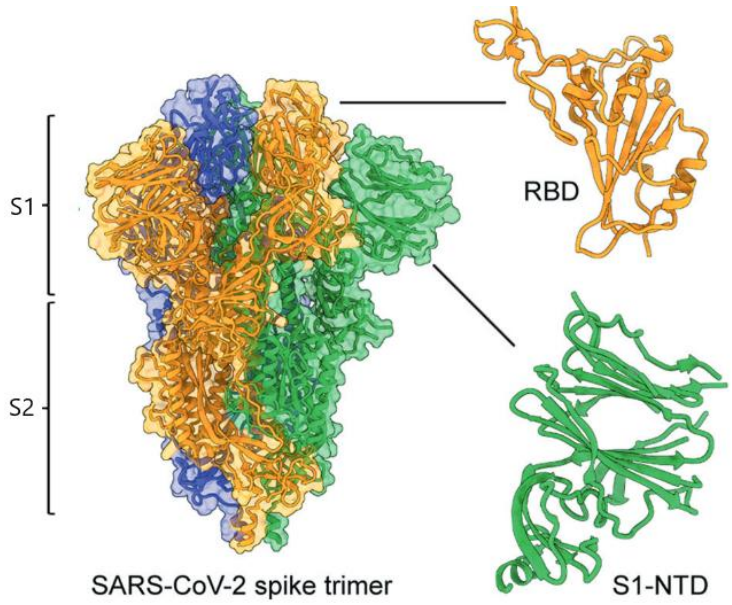


Figure 6: Structure of SARS-CoV-2 spike glycoprotein.
Trimeric spike glycoprotein divided into S1 and S2 subunits with N-terminal domain (NTD) and receptor binding domain (RBD) shown. The three monomers are coloured blue, green and yellow. Figure adapted from: Du *et al.,* 2021 (120).

3.2.3 Viral Evolution and Variants of Concern

In 2019, the first lineage of SARS-CoV-2 was detected in Wuhan, China (100,121). Multiple mutations within the spike glycoprotein have led to the emergence of new SARS-CoV-2 strains (122). These mutations occur through the process of recombination between viral strains, host-mediated RNA editing; a process by which viruses exploit the host cell's RNA enzymes to introduce nucleotide changes allowing for immune escape, and antigenic drift; which occurs at a slower rate than influenza A viruses (123–129). The evolution of SARS-CoV-2 viruses is due to immune pressures from the vast numbers of infections and recent vaccinations, as well as long-term virus shedding in immunocompromised patients (55,56,85,130). The World Health Organization (WHO) has labelled five strains of SARS-CoV-2 as variants of concern (VOCs): Alpha (identified in the United Kingdom), Beta (identified in South Africa), Gamma (identified in Brazil), Delta (identified in India) and Omicron (identified in South Africa) (**Figure 7**) (131).

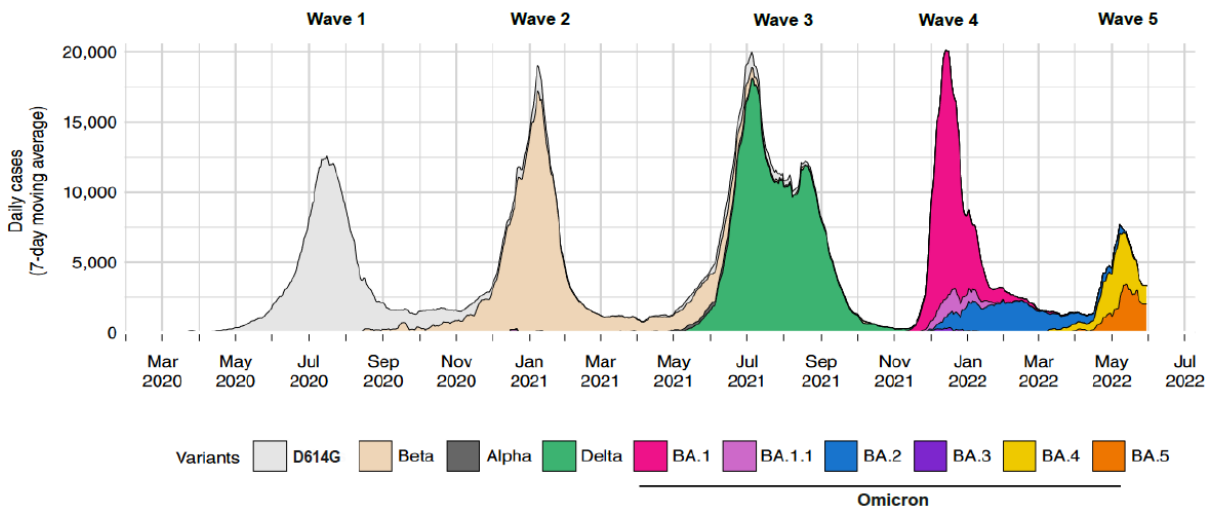


Figure 7: SARS-CoV-2 waves of infection in South Africa from 2020 to 2022.
The 7-day rolling average of daily reported case numbers representing waves of infections and coloured by the proportion of SARS-CoV-2 VOCs responsible for the infections in that period. Figure adapted from: Tegally *et al.,* 2022 (132).

The SARS-CoV-2 VOCs share several key mutations in the spike protein that are biologically and clinically relevant (133). In February 2020, the D614G mutation was reported and it has been associated with increasing spike density on virus particles, and increased infectivity compared to the ancestral Wuhan strain (123,124,134). This mutation has remained present in all evolving SARS-CoV-2 strains (133). Mutations N501Y and E484K are present in the Alpha, Beta and Gamma VOC. N501Y increases hACE2 binding affinity and viral replication in host cells, while E484K results in immune escape (135–143). The K417N mutation found in Beta and implicated in immune escape is also present in a Delta sub-lineage and in the Omicron lineages (144,145). These mutations in combination with several others are associated with the enhanced transmissibility and sometimes increased disease severity, which distinguish VOCs from other strains.

The South African SARS-CoV-2 epidemic has been characterized by five epidemiologic waves between March 2020 and June 2022 (**Figure 7**) (132). The first wave, from March to September 2020, was caused by the Wuhan strain and D614G lineages (139,146). The second wave emerged from October 2020 with daily cases increasing from approximately 2,000 to more than 20,000 cases per day in January 2021. These infections were dominated by the Beta variant (139). The third wave, associated with the Delta variant, had a significant impact with the highest number of cases (>1,3 million) and deaths (>36,000) (147). Omicron

lineages (BA.1, BA.2, BA.3, BA.4 and BA.5) were responsible for the fourth and fifth waves (132). Although fewer cases exist (as low as only 136 sequences obtained in October 2023 in South Africa), Omicron remains the dominant infecting variant. Omicron continues to evolve with the current circulating lineages consisting of XBB sub-lineages (65%), BA.2.86 (29%) and EG.5.1 (6%), in South Africa (146). One of the most important concerns with re-emerging and novel respiratory viruses is the effect on humoral responses following infections and vaccinations.

4. Humoral Immunity

4.1 Antibody Structure

Humoral (antibody) responses of the adaptive immune system are involved in providing protective immunity against viral infections. The antibodies or immunoglobulins (Ig), can either be found as membrane bound B-cell receptors (BCRs) or be secreted into the circulation and tissues by terminally differentiated B-cells (148–151). Upon binding to viral antigens, secreted antibodies are able to block infection and induce antiviral effector functions (150,151). A typical human immunoglobulin G (IgG) antibody (**Figure 8**) is a Y-shaped protein consisting of two identical heavy chains (55 kDa) paired with two identical light chains (24 kDa) joined by disulphide bonds (148,152). Both the heavy and light chains are comprised of variable (VH and VL) and constant (CH and CL) regions. The variable region displays specificity for a large number of antigens. The constant regions are used to classify antibodies into five isotypes namely, IgM, IgD, IgE, IgG and IgA, that are further subdivided into subclasses IgG1, IgG2, IgG3 or IgG4 and IgA1 and IgA2 (150). Additionally, to the variability in the heavy chain, there are two different types of light chains - kappa (κ) and lambda (λ).

Structurally, antibodies can be divided into three functional components, the antigen binding fragments (Fab) and the crystallizable fragment (Fc) region, with the two Fabs linked to the Fc by a flexible hinge (148). The Fab region is formed by the light chains (VL and CL), the VH and CH1 segments, which recognize and bind specific viral antigens (150). The Fc region consists of the CH2 and CH3 segments, which interact with Fc receptors or complement proteins to mediate various cytotoxic functions such as antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent complement deposition (ADCD) and antibody-dependent cellular trogocytosis (ADCT) (153–155). Furthermore, an N-linked glycan is present at position 297 of the CH2 segments as a

result of a highly conserved asparagine. This glycosylation site has a role in regulating Fc receptor binding and function (153,154).

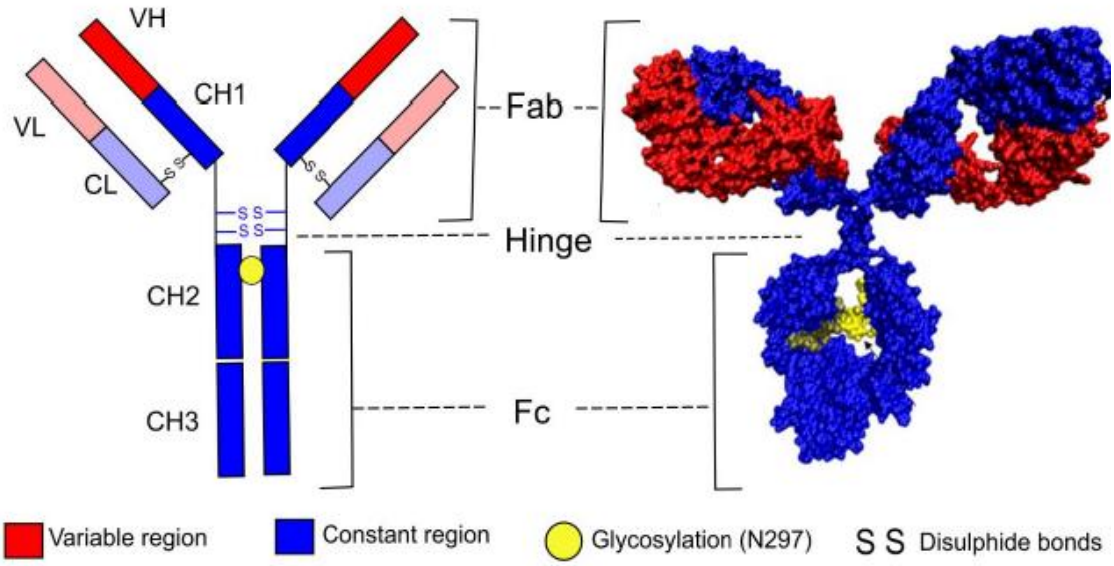


Figure 8: Antibody (IgG) Structure.
An antibody consisting of two heavy chains and two light chains joined by disulphide bonds. The structural domains consist of two Fab regions and one Fc, which are each comprised of variable (red) and constant (blue) regions. A conserved glycan is present in the Fc (yellow). VH = variable heavy, VL= variable light, CH = constant heavy and CL = constant light. Source: Richardson, 2018 (156).

4.2 Antibody Functions

4.2.1 Neutralization

Neutralization is an important function by which antibodies can protect against respiratory viral infections. The Fab region binds to the antigens responsible for recognizing the host cell receptors, preventing cell entry and directly blocks infection (157). This process is essential for acquiring sterile immunity against viral infections (158). Traditionally, neutralization has been regarded as the main correlate of vaccine protection against many viral pathogens. However, high mutation rates in neutralization antigenic targets result in immune escape (159). Several studies have shown that neutralization alone does not always predict the protective capacity of antibodies. Instead, some neutralizing antibodies require Fc gamma receptor (FcγR) engagement against viral pathogens (155,160,161). Furthermore, broadly neutralizing antibodies have been described for several viral pathogens, but these are difficult

to elicit by vaccination in certain diseases such as HIV and influenza (162,163). So in addition to neutralization, Fc effector functions that can potently target conserved antigens are a key focus for broadly protective vaccine design (164–166).

4.2.2 Fc Effector Functions

Various cytotoxic functions including antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent complement deposition (ADCD) and antibody-dependent cellular trogocytosis (ADCT) are able to bridge the innate and adaptive immune systems by linking the potent activity of effector cells and complement proteins with the diversity and specificity of the humoral response.

4.2.2.1 Antibody-dependent Cellular Phagocytosis

Antibody-dependent cellular phagocytosis (ADCP) is primarily mediated by IgG interacting with FcγRI (CD64) and FcγRIIa (CD32A) expressed on mononuclear phagocytes (monocytes, macrophages and dendritic cells) or granulocytes (167). As antibodies bind to virus or virus-infected cells, crosslinking of different FcγRs results in signalling via immunoreceptor tyrosine-based activation motifs (ITAMs), which leads to the engulfing of immune complexes followed by degradation in lysosomes (**Figure 9**) (157). This mechanism results in the accelerated clearance of virus and infected cells but can also facilitate antigen presentation via major histocompatibility complex (MHC) molecules to T-cells (168).

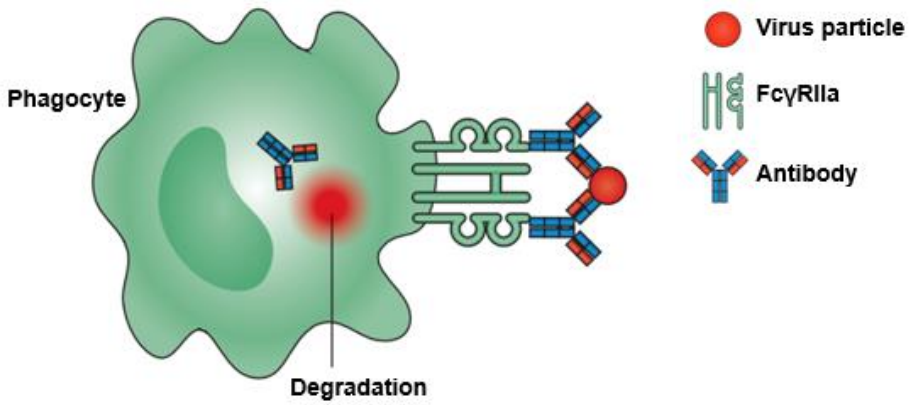


Figure 9: Antibody-dependent Cellular Phagocytosis (ADCP).
Virus particles or infected cells are opsonized by antibodies. Interaction between the phagocyte FcRs and Fc region of antibodies results in the uptake and degradation of immune complexes. Figure adapted from: Lu *et al.,* 2017 (157).

4.2.2.2 Antibody-dependent Cellular Cytotoxicity

Antibody-dependent cellular cytotoxicity (ADCC) typically occurs when there is crosslinking of FcγRIIIa (CD16) expressed on natural killer (NK) cells by the Fc region of IgG antibodies bound to viral antigens on the surface of infected cells. The activated cells release cytotoxic granules perforin and/or granzymes, which induce apoptosis of the infected cell (**Figure 10**) (155). Immune cell activation can also lead to the secretion of other anti-viral cytokines and chemokines. FcγRIIIa is found on the surface of human NK cells, monocytes, and macrophages (169). However, ADCC is predominantly mediated by NK cells. The clearance of virus-infected cells, as well as the release of cytokines, contributes to decreasing virus replication and spread (170).

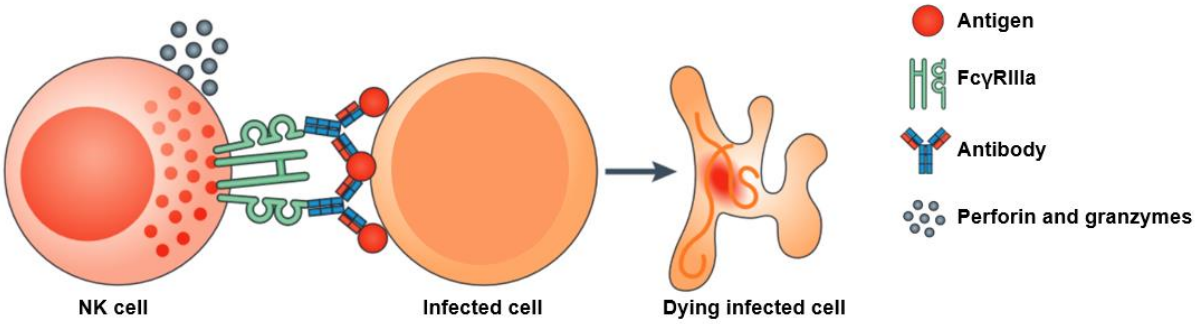


Figure 10: Antibody-dependent Cellular Cytotoxicity (ADCC).
Natural killer (NK) cells are activated through FcγRIIIa binding with the Fc region of antibodies bound to virus infected cells. NK cells release cytotoxic perforin and granzymes that cause the death of the infected cell. Figure adapted from: Lu *et al.,* 2017 (157).

4.2.2.3 Antibody-dependent Complement Deposition

The complement system comprises the classical, lectin and alternative pathways. Antibody-dependent complement deposition (ADCD) occurs through the classical pathway, which is activated when C1q interacts with antibodies, typically IgM and IgG with higher affinities for IgG1 and IgG3, bound to antigens on the surface of virus or virally infected cells (171). Binding of C1q to antigen–antibody complexes leads to formation of C1, which results in a cascade of enzymatic activity involving C3 convertase and deposition of C3b on membrane surfaces (**Figure 11**). This can result in virus clearance by opsonization and phagocytosis, or target cells and enveloped viruses may undergo osmotic lysis due to the insertion of the membrane attack complex (MAC) (171,172).

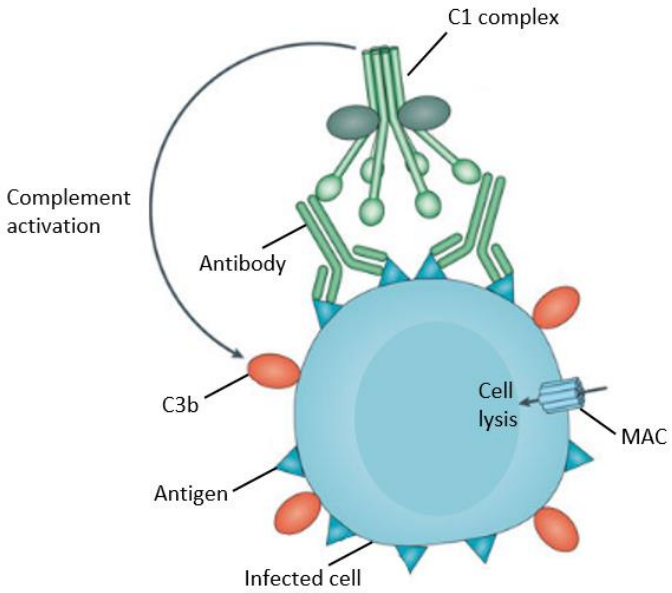


Figure 11: Antibody-dependent Complement Deposition (ADCD).
Infected cells are bound by antibodies. Binding of C1q to the Fc region of these antibodies leads to the assembly of the active C1 complex. Complement activation initiates a cascade of proteolytic activity: C3 convertase cleaves C3 into C3b which is deposited on the surface of the infected cell and serves as a ligand for complement receptors, or goes on to form C5 convertase which along with other proteins forms the membrane attack complex (MAC) that leads to cell lysis. Figure adapted from: Reis *et al.,* 2018 (173).

4.2.2.4 Antibody-dependent Cellular Trogocytosis

The process of removing and transferring fragments of membrane with associated antigen or surface molecules from one cell to another cell is called trogocytosis (174). Most immune cells including monocytes and macrophages can mediate trogocytosis, which suggests that there are various immunological consequences of this process (175). Here, we focus on antibody-dependent cellular trogocytosis (ADCT) mediated by the recognition of antibody-antigen immune complexes on target cells by FcγRs on healthy effector cells via immunological synapses (**Figure 12**) (174). ADCT is distinguished from ADCP as it leads to the transfer of membrane bound immune complexes as opposed to complete engulfment (176). Although limited, studies indicate that FcγRI, FcγRIIa and FcγRIIb are involved in the occurrence of ADCT (177–183). Several studies, including one in HIV, have shown that ADCT results in death of infected cells and efficient transfer of antigen to uninfected cells (177,184–186). However, the ADCT protective capacity or impact on disease progression requires further investigation (155).

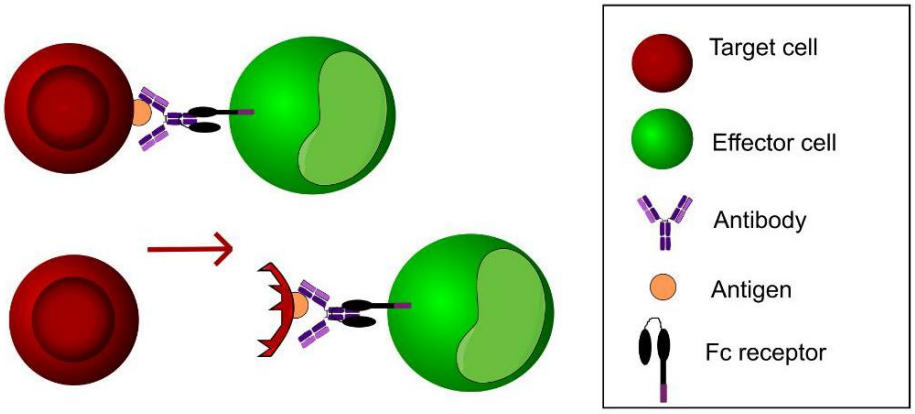


Figure 12: Antibody-dependent Cellular Trogocytosis (ADCT).
Antibodies bind to antigen expressed on the surface of target cells, the Fc portion of the antibody binds to the Fc receptor on the surface of an effector cell. These interactions result in the pulling apart of the target cell plasma membrane for antigen presentation by the effector cell. Source: Richardson, 2018 (156).

4.3 Modulators of Fc Effector Functions

4.3.1 Properties of Isotypes and Subclasses

B-cell activation results in the development of various isotypes through a molecular mechanism known as class switch recombination (CSR) (187). Class-switched B-cells have enhanced response rates to antigens due to alterations in the Fab variable regions (150). Additionally, the antibody isotypes (IgM, IgD, IgG, IgA and IgE) and subclasses (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) (**Figure 13**) substantially affect the Fc functional capacity of antibodies by regulating the binding affinity for different Fc receptors and complement proteins (188).

4.3.1.1 IgM

As a monomer, IgM exists as membrane-bound BCRs on the surface of naïve B-cells. Multimeric (pentameric or hexameric) IgM is the first isotype to be secreted during an immune response and has a half-life of about 5 days (189,190). This unique structure enhances avidity to antigens through the presence of multiple binding sites and allows for higher complement binding through the protruding structure of Fc regions containing a joining chain (J chain) (191,192). IgM functions by opsonizing viruses for phagocytic clearance and fixing complement early in infection (157). Since, this isotype has low affinity maturation, it is typically polyreactive to endogenous antigens to allow for a rapid immune response to a wider range of antigens and to induce other immunoregulatory functions (193).

4.3.1.2 IgD

Similar to IgM, IgD is also a membrane-bound BCR and these isotypes can be co-expressed on the surface of B-cells. Monomeric IgD is secreted in small amounts and has a short half-life of approximately 3 days (194). The function of IgD is unclear, but the presence of these antibodies in the respiratory mucosa and ability to bind basophils, mast cells, monocytes and dendritic cells suggests that this isotype may have an important role in mucosal immunity. The binding to innate immune cells is not dependent on Fc receptors and effector functions have not been described for this isotype (189,195,196).

4.3.1.3 IgE

Associated with allergic diseases and parasitic infections, IgE circulates in blood at the lowest levels and with the shortest half-life when compared to all the isotypes (197). IgE mediates potent Fc effector functions through binding with high affinity to the FcεRI which is expressed on mast cells, basophils, Langerhans cells and eosinophils (198). It also binds with low affinity to FcεRII (CD23), which is expressed on B-cells, NK cells and platelets and has been linked to a non-inflammatory pathway for IgE-allergen complexes, regulating serum IgE levels and playing a role in antigen presentation (199–201).

4.3.1.4 IgA

IgA is present in the blood primarily as a monomer, at higher levels than IgM but lower levels than IgG (189). IgA has an estimated half-life in circulation between 4 and 7 days (202). In the mucosa, secretory IgA antibodies are the most abundant isotype existing as a dimer or higher-order polymers with a J-chain and a secretory component that is derived from a polymeric immunoglobulin receptor (**Figure 13**) (203). Divided into two subclasses, IgA1 has a flexible longer hinge region compared to IgA2, which is more susceptible to proteolytic cleavage resulting in reduced amounts of IgA1 in mucosa (204). The protective functions of IgA include neutralization or Fc effector functions mediated by FcαR (CD89), present on neutrophils, monocytes, eosinophils, some macrophages and dendritic cells. The activation of FcαR induces ADCC or phagocytosis (205,206). IgA lacks the site for C1q binding and therefore does not activate the classical pathway of complement (207).

4.3.1.5 IgG

IgG is the most abundant antibody isotype in human serum. The four subclasses are named IgG1, IgG2, IgG3, and IgG4 in order of decreasing abundance. Despite the conserved amino acid sequence (90% identical), each subclass differs in terms of antigen binding, hinge regions, complement activation, triggering of effector functions, half-life, and placental transport (208).

Viral infections or exposure to protein antigens normally induce IgG1 and IgG3 antibodies while IgG2 antibodies are associated with exposure to bacterial polysaccharide antigens (209,210). In addition to IgE, IgG4 antibodies are elicited in response to allergens following repeated and long term exposures (208). The hinge length of an isotype influences the effector function capacity and half-life of the antibodies. The proportion of IgG1 antibodies is around 65% of the total serum IgG (211). This subclass has moderate binding affinity to Fc receptors and an average half-life of 21 days (212). The subclass with the highest potential for polyfunctionality is IgG3 due to having the longest hinge region (213). However, only some IgG3 antibodies display improved neutralization and Fc effector functions depending on the virus strain and epitope specificity, as shown in HIV (214–216). However, IgG3 has a shorter half-life of around 7 days, linked to reduced binding affinity to neonatal Fc receptor (FcRn) (217,218). Furthermore, IgG1 and IgG3 can efficiently trigger the classical route of complement, but IgG2 and IgG4 are less efficient at triggering this function (189,219).

