



**Functional Assessment on Non-Neutralizing Binding Antibodies in the Blood and Genital Tracts of Women with Breakthrough HIV Infection from the CAPRISA 004 1% Tenofovir Gel Trial.**

**By**

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Submitted in partial fulfilment of the requirements for the degree of Master of Medical Science in the Department of Medical Microbiology, Nelson R Mandela School of Medicine, University of KwaZulu-Natal

Date: 15 December 2017

## PLAGIARISM DECLARATION

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I, Kimone Leigh Fisher, declare that this dissertation has not been published or submitted before, to this or any other tertiary institution. I declare that previously published work mentioned in this dissertation has been adequately referenced and that the research presented is novel.



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Kimone Leigh Fisher

This dissertation was compiled under the supervision of Dr D. Archary, at the Centre for the Aids Programme of Research In South Africa.

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Supervisor: Dr Derseree Archary

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Co-supervisor: Dr Cheryl Baxter

## DEDICATION

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This dissertation is dedicated to my husband Lindsay Fisher, my parents Reuben and Cheryl and my brother Jared Naidoo. They have allowed me to stand on their shoulders and believed that I would accomplish great things in my life. Lindsay Fisher, you have carried me across the finish line, just when I was ready to give up.

Finally, I would like to dedicate this work to Jesus Christ,

“God delights in concealing things; scientists delight in discovering things.” – Proverbs 25:2

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## PRESENTATIONS

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### Poster presentation:

1. Tenofovir gel exposure enhances Antibody Dependent Cell-Mediated Phagocytosis in the Genital Tracts of Women who acquired HIV infection.

KL Fisher, J Mabuka, C Baxter, SS Abdool Karim, L Mansoor, and D Archary

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2. Tenofovir gel exposure enhances Antibody Dependent Cell-Mediated Phagocytosis in the Genital Tracts of Women who acquired HIV infection.

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## **ABBREVIATIONS**

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Ab- antibody

ADCC – Antibody Dependent Cellular Cytotoxicity

ADCP – Antibody Dependent Cellular Phagocytosis

ADCVI – Antibody dependent cell mediated viral inhibition

AIDS – Acquired Immunodeficiency Syndrome

APC – Antigen Presenting Cell

ARV – antiretroviral

BNAb – broadly neutralizing antibody

BCR- B cell receptor

CVL – cervicovaginal lavages

DMPA - depot-medroxyprogesterone acetate

DNA- deoxyribonucleic acid

Env – Surface envelope protein of HIV virus

Fab- fragment binding region

Fc- crystallisable fragment

FcR – crystallisable fragment receptor

FRT- female reproductive tract

Gag – group specific antigen that codes for the core structural proteins of the HIV virus

GC- germinal centre

GT – genital tract

HEPN- Highly exposed persistently seronegative

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

HTS – high throughput screening

ICS – Intracellular Cytokine Staining

IDU- injection drug users

IL- interleukin

KIR – Killer like Receptors

LAI- strain of HIV virus

MAb – monoclonal antibody

MCMV – mouse cytomegalovirus

MHC – major histocompatibility complex

MSM – men who have sex with men

NAb – Neutralizing antibody

Nef – negative regulatory factor protein. It contributes to the virus virulence factor and manipulates the host machinery to allow for infection

NHP –non-human primate

NK – natural killer

nNAb – non-neutralizing antibody

PBS – phosphate buffered saline

pDC – plasmacytoid dendritic cell

PFA – paraformaldehyde

PMN – Polymorphonuclear cells

Pol - DNA polymerase produced by this gene

PrEP – pre-exposure prophylaxis

RFADCC – Rapid fluorescence antibody dependent cellular cytotoxicity

SHM – somatic hypermutation

Tfh                      -T                      follicular                      helper                      cell

## ABSTRACT

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**Introduction:** Pre-exposure prophylaxis (PrEP) can alter antibody kinetics resulting in delayed antibody binding avidity and reduced titres during the early post-HIV infection period. Data from the RV144 vaccine trial have underscored the protective role of non-neutralizing antibodies (nNAbs), likely Fc-mediated, in HIV prevention. Previously published data from the CAPRISA 1 % tenofovir gel trial, showed distinct antibody profiles in the genital tracts, distinguished women with prior tenofovir gel use. The effect of prior PrEP on subsequent nAb function was explored to elucidate whether IgG-specific nAb functions were altered in the plasma and genital tracts of women in the tenofovir compared to those in the placebo arm post-HIV infection.

**Methods:** An *in-vitro* neutrophil-based antibody dependent cellular mediated phagocytic functions (ADCP) assay and a novel intracellular cytokine staining NK cell activated antibody dependent cellular cytotoxicity (ADCC) assay at 3, 6 and 12 months post-infection in the tenofovir (n = 24) and placebo arms (n = 24), were conducted for gp120, gp41, p66 and p24 IgGs in the plasma and genital tracts of women who seroconverted. Statistical analyses involved Wilcoxon Sum Rank test, Wilcoxon Signed rank test and Spearman R correlations.

**Results and Conclusions:** HIV-specific ADCP and NK cell activated ADCC were detected in both the plasma and genital tract compartment. Gp70 V1V2 ADCP was higher in the plasma, while p66 and p24 specific ADCP was higher in the genital tract of women in the tenofovir arm compared to the placebo arm ( $P < 0.01$ ;  $P < 0.1$ ). Gp41 and p66 specific NK cell activated ADCC activity in the genital tract evolved significantly in women in the tenofovir arm ( $P < 0.1$ ), in contrast to ADCP responses which were similar for both compartments ( $P > 0.05$ ) and specificities. Gp120 ADCP and NK cell activated ADCC remained higher in plasma compared to the genital tract ( $P < 0.001$ ). Prior topical tenofovir gel did not diminish Fc-mediated effector ADCP and NK cell activated ADCC functions, as evidenced by the higher NK cell activated ADCC and ADCP specific activity in the genital tract. Together, these data inform combination prevention strategies that parallel PrEP usage can influence humoral immunity and may even enhance; the diverse antibody-mediated effector functions post HIV vaccination, at the site of vulnerability, the genital tract.

## CHAPTER 1

---

### Introduction

Despite strides made in HIV prevention, HIV continues to be a global health challenge. Approximately 1.8 million [1.6 million-2.1 million] new infections occur annually, of which an estimated 64% of these new infections, occur in sub-Saharan Africa (Unaid, 2014b). Despite HIV prevention strategies, the number of new infections is declining too slowly to meet the UNAIDS goal of ending AIDS by 2030 (Unaid, 2017b). Young women (aged 15-24 years) in particular, are more susceptible to HIV infection and account for 26% of new HIV infections despite only making up 10% of the population (Unaid, 2017b).

Pre-exposure prophylaxis is currently one of the most promising biomedical HIV prevention tools. In 2010, results from the CAPRISA 004 1% Tenofovir gel trial, provided evidence that a topical vaginal gel, used as PrEP, can confer protection (Abdool Karim et al., 2010). Several subsequent oral trials proved that oral PrEP could protect against HIV acquisition. However, PrEP efficacies have varied in women ranging from -49 to 75% with sub-optimal adherence being a key factor. Despite this fact, PrEP has been recommended in combination with other HIV prevention strategies and is currently being scaled up in several countries.

Therefore the development of an HIV vaccine, particularly one that can overcome the current adherence challenges and induce a functional protective antibody response against HIV, remains the ultimate HIV prevention strategy for this global epidemic [as reviewed (Safrit and Koff, 2016, Plotkin, 2010)].

Vaccine development, however, has proven challenging. Various factors such as waning antibody response following vaccination, the ability of the induced response to act against viral escape mutants, consensus on whether a vaccine should include multiple or single HIV epitopes, or target conserved or variable regions, contribute to the challenges associated with the development of an HIV vaccine (Rubens et al., 2015, Richman et al., 2003). The

RV144 vaccine trial is, to date, the only HIV vaccine trial, that achieved a modest 31.2% protection from HIV infection (Rerks-Ngarm et al., 2009). In recent years, vaccine research has also focussed on broadly neutralizing antibodies (bNAbs), which bind to various regions of the virus preventing HIV from infecting the cell, by inhibiting viral and target cell membrane or CD4 receptor fusion (Fauci et al., 2008). However, eliciting bNAbs through vaccination has been elusive. Particularly because bNAbs develop slowly, usually 2-3 years after HIV infection and require extensive levels of somatic hypermutation (SHM) in order to develop the breadth and potency required to neutralize various HIV virions (Mikell et al., 2011, Burton et al., 2012, Haynes et al., 2012b).

Interestingly, the observed efficacy in the RV144 trial was achieved in the presence of binding or non-neutralizing antibodies (nNAbs). Analysis of the immune correlates of protection, attributed protection to the induction of gp120 V1V2 specific nNAbs (Haynes et al., 2012a), which mediated antibody dependent cell-mediated cytotoxicity (ADCC) (Yates et al., 2014). Furthermore, ADCC can coordinate polyfunctional effector functions, a characteristic that correlated with HIV risk reduction in the RV144 trial (Chung et al., 2014, Chung et al., 2015). In addition to ADCC, a non-human primate study has shown evidence for the role of other nNAb functions, such as antibody dependent cellular phagocytosis (ADCP) as a correlate of protection (Barouch et al., 2013a). These findings underscore an important role of Fc-mediated functions in HIV protection.

Antibodies in the genital tract generally show close concordance with circulating systemic antibodies, however their relative abundance differ between compartments. Previously published data from Archary *et al.*, (2016) reported that HIV-1 specific binding antibodies to gp120, gp41, p66 and p24, IgG and IgA, were present in plasma and genital tract, in women who experienced breakthrough HIV infections, whilst using 1% tenofovir microbicide gel. Furthermore, HIV specific T cell responses were preserved and significantly increased magnitudes and frequencies of HIV-specific nNAbs were detected in the blood and genital tracts of women who used the tenofovir gel prior to coitus, compared to those women in the placebo arm (Archary et al., 2016). These data support the notion that exposure to PrEP, modulates immune responses in the genital and blood compartments (Castillo-Mancilla et al., 2015, Kersh et al., 2012, Archary et al., 2016). The Fc effector cell interaction in relation

to systemically and mucosally detectable nNABs of women, are largely unknown. Therefore, the objective of this study is to understand if detectability and magnitudes of HIV-specific nNABs have a functional cytotoxic and phagocytic advantage, in the context of prior topical PrEP exposure. This data may then enable us to tease apart putative immune correlates of protection, which may be highly desirable to elicit and block infection at the site of HIV exposure.

Elucidating the effects of PrEP on functional antibody such as IgG mediated ADCP and NK cell activated ADCC activity, may inform on future combination strategies aimed at increasing the qualitative and quantitative humoral immune response [as reviewed in (Corey et al., 2015)], ultimately leading to improved HIV prevention strategies that confer protection and prevent HIV acquisition, such as PrEP with vaccination.

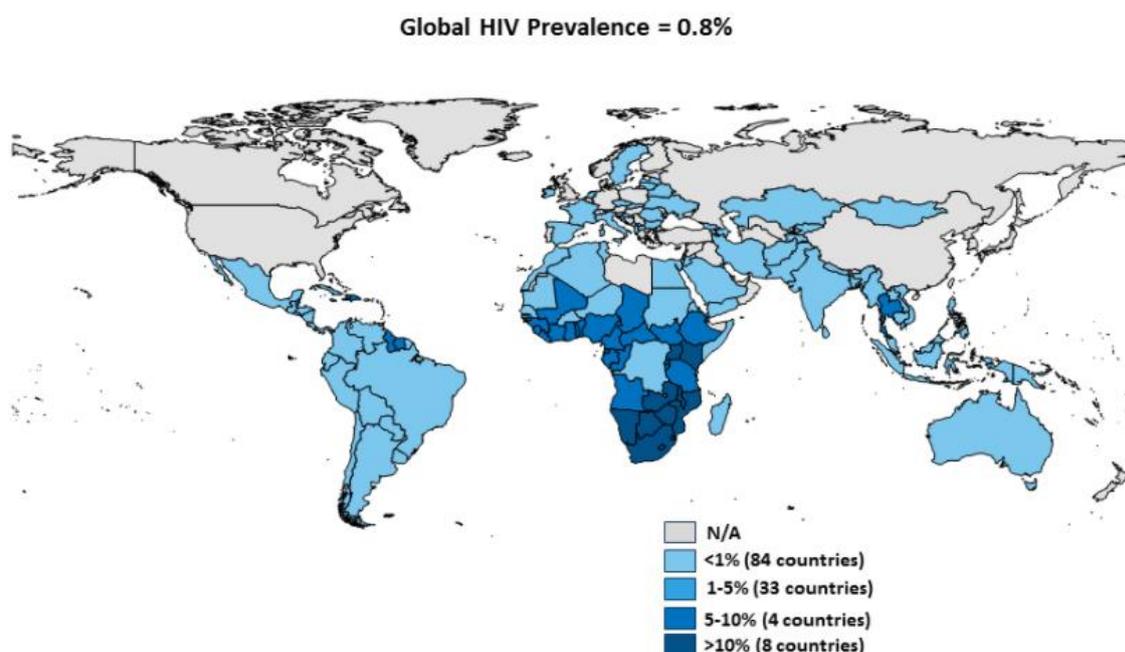
## CHAPTER 2

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### Literature Review

#### 2.1 HIV epidemic worldwide and in sub-Saharan Africa

HIV is a global epidemic affecting approximately 36.7million [34.0–39.8 million] people with a global prevalence rate of 0.8% (**Figure 1**) (Unaids, June 2016). Sub-Saharan Africa is home to approximately 64% of the world's new HIV infections (Unaids, 2014a). According to the most recent UNAIDS report South Africa bears the brunt of the HIV epidemic with approximately 7.1 million [6.4-7.8 million] HIV infected people living in South Africa (Unaids, 2017b).



**FIGURE 1: World Map illustrating the global adult prevalence of (Unaids, 2017b).**

In an effort to reduce new infections, initiatives to increase the distribution of antiretroviral treatment have reduced global new infections by 16% from 1.8 million [1.6-2.1 million] between 2005-2013, respectively (Kharsany and Abdool Karim, 2016). Despite these figures, South Africa is off track to meet the 2030 target of fewer than 500 000 annual new infections, with current estimates at 270 000 [ 240 000- 290 000] (Unaids, 2017a). Women between the ages of 15-24 years of age account for approximately 26% of new infections in

South Africa and are particularly vulnerable to HIV infection (Abdool Karim et al., 1992, Shisana et al., 2009, Unaid, 2017b).

In South Africa, the highest HIV prevalence rates are in the eThekweni district of Kwa-Zulu Natal (KZN) and the Ekurhuleni district in Gauteng. In Durban, KZN, the incidence rate is 6.3 per 100 women years, in woman under the age of 25 years, underscoring the need for effective combination prevention strategies, particularly in these vulnerable populations (Shisana et al., 2009).

Several social, behavioural and biological factors have been identified as contributors to women's susceptibility to HIV infection (Adimora et al., 2013). Multiple sex partners, HIV viral load of the partner, gender based violence, sexually transmitted infections (STIs), the vaginal microbiome and age disparate sexual pairing, increases the HIV acquisition risk in women (Kokab et al., 2010, Gianella et al., 2013, Lisco et al., 2012, Taha et al., 1998, Gregson et al., 2002, Van Der Straten et al., 1998, Dunkle et al., 2004, Abdool Karim, 2011, De Oliveira et al., 2017). HIV can be transmitted in several different ways, such as mother to child transmission, needle stick injuries, transmission by intravenous drug users and blood transfusions (Spinner et al., 2016). However, sexual transmission is the most frequent mode of HIV transmission and predominantly occurs via mucosal portals of entry, such as the genital tracts of females and males, as well as via the rectal mucosa [as reviewed in (Iwasaki, 2010)]. Additionally, biological factors in women, such as the mucous consistency in the female reproductive environment and surface area, may also influence HIV acquisition (Nicolosi et al., 1994). Factors such as endogenous hormones, the menstrual cycle and the use of injectable or oral hormonal contraceptives have been shown to influence the mucosal environment and may contribute to an increased risk of HIV infection (Tucker et al., 2012, Ralph et al., 2015, Morrison et al., 2015). *In vitro* and non-human primate (NHP) studies, have shown that depot-medroxyprogesterone acetate (DMPA), which causes epithelial thinning of the squamous epithelial layer, compromises epithelial barrier integrity (Poonia et al., 2006, Hadzic et al., 2014, Ngcapu et al., 2015). Furthermore, DMPA use in women, alters the innate immune profile of the female genital tract, acting as an immune-suppressive agent further increasing the risk of HIV acquisition in women (Ngcapu et al., 2015). However, such findings of the biological and cellular changes

associated with DMPA use, are yet to be confirmed in human studies designed to answer these questions and remains a topic of ongoing debate (Hild-Petito et al., 1998, Marx et al., 1996, Mitchell et al., 2014, Bahamondes et al., 2014, Mauck et al., 1999, Miller et al., 2000). Therefore, due to the disproportionate risk and burden of HIV in women, strategies to reduce the risk of HIV acquisition in the absence of male partner co-operation are key to limit and control the spread of HIV (Adimora et al., 2013, Mabala, 2006, Ramjee, 2013). Various clinical trials investigating the efficacy of PrEP in the form of oral ARV drugs, subcutaneous ARV administration and topical ARV-containing microbicide gels have been conducted; are still on-going or are in the developmental pipeline, in order to identify the most effective modality of protection for high risk populations, especially women (**Table 1**) (Abdool Karim et al., 2010, McCormack et al., 2016, Gunawardana et al., 2015, Thomson et al., 2016, Baxter and Abdool Karim, 2016). Although protection from HIV infection has yet to be fully achieved, current trials focussing on improving adherence through convenience and multipurpose prevention technologies (MPT) for women in resource-limited countries, are being investigated (Baxter and Abdool Karim, 2016). MPT strategies aim to address various health concerns, such as protection from HIV, other sexually transmitted infection (STI's) and unintended pregnancies simultaneously (Fernández-Romero et al., 2015, Malcolm et al., 2014, Malcolm et al., 2016, Malcolm and Fetherston, 2013, Woodsong and Holt, 2015, Romano et al., 2013).

## 2.2 PrEP in prevention

While MPT strategies are in the pipeline, effective PrEP administration, dosing and combinations, are still being investigated in order to protect young women who continue to be a high-risk population (Baxter and Abdool Karim, 2016).

The efficacy of PrEP in providing adequate protection from HIV is disparate between men and women (**Table 1**). PrEP studies in men have achieved good levels of protection, with oral administration providing up to 86% (95% CI: 39% to 99%) protection from HIV infection (see **Table 1**) (Patterson et al., 2011). However, inconsistent results have been observed for PrEP trials involving women. Various trials have reported no to low efficacy and others range from -49% to 75% effectiveness against HIV (**Table 1**) (Spinner et al., 2016, Rees et al., 2015, Marrazzo et al., 2015).

The CAPRISA 004 1 % tenofovir gel trial had the highest efficacy of 39%, compared to all other PrEP gel trials in women (Van Damme, 2012, Marrazzo et al., 2015, Donnell et al., 2014, Gomez, 2013, Thigpen et al., 2012, Mujugira et al., 2011, Abdool Karim et al., 2010). Poor adherence to PrEP is a major factor impacting PrEP efficacies, as seen with the CAPRISA 004 trial and other studies, which reported high tenofovir gel adherence conferred a 53% efficacy (Van Damme, 2012, Marrazzo et al., 2015, Donnell et al., 2014, Gomez, 2013, Thigpen et al., 2012, Mujugira et al., 2011, Abdool Karim et al., 2010, Baeten et al., 2016). Detectable concentrations of tenofovir or any other PrEP agent, in the blood are key in protection from infection, as this factor was associated with >85% protection in the Partners PrEP study (**Table 1**) (Donnell et al., 2014). Additionally, in an effort to overcome adherence the Ring and ASPIRE trials investigated the potential of a monthly intravaginal ring containing dapivirine. Observed efficacies were between 27-31%, respectively (Baeten et al., 2016, Palanee-Phillips et al., 2015). Further, post-hoc analysis revealed that older women, >21 years of age had higher rates of protection compared to younger women, <21 years, 56% (95% CI, 31 to 71) vs -31% (95% CI 1 to 31%) (***P* < 0.001**) (Baeten et al., 2016).

The discrepant efficacies in women emphasize the need to understand the biological mechanisms that affect PrEP efficacies (Spinner et al., 2016, Baxter and Abdool Karim,

2016). Besides adherence, the low levels of protection observed may have been influenced by hormonal contraceptive use (as previously mentioned), immature mucosae of the young women, high viral load in partners, increased levels of pro-inflammatory cytokines, which recruit target cells and higher exposure of the vaginal surface area to virus (Celum et al., 2015, Chen et al., 2007, Van Damme, 2012, Yi et al., 2013, Nicolosi et al., 1994). Interestingly, a further analysis of the CAPRISA 004 trial revealed that the vaginal microbiome also effects efficacy of tenofovir containing PrEP agents (Klatt et al., 2017). In that sub-study, women with bacterial vaginosis, which is associated with a dysbiotic vaginal microbiome, resulted in reduced efficacy of the tenofovir gel. This was attributed to the presence of *Gardnerella vaginalis* and other anaerobic bacteria, which metabolized tenofovir at a higher rate than the cells, thereby reducing the active metabolite available to inhibit HIV reverse transcriptase activity (Klatt et al., 2017, Abdool Karim et al., 2010, De Clercq, 2007).

Taken together, these factors emphasize the importance of better understanding the vaginal mucosae and contributing risk factors, as these factors have been shown, to significantly, alter the outcome of intended protective modalities. Ultimately, these prevention strategies and outcomes of PrEP trials emphasize the need for an HIV vaccine, which can ultimately overcome these challenges, eliminate new infections all together and provide complete protection from HIV, in both men and women. Furthermore, despite the strides made in some of the PrEP trials in certain populations, due to the relative newness of PrEP, the long-term biological effects are not well described (Pattacini et al., 2015). Following the approval of Truvada® as PrEP and the recent WHO recommendation for PrEP as an additional prevention strategy in high risk populations, knowledge regarding humoral immune responses, in the presence of PrEP is limited (Archary et al., 2016). A recent study by Archary *et al.*, (2016) demonstrated that in women with HIV infection who used the topical 1% tenofovir gel prior to infection; distinct mucosal and blood antibody profiles differentiated them from women in the placebo arm were detected. Therefore, determining the functional abilities of antibody responses, other than neutralization, in the context of PrEP is of paramount importance.

**TABLE 1: Overview of the prospective systemic oral PrEP, topical and other HIV prevention studies [adapted from Kharsany and Karim (2016)].**

Study	Risk population	PrEP Agent	Effect size (95% CI)
<b>CAPRISA 004 (Abdool Karim et al., 2010)</b>	Women	TFV gel	39% (6; 60)
<b>MTN 003 VOICE (Marrazzo et al. 2015)</b>	Women	TFV gel	15% (-21;40)
<b>FACTS 001 – peri-coital tenofovir gel (Rees et al., 2015)</b>	Heterosexual women	TFV gel	0% (-40;30)
<b>MTN020-ASPIRE Intravaginal Ring (Palanee-Phillips et al., 2015)</b>	Women	Dapivirine Ring	27% (1;46)
<b>IPM 027(Nel et al., 2014)</b>	Heterosexual	Dapivirine Ring	30.7% (0.90;51.5)
<b>IPERGAY (Molina et al. 2015)</b>	MSM	TDF/FTC	86% (39;99)
<b>PROUD (McCormack et al. 2016)</b>	MSM	TDF/FTC	86% (62;96)
<b>Partners PrEP daily (TDF/FTC) (Baeten et al. 2012)</b>	HIV-discordant couples	TDF/FTC	75% (55;87)
<b>Partners PrEP daily Tenofovir (Baeten et al. 2012)</b>	HIV-discordant couples	TDF	67% (44;81)
<b>TDF2 (Thigpen et al. 2012)</b>	Heterosexual couples	TDF/FTC	62% (22;83)
<b>iPrEX (Grant et al. 2010)</b>	MSM	TDF/FTC	44% (15;63)
<b>Bangkok Tenofovir Study (Choopanya et al. 2013)</b>	IDU	TDF	49% (10;72)
<b>Fem-PrEP (Van Damme et al. 2012)</b>	Heterosexual women	TDF/FTC	6% ( -52;41)
<b>MTN003/VOICE daily (TDF/FTC) (Marrazzo et al., 2015)</b>	Heterosexual women	TDF/FTC	-4% (-49;27)
<b>MTN003/VOICE daily (Tenofovir) (Marrazzo et al., 2015)</b>	Heterosexual women	TDF	-49% (-129;3)

\*Note: TFV – tenofovir; PrEP – pre exposure prophylaxis; TDF/FTC – truvada (tenofovir disoproxil fumarate and emtricitabine); IDU- intravenous drug users

### **2.3 Biological drivers of HIV acquisition and the effect of PrEP on immune parameters**

Immune activation, genital tract inflammation and the recruitment of activated target T cells are known immunological factors that drive HIV infection risk and HIV disease progression (Masson et al., 2015). The concept of dampening immune responses in the genital tract, to decrease the risk of infection is a topic of ongoing debate [as reviewed in (Card et al., 2013),(Naranbhai et al., 2012)]. Furthermore, in preclinical studies, macaques on oral PrEP showed a reduced inflammation profile and a reduced level of CD4<sup>+</sup> T cell loss during breakthrough SIV infections, compared to controls (Kersh et al., 2012). The study demonstrated that despite HIV infection, prior PrEP improves early immune parameters, which may then impact on the rate of disease progression (Kersh et al., 2012, Castillo-Mancilla et al., 2015, Naranbhai et al., 2012). Similar findings in a sub-study of CAPRISA 004 women were found, reporting the preservation of HIV-specific CD4<sup>+</sup> T cells (Mureithi et al., 2012). CD4<sup>+</sup> T cells play an important role in determining antibody avidity and therefore contribute to shaping the adaptive immune response (Morita et al., 2011, Boswell et al., 2014). Furthermore, in an animal study, topical rectal application of tenofovir gel, reportedly primed T cell responses, supporting the role of PrEP as a prime/boost strategy (Cranage et al., 2008).

Although some studies have shown no evidence of immunological changes in HIV uninfected individuals on oral PrEP (Baeten et al., 2012), other studies report reduced levels of systemic immune activation in HIV-uninfected individuals on oral PrEP (Castillo-Mancilla et al., 2015). Additionally, studies investigating the effect of topical tenofovir gel, reported a reduced immune activation identified as a reduction in activated CD4 T target cells and biomarkers of inflammation (Vibholm et al., 2012, MCGowan et al., 2013), known factors associated with HIV infection risk and disease progression (Masson et al., 2015). The influence of the reduced immune activation on antibody functionality have however, not been described.

Interestingly, studies have also reported that PrEP delays antibody avidity but has no effect on time to seroconversion, in those experiencing breakthrough HIV infection (Curtis et al., 2011, Laeyendecker et al., 2015). However, decreased antibody titres have been associated with the use of ARV's during HIV infection, but the functionality of these antibodies in the

presence of low antibody titres remains unresolved. (Hare et al., 2006, O'sullivan et al., 2002, Payne et al., 2015).

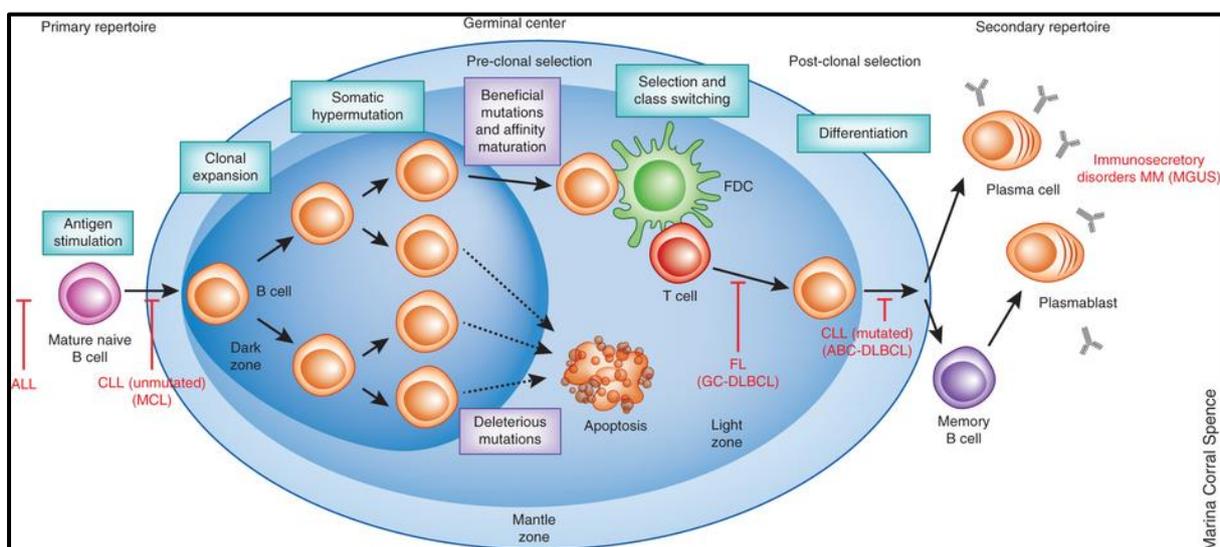
Breakthrough HIV infection amongst those who participate in PrEP trials can directly inform on the effects of PrEP on the immune responses. This is important in order to, better inform on the potential impact of prior PrEP usage on vaccine efficacy, particularly in an era where combination prevention strategies using both vaccines and PrEP are likely. Furthermore, the immune responses that prevail at the female genital mucosal regions, especially in the context of combination therapy, such as vaccines and PrEP, is not well described (Archary et al., 2016).

#### **2.4 Humoral immune responses and HIV**

Antibodies develop at least 1-week post HIV infection in the presence of detectable viremia, however, these antibodies appear to have no effect on modulating viremia due to escape variants (Tomaras et al., 2008). This is due to autologous, strain-specific antibodies, which have narrow breadth and do not neutralize virus isolated from other individuals as well as those that arise through mutation and immune pressure. Transmission of HIV from infected mothers to their infants is not inhibited by neutralizing antibodies (NAbs), due to the transmission of escape variants (Dickover et al., 2006, Wu et al., 2006). Surprisingly, non-neutralizing antibodies showed a role in decreasing transmission of HIV to infants and increasing mortality, due to the activity of antibody mediated cellular cytotoxicity (Mabuka et al., 2012, Milligan et al., 2015). These data add to the mounting evidence that non-neutralizing antibodies may play a role in modulating HIV transmission. In order to understand the role of non-neutralizing antibodies in HIV infection, the developmental process of antibodies and the potential role of a polyfunctional immune response, needs to be explored.

## 2.4.1 The Development of Antibodies

When pathogens are encountered by the immune system the innate immune cells like macrophages, engulf the invading pathogen (**Figure 2**). Macrophages are professional antigen presenting cells (APC) and present certain parts of the pathogen via MHC class II proteins to naïve T cells, which bind and subsequently produce IL-1, resulting in activation of the naïve T cell (**Figure 2**). Once activated, the naïve T cell differentiates and proliferate, and become either a cytotoxic T cell or a T helper cell. The cells produce IL-2, which stimulates T cell proliferation. The T helper cell then activates the mature naïve B cells by stimulation with APC's. B cells, which migrate into the dark zone of the germinal center (GC) located in the lymph nodes (**Figure 2**), undergo clonal expansion, somatic hypermutation (SHM), pre-clonal selection, selection, class switching and differentiation (**Figure 2**). This process ultimately results in the production of antigen specific antibodies (Georgiou et al., 2014). Each of these B cells express unique B cell receptors (BCR) which recognize specific antigens. Inside the dark zone, the B cell forms centroblasts, where affinity maturation occurs by SHM of the fragment binding region (Fab), selecting for the B cell with the highest affinity for a specific antigen (**Figure 2**).

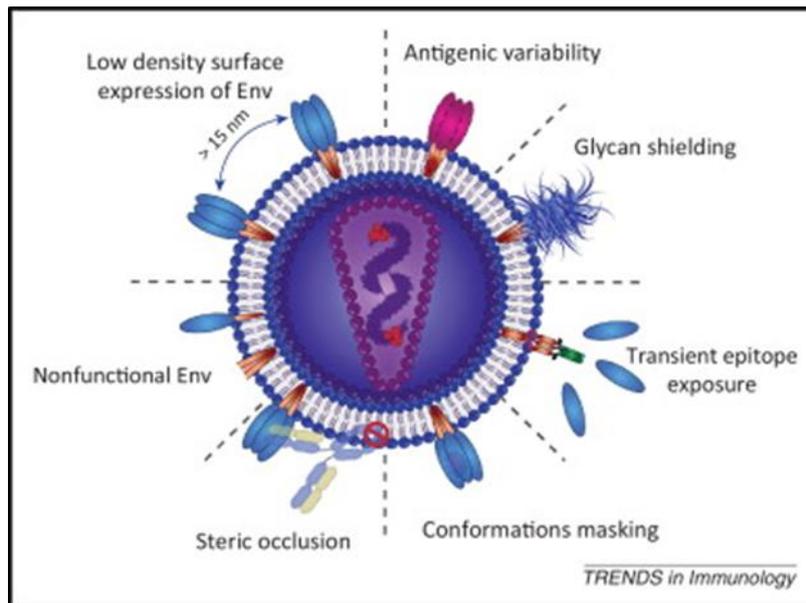


**FIGURE 2: The various stages involved in the development of an antigen-specific B cells responses to a foreign antigen or pathogen in the lymph node [adapted from (Georgiou et al., 2014)].**

During SHM, the expressed BCR mutates via genetic recombination of the genes- which form the VDJ complex- and the BCR, in order to generate the best fit to the pathogen [as

reviewed in (Haynes and McElrath, 2013)]. Throughout this process, T follicular helper cells aid in antibody avidity maturation, selecting against auto reactive B cells, however, this process of tolerance is not fully understood (King et al., 2008). Following SHM, the centroblast moves to the light zone becoming a centrocyte, undergoes class switching of the fragment crystallizable (Fc) portion, after which it will proliferate and become either a plasma cell or memory cell (**Figure 2**). These GCs generate antibodies that are long-lived, with a high antigen affinity to a specific pathogen, usually spanning the lifespan of the individual. After the immune response has concluded, which is usually associated with pathogen clearance, the B cells contract to form memory B cells. Upon reinfection, memory B cells will be stimulated to undergo clonal expansion, to produce many pathogen/antigen-specific antibodies [as reviewed in (Mouquet, 2014), (Georgiou et al., 2014)] .

In the context of HIV infection, the pathogenic characteristics of HIV poses a challenge for the immune system to develop antibodies that are capable of clearing the infection. HIV-1, has rapidly mutating envelope spikes that differ 35-40% in their amino acid sequence between subtypes (Lynch et al., 2009, Gaschen et al., 2002). In addition, the strain-specific antibodies and ensuing immune pressure, creates viral escape mutants, adding to the challenge of developing a vaccine [as reviewed in (Mouquet, 2014)]. The high recombination rate of HIV *in vivo* and lack of a proofreading polymerase enzyme, introduces mutations into the genome, which ultimately affects the expression of the glycoprotein (Ramirez et al., 2008, Zhuang et al., 2002). Effective NAbs require the recognition of a functional HIV-epitopes that are vulnerable to the neutralization. This is often difficult due to highly variable regions and glycosylation which masks envelope (Env) epitopes (Kwong et al., 1998, Richman et al., 2003, Herrera et al., 2003, Chackerian et al., 1997, Wei et al., 2003, Bunnik et al., 2008)]. The carbohydrates or glycans, that cover HIV-1, form rapidly shifting glycan shields (**Figure 3**), which prevent antibodies from binding, through masking recognition of key sites and steric hindrance, resulting in immune evasion [as reviewed in (Overbaugh and Rudensey, 1992, Wyatt, 1998), (Haynes et al., 2012b, Richman et al., 2003, Herrera et al., 2003, Wei et al., 2003, Chackerian et al., 1997)]. Furthermore, the envelope of HIV can present various faces to the immune system with non-functional spikes etc., often baiting the immune system and resulting in antibodies that are inept against the virus (**Figure 3**) (Moore et al., 2006).



**FIGURE 3: Various conformational structures of the HIV envelope.** HIV presents different immunogenic faces to the immune system including shifting glycan shields, conformational masking, non-functional envelope stumps and a low level of Env expression on the surface of the virus that contribute to the virus' pathogenicity, immune evasion and immune dysfunction [adapted from (Mouquet, 2014)].

