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Cell-Mediated and humoral immune responses to trivalent inactivated influenza vaccine in high-risk groups

Malherbe, Alexander Robert

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CELL–MEDIATED AND HUMORAL IMMUNE RESPONSES TO TRIVALENT INACTIVATED INFLUENZA VACCINE IN HIGH-RISK GROUPS

Alexander R Malherbe

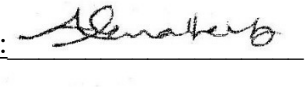
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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

October 2017

DECLARATION

I, Alexander Robert Malherbe declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by the University of the Witwatersrand, Johannesburg will not infringe any third party rights and that I have not previously in its entirety, or in part, submitted it for obtaining any qualification. This thesis 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.

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ABSTRACT

Introduction: Pregnancy is associated with physiological and immunological changes that leave women vulnerable to influenza infection and associated complications. This evolutionary adaptation is not fully understood, but evidence indicates a shift from cell-mediated immunity toward humoral immunity, which places pregnant women at a heightened risk to severe influenza illness, exacerbated further by co-infection with HIV. Tuberculosis (TB) is a major health issue resulting in ill-health among millions of people every year, with approximately one third of the population having latent TB. The considerable gains achieved in reducing the incidence of TB have reversed in recent years due to the emergence of HIV. Currently little data exist on the effectiveness of influenza vaccination in individuals co-infected with HIV and TB. We evaluate and compare the cellular and humoral immune responses to the trivalent inactivated influenza vaccine in these high-risk groups.

Methods: In 2013 we conducted (1) a double-blind randomised controlled trial, involving HIV-infected pregnant women, (2) two prospective, open labelled trials involving HIV-infected non-pregnant women, and HIV-uninfected pregnant and non-pregnant women, respectively, as well as in 2014 (3) a prospective, open labelled four arm trial involving HIV/TB co-infected, HIV-infected TB-uninfected, HIV-uninfected TB-infected and HIV-uninfected TB-uninfected adults. Cell-mediated, as measured by interferon-gamma (IFN-γ) enzyme-linked immunospot (ELISPOT) assay, and humoral, as measured by hemagglutination inhibition (HAI) assay, immune responses to the seasonal

trivalent inactivated influenza vaccine were evaluated and compared between respective study groups at baseline and approximately 1 month post-vaccination.

Results: in this study we report no significant differences in cell-mediated immune (CMI) responses among HIV-infected pregnant and non-pregnant women at both pre-vaccination and post-vaccination. Vaccination improved CMI responses to all three influenza strains, with the only significant increases observed for A/H1N1 in HIV-infected pregnant and non-pregnant women and B/Yamagata in HIV-infected non-pregnant women.

Following stratification of women into low- (LB) and high-baseline (HB) responses we found significantly improved cellular immune responses to the influenza viruses in the LB groups for both HIV-infected and HIV-uninfected pregnant and non-pregnant women, whereas HB women tended to exhibit a declined immune response.

We show significantly enhanced humoral immune responses to the influenza vaccination in TB-infected and TB-uninfected adults living with HIV post-vaccination, with little significant differences between the two groups. We found a similar trend in HIV-uninfected adults infected with and without TB; however HIV-uninfected adults achieved higher geometric mean titers than adults living with HIV.

Conclusion: among the HIV-infected pregnant and non-pregnant women, it appears pregnancy did not play as significant a role in attenuating CMI responses to vaccination as HIV infection. However, a significantly higher percentage of HIV-infected pregnant women were on antiretroviral therapy (ART) at the time of enrolment which may have influenced the role of CD4+ T-cell count on CMI responses and could possibly explain the

similar responses observed in these two study groups. Cellular immune responses to vaccination were significantly greater in HIV-uninfected non-pregnant women compared to HIV-uninfected pregnant women, adding further evidence to the detrimental impact pregnancy has on CMI responses.

Pre-existing immunity to influenza vaccination plays a major role on CMI responses following vaccination. Women with high-baseline responses tended to display decreased responses whereas women with low-baseline responses showed significantly improved responses. We propose that a potential threshold may exist where the quantity of memory T-cells reaches maximal levels in the blood system.

We also show that vaccination with trivalent inactivated influenza vaccine (IIV3) was relatively immunogenic in HIV-infected TB-infected/uninfected adults, albeit not the magnitude observed in healthy populations. However, in adults living without HIV-infection, we showed that influenza vaccination was significantly immunogenic in both adults infected with TB and those without. Additionally, we show that infection with TB does not appear to affect humoral immune responses, even in the HIV-infected population.

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ABBREVIATIONS

USA	United States of America
WHO	World Health Organisation
ACIP	Advisory Committee on Immunisation Practices
IIV3	Trivalent inactivated influenza vaccine
LAIV	Live attenuated influenza vaccine
HA	Hemagglutinin
NA	Neuraminidase
NP	Nucleoproteins
M1/2	Matrix proteins
vRNA	Viral ribonucleic acid
NEP/NSP2	Nuclear export protein
PB1/2	Basic polymerase 1/2
PA	Acidic polymerase protein
vRNP	Viral ribonucleoprotein
cRNA	Complimentary RNA
mRNA	Messenger RNA
ER	Endoplasmic reticulum
IIV	Inactivated influenza vaccine
IIV4	Quadrivalent inactivated influenza vaccine
RIV3	Trivalent recombinant influenza vaccine
HAI	Hemagglutination inhibition assay
HIV	Human immunodeficiency virus
TB	Tuberculosis
TB-infected	individuals living with TB disease
CMI	Cell-mediated immunity
CI	Confidence interval
GMT	Geometric mean titer
HAART	Highly active anti-retroviral treatment
AIDS	Acquired immune deficiency syndrome

T-cell	T-lymphocyte cell
ART	Antiretroviral therapy
CTL	Cytotoxic T lymphocytes
MHC	Major histocompatibility complex
APC	Antigen presenting cell
Th	Helper T-cells
IFN	Interferon
IL	Interleukin
UK	United Kingdom
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
TNF	Tumour necrosis factor
RR	Relative risk
ELISPOT	enzyme-linked immunospot assay
PBMC	peripheral blood mononuclear cell
SFU	spot forming unit
HREC	Human Research Ethics Committee
CHBAH	Chris Hani-Baragwanath Academic Hospital
RMPRU	Respiratory and Meningeal Pathogens Research Unit
PMTCT	prevention of mother-to-child transmission
NICD	National Institute for Communicable Diseases
EAM	ELISPOT assay media
TM	Thawing Media
RT	Room temperature
PHA-M	Phytohemagglutinin
RDE	Receptor destroying enzyme
TRBC	Turkey red blood cell
SD	Standard deviation
BMI	Body mass index
IQR	Interquartile range

LB	Low-baseline response
HB	High-baseline response
SCT	Stem cell transplantation
ELISA	Enzyme-linked immunosorbent assay

1.0 Introduction

1.1 Influenza Epidemiology

Respiratory diseases are a major cause of global morbidity and mortality to which influenza virus infections make significant contributions (1). In the United States of America (USA) an estimated 225 000 hospitalisations and 36 000 deaths per year as a result of seasonal influenza (2,3). In the younger age groups considerable loss of life was associated with the 'moderate' 2009 influenza A/H1N1 pandemic (4). There are roughly 1.1 billion people live in sub-Saharan Africa, an area with high population growth rates, low life expectancy, and many of the world's poorest countries (5). Despite the copious amount of information from numerous areas of the world, there is very little known about influenza epidemiology in sub-Saharan Africa (6).

Influenza viruses have been circulating in the human population for centuries, and is an highly contagious airborne disease normally manifesting as acute febrile respiratory illness with considerable medical and socioeconomic burden that have pandemic potential. The World Health Organisation (WHO) estimates that during annual seasonal epidemics, which occur in the winter months in temperate climates (7), 5% – 15% of the world population is typically infected by influenza virus, with 3 – 5 million cases of severe illness and up to 500 000 influenza-associated deaths per year (8). Influenza-associated morbidity and mortality is highest in the elderly (>65 years of age) with an approximate annual rate of 17 deaths per 100 000, accounting for 88% of the overall estimated annual influenza-associated deaths in the USA (9). In children, annual attack rates can exceed 40% in pre-school and 30% in school-age children (10). Further, the rate of influenza-associated

hospitalisations is high in young children and pregnant women (11,12). Considering work absenteeism as well as direct medical costs, the economic impact of influenza virus infection in the USA has been estimated to approximately US $12 – 14 billion per year (13). Influenza therefore continues to have a drastic worldwide impact, resulting in significant human suffering and economic burden (14).

The incubation period for influenza A and B viruses is generally 1 – 3 days and 1 – 4 days, respectively. The majority of influenza virus infections are self-limited, with the common symptoms being headache, coughing, fever, throat pain and a general discomfort (15). In high-risk groups, particularly those with certain pre-existing medical conditions, influenza infection can have life-threatening complications, including secondary bacterial pneumonia (16-18).

For the past 60 odd years the primary strategy to prevent and control seasonal and pandemic influenza virus is vaccination (19), with the first population-scale use of an inactivated influenza vaccine was in the USA military personnel in 1945 (20). During the early 1960s, the US Surgeon General recommended annual influenza vaccination for individuals with chronic debilitating diseases, people aged 65 years or older and pregnant women (21). This recommendation was made without data of vaccine efficacy or effectiveness in these high-risk groups, but rather based on studies showing efficacy in young, healthy military personnel (22). In 1964, the Advisory Committee on Immunisation Practices (ACIP) reaffirmed this recommendation but noted the absence of efficacy data among high-risk groups (23). Due to longstanding public health recommendation of annual vaccination in high-risk populations, over the last 50 years such patients have generally

been excluded from influenza vaccine clinical trials. The ACIP does support the inclusion of individuals at high-risk of influenza virus infection in placebo-controlled trials as unethical (24).

In 2010, the ACIP recommended seasonal trivalent inactivated influenza vaccine (IIV3) for all individuals ≥6 months, or the live attenuated influenza vaccine (LAIV) for healthy, non-pregnant people between the ages of 2 and 49 years (24). This global influenza vaccination recommendation was a result of a decade of cumulative changes during which the ACIP expanded recommendations to include an increasing proportion of the USA population.

Influenza epidemics and pandemics are a consequence of high genetic variability in the viruses hemagglutinin (HA) and neuraminidase (NA) antigens (25). Hemagglutinin is the major antigen for neutralising antibodies and is involved in the binding of virus particles to host cell receptors; NA is responsible for removal of the cell surface sialic acid receptor and critical for virus release (26). Because influenza A and B viruses are the cause for the majority of seasonal epidemics, current vaccines are generally designed to target these viruses. In contrast, influenza C is less common and mainly causing mild disease in children (27).

Influenza virus A and B differ in the range of animal hosts that they can infect. Influenza B is confined to humans only, whereas influenza A can be found in a much broader range of species. The ancestral host of influenza A is wild aquatic birds and sporadically viruses are transmitted from this host to domestic poultry and mammals, including humans (25).

Currently, H1 and H3 subtypes of influenza A are endemic in humans (28). Given the multiple subtypes, higher mutation rates and diverse host range, influenza A poses the greatest pandemic threat. In contrast, influenza B viruses typically cause a milder form of disease, have a lower mutation rate and have less antigenic diversity (29), thereby posing no pandemic threat (25).

1.2 Family Orthomyxoviridae

The family orthomyxoviridae has a segmented, negative-sense, single-stranded genome with helical symmetry and different size ribonucleoproteins, 50 – 150 nm in length. There are six different genera in the family: the influenza viruses A, B, and C, Thogoto virus, Isa virus and Quaranfil virus (30). The virions are spherical or pleomorphic and approximately 80 – 120 nm in diameter. The virion envelope is derived from the cell membrane, incorporating virus glycoproteins and non-glycosylated proteins. Virion surface glycoprotein projections are 10 – 14 nm in length and 4 – 6 nm in diameter (31). The name Orthomyxoviridae is derived from Greek – *orthos*, meaning correct or right and *myxa*, meaning mucus.

1.2.1 Influenza Virus Classification

The influenza virus genus comprise of three types: A, B and C, defined by the antigenicity of the nucleoproteins (NP) and matrix proteins (M1 and M2) in the viral core (32). Influenza A viruses are further divided into subtypes according to the antigenic properties

of the external glycoproteins (HA and NA). Eighteen antigenically different HAs (H1–18) and eleven different NAs (N1–11) have been identified and their combination designates the virus subtype (33). Influenza B viruses are classified as either Yamagata-like or Victoria-like (34). Viruses belonging to any of the three types of influenza are capable of genetic reassortment, and as a result, readily exchange genetic information. However, reassortment between members of different genera has not been reported. The lack of genetic exchange between viruses of different genera is one indication of speciation as a result of evolutionary divergence (14). The systematic naming of the different influenza virus strains involve their type, the species from which the virus was isolated (omitted if human), the location of the isolate as well as the number of the isolate, the year the virus was isolated, and, in the case of the influenza A viruses, the HA and NA subtypes (35).

1.2.2 Genome Structure

All influenza virus type A and B possess eight viral RNA (vRNA) segments, whereas influenza C has only seven vRNAs. The segmented genome (Figure 1.1) encodes 11 proteins: nine structural and two non-structural proteins (31,36). Virus particles are encapsulated by a lipid envelope derived from the host cellular membrane during the viral budding process, with four viral proteins (HA, NA, M1 and M2) embedded within the lipid bilayer (37). The genetic material consists of single, negative-stranded RNA segments that are complexed with NP and minor amounts of nuclear export protein (NEP, also referred to as non-structural protein, NS2) and three polymerase proteins (basic polymerase 1 (PB1) and 2 (PB2) and acidic polymerase (PA)) (38). Replication and transcription of vRNA

occur within the nucleus of the infected cell, whereas assembly and budding occur on the plasma membrane (26). The 8 gene segments of the influenza virus genome have various functions in the life cycle (Table 1.1).

The HA protein is a homotrimeric type I membrane glycoprotein and plays an important role in the influenza virus life cycle by mediating receptor binding and membrane fusion (39). Cleavage of the HA protein by the host cell proteases determines the pathogenicity of the influenza virus (40,41). The type II transmembrane protein, NA is a homotetramer and plays a role in the release of virions by destroying host cell surface receptors necessary for progeny release (33). The structural protein, M1, functions as a nuclear exporter of vRNA and viral budding, whereas M2 functions as an ion channel for the acidification of the viral core during the uncoating of the virus in endosomes (42). As mentioned, the viral ribonucleoproteins (vRNP) complex is comprised of the vRNA segments, PB1, PB2, PA, NEP and NP (43). PB1 and PB2 are necessary for cap binding and endonuclease activity (44), PA functions as an RNA polymerase subunit (45) and NP is the structural component of the RNP complex. The NEP functions as a nuclear export protein for vRNA in infected cells (46,47). The NS1 and NEP proteins are the only non-structural proteins (48), where NS1 is considered a non-essential virulence factor with multiple accessory functions, such as the inhibition of host immune responses (49).

Table: 1.1: Viral proteins and their functions

Protein	Function
HA (hemagglutinin)	Viral entry into target cell
NA (neuraminidase)	Release of viral particles from target cells; help in dissemination of virion particles throughout respiratory tract
PA (acidic polymerase protein)	Endonuclease activity and help in cap binding mechanism
PB1 (basic polymerase protein 1)	Viral mRNA transcription by 5' cap binding mechanism
PB2 (basic polymerase protein 2)	Unprimes replication of viral mRNA
PB1-F2	Apoptosis of host cell
M1 (matrix protein)	Attachment of vRNP to cell membrane and provide stability
M2 (ion-channel protein)	Release of vRNP from endosome to cytoplasm
NP (nucleoprotein particle)	Nuclear import and export of vRNP and viral replication
NS1 (non-structural protein 1)	Suppress IFN-β and host protein production
NEP/NS2 (nuclear export protein)	Nuclear import of vRNP

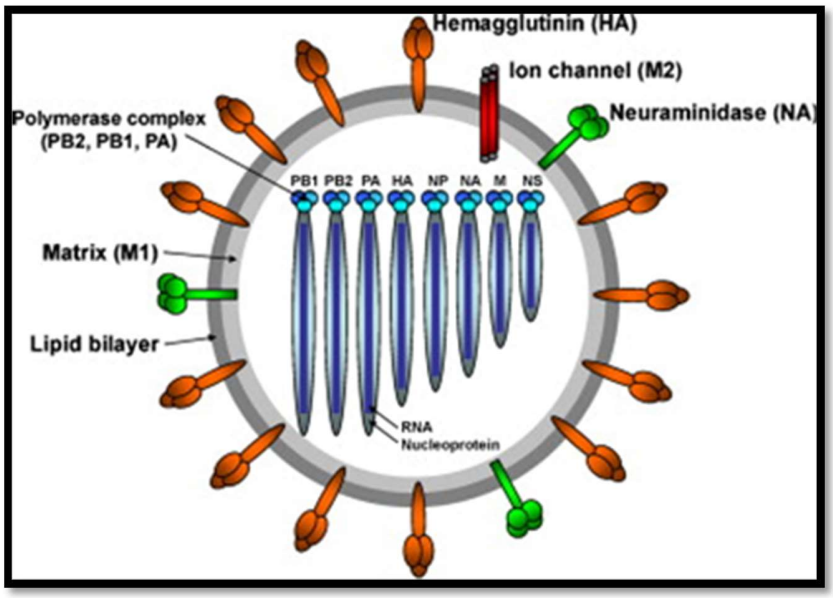


Figure 1.1: The structure of the influenza virus. Eight segments of single-stranded, negative sense RNA molecules are enclosed within the viral envelope. Image from (33).

1.3 Influenza Virus Life Cycle

1.3.1 Attachment

Initially the influenza virus HA attaches to the surface glycoproteins containing sialic acid residue on the host respiratory epithelium cells. The specificity of the sialic acid (α2,3-linked or α2,6-linked sialic acid) and the preferred binding of a particular strain of influenza virus to a specific sialic acid receptor are important determinants for species-specific restrictions of influenza viruses (50). Avian and equine influenza viruses recognise mainly α2,3-linked sialic acid receptors in these species (51,52) and while humans have both types of receptors, human influenza viruses mainly recognise the α2,6-linked sialic acid receptors (53). Pigs also have both types of receptors, which may explain why they are susceptible to viruses from both human and avian origins (54).

1.3.2 Membrane Fusion and Uncoating

Following cell attachment, the influenza virus is internalised into endosomes by means of endocytosis, mediated by host epsin-1, a cargo-specific adaptor for virus entry through the clathrin dependent pathways (55,56). The HA precursor polypeptide (HA0) must first be activated by proteolytic cleavage into HA1 (globular domain) and HA2 (transmembrane domain) by trypsin or a trypsin-like endogenous host protease before it is able to undergo an acid pH-triggered conformational change into a fusogenic form (40). Next, the viral and endosomal membranes fuse, while the ion channel M2 in the viral membrane is activated by endosomal acid pH. This results in an influx of protons into the virion and the dismantling of M1 from the vRNP core (57).The vRNP is released into the cytoplasm of

the host cell as a result of dismantling. The precise timing and location of uncoating (maturity of the endosome) is dependent on the pH-mediated transition of specific HA molecules involved (58). Uptake of vRNP molecules through nuclear pores is an active process, involving the nucleocytoplasmic trafficking machinery of the host cell, where transcription and replication of the viral genome occur (59).

1.3.3 Transcription and Replication

After uncoating, the vRNP are transported into the nucleus and become the template for the transcription of messenger RNA (mRNA) and replicated via complementary RNA (cRNA) (36). The viral polymerase subunits (PB1, PB2, and PA) and NP catalyse both genome replication and transcription. The mRNA products are incomplete copies of the vRNA templates and are capped and polyadenylated, unlike vRNA. The replication process occurs in two steps: firstly, a full length, positive-sense copy of the vRNA is made, known as the cRNA and secondly, this cRNA in turn is used as a template to produce additional vRNA (43).

1.3.4 Assembly and Release

Once replication occurs, the newly formed RNP complexes are assembled in the nucleus and exported into the cytoplasm. Both the M1 and the NEP are involved in assisting the export of RNPs to the cytoplasm. Once in the cytoplasm, the vRNAs are translated into viral proteins (HA, M2 and NA) which are processed in the endoplasmic reticulum (ER),

glycosylated in the Golgi apparatus, and transported to the cell membrane (33). With the help of M1, the production of viral particles begins. Budding initiates and the progeny virus is released from the cell membrane by NA activity which destroys host and viral membrane receptors. This process is essential for progeny release (26).

1.4 Antigenic Variation

The spread of human influenza viruses, and its success, is a direct result of constant antigenic variation to escape host immune responses. In contrast to most other respiratory viruses, influenza viruses possess two different mechanisms of antigenic variation: antigenic drift and antigenic shift (60). Due to the relatively low fidelity of the RNA-dependent RNA polymerase of the influenza virus and the lack of a proof reading mechanism, a rapid rate of mutation occurs in its genome. These mutations result in antigenic variation primarily in the HA and NA proteins (14,61).

1.4.1 Antigenic Drift

Antigenic drift is defined as the gradual evolution of viral strains, due to frequent mutations (62) and generally occurs every 2 – 8 years in response to selection pressure by host immunity (63). Antigenic drift is a result of point mutations in influenza A and B viruses; more specifically, from positive selection of spontaneous mutants by neutralising antibodies where the rate of mutation being about one nucleotide change every copied genome (64). This translates to minor, gradual, antigenic alterations in the HA and NA

proteins. Eventually, these proteins on the virus particle become significantly different to a point where host antibodies are unable to neutralise the virus, resulting in a variant capable of causing illness (25). Antigenic drift is often associated with seasonal epidemics (Figure 1.2), where new strains have amino acid changes in the HA and NA protein coding genes.

1.4.2 Antigenic Shift

The second, far less frequent, yet more profound, type of antigenic variation is known as antigenic shift and occurs only in influenza A viruses. It is defined as the appearance in the human population of a new influenza virus containing a novel HA or NA. This occurs when two different influenza viruses (potentially from different host species) co-infect a single host. Genome segment reassortment results in a new virus that contains elements from both original viruses (Figure 1.2) (61). These newly introduced proteins are immunologically distinct from the proteins expressed by previously circulating strains and these may result in high infection rates of the novel virus in the immunologically naïve population, leading to potential pandemics. Since the beginning of the last century, five antigenic shifts have occurred: in 1918 the appearance of A/H1N1 viruses caused the Spanish Flu pandemic; in 1957, when the A/H1N1 subtype was replaced by A/H2N2 causing the Asian influenza pandemic; in 1968, when A/H3N2 viruses replaced the A/H2N2 leading to the Hong Kong influenza; in 1977, when the A/H1N1 subtype reappeared (Russian influenza); and in 2009, when a novel, antigenically distinct A/H1N1 virus caused a pandemic. This strain has now largely replaced the previous A/H1N1 (14).

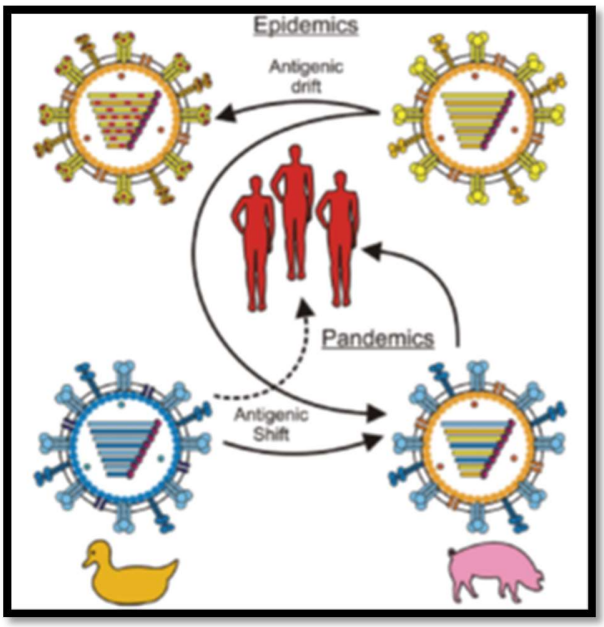


Figure 1.2: Evading immunity by antigenic drift and shift. Antigenic drift: the gradual accumulation of mutations. Antigenic shift: novel subtypes are introduced into the human population and may cause pandemics. Image from (65).

1.5 Influenza Virus Vaccines

Vaccination is the most effective public health intervention against influenza (66). Current influenza vaccines require annual updating and the rate of protection conferred by vaccination at population level varies from season to season being mostly dependent on how good the match is between the circulating viral strains and the strains in the vaccine (67). The WHO, in its position paper on "Vaccines against influenza" in 2012, recommended that annual vaccination should be prioritised for pregnant women at any stage of pregnancy, children aged between 6 months to 5 years, elderly individuals (aged more than 65 years), individuals with chronic medical conditions and health-care workers (69).

Influenza virus strains included in the seasonal vaccine are selected based on the antigenic profile of circulating strains in both hemispheres' winter periods. The chosen strains then normally undergo co-infection of chicken eggs with viruses that are well adapted for growth in this media. Although in recent years there has been a shift away from influenza vaccine propagation in chicken eggs, this technique continues to provide the bulk of vaccine viruses produced today (70). The vaccine viruses are selected are those that retain the required antigenic properties, yet show enhanced yield in chicken eggs. Once propagated, the viruses are purified by means of density gradients or by chromatography, followed by chemical inactivation with formalin or β-propriolactone, and, in most cases, disrupted by lysis of the viral envelope with detergent (7,71). Currently seasonal influenza vaccines are available as inactivated influenza vaccines formulations (IIV) with three-components (trivalent, IIV3) and four-component (quadrivalent, IIV4); live-attenuated influenza vaccines (LIAV); and trivalent recombinant influenza vaccines (RIV3) (68,72).

Due to the constant changing in antigenicity of the two viral proteins in influenza A viruses, HA and NA, influenza vaccines are updated on an annual basis. When the vaccines contain antigens that are well matched to the circulating viral strains, the vaccines are highly efficacious, however if the antigens are not well matched protection is reduced (73). Even in years when one or more of the vaccine strains are the same as the preceding season, vaccination is recommended due to the decline in antibody levels (74). Because influenza vaccines generally take months to manufacture and distribute, vaccine strains need to be selected several months prior to the influenza season in order to provide sufficient time to prepare and distribute the vaccines (75).

The immunogenicity of influenza vaccines can be measured by the level of antibodies against the two major viral surface glycoproteins, HA and NA, in the blood or in nasal secretions. The gold standard for measuring protection against influenza is the hemagglutination inhibition (HAI) antibody assay in serum or plasma (19). The HAI assay measures the ability of antibodies to inhibit binding of the HA protein to sialic-acid on red blood cells present in sera. An HAI titer of ≥1:40 is regarded as a relative measure of sero-protection, with this threshold being predictive of 50% efficacy against influenza illness in healthy adults (76). In vaccine studies individuals displaying post-vaccination HAI titers of ≥1:40 and ≥4-fold increase over baseline HAI titers are referred to as having sero-converted (77).

1.6 Influenza Infection and Vaccination in South Africa

In South Africa it is estimated that the mean annual number of seasonal influenza-associated all respiratory deaths is approximately 450 in children <5 years old and 9 000 among individuals ≥5 years old (78,79). Hospital surveillance in South Africa from 2009 to 2011 for patients with acute lower respiratory tract infection revealed that the influenza detection rate varied by age group, being, 11%, 12%, 9%, 9% and 12% among age groups <1 year, 1 – 4 years, 5 – 24 years, 25 – 44 years, 45 – 64 years, and the elderly, respectively (80). Influenza viral infections are also responsible for 43% – 67% of outpatient visits for influenza-like illness at the peak of the influenza season (81).

Individuals infected with human immunodeficiency virus (HIV) have been shown to be at greater risk for severe influenza illness (80). In South Africa, before widespread use of active antiretroviral therapy (ART) influenza-related excess mortality rate among young adults living with HIV/AIDS was estimated at 570 deaths per 100 000 for all-cause deaths (82). The high prevalence of underlying medical conditions, including HIV and tuberculosis (TB), is a likely contributor to higher influenza-associated mortality in South Africa (83,84).

In South Africa, annual influenza vaccination is recommended as per WHO recommendations (28). There are limited number of free doses available in the public sector for high-risk groups, including the elderly, pregnant or postpartum (within 2 weeks of delivery) women, and persons with underlying medical conditions (such as lung disease and HIV infection). Uptake of influenza vaccine remains low in South Africa with less than 1 million doses distributed in the public and private sectors annually between 2011 and 2014, despite an estimated 20 million South Africans belonging to high-risk groups (85). The number of influenza vaccine doses imported into South Africa is based on the uptake of the vaccine during the previous year. Low uptake of vaccine was, however, not thought to be due to shortage of vaccine (86,87). Despite the relatively low coverage (3.5%) among patients attending general practitioners, vaccination with IIV3 has shown to be effective against influenza-associated respiratory illness, most notably in years when good vaccine strain match was reported (2010, 2011, 2013) (85).

1.7 Influenza Infection and Vaccination in Pregnant Women

Pregnancy is associated with immunologic alterations and physiologic changes that affect the respiratory, cardiovascular, and other organ systems that place women at increased risk for infections and/or associated complications, including severe influenza illness. These physiological changes include increased heart rate, stroke volume, oxygen consumption, and decreased lung capacity (88,89) and possibly death due to influenza infection (90).

The immune system adapts during pregnancy to tolerate a genetically foreign foetus. Although how this adaptation occurs is not fully understood, it includes dampening of cell-mediated immunity (CMI) responses while humoral immune responsiveness is sustained (91). This immunologic adaptation may contribute to the increased risk of complications that are associated with certain infections where CMI plays a critical role in viral clearance, including infection with influenza viruses (92). Heightened susceptibility to severe influenza illness during pregnancy is particularly evident during periods of influenza virus drift and pandemics (14,93,94), which was well documented during the 2009 influenza A/H1N1 pandemic (95,96).

A study from Canada reported that pregnant women in their first, second and third trimester have a 1.7 (95% confidence interval [CI]: 1.0 – 2.8), 2.1 (95% CI: 1.3 – 3.3), and 5.1 (95% CI: 3.6 – 7.3) fold greater risk, respectively, of hospitalisation for influenza-related acute cardio-respiratory illness than non-pregnant women (97). Additionally, influenza illness during pregnancy may increase the risk of premature delivery, foetal distress and emergency caesarean sections (91). To prevent possible complications of influenza infection in pregnant women, the WHO recommends

immunisation with IIV3 at any time during pregnancy (98). Influenza vaccination during pregnancy is also beneficial for the young infants born to vaccinated mothers as they gain protection against influenza infection by transplacental acquisition of antibody from the mother (99).

To date there have been several immunogenicity studies comparing the effect of influenza vaccine in pregnant and non-pregnant women, which have yielded similar sero-conversion rates, however with variations in geometric mean titers (GMT) (100-104). The first study by Bischoff *et al.* aimed at comparing humoral immune responses to MF59®-adjuvanted monovalent A/H1N1 vaccine in pregnant and non-pregnant women at baseline and three weeks post-vaccination. The sero-conversion rate between the two time-points was similar between non-pregnant and pregnant women (98% [95% CI: 91 – 100] vs. 95% [95% CI: 82 – 99], respectively) (100). Similarly, Christian *et al.* showed no significant difference in sero-conversion rates to seasonal IIV3 in pregnant versus non-pregnant women (A/H1N1: 70% [95% CI: 50 – 86] vs. 74% [95% CI: 54 – 89], A/H3N2: 63% [95% CI: 42 – 81] vs. 59% [95% CI: 39 – 78], B/Yamagata: 63% [95% CI: 42 – 81] vs. 74% [95% CI: 54 – 89]), suggesting that pregnancy does not alter humoral responses to influenza vaccination (101). These observations are further supported by a study from Schlaudecker *et al.* where the authors reported that the percentage of women who sero-converted (and achieved sero-protective titers) one month post-IIV3 were similar among pregnant and non-pregnant women, however, the fold increase in HAI titers for A/H1N1 tended to be lower in pregnant women –albeit not significant (p=0.09). Additionally, HAI GMTs were similar in both groups of women prior to IIV3

immunisation, but were significantly reduced in pregnant women to two of the three strains included in the vaccine one month post-vaccination (102).

A clinical randomised controlled trial in Bangladesh from 2004-2005 documented strong antibody responses in pregnant women following IIV3 vaccination, with efficient antibody transfer to their foetus, and similar percentages of mothers and infants at birth with sero-protective titers (104). A more recent randomised placebo-controlled trial in South Africa (103) added further support to the immunogenicity of the influenza vaccines in pregnant women. The authors reported high proportions of HIV-uninfected women post-IIV3 with sero-protective HAI titers (72.5% [95% CI: 64.4 – 79.7] for A/H1N1, 64.8% [95% CI: 56.8 – 72.6] for A/H3N2 and 92.3% [95% CI: 86.6 – 96.1)] for B/Victoria) (103).