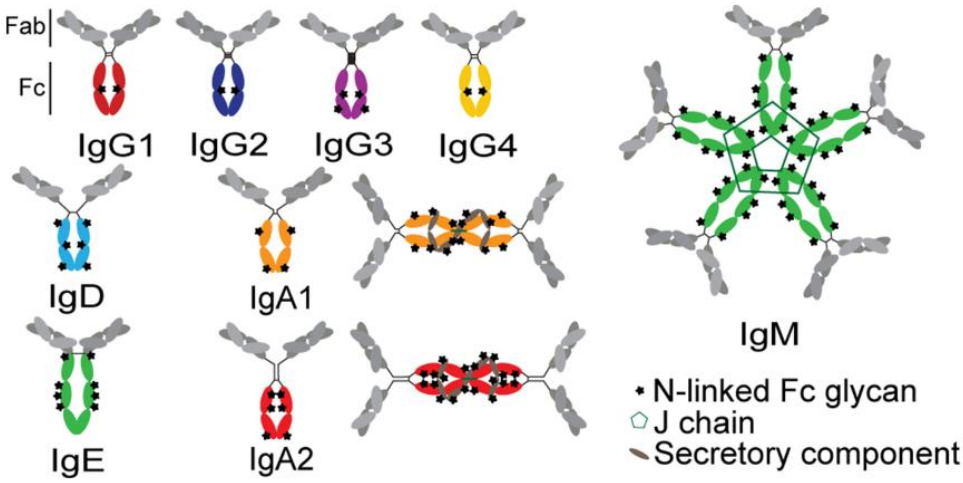


Figure 13: Antibody Isotypes and Subclasses.
Monomeric and multimeric structures of five isotypes (IgM, IgD, IgG, IgA and IgE) and six subclasses (IgG1–4, IgA1 and IgA2) are shown with Fc domains in colour while Fab domains are in gray. Stars indicate N-linked Fc glycans. Source: Boudreau and Alter, 2019 (170).

4.3.2 Glycosylation

Post-translational Fc glycosylation at the conserved asparagine 297 (N297) mediates antibody effector polyfunctionality (220). Factors such as age, sex, pregnancy and chronic comorbidities, such as HIV, substantially influence the glycosylation of antibodies (221). The IgG subclasses each have distinct glycosylation profiles (222,223). Glycan chains are biantennary structures (**Figure 14**), consisting of N-acetylglucosamine (GlcNAc) subunits from which mannose subunits form two branching structures allowing for the addition of galactose and sialic acid. Core fucosylation is the addition of monomeric fucose to the N297 proximal GlcNAc while afucosylation is the removal of this polysaccharide. Several IgG-Fc glycoforms have been identified in humans; based on the composition the glycan delineates the structure of the Fc region allowing for specific interactions with FcγRs and triggering activating or inhibitory signalling (208,224,225).

More specifically, sialylation has been linked to enhanced anti-inflammatory activity (226). Galactosylation also results in anti-inflammatory activity but a recent study of hypergalactosylated monoclonal antibodies enhanced FcγRIIIa binding driving ADCC, suggesting that glycosylation patterns may have varying influences on different effector functions (227). Fucosylation of the Fc glycan has been shown to decrease ADCC activity as a result of steric hindrance interfering with FcγRIIIa binding, while afucosylation stabilizes interactions with FcγRIIIa and boosts ADCC (228,229). The importance of antibody glycosylation in mediating antibody functionality indicates that investigating glycosylation profiles in different populations in response to respiratory viral infections and vaccinations may provide novel insights for the design of improved vaccines.

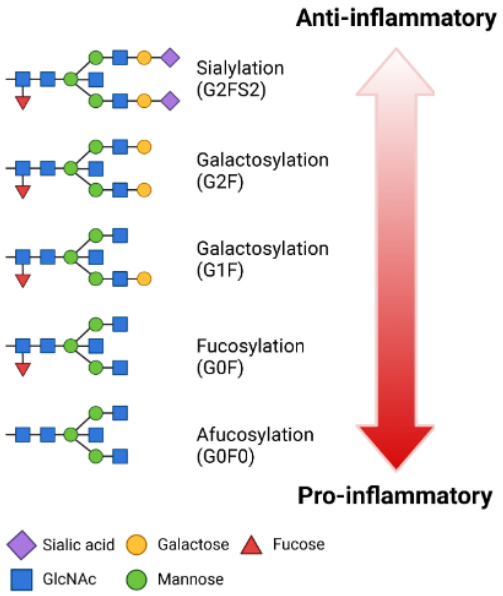


Figure 14: IgG Fc glycan structures.
Fc glycosylation differentially modulates Fc effector functions and therefore inflammation. Overall, the lack of fucose is highly inflammatory while the presence of galactose and sialic acid is anti-inflammatory Figure adapted from: Purcell, 2023 (221).

4.3.3 Fc Receptors

The Fc effector functions for each of the antibody isotypes are also modulated by binding interactions with a particular set of Fc receptors, IgM (FcμR), IgD (FcδR), IgE (FcεR), IgA (FcαR) and IgG (FcγR) (189,230). Of these Fc receptors, the distribution on cells, polymorphisms and signalling activities for FcγRs and binding affinities to IgG is well-studied. In humans, six types of FcγRs have been identified, activating (FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa and FcγRIIIb) or inhibitory (FcγRIIb) (**Figure 15**) (231). The majority of innate immune effector cells, including mast cells, basophils, eosinophils, monocytes, macrophages, dendritic cells (DCs) and neutrophils as well as B-cells and NK cells express FcγRs (232).

FcγRI has three extracellular domains resulting in the highest affinity for monomeric IgG while FcγRII and FcγRIII each have two extracellular domains and bind with lower affinity or require cross-linking of IgG (189). IgG1 and IgG3 bind to all FcγRs with greater affinity than IgG2 and IgG4 antibodies. IgG2 is the only subclass that does not bind to FcγRI (233). A common γ-chain subunit distinguishes FcγRI from the other FcγRs and allows for its stable expression on cells (231). The recruitment of signalling proteins is facilitated by an intracellular immunoreceptor tyrosine-based activating motif (ITAM) for FcγRIIa and FcγRIIc, and an

immunoreceptor tyrosine-based inhibition motif (ITIM) for FcγRIIb (234). FcγRIIa have two polymorphic variants H131 and R131, of which IgG2 has reduced affinity for R131 (233). The co-expression of FcγRIIa and FcγRIIb regulates the activation or inhibition of ADCP, respectively (188). In addition to ADCP, the activating FcγRIIc is expressed at low levels on NK cells and B cells enabling ADCC and B cell activation, respectively (235). FcγRIIIa is predominantly found on NK cells and mediates ADCC with polymorphic variant V158 having a higher affinity to all subclasses than F158 (236). The function of FcγRIIIb is unclear but it can be found on the surface of neutrophils and bound to the cell membrane by a glycosylphosphatidylinositol (GPI) anchors (234). Overall, Fc effector functions are dynamic due to the influence of various factors including the isotype, subclass, glycosylation and Fc receptor binding.

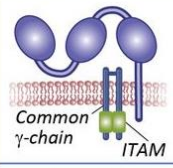
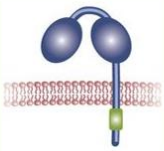
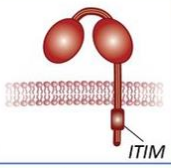
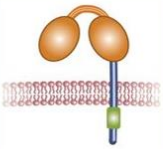
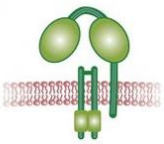
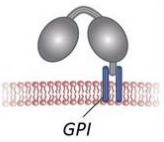
Name	FcγRI CD64	FcγRIIa CD32A	FcγRIIb CD32B	FcγRIIc CD32C	FcγRIIIa CD16A	FcγRIIIb CD16B
Structure						
Function	Activating	Activating	Inhibitory	Activating	Activating	Activating
Affinity	High	Low	Low	Low	Low	Low
Cell Distribution	Macrophages, monocytes, neutrophils, dendritic cells, mast cells	Macrophages, monocytes, neutrophils, eosinophils basophils, dendritic cells, mast cells	Macrophages, monocytes, eosinophils, basophils, B cells, mast cells, dendritic cells[b]	NK cells, monocytes, neutrophils, macrophages, B cells[c]	NK cells, NKT cells, γδ T cells, dendritic cells, macrophages, monocytes, neutrophils, eosinophils	Neutrophils, eosinophils, basophils
Effect of Antibody Binding	ADCP, cytokine release	ADCC, ADCP, vaccinal effect[a]	Inhibits ADCC, ADCP, B cell activation	Enhances ADCC, ADCP, B cell activation	ADCC, ADCP	Decoy receptor that inhibits ADCP[d]

Figure 15: Human IgG-specific FcγRs.
FcγRs differ in their structure, function, cell distribution, signalling motifs and affinity for IgG molecules. Source: Musolino, 2022 (235).

4.4 Fc Effector Functions Are Important for Protection Against Respiratory Disease

The importance of Fc-mediated functions in both influenza virus and SARS-CoV-2 studies has been demonstrated. In animal models, *in vivo* protection against influenza virus challenge has been associated with ADCP, ADCC and ADCD activity (237–241). In humans, seasonal influenza vaccines boost Fc-mediated functions, although to varying degrees depending on the immune state of the individuals (242–244). Fc functionality is highly cross-reactive targeting antigenically drifted strains of influenza viruses (245–247). Additionally, a HA stalk-based vaccine elicited cross-reactive antibodies with Fc functionality in a phase 1 trial (248).

For SARS-CoV-2, Fc effector function has been associated with improved disease outcome (249). While VOCs escape neutralization activity, Fc-mediated functions are retained contributing to protection against severe disease (250). Furthermore, SARS-CoV-2 antibodies with Fc effector function show improved therapeutic potential (161,251,252). Similar to influenza, Fc-mediated functions correlate with protection in animal models and were elicited by the current SARS-CoV-2 vaccines in humans (253–257).

5. Current Vaccination Strategies

Vaccination remains the best tool in the prevention of respiratory viruses (258). In South Africa, providing 1 million seasonal influenza vaccines to high-risk populations averted an estimated >94,000 influenza virus infections and reduced influenza-associated hospitalizations, with the greatest benefit observed among PLWH and persons with other underlying medical conditions (32). Similarly, administering COVID-19 vaccines to approximately 40% of the South African population is estimated to have prevented >9 million infections, >73,000 deaths, and decreased the burden of costs due to reduced hospitalizations (259,260).

5.1 Influenza

5.1.1 Inactivated Influenza Vaccines

Seasonal inactivated influenza vaccines (IIV) are still widely used globally, despite the availability of intranasal live attenuated influenza vaccines (LAIV) and recombinant hemagglutinin (rHA) subunit vaccines (261). There are three types of IIV: whole-virion vaccines, split-virion vaccines, and subunit vaccines (261). These vaccines are manufactured by propagating influenza viruses in eggs, inactivating with formalin or beta-propriolactone and using detergents to split the virion (262). A single dose of IIV is administered annually through intramuscular injection, prior to or during the influenza season (between May and September in South Africa) (263). The WHO Global Influenza Surveillance and Response System (GISRS) is responsible for recommending separate formulations for the Southern and Northern Hemisphere vaccines each year. The chosen formulations are informed by surveillance data obtained by numerous national surveillance programs worldwide (264–266). IIV are produced as either trivalent (TIV) or quadrivalent (QIV) compositions both containing two influenza A strains A/H1N1, A/H3N2 and either one of the influenza B lineages (Victoria or Yamagata lineage) or both influenza B lineages, respectively (267). However,

low- and middle-income countries, including South Africa have had limited access to QIV. Furthermore, since March 2020, influenza B Yamagata has not been detected globally (263). Therefore, as of September 2023, the WHO Southern hemisphere recommendation is to exclude B/Yamagata reverting back to trivalent formulations (268). Currently, the priority groups for receiving TIV in South Africa include healthcare workers, individuals aged ≥ 65 years, individuals with chronic diseases (cardiovascular, diabetes, lung disease or HIV/AIDS) and pregnant women (263).

A major challenge for the use of strain-specific TIV is the high rates of antigenic drift in influenza viruses, which results in mismatches between circulating and vaccine strains, substantially reducing vaccine efficacy (258,264,269–271). In addition to antigenic mismatch, other major concerns include inconsistent vaccine efficacy due to host factors such as age, sex, comorbidities and pre-existing immunity, and the lack of a durable immune response even in healthy individuals (85,272–277). Despite recommendations of annual influenza vaccination, poor immunogenicity in response to these seasonal vaccines in PLWH has been documented (37–41). Most importantly, these vaccines provide limited protection against zoonotic strains with pandemic potential, against which individuals have no natural immunity. Therefore, there is an urgent need to better understand immune responses to current vaccines to inform the development of broadly protective “universal” influenza vaccines.

5.1.2 Universal Influenza Vaccines

In recent years, several advances have been made in the development of universal influenza vaccines. These include vaccines that would provide protection against both group 1 and group 2 influenza A viruses (influenza B would be a secondary target), be at least 75% effective against symptomatic influenza infection and have durable protection over multiple influenza seasons (278,279). Multiple strategies and vaccine platforms are under investigation including targeting conserved viral antigens or epitopes of the HA stalk, M2, NP and NA to induce broadly protective antibodies and developing novel platform technologies, which use virus-like particles (VLPs), nanoparticles, adenoviral vectors and deoxyribonucleic acid (DNA) or messenger RNA (mRNA)-based approaches (280–282). These platforms have had great success in the development of effective COVID-19 vaccines (**discussed in section 5.2.1**). Here, we will discuss one of the main, novel, conserved target strategies: HA stalk-based vaccines.

5.1.2.1 HA Stalk-based Vaccines

Influenza HA stalk-specific antibodies display cross-reactivity within group 1 or group 2 HAs, across the influenza A groups and between influenza A and B HAs, although the latter is rare (83,283). These antibodies have been shown to confer broad protection in mice (284,285). Since these discoveries, several vaccination strategies to induce these antibodies have been developed. To overcome the immuno-subdominance of the HA stalk, approaches include the use of mosaic HA, chimeric HA, headless HA constructs and computationally-optimized broadly reactive antigens (COBRA) (286).

These chimeric HAs (cHAs) consist of the HA stalk domain from current seasonal influenza viruses and an HA head domain from divergent avian viruses (**Figure 16**) (287). Initial vaccination with a cHA vaccine induces antibodies specific to both the head and stalk domains. Sequential immunization with the cHAs with the same HA stalk but different HA head domains elicits a higher immune response against the HA stalk domain due to a memory recall response, eventually leading to broader protection (**Figure 16**) (282). A benefit to this approach is that humans are naïve to the HA head avian strain but have pre-existing HA stalk antibodies. Therefore upon vaccination with cHAs, immune responses are re-directed from the immunodominant HA head to immunosubdominant HA stalk (288). Vaccination with cHA using various vaccine platforms, such as inactivated virus, live-attenuated virus, recombinant protein, viral vectors or nucleotide-based vaccines, has been investigated and cHAs have been utilized for influenza A group 1 and group 2 and influenza B HAs, conferring protection in animal models (248,289–298).

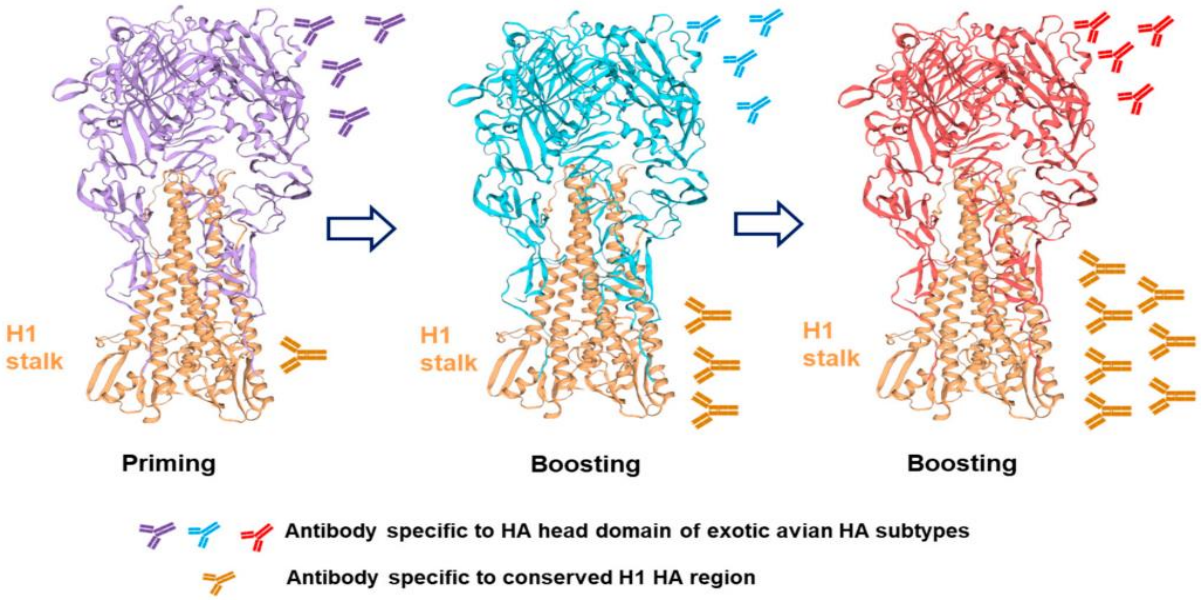


Figure 16: Chimeric HA stalk-based vaccination strategy.
The cHA vaccine contains a stalk domain from H1 HA and head domain from an avian HA subtype. Sequential boosting with cHAs containing the same stalk H1 HA domain with divergent HA head domains directs antibody responses to the H1 HA stalk domain. Source: Nguyen and Choi, 2021 (286).

Recently, results from a phase I clinical trial of cHA as a universal influenza vaccine have been reported (248,299). The cHA vaccines were designed to carry the head domain from H8 and H5 with the H1 stalk, and administered as different prime-boost combinations of live-attenuated virus, inactivated virus, or inactivated virus containing adjuvant AS03. Vaccinated individuals had enhanced titers of HA stalk-specific antibodies with cross-reactivity against several heterologous group 1 HAs, lasting for up 1.5 years. Passive transfer of vaccinee serum to mice provided broad protection, as indicated by the reduction in weight loss following influenza virus challenge (241,248,299). While the cHA vaccine is safe and elicits HA stalk antibodies in humans, further studies are needed to demonstrate the efficacy and protective mechanisms of these HA stalk-specific antibodies against influenza virus infection.

5.2 SARS-CoV-2

5.2.1 Approved Vaccine Platforms and Efficacy

As of March 2023, more than 380 COVID-19 vaccines had been developed, of which 81 vaccines have been evaluated in phase III clinical trials (300). In South Africa, 6 vaccines have been approved for use including BNT162b2, ChAdOx1 nCoV-19, Ad26.COV2.S, BBIBP-CorV, CoronaVac and NVX-2373. These SARS-CoV-2 vaccines had high efficacy

against the ancestral Wuhan or D614G strain (**Table 1**) and were able to elicit robust humoral and cellular responses, thereby reducing the risk of severe COVID-19. BNT162b2 and mRNA-1273 had the highest efficacy (>90%) reported 5-6 months after the second dose (301–304). However, the emergence of SARS-CoV-2 VOCs became a threat to global vaccination efforts. For example, the viral vector vaccines ChAdOx1 nCoV-19 (10,4%) and Ad26. COV2.S (64,7%) had reduced vaccine efficacy against Beta, respectively (305,306). While phase III trials assess the efficacy under controlled conditions and typically assess immune responses in healthy adults, immunocompromised individuals such as PLWH have different responses, which may affect vaccine efficacy. Evidence of this was observed with NVX-2373 trial, vaccine efficacy amongst PWOH was 60% but was reduced to 49.4% upon the inclusion of PLWH (307). This highlights the importance of investigating immunogenicity to respiratory viral vaccinations in PLWH.

Table 1: COVID-19 vaccine efficacy.

Vaccine Manufacturer (Vaccine Name)	Platform	Phase III Trial Efficacy
Pfizer–BioNTech (BNT162b2)	mRNA	91.3%(301), 95%(302)
Moderna/NIAID (mRNA-1273)	mRNA	93.2%(303) and 94.1%(304)
AstraZeneca (ChAdOx1 nCoV-19/ AZD1222/Covishield)	viral vector	66%(308)
Johnson & Johnson (Ad26. COV2.S)	viral vector	69.7%(309)
Sinopharm (BBIBP-CorV)	inactivated virus	78.1%(310)
Sinovac Biotech (CoronaVac)	inactivated virus	83.5%(311)
Novavax (NVX-CoV2373)	Protein subunit	49.4%(307)

6. Humoral Immunity to Influenza in People Living with HIV

6.1 Antibody Responses to Influenza Vaccination in People Living with HIV

6.1.1 Hemagglutination Inhibition and HA Stalk Antibody Titers

Studies investigating antibody responses to seasonal TIV in PLWH are limited. These primarily report on the seroconversion rates of hemagglutination inhibition (HAI) titers (proxy for neutralization) against all strains of the vaccine. We (Paper 1 of this thesis (312)*), and others, have shown that PLWH have lower HAI titers than PWOH in response to seasonal influenza vaccination (39,313). Impaired HAI titers after influenza vaccination have been associated with low CD4+ T-cell counts in PLWH (314–316). In contrast, Kristensen et al. observed similar HAI titers between ART-suppressed PLWH with high CD4+ T-cell counts and PWOH (242). Overall, PLWH have suboptimal HAI responses to seasonal influenza vaccination, despite this, high vaccine efficacy has been reported in this vulnerable population (39–41). This prompted the evaluation of HA stalk antibodies by Dhar et al. using an enzyme linked immunosorbent assay (ELISA), as the HAI assay is unable to detect antibodies directed at the HA stalk (43). We show that HA stalk antibodies are substantially boosted after seasonal TIV irrespective of HIV status, although remaining lower in PLWH than in PWOH (43,312)*. Dhar et al. found that high HA stalk antibody titers are associated with protection against influenza-illness following immunization with seasonal TIV (43). Since HA stalk antibodies have been shown to mediate potent Fc effector functions (160), we next investigated these mechanisms in PLWH and PWOH following seasonal TIV.

6.1.2 Antibody Fc Effector Functions

In this thesis, we report that both HA stalk-specific ADCP and ADCC were higher in ART-controlled PLWH, pre-vaccination. Irrespective of HIV status, HA stalk-specific ADCC was not boosted following vaccination and ADCP was only boosted in PWOH (312)*. We also measured HA stalk-specific ADCD responses, which were significantly boosted regardless of HIV status but remained lower in PLWH. The co-ordination of the these Fc effector functions, important for increased vaccine efficacy (317), differed by HIV status but were only slightly improved by TIV (312)*. Additionally, Fc polyfunctionality was enhanced by vaccination in PWOH and associated with the HA stalk antibody titers. While, in the PLWH, higher pre-vaccination Fc polyfunctionality was sustained post-vaccination but was not linked to the HA

stalk antibody titers (312)*. Here the Fc polyfunctionality z-scores were calculated to account for the contribution of each the measured Fc effector functions equally. This score therefore allows the generalization of overall functional comparisons across individuals. This calculation has been used in other diseases such as HIV (318). The findings of different functional profiles and associations by polyfunctionality z-scores across different populations suggests that the score is biologically relevant. Lastly, Kristensen et al. reported the boosting of ADCC but not ADCP in PLWH after influenza vaccination while in PWOH both these functions were boosted (242). Overall, PLWH have different Fc-mediated responses to PWOH after seasonal TIV, of which the responses are also differentially regulated.

6.2 Novel Correlates of Protection following vaccination

In terms of existing correlates of protection, the HAI assay has a universally accepted threshold (≥1:40) that indicates a 50% reduction in the risk of acquiring influenza-illness in healthy adults against matched influenza virus strains (319). In addition to HAI antibody titers, several other immunological responses have been proposed as potential correlates of protection (320). These include neuraminidase inhibition (NAI) antibody titers, cross reactive CD4+ and CD8+ T-cells and HA stalk antibody titers, which have been shown to be independent correlates of protection in both animal and human studies (43,321–325).

Prior to this thesis, HA stalk-specific Fc effector function association with protection following seasonal TIV had not been shown. However, other research groups have shown seasonal influenza vaccine boosting of HA Fc effector functions in adults ≥65 years old and PLWH (242–244). In this thesis, we show (Paper 2 submitted to Clinical Infectious Diseases, December 2023) that H1 stalk-specific ADCP and ADCD, but not ADCC are boosted by seasonal TIV in a cohort that included PLWH. The study was not powered to assess the protective capacity of these HA stalk-specific Fc-mediated functions by HIV status due to the small number of confirmed influenza cases. However, H1 stalk-specific ADCP (≥250) and ADCD (≥25) were associated with >83% risk reduction against A/H1H1 illness in pregnant women living with or without HIV-1 while ADCC was not associated with protection in this trial. More specifically, the H1 stalk-specific ADCD was protective following immunization with TIV. Overall, our findings suggest that in addition to neutralization or HAI, HA stalk-specific Fc functions contribute to protection against influenza-illness following seasonal TIV in pregnant women, which is an important consideration for future influenza vaccine design, particularly for HA stalk-based approaches.

7. Humoral Immunity to SARS-CoV-2 in People Living with HIV

7.1 Antibody Responses to SARS-CoV-2 Infection in People Living with HIV

7.1.1 Prolonged Viral Shedding

Prolonged SARS-CoV-2 virus shedding in immunocompromised individuals, including people with advanced HIV, during which viral evolution occurs within the host, has been described (54,55,57–59,326–335). Immune dysfunction associated with advanced HIV infection results in the inability to clear SARS-CoV-2. The longest duration of viral shedding reported in a case study of uncontrolled HIV was 9 months (58). Several studies have shown that during the extended period of SARS-CoV-2 infection in PLWH the virus accumulated numerous mutations across the genome, including in the NTD and RBD regions of spike (55,57,58,330–332). Some of these mutations were associated with neutralization escape and were later found in SARS-CoV-2 VOCs (55,57,330). Effective vaccines for PLWH are therefore crucial and investigating SARS-CoV-2 humoral responses in this population may inform implementation.

7.1.2 Binding and Neutralizing Antibodies

Studies comparing the immune responses following SARS-CoV-2 infection between PLWH and PWOH were primarily focused on IgG binding and neutralizing responses but have yielded inconsistent results. We and others have shown that in ART-naïve or HIV viremic PLWH with low CD4 counts <200 cells/ul, IgG binding and neutralizing antibody titers are substantially reduced against SARS-CoV-2 (Paper 3 of this thesis (336)*) (50,60,337,338). In contrast, Alrubayyi et al. reported similar and long-lasting humoral and SARS-CoV-2-specific T-cell responses in both PLWH on ART and PWOH (339). Thereafter, several studies including ours, with cohorts of PLWH majority on ART, have shown similar SARS-CoV-2-specific IgG titers and neutralization responses amongst convalescent COVID-19 patients living with or without HIV (336,340–343)*. Additionally, Snyman et al. reported similar kinetics to peak IgM and IgG responses during acute SARS-CoV-2 infection in ART-controlled PLWH and PWOH (340). However, we observed delayed neutralization in PLWH on ART following D614G infection but no delays with Beta infection, despite similar cohort characteristics (336)*. This suggested that some PLWH on ART may have incomplete restoration of immune

functions, resulting in impaired SARS-CoV-2 responses (21,23). Notably, the differences between PLWH and PWOH observed across studies are dependent on the immune competence of the PLWH included in the cohorts but COVID-19 severity further influences the humoral response in PLWH. Severe COVID-19 induces comparable responses between PLWH and PWOH while symptomatic non-hospitalized disease results in lower magnitude responses in PLWH compared with PWOH (344). Overall, binding and neutralizing responses to SARS-CoV-2 in PLWH are equivalent to the responses in PWOH (336)*. In this thesis, we also assessed the magnitude and kinetics of SARS-CoV-2 antibody Fc effector functions following infection with either D614G or Beta variants in the context of HIV co-infection (discussed below).

