UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

In vitro modelling of the impact of anti-inflammatory drugs on cellular cytotoxicity, activation and inflammation

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Preface

Inflammation has now been recognised as an important risk factor for HIV acquisition and can undermine many prevention and/or treatment strategies. The purpose of this research was to understand how TLR-mediated inflammation influenced HIV infection of CD4+ T cells, and how select anti-inflammatory drugs mitigate this TLR-mediated inflammation and HIV infection of CD4+ T cells. This work is important to understand the potential of immunomodulatory drugs to impact on inflammation and HIV infection for potential incorporation into HIV prevention and treatment strategies.

Declaration

I, Mr Ross Thomas Cromarty, declare that:

1. The work presented in this thesis has not been submitted to UKZN or other tertiary institute for purposes of obtaining an academic qualification, whether by myself or any other party.
2. I also declare that this work is my own work and that where I have consulted others work, I have provided the relevant source. I contributed to the project in conceptualisation of experiments and ran all experimental procedures, data acquisition and statistical analysis. I further declare that I wrote and complied the manuscript.
3. I declare that the contribution of the following were:

   Dr Derseree Archary: Helped with conceptualisation of experiments, generation of HIV plasmid, statistical analysis and writing and editing of manuscript.

   Dr Lenine Liebenberg: Helped with writing and editing of manuscript.

   Dr Alex Sigal: Helped with conceptualisation of experiments.

   Mr Mesuli Mhlongo: Performed the hierarchical cluster analysis for generation of heat maps.

Signed:  Date: 27th August 2020

Dedication

I would like to dedicate this thesis to my two Grandparents, Mrs Mildred Cromarty and the late Mr Ronald Cromarty. Both have/were always there for me in every capacity they could, whether it be emotionally, financially or intellectually. They provided the funds for me to obtain my undergraduate and honours degrees.

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Acronyms

AHRI- African Health Research Institute

AIDS- Acquired Immunodeficiency Syndrome

ART- Antiretroviral Therapy

ARV- Antiretroviral

BCR- B-cell Receptor

BD- Beckman, Dickinson and company

BMS- Betamethasone

bNAbs- broadly neutralizing antibodies

BV- Bacterial Vaginosis

CAPRISA- Centre for the AIDS Programme of Research in South Africa

CCR- C-C Chemokine Receptor

CD- Cluster of Differentiation

COX- Cyclooxygenase

DC- Dendritic Cell

DC-SIGN- Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

DNA- Deoxyribonucleic Acid

FACTS- Follow-on African Consortium for Tenofovir Studies

FCS- Fetal Calf Serum

FGT- Female Genital Tract

FOXP3- Forkhead box P3

GC- Glucocorticoid

GM-CSF- Granulocyte-Macrophage Colony-Stimulating Factor

gp- Glycoprotein

GR- Glucocorticoid Receptor

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HI FCS- Heat Inactivated Fetal Calf Serum

HIV- Human Immunodeficiency Virus

HLA-DR- Human Leukocyte Antigen-antigen D Related

HNP- Human Neutrophil Peptide

HPTN- HIV Prevention Trials Network

HPV- Human Papillomavirus

HSV- Herpes Simplex Virus

IBF- Ibuprofen

IFN- Interferon

Ig- Immunoglobulin

IL- Interleukin

IP-10- IFN-γ-induced protein 10

JNK- c-Jun N-terminal protein Kinase

KRITH- Kwa-Zulu Natal Research Institute for Tuberculosis and HIV

LPS- Lipopolysaccharide

MAPK- Mitogen-Activated Protein Kinase

MHC- Major Histocompatibility Complex

MIP- Macrophage Inflammatory Protein

ml- Millilitre

MSM- Men who have Sex with Men

MTCT- Mother-To-Child Transmission

NaPy- Sodium Pyruvate

NEAA- Non-Essential Amino Acids

NF-κβ- Nuclear Factor- kappa beta

NK- Natural Killer

NSAID- Non-Steroidal Anti-Inflammatory Drug

P/DAMPS- Pathogen/Damage Associated Molecular Patterns

Pam3CSK4- synthetic triacylated lipopeptide

PBMCs- Peripheral Blood Mononuclear Cells

PBS- Phosphate Buffered Solution

PEP- Post-Exposure Prophylaxis

pH- Potential of Hydrogen

PLWH- people living with HIV

PrEP- Pre-Exposure Prophylaxis

PRR- Pathogen Recognition Receptor

R848- Resiquimod

RANTES- Regulated on Activation, Normal T cell Expressed and Secreted

Rpm- Revolutions per Minute

RNA- Ribonucleic Acid

SIV- Simian Immunodeficiency Virus

SLPI- Secretory Leucocyte Protease Inhibitor

SSA- Sub-Saharan Africa

STI- Sexually Transmitted Disease

TCR- T-cell Receptor

TGF- Transforming Growth Factor

Th- T helper cells

TLR- Toll-Like Receptor

TNF- Tumor Necrosis Factor

Tregs- Regulatory T cells

VOICE- Vaginal and Oral Interventions to Control the Epidemic

WT- Wild type

Abstract

The relationship between inflammation and HIV has been a major focus of HIV research. In people living with HIV (PLWH), HIV-associated immune activation drives HIV disease progression. While genital inflammation has been significantly associated with increased risk for HIV acquisition and transmission, immune correlates for reduced HIV risk remain less well defined. In HIV-exposed seronegative individuals, the immune quiescent phenotype, characterised by regulated immune activation and inflammation, has been implicated in reducing HIV acquisition risk. Targeted management of inflammation, therefore, is a plausible strategy to mitigate the risk of HIV infection, and to slow HIV disease progression. Therefore, we sought to investigate how anti-inflammatory drugs affect TLR-mediated inflammation and impact HIV infection of CD4+ T cells. This study utilized an *in vitro* peripheral blood mononuclear cell (PBMC) model. PBMCs were either treated with the anti-inflammatory drugs ibuprofen (IBF) or betamethasone (BMS) or were left untreated. Thereafter they were either left unstimulated or were stimulated with phytohaemagglutinin (PHA) or Toll-like receptor (TLR) agonists Pam3CSK4 (TLR1/2), LPS (TLR4) or R848 (TLR7/8) before exposure to HIV NL4-3 AD8. To assess inflammation, multiplexed ELISA was used to measure 28 proinflammatory, chemotactic, growth-related, adaptive response-related or regulatory cytokines. Flow cytometry was used to measure activation (CD38, HLA-DR and CCR5) and HIV infection (p24 production) of CD4+ T cells. Despite minimal immune activation, TLR stimulation elicited significant cytokine responses ($p<0.05$). TLR4 stimulation significantly reduced HIV infection of CD4+ T cells ($p<0.01$). With the addition of IBF, minimal immunosuppressive effects were observed. In contrast, BMS significantly dampened inflammation ($p<0.05$) and immune activation ($p<0.05$) regardless of the stimulation condition. Regardless of global immunosuppression, only with TLR4 stimulation did BMS significantly reduce HIV infection of CD4+ T cells ($p=0.02$). The finding that TLR4 stimulation reduces rather than increases susceptibility of CD4+ T cells to HIV infection, while BMS only affected HIV infection in the TLR4 condition, strongly suggests that additional factors, and not only inflammation play a powerful, although complex, role in determining HIV infection risk. Together, these data emphasize the importance of understanding signalling pathways induced during inflammation to identify novel targets to mitigate HIV infection.

1 Chapter 1: Introduction

In 1983, the Human Immunodeficiency Virus (HIV), the pathogenic causative agent for the acquired immunodeficiency syndrome (AIDS) was discovered by Françoise Barré-Sinoussi and Luc Montagnier (1, 2). Two major epidemiological trends have emerged in the global distribution of the HIV epidemic, referred to as a 'concentrated' and a 'generalized' epidemic (Reviewed in 3). A 'concentrated' epidemic refers to infections in specific population groups, generally at high risk because of behavioural factors. The 'generalized' trend refers to a self-sustaining epidemic that is not limited to specific groups, however this does not mean the risk of infection is equal across the whole population as there are still high-risk groups. These patterns occur as a result of a combination of biological, behavioural and socio-economic factors. To date, HIV is a global public health challenge and remains one of the leading causes of morbidity and mortality around the world, despite the recent modest downturn in HIV incidence rates (4-6).

The HIV/AIDS epidemic currently affects approximately 38 million people (range 31.6–44.5 million people) with an estimated 1.7 million (range 1.2–2.2 million) new infections for the year of 2019 (6). Despite a relatively late introduction of the virus to sub-Saharan Africa (SSA), it now bears more than half of the world's HIV burden. SSA has approximately 20.7 million infected individuals (range 18.4–23. million), with 730 000 new infections in the region during 2019 (6). South Africa accounts for 7.5 million (range 6.9—8 million) of SSA's infections, with 200 000 new infections during 2019 (7). The disproportionate burden of HIV can also be seen between genders in SSA, with women accounting for more than half (62%) of the infections. Specifically, in South Africa, women (15+ years) accounted for 4.7 million of the infected individuals, with 120 000 new infections (range 110 000—140 000) in 2019 (7). Of concern, young women (15-24 years) accounted for approximately half of these new infections.

Globally there have been an estimated 32.7 million deaths associated with AIDS-related illnesses, with 690 000 thousand (range 500 000— 970 000) deaths in 2019 alone (6), with South Africa accounting for approximately 72 000 (range 58 000—89 000) deaths (7). Encouragingly though, the advent of ART has been critical in transforming the HIV/AIDS epidemic into a manageable disease. As of 2019, 25.4 million HIV-infected people had access to antiretroviral therapy (ART) globally, while in SSA approximately 15 million people had ART access (6). Despite the strides made in prevention through the use of ARVs, social, behavioural and biological factors can undermine the effectiveness of prevention strategies, adding to the complexity of this disease, and the need for new and different prevention strategies.

1.1 Mechanisms and Biology of HIV transmission

There are several modes by which HIV can be transmitted. HIV-1 transmission can occur through transfer of contaminated blood or blood products via intravenous transmission or vertical transmission from mother to child (MTCT) during birth (intra-partum), around birth and delivery (peri-partum) or through breastfeeding (post-partum). The most common route, however, is the horizontal transmission route which occurs through sexual contact, primarily involving exposure to the virus via penetrative vaginal or anal intercourse. While the predominant mode of HIV infection is through mucosal transmission during sexual intercourse (Reviewed in 8), with women more likely to be HIV infected than men (9), several biological and social/behavioural factors further increase HIV acquisition risk.

1.1.1 HIV life cycle

The HIV life cycle can be classified in five main stages: attachment and fusion (Reviewed in 10), reverse transcription (Reviewed in 11), integration (Reviewed in 12), replication (Reviewed in 13), assembly, budding and maturation (Reviewed in 14). The majority of these steps hijacks the host cell metabolites and machinery (15-18) to produce virions to perpetuate the infection/virus production cycle.

1.1.2 HIV Tropism and Target cells

HIV viruses have two predominant tropisms; the R5-tropic strains (which utilise the CCR5 co-receptor) and the X4-tropic strains (which utilise the CXCR4 co-receptor). However, preferential transmission of the R5-tropic phenotype occurs (19-21), with the X4 phenotype more vulnerable to host restrictions (Reviewed in 22). Additional characteristics such as increased dendritic cell interactions and IFN-α resistance enhance transmission probability (23). HIV predominantly infects activated CD4+ T cells expressing the co-receptors (24-26), however, any cellular subtypes expressing these receptors can be infected, such as resting CD4+ T cells (27, 28), natural killer cells (29, 30), B cells (31), macrophages (32, 33) and dendritic cells (34-39). These cellular subsets are generally not productively infected but instead act as a carrier and transporter of the virus (40-46). Additionally, some studies have reported cases of other CD4+ cell subtypes becoming infected (Reveiwed in 47, 48-52). Recently, Stieh et al., (2016) found that a subset of CD4+ T cells, Th17 cells expressing CCR6 and CD4 receptors, were preferentially infected by SIV in a rhesus macaque vaginal challenge model (53).

1.2 Factors that influence the risk of HIV acquisition and transmission

Transmission of HIV in the female genital tract is relatively low between a healthy individual and an infected partner per sex act (between 1:200 to 1:2000 exposures). Among others, several viral characteristics, host adaptive and innate immune responses, and microbial factors are known to influence the risk of HIV acquisition and transmission (54).

1.2.1 Virological Factors

Virological factors influence infection, transmission and pathogenesis. Certain conditions favour HIV acquisition and facilitate transmission, including the selective bottleneck that the virus undergoes to escape the immune system and establish productive infection (55-59). Transmitted variants that have escaped may leave clues for prevention, and adds to the complexity of more than one viral variant causing infection (20, 57, 58, 60, 61). Becoming infected with more than one viral variant has been correlated to the presence of pre-existing sexually transmitted infections (STIs) and hormonal contraception use (58, 62). Recently, replication capacity of the founder virus, and not viral infectivity alone, was shown to drive disease progression (63). Furthermore, infection with more than one virus is associated with faster disease progression (64, 65). In addition, animal studies demonstrated that viruses transmitted during chronic infection stage are often more pathogenic, with a >3000 fold increased replication capacity or high virulence phenotypes than viruses transmitted during the early stage of infection (66).

Viruses that do not have high virulence or high replicative capacity are preferentially transmitted, supporting the concept that the virus "resets" at transmission to its original phenotype (21, 67-69). Taken together these data show that a minor variant may be selectively transmitted suggesting that the process of transmission is not stochastic (55, 56, 62, 70, 71). In addition, >95% of viruses that use the CCR5 co-receptor are also selected for transmission (72-74) and are likely to be less glycosylated and have shorter variable loop sequence lengths (68, 75-81). The less glycosylated phenotype showed enhanced binding to the $\alpha 4\beta 7$ integrin on CD4+ T cells (82-84) increasing efficiency for transmission. Early stage transmitted viruses also display more resistance to IFN-α than chronic stage viruses (23, 85).

The proportion of infections due to cell-free or cell-to-cell transmission, or a combination of these two, is a topic of ongoing study and speculation. Cell-to-cell spread is an efficient process because of the virological synapse that essentially transmits the virus to another cell without the virus becoming soluble or exposed (86). In the context of vertical transmission, transmitting mothers have higher levels of cell-associated HIV in breast milk than non-transmitting mothers, even when controlling for the levels of cell free virus (87). Studies have shown that in terms of prevention, certain select neutralizing antibodies are ineffective in inhibiting cell-to-cell spread of HIV (41, 88-91). It has been repeatedly shown that cell-associated viral transmission and replication is highly resistant to the action of certain classes of ARV's (92-96).

The unique biological properties of the transmitted viruses have been repeatedly demonstrated in many studies. However, the factors that govern the biology and anatomy in the genital mucosae, also play a central role in HIV acquisition (by providing more permeable transmission routes such as the single layered columnar epithelium and more targets for HIV infection) or protection (by the more

impermeable squamous epithelium and possible immunity to hinder HIV infection). Therefore, defining the immune correlates of risk or protection in the genital mucosae in addition to understanding the viral transmission dynamics may further aid in developing combination HIV prevention modalities.

1.2.2 Host biological factors

There are various host immunological factors that can reduce or increase the risk of HIV acquisition. These factors include those from the innate and adaptive immune arms that influence and impact the cellular activation and inflammation status in the systemic or mucosal compartments.

1.2.2.1 Innate immunity

The immune system of the female genital tract (FGT) has characteristics that distinguish it from other mucosal systems, and in particular the systemic immune system (97-99). The mucosal surface of a healthy FGT is a hostile environment for microbial growth with an acidic pH (pH<7) maintained by local commensal bacteria through the production of lactic acid and hydrogen peroxide (100, 101), and a viscous mucous comprised of mucins that can provide an effective barrier to the upper FGT from pathogen invasion (102, 103). Furthermore, secreted innate proteins (Reviewed in 104), like cationic alpha (α)-defensins, made by epithelial cells and leukocytes, inhibit a broad range of bacteria, fungi and viruses, including HIV (105). α-defensins act through direct inactivation of virions, interference of gp120 attachment to CD4 inhibiting viral attachment or entry, down-regulation of co-receptor expression, induction of beta (β)-chemokines, or inhibition of viral fusion and down-regulation of intracellular viral replication (106-111). However, α-defensins made by neutrophils, known as the human neutrophil peptides (HNPs), are also associated with increased HIV risk (112-116) in the presence of pre-existing sexually transmitted infections (117) . These α-defensins may enhance HIV infection by promoting viral entry through an unknown mechanism, can recruit T cells, monocytes and dendritic cells (DCs) and regulate cellular activation and cytokine production (112-116, 118). Human β-defensin 3 also inhibit the inflammatory response induced by lipopolysaccharide (LPS) (119, 120) and tumor necrosis factor (TNF)-α (121), demonstrating the immunoregulatory capacity of β-defensin 3. Another group of secreted acidic proteins, such as secretory leucocyte protease inhibitor (SLPI) (122-124) and elafin (125), have been shown to inhibit HIV movement into the sub-epithelium (54). Despite the innate defences of the mucosal environment of the FGT, HIV and other sexually transmitted infections (STIs) do breach the mucosal barrier to hijack the local immune system, fuelling the inflammatory process causing cellular activation and increasing the availability of targets for the establishment and spread of infection.

1.2.2.2 Adaptive immunity

Activation of the innate immune system leads to subsequent activation of the adaptive immune system, a more specific response (126). The innate immune system drives and controls adaptive immunity by presentation of antigen peptide via MHC molecules and through the expression of cytokines by antigen

presenting cells such as DCs (127-129). The specific milieu of cytokines secreted directs how the adaptive immune response matures and exerts immune function (130, Reviewed in 131). Two main arms of the adaptive immune response exist, namely the cell-mediated and humoral responses (132). The cell-mediated response is dominated by T cells and involves cytotoxic T lymphocytes (CTLs), such as CD8+ T cells. Primate studies demonstrate the importance of cell-mediated immunity in controlling SIV replication (133, 134). Similarly, in humans, strong and early induction of HIV-induced CD8+ T cell responses are crucial for the control of acute HIV infections, leading to lower viral set points (135). Broad Gag-specific CD8+ T cell responses were associated with viral control during primary infections (136), in addition to the secretion of HIV suppressive factors, such as RANTES, MIP-1α and MIP-1β, which compete with HIV for CCR5 co-receptor binding (137). Similarly, in elite controllers, increased levels of MIPs increased resistance to R5, but not X4-tropic viral strains (138). The humoral arm of adaptive immunity is characterised by the production and secretion of antibodies, and can be either T-cell dependent or T-cell independent (139). The T-cell independent humoral response is initiated by TLR engagement and B-cell receptor (BCR) engagement that induce complementary signalling pathways that result in immunoglobulin class switching (140). The T-cell dependent pathway requires three signals: engagement of BCR by antigen, signals from T helper cells (such as CD40L from CD4+ T cells), and signals by cytokines (141, Reviewed in 142). Antibodies have three main mechanisms of action; direct neutralisation, which prevents pathogen adherence; opsonisation, which promotes antibody dependent cell-mediated cytotoxicity or phagocytosis; and complement activation, which either enhances opsonisation or induces lysis of bacteria (139). Neutralisation is the ultimate protective mechanism (143). Broadly neutralizing antibodies (bNAbs) to HIV, which are defined as having both potency and breadth, have the potential to block HIV transmission and supress HIV viremia (144-151). Suppression of viremia could be attributed to the enhanced clearance of cell-free virions (152) as well as HIV infected cells (153). Recently potent and broad bNAbs (CAP256-VRC26 family) against HIV-1 were isolated from a clinical patient of the CAPRISA002 study (154, 155), highlighting the potential of the immune system to naturally control HIV infection.

1.2.2.3 Inflammation

Inflammation is a necessary natural response elicited to control infection and limit tissue damage (Reveiwed in 156, 157). However, persistent inflammation can result in autoimmune or auto-inflammatory disorders (157). Inflammation, generally a symptom of infection, can occur in the absence of infection or tissue damage, a phenomenon known as sterile inflammation. The initial step in the inflammatory process is the recognition of pathogen/damage associated molecular patterns (P/DAMP's) by pathogen recognition receptors (PRR's) such as TLRs (Reviewed in Janeway, 1989), either on the surface or within (Reviewed in Blasius and Beutler, 2010) innate immune cells such as dendritic cells, macrophages and monocytes (Medzhitov and Janeway, 2002, Akira et al., 2006, Kumar et al., 2011). Common PAMPs that are known to have significant immunological effects include

lipopolysaccharide (LPS) recognised by TLR4 (Fang et al., 2004, Porter et al., 2010, Ngkelo et al., 2012, Plociennikowska et al., 2015), single stranded RNA (ssRNA) recognised by TLR7/8 (Xagorari and Chlichlia, 2008, Jounai et al., 2012, Bernard et al., 2014) and bacterial lipopeptides recognised by TLR2 (Mukherjee et al., 2016, Frasnelli et al., 2005, Gambhir et al., 2012). There are two aspects to inflammation: the pro-inflammatory response which upregulates and facilitates activation of the immune system; and the anti-inflammatory response which regulates the pro-inflammatory response to mitigate the development of autoimmune or auto-inflammatory disorders (Reviewed in 158).

1.2.2.4 Genital tract inflammation and risk for HIV acquisition

Genital inflammation increases the risk of HIV transmission and acquisition. In PLWH, increased pro-inflammatory cytokines and immune activation directly correlated with increased HIV viral loads in genital secretions (159-163), increasing HIV transmission. In HIV-uninfected individuals, inflammation resulted in recruitment of HIV target cells and epithelial barrier damage (164-166). Moreover, immune activation and increased cytokines were significantly associated with increased HIV risk in both the blood (167, 168), and the genital tract (117, 169). Multiple studies have reported reduced immune activation in HIV-exposed but seronegative individuals (170-175), underscoring the importance of modulating inflammation or promoting a quiescent immune environment to minimize the risk of HIV acquisition or onward transmission.

Various consequences of inflammation create a conducive environment for HIV acquisition. Nazli et al., (2010) demonstrated that mucosal epithelial cells secreted increased pro-inflammatory cytokines upon exposure to HIV-1 (176). In addition, TNF-α and interferon (INF)-γ correlated to reduced epithelial barrier function, through increased permeability of the mucosal barrier (176-181). TNF-α and IL-1 also directly affect HIV replication through activation of NF-kβ, the transcription factor which binds the HIV promoter region (182). Li et al., (2009) described a target cell recruitment process in which; macrophage inflammatory protein (MIP)-3α and IL-8 recruit plasmacytoid dendritic cells (pDCs) which secrete MIP-1α and MIP-1β to recruit CCR5+ cells in a rhesus macaque model (pathogenic host for SIV infection who progress to an AIDS like state). In this model, inflammation and recruitment of target cells to the genital tract were important preceding events for effective SIV infection following vaginal challenge (183). Unlike rhesus macaques, sooty mangabeys (the natural hosts for SIV that do not progress to an AIDS like state) have lower levels of systemic and mucosal CD4+CCR5+ T cells, and are less likely to get infected with SIV (184). Masson et al., (2015) found that increased genital tract chemokines MIP-1α, MIP-1β, IL-8 and IFN-γ-inducible protein (IP)-10 conferred more than a three-fold increased risk of HIV acquisition (169). Similarly, a follow-up study by Liebenberg et al., (2017) comparing plasma and genital tract cytokine levels showed that increased mucosal concentrations of IP-10, MIP-1β, IL-8 and monocyte chemoattractant protein (MCP)-1 was associated with increased HIV acquisition risk (185). MIP-3α and IL-8 are important chemokines that

facilitate infection through their chemotactic activity involved in the recruitment of HIV target cells (Reviewed in 156, 186). Additionally, IP-10, MIP-1α and MIP-1β have also been shown to recruit HIV target cells (Reviewed in 187, 188-190). Furthermore, MIP-1α-CCR5 interaction was shown to activate the JAK/STAT signalling pathway involved in cellular proliferation (191).

1.2.3 The human microbiome and bacterial dysbiosis

Microbiome refers to the naturally occurring microorganisms that grow within the mucosal environments and plays a central role in the maintenance of a healthy immune system (Reviewed in 192, 193), with the normal flora protecting epithelial cells from pathogens through Toll-Like receptor (TLR) signalling (Reviewed in 194). A healthy vaginal microbiome is generally dominated by *Lactobacillus* species. *Lactobacillus* species generally maintain a low and acidic vaginal mucosal environment through the production of lactic acid and hydrogen peroxide (195), providing hostile conditions for foreign microbes or viruses (Reviewed in 196, 197). However, a disruption of the microbiome, by, among others, antibiotic use (198) and intravaginal practices (199), leads to an increase in bacterial species diversity (dysbiosis) and imbalance and decrease of healthy bacterial communities has been associated with the presence of bacterial dysbiosis in the FGT (Reviewed in 200, 201). This dysbiosis often leads to an inflammatory response and subsequent increase in mucosal permeability, thus increasing the risk of HIV acquisition (202-205). *Prevotella bivia,* for example, a microbe highly associated with bacterial dysbiosis (206), can infect epithelial cells and induce an inflammatory response (Reviewed in 207). Furthermore, the efficacy of the topical 1% tenofovir gel used in the CAPRISA 004 trial was undermined in women who had a non-lactobacillus dominated microbiome (208). This decreased efficacy was attributed to the direct metabolism of tenofovir (TFV) by *Gardnerella vaginalis* in women with a non-lactobacillus dominated vaginal microbiome (208, Reviewed in 209).

1.2.4 Sexually Transmitted Infections

Risk for HIV acquisition has been associated with the presence of pre-existing STIs (210-212), mainly attributed to the induction of inflammation (213, 214). Many laboratory diagnosed STIs are asymptomatic (no clinical symptoms), and are clinically undiagnosed, undetected and untreated and therefore pose risk. These asymptomatic STIs still contribute to elevated genital tract inflammatory cytokines and increased HIV risk (215). Therefore, proper STI diagnosis, and not just syndromic STI management is key to developing effective HIV prevention strategies.

1.3 Prevention strategies against HIV acquisition and transmission

1.3.1 Pre-exposure prophylaxis (PrEP)

Pre-Exposure Prophylaxis (PrEP) has led to effective preventative interventions and treatment regimens (216, 217, Reviewed in 218). Among the PrEP strategies, ARV-containing microbicide gels and intravaginal rings and ARV treatment as prevention have been studied and proposed as options. Microbicide gels showed promise for HIV prevention in 2010 when the CAPRISA 004 1% tenofovir gel trial showed a 39% efficacy (219), with high gel adherers having a 54% efficacy. However, presumably due to poor product adherence, the findings of the two follow-up trials using the 1% tenofovir gel, the Vaginal and Oral Interventions to Control the Epidemic (VOICE) study (220) and the Follow-on African Consortium for Tenofovir Studies (FACTS) 001 study (221) trials did not replicate these findings. However, in a post hoc analysis of the CAPRISA 004 1% tenofovir gel trial, genital inflammation was shown to undermine the efficacy of the microbicide gel, even in high adherers (222). Similar to microbicide gels, ARV containing intra-vaginal rings showed the potential as a means of HIV prevention. In two studies investigating the monthly vaginal ring containing the ARV- dapivirine, the efficacies were 27% (223) and 30.7% respectively (224). Poor adherence here too, attributed to the poor efficacies. The most promising preventative intervention has been daily oral PrEP with Truvada® (tenofovir disoproxil fumarate and emtricitabine) in reducing HIV infections (220, 225-230). Again, PrEP adherence was a key contributor to some of the lower efficacies observed in clinical trials in African women (231, 232). Even though PrEP is the current best practice, many behavioural and biological factors still undermine its effectiveness. Therefore, a comprehensive approach to modify HIV risk may include targeted interventions such as adjunctive treatment and education to prevent infections.

1.3.2 Post-exposure prophylaxis (PeP)

Treatment as prevention as post-exposure prophylaxis (PeP) is also effective at reducing HIV. Cohen et al., (2011) showed a reduction in transmission events by 96% with early initiation of ART in sero-discordant couples in the HIV Prevention Trials Network (HPTN) 052 trial (233). Additionally, Tanser et al., (2013) also showed that high coverage of ART reduced rates of HIV (234). PeP is an effective intervention in preventing establishment of productive infection after HIV exposure, provided it is timeously initiated.

1.3.3 Vaccines

Despite the strides made with PrEP and other preventative strategies, the development of an effective HIV vaccine remains a public health priority. Vaccines prime the immune system to fight infections and immunity is long lived due to induction of long-term immune memory T and B cells. However there are many challenges with developing an effective vaccine to HIV, and many trials failed to show protection (235-238). The RV144 trial was the only vaccine trial to show a moderate efficacy against HIV (239). Recently, the follow-up to RV144, Uhambo HVTN 702 trial, was stopped due to a lack of

efficacy (240). In the absence of efficacious HIV vaccines or potential vaccine candidates, broadly neutralizing antibodies (bNAbs) have become important options for proof of concept in HIV prevention. bNAbs have shown great promise as passive immunity to prevent or treat HIV (241-244) and are currently undergoing extensive clinical trial testing. In addition, bNAbs in combination with ART is more effective than ART alone for suppression and control of HIV-1 (245), demonstrating their importance and potential for therapeutic management of HIV disease. The development of an effective long-lasting vaccine is the ultimate goal to prevent HIV, however development of such a vaccine remains a formidable challenge. In the meantime, in the absence of an effective HIV vaccine, other prevention strategies, which could be adjunctive, can be used effectively to limit transmission or prevent HIV acquisition.

1.3.4 Anti-inflammatories

The prominent link between inflammation and HIV underscores the potential for therapeutic use of drugs to dampen inflammation to manage HIV disease and reduce infection risk (246). Most research with anti-inflammatory drugs has been focussed on reducing HIV-associated immune activation to slow disease progression as well as to reduce co-morbidities (247-249). Chloroquine and the derivative hydroxychloroquine have been used extensively to treat HIV-associated immune activation with positive outcomes (250-253). Aspirin® also reduced HIV-associated immune activation in individuals on ART (254). Furthermore, glucocorticoids, specifically prednisolone, reduced HIV-associated immune activation, slowed CD4+ T cell loss and disease progression (255-260). The use of anti-inflammatory products as a preventative method to reduce HIV risk has been investigated. Daily oral hydroxychloroquine and Aspirin® reduced inflammation and the numbers and activation status of CD4+ T cells systemically and at the FGT (261). Systemic long-term use of anti-inflammatory drugs can have serious adverse effects, therefore topical formulations are preferred because of better safety profiles. A vaginal implant containing hydroxychloroquine was shown to significantly reduce immune activation and inflammation in a small animal model (262). Glycerol monolaurate (GML), a naturally occurring compound also showed anti-inflammatory effects, and reduced SIV infection in rhesus macaques (183, 263). These studies highlight the potential for the use of anti-inflammatory products to prevent disease progression and modulate immunity to mitigate HIV risk.

1.4 Hypothesis

We hypothesised that TLR agonists would induce inflammation and immune activation of CD4+ T cells, thereby increasing their susceptibility to HIV infection. Furthermore, the NSAID; ibuprofen and the glucocorticoid; betamethasone, two anti-inflammatory drugs, modulates the TLR-mediated inflammation and immune activation of CD4+ T cells, thereby reducing the TLR-mediated inflammatory responses and mitigate the susceptibility of CD4+ T cells to HIV infection.

1.5 Aim

The aim of this project was to assess how anti-inflammatory drugs impact TLR-induced inflammation and immune activation, and how this affects TLR-mediated HIV infection of CD4+ T cells.

1.6 Objectives

Primary objective: To assess how TLR agonists mediate inflammation, immune activation and HIV infection of target CD4+ T cells in a PBMC model.

Secondary objective: To assess how the NSAID Ibuprofen and the glucocorticoid modulated TLR-mediated inflammation, immune activation and HIV infection of CD4+ T cells.

1.7 Methods and Materials

This *in vitro* study had been granted ethics approval by the University of KwaZulu-Natal Biomedical Research Ethics Committee (UKZN BREC; Ethics number: BE433/14). Whole blood was obtained from 5 healthy donors enrolled in a volunteer blood study (Ethics number: BE022/13). Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation. PBMCs were then stimulated with TLR agonists Pam3CSK4 (TLR1/2), LPS (TLR4) or R848 (TLR7/8) or stimulated with PHA as a positive control or left unstimulated as a negative control in the absence (Chapter 2) or presence (Chapter 3) of anti-inflammatory drugs ibuprofen or betamethasone. PBMCs were then exposed to the R5 tropic strain of HIV, NL4-3 AD8 (264), at a MOI of 0.9. Cell culture supernatants were used for the assessment of soluble cytokines by multiplex ELISA assays. PBMCs were used for cellular phenotyping and the assessment of immune activation (CD38, HLA-DR and CCR5) of CD4+ and CD8+ T cells by flow cytometry. Titration of antibodies and FMOs are shown in the appendices as supplementary figures 1-3.

1.7.1 Statistical analysis

GraphPad Prism version 7.02 software for Windows (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses and graphical representation of data. For comparisons of cellular activation markers CD38, HLA-DR on CD4+ and CD8+ T cells, between stimulated conditions and the unstimulated control, as well as between anti-inflammatory treated conditions and the untreated control, a repeated measures two-way ANOVA with a Dunnett's multiple comparisons test was performed. Similarly, for CCR5 and cytokine comparisons, an ordinary one-way ANOVA with Dunnett's multiple comparison test was performed. Cellular activation results are displayed as mean percentage (%) ± standard deviation (SD) of CD4+ or CD8+ T cells. Cytokine data was normalized by Log_{10} transformation and is displayed as mean concentration (Log_{10} pg/ml) ± standard deviation (SD). Heat maps were generated by performing a single linkage hierarchical cluster analysis using R version 3.3.3 statistical software (R Foundation for Statistical Computing, Vienna, Austria), to visualize the effect of various TLR agonists and anti-inflammatory drugs on cytokine expression. Radial spider plots were created using Microsoft Excel© 2013 software (Microsoft Corporation, Redmond, WA, USA).

1.8 Bridging chapter

The associations between inflammation and HIV risk have not been fully defined. Genital inflammation significantly modifies HIV acquisition and transmission risk, while HIV-associated inflammation in Persons living with HIV increased disease progression. The biological mechanisms that underpin genital inflammation and HIV risk are not fully elucidated. Immune quiescence, characterised by regulated immune activation and inflammation, has been identified as a potential immune correlate of reduced risk in individuals who are naturally resistant to HIV infection. Toll-Like receptors (TLRs) are important innate pattern recognition receptors for pathogens and initiate immune responses. Therefore, we used three common TLR agonists which bind to TLR1/2, TLR4 and TLR7/8 to induce inflammation and immune cell activation. We then assessed how these TLR agonists impacted on HIV infection of target CD4+ T cells and have published a paper. **This paper, entitled "Diminished HIV Infection of Target CD4+ T Cells in a Toll-Like Receptor 4 Stimulated in vitro Model", has been published on 23rd July 2019 in the Frontiers Journal of Immunology, subsection Viral Immunology (Front. Immunol. 10:1705. doi: 10.3389/fimmu.2019.01705).** This paper formed the basis for the *in vitro* model used to simulate inflammation and provided a segue for the use of anti-inflammatory drugs to modulate inflammation and mitigate HIV infection for chapter 3.

1

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Diminished HIV Infection of Target CD4+ T Cells in a Toll-Like Receptor 4 Stimulated *in vitro* Model

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Genital inflammation is associated with increased HIV acquisition risk. Induction of an inflammatory response can occur through the recognition of pathogenic or commensal microbes by Toll-like receptors (TLRs) on various immune cells. We used a *in vitro* peripheral blood mononuclear cell (PBMC) system to understand the contribution of TLR stimulation in inducing inflammation and the activation of target T cells, and its effect on HIV susceptibility. PBMCs were stimulated with TLR agonists LPS (TLR4), R848 (TLR7/8), and Pam3CSK4 (TLR1/2), and then infected with HIV NL4-3 AD8. Multiplexed ELISA was used to measure 28 cytokines in cell culture supernatants. Flow cytometry was used to measure the activation state (CD38 and HLA-DR), and CCR5 expression on CD4+ and CD8+ T cells. Although TLR agonists induced higher cytokine and chemokine secretion, they did not significantly activate CD4+ and CD8+ T cells and showed decreased CCR5 expression relative to the unstimulated control. Despite several classes of inflammatory cytokines and chemokines being upregulated by TLR agonists, CD4+ T cells were significantly less infectable by HIV after TLR4-stimulation than the unstimulated control. These data demonstrate that the inflammatory effects that occur in the presence TLR agonist stimulations do not necessarily translate to the activation of T cells. Most importantly, the finding that TLR4-stimulation reduces rather than increases susceptibility of CD4+ T cells to HIV infection in this *in vitro* system strongly suggests that the increased chemokine and possible antiviral factor expression induced by these TLR agonists play a powerful although complex role in determining HIV infection risk.

Keywords: Toll-like receptors, inflammation, immune activation, HIV, cytokines, innate antiviral immunity

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INTRODUCTION

HIV and AIDS is a global epidemic that affects approximately 37 million people worldwide, with an additional 1.8 million new HIV infections documented in 2017 (1). Sub-Saharan Africa bears more than half of the global HIV burden, with young adolescent women (aged 15–24 years) twice as likely to be living with HIV compared to men in this region (1). Furthermore, 75% of

2 Chapter 2: Diminished HIV infection of target CD4+ T cells in a Toll-Like Receptor 4 stimulated in vitro model

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Keywords: Toll-like receptors, Inflammation, Immune activation, HIV, Cytokines

Running title: TLR stimulated HIV infection model

2.1 Abstract

Genital inflammation is associated with increased HIV acquisition risk. Induction of an inflammatory response can occur through the recognition of pathogenic or commensal microbes by Toll-like receptors (TLRs) on various immune cells. We used an in vitro peripheral blood mononuclear cell (PBMC) system to understand the contribution of TLR stimulation in inducing inflammation and the activation of target T cells, and its effect on HIV susceptibility. PBMCs were stimulated with TLR agonists LPS (TLR4), R848 (TLR7/8), and Pam3CSK4 (TLR1/2), and then infected with HIV NL4-3 AD8. Multiplexed ELISA was used to measure 28 cytokines in cell culture supernatants. Flow cytometry was used to measure the activation state (CD38 and HLA-DR), and CCR5 expression on CD4+ and CD8+ T cells. Although TLR agonists induced higher cytokine and chemokine secretion, they did not significantly activate CD4+ and CD8+ T cells and showed decreased CCR5 expression relative to the unstimulated control. Despite several classes of inflammatory cytokines and chemokines being upregulated by TLR agonists, CD4+ T cells were significantly less infectable by HIV after TLR4-stimulation than the unstimulated control. These data demonstrate that the inflammatory effects that occur in the presence TLR agonist stimulations do not necessarily translate to the activation of T cells. Most importantly, the finding that TLR4-stimulation reduces rather than increases susceptibility of CD4+ T cells to HIV infection in this *in vitro* system strongly suggests that the increased chemokine and possible antiviral factor expression induced by these TLR agonists play a powerful although complex role in determining HIV infection risk.

