Toll-Like Receptor Responses in Peripheral Blood Mononuclear Cells of HIV Exposed Seronegative Female Commercial Sex Workers from Nairobi Kenya

By

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Abstract

The innate immune system is at the interface between the host's immune system and the initial contact with HIV. Understanding the correlates of innate immune protection against Human Immunodeficiency Virus is an important goal for development of effective anti-HIV therapies or vaccines. Not all exposures to HIV end in infection. The innate immune system has been linked to the reduced susceptibility of HIV-exposed seronegative (HESN) female commercial sex workers in Kenya by a number of studies.

This thesis is a comparison of Toll-like receptor (TLR) responses in different immune cells in peripheral blood mononuclear cells (PBMCs) from HESN and HIV negative (susceptible) female commercial sex workers (CSWs). This study tested the hypothesis that higher TLR8 responsiveness in PBMCs of HESN to ssRNA analogous to HIV's genetic material, would result in higher effector responses capable of making HIV target cells more refractory *in vitro,* compared to susceptible controls. The results showed that PBMCs of HESN were often hypo-responsive to TLR4 and TLR7 stimulations evidenced by often reduced cytokine responses to the corresponding ligands, but hyper-responsive to TLR8 following stimulation with ssRNA analogous to HIV's genetic material. The 'dichotomy' in TLR responsiveness of HESN PBMCs was associated with differential expression of cognate TLRs in PBMCs, and altered activation of TLR signalling pathways.

The opposing pattern of TLR7 and TLR8 responsiveness corresponded to the ability of HIV to infect target cells *in vitro*; where pre-treatment of PBMCs with TLR7 enhanced HIV replication whereas TLR8 stimulation inhibited HIV replication. The differences in outcomes of the HIV infection assays were associated with distinct cytokine profiles, where TLR7 stimulation induced robust type I IFNs responses without proinflammatory TNF-α and IL-12 cytokine responses,

while TLR8 stimulations produced type II IFN responses accompanied by robust proinflammatory responses in both groups. The cytokine milieu of HESN PBMCs prior to and following TLR4 and TLR8 stimulations was more tightly regulated, but was associated with higher activation of $CD8^+$, NK cells, monocytes but not blood DCs.

These results demonstrate that the lower activation or 'quiescent' state of HESN PBMCs did not limit the ability of their cells to recognize ssRNA analogous to HIV derived genetic material and mount potent responses capable of limiting HIV infection *in vitro,* supporting the overall hypothesis tested. This thesis contributes to the growing knowledge on the dichotomous outcomes between TLR7 and TLR8 treatments with respect to HIV infection that could be instrumental in the design of novel HIV inventions such as vaccines or microbicides.

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Dedication

I would like to thank and dedicate this thesis to my lovely wife Stella and sons- Magunga and Wendo for their sacrifice, support and inspiration throughout my studies. I also dedicate this thesis to my late father, mother, brothers and sisters. Thank you all.

Table of Contents

Abstract ii
Acknowledgements iv
Dedication vii
Table of contents viii
List of Tables xiv
List of Figures xv
Publications arising from this thesis xvi

Chapter 1: Introduction 1
1.1 **Human Immunodeficiency Virus** 1
1.1.1. Origin and History of HIV 1
1.1.2. HIV epidemiology 2
1.1.2.1 HIV in Kenya 3
1.1.3. HIV structure and Genome 3
1.1.4. HIV Life cycle 5
1.1.5. HIV Transmission 6
1.1.6. HIV Pathogenesis 8
1.1.7. HIV Prevention 10
1.1.8. The Need for Novel HIV therapies and vaccines 11
1.2. Overview of Human Immune System 14
1.2.1. Adaptive Immune System 14
1.2.1.1. T cells 14
1.2.1.1.1. $CD4^+$ T cells 15
1.2.1.1.2. $CD8^+$ T cells 15
1.2.1.2. B cells 17
1.2.2. Innate immune system 20
1.2.2.1. Components of the Innate Immune system 20
1.2.2.1.1. Monocytes 20
1.2.2.1.2. Dendritic cells 22
1.2.2.1.3. Natural Killer cells 27
1.2.2.2. Innate Recognition 29
1.2.2.3. Toll-Like Receptors 30
1.2.2.3.1. History of Toll-like receptors 30
1.2.2.3.2. TLR structure and PAMP recognition 31
1.2.2.3.3. Cellular and tissue expression of TLRs 33
1.2.2.3.4. TLR gene transcriptional regulation, intracellular trafficking and expression 34
1.2.2.3.5 TLR Signalling Pathways 36
1.2.2.3.5.1 MyD88 dependent pathways 36

1.2.2.3.5.2. TRIF dependent pathways 39
1.2.2.3.6. Negative regulation of TLR signalling pathways 40
1.2.2.3.6.1. Negative regulation through dissociation of adaptor Complexes 40
1.2.2.3.6.2. Degradation of TLR signalling proteins 42
1.2.2.3.6.3 Transcriptional regulation 45
1.2.2.3.7. Role of TLRs in HIV pathogenesis 45
1.2.2.3.7.1. TLR recognition of HIV 45
1.2.2.3.7.2. Effects of TLR signalling on T cells during HIV infection 47
1.2.2.3.7.3. Effect of TLR signalling on B cell responses during HIV Infection 48
1.2.2.3.7.4. TLR expression and responsiveness during HIV disease 49
1.2.2.3.7.5. TLR mediated induction of anti-HIV responses 50
1.3. Correlates of protection against HIV 52
1.3.1 Genetic correlates of protection against HIV 52
1.3.2 Immune correlates of natural protection against HIV 53
1.3.2.1. Innate Immunity to HIV in HESN 54
1.3.2.2. HIV specific T cell responses in HESN 55
1.3.2.3. HIV specific humoral responses in HESN 57
1.3.2.4. Immune quiescence and reduced susceptibility to HIV 58

Chapter 2: Rationale, Hypothesis and Objectives 60
2.1. Rationale 60
2.2. Hypotheses and objectives 61

Chapter 3: Material and Methods 63
3.1 Study Participants 63
3.1.1 Majengo commercial sex worker cohort 63
3.1.2 Winnipeg donors 63
3.1.3 Ethics statement 64
3.2 General Reagents 64
3.2.1 Toll-like receptor agonists and other stimulants 64
3.2.2 Culture media and tissue processing reagents 64
3.2.3 Flow cytometry reagents 65
3.2.4 Luminex assay reagents and kits 66
3.2.5 RNA reagents and PCR kits 66
3.2.6 Western blot reagents, buffers and kits 67
3.2.7 HIV p24 reagents and buffers 68

3.3 Routine procedures 69
3.3.1 Specimen preparation 69
3.3.1.1 Plasma collection 69
3.3.1.2 PBMC isolation 69
3.3.2. TLR stimulation and cell culture 70
3.3.3. HIV serology 71
3.3.4. Storage and shipping of samples 71
3.4 Flow cytometry 71
3.4.1 Flow Cytometry panels 71
3.4.2 Surface staining 72
3.4.3 Intracellular Cytokine staining 73
3.5 RNA isolation and RT-PCR 73
3.5.1 RNA isolation 73
3.5.2 Reverse transcription 74
3.5.3 Real time PCR 74
3.6. Luminex assays 75
3.7. Western blot 76
3.8. HIV Assays 78
3.8.1. HIV viral stock propagation 78
3.8.2. HIV-TLR infection assays 79
3.8.3. HIV-1 p24 ELISA 79
3.9 Data Analysis 81

CHAPTER 4: Dichotomony of TLR7 and TLR8 responses in HESN PBMCs to cognate ligands is associated with HIV infectivity *in vitro* **82**
4.1. Rationale 82
4.2. Hypotheses 82
4.2.1. Hypothesis 1 82
4.2.2. Hypothesis 2 82
4.2.3. Specific Aims to test the hypotheses 83
4.3. Study Outline 83
4.4. Results 84
4.4.1. Higher TLR8 but lower TLR4 and TLR7 cytokine responses by HESN PBMCs 84
4.4.2. Different kinetics of cytokine production by PBMCs of HESN over the Course of TLR4, TLR7 or TLR8 stimulations 88
4.4.3. Higher amplitude of TLR8 cytokines responses by HESN PBMCs over the course of stimulation 90
4.4.4. Cytokine milieu of HESN PBMCs was more tightly regulated with or without TLR stimulations 93

4.4.5. Dichotomy of TLR7 and TLR8 expression in PBMCs of HESN following cognate ligand stimulation corresponding to functional responses 96
4.4.6. Dichotomony in activation of TLR7 and TLR8 signalling pathways in HESN PBMCs 99
4.4.7. Opposing Effects of TLR7 and TLR8 pre-treatment of HESN PBMCs on HIV's infectivity *in vitro* 103
4.5 Summary and discussion 107

CHAPTER 5: Higher CD4+ T cells responses to TLR4 and higher CD8+ T cells to TLR7 and TLR8 in HESN 114
5.1. Rationale 114
5.2. Hypothesis 116
5.3. Specific aims 116
5.4. Results 117
5.4.1. Higher Activation of $CD4^+$ T cells by TLR4 and $CD8^+$ T cell by TLR7 and TLR8 stimulations in HESN 117
5.4.2. Lower activation of resting T cells and higher IFN-γ responses by HESN T cells to TLR4, TLR7 or TLR8 stimulations 126
5.4.3. Correlational Analysis of HESN T cell activation and functions responses to different TLR Stimulations 132
5.4.4. Expression of TLR4 on HESN T cell subsets with TLR4, TLR7 or TLR8 stimulations 135
5.4.5. Role of T cell regulation in HESN responses to TLR stimulation 143
5.4.6. Expression of HIV Co-receptor CCR5 on target cells (CD4+ T cells) of HESN with TLR stimulation 149
5.5. Summary and discussion 151

CHAPTER 6: Higher Monocyte activation but lower Dendritic Cell responses in HESN after TLR4, TLR7 and TLR8 stimulations 156
6.1. Rationale 156
6.2. Hypothesis 159
6.3. Objectives 159
6.4. Results 159
6.4.1. Changes in Monocyte subsets of HESN with TLR stimulation 159
6.4.2. Higher activation of HESN monocytes with TLR stimulation 162
6.4.3. Higher Expression of CD40 ligand (CD154) on T cells of HESN with TLR stimulation 170
6.4.4. Expression of chemokine receptors and adhesion molecules on HESN monocytes with TLR stimulation 173
6.4.5. Expression of cytokines on Monocytes Subsets of HESN with TLR4, 177

TLR7 and TLR8 stimulations
6.4.6. Dendritic cell responses in HESN PBMCs with TLR stimulation 180
6.5. Summary and Discussion 187

CHAPTER 7: Higher responses of Natural Killer cells in HESN to Different TLR stimulations 195
7.1. Introduction 195
7.2. Rationale 197
7.3. Hypothesis 199
7.4. Objectives 199
7.5. Results 199
7.5.1. Changes in NK cell Subsets in HESN PBMC with different TLR stimulations 199
7.5.2. Higher Activation of HESN NK cells with TLR stimulation 202
7.5.3. Expression of NK cell receptors (NKG2, NKG2D, CD16) on NK subsets of HESN with TLR stimulation 204
7.5.4. Higher Degranulation potential of HESN NK cells following TLR7 and TLR8 stimulations 209
7.5.5. Cytokine responses by NK subsets of HESN with TLR stimulation 211
7.5. Summary and Discussion 214

CHAPTER 8: General Discussion, Conclusions, Limitations and Significance 218

8.1 General Discussion 218
8.1.1. The Dichotomous effect of TLR7 and TLR8 stimulation on HIV's infectivity is related to cytokine responses and cytokine milieu. 218
8.1.2. Higher activation of CD40-CD40L Pathway and a higher frequency of virus sensing monocytes in HESN PBMCs 222
8.1.3. Lower activation of HESN blood DC subsets in response to TLR7 and not TLR8 225
8.1.4. Higher activation of antiviral responses in $CD8^+$ T and NK cells of HESN with TLR7 and TLR8 stimulation 226
8.1.5. The proposed relationship between 'Immune Quiescence' and TLR8 Hyperresponsiveness in HESN Female CSWs 231
8.1.6. Model of reduced susceptibility to HIV of HESN based on TLR responsiveness 233
8.2. Conclusion 238
8.3. Limitations 238
8.4. Significance 239

8.5. Future Directions 240

CHAPTER 9: References 242
Appendix 1: List of Abbreviations 266
Appendix 2: Supplementary Table 1- *P-values* for Figure 7 and 8 271
Appendix 3: Spearman's correlation Tables for Figure 9 272

List of Tables

Table 1: Flow cytometry antibody panels **72**

Table 2: TLR PCR array genes **100**

Table 3: Spearman Correlational Analysis between T cell activation and functional responses to TLR4, TLR7, TLR8 and PMA with Ionomycin stimulations in matched HESN and HIV-N samples **133**

Table 4: Spearman's correlational analysis comparing different T cell functional responses and TLR4 expression following various TLR treatments of HESN and HIV-N PBMCs **141**

List of Figures

Figure 1. Human Immunodeficiency Virus 4
Figure 2. TLR signalling Pathways 38
Figure 3. Negative regulation of MyD88 dependent pathway 43
Figure 4. Negative regulation of TRIF dependent pathway 44
Figure 5. Cytokine responses to TLR stimulation in PBMCs from HESN and HIV susceptible controls 85
Figure 6. Fold production of cytokines after TLR4, TLR7, TLR8 or PHA stimulations of PBMCs from HESN and HIV-N 87
Figure 7. Kinetics of Cytokine Production by PBMCs of HESN and HIV-N CSWs 89
Figure 8. Kinetics of fold cytokine production with time by PBMCs of HESN and HIV-N 92
Figure 9. Cytokine milieu of HESN PBMCs with or without TLR4, TLR7 or TLR8 stimulations 95
Figure 10. Comparison of Toll-Like Receptor Expression 98
Figure 11. Gene analysis of TLR signalling and related pathways following TLR7 or TLR8 stimulation of PBMCs 101
Figure 12. HIV infection assays 104
Figure 13. HESN T cell Activation 118
Figure 14. Activation of T cell subsets by TLR4, TLR7 or TLR8 stimulations 120
Figure 15. Fold changes in activation of T cell subsets with TLR4, TLR7 or TLR8 stimulations 123
Figure 16. Comparison of intracellular cytokine, chemokine, degranulation and and proliferative responses in T cells *ex vivo* 128
Figure 17. Kinetics of T cell functional responses to TLR4, TLR7, TLR8 or PMA with Ionomycin stimulations 129
Figure 18. Expression of TLR4 on T cells subsets 137
Figure 19. Regulatory T cell responses to TLR stimulation in HESN 144
Figure 20. *In vitro* T cell degranulation and the relationship to IL-10 expression on T cells 148
Figure 21. Expression of CCR5 on T and B cells with TLR stimulation 150
Figure 22. Changes in monocytes subsets with TLR stimulation in PBMCs of HESN and HIV-N 160
Figure 23. Expression of co-stimulatory molecules on different monocyte subsets with TLR Stimulation 164
Figure 24. Co-expression of co-stimulatory molecules on $CD14^{+}$ (monocytes) and $CD14^{-}$ (other monocytes and DCs) cells 168
Figure 25. Expression of CD40 and CD40L on HESN T and B cells with TLR stimulation 172
Figure 26. Expression of CD54 (intracellular adhesion molecule 1) and chemokine 174

receptors on Monocytes with TLR stimulation
Figure 27. Cytokine responses by monocyte subsets with TLR stimulation 178
Figure 28. Blood DC responses to different TLR stimulations 182
Figure 29. Cytokine responses by DC subsets to TLR4, TLR7 and TLR8 stimulations 185
Figure 30: Changes in NK cell Subsets with TLR stimulation 201
Figure 31. Natural Killer cell activation with TLR stimulation 203
Figure 32. Expression of FcγRIII (CD16) on NK subsets with TLR stimulation 206
Figure 33. Expression of NK cell Receptors (NKRs) on different subsets with TLR Stimulation 208
Figure 34. Degranulation of $CD56^{bright}$ and $CD56^{dim}$ NK populations 210
Figure 35. Cytokines Responses of $CD56^{bright}$ and $CD56^{dim}$ NK cells to different TLR Stimulations 213
Figure 36. Role of Toll-like receptors and NK cell Receptor in immune recognition 230
Figure 37. A conceptual Model of reduced susceptibility in the peripheral blood of HESN to HIV Based on TLR responsiveness 236

Publications Arising From This Thesis

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CHAPTER 1: Introduction

1.1 Human Immunodeficiency Virus

1.1.1 Origin and History of HIV

Human Immunodeficiency Virus (HIV) has two major strains; HIV-1 occurring globally, and HIV-2 found primarily in West Africa. The original human infection with HIV-1 and HIV-2 likely arose from cross-species transmission of the closely related simian immunodeficiency virus in chimpanzees ($SIV_{cpz)}$ and SIV in sooty mangabeys (SIV_{SM})[1, 2]. The earliest SIV transmission events probably occurred when virus present in raw primate meat came into contact with human mucosal tissues leading to establishment of zoonotic infection with what is now known as HIV. HIV-1 has since been categorized into four distinct groups M, N, O and P, arising from four cross-species transmission events; HIV-M, N and O groups arising from zoonotic transmission of SIV from chimpanzees, and P group arising from gorillas. HIV-1 group M is the most widely distributed and is responsible for the global pandemic that began early in the twentieth century, while HIV-1 groups N, O and P only emerged recently and are primarily restricted to West Africa[3]. HIV-1 group M is further divided into nine subtypes based on virus genetics A, B, C, D, F, G, H, J and K, where subtype C predominantly circulates in Africa and Asia accounting for the largest proportion of all HIV-1 infections, while subtype B is restricted mainly to Europe, the Americas and Australia. All HIV-1 groups can cause $CD4^+$ depletion, eventually leading to acquired immunodeficiency syndrome (AIDS) in the abscence of therapeutic intervention[3].

The first description of HIV infection in humans was done 33 years ago in a group of men having sex with men (MSM) who presented with an immune deficiency associated infection-*Pneumocystisis cranii* (now *P. jejunii*) and a rare malignancy-Kaposi`s sarcoma, in New York and San Francisco clinics in the USA[4]. The causative agent was later determined to

be the lentivirus HIV-1 (referred to simply as HIV for purposes of this thesis unless otherwise stated)[5, 6].

1.1.2. HIV epidemiology

At the end of 2013, UNAIDS estimated the number of people living with HIV/AIDS worldwide at between 33.2-37.2 million, the majority of whom lived in Sub-Saharan Africa- 24.7 (23.5-26.1) million- the region hardest hit by the pandemic[7, 8]. An estimated 1.9-2.4 million new HIV infections occurred globally in the same year, representing a 10% drop from the previous year, and a 38% drop in new infections since 2001 and a 33% drop from those reported in 2011. Unfortunately, the majority of these new infections were still occurring in Sub-Saharan Africa. Similarly, AIDS related deaths dropped from 1.7 million in 2011 to 1.6 million in 2012, but HIV/AIDS still remained the leading cause of death among communicable diseases [8].

The historic importance of the HIV/AIDS pandemic, especially with regard to human development, led the United Nations General Assembly to include it in the millennium development agenda under millennium development goal number 6; That targeted to halt or reverse spread of HIV/AIDS, and to provide universal access to HIV treatment to all in need, by the year 2015. The global community is currently contemplating ways of ending the HIV epidemic as the 2015 deadline expires. New, bold and ambitious proposals seeking to expand HIV treatment coverage have been tabled, such as the 90-90-90 strategy that targets to increase to 90% the people living with HIV who are aware of the status, to increase coverage of antiretroviral therapy to 90%, and to achieve 90% viral suppression among those receiving ART, by 2020[9]. The 'three way strategy' is based on the premise that knowledge of one's HIV status, enables health providers to facilitate provision of life saving and health enhancing medication to people living with HIV, while at the same time reducing the risk of HIV

transmission to uninfected individuals. Strategists hope that the 'Three-way strategy' if successfully implemented would ensure that 73% of people living with HIV would be virally suppressed, twice the current rates of those under ART. Attainment of these targets by 2020 has been postulated through modelling to end the epidemic by 2030. While HIV treatment is crucial towards managing and prolonging the health of HIV infected persons, its application alone as a HIV prevention tool is not likely to end the pandemic. Other prevention tools (discussed later) will be needed to augment HIV treatment strategies and to reverse the tide of the HIV pandemic[9]. More research is needed to expand the knowledge on HIV transmission and early immune events following transmission of the virus, which would aid the development of novel prevention tools.

1.1.2.1 HIV in Kenya

Fifteen countries globally account for 75% of all those living with HIV/AIDS. Kenya has the fourth highest number of people living with HIV/AIDS, with a prevalence of 5.6% (KAIS 2012). Kenya's HIV epidemic distribution is both generalized and concentrated. In spite of Kenya's epidemic being largely classified as generalized, there is a concentration of HIV infections in key populations such as sex workers (prevalence of 29.3%), intravenous drug users (18.3%) and MSMs (18.2%) [10].

1.1.3 HIV Structure and Genome

HIV is classified in the *Retrovirodae* family of viruses, subfamily *Orthoretrovirodae* and genus *Lentivirus*. HIV is roughly spherical, enveloped with a diameter of approximately 120nm. The virus envelope consists of a bilayer derived from host cell plasma membrane during budding. The bilayer is interrupted by trimeric glycoprotein projections consisting of a dimeric gp120 head and gp41 stalk (**Figure 1)**. Beneath the viral envelope is layer of matrix protein p17 found above a cone-shaped virus core consisting of capsid protein p24.

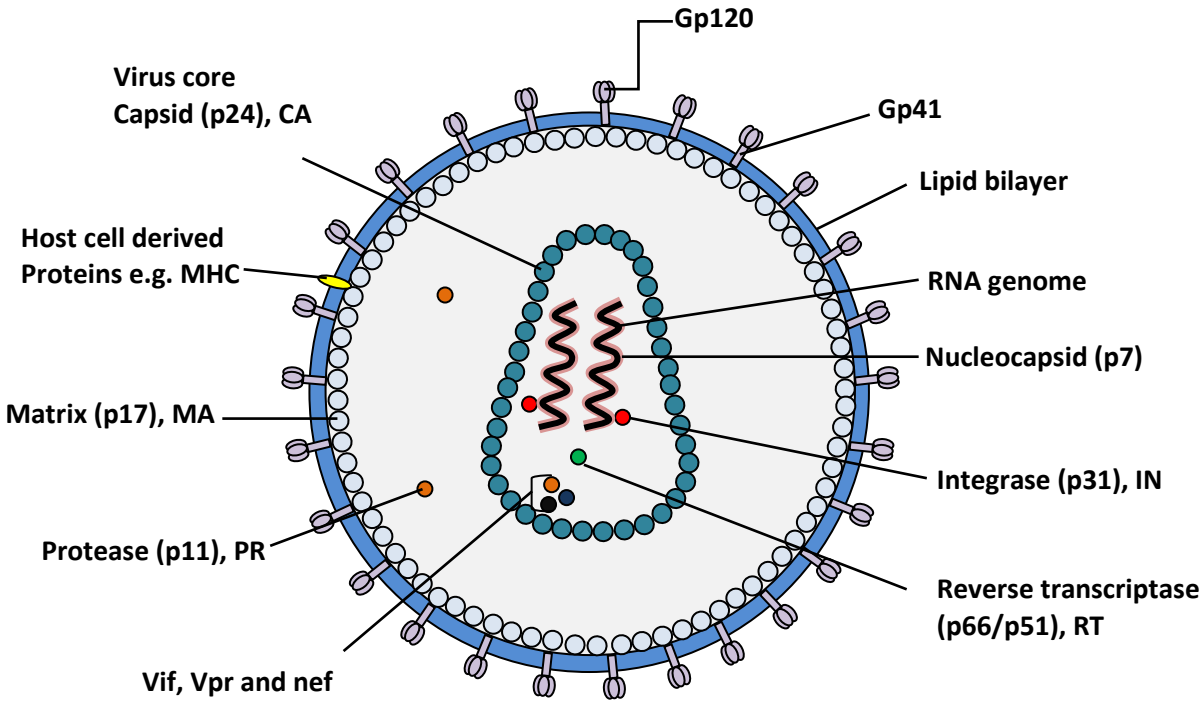


Figure 1. Human Immunodeficiency Virus. HIV is a roughly spherical and enveloped virus with trimeric projections consisting of gp120 (head) and gp41 (stalk) and certain host proteins such as MHC molecules incorporated into the viral envelope. A layer of matrix protein (p17 or MA) found beneath the envelope bilayer surrounds a cone-shaped virus core made up of p24 protein. The virus core contains two positive sense RNA strands encapsulated by nucleocapsid (p7 protein) and enzymes integrase (p31, IN), reverse transcriptase (p66/p51, RT) and protease (p11, PR), and accessory proteins Vif, Vpu, Vpr, Tat and Nef.

Inside the virus core are; two positive sense RNA strands surrounded by a nucleocapsid (p7); three enzymes- integrase (IN), reverse transcriptase (RT) and protease (PR); five accessory proteins- Vif, Vpr, Vpu, Tat and Nef (**Figure 1**). The process of HIV production by an infected host cell, results in the incorporation of a number of host cellular proteins such as heat shock proteins, cellular transport and structural proteins like actin, tubulin, HDAC-1, APOBEC3G among others[11].

HIV genome consists of two single strands of RNA approximately 10kb long, containing 9 open reading frames (ORF)- *gag, pol, vif, vpr, vpu, env, nef, rev* and *tat* encoding 15 different proteins [12]. The *gag* gene encodes Gag protein that is cleaved to produce four structural proteins p17, p24 (capsid), p7 (nucleocapsid) and p6, while *pol* gene encodes three viral enzymes PR, IN and RT, and *env* encodes a polyprotein gp160 that is later cleaved by PR into gp120 and gp41, with the remaining genes encoding for accessory genes products Vif, Vpr, Vpu, Nef, Rev and Tat.

1.1.4. HIV Life Cycle

The replication cycle of HIV can be divided into nine distinct stages; i) binding and Entry ii) uncoating iii) reverse transcription iv) nuclear entry and v) integration vi) transcription of viral DNA vii) viral protein synthesis viii) budding and ix) virion maturation. HIV initiates **entry** into a target cell when gp120 on the surface of the virus binds to a CD4 receptor expressed on $CD4^+$ T cells or macrophages. This is followed by a conformational change in gp120 exposing a chemokine binding domain on gp120, enabling an interaction between CCR5 and the gp120-CD4 complex. The resultant complex is stable and allows the N-terminal end of gp41 to penetrate the plasma membrane. The gp41 protein undergoes a conformational change that draws the viral envelope and plasma membrane of target cell closer together, eventually causing them to fuse. Following this fusion the viral core is

released into cytoplasm, followed by **uncoating** of capsid proteins to release the viral RNA[13, 14]. The core proteins are reconstructed into the reverse transcription complex (RTC) within which viral RNA is converted into DNA by viral reverse transcriptase (RT) through a process called **reverse transcription**. The RTC after reverse transcription changes to a pre-integration complex which is used to transport the viral cDNA into the nucleus (**nuclear entry**), where it is **integrated** into the host genome to form a provirus by the viral enzyme integrase[14]. **Transcription** of the proviral DNA is initiated at a promoter site termed the long terminal repeat (LTR) through a complex process involving viral transactivator of transcription (Tat) and host transcription factors such as NF-κB. The newly transcribed viral mRNA transcripts are exported out of the nucleus as spliced, partially spliced or unspliced RNA, and give rise to different viral proteins. The process of transport of spliced or unspliced viral RNA out of the nucleus is facilitated by Rev a viral protein. Once in the cytoplasm the viral RNA is **translated** by cellular ribosomes. The gag derived protein MA initiates viral assembly on the inner surface of the plasma membrane. The virus core is assembled from the gag-pol protein with incorporation of Vif, Vpr and Nef and genomic viral RNA into the immature virion which then **buds off** the plasma membrane, producing new but immature virions. The budding of the new virion is accompanied by initiation of virus **maturation** during which, the Gag-Pol polyprotein is cleaved by viral protease (PR) to produce structural proteins MA, CA and NC which then make up the viral core, and viral enzymes IN, RT and PR. The mature virion is then capable of infecting a new target cell, thereby initiating a new replication cycle.

1.1.5. HIV Transmission

HIV is considered to be a sexually transmitted disease, because up to 80% of new infections are acquired sexually through heterosexual (penile-vaginal/anal) and homosexual (penile-anal) intercourse. HIV can also be transmitted from a HIV infected mother to child during

delivery, *in utero* to the foetus or through breastfeeding. HIV can be transmitted through sharing infected needles, razors or sharp objects or through transfusion of infected blood. The importance of each mode of transmission varies depending on the region of the World, leading to existence of distinct regional epidemics. Heterosexual transmission accounts for bulk of the new HIV infections in Sub-Saharan Africa, where majority of new infections occurs in girls or women aged between 15-49 years [8]. In other regions, the number HIV/AIDS infected men outweighs that of women, given that most new infections occur in men who frequently visit female sex workers, in men who have sex with men (MSMs), in injection drug users or in transgender persons[8].

Given the ethical challenges in conducting HIV infection studies in humans, bulk of the current knowledge on early events in HIV transmission is derived from *in vitro* infection studies conducted on human genital tissue explants or *in vivo* studies using the SIV-macaque model[15]. The Rhesus Macaques (*Macaca mulatta*)- SIV infection model, allows for manipulation of the infection process and monitoring of pathogenetic development of SIV disease[16]. It is not clear if HIV is transmitted as a cell free or cell associated virus, although, in the SIV model both forms can be transmitted[17]. The mechanism used by HIV to cross the genital epithelial tissues at the preferential site in vaginal/penile/rectal organs where most transmission occurs, is still not clear. However, it is known that cervical mucus slows HIV diffusion across vaginal mucosa[18], and the virus is able to cross epithelial tissues using transcytosis, or between epithelial cells to access underlying HIV target cells. The HIV/SIV transmission event is postulated to involve a single virus or viral strain, that is capable of preferentially infecting and replicating in $CD4^+$ T cells using CCR5 co-receptor, rather than macrophages and monocytes using CXCR4 or other co-receptors [19-21]. The single-virus infection hypothesis has been supported by experiments showing that early in HIV/SIV infection, the genital tissues are percolated with small foci of replicating viruses,

normally near the junction of the endo-and ectocervical tissues. Additional support for this hypothesis, is present in the small number of HIV clonal variants which arise during the acute phase of infection in humans, suggesting a small number of variants establishing infection [22-24]. Studies in the SIV-macaque model, show that productive SIV infection from rectal challenge arises from a single founder virus, thus supporting the use of this model for studying HIV transmission[25]

1.1.6 HIV Pathogenesis

Once the virus crosses the mucosal epithelial barrier and overcomes or evades the host innate defences, it then infects underlying activated $CD4^+$ T cells, multiplying locally to establish a founder population of virus, before spreading systemically[26]. The period between the initial breach of host innate defences, and the first detection of viral DNA-but not RNA- is called the eclipse phase, which lasts 3-4 days, during which there is a rapid local expansion of virus that peaks between 10-14 days post infection[27]. This early phase of HIV disease when there is a small population of replicating virus provides a 'window of opportunity' for eliminating the new infection before it spreads systemically[16].

The systemic spread of HIV is aided by the ability of the virus or viral products to cause endocervival tissues to express inflammatory chemokines, thereby enhancing recruitment of HIV target cells. Plasmacytoid dendritic cells (pDC) are responsible for the recruitment of new target cells to the infection site, aiding the rapid establishment of the new infection[28]. The innate inflammatory process responsible for this recruitment, is exacerbated by sexually transmitted infection (STIs) and innate receptor agonists, all of which have been shown to enhance SIV/HIV acquisition[28, 29]. Eventually, the virus disseminates to the lymph nodes draining the genital tissues where it encounters more HIV target cells, leading to further viral replication prior to systemic spread via the thoracic duct. The founder virus is thought to

initially infect $CD4^+$ T cells, but it can also efficiently infect monocytes/macrophages, or even cells lacking CD4 receptor such as DCs, astrocytes and renal epithelial cells[30, 31]. The robust innate antiviral response mounted by pDCs and other innate cells through production of proinflammatory factors, fails to protect primary $CD4^+$ T cells, but in contrast appears to protect macrophages at this early stage of infection[32].

Certain unique properties of HIV enables it to establish a rapid and persistent lifelong infection unlike other viral infections, for example; the early phase of systemic SIV infection has cytokine and chemokine gene expression proportional to viral load, whereas the acute phase of HIV infection has a cytokine 'storm' present prior to peak vireamia[33]. The peak HIV levels exceeds those seen in other viral infections such as hepatitis B and C[33, 34]. A decline in HIV viral load to a set point is accompanied by the emergence of virus specific innate and adaptive responses, and declines in proinflammatory cytokine levels (recession of the 'cytokine storm'). The massive HIV replication observed immediately following infection is limited by the onset of innate and adaptive immune responses, initially through $CD8^+$ T cells responses against virus infected cells, and later followed by virus specific antibody responses[35, 36]. The first HIV specific responses arise just as viraemia approaches its peak, bulk of the virions in circulation at this point are homogeneous to the founder virus, indicating the absence of immune-driven selection of escape mutants[19, 21]. HIV replication continues throughout the life an infected person, and is accompanied by a dynamic interplay between host immune responses, virus evolution, progressive depletion of $CD4^+$ T cells, and attempts by the host's immune system to replenish cells destroyed by virus[37]. Some individuals express certain human leukocyte antigens (HLA) enabling them to control viral replication more efficiently, through generation of robust, diverse and polyfunctional HIV specific T cell responses[38]. Overtime, a broad range of antibodies capable of neutralizing HIV develop in about 20% of HIV infected individuals, with the development of such

responses being dependent on development of mutant viral strains during the natural course of HIV infection [39-41].

The innate response to HIV-1 is primarily by NK cells, however, escape mutants also emerge restricting the effectiveness of HIV specific NK cells responses[42]. The replicating virus causes death of $CD4^+$ T cells through direct infection, immune activation, syncitia formation, proliferation and senescence. Microbial products such as lipopolysaccharide from the cell walls of gram negative bacteria, and virus derived ssRNA, can enhance production of proinflammatory cytokines which contribute to persistent immune activation present throughout the chronic phase of HIV[43, 44]. The current ART regimes fail to completely reverse the immune activation established during chronic HIV infection, this is due to failure of ART to eliminate virus reservoirs, thereby allowing persistent low-grade viral replication[45].

1.1.7. HIV Prevention

Currently, there is no single effective strategy for preventing HIV infection. As such, HIV prevention encompasses the use of multiple strategies, where the choice of strategy is dependent on; the target population, gender, sexual orientation and global region. The use of multiple prevention strategies is likely to remain in place for as long as there is no effective HIV vaccine or alternate means of effectively preventing HIV transmission. The prevention strategies currently in use, can be categorized into four groups; strategies applicable among individuals with low risk of HIV exposure (e.g. a generalized population); those applicable among high risk individuals (HIV-exposed) pre-coital or coital (e.g. sex workers, MSM, transgender and so on); those applicable among HIV-exposed post-coital (e.g. rape victims, condom burst victims and needle prick cases) and those targeting HIV infected persons. The prevention strategies applicable to low risk or HIV unexposed individuals include;

behavioural modification such as; encouraging condom usage, treatment of sexually transmitted infections and more recently voluntary medical male circumcision (VMMC) capable of reducing risk of HIV acquisition by between 50-66% in circumcised males[46]. Pre-exposure prophylaxis is a relatively new concept of HIV prevention that uses oral ART, once daily Tenofovir combined with emtricitabine, which has been shown to reduced the likelihood of HIV acquisition in HIV discordant couples by 75% in Kenya [47] and 62.2% in Botswana[48], by 44% in MSMs [49], by 48.9% in IDUs [50] and by 39-54% in women receiving 1% tenofovir vaginal gel[51]. Once daily oral pre-exposure prophylaxis failed to reduce the risk of HIV acquisition in women with high risk sexual behaviour, which was linked to low adherence[52, 53]. On the other hand, post-exposure prophylaxis is now widely used as a standard practice for prevention of HIV acquisition in both occupational (needle pricks among healthcare workers and condom bursts among sex workers) and non-occupational exposures to the virus (such as rape) [54]. The combined use of ART, condoms and counselling, has been shown to reduce the likelihood of heterosexual HIV transmission by 96.4% [55]. Broadening ART provision was recently shown to reduce the risk of HIV acquisition by 38% in a community in Kwa Zulu-Natal-South Africa when ART coverage was between 30-40%, when compared to a similar community with 10% lower ART coverage [56]. The success of this strategy depends on the breadth of ART coverage, the uptake of HIV testing and adherence to therapy. Early initiation of ART also reduces peak viraemia during the acute and contagious phase of HIV infection, while also reducing the latent viral reservoirs and virus infected cells. Early initiation of ART also reduces the incidence HIV associated co-morbidities like TB, candidiasis and herpes simplex, consequently lengthening and improving the quality of life of HIV infected persons[55, 57].

1.1.8. The Need for Novel HIV Therapies and Vaccines

Vaccines typify some of few medical interventions that have been used successfully to eradicate diseases[58]. Currently there is no licensed HIV vaccine. To date, six HIV vaccine candidates have been tested in Phase IIb clinical trials; the first two sought to induce antibody responses while another three attempted to induce T-cell based immunity against HIV. Five failed to elicit immune responses capable of providing protection against HIV acquisition. Only one HIV vaccine has been modestly successful to date- ALVAC-AIDSVAX vaccine tested in the RV144 trial, which protected 31% of all vaccinees against HIV acquisition after modified intent treat analysis, through induction of non-neutralizing or weakly neutralizing antibodies[59].

The need for a HIV vaccine still persists. The ideal target for an effective HIV vaccine is one that is capable of eliciting broadly neutralizing antibodies (BnAbs) capable of recognizing conserved regions of the virus[60]. Generating BnAbs against HIV through vaccination has remained challenge so far, due to the rapid rate at which HIV mutates, generating escape mutant viral species that evade the virus specific antibody responses as they develop[41]. New B cell and T cell vaccine designs have shown great promise for use as therapeutic vaccines[61-63]. Novel vaccine vectors, such as cytomegalovirus, have shown great promise of improving the current HIV vaccine constructs offering hope for discovery of an effective vaccine[64]. Other novel strategies in HIV vaccine development include neutralizing antibody cocktails-shown to be capable of limiting or completely eliminating SIV from the genital tracts and rectal tissues in non-human primates[65].

The ultimate goal of an effective vaccine, is to prevent HIV acquisition, or alternatively to clear transient infection[64]. There is a need for development of new strategies of preventing heterosexual transmission, which might include but are not limited to; novel anti-HIV microbicides, improved ART prevention methods, innate vaccines and so on[55, 66].

The reduction in global HIV incidence, cannot be ascribed to a single intervention, but largely can be linked to the rapid scale up of ART globally. Based on mathematical models, a reduction in new HIV infections by one percentage point, requires a 1.7% percent increase in the number of people on ART Worldwide[67]. All ART prevention strategies employed today hope to suppress viral replication in genital tissues, thus lowering the likelihood of HIV transmission. However, this is not always the case, since complete suppression of viral replication in blood by ART, does not eliminate the expression of viral RNA in the male[68] and female [69] genital tracts, nor in the rectal mucosa[70]. There are knowledge gaps on the benefits of ART in preventing HIV transmission among high risk heterosexual couples (e.g. sex workers and their clients); men who have sex with men (MSM) and unsafe injection drug users (IDU). Moreover, the main challenge in implementing the ART 'treatment as prevention strategy' lies in the identification of those at greatest risk of transmitting the virus. Often such individuals are the hardest to reach as majority are not aware of their HIV status[66].

Despite the encouraging progress in expanding ART coverage and the potential of ART based strategies in preventing new HIV infections, these alone may not be sufficient in halting the HIV epidemic. More effective means of preventing transmission in the genital tract and rectal tissues will be needed augment gains made by ART based prevention strategies[13]. There is also a need for novel therapies capable of targeting specific pathogenic aspects of HIV's life cycle for instance targeting HIV infected cells, or preventing the establishment viral reservoirs or seeking to wipe-out viral reservoirs entirely using ART[71]. Development of novel prevention tools for limiting HIV transmission across mucosal tissues can be greatly aided by the expansion of knowledge on the interactions between HIV and the host innate immune system at the point of transmission, particularly with respect to the nature of protective immune responses needed to halt HIV transmission.

1.2 Overview of Human Immune System

The human immune system has evolved to protect body organs and tissues from harmful micro-organisms. In order to effectively carry out this protective function, the immune system has two complementary arms that function in concert to combat microbes- the innate and adaptive immune systems.

1.2.1. Adaptive Immune System

The adaptive immune system is composed primarily of T and B lymphocytes that are responsible for cellular and antibody mediated immune responses. The adaptive immune system is considered to possess two features absent in the innate immune system, specificity and memory. The processing of pathogen derived antigens and the eventual presentation of the same in association with MHC class II or I molecules to $CD4^+$ and $CD8^+$ T cells, respectively, is tightly linked to the pattern recognition of microbial PAMPs, providing the linkage between the innate and adaptive immune system[72, 73]. Recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), and subsequent intracellular TLR signal transduction, terminates in the active transcription of genes encoding antimicrobial peptides, proinflammatory cytokines and co-stimulatory factors that direct the development of adaptive immune responses[73, 74].

1.2.1.1 T cells

The innate immune system functioning through APCs can recognize, process and present antigens from invading pathogens to naive $CD4^+$ or $CD8^+$ T cells in association with HLA class II or I, respectively. The innate signalling processes activated by recognition of viral PAMPs, augments the production of proinflammatory cytokines and expression of co-stimulatory molecules like CD40, CD80 and CD86 on APCs providing a secondary signal necessary in the activation of naive T cells.

1.2.1.1.1. $CD4^+$ T cells

Over the course of HIV disease, the $CD4^+$ T cell compartment is continuously infected and depleted, contributing to development of a persistent immune dysfunction, dysregulation and deficiency[15]. The earliest and most massive depletion of $CD4^+$ T cells takes place in the lamina propria of the gastrointestinal tract, where the depleted cells are never fully replaced even after initiation of ART[75]. The loss of $CD4^+$ T cells preferentially occurs in activated effector memory $CD4^+$ T cells, T-helper 17 cells and mucosa associated invariant T cells (MAIT), whereas resting naive $CD4^+$ T cells are rarely infected[76, 77]. The massive depletion of gut $CD4^+$ T cells, affects the gut defences against commensal and pathogenic bacteria, enhancing gut permeability resulting in microbial translocation and dissemination of microbial products into peripheral blood[43]. The HIV-1 specific $CD4^+$ T cells are not spared during the phase of massive cellular depletion[78]. HIV specific $CD4^+$ T cell responses expand during the acute phase of disease and decline rapidly shortly thereafter[79, 80]. Early initiation of ART can reverse the loss of HIV specific CD4+ T cell responses and results in better control of viraemia[81, 82].

Overall, $CD4^+$ T cell depletion leading to immune suppression, dysfunction and dysregulation of different $CD4^+$ T cells subsets in different tissues, is the hallmark of HIV infection. The role of innate immunity particularly TLRs on $CD4^+$ T cell responses with respect to pathogenesis and protection against, is still widely unexplored. Part of the work presented in this thesis focused investigating nature of T cells responses to different TLR stimulations, with the aim of furthering the understanding of innate recognition in protection against HIV, information that might be useful in the development of HIV vaccines or novel microbicides.

1.2.1.1.2. $CD8^+$ T cells

The primary goal of a $CD8^+$ T cell response during a viral infection is to generate CTLs to kill virus infected cells, and to generate a small subset of cells for longevity to counter future infections of a similar nature. To achieve these goals, virus specific $CD8^+$ T cells require three signals in order to differentiate, clonally expand and efficiently perform effector functions needed to clear viral infections. These are; i) presentation of virus derived antigens through MHC class I by APCs ii) co-stimulation iii) inflammation[83]. Early production of proinflammatory cytokine responses like type I IFNs, IFN-γ, IL-2, IL-12, IL-27 and IL-33, triggered by innate recognition of PAMPs, enhance proliferation, survival and differentiation of CTLs, and is also responsible for contraction of effector $CD8^+$ T cell responses at the tail end of an immune response[84-86]. AN increased intensity of inflammation, TCR signalling or activation of co-stimulatory pathways, results in corresponding increases in CTL and memory T cell responses. Any alterations to these signals, such as the reduction in antigen load or levels of proinflammatory cytokines, results in reduction of CTL function or effector $CD8^+$ T cell responses[87]. Therefore the balance of pro- and anti-inflammatory responses during viral infections can influence the degree to which $CD8^+$ T cells get activated or differentiate into effector or memory profiles.

$CD8^+$ T cell Responses in HIV infection

The Rhesus Macaque-SIV infection model has been instrumental in elucidating aspects $CD8^+$ T cell responses during SIV and HIV infections, given the similarities in pathogenesis between the two diseases[15]. For instance, it is using the NHP SIV model that the critical role of $CD8^+$ T cells responses in SIV replication control was first demonstrated[88]. Ever since, the control of HIV and SIV replication has largely been linked with $CD8^+$ T cell mediated responses[15, 36, 89].

The first HIV specific $CD8^+$ T cell responses emerge 1-2 weeks post infection, just as peak viraemia declines[19, 21]. Following peak viraemia, rapid selection of mutations at discrete

sites of the viral genome occurs, dramatically changing the specificity of $CD8^+$ T cell responses [21]. Virus specific $CD8^+$ T cell responses present in the early stages of infection change from those that recognize epitopes from the founder virus, to those that recognize escape-mutant species. The emergence of new viral mutant species is accompanied by changes in the $CD8^+$ T cell compartment, although, a small percentage of virus escape mutants are not associated with any virus specific $CD8^+$ T cell responses[89]. The current understanding of the role of host genetics in the development of HIV specific $CD8^+$ T cells that is crucial for understanding viral replication control is largely insufficient. Broadening the understanding on the involvement of the innate immune system during development of HIV specific T cell responses will be essential for vaccine design and for complete understanding of virus replication control.

Recent evidence obtained from *in vitro* and *in vivo* studies indicate that TLR agonists can enhance $CD8^+$ T cell proliferation through reversal of $CD4^+$ T_{reg} mediated suppression[90]. The ability of endosomal and surface TLRs to induce activation of $CD8^+$ T cells differed, with higher activation being observed following TLR3, 7 and 8 stimulations *in vivo*[91]. The role of TLRs in influencing the development of $CD4^+$ T cell responses through activation of DCs is well defined, but the role of TLR driven DCs or APCs activation in priming of $CD8^+$ T cells is not clearly understood. Part of the work presented in this thesis sought to understand the influences of TLR signalling pathways on activation and function of different T cell subsets.

1.2.1.2. B cells

HIV does not infect B cells, but infectious virions can bind to complement receptor CR2 (CD21 molecule) expressed on the surface of B cells, thereby aiding the infection of bystander target cells during the acute phase of disease[92]. High levels of viral replication

and proinflammatory factors during the acute phase of HIV infection, results in rapid dysregulation of B cells in multiple tissues, starting from the intestinal mucosa to the secondary lymphoid tissues with viral dissemination[93]. The early direct and indirect effects of viral replication, alters B cell functions irreparably. Abnormalities and dysregulation of B cell responses results in hypergammaglobunemia in HIV infected individuals, a hallmark of HIV infection[94]. Spontaneous production of antibodies similar to HIV associated hypergammaglobunemia, requires dual stimulation through the B- cell receptor (BCR) and TLR and is needed for activation of B cell functions like antigen presentation or cytokine production[95]. Mature B cells possess a clonally re-arranged BCR, and express more than one TLR enabling them to uniquely recognize both antigen-specific epitopes and PAMPs/DAMPs. TLR signalling has only been shown to affect B cell functional responses, but not development and survival of B cells[96]. However, the absence of TLR signalling, such as in individuals lacking MyD88 or IRAK4, results in over-representation of autoreactive B cells, possibly due to alterations in B cell selection process during affinity maturation[97]. The expression of TLRs differs between B cell subsets, consequently the antigen presentation, antibody and cytokine production by different B cell subsets[98, 99]. The variation in B cells expression of TLRs has been implicated in commitment of B cells during development to either innate-like or adaptive B cells, based on their functions and BCR repertoire. MyD88 can orchestrate B cell activating factor (BAFF) dependent signal transduction through TACI (transmembrane activator CAML interactor/ TNFRSF13) leading to activation of NF-κB and activation induced cytidine deaminase(AID), the latter facilitating extra-follicular somatic hyper-mutation and class-switch recombination's[100]. The ability of TLRs to influence TACI-BAFF activity could potentially increase somatic hypermutation and class switch recombinations leading to broadening antiviral antibody specificities. Triggering TLR7 or MyD88 signalling has been shown to enhance CSR during influenza virus infection,

while MyD88 signalling on its own is essential for development of long-term memory B cells responses in polyoma virus infection[101]. Failure of respiratory syncytial virus vaccine to protect infants was associated with production of low affinity antibodies by B cells due low induction of TLR signalling by the vaccine[102]. The ability of B cells to respond and produce antibodies after TLR ligation in the absence of antigen recognition through BCR, may be an evolutionary adaptation that enables the immune system to combat infections through mechanisms like opsonisation or complement activation, prior to development of more specific T and B responses capable of clearing infections[95].

The realization that natural HIV infection in humans can result in production of antibodies with broadly neutralizing capabilities, marked a major turning point in efforts to develop a HIV vaccine[103]. Failure of the humoral response to provide protection against HIV acquisition, as is the case with other viral infections, has been linked to the failure to develop high affinity neutralizing antibodies following the acute stage of disease. Recently, *in vitro* studies showed that co-immunization of HIV viral proteins with TLRs agonists contained in virus like-particles, could facilitate the development of anti-HIV humoral responses[104, 105]. Collectively, these preliminary studies support the premise that co-administration of HIV viral immunogens with TLR agonists as adjuvants, may be a useful strategy worth exploiting to increase CSR events through dual stimulation of B cells (through BCR and TLR) potentially leading to development of high affinity HIV specific antibodies with broadly neutralizing capabilities.

In Summary, HIV infection causes massive depletion of $CD4^+$ T cells, and alters the proportions and function of different T cell and B cell populations, and triggers persistent immune activation linked to the immune suppression and immune dysfunction accompaned by changes to T and B cell populations. Heightened TLR signalling during the acute phase of

HIV infection, has been linked with triggering immune activation. However, the role of TLR recognition of virus derived components toward development of virus specific T cell and B cells particularly during HIV infection is still unknown and has not been widely investigated. Part of the work presented in this thesis investigated aspects of T and B cell responses to different TLR stimulations in an attempt to distinguish the responses of these cells in HESN individuals and susceptible controls.

1.2.2. Innate Immune system.

The innate immune system is evolutionarily the most ancient part of the immune system, given its presence in both the plant and animal kingdoms[106, 107]. The innate system is the first to encounter invading microbes, thus is considered to be the body's first line of immune defence. It also functions to discriminate non-infectious 'self' components from infectious 'non-self' entities. Components of the innate immune system are strategically located at the interface between host tissues and the environment.

1.2.2.1 Components of the Innate Immune system

The innate immune system begins at the first anatomical barrier, and is present at the smallest molecular structure capable of detecting microbial invasion. It is made up of components like mucus-present on mucosal surfaces-capable of trapping microbes and limiting their entry into underlying tissues; intact skin- capable of preventing entry of pathogenic and commensal bacteria (normal flora); enzymes like lysozyme found in tears and digestive enzymes in the gut that are able lyse the microbial membranes and so much more. The innate immune system also consists of a cellular compartment made up of granulocytes- neutrophils, eosinophils and basophils, masts cells, monocytes, DCs, natural killer cells and innate lymphocytes and innate lymphoid cells (ILCs).

1.2.2.1.1 Monocytes

Monocytes make up about 10% of blood leukocytes in humans, and play a major role in linkage between innate and adaptive immunity through inflammation. Monocyte subsets are very heterogeneous varying in size, expression of chemokine and growth factor receptors. Monocytes act as a systemic reservoir for replenishing tissue macrophages and DCs, where the differentiation of monocytes to DCs often occurs under inflammatory conditions, such as during the acute phase of most infections, through yet to be determined mechanisms[108]. Monocytes also play a crucial role in phagocytosis of pathogens, clearance of apoptotic cells and toxic compounds at the terminal end of inflammatory processes, aided by the vast range of scavenging receptors expressed on these cells that can recognize lipids, and which can bind to microorganisms and apoptotic cells facilitating phagocytosis[108]. Monocytes can function as antigen presenting cells, although their antigen presentation is less efficient when compared to that of DCs[109]. Human monocytes can be classified based on the expression of CD14 and CD16 into; classical ($CD14^{+}$ $CD16^{-}$), intermediate ($CD14^{+}$ $CD16^{+}$) or non-classical ($CD14^{dim/-}$ $CD16^{+}$) monocytes.

Role of Monocytes in HIV pathogenesis

HIV can infect monocytes and other cells of the myeloid lineage, using co-receptors that are distinct from those the virus uses when infecting $CD4^{+}$ T cells. Infection of monocytes, macrophages and DCs by HIV is relatively rare compared to infection of $CD4^{+}$ T cells, but when infection occurs it can lead to the establishment of latent HIV reservoirs[110]. The reduced susceptibility of monocytes and macrophages to HIV is linked to their high expression of host restriction factors such as SAMHD1, VIPERIN and APOBEC3G[111, 112]. The level of expression of the different host restriction factors, varies between the monocyte subsets, for instance $CD14^{+}$ $CD16^{-}$ or classical monocytes expresses a lower molecular weight of APOBEC3A and APOBEC3G, while $CD14^{+}$ $CD16^{+}$ intermediate monocytes and $CD14^{dim/-}$ $CD16^{+}$ non-classical monocytes express higher molecular weight

forms of both enzymes[112, 113]. SAMHD1 limits infection of monocytes and macrophages by hydrolyzing deoxyribonucleotides, thus reducing the pool of nucleotides available for viral reverse transcription[114, 115]. The modality of VIPERIN restriction of HIV infection is still unknown, but its expression contributes to activation of TLR signaling through interaction with pathway components such as IRAK1[116], whereas APOBEC3G functions as a cytidine deaminase that catalyzes deamination of cytidine to uridine[112].

In spite of expressing these host restriction factors, HIV still infects cells of mononcyte//macrophage lineage aided by its promiscuous usage of multiple chemokine co-receptors when infecting different myeloid cells. HIV's co-receptor usage is cell type specific, for instance, HIV utilizes CXCR4 to infect macrophages[117], CCR3 and CCR5 to infect microglia in the brain cells[118], CCR5 and rarely CCR2b to infect $CD4^+$ T cells [119, 120]. The contribution and duration of HIV latency in monocytes and macrophages is yet to be definitively demonstrated. It is important to differentiate between persistence and latency, the former referring to long term-stable infection with HIV virus, with the latter referring to the presence of cells producing virus in spite of ART[121].

1.2.2.1.2. Dendritic Cells

Blood DCs are a heterogenous population of cells that can be classified into $CD11c^+$ myeloid or conventional DCs and $CD123^+$ plasmacytoid DCs[122]. Blood DCs lack expression of lineage (Lin) markers-CD3, CD19, CD20 or CD56, but constitutively express MHC class II molecules. Plasmacytoid DCs are Lin^- $HLA\text{-}DR^+$ $CD123^+$ $CD303^+$ (BDCA2), $CD304^+$ (BDCA4), while cDCs are Lin^- $HLA\text{-}DR^+$ $CD11c^+$ (macrophages and monocytes also express CD11c).

DCs are 'sentinels' of the immune system and play a crucial role in immune surveillance[123]. Upon antigen exposure, DCs home to lymphoid organs through the

lymphatic system to localize in the T cell zone where they process and present antigens to the resident T cells, leading the development of potent CTL responses and mediate NK cell activation[124, 125]. Myeloid DCs produce IL-12, IL-15 and IL-18 crucial for development of T_H1 responses thus promoting CTL development, with IL-12 and IL-15 being capable of activating NK cells, where both cytokine responses are critical during viral infections[124, 125].

Changes in DC subsets during HIV

During the acute phase of HIV disease the numbers of DCs- both mDCs and pDCs- is markedly reduced, correlating with disease progression and increasing viral load[126, 127]. The mechanism behind the reduction of DCs during HIV infection is yet to be determined; however, there are a number of different mechanisms for DC reduction proposed; First, the reductions may be due to the direct effects of HIV infecting the DCs, although the experimental evidence from quantifications of DC infections by HIV *in vivo* is lacking, therefore, contradicting this premise[128, 129]. Secondly, declines in DC numbers have been linked with indirect effects of elevated IFN-α production during acute phase of HIV infection. Lastly, the declines may be due to migration of certain DC populations from peripheral blood to lymphoid organs such as lymph nodes and spleen [130-132], given that pDC numbers increase in lymphoid organs during HIV infection[133]. It is important to note that HIV infection has been shown to impair differentiation of monocytes to cDCs[134], yet despite not infecting pDCs, HIV can increase apoptosis of pDCs[135]. There is contradicting evidence with regard to restoration of cDC and pDC numbers by ART, warranting further investigation into the functional status of DCs during ART[133, 136].

HIV recognition and Binding in DCs

Infection of mDCs or cDCs by HIV can be mediated by receptors such as CD4, CCR5, CXCR4 and other receptor like CXCR6, CCR3, CCR8 and CCR9[137]. HIV infects cDCs

using C-type lectins such as DC-specific ICAM3 grabbing non-integrin (DC-SIGN), Langerin (CLEC4K or CD207) and DC immunoreceptor (DCIR or CLEC4A)[137]. The expression of these receptors on cDCs varies depending on sub-type, activation status and tissue localization. HIV binding to cDCs using either DC-SIGN or DCIR results in internalization of virions into endosomes, but the virions are not degraded, thereby enhancing trans-infection of $CD4^+$ T cells in lymphoid organs through increased formation of viral synapses [138, 139]. The survival of internalized virions is enabled by the activation of mammalian target of rapamycin (mTOR) which negatively regulates autophagy through blockade of lysosomal fusion, resulting in survival of HIV in phagosomes and decreasing presentation of HIV antigens[140, 141]. HIV infection of cDCs inhibits autophagy by preventing fusion of endosomes bearing HIV virions after internalization to autophagosomes with lytic components capable of degrading the virus[142]. However, when cDCs bind to HIV using Langerin, the virus is internalized and trafficked to Birbeck granules, where the virions are degraded[143]. These observations indicate that the choice of C-type lectin receptor usage during HIV entry of cDCs has a bearing on the outcome of infection. Whereas pDCs express C-type lectins such as BDCA2 (CLEC4C), HIV does not typically infect these cells, but it can bind to CD4 expressed on these cells using gp120[144].

Unlike other viral infections, HIV infection often fails to induce maturation of infected immature DCs, even when different maturation stimuli are applied to such cells *in vitro,* yet the virus is capable of replicating in such cells[141]. HIV has been shown to equally inhibit maturation of bystander DCs, thereby limiting the ability of these cells to activate other T cells through induction of IL-10 production by T_{regs}[141]. It is important to mention at this point that majority of the infection studies demonstrating infection of DCs have been conducted *in vitro,* the evidence supporting infection DCs *in vivo* has been somewhat contradicting[128, 129]. As such the observations made from *in vitro* HIV infection of DCs

may not be readily translatable to understanding the dysregulation of DCs caused by HIV infection *in vivo*.

Altered DC function during HIV infection

In vitro pDCs have been shown to capture HIV when CD4 molecule expressed on the of these cells surface binds to gp120, while endocytosis of HIV virions can trigger the activation of pDCs through viral RNA-TLR7 interaction [145, 146]. HIV exposed pDCs produce IFN-α capable of enhancing adaptive immune responses, and expression of indoleamine-2,3-dioxygenase that induces differentiation of naive $CD4^+$ T cells into $T_{regs.}$with potential of suppressing HIV specific responses[147-149]. On the contrary, mDC or cDCs fail to become activated following similar exposures but show a defect in IL-12 production[141] corresponding to low levels of IL-12 observed during HIV disease[33]. Myeloid DCs exposed to HIV *in vitro* can also prime virus specific $CD4^+$ and $CD8^+$ T cells[150]. Differences in maturation of pDCs and mDCs after exposure to HIV, have been linked to the lower triggering of TLR7 once HIV virions infect cDCs[151]. However, in spite of inability of HIV to infect pDCs, these cells are able to respond more robustly through TLR7 leading to enhanced maturation[151].

De novo exposure of cDCs to HIV leads internalization of virions into endosomal compartments, triggering DC-SIGN and TLR8 signalling resulting in NF-κB activation and HIV transcription[152]. Both R5 and X4 trophic viruses were recently shown to drive production of IFN-α by pDCs leading to increased expression of BAFF on monocytes, the ligand for BAFF-R expressed on B cells[153]. This indicates that HIV driven pDC responses may have an influence on Ig class switch recombination in B cells through BAFF signalling mediated activation of AID. Individuals capable of naturally controlling HIV replication or 'elite controllers', possess higher numbers of pDCs compared to those who have progressive

HIV disease, but no differences in functions and properties of pDCs between HIV elite controllers and HIV negative individuals, an indication that pDCs may not be very influential in ongoing HIV replication control[154, 155]. The proportion of mDCs was higher in individuals capable of spontaneously controlling viral replication or 'elite controllers' whereas TLR proinflammatory responses by mDCs in such individuals was decreased[156].

DCs contribution to disease progression

Persistent and uncontrolled inflammation is the key driver of HIV pathogenesis, where IFN-α has been implicated as being a key contributor to depletion of $CD4^+$ T cells through upregulation of pro-apoptotic molecules like DR5, FAS-FASL and TRAIL on $CD4^+$ T cells[157, 158]. Higher type I IFN production in women by pDC makes them more likely to develop AIDS faster than men with similar viral loads[159]. Failure of SIV infection in African green monkeys (AGMs) to progress AIDS in spite higher levels of viral replication, has been linked to the limited or completely abscent immune activation, whereas a robust type I IFN response is present in progressive SIV infection of Macaques and in human HIV/AIDS[160]. Lower type I IFN responses in SIV-AGM infection, have been shown to result in lower activation and virus sensing by pDCs,yets such pDCs are capable of enhancing virus sensing and type I IFN production in bystander cells [161]. This indicates that the antiviral response mounted by HIV/SIV activated pDCs during the acute phase of both HIV and SIV infections, may be responsible for enabling the establishment/progression of HIV/SIV infections through initiation of deleterious immune activation[15, 27]. Interestingly, exogenous administration of type I IFN during both chronic HIV and SIV infections decreases viraemia in HIV infected humans, SIV infected AGMs and Sooty Mangabeys (SMs)[162-164].

The role pDC and type I IFN responses in protection against HIV are largely unknown. Part of the work conducted towards this thesis sought to understand aspects of pDC responses to analogues of HIV genetic material, with regard to natural protection against HIV.

1.2.2.1.3. Natural Killer Cells

NK cells are known for their ability to surveil, recognize and kill virus infected, transformed or stressed cells. NK cells mediate anti-tumour and antiviral cytotoxic responses, prior to onset of anti-viral or anti-tumour adaptive immunity, for this NK cells have been largely classified as part of the innate immune system. This could change soon as accumulating evidence in mice and humans indicates that, much like adaptive immune cells, NK cells are 'licensed' during development through interactions involving inhibitory receptors and their ligands, NK can possess antigen specific receptors, can undergo clonal expansion and are capable of transforming into long-lived memory cells[165]. NK cell deficiencies in humans, results in enhanced susceptibility to viral infections such as, herpes simplex virus (HSV), varicella zoster virus (VZV) and cytomegalovirus (CMV)[137, 166].

The primary function of NK cells involves lysing virus infected and tumour cells through three major cytotoxic mechanisms; i) Exocytotic lytic activity, where NK cells upon binding to virus infected or tumour cells, release lytic mediators such as cytoplasmic granules, granzymes and perforins; ii) Induction of apoptosis through Fas-Fas ligand interactions, and; iii) Antibody dependent cellular cytotoxicity (ADCC). The precise mechanism(s) used by NK cells to recognize virally infected cells is very complex and is (are) not fully understood. No receptor on NK cells has been identified to date that uniquely recognizes HIV. Although NK cell responses to HIV derived peptides have been described previously, it is not clear whether such responses are mediated by CD16 or other NK receptors[167, 168].

NK cell Receptors and their Function during viral infections

Unlike B and T cells that possess a single activatory receptor- BCR or TCR, respectively- NK cells lack a single dominant receptor. NK cells express three major classes of NK cell receptors (NKRs) -natural cytotoxic receptors, killer immunoglobulin receptors and NKG2 family, all of which are capable of providing either an inhibitory or activatory signal.

Natural cytotoxic receptors (NCR) that are expressed constitutively and are non-polymorphic, they include; NKp46, NKp30 and NKp44. NKp44 is only expressed on NK cells after undergoing IL-2 mediated activation. The majority of ligands for these receptors have not been identified, although NKp46 and NKp30 have been shown to interact with Influenza virus haemagglutinin (HA), mediating cytolysis of virus infected cells[166]. Killer Immunoglobulin Receptors in humans and C-type lectin Ly49 in mice form the next class of NKRs. Both are structurally very distinct but capable of performing similar functions. The ability of KIRs to provide either inhibitory or activatory signals upon binding to MHC class I molecules, is dependent on the strength of binding between ligand and the cytoplasmic motifs carried on each receptor (as mentioned above and reviewed by Jost and Altfeld)[166]. The NKG2 family of receptors which resemble C-type lectins are type II transmembrane receptors consisting of NKG2A, NKG2C and NKG2E, all of which exist as heterodimers with CD94, except NKG2D that exists as a homodimer. The receptors recognized by NKG2D are expressed by virus infected or malignant cells. HIV negative regulatory factor (NEF), down-regulates ligands for NKG2D such as MICA, ULBP1 and ULBP2 further impairing the NK cell responses during HIV infection[169] Similarly, HIV NEF, can downregulate HLA-A and HLA-B in infected cells but not HLA-C or HLA-E, enabling the virus to evade CTL responses targeting HLA-A and HLA-B, but cannot preventing killing of infected cells expressing inhibitory receptors for HLA-C and HLA-E by NK cells[137, 170-172].

Inhibitory receptors contain on their cytoplasmic tails immunoreceptor tyrosine based inhibitory motifs (ITIMs), which upon engaging their specific ligands become phosphorylated by Src members enabling the recruit SH2 domain containing inositol 5-phosphate -1 (SHP-1 or SHP-2), leading to transmission of an intracellular inhibitory signal. The inhibitory signals suppress NK cell responses such as Ca^{2+} influx, degranulation, proliferation and cytokine production[173]. Long and colleagues, demonstrated that NK cell activation requires ligation of a number of activating or 'co-activating' receptors, with achievement of activation when a signalling threshold is exceeded through synergic function of a number of activating receptors. Majority of these receptors contain a cytoplasmic tail with a constitutively expressed immunoreceptor tyrosine based activating motif (ITAM) capable of delivering an intracellular activatory signal. The only activating NK receptor (NKR) capable inducing NK function without co-activation is CD16, that has a unique transmembrane structure when compared to other activating NKRs[174].

The interaction between inhibitory NKRs and self-MHC class I molecules is important in NK cell development which starts in the bone marrow and continues into the periphery. The NK cell development is dependent on the interaction between NKR interaction with MHC class I molecules expressed on other cells, and is guided by cytokines like IL-15 and IL-18[173]. NK cells also possess the ability to undergo homeostatic division, followed by distribution to lymphoid and non-lymphoid organs, where they are found months following clearance of certain viral infections[175].

1.2.2.2. Innate Recognition

The innate immune system consists of germ-line encoded receptors that function in recognizing conserved pathogen associated molecular patterns (PAMPs) on microbes through pattern recognition receptors (PRRs). Cognate PAMP recognition by a PRR triggers an

intracellular signalling cascade terminating in the activation of transcription factors encoding inflammatory cytokines, chemokines, co-stimulatory and antimicrobial peptides[73].

Several classes of PRRs have been identified that can be categorized into secreted, transmembrane or cytosolic PRRs. The families of PRRs with transmembrane receptors include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), while intracellular PRRs include nucleotide binding oligomerization domain (NOD) like receptors (NLRs) and Retinoic inducible gene (RIG) like receptors (RLRs), while soluble PRRs include collectins, ficolins and pentraxins[74]. The different families of PRRs function in extrinsic or intrinsic recognition of microbial components. There is considerable redundancy in the detection of microbial components, demonstrated by the preservation of adaptive immune responses in the absence of TLR signalling[176].

1.2.2.3. Toll-Like Receptors

1.2.2.3.1. History of Toll-Like Receptors

In 1989, Charles Janeway proposed that pattern recognition of conserved pathogen associated molecules by the innate immune system, was essential for the development of adaptive immunity[72]. At the time, there was limited scientific evidence to back up this hypothesis. However, a series of related discoveries resulted in the definitive identification of the first set of pattern recognition receptors, subsequently revolutionizing the field of innate immunology. Prior to this, the molecular basis of the innate immune system was unknown, with the innate immune system being considered as a crude and unsophisticated part of the immune system that helped the adaptive immune system perform its function of conferring protection against pathogens. The discovery of TLRs led to the rethinking of how T cell activation and pyrogenicity previously linked to IL-1, actually occurred[177]. The cloning of the IL-1 gene in 1988 was the first step towards the discovery of TLRs, the prototypic PRRs. In 1991, the

domain for the IL-1R was shown to be homologous to the *Drosophila melanogaster* Toll gene product, whose only known function at the time was dorso-ventral polarization during embryonic development[178, 179]. Then, it was known that IL-1 activated NF-κB signalling in a similar fashion to *Drosophila melanogaster* protein Dorsal through REL protein homologous to the NF-κB pathway[180, 181]. The first clue on role of Toll and IL-1 in host defence against pathogens was through the discovery sequence homology between amino terminal domain of Tobacco plant N protein that confers resistance to Mosaic virus, and the cytoplasmic domains of Toll and IL-1[182]. The link between Toll, Dorsal and IL-1, led Jules Hoffmann and colleagues to postulate that Toll might have role in regulating immune functions in addition to directing dorso-ventral development. The subsequent demonstration of LPS recognition by TLR4, and a series of studies testing a range of microbial products as possible ligands for the remaining TLRs, furthered the knowledge on TLRs and their ligands.

1.2.2.3.2. TLR Structure and PAMP recognition

Toll-like receptors are the most characterized class of PRRs, other classes of PRRs include NOD like receptor-NLRs, RIG-I like receptors-RLRs, C-type lectin receptors-CLRs, newer ones like secreted PRRs, AIM-2 like receptors-ALRs and intracellular DNA sensors, with discovery of new PRR families on going. Currently, 10 TLRs have been identified in humans (TLR1-10) and 12 TLRs (TLR1-9, 11-13) in mice. TLRs can be expressed on the cell surface or intracellularly; Surface TLRs include TLR1, 2, 4, 6, 10, 11 and 12, while intracellular TLRs include TLR3, 7, 8, 9 and 13[176].

Surface TLRs recognize PAMPs associated with extracellular pathogens or membrane bound microbial components, like proteins, lipids and lipoproteins. TLR4 complexes with MD2 to recognize a component of gram negative bacterial cell wall- LPS, respiratory syncytial virus (RSV) proteins, mouse mammary tumour virus envelope proteins and *Streptococcus*

pneumoniae pneumolysin[183]. TLR2 forms heterodimers with TLR1, TLR6 or TLR10 to recognize a wide range of structurally diverse PAMPs. The TLR2-TLR1 heterodimer recognizes triacylated lipopeptides such as PAM_3CSK_4, cell wall components of gram negative bacteria and *Mycoplasma fermentan* macrophage activating lipopetide 2 (MALP-2). The TLR2-TLR6 heterodimer recognizes diacylated lipopetides of peptidoglycans (lipotechoic acid-LTA), mannan, zymosan and tGPI-mucin[183]. TLR2 collaborates with TLR10 to recognize *Listeria* and Influenza A virus components[184, 185]. TLR5 is only known to recognize monomers of a conserved component of bacterial flagella, the flagellin protein (**Figure 2**)[183].

Intracellular TLRs recognize viral, bacterial and self nucleic acids typically RNA or DNA. TLR3 recognizes double strand RNA from RSV, HSV-1, murine cytomegalovirus (MCMV), encephalomyocarditis and West Nile viruses, small interfering RNAs and self RNAs from damaged cells[186, 187]. TLR3 is thought to be capable of recognizing most positive sense ssRNA viruses, as the majority of these viruses possess dsRNA intermediates in their replication cycles, or due to bidirectional transcription of genomes of ssRNA viruses[188]. TLR7 recognizes small molecules like Imiquimod, Resiquimod and Loxobrine, ssRNA from VSV, Influenza A virus, HIV and RNA from *Streptococcus* B bacteria[183, 189]. TLR8 recognizes viral, bacterial and self ssRNA[190]. TLR9 recognizes bacterial and viral DNA rich in unmethylated cytidine-phosphate-guanosine DNAs (CpG- DNAs) which are absent in mammalian DNA that is mostly methylated, and an insoluble crystal- hemozoin resulting from degradation of haemoglobin in *Plasmodium falciparum* infection[190]. There is a redundancy between TLR3, TLR7 and TLR8 when it comes to the recognition of ssRNA, with TLR3 and TLR9 detecting different products in the replication cycles of DNA viruses[191]. TLR11 recognizes flagellin, a profolin-like molecule from *Toxoplasma gondii* and a yet to be identified proteinaceous component of uropathogenic *E. Coli* (UPEC) [192,

193]. TLR12 found on murine myeloid DCs recognizes profolin from *T. Gondii*, similar to TLR11[194]. Both TLR11 and TLR12, function by forming homodimers or heterodimers[195]. TLR13 recognizes bacterial ribosomal fragment of 23s rRNA and a yet to be identified component of vesicular somatitis virus (VSV)[196].

1.2.2.3.3. Cellular and tissue expression of TLRs

The expression of TLRs varies between tissues and cell types. Majority of cells in the human body express at least one TLR, all of which primarily function to detect PAMPs and/or DAMPs. The TLRs expressed in tissues (other than blood), are associated with pathogenesis of a number of infectious diseases, genetic disorders and autoimmune conditions[197]. The discussion following will be limited to the TLR expression patterns on different blood cells particularly those contained in PBMCs.

The highest TLR expression on blood cells, is on monocytes and DCs. Blood DCs show distinct patterns of TLR expression, some of which is altered with DC maturation; for instance immature DCs (iDCs) express TLR1, 2, 4 and 5, but upon exposure to microbial components, these cells mature into myeloid DCs expressing TLR1, 2, 4, 6 and 8 or pDCs expressing either TLR7 or 9[198, 199]. The expression of TLR7 on mDC has been a subject of controversy with some of studies demonstrating its expression, yet others failing to do so[198, 199]. Monocytes/macrophages express all TLRs except TLR3 with differences in levels of expression of TLRs on different monocyte subsets[108, 199]. TLR expression on lymphocytes is lower than on APCs, including B cells, with innate lymphocytes bearing specific TLR expression patterns[200].