When HIV is transmitted, the virus encounters  $CD4^+$  T cells and undergoes several conformational changes, which enables binding of the gp120 spike of the Env trimer, to  $CD4$  and  $CCR5$  co-receptors on  $CD4^+$  T cells (Burton et al., 2004, Burton et al., 1994). At this point, the virus may be vulnerable to various antibodies, that can neutralize and prevent binding, therefore presenting optimal sites to target for NAb [as reviewed in (Overbaugh and Morris, 2012)]. These regions are conserved due to their functional importance in receptor binding between the virus and target cell (Hendrickson et al., 1998, Wyatt, 1998, Calarese et al., 2003, Huang et al., 2007, Zhou et al., 2007). Effective antibodies require immense breadth (able to recognize a variety of diverse strains), potency (needing small amounts to be effective) and should have high affinity to conserved and their cognate epitopes (Klein et al., 2013, Barouch et al., 2013a, Burton et al., 2004). B cells often produce a large variety of antibodies that often target a wide array of HIV viral proteins.

Env proteins which are expressed on the surface of the virus, are composed of three heterodimers of gp120, anchored into the viral membrane by a gp41 stalk (**Figure 3**) (Stamatatos et al., 2009). Gag proteins also known as p24 antigen, compose the capsid protein structure of the HIV virus (**Figure 3**) and is a marker of infection (Fiebig et al., 2003),

disease progression (Wolf et al., 1988) and immune responses to infection (Mcrae et al., 1991). Other internal proteins include p66 which is produced by the Pol gene that encodes for the reverse transcriptase enzyme (**Figure 3**)- the protein responsible for the replication of viral RNA [as reviewed in (Jacobo-Molina and Arnold, 1991), (Di Marzo Veronese et al., 1986). Nonnucleoside reverse transcriptase inhibitors (ARV drugs), target the enzyme, inhibiting its ability to bind and synthesize new copies of proviral DNA [as reviewed in (De Clercq, 1998), (Sluis-Cremer et al., 2000)]. These viral proteins represent potential targets for HIV vaccine development where both neutralizing antibodies and antibodies mediating Fc-related functions may be exploited.

NABs which develop months after infection, generally target the variable glycosylated region of HIV-1 Env (Rong et al., 2009, Moore et al., 2009). However, antigenic variability prevents recognition between the antibody Fab region and the antigen epitope, also arising from *in vivo* recombination events between envelopes of HIV quasispecies or in some cases, superinfection (Preston et al., 1988, Mansky and Temin, 1995).

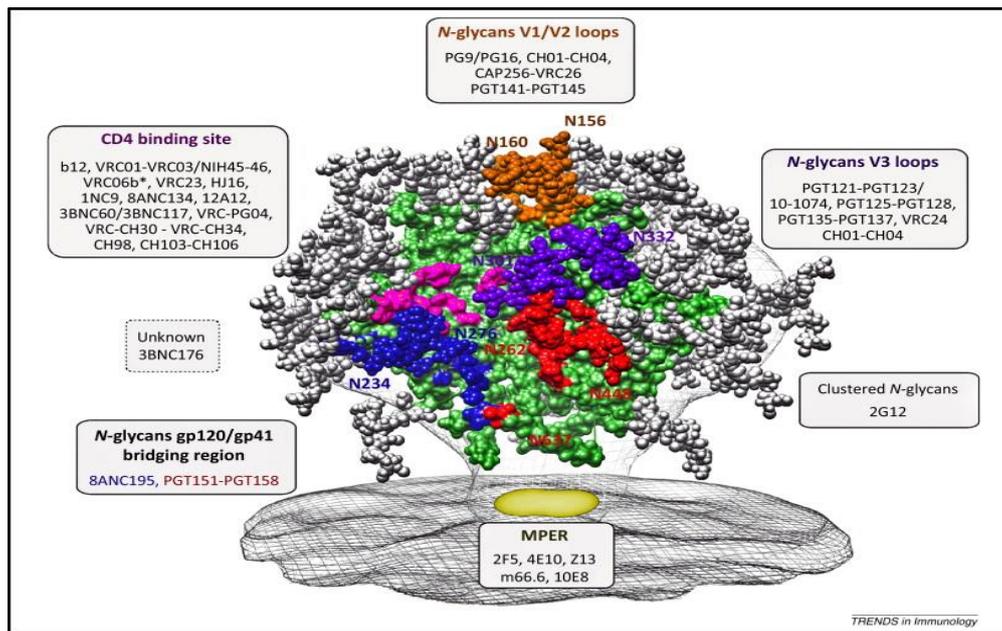
These characteristics of the HIV virus, as well as others such as virus size [as reviewed in (Parren and Burton, 2001)], the proximity of Env surface proteins to each other, the characteristics of the viral proteins i.e. whether one Env surface protein can mediate viral entry, as well as the number of viral surface proteins (Zhu et al., 2003), influence vaccine design. HIV has between 8-10 viral envelope spikes expressed on its surface. But, every single one of these Env spikes need to be blocked or neutralized in order to prevent infection, as one spike can mediate viral entry (Yang et al., 2005b, Zhu et al., 2003). BNABs capable of neutralizing a variety of epitopes, in particular, have the ability to directly inhibit or obstruct the virus and target cell co-receptor from engaging despite mutational differences, between virus envelope proteins (Walker, 2009, Blish et al., 2009). BNABs in particular have the ability to directly inhibit or obstruct the virus and target cell co-receptor from engaging and these are known as entry inhibitors like VRC01 (Wu et al., 2010). Other bNABs include, IgG1 b12 a CD4 binding antibody (Saphire et al., 2001), fusion inhibiting MPER antibodies e.g. 4E10, which directly obstruct fusion between the target cell and HIV virus membrane (Zwick et al., 2001, Zwick et al., 2005, Cardoso et al., 2005).

#### 2.4.2 Broadly Neutralizing Antibodies

BNAbs have two characteristics that distinguish them from other neutralizing antibodies—breadth and potency (Moore et al., 1996, Moog et al., 1997, Beirnaert et al., 2000, Binley et al., 2008, Piantadosi et al., 2009, Sather et al., 2009, Gray et al., 2011, Simek et al., 2009).

However, current knowledge of bNAbs, have reported that these types of antibodies take 2-3 years to develop and furthermore are only present in 20-30% of HIV infected individuals, therefore not providing immediate protection (Binley et al., 2008, Doria-Rose et al., 2009, Sather et al., 2009, Simek et al., 2009, Li et al., 2009). Thus, emphasising the need for novel innovative, scientific approaches that overcome current challenges associated with bNAb development. Novel approaches include, reverse engineering. An approach that involves mimicry of cognate epitopes, the sequence footprint and structure of an HIV envelope, to elicit protective bNAbs [as reviewed in (Burton, 2002, Koff, 2012)].

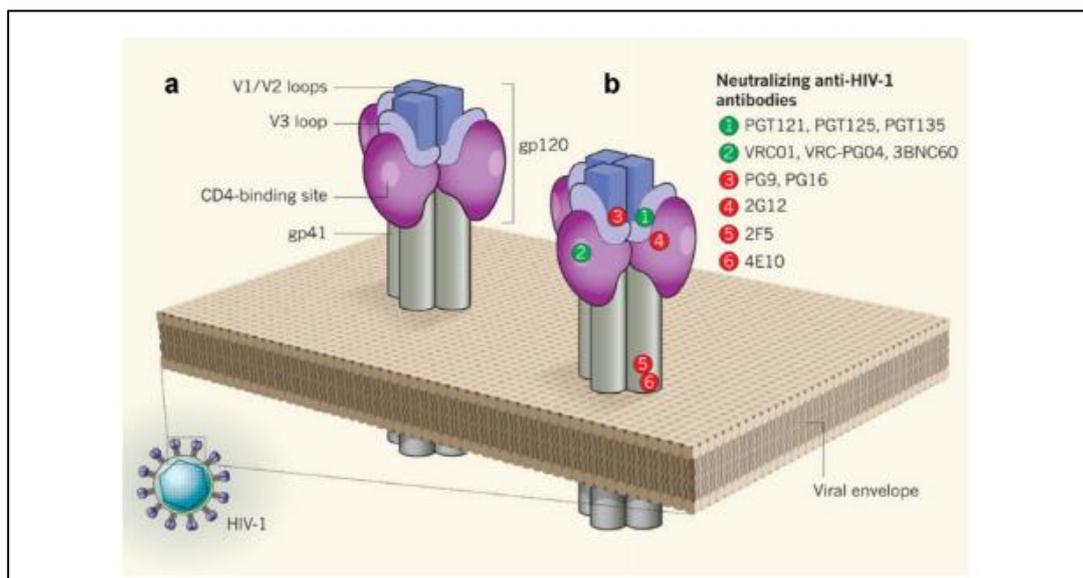
The early 1990's revealed the discovery of what is now known as first generation bNAbs, which included IgG1b12 (b12), 2G12, 2F5 and 4E10, with each antibody binding to different regions of the virus (Walker et al., 2011). B12 binds to a conserved region on the CD4 binding site (Walker et al., 2011, Saphire et al., 2001), 2G12 targets an area rich in mannose residues present on the glycan shield having a unique domain-exchange structure (Trkola et al., 1996, Trkola et al., 1995), 4E10 targets a linear epitope (NWFDIT) in the membrane proximal external region (MPER) of gp41 (Stiegler et al., 2001), and 2F5 targets a ELDKWA motif ectodomain of gp41 (**Figure 4**) (Corti et al., 2010).



**FIGURE 4: A schematic diagram of HIV-1 and the regions of the surface glycoproteins targeted by various broadly neutralizing antibodies.** These include 2G12, the PGT series -targets the N-linked glycan on V3. PG9/16, CAP256 VRC26.25, PGT 141-145-targets the glycans on the V1V2 loops. The CD4 binding site antibodies (b12, VRC01 etc., VRC series and the 3BNC117) and the CH antibodies, the PGT151-158 and 8ANC195 that target the glycans on gp120/gp41 interface. Lastly, the MPER antibodies that include 2F5, 4E10, Z13, m66 and 10E8 3BNC176 binding site remains as yet undefined [adapted from (Mouquet, 2014)].

The development of assays like the microneutralization assay, high throughput single cell, B cell receptor amplification and novel Env selection tools, have resulted in an explosion in the discovery of second generation antibodies, which exhibit increased potency and neutralization breadth [as reviewed in (Koff, 2012)]. These second generation antibodies target CD4 binding site (VRC01, VRC07, 3BCN117) and conserved regions on the V1V2 Env gp120 protein and have given rise to antibodies like PG9 (Huang et al., 2012), PG16 (Walker, 2009) (**Figure 5**) and other antibodies such as CAP256 VRC26.25 (Moore et al., 2011). CAP256 VRC26.25 is a V2 glycan dependent highly potent bNAb, with a bias towards subtype C and A virus neutralization, with exhibiting broadly cross neutralizing capacity against 76% of HIV strains (Moore et al., 2011).

BNABs are unique in that they undergo extensive SHM and some like the CAP256 VRC26.25, have long 30-33 amino acid complementary determining regions on the heavy chain 3 (CDRH3), which likely contributes to the breadth (see **Figure 6**) (Moore et al., 2011). Another potent bNAb with a long CDR region is PGT121, a V3 glycan dependent antibody (Walker et al., 2011). However, in reality, the induction of such antibodies with long CDR regions are problematic, as the germline cells that produce such antibodies are selected against by check-points in the immune system, which function to prevent autoimmune reactivity (Doria-Rose et al., 2014, Mclellan et al., 2011, Wardemann et al., 2003). Insertions and deletions generated by SHM in these variable regions for antigen binding of the antibody, controls for the fit and affinity that occurs between these bNABs and the cognate sites on the Env protein (Sok et al., 2013). Vaccines that strive to elicit the production of bNABs have to overcome the high levels of SHM required in the B cell lineage in the germinal centre in order to develop antibodies with increased neutralization breadth and potency.



**FIGURE 5: HIV epitopes targeted by broadly neutralizing antibodies.** Env spikes composed of a trimer of a heterodimer comprising gp120 and gp41 transmembrane glycoprotein are depicted in purple and grey respectively. The gp120 trimer has variable loops made up of V1 and V3 as well as containing the site for CD binding. The diagram above outlines the regions targeted by the above mentioned broadly neutralizing antibodies, [adapted from(Koff, 2012)].

This would require long regimens of vaccine prime/boosts in order to induce sufficient SHM for generation of these bNABs (Sok et al., 2013, Zhou et al., 2007, Xiao et al., 2009). Despite

these challenges, candidate modified Env immunogens are being pursued to generate these bNAbs, and testing of a potent bNAb or a combination of bNAbs passive administration to prevent HIV infections are currently underway (Rubens et al., 2015).

In passive immunity studies the V3 glycan dependent bNAb PGT121 proved to be potent and able to neutralize approximately 70% of the virus reference strains, in intravaginally challenged macaques (Walker et al., 2011, Barouch et al., 2013a, Barouch et al., 2013b). Previously expressed glycoproteins, have been used to probe HIV-infected sera for unique antibodies, eventually leading to the discovery of VRC01 (Walker et al., 2011), a CD4 binding site antibody, and 3BCN117- also a CD4 binding site antibody (**Figure 5**), which was isolated from a viraemic controller (Scheid et al., 2011). Interestingly, the recent discovery of N6, a potent CD4bs NAb capable of neutralizing 98% of HIV-1 isolates, was discovered and is currently undergoing further investigation for use in future clinical trials (Huang et al., 2016). Passive immunization studies using broadly neutralizing antibodies have shown potential in preventing HIV infection (Armbruster et al., 2004, Mascola et al., 2000, Mascola et al., 1999, Shingai et al., 2014). Furthermore, studies conducted pre-ART era, investigated the potential of passive immunity through, inactivated hyper-immune plasma containing anti-HIV immunoglobulins that likely included both neutralizing and non-neutralizing activities. These studies provided early evidence of passive immunity in ameliorating HIV disease progression and suppressing viral replication [as reviewed in (Stephenson and Barouch, 2016)]. Initial findings showed viral clearance through declining p24 antigen detection (Jackson et al., 1988, Karpas et al., 1990, Karpas et al., 1988). However, other passive immunity studies in contrast showed no impact on the morbidity or mortality of HIV-infected subjects through viral clearance or increased CD4<sup>+</sup> T cell counts (Jacobson et al., 1993). Further conflicting data showed improved clinical parameters like increases in CD4 T cell counts (Levy et al., 1994), and no delay to the progression to AIDS-defining criteria (Vittecoq et al., 1992). Despite the mixed results, passive immunization with bNAbs have shown promise.

Passive immunity studies in the 1990's and early 2000's mainly tested first generation bNAbs like 2G12, 2F5 and 4E10 (Armbruster et al., 2004, Armbruster et al., 2002, Stiegler et al., 2002, Trkola et al., 2005, Joos et al., 2006, Morris et al., 2014, Günthard et al., 1994). These bNAbs at high doses were safe, and well tolerated, and conferred transient viral

suppression in HIV-infected individuals. Proof of concept clinical trials in humans, using passive immunity with the newer, more potent second generation of bNAbs, are currently underway and include bNAbs such as VRC01, VRC07, PGT121 and CAP256 26.25LS. These antibodies have been previously tested in preclinical studies and *in-vivo*, in small animals and *in-vitro* and VRC07 and PGT121 and CAP256 26.25 LS (Rudicell et al., 2014, Dosenovic et al., 2015, Julg et al., 2017, Lynch et al., 2015).

Although bNAbs show promise, eliciting these types of antibodies through vaccination remains a formidable challenge, and while the proof-of-concept studies involving passively infused bNAbs to prevent infection are highly successful and confer complete protection in animal models, the practicality, cost and wide-scale use in humans is a substantial hurdle (Shingai et al., 2014, Mascola et al., 1999).

## **2.5 Vaccines and Vaccine Trials**

The development of an efficacious HIV vaccine remains a challenge and is a public health priority. After three decades of HIV vaccine research focussed on studying the relationship between the virus pathogenicity and the host immunogenicity, the majority of researchers believe that engaging both arms of the adaptive immune system may be the best approach (Chhatbar et al. 2011). Retroviruses such as HIV, have developed mechanisms of evading the immune system like glycosylation and frequent mutation of dominant epitopes, therefore underscoring the need for innovative approaches to vaccine strategies (Gulick et al., 2008, Yang et al., 2005a).

Vaccine immunogen design usually involves the administration of an attenuated or whole inactivated virus, to induce an immune response, known as active immunization [as reviewed in (Law and Hangartner, 2008)]. However, this strategy in the context of HIV, is potentially dangerous as the virus could revert to the wild type, integrating its viral genome into the host DNA, eventually resulting in HIV infection [as reviewed in (Koff, 2012)].

In 1998, researchers genetically engineered a canarypox vaccine as a prime and a protein boost that included Gag and Env proteins from the HIV virus (Belshe et al., 1998, Rubens et al., 2015). NAbs and CD8 T cells directed towards Gag and Env-proteins were detected amongst 64% of volunteers, however, these results were not replicated in a larger clinical

trial (Belshe et al., 1998, Russell et al., 2007). This initial study in 1998, established the concept for the prime-boost strategy, which was used in the RV144 trial (Rubens et al., 2015). However studies that preceded and proceeded the RV144 trial in 2009, such as HVTN P5, HVTN 505, VAX004, and VAX003, HVTN 503 (STEP and Phambili) some of which were recombinant DNA vector virus trials, showed no protection- **(Table 2)**, (Rubens et al., 2015). Trials such as the STEP and PHAMBILI trials had no efficacy **(Table 2)**, with the STEP trial exhibiting an increased risk for HIV infection (Gray et al., 2010, Buchbinder et al., 2008). Specifically, within the STEP trial the increased risk of HIV infection was found following the analysis of a specific population of individuals.

The RV144 trial in 2009, included 16,402 healthy individuals and is the only vaccine trial to achieve a modest efficacy of 31.2% (95% CI, 1.1 to 52.1) (Rerks-Ngarm et al., 2009). The vaccine expressed HIV-1 Gag and Pol (subtype B LAI strain) and CRF01\_AE (subtype E) HIV-1 gp120 (92TH023) (Rerks-Ngarm et al., 2009). The protein boost was AIDS VAX B/E, a bivalent subtype B/E HIV-1 gp120 glycoprotein (Rerks-Ngarm et al., 2009). Ensuing responses from those vaccinated, mediated protection from infection by non-neutralizing, binding antibody responses to the V1V2 regions of the gp120, and Env and Gag CD4+ T cell responses, have also been reported (Tomaras et al., 2012). While the V1V2 binding antibodies were not broadly neutralizing, they did mediate non-neutralizing Fc mediated antibody functions, namely antibody dependent cellular cytotoxicity (ADCC) and elicited NAb's not capable of achieving great breadth (Tomaras et al., 2012). Moreover, the correlates of risk of HIV infection were identified as an enhanced IgA or a reduced gp70 IgG antibody response (Zolla-Pazner, 2014, Haynes et al., 2012a, Bonsignori M, 2012, Chung et al., 2014, Bomsel et al., 2011).

Following the original RV144 Trial, South Africa and South America are currently testing the ALVAC vaccine with a subtype C envelope gp120 **(Table 2)** (Rubens et al., 2015).

**TABLE 2: Efficacies of past and ongoing HIV vaccine trials [adapted from (Rubens et al., 2015)].**

<b>Study</b>	<b>Site</b>	<b>Active/ Passive Vaccine</b>	<b>Volunteers</b>	<b>Vaccine to Placebo randomization</b>	<b>Results</b>
<b>Vax004</b>	USA and Netherlands	AIDSVAX B/B' Env gp120 with alum	5100 MSM and 300 women	2:1	No vaccine efficacy
<b>Vax003</b>	Thailand	AIDSVAX B/E Env gp120 with alum	2500 men and women IDUs	1:1	No vaccine efficacy
<b>HVTN 502 Step trial</b>	North and South America, the Caribbean, and Australia	Merck (MRK) Ad5 HIV-1 Gag/Pol/Nef trivalent vaccine based on adenovirus type 5	3000 MSM and heterosexual women and men	1:1	No vaccine efficacy
<b>RV144</b>	Thailand	Recombinant canarypox vector vaccine (ALVAC-HIV[vCP1521]) and recombinant gp120 subunit vaccine (AIDSVAX B/E)	16 402 community-risk men and women	1:1	31.2% vaccine efficacy at 42 months
<b>HVTN503 Phambili trial</b>	South Africa	Merck (MRK) Ad5 HIV-1 Gag/Pol/Nef trivalent vaccine based on adenovirus type 5	801 heterosexual men and women	1:1	No vaccine efficacy
<b>HVTN 505</b>	USA	6-plasmid DNA vaccine and rAd5 vector boost	2504 men or transgender women who have sex with men	1:1	No vaccine efficacy
<b>HIV-V-A004</b>	USA, Rwanda, South Africa, Thailand and Uganda	Homologous Ad26 mosaic vector regimens or Ad26 mosaic and MVA mosaic heterologous vector regimen, with high-dose, low-dose or no clade C gp140 protein plus adjuvant	400 men and women	-	Ongoing
<b>HVTN 100</b>	South Africa	Clade C Canarypox-HIV (vCP2438) and bivalent subtype C gp120/MF59	252 men and women	5:1	Ongoing
<b>HVTN 702</b>	South Africa	Canarypox-HIV and bivalent subtype C gp120 MF59	5400 men and women	1:1	Ongoing
<b>HVTN 703/ HPTN 081</b>	South America and Sub-Saharan Africa	VRC01 bNAb passive immunity	2400 MSM and transgender and 1500 women	2:1	Ongoing

**Note:** MSM: men who have sex with men; IDUs: Injection drug users



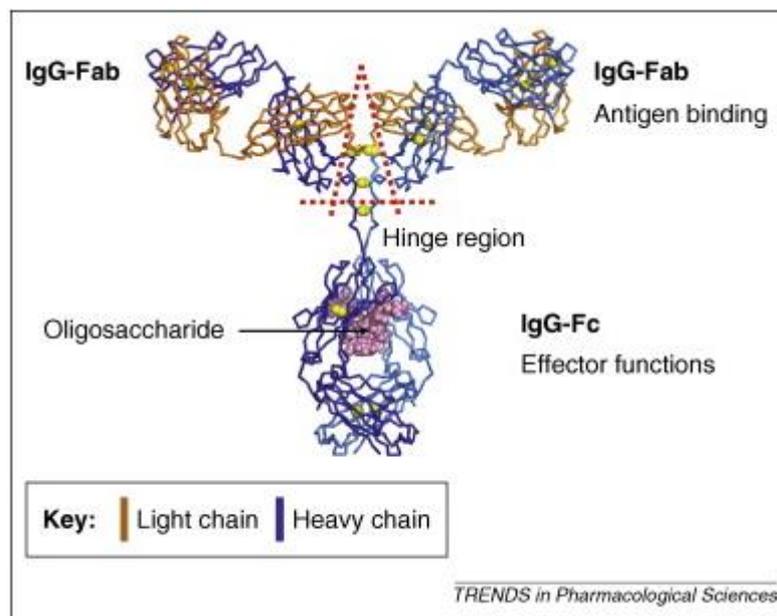
Other immunization strategies such as active immunization, may also elicit antibodies capable of partial protection in the absence of neutralization. Evidence of active immunization in Rhesus macaques vaccinated with a mosaic HIV-1 vaccine showed partial protection after intrarectal SHIV-SF162P3 challenge (Barouch et al., 2013a, Barouch et al., 2012). This mosaic vaccine consisted of an adenovirus/adenovirus vector or an adenovirus/poxvirus vector, with HIV-1 Env/Gag/Pol proteins, elicited Env-specific immune responses (Barouch et al., 2013a). Non-neutralizing antibody functions such as ADCP and NAbS against tier 1 and tier 2 were elicited by the vaccine, as well as Env-specific CD4<sup>+</sup> T cell responses and were associated with protection (Barouch et al., 2013a). These results substantiate a coordinated synergy of multiple and diverse immune responses in preventing HIV infection (Barouch et al., 2013a, Barouch et al., 2012).

Eliciting a sustained B-cell response, CD8<sup>+</sup> and CD4<sup>+</sup> T cell response that can eliminate viruses and contain infection at the site of transmission, remains a substantial hurdle to vaccine development (Rubens et al., 2015). Vaccine strategies have therefore been directed towards stimulating humoral immunity to elicit effective antibody responses capable of neutralization across multiple subtypes, administering passively infused bNAbS or inducing CD8<sup>+</sup> cytotoxic T cell responses or harnessing nNAb effector function (Chhatbar et al., 2011).

## **2.6 Fc-Mediated Antibody Functions – Non-neutralizing antibodies**

Historically, potential vaccine immunogens were chosen based on their ability to elicit neutralizing antibodies and high antibody titres [as reviewed in (Pulendran and Ahmed, 2011)]. The challenge in HIV vaccine trials is the waning of antibody responses, the low potency and breadth of elicited antibodies that failed to prevent HIV-infection or control disease progression (Corey et al., 2015). Although the RV144 vaccine did not elicit cytotoxic T cells, antibody titres and neutralizing antibodies, this vaccine did confer moderate protection from HIV infection (Rerks-Ngarm et al., 2009, Chung et al., 2015). Protective immune responses have been associated with non-neutralizing antibody mediated functions, such as ADCC functions (Chung et al., 2015, Barouch et al., 2015, Hessel et al.,

2007, Bournazos et al., 2014, Dilillo et al., 2016, Kohl and Loo, 1982). Chung *et al.*, (2015), reported that a protective response was associated with “antibody features” including polyfunctional responses and not antibody titres (Hessell et al., 2009a). Besides the Fab region of the antibody that recognizes antigen, the Fc portion (**Figure 6**), engages specific effector cells of the innate immune system to induce a polyfunctional responses such as complement activation, viral inhibition or phagocytosis (Sips et al., 2016).



**FIGURE 6: Crystal representation of the structure of an antibody.** The Fc portion of IgG interacts with FcR on effector cells. The Fab region recognizes the antigen/epitope resulting in either phagocytosis, viral inhibition or cytotoxicity [adapted from (Jefferis, 2009)].

The Fc portion of the antibody is versatile and can engage with various Fc receptors (FcR) on various effector cells, thus linking the innate and humoral immune system (Sips et al. 2016). ADCC is commonly induced by NAb, but can be triggered by nNAb, activating effector cells of the innate immune system such as natural killer (NK) cells, neutrophils, and monocytes (Chung et al. 2009; Hessell et al. 2007). ADCC activation includes the granzyme B pathway, which causes lysis of infected cells as well as the release of immunomodulatory cytokines

[as reviewed in (Russell and Ley, 2002, Berke, 1995), (Kagi et al., 1994)]. Fc receptor-mediated function through nNABs has been shown to be a correlate of protection in vaccinated animals (Barouch et al., 2013a). These findings were further supported by animal and human studies of HIV-1 controllers, showing that nNAb-mediated responses were associated with viral control and reduced risk of infection (Moog et al., 2014, Barouch et al., 2013a). Furthermore, Hessel *et al.*, (2007) showed that Fc-mediated activity but not complement binding, sufficiently conferred protection. The Fc portion is crucial for antiviral activity, for example when b12 was genetically altered to exclude the Fc portion, antiviral activity was completely ablated (Hessell et al., 2009b).

A study by Stromberg and Nussenzweig *et al.*, (2014) investigated combination therapy of a triple cocktail of bNABs and post-exposure prophylaxis in humanized mice and rhesus macaques following HIV infection. Their study drew attention to the role of Fc-mediated antiviral activity in delaying viral rebound, from the latent reservoir (Halper-Stromberg et al., 2014). Additionally, data from Nussenzweig *et al.*, (2014) and Hessel *et al.*, (2007), underscored the importance of engaging effector cells as a means of ameliorating disease, during HIV infection (Halper-Stromberg et al., 2014, Hessell et al., 2007). However, the dynamics of the Fc-FcR interaction and their ability to elicit effector functions may not be as efficient in the genital tract mucosae, compared to the systemic compartment and requires further investigation.

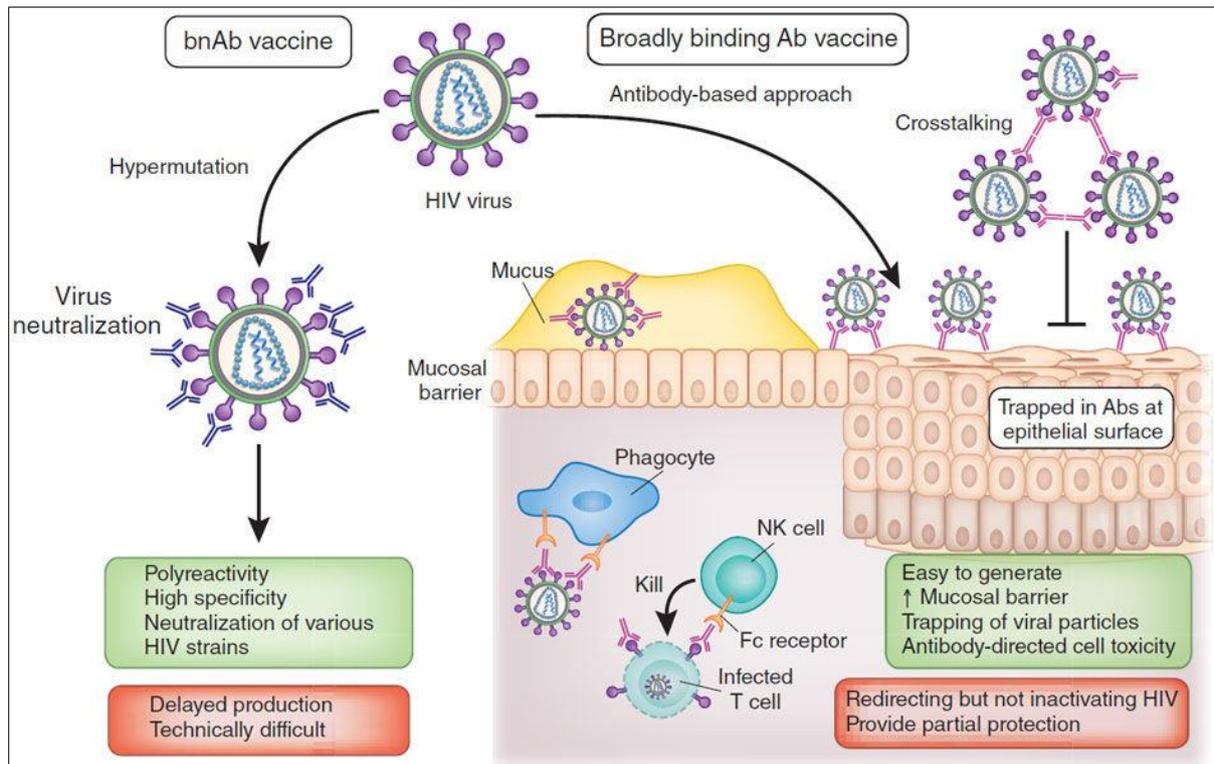
### **2.6.1 Fc-FcR interaction and the potential role of non-neutralizing antibodies in HIV-1 infection**

Where neutralization fails to prevent infection of HIV target cells, various studies have reported the role of non-neutralizing antibody activity, mediated by Fc $\gamma$ -receptor binding, in the control of HIV-1 infection (Mabuka et al., 2012, Barouch et al., 2013a). In animal infection established models, macaques passively infused with SIV-specific non-neutralizing IgG facilitating ADCC activity, was associated with the reduction of SIV infected cells (Banks et al., 2002, Ferrantelli et al., 2003). These data, along with other studies, [reviewed by (Forthal and Moog, 2009)], indicate the role of ADCC activity in altering HIV disease

progression, with increased ADCC activity associated with better disease outcome (**Figure 6 and Figure 7C**) (Hessell et al., 2007, Milligan et al., 2015).

More interestingly, the observed protection in the RV144 trial showed that binding antibodies, directed to the V1V2 region of gp120, in particular the IgG1 (Forthal et al., 2013) and IgG3 subclass, mediated ADCC (**Figure 4**) (Yates et al., 2014). Levels of V1V2 specific-IgG3 were considered correlates of protection, however plasma Env-specific IgA antibodies were correlated with reduced vaccine efficacy (Yates et al., 2014, Vasan et al., 2015). Thus, these findings initiated further scrutiny into factors that may contribute to better protection from HIV, two of which will be discussed in the sections below, namely antibodies and effector cell characteristics.

Effector cell population relative abundance, tissue location, FcR expression and frequency, are key factors that influence nNAb activity (Cheeseman et al., 2016). Innate immune effector cells such as NK cells and macrophages are able to induce cytotoxicity (Ackerman et al., 2013a) and phagocytosis (Ackerman et al., 2013b), respectively. Therefore, the presence of both effector cells and antibodies at mucosal portals of entry (**Figure 7 and 8**), such as the genital mucosae should be considered in vaccine design as both need to be present, in order to provide protection from infection (Cheeseman et al., 2016).



**FIGURE 7: Mechanisms of broadly neutralizing and non-neutralizing antibodies in immune defences at mucosal portals of entry, from HIV infection.** nNAb elicit humoral immune responses, other than neutralization. NNABs are capable of mediating viral inhibition, trogocytosis, cytotoxicity or phagocytosis of infected cells, via Fc-FcR interactions with innate immune effector cells. These activities suggest a role for nNABs in mucosal protection from HIV challenge [adapted from (Hope, 2011)].

Effector cells are commonly found in the sub-epithelial and basal layers of the mucosal tissue as well as in the blood (Cheeseman et al., 2016). Neutrophils, are one of the most abundant white blood cells in the body and are essential in pathogen clearance associated with innate immunity (Amulic et al., 2012). Furthermore these predominant innate cells are capable of eliciting phagocytic activity and are reportedly found in consistent populations in the cervix, in both HIV infected and uninfected women (Nkwanyana et al., 2009). These characteristics of the genital tract should be considered in vaccine design where immunoglobulin subtypes may be harnessed as it has been shown that IgG3 has greater virion uptake capacity than IgG1 (Tay et al., 2016). These neutrophils bind IgG3 more effectively and should these antibodies exist at frequently challenged site, they might provide greater mucosal protection from HIV infection (Sips et al., 2016).

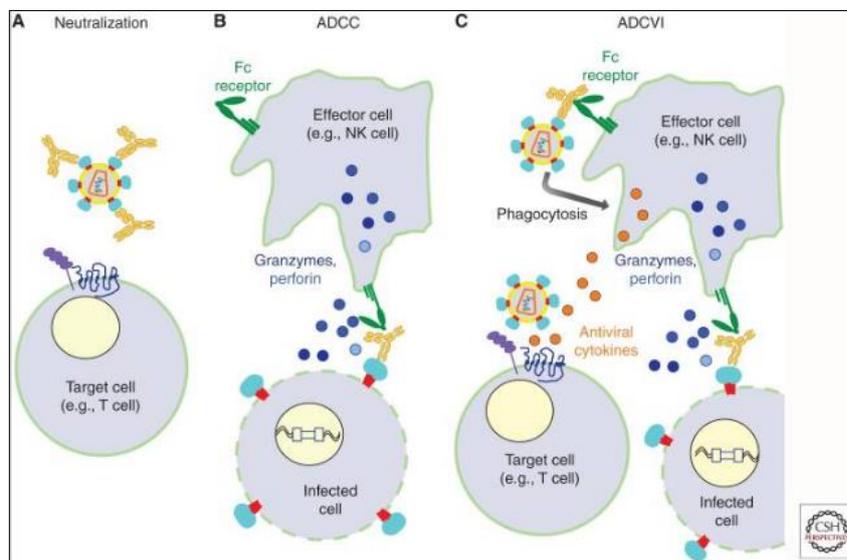
Considering the competitive and inhibitory binding Env-specific IgA had in reducing the efficacy observed in the RV144 trial, vaccine design would need to account for influencing factors, such as antibody isotype and subclass (Haynes et al., 2012a). As seen with the RV144 vaccine, IgG3 exhibited a higher affinity to effector cells than IgG1, which would result in a stronger antiviral response, due to the more rapid release of perforin granzymes and other antiviral cytokines (Smalls-Mantey et al., 2012, Kramski et al., 2013). These differences may dictate the efficacy of the immune-induced responses, which are influenced by immunogen design, active or passive immunization strategies and as well as whether the vaccine is administered intravenously or subcutaneously (Cheeseman et al., 2016).

Furthermore, IgG3 had the highest internalization of HIV-1 antigen compared to IgG1 and IgA1, which corroborated with the correlates of protection analyses from the RV144 trial (Tay et al., 2016, Tomaras et al., 2012). Thus, these findings support recent studies and building momentum regarding the importance that Fc-mediated antiviral activity may have to further protect from HIV-1 infection (Barouch et al., 2013a, Hessel et al., 2007).