2.2 Introduction

HIV and AIDS is a global epidemic that affects approximately 37 million people worldwide, with an additional 1.8 million new HIV infections documented in 2017 (1). Sub-Saharan Africa bears more than half of the global HIV burden, with young adolescent women (aged 15–24 years) twice as likely to be living with HIV compared to men in this region (1). Furthermore, 75% of new HIV infections among adolescents (15–19 years) are in girls (1). Specifically, South Africa accounts for 19% of HIV infected people globally, 15% of new HIV infections annually and 11% of AIDS related deaths worldwide (2). The inception of antiretroviral therapy (ART) has dramatically reduced the risk of HIV related morbidity and mortality and has transformed the epidemic into a manageable chronic disease (3). Strategies to prevent infection are crucial for control and eventual eradication of the HIV epidemic. Various pre-exposure prophylaxis (PrEP) strategies such as oral tablets, microbicides or intra-vaginal rings containing anti-retroviral drugs, have been proposed with various levels of success.

Many social, behavioural and biological factors undermine the efficacy of these prevention strategies (4–6). One important biological factor is female genital tract inflammation. Genital inflammation, defined by the increase in inflammatory and chemotactic cytokines such as IL-1α, IL-1β, TNF-α, MIP-1α, MIP-1β, IP-10, and IL-8, among others, has been associated with an increased risk of HIV acquisition (7–9). One of the mechanisms implicated is that inflammation increases HIV risk by causing activation of HIV target cells (CD4+ T cells), thereby priming the cells for HIV infection (10). Inflammation also leads to increased recruitment of these activated target CD4+ T cells to the environment where infection occurs (11). Additionally, T cell activation profiles in the blood predicted those in the genital tract (12), suggesting that these activation profiles in the blood could be a surrogate indication of activation in the genital tract with subsequent increased risk for HIV. Lastly genital inflammation leads to the disruption of the mucosal barrier, which is not only more permissive to viral translocation (11) but also facilitates infection with less infectious virions (13). Furthermore, genital inflammation has been shown to reduce the protective effect of TFV 1% gel as a vaginal microbicide in the CAPRISA 004 trial (6, 14). Additionally, a dysbiotic microbiome or bacterial vaginosis (BV), which is often associated with genital inflammation (15, 16), also undermined the efficacy of the 1% TFV gel microbicide (17). The reduced efficacy was attributed to the direct metabolism of TFV by Gardnerella vaginalis (17), a microbe which is often associated with BV (18).

Inflammation is the natural biological response for protection against invading pathogens and damaged tissue. Inflammation can be broadly defined into three stages; recognition and release, activation and recruitment, and resolution and repair (19). The causes of genital inflammation have yet to be fully understood, however, sexually transmitted infections (STI) and a dysbiotic microbiome have been implicated (9, 15, 20). The mechanisms underlying the induction of inflammation by these two factors likely involve the recognition of pathogen associated molecular patterns (PAMPs) by Toll-Like

Receptors (TLRs) (21), a cardinal pathway for the induction of an immune response (22). Various TLRs are able to recognize a broad range of antigens, from bacterial wall proteins to various types of bacterial and viral genetic products (23), and each TLR initiates a distinct signalling cascade for the induction of innate immune responses (24, 25). TLRs are expressed to various degrees on most immune cells, with innate antigen presenting cells expressing the widest range (23). Common PAMPs that are known to have significant immunological effects include lipopolysaccharide (LPS) recognized by TLR4 (26, 27), single stranded RNA (ssRNA) recognized by TLR7/8 (28, 29) and bacterial lipopeptides recognized by TLR1/2 (30, 31). TLR-stimulation of mouse splenocytes with R848 (TLR7/8 agonist) increased IL-1α, IL-2 and IL-6 expression, while LPS increased IL-1α, IL-2 and IL-4 expression (32). Additionally, Wang et al. demonstrated that human peripheral blood mononuclear cells (PBMCs) stimulated with LPS induced the production of IL-1β, TNF-α, IL-6, and IL-22 (32). Similarly, in a study investigating Th17 cell induction in human PBMCs, the TLR agonists R848 and LPS elicited production of IP- 10, IL-6, MCP-1, IL-8, MIP-1α, and RANTES, while R848 further induced IL-12(p40), IL-1β, and TNF-α production (33). Furthermore, in the context of vaccine induced immunity, very similar cytokine responses from human monocyte-derived- DC's (MDDCs) and monocyte-derived-macrophages (MDMs) were found with vaccine adjuvants R848 and the TLR4 agonist Glucopyranosyl Lipid Adjuvant (GLA) (34). A strong chemokine response was observed with high expression of MIP-1α, MIP- 1β, RANTES, and IP-10, while the pro-inflammatory cytokine response was less pronounced, with lower expression of IL-1α, IL-1β, and TNF-α compared to the chemokines (34).

TLR agonists have been shown to induce potent inflammatory responses and genital inflammation has been associated with the increased risk of HIV acquisition. Therefore, we sought to recapitulate some of the findings from genital inflammation studies using an *in vitro* PBMC system to understand the contribution of TLR-mediated inflammatory response to the activation and HIV infection of target CD4+ T cells.

2.3 Materials and Methods

2.3.1 Ethics statement

This study was carried out in accordance with the recommendations of the University of KwaZulu-Natal (UKZN) Biomedical Research Ethics Committee (BREC). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the UKZN BREC (BE433/14).

2.3.2 Cell culture media

C10 media was used for all cell culture experiments. C10 media consisted of RPMI 1640 with L-glutamine (Lonza, Basel, Switzerland) containing 10% FCS (non-Hi FCS; Highveld Biological, JHB, SA), 2% L-glutamine, 1% HEPES, 1% NaPy, 1% NEAA (all from Lonza, Basel, Switzerland). Media was sterile filtered through the Filtermax 500 ml (Techno Plastic Products, Trasadingen, Switzerland). IL-2 (PeproTech, Rocky Hill, NJ, USA), was added to C10 media prior to use at a final concentration of 0.01 µg/ml.

2.3.3 Stimulants and HIV strain

The TLR agonists LPS (TLR4), R848 (TLR7/8), and Pam3CSK4 (TLR1/2) (all from Invivogen, San Diego, CA, USA) concentrations were previously optimized in TLR titration experiments. As no significant differences were observed in HIV infections (Supplementary Figure 1) or cytokine profiles (Supplementary Figures 2–4) between the TLR concentrations, a final concentration of 2 µg/ml was used. Phytohaemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO, USA), used as the positive control at a final concentration of 10µg/ml. Unstimulated PBMCs were used as the negative control. The CCR5-tropic HIV-1 NL4-3 AD8 (35) (a gift from Dr. Alex Sigal), was used at a working dilution of 1:20, which corresponded to a MOI of 0.9, which had been previously optimized (data not shown). PHA and unstimulated uninfected conditions were used as controls for HIV.

2.3.4 Cell Culture

Blood was collected from 5 healthy volunteer donors and PBMCs were isolated by density gradient centrifugation. PBMCs were resuspended to $1x10^6$ cells/ml in C10 media and plated into cell culture plates. PBMCs were left either unstimulated (as a negative control) or stimulated immediately after plating with TLR agonists or PHA, which was used as a positive control. Following stimulation, the PBMCs were cultured at 37ºC 5% CO_2 for 48 hours. Following this incubation, the contents of each well was collected into 15ml falcon tubes and an aliquot of 500µl was removed for multiplexed ELISA (culture supernatants) and flow cytometry (PBMCs) for the day 3 time-point (post stimulation, prior to HIV infection). The 15ml falcon tubes were centrifuged, supernatants were discarded, and media replacement was performed with fresh C10 media. PBMCs were plated at $1x10^6$ cells/ml into 24-well cell culture plates, no further TLR or PHA stimulations were performed. Subsequently, HIV infection

was done by the addition of 250µl at a 1:20 dilution of the HIV-1 NL4-3 AD8 viral stocks. PHA and unstimulated uninfected wells were treated with 250µl C10 media. Plates were incubated at 37ºC 5% CO_2 for 48 hours, whereupon multiplexed ELISA (culture supernatants) and flow cytometry (PBMCs) was performed for the day 5 time-point (post HIV infection).

2.3.5 Antibodies

Cellular activation was assessed by measurement of HLA-DR and CD38, similar to previous studies (12, 36, 37). Staining for flow cytometry was performed both extracellularly and intracellularly. The extracellular staining cocktail consisted of LIVE/DEAD Amcyan fixable dye (Thermo Fisher Scientific, Waltham, MA, USA), anti-CD3-APC-H7, anti-CD4-BV605, anti-CD8-BV655, anti-CD14-Pacific blue (all from BD Biosciences, Franklin Lakes, NJ, USA), and anti-CD19- pacific blue (Biolegend, San Diego, CA, USA). The intracellular staining cocktail consisted of anti-CCR5-APC, anti-HLA- DR-PerCP-CY5.5 (all from BD Biosciences, Franklin Lakes, NJ, USA), anti-CD38-PE-CY7 (Biolegend, San Diego, CA, USA) and anti-p24-FITC (Beckman Coulter, Brea, CA, USA). PBMCs were collected at two time-points: day 3 (48 h post stimulation and prior to HIV infection) and day 5 (48 h post infection).

2.3.6 Cell Culture

The cell culture and HIV infection protocol used in this study was adapted from previous studies (38, 39). Blood was collected from 5 healthy volunteer donors and PBMCs were isolated by density gradient centrifugation. PBMCs were resuspended to 1 × 106 cells/ml in C10 media and plated into cell culture plates. PBMCs were left either unstimulated (as a negative control) or stimulated immediately after plating with TLR agonists or PHA, which was used as a positive control. Following stimulation, the PBMCs were cultured at 37◦C 5% CO2 for 48 h. Following this incubation, the contents of each well was collected into 15 ml falcon tubes and an aliquot of 500 µl was removed for multiplexed ELISA (culture supernatants) and flow cytometry (PBMCs) for the day 3 time- point (post stimulation, prior to HIV infection). The 15 ml falcon tubes were centrifuged, supernatants were discarded, and media replacement was performed with fresh C10 media. PBMCs were plated at 1×10^6 cells/ml into 24-well cell culture plates, no further TLR or PHA stimulations were performed. Subsequently, HIV infection was done by the addition of 250 µl at a 1:20 dilution of the HIV-1 NL4-3 AD8 viral stocks at a MOI of 0.9. PHA and unstimulated uninfected wells were treated with 250 µl C10 media. Plates were incubated at 37◦C 5% CO2 for 48 h, whereupon multiplexed ELISA (culture supernatants) and flow cytometry (PBMCs) was performed for the day 5 time-point (post HIV infection).

2.3.7 Flow Cytometry

PBMCs were centrifuged at 3,500 rpm for 5 min to pellet the cells and remove soluble HIV, and the cell culture supernatants were stored at −80◦C for cytokine quantification. PBMCs were washed with sterile PBS supplemented with 2% FCS and then stained

with 100 µl extracellular staining cocktail, fixed, and then stained with 100 µl intracellular staining cocktail. Data was acquired by flow cytometry on a BD LSR Fortessa (BD Biosciences, Franklin Lakes, NJ, USA). Data analysis was performed using FlowJo v10.4.1 software (Tree Star, Ashland, OR, USA), according to the gating strategy (Supplementary Figure 5). For the purpose of this study we reported on four activation phenotypes and defined these as the following; The CD38+HLA-DR+ phenotype was defined as hyper-activated, the CD38+HLA-DR- and CD38- HLA-DR+ phenotypes were defined as intermediately activated, and the CD38-HLA-DR- phenotype was defined as resting or not activated.

2.3.8 Cytokine Quantification

The concentrations of 28 cytokines were assessed from cell culture supernatants using the Bio-Plex Pro Human Cytokine Group I 27-Plex Panel (Bio-Rad Laboratories, Hercules, CA, USA) and the Magnetic Luminex® Assay IL-1α Singleplex Kit (Research and Diagnostic (R&D) systems Inc., Minneapolis, Minnesota, USA) as per manufacturer's instructions. Data was acquired on the Bio-Plex® 200 system (Bio-Rad Laboratories, Hercules, CA, USA). Optimization of standard curves were performed automatically using the Bio-Plex manager software version 6.1 (Bio-Rad Laboratories, Hercules, CA, USA). Values with coefficients of variation <20% and with observed recoveries between 70 and 130% were considered reliable. Values that were below the detectable limit were assigned half of the lowest limit of detection value (LLOD), while values that were above the detectable limit were assigned double the highest limited of detection (HLOD) value.

2.3.9 Statistical Analysis

Statistical analyses and graphical representation of data was performed using the GraphPad Prism version 7.02 software for windows (GraphPad Software, La Jolla, CA, USA). For comparisons of cellular activation markers CD38, HLA-DR on CD4+ and CD8+ T cells, between stimulated conditions and the unstimulated control, a repeated measures two-way ANOVA with a Dunnett's multiple comparisons test was performed. Similarly, for CCR5 and cytokine comparisons, an ordinary one-way ANOVA with Dunnett's multiple comparison test was performed. Cellular activation results are displayed as mean percentage (%) ± standard deviation (SD) of CD4+ or CD8+ T cells. Cytokine data was normalized by Log10 transformation and is displayed as mean concentration (Log10 pg/ml) ± standard deviation (SD). To understand the effect of various TLR agonists on cytokine expression, heat maps were generated by performing a single linkage hierarchical cluster analysis using R version 3.3.3 statistical software (R Foundation for Statistical Computing, Vienna, Austria). Radial spider plots were created using Microsoft Excel© 2013 software (Microsoft Corporation, Redmond, WA, USA).

2.4 Results

2.4.1 TLR Stimulation Did Not Result in the Activation of CD4+ T Cells

Minimal cytotoxic effects were observed with TLR stimulation, apart from R848 where a significant reduction in cell viability was observed (Supplementary Figure 6). As highly activated CD4+ T cells have been shown to be preferentially infected (10), we determined how TLR stimulation impacted on the expression of the activation markers HLA-DR and CD38 on CD4+ T cells. TLR stimulation did not induce significant CD4+ T cell activation compared to the unstimulated control ($p > 0.05$) at day 3 (post stimulation, prior to HIV infection) or day 5 (post infection) (Figure 1). However, when PBMCs were stimulated with the mitogen PHA, distinct increased cellular activation was observed, with all three activation phenotypes significantly increased compared to the unstimulated control on day 3 ($p < 0.05$). Similarly, on day 5 and irrespective of infection status, PHA induced significantly elevated expressions of CD38+HLA-DR+ and CD38+HLA-DR-, but not CD38-HLA-DR+ on CD4+ T cells compared to the unstimulated infected control ($p \leq 0.0001$) (Figure 1B). Representative dot plots of flow cytometric data are shown in Supplementary Figure 7. Relevant mean ± SD for data depicted in Figure 1 are listed in Supplementary Table 1.

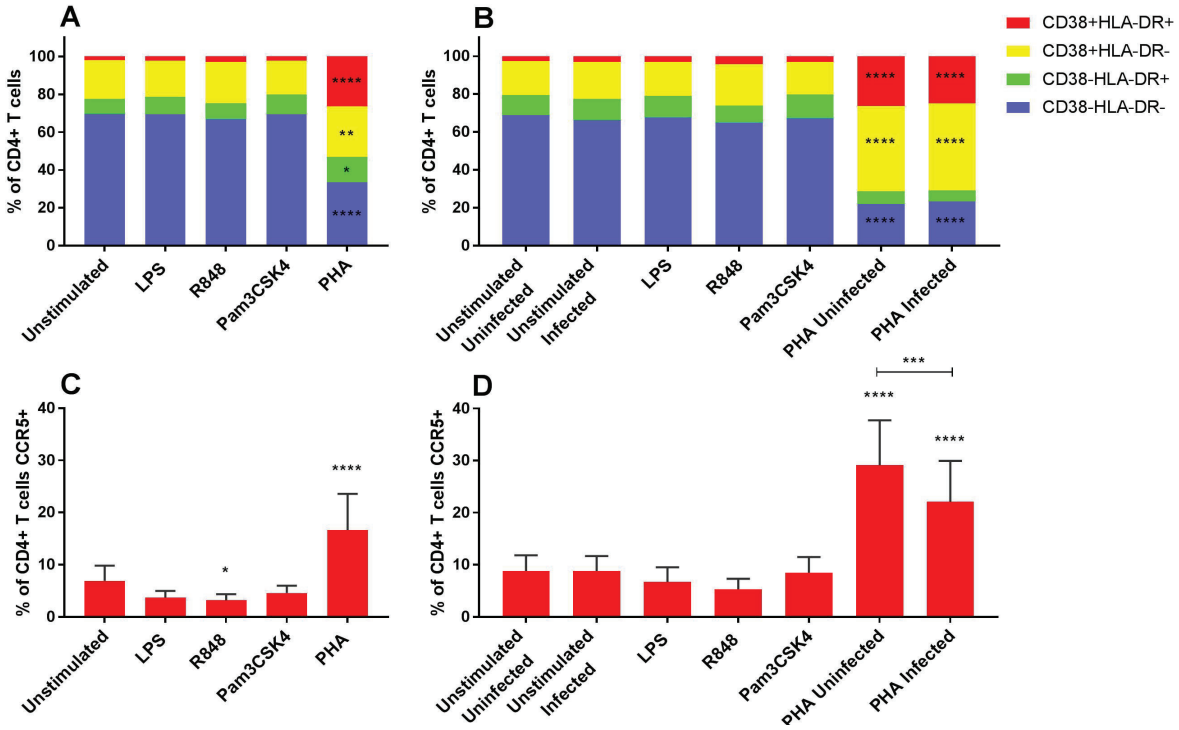

Figure 1: Activation profiles (**A&B**) and CCR5 expression (**C&D**) of CD4+ T cells on day 3 prior to HIV infection (**A&C**) and day 5 post HIV infection (**B&D**). PHA was used in a 1:500 dilution at a working concentration of 5mg/ml. TLR agonists were used at a final concentration of 2ug/ml. A repeated measures two-way ANOVA with Dunnett's multiple comparisons test was used for immune activation, and an ordinary one-way ANOVA with a Dunnett's multiple comparisons test for CCR5 expression. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the unstimulated/unstimulated infected control, unless otherwise shown. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

2.4.2 TLR Activation Downregulated CCR5 Expression on CD4+ T Cells

Since CCR5 is a co-receptor for R5-tropic HIV infection, we determined how TLR activation impacted the expression of CCR5 by CD4+ T cells. R848 stimulation significantly lowered CCR5 expression (3.2 ± 1.2% of CD4+ T cells) compared to the unstimulated control (6.9 ± 2.9% of CD4+ T cells) ($p < 0.05$), while PHA significantly increased the CCR5 expression (16.6 ± 6.9% of CD4+ T cells) ($p \leq 0.0001$) at day 3 (Figure 1C). Of note, CCR5 expression was significantly lower in PHA- stimulated infected condition by day 5 (22.1 ± 7.9% of CD4+ T cells) compared to the PHA-stimulated but uninfected condition (29.1 ± 8.6% of CD4+ T cells) ($p = 0.0003$), although both conditions had significantly higher CCR5 expression than the unstimulated but HIV-infected control (8.8 ± 2.9% of CD4+ T cells) (Figure 1D). Representative dot plots of flow cytometric data are shown in Supplementary Figure 7.

2.4.3 R848 (TLR7/8) Induced Activation of CD8+ T Cells

As CD8+ T cells are important effector cells and are crucial in viral control, we sought to assess the effect of TLR activation on CD8+ T cells. Similar findings were observed in the CD8+ and CD4+ T

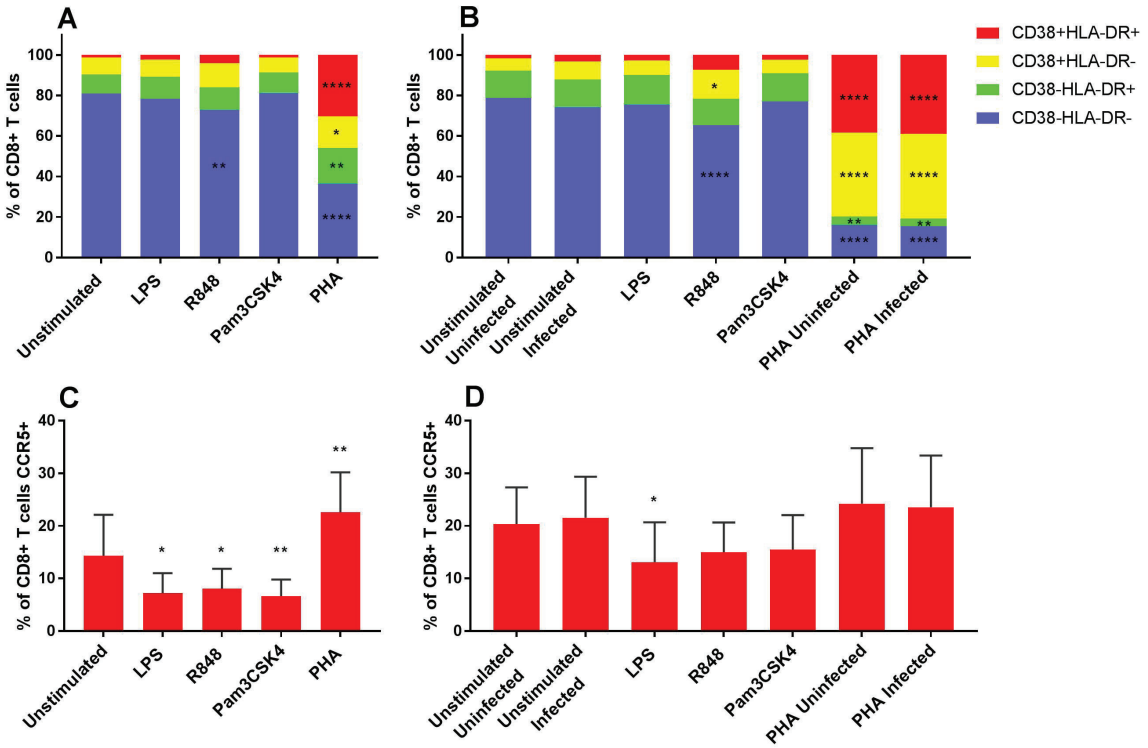

Figure 2: Activation profiles (A&B) and CCR5 expression (C&D) of CD8+ T cells on day 3 prior to HIV infection (A&C) and day 5 post HIV infection (B&D). PHA was used in a 1:500 dilution at a working concentration of 5mg/ml. TLR agonists were used at a final concentration of 2ug/ml. A repeated measures two-way ANOVA with Dunnett's multiple comparisons test was used for immune activation, and an ordinary one-way ANOVA with a Dunnett's multiple comparisons test for CCR5 expression. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the unstimulated/unstimulated infected control, unless otherwise shown. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

cells, with no significant activation observed with LPS or Pam3CSK4 stimulations compared to the unstimulated control at day 3 (Figure 2A). While there was a significant reduction of inactivated (CD38-HLA-DR-) CD8+ T cells with R848-stimulation compared to the unstimulated control ($p < 0.01$), this did not translate to a significant increase in any of the activated phenotypes (Figure 2A). PHA induced significant cellular activation, with all three activation phenotypes significantly increased compared to the unstimulated control ($p < 0.05$) on day 3 (Figure 2A). On day 5, only R848 significantly increased the frequency of CD8+ T cells expressing CD38+HLA-DR- ($p < 0.05$), compared to unstimulated cells ($p \leq 0.0001$) (Figure 2B). PHA, irrespective of infection status, maintained elevated levels of the activation phenotypes CD38+HLA-DR+ and CD38+HLA-DR- ($p \leq 0.0001$) compared to the unstimulated infected control (Figure 2B). Representative dot plots of flow cytometric data are shown in Supplementary Figure 8. Relevant mean ± SD for data depicted in Figure 2 are listed in Supplementary Table 2.

2.4.4 TLR-Mediated Reduction of CCR5 Expression on CD8+ T Cells Is Restored Over Time

CCR5 expression by CD8+ T cells was significantly lower than the unstimulated control (14.3 ± 7.8% of CD8+ T cells) with LPS (7.2 ± 3.8% of CD8+ T cells) ($p < 0.05$), R848 (8.1 ± 3.8% of CD8+ T cells) ($p < 0.05$) or Pam3CSK4 (6.7 ± 3.2% of CD8+ T cells) ($p < 0.01$) on day 3 (Figure 2C). Conversely, significantly elevated CCR5 expression on CD8+ T cells was observed with PHA (22.6 ± 7.6% of CD8+ T cells) compared to the unstimulated control ($p < 0.01$) on day 3. Only LPS (13.1 ± 7.6% of CD8+ T cells) maintained significantly lower CCR5 expression on CD8+ T cells than the unstimulated infected control (21.6 ± 7.8% of CD8+ T cells) on day 5 ($p < 0.05$) (Figure 2D). Representative dot plots of flow cytometric data are shown in Supplementary Figure 8.

2.4.5 LPS (TLR4) and R848 (TLR7/8) Induced Strong Inflammatory Cytokine Responses

Unsupervised hierarchical clustering analysis and Radial spider plots were used to evaluate cytokine production by PBMCs in response to stimulation with various TLR agonists (Figure 3 and Supplementary Figures 9, 10, respectively). Pam3CSK4 (TLR1/2) did not induce much cytokine production and tended to cluster closely with the unstimulated conditions, while LPS, R848, and PHA tended to cluster together, with similarly elevated inflammatory cytokine profiles. Cytokine induction by these TLR agonists appeared to be higher at day 3 than day 5 (Figure 3).

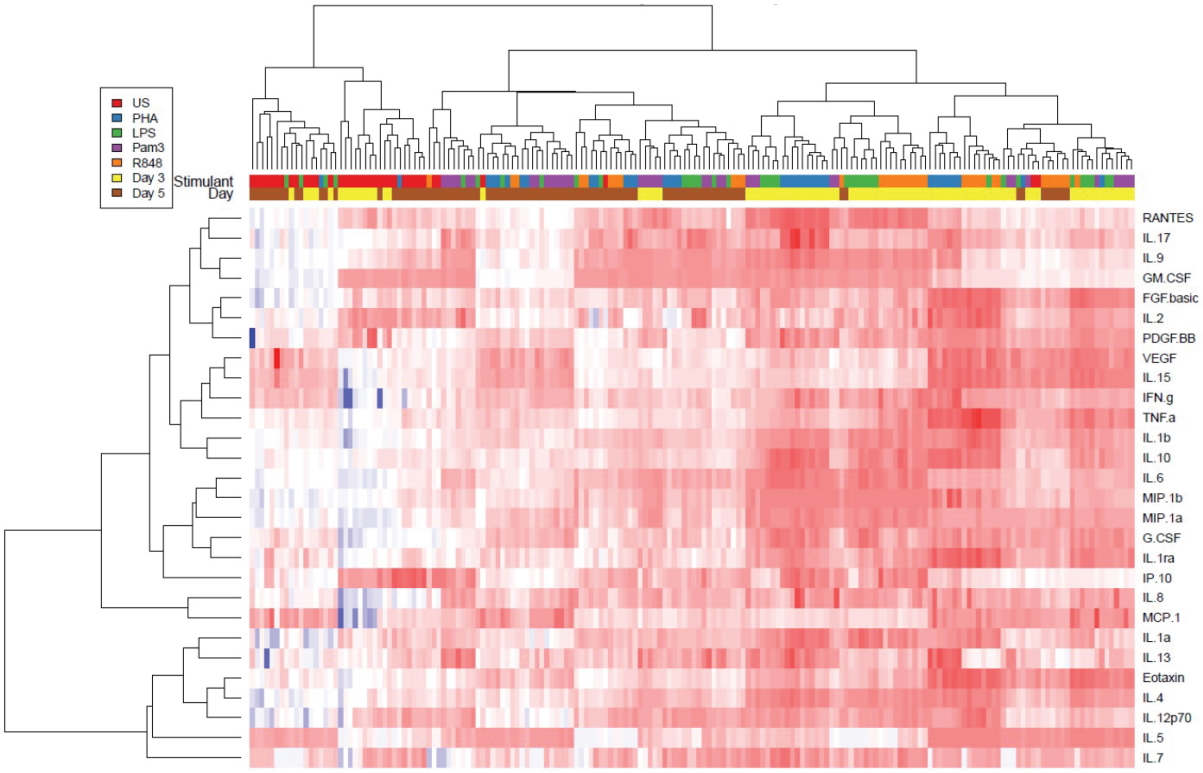

Figure 3: Unsupervised hierarchical cluster heat map analysis of 28 cytokines measured in cell culture supernatants on day 3 (yellow) and day 5 (brown) from the unstimulated (red), PHA (blue), LPS (green), Pam3CSK4 (purple) or R848 (orange) conditions. PHA was used at a 1:500 dilution at a working concentration of 5mg/ml. TLR agonists were used at a final concentration of 2ug/ml. In this heatmap, the redder the colour depicts the higher concentration, while the bluer the colour the lower the concentration. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

2.4.6 TLR4 and TLR7/8 Activation Induced the Greatest Inflammatory Profile, With TLR7/8 Activation Maintaining Inflammatory Cytokine Profile

As previous studies have shown that genital inflammation, defined by increased concentrations of a subset of 12 inflammatory cytokines and chemokines (including IL-1α, IL-1β, IL-6, IL-7, IL-8, IL-10, TNF-α, IP-10, MIP-1α, MIP-1β, MCP-1, and GM-CSF), predicted >3-fold increased risk for HIV acquisition (7), we sought to focus further analysis on the effect of TLR activation on these cytokines and 4 others (IL-12p70, IFN-γ, RANTES, and IL-17) that have crucial immunological roles. Both LPS and R848 induced significant production of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-12p70, IFN-γ, and TNF-α at day 3 ($p \leq 0.0001$ and $p \leq 0.0001$, respectively) compared to the unstimulated control (Figures 4A–F). Pam3CSK4 also elicited significantly elevated pro-inflammatory cytokines compared to the unstimulated control ($p < 0.001$), however these levels were generally lower than those observed with LPS or R848 (Figures 4A–F). Although cytokine induction was declining by day 5, pro- inflammatory cytokines IL-1β and IL-6 remained significantly elevated in the LPS ($p \leq 0.0001$), R848 ($p \leq 0.0001$), Pam3CSK4 ($p \leq 0.0001$), and PHA conditions ($p \leq 0.0001$) compared

to the unstimulated HIV-infected control (Figures 4H,I). IL- 1α was significantly elevated in the R848 and PHA uninfected conditions ($p < 0.05$) (Figure 4G), while IFN-γ and TNF-α were significantly elevated only in the R848 condition ($p < 0.05$) (Figures 4K, L). Relevant mean ± SD for data depicted in Figure 4 are listed in Supplementary Table 3.

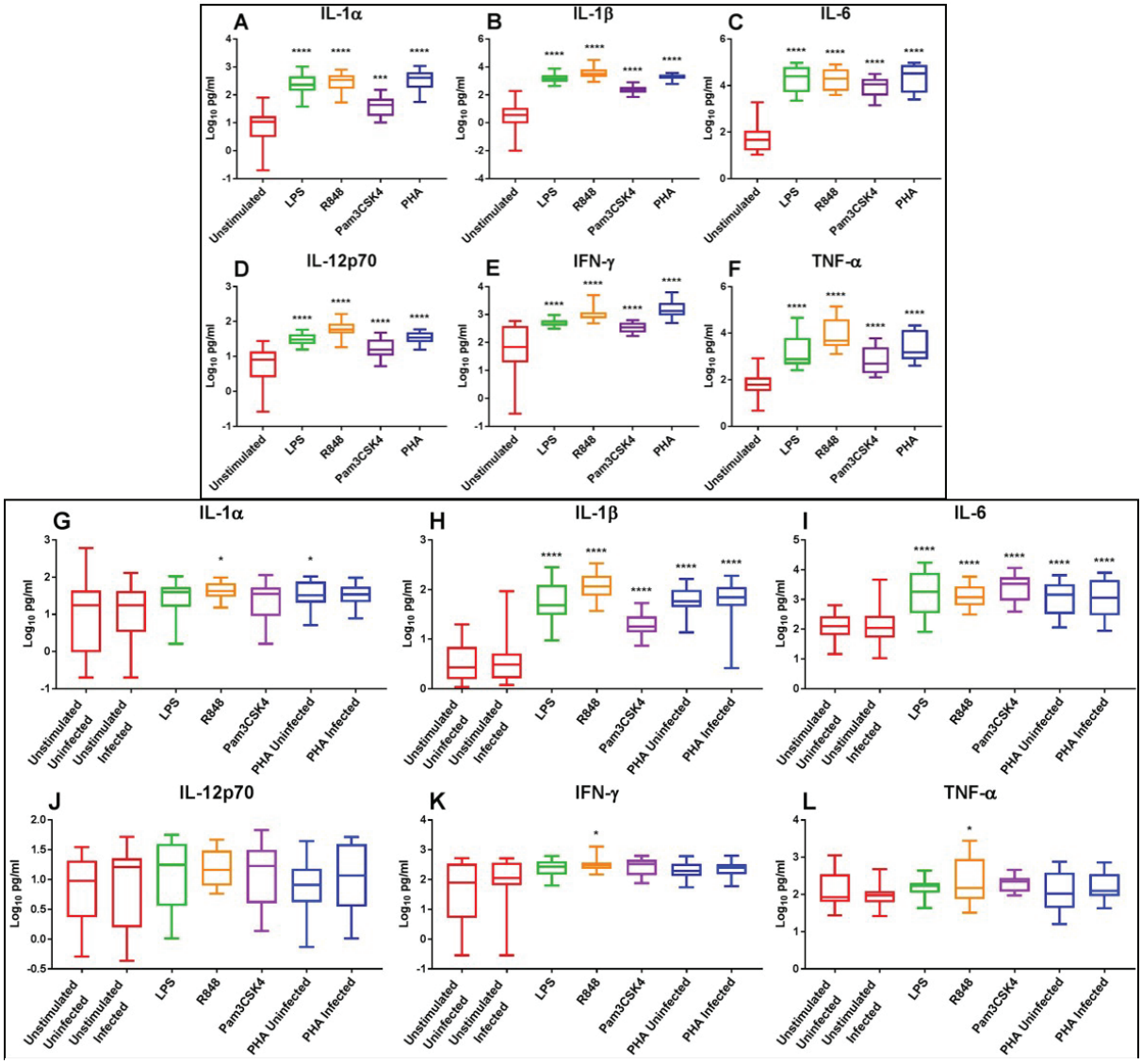

Figure 4: Box and Whisker plots showing mean ± SD Log_{10} concentrations of pro-inflammatory cytokines IL-1α (**A&G**), IL-1b (**B&H**), IL-6 (**C&I**), IL-12p70 (**D&J**), IFN-γ (**E&K**) and TNF-α (**F&L**) from unstimulated (red), LPS (green), R848 (orange), Pam3CSK4 (purple) and PHA (blue) conditions on day 3 prior to HIV infection (**top box: A-F**) and day 5 post HIV infection (**bottom box: G-L**). TLR agonists were used at a final concentration of 2ug/ml. PHA was used at a 1:500 dilution at a working concentration of 5mg/ml. All TLR stimulation conditions were infected. An ordinary one-way ANOVA with a Dunnett's multiple comparisons test was performed. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the unstimulated control. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

2.4.7 Potent Chemokine Response to TLR Activation, With Concomitant Downregulation of IP-10

At day 3, PHA stimulation or TLR activation with LPS, R848 or Pam3CSK4 significantly increased the levels of chemotactic cytokines IL-8 ($p \leq 0.0001$), MIP-1α ($p \leq 0.0001$), MIP-1β ($p \leq 0.0001$), MCP-1 ($p < 0.05$), and RANTES ($p < 0.05$) compared to the unstimulated control (Figures 5A–C,E,F). Additionally, IP- 10 was significantly increased with PHA stimulation compared to the unstimulated

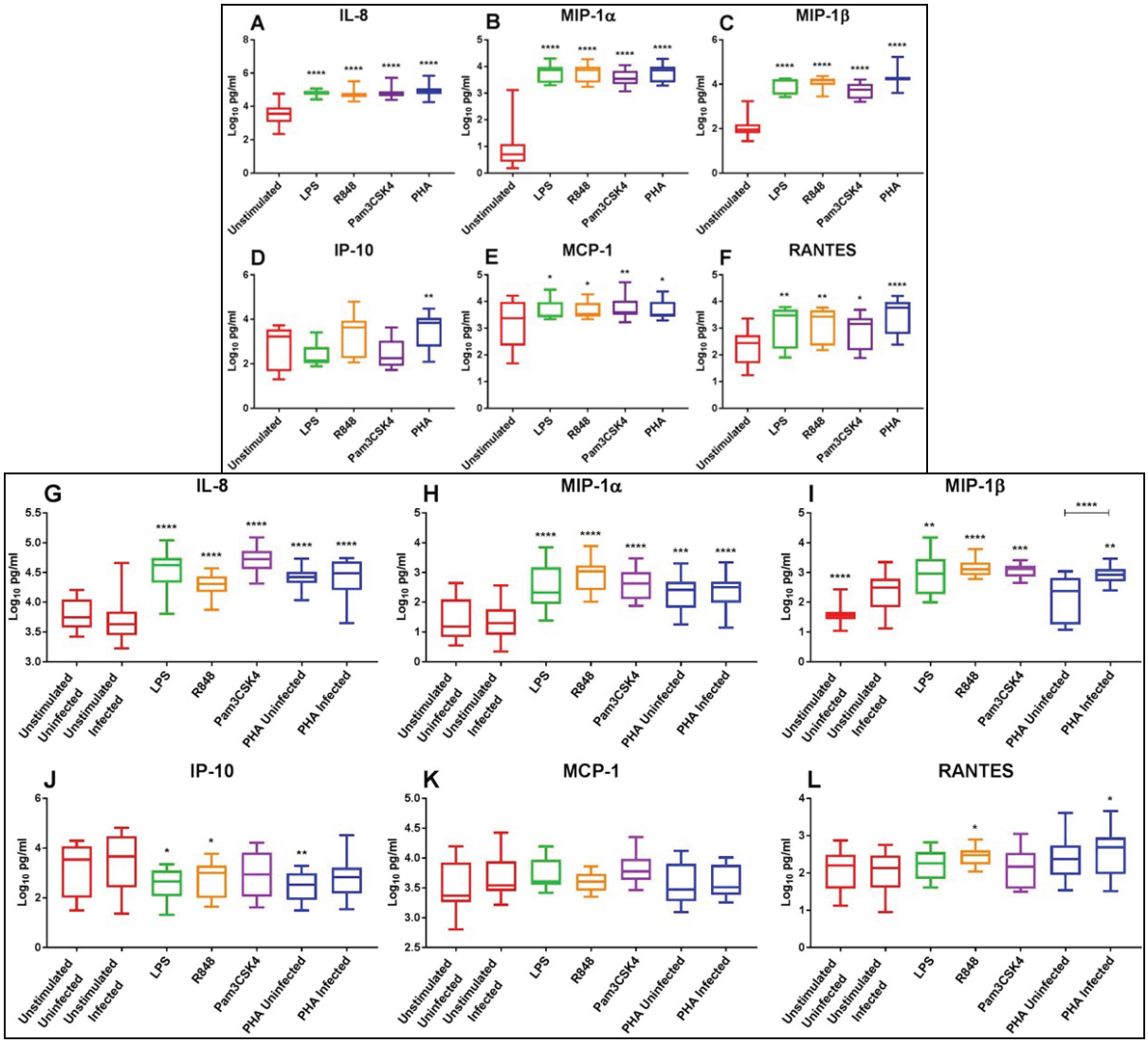

Figure 5: Box and Whisker plots showing mean ± SD Log_{10} concentrations of chemotactic cytokines IL-8 (**A&G**), MIP-1α (**B&H**), MIP-1β (**C&I**), IP-10 (**D&J**), MCP-1 (**E&K**) and RANTES (**F&L**) from unstimulated (red), LPS (green), R848 (orange), Pam3CSK4 (purple) and PHA (blue) conditions on day 3 prior to HIV infection (**top box: A-F**) and day 5 post HIV infection (**bottom box: G-L**). TLR agonists were used at a final concentration of 2ug/ml. PHA was used in a 1:500 dilution at a working concentration of 5mg/ml. All TLR stimulation conditions were infected. An ordinary one-way ANOVA with a Dunnett's multiple comparisons test was performed. Significance displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the unstimulated control. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate

control ($p < 0.01$) (Figure 5D). R848 appeared to be a more potent inducer of IP-10 than either LPS or Pam3CSK4 (Figure 5D). At day 5, the chemokines IL-8 and MIP-1α remained significantly elevated in the LPS ($p \leq 0.0001$), R848 ($p \leq 0.0001$), Pam3CSK4 ($p \leq 0.0001$) and PHA conditions ($p < 0.001$) compared to the unstimulated infected control (Figures 5G,H). TLR activation with LPS ($p < 0.01$), R848 ($p \leq 0.0001$), or Pam3CSK4 ($p < 0.001$) significantly increased MIP-1β compared to the unstimulated infected control (Figure 5I). The PHA infected, but not the uninfected, condition had significantly increased MIP-1β compared to the unstimulated infected control ($p < 0.01$) (Figure 5I). Interestingly, with regards to MIP-1β, the unstimulated and PHA infected conditions had significantly greater concentrations than the matched uninfected conditions ($p \leq 0.0001$) (Figure 5I). Concentrations of RANTES were significantly elevated in the R848 and PHA infected conditions compared to the unstimulated control ($p < 0.05$) (Figure 5L). Conversely, IP-10 concentrations were significantly reduced in the LPS ($p < 0.05$), R848 ($p < 0.05$) and the PHA uninfected ($p < 0.01$) conditions compared to the unstimulated infected control (Figure 5J). Relevant mean ± SD for data depicted in Figure 5 are listed in Supplementary Table 4.