7.1.3 Antibody Fc Effector Functions

Schuster et al. found similar response rates and levels of ADCP in a multinational (USA and Peru) cohort of PWOH and ART-suppressed PLWH (344). These participants had either asymptomatic or symptomatic SARS-CoV-2 infection not requiring hospitalization. The ADCP responses were robust in these individuals considering that the IgG responses were lower in comparison to the few hospitalized PLWH included in the study. This indicated that the ability to elicit ADCP is sustained irrespective of antibody titer and disease severity. Similarly, we observed comparable ADCP levels between hospitalized PWOH and PLWH, irrespective of D614G or Beta variant infection (336)*.

The magnitude and kinetics of SARS-CoV-2 ADCC, ADCD and ADCT have not been described in PLWH. In this thesis, we examined these responses and found that PLWH on ART were capable of eliciting a robust Fc-mediated response after SARS-CoV-2 infection, similar to PWOH (336)*. However, the levels of Fc activity varied depending on which infecting variant triggered the response, as we had previously reported (250). Furthermore, despite delays in some responses, PLWH had triggered an enhanced coordinated Fc effector response in comparison to PWOH (336)*, which has been associated with COVID-19 recovery (249,345). Given that antibody Fc effector functions contribute to protection, it is important that we are able to leverage these responses through vaccination, especially for vulnerable populations.

7.2 Antibody Responses to SARS-CoV-2 Vaccination in People Living with HIV

Several studies have reported similar binding and neutralizing responses between PLWH and PWOH following BNT162b2, mRNA-1273, Ad26.COV2.S and ChAdOx1 nCoV-19 vaccination (60–68). In our study, we observed delayed IgG binding 28 days after the first dose of ChAdOx1 nCoV-19. However, after the second dose, similar to other studies, PLWH achieved equivalent binding levels that were higher than in PWOH at 6-months post-vaccination (336)*. Madhi et al. also demonstrated higher SARS-CoV-2 spike binding antibodies in PLWH with each dose of ChAdOx1 nCoV-19 (65). Initial delays reported did not influence the elicited neutralization responses in PLWH, which were similar throughout despite trending lower than in PWOH. We, and others, have shown that ADCC responses are durable in PLWH and exceed the levels in PWOH after ChAdOx1 nCoV-19 vaccination, highlighting that ADCC may be an important mechanism for protection against SARS-CoV-2 (312,346).

8. Conclusion

In this thesis, we contribute to the current knowledge about crucial Fc effector functions in PLWH during respiratory viral infection and vaccination. In a maternal influenza vaccination cohort, where high levels of HA stalk antibodies were associated with protection, we show that HA stalk-specific Fc-mediated functions are differentially boosted and regulated in PLWH in comparison with PWOH following TIV. These HA stalk-specific responses are not typically measured after seasonal influenza vaccination but the boosting and potency of these functions are essential in conferring protection against influenza virus infection and may be more important for high-risk groups who generally have poorer HAI responses after TIV. We further demonstrated that high pre-existing levels of HA stalk-specific ADCP contributed to the risk reduction of influenza-illness and HA stalk-specific ADCD correlated with protection against influenza-illness following immunization with TIV in pregnant women. Pregnancy has been associated with reduced production of inflammatory antibodies and immunomodulation through functional and structural modifications of antibodies (347,348). These studies are especially relevant where annual influenza vaccination is recommended to these at risk populations, who do have different vaccine-induced responses. Additionally, the study

supports the evaluation of HA stalk-specific Fc effector functions as correlates of protection for future HA stalk-based influenza vaccines that are currently in development.

In addition, this thesis reports on the humoral responses to SARS-CoV-2 infection and vaccination, which displayed different kinetics, in PLWH and PWOH. We also observed that SARS-CoV-2 beta elicited differences in the magnitude of Fc effector functions to D614G. This is supported by previously published data (349), suggesting that SARS-CoV-2 VOCs elicit differential epitope targeting by antibodies influencing their functional capacity. The other main finding here was that PLWH initially had delayed kinetics, but were able to elicit comparable responses with PWOH to SARS-CoV-2 infection and vaccination. This study supports the prioritization of ART distribution and current SARS-CoV-2 vaccine approaches for PLWH.

Given that HIV infection and pregnancy elicit altered antibody responses owing to their inflammatory nature, future work could include measuring antibody Fc glycosylation profiles using various liquid chromatography–mass spectrometry (LC/MS) techniques, to determine regulatory effects on Fc-mediated functions on a population level (350). Limitations of these studies include not knowing the applicability of the influenza vaccination findings to non-pregnant women and men and not knowing whether the SARS-CoV-2 findings described here apply to adults over the age of 40 years. These would require further investigations.

Overall, these studies provided insights into the immune responses and their mechanisms of protection in PLWH following respiratory viral infection and vaccination, which is crucial for reducing the burden of these respiratory viral diseases in a high HIV prevalence setting.

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Part 2: Manuscripts

In **Paper 1** we aimed to investigate and compare hemagglutinin stalk-specific Fc effector functions in people living with or without HIV following immunization with a seasonal trivalent influenza vaccine.

In **Paper 2** we aimed to assess the association between hemagglutinin stalk-specific Fc-mediated functions and protection against influenza-illness after seasonal influenza vaccination in pregnant women.

The aim of the study in **Paper 3** was to investigate the humoral response kinetics of people living with HIV in comparison to people without HIV during SARS-CoV-2 infection or vaccination.

Part 2

Paper 1: Influenza Vaccination Results in Differential Hemagglutinin Stalk-Specific Fc-Mediated Functions in Individuals Living with or without HIV

Part 2: Paper 1

Influenza Vaccination Results in Differential Hemagglutinin Stalk-Specific Fc-Mediated Functions in Individuals Living with or without HIV

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Influenza Vaccination Results in Differential Hemagglutinin Stalk-Specific Fc-Mediated Functions in Individuals Living With or Without HIV

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Influenza virus hemagglutinin (HA) stalk-specific antibodies have been shown to potently induce Fc-mediated effector functions which are important in protection from disease. In placebo-controlled maternal influenza (MatFlu) vaccination trials of pregnant women living with or without HIV, reduced risk of influenza illness was associated with high HA stalk antibody titers following trivalent inactivated vaccination (TIV). However, the mechanisms of immunity conferred by the HA stalk antibodies were not well understood. Here, we investigated HA stalk-specific Fc effector functions including antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent complement deposition (ADCD), and FcγRIIa and FcγRIIIa binding in response to seasonal influenza vaccination. These were measured pre- and 1-month post-vaccination in 141 HIV-uninfected women (67 TIV and 74 placebo recipients) and 119 women living with HIV (WLWH; 66 TIV and 53 placebo recipients). In contrast to HIV-uninfected women, where HA stalk-specific ADCP and FcγRIIa binding were significantly boosted, WLWH showed no increase in response to vaccination. HA stalk-specific ADCC potential and FcγRIIIa binding were not boosted regardless of HIV status but were higher in WLWH compared with HIV-uninfected women prior to vaccination. HA stalk-specific ADCD was significantly increased by vaccination in all women, but was significantly lower in the WLWH both pre- and post- vaccination. Co-ordination between HA stalk-specific ADCP and ADCD in WLWH was improved by vaccination. Fc polyfunctionality was enhanced by vaccination in HIV-uninfected women and driven by the HA stalk antibody titers. However, in the WLWH, higher pre-vaccination Fc polyfunctionality was maintained

post-vaccination but was decoupled from titer. Overall, we showed differential regulation of Fc effector HA stalk responses, suggesting that HIV infection results in unique humoral immunity in response to influenza vaccination, with relevance for future strategies that aim to target the HA stalk in this population.

Keywords: influenza vaccination, Fc effector functions, HIV co-infection, hemagglutinin stalk antibodies, antibody-dependent cellular phagocytosis (ADCP), antibody-dependent complement deposition (ADCD), antibody-dependent cellular cytotoxicity (ADCC)

INTRODUCTION

Seasonal influenza epidemics cause over 56,000 hospitalizations and 11,000 deaths annually in South Africa (1). Immunocompromised individuals such as pregnant women and people living with HIV (PLWH) are especially burdened with severe respiratory disease. Therefore seasonal trivalent inactivated influenza vaccines (TIV) are recommended for these high-risk individuals and have been shown to have a significant impact on public health (2). Whilst TIV efficacy has been confirmed in PLWH, vaccine immunogenicity was suboptimal in these individuals (3–8). Therefore, there is a need to further understand the mechanisms of immunity in PLWH, following seasonal influenza vaccination.

Humoral immune responses elicited by TIVs primarily target the viral hemagglutinin (HA), which is composed of a head and stalk domain. The ability of HA head-specific antibodies to neutralize influenza virus, detected using hemagglutination inhibition (HAI) assays, is considered a relative correlate of protection (9). However, the HA head domain continuously undergoes antigenic drift, allowing escape from HA head-specific antibodies induced from previous viral exposures and vaccinations (10). The immuno-subdominant, but conserved HA stalk domain is a target for the development of broadly protective influenza vaccines (11). In addition to having neutralizing activity, HA stalk antibodies confer protection through Fc-FcγR interactions (12).

Fc effector functions have been associated with protection against influenza virus infection, in experimental challenge models and after vaccination (13–17). Through the interaction of the antibody Fc region with cell surface Fc receptors or complement proteins, cytotoxic functions such as antibody-dependent cellular phagocytosis (ADCP), cellular cytotoxicity (ADCC) and complement deposition (ADCD) occur. In animal models, ADCP, ADCC and ADCD have been associated with protection against infection (18–21). In humans, seasonal influenza vaccination enhances cross-reactive ADCC and ADCP antibodies directed to the HA in healthy individuals and high-risk groups, such as older adults and PLWH (22–24). In these studies, TIV boosted Fc effector functions when head-specific HAI responses were low, highlighting the potential of HA stalk antibodies and their cytotoxic functions for protection in immunocompromised individuals.

In general, PLWH are at a higher risk of deaths associated with severe influenza disease (25). B-cell impairments and reduced HAI antibody levels in response to seasonal TIV have been observed in this group, with pregnancy further increasing susceptibility to severe influenza virus infections (26–30). PLWH on antiretroviral treatment (ART) have lower HAI responses in comparison to HIV-uninfected individuals even when TIV doses were increased or a second dose was administered (5, 6, 8, 31). However, studies focusing on the HA stalk are limited and detailed antibody responses and mechanisms of immunogenicity in this high-risk group are not well understood.

In two randomized, double-blind, placebo-controlled maternal influenza (MatFlu) vaccination trials, lower HAI titers were observed in pregnant women living with HIV (WLWH) compared with pregnant women living without HIV (6). However, the vaccine efficacy against confirmed influenza illness in WLWH trended to being higher (70.6%) than in HIV-uninfected women (54.4%) (6). This indicated that antibody responses to epitopes that lie beyond the HA head may be important in protection in this high-risk group. A follow up study showed that HA stalk antibody titers were associated with reduced risk of influenza virus infection (32). Given that HA stalk-specific antibodies mediate potent Fc effector functions and are known to be protective, we explored the Fc-mediated functions of HA stalk-specific antibodies in this cohort of WLWH.

In this study we observed higher HA stalk-specific *in vitro* ADCP and ADCC as measured by a reporter assay in WLWH prior to vaccination in comparison with HIV-uninfected women. ADCC potential directed at the HA stalk was not boosted regardless of HIV status, whereas ADCP was enhanced only in HIV-uninfected women. In both groups HA stalk-specific ADCD was increased although this function is compromised in WLWH. We also observed differences in the coordination of Fc effector functions post-vaccination. In WLWH the association between HA stalk-specific ADCP and ADCD was enhanced but in the HIV-uninfected women these functions were associated with HA stalk-specific ADCC potential. We also show that Fc polyfunctionality was higher in WLWH prior to vaccination and was not further enhanced, whereas in HIV-uninfected women it was improved. This study suggests that seasonal influenza vaccination may confer protection through different HA stalk targeted mechanisms which may include Fc effector functions for WLWH and HIV-uninfected women.

MATERIALS AND METHODS

Ethics Statement

The 2011 maternal influenza (MatFlu) vaccination trials (approval numbers: 101106 and 101107) and this sub-study

(approval number: M200444) were approved by the Human Research Ethics Committee of the University of the Witwatersrand. All study participants provided written informed consent to have their stored samples used for future studies. All healthy donors in this study provided written informed consent to obtain plasma, isolate IgG and have their samples stored for future use.

Influenza Vaccination Cohort

The two randomized, double-blind, placebo-controlled MatFlu vaccination trials conducted in Soweto, South Africa have been previously described (6). Briefly, women, all of whom were pregnant, between the ages of 18-38 years were stratified according to their HIV status and randomly assigned (1:1) to the placebo or vaccine groups. The trivalent inactivated vaccine (TIV) used in the trials was Vaxigrip, which contained 15 µg each of A/California/7/2009 (A/H1N1/pdm09), A/Victoria/210/2009 (A/H3N2), and a B/Brisbane/60/2008-like virus (B/Victoria) as recommended by the World Health Organization for the Southern Hemisphere in 2011. Active surveillance for respiratory illness and PCR-confirmed influenza-illness was performed. In this study, plasma samples from a sub-set of HIV-uninfected women (74 placebo-recipients and 67 vaccinees) and WLWH (53 placebo-recipients and 66 vaccinees), collected prior to vaccination and one-month post-vaccination, were tested. Data from participants with PCR-confirmed influenza-illness were excluded from this study.

Protein Expression and Pooled Plasma/IgG Preparation

A chimeric recombinant hemagglutinin (cHA) protein, composed of an H6 head and an H1 stalk (cH6/1) was produced as previously described (33). For use as a positive control in all the Fc assays, plasma was pooled from 5 healthy donors with high HA stalk IgG titers. From this pooled plasma IgG was isolated using Protein G (Pierce Biotechnology), according to the manufacturer's instructions and confirmed by IgG enzyme linked immunosorbent assay (ELISA). A cross-reactive HA stalk antibody CR9114 that mediates potent Fc effector function was expressed as a positive control (34). For antibody expression, plasmids encoding heavy or light chain genes were co-transfected into HEK293F cells with PEI-MAX 40,000 (Polysciences) head-to-head. Cells were cultured for six days in 293F Freestyle media at 37°C, 10% CO_2, then harvested supernatants were filtered and purified using Protein G (Thermo Scientific). Antibody concentration was quantified by nanodrop using sequence-specific extinction coefficients as determined by ProtParam (ExPASy) and confirmed by ELISA.

Hemagglutinin Inhibition (HAI) Assay

Modified HAI assays were previously performed (6, 35). Briefly, plasma samples instead of serum were treated with receptor-destroying enzyme (RDE) from *Vibrio cholera* (Denka-Seiken). Then these were diluted 1:10 in saline and subsequent serial 2-fold dilutions of the plasma were used in a standard HAI assay using 4 hemagglutinating units of the antigen and 0.75% turkey red blood cells. Plasma samples with titers ≥10 were considered indicative of immune responses. The antigen used in the assays was A/H1N1/pdm09.

Hemagglutinin Stalk (H1/stalk) IgG ELISA

H1/stalk IgG ELISAs were performed as previously described (32). The cH6/1 recombinant protein described above was utilized and binding against this protein measures antibodies against the H1 stalk domain. Plates were coated with cH6/1 diluted to 2 µg/ml overnight at 4°C. Plates were washed and subsequently blocked with 3% fetal bovine serum (Biowest), 0.5% non-fat dry milk powder (Bio-Rad) for 2 hours at room temperature (RT). After washing, plasma samples were added to the plate diluted to a starting concentration of 1:40, followed by two-fold serial dilution and incubated for 2 hours at RT. Following a wash, anti-human IgG (Fab specific)-peroxidase antibody (Sigma, USA) diluted to 1:3000 in blocking solution was added to the plate. After 1-hour incubation at RT, plates were washed and developed using SigmaFast *o*-phenylenediamine dichloride (OPD) (Sigma, USA) for 10 min at RT. The reaction was stopped by adding 3M HCl and the optical density (OD) was measured at 490 nm. The antibody concentration was quantified against the standard curve included on each plate that consisted of polyvalent human normal immunoglobulin (Polygam) (National Bioproducts Institute, South Africa) with an assigned arbitrary value of 1000 arbitrary units (AU)/ml.

Antibody-Dependent Cellular Phagocytosis (ADCP)

The ADCP assay was performed as previously described (36). Briefly The EZ-Link Sulfo-NHS-LC-Biotin kit (Thermo Scientific) was used to biotinylate cH6/1, which was coated onto fluorescent neutravidin beads (Invitrogen). The coated beads were incubated for 2 hours with mAbs at a final concentration of 50 µg/ml or a 1:100 dilution of plasma sample, prior to overnight incubation with a monocytic cell line, THP-1 cells. The THP-1 cells were obtained from the NIH AIDS Reagent Program and cultured at 37°C, 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum (FBS), 100 units/ml Penicillin and 100 ug/ml Streptomycin (Gibco), referred to as R10, and not allowed to exceed 4 x 10^5 cells/ml. This assay was completed on a FACS Aria II (BD Biosciences). Phagocytic scores were calculated as the geometric mean fluorescent intensity (MFI) of the beads multiplied by the percentage bead uptake minus the no antibody control as background. For this and all functional Fc assays, pooled plasma from 5 healthy donors with previous exposures to influenza, screened for H1 stalk antibodies and CR9114 were used as positive controls and Palivizumab (RSV mAb; MedImmune, LLC) and VRC01 (In house HIV mAb) were used as negative controls. In order to normalize across plates and runs, the pooled plasma positive scores were averaged, divided by the pooled plasma score per plate and this normalizing factor multiplied across the scores.

Antibody-Dependent Cellular Cytotoxicity (ADCC) Reporter Assay

The ability of plasma antibodies to cross-link cH6/1 HA stalk antigen and activate FcγRIIIa on Jurkat-Lucia™ NFAT-CD16 cells (*In vivo*gen) was measured as a proxy for ADCC or cell lysis. These cells *In vivo* were cultured according to the manufacturer's instructions. Adapted from elsewhere (37), high-binding 96 well plates were coated with 1 µg/mL cH6/1 and incubated at 4°C overnight. Plates were then washed with phosphate buffered saline (PBS) and blocked at room temperature for 1 hour with 2.5% bovine serum albumin (BSA)/PBS. After washing, mAbs at a starting concentration of 1 mg/ml or a 1:10 dilution of plasma sample was added and incubated for 1 hour at 37°C. Subsequently, 2x 10^5 cells/well in R10 were added and incubated for 24 hours at 37°C, 5% CO_2. Then 25 µl of supernatant was transferred to a white 96-well plate with 75 µl of reconstituted QUANTI-Luc secreted luciferase and read immediately on a Victor 3 luminometer (PerkinElmer) with 1s integration time. Relative light units (RLU) of a no antibody control were subtracted as background. The pooled plasma and CR9114 were used as positive controls and Palivizumab and VRC01 were used as negative controls. The reported values are the mean of three kinetic reads taken at 0, 2.5, and 5 min. To induce the transgene 1x cell stimulation cocktail (Thermo Scientific) and 2 µg/ml ionomycin in R10 was added as a positive control to confirm sufficient expression of the CD16 Fc receptor. ADCC RLUs were normalised across plates and runs, by averaging the pooled plasma RLUs and multiplying the RLUs across the samples with the normalizing factor.

Antibody-Dependent Complement Deposition (ADCD)

ADCD was measured using a previously described high-throughput bead-based assay (38). Biotinylated cH6/1 was coated 1:1 onto fluorescent neutravidin beads (Invitrogen) for 2 hours at 37°C. The antigen-coated beads were incubated with a 1:10 plasma sample dilution or mAbs at a starting concentration of 100 µg/ml for 2 hours and incubated with guinea pig complement diluted 1 in 50 with gelatin/veronal buffer for 15 minutes at 37°C. Beads were washed in PBS and stained with anti-guinea pig C3b-FITC, fixed and interrogated on a FACS Aria II (BD Biosciences). Complement deposition scores were calculated as the percentage of C3b-FITC positive beads multiplied by the geometric MFI of FITC in this population minus the no antibody or heat inactivated controls. The pooled plasma and CR9114 were used as positive controls and Palivizumab and VRC01 were used as negative controls. ADCD scores were normalised between plates and runs, by dividing the ADCD score of the pooled plasma by the average across the plates and multiplying the scores with this normalizing factor.

Dimeric Fc Gamma Receptor Binding ELISAs

High-binding 96 well ELISA plates were coated with 1 ug/ml cH6/1 in PBS overnight at 4°C. Three wells on each plate were directly coated with 5 ug/ml IgG, isolated from healthy donors, signals from these wells were used to normalize the FcR activity of the plasma samples and pooled plasma was used as a positive control. Plates were washed with PBS and blocked with PBS/1 mM ethylenediaminetetraacetic acid (EDTA)/1% BSA for 1 hour at 37°C. Plates were then washed and incubated with 1:10 diluted plasma for 1 hour at 37°C and then with 0.2 ug/ml of biotinylated FcγRIIa dimer or 0.1 ug/ml of biotinylated FcγRIIIa dimer for 1 hour at 37°C. The dimeric FcγRs were provided by Prof. Mark Hogarth from the Burnet Institute, Melbourne, Australia (39). Subsequently, a 1:10000 dilution of Pierce high-sensitivity streptavidin-horseradish peroxidase (Thermo Scientific) was added for a final incubation of 1 hour at 37°C. Lastly, TMB (3,3,5,5-tetramethylbenzidine) substrate (Sigma-Aldrich) was added, colour development was stopped with 1 M sulfuric acid and absorbance read at 450 nm.

Statistical Analysis

Data were analyzed in Prism (v9; GraphPad Software Inc., San Diego, CA, USA). Non-parametric tests were used for all comparisons. The Mann-Whitney and Wilcoxon tests were used for unmatched and paired samples, respectively. Fc polyfunctionality Z-scores were calculated by subtracting the mean of the Fc function from the individual value and divided by the standard deviation of the mean and then adding all the Z-scores for each function per individual. The proportions of responders and non-responders in each group and the proportions of participants with high or low Fc polyfunctionality Z-scores were compared by Fisher's exact tests. All correlations reported are non-parametric Spearman's correlations. *P* values less than 0.05 were considered statistically significant.

RESULTS

Women Living With HIV (WLWH) Have Lower HAI and H1 Stalk Antibody Titers in Response to Seasonal Influenza Vaccination

In our previous study, HAI titers were significantly boosted by vaccination regardless of HIV status, but WLWH showed significantly lower HAI titers post-vaccination compared to HIV-uninfected women (6). We confirmed this finding in a subset of plasma samples from pregnant vaccinated participants from the MatFlu cohort, including 67 HIV-uninfected women and 66 WLWH, matched in age with a median of 25 years (range 18-39) and 27 years (range 18-38) respectively, and excluded all PCR-confirmed influenza virus infection cases. Plasma samples were collected pre-vaccination and post-vaccination at a median of 31 days (range 28-33) for HIV-uninfected and 30 days (range 28-31) for WLWH (**Supplementary Table 1**). Post-vaccination HAI titers were significantly lower in WLWH compared with HIV-uninfected women (medians 80 vs. 320 A/H1N1 HAI titer) (**Figure 1A**), coupled with decreased boosting levels following vaccination (median fold change 1.5 vs. 4) (**Supplementary Figure 1A**). In these samples, we also confirmed that post-

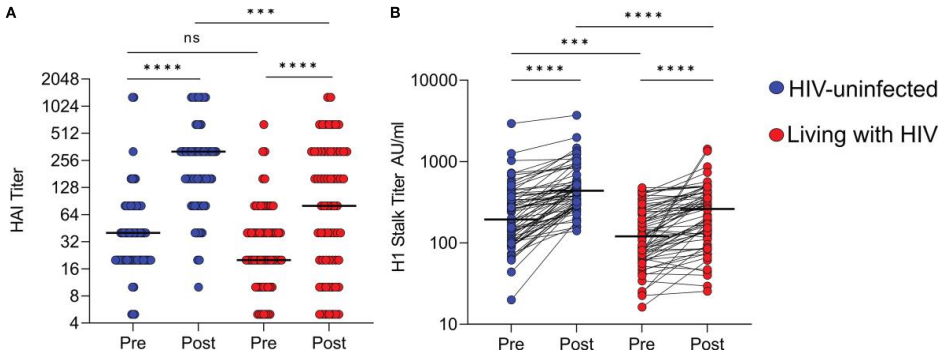

FIGURE 1 | A/H1N1 HAI and H1 stalk antibody responses amongst vaccinated women. **(A)** Pre-vaccination and 1-month post-vaccination hemagglutination inhibition (HAI) titers against A/H1N1 and **(B)** H1 stalk titers by ELISA, with HIV-uninfected participants (n=67), shown in blue and participants living with HIV (n=66), shown in red. The lines represent the median. Wilcoxon matched-pairs signed rank test used to compare pre- and post-vaccination titers. Mann Whitney U test used to compare responses between vaccine groups. Significant associations shown as ****p < 0.0001; ***p < 0.001; ns,not significant.

vaccination H1 stalk antibody titers were significantly lower in WLWH compared with HIV-uninfected women (medians 259.3 vs. 433.5 AU/ml) (**Figure 1B**). This was despite vaccine boosting H1 stalk antibody titers in both groups, which was consistent with the parent study (32). Furthermore, we also confirmed that in contrast to HAI titers, the HIV-uninfected women and WLWH had more comparable fold increases in the H1 stalk antibody titer boosting (median fold change 1.9 vs. 1.7) (**Supplementary Figure 1B**). As expected, no changes in HAI and H1 stalk antibody titers amongst placebo recipients living with or without HIV were observed (**Supplementary Figure 2**).

Women Living With HIV Have Higher Pre-Vaccination ADCP and ADCC Reporter Activity, but These Are Not Boosted by Seasonal TIV

We first assessed whether WLWH differ in terms of pre- and post-vaccination HA stalk antibody Fc-mediated effector functions. To do this, we measured HA stalk-specific ADCP, ADCC reporter activity and ADCD using a chimeric recombinant HA protein with an H6 head, to which humans are generally naïve, and an H1 stalk. TIV administration resulted in significant boosting of HA stalk-specific ADCP in HIV-uninfected women but not in WLWH (**Figure 2A**). Despite this, post-vaccination ADCP activity between the two groups was similar, a consequence of pre-vaccination HA stalk-specific ADCP being significantly higher in WLWH (medians 348.6 vs. 189.7). No ADCP boosting was observed in the placebo groups indicating that these responses were TIV specific (**Supplementary Figure 2**). For ADCC, which was measured using a high throughput FcγRIIIa activation assay, previously shown to correlate with NK degranulation assays (40), there was no significant boosting in either HIV-uninfected women or WLWH (**Figure 2B**). However, like ADCP, pre-vaccination ADCC was significantly higher in the WLWH (medians 154.5 vs. 73.5), and therefore also post-vaccination (medians 170 vs. 83.2). The ADCC activity amongst the vaccinated and placebo groups were similar and remained unchanged since there was no vaccine-mediated boosting of this function (**Supplementary Figure 2**).

