Cytokine production by ME-180 cells and VK2 E6/E7 cells on exposure to *Neisseria gonorrhoeae*, HIV, *N. gonorrhoeae* and HIV

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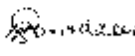
Signed:  Date: 25/04/2019

As the candidate's supervisor, I agree to the submission of this thesis.

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Prof A. W. Sturm

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This dissertation is dedicated to my family

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Abbreviations

µm	Micrometer
AIDS	Acquired Immune Deficiency Syndrome
ATP	Adenosine triphosphate
CDC	Centre for Disease Control
CO_2	Carbon Dioxide
DNA	Deoxyribonucleic Acid
EIA	Enzymes Immunoassays
g	Gram
GTP	Guanosine-5'-triphosphate
HIV	Human Immunodeficiency Virus
IL	Interleukin
IM	Intramuscular
LOS	Lactosyl lipooligosaccharide
M-CSF	Macrophage colony stimulating factor
MOI	Multiplicity of Infection
NAAT	Nucleic Acid Amplification Testing
NK	Natural Killer
Opa	Opacity associated
PID	Pelvic Inflammatory Disease
PMN	Polymorphonuclear leukocyte
PorB	Porin B

RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic Acid
STI	Sexually Transmitted Infection
TGF- β	Transforming growth factor- beta
TNF-α	Tumor necrosis factor-alpha
WHO	World Health Organisation
β	Beta

Abstract

Gonorrhoea is a sexually transmitted disease caused by *Neisseria gonorrhoeae*. Women are more at risk in developing secondary complications due to asymptomatic infections. In 2001, a study was done on the different responses of epithelial cells from three different regions of the lower female genital tract exposed to *N. gonorrhoeae*. Upregulation of cytokines found in cervical and vaginal secretions has been linked with human immunodeficiency virus 1 infection. *In vitro* studies of the immune response following exposure to multiple STI pathogens are relevant as mixed infections are common and not many studies have been done. The aim of this study was to determine the cytokine response in a co-infection model with *N. gonorrhoeae* and HIV using two genital epithelial cell lines.

ME-180 cervical cells and VK2 E6/E7 vaginal cells were infected with *N. gonorrhoeae* and HIV only and with both organisms in different sequence. Infected cells were incubated at 37 °C in 5 % CO_2 for 72 h. The supernatant was assayed for cytokines TNF-α, RANTES, IL-1β, IL-4, IL-6, IL-8, and IL-10 by means of the Bio-Plex Pro Cytokine, Chemokine, and Growth Factor Assay kit.

The spontaneous cytokine release was higher in VK2 E6/E7 cells than in the ME-180 cells. On exposure to single organisms the response to *N. gonorrhoeae* was stronger than to HIV in both cells for IL-10, IL-8 and IL-6. For infection with *N. gonorrhoeae* the VK2 E6/E7 cells had a stronger cytokine response than ME-180 but this was not so for HIV. The response the cells had to exposure to both organisms was independent of the sequence of exposure. Further studies should be done on mixed infections of *N. gonorrhoeae* and HIV with additional STI pathogens.

CHAPTER 1 - INTRODUCTION

A common route of HIV transmission is through sexual intercourse with women contributing to almost half of the HIV infected population (Simon *et al.*, 2006). One of the risk factors for HIV infection is thought to be the presence of other sexually transmitted infections (STIs), in women the greatest risk factors for HIV acquisition were new viral and bacterial STIs (Venkatesh *et al.*, 2011). *Neisseria gonorrhoeae* is a human pathogen causing the disease gonorrhoea. Gonorrhoea is a global health problem as it is the second most common bacterial STI globally (World Health Organization, 2016b).

Pathogenic microorganisms employ different survival strategies to avoid being killed by the host's immune response. Such strategies include preventing activation of host defence mechanisms or activating the host immune response but evading the consequences by different mechanisms. *N. gonorrhoeae* uses the latter strategy by protecting itself from the host's immune response during infection (Hedges *et al.*, 1998). During infection *N. gonorrhoeae* adheres to and invades epithelial cells of the genitalia. Type IV pili present on the organism facilitate adhesion (Jarvis *et al.*, 1999) and stimulate an inflammatory response that is characterized by an intense cellular infiltrate predominantly of neutrophils (Naumann *et al.*, 1997). During initial contact of a pathogen with mucosal surfaces cytokines produced play an important role in the antigen-specific immune response. This has a major impact on the outcome of an infection (Naumann *et al.*, 1997).

Despite cumulating evidence that infection with one or more STI pathogens other than HIV increases acquisition and transmission of the latter (Galvin and Cohen, 2004), *in vitro* studies on interaction between HIV and other pathogens are mainly done in immune cells. Studying co-infections in cells that make up the epithelial lining of the genitalia can potentially add to our knowledge of such infections. This should include cervical and vaginal epithelial cells. It has been shown that these cells produce cytokines associated with pro-inflammatory and anti-inflammatory responses (Fichorova *et al.*, 2001).

In the vagina infection is generally restricted to *Trichomonas vaginalis* and *Candida albicans* with Human Papilloma Virus (HPV) infecting the ectocervix (Quayle, 2002).

In the study presented here ME-180 and VK2 E6/E7 epithelial cells were used. The ME-180 cell line derived from the human cervix was isolated from an omental metastasis of a rapidly spreading cervical carcinoma (Sykes *et al.*, 1970b). A simple epithelium of columnar cells lines the endocervix, major pathogens causing infection at this site are *Neisseria gonorrhoeae* and *C. trachomatis* (Quayle, 2002).

Morphologically the cells have maintained many features of this differentiated stratified squamous epithelium of the endocervix (Tan *et al.*, 1993). This cell line is therefore a suitable model for the study of

cervical infections (Tan *et al.*, 1993). ME-180 cells produce the cytokines Interleukin (IL)-1β, IL-6, IL-8, IL-10, Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) and Tumor Necrosis Factor alpha (TNF-α) (Sabde, 2013).

The VK2 E6/E7 cell line is a human vaginal epithelial cell line that expresses characteristics of stratified squamous non-keratinizing epithelia, which forms a barrier to pathogens (Fichorova, 1997). This non-keratinized squamous epithelium has a thickness of 150-200 μm (Quayle, 2002). The VK 2E6/E7 cell line produces the cytokines IL-1, IL-6, Transforming Growth Factor β (TGF- β) and macrophage colony stimulating factor (M-CSF) (Fichorova *et al.*, 2001).

An infection with *N. gonorrhoeae* may modulate the response to HIV infection at the genital mucosa by activation of immune cells and increasing the number of HIV target cells at the site of infection (Jarvis and Chang, 2012). Also cytokine upregulation of IL-1, IL-6, TNF-α and the chemokine RANTES in cervical and vaginal secretions has been linked with HIV-1 infection (Hill, 1999).

The study presented here aimed to provide insight in the cytokine response during exposure of genital epithelium to *N. gonorrhoea* and HIV.

Objectives

1. To determine which cytokines are produced by ME-180 and VK2 E6/E7.

2. To compare and quantitate the commonly produced cytokines by these cell lines on single exposure to *Neisseria gonorrhoeae* and HIV.

3. To compare and quantitate the commonly produced cytokines by these cell lines on sequential exposure to *Neisseria gonorrhoeae* and HIV and vice versa.

CHAPTER 2 - LITERATURE REVIEW

2.1 History

The disease gonorrhoea has references in the biblical Book of Leviticus making it one of the oldest known human diseases (Edwards and Apicella, 2004; Unemo and Shafer, 2011).
In CE 130, Galen referred to the disease as Greek words *gonor* (seed) and *rhoia* (flow), assuming the disease was linked to the flow of semen (Tønjum and van Putten, 2017). By the thirteenth century, Maimonides identified that the urethral discharge of infected male patients was not semen, but secretions induced by a sexually transmitted disease (Tønjum and van Putten, 2017).

Although the German bacteriologist Albert Neisser observed the presence of gonococci in leukocytes from urethral exudates of infected patients (Tønjum and van Putten, 2017), it was only in 1882 that the organism was cultured by Leistikow and Löffler (Handsfield, 1990).
Before the discovery of antibiotics other treatment options were explored. These included the application of metals and plant extracts (Lancaster *et al.*, 2015). With the use of sulphonamides in 1936 and penicillin in 1943 the prevalence of the disease declined (Handsfield, 1990). In 1978 the United States reported an incidence of more than one million cases per annum (Handsfield, 1990). With the onset of the HIV epidemic in the late 1980s the use of barrier contraceptives lead to decreased gonococcal infections (Knapp and Rice, 1995). This effect was reversed when anti-retroviral drugs became available which lead to decreased condom usage (Phillips *et al.*, 2013).

Gonorrhoea is the second most prevalent global sexually transmitted infection, with an estimated 87 million new cases in 2016 (World Health Organization, 2018). Currently antimicrobial resistance is an international problem due to *Neisseria gonorrhoeae* having the capacity for genetic mutation and to acquire genetic elements from other species (Unemo and Shafer, 2011).

Human immunodeficiency virus (HIV) is the etiological agent of acquired immune deficiency syndrome (AIDS) (Janas and Wu, 2009). AIDS cases were first described in 1981, and in 1983 the causative virus was isolated (Unemo *et al.*, 2013). In 2015, there was an estimate of 36.7 million HIV infected people globally with 2.1 million new cases of infection (UNAIDS, 2016).

2.2 Classification and Morphology

The family *Neisseriaecea* includes the three medically important genera *Kingella*, *Eikenella* and *Neisseria* (Liu *et al*., 2015). Extensive revision of the family taxonomy has been done based on whole genome sequencing and gene sequence analysis of the 16S rRNA gene (Tønjum and van Putten, 2017). The genus *Neisseria* are Gram negative diplococci with adjacent flattened sides that measure 0.6 -1.9 µm. Their G + C content is between 48 and 56 mol % (Prescott *et al*., 2008). They are non-motile, aerobic, oxidase and catalase positive, whilst fimbriae (in *Neisseria* usually referred to as pili) and a capsule can be present (Prescott *et al*., 2008). Optimal growth temperature ranges between 35 – 37 °C (Tønjum and van Putten, 2017).
The genus includes two genetically closely related obligate human pathogens: *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Bonnah *et al.*, 2000). *N. gonorrhoeae* mainly causes infections of the urogenital tract, whereas *N. meningitidis* colonizes the upper region of the respiratory tract as commensal and only strains that are encapsulated cause systemic infection resulting in bacteraemia and meningitis (Ison and Robertson, 1998).

HIV is classified under the family Retroviridae, subfamily Lentiviruses (Unemo *et al.*, 2013). The virion is ~ 100 nm in diameter with a conical capsid. The p24 capsid protein is a main component of the virus. The capsid is covered by a lipid envelope containing 2 glycoproteins, gp120 and gp41 (Unemo *et al.*, 2013). Inside the capsid are 2 copies of genomic RNA (Unemo *et al.*, 2013). HIV has different subtypes worldwide, with some subtypes being more prevalent in certain regions of the world (Unemo *et al.*, 2013). The two major types of HIV are HIV-1 and HIV-2, with HIV-1 being most common dominating the epidemic. HIV-2 is mainly found in West Africa with occasional reported cases throughout the world (Unemo *et al.*, 2013).