2.4.8 Potent Induction of IL-17 Response with TLR Agonists LPS (TLR4), R848 (TLR7/8) and Pam3CSK4 (TLR1/2)

At day 3, the haematopoietic IL-7 and IL-17 were significantly elevated following LPS ($p < 0.01$), R848 ($p < 0.01$), and PHA stimulation ($p < 0.001$) compared to the unstimulated controls, while only IL-17 was significantly elevated with Pam3CSK4 ($p \leq 0.0001$) (Figures 6A,B). Interestingly, IL-17 increased in a dose-dependent manner with R848 stimulation, with this effect more prominent at day 3 than day 5 (Supplementary Figure 11). PHA, but not TLR activation, significantly increased GM-CSF compared to the unstimulated control ($p < 0.05$) (Figure 6C). The anti-inflammatory cytokine IL-10 was significantly elevated by LPS, R848, Pam3CSK4, and PHA stimulation compared to the unstimulated control ($p \leq 0.0001$) (Figure 6D). At day 5, the levels of IL-17 were elevated in the LPS ($p \leq 0.0001$), R848 ($p < 0.05$), Pam3CSK4 ($p < 0.001$), and PHA-stimulated and uninfected ($p < 0.001$) and infected ($p \leq 0.0001$) conditions compared to the unstimulated infected control (Figure 6F). Similarly, IL-10 levels were elevated in the R848 ($p < 0.001$), Pam3CSK4 ($p < 0.05$), and PHA infected ($p < 0.01$) conditions compared to the unstimulated infected control (Figure 6H). Relevant mean ± SD for data depicted in Figure 6 are listed in Supplementary Table 5.

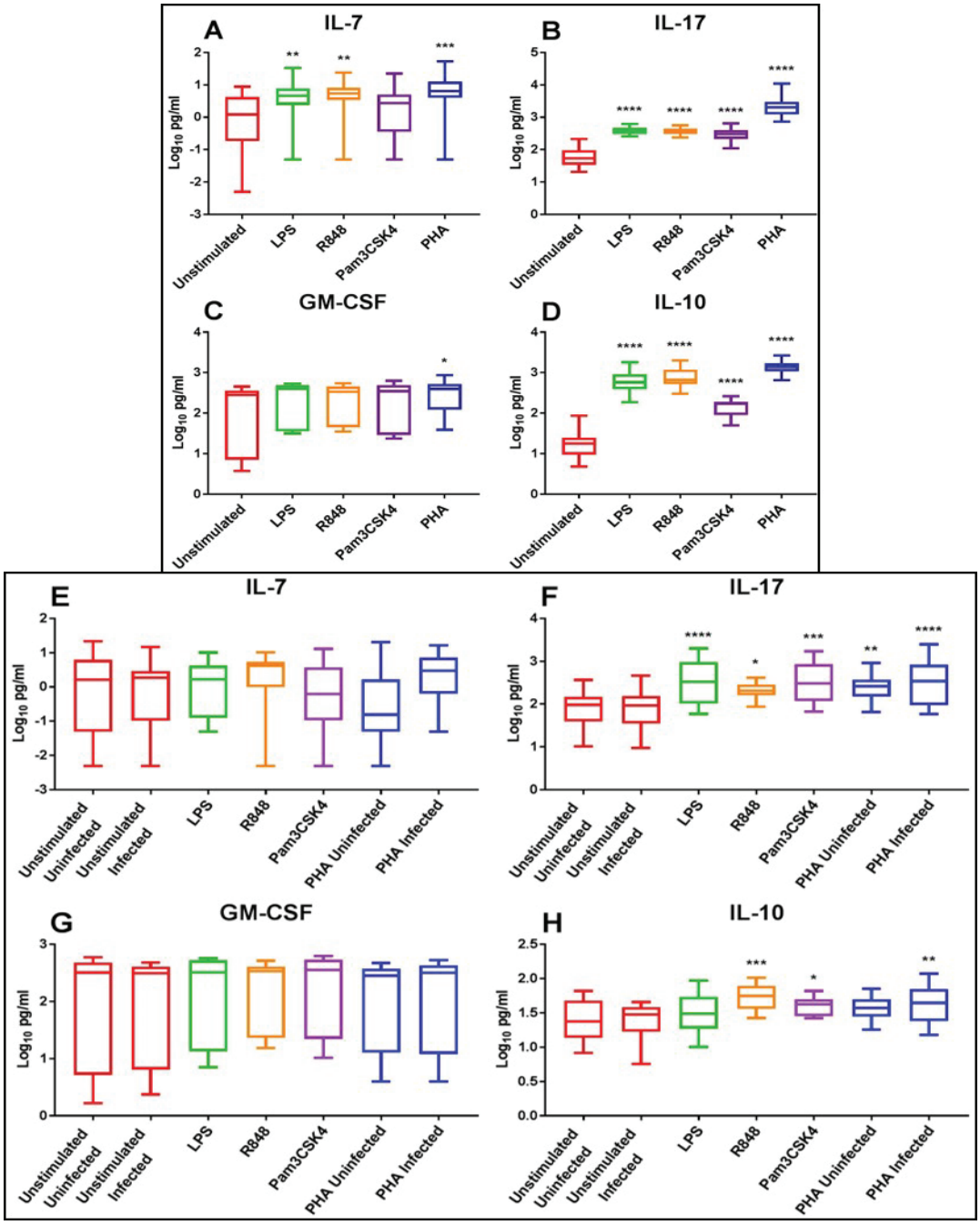

Figure 6: Box and Whisker plots showing mean ± SD Log_{10} concentrations of haematopoietic cytokines IL-7 (**A&E**) and IL-17 (**B&F**), the growth factor GM-CSF (**C&G**) and the anti-inflammatory cytokine IL-10 (**D&H**) from unstimulated (red), LPS (green), R848 (orange), Pam3CSK4 (purple) and PHA (blue) conditions on day 3 prior to HIV infection (**top box: A-F**) and day 5 post HIV infection (**bottom box: G-L**). TLR agonists were used at a final concentration of 2ug/ml. PHA was used at a 1:500 dilution at a working concentration of 5mg/ml. All TLR stimulation conditions were infected. An ordinary one-way ANOVA with a Dunnett's multiple comparisons test was performed. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the unstimulated control. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

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2.4.9 TLR-Induced Inflammation Limits HIV Infection of CD4+ T Cells

We determined the effect of TLR-mediated inflammation on the susceptibility of CD4+ T cells to R5 tropic HIV infection with NL4-3 AD8 HIV. Stimulation with LPS (TLR4; $p < 0.01$), and R848 (TLR7/8) to a lesser extent, reduced HIV infection of CD4+ T cells compared to unstimulated cells (Figure 7). Pam3CSK4 induced infection similar to that of the unstimulated infected control. PHA-stimulation resulted in significantly more infection than all other conditions ($p < 0.001$), with approximately 25% of CD4+ T cells infected (Figure 7). Furthermore, using a combination of TLR agonist and PHA, we found that even in the presence of hyper activation, stimulation with either LPS or R848 protected CD4+ T cells from HIV infection ($p > 0.0001$; Supplementary Figure 12).

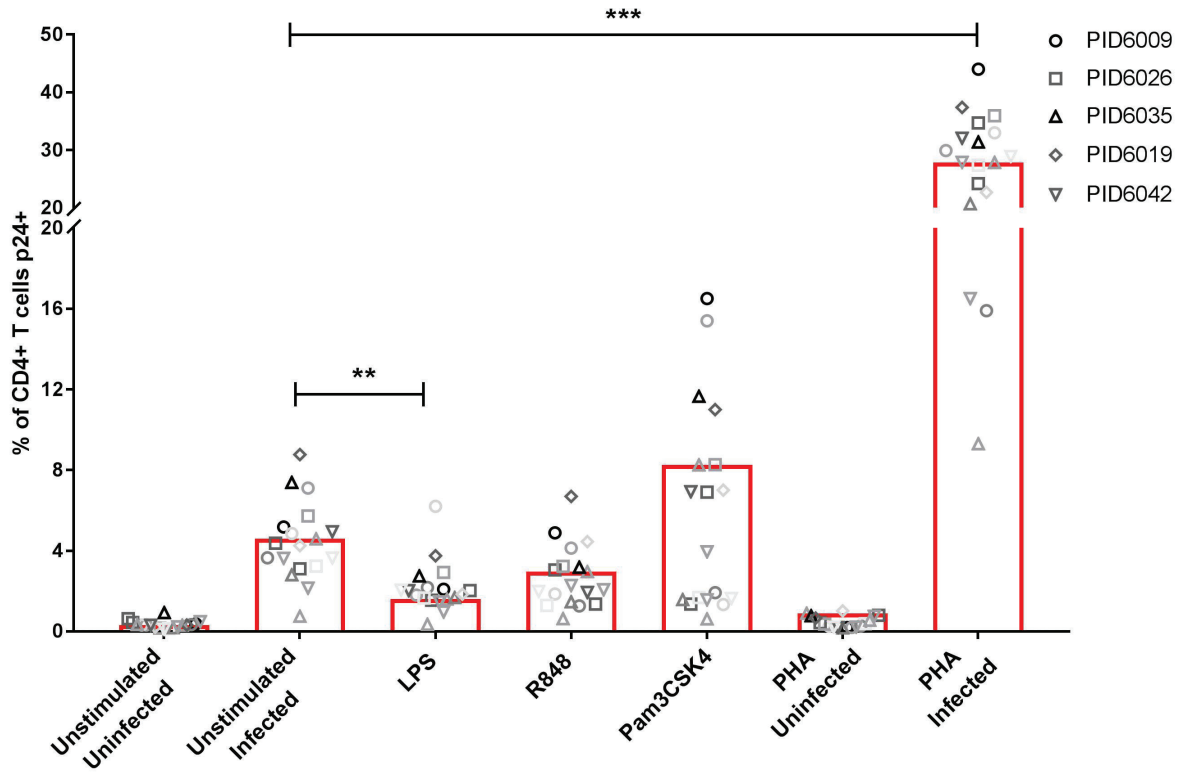

Figure 7: Infection rates (measured by p24 expression) of CD4+ T cells either unstimulated or stimulated with PHA or TLR agonists; LPS (TLR4), Pam3CSK4 (TLR1/2) or R848 (TLR7/8). Each symbol represents a donor, while different shades of each symbol represent repeats for that donor. PHA was used at a 1:500 dilution at a working concentration of 5mg/ml. TLR agonists were used at a final concentration of 2ug/ml. Significance was assessed by two-way ANOVA with Dunnett's multiple comparisons test. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.0001$) compared to the unstimulated infected control. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

2.5 Discussion

The purpose of this study was to identify the effects of TLR agonists on T cell activation, cytokine responses, and the ability of HIV to infect CD4+ T cells. TLR stimulation resulted in limited T cell activation, down-regulation of the CCR5 co- receptor necessary for HIV entry as well as potent inflammatory cytokine responses, creating an environment less conducive to HIV infection of CD4+ T cells. CD4+ T cells have been shown to express various classes of TLRs (40), providing the ability to recognize and respond to TLR agonists. In our study, no significant increase in activation marker expression was observed for either CD4+ or CD8+ T cells when stimulated with agonists targeting TLR1/2, 7/8, or 4. It has been demonstrated that TLR receptors require co- stimulation, needing primary T-cell receptor (TCR) engagement to induce functional T cell responses (41). This is evident with the mitogen, PHA, where significant CD4+ T cell activation was observed following stimulation. PHA is a plant lectin that binds to carbohydrates on the cell surface, including the TCR, thereby inducing proliferation and activation of T lymphocytes (42–45). TLR7/8 agonist R848 induced subtle activation of CD4+ T cells, possibly due to intracellular recognition of R848 that may have stimulated an adaptive Th1 immune response even in the absence of TCR signalling (46). Effector CD8+ T cells are generally highly reliant on CD4+ T cell help for functional and memory responses (47). Thus, the modest activation of CD4+ T cells with R848 was also observed in the CD8+ T cells, providing further evidence of a cytotoxic Th1 response. Distinct activation of both CD4+ and CD8+ T cells was observed with PHA stimulation, likely due to the robust TCR engagement by PHA. A distinct inflammatory response was observed when looking at the cytokine profiles induced by TLR stimulation compared to the unstimulated control. TLR agonists LPS and R848 elicited potent cytokine storms, similar to previous studies that showed increases in pro-inflammatory and chemotactic cytokines after stimulation with these TLR agonists (32–34). R848 also induced the strongest IFN-γ and IL-12p70 response, reminiscent of the anti-viral Th1 response (48). Furthermore, R848 stimulated the prolonged expression of IL-1α, IFN-γ, and TNF-α, further providing evidence of prolonged inflammatory responses associated with the adaptive immune response. Additionally, cytokines associated with immune modulation, such as IL-1RA, IL-4, and IL-10 were upregulated, presumably to prevent a prolonged inflammatory response. These data suggest that the inflammatory responses associated with LPS and R848 stimulation are regulated through immunomodulatory cytokines to counteract the exaggerated pro-inflammatory response. Furthermore, Pam3CSK4-mediated expression of IL-1RA, IL- 4, and IL-10 was much lower than LPS or R848, suggesting that this TLR agonist induces either a Th2 biased or more regulatory cytokine response or a less potent Th1 response. This phenomenon may be in part due to this TLR1/2 agonist being analogous to gram-positive bacteria, which are generally less pathogenic and less inflammatory than gram-negative bacteria (49, 50). The chemokine responses induced by TLR stimulation were potent, with prolonged IL-8, MIP-1α and MIP-1β expression. MIP-1α, MIP-1β, and RANTES are ligands for CCR5 and are associated with the recruitment of CCR5+ cells (51). In the

female genital tract, these chemokines are important factors that are associated with increased risk for HIV acquisition in women (7, 8). Interestingly, MIP-1β was increased in the unstimulated and PHA infected conditions compared to their uninfected controls at day 5, suggesting that HIV itself induces the expression of MIP-1β. Dai and Stevenson (52) similarly showed that HIV-1 Nef induced the production of MIP-1β (52). Alternatively, the increased detection of soluble MIP-1β may be as a result of the competitive binding of HIV to CCR5 precluding MIP-1β binding. Furthermore, the increased expression of IL-8 and MCP-1 at day 3, which are chemotactic for neutrophils (53) and monocytes (54) respectively, provides the basis for the initiation of innate immune responses, which then further potentiate inflammation. Interestingly, higher prolonged IL-8 levels were observed with the bacterial TLR agonists LPS and Pam3CSK4 compared to the viral TLR agonist R848, suggesting that neutrophils would be sufficient for control and clearance of bacterial infections, whereas viral infections generally require a Th1 cytotoxic adaptive immune response to prevent infection. This is supported by the finding that IP-10 was induced at significantly higher concentrations by R848 than the bacterial TLR agonists. Compared to the unstimulated control at day 5, significantly less IP-10 was produced following LPS and R848 stimulation, but not by Pam3CSK4. This further supports the findings above which allude to the induction of an adaptive Th1 response to TLR7/8 activation, but not TLR1/2, while there is potential for the initiation of an adaptive response with continued TLR4 activation. We found no effect of TLR stimulation on the production of GM-CSF, which was surprising given the inflammatory response observed. GM-CSF stimulates granulocyte and macrophage differentiation (55), which we postulated would be key in the innate immune response, especially against the bacterial TLR agonists. However, previous literature suggests that GM-CSF and TLRs appear to have a complex relationship. While GM-CSF stimulation is known to downregulate the expression of TLR1, 2 and 4 on human monocytes (56), it has also been shown to enhance LPS-mediated pro-inflammatory cytokine production in murine microglia via the upregulation of TLR4 and CD14 (57). Similarly, Bauer et al. (58) found that GM-CSF partially restored TLR-mediated functional responses of monocytes from septic patients (58). One possibility for blunted GM-CSF responses was that a PBMC model was used, so the need for GM-CSF may be lost in this lymphocyte-enriched system. Th17 cells have an important role to play in the homeostasis and maintenance of the mucosal barrier (59–62), as well as increased susceptibility to HIV infection (63, 64). One of the limitations of this study is that we did not assess the Th17 cells by flow cytometry, however IL-17, as well as IL-7, are good surrogate indicators for Th17 cell functions (65–67). In our study, we found elevated IL-7 in all stimulation conditions, with the exception of Pam3CSK4, possibly suggesting a dampened or tolerogenic response to this TLR agonist. Furthermore, IL- 17 was also elevated in all stimulated conditions at both day 3 and day 5. Interestingly, a less potent IL-17 response was seen at day 5 in R848 stimulated cells than those stimulated with either LPS or Pam3CSK4. These data suggest that the sensing of bacterial antigens, analogous to microbial translocation, may induce a prolonged and potent Th17 response to maintain homeostasis and integrity of the mucosal barrier. However, the observed significant dose-dependent increase of IL-17 with R848

stimulation suggests a stronger Th17 response with increased viral sensing. Unexpectedly, TLR stimulation did not lead to increased HIV infection of CD4+ T cells. Initially, we assumed that the lack of CD4+ T cell activation could explain this observation, given that activated CD4+ T cells are preferentially and more easily infected (10, 68). However, in preliminary follow up experiments we found that even in a hyper activated setting, where PHA in addition to TLR agonists LPS or R848 were used, HIV infection rates of CD4+ T cells were still lower in the TLR agonist and PHA conditions compared to the PHA only control, thereby indicating other mechanisms at play. One possibility for the reduced HIV infection with LPS or R848 stimulation is through increased CC- binding chemokines which compete with HIV for CCR5 binding (69, 70), which was determined as a mechanism of resistance to R5-tropic viruses in elite controllers (71). This model system supports the concept of increased CC-binding chemokines relative to a decrease in CCR5 expression with a concomitant reduction in HIV infection. Furthermore, activation of TLR4 and TLR7/8, by LPS and R848 respectively, has been shown to induce type 1 interferons (72), which have potent antiviral effects and most likely played a role in the observed protection against HIV infection (73). Similarly, the observed protective effect suggests the induction of an innate antiviral response (74). This innate antiviral response likely involves host factors such as the APOBEC family of proteins, which are known to have nucleic acid editing functions (75–77), and SAMHD1, known to limit intracellular deoxynucleoside triphosphates thereby restricting viral replication (78, 79). This model of TLR stimulation creating an environment less conducive to HIV infection, is similar to the findings of inflammation and partial immune activation in highly exposed sero-negatives (HESN) (80, 81), in contrast to findings in other HESN cohorts showing immune quiescence (82). These data highlight the complex and heterogeneous nature of inflammation and immune activation, that determine HIV risk. To further understand the effects of TLR stimulation on adaptive cellular activation, and address the limitations of this model system, these experiments could be repeated with the addition of a TCR stimulant such as anti-CD3/CD28 beads. Furthermore, we used markers of activation which are more relevant to assessing chronic immune activation (83). Markers of acute cellular activation, such as CD69 (83), may have been more appropriate. Additionally, innate antiviral pathways including interferon stimulated genes, type 1 interferons, APOBEC and SAMHD1 should be assessed to determine their potential roles in the observed protective effect by TLR agonists LPS and R848. Furthermore, the activation of innate immune cells such as monocytes and DCs were not assessed, and these could have provided valuable insight into the mechanisms of a TLR- mediated immunity. Antigen presenting cells such as DCs, macrophages and monocytes are generally the first line of defence in the recognition of pathogens, and subsequently activate the adaptive immune responses (84). However, monocytes constitute approximately 20% of the cell population in PBMCs (85), and so microbial recognition would have occurred. There was an overall decrease in cytokines from day 3 to day 5, which is likely due to the removal of stimulants at day 3 prior to HIV infection. In future, it will be important to assess HIV infection rates in the presence of continued TLR stimulation. Furthermore, we assessed cytokine

expression from culture supernatants and could not distinguish the cellular origin of cytokines. As there was a lack of T cell activation in TLR-stimulated conditions, the observed cytokine responses were likely mediated by innate immune cells such as monocytes and neutrophils. Therefore, performing intracellular cytokine staining (ICS) for a few key cytokines would allow better discrimination of the main cells producing key inflammatory cytokines. Furthermore, ICS would allow better discrimination of cellular functionality, allowing clearer assessment of cellular subsets. While PBMCs may not fully reflect cells in the genital tract of women, this culture system provides valuable insight into the mechanisms of TLR-induced inflammation. Additionally, PBMCs represent circulating cells which are a combination of peripheral and trafficked cells from the tissue, which better reflects an in vivo setting compared to depleted or purified immune cell models or cell lines. Jaspan et al. (12) previously reported that the extent of T cell activation in blood significantly predicted activation of these cells at the cervix (12). Ex vivo samples, such as cervical cells or explants are most biologically representative, however there are still many difficulties in obtaining and assessing immunity even in these types of samples (86–88). These data highlight the inflammatory effects of TLR agonists on PBMCs, and the need for TCR engagement to induce activation of adaptive T cells. These results also provide insight into the nature of the immune responses elicited by various TLR agonists, with specific responses induced to particular pathogenic signals. Together, these data provide important mechanistic insights for HIV acquisition as the types of immune responses induced according to the pathogens or combinations of pathogens sensed, could govern HIV risk.

2.6 Data availability

The datasets generated for this study are available on request to the corresponding author.

2.7 Ethics statement

This study was carried out in accordance with the recommendations of the University of KwaZulu-Natal (UKZN) Biomedical Research Ethics Committee (BREC). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the UKZN BREC (BE433/14.

2.8 Author contributions

SA, J-AP, LL, LM, and DA acquired funding for the study and edited the manuscript. LL, LM, J-AP, AS, and DA assisted in study design and analysis of data. RC designed and ran experimental procedures, acquired and analysed data, wrote and edited the manuscript. AS assisted in study design, provided laboratory space for experimental procedures and edited the manuscript.

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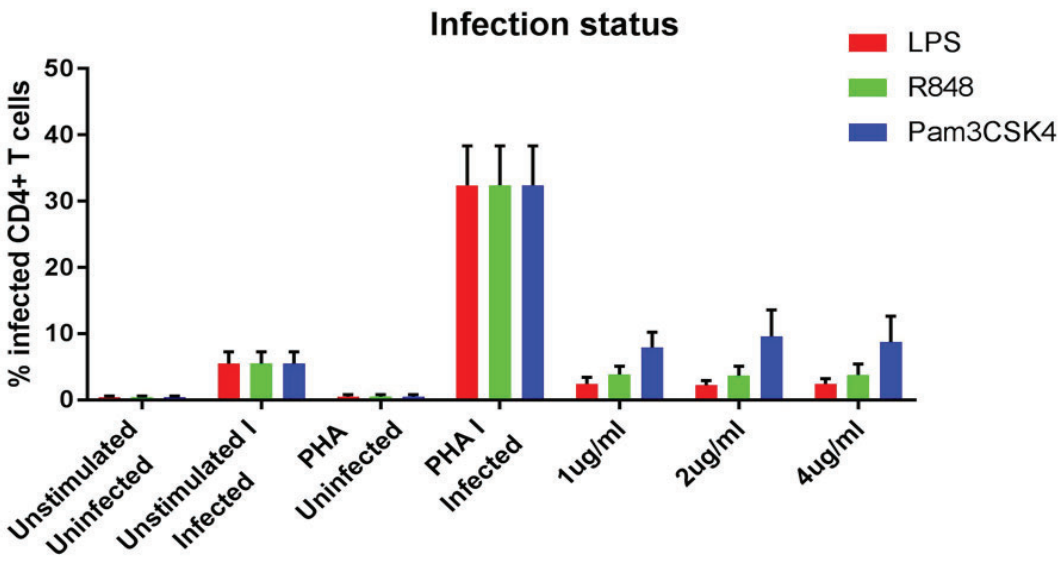
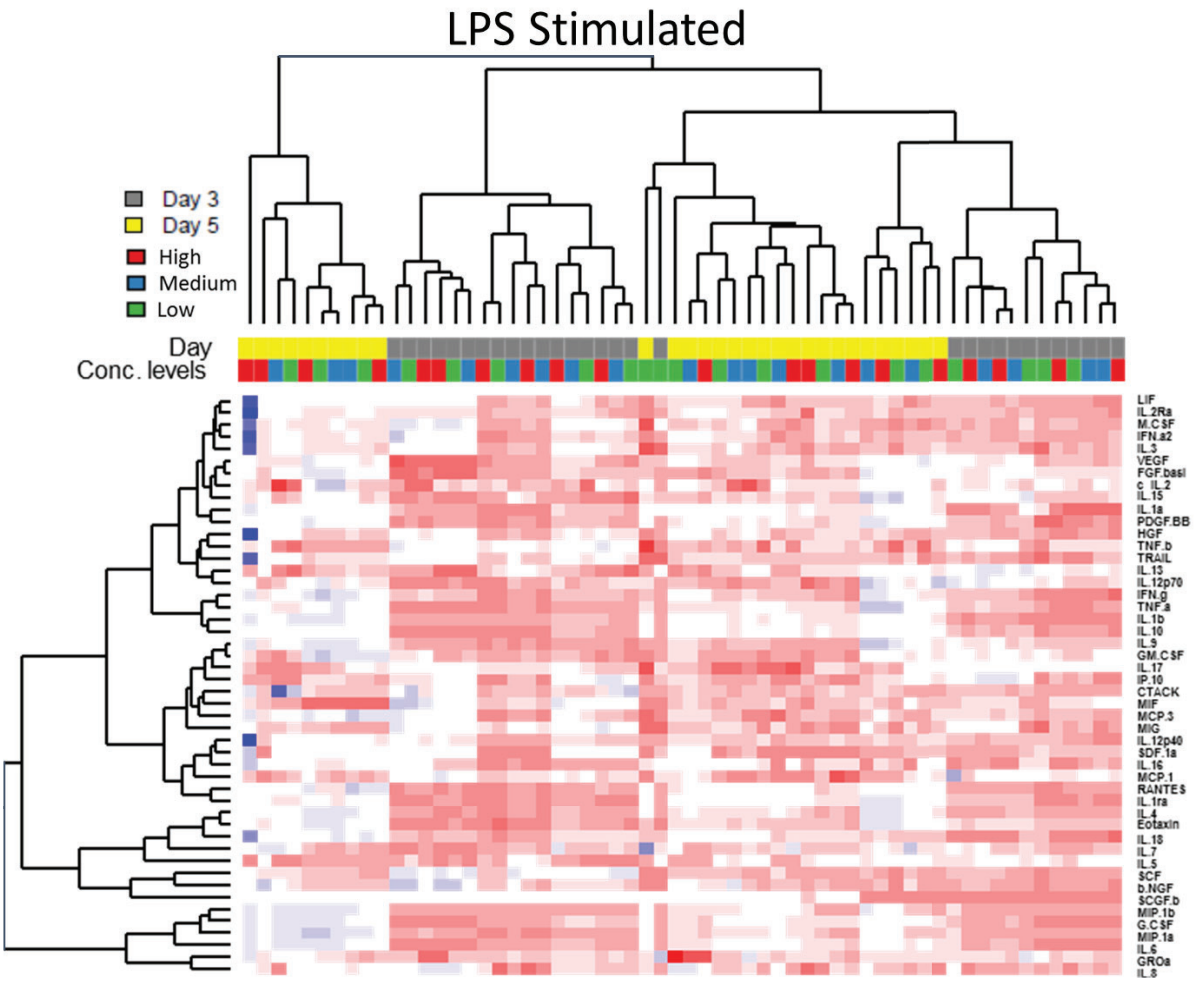
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.12 Supplementary data



Supplementary Figure 1: PBMCs unstimulated or PHA stimulated with or without virus as controls. All TLR stimulation conditions were infected. Infection measured by p24 quantification by flow cytometry at day 5. Sample size, n=5, each donor run in duplicate.



Supplementary Figure 2: Hierarchical cluster heat map analysis of all 48 cytokines from the LPS stimulated condition at LPS concentrations of low (green, 1µg/ml), medium (blue, 2µg/ml) and high (red, 4µg/ml) on day 3 (grey) and day 5 (yellow). In this heatmap, the redder the colour depicts the higher concentration, while the bluer the colour the lower the concentration. Sample size, n=5, each donor run in duplicate.

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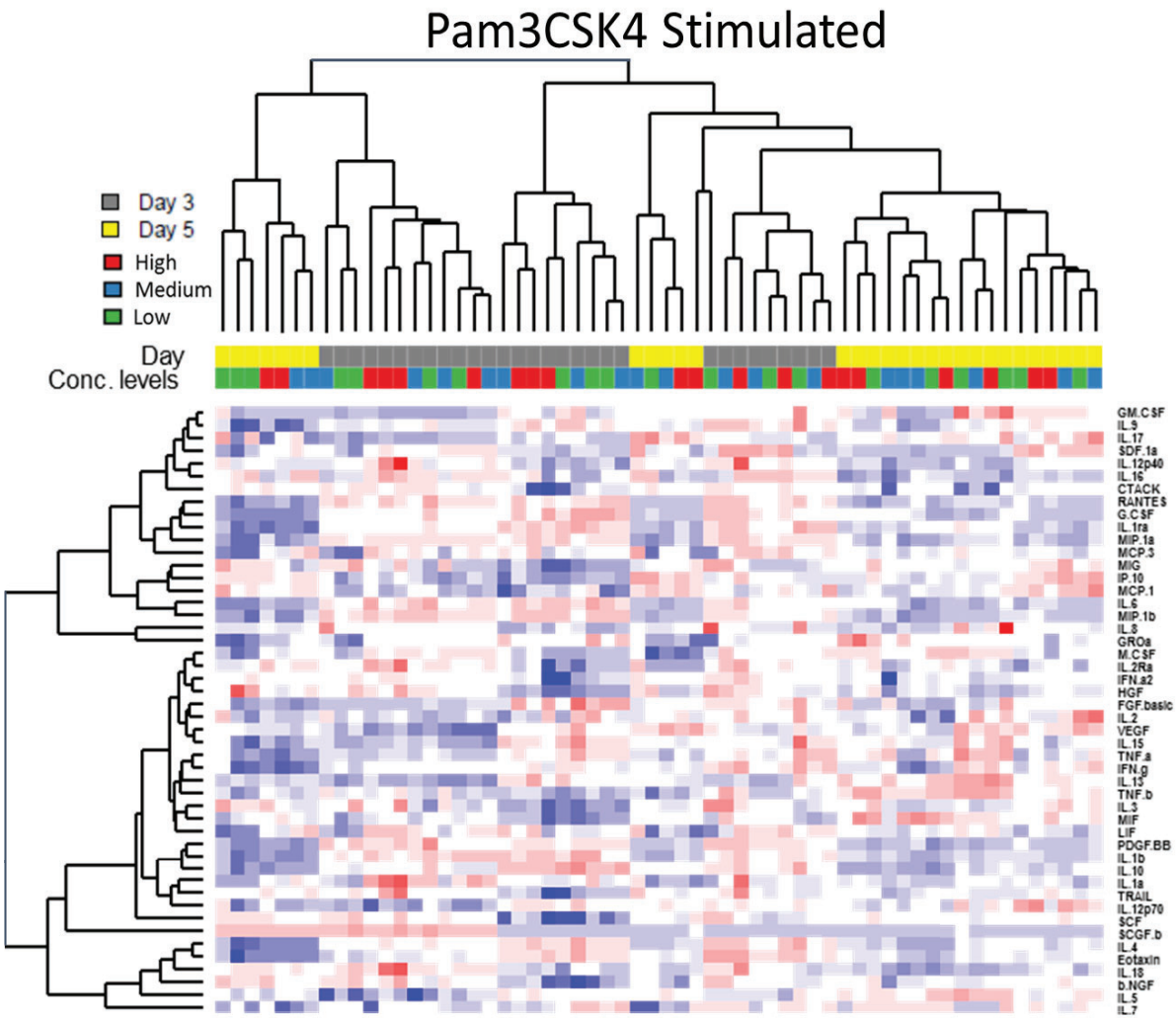

Supplementary Figure 3: Hierarchical cluster heat map analysis of all 48 cytokines from the Pam3CSK4 stimulated condition at Pam3CSK4 concentrations of low (green, 1µg/ml), medium (blue, 2µg/ml) and high (red, 4µg/ml) on day 3 (grey) and day 5 (yellow). In this heatmap, the redder the colour depicts the higher concentration, while the bluer the colour the lower the concentration. Sample size, n=5, each donor run in duplicate.

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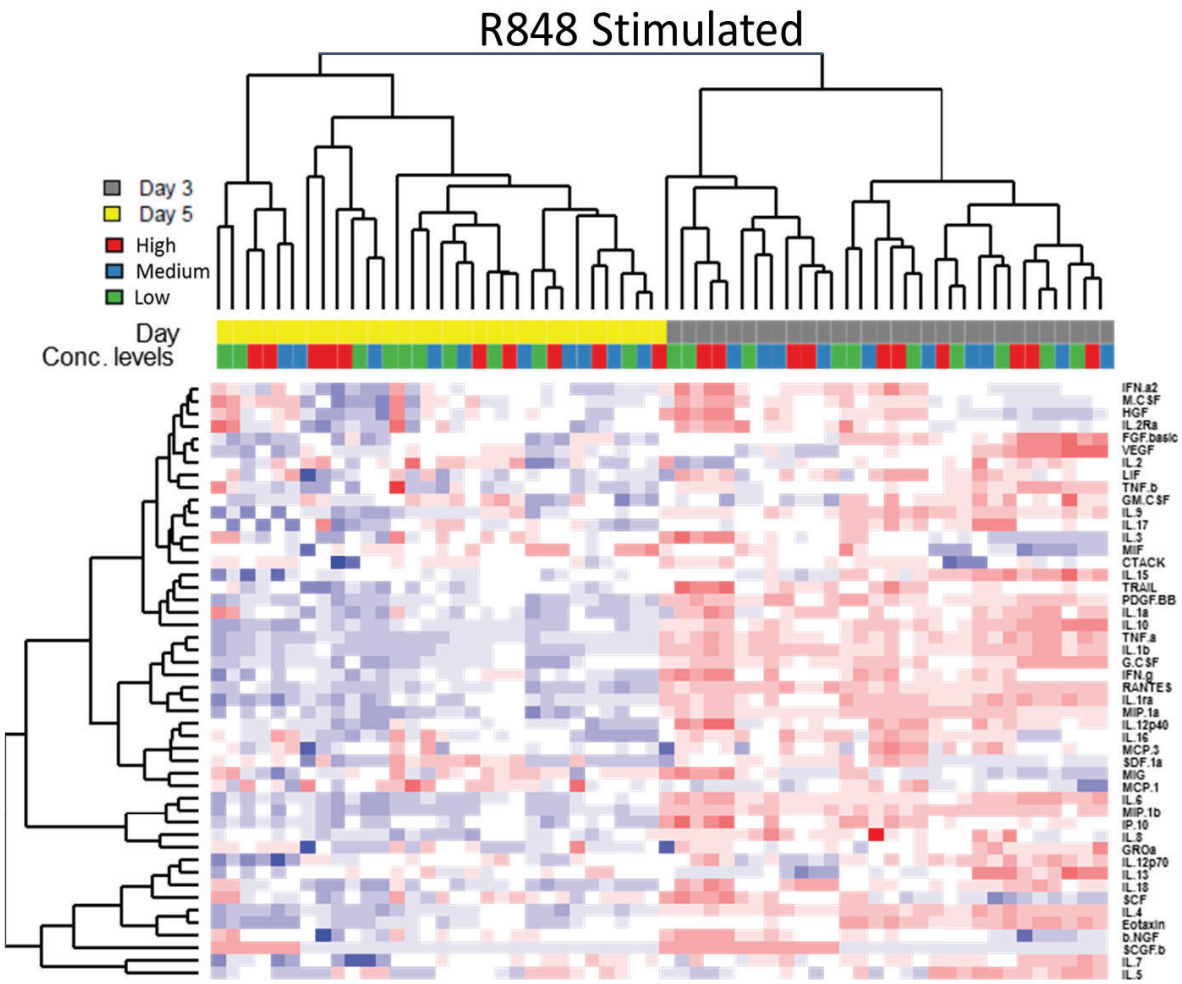

Supplementary Figure 4: Hierarchical cluster heat map analysis of all 48 cytokines from the R848 stimulated condition at R848 concentrations of low (green, 1µg/ml), medium (blue, 2µg/ml) and high (red, 4µg/ml) on day 3 (grey) and day 5 (yellow). In this heatmap, the redder the colour depicts the higher concentration, while the bluer the colour the lower the concentration. Sample size, n=5, each donor run in duplicate.