To assess Fc-FcγR engagement required for ADCP and ADCC activity, plasma samples were tested for the capacity to bind cH6/1 antigen and cross-link recombinant soluble dimeric FcγRIIa or FcγRIIIa, respectively (39). No boosting in FcγRIIa and FcγRIIIa binding was observed in placebo recipients (**Supplementary Figure 2**). However, as observed in the cell-based functional and reporter assays, FcγRIIa binding was boosted by TIV in HIV-uninfected women but not WLWH, whereas for FcγRIIIa binding, no boosting was observed in either group (**Supplementary Figures 3A, B**). Furthermore, strong positive Spearman's correlations were noted between FcγRIIa binding and ADCP scores (r=0.44; p<0.0001), and between FcγRIIIa binding and ADCC RLUs (r=0.55; p<0.0001) (**Supplementary Figures 3C, D**).

Seasonal TIV Substantially Boosts ADCD but Women Living With HIV Have Lower Responses Both Pre- and Post-Vaccination

Since ADCD has been shown to confer protection from influenza virus infection (16, 21), we examined this function in WLWH and HIV-uninfected women, before and after TIV vaccination. ADCD, measured using a bead-based flow cytometry assay, was significantly boosted by TIV in all participants irrespective of HIV status (**Figure 2C**). However, ADCD was substantially impaired in WLWH prior to vaccination and post-vaccination remained significantly lower in comparison to the HIV-uninfected women after TIV boosting (medians 16.2 vs. 225). Placebo recipients showed no increase in ADCD activity (**Supplementary Figure 2**).

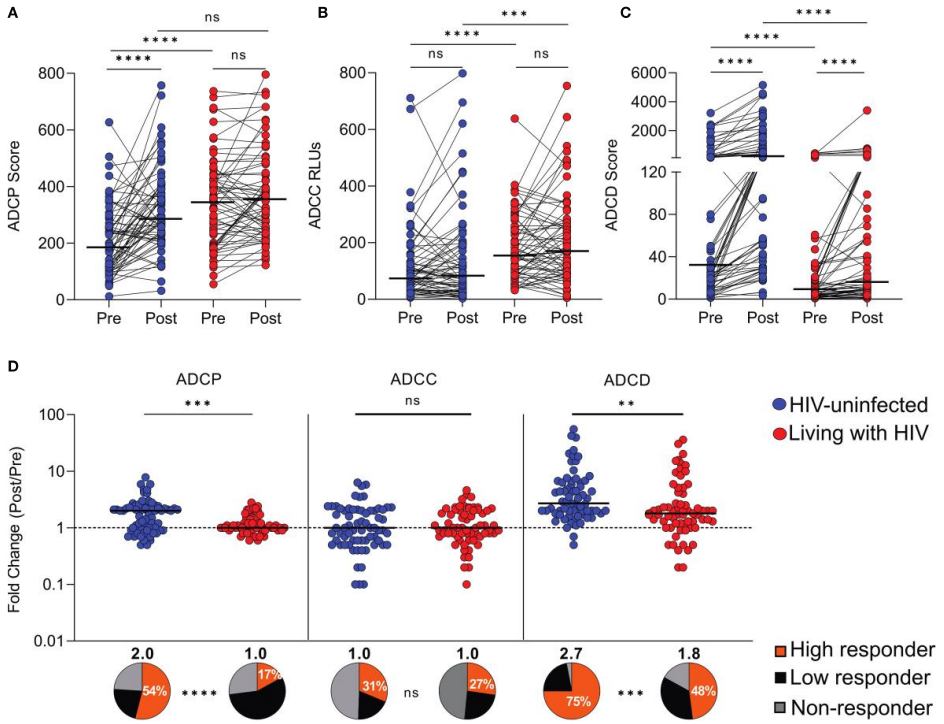

FIGURE 2 | Differential boosting of HA stalk-specific Fc-mediated antibody functions amongst vaccinated women. Pre-vaccination and 1-month post-vaccination H1 stalk-specific **(A)** antibody-dependent cellular phagocytosis (ADCP), **(B)** antibody-dependent cellular cytotoxicity (ADCC) or FcγRIIIa activation and **(C)** antibody-dependent complement deposition (ADCD) are shown where HIV-uninfected participants (n=67), shown in blue and participants living with HIV (n=66), shown in red.**(D)** The fold increases for each of the functions and the proportion (%) of high responders that exceed a 2-fold increase in Fc activity are shown in orange. Low responders, who show reduced enhancement that does not reach a 2-fold increase are shown in black, whilst those not boosted (non-responders) are shown in grey. The lines represent the median. Wilcoxon matched-pairs signed rank tests were used to compare pre-vaccination and post-vaccination functional scores within groups. Mann Whitney U test was used to compare functional scores between the vaccine groups. Fischer's exact tests were used to compare the proportions of high responders. Significant associations are shown as ****p < 0.0001; ***p < 0.001; **p < 0.01; ns,not significant.

The Magnitude of Vaccine-Mediated Boosting of ADCP and ADCD Is Lower in Women Living With HIV

When we compared Fc-mediated functions, we observed differential boosting across the HIV-uninfected women and WLWH for ADCP and ADCD following vaccination (**Figure 2D**). For ADCP the WLWH were not boosted, and the proportion of high responders (individuals that exceeded a 2-fold increase in Fc activity) was significantly lower than in the HIV-uninfected group (17% vs. 54%). Since there was no boosting of ADCC potential, the proportion of high responders was similar between HIV-uninfected women and WLWH (31% vs. 27%). However, for ADCD, in the WLWH the TIV boosting and the proportion of high responders was significantly lower (median fold change 1.8 and high responders 48%) than in the HIV-uninfected women (median fold change 2.7 and high responders 75%). Thus, while ADCD activity was boosted in both groups in response to TIV vaccination, this function was substantially impaired in WLWH prior to vaccination.

Seasonal TIV Does Not Improve Overall Co-Ordination of the Fc-Mediated Responses, Irrespective of HIV Status

Co-ordinated antibody responses elicited by vaccines against other diseases have been associated with increased efficacy (41). We therefore assessed the correlations between HAI titers, stalk-specific responses and Fc-mediated effector functions at baseline and after TIV vaccination and compared these in HIV-uninfected women and WLWH. Prior to vaccination, there was a significant correlation between HAI and HA stalk titers in HIV-uninfected women, but the significant boosting of HAI

antibodies resulted in loss of this correlation post-vaccination (**Figures 3A, B**). In contrast in WLWH, while there was a strong correlation between HA stalk and HAI pre-vaccination (**Figure 3C**), HA stalk titers were boosted simultaneously with HAI titers, as previously reported (32) and remained significantly correlated (**Figure 3D**). In the HIV-uninfected women, ADCP, ADCC, ADCD and FcγRIIa and FcγRIIIa binding were significantly associated with the HA stalk titers before vaccination, and these associations improved slightly following vaccination (**Figures 3A, B**). In WLWH, ADCP, ADCD and FcγRIIa and FcγRIIIa binding were associated with HA stalk titers pre-vaccination, with the FcγRIIIa binding been the only association to not improve, after TIV vaccination (**Figures 3C, D**). We also observed varying relationships amongst the Fc-mediated effector functions. In the HIV-uninfected women, we observed a significant correlation between ADCP and ADCD pre-vaccination, but not post-vaccination. Instead, after vaccination we observed a correlation between ADCC potential and both ADCP and ADCD. The FcγRIIa and FcγRIIIa binding correlated with all the Fc functions both pre- and post-vaccination in the HIV-uninfected women. In contrast, in WLWH the existing association between ADCD and ADCP prior to vaccination was strengthened post-vaccination. There was also an in increase in the FcγRIIa and FcγRIIIa binding associations with all the Fc functions, following vaccination in this group. Therefore, vaccination resulted in nuanced improvements of particular functional relationships that differed between WLWH and HIV-uninfected women, but overall co-ordination of antibody responses and Fc-mediated functions was not significantly improved.

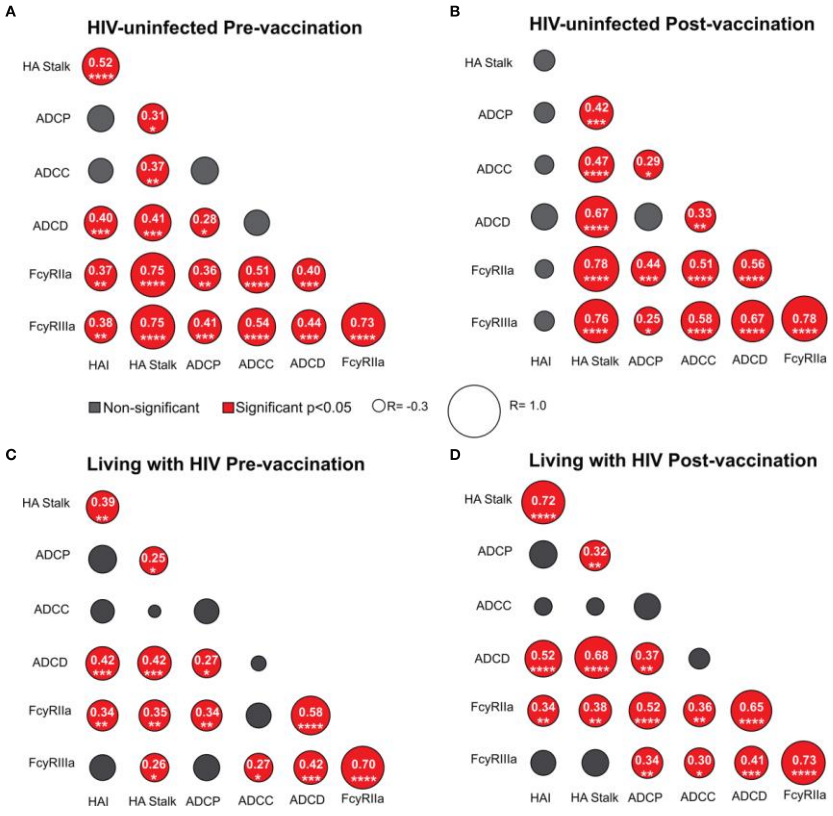

FIGURE 3 | Co-ordination between HAI titers, HA stalk antibodies and HA stalk-specific Fc-mediated functions in response to vaccination. Spearman correlations of Fc effector function and titers in **(A)** HIV-uninfected pre-vaccination, **(B)** HIV-uninfected post-vaccination, **(C)** WLWH pre-vaccination and **(D)** WLWH post-vaccination groups. The asterisks indicate the following statistical significance, ****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05; ns,not significant. Significant correlations are displayed in red. Non-significant correlations are in grey. The size of the circle is proportional to the Spearman correlation coefficients -0.3 been the smallest and 1 been the largest, indicated inside of circles for correlations that were significant.

Stalk-Specific Fc Polyfunctionality of Women Living With HIV Is Not Improved by Vaccination and Is Not Driven by H1 Stalk Antibody Titer

Fc polyfunctionality has been shown to be associated with protective immunity and broader responses in other diseases (41–44). We calculated HA stalk-specific Fc polyfunctionality scores by summing the z-scores of ADCP, ADCC and ADCD for each of the participants. Prior to vaccination, WLWH showed significantly higher Fc polyfunctionality directed at the HA stalk than HIV-uninfected women, as a result of their baseline higher ADCC and ADCP responses (medians 0.89 vs. -0.26) (**Figure 4A**). The proportion of participants with high HA stalk-specific Fc polyfunctionality (the sum of z-scores greater than 0) was also higher for WLWH (76%) than for HIV-uninfected group (45%). Following vaccination, HA stalk-specific Fc polyfunctionality in the HIV-uninfected women significantly increased, reaching the same levels observed in WLWH at baseline. In contrast, WLWH did not show improvement of overall HA stalk-specific Fc-mediated functionality following vaccination.

We next assessed the influence of H1 stalk titer on stalk-specific Fc polyfunctionality after vaccination. In the HIV-uninfected group there was a strong correlation between the HA stalk antibody titers and HA stalk- specific Fc polyfunctionality (r=0,71; p<0.0001) (**Figure 4B**). However, in WLWH we observed no correlation between H1 stalk titers and HA stalk-specific Fc polyfunctionality (r=0,19; p=0.26) (**Figure 4C**). The lack of association in WLWH suggests that in this group, the potency of the Fc–mediated functions is mediated through qualitatively different HA stalk-specific antibodies to those occurring in HIV-uninfected women. Overall, these data indicate a fundamentally different humoral response in WLWH in response to vaccination.

DISCUSSION

HA stalk-specific Fc effector functions have an important complementary role to neutralization in protection against influenza virus infection (45, 46). Therefore, understanding their role in vaccination of high-risk groups, such as PLWH, is crucial but have not been previously studied. In the cohort described here, we observed lower HAI titers and lower HA stalk-specific titers in WLWH than in HIV-uninfected women in response to seasonal influenza vaccination, as reported in our previous studies (6, 32). Despite these lower antibody titers, the vaccine efficacy was higher in WLWH (6). Here, we examined differences in HA stalk-specific Fc-mediated functions between pregnant women living with or without HIV in response to seasonal influenza vaccination in a cohort where the HA stalk antibody titers were previously shown to be protective (32). We observed significant differences in responses; WLWH showed only HA stalk-specific ADCD boosting, whereas in HIV-uninfected women both ADCP and ADCD were significantly enhanced. We also assessed the association between Fc effector functions post-vaccination and found that ADCC or FcγRIIIa activation was coordinated with ADCP and ADCD in HIV-uninfected women but in WLWH only ADCP and ADCD showed improved correlation following vaccination. Furthermore, in HIV-uninfected women overall Fc polyfunctionality was improved following vaccination and was driven by HA stalk antibody titers. In contrast, in WLWH the higher Fc polyfunctionality was not associated with the titers of the HA stalk-specific antibodies. Overall, HA stalk-specific Fc functions were differentially mediated in WLWH in response to seasonal

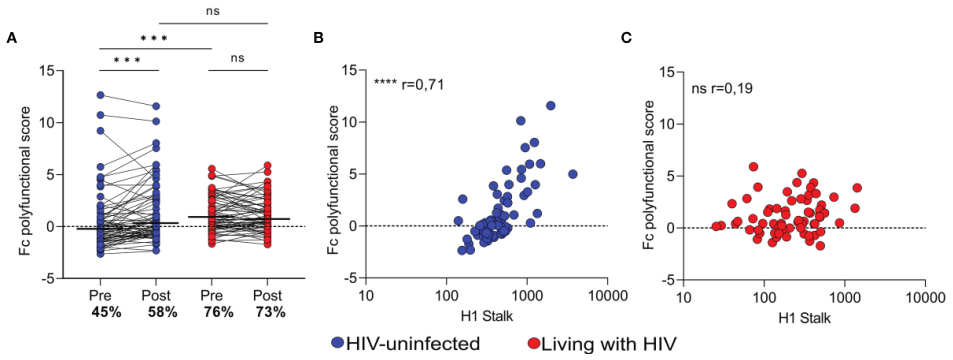

FIGURE 4 | HA stalk-specific Fc polyfunctionality scores of Fc-mediated functions amongst vaccinated women. **(A)** Fc polyfunctionality (determined by the addition of individual standardized Fc function scores), prior to and following vaccination. Dots above 0 indicate Fc polyfunctional individuals, while those below indicate poor Fc polyfunctionality below the mean. The proportion (%) of individuals with Fc polyfunctionality is indicated on the plot. Spearman's correlation between the Fc polyfunctionality score and HA stalk response in **(B)** HIV-uninfected women and **(C)** WLWH. Wilcoxon matched-pairs signed rank test was used to compare pre-vaccination and post-vaccination polyfunctionality scores within groups. Mann Whitney U test was used to compare polyfunctionality scores between the vaccine groups. Significant associations are shown as ****p < 0.0001; ***p < 0.001; ns,not significant.

influenza vaccination, despite similar boosting of HA stalk antibodies.

Although the WLWH and HIV-uninfected groups were matched for age, there were substantial differences in their baseline responses, prior to vaccination. Both ADCP and ADCC potential were higher in WLWH pre-vaccination. This may be due to increased previous exposures to influenza virus infections in WLWH prior to vaccination, which may have broadened the HA stalk-specific Fc-mediated functions (6, 45). Multiple exposures to divergent influenza virus strains has been shown to prime HA-specific antibodies with the ability to mediate ADCP and ADCC (47). Several studies have provided evidence of this concept, with H7N9 infection and vaccination of healthy people, inducing cross-reactive, ADCC mediating HA stalk antibodies (13, 18, 48). In addition, the WLWH were on ART and this may have allowed for the partial restoration of past HA stalk-specific Fc functionality and together with the vaccine, provided sufficient immunity against influenza virus infections (22).

In this trial, irrespective of HIV status, HA stalk-specific ADCC (measured either through a reporter assay or binding to dimeric FcγRIIIa) was not boosted following vaccination. TIV has elicited variable ADCC responses in humans, with similar findings to ours reported in healthy individuals (49–51), but moderate ADCC boosting has been reported in older adults and PLWH (22, 24, 52). The lack of boosting of ADCC potential in our study, compared to others, may be a consequence of high pre-existing levels of ADCC responses from prior infections in both groups. These responses may be at a "ceiling" and cannot be further significantly boosted following vaccination. The reporter assay that we use in this study has been shown to correlate with traditionally used CD107a NK degranulation assays (40) and is therefore unlikely the reason for the observed lack of boosting.

We observed differences in functions that were boosted in response to vaccination by HIV status. Specifically, ADCP and binding to dimeric FcγRIIa were only boosted in HIV-uninfected women, similar to previous studies (22). However, WLWH had ADCP levels prior to vaccination that exceeded that of HIV-uninfected women. Similarly, a weaker ADCP vaccine response directed at HA has been previously associated with higher baseline levels, similar to those observed here in HIV-uninfected women (23). Post-vaccination ADCP levels in HIV-uninfected women did not exceed the levels in the WLWH, indicating a maximum threshold in HA stalk ADCP activity. In contrast, ADCD was boosted regardless of HIV status, but was significantly lower in the WLWH both pre and post-vaccination suggesting that the ability of antibodies to perform this function was impaired in this group. Despite similar increasing of HA stalk antibody boosting in response to vaccination there were distinct differences in the ability to boost stalk-specific Fc responses associated with HIV co-infection. Overall, these differences show distinct Fc function between WLWH and HIV-uninfected women in response to vaccination.

Vaccination that induces coordinated Fc effector responses has been associated with efficacy in other infectious diseases (41). We observed a loss in the association between HA stalk titers and HAI activity post-vaccination in the HIV-uninfected group but it was strengthened in the WLWH group, as shown previously (32). While the simultaneous elicitation of both head and stalk directed antibodies in WLWH post-vaccination may indicate that the antibody functions behave in a synergistic manner in this group, how HA stalk-specific Fc effector function is affected by broad HAI antibodies was not investigated in this study. This was also observed through the Fc-mediated functions correlating with both HAI and HA stalk titers in WLWH, whilst the Fc effector functions and FcγR binding did not correlate at all with HAI post-vaccination in HIV-uninfected women (previously shown for ADCD pre-vaccination). This, coupled with the observation that HA stalk antibody titer alone did not correlate with overall Fc polyfunctionality in WLWH, suggests that the quality of the Fc responses may have been impacted by both HAI and HA stalk responses in this group which requires further investigation. In addition, it is possible that modulators of Fc effector function such as isotype or glycosylation may differ as a result of HIV infection (53–55). These differences may drive polyfunctionality more robustly than titer in WLWH. This further highlights the differences between WLWH and HIV-uninfected women HA stalk mediated Fc effector functions, of which the differences in response to seasonal influenza vaccination may explain the elevated protection observed for WLWH.

Chimeric hemagglutinin-based vaccines aimed at eliciting cross-reactive HA stalk antibodies have the potential to be developed as universal vaccines. Correlates of protection for this approach should include HA stalk-specific Fc-mediated functions (56). In this cohort, levels of HA stalk antibodies were protective however only ADCP and ADCD were boosted in response to the vaccine in HIV-uninfected women and only ADCD in WLWH, suggesting that these antibody functions may be more important than ADCC in protection. Future studies should determine whether the higher levels of HA stalk-specific ADCP and ADCC potential in the WLWH were enough to confer protection against influenza virus infection or disease severity. In summary, we showed that although HIV-uninfected women exhibited significantly improved Fc polyfunctionality post-vaccination that was driven by HA stalk titer, WLWH mounted fundamentally different HAI and HA stalk coordinated responses, likely impacted by high baseline HA stalk-specific Fc effector function. Our study highlights the need to include, in future clinical trials, high-risk groups who may have different responses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of the University of the Witwatersrand. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BM, PM, and SR conceptualized the study. BM performed experiments, analyzed data, generated the figures and wrote the manuscript. ND performed H1 stalk ELISAs. MN and SM established the trials, collected and provided participant samples and the HAI data. FK provided the chimeric hemagglutinin protein (cH6/1). PM and SR assisted in data interpretation, reviewed and edited the manuscript and supervised the research. All authors contributed to the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.873191/full#supplementary-material

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Conflict of Interest: FK reports royalties (Avimex), consulting fees (Pfizer, Seqirus, Third Rock Ventures and Avimex), and payment for academic lectures during the past two years. FK is also named as inventor on IP filed by the Icahn School of Medicine at Mount Sinai for influenza virus vaccines and therapeutics, SARS-CoV-2 vaccines and SARS-CoV-2 serological assays.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

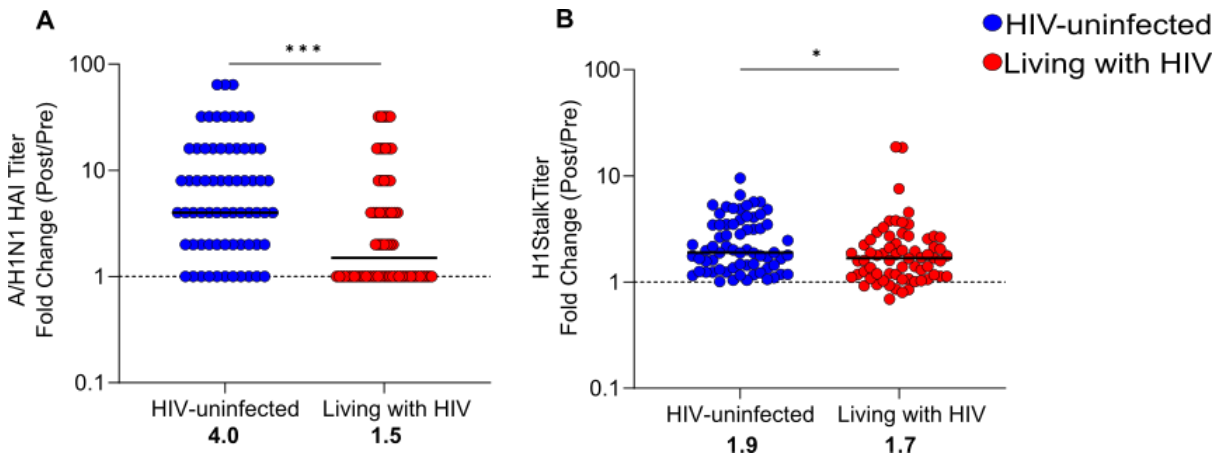
Part 2: Paper 1
Influenza Vaccination Results in Differential Hemagglutinin Stalk-Specific Fc-Mediated Functions in Individuals Living with or without HIV

Supplementary Material

Supplementary Table 17: Age and days between visits of vaccinated women.

Mann Whitney U tests used to compare between the vaccine groups, ns=not significant.

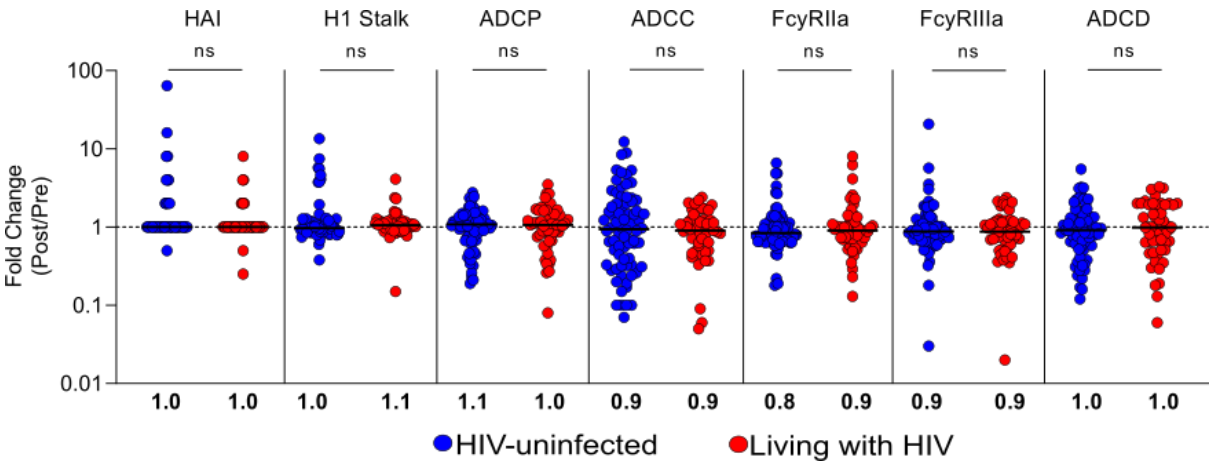
Vaccinated individuals	**HIV-uninfected n=67**	**Living with HIV n=66**	***P Value***
Median age; years [IQR]	25 [18-39]	27 [18-38]	ns 0.1406
Days between pre- and post- vaccination visit; Median [IQR]	31 [28-33]	30 [28-31]	ns 0.3427



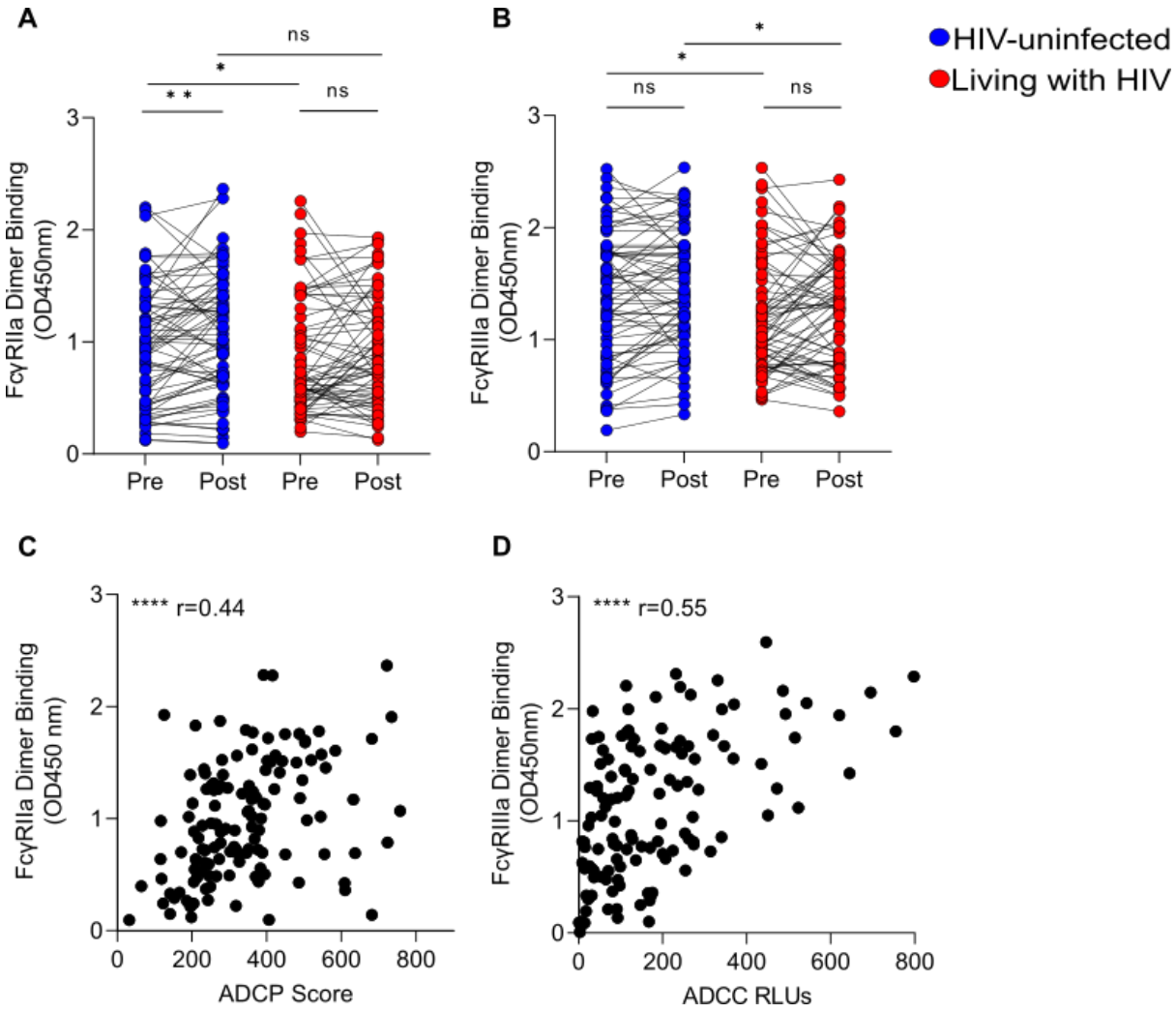
Supplementary Figure 1: Fold changes in A/H1N1 HAI and H1 stalk antibody responses amongst vaccinated women. (A) The fold increases 1-month post-vaccination of hemagglutination inhibition (HAI) titers against A/H1N1 and (B) H1 stalk titers by ELISA, with HIV-uninfected participants (n=67), shown in blue and participants living with HIV (n=66), shown in red. The lines represent the median. Mann Whitney U test used to compare responses between vaccine groups. Significant associations shown as ***p<0.001, *p<0.05.