2.3 Clinical appearance

The epithelial lining of the urethra in both men and women and of the uterine cervix in women are the primary sites of infection for *N. gonorrhoeae*. (Edwards and Apicella, 2004). Other possible sites of infection include the rectal mucosa, pharynx and conjunctiva (Edwards and Apicella, 2004). Although it is uncommon, disseminated disease can cause endocarditis, arthritis dermatitis syndrome and meningitis (Edwards and Apicella, 2004). Exposure to infected secretions during birth can cause ocular infections in neonates which can result in blindness (Tapsall, 2001).

A higher probability of transfer of the infection exists from an infected man to a woman than vice versa (Handsfield, 1990). Infections with *N. gonorrhoeae* are asymptomatic in approximately 50 % of women. Symptomatic women display non-specific symptoms that include an odourless mucopurulent discharge from the vagina, bleeding from the vagina especially after sexual intercourse as well as dyspareunia (McCormack *et al.*, 1977).
Pelvic inflammatory disease (PID) can develop in up to 20 % of infected women which leads to infertility in approximately 15 % of those. Thus early diagnosis and treatment is important in reducing the risk of reproductive complications (Lancaster *et al.*, 2015).

Reports on the prevalence of asymptomatic infections in men vary between 10 and 40 % (Handsfield *et al.*, 1974). Symptomatic men present with a mucopurulent penile discharge and dysuria (Lancaster *et al.*, 2015). The discharge is due to polymorphonuclear leukocyte (PMN) influx and shedding of epithelial urethral cells (Edwards and Apicella, 2004). Acute urethritis is characterised by an inflammatory response caused by the organism itself (Ramsey *et al.*, 1995). Symptoms in men usually occur 2 - 6 days after exposure to the organism. In men with delayed or inadequate treatment epididymitis can develop (Lancaster *et al.*, 2015).

Infection with HIV-1 in the female reproductive tract includes three different major events: entry of HIV via the mucosal epithelium, infection of the subepithelial mononuclear cells, and finally delivery of the virus to lymph nodes initiating systemic infection (Shen, Richter and Smith, 2014).

HIV can be transmitted by exchange of body fluids through sexual contact and contaminated blood or blood products (Unemo *et al.*, 2013). HIV infected pregnant women can transmit the virus to the infant during pregnancy, during delivery or through breast feeding (Unemo *et al.*, 2013).

The typical clinical course of HIV infection occurs in approximately 70 – 80 % of cases (Pantaleo and Fauci, 1996). Signs and symptoms of HIV infection depend on the stage of infection. Patients can be asymptomatic or experience an influenza-like illness which includes headache, fever, rash, or sore throat in the first few weeks after infection (World Health Organization, 2010).

As the infection with HIV progresses it weakens the immune system, and the development of other signs and symptoms occur, such as weight loss, swollen lymph nodes, fever, coughing and diarrhoea (World Health Organization, 2010). Without proper treatment patients could develop severe additional illnesses such as tuberculosis, severe bacterial infections, cryptococcal meningitis, and cancers like lymphomas and Kaposi sarcoma (World Health Organization, 2010).

2.4 *Neisseria gonorrhoeae* laboratory diagnosis

The detection of *N. gonorrhoeae* includes presumptive and confirmatory tests. Presumptive laboratory diagnosis is based on the isolation of an oxidase positive Gram negative diplococcus from urogenital sites that grow on one of the selective media (Ng and Martin, 2005). Confirmatory tests include biochemical, serological, colorimetric tests and nucleic acid methods (Ng and Martin, 2005).

Microscopy

Microscopy of Gram stained smears is used for the direct detection of *N. gonorrhoeae*, in specimens collected from the vagina, cervix, urethra and/or conjunctiva (Ng and Martin, 2005). Swabs containing the specimen must be rolled onto a slide gently to maintain cellular morphology which allows for differentiation between *N. gonorrhoeae* and other species with similar morphology (Ng and Martin, 2005). A significant proportion of the former will be seen intracellularly in the neutrophils.

Urethral specimens from symptomatic men contain intracellular Gram negative, diplococci in PMNs (Janda and Knapp, 2003). If extracellular Gram-negative diplococci are present the specimen will be further tested on culture and/or nucleic acid tests for confirmation (Janda and Knapp, 2003). Endocervical, vaginal and rectal smears are more challenging to interpret due to the presence of other Gram-negative organisms (Weyant, Moss and Weaver, 1996).

Microscopy on urethral specimens from symptomatic male patients have a sensitivity of 90 % and a specificity of 95 % whilst endocervical specimens have a 50 -70 % sensitivity and a reported specificity between 70 and 90 % (Kellogg *et al.*, 1976; Gaydos and Quinn, 1999).

Reliability of results depends on quality of the specimen and experience of the laboratory technician (Ng and Martin, 2005). Most diagnostic laboratories restrict Gram stain microscopy to urethral specimens from male patients since performing this test on other specimens leads to a high frequency of false positive and negative diagnoses which can have major socio-psychological implications (A.W. Sturm, personal communication).

Culture

Culture is the preferred method for isolation, identification and diagnosis of *N. gonorrhoeae* infections (Ng and Martin, 2005). Specimens from patients are inoculated onto a selective agar plates such as

Thayer-Martin, New York City or Martin-Lewis medium (Papp *et al.*, 2014). These media contain antimicrobial agents to inhibit growth of fungi and commensal bacteria (Ng and Martin, 2005). A non-selective medium is usually added for the isolation of gonococci susceptible to one of the antimicrobials used in the selective medium. This can be a chocolate agar or the same medium as the selective one but without antibiotics.

Inoculated plates are incubated at 35 °C – 37 °C with 3 % - 7 % CO_2 (Janda and Knapp, 2003). Cultures are viewed after 18 h - 24 h incubation. Those without suspected colonies are re-incubated overnight and viewed again. Further testing will be done on suspected colonies (Ng and Martin, 2005). This includes identification and susceptibility tests.

Table 1: Carbohydrate (sugar) utilization of different *Neisseria* species (Unemo and Ison, 2013)

	Biochemical activity				
Species	**Glucose**	**Maltose**	**Lactose**	**Sucrose**	**Fructose**
N. gonorrhoeae	+	-	-	-	-
N. meningitidis	+	+	-	-	-
N. lactamica	+	+	+	-	-
N. cinerea	- (+)	-	-	-	-
N. sicca	+	+	-	+	+
N. subflava	+	+	-	+/-	+/-

- (+), mostly positive but negative strains exist; +/-, not consistent for the species

Identification tests included in Table 1 show the different carbohydrate usage and enzyme activity between *Neisseria* species. Isolates should be sub-cultured after initial isolation before being used in diagnostic testing that requires a heavy inoculum (Ng and Martin, 2005). Subcultures should not be incubated beyond 48 hours as the organisms will not survive (Ng and Martin, 2005). Antimicrobial susceptibility testing should be performed to guide treatment in case of treatment failure, for surveillance purposes and preliminary outbreak characterisation (Ng and Martin, 2005).

Microbiological culture methods of *N. gonorrhoeae* are cheap and specific, with a reasonable sensitivity of 85 % – 95 % for both urethral and endocervical infection. (World Health Organization, 2016b). Limitations include prolonged turnaround times and difficulty in maintaining viable specimens during transportation to the laboratory (Lancaster *et al.*, 2015).

Non-culture methods

Non-culture methods to diagnose *N. gonorrhoeae* infections include nucleic acid amplification tests detecting RNA or DNA sequences and enzyme immunoassays (EIAs) that detect gonococcal proteins (Lancaster *et al.*, 2015). Viable organisms are not required for detection with these tests (Lancaster *et al.*, 2015).

Nucleic acid detection

Nucleic acid amplification methods (NAATs) are specific, highly sensitive and rapid (Ng and Martin, 2005). This form of detection permits diagnosis in women from vaginal swabs when endocervical specimen collection proves difficult (Smith *et al.*, 2001).

In addition DNA based NAATs can detect *N. gonorrhoeae* in specimens that have been subjected to long transportation periods or exposure to temperature conditions that result in non-viable organisms (Ng and Martin, 2005).

The CDC recommends the use of NAATs to detect and identify *N. gonorrhoeae* in both symptomatic and asymptomatic men and women. This includes rectal and pharyngeal infections. NAATs have a $\pm$ 35 % higher sensitivity in comparison to EIA tests (Papp *et al.*, 2014).

NAATs do not need bodily fluids collected with invasive techniques, provide results with short turnaround times, and other pathogens can simultaneously be detected (Lancaster *et al.*, 2015). NAATs can be done on urine specimens, however urine specimens from women are not ideal due to suboptimal sensitivity for detection of *N. gonorrhoeae* (Unemo and Ison, 2013).

2.5 HIV laboratory diagnosis

Serological diagnosis

The diagnosis of HIV-1 is based on detection of specific antibodies and antigens in blood specimens or other body fluids (Simon, Ho and Abdool Karim, 2006). Detectable HIV antibodies are elicited ~ 4 – 6 weeks after infection, but in some cases, formation of detectable antibody concentrations may take up to ~ 3 – 6 months (Unemo *et al.*, 2013). This means a negative test result for HIV antibodies cannot exclude infection at 4 – 6 weeks after HIV exposure (Unemo *et al.*, 2013). During the initial phase of virus replication, antibodies are absent. Therefore diagnosis may not be made accurately using only antibody tests (Unemo *et al.*, 2013). During that period tests that directly detect one or more components of the virus such as the p24 antigen or RNA (Unemo *et al.*, 2013) can be used.

Enzyme immunoassays (EIAs)

EIAs are used as a screening assay for HIV and other infectious diseases (Fearon, 2005). HIV specific antibodies are detected in the test making the test highly sensitive and specific (Fearon, 2005; Unemo *et al.*, 2013). Second-generation assays utilize more specific antigens (recombinant proteins or synthetic peptides) but they do not detect early Immunoglobulin M (IgM) antibody responses (Unemo *et al.*, 2013). Thus, a third-generation test with a sandwich format which includes enzyme conjugated antibodies to detect IgM responses was created, reducing the window period for detection (Unemo *et al.*, 2013).

A new fourth-generation reduce the detection window period even further by combining detection HIV antibodies and the p24 viral antigen making this combination assay sensitive in detecting acute HIV infection (Unemo *et al.*, 2013).

Due to the high sensitivity of the tests, false positive results can occur. Therefore the diagnosis is based on two different immunoassays and if there is a discrepancy in results a confirmatory test such as Western blot will be done (Fearon, 2005).