The safety of influenza vaccination during pregnancy has been demonstrated, with no indication of adverse effects of influenza vaccines on pregnancy or birth outcomes (90,105,106). Several studies have also shown that maternal influenza vaccination is a cost-effective intervention in reducing the rates and severity of disease during both seasonal influenza epidemics and pandemics (107,108). Nevertheless, vaccine coverage remains low in pregnant women, especially in low-middle income countries (109).

1.8 Influenza Infection and Vaccination in HIV-Infected Individuals

Influenza infection in individuals with HIV is associated with prolonged duration as well as increased severity of illness compared to the general population (110-115) and annual

vaccination is recommended by many national immunisation guidelines. A temporal association between an increase in the number of deaths during the influenza seasons in USA cities with a high prevalence of HIV infection, coupled with reports of severe influenza-related cases in HIV-infected individuals was the impetus for recommending annual influenza vaccination for HIV-infected individuals by the ACIP in the USA (116). A subsequent study reported that influenza-attributable hospitalisation rates in HIV-infected adults were 48 (95% CI: 16 – 91) per 1 000 persons in 1995 and decreased to 5 (95% CI: 0.5 – 11) per 1 000 persons per year during 1996 through 1999, after the introduction of highly active anti-retroviral treatment (HAART) (117). Although influenza-associated hospitalisations have declined in patients with HIV infection following the introduction of HAART, the rates remained comparable to rates in other high-risk groups for which annual influenza vaccination was recommended (117). A more recent study showed that in the USA, prior to the introduction of HAART, influenza related mortality rate in adults with acquired immune deficiency syndrome (AIDS) was 150 (95% CI: 49 – 460) and 208 (95% CI: 74 – 583) times greater than those observed in the general population for all-cause deaths and pneumonia and influenza deaths, respectively. Although influenza-related mortality in adults with HIV/AIDS dropped 3 – 6-fold after the introduction of HAART, it remained higher than the general population (82).

Although there are case reports of severe influenza illness in HIV-infected adults, in general the spectrum and severity of influenza-associated illness have been described to be similar between HIV-infected and HIV-uninfected adults (112,118,119). There is, however, a trend for HIV-infected adults being more likely to be hospitalised, evaluated in an

emergency room or have an illness of ≥14 days than HIV-uninfected adults (21% vs. 0%, p=0.06) (118). There also does not appear to be any evidence for clinical, immunological or virological progression of HIV during the course of an influenza season (120).

In South Africa before the introduction of antiretroviral therapy, the incidence of severe pneumonia in which influenza virus was identified was 8-fold (95% CI: 5.1 – 12.8) greater in HIV-infected compared to HIV-uninfected children aged <2 years (121). Although the duration of hospitalisation for influenza-associated pneumonia was similar between HIV-infected and HIV-uninfected children (median 4 – 5 days), there was a statistically non-significant increased risk of mortality among HIV-infected children (8.0% vs. 2.2%, p=0.20) (122). Furthermore, differences in the clinical spectrum of influenza-associated pneumonia included HIV-infected children remaining at risk of severe influenza pneumonia beyond early infancy, which is the major age-risk group in HIV-uninfected children, as well as there being more severe chest radiographic changes in HIV-infected children. The latter may be related to impairment of CMI, which is imperative for controlling influenza virus replication and is compromised in HIV-infected individuals (92,123).

There is a paucity of data on the burden of influenza illness in HIV-exposed-uninfected infants, yet these infants are increasingly being recognised as having greater morbidity and mortality than infants born to HIV-uninfected women (124-128). The biological reason for this is not yet known, although aberrations of the immune system of HIV-exposed-uninfected children have been described (129-131). A possibility for the increased morbidity and mortality, primarily experienced during early infancy in

HIV-exposed-uninfected children, may relate to impaired transplacental antibody acquisition compared to new-borns of HIV-uninfected women. This may consequently affect any strategy targeting vaccination of pregnant women in relation to optimising protection against the targeted pathogen in the mother as well as their new-born (132).

Trivalent inactivated influenza vaccines approved for use in immunocompromised patients have shown to be immunogenic in an HIV-infected population with normal CD4+ T-lymphocyte cell (T-cell) counts (133,134). However, immunogenicity is reduced in patients with CD4+ T-cell counts <100/μL (135,136) and as a result might be associated with reduced vaccine-induced protection (118). In a randomised, double-blind, placebo-controlled trial conducted in Johannesburg, South Africa, evaluating the immunogenicity of IIV3 in HIV-infected individuals with CD4+ T-cell counts >100/μL, participants had a moderate humoral responses to the three strains in the vaccine, which was lower than the normally observed in HIV-uninfected adults (137). Furthermore, the authors reported a 75% reduction in influenza-confirmed illness in IIV3-recipients, corroborating the 66% risk reduction estimated in a meta-analysis on IIV3 effectiveness in HIV-infected adults (138).

1.9 HIV Infection in Human Population and in Pregnant Women

Globally, there are approximately 35 million people infected with HIV, including 2.5 million children mainly infected through mother-to-child transmission (139). The overwhelming majority of women living with HIV are in resource-poor countries, and most

of them are unaware of their status until they become pregnant (140). Infection with HIV is associated with a progressive depletion of CD4+ T-cell populations in close association with progressive impairment of cellular immunity and increased susceptibility to opportunistic infections. The progression of HIV disease in untreated humans can take several years and was originally hypothesized to be a consequence of slow, viral-mediated CD4+ T-cell destruction. However, massive CD4+ memory T-cell destruction is now known to occur in the early stages of infection. In most individuals, this initial destruction is countered by CD4+ memory T-cell regeneration that preserves CD4+ T-cell numbers and functions above the threshold associated with overt immunodeficiency. This regeneration, which occurs in the setting of chronic immune activation and immune dysregulation does not, however, restore all functionally important CD4+ T-cell populations and is not stable over the long term. Ultimately, CD4+ memory T-cell homeostasis fails and critical effector populations decline below the level necessary to prevent opportunistic infections. The onset of overt immune deficiency appears to be intimately linked with CD4+ memory T-cell dynamics and reflects the complex interplay of direct viral cytopathogenicity and the indirect effects of persistent immune activation on CD4+ memory T-cell proliferation, differentiation, and survival (377).

Over the past decade, the number of HIV-infected pregnant and breastfeeding women receiving ART has increased dramatically. The expansion of access to and uptake of ART during pregnancy has greatly help drive significant global reductions in mother-to-child HIV transmission (374). The primary concern regarding HIV in pregnancy is the progression of maternal disease and mother-to-child transmission (141). In the absence of

antiretroviral treatment, 15 – 45% of HIV infected pregnant women will pass the infection to their infants. Five to ten percent of these infants would have been infected *in utero*, 10 – 20% from exposure during the peripartum period and 5 – 20% through breastfeeding (142,143). However, robust evidence showed that effective ART use can nearly eliminate mother-to-child-transmission under ideal conditions with less than 2% transmission occurring in resource-limited settings and, since 1995, nearly 1.6 million new HIV infections among children have been prevented in South Africa due to the implementation of prevention of mother-to-child-transmission programmes (375,376).

During pregnancy, immune functions are attenuated in both HIV-infected and -uninfected women (144,145). Pregnancy does not appear to negatively affect HIV progression or survival (146-149), yet dual infection with HIV and malaria has been associated with increased risk of maternal, perinatal and early infant deaths (150). Although HIV RNA levels seem to remain stable during pregnancy, some studies have shown increased viral load in the post-partum period (151). There are conflicting reports on whether maternal HIV-infection is associated with increased rates of adverse pregnancy outcomes such as preterm birth, growth restriction, pre-eclampsia and gestational diabetes (141,152-157).

In developed countries, HIV is rarely associated with maternal mortality due to easy access to specialised health-care. In contrast HIV infection complications are important causes of maternal mortality in Africa (158). In African areas with high prevalence of HIV, the infection has become a major cause of maternal mortality (159,160). Numerous reports from Southern African countries have reported this trend which includes maternal mortality rates that are 5-fold higher in HIV infected women compared with uninfected women

(158). Around 24% of pregnancy-related deaths in sub-Saharan Africa may be attributable to HIV-infection – higher than any other direct obstetric cause (161).

Quantifying the contribution of HIV to maternal mortality is challenging. Attributing the death of a pregnant or post-partum woman to HIV not only requires knowledge of the woman's HIV status, but also decisions about whether it is indirectly attributable to the pregnancy, where the latter is not considered as maternal death. A recent systematic review suggested that HIV does not increase the risk of direct obstetric complications, except for puerperal sepsis (162). Empirical evidence supporting acceleration of HIV disease progression during pregnancy is inconclusive, with some studies on HIV-infected women showing that CD4+ T-cell counts decrease faster during pregnancy (163,164), while one study found that CD4+ T-cell counts rebound postpartum, matching those of non-pregnant women (163).

Antiretroviral therapy (ART) has proven to be a life-saving intervention in HIV-infection. Immune restoration after ART dramatically reduces the incidence and severity of opportunistic infections and mortality. In certain settings, immune restoration may be erratic, leading to acute inflammatory responses shortly after ART initiation, or incomplete with residual inflammation despite chronic treatment, leading to non-infectious morbidity and mortality. ART may not always restore the perfect balance of innate and adaptive immunity in strategic milieus, predisposing HIV-infected persons to complications of acute or chronic inflammation. The best current strategy for fully successful immune restoration is early antiretroviral therapy, which can prevent acquired immunodeficiency syndrome (AIDS)-associated events, restrict cell subset imbalances and dysfunction, while preserving

structural integrity of lymphoid tissues (378). In a setting where ART is readily available, little evidence exists attributing pregnancy to the accelerated progression to HIV/AIDS-defining illnesses, mortality and CD4+ T-cell depletion. In settings without ART availability, all the effect estimates were consistent with pregnancy increasing the risk of progression to HIV/AIDS-defining illnesses and HIV-related or all-cause mortality (165).

The main argument supporting the adverse effect of pregnancy on HIV disease progression stems from the systemic suppression of CMI during pregnancy, which increases susceptibility of infections (92). Infectious diseases such as influenza (166), malaria (167), measles (168) and varicella (169) are more severe during pregnancy (170-172).

1.10 Influenza Infection and Vaccination in HIV-Infected Pregnant Women

To date there is a paucity of data on the immunogenicity of IIV3 in HIV-infected pregnant women. In 2011, HIV-infected and HIV-uninfected pregnant women were enrolled in two maternal influenza vaccination trials conducted in Soweto, South Africa (103). The safety, efficacy and immunogenicity of a single dose IIV3 (administered between 20 and 36 weeks gestation) was investigated. One month post-vaccination, a significantly greater number of HIV-infected women who received IIV3 achieved HAI titers ≥1:40 compared to the placebo group for all vaccine strains (A/H1N1: 42.9% vs. 5.5%, $p<0.001$, A/H3N2: 35.7% vs. 3.6%, $p<0.001$ and B/Victoria: 40.0% vs. 16.4%, $p<0.005$). Vaccine efficacy among the IIV3 recipients against confirmed influenza illness was 70.6%, similar to that reported in a trial of IIV3 in South African HIV-infected individuals conducted in

2008 (75.5%) (137). Although IIV3 was shown to be immunogenic in HIV-infected pregnant women, higher proportions of HIV-uninfected women achieved HAI titers ≥1:40 after IIV3 vaccination for A/H1N1 (72.5%), A/H3N2 (64.8%) and B/Victoria (92.3%) with a corresponding vaccine efficacy against confirmed influenza infection of 54.4% (103).

Another small prospective study evaluating the immunogenicity of influenza vaccination in pregnant women in the USA also found that HIV-infected compared to HIV-uninfected pregnant women had lower GMTs and a lower percentage had a 4-fold titer increase to A/H1N1 and A/H3N2 strains. Antibody responses to influenza B were equally low in both participant groups (173).

Abzug *et al.* evaluating the safety and immunogenicity of a monovalent A/H1N1 vaccine containing 30 µg of HA in HIV-infected pregnant women observed no severe vaccine-related adverse events among the participants and at baseline 21% had HAI titers ≥1:40. Sero-protection and sero-response, 21 days post-vaccination, occurred in 73% and 66% of the women, respectively. Of the women lacking sero-protection at baseline, 66% attained sero-protection post-vaccination (174). These results compare favourably with another study by El Sahly *et al.* where a monovalent vaccine with higher dose of HA (30 µg) increased sero-protection, sero-response and GMTs in HIV-infected adults, but responses were still poorer than in the HIV-uninfected population (175).

1.11 Cell-Mediated Immune Responses to Influenza Vaccine

Protection against influenza infection is mediated by both humoral (antibodies) as well as CMI responses (123,176,177). Neutralising antibodies are capable of preventing infection, whereas CMI is important in clearing infected cells (178,179). Influenza infection induces a cellular response that recruits virus-specific CD4+ and CD8+ T-cells (Figure 1.3). Current vaccine approaches mostly rely on the induction of antibodies to the viral surface proteins HA and NA that neutralise the virus and interfere with the release of newly replicated virus from the host cell (180,181). When the vaccine virus closely matches the infecting virus vaccines are effective; however, the virus frequently undergoes mutations and in so doing the vaccine becomes less effective. This is further exacerbated when a new subtype of influenza virus emerges and there are no cross-reactive antibody sites to HA between the vaccine and circulating influenza strains. The LAIV, which in some study was shown to confer higher protection against influenza illness in children compared with the IIV3, generates robust CMI, but lower humoral response than IIV3 (123), underscoring the importance of CMI in protection against influenza disease. Ideally, influenza vaccines should induce humoral as well as cellular immunity. Influenza virus-specific cytotoxic T-lymphocytes (CTL) have also been shown to limit influenza A virus replication and protect against lethal viral challenge (182).

Activation of CD4+ T-cells occur after recognition of viral epitopes associated with class II major histocompatibility complex (MHC) molecules and interaction with co-stimulatory molecules on antigen presenting cells (APC) (183). Effective CD4+ T-cell activation is reliant on three distinct signals, namely antigen recognition by T-cell receptors,

co-stimulation of the CD28 found on the T-cell and APC-produced co-stimulatory cytokines. Combined, these signals drive the CD4+ T-cell activation, with the cytokine milieu playing a major contributing factor in determining the polarization into type 1 (Th1), type 2 (Th2), or type 17 (Th17) helper T-cells subsets (372). Naïve CD4+ T-cells recognise the virus and are activated in the draining mediastinal lymph nodes during the first few days of infection and differentiate into effectors that migrate to the lung. These naïve CD4+ T-cells differentiate Th1 or Th2 cells (184), where Th1 cells produce interferon-gamma (IFN-γ) and interleukin-2 (IL-2) and are predominantly involved in promoting CTL responses (185), and are essential for the induction of memory CD8+ T-cells (186). While classically the functions of CD4+ T-cells is to assist CD8+ T-cell effector generation, it was shown that such assistance is not essential for an effective primary immune response to influenza (373). Recent studies have however shown the importance of CD4+ T-cell during the priming of CD8+ T-cells in the formation of CD8+ resident memory T-cells in lung airways during influenza infection (372). The Th2 cells produce IL-4, IL-5 and IL-13 and promote activation and differentiation of B cells, resulting in antibody production (187-189). Following induction by primary influenza infection, memory CD4+ T-cells contribute to faster control of subsequent influenza virus infections (190). In addition to helper function, CD4+ T-cells also display cytolytic activity (191,192) and have been shown to play a role in protective immunity against influenza A virus infections in humans (193).

Naïve CD8+ T-cells are activated following recognition of viral epitopes associated with class I MHC molecules on APCs in the draining lymph nodes, and subsequently

differentiate into CTLs. The CTLs then migrate to the infection site and once recognised, eliminate the cells infected with the influenza virus infected cells (194). Influenza specific CTLs are directed against epitopes of the conserved internal viral proteins (M1, NP, PA, PB2), displaying a high degree of cross-reactivity of various subtypes (195,196). A lytic response, mediated by perforin and granzyme, as a result of T-cell receptor activation by specific class I MHC-epitope complex causes apoptosis of the infected cell (197,198). Following infection, a collection of antigen-specific central memory and effector CD8+ T-cells are created and form a basis for the more rapid recall responses upon secondary infection (199-201).

1.12 Tuberculosis, HIV and Influenza

1.12.1 Tuberculosis Epidemiology

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major global health problem (202), resulting in ill-health among millions of people annually and is considered parallel to HIV and malaria as the leading cause of public health issues (203). An estimated 9.6 million new TB cases were reported in 2014 with a reported 1.5 million deaths attributed to TB, of which 400 000 were among HIV-infected people (202).

In most developed countries there has been a considerable decline in the incidence of TB and its mortality over the last century. This decrease was aided by public health measures such as the eradication of tuberculous cattle and mass radiography screening. After 1950, the decline in mortality from TB was quickened by the introduction of anti-TB drugs (204).

The reduction in the incidence of the disease in developed countries led to changes in the ages of the affected patients. In the past TB infection was commonly a disease of the young; however, it soon became largely limited to the elderly in these countries (205). The situation remains different in low-middle income countries, where TB remains one of the most important infectious diseases. Furthermore, the considerable gains that had slowly been achieved were reversed because of the emergence of the HIV/AIDS epidemic and other factors (206). Recently there has been a resurgence of TB, even in groups where HIV/AIDS has yet to make a major impact, possibly due to new levels of urban deprivation and the influx of immigrants and refugees from countries with a high incidence of the disease (207). In the United Kingdom (UK), for example, immigrants from the Indian subcontinent have rates of TB 25-fold higher than the Caucasian population (208). The decline in the incidence of TB in the UK slowed towards 1987 and has subsequently reversed. The situation is similar in many other developed countries (209).

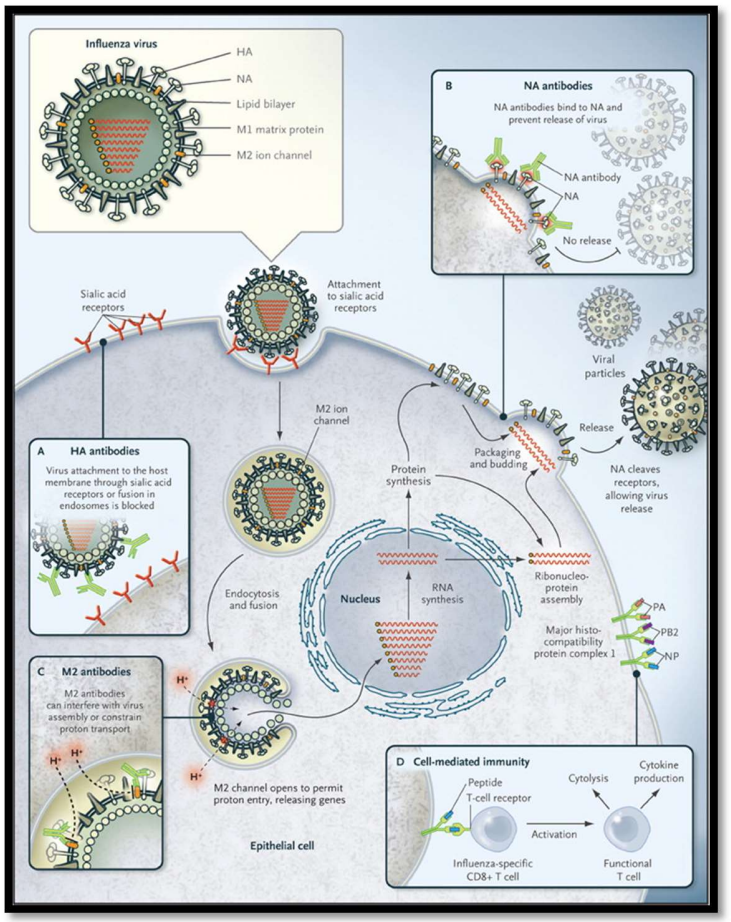


Figure 1.3: The replication cycle of influenza A virus and the adaptive immune response. The influenza HA protein mediates the attachment of the virus to the host cell receptors (A). Antibodies are generated against the NA protein and in so doing limit the release of the virus from the hosts infected cells (B). Antibodies that are generated against the external domain of the M2 protein interfere with the virus assembly (C). CD8+ T-cell responses to conserved influenza virus components enhance clearance of virally infected cells (D). Image from (73).

It is estimated that about one-third of the world's population (approximately 2 billion people) has latent TB, while the prevalence of active disease is approximately 20 million worldwide (210). Despite the prevalence of TB, the human response to infection is good. In the absence of immunosuppressive disorders such as HIV infection, only 10% of those

infected develop clinically evident disease (211). The basis of these patients' susceptibility is not well understood but tobacco smoking is a predisposing cause (212) and genetic factors appear to be involved (213).

1.12.2 Tuberculosis Pathogenesis

Tuberculosis is spread by airborne droplets (1 – 5 μm in diameter) that contain the bacterium *M. tuberculosis*. Due to their small size, aerosol droplets are able to remain airborne for minutes and even hours following release by coughing, sneezing or talking from individuals with pulmonary or laryngeal TB (Figure 1.4) (214-216). Once the droplets are inhaled they can reach the alveoli where they are engulfed by alveolar macrophages. It is at this juncture where the fate of the infection is decided (217). In healthy individuals, the immune system is capable of successfully controlling infection; however individuals with immunocompromised conditions often progress to primary TB infection (218,219). *Mycobacterium tuberculosis* has the ability to inhibit phagosome–lysosome fusion, permitting the bacteria to survive within the host's phagocytes (220,221). Its cell wall is enriched with uncommon lipids and glycolipids, creating a hydrophobic layer that functions as a shield during antibiotic treatment (222).

Early host immune defence involves both alveolar type II pneumocytes and airway epithelial cells and the cytokines they produce (223-225). An influx of activated T-cells and macrophages together with the phagocytised droplets form a structure known as the granuloma: the microenvironment that prevents the spread of *M. tuberculosis* (226). The

granuloma causes destruction of the macrophage and thereby leading to necrosis at the site of attachment in the alveoli (218). Infected dendritic cells migrating to local lymph nodes activate adaptive immune responses in order to halt the bacterium's growth, as well as initiating its entry into the latent phase (227).

Neutrophils are subsequently attracted to these infection sites by the dying infected macrophages, where they exert their killing effect by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent mechanisms (228). If these neutrophils fail to clear the infection, macrophages internalise the TB and process TB-antigens for T-cells, natural killer T-cells and natural killer (NK) cells activation. Dendritic cells are then attracted to activated CD4+ and CD8+ T-cells in lymph nodes (229). Activated CD4+ T-cells are the most abundant cell type recruited, forming organised aggregates surrounding the granuloma (230). Eventually, CD8+ T-cells are recruited and arranged in the periphery of the granuloma while B-lymphocytes occur mostly in the central portion of lymphoid aggregates. The outer margin of the granuloma consists of collagen and various other extracellular matrix components (231).

Typically, when granuloma contain infection successfully, they will shrink with calcification (232), however, in most cases of TB infection, a stand-off is reached between the onset of active disease and the abolition of infection, called latent infection. Such latency is volatile and can be stimulated by exogenous infections, such as HIV or by internal immune incompetency such as malnutrition (233). If such conditions arise, TB may multiply rapidly, resulting in granuloma disintegration, reactivated disease and

disseminated propagation of the pathogen (234). Both the adaptive and innate host immune responses are stimulated during TB infection.

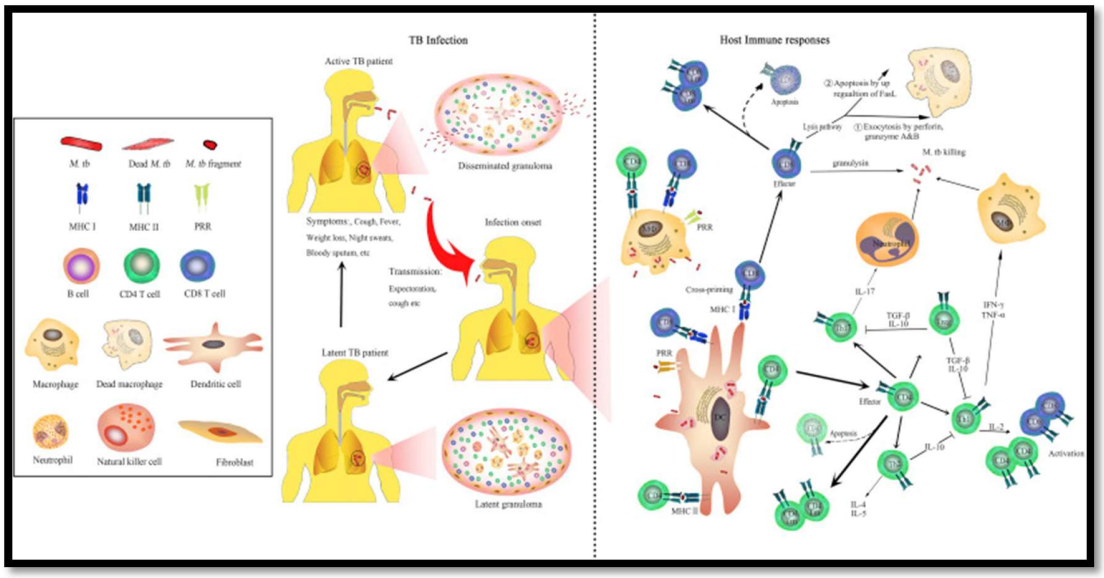


Figure 1.4: Infection with TB and the associated host immune responses. Image from (234).

1.13 Immune Responses to Tuberculosis Infection

1.13.1 Adaptive Immune Responses

Adaptive immune responses involve both humoral and CMI. In most infected individuals, CMI develops 2 – 8 weeks after infection (217). TB antigens are presented by class II MHC to CD4+ T-cells, resulting in their activation (235). TB antigens then activate CD8+ T-cells through the action of class I MHC exposure through a process known as cross-priming (236). Once activation of T-cells has occurred, activated effector T-cells decrease rapidly with only a fraction developing into memory T-cells (237), where some memory T-cells reside in infection sites as effector memory T-cells or migrate to lymph nodes as central

memory T-cells. CD4+ Th1 cells are crucial in fighting intracellular pathogens and secrete IFN-γ and tumour necrosis factor-α (TNF-α) to activate innate immune cells, and IL-2 to activate T-cells (238-240). Concurrently, Th17 produces IL-17 which stimulates recruitment of neutrophils and IFN-γ producing CD4+ T-cells, which, in turn, synergise the function of Th1 (238). Contrary to the protective immune functions of Th1 and Th17, Th2 cells activate the secretion of IL-4, IL-5 and IL-10. These ILs have a negative effect by inhibiting Th1 responses (241). The class I MHC-restricted CD8+ T-cells also play an important role in TB infection control. Activation of CD8+ T-cells eliminates TB directly by releasing granulysin or indirectly by lysing macrophages (242). Two distinct pathways of cytolysis are granule exocytosis, coordinated by perforin and granzyme A or B, and the programmed cell death through Fas aggregation on target cells attracted by FasL (243).

1.13.2 Innate Immune Responses

At the site of entry, TB is controlled by macrophages and neutrophils, which form part of the innate immune response. These macrophages recognise TB by phagocytic receptors (244). Phagocytosis of TB by macrophages exposes the pathogen to a harsh acidic and superoxidic intracellular environment containing oxygen and nitrogen species (222). Tuberculosis is highly evolved and has devised strategies for resisting digestion. It counteracts the killing of macrophages by firstly arresting phagosomes at an early stage in order to avoid fusion with lysosome (245). Secondly, TB catalaseperoxidase protein and alkyl hydroperoxide reductase protein protects it from toxic superoxides (246). Thirdly, virulent TB bypasses apoptosis resulting in necrosis and on-going infection of new

macrophages (247). Lastly, TB manipulates cytokine profiles of host defence mechanisms in order to avoid elimination (248). Dendritic cells recognise TB glycolipids through pattern recognition receptors (such as toll-like receptors) and C-type lectin receptors (249). Following activation, dendritic cells present the "processed" pathogen components to prime naïve T-cells in draining lymph nodes, thereby bridging the innate and adaptive immune responses. Neutrophils also play a protective role in innate responses through specific production of INF-γ (244,250).

1.14 Impact of HIV Infection on Tuberculosis

A compromised immune system is associated with an increased risk of TB disease. In populations that are affected by TB, HIV infection generally exists. This coexistence is influenced by socioeconomic and environmental factors which have the ability to affect TB exposure, risk of infection, the progression of disease, potentially exacerbating the problem (251). Qualitative and quantitative impairments of CMI responses represent the key pathogenic factors responsible for the increased risk of disease (252). HIV infection induces progressive CD4+ T-cell depletion and is therefore associated with increased risk of TB (253). The risk of TB infection is enhanced in HIV-infected individuals throughout the course of HIV infection, even in the early stages of HIV infection when CD4+ T-cell counts are moderately high (254,255). Even with the introduction of ART, which assists in restoring CD4+ T-cell counts in HIV-infected individuals, does not completely reverse the effect of HIV on the risk of TB (256). Co-infection of HIV/TB has been shown to increase

the risk of new additional opportunistic infections and mortality compared to HIV-infected individuals without TB when CD4+ T-cell counts are the same (257-259).

It has been proposed that the primary cause for increased TB susceptibility in HIV-infected individuals is attributed to immunological disruptions of the TB granuloma (260,261), which comprise of an array of organised immunological cells that form in response to *M. tuberculosis* infection (232). The relationship between the granuloma and the bacteria however is complicated. Granulomas can prevent dissemination and kill the bacteria, but can also allow persistence of TB, even be permissive to its growth (262,263). Several studies have indicated that HIV may disrupt this balance by causing granuloma disorganisation, killing resident CD4+ T-cells and deregulating normal T-cells and macrophage function, which leads to increase in susceptibility to both active and disseminated TB disease (260,261,264-268). The mechanism whereby HIV infection promotes TB is probably related to the pattern of cytokines produced by T-lymphocyte subsets. Th1 lymphocytes produce INF-γ and are central to anti-mycobacterial immune defence. However, when peripheral blood lymphocytes from HIV-infected patients with TB are exposed to mycobacterium *in vitro* they produce less INF-γ than lymphocytes from HIV-uninfected patients with TB, suggesting that a reduced Th1 response contributes to HIV-infected patients' susceptibility to TB (269). HIV also affects other immune mediating cells, including macrophages, and influences cytokine production, which usually prevents contracting initial or latent TB infection (270-272). Chemokines induced by TB infection of phagocytic cells may also play a role in recruiting target cells susceptible to HIV to the site of HIV replication, increasing HIV viral replication at TB infection sites (273).

Not surprisingly in view of the interrelationship of HIV and the mycobacterium, the TB associated with HIV infection is particularly aggressive, being characterised by widespread dissemination throughout the body and a poor host response (274).

1.15 Tuberculosis in South Africa

South Africa has one of the highest rates of TB (834 per 100 000 population), which is driven by the high prevalence of HIV (202). The prevalence of HIV in South Africa, although stable over the past few years, is estimated at 12% in the general population and 24% in women of reproductive age. Based on the annual risk of infection with TB in South Africa being approximately 3% – 4%, about three quarters of the adult population would be infected by TB by 18 years of age. The risk of progressing to active TB is greatest within the first year of being infected by TB, and especially high in children under two years of life (43%) compared to a 10% life-time risk in otherwise healthy TB-infected adults (275). However, the risk of TB infection progressing to TB disease is heightened in immunocompromised adults, including an annual risk of 10% in HIV-infected adults not on ART (276). Case fatality ratios of 15% – 35% have been reported in HIV/TB co-infected individuals who had not started ART during TB treatment (277).

Furthermore, TB may occur in the relatively early stages of HIV-infection compared to many other opportunistic infections. Although improved access and management of HIV-infected individuals with ART has resulted in decline in the burden of TB, these individuals remain at a 20-fold greater risk of developing TB compared to the general

healthy population (278). Pre-2012, the South African ART treatment guidelines recommended initiation of ART for TB-HIV co-infected individuals if they had WHO stage 4 disease or if they met specific CD4+ T-cell count criteria: <200 cells/mm^3 in the 2004 guidelines, and <350 cells/mm^3 in the 2010 guidelines (279,280). In 2012, the South African ART program adopted the WHO recommendation that all TB patients begin ART (281).

1.16 Tuberculosis-Influenza Co-Infection

There is currently a paucity of data reporting on the effectiveness of influenza vaccination in persons infected with TB (282), with conflicting study-findings involving TB and influenza co-infection. An interaction between influenza A and pulmonary TB has been suggested with underlying TB considered the identifiable risk factors for severe influenza infection (283,284). This is attributed to immunosuppression by TB as well as the significant percentages of patients treated for TB that present sequela of the disease, which may predispose them to life-threatening influenza A infections (285). In contrast, in a more recent study conducted in Thailand, researchers were unable to identify an increased risk of severe outcomes or mortality in patients co-infected with TB and seasonal influenza compared to those singly infected with TB or influenza (286). This study, however, included a small group of co-infected participants and patients with a more chronic presentation were not enrolled.

De Paus *et al.* investigated the potential relationship between influenza infection and the role it plays in developing active TB, either directly after exposure to TB or through reactivation of latent TB infection (287). They observed no correlation between TB cases and the sero-prevalence of antibodies against influenza viruses. They did find that antibody titers against circulating A/H3N2 influenza virus were slightly enhanced in TB patients compared to controls, and highest in cases of advanced TB, suggesting that TB patients were recently infected with influenza, before clinical manifestation of the TB. Overall they concluded that influenza virus infection is not a major determinant for developing clinically relevant TB (287).