Part 2: Paper 1

Influenza Vaccination Results in Differential Hemagglutinin Stalk-Specific Fc-Mediated Functions in Individuals Living with or without HIV



Supplementary Figure 2: Fold changes in antibody responses, Fc binding and function amongst placebo groups. HIV-uninfected participants (n=74), shown in blue and participants living with HIV (n=53), shown in red. No significant difference in fold change, indicated below plot, across HAI titers, H1 stalk titers, dimeric FcγR binding and Fc-mediated functions, ADCP, ADCC and ADCD. The lines represent the median. Mann Whitney U test compared placebo groups, ns=not significant**.**



Supplementary Figure 3: Dimeric Fc gamma receptor (FcγR) binding correlates with HA stalk ADCP and ADCC. Pre-vaccination and 1-month post-vaccination (A) FcγRIIa dimer binding and (B) FcγRIIIa dimer binding. HIV-uninfected participants (n=67), shown in blue and participants living with HIV (n=66), shown in red. Post-vaccination Spearman's correlations of (C) FcγRIIa dimer binding by ELISA and ADCP activity and (D) FcγRIIIa dimer binding by ELISA and ADCC activity. Wilcoxon matched-pairs signed rank tests for pre- and post-vaccination comparisons. Mann Whitney U test compared vaccine groups. Significant associations shown as ****p<0.0001, **p< 0.01, *p<0.05, ns=not significant.

Paper 2: Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women

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Summary: This study shows that hemagglutinin (H1) stalk-specific Fc-mediated functions are associated with protection against influenza-illness after immunization with a seasonal trivalent inactivated influenza vaccine (TIV) during pregnancy. These data may inform correlates of protection for future HA stalk-based influenza vaccines.

Keywords: influenza, hemagglutinin stalk, Fc effector functions, protection, seasonal influenza vaccine

ABSTRACT

Background

Several current influenza vaccine candidates aim to elicit antibodies against the conserved hemagglutinin (HA) stalk domain. Understanding the protective mechanism of these antibodies, which mediate broad neutralization and Fc-mediated functions, following seasonal vaccination is critical.

Methods

Plasma samples were obtained from a subset of pregnant women living with or without HIV-1 enrolled in a maternal influenza vaccination trial (138 trivalent inactivated influenza vaccine [TIV] and 145 placebo recipients). Twenty-three influenza infection cases were confirmed within 6 months postpartum. We measured HA (H1 sub-type) stalk-specific antibody-dependent cellular phagocytosis (ADCP), complement deposition (ADCD) and cellular cytotoxicity (ADCC) at enrolment and 1-month post-vaccination. The association between these Fc-mediated functions and protection against influenza-illness following vaccination was examined using multiple logistic regression analysis and risk reduction thresholds were defined by the score associated with the lowest odds of influenza-illness.

Results

Amongst TIV and placebo recipients, lower H1 stalk-specific ADCP and ADCD activity was detected for participants with confirmed influenza compared with individuals without confirmed influenza-illness 1-month post-vaccination. Pre-existing ADCP scores ≥250 reduced the odds of A/H1N1 infection by 83% (odds ratio 0.11; p=0.01). Following TIV, ADCD scores of ≥25 and ≥15 were significantly associated with >84% risk reduction against A/H1N1 (0.10; p=0.01) and non-group 1 (0.06; p=0.0004) influenza virus infections, respectively. H1 stalk-specific ADCC potential was not associated with protection against influenza-illness.

Conclusion

H1 stalk-specific ADCD correlates with protection against influenza-illness following influenza vaccination in pregnant women. These findings provide insight into the protective mechanisms of HA stalk antibodies.

INTRODUCTION

Influenza viruses pose a significant threat to public health with seasonal epidemics causing substantial morbidity and mortality globally, and pandemic strains emerging unpredictably in irregular intervals [1,2]. In low and middle income countries, children under the age of 5 years, pregnant women and people living with human immunodeficiency virus 1 (HIV-1) (PLWH) are at an increased risk of severe influenza outcomes [3]. Seasonal trivalent inactivated influenza vaccines (TIV) are the most commonly administered influenza vaccines worldwide, owing to the lack of availability in developing countries. The main concerns with TIV include the variable efficacy (between 19-60% annually) and the short duration of protection [4]. High rates of viral antigenic drift often result in mismatches between the vaccine and circulating influenza virus strains, consequently reducing vaccine efficacy [5]. Furthermore, the hemagglutination inhibition (HAI) antibody titer of ≥1:40 is associated with 50% risk reduction of influenza-illness with matched virus strain [6]. Nevertheless, HAI titers do not provide broad immunity and are less predictive of protection in high-risk groups [7,8]. Consequently, the development of broadly protective influenza vaccines, investigation of novel correlates of protection in current influenza vaccination strategies may improve future vaccine design.

Seasonal TIV primarily targets the viral hemagglutinin (HA), which is composed of a head and stalk domain. Antigenic drift occurs faster in the immunodominant HA head compared with the immunosubdominant HA stalk domain [9]. Therefore, the HA stalk domain is more conserved across subtypes, making it a promising target for the development of broadly protective influenza vaccines [10]. Neutralizing antibodies targeted at the HA head are measured through the HAI assay, which does not detect antibodies directed at the HA stalk. HA stalk-specific antibodies have been shown in several studies to confer protection against influenza virus infection in animals and humans [11–14]. In addition to being broadly

neutralizing, HA stalk antibodies can also mediate Fc effector functions, such as antibody-dependent cellular phagocytosis (ADCP), cellular cytotoxicity (ADCC) and complement deposition (ADCD) [15–18].

In animal models, broadly protective HA stalk antibodies require a range of Fc-Fc receptor interactions to confer *in vivo* protection against influenza virus challenge [14,15]. We and others have shown that seasonal influenza vaccines can enhance HA stalk-directed antibody Fc-mediated functions in healthy individuals and in high-risk groups [17,19–21]. Vaccine elicited ADCC is cross-reactive and after infection, humans can demonstrate significant ADCC breadth extending to the avian strain H7N9 [22–24]. Several HA stalk-based vaccines elicit ADCP and ADCC in animal models and in humans [18,25–27].

In two randomised, double-blind, placebo-controlled maternal influenza vaccination trials we reported moderate (54.4%) and high (70.6%) per-protocol TIV efficacy in people without HIV-1 and PLWH respectively. HAI antibody titers did not fully explain the high efficacy in PLWH, but HA stalk antibody titers were associated with protection and HA stalk-specific Fc-mediated functions were differentially boosted and regulated in PLWH [8,17,28]. However, due to small numbers in confirmed influenza cases the protective capacity of these functions could not be determined in PLWH.

In this study, we analysed HA (H1 sub-type) stalk-specific Fc-mediated functions and whether they were associated with protection from influenza-illness. We observed significantly lower ADCP and ADCD 1-month post-vaccination in individuals who developed influenza-illness compared with those who did not. Pre-existing high ADCP levels of ≥250 were associated with reduced likelihood of A/H1N1 infection. TIV boosted ADCD activity was associated with reduced risk against both A/H1N1 (≥25 score) and non-group 1 (≥15 score) virus infections,

but ADCC potential was not associated with protection against influenza-illness. This study indicates that H1 stalk-specific ADCD confers protection against influenza-illness after influenza vaccination in pregnant women, an important consideration for evaluation of HA stalk-based universal influenza vaccines.

METHODS

Ethics

The 2011 maternal influenza vaccination trials (101106 and 101107) and this sub-study (M200444) were approved by the Human Research Ethics Committee of the University of the Witwatersrand.

Study design

The maternal influenza vaccination trials conducted in Soweto, South Africa have been previously described [8]. Briefly, the trials consisted of pregnant women, including participants living with HIV-1, between the ages of 18-38 years with an estimated gestational age of 27 ± 4 weeks (**Table S1**). The TIV used was Vaxigrip, which contained 15 µg each of A/California/7/2009 (A/H1N1/pdm09), A/Victoria/210/2009 (A/H3N2), and a B/Brisbane/60/2008-like virus (B/Victoria/2/87-like lineage) as recommended by the World Health Organization for the Southern Hemisphere in 2011-2012. Active surveillance for respiratory illness and polymerase chain reaction (PCR) confirmation of influenza-illness was performed weekly until 24 weeks postpartum [8]. Participants in whom influenza-illness occurred before 1-month post-vaccination were excluded, as this would interfere with measuring TIV-specific responses, which typically peak at 2 weeks [29]. HAI and H1 stalk IgG titers were previously measured, and are detailed here in the supplementary materials. In this study, enrolment and post-vaccination plasma samples were collected between 28 to 33 days apart (**Table S1**) from a subset of placebo (n=127) and vaccine (n=133) recipients

and influenza-illness cases (placebo n=18; vaccine n=5). These were tested for H1 stalk-specific antibody-dependent cellular phagocytosis (ADCP), antibody-dependent complement deposition (ADCD) and antibody-dependent cellular cytotoxicity (ADCC) potential.

Antibody-dependent cellular phagocytosis (ADCP) assay

A chimeric recombinant hemagglutinin (cHA) protein, composed of an H6 head (to which humans are naïve) and an H1 stalk from A/California/04/09 was used to measure H1 stalk-specific Fc effector functions [30]. The cH6/1 protein was biotinylated and coated onto fluorescent neutravidin beads, as previously described [31]. Briefly, beads were incubated with monoclonal antibodies (mAbs) at 50 µg/ml or a 1:100 dilution of plasma sample, prior to overnight incubation with monocytic THP-1 cells. The cells were interrogated on a FACSAria II. Phagocytic scores were calculated as the geometric mean fluorescent intensity (MFI) of the beads multiplied by the percentage bead uptake minus the no antibody control as background.

For use as positive controls in all Fc assays, plasma was pooled from 5 healthy donors with high H1 stalk IgG titers and a HA stalk antibody CR9114 that mediates potent Fc function was expressed, as previously described [17]. The normalisation of functional scores between plates and runs was performed using the pooled plasma. Palivizumab (respiratory syncytial virus [RSV] mAb) and VRC01 (HIV-1 mAb) were used as negative controls.

Antibody-dependent complement deposition (ADCD) assay

As previously described [32], biotinylated cH6/1 was coated 1:1 onto fluorescent neutravidin beads. Antigen-coated beads were incubated with a 1:10 plasma sample dilution or mAbs at 100 µg/ml followed by incubation with guinea pig complement diluted 1 in 50 with gelatin/veronal buffer. Beads were washed in phosphate buffered saline (PBS) and stained

with anti-guinea pig C3b-FITC, fixed and interrogated on a FACSAria II. Complement deposition scores were calculated as the percentage of C3b-FITC positive beads multiplied by the geometric MFI of FITC in this population minus the no antibody or heat inactivated controls.

FcγRIIIa reporter assay

The ability of plasma antibodies to cross-link cH6/1 and activate FcγRIIIa on Jurkat-Lucia™ nuclear factor of activated T-cells (NFAT)-CD16 cells (Invivogen) was measured as a proxy for antibody-dependent cellular cytotoxicity (ADCC), and performed as previously described [17]. Plates coated with 1 µg/ml cH6/1 were washed and blocked with 2.5% bovine serum albumin (BSA)/PBS, prior to incubation with mAbs at 1 mg/ml or a 1:10 dilution of plasma sample. Subsequently, NFAT-CD16 cells were added and incubated for 24 hours. Supernatants were then transferred to plates with QUANTI-Luc secreted luciferase and read immediately on a Victor 3 luminometer. Relative light units (RLU) of a “no antibody” control were subtracted as background. The reported values are the mean of three kinetic reads taken at 0, 2.5, and 5 min. To induce the transgene, 1x cell stimulation cocktail and 2 µg/ml ionomycin was added.

Statistical analysis

Data from the trial groups were compared using non-parametric tests. The Fisher's exact test was used to compare categorical variables while the Mann-Whitney test was used for continuous variables. We evaluated differences in the levels and fold changes of each of the antibody responses by performing Kruskal-Wallis test with Dunn’s correction for multiple test comparisons. Calculation of the odds ratio (OR) and 95% confidence intervals (CI) by a multiple logistic regression model was used to assess the association between H1 stalk-

specific ADCP, ADCD and ADCC potential levels and protection against A/H1N1 (n=11) and non-group 1 (n=12 A/H3N2; B/Victoria/2/87-like lineage; B/Yamagata/16/88-like lineage) illnesses. The association was further adjusted for differences in HIV-1 infection status, vaccination status and H1 stalk antibody titers by including these as covariates in the multiple logistic regression model. Fisher's exact tests were performed to compare the Fc levels between participants with or without confirmed influenza-illness, thereby by determining an association with protection. From this analysis, the Fc effector function risk reduction threshold levels were defined by the score with the most significant and lowest odds ratio. Fc levels from participants with or without confirmed influenza-illness were also plotted against the reverse cumulative percentage of pregnant women. The intersecting vertical and horizontal line on the X axis and Y axis at the risk reduction thresholds represents the percentage likelihood of influenza infection. P-values less than 0.05 were considered statistically significant. Analyses were performed in Prism (v9; GraphPad, USA).

RESULTS

Seasonal TIV boosts H1 stalk-specific ADCP and ADCD

We first assessed the TIV boosting of A/H1N1 HAI, H1 stalk antibody titers and H1 stalk-specific Fc functions. We observed no significant increases between the enrolment and 1-month post-enrolment HAI and H1 stalk-specific antibody responses in placebo recipients (**Figure 1**). In contrast, vaccine recipients had boosted HAI titers with a median 4-fold increase 1-month post-vaccination. TIV significantly enhanced H1 stalk antibody titers (median fold increase of 2). Similarly, H1 stalk-specific ADCP and ADCD were significantly boosted each with a median fold increase of 2. The only function not boosted by the seasonal TIV 1-month post-vaccination was H1 stalk-specific ADCC potential. Overall, A/H1N1 HAI,

H1 stalk antibody titers and H1 stalk-specific ADCP and ADCD were boosted by 1-month post-vaccination.

Pre-existing ADCP is associated with protection against A/H1N1 illness

Next, we assessed the association of H1 stalk-specific Fc-mediated functions with protection against group 1 A/H1N1 (n=11) and non-group 1 (n=12 A/H3N2; B/Victoria/2/87-like lineage; B/Yamagata/16/88-like lineage) illnesses. We compared the levels of these responses between cases of confirmed influenza-illness and individuals that did not develop symptomatic influenza-illness during the study period in a multiple logistic regression model. H1 stalk-specific ADCP was significantly lower among individuals developing A/H1N1 illness (median 149) than those who did not develop A/H1N1 illness (236; p=0.02) (**Table 1**). Although not significant, ADCP was also lower in non-group 1 (A/H3N2 or influenza B) illnesses (**Table 1**) compared with those who did not have influenza-illness. ADCP responses were associated with lower odds of acquiring A/H1N1 illnesses (OR 0.20; p=0.03), but this did not remain significant after adjusting for HIV-1 infection status, vaccine status or by H1 stalk titers, as derived from the logistic regression analysis (**Table 1**). Against non-group 1 viruses there was no significant association between ADCP responses and protection from illness. The risk reduction threshold for ADCP activity was a score of 250, which was associated with the lowest odds of A/H1N1 infection (OR 0,11; p=0.01) (**Table S2**) and an 83% likelihood of not developing A/H1N1 illness (**Figure S1A**). A threshold level of ADCP could not be determined for non-group 1 illnesses as there were no significant associations, but the odds of infection decreased as the ADCP score increased, indicating a link between increasing H1 stalk-specific ADCP and cross-protection against divergent strains (**Table S3**). Although vaccine recipients had significantly higher ADCP scores ≥250 (proportion ≥threshold 70%; median 325; p<0.0001) than both the placebo recipients (proportion

≥threshold 24%; 160) and influenza-illness cases (proportion ≥threshold 13%; 165) (**Figure 2A**), adjusting the logistic regression analysis by vaccine status showed no significance suggesting that the association with protection is driven by pre-existing high H1 stalk-specific ADCP instead of TIV boosting against A/H1N1 illness.

ADCD is associated with protection from influenza-illness following TIV

H1 stalk-specific ADCD was also significantly lower for individuals who developed influenza-illness compared with those without influenza symptoms (medians 2 vs 25; p=0.0002), irrespective of the infecting strain (**Table 1**). In the logistic regression analysis, ADCD was associated with lower odds of acquiring influenza virus illness (A/H1N1 OR 0.18; p=0.003 and non-group 1 OR 0.21; p=0.003), which remained significant after adjusting for HIV-1 infection status (A/H1N1 p=0.01 and non-group p=0.006) or vaccine status (A/H1N1 p=0.004 and non-group 1 p=0.008), and when including H1 stalk titers as a covariate (A/H1N1 p=0.04 and non-group p=0.006) (**Table 1**). The risk reduction thresholds significantly associated with the lowest odds of developing A/H1N1 (OR 0.1; p=0.01) (**Table S2**) and non-group 1 illness (OR 0.06; p=0.0004) (**Table S3**) were scores of 25 or 15, respectively. These threshold scores were associated with 84% and 85% reduced risk against A/H1N1 and non-group 1 illnesses respectively (**Figure S1B, C**). The proportion of ADCD scores ≥25 was significantly greater in vaccine recipients (proportion ≥threshold 66%; median 56; p<0.0001) compared with placebo recipients (proportion ≥threshold 36%; 15) and influenza-illness cases (proportion ≥threshold 9%; 2) (**Figure 2B**). Furthermore, placebo recipients without influenza-illness had significantly higher (p=0.0007) ADCD than all individuals in whom influenza-illness occurred. Overall, ADCD was associated with risk reduction of illness against all strains of influenza virus following TIV administration.

ADCC is not associated with protection after seasonal TIV

We observed significantly higher levels of ADCC potential in TIV recipients compared with placebo recipients (**Figure 2C**). However, the lack of ADCC potential boosting following seasonal influenza vaccination (**Figure 1**) suggest the differences were due to pre-existing levels being higher in the vaccine recipients. Therefore, we assessed whether pre-existing ADCC levels were associated with risk reduction of influenza-illness. We observed no significant differences in H1 stalk-specific ADCC potential between individuals with and without influenza-illness (**Table 1**), including when stratified by infecting strains (**Table S2; Table S3**). In this trial, ADCC potential was not associated with protection against influenza-illnesses after vaccination with TIV.

DISCUSSION

Several strategies targeting the conserved HA stalk region are currently being explored in the pursuit of a broadly protective influenza vaccine [33]. Understanding the mechanisms through which vaccine-elicited HA stalk-specific antibodies confer protection against influenza-illnesses is crucial. Here, we report an increase in H1 stalk-specific ADCP and ADCD but not ADCC potential in response to seasonal influenza vaccination. Additionally, we observed that ADCP was associated with a significant risk reduction from group 1 A/H1N1 but not non-group 1 virus illness, albeit not significant after adjusting for HIV-1 status, vaccine status and H1 stalk antibody titers. ADCD levels were associated with significant risk reduction against all influenza virus infecting strains, including in the adjusted analysis. Overall, these data suggest that in addition to neutralization or HAI, HA stalk-specific Fc functions and specifically ADCD contribute to protection against influenza-illnesses following seasonal TIV in pregnant women.

The HAI assay has a universally accepted threshold (≥1:40) that is associated with a 50% reduction in the risk of acquiring influenza infection from a matching strain in healthy adults [6]. Here, we excluded the HAI titers from the multiple regression analysis to determine which of the HA stalk-specific Fc-mediated functions are independently associated with protection. In this trial, H1 stalk-specific ADCP (≥250) and ADCD (≥25) were associated with >83% risk reduction against A/H1N1 illness in pregnant women living with or without HIV-1, whether these Fc-mediated functions significantly contribute to the vaccine efficacy in conjunction with HAI responses remains unclear. We have previously reported that seasonal influenza vaccination results in nuanced co-ordination of H1 stalk antibody titers and H1 stalk-specific Fc-mediated functions, irrespective of HIV- infection status, which may need to be improved by future vaccines for better protection [17,34]. However, there is also evidence from other research groups showing seasonal influenza vaccine boosting of Fc effector functions in adults ≥65 years old and PLWH [19–21]. These studies and our findings highlight the potential of Fc effector functions, which were associated with reduced risk of influenza-illness.

In our study, pre-existing high levels of H1 stalk-specific ADCP were shown to reduce the risk of A/H1N1 illnesses. For ADCP, we previously showed a lack of association with HA stalk antibody titers, high baseline levels, and no boosting in PLWH compared with people without HIV-1, accounting for the lack of association after adjustment by these parameters [17]. While ADCP can be boosted by TIV, previous exposures to influenza may provide sufficient ADCP levels for protection. Additionally, although ADCP was not significantly associated with protection against non-group 1 virus illnesses, the odds trended to be lower with increasing activity. This indicates the potential for cross-reactive protection but requires further investigation.

Our study also analysed H1 stalk-specific ADCD responses showing that it was associated with reducing the odds of influenza-illness, regardless of the infecting strain. ADCD is infrequently studied in the context of influenza vaccination, however, HA-directed ADCD can be boosted by influenza vaccination in humans [17,35,36]. Since most seasonal influenza vaccines are tri- or quadri-valent and there is still considerable protein sequence variability within HA stalk subtypes [33], future work should include the measure of HA subtypes 3 and B stalk-specific ADCD to determine whether there is a stronger association with the homotypic infecting strains and equivalent breadth in protection. Our previous study showed substantial TIV boosting of ADCD in PLWH [17]. In this study, PLWH made up half of the vaccine recipients, and most had levels exceeding the risk reduction threshold. Together, these findings suggest that ADCD could be of greater importance in these high-risk individuals.

In this trial, seasonal influenza vaccination did not result in boost of H1 stalk-specific ADCC potential, and there was no association between ADCC and risk reduction of influenza-illnesses. While others have shown enhanced ADCC following seasonal influenza vaccination in children, healthy adults and high-risk groups, we previously demonstrated a lack of ADCC boosting irrespective of HIV-1 status [16,17,20,21]. This lack of TIV induced ADCC response could be due to high pre-existing ADCC levels from previous influenza virus infections, the vaccine formulation not being optimal for inducing ADCC responses or possibly the impact of pregnancy, associated with immunomodulation [37]. Epitope specificity also has a major role in eliciting different Fc effector functions, therefore ADCC epitopes may have been conformationally restricted in the vaccine immunogens, resulting in reduced protective capacity [38]. Furthermore, we did not assess the isotypes and glycosylation induced after vaccination, both of which modulate Fc effector function [39].

Limitations of this study include the inability to assess the protective capacity of HA stalk-specific Fc-mediated functions in PLWH due to the small number of confirmed influenza cases. We acknowledge that the number of confirmed influenza cases that were vaccine recipients was low (n=5) and therefore these findings should be validated in additional cohorts. Additionally, the applicability of these findings to non-pregnant women and men would require further investigations. In this trial, asymptomatic infections may have been missed due to PCR testing been limited to participants with symptomatic infections [8]. Furthermore, assay standardization for Fc-mediated functions has not been achieved [40], therefore direct comparisons of the risk reduction titers with those from other studies may not be straightforward.

In conclusion, we showed that H1 stalk-specific ADCP and ADCD, but not ADCC potential, were boosted by seasonal TIV. H1 stalk-specific ADCD correlated with protection against influenza-illness in pregnant women following immunization with TIV. Our study findings support the evaluation of HA stalk-specific Fc effector functions as correlates of protection for future HA stalk-based influenza vaccines.