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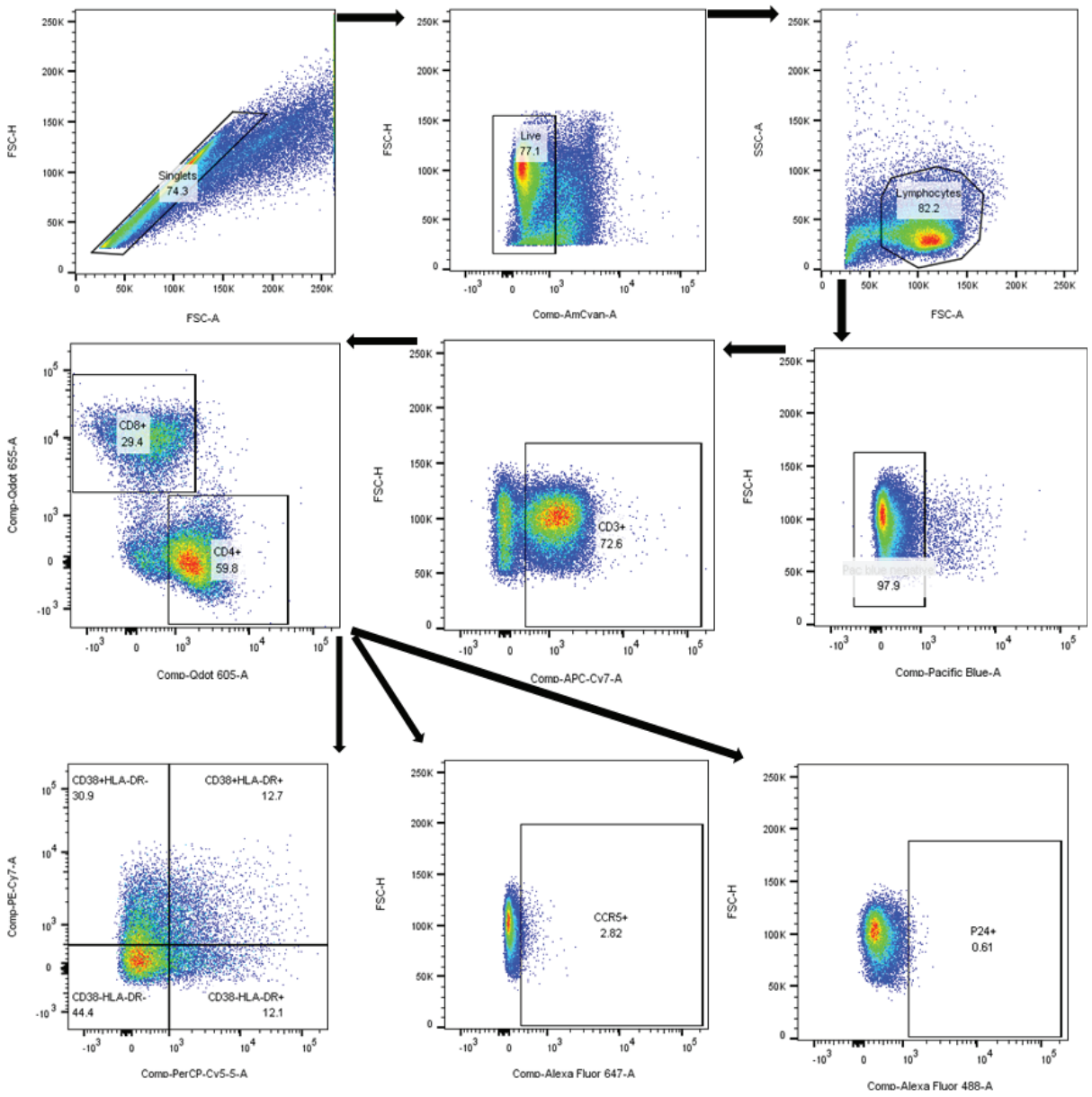

Supplementary Figure 5: Schematic diagram of the gating strategy used for analyses of flow cytometric data

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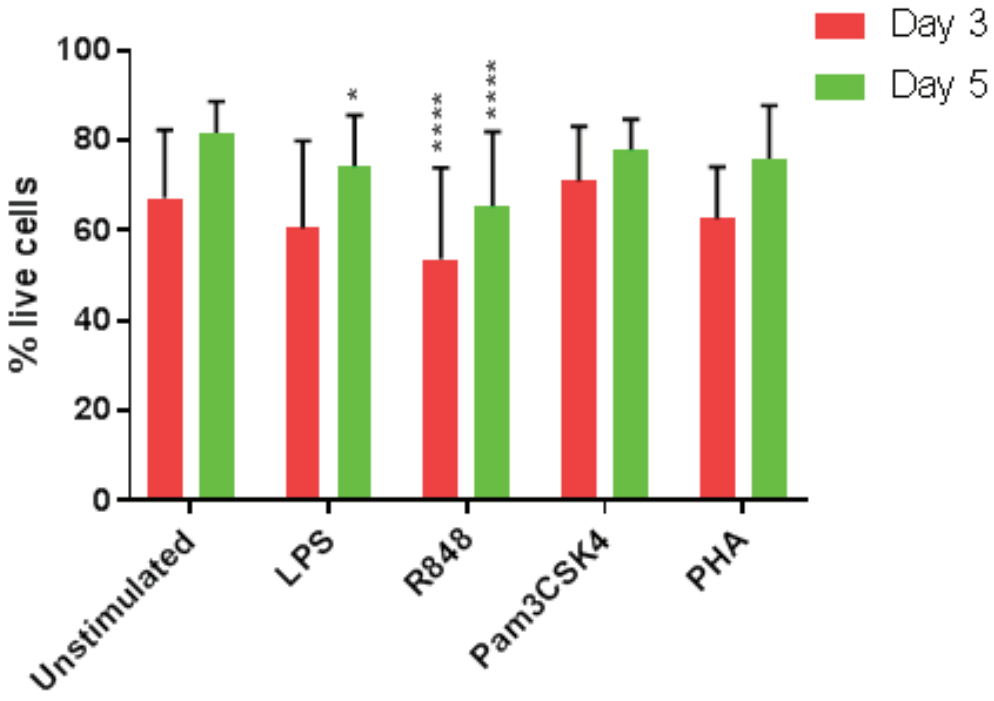
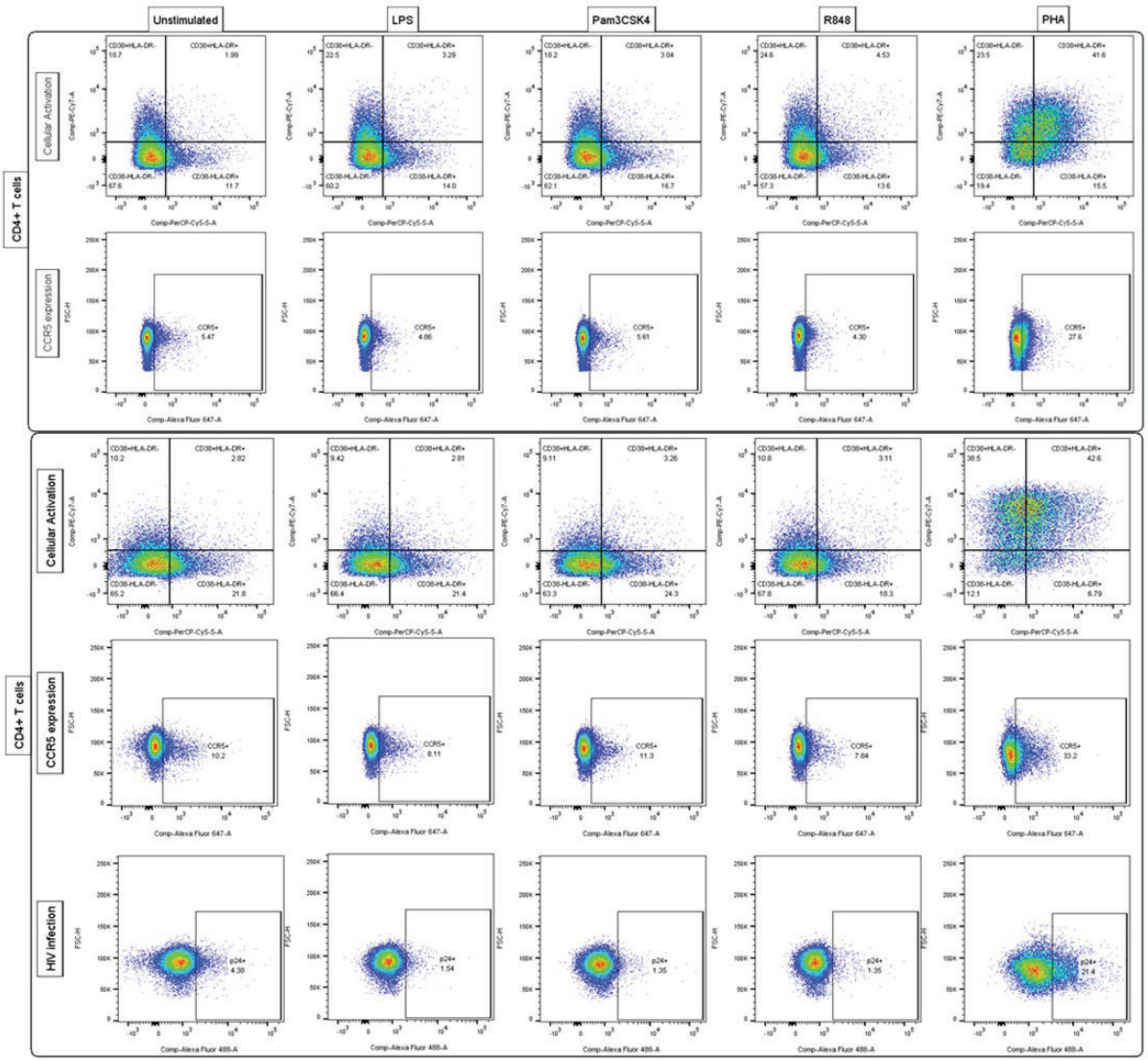


Supplementary Figure 6: Cellular viability profiles for PBMCs either unstimulated or stimulated with TLR agonists LPS, R848 or Pam3CSK4, or the positive control PHA at day 3 (red) and day 5 (green). Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.



Supplementary Figure 7: Representative dot plots of flow cytometric data of cellular activation, CCR5 expression and HIV infection of CD4+ T cells not treated with anti-inflammatory drugs (no AI) prior to HIV infection on day 3 (top box) and post HIV infection on day 5 (bottom box) from the Unstimulated, LPS, Pam3CSK4, R848 and PHA (left to right ordered) conditions.

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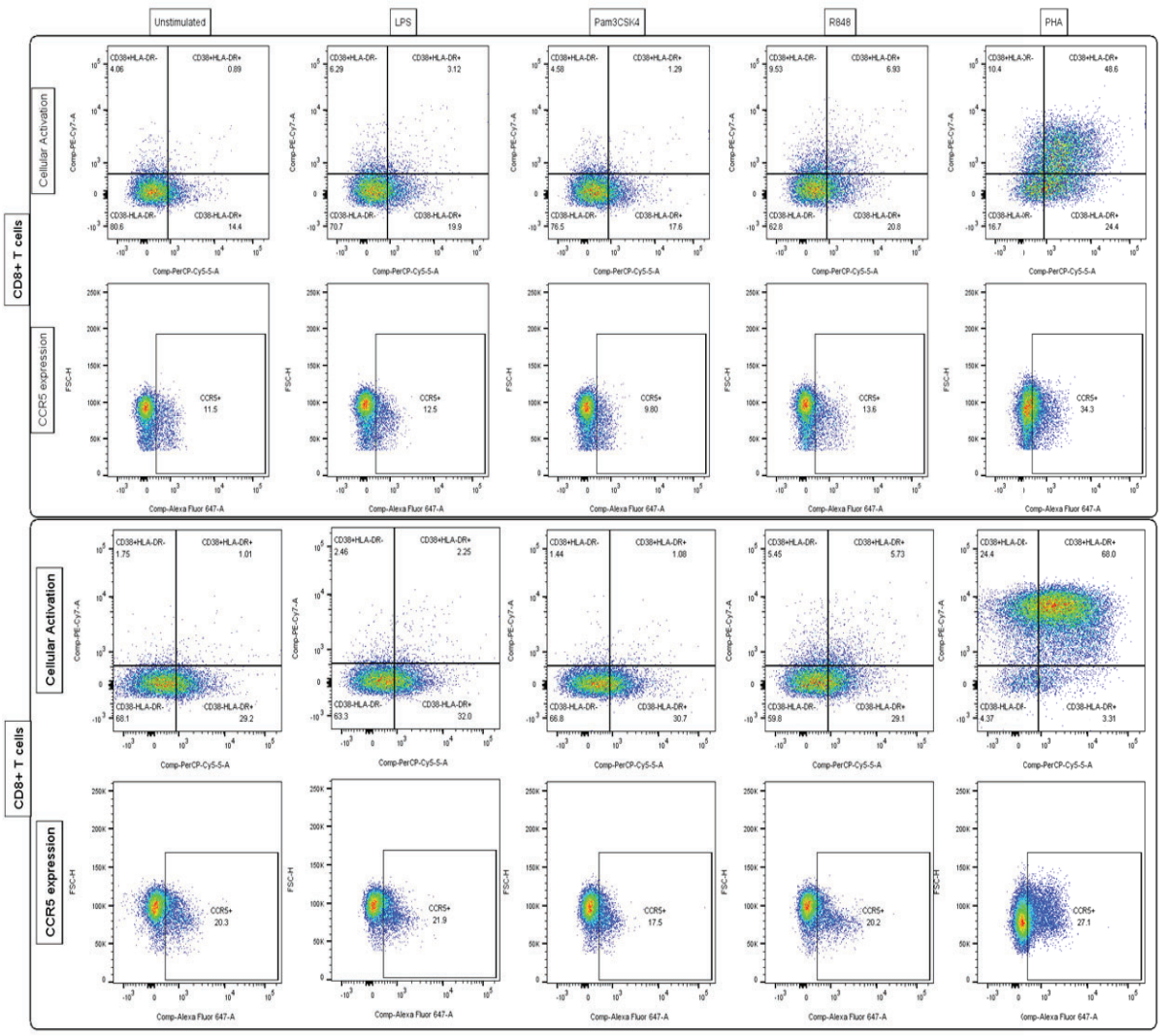

Supplementary Figure 8: Representative dot plots of flow cytometric data of cellular activation and CCR5 expression of CD8+ T cells prior to HIV infection on day 3 (top box) and post HIV infection on day 5 (bottom box) from the Unstimulated, LPS, Pam3CSK4, R848 and PHA (left to right ordered) conditions.

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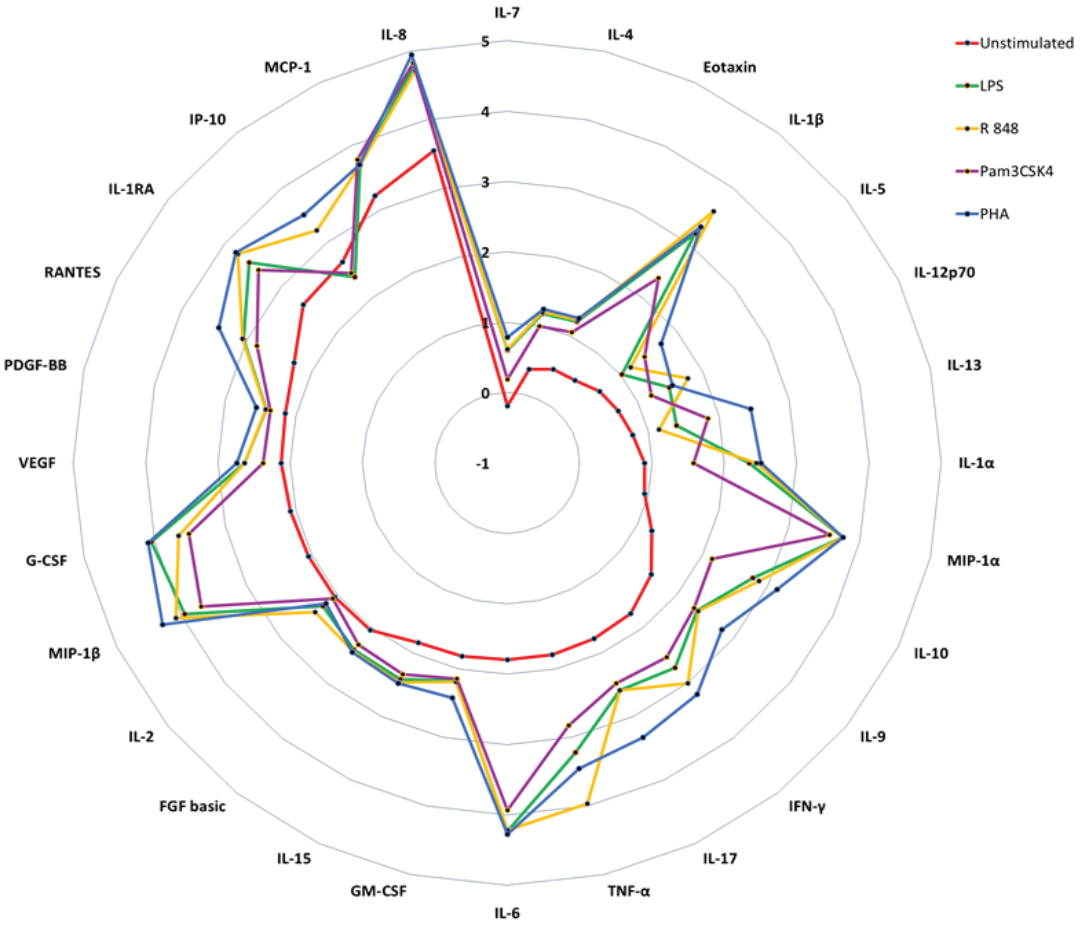

Supplementary Figure 9: Spider plot showing mean Log_{10} concentrations (pg/ml) of 28 cytokines measured in cell culture supernatants at day 3 prior to HIV infection from the unstimulated (red line), LPS (green line), R848 (orange line), Pam3CSK4 (purple line) and PHA (blue line) conditions. Cytokine data was sorted in a clockwise manner on the unstimulated condition from lowest to highest expressed cytokine. PHA was used at a final concentration of 10µg/ml. TLR agonists were used at a final concentration of 2µg/ml. All TLR stimulation conditions were infected with HIV. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

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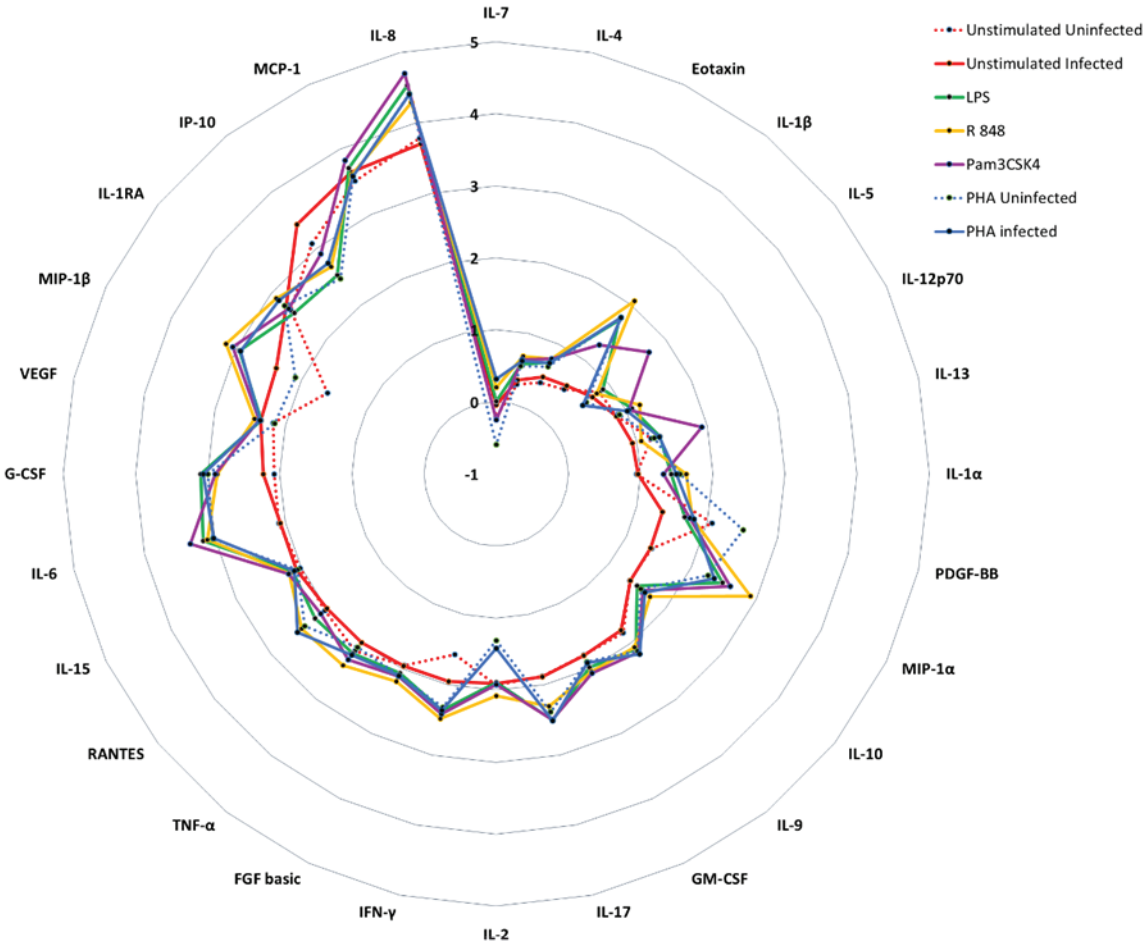

Supplementary Figure 10: Spider plot showing mean Log_{10} concentrations (pg/ml) of 28 cytokines measured in cell culture supernatants at day 5 post HIV infection from the unstimulated uninfected (dotted red line), unstimulated infected (red line) LPS (green line), R848 (orange line), Pam3CSK4 (purple line), PHA uninfected (dotted blue line) and PHA infected (blue line) conditions. Cytokine data was sorted in a clockwise manner on the unstimulated condition from lowest to highest expressed cytokine. PHA was used at a final concentration of 10µg/ml. TLR agonists were used at a final concentration of 2µg/ml. All TLR stimulation conditions were infected with HIV. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

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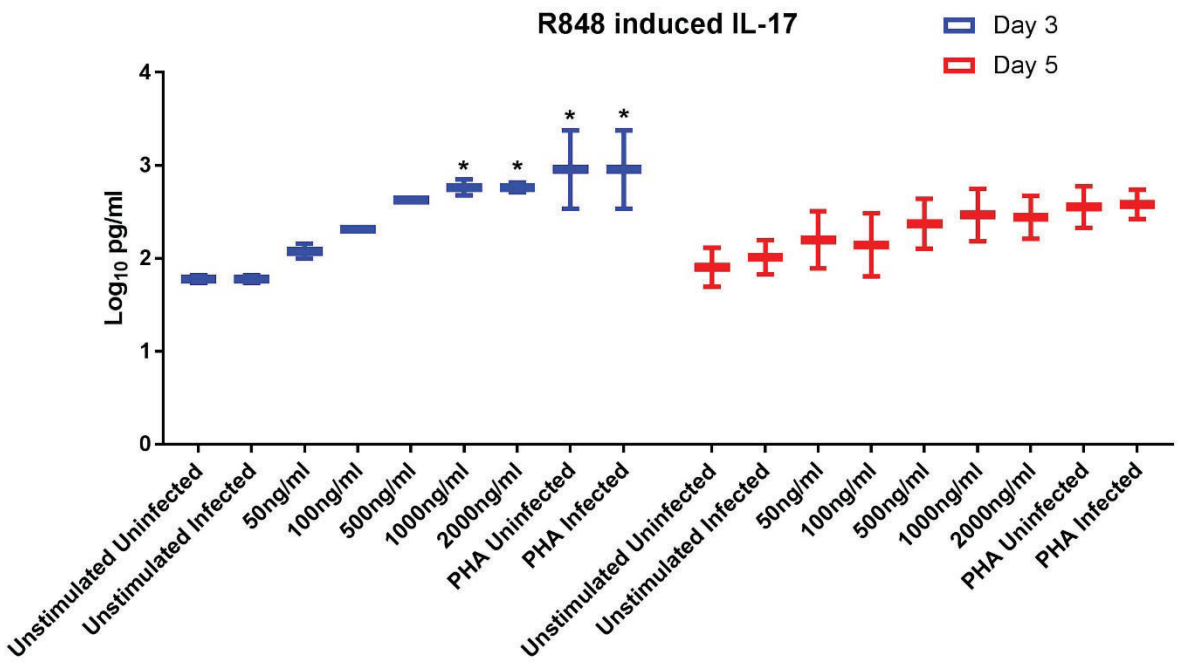

Supplementary Figure 11: Box and Whisker plots showing mean ± SD Log_{10} concentrations of haematopoietic cytokine IL-17 after stimulation with the different concentrations of the TLR7/8 agonist R848 at day 3 prior to HIV infection and day 5 post HIV infection. Concentrations of R848 shown on the X axis. PHA was used at a final concentration of 10µg/ml. All TLR stimulation conditions were infected with HIV. An ordinary one-way ANOVA with a Dunnett's multiple comparisons test was performed. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the unstimulated control. Samples size, n=1 run in duplicate.

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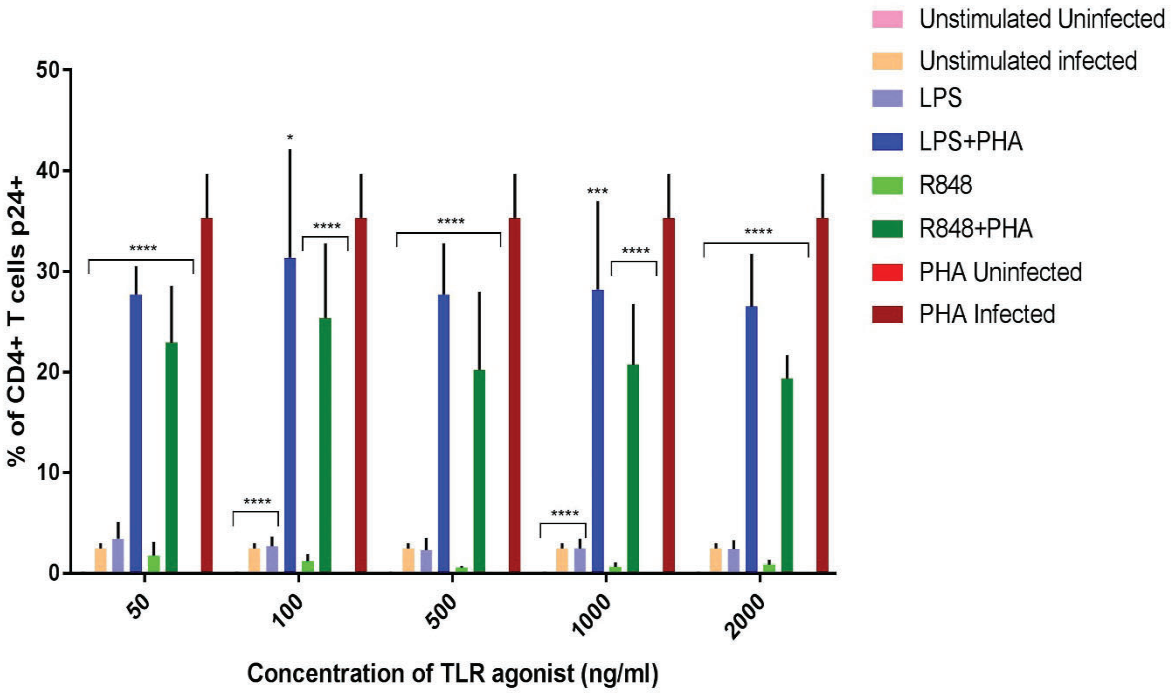

Supplementary Figure 12: Infection rates (measured by p24 expression) of CD4+ T cells either unstimulated of stimulated with PHA and/or TLR agonists; LPS (TLR4) or R848 (TLR7/8) at the concentrations depicted. PHA was used at a final concentration of 10µg/ml. Significance was assessed by two-way ANOVA with Dunnett's multiple comparisons test. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.0001$) compared to the PHA infected control. Sample size, n=1, run in duplicate.

Supplementary Table 1: Mean percentage (%) and standard deviations (SD) of CD4+ T cells expressing cellular activation markers CD38 and HLA-DR in unstimulated or stimulated (LPS, R848, Pam3CSK4 and PHA) conditions at day 3 (top) and day 5 (bottom). Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

Day 3	CD38+HLA-DR+		CD38+HLA-DR-		CD38-HLA-DR+		CD38-HLA-DR-	
	mean	SD	mean	SD	mean	SD	mean	SD
Unstimulated	1.96	1.35	20.43	9.44	8.05	4.19	69.58	12.84
LPS	2.35	1.68	18.93	8.50	9.14	4.56	69.57	12.77
R848	3.08	1.90	21.80	8.79	8.22	4.34	66.93	12.37
Pam3CSK4	2.30	1.68	18.00	7.26	10.16	5.22	69.57	11.99
PHA	26.38	12.82	26.79	9.21	13.33	3.90	33.53	16.81
Day 5	CD38+HLA-DR+		CD38+HLA-DR-		CD38-HLA-DR+		CD38-HLA-DR-	
	mean	SD	mean	SD	mean	SD	mean	SD
Unstimulated Uninfected	2.57	2.81	18.01	9.67	10.47	8.39	68.95	14.15
Unstimulated Infected	3.07	2.81	19.42	10.71	11.08	8.40	66.46	13.50
LPS	3.07	3.17	17.88	9.53	11.19	8.51	67.86	15.17
R848	4.12	3.65	21.82	10.87	8.89	7.24	65.17	14.56
Pam3CSK4	3.17	2.69	17.18	8.95	12.33	8.27	67.32	13.95
PHA Uninfected	26.27	14.85	45.12	8.79	6.63	3.01	22.00	11.55
PHA Infected	24.96	14.60	45.72	8.38	5.95	2.49	23.37	9.96

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Supplementary Table 2: Mean percentage (%) and standard deviations (SD) of CD8+ T cells expressing cellular activation markers CD38 and HLA-DR in unstimulated or stimulated (LPS, R848, Pam3CSK4 and PHA) conditions at day 3 (top) and day 5 (bottom). Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

Day 3	CD38+HLA-DR+		CD38+HLA-DR-		CD38-HLA-DR+		CD38-HLA-DR-	
	mean	SD	mean	SD	mean	SD	mean	SD
Unstimulated	1.28	0.91	8.44	6.40	9.19	6.07	81.09	10.47
LPS	2.20	2.23	8.51	5.27	10.79	6.98	78.50	11.38
R848	4.13	3.41	11.83	6.40	11.06	6.84	73.01	11.82
Pam3CSK4	1.25	0.88	7.29	4.47	10.19	6.50	81.26	9.37
PHA	30.37	14.18	15.50	6.93	17.71	5.27	36.43	17.87
Day 5	CD38+HLA-DR+		CD38+HLA-DR-		CD38-HLA-DR+		CD38-HLA-DR-	
	mean	SD	mean	SD	mean	SD	mean	SD
Unstimulated Uninfected	1.69	1.61	6.07	6.49	13.39	12.49	78.84	15.75
Unstimulated Infected	3.29	3.45	8.85	7.98	13.48	10.97	74.36	15.27
LPS	2.66	2.57	7.32	6.37	14.37	13.30	75.67	16.62
R848	7.40	5.24	14.24	8.34	12.92	11.43	65.46	15.66
Pam3CSK4	2.38	1.87	6.66	5.68	13.84	11.53	77.11	13.69
PHA Uninfected	38.35	16.79	41.41	13.43	4.08	2.53	16.14	9.15
PHA Infected	38.91	16.31	41.87	13.32	3.79	2.29	15.45	9.89

Supplementary Table 3: Mean concentrations (Log_{10} pg/ml) and standard deviations (SD) of pro-inflammatory cytokines in cell culture supernatants at day 3 (top) and day 5 (bottom) from unstimulated, TLR or PHA stimulated PBMCs. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

Pro-inflammatory cytokines Day 3	IL-1α		IL-1β		IL-6		IL-12p70		IFN-γ		TNF
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean
Unstimulated	0.900	0.713	0.503	1.111	1.795	0.711	0.701	0.582	1.738	1.029	1.792
LPS	2.347	0.404	3.170	0.306	4.229	0.597	1.482	0.175	2.722	0.136	3.222
R848	2.441	0.321	3.570	0.418	4.216	0.460	1.771	0.246	3.006	0.273	3.965
Pam3CSK4	1.573	0.366	2.357	0.249	3.935	0.401	1.212	0.260	2.533	0.194	2.828
PHA	2.506	0.348	3.289	0.202	4.279	0.602	1.534	0.163	3.207	0.309	3.456

Pro-inflammatory cytokines Day 5	IL-1α		IL-1β		IL-6		IL-12p70		IFN-γ		TNF
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean
Unstimulated Uninfected	0.943	1.158	0.505	0.371	2.091	0.464	0.845	0.546	1.572	1.238	2.140
Unstimulated Infected	0.966	0.904	0.569	0.463	2.071	0.575	0.849	0.700	1.954	0.810	1.993
LPS	1.426	0.504	1.731	0.401	3.167	0.742	1.087	0.563	2.368	0.293	2.185
R848	1.633	0.213	2.070	0.268	3.107	0.389	1.208	0.302	2.488	0.197	2.403
Pam3CSK4	1.318	0.569	1.286	0.245	3.351	0.464	1.036	0.539	2.421	0.308	2.296
PHA Uninfected	1.546	0.347	1.780	0.246	3.031	0.567	0.900	0.428	2.319	0.287	2.082
PHA Infected	1.498	0.288	1.774	0.456	3.020	0.666	1.018	0.546	2.366	0.255	2.212

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Supplementary Table 4: Mean concentrations (Log_{10} pg/ml) and standard deviations (SD) of chemotactic cytokines in cell culture supernatants at day 3 (top) and day 5 (bottom) from unstimulated, TLR or PHA stimulated PBMCs. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

Chemokines Day 3	IL-8		MIP-1α		MIP-1β		IP-10		MCP-1		RANT
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean
Unstimulated	3.551	0.632	0.950	0.820	2.052	0.479	2.653	0.958	3.217	0.833	2.275
LPS	4.787	0.175	3.751	0.325	3.951	0.338	2.374	0.481	3.693	0.344	3.046
R848	4.720	0.284	3.751	0.330	4.082	0.244	3.228	0.933	3.693	0.301	3.063
Pam3CSK4	4.833	0.339	3.573	0.278	3.696	0.352	2.456	0.668	3.781	0.387	2.844
PHA	4.946	0.395	3.762	0.327	4.290	0.360	3.507	0.713	3.704	0.327	3.428

Chemokines Day 5	IL-8		MIP-1α		MIP-1β		IP-10		MCP-1		RANT
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean
Unstimulated Uninfected	3.781	0.244	1.393	0.648	1.594	0.281	3.093	1.046	3.514	0.404	2.040
Unstimulated Infected	3.697	0.380	1.372	0.644	2.387	0.613	3.426	1.186	3.649	0.303	2.002
LPS	4.534	0.325	2.484	0.784	2.948	0.667	2.531	0.638	3.713	0.238	2.215
R848	4.292	0.194	2.909	0.498	3.159	0.285	2.675	0.716	3.608	0.160	2.446
Pam3CSK4	4.700	0.196	2.599	0.486	3.056	0.238	2.906	0.943	3.832	0.270	2.115
PHA Uninfected	4.415	0.151	2.255	0.589	2.090	0.752	2.466	0.535	3.583	0.316	2.393
PHA Infected	4.408	0.307	2.354	0.521	2.933	0.294	2.742	0.765	3.582	0.260	2.526

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Supplementary Table 5: Mean concentrations (Log_{10} pg/ml) and standard deviations (SD) of haematopoietic and anti-inflammatory cytokines in cell culture supernatants at day 3 (top) and day 5 (bottom) from unstimulated, TLR or PHA stimulated PBMCs. Sample size, n=5, 4 donors run in quadruplicate, 1 donor run in duplicate.