### **2.6.2 Antibody Dependent Cellular Phagocytosis (ADCP) mediated by Polymorphonuclear Neutrophils and Macrophages**

Phagocytosis is the complete internalization of a pathogen or infected cell by phagocytic cells such as neutrophils, macrophages, plasmacytoid dendritic cells (pDC) or eosinophils and result in antigen presentation and the release of cytokines that modulate inflammation (Kramski et al., 2013, Sips et al., 2016). Antibody mediated functions such as phagocytosis have not been explored in detail as studies have focussed on alternate antibody mediated effector functions like direct neutralization or cytotoxicity (Kramski et al., 2013). IgG1 and IgG2 engage with innate cells such as monocytes, neutrophils and macrophages expressing FcRγII on their surfaces and mediate phagocytosis (**Figure 8**) (French et al., 2013, Sips et al., 2016). Phagocytic activity during acute HIV infection may play a key role in pathogen clearance, through early recognition of immune complexes and warrants further research as a mechanism of effective viral control and clearance (Tomaras et al., 2008). However, during HIV infection, phagocytic activity is reduced as a result of the dysregulation of actin

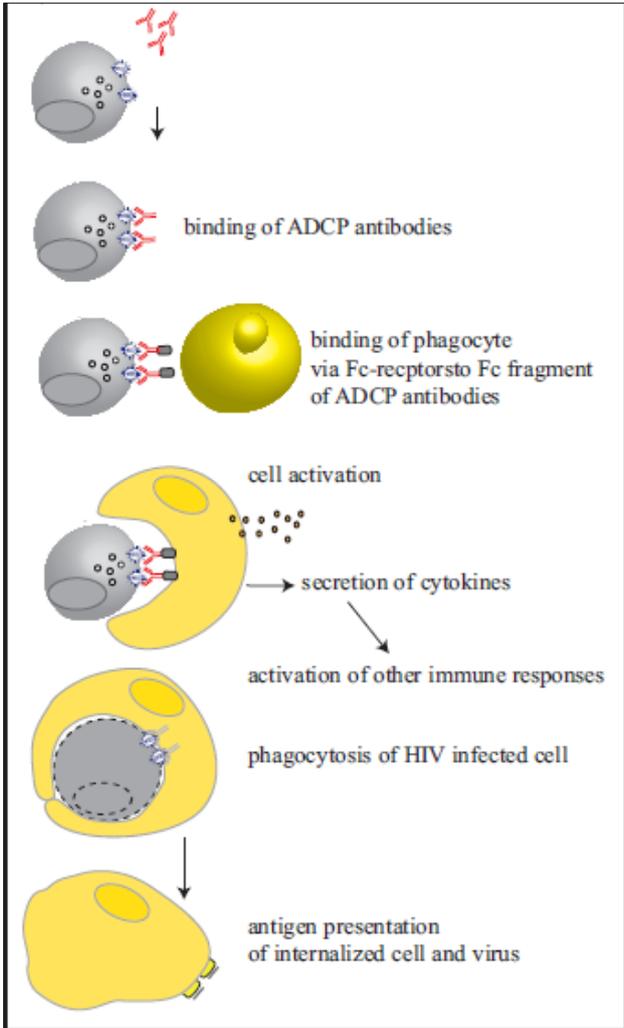
polymerization and the loss of FcγR intracellular signalling molecules of monocytes and macrophages (Kedzierska et al., 2003). Interestingly, in HIV controllers and untreated progressors, ADCP activity is increased and may be attributed to the glycosylation of the Fc portion, which results in increased interaction with phagocytic cells expressing FcγRIIb and FcγRIIa (Ackerman et al., 2013b). Thus, these Fc expressing macrophages, may be one of the “most important functions” required for clearance of HIV infection (Kramski et al. 2013; Kedzierska et al. 2003).



**FIGURE 8: Diagrammatic representation of three various antiviral activities, mediated by antibodies. (A)** Neutralization of cell free viruses. Antibodies bind to specific parts of the virus envelope inhibiting the attachment of the virus to the target cell co-receptors, i.e. CCR5 and CD4. **(B)** Antibody dependent cellular cytotoxicity. Usually mediated by NK cells, by binding of the Fab region with the antigen resulting in the formation of an immune complex. The Fc portion of the bound antibody i.e. IgG engages with the FcR of the effector cell (NK cell) to destroy the infected cell. **(C)** Antibody dependent cellular viral inhibition (ADCVI). A measure of the secondary effects of ADCC activity and the resulting release of anti-viral products such as cytokines that lead to phagocytosis or viral inhibition [adapted from (Overbaugh and Morris, 2012)].

ADCP activity in the mucosal tissue may elicit a stronger protective response than ADCC mediated functions, due to the relative abundance of phagocytic effector cells, such as neutrophils, in the colorectal, penile glans and cervical tissue (Cheeseman et al., 2016). Neutrophils may be key in protecting the female genital tract from HIV infection, due to their stable population abundance during menstrual cycles and inflammation. Interestingly, these effector cells have been associated with the control of HPV viral loads, in the female reproductive tract (FRT), suggesting a potential role for ADCP activity against HIV in the FRT (Sips et al., 2016, Day et al., 2010).

Antibody dependent cellular viral inhibition (ADCVI), another diverse Fc-mediated activity, inhibits viral yield from infected cells and is a measure of antiviral activity mediated by ADCC and ensuing secondary antiviral activity such as phagocytosis and viral inhibition [as reviewed in (Overbaugh and Morris, 2012), (Forthal et al., 2006)]. nNAbs mediate ADCC/ADCVI antiviral activity (**Figure 8B and C**), via crosslinking of the IgG Fc to NK cell FcRγIII which results in NK cell activation [as reviewed in (Daëron, 1997)]. NK cell activation releases cytokines: IFN-γ, TNF-α, MIP1-β and results in degranulation of infected cells [as reviewed in (Scully and Alter, 2016), (Chung et al., 2009, Kramski et al., 2013)]. ADCVI contributes to viral reduction and decrease in viral transmission, acting against both cell free and cell associated virus [as reviewed in (Scully and Alter, 2016)]. Post-challenge animal studies have reported partial protection from SHIV infection as well as viral load reduction and viral clearance due to ADCVI/ADCC activity (Hidajat et al., 2009, Xiao et al., 2010, Florese et al., 2009, Banks et al., 2002, Gómez-Román et al., 2005). Furthermore, in another animal study, ADCVI activity has shown potential in protecting neonates from infection (Forthal et al., 2006). According to a review by Overbaugh and Morris (2012), in the Vaxgen Phase III vaccine study, despite the absence of protection, levels of binding antibody, incidence of infection and ADCVI activity were correlated to lower disease pathogenesis, further supporting the role of non-neutralizing antibody mediated protection in HIV infection [as reviewed in (Overbaugh and Morris, 2012), (Gilbert et al., 2005, Forthal et al., 2010)]. Together these findings highlight the importance of Fc mediated antiviral activity in reducing viral load and providing a level of protection from HIV infection, mediated by either phagocytic or cytotoxic activity.



**FIGURE 9: Phagocytic activity mediated by innate cells like macrophages or neutrophils.** The interactions between the Fc portions of nNABs and surface-bourne Fc receptors innate immune cells, results in phagocytic activity and triggering of the downstream activation of other immune responses subsequent to the secretion of cytokines and leading to the destruction of the pathogen (Kramski et al., 2013).

### 2.6.3 The role of NK cell mediated Antibody Dependent Cellular Cytotoxicity in HIV infection

NK cells play a crucial role in innate immunity because they recognize and destroy infected cells. This activity shapes the adaptive immune response. Harnessing these characteristics may be central in preventing or controlling HIV infection. Antibodies capable of mediating ADCC activities usually develop in the early stages of HIV infection and were shown to be associated with better disease outcome (Milligan et al., 2015, Mabuka et al., 2012). Mabuka *et al.*, (2012) reported that ADCC activity in the breastmilk of HIV infected mothers was associated with reduced vertical HIV transmission. Furthermore in a related study, pre-existing and passively transferred ADCC antibodies through the breastmilk, showed that for every 10% increase in ADCC activity a correlated 49% decrease in childhood mortality occurred (Milligan et al., 2015, Mabuka et al., 2012). This was attributed to pre-existing ADCC activity, present at the oral mucosal surface. Similarly, ADCC activity correlated to reduced HIV RNA levels at the mucosae (Battle-Miller et al., 2002).

Additionally, ADCC activity was correlated to low systemic levels of viremia, in patients initiated on ART treatment and who stopped ART after three years, indicating a sustained nNAb responses [as reviewed in (Scott-Algara, 2015, Scully and Alter, 2016)]. Early ART may preserve ADCC function and may mimic ADCC in HIV elite controllers who display superior ADCC function (Scott-Algara, 2015). In addition, a macaque study, showed that application of topical NAb, nNAb gel or both, before SHIV challenge, resulted in reduced viral loads through ADCC (Moog et al., 2014). Taken together, these data suggest that the presence of nNAb at frequently challenged sites of transmission, such as the genital mucosae, may provide a level of protection against HIV infection.

NK cell populations in the mucosae of penile, cervical and colorectal tissue, may contribute to protection by binding IgG antibodies (Cheeseman et al., 2016). At the subclass level, IgG3 antibodies mediate ADCC activity more effectively (see **Figure 8 and Figure 9**) (Chung et al., 2014). Highly effective NK cells release CD107 and cytokines IFN- $\gamma$  and TNF- $\alpha$ , which in turn recruit more effector cells bolstering antiviral activity (Alter et al., 2004). Methods investigating ADCC function have varied in sensitivity and ease of conduct. To date the rapid

fluorescent antibody dependent cellular cytotoxicity (RFADCC) has been described as a quantitative (Gómez-Román et al., 2006) and more efficient method to analyse ADCC activity in comparison to the chromium release assay (Chung et al., 2009, Ahmad et al., 2001) . However, the intracellular based staining (ICS) ADCC assay which measures NK cell activation, allows for the measurement of various NK cell mediated antiviral activities such as IFN- $\gamma$ , CD107a and MIP-1 $\beta$  expression (Chung et al., 2009, Stratov et al., 2008). This assay utilises HIV antigens, enabling individual peptide responses to be analysed as potential targets for future HIV vaccines eliciting this function (Chung et al., 2009). In addition, the ICS- NK cell activation assay is easier and less laborious, allowing for the use of fresh whole blood for assays, irrespective of whether the blood are from the autologous or healthy donors (Chung et al., 2009). However HIV downregulates HLA through Nef and Vpu proteins, inhibiting NK cell activation and therefore undermines ADCC activity (Cohen et al., 1999). The prior use of PrEP or ongoing ART may be able to rescue NK cell activity through viral loads suppression (Brunetta et al., 2010, Mavilio et al., 2005).

## **2.7 Preliminary data**

Following the recommendation of PrEP to high risk populations which include women between the ages of 15-24 years by the World Health Organization, understanding the kinetics of the immune response in the genital mucosal tract requires further investigation, in order to better inform on future prevention strategies. Recently published data by Archary *et al.*, (2016) demonstrated that seroconverter women from the CAPRISA 004 1% tenofovir microbicide gel trial had significantly higher responses and higher frequencies and titres of HIV-1 specific gp70 V1V2 IgG and Env gp120 respectively. In addition, in the genital tract and in the plasma, these women displayed higher titres of p66 and p24 IgGs and IgA's, compared to women in the placebo group. This discriminant pattern of HIV-specific binding antibodies between the tenofovir and placebo gel users was seen predominantly in the first 12 months post-infection. The close concordance between the plasma and the genital tract was seen for 9 of the 10 HIV-IgG specificities where plasma antibody titres predicted GT levels in the first six months post-HIV infection. However whether the IgG functionality was mirrored between the plasma and genital compartments is an aspect we proposed to

investigate. These published data suggest that in the presence of tenofovir, women who acquired HIV infection had elevated humoral immune responses in the blood and genital tracts of individuals. These data reveal strong associations between the prior use of tenofovir gel, the profile of binding antibodies that prevail and further distinguish the women that used 1% tenofovir gel compared to those who did not, as well as identifying the strong cross compartment association of HIV specific IgG antibodies.

Therefore, the aim of the study was to elucidate the effects of PrEP as an HIV prevention method, on humoral immune responses in the plasma and genital tracts of women who acquired HIV infection, but who had prior exposure to 1 % tenofovir microbicide gel. We investigated effector functions such as ADCC and ADCP activity of non-neutralizing HIV-1 specific IgGs, following the discovery of unique antibody signatures, in the plasma and genital tracts of the women who participated in the CAPRISA 004 trial. These data will be crucial for informing the development of future HIV prevention strategies, in an era where vaccines and PrEP will likely be used in combination, to prevent HIV.

## CHAPTER 3

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### 3.1 Aim

1. To determine the Fc-mediated ADCP and ADCC activities of HIV-1 specific IgG binding antibodies in the blood of women from the CAPRISA 004 1 % Tenofovir gel trial, who acquired HIV during the first 12 months post-infection, in both the tenofovir and placebo arms.
2. To determine the Fc-mediated ADCP and ADCC activities of HIV-1 specific binding antibodies in the genital tracts of women from the CAPRISA 004 1 % Tenofovir gel trial, who acquired HIV during the first 12 months post-infection, in both the tenofovir and placebo arms.
3. To determine if the functional profiles of HIV-specific IgGs mediating ADCP and ADCC were similar between the blood and genital tracts, of women in the tenofovir and placebo arms.

### 3.2 Objectives

1. To investigate Fc-mediated ADCP and ADCC assays using IgGs isolated from the plasma of women who participated in the trial in both the tenofovir and placebo arm.
2. To investigate Fc-mediated ADCP and ADCC activities in the genital tracts of women in both study arms, using CVL samples.
3. To determine the correlation between ADCP and ADCC activity in the plasma and genital tracts of women, at 3 months, 6 months and 12 months post-infection.

### 3.3 Hypothesis

Antibodies isolated from the plasma and genital tracts of women in the CAPRISA 004 trial, elicited functional Fc-mediated ADCP and ADCC activities and were associated with prior tenofovir gel use.

## CHAPTER 4

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### 4.1 Study population and study design

This was a retrospective study of 48 HIV-infected women, who seroconverted during the CAPRISA 004 1% tenofovir microbicide gel trial (Abdool Karim et al., 2010). The CAPRISA 004 microbicide gel trial (Clinical Trials Number #00441298) participants had given written informed consent, to use their stored samples for future research. Approval for the CAPRISA 004 trial was obtained from the University of Kwa-Zulu Natal Biomedical Research Ethics Committee (BREC) (E111/06), Family Health International's protection of Human Subjects Committee (#9946) and the South African Medicines Control Council (#20060835). BREC at the University of KwaZulu-Natal approved this sub-study for post-graduate degree purposes (BE241/16) (appendix A and B).

Twenty-four women from the tenofovir arm and 24 women from the placebo arm were case matched according to their viral loads and CD4<sup>+</sup> T-cell counts at six-months post-infection. Women who were HIV-infected were followed for a median of 36 months after enrolment (range 11.5-53.5 months) however; the samples from 3; 6 and 12 months post-infection were included in this sub-study (**Figure 10**).

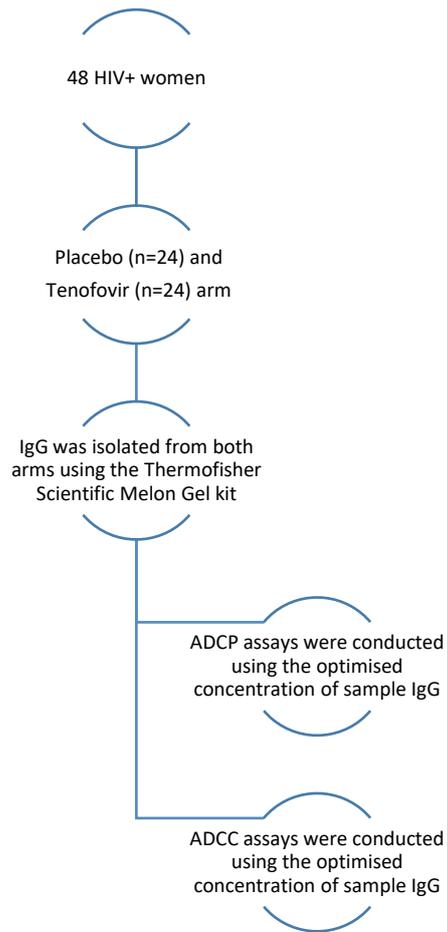
### 4.2 Biological Specimen and Sampling collection

Cervical lavages (CVL) and plasma samples from the women who had experienced breakthrough HIV-1 infection during the CAPRISA 004 1% tenofovir microbicide gel trial were used to achieve the objectives of this sub-study. All women were antiretroviral (ARV) treatment naïve. CVL samples were collected from women at each visit according to the method described (Bebell et al., 2008). CVL were collected by bathing the cervix with 10mL of sterile saline, which was allowed to pool in the posterior fornix before aspirating using a bulb pipette. Upon aspiration, the fluid from the cervicovaginal tract was dispensed into a sterile container and centrifuged at 2700 rpm for 10 minutes. Following this process, aliquots of the CVL supernatant were prepared and stored at - 80°C until required. These

CVL specimens were used undiluted in subsequent assays, unless otherwise stipulated. However, if the participant was menstruating at the proposed time of CVL collection, the collection was postponed until the end of the menses (Bebell et al., 2008). Plasma samples collected by venepuncture into acetate citrate dextran (ACD) vacutainers were spun down to separate the plasma from the packed cells. The plasma was stored at - 80°C until required. HIV statuses of the women were determined by two HIV-1 rapid antibody tests and polymerase chain reaction (PCR) (Roche amplicon v1.5) and confirmed by enzyme immunoassay. Viral loads were determined using a Cobas Amplicor HIV-1 Monitor Test, v 1.5 (Roche diagnostics) and CD4<sup>+</sup> T cell counts were assessed using a FACS Calibur flow cytometer (Becton Dickinson, where from San Jose, CA) (Abdool Karim et al., 2010).

### **4.3 Statistical analysis**

Comparisons of IgG phagocytosis between the study arms at 3 months, 6 months and 12 months, in the plasma and CVL were analysed using the Wilcoxon Rank Sum tests. Wilcoxon-Signed Rank test were used to compare plasma IgG phagocytic scores and CVL IgG phagocytic scores across 3 to 6 months, 6 to 12 months and from 3 to 12 months, using GraphPad Prism version 7, software. This analysis was further stratified by randomization arm. Spearman Rank Correlation analysis was done to determine inter-compartmental relationships between the tenofovir and placebo at each time point, in both arms. In order to identify correlations between phagocytic activity and CD4<sup>+</sup> T cell count and viral load, correlation analysis was done at 6 months post-infection for plasma and genital tract, HIV-specific mediated antibody activity.



**FIGURE 10: Methodology used to investigate the nAb functions of plasma IgG and CVL from women in both the tenofovir (n = 24) and placebo arms (n = 24).** Plasma and CVL samples from the genital tract were collected at 3, 6 and 12 months, for women in both study arms, and were used in ADCP and ADCC assays.

## 4.4 Methods

### 4.4.1 Isolation of IgG from CVL and Plasma samples

In order to determine the Fc-mediated functional capabilities of IgG's isolated from the plasma and CVL samples of women in both the tenofovir and placebo arms were isolated using an IgG isolation kit, in the following manner. 50µl of the plasma/CVL sample, was diluted in 450µl of Purification buffer (Thermofisher Scientific, Waltham, MA). The CVL IgG isolation procedure was discontinued due to the low total immunoglobulins present in the lavage specimens, therefore undiluted CVL specimens were used in the future experiments.

Purification buffer was previously diluted in deionised water from a 10X stock provided in the kit to a 1x working solution. Pierce spin columns-screw caps, with 20-50µl filters, were removed and replaced with the 100-400µl filter (Thermofisher Scientific, Waltham, MA). Melon Gel IgG Purification Support matrix (Thermofisher Scientific, Massachusetts, USA) was reconstituted by repeated swirling to ensure an even suspension before 500µl of Melon Gel (Thermofisher Scientific, Waltham, MA) was dispensed into the pierce columns. The 2ml Eppendorf with the spin column and melon gel, was then spun at 14,000 rpm for 1 minute in a microcentrifuge (Beckman Coulter, Germany). The supernatant was removed and 300µl of Melon Gel Purification buffer (Thermofisher Scientific, Waltham, MA) was added and centrifuged at 14,000 rpm for 1 minute. This process was repeated three times to ensure that the gel was purified and any remnants that may interfere with downstream assays were removed. In order to seal the column, column plugs were inserted onto the bottom ends of the columns and 500µl of each of the diluted samples were added to the remaining melon gel support. The Pierce columns containing the sample and melon gel support was re-suspended by inverting it several times before being mixed using a Hula Mixer (Invitrogen, Oslo, Norway) for 15 min in order to homogenize the suspension with the sample and ensure maximum binding of the gel matrix and the IgG. The pierce columns were then placed in new 2ml Eppendorf tubes and centrifuged for 1 min at 14,000 rpm. The flow through was collected and the concentration of the purified IgG calculated by using a Nanodrop Lite Spectrophotometer (Thermofisher Scientific, Waltham, MA). The isolated IgG was stored at 4 °C until required. The spin column with the remaining Melon Gel Support was reconstituted and used again, by adding 500µl of regeneration buffer from the

Thermofisher Scientific kit and mixed on the Hula Mixer (Invitrogen, Oslo, Norway) for 5 minutes. The column plug was removed and the tubes were centrifuged for 1 minute at 14,000 rpm using the microcentrifuge, to remove the regeneration buffer. The Melon Gel support was then washed 3 times with 300µl of regeneration buffer and centrifuged for 1 minute at 14,000 rpm. 500µl of purification buffer was added and the column plug inserted and the cap screwed on, were stored at 4°C until further use.

#### **4.4.2 R10 Media preparation for cell culture**

The neutrophils were temporarily stored in R10 media. R10 was made in the following manner, RPMI without L-glutamine (Whitehead Scientific, USA), was supplemented with 55 ml of heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich, USA). FCS was heat inactivated by placing it in a water bath that was set at 56°C for one hour. 5ml of 2mM L-glutamine (Whitehead Scientific, USA), 5ml of Pen/Strep, 5ml of 1mM HEPES (Whitehead Scientific, USA) and was thoroughly mixed to ensure an even suspension.

#### **4.4.3 Proteins used in both ADCP and NK cell activation Assay**

In order to gauge the functionality of the IgGs four HIV-1 antigens were used. These included envelope and gag proteins like Con6 gp120 and gp41, Gag p24 and p66 (RT). The antigens used in the assays included Con6 gp120/B (Consensus Group M gp120), gp70\_B CaseA\_V1\_V2 (gp70 V1V2) (Immune Technology, California, USA) only for the ADCP plasma, commercially available antigens included p24 (HIV-1/Clade B/C CN54) (Immune Technology, California, USA) and gp41 (Ectodomain) (HIV-1) (Immune Technology, California, USA) and p66 RT (ImmunoDX, Massachusetts, USA). All experiments were conducted with compliance to good clinical laboratory practice.

#### **4.4.4 Isolation of white blood cells to assay neutrophil mediated phagocytosis**

Neutrophils undergo rapid respiratory burst therefore this cell isolation process was done immediately before the assay. The white blood cells were prepared by pipetting 1mL of EDTA-treated whole blood in a tube containing 9mL of ACK lysis buffer (Life technologies, Grand Island, NY) and incubated for 3-5 minutes at room temperature. White blood cells were collected by centrifugation (Beckman Coulter Allegra, California, USA) at 1500 rpm for 5 minutes at room temperature. The supernatant was aspirated, leaving approximately 50µl to avoid disturbing the pellet. The cells were washed with 5mL of cold phosphate buffered saline (Lonza, Walkersville, MD) to detach the cells from the bottom of the falcon tube. The re-suspended cells were inverted before being pooled and then collected by centrifugation at 1500 rpm for 5 minutes at 4°C. The supernatant was aspirated and re-suspended in approximately 5mL of R10. A haemocytometer was used to count live cells, which were then re-suspended to approximately 50,000 cells/mL in the volume of R10 required for each assay.

#### 4.4.5 Antibody Dependent Cellular Phagocytosis Assay (ADCP) – Neutrophil Assay

The plate layout for a 96 well round bottom tissue culture plate (Sigma-Aldrich) was prepared and designed to include positive, negative and no antibody controls. Assays were run in duplicate with controls run in triplicates. Each plate layout included positive and negative controls, in order to establish a true positive and negative result in comparison to the sample results (**Figure 11**). For the positive control, HIV immunoglobulin (HIVIG) was diluted to a concentration of 0.5mg/ml in a volume of 100µl of R10. For the 'negative control,' 100 µl of PBS was used. 5µl of biotinylated antigen was added to each well in a 96 well round-bottom tissue culture plate and then 100 µl of sample/control were added to each well. The plate was incubated at 37°C for 2 hours in an incubator at 5% CO<sub>2</sub>. 50,000 neutrophils in a total volume of 100 µl of R10 were added to each sample and then incubated at 37°C for 1 hour. The plate was spun at 500 g for 5 min at 4°C. The supernatant was flicked off and the pellet re-suspended in the remaining fluid, by gently running the plate over a rack. Each well was stained with the 1µL of CD3 Alexa Flour 700 (A700) to stain for CD3 negative cells (BD Biosciences New Jersey, USA), 1µL of CD14 Phycoerythrin and cyanine 7 (PE-Cy 7) (BD Biosciences, New Jersey, USA) to stain for monocytes, 1 µL of CD66b Violet 450 (VP450) (BD Biosciences, New Jersey, USA) to stain for neutrophils and 17µl of PBS, approximately 20µL of stain was added per well (**Table 3**). Following staining, the plate was incubated at room temperature for 15 minutes, in the dark before 200 µL of PBS was added to each well. The cells were pelleted by centrifugation at 500 g for 5min and then removed by flicking off the supernatant. 50µL of 4% paraformaldehyde (PFA) was added and incubated at room temperature for 15 minutes in the dark. The cells were pelleted by centrifugation at 500 g for 5 minutes before the supernatant was flicked off. 100 µL of PBS was added to each well and then stored overnight at 4°C until run on the LSR Fortessa (BD Biosciences, California, USA). The total number of events that were acquired for each well was set as 20 000 events.

#### 4.4.6 Biotinylation Protocol

The biotinylation process was conducted according to a previously published method (Ackerman et al., 2011). The neutravidin (Flourosphere<sup>®</sup> Neutravidin<sup>®</sup> Labelled from LTC

Technologies, USA) bead solution was biotinylated (EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format) (ThermoFisher Scientific, Rockford, IL) to one of the five HIV antigens. The neutravidin beads required for 5 $\mu$ l per plate was calculated. The amount of biotinylated antigen is equal to the volume of neutravidin beads multiplied by 4 due to the 1:4 ratio of  $\mu$ l beads to  $\mu$ g of antigen. The antigen-bead solution was prepared by vortexing the beads before transferring neutravidin beads with a pipette into 1.7ml microcentrifuge tube. The required volume of biotinylated antigen was added using a pipette and mixed by repeat pipetting. The tubes were labelled with the date, antigen and operator initials. The beads with the antigen were incubated together in an incubator at 37°C at 5% CO<sub>2</sub> for 2 hours. After the incubation period, the tube was washed with 1ml of 0.1 % phosphate buffered saline (PBS) - bovine serum albumin (BSA) - PBS-BSA solution (KPL, USA) twice before re-suspending it in 0.1% PBS-BSA at a ratio of 1:100 beads: PBS-BSA. The coupled beads and antigen were then stored at 4°C until use- with a maximum storage period of two weeks.

#### 4.4.7 ADCP Antibody Panel

TABLE 3: Antibody panel for the ADCP assay

Antibody	Cell type	Volume (μl)
CD3 AF700	CD3 negative cell	1
CD66 VP450	Neutrophil marker	1
CD14 Pe-Cy7	Macrophages and monocytes	1

#### 4.4.8 Plate design

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

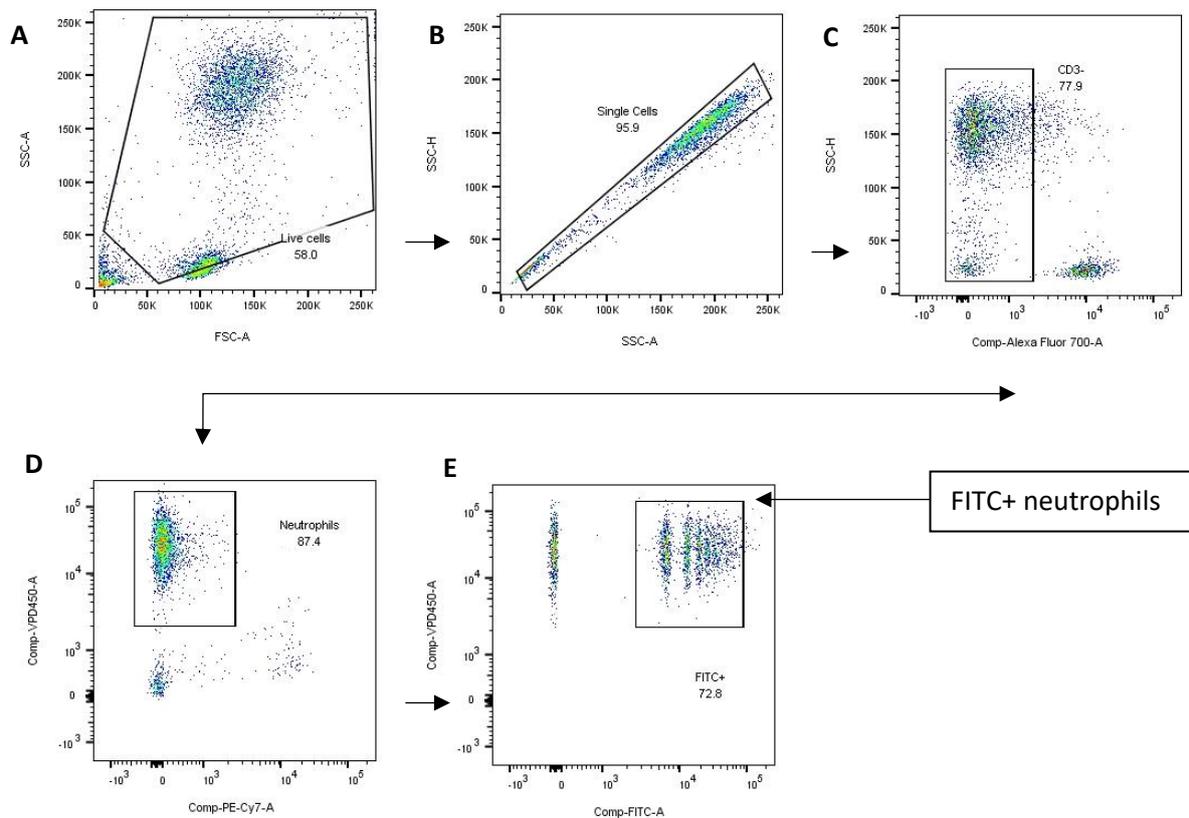
  

	Cells only
	antigen
	sample
	Negative control
	Positive control
	R10

FIGURE 11: Plate design used for both the ADCP and ADCC assays

#### 4.4.9 Gating Strategy for ADCP

To accurately gate for the monocyte populations, the cell size and granularity were used to place the population of interest within the grid, by altering the voltages. Unstained cells were used and the forward and side scatter voltages adjusted accordingly to identify live cells and distinguish them from cellular debris. Positive controls using HIVIG are shown in **Figure 12** and negative controls using PBS are shown in supplementary Figure 2, to accurately gate for ADCP activity. The parameters on the x and y –axis was then altered to SSC-Area and SSC-Height in order to identify single cell populations. Once the single cells were confirmed, the axis was changed again for the x-axis to represent CD3 AF700 and the y-axis to SSC-Height in order to identify the CD3 negative population, in this case the population of interest. Once identified, the x-axis to CD14 PE-Cy 7 and the y-axis to CD66B V450 in order to identify the neutrophils population expressing the CD66B, which is a neutrophil marker. The X-axis was changed to the FITC marker and the Y-axis to CD66B V450 (**Figure 12**), which will aid in calculating the percentage of neutrophils that phagocytosed the neutravidin beads that were conjugated onto the selected antigen, i.e.: gp120. Phagocytic scores were determined by analysing the data using FlowJo V10.0.1 (TreeStar)  $[(\text{percentage of neutrophils taking up beads} \times \text{mean fluorescent intensity})/10,000]$ , which represents the integrated MFI (iMFI). This was proportional to the number of cells and relative to the bead uptake, adapted from (Ackerman et al., 2011).



**FIGURE 12: Gating strategy for the ADCP assay, investigating the phagocytic activity of neutrophils.** Live cells were gated on using FSC-A and SSC-A. Sequential gating from live cells, included singlets, followed by CD3- cell, neutrophils and then neutrophils that phagocytosed antigen coated neutravidin beads.

#### 4.4.10 Intracellular Cytokine Staining of NK cell activated Antibody Dependent Cellular Cytotoxicity (ADCC) Assay

The ADCC assay required isolation of natural killer cells from fresh whole blood collected in citrate tubes, by venepuncture. The assay was conducted according to the protocol outlined by the Ragon Institute, Boston, USA. R10 media composed of 10% heat inactivated FCS, RPMI 1640 (Lonza, Maryland, USA), HEPES (Lonza, Maryland, MD), Penicillin-streptomycin (Whitehead Scientific, Cape Town, SA) and L-Glutamine (Gibco, New York, USA) was prepared. The R10 and ficoll (Histopaque) (Sigma-Aldrich, Dramstadt, Germany) was warmed to room temperature before proceeding with the experiment. A 96 flat bottom

high-binding ELISA plate (Sigma-Aldrich, Dramstradt, Germany) was used to incubate 1.5-3µg/ml of HIV positive proteins per well and an SF PBS-BSA as the background control, in a total volume of 100 µl (made up in 1 x sterile PBS). Assays were run in duplicate with controls run in triplicates. The plate was centrifuged at 1000RPM for 2 minutes. The plate was sealed and incubated for 2 hours at 37°C before it was washed, 3 times with 200 µl of PBS per well, using a multichannel pipette, with blotting in between each wash. Following the removal of excess antigen, 200µl of 5% BSA (10% BSA, KPL, Massachusetts, USA) in PBS was added to prevent non-specific binding. The plate was then incubated overnight at 4°C, or for 1hr at room temperature. During incubation, the samples were prepared at the optimal concentration in PBS as determined by optimisation experiments, which included serial dilutions of purified IgG from 1mg/ml>0.5mg/ml>0.25mg/ml for the plasma. Regarding genital tract CVL samples, neat CVL was used at the same volume used for ADCP assays, which was 50µl of neat CVL, in order to standardise the volume used. Following incubation, the plate was washed 3x with 200µl of PBS and incubated with 50 µl of purified IgG at 0.25mg/ml at 37°C for 2 hours.

During the incubation period, the natural killer (NK) cells were isolated from whole blood on the day of the experiment or the day before, using a RosetteSep (Separations Scientific, Vancouver, ca) as per manufacturing instructions. Briefly, 25µl of RosetteSep per 1 ml of fresh whole blood was added and then incubated at room temperature for 20 minutes. 15 ml of Ficoll (Histopaque) (Sigma Aldrich, Dramstradt, Germany), was added to a 50 ml falcon tube. After the incubation period the 15ml falcon tube containing the blood and Rosettesep beads was topped up with an equal volume of PBS. The blood with the RosetteSep beads, were layered onto the ficoll with care taken to avoid mixing the blood and ficoll. After layering, the ficoll and blood was spun down for 20 minutes at 1200 x g at 25°C, with breaks off, to isolate the NK cells. The NK cells were washed twice with R10, after being centrifuged at 1700 rpm for 5min at 25°C. The NK cells were re-suspended to  $2.5 \times 10^5$  cells/ml in R10. If the cells were incubated overnight, 0.2 µl per ml of IL-15 at a concentration of 1ng/ml (Life Technologies, USA), was added to the culture flask and incubated overnight at 37°C, 5% CO<sub>2</sub>. A full plate requires  $5 \times 10^6$  NK cells in 20 ml of media. An inhibitor cocktail consisting of the following antibodies, 2.5 µl of CD107a-PE-Cy5, 0.4µl of brefeldin and 10µl of Golgi stop (BD Biosciences, San Jose, CA). The Golgi stop was made up in the following manner, 13.6µl of stock Golgi stop +1ml of R10. Notably, the inhibitor cocktail was added to the NK cells in the

culture flask, before they were added in at a volume of 200µl per well. After incubating the antigen coated ELISA plate with the sample, the plate was flicked to remove excess IgG and blotted before it was washed 3 times with 200µl of PBS from Lonza. The plate was covered with a lid from the V-bottom ELISA plate (Sigma-Aldrich, USA) and then incubated for 5 hours at 37°C at 5% CO<sub>2</sub>. The surface stain was prepared to consist of the following antibodies – 0.25µl of CD3 A700, 1µl of CD56 PE-Cy-7, 1 µl of CD16 Allophycocyanin and Cyanine 7(APC-CY 7) (BD Biosciences, San Jose CA) and 7.75µl of PBS. 10µl of the surface stain was added to a new V bottom plate and after the cells-antibody complex incubated, the complex was transferred to the ELISA plate, containing the surface stain. If the NK cells were isolated the day before the experiment and stimulated with IL-15 overnight, the 3µl of the Live/dead aqua stain (Life Technologies, USA), was included in the surface stain, per well. The V-bottom plate was then incubated at room temperature in the dark for 15 minutes. The plate was then spun at 500g for 5 minutes with breaks off and flicked, to remove excess antibodies and 200µl of PBS added, before being spun and flicked again. Following washing, 50µl of Perm A (Thermofisher Scientific, Gerichtsberg) was added to the V-bottom plate and incubated for 15 minutes. After incubation, 100µl of PBS was added and the plate washed, by centrifuging the plate at 500g for 5 minutes. The plate was washed a further 2 times with 200µl of PBS and flicked between each wash. The intracellular stain was prepared in the following format, 1 µl of IFN-γ APC, 1 µl of MIP1-β Phycoerythrin (PE) (BD Biosciences, California, USA) and 48 µl of Invitrogen Perm B (Life Technologies, USA). Following the plate wash, 50 µl of the intracellular stain was added to each well, mixed and incubated at room temperature, for 15 minutes, in the dark. Following incubation, the plate was washed with 200µl of PBS, by centrifuging the plate each time at 500g, for 5 minutes at 25°C and flicked after each wash. 150 µl of BD Cell fix at a 1x concentration, was added to each well and then acquired on FACS Fortessa from BD Biosciences USA, using the high throughput system (BD Biosciences, California, USA). The total number of events that were acquired for each well was set as 20 000 events.