Part 2: Paper 2

Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women

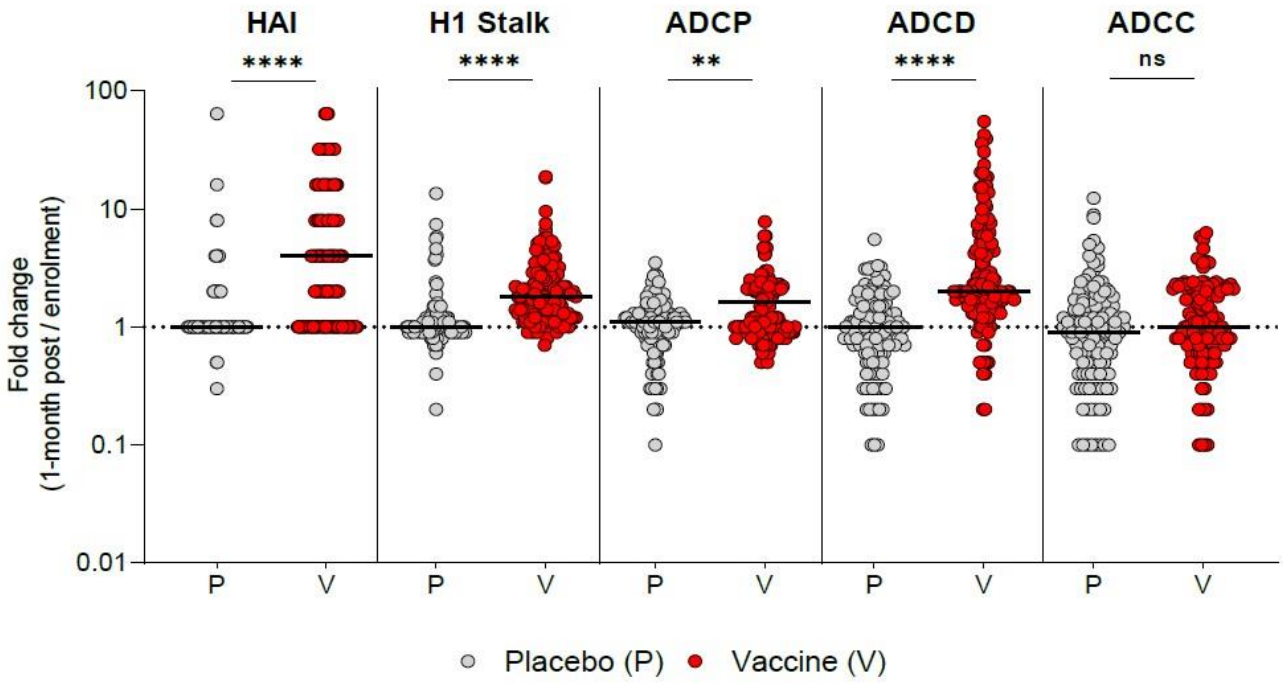

Figure 1: Seasonal TIV boosting of HA antibody responses. Fold changes between enrolment and 1-month post-vaccination A/H1N1 HAI, H1 stalk antibody titers, H1 stalk-specific ADCP, ADCD and ADCC or FcγRIIIa activation in the placebo (grey; n=127) and vaccine (red; n=133) trial groups with matched age and proportion of participants living with or without HIV-1 status. The dotted line indicates no change between sampling time points, above 1 indicates an increase in function and below 1 indicates a decrease in function. The median fold change is indicated by the solid lines. For each of the antibody responses Kruskal-Wallis test with Dunn's correction was calculated for multiple test comparison. Statistical significance: ****p< 0.0001; **p< 0.01 and ns= not significant.

Part 2: Paper 2

Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women

Table 1: Multiple logistic regression to investigate the association between the levels of H1 stalk-specific Fc-mediated functions and confirmed influenza-illness

	Influenza-illness median [IQR]	**No influenza-illness** median [IQR]	[a]	**OR (95% CI)[b]**	**aOR (95% CI)[c]**	**aOR (95% CI)[d]**	**aOR (95% CI)[e]**
Group 1 A/H1N1	**n=11**	**n=272**					
ADCP score	149 [96-236]	236 [140-356]	*	0,20 (0,04-0,88) *	0,23 (0,04-1,43) ns	0,31 (0,04-2,65) ns	0,74 (0,10-6,81) ns
ADCD score	2 [1-3]	25 [6-225]	***	0,18 (0,05-0,48) **	0,24 (0,06-0,64) *	0,19 (0,05-0,51) **	0,26 (0,06-0,82) *
ADCC RLU	89 [49-126]	70 [30-168]	ns	0,78 (0,26-2,53) ns	0,52 (0,16-1,81) ns	1,12 (0,32-4,45) ns	1,01 (0,32-3,31) ns
Non-group 1	**n=12**	**n=271**					
ADCP score	192 [116-224]	237 [140-357]	ns	0,28 (0,05-1,73) ns	0,31 (0,06-1,84) ns	0,77 (0,11-6,70) ns	0.65 (0,09-5,28) ns
ADCD score	2 [1-7]	25 [5-225]	***	0,21 (0,07-0,51) **	0,23 (0,07-0,58) **	0,25(0,07-0,61) **	0,19 (0,05-0,57) **
ADCC RLU	100 [50-199]	70 [30-149]	ns	2,87 (0,8-10,54) ns	2,56 (0,73-10,04) ns	10,2 (2,31-55,4) **	3,67 (1,08-14,15) *

Non-group 1 (A/H3N2 n=6; B/Victoria/2/87-like n=2; B/Yamagata/16/88-like n=4). IQR; Interquartile range. [a] Mann-Whitney test used to compare between individuals with or without confirmed influenza-illness. Placebo (at enrolment) and vaccine recipient (1-month post-vaccination) data included. [b] Unadjusted odds ratio (OR) and 95% confidence intervals (CI) for H1 stalk-specific ADCP, ADCD and ADCC potential levels. [c] Adjusted odds ratio (aOR) and 95%CI for HIV-1 status. [d] aOR and 95%CI for vaccination status. [e] aOR and 95%CI for H1 stalk antibody titers. Statistical significance: ***p<0.001; **p<0.01; *p<0.05 and ns= not significant.

Part 2: Paper 2

Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women

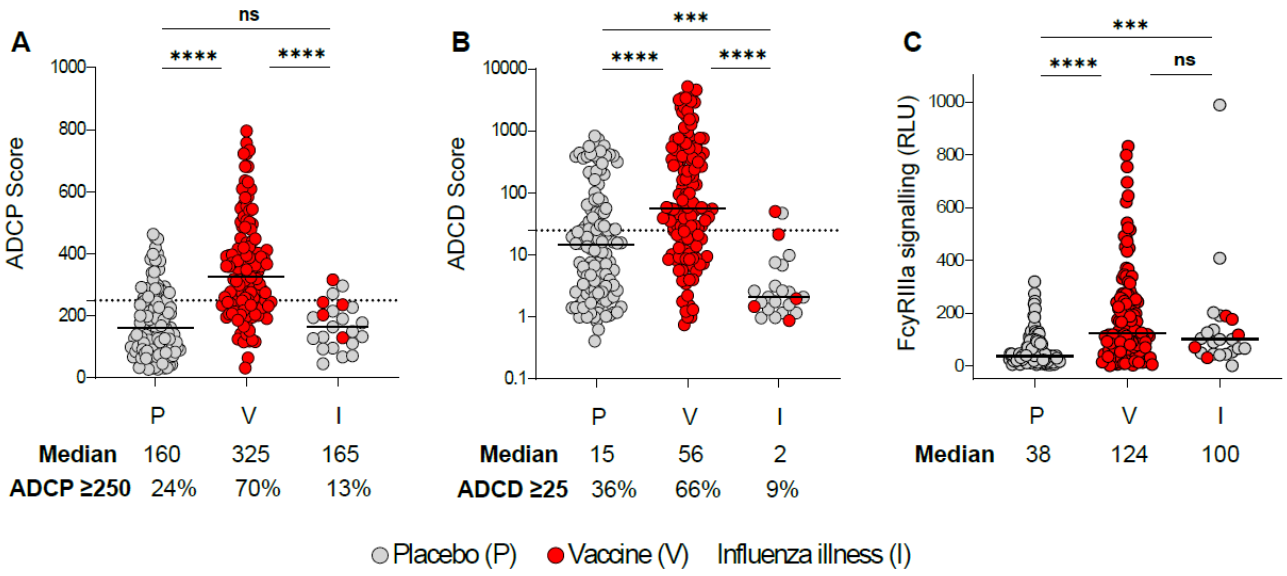

Figure 2: H1 stalk-specific ADCD is associated with protection against A/H1N1 illness after seasonal influenza vaccination. Levels of H1 stalk-specific (A) ADCP, (B) ADCD and (C) ADCC or FcγRIIIa activation in the placebo (grey n=127; at enrolment) and vaccine (red n=133; 1-month post-vaccination) trial groups, as well as for the PCR-confirmed influenza-illness cases (placebo n=17; at enrolment and vaccine n=5; 1-month post-vaccination). The dotted lines indicate the titers associated with the lowest odds of A/H1N1 illness; at 250 for ADCP and 25 for ADCD. The median levels are indicated by solid lines. For each of the antibody responses Kruskal-Wallis test with Dunn's correction was calculated for multiple test comparison. Statistical significance: ****p< 0.0001; ***p< 0.001 and ns= not significant.

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AUTHOR CONTRIBUTIONS

BMM, PLM, and SIR conceptualised the study. BMM performed experiments, analysed data, generated the figures and wrote the manuscript. ND performed H1 stalk ELISAs. MCN and SAM established the trials, collected and provided participant samples and the HAI data. FK provided the chimeric hemagglutinin protein (cH6/1). PLM and SIR assisted in data interpretation, reviewed and edited the manuscript and supervised the research. All authors contributed to the manuscript and approved the submitted version.

POTENTIAL CONFLICT OF INTEREST

FK reports royalties (Avimex), consulting fees (Pfizer, Seqirus, Third Rock Ventures and Avimex, Gritstone), and payment for academic lectures during the past two years. FK is also named as inventor on IP filed by the Icahn School of Medicine at Mount Sinai for influenza

virus vaccines and therapeutics, SARS-CoV-2 vaccines and SARS-CoV-2 serological assays, is co-founder and scientific advisory board member of Castlevax and is working with Dynavax on influenza virus vaccine development.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY

All relevant data are within the manuscript and its supporting information files. Further inquiries can be directed to the corresponding authors.

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Supplementary Material

Hemagglutinin inhibition assay

Modified HAI assays were previously performed [1,2]. Briefly, plasma samples instead of serum were treated with receptor destroying enzyme (RDE) from *Vibrio cholera* (Denka-Seiken). Then these were diluted 1:10 in saline and subsequent serial 2-fold dilutions of the plasma were used in a standard HAI assay using 4 hemagglutinating units of the antigen and 0.75% turkey red blood cells. Plasma samples with titers ≥10 were considered indicative of immune responses. The antigen used in the assays was A/H1N1/pdm09.

Hemagglutinin stalk IgG ELISA

H1 stalk IgG ELISAs were performed as previously described [3]. Plates were coated with cH6/1 diluted to 2 µg/ml overnight. Plates were washed and subsequently blocked with 3% fetal bovine serum, 0.5% non-fat dry milk powder. After washing, plasma samples were added to the plate diluted to a starting concentration of 1:40, followed by two-fold serial dilution and incubated for 2 hours. Following a wash, anti-human IgG (Fab specific)-peroxidase antibody diluted to 1:3000 in blocking solution was added to the plate. After 1-hour incubation, plates were washed and developed using SigmaFast o-phenylenediamine dichloride (OPD) (Sigma, USA). The reaction was stopped by adding 3M HCl and the optical density (OD) was measured at 490 nm. The antibody concentration was quantified against the standard curve included on each plate that consisted of polyvalent human normal immunoglobulin (Polygam) (National Bioproducts Institute, South Africa) with an assigned arbitrary value of 1000 arbitrary units (AU)/ml.

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Part 2: Paper 2

Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women

Supplementary Table 1: Demographic and clinical details of the maternal influenza vaccination trial participants

	Placebo (n=145)	**Vaccine (n=138)**	
HIV-1 status; people living without HIV-1: people living with HIV-1 (%)	77:68 (47%)	68:70 (51%)	ns
Median age; years [IQR]	27 [23-32]	26 [22-30]	ns
Median gestational age at enrolment; weeks ± standard deviation	27 ± 3,8	27 ± 4,3	ns
Days between pre- and post-sampling visits; median [IQR]	31 [28-33]	30 [28-32]	ns
Influenza cases; n (%)	18 (12%)	5 (4%)	*
A/H1N1; n (%)	8 (6%)	3 (2%)	ns
A/H3N2; n (%)	4 (3%)	2 (1%)	ns
B/Victoria/2/87-like or B/Yamagata/16/88-like; n (%)	6 (4%)	0 (0%)	*

Mann-Whitney test used to compare placebo and vaccine recipients. Fisher's exact test used to compare categorical variables. IQR; Interquartile range. Statistical significance: *p <0.05 and ns= not significant.

Part 2: Paper 2

Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women

Supplementary Table 2: The association between the levels of H1 stalk-specific Fc-mediated functions and confirmed influenza A/H1N1 illness

	A/H1N1 illness (n=11) Proportion	No A/H1N1 illness (n=272) Proportion	OR (95% CI)[a]
ADCP Score			
≥**100**	73%	85%	0,46 (0,13-1,66) ns
≥**150**	45%	73%	0,31 (0,10-1,08) ns
≥**200**	27%	63%	0,22 (0,06-0,73) *
≥**250**	9%	47%	0,11 (0,01-0,67) *
≥**300**	9%	32%	0,21 (0,02-1,24) ns
≥**350**	0%	26%	0,00 (0,00-1,05) ns
ADCD Score			
≥**10**	18%	65%	0,12 (0,03-0,48) **
≥**20**	18%	54%	0,19 (0,04-0,75) *
≥**25**	9%	51%	0,10 (0,01-0,57) *
≥**30**	9%	45%	0,12 (0,01-0,72) *
≥**40**	9%	42%	0,14 (0,01-0,82) *
≥**50**	0%	39%	0,00 (0,00-0,58) **
ADCC RLU			
≥**50**	73%	60%	1,81 (0,54-6,42) ns
≥**100**	45%	39%	1,31 (0,44-4,55) ns
≥**150**	9%	26%	0,28 (0,03-1,69) ns
≥**200**	0%	19%	0,00 (0,00-1,63) ns

[a] Odds ratio (OR) and 95% confidence intervals (CI) obtained from Fisher's exact test for H1 stalk-specific ADCP, ADCD and ADCC potential levels. Statistical significance: **p <0.01; *p <0.05 and ns= not significant.

Part 2: Paper 2

Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women

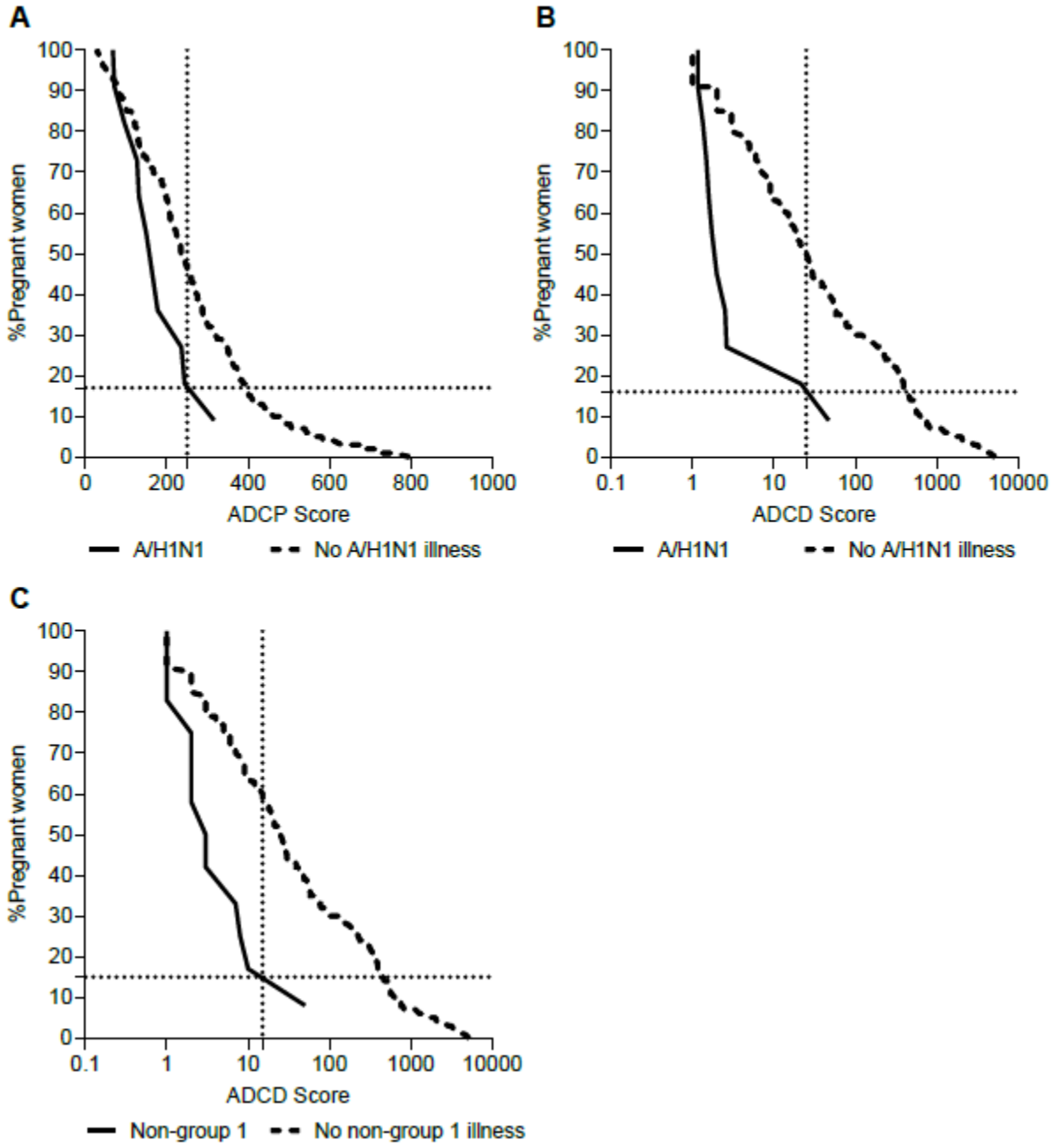
Supplementary Table 3: The association between the levels of H1 stalk-specific Fc-mediated functions and confirmed influenza non-group 1 (A/H3N2 or B) illness

	Non-group 1 illness (n=12) Proportion	No non-group 1 illness (n=271) Proportion	OR (95% CI)[a]
ADCP Score			
≥**100**	83%	85%	0,89 (0,22-4,19) ns
≥**150**	58%	73%	0,53 (0,18-1,50) ns
≥**200**	42%	63%	0,42 (0,15-1,25) ns
≥**250**	17%	47%	0,23(0,05-1,01) ns
≥**300**	0%	33%	0,00 (0,00-0,69) *
ADCD Score			
≥**10**	17%	65%	0,11 (0,02-0,48) **
≥**15**	8%	60%	0,06 (0,01-0,35) ***
≥**20**	8%	54%	0,08 (0,01-0,44) **
≥**30**	8%	45%	0,11 (0,01-0,63) *
≥**40**	8%	42%	0,13 (0,01-0,72) *
≥**50**	8%	39%	0,14 (0,01-0,81) *
ADCC RLU			
≥**50**	75%	59%	2,05 (0,55-7,15) ns
≥**100**	58%	38%	2,25 (0,76-6,36) ns
≥**150**	42%	25%	2,18 (0,76-6,47) ns
≥**200**	25%	18%	1,37 (0,39-5,32) ns
≥**250**	17%	13%	1,31 (0,28-5,21) ns
≥**300**	17%	9%	2,06 (0,43-8,35) ns
≥**450**	17%	5%	3,97 (0,80-18,0) ns

Non-group 1 (A/H3N2 n=6; B/Victoria n=2; B/Yamagata n=4). [a] Odds ratio (OR) and 95% confidence intervals (CI) obtained from Fisher's exact test for H1 stalk-specific ADCP, ADCD and ADCC potential levels. Statistical significance: ***p <0.001; **p <0.01; *p <0.05 and ns= not significant.

Part 2: Paper 2

Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women



Supplementary Figure 1. Reverse cumulative plots of H1 stalk-specific ADCP and ADCD levels from individuals who developed influenza-illness and those who did not display symptoms of influenza. (**A**) ADCP levels and (**B**) ADCD levels for A/H1N1 illnesses (solid line) and no A/H1N1 illnesses (dotted line). Vertical and horizontal line on X axis and Y axis represent ADCP score of 250 at which 83% of the participants are likely to remain A/H1N1-uninfected and ADCD score of 25 at which 84% of the participants are likely to remain A/H1N1-uninfected. (**C**) ADCD levels for non-group 1 illnesses (solid line) and no non-group 1 illnesses (dotted line). Vertical and horizontal line on X axis and Y axis represent ADCD score of 15 at which 85% of the participants are likely to remain A/H3N2 or influenza B-uninfected.

Part 2

Paper 3: Despite delayed kinetics, people living with HIV achieve equivalent antibody function after SARS-CoV-2 infection or vaccination

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Despite delayed kinetics, people living with HIV achieve equivalent antibody function after SARS-CoV-2 infection or vaccination

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Despite delayed kinetics, people living with HIV achieve equivalent antibody function after SARS-CoV-2 infection or vaccination

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The kinetics of Fc-mediated functions following SARS-CoV-2 infection or vaccination in people living with HIV (PLWH) are not known. We compared SARS-CoV-2 spike-specific Fc functions, binding, and neutralization in PLWH and people without HIV (PWOH) during acute infection (without prior vaccination) with either the D614G or Beta variants of SARS-CoV-2, or vaccination with ChAdOx1 nCoV-19. Antiretroviral treatment (ART)–naïve PLWH had significantly lower levels of IgG binding, neutralization, and antibody-dependent cellular phagocytosis (ADCP) compared with PLWH on ART. The magnitude of antibody-dependent cellular cytotoxicity (ADCC), complement deposition (ADCD), and cellular trogocytosis (ADCT) was differentially triggered by D614G and Beta. The kinetics of spike IgG-binding antibodies, ADCC, and ADCD were similar, irrespective of the infecting variant between PWOH and PLWH overall. However, compared with PWOH, PLWH infected with D614G had delayed neutralization and ADCP. Furthermore, Beta infection resulted in delayed ADCT, regardless of HIV status. Despite these delays, we observed improved coordination between binding and neutralizing responses and Fc functions in PLWH. In contrast to D614G infection, binding responses in PLWH following ChAdOx-1 nCoV-19 vaccination were delayed, while neutralization and ADCP had similar timing of onset, but lower magnitude,

and ADCC was significantly higher than in PWOH. Overall, despite delayed and differential kinetics, PLWH on ART develop comparable responses to PWOH, supporting the prioritization of ART rollout and SARS-CoV-2 vaccination in PLWH.

KEYWORDS

humoral response kinetics, people living with HIV (PLWH), SARS-CoV-2 infection, ChAdOx1 nCov-19 vaccination, neutralization, Fc effector functions

1 Introduction

There are more than 38 million people living with HIV (PLWH) globally, two-thirds of whom reside in sub-Saharan Africa (1, 2). South Africa bears the burden of the HIV pandemic with over 7.5 million PLWH, with approximately 2 million not on antiretroviral treatment (ART) (2, 3). Furthermore, PLWH have a 3.2-fold higher probability of testing positive for SARS-CoV-2 and an increased risk for severe COVID-19 and mortality (4–10). PLWH may also be at risk of being reservoirs for the emergence of SARS-CoV-2 variants of concern (VOCs), due to their decreased ability to clear the virus (11–14). Understanding immune responses to SARS-CoV-2 infection and vaccination in PLWH is therefore important for informing vaccine implementation in this population. Binding and neutralizing responses against SARS-CoV-2 infection are reduced in HIV viremic individuals; however, in virally suppressed PLWH, comparable and durable humoral and SARS-CoV-2-specific T-cell responses occur (15–22). Furthermore, COVID-19 severity impacts on the antibody response in PLWH, with severe disease resulting in similar responses and symptomatic non-hospitalized disease in lower magnitude responses compared with people without HIV (PWOH), respectively (23). The BNT162b2, mRNA-1273, Ad26.COV2.S, and ChAdOx1 nCoV-19 vaccines all result in similar binding and neutralizing responses between PLWH and PWOH (17, 24–31). As with infection, stronger vaccine-induced immune responses develop in virally controlled PLWH on ART, compared with ART-naïve PLWH (17, 32–36). However, ART does not fully restore all HIV-specific immune function in PLWH, and additional vaccinations may be required for this at-risk population (37–43).

Beyond neutralization, antibodies mediate cytotoxic functions through the interaction of the antibody Fc region with cellular Fc receptors or complement proteins. These Fc effector functions include antibody-dependent cellular cytotoxicity (ADCC), cellular phagocytosis (ADCP), cellular trogocytosis (ADCT), and complement deposition (ADCD). SARS-CoV-2–specific Fc effector functions have been associated with reduced COVID-19 mortality, are differentially imprinted by SARS-CoV-2 VOCs, are more durable than neutralization activity, and are required for optimal protection conferred by monoclonal antibodies (44–51). Furthermore, Fc effector functions elicited in non-human primates after vaccination correlate with protection (52–57). Apart from a recent study showing similar response rates and levels of ADCP in PLWH and PWOH after infection, the kinetics and longevity of SARS-CoV-2–specific Fc effector function in PLWH are not known (23).

In this study, we investigated SARS-CoV-2 spike-specific Fc functions in hospitalized SARS-CoV-2 vaccine naïve PLWH and PWOH following infection with either D614G or Beta during the acute phase and compared these with ChAdOx1 nCoV-19 vaccinees. ART-naïve PLWH had impaired humoral responses after SARS-CoV-2 infection. Furthermore, subtle differences in Fc-mediated response level and kinetics depending on the infecting variant were observed, and both ADCP and neutralization responses were delayed in PLWH. In comparison with infection, ChAdOx1 nCoV-19 vaccination differed by eliciting delayed binding and higher ADCC in PLWH. Overall, despite early delays in some antibody functions following infection or vaccination, PLWH were able to elicit high levels of humoral immune responses similar to PWOH.

2 Materials and methods

2.1 Study design

D614G and Beta infection convalescent plasma samples were collected from participants enrolled to COVID-19 study cohorts in South Africa. HIV status was tested prior to enrollment, as part of standard of care. The D614G infection samples (39 PWOH and 14 PLWH) were collected during the months of April to September 2020 prior to the emergence of Beta. The Beta infection samples (13 PWOH and 9 PLWH) were collected between October 2020 and May 2021, when Beta accounted for > 90% of infections in the country (58). Participants were admitted to Steve Biko Academic Hospital (Pretoria, South Africa) with mild to moderate infection (WHO scale 3–5). All samples were collected during the acute phase of COVID-19. Patients had PCR confirmed SARS-CoV-2 infection before blood collection, which was done at admission. A second sample was collected a median of 7 days after admission from participants (D614G, n = 37 and Beta, n = 14) that were not discharged early or deceased (D614G, n = 8 and Beta, n = 1). Full demographic and clinical characteristics of the Pretoria COVID-19 study participants are summarized in Supplementary Table S1.

Ethics approval was received from the University of Pretoria, Faculty of Health Sciences' Research Ethics Committee (247/2020). For the ChAdOx1 nCoV-19 vaccine samples, eligible participants were adults living with (n = 13) or without HIV (n = 17) (Supplementary Table S2). Lack of prior infection in these individuals was confirmed by nucleocapsid enzyme-linked immunosorbent assay (ELISA) as previously described (59). Longitudinal sampling was conducted, and plasma was collected 28 days after the 1st dose, at day 42 (14 days after the 2nd dose) and then at day 182 (6 months post-vaccination). The cohort was established at the Chris Hani Baragwanath Hospital as part of the South African safety and efficacy ChAdOx1 nCov-19 randomized trial (28). This study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (ethics reference number: M200501). Written informed consent was obtained from all participants.