During pandemics, individuals most at risk of influenza infection are HIV-infected, TB-infected, and those with chronic hepatitis B or C (24). Increased mortality from TB has been observed during influenza pandemics (288,289). In a recent research article, the relationship between TB and influenza deaths during the A/H1N1 pandemic of 1918 – 19 was reported (290). The data showed an association between TB and influenza mortality (p=0.09), where none was observed in TB-uninfected controls.

A study by Redford *et al.* explored the role of type I interferon signalling pathway on mycobacterial growth in TB-influenza co-infected mice. The authors showed that influenza A virus infection of mice prior to TB infection significantly impaired long-term mycobacterial control in the lungs, leading to a decrease in host survival. In addition, co-infection of mice with TB and influenza A virus enhanced mycobacterial growth in the lungs through a type I IFN signalling dependent pathway (291). The potential mechanisms underlying the detrimental effect of type I IFN during TB infection could include the

down-regulation of the IFN-γ receptors (292), important because macrophage activation by IFN-γ is essential for eliminating intracellular bacterial infections (291). Another study in mice aimed at determining the impact of pulmonary delivery of a recombinant influenza A virus on the induction of TB-specific CD4+ and CD8+ T-cell responses and the resultant protection against TB infection, found that the induction of pulmonary TB epitope-specific CD4+, but not CD8+ T-cells, are essential for protection against acute TB infection in the lung (293).

To date there are limited studies on the role of HIV/TB co-infection in relation to the risk of severe influenza infection and vaccine immune responses. Immune responses induced by influenza vaccination are generally lower in HIV-infected adults. A meta-analysis published in 2006 yielded a pooled relative risk reduction of 66% (95% CI: 36 – 82) for influenza vaccine against influenza illness in HIV-infected adults (294). The meta-analysis, which involved the only four published studies, concluded that substantial methodological shortcomings in the studies provided only limited evidence supporting the efficacy of TIV in HIV-infected adults. One study conducted in South Africa looked at the efficacy of IIV3 in HIV-infected individuals (137). The authors showed an efficacy of IIV3 against confirmed influenza illness was 75% (95% CI: 9.2 – 95.6) and a risk difference of 0.2 per 100 person-weeks in IIV3 recipients. Among the IIV3-recipients, sero-conversion rates for A/H1N1, A/H3N2 and B/Victoria were 52.6%, 60.8% and 53.6%, respectively (137). These findings were in agreement with the meta-analysis on IIV3 in HIV-infected individuals (138).

In South Africa, 10% of cases that died during the 2009 A/H1N1 pandemic were co-infected with TB (295). In a study that modelled the excess influenza-associated mortality to monthly TB and non-TB respiratory deaths, using laboratory-confirmed influenza as a covariate, it was found that TB deaths increase each influenza season (winter) (84). The mean annual influenza-associated TB mortality rate was 164 per 100 000 for HIV-infected and 5 per 100 000 for HIV-uninfected individuals, regardless of age. Among adults less than 65 years, influenza-associated TB mortality risk was increased compared to influenza-associated non-TB respiratory deaths in HIV-infected (relative risk [RR]: 5.2 95% CI: 4.6 – 5.9) and HIV-uninfected (RR: 61.0 95% CI: 41.4 – 91.0) individuals. Observations included an increased risk of influenza-associated mortality in persons with TB compared to non-TB respiratory deaths (84). An additional paper by the same group found that in patients hospitalised with confirmed pulmonary TB and having symptoms ≥7 days in South Africa, TB-influenza co-infection was associated with increased risk of death (RR: 6.1 95% CI: 1.60 – 23.4) compared to TB only, suggesting that influenza infection contributes to the mortality rate among patients with TB, particularly among patients with longer duration of symptoms (282).

1.17 ELISPOT for Quantifying Cellular Immunity

The enzyme-linked immunospot (ELISPOT) assay is one of the most common methods for measuring antigen-specific T-cells responses. It is a highly quantitative method, with high sensitivity to identify a single cell secreting a particular cytokine, can measure a broad range of responses, as well as assessing critical cellular immune-related activities (INF-γ

secretion and granzyme B release). The ELISPOT assay was first described in 1983 by Czerkinsky *et al.* (296) and differs from older techniques by excluding the need for red blood cells and instead, replacing them with a solid plastic surface on which antigens can bind. This method has subsequently been accepted as one of the most validated assays for human clinical trials (297,298). The spatial separation of antigen-specific cells and the use of a visible spot to enumerate the cells allowed for improved quantitation of secreting cells (299). Other advantages include a platform that can accommodate multiple cell types (T- and B-cells) and secretions (cytokines and granzyme B), can be standardised across numerous laboratories, as well as having a lower limit of detection than flow-based immunoassays.

The method involves the isolation of peripheral blood mononuclear cells (PBMC) and the addition of a set number of cells to a capture antibody-coated plate, such as anti-INF-γ. Cells are then stimulated with a pre-determined concentration of specific antigens which causes antigen-specific T-cells to secrete cytokines that are then captured by the antibody coating the plate. Following an incubation period (approximately 16 – 24 hours, depending on the stimulation), the cells are removed by sequential washing and the remaining bound cytokine are then detected using a reagent conjugated to an enzyme label (alkaline phosphatase). This enzyme reagent catalysis the colormetric spot formation when in the presence of a chromogenic substrate (5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine salt). Enumeration of the number of antigen-specific cells through calculation of the number of spot forming units (SFU) allows for quantitative analysis by use of a plate reader (196,299).

There is currently no general consensus as to the nature and threshold of a significant T-cell response needed for immune protection against influenza; however the arbitrary threshold of a 2-fold increase in IFN-γ secreting cells is commonly used to compare T-cell responses. A study by Forrest *et al.* using a protection curve detected that the majority of infants and young children with ≥100 SFU/10^6 PBMC were protected against clinical influenza illness and thereby possibly establishing a target level of CMI for future influenza vaccine development (123). Although the ELSPOT assay has many advantages, limitations are evident: phenotyping cells secreting cytokines are difficult to perform in parallel; the amount of secreted cytokine per cell cannot be quantified and; the assay has a potential to become expensive (299).

To date numerous studies have been conducted that used the IFN-γ ELISPOT assay to determine the immunogenicity of influenza vaccines, with varying results. Most are based on CMI responses in healthy adults (179,300-305), while others focused on CMI responses in HIV-infected children (306-308), healthy children (123,182), HIV-infected adults (309,310) and one study in HIV-infected pregnant women (173). A similar trend was observed among the healthy adult participants when comparing pre- to post-vaccination IFN-γ SFU against various influenza virus or vaccine strains. Iorio *et al.* explored the cellular immunity induced by the seasonal 2007/2008 influenza vaccine in healthy individuals (300). Although the study participant numbers were low, a significant mean SFU increase was observed from baseline to post-vaccination for all three vaccine strain, with mean fold increases of 3.4 for A/H1N1 (p=0.03), 2.7 for A/H3N2 (p=0.03) and 3.2 for B/Yamagata (p=0.03) (300). Similarly, a study by Subbramanian *et al.* found significant

increases in pre- to post-vaccination SFU for A/H1N1 present in the 2007/2008 influenza vaccine (304). A mean fold increase of 1.6 was observed, with 60% of participants having a 2-fold increase two weeks post-vaccination (304). Contrary to these findings, two other studies investigating the CMI responses to IIV3 among healthy individuals found no significant increase in SFU from pre- to post-vaccination (301,305). Notably when the authors stratified the participants' responses based on baseline SFUs they were able to show that low-baseline responses significantly correlated to higher post-vaccination responses against A/H1N1 and A/H3N2 (r^2=0.4, p<0.005 and r^2=0.2, p=0.005, respectively) (305).

Two separate studies by Weinberg *et al.* evaluated the role of CMI responses to IIV3 vaccine (307) and monovalent pandemic influenza vaccine (308) in HIV-infected children and youth, respectively. Both studies confirmed that administration of influenza vaccines to HIV-infected children and youth was associated with a temporary decrease in Th1 CMI as measured by IFN-γ ELISPOT 28 days post-vaccination. Although not statistically significant, Forrest *et al.* noted an upward trend in median IFN-γ ELISPOT SFUs from pre- to 13 days post-IIV3 in healthy young children in response to stimulation with A/H1N1, A/H3N2 and B/Yamagata strains (123).

Agrati *et al.* explored pandemic vaccination efficiency in boosting vaccine-specific T-cell CMI in HIV-infected individuals by comparing T-cell responses pre- and post-vaccination by IFN-γ ELISPOT assay. In this study a response was considered when a 2-fold increase in IFN-γ producing T-cells from baseline to post-vaccination was achieved (309). According to this threshold, a response for A/H1N1 was observed in 68.4% of

HIV-infected participants (309). The authors speculate that the reason why their data do not strongly match those of previous studies (311,312), might be due to high baseline responses to virus antigens, perhaps attributable to previous exposure with seasonal influenza virus.

Finally, a prospective, open label study conducted in Denver, Colorado from 2005 to 2009, evaluated the immunogenicity of IIV3 vaccination in 20 HIV-infected pregnant women (173). An increase in IFN-γ secreting T-cells post-vaccination was observed, however the investigators were unable to demonstrate significant increases in CMI post-vaccination which was speculated to be due to the general immunosuppression associated with pregnancy.

1.18 Study Rationale

A main objective of this project was to measure CMI responses to IIV3 vaccination in population groups who are at high-risk of influenza morbidity, and to compare those responses to that of the more general population. Additionally, the use of ELISPOT and HAI assays performed in this study will provide novel information on the influenza-specific CMI and HAI responses in TB infected and HIV/TB co-infected individuals in the era of highly active antiretroviral therapy (HAART).

This thesis is divided into two different sections. In the first part of the thesis, we report on the cell-mediated immune responses to trivalent inactivated influenza vaccine in HIV-infected pregnant, HIV-infected non-pregnant, HIV-uninfected pregnant and HIV-uninfected non-pregnant women. In the second part, we report on cell-mediated and humoral immune responses to the trivalent inactivated influenza vaccine among HIV/TB

co-infected, HIV-infected TB-uninfected, HIV-uninfected TB-infected and HIV-uninfected TB-uninfected adults. By assessing blood samples taken prior to and one month post influenza vaccination, we were able to compare and evaluate the effect of influenza vaccination among high-risk individuals in a South African setting.

2.0 Methods

2.1 Study Objectives

The objectives of this thesis were to measure and compare cell-mediated immune (CMI) responses to a single-dose of trivalent inactivated influenza vaccine (IIV3) in:

1. HIV-infected pregnant (HIV+Preg) and non-pregnant women (HIV+NP);
2. HIV-uninfected pregnant (HIV-Preg) and non-pregnant women (HIV-NP);
3. HIV-infected (HIV+), TB-uninfected and HIV-uninfected (HIV-) TB-uninfected adults.

As well as to measure and compare humoral immune responses to single-dose of IIV3 in:

1. HIV-infected adults with TB (HIV+TB+) and HIV-infected adults without TB (HIV+TB-);
2. HIV-uninfected adults with TB (HIV-TB+) and HIV-uninfected adults without TB (HIV-TB-).

2.2 Ethical Consideration

This project consists of four different studies that were individually submitted and approved by the Human Research Ethics Committee (HREC) on Human Subjects at the University of the Witwatersrand. The studies were also registered in ClinicalTrials.gov. For all studies signed informed consent for collection of blood and clinical and demographic information was obtained from the study participants. The studies are: 1) “Immunogenicity and safety of different dosing schedules of trivalent influenza vaccine in HIV-infected pregnant

women: a randomised controlled trial" HREC number 111114 and ClinicalTrials.gov NCT01527825 (HIV+Preg women); 2) "Immunogenicity and safety of trivalent influenza vaccine in non-pregnant HIV- infected women: An open label trial" HREC number 130101 and ClinicalTrials.gov NCT01812980 (HIV+NP women); 3) "Immunogenicity and safety of trivalent influenza vaccine in pregnant and non-pregnant HIV- uninfected women: An open label trial" HREC 121109 and ClinicalTrials.gov NCT01816464 (HIV-Preg and HIV-NP women); 4) "Effect of HIV and/or active tuberculosis on the humoral and cell mediated immune responses to unadjuvanted trivalent sub-unit influenza vaccine in adults" HREC number 130102 and ClinicalTrials.gov NCT018111823.

2.3 Study Population

These studies were undertaken in Soweto, Johannesburg, South Africa, an urban low-income community of 1.4 million Black-African inhabitants of a diversity of ethnic backgrounds, including Zulu, Xhosa and Sotho heritage. The majority of households have access to running water; however, at least 25% live in informal settlements and use fossil fuels for heating and cooking. The community is greatly affected by HIV infection, with <5 year old mortality rate of 39 per 1 000 live births in 2014 (313).

There are 23 primary health care (PHC) clinics in the Soweto region and during the period the studies described in this thesis took place there was a single public hospital (Chris Hani-Baragwanath Academic Hospital (CHBAH)) which was the sole referral hospital for all PHC clinics. Prior to delivery, approximately 99% of pregnant women attend one of

PHC antenatal clinics or have antenatal care at CHBAH. Each year, approximately 29 000 births occur in the community, including 23 000 at CHBAH.

All HIV testing was undertaken by trained counsellors with pre- and post-test counselling provided as part of standard of care. The prevalence of HIV infection in women attending antenatal clinics in Soweto has stabilised at 30% since 2005, and the vertical transmission of HIV have declined from 5.9% in 2008 to 1.5% in 2012 due to more effective mother-to-child preventive antiretroviral treatment regimens strategies (314). Mothers and children infected with HIV were provided with free highly active anti-retroviral treatment (HAART) through established HIV clinics in accordance with national guidelines for treatment. In addition to HAART where indicated, pregnant HIV-infected women were provided with zidovudine (300 mg) from 14 weeks of gestational age and a single dose of nevirapine (200 mg), three-hourly zidovudine (300 mg) during labour and single dose (500 mg) Truvada post-partum and newborns were provided with nevirapine within 72 hour of birth and one week of zidovudine. Ninety-five percent of mothers agreed to HIV testing during pregnancy. Evaluation of CD4+ T-lymphocyte cell (T-cell) count was undertaken on all individuals diagnosed as being HIV sero-positive.

In addition to free access to antiretroviral treatment for all HIV-infected individuals (based on national treatment guidelines of 2013); all HIV-infected individuals also had access to cotrimoxazole prophylaxis. Criteria for initiating cotrimoxazole prophylaxis in HIV-infected individuals included a CD4+ T-cell count <200 cells/µL.

2.4 Selection and Enrolment of Participants

For the purpose of this thesis the different trials were divided into two main groups: i) Maternal influenza (MatFlu) studies, which included HIV-infected pregnant women (study 1A), HIV-infected non-pregnant women (study 1B) and HIV-uninfected pregnant and non-pregnant women (study 1C); ii) HIV/TB influenza study (study 2) that included 4 arms, HIV-infected with TB (HIV+TB+), HIV-infected without TB (HIV+TB-), HIV-uninfected with TB (HIV-TB+) and HIV-uninfected without TB(HIV-TB-).

Study 1A, HIV-infected pregnant (HIV+Preg) women were enrolled at Lillian Ngoyi, Mofolo, Diepkloof, Michael Maponya and Chiawelo clinics in Soweto, which offered antenatal services to HIV-infected women.

Study 1B, HIV-infected non-pregnant (HIV+NP) women were identified from screening logs and databases within our research unit (Respiratory and Meningeal Pathogens Research Unit (RMPRU) located at CHBAH). This included adult female past-participants and mothers of infant participants on other non-influenza clinical trials conducted by RMPRU in the past 2 years. Confirmation of HIV status was obtained at least 12 weeks prior to enrolment.

Study 1C, HIV-uninfected pregnant (HIV-Preg) and non-pregnant (HIV-NP) women were enrolled at CHBAH or one of the community antenatal clinics in Soweto. Enrolment into the study occurred following screening of mothers for HIV, which was undertaken as part of a routine, well-functioning existing prevention of mother-to-child transmission (PMTCT) program.

Study 2, IIV3_HIV_TB: Study participants aged 18 to 55 years were identified at HIV and TB clinics at CHBAH and the adjacent Lilian-Ngoyi Clinic. Individuals with HIV infection received routine HIV-related treatment at the clinic in accordance with the South African national treatment guidelines.

2.5 Inclusion and Exclusion Criteria

2.5.1 Study 1 (MatFlu)

Inclusion criteria: All women

(i) Women age ≥18 to <39 years of age.

(ii) Able to understand and comply with planned study procedures.

(iii) Provide written informed consent prior to initiation of study.

Exclusion criteria: All women

(i) Receipt of IIV3, other than through the study, during the current and previous two influenza seasons, documented by medical history or record.

(ii) Receipt of any live licensed vaccine ≤28 days or any other vaccine (except for tetanus toxoid vaccine) ≤14 days prior to study-vaccine.

(iii) Receipt of a non-licensed agent (vaccine, drug, biologic, device, blood product, or medication) ≤28 days prior to vaccination in this study, unless study approval is obtained.

(iv) Any significant (in the opinion of the site investigator) acute illness and/or oral temperature ≥ 38°C ≤24 hours prior to study entry.

(v) Use of anti-cancer systemic chemotherapy or radiation therapy ≤48 weeks of study enrolment, or had immunosuppression as a result of an underlying illness or treatment.

(vi) Long term use of glucocorticoids, including oral or parenteral prednisone ≥20 mg/day or equivalent for more than 2 consecutive weeks (or 2 weeks total) ≤12 weeks of study entry, or high-dose inhaled steroids (>800 mcg/day of beclomethasone dipropionate or equivalent) ≤12 weeks before study entry (nasal and topical steroids are allowed).

(vii) Receipt of immunoglobulin or other blood products (with exception of Rho D immune globulin) ≤12 weeks prior to enrolment in this study or was scheduled to receive immunoglobulin or other blood products.

(viii) Receipt of IL2, IFN, GM-CSF or other immune mediators ≤12 weeks before enrolment.

(ix) Uncontrolled major psychiatric disorder.

(x) History of a severe adverse reaction to previous IIV3.

(xi) Any condition that would, in the opinion of the site investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol.

In addition to the above criteria:

Inclusion criteria for study 1A include:

(i) Gestational age ≥12 weeks to <36 weeks documented by the approximate date of the last menstrual period and corroborated by physical/ sonargraphic exam.

(ii) Documented to be HIV-infected on two assays prior to study-enrolment.

Exclusion criteria for study 1A include:

(i) Features of WHO clinical category 3 or 4 of AIDS at the time of enrolment.

(ii) Receipt of corticosteroids for preterm labour ≤14 days before study entry.

(iii) Pregnancy complications (during the current pregnancy) such as pre-term labour, hypertension (blood pressure (BP) >140/90 in the presence of proteinuria or BP >150/100, with or without proteinuria or where on antihypertensive medication) and pre-eclampsia.

Inclusion criteria for study 1B include:

(i) Documented to be HIV-infected on one assay used in the PMTCT/other program undertaken within 12 weeks of study enrolment.

(ii) Not pregnant at time of enrolment (confirmed by urine testing). If pregnant in past year, participant had to be at least 6 months post-delivery at time of enrolment.

Inclusion criteria for study 1C include:

(i) Documented to be HIV-uninfected on one assay used in the PMTCT/other program.

(ii) Gestational age ≥20 weeks to <36 weeks documented by the approximate date of the last menstrual period and corroborated by physical exam and sonar report if available (pregnant women only).

Exclusion criteria for study 1C include (pregnant women only):

(i) Receipt of corticosteroids for preterm labour ≤14 days before study entry.

(ii) Pregnancy complications (during the current pregnancy) such as pre-term labour, hypertension (BP >140/90 in the presence of proteinuria or BP >150/100, with or without proteinuria or where on antihypertensive medication) or pre-eclampsia.

2.5.2 Study 2 (IIV3_HIV_TB)

Inclusion criteria:

(i) CD4+ T-cell count of >100/µL within the previous 3 months (applicable to HIV-infected participants) prior to enrolment.

(ii) Ability to attend the clinic for immunogenicity and illness visits.

(iii) Having a microbiologic confirmed diagnosis of tuberculosis (TB) (defined as the presence of acid-fast-bacilli (AFB) on a sputum smear or other specimen and/or a positive culture for *M. tuberculosis*) within the previous 120 days (applicable to TB-infected participants) prior to enrolment.

(iv) Aged 18 to 55 years.

Exclusion criteria:

(i) Any contraindication to influenza vaccine.

(ii) Any contraindication to intramuscular injections.

(iii) Any existing grade 3 or grade 4 laboratory or clinical toxicity as per DAIDS toxicity tables.

(iv) Systemic steroid treatment for >21 days within the past 30 days prior to enrolment.

(v) Pregnancy at time of enrolment (confirmed by urine testing on all women of childbearing age).

2.6 Participant Enrolment

Study 1A, HIV+Preg: Women evaluated in this group were enrolled into a randomised controlled trial that evaluated the immunogenicity and safety of different dosing schedules of trivalent influenza vaccine in HIV-infected pregnant women. For this thesis only women who received a single-dose IIV3 (15 µg HA/strain) were evaluated. A total of 200 women between 12 and 36 weeks gestational age were enrolled prior to the onset of the anticipated influenza season in 2013 and received a single-dose IIV3 (figure 2.1A). Blood samples (20 – 26 ml) were collected from each participant pre-vaccination (baseline) and 28 – 35 days post-vaccination (post-IIV3).

Study 1B, HIV+NP: A prospective, open labelled trial evaluating the immunogenicity of a single-dose IIV3 (15 µg HA/strain) in HIV+NP women. A total of 100 HIV+Preg women were vaccinated prior to the onset of the anticipated influenza season in 2013 (figure 2.1B). Blood samples (20 – 26 ml) were collected from each participant pre-vaccination (baseline) and 28 – 35 days post-vaccination (post-IIV3).

Study 1C, HIV-Preg/NP: A prospective, open labelled trial evaluating the immunogenicity of a single-dose IIV3 (15 µg HA/strain) in HIV-Preg and HIV-NP women. A total of 75 HIV-Preg and 75 HIV-NP women were vaccinated with IIV3 post-influenza season in 2013 (figure 2.2). Blood samples (20 – 26 ml) were collected from each participant pre-vaccination (baseline) and 28 – 35 days post-vaccination (post-IIV3).

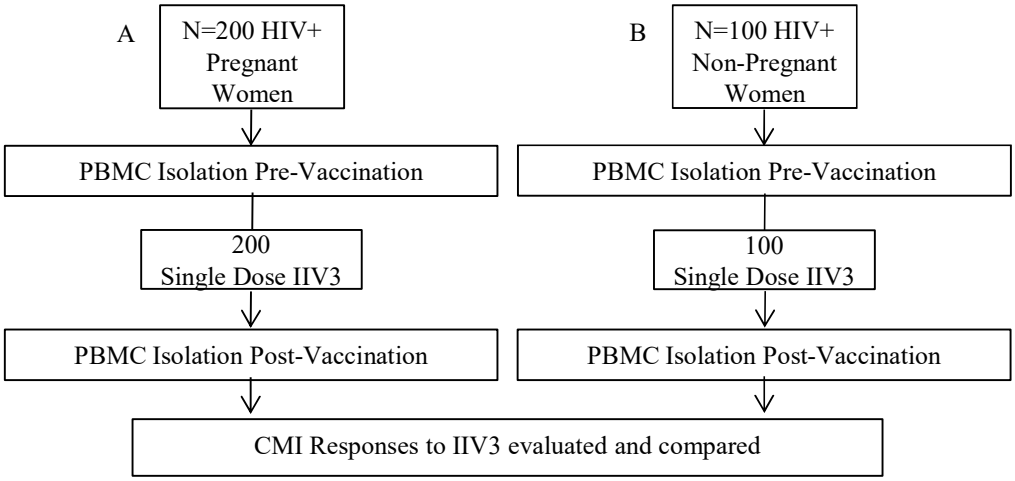


Figure 2.1: Participant enrolment design for MatFlu study 1A and 1B. HIV-infected women were enrolled for the maternal influenza study assessing immunogenicity of IIV3 in 200 HIV-infected pregnant (HIV+Preg) (A) and 100 HIV-infected non-pregnant (HIV+NP) (B) women. PBMC were isolated at baseline and at post-IIV3.

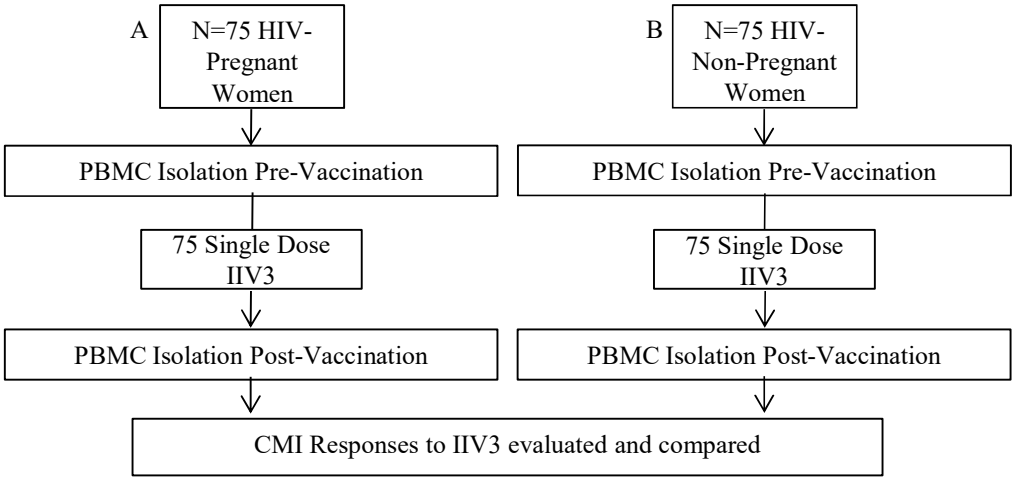


Figure 2.2: Participant enrolment design for MatFlu study 1C. 75 HIV-uninfected pregnant (HIV-Preg) (A) and 75 HIV-uninfected non-pregnant (HIV-NP) (B) women were enrolled for the maternal influenza study assessing immunogenicity of IIV3. PBMC were isolated at baseline and post-IIV3.

Study 2, IIV3_HIV_TB: A prospective, open labelled four arm trial evaluating the immunogenicity of a single-dose IIV3 (15 µg HA/strain) in: 80 HIV+TB+; 80 HIV+TB-; 80 HIV-TB-; and 61 HIV-TB+ individuals, enrolled between March and September 2014 (figure 2.3). Blood samples (20 – 26 ml) were collected from each participant pre-vaccination (baseline) and 28 – 35 days post-vaccination (post-IIV3).

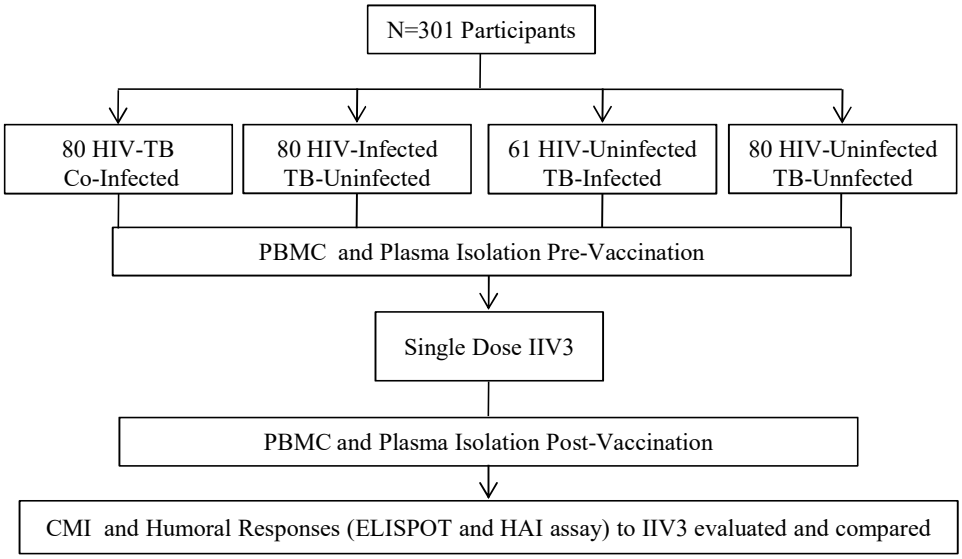


Figure 2.3: Participant enrolment design for IIV3_HIV_TB study. Participants PBMC and plasma were isolated at baseline and post-IIV3 in order to assess the immunogenicity of IIV3.

2.7 Study Vaccines

The IIV3 formulation recommended by World Health Organisation (WHO) for use in the Southern Hemisphere in 2013 was used in Study 1 (MatFlu) and its composition was: A/California/7/2009 NYMC X-179A (A/California/7/2009 [H1N1]pdm09-like); A/Victoria/361/2011 IVR-165 (A/Victoria/361/2011 [H3N2]–like); and B/Hubei-Wujiagang/158/2009 NYMC BX-39 (B/Wisconsin/1/2010-like, Yamagata lineage) (15 µg of each strain specific HA per 0.5 mL dose).

The IIV3 formulation recommended by WHO for use in the Southern Hemisphere in 2014 was used in Study 2 (IIV3_HIV_TB) and the composition was: A/California/7/2009 NYMC X-179A (A/California/7/2009 [H1N1]pdm09 - like); A/Texas/50/2012 NYMC X-223A (A/Texas/50/2012 [H3N2] – like); B/Massachusetts/2/2012 NYMC BX-51B (B/Massachusetts/2/2012-like, Yamagata lineage) (15 µg of each strain specific HA per 0.5 mL dose). Vaccination was administered aseptically by a study-doctor or study-nurse in the deltoid muscle.

2.8 Antigens and Viruses

The viruses for the interferon gamma (IFN-γ) enzyme-linked immunospot (ELISPOT) assays and antigens for the hemagglutination inhibition (HAI) assays were obtained from the National Institute for Communicable Diseases (NICD), South Africa and the Centre of Disease Control and Prevention (CDC), USA, respectively, and were stored in appropriate environments as described by the manufacturer.

Viruses used for the ELISPOT assay were attenuated influenza viruses corresponding to the vaccine strains for 2013: A/California/7/2009 NYMC X-179A (A/California/7/2009 [H1N1]pdm09-like); A/Victoria/361/2011 IVR-165 (A/Victoria/361/2011 [H3N2]–like); and B/Hubei-Wujiagang/158/2009 NYMC BX-39 (B/Wisconsin/1/2010-like) and for 2014: A/California/7/2009 NYMC X-179A (A/California/7/2009 [H1N1]pdm09 -like); A/Texas/50/2012 NYMC X-223A (A/Texas/50/2012 [H3N2] – like); B/Massachusetts/2/2012 NYMC BX-51B (B/Massachusetts/2/2012-like). For simplicity,

the following abbreviations are used throughout this thesis for both 2013 and 2014 strains: A/H1N1, A/H3N2 and B/Yamagata.

Original HA titers for vaccine strains A/H1N1, A/H3N2 and B/Yamagata were 1:64, 1:2048 and 1:64 for the 2013 IIV3 and 1:64, 1:32 and 1:128 for the 2014 IIV3, respectively.

2.9 Optimisation of ELISPOT Experimental Conditions

To ensure that results obtained from the study were comparable with other published ELISPOT studies, a number of quality control measures were assessed. Several experiments were undertaken prior to study sample analyses to establish validity of the ELISPOT experiments as described below (315).

2.9.1 Optimisation of Virus Concentration for IFN-γ ELISPOT Assay

In order to optimise the viruses concentration for valid ELISPOT assay quantification, each of the strains for both 2013 and 2014 vaccines were diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 in ELISPOT assay media (EAM) and tested against PBMC known to respond well to the influenza vaccine strains in both years. The results are shown in figure 2.4A and 2.4B. In this optimisation experiment, it was necessary to choose dilutions that would yield both easily distinguishable spot forming units (SFU). Based on these criteria, the following dilutions were chosen for optimal enumeration: for strains present in the 2013 vaccine 1:16, 1:32 and 1:32 and for the 2014 vaccine strains 1:16, 1:8 and 1:16 for A/H1N1, A/H3N2 and B/Yamagata, respectively.