Haematopoietic and anti-inflammatory cytokines Day 3	IL-7		IL-17		GM-CSF		IL-10	
	mean	SD	mean	SD	mean	SD	mean	SD
Unstimulated	-0.193	0.997	1.772	0.297	1.819	0.870	1.219	0.330
LPS	0.599	0.616	2.588	0.108	2.178	0.572	2.771	0.240
R848	0.618	0.617	2.584	0.110	2.186	0.516	2.865	0.249
Pam3CSK4	0.189	0.812	2.472	0.205	2.142	0.601	2.140	0.212
PHA	0.789	0.652	3.332	0.333	2.421	0.389	3.137	0.151
Haematopoietic and anti-inflammatory cytokines Day 5	IL-7		IL-17		GM-CSF		IL-10	
	mean	SD	mean	SD	mean	SD	mean	SD
Unstimulated Uninfected	-0.206	1.129	1.879	0.411	1.793	0.995	1.372	0.292
Unstimulated Infected	-0.040	0.963	1.887	0.433	1.800	0.930	1.373	0.285
LPS	0.005	0.819	2.509	0.511	1.980	0.795	1.494	0.292
R848	0.203	0.928	2.307	0.191	2.046	0.660	1.729	0.197
Pam3CSK4	-0.253	0.967	2.496	0.472	2.069	0.713	1.599	0.139
PHA Uninfected	-0.597	1.138	2.389	0.295	1.891	0.785	1.557	0.164
PHA Infected	0.317	0.643	2.514	0.491	1.910	0.824	1.636	0.273

2.13 Bridging chapter

TLR-mediated inflammation was protective against HIV infection of CD4+ T cells, contrary to our initial hypothesis. However, we do provide biological plausibility for the mechanism behind this finding. The TLR agonists did not induce significant immune activation, but induced a potent cytokine response, similar to the positive control, PHA. Interestingly, the TLR-mediated immune response *in vitro* partially mimicked an immune quiescent environment seen *in vivo* from HESN cohorts, resulting in minimal immune activation and a specific cytokine signature which was protective against HIV infection. In addition, we still wanted to investigate how two anti-inflammatory drugs; ibuprofen (a NSAID) and betamethasone (a glucocorticoid) would modulate this TLR-mediated inflammation and impact on HIV infection of CD4+ T cells. We hypothesised that these anti-inflammatory drugs would dampen the protective TLR-mediated inflammation, and thus increase HIV infection of CD4+ T cells. **This manuscript, entitled "Betamethasone induces potent immunosuppression and reduces HIV infection in a PBMC *in vitro* model", has been published on 1st October 2020 in the Journal of Investigative Medicine (doi:10.1136/jim-2020-001424).**

OPEN ACCESS

Betamethasone induces potent immunosuppression and reduces HIV infection in a PBMC in vitro model

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ABSTRACT

Genital inflammation is an established risk factor for increased HIV acquisition risk. Certain HIV-exposed seronegative populations, who are naturally resistant to HIV infection, have an immune quiescent phenotype defined by reduced immune activation and inflammatory cytokines at the genital tract. Therefore, the aim of this study was to create an immune quiescent environment using immunomodulatory drugs to mitigate HIV infection. Using an *in vitro* peripheral blood mononuclear cell (PBMC) model, we found that inflammation was induced using phytohemagglutinin and Toll-like receptor (TLR) agonists Pam3CSK4 (TLR1/2), lipopolysaccharide (LPS) (TLR4) and R848 (TLR7/8). After treatment with anti-inflammatory drugs, ibuprofen (IBF) and betamethasone (BMS), PBMCs were exposed to HIV NL4-3 AD8. Multiplexed ELISA was used to measure 28 cytokines to assess inflammation. Flow cytometry was used to measure immune activation (CD38, HLA-DR and CCR5) and HIV infection (p24 production) of CD4+ T cells. BMS potently suppressed inflammation (soluble cytokines, $p<0.05$) and immune activation (CD4+ T cells, $p<0.05$). BMS significantly reduced HIV infection of CD4+ T cells only in the LPS (0.98%) and unstimulated (1.7%) conditions ($p<0.02$). In contrast, IBF had minimal anti-inflammatory and immunosuppressive but no anti-HIV effects. BMS demonstrated potent anti-inflammatory effects, regardless of stimulation condition. Despite uniform immunosuppression, BMS differentially affected HIV infection according to the stimulation conditions, highlighting the complex nature of these interactions. Together, these data underscore the importance of interrogating inflammatory signaling pathways to identify novel drug targets to mitigate HIV infection.

Significance of this study

What is already known about this subject?

- ► HIV is a global epidemic with no vaccine or cure. Due to various social, behavioural and biological factors, women remain particularly vulnerable to HIV.
- ► Genital inflammation significantly increases the risk of HIV acquisition in women.
- ► Genital inflammation has also been shown to significantly reduce the efficacy of topical pre-exposure prophylaxis.

What are the new findings?

- ► In this *in vitro* model, we used various Toll-like receptor (TLR) agonists to simulate inflammation, and two anti-inflammatory drugs (ibuprofen (IBF) and betamethasone (BMS)) were tested to understand their role in modifying HIV infection.
- ► IBF showed minimal immunosuppressive or anti-inflammatory effects, in contrast to other studies, and did not lower HIV infection of CD4+ T cells.
- ► Despite potent uniform immunosuppression, BMS differentially affected HIV infection of CD4+ T cells according to the TLR stimulation condition.

INTRODUCTION

HIV remains a public health challenge with an estimated 1.8 million new infections globally in 2017.[1] South Africa is disproportionately affected by HIV, harboring 20% of the world's HIV-infected population, and women in this region account for 60% of these infections.[2]

Despite high levels of protection in clinical trials testing antiretroviral drugs as pre-exposure prophylaxis (PrEP) in men who have sex with men,[3–6] inconsistent levels of protection have been shown among heterosexual populations, particularly in African women.[7–11] While adherence to PrEP likely undermines protection in women,[12] biological factors such as genital inflammation[13–16] are known to increase women's susceptibility to HIV, even in those using PrEP.[17–20]

Inflammation, a necessary natural response elicited by the body to control infection and limit tissue damage[21 22] is initiated through the recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns, respectively, by pathogen

3 Chapter 3: Betamethasone induces potent immunosuppression and reduces HIV infection in a PBMC *in vitro* model

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Running title: Potent immunosuppression and reduced HIV infection by betamethasone

3.1 Abstract

Genital inflammation is an established risk factor for increased HIV acquisition risk. Certain HIV-exposed seronegative populations, who are naturally resistant to HIV infection have an immune quiescent phenotype defined by reduced immune activation and inflammatory cytokines at the genital tract. Therefore, the aim of this study was to create an immune quiescent environment using immunomodulatory drugs to mitigate HIV infection. Using an *in vitro* peripheral blood mononuclear cell (PBMC) model, inflammation was induced using phytohaemagglutinin (PHA) and Toll-like receptor (TLR) agonists Pam3CSK4 (TLR1/2), LPS (TLR4) and R848 (TLR7/8). After treatment with anti-inflammatory drugs, ibuprofen (IBF) and betamethasone (BMS), PBMCs were exposed to HIV NL4-3 AD8. Multiplexed ELISA was used to measure 28 cytokines to assess inflammation. Flow cytometry was used to measure immune activation (CD38, HLA-DR and CCR5) and HIV infection (p24 production) of CD4+ T cells. Betamethasone potently suppressed inflammation (soluble cytokines, $p<0.05$) and immune activation (CD4+ T cells, $p<0.05$). Betamethasone significantly reduced HIV infection of CD4+ T cells only in the LPS (0.98%) and unstimulated (1.7%) conditions ($p<0.02$). In contrast, ibuprofen had minimal anti-inflammatory and immunosuppressive, but no anti-HIV effects. Betamethasone demonstrated potent anti-inflammatory effects, regardless of stimulation condition. Despite uniform immunosuppression, betamethasone differentially affected HIV infection according to the stimulation conditions, highlighting the complex nature of these interactions. Together, these data underscore the importance of interrogating inflammatory signalling pathways to identify novel drug targets to mitigate HIV infection.

3.2 Introduction

HIV remains a public health challenge with an estimated 1.8 million new infections globally in 2017 (1). South Africa is disproportionately affected by HIV, harbouring 20% of the world's HIV infected population, and women in this region account for 60% of these infections (2). Despite high levels of protection in clinical trials testing antiretroviral drugs as pre-exposure prophylaxis (PrEP) in men who have sex with men (MSM) (3-6), inconsistent levels of protection have been shown among heterosexual populations, particularly in African women (7-11). While adherence to PrEP likely undermines protection in women (12), biological factors such as genital inflammation (13-16) are known to increase women's susceptibility to HIV, even in those using PrEP (17-20).

Inflammation, a necessary natural response elicited by the body to control infection and limit tissue damage (21, 22), is initiated through the recognition of pathogen- and damage- associated molecular patterns (PAMPs and DAMPs, respectively) by pathogen recognition receptors (PRRs). These include toll-like receptors (TLRs) that are expressed both inside and on the cell surface on many cell types, particularly innate immune cells (23-27). Some of the most potent PAMPs, that exert significant immunological and inflammatory responses, include bacterial lipopeptides recognised by TLR2 (28-

30), lipopolysaccharide (LPS) recognised by TLR4 (31-34), and single stranded RNA (ssRNA) recognised by TLR7/8 (35-37). Although inflammation is necessary to mount a successful host defence against pathogens, it can lead to pathology if dysregulated and persistent.

Genital inflammation is associated with immune activation and recruitment of HIV target cells, in addition to disrupting the mucosal barrier. Immune activation and increased concentrations of cytokines in genital tract (14, 38) and blood (39, 40) have directly been associated with increased HIV acquisition risk. Inflammatory cytokines activate CD4+ T cells, targets for HIV (41), which are preferentially and more easily infected than resting CD4+ T cells (42-44). Chemokines secreted by mucosal epithelial cells recruit innate immune cells such as plasmacytoid dendritic cells (pDCs) which in turn produce other chemokines to attract HIV target cells (45). Inflammation and cellular recruitment are important precursors for establishment of SIV infection following vaginal challenge in Rhesus macaques (45). These findings were confirmed in sooty managabeys where protection against SIV infection were associated with lower levels of systemic and mucosal CD4+CCR5+ T cells (46). In humans, increased chemokines in the genital tract conferred >three-fold increased risk for HIV acquisition (14). Similarly, increased mucosal concentrations of inflammatory cytokines compared to plasma was associated with increased HIV risk (47). Inflammation even in HIV negative individuals resulted in recruitment of HIV target cells and epithelial barrier disruption (48-50). Nazli et al., (2010) demonstrated that co-culture of mucosal epithelial cells with infectious HIV stimulated inflammatory cytokines, which in turn compromised the epithelial barrier leading to increased mucosal barrier permeability (51-54). Some known causes of genital inflammation include vaginal microbial dysbiosis and sexually transmitted infections (STIs) (17, 55-61). However, while there are many potential causes of genital inflammation, eliminating these causes may not fully reverse their negative effects, further necessitating additional interventions. Therefore, understanding the complex associations between HIV and the biological factors that drive susceptibility are crucial.

Multiple studies have reported reduced immune activation in HIV-exposed seronegative (HESN) individuals (62-67), which was suggested to confer protection in these individuals against HIV acquisition. Safe, licenced, and easily obtainable drugs that modulate immunity to induce an immune quiescent phenotype to reduce HIV acquisition risk, are a theoretically attractive option. Recently, anti-inflammatory drugs like acetylsalicylic acid (ASA; commonly known as Aspirin®) and hydroxychloroquine (HCQ) were shown to reduce inflammation and immune activation of CD4+ T and Th17 cells systemically and at the mucosa of low-risk uninfected women who were taking these oral drugs daily for six weeks (68). Furthermore, HCQ also reduced systemic inflammatory cytokines (68). Even though the work by Lajoie et al. demonstrated proof of principle that NSAIDs can reduce the proportion of target CD4+ CCR5+ and Th17 cells in women, this study did not investigate the effects of these two anti-inflammatory drugs in preventing HIV infection. Similarly, in HIV infected individuals, chloroquine (CQ) and HCQ significantly reduced HIV associated immune activation (69-

72). Additionally, in a small animal model, an HCQ implant compared to a placebo, reduced recruitment of immune cells to the genital tract, improved mucosal epithelial integrity and reduced T cell activation and inflammatory cytokines (73). Topical application of a glucocorticoid (GC) drug like betamethasone (BMS) (74) or a non-steroidal anti-inflammatory drug (NSAID) like ibuprofen (IBF) (75) have also demonstrated efficacy for treating inflammatory skin conditions and genital inflammation, respectively. Furthermore, a natural product like glycerol monolaurate, which has anti-inflammatory properties, showed efficacy in reducing SIV infections in rhesus macaques (45, 76). These data demonstrate the capacity of anti-inflammatory drugs to reduce immune activation and inflammation as additional modalities toward mitigating HIV risk.

The use of anti-inflammatory drugs to reduce genital inflammation and mucosal immune activation, to mitigate HIV acquisition risk in women, may be plausible in regions with high levels of genital inflammation and HIV burden. The use of such products requires thorough pre-clinical testing to assess the viability, utility and efficacy of such strategies. Using a PBMC-based *in vitro* model for HIV infection, we tested the hypothesis that modulating TLR-induced inflammation with anti-inflammatory drugs; including ibuprofen (IBF) and betamethasone (BMS), reduced inflammatory responses, immune activation and HIV infection.

3.3 Materials and Methods

3.3.1 Ethics statement

This study was approved by the University of KwaZulu-Natal (UKZN) Biomedical Research Ethics Committee (BREC; Ethics number: BE433/14), with written informed consent from all healthy blood donors included in a volunteer donor blood study (Ethics number: BE022/13). Informed consent was obtained from all donors in accordance with the Declaration of Helsinki.

3.3.2 Isolation and culture of peripheral blood mononuclear cells (PBMCs) with HIV

For each experiment, peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood collected from four healthy HIV-negative donors by density gradient centrifugation (77). PBMCs were resuspended to 1x106 cells/ml in C10 media and placed into 24-well cell culture plates. For all cell culture experiments, C10 media consisting of RPMI 1640 with L-glutamine (Lonza, Basel, Switzerland) containing 10% FCS (non-heat inactivated FCS; Highveld Biological, JHB, SA), 2% L-glutamine, 1% HEPES, 1% NaPy, 1% NEAA (all from Lonza, Basel, Switzerland) was used. Interleukin-2 (IL-2) (PeproTech, Rocky Hill, NJ, USA), added to C10 media prior to use, was used at a final concentration of 0.01 µg/ml. Unstimulated PBMCs were used as the negative control and stimulation with phytohaemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control, at a final concentration of 10 µg/ml. The CCR5-tropic HIV-1 NL4-3 AD8 (78) was used at a MOI of 0.9, as previously described (77).

3.3.3 TLR agonists and anti-inflammatory drugs

TLR agonists LPS (TLR4), R848 (TLR7/8) and Pam3CSK4 (TLR1/2) (all from Invivogen, San Diego, CA, USA) were used at a final concentration of 2 µg/ml, as described previously (77). In addition to these TLR agonists, the following anti-inflammatory drugs were used in this study: ibuprofen (IBF) and betamethasone (BMS) (both from Sigma-Aldrich, St. Louis, MO, USA). IBF was resuspended in sterile PBS, while BMS was initially resuspended in 100% ethanol before diluting 1:5 with sterile PBS, and both drugs were used at a final concentration of 1 µg/ml, which was the drug concentration previously optimized in anti-inflammatory drug titration experiments (data not shown).

3.3.4 Treatment of PBMCs with TLRs, anti-inflammatory drugs and HIV

PBMCs were either treated with IBF or BMS or left untreated (negative control) and incubated at 37oC 5% CO2 for 2 hours. Following this incubation period, PBMCs were left either unstimulated (negative control) or stimulated with TLR agonists or PHA (positive control) then incubated for 48 hours at 37oC 5% CO2. Following this incubation (for the day 3 time-point, 48 hours post-stimulation but prior to HIV exposure), both PBMCs and culture supernatants of each well were collected into sterile tubes for flow cytometry analysis and multiplex ELISA experiments, respectively. The tubes containing the remaining PBMCs (that were subsequently exposed to HIV-1 NL4-3 AD8, as described below) were centrifuged, supernatants were discarded, and media replacements were performed with fresh C10 media. PBMCs were then plated at 1x106 cells/ml into 24-well cell culture plates, no further stimulations were performed. Subsequently, 250 µl of 1:20 diluted HIV-1 NL4-3 AD8 viral stocks (a gift from Dr Alex Sigal), corresponding to a MOI of 0.9, was added to expose PBMCs to HIV for infection. PHA and unstimulated uninfected wells were treated with 250 µl C10 media. Plates were incubated at 37oC 5% CO2 for 48 hours, whereupon multiplexed ELISA (culture supernatants) and flow cytometry (PBMCs) was performed for the day 5 time-point (48 hours post-HIV exposure).

3.3.5 Flow cytometry

Cellular activation of PBMCs at two time-points (day 3: 48 hours post-stimulation and prior to HIV exposure and day 5: 48 hours post HIV exposure) was assessed by flow cytometry, focusing on CCR5, HLA-DR and CD38 expression by CD4+ cells, as previously described (79-81), using both extracellular and intracellular staining. The extracellular staining cocktail consisted of LIVE/DEAD Amcyan fixable dye (Thermo Fisher Scientific, Waltham, MA, USA), anti-CD3-APC-H7, anti-CD4-BV605, anti-CD8-BV655, anti-CD14-Pacific blue (all from BD Biosciences, Franklin Lakes, NJ, USA), and anti-CD19-pacific blue (Biolegend, San Diego, CA, USA). The intracellular staining cocktail consisted of anti-CCR5-APC, anti-HLA-DR-PerCP-CY5.5 (all from BD Biosciences, Franklin Lakes, NJ, USA), anti-CD38-PE-CY7 (Biolegend, San Diego, CA, USA) and anti-p24-FITC (Beckman Coulter, Brea, CA, USA).

To pellet the cells and remove soluble HIV, PBMCs were centrifuged at 3,500 rpm for 5 minutes and cell culture supernatants were stored at -80oC for cytokine quantification. PBMCs were washed with sterile PBS supplemented with 2% FCS and then stained with 100 µl extracellular staining cocktail, fixed, and then stained with 100 µl intracellular staining cocktail. Data were acquired by flow cytometry on a BD LSR Fortessa (BD Biosciences, Franklin Lakes, NJ, USA), with 5 x 105 events within the lymphocyte gate collected per sample. Data analysis was performed using FlowJo v10.4.1 software (Tree Star, Ashland, OR, USA). Supplementary Figure 1 shows the gating strategy. In this study, we reported on four activation phenotypes (CD38+HLA-DR+, CD38+HLA-DR-, CD38-HLA-DR+, and CD38-HLA-DR-) and define these as previously described (42, 77, 80). CD4+ T cells expressing CD38+HLA-DR+ were defined as hyper-activated, the CD38+HLA-DR- and CD38-HLA-DR+ phenotypes were defined as intermediately activated, and CD38-HLA-DR- CD4+ T cells were defined as resting or not activated. Representative dot plots of flow cytometric data are shown in Supplementary Figure 2.

3.3.6 Cytokine quantification

From cell culture supernatants the concentrations of 28 cytokines were assessed using the Bio-Plex Pro Human Cytokine Group I 27-Plex Panel (Bio-Rad Laboratories, Hercules, CA, USA) and the Magnetic Luminex® Assay IL-1α Singleplex Kit (Research and Diagnostic (R&D) systems Inc., Minneapolis, Minnesota, USA) as per manufacturer's instructions. Data were acquired on a Bio-Plex® 200 system (Bio-Rad Laboratories, Hercules, CA, USA). Standard curves were optimized using the Bio-Plex manager software version 6.1 (Bio-Rad Laboratories, Hercules, CA, USA). Values with coefficients of variation <20% and with observed recoveries between 70 – 130% were considered reliable. Values that were below the detectable limit were assigned half of the lowest limit of detection value (LLOD), while values that were above the detectable limit were assigned double the highest limit of detection (HLOD) value.

3.3.7 Statistical analyses

GraphPad Prism version 7.02 software for Windows (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses and graphical representation of data. The Shapiro-Wilk normality test was performed to determine the distribution of the data. Cellular activation results are displayed as mean percentage (%) ± standard deviation (SD) of CD4+ T cells. For comparisons of cellular activation markers CD38, HLA-DR on CD4+ T cells, between anti-inflammatory treated conditions and the untreated control, a repeated measures two-way ANOVA with a Dunnett's multiple comparisons test was performed. Similarly, an ordinary one-way ANOVA with Dunnett's multiple comparison test was performed for CCR5 expression and cytokine comparisons. Cytokine data were normalized by log10 transformation and is displayed as mean concentration (log10 pg/ml) ± standard deviation (SD). Heat maps were generated by performing a single linkage hierarchical cluster analysis using R version 3.3.3

statistical software (R Foundation for Statistical Computing, Vienna, Austria), to visualize the effect of various TLR agonists and anti-inflammatory drugs on cytokine expression. Radial spider plots were created using Microsoft Excel© 2013 software (Microsoft Corporation, Redmond, WA, USA).

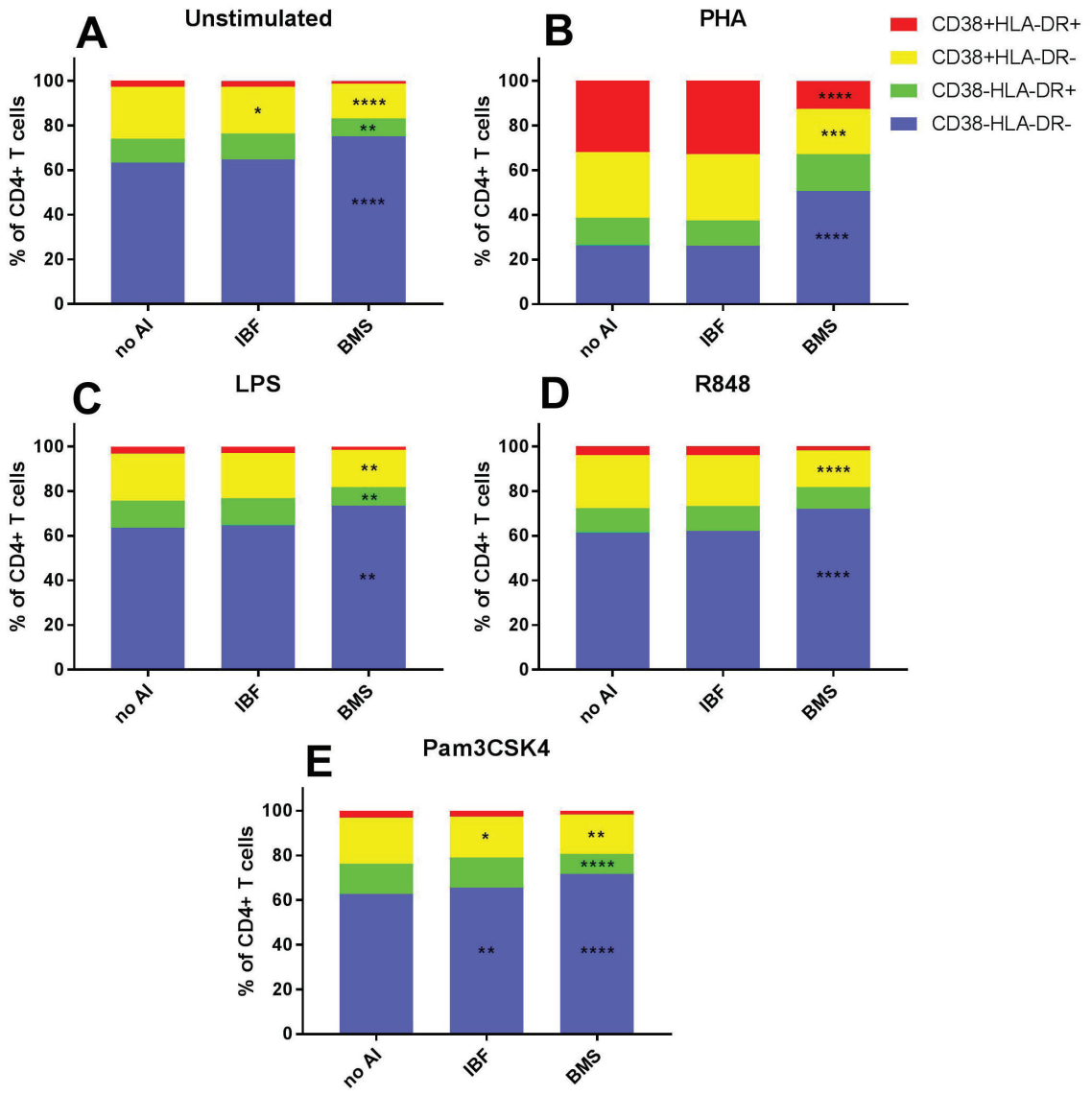

Figure 1: Activation profiles of CD4+ T cells on day 3 prior to HIV exposure either treated with anti-inflammatory drugs ibuprofen (IBF) or betamethasone (BMS) or left untreated (no AI) and then either left unstimulated (A) or stimulated with PHA (B), LPS (C), R848 (D) or Pam3CSK4 (E). PHA was used at a final concentration of 10 µg/ml. TLR agonists were used at a final concentration of 2 µg/ml. Anti-inflammatory (AI) drugs IBF and BMS were both used at a final concentration of 1 ug/ml. A repeated measures two-way ANOVA with Dunnett's multiple comparisons test was performed to assess significant differences between AI conditions within each stimulation condition. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the untreated (no AI) control. Sample size, n=4, donors run in duplicate.

3.4 Results

3.4.1 Reduction of CD4+ T cell activation by BMS but not IBF prior to HIV exposure

As anti-inflammatory drugs can have cytotoxic effects (82), we sought to determine how IBF and BMS impacted on the viability of CD4+ T cells. Prior to HIV exposure (day 3), BMS was slightly toxic to unstimulated cells with a 7.6% reduction of viable cells ($p=0.02$; Supplementary Figure 3A) but improved the number of viable cells stimulated with LPS by 9.2% ($p=0.004$) or R848 by 12.4% ($p=0.0001$; Supplementary Figure 3C&D respectively). Similarly, post HIV exposure (day 5) BMS improved cellular viability in the LPS, R848 and Pam3CSK4-stimulated conditions ($p\leq0.0001$; Supplementary Figure 4C-E).

We sought to determine how anti-inflammatory drugs IBF and BMS impacted the activation status of CD4+ T cells stimulated with TLR agonists, given that activated target cells have been shown to be preferentially infected with HIV (42, 43) and allow more proficient viral replication (83-85). TLR agonists LPS, R848 and Pam3CSK4 had a minimal impact on the activation of CD4+ T cells, unlike the positive control PHA (Figure 1). IBF significantly reduced the frequency of intermediately activated CD38+HLA-DR- CD4+ T cells in the unstimulated ($p=0.02$) and Pam3CSK4-stimulated ($p=0.02$) conditions by 2.35% and 2.36% respectively (Figure 1A&E). Decreases in this subset in the Pam3CSK4-stimulated condition were concomitant with a significantly increased frequency of inactivated CD38-HLA-DR- CD4+ T cells, suggesting that IBF returned CD4+ T cells to their resting state ($p=0.009$; Figure 1E). A similar phenomenon is likely in the unstimulated condition, with a less pronounced increase in the resting CD4+ T cells. Compared to IBF, BMS had potent immunosuppressive effects on CD4+ T cell activation, with increased frequencies of inactivated/resting CD38-HLA-DR- CD4+ T cells across all stimulation conditions ($p\leq0.0001$; Figure 1). As PHA induced significant cellular activation, the frequency of highly activated CD38+HLA-DR+ CD4+ T cells were significantly reduced by 20.8% with BMS treatment ($p\leq0.0001$; Figure 1B). Furthermore, a reduction in the frequency of CD38+HLA-DR- CD4+ T cells was observed across all stimulation conditions ($p<0.01$; Figure 1). Similarly, the frequency of CD38-HLA-DR+ CD4+ T cells were reduced in the unstimulated ($p=0.006$), LPS- ($p=0.005$) and Pam3CSK4-stimulated ($p\leq0.0001$) conditions by 2.78%, 3.8% and 4.48% respectively (Figure 1A, C and E respectively).

3.4.2 Suppression of T cell activation is maintained by BMS after HIV exposure

Similar to the results observed prior to HIV exposure, IBF had minimal immunosuppressive effects in terms of hyperactivated CD4+ T cells, with only a modest 2.76% decrease in the frequency of intermediately activated CD38+HLA-DR- CD4+ T cells in the Pam3CSK4-stimulated condition ($p=0.04$; Figure 2E). Furthermore, an increased frequency of inactivated/resting CD38-HLA-DR- CD4+ T cells were observed in the LPS ($p=0.03$) and Pam3CSK4-stimulated ($p=0.005$) conditions by 2.7% and 3.71% respectively following IBF treatment (Figure 2C&E). As previously observed, BMS

had more potent immunosuppressive activity than IBF, resulting in significantly lower frequencies of highly activated CD38+HLA-DR+ CD4+ T cells following treatment with PHA ($p \leq 0.0001$), LPS ($p=0.02$) and R848 ($p=0.002$) by 20.88%, 2.89% and 4.85% respectively (Figure 2B, C and D). Additionally, BMS also resulted in significantly reduced frequencies of intermediately activated CD38+HLA-DR- CD4+ T cells following PHA ($p \leq 0.0001$) and R848-stimulation ($p=0.004$) by 17.2% and 4.5% respectively (Figure 2B and D). Furthermore, significant reductions in the frequency of

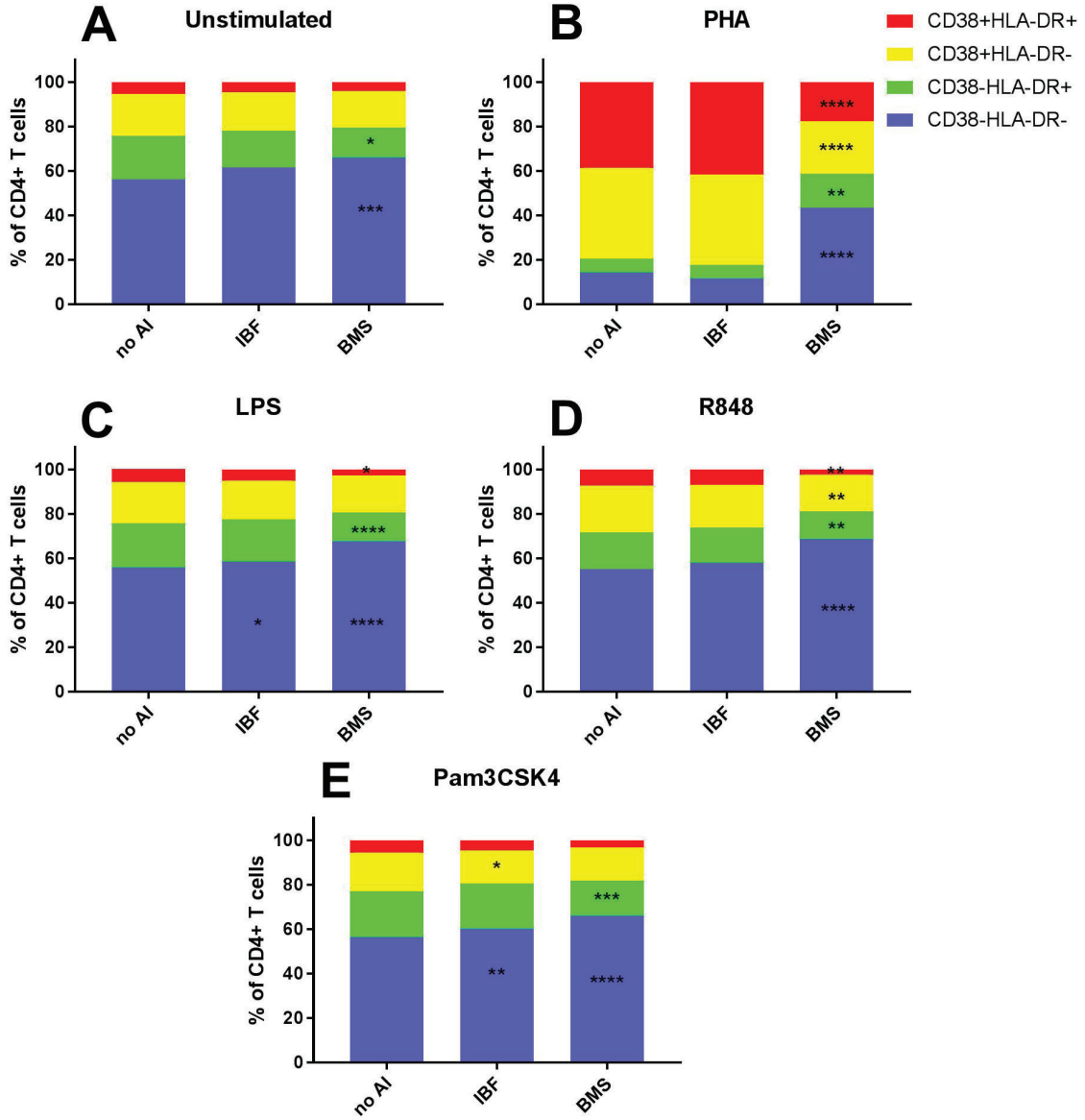

Figure 2: Activation profiles of CD4+ T cells on day 5 post HIV exposure either treated with anti-inflammatory drugs ibuprofen (IBF) or betamethasone (BMS) or left untreated (no AI) and then either left unstimulated (A) or stimulated with PHA (B), LPS (C), R848 (D) or Pam3CSK4 (E). PHA was used at a final concentration of 10 µg/ml. TLR agonists were used at a final concentration of 2 µg/ml. Anti-inflammatory (AI) drugs IBF and BMS were both used at a final concentration of 1 ug/ml. A repeated measures two-way ANOVA with Dunnett's multiple comparisons test was performed to assess significant differences between AI conditions within each stimulation condition. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p \leq 0.0001$) compared to the untreated (no AI) control. Sample size, n=4, donors run in duplicate.

CD38-HLA-DR+ CD4+ T cells ($p<0.05$), and a significant increase in the frequency of CD38-HLA-DR- CD4+ T cells ($p<0.001$) were observed across all stimulation conditions (Figure 2A-E).

3.4.3 Modulation of TLR-mediated CCR5 expression by BMS occurs only at the early time-point

As CCR5 expression on CD4+ T cells is crucial for R5 tropic HIV infection, we sought to assess how the anti-inflammatory drugs IBF and BMS impacted on CCR5 expression following TLR agonist

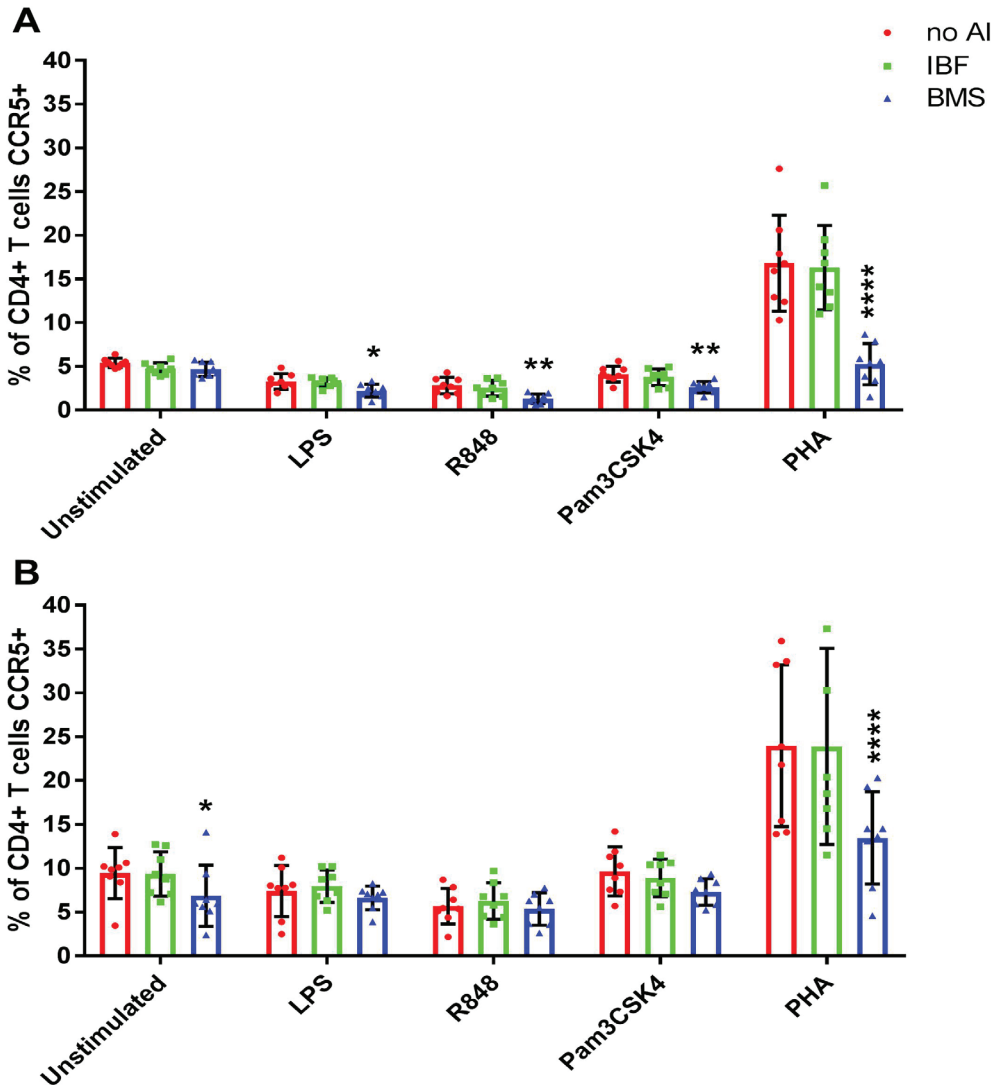

Figure 3: CCR5 expression on CD4+ T cells on day 3 prior to HIV exposure (**A**) or day 5 post HIV exposure (**B**) either treated with anti-inflammatory drugs ibuprofen (IBF, green) or betamethasone (BMS, blue) or left untreated (no AI, red) and then either left unstimulated or stimulated with PHA, LPS, R848 or Pam3CSK4. PHA was used at a final concentration of 10 µg/ml. TLR agonists were used at a final concentration of 2 µg/ml. Anti-inflammatory drugs IBF and BMS were both used at a final concentration of 1 ug/ml. An ordinary one-way ANOVA with a Dunnett's multiple comparisons test was performed to assess significant differences between AI conditions within each stimulation condition. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), **** ($p\leq0.0001$) compared to the untreated (no AI) control within each stimulation condition. Sample size, n=4, donors run in duplicate.

stimulations. Prior to HIV exposure, BMS downregulated CCR5 expression on CD4+ T cells in TLR ($p<0.02$) and PHA-stimulated conditions ($p\leq0.0001$) by 1-1.5% and 11.5% respectively, while IBF had no impact (Figure 3A). Following co-culture with HIV, BMS-mediated downregulation of CCR5 following TLR stimulation was lost, while BMS-reduced CCR5 expression was observed in the unstimulated ($p=0.04$) and PHA-stimulated ($p\leq0.0001$) controls by 2.59% and 10.52% respectively (Figure 3B).