In some instances, the expression of TLRs in different organs, tissues and cells, may be influenced by recognition of cognate ligands, cytokines or microbial invasion, for instance, *Mycobacterium avium* infection induces increased TLR2 expression but on the contrary a

reduction in TLR4 expression in alveolar macrophages[201]. Viral infections can increase expression of TLR1, 2, 3, or 7 through induction of type I IFNs signaling, which is reversed by the removal of the same viral stimuli[202]. Alternatively, TLR expression can be induced directly through signal transduction, for instance, NF-κB activation during TLR or cytokine signalling, results in increased TLR expression[183]. Other cytokines that alter TLR expression include; colony stimulating factor 1 (CSF-1) which down-regulates TLR9 expression, macrophage migration inhibitory factor 1 (MIF) that decreases expression of TLR4 on macrophages and IFN-γ that primes monocytes/macrophages to respond to LPS by increasing TLR4 expression[106]. This overview demonstrates that variation TLR expression on different cell types and tissues, accounts for the differences in functions performed by TLRs at the site of expression, where the expression of TLRs can also be influenced by the prevailing inflammatory or immunological tissue environments.

1.2.2.3.4. TLR gene transcriptional regulation, Intracellular trafficking, and Expression

There are three classes of transcriptional regulators that control the expression of TLR genes and associated response elements; i) Class I transcription factors, that expressed constitutively but are latent, examples include NF-κB, IRF3; ii) Class II transcription factors, these are constitutively expressed, they include CCAAT enhancer-binding protein (C/EBP); iii) Class III TFs are induced during cell differentation, or can be constitutively expressed and active- they include RUNX1 (runt-related transcription factor 1), PU.1 and C/EBP. Inflammatory processes are known to enhance TLR gene expression; however, the primary transcriptional regulators of most TLR gene expression, is yet to be extensively explored[203, 204]. Expression of most TLRs is tissue and cell specific and potentially corresponding to the expression of TLR gene transcription factors; For example TLR4 mRNA expression is limited to a few cell types, where in myeloid cells the TLR4 gene expression is regulated by PU.1 and interferon consensus sequence binding protein or IRF8[205]. However, all TLRs

are synthesized in the endoplasmic reticulum (ER) and trafficked through the Golgi apparatus using the conventional protein secretory pathway, before taking residence on either the plasma or endosomal membranes[206, 207]. The secretory process is highly regulated to prevent intracellular recognition of self components like nucleic acids potentially leading to autoimmune reactions or diseases[176].

A homologue of *Caenorhaditis elegans* UNC93B1 gene has been shown to traffic intracellular TLRs from the ER to endosomes. TLR7 and TLR9 compete for association with UNC93B1, and can regulate the expression of each other, while TLR3 is not party to this competition[208]. TLR9 preferentially binds to UNC93B1 over TLR7, and higher expression of TLR9 is capable of inhibiting TLR7 signalling. On the other hand, TLR8 can inhibit both TLR7 and TLR9 derived from humans and mice (yet the function of murine TLR8 is still unknown) through physical interactions between the different receptors[209]. Migration of TLR1, TLR2, TLR4, TLR5, TLR7 and TLR9 from the ER to the plasma or endosomal membranes is controlled by an ER resident protein PRAT4A [210, 211]. Another ER-resident protein gp96 acts as chaperone for most TLRs including TLR1, 2, 4, 5, 7 and 9[212]. Once in the endosome, the nucleic acid sensing TLRs undergo proteolytic cleavage by asparginyl endopeptidase and cathepsins B, S, L, H and K to give rise to the active receptor forms capable of mediating ligand recognition[207, 213]. The intracellular localization of TLR3 to the early endosomes is dependent on the TIR and transmembrane domains, whereas only the transmembrane domain directs TLR7 and TLR9 intracellular localization. It is not known if intracellular TLRs localize into shared or separate specialized endosomal compartments[191]. The process of acidification of endosomes with maturation is also essential in the activation of TLR3, 7 and 9 to recognize purified ligands[214]. This shows endosomal maturation governs both degradation of microbes and the activation/maturation of TLRs.

1.2.2.3.5. TLR Signalling Pathways

Each TLR consists of a horse shoe shaped ectodomain containing leucine rich repeats (LRRs) which mediate PAMP recognition through formation of homo- or heterodimers, or in collaboration with accessory proteins or co-receptors[215]. The engagement of TLRs by cognate PAMPs activates multiple signalling pathways terminating in the transcription of cytokines, antimicrobial peptides and co-stimulatory factors.

Toll-like receptor signalling is initiated when a PAMP binds to its cognate receptor, causing a conformational change in the Toll/Interleukin 1 receptor (TIR) domain allowing it to bind to different adaptors and activating various downstream pathways (**See Figure 2**). The TIR adaptors include; Myeloid differentiation factor 88 (MyD88), TIR associated protein (TIRAP) also referred to as MyD88 associated like (MAL) adaptor, TIR domain containing adaptor inducing IFN-β (TRIF) and TRIF associated molecule (TRAM)[176, 216]. The nature of conformational change arising from TLR-PAMP engagment influences choice of adaptor recruitment to TIR domain and ultimately downstream signalling.

The first TIR adaptor described- MyD88- is used by all TLRs except TLR3. MyD88 activates the NF-κB and MAPKs complexes, leading to production of inflammatory cytokines. On the contrary, TLR3 and TLR4 utilize TRIF to bind to TIR eventually activating of NF-κB and IRF3 leading to transcription of inflammatory cytokines and type I IFNs, respectively. TIRAP/MAL and TRAM function to recruit different adaptors to TIR; Heterodimers of TLR1-TLR2 and TLR2-TLR6 all recruit MyD88, whereas TLR4 can recruit either MyD88 or TRIF. TLR5, 7, 8 and 9, all use MyD88 to bind to TIR. Based on TIR adaptors usage, TLR signalling pathways are usually divided into two; MyD88 (MyD88 dependent) and TRIF dependent (MyD88 independent) pathways (**Figure 2**)[176, 216].

1.2.2.3.5.1. MyD88 Dependent Pathways

The conformational change arising from TLR-ligand interaction results in MyD88 binding to the TIR domain, enabling MyD88 to recruit IL-1 receptor associated kinase 4 (IRAK4), which in turn activates IRAK1 and IRAK2 leading MAPK and NF-κB signalling[217]. Once MyD88 activates the IRAKs, it dissociates from them, allowing them to bind to TNF receptor associated factor 6 (TRAF6)-an E3 ubiquitin ligase- which then catalyzes the formation of a Ly63 (K63) polyubiquitin chain on IRAK1, NF-κB modulator (NEMO) and itself (**Figure 2**). Alternatively, together with dimeric E2 ubiquitin conjugating enzymes Ubc13 and Uev1A, the K63-linked ubiquitin chain on TRAF6-NEMO complex binds to TAB2 or TAB3, enabling the recruitment of transcription growth factor-β (TGF-β) associated kinase 1 (TAK1) forming a TRAF6-NEMO complex[176, 216]. TRAF6 also promotes the ubiquitination of ECSIT an adaptor protein, resulting in increased generation of cellular and mitochondrial reactive oxygen species (ROS). The K63-linked polyubiquitin chain recruits TAK1, which then phosphorylates the inhibitor of NF-κB kinase β (IKKβ) and mitogen activated-protein kinase 6 (MAPK6), thereby activating both the NF-κB and MAPK signalling pathways, respectively[218]. The NF-κB pathway proceeds through the formation of IKK complex consisting of IKKα and IKKβ bound to TAK1 and NEMO. In resting cells, NF-κB is bound to IκB, and both are sequestered in the cytoplasm. In activated cells, the IKK complex is formed, and it phosphorylates IκB leading to its degradation by the ubiquitin proteosome system and subsequent translocation of NF-κB into the nucleus where it transcribes genes for inflammatory cytokines (**Figure 2**). The phosphorylation of MAPK6 by TAK1 initiates the MAPK signalling cascade leading to activation of ERK1/2, p38 and JNK that can mediate the activation of transcription factor AP-1 (activator protein 1) in the nucleus, alternatively, stabilizing mRNAs that regulate inflammatory responses (**Figure 2**)[176, 183]**.**

Figure 2: TLR signalling Pathways: TLR1, 2, 4, 5, 6 and 11 are expressed on the surface of cells, while TLR3, 7, 8, 9 and 13 are expressed in endosomes. TLRs recognize different pathogen associated molecular patterns (PAMPs) through an ectodormain consisting of leucine rich repeats and a cytoplasmic Toll/IL-1 receptor domain. TLR1-TLR2 heterodimer recognizes triacylated lipopeptides; TLR2-TLR6 heterodimer recognizes diacylated lipopetides; TLR3- double stranded RNA, TLR4-lipopolysaccharide; TLR5-bacterial flagellin; TLR7 and TLR8 recognize imidazoquinolines or Guanine-Uridine rich ssRNA; TLR9- bacterial CpG DNA; TLR11-uropathogenic bacteria and TLR13- ribosomal RNA. Binding of PAMPs to specific TLR conveys a signal through the transmembrane Toll/interleukin-1 receptor (TIR) domain and leading to recruitment of TIR adaptors, that include; MyD88- Myeloid differentiation primary response gene 88 (used in TLR1-2, TLR2-6, TLR4, TLR7/8/9, TLR11 and TLR13), TRIF- TIR domain containing adapter-inducing IFN-β (used in TLR3 and TLR4 signalling), TRAM- TRIF related adapter molecule (only TLR4) and TIRAP/MAL-TIR domain containing adapter protein/MyD88 adapter like (TLR1-2-6 and TLR4). TIR binding to adaptors activates signalling through two distinct pathways, MyD88 and TRIF dependent pathways[176, 219].

Signalling through the MyD88 pathway is tightly intertwined with the migration of receptors through different stages of endosomal development. TLR4 signalling pathway is the only TLR pathway that uses all the four TIR adaptor proteins activating both the MyD88 and TRIF dependent pathways. Following recruitment of the TIR adaptor to the plasma membrane, TLR4 bound to ligand undergoes dynamin-dependent endocytosis and is trafficked to the endosome, where it complexes with TRIF or TRAM, rather than TIRAP and MyD88, leading to activation of TRIF dependent pathway and eventually IRF3 mediated type I IFN production[220]. Therefore, the MyD88 dependent pathway during TLR4 signalling is activated earlier than TRIF dependent, and it is only TLR4 signalling through which both pathways results in production inflammatory cytokines (**Figure 2**)[176]. TLR7 and TLR9 signalling primarily occurs in pDCs in response to viral ssRNA and bacterial DNA, respectively, derived from infectious microbes[108, 199]. Plasmacytoid DCs produce copious amounts of type I IFNs through TLR7 and TLR9 signalling. The production of type I IFNs occurs through the direct binding of MyD88 to IRF7, in a complex containing TRAF3, TRAF6, IRAK4, IRAK1, IKKα, OPNi and Dock2. In this complex IRF7 is phosphorylated by IRAK1 and/or IKKα enabling it to translocate to the nucleus where it transcribes type I IFN genes[221, 222]. On the other hand, the MyD88-IRAK4-TRAF6 axis drives the MyD88 dependent pathway (as described above) leading to the production of inflammatory cytokines. The MyD88-IRAK1-TRAF6-IRF7 complex is formed within lipid bodies by IFN-inducible VIPERIN that activates IRAK1 through K63-linked ubiquitination[223].

1.2.2.3.5.2. TRIF Dependent Pathway

Signalling via this pathway terminates in the production of either type I IFNs or inflammatory cytokines. TRIF can recruit either TRAF3 or TRAF6 (**Figure 2**). TRAF6 recruits RIP-1 kinase through a homotypic interaction motif, leading to its polyubiquitination by K63-linked ligase enabling the activation of TAK1 eventually leading to activation NF-κB and MAPK

signaling which terminate in transcription of inflammatory cytokines. When TRIF activates TRAF3, it recruits the IKK-related kinases TBK1, IKKi and NEMO, leading to phosphorylation of IRF3, which then dimerizes and translocates into the nucleus to transcribe type I IFN genes. There is evidence linking RIP-1 activation through polyubiquitination occurring after phosphorylation of IKKi/TBK1 by Pellino-1 (PELI1), and it also regulates IRF3 activation by binding to DEAF-1 a transcription factor that enables IRF3 to bind to the IFN-β promoter[224].

1.2.2.3.6. Negative Regulation of TLR signalling Pathways.

The negative regulation of TLR signalling prevents excessive and deleterious inflammation through termination or suppression of TLR signalling at multiple levels or in different strata[225]. TLR ligands themselves often turn into negative regulators capable of turning on and off the very signalling pathways they activate[216]. The negative regulation of TLR signalling occurs through three major mechanisms identified so far; i) Through dissociation of adaptor complexes ii) Degradation of signalling proteins or iii) Transcriptional regulation[216]. Some examples of the negative regulators includes; splice variants of adaptors or related proteins, transcriptional regulators, microRNAs, ubiquitin ligases and deubiquitinases[183].

1.2.2.3.6.1. Negative regulation through dissociation of adaptor complexes

Most of the adaptor proteins in the TLR signalling pathway have several variants, for example, the TIR domain has several variants of its adaptors that act as antagonists capable blocking downstream signalling. TRAM a TIR adaptor has a variant called TRAM adaptor with GOLD domain (TAG), which competes with TRAM for the TIR domain, subsequently blocking TLR-TRIF dependent signalling. TAG also plays a role in destabilizing TLR4 as it is required from its degradation, following fusion of the late endosome to lysosomes[226].

Some negative regulators bind to adaptors already bound to the TIR domain, for example sterile alpha-and armadillo containing motif (SARM), which binds to TRIF bound to TIR domain after LPS treatment prevents further downstream signalling. In other instances, the negative regulator protein, binds to TIR linked adaptors such as IRF5 or IRF4 competiting for MyD88 binding; TNF-α induced protein-8 (TNFAIP8) homologous to TNF-α induced protein 8 like 2 (TNFAIP8-2 or TIPE2) which inhibits AP-1 and NF-κB activation, its deletion leads to aberrant inflammation, multiple organ failures or even death[227, 228] (See **Figure 3**). In some instances, the expression of negative regulators can be induced by cognate ligation of its specific TLR. For instance, SHP expression is induced by AMP activated protein kinase (AMPK) whose activation is dependent on influx of Ca^{2+} ions triggered by TLR stimulation[229](see **Figure 3**).

Post-transcriptional modification is another mechanism used to regulate the interaction between adaptors, and ultimately TLR signal transduction. TRAF6 polyubiquitination is needed in NF-κB activation, TANK and TRAF-binding protein have been associated with inhibition of TRAF6 ubiquitination, where TANK deficient cells showed enhanced NF-κB activation and TANK deficient mice developed fatal glomerulonephritis[230]. There are different regulators of polyubiquitination of TRAF6, such as small heterodimer partner (SHP), which suggests the existence of overlapping functions of negative regulators of TLR signalling (**Figure 3**)[229]. Reversal of polyubiquitination restores normalcy in TLR adaptor functions, such as the removal polyubiquitin tail added to TRAF6 when shutting down TLR signalling by A20 and cylindromatosis (CYLD), restores normal TRAF6 function[231, 232]. On the contrary, a number of deubiquitination enzymes and phosphatases negatively regulate TLR signalling by removing ubiquitin and phosphate groups, respectively, from adaptor proteins thereby inactivating TLR signalling. The Src homology 2 domain containing protein tyrosine phosphatase-1 (SHP-1), suppresses IRAK1 and IRAK2 activation thus limiting

signalling through the MyD88 dependent pathway and promoting TRIF dependent signalling, leading to higher type I IFN production (**Figure 3**) [233]. On other hand, SHP-2 negatively regulates TRIF dependent TLR signal transduction by interacting with TBK-1 and dephosphorylates it, consequently limiting type I IFN production(**Figure 4**) [234]. Examples of DUB activity, are the A20 removal of polyubiquitin chain from TRAF6 (as mentioned above), and the removal of the ubiquitin chain by deubiquitinating enzyme A (DUBA) from TRAF3 preventing type I IFN production. These examples demonstrate the role of competitive adaptors, phosphatases and DUBs in disrupting or restoring TLR signal transduction (**Figure 3 & 4**).

1.2.2.3.6.2. Degradation of TLR signalling proteins

Ubiquitination of TLR signalling proteins targets them for proteosomal degradation, allowing for recycling of amino acids for other cellular metabolic processes. TLR4 and TLR9 signalling induces the expression of suppressors of cytokine signalling family 1 and 3 (SOCS1 and SOCS3) proteins that limit excessive inflammatory responses, by shutting down cytokine signaling **(Figure 4)**. Mice deficient of *SOCS1* genes have been shown to be hyper-responsive to LPS through excessive production of TNF-α, IL-12 and IFN-γ associated with endotoxic shock[235, 236]. The suppressive effect of SOCS1 exerted by its E3 ubiquitin ligase activity capable of adding polyubiquitin chains to TIRAP/MAL after it is phosphorylation by Bruton's tyrosine kinase (BTK), leads to its degradation. TLR signalling can activate MAPK signalling leading to activation of Janus Kinase-signal transducers and activator of transcription 1 (JAK-STAT1)[237]. SOCS1 also binds to p65 subunit of NF-κB leading to its degradation, while SOCS3 binds to TRAF6 leading to its degradation. A member of Tripartite-motif containing (TRIM) superfamily Trim30α targets and ubiquitinates TABs proteins of TLR signalling pathway for proteosomal degradation. Trim38 interaction with TRAF6 leads to its polyubiquitination and subsequent degradation(**Figures 3-4)**[238].

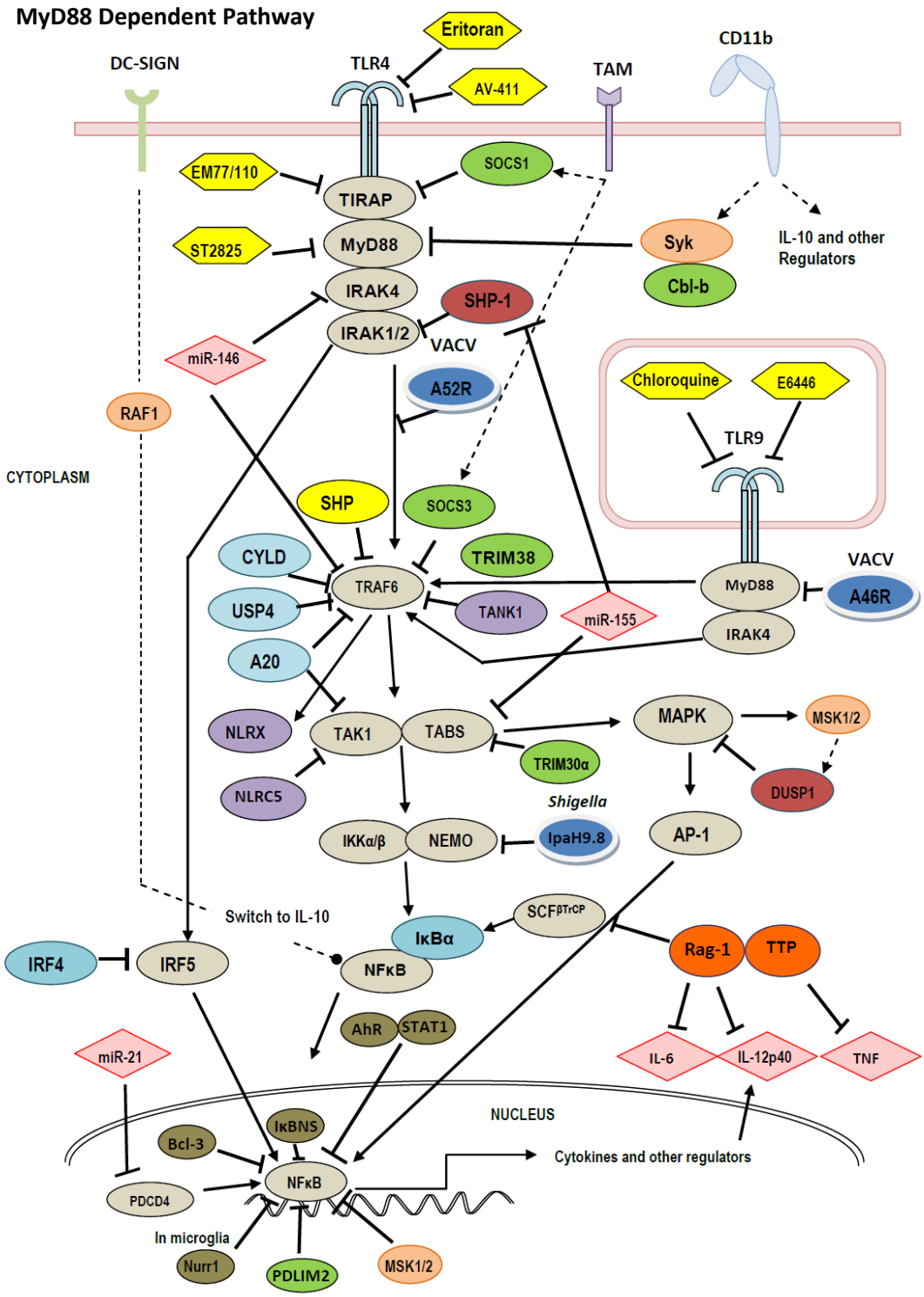


Figure 3. Negative regulation of MyD88 dependent pathway. The figure shows the different mechanisms of negatively regulating the MyD88 pathway. The gray ovals represent signal molecules, light blue ovals-competitors, lime green ovals-ubiquitin ligases or autophagy protein, maroon ovals- deubiquitinase (DUB) or phosphatase, dirty grey ovals-transcriptional regulators, dark orange ovals- regulator of RNA stability, pink diamonds-miRNA, blue ovals- pathogen derived, yellow hexagons- artificial agonist and others categories. Adapted from Kondo, *et al.*2012.

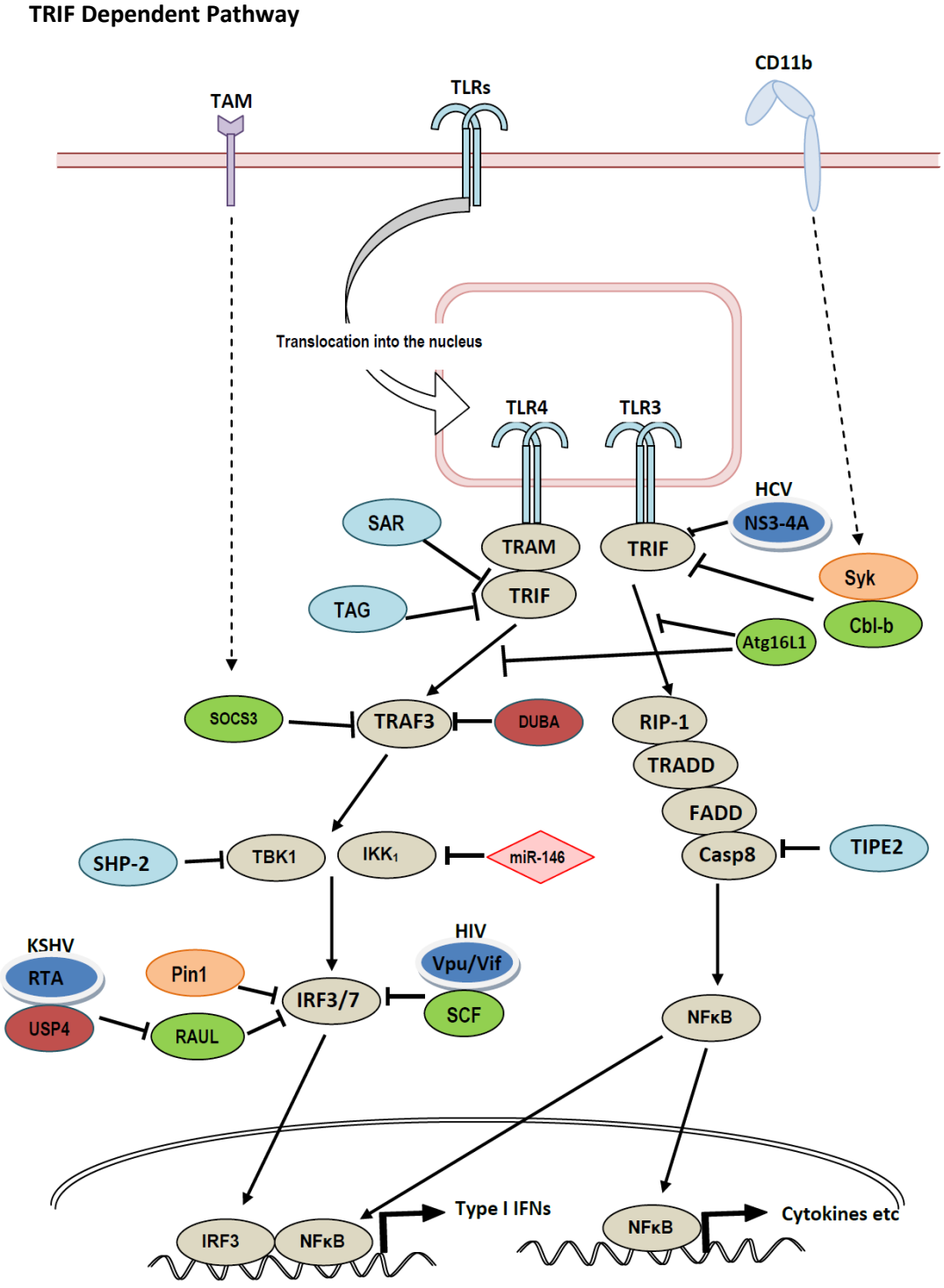


Figure 4. Negative regulation of TRIF dependent pathway. Gray ovals-signal molecules, light blue ovals-competitors, lime green ovals-ubiquitin ligases or autophagy proteins, maroon ovals-DUBs or phosphatases, dirty grey ovals-transcriptional regulators, dark orange ovals- regulators of RNA stability, pink diamonds-miRNAs, blue ovals- pathogen derived, yellow hexagons- artificial agonists and others categories. Adapted from Kondo T. *et a.,l* 2012[216].

Type I IFN production is also regulated by ubiquitination of IRF3 and IRF7 by peptidyl-prolyl isomerase (Pin1) resulting in their proteosomal degradation[238, 239]. An alternate path for degradation of TLR signal proteins other than the ubiquitin-proteasome occurs through autophagy. Loss of autophagy regulatory gene Atg16L1 in Crohn's disease, results in excessive production of reactive oxygen species (ROS), IL-1β and IL-18 through TRIF dependent pathway. This indicates Atg16L1 might have a regulatory role in TRIF dependent cytokine production (**Figure 5**)[240].

1.2.2.3.6.3. Transcriptional regulation

Regulation of TLR activation of gene expression occurs through epigenetic control by factors like cyclic AMP-dependent transcription factor (ATF3) that recruits histone deacetylase (HDAC1), for deacetylation of proinflammatory cytokine genes shutting down their transcription. Other mechanisms of transcriptional regulation of TLR activated genes include; inhibitors of transcriptions factors like IκB and B cell lymphoma 3-encoded protein (Bcl-3), both of which can inhibit NF-κB activation; cytosolic sensors that inhibit STAT1 and NF-κB like aryl hydrocarbon (Ah); RNA binding proteins with CCCH zinc fingers promote decay mRNA of TLR targets genes; non-coding RNAs or micro RNAs like miR155 and programmed cell death proteins capable of inducing cell death in LPS stimulated cells (**Figure 3 & 4**)[216].

Collectively, these examples illustrated that there are multiple overlapping mechanisms of TLR signalling pathway regulation, suggesting the existence of multiple check points in the immune system that limit aberrant or deleterious inflammation.

1.2.2.3.7. Role of TLRs in HIV pathogenesis

1.2.2.3.7.1. TLR recognition of HIV

The human innate immune system has the potential to recognize viral proteins and nucleic acids from the HIV-1 virus[152]. Currently, the mechanisms of innate recognition of cell-free or cell-associated HIV during natural infection are not clearly understood. This in spite the existence of substantial evidence linking HIV to chronic immune activation, the definitive trigger of immune activation is still unknown[15].

Innate recognition of HIV infected cells leads to a more robust production of type I IFNs when compared to recognition cell free virus by pDCs[144]. TLRs rarely recognize cell free HIV-1, but often recognize HIV infected lymphocytes through a number of different mechanisms. The recognition of HIV-1 requires fusion to target cells, but does not require the remaining stages of the viral life cycle such as reverse transcription or integration[241]. The quantity of viral material transferred following viral fusion to target cells, corresponds to the efficiency of recognition of cell associated virus[242]. Recognition of HIV infected cells also requires IRF3 and the TLR7 signalling pathways especially in macrophages and monocyte derived DCs (MDDCs)[151, 243]. Detection of virus infected pDCs and PBMCs requires envelope proteins, with envelope-deleted viruses being poorly recognized by target cells due to lower presentation of viral proteins and lower activation of IRF3 gene signalling pathway[241]. Alternatively, the production of exosomes containing miRNA has been implicated in TLR8 activation in lung alveolar macrophages during HIV infection leading to inflammatory responses[244]. The presentation of cell free virus to immature DCs in the female genital tract, has been shown to enhance expression of antiviral factors such as IFN-β, myxovirus resistance protein A (MxA) and interferon stimulated genes (ISG) leading to enhanced infection of iDCs[245]. Moreover, exposure of iDCs to cell-associated HIV, resulted in increased activation of the IRF3 signalling pathway and expression of tyrosine protein kinase-Lyn, leading to enhanced viral transcription[245]. The recognition of cell-free

HIV by iDCs in the genital tract can also occur through components of the complement signalling pathway particularly CR3, leading to enhanced susceptibility to HIV[245].

Collectively these studies indicate that the innate recognition of virus infected cells results in more robust proinflammatory responses when compared to recognition of cell free virus. This suggests that it is difficult for the innate immune system to recognize incoming HIV virions, before infection of target cells. Once HIV infects target cells, it can robustly activate the innate immune system as a cell-associated virus. Therefore, successful priming of the immune system to recognize incoming cell-free virus prior to infection of target cells and establishment of latency, is an important goal of development of effective anti-HIV microbicides or vaccines.

1.2.2.3.7.2. Effect of TLRs signalling on T cells during HIV infection

TLR signalling can affect the cells of the adaptive immune system directly or indirectly during HIV infection[147-149]. T cells express TLR1-10s but at lower levels compared to NK cells, B cells and APCs[246]. The indirect effects of TLRs signalling on T cell functions, occurs through TLR mediated activation of pDCs and mDCs facilitating presentation of HIV antigens and cytokines priming of T cells, potentially leading to the development of HIV specific T cell responses[247]. Until recently, the direct effect of triggering TLR signalling on HIV's infectivity of $CD4^+$ T cells was unappreciated. Dominguez-Villar, M. *et al.*, demonstrated the anergizing effect of TLR7 ligation of $CD4^+$ T cells, which was dependent on Ca^{2+} ion influx and activation of NFAT resulting in enhanced HIV replication. On the contrary, TLR8 activation through ssRNA failed to produce a similar effect[248]. Separately, a different TLR8 ligand- R-848 an imidazoquinolone- enhanced HIV replication in latently infected monocytes/macrophages by triggering the MAPK pathway components Erk1/2 and through production of TNF-α[249]. Additionally, HIV viraemia associated with increased

TRAIL expression on pDCs and TRAIL-R1 on activated $CD4^+$ T cells has been shown to enhance the targeting of $CD4^+$ T cells for deletion by $TRAIL^+$ pDCs. A situation reversed by ART that increases the expression of inhibiting TRAIL-R4 receptor on $CD4^+$ T cells, thereby limiting the $TRAIL^+$ pDC deletion of activated $CD4^+$ T cells[250]. The ART mediated reversal of $CD4^+$ T cell deletion by $TRAIL^+$ pDCs and the accompanying reduction of viraemia (viral PAMPs or antigens), implies that a higher presence of viral PAMPs or HIV antigens, mediates the increase in TRAIL-R1 expression on $CD4^+$ T cells[250]. HIV activated pDCs induce T_{regs} through TLR signalling dependent on indoleamine 2,3-dioxygenase activation[149], suggesting HIV infection also generates immunoregulatory responses potentially to counter deleterious immune activation triggered by viral replication. HIV-1 infection also increases TLR7 expression and responsiveness in $CD8^+$ T cells dependent on contact with accessory cells, leading to increased production of IFN-γ[251]. Therefore, direct or indirect activation of TLR7 or TLR8 pathways by HIV or its PAMPs can result in differing infection outcomes.

1.2.2.3.7.3. Effect of TLRs signalling on B cell responses during HIV infection

The early dysregulation and impairment of B cells functions in the acute phase of HIV disease, affects the phenotypic distribution and development of antibody responses for limiting HIV disease progression[93]. Soluble factors produced in the acute phase of HIV infection, can affect B cell antibody production by increasing polyclonal B cell antibody production (IL-15), terminal differentiation (IL-6) and by generally enhancing the immune activation (type I IFNs), and are also linked to delays in the development of protective B cell responses[93]. The heightened presence of microbial products and HIV replication during the acute phase of infection drives production of soluble factors responsible for immune activation, which in turns leads to impaired B cells function. Early initiation of ART has been

shown to preserve B cell function potentially by limiting the perturbations arising for activation of the innate immune compartment[252, 253].

1.2.2.3.7.4. TLR Expression and responsiveness during HIV disease

Lester R.T., *et al*., demonstrated the changes in TLR expression in PBMCs over the course of HIV disease. TLR6, 7 and 8 expression increased in the chronic phase of HIV disease, while TLR2, 3 and 4 were significantly elevated in AIDS, with TLR6 and TLR7 expression positively correlating with viral load, where the heightened TLR expression was normalized by HAART[254]. *In vitro* stimulation with ssRNA analogous to HIV's genetic material enhanced the expression of most TLRs quantified, suggesting that HIV viraemia could be accompanied by increases expression of multiple TLRs and enhanced sensitivity to most microbial PAMPs[254]. The effect of HIV on TLR expression and responsiveness is not only dependent on the stage of disease, but also on the TLR expressing immune cell subsets. Two populations of TLR8 expressing cells -monocytes and myeloid DCs- were shown to respond to ssRNA cognate ligand corresponding to viral load and $CD4^+$ T cell counts. Conversely, pDCs responses in infected individuals are known to be lower compared to healthy controls following TLR7 stimulation[255, 256]. An indication that the induction of TLR7 or TLR8 responses in different subsets by HIV virus is not uniform, where some cell types potentially contribute more to persistent of proinflammatory responses arising from viral ssRNA recognition. The absence of pDC but not mDC responsiveness to TLR7 over the course of HIV disease in HIV infected pregnant women but not in unborn foetus, recently demonstrated by others, also supports this premise [257]. The mechanism behind reduced pDCs responsiveness to TLR ligands could involve HIV-1 gp120 binding to CD4 receptor on pDCs[258]. In spite of this, the cytokines produced by pDCs are important in the activation of NK cells when responding to both TLR7 and TLR8 agonists, evidenced by the need for IFN-α, IL-12 and TNF-α produced by pDCs or other APCs in NK cell activation[259]. The

production of proinflammatory cytokines (TNF-α) by monocytes/macrophages following TLR8 stimulation with HIV-1 ssRNA is dependent on endocytosis, MyD88 signalling, and epigenetic control through histone acetyltransferase p300[260].

Collectively, these studies demonstrate that infection with HIV influences the pattern of TLRs expression and responsiveness in different cells types, tissues, and even between sexes.

1.2.2.3.7.5. TLR mediated induction of Anti-HIV responses

TLR driven responses so far are mostly associated with inflammation and breakdown of tolerance resulting in autoimmune diseases, however, this is not always the case. The effects of inflammatory responses due to PAMP triggered TLR signalling can also serve to activate potent antiviral mechanisms during viral infections such as HIV. For instance, triggering of either TLR7 or 8 signalling has been variously shown to result in activation of anti-HIV mechanisms in different immune subsets.

TLR7 and TLR8 signalling induced production of ROS by neutrophils, that in turn enhanced production of neutrophil extracellular traps (NETs)[261]. The NETs once produced, were able to trap HIV-1 for elimination by antiviral proteases- myeloperoxidase and α-defensin produced by neutrophils[261]. Similarly, activation TLR7 and TLR8 signaling in monocytes and macrophages, was able to limit HIV replication *in vitro* [262]. Campbell, G.R. and others, demonstrated that TLR8 mediated HIV inhibition in macrophages which was dependent on induction of cathelicidin microbial peptide, vitamin D receptor and cytochrome p450 factor CYP27B, leading to conversion of an inactive form of vitamin D into an active form, and resulting in autophagy which impaired HIV replication[263]. The capacity of TLR7 and TLR8 signalling to activate anti-HIV mechanisms has been linked to production of yet to be determined factor(s) capable of activating anti-viral mechanisms in blood and lymphoid tissues.

Two studies showed differences in induction of anti-HIV responses between peripheral blood and lymphoid tissues which depended on the activation of either TLR7 or TLR8. In the first study, TLR7 activation induced an unfavourable environment for HIV replication in lymphoid tissues explants with less consistent results in peripheral blood, yet TLR8 activation consistently induced anti-HIV activity in both lymphoid tissues and peripheral blood which was dependent on activation of DCs, NK cells and $CD8^+$ T cells. More importantly, the anti-HIV effect induced by classical CTLs -NK and $CD8^+$ T cells-was only partial in the absence of DCs[264]. Suggesting that the differences in outcomes arising from activation of either TLR7 or TLR8 signalling with respect to anti-HIV responses in the lymphoid tissues and blood, was related to the differences in cellular composition and TLR expression in either tissue compartment, for instance NK cells are nearly absent in the lymphoid tissues but are present in peripheral blood[264]. A second study supported these earlier findings through demonstration of TLR7/8 activation in lymphoid tissues explants resulting in increased resistance to HIV during the acute phase of disease. The proposed mechanism for the increased tissue resistance was shown to possibly involve B cells, and was not reversed by blockade of cytokines associated with TLR7 or 8 activation[265]. This coupled with the fact that expression of TLR7 and 8 genes along with others, increased during the acute phase of HIV infection, suggests that heightened TLR responsiveness may be an innate mechanism to counter viral invasion and may not be entirely linked to deleterious inflammation. The induction of anti-HIV activity may also be dependent on the activation of either TLR7 or TLR8, and the tissue site of the cells expressing either receptor. The primary focus of this thesis is to present findings from an exploration of TLR responsiveness in different immune cell subsets to TLR7 and TLR8 stimulations of PBMCs from sex workers with reduced susceptibility to HIV.

1.3. Correlates of Protection against HIV

Soon after its discovery, HIV was considered a highly infectious and fatal disease, such that all who had sexual contact with a HIV positive individual became infected and eventually died. In time, a number of studies demonstrated that not all sexual exposures to the HIV resulted in infection[266]. There are a number of cohorts in existence to study immunological and genetic mechanisms responsible for protection of commercial sex-workers (CSWs), MSMs, IDUs, HIV discordant couples, haemophiliacs and infants born to HIV infected mothers, who despite high risks of exposure to the virus, remain uninfected or HESN[267]. The evidence accumulated from studies conducted on HESN individuals to date, suggests that there may be unique genetic and immune characteristics in these individuals that protects them from acquiring HIV infection despite repeated exposures.

1.3.1. Genetic Correlates of Protection against HIV

HIV infection in humans is relatively a recent phenomenon, as such the short nature of the interaction between the virus and the host, has limited the ability of the host genetics to adapt and confer protection against HIV[3]. The host genetic makeup influences multiple steps of the viral cycle, such as viral entry, packaging and maturation. However, only one mutation, a 32 base pair deletion in the CCR5 (CKR5) gene which results in expression of a truncated and non-functional CCR5 receptor, confers a high level of protection against HIV acquisition[268]. The CCR5Δ32 mutation is abundant in people of caucasian decent being present in 5-10% of the population, but it is largely absent in other races[269, 270], The complete protective effect of the CCR5Δ32 deletion is only limited to individuals with homozygous allele with partial protection being present among heterozygous individuals. The protective effect of CCR5Δ32 genotype has been observed in a number of HESN cohorts, but this genotype is absent in African populations[271]. Polymorphisms in the regulatory region

of CCR5 gene exist, but none has uniquely been associated with the HESN status[272]. Reduced expression of CCL3L1 the natural ligand for the CCR5 receptor can limit HIV infection of target cells[273], but it has been associated with increased susceptibility to HIV in South African women [274]. An indication that higher expression of *CCL3L1* gene may not be protective against HIV acquisition, but there is limited evidence to support this to date. Similarly, a single nucleotide polymorphism (SNP) in the *SDF-1* gene chemokine whose product binds to HIV co-receptor CXCR4, was reportedly overrepresented in a group of HESN[275], but failed to be associated with HIV resistance in other cohorts[276].

A large number of HLA genotypes have been associated with delayed HIV disease progression including, HLA-B*57:01, HLA-B*58:01, HLA-B27 and HLA-B51, these HLA genotypes better enable T cells to recognize conserved HIV-Gag epitopes in individuals who express them[277]. In the Pumwani sex worker cohort a number of DQB1 alleles and haplotypes have been associated with HIV resistance[278]. HLA discordance between HIV infected mothers and unborn foetuses has been associated with reduced susceptibility to HIV in Kenya[279]. Other genetic markers such protective IRF-1 genotypes, were identified in the Pumwani sex worker cohort, where the level of IRF-1 expression corresponded to IFN-γ responsiveness, suggesting epigenetic regulation of IRF-1 expression[280,281]. More recently, the r1552896 SNP in the FREM1 gene that encodes an extracellular matrix protein expressed on tissues relevant for HIV transmission, uniquely identified with resistance among sex workers from Nairobi[282].

1.3.2. Immune correlates of natural Protection against HIV

The identification of reproducible immunological correlates of protection against HIV remains a challenge. This due to the HIV pandemic existing as multiple regional epidemics; HIV's immense genetic diversity and distributional variation, coupled with the multiple

routes used by HIV to infect humans with each route possessing distinct biology; Presents a major challenge to the identification of immunological correlates of protection, as such, protection against HIV might arise from multiple factors making it difficult to identify or to replicate effect each through vaccine. There also exists the possibility of the mechanisms/factors of protection varying with physiological conditions such as hormonal changes or aging. All these factors have made it particularly difficult to identify the correlates of protection that could be used to inform vaccine design [283, 284].

1.3.2.1. Innate Immunity to HIV in HESN

Innate defence mechanisms present in mucosal tissues act as the first-line of defence against HIV. Antimicrobial peptides like defensins, antiproteases, secretory molecules-elafin/Trappin-2, elevated levels of RANTES (chemokine for CCR5) and IFN-α, have all been associated with reduced risk of HIV acquisition in HESN populations[285-287]. Certain anti-proteases such as serpins and cystatins, capable of inhibiting HIV binding and replication and which in some instances possess anti-inflammatory properties, are overexpressed in the genital tract of HESN. Presumably, these also create an unfavourable environment for the establishment of HIV infection by lowering the activation state of HIV target cells by enhancing anti-inflammatory processes[288, 289]. Host restriction factors like apolipoprotein B mRNA-editing catalytic polypeptide 3 G- (APOBEC3G)[290], were expressed at higher levels in PBMCs and cervical tissues of HESN correlating with higher reduction in susceptibility to HIV infection *in vitro*[291]. This suggests that endogenous host restriction factors could contribute to the reductions in HIV susceptibility as seen in HESN populations.

Pattern recognition of invading HIV may be necessary for the development of immune responses capable of limiting or preventing establishment infection in the genital tissues[292]. Biasin, M., *et al* demonstrated a heightened peripheral TLR responsiveness in

HIV exposed seronegative (HESN) individuals, associated with higher expression of proinflammatory cytokines in PBMCs of HESN. The broadly heightened TLR responsiveness in PBMCs of HESN, encompassed TLR3, TLR4, TLR7 and TLR8, but was not accompanied with differences in expression of $CD14^{+}$/TLR4 and $CD4^{+}$/TLR8 between the HESN and susceptible controls[293]. In our cohort, we observed a lower expression of TLRs in resting cervical epithelial cells (CECs) and cervical mononuclear cells (CMCs) from the genital tract of HESN CSWs. HESN CSWs at baseline had lower expression of TLR2, TLR4, TLR6, TLR7, TLR8, RIG1 and MDA5 in CMCs and CECs, and lower baseline inflammation based on levels of specific chemokines in their genital tracts, which also correlated with the expression of anti-HIV proteases[294, 295]. In spite of having a more 'quiescent' genital tract with regard to inflammation, CMCs of HESN CSWs mounted more robust TNF-α and IL-10 responses to HIV derived ssRNA through TLR8, but not to LPS (TLR4) or Imiquimod (TLR7) *in vitro* [28, 248, 294]. Cervical mononuclear cells of HESN CSWs were also shown to have lower expression of genes encoding proinflammatory cytokines compared to susceptible controls, possibly reducing the levels inflammation and permissiveness of epithelial tissues to HIV entry[296]. HESN CSWs also had fewer Th17- a T cell subset primarily targeted by HIV for depletion in gut mucosa, in CMCs from the female genital tract [297]. The genital tract of HESN CSWs was bathed with cervico-vaginal fluid that contained lower levels of inflammatory cytokines IL-1α, IL-8, MIG and IP-10, majority of which are crucial in the recruitment of T cells into female genital tissues [295]. Cumulatively, the lower expression of PRRs and proinflammatory cytokines in the FGTs of HESN CSW suggested a lowered potential for recruitment of HIV target cells, possibly leading to lowered susceptibility to HIV.

1.3.2.2. HIV-specific T cell responses in HESN

Exposure to HIV in the genital tract, *in utero* or through blood transfusion, have been shown to result in development of HIV-specific T_H responses, HIV-specific CTLs capable of lysing infected cells and serum anti-HIV IgG antibodies in multiple HESN cohorts[298-300]. The presence of HIV-specific T cell responses to viral envelope proteins and HIV-specific antibody responses, was first describe among partners of HIV infected individuals[301]. HIV-specific $CD4^+$ T cells responses in HESN have been associated with higher IL-2[302-304] and TNF-α/β mRNA expression [305], higher levels of CC chemokines particularly RANTES and MIP-1β[306], but lower IL-10 responses[307]. The higher levels of RANTES (CCL5) potentially points to increased competition between HIV and CCR5 ligand, which may lower susceptibility to HIV in HESN. The lower IL-10 production, but higher presence of $CD4^+$ $CD25^+$ T regs in HESN, resulted in dampening of HIV-specific T cell responses[308], without increasing susceptibility to HIV[309]. HIV-specific T helper responses in HESN recognize rare and conserved ENV epitopes, possessed lower percentages of naive T lymphocytes, but had higher Gag Specific central memory T cells responses. These HIV-specific $CD4^+$ T cell responses have been described among female CSWs[310], MSMs[311], HIV exposed healthcare workers[312] and healthy neonates born to HIV infected mothers[313] .

HIV-specific $CD8^+$ T cell responses have been described in peripheral blood[298, 314] and in the genital tracts of HESN female CSWs[315], among IDUs[316] and in discordant couples[317]. The presence of HIV-specific CTLs in peripheral blood and genital mucosal tissues, was accompanied by higher perforin and granzyme production, higher recognition of peculiar HIV viral epitopes, increased percentages of Gag-specific effector and central memory $CD8^+$ T cells, and increased percentages of $CD8^+$ $CD38^{+/}/CD28^+$ T cells (Reviewed by Miyazawa et al) [318]. Development of CTL responses during viral infections usually requires at least one complete cycle of replication virus *in vivo,* thereby allowing presentation

of viral peptides in association with HLA class I molecules by APCs. The presence of CTLs without infection as the case in HESN individuals, could possibly arise from cross-priming of $CD8^+$ T cells by DCs capable of processing and presenting HIV antigens[319]. In such a hypothetical case, the greater the ability of DCs to process and present antigens in HESN individuals, the greater would be the capacity for development of HIV-specific CTLs. More importantly, these HIV-specific T cell responses are now considered to be a result of exposure to the virus, and may not be entirely responsible for protection of HESN individuals against HIV acquisition. This is based on studies showing HIV-specific T cell responses waning, presumably with reduced exposure the virus as was witnessed by the increased HIV susceptibility of Kenyan HESN CSWs after sex work intermissions or 'sex breaks' [283]. Similarly, HIV-specific $CD4^+$ and CTL responses in uninfected children born to HIV infected mothers, waned 6-9 months after birth[313], and following recovery of healthcare workers from needle prick exposures[312]. Moreover, primary HIV target cells in HESN individuals are not refractory to HIV virus[320]. The need for sustained of HIV specific responses in the genital tract and in the peripheral blood indicates that development or eliciting of long-lasting HIV specific memory responses against HIV through vaccination is necessary, but eliciting sustained protective memory T cell responses at mucosal sites remains a challenge[321].

1.3.2.3. HIV-specific Humoral Responses in HESN

HIV-specific humoral responses quantified in HESN populations, can be categorized into two groups; antibodies specific to host cellular proteins involved in HIV infection process and mucosal antibodies specific to HIV antigens. The former consist of antibodies that recognize HLA or CD4 molecules, the presence of these antibodies is associated with prolonged exposure such as occasioned by horizontal or vertical exposure to infectious blood[322, 323]. The second category of antibody consists of mucosal antibodies that are typically of the IgA

isotype, that compete with viruses for binding to epithelial cells, can opsonize or participate in complement-mediated lysis, which can induce ADCC or inhibit virus transcytosis[324].

HIV-specific IgA responses with neutralizing activity and capable of inhibiting HIV transcytosis in human epithelial cells, have been isolated in the mucosal tissues of HESN individuals from a number of cohorts [292, 307, 325-329]. HIV-specific IgA antibodies in HESN can neutralize multiple clades of HIV including clades A, B, C and D[326, 327]. A caveat for the HIV specific IgA antibodies is that the concentration of such antibodies is often very low, and their presence is not always detectable in all HESN populations. The presence of such antibodies has been linked to repeated exposure and not natural resistance to HIV, evidenced by diminished concentrations of HIV specific IgA responses in high risk women following initiation of other HIV prevention measures[330]. More importantly the presence of IgA antibodies in genital mucosa may prevent horizontal transmission of HIV but may not be important in limiting none mucosal transmission of HIV[331, 332].

1.3.2.4. Immune Quiescence and Reduced Susceptibility to HIV

The lack of HIV infection despite repeated exposure has been linked to the more 'quiescent' phenotype of $CD4^+$ T cells of HESN individuals, based on lower expression of activation marker CD69 and gene transcripts accompanied by reduced susceptibility of these cells to HIV infection *in vitro*[309, 333-335]. Although, the lowered state of $CD4^+$ T cell activation in peripheral blood and genital tissues of HESN might reduce HIV targets, HIV remains capable of infecting quiescent cells leading to establishment of long term latent infections. However, infection of quiescent $CD4^+$ T cells does not favour productive HIV replication as compared to infection of activated $CD4^+$ T cells[336, 337]. The immune quiescence hypothesis supports the premise that, even if exposure to HIV occurs in a HESN individual, the presence of lower numbers of activated HIV targets or a higher number of quiescent

$CD4^+$ T cells, coupled with the presence of anti-HIV responses either innate or adaptive responses such as CTLs and/or humoral responses, sufficiently limits establishment of HIV infection[333, 334].

In our cohort, we described lower production of cytokines in unstimulated PBMCs of HESN when compared to those of HIV-N or susceptible CSWs, but PHA stimulation led to similar in cytokine responses between HESN and HIV-N suggesting a 'normalcy' of immune response potential[334]. Studies conducted by others have equally shown reduced susceptibility of HESN $CD4^+$ T cells *in vitro* in unstimulated cells supporting the lowered capacity of HIV to establish infection in quiescent cells, which is reversed following mitogenic stimulation[338]. T cell immune quiescence has also been described by others; among MSMs showing lower expression of activation markers (HLA-DR, CD38, CD70 and Ki67)[339], in discordant couples with lower CD38 expression on T cells in CSWs from Côte d'Ivoire[340] and Central African Republic[338], in IDUs from Vietnam and haemophilliacs in the USA[341].

Majority of the studies that have contributed to the current understanding on HIV pathogenesis and immune responses to HIV, have been conducted using blood cells and not on genital mucosal tissues where most transmissions occur. There are distinct differences in the immune cell subsets, cytokines and cytokine receptors profiles, gene expression patterns and intracellular signalling, between blood and cervical mononuclear cells[342]. Understanding the mechanisms of protection in the genital tract and contribution of innate immune system in protection against HIV is an urgent priority for development of novel anti-HIV microbicides or an effective HIV vaccine. The work of this thesis explored the TLR responsiveness in the peripheral blood of HESN CSWs and attempted to understand the initial response to HIV derived genetic material and other microbial products in HESN.

CHAPTER 2: Rationale, Hypotheses and Objectives

2.1 Rationale

Heterosexual transmission of HIV accounts for the majority of new infections in Sub-Saharan Africa, where women and young girls disproportionately bear the burden of disease. This is despite the expanded ART coverage targeting to prolong the life of HIV infected individuals, to reduce HIV/AIDS related illnesses, and recently for preventing new transmissions. The need for a HIV vaccine persists in spite of the great success of ART strategies in reducing new HIV transmissions, prolonging life and reducing morbidity in infected individuals. This is especially considering that by the end 2013, an estimated 19 million HIV infected individuals were not aware of their status, majority of whom were considered to be in the acute and most infectious stage of disease, and who may be responsible for the sustenance of viral transmission. Recent advancements in the understanding of the role of innate and mucosal immunology during HIV pathogenesis, offer an opportunity for broadening the tools for preventing HIV transmission. However, the immunological correlates of protection against HIV remain unknown. The identification of these is a priority for HIV vaccine development or for formulation of novel interventions such as a microbicides.

Not all exposures to HIV result in infection, especially among individuals engaged in high risk sexual behaviour such as female CSWs. Accumulating evidence from studies conducted in the Majengo sex worker cohort, points to a lowered or 'quiescent' state of immune activation in the blood and genital tracts of HESN female CSWs, being responsible for conferring protection in these women; through reduction in the numbers of activated HIV target cells, thus lowering the likelihood of HIV acquisition.

Previously, we demonstrated the lower expression of specific PRRs including TLR2, TLR6, TLR7, TLR8, RIG-1 and Mda5, and lower IFN-γ production in resting CMCs, yet higher

TNF-α and IL-10 responses to TLR8 and not TLR4 or TLR7 agonists, among HESN female CSWs. These results suggested that a higher responsiveness to HIV related PAMPS through TLR8 in HESN individuals is a novel correlate of protection in the HESN women that separates them from susceptible controls. It also indicated that the innate immune system of these women is able to mount robust responses to HIV derived PAMPs through TLR8 in spite of a quiescent T cells phenotype. In order to further understand the nature of innate responses in HESN CSWs, the work of this thesis evaluated the expression of TLRs, TLR signalling, immune activation and cellular functions in peripheral blood of two groups of CSWs- HESN and HIV-N.

2. 2. Hypotheses and objectives

The work in this thesis tested the following central hypothesis:

HESN female CSWs have higher TLR8 responsiveness specific for recognizing HIV derived ssRNA in their peripheral blood. This is driven by higher TLR8 expression and signalling resulting in higher effector responses capable of making HIV target cells more refractory to HIV infection *in vitro*, compared to susceptible controls.

2.2.1. Sub-Hypotheses

1. **Stimulation of PBMCs by ssRNA with sequence similarity to HIV genetic material, results in higher induction of TLR8 expression and increased signal transduction in HESN PBMCs compared to those HIV-N CSWs**.

   Objectives to test this Sub-Hypothesis:

   i. To compare the effect of TLR4, TLR7 or TLR8 stimulation on the expression of TLR1-10 in PBMCs from the HESN and HIV-N CSWs.

   ii. To compare the patterns of expression of TLR7 and TLR8 signalling pathway components in PBMCs of HESN and HIV-N following cognate ligand stimulations.

2. **Cytokine responses of HESN PBMCs before and after TLR4, 7 and 8 stimulations, are more coordinately regulated compared to those of HIV-N influencing cellular activation and function.**

Objectives to test this sub-hypothesis:

i. To quantify and compare cytokine responses by PBMCs of HESN or HIV-N, following stimulation with TLR4, 7 or 8 ligands.

ii. To compare the cytokine milieu of PBMCs from HESN and HIV-N, in the presence or absence of TLR4, 7 or 8 stimulations.

iii. To assess and compare cellular activation and cytokine production in T cells, NK cells, monocytes and blood DCs in PBMCs, prior to and after TLR4, TLR7 or TLR8 stimulation, in the two groups of CSWs.

3. **Higher TLR8 responsiveness of HESN PBMCs makes them more refractory to infection with HIV *in vitro* compared to susceptible controls.**

Objectives to test this sub-hypothesis:

i. To compare the effect of pre-treating PBMCs with TLR4, 7 or 8 ligands, on the ability of primary HIV isolates to infect PBMCs from the two groups *in vitro*.

CHAPTER 3: Materials and Methods

3.1 Study Participants

The study participants included in this study were drawn from two cohorts:

3.1.1 Majengo Commercial Sex Workers Cohort

The Majengo CSW cohort was established in 1985 and draws most of its members from Nairobi in Kenya and its surroundings. The Majengo cohort is a well characterized cohort used for longitudinal and cross-sectional cohort studies of sexually transmitted infections, particularly HIV/AIDS. It had approximately 6700 female CSWs enrolled with close to 3400 in active follow-up in 2014. Approximately 5% of the actively enrolled female CSWs are consider to be HESN, given they have remained seronegative despite continuous high risk sex work and follow up for a period of no less than 7 years. The cohort participants make at least two scheduled to visits to the study clinic a year for routine clinical and behavioural review.

Fifty HESN CSWs were included in this study, all of whom were HIV negative, without Sexual Transmitted Infections (STIs) and had been continuously enrolled in the cohort for more than 7 years. The control group consisted of 50 healthy HIV negative controls (HIV-N)-free of STIs and were enrolled in the cohort for less than 3 years. The years of follow-up initially used to describe HESN female CSWs was based on previous epidemiological and mathematical modelling studies conducted in the cohort[343]. The duration of follow-up used for the identification HESN CSWs[343], has since been adjusted to reflect the changing HIV incidence and prevalence in Kenya, due to increased condom usage and uptake of ART[10]. The study participants were sampled during clinic visits and they were selected randomly based on availability.

3.1.2. Winnipeg Donors Cohort.

Blood samples used in optimization experiments for flow cytometry performed prior to field visits - such as antibody titration and fluorescence minus one (FMO) assays, was obtained from a volunteer cohort established by students and staff of the Faculty of Medicine at the University of Manitoba, Winnipeg-Canada.

3.1.3. Ethics Statement

Informed consent was obtained from each study participant prior to sample collection, and the study was performed according to Helsinki declaration and guidelines for conduct of research involving human subjects. Ethical approval for this study was obtained from the University of Manitoba institutional review board and the Kenyatta National Hospital/University of Nairobi ethics review board.

3.2. General Reagents

3.2.1. Toll-Like Receptor Agonists and other Stimulants

TLR ligands- used in the study were; *E. coli* lipopolysaccharide-TLR4 agonist; Imiquimod-TLR7 agonist, and ssRNA40/LyoVec-TLR8 agonist (all from Invivogen, San Diego USA**).**

Positive controls: A combination of phorbol 12-Myristate 13-Acetate (Sigma-Aldrich, St Louis-USA) and Ionomycin (Sigma-Aldrich, St Louis-USA), or phytohaemagglutinin (PHA, Sigma), were variously used as positive controls for TLR stimulations.

3.2.2 Culture media and tissue processing reagents

R10 culture Media- was made from RPMI 1640 complemented with 10% Foetal Bovine Serum (heat inactivated at 56^{O} C for 1 hour) and 2% of 100x Antibiotic-antimycotic solution (all from Gibco-Life Technologies-Thermo Scientific, Ontario, Canada).

Dulbecco's Phosphate Buffer Saline (PBS) (Gibco-Invitrogen Thermo Scientific, USA) complemented with 2% FBS heat inactivated at 56^{O} C for 1 hour.

Freezing Media was made from 90% FBS (heat inactivated at 56^{O}C for 1 hour) and 10% Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, St Louis-USA). This was used to freeze PBMCs for storage or for shipment between Nairobi and Winnipeg.

3.2.3. Flow Cytometry Reagents

Antibodies- The following monoclonal antibodies were used for phenotyping and intracellular cytokine staining of T cells, NK cells, B cells, monocytes and dendritic cells in PBMCs; CCR2 APC, CD3 AmCyan, V500 and PE-Cy5; CD4 PE-Cy7 and PE-Cy5; CD8 Pacific Blue and V450; CD11c Pacific Blue or PE-Cy7, CD14 Pacific Blue or V450, CD16 PE, CD18 PE-Cy5, CD19 APC, CD20 Pacific Blue, CD25 PE-Cy7, CD38 PE, CD40 PE-Cy5 or PE-Cy7, CD56 APC or PE-Cy5, CD62 APC, CD69 Alexa Fluor700, CD80 Alexa Fluor 700 or PE, CD83 APC, CD86 FITC, CD107a APC-Cy7 or APC-H7, CD123 PE or FITC, CD154 APC, CD268 FITC, CD282 Alexa Fluor 647, CXCR3 FITC, IL-2 APC, IL-12p40/70 APC, IFNα2b PE, IFN-γ FITC, Ki67 PE-Cy7, MIP1-α PE and TNF-α Alexa Fluor 700 (all antibodies from BD Biosciences, California-USA) and dye Far red 440 nm (Life technologies, New York-USA) (See **Table 1**).

FACS Wash- was used to wash cells before and after extracellular staining. It was prepared from PBS and 2% FBS (heat inactivated at 56^{O}C for 1hour).

Perm/Wash™ Buffer (BD Biosciences, California-USA) is commercially available as a concentrated 10x stock solution. The working solution of 1X Perm/Wash used for washing flow samples, was prepared by diluting the 10X Perm/Wash with FACS Wash in a ratio of 1:10.

Cell Fixing Buffer used consisted of 10% Paraformaldehyde (PFA) solution (Sigma-Aldrich, St Louis-USA). This was prepared by adding 5mg of PFA and 2ml of 5M NaCl (Sigma-Aldrich, St Louis-USA) to 48ml of double distilled H_20. The mixture was heated for 1min, and pH adjusted to 8.3 using 10M NaOH (Sigma-Aldrich, St Louis-USA).

3.2.4. Luminex Assay Reagents and Kits

Three different custom Millipex MAP **Human Cytokine/Chemokine Magnetic Bead Panel Kits designs** (all EMD Millipore, Darmstadt-Germany) were used in this study. Each kit contained the following:

A. Human cytokine/chemokine standards per kit;

   1st Kit (IL-1β, IL-2, IL-4, IL-10, IL-12p40, IFNα2, IFN-γ, MIP1α and TNF-α)

   2nd Kit (IL-1β, IL-2, IL-6, IL-10, IL-12p40, IFNα2, IFN-γ, IP-10, MIP1α and TNF-α)

   3rd Kit (IL-1β, IL-2, IL-6, IL-10, IL-12p40, IL-12p70, IFNα2, IFN-γ, IP-10 and TNF-α)

B. Human cytokine quality controls 1 and 2 (for the cytokines quantified per kit).

C. Serum matrix

D. One 96 well plate with two plate sealers

E. 10X wash buffer

F. Human cytokine detection antibodies

G. Streptavidin-phycoerythrin Antibodies

H. Bead diluent

3.2.5. RNA reagents and PCR Kits

RNA Isolation Kit- RNeasy Plus Mini Kit™ (Qiagen, Maryland-USA) was used for RNA isolation from PBMCs according to manufacturer's instructions.

Reverse transcription kits- reverse transcription of RNA to quantify TLR4, TLR7 and TLR8 expression in PBMCs was done using Quantitect Reverse Transcription Kit™ (Qiagen, Maryland-USA), while reverse transcription to quantify TLR signalling pathway genes was done using RT² First Strand Kit™ (Qiagen, Maryland-USA).

Real Time PCR Kits- QuantiTect Sybr Green PCR^{TM} and RT^{2} SYBR green qPCR mastermix^{TM} kits (both from Qiagen, Maryland-USA), were used for real time quantification of TLR4, TLR7 and TLR8 mRNA, and TLR signalling pathway gene analysis, respectively.

3.2.6. Western blots reagents, buffers and kits

1. **Lysis buffer**

   Prepared using- NP-40 buffer made from 150nM NaCl (Fisher Scientific, USA), 1.0% Triton-x100 (Sigma-Aldrich, USA) and 50mM Tris (hydroxymethyl) aminemethane (or simply Tris) (EMD, Ontario-Canada), adjusted to pH of 5.0 [doi:10.1101/pdb.rec10423Cold Spring Harb Protoc 2006]

2. **Loading Buffer**

   Prepared using- 4% sodium dodecyl sulphate (SDS) (Biorad, Ontario, Canada), 20% Glycerol (Sigma, USA), 10% Mercaptoethanol (Sigma, USA), 125mM Tris- at a pH 6.8; 0.02% Bromothenol blue (Biorad, Canada) and distilled H_2O

3. **Blocking buffer**

   Prepared from- 5% skimmed milk (Gibco-Invitrogen, Ontario-Canada), 0.05% Tween-20 (Sigma, USA) in Tris Buffer Saline

4. **Tris buffered saline (10x TBS stock)**

   Prepared using- 121grams of Tris base (EMD, Canada), 175 grams of NaCl (Fisher Scientific, USA) both of which were dissolved in 2 litres of distilled H_2O and the pH later adjusted to 4.6 using 10N HCl (Fisher Scientific).

5. **Phosphate buffered saline with Tween (PBS-T)**

   Prepared by adding 15.9 grams of PBS powder (Gibco® Life-Technologies, Ontario-USA) and 1ml of Tween-20 (Fisher Scientific, USA) 1 litre of water.

6. **Running buffer (10x)**

The 10x solution was prepared by dissolving 121.2 grams of Tris base (EMD, Canada) and 576.4 grams of glycine (Biorad, Canada) in 4 litres of distilled H_2O. 1x Running buffer used in SDS-PAGE electrophoresis was prepared by diluting 10x running buffer with 890ml of water supplemented by 10ml of SDS.

7. **Transfer buffer**

   For immunoblotting was made from a mixed solution of 100ml of 10x running buffer, 200ml methanol (Fisher Scientific, USA) and 700ml water.

8. **Mild stripping buffer**

   Made from 15 grams of glycine (Biorad, USA), 1gram of SDS (both from Biorad, Canada) and 10ml Tween-20 (Fisher Scientific, USA) added to 1 litre of distilled water, followed by adjustment of the pH to 2.2 using 10N HCl (Fisher Scientific, USA).

9. **Preparation of each SDS-PAGE gel**

   4% stacking gel- was made by mixing 6.1ml distilled H_2O, 1.33ml of 30% bis-acrylamide (Sigma-Aldrich, St Louis-USA), 2.5 ml of 0.5M Tris HCl (Fisher Scientific, USA), 100µL of 10% SDS (Biorad, USA), 100µL of 10% Ammonium persulphate (APS) (Sigma-Aldrich, USA) and 5µL of tetramethylethylenediamine (TEMED)(Sigma-Aldrich, USA) the latter was added last.

   10% separating gel- Made by mixing 4ml distilled H_2O, 4ml of 30% bis-acrylamide, 2.5ml of 1.5M Tris Hcl (Fisher Scientific, USA), 100µL of 10% SDS (Biorad, Canada), 100µL of 10% APS (Sigma-Aldrich, USA), and 10µL of tetramethylethylenediamine (TEMED) (Life Technologies, USA) added last.

3.2.7 HIV p24 reagents and buffers

1. **Blocking Buffer** Used was PBS-T consisting of PBS (Gibco-Invitrogen, Thermo Scientific, USA) with 0.01% Tween-20 (Fisher Scientific, USA) and 2% goat serum albumin (GSA) (Sigma-Aldrich, USA).

2. **Washing Buffer** PBS-T consisting of PBS (Gibco-Invitrogen, USA) and 0.05% Tween-20 (Fisher Scientific, USA).
3. **Diethanolamine buffer (DEA)-** 0.15% w/v $MgCL_2.6H_20$ (Sigma-Aldrich, St Louis-USA) and 492µL diethanolamine (Sigma-Aldrich, St Louis-USA) in 500 ml of distilled H_20.
4. **Substrate**- Para Nitro Phenyl Phosphate hexahydrate tablets (PNPP) (Sigma-Aldrich, St Louis-USA).

3.3. Routine Procedures

3.3.1. Specimen Preparation

Blood samples were collected from study participants of the Majengo CSW cohort during routine clinic visits. 30ml of blood was drawn using sterile needles with syringes and collected in heparinised BD Vacutainer tubes (BD, Pennsylvania-USA), and later used in isolation of PBMCs (section 3.3.1.2).

3.3.1.1. Plasma Collection

Whole blood collected in heparinised vacutainers tubes (30ml per participant) (BD, Pennslyvania-USA), was used in PBMCs isolation using the Ficoll-Histopaque density centrifugation method. Plasma separated from whole blood through centrifugation, was aspirated using sterile pipettes and transferred into non-sterile 2 ml micro-centrifuge tubes for later use in HIV testing.

3.3.1.2. PBMC Isolation

Blood without plasma was diluted in a ratio 1:1 with sterile PBS containing 2% FCS (both Gibco-Invitrogen, USA), and layered onto Lymphoprep (Ficoll-Histopaque) (Stem Cell Technologies, Vancouver-Canada), prior to separation of cellular components using density centrifugation. PBMCs were collected and washed twice using PBS containing 2% FCS, and

suspended in R10 culture media (see Section 3.2.2). The viability testing of isolated PBMCs was done using Trypan Blue (Sigma-Aldrich, St Louis-USA) exclusion method[294]. Three tubes of blood (each 10ml) collected per study participant yielded on average between 40-60x10^6 PBMCs.

PBMC Freezing: After PBMC isolation, the PBMCs were counted using haemocytometer (Neubauer-Neutec, California-USA) using Trypan blue occlusion method. Following the counting, 10 x10^6 PBMCs were suspended in 2ml of freezing media and stored in sterile 2ml vials and gradually frozen to -80^{O}C in Mr Frosty^{TM} containers (Thermo-Scientific, USA) filled with isopropanol (Fischer Scientific, USA).

3.3.2. TLR stimulation and cell culture

Freshly isolated or thawed PBMCs, were cultured in R10 culture media under 5 conditions; a negative or unstimulated control, 0.01µg/ml *E. coli* LPS-TLR4 (Cat No. tlrl-ebpls, Invivogen), 2.5µg/ml Imiquimod-TLR7 (Cat No. Hkb-htlr7,Invivogen), 1.0µg/ml ssRNA40/LyoVec-TLR8 (Cat. No tlrl-lrna40, Invivogen), 5 µg/ml PHA later replaced with 0.001 µg/ml PMA (Cat No. P8139, Sigma-Aldrich, USA) with 0.01 µg/ml Ionomycin (Cat. No I3909, Sigma-Aldrich, USA). Each condition was cultured in duplicate on sterile 96-well round-bottomed plates (Nunc, Thermo-Scientific, USA), and incubated at 37.0^{O}C and 5.0% CO_2 for 24hrs (or different durations for kinetic experiments). Where appropriate, CD107a (LAMP) (BD Biosciences, USA) a marker used in measuring T and NK cell degranulation, was added at the beginning of cultures, or in combination cytokine release blockers. 1.0 µL of GolgiPlug^{TM} and 1.0 µL GolgiStop^{TM} per 200 µL of PBMC or CMC cell suspensions (both BD Biosciences, California-USA), was added six hrs to the end each incubation. Four time points- 6, 12, 18 or 24 hours- were selected for kinetic experiments. PBMC culture supernatants (without protein transport inhibition) were harvested and frozen at -80^{0}C for storage, transportation or for later use in cytokines and chemokines assays.

Equipment used: Centrifuge-Model Allegra^{TM} 6S (Beckman Coulter, USA), Light microscope SM-LUX (Lietz, Ottawa, Canada) and Bio Safety Cabinet (Baker Co, USA)

3.3.3. HIV Serology

All enrollees in the Majengo CSW cohort are routinely tested for HIV during routine clinic visits. The HIV testing is done in two stages, the first stage in the clinic- using rapid HIV antibody tests (Roche, Switzerland), and the second stage of testing is done in the lab using HIV1/2 p24 antigen ELISA(Vironostika BioMeriéux, France) on plasma samples.

3.3.4. Storage and Shipment of Samples

Sample storage- Long term storage of PBMCs was done in liquid nitrogen at between -170 to -177^{O}C, either in Nairobi or Winnipeg. Cell culture supernatants, cell lysates and inactivated viral culture supernatants were frozen at -80^{O}C for storage or shipping to Winnipeg.

Transportation and Shipping-Frozen PBMCs, culture supernatants and cell lysates, were transported from Nairobi to Winnipeg in liquid nitrogen charged dry-shippers. Transportation between laboratories was done using dry ice contained in styrofoam filled boxes.

3.4. Flow cytometry

3.4.1. Flow Cytometry Panels

The monoclonal antibodies listed in 3.2.3 above were used in phenotyping of different populations of T cells, NK cells, Monocytes and Dendritic cells. The antibodies were combined into flow panels as indicated in Table 1 below;

Table 1. Flow Cytometry Antibody Panels

	T cells	NK cells	Monocytes	DCs
Panel 1 Activation	CD3 AmCyan or V500 CD4 PE-Cy7 CD8 AlexaFluor488 CD69 AlexaFluor700 HLA-DR APC-Cy7 CD38 PE CD25 PE-Cy7 TLR2 APC TLR4 FITC Live/Dead far red	CD3 AmCyan or V500 CD56 APC CD16 PE CD69 AlexaFluor700 HLA-DR APC-Cy7	CD3 PE-Cy5 CD19 PE-Cy5 CD56 PE-Cy5 } Dump* CD14 Pacific Blue CD16 V500 HLA-DR APC-Cy7 CD40 PE-Cy7 CD80 AlexaFluor800 CD86 FITC CD54 APC Live/Dead Far Red	CD3 PE-Cy5 CD19 PE-Cy5 CD56 PE-Cy5 } Dump* CD11c Pacific Blue CD123 PE CD80 AlexaFluor700 CD86 FITC CD83 APC
Panel 2 Cytokine/Chemokine	CD3 AmCyan or V500 CD4 PE-Cy5 CD8 Pacific Blue IFN-γ FITC IL-2 APC TNF-α AlexaFluor700 MIP-1α CD107a APC-H7 Ki67 PE-Cy7 Live /Dear Far red	CD3 AmCyan or V500 CD56 PE-Cy5 CD16 PE IFN-γ FITC IL-2 APC TNF-α AlexaFluor700 CD107a APC-H7 Live/Dear Far red	CD3 PE-Cy5 CD19 PE-Cy5 CD56 PE-Cy5 } Dump* CD14 Pacific Blue CD16 V500 HLA-DR APC-Cy7 IL-10 PE IL-12p40/70 APC TNF-α AlexaFluor700 Live/dead Far Red	CD3 PE-Cy5 CD19 PE-Cy5 CD56 PE-Cy5 } Dump* CD11c Pacific Blue CD123 FITC IFNα2b PE IL-12p40/70 TNF-α AlexaFluor700 Live/Dead Far Red
Panel 3 Other	CD3 AmCyan or V500 CD4 PE-Cy5 CD8 Pacific Blue CD25 PE-Cy7 FoxP3 APC IL-10 PE CD107a APC-Cy7	CD3 AmCyan or V500 CD56 PE-Cy5 CD16 PE CD94 APC NKG2D PE-Cy7 Live/Dead Far Red	CD3 PE-Cy5 CD19 PE-Cy5 CD56 PE-Cy5 } Dump* CD14 Pacific Blue CD16 V500 HLA-DR APC-Cy7 CCR2 APC CXCR3 FITC Live/Dead Far Red	

*Dump- All three antibodies used in gating out $CD3^+$, $CD19^+$ and $CD56^+$ cells during monocyte or DC flow analysis.

3.4.2. Surface staining

Freshly isolated or TLR stimulated PBMCs or CMCs, were transferred from culture plates (or centrifuge tubes) into appropriately labelled polypropylene tubes (BD, USA), and washed once with 2 ml FACS Wash at 1500 rpm for 5 mins. This was followed by the addition of a cocktail of monoclonal antibodies for phenotyping surface markers on T cells, NK cells, monocytes or DCs (see **Table 1**). The cells were stained for 30 minutes at room temperature in the dark, and subsequently washed to remove excess antibodies using 1 ml of FACS wash and centrifugation at 1500 rpm for 5 minutes. The stained cells were then fixed using 150µl of 1% paraformaldehyde (PFA) solution (Sigma-Aldrich, USA) prior to flow analysis.

3.4.3. Intracellular cytokine staining (ICS)

The PBMCs cultured with protein transport inhibitors were used in ICS staining. The PBMCs were first stained for surface markers (using the method in section 3.4.2 above); then permeabilized using 150µL of cytoperm/cytofix mixture (BD Biosciences, USA) for 10-15mins; and washed once using 2ml of 1x FACS wash and centrifugation at 1500 rpm for 5 mins. ICS staining followed with the addition of specific cocktails of antibodies for intracellular markers (see **Table 1**), accompanied with a 1 hour incubation in the dark. Excess antibody was removed after staining using a single wash with 1x FACS wash (BD Biosciences, USA) prior to flow analysis. Flow data acquisition was done using FACS DIVA version 6.0 (BD, USA), and flow data analysed using FlowJo version 7.6.5^{TM} (Treestar, USA).

3.5. RNA Isolation and RT-PCR.

3.5.1. RNA Isolation: RNA isolation from PBMCs was done using RNeasy Plus Mini Kit (Cat No. 74316 Qiagen SABiosciences, USA) according to manufacturer's instructions. In brief, cultured PBMCs were transferred from culture plates into labelled microfuge tubes and centrifuged at 300g for 5 minutes (Eppendorf Centrifuge 5410R), the spin supernatant was carefully aspirated and discarded. The precipitated cell pellet was lysed using RLT Plus Buffer (provided in the kit), and the lysate homogenized by pipetting up and down severally. The homogenized lysate was then transferred into genomic DNA Eliminator columns (provided in kit), and spun at 10,000 rpm for 30s. Three hundred and fifty micro-litres of 70% Ethanol (Commercial Alcohols, Ontario-Canada) was then added to the flow-through collected from the gDNA eliminator columns, and the mixture transferred to a RNA Spin column, and centrifuged at 10,000rpm for 15 seconds. This was followed by two sequential spin washes, the first using 700µL of RW1, and a second using 500µL of RPE buffers (both buffers were provided with the kit). The RNA was then eluted from the RNA Spin Column

using 50µL of RNase free water (also provided in kit), and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo scientific, USA).

3.5.2. Reverse transcription: Generation of cDNA was done by reverse transcribing RNA purified from PBMCs using two kits, either the QuantiTect Reverse transcription kit (Cat No.205313) or the RT^2 First Strand kit (Cat No.303404) (both from Qiagen SABiosciences, USA). Briefly, 100ng of purified RNA (isolated as described in section 3.5.1) was treated with gDNA Wipeout buffer for 5 minutes at $42^{O}C$. Following gDNA elimination, the RNA was reverse transcribed using a master mix consisting of Quantiscript Reverse Transcriptase^{TM}, Quantiscript RT buffer^{TM} and RT primer mix^{TM} (all contained in kit) for 30 minutes at $42^{0}C$ and reaction stopped by heating to $92^{O}C$ for 5 minutes.

Complementary DNA (cDNA) for use in custom TLR profiler Arrays (Cat No.PAHS-018A) was synthesized using RT^2 First Strand Kit, by addition of gDNA eliminator mixture to 100ng of purified RNA followed by a 5 minutes incubation at $42^{O}C$. The RNA without gDNA was reverse transcribed at $42^{O}C$ for 30 minutes using a reverse-transcription mix consisting of- 5X Buffer BC3, Control P2, reverse transcriptase mix and RNAse water, and the reaction was terminated by heating to $95^{O}C$ for 5mins. All RT experiments were conducted in a Veriti® 96-Well Fast Thermocycler (Applied Biosystems-Life Technologies, USA).

3.5.3. RT-PCR: Real time quantification of TLRs or TLR signalling pathway genes was done using either the QuantiTect Sybr Green PCR^{TM} or the RT^2 SYBR Green qPCR^{TM} kit (Qiagen-SABiosciences), respectively. In brief, a mixture consisting of 102µL of cDNA, 1248µL RNase free water and 1350µL of Sybr Green was constituted in a reservoir (provided with the kit). 25µL of the PCR mix was then transferred into each well of a PCR or TLR array plate containing primers for 84 target genes, 5 housekeeping genes and 6 controls. The

real time cycling conditions used were; heating to 95^{O}C for 10 minutes to activate Hot Start DNA Taq polymerase, followed by 45 cycles of heating to 95^{O}C for 15 seconds and cooling to 60^{O}C for 1 minute.

Quantification of TLRs and TLR signalling pathway genes was done using ΔΔCT method for absolute quantification of gene expression, and data presented as N-fold change in expression of the ratio of Ct values of target genes and housekeeping genes. The TLR PCR array data analysis was done using an online tool provided by Qiagen SABiosciences (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php), and pathway analysis done using DAVID (http://david.abcc.ncifcrf.gov/)

3.6. Luminex Assay

Three multiplex human cytokine/chemokine kits (all **EMD-Millipore, USA)** were used to quantify IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IFN-γ, IFN-α2, IP-10, MIP-1α and TNF-α responses from TLR treated or untreated PBMC culture supernatants. The assays were performed according to the manufacturer's instructions; briefly, 200µL of wash buffer was added into each well of a 96 well assay plate, and incubated for 10 minutes on a shaker (VWR microplate shaker model 980130) at 20-25^{O}C, then decanted. Standards, controls and samples were then added to appropriate wells and diluted using assay buffer to a 1:1 ratio, then premixed 11-plex cytokine/chemokine beads added into each well and plate incubated overnight at 4^{O}C on a shaker. Following the overnight incubation, the plate was washed twice using 100µL per well of washing buffer (provided in kit) on a BioTek ELx405 Magnetic plate washer, followed by the addition of 25µL of detection antibodies (1 hour incubation), and lastly by the addition of a similar amount of Streptavidin-Phycoerythin labelled antibodies into each well, followed by a 30 minutes incubation at room temperature with agitation. The plate was washed twice prior to the addition of 150µL of sheath fluid into each well, and the

plate was then analyzed on a Luminex 200 machine (**Bio-Rad, USA**). Data acquisition was done using Bioplex data Pro Software (**Bio-Rad, USA**).

3.7. Western Blot Assay

Sample preparation and lysis: Approximately 2 x 10^6 PBMCs/ml harvested from TLR4, TLR7 or TLR8 stimulated or unstimulated cultures, were washed twice using PBS containing 2% FBS, and cells lysed using lysis buffer for 30 mins at room temperature (prepared as described in section 3.2.6). The cell lysates were then stored at -70^{O}C for storage or shipping, or later use in Western blot assays.

Protein quantification: was done using BCA Protein Assay Kit (Biorad), as recommended by the manufacturer.

SDS PAGE electrophoresis: A mixture containing 100µg of protein, diluted with a calculated amount distilled H_20 and 3µL of coomassie dye (loading dye) to give a final volume of 18µL was heated to 100^{O}c for 15 minutes, then cooled. 15µL of the protein mixture or 8 µL Precision Protein Standards (Bio-Rad), was loaded into each well of 15 well precast Norvex® NuGene® 4-12% Bis-Tris Midi Gel (Precast SDS PAGE Gel) (Life Technologies). Alternatively, 15µL of the sample mixture or 8 µL Precision Protein Standards (Bio-Rad) was loaded into appropriate wells of a cast 4%-10% SDS-PAGE gel (prepared as described in section 3.2.6). The precast gels were loaded onto an Invitrogen NuPage® Novex® gel system (**Invitrogen-Life Technologies**), while the cast gels were loaded onto a Mini-Elpho gel electrophoresis system (**Biorad**). 1 litre of 1x running buffer was then poured into each system, and the precast gels were separated at 120 volts for 120 minutes, while cast gels in the Biorad electrophoresis system were first separated for 20 minutes at 80 volts and later for 60 minutes at 120 volts. The gels containing the separated proteins were equilibrated for 10 minutes in transfer buffer before immunoblotting.

Immunoblotting: Transfer of proteins onto 0.2μM Immunoblot™ polyvinylidiene fluoride papers (Biorad, USA), was done by soaking the immunoblot paper in methanol for 10 minutes followed by transfer using the semi-dry immunoblot sandwich setup as follows; wet filter paper, nitrocellulose membrane, gel with separated proteins and finally filter paper. A voltage of 25 volts was applied across the immunoblot sandwich to transfer separated proteins from gel onto the PVDF membrane.

Primary Antibody Incubation: The PVDF membrane containing the transfered proteins was blocked to prevent non-specific binding using 5% no-fat skimmed milk in TBS with 0.05% Tween20 for 120 minutes at room temperature. The blocked blots were then washed using PBS with 0.05% Tween (PBST) for 10 minutes, then incubated overnight at 4^{O}C degrees with primary antibodies specific for TLR4 (Cat. No. SC-10741), TLR7 (SC-30004), TLR8 (25467), MyD88 (SC-11356), IkBα (SC-371), NFκB2 (SC-298) or MEKK1 (SC-252) (all antibodies from Santa Cruz).

Secondary Antibody Incubation: After the overnight incubation, each blot was washed 6 times for approximately 30 minutes using PBS-T to remove unbound antibodies. The membranes were then soaked in a 1% skimmed milk in PBS-T solution with peroxidase conjugated goat anti-rabbit antibody (1:10,000) and incubated for 2 hours with shaking at room temperature. The membranes were then washed 6 times for approximaterly 5 minutes each time using PBS-T.

Detection: Detection of reactive bands was done using Immunobilon Western™ Chemiluminescent HRP substrate (EMD Millipore). In brief, equal parts of Solution A (Luminol) and Solution B (Peroxidase) provided in the kit, were added to a microfuge tube and mixed prior to addition onto washed membranes, this was followed with 5 minutes incubation at room temperature in the dark. Excess detection reagent was drained by lifting membrane using forceps and tapping on absorbent paper. Densitometric analysis on a

Fluorchem™ 8800 imaging system (Biorad) was done shortly thereafter, and resultant immunoblots used for quantification of protein expression.

3.8. HIV Assays

3.8.1. HIV Viral Stock Propagation

Freshly isolated PBMCs (40-60 $x10^6$ cells) in R10 media with IL-2 (20u/ml) was adjusted to $2x10^6$ cells/ml and transferred into a Corning® T75 culture flask (Sigma-Aldrich), then stimulated with 5μg/ml PHA for 72 hrs at 37^OC and 5% CO_2. After 72 hrs, the stimulated PBMCs were washed twice with R10 media at 1400rpm for 10 minutes. The stimulated cells were split in half; one half of the PHA stimulated PBMCs was used for virus production while the other half was kept to act as feeder cells. The first half of the PHA-IL-2 activated cells to be used in infection was transferred to BSL3 laboratory, where the remaining steps of the virus production assay were conducted.

In the BSL3 lab, 3-4 vials of primary HIV-1 isolated from ML1956 was added to the PHA /IL-2 activated PBMCs, swirled and incubated for 4 hrs at 37^OC and 5% CO_2. Post incubation, the volume of R10 + IL-2 (20u/ml) PBMCs and Virus was adjusted to 10mls and incubated overnight at 37^OC and 5% CO_2. On the following day, the flask's contents were transferred to a 50 ml Falcon tube, and centrifuged at 1600 rpm for 5 minutes, and the supernatant aspirated carefully and discarded into 50% bleach (left to inactivate for 48 hours). The infection culture was replenished with 10ml of R10 media with IL-2 (20u/ml) and cultured in a Corning® T75 flasks for 72hrs at 37^OC and 5% CO_2. After 72 hrs of incubation, 1ml aliquots of the culture supernatant were collected and tested for HIV p24 using ELISA. The cultures were then supplemented with 20 $x10^6$ cell/ml PHA/IL-2 stimulated PBMCs, followed by addition of fresh 10-15mls of R10 media with IL-2 (20u/ml). The culture media was monitored daily for colour changes, when an orangish to yellowish colour change was

observed, 5ml of R10 media with IL-2 (20u/ml) was added to the culture flask. After 6 days of incubation, a second 1ml aliquot of the culture supernatant was harvested and later used for HIV p24 assays (see section 3.8.2). If the aliquot obtained on Day 6 was positive for p24, 25-50 cryovials were labelled on Day 7 and 1ml culture supernatant added into each cryovials. This virus harvest procedure was repeated on day 14 of culture.

3.8.2. HIV-TLR Infection assay

The first set of experiments comparing the effect of different TLR treatments on HIV infectivity used two million PBMCs per well, pre-treated with either; 0.01µg/ml *E.coli* LPS - TLR4, 2.5µg/ml Imiquimod-TLR7, 1.0mM ssRNA40/ LyoVec-TLR8, or 0.001 µg/ml PMA with 0.01 µg/ml Ionomycin overnight at 37^{O}c and 5% CO_2. Alternatively, the PBMCs were treated with 0.125mM, 0.25mM, 0.5mM or 1.0mM of ssRNA40/LyoVec (TLR8) overnight to estimate the effect TLR8 ligand dosage on HIV infectivity.

The HIV used for the TLR-HIV infection assays was a primary isolate obtained from a cohort member -ML1956 (HIV^{ML1956}). The amount of virus used was calculated using the 50% tissue culture infectious dose ($TCID_{50}$) of $4.63x10^5$/ml, number of cells (2×10^6 PBMCs/ml) infected and the volume of cell culture suspension (200µL in each well). The multiplicity of infection (MOI) used ranged between ≈0.001 to 0.03. Culture supernatants (100µL) were harvested upon completion of the infection assays 3 and 7 days post infection, and virus particles in culture supernatants inactivated using 100µl of 10x Triton-X-100® (Sigma-Aldrich, USA) per well for 30 minutes prior to freezing at -70^{O}c for storage, transport and later use in p24 ELISA assays.

3.8.3. HIV-1 p24 ELISA

Plate Coating- HIV-1 p24 antibody (NIH HIV reagent program), was diluted in coating buffer to a concentration of 2µg/ml, and 100µL of the diluted antibody added to each well of a 96-well plate and incubated overnight at 4^{O}C.

Blocking-The p24 antibody (NIH HIV reagent program) coated plates, were blocked by addition of 200µL of blocking buffer into each well, followed by a 1 hour incubation at 37^{O}C. Following this incubation, the blocking buffer was decanted, and plate washed twice using tap water and plate tapped to dry on an absorbent paper.

Sample and Standard Addition- 100µL HIV inactivated culture supernatant was added into appropriate wells of the p24 antibody (NIH HIV reagent program) coated and blocked plate. Serial dilution was used to prepare the standards in duplicates with the top standard (20ng/ml) being prepared by adding 10µL p24 standard to 190µL of blocking buffer. The serial dilution was done by transferring 50µL of the top standard to the next standard well containing 150µL of blocking buffer and so on to the 7^{th} dilution. The plate was then incubated at 37^{O}C for 24 hrs in a wet box.

Rabbit anti-p24 antibody-Following the overnight incubation, the plate was washed six times with wash buffer (PBS containing 2% Goat Serum and 0.01% Tween 20) using Biotek ELx405 Plate Washer (Biotek, Vermont-USA). Then, 100µL of rabbit anti-p24 antibody (Abcam, USA) was then added each well and incubated at 37^{O}C for 90 minutes in a wet box. The ratio of rabbit anti-p24 to blocking buffer used was-1:66666 (or 150µL p24-Ab in 10mls blocking buffer).

Biotinlyated Anti-Rabbit-Following the incubation with rabbit anti-p24 antibody, the plate was washed six times with washing buffer, followed by addition of 100 µL biotinlyated anti-rabbit Ab (Abcam, USA) (1:10000) to each well of plate that was then incubated for 90mins at 37^{O}C.

Enzyme addition Streptavidin Alkaline Phosphatase (SAP)- The biotinlyated anti-rabbit Ab (Abcam, USA) was washed off the plate six times using 100µL of washing buffer per well, for each wash, prior to addition of 100µL of SAP solution (a ratio of 1:32,000) to each well on the plate.

Substrate Addition: The plate was washed six times using 100µL of washing buffer per well, per wash. Then 100µL of substrate solution made by dissolving 1 tablet of substrate, in every 5mls of DEA buffer (prepared as described earlier), followed by a 30 minutes of incubation at 37^{O}C in a wet box.

Detection: Each plate was read on a Biotek ELx405® Plate Reader at 407 nm, after 30, 60 or 90 minutes of substrate addition.

3.9. Data and statistical analysis

Statistical analysis and graphing was done using GraphPad Prism 6.01®. Unpaired group comparisons were done using the Mann-Whitney test, while paired comparisons (e.g. between unstimulated and stimulated conditions), was done using Wilcoxon sign rank paired *t* test. Comparisons of grouped linear data, was done using the Holm-Sidak method, while correlational analysis was done by Spearman's correlation. All *P* values lower than 0.05 were considered to be statistically significant and reported.

CHAPTER 4: Dichotomony of TLR7 and TLR8 Responses in HESN PBMCs to Cognate Ligands is associated with HIV Infectivity *in vitro.*

4.1. Rationale

TLR ligand recognition by cognate receptor triggers intracellular signalling events terminating in transcription of cytokines, chemokines and antimicrobial peptides. TLR hyper-responsiveness in the peripheral blood of high-risk discordant HESN couples encompassing TLR3, TLR4, TLR7 and TLR8 has been previously been described[293]. In our cohort, we previously described lower TLR2, TLR4, TLR6, TLR7, TLR8, RIG1 and Mda5 expression in CMCs and CECs of HESN, but higher TLR8 responses in CMCs in the same group[294].

Here, we assessed the effects of TLR4, TLR7 and TLR8 stimulations in peripheral blood mononuclear cells of HESN and HIV-N CSWs by comparing; TLR4, 7 and 8 expression, TLR7 and TLR8 signalling, cytokine responses, cytokine milieu and the infectivity of a circulating strain of HIV virus prior to and after stimulation with LPS-TLR4, Imiquimod-TLR7 or ssRNA40/LyoVec-TLR8.

4.2. Hypotheses

4.2.1. Hypothesis 1

Recognition of ssRNA analogous to HIV's genetic material by TLR8 on HESN PBMCs, results in higher cognate receptor expression, increased TLR8 signal transduction and cytokine responses compared to HIV-N controls.

4.2.2. Hypothesis 2

The cytokine milieu of HESN PBMCs with or without TLR4, TLR7 or TLR8 treatments is more tightly regulated compared to those of HIV-N, influencing cellular activation, function and susceptibility of immune cells subsets to HIV *in vitro.*

4.2.3. Specific Aims to test these hypotheses

1. To compare the change in TLR4, 7 or 8 expression after cognate ligand stimulation between PBMCs from HESN and HIV-N
2. To compare TLR7 and TLR8 signal transduction and activation of related pathways following cognate ligand stimulations in PBMCs of HESN and HIV-N
3. To quantify soluble cytokine responses by PBMCs of HESN and HIV-N to TLR4, 7 or 8 stimulations.
4. To compare the cytokine milieu of HESN and HIV-N PBMCs before and after TLR stimulations.

4.3. Study Outline

Sampling- The experiments in this section used PBMCs collected at three different time points, coinciding with research trips to Nairobi in; 2010 (May-Sept), 2011 (Sept -Jan 2012) and 2014 (May-Sept). The PBMC stimulations and HIV infection assays were conducted in Nairobi-Kenya, whereas the RT-PCR, Western blots and Luminex assays were conducted in Winnipeg using samples shipped to from Nairobi (as described in sections 3.5-3.7). TLR responses were examined in PBMC samples from HESN (n= 25) and HIV-N (n=25) selected using the criteria described in Section 3.1.1.

Freshly isolated PBMCs were stimulated overnight using TLR4, 7 or 8 ligands, and culture supernatants collected following the overnight stimulations, and frozen at -70^{O}c for storage or shipment to Winnipeg where cytokine quantification was done using multiplex bead arrays. Freeze-thawed and TLR stimulated PBMCs were used in the PCR quantification of TLR signalling pathway components. Similarly, quantification of TLR signalling pathway proteins by Western blot was done using lysates of PBMCs first pre-treated with TLR4, 7 or 8 agonists, then lysed and lysates frozen for assays conducted in Winnipeg.

4.4. Results

4.4.1. Higher TLR8 but lower TLR4 and TLR7 cytokine responses by HESN PBMCs

The experiment conducted in this section sought to validate the findings of our previous study that showed higher TNF-α and IL-10 responses in CMC's from HESN women to TLR8 but not TLR4 or TLR7 agonists compared to controls[294]. Based on these observations, we hypothesized that PBMCs from HESN women would also be more responsive to TLR8 but not to TLR4 or TLR7 ligands. For the first time this study was conducted using PBMCs, a compartment that not been fully investigated and that allowed for a more in-depth analysis of different functions in immune subsets. To test this hypothesis, we quantified and compared cytokine and chemokine responses in PBMCs of HESN and HIV-N before and after stimulation with LPS (TLR4), Imiquimod (TLR7), ssRNA40/LyoVec (TLR8) or PMA+ Ionomycin/PHA. We then quantified IFN-γ, IFNα2, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70 MIP1α, IP-10 and TNF-α from culture supernatants of TLR stimulated and unstimulated PBMCs using multiplex bead arrays.

Overnight stimulation of PBMCs with TLR4, TLR7 or TLR8 resulted in production of most of the analytes examined with the exception IL-2 and IL-4 (data not shown) (**Figures 5**). TLR4 stimulation lead to robust IL-1β, IL-6, IL-10, IP-10, IL-12p40/70 and TNF-α responses by PBMCs of both groups, yet induced only low levels of IFNα2 and IFNγ (**Figure 5**). Interestingly, IL-10 responses to TLR4 stimulation was significantly lower in PBMC from HESN compared to controls (Standard error of means -SEMs: **HESN 10479 ± 2175 pg/ml** vs. vs HIV-N 16741 ± 2024 pg/ml, p=0.0474). TLR7 stimulation resulted inrobust production of IL-1β, IL-6, IL-10, IP-10 and IFNα2 but failed to elicit IL-12p40/70, TNFα and IFN-γ in both groups.

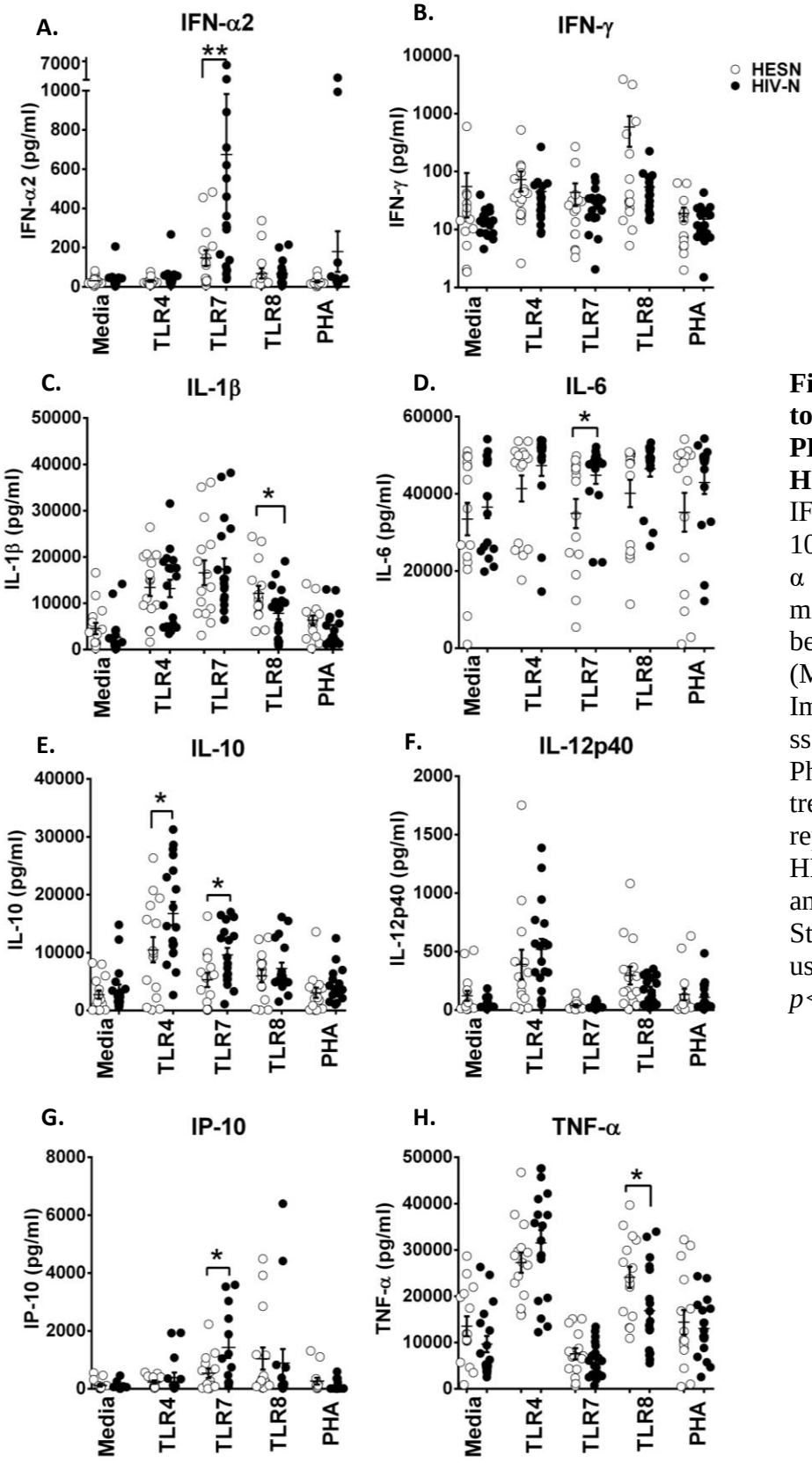


Figure 5. Cytokine responses to TLR stimulations in PBMCs from HESN and HIV susceptible controls. IFNα2, IFN-γ, IL-1β, IL-6, IL-10, IL-12p40, IP-10 and TNF-α expression in pg/ml as measured using a multiplex bead array from unstimulated (Media), *E. coli* LPS-TLR4, Imiquimod-TLR7, ssRNA40/LyoVec-TLR8, and Phytohaemagglutinin (PHA) treated PBMCss. Each dot represents a single individual, HESN (white circles, n=15) and HIV-N (black dots, n=18). Statistical comparison done using Mann-Whitney, all $p<0.05$ are indicated (*).

Once again, TLR7 stimulation resulted in lower IFNα2 (**146.9 ± 39.56 pg/ml** vs. 675.0 ± 308.2 pg/ml, *p*=0.0074), IL-6 (**34896 ± 3800 pg/ml** vs. 44784 ± 2197 pg/ml, *p*= 0.0126), IL-10 (**10479 ± 2175 pg/ml** vs 16741 ± 2024 pg/ml, *p* =0.0299) and IP-10 responses (**539.0 ± 160.3** pg/ml vs. 1431 ± 364.7 pg/ml, *p*= 0.0319) in HESNs compared to controls.

TLR8 stimulation induced similar responses to TLR7 with the exception of IFNα2, in both groups. On the contrary TLR8 stimulation elicited higher IL-1β (**12130 ± 1606 pg/ml** vs. 7840 ± 1306 pg/ml, *p*= 0.0444), TNF-α (**24120 ± 2295 pg/ml** vs 16874 ± 2150 pg/ml, *p*= 0.0330) responses in HESN and a trend for higher IFNγ (**586.0 ± 318.8 pg/ml** vs 54.00 ± 11.78 pg/ml, *p*= 0.07) production, compared to controls (**Figure 5**).

Next we conducted a fold change analysis of the cytokine responses described above. This is considering, cytokine in output (pg/ml) represents the responses to TLR ligation, but does not represent responsiveness to stimulation above media. To determine the fold changes in cytokine production we divided the cytokine responses arising from TLR stimulation with those observed in unstimulated cultures (media) (**Figures 6**). This analysis aimed to determine the magnitude of each cytokine response following TLR stimulation of PBMCs in the two groups of CSWs.

While TLR4 stimulation induced appreciable production of most of the cytokine responses tested above background, the fold change in IFNα2 and IFNγ responses were modest. The fold change analysis revealed that TLR4 stimulation of HESN PBMCs resulted in a lower magnitude of IL-1β (**HESN 4.524 ± 1.01** vs. HIV-N 13.29 ± 2.42, *p*=0.0050), IL-10 (**4.860 ± 0.88** vs. 8.611 ± 1.72, *p*=0.083) and TNF-α (**2.38 ± 0.33** vs. 4.4 ± 0.52, *p*=0.0047) responses. Similarly, TLR7 stimulations resulted in robust IL-1β, IL-6, IL-10, IFNα2 and IP-10, but modest fold production of IFNγ, IL-12p40 and TNFα.

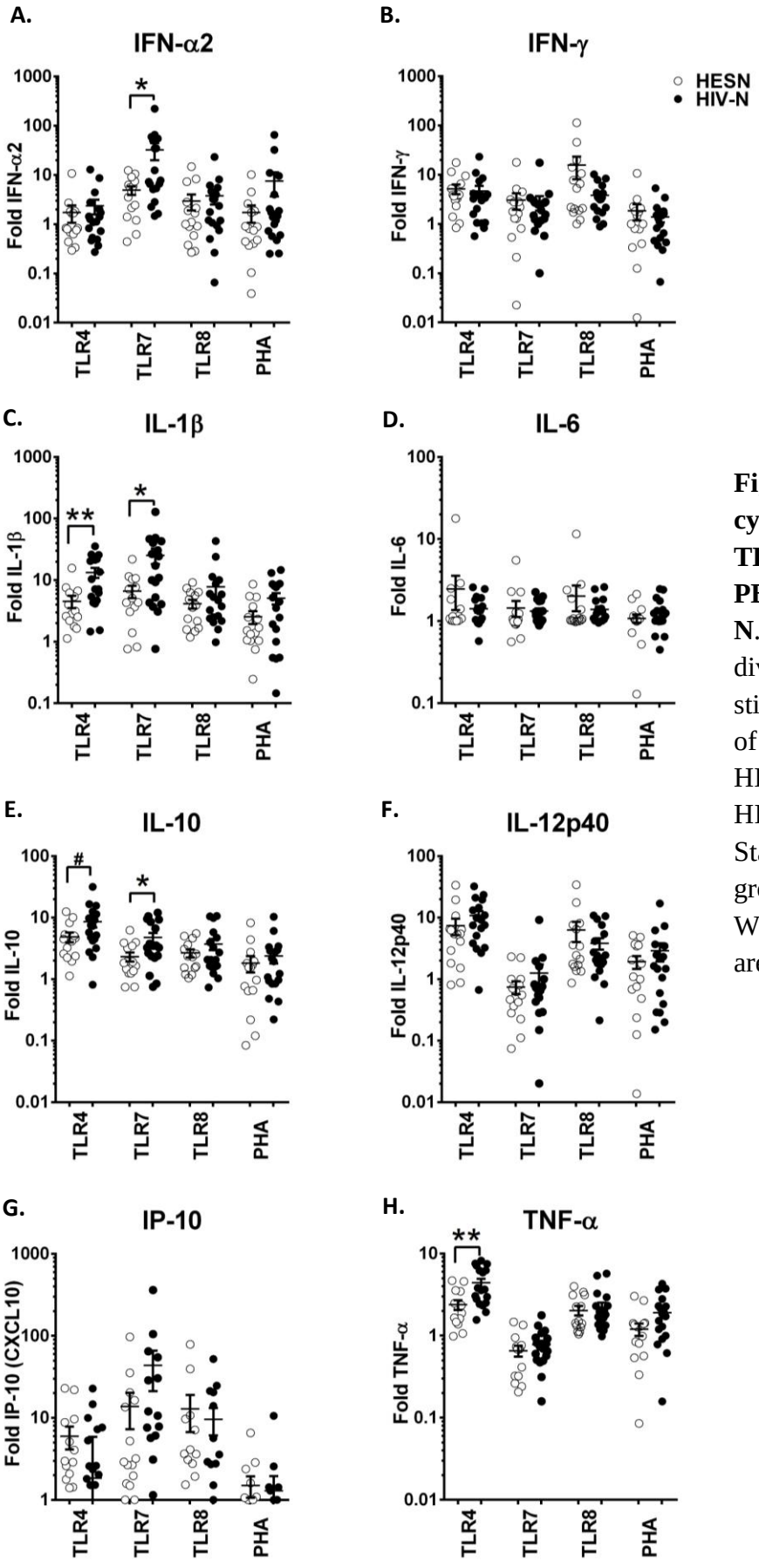


Figure 6. Fold production of cytokines after TLR4, TLR7, TLR8 or PHA stimulations of PBMCs from HESN and HIV-N. Fold responses calculated by division of cytokine responses in stimulated conditions, with those of unstimulated conditions. HESN (white circles, n=15) and HIV-N (black dots, n=18) CSWs. Statistical comparison between groups was done using Mann-Whitney test. All *p*-values <0.05 are represented by asterisks (*).

When the fold responses to TLR7 where assessed, we found a lower magnitude of IFNα2 (**4.884 ± 0.96** vs. 32.11 ± 12.3, *p*= 0.0181), IL-1β (**6.607 ± 1.5** vs. 25.31 ± 7.16, *p*= 0.0246) and IL-10 (**2.33 ± 0.40** vs. 4.79 ± 0.85, *p*= 0.0328) responses in HESN compared HIV-N. The cytokine responses due to TLR8 stimulation were frequently above background levels; however, there were no differences in magnitude of cytokine response to TLR8 between HESN and HIV-N (**Figures 6**).

The cytokine quantification experiments in **Figure 5 & 6** demonstrated that HESN CSWs have higher TLR8 responsiveness but lower responsiveness to TLR4 and TLR7 ligands. These results were consistent with what we previously described in CMCs from the genital tract[294]. Thus, confirming that differences in TLR responsiveness between PBMCs of HESN and HIV-N bore similarity to those seen in the genital tract.

4.4.2. Different kinetics of cytokine production by PBMCs of HESN over the course of TLR4, TLR7 or TLR8 stimulations

To understand the timing and length of each cytokine response during TLR4, TLR7 or TLR8 stimulations, we did a kinetic assessment of cytokine production (IFNα2, IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12p40/70, IP-10 and TNFα) over the course TLR stimulation. These experiments were performed two years following the initial evaluation of overnight cytokine responses using nearly identical samples.

Here, freshly isolated PBMCs from HESN (n=11) and HIV-N (n=10), were stimulated with either 0.01μg/ml *E. coli* LPS-TLR4, 2.5μg/ml Imiquimod-TLR7, 0.1μg/ml ssRNA-TLR8 or with a combination of 0.001μg/ml PMA and 0.01μg/ml Ionomycin, for 6, 12 or 24 hrs and cell culture supernatants harvested for cytokine and chemokine quantification using multiplex bead arrays (**Figure 7**).

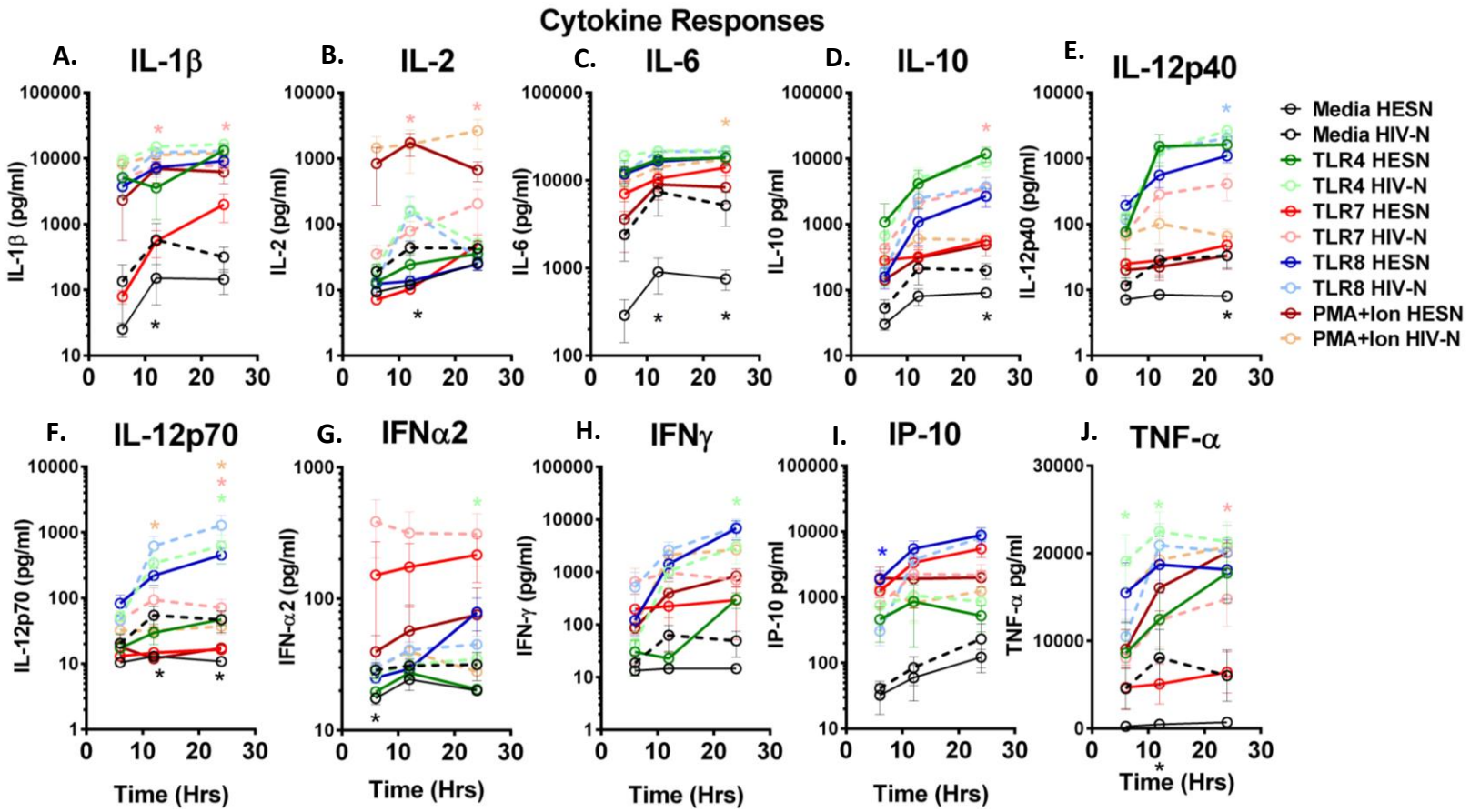


Figure 7. Kinetics of Cytokine Production by PBMCs of HESN and HIV-N CSWs in response to TLR4 (green lines), TLR7 (red lines), TLR8 (blue lines) or PMA with Ionomycin (brown lines) stimulations, while unstimulated (Media) PBMCs are represented by black lines. The cytokine responses were quantified at 6, 12 and 24 hours of stimulation using multiplex bead arrays. The cytokines and chemokines quantified were IL-1β, IL-2, IL-6, IL-10, IL-12p40, IL-12p70 IFNα2, IFN-γ, IP-10 (CXCL10) and TNF-α. Comparisons of grouped linear data was done using Holm-Sidak method with α=5%, all $P<0.05$ are indicated with an asterisk (*).HESN (n=11) represented by darker lines and asterisks while HIV-N (n=10) represented by lighter lines and asterisks.

The results showed that unstimulated PBMCs (media) from HESN produced significantly lower amounts of IL-1β at 12hrs (means: **2567.76 pg/ml** vs 18147.9 pg/ml, *p*= 0.001), IL-2 at 12hrs (means: **12.04 pg/ml** vs 44.0 pg/ml, *p*=0.003), IL-6 between 18-24hrs (means: **901.6 pg/ml** vs 7416 pg/ml, *p*= 0.01; and **751.2 pg/ml** vs 5187.3 pg/ml, *p*=0.02), IL-10 at 24hrs (means: **90.9 pg/ml** vs 199.0 pg/ml, *p*=0.03), IL-12p40 at 24hrs (means: **8.04 pg/ml** vs 33.3, *p*=0.015), IL-12p70 between 12-24hrs (means: **12.9 pg/ml** vs 54.7 pg/ml *p*=0.02 ; and **10.8 pg/ml** vs 46.8, *p*= 0.04), IFNα2 at 6hrs (means: **17.5 pg/ml** vs 28.75 pg/ml, *p*=0.01) and TNF-α at 12hrs (mean **477.9 pg/ml** vs 8080.6 pg/ml, *p*=0.01) during culture(**Figure 7A-G**).

Similarly, over the course of TLR4 stimulation, HESN PBMCs produced significantly lower levels of IL-12p70 at 24 hours (means: **47.1 pg/ml** vs 617.4 pg/ml, *p*=0.05), IFNα2 at 6 hours (mean **20.5 pg/ml** vs 34.9 pg/ml, *p*=0.006), IFN-γ at 24 hours (means: **299.9 pg/ml** vs 3035.8 pg/ml, *p*=0.009) and TNF-α between 18-24 hours (means: **8607.2 pg/ml** vs 19078.1 pg/ml, *p*=0.03; and **12646.4 pg/ml** vs 22487.1 pg/ml, *p*=0.02) (**Figures 7F, G, H and J).** The production of IL-1β between 12-24 hours (SEMs **555.2 pg/ml** vs 7651.9 pg/ml, *p*=0.01 ; and **1982.2 pg/ml** vs7785.1 pg/ml, *p*= 0.03), IL-10 at 24 hours (mean **561.7 pg/ml** vs 3485.8 pg/ml, *p*=0.007), IL-12p70 at 24 hours (SEMs **16.4 pg/ml** vs 70.9 pg/ml, *p*=0.03) and TNF-α at 24 hours (mean **6421.3 pg/ml** vs 14799.3 pg/ml, *p*=0.04) were also significantly lower in HESN PBMCs over the course of TLR7 stimulation(**Figures 7A-J)**. On the contrary, the production of IP-10 was higher after 24 hours of TLR8 stimulation in HESN (mean **1855.6 pg/ml** vs 304.45 pg/ml, *p*=0.03) compared to HIV-N.

4.4.3. Higher amplitude of TLR8 cytokine responses by HESN PBMCs over the course stimulation

To compare the magnitude of the cytokine responses between the two groups of CSW over the course of TLR stimulation of PBMCs, we calculated the fold production of cytokines by

dividing the quantities of cytokines produced in response to the different stimulated conditions to those produced by unstimulated cells (**Figures 8A-J**).

TLR4 stimulation increased the fold production of IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IFN-γ and TNF-α but failed to increase the production of IL-2, IFNα2 and IP-10 in PBMCs from both CSW groups. TLR7 stimulation on the other hand, led to significant fold increases in IL-1β, IL-10, IFNα, IFNγ, IP-10 and TNF-α, but barely increased the fold production of IL-2, IL-6, IL-12p40 or IL-12p70, while TLR8 stimulation resulted in significant fold increase of all the analytes except IL-2 and to a lesser extent IFNα2.

A comparison of fold cytokine responses between the groups, revealed that over the course of TLR4 stimulation, PBMCs of HESN had higher fold production of IL-6 between 6-24 hours (fold means: 6 hrs- **89.15** vs 9299.0, p=0.015; 12hrs- **901.0** vs 15628 pg/ml, p= 0.0002; and 24hrs **751.2** vs 9871.5; p=0.009) and IL-10 at 24 hours (fold means: **183.7** vs 32.47, p=0.013) , while the fold IL-12p70 at 24 hours (fold mean: **4.6** vs 10.8, p=0.03) was lower in compared to controls.

TLR7 stimulation led to higher production of IL-6 at 6 hours (fold mean: **190.6** vs 21.6, p=0.014) and IP-10 between 12-24 hours (fold mean: **49.01** vs 4.72, p=0.038; and **61.2** vs 5.5, p=0.018), but lower induction of IFNα2 after 6 hours (mean fold: **2.0** vs 12.7, p=0.05) of TLR7 stimulation compared to HIV-N (**Figures 8C, G and I**).

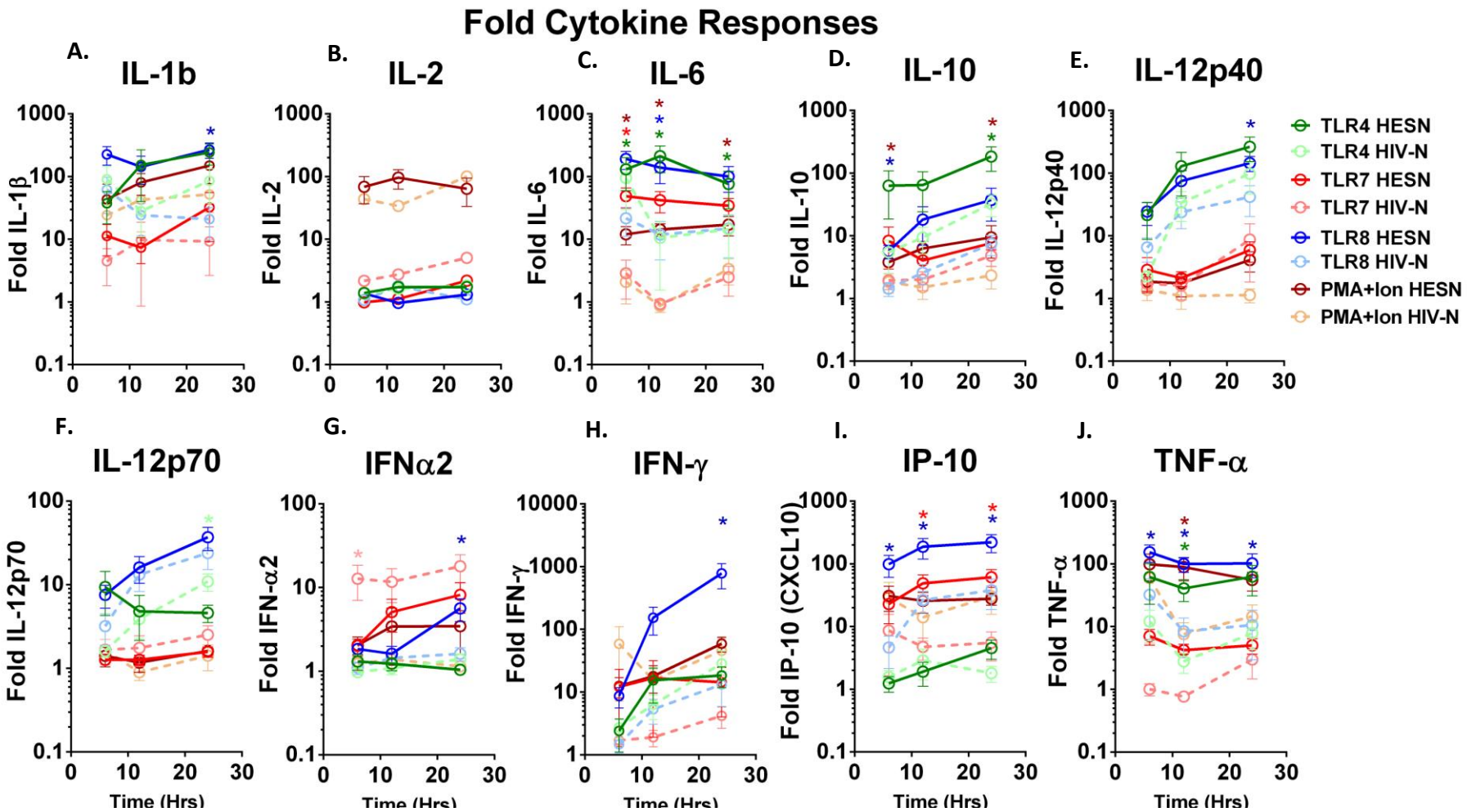


Figure 8: Kinetics of fold cytokine production with time in PBMCs of HESN and HIV-N, in response to TLR4 (green lines), TLR7 (red lines), TLR8 (blue lines) and PMA with Ionomycin (brown lines) stimulations. A-J) Line graphs represent fold production of IL-1β, IL-2, IL-6, IL-10, IL-12p40, IL-12p70 IFNα2, IFN-γ, IP-10, and TNF-α. HESN (n =11) represented by darker lines and asterisks (*) and NN (n =10) represented by dashed lighter lines and asterisks. Comparison of lines done using Holm-Sidak method with α=5%, *P<0.05.

TLR8 stimulation led to higher fold changes in production of majority of the cytokines in HESN PBMCs compared to controls, these included the production of IL-1β at 24hrs (fold mean: **268.7** vs 20.8, p=0.004), IL-6 between 6-24hrs (fold mean: **211.5** vs 10.5, p=0.031), IL-10 at 6 hours (fold mean: **5.65** vs 1.43, p=0.008), IFN-γ at 24hrs (fold mean: **778.6** vs 13.24, p=0.001), IP-10 between 6-24 hours (fold means: **98.6** vs 4.63, p=0.017; **187.06** vs 26.4, p=0.017 and **221.1** vs 37.7, p=0.018) and TNF-α between 6-24hrs (fold means: 153.2 vs 32.1 p=0.027; 99.9 vs 8.4 p=0.001; and 101.9 vs 10.5, p=0.022) (**Figures 8A, C, D, H, I and J**).

To recap, HESN PBMCs were shown to have lower fold IL-12p70 production but higher IL-6 and IL-10 responses over the course of TLR4 stimulation, lower IFNα2 and higher IL-6 and IP-10 responses to TLR7 stimulation, but higher IL-1β, IL-6, IL-10, IFNγ, IP-10 and TNFα responses to TLR8 stimulation, compared to HIV-N. However, over the duration of culture, unstimulated PBMCs of HESN largely produced lower amounts cytokine responses compared to controls corresponding to a more 'quiescent' immune phenotype. More importantly, PBMCs of HESN mounted robust fold changes in cytokine responses especially to TLR8 ligand ssRNA analogous HIV's genetic material.

Collectively, the quantification of cytokine responses following different TLR stimulations (Figures 5-8), often lower TLR4 and TLR7 responses, but higher TLR8 cytokine response between PBMCs of HESN and HIV-N.

4.4.4. Cytokine milieu of HESN PBMCs was more tightly regulated with or without TLR stimulations.

Next we used correlational analyses to compare the balance of cytokine responses before and after TLR stimulations from culture supernatants of HESN and HIV-N PBMCs. The expression levels of the 10 cytokines examined- IFNα2, IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12p40, IP-10, MIP-1α and TNF-α, were correlated with each other using Spearman's

correlations and results presented as heat maps of *p*-values of Spearman's coefficients (**Figures 9A-D** and Table of Spearman's co-efficients contained in Appendix 3**)**.

When we examined the baseline correlations of cytokines, we found that, IFNα2 levels positively correlated with the levels of IFN-γ, IL-12p40, IL-1β, IL-10 and IP-10 in unstimulated cultures from HESN study participants but not in those of HIV-N controls (**Figure 9A)**. Similarly, the levels of IP-10 correlated with all cytokines examined except MIP-1α, in HESN with the opposite being observed in HIV-N (**Figure 9A)**. The levels of IL-10, a key anti-inflammatory cytokine [344, 345], positively correlated to levels of IFN-γ, IL-12p40, IL-1β, IL-2 and IP-10 in unstimulated PBMC cultures from HESN, but not in those of HIV-N where it correlated with TNF-α and MIP1α (**Figure 9A)**. However, the relationships between IFN-γ and IL-1β /IL-12p40; IL-12p40 and IL-1β/TNFα; IL-6, TNFα and MIP-1α, was similar between the two groups (**Appendix 3 Table 1**) Cytokine production in unstimulated cultures of HESN PBMCs was more co-ordinately balanced than those of HIV-N individuals given the high number of positive correlations in cytokines levels.

Upon TLR4 stimulation, the levels of IFNα2 were found to positively correlate with IL-1β, IL-2 and IL-10 in supernatants from HESN cultures, while IL-10 responses by HESN PBMCs corresponded to IFNα2, IL-12p40, IL-1β, IL-2 and IL-6 responses in HESN but not in HIV-N PBMCs. Similarly, HESN PBMCs produced lower IL-1β that correlated with IL-12p40 and IP-10 but not in HIV-N cultures, and lower TNF-α responses following TLR4 stimulation, with corresponding IFN-γ and IL-12p40 responses in both groups (**Figure 9B)**. (ref. **Appendix 3 Table 2**).

When we examined the effects of TLR7 stimulation we found reduced IFNα2 responses in HESN PBMCs which correlated with IL-1β production in HESN, but positively correlated with IP-10 levels in HIV-N (**Figure 9C)**.

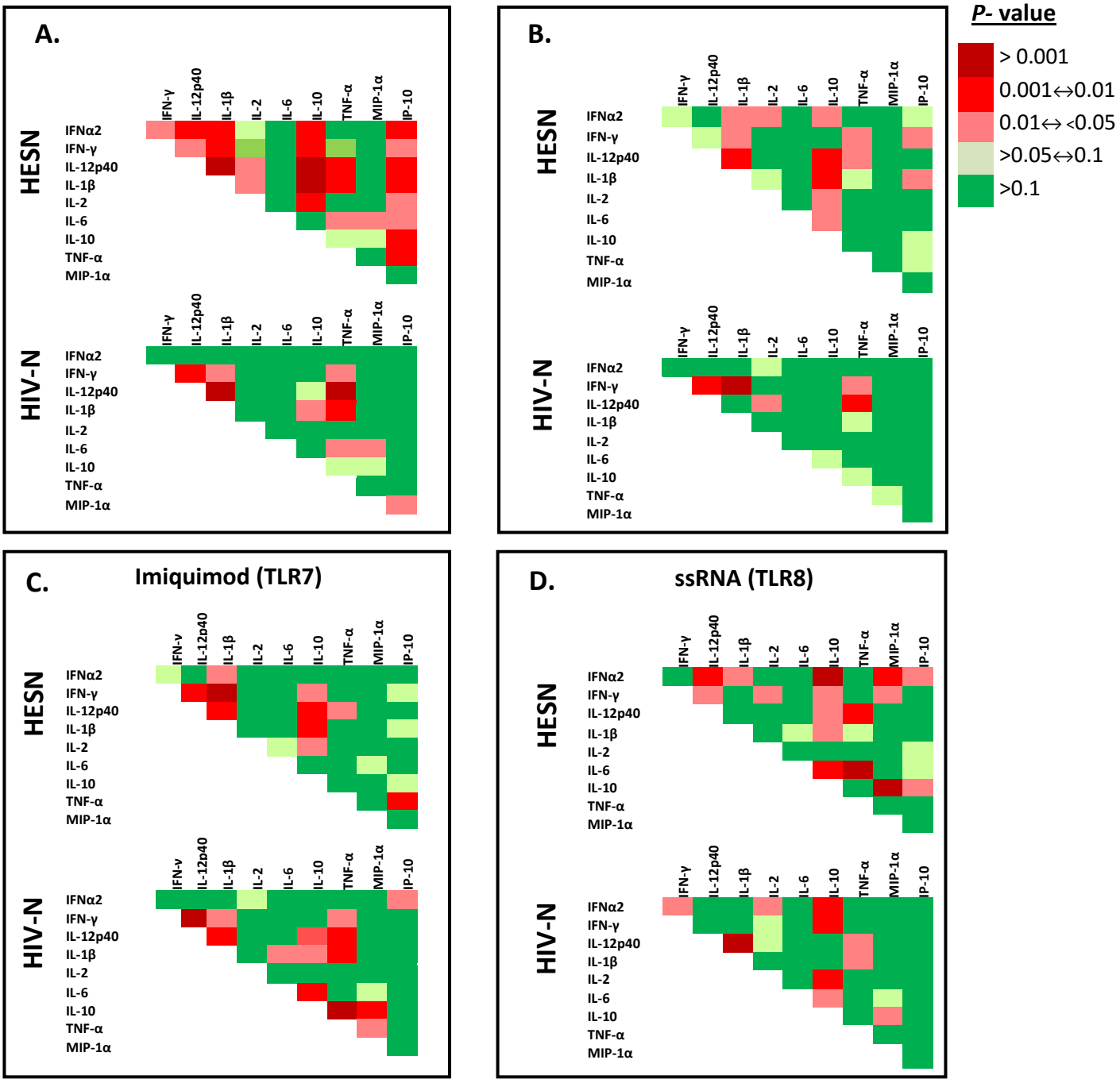


Figure 9. Cytokine milieu of HESN PBMCs with or without TLR4, TLR7 or TLR8 stimulations. The quantities of IFNα2, IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12p40, TNF-α and MIP1α, was determined using multiplex bead arrays from supernatants of untreated PBMCs Media (unstimulated) and those treated with, *E.coli* LPS-TLR4, Imiquimod-TLR7 and ssRNA40/LyoVec-TLR8 from HESN and HIV-N samples. Paired comparisons of cytokines were done using *Spearman's* correlation and the *p*-values obtained from correlations used to construct heat maps (A-D).

On the other hand, TLR7 stimulation led to IL-10 responses correlating with IFN-γ, IL-12p40, IL-1β, and IL-2 responses in HESN, but only IL-12p40 and IL-1β in HIV-N along with IL-6, MIP-1α and TNF-α. In addition, IL-1β levels correlated with IFN-γ, IL-12p40 and IL-10 in both groups after TLR7 stimulations, but only with IL-6 and TNF-α in HIV-N PBMC cultures (**Figure 9C)**. Finally, TLR7 stimulation resulted in lower TNF-α production by HESN PBMCs which correlated with IL-12p40 and MIP-1α, and IFN-γ, IL-1β, IL-10 and MIP-1α in HIV-N (**ref Appendix 3 Table 3**)

TLR8 stimulation of PBMCs in both HESN and HIV-N lead to IL-10 production that correlated IL-12p40 and IL-1β levels, but only IFN-γ and IL-2 levels in HESN, and only IL-6 in HIV-N. TLR8 stimulation produced higher IL-1β in HESN and its levels corresponded to levels of IFNα2 and IL-10 by PBMCs from the two groups, but only IL-12p40, MIP-1α and IP-10 in HESN cultures and TNF-α in HIV-N cultures (**Figures 9C and D**).

Taken together, this correlational analysis revealed that differences observed in quantities and magnitude of cytokine responses impacted the cytokine milieu generated by TLR4, TLR7 or TLR8 stimulations in HESN when compared to HIV-N controls. The cytokine milieu generated by PBMCs of HESN appeared to be more coordinately regulated in unstimulated, TLR4 or TLR8 PBMCs, but less so in TLR7 stimulated PBMCs. This suggest that HESN PBMCs the choice of TLR ligand influences the balance ytokine responses by HESN PBMCs.

4.4.5. Dichotomy of TLR7 and TLR8 expression in PBMCs of HESN following cognate ligand stimulation corresponding to functional responses

To test if levels of TLR expression could explain the differential cytokine production patterns shown in **Figures 5-8**, we quantified and compared the changes in RNA and protein expression of TLR4, TLR7 and TLR8, using RT-PCR and Western blots. Here, PBMCs from

HESN (n=5) and HIV-N (n=5) were stimulated overnight with TLR4-LPS, TLR7-Imiquimod or TLR8-ssRNA (**Figure 10)**. Messenger RNA was isolated from unstimulated, TLR4, 7 or 8 stimulated PBMCs, reverse transcribed and cDNA transcripts quantified for RT-PCR (details described in **section 3.5**).

When we examined the effects of overnight stimulation with LPS-TLR4 on PBMCs from HESN and HIV-N, we found that LPS did not appreciably alter TLR4 expression in either group. However, TLR7 stimulation enhanced TLR mRNA above 1 fold in HIV-N while TLR8 stimulation, tended to increase the expression TLR4 in HESN, but there were no statistical difference between the two groups (**Figure 10A).**

TLR7 stimulation of HESN PBMCs resulted in significantly lower expression of TLR7 in PBMCs of HESN compared to those of HIV-N (N-fold SEM: **0.39 ± 0.13** vs. 1.43 ± 0.60, p=0.0232) and a trend for higher TLR8 expression (N-fold SEM: **29.65 ± 18.32** vs. 0.6005 ± 0.18, p=0.0532) (**Figure 10B).**

On the other hand, TLR8 stimulation of PBMCs resulted in a significantly higher expression of TLR8 mRNA in HESN compared to in HIV-N (N-fold SEM: **5.173 ± 0.76** vs 1.636 ± 0.45, p= 0.0358) with no differences in the TLR4 and TLR7 mRNA expression between the two groups **(Figure. 10C**).

Next we assessed the pattern of TLR7 and TLR8 expression at the protein level using Western blots. Here cell lysates obtained from PBMCs stimulated with TLR4, 7 or 8 for 24hrs, were separated using SDS-PAGE electrophoresis and transferred by immunoblotting for protein detection and quantification (as described in **section 3.7**) at the RNA level matched those seen at the protein level (**Figures 10D and E**).

The results showed that the expression of TLR7 and TLR8 at the protein level was similar in the representative blots of the two gourps.

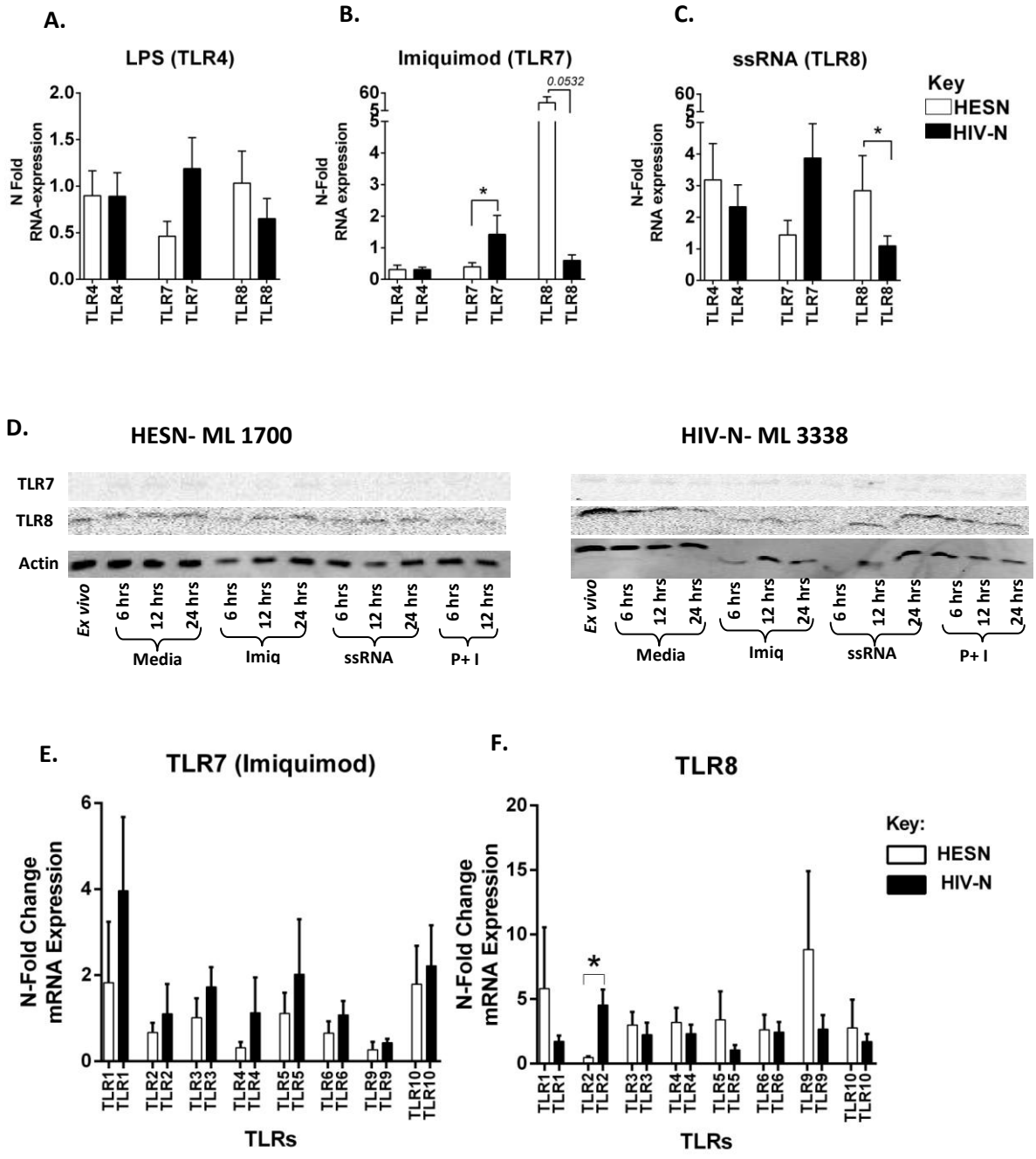


Figure 10. Comparison of Toll-Like Receptor Expression as quantified in PBMCs of HESN (white bar) and HIV-N (black bars) by real time-PCR and Western Blot. (A-C) The expression of TLR4, TLR7 and TLR8 R mRNA in *E. coli* LPS-TLR4, Imiquimod-TLR7 and ssRNA-TLR8 stimulated PBMCs between the two groups. (D) Expression of TLR7 and TLR8 protein over time in lysates from PBMCs of a HESN and HIV-N individual cultured in Media, TLR7 (Imiq-Imiquimod), TLR8 (ssRNA40/LyoVec) and PMA+ Ionomycin (P+I). E-G) Expression of TLR1-10 RNA with Imiquimod-TLR7 and ssRNA40/LyoVec- TLR8 stimulations, the latter resulted in lower expression of TLR2 mRNA in HESN PBMCs compared to in HIV-N. The statistical comparisons between groups were done using Mann-Whitney test and all $p<0.05$ indicated with (*).

Next we examined the effect of TLR7 or TLR8 stimulation, on expression of TLR1-10 mRNA. Here, PBMCs stimulated with TLR7 and TLR8 were lysed, mRNA isolated and reverse transcribed prior to cDNA amplification and quantification using RT-PCR (section 3.5). This examination revealed that TLR8 stimulation resulted in lower TLR2 mRNA expression in PBMCs of HESN (N-fold SEM: **0.47 ± 0.10** vs. 4.54 ± 1.20, p=0.0159), but there were no differences in TLR1-10 mRNA expression following TLR7 stimulation (**Figures 10F and G**).

In summary, the quantification of TLR expression with stimulation, revealed no difference in TLR4 expression between the two groups while TLR7 mRNA expression in PBMCs of HESN was lower when that of TLR8 tended to be higher(**Figure 5-8**), Whereas TLR8 expression was higher in PBMCs of HESN following ssRNA stimulation. The higher TLR8 mRNA expression in HESN PBMCs following TLR8 ligation corresponded to the higher cytokine responses (IL-1β, IFN-γ and TNF-α) following TLR8 stimulations, while the lower TLR7 mRNA expression after Imiquimod stimulation was accompanied with lower cytokine responses (IL-1β, IL-10, IFNα2 and TNF-α). In both cases the patterns of TLR7 or TLR8 expression in PBMCs of both HESN and HIV-N was opposed each other.

4.4.6. Dichotomony in activation of TLR7 and TLR8 signalling pathways in HESN PBMCs with cognate ligation corresponding to functional responses

To investigate if the patterns observed in TLR7 and 8 mRNA expression and cytokine responses matched the activation of TLR7 and TLR8 signalling/related pathways components (**Table 2**), we assessed the expression of different pathway components using TLR PCR arrays. The assessment was focused on TLR7 and TLR8 pathways, because these have been previously linked with the recognition of HIV ssRNA, and would be more relevant in detection of HIV potentially influencing immunity. We quantified the expression of TLR

signalling pathway genes in PBMCs from HESN (n=5) and HIV-N (n=5) following TLR7 and TLR8 stimulations. Messenger RNA was quantified using a commercial TLR PCR array kit for quantifying the expression of 84 genes as indicated in **Table 2** (details are described in **section 3.5**).The results are presented N-fold changes in expression of the pathway genes, arising from a comparison of expression unstimulated PBMCs versus in either TLR7 or TLR8 stimulated PBMCs.

Table 2: TLR PCR Array Genes

Associated Signalling Pathway	Gene
Toll-Like Receptor	TLR 1, 2, 3, 4, 5, 6, 7, 8, 9, 10
TLR signalling	IRAK1, IRAK2, IRAK4 TAB1, MYD88, TBK1, TICAM2 (TRAM), TIRAP, TRAF6, TICAM1 (TRIF)
MAPK and JNKinases	MAP2K3,MAP2K4, MAP3K1, MAP3K7, MAP4K4, MAPK8, MAPK8IP3, ELK1, FOS, ECSIT, PELI,
Cytokines, Chemokines and growth factors	IL-10,IL-1α,IL-1β,IL-2, IL-8, IL-12A, IFNG, IFNA1, IFNB1, CCL2, CSF2 (GM-CSF), CSF3 (G-CSF), TNF, TNFRSF1A, LTA (Lymphotoxin), CXCL10 (IP-10)
Transcription factors and related genes	IRF-1, IRF3, JUN (AP-1), NFKB1, NFKBIA, CHUK (IKKα), NFKBIL1, NFRKB, IKBKB NR2C2, PPARA, REL, RELA
Adaptive Immune response	CD14, CD86, CD80, CD180, LY86 (MD1), LY96 (MD2) BTK
Negative regulators of TLR signalling	SARM1, SIGIRR, TOLLIP, UBE2N, UBE2V1, HMGB1
Other PRRs and apoptotic factors	EIF2AK2 (PKR), CASP8, CARD3 (RIPK2), FADD, PTGS2 (COX2), CLEC4E, HRAS, HSPA1, HSPA1A

TLR7 stimulation led to lower expression of TLR7 signalling pathways components in HESN PBMCs compared HIV-N, including; MyD88 (N-fold SEM: **HESN 0.41 ± 0.19** vs. HIV-N 3.47 ± 2.27, p=0.031), TAB1(N-fold SEM: **0.52 ± 0.18** vs. 1.54 ± 0.31, p=0.03) and JUN /AP-1(N-fold SEM: **0.26 ± 0.07** vs. 5.22 ± 0.95, p=0.035) (**Figure 11A)**; Lower expression of NF-κB complex genes-NFKB2 (N-fold SEM: **0.73 ± 0.30** vs. 3.22 ± 0.68, p=0.02), NFKBIA (N-fold SEM: **0.68 ± 0.26** vs. 2.37 ± 0.99, p=0.02) and IKBKB (N-fold median: **0.34 ± 0.09** vs. 2.43 ± 0.86, p=0.008); Lower expression of MAPK genes-MAP3K1(N-fold SEM: 0.56± 0.15 vs. 35.41 ± 33.84, p=0.015), MAP2K3 (**0.47 ± 0.14** vs. 5.36e+010 ± 5.36e+010, p=0.01), MAPK8IP3 (N-fold SEM: **0.50 ± 0.20** vs. 4.16 ± 1.85, p=0.02), PELI

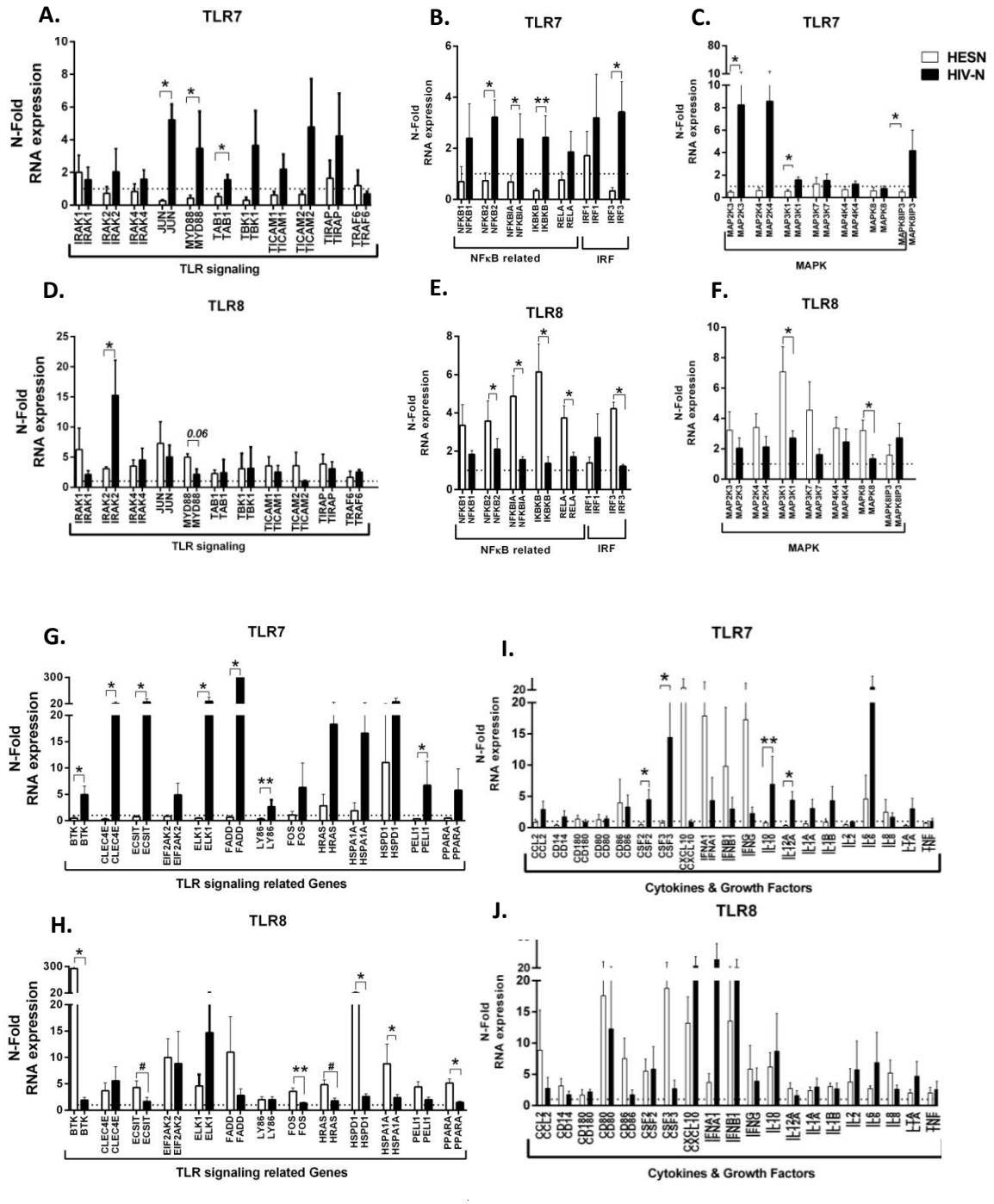


Figure 11: Gene analysis of TLR signalling and related pathways following TLR7 and TLR8 stimulation of PBMCs. A commercial TLR PCR array measuring a panel of 84 genes was used to compare the changes in expression of TLR signalling and related pathway genes with TLR7 and TLR8 stimulation of PBMCs (HESN-white bars and HIV-N-black bars). Only differentially expressed genes in the 84 quantified are presented here. The changes in RNA expression are presented as N-fold changes, calculated by ΔΔCT method of absolute quantification of genes. HESN (n=5) and HIV-N (n=5).

(N-fold SEM: **0.32 ± 0.05** vs. 6.70 ± 4.57, *p*=0.01), ECSIT (N-fold SEM: **0.68 ± 0.30** vs. 38.12 ± 34.64, *p*=0.03) and ELK1 (N-fold SEM: **0.48 ± 0.13** vs. 47.23 ± 45.02, *p*=0.01) (**Figure 11B);** and lower Interferon regulatory factor -3 (IRF3) (N-fold SEM: **0.33 ± 0.14** vs. 3.41 ± 1.21, *p*=0.01) compared to those of HIV-N (**Figure 11C**).

Additionally, HESN PBMCs had lower expression of BCR signalling gene BTK (N-fold SEM: **0.46 ± 0.32** vs. 4.96 ± 1.60, *p*=0.03), C-type lectin family 4 member E (CLEC4E) (N-fold SEM: **0.24 ± 0.22** vs. 8.016e+010 ± 8.016e+010, *p*=0.01), Ly86 (N-fold SEM: **0.30 ± 0.05** vs. 2.62 ± 1.31, *p*=0.008) and apoptotic factor FADD (N-fold SEM: **0.64 ± 0.28** vs. 1.744e+011 ± 1.744e+011, *p*=0.01) (**Figure 11G)**.

TLR8 stimulated PBMCs of HESN had lower expression of IRAK2 (N-fold SEM: **3.09 ± 0.31**, *p*=0.02) but higher MyD88 expression (N-fold SEM: **4.98 ± 0.58** vs. 2.16 ± 0.84, *p*=0.035); higher expression of MAPK pathway components MAPK8/JNK1 (N-fold SEM: **3.22 ± 0.68** vs 1.34 ± 0.28, *p*=0.03), MAP3K1(N-fold SEM: **7.07 ± 1.64** vs 2.70 ± 0.49, *p*=0.02), c-FOS (N-fold SEM: **3.55 ± 0.64** vs 1.38 ± 0.15, *p*=0.008) and PPARA (N-fold SEM: **5.15 ± 0.77**, *p*=0.01); equally higher expression of heat shock proteins HSPD1(N-fold SEM: **20.29 ± 12.29** vs 2.59 ± 0.53, *p*=0.01) and HSPA1A (N-fold SEM: **8.79 ± 3.74** vs 2.36 ± 0.60, *p*=0.01). HESN PBMCs also had higher expression of IKK and NF-κB related proteins following TLR8 stimulation, these included; IKBKB (N-fold SEM: **6.15 ± 1.46** vs 1.37 ± 0.35, *p*=0.02), IκBα/NFKBIA (N-fold SEM: 4**.87 ± 1.08** vs 1.57 ± 0.14, *p*=0.035), NFkB2 (N-fold SEM: **4.42 ± 0.82** vs 2.11 ± 0.55, *p*=0.03) and RELA (N-fold SEM: **3.74 ± 0.63** vs 1.71 ± 0.23, *p*=0.01)[346]. Other TLR8 signalling related genes were highly expressed in TLR8 stimulated PBMCs of HESN and these included IRF3 (N-fold SEM: **4.22 ± 0.34** vs 1.20 ± 0.13, *p*=0.02) and B cell receptor adaptor BTK (N-fold SEM: **280.3 ± 202.8** vs. 1.88 ± 0.52, *p*=0.03) (**Figures 11D, E, F and H).**

There were no differences in the expression of negative regulators of TLR signalling such as; Toll interacting protein-TOLLIP[347]; decoy receptor-Single Immunoglobulin Interleukin 1 related protein-SIGIRR[348, 349]; regulator Sterile alpha and TIR motif containing 1-SARM1[350]; ubiquitin-Conjugating Enzyme E2N UBE2N [351] and HMGB1[352] between the two groups either following TLR7 or TLR8 stimulations.

The TLR signalling analysis demonstrated the limited activation of TLR7 signalling pathway in HESN following Imiquimod stimulation, but higher activation of TLR8 pathways after ssRNA stimulation, corresponding to TLR expression and cytokine production (**Figures 5-8 and 10**).

4.4.7. Opposing Effects of TLR7 and TLR8 pre-treatment of HESN PBMCs on HIV's infectivity *in vitro*

The final set of experiment in this chapter tested the effect of pre-treatment of PBMCs with different TLR agonists, on HIV's infectivity *in vitro*. In the first set of experiments, freshly isolated PBMCs ($2x10^6$ cells/ml) were stimulated overnight with either TLR4, TLR7, TLR8 or PMA with Ionomcyin, washed twice and infected for 4 hours with replication competent primary HIV isolates (cultured from an HIV infected cohort member ML1956- HIV^{ML1956}). The infected cells were then washed twice and incubated for 3-7days (for details see **section 3.8**). The concentration of virus was determined using a multiplicity of infection calculated from $TCID_{50}$/ml of $4.63x10^5$ with the amount used encompassing the physiological range of primary HIV infection *in vivo*[248]. The culture supernatants were harvested on days 3 and 7 and used for quantification of HIV capsid protein p24 (used as the surrogate marker for infection) by ELISA (**Figure 12**).

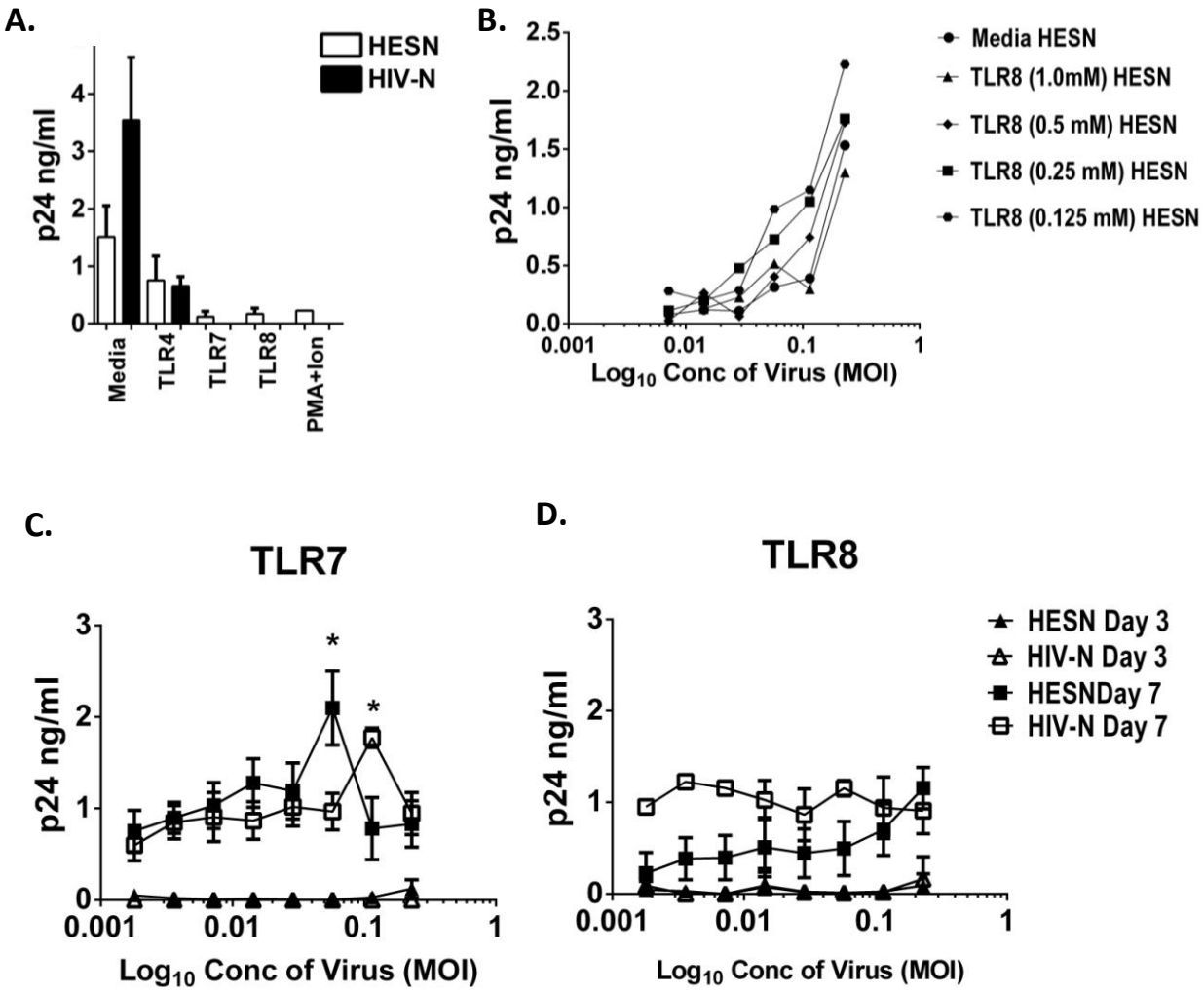


Figure 12. HIV infection assays. A) Freshly isolated PBMCs (2.0 x 10^6cells/ml) from HESN (white) and HIV-N (bar) were stimulated with 0.01µg/ml *E. coli* LPS (TLR4), 2.5µg/ml Imiquimod (TLR7), 1.0mM ssRNA40/LyoVec (TLR8) or 0.001µg/ml PMA+ 0.011µg/ml Ionomycin for 24hours, before being infected for 4 hours with primary HIV isolated from a cohort member ML1956 (HIV^{1956}), washed, and cultured for 3-7 days at 37^OC and 5% CO_2. B) PBMCs (2.0 x 10^6cells/ml) from HESN were treated overnight with different concentrations of ssRNA40/LyoVec, ranging from 0.125mM to 1.0mM. C&D) PBMCs (2.0 x 10^6cells/ml) of HESN and HIV-N were treated with 2.5µg/ml Imiquimod (TLR7) (C) and 1.0mM ssRNA40/LyoVec (TLR8) overnight and infected with primary HIV isolated virus MOI ranging from (0.002-0.23) following the protocol described in methods. (HESN n =5, HIV n =5), Mann-Whitney test used to compare group data and the Holm-Sidak method used for statistical comparison of grouped linear data. All *p*-values <0.05 indicated with (*).

The quantification of p24 production at day 7 showed that pre-treatment of PBMCs with either TLR4, 7, 8 or PMA+ Ionomycin prior to infection with HIV, reduced the capacity of HIV^{ml1956} virus to infect target cells in PBMC *in vitro*, while untreated PBMCs were more robustly infected, but perhaps to lesser extent in HESN compared to controls, although this was not significant (MOI $p = 0.09$) (**Figure 12A**).

The effect of TLR mediated inhibition of HIV infection in PBMCs was more profound in TLR7 and TLR8 stimulated PBMCs, but relatively modest in TLR4 stimulated cells (**Figure 12A**).

Previous studies have shown that pre-treating PBMCs with either TLR7 or TLR8 produced opposing results with regard to HIV's infectivity of target cells *in vitro*[248, 264, 353]. Where pre-treatment of purified $CD4^+$ T cells using Imiquimod TLR7 agonist- enhanced HIV infection and replication of $CD4^+$ T cells through induction of anergy caused by influx of Ca^{2+} ions, while pre-treatment of PBMCs with TLR8 was shown to variously inhibit HIV infection and replication *in vitro*[248, 264, 353].

The next set of experiments tested the effect of pre-treating PBMCs with different concentrations of TLR8 ligand ssRNA40/LyoVec (0.125, 0.25, 0.5 and 1.0) mM, on the infectivity of HIV^{ML1956} strain. The caveat for the results presented in **Figure 12B** above**,** is that the data is from a single of replicate, and therefore the data should be viewed more as proof of concept on the effect of TLR8 ligand pretreatment on reduction of HIV's infectivity. We found that higher concentrations of ssRNA40/LyoVec inhibited infection of PBMCs with primary HIV isolates better than lower ones in a dose dependent manner, consistent with previous studies[263, 264, 354]. The reducted ability of HIV^{ML1956} strain to infect PBMCs was more discernible at higher concentrations ssRNA40/LyoVec or TLR8 (1.0mM/ml) ligand compared to 0.5,mM/ml, 0.25mM/ml or O.125mM/ml.

Next we tested the effect of varying the concentration of virus (MOI range ≈ 0.002-0.03) on the capacity of HIV^{ML1965} strain to infect PBMCs pre-treated with the experimental concentrations of TLR7 agonist Imiquimod (2.5μg/ml) or TLR8 ligand ssRNA40/LyoVec (1.0mM). The infection rate of TLR7 pretreated PBMCs (based on p24 levels) was barely detectable after 3 days of culture, but rose steadily after 7 days of infection with increasing virus titres, peaking at MOIs ≈ 0.057 and 0.115 in HESN and HIV-N, respectively, before dropping rapidly to almost background levels (**Figure 12C**).

Pre-treatment with 2.5μg/ml Imiquimod (TLR7) and followed by infection with various virus titres using serial dilution, resulted in higher level of infection in HESN PBMCs at an MOI of 0.057 (mean p24 levels: **HESN 2.098pg/ml** vs.HIV-N 0.967 pg/ml, p=0.043), which then dropped significantly as the titre of virus was increased to an MOI of 0.115 (mean p24 level: **0.78pg/ml** vs 1.77pg/ml, p=0.031) compared to controls (**Figure 12C**). Although there was a higher p24 production in HESN study partcipants at some points, HIV appeared to replicate equally well in HESN as it did HIV-N controls.

When we pretreated PBMCs with 1.0mM/ml ssRNA40/LyoVec-TLR8 ligand, and infected with various MOI's we also found limited p24 production at 3 days post infecetion (**Figure 12D)**. However, the level of p24 production by PBMCs pre-treated with 1.0mM/ml ssRNA40/LyoVec-TLR8 ligand after 7 days of culture with HIV^{ML1956} was readily detectable at 7 days. We found that at the lower MOI's that HESN cells were much less likely to produce p24 after HIV infection of TLR8 treated cells, but this difference between populations was lost when infection was done using higher titres of virus. Furthermore, TLR8 pretreated PBMCs of HESN appeared to have lower rates of infections when infected with lower titres of virus, although this was not significant possibly due to the low number of replicates (HESN n=5, HIV-N n= 4) (**Figure 12D**).

Collectively, the findings of the HIV infection assays testing the effect of pre-treating PBMCs with either TLR7 or TLR8 ligand, demonstrated that Imiquimod (TLR7) enhanced HIV infection of HESN PBMCs at certain concentrations of virus, whereas TLR8 ligand ssRNA40/LyoVec pre-treatment of PBMCs was more efficient in limiting HIV infection in the same group particularly when lower virus titres was used for infections. These results show that pretreatment with TLR8 ligand analogous to HIV's genetic material treatment of PBMCs made HIV target cells in HESN PBMCs more refractory to infection with a circulating strain of HIV through yet to be defined mechanisms. However, the data presented above suggests that this may be due to the altered TLR8 responsiveness with respect to cytokine production in HESNs.

4.5. Summary and Discussion

Toll-like receptors are important innate sensors responsible for initializing the host immune response against invading organisms. TLR recognition of microbial components, initiates a well characterized signalling cascade terminating in the activation of transcription factors that transcribe genes for cytokine, chemokines and other antimicrobial factors[72, 355]. Cytokines mediate immune response during an infection, often directing cellular function, signalling, proliferation and differentiation. Dysfunction or variation in innate recognition has been shown to have consequences on the outcome of exposures to certain bacterial and viral infections[197].

Understanding the innate mechanisms of protection against the HIV is crucial for vaccine or microbicide development. Two recent studies have shown differences in innate responsiveness between HESN and susceptible individuals [293, 294]. Broader and higher TLR hyper-responsiveness to TLR3, TLR4, TLR7 and TLR8 ligands was described in a cohort of high-risk discordant HESN couples [293]. In the Pumwani cohort, we previously

described lower TLR2, TLR4, TLR6, TLR7, TLR8, RIG1 and MDA5 in CMCs and CECs expression in HESN, but higher TLR8 and not TLR4 or TLR7 responses in CMCs in the genital tracts of HESN female CSWs[294]. The differences in observations between the two cohorts could possibly arise from the nature of the cohorts, the former being a cohort of discordant couples including both sexes with potentially few exposures from multiple partners, while the latter is entirely a cohort of female sex workers. Gender has previously been shown to impact on TLR responsiveness, with women having higher TLR9 responses in pDCs compared to men, influencing disease progression[159].

In the present study, HESN PBMCs without TLR perturbations generally produced lower amounts of specific pro-inflammatory cytokines, strongly supporting the concept of a quiescent immune system, consistent with what was previously described in the same cohort [309]. It was also noted that the separation between the cytokine production between HESN and controls, frequently started at around the 12 hours of culture with cytokine production after 24 hours not matching earlier time points, also suggesting differences in timing of cytokine responses between HESN and HIV-N. Previous studies have shown that the process of separation of PBMCs from whole blood or venipuncture increased the activation state of blood cells substantially[356]. Therefore the lower activation state of HESN PBMC in spite of culture suggests that the cells maybe less activated by extraneous environmental changes and therefore can be said to be more 'quiescent' compared to those of controls. Additionally, for first time this study demonstrated a specifically stronger TLR8 responsiveness in the peripheral blood of HESN individuals based on production of cytokines and chemokines to TLR8 ligands analogous to HIV's genetic material, and ofter lower responses to TLR4 or TLR7 ligands not associated with HIV. The lower TLR7 and higher TLR8 responsiveness corresponded to cognate TLR expression and downstream activation of TLR signalling or related pathways. This was accompanied by lower IL-1β, IL-10 and TNFα to TLR4 ligand

LPS and lower IFNα2, IL-1β, IL-6, IL-10, and IL-12p70 to TLR7 ligand Imiquimod but higher TLR8 cytokine responses such as IL-1β, IL-6, IL-10, IL-12p40, IFNα2, IFNγ IP-10 and TNFα by HESN PBMCs. Although subtle differences were observed between the results of the overnight and kinetic cytokine quantification experiments, there was great similarity in the higher TLR8 responsiveness between the both sets of experiments, strongly suggesting that differences observed may be more representative of HESN phenotype than experimental variation. Further strengthening these observations was the fact that there were very few distinctions in the responses to the positive controls - PHA or PMA+ Ionomycin between the two groups.

This study for the first time tested the effect of pretreatment of PBMCs with TLR4, TLR7 or TLR8 ligands on HIV's infectivity *in vitro* in an HESN population. Prior to this, a number of studies had been conducted by others testing the effects of pre-treatment of PBMCs with either TLR7 or 8 ligands, on HIV's infectivity *in vitro*[248, 263-265, 353]. The first of these studies, showed an inhibitory effect of TLR7/8 stimulation (using ssRNA as the agonist) on HIV replication *in vitro* associated with the induction of anti-HIV $CD8^+$ T cells and NK cells responses by DCs[264]. A second study showed TLR8 stimulation induced cathelicidin microbial peptide (cAMP), vitamin D receptor (VDR) and cytochrome p450 member CYP27B1, which mediated conversion of an inactive Vitamin D metabolite into an active form, leading to autophagy capable of inhibiting HIV replication in macrophages *in vitro* [263]. On the contrary, TLR7 stimulation with Imiquimod enhanced susceptibility of purified $CD4^+$ T cells to HIV infection, through induction of Ca^{2+} influx leading activation anergic gene-expression dependent on NFATc2[248]. Switching TLR7 agonists from Imiquimod to Gardiquimod, made macrophages and PBMCs more refractory to both X4 and R5 trophic viruses, an indication that the type of TLR7 ligand may have a bearing on the direction of HIV infectivity[353]. *In vivo*, topical application of an Imiquimod based microbicide (intra-

vaginally) enhanced susceptibility to SIV in non-human primates, through induction of β-chemokines and IFN-α responsible for massive recruitment of SIV target cells, thereby enabling establishment of infection[28]. Mechanistically, the variation in outcomes HIV's infectivity studies testing different TLR7 agonist's, may be related to TLR7 ligand-receptor interactions, which in turn direct the TLR7 signal transduction and effector responses. Characterization of TLR7 structure may help delineate the differences in outcomes of HIV infection studies arising from use of different TLR7 ligands, as this would allow an assessment of receptor-ligand intereactions. Crystalization of TLR7 is an achieveable goal given the recent determination of TLR8 receptor structure[219]. The findings from this and other similar studies suggest that cytokine responses, particularly from DCs or other APCs may be important in directing the development of anti HIV responses in by PBMCs *in vitro.*

In our assessment, the distinct dichotomy of TLR7 and TLR8 cytokine responses in HESN PBMCs may have influenced the infectivity of a circulating strain of HIV *in vitro* in the two populations of CSW. This is considering, TLR7 stimulation induced copious production of IFNα2 but failed to elicit TNF-α and IL-12p40 in either group. Whereas, TLR4 and TLR8 stimulations resulted in lower IFNα2 responses, which was consistent with previously described TLR7 driven production of IFNα2 primarily in pDCs, that lack TLR4 and TLR8[357, 358]. The induction of IFNα2 in pDCs has been shown to occur in a MyD88 dependent manner leading to NFκB activation followed by concomitant binding of IRF3 or IRF7 to the *IFNA1* promoter[359]. A number of groups have previously shown that IFNα production alone, was not sufficient to provide protection against HIV both *in vitro* and *in vivo*[28, 248]. From this we inferred that, based on previously shown inability of IFNα2 responses to reduce the infectivity of HIV *in vitro* following TLR7 pre-treatment of PBMCs, the higher TLR7 responses by PBMCs HIV-N may increase the susceptibility HIV targets cells in virus infecting dose dependent manner. However, IFNα2 has been shown to be a

potent inducer of post-entry antiviral mechanisms capable of limiting HIV replication *in vitro*, more recently through induction of MX2 and MXB proteins[361, 362]. We observed reduced p24 production by TLR7 pre-treated PBMCs when infected with highest virus concentrations, this could be due to HIV induced apoptosis occasioned by higher virus titres rather than antiviral mechanisms, but further investigation is needed to better understand these results. On the other hand, TRAF6 or TRAF3 activation leads to the production of IL-6, IL-10 and IP-10 in a NFκB dependent manner[360]. Both NFκB and IRF3 expression in HESN PBMCs was lower following TLR7 stimulation with correspondingly lower expression of CSF2, CSF3, IL10 annd IL-12 mRNA, yet there was no difference in TRAF6 mRNA expression in between the two groups.

An interesting observation made in this study, was the surprisingly higher TLR8 expression in TLR7 stimulated HESN PBMCs (**Figures 11B**). This led us to speculate that this higher expression may responsible for the inhibited expression of TLR7, as TLR8 inhibition of TLR7 expression has previously been described by Wang and colleagues[28]. Who observed that interactions between TLR8 and evolutionarily closely related TLR7 and TLR9, resulted to the inhibition of TLR7 and TLR9 expression[209]. In contrast, TLR8 stimulation of HESN PBMCs produced higher cytokine responses (IL-1β, IL-6, IL-10, IL-12p70, IFNα2, IFN-γ, IP-10 and TNF-α), that corresponded to the higher expression of TLR8 but not TLR7 receptors, and higher induction of specific TLR8 signal transduction components. The higher TLR8 signal transduction in HESN PBMCs was through higher expression of components in the MyD88-MAPK-JNK/ERK-AP-1 or MyD88-TAK/TAB1-NFκB pathways, evidenced by the higher expression of MyD88, NFκB complex and MAPK pathway components. The MyD88-NFκB or MyD88-MAPK-AP-1 pathways were activated by TLR8 stimulation pathways, drive higher IL-1β, IL-6, IL-10, IL-12p40/70 and TNF-α production in HESN. Given that the quantification of mRNA was done after 24hrs of stimulation, there is a

possibility of autocrine amplification of a number cytokine responses occurring[363]. The cytokine environment generated by TLR8 stimulations was also associated with tendancy for lower HIV infection in a dose dependent manner, more so when lower viral titres were used in infections of HESN PBMCs. The TLR8 cytokine environments contained IFN-γ, IL-12p40, IL-12p70 and TNF-α, all of which were lowly produced or absent in TLR7 stimulated PBMC responses. Taken together, the HIV analogue ssRNA induced higher TLR8 expression and TLR8 signalling in HESN PBMCs, resulting in better inhibition of HIV infection *in vitro* compared TLR7 stimulation with Imiquimod.

The expression of certain TLR signalling pathway components corresponded to the cytokine responses in PBMCs of HESN and HIV-N folllowing TLR7 and TLR8 stimulations. This was evident in the opposing patterns of expression of TLR7 or TLR8 signalling pathway components, for example; MyD88 a critical regulator of TLR signalling, MAPK signaling component MAP3K1 (MEKK1), BTK a B- cell receptor signalling pathway adaptor linked to TLR signalling pathway and transcription factors IRF3 and NFκB factor-NFKB2, NFKBIA (IκKα) and IKBKB (IKKβ). Given there were no differences in expression of negative regulators of the TLR such as TOLLIP, it is our view that these may not have accounted for lower TLR7 responsiveness or higher TLR8 responsiveness in HESN as observed in this study. However, TLR8 signalling in HIV-N may have been under control of IRAK2 given its higher expression in TLR8 stimulated PBMCs from this group, as over-expression of splice variants of *Irak2* gene have been shown to inhibit TLR4 signalling downstream of MyD88[364].

The cytokine profiles following the different TLR stimulations, can be accounted for by the differences in TLR expression patterns on immune cells within PBMCs[246, 365]. (This shall be discussed late in chapter 6).

Lastly, we published a study showing lower expression of TLR4, lower TNF-α and IL-10 production by CMCs in the genital tract of HESN compared to HIV-N. In the present study the production of IL-1β, IL-10, IL-12p40, IL-12p70 and TNF-α was lower in TLR4 stimulated PBMCs of HESN compared to those of HIV-N, yet the TLR4 mRNA expression between the two groups was similar. This indicates the pattern of TLR4, TLR7 or TLR8 responsiveness in the genital of HESN, was similar what was observed in the genitral tract Suggesting that the TLR4, 7 or 8 cellular responses, whether in PBMC or CMCs, are programmed intracellularly and may not be entirely tissue dependent. However, neither of these studies tested the impact of tissues localization on PBMCs or CMCs responsivness to different TLRs agonists in HESN.

In summary, HESN PBMCs were shown to have higher cytokine responses to TLR8 but not always to TLR4 or TLR8 stimulations. However, unstimulated PBMCs of HESN had lower cytokine responses compared to controls corresponding to a more 'quiescent' immune phenotype. The cytokine milieu of HESN PBMCs was also more tightly or co-ordinately regulated compared to susceptible controls. More importantly, pre-treatment of HESN PBMCs with Imiquimod-TLR7 enhanced susceptibility to infection with primary HIV isolates, whereas similar treatment with TLR8 made PBMCs from the same group more refractory to infection with HIV *in vitro*. For the first time, this study demonstrated a dichotomy in TLR responsiveness of HESN PBMCs to TLR7 or 8 based on opposing patterns of cytokine production that was related to equally opposing receptor expression and the downstream TLR signalling. These findings have important implications on the development of TLR based HIV therapeutics, and might be useful in guiding the choice(s) of TLR7 or TLR8 agonists/antagonists for HIV microbicide development or vaccine design.

CHAPTER 5: Higher $CD4^+$ T cells responses to TLR4 and higher $CD8^+$ T cells responses to TLR7 and TLR8 in HESN

5.1 Rationale

Innate recognition through TLRs is at the interface of innate and adaptive immunity, playing a crucial role in the development of effective virus specific adaptive immune responses primarily through inflammation[74]. In the past, the functions of the innate and adaptive immune systems have been considered to be very distinct during infection and non-infectious inflammation. The discovery of PRRs and the subsequent demonstration of their expression on cells adaptive immune system, shifted the view on the role of innate and adaptive immune systems[72, 73]. Currently, the function of PRRs expressed on T cells is poorly understood, and is currently an area of great research interest. However, triggering of PRRs in T cells has been shown to activate transcription factors NF-κB, IRFs and NFAT, directly or indirectly, thereby influencing the development of T helper responses in $CD4^+$ T cells and cytotoxic lymphocytic functions in $CD8^+$ T cells[248]. Alternatively, T cells can modulate the expression of cytokines, chemokines and co-stimulatory factors on antigen presenting cells (APCs), enhancing their capacity to respond to pathogen derived components[366]. Indirect T cell activation following pattern recognition occurs through cytokine production and expression of co-stimulatory factors by APCs[197], whereas direct TLR recognition of PAMPs by T cells in itself can activate different functions on T cells dependent on the TLRs expressed.

Previous studies conducted in our cohort have shown a lowered state of immune activation particularly with respect to CD69 expression on T cells, and in the production of proinflammatory factors in the genital tract and peripheral blood of HESN CSWs. A 'quiescent' immune system hypothetically lowers the risk of HIV infection through reduction

of HIV target cells- mainly $CD4^+$ T cellls. On the flip side, a heightened state of immune activation enhances the risk of HIV transmission possibly by increasing the number of HIV target cells.

The effect of directly triggering innate signalling in HIV primary targets- $CD4^+$ T cells- was recently shown to enhance replication of HIV[248]. This serves as a strong indication of the potential of activating certain TLR signalling pathways on viral pathogenesis. The HIV replication enhancing effect in $CD4^+$ T cells occurred by triggering TLR7 using Imiquimod, leading an anergic gene transcription of NFATc2 driven by Ca^{2+} influx[248]. Ablation of TLR7 signalling restored the ability of $CD4^+$ T cells to mount effective responses capable of limiting HIV infection *in vitro*[248]. Additionally, TLR8 triggering through ssRNA has also been shown to induce anti-HIV effects through increased vitamin D driven autophagy or cytotoxic $CD8^+$ T and NK cell activity[263, 264]. The opposing effects of TLR7 and TLR8 stimulation with respect to HIV infection of $CD4^+$ T cells, indicates that there is a need to further understand the effects of triggering either pathway particularly within the context of protection and susceptibility to HIV. Whereas both TLR7 and TLR8 can recognize GU rich ssRNA, accumulating evidence points to the differences in T cell responses arising from selectively triggering either pathway using cognate ligands.

Other studies have shown that triggering of TLR8 signalling is able to reverse the $CD4^+$ regulatory T cell mediated peripheral tolerance, leading to increased antitumor activity[367, 368]. The ability of TLR signalling to induce T_{regs} or to break tolerance to self antigens, raised concerns about the potential dangers in the use of TLR based agonists as adjuvants. However, considering that most inflammatory process are short-lived and are tightly regulated under non-pathogenic conditions, it is probable that the same process initiated by TLR signalling leading to inflammation may be beneficial to the development of HIV

specific responses. The regulatory mechanisms accompanying inflammatory processes such as the induction of T_{regs} by TLR signalling have not been fully elucidated, especially during viral infections such as HIV. Previously, studies in our cohort have discovered higher frequencies of $CD4^+$ $Foxp3^+$ $CD4^+$ T cells T_{regs} in resting PBMCs of HESN CSWs, however; there is limited information on changes in the T_{reg} compartment following exposure to viral PAMPs in HESN. Considering the ability of TLR8 signalling to induce regulatory T cells, part of the experiments in this section sought to compare the ability of TLR4, 7 and 8 to induce or influence T regs functions.

Based on the observations presented in the previous chapter showing lower TLR4 and TLR7, but higher TLR8 responsivenss in HESN PBMCs, and based on what is known in literature, in this chapter we sought to quantify T cell responses in HESN and HIV-N female CSWs following TLR4, TLR7 and TLR8 stimulations. The goal of this investigation was to identify potential correlates of protection against HIV by evaluating T cell activation, cytokine functions, expression of specific TLRs and changes in T regulatory cell frequencies with TLR4, 7 or 8 stimulations of PBMCs from HESN and HIV-N.

5.2 Hypothesis

This section of the thesis tested the following hypothesis:

Peripheral blood T cells of HESN have higher activation and functional responses to TLR8 not TLR4 or TLR7 stimulations compared to controls.

5.3. Specific Aims

i. To compare the activation of T cell subsets in PBMCs of HESN and HIV-N after TLR4, TLR7 or TLR8 stimulations.
ii. To quantify and compare T cell functions like cytokine production, proliferation and degranulation after TLR4, TLR7 and TLR8 stimulation in the two groups.

iii. To compare the changes in TLR4 expression on T cell subsets with different TLR stimulations between HESN and HIV-N.

iv. To assess the changes in $CD4^+$ $CD25^+$ $Foxp3^+$ or T regulatory cell frequencies and functions with TLR stimulation in the two groups.

v. To correlate the TLR4 expression, cellular activation and functional responses by T cell subsets to TLR4, 7 or 8 stimulations in HESN and HIV-N.

vi. To compare the changes in expresssion of HIV co-receptor CCR5 on $CD4^+$ T cells with TLR4, TLR7 and TLR8 stimulations.

5. 4. Results

5.4.1 Higher Activation of $CD4^+$ T cells by TLR4 and $CD8^+$ T cells by TLR7 or TLR8 stimulations in HESN

To compare the effect of TLR stimulation on T cell activation, we quantified the changes in frequencies and surface expression (based on mean fluorescent intensities) of activation markers CD38, CD69 and HLA-DR on T cells, following TLR4 *E.coli*-LPS, TLR7 Imiquimod, TLR8-ssRNA40/LyoVec or PHA stimulations (later switched to PMA with Ionomycin due to inefficiency of PHA to upregulate expression actiavtion markers on T cells). This evaluation sought to compare the ability of the different TLR ligands to increase expression of activation markers on $CD4^+$ and $CD8^+$ T cells in the two CSW groups. The assessment included $CD4^+$ T cells that are the primary target cells of HIV and CD8+ T cells that have been implicated in control of viral replication during HIV disease.

Experiments were conducted on fresh and frozen PBMCs samples. Experiments using fresh samples were conducted in Nairobi over two visits in 2010 and 2011. While the experiments using freeze-thawed PBMCs samples were conducted in Winnipeg using samples collected in 2007-2008. Many of the samples were from matched study participants, thus enabling evaluation of T cell responses and function at multiple sampling points.

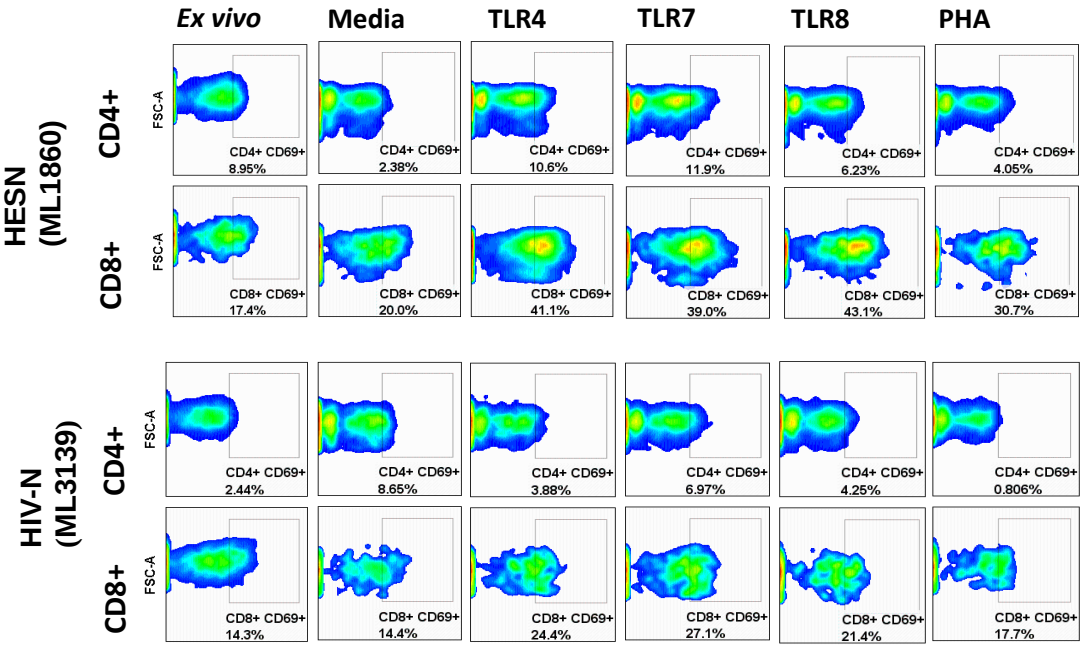


Figure 13. HESN T cell Activation. Representative flow plots to compare the expression of activation induce marker (AIM or CD69) on T cells subsets with TLR4, 7, 8 or PHA stimulations, from a HESN (ML 1860) and HIV-N (ML3139) study participant each.

The results showed that $CD8^+$ T cells expressed more CD69 compared to $CD4^+$ T cells before and after TLR stimulation. HESN $CD4^+$ T cells and $CD8^+$ T cells of ML1860 had higher frequencies of cells expressing CD69 compared to HIV-N 3139 following TLR4 (**10.6%** vs. 3.88%; **41.1%** vs. 24.4%), TLR7 (**12.0%** vs. 6.97%; **39.0%** vs. 27.1%) and TLR8 (**6.23%** vs. 4,25%; **43.1%** vs. 21.4%) stimulations respectively (**Figure 13**).

Next we compared the change in expression CD38, CD69 and HLA-DR on $CD4^+$ and $CD8^+$ T cells of HESN (n=25) and HIV-N (n=25) after TLR4, 7 or 8 stimulations (**Figure 14).** In freeze-thawed PBMC samples collected between 2007-2008, the expression CD38 on $CD4^+$ T cells was higher (30%- 48%) than on $CD8^+$ T cells (5-25%) in both groups before or after TLR4, 7 or 8 stimulationz, but there were no differences between HESN and HIV-N irrespective of the stimulation(**Figures 14A and D**).The expression of CD69 on resting $CD4^+$ T cells was similar between the two groups, but the frequencies of $CD8^+$ $CD69^+$ T cells in HESN as lower compared to HIV-N (**6.23 ± 1.05%** vs. 9.70 ± 1.10%, p=0.029, Mann-Whitney test)(**Figures 14B and G).**

Escherchia coli LPS TLR4 stimulation enhanced the expression of CD69 on $CD4^+$ T cells and $CD8^+$ T cells of HESN (***p*=0.0033**; p=**0.0035**, Wilcoxon signed ranked test) but not in those of HIV-N (p=0.5397; p=0.6120). TLR4 stimulation led to higher expression of CD69 on $CD4^+$ T cells of HESN (SEM: **14.34 ± 1.31%** vs. 9.52 ± 1.75%, p=0.0075), and higher expression of the same on $CD8^+$ T cells of HESN (SEM: **22.26 ± 1.91%** vs. 16.44 ± 1.95%, p=0.0399) compared to HIV-N (**Figures 14B and E**). TLR7 stimulation of PBMCs (2007-2008) increased frequency of CD69 expressing $CD4^+$ and $CD8^+$ T cells in HESN (p=0.0003; 0.0035, Wilcoxon signed rank test) but not so in T cells of HIV-N (p=0.0539; 0.098). TLR7 stsimulation resulted in higher frequencies of CD69 expressing $CD8^+$ T cells in HESN (**22.19 ± 2.23%** vs. 15.34 ± 1.92%, p= 0.0465) (**Figures 14E**).

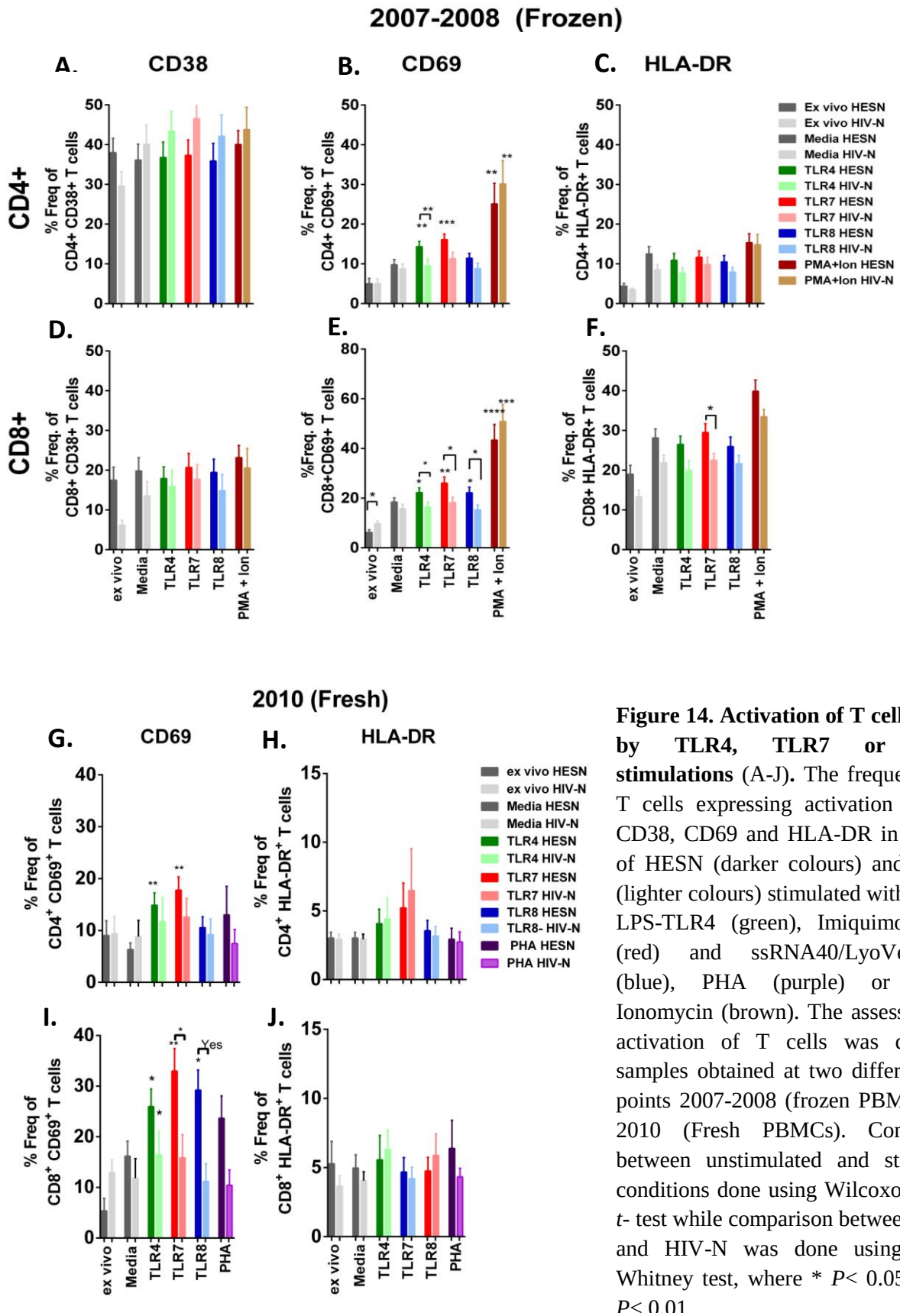


Figure 14. Activation of T cell subsets by TLR4, TLR7 or TLR8 stimulations (A-J)**.** The frequencies of T cells expressing activation markers CD38, CD69 and HLA-DR in PBMCs of HESN (darker colours) and HIV-N (lighter colours) stimulated with; *E. coli* LPS-TLR4 (green), Imiquimod-TLR7 (red) and ssRNA40/LyoVec-TLR8 (blue), PHA (purple) or PMA+ Ionomycin (brown). The assessment of activation of T cells was done on samples obtained at two different time points 2007-2008 (frozen PBMCs) and 2010 (Fresh PBMCs). Comparison between unstimulated and stimulated conditions done using Wilcoxon Paired *t*- test while comparison between HESN and HIV-N was done using Mann-Whitney test, where * $P< 0.05$ and ** $P< 0.01$

TLR8 stimulation did not increase the frequency of CD69 expressing $CD4^+$ T cells in either group, but significantly increased the frequencies of CD69 expressing $CD8^+$ T cells in HESN compared to HIV-N (**26.06 ± 2.47%** vs. 18.22 ± 2.17%, p=0.035).

TLR4, TLR7 or TLR8 stimulations failed to increase the frequency of HLA-DR expressing activation markers in either T cell subset, but a difference in frequency of HLA-DR expressing $CD8^+$ T cells was observed following TLR7 stimulation (**29.50 ± 2.21%** vs. 22.51 ± 1.71%, p=0.0315) (**Figures 14C and F**).

To validate the findings made from experiments utilizing freeze-thawed PBMCs, we repeated the experimental procedure (above) using fresh PBMCs from HESN (n=11) and HIV-N (n=10) collected in 2010. This time the evaluation of T cell activation was limited to CD69 and HLA-DR, with the quantification CD38 expression being dropped as its expression was not altered by TLR stimulation in the first set of experiments.

The frequencies of CD69 expressing $CD4^+$ T cells after TLR4, 7 and 8 stimulations followed a similar pattern, as was observed in freeze-thawed samples. Where, there was no difference in frequencies of CD4 and CD8 T cells expressing CD69 *ex vivo*, although a trend for lower frequencies of CD69 expressing $CD8^+$ T cells of HESN was noted (median: **5.38 ± 2.45%** vs. 12.96 ± 2.50%, p=0.052) (**Figures 14G and I**).

TLR4 and TLR7 stimulations increased the frequency of CD69 expressing $CD4^+$ T cells in HESN (***p*=0.0020; 0.0039**, Wilcoxon signed ranked test) but not HIV-N (p=0.125; 0.56), although the difference between the two groups after either stimulation was not significant. TLR8 stimulation on the other hand failed to enhance the frequency of CD69 expressing $CD4^+$ T cells in either group (**Figures 14G and I**). On the contrary, TLR4, TLR7 and TLR8 ligands, significantly increased the frequencies of CD69 expressing $CD8^+$ T cells in HESN (p=0.0059; 0.0049; 0.0040), but not in HIV-N (p=0.013; 0.148; 0.269, all Wilcoxon signed

ranked test). A comparison between the groups revealed that TLR7 and TLR8 stimulations increased the frequencies of CD69 expressing $CD8^+$ T cells significantly more in HESN compared to HIV-N (**32.99 ± 4.43%** vs. 15.84 ± 4.59%, p=0.0245; and **29.22 ± 3.98 %** vs 11.19 ± 3.53, p= 0.0153, respectively) (**Figures 14G and I**).

This first set of experiments demonstrated a consistency in T cell responses to TLR4, 7 and 8 stimulations that was not affected by cryopreservation. HESN had higher increases in frequencies of CD69 expressing $CD4^+$ T cells following TLR4 stimulation, and consistently higher frequencies of $CD69^+$ $CD8^+$ T cells following TLR7 and TLR8 stimulations compared to controls.

In order, to get a better understanding of the amplitude of individual T cell responses to the different TLR stimulations, we performed a fold analysis (**Figure 15)** of the data presented in **Figure 13** and **14** above. This was done by the division of the frequencies of T cells expressing activation markers CD38, CD69 or HLA-DR in stimulated conditions, with those expressing corresponding markers in unstimulated cultures. Here, cryopreserved and freshly isolated PBMCs of HESN (n=18) and HIV-N (n=16) collected between 2007-08 or 2010, respectively were stimulated using TLR4(LPS), TLR7(Imiquimod), TLR8 (ssRNA40/LyoVec), PHA or PMA with Ionomycin, for no less than 20 hours and not exceeding 24 hours.

In cryopreserved samples collected between 2007-2008, TLR4, TLR7 and TLR8 stimulations of HESN PBMCs did not cause any differences in fold changes in CD38 expression on $CD4^+$ T cells between the two groups (**Figures 15A and B**), but resulted in lower fold changes in frequencies of $CD8^+$ T cells expressing CD38 in HESN following TLR4 (fold SEM: 0.92 ± 0.07 vs. 1.21 ± 0.10, p=0.024) and TLR7 (**1.066 ± 0.06** vs. vs. 1.374 ± 0.10, p=0.0152) (**Figures 15C and D**).

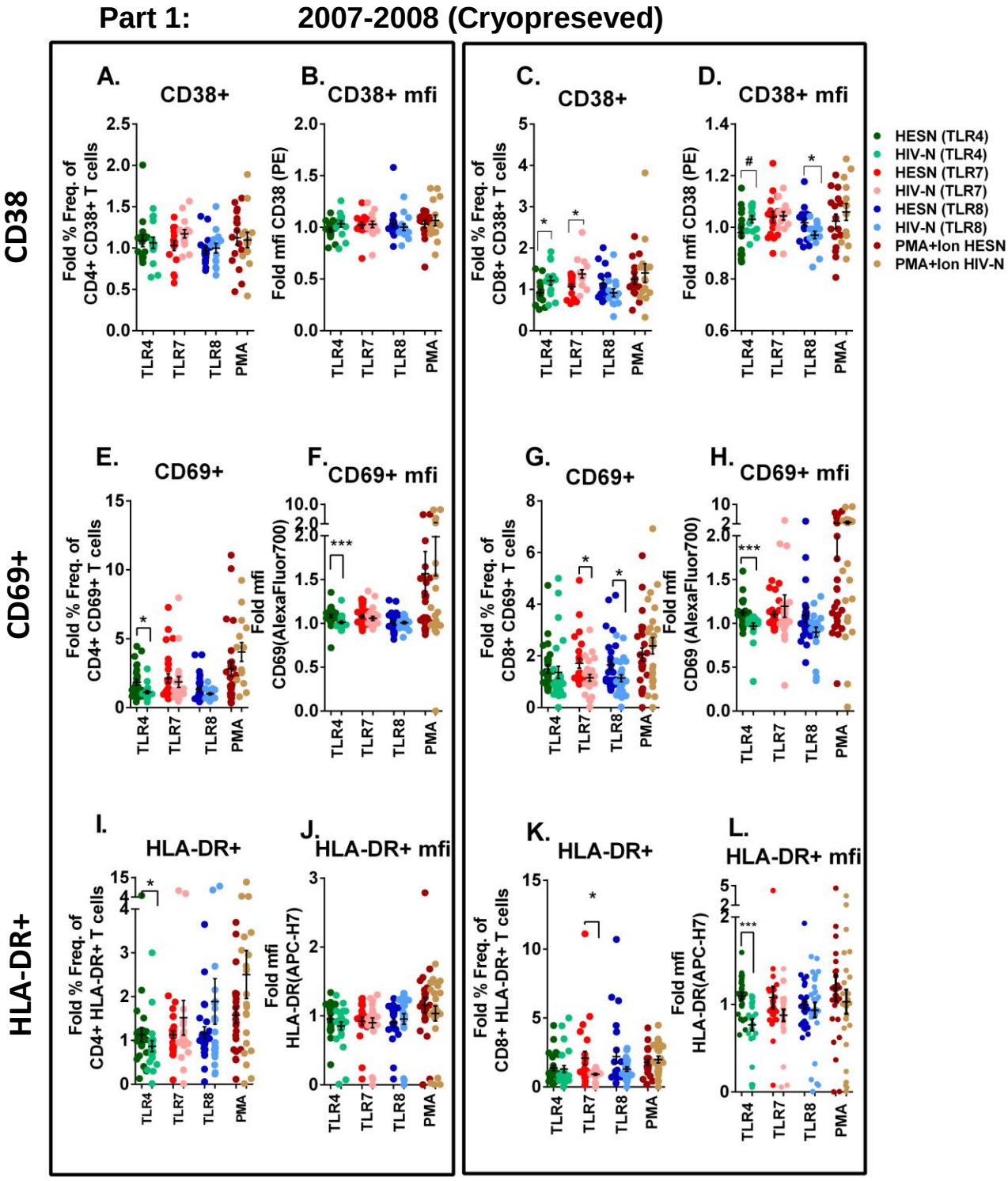


Figure 15 (Part 1). Fold changes in activation of T cell subsets with TLR4, TLR7 or TLR8 stimulations (2007-2008). (A-P) Fold comparisons of changes in frequency and mean fluorescent intensity of CD38, CD69 and HLA-DR in $CD4^+$ and $CD8^+$ T cells in freeze-thawed or fresh PBMCs of HESN (darker colours) and HIV-N (lighter colours) stimulated with; *E. coli* LPS-TLR4 (green), Imiquimod-TLR7 (red) and ssRNA40/LyoVec-TLR8 (blue), PHA (purple) or PMA+ Ionomycin (brown). The assessment of fold change in activation of T cells was done on samples obtained at two different sampling time points 2007-2008 (frozen PBMCs) and 2010 (Fresh PBMCs). Comparisons between grouped data was done using Mann-Whitney test, where * represents $P<0.05$, ** $P<0.01$ and ***$P<0.001$. HESN (n=18) and HIV-N (n=18)

Part 2: 2010 (Fresh)

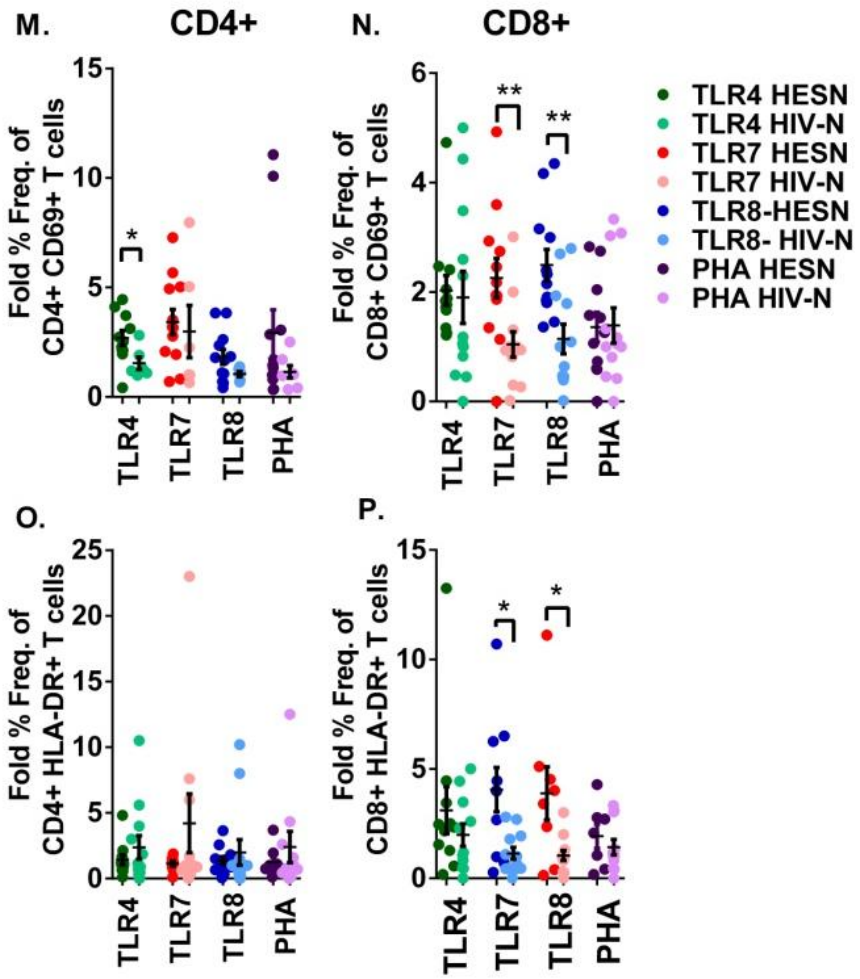


Figure 15 (Part 2). Continued- Fold changes in activation of T cell subsets with TLR4, TLR7 or TLR8 stimulations (2010) (M-P). Fold comparisons of changes in frequency and mean fluorescent intensity of CD38, CD69 and HLA-DR in $CD4^+$ and $CD8^+$ T cells in freeze-thawed or fresh PBMCs of HESN (darker colours) and HIV-N (lighter colours) stimulated with; *E. coli* LPS-TLR4 (green), Imiquimod-TLR7 (red) and ssRNA40/LyoVec-TLR8 (blue), PHA (purple). HESN (n=12) and HIV (n=12).

On the contrary, the fold change in surface expression of CD69 on $CD8^+$ T cells after TLR4 stimulation was higher in HESN (fold SEM: **1.12 ± 0.03** vs. 0.97 ± 0.03, p=0.0002*)* but not in frequencies of $CD8^+$ $CD69^+$ T cells (fold SEM: 1**.49 ± 0.16** vs. 1.36 ± 0.24, p=0.0626) compared to those of HIV-N (**Figures 15G and H**).

TLR7 or TLR8 stimulations of HESN PBMCs resulted in higher fold changes in frequencies of CD69 expressing $CD8^+$ T cells compared to HIV-N (fold SEMs: **1.71 ± 0.19** vs. 1.15 ± 0.13, p=0.0167; **1.66 ± 0.20** vs. 1.13 ± 0.13, p= 0.0373), but faild to produce significant differences in fold CD69 MFI expression on $CD8^+$ T cells between the groups(**Figures 15G and H)**.

Next we evaluated the change in HLA-DR expression on T cells from the two CSW groups following stimulation with TLR4, 7 or 8 ligands. Higher fold changes in frequencies of HLA-DR expression on $CD4^+$ T cells of HESN after TLR4 stimulation was observed compared those of HIV-N (fold SEM: **1.13 ± 0.16** vs. 0.87 ± 0.13, p=0.023). However, there were no differences in fold changes in frequencies or MFI of HLA-DR on $CD4^+$ T cells after either TLR7 or TLR8 stimulations between the two groups (**Figures 15I, and J).** HESN had higher surface expression of HLA-DR on $CD8^+$ T cells (fold SEM: **1.10 ± 0.04** vs. 0.77 ± 0.07, p=0.0001) after TLR4 stimulation. Only TLR7 stimulation resulted in higher fold changes in frequencies of HLA-DR^+ $CD8^+$ T cells (fold SEM: **2.09 ± 0.50** vs. 0.92 ± 0.07, p=0.04) in HESN compared to HIV-N (**Figures 15K and L**).

The fold analysis was also conducted by reanalyzing the data obtained from the frequency and MFI quantification of T cell activation markers- CD38, CD69 and HLA-DR- expression with TLR4, TLR7 or TLR8 stimulations of fresh PBMC samples collected in 2010. A similar pattern of higher activation of $CD4^+$ T cells in HESN following TLR4 ligand LPS stimulation based on fold changes in CD69 expression (fold SEM: **2.69 ± 0.34** vs. 1.54 ± 0.29, p=0.036)

was observed(**Figure 15M**). $CD8^+$ T cells of HESN also had a consistent pattern of higher fold changes in frequencies of cells expressing CD69 after TLR7 (fold SEM: **2.49 ± 0.28** vs. 1.143 ± 0.27, p=0.0036) and TLR8 (fold SEM: **2.26 ± 0.36** vs. 1.04 ± 0.23, p=0.0079) stimulations, and higher fold changes in frequencies of cells expressing of HLA-DR after TLR7 (fold SEM: **4.06 ± 1.01** vs. 1.14 ± 0.27, p=0.036) and TLR8 (fold SEM: **3.89 ± 1.22** vs. 1.04 ± 0.23, p=0.020) stimulations (**Figures 15M-P**).

These results revealed a consistency in the higher pattern of $CD4^+$ T cell responses to TLR4 stimulation and higher $CD8^+$ T cell responses to TLR7 or TLR8 agonists in HESN both in freeze-thawed and fresh samples (**Figures 13-15**). Additionally, there were no differences in the expression of activation markers on T cells of HESN and HIV-N with PHA and PMA with Ionomycin treatments.

5.4.2. Lower activation of resting T cells and higher IFN-γ responses by HESN T cells to TLR4, TLR7 or TLR8 stimulations

TLR signalling in T cells activates transcription of cytokines and chemokines[200]. Previous studies in our cohort, showed that T cells of HESN CSWs express lower levels of activation marker CD69[309] and lower levels of cellular activation genes transcripts, but similar levels of cytokine responses to mitogenic or antigenic stimulation between T cells of HESN and susceptible CSWs [334]. These observations suggest that T cells of HESN are 'quiescent' or bear a lower state of immune activation, yet possess similar capacity to respond to non-specific or recall stimulation.

Here we determined if TLR4, 7 or 8 stimulations of PBMCs from HESN and HIV-N CSWs produced differences in expression of different cytokines and chemokines on T cells *in vitro*. To test this, we quantified the expression of IFN-γ, IL-2, TNFα, MIP1α, marker for degranulation potential LAMP1 or CD107a and proliferation marker Ki67 on CD4 and CD8

T cells contained in PBMCs from HESN and HIV-N before and after TLR4, 7, 8 and PMA + Ionomycin using flow cytometry (as described in **section 3.3.2**). The PBMCs used in these experiments had release of cytokines and chemokines blocked with protein transport inhibitors (GolgiPlug^{TM} and GolgiStop^{TM}) 6 hours prior to flow quantification of cytokines and chemokines expression (**Figure 16-18**) [369-371].

The results showed that *ex vivo* $CD4^+$ and $CD8^+$ T cells of HESN had lower expression of the marker for degranulation potential LAMP1 or CD107a (**0.31 ± 0.18%** vs 3.98 ± 0.81%, *p*= 0.001: **0.34± 0.20%** vs 2.76 ± 0.58%, *p*= 0.0001, respectively), and lower frequencies of T cells expressing TNF-α in both subsets (**1.03 ± 0.40%** vs 2.21 ± 0.61%, *p*= 0.0207; **0.524 ± 0.19%** vs 1.92 ± 0.31%, *p*=0.0053, respectively) compared to HIV-N. Similarly, lower frequencies of $CD8^+$ T cells in HESN expressed IL-2 compared to HIV-N (**1.22 ± 0.72%** vs 4.23 ± 1.59%, *p*= 0.0030) (**Figures 16A, B, F, G and H**). These findings show that HESN had lower expression of proinflammatory factor TNF-α and lower potential for degranulation based on CD107a expression in both T cell subsets, suggesting a lower immune activation state in T cells of HESN.

Next we measured the kinetics of T cell cytokine, chemokine and other functional responses over the course of different TLR stimulations using multicolour flow cytometry (**Figure 17**). These experiments were performed in parallel with the assessment of T cell activation (**Figures 13-15)**. Here, different intracellular functional T cell markers -CD107, IFN-γ, IL-2, TNF-α, MIP-1α, and Ki67- were quantified from TLR4, TLR7, TLR8 or PMA+ Ionomyin stimulated PBMCs cultured with protein transport inhibitors.

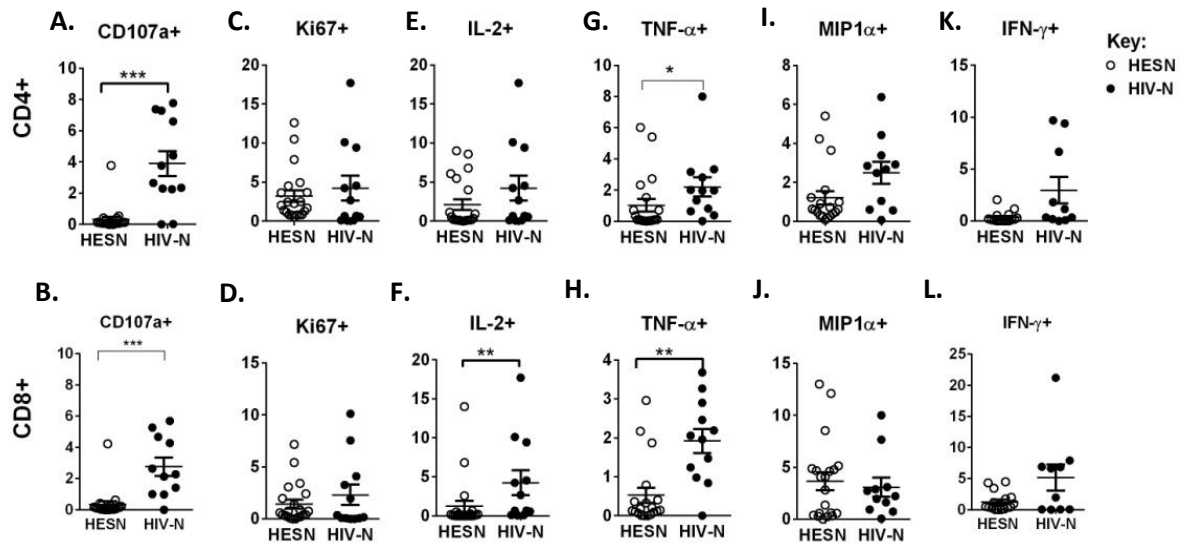


Figure 16. Comparison of intracellular cytokines, chemokine, degranulation and proliferation responses in T cells *ex vivo*. Freshly isolated PBMCs from HESN (white circles) and HIV-N (black circle) were stained interacellularly for CD107a, Ki67, IL-2, TNF-α, MIP-1α or IFNγ, and the expression of these markers quantified by multicolour flow cytometry. Comparison between groups was done using Mann-Whitney test and all significant differences indicated with an asterisk, where *p <0.05, **p <0.01 and ***p < 0.001.

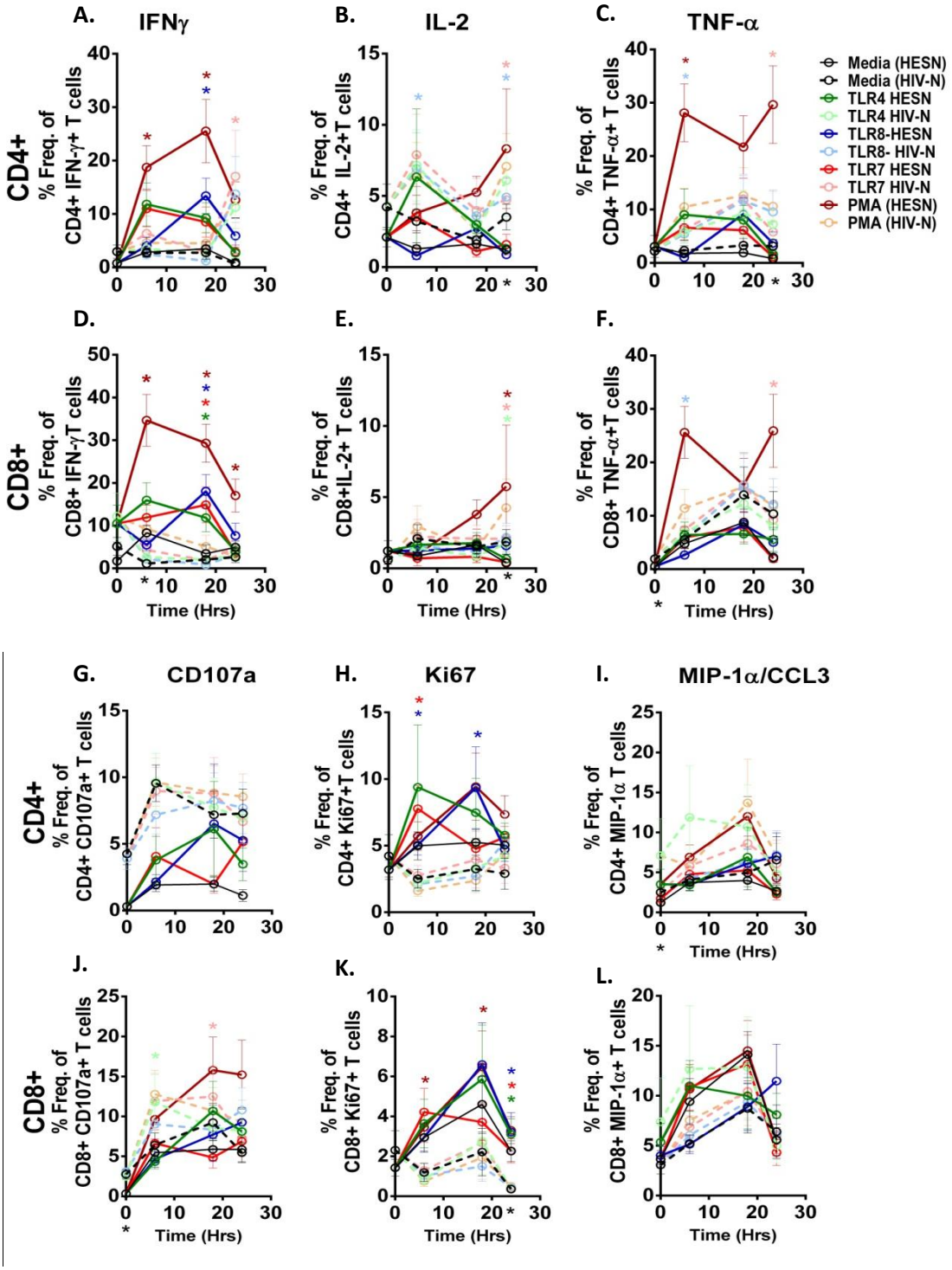


Figure 17: Kinetics of T cell functional responses to TLR4, TLR7, TLR8 or PMA with Ionomycin stimulations. Freshly isolated PBMCs were cultured with or without; 0.01μg/ml of *E. Coli* LPS-TLR4 (green lines), 2.5μg/ml Imiquimod-TLR7 (red lines), 1.0mM ssRNA40/LyoVec-TLR8 (blue lines) or 0.001μg/ml PMA with 0.01μg/ml Ionomycin (brown lines). Intracellular cytokine staining was used to evaluate different $CD4^+$ and $CD8^+$ T cells functions using 10- colour flow cytometry at 0, 6, 18 and 24 hours. The functions assessed included; levels of IFN-γ (A and D), IL-2 (B and E), TNF-α (C and F), potential of degranulation (CD107a or lysosomal associated membrane protein 1-LAMP1) (G and J), proliferation (Ki67) (H and K) and MIP-1α/CCL3 (I and L). HESN (n=19) represented with darker colours and HIV-N (n=15) by lighter colours. The comparison of grouped linear data done using Holm-Sidak method alpha = 5.0%, $P<0.05$ indicated using asterisks (* the colour matches the group with the higher mean).

We observed the increased expression of IFN-γ on $CD4^+$ and $CD8^+$ T cells after TLR4, TLR7 or TLR8 stimulations, where the increases in IFN-γ expression occured earlier in HESN (0-6 hours) compared to HIV-N (18-24 hours). The expression of IFN-γ in both T cell subsets of HESN dipped after 24 hours, just as production of the same started to increase in HIV-N, with $CD4^+$ T cells of HIV-N producing higher amounts of IFN-γ after 24 hours of TLR7 stimulation (**Figure 17A**). The frequency of IFN-γ expressing $CD4^+$ T cells was higher in HESN after 18 hours of TLR7 stimulation (**2.90 ± 1.29%** vs. 17.03 ± 8.63%, p=0.02) and TLR8 stimulation (**13.3± 3.31%** vs. 1.23± 0.40%, p= 0.009) compared to HIV-N (**Figures 17A and D**). Similarly, the frequency of IFN-γ^+ $CD8^+$ T cells was higher in HESN between 6-18 hours of TLR4 (6hrs-**15.9 ± 4.10%** vs. 2.58 ± 1.15%, p=0.02; 18hrs- **11.9 ± 3.258%** vs 2.11± 1.38%, p= 0.03), after 18 hours of TLR7 (**14.8 ±4.20%** vs. 1.87±1.02%, p=0.02) and after 18 hours of TLR8 (**18.9 ±3.94%** vs 0.9 ± 0.28%, p=0.002) stimulations, compared to in HIV-N (**Figure 17D**).

Interleukin-2 (IL-2) expression was relatively unaffected by the different TLR stimulations. Although, $CD4^+$ T cells of HESN had lower numbers of cells producing IL-2 after 24 hours of TLR7 stimulation (**1.5 ± 0.721%** vs. 4.73 ±1.50 %, p=0.04) and after 6 or 24 hours TLR8 (**0.83** ± 0.28 **%** vs. 6.93 ± 2.52%, p= 0.003; **0.88** ±0.17**%** vs 4.92± 1.33%, p= 0.003) compared to HIV-N (**Figure 17B)**. $CD8^+$ T cells of HESN had lower frequencies of IL-2 expressing cells after 24 hours of TLR7 stimulation compared to controls (**0.43 ± 0.22%** vs 2.08 ± 0.91, p=0.033).

On the contrary, all the three TLR ligands enhanced expression of TNF-α on both $CD4^+$ and $CD8^+$ T cells. However, HESN had lower frequencies of $CD4^+$ T cells expressing TNF-α after 24 hours of TLR7 stimulation (SEMs: **1.04 ± 0.40%** vs. 5.45 ± 1.72%, p=0.004) and after 6 hours of TLR8 stimulation (SEMs: **1.14 ± 0.42%** vs. 5.79 ± 1.89%, p=0.003).

Similarly, HESN had lower frequencies of $CD8^+$ T cells expressing TNF-α after 24 hours of TLR7 stimulation (**1.98 ± 0.54%** vs. 9.25 ± 3.32%, p=0.008) and after 6 hours of TLR8 stimulations compared to HIV-N (**2.65 ± 0.69%** vs. 6.06 ± 1.74%, p=0.03) (**Figures 17C and F**).

TLR4, TLR7 and TLR8, all induced the expression of CD107a on both $CD4^+$ and $CD8^+$ T cells, but TLR4, 7 and 8 stimulations did not result in differences in frequencies of CD107a expressing $CD4^+$ T cells between the two groups. On the other hand, a lower frequency of $CD8^+$ T cells in HESN expressed CD107a after 6 hours of TLR4 stimulation (**4.33 ± 0.90%** vs. 11.78 ± 3.667%, p=0.01) and 18 hours of TLR7 stimulation (**4.85 ± 1.35 vs.** 12.49 ± 3.70, p=0.029), suggesting a reduced potential for degranulation in $CD8^+$ T cells of HESN with TLR4 and 7 stimulations (**Figures 17G and J**).

The expression of Ki67 a marker for proliferation potential, increased in both $CD4^+$ and $CD8^+$ T cells with all three TLR stimulations, but the increases were more evident on T cells of HESN compared to HIV-N which remained almost unaltered. HESN had higher numbers of $CD4^+$ T cells expressing Ki67 following 6 hours of TLR7 stimulation (**9.39 ± 4.66%** vs, 2.17 ± 0.49%, p=0.04) or 6 hours after TLR8 stimulation (**5.05 ± 0.74% vs.** 2.11 ± 0.51%, p=0.009), and higher frequencies of $CD8^+$ T cells after 24 hour of TLR4 (**3.10 ± 0.78% vs** 0.39 ± 0.17%, p=0.01), TLR7 (**2.27 ± 0.60% vs.** 0.33 ± 0.12%, p=0.012) or TLR8 stimulations (**3.21 ± 0.97% vs.** 0.44 ± 0.20%**,** p= 0.04) (**Figures 17H and K**).

The production of MIP-1α/CCL3 by both T cells was robust in response to all TLR stimulation conditions; although, there were no differences in its production between the two groups (**Figures 17I and L**).

In summary, the kinetic assessment of T cell functions with different TLR stimulations, revealed differences in IFN-γ and TNF-α responses between HESN and HIV-N some of

which had similarity to the patterns of soluble cytokine responses quantified in 2010 and 2014 (**Figure 5-8**). Specifically, the higher IFN-γ responses by HESN T cells to TLR8 stimulation corresponded to the higher production of the same in culture supernatants as quantified by Luminex assays (data presented in chapter 4). This demonstrates that T cells may be the principle cells contributing to the higher IFN-γ responses in HESN PBMCs.

5.4.3. Correlational Analysis of HESN T cell Activation and functions responses to different TLR stimulations

Next, we sought to reconcile the differences observed in T cell activation (**Figures 14-16**) to those observed in T cell functional responses following different TLR stimulations between the two groups of CSW using correlational analyses (**Table 3**). Considering that activation of TLR signalling pathways leads to increased transcription of cytokines and anti-microbial factors, yet the relationship of cellular activation and cytokine production, has not been well defined especially within the context of protection or susceptibility to HIV. The decision to use of correlational rather than multiparametric flow analyses, was necessitated by the separation of flow panels for T cells activation and cytokine/chemokine (see **Table 1**).

We used Spearman's correlation analysis to compare the T cell activation (based on CD38, CD69 and HLA-DR expression) and functional response data (CD107a, IFNγ, IL-2, TNFα, Ki67 and MIP-1α), to determine if there were significant relationships. Both data sets were obtained from samples used in kinetics experiments conducted under similar conditions and matched to study participants. The results of this analysis are contained in **Table 3**. The correlation analysis revealed numerous relationships, however, for purposes of trying to understand the true differences in T cells responses between HESN and HIV-N, our discussion will be restricted to the relationships established between T cell responses that were different between the two groups in previous experiments.

Table 3: Spearman Correlational analysis between T cell Activation and Functional Responses to TLR4, TLR7, TLR8 and PMA with Ionomycin stimulations in matched HESN and HIV-N samples

		IFN-γ				IL-2				TNFα			
		CD4		CD8		CD4		CD8		CD4		CD8	
		HESN	HIV-N	HESN	HIV-N	HESN	HIV-N	HESN	HIV-N	HESN	HIV-N	HESN	HIV-N
Ex vivo	CD38	0.015	0.201	0.038	0.342	0.043	0.766	0.231	0.001	0.002	0.619	0.264	0.185
	CD69	0.213	0.110	0.285	0.067	0.928	0.012	0.191	0.369	0.632	0.974	0.095	0.635
	HLA-DR	0.056	0.067	0.011	0.056	0.131	0.014	0.069	0.0001	0.087	0.619	0.442	0.193
TLR4	CD38	0.304	0.174	0.452	0.197	0.871	0.011	0.023	0.0001	0.183	0.0001	0.183	0.011
	CD69	0.260	0.084	0.580	0.877	0.376	0.295	0.158	0.032	0.020	0.217	0.020	0.954
	HLA-DR	0.254	0.000	0.262	0.148	0.752	0.649	0.308	0.006	0.985	0.164	0.985	0.386
TLR7	CD38	0.029	0.018	0.843	0.020	0.195	0.254	0.650	0.032	0.043	0.000	0.046	0.548
	CD69	0.095	0.375	0.003	0.743	0.084	0.217	0.079	0.170	0.024	0.565	0.0001	0.992
	HLA-DR	0.257	0.001	0.758	0.082	0.997	0.560	0.037	0.394	0.871	0.193	0.321	0.080
TLR8	CD38	0.029	0.019	0.015	0.164	0.025	0.003	0.003	0.0001	0.021	0.002	0.000	0.063
	CD69	0.197	0.037	0.781	0.424	0.300	0.069	0.568	0.234	0.129	0.620	0.355	0.706
	HLA-DR	0.997	0.001	0.031	0.726	0.370	0.857	0.074	0.183	0.073	0.036	0.019	0.221
PMA + Ion	CD38	0.062	0.015	0.467	0.217	0.336	0.362	0.028	0.178	0.000	0.049	0.005	0.795
	CD69	0.001	0.974	0.011	0.197	0.000	0.194	0.011	0.012	0.018	0.768	0.238	0.834
	HLA-DR	0.439	0.265	0.960	0.312	0.715	0.908	0.870	0.137	0.544	0.224	0.908	0.084

		CD107a				Ki67				MIP-1α/CCL3			
		CD4		CD8		CD4		CD8		CD4		CD8	
		HESN	HIV-N	HESN	HIV-N	HESN	HIV-N	HESN	HIV-N	HESN	HIV-N	HESN	HIV-N
Ex vivo	CD38	0.684	0.079	0.976	0.864	0.212	0.869	0.961	0.067	0.730	0.297	0.479	0.700
	CD69	0.613	0.573	0.112	0.400	0.585	0.991	0.011	0.630	0.052	0.002	0.010	0.122
	HLA-DR	0.294	0.342	0.597	0.961	0.332	0.974	0.896	0.082	0.619	0.006	0.181	0.104
TLR4	CD38	0.075	0.005	0.693	0.137	0.039	0.198	0.000	0.161	0.109	0.591	0.903	0.938
	CD69	0.028	0.439	0.428	0.042	0.865	0.814	0.094	0.002	0.304	0.354	0.211	0.234
	HLA-DR	0.443	0.616	0.924	0.375	0.084	0.004	0.128	0.150	0.091	0.765	0.919	0.234
TLR7	CD38	<0.0001	0.156	0.001	0.923	0.464	0.003	0.025	0.007	0.758	0.183	0.933	0.223
	CD69	0.001	0.734	0.001	0.103	0.071	0.279	0.024	0.022	0.751	0.877	0.396	0.221
	HLA-DR	0.218	0.188	0.148	0.932	0.566	0.018	0.220	0.562	0.064	0.519	0.246	0.091
TLR8	CD38	0.015	0.001	0.211	0.122	0.073	0.258	0.000	0.069	0.625	0.223	0.711	0.905
	CD69	0.009	0.221	0.904	0.528	0.283	0.966	0.446	0.005	0.330	0.054	0.582	0.335
	HLA-DR	0.038	0.119	0.889	0.676	0.162	0.008	0.086	0.187	0.042	0.261	0.442	0.127
PMA+ Ion	CD38	0.019	0.048	0.066	0.545	0.286	0.024	0.009	0.026	0.675	0.004	0.062	0.411
	CD69	0.953	0.402	0.400	0.045	0.634	0.752	0.753	0.231	0.050	0.326	0.283	0.919
	HLA-DR	0.585	0.280	0.767	0.639	0.765	0.429	0.482	0.085	0.093	0.625	0.084	0.099

* P <0.05 ** P <0.01 ***P <0.001 ****P <0.0001

Unstimulated T cells of HESN had lower expression of CD107 and TNF-α, however these did not correlate with the expression CD69 or HLA-DR, but TNF-α expression corresponded to CD38 expression on $CD4^+$ T cells of HESN. Equally, resting $CD8^+$ T cells of HESN expressed lower levels of CD69, which postively with IL-2 correlated in HIV-N and with expression of Ki67 and MIP-1α in HESN. IL-2 expression which was lower on $CD8^+$ T cells of HESN its expression corresponded to that of CD38 and HLA-DR in HIV-N but not in HESN (**Table 3**).

TLR4 stimulation consistently led to higher expression of CD69 on $CD4^+$ T cells of HESN (**Figures 13-15**), this positively correlated with the expression of proinflammatory factors TNF-α and CD107a. Moreover, TLR4 stimulation lead to lower expression of CD38 on $CD4^+$ T cells of HESN which positively correlated with the expression of proliferation marker Ki67, while in HIV-N in correlated with expression of IL-2, TNF-α and CD107a expression (**Table 3**).

TLR7 and TLR8 stimulations on the other hand, alternately resulted in higher activation of $CD8^+$ T cells in HESN based on CD38, CD69 and HLA-DR expression. The frequency of cells expressing $CD8^+$ $CD38^+$ T cells was lower in HESN following TLR7 stimulations, this correlated with the expression levels of CD107a and TNF-α, but with IFN-γ and IL-2 expression in HIV-N, and with Ki67 in both groups. The expression of CD69 on $CD8^+$ T cells of HESN following TLR7 stimulation correlated with the expression of IFN-γ, TNF-α and CD107a expression, but it correlated to Ki67 expression in both groups. TLR7 stimulation also resulted in significantly higher expression of HLA-DR on $CD8^+$ T cells of HESN corresponding to the expression of IL-2 but not in HIV-N.

TLR8 stimulation led to lower CD38 expression on $CD8^+$ T cells of HESN which correlated with IFN-γ, CD107a and TNFα expression in the same subset, while in both groups its expression correlated IL-2 and Ki67. On the contrary, TLR8 stimulation consistently resulted in higher expression of CD69 on $CD8^+$ T cells of HESN, it expression positively correlated with the expression of proliferation marker Ki67 in both groups. HLA-DR was highly expressed by $CD8^+$ T cells of HESN following TLR8 stimulations, it expression correlated with the expression of IFN-γ and TNF-α production.

In summary, this correlation analysis of T cell activation and functional response data after TLR4, TLR7 and TLR8 stimulations of PBMCs, discovered higher number of positive

correlations between expression of T cell activation and functional markers on T cells in HESN compared to HIV-N. This suggests that selectively triggering different TLR pathways results in different outcomes with regard to T cell activation between groups of CSWs. This information may be relevant for understanding the quality of T cells responses needed in rotection against HIV acquisition among female CSWs.

5.4.4. Expression of TLR4 on HESN T cell subsets with TLR4, TLR7 or TLR8 stimulations

Expression of TLRs on T cells varies depending subset, cellular activation and tissue localization. Previously, peripheral T cells have been shown to express TLR1-10 mRNA but at lower levels compared to APCs[246, 372]. TLR expression on T cells also varied with cellular activation, evidenced by the higher expression of TLR8 on more activated $CD25^{high}$ $CD4^{+}$ T cells compared to $CD25^{-}$ $CD4^{+}$ T cells [367]. The expression of the TLRs on T cells has been shown to vary depending on the tissue localization, for instance tonsillar T cells express mRNA for TLR1, 2, 3, 4, 5, 9 and 10, unlike peripheral T cells that express TLR1-10. Differences in TLR expression between $CD4^{+}$ and $CD8^{+}$ T cell populations has also been descibed. The expression of TLRs on T cells can be enhanced by specific ligation[200].

In this study, we quantified the changes in expression of TLR4 on T cells, before and following stimulation with TLR4 specific ligand LPS, and non-specific ligands Imiquimod (TLR7) and ssRNA40/LyoVec (TLR8), using flow cytometry. TLR4 expression on T cells was assessed due to the linkage of its ligand-LPS with immune activation[373] in a mechanism possibly involving LPS drive enhancement of viral replication[374]. TLR7 and TLR8 quantification on T cells by flow cytometry was not possible during this study, due to the inherent difficulty of staining for both receptors for flow cytometry evaluation. Moreover, these experiments allowed for multiple evaluations on the effects of the three stimulations on

TLR4 expression on different T cells subsets, especially on $CD25^+$ (IL-1 receptor α) T cells associated with regulatory functions, in the two groups of CSWs. Therefore in this section we compared the expression of TLR4 on CD25 positive and negative cells, this comparison sought to determine the effect of TLR4 expression on activation and functional responses of T cells.

First, we compared the changes in frequencies of $CD4^+$, $CD4^+$ $CD25^+$, $CD4^+$ $CD25^-$, $CD8^+$, $CD8^+$ $CD25^+$ and $CD8^+$ $CD25^-$ T cells in PBMCs of HESN (n=20) and HIV-N (n=18) afther either TLR4, TLR7 or TLR8 stimulations. The results showed, prior to and after TLR4, TLR7 or TLR8 stimulations, all T cells subsets including $CD4^+$, $CD4^+$ $CD25^+$, $CD4^+$ $CD25^-$, $CD8^+$, $CD8^+$ $CD25^+$ and $CD8^+$ $CD25^-$ T cells, expressed TLR4 in both CSW groups, but to varying degrees (**Figure 18**).

Prior to any TLR stimulation, $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ T cells in either CSW group, had higher frequencies of cells expressing TLR4 when compared to $CD4^+$ $CD25^-$ T cells (HESN p=0.0104; HIV-N p=0.0203, Wilcoxon matched pair rank test) or $CD8^+$ $CD25^-$ T cells (HESN p=0.0004; HIV-N p=0.0004, Wilcoxon matched pair rank test) (**Figure 18A**). A comparison of TLR4 expression between the two study populations showed that HESN CSWs had lower frequencies of TLR4 expressing $CD4^+$ T cells (**HESN 3.14% ± 0.625** vs. HIV-N 7.53% ± 1.64, p=0.0138, Mann-Whitney test), $CD4^+$ $CD25^+$ T cells (**3.5% ± 1.035** vs. 13.38% ± 3.05, p=0.0155), $CD4^+$ $CD25^-$ T cells (**1.37% ± 0.53** vs.4.92% ± 1.39, p=0.0043) and $CD8^+$ $CD25^-$ T cells (**1.87% ± 0.56** vs. 4.15% ± 0.96, p=0.0397) compared to HIV-N (**Figure 18A**).

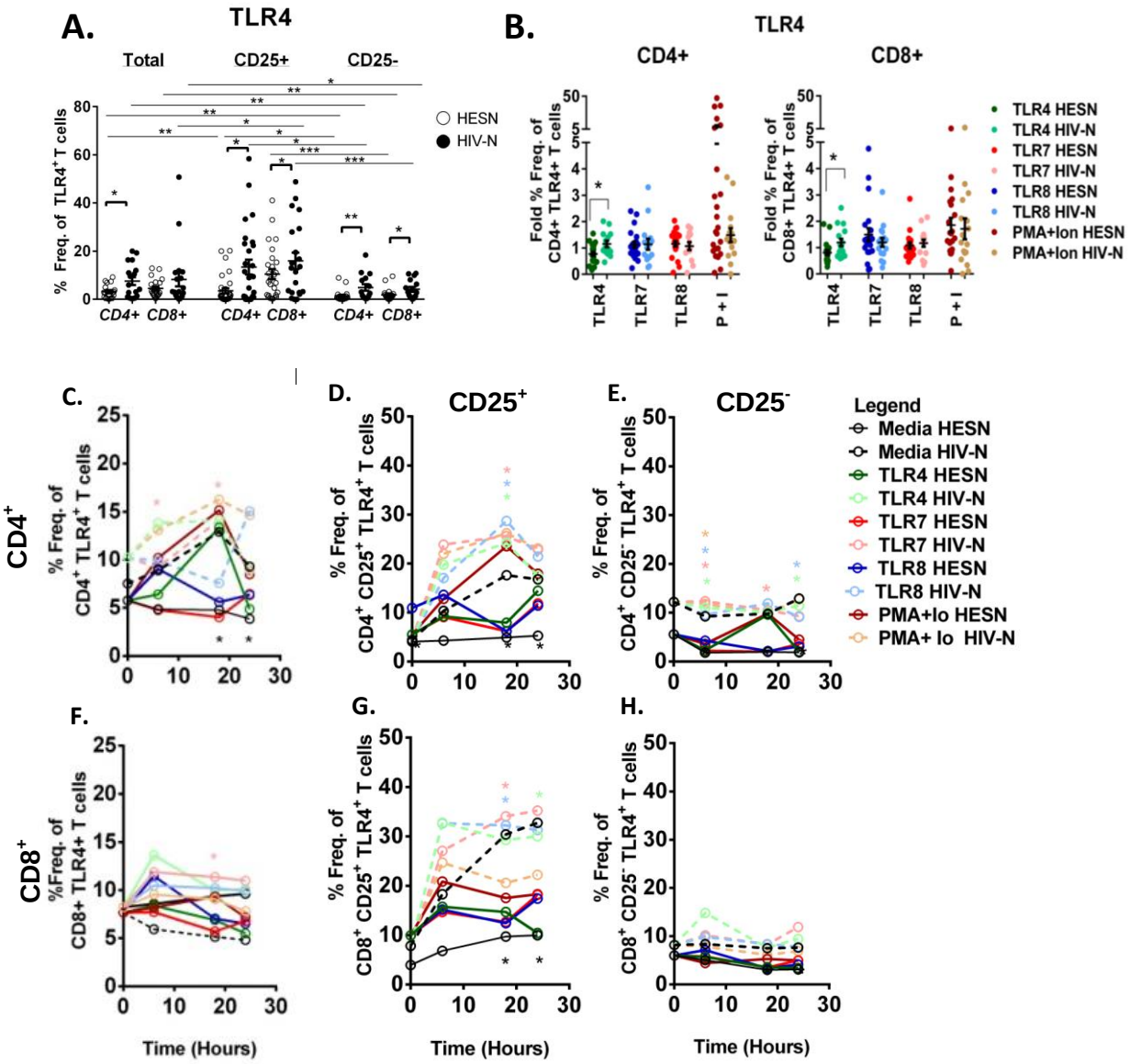


Figure 18: Expression of TLR4 on T cells subsets was quantified using flow cytometry. Baseline expression of (A) TLR4 on $CD4^+$, $CD4^+$ $CD25^{+/-}$, $CD8^+$ and $CD4^+$ $CD25^{+/-}$ T cells of HESN (clear/white) and HIV-N (black). B) Fold changes in TLR4 expression (C-H) Kinetics of TLR4 expression on $CD4^+$ and $CD8^+$ T cells with LPS-TLR4 (green), Imiquimod-TLR7 (red), ssRNA40/LyoVec-TLR8 (blue) and PMA with Ionomycin stimulations (brown). HESN represented by darker lines and HIV-N lighter dashed lines. Holm-Sidak method, with alpha=5.0%.

Next we tested the effect of specific and non-specific TLR stimulations, on the expression of TLR4 on T cells from the two groups of CSW. Once again, PBMCs were stimulated using LPS-TLR4, Imiquimod-TLR7, ssRNA40/LyoVec-TLR8 or PMA+ Ionomycin for between 20-24 hours. The expression of TLR4 on $CD4^+$ and $CD8^+$ T cells was quantified using multicolour flow cytometry, and the results are presented as fold changes in expression to demonstrate the actual changes in TLR4 expression with the different TLR stimulations (**Figure 18B**).

Only LPS-TLR4 stimulation, not TLR7 or TLR8 stimulations, resulted in significant differences in fold change in TLR4 expression in both $CD4^+$ and $CD8^+$ T cells of HESN, which had lower fold changes in TLR4 expression compared to controls (p=0.0237; 0.0135) (**Figure 18B**).

Next we used a kinetic assessment of TLR4 expression to determine the effect of the three TLR stimulations on TLR4 expression on T cells over the course of TLR4, TLR7 and TLR8 stimulations. Overall the pattern of TLR4 expression in T cell subsets during LPS (TLR4), Imiquimod (TLR7) and ssRNA40/LyoVec (TLR8) stimulation in the two groups, was similar to what was observed *ex vivo*. The expression of TLR4 was comparable on $CD4^+$ and $CD8^+$ T cells, but higher frequencies of $CD25^+$ T cells expressed TLR4 compared to $CD25^-$ T cells before and over the course of different TLR stimulations.

The frequencies of both $CD4^+$ $TLR4^+$ and $CD8^+$ $TLR4^+$ T cells, was lower in HESN over course of TLR4, TLR7 and TLR8 stimulations. In unstimulated T cells, HESN had lower frequencies of $CD4^+$ $TLR4^+$ T cells compared to controls between 18-24 hours of culture (**4.78 ± 0.99%** vs. 12.91 ± 3.65%, p=0.034; **3.85 ± 1.21%** vs. 9.28 ± 2.28%, p=0.039). Similarly, TLR7 led to lower frequencies of $CD4^+$ $TLR4^+$ between 6-18 hours of stimulation (**4.78 ± 0.98%** vs. 9.22 ± 1.88%, p=0.027; **4.04 ± 1.05%** vs. 14.15 ± 4.02%, p=0.017), and

lower frequencies of $CD8^+$ $TLR4^+$ T cells after 18 hours of stimulation in HESN compared to controls (**6.78 ± 2.71%** vs. 10.98 ± 4.28%, p=0.014) .

The frequency of $CD4^+$ $CD25^+$ $TLR4^+$ T cells in unstimulated PBMCs of HESN was similar to those of HIV-N between 0-6 hours of culture, but which reduced between 18-24 hours of culture compared to those of HIV-N (SEM: **4.89 ± 1.60%** vs. 17.62 ± 4.55%, p=0.0056; **5.26 ± 2.31%** vs. 16.78 ± 3.52%, p=0.011). TLR4, TLR7 and TLR8 stimulations of PBMCs all led to lower frequencies of $CD4^+$ $CD25^+$ $TLR4^+$ T cells in HESN after 18 hours of stimulation (SEMs **9.22 ± 3.17%** vs. 19.75 ± 6.09%, p=0.009; **6.19 ± 2.10%** vs. 25.62 ± 4.04%, p=0.0002; **6.186 ± 2.22%** vs. 28.71 ± 6.61%, p=0.003, respectively). HESN also had lower frequencies of $CD8^+$ $CD25^+$ $TLR4^+$ T cells after 24 hours of TLR4 ligand stimulation (SEMs: **10.44 ± 4.40%** vs. 30.03 ± 4.74%, p=0.006), lower frequencies of the same after 18 hours of TLR7 and TLR8 stimulation (SEM: **12.65 ± 4.17%** vs. 34.07 ± 4.72, p=0.002: **12.42 ± 4.15%** vs. 32.24 ± 5.41%, p=0.006), compared to those of HIV-N (**Figures 18D, 18E and 18H**).

Unstimulated PBMCs of HESN and HIV-N had similar frequencies of $CD4^+$ $CD25^-$ $TLR4^+$ T cells over the duration of culture. However, TLR4 stimulation led to lower frequencies of $CD4^+$ $CD25^-$ $TLR4^+$ T cells in HESN at 6 and 24 hours of stimulation (**2.27 ± 0.74%** vs. 10.99 ± 3.70%, p=0.015: **2.05 ± 0.65%** vs. 11.43 ± 3.50%, p=0.006). Non-specific TLR7 stimulation of HESN PBMCs resulted in lower frequencies of $CD4^+$ $CD25^-$ $TLR4^+$ T cells between 6 and 18 of stimulation (**2.23 ± 0.63** vs. 12.38 ± 4.86% , p=0.03; **2.00 ± 0.60%** vs. 10.53 ± 3.66%, p=0.03) and compared HIV-N. Similarly, TLR8 stimulation of HESN PBMCs led to lower frequencies of $CD4^+$ $CD25^-$ $TLR4^+$ T cells after 6 and 24 hours (**4.26 ± 2.02%** vs. 9.56 ±4.70, p=0.01: **3.08 ± 1.24%** vs. 9.13 ± 2.97, p=0.006) compared to controls (**Figures 18D, E and H**).

In summary, both $CD4^+$ and $CD8^+$ T cells had the highest expression of TLR4 on $CD25^+$ cells. Often the expression of TLR4 was found to be lower on T cells of HESN compared to HIV-N prior and over the course of specific TLR4 stimulation with LPS, and over the course of non-specific stimulation through TLR7 and TLR8.

Table 4: Spearman's correlational analysis comparing different T cell functional responses and TLR4 expression following various TLR treatments of HESN and HIV-N PBMCs

		CD107a				IFNγ				TNFα			
		CD4		CD8		CD4		CD8		CD4		CD8	
		HESN	NN	HESN	NN	HESN	NN	HESN	NN	HESN	NN	HESN	NN
Ex vivo	TLR4	0.3437	0.3663	0.7491	0.8042	0.0051	0.9210	0.0083	0.0708	0.0048	0.0003	0.0721	0.9388
TLR4	TLR4	0.5781	0.0004	0.0746	0.0290	0.0090	0.4417	0.6402	0.1376	0.0229	0.0173	0.0229	0.1471
TLR7	TLR4	0.0001	0.0001	0.0016	0.0042	0.0001	0.4239	0.0528	0.4117	0.0025	0.0007	0.0124	0.0301
TLR8	TLR4	0.0689	0.0004	0.1024	0.0968	0.0001	0.9299	0.2514	0.3947	0.0058	0.0016	0.0321	0.0778
PMA	TLR4	0.1522	0.0115	0.0114	0.0275	0.1876	0.2057	0.0033	0.5370	0.0026	0.0019	0.0215	0.0054
		IL-2				MIP1α				Ki67			
		CD4		CD8		CD4		CD8		CD4		CD8	
		HESN	NN	HESN	NN	HESN	NN	HESN	NN	HESN	NN	HESN	NN
ex vivo	TLR4	0.3517	0.0323	0.1224	0.357	0.4907	0.4708	0.9585	0.499	0.4153	0.8692	0.8481	0.5008
TLR4	TLR4	0.214	0.8388	0.2344	0.93	0.3754	0.0112	0.3131	0.0957	0.0989	0.9863	0.0926	0.0217
TLR7	TLR4	0.1185	0.2327	0.6481	0.525	0.9646	0.0459	0.7052	0.002	0.4439	0.3522	0.2468	0.6099
TLR8	TLR4	0.0143	0.1905	0.1338	0.443	0.0242	0.0472	0.2262	0.0215	0.496	0.8557	0.4539	0.0064
PMA+ I	TLR4	0.3089	0.1478	0.0002	0.02	0.9955	0.0254	0.555	0.0004	0.7785	0.8633	0.09	0.411

Highlighted boxes - $P< 0.05$

Given the differences in proportions of T cells expressing functional markers and the differences in TLR4 expression between HESN and HIV-N described above; we sought to establish if there was relationship between TLR4 expression and the functional responses on T cells HESN and HIV-N using correlational analyses (**Table 4**). The use of correlational analysis was necessitated by a separation of the panels used to quantify TLR4 expression and functional responses (ref. **Table 1**). The analysis compared the TLR4 expression on T cells subsets (**Figure 18)**, with data on intracellular cytokine expression, proliferation or degranulation of T cells (**Figures 16 and 17)** obtained from HESN and HIV-N PBMCs stimulated using TLR4, TLR7 and TLR8 at the same time point. Here we hypothesized that functional responses of T cells to TLR4, TLR7 and TLR8 ligands in either CSW group would be dependent on TLR4 expression in PBMCs of either CSW group.

This analysis revealed that *ex vivo*, the proportion of $CD4^+$ IFN-γ^+ T cells in HESN, $CD4^+$ IL-2^+ T cells in HIV-N, and $CD4^+$ TNF-α^+ T cells in both groups, positively correlated with the frequencies $CD4^+$ $TLR4^+$ T cells (**Table 4**). Following cognate stimulation with LPS, the frequencies of $CD4^+$ $TLR4^+$ T cells correlated with those of $CD4^+$ IFN-γ^+ T cells and $CD4^+$

TNF-α^+ in HESN, whereas in HIV-N it correlated with frequencies $CD4^+$ $CD107a^+$ T cells and $CD4^+$ TNF-α^+ T cells.

The frequency of $CD8^+$ $TLR4^+$ T cells positively correlated with those expressing IFN-γ and TNF-α in $CD8^+$ T cells in HESN, and with those expressing CD107a and TNF-α in HIV-N. TLR7 stimulation resulted in frequencies of $CD4^+$ $TLR4^+$ T cells that positively correlating with those of $CD4^+$ $CD107a^+$ T cells and $CD4^+$ TNF-α^+ T cells in both groups, but only with $CD4^+$ IFN-γ^+ T cells in HESN and $CD4^+$ MIP1α^+ in HIV-N. Similarly, TLR7 stimulation resulted in a positive relationship between frequencies of $CD8^+$ $TLR4^+$ T cells on one hand and $CD8^+$ $CD107a^+$ T cells or $CD8^+$ TNF-α^+ T cells on the other in both CSW groups, but only MIP1α in HIV-N. TLR8 stimulation resulted in frequencies of $CD4^+$ $TLR4^+$ T cells corresponding to those of $CD4^+$ TNF-α^+ and $CD4^+$ MIP1α^+ T cells in both CSW groups. However, the frequencies of $CD4^+$ $TLR4^+$ T cells in TLR8 stimulated PBMCs corresponded to those $CD4^+$ IFN-γ^+ and $CD4^+$ IL-2^+ T cells in HESN, and $CD4^+$ $CD107a^+$ T cells in HIV-N. Lastly, the frequencies of $CD8^+$ $TLR4^+$ T cells corresponded to those of $CD8^+$ TNF-α^+ T cells in HESN and $CD8^+$ $Ki67^+$ T cells in HIV-N (**Table 4**).

These correlational analyses, revealed a vast number of the positive correlations between frequencies of TLR4 expressing T cells corresponding to those of IFN-γ expressing T cells, especially considering HESN had higher IFN-γ responses to most TLR treatments particularly TLR8 when compared to HIV-N. These findings indicate that TLR stimulation might increase cellular protein synthesis leading to increased transcriptional activity; however such activation is not random, but dependent on TLR expression and subsequent TLR signal transduction.

5.4.5. Role of T cell regulation in HESN responses to TLR stimulation

The immune regulation of inflammatory responses during an infection prevents tissue injury potentially resulting in chronic inflammatory or autoimmune diseases[225]. The cytokine environment established upon exposure to microbial components has been shown to have a bearing on ability of HIV infect target cells. An assessment of the cytokine milieu of HESN PBMCs before and following TLR4 and TLR8 not TLR7 stimulations, revealed a higher number relationships between certain cytokine responses and IL-10 in HESN when compared to in HIV-N (**Figure 9**). This suggests that a tighter regulation of cytokine responses in HESN PBMCs in the absence of TLR stimulations or following TLR4 or TLR8 stimulations. Moreover, IL-10 responses were higher with TLR8 stimulation but lower in response to TLR4 and TLR7 stimulations (**Figures 5-8**).

Next we investigated if the higher IL-10 responses or the more balanced cytokine responses in HESN cytokine corresponded to higher frequencies of regulatory T cell ($CD4^+$ $CD25^+$ Forkhead box $P3^+$). This led us to hypothesize that, higher frequencies of regulatory T cell and higher production of IL-10 by regulatory T cells in HESN, corresponded to the tighter regulation of cytokine responses *in vitro.* To test this hypothesis we compared the frequencies of IL-10 producing $CD4^+$ and $CD8^+$ T cells, and further broke down this comparison to IL-10 production into different T cells subsets expressing or lacking either CD25 or FoxP3. This is considering that the level of expression of both have been linked to increased or decreased T_{reg} suppressor activity[375]. The regulation of proinflammatory cytokines through IL-10 production can be performed by different T cell populations depending on immunological environment, dictated by the cytokine milieu and tissue localization[375, 376]. Once more, PBMCs from HESN (n=11) and HIV-N (n=10) CSWs were stimulated for 20-24hrs using TLR4, TLR7, TLR8 or PMA with Ionomycin, and the expression of CD25, Foxp3, CD107a and IL-10 expression.

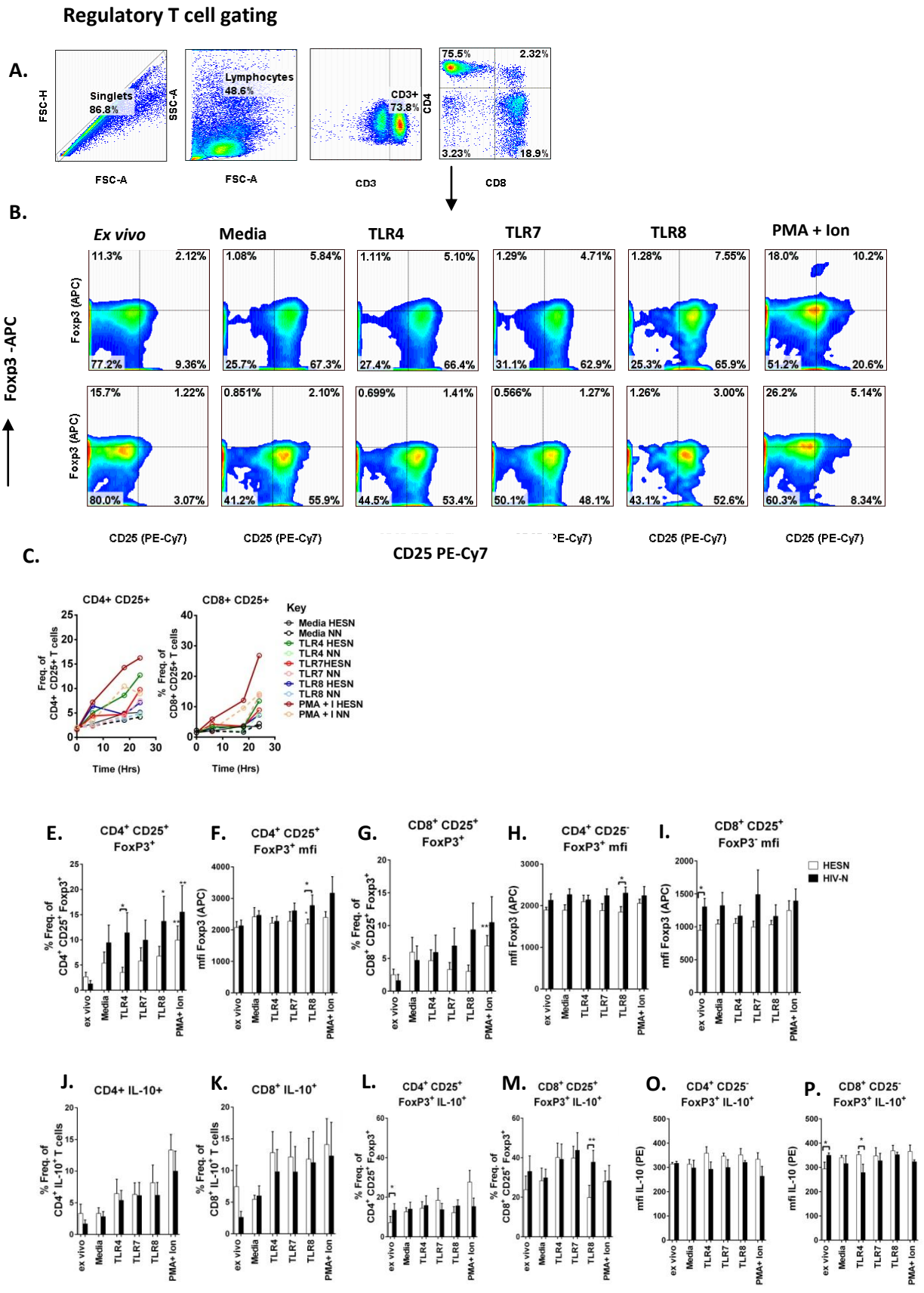


Figure 19. Regulatory T cell responses to TLR stimulation in HESN as quantified by 10-colour flow cytometry from PBMCs of HESN and HIV-N isolated from heparinized blood, then treated with either *E. Coli* LPS- TLR4, Imiquimod-TLR7, ssRNA40/LyoVec-TLR8 or PMA with Ionomycin overnight. A) The gating of $CD4^+$ $CD25^+$ $Foxp3^+$ T_{regs}, $CD4^+$ $CD25^+$

$Foxp3^-$, $CD4^+$ $CD25^-$ $Foxp3^+$, $CD8^+$ $CD25^+$ $Foxp3^+$, $CD8^+$ $CD25^+$ $Foxp3^-$ and $CD8^+$ $CD25^-$ $Foxp3^+$ T cells is shown in, while the changes in these subsets in TLR stimulation in (B-I). The production of IL-10 by the different T cells subsets was quantified by ICS (see methods) (J-P). Pair-wise comparisons were done using Wilcoxon *t*-test, while unpaired comparisons done using Mann Whitney *t*-test. *P* values determine* P <0.05, ** P <0.01, *** P <0.001.

The gating used to define the different T cell populations was based on CD25 and Foxp3 expression is indicated in **Figure 19A**. The results revealed that the expression CD25 and Foxp3 on both T cell subsets increased in some individuals but not all with TLR4, TLR7 and TLR8 stimulations (**Figures 19A-C)**, in line with earlier descriptions of similar increases in expression of both markers on T cells with cellular activation *in vitro*[377].

The number of T_{regs} or $CD4^+CD25^+$ $Foxp3^+$ T cells in resting PBMCs of HESN individuals trended to be higher than in HIV-N (**2.65 ± 0.92**% vs. 1.25 ± 0.67%, p= 0.06), similar to what had previously been described in the same cohort[309]. TLR4 stimulation did not increase numbers of T_{regs} in either groups when compared to unstimulated T cells in media. Although significantly lower numbers $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (**3.52 ± 1.03%** vs. 11.39 ± 4.00%, p=0.03) and a lower surface expression of Foxp3 on $CD4^+$ $CD25^-$ T cells (MFI: **2192 ± 146.3** vs. 2774 ± 329.1, p=0.0387) was seen in HESN PBMCs following TLR8 treatment (**Figures 19E-H**). The surface expression Foxp3 on $CD8^+$ $CD25^+$ T cells of HESN was also lower prior to TLR treatments (MFI: **947.6 ± 77.28** vs. 1304 ± 126.4, p=0.0317), but there was no difference in its expression following TLR4, TLR7 or TLR8 treatments between the two groups.

The expression of IL-10 in $CD8^+$ T cell subsets increased after TLR4, TLR7, TLR8 and PMA+ Ionomycin treatments, with higher frequencies of $CD8^+$ T cells expressing IL-10 between the two T cell subsets (**Figures 19J-L**). *Ex vivo* the proportion of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (T_{regs}) expressing IL-10 (**7.19 ± 3.15%** vs. 13.44 ± 3.34%, p=0.0428) and those expressing IL-10 on $CD8^+$ $CD25^-$ $Foxp3^+$ T cells (MFI: **293.6 ± 28.16** vs. 350.0 ±

9.609, p=0.0247) was lower in HESN. TLR4 treatment resulted in higher IL-10 expression by $CD8^+$ $CD25^-$ $Foxp3^+$ T cells of HESN (MFI: **352.6 ± 12.18** vs. 278.5 ± 34.63, p=0.03), while TLR8 treatment resulted in higher IL-10 production by $CD8^+$ $CD25^+$ $Foxp3^+$ T cells in the same group compared to HIV-N (**19.81 ± 6.27**% vs. 37.80 ± 5.92%, p=0.0049) (**Figures 19M-P**).

Taken together, the level of IL-10 production in response to different TLR stimulations by the different T cell subsets including T_{regs} was modest. This corresponded to what has been previously reported in the literature, showing that cells from the monocyte/macrophage lineage are the most prolific producers of IL-10 in responses to pathogen derived components[378]. The production of IL-10 in monocytes was evaluated in this study (the data presented later), and indeed the responses in those cells were more robust compared to those observed to in T cells. However, it is important to note there were differences in the proportions of different T cell populations producing IL-10 and in the proportions of regulatory T cells, particularly of interest were the differences observed following stimulations with HIV analogous ssRNA-TLR8.

Next we sought to understand the relevance of the differences in T_{reg} proportions and IL-10 producting cells, with regard to other T cell functional responses associated with anti-HIV activity. Previous studies have linked $CD8^+$ T cells and NK cell degranulation in response to TLR8 stimulation with HIV inhibition *in vitro,* whereas induction Ca^{2+} ions influx in $CD4^+$ T cells by Imiquimod-TLR7 has recently been shown to induce anergy in T cells capable of enhancing replication in $CD4^+$ T cells[248, 264]. We hypothesized that IL-10 production matched inflammatory processes like degranulation, thereby modulating the excessive inflammatory responses by T cells when stimulated with different TLR agonists *in vitro*. To test this hypothesis we conducted a pair-wise correlational analysis comparing the IL-10

expression to degranulation of T cells in samples stimulated with TLR4, TLR7 or TLR8 (**Figure 20**).

Overnight treatment of PBMCs with TLR7 ligand resulted in a lower expression of CD107a on $CD8^+$ T cells of HESN compared to HIV-N, corresponding to what was observed over the course of TLR7 stimulation in kinetic experiments (**Figures 16J and 20A-D**). There no correlation between CD107a expression and IL-10 production in resting $CD4^+$ and $CD8^+$ T cells (this analysis combined HESN and HIV-N data). On the contrary, TLR4, TLR7, TLR8, but not PMA + Ionomycin treatments of PBMCs from both groups resulted in IL-10 production in $CD4^+$ T cells that corresponded $CD4^+$ T cell potential for degranulation (**Figure 20E)**.

The IL-10 expression appeared to be elevated in T cells following exposure to TLR4, TLR7 or TLR8 ligands, but not in resting cells, corresponding to cellular degranulation, possibly as consequence of induction of homeostatic regulatory processes. TLR recognition and signalling has been shown to result in activation of the MAPKs and JNK pathways leading to increased STAT3 activity and IL-10 production, while cellular degranulation is controlled by NF-kB signalling, thus indicating both processes may be as a consequence of TLR signalling and homeostatic processes.

The differences in correlation of CD107a expression and IL-10 production by $CD4^+$ T cells but not $CD8^+$ T cells, is difficult to explain without further experimentation (**Figure 20E**). Considering $CD8^+$ T cells degranulation has previously been implicated in anti-HIV activity following TLR8 stimulations, it appears IL-10 may not be involved in regulation of such responses in the generation of anti-HIV activity. IL-10 responses and $CD4^+$ T cell degranulation appear to go hand in hand therefore we speculate that this may be related to the ability of these cells to induce direct anti-HIV responses.

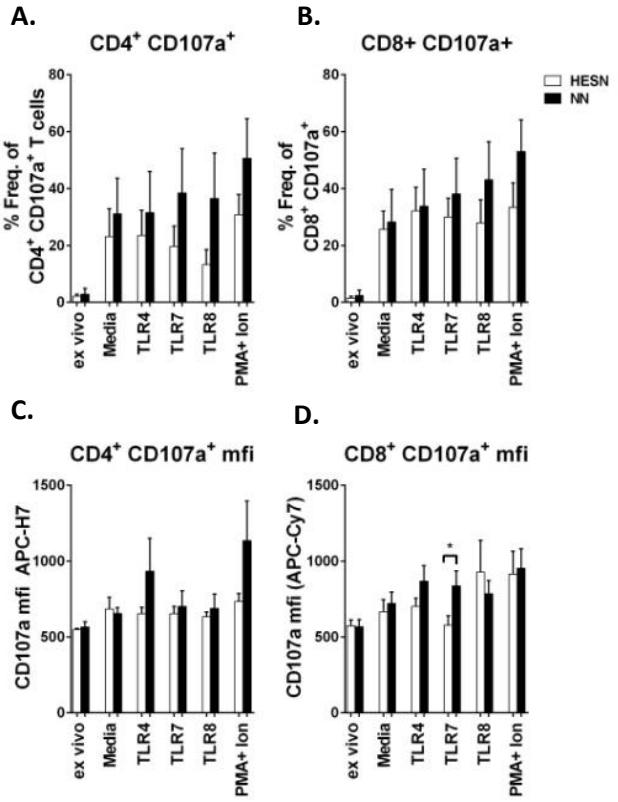
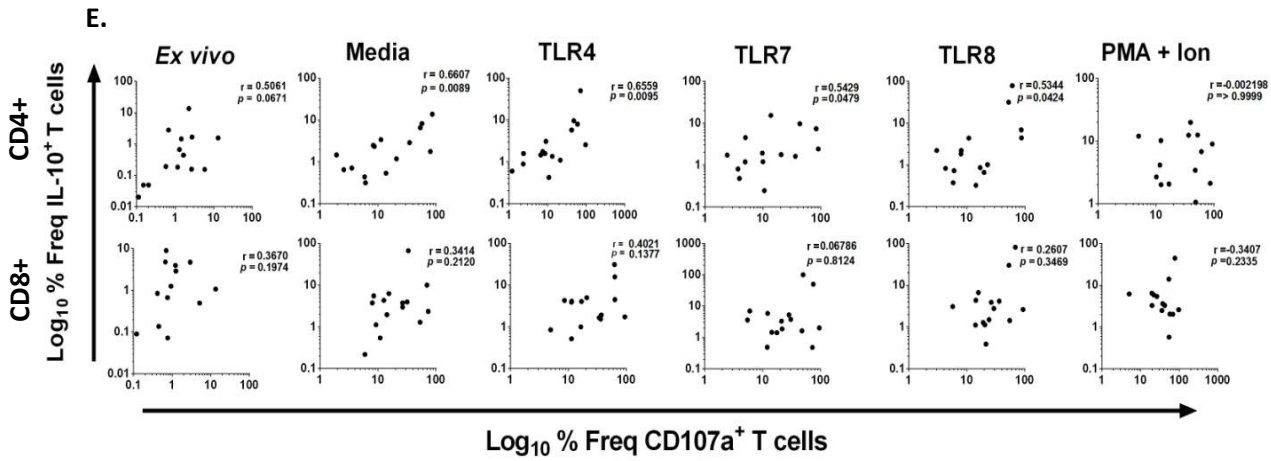


Figure 20: *In vitro* T cell degranulation and the relationship to IL-10 expression on T cells. (A-D) Flow cytometry was used to measure degranulation based on expression of Lysosomal associated membrane protein 1 (LAMP1 or CD107a) and IL-10 production, both were measured by intracellular cytokine staining from *E. Coli*-LPS (TLR4), Imiquimod (TLR7), ssRNA40/LyoVec (TLR8) and PMA + Ionomycin stimulated PBMCs of HESN (white bars) and HIV-N (black bars). Spearman's correlation was used analysis of the relationship between CD107a expression and IL-10 production by T cells.



5.4.6. Expression of HIV co-receptor CCR5 on target cells ($CD4^+$ T cells) of HESN with TLR stimulation

The final set of experiments in this chapter evaluated the consequences of higher TLR8 responsiveness and lower TLR4 or TLR7 responsiveness by HESN PBMCs, on the potential of susceptibility of $CD4^+$ T cells based on expression of HIV-co-receptor CCR5 used by HIV to infect $CD4^+$ T cells [19-21]. A CCR5 specific antibody was used to quantify the receptor expression in $CD4^+$ and $CD8^+$ T cells by flow cytometry in PBMCs stimulated using TLR4, TLR7, TLR8 or PMA with Ionomycin (**Figure 22**).

The comparison of CCR5 expression on $CD4^+$ T cells, before and following TLR4, TLR7 or TLR8 stimulations, revealed no differences in expression between the two groups (**Figure 21A**). Possibly indicating that HESN T cells may have higher $CD4^+$ T cell activation following LPS stimulation which does not increase the susceptibility of $CD4^+$ T cells to HIV based on CCR5 expression. However, additional experiments are needed to confirm the effect of TLR4, TLR7 or TLR8 stimulations no HIV's infectivity on HESN CD4+ T cells. Such experiments may use pure or sorted $CD4^+$ T cells separated from HESN PBMCs infected with HIV in vitro following the three TLR stimulations.

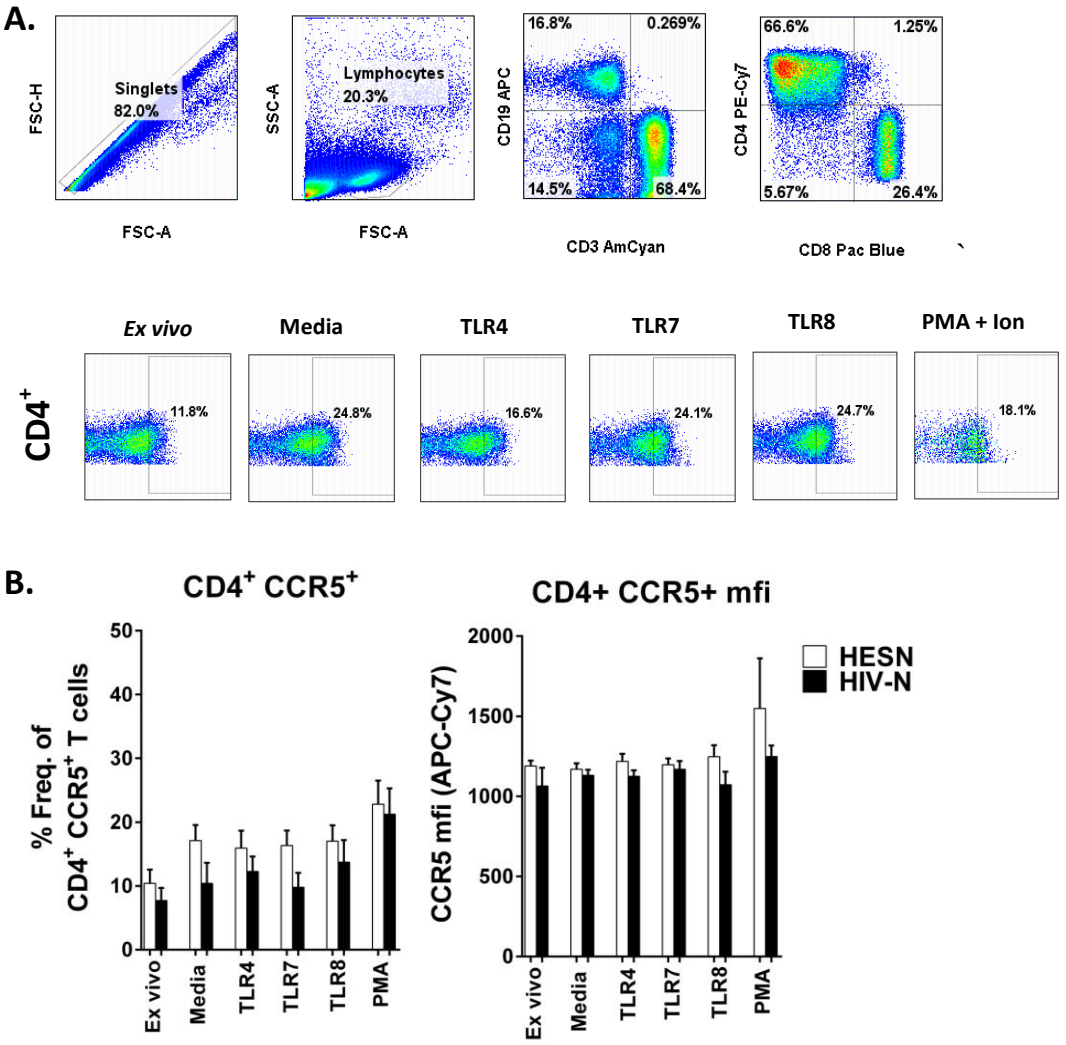


Figure 21. Expression of CCR5 on T cells with TLR stimulation. A) Flow cytometry gating strategy used to quantify the frequency of $CCR5^+$ cells and surface expression using mean fluorescence intensity in $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells in PBMCs from HESN (White bars) and HIV-N (black bars). Comparison between groups done using Mann-Whitney test, all P <0.05 are indicated using an asterisk (*).

5.5. Summary and Discussion

Immune quiescence in resting T cells is a recurring theme in most of the studies conducted on the peripheral blood and genital tracts of HESN CSWs from Pumwani. The lack of HIV infection despite repeated exposure has been linked to the 'quiescent' phenotype of $CD4^+$ T cells in HESN individuals, based on the lower expression of activation marker CD69 and lowere cellular activation of gene transcripts accompanied by reduced susceptibility of these cells to HIV infection *in vitro*[309, 333-335].

In this study, we observed lower production of proinflammatory cytokine TNF-α, lower expression of cellular degranulation marker CD107a, and lower frequencies of $CD8^+$ $CD69^+$ and TLR4 expression in unstimulated T cells of HESN. However, for the first time we described higher frequencies $CD4^+$ $CD69^+$ T cells after TLR4 stimulation, and equally higher frequencies of $CD8^+$ $CD69^+$ T cells following TLR7 and TLR8 stimulation in HESN. Thus, revealing a higher responsiveness by HESN $CD8^+$ T cells to TLR7 or TLR8 stimulations, and higher $CD4^+$ T cell responsiveness to TLR4 at multiple sampling time points. The higher $CD8^+$ T cell responsiveness in HESN was demonstrated by the higher changes in expression of activation markers CD69 and HLA-DR, and production of IFN-γ in this subset in response to TLR7 or TLR8 stimulations. The quantification of T cell activation with TLR stimulation was done in samples obtained from multiple time points, and the patterns of responses remained consistent, indicating that the pattern of responses observed may be representative of the HESN phenotype. Some of the responses quantified by flow assessment of T cell functions, were confirmed using other methods like multiplex bead arrays or RT-PCR. One such example, was the higher production of IFN-γ in response TLR8 stimulation; this was observed in $CD8^+$ T cells as quantified by flow cytometry, and from culture supernatants when quantified using multiplex bead arrays (see chapter 4). The confirmation of the differences between HESN and HIV-N responses to different TLR stimulations, using

multiple experimental techniques and at various sampling time-points, strengthened the deductions made from these results.

It is logical to assume that the level of TLR expression on responding T cells corresponded to activation of intracellular signalling pathways and resulting cellular functions. In the chapter, we described that this was not always the case, for instance, HESN $CD4^+$ T cells had consistently higher activation based on CD69 expression in both fresh and frozen PBMC samples, and in response to TLR4 ligand LPS, yet the same group had lower expression of TLR4 on T cells over the course of cognate stimulation. The level of TLR4 expression and cellular activation appeared to bear no relationship in HESN individuals, particularly in the $CD25^+$ T cells, the T cell subset with the highest expression of TLR4. The expression of TLR4 highly corresponded to cytokine and chemokine responses, along with the potential for cellular degranulation and proliferation in CD4 and CD8 T cells with various TLR stimulations. A major limitation of to this study was the inability to quantify endosomal TLR7 and 8 by flow cytometry; however, expression of the same was evaluated in bulk PBMCs (presented in **chapter 4**). However, recent developments in fluorescent labelling of TLR7 and 8 will make such quantification by flow cytometry possible in the future. In spite of this, we were able to demonstrate differences in specific and non-specific effects of different TLR stimulations on T cell expression of TLR4 between the two CSW groups. Using correlation analyses of TLR4 expression and functional responses to TLR4, TLR7 and TLR8 stimulations, we demonstrated a vast number of the positive correlations between the frequencies of TLR4 expressing cells and those of IFN-γ expressing cells. This was intriguing considering that HESN had higher IFN-γ responses to most TLR treatments, particularly after TLR8 stimulation compared to HIV-N (**Table 4**). The subtle differences in the relationships between the expression of functional markers-such as cytokines, or markers for potential of proliferation and degranulation with TLR4 expression on T cells, depended on the TLR

pathway triggered and on the sex-work group. In addition, these observations contributed to the growing knowledge on the differences in cellular outcomes arising from specific triggering of either TLR7 or TLR8 pathways. Occasionally, we observed subtle difference between T cell and overall PBMC responses, these were related to secondary activation of lymphocytes such as T cells, by cells that primarily respond to TLRs, typically APCs. This is similar to the recently described 'two tiered' immune response, in which APCs (monocytes, macrophages and DCs) initially respond to PAMPs or DAMPs forming the 1^{st} Tier of the immune response. This eventually activates different lymphocyte populations including T cells, NK cells, innate lymhocytes and innate lymphoid cells, which form a 2^{nd} tier of the immune response[197].

In the previous chapter, the cytokine milieu of HESN PBMCs was observed to be more tightly or co-ordinately regulated given the higher number of positive correlations with or without TLR4 or 8 stimulations. Generally, the cytokine microenvironment created by innate recognition of microbial PAMPs, influences the nature, quality and magnitude of T cells helper responses[366]. Activation of TLR signalling pathways drives both inflammatory and anti-inflammatory processes which function to modulate deleterious injury to the tissues and organs[225]. Regulatory T cells produce IL-10 and transforming growth factor-beta (TGF-β) both of which play an important role in cell-extrinsic regulation of inflammatory responses, that is often autocrine-paracrine in nature[225]. This study evaluated the production of IL-10 from T cells in the peripheral blood, focusing on the IL-10 responses by T_{regs} and other T cell subsets expressing or lacking Foxp3 or CD25. The production of IL-10 by $CD4^+$ and $CD8^+$ T cells with TLR stimulation was modest, rarely exceeding the background (media) levels of production, however, overnight culture of T cells significantly increased the IL-10 production levels above what was seen in resting cells (ex vivo). Considering that T cells are primarily involved in the adaptive immune response their role in pathogen recognition or innate

responses, may be limited compared to APCs such as monocytes, DCs and neutrophil[379]. There were no differences in production IL-10 between bulk CD4 and CD8 T cells of HESN and HIV-N. Although a finer examination of IL-10 responses in T cells subsets based on Foxp3 and CD25 expression, revealed the lower production of IL-10 by HESN $CD4^+$ $CD25^+$ T_{regs}, in spite of tending to have higher numbers of $CD4^+$ $CD25^+$ T_{regs} *ex vivo*. TLR4 and TLR8 stimulations resulted in significantly lower numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ T_{regs} in HESN, while TLR8 stimulations only caused lower expression of Foxp3 on $CD4^+$ $CD25^-$ T cells in the same group. The expression of Foxp3 is crucial for the suppressor activity of T_{regs}[380], as such its lower expression on $CD4^+$ T cells with either TLR4 and TLR8 stimulation could be indicative of lower T_{reg} suppressor activity in HESN cultures, enabling more robust activation of $CD4^+$ and $CD8^+$ T cells of HESN by TLR8 stimulation compared to those of HIV-N. The lower expression of Foxp3 on $CD4^+$ $CD25^-$ T cells, the higher proliferation in both T cell subsets and higher production of IFN-γ responses by $CD8^+$ T cells of HESN, lends credence to the deduction that loss of Foxp3 expression is accompanied by higher T cells responses *in vitro*, as previously described[381]. In this study, resting T cells had IL-10 production that was not related to $CD4^+$ and $CD8^+$ T cell degranulation, however with TLR4, 7 and 8 stimulations, the levels of cellular degranulation matched the expression of IL-10. This indicated that in our experimental set up, increases in a proinflammatory process (cell degranulation) could have resulted in the induction of anti-inflammatory IL-10 production possibly triggered by TLR signalling. An alternate explanation would be that, the induction of both T cell functions- degranulation and IL-10 production- was as a result of TLR signalling driven processes. Determination of the mechanism in play will require further experimentation, but what is evident is the kicking in of proportionate IL-10 production with the increase of an inflammatory process, consistent with previously described mechanisms of IL-10 response induction (as discussed above).

The discussion on the possible regulatory role of $CD25^+$ on T cells in both subsets cannot go without mention of the higher TLR4 expression in this subset, where this higher expression of both TLR4 on $CD25^+$ T cells coincided with higher expression of activation markers CD38, CD69 and HLA-DR (data not shown).

In conclusion, we demonstrated that resting T cells of HESN were 'quiescent' or had lower expression of CD107a and TNF-α, but had higher $CD4^+$ T cell activation after TLR4 stimulation and higher $CD8^+$ T cells to TLR7 and TLR8 stimulations based on CD69 and HLA-DR expression. *Ex vivo* HESN T cells had lower potential for degranulation based on CD107a expression and lower proinflammatory TNFα expression, but had higher frequencies of cells expressing IFN-γ following with TLR4, TLR7 or TLR8 stimulations. On the contrary HESN had lower TLR4 expression following cognate ligand LPS stimulation, and during TLR7 or TLR8 stimulations. The expression of TLR4 on T cells of HESN had mixed relationships with the expression of T cell activation and functional markers. Lastly, the heightened $CD4^+$ T cell activation potentially did not enhance the susceptibility to HIV-1 based on expression of HIV co-receptor CCR5. Taken together, these results suggest that the processes governing T cell activation, functions and TLR expression in HESN are more very complex, probably influenced by the cytokine and chemokine responses of other cells contained in PBMCs. More importantly, there were distinct differences between HESN and HIV-N T cell responses to different pathogen derived components.

C**hapter 6: Higher Monocyte activation but lower Dendritic Cell Responses in HESN after TLR4, 7 and 8 stimulations.**

6.1. Rationale

Monocytes are the primary drivers of inflammation during infection, while dendritic cells play a sentinel role of sampling, processing and presenting antigens to T cells leading to the development of adaptive immune responses[366]. Monocytes and DCs are both heterogeneous populations of cells that can be infected by HIV, albeit to a lesser degree compared to the primary target cells- $CD4^+$ T cells[382]. The lowered susceptibility of monocytes/macrophages to HIV infection has been linked to the higher expression of host restrictions factors-SAMDH1, VIPERIN and APOBEC3G[111, 112]. Currently, the role of monocytes during HIV infection is poorly understood, primarily due the challenge of isolating HIV infected monocytes. As such the majority of studies seeking to understand the role of monocytes during HIV pathogenesis have been conducted in humanized mice or NHPs[382]. Despite expressing host restriction factors, HIV can still infect cells of mononcyte/macrophage lineage aided by a promiscuous usage of multiple chemokine co-receptors for viral entry[117, 118]. Cell specific HIV's co-receptor usage and the ability of monocytes to exit blood vessels and differentiate into macrophages, facilitates the spread of HIV from peripheral circulation into tissues such as the lungs[117] and brain [118]. HIV infection also causes changes in proportions and distribution monocyte and DC subsets, increasing the frequencies of certain subsets such as $CD14^{dim}$ $CD16^+$ which are associated with disease progression[382, 383]. However, the role of different monocyte subsets in protection or susceptibility against HIV acquisition is largely unknown.

Recent studies conducted in mice, have shown that non-classical or $CD14^{dim/-}$ $CD16^+$ monocytes, patrol and sense of HIV ssRNA through TLR8, and the same subset possessed

the greatest phagocytic capacity among monocyte subsets[378, 384]. $CD14^{dim}$ $CD16^{-}$ monocytes are also preferentially infected by HIV during sustained viral suppression by ART, and harbour variants of genetically distinct viruses compared those present in $CD4^{+}$ T cells[385]. It is not clear if the enhanced infection of this subset is linked to the virus sensing role played by $CD14^{dim}$ $CD16^{+}$ monocytes. Alternatively, the higher phagocytic potential in this subset accompanied by alterations in cellular functions such as pattern recognition, antigen processing or presentation during HIV disease, may increase the susceptibility of this subset to HIV infection. The ability of monocytes to recognize HIV derived PAMPs like ssRNA through TLR7 or TLR8 leading to upregulation of co-stimulatory factors or production of cytokines by APCs, may also be essential for the development or activation of anti-HIV innate and adaptive mechanisms[283, 325, 326, 329, 386].

Toll-like receptor mediated activation of APCs, enhances the expression of co-stimulatory factors and their ligands, where CD40-CD40L co-stimulation can induce macrophages to produced β- chemokines like CCR2 and CCR5 capable of anti-HIV activity[387]. The expression of CD40L on T cells increases with cellular activation, and CD40-CD40L interaction is important for generation of antiviral humoral responses against VSV, HSV and influenza virus through B cell licensing [388-390]. CD40-CD40L interactions were equally crucial for the induction of β chemokines in macrophages leading to recruitment of antiviral $CD8^{+}$ T cells to the sites of viral infection[391]. The expression of CD40 on T cells albeit low has previously been described, although its relevance in defence against viral infections particularly HIV, remains to be explored[392]. B cells can also express costimulatory molecules and function as antigen presenting cells when activated by T cells.

Dendritic cells function as sentinels of the immune system, constantly sampling tissues for foreign antigens and homing to lymphoid organs to present such antigens to resident T cells.

HIV infection of DCs is facilitated by a large number of co-receptors, but HIV typically infects mDC (cDCs) using C-type lectins such as DC-SIGN, Langerin and DCIR or CLEC4A[139, 393, 394]. On the contrary, pDCs are not frequently infected by HIV, but these cells can bind to HIVgp120 using the CD4 receptor expressed on them[145]. The fate of HIV virions following internalization by mDCs, is dependent on the co-receptor used during viral entry. When infection of mDCs occurs through DC-SIGN or DCIR the internalized virions are not degraded, leading to creation of viral synapses that facilitate trans-infection of $CD4^+$ T cells[138, 139]. On the contrary, when HIV infection of mDC occurs through Langerin, the virions are targeted to Birbeck granules where the virions are degraded[143]. HIV infection fails to induce maturation of infected immature DCs, this despite the virus being able to replicate in such cells. DC maturation is dependent on activation of TLR dependent signalling pathways; however, exposure to mDCs and pDCs to HIV *de novo* differentially affects the maturation of the mDCs but not pDCs. The failure of mDCs to mature when exposed to HIV has been associated with lower TLR7 signalling in them as compared to pDCs. Higher activation of pDCs during pathogenic HIV (human) or SIV-macaque infections but not non-pathogenic SM/AGM SIV infections, has been linked to TLR7 driven deleterious chronic immune activation responsible for progression to AIDS.

In this chapter, we evaluated TLR4, TLR7 and TLR8 responses in different monocytes and blood DCs subsets, found in peripheral blood of HESN and HIV-N CSWs. This evaluation was motivated by the differences observed in cytokine responses generated by TLR4, TLR7 and TLR8 stimulations of CMCs from the genital tract of HESN compared to those of HIV-N CSWs[294]. We sought to determine if triggering TLR signalling in monocytes and DCs could potentially alter subset proportions, cellular activation based on expression of co-stimulatory factors and on expression of cytokines in HESN and HIV-N.

6.2 Hypothesis

Antigen presenting cells from HESN (Monocytes, DCs and B cells) are more responsive to TLR8 stimulation based on expression of co-stimulatory factors, cytokines and chemokines when compared to those of HIV-N controls *in vitro.*

6.3 Objectives

1. To compare changes in frequencies of different monocyte and DC subsets in PBMCs with TLR4, 7 or 8 treatments between HESN and HIV-N.
2. To compare the changes in expression of co-stimulatory molecules CD40, CD80, CD86 and CD83 on monocytes and DCs with TLR4, 7 and 8 stimulations between PBMCs of HESN and HIV-N.
3. To compare the changes in expression of chemokine receptors and ICAM1 on different monocyte subsets with TLR stimulation between the two groups.
4. To quantify and compare the cytokine expression in monocytes and DCs following TLR4, 7 or 8 stimulations in PBMCs of HESN and HIV-N CSWs.
5. To compare the expression of CD40 and CD40L on lymphocytes (T cells and B cells) with TLR stimulation in HESN and HIV-N.

6.4 Results

6.4.1. Changes in Monocyte Subsets of HESN with TLR stimulation

First we compared the changes in frequencies of $CD14^+$ $CD16^-$ (classical), $CD14^+$ $CD16^+$ (intermediate) and $CD14^-$ $CD16^+$ (non-classical) monocytes in the two study populations with TLR4, TLR7, TLR8 or PMA with Ionomycin treatments (**Figure 22**). This assessment sought to understand the impact of TLR4, TLR7 or TLR8 stimulations on proportions of monocyte subsets. Here, freshly isolated PBMCs of HESN (n=16) and HIV-N (n=16) suspended in R10 media, were stimulated for 6 hours, or over a time course for 6, 18 or 24 hours with LPS-TLR4, Imiquimod-TLR7, ssRNA/LyoVec-TLR8 or PMA + Ionomycin (described in **section 3.3.2**). These assessments were done at multiple sampling time points- 1st period (October 2011- January 2012), and 2nd period (May-August 2014).

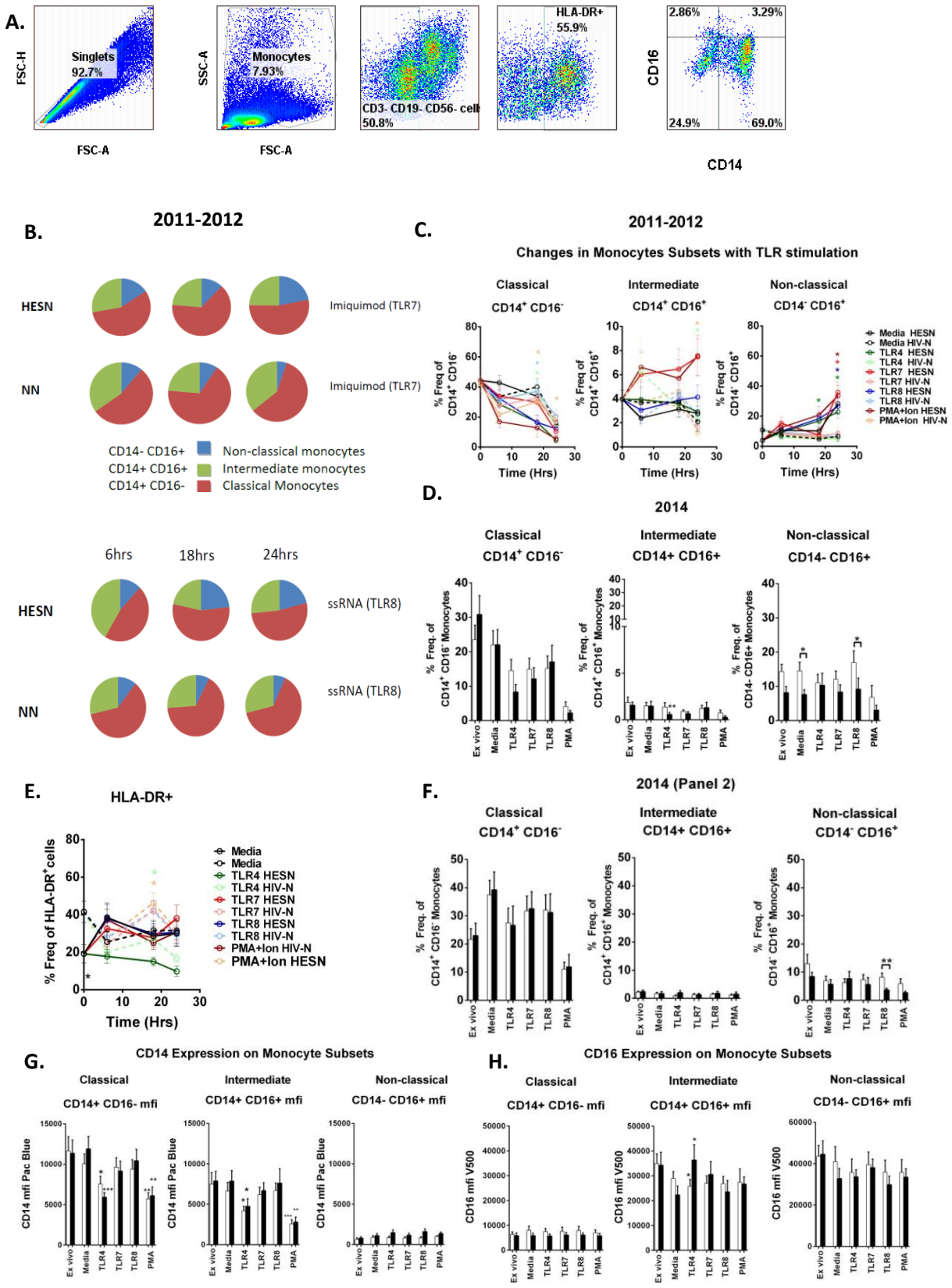


Figure 22. Changes in Monocytes subsets with TLR stimulation in PBMCs of HESN and HIV-N. Monocyte subsets in PBMCs were distinguished using multi-color flow cytometry based on expression of CD14 and CD16 into; classical ($CD14^+$ $CD16^-$), intermediate ($CD14^+$ $CD16^+$) or non-classical monocytes ($CD14^-$ $CD16^+$). A) The gating

scheme used to characterize the monocytes is shown, (B) pie charts demonstrating the changes in monocyte subsets (each colour sections represents a monocytes subset) over the course TLR7 (Imiquimod) and TLR8 (ssRNA40/LyoVec) stimulations. C) Line graphs represent the changes in monocytes with TLR4 (green lines), TLR7 (red lines), TLR8 (blue) or PMA with Ionomycin (brown) stimulations, HESN (darker colours) and HIV-N (lighter colours). Significant differences (P <0.05) are indicated with asterisks (*) bearing the colour of the group with the highest mean. F-H) The differences in monocyte subsets in HESN (white) and HIV-N (black) PBMCs with TLR stimulation evaluated in 2014, together with the changes in surface expression based on mean fluorescence intensity of CD14 (Pacific Blue) and CD16 (V500) (G and H). The change in MHC class II (HLA-DR) was also quantified using flow cytometry, (E). Comparison of linear data was done using Holm-Sidak method with α =5.0%, paired comparisons done using Wilcoxon signed rank test and unpaired group comparisons done using Mann-Whitney test. HESN (n=25) and HIV-N (n=25)

The gating strategy used to phenotype the different monocyte populations was based on lack of CD3- CD19 and CD56- expression, and expression of HLA-DR, CD14 and CD16 on the different monocyte subsets (**Figure 22A**). The percentage of classical monocytes ($CD14^+$ $CD16^-$) declined (HESN **44.3 ± 8.3%** vs HIV-N 41.9 ± 5.3%) between 0-24 hours in media (**14.3 ± 6.5%** vs. 16.2 ± 4.4%, p=ns), TLR4 (**4.6 ± 2.0%** vs. 13.1 ± 3.8%, p=ns), TLR7 (**10.4 ± 3.9%** vs. 17.0 ± 4.7%, p=ns) and TLR8 stimulations (**12.1 ± 5.3%** vs. 20.5 ± 4.6%, p=0.25) (**Figures 22B, 22C**). The frequency of intermediate monocytes ($CD14^+$ $CD16^+$) (**3.9 ± 0.7%** vs 3.9 ± 0.7%, p=ns) remained relatively stable in media (**2.7 ± 0.5%** vs. 2.1 ± 0.7, p= ns), TLR4 (**2.9 ± 0.8%** vs. 2.3 ± 1.1%, p=ns) and TLR8 (**4.1 ± 1.0%** vs. 2.7 ± 0.8, p=ns) stimulations, but increased more with TLR7 stimulations in HESN (**7.5 ± 1.5%** vs. 1.7 ± 0.5%, p=0.002). The numbers of non-classical ($CD14^-$ $CD16^+$) monocytes increased significantly in HESN from *ex vivo* (**3.8 ± 1.3%** vs. 10.8 ± 2.6%, p=ns) to (**28.2 ± 6.1%** vs. 6.6 ± 3.1%, p=0.003) in unstimulated cells, (**22.8 ± 4.2%** vs. 4.9 ± 2.6%, p=0.001) after TLR4, (**35.8 ± 7.1%** vs. 8.6 ± 4.4%, p=0.003**)** after TLR7 and (**26.5 ± 5.3%** vs. 6.2 ± 1.9%, p=0.001) TLR8 stimulations (**Figure 22B derived from 22C)**. Quantification of changes in monocytes subsets in PBMCs stained with extracellular antibodies in 2014 (**Figure 22D**), showed significantly higher frequencies of $CD14^-$ $CD16^+$ in HESN PBMCs after TLR8

stimulation (**17.0 ± 3.4 vs.** 9.17 ± 3.2, p=0.01), likewise in the same subset after intracellular staining in 2014 (8.2 ± 1.5% vs. 3.6 ± 0.5%)(**Figures 22F**).

When we examined HLA-DR expression (MHC class II- associated with antigen presenting function to $CD4^+$ T cells[395]), we found that the number of cells expressing HLA-DR was lower over the course of TLR4 stimulation in $CD3^-$ $CD19^-$ $CD56^-$ cells of HESN (**25.0%** vs 46.2%, p=0.002) (**Figure 22E)**.

Notably, the expression of CD14 was highest on classical ($CD14^+$ $CD16^-$) monocytes followed by intermediate ($CD14^+$ $CD16^+$) monocytes with least expression on non-classical monocytes ($CD14^-$ $CD16^+$)(**Figures 22G and H**). CD14 expression declined significantly on the surface of classical and intermediate monocytes of both study groups with LPS and PMA with Ionomycin stimulations (**Figures 22G and H**). The expression of CD16 was absent on classical monocytes, but its expression increased on intermediate monocytes with LPS stimulation in both study groups (**Figure 22H)**. This pattern of monocyte subset changes, was maintained in samples obtained in 2010 and 2014.

Taken together, the evaluation of changes in monocytes subsets and the expression of definitive receptors with TLR4, 7 or 8 stimulations, was consistent overtime and followed patterns previously described by others. More importantly, these results demonstrated that HESN individuals had significantly higher increases in nonclassical ($CD14^-$ $CD16^+$) monocyte subset with TLR8 stimulation, a subset previously linked with detection of nucleic acid from viruses in murine studies, particularly through TLR7 or TLR8.

6.4.2. Higher activation of HESN Monocytes with TLR stimulation.

The next objective was to investigate if there were differences in the functional responses of monocytes subsets prior to and after TLR stimulations between the two study groups.

Considering the HESN phenotype is associated with reduced susceptibility to HIV, the hypothesis tested here was; HESN CSWs have a higher TLR8 responsiveness in monocytes based on expression of co-stimulatory factors, cytokine responses and other monocyte functions that may affect HIV susceptibility, as compared to HIV-N.

To address this hypothesis, freshly isolated PBMCs from HESN (n= 20) and HIV-N (n=20) CSWs were stimulated using LPS-TLR4, Imiquimod-TLR7 and ssRNA/LyoVec-TLR8 for 6 hours or for 0, 6, 18 and 24 hours. The functional responses in monocytes evaluated included; expression of co-stimulatory factors (CD40, CD80 and CD86), expression of chemokine receptors (CCR2 and CXCR3) and expression of cytokines (IL-10, IL-12p40/70 and TNF-α) using multicolour flow cytometry (**Figure 23**). These responses were evaluated at three separate time points, 1st- May-August 2010), 2nd- (October 2011 to January 2012) and lastly between May-July 2014. The expression of co-stimulatory factors was done at two time points, May-August 2010 and October 2011-January 2012, while cytokine/chemokine responses and chemokine receptor expression, was only evaluated in 2014. The multiple evaluations coincided with scheduled field visits to Nairobi, such that the scheduled experiments were conducted using fresh samples.

The evaluation of monocyte functional responses with TLR stimulation began with the quantification of co-stimulatory factors on CD14^{+} cells (all monocytes subsets).This first assessment served as a broad screen for identification of potential differences expression of monocytes of HESN and HIV-N, to be used as a justification for more in depth analysis of monocyte subset functions at a later time point.

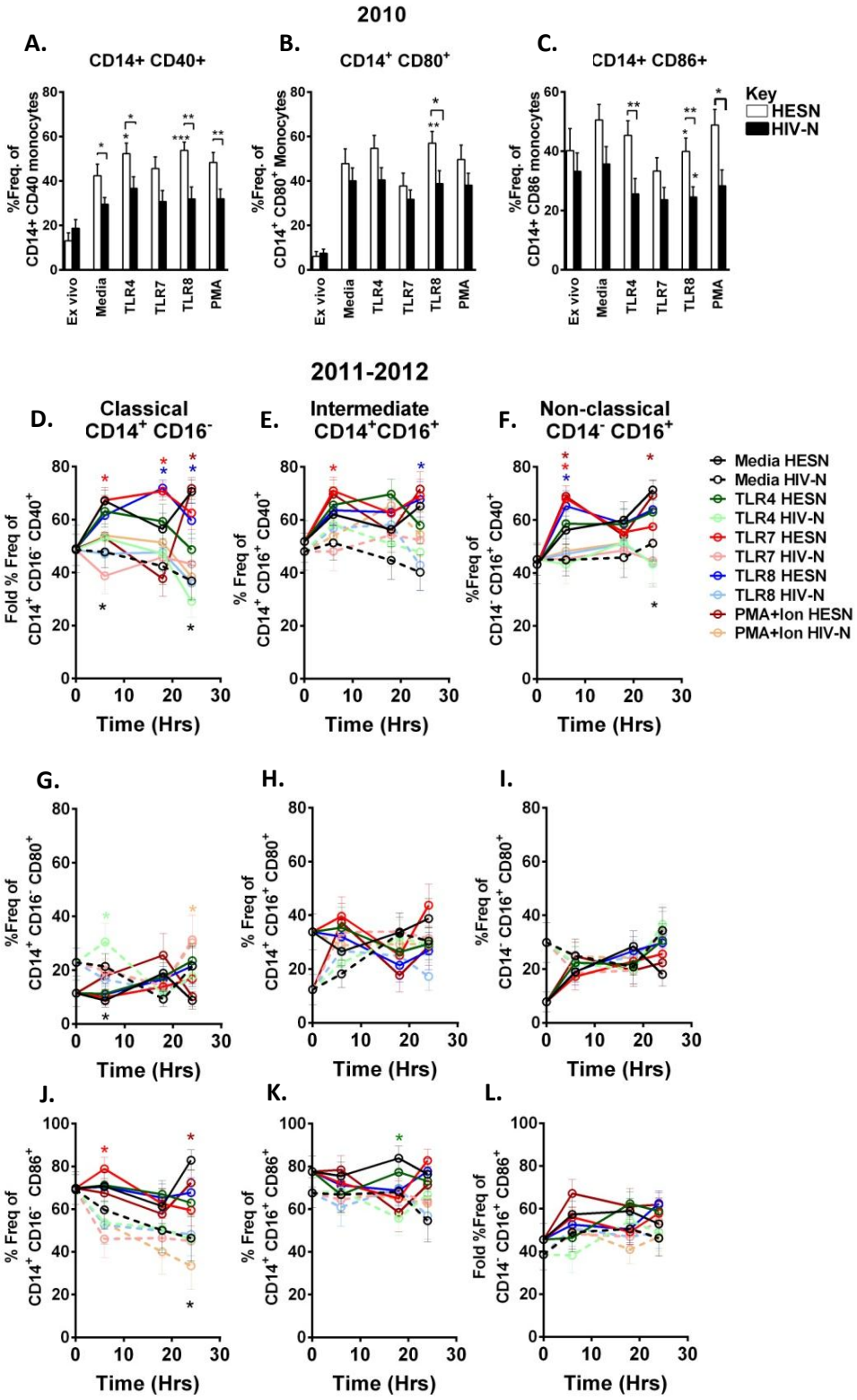


Figure 23. **Expression of co-stimulatory molecules on different monocyte subsets with TLR stimulation.** Freshly isolated PBMCs from HESN (white bars or darker solid lines) and HIV-N (black or lighter dashed lines) were stimulated with *E.coli* LPS-TLR4 (green), Imiquimod-TLR7 (red), ssRNA40/LyoVec- TLR8 (blues), phytohaemagglutin (PHA) or PMA with Ionomycin overnight at 37^{O}c and 5% CO_2 or for 6, 18 and 24 hours for kinetic evaluation of CD40, CD80 and CD86 expression by 10-colour flow cytometry. The quantification of co-stimulatory molecules was done at two different time points; 2010 and between October 2011-January 2012. HESN (n=18) and HIV (n=18)

The results from first quantification of co-stimulatory factor expression, showed that TLR4 stimulation (means: **42.4% to 52.2%, *p*=0.01**), Wilcoxon rank test) and TLR8 stimulations (means: **42.4% to 53.2%, *p*=0.001**) increased the frequencies of $CD14^+$ $CD40^+$ monocytes in HESN individuals but not in HIV-N (29.2% to 36.6%, p=0.0537 and 29.2% to 31.2% p=0.0532, respectively) (**Figure 23A**). TLR8 stimulation lead to higher frequencies of $CD14^+$ $CD80^+$ in HESN but not in HIV-N (mean: **47.7% to 57.0%, *p*=0.006** vs p=0.678), whereas similar stimulation led to declines in of both CD80 and CD86 expression on $CD14^+$ monocytes of HESN (SEMs: **50.5% to 39.3%, *p*=0.032**) and HIV-N (SEMs: 35.6% to 24.5%, p=0.047) (**Figures 23 B and C**).

HESN had a higher frequencies of $CD14^+$ $CD40^+$ monocytes following overnight stimulation with TLR4 (**52.2 ± 4.7 % vs.** 36.6 ± 5.2%, p=0.015) and TLR8 stimulation (SEMs: **53.8 ± 3.6%** vs 31.9 ± 5.3%, p= 0.003) compared to HIV-N (**Figure 23A**). The higher frequencies of $CD14^+$ $CD40^+$ monocytes was also observed on PBMCs cultures without TLR treatments, possibly due activation of monocytes by culture conditions, as monocytes are highly excitable[396](**Figure 23A**). The frequencies of $CD14^+$ $CD80^+$ monocytes was higher in PBMCs of HESN after TLR8 stimulation (SEMs**: 57.0 ± 5.3%** vs. 38.8 ± 5.7%, p=0.030), while the frequencies of $CD14^+$ $CD86^+$ monocytes was also higher in HESN after TLR4 stimulation (SEMs: **45.3 ± 4.9%** vs. 25.6 ±5.1%, p=0.006) and TLR8 stimulation (SEMs: **39.6 ± 4.4%** vs. 24.5 ± 3.4%, p=0.009), but not TLR7 stimulations (**Figures 23B and C**). Our quantification of changes in co-stimulatory factor expression on $CD14^+$ monocytes, revealed a higher expression of co-stimulatory factors in $CD14^+$ monocytes of HESN following TLR4 and TLR8 but not after TLR7 stimulation.

A repeat evaluation of co-stimulatory factor expression was done in a second set of samples obtained in 2011. This time the monocytes were divided into subsets -classical ($CD14^+$

$CD16^-$), intermediate ($CD14^+$ $CD16^+$) and non-classical monocytes ($CD16^-$ $CD16^+$)- and the evaluation co-stimulatory factor expression was conducted using time course experiments with TLR4, TLR7, TLR8 and PMA with Ionomycin stimulations (switched from PHA used in the first set of experiments) (**Figures 23G-L)**.

This secondary evaluation of co-stimulation molecules expression on monocytes revealed that CD40 expression on $CD14^+$ $CD16^-$ classical monocytes was higher in HESN between 6-18 hours of TLR7 stimulations (means**: HESN 67%** vs HIV-N 38.7%, p=0.001 and **70.7%** vs 48.5%, p=0.0006) and after 24 hours of TLR8 stimulation (means: **71.8%** vs 38.4%, p=0.0003) (**Figure 23D**). In intermediate monocytes $CD14^+$ $CD16^+$ of HESN had higher frequencies of CD40 expressing cells after 6 hours of TLR7 stimulation (means: **71.1%** vs. 48.4%, p=0.01) and after 24 hours of TLR8 stimulations (means: **67.7%** vs 42.9%, p=0.03) (**Figure 23E**). Non-classical or $CD14^-$ $CD16^+$ $CD40^+$ monocytes in HESN were higher in proportions after 6 hours of TLR7 (means: **68.0%** vs. 48.3%, p=0.0006) and TLR8 stimulations (means: **65.2%** vs. 47.1%, p= 0.04) (**Figure 23F**).

There were no outstanding differences in the expression of CD80 and CD86 between the two groups over the course of different TLR treatments (**Figures 23G-L)**. Minor differences observed were in the lower expression of CD80 on classical monocytes ($CD14^+$ $CD16^-$) of HESN after 6 hours of TLR4 stimulation (means: **11.2%** vs. 30.4%, p=0.01). HESN also had higher CD86 expression on classical ($CD14^+$ $CD16^-$) after 6 hours of TLR7 stimulation (means: **78.8%** vs. 46.0%, p=0.0001) and on intermediate monocytes ($CD14^+$ $CD16^+$) after 18 hours of TLR4 stimulation (means: **77.2%** vs 55.7%, p=0.03) (**Figure 23G-L**).

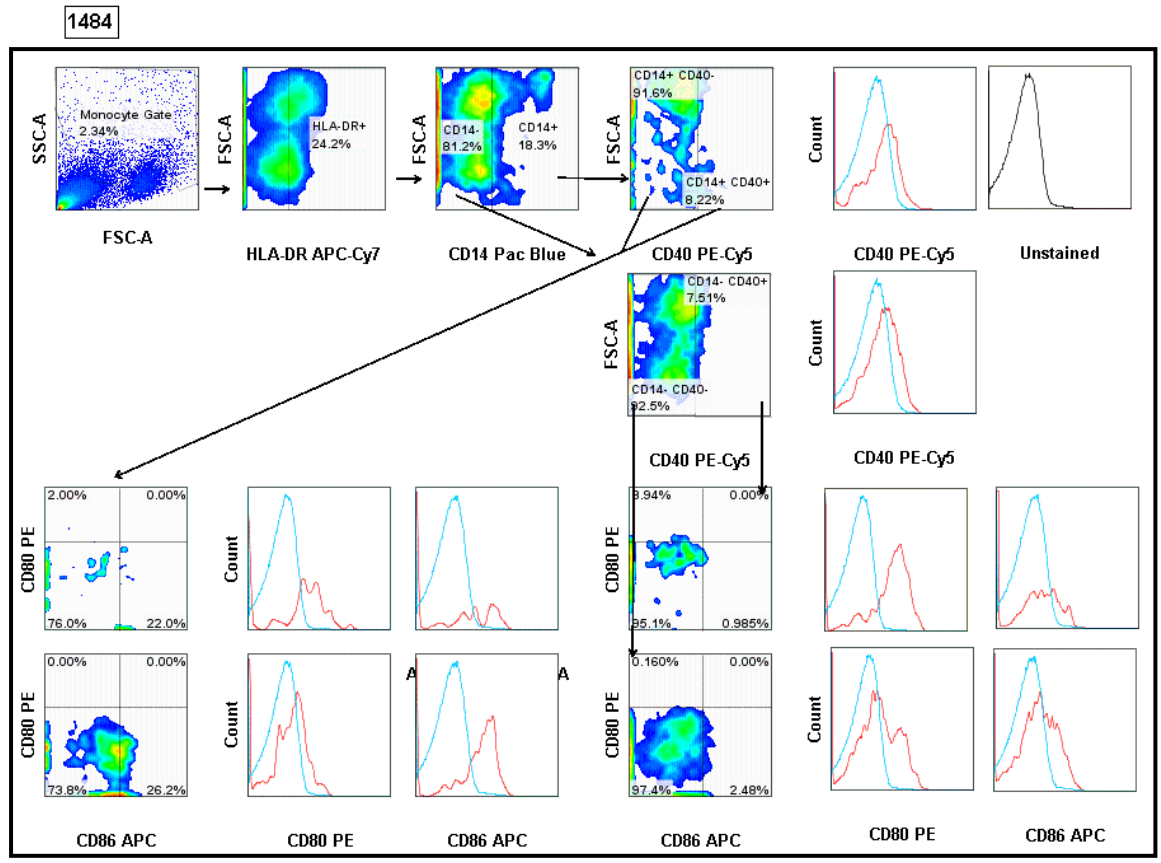
After the two evaluations of co-stimulatory factor expression on HESN monocytes, it was apparent that the expression of CD40 in ex vivo monocytes did not differ between the two study populations, but upon stimulation with the different TLR ligands, HESN monocytes

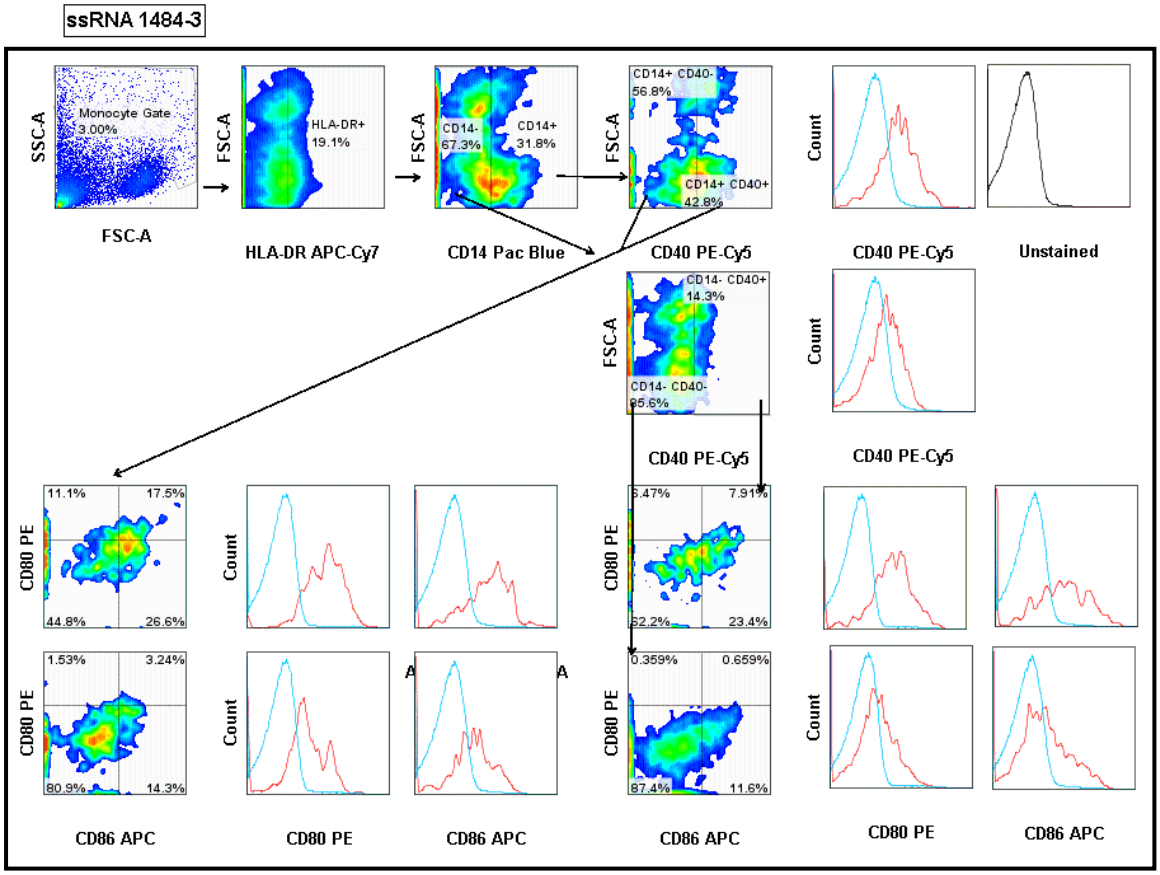
consistently had a higher upregulation of CD40 expression, but were less consistent in the pattern of CD80 and CD86 expression with TLR stimulation. It was also apparent that all the monocyte subsets contributed to the higher CD40 expression observed on bulk $CD14^+$ cells of HESN at different times over the course of the different TLR stimulations. The fact that these observations were made at more than one sampling point, suggests that HESN monocytes have a greater capacity to increase CD40 expression upon exposure to certain microbial components when compared to controls.

To further our understanding on the expression of co-stimulatory molecules on HESN monocytes, we conducted an unbiased Boolean gating analysis to determine the co-expression of the co-stimulatory molecules on all $CD14^+$ cells (see **Figure 23**). This was conducted by reanalysing flow data obtained from the experiments described above (**Figure 23A-C**) samples collected in 2010. The Boolenan gating strategy used in this analysis used HLA-DR^+ $CD14^+$ $CD40^+$ or HLA-DR^+ $CD14^-$ $CD40^+$ as the parent gate, for subsequent determination of CD80 and CD86 expression as presented in **Figure 24A**. We used this system of gating because biologically CD40 receptor expressed on APCs (such as DCs), can couple with CD40L on $CD4^+$ T cell resulting in upregulation of CD80 and CD86 for optimal activation of $CD8^+$ T cells, through process known as licensing[397].

The co-expression analysis revealed that HESN PBMCs had higher frequencies of $CD14^+$ $CD40^+$ $CD80^+$ $CD86^+$ cells following TLR4 (SEMs: **50.8 ± 6.9%** vs. 26.8 ± 3.8%, p=0.0039) and TLR8 stimulations (**52.1 ± 5.4%** vs. 31.5 ± 4.6%, p=0.0088). Similarly HESN had higher frequencies of $CD14^+$ $CD40^-$ $CD80^+$ $CD86^+$ after TLR4 (**21.9 ± 4.2%** vs. 12.5 ± 2.3%, p=0.022) and TLR8 stimulation (**22.6 ± 5.6%** vs. 10.3 ± 2.5%, p=0.047). On the contrary, HESN had lower frequencies of $CD14^+$ $CD40^+$ $CD80^-$ $CD86^-$ cells, following all TLR4 (**8.1 ± 2.8%** vs 41.7 ± 6.2%, p=0.0001), TLR7 (**9.3 ± 1.8%** vs. 47.0 ± 6.0%, $p < 0.0001$) and TLR8 (**5.9 ± 1.4** vs. 39.5 ± 6.4, p=0.0003) stimulations compared to HIV-N (**Figure 24B**).

A, Monocyte Boolean Gating Strategy





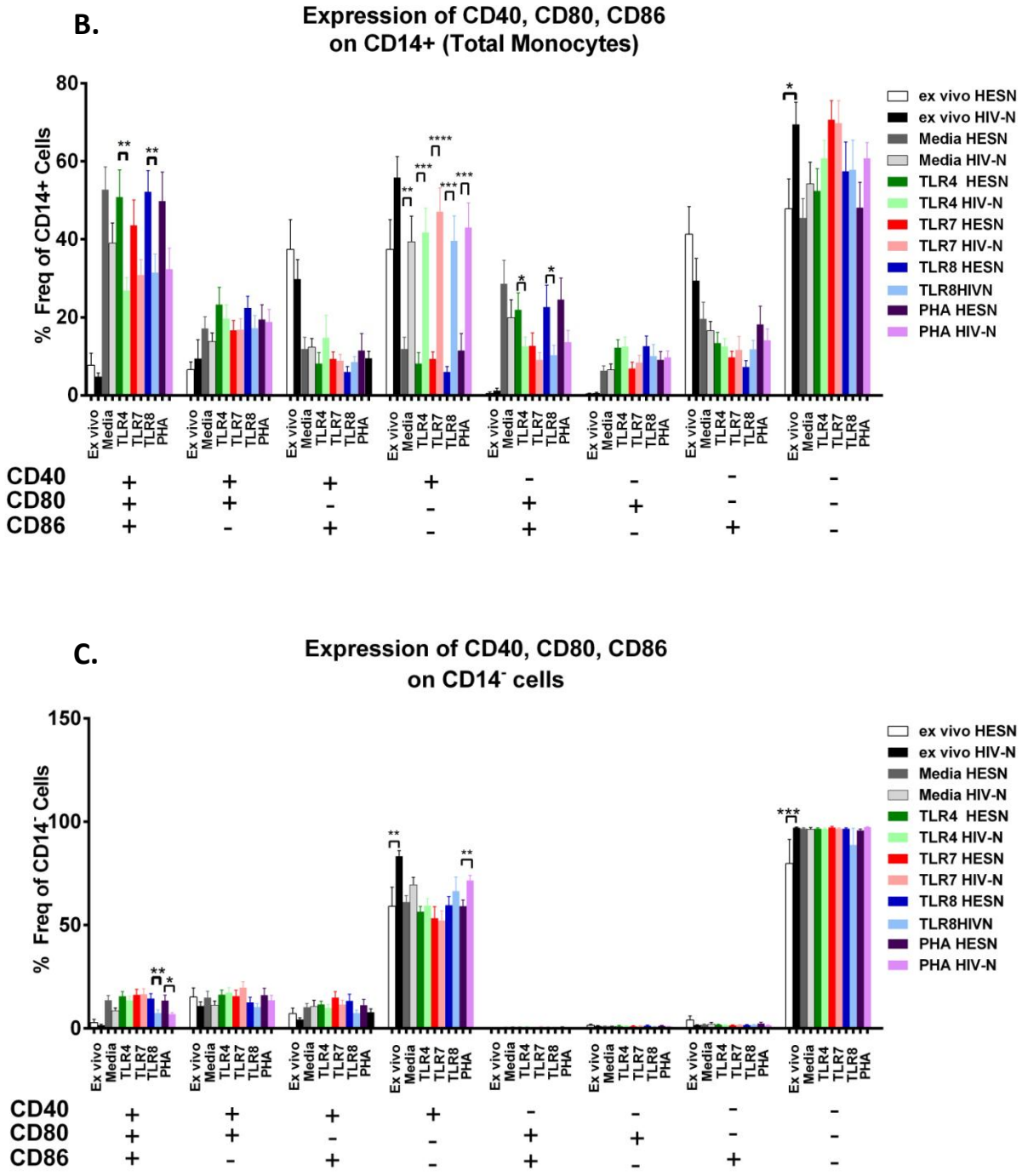


Figure 24. **Co-expression of co-stimulatory molecules on CD14$^+$ (monocytes) and CD14$^-$ (other monocytes and DCs) cells**. A) Boolean gating was used to deterimine the co-expression of CD40, CD80 and CD86 on HLA-DR$^+$ CD14$^+$ and HLA-DR$^+$ CD14$^-$ cells in PBMCs from HESN (darker colours, n=18) and HIV-N (lighter colours, n=18) were stimulated using either LPS-TLR4 (green bars), Imiquimod-TLR7 (red bars), ssRNA40/LyoVec-TLR8 (blue bars) or Phytohaemagglutin (PHA-purple bars). B and C) A comparison of the frequencies of CD14$^+$ or CD14$^-$ co-expressing or singular expressing CD40, CD80 and CD86 cells done using Mann-Whitney test and *P*-values are indicated using * $P<0.05$ ** $P<0.01$.

This co-expression analysis was also extended to $CD14^-$ cells, considering that non-classical or $CD14^{-/dim}$ $CD16^+$ monocytes express very low levels CD14 or none at all. The frequency of $CD14^-$ $CD40^+$ $CD80^+$ $CD86^+$ cells was higher in HESN PBMCs following TLR8 (**14.4 ± 2.3%** vs. 7.4 ± 1.5%, p=0.0083) stimulations (**Figure 24C**). HESN PBMCs had lower frequencies of $CD14^-$ $CD40^+$ $CD80^-$ $CD86^-$ cells following *ex vivo* isolation (**79.82 ± 11.7%** vs. 97.10 ± 0.3%, p=0.0007) from whole blood (**Figure 24C**).

The results of the co-expression analysis demonstrated that the $CD14^+$ cells in HESN CSWs were more 'wholesomely activated' through co-expression of CD40, CD80 and CD86 in response to TLR4 and TLR8 but not TLR7, whereas those of HIV-N individuals had higher singular expression of CD40 without CD80 or CD86 co-expression. This suggests that when monocytes of HESN are activated by TLR4 and TLR8 stimulations they co-express CD40, CD80 and CD86 more than those of HIV-N, potentially influencing the capacity of these cells to provide costimulation to the adaptive immune system.

6.4.3 Higher expression of CD40 Ligand (CD154) on T cells of HESN with TLR stimulation.

Costimulatory factors provide a third signal needed in T cell activation[366] and can be expressed either constitutively or upon activation[397]. Those belonging to the TNF family like CD40L are expressed transiently on T cells upon activation, and subsequently play fundamental roles in T and B cell functions[398]. Certain viruses, TLR3 or TLR9 agonists, can enhance $CD4^+$ T helper dependent CTLs, through upregulation of CD40L[399]. Similarly, different combinations of TLR agonists including TLR3, TLR4 and TLR8, have been shown to enhance CD40L mediated T helper mediated activation of DCs[400]. Therefore, we hypothesized that, higher $CD8^+$ T cell activation in HESN PBMCs after TLR7 and TLR8 stimulations (as described in the previous chapter) corresponded to the higher

expression of co-stimulatory receptors CD40 on APCs and CD40 ligand on T cells, both of which are driven by direct or indirect effects of higher TLR8 responsiveness of HESN PBMCs *in vitro*. To test this hypothesis we quantified the expression of CD40L and CD40 receptor on T cell and B cells before and after TLR4, 7 or 8 stimulations in PBMCs of HESN and HIV-N CSWs.

First, we evaluated changes in expression of co-stimulatory ligand CD40L on T cells and B cells with TLR4, 7 or 8 stimulations (**Figure 25**). This was done by stimulating freshly isolated PBMCs of HESN and HIV CSWs for 6hrs using TLR4-LPS, TLR7-Imiquimod, TLR8-ssRNA40/LyoVec or PMA+ Ionomycin. Then we quantified expression of CD40L (CD154) and CD40 on $CD3^+$ $CD4^+$ T cells, $CD3^+$ $CD8^+$ T cells and $CD3^-$ $CD19^+$ B cells using flow cytometry ,in PBMCs of HESN (n =18)and HIV-N (n=15) (**Figure 25**).

The quantification of CD40 Ligand (CD154) expression on T and B cells, revealed that its expression was higher on T cell than on B cells, consistent with what had previously been described[401]. HESN had a higher expression of CD40L on $CD4^+$ T cells after TLR7 stimulation (**12.8 ± 2.1%** vs 3.9 ± 0.8%, p= 0.0056), and on $CD8^+$ T cells after TLR7 (**9.8 ± 1.8** vs. 2.2 ± 0.6, p=0.0062) or TLR8 (**9.9 ± 1.6** vs. 3.4 ± 0.4, p=0.014) stimulations compared to those of HIV-N (**Figures 25B**).

CD40 expression on DCs and B cells is well established, however, the function of CD40 expressed on $CD8^+$ T cells has only been been linked generation of memory responses[402]. The effect of TLR4, 7 or 8 stimulations on CD40 expression T cells had not been described hitherto this. The higher expression of CD40 on monocytes of HESN with TLR4 and TLR8 stimulations, was not present in T cell and B cells following similar TLR stimulations (**Figure 25C**).

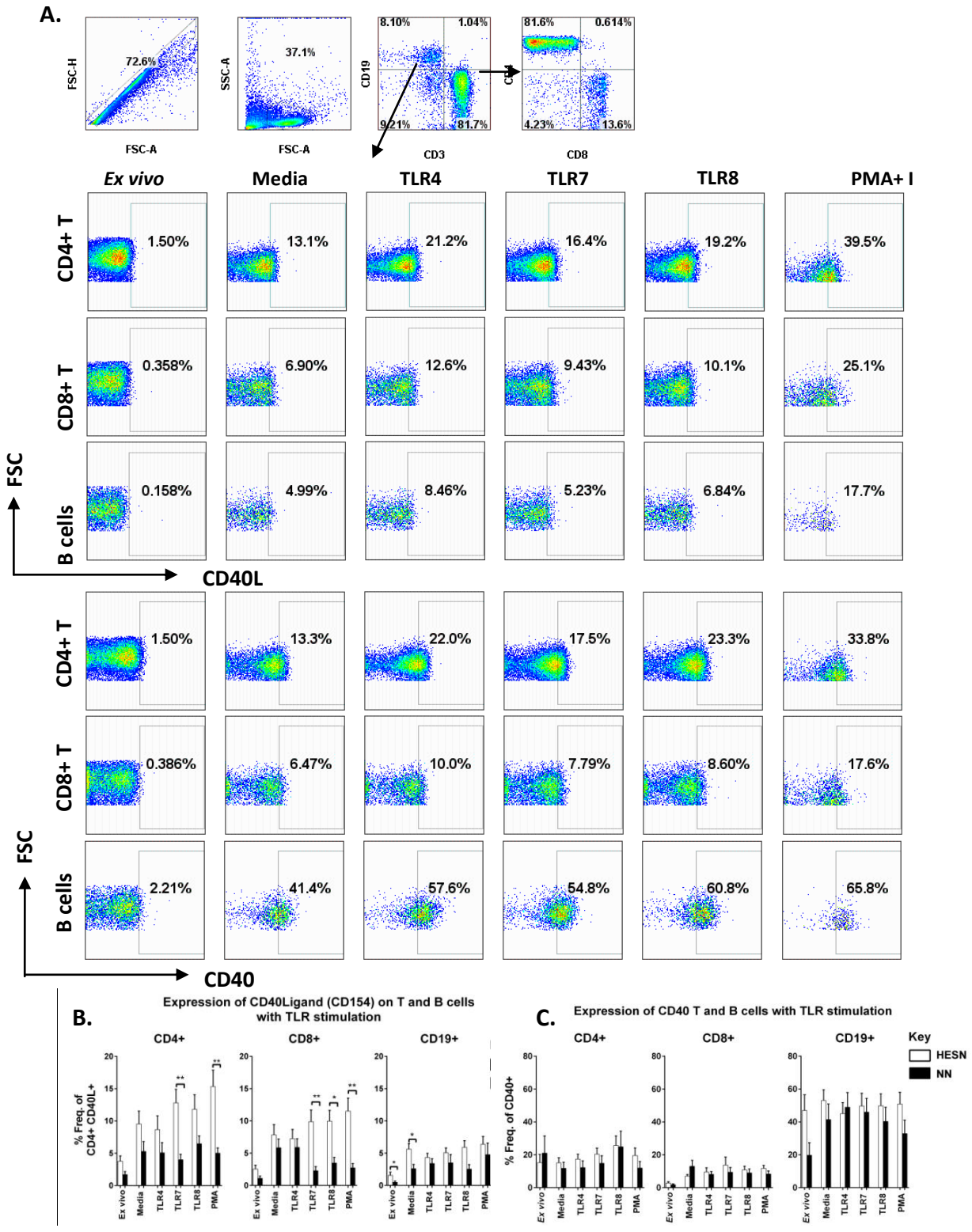


Figure 25: Expression of CD40 and CD40L on HESN T and B cells with TLR stimulation. A) Flow gating of CD40 and CD40 ligand (CD154) expression was quantified on lymphocytes in PBMCs before or after stimulation with either *E. coli*-LPS-TLR4, Imiquimod-TLR7, ssRNA40/LyoVec-TLR8 or PMA with Ionomycin overnight. A)The phenotyping of $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$ T cells and $CD3^-$ $CD19^+$ B cells and changes in expression of CD40 and CD40L on these cells with stimulation (B-C). Statistical analysis was done using Mann-Whitney test, $*P < 0.05$, $**P < 0.01$. HESN (n=10) and HIV-N (n=10)

Combining the results obtained from CD40 expression on APCs (described in the previous **section 6.4.2),** which showed a higher expression of CD40 in HESN monocytes in response to TLR4 and TLR8 stimulations, and the results obtained in this section that showed a higher expression of CD40L on HESN $CD4^+$ and $CD8^+$ T cells with TLR7 and TLR8 stimulations; It appeared HESN individuals had a higher activation of the CD40-CD40L following TLR8 stimulation. Expanding the assessment of the expresion, activation and functioning of different co-stimulatory pathways during TLR responses in HESN could be very informative.

6.4.4. Expression of chemokine receptors and adhesion molecules on HESN monocytes with TLR stimulation

Chemokine receptors expression is closely linked to monocyte function[109, 403, 404]. The differential expression of chemokine receptors on the three monocyte subsets can be used to differentiate between them, and to distinguish the functions of the subsets during inflammatory processes. Monocyte subset functions, are also linked to the pattern of integrin and adhesion marker expression, both of which are important in facilitating motility of these cells within blood vessels and during extravasations of monocytes out of capillaries into tissues, leading to formation of macrophages[403, 405].

The chemokine receptors evaluated in this section were; CCR2- essential for bone marrow release of monocytes and in homing of classical monocytes ($CD14^+$ $CD16^-$); CXCR3 a receptor for the chemokine IP-10 (CXCL10), MIG (CXCL9) and CXCL11 needed in development of T_H1 responses and trafficking (T_H1, CD8 and NK cells) [406]. In the previous chapters, we showed that IP-10 (CXCL10) production by HESN PBMCs was lower in response to TLR7 (**Figure 5**) but higher in response to TLR8 (**Figure 8**), while in resting PBMCs IP-10 levels uniquely correlated to the levels of most cytokines quantified which was in stark contrast to what was observed in controls (**Figure 9**).

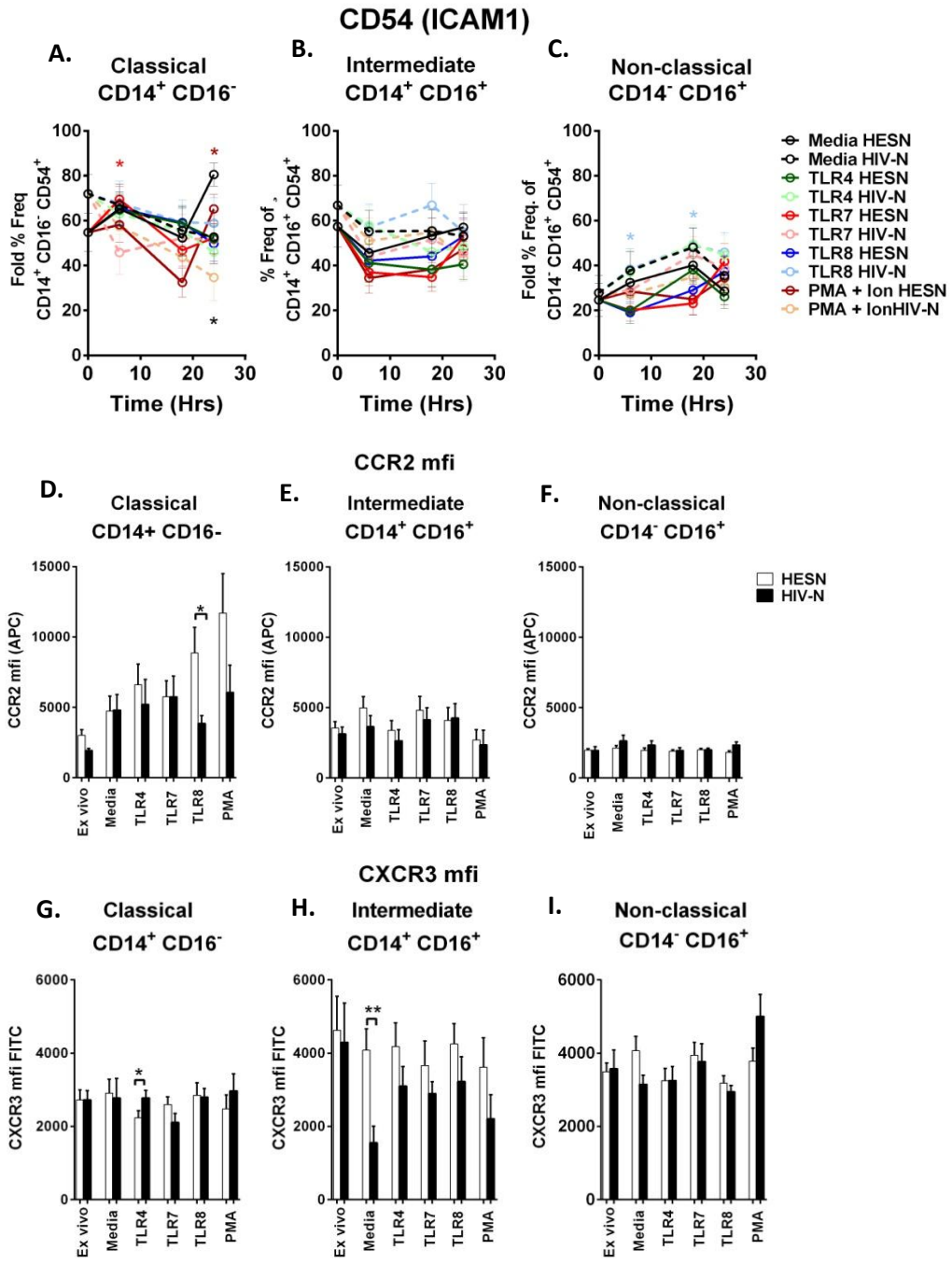


Figure 26. Expression of CD54 (Intracellular adhesion molecule) and chemokine receptors on monocytes with TLR stimulation (A-C) Freshly isolated PBMCs (2 x10^6cells/ml) from HESN (darker solid lines) and HIV-N (light dash lines) CSWs, were stimulated with *E.coli* LPS-TLR4 (Green), Imiquimod-TLR7 (red), ssRNA40/LyoVec-TLR8 (blue) or PMA with Ionomycin (brown). The expression of CD54 (ICAM1)(D-I) and the expression of CCR2 and CXCR3 was quantified from different monocyte subsets; classical ($CD14^+$ $CD16^-$), intermediate ($CD14^+$ $CD16^+$) and non-classical monocytes ($CD14^-$ $CD16^+$). Statistical comparison of linear grouped data done using Holm-Sidak method with α=5.0% and grouped comparison done using- Mann-Whitney test P<0.05. HESN (n=18) and HIV-N (n=18)

Monocytes are the principle producers of IP-10 (CXCL10); therefore we investigated if its receptor -CXCR3- was differentially expressed on monocytes of HESN and HIV-N after TLR4, 7 or 8 stimulations. We also chose to evaluate the expression of intracellular adhesion molecule 1 (ICAM1 or CD54), that can be incorporated by the HIV onto its surface and subsequently used in infection of target cells[407].

Here, we present data from experiments in which we tested the effect of TLR4, TLR7 or TLR8 stimulations on PBMCs of HESN (n=20) and HIV-N (n=16), with regard to the expression of the chemokine receptors CCR2 and CXCR3, and adhesion marker CD54 (ICAM1), in classical ($CD14^+$ $CD16^-$), intermediate ($CD14^+$ $CD16^+$) and non-classical monocytes ($CD14^+$ $CD16^+$) (**Figure 26**).

The results showed there was no difference in the frequency of monocytes expressing of CD54 (ICAM1) between the two groups with TLR4 stimulation. However, after 6 hours of TLR7 stimulation, HESN had higher frequencies of CD54 expressing classical monocytes ($CD14^+$ $CD16^-$) (means: **69.5%** vs 48.5%, p=0.03), and HESN also had lower expression of CD54 (ICAM1) on non-classical monocytes ($CD14^+$ $CD16^+$) between 6-18 hour of TLR8 stimulation to HIV-N controls (means: **19.0%** vs. 38.3%, p=0.04; **29.1%** vs. 48.9%, p=0.03) (**Figures 26A-C**).

We also examined the changes in frequencies and surface expression (based MFI) of CCR2, in classical ($CD14^+$ $CD16^-$), intermediate ($CD14^+$ $CD16^+$) and non-classical ($CD14^+$ $CD16^+$) monocytes, before and after TLR4, TLR7 or TLR8 stimulations in the PBMCs from the two groups. There were no differences in frequencies of $CD14^+$ $CD16^-$ $CCR2^+$ (classical), $CD14^+$ $CD16^+$ $CCR2^+$ (intermediate) and $CD14^+$ $CD16^+$ $CCR2^+$ (non-classical) monocytes between the two groups before and after TLR4, 7 or 8 stimulation (data not shown). However, based on the surface expression of CCR2 (based on MFI), classical monocytes ($CD14^+$ $CD16^-$) had

the highest expression of CCR2, followed by intermediate monocytes ($CD14^+$ $CD16^+$), with the lowest expression being in non-classical monocytes ($CD14^-$ $CD16^+$), consistent with what has been described previously (**Figures 26D-F**)[405]. Only TLR8 stimulation resulted in higher surface expression of CCR2 based on MFI on classical ($CD14^+$ $CD16^-$) monocytes of HESN (MFI: **8852 ± 1829** vs. 3864 ± 556, p=0.012) (**Figure 26D**).

Next we quantified the expression of CXCR3-a receptor for IP-10 (CXCL10), MIG (CXCL9) and CXCL11, on the three monocytes subsets in PBMCs of HESN and HIV-N stimulated with TLR4, TLR7 or TLR8. The surface expression of CXCR3 was similar across the three monocyte subsets, however, TLR4 stimulation resulted in the lower expression of CXCR3 on classical monocytes of HESN compared to controls (MFIs: **2235 ± 193.4** vs 2279 ± 208.7, p=0.019), while intermediate monocytes of HESN also expressed higher amounts of the CXCR3 in unstimulated cells (**Figures 26G-I**).

These results demonstrated that classical monocytes of HESN are capable of expressing the higher amounts of CCR2, a receptor critical for the functioning of this subset, following TLR8 stimulation. The expression of CXCR3 was only lower in response to TLR4 but not TLR7 or TLR8 stimulation PBMCs. This showed the expression CXCR3 receptor did not differ much between the two groups as did the production of the cognate chemokine IP-10. Although the evaluation of chemokine receptors and adhesion markers in this study was not exhaustive, the results demonstrate that differences in expression of functional receptors on monocyte subsets exist between HESN and HIV-N, possibly influencing the capacity of the different monocyte subsets in HESN to home and enter infection sites. Expanding the analysis of chemokines, adhesion markers and chemokine responses in HESN monocytes with TLR stimulations in the future, would build onto this.

6.4.5. Expression of Cytokines on Monocytes Subsets of HESN with TLR4, TLR7 and TLR8 stimulations

Finally we conducted a quantification of cytokine production by the different monocyte subsets in response to TLR4, TLR7 or TLR8 stimulations in PBMCs from the two CSW groups. Previous studies have demonstrated distinct cytokine responses in the different monocyte subsets, which was dependent on TLR stimulation[378]. For instance TLR4 stimulation has previously been shown to result in production of IL-10 rather than TNF-α or IL-1 in classical or $CD14^+$ $CD16^-$ monocytes[378].

Here we quantified the expression of IL-10, IL-12p40/70 and TNF-α in $CD14^+$ $CD16^-$ (classical), $CD14^+$ $CD16^+$ (intermediate) or $CD14^-$ $CD16^+$ (non-classical) monocytes in HESN and HIV-N following TLR4-LPS, TLR7-Imiquimod, TLR8-ssRNA40/LyoVec or PMA with Ionomycin stimulations for 6 hours. The cytokine quantification was done using intracellular cytokine staining for multicolour flow cytometry. The PBMCs used in these assays were treated with protein inhibitors (GolgiPlug™ and GolgiStop™) to prevent release of cytokines over the course of the stimulations, thereby easing flow quantification of cytokines (**Figures 27A-R**).

We observed the highest number of IL-10 expressing cells in the $CD14^+$ $CD16^+$ intermediate monocytes subset, with nearly equal proportions of IL-10 expressing cells in $CD14^+$ $CD16^-$ and $CD14^-$ $CD16^+$ monocytes (**Figures 27A-F**). IL-12p40/70 expression was highest in classical ($CD14^+$ $CD16^-$) and intermediate ($CD14^+$ $CD16^+$) monocytes with very few $CD14^-$ $CD16^+$ monocytes expressing the same.

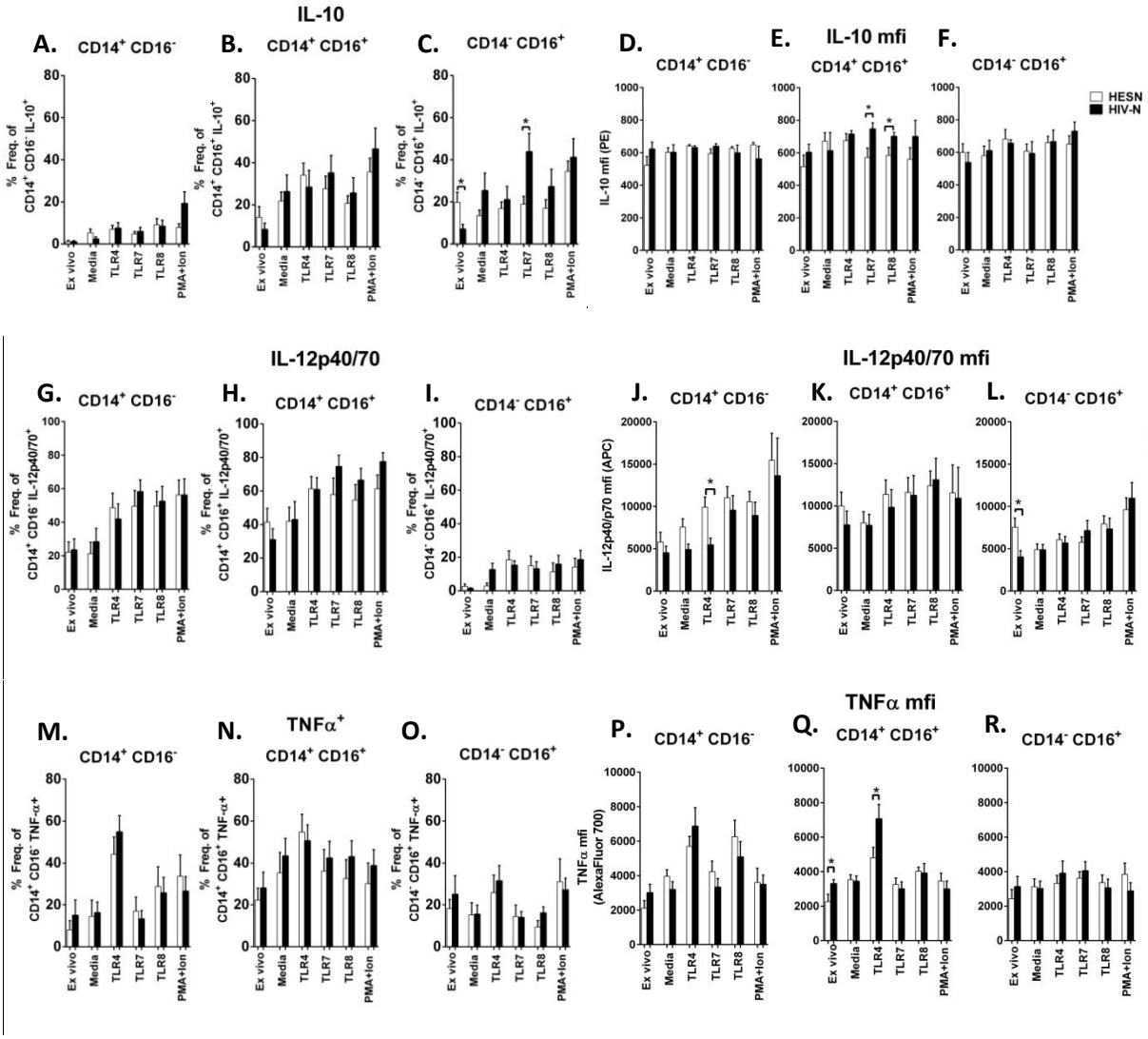


Figure 27. Cytokine responses by monocyte subsets with TLR stimulation. Peripheral blood mononuclear cells from HESN (n=16) and HIV-N (n=16) CSW, were stimulated with LPS-TLR4, Imiquimod-TLR7, ssRNA40/LyoVec-TLR8 or PMA with Ionomycin for 6 hours together with cytokines release blockers, and cultured at 37^{O}c and 5% CO_2 . The cytokine responses are presented as frequencies or mean fluorescence intensity (mfi) of $CD14^{+}$ $CD16^{-}$ or classical, $CD14^{+}$ $CD16^{+}$ or intermediate or $CD14^{-}$ $CD16^{+}$ or non-classical monocytes, expressing IL-10 (A-F), IL-12p40/70 (G-L) or TNF-α (M-R). Statistical comparison between groups done using Mann-Whitney test, all P<0.05 indicated (*).

Robust TNF-α expression (frequencies and mfi) was observed in $CD14^+$ $CD16^-$ (classical) and $CD14^+$ $CD16^+$ (intermediate) monocytes in response to TLR4 and TLR8 stimulations, while Imiquimod or TLR7 stimulation of failed to induce expression of TNF-α above the background responses. The pattern of TNF-α expression by monocytes resembled that the pattern of soluble TNF-α quantified from the culture supernatants using multiplex bead assays, which showed TLR7 stimulation failed to induce expression of TNF-α (**Figures 5-8**).

Notably, a higher frequency of unstimulated non-classical monocytes ($CD14^-$ $CD16^+$) in HESN expressed of IL-10 (**19.8 ± 4.8%** vs. 7.0 ± 2.1%, p=0.033), but lower frequencies of IL-10 expressing cells in the same subset was observed in HESN with TLR7 stimulation (**18.9 ± 3.6%** vs. 43.8 ± 8.7%, p= 0.012). The surface expression (MFI) of IL-10 on intermediate monocytes ($CD14^+$ $CD16^+$) of HESN was lower both after Imiquimod-TLR7 (MFI: **570.6 ± 58.1** vs. 747.1 ± 37.2, p=0.0303) and ssRNA40/LyoVec-TLR8 stimulations (MFI: **584.2 ± 49** vs. 701.2 ± 24.6, p=0.019) compared to HIV-N (**Figures 27C and E**).

Ex vivo non-classical $CD14^-$ $CD16^+$ monocytes of HESN had higher expression of IL-12p40/70 compared to those of HIV-N (MFI: **7531 ± 1072** vs. 4014 ± 749, p=0.026). TLR4 stimulation led to higher IL-12p40/70 expression in classical ($CD14^+$ $CD16^-$) monocytes in HESN compared to those of HIV-N (MFI: 9915 ± 1212 vs. 5463 ± 788.2, p=) (**Figures 27G-L**). This was consistent with the levels of IL-12p70 but not IL-12p40 quantified from culture supernatants of PBMCs stimulated with TLR4 (**Figure 7-8**). .

Finally, surface expression of TNF-α on intermediate monocytes ($CD14^+$ $CD16^+$) of HESN was lower *ex vivo* (MFI: **2257 ± 428.2** vs. 3301 ± 238.5, p=0.049) and following TLR4 stimulation compared to HIV-N (MFI: **4805 ± 607.2** vs. 7065 ± 834.7, p=0.036) (**Figure 27M-R**). This corresponded to the lower responses soluble TNF-α observed following TLR4 stimulation of HESN PBMCs. Expression of TNF-α in response to TLR8 stimulation was

most visible in classical monocytes, although expression of TNF-α tended to higher in HESN it was not statistically significant (**Figures 27M-R**).

In summary, evaluation of cytokine responses in the three monocytes with TLR stimulation showed similarities to the patterns of the cytokine responses observed in soluble cytokine quantified from culture supernatants. Ex vivo monocytes of HESN had higher expression of IL-10 but lower expression of proinflammatory TNF-α, but upon TLR7 or TLR8 stimulation expressed lower levels of IL-10, but higher levels of IL-12p40/70 but lower levels of TNF-α after TLR4 stimulation.

6.4.6. Dendritic Cell responses in HESN PBMCs with TLR stimulation

Dendritic cells are a heterogeneous population of cells which express high levels of TLRs, and play an integral role in linkage of the innate and adaptive immune systems. TLR stimulation of immature DCs leads to DC maturation characterized by increased expression co-stimulatory molecules and copious production of cytokines. Blood DCs can be divided into $CD11c^{-}$ $CD123^{++}$ plasmacytoid DCs (pDCs) which express high levels of TLR7 and TLR9 and $CD11c^{++}$ $CD123^{-}$ myeloid DCs (mDCs), that express TLR1, 2, 3, 4, 6, 7 (contentious) 8, 10, but lack TLR9 expression[108, 137, 199, 408]. The functional responses of DCs vary depending on TLRs expressed on the responding subset. Ito *et al.* 2002 demonstrated the differential production of IFN-α and IL-12p40/70 by pDCs and mDCs, in response to TLR7 and TLR8 ligands, respectively [199]. In chapter 4, we presented data showing an absence of IL-12 and TNF-α responses in Imiqimod or TLR7 stimulated of PBMCs in either study group, yet the same treatment led to the robust production of IFNα2 (**Figures 6-9**).

The experiments conducted in this chapter attempted to trace the source of the elevated IFNα2 responses observed in TLR7 stimulated PBMCs and IL-12p40 and 70 produced by

TLR4 and TLR8 stimulations. The hypothesis tested was that HESN DCs are less responsive to TLR7 but more responsive to TLR8 stimulation leading higher cytokine responses corresponding those quantified from PBMC cultures (**Chapter 4**). Here, PBMCs from HESN (n=18) and HIV-N (n=16), were stimulated with TLR4-LPS, TLR7-Imiquimod, TLR8 ssRNA40/LyoVec or PMA with Ionomycin for 6 hours, and the expression of cytokines (IFNα2, IL-12p40/70 and TNF-α), co-stimulatory (CD80 and CD86) and DC maturation marker (CD83) in blood DCs subsets done using multicolour flow cytometry.

Considering there is no single marker that uniquely identifies DCs, we used the expression of two extracellular proteins CD11c and IL-3 receptor (CD123), to categorize DCs into three subsets; Myeloid or conventional DCs ($CD11c^{+}$ $CD123^{-}$), plasmacytoid DCs ($CD11c^{low/-}$ $CD123^{++}$) and other myeloid cells ($CD11c^{++}$ $CD123^{+}$) (**Figure 28A)**. Myeloid DCs made up 20-50% of all Lin^{-} cells in PBMCs, while pDCs made up 1-2% of all $CD3^{-}$ $CD19^{-}$ $CD56^{-}$ cells. The proportions of DC subsets remained relatively unperturbed by TLR4, 7 or 8 stimulations (**Figures 28A-D**).

DC maturation was measured by changes in expression of the surface glycoprotein CD83; using the expression of co-stimulatory markers CD80 and CD86; and using cytokines expression IFNα2b, IL-12p40/70 and TNF-α. All DC subsets expressed low levels of CD83 and CD80 prior to culture or stimulation with different TLR ligands, suggesting that resting DCs were immature DCs (**Figures 28A-V**). All TLR stimulations used enhanced the expression of CD83 (marker for DC maturation). However, myeloid DCs ($CD11c^{+}$ $CD123^{-}$) failed to up-regulate the expression of CD83, although the surface expression of CD83 (mfi) was lower on mDCs ($CD11c^{+}$ $CD123^{-}$) cells of HESN after TLR4 (MFIs: **1270 ± 26.9** vs. 1523 ± 77.0, p=0.0089) and TLR7 (MFIs: **1231 ± 80.4** vs. 1546 ± 118.9, p=0.01) stimulations compared to HIV-N (**Figures 28E and H**).

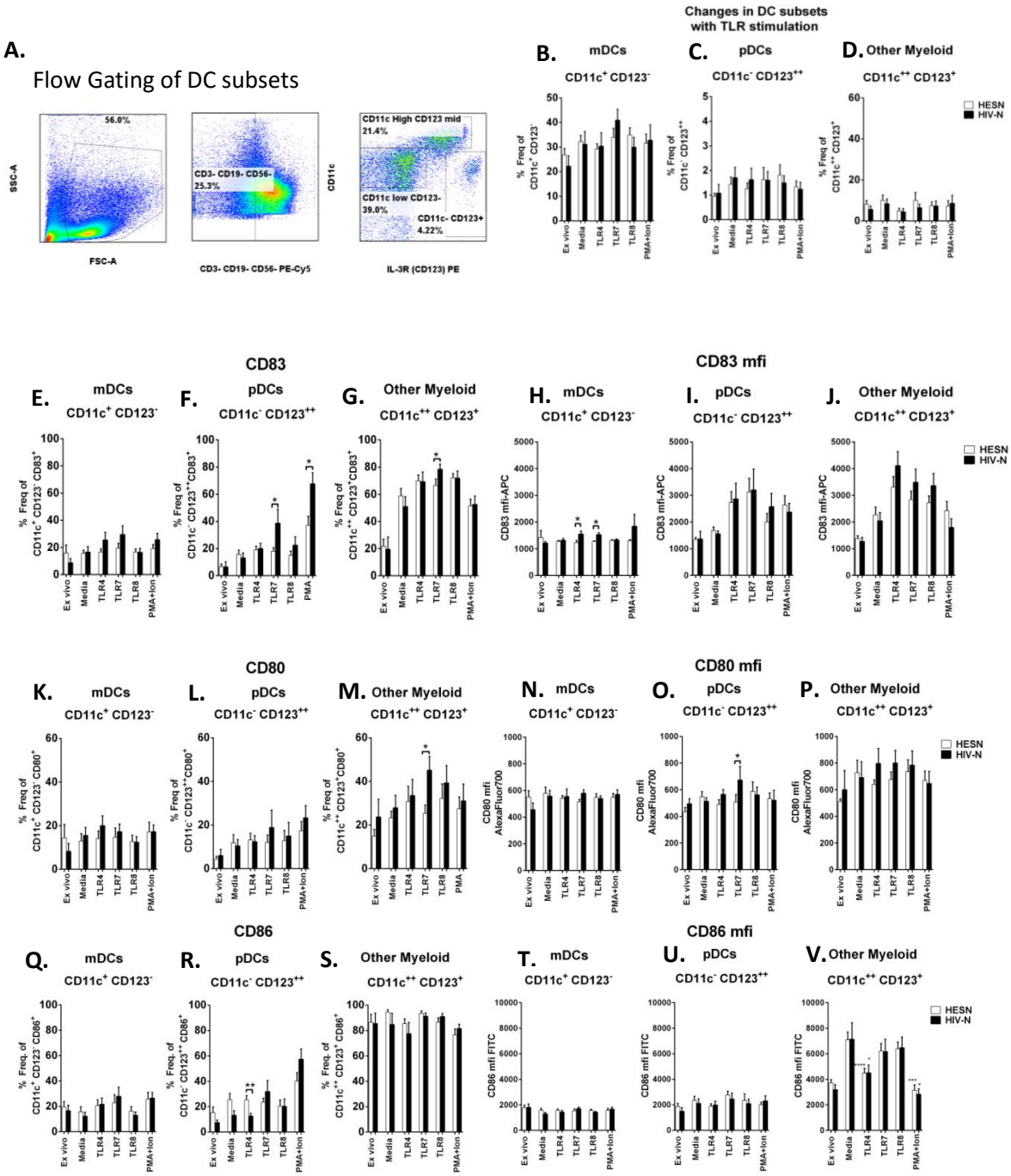


Figure 28. Blood DC responses to different TLR stimulations. (A) DCs subsets in PBMCs of HESN (white bars) and HIV-N (black bars), were phenotyped based on expression CD11c and CD123 (IL-3R) before and after stimulation with TLR4, TLR7, TLR8 or PMA with Ionomcyin. (B-D) Limited changes in the proportions of DC subsets; $CD11c^{+}$ $CD123^{-}$ (myeloid DCs) $CD11c^{-}$ $CD123^{++}$ (pDCs) or $CD11c^{++}CD123^{+}$ (other Myeloid cells) was observed following the different TLR treatments. (E-J) Statistical comparison between groups was done using Mann-Whitney test *P<0.05. HESN (n=18) and HIV-N (n=16).

The frequency of CD83 expressing pDCs ($CD11c^{-}$ $CD123^{++}$) (**18.04 ± 2.5%** vs. 38.7 ± 10.2%, p=0.032) and other myeloid cells ($CD11c^{++}CD123^{+}$) (**66.7 ± 4.4%** vs. 78.4 ± 3.9%, p=0.0315) was significantly lower in HESN following TLR7 stimulation (**Figures 28F and G**).

The expression of CD80 on both pDCs ($CD11c^{-}$ $CD123^{++}$)(MFIs: **507.5 ± 55.52** vs. 672.3 ± 103.1, p= 0.015) and other myeloid cells ($CD11c^{++}$ $CD123^{+}$) (**25.43 ± 3.811%** vs. 45.08 ± 6.414% , p=0.0237) was lower in HESN after TLR7 stimulation (**Figures 28M and O**).

The expression of CD86 on mDCs and pDCs was lower than on other myeloid cells, that constitutively expressed it and but lost its expression after TLR4 and PMA + Ionomycin stimulations (**Figures 28Q-V**). This pattern was similar to what was observed on monocytes, strongly suggesting that 'other myeloid cells' may include monocytes (**Figure 23C**). The surface expression of CD86, based on mean fluorescence intensity was significantly lower on TLR4 and PMA + Ionomycin stimulated other myeloid cells from both groups (**Figure 28V**). In spite of this, HESN individuals had higher frequencies of pDCs expressing CD86 on pDCs with TLR4 stimulation compared to HIV-N (**25.4 ± 3.4%** vs. 12.4 ± 2.3%, p=0.0093) (**Figures 28M, O and R**).

Thus far, the maturation of HESN mDCs and pDCs was lower based on CD80 and CD83 expression following TLR7 stimulation, but higher on mDCs based on CD86 expression following TLR4 stimulation, compared to controls. These results suggest that HESN DCs may not undergo full maturation following stimulation by TLR4 or TLR7 ligands *in vitro*.

The next set of experiments assessed the intracellular cytokine responses by different DC subsets to TLR4, 7 or 8 stimulations (**Figure 29**). The results showed that, e*x vivo* HESN had higher frequencies of mDCs expressing IFNα2b compared to in HIV-N (**11.0 ± 2.9%** vs. 4.1 ± 0.9%, p=0.012) (**Figure 29A**). While both TLR4 and TLR8 stimulations increased the

frequencies of IFNα2b expressing pDCs ($CD11c^{-}$ $CD123^{++}$) in HESN (*p*=**0.015; 0.021**, Wilcoxon signed rank test) both failed to do so in HIV-N (*p*=0.08; 0.340). On the contrary, TLR7 significantly increased the frequency of IFNα2b expressing pDCs in HIV-N (*p*=0.049, Wilcoxon rank test) but not in HESN (***p*=0.292**) (**Figure 29B**). Both mDCs ($CD11c^{+}$ $CD123^{-}$) and pDCs ($CD11c^{-}$ $CD123^{++}$) in HESN had higher expression of IFNα2b after TLR8 stimulation (MFIs: **543.9 ± 19.1** vs. 451.9 ± 36.6, *p*=0.024 and **607.1 ± 27.52** vs 465.5 ± 53.96, *p*=0.018, respectively) (**Figures 29D and E**).

Contrastingly, HESN had lower fold changes in expression of IFNα2b on mDCs ($CD11c^{+}$ $CD123^{-}$) (fold MFIs: **0.98 ± 0.011** vs. 1.021 ± 0.017, *p*=0.04) and pDCs ($CD11c^{-}$ $CD123^{++}$) (fold MFIs: **0.98 ± 0.02** vs. 1.04 ± 0.02, *p*=0.022) compared to susceptible controls following TLR7 stimulation (**Figures 29G-I**). The pattern of IFNα2b expression in pDCs resembled that of soluble IFNα2 quantified from cell culture supernatants at the same time, where TLR7 stimulation resulted in lower production IFNα2 and TLR8 led to higher production of the same in HESN PBMCs (refer **Figures 5-8**).

The mDCs and pDCs had lower frequencies of cells expressing of IL-12p40/70 in response to the three TLR stimulations. However, the frequency of other myeloid cells ($CD11c^{++}$ $CD123^{+}$) expressing IL-12p40/70 in HESN after both TLR7 and TLR8 stimulations was lower that in HIV-N (**39.1 ± 7.5%** vs. 61.2 ± 7.3, *p*=0.045 and **39.8 ± 7.3%** vs. 67.4 ± 8.3%, *p*=0.022) (**Figures 29J-L**). The fold change in IL-12p40/70 expression in other myeloid cells ($CD11c^{++}$ $CD123^{+}$) was lower in HESN compared to HIV-N following TLR7 stimulation (**Figure 29O**). Again these responses matched the pattern of soluble IL-12p40 and p70 production by bulk PBMCs as quantified by multiple bead assays, where HESN PBMCs produced lower amounts of IL-12p40 and IL-12p70 in response to TLR8 stimulation, but not TLR7 stimulations (ref. **Figures 7-8**).

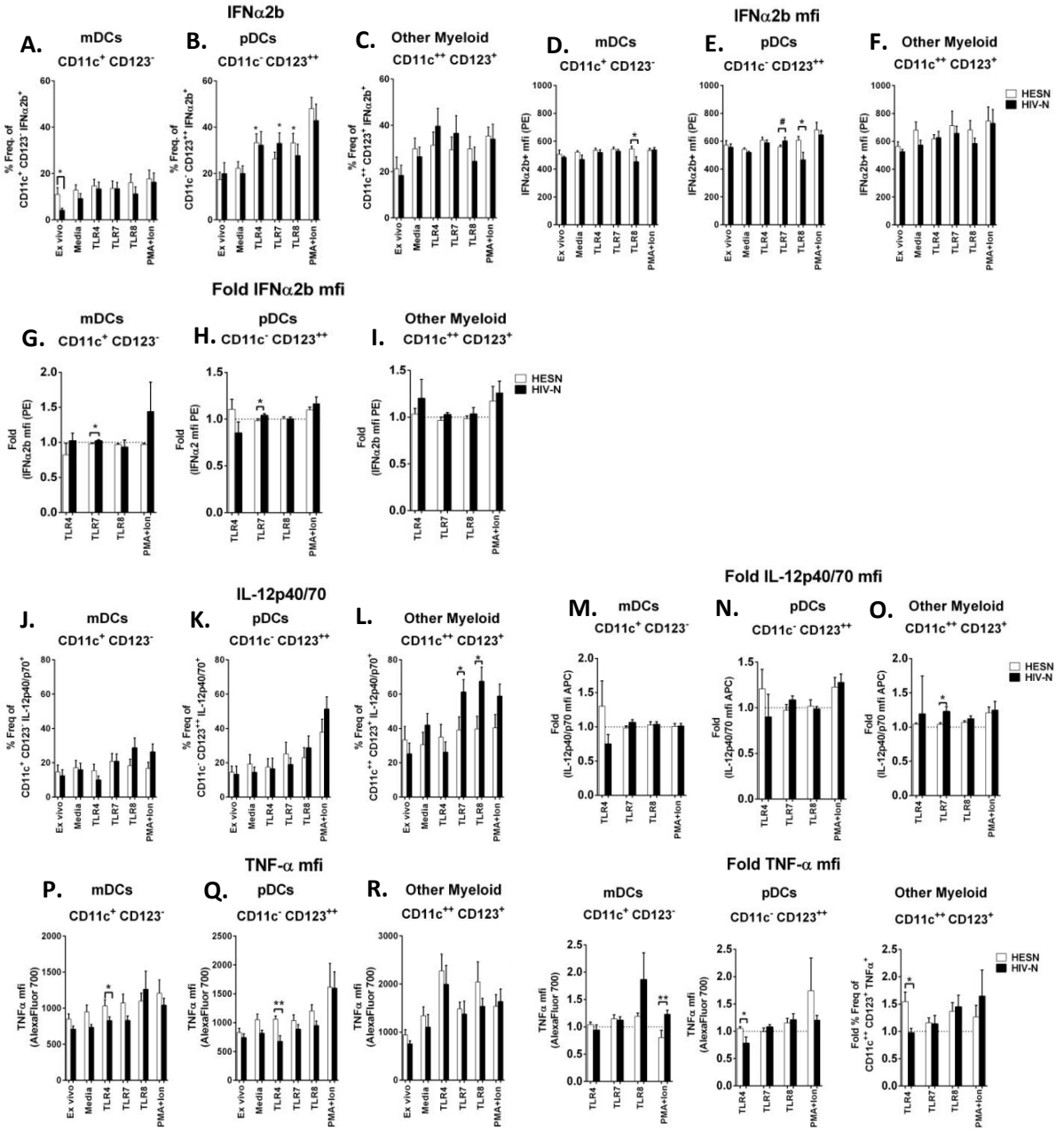


Figure 29. Cytokine responses by DC subsets to TLR4, TLR7 and TLR8 stimulations. Freshly isolated PBMCs (2 x10^6 cell/ml) from HESN (white bars) and HIV-N (black bars) CSWs were stimulated with LPS-TLR4, Imiquimod-TLR7, ssRNA40/LyoVec-TLR8 and PMA+ Ionomycin for 6 hour at 37^{O}C and 5% CO_2, together with cytokine release blockers. Flow cytometry was used to quantify IFNα2b (A-F), IL-12p40/70 (G-L) or TNF-α (M-R) expression. The results of the cytokine quantification are presented as frequencies, MFIs or fold changes in; $CD11c^+$ $CD123^-$ (myeloid DCs), $CD11c^-$ $CD123^{++}$ (plasmacytoid DCs) and $CD11c^{++}CD123^+$ (other myeloid cells). Comparison between groups done using Mann-Whitney test, where *P<0.05, **P<0.01. HESN (n=18) and HIV-N (n=16).

Finally, there were no differences frequencies of TNFα expressing mDCs ($CD11c^{+}$ $CD123^{-}$), pDCs ($CD11c^{-}$ $CD123^{++}$) and other myeloid cells ($CD11c^{++}CD123^{+}$) after TLR4, TLR7 and TLR8 stimulation (data is not shown). However, TNF-α expression (based on MFI) in other myeloid cells of HESN was highest in response to TLR4 and TLR8 stimulation, with the expression of the same being below background responses following TLR7 stimulation (**Figures 29P-R**). TLR4 stimulation resulted in higher TNF-α expression in HESN mDCs ($CD11c^{+}$ $CD123^{-}$) (MFIs: **1034 ± 83.2** vs 826.2 ± 54.9, p=0.048) and pDCs ($CD11c^{-}$ $CD123^{++}$) (MFIs: 1061 ± 54.8 vs. 674.4 ± 98.5, p=0.0012) compared to controls (**Figure 29P**). Correspondingly, the fold changes in TNF-α expression on pDCs ($CD11c^{-}$ $CD123^{++}$) and other myeloid cells ($CD11c^{++}CD123^{+}$) of was higher compared to those of HIV-N (fold MFIs: **1.051 ± 0.03** vs. 0.78 ± 0.11, p=0.015 and **1.54 ± 0.18** vs. 0.98 ± 0.08, p=0.013). The fold changes in TNF-α responses were also in harmony with those seen in multiplex bead array quantification of cytokine responses in TLR stimulated PBMCs culture supernatants (**Figure 8J**).

The functional analysis of DC subset responses to different TLR stimulations, demonstrated that mDCs and pDCs in HESN underwent lower maturation compared to those of HIV-N, based on CD83 and CD80 expression following TLR4 and TLR7 stimulations, and consequently had lower expression of IL-12p40/70 by mDCs and IFNα2b by both DC subsets. Although, the TLR8 stimulation produced higher IFNα2b responses in both mDCs and pDCs of HESN compared to those of controls, its expression did not correspond to the maturation of either DC subset between the two groups. On the other hand, TLR4 stimulations of PBMCs resulted in higher production of TNF-α by mDCs and pDCs of HESN and higher expression of CD86 (pDCs only).

6.5. Summary and Discussion

Blood Monocytes and DCs are heterogeneous populations united by a common ancestry, resulting in great overlap in the immunological functions between the two cell types. The definition of monocyte and DC lineages in this chapter was based on the most current literature, where the term 'lineage' was used to refer to dedicated functions performed by different subsets within monocyte and DC populations. As such monocytes were divided into classical monocytes ($CD14^+$ $CD16^-$), intermediate ($CD14^+$ $CD16^+$) and non-classical ($CD14^-$ $CD16^+$) monocytes, and DCs divided into mDCs ($CD11c^+$ $CD123^-$), pDCs ($CD11c^-$ $CD123^{++}$) and other myeloid cells ($CD11c^{++}$ $CD123^+$). The results presented in this chapter showed that monocytes and DCs of HESN responded differently to those of HIV-N when stimulated with TLR4, TLR7 or TLR8 ligands. Certain monocyte functions were assessed at multiple sampling points in both groups of CSWs, which included; the assessment of changes in frequencies of monocyte subsets and expression of co-stimulatory molecules on monocytes with TLR stimulation of PBMCs in HESN and HIV-N. A vast number of these functional responses were consistent overtime, while others were not.

In the first set of experiments, we demonstrated increases in numbers of non-classical monocytes that were accompanied by equal declines in the numbers of $CD14^+$ $CD16^-$ classical monocytes from 40-60% to 10-30%, coupled with increases in $CD14^+$ $CD16^+$ intermediate monocytes from 2-4% to 8-10% and proportional increases in $CD14^-$ $CD16^+$ non-classical monocytes from 0-5% to 10-20%, in response to all TLR stimulations (**Figure 22**). This observation was made using samples obtained at multiple time points and in samples used for both intracellular and extracellular flow analysis. These results strongly suggested that $CD14^+$ $CD16^+$ classical monocytes potentially differentiated into either $CD14^+$ $CD16^+$ or $CD14^-$ $CD16^+$ monocytes by increasing CD16 expression or loosing expression of

CD14. Declines in frequencies of classical and intermediate monocytes were registered after both TLR7 and TLR8 stimulations, probably due to increased differentiation of both subsets into non-classical monocytes thereby enhancing capacity for detection of ligands recognized by either receptor in this subset. The differentiation of monocytes resulted in increased proportions of virus sensing non-classical monocytes ($CD14^{-}$ $CD16^{+}$) after TLR4, TLR7 and TLR8 stimulations *in vitro*. The virus sensing function of monocytes has been associated with a specific monocyte subset- $CD14^{dim/-}$ $CD16^{+}$ monocytes that patrols or crawls on the surface of blood vessels sensing viral PAMPs such as ssRNA through TLR7 and TLR8, and is absent in classical ($CD14^{+}$ $CD16^{-}$) or intermediate ($CD14^{+}$ $CD16^{+}$) monocytes that tend to be found in circulation[378]. The proportion of the virus sensing monocyte subset, rose significantly faster in HESN PBMCs immediately following exposure to all TLR ligands, but more importantly after TLR8 stimulation.

The changes in proportions of monocyte subsets were also accompanied by changes in surface expression of CD14, a feature previously associated with ligand driven internalization of TLRs during phagocytosis (**Figure 22F**). A reduction in CD14 expression was seen in classical ($CD14^{+}$ $CD16^{-}$) and intermediate ($CD14^{+}$ $CD16^{+}$) monocytes following TLR4 stimulation as previously described[409], but not with TLR7 or TLR8 stimulations. The decline in TLR4 expression is dependent on the internalization of the TLR4-CD14 complex when bound to LPS into cytosolic membrane bound vesicles, facilitating the initiation of TLR4 signalling through TRIF/TRAM pathway (see **Figure 2**)[409]. Therefore reductions in CD14 expression correspond to reductions in surface TLR4 expression, however, this is dependent on the cell type and on the level of CD14 surface expression [410]. This could possibly explain the stable expression of TLR4 on T cells in response to LPS described earlier (**Figure 18**), as lymphocytes do not to express CD14, hence do not internalize TLR4 [411].

Expression of HLA-DR or MHC-class II on APCs is important for antigen presentation to $CD4^+$ T cells. An increase in the frequencies of HLA-DR expressing cells occurred earlier in CD3- CD19- CD56- cells of HESN compared to those of HIV-N, while a reduction in the frequencies of $HLA\text{-}DR^+$ CD3- CD19- CD56 cells was only observed in with LPS stimulation. Similar reductions in HLA-DR expression have been previously associated with reduction TNF-α responses to LPS[412], in this study HESN PBMCs produced lower soluble TNF-α expression, while $CD14^+$ $CD16^+$ monocytes in the same group expressed lower amounts of the same in response to LPS stimulation.

Costimulatory factors provide a third signal needed in T cell activation[366]. In this study we quantified the changes in expression costimulatory factors in samples obtained at two time points. We found that TLR4 and TLR8 stimulants significantly enhanced CD40 expression on $CD14^+$ monocytes of HESN than in HIV-N controls, a pattern that was retained when the costimulatory factor expression analysis was broken down to subset level. The differences in expression co-stimulatory markers CD80 and CD86 between the two study populations were less consistent, but differences were present. Consistently, CD40 expression was higher on monocytes of HESN in response to TLR8 ligand and analogue of HIV genetic material, which motivated us to assess the expression of CD40L (CD154) on T cells. We observed an upregulation of CD40L expression on T cells following TLR7 and TLR8 stimulation, with $CD8^+$ T cells expressing higher levels of the same compared to $CD4^+$ T cells, this was similar to what has been described by others[399]. The expression of CD40L was found to be highest in HESN $CD4^+$ T cells following TLR7 stimulation using Imiquimod and on $CD8^+$ T cells in the same group after either TLR7 or TLR8 treatments. The higher expression of CD40 on monocytes corresponded with higher expression of CD40L on T cells particularly in response to TLR7 and TLR8, suggesting that HESN individuals may have a higher activation of CD40-CD40L co-stimulatory pathway through TLR8 (TLR7 did not cause higher CD40),

potentially leading to higher $CD8^+$ T cells activation upon exposure to HIV genetic material *in vivo*. It is important to mention that these *in vitro* observations may be an oversimplification of the actual responses of monocytes upon exposure to HIV or HIV derived components *in vivo,* but these results provide a strong indication of the inherent differences in capacities of monocytes from HESN and HIV-N to respond to such exposures.

Monocytes are principally inflammatory cells and their functions are tightly linked to their ability to express homing markers or chemokine receptors[109]. Classical ($CD14^+$ $CD16^-$) monocytes express higher levels of chemokine receptor CCR2 compared to intermediate or non-classical monocytes. Binding of CCR2 to its cognate chemokine CCL2, causes classical monocytes to extravasate through blood vessel walls and enter infected tissues, a process that is important in the recruitment and function of classical ($CD14^+$ $CD16^-$) monocytes[405, 413]. In the experiments presented in this chapter, TLR8 stimulation resulted in a higher expression of CCR2 on classical monocytes of HESN, while the expression of CXCR3 was lower in the same subset following TLR4 stimulation in the same group. The similar expression of CXCR3 between the three monocytes subsets and between the two groups, possibly indicates that the quantity of chemokines that bind to CXCR3 i.e. CXCL4, CXCL9 MIG, CXCL10/IP-10 and CXCL11and not receptor expression itself, may be the key influences on functional responses of the monocyte subsets with regard to CXCR3. This is considering the production of CXCL10 or IP-10 a chemokine specific for CXCR3, was lower in TLR7 stimulated PBMCs of HESN but higher in TLR8 stimulated PBMCs of the same group compared to controls (**Figures 6 and 8**).

Intracellular adhesion molecule 1 (ICAM1) functions in adhesion of monocytes to luminal walls of blood vessels by binding to lymphocyte function associated antigen-1 (LFA-1; CD11a/CD18) expressed on endothelial cells. The binding of monocytes to LFA-1 on

endothelial surfaces is known to be responsible from the crawling or rolling activity of monocytes in intravenous blood of mice[384]. The increases in proportions of $CD14^{-}$ $CD16^{+}$ monocytes or the virus sensing subset, was accompanied by loss adhesive function, particularly in HESN PBMCs in response to TLR8 (**Figure 22**), such that over the course of TLR8 stimulation, non-classical ($CD14^{-}$ $CD16^{+}$) monocytes of HESN expressed lower levels of ICAM1. This suggests that the higher recognition of HIV derived genetic material, as was seen in HESN, could have resulted in loss of adhesive function of $CD14^{-}$ $CD16^{+}$ monocytes, potentially enhancing the ability of these cells to move or enter tissues, and differentiate into other cell types to perform different functions (ref **Figure 26**).

The differences observed in proportions of monocyte subsets, expression of costimulatory factors and adhesive functions on monocytes in PBMCS of HESN compared to HIV-N, were also present in cytokine expression in monocytes. Where intermediate and non-classical monocytes of HESN expressed lower levels of IL-10 and IL-12p40/70 following either TLR7 or TLR8 stimulation and lower levels of TNF-α in response to TLR4 and with the tendency for higher TNF-α expression in response to TLR8. Notably, TLR7 stimulation with Imiqimod failed to elicit the expression of TNF-α, when quantified using intracellular cytokine staining from both monocytes and DCs, and when quantified in culture supernatants (**Figure 5-8**). The magnitude of cytokine responses between the monocyte subsets varied with IL-10 production being highest in intermediate and non-classical monocytes following either TLR4 or TLR7 stimulations, respectively, whereas IL-12p40/70 responses were highest in classical and intermediate monocytes after TLR7 and 8 stimulations. Delineating the production of the two IL-12 subunits using a single flow cytometry antibody proved very difficult; in the future it would necessary to repeat this quantification using antibodies specific for the different IL-12 subunits, especially considering the differences in production of IL-12 subunits with TLR4 stimulation; where HESN produced higher amounts of soluble IL-12p40 but lower

amounts of IL-12p70 (**Figures 7 and 8**). Lastly, TNF-α responses were present in classical and intermediate monocytes with TLR4 and 8 but not TLR7 stimulations. These results resembled previous observations made by Cros., *et al* 2010, where intermediate monocytes expressed the highest amounts of TNF-α and IL-1β, while classical and intermediate monocytes expressed the highest amounts of IL-10 in response to LPS[378]. The differences in cytokine expression between the $CD14^{+}$ and $CD14^{dim}$ monocyte subsets was linked to differences in activation of intracellular TLR signalling by TLR7 or TLR8 ligands.

Dendritic cells are closely related to monocytes and are derived from a common progenitor. *In vitro* monocytes can differentiate into DCs in the presence of GM-CSF and IL-4, or into macrophages in the presence of IL-6 and IFN-γ[404, 414]. The mechanism, through which monocytes differentiate to DCs *in vivo,* is still unknown. The primary reason for evaluating cytokine responses and functions in these two cell types was to try and identify the cells responsible for the differential cytokine production in supernatants from PBMCs of HESN and HIV-N after the three TLR stimulations (**Figures 5-8**). This is considering that monocytes and DCs express the highest levels of TLRs, and equally play an important role in the development of adaptive immune responses and immune regulation. The variable expression profiles of TLRs on monocytes and DCs separates the functions performed by these cells during infection. Monocytes express TLR1, 2, 4-10 but fail to express TLR3, while pDCs express TLR7 and TLR9 but lack TLR4 and TLR7, and mDCs express TLR1, 2, 3, 6, 7, 8 but lack TLR9 [108, 199, 378].

Higher pDC maturation and NK cell activation has been described among HESN IDUs, and in individuals who recently had high risk sexual contact[415]. This study also evaluated different aspects of DC activation- maturation, expression of co-stimulatory factors and cytokine production in response to TLR4, 7 or 8 stimulations in HESN CSWs. The results

from this evaluation revealed a lower maturation of HESN pDCs ($CD11c^{-}$ $CD123^{++}$), mDCs ($CD11c^{++}$ $CD123^{-}$) and other myeloid cells ($CD11c^{++}$ $CD123^{+}$) following TLR7 stimulation based on expression of maturation marker CD83, co-stimulatory factor CD80, IFNα2b and IL-12p40/70 responses. Conversely, TLR4 stimulation of HESN PBMCs resulted in higher activation pDCs with regard to CD86 expression and higher TNF-α production.

The differences in cytokine responses in DC subsets described here resembled what has been previously documented, where mDCs primarily expressed higher amounts of IL-12p40/70 and TNF-α, while pDCs typically express IFNα2b after TLR7 stimulation[199]. However, TLR8 ligand stimulation led to significantly higher levels of IFNα2b expression in pDCs of HESN yet these cells do not to express TLR8. This aberration was linked to the ability of TLR7 to recognize both Imiquimod and ssRNA in the absence of TLR8[416], demonstrating that different TLR7 ligands stimulating the same receptor, can produce opposing results in pDCs of HESN and HIV-N CSWs. The plausible explanations for this phenomenon could include; differences in TLR7 signal transduction arising from variable receptor-ligand interactions resulting in differential transcription of cytokine genes. Receptor-ligand conformational rearrangements direct the recruitment of adaptor molecules to the TIR domain (TRAM or MyD88), subsequently affecting downstream signal transduction. Such variation in receptor-ligand interactions could be introduced into TLR7 receptor protein by genetic variation through single nucleotide polymorphisms (SNPs), such as TLR7 rs179012 SNP associated with reduced HIV acquisition in a Kenyan population[417]. Alternatively, IFNα2 expression in response to TLR8 could be driven by other soluble factors such as cytokines (IFN-γ) produced by other cells expressing and responding to the same TLR8 ligand. Much as IFNα2 production is important in antiviral responses, its induction by TLR7 has previously been shown to have no impact on reducing the risk of infection with SIV *in vivo* or HIV infection *in vitro*[28, 248]. The lower responsiveness of HESN pDCs to TLR7

stimulation based on IFNα2 production, could confer protection by reducing or shutting down recruitment of HIV targets in sub-epithelial layers of the cervix in the event of exposure to the virus[418].

In total, the experiments in this section demonstrated for the first time that differences exist in the differentiation, activation and maturation of monocytes and DCs in PBMCs of HESN and HIV-N, when stimulated with different TLR ligands. Non-classical or $CD14^{-}$ $CD16^{+}$ monocytes in HESN increased in number upon exposure to microbial components, and this cell subset is known to increase during in infection or inflammation. On the other hand, HIV-N had higher activation of DCs especially in response to TLR7, considering that DCs are less inflammatory and more efficient in guiding development of T cells responses in lymphoid tissues, higher activity in this subset upon exposure to HIV could increase the risk of DCs shuttling invading virions to lymphoid tissues, thereby enabling the establishment of the new infection. On the other hand higher activation of monocytes as was the case in HESN, may be more protective, as monocytes are less efficient in antigen presentation[109] but more efficient in directing inflammatory processes, sampling tissues for foreign antigens as macrophages, and in killing virus infected cells through potent effector responses such as production ROS, iNOS and peroxidases[405]. Therefore we speculate the higher activation of virus sensing monocytes could lead to enhanced clearance of tissue infections with low doses HIV through monocyte mediated effector mechanisms or through recruitment of other actors like $CD8^{+}$ T cells and NK cells capable of killing infected cells prior to dissemination. These findings have potentially useful therapeutic implications, given that the selective activation of the TLR7 or TLR8 signalling pathways through a microbicide could provide new alternative means for preventing HIV transmission from the current methods in use.

CHAPTER 7: Higher Responses of Natural Killer Cells in HESN to Different TLR Stimulations.

7.1. Introduction

The absence of immune correlates of protection against HIV in NK cells, necessitates the use of HIV pathogenesis as a means of understanding the roles played by NK cells during HIV infection[166]. Natural killer cells are capable rapidly killing HIV infected cells, deleting autologous $CD4^+$ and $CD8^+$ T cells during chronic infections like LCMV, and killing over-reactive cells in chronic inflammatory diseases [419, 420]. HIV infection leads to functional impairment of NK cells starting in the acute phase of infection and worsening during the chronic phase of disease[137]. NK cell mediated suppression of HIV replication in autologous $CD4^+$ T cells of HIV infected indivduals has been show to be principally mediated by CC chemokines, and is inversely correlated with viraemia[421]. Reductions in perforin or granzyme A production, aberrant/decreased expression of activatory and inhibitory NK cell receptors (NKRs), results in reduced NK cytotoxicity in HIV infected individuals[137]. NK cells can identify HIV infected cells through altered expression of markers like TNF related apoptosis inducing ligand (TRAIL), or when such cells fail to express certain MHC class I molecules. Increased immune activation during the acute phase of HIV is accompanied by increased expression of TRAIL on T and B cells[422].The increased expression of TRAIL on T and B cells also targets them for deletion by NK cells, bystander apoptosis or immune-suppression[422]. By deleting these virus infected or excessively inflammatory T cells, NK cells perform a regulatory function[423].

NK cell activity through activatory and inhibitory KIR3DS1 and KIR3DL1, respectively, aids in the attainment of viral set point and delays disease progression through recognition of HLA-B 80Ile variant Bw4 motif in individuals expressing these HLA-class I haplotypes[35,

424]. Higher frequencies of individuals with homozygous KIR3DS1, have been described among HIV exposed seronegative individuals when compared to HIV infected individuals[425]. NK cell responses expand during the acute phase of HIV disease, enhancing the killing of HIV-1 infected cells[426]. The pressure exerted by KIR3D mediated NK cell killing activity during HIV disease has been shown to influence virus sequence evolution[42]. HIV has evolved to reduce the expression of NKR ligands in infected cells[427]. Additionally, during the chronic phase of HIV infection, NK cells have higher but incomplete activation state based on higher expression of HLA-DR and CD69, but not CD25 and NKp44 (NCR2)[428]. Presently, the understanding of NK cell function during HIV infection supports the premise that increased NK activity favours protection and slowed disease progression, however, *in vivo* data needed to support this is currently unavailable.

NK cells produce cytokines and chemokines that condition DCs functions in directing antiviral T cell responses[426, 429-431]. NK cells also bolster and expand effector T cell responses through production of IL-2, leading to enhanced proliferation of antigen specific T cells, in turn enhancing NK proliferation during viral infections [432]. Proinflammatory cytokines like IL-12, IL-15 and IL-18 produced by monocytes and DCs in response to pathogen derived components, can enhance the ability of NK cells to respond to IL-2, by enhancing IL-2Rα expression on NK cells[42]. Production of IL-12 and IL-18 by DCs, can enhance IFN-γ production by NK cells, while type I IFN production by pDCs when in contact with NK cells, promotes NK cytotoxic and proliferative functions[430-432]. Exogenous IL-15/IL-15Rα complex, has recently been shown to enhance NK cell mediated cytotoxic targeting of HIV infected cells *in vitro,* and can also increase anti-HIV activity in HIV infected humanized mice[433]. This suggests that IL-15 produced by APCs during HIV infection could potentially enhance anti-HIV activity by NK cells, although the effect of HIV on IL-15 production by APCs has not been fully determined.

7.2. Rationale

Heightened NK cell activity has been described in individuals with reduced susceptibility to HIV (HESN), suggesting a protective role of NK cells against HIV acquisition. A number of studies have compared the expression of genetic NK cells factors associated with reduced susceptibility to HIV- such as protective KIRs- KIR3DSl and KIR3DL genotypes[35, 425, 434] and their impact on susceptibility to HIV using the HESN model. The findings of these studies have been mixed, in some instances the protective effect of KIRs have been dependent on the HESN population being studied. For instance, the expression of protective yet inhibitory KIR3DL1 and KIR3DL1(+) HLA-Bw4 (+), was significantly lower in HESN, while protective and activatory KIR3DS1 and Bw4 (+) DL1(-) DS1(+) was overrepresented in HESN individuals[435]. Similarly, in a Canadian cohort of IDUs, a higher prevalence of protective KIR3DL1 and KIR3DS1 was observed in HESN IDUs compared to HIV infected IDUs from the same geographical area, but the protective effect of these KIRs was absent in African populations from Cote d'Ivorie, potentially due to the low prevalence of KIR3DL1 genotype in African populations[425, 436]. These genetic studies reveal that a number of KIR alleles associated with protection against HIV could be present in specific HESN populations, but not all [415].

Some of the genetic associations mentioned above have been linked to immunological functions in NK cells. NK cells that expressed the KIR3DS1 also produced higher amounts of antiviral cytokines and were more potent at inhibiting HIV replication[437, 438]. The frequency NK cells bearing protective KIRs has been shown to expand in the early phase of certain viral infections, prior to the development of virus specific adaptive immune responses *in vivo*[137].

HIV exposed intravenous drug users (IDU) in Vietnam were the first to be shown to possess heightened NK activity based on increased cytotoxicity and production antiviral cytokines[434]. Elevated production of IFN-γ has been described in the genital tract of HESN individuals following sex with a HIV-1 positive partners[439]. Higher NK cell activation and degranulation based on expression of CD69 and CD107, respectively, has been observed in children born to HIV infected mothers in several cohorts[415]. On the contrary, a recent study conducted among female sex workers from Majengo, demonstrated a lower state of immune activation in NK cells of HESN *ex vivo* based on CD69, PD-1, TIM-3 and LAG3 expression[440]. The higher CD107a expression has been shown to correspond to higher NK cytolytic activity, suggesting that NK cells in HESN individuals possess a higher capacity to mount cytolytic NK responses, potentially capable of limiting HIV infection *in vitro*[264].

NK cell cytolytic activity can be augmented by cytokines such as IFN-α, IL-2, IL-12, IL-15, IL-18 and IL-21. The majority of these cytokines are produced by APCs in response to different PAMPs. NK cells are activated soon after infection with HIV and remain so throughout the chronic phase of disease[166]. These cells potentially become activated in the presence of $CD14^{+}$ monocytes and pDCs responding HIV derived genetic material, leading to a 'cytokine storm' containing of IL-12, IFN-α and TNF-α capable of activating NK cells[26, 27, 33]. Previously, a study in our cohort demonstrated a greater iDC mediated enhancement of NK cell degranulation based on expression of CD107a and antiviral cytokine production (IFN-γ and IL-12) in HESN compared to HIV-N and HIV-positive individuals[441]. *In vitro* the activation of NK cells in the context of TLR7/8 stimulation of PBMCs has been shown to occur in the presence of cytokine mediators produced by APCs, this is despite NK cells expressing TLR1-6 and TLR8-10, but lacking TLR7[259].

Hitherto this, there has never been a study comparing the responsiveness of NK cells in PBMCs of HESN and susceptible controls to different TLR treatments. In this chapter, we quantified and compared different NK cells functions in PBMCs from HESN and HIV-N CSWs prior to and following treatment with different TLR4, TLR7 and TLR8 ligands. We then proceeded to compare the NK cell responses presented in this chapter to the cytokine responses presented from chapter 4, and APC functions from Chapter 6. This was motivated by the known importance of APCs functions in direction of NK cells responses *in vitro*[259].

7.3. Hypothesis

Natural Killer cells in HESN PBMCs when treated with TLR8 agonist ssRNA become more activated, express higher levels of antiviral cytokines and possess greater degranulation potential compared to those of HIV-N.

7.4. Objectives

The following objectives were developed to address the hypothesis above:

1. To quantify the changes in $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets in PBMCs of HESN and HIV-N before and after TLR4, 7 or 8 stimulations.
2. To measure the expression of activation markers CD69 and HLA-DR on different NK cell subsets in PBMCs of HESN and HIV-N before and after TLR4, TLR7 or TLR8 stimulations.
3. To quantify the production of IFN-γ, IL-2 and TNF-α by NK cell subsets before and after TLR4, TLR7 or TLR8 stimulations.
4. To compare the expression of NK receptors (NKRs) - NKG2D, CD94 and CD16 on different NK cell populations with TLR4, TLR7 or TLR8 stimulations.
5. To compare the potential of NK cells of HESN and HIV-N to degranulate based on expression of CD107a (LAMP1) on NK cell subsets with TLR4, TLR7 and TLR8 stimulations.

7.5. Results

7.5.1. Changes in NK cell subsets in HESN PBMCs with different TLR stimulations

To quantify the changes in NK subsets in PBMCs of HESN and HIV-N with TLR stimulation, freshly isolated PBMCs were stimulated overnight with either LPS-TLR4, Imiquimod-TLR7, ssRNA40/LyoVec-TLR8, PHA or PMA with Ionomycin. First, we quantified and compared the changes in CD3- $CD56^{bright}$ and $CD3^{-}$ $CD56^{dim}$ NK cells in PBMCs of HESN and HIV-N prior to and following stimulations, using multicolour flow cytometry. The changes in NK cell subsets were evaluated at two sampling time points, in 2010 and 2014 which coincided with research field trips to Nairobi (**Figure 30**).

Two different flow cytometry gating strategies were used to assess different NK cell subsets guided by the flow panel designs (**Table 1**). The first gating method used was to phenotype NK cells in PBMCs collected in 2010. It started from a dot plot derived from the lymphocyte gate with CD3 (on X-axis) and CD56 (y-axis) that was used to gate $CD3^{-}$ $CD56^{+}$ cells, and a second plot used to separate $CD56^{Bright}$ from $CD56^{dim}$ NK cells (**Figure 30A**). The first gating strategy produced frequencies of $CD56^{Bright}$ NK cells between 10-20% and $CD56^{dim}$ 70-90% calculated from all $CD3^{-}$ $CD56^{+}$ cells, and the surface expression of CD56 on the bright NK population was higher compared than on the dim NK population(**Figures 30B-E**), consistent with what is in the literature[137].

A second gating strategy was used to identify NK cell subsets in samples collected in 2014. Here, $CD3^{+}$ (T cells) were first separated from $CD3^{-}$ cells using a first plot, and a second plot used to separate $CD3^{-}$ cells into $CD56^{Bright}$ and $CD56^{dim}$ NK populations (**Figure 30G)**. The second gating strategy produced frequencies of $CD56^{Bright}$ NK cells (1-3% of all $CD3^{-}$ cells) and $CD56^{dim}$ NK cells (15-40% of all $CD3^{-}$ cells) to give a ratio of 1; 9 $CD56^{bright}$:$CD56^{dim}$. Once again the CD56 surface expression was higher on the bright NK population compared to the dim (**Figures 30H-P**).

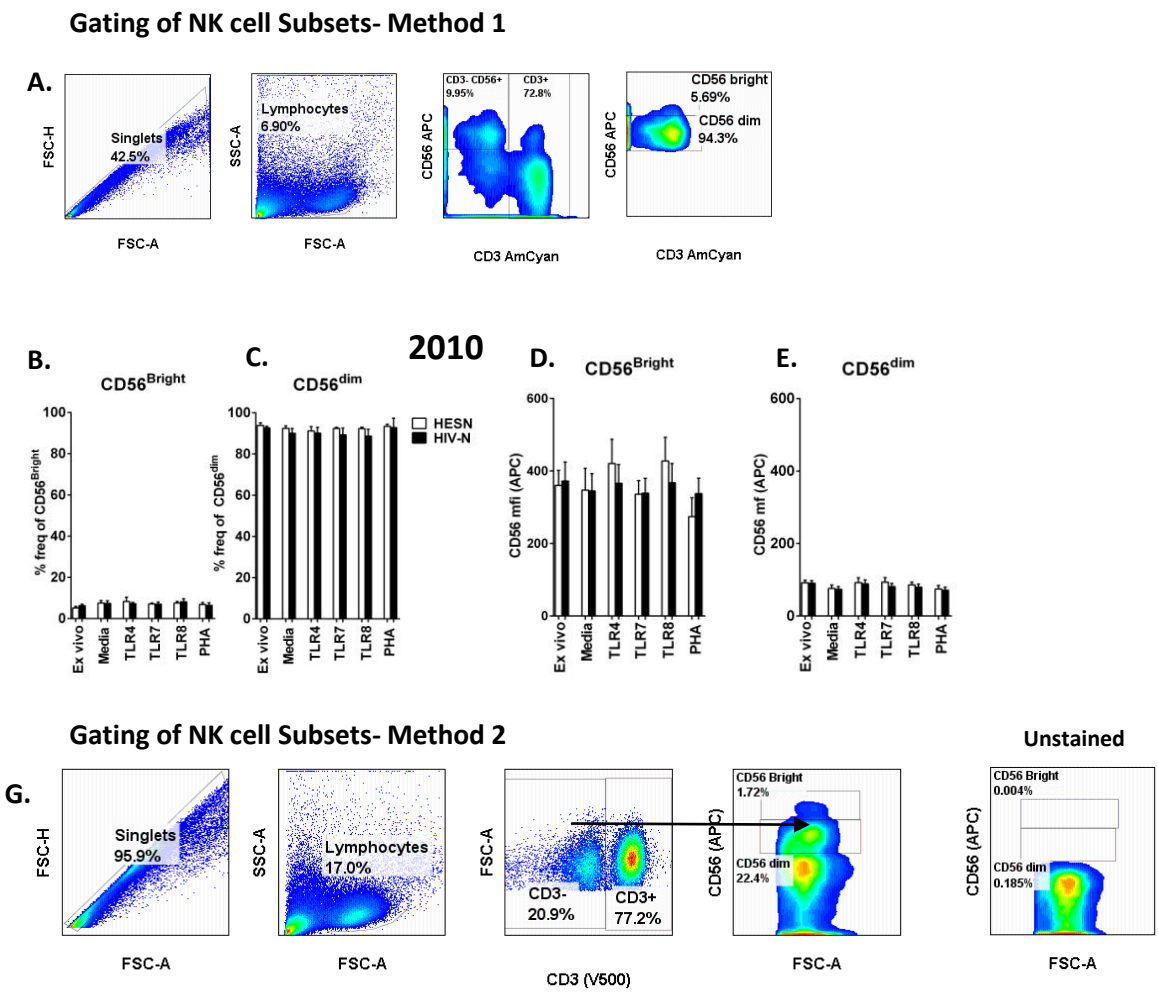


Figure 30: Changes in NK cell Subsets with TLR stimulation. Freshly isolated PBMCs were treated with LPS (TLR4), Imiquimod (TLR7), ssRNA40/LyoVec (TLR8) or PMA with Ionomycin, overnight. A) Gating of $CD56^{bright}$ and $CD56^{dim}$ NK subsets during flow cytometry. B-E) Comparison of NK subset frequencies with TLR stimulation in HESN (white) and HIV-N (black). H-K) Changes in NK cell subsets in extracellular flow panel. M-P) Changes in NK cell subsets during intracellular cytokine staining (ICS). Statistical analysis was done using Mann-Whitney test for grouped comparisons and Wilcoxon signed rank test $P<0.05$ indicated with *.

In 2010, none of the TLR treatments altered the proportions of $CD56^{Bright}$ or $CD56^{dim}$ NK cells in either group, nor produced differences between the CSW groups. However in 2014, *ex vivo* and in unstimulated cultures (media), HESN had higher frequencies of $CD56^{bright}$ NK cells compared to HIV-N controls (**0.92 ± 0.2%** vs. 0.3 ± 0.1, p=0.017 and **1.7 ± 0.4**% vs. 0.7 ± 0.2, p=0.0090, respectively). The surface expression of CD56 on $CD56^{Bright}$ NK cells, increased after TLR8 stimulations, but with no significant differences between the CSW groups. Overall, TLR simulations of PBMCs did not significantly cause changes to the proportions of either NK subsets in HESN or HIV-N, but in some instances TLR8 stimulation enhanced the expression CD56 on $CD56^{bright}$ NK cells (**Figures 30A-P**).

7.5.2. Higher activation of HESN NK cells with TLR stimulation

Higher activation and degranulation of NK cell subsets based on expression of CD69 and CD107a has previously been linked to the HESN phenotype[441]. TLR7/8 stimulation has been shown to up-regulate CD69 expression on NK cells in mixed PBMCs, but not in purified NK cells[259]. The capacity of NK cells to be activated in the presence of TLR7/8 ligand ssRNA, needs contact with monocytes and pDCs but not mDCs[259]. Therefore, all the experiments evaluating NK functions in this study were conducted using mixed PBMCs.

In this study we quantified the expression of CD69 and HLA-DR on $CD56^{Bright}$ and $CD56^{dim}$ NK cells before and after TLR4, TLR7 or TLR8 stimulations using multicolour flow cytometry. This tested the hypothesis that TLR8 stimulation of PBMCs would result in higher activation based on CD69 and HLA-DR expression in NK cells of HESN (n=15) compared to HIV-N (n=15) (**Figure 31**).

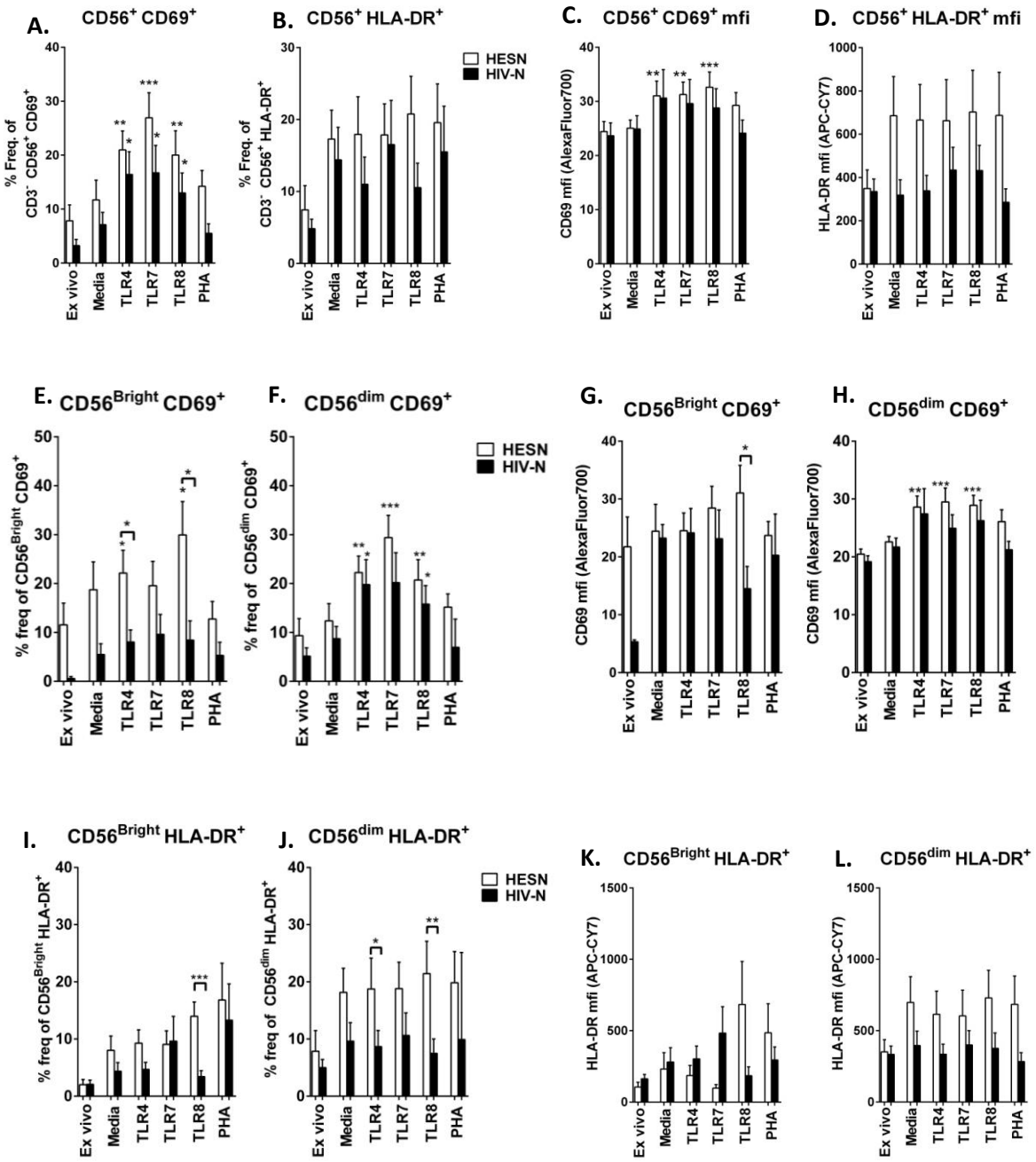


Figure 31. Natural Killer cell activation with TLR stimulation. The expression of activation induced marker (AIM or CD69) and MHC class II molecule (HLA-DR) on NK cell subsets, was quantified using flow cytometry. Freshly isolated PBMCs from HESN and HIV-N CSWs were stimulated overnight using LPS (TLR4), Imiquimod (TLR7), ssRNA40/LyoVec (TLR8) or PMA with Ionomycin. Upregulation CD69 but not HLA-DR was more pronounced on both $CD56^{bright}$ and $CD56^{dim}$ NK cells of HESN compared to those of HIV-N. Statistical comparison unpaired grouped data was done using Mann-Whitney test and paired comparsions done using Wilcoxon signed-rank *t*-test. All $p<0.05$ are indicated with *. HESN (n=15) and HIV-N (n=15).

The results showed that stimulation of NK cells in mixed PBMCs with TLR4, TLR7 or TLR8 and not PHA, enhanced both the frequency of cells and surface expression of CD69 in $CD3^-$ $CD56^+$ (all NK cells). On the contrary, the expression of HLA-DR (MHC class II) increased in some individuals with TLR4, 7 or 8 stimulations, although this was not significant (**Figures 31A-D**). At the NK subset level, the frequency of CD69 expressing $CD56^{Bright}$ NK cells was higher in HESN following TLR4 (**22.13 ± 4.7%** vs. 8.03 ± 2.59, p=0.022) and TLR8 stimulations (**29.95 ± 6.8**% vs. 8.43 ± 3.9, p=0.028) compared to HIV-N controls (**Figure 31E**). The surface expression of CD69 on $CD56^{bright}$ NK cells of HESN was equally higher following TLR8 stimulation (SEM MFIs: **31.04 ± 4.8** vs. 14.51 ± 3.8, p= 0.04) (**Figure 31G**). Similarly, HESN had significantly increased frequencies of $CD56^{dim}$ NK cells expressing CD69 after TLR4 (p=0.0049, Wilcoxon rank test), TLR7 (p=0.0010) and TLR8 (p=0.0034) stimulations. Whereas only TLR4 (p=0.011) and TLR8 (p=0.0195) stimulations increased the frequencies of CD69 expressing cells in the $CD56^{dim}$ NK subset of HIV-N, with no differences between the two groups (**Figure 31F and H**).

When we examined the HLA-DR expression on $CD56^{bright}$ and $CD56^{dim}$ NK cells, we found that TLR4 stimulation lead to higher frequencies of HLA-DR expressing cells in $CD56^{Bright}$ NK cells, but TLR8 stimulations caused significantly higher frequencies of HLA-DR cells in both NK subsets of HESN compared to those of HIV-N, (**Figure 32I and J**).

In summary, these results demonstrated that the NK cells of HESN became more activated when exposed to different microbial PAMPs, and this was primarily restricted to the $CD56^{Bright}$ NK subset of HESN compared to those of HIV-N.

7.5.3. Expression of NK cell receptors (NKG2, NKG2D and CD16) on NK subsets of HESN with TLR stimulation

The ability of NK cells to recognize virus infected, stressed or transformed cells, is dependent on the activity of NKRs, such as NKG2 family of receptors and natural cytotoxic receptors like CD16. Natural killer cells express CD16 (FcγRIII), a receptor first identified for its ability to spontaneously trigger ADCC and cytotoxicity[442, 443]. First, we compared the changes in expression of CD16 (FcγRIII) using multicolor flow cytometry on the two NK subsets - $CD3^-$ $CD56^{dim}$ and $CD3^-$ $CD56^{bright}$ NK cells - after TLR4, 7 or 8 stimulation in PBMCs from the HESN (n=15) and HIV-N (n=15).

The frequencies and surface expression of CD16 on $CD56^{bright}$ and $CD56^{dim}$ in both groups remained unaltered with TLR4, 7 or 8 stimulations. The expression CD16 on $CD56^{dim}$ NK cells was higher than on $CD56^{bright}$ NK cells, although the surface expression of CD16 on $CD56^{dim}$ NK cells of HESN tended to be higher compared to those of HIV-N, but this was not significant (**Figures 32A-Q**). The higher CD16 expression observed on $CD56^{dim}$ NK cells compared to $CD56^{bright}$ NK cells was consistent with what has been previously described (**Figures 32A-Q**)[137].

However, *ex vivo* $CD56^{bright}$ NK cells of HESN had higher expression of CD16-based on MFIs (MFI: in 2010-**2990 ± 557.9** vs. 1379 ± 263.7, p=0.0142; in 2014- **22025 ± 4649** vs 7956 ± 1442, p=0.023) and higher frequencies $CD56^{bright}$ $CD16^+$ NK cells (in 2014- **45.6 ± 4.0%** vs. 24.6 ± 5.3%, p=0.0036) compared to susceptible controls at two different time points (**Figure 32**). The frequencies of $CD56^{dim}$ $CD16^+$ and $CD56^{bright}$ $CD16^-$ NK cells were lower in HESN following TLR4 (**54.13 ± 6.9%** vs. 70.5 ± 3.9%, p=0.041) and TLR7 (**47.09 ± 5.0%** vs. 65.92 ± 3.8%, p=0.0060) stimulations in 2010, but not in 2014 (**Figures 32B and C**).

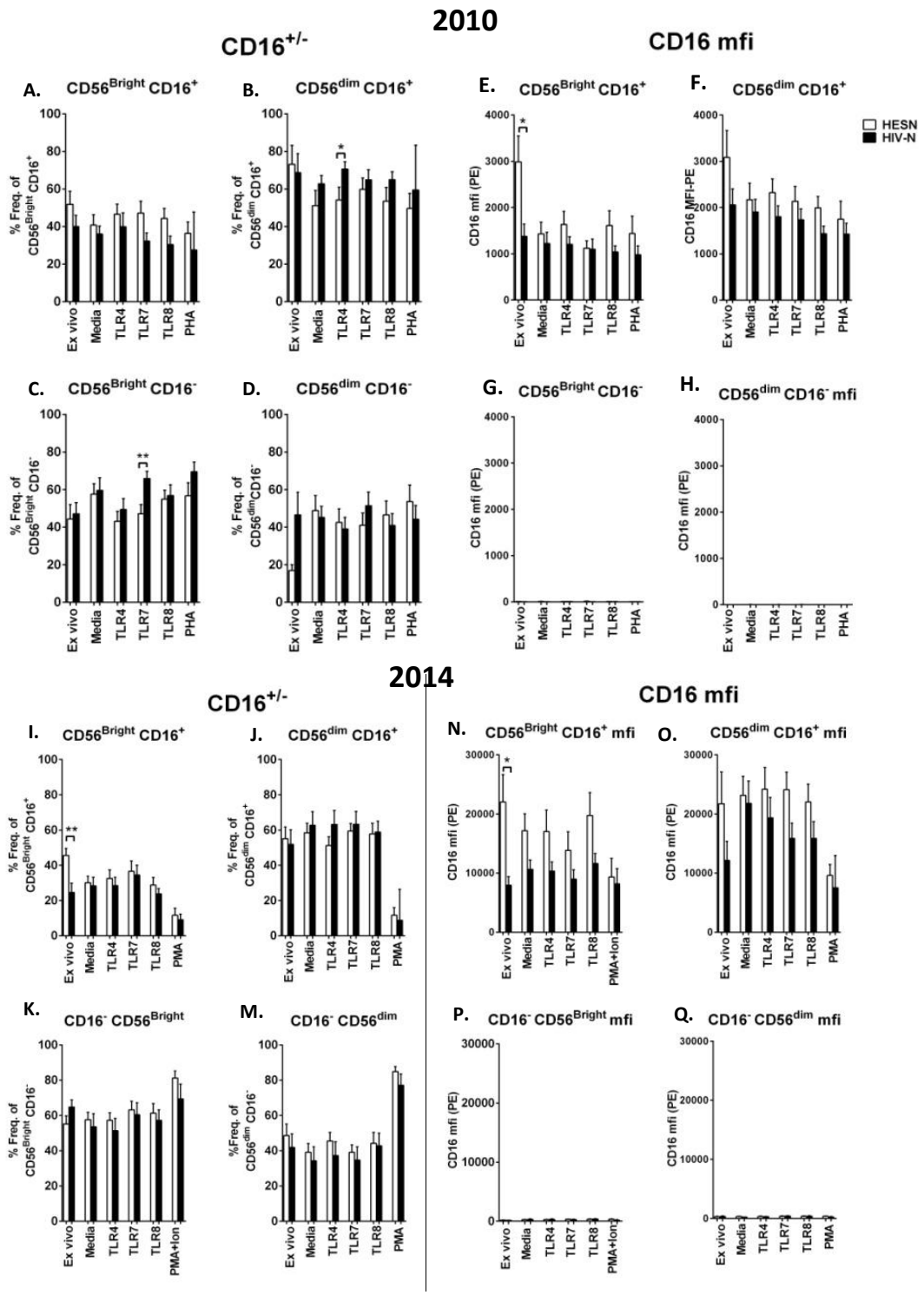


Figure 32. Expression of FcγRIII (CD16) on NK subsets with TLR stimulation. ADCC function of NK cells is dependent on the expression of CD16. The expression of CD16 on $CD56^{bright}$ and $CD56^{dim}$ NK subsets was evaluated on PBMCs from HESN and HIV-N, in the presence or absence of different stimulation conditions (TLR4, TLR7, TLR8, PHA or PMA with Ionomycin). This evaluation was conducted at two different time points- 2010 and 2014. Expression of CD16 was found to be highly expressed on $CD16^{Bright}$ NK cells of HESN at more than one time point, and the expression of CD16 varied little with TLR stimulation in both study groups. Comparison of groups done using Mann-Whitney *t*-test $P<0.05$ are indicated with *.HESN (n=15) and HIV-N (n=15)

Pathogen derived components are also capable of indirectly inducing NK cell killing through NKG2D. TLR3 signalling in murine macrophages has been shown to induce the expression of NKG2D ligand- retinoic acid early transcript 1 (Rae1)[444]. TLR7 stimulation can induce NK cells to produce IFN-γ and its production can be enhanced by the co-stimulatory effect of NKG2D binding to its ligand MHC class I chain related protein A (MICA) when expressed on tumour cells[445]. NKG2D is an activatory receptor, and binding to its ligand expressed on infected or transformed cells, leads to NK killing activity. Healthy cells typically lack expression of ligands recognized by NKG2D, but the expression of such ligands is more pronounced on stressed, transformed or infected cells [446].

In this study the changes in expression of NKG2 and NKG2D (CD94) were evaluated on $CD56^{bright}$ and $CD56^{dim}$ NK cells in mixed PBMCs of HESN and HIV-N, before and after TLR4, TLR7 or TLR8 stimulations using multicolour flow cytometry (**Figure 33**). The flow gating used was indicated in **Figure 33A.** Both $CD56^{bright}$ and $CD56^{dim}$ NK cells expressed NKG2, with the frequency of $CD56^{bright}$ $NKG2^{+}$ NK cells ranging between 69-96.4% compared to the frequency of $CD56^{dim}$ $NKG2^{+}$ NK cells (40-60%) in both CSW groups (**Figures 33A-E**).

The frequencies of $CD56^{bright}$ $NKG2^{+}$ and $CD56^{dim}$ $NKG2^{+}$ NK cells remained relatively stable after TLR4, TLR7, TLR8 or PMA+Ion stimulations with no differences in expression of NKG2 between the two CSW groups (**Figures 33B-E**). We found that NKG2D (CD94) was expressed on both $CD56^{bright}$ and $CD56^{dim}$ NK cells, although, the former expressed higher amounts of NKG2D compared to the latter in terms of numbers of cells and surface expression (MFI data not shown) (**Figures 33D and E**). None of the TLR stimulations used produced significant increases in NKG2D expression on $CD56^{bright}$ and $CD56^{dim}$ NK cells, or differences in frequencies of cells expressing NKG2D in both NK cells subsets significant between HESN and HIV-N.

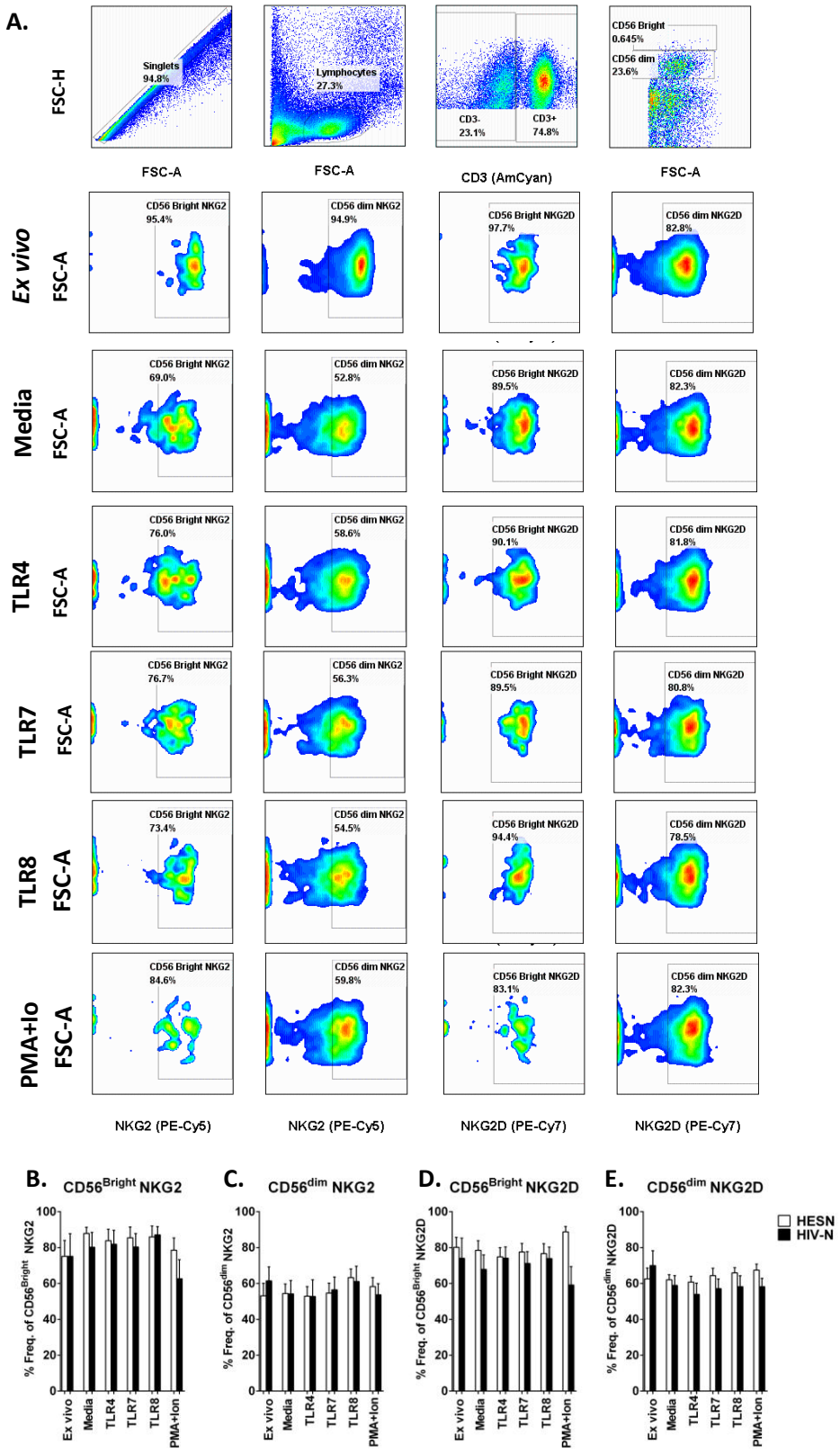


Figure 33. Expression of NK cell Receptors (NKRs) on different NK subsets with TLR stimulation. The expression of NKRs; NKG2D- an activating receptor and CD94- an activating/ inhibiting receptor on $CD56^{bright}$ and $CD56^{dim}$ NK cells in mixed PBMCs before and after TLR4 (LPS), TLR7 (Imiquimod), TLR8 (ssRNA) or PMA+ Ionomycin stimulations. A) Flow gating and expression of NKG2 and NKG2D on $CD56^{bright/dim}$ NK cells with different stimulated conditions. B-E) Bar graphs comparing the expression NKG2 and NKG2D on NK cells of HESN and HIV-N. HESN (n=15) and HIV-N (n=15)

In summary, *ex vivo* HESN had higher frequencies of $CD56^{bright}$ $CD16^{+}$ NK cells in PBMCs, while the frequencies of $CD56^{dim}$ $CD16^{+}$, and both $CD56^{bright}$ and $CD56^{dim}$ NK cells lacking CD16 expression was similar between HESN and HIV-N. An evaluation of other NKRs revealed that NKG2D were expressed on both $CD56^{bright}$ and $CD56^{dim}$ NK cells almost to a similar degree. On the other hand, the frequencies of NKG2 expressing $CD56^{bright}$ NK cells was higher than $CD56^{dim}$ $NKG2^{+}$ NK cells, with no TLR enhancing effects or differences in NKG2 expression on the two NK subsets between HESN and HIV-N. In all cases, the frequencies of $CD56^{bright}$ and $CD56^{dim}$ expressing NKG2, NKG2D or CD16 in both HESN and HIV-N was not altered by TLR4, TLR7 or TLR8 stimulations.

7.5.4. Higher Degranulation potential of HESN NK cells following TLR7 and TLR8 stimulations

Signalling through the Natural killer cell receptor (NKR)- CD16, induces exocytosis of cytolytic granules capable of killing antibody coated pathogens through ADCC[447]. We compared the changes in LAMP1 or CD107a expression as a surrogate marker for cellular degranulation on $CD16^{+/-}$ NK cells with TLR stimulation (**Figure 34)[447]**. This tested the hypothesis that, TLR8 activation using cognate ligands would result in greater degranulation of NK cells in HESN compared to susceptible female CSWs. Once again, PBMCs from HESN (n=19 and HIV-N (n=14), were stimulated with TLR4-LPS, TLR7-Imiquimod, TLR8-ssRNA40/LyoVec or PMA with Ionomycin for between 20-24 hours, and CD107a expression measured by multicolour flow cytometry. Respresentative gating plots used in defining $CD56^{bright}$ and $CD56^{dim}$ NK cell populations shown in **Figure 34A**.

The results showed that, TLR4, 7 and 8 stimulations lead to increases in frequency of $CD56^{dim}$ $CD107a^{+}$ NK cells, in HIV-N but not in HESN, probably due to the higher frequencies of CD107a expressing $CD56^{dim}$ in unstimulated cultures of HESN (**39.6 ± 4.9%** vs. 17.9 ± 3.4%, p=0.0062).

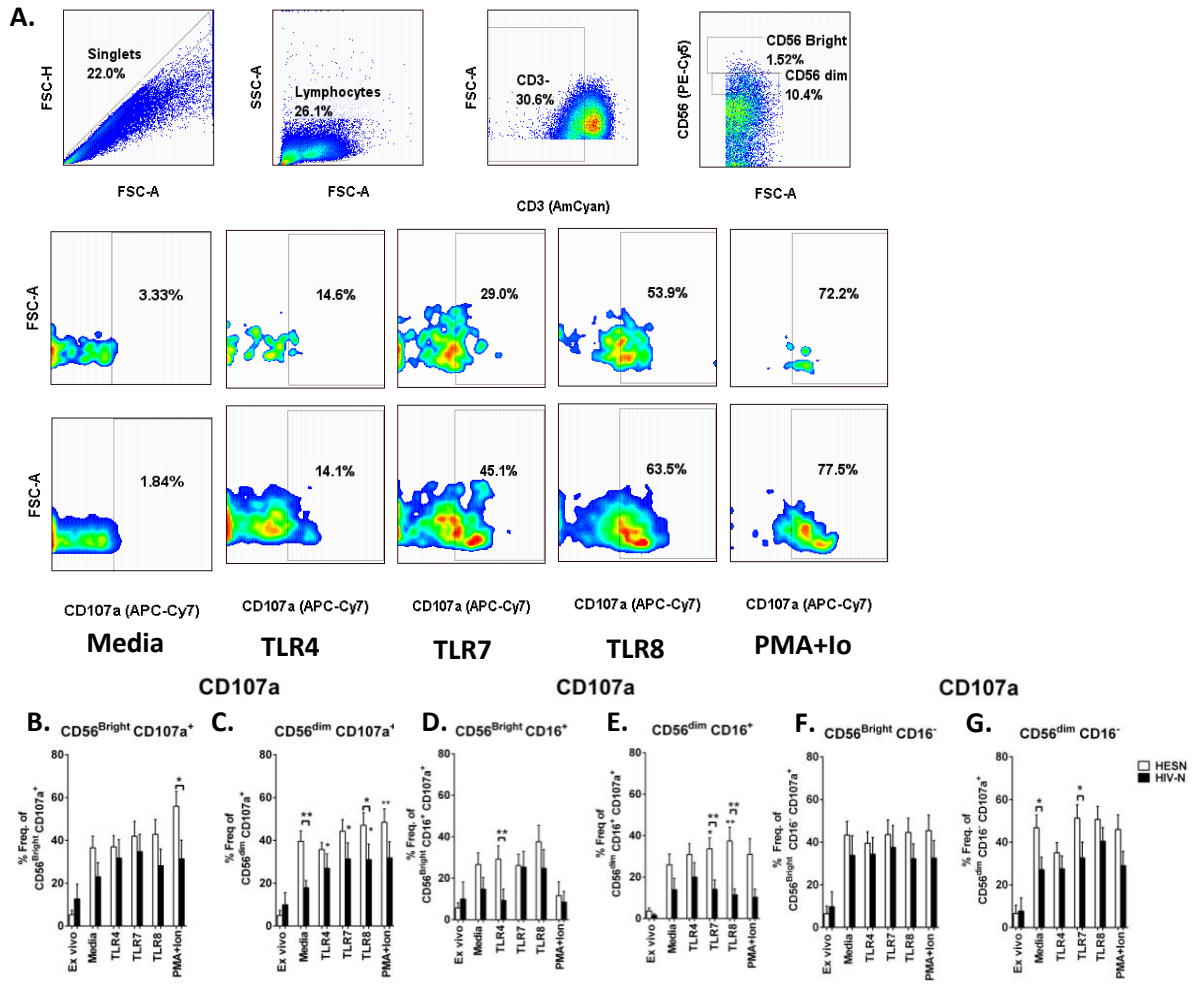


Figure 34. Degranulation of CD56^{bright} and CD56^{dim} NK cell populations. The expression of LAMP1 or CD107a is expressed was quantified on CD56^{bright} and CD56^{dim} NK cells in PBMCs from HESN and HIV-N before and after stimulation with LPS-TLR4, Imiquimod-TLR7, ssRNA40/LyoVec-TLR8 or PMA+ Ionomycin for between 20-24hrs. A) The gating of CD107a expression on CD56^{bright} and CD56^{dim} NK cells and changes in proportion of CD56^{bright} CD107a^{+} and CD56^{dim} CD107^{+} NK cells with TLR stimulation is shown. B-G) Bar graphs showing comparisons of CD107a expression on different NK cell subsets. Statistical comparison done using Mann-Whitney-test, all p<0.05 indicated with (*). HESN (n=15) and HIV-N (n=15).

However, TLR8 treatment resulted in higher increases in frequencies of $CD56^{dim}$ NK cells expressing CD107a in HESN but not in HIV-N (**47.04 ± 5.9%** vs. 31.00 ± 7.3%, p=0.044) (**Figures 34B**). A comparison of CD107a expression on $CD56^{dim}$ and $CD56^{bright}$ NK subsets expressing or lacking CD16, revealed that TLR4 stimulation resulted in higher frequencies of $CD56^{bright}$ $CD16^{+}$ $CD107a^{+}$ NK cells in HESN (**29.16 ± 6.5%** vs. 9.319 ± 5.5%, p=0.0088) following TLR4 stimulation. Similarly, TLR7 and TLR8 stimulations resulted in higher frequencies of CD107a expressing $CD56^{dim}$ $CD16^{+}$ (**37.4 ± 6.7**% vs. 11.5 ± 2.8% p=0.0090; **33.65 ± 5.3%** vs. 14.11 ± 4.5%, p=0.0087, respectively), and higher $CD56^{dim}$ $CD16^{-}$ NK cells in HESN compared to HIV-N (**51.3 ± 6.3%** vs. 32.6 ± 7.4%, p=0.0498) (**Figures 34D-G**).

Collectively, these results demonstrated that all TLR agonists tested enhanced the potential for NK cell degranulation based on CD107a expression. The enhancements of cellular degranulation potential were more pronounced on $CD56^{bright}$ and $CD56^{dim}$ NK cells of HESN especially with TLR7 or TLR8 stimulations. These results suggest that TLR7 and TLR8 stimulations enhanced the cellular degranulation capacity or NK cell activation based on CD107a expression in HESN when compared to susceptible controls. The degree of NK cell activation was best predicted by activation markers CD69, and degranulation based on CD107a expression.

7.5.5. Cytokine responses by NK subsets of HESN with TLR stimulation

NK cells play a crucial role in production of antiviral cytokines like IFN-γ, which are crucial for killing or destruction of infected, stressed or transformed cells. NK cell production of IFN-γ occurs prior to development of pathogen specific adaptive immune responses, informs the development of T_H1 responses needed for clearance of most viral infections[448]. Previous studies in our cohort described higher IFN-γ and IL-12 cytokine responses, and higher degreanulation based on CD107a in NK cells of HESN compared to HIV-N and HIV-

P[441]. We hypothesized that NK cells of HESN in PBMCs would produce higher levels of antiviral cytokines (IFN-γ, IL-2 and TNFα) in response to TLR8 stimulation compared to susceptible controls. To test this, we quantified and compared the changes in expression of cytokines -IFN-γ, IL-2 and TNF-α- typically produced by NK cells using multicolour flow cytometry from PBMCs of HESN and HIV-N CSWs stimulated TLR4, TLR7 or TLR8 ligands (**Figure 36**).

This analysis revealed that, the expression of IFN-γ was enhanced by TLR7 stimulation in both $CD56^{dim}$ NK cells (p=0.0192, Wilcoxon rank test) and $CD56^{bright}$ NK cells of HESN, and after TLR7 (p=0.0342) and TLR8 (p=0.0159) treatments of based on MFIs. Although, there were no differences in IFN-γ expression on either NK cell subset between the two study populations (**Figures 35A-D**).

Expression of IL-2 in HESN had great intra-group variability, where some individuals had higher IL-2 responses than others, though in all instances intergroup differences in its expression in $CD56^{bright/dim}$ NK cells populations were not significant (**Figure 36E-G**). *Ex vivo,* the expression of IL-2 on both $CD56^{bright/dim}$ NK cells of HESN, was higher than in matching cells of HIV-N (**Figures 35E and F**).

On the other hand, TNF-α responses were more robust and were detectable in both NK subsets after TLR4, TLR7 and TLR8 stimulations. HESN had higher frequencies of $CD56^{bright}$ TNF-α^{+} NK cells following TLR7 (**69.73 ± 5.4%** vs. 54.22 ± 6.1%, p=0.0139) and TLR8 stimulations (**70.98 ± 2.7%** vs. 57.39 ± 6.8%, p=0.039) compared to HIV-N (**Figure 36I-L**). Similarly, the surface expression of TNF-α was higher in $CD56^{bright}$ NK cells of HESN following TLR7 (MFIs: **667.4 ± 66.4** vs. 489.8 ± 53.0, p=0.0140) and TLR8 treatments (MFIs: **665.0 ± 34.6** vs. 500.4 ± 67.2, p= 0.0232).

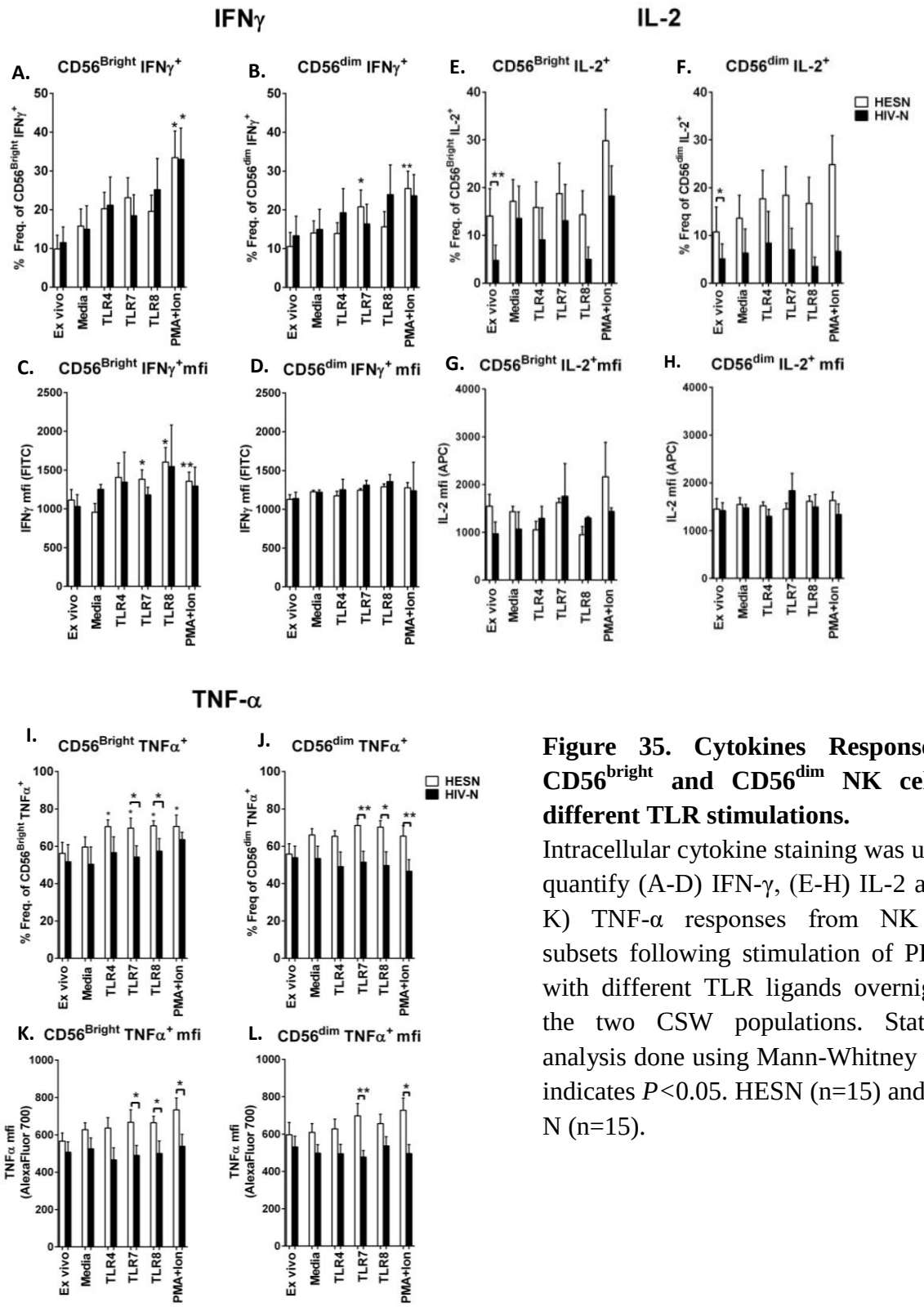


Figure 35. Cytokines Responses of CD56^{bright} and CD56^{dim} NK cells to different TLR stimulations.

Intracellular cytokine staining was used to quantify (A-D) IFN-γ, (E-H) IL-2 and (I-K) TNF-α responses from NK cells subsets following stimulation of PBMCs with different TLR ligands overnight in the two CSW populations. Statistical analysis done using Mann-Whitney test, * indicates $P<0.05$. HESN (n=15) and HIV-N (n=15).

HESN also had higher frequencies of $CD56^{dim}$ $TNF\alpha^{+}$ NK cells after TLR7 (**67.62 ± 4.8%** vs. 51.47 ± 5.9%, p=0.0069) and TLR8 stimulations (**66.73 ± 4.84%** vs. 49.70 ± 7.35%, p=0.045) compared to HIV-N. The surface of expression of TNFα on $CD56^{dim}$ NK cells of HESN was higher in following TLR7 stimulation (**697.5 ± 66.70** vs. 476.7 ± 35.07, p=0.0047).

In summary, *ex vivo* HESN had higher frequencies of $CD56^{bright}$ and $CD56^{dim}$ NK cells expressing IL-2. Similarly, both $CD56^{bright}$ and $CD56^{dim}$ NK cells of HESN had higher proinflammatory capacity based on higher expression of TNFα and CD107a following TLR4, TLR7 and TLR8 stimulations. This suggested that HESN NK cells in PBMCs have higher activity in response to TLR ligands compared to those of HIV-N.

7.6. Summary and Discussion

Natural killers cells recognize and kill virus infected, transformed or stressed cells based on MHC class I molecule expression ('missing' self hypothesis'). In this chapter we presented results from a number of experiments that quantified changes in proportions of NK subsets, expression of NKRs and NK effector functions, in response to different TLR stimulations in PBMCs of two groups of CSWs.

The findings of this study showed that TLR4, TLR7 or TLR8 stimulations neither increased the proportions of $CD56^{bright}$ nor $CD56^{dim}$ NK cells, however, in some instances TLR4 stimulation led to increased numbers of $CD56^{dim}$ NK cells. We also found that *ex vivo* HESN individuals had higher numbers of $CD56^{bright}$ NK cells in PBMCs a pattern maintained overtime. HESN equally had higher activation of the $CD56^{bright}$ NK cell population upon either TLR4 or TLR8, but not TLR7 stimulations based on CD69 and not HLA-DR expression. The three TLR stimulations neither altered the expression of NK cell receptors (NKRs)- NKG2D, NKG2 and CD16 nor resulted in differential expression of NKRs between

the two study groups with TLR stimulation. However, HESN had higher frequencies of $CD56^{dim}$ $CD107a^{+}$ NK cells with TLR8 stimulation, higher frequencies of $CD56^{dim}$ $CD16^{+}$ $CD107a^{+}$ and $CD56^{dim}$ $CD16^{-}$ $CD107a^{+}$ NK cells following TLR7, TLR8, or both stimulations. *Ex vivo*, HESN had NK cells expressing higher IL-2 levels in both $CD56^{bright/dim}$ NK cells populations, similarly both populations had higher expression of TNF-α, variously after TLR7 or 8 stimulations, with the higher NK cell activation being independent of NKR (CD16) expression (data not shown).

The experiments used to evaluate the different effects of TLR stimulations on NK cell responses, were performed using mixed PBMCs. Direct stimulation of NK cells without NKR-NKR ligand engagement and cytokine priming by APCs (like monocytes and DCs), limits NK cell activation[166]. Additionally, activation of NK cells based on CD69 expression and production of NK associated cytokines has also been shown to occur in response to HIV analogous GU rich ssRNA [259], thus providing some validation for observations made in this study. Higher activation of NK cells in HESN after different TLR stimulations, contrasted the lower immune activation state of NK cells *ex vivo* described by a separate study in the same cohort[440]. Although, to the best of our knowledge this is the first study comparing the NK cells responses to different TLR treatments among HESN female CSWs alongside monocytes and DCs responses. In addition, it is also the first study to compare the changes in NK subsets with different TLR treatments at multiple sampling points in a HESN population.

There are a numbers of previous studies quantifying and comparing NK cell functions in different HESN populations, including IDUs, children born to HIV infected mothers, discordant couples and commercial sex workers, but majority of these have been conducted on resting cells or using stimulants other than TLR ligands[415, 449]. A recurrent theme in

the findings of this and others studies is the heightened NK cell activity in HESN individuals compared to susceptible controls. Majority of studies- with the exception of one- have found that resting NK cells of HESN individuals had higher activation based on CD69, higher degranulation potential based on CD107a expression[440]. While the higher of expression of type I IFN by pDCs in response to TLR7 could have contributed to the higher activation of NK cells, consistent with what is know in the literature[415]. Higher antiviral responses such as IFN-γ have also been described in the genital tracts of individuals who have had sex with HIV positive partners[439]. Others have describe the overrepresentation of certain protective KIRs such as KIR3DL1 and KIR3DS1 in HESN Caucasian cohorts, that were absent in an African population[425, 436, 450].

Constantly we observed higher numbers of $CD56^{bright}$ NK cells expressing cytolytic NKR CD16 in HESN, where this subset has previously been shown to be responsible for production of IFN-γ, TNF-α and GM-CSF in response to monokines [451]. This study found that both $CD56^{bright}$ and $CD56^{dim}$ NK subsets were more activated in HESN based on CD69 expression in response to TLR4, 7 or 8 stimulations, but only based on HLA-DR expression after TLR4 and TLR8 stimulation. The $CD56^{dim}$ NK subset normally found in circulation and considered to play a greater role in NK killing of target cells, was overrepresented in HESN PBMCs following TLR4 stimulation. The $CD56^{dim}$ NK cell subset has previously been shown to be more potent in lysing NK sensitive targets (K562 cells) compared to $CD56^{bright}$ NK cells[452].

This study also evaluated the expression of three NKRs- CD16, NKG2 and NKG2D- in different NK subsets of HESN and HIV-N prior to and following TLR4, 7 and 8 stimulations. This considering the important role played by NKRs in identification of virus infected or transformed cells, leading activation of the natural killer functions once a threshold is

exceeded. Both NKG2 family of receptors -NKG2 and NKG2D-, were expressed more on $CD56^{bright}$ NK cells compared to $CD56^{dim}$ NK cells, consistent with what has been described in the literature[446]. However, all TLR stimulations tested produced little variation in the expression of these receptors on the two NK subsets. CD16 the only NKR capable of inducing killer NK cell activity without co-activation with other NKRs, showed little variation in expression on the different NK subsets over time, following different TLR stimulations and between the two study populations. Majority of the NK functions evaluated in this study were independent of CD16 expression (data not shown), an indication that direct or indirect activation of NK cells by APCs after TLRs stimulations, may have little effect on CD16 expression and function. We speculate that CD16 may be more important in NK mediated killing of antibody coated pathogens, but its expression may not be very relevant in responses where NK cells are primed by monocytes and DCs as when responding to intracellular pathogens or following TLR stimulation (**Figure 36**).

In the last set of experiments, we evaluated the expression cytokines in NK cells in response to the three TLR stimuli. The results showed similarities the pattern and degree of cytokine responses by $CD56^{bright}$ and $CD56^{dim,}$ NK cells. Where the expression of TNF-α was almost always higher in NK cells of HESN compared to those of HIV-N, yet there were no differences in IFN-γ responses between the two groups.

In conclusion, experiments in this section, clearly demonstrated functional differences in NK cell responses between HESN and susceptible controls, majority of which were in congruence with previously described heightened NK cell activity in HESN individuals by studies conducted in other cohorts. This study adds a new dimension to heightened NK cell activity in a HESN population due to stimulation with different microbial components particularly ssRNA analogous to HIV's genetic material.

CHAPTER 8: General Discussion, Conclusions, Limitations and Significance

8.1. General Discussion

Not all exposures to HIV result in infection[266]. The need to identify the correlates of protection against HIV is still a priority for vaccine development, and is the central goal for numerous studies conducted in HESN populations seeking to understand the genetic and immunological parameters of protection. The evaluation of HESN TLR expression, signaling and responsiveness in this study, was done on bulk PBMCs, while different functional aspects of immune cells present in PBMCs was assessed using multicolor flow cytometry. This study demonstrated differences in functional responses of PBMCs from HESN female CSWs compared to controls in samples collected at multiple times points, following TLR4, TLR7 and TLR8 stimulations *in vitro*. Previously, we conducted a similar study using cervical mononuclear cells from the genital tracts of female CSWs in the same cohort. In that study, we found a lower expression of a number of PRRs on cervical epithelial cells (CEC) and cervical mononuclear cells (CMC) from HESN, yet higher TNF-α and IL-10 responses in TLR8 stimulated CMCs of HESN[294]. This provided the rationale for the present study, which sought to expand the evaluation of TLR responses in HESN to PBMCs.

8.1.1. The Dichotomous effect of TLR7 and TLR8 stimulations on HIV's infectivity is related to quality and magnitude cytokine responses by HESN PBMC

The findings of this study showed that PBMCs of HESN had a higher TLR8 responsiveness specific for ssRNA analogous to HIV genetic material, and often lower responsiveness to TLR4 and TLR7 compared to controls in PBMC samples collected at multiple time points. The heightened TLR8 responsiveness corresponded to higher TLR8 mRNA expression post stimulation, while the often lower TLR4 and TLR7 responsiveness was associated with the lower expression of cognate receptors on T cells and PBMCs, respectively. TLR7 stimulation

of HESN PBMCs resulted in lower expression of TLR7 (relative to unstimulated PBMCs) that was accompanied by higher TLR8 expression. The opposite pattern was observed in TLR8 stimulated PBMCs of HESN that had higher TLR8 expression but lower TLR7 expression relative to controls. The opposing patterns of TLR7 and TLR8 expression may be explained by the previously described TLR8 specific inhibition of TLR7 expression[28]. The lower activation of TLR7, and higher activation TLR8 signalling pathways corresponded to receptor expression; where TLR7 ligation of HESN PBMCs led to lowered induction of the MyD88-TAB1-NFκB and MyD88-MAPK-MAPKK-JNK-AP-1 pathways, whereas TLR8 stimulation resulted in higher induction of MyD88- NFκB/IRF3 or MyD88-MAPK-AP-1 pathways. The net effect of the differentially activated TLR7 and TLR8 signalling pathways was the lower cytokine responses to TLR7 ligand Imiquimod but higher TLR8 responses to GU-rich HIV analogous ssRNA.

The cytokine milieu HESN of PBMCs appeared to be more coordinately or tightly regulated compared to those of HIV-N controls in the absence of TLR stimulation, a pattern that was maintained when responding to TLR4 and TLR8 but not TLR7 ligands. These tightly regulated cytokine micro-environments of HESN PBMCs contained $CD8^+$ T cells and NK cells that were highly activated by TLR7 or TLR8 ligands, where both immune subsets are capable of mounting potent antiviral responses. In addition, the co-ordinately or tightly regulated HESN PBMC cytokine microenvironment did not increase expression of HIV co-receptor CCR5 in the primary HIV targets- $CD4^+$ T cells of HESN when compared to HIV-N controls. The tighter or more co-ordinate regulation of the cytokine milieus in HESN PBMCs, was particularly evident with regard to the correspondence of IL-10 levels, a key regulatory cytokine, and the levels of other cytokine responses. This was potentially influenced the pattern of immune subset activation within PBMCs when responding to the TLR stimulations. Moreover, the cytokine milieu of HESN PBMCs after TLR8 treatment the

tended to make the HIV target cells less susceptible to a circulating Kenyan strain of HIV, whereas pre-treatment of PBMCs with TLR7 differentially enhanced the capacity of primary HIV isolates to infect PBMCs from either group depending on the concentration of virus used *in vitro*. This is the first time pre-treatment of PBMCs with a TLR8 ligand has been shown to reduce on susceptibility of HIV targets cells to primary HIV isolates, in a HESN population.

The differences in outcomes of TLR7 and TLR8 pretreatment of HESN PBMCs with regard to HIV's infectivity was consistent with cytokine responses observed after TLR7 and TLR8 treatments of PBMCs in the same group. In the first instance, TLR7 stimulation induced type I IFN (IFNα2) production accompanied by lower or undetectable IFN-γ, IL-12 and TNF-α responses, whereas TLR8 stimulations resulted in a wider breadth of cytokine responses including type II IFN (IFN-γ), IL-12p40, IL-12p70, and TNF-α in both HESN and HIV-N PBMCs. The production of IFNα2 was lower in PBMCs of HESN following Imiquimod or TLR7 ligand stimulation, while type II IFN or IFN-γ responses were more robust after ssRNA or TLR8 ligand stimulations of PBMCs from the same group compared to controls. These responses represented a 'dichotomy' of type I (IFNα2) and type II IFN (IFN-γ) responses by HESN PBMCs to TLR7 and TLR8 stimulations, respectively, with this pattern being maintained over multiple sampling points, strongly suggesting that this may be representative of the HESN phenotype. The production of both IFN-γ and IL-12p40 in response to TLR8 was higher in HESN PBMCs with positively correlating levels of both cytokines. Additionally, TLR8 stimulations also resulted to higher proinflammatory cytokine responses such as IL-1β, IL-6, IL-12p40 and TNF-α by HESN PBMCs compared to controls.

The opposing patterns of TLR4, TLR7 and TLR8 cytokine responses in HESN PBMCs was associated with differential activation of immune subsets in PBMCs by the different TLR ligands. The primary responders in LPS-TLR4 stimulated PBMCs are known to be monocytes and mDCs (cDCs) capable of producing majority of the cytokines quantified in

this study. On the other hand, type I IFN production in response to TLR7 occurs primarily through pDCs, whereas mDC(cDCs) and monocytes are the main responders in TLR8 stimulated PBMCs[359]. Both IL-12 subunits -p40 and 70- are usually produced by mDCs but not pDCs in response to TLR8 and not TLR7, consistent with our observations[199, 365]. The mechanism of induction IFN-γ in T and NK cells has been shown to be dependent on IL-12 production by APCs (supporting a two tiered immune response hypothesis)[197]. Where IFN-γ production by T cells and NK cells is initiated when IL-12 binds to its receptor IL-12β1/2 triggering Jak2/Tyk2-STAT4 and MAPK-NFκB/AP-1 signalling[453, 454]. The observations made in this study, strongly suggest that the quality of cytokine responses generated by TLR7 or TLR8 stimulation of PBMCs may have a bearing on the 'dichotomous' outcomes of HIV infection assays, following pretreatment of PBMCs. Therefore, it is reasonable to presume that the reduced susceptibility of HIV target cells in HESN and HIV-N PBMCs following TLR8 ligand pre-treatment and not so much for TLR7 ligand, may be due to the quality of cytokine responses (indirect effects TLR8 signalling- described above) or possiby due to other intracellular processes (direct-effects of TLR signalling- described below).

Alternatively, TLR8 mediated reduction of PBMCs susceptibility to HIV in HESN, may be a consequence of what we called the 'over-engaging hypothesis'. In this hypothesis, higher activation of TLR signalling pathways- as was seen in HESN PBMCs following TLR8 stimulations- could have resulted in increased engagement of cell signalling machinery and metabolic processes, to point that those needed by HIV for virus entry and replication(such as NF-κB), become unavailable for virus infection or production[455]. The idea of 'over-engagement' of cellular machinery by TLR signalling pathways or innate signalling pathways, has gained much traction recently[456]. On the contrary, induction of alternate cellular processes by TLR7 stimulation could enhance HIV replication, for instance the

induction of anergic signaling profiles due excessive Ca^{2+} influx triggered by Imiquimod stimulation, was recently shown to increase susceptibility of $CD4^+$ T cells to infection with HIV *in vitro* [248]. On the other hand, induction of other metabolic cellular processes by TLR8 signalling, for example conversion of an inactive form of vitamin D into active intermediates, resulted in increased resistance of macrophages to HIV infection *in vitro* [319]. When the effects of the 'over-engagement hypothesis' are combined with classical antiviral responses such as $CD8^+$ T and NK cell mediated cytotoxicity, these could contribute to inhibition of HIV replication *in vitro* [263, 264]. Any or all of these mechanisms, could potentially contribute to the low-grade inhibition of HIV infectivity in TLR8 stimulated PBMCs particularly in HESN or increased susceptibility of PBMCs as induced by TLR7. Further investigations into the effect of cellular signalling and metabolic processes induced by TLR7 or 8 signalling in relation to expression of HIV entry proteins or proviral factors, may be useful for the elucidation of the dichotomous effects of TLR7 ligand -Imiquimod and TLR8 ligand ssRNA, on HIV's infectivity.

8.1.2. Higher activation of CD40-CD40L Pathway and a higher frequency of virus sensing monocytes in HESN PBMCs

An enhanced capacity to sense and respond to HIV derived genetic components could indicate a greater capacity to generate anti-HIV mechanisms or immune responses in the setting of an HIV exposure. Viral infections are initially sensed through innate recognition of viral PAMPs by PRRs, resulting in activation and enhanced development of innate and adaptive virus specific responses capable of blocking infection or destroying invading viruses. In the case of HIV, protection against infection should arise before the virus can establish itself, triggering the detrimental and irreversible destruction of the immune system. The identification of a patrolling and virus sensing $CD14^{dim}$ $CD16^+$ monocyte subset in mice[378], led us to hypothesize that this subset in HESN individuals may possess a better

capacity to recognize and respond to HIV derived components through either TLR7 or TLR8 compared to controls. To test this hypothesis, we compared the changes in expression of co-stimulatory factors, markers of adhesion, chemokine receptors and cytokine production in this and other monocyte subsets in PBMCs of HESN treated with TLR4, TLR7 or TLR8.

This primary virus sensing monocyte subset or non-classical $CD14^{dim}$ $CD16^{+}$ monocytes was over-represented in HESN PBMCs before and after TLR8 stimulations. In addition, this virus sensing monocyte subset of HESN had higher CD40 but not CD80 or CD86 expression, but lower expression of adhesion marker ICAM1 or CD54 in response to TLR8 stimulation. Suggesting that following recognition of virus derived components, these cells become more activated but less adhesive, potentially allowing them to migrate more freely in HESN compared to HIV-N. Studies conducted in mice show that the non-classical monocyte subset patrols the luminal surface of blood vessels while loosely adhering endothelial cells, whereas, classical monocytes $CD14^{+}$ $CD16^{-}$ are readily found in circulation, and mostly extravasate blood vessels to differentiate into tissue macrophages[378, 384]. The lowered expression of adhesion marker ICAM1 (CD54) on non-classical monocytes in response to TLR8 stimulation, suggests that this subset in HESN may have a reduced capacity to adhere to endothelial surfaces upon exposure to HIV derived genetic material in the setting of an infection *in vivo*. The mechanism of how this might occur would be difficult study in humans, but the use of animal models like humanized mice or non-human primate studies could be very informative.

The other monocyte subsets -classical $CD14^{+}$ $CD16^{-}$ and intermediate $CD14^{+}$ $CD16^{+}$- were also more activated by TLR8 stimulation in HESN compared to in controls, based co-expression or singular expression of CD40, CD80 and CD86, however, it is only CD40 expression that was persistently higher with TLR8 stimulation. This motivated a further investigation into the usage of CD40-CD40L pathway by HESN monocytes and T cells.

Indeed, the higher expression of CD40 on monocytes corresponded to the higher expression CD40L on T cells, strongly suggesting that this pathway may be more activated in HESN monocytes and T cells. An expanded evaluation of other co-stimulatory pathways would be instrumental for understanding the mechanisms of T and B cells activation and functions following TLR stimulations *in vitro*.

Monocytes are the principle mediators of inflammatory responses through production of cytokines and chemokines capable of recruiting effector cells to sites of infection. This study quantified the proinflammatory responses by different monocytes subsets. Majority of responses to TLR8 stimulation were similar between HESN PBMCs and controls, but TLR4 and TLR7 cytokine responses were generally lower in monocytes of HESN, for instance HESN had lower production IL-10, IL-12p40/70 and TNF-α in response to TLR4 and TLR7 stimulation as quantified from both by monocytes by ICS and in culture supernatants of bulk PBMCs. These results demonstrated that $CD14^-$ $CD16^+$ monocytes may be better at sensing invading virions and in activating T cells through CD40-CD40L in PBMCs HESN, but less potent in driving overall inflammatory processes in response to non-HIV associated components like LPS and Imiquimod.

Overall, HESN PBMCs had lower responses to non-HIV specific microbial components, suggesting a lowered capacity to become activated non-HIV components. The lowered capacity to respond to non-HIV related components (LPS and Imiquimod) and the hypersponsiveness to ssRNA by HESN PBMCs, however this did not encompass all monocyte functions. This was demonstrated by the similar expression of certain chemokine receptors like CXCR3, but not others like CCR2 on classical monocytes of HESN when responding to TLR8. Much as the CXCR3 receptor was equally expressed on the different monocyte subsets, opposing production patterns of its ligand CXCL10 or IP-10 by HESN PBMCs was observed in response to TLR7 and TLR8, respectively. Chemokines and

chemokine receptors are crucial in the trafficking and homing or monocytes during inflammation[403, 405, 413, 457]. CCR2, one such receptor has been shown to play an important role in the egress and homing of classical monocytes ($CD14^+$ $CD16^-$) from the bone marrow to inflammatory tissues [457]. TLR8 stimulation resulted in a higher expression of CCR2 on classical monocytes suggesting that upon exposure to HIV derived components, this subset in HESN individuals may be better placed to migrate into tissues in the setting of an exposure to HIV compared to controls. These findings provide a strong rationale for an expanded and in-depth evaluation of chemokine receptor expression and chemokine responses to different TLR ligands particularly HIV derived ssRNA in HESN in the future.

8.1.3. Lower activation of HESN blood DC subsets in response to TLR7 and not TLR8

Blood DCs primarily perform a immunosurveillance function due to a higher capacity of these cells to recognize, engulf, process, shuttle and present antigens to T cells through blood or the lymphatics system [458]. The physiological presence of these cells in both the blood and lymphatics separates them from monocytes which differentiate into macrophages upon exiting blood vessels and entering tissues or lymphatic system. As such DCs have been shown to be very potent at inducing pathogen specific T cell responses[459]. On the flip-side, the enhanced capacity of DCs to shuttle and present antigens to T cells in lymphoid tissues, aids dissemination HIV virions leading to establishment of new infections[27]. Initially we hypothesized that DC responses would correspond to the heightened TLR8 responsiveness congruent with the overall hypothesis tested in this study. To address this hypothesis we compared the activation and maturation of different DC subsets present in PBMCs in response to different TLR agonists. In line with our expectations, a higher TLR8 responsiveness was observed in pDCs from HESN with regard to IFNα2 production, but much more pronounced was the lower TLR7 responsiveness observed in HESN pDCs and mDCs based on CD80, CD83 and IFNα2b expression. The lower production of IFNα2b by

pDCs in response to TLR7 was potentially responsible for the equally lower IFNα2 responses quantified from culture supernatants of TLR7 stimulated PBMCs of HESN.

The findings from the monocyte and DC studies demonstrated that TLR7 responses in both cells types was lower in HESN compared to controls while majority of the heightened TLR8 responses were on monocytes and not DCs. These findings suggest that the capacity of the TLR7 ligand Imiquimod to enhance infection in PBMCs as described by others *in vitro* and *in vivo*[28, 248], may be altered in HESN and related to a lowered capacity of HESN immune cells to be activated by TLR7 stimulation. On the contrary, TLR8 stimulation was able to enhance APC activity that is related to virus sensing and development of T cell responses rather than driving massive inflammation, perhaps resulting in enhanced HIV inhibition *in vitro*. All this occurred in a background of tighter and more balanced regulation of cytokine responses in HESN PBMCs when compared to controls.

8.1.4. Higher activation of antiviral responses in $CD8^+$ T and NK cells of HESN with TLR7 and TLR8 stimulation

Next, we sought to understand the impact of the differences in APCs responses by HESN and HIV-N to different TLR stimulations, on the activation and function of different lymphocyte (T and NK cells) populations in PBMCs. A number of previous studies have shown that lymphocytes (T and NK cells) express TLR1-10, with TLR expression being generally lower on lymphocytes compared to APCs[246]. Naturally, one would expect that the cells with the highest levels of TLR expression (APCs), would be evolutionarily and functionally the most relevant for recognizing pathogen derived components. The role of the TLRs expressed on lymphocytes particularly T cells and NK cells, has not been widely investigated.

In this study, $CD8^+$ T cells of HESN *ex vivo* expressed lower levels of activation marker CD69, while both T cell subsets expressed lower levels of proinflammatory cytokine TNF-α

and marker for degranulation CD107a. These results supported previous findings within the same cohort showing HESN individuals have a 'quiescent' T cell phenotype in resting T cells based on lower expression of activation marker CD69, lower production of proinflammatory cytokines, lower activation of cellular signalling pathways, and more importantly possessed higher frequencies of regulatory T cells potentially responsible for this 'quiescent phenotype[309, 333-335]. The 'quiescent' phenotype of resting T cells of HESN individuals did not inhibit the capacity of these cells in PBMCs to respond robustly to different TLR treatments. For instance $CD8^+$ T cells of HESN CSWs were more activated based on expression of CD69 and HLA-DR following TLR7 and TLR8 stimulations, while $CD4^+$ T cells of HESN were more activated by TLR4 stimulation compared to controls. The higher activation both T cell subsets was extended to production of IFN-γ and expression of proliferation marker-Ki67 following TLR4, TLR7 or TLR8 stimulations. Additionally, the higher expression of activation marker CD69 but not MHC class II, correlated with production of IFN-γ and TNF-α, suggesting a greater potential of T cells of HESN to mount T_H1 responses to microbial components.

We also investigated the role of regulatory T cells ($CD4^+$ $C25^+$ $Foxp3^+$) in modulating TLR responses in the two groups. The results of this evaluation showed TLR8 stimulation resulted in lower expression of production of IL-10, possibly allowing the greater activation of $CD8^+$ T cells in HESN as compared to controls who had higher T_{regs} frequencies, especially considering that regulatory T cells are known to limit or control activation of T cells *in vitro* and *in vivo*[375]. IL-10 production in response to all TLR stimulations corresponded to cellular degranulation based on CD107a expression in $CD4^+$ T cells suggesting a tight coupling of inflammatory and anti-inflammatory processes in HESN possibly to limit excessive inflammation. On the contrary, HIV-N controls appeared to have an imbalance between proinflammatory and anti-inflammatory responses based on the fewer correlational

relationships between cytokine quantities following the different TLR stimulations, as discussed earlier. HESN T cells had lower expression of TLR4 before and after stimulation with specific ligand LPS, but not with non-specific TLR7 or TLR8 stimulation. Interestingly, this correlated with T cell functional responses but not expression of activation markers. This suggests that, T cell activation and functional responses are influenced differentially, where the function of lowly expressed TLR4 was probably influenced cytokines and chemokines produced by other responding cells possibly APCs, consistent with what is known in the literature[197].

Lastly, this study evaluated the NK cell activation and functional responses in mixed PBMCs. This was motivated by a desire to understand the quality of NK cells in HESN upon exposure to microbial components. In this study, the assessment of NK functions was done using PBMCs and not purified cells; this is considering purified NK cells tend to be unresponsive to different TLR stimulations in spite of NK cells expressing various TLRs[259]. A recent study in the Majengo cohort demonstrated a lower expression of markers of cellular activation -CD69, and exhaustion markers-LAG3, PD-1 and TIM3 on NK cells of HESN compared to controls[440]. In this study we found that *ex vivo* HESN had higher numbers of $CD56^{bright}$ and IL-2 expressing NK cells, with higher CD69 expression in the bright NK subset in response to TLR7 or TLR8 stimulations. On the other hand the $CD56^{dim}$ NK subset of HESN produced higher amounts proinflammatory cytokines and had higher cellular degranulation following TLR7 or TLR8 stimulations compared to controls.

On the contrary, the expression of CD16 an activatory NKR and other NKRs- NKG2D (CD94) and NKG2, remained unaffected by all TLR treatments, in either NK subset of HESN or HIV-N. Majority of the NK cell functions evaluated were equally similar in magnitude between $CD56^{bright}$ and $CD56^{dim}$ NK cells especially following the different TLR stimulations. The overlap was contrary to previous studies that have shown $CD56^{bright}$ NK

cell subsets usually localize to peripheral lymphoid tissues and produce higher cytokine responses when compared to the $CD56^{dim}$ NKs that are usually found in circulation and are associated with bulk of NK killer activity.

NK cells need direct contact with monocytes and DCs, in addition to soluble mediators produced by APCs such as IL-12, IL-15, IL-18 or type I IFNs to in order to be activated *in vitro* (**Figure 36**). In turn NK cells need to express the prerequisite cytokine receptors in order detect these soluble factors produced by APCs to be completely activated. The dependency of NK activation on APC responses, has been show to be independent of TLR expression on NK cells[259].

In vivo, the mechanism of 'arming' or 'disarming' NK cells during recognition of transformed, infected or stressed cells, requires two signals; the first being binding of activatory or inhibitory NKRs to their specific ligands, and the second being cytokine production by activated monocytes and DCs (**Figure 36**). NK cell activation pattern recognition of PAMPs, has previously been shown to be mediated by APC-derived cytokines and contact to APCs [42, 259, 460]. In this study, the differences in cytokine responses and millieu between HESN and HIV-N depended on TLR4, TLR7 or TLR8 stimulations. The mechanism of *in vitro* activation of NK cells through TLR stimulations has previously been shown to include upregulation of NKR ligands such as Rae1 by TLR3[445]. Much as this study did not investigate the expression of all known NKR ligands, the similarity in NKG2D and CD94 expression between HESN and HIV-N, provides a strong indication that the differences in NK activity may arise from the secondary signals needed in NK activation, rather than NKR-ligand interactions.

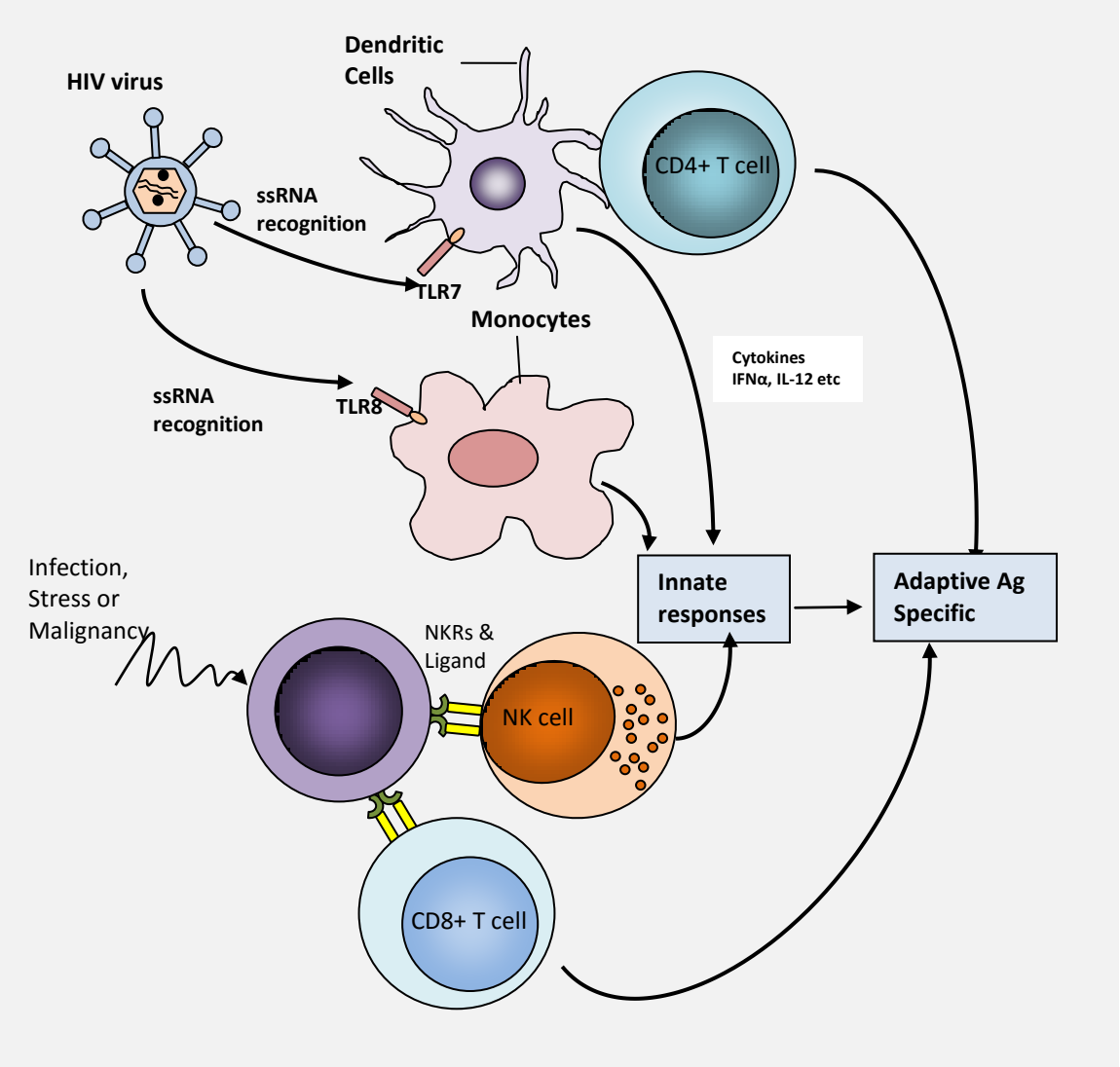


Figure 36. Role of Toll-like receptors and NK cell Receptors in immune recognition. Both the TLRs and NKRs are innate receptors capable of enhancing adaptive immune responses. TLR recognize PAMPs derived from microbial products while NKRs recognize ligands expressed on infected cells, stressed or transformed cells (malignant cells). TLR can enhance the expression of ligands of NKRs but not expression of NKRs themselves synergistically enhancing the adaptive immune response[446].

NK cell activation is highly related to monocyte and DC functions like IL-12 and IFNα cytokine production, which are important in the activation of NK cells (**Figure 36**). IFNα2 responses were robustly detected in TLR7 treated PBMCs with HESN responses being lower compared to those of HIV-N. The two subunits of IL-12 were lowly expressed by PBMCs of HESN following stimulation with TLR4 but more highly expressed in TLR8 stimulated PBMCs from the same group. The higher NK cell activation in TLR8 stimulated PBMCs of HESN, corresponded to the higher IL-12p40, IL-12p70, TNF-α and IFNγ responses in PBMCs following the same stimulation. Whereas the higher NK activity in PBMCs of the same group following TLR7 stimulation was in dissonance with lower IFNγ, IL-12p40/70 and TNF-α cytokine responses quantified in the HESN group. The mismatch in TLR7 responses in bulk PBMCs with NK activity can possibly be explained by the multifactorial regulation of NK activation when responding to microbial components. In this study, we demonstrated differences in NK cell functions between HESN and controls, majority of which were in congruence with previously described heightened NK cell activity in HESN individuals. However, this study for the first time demonstrated heightened NK cell activity in a HESN population based on responses to TLR7 and TLR8 ligands.

8.1.5. The proposed relationship between immune 'quiescence' and TLR8 hyperresponsivenss in HESN Female CSWs

HIV infection favours activated rather than resting $CD4^+$ T cells, the latter being infected to a lower extent[20, 336]. The immune quiescence hypothesis postulates that a lowered immune activation state lowers the risk of HIV acquisition in HESN individuals, by lowering the capacity of HIV to infect target cells in the event of an exposure and allowing for anti-HIV adaptive responses to clear or limit virus establishment[334]. The mechanism of development of HIV specific responses, that have been demonstrated in a number of studies, is unknown (**ref. Sections 1.3.2.2 and 1.3.2.3**). The higher TLR8 responsivenesss in the peripheral blood

and gential tracts of HESN female CSWs in our cohort suggests that more robust innate responses may be critical for development adaptive immunity in HESN. Based on the findings of this study, we propose that the mechanism of development of HIV specific adaptive immunity may involve; exposure to HIV or HIV PAMPs such as ssRNA resulting in robust but tightly regulated inflammation capable of exceeding an immune threshold necessary for development of HIV specific adaptive immune responses. The development of productive immunity against any pathogen requires an initial inflammatory response that exceeds the underlying homeostatic anti-inflammatory processes that can be thought of as an immune threshold[225, 461]. The contrast between the 'quiescent' resting state and the higher activation of different immune cells following TLR8 agonist ssRNA stimulation of peripheral blood and genital immune cells in HESN CSW; points to the existence of an immune threshold for development of protective immunity against HIV.

Ideally, the innate immune system of both HESN and susceptible individuals should able to recognize invading HIV virions resulting in an inflammatory response. Hypothetically, the capacity of the innate immune system to prime the adaptive response may be dependent on a number of factors, which may include but are not limited to; the dose of infecting virus, level of PRR expression and among others, which would dictate the magnitude of inflammation generated in response HIV or HIV derived PAMPs.

In such a situation a tightly regulated innate response may wane with decline or removal of the stimulus or with reduced exposure to HIV, accompanied by declines in HIV specific adaptive immunity. A number of studies support this premise, where reductions in HIV specific adaptive immunity has been observed in HESN populations with interruptions of activities associated with high risk of HIV exposure, such as sex work intermissions[283], recovery from needle-prick exposures in health workers and waning of CTLs in uninfected infants born to HIV positive mothers[313]. It is not yet clear if components of the innate

system acting through the inflammatory response or the virus specific adaptive immunity (which would develops much later), are principally responsible for limiting or complete clearance of HIV in the event of an exposure. Presumably, both arms of the immune system work in concert, or together with other unknown factors to limit or clear the viral infection.

8.1.6. Model of reduced Susceptibility to HIV of HESN based on TLR Responsiveness

Previously we demonstrated lower immune activation in the genital tract of female HESN CSWs based on expression of different PRRs[294]. In spite of this lowered immune activation state in the genital tract, CMCs of the HESN women were able to mount a potent response to TLR8 but not TLR4 or TLR7 agonists based on production of TNF-α and IL-10 production. Using the findings from that previous study, we developed a conceptual model for reduced susceptibility to HIV in the FGT of HESN female CSWs.

In that model, we proposed that the female genital tract of HESN woman is bathed with cervico-vaginal (CVL) fluid containing lower levels of proinflammatory cytokines IL-1β, IL-8 and RANTES, but higher levels of antiviral proteins Trappin2 and Elafin (Tr/E)[287]. The HESN FGT also contained CECs (epithelial cells) expressing lower levels of PRRs-TLR2, TLR4, RIG-1, Mda-5 but with higher expression of TLR3, TLR7 and TLR8. The female genital tract of HESN equally contained CMCs (T, B, macrophages and DCs), that expressed lower levels of PRRs- TLR2, TLR4, TLR6, TLR8 RIG-1, Mda-5 and PRR response regulators IDO and UNC93B1, and lower levels of proinflammatory and anti-inflammatory cytokines TNF-α and IL-10, and antiviral IFN-γ. However, upon exposure to TLR8 ligand ssRNA and not TLR4 or TLR7 ligands, CMCs of HESN produced higher IL-10 and TNF-α responses compared to HIV-N[294]. Based on these observations, we concluded that the genital tract of HESN has a 'quiescent' phenotype based on lower expression of PRRs and proinflammatory cytokines by stromal cells and CMCs. However, the HESN female CSWs

could mount a potent antiviral response upon activation of TLR8 pathway by HIV specific PAMPs.

The work presented in this thesis, continued the evaluation of the innate responses in HESN CSWs, but this time focussing on peripheral blood mononuclear cells. Based on the findings presented in this thesis, we developed a conceptual model for 'Reduced susceptibility in HESN based on TLR responsiveness in the peripheral blood' (**Figure 37)**.

In this expanded model, lymphocytes of HESN were shown to have lower or 'quiescent' immune activation state compared to susceptible controls[309, 333]. This was based on a lower immune activation of T cells in the absence of TLR stimulations, lower cytokine responses by HESN PBMCs supernatants, lower expression of different activation and co-stimulatory markers on immune cells. The 'quiescent' or lower immune activation state, presumably lowers the risk of HIV infectioning target cells in the event of a hypothetical exposure. However, during such a hypothetical HIV exposure, the immune system of HESN more efficiently recognizes the incoming viral ssRNA through TLR8, leading to significantly higher production of proinflammatory cytokines (IL-1β, IL-6, IL-12p70 and TNF-α) and type II IFN (IFNγ), but correspondingly higher production of anti-inflammatory IL-10 in a tightly coupled immune response. The higher TLR8 responses to HIV's genetic material in HESN would arise from a higher TLR8 receptor expression and signaling, with the opposite being observed after TLR7 stimulation. Likewise, TLR8 recognition of HIV's genetic material, analogous to the ssRNA used stimulations experiments in this study, would probably result in higher activation of monocytes especially the virus sensing $CD14^{dim}$ $CD16^{+}$ monocyte subset. The higher activation of peripheral immune cells was unique to ssRNA analogous to HIV genetic material through TLR8 in HESN, but not to non-HIV associated PAMPs. The higher TLR8 responses were reflected in different functional aspects of immune cells contained in peripheral blood, with APCs presumably playing a crucial role in PAMP recognition,

activation and conditioning of the different lymphocyte populations (T and NK cells). More importantly, the higher TLR8 responsiveness by HESN PBMCs would induces an anti-HIV environment capable of limiting HIV infection of target cells, consistent with what is known in the literature **Figure 37)**[263, 264].

On the other hand, TLR7 stimulation with Imiquimod could enhance infection and replication of HIV in HESN, due to creation of an unfavourable cytokine environment lacking potentially key cytokines- IL-12p40/70, IFN-γ and TNFα, which are critical for conditioning other antiviral functions in $CD8^+$ T cells and NK cells. Similarly, exposure of HESN to the non-HIV related TLR ligands such as bacterial LPS and synthetic antiviral compound Imiquimod would lead to lower production of IL-1β, IL-6, IL-10 and type I IFN (IFNα2) by HESN PBMCs when compared to susceptible controls. The lower cytokine responses were associated with lowered activation of various DCs and monocyte functions. The lower cytokine responses to TLR7 ligand, matched the lower TLR7 receptor expression and signal transduction. The lower TLR7 signaling in HESN could be due to higher concurrent TLR8 receptor expression following TLR7 stimulations, where the higher TLR8 expression potentially inhibits TLR7 expression and downstream signalling in HESN. More importantly TLR7 ligand Imiquimod appeared to differentially enhance susceptibility of PBMCs to infection with HIV *in vitro.* This is consistent with previous studies showing the Imiquimod enhancing effects on HIV's susceptibility *in vitro* and *in vivo*ts[28]. We prospose that TLR7 enhanced susceptibility could be associated with failure of Imiquimod to generate IL-12, IFN-γ and TNF-α responses that are important for development of antiviral responses (**Figure 37**).

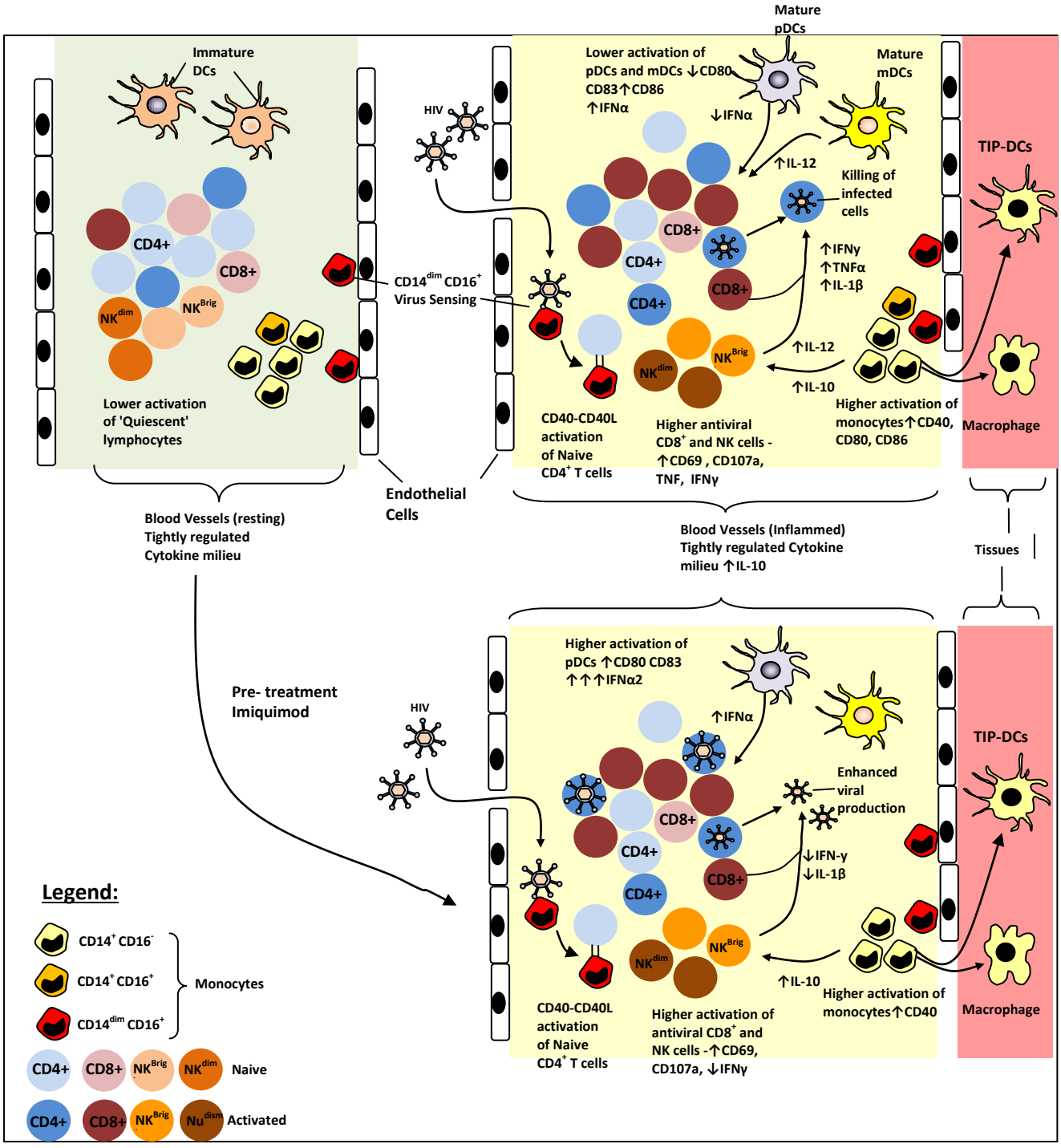


Figure 37. A conceptual model for reduced susceptibility to HIV in peripheral blood of HESN based on TLR Responsiveness. Resting PBMCs of HESN contain few activated T cells and monocytes modulated by lower presence of proinflammatory cytokines and higher IL-10 production by T_{regs} and monocytes. The resting state also contained higher proportions of virus sensing monocytes and $CD56^{bright}$ NK cells, bathed in a tightly regulated cytokine environment. Upon exposure to HIV, the monocytes (including viruses sensing) with the highest expression of TLR7 and TLR8, become highly activated to produce proinflammatory cytokines (IL-1β, IL-6, IL-12 and TNF-α) capable of activating antiviral responses in lymphocytes ($CD8^+$ and NK cells) which then produce IFN-γ and undergo degranulation

(CD107a) capable of limiting spread of virus infected cells. The higher TLR8 responses were due to higher expression of TLR8 receptor and signalling particularly in monocytes and not in DCs. On the contrary, TLR7 stimulation HESN PBMCs resulted in lower cytokine responses (IL-1β, IL-6, IL-10 and IFNα2) and failed to induce certain cytokine responses (IFN-γ, IL-12p40/70 and TNF-α) crucial for development of antiviral T and NK responses (T_H1). The TLR7 generated cytokine environment was primarily influenced by mDCs and pDCs and not monocytes, limiting the induction antiviral T and NK cell responses. The production of IFNα2 was robust but largely insufficient for limiting infection of target cells due to the absence of key components needed in development of beneficial antiviral responses, hence enhanced susceptibility to HIV *in vitro*.

The contrast between lower or 'quiescent' resting state and the higher activation of different immune cells in the peripheral blood and genital tracts of HESN CSW; possibly indicates an immune threshold of protection against HIV (discussed in **section 8.1.6**). An immune threshold is needed for development of productive immunity against most pathogens, and this usually requires a tightly regulated inflammatory response.

8.2. Conclusion

The studies conducted in this thesis evaluated the TLR responsiveness in peripheral blood mononuclear cells of HESN and HIV-N (susceptible) female CSWs in a well established cohort. The results showed that persistently HESN PBMCs had a higher responsiveness to ssRNA via TLR8 but often lower responses to TLR4 and TLR7 ligands. The higher TLR8 responsiveness in PBMCs was asssociated with higher receptor expression and TLR8 signal transduction with the opposite being observed with TLR7 stimulation. The higher TLR8 responses were also present in the different functional aspects of immune cells contained PBMCs, with activation of the different immune subsets appearing to be more dependent on the pervading cytokine responses generated by the TLR stimulations. More importantly, pre-treatment of HESN PBMCs with TLR8 ligand made them more refractory to infection with primary HIV isolates, whereas TLR7 stimulation enhanced infection of PBMCs *in vitro.*

8.3 Limitations

The experiments conducted in this study were all done *in vitro*. Considering the complex nature of events surrounding HIV transmission and disease progression, the findings of this study can only serve as postulations or as a rationale for trial of TLR8 agonists in therapeutics or for use as adjuvants to boost HIV vaccines. As such the following areas were considered to be limitations of this study.

- This study did not qualify the expression of TLR7 and TLR8 in the different immune subsets of PBMCs. This expression data would have been extremely useful in understanding the differences in responses between the two study populations at the subset level. This was due to lack of flow cytometry antibodies capable of labeling either TLR7 or TLR8 on the different immune subsets.

- Due to time constrains we were unable to conduct trans-well experiments to ascertain the mechanism of activation of lymphocyte populations in mixed PBMCs from the two study groups, such studies would have made it possible to understand the meaning of the tightly regulated cytokine environment in relation to HIV infectivity *in vitro*.
- The expansion of ART coverage in Kenya and its usage as a means for preventing HIV transmission is likely to reduce the overall risk of exposure to HIV in the general population and among most at risk populations such as HESN CSWs. This has consequences on similar studies within HESN populations in the future, especially with regard to separating new HESN individuals from susceptible controls. Fortunately the present study, utilized a well established cohort with members dating back to the early days of HIV's discovery when the risk of exposure to HIV was at its peak in Kenya.

8.4. Significance

Selectively activating TLR signalling pathways can modulate immunological functions, and has great potential for development of TLR based therapeutics for treating allergies, malignancies, infectious and autoimmune diseases. This study demonstrated that simulating the recognition of HIV through TLR8 agonists such as ssRNA and not TLR7, has the potential of priming the innate and adaptive immune system to prevent infection with HIV. In order to realize this goal, there is a need for greater understanding of the nature of protective TLR7 or TLR8 responses, as a means for guiding the selection of suitable TLR7 or 8 agonists/ antagonist for microbicide development. A number of studies have demonstrated the HIV inhibitory effect of TLR8 agonist ssRNA, and the HIV infection enhancing effects of TLR7-Imiquimod treatments *in vitro* and *in vivo*. This study is the first to compare the effects of selectively activating TLR7 or TLR8 signalling pathways on HIV's infectivity in a HESN population. The TLR7 enhancing effects on HIV infection *in vitro* described by others, was also present in the HESN individuals, while triggering TLR8 reduced susceptibility to HIV in

HESN. This is also the first study to demonstrate differences in cytokine production profiles following TLR7 or TLR8 stimulation and their potential importance these on HIV's infectivity *in vitro.* Especially with regard to TLR8 reducing susceptibility of HESN PBMCs HIV, albeit modestly.

This study is also the first to characterize and compare the immune responses in different immune subsets of a HESN population to different TLR treatments. The major finding from this was the higher activation of antiviral functions in $CD8^+$ T cells and NK cells of HESN PBMCs when compared to controls, with potential consequences on limiting of HIV infectivity *in vitro*. Finally, it is the first time differences in profiles of activation of APCs-monocytes and DCs has been linked to susceptibility to HIV *in vitro*. Where the persistently higher activation of monocytes in response to TLR8 in HESN was evident compared to in controls who had higher activation of blood DCs. Given the recent discovery of the viral sensing role by monocytes, the higher activation of monocytes by HIV protective TLR8 ligand-GU-rich ssRNA but not HIV enhancing TLR7- ligand Imiquimod, clearly demonstrates that the choice of ligand and immune subset it activates has consequences on HIV susceptibility.

8.5. Future Directions

The main finding of this study was the discovery of the dichotomous effects of TLR7 and TLR8 stimulation with respect to HIV's infectivity *in vitro* in PBMCs of HESN female CSWs. There are many questions arising from this, many which could not have been exhaustively addressed by this study alone. Therefore, we would like to propose some future directions which may be benefical in advancing the findings of this study. This could include, a complete proteomic assessment of activation of TLR signalling pathway components especially following TLR7 and TLR8 stimulations in the PBMCs, and CECs or CMCs from the genital tract. These assessments need not be limited to a comparison between HESN and HIV-N populations, but it can be conducted in other populations.

Secondly, thorough mechanistic studies there is a need to understand the interaction between TLR7 and TLR8, which potentially has a critical influence on the dichotomy of the TLR signaling, cytokine production and HIV infectivity observed in this study. Thirdly, there is a need to expand the evaluation of co-stimulatory factor and ligand expression on APCs and lymphocytes with TLR7 and TLR8 in PBMCs as a starting point for understanding the mechanisms APC direction of lymphocyte responses. Forthly, there is a need to conduct transwell experiments to test the effect of TLR7 and 8 stimulations on PBMCs in comparison to responses of purified T cells, NK cells, B cells and APCs. Lastly, most of the clinical trials conducted to date using either TLR7 or 8 adjuvants, have used Imiquimod that could increase susceptibility to HIV, this indicates that there is an urgent need to develop new TLR agonists that could be used in development of HIV vaccines. It is our hope that this study will demonstrate the need for novel TLR8 agonists that can be used in therapeutics or in adjuvanting HIV vaccines.

CHAPTER 9: REFERENCES

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Appendix 1: List of Abbreviations

Ah6- aryl hydrocarbon 6
AID- activation induced cytidine deaminase
AIDS-Acquired immune deficiency syndrome
ALR- AIM like receptor
AMPK- adenosine monophosphate activated protein kinase (AMPK
AP-1- activated protein 1
APC- allophycocyanin
APC- antigen presenting cell
APOBEC3G- apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3 G
APRIL- a proliferation-inducing ligand
ART- antiretroviral therapy
ARV- antiretroviral(s)
ATF3- AMP-dependent transcription factor
BAFF- B cell activating factor
BATF- Basic leucine zipper transcription factor
Bcl-3- B- cell lymphoma 3 protein
BCR- B-cell receptor
BTK- Bruton's tyrosine kinase
CASP1- caspase 1
CCL2- chemokine (C-C) ligand 2
CCR5- C-C chemokine receptor type 5
CD4- cluster differentiation factor 4
CD8-cluster differention factor 8 (and so on)
cDNA- complementary DNA
CEC- cervical epithelial cells
CLEC4E- C-type lectin family 4 member E
CLR- C-type lectins
CMC- cervical mononuclear cells
CMV- cytomegalovirus
CNS- central nervous system
CpG-DNA- cytidine-phosphate-guanosine DNA
CREB- cAMP response element-binding protein
CSR-class switch recombination
CSWs- commercial sex worker
CTL- cytotoxic lymphocytes
CX_3CR1- CX3C chemokine receptor
CXCL10- C-X-C chemokine 10
CXCR4- C-X-C chemokine receptor type 4
CYLD-cylindromatosis
DAMP- danger associate molecular patterns
DIMS- DNA-base immune modulatory sequences
DMSO- diethyl sulphoxide
DNA- deoxyribonucleic acid
dsRNA- double strand ribonucleic acid

DUBA- deubiquitinating enzyme A
DUSP1-dual specificity protein phosphatase 1
ECSIT- evolutionarily conserved intermediate in the Toll/IL-1 signal transduction
EIF2AK2- eukaryotic translation initiation factor 2-alpha kinase 2 or protein kinase R
ELK1- ETS domain-containing protein 1
EOMES- eomesodermin
ER- endoplasmic reticulum
ERK1-Extracellular signal-regulated kinases
FADD- Fas-Associated protein with Death Domain
FITC-fluorescein isothiocyanate
Foxp3- forkhead box 3
gag- group specific antigen
GATA- globin transcription factor
GM-CSF- granulocyte monocyte colony stimulating factor (also colony stimulating factor 2)
gp120- glycoprotein 120
HA- hyaluronic acid
HDAC-1- histone deacetylase 1
HIV- Human immunodeficiency virus
HLA- human leukocyte antigen
HMGB1- high mobility group box 1
HPV- human papilloma virus
HSP70- heat shock protein 70
HSV- herpes simplex virus
hToll- human Toll
IDU- injection drug users
IFNα- interferon alpha
IgG- Immunoglobulin G
IgM- immunoglobulin M
IKBKB-see IKKβ
IKKα/β- Inhibitor of κ B kinase kinase subunit alpha or beta
IL-1β- interleukin 1 beta (and so on)
ILC- innate lymphoid cells
IMO- immunomodulator oligonucleotides
IN- integrase
IP-10- interferon gamma induced protein 10
IRAK- IL-1 receptor associated kinase 4
IRF- interferon regulator factor
ISGs- interferon stimulated genes
ISS- immunostimulatory sequences
JNK- Janus Kinase
KAIS- Kenya AIDS indicator Survey
KLGR1- killer cell lectin-like receptor G1
LAMP1/2- lysosomal associated membrane protein protein 1 or 2
LPS-lipopolysaccharide

LRO- lysosome related organelles
LRR- leucine rich repeats
MALP-2- macrophage activating lipopetide 2
MAP2K3- also MKK3
MAP2K4- also MKK4
MAP3K1- Mitogen-activated protein kinase kinase kinase 1 (MAPKKK1)
MAP4K4- Mitogen-activated protein kinase kinase kinase kinase 4 (MEKKK4)
MAPK- mitogen activated protein kinases
MAPK8IP3- C-jun-amino-terminal kinase-interacting protein 3
MCMV- murine cytomegalovirus
MCP-1- monocyte chemotactic protein 1
MD2- lymphocyte antigen 96
mDC- myeloid dendritic cell
MHC- major histocompatibility complex
MICA-MHC class I chain related protein
MIP1α- macrophage inflammatory protein 1 alpha
MIP3α-macrophage inflammatory protein 3 alpha
miRNA- MicroRNA
MKK3-mitogen activate prokein kinase kinase 3
MSM- men having sex with Men
MyD88- myeloid differentiation factor 88
Nef-negative regulatory factor
NEMO- NFκB- modulator
NFAT- nuclear factor of activated T cells
NFKB2- Nuclear factor NF-kappa-B p100 subunit
NFKBIA-also IkBα
NFκB- nuclear factor kappa beta
NKG2D- natural killer cell
NLR- nucleotide binding oligomerization domain (NOD)- like receptor
NLRX- NOD like receptor X
PAMP- Pathogen associated molecular pattern
PBMC-peripheral blood mononuclear cells
PBS- phosphate buffered saline
PD-1- programmed cell death 1
pDC- plasmacytoid dendritic cells
PE-Cy5- phycoerythin cyananin 5
PELI1- Protein pellino homolog 1
PE-phycoerytherin
PHA-phytohaemagglutinin
Pin1- peptidyl-prolyl isomerase
PMA- Phorbol 12-myristate 13-acetate
PMNs- polymorphonuclear cells
pol- polymerase
Poly I:C- polyinosinic:polycytidylic acid
PPARA- Peroxisome proliferator-activated receptor alpha
PRAT4A- protein associated with TLR 4

PR-protease
PRR-pattern recognition receptor
PVDF- polyvinylidiene fluoride
RAF-1- c-Raf oncogene
RAG-1-recombinase activating gene 1
RAGE- receptor for advanced glycation endproducts
REL- proto-oncogene c-Rel
RELA- nuclear factor NF-kappa-B p65 subunit
RIP-1-Receptor interacting protein 1
RLR- retinoic inducible gene (RIG) like receptor
RNA-ribonucleic acid
RSV-respiratory syncytial virus
RT- reverse transcriptase
RTC- reverse transcriptaase complex
SAR- sar1A protein
SARM1- sterile alpha-and armadillo containing motif 1
SAMHD1-Sterile alpha motif histidine aspartic acid domain containing protein 1
SCF- stem cell factor
SDS-PAGE- sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHP-1- Src homology region 2 domain-containing phosphatase-1
SIGIRR- Single Immunoglobulin Interleukin 1 related protein
SLAN -Sulpho 6-LacNAc
SLE- systemic lupus erythematosus
SOCS1- suppressor of cytokine signalling 1
ssRNA- single strand ribonucleic acid
STAT1- signal transducer and activator of transcription 1
STI- sexually transmitted infections
Syk- spleen tyrosine kinase
TAB1- transforming growth factor-beta-activated kinase 1/MAP3K7 binding protein 1
TACI- transmembrane activator CAML interactor/ TNFRSF13
TAG- TRAM adaptor with GOLD domain
TANK- TRAF family member-associated NF-kappa-B activator
tat-transactivator of transcription
Tbet-T box 21
TBK1- TANK binding kinase 1
TCR- T cell receptor
TGFβ- transforming growth factor beta
T_H1- T helper 1cells
TICAM1- TIR domain-containing adapter molecule 1 see TRIF
TICAM2- TIR domain-containing adapter molecule 2 see TRAM
TIP DCs- TNF and induced nitric oxide synthase producing DCs
TIPE2- Tumour necrosis factor-α-induced protein-8 like-2
TIR- Toll/Interleukin 1 receptor
TIRAP- TIR associated protein
TLR- Toll-like receptor

TNFAIP8- TNF-α induced protein-8
TNFAIP8-2-TNF-α induced protein 8 like 2
TNFα- tumour necrosis factor alpha
TOLLIP- Toll interacting protein
TRADD- Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF6- TNF receptor associate factor 6
TRAIL- TNF-related apoptosis-inducing ligand
TRAM- TLR associated molecule
T_{reg}- regulatory T cell
TRIF- TIR domain containing adaptor inducing IFNβ
TRIM38- tripartite motif 38
TTP- thymidine triphosphatase
UBE2N- ubiquitin-Conjugating Enzyme E2N
ULBP1-UL16 binding protein 1
USP4- ubiquitin specific protease 4
VAMP3- vesicle-associated membrane protein 3
VIPERIN-Virus inhibiting protein endoplasmic reticulum-associated IFN inducible
Vif- viral infectivity factor
Vpr- viral protein R
vpu-viral protein unique
VSV- vesicular somatitis virus

Appendix 2: Supplementary Table 1- *p*-values for Figure 7 and 8

		HESN vs HIV-N								
		Cytokine Responses (pg/ml)					Fold Cytokine Responses			
Cytokine	Hours	Media	TLR4	TLR7	TLR8	PMA	TLR4	TLR7	TLR8	PMA
IL-1β	6	0.199	0.294	0.090	0.539	0.093	0.427	0.287	0.060	0.579
	12	0.174	**0.001**	**0.012**	0.078	0.240	0.311	0.800	0.116	0.383
	24	0.186	0.428	**0.032**	0.225	0.075	0.154	0.351	**0.005**	0.220
IL-2	6	0.086	0.353	**0.047**	0.800	0.525	0.943	0.145	0.339	0.513
	12	**0.004**	0.184	**0.032**	0.172	0.945	0.903	0.217	0.166	0.096
	24	0.218	0.271	0.260	0.547	0.143	0.820	0.207	0.635	0.420
IL-6	6	0.341	0.220	0.322	0.851	0.109	0.722	**0.023**	0.014	**0.032**
	12	0.171	0.346	0.167	0.215	0.263	**0.032**	**0.029**	0.067	**0.000**
	24	0.718	0.363	0.351	0.491	**0.046**	0.179	**0.013**	0.087	**0.046**
IL-10	6	0.199	0.679	0.888	0.730	0.341	0.345	0.254	**0.008**	**0.027**
	12	0.077	0.766	0.090	0.307	0.171	0.357	0.052	0.197	**0.026**
	24	**0.030**	0.424	**0.007**	0.537	0.718	**0.013**	0.229	0.176	0.182
IL-12p40	6	0.170	0.577	0.195	0.357	0.316	0.129	0.508	0.094	0.509
	12	0.062	0.767	0.091	0.150	0.144	0.291	0.566	0.197	0.430
	24	**0.015**	0.221	0.054	0.186	0.164	0.205	0.688	**0.036**	0.063
IL-12p70	6	0.206	0.206	0.060	0.239	0.248	0.163	0.168	0.116	0.723
	12	**0.025**	0.150	0.098	0.144	**0.021**	0.773	0.493	0.708	0.423
	24	**0.042**	0.054	**0.032**	0.131	**0.049**	**0.037**	0.198	0.380	0.708
IFNα2	6	**0.018**	0.075	0.305	0.350	0.474	0.107	0.107	0.107	0.094
	12	0.270	0.463	0.410	0.102	0.612	0.199	0.823	0.199	0.215
	24	0.136	**0.006**	0.553	0.176	0.308	**0.016**	0.065	**0.016**	0.175
IFNγ	6	0.460	0.992	0.410	0.409	0.900	0.211	0.831	0.974	0.346
	12	0.064	0.359	0.169	0.374	0.128	0.088	0.314	0.529	0.353
	24	0.101	**0.009**	0.295	0.919	0.181	0.223	0.583	**0.001**	0.366
IP-10	6	0.672	0.597	0.524	**0.033**	0.524	0.340	0.326	**0.017**	0.937
	12	0.643	0.834	0.544	0.440	0.544	0.558	**0.039**	**0.018**	0.378
	24	0.447	0.544	0.083	0.864	0.083	0.119	**0.019**	**0.019**	0.866
TNF-α	6	0.068	**0.039**	0.417	0.291	0.109	0.251	0.587	**0.027**	0.581
	12	**0.012**	**0.026**	0.069	0.592	0.376	**0.044**	0.235	**0.002**	**0.034**
	24	0.059	0.403	**0.044**	0.663	0.827	0.139	0.421	**0.023**	0.056

Appendix 3: Spearman's correlation Tables for Figure 9

TABLE 1: Cytokine correlation matrix in unstimulated PBMCs of HESN and HIV-N

HESN	IFN-g	IL-12p40	IL-1b	IL-2	IL-6	IL-10	TNF-a	MIP-1a	IP-10
IFN-a2	r^2= 0.5344 p=0.0424*	r^2=0.7491 p=0.0020**	r^2=0.7250 p=0.0031**	r^2=0.4781 p=0.0740	r^2= 0.061 p=0.8324	r^2=0.6964 p=0.0051**	r^2=0.3536 p=0.1964	r^2=0.0250 p=0.9336	r^2=0.7382 p=0.0024**
IFN-g		r^2=0.6434 p=0.0114*	r^2=0.7078 p=0.0041**	r^2=0.3753 p=0.1676	r^2=0.05004 p=0.8598	r^2=0.6774 p=0.0069**	r^2=0.3610 p=0.1853	r^2=0.04290 p=0.8800	r^2=0.5451 p=0.0379*
IL-12p40			r^2=0.9286 p=< 0.00***	r^2= 0.5893 p=0.0232*	r^2=0.3429 p=0.2111	r^2=0.8071 p=0.0005***	r^2=0.7036 p=0.0045**	r^2=0.1893 p=0.4983	r^2=0.7250 p=0.0031**
IL-1b				r^2= 0.5321 p=0.0438*	r^2=0.1929 p=0.4901	r^2=0.8750 p=<0.00***	r^2=0.6571 p=0.0094**	r^2= 0.1643 p=0.5580	r^2=0.7286 p=0.0029**
IL-2					r^2=0.3953 p=0.1455	r^2=0.6023 p=0.0203*	r^2=0.1619 p=0.5628	r^2=0.3313 p=0.2270	r^2=0.5270 p=0.0465*
IL-6						r^2=0.2714 p=0.3269	r^2=0.5786 p=0.0263*	r^2=0.5393 p=0.0406*	r^2=0.5393 p=0.0406*
IL-10							r^2=0.4571 p=0.0888	r^2=0.4821 p=0.0711	r^2=0.7679 p=0.0013**
TNF-a								r^2=0.1464 p=0.6024	r^2=0.7250 p=0.0031**
MIP-1a									r^2=0.2821 p=0.3074
HIV-N	**IFN-g**	**IL-12p40**	**IL-1b**	**IL-2**	**IL-6**	**IL-10**	**TNF-a**	**MIP-1a**	**IP-10**
IFN-a2	r^2=0.1432 p=0.5707	r^2=0.05475 p=0.8292	r^2=0.1570 p=0.5338	r^2= 0.2141 p=0.3935	r^2=0.1960 p=0.4989	r^2=0.3771 p=0.1230	r^2= 0.3202 p=0.1951	r^2=0.3137 p=0.2352	r^2=0.1281 p=0.6341
IFN-g		r^2=0.5913 p=0.0097**	r^2=0.5542 p=0.0170*	r^2= -0.209 p=0.4057	r^2=0.3363 p=0.2399	r^2=0.2879 p=0.2466	r^2=0.4881 p=0.0399*	r^2=0.1971 p=0.4632	r^2=-0.012 p=0.9694
IL-12p40			r^2=0.7379 p=0.0005***	r^2=0.2481 p=0.3209	r^2=0.1297 p=0.6595	r^2=0.4056 p=0.0950	r^2=0.7214 p=0.0007***	r^2= 0.0059 p=0.9869	r^2=0.02059 p=0.9432
IL-1b				r^2=-0.1881 p=0.4548	r^2= 0.3802 p=0.1808	r^2=0.4138 p=0.0878	r^2=0.6285 p=0.0052**	r^2=-0.08529 p=0.7545	r^2= -0.2000 p=0.4564
IL-2					r^2=-0.3574 p=0.1808	r^2=0.05634 p=0.8243	r^2=0.05843 p=0.8179	r^2=0.1941 p=0.4695	r^2=0.3436 p=0.1915
IL-6						r^2=0.6879 p=0.0082**	r^2=0.2703 p=0.3492	r^2=0.5868 p=0.0303*	r^2=-0.0066 p=0.9879
IL-10							r^2=0.6285 p=0.0052**	r^2=0.6588 p=0.0068***	r^2=0.2500 p=0.3491
TNF-a								r^2=0.4941 p=0.0540	r^2=0.2029 p=0.4496
MIP-1a									r^2=0.6029 p=0.0153*

TABLE 2: Cytokine correlations matrix in TLR4 stimulated PBMCs of HESN and HIV-N

HESN	IFN-γ	IL-12p40	IL-1β	IL-2	IL-6	IL-10	TNF-α	MIP-1α	IP-10
IFNα2	r^2= 0.5179 p=0.0506	r^2=0.2750 p=0.3203	r^2= 0.5500 p=0.0362*	r^2= 0.5385 p=0.0404*	r^2= 0.1071 p=0.7049	r^2= 0.6071 p=0.0186*	r^2= 0.1000 p=0.7241	r^2= 0.2088 p=0.4731	r^2= 0.4429 p=0.1002
IFN-γ		r^2=0.4464 p=0.0972	r^2=0.6286 p=0.0141*	r^2=0.1036 p=0.7144	r^2= -0.3107 p=0.2592	r^2= 0.3071 p=0.2650	r^2= 0.5679 p=0.0297*	r^2=0.01786 p=0.9540	r^2=0.6071 p=0.0186*
IL-12p40			r^2=0.7393 p=0.0023**	r^2= 0.1964 p=0.4819	r^2=0.1464 p=0.6024	r^2=0.6893 p=0.0057**	r^2=0.6321 p=0.0134*	r^2=-0.4000 p=0.1408	r^2= 0.4179 p=0.1227
IL-1β				r^2=0.5179 p=0.0506	r^2=0.05714 p=0.8425	r^2=0.7107 p=0.0040**	r^2=0.4750 p=0.0759	r^2=-0.057 p=0.8425	r^2=0.5500 p=0.0362*
IL-2					r^2=0.1973 p=0.4784	r^2=0.6123 p=0.0170*	r^2=-0.2268 p=0.3470	r^2=0.1605 p=0.5657	r^2=0.1844 p=0.5081
IL-6						r^2=0.6071 p=0.0186*	r^2=-0.01786 p=0.9540	r^2=0.2857 p=0.3012	r^2=0.1536 p=0.5844
IL-10							r^2=0.3607 p=0.1870	r^2=0.1536 p=0.5844	r^2=0.4964 p=0.0623
TNF-α								r^2=-0.1929 p=0.4901	r^2=0.4607 p=0.0861
MIP-1α									r^2=-0.008 p=0.9847
HIV-N	**IFN-γ**	**IL-12p40**	**IL-1β**	**IL-2**	**IL-6**	**IL-10**	**TNF-α**	**MIP-1α**	**IP-10**
IFNα2	r^2= -0.0423 p=0.8676	r^2=-0.2157 p=0.3900	r^2= 0.1455 p=0.5645	r^2= -0.4545 p=0.0581	r^2= 0.1446 p=0.5789	r^2= 0.3044 p=0.2193	r^2=0.2198 p=0.3808	r^2= -0.00588 p=0.9869	r^2= -0.068 p=0.8051
IFN-γ		r^2=0.5975 p=0.009**	r^2= 0.7915 p=< 0.00****	r^2=0.3540 p=0.1495	r^2=0.1297 p=0.6595	r^2=0.3003 p=0.2260	r^2= 0.5521 p=0.0175*	r^2=0.08529 p=0.7545	r^2=0.2588 p=0.3319
IL-12p40			r^2=0.3829 p=0.1168	r^2= 0.5466 p=0.0189*	r^2=-0.3187 p=0.2666	r^2=0.2673 p=0.2836	r^2=0.6966 p=0.0013**	r^2=0.2912 p=0.2731	r^2=0.3000 p=0.2583
IL-1β				r^2=0.07453 p=0.7688	r^2=0.2527 p=0.3825	r^2=0.2673 p=0.2836	r^2=0.4159 p=0.0861	r^2= -0.065 p=0.8137	r^2=0.1353 p=0.6170
IL-2					r^2=0.1084 p=0.7109	r^2=-0.1771 p=0.4820	r^2=0.3977 p=0.1022	r^2=-0.073 p=0.7766	r^2=0.09454 p=0.7263
IL-6						r^2= 0.5209 p=0.0591	r^2=0.1385 p=0.6375	r^2=0.1253 p=0.6706	r^2=-0.1824 p=0.5321
IL-10							r^2=0.4469 p=0.0630	r^2=0.3882 p=0.1382	r^2=-0.027 p=0.9258
TNF-α								r^2=0.4324 p=0.0961	r^2=0.4235 p=0.1036
MIP-1α									r^2=-0.1176 p= 0.6645

TABLE 3: Cytokine correlations matrix in Imiquimod (TLR7) stimulated PBMCs of HESN and NN

HESN	IFN-γ	IL-12p40	IL-1β	IL-2	IL-6	IL-10	TNF-α	MIP-1α	IP-10
IFNα2	r^2=0.3843 p=0.1573	r^2=0.7464 p=0.0020**	r^2=0.5643 p=0.0310*	r^2=0.05714 p=0.8425	r^2=0.2536 p=0.3607	r^2=0.8000 p=0.0006***	r^2=0.3000 p=0.2767	r^2=0.7679 p=0.0013**	r^2=0.6357 p=0.0128*
IFN-γ		r^2=0.6363 p=0.0125*	r^2=0.4343 p=0.1068	r^2=0.5773 p=0.0264*	r^2=-0.08937 p=0.7458	r^2=0.5290 p=0.0447*	r^2=0.4397 p=0.1021	r^2=0.6238 p=0.0149*	r^2=0.03753 p=0.8955
IL-12p40			r^2=0.2929 p=0.2888	r^2=0.09286 p=0.7435	r^2=-0.1250 p=0.6575	r^2=0.6000 p=0.0203*	r^2=0.6929 p=0.0054**	r^2=0.3821 p=0.1607	r^2=0.3821 p=0.1607
IL-1β				r^2=-0.03932 p=0.8853	r^2=0.4772 p=0.0738	r^2=0.5964 p=0.0213*	r^2=0.5179 p=0.0506	r^2=0.1536 p=0.5844	r^2=0.4321 p=0.1094
IL-2					r^2=-0.2065 p=0.4278	r^2=0.06126 p=0.8281	r^2=-0.1171 p=0.6518	r^2=0.003604 p=0.9871	r^2=0.4847 p=0.0690
IL-6						r^2=0.6649 p=0.0083**	r^2=0.8150 p=0.000***	r^2=0.4236 p=0.1165	r^2=0.4643 p=0.0834
IL-10							r^2=0.2857 p=0.3012	r^2=0.7964 p=0.00***	r^2=0.5857 p=0.0242*
TNF-α								r^2=0.3893 p=0.1525	r^2=0.3107 p=0.2592
MIP-1α									r^2=0.5179 p=0.0506
HIV-N	**IFN-γ**	**IL-12p40**	**IL-1β**	**IL-2**	**IL-6**	**IL-10**	**TNF-α**	**MIP-1α**	**IP-10**
IFNα2	r^2=0.5503 p=0.0180*	r^2=0.2487 p=0.3196	r^2=0.1971 p=0.4331	r^2=0.4757 p=0.0460*	r^2=0.4418 p=0.1158	r^2=0.6594 p=0.0029**	r^2=0.3767 p=0.1234	r^2=0.4265 p=0.1011	r^2=0.2618 p=0.3262
IFN-γ		r^2=0.3077 p=0.2142	r^2=0.2282 p=0.3624	r^2=0.4223 p=0.0808	r^2=0.2640 p=0.3590	r^2=0.6495 p=0.0035**	r^2=0.2819 p=0.2571	r^2=0.4224 p=0.1041	r^2=0.1339 p=0.6188
IL-12p40			r^2=0.8411 p=< 0.00****	r^2=0.4256 p=0.0782	r^2=0.3099 p=0.2806	r^2=0.2806 p=0.1168	r^2=0.5212 p=0.0266*	r^2=-0.1500 p=0.5786	r^2=0.2060 p=0.4409
IL-1β				r^2=0.1591 p=0.5283	r^2=0.04615 p=0.8796	r^2=0.1496 p=0.5534	r^2=0.5480 p=0.0186*	r^2=0.08824 p=0.7462	r^2=0.02060 p=0.9409
IL-2					r^2=0.3669 p=0.1962	r^2=0.6836 p=0.0018**	r^2=0.02840 p=0.9109	r^2=0.08748 p=0.7462	r^2=0.3257 p=0.2164
IL-6						r^2=0.6308 p=0.0181*	r^2=0.1648 p=0.5733	r^2=0.5209 p=0.0591	r^2=0.01978 p=0.9517
IL-10							r^2=0.3086 p=0.2128	r^2=0.5029 p=0.0493*	r^2=-0.1029 p=0.7049
TNF-α								r^2=0.2647 p=0.3207	r^2=-0.1265 p=0.6405
MIP-1α									r^2=0.2294 p=0.3914

TABLE 4: Cytokine correlations matrix in ssRNA (TLR8) stimulated PBMCs of HESN and HIV-N

HESN	IFN-γ	IL-12p40	IL-1β	IL-2	IL-6	IL-10	TNF-α	MIP-1α	IP-10
IFNα2	r^2= 0.5071 p=0.0562	r^2=0.08929 p=0.7532	r^2= 0.5893 p=0.0232*	r^2=-0.009 p=0.9667	r^2= -0.2929 p=0.2888	r^2=0.2000 p=0.4738	r^2= -0.2286 p= 0.4114	r^2= 0.03214 p=0.9132	r^2=0.2179 p=0.4343
IFN-γ		r^2=0.7000 p=0.0048**	r^2=0.8571 p=< 0.00****	r^2=0.1056 p=0.7065	r^2= -0.1786 p=0.5235	r^2= 0.5214 p=0.0488*	r^2=0.3321 p=0.2264	r^2=-0.08571 p=0.7630	r^2=0.4786 p=0.0735
IL-12p40			r^2=0.7500 p=0.0019**	r^2= 0.1683 p=0.5460	r^2=-0.1607 p= 0.5667	r^2=0.7786 p=0.0010**	r^2=0.4250 p=0.1159	r^2=-0.1679 p=0.5492	r^2= 0.3964 p=0.1446
IL-1β				r^2=0.1110 p=0.6921	r^2=-0.3286 p=0.2317	r^2=0.2317 p=0.0222*	r^2=0.2607 p=0.3469	r^2=-0.09286 p=0.7435	r^2=0.4500 p=0.0944
IL-2					r^2=0.4749 p=0.0755	r^2=0.5229 p=0.0479*	r^2=-0.3585 p=0.1465	r^2=0.3862 p=0.1547	r^2=-0.1478 p=0.5101
IL-6						r^2=0.1607 p=0.5667	r^2=-0.1357 p=0.6297	r^2=0.4500 p=0.0944	r^2=-0.3393 p=0.2161
IL-10							r^2=0.1393 p=0.6205	r^2=-0.02143 p=0.9438	r^2=0.3179 p=0.2480
TNF-α								r^2=0.02143 p=0.9438	r^2=0.6571 p=0.0094**
MIP-1α									r^2=-0.1250 p=0.6575
HIV-N	**IFN-γ**	**IL-12p40**	**IL-1β**	**IL-2**	**IL-6**	**IL-10**	**TNF-α**	**MIP-1α**	**IP-10**
IFNα2	r^2= 0.1218 p=0.6301	r^2= 0.05576 p=0.8261	r^2=0.1558 p=0.5369	r^2= 0.07649 p=0.7629	r^2=-0.1956 p=0.5022	r^2=-0.0092 p=0.9708	r^2=0.2797 p=0.2610	r^2=0.09706 p=0.7213	r^2=0.5372 p=0.0339*
IFN-γ		r^2=0.7805 p=0.0001***	r^2=0.5307 p=0.0235*	r^2=0.1898 p=0.4507	r^2=0.07921 p=0.7877	r^2=0.7877 p=0.2015	r^2= 0.4915 p=0.0383*	r^2=0.2502 p=0.3472	r^2=0.3962 p=0.3962
IL-12p40			r^2=0.6897 p=0.0015**	r^2=0.2606 p =0.2963	r^2=0.3011 p=0.2951	r^2=0.4760 p=0.0459*	r^2=0.6185 p=0.0062**	r^2=0.3400 p=0.1967	r^2=0.3726 p=0.1548
IL-1β				r^2=-0.05995 p=0.8132	r^2=0.5473 p=0.0458*	r^2=0.5769 p=0.0122*	r^2=0.6037 p=0.0080**	r^2=0.1647 p=0.5412	r^2=0.1560 p=0.5612
IL-2					r^2=0.4199 p=0.1356	r^2=0.1409 p=0.5772	r^2=0.1036 p=0.6826	r^2=0.2349 p=0.3783	r^2=0.4287 p=0.0983
IL-6						r^2=0.7451 p=0.0031**	R ^{2}=0.4549 p=0.1044	r^2=0.4637 p=0.0973	r^2=0.1804 p=0.5345
IL-10							r^2=0.7936 p=< 0.00****	r^2=0.6824 p=0.0046**	r^2=0.05445 p=0.8415
TNF-α								r^2=0.5941 p=0.0172*	r^2=0.08094 p=0.7650
MIP-1α									r^2=0.1192 p=0.6584