#### 4.4.11 Antibody Panel for NK cell activated ADCC Assay

The same plate design as the ADCP assay was used in the ADCC assays. The NK cells were isolated and used on the same day, therefore forward and side scatter was used to identify the live population (**Table 4**).

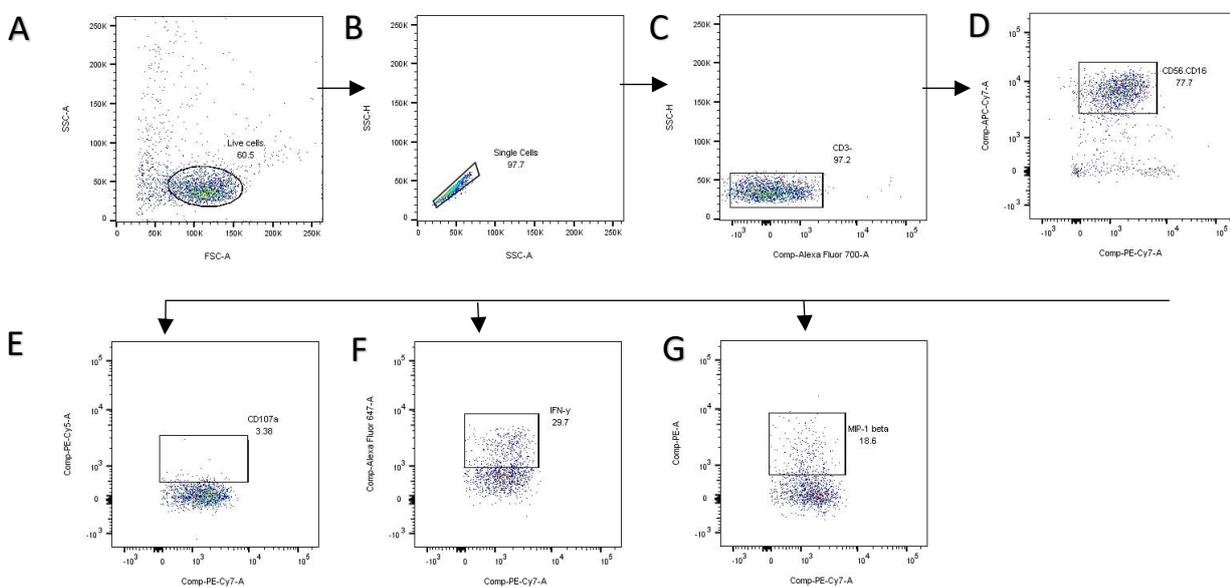
**TABLE 4: Antibody panel for the ADCC assay the ADCC assay**

Antibody Inhibitor cocktail	Cell type	Volume ( $\mu$ l)
CD107a –PE-Cy5	Marker for degranulation	2.5
Brefeldin (BFA)	Inhibits protein transport from Endoplasmic Reticulum to golgi	0.4
Golgi Stop	Accumulates cytokine production in the cell	10
<b>Surface Stain</b>		
CD3 A700	CD3 negative population	0.25
CD56 PE-Cy7	NK cell marker of activation	1
CD16 APC	Fc receptor marker on NK cells	1
<b>Intracellular Stain</b>		
IFN- $\gamma$ APC	Cytokine marker	1
MIP- 1 $\beta$ PE	Chemokine marker produced to signal other NK cells	1

#### 4.4.12 Gating Strategy for ADCC assay

Compensation for each experiment was run and set to ensure that the peaks for each fluorescent label were within range. This was done to ensure reproducibility and accuracy of each experiment according to those set by the rainbow beads, of a previously optimized ADCC experiment. To accurately gate for the NK cell population the voltages that are determined by the cell size and granularity were altered to place the population of interest within the grid. Unstained cells were used and the forward and side scatter voltages adjusted accordingly. The gating strategy was determined by both positive controls using HIVIG (**Figure 13**) and negative controls using PBS (supplementary Figure 3) in order to accurately identify NK cell mediated ADCC activity. The x-axis parameter was change to SSC-H and the y-axis to SSC-A to gate for singlet populations. To gate for CD3 negative NK cells only and to exclude NKT cells, CD3 AF700 was selected for the x-axis ad the y-axis to SSC-A to identify the CD3 negative population. CD16 receptor expression define the NK cell population, therefore the y-axis was changed to CD16- APC-Cy7 and the x-axis to CD56 PE-

Cy7. Upon activation CD16 MFI significantly decreases relating to ADCC activity (Stratov et al., 2008). Following this gating, the x-axis is changed to CD107 PE-Cy5 and the y-axis to CD16 APC-Cy7 to gate on the expression of the degranulation marker which indicates NK cell activity. Further gating to identify NK cell activity was done by changing the x-axis to IFN- $\gamma$  AF647 and then to MIP-1 $\beta$  PE, while maintaining the y-axis on CD16-APC Cy7 (**Figure 13**). “The percentage of NK cell CD107a, IFN- $\gamma$  and MIP-1 $\beta$  expressions were used to calculate the cytotoxic activities using FlowJo V10.0.1 (Treestar).”



**FIGURE 13: Gating strategy for the ICS NK cell activated ADCC assay. (A)** Live cells were gated on using FSC-A and SSC-A. Following identification of live cells in this bio-assay, **(B)** singlets were gated on, **(C)** followed by CD3- cells, **(D)** CD56+CD16dim NK cells and the subsequent cytokine productions to identify NK cell activity **(E)** CD107a, **(F)** IFN- $\gamma$  and **(G)** MIP-1 $\beta$ .

## CHAPTER 5

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### Results

#### 5.1 Demographics of study participants in the CAPRISA 004 1 % Tenofovir gel trial

This sub-study included 48 women who acquired HIV infection while participating in the CAPRISA 004 1% tenofovir gel trial (Abdool Karim et al., 2010). In order to account for CD4<sup>+</sup> T helper cells and the viral loads that could confound analysis, the women from both arms were matched on CD4<sup>+</sup> T cell counts and viral loads at 6 months post-infection. Therefore, there were no significant differences in either the CD4<sup>+</sup> T cell counts or HIV viral loads between the arms. Twenty-four women from the tenofovir gel (cases) arm and 24 women from the placebo (controls) arm were included in this sub-study. Over half of the women came from rural KwaZulu-Natal area (58.3%, **Table 5**). Overall, the median age of women in this study was (23; IQR = 22 - 28 years) with no significant differences noted between the ages of the women in the tenofovir (24; IQR = 22 - 28 years) and placebo arms (22; IQR = 22 - 23 years). Delay in time to infection was significantly longer among women who used the tenofovir gel (12.8 months; IQR = 6.6-16.6) compared to those who used the placebo gel (7.4 months; IQR = 3.3 - 10.6) (**P = 0.02**), with an average difference of 5.4 months between the arms. There were no significant differences for education level or hormonal contraceptive use between the women in both arms (**Table 5**). With regard to condom use, 33 (68.8%) women reported using condoms at the last sex act and 45 (93.8%) women reported 0 - 1 sexual partners in the past 3 months, however there was no statistically significant differences observed between women who used condoms in the tenofovir or placebo arm. Overall, the women in this study were similar across each demographic category except for the time to HIV infection (**Table 5**).

**Table 5: Demographics and clinical characteristics of the sub-set of women who acquire HIV infections during the CAPRISA 004 trials (n = 48)**

Characteristics	All (n = 48)	Tenofovir (n = 24)	Placebo (n = 24)	P - value
Rural % (n)	58.3% (28)	58.3% (14)	58.3% (14)	ns
Median age in years (IQR)	23 (22 - 25)	24 (22 - 28)	22 (22 - 23)	ns
Median days PI at enrolment (IQR)	38 (24 - 65)	35 (27 - 63)	45 (23 - 65)	ns
Median CD4 count (cells/ $\mu$ l) (IQR)	498 (434 to 655)	468(444 - 569)	515 (433 - 685)	ns
Median viral load (copies/ml) (IQR)	59,0505 (17,300 - 135,500)	80,600 (22,000 - 130,000)	54.800 (13,600 - 148,000)	ns
Time to HIV infection from enrolment in months (IQR)	9.2 (4.9 - 14.1)	12.8 (6.6 - 16.6)	7.4 (3.3 - 10.6)	<b>0.02**</b>
Completed high school % (n)	54.2% (26)	41.7% (10)	66.7% (16)	ns
Hormonal contraceptive use <sup>b</sup>	97.9% (47)	100% (24)	95.8% (23)	ns
Marital Status % (n)				
Stable/married partner	79.2% (38)	87.5% (21)	70.8 (17)	ns
Single	18.7% (9)	12.5% (3)	25.0% (6)	ns
>2 partners	2.1% (1)	0.0% (0)	4.2% (1)	ns
Numbers of reported sexual partners in the last 3 months % (n)				
0 to 1	93.8% (45)	95.8% (23)	91.7% (22) <sup>a</sup>	ns
2 to 5	6.2% (3)	4.2% (1)	8.3% (2)	ns
Reported condom used at last sex act % (n)	68.8% (33)	75.0% (18)	62.5% (15)	ns

IQR = interquartile range, PI = post-infection.

Significant values were defined as **\*\*( $P \leq 0.05$ )** and those that trended as **\*( $P < 0.1$ )**

<sup>a</sup> Two non-rapid progressing women had missing sexual partner data (in previous 3 months) as they refused to answer the question.

<sup>b</sup> Hormonal contraception use included the injectable norethisterone and depot medoxyprogesterone acetate, and oral contraception. One woman in the placebo arm of the study was using an intrauterine device.

## 5.2 Antibody dependent cellular phagocytosis mediated by IgG's isolated from the plasma and genital tracts of study participants

### 5.2.1 Plasma IgG was more detectable and had higher concentrations than that of the genital tract

Plasma IgG concentrations in the tenofovir and placebo arm were similar at 3 months (2.8mg/ml for both arms), 6 months (2.5mg/ml for both arms) and at 12 months (tenofovir arm = 2.9mg/ml; placebo = 2.9mg/ml). IgG concentrations were determined after purifying IgG from 50µl volume of the plasma samples from the HIV infected women were diluted with 450 µl of IgG purification buffer, representing a 10-fold dilution of the plasma IgG concentrations, which roughly translates to between 25-28mg/ml. In the CVL, the concentrations of IgG were similar between both arms and across time points. However, the CVL concentrations irrespective of arm and time points were much lower than the IgG quantified in the plasma (**Table 6**). Furthermore, there were significant differences between IgG levels in the plasma compared to those in the genital tract, across time points and irrespective of arm assignment ( **$P < 0.0001$** ). For these analyses, the concentrations were adjusted to similar units of measurement i.e. mg/ml for the concentration of IgG quantified in each compartment. Notably, in the tenofovir arm, the plasma IgG concentrations were 96.6-fold higher at 3 months, 125.0-fold higher at 6 months and 193.3-fold higher at 12 months, than that in the genital tract. A similar trend followed for the placebo arm with the plasma IgG concentrations being 140.0-fold higher at 3 months, 138.9-fold higher at 6 months and 117.4-fold higher at 12 months, than those in the genital tract. Plasma and CVL IgG unit concentrations were represented differently, in order to best portray the data i.e. plasma IgG concentrations in mg/ml and CVL IgG concentrations in µg/ml. The IgG isolated from the plasma and genital tracts of women in the study, had similar concentrations regardless of study arm, at each time point (**Table 6**).

**TABLE 6: Plasma and Genital tract IgG concentrations for women in the Tenofovir and Placebo arms**

Months Post-infection	Plasma median IgG (mg/ml) (IQR)		CVL median IgG (IQR) (µg/ml)		Plasma vs CVL IgG concentration P - value		Fold differences between Plasma IgG and CVL IgG ***		Tenofovir vs Placebo P-value	
	Tenofovir	Placebo	Tenofovir	Placebo	Tenofovir	Placebo	Tenofovir	Placebo	Plasma	CVL
<b>3</b>	2.8 (2.3 - 3.2)	2.8 (2.5 - 3.2)	29.0 (18.0 - 36.5)	20.0 (11.0 - 28.0)	0.0001**	< 0.0001**	96.6	140.0	0.807	0.104
<b>6</b>	2.5 (2.3 - 2.6)	2.5 (2.2 - 2.8)	20.0 (13.5 to 30.5)	18.0 (15.0 - 35.0)	< 0.0001**	< 0.0001**	125.0	138.9	0.715	0.857
<b>12</b>	2.9 (2.3 - 3.0)	2.7 (2.5 - 3.5)	15.0 (6.0 - 28.0)	23.0 (18.0 - 27.0)	< 0.0001**	< 0.0001**	193.3	117.4	0.948	0.503

\*\*indicates significant values defined as  $P \leq 0.05$

\*\*\*fold difference was calculated by dividing the plasma median IgG concentrations (mg/ml) in each arm and time point by the corresponding median IgG concentration in the genital specimens (CVL) (µg/ml). The mg/ml concentrations in the plasma were converted to (µg/ml) in order to calculate differences between the plasma and genital tract

### 5.2.2 Tenofovir gel use did not diminish the detectability of the phagocytic function in the genital tracts of women who used the gel

To determine whether antibody functions were different in women who had prior tenofovir gel use compared to the placebo gel users, the neutrophil phagocytic activity mediated through the antibodies isolated from the genital tract and the plasma, were performed. Phagocytic responses above detectability were defined as scores greater than the background as indicated by the negative control [negative control HIV-1 gp120 > 11.24; gp41 >11.22; p66 > 9.32; p24 >29.5]. In the plasma, based on the phagocytic scores above the background, most women in the tenofovir and placebo arms had detectable phagocytic function, which did not diminish over time (**Table 7**). Notably at 12 months, there was an increased detection for phagocytic function, in the plasma to all the HIV-1 proteins in both arms (**Table 7**). In particular, in the tenofovir arm, there was a 100% detection to both p66 and gp41 from 3 months and 6 months, up until 12 months, where the phagocytic response declined to 82.4% and 88.2% for p66 and gp41 respectively.

Phagocytic activities in both compartments and arms were highly detectable at 3 months, after which responses seemed to increase or decrease sporadically in both arms, over the 6 months and 12-month period for which these analyses were done (**Table 7 and Table 8**).

To assess the impact of IgG titres on ADCP activities, titrations of IgG isolated from the plasma of women in both arms were performed. No differences in ADCP specific activities were found between 1mg/ml and 0.5mg/ml ( $P > 0.1$ ) (supplementary Figure 1A). Therefore, the sample concentrations were diluted to a concentration of 0.5mg/ml to be used in the ADCP assays.

**TABLE 7: Detection of HIV-specific phagocytic activity in the tenofovir and placebo arms in the plasma**

HIV Proteins	Tenofovir Arm			Placebo Arm		
	3 months n/N = 15 (%)	6 months n/N = 23 (%)	12 months n/N = 17 (%)	3 months n/N = 16 (%)	6 months n/N = 25 (%)	12 months n/N = 22 (%)
<b>gp120</b>	12 (80)	18 (78.3)	14 (82.4)	13 (81.3)	23 (92.0)	22 (100.0)
<b>gp41</b>	15 (100.0)	23 (100.0)	15 (88.2)	15 (93.8)	24 (96.0)	22 (100.0)
<b>p66</b>	15 (100.0)	23 (100.0)	14 (82.4)	16 (100.0)	25 (100.0)	22 (100.0)
<b>p24</b>	11 (73.3)	18 (78.3)	16 (94.1)	14 (87.5)	22 (88.0)	21 (95.5)

**TABLE 8: Detection of HIV-specific phagocytic activities in the genital tracts (CVL) of women in both the tenofovir and placebo arms**

HIV Proteins	Tenofovir Arm			Placebo Arm		
	3 months n/N = 15 (%)	6 months n/N = 23 (%)	12 months n/N = 17 (%)	3 months n/N = 16 (%)	6 months n/N = 25 (%)	12 months n/N = 22(%)
<b>gp120</b>	12 (80)	16 (69.6)	14 (82.4)	15 (93.8)	23 (92.0)	19(86.4)
<b>gp41</b>	14 (93.3)	20 (86.9)	15 (88.2)	14 (87.5)	19 (76.0)	16(72.7)
<b>p66</b>	14 (93.3)	20 (86.9)	14 (82.4)	15 (93.8)	24 (96.0)	21(95.5)
<b>p24</b>	15 (100)	22 (95.7)	16 (94.1)	15 (93.8)	22 (88.0)	22(100.0)

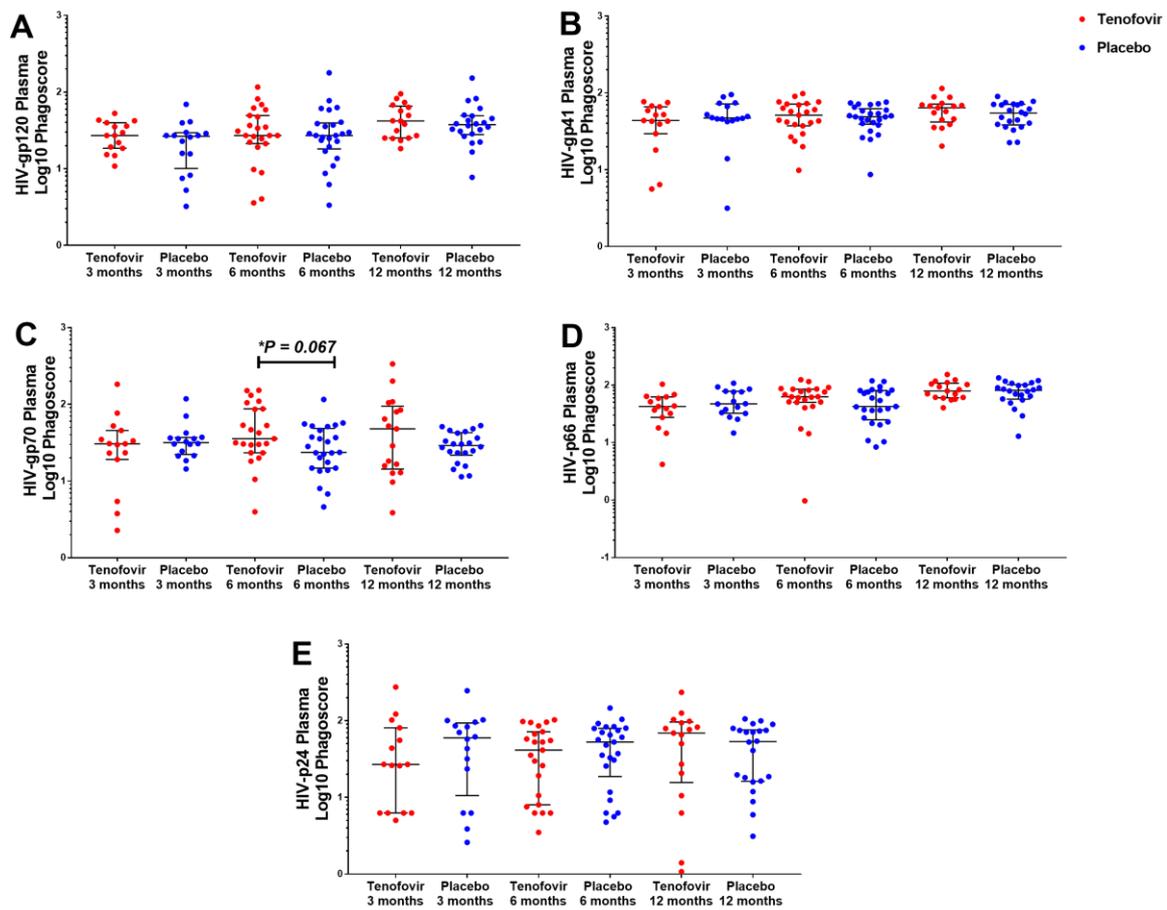
### 5.2.3 Phagocytic activities to HIV-1 gp120 and p66, increased from 3-12 months post-infection in the plasma

To determine if there were differences between arms for the phagocytic functions mediated by the HIV-specific antibodies, the phagocytic activities between arms were compared at the three different time points. There were no significant differences between the tenofovir and placebo phagocytic responses to gp120 in the plasma, at 3 months ( $P = 0.295$ ), 6 months ( $P = 0.760$ ) or 12 months ( $P = 0.664$ ). Phagocytic responses to gp41 remained similar at 3 months ( $P = 0.446$ ), 6 months ( $P = 0.759$ ) and 12 months ( $P = 0.644$ ) when the placebo and the tenofovir arms were compared (**Figure 14B**). However, phagocytic activities to gp70 was higher in the tenofovir compared to the placebo arm at 6 months (tenofovir 35.70; IQR = 23.31 - 87.37, placebo 23.68; IQR = 14.74 - 48.52) ( $P = 0.067$ ). Plasma phagocytic activities to p66 between arms at 3 months ( $P = 0.338$ ), 6 months ( $P = 0.210$ ) and 12 months ( $P = 0.664$ ) was not statistically significant. Furthermore, there were no statistically significant differences between arms for p24 at 3 months ( $P = 0.512$ ), 6 months ( $P = 0.609$ ) and at 12 months ( $P = 0.457$ ) (**Figure 14E**, supplementary Table 1).

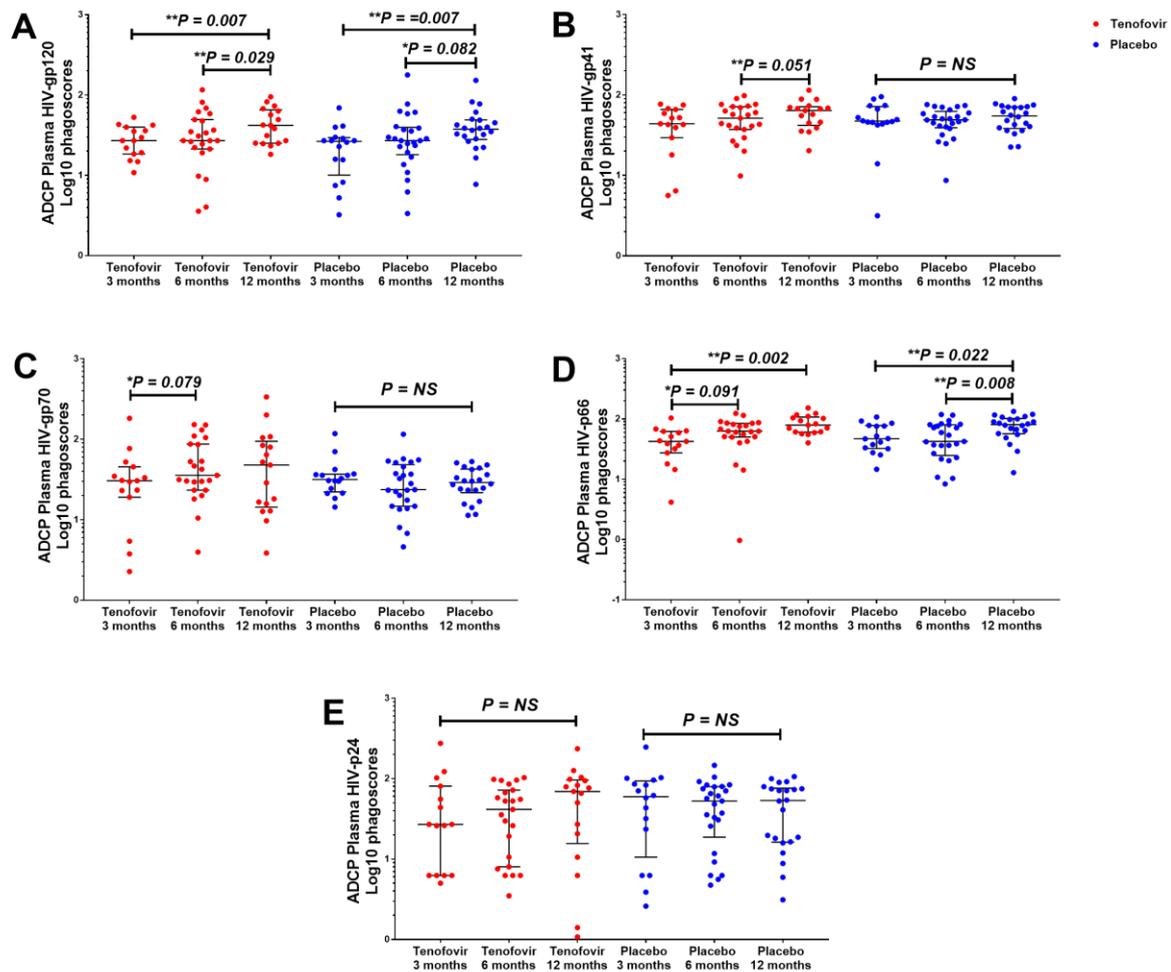
### 5.2.4 Increasing phagocytic activity to HIV-Env and p66 over time, in both arms

Longitudinal analyses of HIV- gp120 phagocytic activities in the tenofovir arm in the plasma, were significantly lower at 3 months (27.12; IQR = 18.49 - 39.85), compared to 12 months (41.99; IQR = 25.17 - 66.51) ( $P = 0.007$ ) (**Figure 15A**). This significantly increasing response was observed from 6 months (27.12; IQR = 18.14 - 39.65), to 12 months ( $P = 0.029$ ). However, when only the detectable responses for the gp120 specific phagoscores were included for the tenofovir arm, (that is all phagoscores below the negative control were removed from analyses), a difference from 3 months to 6 months was observed ( $P = 0.067$ ) and an increase from 3 months to 12 months was observed ( $P = 0.001$ ) (supplementary Figure 5A). Phagocytic activities to gp120 in the placebo arm significantly increased from 3 months (26.52; IQR = 10.08 - 29.56) to 12 months (37.52; IQR = 28.00 - 49.11) ( $P = 0.007$ , **Figure 15A**). Additionally, the increase in phagocytic activities were maintained from 6

months (27.12; IQR = 18.14 - 39.65) to 12 months (37.52; IQR = 28 - 49.11), in the placebo arm to gp120, although this increase trended to significance ( **$P = 0.082$** , **Figure 15A**) and this difference was more obvious when only detectable gp120 responses were analysed (supplementary Figure 5A). Responses to gp41 revealed no significant increase from 3 months to 6 months in the tenofovir ( $P = 0.535$ ) or placebo arms ( $P = 0.721$ ) (**Figure 15B**). However, in the tenofovir arm a significant increase was observed from 6 months (51.52; IQR= 37.26 - 71.69) to 12 months (64.1; IQR = 41.91 - 71.59) ( **$P = 0.051$** , **Figure 15B**) (supplementary Figure 5B). No further significant differences were observed to gp41 in the placebo arm from 6 months to 12 months ( $P = 0.519$ ) or from 3 months to 12 months in the tenofovir ( $P = 0.114$ ) or placebo arms ( $P = 0.988$ ) (**Figure 15B**). Interestingly, gp70 specific ADCP activity in the tenofovir arm only, increased from 3 months (30.56; IQR = 19.1 – 45.58) to 6 months (35.7; IQR = 23.31 – 87.37) ( **$P = 0.079$** ) (**Figure 15C**), but this difference was lost when looking at detectable gp70 ADCP only (supplementary Figure 5C). In the tenofovir arm, phagocytic activity to p66 increased (**Figure 15D**), that trended to significance, from 3 months (43.25; IQR = 27.40 - 62.37) to 6 months (62.76; IQR = 50.15 - 84.58), ( **$P = 0.091$** ). This profile also significantly increased in the same arm from 3 months (42.35; IQR = 27.4 - 62.37) to 12 months (79.01; IQR = 60.21 - 107.5) ( **$P = 0.002$** ) (**Figure 15D**). Analysis in the placebo arm to HIV-p66, in the plasma, exhibited significant differences from 6 months (42.41; IQR = 24.87 - 80.24) to 12 months (80.89; IQR = 56.81 - 101.90) post-infection ( **$P = 0.008$** ). Further differences for HIV-specific p66 responses were observed at 3 months (47.16; IQR = 32.4 - 77.85) to 12 months (80.89; IQR = 56.81 - 101.9) ( **$P = 0.022$** ) post-infection (**Figure 15D**) (supplementary Figure 5D). This HIV-p66 specific pattern was also seen in the tenofovir arm with increasing activity from 3 months to 6 months ( **$P = 0.091$** ), and from 3 months to 12 months ( **$P = 0.002$** , **Figure 15D**). P24-specific phagocytic activities in the plasma of women in both the tenofovir and placebo arms showed no significant trends, when longitudinal analyses were performed (**Figure 15E**). Phagocytic activities were similar at 3 to 6 months in the tenofovir ( $P > 0.999$ ) and placebo arms ( $P = 0.670$ ), as well as from 6 months to 12 months in the tenofovir ( $P = 0.274$ ) and placebo arms ( $P = 0.622$ ) and from 3 months to 12 months in the tenofovir ( $P = 0.365$ ) and placebo arms ( $P = 0.188$ ) (**Figure 15E**). In contrast, in the detectable only analyses, a significant increase in p24 phagocytic activities in the placebo arm were observed, from 3 months to 12 months ( **$P = 0.048$** ) (supplementary Figure 5E).



**FIGURE 14: Comparisons of plasma IgG HIV-specific phagocytic activities between the tenofovir and placebo arms at 3 months, 6 months and 12 months.** Phagocytic activities (Log<sub>10</sub> phagosome scores) to HIV A) gp120, B) gp41, C) gp70, D) p66 and E) p24. Solid lines in either the tenofovir or the placebo arm indicate the minimum and maximum values of box and whisker plots, with black solid lines across the dots indicating the median of each time point and arm, accordingly. Horizontal lines across bars, indicate statistical differences between arms at respective time points and values that trended to significance as  $*P < 0.1$ . Statistical differences were determined by Wilcoxon Sum Rank tests.



**FIGURE 15: Longitudinal analyses of plasma IgG HIV-specific phagocytic activities in both the tenofovir and the placebo arms across 3 months, 6 months and 12 months.** Phagocytic activities (Log<sub>10</sub> phagocytic scores) to HIV **A)** gp120, **B)** gp41, **C)** gp70, **D)** p66 and **E)** p24. Solid lines indicate the minimum and maximum values of box and whisker plots, with black solid lines across the dots indicating the median of each time point and study arm, accordingly. Horizontal lines across dot plots indicate statistical differences between the tenofovir and placebo arm, at respective time points. **\***( $P < 0.1$ ) indicate values that trended to significance. Those that were statistical differences were defined as **\*\***( $P \leq 0.05$ ) and were determined by Wilcoxon Signed Rank test.

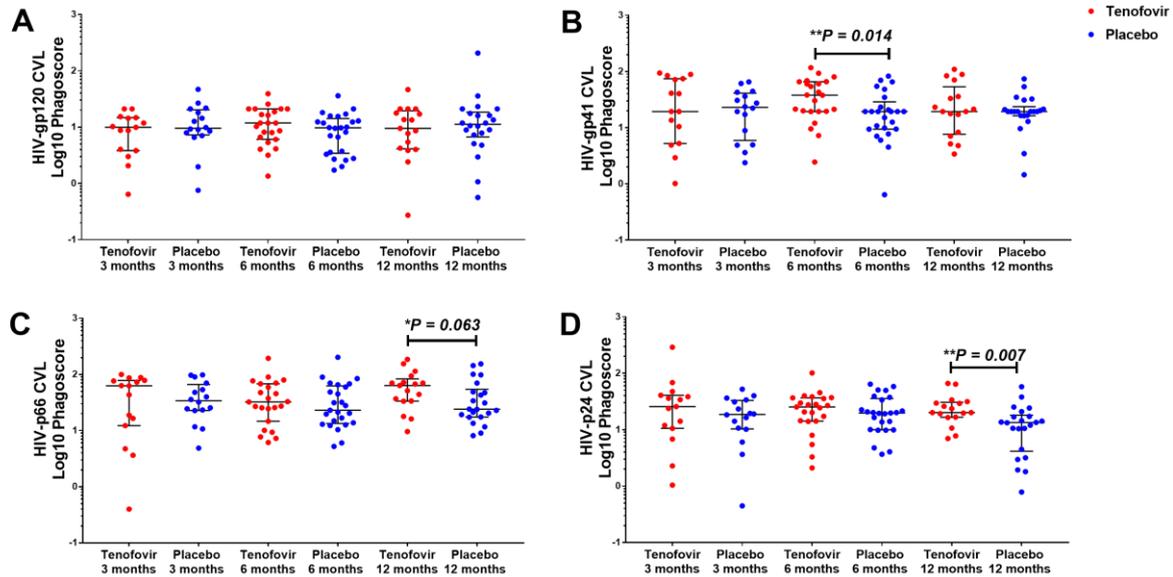
### **5.2.5 CVL phagocytic activity to p66 and p24 significantly increased only at 12 months in the tenofovir arm compared to the placebo arm**

Phagocytic responses to gp120 were not significantly different at 3 months ( $P = 0.572$ ), 6 months ( $P = 0.235$ ) or 12 months ( $P = 0.721$ ) between placebo and tenofovir arms. However, phagocytic activity to gp41 was significantly increased in the tenofovir arm (37.92; IQR = 19.4 - 65.60) compared to the placebo arm (19.4; IQR = 9.43 - 28.93) at 6 months ( **$P = 0.014$** ) (**Figure 16B**). Following this analysis, no significant differences were observed at any other time point between the two study arms for gp41 at 3 months ( $P = 0.579$ ) and 12 months ( $P = 0.642$ ) (**Figure 16B**). Cross-sectional analysis of phagocytic responses to p66 revealed that responses were significantly elevated in the tenofovir arm (63.58; IQR = 33.48 - 83.09) compared to the placebo arm (24.03; IQR = 17.44 - 54.16) ( **$P = 0.063$** ) (**Figure 16C**) at 12 months post-infection. No significant differences were observed at 3 months ( $P = 0.892$ ) and 6 months ( $P = 0.570$ ). Similarly, phagocytic activity to p24 was significantly increased only in the tenofovir arm at 12 months (26.15; IQR = 16.61 - 30.62) compared to the placebo arm (13.42; IQR = 4.16 - 18.11) ( $P = 0.01$ ) (**Figure 16D**). No other significant differences were observed between arms at 3 months ( $P = 0.358$ ) and 6 months ( $P = 0.461$ ) for p24 (supplementary Table 2). Similar responses were observed in the detectable cross-sectional analyses for the HIV-specific phagocytic activities in the genital tract (supplementary Figure 6A-D).