Rapid tests

Rapid tests allow for HIV diagnosis in non-laboratory settings. These tests have two different formats, immuno-concentration devices and lateral flow cassette/strips (Unemo *et al.*, 2013). Rapid tests are optimized to accelerate antigen-antibody interaction thus detecting HIV antibodies within a few minutes (1–15 min). In contrast, EIAs, which may take up to 2–4 hours. Rapid tests can provide results for remote

populations and pregnant women during ANC visits. HIV rapid tests can be done using plasma, serum or whole blood (Unemo *et al.*, 2013).

2.6 Pathogenesis and Immune response

Neisseria gonorrhoeae

N. gonorrhoeae colonizes and invades epithelial cells of the genital mucosa resulting in an inflammatory response (Nassif *et al.*, 1999). Infection of epithelial cells involves adhesins including pili, opacity-associated (Opa) proteins, lactosyl lipooligosaccharide (LOS) and porin proteins (Jarvis and Chang, 2012).

Type IV pili extends from the bacterial surface facilitating initial adhesion. Pili are filamentous structures made up of protein subunits (Nassif *et al.*, 1999). The PilC is a 110 kDa molecule that is responsible for the pilus mediated adhesion to the complement regulatory protein CD46 which is the pilus receptor for pathogenic *Neisseria* (Källström and Jonsson, 1998).

The opacity (Opa) outer membrane proteins mediate cellular interactions and invasion of host cells (Nassif *et al.*, 1999). Opa proteins interact with PMNs resulting in an opsonin-independent uptake via phagocytes (Fischer and Rest, 1988). Opa proteins consist of two groups, the first interacts with a heparan sulphate proteoglycan (Van Putten and Paul, 1995) followed by adhesion and internalization of *N. gonorrhoeae* by epithelial cells (Nassif *et al.*, 1999). The second group of Opa proteins interacts with eukaryotic cells receptor CD66 present on endothelial and epithelial cells and on PMNs (Gray-Owen *et al.*, 1997; Nassif *et al.*, 1999).

Porins PorA and PorB are produced by pathogenic *Neisseria* species. They function as pores in the plasma membrane for ion exchange and cause a transient change in membrane potential and cell signalling interference (Ulmer *et al.*, 1992). LOS is a major immunogenic and antigenic component that induces the production of cytokines IL-1β, IL-6, IL-8 and TNF-α in primary urethral epithelial cells (Harvey *et al*., 2002). Infection with pathogenic *Neisseria* induces an inflammatory response. The pili, Opa proteins, LOS and porin proteins induce cytokine production by activating toll-like receptor 2 (TLR2) and TLR4 (Jarvis and Chang, 2012).

The innate immune response to bacterial infection is the influx of PMNs which phagocytose and kill microorganisms (Borregaard, 2010). In keeping with this, during infection with *N. gonorrhoeae* the inflammatory response is characterized by the recruitment and influx of PMNs (Johnson and Criss, 2011).

PMNs follow chemotactic signals resulting in migration to the site of infection (Johnson and Criss, 2011). Resident immune and mucosal epithelial cells release chemokines for PMNs. These include IL-1, IL-6,

IL-8 and TNF-α (Borregaard, 2010). PMNs contain receptors that bind and engulf complement and antibody opsonized particles such as complement receptor 3 (CR3) and Fc receptor, killing the microorganism (Johnson and Criss, 2011). As a result the bactericidal activity of PMNs limits bacterial invasion into deeper layers (Fisette *et al.*, 2003; Maisey *et al.*, 2003). Although there is an influx of PMNs at the site of infection, viable gonococci can still be cultured from exudate taken from infected patients (Wiesner and Thompson, 1980). Thus, the PMN immune response to *N. gonorrhoeae* infection is not effective in clearing the organism at the site of infection (Johnson and Criss, 2011; Stevens and Criss, 2018) . The persistence of *N. gonorrhoeae* in the presence of PMNs enables long term colonization of the human host, creating an opportunity for both dissemination and transmission (Johnson and Criss, 2011; Stevens and Criss, 2018). In a limited subset of patients gonococci invade the sub-epithelial tissue and the bloodstream resulting in deep-seated infections like arthritis (Edwards and Apicella, 2004).

Investigations into the anti-gonococcal immune response have focused mainly on the humoral response (Hedges *et al.*, 1998, 1999). Repeated exposure to an organism's antigens should enhance the immune response by evoking memory within the immune system (Hedges *et al.*, 1999). During infection with *N. gonorrhoeae* anti-gonococcal antibodies were detected in secretions and serum from infected patients but their levels were very low (Hedges *et al.*, 1998). This suggests that the level of anti-gonococcal antibodies induced during infections may be inadequate in providing protection against re-infection and this may explain the lack of immunity (Hedges *et al.*, 1999).

Pili proteins I (PI)and II (PII), H.8 protein, immunoglobulin A1 (IgA1) protease and LOS antigens induce antibody production during infection (Imarai *et al.*, 2011). Pili proteins appear to be the main antigen in women, whilst men appear to have higher levels of antibodies directed against Opa and porin proteins (Brooks and Lammel, 1989).

Pathogenic organisms can evade the host's immune response using different mechanisms including antigenic variation of surface antigens and LOS (Meyer, Gibbs and Haas, 1990), production of IgA1 protease (Plaut *et al.*, 1975) and resistance to complement-mediated bacteriolysis (Rice *et al.*, 1986; Smith *et al.*, 1992).

The cumulating effect of incomplete clearance of *N. gonorrhoeae* by PMNs and production of ineffective antibodies results in a state of non-protective immunity. This is supported by clinical data which indicate that prior infections with the organism do not provide an improved immune response thus allowing for repeated infection with *N. gonorrhoeae* (Hedges *et al.*, 1999).

Human Immunodeficiency Virus

The initial step in HIV infection is when the glycoproteins of the virus bind to CD4 carrying cells, generating a host immune response (Unemo *et al.*, 2013). HIV contains a gp120 envelope protein that attaches to the CD4 glycoprotein surface receptor on CD4+ T cells, monocytes, macrophages and dendritic cells (Prescott, Harley and Klein, 2008). A co-receptor in addition to the CD4 receptor is required for virus entry. HIV macrophage tropic strains that predominate early in disease, infect cells of the macrophage lineage. To enter these cells, the virus requires the CCR5 chemokine receptor protein as well as CD4 (Prescott, Harley and Klein, 2008). A second chemokine coreceptor CXCR-4 (fusion) is generally used by T-cell tropic strains to enter these cells. Such strains are mainly found in later stages of infection (Prescott, Harley and Klein, 2008). Fusion of the viral envelope and host cell plasma membrane results in the virus entering and releasing its core containing two RNA strands into the cytoplasm (Prescott, Harley and Klein, 2008). The RNA is copied into a single strand of DNA by the RNA-dependent DNA polymerase activity of the reverse transcriptase enzyme (Prescott, Harley and Klein, 2008). The RNA is then degraded by ribonuclease H and the DNA strand is duplicated to form a double stranded DNA copy of the original RNA genome (Prescott, Harley and Klein, 2008). The proviral DNA and integrase enzymes move into the nucleus and integrate the DNA into the host cell's DNA. The integrated provirus can either be latent with no clinical signs or force the cell to synthesize viral mRNA (Prescott, Harley and Klein, 2008). RNA is translated to produce viral proteins, these viral proteins and the HIV-1 RNA genome are assembled into new virions that bud from the infected cell and eventually results in lysis of the host cell. Both budding and lysis contribute to spreading of the infection in the host's body (Prescott, Harley and Klein, 2008).

Cellular immunity plays an important role in fighting or eliminating an infectious agent (Levy, 1993). During the acute stage of infection the virus multiplies rapidly and disseminates to the lymphoid tissues throughout the body until an acquired immune response (antibodies and cytotoxic T cells) can be generated to bring virus replication under control (Prescott, Harley and Klein, 2008). The HIV envelope is the main target for the humoral antibody response (Levy, 1993). Other cellular immunity responses include cytotoxic Natural Killer (NK) cells and CD4+ cell responses (Levy, 1993). HIV infected cells are killed by NK cells by the recognition of antibodies bound to the viral envelope proteins on the cell surface (Levy, 1993).

Human CD4+ T cells can be divided into Th1 and Th2 subsets, Th1 cells secrete IL-2 and IFN-γ and Th2 cells produce IL-4, IL-6, and IL-10 (Levy, 1993). During the chronic stage of HIV infection, gonococcal infection has been linked with a transient increase in plasma viremia and plasma Th2 cytokines IL-4 and IL-10, and decreased CD4+ T cell counts (Anzala *et al.*, 2000).

Genital epithelial cells support transcytosis of extra-cellular HIV across the epithelium in primary human endocervical epithelial cells and genital mucosa derived cell lines (Stoddard *et al.*, 2007, 2010). These include Endo1/E6E7, Ect1/E6E7, VK2/ E6E7 and HEC1A cell lines that are derived from the endocervical, ectocervical, vaginal and endometrial tissue, respectively (Stoddard *et al.*, 2007, 2010).

Primary cervical and endometrial epithelial cells interact with HIV-1 via gp120, eliciting pro-inflammatory cytokine production such as TNF-α (Nazli *et al.*, 2010). The production of TNF-α leads to barrier function impairment of the epithelium, allowing HIV-1 entry across the epithelial lining (Nazli *et al.*, 2010). Also, the cervix derived epithelial cell line (ME-180) can be infected with HIV (Tan *et al.*, 1993).

2.7 Treatment

Antimicrobial resistance to first-line treatment with 3rd generation cephalosporins and azithromycin of *N. gonorrhoeae* is increasing globally (Lancaster *et al.*, 2015). The WHO recommends treatment with dual or single agents based on local resistance data. When data is not available dual therapy is recommended (World Health Organization, 2016b). Dual therapy includes: 250 mg intramuscular (IM) administered ceftriaxone plus 1g oral azithromycin or 400 mg oral cefixime plus 1 g oral azithromycin (World Health Organization, 2016b). Single therapy includes: 250 mg ceftriaxone IM, 400 mg oral cefixime or 2 g spectinomycin IM (World Health Organization, 2016b). When treatment fails different combination treatments are recommended as per the WHO guidelines (World Health Organization, 2016b). Local data on antimicrobial resistance are shown in Table 2 (Rambaran *et al.*, 2018).

HIV infection is treated with antiretroviral agents. The WHO recommends immediate anti-retroviral treatment (ART) for all people diagnosed with HIV without restrictions of CD4 counts (World Health Organization, 2016a). First-line treatment should include one non-nucleoside reverse transcriptase inhibitor (NNRTI) plus two nucleoside reverse transcriptase inhibitors (NRTIs) or an integrase inhibitor (INSTI) (World Health Organization, 2016a).