2.1 Spike plasmid and antigen production

The SARS-CoV-2 Wuhan-1 spike was mutated to include D614G or lineage defining mutations for Beta (L18F, D80A, D215G, 242-244 del, K417N, E484K, N501Y, D614G, and A701V).

For ELISA and Fc effector function assays, SARS-CoV-2 D614G and Beta variant full spike proteins were expressed in human embryo kidney (HEK) 293F suspension cells by transfecting the cells with the respective expression spike plasmids. The cells were incubated for a period of 6 days at 37°C, 70% humidity and 10% CO_2. Proteins were purified using a nickel-charged resin followed by size-exclusion chromatography. The relevant fractions were collected and stored at −80°C until use.

2.2 Cell lines

HEK293F suspension cells were cultured in 293 Freestyle media (Gibco BRL Life Technologies, Ontario, CA) and incubated shaking at 37°C, 5% CO_2 and 70% humidity at 125 rpm. HEK293T cells were cultured at 37°C, 5% CO_2, in DMEM (Gibco BRL Life Technologies, Ontario, CA) containing 10% heat-inactivated fetal bovine serum (FBS) and supplemented with 50 mg/ml Gentamicin. Cells were disrupted at confluence with 0.25% trypsin in 1 mM EDTA every 48h–72h. HEK293T/ACE2.MF cells were maintained as for HEK293T cells but were supplemented with 3 mg/ml Puromycin for selection of stably transduced cells. THP-1 cells were used for the ADCP assay and obtained from the AIDS Reagent Program, Division of AIDS, NIAID, NIH contributed by Dr. Li Wu and Vineet N. Kewal Ramani. Cells were cultured at 37°C, 5% CO_2 in RPMI (Gibco BRL Life Technologies, Ontario, CA) containing 10% heat-inactivated FBS with 1% Penicillin-Streptomycin (Pen/Strep) and 2-mercaptoethanol to a final concentration of 0.05 mM and not allowed to exceed 4×10^5 cells/ml to prevent differentiation. Jurkat-Lucia™ NFAT-CD16 cells (Invivogen, USA) were maintained in IMDM (Gibco BRL Life Technologies, Ontario, CA) media with 10% heat-inactivated FBS, 1% Pen/Strep, and 10 mg/ml of Blasticidin and 100 mg/ml of Zeocin were added to the growth medium every second passage to allow the selection of CD16 expressing cells.

2.3 SARS-CoV-2 spike enzyme-linked immunosorbent assay

Two microgram per mililiter of spike protein (D614G or Beta) was used to coat 96-well, high-binding plates and incubated overnight at 4°C. The plates were incubated in a blocking buffer consisting of 5% skimmed milk powder, 0.05% Tween 20, 1× phosphate buffered saline (PBS). Plasma samples were diluted to 1:100 starting dilution in a blocking buffer and added to the plates. IgG secondary antibody was diluted to 1:3000 in blocking buffer and added to the plates followed by TMB substrate (Thermo Fisher Scientific, USA). Upon stopping the reaction with 1 M H_2SO_4, absorbance was measured at a 450-nm wavelength. In all instances, mAbs CR3022 and BD23 (a differential control for Beta) were used as positive controls and Palivizumab was used as a negative control.

2.4 Lentiviral pseudovirus production

Pseudoviruses were prepared as previously described (60). Briefly, pseudotyped lentiviruses were prepared by co-transfecting the HEK293T cell line with the SARS-CoV-2 variant spike D614G or the Beta spike (L18F, D80A, D215G, 242-244 del, K417N, E484K, N501Y, D614G, and A701V) plasmids in conjunction with a firefly luciferase encoding lentivirus backbone (HIV-1 pNL4.luc) plasmid for 72h. Culture supernatants were clarified of cells by a 0.45 mM filter and stored at −70°C. Other pcDNA plasmids were used for the ADCT assay.

2.5 Pseudovirus neutralization assay

For all assays, including the neutralization assay, plasma samples were heat inactivated and clarified by centrifugation. IgG was isolated from the plasma samples of PLWH on ART, using Protein G (Pierce Biotechnology, USA), according to the manufacturer's instructions and confirmed by IgG ELISA. The plasma samples or IgG preparations from study participants were incubated with the SARS-CoV-2 pseudotyped virus for 1h at 37°C and 5% CO_2. These samples were also tested against murine leukaemia virus pseudovirus to ensure that there was no interference from ART. Subsequently, 1×10^4 HEK293T cells engineered to overexpress ACE-2 (293T/ACE2.MF) [kindly provided by M. Farzan (Scripps Research)] were added and incubated at 37°C and 5% CO_2 for 72h upon which the luminescence of the luciferase gene was measured. Titers were calculated as the reciprocal plasma dilution (ID_{50}) causing 50% reduction of relative light units (RLUs). CB6 and CA1 were used as neutralization controls for D614G and Beta.

2.6 Antibody-dependent cellular phagocytosis (ADCP) assay

SARS-CoV-2 full spike proteins were biotinylated using the EZ-Link Sulfo-NHS-LC-Biotin kit (Thermo Scientific, USA) and coated onto fluorescent neutravidin beads as previously described (61). Briefly, beads were incubated for 2h with monoclonal antibodies at a starting concentration of 20 mg/ml or plasma at a single 1 in 100 dilution. Opsonized beads were incubated with the monocytic THP-1 cell line overnight, fixed and interrogated on the FACSAria II. Phagocytosis score was calculated as the percentage of THP-1 cells that engulfed fluorescent beads multiplied by the geometric mean fluorescence intensity of the population less the no antibody control. For this and all subsequent Fc effector assays, pooled plasma from five PCR-confirmed SARS-CoV-2–infected individuals and CR3022 were used as positive controls and plasma from five pre-pandemic healthy controls and Palivizumab were used as negative controls. 084-7D, a mAb isolated from a donor following SARS-CoV-2 infection, was used as a positive control for Beta. ADCP scores for different spikes were normalized to each other and between runs using CR3022.

2.7 FcgRIIIa reporter assay

The ability of plasma antibodies to cross-link spike SARS-CoV-2 protein and activate FcgRIIIa on Jurkat-Lucia™ NFAT-CD16 cells (Invivogen, USA) was measured as a proxy for ADCC. High-binding 96-well plates were coated with 1 mg/ml spike SARS-CoV-2 protein and incubated at 4°C overnight. Plates were then washed with PBS and blocked at room temperature for 1h with 2.5% bovine serum albumin (BSA)/PBS. After washing, heat-inactivated plasma (1:100 final dilution) or monoclonal antibodies (final concentration of 100 mg/ml) in RPMI media supplemented with 10% FBS 1% Pen/Strep were added to the wells and incubated for 1h at 37°C. Jurkat-Lucia™ NFAT- CD16 2 × 10^5 cells per well were added and incubated for 24h at 37°C and 5% CO_2. Twenty microliter of supernatant was then transferred to a white 96-well plate with 50 ml of reconstituted QUANTI-Luc secreted luciferase and read immediately on a Victor 3 luminometer with 1 s integration time. The RLUs of a no antibody control were subtracted as background. Palivizumab was used as a negative control, while CR3022 was used as a positive control, and P2B-2F6 to differentiate the Beta from the D614G variant. To induce the transgene, 1× cell stimulation cocktail (Thermo Fisher Scientific, Oslo, Norway) and 2 mg/ml ionomycin in R10 was added. RLUs for spikes were normalized to each other and between runs using CR3022.

2.8 Antibody-dependent complement deposition (ADCD) assay

ADCD was measured as previously described (62). Biotinylated spike protein was coated 1:1 onto red fluorescent 1 mM neutravidin beads (Molecular Probes Inc., USA) for 2h at 37°C. These were incubated with a single 1:10 plasma sample dilution or fivefold titration of mAb at a starting concentration of 100 mg/ml for 2h and guinea pig complement diluted 1 in 50 with gelatin/veronal buffer for 15 min at 37°C. Beads were washed in PBS and stained with anti-guinea pig C3b-FITC, fixed and interrogated on a FACSAria II. Complement deposition score was calculated as the percentage of C3b-FITC–positive beads multiplied by the geometric mean fluorescent intensity of FITC in this population less the no antibody or heat-inactivated controls. ADCD scores for D614G and Beta spikes were normalized to each other and between runs using CR3022.

2.9 Antibody-dependent cellular trogocytosis (ADCT) assay

ADCT was performed as described in and modified from a previously described study (63). HEK293T cells transfected with a SARS-CoV-2–spike pcDNA vector as above were surface biotinylated with EZ-Link Sulfo-NHS-LC-Biotin as recommended by the manufacturer. Fifty-thousand cells per well were incubated with fivefold titration of mAb starting at 25 mg/ml or single 1 in 100 dilutions for 30 min. Following an RPMI media wash, these were then incubated with carboxyfluorescein succinimidyl ester (CFSE)–stained THP-1 cells (5 × 10^4 cells per well) for 1h and washed with 15 mM EDTA/PBS followed by PBS. Cells were then stained for biotin using Streptavidin-PE and read on a FACSAria II. Trogocytosis score was determined as the proportion of CFSE-positive THP-1 cells also positive for streptavidin-PE less the no antibody control with infecting variants run head to head.

2.10 Dimeric Fc gamma receptor-binding ELISAs

High-binding 96-well ELISA plates were coated with 1 µg/ml spike protein in PBS overnight at 4°C. Three wells on each plate were directly coated with 5 µg/ml IgG, isolated from healthy donors, and signals from these wells were used to normalize the Fc receptor activity of the plasma samples. Plates were washed with PBS and blocked with PBS/1 mM EDTA/1% BSA for 1h at 37°C. Plates were then washed and incubated with 1:10 diluted plasma for 1h at 37°C and then with 0.2 µg/ml or 0.1 µg/ml of biotinylated FcgRIIa or FcgRIIIa dimer, respectively (constructs kindly provided by Prof. Mark Hogarth from the Burnet Institute, Australia), for 1h at 37°C (64). Subsequently, a 1:10000 dilution of Pierce high-sensitivity streptavidin-horseradish peroxidase (Thermo Scientific, USA) was added for a final incubation for 1h at 37°C. Last, TMB substrate (Sigma-Aldrich) was added, and color development was stopped with 1 M H_2SO_4 and absorbance read at 450 nm.

2.11 Quantification and statistical analysis

Analyses were performed in Prism (v9; GraphPad Software Inc, San Diego, CA, USA). Non-parametric tests were used for all

comparisons. Fisher's exact test was used to compare categorical variables between the study participants living with or without HIV, when assessing the clinical data. The Mann-Whitney test was used for unmatched samples. The Kruskal-Wallis test with Dunn's correction was conducted for multiple comparisons. All correlations reported are non-parametric Spearman's correlations. *P*-values less than 0.05 were considered to be statistically significant.

3 Results

3.1 People living with HIV have similar levels of humoral responses irrespective of the SARS-CoV2 infecting variant

We first compared antibody responses in SARS-CoV-2–infected PLWH with PWOH and assessed whether the infecting variant differentially triggered antibody functions. We used plasma from patients hospitalized during South Africa's first (PWOH, n = 39 and PLWH, n = 14) and second (PWOH, n = 13 and PLWH, n = 9) SARS-CoV-2 infection waves, which were dominated by the D614G and Beta variants, respectively. PWOH and PLWH were matched for age, sex assigned at birth, comorbidities, and SARS-CoV-2 disease severity for each of the infecting variants (Supplementary Table S1). Samples were collected at admission, which was a median of 2 days after a positive SARS-CoV-2 PCR test. During the acute infection, a second sample was taken a median of 7 days later, for a subset of patients who had not been discharged or were not deceased by this time (Supplementary Table S1).

SARS-CoV-2 IgG autologous spike binding (binding to the D614G spike for D614G infections and Beta spike for Beta infections) was measured at admission using an ELISA. The magnitude of IgG antibodies was similar irrespective of infecting variant and by HIV status [D614G geometric mean titers (GMTs) PWOH 349 vs. PLWH 328 and Beta GMTs PWOH 313 vs. PLWH 257] (Figure 1A). There were also no significant differences in pseudovirus neutralization titers by HIV status or across the infecting variants of SARS-CoV-2 infection (D614G GMTs PWOH 279 vs. PLWH 247 and Beta GMTs PWOH 726 vs. PLWH 332) (Figure 1B). SARS-CoV-2 spike-specific Fc effector responses were similar among the patients regardless of their HIV status (Figures 1C–F). While ADCP was not different across the infecting variants (Figure 1C), the Beta variant induced lower ADCC activity in PLWH (medians Beta 75 vs. D614G 392 p = 0.037) (Figure 1D), higher ADCD in PWOH (medians Beta 129 vs. D614G 27 p = 0.024) (Figure 1E) and higher levels of ADCT in both PWOH (medians Beta 5 vs. D614G 3 p = 0.007) and PLWH (medians Beta 4 vs. D614G 2 p = 0.022) (Figure 1F). Overall, PLWH had similar functional responses to PWOH during infection by either variant. However, the Beta variant differentially induced Fc-mediated functions similar to our previous findings (44).

As Fc effector function is modulated by Fc receptor binding, we examined the ability of antibodies from D614G and Beta infections to cross-link dimeric FcgRIIa or FcgRIIIa receptors (which modulate ADCP and ADCC, respectively) and the D614G or Beta spike protein by ELISA. As expected, Spearman's correlations > 0.5 p < 0.001 were noted between FcgRIIa binding and ADCP score, and between FcgRIIIa binding and ADCC reporter assay against D614G spike (Supplementary Figures S1A, B). Similar to the functional readouts, there were no significant differences in FcgRIIa binding (Supplementary Figure S1C) by HIV status. For the Beta infections, we observed lower FcgRIIa binding in comparison with the D614G infections (p = 0.021). However, no differences were noted between Beta- and D614G-triggered ADCP (Figure 1C), suggesting the potential impact of variation in antigen presentation on functional bead-based and soluble protein assays. The FcgRIIIa cross-linking (Supplementary Figure S1D) and ADCC reporter assay, where FcgRIIIa is expressed on a target cell, both showed similar responses in hospitalized patients, regardless of HIV status, but the Beta variant compared with D614G resulted in significantly reduced FcgRIIIa binding (p = 0.010).

3.2 SARS-CoV-2 humoral responses are compromised in people living with HIV not on antiretroviral treatment

PLWH were stratified according to whether they were on ART (n = 18, all of whom had CD4 T-cell counts greater than 100 cells/μl) or ART-naïve (n = 5, all with CD4 T-cell counts lower than 100 cells/μl). The ART-naïve PLWH had significantly lower IgG binding (p = 0.037), neutralization (p = 0.006), and ADCP (p = 0.012) activity than the PLWH on ART (Figure 1G). Additionally, both ADCC and ADCD responses were lower in ART-naïve individuals, although not significantly so, while ADCT responses were comparable between the two groups. Overall, this indicates that PLWH who do not receive ART have impaired immune responses to SARS-CoV-2 infection, as has been previously reported (15–18).

3.3 People living with HIV have delayed neutralization and ADCP SARS-CoV-2 responses following D614G infection

We next investigated the kinetics of humoral responses between admission and 1 week of hospitalization in PLWH and PWOH following infection with D614G (PWOH, n = 27 and PLWH, n = 10) or Beta (PWOH, n = 9 and PLWH, n = 5). This analysis excluded patients that were either discharged early or deceased. Overall, humoral responses increased between admission and 1 week of hospitalization, as indicated by a fold change greater than 1 (week 1/admission responses), regardless of HIV status and the infecting variant (Figure 2). There were no significant differences in the median fold changes of IgG binding, ADCC, ADCD, and ADCT by HIV status, indicating that these functional responses increased at the same rate in PLWH as in PWOH. However, during D614G

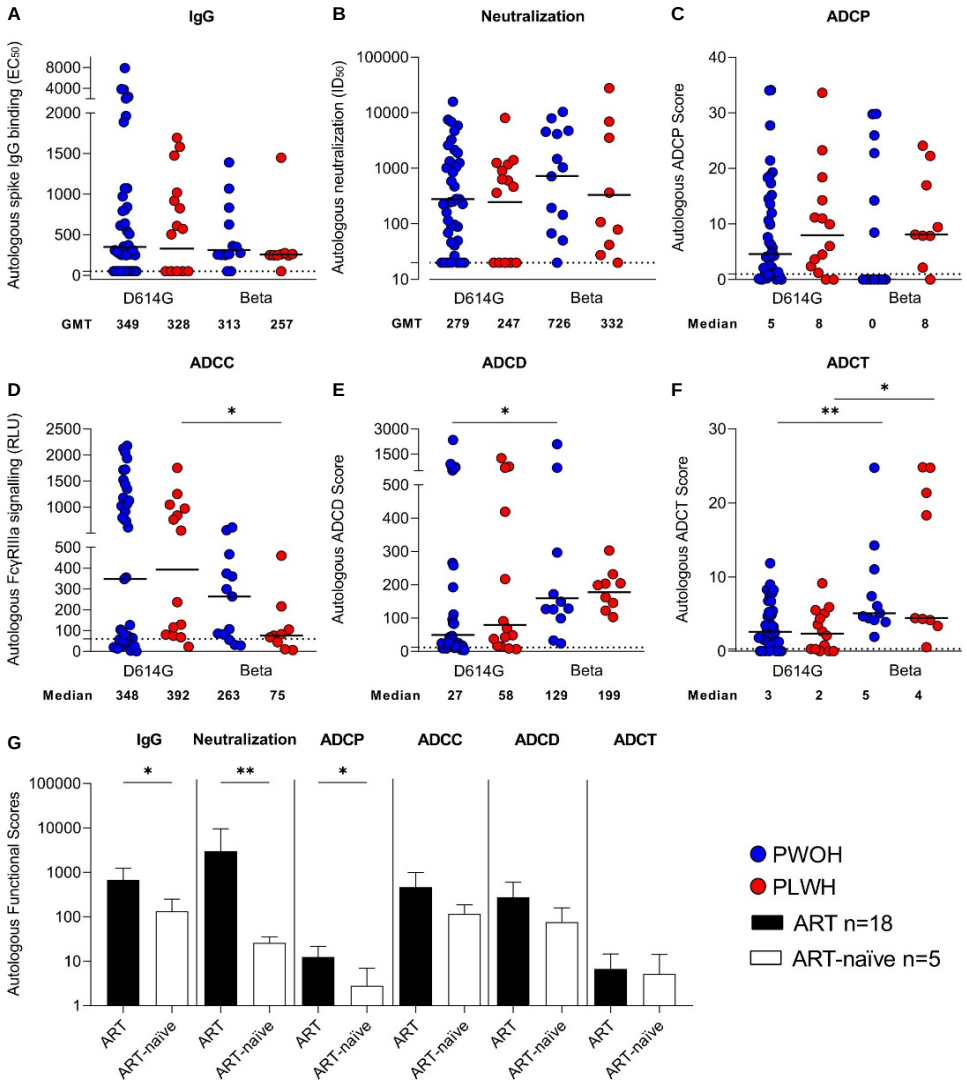

FIGURE 1
Similar antibody responses in hospitalized patients living with or without HIV during SARS-CoV-2 D614G and Beta infections. Autologous antibody responses at admission against D614G spike for D614G infections and Beta spike for Beta infections in people without HIV (PWOH) (D614G, *n* = 39; Beta, *n* = 13) are shown in blue and for people living with HIV (PLWH) (D614G, *n* = 14; Beta, *n* = 9) are shown in red. (A) IgG binding by ELISA against autologous full-length spikes. (B) Neutralization of D614G or Beta pseudoviruses. (C) Antibody-dependent cellular phagocytosis (ADCP) scores, as the percentage of monocytic cells that take up spike-coated beads multiplied by their geometric mean fluorescence intensity (MFI). (D) ADCC shown as relative light units (RLUs) signaling through FcgRIIIa-expressing cells. (E) ADCD is shown as the MFI of C3b deposition on spike-coated beads and (F) ADCT represented as the relative proportion of biotinylated spike-expressing cell membrane on carboxyfluorescein succinimidyl ester (CFSE)–positive monocytic cells. The lines represent geometric mean titers for the IgG and neutralization responses, and the median for the Fc-mediated responses. Limits of detection are shown with dotted lines. Kruskal-Wallis test with Dunn's correction was calculated for multiple test comparisons. (G) Autologous functional scores, referring to the activity of either the IgG-binding EC_{50}, neutralization ID_{50}, ADCP score, FcgRIIIa signaling RLU, ADCD score, or ADCT score measured against spike protein matching the infecting variant, among PLWH on antiretroviral treatment (ART) or ART-naïve at admission. PLWH on ART (*n* = 18), data shown as filled bars and ART-naïve participants (*n* = 5), data shown as unfilled bars. Error bars indicate standard deviation of the mean of participant data. Mann Whitney U test was used to compare groups. Statistical significance: $^{**}p < 0.01$; $^{*}p < 0.05$.

infection, neutralization ($p = 0.042$) and ADCP responses ($p = 0.032$) had significantly lower fold changes in PLWH, indicating that these responses were delayed compared with PWOH. The Beta variant had slower ADCT responses in both PWOH ($p = 0.001$) and PLWH ($p = 0.021$), and in contrast to D614G, Beta rapidly elicited ADCP in PLWH (Figure 2). These data suggest that some humoral responses are delayed in PLWH following acute SARS-CoV-2 infection and that different variants elicit altered levels of activity and also affect the kinetics of humoral responses.

3.4 People living with HIV show stronger coordination between SARS-CoV-2 IgG binding, neutralization, and Fc effector functions following infection

A coordinated antibody response against other diseases has been shown following vaccination and for HIV associated with improved protection (53, 65–67). We therefore assessed the correlations between antibody functions in PLWH (*n* = 10) and PWOH (*n* =

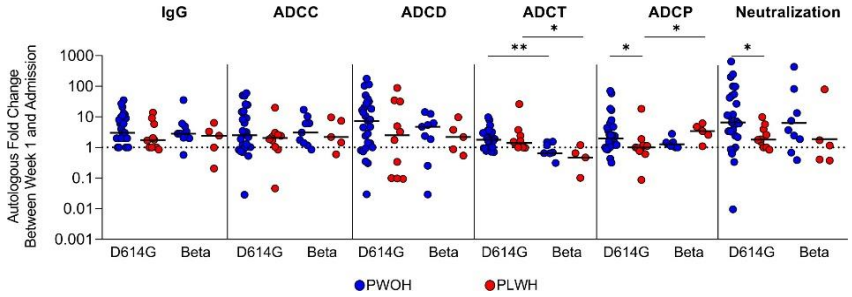

FIGURE 2
People living with HIV have delayed humoral response kinetics following SARS-CoV-2 D614G infection. Fold changes in autologous antibody responses a week after admission during SARS-CoV-2 D614G or Beta infection in PWOH (D614G, $n = 27$; Beta, $n = 9$) shown in blue and for PLWH (D614G, $n = 10$; Beta, $n = 5$) shown in red. The dotted line indicates no change between sampling time points, above 1 indicates an increase in function and below 1 indicates a decrease in function. The median of the fold changes is indicated by solid lines. For each of the antibody responses Kruskal-Wallis test with Dunn's correction was calculated for multiple test comparison. Statistical significance: $^{**}p < 0.01$; $^{*}p < 0.05$.

27) 1 week after admission following D614G infection. In PWOH, the strongest significant associations ($r > 0.5$ $p < 0.05$) with IgG binding were ADCC ($r = 0.61$ $p < 0.001$), ADCT ($r = 0.50$ $p < 0.01$), and FcgRIIa binding ($r = 0.67$ $p < 0.001$) (Figure 3A). In PLWH, SARS-CoV-2 spike IgG binding had a strong significant association with neutralization ($r = 0.83$ $p < 0.01$), ADCC ($r = 0.83$ $p < 0.01$), ADCD ($r = 0.84$ $p < 0.01$), and FcgRIIIa binding ($r = 0.67$ $p < 0.05$) (Figure 3B). The most striking difference between the groups was that neutralization activity was significantly associated with ADCC ($r = 0.77$ $p < 0.05$) and ADCD ($r = 0.69$ $p < 0.05$) in PLWH, but there were no significant associations between the neutralization activity and other functions in PWOH. Similarly, ADCP strongly correlated with ADCD ($r = 0.65$ $p < 0.05$), ADCT ($r = 0.82$ $p < 0.01$), and FcgRIIIa binding ($r = 0.65$ $p < 0.05$), and both ADCD and ADCT correlated with FcgRIIa ($r = 0.76$ $p < 0.05$ and $r = 0.75$ $p < 0.05$ respectively) and FcgRIIIa binding ($r = 0.7$ $p < 0.05$ and $r = 0.78$ $p < 0.05$, respectively) in PLWH. In comparison with the Fc-mediated functions of PLWH, fewer significant associations were observed in PWOH. Strong correlations were only observed for ADCC with FcgRIIa ($r = 0.69$ $p < 0.001$) and FcgRIIIa binding ($r = 0.56$ $p < 0.01$), and ADCT with FcgRIIa binding ($r = 0.55$ $p < 0.01$). Thus, antibody functional relationships differ between PLWH and PWOH, and PLWH in this study had a more coordinated functional response to SARS-CoV-2 infection, despite slower neutralization and ADCP kinetics in D614G infection.