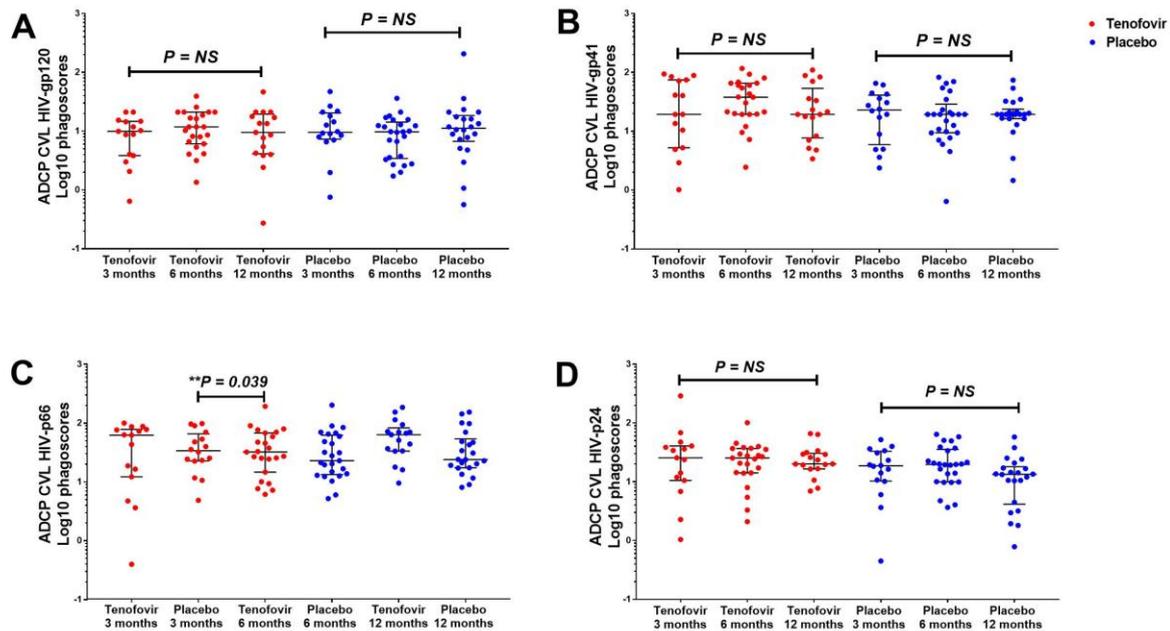
### **5.2.6 HIV-p66 specific phagocytic activity in the genital tract evolved significantly, in those women who used tenofovir, prior to HIV infection**

To identify trends in each arm over time, longitudinal analyses were performed. Analysis of phagocytic responses revealed varying differences and increasing (in some cases) responses to different HIV proteins over time. Interestingly, the only observable difference in phagocytic activities occurred in the tenofovir arm to p66, with responses increasing from 6 months (32.33; IQR = 14.68 - 67.99) to 12 months (63.58; IQR = 33.48 - 83.09) ( **$P = 0.039$** ) (**Figure 17C**). In contrast, the p66 specific responses in the placebo arm remained similar from 3 months to 12 months ( $P > 0.1$ ). Phagocytic activity in the genital tract to gp120

(Figure 17A), gp41 (Figure 17B) and p24 (Figure 17D) were similar across all time points, irrespective of study arm ( $P > 0.1$ ). Similar responses were observed in the detectable analyses (supplementary Figure 7A-D).



**FIGURE 16: Cross-sectional comparisons of ADCP responses in the genital tracts (CVL) of women in the tenofovir and placebo arms, for all HIV proteins, at 3, 6 and 12 months.** Phagocytic activities (Log10 phagosome scores) to **A)** gp120, **B)** gp41, **C)** p66 and **D)** p24 illustrated by box and whisker plots. Horizontal solid lines within each arm at respective time points indicate the maximum and minimum percentiles, as well as the median phagosome scores. Data collected did not display a Gaussian distribution therefore the Wilcoxon Sum Rank test tests were used. Differences that trended to significance were defined as  $*(P < 0.1)$  and those that were significant, as  $** (P \leq 0.05)$ .



**FIGURE 17: Longitudinal analyses of HIV-specific phagocytic activities in the genital tracts (CVL) of women in the tenofovir and placebo arms across 3 months, 6 months and 12 months.** Phagocytic activities (Log<sub>10</sub> phagocytosis scores) to HIV **A**) gp120, **B**) gp41, **C**) p66 and **D**) p24. Solid lines indicate the minimum and maximum values of box and whisker plots, with black solid lines across the dots indicating the median of each time point and study arm, accordingly. Horizontal lines across dot plots indicate statistical differences between the tenofovir and placebo arm, at respective time points. **\***( $P < 0.1$ ) Indicate values that trended to significance. Those that were statistically different were defined as **\*\*** ( $P \leq 0.05$ ) and were determined by Wilcoxon Signed rank test.

### 5.2.7 Tenofovir gel enhanced functional phagocytic activities in the genital tract, which were mediated by non-neutralizing antibodies

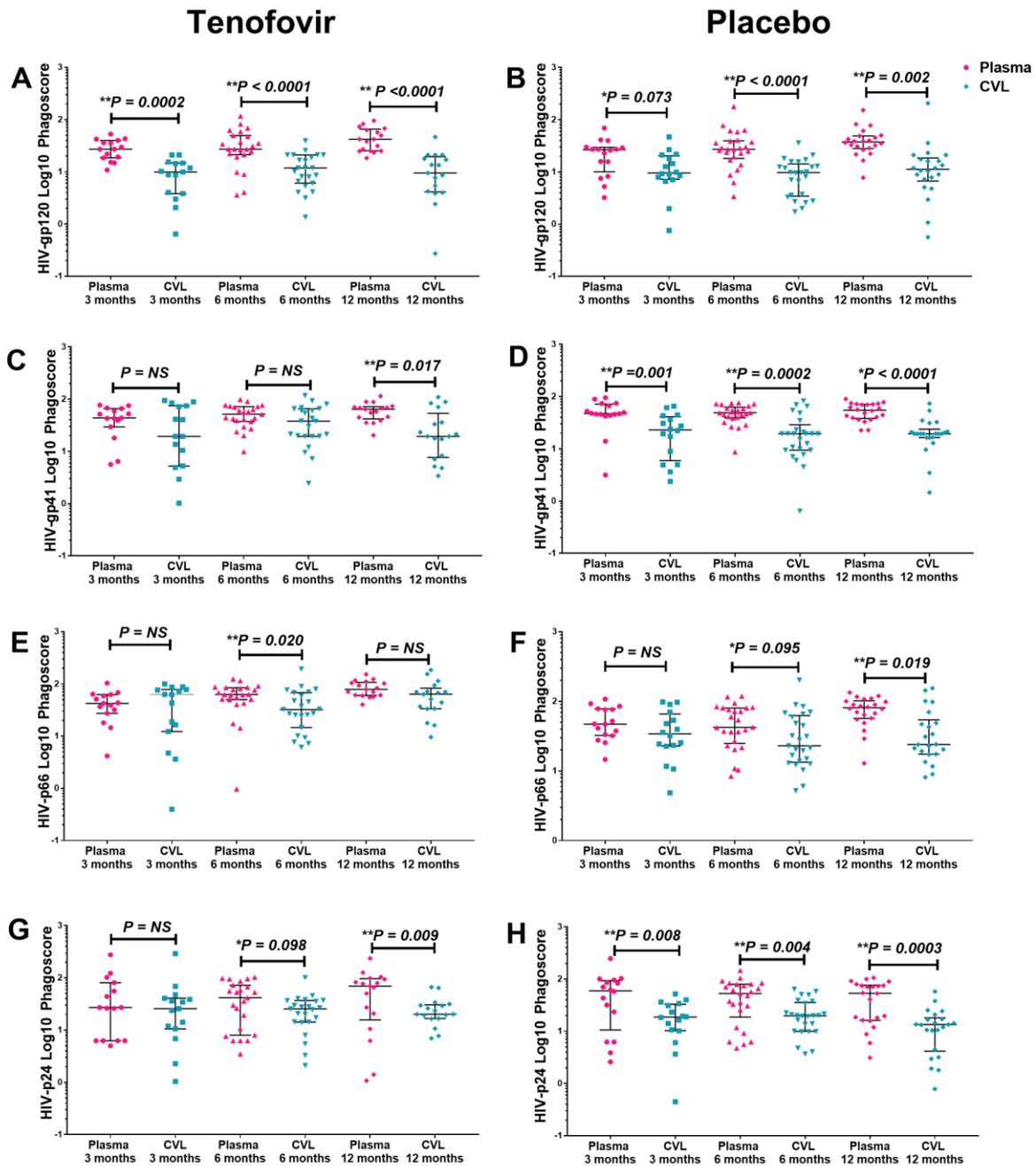
In order to compare ADCP activities between the systemic and genital tract, the CVL and plasma ADCP responses to HIV-1 proteins (gp120, gp41, p66 and p24) in both arms were analysed at 3 months, 6 months and at 12 months. In the tenofovir arm phagocytic activities to gp120 were greater at 3 months in the plasma (27.12; IQR = 18.49 - 39.85) compared to the CVL (9.95; IQR = 3.83 - 14.72) ( $P = 0.0002$ , **Figure 18A**). These significantly increased responses were consistently observed at 6 months [plasma 27.12 (IQR = 21-32 - 49.47); CVL 11.82 (IQR = 6.10 - 21.10)] ( $P < 0.001$ ) and at 12 months [plasma 41.99 (IQR = 25.17 - 65.51); CVL 9.51 (IQR = 4.12 - 19.44)] ( $P < 0.0001$ , **Figure 18A**). In the placebo arm, gp120 responses were higher at 3 months in the plasma (10.08; IQR = 3.23 - 29.56), compared to the CVL

(9.54; IQR = 7.30 - 20.35) (***P* = 0.073, Figure 18B**) but this difference only trended to significance. However, when only detectable phagoscores were compared in the placebo arm, similar responses at 3 months between the plasma and genital tract were found (*P* > 0.1, supplementary 8B). Furthermore, in the same arm, the observed disparity in compartment responses increased significantly, and became greater at 6 months in the plasma, (27.12; IQR = 18.14 - 39.65) compared to the CVL (9.73; IQR = 3.43 - 14.23) (***P* < 0.0001**), as well as at 12 months (plasma 37.52; IQR = 28.00 - 49.11; CVL 11.21; IQR = 6.71 - 18.45) (***P* = 0.002**) (**Figure 18B**).

HIV-gp41 specific phagocytic activity in the tenofovir arm, in the plasma, showed close concordance with those responses observed in the genital tract at 3 months and 6 months (*P* > 0.1). However, at 12 months plasma phagocytic activity (64.1; IQR = 41.91 - 71.59) was significantly greater than the CVL (19.4; IQR = 7.69 - 53.40) (***P* = 0.017**) (**Figure 18C**). Placebo arm responses to gp41 at 3 months in the plasma (47.26; IQR = 44.08 - 71.82) were greater compared to those observed in the genital tract (23.04; IQR = 5.94 - 41.09) (***P* = 0.001**) (**Figure 18D**). Furthermore, in the placebo arm, gp41 responses in the plasma at 6 months (49.00; IQR = 39.12 - 62.56) were greater than those in the genital tract (19.4; IQR = 9.43 - 28.93) (***P* = 0.0002**). This pattern was observed at 12 months in the same arm, with greater responses observed in the plasma (54.74; IQR = 38.35 to 71.13) compared to the genital tract (19.40; IQR = 16.39 - 23.75) (***P* < 0.0001**) (**Figure 18D**), and these differences were still preserved when only the detectable responses were included (supplementary Figure 8D).

Interestingly ADCP activity to p66 was similar between compartments at 3 months in the tenofovir arm (*P* = 0.525). However, at 6 months phagocytic activity was higher in the plasma (62.76; IQR = 50.15 - 84.58) compared to the genital tract (32.33; IQR = 14.68 - 67.99) (***P* = 0.020**), a difference that was not seen at 12 months (*P* = 0.284) (**Figure 18E**). In the placebo arm, phagocytic activity to p66 at 3 months (*P* = 0.433) was similar between compartments, but a trend was observed at 6 months in placebo arm, with phagocytic activity being greater in the plasma (42.42; IQR = 24.87 - 80.24) compared to the CVL (22.98; IQR = 13.37 - 62.46) (***P* = 0.095**). The increasing difference between systemic and genital tract phagocytic activity, became more pronounced at 12 months with the plasma exhibiting greater responses (80.89; IQR = 56.81 - 101.9) than in the CVL (24.03; IQR = 17.44 - 54.16) (***P* = 0.019**) (**Figure 18F**). HIV-p24 specific responses in the tenofovir arm were similar between the plasma and CVL at the 3 months (*P* = 0.978). However, at 6 months plasma were higher,

(plasma 41.51; IQR = 8.02 - 71.99, CVL 25.4; IQR = 14.2 - 36.81), and by 12 months was significantly different (plasma 53.48; IQR = 16.24 - 76.11, CVL 13.41; IQR = 4.16 - 18.11) (***P* = 0.009**) (**Figure 18G**). P24 specific responses in the placebo arm at 3 months were significantly different, with greater phagocytic activity observed in the plasma (59.78; IQR = 10.59 - 93.42) compared to the CVL (18.71; IQR = 10.32 - 33.13) (***P* = 0.008**) (**Figure 18H**). Similar differences were observed at 6 months with greater plasma median phagocytic activity (52.79; IQR = 18.71 - 79.68) compared to the CVL (19.81; IQR = 9.99 - 35.65) (***P* = 0.004**). At 12 months post-infection in the placebo arm, this difference was more evident, with greater p24 specific phagocytic activity in plasma, (53.48; IQR = 16.24 - 76.11) compared to the CVL (13.41; IQR = 4.16 - 18.11) (***P* = 0.0003**) (**Figure 18H**).



**FIGURE 18: Compartmental comparisons between plasma and genital tracts (CVL) of all women (n = 48), across 3 months, 6 months and 12 months, for all HIV-proteins, in the tenofovir and placebo arms. Inter-compartmental analysis, for phagocytic activities (Log10 phagosome scores) in the tenofovir arm on the left and the placebo arm on the right to gp120 (A and B), gp41 (C and D), p66 (E and F) and p24 (G and H). Wilcoxon Sum rank tests were done to determine statistical differences between phagocytic activities within each arm of the study and between compartments, for each protein. Statistically significant values were defined as **\*\*P ≤ 0.05**, values that trended as **\*P < 0.1** and **P = NS** for differences that were not significant. Cervicovaginal lavages (CVL) were collected from the genital tract.**

### 5.2.8 Genital tract and plasma phagocytic activity to p66 is inversely correlated

In order to establish whether the the phagocytic activity in the plasma correlated with the phagocytic activity in the genital tract cross-compartmental Spearman R correlation analyses for both the tenofovir and placebo arms were performed for all women. Gp120 specific ADCP at 6 months in the tenofovir arm exhibited a weak, positive correlation between the systemic and genital tract phagocytic activity ( $R = 0.352$ ;  $P = 0.099$ ) (Table 9). At 12 months in the placebo arm, gp120 responses showed a weak negative correlation between the systemic and genital tract ( $R = -0.368$ ;  $P = 0.092$ ). Additionally, regarding HIV envelope responses, systemic and genital tract phagocytic activity to gp41 exhibited a positive correlation in both the tenofovir and placebo arms, however this association was not significant (*tenofovir*:  $R = 0.454$ ;  $P = 0.092$ ; *placebo*:  $R = 0.432$ ;  $P = 0.096$ ). Interestingly, at 12 months post-infection in the placebo arm, phagocytic activity to p66, showed a significantly inverse correlation between the systemic and genital tract compartments ( $R = -0.481$ ;  $P = 0.024$ ). HIV-p24 phagocytic responses did not exhibit any significant cross-compartmental associations for either arm, at any of the time points (Table 9). Furthermore, when only detectable phagoscores were correlated between compartments, gp120 activity negative correlated only at 12 months in the placebo arm ( $R = -0.603$ ;  $P = 0.012$ ). Additional negative correlations were found only in the placebo arm to gp41 ( $R = 0.511$ ;  $P = 0.054$ ) and p66 ( $R = -0.442$ ;  $P = 0.051$ ) (supplementary Table 3).

**TABLE 9: Compartmental correlations of phagocytic activities between the plasma and the genital tracts (CVL) of women, in the Tenofovir and Placebo arms, for all HIV-proteins**

Protein	Arm	3 months		6 months		12 months	
		<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
gp120	Tenofovir	-0.034	0.905	<b>0.352</b>	<b>0.099*</b>	0.012	0.966
	Placebo	-0.268	0.313	0.047	0.8223	<b>-0.368</b>	<b>0.092*</b>
gp41	Tenofovir	<b>0.454</b>	<b>0.092*</b>	-0.195	0.372	-0.004	0.991
	Placebo	<b>0.432</b>	<b>0.096*</b>	-0.062	0.769	0.259	0.245
p66	Tenofovir	0.046	0.873	0.260	0.231	-0.297	0.246
	Placebo	-0.147	0.586	-0.257	0.215	<b>-0.481</b>	<b>0.024**</b>
p24	Tenofovir	0.088	0.753	-0.340	0.113	-0.172	0.509
	Placebo	-0.167	0.536	0.030	0.887	-0.053	0.817

\*indicates values that trended to significance, defined as  $P < 0.1$

\*\*indicates significant values defined as  $P \leq 0.05$

### 5.2.9 Plasma phagocytic responses to HIV-gp70 was positively correlated with CD4<sup>+</sup> T cell counts

CD4<sup>+</sup> T cells can influence B cell activity, as well as antibody titres. Therefore, in order to determine if CD4<sup>+</sup> T-cell count at 6 months post-infection impacted on the HIV-antibody specific phagocytic activities, correlation analyses were performed (**Table 10**). In the tenofovir arm, there were no observable correlations between the systemic CD4<sup>+</sup> T cell counts and any of the HIV-antibody specific phagocytic activities. However, in the placebo arm a positive correlation between the CD4<sup>+</sup> T cell count and gp70 antibody specific phagocytic activity was found ( $R = 0.465$ ;  $P = 0.025$ ). In the genital tract, overall, there were no significant associations observed between the systemic levels of CD4<sup>+</sup> T cells, and the HIV-antibody specific phagocytic activities (**Table 10**).

**TABLE 10: Correlations between CD4<sup>+</sup> T cells and phagocytic activities for HIV-specific antibodies at 6 months, in the plasma and genital tract (CVL)**

HIV antigens	CD4 <sup>+</sup> T cells vs Plasma phagocytic scores (Tenofovir) n = 24		CD4 <sup>+</sup> T cells vs Plasma phagocytic scores (Placebo) n = 24		CD4 <sup>+</sup> T cells vs CVL phagocytic scores (Tenofovir) n = 24		CD4 <sup>+</sup> T cells vs CVL phagocytic scores (Placebo) n = 24	
	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
gp120	0.080	0.724	-0.236	0.278	-0.026	0.908	0.147	0.503
gp41	0.105	0.644	0.229	0.294	0.003	0.988	-0.072	0.746
gp70	0.248	0.266	<b>0.465</b>	<b>0.025**</b>	-	-	-	-
p66	0.266	0.233	-0.020	0.929	-0.005	0.982	0.110	0.617
p24	-0.174	0.440	-0.041	0.854	0.116	0.608	0.310	0.149

\*\*indicates significant values defined as  $P \leq 0.05$

-no data for gp70 HIV-IgG specific activity due to limited sample availability

### 5.2.10 HIV-gp120 specific phagocytic activities negatively correlated with viral loads, in the genital tracts of women in the placebo arm

Viral load in infected individuals is a marker of disease progression. Therefore, in order to elucidate the impact of phagocytic activities in the plasma and genital tracts on viral load, Spearman R correlation analyses were performed (**Table 11**). In the placebo arm only, HIV-gp120 specific activity, in the genital tract negatively correlated with plasma viral load ( **$R = -0.418$ ;  $P = 0.047$** ). There were no further significant correlations observed between viral loads and any HIV-specific phagocytic activities, in either arm of the plasma or genital tract ( $P > 0.1$ ).

**TABLE 11: Correlations between viral loads and phagocytic activity for HIV-specific antibodies at 6 months post-infection, in the plasma and genital tract (CVL)**

HIV antigens	Viral load vs Plasma phagocytic scores (Tenofovir) n = 23		Viral load vs Plasma phagocytic scores (Placebo) n = 24		Viral load vs CVL phagocytic scores (Tenofovir) n = 23		Viral load vs CVL phagocytic scores (Placebo) n = 24	
	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
<b>gp120</b>	-0.298	0.168	0.126	0.567	-0.169	0.440	<b>-0.418</b>	<b>0.047**</b>
<b>gp41</b>	-0.213	0.329	-0.254	0.242	0.278	0.199	0.202	0.356
<b>gp70</b>	-0.243	0.264	-0.13	0.555	-	-	-	-
<b>p66</b>	-0.305	0.157	-0.227	0.298	-0.063	0.774	-0.097	0.659
<b>p24</b>	0.317	0.140	-0.089	0.684	-0.267	0.218	-0.101	0.647

\*\*indicates values that were significant, defined as  $P \leq 0.05$

-no data for gp70 HIV-specific activities due to limited sample availability

### 5.2.11 Gp41 IgG titres in the tenofovir arm and p66 IgG titres in the placebo arm, exhibited inverse correlations to IgG mediated phagocytic activities at 6 months post-infection, in the plasma

In order to identify an association between antibody titres measured in a previously published study (Archary et al., 2016) and antibody activities obtained in this sub-study, the analyses were done using the Spearman R correlations. Interestingly, a strong correlation was detected to HIV-gp41 in the tenofovir arm, which indicated that as systemic IgG mediated phagocytic activity increased, antibody gp41-specific titres decreased ( $R = -0.499$ ;  $P = 0.015$ ). Furthermore, a moderate correlation was observed for p66 in the placebo arm and although this trended ( $R = -0.404$ ;  $P = 0.062$ ), it suggests that as systemic IgG mediated phagocytic activity increased, antibody p66-specific titres decreased. No further correlations were observed to any of the other HIV-proteins in either the plasma or the genital tract, in either study arm ( $P > 0.01$ ) (Table 12).

**TABLE 12: Correlations between HIV-specific IgG titres and phagocytic activities for HIV-specific antibodies at 6 months post-infection, in the plasma and the genital tract (CVL)**

HIV antigens	IgG titres (Log10) vs plasma ADCP (Tenofovir) n = 23		IgG titres (Log10) vs plasma ADCP (Placebo) n = 23		IgG titres (Log10) vs CVL ADCP (Tenofovir) n = 23		IgG titres (Log10) vs CVL ADCP (Placebo) n = 24	
	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
gp120	-0.026	0.907	0.097	0.659	0.105	0.635	0.070	0.750
gp41	<b>-0.499</b>	<b>0.015**</b>	0.016	0.942	-0.083	0.705	0.484	0.181
Gp70	-0.033	0.883	-0.114	0.606	-	-	-	-
p66	0.262	0.227	<b>-0.404</b>	<b>0.062*</b>	0.050	0.824	-0.181	0.407
p24	0.234	0.283	0.320	0.147	-0.226	0.325	-0.239	0.272

\*indicates values that trended to significance defined as  $P < 0.1$

\*\*indicates values that were significant defined as  $P \leq 0.05$

-no data for gp70 HIV-IgG titres due to limited sample availability

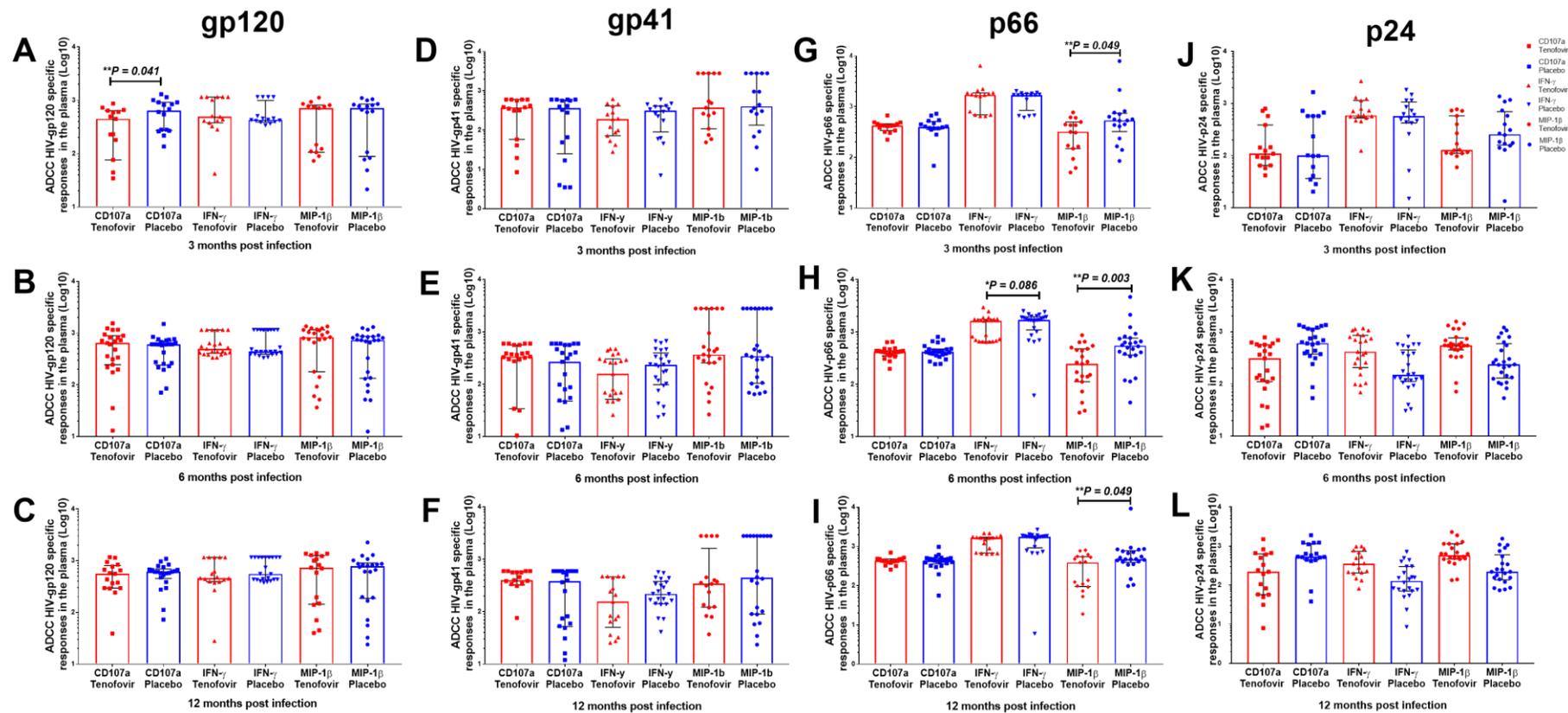
### 5.3 Functional NK cell activated Antibody Dependent Cellular Cytotoxicity using an Intracellular Cytokine Staining based assay to investigate plasma nAb IgG functionality

To assess the impact of IgG titres on NK cell activated ADCC activities, titrations of IgG isolated from the plasma of women in both arms were performed. No differences in NK cell activated ADCC specific activity were found between 1 - 0.5mg/ml, 0.5 – 0.25mg/ml and 1- 0.25mg/ml ( $P > 0.1$ ) (supplementary Figure 1B).

#### 5.3.1 Cross-sectional and longitudinal analysis of plasma nAb activities, identified an increase in IgG mediated NK cell activated ADCC at 6 and 12 months to HIV-gp41, p66 and from 3-12 months to HIV-gp120

To determine functional nAb differences between women who used either tenofovir or the placebo gel, cross-sectional analyses of NK cell activated ADCC activity to the four HIV-proteins at various time points were done. Cytotoxic activities were identified as CD107a expression and IFN- $\gamma$  and MIP-1 $\beta$  production as NK cell ADCC antiviral activity. Gp120 specific cytotoxic activity at 3 months in the tenofovir arm (449.5; IQR = 76.5 - 640.5) was

significantly lower than the placebo arm (644.8; IQR = 272.5 - 918.3) (**Figure 19A**). NK cell activated ADCC gp120 specific activity at 6 and 12 months and antiviral cytokine production at 3, 6 and 12 months were similar between the tenofovir and placebo arms ( $P > 0.1$ ) (**Figure 19C**). Cytotoxic activity to p66 was similar at 3, 6 and 12 months ( $P > 0.1$ ). However, at 6 months only, post-infection IFN- $\gamma$  was greater in the placebo arm (548.3; IQR = 365.8 - 764.3) compared to the tenofovir arm (246; IQR = 112.5 - 478.5) ( $P = 0.086$ ) (**Figure 19H**). P66 specific MIP-1 $\beta$  chemokine responses were greater in the placebo arm, compared to the tenofovir arm at 3 months (tenofovir 317.5; IQR = 149 - 500, placebo 535; IQR = 323.4 - 740.8) ( $P = 0.049$ ) (**Figure 19G**). This p66 specific response was maintained at 6 months (tenofovir 246; IQR = 112.5-478.5, placebo 548.3; 365.8-764.3) ( $P = 0.003$ ) and at 12 months (tenofovir 391; IQR = 95.25 - 549.5, placebo 473.3; IQR = 391.8 - 776.3) ( $P = 0.049$ ) (**Figure 19I**). Gp41 and p24 specific ADCC and antiviral cytokine production were similar between both study arms across the time points assessed ( $P > 0.1$ ) (supplementary Table 4) (**Figure 19D-F and 19J-I**, supplementary Table 4).

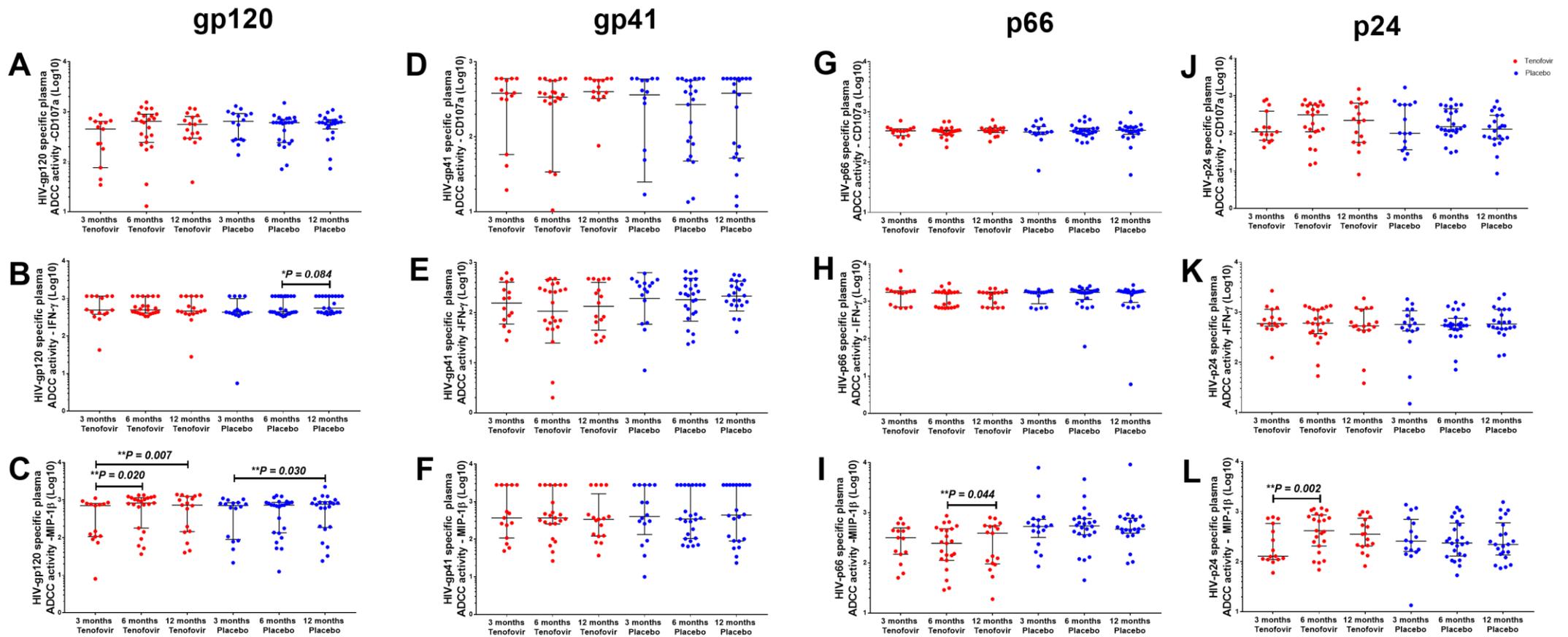


**FIGURE 19: Cross-sectional comparisons of plasma IgG activities mediated by NK cells isolated from fresh whole blood, between the tenofovir and placebo arms, across 3 months, 6 months and 12 months. ADCC activities (Log10 ADCC) measured by CD107a, IFN- $\gamma$  and MIP-1 $\beta$  production in both the tenofovir and placebo arm to HIV gp120- (A-C), gp41 (D-F), p66 (G-I) and p24 (J-L). Graphs read from top to bottom, to observe differences in ADCC responses over time, from 3-12 months post-infection. Solid lines in either the tenofovir or the placebo arm above the column indicate the maximum geometric mean of each cytokine detected in the ICS assay. Horizontal lines across bars, indicate statistical differences between arms at respective time points and statistical differences were defined as **\*\*( $P \leq 0.05$ )** and values trending to significance were defined as **\*( $P < 0.1$ )** (Wilcoxon Sum Rank test)**

### 5.3.2 Longitudinal plasma IFN- $\gamma$ and MIP-1 $\beta$ production increased over time to HIV gp120, p66 and p24 mainly in the Tenofovir arm

Furthermore, in order to elucidate the potential evolution of cytotoxic and antiviral cytokine production over time in either study arm, longitudinal analyses were performed. There was no significant difference in NK cell activated ADCC activity in both study arms to gp120 across all time points ( $P > 0.1$ ) (Figure 20A). IFN- $\gamma$  responses to gp120 in the placebo arm only, increased from 6 months (441.5; IQR = 393.5 - 1158) to 12 months (553.8; IQR = 430.9 - 1158) ( $P = 0.084$ ) (Figure 20B). Furthermore, MIP-1 $\beta$  production only increased in the tenofovir arm from 3 months (494; IQR = 380.5 - 1158) to 6 months (644; IQR = 245 - 895) ( $P = 0.020$ ) (Figure 20C). Additional differences were observed in the tenofovir arm from 3 months to 12 months (733.5; IQR = 144 - 1256) ( $P = 0.007$ ) (Figure 20C), exhibiting a significant increase in MIP-1 $\beta$  production. Similarly, MIP-1 $\beta$  production increased in the placebo arm, only from 3 months (719.3; IQR = 89.25 - 849.4) compared to 12 months (777.8; IQR = 186.6 - 907.4) ( $P = 0.030$ ) (Figure 20C).

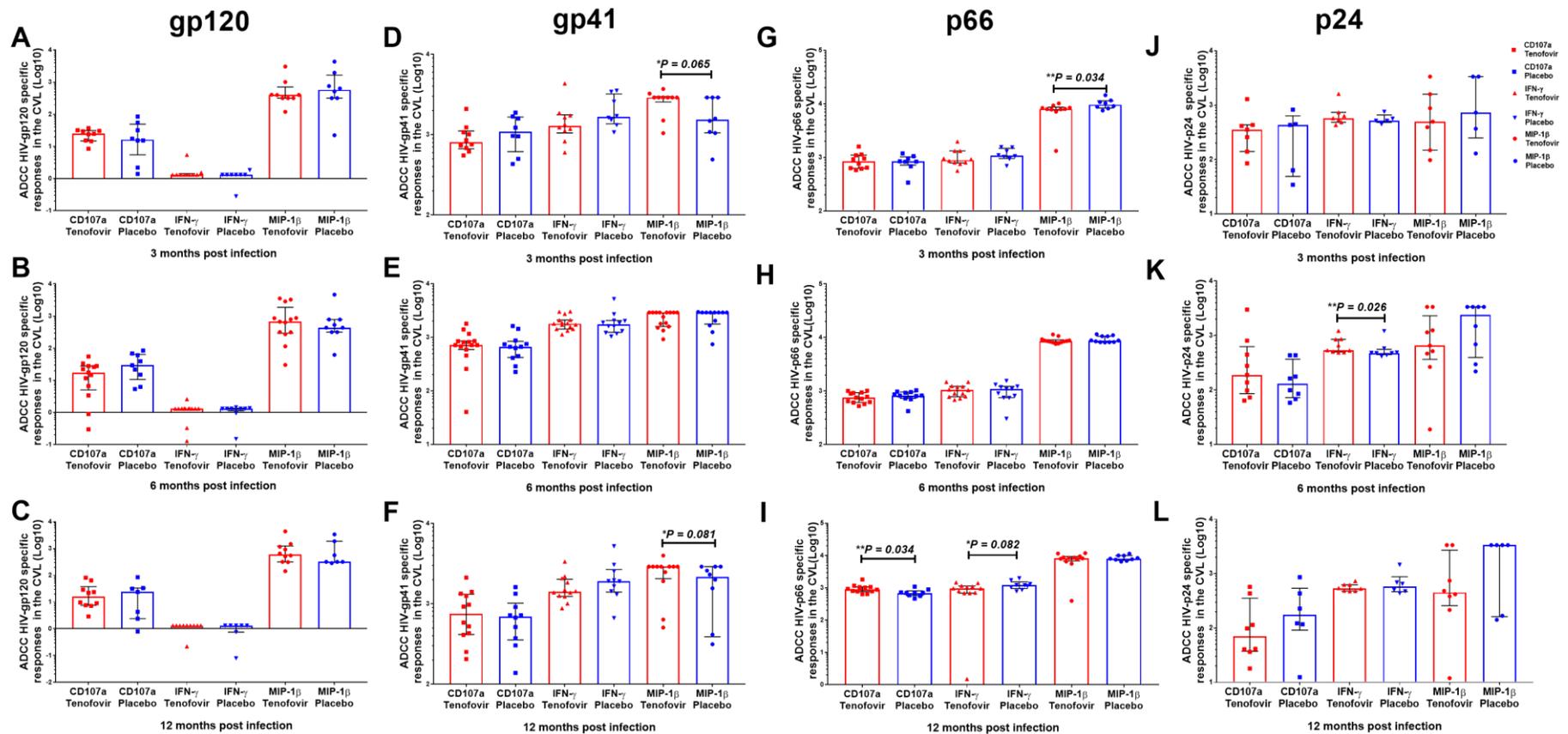
Furthermore, only MIP-1 $\beta$  antiviral production to p66 in the tenofovir arm, demonstrated a significant increase from 6 months (246; IQR = 122.5 - 478.5) to 12 months (391; IQR = 95.25 - 549.5) ( $P = 0.044$ ) (Figure 20I). No further differences were observed for p66 and p24 CD107a expression and IFN- $\gamma$  production. However, only p24 specific MIP-1 $\beta$  production in the tenofovir arm, exhibited a significant increase from 3 months (129; IQR = 111 - 580.5) to 6 months (415.5; IQR = 206 - 855) ( $P = 0.002$ ) (Figure 20L). NK cell activated ADCC activity and antiviral cytokines to gp41 in the plasma were similar in both study arms across all time points ( $P > 0.1$ , supplementary Table 4).