Table 2: MIC (mg/L) distribution (%) of *N. gonorrhoeae* isolates (n=319)

Antimicrobial agent	≤0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	≥32
Penicillin				2	10	13	10	5	11	17	32		
Cefixime	52	17	17	11	2	1							
Ceftriaxone	77	15	6.5	1	0.5								
Azithromycin					1	4	28	41	25	1			
Ciprofloxacin	16	9	4	1	1	3	4	15	8	38	1		
Ofloxacin	13	12	4	1	1	1	2	4	14	41	7		
Tetracycline												4	96

2.8 HIV Co-infection

As of 2015, an estimate of 36.7 million people were infected with HIV with 2.1 million new infections occurring worldwide (UNAIDS, 2016).
The main route of HIV transmission is sexual intercourse, yet transmission efficacy by this route is relatively low as seroconversion occurs in 1 in every 1000 episodes of sexual intercourse with an infected partner (Galvin and Cohen, 2004). Observational studies have indicated a strong link between the acquisition of HIV-1 and other STIs (Buvé *et al.*, 2001).

Replication rates differ between patients. Mechanisms that enhance replication rates are unclear but may be linked to increased numbers of activated CD4+ T lymphocytes due to concomitant infections. (Levine *et al.*, 1998).

A difference in HIV-1 plasma viremia levels in infected individuals could be due to various factors including the number of cells infected with HIV-1, level of cellular activation transcription of the HIV-1 genome and the cellular and humoral immune responses to HIV-1 (Anzala *et al.*, 2000). Cellular transcriptions factors are controlled by cellular activation via the action of inflammatory cytokines and mediators (Anzala *et al.*, 2000). This is induced by other infections, including gonorrhoea.

A longitudinal STI study conducted on female sex workers in Nairobi, Kenya presented data referred to as natural "challenge" as acute STIs could have resulted from re-activation of persistent infections or new infections (Anzala *et al.*, 2000). The main finding of the study identified that during dual HIV and gonococcal infection, HIV-1 RNA copy number in plasma as well as Th2 cytokines increase while the number of CD4+ T cells decrease (Anzala *et al.*, 2000). Women experiencing acute PID showed an increase in plasma cytokines and plasma viremia but no increase in CD4 T cell and CD8 T cell counts (Anzala *et al.*, 2000). A direct activation of HIV-1 replication via the action of bacterial products could also explain the increased plasma viremia during PID and gonococcal infection (Anzala *et al.*, 2000)

Thus, the data strongly suggests a linkage between gonococcal infection and plasma viremia. LOS has been reported to stimulate replication of HIV *in vitro*, this upregulation occurs via the activation of the NF-κβ pathway (Anzala *et al.*, 2000). Studies conducted on men did not result in change in the plasma viremia during gonococcal infection suggesting there are different systemic effects in men and women (Anzala *et al.*, 2000). This highlights the complexities of studying co-infections.

Immortalized human epithelial cells derived from the endocervical, ectocervical and vaginal tissue were infected with *Mycoplasma genitalium* (Mcgowin, Popov and Pyles, 2009). Post infection all three

epithelial cells expressed significant secretion of IL-6 and IL-8. It was suggested that IL-6 secretion and other cytokines could contribute to HIV pathogenesis (Mcgowin, Popov and Pyles, 2009).

2.9 Cytokine Assays

Enzyme-linked immunosorbance assay (ELISA)

ELISA's are considered the standard method for cytokine measurement, ELISA kits are commercially available and are used in biomedical research and clinical laboratories (Leng *et al.*, 2008). This methodology allows for the detection of secreted cytokines at protein level (Amsen, de Visser and Town, 2009). In a double antibody sandwich ELISA, the antibody is attached to the bottom of the well providing for both cytokine-antigen capture and immune specificity, while another antibody linked to an enzyme provides detection and amplification (Leng *et al.*, 2008). Advantages include sensitive and accurate detection of the desired cytokine, and highly quantitative and reproducible results (Leng *et al.*, 2008). Disadvantages of an ELISA is that the kit performance is dependent on antibody quality, operator experience and skills and that the measurement of only one cytokine at a time in a given sample can be done (Leng *et al.*, 2008).

Flow cytometry

Flow cytometry uses beads coated with a specific capture antibody which capture a cytokine. Fluorescent labelled detection antibodies bind to a specific cytokine-capture antibody complex on a bead (Leng *et al.*, 2008). The above allows for multiple cytokines to be measured in the same sample by using beads with different capture and detection antibodies with different fluorogenic substances resulting in chromogenic emissions measured by flow cytometric analysis (Leng *et al.*, 2008). Other advantages include smaller sample volume required and time efficiency (Leng *et al.*, 2008) Some of the disadvantages include the high cost (Amsen, de Visser and Town, 2009) and that multiplex data interpretation can be challenging (Leng *et al.*, 2008).

Bio-Plex Assay

This system employs the xMAP technology allowing a multiplexing of up to 100 analytes (Houser, 2012). This multiplex system looks at analytes simultaneously providing more information from a smaller sample volume in a time efficient manner (Houser, 2012). The principle is similar to an ELISA, where the

xMAP uses an antibody sandwich for the detection but is different in the capture substrate and detection method (Houser, 2012). The Bio-Plex uses bead sets as a substrate capturing analytes in solution and uses a fluorescent method of detection (Houser, 2012). The bead sets identify the analytes and detection antibodies are used to measure the quantity of the analyte (Houser, 2012). The use of differentially detectable beads enables the simultaneous identification and quantification of many analytes in the same sample (Houser, 2012).

CHAPTER 3 - METHODOLOGY

3.1 Pathogenic microorganisms

A stored isolate of *Neisseria gonorrhoeae* from a previous study conducted at the Department of Medical Microbiology, Nelson R Mandela School of Medicine, UKZN was used.
Serum containing HIV-1 at a concentration of 3 x 10^8 copies/ml was kindly donated by Dr Alex Segal of Africa Health Research Institute (AHRI), K-RITH Tower Building, Nelson R Mandela School of Medicine.

The study was approved by the Biomedical Research Ethics Committee (BREC), University of Kwa-Zulu-Natal (UKZN) – ethics number BE220/13.

3.2 Propagation, subculture and storage of *N. gonorrhoeae*

A vial with frozen suspension of *N. gonorrhoeae* was removed from the biofreezer. A sterile loop was twirled in the frozen suspension to pick up a bead containing the organism. This was immediately streaked onto pre-warmed non-selective New York City (NYC) agar plates. Streaked agar plates were incubated at 37 °C in 5 % CO_2 for 24 – 48 hours. Single colonies were sub-cultured onto fresh agar plates, colonies could either be used for experimental work or stored once grown.

For non-selective NYC medium preparation 36 g of GC agar base (Oxoid, England) was dissolved in 860 ml distilled water and autoclaved at 121 °C for 15 minutes. Thereafter 0.5 g of saponin (Sigma, USA) was dissolved in 10 ml distilled autoclaved water and filter sterilised. The saponin mixture was added to 100 ml of pre-dispensed horse blood in an autoclaved bottle and placed on the bench for 30 minutes. Yeast autolysate (Oxiod, England) supplements were reconstituted with distilled autoclaved water. The yeast autolysate supplement and blood were added to the media once it was cooled to 55 °C. Media was poured into 90 mm Petri dishes and stored at 4 °C.

For storage of the organism a loop full of growth was suspended into 1 ml of storage broth (Appendix 1) in a cryo-preservation vial containing sterile glass beads. The vial contents was vortexed to break up any clumps before storage at -70 °C until needed.

3.3 Cell Culture

Cell lines and culture media

The ME-180 cervical cell line (ATCC® HTB-33™) and VK2 E6/E7 (ATCC® CRL-2616™) vaginal cell line were used. Both cell lines had nutritional requirements that differed. The recommended medium for ME-180 cells was McCoy's 5a Modified Medium containing L-glutamine and 25 mM Hepes (BioWhittaker, USA) supplemented with 10 % Fetal Bovine Serum (FBS) (BioWest, France).
VK2 E6/E7 cells were grown in Keratinocyte Cell Basal Medium (KBM) -Gold media (Clonetics, USA) supplemented with 0.1 % $CaCl_2$. All media were warmed prior to usage. Cell growth required incubation at 37 °C in 5 % CO_2.

Propagation of cells

Cells stored at -70 °C were thawed in a 37 °C water bath, the outer surface of the vial was wiped with 70 % ethanol. The cells were seeded into a 75 cm^2 tissue culture flask containing the culture medium required for each cell type. Incubated flasks were monitored daily for cell growth, colour change of media and possible contamination. Culture medium was changed 2-3 times a week or earlier, spent media was discarded, cells were rinsed with phosphate buffered saline (PBS) (Oxoid, England) (Appendix 1) to remove all unattached cells and fresh culture medium added.

Once cells were 80 – 90 % confluent they were passaged. The passage procedure differed between the cells. Spent culture medium was discarded and the cells rinsed with PBS thrice. For ME-180 cells 1 ml of a trypsin solution containing 0.05 % trypsin and 0.02 % versine (EDTA) (BioWhittaker, USA) was added while for VK2 E6E7 2 ml was used. The flask was rotated to ensure even distribution and excess solution was discarded. After 1 - 2 minutes incubation for ME-180 cells and 3 - 4 minutes for VK2 E6/E7 cells, the flask was gently tapped to detach cells.

To stop the action of trypsin 1 ml of FBS was added to ME-180 cells and 6 ml of blocking solution (Appendix 1) was added to VK2 E6/E7 cells and distributed evenly over the cell layer. The detached cells were transferred into centrifuge tubes. To remove the blocking solution, the VK2/ E6E7 cells were centrifuged at 1300 rpm for 10 minutes, the supernatant discarded, and the pellet re-suspended in 1 ml KBM-Gold media. The cells were either transferred into a new flask with fresh media, seeded onto 24-well collagen coated plates or cryopreserved depending on the requirements.

Cryo-preservation

Cryopreservation fluid differed for each cell line. An equal volume of the appropriate cryopreservation fluid (Appendix 1) was added dropwise and gently swirled to ensure a homogenous suspension. Aliquots of 1 ml were dispensed into cryovials and stored at -70 °C for both cell types.

3.4 Infection of cell lines ME-180/ VK2 E6/E7

ME-180 or VK2 E6/E7 cells were seeded to reach a confluency of approximately 2 x 10^5 cells/ml into the wells of 24-well collagen coated plates and incubated for 24 hrs at 37°C in 5% CO_2. The monolayers were then rinsed with PBS to remove unattached cells.

N. gonorrhoeae grown on antibiotic free NYC media was suspended in enriched Brain Heart Infusion (BHI) Broth (Oxoid, England) (Appendix) to reach an OD of 1 at 450 nm (~ 4.69 x 10^8 CFU/ml). This was measured with the Bio-Rad iMark microplate reader (Bio-Rad, USA). The suspension was incubated at 37 °C, 5 % CO_2 for 90 minutes, thereafter the undisturbed upper layer was collected, vortexed and used for the experiments. Serial 10-fold dilutions of this *N. gonorrhoeae* suspension was plated out to confirm its density by colony counts. At the time of infection the HIV stock solution of 3 x 10^8 copies/ml was diluted with cell type specific media to obtain an MOI of 1 (~ 2 x 10^5 copies/ml).