3.5 ChAdOx1 nCoV-19 vaccination elicits higher IgG binding and ADCC over time in people living with HIV

The initial SARS-CoV-2 vaccines were based on the ancestral spike sequence, enabling us to examine IgG binding, neutralization, and Fc effector functions following ChAdOx1 nCoV-19 vaccination, to determine whether the kinetics are similar to D614G infection responses in both PLWH and PWOH. Thirty participants stratified by HIV status (PWOH, $n = 17$ and PLWH, $n = 13$) received two doses of the vaccine and plasma samples were collected at days 28 (after first dose), 42 (after second dose) and 182 post-vaccination (Figure 4A). The PWOH and PLWH were

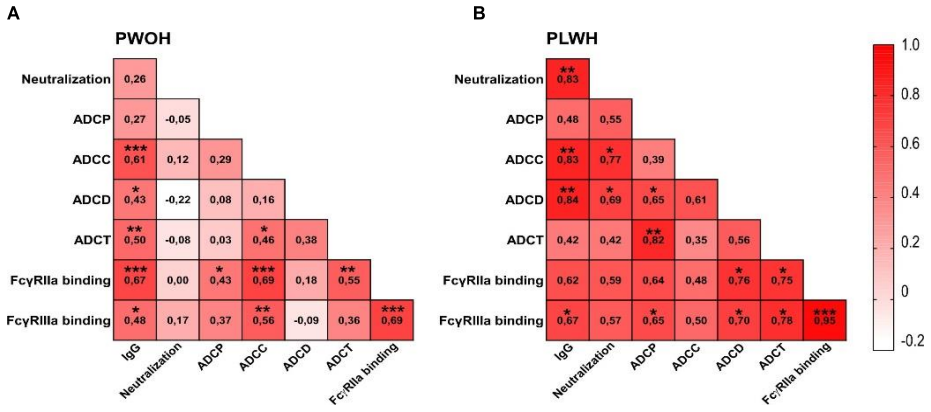

FIGURE 3
Differential associations between antibody responses in hospitalized patients living with or without HIV. Spearman correlations for (A) PWOH ($n = 27$) and (B) PLWH ($n = 10$) 1-week post-admission during SARS-CoV-2 D614G infection. Color intensity from white to red is associated with increasing correlation, indicated within the blocks. Statistical significance: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

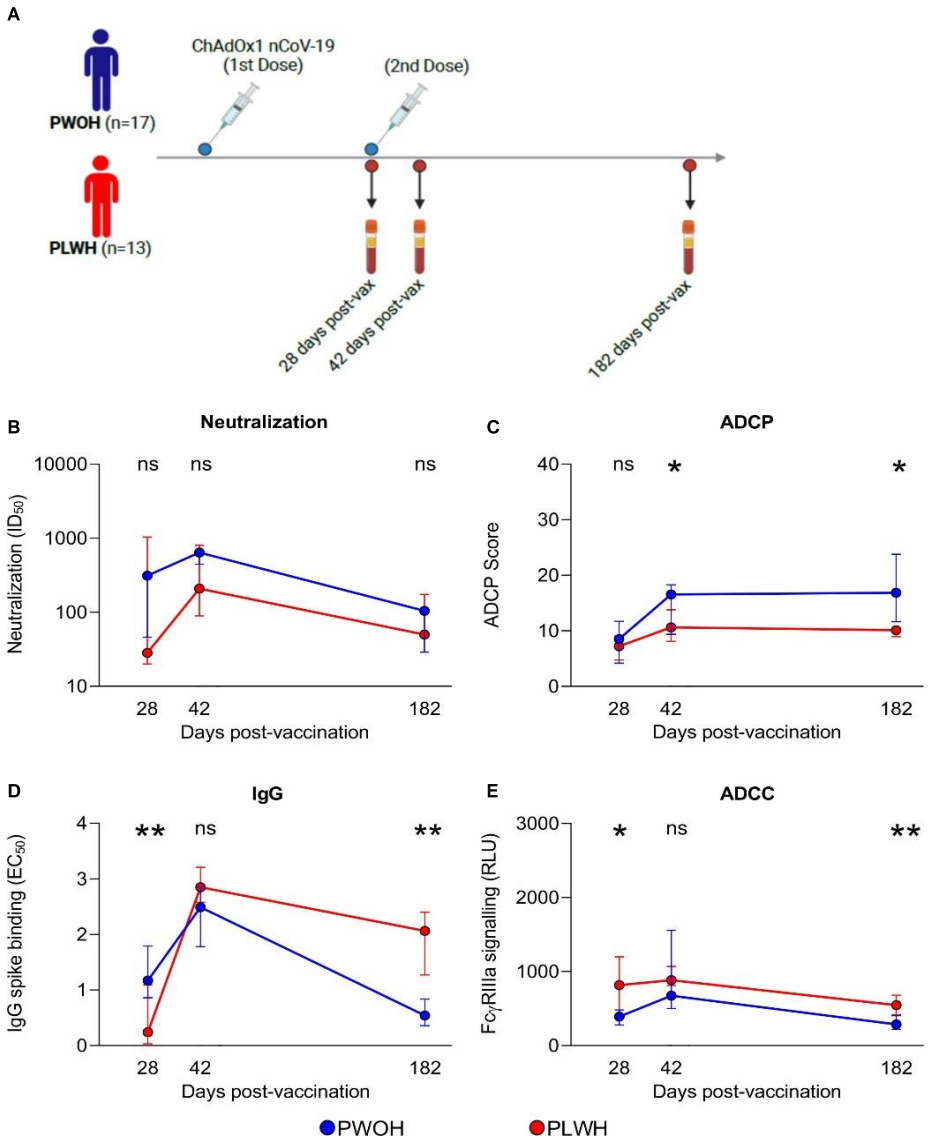

FIGURE 4
People living with HIV have durable humoral responses following ChAdOx1 nCoV-19 vaccination. (A) Thirty participants received two doses of the ChAdOx1 nCoV-19 vaccine, antibody responses were assessed for days 28, 42, and 182 post-vaccinations when plasma samples were collected from PWOH (n = 17) shown in blue and PLWH (n = 13) shown in red. (B) Neutralization of D614G pseudovirus, (C) ADCP scores, (D) IgG binding by ELISA, and (E) ADCC FcgRIIIa signalling were measured. The lines represent the medians with error bars, indicating standard deviation of participant data. Mann Whitney U test was calculated to compare groups at each of the time points. Statistical significance: $^{**}p < 0.01$; $^{*}p < 0.05$; ns, not significant.

matched for age, but PLWH had a lower proportion of males (Supplementary Table S2). Among the PLWH, the majority (77%) were on ART and had CD4 T-cell counts greater than 250 cells/µl. The onset of neutralization responses was similar regardless of HIV status, although this functional response trended lower in PLWH (Figure 4B). At 28 days post-vaccination, the onset of ADCP responses was similar between the groups, but the maturation of ADCP following the second dose in PLWH was slowed, resulting in significantly lower activity at days 42 (p = 0.038) and 182 (p < 0.011) (Figure 4C).

In contrast to infection, IgG binding was the only response significantly delayed at day 28 (p = 0.003) in PLWH. However, following the second dose, PLWH achieved comparable levels and then exceeded the titers observed in PWOH at 182 days (p = 0.002) (Figure 4D). In comparison with the other functions, ADCC was rapidly elicited in PLWH with significantly higher levels at day 28 (p = 0.035) than in the PWOH, and following the second dose, these higher levels persisted through to day 182 (p = 0.005) (Figure 4E). Last, we observed similar antibody kinetics between ART-treated and ART-naïve PLWH, likely due to small participant numbers, following ChAdOx1 nCoV-19 vaccination (data not shown). In contrast to infection with D614G, there was an initial delay in IgG binding, which may have compromised the levels and kinetics of neutralization and ADCP responses in PLWH, although not

significantly. Despite early delays in antibody functions, ChAdOx1 nCoV-19 vaccination was able to induce higher IgG binding and ADCC in PLWH than in PWOH.

4 Discussion

PLWH are at high risk of COVID-19 morbidity and mortality (5–10), therefore investigating the kinetics of antibody responses is essential for understanding protection against SARS-CoV-2 and informing vaccine implementation. Here, we confirm that ART-naïve individuals showed compromised SARS-CoV-2 immunity. We show that the infecting variants triggered nuanced differences in the levels and kinetics of Fc effector functions, as we have previously reported (44). Neutralization and ADCP responses were significantly delayed in PLWH, but despite these delays, a more coordinated antibody functional response was observed in comparison with PWOH. Following ChAdOx1 nCoV-19 vaccination, PLWH overall had slightly impaired neutralization and ADCP responses, and spike binding was delayed; however, ADCC was significantly higher. This study highlights that the kinetics of Fc-mediated functions differ by SARS-CoV-2 infection and vaccination in PLWH and despite initial delays, PLWH are able to reach an equivalent humoral immune response to PWOH.

Our observation that ART-naïve PLWH (CD4 counts less than 100 cells/μl) had significantly lower IgG binding, neutralization, and ADCP responses suggests impaired SARS-CoV-2 immunity in this immune-compromised population. This corroborates previous studies that showed lower IgG and pseudovirus neutralizing antibody titers in virally unsuppressed PLWH with low CD4 counts between 200 and 500 cells/ul (15–18). In contrast, several studies reported unimpaired binding and neutralization in the context of ART-controlled HIV, who had even higher CD4 counts than participants in our study (19, 20). Most PLWH in our cohort accessed ART, likely explaining why we saw no overall impairment in humoral responses (68–70).

Our study also investigated Fc effector functions ADCC, ADCD, and ADCT, which have not previously been measured in PLWH following SARS-CoV-2 infection. We found no differences in Fc effector functions compared with PWOH. This confirms a previous finding that ADCP in PLWH after SARS-CoV-2 infection showed no difference compared with PWOH (23). Thus, PLWH on ART were capable of eliciting a robust Fc-mediated response after SARS-CoV-2 infection, similar to PWOH.

However, differences in the magnitude of Fc effector functions were observed depending on which infecting variant triggered the response. Beta induced significantly lower ADCC activity in PLWH and also trended lower in PWOH. In PLWH, ADCD trended higher and was shown to be significantly higher in PWOH following Beta infection. These observations were similar to our previous findings in a different cohort of PWOH, in which ADCC was reduced and ADCD was substantially higher in individuals infected with Beta (44). Here, Beta elicited higher levels of ADCT in both PWOH and PLWH, but in our previous study, responses were unaffected in comparison with D614G (44). This difference in studies suggests that other factors, such as disease severity, may also impact variant imprinting. Here, and in our previous study, we conclude that the sequence of infecting variants may alter antibody quality, perhaps by eliciting antibodies with varying glycosylation and/or isotype, a finding which requires further investigation.

While no quantitative differences in the level of responses were noted at enrollment, both neutralization and ADCP were delayed in PLWH following D614G infection. These delays were not observed for PLWH infected with Beta despite similarities in age, CD4 counts, and the proportion of individuals on ART, when compared with PLWH infected with D614G. This indicates that there is an impairment in SARS-CoV-2 responses in PLWH, perhaps due to the incomplete restoration of immune function following ART (37, 38). Delayed responses could increase the risk of re-infections or vaccine breakthrough infections and have been associated with SARS-CoV-2 RNAemia and COVID-19 progression (71). However, these same delays were not noted following ChAdOx1 nCoV-19 vaccination. Vaccinated PLWH rapidly elicited robust ADCC responses exceeding the levels in PWOH after the first dose and these levels remained higher 6 months post-vaccination. ChAdOx1 nCoV-19–induced ADCC in PWOH showed greater cross-reactivity against Delta and lasted longer than binding and neutralizing antibodies as previously reported (72). This highlights that ADCC may also be an important mechanism for durable and broad vaccine-induced immunity in PLWH but requires further investigation into how this functional response is differentially regulated in these individuals. In contrast to comparable binding kinetics observed for D614G infection, binding responses were initially delayed in ChAdOx1 vaccinated PLWH. This could be due to lower antigen exposure in vaccination, as the only difference in spike immunogen sequences is the D614G mutation. However, these binding responses increased to equivalent levels in PWOH after the second vaccine and result in higher binding at 6 months post-vaccination. In an immunogenicity study for the ChAdOx1 vaccine, including larger numbers of PLWH, higher SARS-CoV-2 spike binding antibodies were reported with each vaccine dose in PLWH (28). The durability and higher binding is potentially due to the late onset of this response in PLWH compared with PWOH. While neutralization and ADCP were delayed in infection, after vaccination, these responses had similar kinetics but lower levels in PLWH, although not significantly so for neutralization. Overall, vaccination induces different effects compared with D614G infection despite a similar antibody-targeted immunogen.

In addition to delayed responses following infection, PLWH had a surprisingly better coordination of antibody functional responses. For SARS-CoV-2, early infection that triggers an enhanced coordinated Fc effector response has been associated with COVID-19 recovery (47, 48). Here, our data suggest that PLWH have a unique trajectory in humoral responses against SARS-CoV-2. This is not restricted to SARS-CoV-2 responses, as we have previously reported on a distinct coordination of antibody responses in PLWH in comparison with PWOH after influenza vaccination (66). However, the mechanisms that drive this more coordinated response in PLWH require further investigation. Given that there was no difference in severity or death between PLWH and PWOH in this cohort, it is possible that a more balanced or synergistic immune response, despite delays in their development, may be required for protection in this population.

Limitations of this study include the lack of sequences for the infecting variant; however, this was mitigated by sampling when the circulating variant accounted for > 90% of infections in the population. For Beta-triggered neutralization responses, PWOH had higher responses; however, the lack of statistical significance when compared with responses in PLWH is possibly a result of small participant numbers. In several cases, due to small numbers of ART-naïve PLWH, we pooled data from both waves, limiting our ability to assess the impact of the infecting variant. In addition, HIV viral loads were not available for all participants and, as a result, viral suppression could not be defined. Furthermore, sample sizes for the kinetics and coordination analysis were reduced due to the lack of follow-up samples from some patients. The SARS-CoV-2 infection humoral kinetics reported in this study are restricted to the first week following infection, which corresponds to the acute phase. Therefore, unlike in vaccination where longitudinal samples were obtained, a more complete analysis of the infection humoral kinetics was not possible.

In conclusion, our study shows that SARS-CoV-2 infection induces different kinetics of responses to vaccination between PLWH and PWOH. These data highlight the importance of rapid ART rollout and support the current SARS-CoV-2 vaccine implementation strategies in PLWH. Despite delayed kinetics, PLWH were able to elicit comparable responses to SARS-CoV-2 infection and vaccination. This study highlights the importance of investigating the kinetics of humoral immune responses against SARS-CoV-2 in PLWH, as these provide insights into the mechanisms required for immunity against severe disease and vaccine efficacy in this population.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by University of Pretoria, Faculty of Health Sciences' Research Ethics Committee (247/2020) and Human Research Ethics Committee of the University of the Witwatersrand (ethics reference number: M200501). The patients/participants provided their written informed consent to participate in this study.

Author contributions

BM, PM, and SR conceptualized the study. BM performed experiments, analysed data, generated the figures and wrote the manuscript. NM and SR performed Fc experiments and analysed data. HK and PK performed neutralization assays supervised by TH. FAy and ZM performed ELISA assays supervised by TM-G. MM processed samples and FAb, MB, VU, and TR established the Pretoria COVID-19 study, which provided participant samples from the Tshwane District Hospital. SM provided samples and clinical data for the ChAdOx1-nCoV19 vaccination trial. PM and SR assisted in data interpretation, reviewed and edited the manuscript and supervised the research. All authors contributed to the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1231276/full#supplementary-material

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Part 2: Paper 3

Despite delayed kinetics, people living with HIV achieve equivalent antibody function after SARS-CoV-2 infection or vaccination

Supplementary Material

Table S1: Demographic and clinical details of the D614G and Beta COVID-19 infection cohorts, stratified by HIV status

	D614G			Beta		
	PWOH (n=39)	**PLWH (n=14)**		**PWOH (n=13)**	**PLWH (n=9)**	
Demographics						
Median age; years [IQR]	47 [42-56]	46 [44-52]	ns	46 [42-52]	37 [36-56]	ns
Gender Male: Female (% Female)	24:15 (38%)	[a] 4:10 (71%)	ns	6:7 (54%)	2:7 (78%)	ns
Clinical						
Comorbidities; n (%)						
Hypertension	23 (59%)	4 (29%)	ns	5 (38%)	4 (44%)	ns
Diabetes	16 (41%)	3 (20%)	ns	2 (15%)	1 (11%)	ns
Obesity	21 (54%)	6 (43%)	ns	5 (38%)	3 (33%)	ns
History of tuberculosis	1 (3%)	2 (13%)	ns	0	0	ns
Chronic kidney disease	3 (8%)	0	ns	2 (15%)	0	ns
Cardiovascular disease	8 (21%)	2 (13%)	ns	2 (15%)	1 (11%)	ns
Cancer	1 (3%)	0	ns	0	1 (11%)	ns
>1 comorbidity	24 (62%)	[b] 4 (29%)	ns	6 (46%)	[b] 4 (44%)	ns
No comorbidities	7 (18%)	[c] 2 (13%)	ns	5 (38%)	[c] 5 (55%)	ns
HIV infection						
ART, n (%)	n/a	11 (73%)	n/a	n/a	7 (78%)	n/a
Median CD4+ count; cells/µl [IQR]	n/a	260 [113-352]	n/a	n/a	137 [70-514]	n/a
SARS-CoV-2 infection						
[d] PCR positivity	39 (100%)	14 (100%)	n/a	13 (100%)	9 (100%)	n/a
Test to admission; days [IQR]	2 [1-3]	2 [0-5]	ns	1 [0-3]	0 [0-2]	ns
Post-symptom onset at admission; days [IQR]	8 [7-11]	9 [6-11]	ns	7 [4-9]	5 [3-11]	ns
Duration of admission; days [IQR]	9 [6-13]	9 [7-14]	ns	7 [5-9]	7 [4-8]	ns
Inpatients for ≥ 1 week	27 (69%)	10 (71%)	ns	9 (69%)	5 (56%)	ns
Disease severity at admission						
[e] WHO Scale 3 mild	5 (13%)	2 (13%)	ns	3 (23%)	4 (44%)	ns
[f] WHO Scale 4 moderate	23 (59%)	10 (73%)	ns	9 (69%)	5 (56%)	ns
[g] WHO Scale 5 moderate	6 (15%)	0	ns	0	0	ns
Deaths	7 (18%)	1 (7%)	ns	0	1 (11%)	ns

Mann-Whitney test used to compare between the study participants living without or with HIV. Fisher's exact test used to compare categorical variables between the study participants living without or with HIV.

[a] Pregnant (1)

[b] More than 1 co-morbidity in addition to HIV

[c] No co-morbidities in addition to HIV

[d] Variant not sequenced but all tests conducted between April and September 2020 (South Africa's 1st wave; D614G dominance) and between October 2020 to May 2021 (South Africa's 2nd wave; Beta dominance)

[e] Ambulatory mild, symptomatic assistance needed

[f] Hospitalized moderate, no oxygen therapy

[g] Hospitalized moderate, oxygen by mask or nasal prongs

Statistical significance: ns=not significant; n/a=not applicable

Part 2: Paper 3

Despite delayed kinetics, people living with HIV achieve equivalent antibody function after SARS-CoV-2 infection or vaccination

Table S2: Demographic and clinical details of the ChAdOx1 nCoV-19 vaccine trial participants, stratified by HIV status

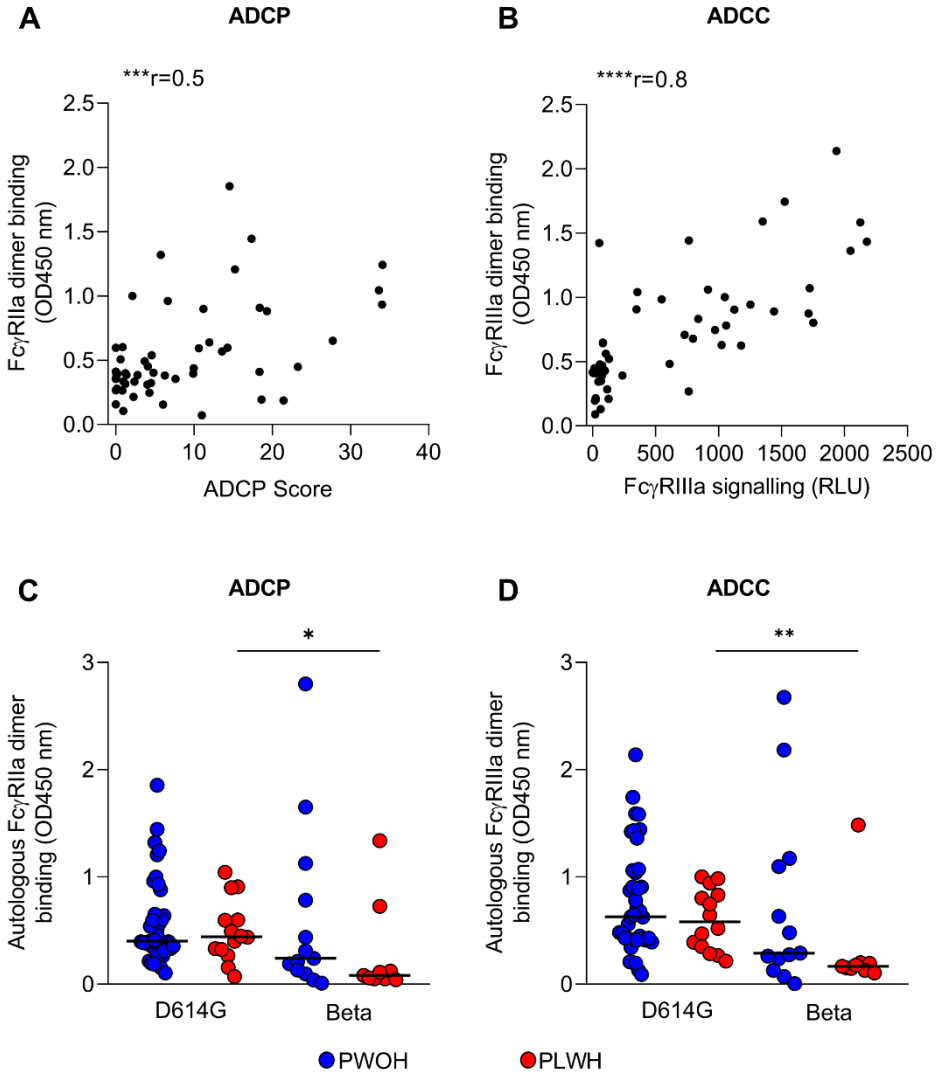
	PWOH (n=17)	**PLWH (n=13)**	
Demographics			
Median age; years [IQR]	31 [24-37]	35 [31-44]	ns
Gender Male: Female (% Female)	10:7 (41%)	2:11 (85%)	*
HIV infection			
ART, n (%)	n/a	10 (77%)	n/a
Median CD4+ count; cells/µl [IQR]	n/a	737 [615-901]	n/a

Mann-Whitney test used to compare between the study participants living without or with HIV. Fisher's exact test used to compare categorical variables between the study participants living without or with HIV.

Statistical significance: *p <0.05; ns=not significant; n/a=not applicable

Part 2: Paper 3

Despite delayed kinetics, people living with HIV achieve equivalent antibody function after SARS-CoV-2 infection or vaccination



Supplementary Figure 1. Fc gamma receptor (FcγR) binding correlates with functional activity against SARS-CoV-2. Spearman's correlations of **(A)** FcγRIIa dimer binding by ELISA and ADCP score by flow cytometry-based assay and **(B)** FcγRIIIa dimer binding by ELISA and FcγRIIIa signalling relative light units (RLU) by ADCC reporter assay of D614G infection plasma samples (n=53) against D614G spike. **(C)** FcγRIIa and **(D)** FcγRIIIa dimer binding against autologous spikes, D614G and Beta. FcγR dimer binding for PWOH shown in blue (D614G n=39 and Beta n=13) and for PLWH shown in red (D614G n=14 and Beta n=9). The lines represent the median. Kruskal-Wallis test with Dunn's correction was calculated for multiple test comparisons. Statistical significance: ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

Appendices

Appendix 1: Plagiarism Declaration

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Boitumelo Motsoeneng (Student number: 474346) am a student registered for the degree of Doctor of Philosophy in the academic year 2023.

I hereby declare the following:

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

Signature:  Date: 18/12/2023

Appendix 2: Turnitin Report

Turnitin Originality Report

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Appendix 3: Change of Title

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

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

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

15 March 2023
Person No: 474346
TAA

Miss BM Motsoeneng
PO Box 921
Wendywood
2144
South Africa

Dear Miss Boitumelo Motsoeneng

Doctor of Philosophy: Change of title of research

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

From:	**Functional and genetic characteristics of hemagglutinin stalk antibodies induced by influenza vaccination and infection**
To:	**Defining Fc-mediated functions in people living with HIV during respiratory viral infection and vaccination**

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

Appendix 4: Ethics Clearance

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Ms BM Motsoeneng

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M200444

NAME: (Principal Investigator)	Ms BM Motsoeneng
DEPARTMENT:	School of Pathology Department of Virology Medical School University
PROJECT TITLE:	Functional and genetic characteristics of hemagglutinin stalk antibodies induced by influenza vaccination and infection
DATE CONSIDERED:	2020/04/24
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Professor P Moore and Dr S Richardson
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/09/11

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **April** and will therefore reports and re-certification will be due early in the month of **April** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

2023/06/22

Professor P Moore, et al
School of Pathology
Department of Virology
Centre for HIV and STI's
National Institute for Communicable Diseases
Sandringham

Sent by e-mail to: carolc@nicd.ac.za

Dear Professor Moore

Re: **Protocol Ref No:** M2111103
Protocol Title: *Defining SARS-CoV-2 humoral responses in COVID-19 vaccinee sera in Southern Africa*
Principal Investigators: Professor P Moore, et al

Thank you for your letter of 2023/06/15.

We note and approve of your progress report. Congratulations on your publications in some high impact journals.

Thank you for keeping us informed.

Yours Sincerely

.....
Mr I Burns
For the Human Research Ethics Committee (Medical)

.....
Dr CB Penny, Chairperson, Human Research Ethics Committee (Medical)

Appendix 5: Letter of Support for Publications

NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES
Division of the National Health Laboratory Service

Antibody Immunity Research Unit (AIRU)
HIV / SARS-CoV-2 Virology Section, Centre for HIV & STI's
1 Modderfontein Road, Sandringham, 2031
Email: pennym@nicd.ac.za
Tel: 011 386 6362

14 December 2023

Faculty of Health Sciences
University of the Witwatersrand

To whom it may concern

Letter of support: Published papers included in PhD thesis: Boitumelo M. Motsoeneng

We are writing to confirm Boitumelo M. Motsoeneng's substantial role in the generation of three multi-authored manuscripts, of which two have been formally published and one submitted to a journal this December. These form part of Boitumelo's PhD thesis and all co-authors agree to this.

Boitumelo has led data generation for all the publications and has written all manuscripts, generated all figures and performed all analysis. She is therefore first author on each manuscript.

The manuscripts included in this submission are:

1. Boitumelo M. Motsoeneng, Nisha Dhar, Marta C. Nunes, Florian Krammer, Shabir A. Madhi, Penny L. Moore and Simone I. Richardson. Influenza vaccination results in differential hemagglutinin stalk-specific Fc-mediated functions in individuals living with or without HIV. Frontiers in Immunology, 2022, doi.org/10.3389/fimmu.2022.873191

2. Boitumelo M. Motsoeneng, Nisha Dhar, Marta C. Nunes, Florian Krammer, Shabir A. Madhi, Penny L. Moore and Simone I. Richardson. Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women. Submitted to Clinical Infectious Diseases, 12 December 2023.

3. Boitumelo M. Motsoeneng, Nelia P. Manamela, Haajira Kaldine, Prudence Kgagudi, Tandile Hermanus, Frances Ayres, Zanele Molaudzi, Thandeka Moyo-Gwete, Mieke A. van der Mescht, Fareed Abdullah, Michael T. Boswell, Veronica Ueckermann, Theresa M. Rossouw, Shabir A. Madhi, Penny L. Moore and Simone I. Richardson. Despite delayed kinetics, people living with HIV achieve equivalent antibody function after SARS-CoV-2 infection and vaccination. Frontiers in Immunology, 2023, doi.org/10.3389/fimmu.2023.1231276

Boitumelo's significant contribution to these papers is testament to the high quality of her research.

Yours sincerely

Dr Simone Richardson (Supervisor)
Senior Medical Scientist, AIRU
Senior Researcher, Wits

Prof. Penny Moore (Supervisor)
Research Professor
Academic Head: Divisions of Immunology and Virology, University of the Witwatersrand
DST/NRF South African Research Chair of Virus-Host Dynamics
Director: Antibody Immunity Research Unit (AIRU), an extramural unit of the South African Medical Research Council and NICD
Honorary Senior Scientist: Centre for the AIDS Programme of Research in South Africa (CAPRISA), University of KwaZulu-Natal

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