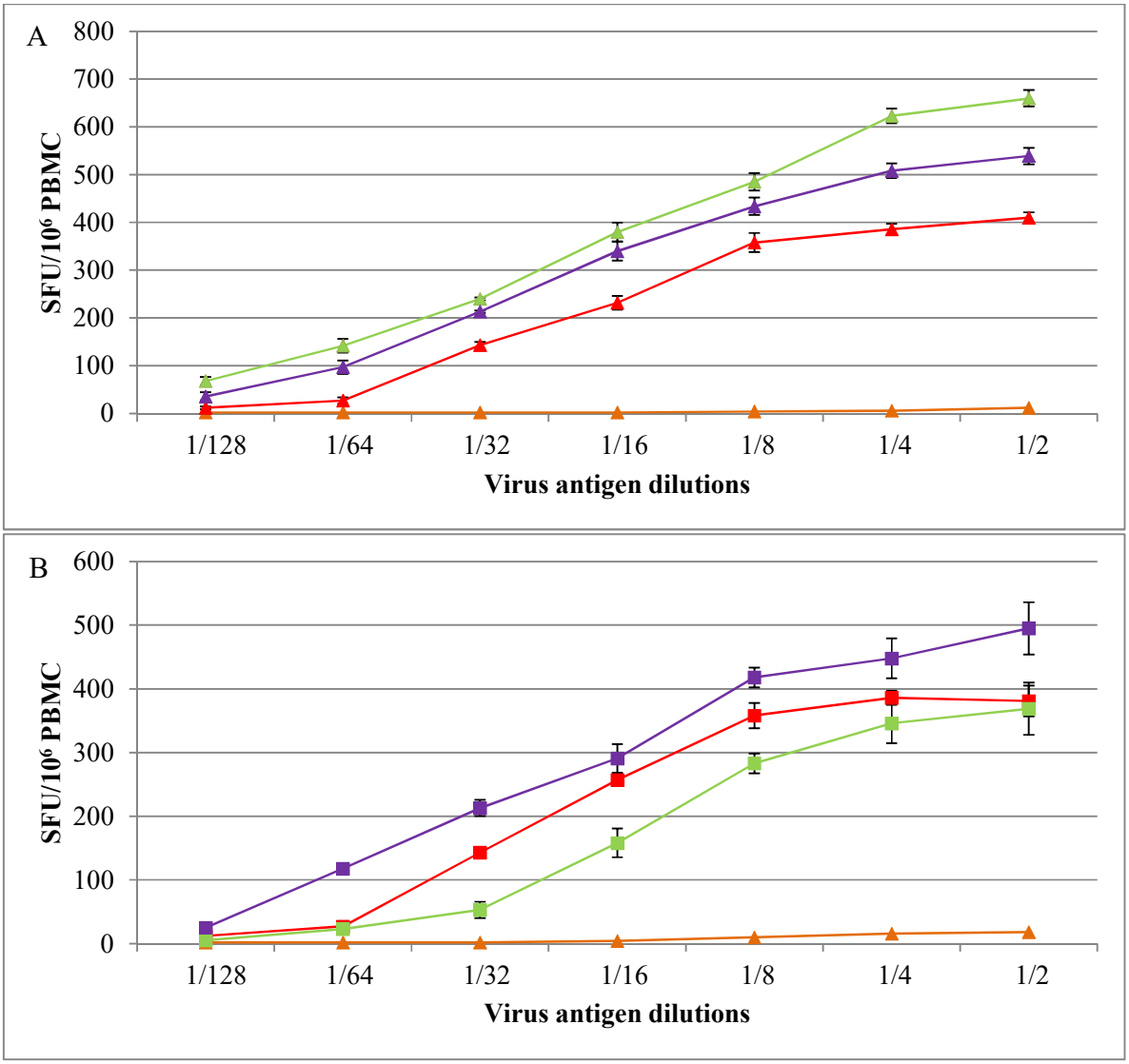


Figure 2.4: Effect of varying the virus concentrations on ELISPOT assay response for strains present in the 2013 (A) and 2014 (B) Southern Hemisphere IIV3. PBMC known to elicit responses to the three strains (2013: A/H1N1, A/H3N2 and B/Yamagata; 2014: A/H1N1, A/H3N2 and B/Yamagata) was used to determine the optimum antigen concentration. Antigens were diluted in EAM and assayed in duplicate, and normalised by subtracting background ( ) SFU. Results expressed as SFU/10^6 PBMC. Error bars indicate standard deviation from the mean.

2.9.2 Optimisation of Cell Concentration for IFN-γ ELISPOT Assay

In previous studies cell concentrations or absolute number of cells per well used in ELISPOT assays vary depending on the antigens and cell populations tested, but are generally between 2 x 10^5 – 5 x 10^5 cells/well. Critical features to have in consideration when determining the amount of cells to use include: having sufficient cells to detect a response, not having excess cells (which may result in non-specificity) and cell viability and the number of cells available for the assay. For this project cell availability was a concern and it was necessary to determine the optimal concentration of cells per well to accommodate this threshold. According to the manufacturer's (BD® Biosciences) recommendations, a cell concentration of 5 x 10^3 – 10 x 10^6 cells per well was suggested, due to this wide range, optimisation was essential for clear, accurate quantification. Agrati *et al.* optimised their ELISPOT cell concentrations at 3 x 10^5 cells/well (309), and using this as a reference point we used dilutions ranging from 1 x 10^5 – 6 x 10^5 cells/well, with the already established antigen concentrations stated earlier. This was further supported by Hobeika *et al.* and Britten *et al.* who stated that cell numbers of 1 x 10^5 - 4 x 10^5 cells/well yielded the highest ratio of spots to cells number (316,317). Isolation of PBMC from two participants known to respond well to the influenza virus stains were frozen (as described below) and stored until required for cell concentration optimisation determination. It was clear from figure 2.5A and 2.5B that responses were detected at all concentrations for both participants PBMC. Overall responses were lower when 1 x 10^5 cells/well was used, raising concern regarding sensitivity. However, sensitivity was greater at higher cell concentrations with a clear plateau effect noticeable at 4 x 10^5 cells/well, with the acceptable specificity concentration chosen at 3 x 10^5 cells/well.

Additionally, using this concentration ensured that there were sufficient cells from all participants to perform the ELISPOT assay in duplicates.

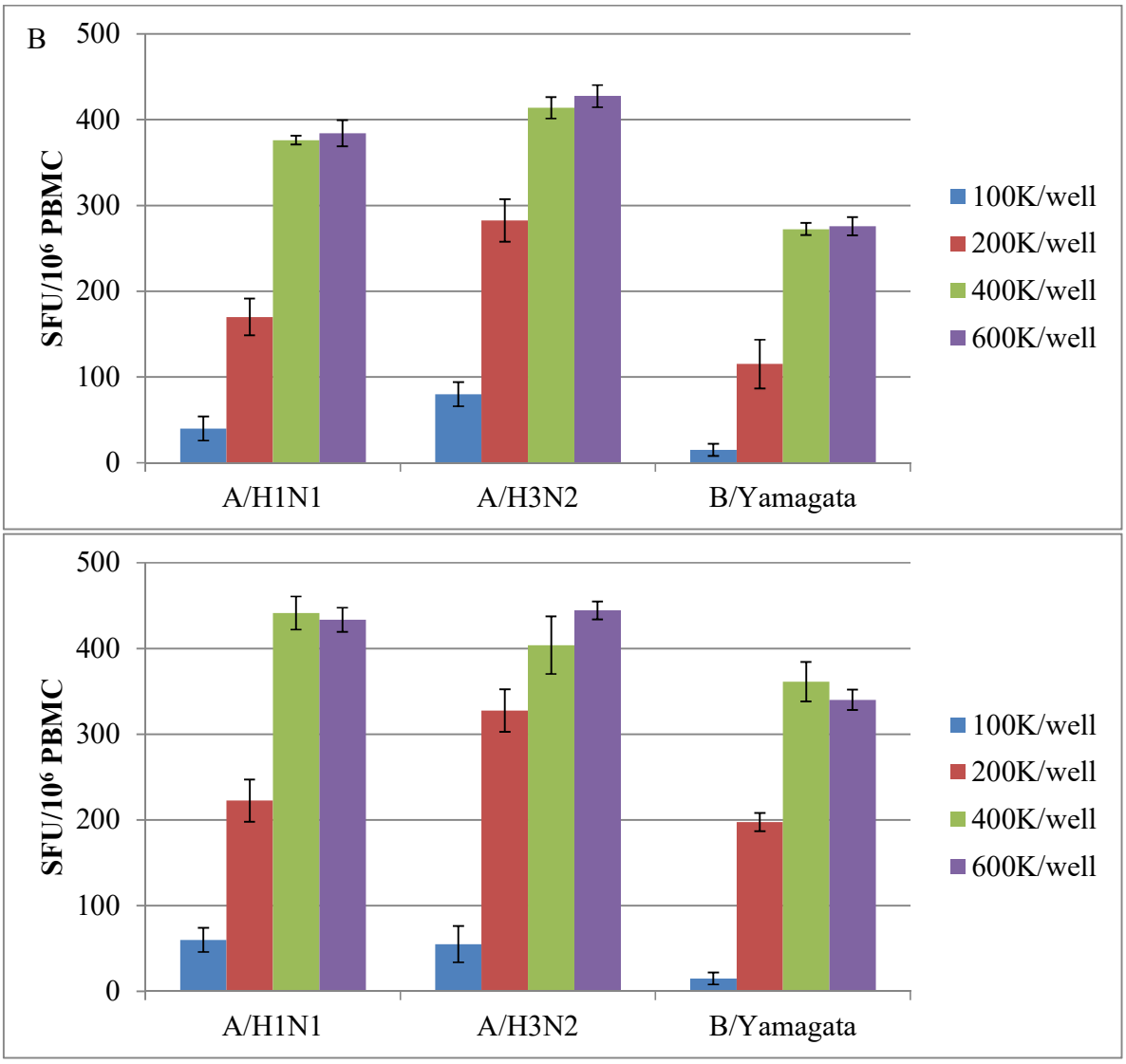


Figure 2.5: Effect of varying cell concentrations on ELISPOT assay responses for participant 1 (A) and participant 2 (B). Each participant cells were exposed to the three strains present in the 2013 Southern Hemisphere IIV3 at antigen dilutions of 1:16, 1:32 and 1:32 for A/H1N1, A/H3N2 and B/Yamagata, respectively. Antigens and varying cell concentrations were diluted in EAM and assayed in duplicate, and normalised by subtracting background SFU. Results expressed as SFU/10^6 PBMC. Error bars indicate standard deviation from the mean.

2.10 Peripheral Blood Mononuclear Cells and Plasma Isolation

Blood samples from participants were centrifuged at 400 g for 10 minutes within 4 - 6 hours of collection. In a laminar flow hood plasma was removed from the top layer using a sterile pipette. Plasma was aliquoted into cryopreservation tubes and stored at -80°C. The remaining blood cells were then diluted with PBS (1:1) and resuspended thoroughly in 50 mL conical polypropylene (Falcon, BD™ Biosciences) tubes. In separate 50 mL conical polypropylene tubes containing 15 mL of Ficoll-Hypaque (Sigma) 30 mL of diluted blood was overlayed; tubes were then centrifuged at 900 g for 15 minutes without centrifuge brakes to preserve the different layers formed. The PBMC were then removed from the overlay and transferred to new sterile conical polypropylene tubes. Peripheral blood mononuclear cells were washed twice with PBS and centrifuged at 400 g for 10 minutes with centrifuge brakes. The supernatant was aspirated and the pellet diluted into 1 mL of PBS, 10 µL of the suspension were transferred to a U-bottom plate well and mixed with 10 µL of 0.4% w/v Trypan Blue. The PBMC-Trypan Blue suspension (10 µL) was transferred to a Counting Slide (Bio-Rad) and viability and cell concentration determined using a TC20™ Automated Cell Counter (Bio-Rad). Once the cells were counted, the polypropylene tubes were filled with PBS and centrifuged. The final pellet was resuspended in Freezing Media (10% DMSO, HI-FBS) at a concentration of 1 x 10^6 cells/mL and aliquoted in 1 mL aliquots in cryovials, that were subsequently placed in a Mr Frosty Control Freeze container (Nalgene) for overnight slow freezing at -70°C before being transferred to liquid nitrogen.

2.11 Thawing of Peripheral Blood Mononuclear Cells

Prior to thawing, Thawing Media (TM) was warmed to 37°C and 1 mL was added to 15 mL sterile polypropylene (Falcon, BD™ Biosciences) tubes. One 15 mL sterile centrifuge tube was required for each PBMC cryovial. Each 15 mL tube was labelled accordingly. The PBMC cryovials were removed from the liquid nitrogen (4 vials at a time) and placed in a 37°C water bath for approximately 1 – 3 minutes, or until a small amount of ice remained. Once thawed the vials were briefly submerged in 70% alcohol to sterilise the exterior surface before opening. The cells were then transferred drop-wise to the 15 mL tube containing pre-warmed TM. After 1 minute, 500 µL of pre-warmed TM were added drop-wise to the PBMC. After waiting 1 minute, another 1 mL of TM was added to the PBMC. After 1 minute an additional 1 mL of TM was added to the cell suspension (repeated three times). The initial process was completed by filling the 15 mL tube with TM to a total of 10 ml. The cell suspension was then mixed by gently inverting the tubes and subsequently centrifuged at 400 g for 10 minutes. The supernatant was removed and the pellet resuspended in 10 mL TM and centrifuged at 400 g for 10 minutes. The supernatant was once again removed and the pellet resuspended in 10 mL of TM and thoroughly mixed. Ten microliters of the suspension were transferred to a U-bottom plate well and mixed with 10 µL of 0.4% w/v Trypan Blue. The PBMC-Trypan Blue suspension (10 µL) was transferred to a Counting Slide and viability and cell concentration determined using the TC20™ Automated Cell Counter. The cells were then centrifuged at 400 g for 10 minutes, supernatant removed, and diluted into TM to a final concentration of 1×10^6 cells/mL for overnight resting in a 37°C, 5% CO_2 humidified incubator.

2.12 IFN-γ ELISPOT Assay

The IFN-γ ELISPOT assay was performed according to the manufacturer's instruction (BD™ Biosciences). Each test and control wells were performed in duplicate and both visits (baseline and post-IIV3) from the same participant were performed on the same plate on the same day to minimise variability.

Day one consisted of pre-treatment of ELSPOT plates and involved coating the wells overnight at 4°C with 100 µL diluted (1:200, final concentration of 5 µg/ml) Capture Antibody (Purified NA/LE Anti-human IFN-γ) in PBS.

On day two the plates were washed once with 100 µL EAM, and subsequently "blocked" with 200 µL EAM for 2 hours at room temperature (RT). All media were brought to RT while positive control, negative control and viruses were prepared during this 2 hour waiting step as follows:

The positive control used in this assay was reconstituted phytohemagglutinin (PHA-M, lyophilised, Sigma-Aldrich) in deionised water (dH_2O) to a final concentration of 5 µg/ml, EAM was used to detect spontaneous cytokine production (background, negative control) and viruses were diluted in EAM according to their optimum concentration as determined earlier.

The PBMC that were incubated overnight were centrifuged at 400 g for 10 minutes and the pellet was diluted into 1 mL of EAM in preparation for cell count and viability testing as done earlier. The PBMC were centrifuged at 400 g for 10 minutes and diluted in EAM to a final working concentration of 3×10^5 PBMC/well.

The “Blocking” solution was discarded and 50 µL of positive control (PHA-M), negative control (EAM) and diluted viruses (A/H1N1, A/H3N2 and B/Yamagata) were added to their respective wells (Figure 2.6). Participant PBMC suspensions were added (50 µL/well) at a concentration of 3 x 10^5 cells/well to a final volume of 100 µL/well. Special care was taken at all steps not to accidently damage the membrane at the bottom of the well. Prior to incubation the lid was replaced to avoid any potential evaporation, and the ELISPOT plate (including the lid) placed bottom side down onto a piece of aluminium foil approximately 10 x 15 cm in size. This was tucked around the base of the plate and remained *in-situ* until after removal of the chromogen solution. The purpose of using aluminium foil was to reduce the possibility of “edge-effect”, whereby the external wells of the 96-well plate produce unreliable results (318). The ELISPOT plate was placed in a 37°C, 5% CO_2 humidified incubator overnight (20 – 24 hours). Care was taken to ensure that the plates were not disturbed while incubating overnight in order to avoid “smudging”, which may occur when agitating IFN-γ-producing cells.

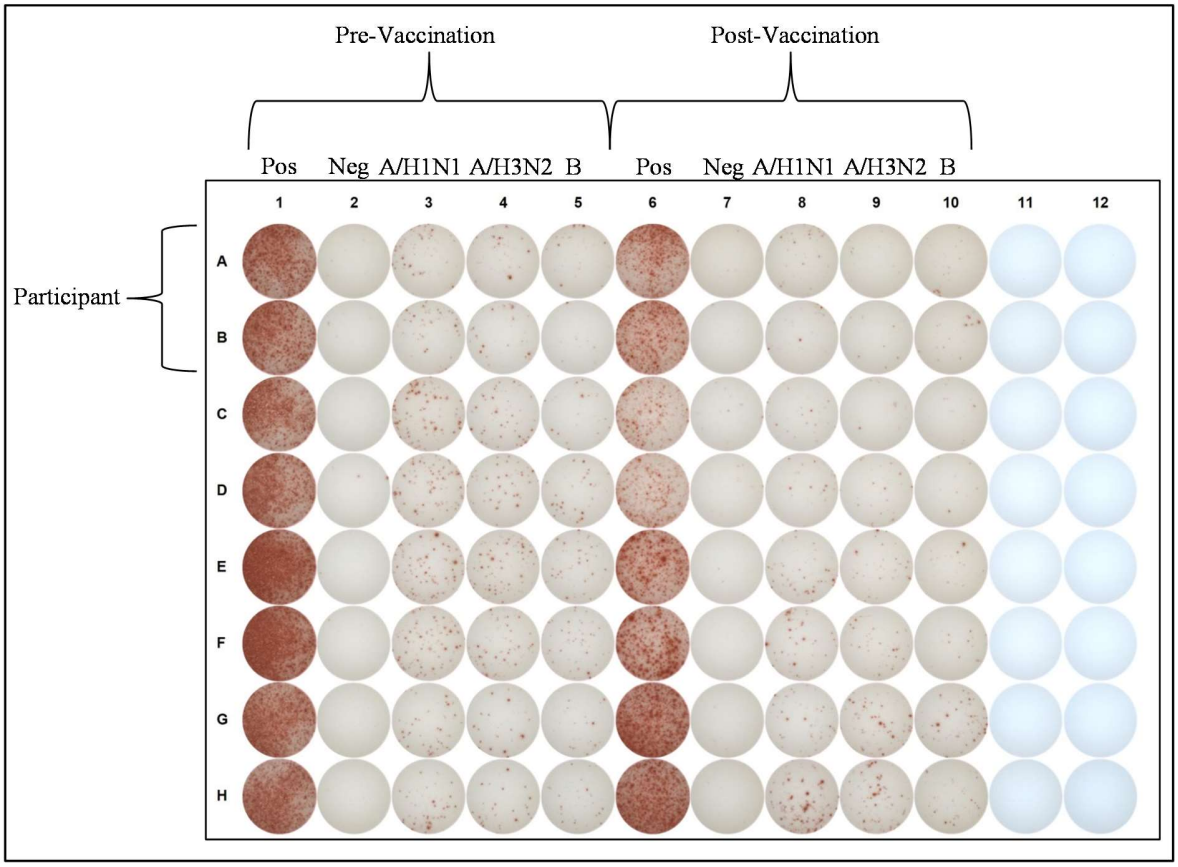


Figure 2.6: Representative ELISPOT assay plate layout used for all assays performed. Duplicates were performed for all participants, with PBMC from baseline and post-IIV3 assayed on the same plate to avoid potential variability. A total of four participants were assayed in one plate, each with a positive control (PHA-M), negative control (EAM) and test wells containing A/H1N1, A/H3N2 and B/Yamagata, respectively.

On day three the cell suspensions were discarded and plates washed (5 times) by manually filling each well with 200 µL PBS for 1 minute per wash. After each washing step, the plates were inverted and blotted against clean paper towels. During the final wash the Detection Antibody (Biotinylated anti-human IFN-γ) was diluted (1:250, final concentration 2 µg/ml) in Dilution Buffer (1 x PBS, 10% HI-FBS) according to the manufacturer's instructions. One hundred microliters of the Detection Antibody were added to each well and incubated at RT for 2 hours. The Detection Antibody solution was then

discarded and the wells washed (3 times) with PBS. Again, during the final washing step the Enzyme Conjugate (Streptavidin-HRP) was prepared according to the manufacturer's instruction by diluting (1:100) in Dilution Buffer and 100 µL were added to each well and incubated at RT for 1 hour. The Enzyme Conjugate was discarded and the plate wells washed (6 times) as previously described. The Final Substrate Solution (a stabilised chromogen mixture of 3-amino-9-ethylcarbazole [AEC] and 2.2% N,N-dimethylformamide [DMF], prepared according to the manufacturer's instructions) was added to each well (100 µL). Spot formation was monitored for a maximum of 5 minutes before discarding the Final Substrate Solution and the reaction stopped by rinsing the plate wells with dH_20. The aluminium foil and flexible plastic casing were removed, the bottom of the plate wiped thoroughly with clean paper towels and the plate was left to air-dry completely at RT for at least 2 hours. The plates were then ready for spot enumeration.

2.13 Reading of ELISPOT Plates

Plates were sent in batches of 50 to Cellular Technology Ltd. (CTL-Europe GmbH, Bonn, Germany) for scanning and images were available for digital download and analysed using ImmunoSpot® Professional Software (version 5.3.21). Specific parameters for optimal spot counting analyses were as follows: Normalise Counts of Mask: Off; Sensitivity: 180; Minimum Spot Size: 0.0020 mm^2; Maximum Spot Size: 0.1032 mm^2; Spot Separation: 3.0; Diffuseness: Normal; Background Balance: On (80); Edge Compensation Level: 1.0. Uniform settings were used for all experiments to enable comparisons. Each well was manually reviewed using the software's built-in quality control functions to exclude the

possibility of aberrant counting. Absolute numbers of spots were counted and the results exported to Excel (Microsoft) for further analysis. Results were reported as geometric mean (GM) SFU/10^6 PBMC and only experiments that fulfilled the following criteria at both baseline and post-IIV3 were included in the final analysis:

i) test wells (prior to subtraction of background SFUs) had to exhibit SFUs ≥2 times the background;

ii) test wells had to have ≥10 SFU/10^6 PBMC following subtraction of background SFUs and

iii) in the background wells SFUs needed to be <50 SFU/10^6 PBMC.

Furthermore a significant response to vaccination was defined as ≥2-fold increase in SFUs from baseline to post-IIV3.

2.14 Hemagglutination Inhibition Assay

The following steps were undertaken sequentially for each assay day:

Receptor destroying enzyme treatment and inactivation

Plasma collected from participants were thawed at RT and diluted (1:4) with receptor destroying enzyme (RDE) and incubated at 37°C overnight. The RDE was then inactivated by incubating the samples at 56°C for 30 minutes.

Turkey red blood cell standardisation

Standardisation of turkey red blood cells (TRBC) was performed during the 30 minute wait for the inactivation incubation of the plasma-RDE mix. In a biosafety hood, 5 mL of TRBC were added to a 15 mL conical polypropylene (Falcon, BD™ Biosciences) tube and filled with sterile PBS to a final volume of 15 ml. The mixture was inverted, centrifuged at 2 200 rpm for 10 minutes and the supernatant was discarded. The TRBC at the bottom of the tube were washed three times by adding PBS, centrifuging and aspirating the supernatant, finally 25 µL of washed TRBC were added to 300 µL of sterile saline solution. The plasma-RDE mix following inactivation was added to the 325 µL of this 0.5% TRBC, mixed thoroughly and then incubated at 4°C for 1 hour in order to remove non-specific agglutinins. The mixture was inverted every 15 minutes during this 1 hour incubation. The mixture was then centrifuged at 900 g for 5 minutes and the supernatant removed and aliquoted into 96-well U bottom plates (2 wells per plasma sample).

Detection of non-specific agglutinins

In a separate 96-well V-bottom plate (figure 2.7), 25 µL PBS were added to wells B through H for every plasma specimen tested, with an additional column for the cell control (A12). Treated plasma (50 µL) was added to each well in row A (1 – 11), while 50 µL PBS was added to well A12 as the TRBC control. Serial dilutions of plasma were prepared by transferring and mixing 25 µL from row A through H, discarding the remaining 25 µL after well H, each well then received 25 µL PBS and the contents mixed gently by agitating the plate. Packed TRBC (50 µL) was diluted in 10 mL PBS for a 0.5% TRBC suspension. Fifty microliters of this suspension was added to every well, mixed, and

incubated for 30 minutes at RT. Successful results were indicated by the presence of a "halo" of TRBCs in each well.

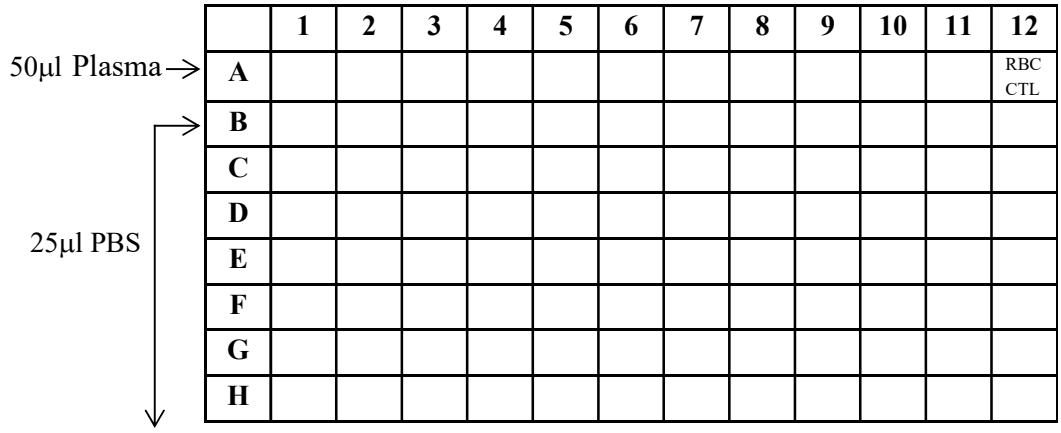


Figure 2.7: Plate layout for detection of non-specific agglutination. See text for details.

Haemagglutinin titration of antigens

Antigens that corresponded with the strains present in the 2014 Southern Hemisphere IIV3 were received from the CDC, USA. The antigens were diluted 1:10 in PBS. In a 96-well V-bottom plate (figure 2.8), 50 µL PBS was added to each well, excluding row 1. Titration was performed in duplicates for each strain. Diluted antigen (100 µL) was added to row 1 and serially diluted by transferring 50 µL from well to well, making sure to mix the antigens before each dilution series. After row 10 the remaining 50 µL were discarded. To each well, 50µL 0.5% TRBC was added and gently mixed by agitating the plate and finally the plate was left to incubate for 30 minutes at RT. The highest dilution of antigens that causes complete agglutination was regarded as the titration endpoint. This endpoint dilution was divided by 8, and the stock antigen diluted to this value (e.g. if the highest dilution was 320, then 320/8=40, hence the stock antigen was diluted 1:40).

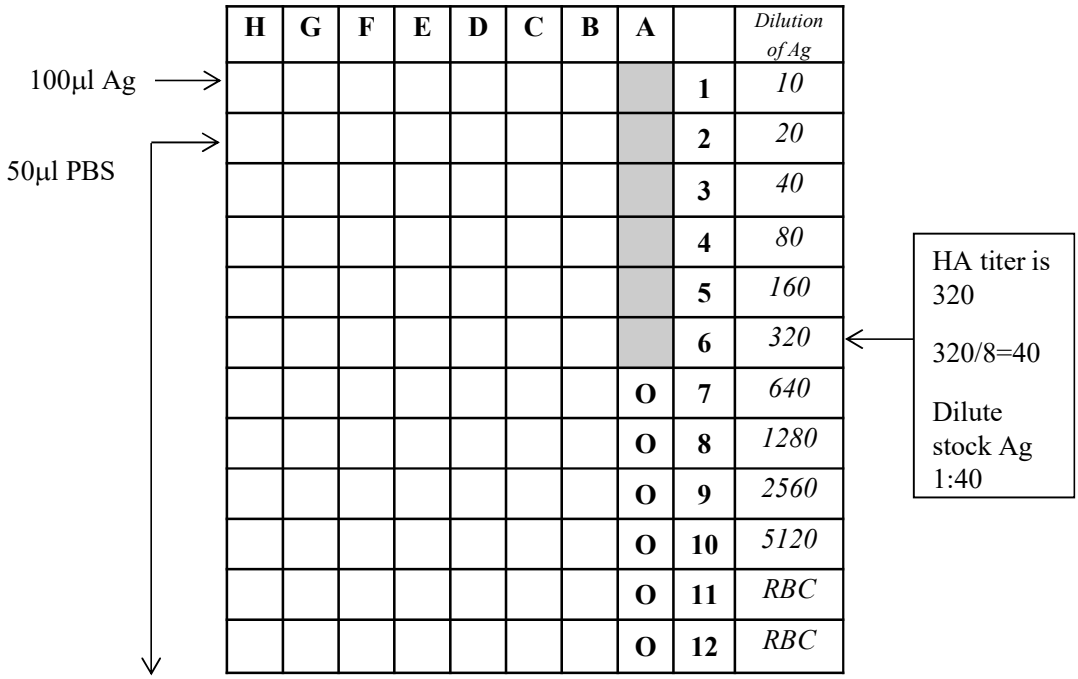


Figure 2.8: Plate layout for haemagglutinin titration of antigens. See text for details.

Back titration

In a new 96-well V-bottom plate, 50 µL PBS was added to wells 2 – 12 for each column used in triplicate. Then, 100 µL of the standardised antigen was added to wells 1 for each lettered column. A serial dilution was performed by transferring 50 µL of standardised antigen from wells 1 through to wells 10. Wells 11 and 12 were used as controls. Fifty microliters of 0.5% TRBC was added to each well and mixed by gently agitating the plate, and incubated for 30 minutes at RT. Results were considered positive if the back titration agreed with the original titration.

HAI Assay

25 µL PBS was added to wells B through H of each numbered column in a new 96-well V-bottom plate (figure 2.9). Plasma samples (50 µL) were added to the appropriate wells in each row A. The control in well A12 received 50 µL PBS. Serial dilutions were made by transferring 25 µL from wells in row A to successive well and mixed thoroughly after each dilution step. The remaining 25 µL was discarded after row H. Standardised antigen was added (25 µL) to each well, except the control well in column 12. The contents were mixed by gently agitating the plate, and incubated for 15 minutes at RT. Following incubation, 50 µL of 0.5% TRBC were added to each well, mixed by agitating the plate and incubated for 30 minutes at RT. The results were recorded where the HAI titer was the last dilution of plasma that completely inhibited hemagglutination (as shown by a "halo" of TRBC). For plasma that displayed no "halos", a result of <1:10 was recorded, whereas complete agglutination of all dilutions, a recorded result of ≥1:1280 is given.

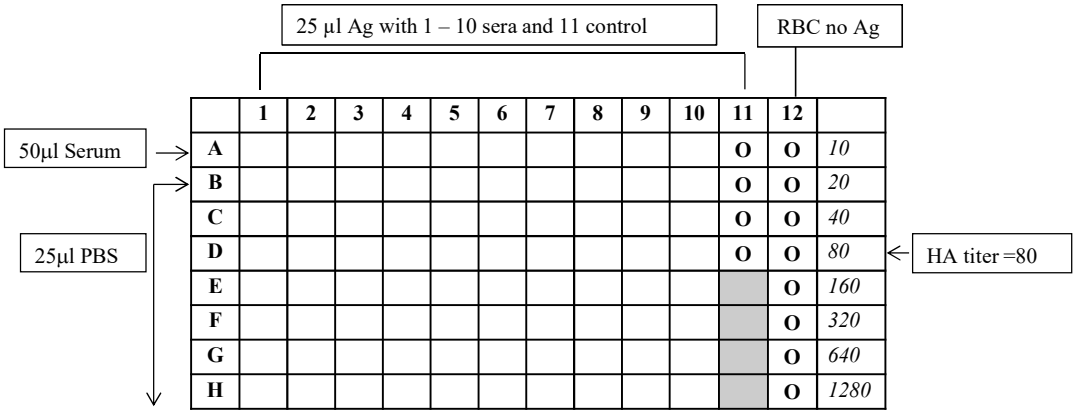


Figure 2.9: Plate layout for HAI assay. See text for details.

2.15 Statistical Analysis

To compare demographic data between study groups (HIV+Preg vs. HIV+NP; HIV-Preg vs. HIV-NP; HIV+TB+ vs. HIV+TB-; and HIV-TB+ vs. HIV-TB- participants) the unpaired Student's t-test was used. Chi-square or Fisher's exact test was used to compare categorical data. Only participants that adhered to the inclusion criteria were analysed. Demographic data was described using means, medians, interquartile ranges and standard deviations, where applicable. Where applicable, *p*-values were adjusted using logistic and linear regression analyses for significant differences in baseline characteristics among study groups and are stated only if a change in significance resulted.

All data obtained from the HAI and ELISPOT assays were logarithmic converted to approach a normal distribution, and presented as geometric means with 95% confidence interval (CI). When comparing baseline to post-IIV3, a paired Students t-test was used for analysis within the same groups. When comparing across groups in terms of baseline and post-IIV3, the unpaired Student's t-test was used. Where relevant, the Chi-square or Fisher's exact test was used to compare categorical data reported as percentages.