The following experiments were done with each cell type: (i) *N. gonorrhoeae* alone, (ii) HIV alone, (iii) *N. gonorrhoeae* and HIV together, (iv) HIV first and *N. gonorrhoeae* 2 hours later, (v) *N. gonorrhoeae* first and HIV 2 hours later. Uninfected epithelial cells were used as controls. Infected cells were incubated for at 37 °C in 5 % CO_2 for 72 h. After this incubation period, culture medium supernatants were collected from each well and centrifuged at 1,000 g for 15 minutes at 4 °C. Since the VK2 E6/E7 cell line was cultured in serum free culture media a final concentration of 0.5 % BSA was added as a carrier protein to stabilize the protein analytes and prevent adsorption to labware, this is according to the Bio-plex kit instructions. Experiments were done three times in triplicate for each cell line.

3.5 Cytokine Assay

The instruction manual as per the Bio-Plex Pro Cytokine, Chemokine, and Growth Factor Assay was followed. A lyophilized standard was reconstituted with 125 µl of cell specific culture media, vortexed for 5 seconds and incubated for 30 minutes on ice. A fourfold dilution of the standard was done using the cell specific culture media as the diluent.

The Bio-Plex Human Cytokine 7 Plex (Bio-Rad, USA) coupled magnetic beads were used in the assay. Capture antibodies directed against the desired cytokines TNF-α, RANTES, IL-1β, IL-4, IL-6, IL-8, and IL-10 are covalently coupled to these beads. Into each well 50 µl of coupled magnetic beads was dispensed, and the plate was washed twice with 100 µl of Bio-Plex wash buffer using the Bio-Plex Pro Wash Station (Bio-Rad, USA). A volume of 50 µl of the diluted standard, uninoculated cultured media (blank) and culture medium from the experiments were added to wells. The plate was sealed with aluminium film and incubated on a shaker (Shaker-Incubator Stat Fax-2200, Bio-Rad, USA) at 850 rpm for 30 minutes, all incubation steps on the shaker were done at room temperature. After the incubation step the sealing film was removed and the beads were washed thrice with 100 µl wash buffer.

Into each well 25 µl of detection antibodies was added. Thereafter the plate was sealed and incubated on the shaker at 850 rpm for 10 minutes. This was followed by the addition of 50 µl of Streptavidin-PE (SA-PE) per well. The SA-PE contains the Phycoerythrin Fluorescent Reporter. The plate was sealed again and incubated on the shaker at 850 rpm for 10 minutes. Into each well 125 µl of assay buffer was added, the plate was covered with a sealing film and shaken at 850rpm for 30 seconds. The sealing film was removed, and the fluorescence intensity measured using the Bio-Plex 200 System (Bio Rad, USA). The concentration of the cytokine bound to each bead is proportional to the concentration intensity of the reporter signal.

3.6 Statistical Analysis

Statistical analysis was done using STATA. A p value of < 0.05 indicated significance.
Cytokine levels were summarized using median and Inter Quartile Range (IQR).
Two group comparisons were made using a Wilcoxon rank sum test whilst three group comparisons were tested first using a Kruskal Wallis test and if significant followed Dunn's pairwise comparisons with Sidak adjustment for multiple comparisons. The data was analyzed using STATA v13.1.

CHAPTER 4 - RESULTS

Cytokine production of uninfected cells

Table 3 compares the cytokine release of the ME-180 cells with that of VK2 E6/E7. The median cytokine production by the VK2 vaginal cells was significantly higher for all cytokines tested. This difference reached statistical significance for all cytokines produced. For both cells the highest levels of spontaneous production were seen with IL-8 and RANTES.

Table 3: Cytokine production (pg/ml) by uninfected ME-180 and VK2 E6/E7 cells

Cytokine	**Cell line**				
	ME-180		**VK2 E6/E7**		
	Median	**IQR**	**Median**	**IQR**	**p value**
IL-1β	0.01	0 - 0.18	0.07	0.07-0.07	0.04
IL-6	0.04	0.01- 0.05	0.2	0.2- 0.25	0.046
IL-8	6.07	5.91 - 8.51	62.34	61.27 - 115.59	0.0495
RANTES	1.58	1.56 - 1.68	21.74	13.83 - 26.65	0.0495
TNF-α	0.71	0.71 - 0.71	1.71	1.56 - 2.28	0.04
IL-4	0.02	0.02 - 0.02	0.05	0.05 - 0.05	0.03
IL-10	0.23	0.03 - 0.32	0.48	0.44 - 0.54	0.0495

ME-180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

The median production of the combined pro-inflammatory cytokines by ME-180 cells was 0.71 (IQR 0.02 - 1.68) and 1.71 (IQR 0.2 - 26.65) for VK2 E6/E7 cells (p=0.051). For anti-inflammatory cytokines these values were 0.03 (IQR 0.02 - 0.23) and 0.25 (IQR 0.05 - 0.48) respectively (p=0.051) (Appendix 2 Table 13).

In the following tables results are presented as measured cytokine production minus the amount of cytokine produced by uninfected cells (Table 3).

Cytokine production of ME-180 cells infected with Neisseria gonorrhoeae or HIV only

Table 4 compares cytokine release of ME-180 cells when exposed to *N. gonorrhoeae* and HIV separately for 72 hours. The median cytokine production was higher for IL-1β when infected with *N. gonorrhoeae* as compared to HIV infection but when infected with HIV higher cytokine medians were present for IL-6 and IL-10 production. However, none of these reached statistical significance.

Table 4: Cytokine production (pg/ml) by ME-180 cells exposed to *Neisseria gonorrhoeae* or HIV

Cytokine	***N. gonorrhoeae***		**HIV**		
	Median	**IQR**	**Median**	**IQR**	**p value**
IL-1β	0.04	0.03 - 0.07	0.02	0 - 0.03	0.13
IL-6	0	-0.02 to 0.03	0.05	0 - 0.08	0.2
IL-8	0	-0.24 to 1.28	-0.7	-1.5 to 0.09	0.3
RANTES	-0.25	-0.31 to -0.24	-0.2	-0.3 to -0.1	0.4
TNF-α	0	0 to 0	0	0 to 0	n/a
IL-4	0	0 to 0	0	0 to 0	n/a
IL-10	-0.12	-0.23 to 0.16	0.06	-0.2 to -0.07	0.8

ME-180 - Cervical cell line, IQR - Inter Quartile Range
Cytokine levels represent the difference between infected and uninfected cells (baseline).
Median = 0: cytokine production remained the same in uninfected and infected cells

A decrease of spontaneous cytokine release was observed for RANTES on exposure to both *N. gonorrhoeae* and HIV separately. Also, a decrease in IL-8 release occurred when exposed to HIV but not to *N. gonorrhoeae,* but none of these observations were statistically significant.

If, on exposure to the infectious agents, cytokine production remained the same as by uninfected cells the median value is reflected as 0.

The median production of the combined pro-inflammatory cytokines by ME-180 cells exposed to *N. gonorrhoeae* as well as to HIV was the same as that of uninfected cells (mean 0; IQR -0.2 - 0.03 and 0 (IQR -0.2. - 0.03 respectively) (p=0.7). Anti-inflammatory cytokine mean values were also 0 for both microbes (p=0.9) with IQR -0.1 to 0 for *N. gonorrhoeae* and IQR 0 - 0.06 for HIV (Appendix 2 - Table 14).

Cytokine production of VK2 E6/E7 cells infected with Neisseria gonorrhoeae or HIV only

Table 5 depicts cytokine release of VK2 E6/E7 cells when exposed to *N. gonorrhoeae* or HIV separately for 72 hours. Cytokine production was higher for IL-10 when exposed to *N. gonorrhoeae* as compared to HIV infection (p=0.0495).

Table 5: Cytokine production (pg/ml) by VK2 E6/E7 cells exposed to *Neisseria gonorrhoeae* or HIV

Cytokine	***N. gonorrhoeae***		**HIV**		
	Median	**IQR**	**Median**	**IQR**	**p value**
IL-1β	-0.04	-0.05 to 0.0	0	0 to 0	0.12
IL-6	0.23	-0.02 to 0.29	0.01	-0.01 to 0.19	0.5
IL-8	193.95	32.2 to 237.55	66.18	-8.8 to 188.6	0.3
RANTES	13.63	-4.3 to 18.16	-4.1	-9.1 to 21.8	0.8
TNF-α	2.88	0.43 to 4.04	0.28	-0.3 to 2.16	0.13
IL-4	0	0 to 0	0	0 to 0	n/a
IL-10	0.06	0.04 to 0.15	-0.01	-0.03 to 0	0.0495

VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range
Cytokine levels represent the difference between infected and uninfected cells (baseline).
Median = 0: cytokine production remained the same in uninfected and infected cells

The median cytokine production was higher for IL-6, IL-8 and RANTES with *N. gonorrhoeae* but without reaching statistical significance. Although not significant the highest levels of cytokine production were seen for IL-8 and IL-6 respectively when exposed to *N. gonorrhoeae*.
Decreased spontaneous cytokine release occurred for IL-1β on exposure to *N. gonorrhoeae* and for IL-10 and RANTES when exposed to HIV. None of these observations were statistically significant.
The median production of the combined pro-inflammatory cytokines by VK2 E6/E7 cells exposed to *N. gonorrhoeae* was 0.43 (IQR -0.02 - 18.2) and 0 (IQR -0.03 - 2.2) when infected with HIV (p=0.2). For anti-inflammatory cytokines these values were 0.02 (IQR 0 - 0.06) when exposed to *N. gonorrhoeae* and 0 (IQR -0.01 - 0) when exposed to HIV, reaching statistical significance (p=0.03) (Appendix 2 – Table 15).

Comparison of cytokine production by the two cell lines when infected with Neisseria gonorrhoeae only

Table 6 compares the cytokine production by ME-180 and VK2 E6/E7 cells when exposed to *N. gonorrhoeae* for 72 hours. Statistical significance was just reached for IL-1β and IL-8 cytokine production p=0.0459 whereas IL-1β levels were higher in ME-180 and IL-8 higher in VK2 E6/E7 cells when exposed to *N. gonorrhoeae.*

Table 6: Production of cytokines (pg/ml) by ME-180 and VK2 E/E7 exposed to *N. gonorrhoeae*

Cytokine	**ME-180** *N. gonorrhoeae*		**VK2 E6/E7** *N. gonorrhoeae*		
	Median	**IQR**	**Median**	**IQR**	**p value**
IL-1β	0.04	0.03 - 0.07	-0.04	-0.05 to 0.0	0.0495
IL-6	0	-0.02 to 0.03	0.23	-0.02 to 0.29	0.4
IL-8	0	-0.24 to 1.28	193.95	32.2 to 237.55	0.0495
RANTES	-0.25	-0.31 to -0.24	13.63	-4.3 to 18.16	0.5
TNF-α	0	0 to 0	2.88	0.43 to 4.04	0.04
IL-4	0	0 to 0	0	0 to 0	n/a
IL-10	-0.12	-0.23 to 0.16	0.06	0.04 to 0.15	0.5

ME-180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range
Cytokine levels represent the difference between infected and uninfected cells (baseline).
Median = 0: cytokine production remained the same in uninfected and infected cells

A statistical significance difference was also observed for TNF-α cytokine levels with higher cytokine production by the VK2 E6/E7 cells (p=0.04).