3.4.4 BMS treatment potently reduces global cytokine and chemokine secretion

Unsupervised hierarchical clustering analysis showed a pattern that overall, concentrations of all cytokines were reduced with BMS treatment compared to the untreated and IBF-treated conditions (Supplementary figure 5). Prior to HIV exposure (day 3), concentrations of IL-1α was significantly increased by 0.45 Log10pg/ml with IBF treatment compared to the untreated control in the unstimulated condition ($p=0.03$; Figure 4A). Conversely, BMS reduced IL-1α levels in both the TLR- ($p<0.005$) and PHA-stimulated ($p\leq0.0001$) conditions by 0.6-1.78 and 1.5 Log10pg/ml respectively (Figure 4A). Similarly, BMS significantly reduced IL-1β ($p\leq0.0001$), IL-6 ($p<0.01$), IL-12(p70) ($p\leq0.0001$) and TNF-α ($p\leq0.0001$) compared to the untreated control for all conditions (Figure 4B, C, D and F). Furthermore, IFN-γ production was also significantly reduced by BMS in the unstimulated ($p\leq0.0001$), LPS- ($p=0.02$), R848- ($p=0.005$) and PHA-stimulated ($p\leq0.0001$) conditions by 1.8, 0.15, 0.17 and 0.6 Log10pg/ml respectively (Figure 4E). After HIV exposure (day 5), BMS treatment reduced IL-1α levels in the LPS ($p=0.003$), R848 ($p=0.0008$) and PHA-stimulated ($p=0.003$) conditions by 0.99, 1.11 and 0.98 Log10pg/ml respectively, but not the Pam3CSK4 condition, compared to the untreated control (Figure 4G). Consistent with the results prior to HIV exposure, the levels of IL-1β ($p\leq0.0001$), IL-6 ($p\leq0.0001$) and TNF-α ($p=0.03$) were reduced with BMS treatment compared to the untreated cells, across all conditions (Figure 4H, I and L). Similarly, BMS dampened the production of IL-12(p70) in R848- ($p=0.01$), Pam3CSK4- ($p=0.008$) and PHA-stimulated ($p=0.02$) conditions by 0.45, 0.46 and 0.42 Log10pg/ml respectively, while IFN-γ was also dampened in the unstimulated condition ($p\leq0.0001$) by 0.46 Log10pg/ml (Figure 4J and K).

Similar to the pro-inflammatory cytokines, BMS significantly reduced IL-8 ($p\leq0.0001$), MIP-1α ($p<0.05$), MIP-1β ($p\leq0.0001$) and IP-10 ($p<0.005$) production in all conditions compared to the untreated control, prior to HIV exposure (Figure 5A-D). Furthermore, MCP-1 was reduced by 2.12 Log10pg/ml following BMS treatment in the unstimulated condition only ($p<0.0001$; Figure 5E). RANTES production was also significantly reduced following BMS treatment in all the TLR- ($p\leq0.002$)

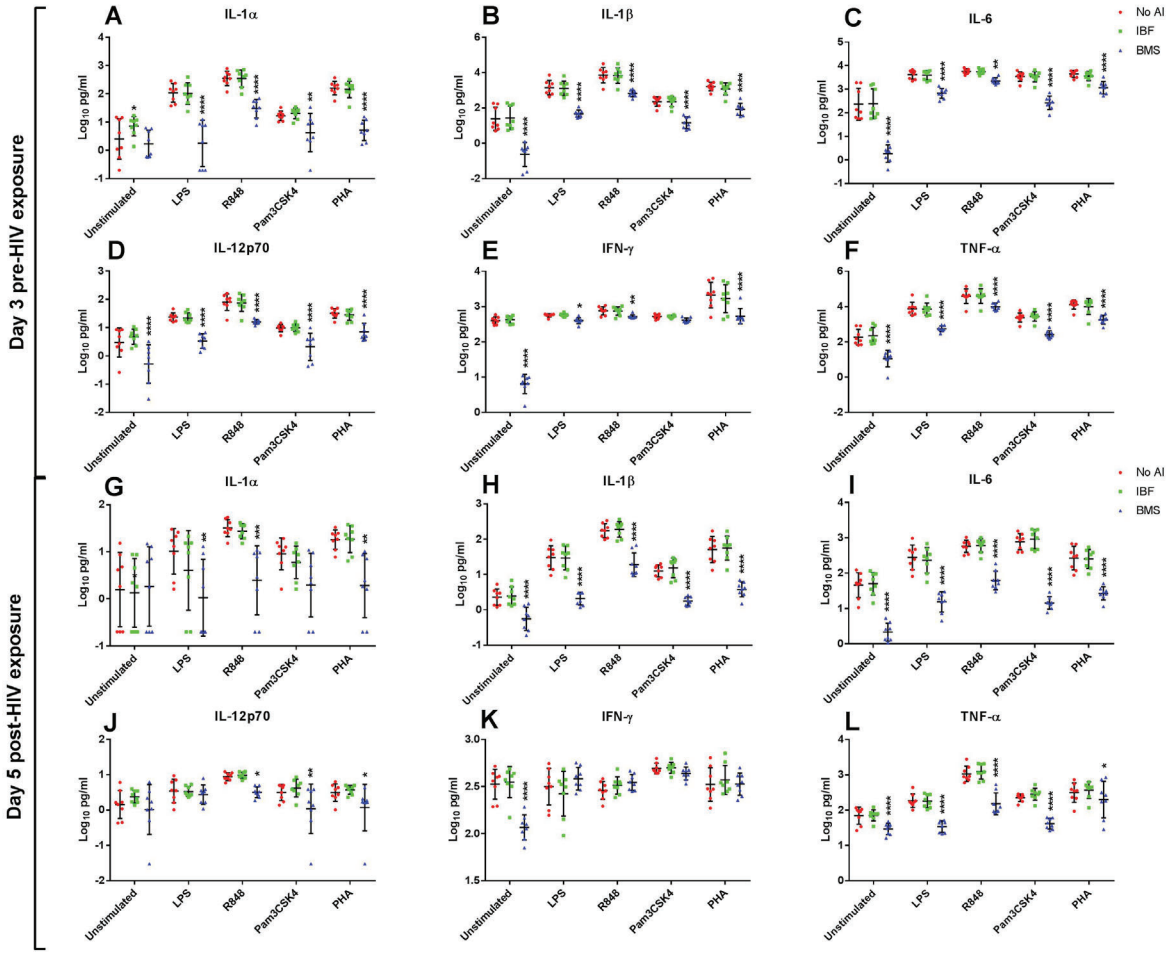

Figure 4: Box and Whisker plots showing mean ± SD Log_{10} concentrations of soluble pro-inflammatory cytokines IL-1α (**A&G**), IL-1β (**B&H**), IL-6 (**C&I**), IL-12p70 (**D&J**), IFN-γ (**E&K**) and TNF-α (**F&L**) from PBMCs either left untreated (no AI, red) or treated with anti-inflammatory drugs ibuprofen (IBF, green) or betamethasone (BMS, blue) and then either left unstimulated or stimulated with LPS, R848, Pam3CSK4 or PHA on day 3 prior to HIV exposure (**A-F**) and day 5 post HIV exposure (**G-L**). PHA was used at a final concentration of 10 µg/ml. TLR agonists were used at a final concentration of 2 µg/ml. Both IBF and BMS were used at 1 µg/ml. An ordinary one-way ANOVA with a Dunnett's multiple comparisons test was performed to assess significant differences between AI conditions within each stimulation condition. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the untreated (no AI) control. Sample size, n=4, donors run in duplicate.

and PHA-stimulated ($p\leq0.0001$) conditions by 0.26-0.64 and 0.6 Log10pg/ml compared to the untreated controls (Figure 5F). Similarly, post HIV exposure, IL-8 ($p\leq0.0001$) and MIP-1α ($p\leq0.001$) levels were significantly reduced with BMS treatment compared to the untreated control across all conditions (Figure 5G&H). Furthermore, MIP-1β and RANTES levels were significantly reduced following BMS treatment compared to the untreated control in the TLR- and PHA-stimulated conditions after HIV exposure ($p\leq0.0001$; Figure 5I&L). Similarly, MCP-1 levels were significantly reduced with BMS treatment by 0.35 Log10pg/ml compared to the untreated control in the unstimulated condition ($p\leq0.0001$). However, BMS treatment increased MCP-1 levels produced in response to R848 stimulation by 0.17 Log10pg/ml compared to untreated cells ($p=0.02$; Figure 5K). IP-10 levels were

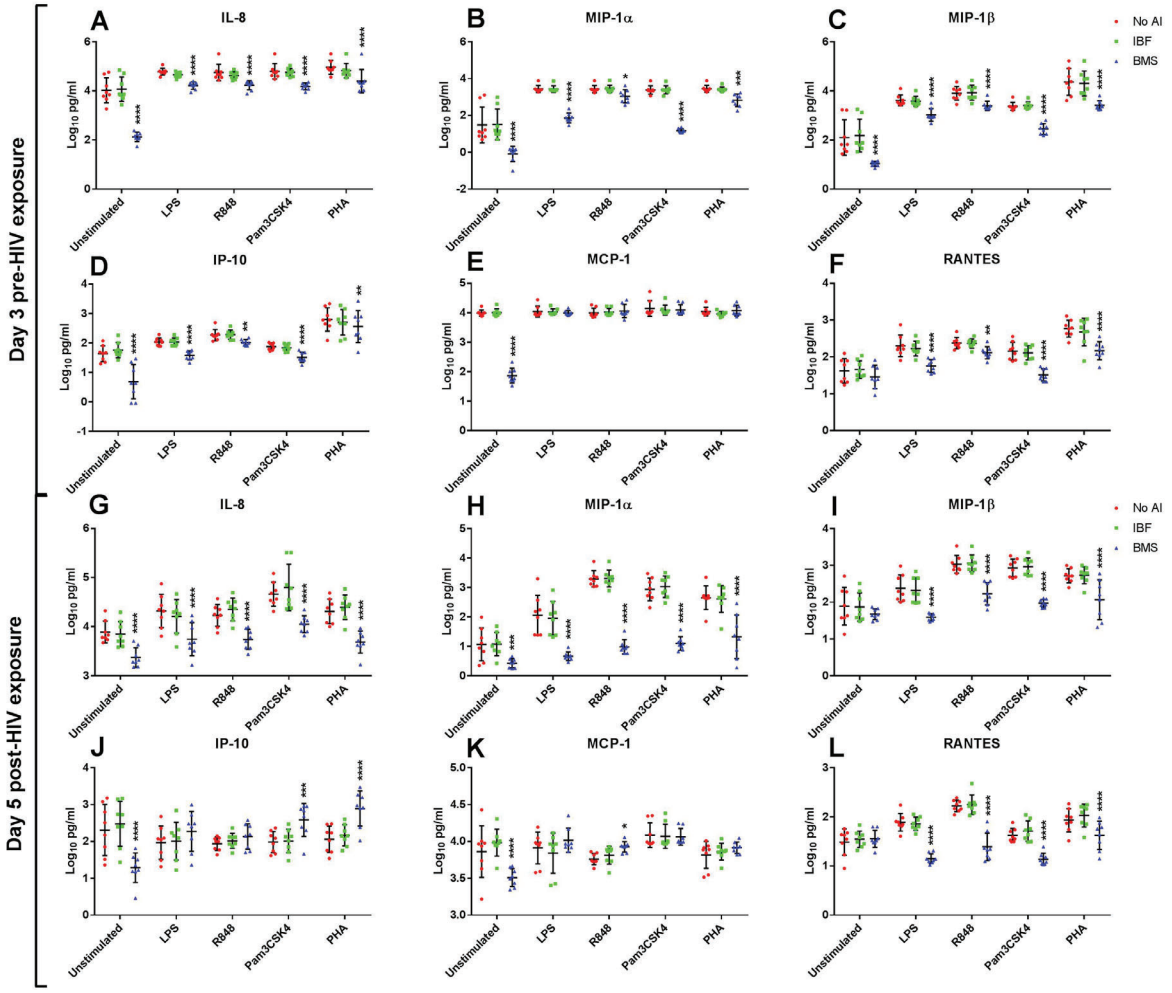

Figure 5: Box and Whisker plots showing mean ± SD Log_{10} concentrations of soluble chemotactic cytokines IL-8 (**A&G**), MIP-1α (**B&H**), MIP-1β (**C&I**), IP-10 (**D&J**), MCP-1 (**E&K**) and RANTES (**F&L**) from PBMCs either left untreated (no AI, red) or treated with anti-inflammatory drugs ibuprofen (IBF, green) or betamethasone (BMS, blue) and then either left unstimulated or stimulated with LPS, R848, Pam3CSK4 or PHA on day 3 prior to HIV exposure (**A-F**) and day 5 post HIV exposure (**G-L**). PHA was used at a final concentration of 10 µg/ml. TLR agonists were used at a final concentration of 2 µg/ml. Both IBF and BMS were used at 1 µg/ml. An ordinary one-way ANOVA with a Dunnett's multiple comparisons test was performed to assess significant differences between AI conditions within each stimulation condition. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the untreated (no AI) control. Sample size, n=4, donors run in duplicate.

significantly reduced in the unstimulated condition by 1.02 Log10pg/ml ($p\leq0.0001$), while they were increased in the Pam3CSK4- ($p=0.0002$) and PHA-stimulated ($p\leq0.0001$) conditions by 0.6 and 0.84 Log10pg/ml respectively with following BMS treatment compared to the untreated control (Figure 5J).

Regulatory cytokines like IL-17 are secreted primarily by Th17 cells that maintain mucosal barrier homeostasis (86-88). Prior to HIV exposure, regulatory cytokines IL-17 and GM-CSF levels were reduced following BMS treatment compared to the untreated control in the unstimulated condition by

1.3 and 1.57 Log10pg/ml, and conditions stimulated with LPS by 0.83 and 0.7 Log10pg/ml, Pam3CSK4

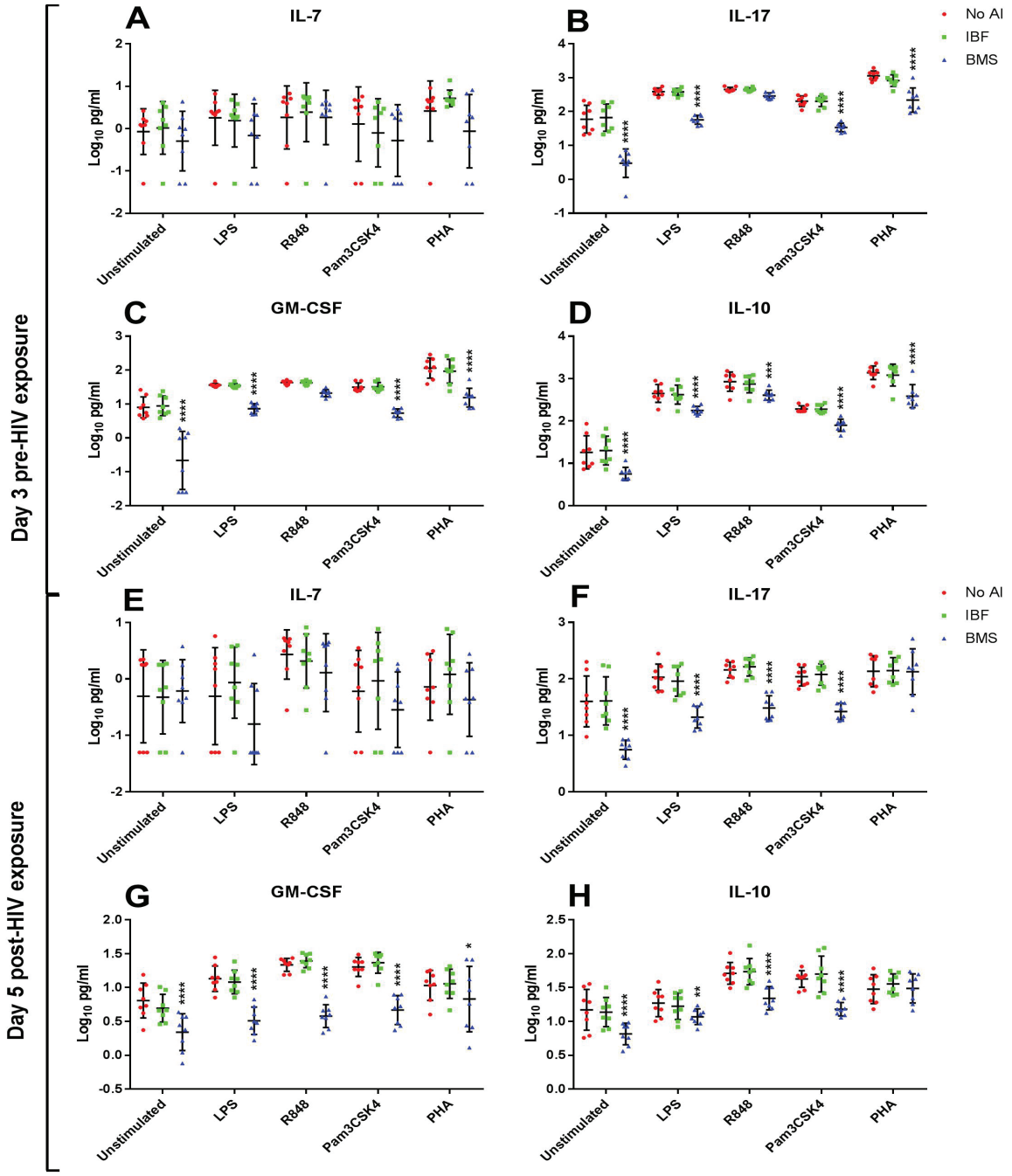

Figure 6: Box and Whisker plots showing mean ± SD Log_{10} concentrations of soluble haematopoietic cytokines IL-7 (**A&E**) and IL-17 (**B&F**), the growth factor GM-CSF (**C&G**) and the anti-inflammatory cytokine IL-10 (**D&H**) from PBMCs either left untreated (no AI, red) or treated with anti-inflammatory drugs ibuprofen (IBF, green) or betamethasone (BMS, blue) and then either left unstimulated or stimulated with LPS, R848, Pam3CSK4 or PHA on day 3 prior to HIV exposure (**A-D**) and day 5 post HIV exposure (**E-H**). PHA was used at a final concentration of 10 µg/ml. TLR agonists were used at a final concentration of 2 µg/ml. Both IBF and BMS were used at 1 µg/ml. An ordinary one-way ANOVA with a Dunnett's multiple comparisons test was performed to assess significant differences between AI conditions within each stimulation condition. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p\leq0.0001$) compared to the untreated (no AI) control. Sample size, n=4, donors run in duplicate.

by 0.78 and 0.78 Log10pg/ml and PHA by 0.72 and 0.87 Log10pg/ml ($p \leq 0.0001$; Figure 6B&C). Similarly, IL-10 levels were also significantly reduced with BMS treatment compared to the untreated control across all conditions ($p < 0.001$; Figure 6D). Post HIV exposure, IL-17 and IL-10 levels were still reduced with BMS treatment compared to the untreated control in the unstimulated ($p \leq 0.0001$) and TLR-stimulated ($p \leq 0.009$) conditions (Figure 6F&H). Additionally, GM-CSF levels were reduced with BMS treatment compared to the untreated control across all conditions ($p < 0.05$; Figure 6G).

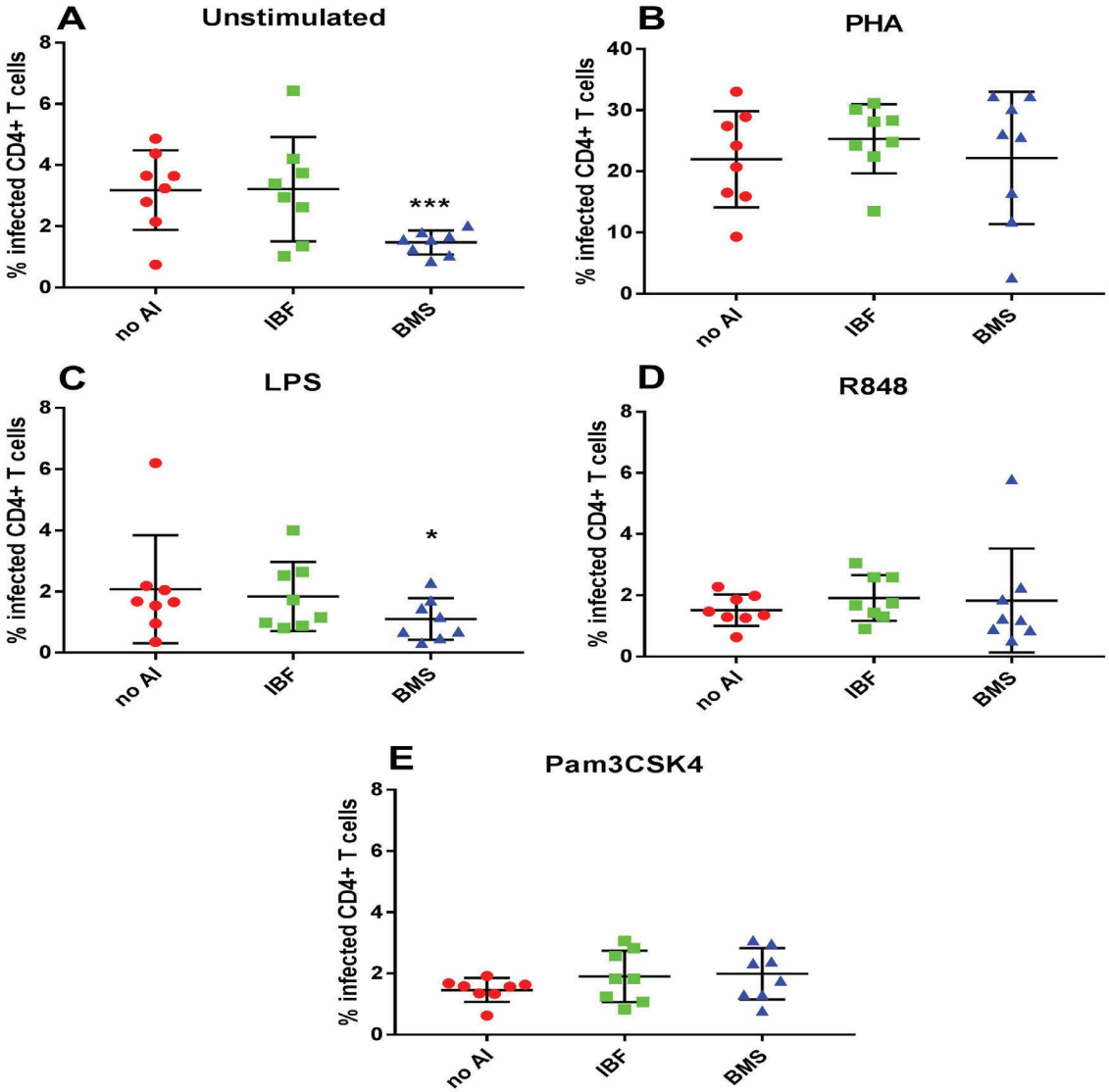

Figure 7: Frequency of HIV infected CD4+ T cells (measured by p24 expression) either left untreated (no AI, red) or treated with anti-inflammatory drugs ibuprofen (IBF, green) or betamethasone (BMS, blue) and then either left unstimulated (A) or stimulated with PHA (B), LPS (C), R848 (D) or Pam3CSK4 (E). PHA was used at a final concentration of 10 µg/ml. TLR agonists were used at a final concentration of 2 µg/ml. Both IBF and BMS were used at 1 µg/ml. An ordinary one-way ANOVA with a Dunnett's multiple comparisons test was performed to assess significant differences between AI conditions within each stimulation condition. Significance is displayed as * ($p < 0.05$), *** ($p < 0.001$) compared to the untreated (no AI) control. Sample size, n=4, donors run in duplicate.

3.4.5 BMS-mediated reduction of HIV infection occurs in the unstimulated and LPS stimulated conditions

Significant reductions of HIV infections were found in the BMS-treated unstimulated ($p=0.0002$) and LPS-stimulated ($p=0.02$) conditions by 1.7% and 0.98% respectively, compared to the untreated control (Figure 7A&C). No significant differences were observed with BMS treatment in the PHA-, R848- or Pam3CSK4-stimulated conditions ($p>0.05$; Figure 7B, D&E respectively), suggesting some differential interactions occurring in the unstimulated and LPS-stimulated conditions as opposed to the PHA-, R848- and Pam3CSK4-stimulated conditions with BMS. Additionally, no significant differences in HIV infection were observed with IBF treatment in any of the stimulation conditions ($p>0.05$; Figure 7).

3.5 Discussion

Genital inflammation is associated with increased HIV acquisition risk (14, 16, 47), while immune quiescence is an established correlate of protection for reduced risk in HESN populations (64, 89, 90). Therefore, the utility of immunomodulatory drugs to augment immune quiescence is attractive to reduce HIV susceptibility. Using a PBMC-based culture system, this study aimed to investigate the effect of two licenced anti-inflammatory drugs; the glucocorticoid (GC) BMS and the non-steroidal anti-inflammatory drug (NSAID) IBF, in limiting TLR-induced inflammatory cytokine productions, cellular activation and susceptibility to HIV infection. While IBF demonstrated only modest immunosuppression and no anti-inflammatory or anti-HIV activity in this model, BMS showed potent immunosuppression and anti-inflammatory effects, with reduced HIV infection.

Consistent with our previous findings (77), TLR2 (Pam3CSK4) and TLR4 (LPS) stimulation did not induce significant CD4+ T cell activation, while TLR7/8 (R848) activation was moderately more effective. PHA induced the greatest cellular activation, likely due to activation of the T-cell receptor (TCR) on CD4+ T cells (91). All TLR agonists induced a strong pro-inflammatory cytokine response at day 3, with R848 inducing the strongest inflammatory response over time (77). PHA induced a similar pro-inflammatory profile with higher concentrations of growth factor, anti-inflammatory and adaptive responses, and chemokines (IP-10, MIP-1β and RANTES) (77).

With IBF treatment, minimal immunosuppressive effects were observed, with small reductions in frequencies of intermediately activated CD38+HLA-DR- CD4+ T cells in the unstimulated and Pam3CSK4 stimulated conditions. Furthermore, IBF treatment had no discernible impact on frequencies of T cells expressing CCR5. This lack of immunosuppression may be attributed to T cells being unable to produce prostaglandins, likely a result of non-functional cyclooxygenase (COX) enzymes (92, 93). However, conflicting data show NSAID-reduced T cell activation through the

inhibition of COX enzymes resulting in blocking of TCR dependent p38 MAPK activation (94). Therefore, IBF may have interfered with the signalling pathways involved in immune activation in the unstimulated and Pam3CSK4 stimulated conditions here. Lajoie et al., (2018) showed reduced levels of systemic and mucosal HIV target and Th17 cells in women treated with oral acetylsalicylic acid (ASA) daily for six weeks, while hydroxychloroquine (HCQ), mimicking the regime of ASA, reduced systemic CD4+CCR5+ and Th17 cells. Additionally, they showed that mucosal Th17 cells expressed lower CCR5 and CD69 following ASA treatment (68), highlighting that such commonly used NSAIDS may be effective in mitigating immune activation in vivo. IBF treatment had no effect on cytokine production here, in contrast to observations of reduced IL-1β and IL-6 levels a human skin model (95) and similar findings of reduced systemic inflammatory cytokines with oral ASA and HCQ (68). Conversely, in human PBMCs, IBF enhanced TNF-α, IL-6 and IL-1β, but inhibited IL-1RA and IL-10 (96), while ASA augmented IL-2 and IFN-γ (97). These data highlight the complex and heterogeneous immune profiles associated with different drugs. IBF had no effect on HIV infection, regardless of the stimulation conditions whereas chloroquine (CQ), a NSAID, limited HIV replication in CD4+ T cells both *in vitro* and *in vivo*, through limiting DC-SIGN mediated viral transfer to CD4+ T cells (98).

Unlike IBF, BMS had potent immunosuppressive and anti-inflammatory effects. CCR5 expression on CD4+ T cells was reduced by BMS prior to HIV-exposure in all stimulated conditions and the mechanisms underlying reduced CCR5 expression remains undefined. However, the effect of BMS reducing CCR5 expression in all TLR-stimulated conditions was lost after HIV exposure. Similarly, others also showed that GC treatment resulted in dramatic reduction of renal CCR5+CD3+ T cells (99). In contrast, upregulation of the chemokine receptor CCR2 (which binds MCP) was found on human monocytes with GC treatment, leading to increased HIV susceptibility (100). CCR2, like CCR5, has been shown to have functional importance for HIV infection and disease progression by acting as a co-receptor for HIV (101-104). However, the heterogenous effects of BMS on CCR5 expression before and after HIV exposure was unexpected and the mechanisms underlying these differential effects need to be elucidated. Therefore, these findings necessitate the characterization of HIV co-receptor expression on target T cells especially if GC therapy is proposed as a means to mitigate HIV acquisition risk. BMS displayed potent immunosuppression and anti-inflammatory effects in all stimulation conditions, likely through the interference of gene transcription and signalling pathways (105-108). BMS was generally less effective with PHA-stimulation, likely due to robust TCR activation by PHA (109). In concordance with our data, human studies have shown that GCs effectively reduced inflammatory cytokines (110-113). In contrast, Frank et al., (2010) found that pre-treatment with GCs, prior to LPS challenge, augmented inflammatory cytokine production (TNF-α, IL-1β and IL-6) (114). However, when GCs were administered post LPS challenge, the inflammation was supressed suggesting the temporal dynamics of anti-inflammatory action is likely to be important in determining their potency (114). These results suggest that there is differential sensitivity to GCs, which may be tissue or

compartment specific. Another postulate for the immunoregulatory mechanism of GCs is the up-regulated transcription of anti-inflammatory genes, such as IL-10 via the GC receptor (115-117) and increased soluble IL-10 concentrations (112, 113). However, in our study, IL-10 production was reduced by BMS treatment, consistent with the global anti-inflammatory effects of GCs. BMS likely inhibited TLR-mediated induction of gene expression through NF-κβ or AP-1 blockade by the GC receptor (118, 119). GCs have been shown to impact HIV replication by interfering with viral transcription, mediated through the GC receptor (120). In the unstimulated and LPS-stimulated conditions only, BMS likely inhibited NF-κβ mediated gene transcription which reduced HIV infection, whereas R848 and Pam3CSK4 agonists likely use different signalling pathways (121), or have compensatory pathways with redundant functions. Despite the effective immunosuppression by BMS in the PHA condition, no impact on HIV infection was observed.

Our model system has some limitations that need to be acknowledged (77). We used a PBMC model instead of a vaginal epithelial cell line or ex vivo samples such as cervical mononuclear cells or explants. Despite inherent deficiencies with this model, PBMCs are more biologically representative than cell lines, depleted or purified immune cell models or explants which are notoriously difficult to obtain and standardize (49, 122, 123). PBMCs contain both peripheral and trafficked cells to or from tissues and the activation status of these cells correlated between these compartments (79). A further limitation was the lack of cellular activation and minimal HIV infection observed following TLR stimulation. In contrast, the PHA stimulated model showed higher HIV infection, as activated T cells are more efficiently and preferentially infected (42-44). However, strong inflammatory cytokine responses were induced by TLR agonists, highlighting their roles in initiating inflammation to drive immunity. In the genital tract, continuous TLR stimulation by pathogenic microbes drive immune activation and genital inflammation (59) which is associated with increased HIV risk (14, 124). To simulate similar conditions in a PBMC model, future experiments should include a TCR activator, such as anti-CD3 and anti-CD28 beads, to mimic antigen presentation in combination with TLR stimulation to provide more robust immune activation. The TCR activated model may be more appropriate for testing of anti-inflammatory drugs for their effects on immunosuppression and subsequent HIV infection. A further limitation was despite the potent immunosuppression by BMS in the PHA condition, there were no reductions in HIV infections, and we postulate that BMS impacts HIV infection independently of immunosuppression. This concept is reflected by the reduced HIV infection with BMS in the LPS and unstimulated conditions. Insight into the action of BMS on the GC receptor and HIV transcription pathways, may give clarity into the mechanisms of reduced HIV infection in these two conditions. While IBF effects were inferior to BMS which we speculate may be related to the anti-inflammatory pathways each anti-inflammatory drug targets, we did not measure levels of COX enzymes, prostaglandins or signalling proteins to verify possible mechanisms responsible for IBF's relative inferior immunosuppressive and anti-inflammatory capabilities. While it would have been interesting to investigate in more depth the

temporal impact of anti-inflammatory drugs in relation to HIV co-culture, our study focussed on pre-treatment with anti-inflammatory drugs prior to stimulation. In so doing we endeavoured to identify plausible drug candidates to mitigate genital inflammation in populations at increased risk for HIV acquisition. This approach has precedence given that glycerol monolaurate, a topically applied vaginal microbicide, reduced inflammation and prevented SIV infections in rhesus macaques (45, 76). Both BMS and IBF are also licenced as topical formulations (74, 75, 125, 126), making them attractive drug candidates. However, we acknowledge that long-term use of anti-inflammatory drugs do have unwanted and off-target adverse effects (127-130) that should be considered. Topical anti-inflammatory formulations may be subject to the same limitations of adherence that undermined topical PrEP (12). However, various HIV prevention options need to be explored to accommodate the varying and changing needs of the HIV affected communities.

To our knowledge, this is the first study investigating the effects of NSAIDs or GC treatment on HIV infection using an *in vitro* model. Other studies have investigated the effects of these drugs on inflammation/immune activation and HIV-mediated immune activation/replication in disease progression. This study provides important information on NSAID and GC effects on TLR-mediated immune responses and HIV infection, as well as underscoring the need to interrogate the inflammatory signalling pathways to identify novel drug targets. Together, these data may inform on the use of anti-inflammatory drug candidates as adjunctive prophylactic therapies in high risk populations for HIV.

3.6 Conflict of Interest Declarations

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

3.7 Author contributions

SSAK, JSP, LJPL, LRM and DA acquired funding for the study and edited the manuscript. LJPL, LRM, JSP, AS and DA assisted in study design and analysis of data. RC designed and ran experimental procedures, acquired and analysed data, wrote and edited the manuscript. AS assisted in study design, provided laboratory space for experimental procedures and edited the manuscript.