**FIGURE 20: Longitudinal analysis of plasma IgG mediated NK cell activated-ADCC activities, in the tenofovir and the placebo arms, across 3 months, 6 months and 12 months.** ADCC activities (Log10 ADCC) measured by CD107a, IFN- $\gamma$  and MIP-1 $\beta$  production in both the tenofovir and placebo arm to HIV gp120 (A-C), gp41 (D-F), p66 (G-I) and p24 (J-L). Solid lines in either the tenofovir or the placebo arm within the dot plots indicate the maximum geometric mean of each cytokine detected in the ICS assay. Horizontal lines across bars indicate statistical values between arms at over time. Wilcoxon-Signed Rank test was used to analyse the data and statistical differences were defined as **\*\* ( $P \leq 0.05$ )**.

### 5.3.3 HIV-gp41, p66 and p24 NK cell activated ADCC and antiviral activities varied between study arms in the genital tract

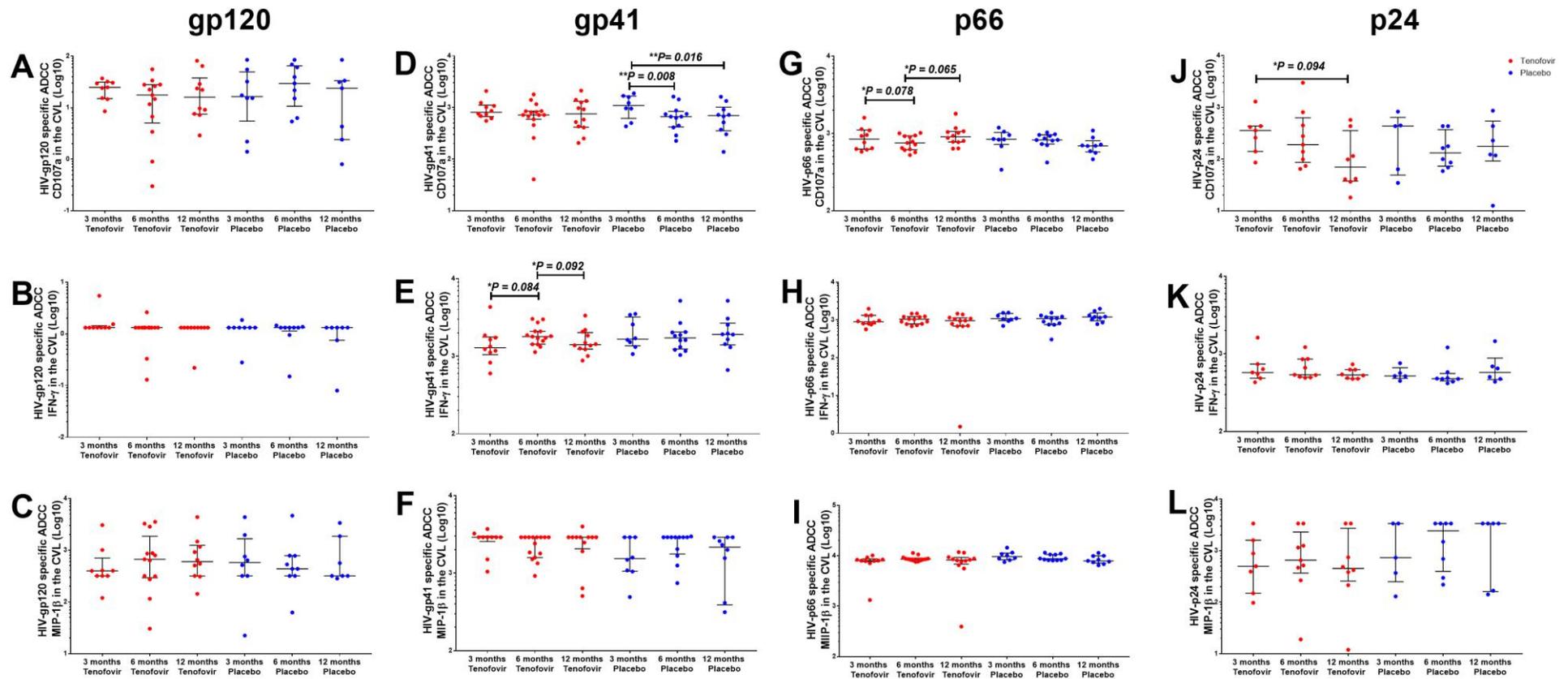
To elucidate the impact of tenofovir on genital tract nNAb NK cell activated ADCC activity, cross-sectional analyses were done. Gp41 specific MIP-1 $\beta$  responses were higher in the tenofovir arm compared to the placebo arm, at 3 months (tenofovir 2905; IQR = 2555 - 2990, placebo 1539; IQR = 1056 - 2905) (***P* = 0.065**) (**Figure 21D**) and at 12 months (tenofovir 2905; IQR = 2055 - 2905; placebo 2155; IQR = 389 - 2905) (***P* = 0.081**) (**Figure 21F**). P66 specific cytotoxic activity at 12 months, was higher in the tenofovir arm (903.3; IQR = 774.6 - 1061) compared to the placebo arm (691.5; IQR = 575.5 - 801.5) (***P* = 0.034**) (**Figure 21G**). In contrast, IFN- $\gamma$  production in the tenofovir arm was lower (954.8; IQR = 687.9 - 11.35) compared to that of the placebo arm (1198; IQR = 948.8 - 1525) (***P* = 0.082**) (**Figure 21I**) at 12 months. Furthermore, chemokine p66 specific MIP-1 $\beta$  responses at 3 months in the tenofovir arm was greater than that of the placebo arm (tenofovir 9654; IQR = 8361 - 11372, placebo 8568; IQR = 8108 - 9041) (***P* = 0.034**) (**Figure 21G**). P24 specific IFN- $\gamma$  antiviral responses were only discernibly greater in the tenofovir arm (538; IQR = 502 - 857.5) compared to the placebo arm (478.5; IQR = 448.3 - 559) (***P* = 0.026**) (**Figure 21K**) at 6 months. Cytotoxic activity mediated by CVL antibodies to gp120 at 3, 6 and 12 months were similar between tenofovir and placebo arms for CD107a, IFN- $\gamma$  and MIP-1 $\beta$  (***P* > 0.1**) (**Figure 21A-C, supplementary Table 5**).



**FIGURE 21: Cross- sectional comparisons of CVL IgG ADCC activities mediated by NK cells isolated from fresh whole blood, between the tenofovir and the placebo arms across 3 months, 6 months and 12 months. ADCC activities (Log10 ADCC) measured by CD107a, IFN- $\gamma$  and MIP-1 $\beta$  production in both the tenofovir and placebo arm to HIV gp120 (A-C), gp41 (D-F), p66 (G-I) and p24 (J-L). Graphs read from top to bottom in order to observe differences in ADCC responses over time, from 3-12 months post-infection. Solid lines in either the tenofovir or the placebo arm above the column indicate the maximum geometric mean of each cytokine detected in the ICS assay. Horizontal lines across bars, indicate statistical differences determined by Wilcoxon Sum rank tests, between arms at respective time points and statistical differences were defined as **\*\*( $P \leq 0.05$ )** and values that trended toward significance were defined as **\*( $P < 0.1$ )**.**

### 5.3.4 Increased HIV-gp41 and p66 cytotoxic and antiviral activities in the genital tracts of women who used tenofovir

In order to ascertain the evolution in nNAb mediated NK cell activated ADCC activity over time, longitudinal analyses from 3 months to 12 months were done. Placebo arm responses to gp41, decreased from 3 months (1096; IQR = 614.4 - 1663) to 6 months (666; IQR = 421 - 842.8) ( $P = 0.008$ ) and from 3 months to 12 months (697; IQR = 355.8 - 1019) ( $P = 0.016$ ) (Figure 22D-F). In contrast, cytotoxic responses to gp41 in the genital tract were similar over time in the tenofovir arm ( $P > 0.1$ ). However, in the tenofovir arm, gp41 specific antiviral IFN- $\gamma$  production increased from 3 months (1288; IQR = 1048 - 1768) to 6 months (1797; IQR = 1430 - 2103) ( $P=0.084$ ) and decreased from 6 to 12 months (1410; IQR = 1238 - 2020) ( $P = 0.092$ ) (Figure 22E). P66 specific NK cell activated ADCC activity in the tenofovir arm only, was lower at 3 months (84; IQR = 626.5 - 1106) compared to 6 months (754.5; IQR = 615.3 - 931.3) ( $P = 0.078$ ) (Figure 22G). However, p66 specific cytotoxic activity increased in the tenofovir arm from 6 months to 12 months (903.3; IQR = 774.6 - 1061) ( $P = 0.065$ ) (Figure 22G) but this was not significantly different. Longitudinal analysis of p24 specific cytotoxic activity in the genital tract of women who used tenofovir only, significantly decreased from 3 months (356.5; IQR = 140.5 - 34.5) to 12 months (70; IQR = 37.5 - 354.5) ( $P = 0.094$ ) (Figure 22J). Responses to gp120 in the genital tract were similar in both arms over time for CD107a, IFN- $\gamma$  and MIP-1 $\beta$  ( $P > 0.1$ ) (Figure 22A –C, supplementary Table 5).



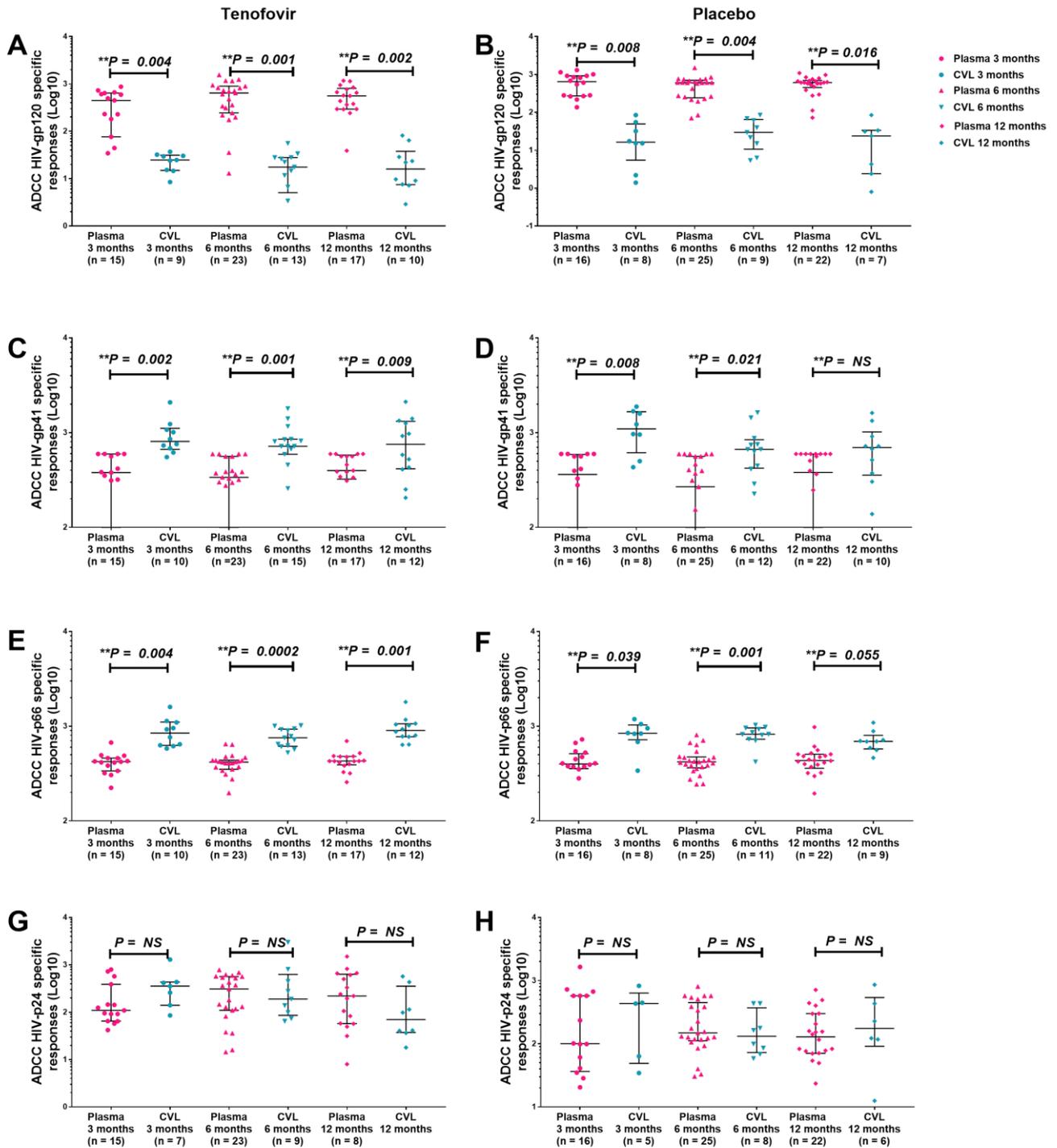
**FIGURE 22: Longitudinal analyses of CVL IgG mediated NK cell activated ADCC activities, in both the tenofvir and placebo arms across 3 months, 6 months and 12 months.** ADCC activities (Log<sub>10</sub> ADCC) measured by CD107a, IFN- $\gamma$  and MIP-1 $\beta$  production in both the tenofvir and placebo arm to HIV gp120 (A-C), gp41 (D-F), p66 (G-I) and p24 (J-L). Solid lines in either the tenofvir or the placebo arm within the dot plots indicate the maximum geometric mean of each cytokine detected in the ICS assay. Horizontal lines across bars indicate statistical values between arms at over time. Wilcoxon Signed Rank tests were performed to elucidate statistical differences, which were defined as **\*\* ( $P \leq 0.05$ )** and values trending towards significance, were defined as **\* ( $P < 0.1$ )**.

### 5.3.5 NK cell activated ADCC activities in the genital tract were significantly higher than those of the plasma for gp41 and p66 at 3 months, 6 months and 12 months, irrespective of study arm

To determine if there was concordance between NK cell activated ADCC activities in the plasma and genital mucosae, responses in both arms were compared at 3 months, 6 months and 12 months for HIV-gp120, gp41, p66 and p24. Cytotoxic activity (CD107a production) to gp120 in the tenofovir arm, was greater in the plasma at 3 months (449.5; IQR = 76.5 - 640.5) compared to the CVL (24.8; IQR = 14.95 - 31.25) ( $P = 0.004$ ). This pattern was consistent at 6 months [plasma 644; (IQR = 245 - 895); CVL 17.6; (IQR = 5.05 - 27.95)] ( $P = 0.001$ ) and at 12 months [plasma 559.5 (IQR = 294.5 - 805.8); CVL 15.95; (IQR = 7.5 - 37.73)] ( $P = 0.002$ , Figure 23A). Similarly, in the placebo arm, responses to gp120 were significantly higher in the plasma at 3 months [plasma 644.8; (IQR = 272.5 - 918.3); CVL 16.35; (IQR = 5.475 - 49.28)] ( $P = 0.008$ ), at 6 months [plasma 604; (IQR = 242.3 - 9703.5); CVL 29.6; (IQR = 10.7 - 64.8)] ( $P = 0.004$ ) and at 12 months [plasma 609; (IQR = 453 - 694.1); CVL 23.9; IQR = 2.4 - 33.4)] ( $P = 0.016$ , Figure 23B). In contrast, the genital tract shows a different pattern of gp41 specific NK cell activated ADCC activities in both arms. In the tenofovir arm, responses were greater in the CVL at 3 months compared to those observed in the plasma at 3 months [plasma 379.5; (IQR = 58 - 596.5); CVL 808; (IQR = 667 - 1113)] ( $P = 0.002$ ). This greater CVL response was maintained at 6 months [plasma 337.5; (IQR = 34 - 562); CVL 718; (IQR = 591 - 853.5)] ( $P = 0.001$ ) and at 12 months [plasma 398; (IQR = 323.3 - 579.3); CVL 753.3; (IQR = 415.5 - 1317)] ( $P = 0.009$ , Figure 23C). Similarly, in the placebo arm, responses to gp41 were greater in the CVL at 3 months [plasma 362; (IQR = 25 - 587.9); CVL 1096 (IQR = 614.4 - 1663)] and 6 months [plasma 268.5; (IQR = 47.5 - 562)]. Only at 12 months responses to gp41 in the placebo arm, between the plasma and genital tract (CVL) were similar ( $P = 0.194$ , Figure 23D).

NK cell activated ADCC activity to p66 in the tenofovir arm were greater in the CVL at 3 months [plasma 422.5; IQR = 338 - 461]; CVL 846; (IQR = 626.5 - 1106)] ( $P = 0.004$ ). These responses were maintained at 6 months [plasma 417; (IQR = 351 - 437); CVL 754.5; IQR = 612.3 - 931.3)] ( $P = 0.0002$ ) and at 12 months [plasma 428.5; IQR = 390.5 - 479.8]; CVL 903.3; IQR = 774.6 - 1061)] ( $P = 0.001$ , Figure 23E). Similarly, in the placebo arm responses

to p66 were greater in the genital tract compared to the plasma at 3 months [plasma 398.5; (IQR = 356.9 - 512.9); CVL 842.3; (IQR = 720.1 - 1029)] (***P* = 0.039**) and at 6 months [plasma 420; (IQR = 363.8 - 473.8); CVL 824; (IQR = 729.7 - 957.5)] (***P* = 0.001**). This p66 specific response was maintained at 12 months [plasma 433.8; (IQR = 359.6 - 505.6); CVL 691.5; (IQR = 575.5 - 801.5)] (***P* = 0.055, Figure 23F**). Interestingly, NK cell activated ADCC activity to p24 in both the tenofovir and placebo arms (**Figure 23G and H**), were similar between the plasma and genital tract (*P* > 0.1).



**FIGURE 23: Compartmental comparisons of NK cell activated ADCC activities between the plasma and genital tracts (CVL) of women at 3 months, 6 months and 12 months, between the tenofovir and placebo arms, for each HIV-protein.** Inter-compartmental analysis for cytotoxic activities (Log10 ADCC) in the tenofovir arm on the left and the placebo arm on the right to gp120 (A and B), gp41 (C and D), p66 (E and F) and p24 (G and H). Wilcoxon Sum Rank paired tests, were done to determine statistical differences between phagocytic activities within each arm of the study and between compartments, for each protein. Statistically significant values were defined as **\*\*P ≤ 0.05** and those that trended as **\*P < 0.1**.

### 5.3.6 NK cell activated ADCC gp41 and p66 specific responses, exhibit strong correlations between the plasma and genital tract

To determine whether transudation of nNAb mediating HIV-specific NK cell activated ADCC occurred in either arm, Spearman R correlation analyses were done. Interestingly, ADCC activity in the plasma positively correlated with ADCC activity in the genital tracts of women in the tenofovir arm ( $R = 0.819$ ;  $P = 0.002$ ) (Table 13). In the placebo arm for NK cell activated ADCC p66-specific responses at 3 months, in the plasma negatively correlated with those observed in the genital tract ( $R = -0.643$ ;  $P = 0.096$ ). However, in contrast, at 6 months, in the plasma, p66 specific NK cell activated ADCC activity strongly correlated with those in the genital mucosa ( $R = 0.809$ ;  $P = 0.004$ ) (Table 13). No further significant correlations were observed for gp120 and p24.

**TABLE 13: Compartmental correlations of cytotoxic activity between the plasma and the genital tract (CVL), for the tenofovir and placebo arms, for all HIV-proteins**

Protein	Arm	3 months		6 months		12 months	
		<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
gp120	Tenofovir	-0.300	0.437	-0.236	0.437	-0.358	0.313
	Placebo	-0.084	0.849	0.35	0.359	-0.631	0.143
gp41	Tenofovir	0.043	0.918	0.281	0.307	<b>0.819</b>	<b>0.002**</b>
	Placebo	0.464	0.255	0.057	0.862	0.068	0.856
p66	Tenofovir	-0.333	0.349	-0.148	0.630	0.175	0.588
	Placebo	<b>-0.643</b>	<b>0.096*</b>	<b>0.809</b>	<b>0.004**</b>	0.267	0.493
p24	Tenofovir	-0.300	0.683	0.036	0.964	-0.100	0.950
	Placebo	0.5	> 0.999	-0.257	0.658	0.8	0.333

\*\*indicates significant values defined as  $P \leq 0.05$

### 5.3.7 CD4<sup>+</sup> T cell count correlates negatively with HIV-gp41 and positively with HIV-p66 specific NK cell activated ADCC activity in the tenofovir arm at 6 months post-infection

In order to determine if NK cell activated ADCC activity in the plasma correlated with CD4<sup>+</sup> T cell counts in the plasma and genital tract correlation analyses were performed. Analysis revealed a significant negative correlation in the plasma, between CD4<sup>+</sup> T cell count and gp41 specific NK cell activated ADCC activity in the tenofovir arm (***R = -0.501; P = 0.018***) (Table 14). Interestingly a positive but weak correlation trended for HIV-p24 NK cell activated ADCC, in the plasma tenofovir arm (***R = 0.397; P = 0.067***). No further differences or similarities were observed when CD4<sup>+</sup>T cells were compared to HIV-specific gp120, p66 in the plasma tenofovir or placebo arm. In the genital tract, no significant correlations were observed for HIV protein specific NK cell activated ADCC activity and blood CD4<sup>+</sup> T cell counts for either arm (Table 14).

**TABLE 14: Correlations between CD4<sup>+</sup> T cells and NK cell activated ADCC activities for all HIV-specific antibodies at 6 months post-infection, in the plasma and the genital tract (CVL)**

HIV antigens	CD4 <sup>+</sup> T cell count vs Plasma ADCC by CD107a (Tenofovir) n = 22		CD4 <sup>+</sup> T cell count vs Plasma ADCC by CD107a (Placebo) n = 23		CD4 <sup>+</sup> T cell count vs CVL ADCC by CD107a (Tenofovir) ^ n = 12		CD4 <sup>+</sup> T cell count vs CVL ADCC by CD107a (Placebo) ^ n = 10	
	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
gp120	0.081	0.719	-0.031	0.889	0.245	0.443	-0.383	0.313
gp41	<b>-0.501</b>	<b>0.018**</b>	0.243	0.264	-0.021	0.952	0.035	0.921
p66	-0.051	0.821	-0.330	0.124	-0.231	0.471	-0.5	0.122
p24	<b>0.397</b>	<b>0.067*</b>	-0.274	0.207	0.233	0.552	0.204	0.629

\*indicates values that trended to significance, defined as ***P < 0.1***

\*\*indicates significant p-values which were classified as ***P ≤ 0.05***

^CVL was not available for 10 participants in the tenofovir arm and 12 participants in the placebo arm. Therefore, the values represented were from the available CVL samples to test for each protein.

### 5.3.8 HIV-gp41 specific NK cell activated ADCC activity in the plasma placebo arm, negatively correlated with viral load at 6 months post-infection in the plasma

To identify the impact of NK cell activated ADCC activity in the plasma on viral loads at 6 months post-infection, in the tenofovir and placebo arm, correlation analyses were performed. The analyses showed a significant weak negative correlation between HIV-gp41 specific nNAb mediating cytotoxic activity in the plasma of the placebo arm and viral load ( $R = -0.433$ ;  $P = 0.039$ ). Thus, as HIV-gp41 NK cell activated ADCC activity increased, viral load decreased, possibly due to cytotoxic activity. No further correlations were observed between any other HIV-protein specific NK cell activated ADCC activities analysed and plasma viral loads (Table 15). Many samples were unavailable for certain time points, therefore many CD4<sup>+</sup> T cell correlations with genital tract HIV specific NK cell activated ADCC activities had to be excluded, which precluded a more complete analysis of the data.

**TABLE 15: Correlations between the viral loads and NK cell activated cytotoxic activities mediated by HIV-specific antibodies, at 6 months post-infection, in the plasma and the genital tract (CVL)**

HIV antigens	Viral load (Log10) vs plasma ADCC - CD107a (Tenofovir) n = 23		Viral load (Log10) vs plasma ADCC - CD107a (Placebo) n = 23		Viral load (Log10) vs CVL ADCC - CD107a (Tenofovir) ^ n = 12		Viral load (Log10) vs CVL ADCC - CD107a (Placebo) ^ n = 10	
	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
gp120	0.205	0.348	0.268	0.216	0.313	0.298	0.05	0.912
gp41	-0.056	0.800	<b>-0.433</b>	<b>0.039**</b>	-0.361	0.185	-0.109	0.737
p66	0.048	0.826	-0.043	0.844	-0.028	0.931	0	>0.999
p24	0.205	0.348	-0.032	0.886	-0.35	0.359	-0.108	0.806

\*\*indicates significant p-values which were classified as  $P \leq 0.05$

^ CVL was not available for 11 participants in the tenofovir arm and 13 participants in the placebo arm. Therefore, the values represented were from the available CVL samples to test for each protein.

### 5.3.9 HIV-p24 IgG titres negatively correlated with HIV-p24 specific NK cell activated ADCC activity in the tenofovir arm at 6 months post-infection

Correlation analyses were performed in order to ascertain the association between previously reported antibody titres (Archary et al., 2016) and HIV specific NK cell activated ADCC activities. At 6 months post-infection and only in the tenofovir arm, HIV-p24 IgG titres correlated with ADCC activity in the plasma ( $R = -0.430$ ;  $P = 0.041$ ) and in the genital tract ( $R = -0.174$ ;  $P = 0.058$ ) (Table 16). Neither the tenofovir nor the placebo arms demonstrated significant correlations between IgG titres and NK cell activated ADCC activity to gp120, gp41 and p66.

**TABLE 16: Correlations between HIV-specific IgG titres and HIV-specific antibody mediated cytotoxic activities at 6 months post-infection, in the plasma and the genital tract (CVL)**

HIV antigens	IgG titres (Log10) vs plasma ADCC - CD107a (Tenofovir) n = 23		IgG titres (Log10) vs plasma ADCC - CD107a (Placebo) n = 23		IgG titres (Log10) vs CVL ADCC- CD107a (Tenofovir) ^ n = 12		IgG titres (Log10) vs CVL ADCC- CD107a (Placebo) ^ n = 10	
	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
gp120	-0.021	0.925	0.034	0.879	-0.160	0.600	0.274	0.476
gp41	-0.069	0.755	-0.188	0.390	-0.162	0.560	-0.081	0.894
p66	-0.270	0.212	0.121	0.584	0.416	0.181	0.523	0.103
p24	<b>-0.430</b>	<b>0.041**</b>	0.087	0.692	<b>-0.174</b>	<b>0.058*</b>	0.120	0.779

\*indicates values that trended to significance defined as  $P < 0.1$

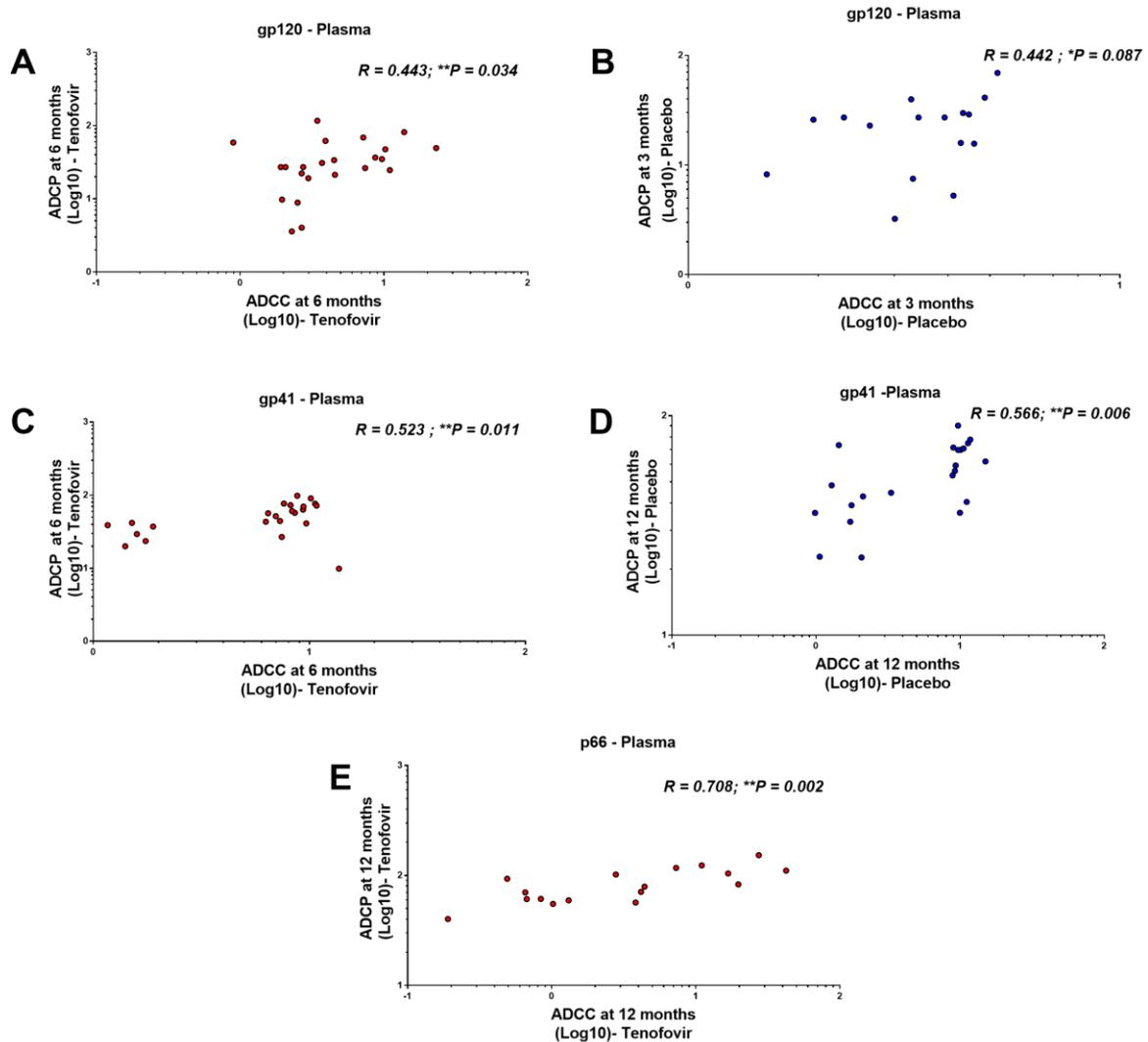
\*\*indicates significant p-values which were classified as  $P \leq 0.05$

^ CVL was not available for 11 participants in the tenofovir arm and 13 participants in the placebo arm. Therefore, the values represented were from the available CVL samples to test for each protein.

### 5.3.10 Moderate to strong correlations between ADCP and NK cell activated ADCC activity in the plasma, to HIV-gp120, gp41 and p66

In order to elucidate any correlations between the two different nAb functions assessed in this study and to address the final objective, correlation analyses of HIV-specific IgG-mediated ADCP and NK cell activated ADCC activities in the systemic compartment and in the genital tract were performed. In the plasma HIV-gp120 specific ADCP activity directly correlated with NK cell activated ADCC activity at 6 months in the tenofovir arm ( $R = 0.422$ ;  $P = 0.035$ ) (Figure 24A) and at 3 months in the placebo arm ( $R = 0.442$ ;  $P = 0.087$ ) (Figure 24B, supplementary Table 9). Interestingly, HIV-gp41 nAb ADCP activity in the tenofovir arm at 6 months, positively correlated with NK cell activated ADCC at the same time point ( $R = 0.523$ ;  $P = 0.011$ ) (Figure 24C). Similarly, at 12 months in the placebo arm, ADCP and NK cell activated ADCC activity to HIV-gp41, exhibited a strong, significant, positive correlation ( $R = 0.566$ ;  $P = 0.006$ ) (Figure 24D). Furthermore, nAb functions to p66 at 12 months post-infection in the tenofovir arm also showed strong, direct and significant correlations ( $R = 0.708$ ;  $P = 0.002$ ) (Figure 24E), indicating that in the plasma, both nAb functions may occur simultaneously with similar magnitudes of activities, no correlations were observed for p24 specific activity (supplementary Table 8).

Interestingly, when ADCP in the genital tract were correlated to NK cell activated ADCC activities in the genital tract, no significant associations were observed (supplementary Table 9) for any of the HIV-specificities.



**FIGURE 24: Correlations comparing ADCP and NK cell activated ADCC activities in the plasma and genital tracts (CVL) of women in both the tenofovir and the placebo arms.** HIV-specific activities (both Log10) **A)** gp120 responses at 6 months in the tenofovir arm, **B)** gp120 responses at 3 months in the placebo arm, **C)** gp41 responses at 3 months in the tenofovir arm, **D)** gp41 responses at 12 months in the placebo arm, **E)** p66 responses in the tenofovir arm in the plasma. Spearman R correlation analyses were performed and patient nAb HIV-specific functions were matched in the plasma and in the genital tracts of women. Significant values were identified as **\*\*  $P \leq 0.05$**  and those that trended as **\*  $P < 0.1$** .

## CHAPTER 6

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### Discussion

In recent years, there has been emerging evidence strongly supporting the role of non-neutralizing antibodies in preventing, controlling and altering HIV disease progression (Haynes et al., 2012a, Halper-Stromberg et al., 2014). However, in the era of PrEP as a new HIV prevention strategy in combination with HIV vaccines, the subsequent effect of PrEP on immunity, in particular antibody functionality has yet to be determined. In this novel study, we investigated the effect of prior PrEP on ensuing nNAb functions in both the blood and genital tracts of women from the CAPRISA 004 1 % tenofovir gel trial. NNAb assessed included Fc-mediated ADCP using a neutrophil-based assay, as well as a NK cell activated ADCC, using a novel intracellular cytokine staining based assay. IgG isolated from the plasma samples and matching genital tract CVL supernatants were investigated for nNAb functionality to HIV-1 proteins, from 24 women who had used either 1 % tenofovir gel or the placebo gel respectively, prior to seroconversion (Abdool Karim et al., 2010). We report that prior use of the topical 1% tenofovir microbicide gel impacted antibody functionality in women who used the gel prior to HIV-1 infection compared to the placebo gel users. Furthermore, this study showed that prior use of the gel did not diminish antibody detectability or functionality both systemically or at the genital tract

In this study, plasma IgG concentrations were much higher compared to those in the genital tract, irrespective of arm assignment of women, corroborating with previously reported data by Archary *et al.*, (2016). The quantity of IgG's in the genital tract depends on whether IgG is locally produced (Bélec et al., 1995) or is transudated across the epithelial barrier (Li et al., 2011). Various factors influence transudation dynamics such as FcRn expression on epithelial cells, the glycosylation properties of the IgG Fc portion (Ackerman et al., 2013a), the affinity interaction between antibody Fc-FcRn on epithelial cells (Gupta et al., 2013, Li et al., 2011) and the pH of the genital tract [as reviewed by (Cocklin and Schmitz, 2014)]. Furthermore, IgG compared to IgA is well known to be the most dominant antibody isotype in the female genital tract (Bomsel et al., 2011, Bélec et al., 1995, Archary et al., 2016, Johansson and Lycke, 2003). Indeed, this Ig isotype in particular, may have functional

relevance, given that Env-specific IgG1 and IgG3 mediate enhanced ADCP (Tay et al., 2016, Liu et al., 2016). In this study, HIV-specific IgG phagocytic function was highly detected in both the plasma and the genital tract in women irrespective of study arm. Notably, ARV treatment has been associated with reduced antibody titres (Hare et al., 2006, Payne et al., 2015, O'sullivan et al., 2002) and reduced antibody avidity (Laeyendecker et al., 2015) and to date the effect on antibody functionality is not known. In this study, we observed inverse correlations in both arms of the study, between HIV-specific IgG titres and nNAb functions. The inverse correlations between gp41 and p24 IgG titres and the HIV-specific phagocytic activities, in the tenofovir and placebo arm respectively in the plasma, indicate that even in the presence of low titre HIV-specific antibodies, nNAbs may still be able to elicit potent antiviral ADCP functions. Our findings corroborate other studies, which report that it is the quality of the elicited antibodies and not necessarily the magnitude, that is key to conferring protection (Hessell et al., 2009a, Mascola et al., 2000, Hessell et al., 2009b).