Decreased cytokine production was observed for IL-10 and RANTES by ME-180 cells as well as for IL-1β by VK2 E6/E7 cells. These observations were not statistically significant.

Statistical significance was observed for the combined pro-inflammatory cytokine release by ME-180 and VK2 E6/E7 cells exposed to *N. gonorrhoeae* (mean 0; IQR 0.2 - 0.03 and 0.43; IQR -0.02 – 18.2 respectively; p=0.02). No statistical significance was observed for the combined anti-inflammatory cytokine production (ME-180: mean 0; IQR -0.1 – 0); VK2 E6/E7: mean 0.02; IQR 0 - 0.06; p=0.2) (Appendix 2 – Table 16).

Comparison of cytokine production by the two cell lines when infected with Neisseria gonorrhoeae only

A comparison of cytokine production by ME-180 and VK2 E6/E7 cells when exposed to HIV for 72 hours is shown in Table 7. A higher expression of TNF-α and IL-8 was observed in VK2 E6/E7 cells compared to ME 180 cells, but this did not reach significance.

Table 7: Production of cytokines (pg/ml) by ME-180 and VK2 E/E7 exposed to HIV

Cytokine	**ME-180 HIV**		**VK2 E6/E7 HIV**		
	Median	**IQR**	**Median**	**IQR**	**p value**
IL-1β	0.02	0 to 0.03	0	0 to 0	0.12
IL-6	0.05	0 to 0.08	0.01	-0.01 to 0.2	0.8
IL-8	-0.7	-1.47 to 0.09	66.18	-8.76 to 188.65	0.51
RANTES	-0.2	-0.31 to -0.1	-4.1	-9.14 to 21.8	0.5
TNF-α	0	0 to 0	0.28	-0.29 to 2.16	0.49
IL-4	0	0 to 0	0	0 to 0	n/a
IL-10	0.06	-0.24 to 0.07	-0.01	-0.03 to 0	0.5

ME-180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range
Cytokine levels represent the difference between infected and uninfected cells (baseline).
Median = 0: cytokine production remained the same in uninfected and infected cells

When exposed to HIV the ME-180 cells showed higher cytokine expression for IL-1β, IL-6, and IL-10 but no statistical significance was reached.

Decreased cytokine expression was observed for RANTES for both cells, decreases also occurred for IL-8 in the ME-180 cells as well as IL-10 in the VK2 E6/E7 cells. None of these observations reached statistical significance.

The median production of both the combined pro-inflammatory and anti-inflammatory cytokines by both type of cells when exposed to HIV was 0. The pro-inflammatory response had an IQR -0.2 – 0.03 for ME-180 cells and IQR -0.3 – 2.2 for VK2 E6/E7 cells (p=0.5). The anti-inflammatory response had an IQR 0 - 0.03 for ME-180 and IQR -0.01 – 0 for VK2 E6/E7 cells p= 0.28 (Appendix 2 – Table 17).

Cytokine production of ME-180 cells infected combinations of Neisseria gonorrhoeae and HIV in different order of exposure

Table 8 compares cytokine release of ME-180 cells when infected with *N. gonorrhoeae* and HIV in different sequence. The first columns show the results when cells were first exposed to *N. gonorrhoeae* for 2 hours after which HIV was added for the remaining 72 hour incubation period. The second set of columns show the results when the sequence of exposure was reversed while the last columns show the effect of simultaneous exposure. No differences in production of any of the cytokines was found.

Table 8: Cytokine production (pg/ml) by ME-180 cells exposed to *N. gonorrhoeae* and HIV

Cytokine	***N. gonorrhoeae* followed by HIV**		**HIV followed by *N. gonorrhoeae***		***N. gonorrhoeae* and HIV together**		
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Overall p value**
IL-1β	0.03	(0 - 0.04)	0.05	(0.02 - 0.05)	0.02	(0.01 - 0.07)	0.7
IL-6	0.06	(-0.02 to 0.08)	0.02	(-0.03 to 0.04)	-0.02	(-0.02 - 0.12)	0.7
IL-8	0.5	(-3.9 to 2.8)	-1.14	(-4.3 - 6.2)	-1.67	(-2.7 to -0.07)	0.8
RANTES	-0.13	(-0.3 to 0.2)	-0.14	(-0.4 to -0.1)	-0.13	(-0.3 to -0.05)	0.4
TNF-α	0	(0 to 0)	0	(0 to 0)	0	(0 to 0)	0.9
IL-4	0	(0 to 0)	0	(0 to 0)	0	(0 to 0)	0.9
IL-10	0.06	(0.02 to 0.08)	0.18	(-0.2 to 0.3)	-0.18	(-0.2 to 0.3)	0.5

ME-180 - Cervical cell line, IQR - Inter Quartile Range
Cytokine levels represent the difference between infected and uninfected cells (baseline).
Median = 0: cytokine production remained the same in uninfected and infected cells

The combined pro-inflammatory cytokines produced by ME-180 cells when exposed to *N. gonorrhoeae* followed by HIV was the same as by uninfected cells (mean 0; IQR -0.02 - 0.08). The same was observed when HIV exposure was followed by *N. gonorrhoeae* (IQR -0.1 - 0.04) (p=0.3). When the cells were infected with both *N. gonorrhoeae* and HIV at the same time spontaneous cytokine production was slightly inhibited (mean -0.02; IQR -0.1 - 0.01) but this did not reach clinical significance (p=0.3). Mean anti-inflammatory cytokine values were 0.01 (IQR 0 - 0.06) when exposed to *N. gonorrhoeae* followed by HIV, 0 (IQR 0 - 0.2) when exposed to HIV followed by *N. gonorrhoeae* and 0 (IQR 0.2 - 0) when exposed to *N. gonorrhoeae* and HIV together (p=0.5) (Appendix 2 – Table 18).

Cytokine production of VK2 E6/E7 cells infected with combinations of Neisseria gonorrhoeae and HIV in different order of exposure

In Table 9 cytokine release is compared when VK2 E6/E7 cells were infected with *N. gonorrhoeae* and HIV in different sequence. Cells were exposed as follows: *N. gonorrhoeae* for 2 hours followed by HIV, HIV for 2 hours followed by *N. gonorrhoeae* and *N. gonorrhoeae* and HIV together. No differences in production of cytokines were found.

Table 9: Cytokine production (pg/ml) by VK2 E6/E7 cells exposed to *N. gonorrhoeae* and HIV

Cytokine	***N. gonorrhoeae* followed by HIV**		**HIV followed by *N. gonorrhoeae***		***N. gonorrhoeae* and HIV together**		
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Overall p value**
IL-1β	-0.02	(-0.07 to 0)	-0.03	(-0.04 to 0)	0	(0 to 0)	0.3
IL-6	0.32	(0.04 to 0.5)	0.26	(-0.1 to 0.3)	0.06	(-0.02 to 0.2)	0.4
IL-8	298.64	(60.6 to 345.9)	268.94	(-108.4 to 279.9)	50.4	(29.0 to 214.8)	0.3
RANTES	16.63	(4.2 to 54.8)	23.56	(-26.2 to 37.5)	7.34	(-5.5 to 7.5)	0.6
TNF-α	5.05	(0.9 to 8.7)	4.47	(-1.4 to 5.3)	1.59	(0 to 3.2)	0.5
IL-4	0	(0 to 0)	0	(0 to 0)	0	(0 to 0)	0.9
IL-10	0.06	(0 to 0.06)	0.03	(-0.03 to 0.04)	-0.06	(-0.1 to 0.07)	0.6

VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range
Cytokine levels represent the difference between infected and uninfected cells (baseline).
Median = 0: cytokine production remained the same in uninfected and infected cells

The combined pro-inflammatory cytokines produced by VK2 E6/E7 cells when exposed to *N. gonorrhoeae* first was 4.2 (IQR 0.04 – 54.8), HIV first 0.26 (IQR -0.1 – 23.6) and *N. gonorrhoeae* and HIV exposure at the same time 0.19 (IQR 0 – 7.5) with no overall statistical significance (p=0.3). The mean anti-inflammatory cytokine values were 0 for all three experiments (IQR 0 - 0.06 for *N. gonorrhoeae* first, IQR 0 - 0.03 for HIV first and (IQR -0.06 - 0 when exposed together; p=0.5) Appendix 2 – Table 19).

Cytokine production of cells infected with Neisseria gonorrhoeae followed by HIV

The comparison of cytokine production of ME-180 and VK2 E6/E7 cells when exposed to *N. gonorrhoeae* followed by HIV is shown in Table 10. A higher cytokine expression occurred for TNF-α in the VK2 E6/E7 cells as compared to the ME-180 cells (p=0.04). With the latter, cytokine production remained the same as by uninfected cells.

Table 10: Cytokine production (pg/ml) of ME-180 and VK2 E6/E7 cells exposed to *N. gonorrhoeae* followed by HIV

Cytokine	**ME-180**		**VK2 E6/E7**		
	Median	**IQR**	**Median**	**IQR**	**Overall p value**
IL-1β	0.03	(0 - 0.04)	-0.02	(-0.07 to 0)	0.08
IL-6	0.06	(-0.02 to 0.08)	0.32	(0.04 to 0.5)	0.3
IL-8	0.5	(-3.9 to 2.8)	298.64	(60.6 to 345.9)	0.0495
RANTES	-0.13	(-0.3 to 0.2)	16.63	(4.2 to 54.8)	0.0495
TNF-α	0	(0 to 0)	5.05	(0.9 to 8.7)	0.04
IL-4	0	(0 to 0)	0	(0 to 0)	n/a
IL-10	0.06	(0.02 to 0.08)	0.06	(0 to 0.06)	0.5

ME-180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range
Cytokine levels represent the difference between infected and uninfected cells (baseline).
Median = 0: cytokine production remained the same in uninfected and infected cells

The highest cytokine expression overall was observed for IL-8 in VK2 E6/E7 cells while the expression of RANTES was also higher in VK2 E6/E7 cells as compared to ME-180 cells (p = 0.0495). Although higher expressions of IL-6 were seen for VK2 E6/E7 cells, this did not reach statistical significance. Decreases in cytokine production occurred for IL-1β in VK2 E6/E7 cells and RANTES in the ME-180 cells. Cytokine expression of IL-10 remained the same for both cells when compared with uninfected cells.

Exposure of *N. gonorrhoeae* followed by HIV resulted in an overall increase in pro-inflammatory cytokine production by VK2 E6/E7 cells while the production by ME-180 cells remained at baseline. 0 (-0.02 – 0.08) and 4.2 (IQR 0.04 -54.8) for ME-180 and VK2 E6/E7 cells respectively this reached statistical significance (p =0.003). The anti-inflammatory cytokine response: did not differ between the cell types (p=0.6) (Appendix 2 – Table 20).