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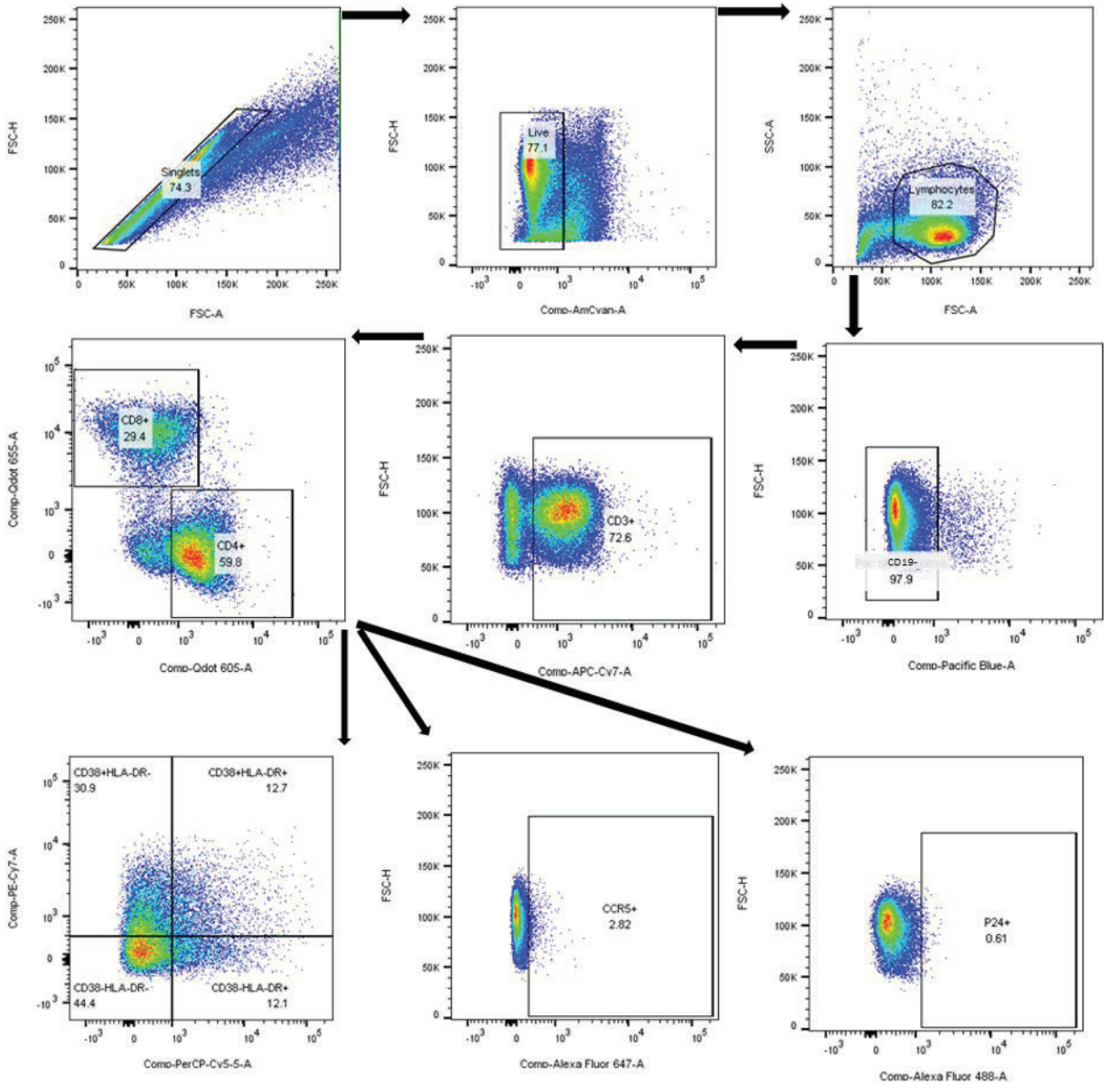
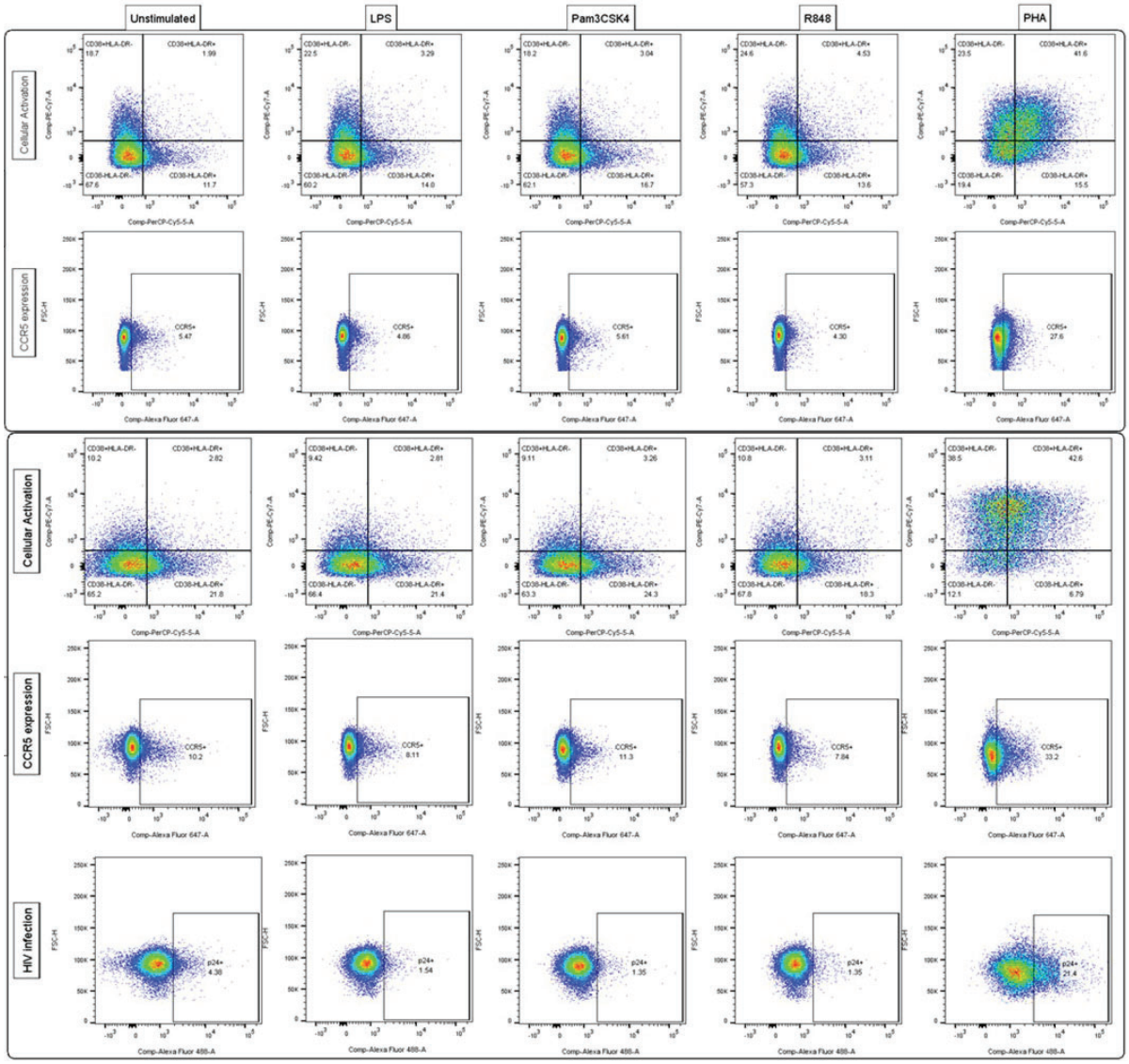
3.10 Supplementary data

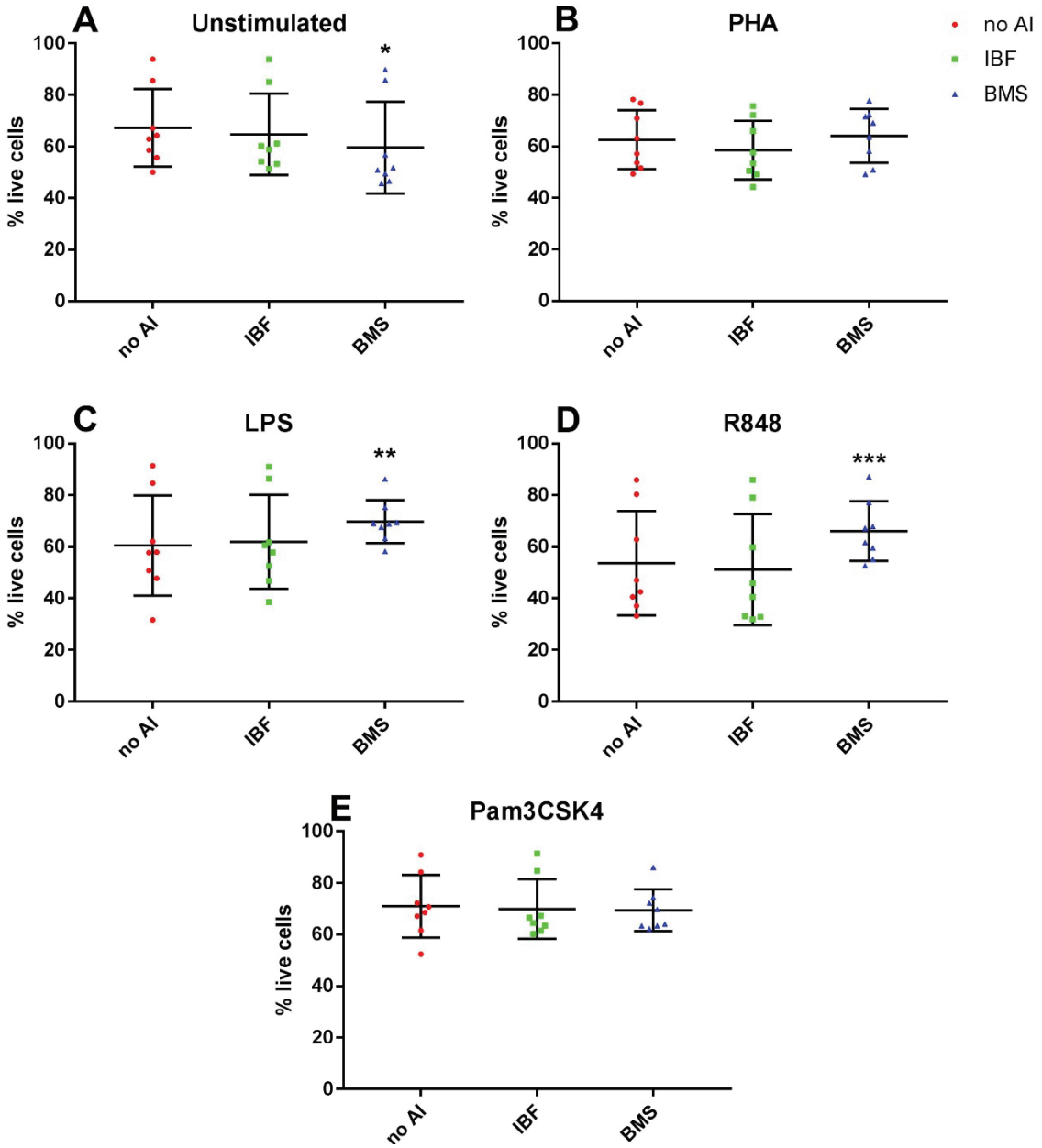


Supplementary Figure 1: Schematic diagram of the gating strategy used for analyses of flow cytometric data



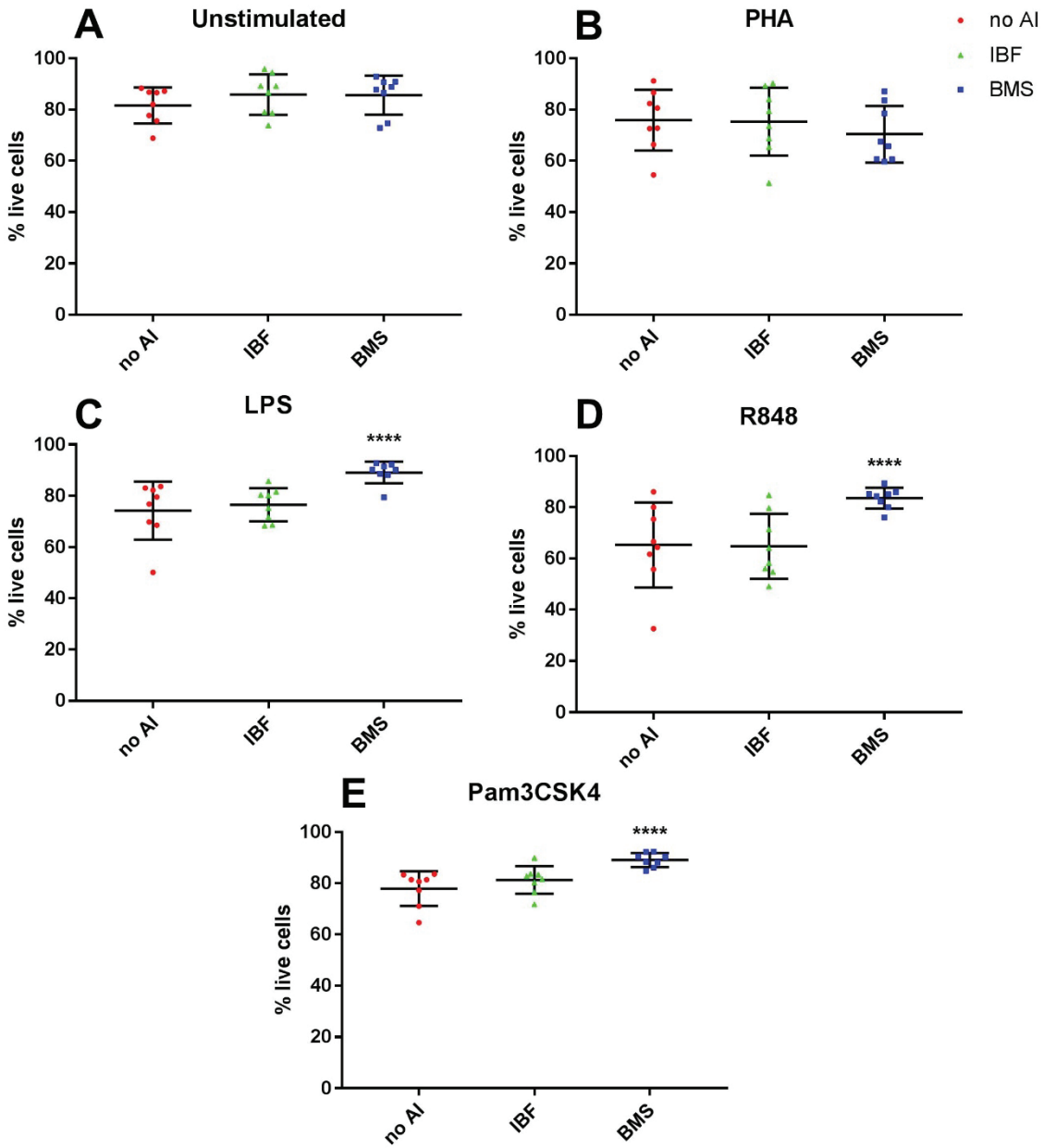
Supplementary Figure 2: Representative dot plots of flow cytometric data of cellular activation, CCR5 expression and HIV infection of CD4+ T cells not treated with anti-inflammatory drugs (no AI) prior to HIV infection on day 3 (top box) and post HIV infection on day 5 (bottom box) from the Unstimulated, LPS, Pam3CSK4, R848 and PHA (left to right ordered) conditions.

1



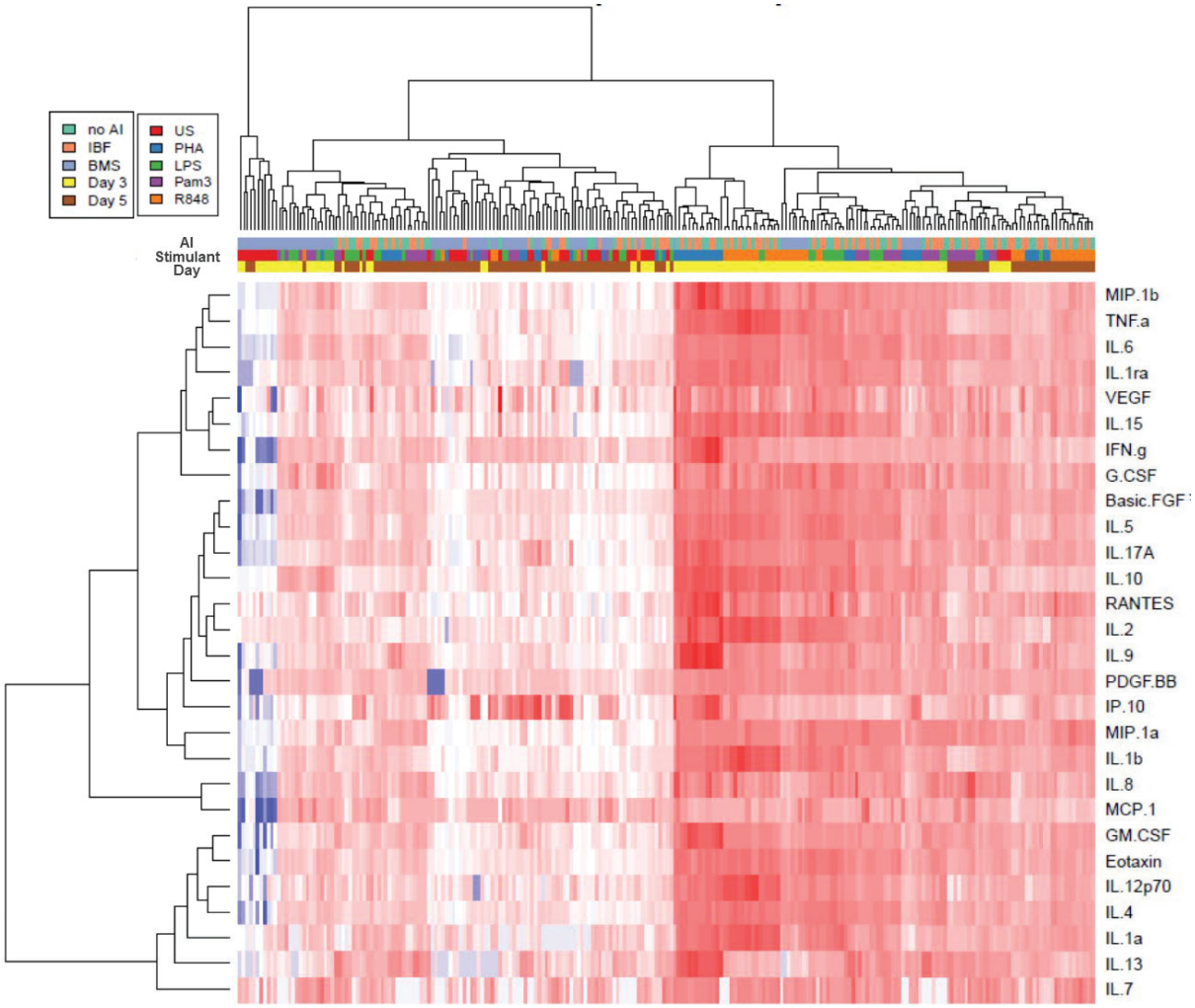
Supplementary Figure 3: Toxicity profiles of PBMCs treated with Ibuprofen (IBF, green) or Betamethasone (BMS, blue) or left untreated (no AI, red) and either left unstimulated (A) or stimulated with PHA (B) or TLR agonists LPS (C), R848 (D), or Pam3CSK4 (E) at day 3 prior to HIV infection. PHA was used at a final concentration of 10µg/ml. TLR agonists were used at a final concentration of 2µg/ml. Anti-inflammatory (AI) drugs IBF and BMS were both used at a final concentration of 1ug/ml. A repeated measures two-way ANOVA with Dunnett's multiple comparisons test was performed to assess significant differences between AI conditions within each stimulation condition. Significance is displayed as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) compared to the untreated (no AI) control. Sample size, n=4, donors run in duplicate.

1



Supplementary Figure 4: Toxicity profiles of PBMCs treated with Ibuprofen (IBF, green) or Betamethasone (BMS, blue) or left untreated (no AI, red) and either left unstimulated (A) or stimulated with PHA (B) or TLR agonists LPS (C), R848 (D), or Pam3CSK4 (E) at day 5 post HIV infection. PHA was used at a final concentration of 10µg/ml. TLR agonists were used at a final concentration of 2µg/ml. Anti-inflammatory (AI) drugs IBF and BMS were both used at a final concentration of 1ug/ml. A repeated measures two-way ANOVA with Dunnett's multiple comparisons test was performed to assess significant differences between AI conditions within each stimulation condition. Significance is displayed as **** ($p \leq 0.0001$) compared to the untreated (no AI) control. Sample size, n=4, donors run in duplicate.

1



Supplementary Figure 5: Unsupervised hierarchical cluster heat map analysis of 28 cytokines measured in cell culture supernatants on day 3 (yellow) and day 5 (brown) from the unstimulated (red), PHA (blue), LPS (green), Pam3CSK4 (purple) or R848 (orange) conditions either left untreated (light green) or treated with anti-inflammatory drugs ibuprofen (IBF, light orange) or betamethasone (BMS, light blue). PHA was used at a final concentration of 10 µg/ml. TLR agonists were used at a final concentration of 2 µg/ml. Both IBF and BMS were used at 1 µg/ml. In this heatmap, the redder the colour depicts the higher concentration, while the bluer the colour the lower the concentration. Sample size, n=4, donors run in duplicate.

1

3.11 Bridging chapter

The glucocorticoid drug, betamethasone, reduced HIV infection of CD4+ T cells in the TLR4 (LPS) stimulated condition, but not in the other stimulation conditions (TLR1/2, TLR7/8 and PHA). This was unexpected as the immunological profiles between TLR-stimulation conditions were similar, with potent immunosuppression across all stimulation conditions. These results suggest that it is not the suppression of immune activation and inflammation alone that is conferring this protective effect observed in this model. Rather these results suggest that the specific signalling pathways that were activated by TLR4 stimulation were affected by BMS treatment, thereby reducing productive HIV infection. These data highlight the complex nature of the inflammatory response, and how anti-inflammatory drugs may impact on HIV susceptibility apart from their traditional immunosuppressive effects. However, we know that inflammation and immune activation increase HIV acquisition risk, as well as increasing the likelihood of HIV disease progression to AIDS. Therefore, we investigated a variety of immunomodulatory products that could be used to modulate inflammation in order to reduce HIV susceptibility and slow HIV disease progression. **This review entitled "Inflammation, HIV and immune quiescence: leveraging immunomodulatory products to reduce HIV susceptibility" has been published on the 27th October 2020 in the Hindawi journal AIDS Research and Treatment for review (doi.org/10.1155/2020/8672850).**

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Review Article

Inflammation, HIV, and Immune Quiescence: Leveraging on Immunomodulatory Products to Reduce HIV Susceptibility

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The relationship between inflammation and HIV has been a focus of research over the last decade. In HIV-infected individuals, increased HIV-associated immune activation significantly correlated to disease progression. While genital inflammation (GI) has been shown to significantly increase the risk of HIV acquisition and transmission, immune correlates for reduced risk remain limited. In certain HIV-exposed seronegative individuals, an immune quiescent phenotype characterized reduced risk. Immune quiescence is defined by specific, targeted, highly regulated immune responses that hinder overt inflammation or immune activation. Targeted management of inflammation, therefore, is a plausible strategy to mitigate HIV risk and slow disease progression. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as hydroxychloroquine and aspirin have shown encouraging preliminary results in low-risk women by reducing systemic and genital immune activation. A topical NSAID, containing ibuprofen, is effective in treating vulvovaginal inflammation. Additionally, the glucocorticoids (GCs), prednisolone, and dexamethasone are used to treat HIV-associated immune activation. Collectively, these data inform on immune-modulating drugs to reduce HIV risk. However, the prolonged use of these pharmaceutical drugs is associated with adverse effects, both systemically and to a lesser extent topically. Natural products with their reduced side effects coupled with anti-inflammatory properties render them viable options. Lactic acid (LA) has immunomodulatory properties. LA regulates the genital microbiome by facilitating the growth of *Lactobacillus* species, while simultaneously limiting bacterial species that cause microbial dysbiosis and GI. Glycerol monolaurate, besides being anti-inflammatory, also inhibited SIV infections in rhesus macaques. The proposed pharmaceutical and natural products could be used in combination with either antiretrovirals for treatment or preexposure prophylaxis for HIV prevention. This review provides a summary on the associations between inflammation, HIV risk, and disease progression. Furthermore, we use the knowledge from immune quiescence to exploit the use of pharmaceutical and natural products as strategic interventions to manage inflammation, toward mitigating HIV infections.

1. Introduction

Human immunodeficiency virus (HIV), which causes acquired immunodeficiency syndrome (AIDS), is a global epidemic affecting approximately 37.9 (range 32.7–44) million people with an estimated 1.7 (range 1.4–2.3) million new infections for the year of 2018 [1]. Currently, sub-Saharan Africa (SSA) is the worst affected region with 20.6 (range 18.2–23.2) million infected individuals, with 800,000 new infections in the region during 2018 [1]. Furthermore, young women (15–24 years) are of particular concern in SSA as they account for over half of new HIV infections in this region [2]. The roll out of antiretroviral (ARV) drugs for infected populations has significantly altered the trajectory of the disease and the epidemic, transforming it into a manageable chronic condition for the majority of infected individuals [3]. The use of ARVs as preexposure prophylaxis (PrEP) for prevention has shown promise in men who have sex with men (MSM) populations taking oral PrEP [4–7]. However, variable degrees of success with oral PrEP have

4 Chapter 4: Inflammation, HIV and immune quiescence: leveraging immunomodulatory products to reduce HIV susceptibility

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4.1 Abstract

The relationship between inflammation and HIV has been a focus of HIV research over the last decade. In HIV infected individuals, HIV-associated immune activation is associated with disease progression to AIDS. While genital inflammation has been shown to significantly increase the risk of HIV acquisition and transmission, immune correlates for reduced HIV risk remain less well defined. In HIV-exposed seronegative individuals, immune quiescence was the phenotype that characterised reduced risk for HIV infection. Immune quiescence was defined by specific targeted, highly regulated immune responses that do not cause overt inflammation or immune activation. Immune quiescence was also shown in sooty mangabeys, the natural host for SIV that do not progress to AIDS. Targeted management of inflammation, therefore, is a plausible strategy to mitigate HIV acquisition risk, and slow HIV disease progression. Many pharmaceutical products such as non-steroidal anti-inflammatory drugs and glucocorticoids are commonly used to treat inflammation. Furthermore, many have been formulated into topical products. However, the prolonged use of these pharmaceutical drugs, is often associated with adverse effects, both systemically and to a lesser extent topically. Natural products are also a viable option as they have less adverse effects and have been shown to have anti-inflammatory properties. The proposed products could also be used in combination with either ART for treatment of HIV disease or pre-exposure prophylaxis for HIV prevention. This review reaffirms the links between inflammation and HIV disease progression, the protective effect of an immune quiescent environment, and possible pharmaceutical and natural products that could be used either alone or in combination to manage inflammation.

4.2 Introduction

Human immunodeficiency virus (HIV) which causes acquired immunodeficiency syndrome (AIDS), is a global epidemic affecting approximately 37.9 million people (range 32.7–44 million people) with an estimated 1.7 million (range 1.4–2.3 million) new infections for the year of 2018 [1]. Currently, sub-Saharan Africa (SSA) is the worst affected region with 20.6 million infected individuals (range 18.2–23.2 million), with 800 000 new infections in the region during 2018 [1]. Furthermore, young women (15-24 years) are of particular concern in SSA as they account for over half of new HIV infections in this region [2]. The roll out of antiretroviral (ARV) drugs for infected populations has significantly altered the trajectory of the disease and the epidemic, transforming it into a manageable chronic condition for the majority of infected individuals [3]. The use of ARVs as Pre-Exposure Prophylaxis (PrEP) for prevention, has shown promise in men who have sex with men (MSM) populations taking oral PrEP [4-7]. However, variable degrees of success with oral prep have been found in heterosexual populations [8-11]. The use of PrEP topically, in formulations such as microbicides gels and vaginal rings among others, has also shown some promise [12, 13]. However, despite the relative successes of these PrEP trials, behavioural factors like PrEP adherence [14] and biological factors such as genital inflammation [15] and a dysbiotic vaginal microbiome [16] have been shown to undermine these prevention strategies [17]. This review summarizes our current knowledge on the interplay between HIV and inflammation and the causes and consequences of inflammation. We also provide insight into immune quiescence as a protective factor against HIV acquisition with special emphasis on the putative use of pharmaceutical or natural products toward inducing a quiescent genital immune environment.

4.3 Inflammation and HIV

Inflammation has been associated with an increased risk of HIV transmission and acquisition [18-21]. In HIV-infected individuals, increased pro-inflammatory cytokines and immune activation directly correlated with increased HIV viral loads in genital secretions [20-23], thereby increasing the probability of onward transmission. Inflammatory cytokines: tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) were shown to directly affect HIV replication by activation of NF-κβ transcription factor, which bind to the HIV promoter region [24]. Furthermore, in a non-human primate (NHP) model of simian immunodeficiency virus (SIV) infection, higher monokine induced by interferon-γ (MIG) and interferon-γ-induced protein 10 (IP-10) mRNA levels in lymph nodes were positively correlated with more-rapid disease progression [25]. Similarly, antibody blocking of MIG was shown to reduce HIV-1 replication in an ex vivo human cervical tissue model [26]. In women increased cervical concentrations of TNF-α, IL-1β, IL-6 and IL-8 were found who were detectably shedding HIV compared to those that were not detectably shedding HIV in the genital tract [27]. Furthermore, monocyte chemoattractant protein 1 (MCP-1) was found to be positively correlated with viral loads, as well as promoting X4-tropic HIV infection of resting CD4+ T cells [28].

There are various mechanisms whereby inflammation creates a conducive environment for HIV acquisition. In HIV-uninfected individuals, inflammation resulted in recruitment of HIV target cells and epithelial barrier damage [29-31]. Moreover, immune activation and increased cytokines were directly associated with increased HIV acquisition risk in both the blood [32, 33], and the genital tract [19, 34]. Nazli et al., (2010) demonstrated that mucosal epithelial cells secreted increased pro-inflammatory cytokines upon exposure to HIV-1. In addition, TNF-α and interferon (IFN)-γ have been implicated in reduced epithelial barrier function, thereby increasing permeability of the mucosal barrier [35-40]. Li et al., (2009) described a process for target cell recruitment; macrophage inflammatory protein (MIP)-3α and IL-8 expression recruit plasmacytoid dendritic cells (pDCs) which in turn secrete MIP-1α and MIP-1β which recruit CCR5+ cells. Using an in vivo rhesus macaque model, they showed that inflammation and recruitment of target cells to the genital tract were important events for seeding and forming foci of SIV infection following vaginal challenge [41]. A study by Masson et al., (2015) showed that elevated genital tract chemotactic cytokines MIP-1α, MIP-1β, IL-8 and IP-10 which formed part of the definition for genital inflammation, conferred a more than three-fold increased risk for HIV acquisition [19]. Similarly, a follow-up study by Liebenberg et al., (2017) comparing plasma and genital tract cytokine levels showed that increased mucosal concentrations of IP-10, MIP-1β, IL-8 and monocyte chemoattractant protein (MCP)-1 were associated with increased HIV acquisition risk [18]. MIP-3α and IL-8 are important chemokines that facilitate infection through their chemotactic activity involved in the recruitment of HIV target cells [42, 43]. Additionally, IP-10, MIP-1α and MIP-1β have also been shown to recruit HIV target cells [44-47]. The MIP-1α-CCR5 interaction was also shown to activate the JAK/STAT signalling pathway which is also key to initiating cellular proliferation [48], and the inflammatory cascade [49, 50].

4.4 Causes of genital inflammation

Various biological and behavioural factors have been implicated in causing inflammation in the genital tract. Biological factors include sexually transmitted infections (STIs) and a dysbiotic vaginal microbiome. Risk for HIV acquisition has been associated with presence of pre-existing STIs [51-53], likely due to the inflammatory and immune responses against the causative pathogens [54-56]. Furthermore, asymptomatic STIs can further exacerbate inflammation through elevated genital tract inflammatory cytokine profiles and increase the risk for HIV acquisition [57]. Common STIs associated with increased HIV acquisition risk include the Herpes simplex virus (HSV) [58, 59], human papillomavirus (HPV) [60, 61], Neisseria Gonorrhoeae [62], Chlamydia trachomatis [63] and Trichomonas vaginalis [64, 65].

A dysbiotic vaginal microbiome, commonly referred to as bacterial vaginosis (BV), occurs when there is a shift from a Lactobacillus dominant to a non-Lactobacillus dominant genital mucosal environment with highly diverse bacterial communities [66]. This dysbiosis often leads to an inflammatory response

and subsequent increase in the permeability of the mucosal epithelia [67-69], thus increasing the risk of HIV acquisition [70-74]. Furthermore, a recent study demonstrated that the efficacy of the topical 1% tenofovir gel used in the CAPRISA 004 trial was undermined in women who had a non-lactobacillus dominated microbiome [16]. This decreased efficacy was attributed to the direct metabolism of tenofovir (TFV) by Gardnerella vaginalis in women with a non-lactobacillus dominated vaginal microbiome [16, 75].

Vaginal practices have been noted in certain populations of women, which include practices for intimate female hygiene [76] and to enhance sexual pleasure [77]. These practices include washing, douching, and insertion of products, among others [78]. While no studies have been powered to investigate the link between vaginal practices and HIV risk, and no compelling evidence [79, 80], there is however, biological plausibility [81]. Studies have shown that women who practice various forms of vaginal hygiene may impact the vaginal microflora [82, 83], which could lead to a dysbiotic microbiome [84, 85] and a subsequent inflammatory response in the genital tract [67, 68, 86]. Furthermore, although there is no direct evidence, inserting products into the vaginal tract is likely to compromise the mucosal barrier through causing micro abrasions for easier HIV viral translocation.

Together, STIs, a dysbiotic vaginal microbiome and vaginal practices have been shown as major factors driving inflammation in the FRT. However, these factors alone are not solely responsible for causing genital inflammation, and further studies are warranted to define the complex immunology of this vulnerable site.

4.5 HIV-Exposed Seronegative (HESN)

The risk of HIV infection is heterogenous across a population. Individuals that are continually exposed to HIV without becoming productively infected over a long period of observation are called HIV-exposed seronegative (HESN). HESN individuals display particular immunological phenotypes that have been posited as immune correlates of protection against HIV [87-89]. Genetic polymorphisms for example conferred significant protection against HIV in certain HESN individuals [90-95]. One particular genetic polymorphism was the delta 32 mutation in the CCR5 encoding region in the genome [96-98]. Other correlates of protection discovered were the presence or induction of particular immune responses [99] of both innate [100-104] and adaptive immunity [105-109], that were able to control acute infection by either neutralizing the virus [110-112] or killing infected cells [113-115] before viral propagation could occur. An additional correlate for reduced HIV susceptibility *in vitro* showed significantly greater sterol metabolism, possibility related to the induction of type-1 interferon genes, in PBMCs from HESN individuals compared to healthy controls [116]. These types of immune responses, which are generally triggered through TLR signalling [117], have increased the interest in using TLR agonists as adjuvants in vaccine research [118, 119]. However, it should be noted that there

is heterogeneity between HESN populations as they do not all display the same immune correlates of protection, which makes the comparisons across studies difficult. A particular cohort of HESN commercial sex workers (CSWs) from the Pumwani district in Nairobi, Kenya have been followed and studied extensively since 1984 [120]. Reduced immune activation and inflammation, commonly termed as immune quiescence, was identified in this group of HESNs. The concept of immune quiescence as a correlate of protection for HIV acquisition has largely stemmed from biologic and behavioural studies on this particular cohort.

4.6 Immune quiescence and HIV risk

The concept of immune quiescence not only unique in HESNs but has also been observed in sooty mangabeys, the natural host for SIV. Despite these animals being infected with SIV, with high levels of viral replication and depletion of gut CD4+ T cells, sooty mangabeys do not progress to AIDS [121]. Lower levels of systemic and mucosal CD4+CCR5+ T cells [122], reduced type 1 IFN responses [123], lower Th17 cells [124], and better management of immune activation through IL-10 and regulatory T cell (Treg) upregulation [121] attributed to their quiescent state despite ongoing SIV replication and high viral loads.

Multiple studies have reported reduced immune activation in HIV-exposed but seronegative individuals [89, 104, 125-128] underscoring the importance of modulating inflammation and immune activation or having an immune quiescent environment in an effort to minimize the risk of acquiring HIV. Reduced immune activation, defined by CD69 expression on CD4+ and CD8+ T cells, was found to be a correlate of protection in HESN CSWs compared to HIV-uninfected CSWs, and this reduced immune activation was associated with increased frequencies of regulatory T cells [129, 130]. Furthermore, in a HESN MSM cohort, low frequencies of CD4+ and CD8+ T cells expressing HLA-DR, CD38, CD70 and Ki67 were found [131]. Similarly, the uninfected partners of serodiscordant couples, had reduced expression of CD38, HLA-DR and CCR5 on CD4+ T cells [132, 133], the target cells for HIV infection. Our *in vitro* data show that PBMCs stimulated with LPS were less susceptible to HIV infection than the unstimulated negative control [134]. LPS stimulation elicited a strong cytokine response with very limited immune activation (defined by CD38 and HLA-DR expression on CD4+ and CD8+ T cells) [134], partially reminiscent of an immune quiescent environment. These data highlight the potential of TLR agonists to induce protective immune responses, however, the continued management of these immune responses will be necessary to avoid overt inflammation.

Furthermore, molecular studies investigating the function of CD4+ T cells from HESN CSWs found lower gene expression in peripheral blood mononuclear cells (PBMCs) and whole blood from HESN CSWs compared to HIV-uninfected susceptible CSWs [127, 135]. The most under expressed genes identified in HESN CSWs were involved in T cell receptor signalling and host factors required for HIV

replication [127, 135]. Additionally, McLaren et al., (2010) found that unstimulated PBMCs from HESN CSWs produced lower levels of cytokines than HIV-uninfected susceptible CSW PBMCs, however this difference was lost after stimulation [127]. This suggests that PBMCs from HESN CSWs are not immunosuppressed, but rather have lower baseline expression of cytokines. Similarly, even post PBMC stimulation, lower levels of IL-17 and IL-22 were still observed in HESN CSWs, suggesting that HESN individuals have a blunted Th17 response [125]. Th17 cells are an important subset of CD4+ T cells and play an important role in homeostasis of mucosal tissues [136-138]. However, as essential as Th17 cells are for mucosal barrier integrity, they are preferentially hijacked for HIV infection [139, 140]. The duality of Th17 cells have been shown to be important targets and are particularly susceptible to infection in a SIV NHP model [141, 142], while remaining susceptible to preferential depletion in HIV-infected individuals [143, 144].

As the majority of HIV infections occur at the FGT, the immune environment within this compartment will be important in determining HIV risk. In the study by Chege et al., (2012), cervical mononuclear cells (CMCs) expressed lower IL-17 and IL-22 after stimulation, suggesting that a blunted TH17 response [125] maybe protective against HIV infection. Furthermore, reduced expression of pattern recognition receptors (PRRs), and low cytokine production in culture was found in HESN CMCs [104]. Despite this, these CMCs produced a strong anti-viral responses post TLR7/8 stimulation, suggesting that cells with immune quiescent phenotypes from HESNs can still elicit protective antiviral responses against HIV [104]. Lajoie et al., (2012), investigating differences between HESN CSWs, HIV-infected CSWs and HIV-uninfected CSWs, found that HESN individuals had lower expression levels of the pro-inflammatory cytokine IL-1α and IFN-γ regulated chemokines MIG and IP-10. Furthermore, the reduced cytokine expression correlated with higher levels of mucosal antiproteases in HESN individuals, suggesting unique expression patterns of mucosal immune mediators which creates an environment less conducive to HIV acquisition [89]. Furthermore, MIG and IP-10 bind to CXCR3 which induces the recruitment of activated T cells [89], suggesting that these two chemokines play an important role in modifying HIV risk. These studies describe immune quiescence in the mucosal compartment, which leads to reduced recruitment of HIV target cells and therefore reduced HIV infection risk. Therefore, inducing an immune quiescent mucosal environment is a biologically plausible strategy to reduce risk of HIV infection. We suggest that the use of immunomodulatory products, possibly in combination with ARVs after thorough and rigorous scientific and clinical trial testing, may be an additional strategy to incorporate into the currently limited HIV prevention options.

4.7 Immunomodulatory products

As genital inflammation is regarded as a significant risk factor for HIV infection, and immune quiescence was attributed as a correlate of protection in certain populations, products that modulate inflammation are attractive additive HIV prevention options. Increased co-morbidities in HIV-infected

individuals [145, 146], warrant the use of anti-inflammatory therapies to stem HIV-associated inflammation and immune activation [147-151] to ameliorate disease.

4.7.1 Antiretroviral drugs

Interestingly, ARVs have been associated with reduced immune activation. In a rhesus macaque model of rectal SHIV infection, monkeys that were given oral PrEP had reduced levels of cytokines: IL-15, IL-18 and IL-RA [152]. Healthy individuals taking daily PrEP for 30 days had lower systemic CD8 T cell activation (CD38/HLA-DR co-expression) compared to their baseline before PrEP initiation, however cytokines and other markers of inflammation in the blood were not affected [153]. In high-risk heterosexual HIV serodiscordant African couples, daily oral PrEP also did not modulate HIV-specific immune responses [154]. There may, however, be a heterogenous effect of ARVs on immunity depending on exposure to HIV or HIV infection itself.

4.7.2 Non-Steroidal Anti-Inflammatory Drugs

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are the most common anti-inflammatory drugs prescribed for reducing inflammation and through this process, supress pain [155]. NSAIDs account for approximately 5-10% of prescribed medication each year [156]. The main mechanism for NSAIDs is through the inhibition of the cyclooxygenase (COX) enzymes which convert arachidonic acid into prostaglandins [157]. Prostaglandins in turn exhibit varied and seemingly opposite functions such as induction and resolution of inflammation [158].

4.7.2.1 Aspirin

Acetylsalicylic acid (ASA), commonly known as Aspirin®, is a very common NSAID with fairly good safety profiles [159]. Aspirin® is Food and Drug Administration (FDA) in the United States of America approved and is also readily available as an over the counter drug. ASA is commonly used to treat headaches [160], to prevent of cardiovascular disease [161-163] and to reduce the risk of breast [164] and colorectal cancer [165].

A study of daily oral Aspirin® in low-risk Kenyan women found reduced the levels of systemic and mucosal HIV target CD4+ T cells and Th17 cells, as well as reduced systemic inflammatory cytokines [166]. Even in HIV-infected virologically suppressed patients, low-dose Aspirin® was shown to reduce platelet count, T cell and monocyte activation [167], thereby reducing the risk of non-AIDS related morbidities, such as cardiovascular diseases [145].

4.7.2.2 Ibuprofen

Ibuprofen (IBF) is a frequently prescribed and commonly used NSAID with prominent analgesic and antipyretic properties [168, 169]. IBF has similar efficacies as ASA for the treatment of conditions such as headaches [170]. Furthermore, IBF is used for the treatment of various inflammatory, musculoskeletal and rheumatic disorders [171]. IBF has also been shown to increase the efficacy of

nucleoside reverse transcriptase inhibitors (NRTI) by reducing drug transporter proteins, and this limits the transport of NRTI's out of the cells [172, 173]. IBF is available commercially as a topical anti-inflammatory [Ginenorm® (ibuprofen isobutanolammonium)] as is a vaginal douche. Ginenorm® is effective for treating the inflammatory condition vulvovaginitis, and has a superior action compared to other NSAID topical options such as benzydamine [174].