Due to nNAb engagement with innate immune cells, effector cell populations at sites of infection influence Fc-mediated antiviral activity (Cheeseman et al., 2016, Sips et al., 2016). Furthermore, neutrophils and monocytes/ macrophages may confer superior protection due to their relative abundance compared to other immune cells in the female genital tract (Sips et al., 2016, Tay et al., 2016). In this study, we report phagocytic activities in the genital tract to levels comparable to the plasma in women who used tenofovir gel. During HIV-infection, in the presence of the tenofovir or placebo gel, neutrophil-mediated gp120 specific ADCP increased over time in the blood. Furthermore, gp41 and gp120 specific ADCP activity in the genital tract were directly associated with those systemically, in both arms. In the genital tracts of women in the placebo arm, gp120 specific phagocytic activities negatively correlated with viral loads, at 6 months post-infection. This data may indicate that Env-specific phagocytosis may be undermined in the genital tract with increasing plasma viral loads, possibly due to circulating gp120-mediated specific ADCP, resulting in less gp120-specific antibodies transudating into the genital tract or that gp120 antibodies showed delayed kinetics in the genital tract. Our data indicates that gp120 ADCP and NK cell activated ADCC remained higher in the plasma than the genital tract compared to other HIV-specific antibodies mediating either functions. Therefore, the detectability or increases in HIV-specific antiviral activities suggest that prior tenofovir gel use did not dampen these responses. Interestingly, women from the tenofovir arm had distinct functional differences

from those in the placebo arm with enhanced gp70 V1V2, gp41 and p66 ADCP systemically, over time. This enhanced HIV-specific IgG mediated phagocytic activity in the tenofovir arm may suggest immune priming due to continuous antigen exposure over a long period of time prior to infection (Archary et al., 2016, Haynes et al., 2012a, Cranage et al., 2008). Notably, in this study phagocytic responses to gp70 correlated with an increase in CD4<sup>+</sup> T cell counts and not viral loads, which may impact humoral immune nNAb responses due to preserved CD4<sup>+</sup> T cells and resulting B cell interaction. Other studies, have suggested that antibodies that bind to variable regions of the HIV envelope, may impede infection (Pinter et al., 1998, Gorny et al., 1993, Corti et al., 2010, Mouquet et al., 2011, Rerks-Ngarm et al., 2009). Additionally, gp70 directed antibodies have also exhibited cross-clade reactivity with other HIV subtypes as well as to HIV-subtype C, the most prevalent subtype in South Africa (Zolla-Pazner, 2014), further underscoring the importance of the inclusion of Env as a vaccine immunogen (Haynes et al., 2012a). Interestingly, gp70 antibody specific profiles have also been linked to enhanced phagocytic activity associated with IgG3 (Zolla-Pazner, 2014, Chung et al., 2015), which corroborates our gp70 specific ADCP activity. However, whether this can be attributed to a IgG specific subclass was not investigated in this study. Notably, ADCP activity has also been identified as a correlate of protection in the RV144 trial (Chung et al., 2014), therefore the increased phagocytic activities observed in this study, suggest the potential benefits of PrEP in enhancing nNAb associated protection from HIV infection, in the absence of neutralization. These findings further underscore the importance of the inclusion of Env targets (Haynes et al., 2012a), not only as a bNAb vaccine immunogen but also to elicit nNAb functions, as correlates of protection and post-infection viral control mechanisms (Barouch et al., 2015, Bournazos et al., 2014, Hessel et al., 2007, Chung et al., 2015).

In addition to ADCP, NK cell mediated ADCC has been recognized as a correlate of protection, effecting viral control in elite controllers (Lambotte et al., 2009) and delaying disease progression (Baum et al., 1996a, Gómez-Román et al., 2005, Aasa-Chapman et al., 2005, Ljunggren et al., 1990, Ahmad et al., 1994). More importantly, ADCC activity was identified as a correlate of protection in the only vaccine trial to exhibit a 31.2 % efficacy (Haynes et al., 2012a, Rerks-Ngarm et al., 2009). The main effector cells mediating ADCC antibodies, are NK cells which engage and destroy infected cells by recognition of stress ligands [as reviewed by (Moretta et al., 2001)]. ADCC gp120 and p66 specific IFN- $\gamma$  and MIP-

1 $\beta$  production, for both arms, exhibited increased antiviral production over time. Subsequent to acute infection, cellular and humoral responses to HIV regulatory proteins in a NHP study, reportedly contributed to HIV control (Florese et al., 2009). In addition to p66 responses, p24 specific CD107a NK cell activated ADCC was directly associated with CD4<sup>+</sup> T cell counts and in the tenofovir arm, we observed an increase in MIP-1 $\beta$  production. We report that NK cell activated ADCC activity, in the plasma to p66 was positively associated with CD4<sup>+</sup> T cells in women who used tenofovir prior to infection, these data provide additional evidence for the role of nNABs in altering disease progression. Additionally, p24 specific CD107a NK cell activated ADCC was directly associated with CD4<sup>+</sup> T cell counts in the tenofovir arm. These data corroborate findings that preserved Gag-specific CD4<sup>+</sup> T cells in the context of topical PrEP (Mureithi et al., 2012), can confer help to B cells, a hypothesis that was tested by Archary *et al.*, (2016). In the context of topical PrEP, Gag specific CD4<sup>+</sup> T cells in women who used tenofovir gel were reportedly preserved 3 months post-infection in the plasma (Mureithi et al., 2012) and our data suggest that these Gag specific NK cell activated ADCC responses continued until 6 months post-infection. CD4<sup>+</sup> antigen specific T cells significantly influence antibody development, allowing for antigen presentation and contributing significantly to antigen specific B cells and antibody development (Moir et al., 2003). It is well known that HIV infection, results in dysfunctional B cell responses (Moir et al., 2003, Busmann et al., 2010, Nicholas et al., 2013) and various immune responses, which in the presence of PrEP, may have been rescued and preserved (Mureithi et al., 2012, Abdool Karim et al., 2010, Archary et al., 2016). The resulting cellular and humoral interactions possibly allowed for affinity maturation, due to preserved antigen specific CD4<sup>+</sup> T cell populations, leading to increased ADCC activity 6 months post-infection (Nicholas et al., 2013, Mureithi et al., 2012, Archary et al., 2016). Furthermore, gp41 and p24 antibody responses have been correlated with delayed disease progression (Schmidt et al., 1989, Fernández-Cruz et al., 1990, Mertens et al., 1990, Allain et al., 1991, Sheppard et al., 1991, Cheingsong-Popov et al., 1991, Chargelegue et al., 1993a, Zwart et al., 1994, Chargelegue et al., 1993b, Chargelegue et al., 1995, Morand-Joubert et al., 1995, Garland et al., 1996, Thomas et al., 1996, Farzadegan et al., 1992). The increase and decrease of gp41 responses from 3 months to 12 months, may be attributed to antibody responses stimulated due to the presence of antigen, and the subsequent decline due to the decrease in viral protein production, as suggested in a previous study (Mcrae et al., 1991). Specifically, data from a

study by McRae *et al.*, 1991 indicated that fluctuations in p24 antigen may be due to the decrease in viral protein production often associated in HIV-disease, and therefore it is plausible that any fluctuations in HIV viral loads, could impact other HIV antigens [like gp41, or p66] and therefore related humoral or other immune functions. These gp41 and p24 responses suggest that stimulating the production of certain antibodies through antigen specific T cell help may aid in nNAb activity. The elevated mucosal HIV-gp41 NK cell activated ADCC activity in the genital tract of women in the tenofovir arm further infer that gp41 specific Ab responses may have been a result of cumulative immune priming (Cranage *et al.*, 2008) and pre-existing cross reactivity with commensal bacteria (Trama *et al.*, 2014, Williams *et al.*, 2015), even in the presence of tenofovir gel. In the placebo arm, a negative correlation between gp41 specific NK cell activated ADCC and viral loads at 6 months post-infection were observed in the plasma. Therefore, in the absence of prior topical PrEP, circulating nNAbs are capable of antiviral activity, which in macaque studies have been associated with correlates of protection (Bomsel *et al.*, 2011, Hessel *et al.*, 2010).

NK cells in the gut and genital tract during HIV infection have been reported to be phenotypically similar [as reviewed by (Roy, 2000)]. Furthermore, following HIV infection, NK cells were reported to expand in the intraepithelial lining and lamina propria at the mucosae and was directly shown to be associated with CD4<sup>+</sup> T cell loss (Sips *et al.*, 2012). In the presence of ARVs where CD4<sup>+</sup> T cell counts were still not completely recapitulated, corresponding NK cell expansions were observed (Sips *et al.*, 2012). NK cells in the gut expand in the intraepithelial lining and lamina propria at the mucosae, due to extensive CD4<sup>+</sup> T cell loss and incomplete CD4<sup>+</sup> T cell restoration associated with ARV treatment (Sips *et al.*, 2012). Therefore, it is plausible that perhaps NK cells in the lamina propria in the genital tract expand during infection to target infected cells. This suggests that the observed NK cell mediated ADCC activity observed in the genital tracts of women in this study, irrespective of arm, might have increased in responses to NK cell expansion at the site of transmission. However, this requires further investigation into NK cell-mediated activities in response to infection, specifically in the female genital tract. Moreover, NK cell-mediated ADCC activities were shown to control viral loads in elite controllers and were a correlate of protection in highly exposed seronegative women (HESN) (Baum *et al.*, 1996b, Lambotte *et al.*, 2009). These ADCC-mediating nNAbs were also found at early stages of HIV infection (Connick *et al.*, 1996, Forthal *et al.*, 2001). Furthermore, in the context of menstrual cycles,

NK cells are reportedly stable (Martin et al., 2002, Alter et al., 2007a), underscoring the importance of harnessing NK mediated anti-HIV activity at the most vulnerable surface - the female genital tract (Martin et al., 2002, Alter et al., 2007b).

However, NK cells are present at low frequencies in vaginal tissue (Sips et al., 2016) and should there be lower titres of HIV-specific IgG's mediating NK ADCC in the genital secretions, NK cell mediated activity alone, may likely be inadequate in reducing genital tract viral loads or preventing HIV infection (Cheeseman et al., 2017). In addition to NK cell activity, the IFN- $\gamma$  production observed in response to gp120, gp41 and p66 in both arms, at varying time points and in both compartments, suggests that this antiviral cytokine may have played a role in augmenting NK cell activity. The stimulatory function of IFN- $\gamma$  (Welsh, 1984, Boehm et al., 1997) may likely be involved in post-infection control of viremia and furthermore influences Ig class switching and thus antibody production (Mills et al., 1990). NK cells produce MIP-1 $\beta$ , a chemotactic cytokine which may magnify the immune responses through NK cell recruitment (Taub et al., 1995, Pincetic et al., 2014). In the genital tract, while the MIP-1 $\beta$  production was secondary to gp41 specific NK cell activated ADCC, and CD107a NK cell activated ADCC for p66 was elevated in the tenofovir arm, the CD107a gp41 NK cell activated ADCC diminished significantly in the placebo arm over time. Indeed, innate immune functions involving effector cells and synergized innate antibody recruitment have been associated with elite controllers (Ackerman et al., 2016). NHP studies have shown a strong and direct association between the Env IgG titres and ADCC activity (in Env prime boost strategies) which conferred protection to SHIV infection (Xiao et al., 2010, Florese et al., 2009). In this study however, a negative association was found between p24 CD107a NK cell activated ADCC activity and p24 specific IgG titres both in the blood and genital compartments, and this may suggest that even in the presence of low IgG-specific titres, antibody function is not compromised. Previously Archary *et al.*, (2016) did show that p24 specific IgG binding titres were associated with the placebo rather than the tenofovir arm. These data may further suggest that despite low p24 specific titres in the genital tract or blood of tenofovir gel exposed women, the p24-specific NK cell activated ADCC function is preserved. Therefore, there is rationale to harness nNAb targets that effect ADCC function for example, even if available in low titres, which likely may exert functional activity on contemporaneous virus and may circumvent the need to focus solely on only developing epitope based neutralizing recognition [as reviewed by (Overbaugh and Morris, 2012)].

Whether both HIV-specific ADCC and ADCP functions need to be equally exerted in the genital tract and systemically remains a point of interest.

The magnitudes of ADCP and ADCC functions directly correlated to those in the plasma for gp120, gp41 and p66-specific activities. These data highlight the plausibility of inducing an immune response capable of targeting multiple epitopes, which may contribute to reducing viral loads (Moog et al., 2014). Our data showed that ADCP and NK cell activated ADCC activities for any of the HIV-antibody specificities were not correlated in the genital tract, indicating that certain HIV-specific antibodies that may elicit redundant nNAb functions may occur simultaneously, although they may not occur at the same magnitude, or in the same compartment but may act in a coordinated manner (Ackerman et al., 2016). ADCC and ADCP functions may be differentially regulated possibly to avoid redundancies of nNAb functions, and supports the notion that various immunological mechanisms of protection occupy specific compartmental spaces in pathogenic control (Tay et al., 2016, Liu et al., 2013). Whether this profile of a coordinated functional relationship is restricted to, certain other HIV-IgG specificities not covered in this study and to the genital tract remains to be determined. IgG predominates the genital tract and is associated with ADCC activity (Mestecky et al., 2011, Jackson et al., 1999, Stratov et al., 2008) and reduced genital tract viral loads (Nag et al., 2004) and may likely play a role in ameliorating disease progression (Pincetic et al., 2014). The data from our study showed that plasma gp41 specific NK cell activated ADCC activity was negatively associated with plasma viral loads. Additionally, other studies have shown strong associations between ADCC activity in mucosal fluids such as breastmilk of infected mothers, and decreased infant mortality and HIV transmission (Milligan et al., 2015, Mabuka et al., 2012), further supporting the role of mucosal associated ADCC activity in reducing HIV disease progression. Furthermore, localization of effector cells (Cheeseman et al., 2016) and immune cell trafficking, may influence the predominant Fc-mediated activity prevailing, depending on the expression of antibody subclass specific FcR expressed on effector cells (Cheeseman et al., 2016, Dugast et al., 2011). Interestingly, viral suppression has been associated with a more enhanced effector cell activity suspected to influence antibody glycosylation and thus nNAb functionality, which may in part be due to B cell education (Ackerman et al., 2013a). Therefore, vaccination with immunogens that elicit nNAbs may provide increased antiviral control in concert with NAbs (Moog et al., 2014). Several studies have suggested that Fc-associated

functions enhance bNAb responses (Bournazos et al., 2014, Hessel et al., 2007, Halper-Stromberg et al., 2014). Therefore, elucidating the non-neutralizing functions that augment bNAb functions may be central to mucosal protection and also in limiting systemic spread of the virus, an important consideration for HIV reservoir and cure studies.

Although this study investigated the functional activity of IgG using peripheral neutrophils and NK cells, the study's findings support the role of tissue resident neutrophils in mediating phagocytosis (Sips et al., 2016). Furthermore, the fairly consistent populations of neutrophils and NK cells in tissue during inflammation (Sips et al., 2016), may be harnessed by nNAb to elicit antiviral activity. Additionally, a polyfunctional antibody response mediated by IgG1 and IgG3 has been shown to recruit and engage other effector functions to induce a synergistic nNAb response that maintains viremia at levels below detectability (Ackerman et al., 2016). However, this study did not exploit the relative contributions of subclass HIV-specific IgG1 versus IgG3 ADCC or ADCP activities and remains undefined. The panel of HIV-specific antibodies used to test for NK cell activated ADCC and ADCP activities were premised on the previous publication that showed distinct antibody profiles associated with prior topical PrEP (Archary et al., 2016), therefore the panel of HIV-IgG specificities tested for, was not exhaustive. In this study, women with prior topical PrEP compared to placebo gel users, had increased HIV-gp41 and p66 cytotoxic and antiviral activity in the genital tracts indicating preservation of nNAb functions despite HIV infection. In addition, NK cell activated ADCC activities in the genital tract were significantly higher, compared to circulating plasma NK cell activated ADCC activities for gp41 and p66 over time, indicating compartmental differences for these HIV-specific IgG functions. In the context of PrEP, long term ARV treatment has been shown to partially reconstitute the immune system (Connick et al., 2000), and improve antiviral responses to HIV infection (Ackerman et al., 2016) despite the delays in antibody binding avidity (Laeyendecker et al., 2015). Improving nNAb function can be engineered through altering the Fc portion of various bNAbs which has shown to synergistically enhance Fc-mediated antiviral activity [as reviewed in (Jefferis et al., 1998),(Lazar et al., 2006, Sips et al., 2016, Hessel et al., 2007)], suggesting that for vaccine development there is rationale for perhaps inducing bNAbs capable of augmented Fc-mediated activities.

Our study focussed on IgG mediated nNAb activities particularly because IgG is more common in vaginal secretions than IgA (Bomsel et al., 2011) and was a correlate of

protection in the RV144 trial (Haynes et al., 2012a, Yates et al., 2014). One of the limitations of this study was that due to the low yield of IgGs in the genital compartment and the limited sample volumes available to do the assays the standardization of the concentrations of CVL IgGs could not be performed. We therefore used the undiluted CVLs (a sample more closely representing *in vivo* Fc-mediated activities), previously shown to mediate ADCC activity as an unpurified IgG mucosal sample (Nag et al., 2004). Therefore, using this method allowed us to assess both the ADCP and NK cell activated ADCC activities of genital tract specimens. We did however, standardize for the volume of undiluted CVL used in these assays. To address this limitation, we suggest that the soft-cup method of genital specimen collection which has been proven to display superior and concentrated amounts of total immunoglobulins (Ig) compared to CVL should be used (Archary et al., 2015, Mkhize et al., 2016). Therefore, future studies should be done using a less diluted genital tract specimen like that obtained in the soft-cup sample in order to make an accurate and direct comparison between the genital tract and the blood. We acknowledge that this is a limitation of our current method which depended on a diluted CVL specimen, which was collected routinely as part of the genital tract specimens in the CAPRISA 004 clinical trial study. A further limitation of this study was the lack of comparison for the NK cell activation ADCC assays with the RFADCC assay, mostly due to the lack of sample availability. Another limitation is that we did not evaluate the impact of PrEP on IgA nNAb activities in the blood or genital tracts of women. Various studies have shown that gp41 specific IgA's prevent viral transcytosis of HIV across mucosal tissue (Bomsel et al., 2011, Alfsen et al., 2001, Tudor et al., 2009, Yates et al., 2013, Devito et al., 2000). However, due to the lack of availability of genital specimens for further experiments, such experimental analyses cannot be undertaken and should perhaps be interrogated in other PrEP studies. In chronic HIV infection NK cells are dysfunctional [as reviewed by (Fauci et al., 2005)], in the absence of contemporaneous, autologous NK cells or neutrophils from the study's participants, we cannot confirm any cellular dysfunction. However, the strengths of the study include the longitudinal analysis of the matching plasma and genital tract specimens that allowed us to cross-examine both mechanistic functions of the IgG's over time and in both compartments and relate these findings to HIV-specific binding antibody titres from previously published data. This study was designed to be a case-matched control study based on plasma viral loads and CD4<sup>+</sup> T cell counts at 6 months post-infection. NNAb differences observed

between the tenofovir and placebo arms were not confounded by differences between the groups, for markers of disease progression. However, the effect of NK cell activated ADCC activities on viral loads in the genital tract were not assessed and warrants further investigation to determine the contribution of local nNAb activity on genital tract viral load control. In addition, in this study effector cells from healthy individuals were used, which has previously been shown to augment nNAb cell mediated antiviral activity (Chung et al., 2011). Therefore, we suggest that if PrEP preserves NK cell function, it may lead to better protection from HIV infection at sites of transmission. However, the effects of PrEP on autologous NK cells and other effector cells associated with innate immunity, remains a gap in our knowledge. In light of several limitations, we were able to show that there are compartment specific differences for NK cell activated ADCC and ADCP functions, where higher NK cell activated ADCC activity in the genital tract may translate to lower ADCP for the same HIV specificity mediating nNAb functions. Therefore, these data will have to be more thoroughly investigated in larger studies.

### **Conclusions**

Our findings suggest that prior topical PrEP usage did not dampen but rather primed and augmented certain humoral nNAb activities in the plasma and genital tracts of women who used 1 % tenofovir gel and underscores the potential role of nNAbs that target a variety of HIV epitopes in vaccine design. These data indicate that certain HIV-specific nNAb immune activities, which may contribute to HIV protection, also show differences in magnitudes for these activities and may be compartment specific possibly to avoid immunological redundancies and avert extra inflammation associated with nNAb functions. Moreover, the simultaneous assessment of these two nNAb functions as well as other functions such as ADCVI requires further investigation for the contribution of polyfunctional immune mechanisms to better protection. Taken together, these data provide more evidence for the role of nNAbs as quintessential to a functional and protective immune profile, especially in the area of vulnerability, the female genital tract. Further studies should be undertaken to assess the impact of oral PrEP on humoral and cellular immunity for correlates of risk and protection in the genital tract, especially if combination PrEP and vaccines are to be used as HIV prevention strategies.

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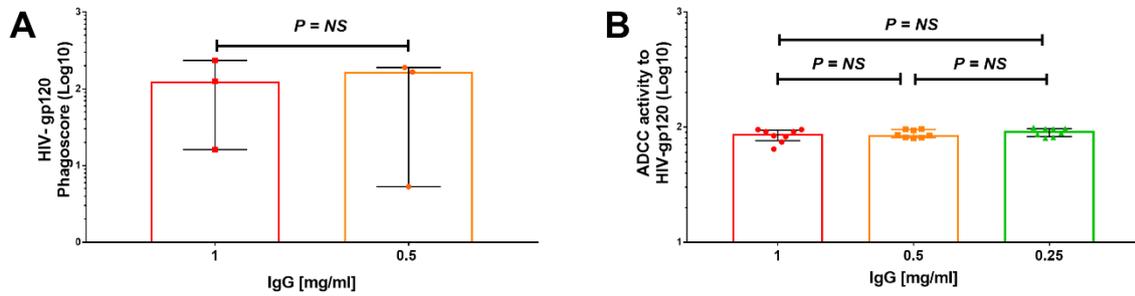
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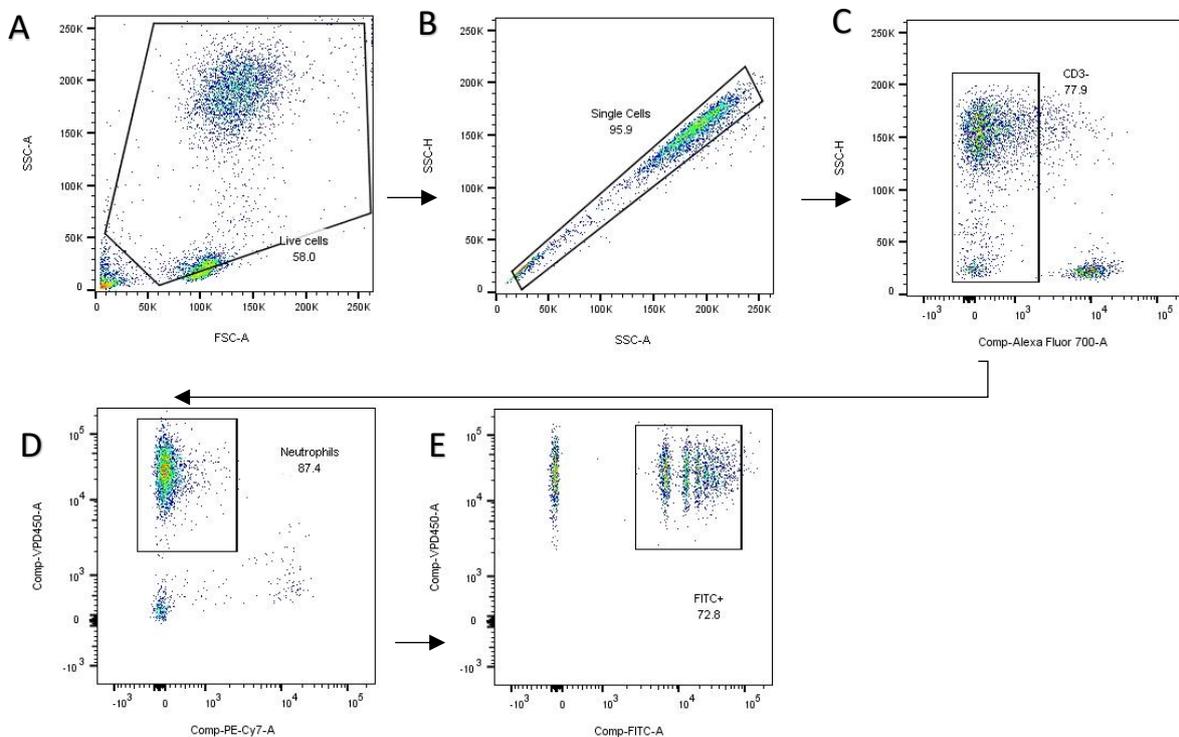
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## APPENDIX

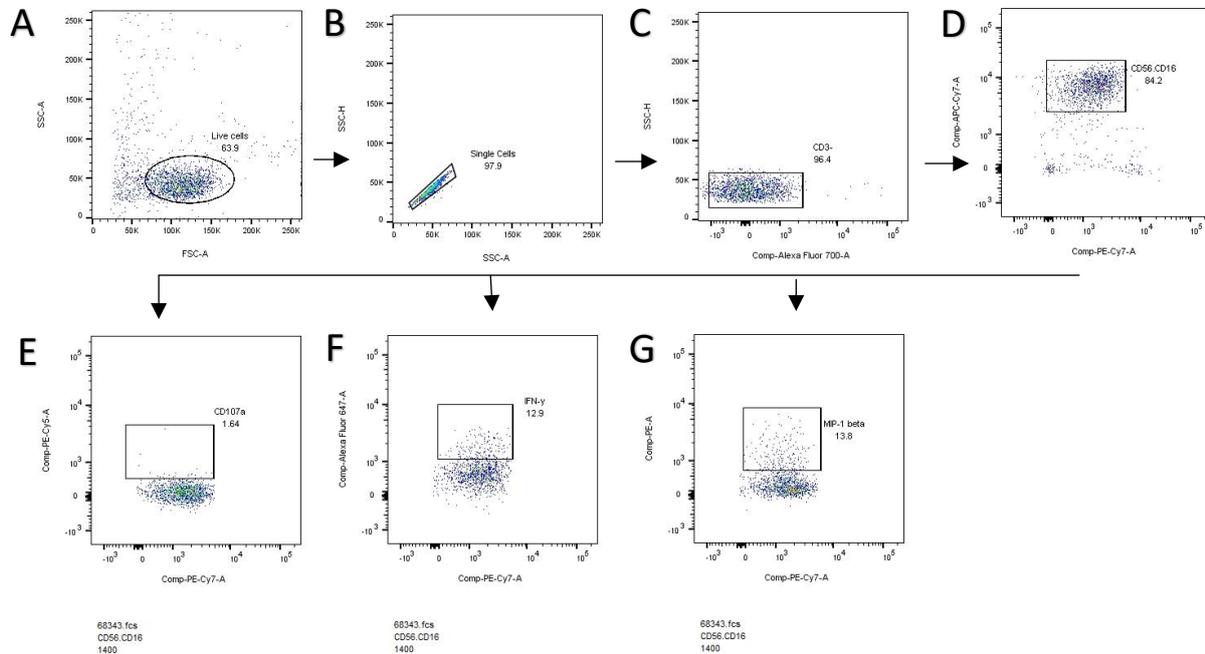


**SUPPLEMENTARY FIGURE 1: Titration of IgG isolated from the plasma of women in the CAPRISA 004 trial assessing how different IgG titres affect ADCP and ADCC nAb functional assays.** Wilcoxon Sum Rank tests were performed in order to determine differences between IgG concentrations and their relative functional responses. HIVIG was used to determine optimal IgG concentrations to be used in the assays. **(A)** ADCP phagocytic activities were similar between dilutions ( $P > 0.999$ ). **(B)** ADCC Cytotoxic activities (CD107a) between 1mg/ml to 0.5mg/ml ( $P = 0.721$ ), 0.5mg/ml to 0.025mg/ml ( $P = 0.169$ ) and 1mg/ml to 0.025mg/ml ( $P = 0.235$ ), were similar.  $P = NS$  defined values that were  $P > 0.1$ .

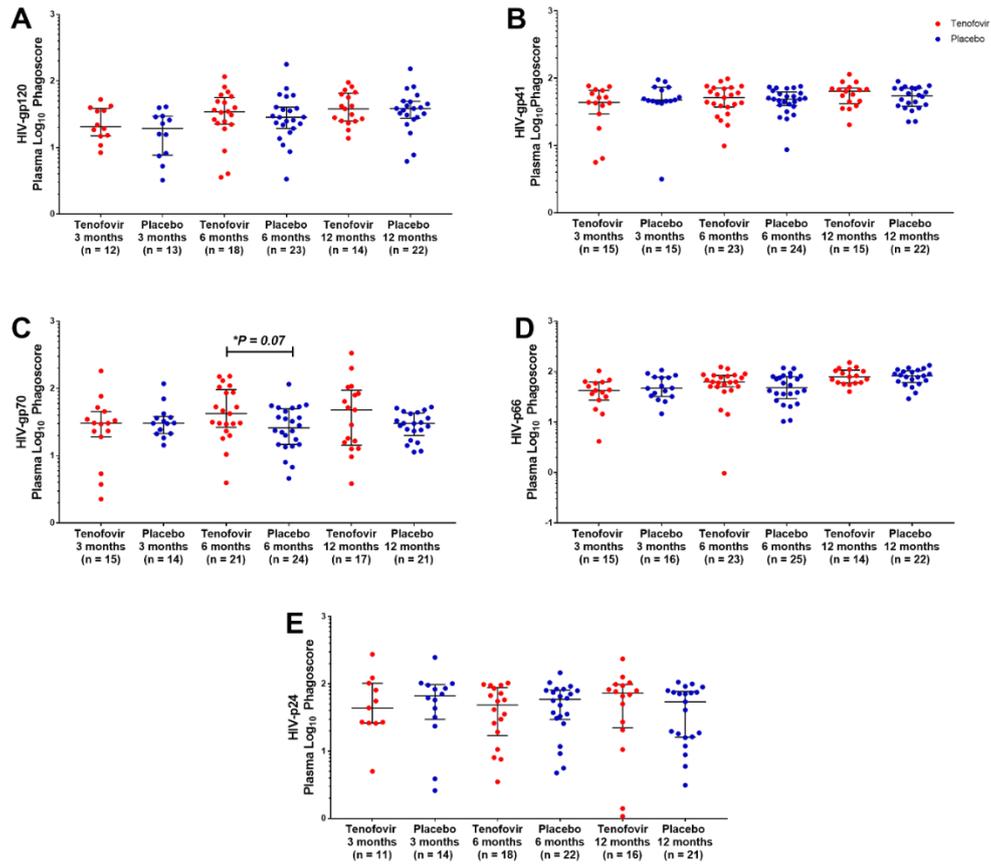


**SUPPLEMENTARY FIGURE 2: Gating strategy for the ADCP assay, investigating the phagocytic activity of neutrophils using PBS as a negative control, to indicate the level of background ADCP**

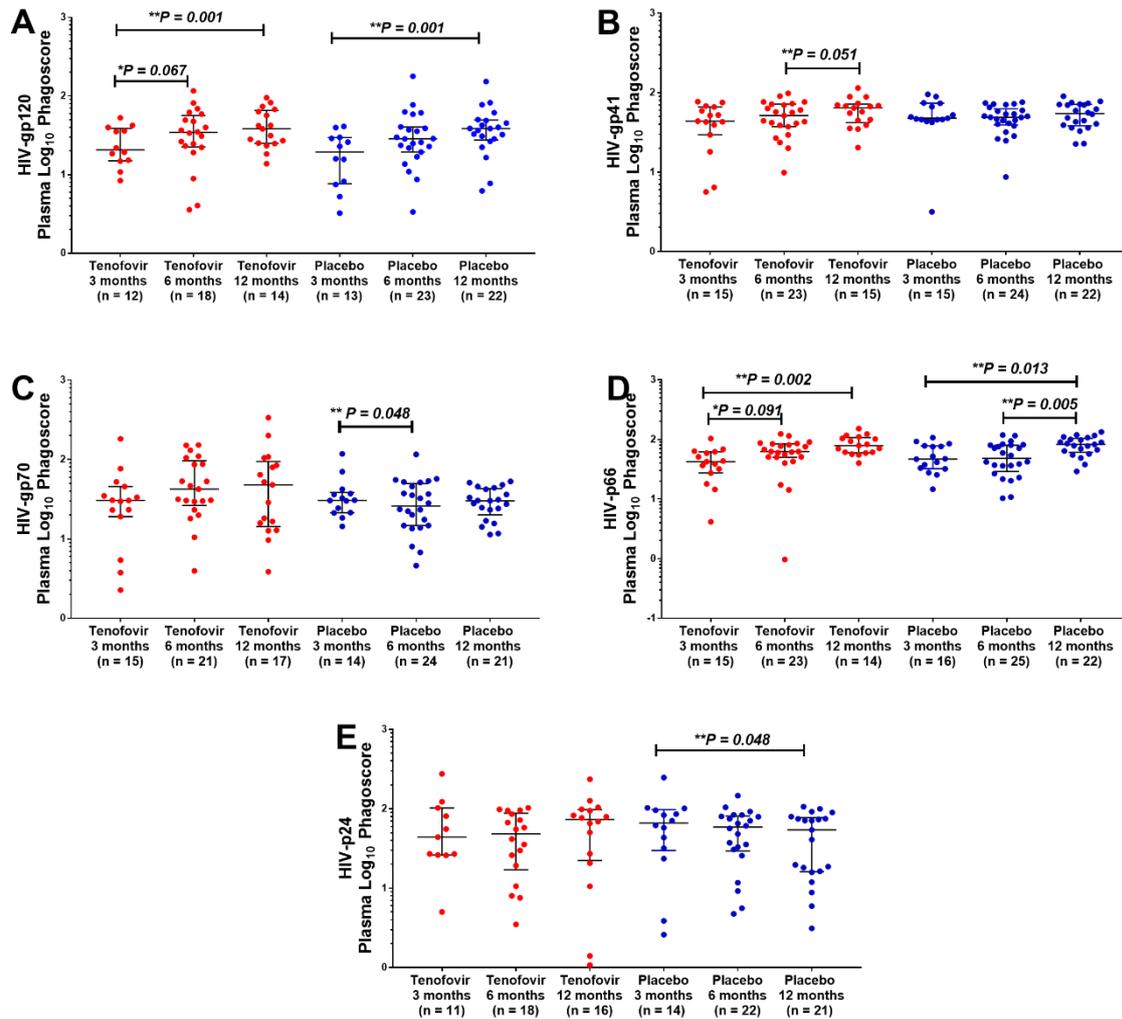
**activity.** Live cells were gated on using FSC-A and SSC-A. Sequential gating from live cells, included singlets, followed by CD3- cell, neutrophils and then neutrophils that phagocytosed antigen coated neutravidin beads.