For ELISPOT assay data, a fold increase index was used after stratification by low-baseline and high-baseline responses where SFU/10^6 PBMC from post-IIV3 was divided by the baseline SFU/10^6 PBMC and analysed using the non-parametric Mann-Whitney test.

Haemagglutinin inhibition antibody titers ≥1:40 were considered evidence of immunity (sero-protection). Participants with a ≥4-fold increase from baseline to post-IIV3 and with post-IIV3 titers ≥1:40 were considered as sero-converters. For each virus strain, the

proportion of participants in each group with HAI antibody titers ≥1:40 were compared between baseline and post-IIV3 using the Chi-square or Fisher's exact test where applicable. HAI titers <1:10 were considered undetectable and were assigned a value of 1:5.

Differences were considered significant when the *p*-value was less than 0.05. All statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) and STATA 13 (Stata Corp., College Station, TX).

3.0 Cell-Mediated Immune Responses to Trivalent Inactivated Influenza Vaccine in HIV-Infected Pregnant and Non-Pregnant Women

3.1 Results

3.1.1 Baseline Characteristics

In 2013 we enrolled 200 pregnant (HIV+Preg) and 100 non-pregnant (HIV+NP) women infected with HIV; all participants received one single dose (15 μg per antigen) of trivalent inactivated influenza vaccine (IIV3). Enrolment of the pregnant cohort started 11 February and was concluded 5 June and non-pregnant women were enrolled from 2 May to 4 June. At enrolment the pregnant women were slightly younger (mean age 29.4 years [standard deviation (SD): 5.2]) than non-pregnant women (mean age 31.3 years [SD: 4.7], p=0.003), and a higher percentage of HIV+Preg women were receiving antiretroviral therapy (ART) compared to HIV+NP women (98.0% vs. 59.0%, p<0.001). Overall the median CD4+ T-cell count was 428 cells/mm^3 and the median HIV viral load was 5780 copies/ml and this was similar between the two cohorts. At enrolment the mean gestational age among HIV+Preg women was 23.5 weeks (Table 3.1). The mean time between the two study visits was 29.4 days. All 200 of the HIV+Preg women and all 100 of the HIV+NP women completed the study.

A comparison of baseline characteristics between HIV-infected pregnant and non-pregnant women who qualified for enzyme-linked immunospot (ELISPOT) analysis is available in Table 3.2 (see section 3.1.2). Ninety HIV+Preg and 71 HIV+NP women qualified for ELISPOT analysis (Table 3.5). As in the overall cohort HIV+Preg women were slightly younger than HIV+NP women (31.5 years [SD: 4.7] vs. 29.2 [SD: 5.0], p=0.003) and higher percentages of HIV+Preg women compared to HIV+NP women were on ART and

had CD4+ T-cell counts greater than 350 cells/mm^3. The mean time between visits was 29.2 days (SD: 4.3).

Table 3.3 and Table 3.4 show the comparison for baseline demographic characteristics between women who qualified for ELISPOT analysis and those that did not in the HIV+Preg and HIV+NP cohorts, respectively. No significant differences in baseline characteristics were recorded, except for CD4+ T-cell count among HIV+Preg women, where those analysed by ELISPOT assay had a higher median count compared to women not analysed (470 cells/mm^3 vs. 361 cell/mm^3, p=0.007).

Table 3.1: Baseline characteristics of the HIV-infected pregnant and non-pregnant women enrolled into the study in 2013

	Overall	HIV+Preg	HIV+NP	P value
Total no. women	300	200	100	-
Mean days between visits	29.4 ± 5.1	29.0 ± 4.4	30.0 ± 6.2	0.11
Ethnicity (%)				
Black	300 (100)	200 (100)	100 (100)	
Mean age - years	30.0 ± 5.1	29.4 ± 5.2	31.3 ± 4.7	0.003
Median BMI (IQR)	29.4 (25.1 – 34.0)	29.2 (25.1 – 33.7)	29.5 (25.1 – 34.6)	0.77
Mean gestational age - weeks	-	23.5 ± 5.5	-	-
No. Primigravid (%)	-	31 (15.5)	-	-
Median gravidity (IQR)	-	2.0 (2.0 – 3.0)	-	-
Median parity (IQR)	-	1.0 (1.0 – 2.0)	-	-
No. on ART (%)	255 (85.0)	196 (98.0)	59 (59.0)	<0.001
Median CD4+ T-cell count cells/mm^3 (IQR)	428 (281 – 602)	403 (265 – 578)	488 (344 – 636)	0.13
No. CD4+ T-cell>350 cell/mm^3 (%)	155 (51.7)	115 (57.5)	40 (40.0)	0.05
Median HIV viral load copies/ml (IQR)	5780 (504 – 36398)	6030 (652 – 35614)	381 (94 – 40298)	0.48
No. HIV viral load<40 copies/ml	27 (9.0)	21 (10.5)	6 (6.0)	0.28

Plus-minus values are means ± standard deviation
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
ART, antiretroviral therapy
HIV, human immunodeficiency virus
No., number of

Table 3.2: Baseline characteristics of the HIV-infected pregnant and non-pregnant women analysed by ELISPOT assay

	Overall	HIV+Preg	HIV+NP	P value
Total no. women	157	90	71	-
Mean days between visits	29.2 ± 4.3	28.9 ± 3.3	29.6 ± 5.3	0.33
Ethnicity (%)				
Black	161 (100)	90 (100)	71 (100)	1.0
Mean age - years	30.2 ± 5.0	29.2 ± 5.0	31.5 ± 4.7	0.003
Median BMI (IQR)	29.7 (25.7 – 33.9)	29.6 (25.3 – 33.0)	30.0 (26.0 – 34.1)	0.31
Mean gestational age - weeks	-	23.8 ± 5.5	-	-
No. Primigravid (%)	-	16 (17.8)	-	-
Median gravidity (IQR)	-	2.0 (2.0 – 3.0)	-	-
Median parity (IQR)	-	1.0 (1.0 – 2.0)	-	-
No. on ART (%)	132 (82.0)	89 (98.9)	43 (60.1)	<0.001
Median CD4+ T-cell count cells/mm^3 (IQR)	478 (312 – 683)	470 (293 – 688)	501 (357 – 690)	0.56
No. CD4+ T-cell>350 cell/mm^3 (%)	100 (62.2)	58 (64.4)	29 (40.8)	0.004
Median HIV viral load copies/ml (IQR)	9205 (485 – 39339)	9475 (830 – 35864)	343 (78 – 49241)	0.48
No. HIV viral load<40 copies/ml	13 (8.3)	7 (7.8)	6 (8.5)	1.00

Plus-minus values are means ± standard deviation.
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
ART, antiretroviral therapy
HIV, human immunodeficiency virus

Table 3.3: Baseline characteristics of the HIV-infected pregnant analysed and not analysed

	Overall	Analysed	NA	P value
Total no. women	200	90	110	-
Mean days between visits	29.0 ± 4.4	28.9 ± 3.3	29.1 ± 5.2	0.80
Ethnicity (%)				
Black	200 (100)	90 (100)	110 (100)	1.0
Mean age - years	29.4 ± 5.2	29.2 ± 5.0	29.6 ± 5.4	0.54
Median BMI (IQR)	29.2 (25.1 – 33.7)	29.6 (25.3 – 33.0)	28.8 (25.1 – 34.0)	0.84
Mean gestational age - weeks	23.5 ± 5.5	23.8 ± 5.5	23.4 ± 5.5	0.62
No. Primigravid (%)	31 (15.5)	16 (17.8)	15 (13.6)	0.44
Median gravidity (IQR)	2.0 (2.0 – 3.0)	2.0 (2.0 – 3.0)	2.0 (2.0 – 3.0)	0.63
Median parity (IQR)	1.0 (1.0 – 2.0)	1.0 (1.0 – 2.0)	1.0 (1.0 – 2.0)	0.28
No. on ART (%)	196 (98.0)	89 (98.9)	107 (97.3)	0.63
Median CD4+ T-cell count cells/mm^3 (IQR)	403 (265 – 578)	470 (293 – 688)	361 (241 – 536)	0.007
No. CD4+ T-cell>350 cell/mm^3 (%)	115 (57.5)	58 (64.4)	57 (51.8)	0.12
Median HIV viral load copies/ml (IQR)	6030 (652 – 35614)	9475 (830 – 35864)	5088 (595 – 35708)	0.52
No. HIV viral load<40 copies/ml	21 (10.5)	7 (7.8)	14 (12.3)	0.35

Plus-minus values are means ± standard deviation.
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
ART, antiretroviral therapy
HIV, human immunodeficiency virus

Table 3.4: Baseline characteristics of the HIV-infected non-pregnant analysed vs. not analysed

	Overall	Analysed	NA	P value
Total no. women	100	71	29	-
Mean days between visits	30.0 ± 6.2	29.6 ± 5.3	31.1 ± 8.0	0.28
Ethnicity (%)				
Black	100 (100)	71 (100)	29 (100)	1.00
Mean age - years	31.3 ± 4.7	31.5 ± 4.7	30.6 ± 4.6	0.40
Median BMI (IQR)	29.5 (25.1 – 34.6)	30.0 (26.0 – 34.1)	27.4 (22.2 – 34.9)	0.11
No. on ART (%)	59 (59.0)	43 (60.1)	16 (55.2)	0.13
Median CD4+ T-cell count cells/mm^3 (IQR)	478 (329 – 618)	501 (357 – 690)	404 (296 – 608)	0.24
No. CD4+ T-cell>350 cell/mm^3 (%)	59 (59.0)	29 (40.8)	17 (58.6)	1.00
Median HIV viral load copies/ml (IQR)	381 (94 – 40298)	343 (78 – 49241)	506 (168 – 13650)	0.37
No. HIV viral load<40 copies/ml	6 (6)	6 (8.5)	0 (0)	-

Plus-minus values are means ± standard deviation.
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
ART, antiretroviral therapy
HIV, human immunodeficiency virus

3.1.2 Participants Selection

Participants with HIV-infection enrolled in the MatFlu study in 2013 (HIV+Preg, N=200; HIV+NP, N=100) were immunised with a single dose of IIV3 to determine cell-mediated immune (CMI) responses one month post-vaccination (post-IIV3), assessed by detecting interferon-gamma (IFN-γ) production using ELISPOT assay. Blood was collected on the day of vaccination, just prior to IIV3 administration (baseline) and one month post-IIV3. For participants to qualify for EISPOT analyses they needed to have peripheral blood mononuclear cells (PBMC) viability ≥75% and have sufficient number of cells to perform duplicate assays (319) at both baseline and post-vaccination visits. Table 3.5 shows the total numbers of participants in each study cohort that qualified for IFN-γ ELISPOT assessment based on these criteria. Within these subpopulations, only experiments that fulfilled the following criteria at both baseline and post-IIV3 were included in the final

analysis: i) test wells, prior to subtraction of background spot forming units (SFU) had to exhibit SFU ≥2 times the background, ii) test wells had to have ≥10 SFU/10^6 PBMC following subtraction of background SFU and iii) in the background wells SFU needed to be <50 SFU/10^6 PBMC. Furthermore a significant response to vaccination was defined as ≥2-fold increase in SFU from baseline to post-IIV3.

Table 3.5: Number of participants eligible for IFN-γ ELISPOT analysis

			No. participants (%); [95% CI]		
Study Group	**Study Population**	**Viability ≥75%‡**	**A/H1N1§**	**A/H3N2 §**	**B/Yamagata§**
HIV+Preg	200	90 (45.0); [38.0 – 52.2]	55 (61.1); [50.3 – 71.2]	50 (55.6); [44.7 – 66.0]	44 (48.9); [38.2 – 59.7]
HIV+NP	100	71 (71.0); [61.1 – 79.6]	46 (64.8); [52.5 – 75.8]	25 (35.2); [24.2 – 47.5]	23 (32.4); [21.8 – 44.5]

‡ Participants with ≥75% viable live-cells prior to ELISPOT assay

§ Participants displaying test wells SFU ≥2 times background SFU, ≥10 SFU/10^6 PBMC after background subtraction, and background wells with <50 SFU/10^6 PBMC per vaccine strain

3.1.3 Cell-Mediated Immune Responses

Baseline

Of the 200 HIV+Preg and the 100 HIV+NP women enrolled, 90 (45.0%) and 71 (71.1%), respectively, had cell viability ≥75% at both study visits and qualified for the analyses of CMI responses to IIV3 as measured by IFN-γ ELISPOT assay (Table 3.5). At baseline, geometric mean (GM) SFU/10^6 PBMC were higher in HIV+Preg compared to HIV+NP women for A/H3N2 (56.7 [95% confidence interval (CI): 41.7 – 77.2] vs. 32.3 [95% CI: 22.7 – 45.7], p=0.03) and a similar trend was observed for the other two vaccine strains (Figure 3.1A and Table 3.6). We further performed age and ART adjusted analysis for each vaccine strain and similar results to the non-adjusted analysis were obtained (for A/H1N1

adjusted: p=0.61 and B/Yamagata adjusted: p=0.25 while the adjusted p-value for A/H3N2 remained significant p=0.003).

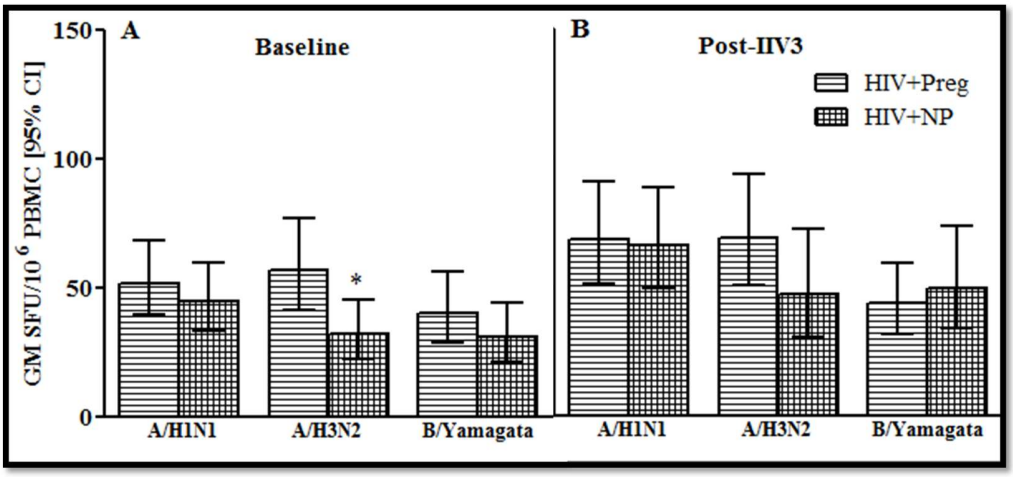


Figure 3.1: Cell-mediated immune responses to the different influenza vaccine strains in HIV-infected pregnant (HIV+Preg) and HIV-infected non-pregnant (HIV+NP) women at baseline (A) and post-vaccination (B). The number of A/H1N1, A/H3N2, and B/Yamagata specific IFN-γ producing peripheral blood mononuclear cells was evaluated at baseline and post-vaccination by ELISPOT assay. Statistical analysis was performed using unpaired t-test. *p=0.03.

Post-vaccination

An increase in GM SFU/10^6 PBMC from baseline to post-IIV3 was observed in both study groups for all three vaccine strains. In HIV+Preg women the increase was, however, statistically significant only for A/H1N1 (52.0 [95% CI: 39.6 – 68.2] at baseline vs. 68.5 [95% CI: 51.5 – 91.0] at post-IIV3, p=0.03) and in HIV+NP women for A/H1N1 (44.6 [95% CI: 33.3 – 59.8] at baseline vs. 66.4 [95% CI: 49.7 – 88.6] at post-IIV3, p=0.005) and B/Yamagata (30.9 [95% CI: 21.5 – 44.4] at baseline vs. 49.8 [95% CI: 33.7 – 73.5] at post-IIV3, p=0.02) (Figure 3.2, Table 3.6).

Post-IIV3 both HIV+Preg and HIV+NP women achieved similar GM SFU/10^6 PBMC for the three vaccine strains in both unadjusted and adjusted analyses (Figure 3.1B, Table 3.6). Similarly, HIV+Preg compared to HIV+NP women had similar GM fold-increases from baseline to post-IIV3 all for strains (Table 3.6). Cell-mediated immune responses to IIV3, as defined by ≥2-fold increase in GM SFU/10^6 PBMC from baseline to post-IIV3, were achieved by 27.3%, 22.0%, and 25.0% of HIV+Preg women for A/H3N2, A/H1N1 and B/Yamagata, respectively; the corresponding proportions of HIV+NP women were 34.8%, 32.0% and 34.8%, respectively (Table 3.6). The difference in the percentage of women who had a ≥2-fold increase in GM SFU/10^6 PBMC between the study groups was not significant. Among HIV+NP women, significantly higher GM SFU/10^6 PBMC were achieved among those on ART compared to those not on ART at post-vaccination for A/H1N1 only (83.8 [95% CI: 57.8 – 121.6] vs. 46.1 [295 – 72.3], p = 0.03).

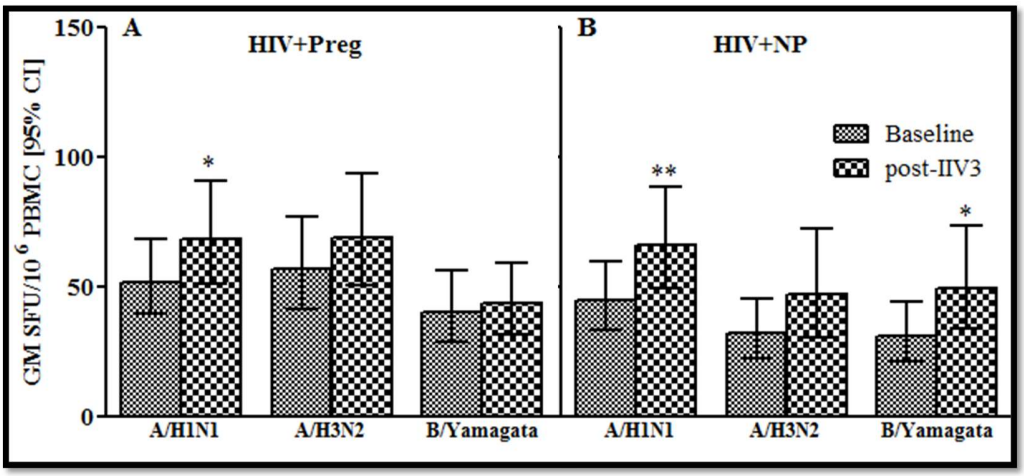


Figure 3.2: Cell-mediated immune responses to the different influenza vaccine strains at baseline and post-IIV3 in HIV-infected pregnant (HIV+Preg) (A) and HIV-infected non-pregnant (HIV+NP) (B) women measured by IFN-γ ELISPOT assay. Statistical analysis was performed using paired t-test. *p<0.05; **p=0.005.

Table 3.6: Cell-mediated immune responses to inactivated influenza vaccine in HIV-infected women

Measure	A/H1N1			A/H3N2			B/Yamagata		
	HIV+Preg	HIV+NP	*P* Value§	HIV+Preg	HIV+NP	*P* Value§	HIV+Preg	HIV+NP	*P* Value§
No. participants	55	46		50	25		44	23	
GM SFU/10^6 PBMC [95% CI]									
Baseline	52.0 [39.6 – 68.2]	44.6 [33.3 – 59.8]	0.45/ *0.61*	56.7 [41.7 – 77.2]	32.3 [22.7 – 45.7]	0.03/ *0.003*	40.5 [29.1 – 56.3]	30.9 [21.5 – 44.4]	0.30/ *0.25*
Post-IIV3	68.5 [51.5 – 91.0]	66.4 [49.7 – 88.6]	0.88/ *0.55*	69.0 [50.7 – 93.9]	47.2 [30.7 – 72.4]	0.15/ *0.24*	43.6 [31.9 – 59.5]	49.8 [33.7 – 73.5]	0.60/ *0.81*
P value‡	0.03	0.005		0.15	0.12		0.64	0.02	
GM factor increase [95% CI]	1.3 [1.0 – 1.7]	1.5 [1.1 – 2.0]	0.52	1.2 [0.9 – 1.6]	1.5 [0.9 – 2.4]	0.79	1.1 [0.8 – 1.5]	1.6 [1.1 – 2.4]	0.16
No. ≥2 fold increase post-IIV3 (%); [95% CI]	15 (27.3); [16.1 – 41.0]	16 (34.8); [21.4 – 50.3]	0.52/ *0.05*	11 (22.0); [11.5 – 36.0]	8 (32.0); [14.9 – 53.5]	0.40/ *0.07*	11 (25.0); [13.2 – 40.3]	8 (34.8); [16.4 – 57.3]	0.58/ *0.45*

‡, *P* values comparing baseline vs. post-IIV3 within groups

§, Unadjusted/*adjusted P* values comparing HIV+Preg vs. HIV+NP

§, Adjusted P values for age, number of women on ART and number of women with CD4 T-cell count >350 cells/mm^3

IIV3, trivalent inactivated influenza vaccine

SFU, spot forming units

GM, geometric mean with 95% confidence interval

No., number of

3.1.4 Trivalent Inactivated Influenza Vaccine-Specific Cell Mediated Immunity According to Baseline Responses

Similar to studies by Agrati *et al.* (309), He *et al.* (320), Co *et al.* (305) and Subbramanian *et al.* (304), we investigated if CMI responses to IIV3 were associated with baseline SFU/10^6 PBMC. Participants were stratified evenly according to their individual baseline SFU/10^6 PBMC into low-baseline (LB) and high-baseline (HB) responses (304). In situations where an uneven number of participants occur, the remainder participant was allocated to the high-baseline response group. In the HIV+Preg group for A/H1N1, A/H3N2 and B/Yamagata 27, 25 and 22 women were classified as LB and 28, 25 and 22 as HB, for each strain respectively. In the HIV+NP group the corresponding numbers were 23, 12 and 11 for LB and 23, 13 and 12 for HB, respectively.

Lower baseline GM SFU/10^6 PBMC were associated with significant increase post-IIV3 response in HIV+Preg and HIV+NP women for the three vaccine strains (p<0.05 for all comparisons, Figure 3.3A and 3.3B). Interestingly, higher baseline GM SFU/10^6 PBMC were associated with non-significant decreased responses post-IIV3 for all three vaccine strains in HIV+Preg women (Figure 3.3C and 3.3D).

Although statistically significant increases from baseline to post-IIV3 were observed in the LB sub-groups, LB participants did not reach the levels of GM SFU/10^6 PBMC observed in HB participants post-IIV3 (Table 3.7).

Receipt of IIV3 resulted in significantly higher GM SFU/10^6 PBMC fold-increases in the LB compared to the HB sub-group in HIV+Preg women for A/H1N1 (LB: 2.0 [95% CI: 1.4 – 2.8] vs. HB: 0.9 [95% CI: 0.7 – 1.2], p=<0.001), A/H3N2 (LB: 1.7 [95% CI: 1.2 – 2.4]

vs. HB: 0.9 [95% CI: 0.6 – 1.3], p=0.009) and B/Yamagata (LB: 1.7 [95% CI: 1.2 – 2.3] vs. HB: 0.7 [95% CI: 0.4 – 1.1], p=0.003) (Figure 3.4A). In HIV+NP women GM SFU/10^6 PBMC fold-increases were significantly higher in the LB compared to the HB sub-group for A/H1N1 (LB: 2.4 [95% CI: 1.7 – 3.4] vs. HB: 0.9 [95% CI: 0.7 – 1.3], p<0.001) and for A/H3N2 (LB: 2.4 [95% CI: 1.2 – 4.7] vs. HB: 0.9 [95% CI: 0.5 – 1.9], p=0.02), with a similar non-significant trend observed for B/Yamagata (Figure 3.4B).

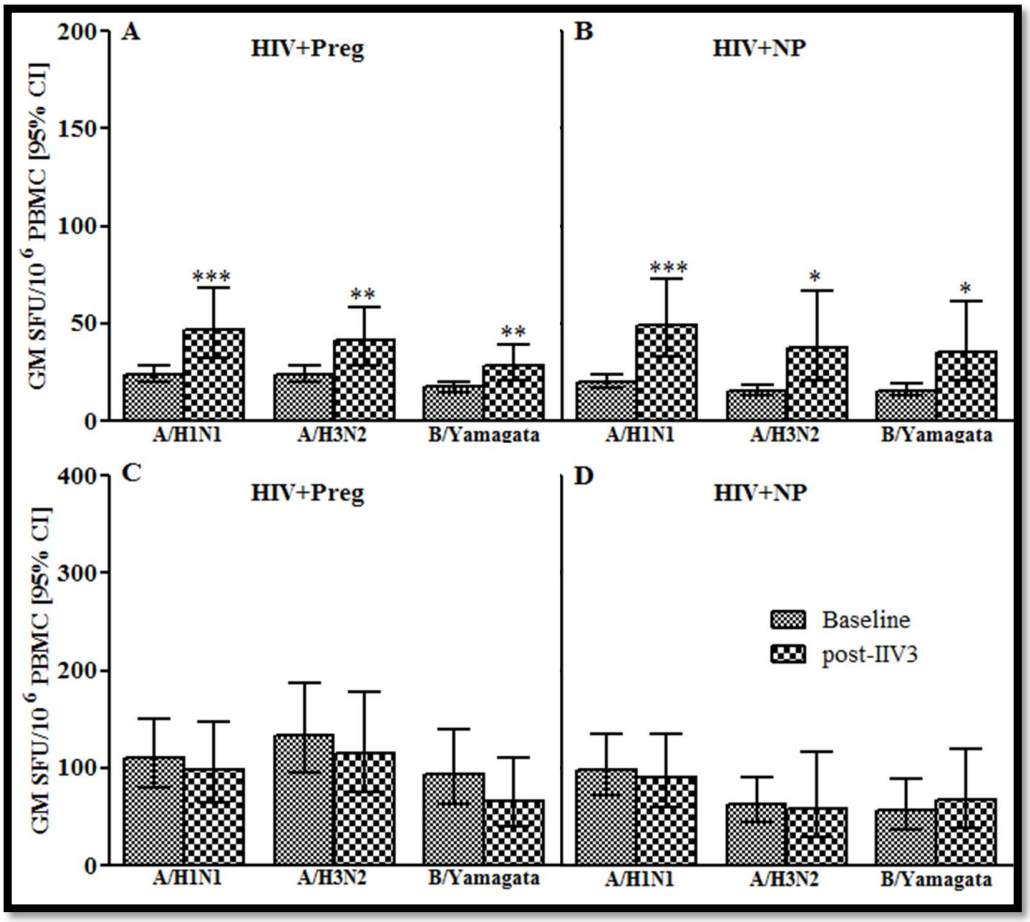


Figure 3.3: Cell-mediated immune responses post-IIV3 according to the numbers of IFN-γ secreting peripheral blood mononuclear cells at baseline. HIV+Preg (A and C) and HIV+NP (B and D) women were stratified evenly according to their individual baseline responses into low-baseline (LB) response (A and B) and high-baseline (HB) response (C and D). Statistical analysis performed using paired t-test. *p<0.05; **p<0.01; ***p<0.001.

Table 3.7: Cell-mediated immune responses to inactivated influenza vaccine in HIV-infected women stratified according to low- and high-baseline responses

		A/H1N1				A/H3N2				B/Yamagata			
Group		N	Baseline	Post-IIV3	*P* value§	N	Baseline	Post-IIV3	*P* value§	N	Baseline	Post-IIV3	*P* value§
HIV+Preg	LB	27	23.8 [20.1 – 28.2]	47.1 [32.6 – 68.1]	<0.001	25	24.0 [20.0 – 28.8]	41.1 [28.8 – 58.7]	0.003	22	17.4 [14.8 – 20.4]	28.7 [21.1 – 39.0]	0.007
	HB	28	110 [80.6 – 151]	98.1 [65.3 – 147]	0.44	25	134 [95.9 – 187]	116 [74.9 – 178]	0.45	22	94.4 [63.7 – 140]	66.1 [39.8 – 110]	0.14
P Value‡			<0.001	0.008			<0.001	<0.001			<0.001	0.006	
HIV+NP	LB	23	20.2 [17.2 – 23.8]	49.0 [33.0 – 72.7]	<0.001	12	15.5 [13.2 – 18.1]	37.3 [20.9 – 66.8]	0.01	11	15.8 [13.1 – 19.1]	35.7 [20.7 – 61.4]	0.02
	HB	23	98.6 [72.2 – 135]	90.0 [59.6 – 136]	0.58	13	63.5 [44.9 – 89.9]	58.6 [29.5 – 116]	0.80	12	57.2 [37.0 – 88.6]	67.6 [38.2 – 120]	0.49
P Value‡			<0.001	0.03			<0.001	0.29			<0.001	0.09	

All values are expressed as geometric mean SFU/ 10^6 PBMC with 95% confidence interval

§, *P* values comparing baseline vs. post-IIV3 within groups

‡, *P* values comparing LB vs. HB participants

LB, low-baseline response

HB, high-baseline response

N, number of participants within each group

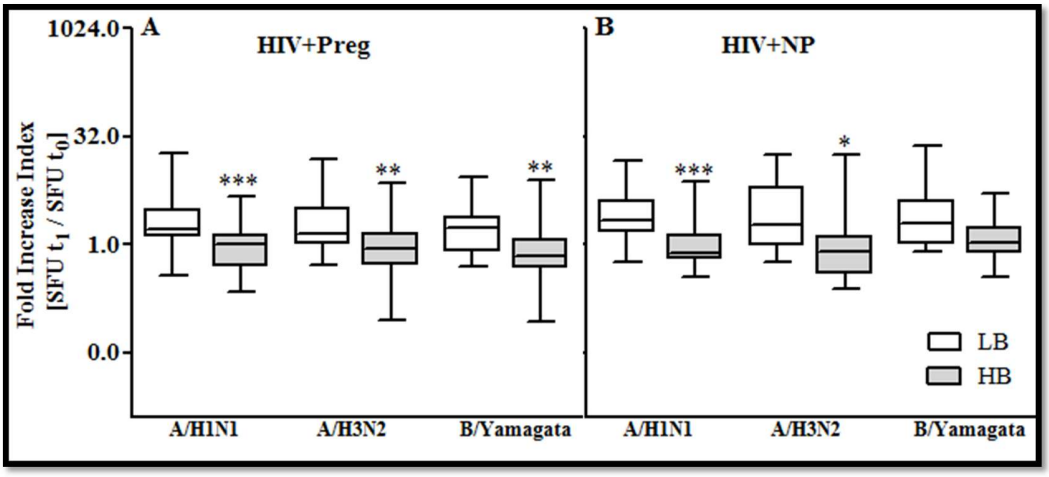


Figure 3.4: Geometric mean SFU/10^6 PBMC fold-increase from baseline (t0) to post-IIV3 (t1) for the vaccine strains A/H1N1, A/H3N2 and B/Yamagata stratified by low-baseline (LB) and high-baseline (HB) responses in HIV-infected pregnant (HIV+Preg) (A) and HIV-infected non-pregnant (HIV+NP) (B) women. Values are presented as a logarithmic (log2) scale of fold-increases and vertical bars represent minimum and maximum values. Statistical analysis was performed using the Mann-Whitney test. *p=0.02; **p<0.01; ***p<0.001.

3.2 Discussion

In this unique study we compared CMI responses to IIV3 using IFN-γ ELISPOT assay in HIV-infected pregnant and non-pregnant women in a low-income South African setting. While HIV infection immunosuppresses the host, pregnancy adds an extra pressure in the immune system that may increase the risk of infections in this already vulnerable population. During pregnancy the maternal immune system modulates itself for the prevention of foetal rejection, while similarly protecting both mother and foetus from various infections (321). The substantial immune alterations that occur during pregnancy involve (among other) changes in hormonal levels, leading to a shift from CMI toward humoral (antibody) immunity (91). These immune adaptations may contribute to increased

risk of complications associated with influenza infection, where CMI would play a critical role in viral clearance (92).

Observational studies have suggested that HIV-infection is associated with varying rates of adverse pregnancy outcomes including spontaneous abortions, still-births, perinatal and infant mortality and low birth weight (322-324). Due to easily accessible health and specialist care in developed countries, HIV is rarely associated with maternal mortality; however HIV infection complications are important causes of maternal mortality in developing countries (158). Typically around 25% of pregnancy-related deaths in the sub-Saharan Africa may be attributed to HIV (162); with reports from Southern Africa showing maternal mortality rates 5-fold higher in HIV-infected women compared to uninfected women (158). Given the immunosuppressive condition associated with HIV-infection (325) combined with immune attenuation during pregnancy (326), HIV-infected pregnant women are most likely at an even higher risk of influenza-related complications and infection compared to healthy, non-pregnant women.