Cytokine production of cells infected with HIV followed by Neisseria gonorrhoeae

The cytokine production of ME-180 and VK2 E6/E7 cells when exposed to HIV followed by *N. gonorrhoeae* is seen in Table 11.

Table 11: Cytokine production (pg/ml) of ME-180 and VK2 E6/E7 cells exposed to HIV followed by *N. gonorrhoeae*

Cytokine	**ME-180**		**VK2 E6/E7**		
	Median	**IQR**	**Median**	**IQR**	**Overall p value**
IL-1β	0.05	(0.02 - 0.05)	-0.03	(-0.04 to 0)	0.0495
IL-6	0.02	(-0.03 to 0.04)	0.26	(-0.1 to 0.3)	0.5
IL-8	-1.14	(-4.3 to- 6.2)	268.94	(-108.4 to 279.9)	0.5
RANTES	-0.14	(-0.4 to -0.1)	23.56	(-26.2 to 37.5)	0.5
TNF-α	0	(0 to 0)	4.47	(-1.4 to 5.3)	0.5
IL-4	0	(0 to 0)	0	(0 to 0)	n/a
IL-10	0.18	(-0.2 to 0.3)	0.03	(-0.03 to 0.04)	0.5

ME-180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range
Cytokine levels represent the difference between infected and uninfected cells (baseline).
Median = 0: cytokine production remained the same in uninfected and infected cells

A higher cytokine median for IL-1β response occurred in ME-180 cells when compared to VK2 E6/E7 cells (p=0.0495). Also increases in cytokine expression were observed for IL-10 in ME-180 cells and IL-6, IL-8, TNF-α and RANTES in VK2 E6/E7, but none of these reached statistical significance.

The highest cytokine expression occurred for IL-8 in the VK2 E6/E7 cells. Exposure to microbes in this sequence lead to decreased cytokine expression for IL-8 and RANTES in the ME-180 cells and IL-1β in the VK2 E6/E7 cells (p=0.0495).

The pro-inflammatory cytokine response remained for this exposure sequence at baseline for ME-180 and was slightly elevated (0.26; IQR -0.1 – 23.6) for VK2 E6/E7 (p =0.3). The anti-inflammatory cytokine response remained the same as of unexposed cells for both ME-180 and VK2 E6/E7 (p =0.8) (Appendix 2 – Table 21).

Cytokine production of cells infected with Neisseria gonorrhoeae and HIV together

Table 12 summarizes the cytokine production of ME-180 and VK2 E6/E7 cells when exposed to *N. gonorrhoeae* and HIV together.

Table 12: Cytokine production (pg/ml) of ME-180 and VK2 E6/E7 cells exposed to *N. gonorrhoeae* and HIV together

Cytokine	**ME-180**		**VK2 E6/E7**		
	Median	**IQR**	**Median**	**IQR**	**Overall p value**
IL-1β	0.02	(0.01 to 0.07)	0	(0 to 0)	0.04
IL-6	-0.02	(-0.02 to 0.12)	0.06	(-0.02 to 0.2)	0.5
IL-8	-1.67	(-2.7 to -0.07)	50.4	(29.0 to 214.8)	0.0495
RANTES	-0.13	(-0.3 to -0.05)	7.34	(-5.5 to 7.5)	0.5
TNF-α	0	(0 to 0)	1.59	(0 to 3.2)	0.12
IL-4	0	(0 to 0)	0	(0 to 0)	n/a
IL-10	-0.18	(-0.2 to 0.3)	-0.06	(-0.1 to 0.07)	0.5

ME-180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range
Cytokine levels represent the difference between infected and uninfected cells (baseline).
Median = 0: cytokine production remained the same in uninfected and infected cells

IL-1β cytokine release was higher in ME-180 cells than in VK2 E6/E7 cells (p=0.04). A higher IL-8 cytokine expression was observed in VK2 E6/E7 cells as compared to ME-180 cell (p=0.0495).
The VK2 E6/E7 cells had a higher cytokine expression for IL-6 and TNF-α but this did not reach statistical significance.
Decrease cytokine expression mostly occurred for the cytokines IL-6, IL-8, and IL-10 in ME-180 cells and IL-10 in VK2 E6/E7 cells with no statistical significance.
The combined pro-inflammatory cytokine response of VK2 E6/E7 was with a mean of 0.19 (IQR 0 – 7.5) significantly higher than that of ME-180 cells (p =0.006). The anti-inflammatory cytokine response remained the same for both ME-180 and VK2 E6/E7 (p=0.8) (Appendix 2 – Table 22).

CHAPTER 5 - DISCUSSION & CONCLUSION

Pathogens that are sexually transmitted cause extensive morbidity and mortality (Fichorova and Anderson, 1999). *In vitro* studies of the immune response following exposure to multiple pathogens are relevant in studies of STIs as mixed infections are common and not many studies have been done. Furthermore, sexually transmitted pathogens can increase susceptibility to infection with HIV and HIV shedding (Anzala *et al.*, 2000). This emphasizes the importance of the study of mixed infections with sexually transmitted pathogens.

The aim of our study was to determine the change in production of cytokines by epithelial cells of the female genital tract when exposed to *N. gonorrhoeae* and HIV. This was done by determining which cytokines were produced by endocervical (ME-180) and vaginal (VK2 E6/E7) epithelial cells without exposure to microbes and comparing this qualitatively and quantitatively with the cytokines produced when exposed to *N. gonorrhoeae* and HIV in different sequence. To the best of our knowledge this study is novel with regard to the combination of organisms and sequence of exposure.

N. gonorrhoeae is one of the organisms that forms aggregates in fluid. A suspension of the organism with as few clumps as possible is desirable for use in tissue culture-based experiments. By allowing the microbial suspension to stand, the larger clumps settle at the bottom and the upper layer was used for infection experiments.

The ME-180 and VK2 E6/E7 epithelial cells were exposed to the organisms in three different sequences: (i) HIV followed by *N. gonorrhoeae,* (ii) *N. gonorrhoeae* followed by HIV and (iii) HIV and *N. gonorrhoeae* at the same time. Cells were also exposed to *N. gonorrhoeae* and HIV alone the results of which were used for comparison. For each experiment cytokine expression levels were measured for TNFα, RANTES, IL-1β, IL-4, IL-6, IL-8, IL-10. These cytokines were chosen based on the overlap of the commonly produced cytokines by the ME-180 and VK2 E6/E7 epithelial cells.

An infection with pathogenic organisms activates host transcription factors and production of immunomodulatory cytokines. Epithelial cells and other immune cells containing specific genes encoding inflammatory cytokines are controlled by the transcriptional factor Nuclear Factor Kappa Beta (NF-κβ) (Naumann *et al.*, 1997). Ramsey *et al.* (1995) reported that infection with *N. gonorrhoeae* induces the release of cytokines IL-1β, IL-6, IL-8 and TNF-α, which results in neutrophil influx to the site of infection. However, *N. gonorrhoeae* escapes kill by neutrophils (Johnson and Criss, 2011). Not only

neutrophils but also other immune cells like macrophages and lymphocytes are recruited while, due to inflammation, the amount of exudate also increases. The resulting increase in secretions may amplify and aid the spread of the *N. gonorrhoeae*. In mixed infections, concomitant pathogens will also be more effectively transmitted due to these increased secretions. In addition, it has been postulated that the presence of increased numbers of immune cells in the genitalia allows for more effective acquisition of HIV (Ostrowski *et al.*, 1998; Shen *et al*., 2014).

ME-180 and VK2 E6/E7 epithelial cells represent different compartments of the female genital tract. The vagina is composed by stratified squamous non-keratinizing epithelium forming a physical barrier to pathogens (Fichorova, 1997). The endocervical epithelium contains a single layer of columnar epithelial cells which plays a significant role in mucosal immunity (Fichorova, 1997). The main site of gonococcal infection in women is the endocervix (Stevens and Criss, 2018) but vaginal cells are also exposed during the acquisition of the infection as well as during the infection when cervical secretions containing the pathogen are shed into the vagina.

Spontaneous release of cytokines was tested in both types of epithelial cells. This spontaneous release by vaginal epithelial cells was higher for both the pro-inflammatory and anti-inflammatory cytokines. Our findings differ from those of Fichorova and Anderson, (1999) who reported that endocervical epithelial cells had a higher spontaneous cytokine release than vaginal epithelial cells. The two studies differed in the endocervical cell line used. The Fichorova study used a primary endocervical epithelial cell line that they immortalized by expression of E6 and E7 genes of the human papilloma virus type 16 (End/E6E7) (Fichorova, 1997). We used the ME-180 cell line which is derived from the epithelial human cervix which was established from an omental metastasis of a cervical carcinoma (Sykes *et al.*, 1970a)**.**

Further work by Fichorova, (2001) reported a marked increase of IL-6, and IL-8 in cervical and vaginal epithelial cells when exposed *to N. gonorrhoeae* (Fichorova *et al.*, 2001). Similarly, our experiments with exposure of *N. gonorrhoeae* only for both epithelial cell lines resulted in a strong cytokine response for IL-1β, IL-8, and TNF-α.

When both cell types were exposed to HIV, cervical cells showed a higher cytokine expression than vaginal cells for IL-1β, IL-6, and IL-10 but with no statistical significance.
In comparison, on single exposure a stronger cytokine response to *N. gonorrhoeae* than HIV is evident. In addition, the pro-inflammatory response was also higher for *N. gonorrhoeae* exposure when compared to

HIV. Since these cells are not the primary target for HIV, a lower response to this virus could be due to relatively low levels of HIV adhesion to the epithelial cell surface.

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The ability of *N. gonorrhoeae* to elicit expression of IL-6 and IL-8 by epithelial cells of the lower region of the female genital tract may contribute directly to the inflammatory infiltrate characteristic for this organism (King, James and Swanson, 1978). IL-8 attracts and activates polymorpho-nuclear leukocytes (Eckmann, Kagnoff and Fierer, 1993) and the function of IL-6 is neutrophil priming to chemotactic factors (Linder *et al.*, 1990). The anti-inflammatory cytokine IL-10 inhibits the activity of macrophages, Th1 lymphocytes and NK cells during infection. These cells are needed for optimal pathogen clearance but they can also contribute to tissue damage (Couper, Blount and Riley, 2008).

A study by Hedges, (1998) found that women with gonococcal cervicitis did not demonstrate elevated cytokines levels of IL-1, IL-6 and IL-8 in serum and cervical mucus specimens. This is in contrast with the work of Fichorova, (2001) and ourselves.

Experimental gonococcal inoculation of the urethra in male volunteers conducted by Ramsey, (1995) an increase of IL-8, IL-6 and TNF-α was found in urine specimens. This differs from the findings by Hedges, (1998) but is similar with our findings. Harvey *et al* (2002) found that the LOS component of *N. gonorrhoeae* elicits secretion of TNF-α, IL-1β, IL-6, and IL-8 from primary urethral epithelial cells. We found similar activity by epithelial cells from the female genital tract.