4.7.2.3 Indomethacin

The NSAID indomethacin is primarily used for the treatment of rheumatoid arthritis, but also limits HIV replication [175, 176]. In the presence of indomethacin, but not other NSAIDS (Aspirin®, indoprofen, IBF or naproxen), MT-4 cells (CD4+ T cell line) displayed reduced HIV replication, measured by ELISA as reduced p24 production [175]. Furthermore, indomethacin suppressed HIV replication further when used in combination with an antiviral plant protein called MAP30 [176]. Additionally, similar to IBF, indomethacin improved NRTI efficacy by reducing drug transporter proteins, thus reducing the efflux of these drugs out of cells [172, 173].

4.7.2.4 Chloroquine and Hydroxychloroquine

The use of Chloroquine (CQ) and Hydroxychloroquine (HCQ) have been investigated fairly extensively in HIV-infected populations as well. Both of these drugs, taken orally, have been shown to significantly reduce HIV associated immune activation [148-151]. Furthermore, chloroquine was also shown to directly limit HIV replication and DC-SIGN mediated HIV viral transfer to CD4+ T cells both *in vitro* and *in vivo* [177].

Daily HCQ use was shown to reduce the numbers of circulating CD4+CCR5+ and Th17 cells, while mucosal Th17 cells expressed lower CCR5 and CD69 [166]. Oral HCQ administration reduced systemic, but not mucosal, IP-10 and IL-2RA [166]. Furthermore, an HCQ vaginal implant was tested in a rabbit and mouse model, and its immunomodulatory effects were tested in the presence of nonoxynol-9 (N9) induced inflammation. N9, originally designed as a spermicide, was also hypothesized to reduce HIV infection through disrupting the viral membrane. However, N9 increased HIV risk through causing inflammation [178-180]. In contrast, the HCQ implant alone was able to reduce the recruitment of immune cells, improve mucosal epithelial integrity, reduce T cell activation and reduce inflammatory cytokine production [181] suggesting that an implant containing an anti-inflammatory drug may reduce the risk of HIV infection.

4.7.3 Glucocorticoids

Glucocorticoids (GCs), also commonly referred to as corticosteroids although technically GCs are part of the corticosteroid class of drugs. GCs are produced endogenously in the adrenal glands and other tissues [182] as hormonal compounds and are essential to everyday life to regulate and support physiological process throughout the body [183]. GCs modulate immunity through the interference of

gene transcription, resulting in impaired signalling pathways [184, 185], as well as through non-genomic effects such as interactions with cellular membranes and receptors to initiate or inhibit signalling responses [186]. GCs have also been shown to induce apoptosis of T cells [187], a potential mechanism for immunomodulation. A major hallmark of AIDS is the steady decline in the numbers of CD4+ T cells through a combination of cellular apoptosis, exhaustion, and subsequent immune system dysfunction [188]. Triggering of the glucocorticoid induced tumour necrosis factor receptor family related (GITR) protein has been shown to limit T cell apoptosis, thereby improving immune function, measured by cytokine expression [189], highlighting the potential of GCs to slow HIV disease progression by limiting CD4+ T cell loss.

4.7.3.1 Dexamethasone

Dexamethasone (DEX) is a commonly used glucocorticoid. DEX has been shown to reduced cytokines associated with a TH1 response, with concomitant increases cytokines associated with a TH2 response in human PBMCs [190]. Apart from the traditional GC effects on gene transcription, DEX has additional posttranscriptional regulatory effects [191, 192], enhancing its immunomodulatory effects. Furthermore, DEX has also been shown to reduce arachidonic acid derived from the cellular membranes of epithelial cells [193], as well as suppression of COX-2 and prostaglandin E2 expression [194], highlighting the additional immunomodulatory effects of this drug. Similar to indomethacin, DEX has shown to inhibit HIV replication in an MT-4 cell line, an effect potentiated by concurrent MAP30 treatment [176]. DEX also suppressed the HIV promoter region, thus inhibiting viral transcription and subsequent replication [195]. However, DEX inhibited the killing of HIV infected CD4+ T cells by macrophages, mediated through antibody dependent cellular cytotoxicity, in PBMCs from both HIV infected and uninfected individuals [196], highlighting that DEX, and likely most glucocorticoids, can be overtly immunosuppressive and dampen protective responses too.

4.7.3.2 Betamethasone

Betamethasone (BMS) is another common GC which is similar to dexamethasone. BMS is commonly used topically, and these topical formulations have been around for years [197]. In a mouse model, topical BMS reduced the expression of IFN-γ, IL-1β, TNF-α, IL-17, IL-22 and IL-13 induced by TLR7/8 stimulation [198]. Similarly, a topical beclomethasone dipropionate inhibited allergen-induced T cell production of IL-3, IL-5 and GM-CSF [199]. Data from our group shows that BMS was potently immunosuppressive in human PBMCs stimulated with TLR agonists LPS, R848 and Pam3CSK4 and the mitogen PHA, and even in our unstimulated condition (R Cromarty, unpublished results). Furthermore, despite global immunosuppression, BMS significantly reduced HIV infected CD4+ T cells in the unstimulated and LPS stimulated conditions, but not in the R848, Pam3CSK4 or the PHA conditions (R Cromarty, unpublished results). These results suggest that it may be prudent to understand

the inflammatory response at the gene transcription level to understand potential drug targets which lead to the discovery and formulation of appropriate drugs.

4.7.3.3 Prednisolone

Prednisolone, another common GC, has been used extensively in reducing HIV-associated immune activation to slow the progression to AIDS [200]. The use of prednisolone has been shown to reduce HIV viral loads and the chemokine MCP-1 [201], as well as HIV associated immune activation [202]. Furthermore, prednisolone slows the loss of CD4+ T cells and inhibits apoptosis of activated CD4+ T cells in ARV treated patients and during structured therapy interruption [203-205], hindering the progression to AIDS. Conversely, prednisolone treatment in HIV infected ARV treatment naïve patients showed no effect on disease progression with continued high viral loads despite reduced immune activation, likely due to increased target CD4+ T cells supporting ongoing viral replication [206].

4.7.4 Natural Compounds

Anti-inflammatory drugs do have unwanted and off-target adverse effects [207, 208]. Chronic use of NSAIDs have adverse effects on the gastrointestinal tract [209, 210], kidney [211] and the cardiovascular system [212]. Similarly, chronic GC use can increase risk for cardiovascular [213, 214] and metabolic disease [215] and also neurodegeneration [216]. Although, topical NSAID and GC treatments have dramatically less common adverse effects, systemic effects have been reported with continued use [217, 218], especially in elderly patients [219]. Mucosal surfaces being more permeable than skin, are especially susceptible to potential adverse events [220]. Therefore, natural products that may have minimal, if any side effects, either in combination or alone may provide an alternative for certain indications. Three such products are discussed below as these have already been formulated for topical use and have shown promising results from *in vitro* and animal studies.

4.7.4.1 Vitamin D

Vitamin D deficiency has been associated with a myriad of diseases such as cardiovascular disease, cancers, chronic lung disease, diabetes and autoimmune diseases in addition to its well-known role in reduced bone homeostasis [221, 222]. Vitamin D has numerous physiological effects on the immune system [223] as its primary active metabolite is a steroid hormone [224]. Supplementation with the active compound of Vitamin D, calcitriol, has proven to be effective in preventing both the initiation and progression of various autoimmune diseases in humanized mice models [225-227]. Vitamin D is available as a topical formulation to treat psoriasis [228]. Vitamin D analogues are known to upregulate Th-2 and Treg responses and may counterbalance against the adverse effects of GCs [229] which cause global immunosuppression. Combination therapies utilising BMS and another vitamin D analogue, Calcipotriol (CAL), were shown to be highly and more effective for treating psoriasis than BMS monotherapy alone [230].

Patients with vitamin D deficiency display a similar immune dysfunction profile to that of HIV infected patients. A hallmark of HIV disease progression is dysregulated immune activation [231]. Since Vitamin D has immunoregulatory properties [223], vitamin D supplementation may be a suitable adjunctive therapy to slow disease progression and possibly lower inflammation and immune activation to limit HIV replication. A clinical trial (http://clinicaltrials.gov identifier NCT03426592) is currently in progress to assess the impact of Vitamin D supplementation on HIV latency. Furthermore, the association between the use of certain ARVs and reduced vitamin D levels [231], highlights the need for further studies to identify mechanisms for Vitamin D depletion in HIV infected populations on ARVs. These data may be important at a public health level for vitamin D supplementation into ARV regimens in HIV endemic populations.

4.7.4.2 Glycerol Monolaurate

The most successful non-ARV based microbicide is Glycerol Monolaurate (GML) which is also commonly used in cosmetic products. Two studies in SIV Rhesus macaque models demonstrate the effect of GML in preventing SIV infection [41, 232]. Two mechanisms of action were identified; firstly, GML is a fatty acid monoester which assists with membrane stabilization by blocking bacterial induced pore formation and T cell activation [233-236]. Secondly GML disrupts T cell signalling and function [237] and inhibits cytokine and chemokine production thereby preventing the recruitment and activation of HIV target cells, important preceding events for establishment of SIV infection [41]. Furthermore, GML was shown to inhibit Candida and Gardnerella vaginalis in women [238], the overgrowth of these two microbes are associated with BV [66] and subsequent inflammation in the genital tract [54, 67, 68], and did not impact on the Lactobacilli sp. [238], the bacterial species generally associated with a healthy vaginal microbiome [66]. GML also inactivates HSV-2 [239] and Chlamydia trachomatis [240]. GML with its low side effects profile and its ability, at least in preclinical studies, to prevent SIV infections, is an attractive candidate for topical formulation as an HIV prevention modality.

4.7.4.3 Lactic acid

Lactic acid (LA) is a naturally occurring compound commonly found in the female genital tract that is produced by Lactobacillus species [241, 242]. The amount of LA depends on the dominance of the Lactobacillus species. A vaginal microbiome that is dominated by Lactobacillus species, with low abundance of microbial diversity, are often termed a “healthy” vaginal microbiome [243]. Research has been focussed on the role that LA plays in the female reproductive tract. Both the L and D isomers of LA have potent anti-inflammatory effects, with suppressed expression of inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α, RANTES and MIP-3α, and increased expression of the anti-inflammatory cytokine IL-1RA from cervicovaginal epithelial cells, even in the presence of TLR stimulation and seminal plasma [244]. Furthermore, LA has been shown to inactivate HIV *in vitro*, with the L- isoform more potent than the D- isoform, with this effect not solely due to pH [245]. Similarly, this anti-viral

effect of LA has been shown from clinical samples, whereby cervicovaginal fluid from women with lactobacillus-dominated microbiomes was shown to inactivate HIV ex vivo [246].

Furthermore, topical LA is versatile and is used for the treatment of various skin and oral complications such as acne vulgaris, melanogenesis and recurrent aphthous ulcerations respectively [247-250]. An over the counter LA containing vaginal douche was assessed for its impact on vaginal microbiota, with adverse findings of ~ three-fold-increased risk for acquiring diverse vaginal microbial species through douching with this product during menses [251]. However, the diverse and dysbiotic vaginal microbiome may arise through a combination of douching [83] and menses [252], and may not be the effect of LA itself, as the majority of the women in this study had a Lactobacillus dominant vaginal microbiome at the start of the study [251]. An LA based vaginal gel is also currently under investigation for its effectiveness in treating BV compared to the current standard-of-care, Metronidazole [253]. As there is a high recurrence rate of BV after Metronidazole treatment, a Lactobacillus crispatus containing vaginal gel used post Metronidazole treatment was effective in preventing BV recurrence [254].

4.7.4.4 Alternative natural products

There are many other natural products that could be considered as possible adjunctive therapy due to their anti-inflammatory effects. Curcumin, a curcuminoid contained in turmeric, is one such natural product. Curcumin has shown potent anti-inflammatory and anti-microbial effects [255-258], as well as anti-viral activity against HIV-1 and HSV-2 [258, 259]. Garlic is another such product that has been shown to display anti-inflammatory effects [260-262]. Similarly, consistent with the growing global acknowledgement of medicinal properties of Cannabis [263-265], this plant has been shown to have anti-inflammatory properties [266], mainly attributed to the cannabinoid metabolites contained within the plant [264, 267-270]. Cannabis was found to reduce the level of circulating CD16+ monocytes as well as levels of IP-10, compared to individuals who did not use cannabis [271]. Similarly, heavy cannabis use in HIV-infected individuals was associated with reduced frequencies of activated CD4+ and CD8+ T cells, intermediate and non-classical monocytes and cytokine producing antigen presenting cells [272], highlighting the immunomodulatory potential of cannabis in preventing inflammation and immune activation. As attractive as these products may be in modulating inflammation (based largely on *in vitro* data), their safety and side effects have to be rigorously, scientifically tested.

4.8 Conclusion

Genital inflammation significantly modifies the risk for HIV acquisition, although the causes of genital inflammation and exact biological mechanisms need to be further defined. Inflammation leads to the recruitment and activation of CD4+ T cells, which serve as target cells for HIV infection, with a concomitant disruption of the mucosal barrier allowing for easier viral translocation. HIV replicates more efficiently in activated target cells. Conversely, in the era preceding ARVs and PrEP, immune

quiescence has been identified as an immune correlate of protection against HIV infection in some high-risk populations. The use of anti-inflammatories to reduce HIV transmission is therefore not a new concept and dampening inflammation to induce an immune quiescent phenotype in high risk populations is attractive as adjunctive therapy in combination with PrEP, or in areas where PrEP access is limited. Therefore, the purpose of this review was to reaffirm the links between inflammation and increased HIV risk, immune quiescence and HIV, and to propose products that may be used to induce immune quiescence to reduce the risk of HIV acquisition. Many pharmaceutical anti-inflammatory drugs have known adverse effects, therefore we also proposed natural products that may be used either in combination or alone to mitigate HIV risk by reducing genital inflammation. However, inflammation is a necessary and protective response against invading pathogens and damaged tissues. The modulation of specific immune responses that initiate and drive the inflammatory cascade may be key in preserving a certain threshold of inflammation that is protective. Therefore, interrogating the cellular transcriptional signalling pathways during inflammation will be an important first step in understanding which immunomodulatory products would be appropriate to use to mitigate overt inflammation, while allowing protective inflammatory responses to continue.

4.9 Conflict of Interest Declarations

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

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5 Chapter 5: Synthesis

This synthesis chapter comprises of three main sections:

The first part covers the effect of various TLR agonists on immune activation and inflammation pathways and their effects on HIV infection of target CD4+ T cells. The second part covers the effects of two anti-inflammatory drugs in modulating the various TLR-mediated immune activation and inflammatory responses and HIV infections of target CD4+ T cells. The final part reviews immunomodulatory pharmaceutical and natural products as putative adjunctive prophylaxis/therapies to modulate inflammatory responses in order to mitigate HIV risk.

The link between inflammation, immune activation, and increased risk of HIV acquisition is well established. Various biological and behavioural factors together create immunological responses (205) conducive for HIV infection. The ensuing inflammatory immune responses drive the recruitment and activation of HIV target cells (185, 265), and also disrupt the mucosal barrier, allowing for easier viral translocation (166, 266). Even in the presence of topical PrEP, genital inflammation can undermine drug efficacy (222). A cardinal initiator of inflammation and immune responses is the recognition of pathogen and damage associated molecular patterns (P/DAMPs) by pathogen recognition receptors (PRRs) such as TLRs (267). We hypothesised that TLR agonists (LPS (TLR4), R848 (TLR7/8) and Pam3CSK4 (TLR1/2)) would create an inflammatory environment which would be conducive to HIV infection of target CD4+ T cells. Therefore, in our study we used TLR agonists to induce inflammation and immune activation using PBMCs, similar to a previous study from our laboratory (268). These TLR agonists were used to mimic previous *in vivo* findings of inflammation associated with pathogenic infections or a dysbiotic microbiome (205). We then assessed how these TLR-mediated responses impacted on HIV infection of CD4+ T cells. We found that TLR agonists induced significant inflammatory cytokine responses, limited immune activation and down regulated the HIV co-receptor CCR5 which created a less conducive environment for HIV infection of target CD4+ T cells. The co-stimulatory nature of TLRs likely resulted in the lack of CD4+ T cell activation while robust activation generally requires a TCR activator in combination with TLR stimulation (269). Contradictory to our hypothesis, we found that LPS-mediated inflammation significantly reduced HIV infection. While there was no evidence of reduced HIV infection with Pam3CSK4 and R848 stimulation, these results suggest the specific response to LPS, is likely to be a heightened innate anti-viral response which played an important role in reducing HIV infection of CD4+ T cells. These results partially mimic findings in certain HESN cohorts where targeted, robust immune and potent cytokine responses were observed (270-272) which likely contributed to the reduced likelihood of HIV infection in these individuals. Furthermore, stimulation of TLRs induced more robust immune responses in these HESN individuals (270). However, other studies have established an association between these inflammatory cytokine responses and increased risk of HIV acquisition in the genital mucosal environment (166, 169, 185).

Contrary to these data, in other HESN cohorts, another correlate of reduced risk of HIV infection was immune quiescence. The immune quiescent phenotype is defined as a reduced or highly regulated inflammatory and immune activation response/s (273). The use of anti-inflammatory drugs to induce immune quiescence and/or limit inflammation has been a topic of ongoing debate in the HIV prevention field, with one such study showing that the use of anti-inflammatory drugs reduced inflammation and immune activation (261).

The assessment of two different classes of anti-inflammatory drugs were therefore investigated to understand their effects on TLR-mediated immune responses and HIV infection of CD4+ T cells. The NSAID; ibuprofen (IBF), and the glucocorticoid; betamethasone (BMS) were the two candidate drugs tested. We observed no significant differences between the TLR conditions in this chapter (supplementary figure 4 and supplementary table 1 in the appendices). These results were interesting as we did see a significant reduction in HIV infection with TLR4 stimulation in chapter 2, therefore these interaction need to be further analyses with RNAseq analysis. Following on from this we sought to assess how the two different classes of anti-inflammatory drugs impact on these TLR-mediated immune responses and HIV infection. IBF, a commonly used NSAID world-wide, did not have a strong anti-inflammatory effect in this model. Despite the ineffectiveness of IBF in our model, this drug has been formulated as a topical vaginal douche for the effective management and treatment for an inflammatory condition in the genital tract, vulvovaginitis (274, 275). BMS, on the other hand, showed strong anti-inflammatory and immunosuppressive potential in our *in vitro* model, and has already been formulated as a topical ointment for treatment of inflammatory skin conditions (276-278). Furthermore, BMS significantly limited HIV infection of CD4+ T cells in the LPS stimulated and unstimulated conditions, but not the other TLR (Pam3CSK4 and R848) or PHA stimulated conditions. These findings highlighted that in our model BMS was not exerting its protective mechanism through immunosuppression, as the reduction in HIV infection is only seen in the LPS and unstimulated conditions, despite potent immunosuppression across all conditions. Glucocorticoids (GC) such as BMS generally inhibit the induction of gene expression through NF-κβ or AP-1 blockade by the GC receptor (279, 280). Furthermore, GCs have been shown to interfere with viral replication, mediated through the GC receptor, thus hindering HIV's ability to further replicate (281). In the unstimulated and LPS-stimulated conditions only, BMS likely inhibited NF-κβ mediated gene transcription which reduced HIV replication (measured by p24 production). Whereas in the R848 (TLR7/8) and Pam3CSK4 (TLR1/2) stimulated conditions different signalling pathways were likely used (282). An alternative explanation is that compensatory pathways with redundant functions were activated. These results suggest that there are specific signalling pathways activated with each stimulation condition, and these were differentially affected by BMS, which determined the HIV infections observed. However, confirmatory follow-up studies focussing on transcriptional pathways in these different stimulation conditions would need to

be conducted. Therefore, assessment of inflammatory signalling pathways in the mucosal environments would play an important role in determining the most effective intervention.

Although our studies did not replicate *in vivo* inflammation and immune activation associated with increased HIV infection, our previous clinical studies confirmed the link between genital inflammation and increased risk of HIV acquisition in women (166, 169, 283), while in some studies on HESN individuals, immune quiescence has been identified as a correlate of reduced risk against HIV infection (172, 273, 284, 285). However, other studies in different HESN individuals found that immune activation was present, and that this immunological activation provided protection against HIV infection (270-272). Together these studies demonstrate that a local immunological response that is specific against HIV or viral infections in general can be protective. However, this response needs to be highly regulated and not create an overt inflammatory environment, which is conducive to HIV infection (169, 286). Therefore, the use of immunomodulatory products to mediate, but not fully suppress, the mucosal inflammatory environment could be a plausible prevention strategy. One such study using a natural product, glycerol monolaurate (GML), has shown promising results. GML was shown to interfere with the inflammatory response and prevent SIV infection even after high-dose repeated viral exposures in pre-clinical trials on non-human primates (183, 263). However, the current trend is the utilisation of easily accessible and generally safe to use pharmaceutical products (261). These pharmaceutical immunomodulatory products can have adverse unwanted effects, especially after long term and systemic use, and the long-term safety of these products need to be assessed. Natural products that have immunomodulatory properties, do not appear to have adverse side effects, although clinical trials need to be conducted to confirm this.

5.1 Conclusions and recommendations

We found that TLR4, and to an extent TLR7/8 stimulation induced an inflammatory response that reduced HIV infection of target CD4+ T cells. Furthermore, the glucocorticoid betamethasone, and not the non-steroidal anti-inflammatory drug ibuprofen, reduced HIV infection of target CD4+ T cells only when TLR4 was stimulated or left unstimulated, with no such effects observed in the other stimulation conditions. Furthermore, these results were independent of immune activation. Therefore, these results suggest that the inflammatory signalling pathways elicited, in response to TLR4 and modulated in the presence of betamethasone, reduced HIV infection in this model. Data from human and animal studies have established the associations between inflammation/immune activation and increased risk of HIV acquisition, as well as disease progression. In this regard, immunomodulating products are an attractive strategy to manage inflammation and HIV acquisition risk, and slow HIV disease progression. The use of anti-inflammatories to reduce HIV transmission is not a new concept. However, many

pharmaceutical anti-inflammatory drugs that are indicated for oral use have adverse effects due to the prolonged presence of drugs in the systemic circulation. The use of natural or pharmaceutical topical immunomodulatory products may be plausible to limit such adverse effects. However, any such immunomodulatory products would need to undergo rigorous human clinical trials to assess their safety and efficacy. Despite the limitations of the model system used, our study is the first, to show reduced HIV infection with an anti-inflammatory drug. Our findings also support the notion that various stimulation conditions augment differential patterns of inflammation and immune activation. The use of transcriptional profiling to assess the inflammatory signalling pathways would be key to the identification of these new drug targets, as well as to better understand the immune responses specific to various conditions. Together, these data may inform on the use of anti-inflammatory drug candidates as adjunctive prophylactic strategies in high risk populations for HIV.

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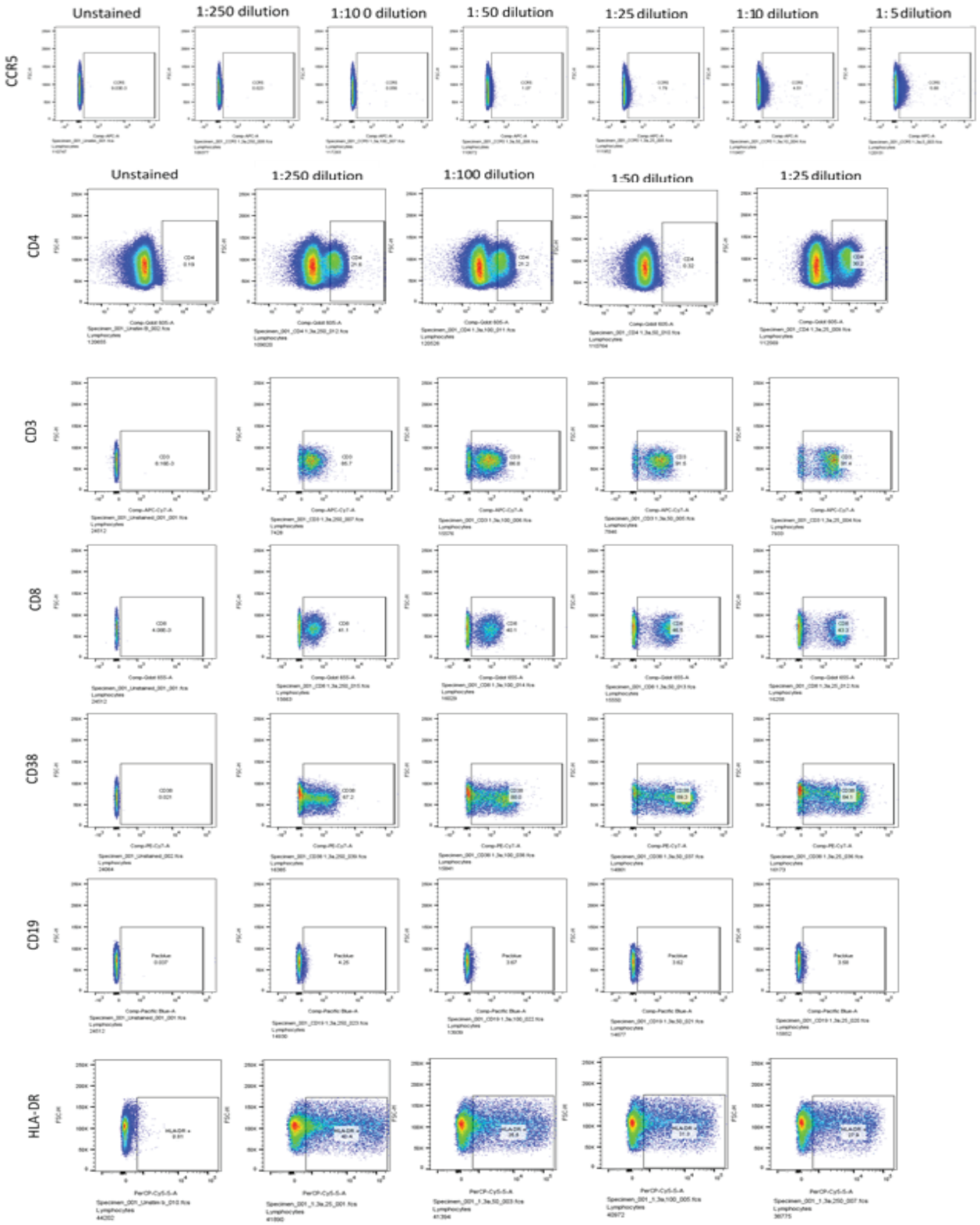
7 Appendices



Supplementary figure 1: Dot plots representing antibody titration for CCR5, CD4, CD3, CD8, CD38, CD19 and HLA-DR to assess optimal antibody dilutions.

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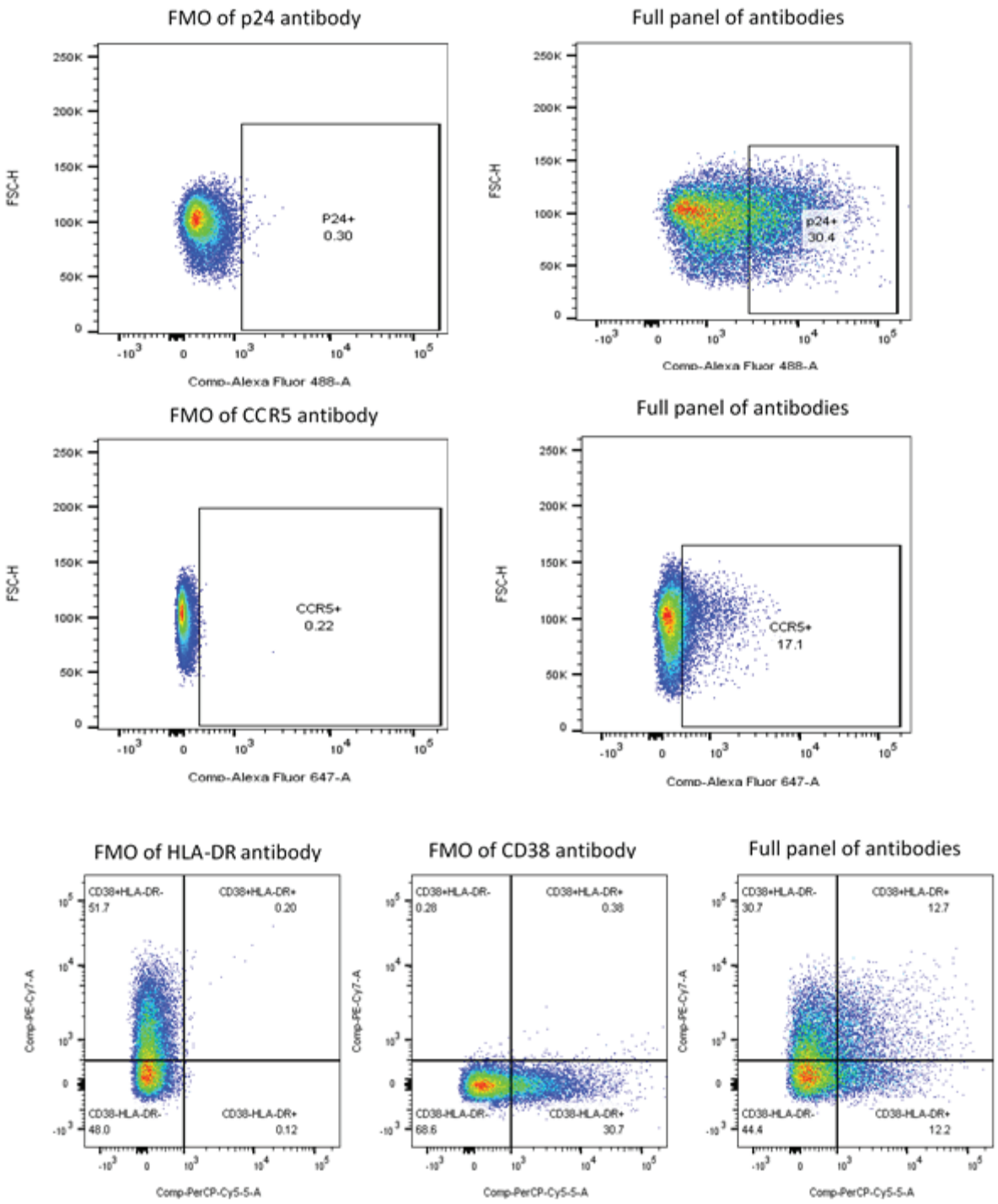
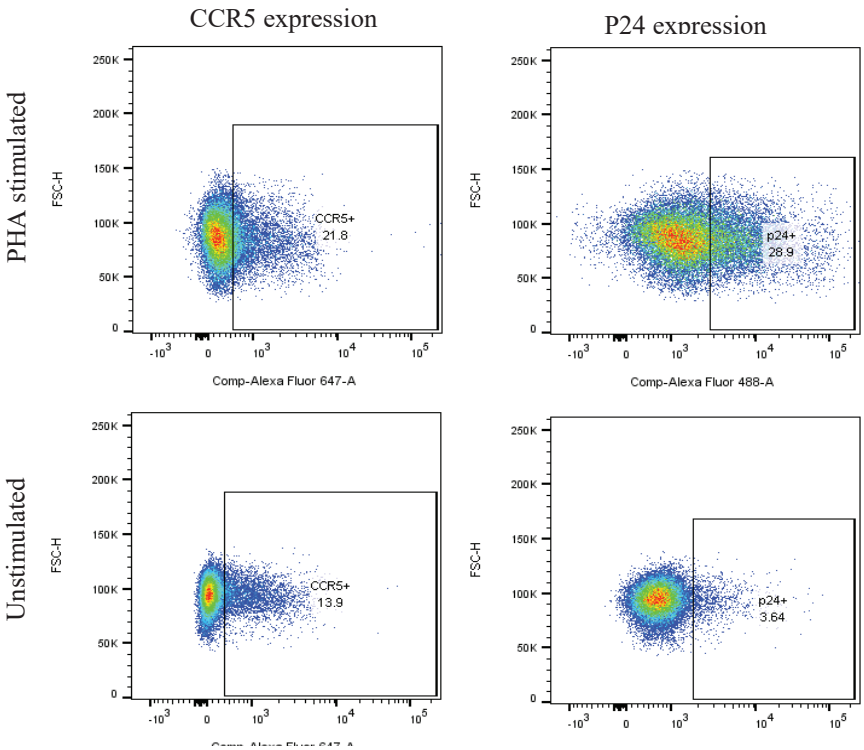
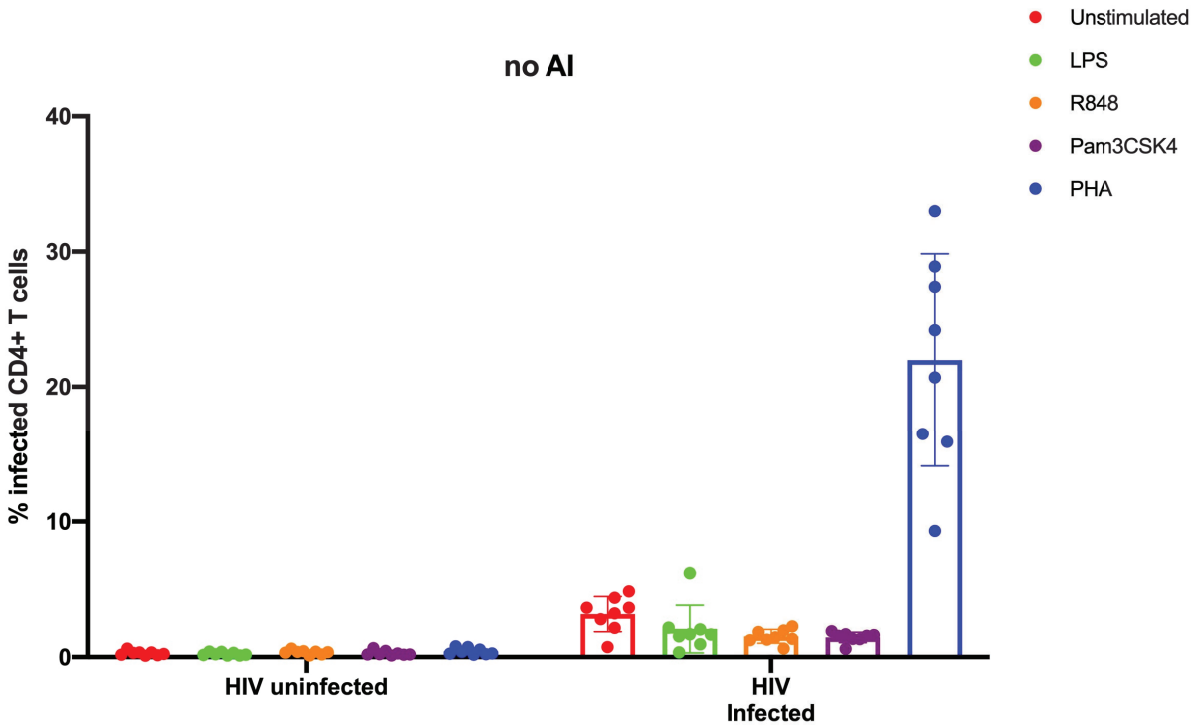


Supplementary figure 2: Dot plots of fluorescence minus one (FMO) experiments for the difficult to distinguish antibodies p24, CCR5, CD38 and HLA-DR.



Supplementary figure 3: Representative dot plots of stimulated (top) and unstimulated (bottom) expression of CCR5 (left) and p24 (right).



Supplementary Figure 4: HIV infection rates (measured by p24 expression) of CD4+ T cells either unstimulated (red) or stimulated with PHA (blue) or TLR agonists; LPS (green), Pam3CSK4 (purple) or R848 (orange). PHA was used at a 1:500 dilution at a working concentration of 5mg/ml. TLR agonists were used at a final concentration of 2ug/ml. Sample size, n=4, each donor run in duplicate

Number of families	1							
Number of comparisons per family	4							
Alpha	0.05							
Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	A-?		
Unstimulated vs. LPS	0.5769	-10.99 to 12.15	No	ns	0.7348	B	LPS	
Unstimulated vs. R848	0.7969	-18.26 to 19.85	No	ns	0.7993	C	R848	
Unstimulated vs. Pam3CSK4	0.86	-18.02 to 19.74	No	ns	0.7689	D	Pam3CSK4	
Unstimulated vs. PHA	-9.471	-213.5 to 194.5	No	ns	0.7618	E	PHA	
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Unstimulated vs. LPS	1.735	1.158	0.5769	0.5294	2	2	1.09	1
Unstimulated vs. R848	1.735	0.9381	0.7969	0.8719	2	2	0.914	1
Unstimulated vs. Pam3CSK4	1.735	0.875	0.86	0.8638	2	2	0.9957	1
Unstimulated vs. PHA	1.735	11.21	-9.471	9.333	2	2	1.015	1

Supplementary table 1: Repeated measures one-way ANOVA results of comparisons between stimulation conditions within the untreated (no AI) group.

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16 March 2020

Mr Ross Cromarty
Private Bag X7
Congella
Durban
4013
ross.cromarty@caprisa.org

Dear Mr Cromarty

PROTOCOL: *In vitro* modelling of the impact of anti-inflammatory drugs on cellular cytotoxicity, activation and inflammation: Degree Purposes (MMedSc). BREC REF: BE433/14.

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	12 February 2020
Expiration of Ethical Approval:	11 February 2021

I wish to advise you that your application for Recertification dated 04 March 2020 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 at its next meeting to be held on 14 April 2020.

Yours sincerely

[redacted]

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

12 April 2013

Dr. K Wallengren
KwaZulu- Natal Research Institute for TB and HIV (K-RITH)
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

PROTOCOL: Collection of Sputum, Urine and Blood samples for research at K- RITH.
REF: BE022/13.

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 11 January 2013.

The study was provisionally approved pending appropriate responses to queries raised. Your responses dated 08 March 2013 to queries raised on 06 February 2013 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 12 April 2013 at **King Dinuzulu Hospital (formerly King George V Hospital).**

This approval is valid for one year from **12 April 2013.** 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 (2004), 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 next meeting taking place on **14 May 2013.**

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

Yours sincerely

[redacted]

Professor D.R Wassenaar
Chair: Biomedical Research Ethics Committee

Professor D Wassenaar (Chair)
Biomedical Research Ethics Committee
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

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Website: http://research.ukzn.ac.za/ResearchEthics/BiomedicalResearchEthics.aspx

09 February 2018

Dr Alex Pym
c/o Farina Karim
KwaZulu-Natal Research Institute for TB and HIV (K-RITH)
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

PROTOCOL: Collection of Sputum, Urine and Blood samples for research at K- RITH.
REF: BE022/13.

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	12 April 2018
Expiration of Ethical Approval:	11 April 2019

I wish to advise you that your application for Recertification received on 16 January 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 approval will be **ratified** by a full Committee at a meeting to be held on **13 March 2018.**

Yours sincerely

[redacted]

Ms A Marimuthu
Senior Administrator: Biomedical Research Ethics

Cc: Taryn.Naidoo@ahri.org

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PhD Thesis

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