In our study HIV-infected pregnant and HIV-infected non-pregnant women had similar CMI responses before vaccination for A/H1N1 and B/Yamagata. The GM SFU/10^6 PBMC was however higher for A/H3N2 among pregnant women (p=0.03). Post-IIV3 responses were slightly increased over baseline, yet in pregnant women only for A/H1N1 was there a significant increase in GM SFU/10^6 PBMC. These findings are surprising given the immunosuppressive condition associated with pregnancy: in a previous study assessing CMI responses to a pandemic monovalent A/H1N1 vaccine, Weinberg *et al.* reported a decrease in SFU/10^6 PBMC following vaccination among HIV-infected pregnant women

(327). The study did not include, however, an HIV-infected non-pregnant control group. In another study assessing interferon responses to the 2009 pandemic A/H1N1 influenza virus strain in HIV-uninfected unvaccinated women, pregnant women had reduced interferon producing PBMC compared to non-pregnant women (328). Although vaccination improved this response interferon production was still non-significantly reduced in pregnant women (328).

In our study we found that receipt of IIV3 increased the number of IFN-γ producing cells after stimulation with A/H1N1 in pregnant women and A/H1N1 and B/Yamagata in non-pregnant women. Although current data on CMI responses to influenza vaccination among HIV-infected individuals are inconclusive (306,307,329), several studies have shown increases among healthy, HIV-uninfected individuals following vaccination (123,301,320). Richardson *et al.*, reported non-significant increases in CMI responses to pooled influenza vaccine viruses among HIV-infected pregnant women 6 weeks post-vaccination using IFN-γ ELISPOT assay (173). A study assessing the cellular immune responses to a trivalent MF59-adjuvanted seasonal influenza vaccine in HIV-infected and HIV-uninfected adults also reported a non-significant increase one month post-vaccination in HIV-infected adults whereas a significant increase was seen for HIV-uninfected participants (330). We report an increase in CMI responses among HIV-infected non-pregnant women only for A/H1N1 and B/Yamagata post-IIV3. A possible reason for the discrepancy in results between our study and those reported by Fabbiani *et al.* may be due to the experimental techniques used: our study looked at IFN-γ secretions by means of the ELIPSOT assay, whereas Fabbiani *et al.* tested an array of individual cytokine

production following influenza-specific stimulation by means of a commercially available multiplex kit (330). Additionally, the authors demonstrate a significant association between lower CD4 T-cell counts and impaired Th1 response, which may be extrapolated to pregnancy, where Th1 responses are impaired and Th2 responses are increased in order to accommodate a foreign foetus (331).

In a prospective trivalent virosomal vaccine trial, researchers aimed to assess CMI responses to the vaccine using IFN-γ ELISPOT assay in HIV-infected and HIV-uninfected individuals (332). The authors showed that HIV-infected individuals with CD4+ T-cell counts <350/µl displayed impaired IFN-γ secretions compared to HIV-infected individuals with >350/µl one month post-vaccination. Significant increases in response were observed in both healthy controls as well as HIV-infected individuals with CD4+ T-cell counts >350/µl (but not in those with <350/µl). These results may partly explain those observed in our study with regard to the low number of women achieving significant response to vaccination. A study from Sweden also found no significant CMI response to influenza vaccination in an immunocompromised cohort (333). A total of 18 healthy controls and six stem cell transplantation (SCT) patients were immunised with IIV3 and CMI responses measured one month post-IIV3 using the IFN-γ ELISPOT assay. As expected, the healthy controls elicited significant increases in SFU/10^6 PBMC post-IIV3 but there was no increase in SFU/10^6 PBMC for SCT patients following receipt of vaccine to any of the influenza strains. Interestingly, a previous study assessing humoral immune responses to influenza vaccination in SCT patients showed poor antibody responses in those individuals with depleted T-cell counts (334), adding further evidence that low CD4+ T-cell counts

may play a role in decreased immune responses to influenza vaccination, not only cell-mediated but also humoral immunity.

Agrati *et al.* performed an analysis on presumably influenza virus-unexposed HIV-infected individuals and healthy controls and assessed CMI responses using the IFN-γ ELISPOT assay against the A/H1N1 and A/H3N2 virus strains (335). The authors describe a significant response to the vaccine strains as >40 SFU/10^6 PBMC post-vaccination, and showed that <37% of HIV-infected individuals achieved this threshold. No significant difference between healthy controls and HIV-infected individuals were reported for A/H3N2, however a significantly higher proportion of healthy controls (69% vs. 37%, p=0.002) achieved this threshold compared to HIV-infected individuals for A/H1N1. Furthermore, no significant difference in SFU/10^6 PBMC were reported among the two study groups for A/H3N2, but were significantly higher in healthy controls for A/H1N1 (p<0.001). Although the participants of the study were not vaccinated, the data suggest that HIV-infection has a detrimental effect on CMI responses to the pandemic A/H1N1 virus compared to a healthy population. The author's also assessed humoral immune responses using HAI assay against the same vaccine strains and, in line with previous observations (300,309,336,337), reported no significant correlation between CMI and humoral responses (335).

Contrary to the findings in this study where we report lower IFN-γ production in non-pregnant women compared to pregnant women, a recent evaluation of PBMC from pregnant and non-pregnant women, stimulated *ex vivo* with A/H1N1 and A/H3N2 virus, showed that pregnant women had lower IFN-γ production (measured by intracellular

cytokine staining) compared to the non-pregnant counterparts (339). A possible explanation for this difference may be due to different viruses, different study years as well as using a different assay for analyses. Interestingly, following influenza infection both NK and T-cells from pregnant women significantly increased. However vaccination did not significantly affect NK or T-cell cytokine and chemokine responses in pregnant and non-pregnant women (339).

Another study compared the induction of cytokines by ELISA, cytometric bead array, and mRNA levels between pregnant and non-pregnant women following stimulation of PBMC with A/H1N1 (328). The production of IFN-γ was significantly reduced in unvaccinated pregnant women compared to non-pregnant women, suggesting that pregnancy inhibits IFN-γ induction. Following vaccination however, pregnant women displayed normalised IFN-γ production as compared to their non-pregnant counterpart. These observations may add strength to our current findings, where vaccinating HIV-infected pregnant women showed increased CMI responses to all three vaccine strains.

It has been previously suggested that, when using ELISPOT assay results as a potential correlate of protection, fold-changes between baseline and post-vaccination would be more informative than to use specific ELISPOT values (196). Here we report similar fold-increases among HIV+Preg and HIV+NP women for all three vaccine strains, ranging from 1.1 – 1.3 and 1.5 – 1.6, respectively. The proportion of participants yielding a significant response to vaccination, defined by ≥2-fold increase in GM SFU/10^6 PBMC over baseline, was achieved by <27% and <35% of HIV+Preg and HIV+NP women, respectively, for all three vaccine strains. It should be noted that the 2-fold increase in

IFN-γ secreting cells used to compare CMI responses is an arbitrary threshold as no general consensus currently exists as to the nature and threshold of a significant CMI responses needed for immune protection (304).

Lastly, we explored the relationship between individual baseline GM SFU/10^6 PBMC and the response to vaccination (304,305,309,340). We discovered that pre-existing CMI to the vaccine strains significantly affected vaccine responses. Participants were evenly stratified (where possible) into LB and HB responses (304). Significant increases at post-IIV3 to all three vaccine strains were observed in both HIV+Preg and HIV+NP LB women (p<0.05 for all comparisons). Additionally, significantly greater fold-increases were achieved by LB women compared to HB women from baseline to post-IIV3 for at least two of the three vaccine strains.

Although significant increases were seen in LB, but not HB women post-IIV3, LB women were unable to achieve the levels of responses observed in HB participants for all three strains. As expected, HB women had significantly higher GM SFU/10^6 PBMC compared to LB women at baseline for all three vaccine strains (p<0.001). Among the HIV+Preg women, post-IIV3 responses were also significantly higher in HB women compared to LB women for all three vaccine strains (p=0.008). A similar trend was observed for HIV+NP women, where HB women achieved significantly higher post-IIV3 responses to A/H1N1 (p=0.03) and non-significantly higher responses to A/H3N2 and B/Yamagata than did LB women. He *et al.* conducted a study involving healthy children and adults and their cellular immune responses to either IIV3 or live attenuated influenza vaccine (LAIV) vaccination (340). The authors also stratified the participants into low and high baseline responses and

reported findings similar to those achieved in this study: a lower baseline response was directly and significantly related to immune responses post-vaccination (340). Since most adults have previous exposure to influenza infection or vaccination (320) they therefore have a certain amount of influenza-specific memory T-cells which could explain why those with higher baseline values did not significantly increase post-IIV3 and why those with lower baseline values displayed significant increases. Other potential reasons for the results seen in this study are brought to light in a study by Agrati *et al.* (309). The authors, who also stratified their HIV-infected participants according to low and high baseline responses found a significant increase in CMI responses following receipt of vaccine in the low baseline sub-group. Their explanation for this phenomenon included hyperstimulation-induced T-cell anargy, expansion/deletion of T-cell clones, and trafficking to mucosal surfaces (309). Previous observations have shown that stimulation of pre-activated T-cells with high doses of antigen can induce T-cell anargy or perhaps activation-induced cell death (336,341). Additionally, it has been shown that repeated exposure to the same influenza strain leads to limited boosting of CMI responses (342), which correlates well with the results we obtained in the HB group of this study.

4.0 Cell-Mediated Immune Responses to Trivalent Inactivated Influenza Vaccine in HIV-Uninfected Pregnant and Non-Pregnant Women

4.1 Results

4.1.1 Baseline Characteristics

In 2013 we enrolled 75 pregnant (HIV-Preg) and 75 non-pregnant (HIV-NP) women not infected with HIV; all participants received one single dose (15 μg per antigen) of trivalent inactivated influenza vaccine (IIV3). Enrolment of both the pregnant and non-pregnant cohorts started 16 September and was concluded 7 October. At enrolment HIV-Preg women (mean age 26.4 years [standard deviation (SD): 4.9]) were slightly older than HIV-NP women (mean age 24.0 years [SD: 5.0], p=0.05) and the median body mass index (BMI) was 29.9 and 27.8 (p=0.17) among HIV-Preg and HIV-NP women, respectively. The mean days between baseline and post-IIV3 visits were 28.6 days overall and this was similar among HIV-Preg and HIV-NP women. The mean gestational age at enrolment among pregnant women was 27.3 weeks (Table 4.1). Of the total 75 HIV-Preg women, five did not return for the second visit and did not complete the study, and of the 75 HIV-NP women, one did not complete the study.

The number of participants who qualified for enzyme-linked immunospot (ELISPOT) analysis was 63 in the HIV-Preg and 72 in the HIV-NP group (Table 4.5). A comparison of baseline characteristics between pregnant and non-pregnant women who qualified for ELISPOT analysis is available in Table 4.2 and in Tables 4.3 and 4.4 participants who qualified for ELISPOT analysis are compared to participants that did not qualify. No

significant differences in baseline characteristics were reported in either of the analyses, and only in the non-pregnant group the 3 women who did not qualify for ELISPOT analysis had a significant longer time between the two study visits (30.9 mean days [SD: 9.7]) compared to the women who qualified (28.4 mean days [SD: 1.3], p=0.035).

Table 4.1: Baseline characteristics of the HIV-uninfected pregnant and non-pregnant women enrolled into the study in 2013

	Overall	**HIV-Preg**	**HIV-NP**	***P* value**
Total no. women	150	75	75	
Mean days between visits‡§	28.6 ± 3.0	28.4 ± 1.7	28.8 ± 3.9	0.45
Ethnicity (%)				
Black	148 (98.7)	74 (98.7)	74 (98.7)	-
Other	2 (1.3)	1 (1.3)	1 (1.3)	-
Mean age - years	25.2 ± 5.1	26.4 ± 4.9	24.0 ± 5.0	0.05
Median BMI (IQR)	29.0 (26.0 – 34.2)	29.9 (26.7 – 34.6)	27.8 (25.0 – 32.8)	0.17
Mean gestational age - weeks	-	27.3 ± 4.1	-	-
No. Primigravid (%)	-	20 (26.7)	-	-
Median gravidity (IQR)	-	2.0 (1.0 – 3.0)	-	-
Median parity (IQR)	-	1.0 (0.0 – 1.0)	-	-

Plus-minus values are means ± standard deviation
‡, based on 70 HIV-Preg, 5 women did not complete visit 2
§, based on 74 HIV-NP, 1 woman did not complete visit 2
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
HIV, human immunodeficiency virus
No., number of

Table 4.2: Baseline characteristics of the HIV-uninfected pregnant and non-pregnant women analysed by ELISPOT assay

	Overall	HIV-Preg	HIV-NP	*P* value
Total no. women	135	63	72	-
Mean days between visits	28.4 ± 1.6	28.5 ± 1.8	28.4 ± 1.3	0.78
Ethnicity (%)				
Black	133 (98.5)	62 (98.4)	71 (98.6)	1.0
Other	2 (1.5)	1 (1.6)	1 (1.4)	1.0
Mean age - years	25.1 ± 5.3	26.1 ± 5.0	24.3 ± 5.4	0.05
Median BMI (IQR)	28.9 (26.0 – 33.2)	29.6 (23.1 – 28.5)	27.4 (24.9 – 32.4)	0.13
Mean gestational age - weeks		27.5 ± 4.3	-	-
No. Primigravid (%)	-	16 (25.4)	-	-
Median gravidity (IQR)	-	2.0 (1.0 – 3.0)	-	-
Median parity (IQR)	-	1.0 (0.0 – 1.0)	-	-

Plus-minus values are means ± standard deviation
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
HIV, human immunodeficiency virus
No., number of

Table 4.3: Baseline characteristics of the HIV-uninfected pregnant analysed vs. not analysed

	Overall	Analysed	Not Analysed	*P* value
Total no. women	75	63	12	-
Mean days between visits	28.4 ± 1.7	28.5 ± 1.8	28.1 ± 0.3	0.49
Ethnicity (%)				
Black	74 (98.7)	62 (98.4)	12 (100)	1.0
Other	1 (1.3)	1 (1.6)	0	
Mean age - years	26.4 ± 4.9	26.1 ± 5.0	28.1 ± 4.6	0.14
Median BMI (IQR)	29.9 (26.7 – 34.6)	29.6 (23.1 – 28.5)	31.7 (26.9 – 37.5)	0.52
Mean gestational age - weeks	27.3 ± 4.1	27.5 ± 4.3	27.2 ± 3.8	0.80
No. Primigravid (%)	20 (26.7)	16 (25.4)	5 (41.7)	0.30
Median gravidity (IQR)	2.0 (1.0 – 3.0)	2.0 (1.0 – 3.0)	2.0 (1.0 – 3.0)	0.95
Median parity (IQR)	1.0 (0.0 – 1.0)	1.0 (0.0 – 1.0)	0.5 (0.0 – 2.0)	0.90

Plus-minus values are means ± standard deviation
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
HIV, human immunodeficiency virus
No., number of

Table 4.4: Baseline characteristics of the HIV-uninfected non-pregnant analysed vs. not analysed

	Overall	Analysed	Not Analysed	*P* value
Total no. women	75	72	3	
Mean days between visits	28.8 ± 3.9	28.4 ± 1.3	30.9 ± 9.7	0.035
Ethnicity (%)				
Black	74 (98.7)	71 (98.6)	3 (100)	1.0
Other	1 (1.3)	1 (1.4)	0	1.0
Mean age - years	24.0 ± 5.0	24.3 ± 5.4	22.7 ± 2.9	0.33
Median BMI (IQR)	27.8 (25.0 – 32.8)	27.4 (24.9 – 32.4)	27.4 (24.9 – 32.4)	0.49

Plus-minus values are means ± standard deviation
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
HIV, human immunodeficiency virus
No., number of

4.1.2 Participants Selection

Participants without HIV-infection enrolled in the MatFlu study in 2013 (HIV-Preg, N=75; and HIV-NP, N=75) were immunised with a single dose of IIV3 to determine cell-mediated immune (CMI) responses one month post-vaccination (post-IIV3), assessed by detecting interferon-gamma (IFN-γ) production using enzyme linked immunospot (ELISPOT) assay. Collection of blood was conducted pre-vaccination (baseline) and at one month post-IIV3. For participants to qualify for EISPOT analyses they needed to have peripheral blood mononuclear cell (PBMC) viability ≥75% and have sufficient number of cells to perform duplicate assays (319) at both baseline and post-IIV3 visits. Table 4.5 shows the total numbers of participants in each study group that qualified for IFN-γ ELISPOT assessment for both study visits based on these criteria. Within these subpopulations, only experiments that fulfilled the following criteria at both baseline and post-IIV3 were included in the final analysis: i) test wells, prior to subtraction of background spot forming units (SFU) had to

exhibit SFU ≥2 times the background, ii) test wells had to have ≥10 SFU/10^6 PBMC following subtraction of background SFU and iii) in the background wells SFU needed to be <50 SFU/10^6 PBMC. Furthermore a significant response to vaccination was defined as ≥2-fold increase in SFU from baseline to post-IIV3.

Table 4.5: Number of participants eligible for IFN-γ ELISPOT analysis

			No. participants (%); [95% CI]		
Study Group	**Study Population**	**Viability ≥75%‡**	**A/H1N1§**	**A/H3N2 §**	**B/Yamagata§**
HIV-Preg	75	63 (84.0); [73.3 – 91.5]	43 (68.3); [55.3 – 79.4]	37 (58.7); [45.6 – 71.0]	25 (39.7); [27.6 – 52.8]
HIV-NP	75	72 (96.0); [88.8 – 99.2]	60 (83.3); [72.7 – 91.1]	52 (72.2); [60.4 – 82.1]	50 (69.4); [57.5 – 79.8]

‡ Participants with ≥75% viable live-cells prior to ELISPOT assay

§ Participants displaying test wells SFU ≥2 times background SFU, ≥10 SFUs/10^6 PBMC after background subtraction, and background wells with <50 SFU/10^6 PBMC per vaccine strain

4.1.3 Cell-Mediated Immune Responses

Baseline

Of the 75 HIV-Preg and the 75 HIV-NP women enrolled, 63 (84.0%) and 72 (96.0%, p=0.030), respectively, had cell viability ≥75% at both study visits and qualified for the analyses of CMI responses to IIV3 as measured by IFN-γ ELISPOT assay (Table 4.5). At baseline, HIV-NP women showed significantly higher geometric mean (GM) SFU/10^6 PBMC compared to HIV-Preg women for A/H1N1 (85.1 [95% confidence interval (CI): 65.0 – 111] vs. 44.8 [95% CI: 34.1 – 59.0], p=0.002) and A/H3N2 (61.2 [95% CI: 47.3 – 79.1] vs. 32.9 [95% CI: 24.4 – 44.3], p=0.002), and a similar trend was observed for B/Yamagata (Table 4.6 and Figure 4.1A).

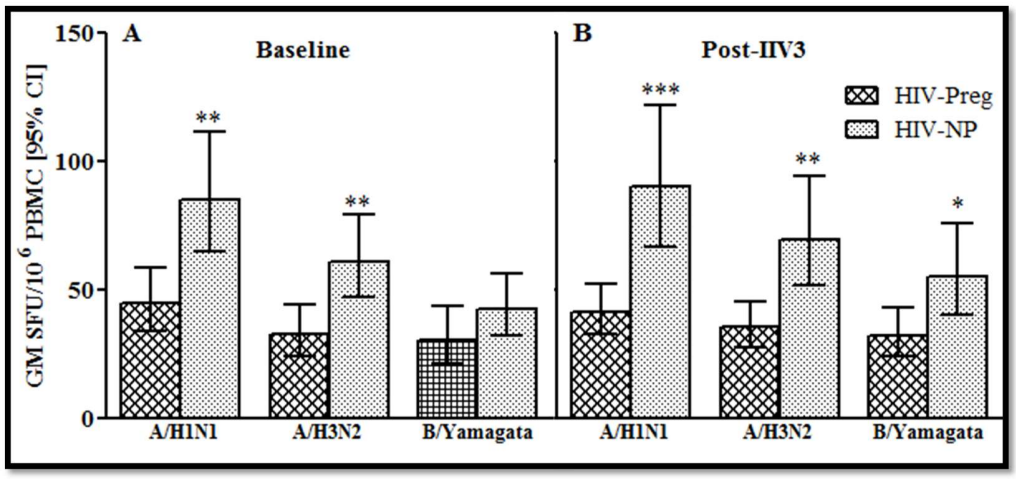


Figure 4.1: Cell-mediated immune responses to the different influenza vaccine strains in HIV-uninfected pregnant (HIV-Preg) and HIV-uninfected non-pregnant (HIV-NP) women at baseline (A) and post-vaccination (B). The number of A/H1N1, A/H3N2, and B/Yamagata specific IFN-γ producing peripheral blood mononuclear cells was evaluated at baseline and post-vaccination by ELISPOT assay. Statistical analysis was performed using unpaired t-test. *p=0.03; **p <0.01; ***p<0.001.

Post-vaccination

Receipt of IIV3 resulted, in general, in a very weak increase in CMI responses from baseline to post-IIV3 in both study groups. For HIV-Preg women there was, however, a slight non-significant decrease in GM SFU/10^6 PBMC from baseline to post-IIV3 for A/H1N1 (Table 4.6 and Figure 4.2). Post-IIV3 GM SFU/10^6 PBMC were significantly higher in HIV-NP compared to HIV-Preg women for A/H1N1 (90.3 [95% CI: 66.9 – 122] vs. 41.6 [95% CI: 32.9 – 52.5], p<0.001), A/H3N2 (69.8 [95% CI: 51.7 – 94.2] vs. 35.6 [95% CI: 27.9 – 45.3], p=0.001) and B/Yamagata: (55.4 [95% CI: 40.4 – 76.0] vs. 32.6 [95% CI 24.4 – 43.4], p=0.03) (Table 4.6 and Figure 4.1B). Similar GM fold-increases from baseline to post-IIV3 were observed in both study groups (Table 4.6).

Following administration of IIV3, 18.6%, 27.0% and 20.0% of HIV-Preg and 38.3%, 26.9% and 42.0% of HIV-NP women achieved a ≥2-fold increase from baseline for A/H1N1, A/H3N2 and B/Yamagata, respectively, and this was significantly higher in HIV-NP compared to HIV-Preg women for A/H1N1 (p=0.03) (Table 4.6).

4.1.4 Trivalent Inactivated Influenza Vaccine-Specific Cell Mediated Immunity According To Baseline Responses

Using the same criteria described previously for HIV-infected women (see: *3.1.4 IIV3-specific CMI according to baseline responses in HIV-infected women*), we investigated if CMI responses to IIV3 were associated with baseline SFU/10^6 PBMC. Participants were again stratified evenly (where possible) according to their individual baseline SFU/10^6 PBMC into low-baseline (LB) and high-baseline (HB) responses (304). For A/H1N1, A/H3N2 and B/Yamagata: 21, 18 and 12 were classified as LB and 22, 19 and 13 as HB in the HIV-Preg group, while 30, 26 and 25 were classified as LB and 30, 26 and 25 as HB in the HIV-NP group, respectively.

In both study groups, lower baseline CMI responses were associated with significant increases post-IIV3 for A/H1N1, A/H3N2 and B/Yamagata (p<0.05 for all comparisons, Figure 4.3A and Figure 4.3B, Table 4.7). Similar to what was observed in HIV-infected women, higher baseline GM SFU/10^6 PBMC was associated with significant decreases in CMI responses post-IIV3 for all three IIV3 vaccine strains in HIV-Preg (p<0.05 for all comparisons) and for A/H1N1 in HIV-NP women (197 [95% CI: 157 – 247] at baseline vs.

91.8 [95% CI: 58.1 – 145] at post-IIV3, p=0.006), with non-significant decreases observed for A/H3N2 and B/Yamagata in HIV-NP women (Figure 4.3C and Figure 4.3D, Table 4.7).

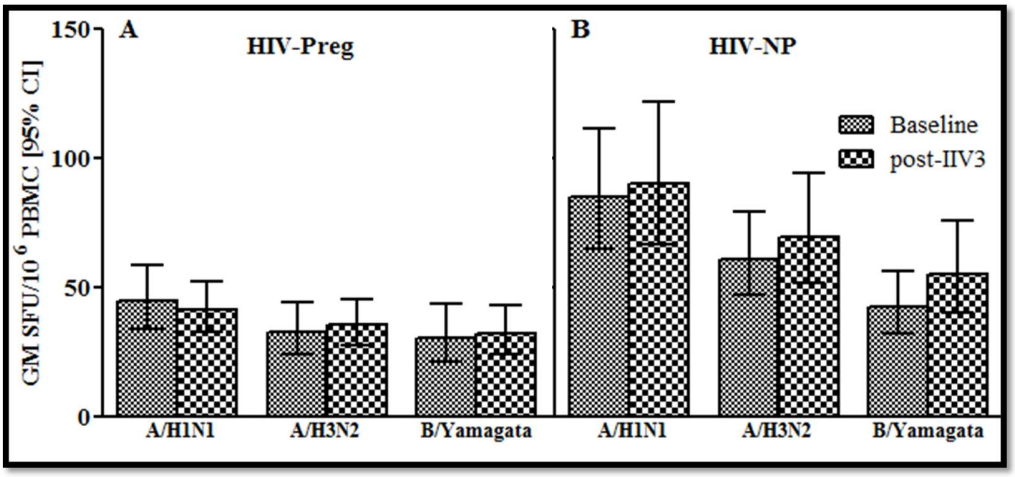


Figure 4.2: Cell-mediated immune responses to the different influenza vaccine strains at baseline and post-IIV3 in HIV-uninfected pregnant (HIV-Preg) (A) and HIV-uninfected non-pregnant (HIV-NP) (B) women measured by IFN-γ ELISPOT assay. Statistical analysis was performed using paired t-test.

As expected baseline GM SFU/10^6 PBMC were significantly higher in the HB compared to LB group for all three IIV3 vaccine strains in both HIV-Preg and HIV-NP women (p<0.001 for all comparisons), and higher GM SFU/10^6 PBMC were observed in HB compared to LB for both groups at post-IIV3 for all three strains, but none reached statistical significance (Table 4.7).

Table 4.6: Cell-mediated immune responses to inactivated influenza vaccine in HIV-uninfected women

Measure	**A/H1N1**			**A/H3N2**			**B/Yamagata**		
	HIV-Preg	HIV-NP	*P* Value§	HIV-Preg	HIV-NP	*P* Value§	HIV-Preg	HIV-NP	*P* Value§
No. participants	43	60		37	52		25	50	
GM SFU/10^6 PBMC [95% CI]									
Baseline	44.8 [34.1 – 59.0]	85.1 [65.0 – 111]	0.002	32.9 [24.4 – 44.3]	61.2 [47.3 – 79.1]	0.002	30.6 [21.6 – 43.6]	42.6 [32.2 – 56.4]	0.16
Post-IIV3	41.6 [32.9 – 52.5]	90.3 [66.9 – 122]	<0.001	35.6 [27.9 – 45.3]	69.8 [51.7 – 94.2]	0.001	32.6 [24.4 – 43.4]	55.4 [40.4 – 76.0]	0.032
P value‡	0.57	0.77		0.64	0.50		0.71	0.13	
GM factor increase [95% CI]	0.9 [0.7 – 1.2]	1.1 [0.7 – 1.6]	0.38	1.1 [0.8 – 1.5]	1.1 [0.8 – 1.7]	0.92	1.1 [0.8 – 1.5]	1.3 [0.9 – 1.8]	0.15
No. ≥2 fold increase post-IIV3 (%); [95% CI]	8 (18.6); [8.4 – 33.4]	23 (38.3); [26.1 – 51.8]	0.031	10 (27.0); [13.8 – 44.1]	14 (26.9); [15.6 – 41.0]	1.00	5 (20.0); [6.8 – 40.7]	21 (42.0); [28.2 – 56.8]	0.07

‡, *P* values comparing baseline vs. post-IIV3 within groups
§, *P* values comparing HIV-Preg vs. HIV-NP
IIV3, trivalent inactivated influenza vaccine
SFU, spot forming units
GM, geometric mean with 95% confidence interval
No., number of

Following administration of IIV3, significantly higher GM SFU/10^6 PBMC fold-increases in LB compared to HB were observed in HIV-Preg for A/H1N1 (LB: 1.6 [95% CI: 1.2 – 2.1] vs. HB: 0.6 [95% CI: 0.4 – 0.8], p<0.001), A/H3N2 (LB: 2.0 [95% CI: 1.4 – 2.9] vs. HB: 0.6 [95% CI: 0.4 – 0.9], p<0.001) and B/Yamagata (LB: 1.8 [95% CI: 1.2 – 2.7] vs. HB: 0.6 [95% CI: 0.4 – 0.9], p<0.001) (Figure 4.4A). Similarly in HIV-NP women LB participants had significantly higher GM SFU/10^6 PBMC fold-increases compared to HB participants for A/H1N1 (LB: 2.4 [95% CI: 1.5 – 3.8] vs. HB: 0.5 [95% CI: 0.3 – 0.8], p<0.001), A/H3N2 (LB: 2.1 [95% CI: 1.2 – 3.5] vs. HB: 0.6 [95% CI: 0.4 – 0.9], p=0.003) and B/Yamagata (LB: 2.2 [95% CI: 1.6 – 3.0] vs. HB: 0.8 [95% CI: 0.5 – 1.4], p=0.007) (Figure 4.4B).

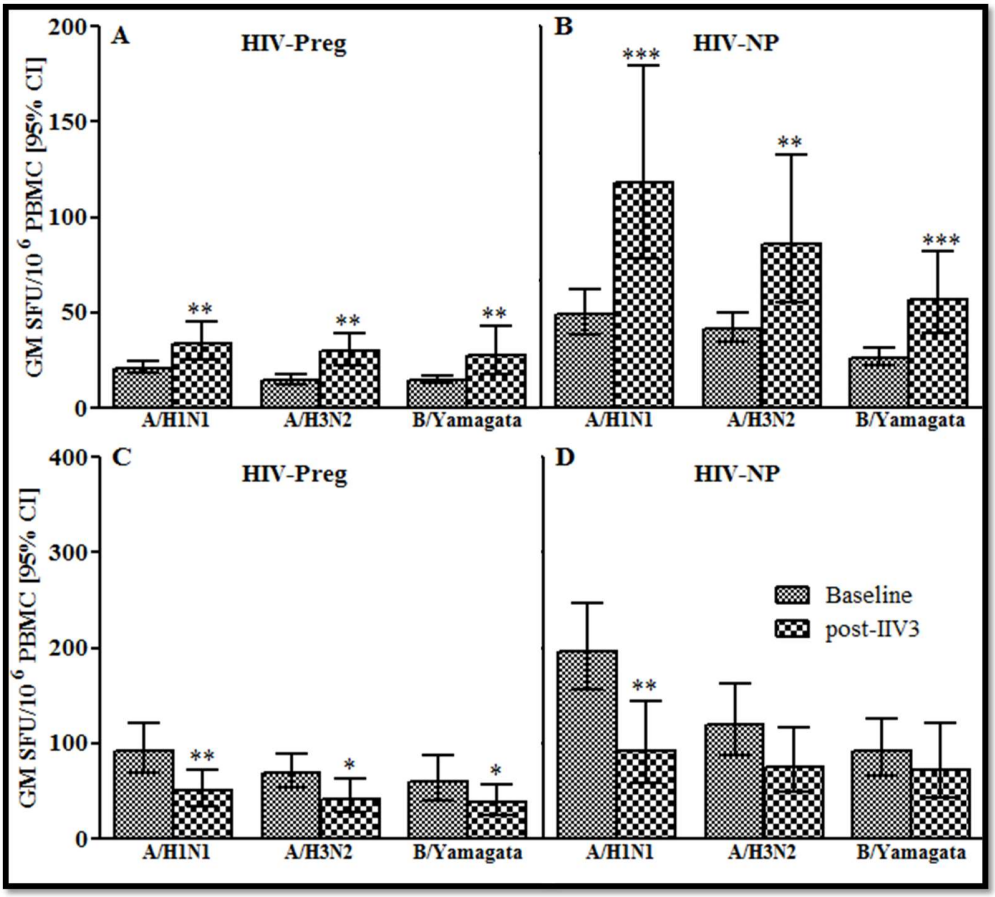


Figure 4.3: Cell-mediated immune responses post-IIV3 according to the numbers of IFN-γ secreting peripheral blood mononuclear cells at baseline. HIV-Preg (A and C) and HIV-NP (B and D) were stratified evenly according to their individual baseline responses into low-baseline (LB) response (A and B) and high-baseline (HB) response (C and D). Statistical analysis performed using paired t-test. *p<0.05; **p<0.005; ***p<0.001.