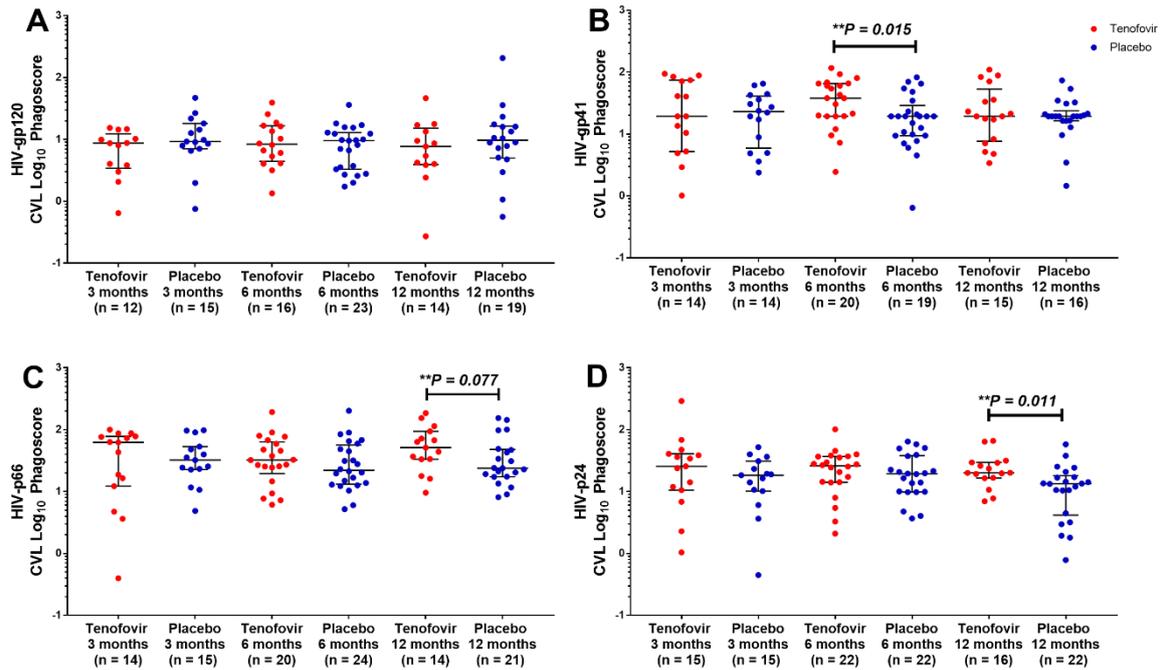
**SUPPLEMENTARY FIGURE 3: Gating strategy for the ICS NK cell activated ADCC assay using PBS as a negative control, to indicate the level of NK cell activated ADCC background activity. (A)** Live cells were gated on using FSC-A and SSC-A. Following identification of live cells in this bio-assay, **(B)** singlets were gated on, **(C)** followed by CD3- cells, **(D)** CD56+CD16dim NK cells and the subsequent cytokine productions to identify NK cell activity **(E)** CD107a, **(F)** IFN- $\gamma$  and **(G)** MIP-1 $\beta$ .



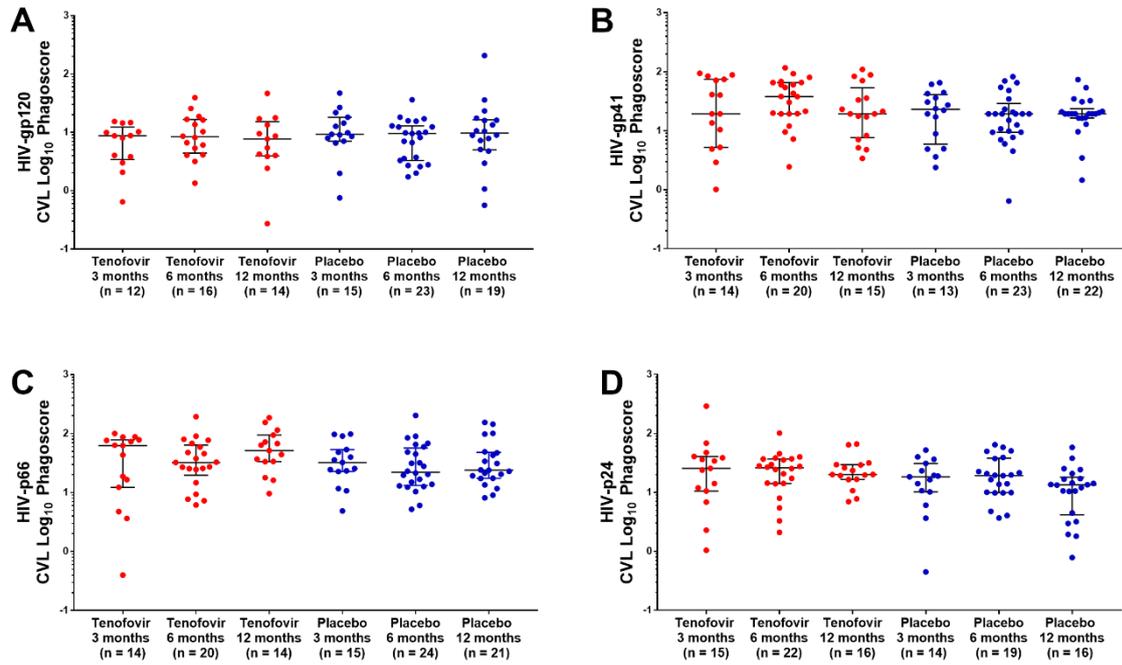
**SUPPLEMENTARY FIGURE 4: Comparisons of detectable phagocytic activities in the plasma to HIV-proteins at 3, 6 and 12 months.** Phagocytic activity below background phagocytic activity were removed, in order to adjust for outliers, for each of the HIV proteins **A)** gp120, **B)** gp41, **C)** gp70 and **D)** p66 and **E)** p24. Horizontal solid lines within each arm and time point indicate maximum and minimum values with the central solid line indicating the median. Horizontal lines across boxes, between arms and time points, if any, identify differences between tenofovir and placebo arm medians using the Wilcoxon Sum Rank test.



**SUPPLEMENTARY FIGURE 5: Longitudinal analyses of detectable phagocytic activities in the plasma to HIV-proteins from 3 months to 12 months, post-infection, in both study arms.** Phagocytic activities below background phagocytic activity were removed, in order to adjust for outliers, for each of the HIV proteins **A)** gp120, **B)** gp41, **C)** gp70 and **D)** p66 and **E)** p24. Horizontal solid lines within each arm and time point indicate maximum and minimum values with the central solid line indicating the median. Horizontal lines across boxes, between arms and time points, if any, identify differences between tenofovir and placebo arm medians using the Wilcoxon Signed Rank test. Statistically significant values were defined as **\*\*P ≤ 0.05**, and those that trended as **\*P < 0.1**.



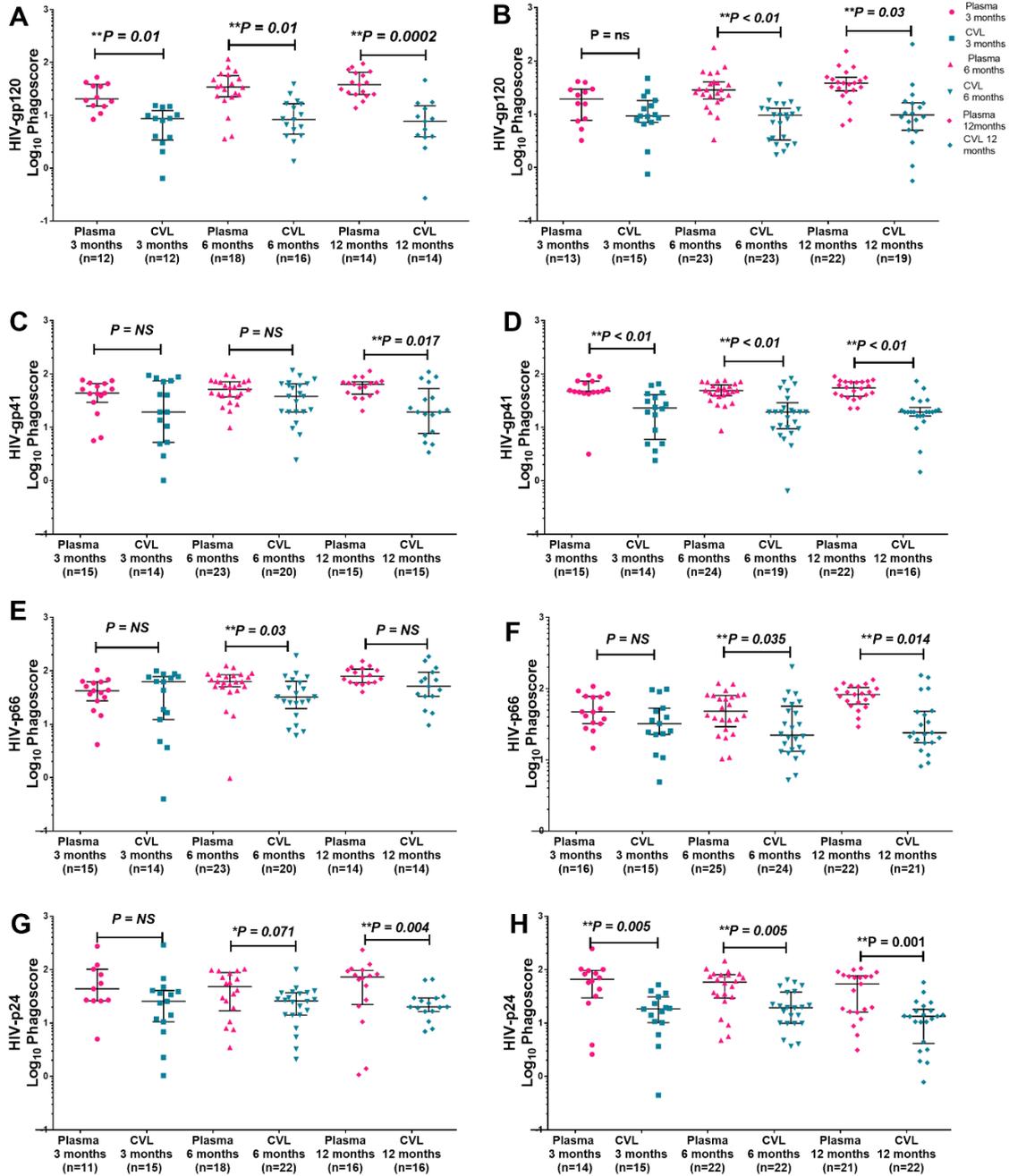
**SUPPLEMENTARY FIGURE 6: Comparisons of detectable phagocytic activities in the genital tracts (CVL) of women in the tenofovir and placebo arms at 3, 6 and 12 months, post-infection, for all HIV-proteins. Phagocytic activities below the background score were removed. Phagocytic scores to A) gp120, B) gp41, C) p66 and D) p24 shown in box and whisker plots, with horizontal lines indicating the median, maximum and minimum values. Solid black horizontal lines indicate statistical analysis using Wilcoxon Sum Rank tests. Significant values were defined as **\*\*P ≤ 0.05** and those that trended as **\*P < 0.1**.**



**SUPPLEMENTARY FIGURE 7: Longitudinal analyses of detectable phagocytic activities in the genital tracts (CVL) of women in the tenofovir and placebo arms, 3 months to 12 months post-infection, for all HIV-proteins.** Phagocytic activities below the background score were removed. Phagocytic scores to **A) gp120**, **B) gp41**, **C) p66** and **D) p24** shown in box and whisker plots, with horizontal lines indicating the median, maximum and minimum values. Solid black horizontal lines indicate statistical analysis using Wilcoxon-Signed Rank test.

## Tenofovir Arm

## Placebo Arm



**SUPPLEMENTARY FIGURE 8: Inter-compartmental analyses of detectable phagocytic activities between the tenofovir and placebo arms.** Phagocytic activity between the plasma and genital mucosae were analysed using a Wilcoxon Rank-Signed test, in order to determine any significant differences. HIV-specific phagocytic activities were separated into tenofovir (left) and placebo (right) arms according to the respective HIV proteins with **A**) gp120 tenofovir, **B**) gp120 placebo, **C**) gp41 tenofovir, **D**) gp41 placebo **E**) p66 tenofovir, **F**) p66 placebo, **G**) p24 tenofovir and **H**) p24 placebo. **\*\*P ≤ 0.05** were defined as significant, and those that trended as **\*P < 0.1** with pink dots indicating plasma phagocytic activity and turquoise indicating genital mucosal (CVL) phagocytic activity. Maximum and minimum values are shown by solid lines in the box and whisker plots as well as median phagocytic activity for each compartment, at different time points. Horizontal solid lines above the box and whisker plots identifying significant values.

**SUPPLEMENTARY TABLE 1: Cross-sectional analyses of plasma tenofovir and placebo phagocytic activities to HIV-gp120, gp41, p66 and p24, at 3, 6 and 12 months, post-infection**

	3 months			6 months			12 months		
	Tenofovir	Placebo	<i>P - value</i>	Tenofovir	Placebo	<i>P - value</i>	Tenofovir	Placebo	<i>P value</i>
<b>HIV protein</b>	<b>Median (IQR)</b>			<b>Median (IQR)</b>			<b>Median (IQR)</b>		
<b>Gp120</b>	27.12 (18.49-39.85)	26.52 (1008 – 29.56)	0.295	27.12 (21.32 – 49.47)	27.12 (18.14 – 39.65)	0.759	41.99 (25.17 – 65.51)	37.52 (28-49.11)	0.664
<b>Gp41</b>	43.85 (29.48 – 66.04)	47.26 (44.08 – 71.82)	0.446	51.52 (37.26 – 71.69)	49 (39.18 – 62.56)	0.759	64.1 (41.91 – 71.59)	54.74 (38.35 – 71.13)	0.644
<b>P66</b>	42.35 (27.4 – 62.37)	47.12 (32.4 – 77.85)	0.338	62.76 (50.15 – 84.58)	41.41 (24.87 – 80.24)	0.210	79.01 (60.21 – 107.5)	80.89 (56.81 – 101.9)	0.664
<b>P24</b>	27.01 (6.26 – 80.82)	59.78 (10.59 – 93.42)	0.512	41.51 (8.018 – 71.99)	52.79 (18.71 – 79.68)	0.609	69.16 (15.63 – 96.35)	53.48 (16.24 – 76.11)	0.457

\*indicates values that trended towards significance, defined as  $P < 0.1$

\*\*indicates significant values defined as  $P \leq 0.05$

**SUPPLEMENTARY TABLE 2: Cross-sectional analyses of phagocytic activities in the genital tracts of women in the tenofovir and placebo arms at 3, 6 and 12 months, post-infection.**

	3 months			6 months			12 months		
	Tenofovir	Placebo	<i>P - value</i>	Tenofovir	Placebo	<i>P - value</i>	Tenofovir	Placebo	<i>P value</i>
HIV protein	Median (IQR)			Median (IQR)			Median (IQR)		
<b>Gp120</b>	9.95 (3.831 – 14.72)	9.54 (7.298 – 20.35)	0.572	11.81 (6.07 – 21.1)	9.735 (3.431 – 14.23)	0.235	9.50 (4.123- 19.44)	11.21 (6.712 – 18.450)	0.721
<b>Gp41</b>	19.4 (5.243 – 65.6)	23.04 (5.942 – 41.09)	0.579	37.92 (19.4 – 65.6)	19.4 (9.43 – 28.93)	<b>0.014**</b>	19.4 (7.691 – 53.4)	19.4 (16.39 – 23.75)	0.642
<b>P66</b>	62.56 (12.24 – 78.28)	34.01 (22.82 – 65.93)	0.173	32.33 (14.68 – 67.99)	22.98 (13.37 – 62.46)	<b>0.039**</b>	63.58 (33.48 – 83.09)	24.03 (17.44 – 54.16)	0.622
<b>P24</b>	25.67 (10.54 – 40.76)	18.71 (10.32 – 33.13)	0.326	25.4 (14.2 – 36.81)	19.81 (9.989 – 35.65)	0.860	20.15 (16.61 – 30.61)	13.41 (4.16 – 18.11)	0.569

\**P* < 0.1 indicate values that trend to significance

\*\* *P* ≤ 0.05 values that are significant

SUPPLEMENTARY TABLE 3: Correlations between detectable phagocytic activities in the plasma and genital tracts for all HIV-proteins, in the tenofovir and placebo arms.

Protein	Arm	3 months		6 months		12 months	
		R-value	P-value	R-value	P-value	R-value	P-value
gp120	Tenofovir	0.283	0.463	-0.027	0.935	0.324	0.280
	Placebo	-0.218	0.521	0.107	0.642	<b>-0.603</b>	<b>*0.012</b>
gp41	Tenofovir	0.454	0.092	-0.207	0.355	-0.004	0.989
	Placebo	<b>0.511</b>	<b>0.054**</b>	-0.062	0.769	0.259	0.245
p66	Tenofovir	0.046	0.873	0.36	0.105	-0.268	0.334
	Placebo	-0.093	0.744	-0.208	0.342	<b>-0.442</b>	<b>0.051**</b>
p24	Tenofovir	0.118	0.735	-0.287	0.264	-0.132	0.639
	Placebo	-0.071	0.821	-0.044	0.859	-0.074	0.750

\*indicates values that trended towards significance, defined as  $P < 0.1$

\*\*indicates significant values defined as  $P \leq 0.05$

**SUPPLEMENTARY TABLE 4: Cross-sectional analyses of plasma ADCC activities mediated by IgG from women in the tenofovir and placebo arms, at 3, 6 and 12 months, post-infection**

3 months									
	median (IQR) TFV n = 15			median (IQR) placebo n = 16			P-value		
	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$
<b>gp120</b>	449.5 (76.5-640.5)	494 (380.5-1158)	713.5 (106.5-814)	644.8 (272.5-918.3)	430.3 (389.5-995.1)	719.3 (89.25-849.4)	0.041	0.686	0.778
<b>gp41</b>	379.50 (58-596.5)	190.5 (72-411)	370.5 (108.5-2792)	362 (25-587.9)	315.3 (90.38-406.4)	403 (133.6-2792)	0.616	0.429	0.961
<b>p66</b>	422.5 (338-461)	1706 (691.5-1880)	317.5 (149-500)	398.5 (356.9-512.9)	1692 (815.5-1818)	535 (323.4-740.8)	0.741	0.626	<b>0.049**</b>
<b>p24</b>	109.5 (65-388.5)	595 (527.5-1147)	129 (111-580.5)	100.4 (36.5-575.3)	569.5 (424.1-1074)	257.5 (159.6-699.4)	0.792	0.423	0.120
6 months									
	median (IQR) TFV (n=23)			median (IQR) Placebo (n=25)			P - value		
	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$
<b>gp120</b>	644 (245-895)	497 (409-1158)	837 (179.5-1154)	604 (242.3-703.5)	441.5 (393.5-1158)	730.5 (134-865.5)	0.315	0.847	0.384
<b>gp41</b>	337.5 (34-562)	157.5 (51.5-304.5)	368.5 (256-2792)	268.5 (47.5-562)	233.5 (98.25-395.5)	345.5 (104.8-2792)	0.786	0.317	0.930
<b>p66</b>	417 (351-437)	1639 (660-1745)	246 (112.5-478.5)	420 (363.8-473.8)	1711 (1101-1966)	548.3 (365.8-764.3)	0.613	<b>0.086*</b>	<b>0.003*</b>
<b>p24</b>	310 (110-566)	606 (374-1147)	415.5 (206-855)	149.0 (111.6-449)	549 (451.8-763)	236.5 (129.8-591.5)	0.556	0.744	0.144
12 months									
	median (IQR) TFV (n=17)			median (IQR)Placebo (n=22)			P - value		
	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$
<b>gp120</b>	559.5 (294.5-805.8)	461 (392.8-1158)	733.5 (144-1256)	609 (453-694.1)	553.8 (430.9-1158)	777.8 (186.6-907.4)	0.778	0.119	0.763
<b>gp41</b>	398 (323.3-579.3)	155.5 (50.25-461.2)	340 (122-1623)	380.8 (52-596.5)	218.8 (141.6-379)	442.3 (90.13-2792)	0.749	0.276	0.558
<b>p66</b>	428.5 (390.5-479.8)	1654 (679.8-1731)	391 (95.25-549.5)	433.8 (359.6-505.6)	1741 (923.6-1864)	473.3 (391.8-776.3)	0.856	0.172	<b>0.049**</b>
<b>p24</b>	220 (57.5-634.5)	536 (444.8-1161)	354.5 (206-734.5)	128.8 (70.75-300.1)	582.3 (466-1147)	221 (136.5-595.8)	0.295	0.922	0.333

indicates values that trended towards significance, defined as  $P < 0.1$

\*\*indicates significant values defined as  $P \leq 0.05$

**SUPPLEMENTARY TABLE 5: Cross-sectional analyses of ADCC activities mediated by IgG from the genital tracts of women in the tenofovir and placebo arms, at 3, 6 and 12 months, post-infection**

3 months									
	median (IQR) TFV n=15			median (IQR) placebo n=16			<i>P-value</i>		
	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$
<b>gp120</b>	24.8 (14.95 – 31.25)	1.32 (1.32 – 1.43)	398.4 (318.6 – 708.4)	16.35 (5.475 – 49.28)	1.32 (1.32 – 1.32)	577.4 (318.6 – 1663)	0.835	0.559	0.536
<b>gp41</b>	808 (667 – 1113)	1288 (1048 – 1768)	2905 (2555-2990)	1096 (614.4 – 1663)	1665 (1368 -3210)	1539 (1056 – 2905)	0.573	0.274	<b>0.065*</b>
<b>p66</b>	846 (626.5 – 1106)	891 (790.4 – 1322)	8010 (7652 – 8655)	842.3 (720.1 – 1029)	1079 (957.8 – 1478)	9654 (8361 – 11372)	0.965	0.274	<b>0.034*</b>
<b>p24</b>	356.5 (140.5 – 434.5)	576 (487 – 738)	498 (149 – 1595)	434.5 (49 – 637.5)	518 (482.5 – 662.5)	731 (250 – 3361)	> 0.999	0.639	0.751
6 months									
	median (IQR) TFV (n=23)			median (IQR) Placebo (n=25)			<i>P - value</i>		
	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$
<b>gp120</b>	17.6 (5.05 – 27.95)	1.32 (1.32 – 1.32)	668.4 (293.9 – 1868)	29.6 (10.7 -64.8)	1.32 (1.135 – 1.345)	436.4 (318.6 – 783.4)	0.144	0.907	0.682
<b>gp41</b>	718 (591 – 853.5)	1797 (1430 – 2103)	2905 (1590 – 2905)	666 (421 – 842.8)	1727 (1240 – 2051)	2905 (1766- 2905)	0.486	0.614	0.510
<b>p66</b>	754.5 (615.3 – 931.3)	1043 (783.5 – 1221)	8568 (8108 – 9041)	824 (729.7 – 957.5)	1088 (750.8 – 1221)	8784 (8174 – 10591)	0.361	>0.999	0.424
<b>p24</b>	189.5 (86 – 628)	538 (502 – 857.5)	654 (367 – 2306)	131.5 (72.75 – 370.3)	478.5 (448.3 – 559)	2426 (396.8 – 3361)	0.357	<b>0.026*</b>	0.302
12 months									
	median (IQR) TFV (n=17)			median (IQR)Placebo (n=22)			<i>P - value</i>		
	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$	CD107a	IFN- $\gamma$	MIP-1 $\beta$
<b>gp120</b>	15.95 (7.5 – 37.73)	1.32 (1.32 – 1.32)	599.9 (316.3 – 1245)	23.9 (2.4 – 33.4)	1.32 (0.75 – 1.32)	318.6 (318.6 – 1869)	0.962	0.434	0.756
<b>gp41</b>	753.3 (415.5 – 1317)	1410 (1238 – 2020)	2905 (2055 – 2905)	697 (355.8 – 1019)	1914 (1401 – 2672)	2155 (389 -2905)	0.628	0.228	<b>0.081*</b>
<b>p66</b>	691.5 (575.5 – 801.5)	954.8 (687.9 -1135)	8244 (6793 – 9255)	691.5 (575.5 – 801.5)	1198 (948.8 – 1525)	7953 (7145 – 9973)	<b>0.034*</b>	<b>0.082*</b>	>0.999
<b>p24</b>	70 (37.5 – 354.5)	536 (481.5 – 734)	451.5 (257.3 – 2707)	175.5 (91.25 – 541.3)	577.5 (466.3 – 882.3)	3361 (161.5 – 3361)	0.298	0.881	0.453

\*indicates values that trended towards significance, defined as  $P < 0.1$

\*\*indicates significant values defined as  $P \leq 0.05$



**SUPPLEMENTARY TABLE 6: Comparison analyses of ADCP and ADCC (CD107a) activities in the plasma of women in the tenofovir and placebo arms, for HIV-gp2120, gp41, p66 and p24.**

3 months						
Tenofovir			Placebo			
median (IQR) (n=15)			median (IQR) (n=16)			
	ADCC activity (CD107a)	Phagocytic Scores	<i>P</i> - value	ADCC activity (CD107a)	Phagocytic Scores	<i>P</i> - value
<b>HIV proteins</b>						
<b>gp120</b>	2.8 (2.325 – 5.01)	27.12 (18.49 – 38.85)	< 0.0001**	3.67 (2.729 – 4.435)	26.52 (10.08 – 29.56)	< 0.0001**
<b>gp41</b>	9.245 (2.42 – 9.52)	43.85 (29.48 – 66.04)	0.0003**	8.413 (1.456 – 11.55)	47.26 (44.08 – 71.82)	< 0.0001**
<b>p66</b>	6.29 (0.63 – 26.45)	42.35 (27.4 -62.37)	0.003**	8.08 (4.135 – 17.02)	47.16 (32.4 – 77.85)	0.0001**
<b>p24</b>	5.1 (4.63 – 7.435)	27.01 (6.26 – 80.82)	0.0001**	6.52 (5.075 – 8.585)	59.78 (10.59 – 93.42)	0.0004**
6 months						
median (IQR) (n = 23)			median (IQR) (n = 25)			
	ADCC activity CD107a	Phagocytic Scores	<i>P</i> - value	ADCC activity (CD107a)	Phagocytic Scores	<i>P</i> - value
<b>HIV proteins</b>						
<b>gp120</b>	3.705 (2.505 – 8.69)	27.12 (21.32 – 49.47)	< 0.0001**	3.85 (3 – 6.065)	27.12 (18.14 – 39.65)	< 0.0001**
<b>gp41</b>	7.62 (1.89 – 9.385)	51.52 (37.26 – 71.69)	< 0.0001**	9.25 (1.875 – 11.98)	49 (39.18 – 62.56)	< 0.0001**
<b>p66</b>	5.25 (0.655 -24.05)	62.76 (50.15 – 84.48)	< 0.0001**	10.74 (2.686 – 20.72)	42.41 (24.87 – 80.24)	< 0.0001**
<b>p24</b>	6.25 (5.275 – 8.595)	41.51 (8.018 – 71.99)	< 0.0001**	6.53 (5.556 – 7.093)	52.79 (18.71 – 79.68)	0.0004**
12 months						
median (IQR) (n = 17)			median (IQR) (n = 22)			
HIV proteins	ADCC activity CD107a – median (IQR)	Phagocytic Scores	<i>P</i> - value	ADCC activity (CD107a)	Phagocytic Scores	<i>P</i> - value
<b>Gp120</b>	4.375 (2.883 – 12.31)	41.99 (25.17 – 65.51)	< 0.0001**	4.348 (2.529 – 10.99)	37.52 (28 – 49.11)	< 0.0001**
<b>Gp41</b>	8.855 (2.305 -9.903)	64.1 (41.91 – 71.59)	< 0.0001**	9.08 (1.756 – 10.21)	54.74 (38.35 – 71.13)	< 0.0001**
<b>P66</b>	3.82 (0.755 – 13.87)	79.01 (60.21 – 107.5)	< 0.0001**	9.2 (6.33 – 33.51)	80.89 (56.81 – 101.9)	< 0.0001**
<b>P24</b>	7.17 (5 – 9.555)	69.16 – 15.63 – 96.35)	0.0002**	6.88 (5.02 – 9.253)	53.48 (16.24 – 76.11)	< 0.0001**

\*indicates values that trend towards significance, defined as  $P < 0.1$   
\*\* indicates values that are significant, defined as  $P \leq 0.05$

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SUPPLEMENTARY TABLE 7: Comparison analyses of ADCP and ADCC (CD107a) activities in the genital tracts of women in the tenofovir and placebo arms, for HIV-gp2120, gp41, p66 and p24.

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3 months

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**SUPPLEMENTARY TABLE 8: Spearman R correlation analyses of ADCC and ADCC (CD107a) activities in the plasma of women in the tenofovir and placebo arms at phase 3, 6 and 12 months post-infection, for all HIV-proteins.**

	median (IQR) TFV ^ (n = 12)			median (IQR) TFV ^ (n=12)		
	ADCC activity (CD107a)	Phagocytic Scores	P - value	ADCC activity (CD107)	Phagocytic Scores	P - value
<b>HIV proteins</b>						
<b>gp120</b>	<b>24.8 (14.95 – 31.25)</b>	<b>9.952 (3.831 – 14.72)</b>	<b>0.004**</b>	<b>16.35 (5.475 – 49.28)</b>	<b>9.544 (7.298 – 20.35)</b>	<b>0.742</b>
<b>gp41</b>	808 (667 – 1113)	19.4 (5.243 – 74.55)	0.002**	1096 (614.4 – 1663)	23.04 (5.942 – 41.09)	<b>0.008**</b>
<b>p66</b>	846 (626.5 – 1106)	62.56 (12.24 – 78.28)	<b>0.002**</b>	842.3 (720.1 -1029)	34.01 (22.82 – 65.93)	<b>0.008**</b>
<b>p24</b>	356.5 (140.5 – 434.5)	25.67(10.54 – 40.76)	<b>0.016**</b>	434.5 (49 – 637.5)	18.71 (10.32 – 33.13)	<b>0.063*</b>
<b>6 months</b>						
	Median (IQR) ^ (n = 17)			Median ^ (IQR) (n = 18)		
	ADCC activity CD107a	Phagocytic Scores	P - value	ADCC activity (CD107a)	Phagocytic Scores	P - value
<b>HIV proteins</b>						
<b>gp120</b>	20 (10.45 – 29.43)	11.81 (6.073 – 21.1)	0.193	29.6 (10.7 – 64.8)	9.725 (3.431 – 14.23)	<b>0.039**</b>
<b>gp41</b>	718 (591 – 853.5)	37.92 (19.4 – 65.6)	<b>0.0001**</b>	666 (421 -842.8)	19.4 (9.43 – 28.93)	<b>0.0005**</b>
<b>p66</b>	754.5 (615.3 – 931.3)	32.33 (14.68 – 67.99)	<b>0.0002**</b>	824 (729.7 – 957.5)	22.98 (13.37 – 62.46)	<b>0.001**</b>
<b>p24</b>	189.5 (86 – 628)	25.4 (14.2 – 36.81)	<b>0.004**</b>	131.5 (72.75 – 370.3)	19.81 (9.989 – 35.65)	<b>0.008**</b>
<b>12 months</b>						
	Median (IQR) ^ (n = 14)			Median ^ (IQR) (n =15)		
HIV proteins	ADCC activity CD107a – median (IQR)	Phagocytic Scores	P - value	ADCC activity (CD107a)	Phagocytic Scores	P - value
<b>Gp120</b>	15.95 (7.5 – 37.73_	9.499 (4.123 – 19.44)	0.432	23.9 (2.4 – 33.4)	11.21 (6.712 – 18.45)	> 0.999
<b>Gp41</b>	753.3 (415.5 – 1317)	19.4 (7.691 – 53.4)	<b>0.001**</b>	697 (355.8 – 1019)	19.4 (16.39 -23.75)	<b>0.002**</b>
<b>P66</b>	903.3 (774.6 – 1061)	63.58 (33.48 – 83.09)	<b>0.001**</b>	691.5 (575.5 – 801.5)	24.03 (17.44 – 54.16)	<b>0.004**</b>
<b>P24</b>	70 (37.5 – 354.5)	20.15 (16.61 – 30.61)	<b>0.023**</b>	122.5 (65 – 331.5)	13.41 (4.16 – 18.11)	<b>0.063*</b>

\*indicates values that trend towards significance, defined as  $P < 0.1$

\*\* indicates values that are significant, defined as  $P \leq 0.05$

^ CVL was not available for some participants in the tenofovir and placebo arms. Therefore, the sample sizes (n) represented were the average number of participant CVL samples available to test for each protein.

	3 months Tenofovir		3 months Placebo		6 months Tenofovir		6 months Placebo		12 months Tenofovir		12 months Placebo	
	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
<b>gp120</b>	0.132	0.639	<b>0.442</b>	<b>0.087*</b>	<b>0.443</b>	<b>0.034**</b>	0.155	0.461	-0.174	0.503	-0.058	0.797
<b>gp41</b>	0.090	0.749	0.260	0.331	<b>0.523</b>	<b>0.011**</b>	0.003	0.988	0.767	0.0005	<b>0.566</b>	<b>0.006**</b>
<b>p66</b>	-0.216	0.440	0.231	0.408	-0.153	0.485	-0.002	0.994	<b>0.708</b>	<b>0.002**</b>	0.166	0.471
<b>p24</b>	0.391	0.151	-0.082	0.772	0.316	0.142	0.187	0.381	0.348	0.171	0.239	0.297

\*indicates values that trend towards significance, defined as  $P < 0.1$

\*\* indicates values that are significant, defined as  $P \leq 0.05$

**SUPPLEMENTARY TABLE 9: Spearman R correlation analyses of ADCP and ADCC (CD107a) activities in the genital tracts of women in the tenofovir and placebo arms, at 3, 6 and 12 months post-infection, for all HIV-proteins.**

	3 months Tenofovir		3 months Placebo		6 months Tenofovir		6 months Placebo		12 months Tenofovir		12 months Placebo	
	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>	<i>R-value</i>	<i>P-value</i>
<b>gp120</b>	0.355	0.349	-0.436	0.280	-0.311	0.379	0.433	0.250	-0.350	0.324	-0.036	0.964
<b>gp41</b>	0.128	0.726	0.170	0.688	-0.429	0.113	-0.088	0.788	0.042	0.904	0.200	0.584
<b>p66</b>	0.54	0.107	0.184	0.663	0.049	0.878	0.182	0.595	-0.459	0.135	0.167	0.678
<b>p24</b>	0.072	0.878	0.032	0.959	0.183	0.644	-0.072	0.872	0.381	0.360	0.500	0.450

\*indicates values that trend towards significance, defined as  $P < 0.1$

\*\* indicates values that are significant, defined as  $P \leq 0.05$



31 May 2016

Ms K Naidoo (210507122)  
 Discipline of Medical Microbiology  
 School of Laboratory Medicine and Medical Sciences  
 Health Sciences  
[kinleightn@gmail.com](mailto:kinleightn@gmail.com)

Protocol: Functional assessment on non-neutralizing binding antibodies in the blood and genital tracts of women with breakthrough HIV infection from the CAPRISA 004 tenofovir 1% gel trial.  
 Degree: MMedSc  
 BREC reference number: BE241/16 (sub-study of BE013/04)

The Biomedical Research Ethics Committee has considered and noted your application received on 08 April 2016.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 18 May 2016 to queries raised on 16 May 2016 have been noted and approved by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval.

This approval is valid for one year from 31 May 2016. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at <http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx>.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be RATIFIED by a full Committee at its meeting taking place on 12 July 2016.

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni  
 Chair: Biomedical Research Ethics Committee

cc: Supervisor: [osh.archana@caprisa.org](mailto:osh.archana@caprisa.org)  
 Administrator: [dudhra@pmedic.ac.za](mailto:dudhra@pmedic.ac.za)

Biomedical Research Ethics Committee  
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28 March 2017

Ms K Naidoo (210507122)  
 Discipline of Medical Microbiology  
 School of Laboratory Medicine and Medical Sciences  
 Health Sciences  
[kimleighn@masfl.com](mailto:kimleighn@masfl.com)

Dear Ms Naidoo

Protocol: Functional assessment on non-neutralizing binding antibodies in the blood and genital tracts of women with breakthrough HIV infection from the CAPRISA 004 tenofovir 1% gel trial. Degree: MMedSc. BREC reference number: BE241/16 (sub-study of BE013/04).

#### RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 31 May 2017  
 Expiration of Ethical Approval: 30 May 2018

I wish to advise you that your application for Recertification received on 16 March 2017 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The approval will be ratified by a full Committee at a meeting to be held on 11 April 2017.

Yours sincerely

Mrs A Marimuthu  
 Senior Administrator: Biomedical Research Ethics