When both epithelial cell types were exposed to *N. gonorrhoeae* followed by HIV, increase of IL-8, RANTES and TNF-α reached statistical significance, whilst when cells were exposed to HIV followed by *N. gonorrhoeae* there was a significant IL-1β response. When cells were exposed to both *N. gonorrhoeae* and HIV at the same time only the responses of IL-1β and IL-8 were significant,while no response was seen with RANTES and TNF-α. Overall comparison of the three experiments (Table 8-9) with the combined co-infections no differences were found for both cell types. Perhaps more isolates of *N. gonorrhoeae* need to be included as the organism has the ability to change its antigenic make-up and this impacts on interaction with human cells. Also testing of a variety of genotypes of the organism (Moodley, 2001) could result in differences in observation between these genotypes.

The responses in all three experiments with both organisms in combination follow the trend of exposure to *N. gonorrhoeae* only.

Pro-inflammatory cytokines such as IL-6 and IL-8 are controlled by the NF-κβ pathway and this pathway is induced *in vivo* as well as *in vitro* in many cell types by IL-1 or TNF-α, produced by activated immune

cells, or by direct contact with pathogenic bacteria or bacterial products (endotoxin or LOS) (Holtmann *et al.*, 1999). However, whether those responses will be the same and of the same magnitude *in vivo* as compared to *in vitro* is difficult to establish if possible at all. A tissue culture model that includes multiple cell types in layers mimicking complete tissues (Ryndak, Chandra and Laal, 2017) may result in *in vitro* responses closer to those *in vivo*.

Cytokine production of IL-6 and IL-8 by epithelial cells could amplify the immune response by recruiting PMNs to the site of infection which release soluble mediators. Large amounts of soluble mediators can result in cell death destroying the protective cell layer allowing for further invasion (Fisette *et al.*, 2003).

A limitation of the study is that cell viability was only assessed at the start of the experiment and not during the course of the experiment or at the end. Cell death during the experiments could have influenced the results. However, microscopic viewing of the cells did not indicate significant loss of viability.

In conclusion, from the results of this study it is clear that the vaginal cells are more responsive than cervical cells. We also found that the response to *N. gonorrhoeae* exposure is stronger than to HIV in both cell types and that on dual exposure the response does not differ from single exposure to *N. gonorrhoeae*. Further studies should be done on mixed infections of *N. gonorrhoeae* and HIV with additional pathogens.

CHAPTER 6 - REFERENCES

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APPENDIX 1 – REAGENTS AND MEDIA

Phosphate Buffered Saline

1 PBS tablets

100 ml distilled water

One PBS tablet was added to every 100 ml distilled water and mixed until dissolved. This was autoclaved for 10 minutes at 121 °C. Once cooled 20 ml aliquots were refrigerated.

ME-180 cell cryo-preservation fluid

(80 % McCoy's 5a, containing 10 % FBS and 10 % DMSO)

16 ml McCoy's 5a medium

2 ml FBS

2 ml DMSO

Sixteen millilitres of McCoy's 5a medium was added to 2 ml FBS and 2 ml DMSO. The solution was filter sterilised into a sterile container.

VK2/ E6E7 cell cryo-preservation fluid

(85 % DMEM-F12, containing 10 % FBS and 5 % DMSO)

17 ml DMEM-F12

2 ml FBS

1 ml DMSO

Seventeen millilitres of DMEM-F12 was added to 2 ml FBS and 1 ml DMSO. The solution was filter sterilised into a sterile container.

Blocking solution for VK2/ E6E7 cells

5.7 ml DMEM-F12 media

300 ul FBS

Three hundred microliters was added to 5.7 ml DMEM-F12 media and dispensed into a sterile tube for single use.

GC Agar

36 g GC agar base

Distilled water

2 x yeast autolysate

0.5 g saponin

100 ml of horse blood

Thirty six grams of GC agar base was weighed and dissolved in 860 ml of distilled water and autoclaved for 15 minutes at 121 °C. 0.5 g of saponin was dissolved in 10 ml of distilled autoclaved water and filtered sterilised. The saponin mixture was added to 100 ml of pre dispensed horse blood in an autoclaved bottle and placed on the benchtop for 30 minutes. 2 yeast autolysate supplements was suspended with 15 ml of distilled autoclaved water per vial. The yeast autolysate supplements and blood were added to the media once it was cooled to 55 °C.

Storage broth for *N. gonorrhoeae* isolates

3.7 g BHI

80 ml distilled water

20 ml glycerol

To 80 ml od distilled water 3.7 g of BHI broth was added and mixed, this was autoclave for 15 minutes at 121 °C. 20 ml of glycerol was added to the broth by filter sterilisation.

Enriched BHI for *N. gonorrhoeae* suspension

3.7g BHI

100 ml distilled water

4 % Yeast Extract

5% Haemin

10 % Vitamin K

Into 100 ml distilled water 3.7 g BHI and 4 % Yeast Extract was added and autoclaved for 15 minutes at 121 °C. Once cooled 5% Haemin and 10 % Vitamin K were filter sterilised and added to the broth.

APPENDIX 2 – PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINE DATA

Table 13: Inflammatory cytokine production (pg/ml) by uninfected ME180 and VK2 E6/E7

	Cell line						
	ME180			VK2			
	n	median	IQR	n	median	IQR	p value
Pro-inflammatory cytokines	15	0.71	0.02 - 1.68	15	1.71	0.2 - 26.65	0.051
Anti-inflammatory cytokines	6	0.03	0.02 - 0.23	6	0.25	0.05 - 0.48	0.051

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

Table 14: Inflammatory cytokine production (pg/ml) by ME180 cells exposed to *Neisseria gonorrhoeae* or HIV

	ME180						
	N. gonorrhoeae			HIV			
	n	median	IQR	n	median	IQR	p value
Pro-inflammatory cytokines	15	0	(-0.2 to 0.03)	15	0	(-0.2 to 0.03)	0.7
Anti-inflammatory cytokines	6	0	(-0.1 to 0)	6	0	(0 to 0.06)	0.9

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

Table 15: Inflammatory cytokine production (pg/ml) by VK2 E6/E7 cells exposed to *Neisseria gonorrhoeae* or HIV

	VK2 E6/E7						
	N. gonorrhoeae			HIV			
	n	median	IQR	n	median	IQR	p value
Pro-inflammatory cytokines	15	0.43	(-0.02 to 18.2)	15	0	(-0.03 to 2.2)	0.2
Anti-inflammatory cytokines	6	0.02	(0 to 0.06)	6	0	(-0.01 to 0)	0.03

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

Table 16: Inflammatory cytokine production (pg/ml) by ME180 and VK2 E6/E7 cells exposed to *Neisseria gonorrhoeae*

	N. gonorrhoeae						
	ME180			VK2 E6/E7			
	n	median	IQR	n	median	IQR	p value
Pro-inflammatory cytokines	15	0	(0.2 to 0.03)	15	0.43	(-0.02 to 18.2)	0.02
Anti-inflammatory cytokines	6	0	(-0.1 to 0)	6	0.02	(0 to 0.06)	0.2

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

Table 17: Inflammatory cytokine production (pg/ml) by ME180 and VK2 E6/E7 cells exposed to HIV

	HIV						
	ME180			VK2 E6/E7			
	n	median	IQR	n	median	IQR	p value
Pro-inflammatory cytokines	15	0	(-0.2 to 0.03)	15	0	(-0.3 to 2.2)	0.5
Anti-inflammatory cytokines	6	0	(0 to 0.06)	6	0	(-0.01 to 0)	0.28

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

Table 18: Inflammatory cytokine production (pg/ml) by ME180 cells exposed to *Neisseria gonorrhoeae* and HIV

	ME180									
	N. gonorrhoeae followed by HIV			HIV followed by *N. gonorrhoea*			*N. gonorrhoeae* and HIV together			
	n	median	IQR	n	median	IQR	n	median	IQR	p value
Pro-inflammatory cytokines	15	0	(-0.02 to 0.08)	15	0	(-0.1 to 0.04)	15	-0.02	(-0.1 to 0.01)	0.3
Anti-inflammatory cytokines	6	0.01	(0 to 0.06)	6	0	(0 to 0.2)	6	0	(0.2 to 0)	0.5

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

Table 19: Inflammatory cytokine production (pg/ml) by VK2 E6/E7 cells exposed to *Neisseria gonorrhoeae* and HIV

	VK2 E6/E7									
	N. gonorrhoeae followed by HIV			HIV followed by *N. gonorrhoeae*			*N. gonorrhoeae* and HIV together			
	n	median	IQR	n	median	IQR	n	median	IQR	Overall p value
Pro-inflammatory cytokines	15	4.2	(0.04 to 54.8)	15	0.26	(-0.1 to 23.6)	15	0.19	(0 to 7.5)	0.3
Anti-inflammatory cytokines	6	0	(0 to 0.06)	6	0	(0 to 0.03)	6	0	(-0.06 to 0)	0.5

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

Table 20: Inflammatory cytokine production (pg/ml) by ME180 and VK2 E6/E7 cells exposed to *Neisseria gonorrhoeae* followed by HIV

	ME180			VK2 E6/E7			
	n	median	IQR	n	median	IQR	p value
Pro-inflammatory cytokines	15	0	(-0.02 to 0.08)	15	4.2	(0.04 to 54.8)	0.003
Anti-inflammatory cytokines	6	0.01	(0 to 0.06)	6	0	(0 to 0.06)	0.6

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

Table 21: Inflammatory cytokine production (pg/ml) by ME180 and VK2 E6/E7 cells exposed to HIV followed by *Neisseria gonorrhoeae*

	ME180			VK2 E6/E7			
	n	median	IQR	n	median	IQR	p value
Pro-inflammatory cytokines	15	0	(-0.1 to 0.04)	15	0.26	(-0.1 to 23.6)	0.3
Anti-inflammatory cytokines	6	0	(0 to 0.2)	6	0	(0 to 0.03)	0.8

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

Table 22: Inflammatory cytokine production (pg/ml) by ME180 and VK2 E6/E7 cells exposed to *Neisseria gonorrhoeae* and HIV together

	ME180			VK2 E6/E7			
	n	median	IQR	n	median	IQR	p value
Pro-inflammatory cytokines	15	-0.02	(-0.1 to 0.01)	15	0.19	(0 to 7.5)	0.006
Anti-inflammatory cytokines	6	0	(-0.2 to 0)	6	0	(-0.1 to 0)	0.8

ME180 - Cervical cell line; VK2 E6/E7 - Vaginal cell line; IQR - Inter Quartile Range

APPENDIX 3 – BREC APPROVAL

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04 September 2018

Prof P Moodley
719 Umbilo Road
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Dear Prof Moodley

PROTOCOL: Surveillance for Sexually Transmitted Infections in KZN. REF: BE220/13.

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 16 September 2018
Expiration of Ethical Approval: 15 September 2019

I wish to advise you that your application for Recertification received on 28 August 2018 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 committee will be notified of the above approval at its next meeting to be held on 09 October 2018.

Yours sincerely

pp
Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc: Prof AW Sturm