Table 4.7: Cell-mediated immune responses to inactivated influenza vaccine in HIV-uninfected women stratified according to low- and high-baseline responses

		A/H1N1				A/H3N2				B/Yamagata			
Group		N	Baseline	Post-IIV3	*P* value§	N	Baseline	Post-IIV3	*P* value§	N	Baseline	Post-IIV3	*P* value§
HIV-Preg	LB	21	21.1 [18.3 – 24.3]	34.2 [25.6 – 45.6]	0.001	18	15.0 [12.8 – 17.7]	29.8 [22.6 – 39.4]	0.002	12	14.9 [12.9 – 17.2]	27.4 [17.5 – 42.9]	0.007
	HB	22	92.0 [69.7 – 121]	50.2 [34.7 – 72.6]	0.001	19	69.0 [53.2 – 89.5]	42.0 [28.2 – 62.7]	0.021	13	59.5 [40.3 – 87.8]	38.1 [25.2 – 57.7]	0.024
P Value‡			<0.001	0.10			<0.001	0.15			<0.001	0.25	
HIV-NP	LB	30	36.8 [29.1 – 46.6]	88.8 [58.5 – 135]	<0.001	26	31.3 [26.0 – 37.6]	64.5 [41.7 – 99.8]	0.010	25	19.8 [16.7 – 23.5]	42.6 [29.5 – 61.4]	<0.001
	HB	30	197 [157 – 247]	91.8 [58.1 – 145]	0.006	26	120 [87.8 – 164]	75.5 [48.7 – 117]	0.06	25	91.6 [66.9 – 126]	72.1 [42.9 – 121]	0.39
P Value‡			<0.001	0.91			<0.001	0.60			<0.001	0.09	

All values expressed as geometric mean SFU/ 10^6 PBMC with 95% confidence interval
§, *P* values comparing baseline vs. post-IIV3 within groups
‡, *P* values comparing LB vs. HB participants
LB, low-baseline response
HB, high-baseline response
N, number of participants within each group

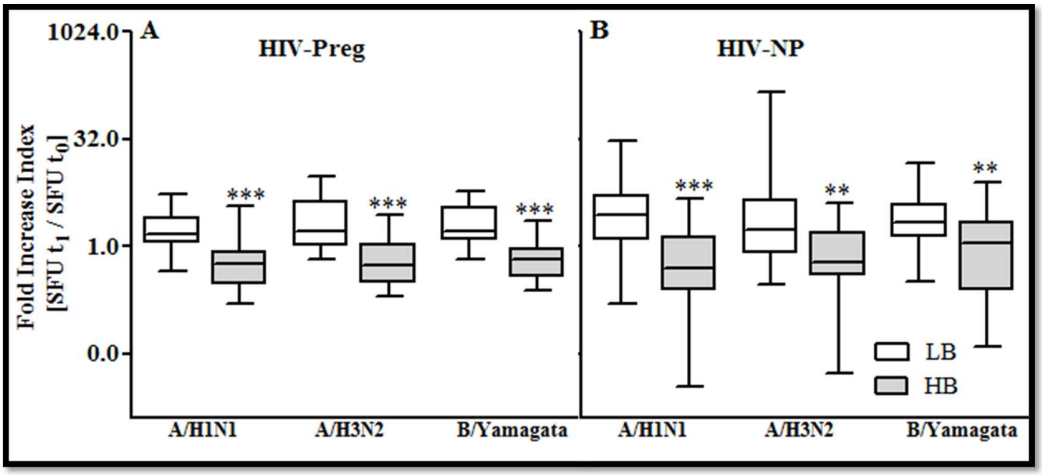


Figure 4.4: Geometric mean SFU/10^6 PBMC fold-increase from baseline (t0) to post-IIV3 (t1) for the vaccine strains A/H1N1, A/H3N2 and B/Yamagata stratified by low-baseline (LB) and high-baseline (HB) responses in HIV-uninfected pregnant (HIV-Preg) (A) and HIV-uninfected non-pregnant (HIV-NP) (B) women. Values are presented as a logarithmic (log2) scale of fold-increases and vertical bars represent minimum and maximum values. Statistical analysis was performed using the Mann-Whitney test. ** $p<0.01$; *** $p<0.001$.

4.2 Discussion

In this chapter we compared the CMI responses to IIV3 in pregnant and non-pregnant women without HIV-infection in a low-income African setting. Pregnant women are at high risk for complications due to influenza virus infection (343), attributed to physiological and immunological changes that occur during pregnancy (170). With the progression of pregnancy, hormonal levels change and there is a shift from CMI toward humoral immunity (344). Given the attenuation of CMI during pregnancy the majority of the studies evaluating immune responses to influenza vaccination have been focused on antibody production (345-347); with only a few immunogenicity studies assessing CMI responses and reporting variable results (173,300,328,333,348).

Currently the inactivated influenza vaccine is recommended for pregnant women (349), which has shown to elicit poor CMI responses compared to the live attenuated influenza vaccine (LAIV) in children (182). In healthy adults however, Subbramanian *et al.*, reported that inactivated influenza vaccine (IIV) elicited good CMI responses post-vaccination, albeit to a lesser degree than LAIV (304). Additionally, the authors reported that IIV elicited significant hemagglutination inhibition (HAI) geometric mean titers (GMT) increases measured post-vaccination, whereas LAIV did not, concluding that antibody and CMI responses are likely independent parameters in the host immune responses against influenza (304). In this study we aimed to measure cell-mediated immune (CMI) responses to the 2013 Southern Hemisphere IIV3 at baseline and one month following vaccination by means of the IFN-γ ELISPOT assay in HIV-Preg women and compare these responses with HIV-NP women. We found that IIV3 elicited variable CMI responses in HIV-Preg and HIV-NP women to the three influenza vaccine strains accordingly to their pre-vaccination responses.

Pregnant women had significantly lower CMI responses to A/H1N1 and A/H3N2 at baseline and to A/H1N1, A/H3N2 and B/Yamagata post-IIV3 compared to HIV-NP women. These observations are consistent with a report by Vanders *et al.*, who explored IFN-γ secretion from PBMC isolated from unvaccinated pregnant and non-pregnant women when stimulated with the A/H1N1 influenza virus. In that study the analysis was done using flow cytometry and the authors reported a decreased response to A/H1N1 in pregnant compared to non-pregnant women (p<0.04) (348). Similarly, Forbes *et al.* reported significantly less IFN-γ secretion from PBMC isolated from unvaccinated pregnant women

compared to unvaccinated non-pregnant women following stimulation with A/H1N1 influenza virus (p<0.01). This impaired response was, however, improved in pregnant women by influenza vaccination, yet still showed a reduced IFN-γ response compared to non-pregnant vaccinated women (328). Although these studies support our findings, it is important to note that in our study both study groups were enrolled and vaccinated following the influenza season (September – October) and therefore may have been exposed to the circulating influenza strains before vaccination. This pre-exposure to influenza strains explanation was also suggested in the study by Nayak *et al.*, whose participants were enrolled prior to the onset of influenza season and reported a significant CMI response to the monovalent A/H1N1 vaccine (p=0.002) (350). This, together with the pregnancy-associated attenuation of CMI and general immunosuppression associated with pregnancy (344), might explain the disproportionately higher responses in HIV-NP, but poor responses by HIV-Preg women at both baseline and post-IIV3.

Fold-increases in CMI responses from baseline to post-IIV3 were similar for all three strains in both study groups. While less than 28% of HIV-Preg women achieved ≥2-fold increase over baseline for at least one strain, 38% and 42% of HIV-NP women achieved a ≥2-fold increase for A/H1N1 and B/Yamagata, respectively. Nevertheless, it should be emphasized that the 2-fold increase in IFN-γ secreting cells used to compare CMI responses is an arbitrary threshold as no general consensus currently exists as to the nature and threshold of a significant CMI responses needed for immune protection (304). Studies by Iorio *et al.* and Avetisyan *et al.* have shown that 59% and 72%, respectively, of the healthy participants in their studies achieved a ≥2-fold increase in CMI responses post-IIV3

(300,333). A possible reason for the lower proportion of HIV-NP women achieving a ≥2-fold increase in our study may be, as mentioned, the time of enrolment post-influenza season, which would have exposed participants to circulating influenza virus strains and as a result, IFN-γ responses was already maximal (328). The proportions we observe in HIV-Preg women can possibly be attributed to the same reason and to the shift from CMI to humoral immunity during pregnancy.

Although HIV-NP women had slightly improved responses to all three strains compared to HIV-Preg women, they were also unable to reach significant increases post-IIV3. These findings correlate with the results from Richardson *et al.*, who explored IFN-γ secreting PBMC from HIV-uninfected pregnant women following administration of IIV3 and reported no significantly increased response 6 weeks post-vaccination (173). Co *et al.* also reported an overall moderate non-significant increase in the number of IFN-γ producing cells post-IIV3 for both A/H1N1 and A/H3N2 strains in healthy adults (305). Importantly, although HIV-Preg participants' CMI responses to IIV3 were poor compared to HIV-NP women, and that no significant increase occurred from baseline to post-IIV3, this does not necessarily equate to a lack of protection. Numerous studies on the immunogenicity of the influenza vaccine have been conducted measuring humoral responses and have shown significant protection following receipt of vaccine (100,103,104,347,351,352)

Finally, we explored the possible relationship between baseline GM SFU/10^6 PBMC and post-IIV3 responses. Although both groups had no significant increases in CMI responses following vaccination, subsets of participants demonstrated distinct responses. We found that pre-existing CMI to the vaccine strains affected the ability of vaccination to induce

significant increases post-IIV3. When participants were stratified into LB and HB responses (304), both HIV-Preg and HIV-NP LB women achieved significant increases post-IIV3 ($p<0.05$ for all comparisons), as well as displaying significantly greater fold-increases compared to HB women from baseline to post-IIV3 for all three vaccine strains. Previous studies involving influenza vaccination in healthy adults and children have found similar results (304,305,309,320). Agrati *et al.* stratified participants into low responders (<60 SFU/10^6 PBMC) and high responders (>60 SFU/10^6 PBMC) based on individual baseline CMI responses. By using an "Efficacy Index" (SFU at baseline/SFU at post-IIV3), the authors found a non-significant association between lower baseline and higher post-vaccination responses in health care workers (309). Another example of this association was reported by He *et al.*, where, after receipt of the LAIV, 58% of adult participants had significantly increased CMI responses, whereas 42% had decreased CMI responses. Participants in the first group had significantly lower baseline responses compared to the second group ($p<0.05$), strengthening the possibility that a lower baseline CMI response is associated with a higher post-vaccination CMI response (320). A potential explanation for this trend reported in our study and others might be that in individuals with low baseline-high post-vaccination responses, IFN-γ producing T-cells may be responding to the HA and the NA proteins contained in the inactivated vaccine (305). The reason behind this possible explanation comes from studies reporting having found a considerable amount of nucleoprotein and M1 protein in inactivated influenza vaccines, which may be additional targets for CD8+ T-cell responses (302,353,354). Additionally, the reason for low responses post-vaccination in individuals with high baseline responses may be due to the presence of CD8+ and CD4+ influenza specific T-cells, which may rapidly eliminate

antigen presenting cells containing vaccine antigens, thereby limiting subsequent activation of T-cells upon re-exposure (305). Other mechanisms have been suggested, including hyper-stimulation-induced T-cell anargy, expansion/deletion of T-cell clones and trafficking to mucosal surfaces (309).

5.0 Humoral and Cell Mediated Immune Responses to Trivalent Inactivated Influenza Vaccine in HIV/TB Infected Individuals

5.1 Results

5.1.1 Baseline Characteristics

At enrolment HIV/TB-uninfected (HIV-TB-) study participants were younger (25.6 years standard deviation [SD]: 6.6) than the other participants and had higher median baseline body-mass index (BMI) compared to HIV-uninfected/TB-infected (HIV-TB+) and HIV/TB-co-infected (HIV+TB+), but lower than the HIV-infected/TB-uninfected (HIV+TB-) participants. Among the HIV-infected participants those infected with TB (HIV+TB+) were younger, had a lower BMI, lower CD4+ T-cell count and a lower percentage were on antiretroviral therapy compared to HIV+TB- (Table 5.1).

The baseline characteristics of the participants that underwent hemagglutination inhibition (HAI) assays are shown in Table 5.2. Only participants who had pair blood samples pre-vaccination and at one month post-vaccination available for HAI assay were included in the analysis. At enrolment HIV-TB- participant were younger (26.3 years [SD: 6.8]) than HIV+TB+ and HIV-TB+ participants and had greater median BMI compared to HIV-TB+ participants. Among the HIV-infected participants, those infected with TB were significantly younger (37.0 [SD: 8.5]), had a greater BMI and higher median CD4+ T-cell count than those without TB-infection (Table 5.2).

Baseline characteristics of the TB-uninfected participants who fulfilled the criteria to be included in the cell-mediated immune (CMI) response experiments are shown in Table 5.3.

HIV-infected participants (HIV+TB-) were significantly older (41.2 [SD: 7.7] vs. 26.1 [SD: 7.0], $p<0.001$) than HIV-infected (HIV+TB-) participants.

5.1.2 Participants Selection

All the participants enrolled in the IIV3_HIV_TB study in 2014, including 80 HIV+TB+, 80 HIV+TB-, 61 HIV-TB+ and 80 HIV-TB- adults, were immunised with a single dose (15 μg per antigen) of trivalent inactivated influenza vaccine (IIV3) to determine humoral and CMI responses one month post vaccination (post-IIV3). Humoral responses were assessed by HAI assay in all study groups, and CMI responses were measured by detecting interferon-gamma (IFN-γ) production using enzyme-linked immunospot (ELISPOT) assay in the HIV+TB- and HIV-TB- participants. Collection of blood was conducted on the day of vaccination, just prior to IIV3 administration (baseline) and at one month post-IIV3. For participants to qualify for EISPOT analyses they needed to have peripheral blood mononuclear cell (PBMC) viability ≥75% and have sufficient number of cells to perform duplicate assays (319) at both baseline and post-vaccination visits. In table 5.4 the total numbers of participants in each study group that qualified for IFN-γ ELISPOT assessment for both study visits based on these criteria are shown. Within this subpopulation, only experiments that fulfilled the following criteria per vaccine strain at both baseline and post-IIV3 visits were included in the final analysis: i) test wells, prior to subtraction of background spot forming units (SFU) had to exhibit SFU ≥2 times the background, ii) test wells had to have ≥10 SFU/10^6 PBMC following subtraction of background SFU and iii) in the background wells SFU needed to be <50 SFU/10^6 PBMC. Furthermore a significant response to vaccination was defined as ≥2-fold increase in SFU from baseline to post-IIV3.

Table 5.1: Baseline characteristics of HIV/TB study participants in 2014

	Overall	HIV+TB+	HIV+TB-	*P* value	HIV-TB+	HIV-TB-	*P* value
Total no. participants	301	80	80	-	61	80	-
Mean days between visits¶	30.2 ± 4.8	28.8 ± 1.8	31.2 ± 6.1	0.002	29.8 ± 4.8	30.9 ± 5.0	0.21
Ethnicity (%)							
Black	299 (99.3)	80 (100)	80 (100)	1.00	60 (98.4)	79 (98.8)	1.00
Other	2 (0.7)	0 (0)	0 (0)		1 (0.6)	1 (0.2)	
Mean age - years	34.0 ± 9.9	36.8 ± 8.4	40.8 ± 7.4	0.001	32.1 ± 10	25.6 ± 6.6	<0.001
Median BMI (IQR)‡	23.5 (21.0 – 27.2)	22.9 (20.8 – 26.2)	24.9 (22.3 – 31.1)	0.002	21.2 (19.5 – 24.7)	23.9 (21.5 – 30.3)	0.003
Median CD4+ T-cell Count cells/mm^3(IQR)§	-	250 (158 – 404)	513 (341 – 659)	<0.001	-	-	-
No. on ART (%)	-	36 (45.0)	64 (80.0)	<0.001	-	-	-
No. on TB treatment (%)	-	70 (87.5)	-	-	58 (95.1)	-	-

Plus minus values are means ± standard deviation

¶, 5 HIV-TB-, 1 HIV+TB-, 6 HIV-TB+ and 5 HIV+TB+ participants did not complete visit 2

‡, 3 HIV+TB+ participants did not have BMI data

§, 1 HIV+TB- participant did not have CD4+ data

IIV3, trivalent inactivated influenza vaccine

BMI, body mass index (weight in kilograms divided by the square of height in meters)

IQR, interquartile range

HIV, human immunodeficiency virus

TB, tuberculosis

ART, antiretroviral therapy compared

No., number of

Table 5.2: Baseline characteristics of HIV/TB study participants in 2014 analysed by HAI

	Overall	HIV+TB+	HIV+TB-	*P* value	HIV-TB+	HIV-TB-	*P* value
Total no. participants	263	74	71	-	51	67	-
Mean days between visits¶	29.3 ± 2.2	28.8 ± 1.9	30.0 ± 2.4	<0.001	28.7 ± 1.7	29.5 ± 2.3	0.037
Ethnicity (%)							
Black	261 (99.2)	74 (100)	71 (100)	1.00	50 (98.0)	66 (98.5)	1.00
Other	2 (0.8)	0	0	-	1 (2.0)	1 (1.5)	1.00
Mean age - years	34.2 ± 9.9	37.0 ± 8.5	40.7 ± 7.5	0.006	31.8 ± 10.3	26.3 ± 6.8	<0.001
Median BMI (IQR)‡	23.6 (21.1 – 27.7)	23.1 (20.8 – 26.2)‡	24.9 (22.4 – 31.2)	0.001	21.5 (19.5 – 24.6)	24.0 (21.8 – 30.6)	<0.001
Median CD4+ T-cell Count cells/mm^3(IQR)§	-	262 (158 – 408)	510 (331 – 655)§	<0.001	-	-	-
No. on ART (%)	-	32 (43.3)	56 (78.9)	<0.001	-	-	-
No. on TB treatment (%)	-	73 (98.6)	-	-	50 (98.0)	-	-

Plus minus values are means ± standard deviation
‡, 1 participants did not have BMI data
§, 1 participant did not have CD4+ data
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
HIV, human immunodeficiency virus
TB, tuberculosis
ART, antiretroviral therapy compared
No., number of

Table 5.3: Baseline characteristics of study participants enrolled in CMI sub-study

	Overall	HIV+TB-	HIV-TB-	*P* value
Total no. participants	116	56	60	-
Mean days between visits	30.9 ± 4.8	30.9 ± 5.5	30.9 ± 4.2	0.98
Ethnicity (%)				
Black	115 (99.1)	56 (100)	59 (98.3)	1.00
Other	1 (0.9)	0 (0)	1 (1.6)	-
Mean age - years	33.4 ± 11.0	41.2 ± 7.7	26.1 ± 7.0	<0.001
Median BMI (IQR)	24.0 (21.5 – 30.7)	23.8 (21.6 – 30.3)	24.3 (21.5 – 31.0)	0.95
Median (IQR) CD4+ T-cell count cells/mm^3	-	516 (372 – 656)	-	-
No. on ART (%)	-	46 (82.1)	-	-

Plus-minus values are means ± standard deviation
IIV3, trivalent inactivated influenza vaccine
BMI, body mass index (weight in kilograms divided by the square of height in meters)
IQR, interquartile range
HIV, human immunodeficiency virus
TB, tuberculosis
ART, antiretroviral therapy compared
No., number of

Table 5.4: Number of participants eligible for IFN-γ ELISPOT analysis

			No. participants (%); [95% CI]		
Study Group	**Study Population**	**Viability ≥75%‡**	**A/H1N1§**	**A/H3N2 §**	**B/Yamagata§**
HIV+TB-	80	56 (70.0); [58.7 – 79.7]	35 (43.8); [32.7 – 55.3]	33 (41.3); [30.4 – 52.8]	33 (41.3); [30.4 – 52.8]
HIV-TB-	80	60 (75.0); [64.1 – 84.0]	29 (36.3); [25.8 – 478.8]	17 (21.3); [12.9 – 31.8]	27 (33.8); [23.6 – 45.2]

‡ Participants with ≥75% viable live-cells prior to ELISPOT assay
§ Participants displaying test wells SFU ≥2 times background SFU, ≥10 SFU/10^6 PBMC after background subtraction, and background wells with <50 SFU/10^6 PBMC per vaccine strain

5.1.3 Humoral Immune Responses to Trivalent Inactivated Influenza Vaccine in HIV/TB Co-Infected and HIV-Infected TB-Uninfected Participants

Baseline

Humoral responses to IIV3 were assessed in 74 HIV+TB+ and 71 HIV+TB- participants using HAI assay. Baseline geometric mean titers (GMT) were similar among HIV+TB+ and HIV+TB- participants for A/H1N1 and A/H3N2, while HIV+TB+ participants had

significantly higher baseline GMT compared to HIV+TB- for B/Yamagata (18.6 [95% CI: 16.1 – 21.6] vs. 14.2 [95% CI: 12.7 – 16.0], p=0.008) (Table 5.5). At baseline the percentage of participants with sero-protective titers (≥1:40) ranged from 8.5% to 27.0% in the HIV+TB- group and from 16.2% to 27.0% in the HIV+TB+ group. This percentage was similar in the HIV+TB+ and HIV+TB- group for A/H1N1 (16.2% vs. 21.1%) and A/H3N2 (27.0% vs. 19.7%), but significantly higher in HIV+TB+ participants for B/Yamagata (27.0% vs. 8.5%, p=0.005, Table 5.5). Following multi-variant adjusted analysis for the baseline characteristics (age, BMI, CD4+ T-cell counts and number of participants on ART), we found no change in significance for any of the vaccine strains.

Post-vaccination

Post-IIV3 significant increases in GMTs from baseline were noted in both study groups for all strains (p<0.01 for all comparisons, Table 5.5). HIV+TB+ and HIV+TB- participants achieved similar GMTs post-IIV3 for A/H1N1 and A/H3N2; for B/Yamagata higher titers were achieved in HIV+TB+ compared to HIV+TB- participants (29.8 [95% CI: 25.0 - 35.5] vs. 19.8 [95% CI: 16.3 – 23.9], p<0.001). The geometric mean fold-increase ranged from 1.2 for B/Yamagata to 4.5 for A/H1N1 and was similar between study groups except for A/H3N2 with HIV+TB+ participants having a lower fold-increase compared to HIV+TB- (2.7 vs. 4.4, p=0.008); however, after adjustment for baseline characteristics (age, BMI, CD4+ T-cell counts and number of participants on ART) the association was no longer significant (adjusted p-value=0.26).

A significant increase in the proportion of participants with sero-protective titers from baseline to post-IIV3 was observed in both study groups (p<0.005 for all comparisons),

with less than 79% of participants achieving sero-protective titers for at least one strain. A higher percentage of HIV+TB+ participants compared to HIV+TB- had sero-protective titers for B/Yamagata (55.4% vs. 28.2%, p=0.001) and a trend in the opposite direction was detected for A/H3N2 (59.5% vs. 78.9%, respectively, p=0.05); following adjustment for baseline characteristics (age, BMI, CD4+ T-cell counts and number of participants on ART), the percentage of HIV+TB- participants that had sero-protective titers post-vaccination became significantly higher compared to HIV+TB+ participants for A/H3N2 (adjusted p-value=0.012).

Sero-conversion rates to at least one strain were observed in less than 62% of the participants. No significant differences in the proportion of HIV+TB+ and HIV+TB- participants that sero-converted was detected for A/H1N1 (60.8% vs. 62.0%) and B/Yamagata (13.5% vs. 18.3%), whereas for A/H3N2, a significantly higher proportion of HIV+TB- participants sero-converted post-IIV3 compared to HIV+TB+ participants (35.1% vs. 54.9%, p=0.020); this significant association could no longer be detected following adjustment for baseline characteristics (age, BMI, CD4+ T-cell counts and number of participants on ART) (adjusted p-value=0.07).

Table 5.5: Humoral immune responses to inactivated influenza vaccine in HIV-infected participants

Measure	A/H1N1			A/H3N2			B/Yamagata		
	HIV+TB+	HIV+TB-	*P* Value§	HIV+TB+	HIV+TB-	*P* Value§	HIV+TB+	HIV+TB-	*P* Value§
No. participants	74	71		74	71		74	71	
HAI GMT [95% CI]									
Baseline	14.5 [12.3 – 17.2]	16.9 [13.9 – 20.6]	0.28/ *0.31*	20.5 [16.1 – 26.2]	17.6 [14.6 – 21.2]	0.77/ *0.99*	18.6 [16.1 – 21.6]	14.2 [12.7 – 16.0]	0.008/ *0.019*
Post-IIV3	64.6 [46.1 – 90.5]	75.4 [55.4 – 103]	0.44/ *0.59*	55.8 [39.7 – 78.4]	78.1 [57.5 – 106]	0.14/ *0.12*	29.8 [25.0 – 35.5]	19.8 [16.3 – 23.9]	<0.001/ *0.009*
P value‡	<0.001	<0.001		<0.001	<0.001		<0.001	0.007	
Factor increase in GMT [95% CI]	4.5 [3.3 – 6.1]	4.5 [3.42 – 5.82]	0.70	2.7 [2.1 – 3.5]	4.4 [3.2 – 6.1]	0.008	1.6 [1.4 – 1.8]	1.4 [1.2 – 1.7]	0.79
HAI titre ≥1:40 –No. (%); [95% CI]									
Baseline	12 (16.2); [8.7 – 26.6]	15 (21.1); [12.3 – 32.4]	0.52/ *0.40*	20 (27.0); [17.4 – 38.6]	14 (19.7); [11.2 – 30.9]	0.33/ *0.86*	20 (27.0); [17.4 – 38.6]	6 (8.5); [3.2 – 17.5]	0.005/ *0.014*
Post-IIV3	51 (68.9); [57.1 – 79.2]	52 (73.2); [61.4 – 83.1]	0.59/ *0.97*	44 (59.5); [47.4 – 70.7]	56 (78.9); [67.6 – 87.7]	0.05/ *0.012*	41 (55.4); [43.4 – 67.0]	20 (28.2); [18.1 – 40.1]	0.001/ *0.004*
P value‡	<0.001	<0.001		<0.001	<0.001		<0.001	0.004	
No. SC post-IIV3 (%); [95% CI]	45 (60.8); [48.8 – 72.0]	44 (62.0); [49.7 – 73.2]	1.00/ *0.60*	26 (35.1); [24.4 – 47.1]	39 (54.9); [42.7 – 66.8]	0.020/ *0.07*	10 (13.5); [6.7 – 23.5]	13 (18.3); [10.1 – 29.3]	0.50/ *0.49*

‡, *P* values comparing baseline vs. post-IIV3 within groups
§, Unadjusted/*adjusted P* values comparing HIV+TB+ vs. HIV+TB-
§, Adjusted P values for age, BMI, median CD4+ T-cell count and number of adults on ART
IIV3, trivalent inactivated influenza vaccine
HAI, hemagglutination inhibition
GMT, geometric mean titer with 95% confidence interval
No., number of
SC, sero-converted

5.1.4 Humoral Immune Responses to Trivalent Inactivated Influenza Vaccine in HIV-Uninfected TB-Infected and HIV-Uninfected TB-Uninfected Participants

Baseline

Analysis of humoral immune responses by HAI assay to IIV3 was conducted in 51 HIV-TB+ and 67 HIV-TB- participants. Baseline GMT were significantly higher in HIV-TB+ compared to HIV-TB- participants for B/Yamagata (21.1 [95% CI: 17.9 – 24.7] vs. 13.0 [95% CI: 11.7 – 14.5], $p<0.001$), and similar for the other two strains. Similarly a higher percentage of HIV-TB+ compared to HIV-TB- participants had sero-protective titers for B/Yamagata (7.5% vs. 25.5%, $p=0.009$) while no differences were detected for A/H1N1 (35.3% vs. 43.3%, $p=0.45$) and A/H3N2 (39.2% vs. 37.3%, $p=0.85$) (Table 5.6).

Post-vaccination

Both HIV-TB+ and HIV-TB- participants achieved significant increases in GMTs post-IIV3 for all three vaccine strains ($p<0.001$ for all comparisons, Table 5.6). Receipt of IIV3 resulted in significantly higher GMT in HIV-TB+ compared to HIV-TB- for A/H1N1 (233 [95% CI: 158 – 343] vs. 166 [95% CI: 128 – 216], $p=0.02$) and B/Yamagata (80.4 [95% CI: 60.8 – 106] vs. 33.6 [95% CI: 28.2 – 40.2], $p<0.001$). Similar GMT were achieved among HIV-TB+ and HIV-TB- participants for A/H3N2 (Table 5.6). The geometric mean fold-increase post-IIV3 was significantly greater in HIV-TB+ compared to HIV-TB- participants for A/H1N1 (9.6 vs. 5.2, $p=0.02$) and B/Yamagata (3.8 vs. 2.6, $p=0.04$), yet similar for A/H3N2 (6.6 vs. 6.6, $p=0.83$). Once adjusted for baseline characteristics (age and BMI) however, the difference between HIV-TB+ and HIV-TB- participants for A/H1N1 was no longer significant (adjusted p-value=0.43).

IIV3 vaccination resulted in >90% of HIV-TB+ and HIV-TB- achieving sero-protective titers for A/H1N1 and A/H3N2. This was less pronounced for B/Yamagata, where a significantly higher proportion of HIV-TB+ participants reached sero-protective titers than HIV-TB- participants (84.3% vs. 59.7%, p=0.004) (Table 5.6), but this was non-significant after adjustment for baseline characteristics (age, BMI, CD4+ T-cell counts and number of participants on ART) (adjusted p-value=0.09).

Sero-conversion rates ranged from 42% for B/Yamagata to 80% for A/H1N1 and were similar among HIV-TB+ and HIV-TB- participants for A/H3N2 (68.6% vs. 73.1%, p=0.68) and B/Yamagata (58.8% vs. 41.8%, p=0.09) (Table 5.6). For A/H1N1 a higher percentage of HIV-TB+ participants compared to HIV-TB- participants seroconverted (80.4% vs. 58.2%, p=0.016), however this was non-significant after adjustment for baseline characteristics (age and BMI) (adjusted p-value=0.06).

Table 5.6: Humoral immune responses to inactivated influenza vaccine in HIV-uninfected participants

Measure	A/H1N1			A/H3N2			B/Yamagata		
	HIV-TB+	HIV-TB-	*P* Value§	HIV-TB+	HIV-TB-	*P* Value§	HIV-TB+	HIV-TB-	*P* Value§
No. participants	51	67		51	67		51	67	
HAI GMT [95% CI]									
Baseline	24.3 [18.4 – 32.2]	31.7 [24.0 – 41.9]	0.20/ *0.96*	31.7 [22.6 – 44.4]	27.8 [21.9 – 35.2]	0.80/ *0.43*	21.1 [17.9 – 24.7]	13.0 [11.7 – 14.5]	<0.001/ *<0.001*
Post-IIV3	233 [158 – 343]	166 [128 – 216]	0.021/ *0.038*	209 [146 – 299]	183 [144 – 233]	0.26/ *0.46*	80.4 [60.8 – 106]	33.6 [28.2 – 40.2]	<0.001/ *<0.001*
P value‡	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001	
Factor increase in GMT [95% CI]	9.6 [6.6 – 13.9]	5.2 [3.7 – 7.3]	0.021	6.6 [4.5 – 9.7]	6.6 [4.8 – 9.2]	0.83	3.8 [2.8 – 5.1]	2.6 [2.1 – 3.2]	0.04
HAI titre ≥1:40 – No. (%); [95% CI]									
Baseline	18 (35.3); [22.4 – 49.9]	29 (43.3); [31.2 – 56.0]	0.45/ *0.72*	20 (39.2); [25.8 – 53.9]	25 (37.3); [25.8 – 50.0]	0.85/ *0.84*	13 (25.5); [14.3 – 39.6]	5 (7.5); [2.5 – 16.6]	0.009/ *0.09*
Post-IIV3	46 (90.2); [78.6 – 96.7]	62 (92.5); [83.4 – 97.5]	0.74/ *0.24*	46 (90.2); [78.6 – 96.7]	65 (97.0); [89.6 – 99.6]	0.24/ *0.71*	43 (84.3); [71.4 – 93.0]	40 (59.7); [47.0 – 71.5]	0.004/ *<0.001*
P value‡	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001	
No. SC post-IIV3 (%); [95% CI]	41 (80.4); [66.9 – 90.2]	39 (58.2); [45.5 – 70.2]	0.016/ *0.06*	35 (68.6); [54.1 – 80.9]	49 (73.1); [60.9 – 83.2]	0.68/ *0.94*	30 (58.8); [44.2 – 72.4]	28 (41.8); [29.8 – 54.5]	0.09/ *0.05*

‡, *P* values comparing baseline vs. post-IIV3 within groups
§, Unadjusted/*adjusted P* values comparing HIV-TB+ vs. HIV-TB+
§, Adjusted P values for age and BMI
IIV3, trivalent inactivated influenza vaccine
HAI, hemagglutination inhibition
GMT, geometric mean titer with 95% confidence interval
No., number of
SC, sero-converted

5.1.5 Cell-Mediated Immune Responses to Trivalent Inactivated Influenza Vaccine in HIV-Infected TB-Uninfected and HIV-Uninfected TB-Uninfected Participants

Baseline

Cell-mediated immune responses were assessed in 56 HIV+TB- and 60 HIV-TB- participants at baseline and one month post-IIV3, as measured by IFN-γ ELISPOT assay. At baseline GM SFU/10^6 PBMC were significantly higher in HIV+TB- compared to HIV-TB- participants for A/H1N1 (205 [95% CI 145 – 290] vs. 97.5 [95% CI 72.0 – 132], p=0.002) and B/Yamagata (166 [95% CI 120 – 228] vs. 89.6 [95% CI 64.3 – 125], p=0.009), while a similar trend, yet non-significant was observed for A/H3N2 (Figure 5.1A, Table 5.7).

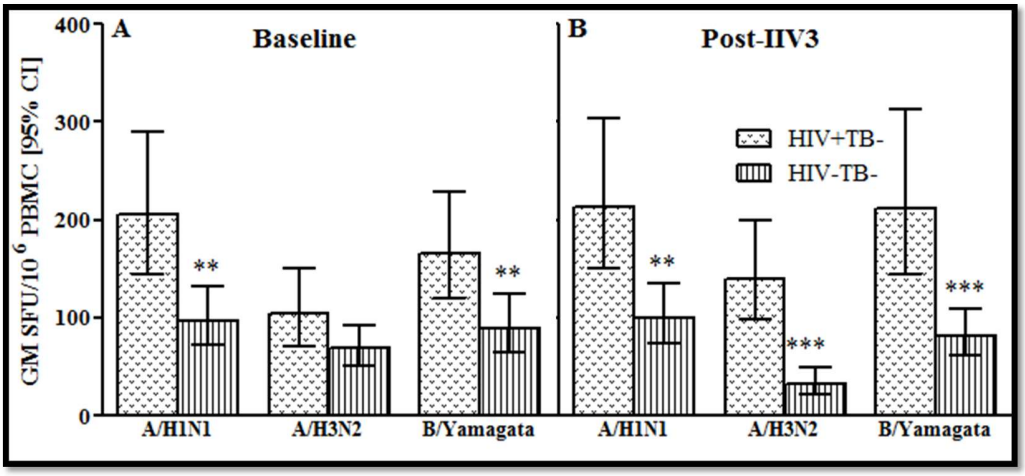


Figure 5.1: Cell-mediated immune responses to the different influenza vaccine strains in HIV-infected TB-uninfected (HIV+TB-) and HIV-uninfected TB-uninfected (HIV-TB-) participants at baseline (A) and post-IIV3 (B). The number of A/H1N1, A/H3N2, and B/Yamagata specific IFN-γ producing peripheral blood mononuclear cells was evaluated at baseline and post-vaccination by ELISPOT assay. Statistical analysis was performed using unpaired t-test. **p<0.01; ***p<0.001.

Post-vaccination

Post-IIV3 HIV+TB- participants had a minimal non-significant increase in GM SFU/10^6 PBMC for A/H3N2 and B/Yamagata and no change was observed for A/H1N1; whereas HIV-TB- participants had a significant decrease in GM SFU/10^6 PBMC for A/H3N2 from baseline to post-IIV3 (68.6 [95% CI: 50.9 – 92.6] at baseline vs. 32.3 [95% CI: 21.1 – 49.4] at post-IIV3, $p<0.001$) and no change for A/H1N1 and B/Yamagata (Figure 5.2, Table 5.7). Post-IIV3 GM SFU/10^6 PBMC were significantly higher in HIV+TB- compared to HIV-TB- participants for A/H1N1 (214 [95% CI: 151 – 303] vs. 99.6 [95% CI: 73.3 – 135], $p=0.002$), A/H3N2 (140 [95% CI: 98.0 – 200] vs. 32.3 [95% CI: 21.1 – 49.4], $p<0.001$) and B/Yamagata (212 [95% CI:144 – 313] vs. 81.5 [95% CI: 61.0 – 109], $p<0.001$) (Figure 5.1B, Table 5.7). From baseline to post-IIV3 HIV+TB- compared to HIV-TB- participants had similar GM fold-changes for A/H1N1, but higher GM fold-changes were observed for A/H3N2 (1.4 [95% CI: 1.0 – 1.9] vs. 0.5 [95% CI: 0.3 – 0.7], $p<0.001$) and B/Yamagata (1.3 [95% CI: 0.9 – 1.8] vs. 0.9 [95% CI: 0.6 – 1.3], $p=0.05$) (Table 5.7).

The proportion of participants achieving ≥2 fold increase from baseline to post-IIV3 was similar between HIV+TB- and HIV-TB- for A/H1N1 (14.3% vs. 17.2%, $p=1.00$) and B/Yamagata (27.3% vs. 14.8%, $p=0.35$) but significantly greater in HIV+TB- participants for A/H3N2 (42.4% vs. 5.9%, $p=0.009$). Comparisons of immune responses between the two groups did not change following adjustment for age at both baseline and post-vaccination.

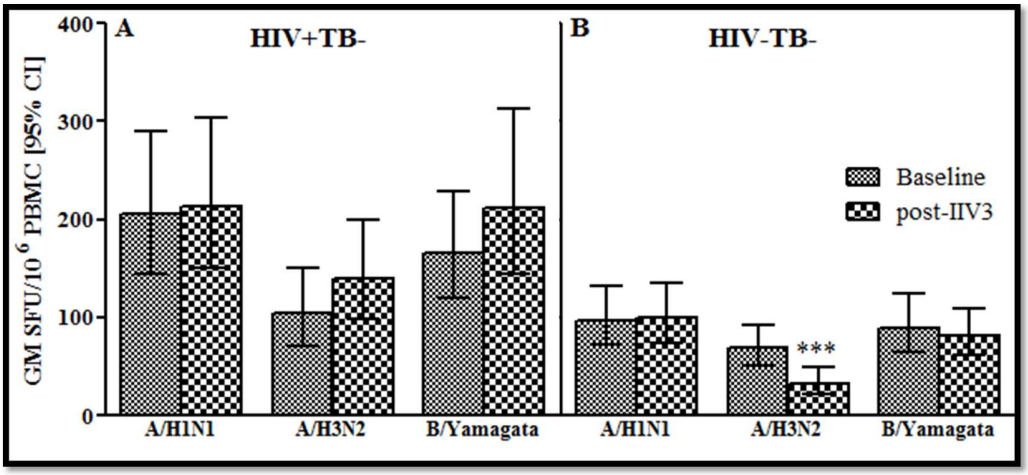


Figure 5.2: Cell-mediated immune responses to the different influenza vaccine strains at baseline and post-IIV3 in HIV-infected TB-uninfected (HIV+TB-) (A) and HIV-uninfected TB-uninfected (HIV-TB-) (B) participants measured by IFN-γ ELISPOT assay. Statistical analysis was performed using paired t-test. ***$p<0.001$.

Table 5.7: Cell-mediated immune responses to inactivated influenza vaccine in HIV-infected TB-uninfected and HIV-uninfected TB-uninfected participants

Measure	**A/H1N1**			**A/H3N2**			**B/Yamagata**		
	HIV+TB-	HIV-TB-	*P* Value§	HIV+TB-	HIV-TB-	*P* Value§	HIV+TB-	HIV-TB-	*P* Value§
No. of participants	35	29		33	17		33	27	
GM SFU/10^6 PBMC [95% CI]									
Baseline	205 [145 – 290]	97.5 [72.0 – 132]	0.002	104 [71.4 – 151]	68.6 [50.9 – 92.6]	0.14	166 [120 – 228]	89.6 [64.3 – 125]	0.009
Post-IIV3	214 [151 – 303]	99.6 [73.3 – 135]	0.002	140 [98.0 – 200]	32.3 [21.1 – 49.4]	<0.001	212 [144 – 313]	81.5 [61.0 – 109]	<0.001
P value‡	0.79	0.91		0.07	<0.001		0.14	0.62	
GM factor increase [95% CI]	1.0 [0.8 – 1.4]	1.0 [0.7 – 1.5]	0.55	1.4 [1.0 – 1.9]	0.5 [0.3 – 0.7]	<0.001	1.3 [0.9 – 1.8]	0.9 [0.6 – 1.3]	0.05
No. ≥2 fold increase post-IIV3 (%); [95% CI]	5 (14.3); [4.8 – 30.3]	5 (17.2); [5.8 – 35.8]	1.00	14 (42.4); [25.5 – 60.8]	1 (5.9); [1.5 – 28.7]	0.009	9 (27.3); [13.3 – 45.5]	4 (14.8); [4.2 – 33.7]	0.35

‡, *P* values comparing baseline vs. post-IIV3 within groups
§, *P* values comparing HIV+TB- vs. HIV-TB- participants
IIV3, trivalent inactivated influenza vaccine
SFU, spot forming units
GM, geometric mean with 95% confidence interval
No., number of

5.2 Discussion

In this study we report on the immunogenicity of the trivalent inactivated influenza vaccine (IIV3) by assessing both humoral and cell-mediated immune (CMI) responses in TB/HIV infected adults, prior to immunization and one month post-vaccination. We compare the humoral immune responses to IIV3 among: (1) HIV/TB co-infected (HIV+TB+) and HIV-infected TB-uninfected (HIV+TB-) adults and; (2) HIV-uninfected TB-infected (HIV-TB+) and HIV-uninfected TB-uninfected (HIV-TB-) adults, as well as to compare the CMI responses to IIV3 among HIV+TB- and HIV-TB- adults. Humoral immune responses were measured and compared using the hemagglutinin inhibition assay (HAI) and CMI responses were measured and compared by enzyme-linked immunospot assay (ELISPOT) at both pre-vaccination and one month post-vaccination.

The immunogenicity of IIV3, measured by either HAI or ELISPOT in healthy adults and children has been documented (123,307). There is, however, little information about the immunogenicity of this vaccine in high-risk groups (such as infants, pregnant women, HIV-infected and HIV/TB co-infected adults).

Tuberculosis (TB) is a major global issue resulting in adverse health among millions of individuals and ranks parallel to HIV and malaria as a leading cause of public ill-health (203), with an estimated 9.6 million new cases reported in 2014 (202). A compromised immune system severely increases the risk of TB disease, and in Southern Africa both socioeconomic and environmental factors play a role in potentially exacerbating the disease progression (251). The risk of TB infection is elevated most notably among patients with HIV, even during the first years after viral acquisition when CD4+ T-cell counts are still

high (254,255). Even with the introduction of ART in HIV-infected individuals, the risk of TB progression is not entirely reversed (256). Co-infection of HIV/TB has been shown to increase the risk of new additional opportunistic infections and of death compared to HIV-infected individuals without TB when CD4+ T-cell counts are similar (257-259). The relationship between HIV/TB co-infection is particularly aggressive, with both diseases being characterised by widespread dissemination throughout the human body as well as exhibiting poor host immune responses (274).

South Africa has one of the highest rates of TB, further compounded by co-existing high prevalence of HIV (355). The increased risk of HIV-infected individuals developing TB, including early in the clinical course of HIV-infection, relates to the gradual impairment of multi-faceted arms of their immune systems (356). Foremost among these is the decrease in CD4+ T-cells and their impaired function, which contributes to impaired CMI responses which occur at a relatively early stage following HIV-infection (357). This gradual decline in the host immune system, coupled with other factors may stimulate the reactivation of dormant TB in latently infected individuals. In addition, the impaired immune system is also less able to cope with any new environmental exposure/infection by TB, resulting in a greater likelihood of infection progressing to disease compared to otherwise healthy individuals (357). The depletion of CD4+ T-cells and impaired macrophage function in HIV-infected individuals contributes to the susceptibility of co-infection with TB (358) and influenza (359). Currently there are no data regarding the immunogenicity of IIV in HIV/TB co-infected individuals (295,360).

In our study we first assessed the humoral immune responses to IIV3 among HIV/TB co-infected (HIV+TB+) and HIV-infected adults without TB (HIV+TB-). At baseline, HIV+TB+ were significantly younger and had a lower body-mass index (BMI) and median CD4+ count compared to the HIV+TB- participants (Table 5.2). Additionally, a lower percentage of HIV+TB+ adults were on ART than HIV+TB- adults (43.3% vs. 78.9%, $p<0.001$). Prior to immunisation, geometric mean titers (GMT) were comparable among study participants for A/H1N1 and A/H3N2, whereas greater GMT were obtained among the HIV+TB+ participants (Table 5.5), even after adjustment for baseline characteristics. Among the HIV+TB+ participants, HAI titers ≥1:40 were observed in 16.2%, 27.0% and 27.0% for A/H1N1, A/H3N2 and B/Yamagata, respectively. The corresponding percentage of HIV+TB- adults having HAI titers ≥1:40 were 21.1%, 19.7% and 8.5%, respectively.

In this study we found that vaccination with IIV3 elicited significant increases in GMT for all three vaccine strains in HIV+TB+ and HIV+TB- participants ($p<0.01$ for all comparisons, Table 5.5), with increases from baseline by a factor of 1.6 – 4.5 among HIV+TB+ participants and 1.4 – 4.5 for HIV+TB- participants. Although higher GMT were achieved in HIV+TB- participants, interestingly no significant differences in GMT post-vaccination was observed between the HIV+TB+ and HIV+TB- participants for A/H1N1 and A/H3N2. Contrary to what we expected, HIV+TB+ participants achieved significantly greater GMT for B/Yamagata compared to HIV+TB- participants ($p<0.001$), even after adjusting for baseline characteristics.

Based on the current information available on the association between immune responses and HIV/TB co-infection, we expected HIV+TB+ adults to have a significantly lower

immune response to IIV3 than HIV+TB- participants: TB infection is often associated with a collection of cellular activations and irregularities in both cytokine and chemokine pathways that are permissive of HIV replication (258,361) and although clinical and experimental evidence suggests active TB accelerates HIV disease (362) it has been shown that TB treatment in HIV-infected populations remains as effective as that in HIV-uninfected populations (363). This may explain the similar immune responses observed in the HIV-infected participant groups, where 98.6% of HIV+TB+ adults in this study were on TB-treatment (Table 5.2).

The percentage of participants having sero-protective HAI titers (≥1:40) post-vaccination increased significantly for all three strains in both HIV+TB+ and HIV+TB- participant groups (p<0.001 for all comparisons), albeit to a lower degree than those observed in healthy populations (300). There was no significant difference in the percentage of participants having sero-converted for A/H1N1 and B/Yamagata following vaccination between the two groups, however for A/H3N2 a significantly greater percentage of HIV+TB- participants sero-converted compared to HIV+TB+ participants (54.9% vs. 35.1%, p=0.020). These observations are consistent with findings from a previous study: Crum-Cianflone *et al.* reported HIV-infected individuals showing significantly reduced sero-conversion (54% vs. 75%, p=0.021) and sero-protection (67% vs. 83%, p=0.005) compared to HIV-uninfected individuals one month after receiving the monovalent pandemic influenza vaccine (364). Bickel *et al.* however reported a far more successful rate among HIV-infected patients where, after one round of vaccination 68% of patients (with mean CD4+ counts of 514 cells/mm^3) had sero-converted (311). Although the results

obtained in our study were unexpected, it is interesting to note that humoral immune responses to IIV3 appeared not to be affected by TB-infection among an HIV-infected population on TB-treatment (365). Additionally, in a previous study from 2009, researchers showed that a single dose of the monovalent A/H1N1 vaccine was immunogenic in both healthy and HIV-infected individuals with CD4+ cell counts >200 cells/mm^3 (366), which may add strength to the findings in this study, given that HIV+TB+ and HIV+TB- participants had a median CD4+ count of 262 cells/mm^3 and 510 cells/mm^3, respectively.

Next, we assessed humoral immune responses to IIV3 in HIV-uninfected TB-infected (HIV-TB+) and HIV-uninfected TB-uninfected (HIV-TB-) adults. At baseline HIV-TB+ participants were significantly older and had a significantly lower BMI compared to HIV-TB- participants and 98.0% of HIV-TB+ participants were on TB-treatment (Table 5.2). In this study we found that IIV3 elicited good humoral immune responses in HIV-TB+ and HIV-TB- adults. Baseline GMT were similar among the study groups for A/H1N1 and A/H3N2, while HIV-TB+ participants had significantly higher GMT for B/Yamagata. These findings are slightly higher to those previously described in healthy adults (302,367) yet similar to those observed in pregnant women (103). Sero-protective GMT at baseline ranged from 25.5% - 39.2% and 7.5% - 43.3% in HIV-TB+ and HIV-TB- participants, respectively, with no significant differences between the two groups for A/H1N1 and A/H3N2. However, a significantly greater percentage of HIV-TB+ adults had sero-protective titers against B/Yamagata.

One month after vaccination the percentage of participants who had sero-protective titers significantly increased, with >90% of HIV-TB+ and HIV-TB- adults demonstrating GMT

≥1:40 to at least two of the three vaccine strains. Sero-protection against B/Yamagata however remained <60% among HIV-TB- adults, while 84.3% of HIV-TB+ participants achieved GMT ≥1:40. Sero-conversion rates were similar between the study groups for A/H3N2 and B/Yamagata, but a significantly greater percentage of HIV-TB+ adults sero-converted to A/H1N1 (80.4% vs. 58.2%, p=0.016), however, after adjusting for differences in baseline characteristics (), this difference was no longer significant. These findings are in line with results from a study performed in 2009, where HIV-uninfected adults achieved a 78.8% sero-conversion rate (366) and from a study performed in Indonesia in 2004, where researchers aimed at determining if influenza virus infections promote the development of TB disease. The authors reported significantly higher GMT for A/H3N2 in TB-infected participants compared to the healthy controls (244 vs. 145, p=0.002) and a non-significantly higher GMT to A/H1N1 (72 vs. 51, p=0.33) (287). A possible reason for this result may be due to the relationship that exists between the influenza virus and the clinical manifestation of TB, but caution that this association may be confounded: influenza infection in patients with TB may be more severe or possibly longer lasting due to damage caused in the lung by *M. tuberculosis*, and as a result greater quantities of antibodies against influenza are generated (287). Furthermore, it was previously shown that prior exposure to influenza A virus in mice, followed by TB infection lead to enhanced mycobacterial growth and decreased survival and following co-infection, mycobacterial growth was enhanced by a type I interferon signalling pathway (291).

Post-vaccination GMT were significantly higher among HIV-TB+ adults for A/H1N1 and B/Yamagata with increases from baseline by a factor of 3.8 – 9.6 in HIV-TB+ adults and 2.6 – 6.6 in HIV-TB- adults for all three strains. These observations, although slightly less in our study, are consistent with findings from Tiu *et al.* who showed a 13.7-fold GMT increase 4 to 6 weeks post-vaccination among HIV-uninfected participants (366).

Finally, we assessed and compared CMI responses to IIV3 using IFN-γ ELISPOT assay in a sub-set of HIV-TB+ (N=56) and HIV-TB- (N=60) participants. A substantial body of work on the immunogenicity to IIV in healthy adults and children (measured mainly by HAI assay to assess humoral responses) already exists in the literature (123,304). Advanced HIV infection is associated with greater seasonal influenza-related morbidity and mortality (113,359,368). Studies assessing seasonal influenza vaccination showed good efficacy both in HIV-infected persons (369) as well as in healthy adults (309). HIV-infected individuals are characterised by a compromised immune cell function due to the loss of CD4+ helper T-cells specifically infected by HIV, which are necessary for the activation of B-cells and cytotoxic T-cell immune responses (330), as well as affecting macrophages (260).

At baseline HIV+TB- adults were significantly older than HIV-TB- adults (41.2 years [SD: 7.7] vs. 26.1 years [SD: 7.0], $p<0.001$) and 82.1% of HIV+TB- adults were on ART and had a median CD4+ count of 516 cell/mm^3 (Table 5.3). HIV+TB- adults compared to HIV-TB- adults prior to vaccination had significantly greater CMI responses to A/H1N1 and B/Yamagata and post-IIV3 to all three vaccine strains (Table 5.7). This is in contrast with previous findings, where generally HIV-infected adults and children exhibit impaired

or similar cellular immune responses to influenza vaccination compared to healthy, HIV-uninfected populations (306,308,309,310,330,335).

Approximately one month post-vaccination CMI responses were slightly increased over baseline in HIV+TB- adults for all three strains, but none reached statistical significance. Among HIV-TB- adults however, decreased CMI responses were observed for A/H3N2 and B/Yamagata. The fold-increases over baseline were in the range of 1.0 – 1.4 in HIV+TB- and 0.9 – 1.0 in HIV-TB- adults for the vaccine stains. A small percentage of participants achieved a ≥2-fold increase over baseline for all three vaccine stains, ranging from 14% – 42% in HIV+TB- and from 65 – 17% in HIV-TB- adults. Our results contradict those previously reported among healthy participants, where Iorio *et al.* and Avetisyan *et al.* showed 59% and 72%, respectively, of study participants achieving ≥2-fold increase in SFU post-vaccination (300,333). In another study, Agrati *et al.* reported that 52.2% of health care workers versus 68.4% of HIV-infected patients achieved ≥2-fold increase over baseline for A/H1N1 following vaccination (309). It should be noted that a ≥2-fold increase post-vaccination is an arbitrary threshold indicating a significant response to IIV3 (196).

In a study by Fabbiani *et al.* (330,370) HIV-infected individuals vaccinated with an adjuvanted seasonal influenza vaccine showed sustained humoral responses (measured by HAI assays) but cellular responses were impaired (330). Interestingly, humoral immune responses to IIV3 among HIV+TB- (Table 5.5) and HIV-TB- (Table 5.6) participants were shown to be robust, yet CMI responses reported for this sub-group were attenuated (Table 5.7). We hypothesise that the results obtained in this study, whereby we show

comparatively good responses to IIV3 in HIV-TB+ adults, is probably due to the effectiveness of TB treatment, with 98.0% on TB treatment. The findings in this study appear to show no correlation between the sero-prevelance of antibodies against the influenza vaccine strains and the existence of TB-infection. Although antibodies play a critical role in protection against influenza infection, activation of T-cells, particularly the CD4+ helper subpopulation is essential for supporting virus-specific effector cell functions (371).

6.0 Conclusion

In this study we aimed to determine the cell-mediated immune (CMI) responses to the seasonal trivalent inactivated influenza vaccine (IIV3) in high-risk groups.

There are several important findings generated from our study, as well as results that further strengthen observations already reported elsewhere. For simplicity, we divided the results chapter of this thesis into three major sections. In the first section we report on the comparison of CMI responses to IIV3 between HIV-infected pregnant and non-pregnant women. Vaccination improved CMI responses in both groups, however only significantly for A/H1N1 in both groups and for B/Yamagata in non-pregnant women. We found no significant differences in CMI responses to the influenza strains contained in the 2013 IIV3 formulation at both baseline and post-vaccination between the study groups; however, HIV-infected pregnant women did display slightly higher GM SFU/10^6 PBMC than HIV-infected non-pregnant women at both time points. However, although non-significant, non-pregnant women tended to have higher fold-increases and a higher percentage achieved ≥2-fold increase from baseline to post-IIV3 compared to pregnant women. Prior to initiating this study, we hypothesised that HIV-infected pregnant women would show diminished CMI responses to the influenza vaccine strains at both baseline and post-vaccination compared to the HIV-infected non-pregnant women. Contrary to this predicted outcome, there was no significant difference in CMI responses between the study groups for any of the three vaccine strains. This observation may be explained by the higher

percentage of HIV-infected pregnant women on ART, as well as a significantly larger percentage having CD4+ T-cell counts >350 cell/mm^3 at baseline. We speculate that ART treatment in >98% of the HIV-infected pregnant women enrolled in this study contributed to the improved responses observed, when compared to 60.1% of HIV-infected non-pregnant women on ART.

In the second chapter, we described CMI response in HIV-uninfected pregnant and non-pregnant women. Since the HIV-infected and HIV-uninfected groups were enrolled over different periods we were unable to directly compare the results obtained in both groups. Following vaccination we found no significant increases in responses in either HIV-uninfected pregnant and non-pregnant women. A potential reason for this observation is the time of participant enrolment being post-influenza season. Participants were most likely exposed to the circulating strains and might have developed naturally acquired cellular immunity. Compared to pregnant women, non-pregnant women did achieve significantly higher CMI responses to all three influenza vaccine strains at both baseline and post-vaccination. Fold-increases were similar between the two groups and other than for A/H1N1, no significant differences in women achieving a ≥2-fold increase were observed. The results reported are in line with our expected outcomes, where pregnant women would show attenuated CMI responses to vaccination compared to HIV-uninfected non-pregnant women.

We also show in this study that pre-existing cellular immunity to influenza viruses has a major influence on responses following vaccination. After stratification we discovered that women with low baseline responses had significantly improved responses post-vaccination,

suggesting that having little or no pre-existing immunity plays a significant role in the responses to vaccination. Whereas, having pre-existing CMI to the vaccine strains negatively impacted the responses post-vaccination. Women with higher baseline responses to the three vaccine strains were unable to reach significant increases post-vaccination, where in fact decreases in responses were observed. A possible explanation for this observation may be attributed to the maximal quantity of influenza-specific memory T-cells available. A threshold may exist where the amount of T-cells circulating in the body, such as in the high-baseline response participants, could potentially explain why high-baseline responders were unable to achieve significant increases post-vaccination. A possible explanation may be due to the regulatory responses that can potentially induce or limit the magnitude of response. More often than not, women with high-baseline responses showed a reduced response post-vaccination, which may add further evidence that a potential threshold exists in individuals with pre-existing CMI, where a drop off may occur. Although women with low-baseline responses achieved significantly increased responses, they were unable to reach the levels seen in the high-baseline groups. This may add further evidence that a potential threshold exists; we observed significant increases in the low-baseline response groups, yet it is unclear how far that increase may reach, unless we take into account that the high-baseline groups peaked with regard to their CMI responses and resulted in a decrease one month after receiving the influenza vaccine. A potential exploratory study could be to analyse CMI responses with shorter time intervals. This could shed light on the possibility that cellular immunity is short-lived, which could explain the potential threshold. If women in the low-baseline groups significantly increased their CMI responses, they may not have yet reached this threshold, which is why we do not observe a

decrease in responses as was seen in the high-baseline response groups. The high-baseline response groups may have reached this threshold sooner than the low-baseline groups, and could explain why the decrease was observed. Furthermore, we observed significantly greater fold-increases among the low-baseline groups than the high-baseline groups for all vaccine strains, adding evidence that having little or no pre-existing T-cells may positively influence the CMI response to vaccination.

In the third and final section, we assessed humoral and cell mediated immune responses in a cohort of adults living with or without HIV and TB infection. Among the HIV-infected group, individuals with TB infection had significantly lowered CD4+ T-cell counts and a lower percentage were on ART compared to the adults without TB. 98.6% of HIV-infected adults with TB adults were on TB treatment. Among the HIV-infected adults humoral immune responses were similar between those with and without TB at both baseline and post-vaccination except for responses to B/Yamagata, which were higher in individuals with TB. Vaccination increased the antibody titers against the vaccine strains in both HIV-infected groups, however participants were unable to reach the levels of sero-conversion and sero-protection reported in healthy adults. An important finding in this study was that TB infection did not negatively impact humoral immune responses to influenza. Furthermore, vaccination significantly increased the number of adults achieving sero-protection and sero-conversion in both groups.

Among the HIV-uninfected groups, 98.0% of the participants with TB were on TB treatment, this could potentially explain why adults with TB achieved a good humoral response post-vaccination. As stated earlier, it appears that TB infection, if treated, does not

negatively impact the immune responses to IIV3. This is evidenced by the fact that our results in this group strongly correlate with results obtained in a healthy population.

Within the sub-group analysed by ELISPOT assay, we found that HIV-infection does not appear detrimental to cellular immunity, as compared to healthy, HIV-uninfected adults. Adults with HIV-infection showed significantly higher CMI responses at both baseline and after immunisation compared to HIV-uninfected adults. Further, no significant increases in CMI responses were observed for either group following vaccination and surprisingly HIV-uninfected adults without TB were unable to achieve the level of immune responses seen in HIV-infected adults.

As a secondary observation from our study, it is apparent that CMI measurements are problematic in high-risk groups. The ELISPOT assay relies heavily on the quality of isolated PBMC. We found that HIV infection appeared to negatively affect the quality of PBMC. This is clear from the percentages of viable PBMC we were able to use, in order to conduct accurate measurements. The lowest percentage of participants with viable PBMC (live cell count >70%) were in the HIV-infected groups, with only 45% HIV-infected pregnant women's PBMC reaching this quality where we were able to conduct the ELISPOT assay. The highest percentages of usable PBMC were from the HIV-uninfected groups, with >84% of the PBMC isolated having reached the quality that allows for ELISPOT analysis. Secondly, no consensus currently exists as to the nature and threshold of a significant CMI response representative of protection. With such variations in the methodology of the ELISPOT assay reported, including different virus and cell concentrations used for each assay, as well as different incubation times, it is more prudent

to rely on fold-increases as the best strategy for gauging responses, rather than to rely on absolute spot forming units. Further studies are needed to standardise the ELISPOT method. Furthermore, it would be advantageous to conduct parallel studies using both the HAI assay, where a titer ≥1:40 is predictive of an anticipated vaccine efficacy of 50% in adults, and ELISPOT assays in order to determine a potential quantitative threshold for protection when using the ELISPOT assay.

In conclusion, the results reported in this thesis clearly suggest that pregnancy may more negatively influence cellular immunity than infection with HIV, most notably when pregnant women living with HIV are on ART and have a CD4+ T-cell count >350 cells/mm^3. We also show that pre-existing memory T-cells have a major impact on the magnitude of response to vaccination, regardless of time of vaccination. IIV3 was relatively immunogenic in HIV-infected adults with and without TB, albeit not the magnitude observed in healthy populations. However, in HIV-uninfected adults we showed that influenza vaccination was immunogenic in both with and without TB groups. Additionally, we show that infection with TB does not appear to affect CMI responses, even in the HIV-infected population. However, this observation should not overshadow the effect that HIV does have on vaccine response: although TB infection did not appear to play as large a role on immune responses as we expected, the results reported for HIV/TB co-infected were dramatically lower than those observed in healthy populations. We also show that there appears to be no correlation, albeit an indirect observation, between CMI and humoral immune responses to IIV3, as evidenced by the ELISPOT assay results obtained for the participants without TB and the corresponding humoral immune responses.

7.0 References

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8.0 Appendices

8.1 Laboratory Methods

8.1.1 Instruments

Automatic Cell Counter (TC20™) and Slides	Bio-rad, SA
Cell Culture Incubator	SANYO electric Co. Ltd.
Centrifuge 5810	Eppendorf, Hamburg
Laminar Flow	Airvolution (PTY) Ltd.
Waterbath YCW-01 22L	Gemmy Industrial Corp
Freezer (-150°C)	ThermoScientific, USA
BD™ ELISPOT Assay Kit	BD™ Biosciences
BD™ AEC Substrate Solution Kit	BD™ Biosciences
LuecoSeps™ Centrifuge Tubes	Greiner Bio-One
Autoclave	Gemmy Industrial Corp
Liquid Nitrogen Container	Chart Industries Inc.

8.1.2 Solutions and Reagents

Ficoll-Hypaque	Sigma-Aldrich
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
96-well Microtiter Plate	BD™ Biosciences
96-well U-Bottom Plates	Nunc™
96-well V-Bottom Plates	Nunc™
Dilute Antibody Solution	BD™ Biosciences
Biotinylated anti-human IFN-γ	BD™ Biosciences

Enzyme Conjugate (Streptavidin-HRP)	BD™ Biosciences
Substrate Solution	BD Biosciences
PHA-M at 5 mg/mL	Sigma-Aldrich
Fetal Bovine Serum (FBS)	Biowest
RPMI 1640 (w/glutamine)	Gibco®
Penicillin-Streptomycin (10 000 U/ml)	Gibco®
HEPES (1 M)	Gibco®
Turkey red blood cells in Alsever's solution	CO Serum Company
Receptor Destroying Enzyme	Denke Seiken Co. Ltd.
Physiologic Saline Solution (0.9% NaCl)	

Phosphate Buffered Saline (PBS) pH 7.2 (Autoclave Sterilised), 1 litre

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
dH_2O	1 L

Freezing medium (Filter Sterilised), 50 ml

Heat inactivated (HI) FBS (Biowest)	45 ml
DMSO (Sigma-Aldrich)	5 ml

Thawing Medium (Filter Sterilised), 500 ml

RPMI 1640 w/glutamine (Gibco®)	450 ml
HI-FBS (Biowest)	50 ml

ELISPOT Assay Medium (Filter Sterilised), 500 ml

RPMI 1640 w/glutamine (Gibco®)	440 ml
HI-FBS (Biowest)	50 ml
Penicillin-Streptomycin (10 000 U/ml) (Gibco™)	5 ml
HEPES (1 M) (Gibco™)	5 ml

Dilution Buffer (Filter Sterilised), 1 litre

PBS	100 ml
HI-FBS (Biowest)	900 ml

R14/49 Mr Alexander Malherbe

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M161050

NAME: (Principal Investigator)	Mr Alexander Malherbe
DEPARTMENT:	Clinical Microbiology and Infectious Diseases Respiratory and Meningeal Pathogens Research Unit University of the Witwatersrand Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Humoral and Cell Mediated Immune Responses to Inactivated Influenza Vaccine in High-Risk Groups
DATE CONSIDERED:	28/10/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Marta Nunes
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	29/03/2017

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor Senate House/2nd floor, Phillip Tobias Building, Parktown. University of the Witwatersrand. I/We fully understand th the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake t ensure compliance with these conditions. Should any departure be contemplated,from the research protocol a approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date fo annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. i this case, the study was initially review October and will therefore be due in the month of October each yea Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

31 MARCH 2017
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES