CHARACTERIZING THE ROLE OF TOLL-LIKE RECEPTOR 2 IN SENSING AND REGULATING HUMAN IMMUNDEFICIENCY VIRUS-1 INFECTION FROM MOTHER-TO-CHILD THROUGH BREAST MILK

By

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—ABSTRACT—

Breastfeeding from HIV-infected mothers is one of the major sources of pediatric HIV-1 infection; however, an intervention that promotes exclusive breastfeeding has significantly reduced vertical HIV transmission rates and infant mortality. The mechanisms underlying this phenomenon remain unknown; however, have been closely linked to high levels of innate immune factors in breast milk. Indeed, the level of several innate factors in breast milk correlate with protection and/or have direct anti-viral properties *in vitro.* The innate immune factor, soluble TLR2 (sTLR2) is found in high concentration in breast milk and has previously been investigated for its anti-bacterial properties; however, its anti-viral properties remain poorly understood. Thus, the research presented in this thesis extended our understanding of sTLR2 by characterizing the mechanisms by which sTLR2 inhibited HIV-induced inflammation and infection. Chapter 2 examined the predominant forms of sTLR2 in breast milk from different women, its cellular source, bioavailability and kinetics postpartum. Functionally, we confirmed sTLR2's anti-bacterial properties and extended to show, for the first time, that sTLR2 directly inhibited HIV infection *in vitro.* Chapter 3 documented a potential mechanism of sTLR2's direct inhibition of HIV infection *in vitro* and, investigated sTLR2 and TLR2 expression in HIV uninfected compared to HIV infected breast milk and breast milk cells, respectively. Chapter 4 investigated the role of TLR2's recognition of novel HIV pathogen associated molecular patterns (PAMPs), and whether TLR2 expression increased HIV infection and integration. Taken together, we present novel anti-viral functions of sTLR2 by demonstrating that sTLR2 bound to specific HIV PAMPs, which led to significantly decreased HIV-induced inflammation, co-receptor expression, and HIV infection. Furthermore, we demonstrated, for the first time, that TLR2 recognizes specific HIV PAMPs, which led to significantly increased pro-inflammatory cytokine production, co-receptor expression and HIV infection. Thus, sTLR2 and TLR2 represent innate immune factors that might have preventative and therapeutic applications for both infants and adults in the future.

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— LIST OF ABBREVIATIONS —

Ab	antibody
AIDS	Acquired Immunodeficiency Syndrome
AP-1	activator protein-1
ARS	acute retroviral syndrome
ARV	antiretroviral
AZT	zidovudine
B cell	bone marrow derived lymphocyte
CA	capsid protein (p24)
Caco-2	human intestinal epithelial cell line
CAV	cell-associated virus
CBA	cytometric bead array
cDNA	complementary deoxyribonucleic acid
CCR5	C-C chemokine receptor type 5
CD4	cluster of differentiation 4
CD4bs	cluster of differentiation binding site
CD74	cluster of differentiation 74 (major histocompatibility complex, class II invariant chain)
CFV	cell free virus
CLR	c-type lectin receptor
CMV	cytomegalovirus
CXCR1	C-X-C chemokine receptor type 1
CXCR4	C-X-C chemokine receptor type 4
COX-2	cyclooxygenase 2
DCs	dendritic cells
DC-SIGN	dendritic cell specific intracellular adhesion molecule
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
EBF	exclusive breastfeeding
EBV	Epstein Barr Virus
ELISA	enzyme-linked immunosorbant assay
ENV	envelope glycoprotein
FACS	flow cytometry
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAG	glucosaminoglycan
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
gp	glycoprotein
gp41	glycoprotein 41
gp120	glycoprotein 120
gp160	glycoprotein 160

HAART	highly active antiretroviral therapy
HCV	hepatitis C virus
HEK293	human embryonic kidney cell
HESN	highly exposed seronegative
HIV	human immunodeficiency virus refers to HIV-1 unless specifically stated since cases of mother-to-child transmission of HIV-2 are rare.
HSV	herpes simplex virus
IFNα	interferon alpha
IFNγ	interferon gamma
Ig	immunoglobulin
IL-8	interleukin 8
IL-15	interleukin 15
IN	integrase
LAG-3	lymphocyte activating gene-3
LPS	lipopolysaccharide
LTRs	long terminal repeat
MA	matrix protein (p17)
MCF-10A	mammary epithelial cell line
MDA-5	melanoma differentiation associated protein 5
MECs	mammary epithelial cells
mRNA	messenger RNA
MTCT	mother-to-child transmission
MUC1	mucin 1
NC	nucleocapsid (p6)
NEBF	non-exclusive breastfeeding
NFAT	nuclear factor associated T cells
NFκB	nuclear factor kappa light chain enhancer of B-cell
NK	natural killer
NS3	non-structural protein 3 (p-70)
NVP	neviripine
Pam_3CSK_4	synthetic bacterial triacylated lipoprotein
PAMPs	pathogen associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PD-1	programmed death-1
POL	HIV polymerase gene
PMA	phorbol 12-myristate 13-acetate
PMTCT	prevention of mother-to-child transmission
PR	protease
PR55(GAG)	GAG precursor protein
PRRs	pattern recognition receptors
RANTES	chemokine ligand 5 (CCL5)
REB	research ethics board
RIG-I	retinoic acid-inducible gene 1

RPL13A	ribosomal protein
RPMI 1640	Rosswell Park Memorial Institute media
RNA	ribonucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SIV	simian immunodeficiency virus
SIVcpz	simian immunodeficiency virus chimpanzee
SIVgor	simian immunodeficiency virus gorilla
SLPI	secretory leukocyte protease inhibitor
SNPs	single nucleotide polymorphisms
Sp1	specificity protein 1
STIs	sexually transmitted infection
siRNA	small interfering ribonucleic acid
ssRNA	single stranded ribonucleic acid
sTLR	soluble toll-like receptor
T cell	thymus derived lymphocyte
TAT	transactivator binding protein
TEER	transepithelial electrical resistance
THP-1	human monocyte cell line
Tim-3	T cell immunoglobulin and mucin domain-containing molecule-3
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
TZMbl	CD4, CCR5, and CXCR4 stably transfected cervical epithelial cells
UNAIDS	United Nations Program on HIV/AIDS
US	United States of America
U937	human monocyte cell line
VIF	viral infectivity factor
VLPs	virus like particles
VPR	viral protein r
VPU	viral protein U
WHO	World Health Organization

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— PREAMBLE —

The research presented in this thesis includes material that has been previously published or has been submitted for publication. Chapter 1 provides an overview of human immunodeficiency virus (HIV) including the origins, global impact, structure, infection cycle, immunopathogenesis. As well, it highlights mother-to-child transmission (MTCT) of HIV and focuses on breast milk immunology as a protective mucosal fluid. Chapters 2 through 4 present three peer-reviewed scientific manuscripts that, as of July 2013, are published or have been submitted. Completion of the research for the each manuscript required collaboration with colleagues and, therefore, resulted in multiple authors.

CHAPTER 2 Henrick BM, Nag K, Yao X-D, Drannik AG, Aldrovandi GM, Rosenthal KL. Milk matters: soluble Toll-like receptor 2 (sTLR2) in breast milk significantly inhibits HIV-1 infection and inflammation. PLoS ONE 2012 July 6; 7:e40138.

This work was conducted from 2008 to 2010. I designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. Drs. Kakon Nag (post-doctoral fellow), Xiao-Dan Yao (research scientist), and Anna Drannik (PhD candidate), all at McMaster University, provided guidance in the design of experiments, assisted with experiments and offered scientific input. Our collaborator, Dr. Grace Aldrovandi (University of California Los Angeles) provided multiple breast milk samples for use in this study. In addition, we started the Hamilton Breast Milk Cohort (Research Ethics Board #08-176) to collect HIV-uninfected breast milk samples from the area. Dr.

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CHAPTER 3 Henrick BM, Yao X-D, Nag K, Abumiku A, Rosenthal KL. Direct binding of sTLR2 to HIV-1 structural proteins reduces cellular activation and inhibits HIV-1 infection.

This work was conducted from 2011 to 2013. I designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. Dr. Xiao-Dan Yao (research scientist) provided novel technology and offered scientific input. Dr. Kakon Nag (post-doctoral fellow) provided novel technology. Our collaborator Dr. Alash'le Abumiku (Institute of Virology, University of Maryland, USA; Institute of Human Virology, Nigeria) provided breast milk samples. Dr. Kenneth Rosenthal provided guidance and supervision for the preparation of the manuscript.

CHAPTER 4 Henrick BM, Yao X-D, Rosenthal KL. Unlikely Suitors: Identification of HIV-1 Ligands that Activate Through TLR2 and Significantly Increase HIV Infection/Integration. Submitted to *Journal of Virology.*

This work was conducted from 2011 to 2013. I designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. Dr. Xiao-Dan Yao (research scientist) provided novel technology and offered scientific input. Dr. Kenneth Rosenthal provided supervision, ideas and general guidance throughout the study.

— CHAPTER 1 —

1.0 HIV: Origins, Global Impact and Response

Human immunodeficiency virus (HIV) is a lentivirus, originating from the retroviridae family, and is the etiological agent of human acquired immunodeficiency syndrome (AIDS). Lentiviruses cause slow, persistent infections unique to their mammalian hosts (i.e. equine, bovine, ovine, lagomorphine, feline, and simian) [1,2]. The human lentivirus, HIV, which is categorized into two main types (HIV-1 and HIV-2) is thought to have entered into the human population as a result of multiple zoonotic transfers from primates infected with the lentivirus, simian immunodeficiency virus (SIV). The most accepted explanation, 'the natural transfer theory', describes multiple human cutaneous and mucosal exposures with SIV-infected body fluids, which are a common occurrence during bush meat hunting [3]. Indeed, epidemiological studies indicate that a substantial percentage of people living in rural areas and participating in bush meat hunting, have been exposed to SIV [4]. The exact mechanism that enabled SIV to mutate in order to propagate in humans remains poorly understood, although it likely has to do with the error prone reverse transcription of the virus and its astonishing ability to evolve in relation to mammalian DNA [5].

HIV-1 is categorized into four major groups (M, N, O, P), each of which is a result of an individual cross-species transfer event. Specifically, groups M and N share the closest genetic relationship to SIV infecting chimpanzees (SIVcpz) [6], while groups P and O are closely related to SIV infecting gorillas (SIVgor) [7]. Group M, which is

responsible for the pandemic of HIV-1, is believed to have crossed species between 1910-1930 in colonial West Africa near Kinshasa (formally Leopoldville, Republic of the Congo) [8,9], as it was here that the first documented case of HIV-1 was identified in historic blood samples obtained between 1959 and 1960 [10].

HIV infection went largely unnoticed until it presented itself in the United States (US) in the early 1980's. It was during this time that physicians began documenting rare opportunistic infections, including *Pneumocystis carinii* pneumonia, *candida* mucosal infections, and cytomegalovirus infections, in young homosexual men from Los Angeles, CA who had previously been healthy [11]. Following these reports, clusters of other unusual opportunistic infections that were previously exclusively unique to immunosuppressed individuals [12], including Kaposi's Sarcoma and lymphadenopathy, were identified in men living in major urban centres in the US. In the summer of 1982, the disease was referred to as Acquired Immune Deficiency Syndrome (AIDS) and, by 1983, Dr. Luc Montagnier, Fancoise Barre-Sinoussi, and Robert Gallo had independently identified the etiological agent that caused AIDS. By 1986, the causative agent was termed HIV [13]. This name was derived from the virus' ability to incapacitate the immune system, thus rendering infected individuals unable to mount an immune response to opportunistic infections, most of which were lethal.

Since HIV was first recognized, an estimated 60 million people worldwide have become infected with the virus. Current estimations report approximately 33.2 million

HIV-1-infected people globally and, without greater universal access to testing, counseling, and treatment, the number is expected to surpass 60 million by 2050 [14]. The most endemic areas, including sub-Saharan Africa, account for approximately 70% of the global pandemic. More alarming, in the high endemic area in sub-Saharan African countries where infection rates are at an estimated 23-26%, including Botswana and Swaziland, reports estimate that almost a fifth of their adult population will die prematurely from AIDS [14,15]. However, despite these disturbing statistics, the prevalence of HIV-1 infection globally has stabilized and begun to decline in many high incidence countries, which is likely due to the unprecedented application of universal antiretroviral (ARV) drug therapy, increased access to testing and education about prevention [16].

Children account for approximately 2.5 million of the global HIV-1 burden [16] which is primarily transmitted from their HIV-1-infected mothers during pregnancy, delivery, and/or breastfeeding. HIV-2 is very rarely transmitted to infants; therefore, the vast majority of research investigates mother-to-child transmission (MTCT) of HIV-1 [17]. Ninety percent of all HIV-infected children live in sub-Saharan Africa and studies indicate that one in three newborns infected with HIV-1 will die before the age of one, over one half of these will die before reaching their second birthday, and the majority of such infected children will die before they are five years old [18]. Notably, however, significant progress has been made in the past decade in the prevention of mother-to-child transmission. The number of infants infected during pregnancy and delivery has

dramatically declined, which is largely due to short-course ARV therapy given around the time of delivery [16]. However, in 2011, only 57% of women in low to middle income countries received a World Health Organization (WHO)-recommended regimen of ARV therapy [19], which rarely continues postnatally [20]. For this reason, as well as the fact that breastfeeding is associated with a 50% reduction in hospitalization for diarrheal and 30% reduction for respiratory disease, the WHO has endorsed a prevention therapy that promotes exclusive breastfeeding irrespective of the HIV status of the mother when safe and accessible feeding alternatives are not available [21]. While the risk of HIV transmission from mother-to-child (also known as vertical transmission) exists, the risk is significantly reduced in mothers that exclusively compared to mix breast feed [22-25], and, importantly, EBF is a feasible preventative strategy in resource poor areas.

1.2 HIV-1 Structure

HIV-1 is a 100 nm diameter spherical retrovirus with nine genes (ENV, GAG, POL, nef, rev, tat, vif, vpu and vpr) that encode for 15 distinct proteins (gp120, gp41, p24, p17, p7, p6, integrase, protease, reverse transcriptase, vif, vpu, vpr, nef, rev and tat) [2,26]. The genes are classified into two major groups: structural genes (GAG, POL, and ENV) and regulatory/accessory genes (nef, rev, tat, vif, vpu, and vpr). Additionally, as a provirus, each end of the genome contains long-terminal repeats sequences (LTRs) along with promoters, enhancers, and other gene sites that facilitate binding to host cellular transcription factors including nuclear factor kappa light chain enhancer of B cells (NFκB), activator protein (AP)-1, and nuclear factor of activated T cells (NFAT) [27].

Mature virions consist of a viral membrane, which is a host-derived phospholipid bilayer containing approximately 10-100 surface glycoprotein spikes [28] (Fig.1). The glycoprotein spike, known as the envelope protein (ENV) is a trimer composed of triplicate copies of two non-covalently associated proteins: surface glycoprotein, gp120, which interacts with host cellular receptors, and gp41, the transmembrane protein responsible for viral and target membrane fusion [29]. The viral membrane is stabilized internally by the viral matrix protein (MA, p17) [30,31]. Within the viral envelope, there is a cone-shaped capsid comprised of capsid protein (CA, p24), which encapsulates two single-stranded, positive-sense RNA molecules and enzymatic proteins critical to viral integration, including: (1) reverse transcriptase (RT), (2) integrase (IN), and (3) protease (PR). Additionally, the capsid contains accessory and regulatory proteins (vif, vpu, vpr,

nef), as well as cellular factors [32] (Figure 1; [33]). To thoroughly understand and complement the data defining Toll-like receptor (TLR) 2-dependent, HIV-1-induced cellular activation and soluble TLR (sTLR) 2 inhibition of HIV-1 infection described in Chapters 2 through 4, the HIV-1 structural proteins studied in this thesis will be described in greater detail than the accessory and regulatory proteins.

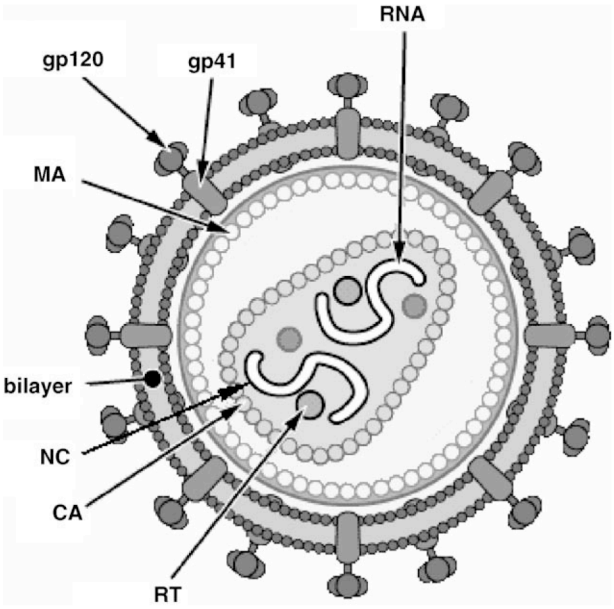

Figure 1. ***Figure of a mature virion***. The phospholipid bilayer contains 10-100 ENV (gp120 and gp41) spikes. The matrix proteins (MA; p17) provide the inner core, capsid proteins (CA; p24) comprise the inner capsid surrounding the two ssRNA, nucleocapsid protein (NC; p7), reverse transcriptase (RT), protease (PR), and integrase (IN). Figure adapted from Sierra *et al.*, 2005.

1.2.1 Envelope (ENV)

The ENV protein is comprised of two non-covalently bound glycoproteins, gp120 and gp41. Glycoprotein 120 (gp120) is the sole surface viral antigen and mediates viral-host interaction essential to viral entry. This glycoprotein has conserved inner and outer portions which are retained among all primary immunodeficiency viral subtypes; and these permit the virus to bind to host cluster of differentiation (CD) 4 and co-receptors, C-C chemokine receptor type (CCR) 5 and C-X-C chemokine receptor type (CXCR) 4 [34]. Notably, gp120 is highly variable (largely restricted to the variable loops (V1-V5)), which are key to HIV's success in circumventing host adaptive immune responses (reviewed in [35]. Importantly, gp120 peptides are known to dysregulate the biological activity of many cell types, including the loss of tight junctions in the female genital tract tissues [36], as well as being responsible for the immunostimulatory effects related to HIV-1-associated dementia [37,38].

Glycoprotein 41 (gp41) is the transmembrane glycoprotein component of ENV that mediates viral-host fusion through C-terminal interactions rooted in the viral membrane and the N-terminal spike or ectodomain fusing with the host plasma membrane. As a mature virion, gp41 lies underneath gp120 in its pre-hairpin state (Fig.2), and once revealed after a conformational change in the envelope complex, the interaction between gp120 and host CD4 and CCR5 receptors occurs (Fig.2) and it inserts its N-terminus peptides into the host cell membrane. Subsequently, gp41 conformationally rearranges by bringing the N-terminal and C-terminal ends towards

each other, thus pulling the host and viral membranes into close proximity to complete the fusion process [39] (Fig.2; step 4). Additionally, gp41 has been shown to regulate cellular functions by interacting with a number of cellular proteins [40], and manipulates the expression of chemokine receptors on monocytes [41]. Additionally, gp41 significantly enhances HIV-1 infection and replication by binding to CD74 and triggering cellular division through the MAPK/ERK pathway [42]. Furthermore, we showed that gp41 significantly increased cellular activation, pro-inflammatory cytokine production, and CCR5 expression in macrophages through a TLR2/1-dependent mechanism (Henrick *et al.,* 2013; submitted, Chapter 4) that was readily inhibited by sTLR2 (Henrick *et al*., 2013b; submitted, Chapter 3).

1.1.2 GAG

The precursor GAG polyprotein (Pr55GAG) self-assembles on the inner surface of an immature virion, and during maturation is proteolytically cleaved by viral protease into four proteins: p17 (matrix protein, MA), p24 (capsid protein, CA), p6 (nucleocapsid protein, NC), and p7, which are all critically important in the formation of a functional infectious virion [43]. Pr55GAG has previously been referred to as the 'particle-making machine' as it will continuously form virus-like particles (VLPs) in the absence of other viral proteins or RNA [44,45]. Due to this intrinsic feature, soluble GAG proteins are often found at high levels systemically and have been shown to correlate with disease progression [46,47].

p17 (MA) is a 132 amino acid myristoylated polypeptide originating from the N-terminal end of the Pr55GAG polyprotein [30]. Using its myristoylated N-terminus, p17 anchors Pr55GAG to the inner surface of host cells [48], thus directing GAG polyproteins to the plasma membrane during assembly and budding [43]. Mutations to p17, including the N-terminal myristoylation, severely inhibit viral budding and lead to the accumulation of viral particles in the cytoplasm of infected cells [45]. Importantly, it has recently been shown that p17 enhances HIV-1 infection and replication in permissive cells by triggering cellular activation possibly through CXC chemokine receptor 1 (CXCR1) [49,50]. In addition, we showed that p17 activates cells through heterodimer TLR2/1 leading to increased NFκB activation, IL-8 production, and CCR5 expression (Henrick *et al.,* 2013; submitted, Chapter 4). Furthermore, sTLR2 directly interacts with p17, thus inhibiting its induced cellular activation (Henrick *et al*., 2013b; submitted, Chapter 3).

p24 (CA) forms the cone-shaped capsid core surrounding the viral genome and core-associated proteins [44,51]. This protein plays an important role in post-entry integration, assembly, budding, and maturation of new virions [44,51]. Furthermore, high circulating levels of p24 correlate with disease progression [46,47] and have been shown to stimulate peripheral blood mononuclear cells (PBMCs) of HIV-1-infected individuals receiving highly active anti-retroviral therapy (HAART) [52]. Our recent data indicated that p24 binds to TLR2/6 leading to increased cellular activation. Importantly, however, in cells lacking TLR6 expression, p24 readily blocked gp41 and p17-induced activation

(Henrick *et al.,* 2013; submitted, Chapter 4). Furthermore, p24 interacts directly with sTLR2 *in vitro* and significantly correlates with sTLR2 levels in HIV-1-infected breast milk (Henrick *et al*., 2013b; submitted, Chapter 3).

Surprisingly, however, HIV-1 proteins have not been well studied for their ability to serve as viral pathogen associated molecular patterns (PAMPs), despite numerous publications that emphasize their immunostimulatory effects. Specifically, gp120 has been shown to stimulate the production of pro-inflammatory cytokines related to HIV-1-associated dementia [37,38]. Additionally, a seminal publication by Nazli *et al.,* (2010) highlighted the role of gp120-specific impairment of tight junctions in genital and rectal mucosae, which might be critical to transmission and acute infection [36]. Furthermore, gp41 binds to CD74 and significantly enhances HIV-1 infection and replication [42]. Additionally, p24 exposure stimulated PBMCs obtained from HIV-1-infected individuals receiving HAART to produce increased IL-10 [52]. Furthermore, p17 possesses potent immunostimulatory properties and increased HIV-1 replication in activated PBMCs, [50,53]. Thus, HIV-1 creates a more suitable environment for its own integration and replication via virus-induced innate immune activation.

1.2 Viral Infection Cycle

HIV infection and replication is an intricate biological process that involves several molecular phases as reviewed by Sierra *et al.*, 2005 [33], including (1) viral-host binding and entry, (2) reverse transcription of viral RNA; (3) proviral integration into host DNA; (4) viral DNA transcription; (5) mRNA translation; (6) assembly, budding, and maturation of virions.

HIV binding and entry into permissible cells is the first critical phase in the viral infection cycle and can be divided into four major steps. Initially, viral-host interactions are relatively non-specific. Here, viral envelope proteins (ENV or non-specific host cellular membrane proteins previously incorporated into the virion bilayer) attach to negatively charged cell surface proteoglycans [54]. As well, more specific interactions between HIV and $\alpha 4\beta 7$ integrins and/or dendritic cell-specific intercellular adhesion molecule-2-grabbing non-integrin (DC-SIGN) have been documented [55]. These non-canonical interactions facilitate viral ENV proximity with the primary host receptor, CD4. Next, gp120 binds to CD4 (Fig.2, step 2), using a specific CD4 binding site (CD4bs), which triggers a conformational change within gp120 causing its variable loop regions to rotate outward [34]. The third step involves gp120 attaching to co-receptor CCR5 or CXCR4 (Fig.2, step 3). Notably, HIV strains that use CCR5 as a co-receptor are classified as R5 virus, while virus strains that preferentially use CXCR4 are termed X4 virus [56,57]. Upon co-receptor attachment, the ENV protein ejects the transmembrane fusion protein, gp41, into the host cell membrane (Fig.2, step 4). The N-terminal peptides

of gp41 attach to the target cell and fuse the virus and host membranes [58] (Fig. 2; [56]. Once fusion has taken place, the virion capsid is released into the host cell [56]. Inside the host cell, the virus requires the use of its RT, which is transported inside the viral capsid, to transcribe its ssRNA into proviral dsDNA [59]. RT is one of the defining features of all retroviruses and is paramount to the variation of HIV virions. Reverse transcription is error-prone and lacks any proofreading function; therefore, during this step mutations frequently occur at a rate of about once every genome transfer [59]. The ability of the virus to diverge rapidly plays an important role in its ability to avoid stimulating the adaptive immune system and it quickly becomes resistant to ARV therapies [60].

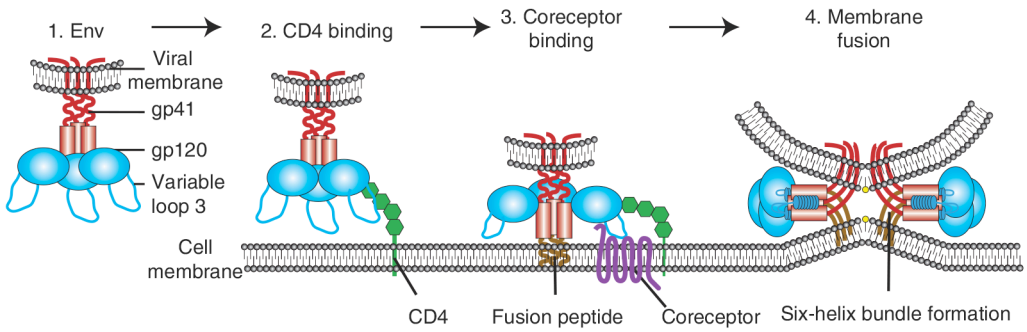

Figure 2. ***Viral Binding to Host Cell***. Overview of viral entry into permissible cell in which (1) HIV ENV (gp120 and gp41); (2) Attaches to host CD4; (3) ENV undergoes a conformational change allowing gp120 to bind to co-receptor (CCR5 or CXCR4); (4) gp41 inserts into the host membrane allowing fusion of both membranes. Figure adapted from Wilen *et al.,* 2012.

After RT, proviral cDNA is incorporated into the host DNA using the viral integrase (IN) enzyme, which is a necessary step in the retroviral infection cycle [33,60]. Once integrated, proviral DNA is transcribed, starting LTR sequence, which functions as a promoter [33]. The LTR contains the transcriptional promoter TATA box and two specificity protein (Sp) 1 transcriptional factor sites [61]. Despite these transcriptional promoters, viral transcription is not efficient without HIV's viral trans-activator protein (TAT), which significantly enhances viral transcription [62,63]. Notably, upstream of the viral promoter are transcriptional enhancers, NFκB, AP-1 and NFAT which, when activated following PAMP recognition, further promotes viral transcription [64]. After viral transcription, viral mRNA is exported to the cytoplasm and translated into three precursor proteins, Pr55(GAG), ENV, and GAG-POL polyproteins [33]

The assembly of new virions at the plasma membrane initiates the final stage of the viral infection cycle which is largely directed by Pr55GAG polyprotein [44,45]. During assembly, Pr55GAG polyproteins associate with the inner surface of the cell membrane along with ENV proteins and HIV genomic RNA [33]. Once this happens, the virion starts forming into an immature HIV capsid and buds from the host cell taking a portion of the host cell's phospholipid bilayer as its viral envelope [65]. After budding from the host cell, the virion matures as the Pr55GAG polyprotein is cleaved by viral PR, thus, resulting in the generation of structural proteins, p17 (MA), p24 (CA), p6 (NC) and p7 [63]. Together, these proteins produce a mature virion that is capable of infecting permissible cells.

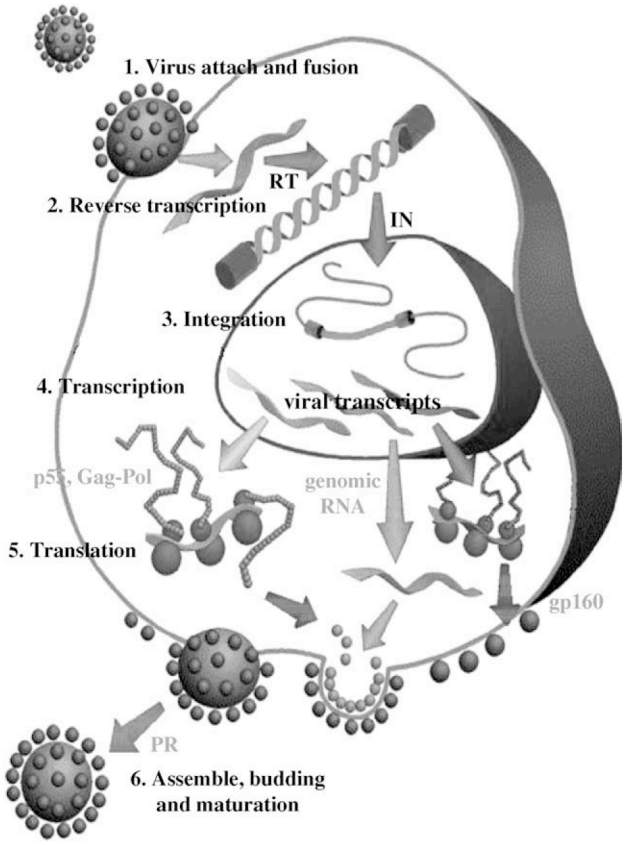

Figure 3. ***Viral Infection Cycle.*** The main steps of HIV replication include, (1) Viral binding, fusion, and capsid release into host cell; (2) Reverse transcription of viral RNA; (3) Integration of viral DNA into host genome; (4) Transcription of viral mRNA; (5) Translation of viral proteins; (6) Assembly, budding and maturation of new virions. Figure adapted from Sierra *et al.*, 2005.

1.3 Transmission

An estimated 2.7 million people transmitted HIV infection in 2010 resulting from viral exposure across mucosal membranes, from mother-to-child or direct percutaneous injection through intravenous drug use and hypodermic needle accidents [16]. The vast majority of transmission (80%) occurs through sexual contact when infected seminal or vaginal fluid comes into contact with mucosal membranes [66]. HIV transmission probability *per coital* act is extremely low overall; however, confounding factors can significantly increase acquisition incidence.

1.3.1 Confounding Factors

Sexually transmitted infections (STIs) that cause genital ulcers or local inflammation lead to significant increases in HIV acquisition in susceptible individuals [67]. These coexisting infections are thought to abrogate barrier defenses and permit virions to directly enter the mucosa. As well, STI-induced inflammation might attract permissible cells in the genital mucosa environment, thus leading to an increase in activated target cells and a two to 11-fold increase in HIV susceptibility [67]. Additionally, HIV-infected people who also have an STI shed more HIV-1 virus in semen and vaginal fluids [68,69]. Male circumcision lowers the risk of HIV acquisition by 60%, although the exact mechanism accounting for this is not clear. It has been proposed that removal of the foreskin, which is rich in Langerhans and dendritic cells (DCs), CD4+ T cells, and macrophages, might simply remove cells permissible from infection [70,71]. Moreover, circumcised men are less likely to have micro-tears in the penile mucosa

during intercourse compared to uncircumcised men [72]. A study showed women are significantly more likely to acquire HIV if they are pregnant or breastfeeding compared to non-pregnant and non-lactating women. This increased risk might be attributable to hormonal changes that likely affect the genital mucosa and/or immune response [73]. The clinical stage of HIV infection can increase the risk of transmission since acutely infected individuals often have the highest viral burden with little immune control, and highly infectious virus [74,75]. Importantly, the most accurate predictor of transmission is viral load. Indeed, in discordant couples, a 2.5-fold increase in transmission was documented when the HIV-infected partner's viral load went up 10-fold [76]. A similar effect has been documented with MTCT of HIV through breast milk (discussed in Section 3.1).

1.3.2 HIV Bottleneck and Founding Viruses

Although a chronically infected individual has a heterogeneous pool of HIV, only one or a few founder viruses are successfully transmitted to the susceptible individual [77]. The factors underlying this 'transmission bottleneck' are incompletely understood. In a study of 300 acutely infected individuals, approximately 80% of heterosexual subjects were infected by a single viral genome [78]. While approximately 68% of infants were infected by their mothers, 60% of men who have sex with men and 40% of intravenous drug users were infected by a single virus [79-84]. The selection of one founder virus remains unclear but is likely related to host biological mechanisms and/or the fitness of the virus itself [77]. It is well established, however, that founding viruses

are predominantly CCR5 tropic [85]. This phenomena is irrespective of the route of transmission and the fact that cellular targets for X4 tropic virus are readily available during acute infection [80]. Indeed, individuals with a CCR5 mutation, due to a 32 base pair deletion (known as CCR5Δ32), are largely protected from acquiring HIV infection [85]. Given the inefficiency of transmission and the overwhelming evidence that one founder virus establishes new infection in the host, further understanding of acute infection and founder virus remains an important objective to effectively prevent HIV transmission.

1.4 Course of Infection

The natural course of untreated HIV infection in adults is well characterized and described below in Fig. 4; however, very little is known about the course of infection in infants and children. Furthermore, the exact mechanism of transmission across the mucosal barrier (whether vaginal, penile, rectal, oral or gastrointestinal) remains poorly understood. Vaginal HIV transmission is the most widely investigated route of transmission and several routes across the mucosal barrier have been proposed based on *in vitro* and *ex vivo* experiments including direct infection of mucosal epithelium [86], passage through tissue micro-tears [87], viral capture by the dendrites of Langerhans' cells [88], passage between cells after loss of tight junctions [36], and transcytosis [89]. To my knowledge, only one publication has investigated routes of MTCT *in vivo* using a humanized mouse model in which breast milk completely inhibited oral transmission of HIV [90]. Importantly, with the advent of this model, the characterization of MTCT of HIV will likely be studied in greater detail in the future; however, this section (1.4 Course of Infection) relies on data available detailing the course of infection in adults and SIV infection in non-human primates.

1.4.1 Acute Infection

Acute infection, commonly referred to as the primary infection or the initial phase, occurs in the first three to six weeks following transmission and is characterized by an exponential increase in systemic viral load (up to 10^7copies/mL HIV RNA systemically) and destruction of substantial proportions of mucosal and systemic CD4+ T cells. During

this time, diseased individuals are highly infectious, however only about half of all acutely infected individuals exhibit general symptoms associated with acute retroviral syndrome (ARS) including fever, diarrhea, night sweats, and general malaise [91]. Evidence from macaque primate models of acute SIV infection indicate that once virus has crossed the mucosa, CD4+ T cells and Langerhans cells are the first viral targets [1,92], although other DCs likely play an important role in transferring virus via DC-SIGN [55]. Within the first few days after transmission, the high viremia facilitates widespread dissemination of HIV to lymphoid tissue, including the gut associated lymphoid tissue (GALT). It is here that the most substantial loss of CCR5+ CD4+ T cells occurs. Specifically, within the first three weeks of infection, up to 80% of CD4+ T cells are depleted from the gut and remain depleted evermore [93]. Despite the subsequent activation of DCs, natural killer (NK), NK T cells, and seroconversion, plasma viremia continues to rise, peaking at around 21-28 days post infection. Importantly, following peak viremia, substantial increases in immune activation occur [94,95](Fig. 4).

1.4.2 Asymptomatic Infection

The asymptomatic or chronic phase is characterized with the dramatic decline of viremia from the acute peak to a relatively stable level, termed the viral set point, in which CD8+ T cells are largely responsible for virus control [96]. Importantly, the viral set point has been shown to correlate with the rate of disease progression to AIDS, which in untreated infection, results in a slow, steady CD4+ T cell decline and CD8+ T cell exhaustion in an average of 2-7 years [95]. This disease phase is largely asymptomatic;

however, the immune system is irreparably damaged through persistent immune activation (discussed in Section 2.2), low-level virus production (viral reservoirs), dysregulated expression and responsiveness of TLRs [97,98], high CD4+ turnover [99], and CD8+ cell exhaustion, as evidenced by the presence of activation markers HLA-DR and CD38, which are considered the most predictive of disease progression [100]. Ultimately, this sustained immune activation leads to immune dysfunction and the onset of opportunistic infections that are characteristic of AIDS.

1.4.3 AIDS

The outcome of systemic CD4+ T cells counts below 200/mm^3 generally signifies the onset of AIDS [101]. Concurrently, there is a rapid rise in viremia (Fig. 4) and opportunistic infections begin to appear, most commonly *Pneumocystis pneumonia,* re-emergence of cytomegalovirus (CMV), and Epstein Barr Virus (EBV) [102]. Typically, these infections coincide with weight loss, fatigue, fever, and sweating and, if left untreated, will eventually lead to death.

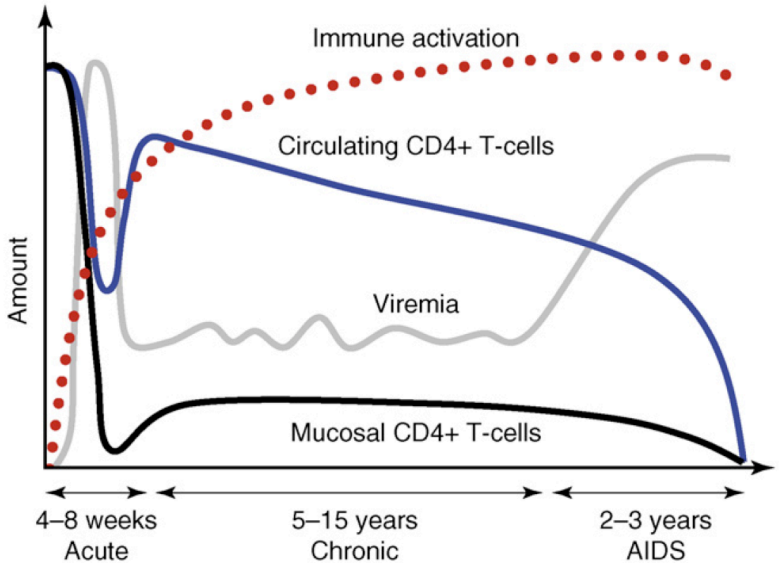

Figure 4. A schematic representation of untreated course of HIV infection. Adapted from Forman *et al.,* 2008.

2.0 Pattern Recognition Receptors and HIV Immunopathogenesis

There is a growing body of evidence that supports the idea that chronic immune activation drives HIV-specific immune dysfunction [95]; however, we still do not have a clear understanding of what fuels HIV immunopathogenesis. Historically, research has focused on HIV-specific adaptive immune responses, whereas the role of innate immune responses have been largely overlooked, despite the fact that it provides the first line of defense and shapes adaptive responses. Innate immune activation begins with the recognition of PAMPs leading to inflammatory and antiviral responses. The identification of HIV PAMPs that are recognized by innate pattern recognition receptors (PRRs) remain poorly elucidated, although they likely play a critical role in driving chronic immune activation. To complement our data identifying novel recognition of HIV structural proteins through TLR2, HIV PAMP-induced immune activation and infection studied in Chapters 4, this Section focuses on innate responses resulting from the recognition and associated immunopathogenesis specific to HIV infection.

2.1 Viral Recognition

Although our knowledge of HIV infection and pathogenesis continues to expand, our understanding of how HIV is recognized by the innate immune system remains poorly understood. Indeed, innate immune recognition of PAMPs by PRRs is essential for an effective host response against invading pathogens [103,104], including HIV. To date, the primary sensing model of HIV relies on the recognition of uridine-rich HIV ssRNA through endosomal and cytosolic PRRs [105]. Multiple families of intracellular, germline-encoded PRRs are responsible for triggering an innate immune response against HIV, including endosomal TLRs and cytosolic PRRs retinoic acid-inducible gene-like receptors (RIG-I) and myeloma differentiation-associated gene 5 (MDA5). Currently, a total of 10 TLRs have been identified in humans that have proven fundamental in our understanding of early recognition and subsequent activation of immune responses to many pathogens. The TLRs are categorized into two main categories: extracellular and endosomal. The extracellular TLRs (TLR1, 2, 4, 5, 6, and 10) characteristically recognize bacterial, fungal and parasitic PAMPs, whereas, endosomal TLRs (TLR 3, 7/8, and 9) sense viral dsRNA and ssRNA, respectively [105-107]. Cytosolic PRRs, RIG-I and MDA-5 RNA helicases are pivotal in the recognition of cytoplasmic viral RNA. Specifically, during HIV infection, RIG-I and MDA-5 sense HIV entry and translation, respectively [108]. Specific to HIV infection, DC-SIGN, a C-lectin-like receptor (CLR), have been shown to mediate antigen presentation between DCs and CD4+ T cells [55].

Although TLR2 is classically considered in the context of bacterial recognition, the breadth of ligand recognition has not been fully elucidated. Indeed, since it was first identified in 1998 [109], it has been shown to sense specific PAMPs from a variety of phyla including viruses, bacteria, fungi, and parasites [110]. The reason for this wide breadth of pathogen recognition comes from its unique ability to heterodimerize with other members of the TLR1 superfamily including TLR1, 2, and 10, and non-TLR cellular molecules [110,111]. The crystal structure for TLR2/1 and TLR2/6 has been determined in which the extracellular domains of each heterodimer forms an 'm'-shaped complex with specific bacterial ligands held in the crevice between the two TLRs [112]. Specifically, these structures indicate that TLR2/1 recognizes triacylated bacterial lipoproteins, while TLR2/6 senses diacylated bacterial lipoproteins [112]. In this way, the ligand is required for the heterodimeric interaction and downstream signaling [112,113]. Additionally, publications describe a TLR2/10 complex, although the ligand(s) and function of this heterodimer remains undetermined [114].

Of particular interest to our studies is the number of viral proteins that have been identified as PAMPs for TLR2 including those from cytomegalovirus (CMV) [115], herpes simplex virus (HSV) [116], hepatitis C virus (HCV) [117], and measles virus [118]. Specifically, CMV glycoproteins B and H have been shown to directly interact with the TLR2/1 heterodimer, leading to activation of NFκB, thereby initiating pro-inflammatory cytokine production which supports productive infection [115,119]. Similarly, a recent publication indicated that HSV glycoproteins gH/gL and gB co-

immunoprecipitated with TLR2, while only gH/gL led to downstream NFκB activation [116]. Furthermore, TLR2/1 and TLR2/6 were shown to be involved in sensing HCV core and NS3 proteins, respectively, which led to NFκB activation and significant increases in cytokine production in human macrophages and cell lines [117]. Importantly, TLR2-/- mice did not produce pro-inflammatory cytokines compared to wild type mice after exposure to HCV core and NS3 proteins [117]. Additionally, the hemagglutinin protein of measles virus significantly increased cellular activation in human monocytic cells but, more importantly, facilitated virus spread by increasing the surface expression of the measles receptor, CD150 through a TLR2-dependent mechanism [118]. Several HIV structural proteins have immunomodulatory functions (described in detail in Section 1.1) that are likely critical for immunopathogenesis. We recently showed that HIV proteins, p17, p24 and gp41 interacted with TLR2 leading to substantial increases in NFκB activation. Specifically, p17 and gp41 interacted with TLR2/1, while p24 was sensed by TLR2/6 (Henrick *et al*, 2013; submitted, Chapter 4). Taken together, these publications highlight an understudied function of TLR2 and its heterodimers as important extracellular PRRs for viral PAMPs recognition that lead to increased cellular activation and facilitate viral entry.

2.2 Chronic Immune Activation

Increasing evidence supports the notion that chronic immune activation is a fundamental driver and a hallmark of HIV-1. The mechanisms underlying chronic activation are believed to be primarily driven by translocation of bacterial and virus components from the gastrointestinal tract and low level viremia [99,120]. Indeed, increased lipopolysaccharide (LPS) levels in sera, a result of microbial translocation, strongly correlate with immune activation in chronically HIV-infected individuals [120]. However, we and others have proposed a direct contribution of HIV itself to immune activation. Indeed, the initiation of HAART therapy and subsequent reduced levels of viremia led to an almost immediate decline in correlates of immune activation [98]. Furthermore, a number of laboratories, including ours, showed that multiple HIV proteins can directly activate cells *in vitro* leading to increased production of pro-inflammatory cytokines, significantly increased co-receptor (CCR5) expression and HIV infection [36,38,121,122] (Henrick *et al.*, 2013; submitted, Chapter 4). It is unlikely, however, that immune activation is exclusively dependent on viral load, since natural simian immunodeficiency virus (SIV) hosts have limited immunopathology despite high levels of viremia [123].

Regardless of the exact antigenic stimulation, however, persistent immune stimulation during HIV infection leads to immune exhaustion, a phenomenon where T cells become dysfunctional, lose their proliferative capacity, and become functionally unresponsive to further antigenic stimulation [124]. Several cellular and serum markers

have been identified to quantify the level of T cell activation, including CD38, HLA-DR, CD25, CD69, CD70, neopterin, and β_2-microglobulin [125-127]. However, the most accurate marker of T cell activation, CD38, is up regulated during early T cell activation, correlates with increased cytokine production, and CD4+ proliferation [95,127]. Furthermore, several markers of immune exhaustion have been identified, including programmed death-1 (PD-1), lymphocyte activating gene-3 (LAG-3) and T cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3) [124]. Over time, chronic immune activation leads to immunosenescence and overall immune deficiency, thus rendering the individual incapable of developing an immune response to opportunistic infections that eventually lead to death.

2.3 Regulation of TLR Mediated Immune Activation

Since the discovery of PRRs, research primarily focused on the engagement of PAMPs that trigger innate immunity and enhanced adaptive immune responses against pathogens; however; alternatively control of aberrant immune activation and signaling are equally important. Without proper regulation, PRR activation can lead to undesirable consequences and, indeed, overactivation of TLRs is directly involved in the pathogenesis of autoimmune diseases and plays a crucial role in chronic activation particular to HIV disease (discussed in Section 2.2). Multiple stages of intrinsic extracellular and intracellular regulatory mechanisms balance TLR-dependent immune responses. Indeed, extracellular regulatory mechanisms include the production of soluble TLRs (sTLRs) that act as decoy receptors and inhibit TLR-PAMPs engagement. Furthermore, once TLR-ligand interaction occurs there are multiple intracellular regulators that inhibit signaling pathways, including negative feedback loops, downregulation of TLR expression, degradation of TLR proteins or, as a last resort, activation-induced cell death. Although intracellular mechanisms play an important role in regulating cellular activation, to thoroughly understand and compliment the data defining sTLR2 and its role in regulating HIV-induced activation and infection as it pertains to the manuscripts presented in Chapters 2 and 3, this section will focus on soluble TLRs.

sTLRs provide the most direct attenuation of innate immune responses to pathogens by binding to PAMPs before they engage membrane-bound TLRs, thus effectively inhibiting PAMP-PRR engagement [107]. To date, four sTLRs have been

identified in humans (sTLR1, sTLR2, sTLR4, and sTLR6), and primarily have been identified for their role in inhibiting Gram-negative and Gram-positive bacterial-induced cellular activation. LeBouder *et al.* (2003) were the first to identify forms of sTLR2 in breast milk and plasma. Computational molecular docking revealed that a cylindrical N-terminus to C-terminus arrangement between sTLR2 and soluble CD14 (sCD14) encapsulated synthetic bacterial lipoprotein, Pam_3CSK_4, thus, preventing bacterial-induced cellular activation through membrane-bound TLR2 [128]. Furthermore, other publications have highlighted sTLR2's role in significantly inhibiting bacterially-induced pro-inflammatory cytokine production *in vitro* in oral epithelial cells, placental tissue explants and human intestinal epithelial cells [129-131]. As well, sTLR2 significantly reduced bacterially-associated inflammation in mice without impairing microbial clearance [132]. Together, these publications indicate that sTLR2 is critically important in regulating bacteria-induced cellular activation.

sTLR2-dependent regulation of immune activation during virus infection remains poorly understood. However, accruing evidence indicates that the immune system uses a range of soluble molecules, including defensins, anti-proteases, IFNs, and chemokines to suppress and control viral infections [133,134]. Indeed, elafin/trappin-2 has been shown to directly interfere with viral PAMPs/host engagement, thus modulating immune responses [135]. Likewise, we showed that sTLR2 directly interacted with HIV PAMPs, including p17, p24, and gp41, leading to significantly reduced NFκB activation, IL-8 production, CCR5 expression and HIV-infection in a dose-dependent manner.

Importantly, sTLR2 levels were significantly increased in HIV-infected compared to uninfected breast milk samples, and significantly correlated with p24 (a marker of disease progression) [47], Henrick *et al.*, 2013b; submitted). Furthermore, *in vitro* mammary epithelial cells (MECs) exposed to HIV PAMPs induced production of sTLR2*,* indicating that the breast might provide a local innate compensatory response to HIV-1-induced activation and infection. Taken together, sTLR2 plays a dual role in regulating immune activation by directly inhibiting bacterial and virus PAMP engagement with TLR2. In this way, it acts as a decoy receptor and is crucial in regulating aberrant immune activation.

3.0 Mother-to-child HIV Transmission

The vast majority of the 2.5 million children that are infected with HIV globally acquire the disease from their HIV-infected mother *in utero*, *intrapartum*, and/or *postpartum* through breastfeeding. Ninety percent of all HIV-infected children globally live in sub-Saharan Africa and have very limited access to antiretroviral therapies [16] which likely plays a major role in the poor life expectancy of HIV-infected children. Statistics indicate that one in three newborns infected with HIV will die before the age of one, over one half of these will die before reaching their second birthday, and the vast majority of infected children will die before they are five years old [18]. The risk of MTCT is strongly associated with the mother's disease status (including viral load), fetal exposure to infected maternal fluids, and breastfeeding.

In the absence of any preventative intervention, MTCT of HIV occurs in 11-42% of all HIV-infected-mother-infant dyads [136,137]. Taken statistically, approximately 1000 children acquire HIV infection from their HIV-infected mothers every day [16]. To comprehensively understand the data defining sTLR2 inhibition of cellular activation and HIV infection described in Chapters 2 and 3, postnatal HIV MTCT through infected breast milk will be described in greater detail than *in utero* and *intra-partum* transmission.

The benefits of breastfeeding for infants are undeniable due largely to the unique composition breast milk, which delivers complete nutrition as well as provides passive protection through maternal immunological factors, and imparts tolerance to beneficial

gut microbiota. Indeed, breastfeeding is well recognized to protect against gastrointestinal and respiratory infections, diarrheal diseases, and provides long-term health benefits to the infant [138,139]. In resource poor areas, breastfeeding is particularly important since replacement feeding is cost prohibitive and access to clean water needed to hydrate powdered formula milk is limited. Therefore, the risk of acute, life-threatening diseases from formula feeding outweighs the danger associated with HIV-infected breastfeeding. Thus, the WHO recommends that women, despite their HIV status, exclusively breastfeed their infants for the first six months postpartum if adequate and sustainable replacement feeding is not available [140]. Furthermore, the immediate benefits of breastfeeding extend to mothers and families as it promotes child spacing, social acceptance for women and is cost effective [141,142].

However, breast milk also provides a medium for cell-free and cell-associated HIV transmission to the breastfeeding infant. Indeed, it is estimated that an infant can ingest up to 228,000 copies/mL of cell-free virus (CFV) and 12-58 HIV-infected cells/10,000 breast milk cells [143,144] when breastfeeding from their HIV-infected mother. Additionally, the amount of CFV and cell-associated virus (CAV) in breast milk is strongly dependent on the systemic viral burden and immunosuppression status of the mother (predicted by CD4+ T cell level) [145]. Importantly, ARV therapy significantly reduces viral burden in breast milk but is often not available in resource poor areas for the duration of breastfeeding [20].

The exact mechanism of postnatal MTCT of HIV transmission remains unclear. However, several laboratories have shown virus production in cells common to the mammary gland, including MECs [79], CD4+ cells [146], and breast milk macrophages [147]. Multiple target sites in the infant's gastrointestinal tract, including the oral, esophageal, and intestinal mucosal epithelium have been proposed. Specifically, the oral epithelium has been shown to be permissive to both CFV and CAV *in vitro* [148]. Yet, the oral environment also has strong anti-HIV properties [149] making MTCT HIV transmission possible, albeit at a very low incidence [148]. Moreover, a recent publication describing an *in vivo* infection model demonstrated that humanized mice were readily infected with HIV through the oral cavity; however, breast milk had strong inhibitory effects on both CFV and CAV [90]. A more compelling argument has been made for MTCT HIV transmission through the infant's intestine since multiple studies have shown that mixed feeding doubles the risk of an infant acquiring the disease compared to exclusively breastfeeding [22-25]. Indeed, we showed that bacterial ligands increased cellular activation in intestinal cells [131]. Furthermore, HIV transcytosis from apical to basolateral poles of intestinal epithelial cells was demonstrated *in vitro* [150] and gastric pH, which is neutral at birth, [151] likely would not deactivate CFV or CAV. Although multiple immune factors in breast milk have been shown to correlate with protection or directly inhibit HIV infection *in vitro*, the fact remains that increased viral burden can obviate host defenses to establish infection in the infant. Thus, prevention strategies that reduce breast milk virus levels will ultimately be the most effective in reduction MTCT of HIV.

3.1 Anatomy of the Mammary Gland

Mammary glands are thought to have evolved from the innate immune system and the nutritional value of milk developed later [152]. Each breast is considered a mammary gland and contains 15-20 lobes. Each lobe contains differentiated lobules of grape-like clusters of milk-secreting glands known as alveoli. These alveoli contain MECs that are responsible for the production of milk. Once the milk has left the alveoli, it travels towards the mammary ducts with the help of myoepithelial cells and is propelled towards the nipple upon stimulation through suckling [153]. Mammary glands also contain high numbers of leukocytes (discussed in Section 3.6.2) that migrate to the breast from the intestine during the later stages of pregnancy. These cells reside within mammary glands during lactation or traffic paracellularly or transcellularly through the normally impermeable MEC monolayer into the milk [154].

3.2 Risk Factors

The maternal viral load is the most consistent predictor of vertical transmission among all stages of pregnancy, delivery, and breastfeeding [144,155,156] (Fig. 5). Indeed, the highest rates of transmission are noted in women who have viral loads of more than 100,000 copies/ml in their blood, while MTCT is relatively rare when the mother's viral load is undetectable [157]. A low maternal CD4+ T cell level [158], an absence of ARV therapy [159], the use of tobacco and illegal drugs (particularly cocaine) [160] and untreated STIs [161] are associated with higher rates of MTCT of HIV. Also, infants who have a prolonged exposure (> 4 hours) to ruptured placental membranes [162], are premature, have a low birth weight [163], are vaginally delivered and/or are exposed to HIV-infected breast milk greater than six months are more likely to acquire the disease from their HIV-infected mothers [164]. Furthermore, specific breast pathologies, including mastitis and nipple cracking are associated with significantly increased postnatal HIV transmission [165-167]. More recently, it has been documented that weaning, which increases breast milk viral load significantly correlates with higher incidence of MTCT [168]. Therefore, preventative strategies that can control risk factors would play a significant role in reducing MTCT of HIV.

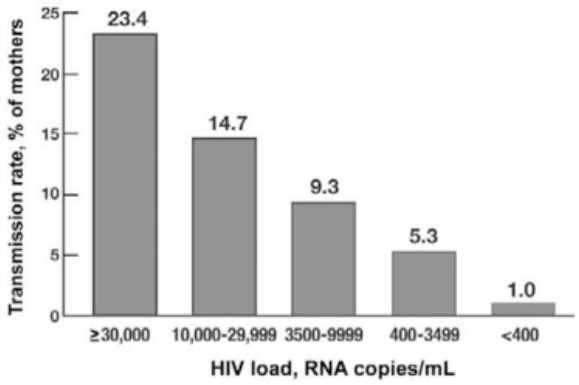

Figure 5: A schematic graph identifying the association between maternal plasma HIV RNA levels and risk of mother-to-child transmission of HIV. Figure is adapted from Cooper *et al.,* 2002.

3.3 Viral Determinants of HIV transmission through breast milk

Our understanding of transmitted/founder viruses in breast milk is not clearly defined [169,170]. Similar to other mucosally-transmitted/founder viruses, post-natal acquisition is primarily CCR5-tropic [169]. Phylogenetic comparison of milk and plasma ENV sequences revealed that monotypic viruses are significantly more common in milk as compared to plasma from the same mother [169,170], thus suggesting that the majority of breast milk viruses are produced by infected cells of the mammary gland. Conversely, other studies suggest that virus variants found in the breast milk and plasma of infected mothers are genetically similar [171]. Therefore, a hypothesis to explain these divergent observations is that the breast is continuously replenished with systemic CFV or CAV that can readily be transmitted to the breastfeeding infant and/or undergoes local replication in the mammary compartment [169].

Despite our increasing knowledge of breast milk virus, it remains unclear whether CFV or CAV is responsible for HIV acquisition in the infant. Indeed, both CFV HIV RNA and CAV proviral DNA can be found in HIV-infected breast milk (when the mother is not receiving ARV therapy), and both levels correlate with breast milk MTCT of HIV [172,173]. Importantly, it has been shown that multiple immune factors that are endogenous to breast milk, including mucin, secretory leukocyte proteinase inhibitor (SLPI), sTLR2, lactoferrin, lysozyme, and oligosaccharides can effectively inactivate CFV infection *in vitro* [131,149,174-176], whereas innate immune factors seemingly have little to no affect on CAV infection *in vitro* [176,177]. Indeed, CAV HIV infection

has been shown to be more efficient compared to CFV infection [178], and is significantly more difficult to neutralize [179], thus indicating that CAV may be responsible for transmission. Conversely, ARV therapy significantly decreases HIV RNA and correlates with reduction in breast milk transmission rates [180,181], while proviral DNA levels remain largely unaltered [143,182]. These observations suggest that CFV likely plays an important role in breast milk transmission. Given these contradictory studies, it could be argued that multiple factors including overall maternal viral load, breast health (e.g. mastitis), immune factor levels, as well as feeding practices, all contribute to the founding virus infection in the infant.

3.4 Prevention of Mother-to-Child HIV Transmission through Breast Milk

Without intervention, about 11-42% of infants breastfeeding from HIV-infected mothers will acquire HIV through breast milk [136,137]. However, recently, significant progress has been achieved in the prevention of MTCT (PMTCT) of HIV globally through improved access to ARV therapies for pregnant, birthing, and lactating women. For the first time, the elimination of MTCT of HIV is considered a realistic public health goal [19].

3.4.1 Antiretroviral Therapies

In low-and-middle-income countries, prenatal and perinatal ARV therapies, namely single dose neviripine (NVP) have been used for almost 15 years. This preventative therapy, given to the mother during delivery and the infant within 72 hours *postpartum,* has proven effective and has undoubtedly played an important role in the dramatic decrease of 800,000 cases of MTCT of HIV in 2002 to 300,000 cases in 2011 [16]. Due to its success, in 2010 the WHO created new guidelines for increased ARV therapy use in pregnant and breastfeeding mothers globally in which they recommend the following:

Maternal CD4+ T cell count:

- <350 mm3: Highly activate antiretroviral therapy (HAART) for life.
- >350 mm3: Zidovudine (AZT) prenatal and NVP *peripartum* or HAART through duration of breastfeeding.

Infant:

- NVP for 6 weeks.

However, in 2011, only 57% of pregnant or lactating HIV-infected women were receiving any ARV therapy [16] due to the cost, lack of health care workers and inconsistent supply [183]. Given the success of ARV therapy, greater resources and infrastructure in resource-poor areas could effectively eliminate MTCT of HIV.

3.4.2 Exclusive Breast Feeding

It is well accepted that exclusive breastfeeding is advantageous to babies who are born to healthy HIV-uninfected mothers. This is associated with decreased risk of diarrheal and respiratory infections in developed and developing nations. Furthermore, it facilitates normal production of milk leading to decreased breast pathologies, including mastitis [184], and correlates with long-term benefits for both mother and child (discussed in Section 3.5). However, for HIV-infected women, breastfeeding becomes uncertain since breast milk can contain high levels of HIV (discussed in Section 3.0). Indeed, the method of infant feeding undoubtedly correlates with postnatal MTCT of HIV. Paradoxically, exclusive breastfeeding significantly decreased postnatal MTCT of HIV compared to mixed feeding or non-exclusive breastfeeding despite the prolonged and repeated exposure to CFV and CAV [22,24,25,185]. Specifically, in four large cohort studies, exclusive breastfeeding reduced HIV MTCT of HIV by 4-10 fold compared to non-exclusive breastfeeding or mixed feeding. Kuhn *et al.* (2007) showed that non-exclusive breastfeeding more than doubled the risk of postnatal HIV transmission, while Iliff *et al.* (2005) showed transmission rates as low as 1.3 % in women who were exclusive breastfeeding up to 6 months [22,25]. Importantly, there

were no significant differences in disease severity between the mothers and, when confounding factors including maternal viral load, CD4+ T cell levels, STI screening and low birth weight were taken into account, the differences between transmission rates of exclusive breastfeeding and non-exclusive breastfeeding remained significant [22,23,25]. Indeed, this preventative method is so effective in the reduction of MTCT HIV transmission, as well as protection against enteric infections, the WHO promotes exclusively breastfeeding despite the HIV status of the mother when safe and sustainable alternative feeding is unavailable [140]. Although exclusive breastfeeding decreases vertical transmission, the mechanism underlying this protection remains unclear.

3.5 The Role of Breast Milk Components in Mother-to-Child Transmission of HIV

Neonates are immunologically naïve when they are born and, thus, are highly vulnerable to opportunistic infections early in life. Numerous reports, originating with Grulee *et al* (1935), have highlighted the beneficial effects of breast milk in providing passive immunity to the infant against a wide range of environmental pathogens [186-193]. Indeed, gastrointestinal and respiratory infections are significantly less common in breast fed infants compared to formula fed infants [138]. Moreover, breast fed infants benefit from long term effects including markedly reduced incidence of type 1 diabetes, multiple sclerosis, eczema, Crohn's disease, rheumatoid arthritis, inflammatory bowel diseases and allergies when compared to non-breast fed infants [138,139]. Such protective effects are incompletely understood yet are thought to be a result of the ingestion of a milieu of innate, adaptive, and non-immune factors with direct antimicrobial, anti-inflammatory and immunomodulatory functions in breast milk (Fig. 6). To complement the data in Chapter 2 and 3 defining sTLR2-dependent reduction in bacterial and viral-induced cellular activation and direct inhibition of HIV infection, this Section will focus on maternal immune factors in breast milk that have anti-HIV properties.

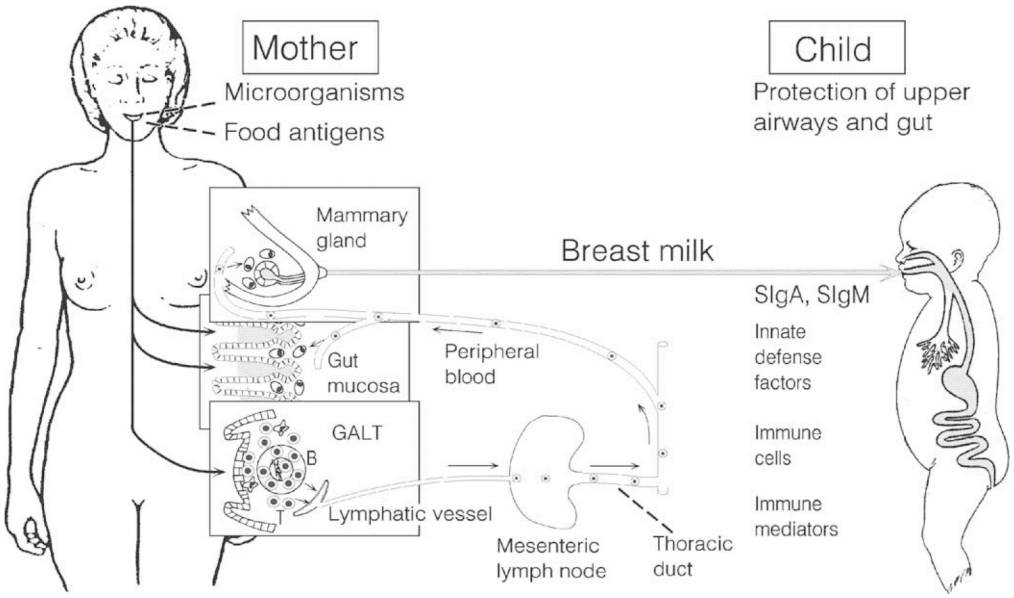

Figure 6: Schematic representation of the integration of maternal immunity with breastfeeding infant with an emphasis on migration and transfer of maternal leukocytes through breast milk. Figure adapted from Brandtzaeg *et al.,* 2003.

3.5.1 Non-cellular components

A number of non-cellular factors in breast milk have been attributed to the breastfeeding infant's protection from HIV, including innate factors, cytokines and oligosaccharides [149,176,194], whereas data are conflicting for other factors such as HIV-specific antibodies [195,196]. Many breast milk factor levels correlated with protection from MTCT of HIV and/or have direct anti-HIV function *in vitro.* Here, I discuss particular innate factors that were evaluated or directly relate to our studies including lactoferrin, secretory leukocyte protease inhibitor (SLPI), mucin and sTLR2 all of which have documented HIV inhibitory properties [131,197-199]. Specifically,

lactoferrin has been shown *in vitro* to bind to the V3 loop of gp120, thus inhibiting gp120 interaction with host CD4 receptor [192], and breast milk lactoferrin levels have been shown to correlate with reduced MTCT of HIV [200]. Moreover, lactoferrin has been shown to inhibit bacterial-induced inflammation [201,202]. Similarly, SLPI levels correlated with decreased MTCT HIV transmission through breast milk [203], and *in vitro* studies have indicated that it interacts with target cells to inhibit viral entry [204]. Mucin 1 (MUC1), which is abundant in breast milk, has been shown to inhibit HIV infection *in vitro* by preventing DC-SIGN-mediated transmission of HIV from DCs to activated CD4+ T cells [197]. Breast milk oligosaccharides have little to no nutritional value for the infant; however, they provide a prebiotic for the growth of commensal bacteria, and have been shown to act as soluble decoy receptors to inhibit pathogen adhesion [205]. One study identified that HIV-infected mothers with higher than median levels of oligosaccharides were less likely to transmit HIV through breast milk [205]. Additionally, we recently reported that breast milk sTLR2 directly interacts with specific structural proteins, namely p17, p24, and gp41, thus inhibiting cell-free HIV infection *in vitro* [131]. Furthermore, sTLR2 has known anti-microbial properties that significantly inhibit pro-inflammatory cytokine production in human intestinal epithelial cells, as well as reducing bacterial-associated inflammation in mice without impairing microbial clearance [131,132]. These factors function concomitantly to control aberrant microbial-induced inflammation as well as inhibit HIV-host interaction and, thus, are critical in protecting breastfeeding infants from acquiring HIV through breast milk.

Breast milk contains a range of cytokines, some of which could potentially influence immune function and directly correlate with MTCT HIV transmission. Specifically, the pro-inflammatory chemokine ligand 5 (RANTES/CCL5) indirectly inhibits HIV infection *in vitro* by binding to its ligand CCR5, thus inhibiting gp120 to binding to its co-receptor which is integral to host-viral attachment [206]. However, RANTES levels in breast milk positively correlated with increased MTCT of HIV [207]. Conversely, levels of breast milk interleukin (IL) 15, a pleotropic cytokine involved in activating CD8+ T and NK cells, positively correlated with protection from MTCT of HIV [208]. Furthermore, we showed a positive correlation of sTLR2 and IL-15 levels in breast milk (Henrick *et al.*, 2013b; submitted, Chapter 3), thus indicating that these protective factors can function in concert to reduce MTCT of HIV.

3.5.2 Cellular Components

The biological relevance of breast milk cells in MTCT of HIV remains unclear. Indeed, there are arguments that infected cells both facilitate and protect against HIV transmission in breastfeeding infants [147,209,210]. Depending on the stage of lactation, the predominant cells types in milk consist of a variety of leukocytes in colostrum ($4x10^6$/ml) and mature breast milk (10^5-10^6/ml) and MECs. The majority of leukocytes in breast milk are of an activated phenotype [211] and are comprised of macrophages (55-60%) and neutrophils (30-40%), while 5-10% are lymphocytes (~65% CD8+ T cells, 15% CD4+ T cells, 20% B cells) [212-214]. Macrophages and MECs are thought to facilitate MTCT of HIV. First, macrophages comprise the majority of leukocytes in breast milk

[212], are readily infected with HIV, and express DC-SIGN, a DC-specific receptor for HIV, that facilitates HIV infection *in vitro* [147]. In addition, oral administration of macrophages to newborn mice survived several hours and were found in the neonatal intestine [210]. Second, MECs make up a substantial portion of all of the cells in breast milk [215]. Importantly, these cells express several canonical HIV receptors, including CD4 and CCR5, readily endocytose cell-free HIV and can act as a viral reservoir [79,216]. Conversely, however, a recent publication reported that breast milk HIV-gag-specific interferon gamma (IFNγ) cellular responses correlated with decreased MTCT of HIV [209]. Taken together, the role of breast milk cells in MTCT of HIV remains vague.

4.0 Hypothesis & Objectives

Hypothesis

Based on the evidence presented above, we hypothesized that sTLR2 plays a pivotal role in preventing mother-to-child HIV transmission through breast milk by inhibiting cellular activation and HIV infection. If sTLR2 inhibits HIV cellular activation and infection, then we further hypothesize that TLR2 recognizes HIV structural proteins leading to increased cellular activation and HIV infection.

Objectives

The research presented in Chapters 2 and 3 was designed to test our hypotheses and expand our knowledge regarding the direct anti-HIV properties of sTLR2 and the potential mechanism in inhibiting HIV infection. Furthermore, we investigated the role of TLR2 in recognizing HIV specific structural proteins and the role viral-induced cellular activation played in infection.

In the first manuscript presented in this thesis (Chapter 2), we characterized forms and concentrations of sTLR2 in breast milk from HIV-uninfected women, determined sTLR2 kinetics, its cellular source and determined its effect on bacterially-induced inflammation and HIV-infection. To accomplish this, breast milk was collected at one week and one, three, and six months postpartum and fractions were separated (lipid, supernatant, and cell layers). Supernatant layers from each sample were evaluated for

sTLR2 forms, concentration, degradation, and expression kinetics over time postpartum. Furthermore, breast milk cells were evaluated for production of sTLR2. We used human embryonic kidney cells (HEK293) stably transfected with TLR2, the monocyte cell line (U937) and a human intestinal cell line (Caco-2) to test whether mock-depleted or sTLR2-depleted HIV-uninfected breast milk could inhibit Pam_3CSK_4-induced inflammation. Also we specifically determined whether mock or sTLR2-depleted breast milk could inhibit HIV infection in a HIV infection indicator assay (CD4, CCR5, and CXCR4 stably transfected cervical epithelial cells (TZMbl)). We focused on the sTLR2-specific inhibition of pro-inflammatory, IL-8, production after TLR2 ligand, Pam_3CSK_4, challenge and the inhibition of HIV-infection. Here we identified novel predominant forms of sTLR2 in breast milk that closely resembled forms previously reported in saliva and amniotic fluid [129,130]. Furthermore, we determined that these forms were quickly degraded at room temperature and were produced, at least in part, by MECs. Functionally, our studies showed that sTLR2 inhibited Pam_3CSK_4-induced inflammation in intestinal cells and, for the first time, that sTLR2 directly inhibited cell-free R5 HIV infection *in vitro*.

In the second manuscript (Chapter 3), we built on findings from Chapter 2. Here, we investigated the mechanism by which sTLR2 inhibited HIV-induced cellular activation and infection *in vitro*, evaluated sTLR2 levels in HIV-infected and uninfected breast milk, as well as TLR2 expression levels from breast milk cells. We determined TLR2 expression in HIV-infected and HIV-uninfected breast milk cells. Furthermore, we

evaluated sTLR2 levels in HIV-uninfected and infected breast milk and its correlation to HIV disease progression and pro-inflammatory markers. *In vitro,* we investigated sTLR2's ability to interact with specific HIV structural proteins, decrease HIV-induced NFκB activation, IL-8 production, CCR5 expression, and HIV infection. The data reported in Chapter 3 showed a possible mechanism by which sTLR2 directly inhibited HIV-induced NFκB activation and IL-8 expression in dose-dependent manner through direct interaction with HIV structural proteins, including p17, p24, and gp41. Additionally, we evaluated breast milk cells for TLR2 expression and sTLR2 concentration in uninfected and HIV-infected breast milk and found significantly increased levels in HIV infected compared to uninfected women. sTLR2 concentrations correlated with both p24 and IL-15 levels in breast milk and could be induced *in vitro* in MECs and macrophages after exposure to HIV structural proteins. These findings indicated novel mechanisms by which sTLR2 played a critical role in inhibiting MTCT of HIV.

Based on the data collected in our first and second manuscripts (Chapters 2 and 3) that indicated that sTLR2 directly interacted with specific HIV structural proteins, we sought to investigate whether TLR2 recognized HIV and led to increased cellular activation and HIV-infection. Here, we stably-transfected the well-characterized HIV infection indicator assay, TZMbl, with TLR2 (TZMbl-2) and evaluated the differences in direct HIV structural protein interaction, HIV-specific NFκB activation, tumour necrosis factor alpha (TNF-α) and IL-8 production and HIV infection rates between the two cell

lines. In this study, we also identified specific TLR2 heterodimers that interacted with particular HIV structural proteins and led to cellular activation. Specifically, we demonstrated that HIV structural proteins, including p17, p24, and gp41 act as viral pathogen-associated molecular patterns (PAMPs) that are recognized by TLR2 and its heterodimers leading to significantly increased NFκB activation and IL-8 production. TLR2/1 heterodimer recognized p17 and gp41, while p24 signaled through TLR2/6. However, p24 blocked p17 and gp41-induced cellular activation in a TLR2-dependent manner, thus providing a novel mechanism by which HIV can manipulate extracellular innate sensing. Importantly, TLR2-dependent cellular activation led to significantly increased CCR5 expression in macrophages, which mechanistically explains why HIV integration was significantly increased in TZMbl-2 cells compared to TZMbl cells. Our results identify, for the first time, novel HIV PAMPs that play a role in cellular activation via TLR2, and showed that cellular TLR2 expression significantly increased HIV infection/integration. These results have important implications for our fundamental understanding of HIV immune activation and immunopathogenesis.

—CHAPTER 2 —

Milk Matters: Soluble Toll-like Receptor 2 (sTLR2) in Breast Milk Significantly Inhibits HIV-1 Infection and Inflammation

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This manuscript investigated multiple forms of sTLR2 in HIV-uninfected breast milk and their effect on synthetic bacterial-ligand induced inflammation and HIV-1 infection *in vitro.* To better understand breast milk sTLR2's role in inhibiting inflammation and directly inhibiting HIV-1 infection, we utilized immunodepletion assays to remove sTLR2 from breast milk. From this study, we discovered that sTLR2 concentrations differ dramatically among women and specific forms decrease over time postpartum. Additionally, it was determined that sTLR2 is an essential innate immune factor critical to the inhibition of bacterial ligand induced inflammation. Furthermore, for the first time, our data strongly suggested that sTLR2 inhibits HIV-1 infection *in vitro.* The results of these experiments showed that breast milk sTLR2 played a dual role in inhibiting inflammation and HIV-1 infection *in vitro.*

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Milk Matters: Soluble Toll-Like Receptor 2 (sTLR2) in Breast Milk Significantly Inhibits HIV-1 Infection and Inflammation

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Abstract

The majority of infants who breastfeed from their HIV-positive mothers remain uninfected despite constant and repeated exposure to virus over weeks to years. This phenomenon is not fully understood but has been closely linked to innate factors in breast milk (BM). Most recently we have focused on one such innate factor, soluble Toll-like receptor 2 (sTLR2) for its significant contribution as an inhibitor of inflammation triggered by bacterial and viral antigens. We hypothesized that sTLR2 in BM inhibits immune activation/inflammation and HIV-1 infection. sTLR2 protein profiles were analyzed in HIV-uninfected BM and showed dramatic variability in expression concentration and predominant sTLR2 forms between women. sTLR2 immunodepleted BM, versus mock-depleted BM, incubated with Pam_3CSK_4 lead to significant increases in IL-8 production in a TLR2-dependant fashion in U937, HEK293-TLR2, and Caco-2. Importantly, TLR2-specific polyclonal and monoclonal antibody addition to BM prior to cell-free R5 HIV-1 addition led to significantly ($P<0.01$, $P<0.001$, respectively) increased HIV-1 infection in TZM-bl reporter cells. To confirm these findings, sTLR2-depletion in BM led to significantly ($P<0.001$) increased HIV-1 infection in TZM-bl cells. Notably, immunodepletion does not allow for the complete removal of sTLR2 from BM, thus functional testing shown here may underestimate the total effect elicited by sTLR2 against HIV-1 and synthetic bacterial ligand. This study provides evidence for the first time that sTLR2 in BM may provide a dual protective role for infants breastfeeding from their HIV-infected mothers by; (1) immunomodulating pro-inflammatory responses to bacterial ligands, and (2) directly inhibiting cell-free HIV-1 infection. Thus, sTLR2 in BM may be critical to infant health and prove beneficial in decreasing vertical HIV-1 transmission to infants.

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Introduction

Breast milk (BM) is unique in its ability to fulfill infant nutritional requirements and, arguably more importantly, protect the newborn from environmental and infectious agents during the early stages of life. This protection comes as a complex milieu of innate and adaptive immune components in BM including maternal antibodies, immune cells and innate antimicrobial and immunomodulatory factors [1]. Importantly, many uncharacterized innate factors in BM have not had their functions fully elucidated, although they undoubtedly cooperate to protect the immunologically naïve infant. Numerous publications, originating in 1935 by Grulee *et al.*, [1] have correlated the beneficial role of breastfeeding with significant decreases in infant morbidity and mortality. More contemporary publications show decreased prevalence of specific childhood diseases, including respiratory and gastrointestinal infections, particularly in developing nations [2–4], making exclusive breastfeeding (EBF) an optimal option for most mothers. Notwithstanding, when a mother is HIV-positive a dilemma arises since there is a risk of vertical HIV- transmission through BM. Paradoxically, however, a number of cohort studies have shown significantly reduced HIV-transmission rates when HIV-infected mothers exclusively breastfed (EBF) compared to mothers who mixed fed their infants [5–8]. This phenomena is not fully understood but may be linked to short-lived innate factors in BM that contribute to both reduced immune activation and inhibition of R5 HIV-1 [9,10], the predominant phenotype transmitted from mother-to-child [11–14]. Importantly, activation of the immune system, namely inflammation, is a strong prognostic marker of HIV infection [15], and has been associated with increased HIV acquisition risk caused by activation in the

genital tract due to pre-existing sexually transmitted infections (STI) [16–19].

Toll-like receptors (TLRs) are innate sensing molecules, known as pattern-recognition receptors (PRRs), that sense 'danger' signals or pathogen associated molecular patterns (PAMPs) [20]. TLR2 has a special place among all TLRs in its well characterized recognition of a large breadth of pathogens, including bacteria, viruses, fungi, mycobacteria, and parasites [21]. In contrast to the majority of TLRs, TLR2 has a soluble form (sTLR2) that is produced through post-translational modification [22,23]. It was first shown to be present in high concentration in BM [23] and was further described in various natural sTLR2 forms in amniotic fluid and saliva with significant immunomodulatory functions to known TLR2 agonists [22–26].

In the present study, we confirm and extend our understanding of BM sTLR2 as an important immunomodulator of PAMP-induced proinflammatory cytokine production in the nursing infant's intestinal mucosa [24]. Further, we demonstrate, for the first time, that BM sTLR2 plays a dual role in inhibiting R5 HIV-1 infection and inflammation, which may prove important in understanding reduced transmission rates to EBF infants from HIV-infected mothers.

Methods

Study Cohort and Breast Milk

study was approved by McMaster Research Ethics Board (REB Approval #08-176), and the CCI of Children's Hospital, Los Angeles. All participants provided voluntary written informed consent. Inclusion criteria included breastfeeding women who were HIV-uninfected and did not report complications *in utero* during their full-term pregnancies or *intra partum*. Women were excluded if they had had caesarean sections, their pregnancies were not full-term, or they were diagnosed with mastitis *post partum*. Samples included in these analyses were taken from women who were not taking medications other then vitamin supplements *intra* or *post partum*, and did not receive an epidural *intra partum*. Three mothers reported minor colic and one reported silent reflux in their infants. Milk samples were self-collected into sterile tubes within the first week and at one month, three months and six months postpartum, and immediately shipped on ice for processing in our laboratory. Samples were separated into lipid, supernatant, and cellular fractions and stored at −80°C and liquid nitrogen, respectively. One-month postpartum samples were also collected from women in a Los Angeles cohort. Specific demographics are provided in Table 1. BM supernatant fractions were used for Western blotting or were filter-sterilized (0.45 µm) for functional assays to avoid cellular contamination.

Cell Lines and Reagents

Human embryonic kidney (HEK293), HEK293-TLR2, and human monocytic U937 cells were cultured in DMEM or RPMI supplemented with 10% FBS (Invitrogen), 10 µM HEPES (Invitrogen), 2 µM L-glutamine (Invitrogen), 100 units/mL penicillin/streptomycin (Sigma-Aldrich), respectively and maintained at 37°C and 5% CO_2. HEK293-TLR2 additionally required selection media with 10 µg/mL blasticidin. All cell lines were plated to reach 5×10^4 cells/well for each experiment in 96-well plates. Caco-2 intestinal epithelial cell line were cultured as described previously [27] and plated at 2.5×10^4 cells/well and grown overnight.

Table 1. Characteristics of Healthy HIV-uninfected Women Participants.

Samples Collected:	
< one week	13
> two weeks	5
Median Maternal Age	31.5
Mean Maternal Age	31.78
Median Parity	2
Mean Parity	2.22
Clinical Diagnosis of Mastitis	0
Racial Background:	
African American	1
Asian	1
Caucasian	14 (84.6%)
Jamaican	1 (8.3%)
Indian	1 (8.3%)
Exclusively Breast Feeding:	
< one week samples	13/13 (100%)
> two weeks	3/5 (60%)

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Western Blot

BM was evaluated for total protein concentration using a NanoVue luminometer (GE Healthcare) and at equal concentration was boiled with 5× Laemmli reducing buffer and resolved in SDS-PAGE as per standard protocol. Signals were developed and detected as previously described [23]. sTLR2 bands were analyzed using Un-Scan-It image digitizing software (Silk Scientific Inc.). Recombinant sTLR2 was used as reference standard.

Antibodies

The following anti-sTLR2 antibodies were used: goat polyclonal IgG, N-17 (sc-8689); mouse monoclonal IgG2a, TL2.1 (sc-21759), TL2.3 (sc-21760); mouse monoclonal IgG1, T2.5 (sc-52736) (Santa Cruz Biotechnology) and mouse monoclonal IgG1 ascites, 1030A5.138 (ebioscience). Secondary antibodies included: donkey anti-goat IgG and chicken anti-mouse IgG (Santa Cruz). Isotype controls included: goat and mouse (Dako Canada Inc.).

Immunodepletion of sTLR2 from Breast Milk

Immunodepletion of sTLR2 from mucosal samples using N-17 antibody and Protein G-Sepharose (GE Healthcare) was described previously [22,23]. Mock-depleted samples were treated simultaneously and with similar reagents without specific antibodies. Cyanogen-bromide (CNBr) was also used for immunodepletion of sTLR2 forms as per manufacturer's instructions (Pharmacia Biotech). TLR2 antibody N-17 or T2.5 was linked to conjugated CnBr beads as per manufacturer's instructions. Bead washing up to 10 washes were tested using Western blotting to ensure no free antibody remained in the system (data not shown).

Immunoassays

ELISA Duoset was used to measure IL-8 levels in cell culture systems according to manufacturer's instructions (R&D Systems). sTLR2 cytometric bead assay (CBA) was developed in our lab as per manufacturer's instructions (BD Biosciences). Capture beads were covalently bound with T2.5 Ab and N-17-Phycoerythrin (PE)

Ab was used as detection antibody (Santa Cruz). Human IL-8 flex-set (BD Biosciences) was used to determine IL-8 production in Caco-2 supernatants.

HIV Preparation and Reporter Assay

R5-tropic ADA or BaL virus was prepared and tissue culture infectious dose (TCID) of pooled supernatants as well as *in vitro* infection functional assays were determined using TZM-bl cells as described previously [28]. Briefly, 5×10^4 cells/well were plated in a 96-well plate with 25 μg/mL of diethylaminoethyl-dextran (Sigma). Luciferase activity from TZM-bl (JC53-BL) cells (kindly provided by Dr. D. Montefiori, Duke University, North Carolina) was measured using Bright-Glo reagents (Promega) and luminescence was read using the Veritas luminometer (Promega) and reported as relative light units (RLU).

MTT Viability Assay

MTT assay was used to determine viability of TZM-bl reporter cells exposed to human breast milk. The assay was performed using the manufacturer's instructions (Biotium Inc., CA, USA). TZM-bl cells (5×10^5) were plated in a 96-well plate, exposed to 2-fold serial dilutions of human breast milk in triplicate, and incubated at 37°C for 24 hours. 10 μl of MTT solution was added and incubated for 4 hours at 37°C. After incubation, the media was discarded and 200 μl DMSO (dimethyl sulfoxide) was added. Optical density (OD) was tested at 570 nm (reference 630 nm) in an ELISA plate reader.

cDNA Analysis

Total RNA was extracted from intestinal epithelial cells, human Caco-2 and fetal FHs-074, using TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA was reverse transcribed. Primers used to amplify TLR2 and 18 S cDNA were described previously [29].

Statistical Analysis

Data were plotted and analyzed using Prism (GraphPad Software). Non-parametric tests were used including Mann–Whitney U-tests and Student t-test for unmatched comparisons and ANOVA for multiple comparisons. P was considered statistically significant if <0.05.

Results

Predominant sTLR2 Polypeptides Profile in Breast Milk

We set out to examine the sTLR2 protein profiles in breast milk (BM) samples from healthy HIV-uninfected women using Western and Native blot analysis. Using reducing methods and a variety of TLR-specific antibodies, our data show three major polypeptide bands of ~83 kDa, ~38 kDa and ~26 kDa and additional minor polypeptides of ~130 kDa, and ~66 kDa (Fig.1A). These data compliment previously published reports that identified similar sTLR2 polypeptide bands, including ~83 kDa, ~66 kDa, ~38 kDa, and ~26 kDa [23] under reducing conditions. However, the predominant sTLR2 polypeptide forms described here (namely 38 kDa and 26 kDa) were substantially different in size compared to a previously reported predominant sTLR2 polypeptides in BM (66 kDa) [23]. Additionally, polypeptide band patterns varied among samples tested (Fig.1A). Data indicate the polyclonal antibody (pAb) N-17 was specific for the ~83 kDa and ~38 kDa sTLR2 polypeptide bands. Multiple TLR2-specific monoclonal antibodies (mAbs) detected a similar ~83 kDa band as well as a separate and unique ~26 kDa band (Fig.1A). Notably, pAb detected the presence of commercially available rsTLR2, while anti-TLR2 mAbs were unable to detect the recombinant protein (Fig. 1A). Further, using Native blot analysis, we identified three predominant sTLR2 protein complexes found in BM (Fig. 1B). Specifically, N-17 and T2.5 Abs could detect identical native proteins indicated by arrow 1 and 2. Additionally, T2.5 Ab detected a unique sTLR2 protein identified with arrow 3, which may be specific for a C-terminal portion of TLR2 extracellular domain as has been previously suggested [30].

Variation in sTLR2 Polypeptides between Different Women

Multiple BM samples collected within one week (N = 13) and at one month (N = 5) post partum were tested under reducing conditions to further investigate the extent of sTLR2 polypeptide variation among women. Two major observations were noted: (1) The intensities of the two predominant sTLR2 bands were remarkably variable, showing high (samples H3–6, H8–10, and H13), intermediate (H1, H11, LA1, LA2, and LA5) and very low (H2, H7, H12, LA3, and LA4) levels of sTLR2 expression despite equivalent amount of protein for each sample; (2) sTLR2 forms varied considerably among different women's BM. Under our detection condition, the 38 kDa polypeptide band appeared at a substantially greater concentration than the 26 kDa sTLR2 polypeptide band in about half the women (samples H3, H6, H8–10, H13, LA2, LA3, and LA5) while in approximately 25% of BM samples higher concentrations of 26 kD were observed (samples H1, H11, LA1, and LA4) (Fig. 2).

Specificity of Anti-sTLR2 Antibodies

In light of these observations, we evaluated the specificity of anti-TLR2 Abs. N-17 specificity was confirmed using a competition assay with the corresponding peptide (N-17P), shown previously [22,26]. Pre-incubation of pAb with N-17P negated detection of the 38 kDa, and a substantial amount of 83 kDa polypeptide bands in BM when evaluated using N-17 Ab (Fig. 3A). To build upon previous reports confirming the specificity of mAb [25,30,31], we used a sandwich cytometric bead array (CBA) (Fig. 3B) designed in our laboratory. A bead complex using a capture antibody (mAb T2.5) and detection antibody (pAb N-17-PE) confirmed mAb specificity for sTLR2 in BM. However, the CBA did not detect commercially available recombinant extracellular TLR2 (rsTLR2) (Fig. 3C), which was consistent with our TLR2 profiling Western blot data (Fig. 1).

Expression Kinetics, Source, and Bioavailability of sTLR2 Expression

Western blot analyses of BM collected at different time points following delivery revealed sTLR2 expression is regulated postpartum since the expression of the 38 kDa decreased gradually (Fig. 4A, top panel), thus complimenting previous literature showing decreases in major sTLR2 polypeptides post partum [22,23]. In contrast, the expression of the 26 kDa polypeptide remained unchanged over six months (Fig. 4A, bottom panel).

To determine the source of sTLR2 polypeptides, supernatants collected from BM cells and a mammary epithelial cell line, MCF-10A were examined. Our results revealed that the 26 kDa polypeptide is, at least in part, produced by the MCF-10A and BM cellular fraction (Fig. 4B). Surprisingly, however, 38 kDa sTLR2 was not detected in these cell supernatants (Fig. 4B). These findings lead to two possibilities–(1) the 38 kDa form is not produced by the cells tested here or (2) the protein is not stable in cell culture conditions. Therefore, we next tested the stability of 38 kDa and 26 kDa sTLR2 in BM over a 15-day period, and

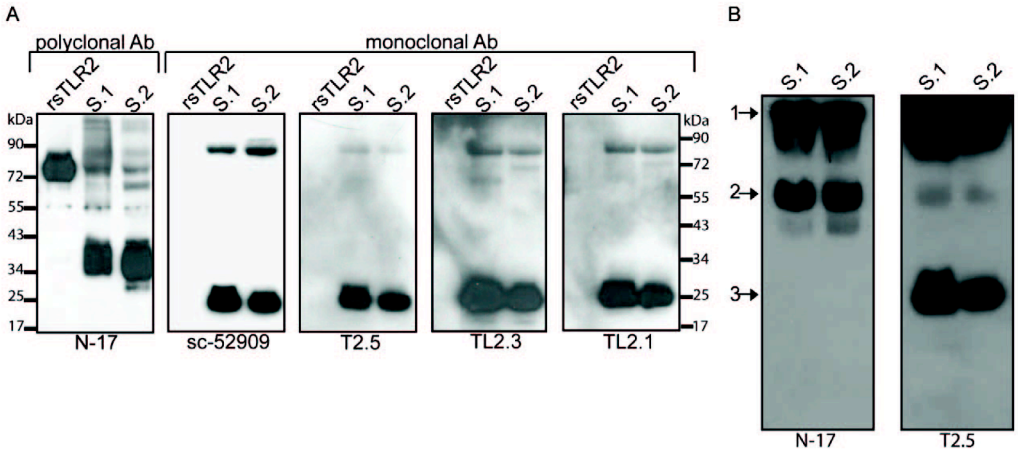

Figure 1. Predominant sTLR2 polypeptides profiles in breast milk. Predominant sTLR2 polypeptide profiles found in multiple breast milk (BM) samples using Western and Native blot analysis. (**A**) BM samples (10 µg total milk protein) with commercial rsTLR2 were evaluated by Western blot analysis with N-17 pAb and 4 mAb (sc-52909, T2.5, TL2.3, TL2.1). pAb N-17 detected commercial rsTLR2 as well as multiple bands in BM; the predominant BM forms were ~83 kDa and ~38 kDa sTLR2 forms. In contrast, mAbs did not detect the commercial rsTLR2. In BM mAbs detected the ~83 kD band, as well as a unique ~26 kDa sTLR2 form, which was not detected with the N-17 pAb. (**B**) N-17 pAb and T2.5 mAb were used in Native blot analysis of two BM samples. N-17 pAb detected two large proteins (arrow 1 & 2), while T2.5 mAb detected 3 proteins (arrow 1, 2 & 3). A representative data set from three experiments is shown.
doi:10.1371/journal.pone.0040138.g001

observed that the 38 kDa form of sTLR2 was not detectable when stored at room temperature (24°C), yet was detectable to 7 days when stored at 4°C (Fig. 4C). Conversely, the 26 kDa sTLR2 was detectable at all time points (Fig. 4C) indicating that 38 kDa is less stable then 26 kDa sTLR2 in BM.

sTLR2-mediated Augmentation of Pro-inflammatory Cytokines during Bacterial Lipoprotein Exposure

sTLR2 is an established inhibitor of pro-inflammatory responses to a variety of microbial pathogens (reviewed in [32]). In order to test the functional role of BM sTLR2, we immunodepleted natural forms of sTLR2 from BM using TLR2-specific antibodies and tested prototypic inflammatory cytokine, IL-8, production in cell lines that endogenously express TLR2, including U937 [33] and HEK293-TLR2 in response to the synthetic bacterial lipoprotein, Pam_3CSK_4. Qualitative and quantitative evaluation of sTLR2 immunodepletion using Western blot and ELISA analysis, respectively, indicate that a significant amount of sTLR2 was depleted from sTLR2-depleted (sTLR2-D) compared to mock-depleted (mock-D) BM (Fig. 5A). Further, digital semi-quantification analysis revealed that roughly 75% of 83 kDa and 71% of 38 kDa sTLR2 were depleted from BM.

As postulated, sTLR2-D BM did not significantly inhibit the Pam_3CSK_4-induced production of pro-inflammatory IL-8 compared to the mock-D BM in U937 and HEK293-TLR2 (1:20 dilution: $P<0.01$, $P<0.001$; respectively), in a dose-dependent manner (1:40 dilution: $P<0.001$, $P<0.001$, respectively) (Fig. 5B & C). Importantly, HEK293 cells, which are devoid of membrane bound TLR2 [34], demonstrated no significant differences in IL-8 production among groups (Fig. 5D). These data indicate that inhibition of Pam_3CSK_4-induced pro-inflammatory response by BM is largely mediated by membrane-bound TLR2.

We next examined the use of recombinant sTLR2 (rsTLR2) in inhibiting pro-inflammatory cytokine production in the HEK293-TLR2 cell line in response to TLR2 ligand, Pam_3CSK_4. Multiple reports demonstrate cellular responses against microbial agonists triggered through TLR2 are extremely low without co-receptor

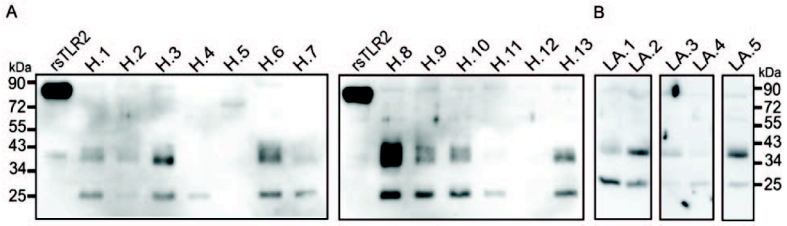

Figure 2. Variation in sTLR2 polypeptides between different women. Breast milk samples (10 µg total protein) from HIV-uninfected women were evaluated using a cocktail of anti-sTLR2 antibodies (N-17 pAb and T2.5 mAb) using Western blot analysis. Results show dramatic variation in predominant sTLR2 polypeptide (~38 kDa and ~26 kDa) expression in milk from different women. (A) Samples taken within one week postpartum. (B) Samples taken within one month postpartum.
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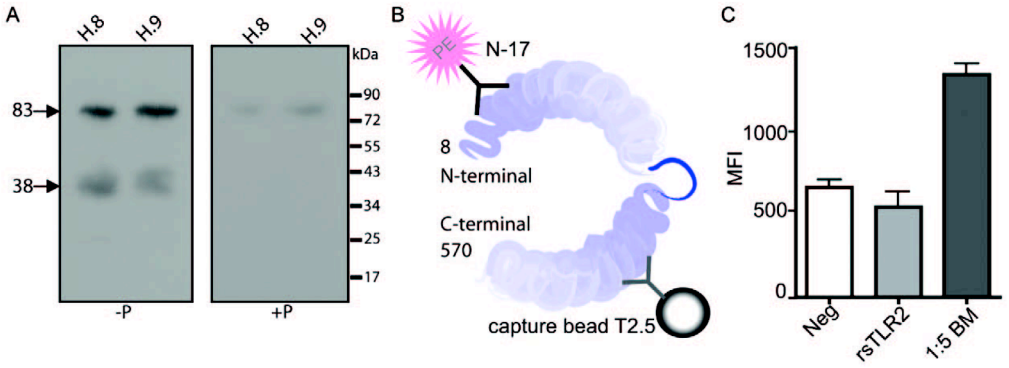

Figure 3. Specificity of anti-sTLR2 antibodies. The specificity of anti-sTLR2 antibodies was confirmed using a peptide competition assay and CBA. (A) N-17 was preincubated without (-P) or with (+P) 5× molar excess of peptide (N-17P) prior to immunoblotting. Pre-incubation with excess peptide markedly reduced both the ~83 kDa and ~38 kDa isoforms of sTLR2. Results representative of breast milk (BM) samples from different donors tested. (B) Schematic of cytometric bead array shows that beads coated with T2.5 mAb pulled natural sTLR2 out of milk and were detected with phycoerythrin (PE) labeled N-17 detection antibody. (C) CBA analysis of commercial rsTLR2 and BM dilution clearly shows system can detect natural sTLR2 but cannot detect commercial rsTLR2. A representative data set from triplicate experiments is shown.
doi:10.1371/journal.pone.0040138.g003

sCD14 [35,36]. Therefore, we demonstrate that at varying concentrations, with or without sCD14, rsTLR2 was unable to reduce IL-8 or IL-6 production (Fig. 5 E & F, respectively). These data precluded the use of recombinant sTLR2 in further *in vitro* experiments.

sTLR2 Forms Function Similarly in Human Intestinal Epithelial Cells

Given the key role BM has on the development and function of the neonatal gut (reviewed in [37]), the established role of TLR2 in the preservation of intestinal epithelial cell (IEC) barrier integrity [27,38], as well as our data showing multiple forms of sTLR2 in BM (Fig. 1), we considered the functional role of predominant sTLR2 polypeptides on human IEC in response to known TLR2 ligand, Pam_3CSK_4. Caco-2, a well-characterized IEC line that expresses TLR2 shown here (Fig. 6A) and elsewhere [39], was chosen since an infant IEC cell line is not commercially available. Fetal intestinal epithelial cell line (FHs 074) was considered for use, however proved unsuitable, as TLR2 cDNA was not detected (Fig. 6A).

Western blot analysis shows that the majority of sTLR2 was removed using T2.5 mAb immunodepletion (Fig. 6B). Optical densitometry confirmed that predominant sTLR2 forms were depleted in sTLR2-D compared to mock-D BM by the following percentages: 95% of 83 kDa, 64% of 38 kDa, and 99% of 26 kDa form in this batch preparation (Fig. 6A). The 38 kDa appears partially immunodepleted along with the majority of 83 kDa and 26 kDa forms, and may be due to the co-identification of two

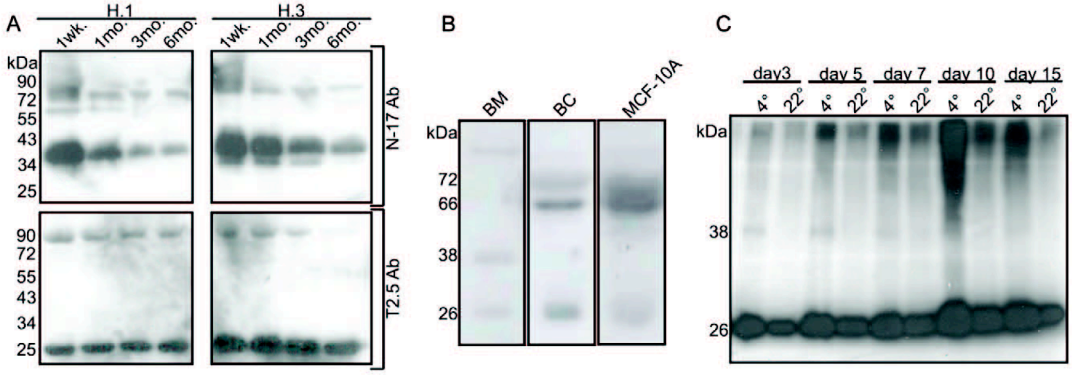

Figure 4. Expression kinetics, source, and bioavailability of sTLR2. sTLR2 expression kinetics, source and bioavailability in breast milk (BM) (10 μg total milk protein) were evaluated using Western blot analysis. (**A**) BM evaluated at one week to six months using N-17 pAb (top panel) and T2.5 mAb (bottom panel) indicated a reduction of the ~38 kDa and consistent expression of the ~26 kDa sTLR2 polypeptide postpartum. A representative data set from four different donors is shown. (B) BM, BM cells (BC), and MCF-10A supernatant were tested. ~38 kDa sTLR2 was observed in BM, but absent from BC and MCF-10A. ~26 kDa sTLR2 form was observed in BM, BC, and MCF-10A. (C) BM stored at 4°C or 24°C revealed ~38 kDa sTLR2 polypeptide was quickly degraded compared to the slight degredation of the ~26 kDa form. Western blot analyses were developed with a cocktail of N-17 pAb and T2.5 mAb. A representative data set from triplicate experiments is shown.
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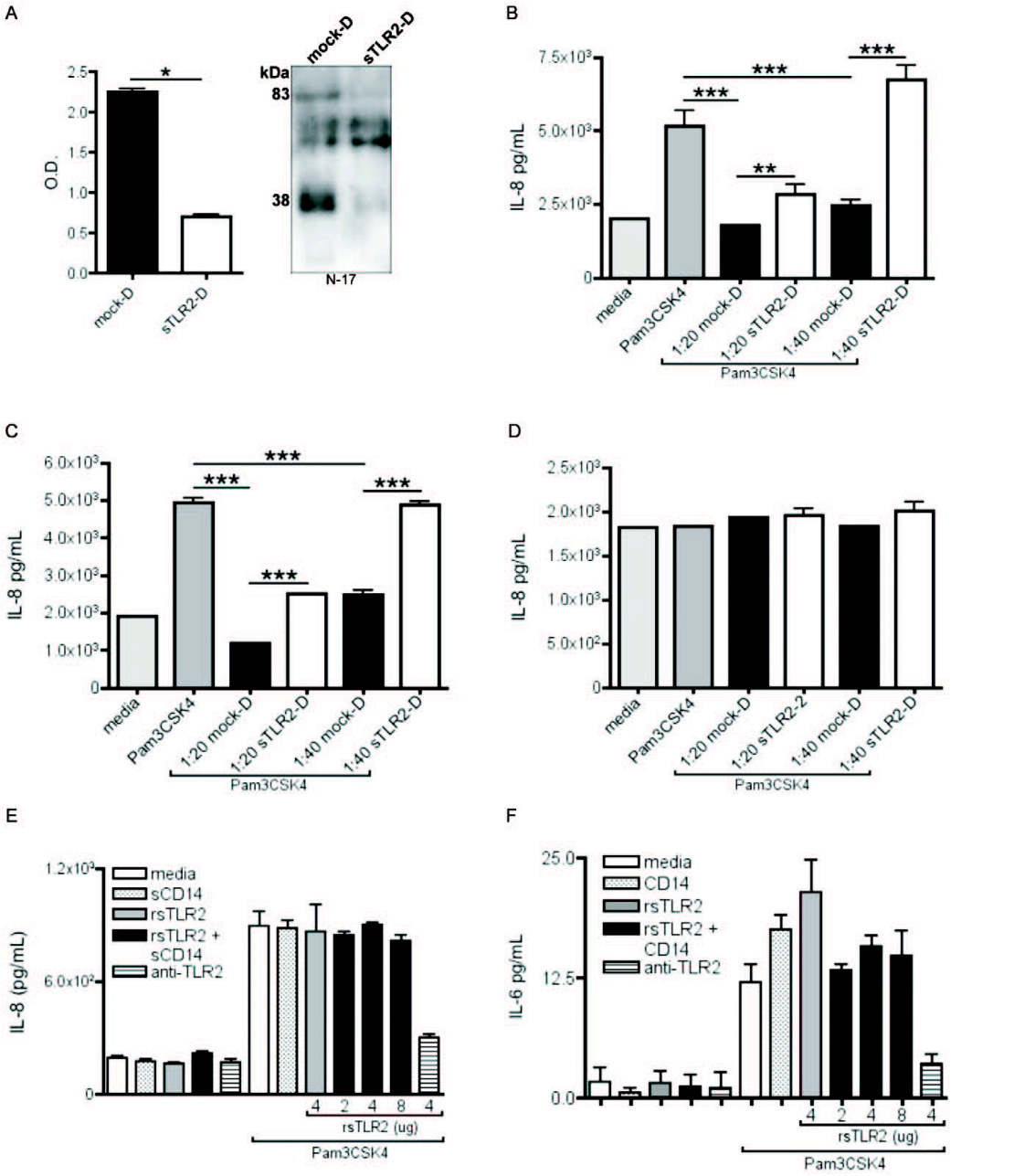

Figure 5. sTLR2-mediated augmentation of pro-inflammatory cytokines during bacterial lipoprotein exposure. (A) Immunodepletion of ~38 kDa sTLR2 in breast milk (BM) described in materials and methods. Quantitative analysis using TLR2 ELISA indicated a significant ($P<0.05$) decrease in sTLR2-depleted (sTLR2-D) compared to mock-D breast milk (BM). Western blot analysis revealed that ~38 kDa and ~83 kDa sTLR2 were markedly reduced compared to mock-D BM. (B-D) 500 ng/mL Pam_3CSK_4 was incubated with media or BM that was either mock-D or sTLR2-D for 1 hr at 37°C before being placed on cells. Supernatants were collected for IL-8 ELISA after 18 hours. Results represent (B) U937, (C) HEK293-TLR2 and (D) HEK293. (E) Commercial rsTLR2 was used at varying concentration with or without sCD14 and showed no inhibition of IL-8 or (F) IL-6 production in HEK293-TLR2 cells. Significant increases in pro-inflammatory cytokine, IL-8, was observed in sTLR2-D compared to mock-D BM. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Errors bars, SEM. A representative data set from triplicate experiments is shown.
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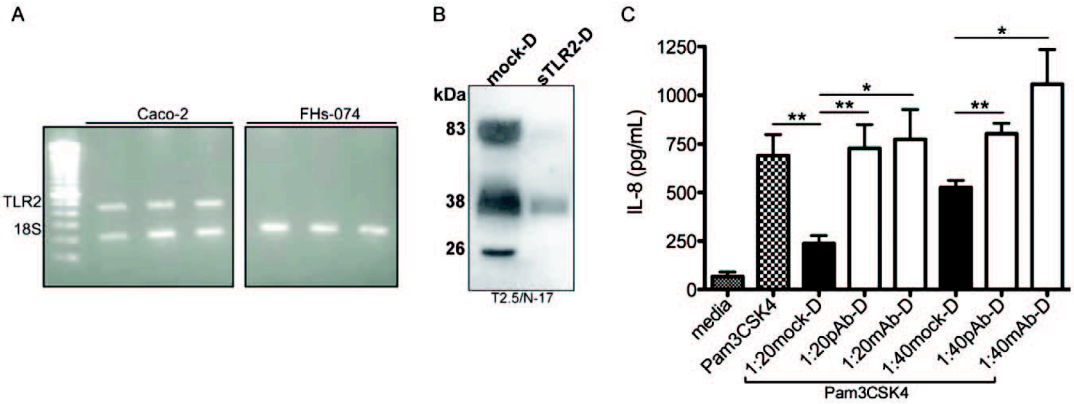

Figure 6. sTLR2 forms function similarly in human intestinal epithelial cells. (A) PCR analysis indicated that Caco-2, intestinal epithelial cells (IEC), expressed TLR2 mRNA while FHs-074 IEC did not. (B) Western blot analysis indicated that the majority of ~83 kDa and ~26 kDa sTLR2 forms were removed from sTLR2-depleted (sTLR2-D) compared to mock-D breast milk (BM). (C) *In vitro* testing of Caco-2 cells pre-incubated with mock-D BM for 1 hour at 37°C significantly inhibited IL-8 production (1:20 $P<0.001$; 1:40 $P<0.001$) following exposure to Pam_3CSK_4 (5 ng/mL). A significant IL-8 increase was observed in pAb-D (N-17 pAb) BM ($P<0.01$) and mAb-D (T2.5) BM ($P<0.05$) following Pam_3CSK_4 exposure. **$P<0.01$, ***$P<0.001$. Errors bars, SEM. A representative figure of the experiment completed in triplicate is shown.
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sTLR2 forms with both antibodies as shown previously in Native blots (Fig. 1B). Similar to experiments shown previously, mock-D BM, pAb-D, or mAb-D BM was incubated with Pam_3CSK_4 prior to addition to the IECs. As postulated, pAb-D and mAb-D BM incubated with Pam_3CSK_4 lead to significantly ($P<0.01$, $P<0.05$, respectively) increased IL-8 production compared to mock-D BM. These data indicate that predominant sTLR2 forms are involved in inhibiting Pam_3CSK_4-induced inflammation in IECs.

sTLR2-significantly Inhibits HIV-1 Infection in Reporter Assay

The majority of infants breastfed by HIV-positive mothers remain uninfected despite constant and repeated exposure to virus over weeks, months or even years [5–8]. Given the large quantity of sTLR2 in BM observed in our laboratory as well as others [23], and the role of TLR2 in sensing and activating anti-viral responses to a number of viruses [40–44], we hypothesized that BM sTLR2 may play a role in inhibiting HIV-1 infection.

We first determined that BM was not toxic to TZM-bl cells (Fig. 7A). To test our hypothesis, TLR2-specific mAb or pAb was incubated with BM prior to the addition of R5 virus and then placed on TZM-bl cells. Our results show that positive controls T20 and 2F5IgG significantly ($P<0.001$) inhibited HIV-1 infection (Fig. 7B) as shown previously [28]. Likewise, 1:100 BM significantly ($P<0.001$) inhibited HIV-1 infection (Fig. 7B), indicating that 1:100 BM could be used as a positive control in further experiments, as shown previously [10]. Conversely, when TLR2-specific pAb or mAb were incubated with BM prior to addition of cell-free virus, there was a significant increase ($P<0.001$) in HIV infection (Fig. 7B). Additionally, we found that neither antibodies alone nor isotype controls had any significant effect on HIV-1 infection (Fig. 7B).

To further confirm that sTLR2 was involved in inhibiting HIV-1, we incubated mock-D or sTLR2-D BM with virus prior to being placed on TZM-bl cells. Our results indicated that our positive control, mock-D BM, significantly decreased cell-free HIV-1 infection at a 1:100 dilution ($P<0.001$) (Fig. 7C), which is consistent with a previous publication [10]. However, when BM was depleted of sTLR2, cell-free HIV-1 infection was significantly increased ($P<0.001$). Conversely, rsTLR2 that was tested with or without sCD14 did not potently inhibit cell-free HIV-1 infection (Fig. 7C & D). Further, we examined the effect of pre-incubating TZM-bl cells with either mock-D or sTLR2-D BM prior to adding virus, and found no significant difference in HIV-1 infection (Fig. 7B), which indicates that sTLR2 may directly inhibit cell-free HIV-1.

Discussion

Here we report on predominant forms of sTLR2 in breast milk (BM) that vary between women and show that they have direct effects on inflammatory responses and HIV-1 infection. Results confirm previous findings that indicate sTLR2 is critical in suppressing inflammatory responses to bacterial PAMPs [22,23,25,26], and extend to show direct inhibition of cell-free R5 HIV-1 infection. Thus, sTLR2 may be an important innate factor that protects infants breastfeeding from HIV positive mothers by (1) directly inhibiting cell-free R5 HIV-1 infection, and (2) inhibiting immune activation, namely inflammation of the nursing infant's gut.

To our knowledge, this is the first time that the major 38 kDa and 26 kDa sTLR2 polypeptides have been reported as predominant sTLR2 forms in BM, although similar-sized sTLR2 forms have been observed in both amniotic fluid, saliva [22,26], and cervicovaginal fluid (Henrick et al., unpublished data). Variation in predominant sTLR2 polypeptides between different women's BM samples may be the result of racial, ethnic or genetic variability, which may explain the differences in predominant sTLR2 forms identified between our cohort and the one described by LeBouder *et al* (2003), despite the similarity in sample collection, times postpartum, processing, and Western blot analysis, including antibodies used. Notably, we provide data that multiple TLR2-specific mAbs were unable to detect commercially available human rsTLR2 indicating important structural, and/or conformational differences that were further associated with altered immune functionality *in vitro*. Indeed, rsTLR2 is produced in mouse myeloma cell lines,

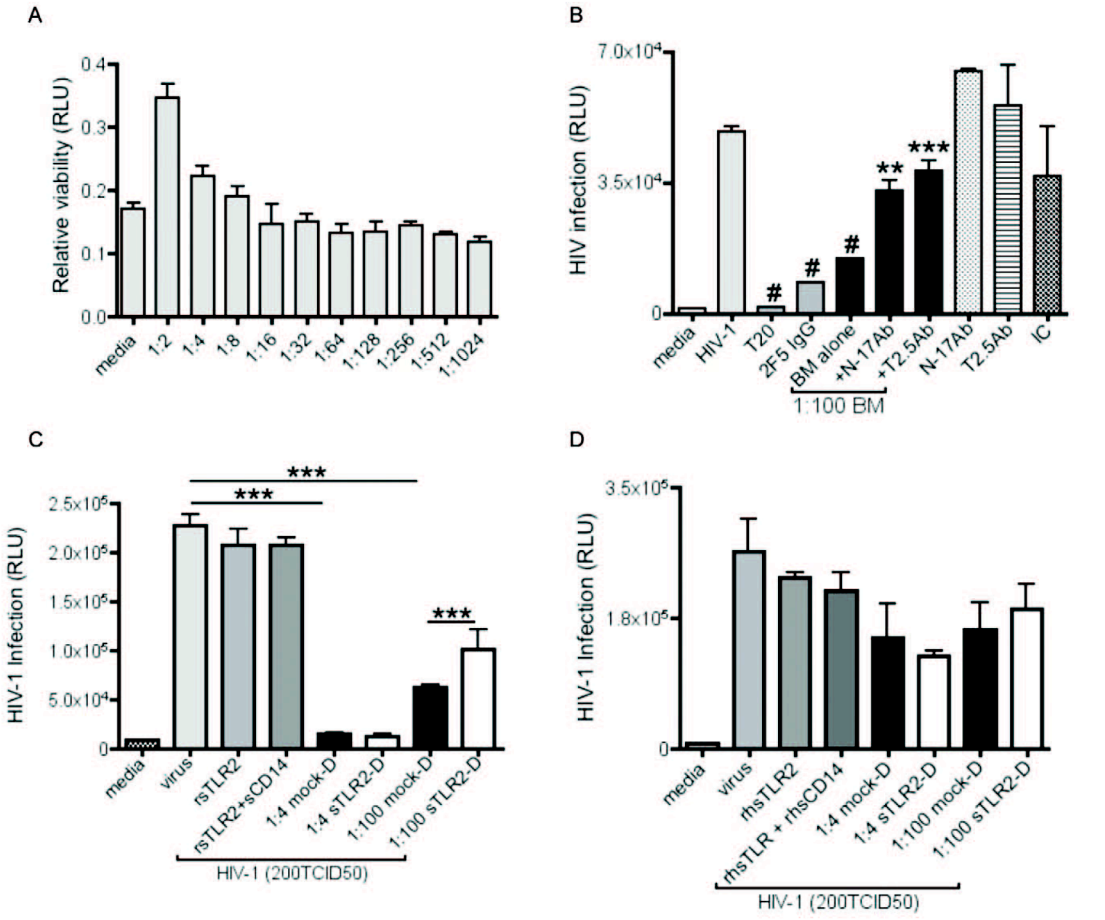

Figure 7. sTLR2 significantly inhibits HIV-1 infection in reporter assay. (A) MTT assay indicates that HIV-uninfected breast milk (BM) was not toxic to TZM-bl cells. (B) HIV-uninfected BM was incubated with either N-17 pAb or T2.5 mAb (200 before R5 HIV-1 (200 $TCID_{50}$) and then placed on TZM-bl cells. T20, 2F5IgG, and 1:100 BM significantly inhibited infection (*P<0.001).* A significant increase (*P<0.001, 0.01,* respectively*)* in HIV-1 infection was shown when sTLR2-specific N-17 or T2.5 Ab were pre-incubated with BM. N-17 pAb and T2.5 mAb alone and isotype control (200 ng/mL) did not inhibit HIV-1 infection. (C) rsTLR2+/− sCD14 or pooled HIV-uninfected mock-D or sTLR2-D BM (described in materials and methods) were incubated with R5 HIV-1 (200 $TCID_{50}$) before addition to TZM-bl cells for 48 hours. A significant decrease (*P<0.001*) in HIV infection was observed in cells exposed to mock-D BM. However, a significant increase (*P<0.001*) in HIV infection was detected with sTLR2-depleted BM. (D) rsTLR2+/− sCD14, mock-D, or sTLR2-D was incubated with cells for 1 hour at 37°C, washed with PBS, and then exposed to R5 HIV-1 (200 $TCID_{50}$) did not alter HIV-1 infection. ** *P<0.01*, ***P<0.001*. Errors bars, SEM. A representative data set from four experiments is shown.
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which may alter glycosylation patterns as opposed to native proteins, and may prove pivotal given the undoubted importance of N-linked glycosylation in proper sTLR2 synthesis, secretion, and function [45,46]. These data indicate that caution should be used when interpreting data in which commercially available rsTLR2 is used as a replacement for natural sTLR2 forms. Further, interpretation of our data leads us to believe that intermolecular non-covalent interactions between the predominant sTLR2 polypeptide 38 kDa and 26 kDa are responsible for sTLR2 protein complexes that function in a coherent manner to inhibit bacterial PAMP-induced production of pro-inflammatory cytokine production and R5 HIV-1 infection.

Further, experiments into the regulation of major sTLR2 polypeptides in BM may be important in fully appreciating the intricacies of intestinal microbiome development in infants. Our observations indicated that multiple cells of the mammary gland and BM contribute to the generation of 26 kDa sTLR2, however the source of the 38 kDa form remains elusive. Data indicate that 38 kDa sTLR2 production is down-regulated over time postpartum and is susceptible to degradation at room temperature. These observations may correlate with the infant's maturing immune system [47], and assist in the development of tolerance as well as proper IEC epithelial barrier function [27,38].

The function of BM sTLR2 has been shown to be immunomodulatory by providing direct attenuation of signaling through

membrane TLR2 while still allowing for effective elimination of the pathogen in a mouse model [25]. Here, we demonstrated that sTLR2 in BM has dual utility by providing both anti-inflammatory and anti-viral functions: (1) sTLR2 depleted BM led to significantly increased IL-8 pro-inflammatory cytokine production in U937, HEK293-TLR2 and human IECs exposed to PAM_3CSK_4; (2) Inhibition of sTLR2 through the addition of TLR2-specific antibodies as well as sTLR2-depleted BM led to significantly increased HIV-1 infection in TZM-bl reporter assays. To the best of our knowledge, this is the first study to demonstrate that sTLR2 in BM can directly inhibit cell-free R5 HIV-1 infection. However, previous studies have demonstrated a strong correlation between decreased sTLR2 concentrations and HIV-1 progression [48], and high levels of TLR2 expression on monocytes taken from HIV-1 infected patients [49]. Indeed, there are numerous publications indicating that many viruses including measles, VSV, CMV, hepatitis C and herpes virus activate TLR2 through viral glycoproteins leading to the production of proinflammatory cytokines [40–44,50]. Further, we previously showed significantly increased TLR2 expression in peripheral blood mononuclear cells of Kenyan commercial sex workers with AIDS, which is reduced with HAART treatment [29]. Our data is timely given a recent publication confirming increased levels of HIV-1 RNA in BM positively correlate with vertical transmission [51,52].

Notably, immunodepletion does not allow for the complete removal of sTLR2 from BM, thus functional testing shown here may underestimate the total effect elicited by sTLR2 against HIV-1 and synthetic bacterial ligand. Likewise, this technique has experimental limitations that do not preclude the possibility that an unknown binding partner of sTLR2 may also contribute for the reduction of pro-inflammatory cytokine production in response to bacterial ligands and HIV-1 inhibition shown here. Future research is warranted to determine the exact mechanism of sTLR2 functionality including the involvement of cofactor CD14 in the direct binding and inhibition of HIV, as the necessity of the cofactor differs between viral agonists [40,42,50].

In conclusion, the current study provides evidence for the first time that sTLR2 in BM may provide a dual protective role for infants breastfeeding from their HIV-infected mothers. We confirm that BM sTLR2 complexes inhibit production of pro-inflammatory cytokines during bacterial ligand exposure, and extend the functional role of BM sTLR2 complexes to include inhibition of cell-free R5 HIV-1 infection. As discussed previously, immune activation has marked effects on HIV-1 acquisition (reviewed in [15]), therefore, data shown here may prove critical in understanding vertical HIV-1 transmission through BM, and indicates sTLR2 may be an important factor in a complex milieu of immunomodulatory and anti-viral factors that help protect the majority of infants from becoming infected despite repeated and prolonged exposure to HIV-1 infected BM.

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Author Contributions

Conceived and designed the experiments: BMH KN XDY KLR. Performed the experiments: BMH KN XDY. Analyzed the data: BMH KN XDY AD. Contributed reagents/materials/analysis tools: GMA. Wrote the paper: BMH KN KLR.

References

1. Grulee CG, Sanford HN, Schwartz H (1935) Breast and Artificially Fed Infants. Jour AMA: 1–3.
2. Beaudry M, Dufour R, Marcoux S (1995) Relation between infant feeding and infections during the first six months of life. J Pediatr 126: 191–197.
3. Kramer MS (2010) "Breast is best": The evidence. Early Hum Dev 86: 729–732. doi:10.1016/j.earlhumdev.2010.08.005.
4. Arifeen S, Black RE, Antelman G, Baqui A, Caulfield L, et al. (2001) Exclusive breastfeeding reduces acute respiratory: 1–10. doi:10.1542/peds.108.4.e67.
5. Coovadia HM, Rollins NC, Bland RM, Little K, Coutsoudis A, et al. (2007) Mother-to-child transmission of HIV-1 infection during exclusive breastfeeding in the first 6 months of life: an intervention cohort study. Lancet 369: 1107–1116. doi:10.1016/S0140-6736(07)60283-9.
6. Coutsoudis A, Kuhn L, Pillay K, Coovadia HM (2002) Exclusive breast-feeding and HIV transmission. AIDS 16: 498–499.
7. Kuhn L, Sinkala M, Kankasa C, Semrau K, Kasonde P, et al. (2007) High uptake of exclusive breastfeeding and reduced early post-natal HIV transmission. PLoS ONE 2: e1363. doi:10.1371/journal.pone.0001363.
8. Iliff PJ, Piwoz EG, Tavengwa NV, Zunguza CD, Marinda ET, et al. (2005) Early exclusive breastfeeding reduces the risk of postnatal HIV-1 transmission and increases HIV-free survival. AIDS 19: 699–708.
9. Kazmi SH, Naglik JR, Sweet SP, Evans RW, O'Shea S, et al. (2006) Comparison of human immunodeficiency virus type 1-specific inhibitory activities in saliva and other human mucosal fluids. Clin Vaccine Immunol 13: 1111–1118. doi:10.1128/CDLI.00426-05.
10. Lyimo MA, Howell AL, Balandya E, Eszterhas SK, Connor RI (2009) Innate factors in human breast milk inhibit cell-free HIV-1 but not cell-associated HIV-1 infection of CD4+ cells. J Acquir Immune Defic Syndr 51: 117–124. doi:10.1097/QAI.0b013e3181a3908d.
11. Salvatori F, Scarlatti G (2001) HIV type 1 chemokine receptor usage in mother-to-child transmission. AIDS Res Hum Retroviruses 17: 925–935. doi:10.1089/088922201750290041.
12. Matala E, Hahn T, Yedavalli VR, Ahmad N (2001) Biological characterization of HIV type 1 envelope V3 regions from mothers and infants associated with perinatal transmission. AIDS Res Hum Retroviruses 17: 1725–1735. doi:10.1089/08892220152741423.
13. Ahmad N (2011) Molecular mechanisms of HIV-1 mother-to-child transmission and infection in neonatal target cells. Life Sci 88: 980–986. doi:10.1016/j.lfs.2010.09.023.
14. Pádua E, Parreira R, Tendeiro R, Nunes B, Castela J, et al. (2009) Potential impact of viral load and genetic makeup of HIV type 1 on mother-to-child transmission: characterization of env-C2V3C3 and nef sequences. AIDS Res Hum Retroviruses 25: 1171–1177. doi:10.1089/aid.2009.0103.
15. Lawn SD, Butera ST, Folks TM (2001) Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection. Clin Microbiol Rev 14: 753-77, table of contents. doi:10.1128/CMR.14.4.753–777.2001.
16. Plummer FA, Simonsen JN, Cameron DW, Ndinya-Achola JO, Kreiss JK, et al. (1991) Cofactors in male-female sexual transmission of human immunodeficiency virus type 1. J Infect Dis 163: 233–239.
17. Plummer FA (1998) Heterosexual transmission of human immunodeficiency virus type 1 (HIV): interactions of conventional sexually transmitted diseases, hormonal contraception and HIV-1. AIDS Res Hum Retroviruses 14 Suppl 1: S5–10.
18. Corbett EL, Steketee RW, Kuile ter FO, Latif AS, Kamali A, et al. (2002) HIV-1/AIDS and the control of other infectious diseases in Africa. Lancet 359: 2177–2187. doi:10.1016/S0140-6736(02)09095-5.
19. Laga M, Manoka A, Kivuvu M, Malele B, Tuliza M, et al. (1993) Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. AIDS 7: 95–102.
20. Medzhitov R, Preston-Hurlburt P, Janeway CA (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388: 394–397. doi:10.1038/41131.
21. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 783–801. doi:10.1016/j.cell.2006.02.015.
22. Dulay AT, Buhimschi CS, Zhao G, Oliver EA, Mbele A, et al. (2009) Soluble TLR2 is present in human amniotic fluid and modulates the intraamniotic inflammatory response to infection. J Immunol 182: 7244–7253. doi:10.4049/jimmunol.0803517.
23. LeBouder E, Rey-Nores JE, Rushmere NK, Grigorov M, Lawn SD, et al. (2003) Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. J Immunol 171: 6680–6689.
24. LeBouder E, Rey-Nores JE, Raby A-C, Affolter M, Vidal K, et al. (2006) Modulation of neonatal microbial recognition: TLR-mediated innate immune responses are specifically and differentially modulated by human milk. J Immunol 176: 3742–3752.

25. Raby A-C, Le Bouder E, Colmont C, Davies J, Richards P, et al. (2009) Soluble TLR2 reduces inflammation without compromising bacterial clearance by disrupting TLR2 triggering. J Immunol 183: 506–517. doi:10.4049/jimmunol.0802909.
26. Kuroishi T, Tanaka Y, Sakai A, Sugawara Y, Komine K-I, et al. (2007) Human parotid saliva contains soluble toll-like receptor (TLR) 2 and modulates TLR2-mediated interleukin-8 production by monocytic cells. Mol Immunol 44: 1969–1976. doi:10.1016/j.molimm.2006.09.028.
27. Cario E, Gerken G, Podolsky DK (2004) Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. Gastroenterology 127: 224–238.
28. Jain S, Rosenthal KL (2011) The gp41 epitope, QARVLAVERY, is highly conserved and a potent inducer of IgA that neutralizes HIV-1 and inhibits viral transcytosis. Mucosal Immunology: 1–15. doi:10.1038/mi.2011.21.
29. Lester RT, Yao X-D, Ball TB, McKinnon LR, Kaul R, et al. (2008) Toll-like receptor expression and responsiveness are increased in viraemic HIV-1 infection. AIDS 22: 685–694. doi:10.1097/QAD.0b013e3282f4de35.
30. Meng G (2004) Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. J Clin Invest 113: 1473–1481. doi:10.1172/JCI200420762.
31. Brüll F, Mensink RP, van den Hurk K, Duijvestijn A, Plat J (2010) TLR2 activation is essential to induce a Th1 shift in human peripheral blood mononuclear cells by plant stanols and plant sterols. J Biol Chem 285: 2951–2958. doi:10.1074/jbc.M109.036343.
32. Liew FY, Xu D, Brint EK, O'Neill LAJ (2005) Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol 5: 446–458. doi:10.1038/nri1630.
33. Greene CM, McElvaney NG, O'Neill SJ, Taggart CC (2004) Secretory leucoprotease inhibitor impairs Toll-like receptor 2- and 4-mediated responses in monocytic cells. Infect Immun 72: 3684–3687. doi:10.1128/IAI.72.6.3684-3687.2004.
34. Razonable RR, Henault M, Watson HL, Paya CV (2005) Nystatin induces secretion of interleukin (IL)-1beta, IL-8, and tumor necrosis factor alpha by a toll-like receptor-dependent mechanism. Antimicrob Agents Chemother 49: 3546–3549. doi:10.1128/AAC.49.8.3546-3549.2005.
35. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, et al. (1999) Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 163: 1–5.
36. Nilsen NJ, Deininger S, Nonstad U, Skjeldal F, Husebye H, et al. (2008) Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling: role of CD14 and CD36. J Leukoc Biol 84: 280–291. doi:10.1189/jlb.0907656.
37. Verhasselt V (2010) Neonatal tolerance under breastfeeding influence. Curr Opin Immunol 22: 623–630. doi:10.1016/j.coi.2010.08.008.
38. Cario E, Gerken G, Podolsky DK (2007) Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology 132: 1359–1374. doi:10.1053/j.gastro.2007.02.056.
39. Cario E, Rosenberg IM, Brandwein SL, Beck PL, Reinecker HC, et al. (2000) Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. J Immunol 164: 966–972.
40. Bieback K, Lien E, Klagge IM, Avota E, Schneider-Schaulies J, et al. (2002) Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. J Virol 76: 8729–8736.
41. Boehme KW, Guerrero M, Compton T (2006) Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells. J Immunol 177: 7094–7102.
42. Compton T, Kurt-Jones EA, Boehme KW, Belko J, Latz E, et al. (2003) Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. J Virol 77: 4588–4596.
43. Michaud F, Coulombe F, Gaudreault E, Kriz J, Gosselin J (2010) Involvement of TLR2 in recognition of acute gammaherpesvirus-68 infection. PLoS ONE 5: e13742. doi:10.1371/journal.pone.0013742.
44. Sato A, Linehan MM, Iwasaki A (2006) Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells. Proc Natl Acad Sci USA 103: 17343–17348. doi:10.1073/pnas.0605102103.
45. Weber ANR, Morse MA, Gay NJ (2004) Four N-linked glycosylation sites in human toll-like receptor 2 cooperate to direct efficient biosynthesis and secretion. J Biol Chem 279: 34589–34594. doi:10.1074/jbc.M403830200.
46. Kataoka H, Yasuda M, Iyori M, Kiura K, Narita M, et al. (2006) Roles of N-linked glycans in the recognition of microbial lipopeptides and lipoproteins by TLR2. Cell Microbiol 8: 1199–1209. doi:10.1111/j.1462-5822.2006.00702.x.
47. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, et al. (2011) Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci USA 108 Suppl 1: 4578–4585. doi:10.1073/pnas.1000081107.
48. Heggelund L, Flo T, Berg K, Lien E, Mollnes TE, et al. (2004) Soluble toll-like receptor 2 in HIV infection: association with disease progression. AIDS 18: 2437–2439.
49. Heggelund L, Müller F, Lien E, Yndestad A, Ueland T, et al. (2004) Increased expression of toll-like receptor 2 on monocytes in HIV infection: possible roles in inflammation and viral replication. Clin Infect Dis 39: 264–269. doi:10.1086/421780.
50. Dolganiuc A, Oak S, Kodys K, Golenbock DT, Finberg RW, et al. (2004) Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. Gastroenterology 127: 1513–1524.
51. Neveu D, Viljoen J, Bland RM, Nagot N, Danaviah S, et al. (2011) Cumulative exposure to cell-free HIV in breast milk, rather than feeding pattern per se, identifies postnatally infected infants. Clin Infect Dis 52: 819–825. doi:10.1093/cid/ciq203.
52. Rousseau CM, Nduati RW, Richardson BA, Steele MS, John-Stewart GC, et al. (2003) Longitudinal analysis of human immunodeficiency virus type 1 RNA in breast milk and of its relationship to infant infection and maternal disease. J Infect Dis 187: 741–747. doi:10.1086/374273.

— CHAPTER 3 —

sTLR2 is significantly elevated in HIV-1 infected breast milk and inhibits HIV-1 induced cellular activation, inflammation and infection

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This manuscript investigated the mechanism of sTLR2 in inhibiting HIV-induced cellular activation and viral infection *in vitro.* These experiments were an extension of our previous findings, which strongly suggested that sTLR2 directly inhibited HIV infection *in vitro.* Here we evaluated TLR2 expression and sTLR2 concentration in HIV uninfected and infected breast milk, and determined a possible mechanism by which sTLR2 directly inhibits HIV-1-induced cellular activation and infection *in vitro.* Notably, we showed that sTLR2 concentration positively correlates with p24 levels in HIV infected breast milk. We also demonstrated that sTLR2, produced *in vitro*, inhibited HIV-1-induced NFκB activation and downstream IL-8 production in TLR2-bearing cells. Furthermore, we determined that MEC exposed to HIV-1 viral proteins increased sTLR2 production, which significantly decreased elevated CCR5 expression, and led to lower HIV-1 infection *in vitro*. Together, these results indicated that sTLR2 played a critical

role in inhibiting MTCT of HIV through breast milk.

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Running Title: sTLR2 inhibits HIV in breast milk

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Key words: breast milk (BM); soluble TLR2 (sTLR2); human immunodeficiency virus (HIV); inflammation; innate immunity; cellular activation; p17; p24; gp41; gp120.

ABSTRACT

Background: We previously demonstrated that breast milk soluble Toll-like receptor 2 (sTLR2) directly inhibited HIV infection, and have identified HIV-1 pathogen-associated molecular patterns (PAMPs) that activate through TLR2. We extended these findings to identify mechanisms by which sTLR2 inhibits HIV-induced activation and infection.

Methods: Milk was collected from HIV-infected and uninfected women and mRNA expression of TLR2 and sTLR2 levels and their correlation to p24 and IL-15 was evaluated. Inhibition of HIV-1 PAMP-induced cellular activation, inflammation, CCR5 expression and infection were assessed.

Results: HIV-1-infected milk had significantly elevated TLR2 expression and sTLR2 levels compared to uninfected samples. In HIV-infected milk sTLR2 significantly correlated with p24 and IL-15, and sTLR2 directly interacted with HIV-1 PAMPs. Pam3CSK and HIV-1 PAMPs significantly increased sTLR2 levels *in vitro*, which inhibited NFκB activation, IL-8 production and increased CCR5 expression. Lastly, sTLR2 significantly inhibited cell-free R5 HIV-1 infection and inflammation, and this effect was abrogated with TLR2-specific antibodies.

Conclusions: Our data indicate an innate compensatory response to HIV-1-induced activation and infection in the mammary gland that significantly elevated sTLR2 levels. We show for the first time a mechanism by which sTLR2 inhibits HIV activation,

inflammation and infection thus contributing to the inhibition of mother-to-child HIV transmission.

INTRODUCTION

Breast milk (BM) evolved to provide the optimal source of nutrition for the developing infant and confers protection against a wide range of pathogens via innate and adaptive immune factors. An intervention that promotes exclusive breastfeeding, regardless of the mother's HIV status, led to significantly decreased mother-to-child transmission (MTCT) compared to mixed feeding [1-4]. Although it is poorly understood how infants breastfeeding from HIV-infected mothers remain uninfected despite repeated and prolonged exposure to HIV-1, the protection is attributed to innate factors in milk that possess potent anti-viral activities [5,6] including lactoferrin, secretory leukocyte protease inhibitor (SLPI), and soluble Toll-like receptor 2 (sTLR2); all of these are found at high levels in BM and have HIV-1 inhibitory properties [7-9]. Our recent data, using neutralization and immunodepletion of sTLR2 from BM, indicated that sTLR2 directly interacted with the virus, thus inhibiting cell-free HIV-1 infection *in vitro*. Moreover, our results showed that MECs and breast milk cells (BMCs) produced sTLR2 [9].

Soluble forms of TLRs play a fundamental role in regulating pro-inflammatory responses that trigger innate immunity. sTLR2, which is produced through post-translational modification of the extracellular domain of TLR2, has direct anti-microbial modulatory effects by binding pathogen-associated molecular patterns (PAMPs) that signal through TLR2 [10-12]. Through computational molecular docking, a cylindrical N-terminus to C-terminus arrangement between sTLR2 and soluble cluster of differentiation 14 (sCD14) that encapsulated synthetic bacterial lipoprotein, Pam_3CSK_4

[12], thus preventing bacterial-induced cellular activation through membrane-bound TLR2.

Although TLR2 is classically considered in the context of bacterial-associated PAMP recognition, multiple viral proteins have been identified that trigger cellular activation in a TLR2-dependent manner. Specifically, cytomegalovirus glycoproteins, hepatitis C core and structural proteins, and measles hemagglutinin protein trigger TLR2-dependent pro-inflammatory responses [13]. The outcome of virus-specific cellular activation mediates a range of responses including acceleration of anti-viral clearance to establishing a favorable microenvironment that facilitates viral integration and replication [14], or inducing inflammation thereby recruiting more target cells. Cellular activation promotes HIV-1 replication due to its NFκB binding sites [15]. NFκB is critical to a variety of host immune responses [16] and is activated through multiple TLRs, including TLR2, signaling [17]. We recently identified specific HIV-1 structural proteins that serve as TLR2 PAMPs triggering NFκB activation, increased pro-inflammatory cytokine production and CCR5 expression and, importantly, significantly increased viral infection/integration (Henrick *et al*., submitted, 2013), thus indicating that HIV-1 manipulates host innate immune responses to promote a desirable microenvironment to accommodate HIV-1 infection and replication.

The primary aim of the present study was to identify a mechanism by which sTLR2 inhibits virally-induced cellular activation and HIV-1 infection *in vitro*. The

secondary aim was to characterize sTLR2 concentrations in HIV-uninfected and HIV-infected BM and its association with correlates of disease progression.

RESULTS

Significantly increased TLR2 expression in breast milk cells and sTLR2 in HIV-infected breast milk

We recently showed that TLR2 recognized novel HIV-1 PAMPs leading to increased cellular activation, CCR5 expression and infection (Henrick *et al*, submitted, 2013). In addition, we showed increased TLR2 expression in peripheral blood mononuclear cells (PBMCs) over the course of untreated HIV infection [18]. Therefore, we evaluated the influence HIV-1 infection might have on TLR2 expression levels in breast milk cells (BMCs). Our results showed increased TLR2 expression in BMCs from HIV-infected Nigerian women compared to uninfected Nigerian and Canadian women BMCs (Fig. 1A; *P=0.0010, P=0.0022*, respectively). Similarly, sTLR2 levels were elevated in milk from HIV-infected Nigerian women compared to HIV-uninfected women from Nigeria and Canada (Fig. 1B; *P=0.0011, P=0.0002*, respectively).

sTLR2 levels in HIV-1 infected breast milk correlates with p24 and IL-15 concentration.

HIV-1 p24 antigen levels correlate with HIV disease progression [19], and we recently showed that p24 is an HIV-specific PAMP signaling through TLR2/6 (Henrick *et al*, submitted 2013). Therefore, we evaluated the association between p24 and sTLR2 concentrations in BM, and results revealed that the p24 concentration positively correlated with sTLR2 levels in HIV-infected BM (Fig. 1C; *P=0.0158, R=0.3791*).

A protective association between IL-15 concentrations in BM and postnatal HIV transmission via breastfeeding has been reported [20]. Therefore, we determined whether IL-15 and sTLR2 levels correlate in BM. Our data demonstrated a strong correlation between sTLR2 and IL-15 levels in HIV-infected BM (Fig. 1D; *$P<0.0001$, $r=0.5698$*). These results suggest a compensatory innate mechanism by which sTLR2 levels are significantly increased in HIV-infected milk as the p24 concentration increased which positively correlated with IL-15 levels in infected BM.

HIV-1 PAMPs significantly elevate sTLR2 concentration *in vitro*

Previously, we and others showed that MECs and macrophages produce sTLR2 [9,12]. Given the increase of sTLR2 in HIV-infected BM, we evaluated whether MECs and/or macrophages exposed to HIV-1 PAMPs (p17, p24, and gp41) and a TLR2 ligand (Pam_3CSK_4) influenced sTLR2 levels. Qualitative and quantitative analysis of MEC supernatants revealed that HIV-1 PAMPs, as well as Pam_3CSK_4, increased supernatant sTLR2 levels compared to medium alone (Fig. 2A & B; *$P=0.0135$, 0.0116, $0,0390$,* respectively). Similarly, macrophages exposed to Pam_3CSK_4 or HIV-1 PAMPs elevated supernatant sTLR2 levels compared to medium (Fig. 2C; *$P=0.0008$, 0.0055, 0.0131, 0.0263,* respectively). These data suggest that elevated sTLR2 levels in HIV infected BM might be a result of local, virally-induced release and/or production of sTLR2 from resident macrophages and MECs.

sTLR2 binds directly to HIV-1 PAMPs

We tested our hypothesis that sTLR2 inhibits infection through direct interaction with HIV-1 proteins. Recombinant HIV-1 components (p17, p24, gp41, gp120, nef and ssRNA40) were blotted onto a nitrocellulose membrane, along with positive controls (sCD14, Pam_3CSK_4), a membrane control (recombinant sTLR2), and negative carrier controls (urea solution, PBS). Viral protein-sTLR2 interactions were identified using anti-TLR2 antibodies and indicated that HIV-1 structural proteins p17, p24, and gp41 directly interacted with sTLR2, whereas no interaction was identified between sTLR2 and viral components, gp120, nef, or ssRNA40 (Fig. 3A). Interactions were also detected between sTLR2 and the positive control, Pam_3CSK_4 and sCD14. The membrane control indicated that anti-TLR2 antibodies were at a sufficient concentration to detect sTLR2 if present (Fig. 3A). When sTLR2 was immunodepleted from BM, no interaction was observed between p17, p24, and gp41 (Fig. 3B). However, a slight interaction between Pam_3CSK_4 and remaining sTLR2 was observed under our experimental conditions, thus indicating that immunodepletion methods left trace amounts of sTLR2. These results demonstrated that sTLR2 directly interacted with HIV-1 PAMPs (p17, p24, and gp41).

sTLR2 inhibits HIV-induced NFκB-dependent cellular activation

Given the importance of cellular activation in HIV-1 infection [21,22], we tested whether sTLR2 containing supernatants (sTLR2-sup.), produced *in vitro*, inhibited HIV-1 PAMP induced NFκB activation. Pam_3CSK_4 or HIV-1 PAMPs (p17, p24 and gp41) were incubated with sTLR2 sup. or sTLR2-free supernatant (sTLR2-free sup.) for one hour prior to the addition to TZMbl-2 cells. Cells exposed to sTLR2-free sup. and Pam_3CSK_4

or HIV-1 PAMPs (p17 or gp41) showed increased nuclear p65 (a subunit of NFκB) translocation while sTLR2-sup inhibited nuclear p65 translocation (Fig. 4), thus indicating an important function of sTLR2 in suppressing HIV-1 PAMP-induced NFκB-dependent activation.

sTLR2 inhibits HIV-1 PAMP-induced inflammation

Using sensitive qRT-PCR quantification, we tested IL-8 expression after TZMbl-2 cells were exposed to HIV-1 PAMPs with sTLR2-sup. or sTLR2-free sup. TZMbl-2 cells, which stably express TLR2 and endogenously express TLR1, allowed us to specifically determine whether sTLR2 inhibited TLR2-dependent activation. sTLR2 and sTLR2-free supernatants, at various concentrations, were incubated with HIV-1 PAMPs (p17 or gp41) or Pam_3CSK_4 for one hour before addition to TZMbl-2 cells. cDNA analyses of exposed cells after 4 hours indicated that sTLR2 sup. inhibited Pam_3CSK_4-induced IL-8 production in a dose-dependent manner compared to sTLR2-free sup. (Fig. 5A; *P=0.0115, 0.05*). Additionally, sTLR2 sup. inhibited HIV-1 PAMPs (p17 and gp41)-induced IL-8 expression in a dose-dependent manner compared to sTLR2-free sup. (Fig. 5B & C; *P=0.0032, 0.0036, 0.0041;P=0.0086, 0.0023, 0.0007*, respectively). Collectively, these data indicated that sTLR2 inhibits HIV-1 PAMP-induced cellular activation by competitively suppressing ligand interaction with TLR2.

sTLR2 inhibits increased CCR5 expression

We and others showed an increase in CCR5 expression through TLR2 [23,24, Henrick *et al*, submitted 2013]. Given the importance of this chemokine receptor to R5-specific HIV-1 infection, we determined whether sTLR2 inhibited increased expression of CCR5. qRT-PCR analysis revealed that CCR5 expression was increased at four hours in macrophages (differentiated THP-1 cells) exposed to HIV-1 PAMPs (p17, p24 and gp41) or Pam_3CSK_4 that were pre-incubated with sTLR2-free sup. (Fig. 6A; *P=0.0092*). While CCR5 expression remained unchanged after exposure to HIV PAMPs or Pam_3CSK_4 in the presence of sTLR2-sup (Fig.6A). These data indicated a possible novel mechanism by which sTLR2 acted as a decoy receptor to inhibit HIV-1 protein-induced increase in CCR5 expression.

sTLR2 significantly inhibits cell-free R5 HIV-1 inflammation and infection

We determined whether sTLR2 inhibited pro-inflammatory cytokine production after exposure to cell-free R5 virus. HIV-1 was incubated with sTLR2-sup. or sTLR2-free sup. for one hour before its addition to TZMbl cells. Forty-eight hours after infection, IL-8 production was decreased in cells exposed to HIV-1 in the presence of sTLR2-sup. compared to sTLR2-free sup. (Fig. 6B; *P=0.001*). When sTLR2 was neutralized, using TLR2-specific monoclonal antibodies (as shown previously [9]), IL-8 production significantly increased after exposure to HIV-1 (Fig. 6B; *P=0.0151*).

We tested the ability of sTLR2 to inhibit HIV-1 infection. Cell-free R5 HIV-1, at varying concentrations ($TCID_{50}$) was pre-incubated with sTLR2-sup. or sTLR2-free sup.

for one hour before being added directly to TZMbl cells. Our results indicated that cells incubated with sTLR2-sup. had significantly lower HIV-1 infection rates at various infectious doses compared to sTLR2-free supernatants (Fig. 6C; *P=0.014, P=0.0004*, respectively). To evaluate whether sTLR2 was directly responsible for the inhibition of HIV-1 infection, sTLR2 was neutralized using TLR2-specific antibodies prior to the addition of cell-free virus, which resulted in significantly increased HIV compared to sTLR2 supernatant (Fig. 6C; *P=0.037, P=0.018*).

DISCUSSION

The low rate of transmission in infants breastfeeding from HIV-infected mothers is suggestive of the multitude of protective innate factors that are present in high concentrations in BM [5,6]. The innate factor, sTLR2, has recognized anti-microbial properties [10-12] and we previously showed that sTLR2 significantly inhibited cell-free HIV-1 infection *in vitro* [9]; however, the mechanism remained undetermined. Presently, we demonstrated that sTLR2, produced *in vitro*, directly bound to HIV-1 PAMPs, inhibited HIV-1-induced cellular activation, inhibited increased CCR5 expression and decreased HIV-1 infection *in vitro*. *In vitro* MEC and macrophages exposed to HIV-1 PAMPs increased sTLR2 production, thus providing an innate immune mechanism in which the mammary gland compensates for HIV infection. These data indicated that manipulation of local innate immune defenses might provide important prophylactic and therapeutic strategies for prevention and care of HIV-1 infection.

Chemokine receptors serve as cofactors for viral entry and HIV-1 infection [25]. CCR5 is endogenously expressed in many innate immune cells [26], mammary [27], tonsil [28] and intestinal epithelial cells [29]. We recently showed that HIV-1 PAMPs significantly increased CCR5 expression in a TLR2-dependent manner (Henrick *et al*, submitted 2013). Here, we extend these findings and show that sTLR2 inhibited CCR5 expression in MEC exposed to HIV-1 PAMPs. These findings are important since MEC might act as a viral reservoir [27]. Thus, sTLR2 might affect virus levels in BM.

TLR2 is classically considered in the context of bacterial recognition and signaling; however, evidence indicates that TLR2 is involved in viral sensing [13]. We recently provided evidence that TLR2 recognizes specific HIV-1 structural proteins (Henrick *et al*, submitted, 2013). TLR2 activation has been shown to enhance HIV infection in T cells [30], viral replication in macrophages of HIV-1 infected patients, and TLR2 expression is significantly increased in monocytes from HIV-infected individuals [23]. Here, cells from HIV-1-infected BM had elevated TLR2 expression compared to uninfected BMCs. Given that macrophages are a major cell population in BM [31] and are thought to be a predominant cell type involved in vertical HIV transmission [32], our current findings indicating increased TLR2 expression in BMCs highlights a novel HIV PAMP-induced alteration in innate immune signaling in BM.

Soluble TLRs provide a first line regulatory mechanism by binding PAMPs before recognition by their membrane-bound counterparts, thus preventing aberrant cellular activation [33]. This function might be particularly important in breastfeeding infants since neonatal lymphocyte activation is required for HIV-1 infection [34]. Viral entry itself might not equal transmission until viral integration is established in permissive cells [35]. Our finding that sTLR2 concentration was significantly elevated in HIV-1-infected BM is intriguing for three reasons: (1) sTLR2 has known anti-inflammatory properties to bacterial-induced ligands, and, we show here, significantly inhibits HIV-1-induced NFκB activation and IL-8 production in a dose-dependent manner; (2) sTLR2 directly binds to

HIV-1 structural proteins; and (3) sTLR2 inhibits TLR2-dependent, HIV-1-induced increases in CCR5 co-receptor expression, thus contributing to significantly decreased infection shown here.

sTLR2 levels correlated with IL-15 in HIV-1 infected BM. This result is intriguing since IL-15 is associated with protection against breastfeeding HIV transmission [20]. Our findings that HIV-1 PAMPs induced TLR2-dependent cellular activation are similar to previous results indicating that exposure of macrophages to HSV envelope glycoproteins stimulated pro-inflammatory production and IL-15 release through a TLR2-dependent mechanism [36,37]. Given that macrophages are a major cell type in BM [31], release sTLR2 when stimulated with HIV-1 PAMPs, and produce IL-15 through a TLR2-dependent viral protein-induced mechanism [36], we propose that BM macrophages provide crucial, compensatory innate immune protection that plays a role in inhibiting HIV MTCT. Moreover, IL-15 increases proliferation and function of NK cells which have previously been shown to control HIV replication [38]. Given the strong correlation between sTLR2 and IL-15 in BM, we speculate that these innate factors act in concert to help control HIV infection.

To the best of our knowledge, this is the first study to demonstrate that sTLR2 inhibits viral protein-induced NFκB activation. This is important since multiple viruses, including HIV-1 (Henrick *et al*, submitted, 2013), induce NFκB activation through a TLR2-dependent mechanism [13]. Specifically, sTLR2-dependent inhibition of HIV-1-

induced p65 nuclear translocation shown here is important since this factor is directly associated with secretion of pro-inflammatory cytokines, HIV-1 replication and pathogenesis [39]. These findings are similar to previous reports indicating that SLPI can affect NFκB nuclear translocation and gene expression [40]. However, contrary to SLPI, we showed direct protein-to-protein interaction between sTLR2 and HIV-1 PAMPs. Our data indicated that sTLR2 binds directly to HIV-1 proteins inhibiting infection in at least two possible ways: (1) sTLR2 inhibits HIV-1 from inducing cellular activation, thus retaining low CCR5 expression levels, typical of unstimulated cells [41] and, (2) sTLR2-gp41 binding directly impeded virus-host membrane fusion critical to HIV entry and infection [42].

sTLR2 positively correlated with p24 levels in BM, and our *in vitro* data indicated that MECs and macrophages exposed to HIV-1 PAMPs increased sTLR2 levels in cell supernatants. Indeed, monocyte/macrophage cell lines, and placental explants exposed to Pam_3CSK_4 increased sTLR2 secretion in cell supernatants [10,12]. We extended these findings to show that HIV-1-specific cellular activation promoted sTLR2 release from BMCs. This is in contrast to Heggelund *et al.* (2004) whose results did not indicate significant differences in sera sTLR2 levels between HIV-uninfected and HIV-infected patients [43]. We speculate that this observation is specific to the mammary tissue and is intriguing since it suggests a host homeostatic mechanism of the innate immune system particular to the breast. Here, HIV-1 infection stimulated increased sTLR2 release that led to decreased HIV infection, thus explaining why HIV-infected BM sTLR2

concentration positively correlated with p24 levels. Indeed, similar findings have been reported for α-defensin level, which correlated with viral burden in BM [44].

In summary, we demonstrated, for the first time, that sTLR2 inhibited HIV-1 PAMP-induced cellular activation, increased CCR5 expression and viral infection through direct interaction with HIV-1 structural proteins. Moreover, our data demonstrated that sTLR2 is significantly elevated in HIV-infected BM and positively correlated with p24 and IL-15. Importantly, MECs and macrophages exposed to HIV-1 PAMPs led to significantly increased sTLR2 levels *in vitro*. These data highlight a need to further investigate the mammary gland for critical compensatory mechanisms that are likely pivotal in the prevention of HIV-1 MTCT, and underscore the importance of BM sTLR2 in infant health and development.

MATERIALS & METHODS

Study cohort and breast milk

This study was approved by the McMaster Research Ethics Board (REB Approval #08-176), the University of Maryland Institutional Review Board and the Plateau State Specialist Hospital Ethics Committee. All participants provided voluntary written informed consent. Samples included in these analyses were obtained from women who were not taking medications (other than neviripine for HIV-infected women or vitamin supplements *intra* or post partum) and did not receive epidural intra partum. All HIV-infected Nigerian women were provided with single-dose nevirapine (200 mg tablet) at the onset of labor and to the neonate (2 mg/kg) by 48 hours of life according to the Nigerian National guideline at the time of the study. Milk samples were self-collected into sterile tubes and shipped on ice for processing in our laboratory. Samples were separated into lipid, supernatant, and cellular fractions and stored at -80°C and liquid nitrogen, respectively. BM supernatant fractions were used for dot blots and ELISA while cellular fractions were used for evaluation of TLR2 expression.

Cell Lines

Establishment of TZMbl-2 from TZMbl cell line (kindly provided by Dr. Montefiori, Duke University) was described previously (Henrick, *et al.* submitted, 2013). TZMbl and TZMbl-2 were cultured in DMEM, THP-1 (Dr. D. Bowdish, McMaster University) were cultured in RPMI 1640 supplemented with 10% FBS (Invitrogen), 10mM HEPES (Invitrogen), 2mM L-glutamine (Invitrogen), 100 units/mL

penicillin/streptomycin (Sigma-Aldrich), G418 (TZMbl-2 only). MCF-10A cells were cultured as previously described [45]. All cells were maintained at 37°C and 5% CO2. THP-1 cells were differentiated into macrophages using 50ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) supplemented media and cultured for 48 hours before use.

Reagents and antibodies

HIV-1 components included p17 (Virogen, Mississauga, Ontario, Canada), p24 (Genway Biotech, Inc., San Diego, California, USA), gp41 (Genway), gp120 (NIH AIDS Reference & Reagent Program), nef (Genway) and ssRNA40 (Mobix, McMaster University, Hamilton, Ontario, Canada). Pam_3CSK_4 (InvivoGen, Burlington, Ontario, Canada and Poly I:C (Sigma-Aldrich) was reconstituted in phosphate-buffered saline (PBS) and used at concentrations shown in the figures. The following anti-sTLR2 antibodies were used for western blots and neutralization: goat polyclonal IgG, N-17 (Santa Cruz Biotechnology; Santa Cruz, California, USA); mouse monoclonal IgG1, T2.5 (Santa Cruz Biotechnology was used for neutralization of sTLR2 in immunoassays at 200ng/mL.

Supernatants

Supernatants were collected from TZMBl-2 and TZMbl cells and tested for sTLR2 concentration using TLR2 ELISA (R&D). sTLR2 was stably produced at approximately 430pg/mL in TZMbl-2 cells while sTLR2 was not detected in supernatants from TZMbl cells (Supplementary Figure 1). Supernatants were concentrated using

Amicon 10K concentrators (Millipore, Burlington, Ontario, Canada) and sTLR2 levels were tested using TLR2 ELISA (R&D).

Nuclear extraction

TZMbl-2, THP-1, and MCF-10A cells were grown in 12-well plates and treated with HIV-1 proteins or Pam_3CSK_4 in the presence of sTLR2 medium or sTLR2-free medium for one hour at 37°C and nuclei were isolated as described previously (Henrick, *et al.* submitted, 2013).

Western Blot

Nuclear and cell lysate samples were loaded at 10 μg protein per lane and separated using SDS-PAGE in 4-15% Mini-Protean TGX gels (Bio-Rad) and transferred onto PVDF membranes as previously described [9]. Optical densitometry was determined using Un-Scan-It image digitizing software and fold expression was normalized to loading control and medium (Silk Scientific Inc., Orem, Utah, USA).

Immunodepletion of sTLR2

Immunodepletion of sTLR2 from HIV-uninfected BM samples using N-17 and non-specific normal goat IgG (Santa Cruz Biotechnologies) antibodies with Protein G magnetic Beads (Invitrogen) was described previously [9].

Dot blot

Nitrocellulose membranes were blotted with 20 pmol of viral components, negative controls, and positive controls as previously described in detail previously (Henrick, *et al.* submitted, 2013) and incubated with 1:20 diluted mock-depleted or sTLR2-depleted HIV-uninfected pooled BM, washed and incubated with anti-TLR2 antibodies (R&D), before exposure to enzyme conjugate and substrate as described for western blotting [9].

Quantitative reverse-transcriptase real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from BMCs pellets in Trizol according to the manufacturer's instructions (Invitrogen, USA), treated with DNAse I (DNA-free, Ambion, USA), reverse transcribed and qRT-PCR was completed as previously described [18]. PCR primers for IL-8, TLR2, and RPL13A were designed using the program, Primer 3.0 (http://frodo.wi.mit.edu), and were supplied by Mobix (McMaster University). The organized data was a ratio of gene quantity and normalized to RPL13A quantity, defined as relative expression.

Immunoassays

OptiEIA was used to measure IL-8 levels in cell culture supernatants according to manufacturer's instructions (BD Biosciences, Oakville, Ontario, Canada). TLR2 ELISA was used to determine sTLR2 levels in cell supernatants according to manufacturer's instructions (R&D Biosciences, Burlington, Ontario, Canada). p24 ELISA was used to determine p24 levels in HIV-infected BM according to manufacturer's instructions

(Advanced Bioscience Laboratories, Burlington, Ontario). Interleukin (IL)-15 concentration in BM supernatants was measured in a multiplex ELISA-based assay according to manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD).

HIV Preparation and Reporter Assay

R5-tropic ADA or BaL virus was prepared and tissue culture infectious dose (TCID50) of pooled supernatants as well as *in vitro* infection functional assays were determined using TZM-bl cells as described previously [46] and reported as relative light units (RLU).

Statistical analysis

Data were plotted and analyzed using Prism 5.0 software (GraphPad Software, USA). Non-parametric tests were used including Student t-test, t-tests, and correlation with Welch's correction for unmatched comparisons. Statistical significance was obtained if $P<0.05$.

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CONFLICT OF INTEREST

All authors have declared that there are no conflicts of interest.

REFERENCES

1. Coovadia HM, Rollins NC, Bland RM, et al. Mother-to-child transmission of HIV-1 infection during exclusive breastfeeding in the first 6 months of life: an intervention cohort study. Lancet **2007**; 369:1107–1116.

2. Kuhn L, Sinkala M, Kankasa C, et al. High uptake of exclusive breastfeeding and reduced early post-natal HIV transmission. PLoS ONE **2007**; 2:e1363.

3. Iliff PJ, Piwoz EG, Tavengwa NV, et al. Early exclusive breastfeeding reduces the risk of postnatal HIV-1 transmission and increases HIV-free survival. AIDS **2005**; 19:699–708.

4. Coutsoudis A, Kuhn L, Pillay K, Coovadia HM. Exclusive breast-feeding and HIV transmission. AIDS **2002**; 16:498–499.

5. Kazmi SH, Naglik JR, Sweet SP, et al. Comparison of human immunodeficiency virus type 1-specific inhibitory activities in saliva and other human mucosal fluids. Clin. Vaccine Immunol. **2006**; 13:1111–1118.

6. Lyimo MA, Howell AL, Balandya E, Eszterhas SK, Connor RI. Innate factors in human breast milk inhibit cell-free HIV-1 but not cell-associated HIV-1 infection of CD4+ cells. J. Acquir. Immune Defic. Syndr. **2009**; 51:117–124.

7. Groot F, Geijtenbeek TBH, Sanders RW, et al. Lactoferrin prevents dendritic cell-mediated human immunodeficiency virus type 1 transmission by blocking the DC-SIGN--gp120 interaction. Journal of Virology **2005**; 79:3009–3015.

8. McNeely TB, Dealy M, Dripps DJ, Orenstein JM, Eisenberg SP, Wahl SM. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. J. Clin. Invest. **1995**; 96:456–464.

9. Henrick BM, Nag K, Yao X-D, Drannik AG, Aldrovandi GM, Rosenthal KL. Milk matters: soluble Toll-like receptor 2 (sTLR2) in breast milk significantly inhibits HIV-1 infection and inflammation. PLoS ONE **2012**; 7:e40138. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=22792230&retmode=ref&cmd=prlinks.

10. Dulay AT, Buhimschi CS, Zhao G, et al. Soluble TLR2 is present in human amniotic fluid and modulates the intraamniotic inflammatory response to infection. J. Immunol. **2009**; 182:7244–7253.

11. Kuroishi T, Tanaka Y, Sakai A, Sugawara Y, Komine K-I, Sugawara S. Human

parotid saliva contains soluble toll-like receptor (TLR) 2 and modulates TLR2-mediated interleukin-8 production by monocytic cells. Mol. Immunol. **2007**; 44:1969–1976.

12. LeBouder E, Rey-Nores JE, Rushmere NK, et al. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. J. Immunol. **2003**; 171:6680–6689.

13. Worobey M, Bjork A, Wertheim JO. Point, Counterpoint: The Evolution of Pathogenic Viruses and their Human Hosts. Annu. Rev. Ecol. Evol. Syst. **2007**; 38:515–540.

14. Mogensen TH, Paludan SR. Reading the viral signature by Toll-like receptors and other pattern recognition receptors. J. Mol. Med. **2005**; 83:180–192.

15. Nabel G, Baltimore D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature **1987**; 326:711–713.

16. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. **1998**; 16:225–260.

17. O'Neill LAJ, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat. Rev. Immunol. **2007**; 7:353–364.

18. Lester RT, Yao X-D, Ball TB, et al. Toll-like receptor expression and responsiveness are increased in viraemic HIV-1 infection. AIDS **2008**; 22:685–694.

19. Ledergerber B, Flepp M, Böni J, et al. Human immunodeficiency virus type 1 p24 concentration measured by boosted ELISA of heat-denatured plasma correlates with decline in CD4 cells, progression to AIDS, and survival: comparison with viral RNA measurement. The Journal of Infectious Diseases **2000**; 181:1280–1288.

20. Walter J, Ghosh MK, Kuhn L, et al. High Concentrations of Interleukin 15 in Breast Milk Are Associated with Protection against Postnatal HIV Transmission. The Journal of Infectious Diseases **2009**; 200:1498–1502.

21. Deeks SG. Immune dysfunction, inflammation, and accelerated aging in patients on antiretroviral therapy. Top HIV Med **2009**; 17:118–123.

22. Hazenberg MD, Otto SA, van Benthem BHB, et al. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. AIDS **2003**; 17:1881–1888.

23. Heggelund L, Müller F, Lien E, et al. Increased expression of toll-like receptor 2

on monocytes in HIV infection: possible roles in inflammation and viral replication. Clin. Infect. Dis. **2004**; 39:264–269.

24. Ding J, Chang TL. TLR2 Activation Enhances HIV Nuclear Import and Infection through T Cell Activation-Independent and -Dependent Pathways. J. Immunol. **2011**;

25. Dittmar MT, McKnight A, Simmons G, Clapham PR, Weiss RA, Simmonds P. HIV-1 tropism and co-receptor use. Nature **1997**; 385:495–496.

26. Zaitseva M, Blauvelt A, Lee S, et al. Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection. Nat. Med. **1997**; 3:1369–1375.

27. Dorosko SM, Connor RI. Primary human mammary epithelial cells endocytose HIV-1 and facilitate viral infection of CD4+ T lymphocytes. J. Virol. **2010**; 84:10533–10542.

28. Kumar RB, Maher DM, Herzberg MC, Southern PJ. Expression of HIV receptors, alternate receptors and co-receptors on tonsillar epithelium: implications for HIV binding and primary oral infection. Virol. J. **2006**; 3:25.

29. Meng G, Wei X, Wu X, et al. Primary intestinal epithelial cells selectively transfer R5 HIV-1 to CCR5+ cells. Nat. Med. **2002**; 8:150–156.

30. Ding J, Rapista A, Teleshova N, et al. Neisseria gonorrhoeae enhances HIV-1 infection of primary resting CD4+ T cells through TLR2 activation. J. Immunol. **2010**; 184:2814–2824.

31. Goldman AS. Human milk, leukocytes, and immunity. J. Pediatr. **1977**; 90:167–168.

32. Satomi M, Shimizu M, Shinya E, et al. Transmission of macrophage-tropic HIV-1 by breast-milk macrophages via DC-SIGN. The Journal of Infectious Diseases **2005**; 191:174–181.

33. Liew FY, Xu D, Brint EK, O'Neill LAJ. Negative regulation of toll-like receptor-mediated immune responses. Nat. Rev. Immunol. **2005**; 5:446–458.

34. Legrand FA, Nixon DF, Loo CP, et al. Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants and neonates revealed after regulatory T cell removal. PLoS ONE **2006**; 1:e102.

35. Kourtis AP, Lee FK, Abrams EJ, Jamieson DJ, Bulterys M. Mother-to-child transmission of HIV-1: timing and implications for prevention. Lancet Infect Dis **2006**; 6:726–732.

36. Ahmad A, Ahmad R, Toma E, Morisset R, Menezes J. Impaired induction of IL-15 in response to herpes simplex virus type 1 infection in peripheral blood mononuclear cells of HIV-infected patients. AIDS **2000**; 14:744–746.

37. Leoni V, Gianni T, Salvioli S, Campadelli-Fiume G. Herpes Simplex Virus Glycoproteins gH/gL and gB Bind Toll-Like Receptor 2, and Soluble gH/gL Is Sufficient To Activate NF-κB. J. Virol. **2012**; 86:6555–6562.

38. Oliva A, Kinter AL, Vaccarezza M, et al. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. J. Clin. Invest. **1998**; 102:223–231.

39. Pereira LA, Bentley K, Peeters A, Churchill MJ, Deacon NJ. A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. Nucleic Acids Res. **2000**; 28:663–668.

40. Taggart CC, Cryan S-A, Weldon S, et al. Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding. J. Exp. Med. **2005**; 202:1659–1668.

41. Viard M, Parolini I, Sargiacomo M, et al. Role of cholesterol in human immunodeficiency virus type 1 envelope protein-mediated fusion with host cells. Journal of Virology **2002**; 76:11584–11595.

42. Chan DC, Kim PS. HIV entry and its inhibition. Cell **1998**; 93:681–684.

43. Heggelund L, Flo T, Berg K, et al. Soluble toll-like receptor 2 in HIV infection: association with disease progression. AIDS **2004**; 18:2437–2439.

44. Bosire R, John-Stewart GC, Mabuka JM, et al. Breast Milk α-Defensins Are Associated with HIV Type 1 RNA And CC Chemokines in Breast Milk But Not Vertical HIV Type 1 Transmission. AIDS Res. Hum. Retroviruses **2007**; 23:198–203.

45. Debnath J. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods **2003**; 30:256–268.

46. Jain S, Rosenthal KL. The gp41 epitope, QARVLAVERY, is highly conserved and a potent inducer of IgA that neutralizes HIV-1 and inhibits viral transcytosis. Mucosal Immunology **2011**; :1–15.

Figure 1

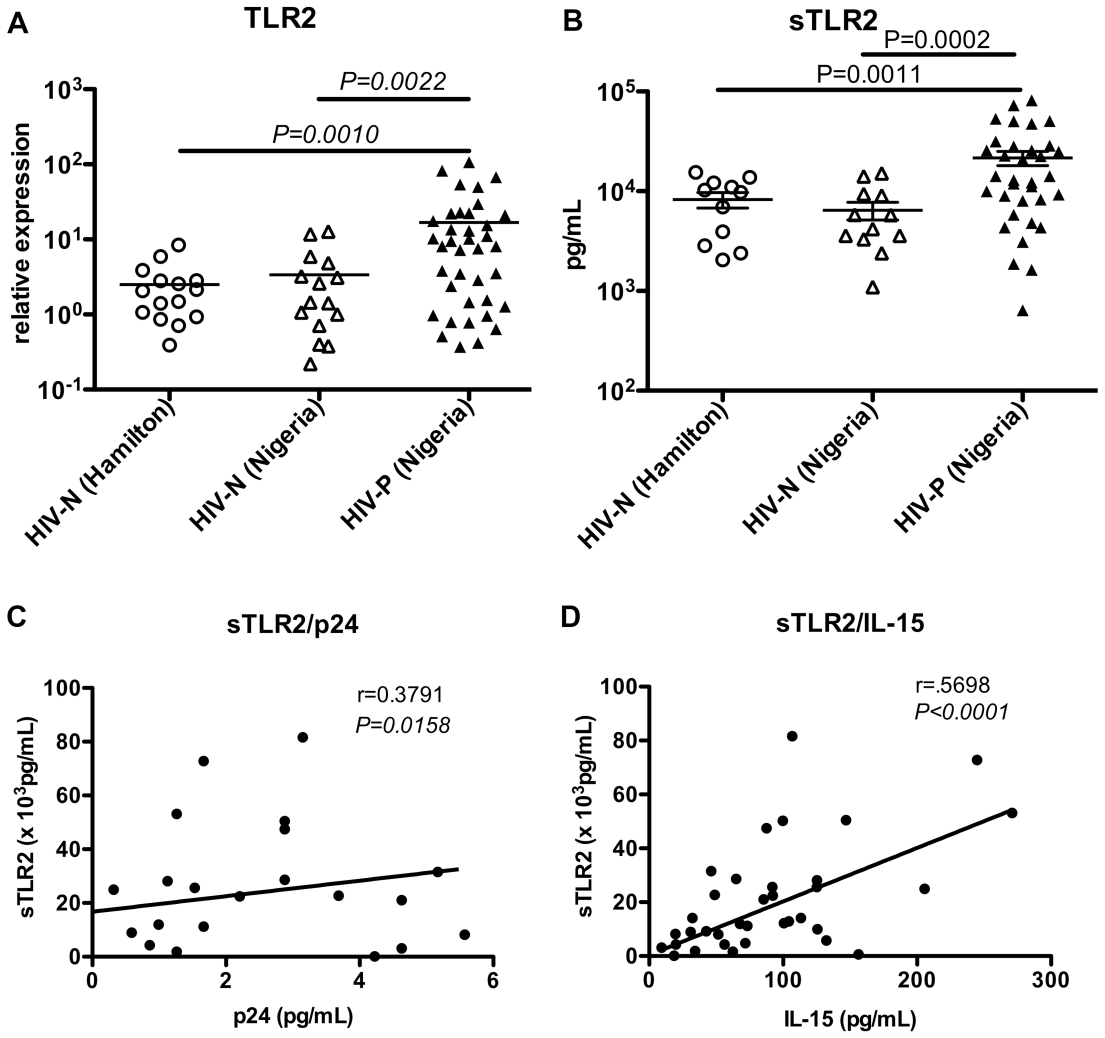
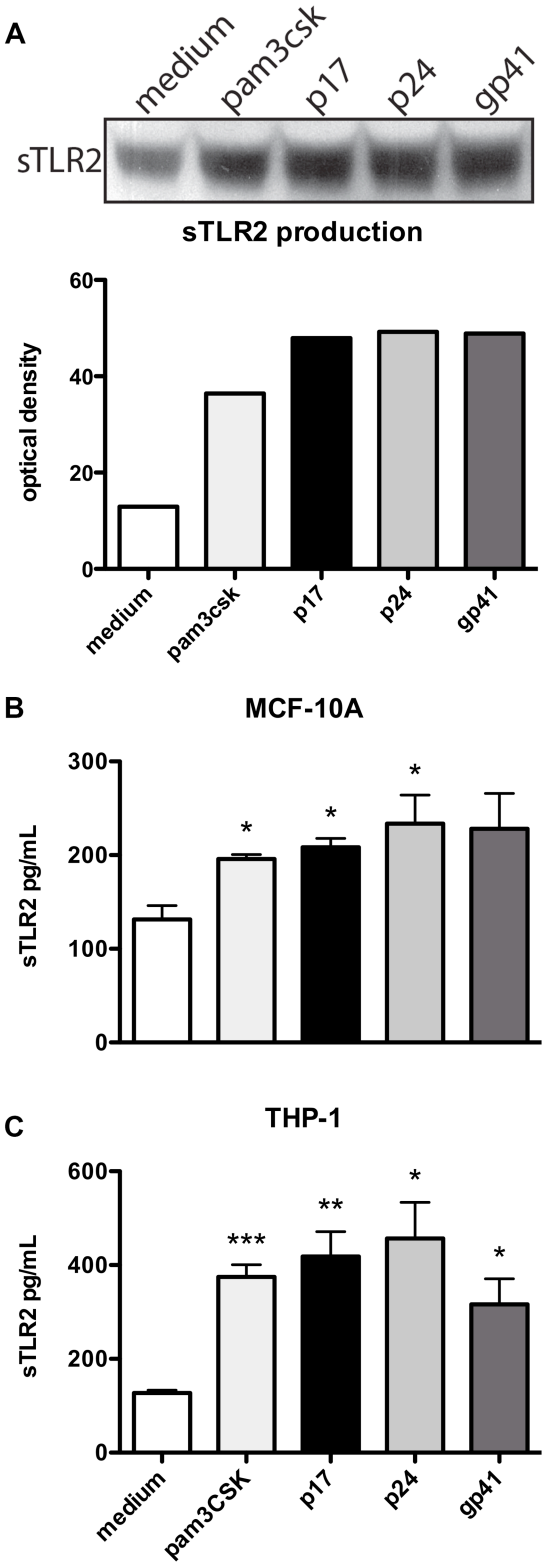


Figure 1. Significantly elevated TLR2 expression in BMCs and sTLR2 concentration in HIV-infected BM correlates with p24 and IL-15 levels. (A) Expression of TLR2 was significantly elevated in HIV-1 infected versus HIV-1 uninfected BMCs (*P=0.0291, P=0.0129).* (B) sTLR2 concentration was significantly elevated in HIV-1 infected compared to HIV-1 uninfected BM (*P=0.043, P=0.062*). (C) sTLR2 concentration correlated with p24 levels in HIV-1 infected BM (*P=0.0186*). (D) sTLR2 concentration correlated with IL-15 levels in HIV-1 infected BM (*P<0.0001).*

Figure 2


A
medium
pam3csk
p17
p24
gp41
sTLR2
sTLR2 production
optical density
60
40
20
0
medium
pam3csk
p17
p24
gp41
B
MCF-10A
sTLR2 pg/mL
300
200
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THP-1
sTLR2 pg/mL
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*
*
medium
pam3CSK
p17
p24
gp41

Figure 2. HIV-1 PAMPs significantly elevate sTLR2 concentration *in vitro.* (A) Western blot analysis of MEC exposed to HIV-1 proteins (p17 and p24) for 20 hours produced increased sTLR2 protein compared to medium alone. (B) Enzyme-linked immunosorbant assay (ELISA) quantification of sTLR2 in cell supernatants exposed as in (A). (*P=0.0396, 0.0117*). (C) Activated THP-1 cell supernatants exposed as in (A). *P<0.05, **P<0.01, ***P<0.001. Errors bars, SEM. A representative data set from triplicate experiments is shown. MCF-10A-mammary epithelial cell line. THP-1-Human monocyte cell line.

Figure 3

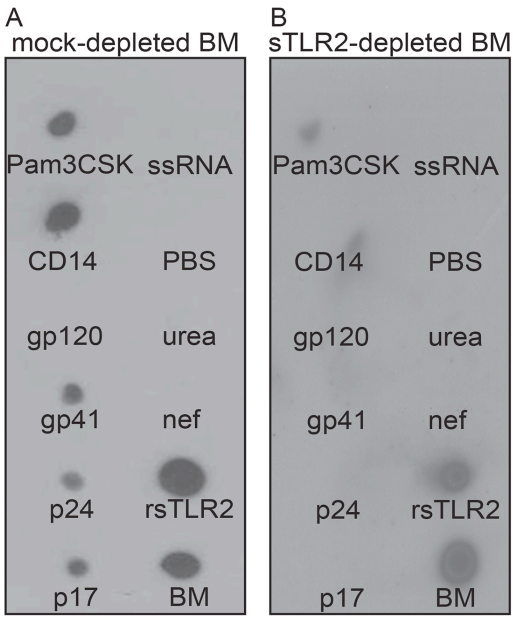

Figure 3. sTLR2 binds directly to HIV-1 structural proteins**.** (A) Diluted mock-depleted HIV-1 uninfected BM was incubated with nitrocellulose membranes previously blotted with positive controls (660 pmol Pam_3CSK_4, 20 pmol sCD14), HIV-1 components (20pmol p17, p24, gp41, gp120, ssRNA40 and nef), negative controls (1 μL PBS and 1 μL 1M urea), and membrane controls (1 ng rsTLR2, and 1:10 dilution of pooled HIV-uninfected BM) and developed using anti-TLR2 goat IgG. (B) Diluted sTLR2-depleted HIV-1 uninfected BM treated as in (A). Secondary antibodies for membranes were HRP-donkey anti-goat IgG. A representative data set of at least three independent experiments in shown.

Figure 4

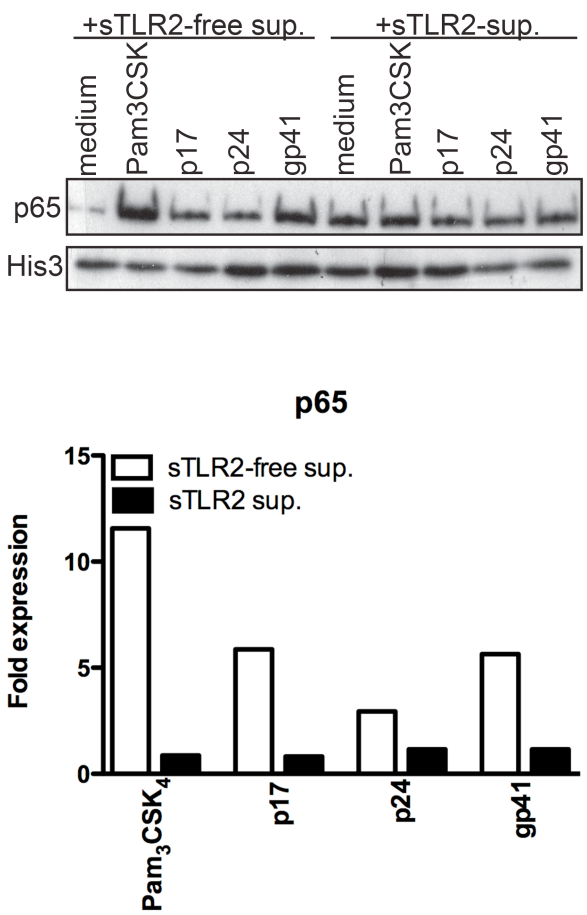

Figure 4. sTLR2 inhibits HIV-induced NFκB-dependent cellular activation**.** Supernatants from TZMbl (sTLR2-free sup.) and TZMbl-2 (sTLR2 sup.) were incubated with HIV-1 proteins (p17, p24, and gp41) or TLR2 ligand (Pam_3CSK_4) for one hour at 37°C before being placed on TZMbl-2 cells for 2 hours, and nuclear fractions were evaluated for p65 nuclear translocation. (A) SDS-PAGE gel indicates that p17 and gp41, as well as Pam_3CSK_4 incubated with sTLR2-free sup. activated nuclear translocation of p65, whereas in the presence of sTLR2 there was little to no p65 nuclear translocation for Pam_3CSK_4, p17 or gp41. Optical densitometry shows decreased p65 nuclear translocation in TZMbl-2 cells exposed to Pam_3CSK_4, p17, and gp41 in the presence of sTLR2 sup. Fold expression is pixel density normalized to loading control (histone 1) and medium. A representative figure set of at least three independent experiments in shown.

Figure 5

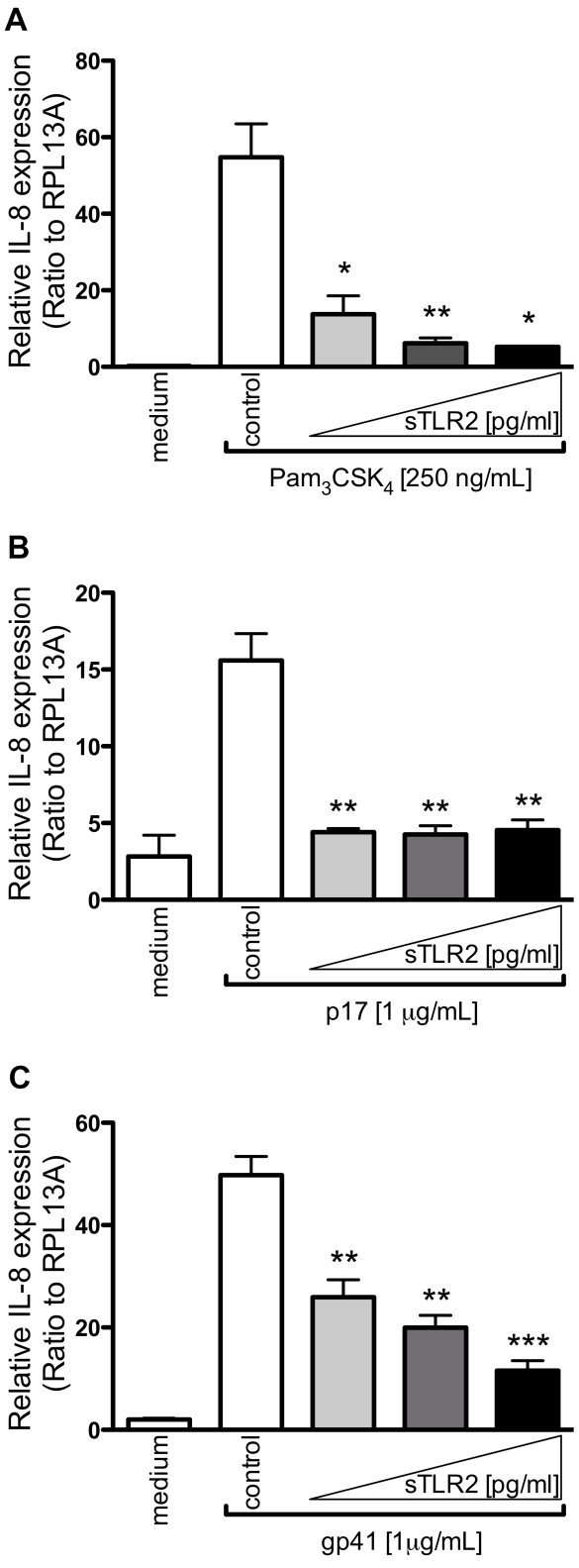
A
Relative IL-8 expression (Ratio to RPL13A)
80
60
40
20
0
*
**
*
medium
control
sTLR2 [pg/ml]
Pam3CSK4 [250 ng/mL]
B
Relative IL-8 expression (Ratio to RPL13A)
20
15
10
5
0
**
**
**
medium
control
sTLR2 [pg/ml]
p17 [1 μg/mL]
C
Relative IL-8 expression (Ratio to RPL13A)
60
40
20
0
**
**
***
medium
control
sTLR2 [pg/ml]
gp41 [1μg/mL]

Figure 5. sTLR2 inhibits HIV-1 PAMP protein-induced inflammation. TZMbl-2 supernatant (sTLR2 sup.) was collected, concentrated, and sTLR2 concentration was evaluated before being pre-incubated at various dilutions with Pam_3CSK_4, p17, and gp41 for one hour at 37°C and placed on TZMbl-2 cells. (A) IL-8 cDNA analysis after 4 hours indicated that sTLR sup. and various concentration significantly inhibited Pam_3CSK_4-induced IL-8 production in a dose dependent manner (*P=0.022, P=0.0054, P=0.0148*). (B) p17 treated as in (A; *P=0.0041, 0.0036, 0.0032*). (C) gp41 treated as in (A; *P=0.0007, P=0.0023, P=0.0086*). Data set is representative of at least three different experiments completed in triplicate and are considered statistically significant if **P<0.05, **P<0.01, ***P<0.001*.

Figure 6

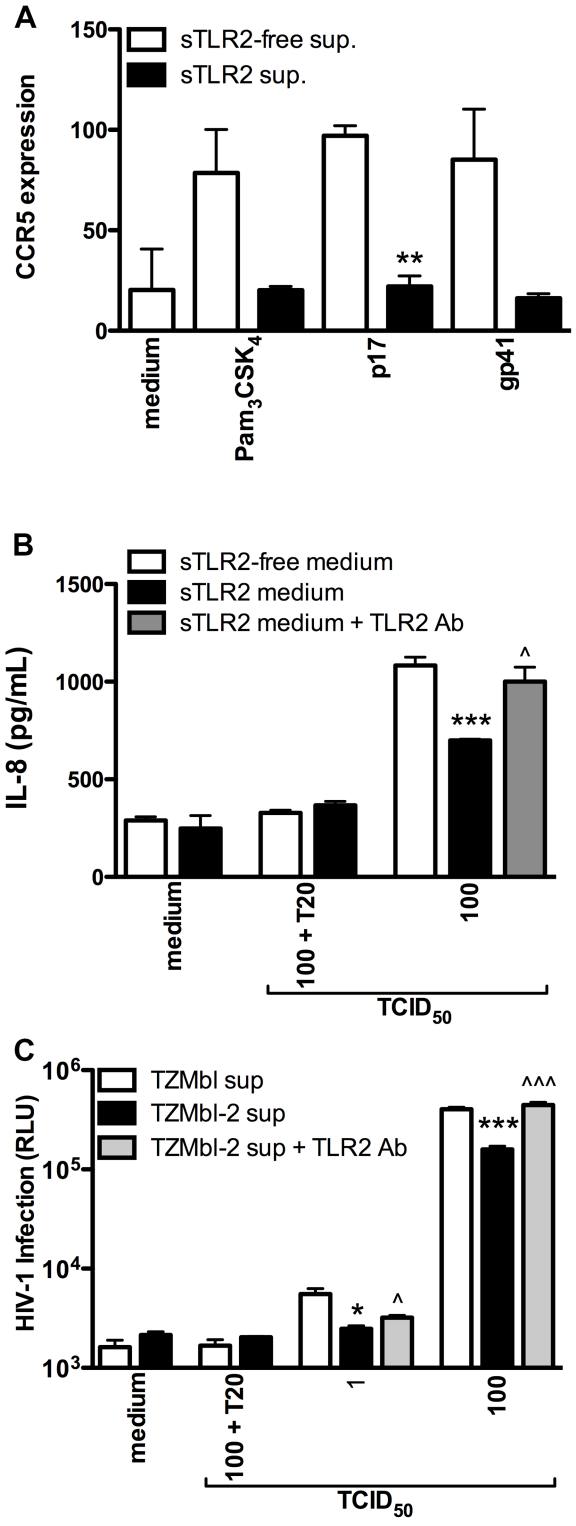
A
CCR5 expression
150
100
50
0
sTLR2-free sup.
sTLR2 sup.
**
medium
Pam3CSK4
p17
gp41
B
IL-8 (pg/mL)
1500
1000
500
0
sTLR2-free medium
sTLR2 medium
sTLR2 medium + TLR2 Ab
***
^
medium
100 + T20
100
TCID50
C
HIV-1 Infection (RLU)
10^6
10^5
10^4
10^3
TZMbl sup
TZMbl-2 sup
TZMbl-2 sup + TLR2 Ab
*
^
***
^^^
medium
100 + T20
1
100
TCID50

Figure 6. sTLR2 significantly inhibits cell-free R5 HIV-1 inflammation and infection. (A) CCR5 cDNA expression was analyzed 4 hours after exposure to HIV-1 PAMPs (p17, gl41), or positive control, TLR2 ligand (Pam_3CSK_4) with cell supernatant that did or did not contain sTLR2 (TLR2-free sup. or sTLR2 sup., respectively). Data indicated that sTLR2 sup. significantly inhibited HIV-1 PAMPs and TLR2 ligand-induced increase in CCR5 expression (*P=0.0024*). (B) IL-8 production after 48 hours was investigated in TZMbl cells exposed to cell-free R5 virus with or without supernatant containing sTLR2, and sTLR2 sup. show a significant decrease in IL-8 production after HIV-1 exposure compared to sTLR2-free sup (*P=0.0010*), and significantly increased when sTLR2 was neutralized with specific antibody (*P=0.0151*). (C) HIV-1 infection was evaluated in TZMbl cells exposed to various doses of cell-free R5 virus with or without sTLR2-containing supernatant, and shows a significant decrease in HIV-1 infection in the presence of sTLR2 sup. compared to sTLR2-free sup. (*P=0.0004*), which was neutralized using TLR2-specific antibody (*P=0.0008*).

Figure S1

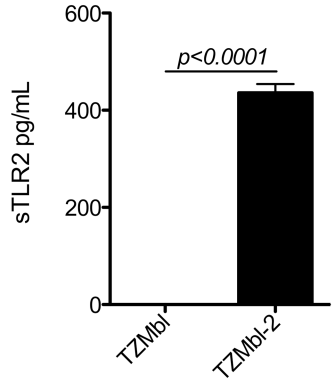

Figure S1. TZMbl and TZMbl-2 cells were grown to confluency and supernatants were tested for sTLR2 concentration using a TLR2 ELISA. Data indicated that TZMbl-2 cells produced 432 pg sTLR2/mL supernatant, which was significantly elevated compared sTLR2 levels in TZMbl supernatant. Data set is representative of at least three different experiments completed in triplicate and are considered statistically significant if **P<0.05, **P<0.01, ***P<0.001*.

— CHAPTER 4 —

Unlikely Suitors: Identification of HIV-1 Ligands that Activate Through TLR2 and Significantly Increase HIV Infection/Integration[1]

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This manuscript investigated the role of TLR2 in sensing HIV-1 structural proteins and increasing HIV integration. Specifically, we stably transfected an HIV infection reporter assay, TZMbl, to express non-endogenous TLR2, and then assessed cellular activation after exposure to HIV structural proteins. Further, we evaluated HIV integration rates in TZMbl-2 compared to TZMbl cells. From this study, we discovered that HIV structural proteins, p17, p24 and gp41, bind to TLR2. Furthermore, p17 and gp41 induced NFκB-dependent cellular activation through a TLR2/1 heterodimerization, which could be blocked by p24. However, p24 promoted inflammation through TLR2/6 heterodimers. Importantly, HIV proteins that induced cellular activation significantly increased CCR5 expression thus providing a mechanism by which HIV integration was significantly elevated in cells expressing TLR2 compared to those that did not. Taken together, these data indicated that TLR2 expression played a role in sensing of HIV-1 proteins, inducing cellular activation, and increasing HIV-1 infection

Unlikely Suitors: Identification of HIV-1 Ligands that Activate Through TLR2 and Significantly Increase HIV Infection/Integration[1]

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Running Title: Identification of Novel HIV PAMPs for TLR2

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[1]Abbreviations used in this article: HAART, highly active antiretroviral therapy; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; sTLR2, soluble Toll-like receptor 2.

word count (abstract): 236

word count (body including methods & materials): 5819

ABSTRACT

Immune activation is a critical driver of HIV infection and pathogenesis; however, our understanding of HIV innate immune activation remains incomplete. Here, we investigated TLR2 as an innate PRR for HIV-1 proteins using TZMbl cells stably expressing TLR2 (TZMbl-2). Our results demonstrate that HIV-1 structural proteins p17, p24, and gp41 act as viral pathogen-associated molecular patterns (PAMPs) and are recognized by TLR2 leading to significantly increased cellular activation via the NFκB signaling pathway. Initial studies demonstrated direct protein interactions between p17, p24, gp41 with TLR2, while only p17 and gp41 bound with TLR1. Specifically, TLR2/1 heterodimer recognized p17 and gp41 leading to cellular activation, while p24 signaled through TLR2/6. These results were confirmed using TLR2/1 siRNA knock down assays which ablated p17 and gp41-induced IL-8 production and through studies of HEK293 cells expressing selected TLRs. Interestingly, p24 blocked p17 and gp41-induced cellular activation in a TLR2-dependent manner, thus providing a novel mechanism by which HIV can manipulate innate sensing. Importantly, significantly increased HIV-1 infection/integration occurred in TZMbl-2 cells compared to cells lacking TLR2, and mechanistically this appeared to be due to increased expression of CCR5. Taken together, our results identify, for the first time, novel HIV-1 PAMPs that play a role in cellular activation via TLR2, and show that cellular TLR2 expression significantly increased HIV infection/integration. These results have important implications for our fundamental understanding of HIV immune activation and pathogenesis, and for HIV vaccine development.

INTRODUCTION:

Chronic immune activation is a fundamental driver of HIV-1 infection, replication, and pathogenesis (1, 2). Although our knowledge of HIV-1 infection and pathogenesis continues to expand, our understanding of how HIV-1 is recognized by the innate immune system remains poorly understood. Currently, HIV-1 immune activation is believed to be primarily driven by translocation of bacterial and viral components from the gut (3). Indeed, increased lipopolysaccharide (LPS) levels in sera, a result of microbial translocation through the gut-associated lymphoid tissue (GALT), were strongly correlated with immune activation in chronically HIV-1-infected individuals (3). However, HIV-1 itself is an important contributor to chronic immune activation. In untreated HIV-1-infected individuals we showed that significant and progressive increases in Toll-like receptor (TLR) expression occurred and became progressively dysfunctional (4). Moreover, following antiretroviral therapy (ARV), TLR expression was normalized, suggesting that HIV itself was involved in increased expression (4). Thus, we hypothesized that HIV-1 components play a fundamental role in facilitating immune activation that is central to this infection.

HIV-1, a single-stranded ribonucleic acid (RNA) virus from the lentivirus genus, is composed of multiple structural proteins including envelope glycoproteins 120 (gp120) and gp41, capsid protein p24 and matrix protein p17 (5). Importantly, the vast majority of viral particles released from HIV-infected cells are considered non-infectious (6), thus creating a milieu of viral antigens that are potentially highly immunogenic and possess differing abilities to activate the innate immune system (7). Specifically, gp120 is known

to deregulate the biological activity of many cell types, as well as being responsible for the immunostimulatory effects related to HIV-1-associated dementia (8). gp41 significantly enhanced HIV-1 infection and replication (9). p24 stimulated peripheral blood mononuclear cells (PBMCs) of HIV-1-infected individuals receiving HAART (10). Further, p17 possesses potent immunostimulatory properties, and *in vitro* increased HIV replication in activated PBMCs, possibly through a recently-described interaction with CXCR1 (11, 12). Thus, HIV-1 creates a more suitable environment for its own integration and replication via virus-induced innate immune activation.

Surprisingly, HIV proteins have not been well studied for their ability to serve as viral pathogen-associated molecular patterns (PAMPs). To date, despite the potent ability of HIV-1 to initiate the signaling events responsible for immune cellular activation, the only HIV-1 PAMP identified is uridine-rich HIV-1 ssRNA (13).

Currently, 10 TLRs have been identified in humans (14). TLRs have proven fundamental in the early recognition of many pathogen classes and are largely responsible for activating innate immunity and shaping subsequent adaptive immune responses (15, 16). Typically, the recognition of viral PAMPs via TLR engagement triggers a signaling cascade resulting in the activation of transcription factor, nuclear factor kappa B (NFκB), leading to the downstream production of anti-viral and pro-inflammatory cytokines (17). While, untreated HIV-1 infection has been associated with progressively increased TLR expression (4) and induction of NFκB, the particular components that engage TLRs are only beginning to be elucidated. Furthermore, given that NFκB is particularly

important during HIV infection, for host immune activation as well as viral replication due to the presence of NFκB binding sites found within HIV-1's long terminal repeats (18), it is necessary to understand the interaction between HIV PAMPs and TLR engagement.

While classically considered in the context of bacterial recognition, TLR2 is unique among the TLR family in that it can heterodimerize with co-receptors TLR1, 6 and 10 (19, 20). These co-receptors allow TLR2 to markedly increase the diversity of PAMPs recognized. Of particular interest, a number of viral proteins have been identified as novel PAMPs for TLR2 including cytomegalovirus (CMV) glycoprotein B (21), herpes simplex virus (HSV) gH/gL and gB (22), hepatitis C virus (HCV) core protein (23), and measles virus hemagglutinin A glycoprotein (24). In addition, the extracellular portion of TLR2 can be found in soluble form systemically and in mucosal fluids. Indeed, we and others (25-28), showed that soluble TLR2 (sTLR2) can inhibit production of pro-inflammatory cytokines. Importantly, we also demonstrated that sTLR2 directly inhibited cell-free HIV-1 infection *in vitro* (28), thus indicating that sTLR2 might directly interact with HIV-1. These findings motivated us to hypothesize that TLR2 acts as an innate PRR for the recognition of HIV-1 structural proteins.

In the present study, we investigated the engagement between TLR2 and HIV-1 proteins and demonstrated that HIV-1 proteins, p17, p24 and gp41 represent a novel cluster of viral PAMPs that are recognized by TLR2 heterodimers. Interestingly, while engagement of TLR2/1 by HIV-1 proteins p17 and gp41 led to significant increases in pro-inflammatory cytokine production via NFκB activation, p24 did not activate innate

responses. Instead, p24 blocked p17 and gp41-induced pro-inflammatory cytokine production. Importantly, TLR2-expressing cells showed significantly increased HIV infection/integration levels compared to cells that did not express TLR2. Taken together, our results identify, for the first time, novel HIV-1 PAMPs that play an important role in cellular activation via TLR2, which in turn, significantly increased HIV-1 infection and integration.

MATERIALS & METHODS

Cell lines and Toll-like Receptor Ligands

TZMbl (JC53-BL) cells (kindly provided by Dr. D. Montefiori, Duke University, North Carolina) were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen, Burlington, Ontario, Canada), 10 mM HEPES (Invitrogen), 2mM L-glutamine (Invitrogen), 100 units/mL penicillin/streptomycin (Sigma-Aldrich, Oakville, Ontario, Canada). TZMbl-2 cells required 10% FBS (Invitrogen), 10 mM HEPES (Invitrogen), 2mM L-glutamine (Invitrogen), 100 units/mL penicillin/streptomycin (Sigma-Aldrich), 0.8mg/mL Geneticin (G418; Invitrogen) selection media. HEK293-TLR2, TLR2/1, and TLR2/6 stably transfected human embryonic kidney cells (InvivoGen, Burlington, Ontario, Canada) were cultured in DMEM supplemented with 10% FBS, 10mM HEPES, 2 mM L-glutamine, 100 units/mL penicillin/streptomycin, 10 mg/mL blasticidin (InvivoGen). All cell lines were maintained at 37°C in 5% CO_2.

HIV-1 components included p17 (Virogen, Mississauga, Ontario, Canada), p24 (Genway Biotech, Inc., San Diego, California, USA), gp41 (Genway), gp120 was kindly provided by NIH AIDS Research and Reference Reagent Program, nef (Genway) and ssRNA40 (Mobix, McMaster University, Hamilton, Ontario, Canada). Pam_3CSK_4 (InvivoGen, Burlington, Ontario, Canada and Poly I:C (Sigma-Aldrich, Oakville, Ontario, Canada) was reconstituted in phosphate-buffered saline (PBS) and used at concentrations shown in the Figs.

Establishment of a stable TLR2 transfected TZMbl-2 cell line

cDNA constructs for human TLR2 were generated from PBMC by RT-PCR and cloned into pcDNA3.1(+)-002 (kindly provided by Dr. Jonathan Bramson, McMaster University). The plasmid was operationally named phTLR2. phTLR2 was used as a template with XhoI and BamHI endonucleases at either end, respectively, and PCR-amplified before insertion into pIRES2-ZsGreen1 vector (Clontech, Burlington, Ontario, Canada). DNA sequencing confirmed the structure of the recombinant plasmid pIhT2G, and TLR2 protein expression was confirmed using western blot analysis. pIhT2G was transfected into TZMbl cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Selection of transfected cells was completed every 48 hours post-transfection by serial dilutions with medium containing 0.8mg/mL G418. Long-term expression of TLR2 in these stably transfected TZMbl cells (TZMbl-2) was confirmed using RT-PCR and western blot analyses up to passage 15, and all testing was completed in cells before this mark.

Transient transfection of TZMbl cell line

TZMbl cells were plated in 24-well plates and left overnight. At approximately 80% confluence cells were transfected for 24 hours with Lipofectamine 2000 (Invitrogen) containing 0.5 μg/well of plasmid phTLR2, phTLR3 or vector pcDNA3.1(+)-002, respectively. The cells were superinfected with 100TCID50 HIV-1 BaL for 48 h. Total cellular DNA was extracted for analysis of proviral DNA quantity.

Viral stocks and reporter assay

HIV-1 R5-tropic BaL was prepared and tissue culture infectious doses (TCID) of

pooled supernatants as well as *in vitro* functional assays were determined using TZMbl and TZMBL-2 cells, as previously described (29). Briefly, 3.5 x 10^4 cells/well were plated in 96-well plates with 25μg/ml diethylaminoethyl-dextran (Sigma). Luciferase activity from TZMbl and TZMbl-2 cells were measured using Bright-Glo reagents (Promega, Madison, Wisconsin, USA), analyzed using a Veritas luminometer (Promega) and reported as relative light units (RLU).

Quantitative reverse-transcriptase real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from Trizol samples according to the manufacturer's instructions (Invitrogen, USA) and treated with DNAse I (DNA-free, Ambion, USA). Reverse transcription (RT) reactions were prepared by adding 500 ng DNA-free total RNA to 20 μL manufacturer's reagents (Invitrogen). RT was conducted with incubations at 25°C for 5 min, 50°C for 50 min and 70°C for 15 min. cDNA was prepared at various dilutions for qRT–PCR of selected genes and an internal control, RPL13A. PCR primers for COX-2, TNF-α, and HIV Pol were designed using the program, Primer 3.0 (http://frodo.wi.mit.edu), and were supplied by Mobix (McMaster University). qRT–PCR, with one RT sample in duplicate, was performed in a 20 μl reaction containing 2 μl diluted complimentary DNA, 200 nmol forward primer, 200 nmol reverse primer, and 10 μL KAPA SYBR qPCR Master Mix (KAPA Biosystems, Burlington, Ontario, Canada) in a 96-well plate with the sequencing detection system 7900HT (Applied Biosystems, Burlington, Ontario, Canada). The organized data was a ratio of gene quantity and normalized to RPL13A quantity, defined as relative expression.

siRNA knockdown

TZMbl-2 cells ($2x10^4$) were transfected with Lipofectamine RNAiMAX (Invitrogen) and 20 nM siRNA individually, including a negative control siRNA (Invitrogen, Cat. # 129201 H07/129296 H05), TLR1 siRNA (Sigma-Aldrich, Cat. # SASI_Hs01_00162170/AS) and TLR2 siRNA (Sigma-Aldrich, Cat. # SASI_Hs01_ 00081589/AS) or with 15 nM of both TLR1 siRNA and TLR2 siRNA together in a 96-well plates in reverse manner according to the manufacturer's instructions. 48 hr post-transfection, the cells were either harvested for detection of protein expression using western blot analyses or further treated with HIV-1 proteins for 18 h to test IL-8 production levels using enzyme-linked immunosorbant assay (ELISA) (BD Biosciences, Mississauga, Ontario, Canada).

Nuclear extraction

TZMbl-2 cells were grown in 12-well plates and treated with HIV-1 proteins and Pam_3CSK_4 for selected periods and nuclei were isolated after cells were washed with dilute PBS, immersed in 160 µl of a hypotonic buffer, and then 200 µl of 1% Triton X-100 buffer to rupture cellular membranes. After centrifugation at 12000 ×g for 3 min, the nuclear pellets were dissolved in a high salt buffer containing protease inhibitor and phosphotase inhibitor cocktails (PhosStop Mini Complete; Roche Applied Science, Burlington, Ontario, Canada). The supernatants were collected and protein concentrations were determined using the DC assay (Bio-Rad, Burlington, Ontario, Canada).

Western Blot

TZMbl-2 and TZMbl total cell lysates, membranes, and cytosol were evaluated

for total protein concentration using DC assay prior to being boiled in 5x Laemmli reducing buffer and resolved in a SDS-PAGE gel for the evaluation of TLR protein expression and phosphorylation of IκBα. Signals were detected and developed as previously described (28). Briefly, TLR-specific primary antibodies included: anti-TLR1 goat polyclonal IgG (R&D Systems, Burlington, Ontario, Canada); anti-TLR2 goat polyclonal IgG (R&D Systems) and anti-TLR2 goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, California, USA); anti-TLR6 goat polyclonal IgG (Santa Cruz Biotechnology). The secondary reagents used in these assays included: HRP-labeled donkey anti-goat IgG (Santa Cruz Biotechnology), HRP-labeled mouse anti-rabbit IgG (Pierce Biotechnology Inc, Mississauga, Ontario, Canada), HRP-labeled chicken anti-mouse IgG (Santa Cruz Biotechnology). Optical densitometry was determined using Un-Scan-It image digitizing software and normalized to β-actin levels (Silk Scientific Inc., Orem, Utah, USA).

Nuclear samples were loaded at 10 μg protein per lane and separated using SDS-PAGE in 4-15% Mini-Protean TGX gels (Bio-Rad) and transferred onto PVDF membranes. After blocking with 5% skim milk powder-Tris buffered saline-0.1% Tween-20 (TBS-t), the membranes were exposed to primary antibodies including: anti-IκBα rabbit polyclonal IgG (Cell Sciences Inc, Burlington, Ontario, Canada). β-actin primary antibodies included, anti-β-actin mouse monoclonal IgG (Cell Sciences) in 5% skim milk powder-TBS-t at 4^{o}C overnight. The membranes were extensively washed and reacted with goat anti-rabbit IgG-HRP (Bio-Rad), in 5 % skim milk powder-TBS-t at RT for 1 hr,

washed, and stained with either Super Signal West Pico Chemiluminescent Substrate or Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, Illinois, USA) before being exposed to CL-X Posure film (Thermo).

Dot blot

Nitrocellulose membranes were blotted with 20 pmol of viral components, p17 (Virogen), p24 (Genway), gp41 (Genway), gp120 (kindly provided by NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), and ssRNA40 (Mobix, McMaster University). As negative controls, all solutions (Urea) and glutathione transferase enzyme tag (GST, Virogen) were similarly blotted. Positive protein control including, sCD14 (Cell Sciences, Burlington, Ontario, Canada) was blotted at 20 onto the nitrocellulose membrane. Once dried and blocked, the membranes were probed with the cell membrane fractions of either TZMbl or TZMbl-2, and incubated with detection antibodies, anti-TLR1 or anti-TLR2, before exposed to enzyme conjugate and substrate as described for the western blot (Material and Methods above).

Endotoxin

Endotoxin detection assay (kindly provided by Dr. Bowdish, McMaster University) was completed according to the manufacturer's instructions (Lonza, Burlington, Ontario, Canada). Briefly, the supplied endotoxin standard was prepared into serial dilutions. 100μL of standard, blank, and samples were added in duplicate to separate wells in a 96-well plate. The plate was pre-incubated in the reader at 37°C for a

minimum of ten minutes. The working reagent contained a 5:4:1 ratio of fluorogenic substrate, assay buffer, and rFC enzyme solution was added to each well and fluorescence was determined at time zero. The plate was incubated for one hour and examined again. The difference between fluorescence at time zero and 60 mins was corrected to the blank and plotted against the log of endotoxin concentration as a linear regression curve. Endotoxin concentrations in the samples were determined using the standard curve.

Immunoassays

OptiEIA was used to measure IL-8 levels in cell culture supernatants according to manufacturer's instructions (BD Biosciences). Briefly, plates were incubated with capture antibody overnight at 4°C before being extensively washed with PBS 0.05% tween-20 and blocked with 10% FBS in PBS. Samples and standards were diluted in blocking buffer and aliquoted into 96-well plates for 2 hours at room temperature before being washed and coated with detection antibodies. After 1 hour incubation at room temperature, the plates were washed and substrate was added for up to 20 mins. 2N sulfuric acid was added to halt the reaction and absorbance was determined using at BIO-TEK EL800 at 450 nanometers.

Statistical analysis

Data were plotted and analyzed using Prism 5.0 software (GraphPad Software, USA). Non-parametric tests were used including Student t-test and t-tests with Welch's correction for unmatched comparisons. Statistical significance was obtained if $P<0.05$.

RESULTS

Characterization of TLR expression in TZMbl cells and establishment of functional stably transformed TLR2 cell line, TZMbl-2

In order to investigate the effect cellular TLR2 expression had on host innate responses to HIV-1 proteins and infection, we initially set out to utilize the well-characterized HIV-1 luciferase reporter assay, TZMbl cell line. Early results showed that TZMbl cells do not endogenously express TLR2, consequently we established a stably transformed TZMbl cell line, TZMbl-2, that showed a substantial increase in TLR2 transcription and protein expression (Fig. 1A and B). In addition TLR1 protein was detected in TZMbl and TZMbl-2 cytosol fractions (Fig. 1B). We also observed low levels of cDNA expression of TLR6 but not at the protein level (Fig. 1A). TZMbl-2 exposed to TLR2 ligand, Pam_3CSK_4, produced significantly increased IL-8 levels in a dose-dependent manner compared to TZMbl (Fig. 1C, *$P<0.05, 0.001, 0.01$, respectively*). Taken together, these results indicated that TZMbl-2 cells expressed TLR2 and TLR1, and developed a proinflammatory response to synthetic bacterial TLR2/1 ligand, Pam_3CSK_4.

HIV-1 proteins bind TLR2 from TZMbl-2 cell lysates

Physical interaction of TLR2 and specific HIV-1 proteins were determined by utilizing a previously described dot blot detection method (30). Recombinant HIV-1 proteins (p17, p24, gp41, gp120, and nef), positive controls CD14 and 1:20 diluted human breast milk, which contains high levels of sTLR2 (25, 28), as well as negative carrier

controls (urea solution, PBS, GST) were blotted onto a nitrocellulose membrane. After blocking, the membranes were exposed to TZMbl or TZMbl-2 cell lysates and viral protein-TLR interactions were identified using TLR2 and TLR1-specific antibodies. The results indicated HIV-1 proteins p17, p24, and gp41 directly interacted with membrane bound TLR2 (Fig. 2C), whereas no interaction was detected between TLR2 and gp120, nef, or ssRNA40 (Fig. 2C). As well, very strong interactions between TLR2 and the positive control, CD14 were detected (Fig. 2C), which was critical because it indicated direct protein-to-protein interactions. TZMbl lysate incubation did not reveal a TLR2-viral protein interaction, and indirectly demonstrated that there was no non-specific antibody binding between blotted proteins and antibodies used (Fig. 2B). Dot blots exposed to TZMbl lysates probed with anti-TLR1 antibodies indicated strong interactions between TLR1 and viral proteins p17 and gp41, but not with p24 or other viral components (Fig. 2A). In addition, TLR1 did not interact with CD14, which is important since CD14 is a TLR2-specific signaling co-factor (Fig. 2A). These results demonstrated direct protein-to-protein interactions between TLR2/1 and HIV-1 structural proteins but not regulatory protein, nef. Specifically, TLR2 preferentially bound p17, p24, and gp41 while TLR1 only interacted with p17 and gp41.

HIV-1 proteins activate phosphorylation of IκBα and translocation of p65

PAMPs recognized by TLRs typically induce pro-inflammatory responses via the phosphorylation of IκBα and subsequent translocation of NFκB transcription factors (17). Given the central role of NFκB to immune responses, we sought to qualitatively assess the impact of HIV-1 protein exposure on the phosphorylation of IκBα in TZMbl-2 cells.

Following exposure to HIV-1 proteins (p17, p24, gp41, and gp120), positive control (Pam_3CSK_4), and negative control (medium), western blot analysis of TZMbl-2 cell lysates showed a substantial increase in phosphorylated IκBα in cells exposed to p17, gp41, and Pam_3CSK_4, compared to medium and gp120 (Fig. 3A). Interestingly, p24 did induce a moderate increase in phosphorylated IκBα (Fig. 3A). Further, assessment of NFκB subunit p65 nuclear translocation in TZMbl-2 cells revealed substantially increased p65 in nuclear fractions of TZMbl-2 cells that were exposed to p17, gp41 and Pam_3CSK_4, but not in cells exposed to medium or p24 (Fig. 3A).

Given that HIV-1 infection induces COX-2 in multiple cell types (31, 32), we examined COX-2 mRNA in TZMbl-2 cells exposed to HIV-1 proteins (p17, p24, gp1, and gp120), positive control (Pam_3CSK_4), and negative control (medium). Our results showed significantly increased COX-2 cDNA expression levels in TZMbl-2 cells exposed to p17 and gp41, and Pam_3CSK_4 compared to medium (Fig. 3B; *P=0.0033; P=0.0022; P=0.0002 respectively*). Since TNF-α is strongly implicated in HIV-1 pathogenesis (33), we examined cDNA expression using qRT-PCR, and showed significantly increased TNF-α expression in TZMbl-2 cells exposed to p17 and gp41, and Pam_3CSK_4 compared to medium (Fig. 3C; *P=0.0058, P=0.0064, P=0.0051*, *respectively*).

We next determined the effect of viral protein exposure on pro-inflammatory cytokine production. TZMbl-2 and TZMbl cells were exposed to various concentrations of viral proteins (p17, p24, gp41, and gp120), positive control (Pam_3CSK_4) and negative controls (medium and protein tag glutathione transferase; GST). Results showed

significantly increased IL-8 production in TZMbl-2 after exposure to p17, gp41, and Pam_3CSK_4 compared to medium (Fig. 3D, *P=0.0002, P=0.0004, P<0.0001, respectively*), but not in TZMbl cells (Fig. S1). p24 and gp120 protein exposure did not substantially increase IL-8 production in either TZMbl-2 (Fig. 3D) or TZMbl cell lines (Fig. S1).

To rule out the possibility that induction of IL-8 might be due to lipoprotein contamination all components were tested for the presence of endotoxin, and data indicated that all recombinant proteins, including Pam_3CSK_4, p17, p24, gp41, gp120, and GST had undetectable to very low levels of endotoxin that were well below levels found in fetal bovine serum used in cell medium (Table S1).

Collectively, these data indicate that HIV-1 structural proteins, p17 and gp41, induced a TLR2-dependent pro-inflammatory response via NFκB signaling pathway.

HIV-1 proteins, p17 and gp41, signal through TLR2/1 heterodimer

To understand the mechanism by which HIV-1 proteins signal through TLR2 and/or its binding partner, TLR1, we performed a series of siRNA knockdown assays. Western blot analyses of total cell lysates showed that specific siRNA knockdown substantially reduced endogenous TLR1 and TLR2 protein expression in TZMbl-2 cells compared to non-specific control (Fig. 4A). After siRNA knockdown, TZMbl-2 cells were exposed to viral proteins overnight and IL-8 levels were assessed. Our results indicated that single siRNA knockdown of TLR1 or TLR2 ablated viral p17 and gp41-induced IL-8 production, as well as significantly reduced Pam_3CSK_4-induced

IL-8 levels (Fig. 4B and C). In the absence of both TLR1 and TLR2 expression, little to no IL-8 response was elicited in the presence of p17 or gp41, and a significant reduction in Pam_3CSK_4–induced cytokine production (Fig. 4D), thus confirming that both TLR1 and TLR2 are involved in recognizing these HIV-1 structural proteins.

To confirm that particular HIV proteins elicited pro-inflammatory responses specifically through TLR2 and its co-receptors, HEK293 cells expressing TLR2, TLR2/1 or TLR2/6 were evaluated. HEK293-TLR2 cells were shown to express low levels of cDNA for TLR1 and TLR2 (Fig. 5A, insert). Exposure to various concentrations of gp41 significantly increased IL-8 levels in a dose-dependent manner; whereas no other viral protein produced substantial increases in IL-8 levels (Figure 5A; *P=0.0042, 0.0136, respectively*). TLR2/1 cDNA expression levels were extremely high in HEK293-TLR2/1 cells (Fig. 5B insert), and they produced significantly elevated IL-8 levels after exposure to p17 and gp41 compared to medium, while, p24 and gp120 did not induce a pro-inflammatory response (Fig. 5B; *P=0.0062; P=0.0143, 0.0024, 0.0411; respectively*). HEK293-TLR2/6 cell line expressed cDNA for TLR2, and both heterodimers TLR1 and TLR6 (Fig. 5C, insert). Exposure to various concentrations of recombinant viral proteins (p17, gp41, and p24) induced significantly increased levels of IL-8, while gp120 did not induce IL-8 compared to medium (Fig. 5C; *P<0.0001; P=0.005, P=0.0106, P=0.0151; P=0.0127, P=0.0097; P=0.0003, P=0.0002, P=0.0012, respectively*). IL-8 was profoundly induced with gp41, probably due to higher amounts of TLR1/2/6 expression in HEK293-TLR2/6 cell line. Pam_3CSK_4 (positive control) increased IL-8 production in all three cell types (Fig. 5A, B, C; *P<0.0001, P=0.0044, P<0.0001*). Taken together,

these data indicated specific synergistic effects of the TLR2/1 heterodimer in sensing HIV proteins, p17 and gp41, and demonstrated that TLR2/6 heterodimer senses p24 leading to cellular activation and production of pro-inflammatory cytokine.

p24 blocks p17 and gp41 induced production of pro-inflammatory cytokines

Our previous data showed a strong protein-to-protein interaction between p24 and TLR2, yet failure to induce a pro-inflammatory response in TZMbl-2 cells. Therefore, we next sought to determine whether p24 was interacting with TLR2 in a manner that impacted TLR2-dependent pro-inflammatory activation induced by p17 and gp41.

TZMbl-2 cells were incubated with increasing concentrations of p24 for one hour prior to the addition of cellular activators, Pam_3CSK_4, p17, or gp41, and resulted in a significant dose-dependent attenuation in IL-8 production following exposure to p17 and gp41 (Fig. 6B & C; *P=0.0054, P=0.0047, P=0.0016, P=0.0034; P=0.011, P=0.0028, P=0.0006, P=0.0003, respectively*), yet had little to no effect on the inhibition of Pam_3CSK_4-induced production of IL-8 (Fig. 6A).

Next, TZMbl-2 cells simultaneously exposed to p24 and Pam_3CSK_4, p17, or gp41 indicated that p24 did not inhibit Pam_3CSK_4 or gp41 cellular activation (Fig. 6D & F). However, p24 did significantly block p17-induced production of IL-8 in a dose-dependent manner (Fig. 6E, *P=0.0135, P=0.0473, P=0.0421, P=0.0345, respectively*).

Collectively, these data suggest that p24 can block activation by p17 and gp41 HIV structural proteins, but not Pam_3CSK_4, and taken together with our dot blot assay

indicates that HIV viral proteins are capable of manipulating innate immune signaling through a TLR2-dependent mechanism.

Cellular TLR2 expression is associated with increased HIV-1 infection/integration

Given that multiple viruses induce cellular activation via TLR2 as a means to facilitate entry (21, 23, 24), and data here showing an interaction between TLR2 and specific HIV-1 structural proteins, we next sought to explore our hypothesis that cellular expression of TLR2 increased cell-free HIV-1 infection and integration.

Previously, Heggelund *et al.* (2004) demonstrated that TLR2 stimulation increased CCR5 protein expression on monocytes (34). Given the importance of the HIV-1 co-receptor CCR5 expression to viral entry and integration, we determined whether THP-1, a monocyte/macrophage cell line, exposed to viral proteins (p17, p24, gp41, gp120) or Pam_3CSK_4 increased CCR5 expression. Evaluation of cDNA indicated that CCR5 expression was significantly elevated after four-hour exposure to p17 and gp41, as well as Pam_3CSK_4 compared to medium (Fig. 7A; *P=0.0014, 0.0256, 0.0027,* respectively).

To determine whether TLR2 expression could significantly increase HIV-1 integration into permissive cells, we first transiently-transfected empty plasmid, TLR2, or TLR3 into TZMbl cells and infected the cells for 48 hours. Our results indicated that TLR2-transfected cells showed significantly increased HIV-1 proviral integration compared to empty-plasmid control, whereas no significant increases in integration were identified in empty plasmid or TLR3-transfected TZMbl cells (Fig. 7B; *P=0.0256*).

Next, we determined the optimal time of HIV-1 integration following infection of TZMbl cells using qRT-PCR analysis for the proviral gene Pol, and found integration levels peaked at 8 hours post exposure in TZMbl cells (Fig. S2). Using these data, TZMbl-2 and TZMbl were infected with cell-free R5 virus and DNA evaluated at 8 hours post infection indicated that TZMbl-2 had significantly increased HIV-1 integration rates compared to TZMbl cells at various infectious doses (Fig. 7C; *P=0.0044, P=0.017, P=0.0132*, respectively).

Taken together these data indicated that cellular activation via TLR2 played an important role in increased expression of HIV-1 co-receptor CCR5 and led to significantly increased HIV infection/integration rates compared to cells that did not express TLR2.

DISCUSSION

Increasing evidence supports the notion that chronic immune activation is a central characteristic of HIV-1 infection and the progression to AIDS (1, 2). Despite this understanding, the ability of HIV to alter innate immune responses as a means of perpetuating chronic activation is only beginning to be fully elucidated. Currently, a key driver of chronic immune activation is bacterial translocation from the HIV damaged intestinal tract (3). Indeed, during acute HIV infection mucosal CD4 T cells are dramatically depleted, especially in the intestine (35), and increased systemic LPS levels strongly correlate with immune activation in chronically infected individuals (3). However, we previously showed that initiation of ART normalized TLR expression despite increased serum LPS levels (36), indicating that although bacterial translocation is an important immune activator, HIV-1 itself might play an important role in driving chronic immune activation (4). Indeed, HIV-1 ssRNA triggers cellular activation through endosomal RIG-I and TLR7/8 sensing (37). However, we and others previously showed that TLR expression, including TLR2, significantly and progressively increased as untreated HIV infection advanced (4). Several reports also highlight the importance of TLR2 expression in sensing multiple viral proteins which lead to increased cellular activation and facilitate viral entry (21-24). In addition, we recently reported that sTLR2 directly inhibited HIV-1 infection *in vitro* (28). Given the correlation between TLR2 expression and HIV disease progression, the critical role of TLR2 in recognizing multiple viral proteins, and the direct inhibition of infection by sTLR2, led us to hypothesize that HIV-1 proteins might play a fundamental role in driving innate immune activation and

lead to increased viral infection. Thus, in the present study, we identified a number of novel HIV-1 PAMPS, demonstrating that specific HIV proteins can engage and activate membrane bound TLR2, while others block TLR2 activation. Moreover, we demonstrated that TLR2 expression significantly increased HIV-1 infection/integration *in vitro*. Collectively, we provide an illustrated summary of our results of HIV PAMPs interactions with PRRs and TLR2 heterodimer signaling in Fig. 8.

To investigate the role of TLR2 in sensing HIV-1 structural proteins, we developed a TLR2-stably transfected TZMbl (TZMbl-2) cell line. TZMbl cells were a rationale choice given their stable expression of canonical HIV-1 receptors and Tat-regulated reporter genes, allowing for sensitive and reproducible quantification of HIV-1 integration (38). As well, stable expression of the PRR allowed us to focus our studies specifically on TLR2 signaling and validate our findings with reasonably designed controls. TZMbl-2 cells express TLR2/TLR1heterodimer at the mRNA and protein level, and together recognize HIV p17 and gp41. Given that TLR2/1 classically recognizes triacylated lipopeptides by forming M-like shapes (39), as well as our findings that HIV proteins do not block Pam_3CSK_4-induced activation, the heterodimer configuration that recognizes HIV-1 proteins requires further investigation. In addition, HEK293 and its derived cell lines expressing TLR2 and TLR2 co-receptors, HEK293 TLR2, HEK293 TLR2/1 and HEK293 TLR2/6 were also used in this study, and the experimental data from these cell lines strongly support our findings. Indeed, further investigation of TLR2's role in HIV-1 sensing and immunopathology is an important endeavor and

requires further investigation using primary cells and *ex vivo* samples from HIV-1 infected individuals.

Previous publications have provided evidence that multiple HIV-1 proteins play key roles in altering cellular activity. Indeed, p17 has previously been shown to induce the production of pro-inflammatory cytokines *in vitro* and *in vivo* (40, 41), and act as an adjuvant in vaccine strategies tested in animal models (42). Additionally, a recent publication showed that gp41 activated NFκB in exposed lymphocytes (43), and gp120 altered both pro- and anti- inflammatory responses *in vitro* (44-46). Taken together with data indicating HIV-1 infected cells shed enormous amounts of viral proteins (6), it seemed critical to determine the role this milieu provided in driving immunopathogenesis. In line with previous publications, our data confirms and extends these findings by demonstrating a direct role of TLR2/1 recognition of p17 and gp41 that induced activation of a key transcription factor, NFκB. This is an important step in not only the TLR2 signaling cascade pathway but is also required for induction of HIV-1 gene expression via viral long terminal repeat (LTR) binding (18). Nuclear translocation of p65 led to significantly increased pro-inflammatory gene expression and production of pro-inflammatory cytokine, IL-8, in a TLR2-dependent manner that might correlate with the increased IL-8 levels observed during HIV-1 infection (47). Importantly, endotoxin testing revealed little to no LPS contamination in protein preparations. Additionally, siRNA knockdown of TLR2 and/or TLR1 but not TLR4 ablated the production of pro-inflammatory cytokines after HIV-1 PAMP exposure, and HIV-1 PAMPs did not induce

production of IL-8 in TZMbl cells which endogenously express TLR4 but not TLR2. Moreover, HEK293 cells lines do not express TLR4, yet responded to specific HIV proteins in a TLR2-dependent manner. Taken together, these results indicate a novel mechanism in which HIV-1 structural proteins induce cellular activation and increased HIV-1 integration through TLR2 that was not due to endotoxin contamination.

Surprisingly, gp120 had little to no effect on cytokine production or IκBα phosphorylation, which were unexpected given recent publications showing the induction of pro-inflammatory cytokines in genital epithelial cells (44), and indicate that cellular differences, including PRR expression, might be critical for viral pathogenesis and requires further investigation. To our knowledge, investigating whether HIV proteins can engage and activate membrane-bound TLR2 has not previously been demonstrated, however CXCR1 has recently been identified as a p17 receptor that triggered adhesion and chemotactic-like migration in monocytes (11). Given that CXCR1 is not expressed on many epithelial cell types, including HeLa (48), the data provided here revealed TLR2 as a novel innate HIV structural protein PRR. Further, p24 did not induce IL-8 production in TZMbl-2 cells, despite the strong protein-to-protein interaction observed in our TLR2 dot blot analyses, which indicated that p24 did not interact with TLR1. However, HEK293-TLR2/6 cells, which express TLR1, TLR2, and TLR6 at the protein level (49), produced significantly increased IL-8 levels after exposure to p24 in a dose-dependent manner. Thus indicating that TLR6 is a required TLR2 heterodimer in order to recognize p24 and stimulate cellular activation. Importantly, in the absence of TLR6 expression, p24 blocked p17- and gp41-induced pro-inflammatory cytokine production in

a dose-dependent manner. These data are particularly intriguing since they provide evidence that HIV has a mechanism by which it can manipulate the innate immune system balance between viral protein-induced cellular activation and quiescence in a TLR2-dependent manner. However, p24 was unable to block Pam_3CSK_4-induced cellular activation, which is intriguing as it is well documented that Pam_3CSK_4 binds in the crevice of the m-shaped heterodimer produced through the interaction of TLR2 and TLR1 (39, 50). Therefore, we speculate that HIV proteins bind to alternate conformations or in different regions of this heterodimer than previously described for Pam_3CSK_4.

Previous publications showed a TLR2-dependent increase in surface HIV co-receptor, CCR5 expression on permissible cells (51, 52). With this in mind, we tested CCR5 expression after p17, gp41, and p24 exposure and found significantly increased expression of CCR5 after cellular exposure to p17. These data showed an important innate immune mechanism by which HIV-1 proteins can promote infection by significantly increasing co-receptor expression on target cells. Several publications demonstrated a TLR2-mediated enhancement of HIV infection/integration in resting T cells (52). Interestingly, our evaluation of PRR expression in a population of highly exposed seronegative (HESN) female commercial sex workers (CSWs) showed significantly lower expression of TLR2 in cervical epithelial and cervical mononuclear cells compared to HIV-uninfected and HIV-infected CSWs from the same cohort (Yao et al, 2013 *manuscript submitted*). Thus, data here may partially account for a mechanism by which HESN CSWs remain negative despite working in an HIV endemic area.

In conclusion, the present investigation extends our current understanding of innate sensing of HIV-1 and for the first time reveals novel HIV-1 PAMPs, including p17, p24 and gp41, that can manipulate innate sensing and immune activation. Further, our finding that TLR2 expression significantly increased HIV integration via increased CCR5 expression provides a mechanism by which HIV-1 can regulate host infection and persistence. Thus, perhaps by manipulating innate activation, one can flush out the reservoir of latently HIV-infected cells. Additionally, the data shown here indicated that TZMbl-2 cells are more highly activated after exposure to HIV-1 proteins and have significantly increased HIV infection/integration rates compared to TZMbl cells. Therefore, TZMbl-2 cells might be more suitable for assessing HIV-1 neutralizing antibodies and microbicides. Lastly, identification of these novel HIV PAMPs could be utilized as adjuvants or delivery systems to promote immunogenicity of HIV vaccines. Thus, these results have important implications for our understanding of fundamental innate immune activation by HIV-1 and may provide insight into the design of novel vaccine strategies.

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REFERENCES

1. **Hazenberg MD**, **Otto SA**, **van Benthem BHB**, **Roos MTL**, **Coutinho RA**, **Lange JMA**, **Hamann D**, **Prins M**, **Miedema F**. 2003. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. AIDS **17**:1881–1888.
2. **Deeks SG**, **Kitchen CMR**, **Liu L**, **Guo H**, **Gascon R**, **Narváez AB**, **Hunt P**, **Martin JN**, **Kahn JO**, **Levy J**, **McGrath MS**, **Hecht FM**. 2004. Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. Blood **104**:942–947.
3. **Brenchley JM**, **Price DA**, **Douek DC**. 2006. HIV disease: fallout from a mucosal catastrophe? Nat. Immunol. **7**:235–239.
4. **Lester RT**, **Yao X-D**, **Ball TB**, **McKinnon LR**, **Kaul R**, **Wachihi C**, **Jaoko W**, **Plummer FA**, **Rosenthal KL**. 2008. Toll-like receptor expression and responsiveness are increased in viraemic HIV-1 infection. AIDS **22**:685–694.
5. **Freed EO**. 1998. HIV-1 gag proteins: diverse functions in the virus life cycle. Virology **251**:1–15.
6. **Dimitrov DS**, **Willey RL**, **Sato H**, **Chang LJ**, **Blumenthal R**, **Martin MA**. 1993. Quantitation of human immunodeficiency virus type 1 infection kinetics. Journal of Virology **67**:2182–2190.
7. **Wagner R**, **Fliessbach H**, **Wanner G**, **Motz M**, **Niedrig M**, **Deby G**, **Brunn von A**, **Wolf H**. 1992. Studies on processing, particle formation, and immunogenicity of the HIV-1 gag gene product: a possible component of a HIV vaccine. Arch. Virol. **127**:117–137.
8. **Yang B**, **Akhter S**, **Chaudhuri A**, **Kanmogne GD**. 2009. HIV-1 gp120 induces cytokine expression, leukocyte adhesion, and transmigration across the blood-brain barrier: modulatory effects of STAT1 signaling. Microvasc. Res. **77**:212–219.
9. **Zhou C**, **Lu L**, **Tan S**, **Jiang S**, **Chen Y-H**. 2011. HIV-1 glycoprotein 41 ectodomain induces activation of the CD74 protein-mediated extracellular signal-regulated kinase/mitogen-activated protein kinase pathway to enhance viral infection. Journal of Biological Chemistry **286**:44869–44877.
10. **Terzieva V**, **Popova D**, **Kicheva M**, **Todorova Y**, **Markova R**, **Martinova F**, **Elenkov I**, **Yankova M**. 2009. Correlation between the degree of immune activation, production of IL-2 and FOXP3 expression in CD4+CD25+ T regulatory cells in HIV-1 infected persons under HAART. Int. Immunopharmacol. **9**:831–836.
11. **Giagulli C**, **Magiera AK**, **Bugatti A**, **Caccuri F**, **Marsico S**, **Rusnati M**, **Vermi W**, **Fiorentini S**, **Caruso A**. 2012. HIV-1 matrix protein p17 binds to the IL-8 receptor CXCR1 and shows IL-8-like chemokine activity on monocytes through Rho/ROCK activation. Blood **119**:2274–2283.
12. **De Francesco MA**, **Caruso A**, **Fallacara F**, **Canaris AD**, **Dima F**, **Poiesi C**, **Licenziati S**, **Corulli M**, **Martinelli F**, **Fiorentini S**, **Turano A**. 1998. HIV p17 enhances lymphocyte proliferation and HIV-1 replication after binding to a human

serum factor. AIDS **12**:245–252.
13. **Heil F**, **Hemmi H**, **Hochrein H**, **Ampenberger F**, **Kirschning C**, **Akira S**, **Lipford G**, **Wagner H**, **Bauer S**. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science **303**:1526–1529.
14. **Connolly DJ**, **O'Neill LAJ**. 2012. New developments in Toll-like receptor targeted therapeutics. Curr Opin Pharmacol **12**:510–518.
15. **Aderem A**, **Ulevitch RJ**. 2000. Toll-like receptors in the induction of the innate immune response. Nature **406**:782–787.
16. **Janeway CA**, **Medzhitov R**. 2002. Innate immune recognition. Annu. Rev. Immunol. **20**:197–216.
17. **Kawai T**, **Akira S**. 2007. Antiviral signaling through pattern recognition receptors. J. Biochem. **141**:137–145.
18. **Nabel G**, **Baltimore D**. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature **326**:711–713.
19. **Guan Y**, **Ranoa DRE**, **Jiang S**, **Mutha SK**, **Li X**, **Baudry J**, **Tapping RI**. 2010. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. J. Immunol. **184**:5094–5103.
20. **Ozinsky A**, **Underhill DM**, **Fontenot JD**, **Hajjar AM**, **Smith KD**, **Wilson CB**, **Schroeder L**, **Aderem A**. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc. Natl. Acad. Sci. U.S.A. **97**:13766–13771.
21. **Boehme KW**, **Guerrero M**, **Compton T**. 2006. Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells. J. Immunol. **177**:7094–7102.
22. **Leoni V**, **Gianni T**, **Salvioli S**, **Campadelli-Fiume G**. 2012. Herpes Simplex Virus Glycoproteins gH/gL and gB Bind Toll-Like Receptor 2, and Soluble gH/gL Is Sufficient To Activate NF-κB. J. Virol. **86**:6555–6562.
23. **Dolganiuc A**, **Oak S**, **Kodys K**, **Golenbock DT**, **Finberg RW**, **Kurt-Jones E**, **Szabo G**. 2004. Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. Gastroenterology **127**:1513–1524.
24. **Bieback K**, **Lien E**, **Klagge IM**, **Avota E**, **Schneider-Schaulies J**, **Duprex WP**, **Wagner H**, **Kirschning CJ**, **Meulen Ter V**, **Schneider-Schaulies S**. 2002. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. J. Virol. **76**:8729–8736.
25. **LeBouder E**, **Rey-Nores JE**, **Rushmere NK**, **Grigorov M**, **Lawn SD**, **Affolter M**, **Griffin GE**, **Ferrara P**, **Schiffrin EJ**, **Morgan BP**, **Labéta MO**. 2003. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. J. Immunol. **171**:6680–6689.
26. **Kuroishi T**, **Tanaka Y**, **Sakai A**, **Sugawara Y**, **Komine K-I**, **Sugawara S**. 2007. Human parotid saliva contains soluble toll-like receptor (TLR) 2 and modulates TLR2-mediated interleukin-8 production by monocytic cells. Mol. Immunol. **44**:1969–1976.
27. **Dulay AT**, **Buhimschi CS**, **Zhao G**, **Oliver EA**, **Mbele A**, **Jing S**, **Buhimschi IA**.

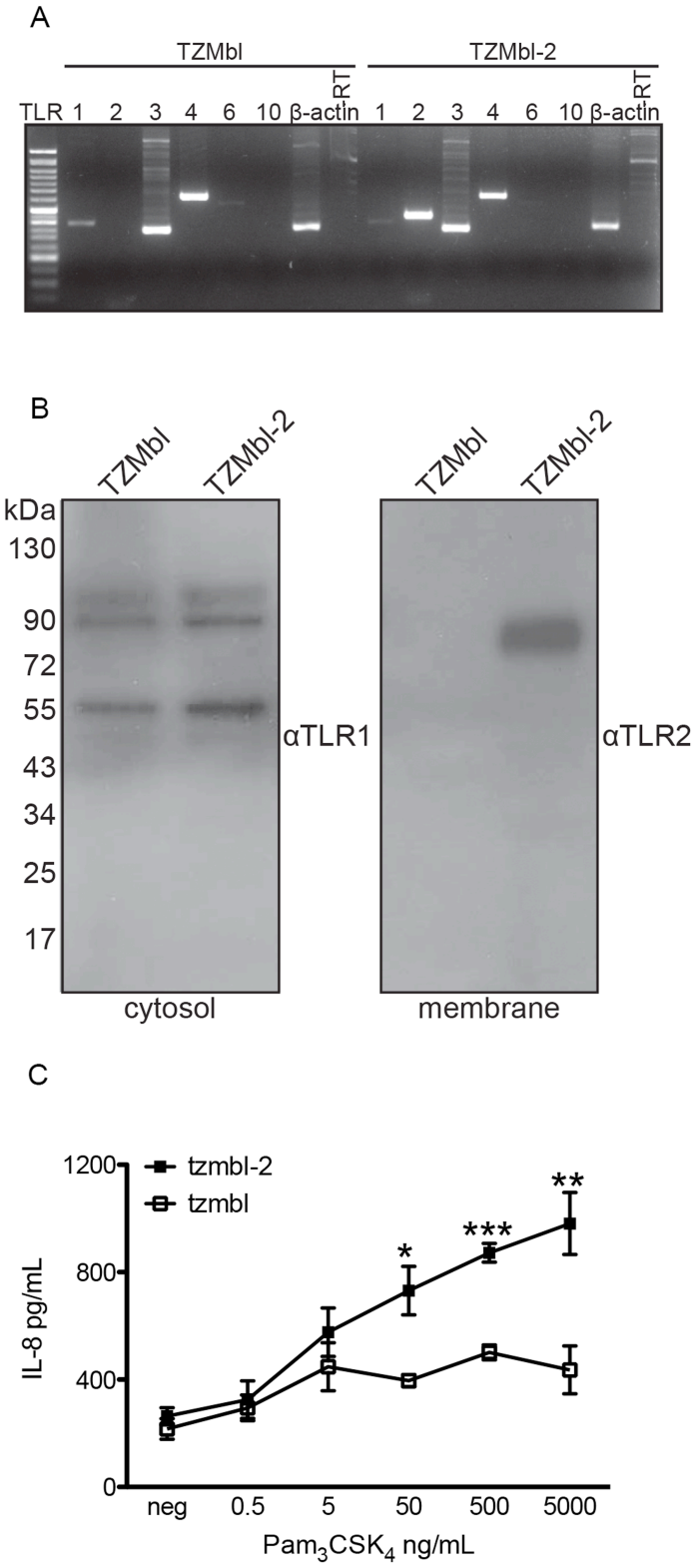
2009. Soluble TLR2 is present in human amniotic fluid and modulates the intraamniotic inflammatory response to infection. J. Immunol. **182**:7244–7253.

28. **Henrick BM**, **Nag K**, **Yao X-D**, **Drannik AG**, **Aldrovandi GM**, **Rosenthal KL**. 2012. Milk matters: soluble Toll-like receptor 2 (sTLR2) in breast milk significantly inhibits HIV-1 infection and inflammation. PLoS ONE **7**:e40138.
29. **Jain S**, **Rosenthal KL**. 2011. The gp41 epitope, QARVLAVERY, is highly conserved and a potent inducer of IgA that neutralizes HIV-1 and inhibits viral transcytosis. Mucosal Immunology 1–15.
30. **Thacker TC**, **Johnson FB**. 1998. Binding of bovine parvovirus to erythrocyte membrane sialylglycoproteins. J. Gen. Virol. **79 (Pt 9)**:2163–2169.
31. **Pereira CF**, **Boven LA**, **Middel J**, **Verhoef J**, **Nottet HS**. 2000. Induction of cyclooxygenase-2 expression during HIV-1-infected monocyte-derived macrophage and human brain microvascular endothelial cell interactions. J. Leukoc. Biol. **68**:423–428.
32. **Ramis I**, **Roselló-Catafau J**, **Gómez G**, **Zabay JM**, **Fernández Cruz E**, **Gelpí E**. 1991. Cyclooxygenase and lipoxygenase arachidonic acid metabolism by monocytes from human immune deficiency virus-infected drug users. J Chromatogr A **557**:507–513.
33. **Fauci AS**, **Schnittman SM**, **Poli G**, **Koenig S**, **Pantaleo G**. 1991. NIH conference. Immunopathogenic mechanisms in human immunodeficiency virus (HIV) infection. Ann. Intern. Med. **114**:678–693.
34. **Heggelund L**, **Damås JK**, **Yndestad A**, **Holm AM**, **Mūller F**, **Lien E**, **Espevik T**, **Aukrust P**, **Frøland SS**. 2004. Stimulation of toll-like receptor 2 in mononuclear cells from HIV-infected patients induces chemokine responses: possible pathogenic consequences. Clin. Exp. Immunol. **138**:116–121.
35. **Sandler NG**, **Douek DC**. 2012. Microbial translocation in HIV infection: causes, consequences and treatment opportunities. Nat. Rev. Microbiol. **10**:655–666.
36. **Lester RT**, **Yao X-D**, **Ball TB**, **McKinnon LR**, **Omange WR**, **Kaul R**, **Wachihi C**, **Jaoko W**, **Rosenthal KL**, **Plummer FA**. 2009. HIV-1 RNA dysregulates the natural TLR response to subclinical endotoxemia in Kenyan female sex-workers. PLoS ONE **4**:e5644.
37. **Iwasaki A**. 2012. Innate Immune Recognition of HIV-1. Immunity **37**:389–398.
38. **Montefiori DC**. 2009. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol. Biol. **485**:395–405.
39. **Jin MS**, **Kim SE**, **Heo JY**, **Lee ME**, **Kim HM**, **Paik S-G**, **Lee H**, **Lee J-O**. 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell **130**:1071–1082.
40. **Fiorentini S**, **Giagulli C**, **Caccuri F**, **Magiera AK**, **Caruso A**. 2010. HIV-1 matrix protein p17: a candidate antigen for therapeutic vaccines against AIDS. Pharmacol. Ther. **128**:433–444.
41. **De Francesco MA**, **Baronio M**, **Fiorentini S**, **Signorini C**, **Bonfanti C**, **Poiesi C**, **Popovic M**, **Grassi M**, **Garrafa E**, **Bozzo L**, **Lewis GK**, **Licenziati S**, **Gallo RC**, **Caruso A**. 2002. HIV-1 matrix protein p17 increases the production of proinflammatory cytokines and counteracts IL-4 activity by binding to a cellular

receptor. Proc. Natl. Acad. Sci. U.S.A. **99**:9972–9977.

42. **Becker PD**, **Fiorentini S**, **Link C**, **Tosti G**, **Ebensen T**, **Caruso A**, **Guzmán CA**. 2006. The HIV-1 matrix protein p17 can be efficiently delivered by intranasal route in mice using the TLR 2/6 agonist MALP-2 as mucosal adjuvant. Vaccine **24**:5269–5276.
43. **Postler TS**, **Desrosiers RC**. 2012. The Cytoplasmic Domain of the HIV-1 Glycoprotein gp41 Induces NF-κB Activation through TGF-β-Activated Kinase 1. Cell Host and Microbe **11**:181–193.
44. **Nazli A**, **Chan O**, **Dobson-Belaire WN**, **Ouellet M**, **Tremblay MJ**, **Gray-Owen SD**, **Arsenault AL**, **Kaushic C**. 2010. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. PLoS Pathog. **6**:e1000852.
45. **Borghi P**, **Fantuzzi L**, **Varano B**, **Gessani S**, **Puddu P**, **Conti L**, **Capobianchi MR**, **Ameglio F**, **Belardelli F**. 1995. Induction of interleukin-10 by human immunodeficiency virus type 1 and its gp120 protein in human monocytes/macrophages. Journal of Virology **69**:1284–1287.
46. **Schneider J**, **Kaaden O**, **Copeland TD**, **Oroszlan S**, **Hunsmann G**. 1986. Shedding and interspecies type sero-reactivity of the envelope glycopolypeptide gp120 of the human immunodeficiency virus. J. Gen. Virol. **67 (Pt 11)**:2533–2538.
47. **Matsumoto T**, **Miike T**, **Nelson RP**, **Trudeau WL**, **Lockey RF**, **Yodoi J**. 1993. Elevated serum levels of IL-8 in patients with HIV infection. Clin. Exp. Immunol. **93**:149–151.
48. **Kampik D**, **Schulte R**, **Autenrieth IB**. 2000. Yersinia enterocolitica invasin protein triggers differential production of interleukin-1, interleukin-8, monocyte chemoattractant protein 1, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor alpha in epithelial cells: implications for understanding the early cytokine network in Yersinia infections. Infect. Immun. **68**:2484–2492.
49. **Chang S**, **Dolganiuc A**, **Szabo G**. 2007. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. J. Leukoc. Biol. **82**:479–487.
50. **Kang JY**, **Lee J-O**. 2011. Structural biology of the Toll-like receptor family. Annu. Rev. Biochem. **80**:917–941.
51. **Heggelund L**, **Müller F**, **Lien E**, **Yndestad A**, **Ueland T**, **Kristiansen KI**, **Espevik T**, **Aukrust P**, **Frøland SS**. 2004. Increased expression of toll-like receptor 2 on monocytes in HIV infection: possible roles in inflammation and viral replication. Clin. Infect. Dis. **39**:264–269.
52. **Ding J**, **Chang TL**. 2011. TLR2 Activation Enhances HIV Nuclear Import and Infection through T Cell Activation-Independent and -Dependent Pathways. J. Immunol.

Figure 1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TZMbl
TZMbl-2
TLR 1 2 3 4 6 10 β-actin RT
1 2 3 4 6 10 β-actin RT
B
TZMbl
TZMbl-2
TZMbl
TZMbl-2
kDa
130
90
72
55
43
34
25
17
αTLR1
αTLR2
cytosol
membrane
C
tzmbl-2
tzmbl
1200
800
400
0
IL-8 pg/mL
*
***
**
neg
0.5
5
50
500
5000
Pam3CSK4 ng/mL

Figure 1. Establishment of a functional TLR2 stably transfected cell line, TZMbl-2. (A) Total RNA was harvested from TZMbl and TZMbl-2 cells and mRNA levels for TLR1, 2, 3, 4, 6, and 10 were assessed using conventional PCR. (B) TLR1 and TLR2 cellular protein expression in TZMbl and TZMbl-2 cell fractions were evaluated by western blot analyses using anti-TLR1 antibodies (α TLR1) and anti-TLR2 antibodies (α TLR2). (C) TZMbl and TZMbl-2 cell supernatants were assessed for IL-8 production after exposure to various concentrations of Pam_3CSK_4. *P<0.05, **P<0.01, ***P<0.001. Errors bars, SEM. A representative data set from triplicate experiments is shown.

Figure 2

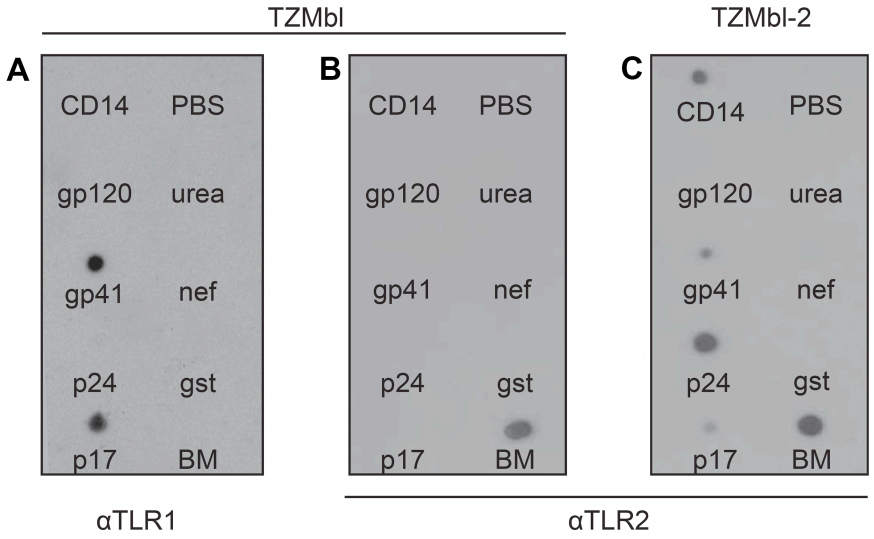


Figure 2. HIV-1 proteins bind TLR2 from TZMbl-2 cell lysates. (A) TZMbl cell lysates incubated with nitrocellulose membranes previously blotted with positive control (20 pmol sCD14, and 1 μL 1:20 diluted breast milk (BM), HIV-1 components (20 pmol; p17, p24, gp41, gp120 and nef), protein tag (20 pmol GST) and negative controls (1 μL PBS and 1 μL 1M urea) and developed using anti-TLR1 goat IgG. (B) TZMbl cell lysates were treated as in (A) and developed using anti-TLR2 goat IgG. (C) TZMbl-2 cell lysates were treated as in (A) and developed using anti-TLR2 goat IgG. Secondary antibodies for all membranes were HRP-donkey anti-goat IgG. A representative data set of at least three independent experiments in shown.

Figure 3

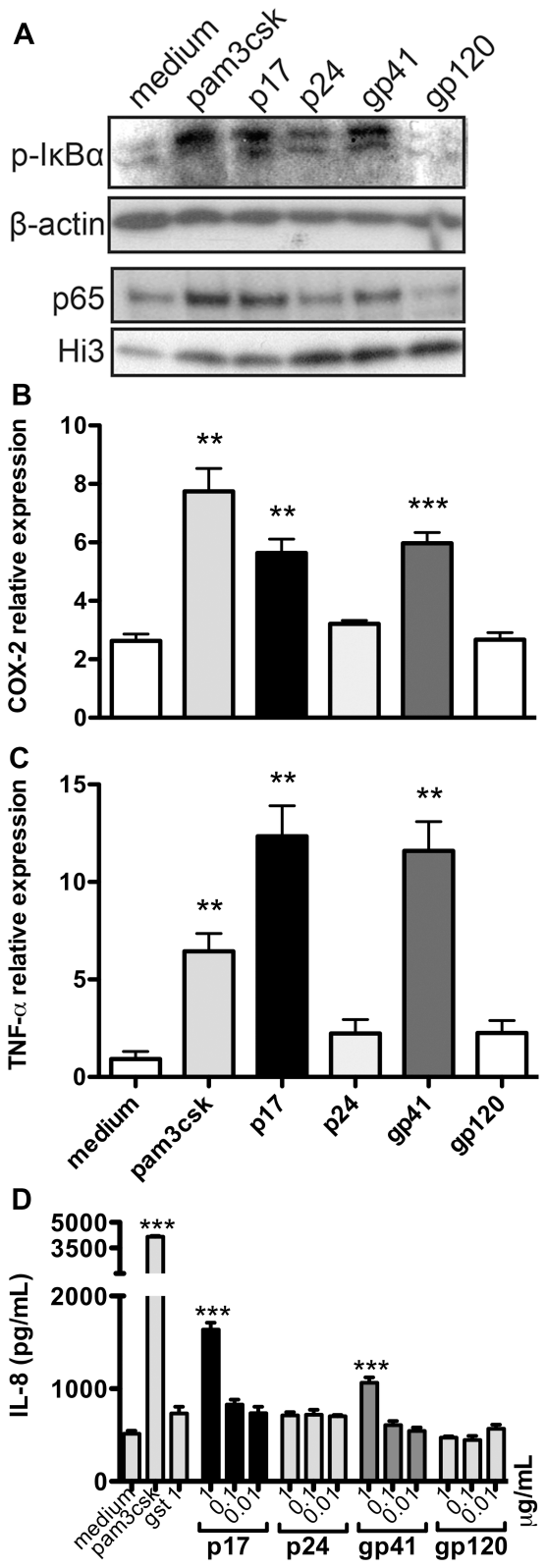

A
medium
pam3csk
p17
p24
gp41
gp120
p-IκBα
β-actin
p65
Hi3
B
COX-2 relative expression
C
TNF-α relative expression
D
IL-8 (pg/mL)
μg/mL
p17
p24
gp41
gp120

Figure 3. HIV-1 proteins activate phosphorylation of IκBα and trigger pro-inflammatory cytokine production in TZMbl-2 cells. (A) Cell lysates from TZMbl-2 were evaluated for phosphorylated IκBα and p65 nuclear translocation (except for gst and Poly I:C) using western blot analyses after treatment with 500ng/mL positive control (Pam_3CSK_4), 1μg/mL HIV-1 proteins (p17, p24, gp41, gp120), and negative control (medium) for 2 and 4 hours, respectively (B) Evaluation of COX-2 expression in TZMbl-2 treated as in (A). (C) Evaluation of TNF-α expression in TZMbl-2 treated as in (A). (D) TZMbl-2 cells were treated with 500 ng/mL TLR2 positive control (Pam_3CSK_4), various concentrations (1-0.01 μg/mL) of HIV-1 proteins (p17, p24, gp41, gp120), 1μg/mL protein tag (GST), or negative control (medium). Data in (D) represent IL-8 levels in supernatants, measured by enzyme-linked immunosorbant assay (ELISA) after overnight culture, and are the mean of triplicate samples ± SEM. *P<0.05, **P<0.01, ***P<0.001. For data in (A-D), no less than three independent experiments were analyzed.

Figure 4

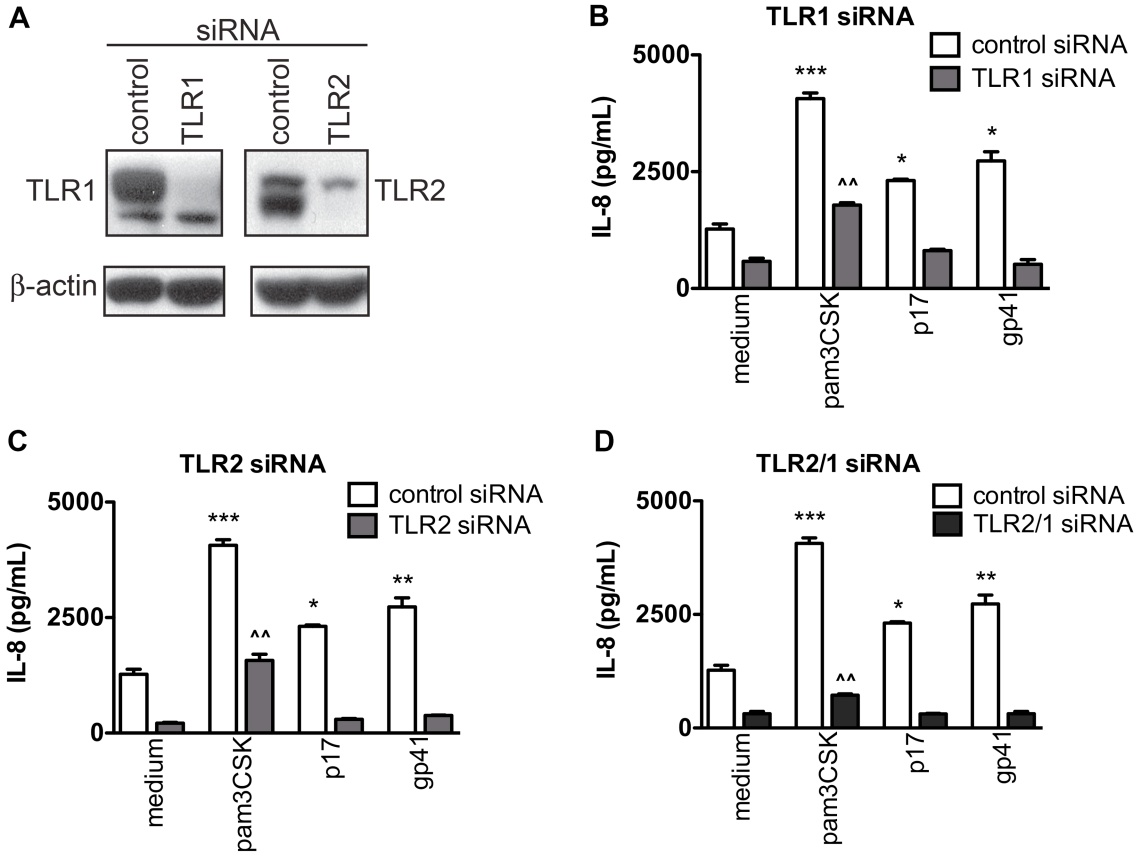


Figure 4. HIV-1 p17- and gp41-induced IL-8 stimulation was significantly decreased in both TLR1 and TLR2 knocked down cells. TZMb1-2 cells were transfected with 20 nM control siRNA or (A) TLR1 siRNA, (B) TLR2 siRNA, (C) TLR2/1 siRNA for 2 days followed by medium containing 500 ng/ml Pam_3CSK_4 or 1 μg/ml HIV-1 proteins, p17 or gp41. Data in (A), (B), and (C) represent IL-8 levels in supernatants, measured by an ELISA after overnight culture, and are the mean of triplicate samples ± SEM. *P<0.05, **P<0.01, ***P<0.001. A representative data set of at least two independent experiments is shown completed in triplicates.

Figure 5

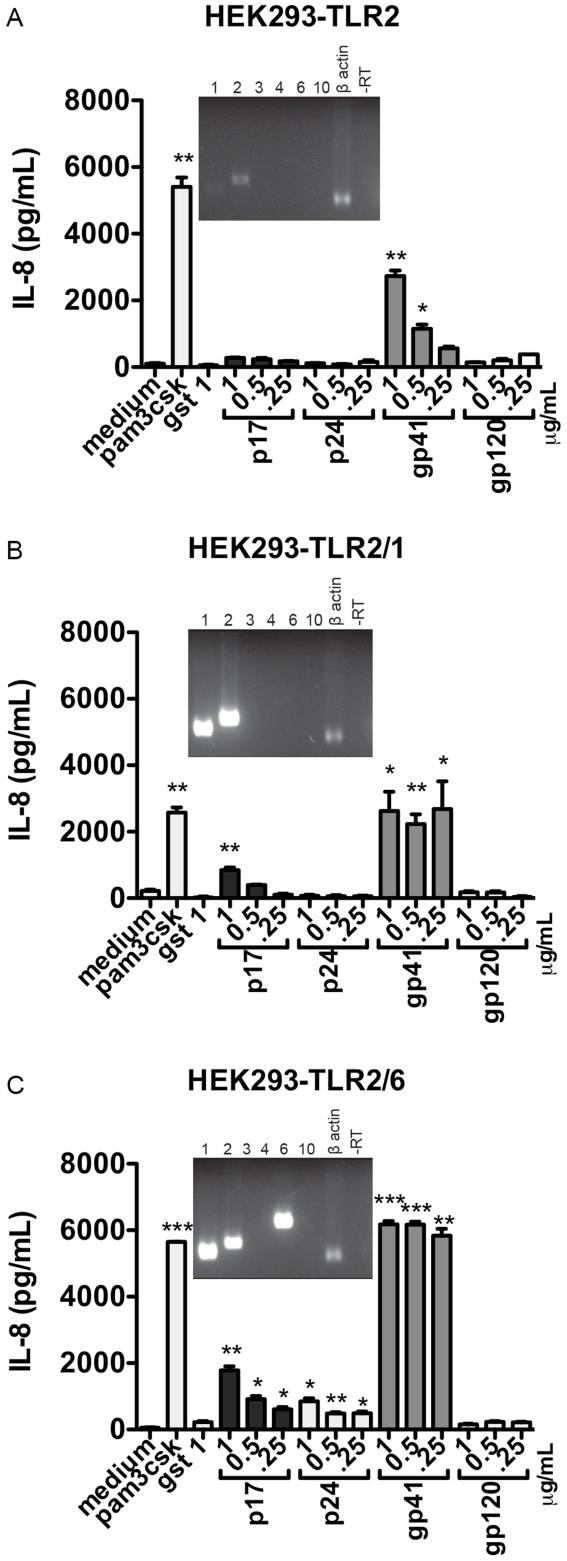

A
HEK293-TLR2
B
HEK293-TLR2/1
C
HEK293-TLR2/6
IL-8 (pg/mL)
8000
6000
4000
2000
0
1 2 3 4 6 10 β actin -RT
medium
pam3csk
gst 1
1
0.5
.25
p17
p24
gp41
gp120
μg/mL

Figure 5. HIV-1 proteins-induced IL-8 stimulation was significantly increased in TLR2 transgene cell lines. (A) HEK293-TLR2 cells were treated with 500 ng/mL TLR2 ligand (Pam_3CSK_4), 1 μg/mL HIV-1 proteins (p17, p24, gp41, gp120), protein tag (GST), or in medium (control). (B) HEK293-TLR2/1 treated as in (A). (C) HEK293-TLR2/6 treated as in (A). Data in (A), (B), and (C) represent IL-8 concentration in supernatants that were measured in ELISAs after overnight culture, and are the mean of triplicate samples ± SEM. *P<0.05, **P<0.01, ***P<0.001. Inserts: mRNA expression of TLR1, TLR2, TLR3, TLR4, TLR6 and TLR10 was detected in the cell lines with conventional RT-PCR as shown by electrophoresis agarose gels. A representative data set of at least three independent experiments is shown.

Figure 6

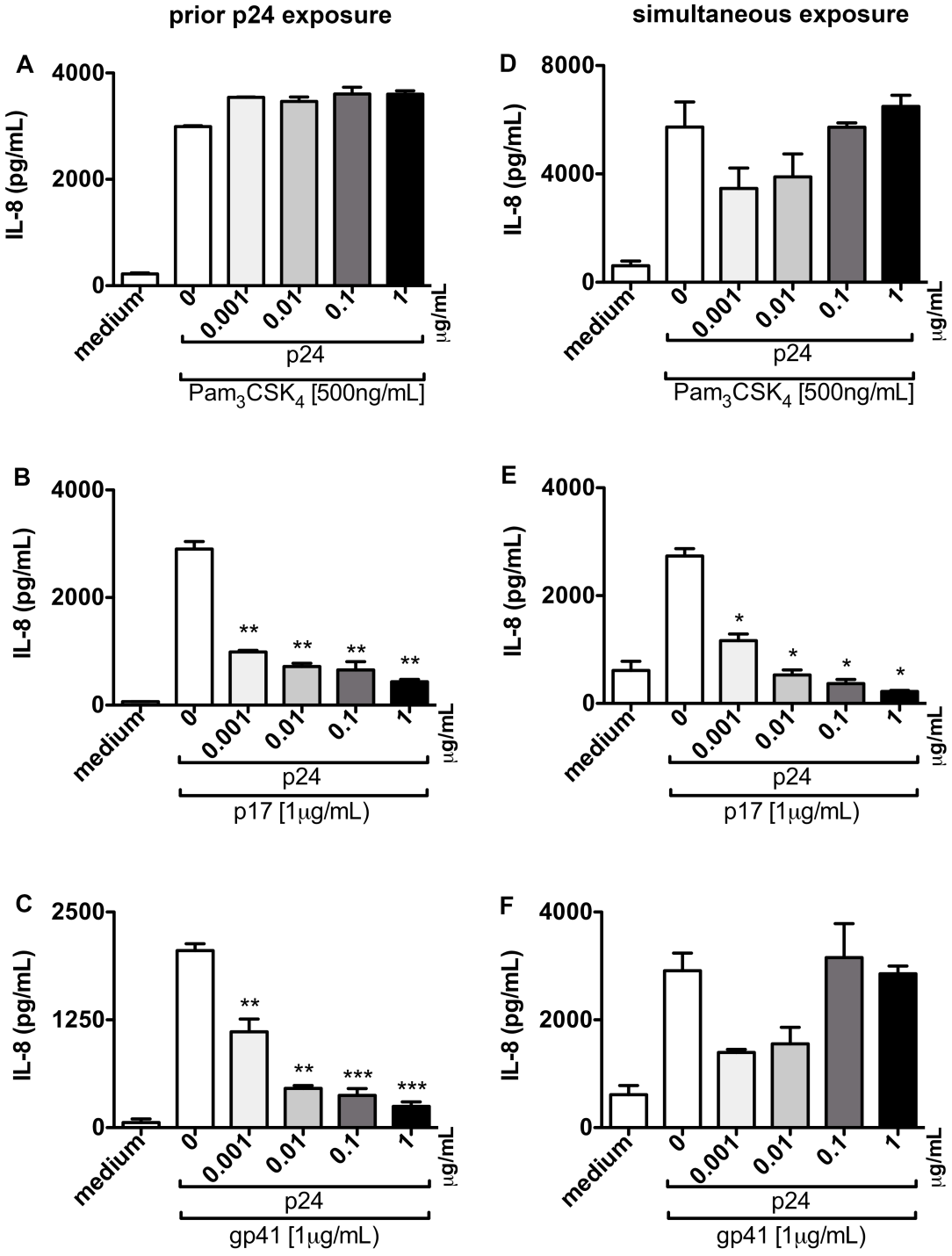
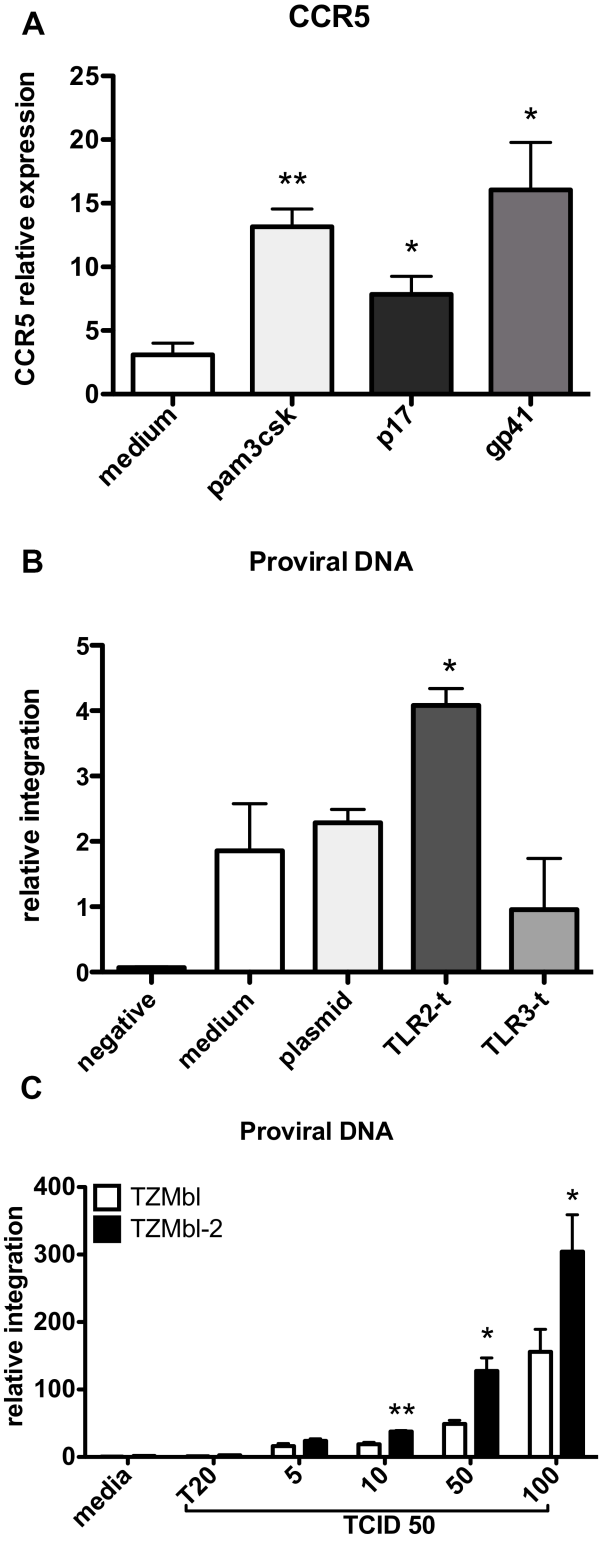

prior p24 exposure
simultaneous exposure
A
B
C
D
E
F
IL-8 (pg/mL)
4000
2000
0
8000
2500
1250
medium
0
0.001
0.01
0.1
1
μg/mL
p24
Pam3CSK4 [500ng/mL]
p17 [1μg/mL]
gp41 [1μg/mL]
*
**
***

Figure 6. p24 blocks p17 and gp41 induced production of pro-inflammatory cytokines. (A-C) TZMbl-2 cells were exposed to various doses ranging from 0-1 μg/mL of p24 before 500 ng/mL TLR2 ligand, (Pam_3CSK_4) or 1μg/mL HIV-1 structural proteins, p17 or gp41 were added. Data in (A-F) represent IL-8 concentrations in supernatants that were measured in ELISAs after overnight culture, and are the mean of triplicate samples ± SEM. *P<0.05, **P<0.01, ***P<0.001. A representative data set from three independent experiments is shown.

Figure 7


A
CCR5
CCR5 relative expression
25
20
15
10
5
0
**
*
*
medium
pam3csk
p17
gp41
B
Proviral DNA
relative integration
5
4
3
2
1
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*
negative
medium
plasmid
TLR2-t
TLR3-t
C
Proviral DNA
relative integration
400
300
200
100
0
TZMbl
TZMbl-2
**
*
*
media
T20
5
10
50
100
TCID 50

Figure 7. Cellular TLR2 expression is associated with increased HIV-1 infection in TZMbl cells.

(A) Total RNA was harvested from TZMbl-2 cells after overnight exposure to 1μg/mL TLR2 ligand (Pam_3CSK_4), 4 μg/mL HIV-1 proteins (p17, p24, gp41, gp120), or in medium (control), and CCR5 mRNA levels were assessed using qRT-PCR. (B) TZMbl cells were transiently transfected with empty plasmid, TLR2 or TLR3 DNA and 24 hours later were infected with 100 $TCID_{50}$ of BAL (R5) virus. 48 hours post infection, DNA was isolated and analyzed for HIV-1 Pol integration. (C) HIV-1 Pol integration evaluated in TZMbl and TZMbl-2 exposed to various concentrations of cell free R5 HIV-1 virus at eight hours, and are the mean of triplicate samples ± SEM. *P<0.05, **P<0.01, ***P<0.001. A representative data set from three independent experiments is shown.

Figure 8

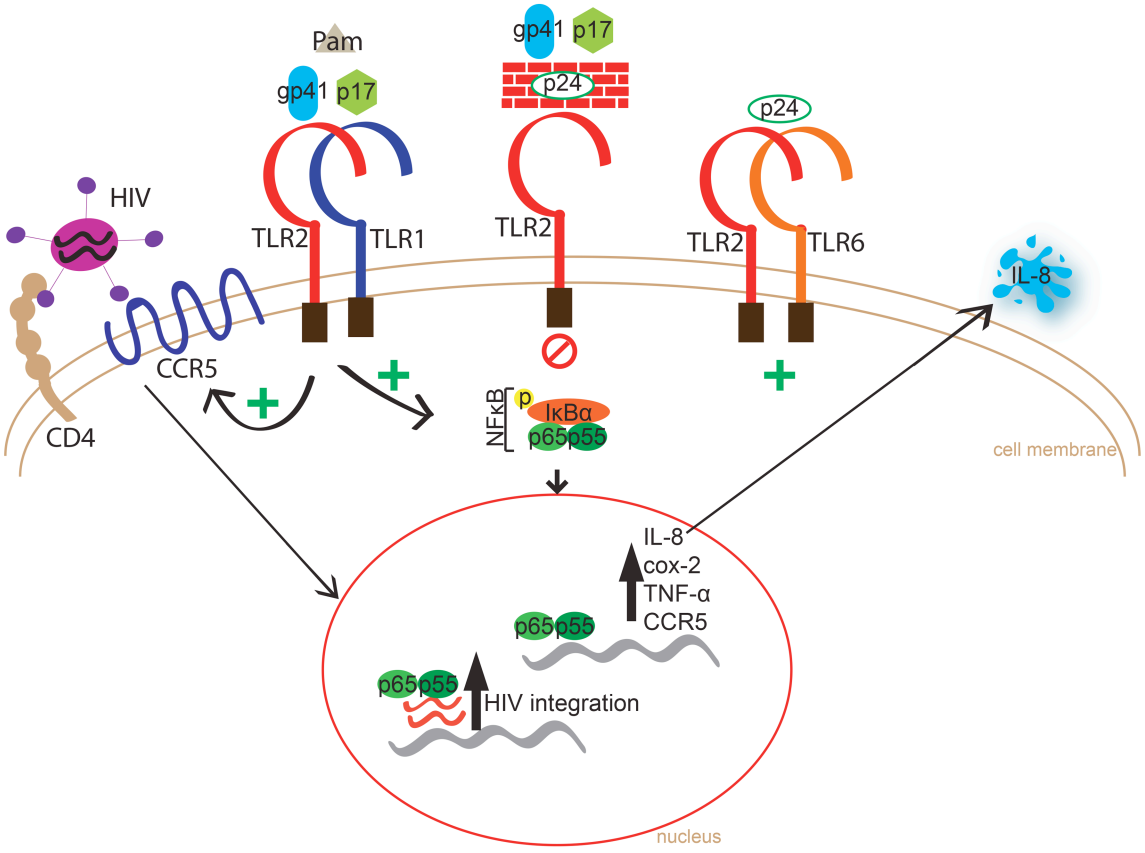
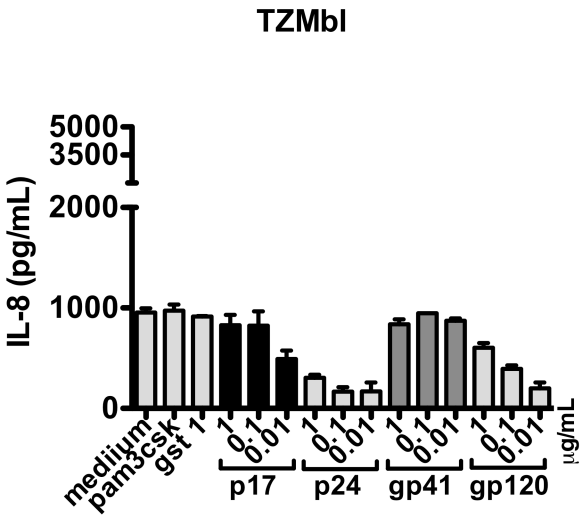


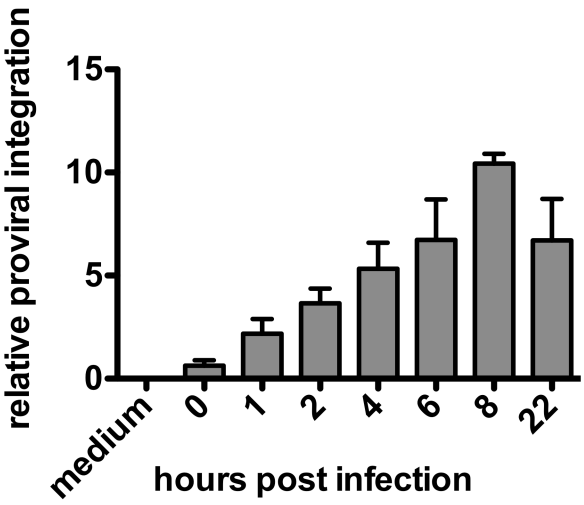
Figure 8. Schematic representation of HIV PAMPs Interaction and Cellular Activation via TLR2. Innate immune recognition of HIV-1 proteins, p17 and gp41, through TLR2/1 results in phosphorylation of IκBα leading to amplified transcription of IL-8, COX-2, TNF-α, and CCR5, and increased IL-8 levels. In the absence of TLR6 expression, p24 blocked p17 and gp41-induced production of pro-inflammatory cytokine, yet induced the production of IL-8 in cells expressing the TLR2/6 heterodimer. TLR2 expression increased HIV integration, possibly due to an increase in CCR5 co-receptor expression.

Figure S1



Supplemental Figure 1. HIV-1 PAMPs do not induce IL-8 production in cells that lacked TLR2 expression. TZMbl cells were treated overnight with 500 ng/mL TLR2 positive control (Pam_3CSK_4), various concentrations (1-0.01 μg/mL) of HIV-1 proteins (p17, p24, gp41, gp120), 1μg/mL protein tag (GST), or negative control (medium) and IL-8 levels in supernatants were measured by enzyme-linked immunosorbant assay (ELISA). Data are the mean of triplicate samples ± SEM. *P<0.05, **P<0.01, ***P<0.001 in which no less than three independent experiments were analyzed.

Figure S2



Supplemental Figure 2. Optimization of HIV-1 integration in TZMbl Cell Line. TZMbl cells were exposed to 100 $TCID_{50}$ of BAL (R5) virus and DNA was isolated at various time points (0, 2, 4, 6, 8, 22 hours) post infection and HIV-1 integration determined by qRT-PCR. Data are the mean of triplicate samples ± SEM. *P<0.05, **P<0.01, ***P<0.001 in which no less than two independent experiments were analyzed.

Table S1

Endotoxin Concentration		
samples	**EU/mL**	**working quantity (EU/mL)**
pam3CSK	0.01	5×10^{-6}
p17	13.5	1.35×10^{-2}
p24	0.25	2.5×10^{-4}
gp41	0.44	4.4×10^{-4}
gp120	0.07	7.0×10^{-5}
gst	0.09	9.0×10^{-5}
FBS	≤ 50	5

Supplemental Table 1. Endotoxin levels in recombinant proteins were assessed using immunoassay. Recombinant proteins including Pam_3CSK_4, p17, p24, gp41, gp120, and gst were assessed for endotoxin concentration using an enzyme-linked immunoassay (ELISA) and levels are shown at working concentrations. Fetel bovine serum (FBS) levels are based on manufacturers maximum allowable endotoxin concentrations.

– CHAPTER 5 –

DISCUSSION

1.0 Preamble

The correlation between breastfeeding and healthy infants is well documented. A seminal publication by Grulee *et al.*, (1935) was one of the first publications to highlight decreased morbidity and mortality in infants who were breastfed compared to those who were not [186]. Breast milk contains a plethora of broad-range antimicrobial and anti-inflammatory factors, which compensate for the naïve immune system of the infant, and is currently the most effective intervention for preventing infant disease [201,217,218]. Notwithstanding the proven benefits of breastfeeding, breast milk also provides a medium for vertical transmission of HIV. Without prophylactic strategies, which are rarely available for the duration of breastfeeding in developing nations [20], MTCT through breastfeeding accounts for approximately 11-42% of all pediatric HIV infections [136,137]. Paradoxically, however, an intervention that promotes exclusive breastfeeding, regardless of the mother's HIV status, has led to significantly decreased MTCT compared to mixed feeding [22,24,25,185]. Indeed, this preventative method is so effective in the reduction of MTCT HIV transmission, as well as protection against enteric infections, that the WHO promotes exclusive breastfeeding despite the HIV status of the mother when safe and sustainable supplemental feeding is unavailable [140]. The low rate of transmission is suggestive of the multitude of protective innate immune factors which are present in high concentrations in breast milk [149,176]. The innate factor, sTLR2, has recognized anti-microbial properties [128-130,219]; however, its anti-

HIV properties were not previously documented. Given the association between innate factor levels and the overall protective effect of breast milk in preventing the majority of infants from acquiring HIV from their infected mothers, the identification and characterization of soluble factors with anti-viral and immunomodulatory properties might have novel applications for therapeutics and/or prophylactic strategies in the future.

Thus, the objective of the research presented here was to characterize the role of sTLR2 in inhibiting HIV-induced cellular activation and infection and to identify the role of TLR2 in sensing HIV. Specifically, the manuscripts presented in Chapters 2 and 3 examined sTLR2 forms and concentrations among women's breast milk, and assessed a mechanism of inhibition for bacterial and viral-induced cellular activation and HIV infection *in vitro.* Furthermore, sTLR2 concentration in HIV-infected breast milk was evaluated and compared with pro-inflammatory cytokine and viral antigenemia levels. Chapter 4 investigated the role of TLR2, and its heterodimers, TLR1 and TLR6 in sensing HIV-1, which led to increased cellular activation, co-factor expression, and infection *in vitro*. Additionally, results not included in our manuscripts were incorporated into this thesis to further support our hypotheses. Together, these data highlight the importance of sTLR2 and TLR2 in inhibiting HIV infection and activating immunopathogenesis, respectively. These data are presented with the hope that a better understanding of natural inhibitors of HIV infection and further insight into what is driving immunopathogenesis once infection has been established might lead to the development of novel immunotherapeutics.

2.0 Experimental Models

2.1 Immunodepletion of sTLR2

Our initial experiments utilized immunodepletion methods to remove sTLR2 from HIV-uninfected breast milk. This technique was beneficial to evaluate the specific role of sTLR2 in breast milk since we discovered that the commercially available recombinant sTLR2 was not functional *in vitro*. However, our immunodepletion techniques were unable to remove all of the sTLR2 from breast milk; therefore, our results might not portray the full effectiveness of sTLR2 at physiological levels. Furthermore, breast milk is a highly complex mucosal fluid containing abundant innate and adaptive immune components, many of which have not had their functions fully elucidated. Consequently, we were unable to rule out the possibility that undiscovered immunological factors might have played a role in the significant inhibition of cell-free R5 HIV infection *in vitro*. Therefore, we supported our original findings by further investigating the function of sTLR2 produced from TLR2 stably transfected TZMbl-2 cells to inhibit bacterially and virally-induced pro-inflammatory responses and directly inhibit HIV infection and integration.

2.2 Dot Blot

Further in our studies, we utilized a dot blot detection method to visualize protein-to-protein interactions between HIV-1 proteins and sTLR2, TLR2, or TLR1. This method, described previously [220], was critically important to the identification of viral-host protein interactions that have not been previously reported. Although this method is

not used extensively, its advantages (the ability to evaluate several protein-to-protein interaction with high objectivity) outweighed the disadvantages (information regarding location of the interaction and inability to quantify) as a preliminary experiment. Subsequent experiments using neutralization of sTLR2, short interfering RNA (siRNA) knockdowns of TLR2 and TLR1, and stably transfected cell lines expressing TLR2, TLR2/1, and TLR2/6 verified and extended our findings from the dot blot method.

2.3 Endotoxin Testing of Reagents

We identified several novel HIV-1 PAMPs that specifically signal through TLR2. These findings were met with some caution since historically mistakes regarding the proper identification of TLR2 ligands have been made. Specifically, LPS was incorrectly identified as an agonist for TLR2 [221,222] and later found to be due to contaminants from incompletely purified recombinant LPS [223]. Therefore, all recombinant proteins used in our assays were tested for endotoxin contamination and had levels that were undetectable or well below LPS levels found in fetal bovine serum (FBS) (which is commonly included in cell culture medium). Furthermore, siRNA knockdown of TLR2 and TLR1 in cells endogenously expressing TLR4 negated the production of IL-8 after HIV protein exposure, and cells not expressing TLR4 only produced IL-8 when TLR2, TLR2/1, or TLR2/1/6 were expressed. Taken together, the data indicated that the results were not due to endotoxin contamination.

2.4 Hamilton Breast Milk Cohort

We started a Breast Milk Cohort (approved on 31 July 2008 by the McMaster Research Ethics Board; REB Approval #08-176) to collect breast milk from HIV-uninfected mothers. All participants provided voluntary written informed consent. Inclusion criteria included HIV-uninfected women with full-term pregnancies that planned to breastfeed for at least six months postpartum, and were willing to self-collect >15mL of breast milk within the first week, and at one, three, and six months after giving birth vaginally. Once at our laboratory, the samples were separated into lipid, supernatant, and cellular fractions. The cellular fractions were frozen in liquid nitrogen vapour phase in freezing medium (95% FBS, 5% dimethyl sulfoxide; DMSO) and lipid and supernatant fractions were stored at -80°C. However, given the low number of HIV-infected mothers in Ontario and the fact that they are counseled to not breastfeed, our HIV-infected breast milk samples came from Nigerian mothers. Although Nigerian HIV-uninfected samples were used as controls, these samples might not have been processed exactly the way they were in our laboratory. Additionally, some caution has to be used when directly comparing HIV-uninfected Hamilton and Nigerian samples as it remains unclear how genetic, racial and environmental differences affect TLR2 and sTLR2 expression. Indeed, Texereau *et al.,* (2005) have identified TLR2 polymorphisms that might impair host responses to *Mycobacterium tuberculosis* and *leprae* [224]. Therefore, mutations in the TLR2 protein might affect cellular responses to PAMPs and/or sTLR2 expression thus requires further evaluation in larger cohorts.

2.5 TZMbl-2 Cell Line

The TZMbl cell line was developed by Dr. Montefiori's Lab (Duke University) to quantify HIV infection *in vitro*. The cell line originated from HeLa cells that were stably transfected with canonical HIV entry receptors (CD4, CCR5, and CXCR4) and luciferase and β-galactosidase genes that are controlled by the HIV TAT accessory protein. The addition of luciferase and β-galactosidase genes enable quantification of HIV infection *in vitro*. However, TZMbl cells do not endogenously express TLR2 but do express TLR1. Consequently, we established a stably transformed TZMbl cell line, TZMbl-2, which expressed TLR2 at the protein level. TZMbl cells were a rationale choice given their stable expression of canonical HIV receptors and TAT-regulated reporter genes, allowing for sensitive and reproducible quantification of HIV integration [225]. As a model, TZMbl-2 allowed us to focus our studies specifically on TLR2 signaling and validate our findings with reasonably designed controls.

3.0 sTLR2: key findings and direct anti-viral mechanisms

The inhibition of MTCT of HIV has been attributed to the plethora of innate immune factors in breast milk [149,176]. Indeed, breast milk contains SLPI, lactoferrin, mucin, and lysozyme and these innate factors have documented anti-HIV properties [149,192,226,227]. Previously, sTLR2 was characterized as an innate immune factor that was involved unilaterally in the recognition of bacterial ligands. This bacterial ligand-sTLR2 engagement regulated the amount of ligand available to bind to TLR2, thus inhibiting bacterial-induced cellular activation and inflammation [128] without hindering bacterial clearance [132]. Our key findings clearly established, for the first time, a beneficial role and mechanism of action for sTLR2 in preventing HIV-induced cellular activation and infection *in vitro*. As well, our data highlighted important differences in sTLR2 forms and concentrations among women, identified cellular sources, post-partum kinetics and provided novel information of sTLR2 concentrations during HIV disease. The following Discussion elaborates these findings as described in our manuscripts presented in Chapters 2 and 3.

Classically sTLR2 has been studied for its role in modulating Gram-positive bacterial-induced cellular activation. In 2003, LeBouder *et al.* were the first to identify six major forms of sTLR2, predominantly 66 kDa, in plasma and breast milk [128]. They further characterized sTLR2's role in immunomodulating bacterially-induced pro-inflammatory cytokine production by PBMCs [128]. Subsequent studies showed sTLR2 immunodepletion from saliva and amniotic fluid led to significantly increased pro-

inflammatory cytokine production in oral epithelial cells and placental villous explants, respectively [129,130]. Our manuscript presented in Chapter 2 demonstrated previously unreported forms of sTLR2 in breast milk that differed in concentration among women, had a short half-life at physiological temperatures and the expression levels decreased over time postpartum. These findings were intriguing as the predominant sTLR2 forms in our breast milk cohort including the ~38 kDa and ~26 kDa masses were substantially different from the previously reported breast milk forms [128], and closely mirrored the predominant forms found in saliva, amniotic fluid [129,130] and in cervical fluid (Fig. 8). Despite the similarities between LeBouder *et al*., (2003) and our breast milk sample collection, times postpartum, processing, and western blot analyses, we proposed that variations in predominant sTLR2 polypeptides might be the result of racial, ethnic or genetic variability. Furthermore, our data highlighted the progressive decline of sTLR2 levels over time postpartum which were similar to other milk proteins [228]. These decreases might correspond to the infant's increased ingestion of breast milk with age, therefore providing the optimal level of sTLR2 over the entire breastfeeding period.

Furthermore, our manuscript presented in Chapter 2 supported the previous finding that breast milk sTLR2 significantly inhibited bacterially-induced inflammation. Using our *in vitro* model, sTLR2-immunodepleted breast milk led to significantly increased bacterially-induced inflammation in TLR2-stably transfected HEK293 cells, monocytes, and intestinal epithelial cells. These findings are particularly important as they revealed specific sTLR2 control of bacterially-induced inflammation in cells that are

likely involved in MTCT of HIV (discussed in Section 3.5.2). Furthermore, using sTLR2-depleted or mock-depleted breast milk, we identified a novel function of sTLR2 to significantly inhibit HIV-1 infection in a dose-dependent manner *in vitro*. Taken together, these data suggest that sTLR2, similar to other innate immune factors found in breast milk, have anti-bacterial, anti-viral, and immunomodulatory properties, thus indicating that sTLR2 is involved in breast milk-specific inhibition of HIV.

The manuscript presented in Chapter 3 extended these findings to evaluate the specific mechanism of sTLR2-dependent inhibition of HIV-induced inflammation and infection. Specifically, evaluation of protein-to-protein interactions using the dot blot method demonstrated that sTLR2 binds directly to specific HIV proteins (p17, p24, and gp41). This finding was similar to lactoferrin, which binds directly to the GPGRAF peptide of the V3 loop (gp120) resulting in inhibition of viral-host fusion [229]. Furthermore, sTLR2 inhibited Pam_3CSK_4, p17, or gp41-induced NFκB activation and IL-8 expression in a dose-dependent manner. This inhibitory action is similar to the innate factors, SLPI and trappin-2/elafin, which have been shown to inhibit NFκB translocation and gene expression [135,230]. This finding is particularly important in breastfeeding infants since neonatal lymphocyte activation is required for HIV infection [231]. In addition, our data indicated that sTLR2 suppressed HIV-PAMP-induced increases in CCR5 expression. Moreover, sTLR2 significantly decreased HIV infection *in vitro,* which was abrogated using TLR2-specific antibodies (Ab), thus indicating that this inhibitory effect was sTLR2-dependent. Given the importance of CCR5 to R5-specific

HIV infection, these data revealed a novel mechanism by which sTLR2 acted as a decoy receptor and inhibited HIV integration in two possible ways: (1) reduced HIV PAMP-induced cellular activation and CCR5 expression and, (2) inhibited viral-host membrane fusion by binding directly to gp41. Taken together, our findings provided novel data highlighting the important contribution of sTLR2 to breastfeeding infants as a first line regulatory mechanism preventing bacterially and virally-induced cellular activation and HIV infection.

It is debatable whether MECs represent a direct target for HIV infection or simply endocytose virions [79,216]. Similar to findings by Dorosko *et al*., (2010), we showed, using p24 ELISA, that cell-free R5 HIV-1 crossed the MEC monolayer from the basolateral to the apical compartment (Appendix I, Fig. 7A), without affecting tight junctions, as shown by transepithelial electrical resistance (TEER) (Appendix I, Fig. 7B). Furthermore, HIV did not integrate into the MEC genome (Appendix I, Fig. 8). The ability of MECs to directly recognize and respond to HIV might largely determine the infant's risk of HIV acquisition through breast milk. Indeed, we showed that BMCs from HIV-infected mothers had significantly elevated expression of TLR1, TLR2, TLR3, TLR4, TLR8 and TLR10 (Appendix I, Fig. 9). As shown in our manuscript presented in Chapter 3, MECs exposed to HIV PAMPs led to significantly increased sTLR2 production *in vitro,* and MECs exposed to cell-free HIV-1 on the basolateral surface led to significantly increased pro-inflammatory cytokine production in both the apical (breast milk side) and basolateral compartments (Fig. 10). Indeed, we found significantly

increased sTLR2 concentration in HIV-infected breast milk, which correlated to HIV-1 PAMP (p24) levels and pro-inflammatory cytokine, IL-15. The finding that sTLR2 correlated with p24 concentration is similar to a previous publication showing that breast milk α-defensin levels correlate with HIV disease progression [232]. Additionally, breast milk IL-15 levels reportedly correlate with protection from mother-to-child HIV transmission, which might be due to its ability to activate CD8+ T cells and NK cells [233,234]. Nonetheless, given the correlation between sTLR2 and IL-15, we speculate that these factors operate in concert to facilitate decreased immune activation while eliciting specific innate cellular responses.

In summary, our manuscripts provided the first evidence that sTLR2 played a multifactorial role in breast milk by providing anti-bacterial, anti-viral and immunomodulatory activity. Importantly, these observations compliment previous reports highlighting the anti-bacterial activity of sTLR2, and expand our knowledge by providing evidence that sTLR2 binds specific HIV structural proteins, thus leading to decreased inflammation and infection *in vitro.* As well, the characterization of sTLR2 in HIV-infected breast milk highlighted a local, innate compensatory mechanism to control HIV-induced cellular activation in breast milk, which might be exploited in future therapies to control immunopathogenesis.

4.0 A new trick for TLR2

The TLRs are a family of PRRs that initiate innate immune responses after recognition of a broad-range of PAMPs specific to bacteria, virus, or fungus. TLR2 has been thoroughly studied as a bacterial-sensing PRR, specifically recognizing diacylated and triacylated lipoproteins after heterodimerization with TLR6 or TLR1, respectively [112]. Nonetheless, a number of viral proteins have been identified as novel TLR2 PAMPs including CMV glycoprotein B and H [115], HSV gH/gL and gB [116], HCV core protein [235], and measles hemagglutinin A glycoprotein [118]. Furthermore, virally-induced TLR2-dependent cellular activation has been shown to contribute to viral spread and pathogenesis due to increased production of pro-inflammatory cytokines, recruitment of additional target cells and increased viral entry receptors [116,118,119]. Our manuscript presented in Chapter 4 investigated the role of TLR2 and its heterodimers (TLR1 and TLR6) in sensing HIV-1. In line with previous findings, our key data clearly established a role for TLR2 in sensing HIV-1 PAMPs, which led to increased cellular activation, co-receptor expression, and infection/integration *in vitro*. As well, our results identified unique HIV PAMPs, and were suggestive of a novel mechanism in which HIV can manipulate innate sensing with specific viral proteins. The following Discussion elaborates these findings as described in our manuscript presented in Chapters 4.

Our data indicated that TLR2 binds directly to specific HIV-1 structural proteins, p17, p24, and gp41, whereas other viral components including gp120, nef and ssRNA (the quintessential HIV PAMP) did not participate in the interaction. Further, the TLR2

heterodimer, TLR1, only interacted with p17 and gp41, whereas it did not interact with p24. These interactions had specific consequences on downstream TLR2-specific cellular activation. Most notably, p17 and gp41 induced TLR2/1-dependent activation and nuclear translocation of NFκB, which led to significantly increased pro-inflammatory cytokine production. These data are similar to previous publications showing that multiple HIV-1 proteins altered cellular activity. Specifically, p17 has been shown to induce the production of pro-inflammatory cytokines *in vitro* and *in vivo* and act as an adjuvant in vaccine strategies tested in animal models [49,236,237]. Additionally, a recent publication showed that gp41 exposure activated NFκB in lymphocytes [238], while gp120 has been shown to effect both pro- and anti- inflammatory responses *in vitro* [36,239,240]. Importantly, in the absence of TLR6 expression, p24 blocked p17- and gp41-induced pro-inflammatory cytokine production in a dose-dependent manner. These data are particularly intriguing since they provide evidence, for the first time, that HIV employs an extracellular mechanism, which manipulates viral sensing through TLR2. Indeed, HIV has several intracellular mechanisms to suppress receptor expression and circumvent host restriction factors including Vpu and Vif, respectively [241,242]. Taken together, these data are timely since it is becoming increasingly clear that chronic immune activation is central to HIV pathogenesis and the progression to AIDS [95,127]. Yet, despite this understanding, the ability of HIV to alter innate immune responses is not completely understood. Our current model of chronic immune activation relies on bacterial translocation from the HIV-damaged intestinal tract [120] caused by the devastation of mucosal CCR5+ CD4+ T cells during acute HIV infection. Indeed,

systemic LPS have been shown to strongly correlate with immune activation in chronically infected individuals [120]. However, we previously showed that initiation of ART normalized TLR expression despite increased serum LPS levels [97]. Furthermore, high-levels of non-infectious HIV particle production and persistent levels of viral structural and glycoprotein levels in lymph nodes during HIV infection have been previously documented [243,244]. Thus, taken together, these date indicate that HIV-1 itself might play an important role stimulating chronic immune activation over the course of the disease.

The primary consequence of viral recognition involves production of pro-inflammatory cytokines and the subsequent recruitment of target cells. However, we also observed a significant increase in CCR5 expression in macrophages exposed to HIV-1 PAMPs (p17 and gp41). The consequence of increased co-receptor expression led to significantly increased cell-free R5 HIV infection *in vitro*. These results are similar to other viral proteins that caused cellular activation through a TLR2-mechanism. Indeed, the measles extracellular hemagglutinin protein led to significantly increased co-receptor, CD150, expression through a TLR2-dependent mechanism [118]. Furthermore, previous publications have shown a TLR2-dependent increase in CCR5 expression on permissible cells which led to significantly increased HIV infection [245,246]. These data highlight, for the first time, an important innate immune mechanism by which HIV-1 PAMPs promote viral infection in permissive cells.

The data presented in Chapter 4 demonstrated that novel HIV-1 PAMPs promoted cellular activation *in vitro,* which might be critically important to understanding aberrant immune activation during the course of HIV disease. Given TLR2's critical role in the innate immune system and its ubiquitous expression on lymphocytes and mucosal epithelium, it might be the best PRR to facilitate cellular activation which is required for persistent HIV infection. However, building on novel data presented here might facilitate the production of therapeutics that control aberrant cellular activation caused by HIV.

— CHAPTER 6 —

CONCLUDING REMARKS

The majority of infants breastfeeding from their HIV-infected mothers do not acquire HIV. Indeed, exclusive breastfeeding has been one of the most successful interventions in protecting infants from a broad range of infectious diseases. Although the reason for this remains unclear, coordination of a number of innate immune factors in breast milk seem crucial for providing protection when infants are most vulnerable. As demonstrated in this thesis, we identified several novel properties and mechanisms of the innate immune factor, sTLR2, that advance our understanding of this molecule in providing indirect and direct protection against HIV for the breastfeeding infant. Furthermore, we identified novel HIV-1 PAMPs that signal through TLR2 and these might account, in part, for aberrant immune activation during the course of HIV disease.

HIV infection is characterized by a profound depletion of gastrointestinal (GI) effector T cells, systemic microbial translocation and chronic immune activation. Therefore, our sTLR2 data showing direct inhibition of HIV-induced or bacterial-induced cellular activation suggests that sTLR2 could possibly be used as an immunotherapeutic for HIV infected individuals. Oral administration of sTLR2 might directly inhibit viral- and bacterial-induced inflammation and cellular activation in the GI tract, as we showed *in vitro*. Moreover, sTLR2 has the potential to be used in microbicides that are applied to the vaginal or rectal mucosa before sexual intercourse. The ability of sTLR2 to inhibit

inflammation at mucosal sites could potentially reduce HIV transmission by inhibiting the virus's ability to penetrate the mucosal barrier and subsequent recruitment of host target cells. Importantly, the concentration and forms of sTLR2 at mucosal sites might provide important correlates of HIV protective immunity. Indeed, our evaluation of sTLR2 in breast milk indicated striking differences in forms and concentrations between women. Taken together with a previous publication that showed that single-nucleotide polymorphisms (SNPs) enhanced the risk of acquiring infections [224], it is important that future research evaluates whether SNPs affect sTLR2 function against HIV infection.

Our data revealed, for the first time, the ability of TLR2 to recognize specific HIV PAMPs, which led to significantly increased NFκB activation, inflammation and HIV infection. These results advance our fundamental understanding of HIV sensing and pathogenesis by providing evidence that HIV itself perpetuates chronic immune activation. Importantly, future experiments need to evaluate whether these novel HIV PAMPs are correlates of chronic immune activation during HIV disease *in vivo*. A previous report [244] indicated that several HIV structural proteins persist in lymphoid tissue despite an undetectable viral load. Taken together, these HIV PAMPs might provide persistent antigenic stimulation required for chronic immune activation, a hallmark of HIV disease. Alternatively, these PAMPs might provide a novel therapy to systemically activate latent viral reservoirs in chronically infected individuals and/or serve as unique HIV vaccine immunogens that possess auto-adjuvant activity. Furthermore, it is important that future experiments characterize the interaction and exact

location of HIV PAMP binding on the TLR2 heterodimer complex. These data could inform the development of novel antagonists that prevent viral PAMP-TLR2 binding and thus inhibit HIV-induced immune activation. Although several limitations remain, the data presented here provide novel and fundamental evidence contributing to our understanding of HIV pathogenesis and inhibition of HIV infection that could translate into effective immunotherapeutics in the future.

— CHAPTER 7 —

REFERENCES (CHAPTERS 1, 5 AND 6)

1. Van Heuverswyn F, Peeters M. The origins of HIV and implications for the global epidemic. Curr Infect Dis Rep **2007**; 9:338–346.

2. Turner BG, Summers MF. Structural biology of HIV. J. Mol. Biol. **1999**; 285:1–32.

3. Peeters M, Courgnaud V, Abela B, et al. Risk to human health from a plethora of simian immunodeficiency viruses in primate bushmeat. Emerging Infect. Dis. **2002**; 8:451–457.

4. Kalish ML, Wolfe ND, Ndongmo CB, et al. Central African hunters exposed to simian immunodeficiency virus. Emerging Infect. Dis. **2005**; 11:1928–1930.

5. Li WH, Tanimura M, Sharp PM. Rates and dates of divergence between AIDS virus nucleotide sequences. Mol. Biol. Evol. **1988**; 5:313–330.

6. Gao F, Bailes E, Robertson DL, et al. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature **1999**; 397:436–441.

7. Van Heuverswyn F, Li Y, Neel C, et al. Human immunodeficiency viruses: SIV infection in wild gorillas. Nature **2006**; 444:164.

8. Korber B, Muldoon M, Theiler J, et al. Timing the ancestor of the HIV-1 pandemic strains. Science **2000**; 288:1789–1796.

9. Vidal N, Peeters M, Mulanga-Kabeya C, et al. Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. Journal of Virology **2000**; 74:10498–10507.

10. Zhu T, Korber BT, Nahmias AJ, Hooper E, Sharp PM, Ho DD. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. Nature **1998**; 391:594–597.

11. Centers for Disease Control (CDC). Pneumocystis pneumonia--Los Angeles. MMWR Morb. Mortal. Wkly. Rep. **1981**; 30:250–252.

12. Walzer PD, Perl DP, Krogstad DJ, Rawson PG, Schultz MG. Pneumocystis carinii pneumonia in the United States. Epidemiologic, diagnostic, and clinical

features. Ann. Intern. Med. **1974**; 80:83–93.

13. Coffin J, Haase A, Levy JA, et al. What to call the AIDS virus? Nature **1986**; 321:10.

14. UNAIDS. Report on the Global AIDS Epidemic. **2012**; :1–110. Available at: http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2012/gr2012/20121120_UNAIDS_Global_Report_2012_en.pdf.

15. UNAIDS. 2008 Report on the global AIDS epidemic. **2008**; :1–362. Available at: http://www.unaids.org/en/dataanalysis/knowyourepidemic/epidemiologypublications/2008reportontheglobalaidsepidemic/.

16. Global HIV/AIDS Response. **2011**; :1–229.

17. De Cock KM, Zadi F, Adjorlolo G, et al. Retrospective study of maternal HIV-1 and HIV-2 infections and child survival in Abidjan, Côte d'Ivoire. BMJ **1994**; 308:441–443.

18. UNAIDS, World Health Organization. AIDS epidemic update. **2009**; :1–100.

19. World Health Organization. Antiretroviral Drugs For Treating Pregnant Women and Preventing HIV Infection in Infants. **2011**; :1–117.

20. Schramm DB, Kuhn L, Gray GE, Tiemessen CT. In vivo effects of HIV-1 exposure in the presence and absence of single-dose nevirapine on cellular plasma activation markers of infants born to HIV-1-seropositive mothers. J. Acquir. Immune Defic. Syndr. **2006**; 42:545–553.

21. WHO Organization. HIV and infant feeding. **2010**; :1–58. Available at: http://whqlibdoc.who.int/publications/2010/9789241599535_eng.pdf.

22. Iliff PJ, Piwoz EG, Tavengwa NV, et al. Early exclusive breastfeeding reduces the risk of postnatal HIV-1 transmission and increases HIV-free survival. AIDS **2005**; 19:699–708.

23. Coovadia HM, Rollins NC, Bland RM, et al. Mother-to-child transmission of HIV-1 infection during exclusive breastfeeding in the first 6 months of life: an intervention cohort study. Lancet **2007**; 369:1107–1116.

24. Coutsoudis A, Kuhn L, Pillay K, Coovadia HM. Exclusive breast-feeding and HIV transmission. AIDS **2002**; 16:498–499.

25. Kuhn L, Sinkala M, Kankasa C, et al. High uptake of exclusive breastfeeding and reduced early post-natal HIV transmission. PLoS ONE **2007**; 2:e1363.

26. Frankel AD, Young JA. HIV-1: fifteen proteins and an RNA. Annu. Rev. Biochem. **1998**; 67:1–25.

27. Nabel G, Baltimore D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature **1987**; 326:711–713.

28. Chertova E, Bess JW, Crise BJ, et al. Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), Is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus. Journal of Virology **2002**; 76:5315–5325.

29. Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **1984**; 312:763–767.

30. Göttlinger HG, Sodroski JG, Haseltine WA. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. U.S.A. **1989**; 86:5781–5785.

31. Hoshikawa N, Kojima A, Yasuda A, et al. Role of the gag and pol genes of human immunodeficiency virus in the morphogenesis and maturation of retrovirus-like particles expressed by recombinant vaccinia virus: an ultrastructural study. J. Gen. Virol. **1991**; 72 (Pt 10):2509–2517.

32. Fields BN, Knipe DM, Howley PM. Fields Virology , 5e. Lippincott Williams & Wilkins, 2007.

33. Sierra S, Kupfer B, Kaiser R. Basics of the virology of HIV-1 and its replication. J. Clin. Virol. **2005**; 34:233–244.

34. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **1998**; 393:648–659.

35. Merk A, Subramaniam S. HIV-1 envelope glycoprotein structure. Curr. Opin. Struct. Biol. **2013**;

36. Nazli A, Chan O, Dobson-Belaire WN, et al. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. PLoS Pathog. **2010**; 6:e1000852.

37. Kanmogne GD, Kennedy RC, Grammas P. HIV-1 gp120 proteins and gp160 peptides are toxic to brain endothelial cells and neurons: possible pathway for HIV entry into the brain and HIV-associated dementia. J. Neuropathol. Exp. Neurol. **2002**; 61:992–1000.

38. Yang B, Akhter S, Chaudhuri A, Kanmogne GD. HIV-1 gp120 induces cytokine expression, leukocyte adhesion, and transmigration across the blood-brain barrier: modulatory effects of STAT1 signaling. Microvasc. Res. **2009**; 77:212–219.

39. Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. Cell **1997**; 89:263–273.

40. Cohen CR, Moscicki A-B, Scott ME, et al. Increased levels of immune activation in the genital tract of healthy young women from sub-Saharan Africa. AIDS **2010**; 24:2069–2074.

41. Ueda H, Howard OM, Grimm MC, et al. HIV-1 envelope gp41 is a potent inhibitor of chemoattractant receptor expression and function in monocytes. J. Clin. Invest. **1998**; 102:804–812.

42. Zhou C, Lu L, Tan S, Jiang S, Chen Y-H. HIV-1 glycoprotein 41 ectodomain induces activation of the CD74 protein-mediated extracellular signal-regulated kinase/mitogen-activated protein kinase pathway to enhance viral infection. Journal of Biological Chemistry **2011**; 286:44869–44877.

43. Freed EO. HIV-1 gag proteins: diverse functions in the virus life cycle. Virology **1998**; 251:1–15.

44. Wills JW, Craven RC. Form, function, and use of retroviral gag proteins. AIDS **1991**; 5:639–654.

45. Gheysen D, Jacobs E, de Foresta F, et al. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. Cell **1989**; 59:103–112.

46. Pascale JM, Isaacs MD, Contreras P, et al. Immunological markers of disease progression in patients infected with the human immunodeficiency virus. Clin. Diagn. Lab. Immunol. **1997**; 4:474–477.

47. Ledergerber B, Flepp M, Böni J, et al. Human immunodeficiency virus type 1 p24 concentration measured by boosted ELISA of heat-denatured plasma correlates with decline in CD4 cells, progression to AIDS, and survival: comparison with viral RNA measurement. The Journal of Infectious Diseases **2000**; 181:1280–1288.

48. Nermut MV, Hockley DJ, Jowett JB, Jones IM, Garreau M, Thomas D. Fullerene-like organization of HIV gag-protein shell in virus-like particles produced by recombinant baculovirus. Virology **1994**; 198:288–296.

49. De Francesco MA, Baronio M, Poiesi C. HIV-1 p17 Matrix Protein Interacts with Heparan Sulfate Side Chain of CD44v3, Syndecan-2, and Syndecan-4 Proteoglycans Expressed on Human Activated CD4+ T Cells Affecting Tumor Necrosis Factor and Interleukin 2 Production. Journal of Biological Chemistry **2011**; 286:19541–19548.

50. Giagulli C, Magiera AK, Bugatti A, et al. HIV-1 matrix protein p17 binds to the IL-8 receptor CXCR1 and shows IL-8-like chemokine activity on monocytes through Rho/ROCK activation. Blood **2012**; 119:2274–2283.

51. Gelderblom HR, Hausmann EH, Ozel M, Pauli G, Koch MA. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. Virology **1987**; 156:171–176.

52. Terzieva V, Popova D, Kicheva M, et al. Correlation between the degree of immune activation, production of IL-2 and FOXP3 expression in CD4+CD25+ T regulatory cells in HIV-1 infected persons under HAART. Int. Immunopharmacol. **2009**; 9:831–836.

53. De Francesco MA, Caruso A, Fallacara F, et al. HIV p17 enhances lymphocyte proliferation and HIV-1 replication after binding to a human serum factor. AIDS **1998**; 12:245–252.

54. Saphire AC, Bobardt MD, Zhang Z, David G, Gallay PA. Syndecans serve as attachment receptors for human immunodeficiency virus type 1 on macrophages. Journal of Virology **2001**; 75:9187–9200.

55. Geijtenbeek TB, Kwon DS, Torensma R, et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell **2000**; 100:587–597.

56. Wilen CB, Tilton JC, Doms RW. HIV: cell binding and entry. Cold Spring Harb Perspect Med **2012**; 2.

57. Berger EA, Doms RW, Fenyö EM, et al. A new classification for HIV-1. Nature **1998**; 391:240.

58. Chan DC, Chutkowski CT, Kim PS. Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. Proc. Natl. Acad. Sci. U.S.A. **1998**; 95:15613–15617.

59. Wain-Hobson S. The fastest genome evolution ever described: HIV variation in situ. Curr. Opin. Genet. Dev. **1993**; 3:878–883.

60. Coffin JM, Hughes SH, Varmus HE, Swanstrom R, Wills JW. Synthesis,

Assembly, and Processing of Viral Proteins. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, 1997.

61. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. Cell **1988**; 55:1189–1193.

62. Greene WC, Peterlin BM. Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. Nat. Med. **2002**; 8:673–680.

63. Goff SP. Host factors exploited by retroviruses. Nat. Rev. Microbiol. **2007**; 5:253–263.

64. Barboric M, Nissen RM, Kanazawa S, Jabrane-Ferrat N, Peterlin BM. NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II. Mol. Cell **2001**; 8:327–337.

65. Balasubramaniam M, Freed EO. New insights into HIV assembly and trafficking. Physiology (Bethesda) **2011**; 26:236–251.

66. Royce RA, Seña A, Cates W, Cohen MS. Sexual transmission of HIV. N. Engl. J. Med. **1997**; 336:1072–1078.

67. Galvin SR, Cohen MS. The role of sexually transmitted diseases in HIV transmission. Nat. Rev. Microbiol. **2004**; 2:33–42.

68. Plummer FA. Heterosexual transmission of human immunodeficiency virus type 1 (HIV): interactions of conventional sexually transmitted diseases, hormonal contraception and HIV-1. AIDS Res. Hum. Retroviruses **1998**; 14 Suppl 1:S5–10.

69. Kaul R, Pettengell C, Sheth PM, et al. The genital tract immune milieu: an important determinant of HIV susceptibility and secondary transmission. J. Reprod. Immunol. **2008**; 77:32–40.

70. Gray L, Fiscus S, Shugars D. HIV-1 variants from a perinatal transmission pair demonstrate similar genetic and replicative properties in tonsillar tissues and peripheral blood mononuclear cells. AIDS Res. Hum. Retroviruses **2007**; 23:1095–1104.

71. Auvert B, Taljaard D, Lagarde E, Sobngwi-Tambekou J, Sitta R, Puren A. Randomized, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 Trial. PLoS Med **2005**; 2:e298.

72. Gray RH, Kigozi G, Serwadda D, et al. Male circumcision for HIV prevention in men in Rakai, Uganda: a randomised trial. Lancet **2007**; 369:657–666.

73. Gray RH, Li X, Kigozi G, et al. Increased risk of incident HIV during pregnancy in Rakai, Uganda: a prospective study. Lancet **2005**; 366:1182–1188.

74. Richman DD, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc. Natl. Acad. Sci. U.S.A. **2003**; 100:4144–4149.

75. Wawer MJ, Gray RH, Sewankambo NK, et al. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. The Journal of Infectious Diseases **2005**; 191:1403–1409.

76. Quinn TC, Wawer MJ, Sewankambo N, et al. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. N. Engl. J. Med. **2000**; 342:921–929.

77. Derdeyn CA, Decker JM, Bibollet-Ruche F, et al. Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. Science **2004**; 303:2019–2022.

78. Keele BF, Giorgi EE, Salazar-Gonzalez JF, et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc. Natl. Acad. Sci. U.S.A. **2008**; 105:7552–7557.

79. Toniolo A, Serra C, Conaldi PG, Basolo F, Falcone V, Dolei A. Productive HIV-1 infection of normal human mammary epithelial cells. AIDS **1995**; 9:859–866.

80. Margolis L, Shattock R. Selective transmission of CCR5-utilizing HIV-1: the 'gatekeeper' problem resolved? Nat. Rev. Microbiol. **2006**; 4:312–317.

81. Margolis L, Shattock R. Selective transmission of CCR5-utilizing HIV-1: the 'gatekeeper' problem resolved? Nat. Rev. Microbiol. **2006**; 4:312–317.

82. Li H, Bar KJ, Wang S, et al. High Multiplicity Infection by HIV-1 in Men Who Have Sex with Men. PLoS Pathog. **2010**; 6:e1000890.

83. Bar KJ, Li H, Chamberland A, et al. Wide variation in the multiplicity of HIV-1 infection among injection drug users. J. Virol. **2010**; 84:6241–6247.

84. Russell ES, Kwiek JJ, Keys J, et al. The genetic bottleneck in vertical transmission of subtype C HIV-1 is not driven by selection of especially neutralization-resistant virus from the maternal viral population. J. Virol. **2011**; 85:8253–8262.

85. Huang Y, Paxton WA, Wolinsky SM, et al. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat. Med. **1996**; 2:1240–1243.

86. Howell AL, Edkins RD, Rier SE, et al. Human immunodeficiency virus type 1 infection of cells and tissues from the upper and lower human female reproductive tract. Journal of Virology **1997**; 71:3498–3506.

87. Norvell MK, Benrubi GI, Thompson RJ. Investigation of microtrauma after sexual intercourse. J Reprod Med **1984**; 29:269–271.

88. Kawamura T, Cohen SS, Borris DL, et al. Candidate microbicides block HIV-1 infection of human immature Langerhans cells within epithelial tissue explants. J. Exp. Med. **2000**; 192:1491–1500.

89. Bomsel M. Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. Nat. Med. **1997**; 3:42–47.

90. Wahl A, Swanson MD, Nochi T, et al. Human Breast Milk and Antiretrovirals Dramatically Reduce Oral HIV-1 Transmission in BLT Humanized Mice. PLoS Pathog. **2012**; 8:e1002732.

91. Gay C, Dibben O, Anderson JA, et al. Cross-sectional detection of acute HIV infection: timing of transmission, inflammation and antiretroviral therapy. PLoS ONE **2011**; 6:e19617.

92. Hladik F, Hope TJ. HIV infection of the genital mucosa in women. Curr HIV/AIDS Rep **2009**; 6:20–28.

93. Brenchley JM. CD4+ T Cell Depletion during all Stages of HIV Disease Occurs Predominantly in the Gastrointestinal Tract. Journal of Experimental Medicine **2004**; 200:749–759.

94. Appay V, Sauce D. Immune activation and inflammation in HIV-1 infection: causes and consequences. J. Pathol. **2008**; 214:231–241.

95. Deeks SG, Kitchen CMR, Liu L, et al. Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. Blood **2004**; 104:942–947.

96. Liu Z, Cumberland WG, Hultin LE, Kaplan AH, Detels R, Giorgi JV. CD8+ T-lymphocyte activation in HIV-1 disease reflects an aspect of pathogenesis distinct from viral burden and immunodeficiency. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **1998**; 18:332–340.

97. Lester RT, Yao X-D, Ball TB, et al. HIV-1 RNA dysregulates the natural TLR response to subclinical endotoxemia in Kenyan female sex-workers. PLoS ONE **2009**; 4:e5644.

98. Lester RT, Yao X-D, Ball TB, et al. Toll-like receptor expression and

responsiveness are increased in viraemic HIV-1 infection. AIDS **2008**; 22:685–694.

99. Rajasuriar R, Khoury G, Kamarulzaman A, French MA, Cameron PU, Lewin SR. Persistent immune activation in chronic HIV infection. AIDS **2013**; :1.

100. Haase AT. Early events in sexual transmission of HIV and SIV and opportunities for interventions. Annu. Rev. Med. **2011**; 62:127–139.

101. Mellors JW, Rinaldo CR, Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science **1996**; 272:1167–1170.

102. Moore RD, Chaisson RE. Natural history of HIV infection in the era of combination antiretroviral therapy. AIDS **1999**; 13:1933–1942.

103. Janeway CA, Medzhitov R. Innate immune recognition. Annu. Rev. Immunol. **2002**; 20:197–216.

104. Medzhitov R, Janeway CA. Decoding the patterns of self and nonself by the innate immune system. Science **2002**; 296:298–300.

105. Heil F, Hemmi H, Hochrein H, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science **2004**; 303:1526–1529.

106. Beignon A-S, McKenna K, Skoberne M, et al. Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions. J. Clin. Invest. **2005**; 115:3265–3275.

107. Liew FY, Xu D, Brint EK, O'Neill LAJ. Negative regulation of toll-like receptor-mediated immune responses. Nat. Rev. Immunol. **2005**; 5:446–458.

108. Kawai T, Akira S. Antiviral signaling through pattern recognition receptors. J. Biochem. **2007**; 141:137–145.

109. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc. Natl. Acad. Sci. U.S.A. **1998**; 95:588–593.

110. Zähringer U, Lindner B, Inamura S, Heine H, Alexander C. TLR2 - promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. Immunobiology **2008**; 213:205–224.

111. Matsushima N, Tanaka T, Enkhbayar P, et al. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. BMC Genomics **2007**; 8:124.

112. Jin MS, Kim SE, Heo JY, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell **2007**; 130:1071–1082.

113. Kang JY, Nan X, Jin MS, et al. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. Immunity **2009**; 31:873–884.

114. Guan Y, Ranoa DRE, Jiang S, et al. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. J. Immunol. **2010**; 184:5094–5103.

115. Boehme KW, Guerrero M, Compton T. Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells. J. Immunol. **2006**; 177:7094–7102.

116. Leoni V, Gianni T, Salvioli S, Campadelli-Fiume G. Herpes Simplex Virus Glycoproteins gH/gL and gB Bind Toll-Like Receptor 2, and Soluble gH/gL Is Sufficient To Activate NF-κB. J. Virol. **2012**; 86:6555–6562.

117. Chang S, Dolganiuc A, Szabo G. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. J. Leukoc. Biol. **2007**; 82:479–487.

118. Bieback K, Lien E, Klagge IM, et al. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. J. Virol. **2002**; 76:8729–8736.

119. Compton T, Kurt-Jones EA, Boehme KW, et al. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. J. Virol. **2003**; 77:4588–4596.

120. Brenchley JM, Price DA, Douek DC. HIV disease: fallout from a mucosal catastrophe? Nat. Immunol. **2006**; 7:235–239.

121. Chen YH, Böck G, Vornhagen R, Steindl F, Katinger H, Dierich MP. HIV-1 gp41 enhances major histocompatibility complex class I and ICAM-1 expression on H9 and U937 cells. Int. Arch. Allergy Immunol. **1994**; 104:227–231.

122. Koutsonikolis A, Haraguchi S, Brigino EN, Owens UE, Good RA, Day NK. HIV-1 recombinant gp41 induces IL-10 expression and production in peripheral blood monocytes but not in T-lymphocytes. Immunol. Lett. **1997**; 55:109–113.

123. Silvestri G, Sodora DL, Koup RA, et al. Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. Immunity **2003**; 18:441–452.

124. Khaitan A, Unutmaz D. Revisiting immune exhaustion during HIV infection. Curr HIV/AIDS Rep **2011**; 8:4–11.

125. Salazar-Gonzalez JF, Martinez-Maza O, Nishanian P, et al. Increased immune activation precedes the inflection point of CD4 T cells and the increased serum virus load in human immunodeficiency virus infection. The Journal of Infectious Diseases **1998**; 178:423–430.

126. Fahey JL, Taylor JM, Detels R, et al. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. N. Engl. J. Med. **1990**; 322:166–172.

127. Hazenberg MD, Otto SA, van Benthem BHB, et al. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. AIDS **2003**; 17:1881–1888.

128. LeBouder E, Rey-Nores JE, Rushmere NK, et al. Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast milk. J. Immunol. **2003**; 171:6680–6689.

129. Kuroishi T, Tanaka Y, Sakai A, Sugawara Y, Komine K-I, Sugawara S. Human parotid saliva contains soluble toll-like receptor (TLR) 2 and modulates TLR2-mediated interleukin-8 production by monocytic cells. Mol. Immunol. **2007**; 44:1969–1976.

130. Dulay AT, Buhimschi CS, Zhao G, et al. Soluble TLR2 is present in human amniotic fluid and modulates the intraamniotic inflammatory response to infection. J. Immunol. **2009**; 182:7244–7253.

131. Henrick BM, Nag K, Yao X-D, Drannik AG, Aldrovandi GM, Rosenthal KL. Milk matters: soluble Toll-like receptor 2 (sTLR2) in breast milk significantly inhibits HIV-1 infection and inflammation. PLoS ONE **2012**; 7:e40138. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=22792230&retmode=ref&cmd=prlinks.

132. Raby A-C, Le Bouder E, Colmont C, et al. Soluble TLR2 reduces inflammation without compromising bacterial clearance by disrupting TLR2 triggering. J. Immunol. **2009**; 183:506–517.

133. DeVico AL, Gallo RC. Control of HIV-1 infection by soluble factors of the immune response. Nat. Rev. Microbiol. **2004**; 2:401–413.

134. Drannik AG, Henrick BM, Rosenthal KL. War and peace between WAP and HIV: role of SLPI, trappin-2, elafin and ps20 in susceptibility to HIV infection. Biochem. Soc. Trans. **2011**; 39:1427–1432.

135. Drannik AG, Nag K, Yao X-D, Henrick BM, Sallenave J-M, Rosenthal KL.

Trappin-2/elafin modulate innate immune responses of human endometrial epithelial cells to PolyI:C. PLoS ONE **2012**; 7:e35866. Available at: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=22545145&retmode=ref&cmd=prlinks.

136. Breastfeeding and HIV International Transmission Study Group, Coutsoudis A, Dabis F, et al. Late postnatal transmission of HIV-1 in breast-fed children: an individual patient data meta-analysis. The Journal of Infectious Diseases **2004**; 189:2154–2166.

137. Kourtis AP, Lee FK, Abrams EJ, Jamieson DJ, Bulterys M. Mother-to-child transmission of HIV-1: timing and implications for prevention. Lancet Infect Dis **2006**; 6:726–732.

138. Brandtzaeg P. Mucosal immunity: integration between mother and the breast-fed infant. Vaccine **2003**; 21:3382–3388.

139. Hanson LA, Korotkova M, Telemo ER. Breast-feeding, infant formulas, and the immune system. **2003**; 90:59–63.

140. World Health Organization. Global HIV/AIDS Response. World Health Organization Progress Report 2011 **2011**; :1–233.

141. Kramer MS, Chalmers B, Hodnett ED, et al. Promotion of Breastfeeding Intervention Trial (PROBIT): a randomized trial in the Republic of Belarus. JAMA **2001**; 285:413–420.

142. Thapa S, Short RV, Potts M. Breast feeding, birth spacing and their effects on child survival. Nature **1988**; 335:679–682.

143. Kantarci S, Koulinska IN, Aboud S, Fawzi WW, Villamor E. Subclinical mastitis, cell-associated HIV-1 shedding in breast milk, and breast-feeding transmission of HIV-1. J. Acquir. Immune Defic. Syndr. **2007**; 46:651–654.

144. Pillay K, Coutsoudis A, York D, Kuhn L, Coovadia HM. Cell-free virus in breast milk of HIV-1-seropositive women. J. Acquir. Immune Defic. Syndr. **2000**; 24:330–336.

145. Fiscus SA, Aldrovandi GM. Virologic determinants of breast milk transmission of HIV-1. Adv. Exp. Med. Biol. **2012**; 743:69–80.

146. Petitjean G, Becquart P, Tuaillon E, et al. Isolation and characterization of HIV-1-infected resting CD4+ T lymphocytes in breast milk. J. Clin. Virol. **2007**; 39:1–8.

147. Satomi M, Shimizu M, Shinya E, et al. Transmission of macrophage-tropic HIV-

1 by breast-milk macrophages via DC-SIGN. The Journal of Infectious Diseases **2005**; 191:174–181.

148. Moore JS, Rahemtulla F, Kent LW, et al. Oral epithelial cells are susceptible to cell-free and cell-associated HIV-1 infection in vitro. Virology **2003**; 313:343–353.

149. Kazmi SH, Naglik JR, Sweet SP, et al. Comparison of human immunodeficiency virus type 1-specific inhibitory activities in saliva and other human mucosal fluids. Clin. Vaccine Immunol. **2006**; 13:1111–1118.

150. Bomsel M, Alfsen A. Entry of viruses through the epithelial barrier: pathogenic trickery. Nat. Rev. Mol. Cell Biol. **2003**; 4:57–68.

151. Paxson CL, Cress CC. Survival of human milk leukocytes. J. Pediatr. **1979**; 94:61–64.

152. Vorbach C, Capecchi MR, Penninger JM. Evolution of the mammary gland from the innate immune system? Bioessays **2006**; 28:606–616.

153. Neville MC, Keller R, Seacat J, et al. Studies in human lactation: milk volumes in lactating women during the onset of lactation and full lactation. Am. J. Clin. Nutr. **1988**; 48:1375–1386.

154. Hanson LA, Korotkova M. The role of breastfeeding in prevention of neonatal infection. Semin Neonatol **2002**; 7:275–281.

155. Burns DN, Landesman S, Wright DJ, et al. Influence of other maternal variables on the relationship between maternal virus load and mother-to-infant transmission of human immunodeficiency virus type 1. The Journal of Infectious Diseases **1997**; 175:1206–1210.

156. Ioannidis JP, Abrams EJ, Ammann A, et al. Perinatal transmission of human immunodeficiency virus type 1 by pregnant women with RNA virus loads. The Journal of Infectious Diseases **2001**; 183:539–545.

157. Garcia PM, Kalish LA, Pitt J, et al. Maternal levels of plasma human immunodeficiency virus type 1 RNA and the risk of perinatal transmission. Women and Infants Transmission Study Group. N. Engl. J. Med. **1999**; 341:394–402.

158. O'Shea S, Newell ML, Dunn DT, et al. Maternal viral load, CD4 cell count and vertical transmission of HIV-1. J. Med. Virol. **1998**; 54:113–117.

159. Melvin AJ, Burchett SK, Watts DH, et al. Effect of pregnancy and zidovudine therapy on viral load in HIV-1-infected women. J. Acquir. Immune Defic. Syndr.

Hum. Retrovirol. **1997**; 14:232–236.

160. Bulterys M, Landesman S, Burns DN, Rubinstein A, Goedert JJ. Sexual behavior and injection drug use during pregnancy and vertical transmission of HIV-1. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **1997**; 15:76–82.

161. Mwapasa V, Rogerson SJ, Kwiek JJ, et al. Maternal syphilis infection is associated with increased risk of mother-to-child transmission of HIV in Malawi. AIDS **2006**; 20:1869–1877.

162. Minkoff H, Burns DN, Landesman S, et al. The relationship of the duration of ruptured membranes to vertical transmission of human immunodeficiency virus. Am. J. Obstet. Gynecol. **1995**; 173:585–589.

163. Chazotte C, Youchah J, Freda MC. Cocaine using during pregnancy and low birth weight: the impact of prenatal care and drug treatment. Semin. Perinatol. **1995**; 19:293–300.

164. Van de Perre P, Simonon A, Hitimana DG, et al. Infective and anti-infective properties of breastmilk from HIV-1-infected women. The Lancet **1993**; 341:914–918.

165. Embree JE, Njenga S, Datta P, et al. Risk factors for postnatal mother-child transmission of HIV-1. AIDS **2000**; 14:2535–2541.

166. John GC, Nduati RW, Mbori-Ngacha DA, et al. Correlates of mother-to-child human immunodeficiency virus type 1 (HIV-1) transmission: association with maternal plasma HIV-1 RNA load, genital HIV-1 DNA shedding, and breast infections. The Journal of Infectious Diseases **2001**; 183:206–212.

167. Semba RD, Neville MC. Breast-feeding, mastitis, and HIV transmission: nutritional implications. Nutr. Rev. **1999**; 57:146–153.

168. Kuhn L, Kim H-Y, Walter J, et al. HIV-1 Concentrations in Human Breast Milk Before and After Weaning. Sci Transl Med **2013**; 5:181ra51.

169. Salazar-Gonzalez JF, Salazar MG, Learn GH, et al. Origin and evolution of HIV-1 in breast milk determined by single-genome amplification and sequencing. J. Virol. **2011**; 85:2751–2763.

170. Gantt S, Carlsson J, Heath L, et al. Genetic analyses of HIV-1 env sequences demonstrate limited compartmentalization in breast milk and suggest viral replication within the breast that increases with mastitis. J. Virol. **2010**; 84:10812–10819.

171. Henderson GJ, Hoffman NG, Ping LH, et al. HIV-1 populations in blood and

breast milk are similar. Virology **2004**; 330:295–303.

172. Rousseau CM, Nduati RW, Richardson BA, et al. Association of levels of HIV-1-infected breast milk cells and risk of mother-to-child transmission. The Journal of Infectious Diseases **2004**; 190:1880–1888.

173. Rousseau CM, Nduati RW, Richardson BA, et al. Longitudinal analysis of human immunodeficiency virus type 1 RNA in breast milk and of its relationship to infant infection and maternal disease. The Journal of Infectious Diseases **2003**; 187:741–747.

174. Habte HH, de Beer C, Lotz ZE, Tyler MG, Kahn D, Mall AS. Inhibition of human immunodeficiency virus type 1 activity by purified human breast milk mucin (MUC1) in an inhibition assay. Neonatology **2008**; 93:162–170.

175. Semba RD. Mastitis and transmission of human immunodeficiency virus through breast milk. Ann. N. Y. Acad. Sci. **2000**; 918:156–162.

176. Lyimo MA, Howell AL, Balandya E, Eszterhas SK, Connor RI. Innate factors in human breast milk inhibit cell-free HIV-1 but not cell-associated HIV-1 infection of CD4+ cells. J. Acquir. Immune Defic. Syndr. **2009**; 51:117–124.

177. Yamaguchi K, Sugiyama T, Takizawa M, Yamamoto N, Honda M, Natori M. Viability of infectious viral particles of HIV and BMCs in breast milk. J. Clin. Virol. **2007**; 39:222–225.

178. Groot F, Welsch S, Sattentau QJ. Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses. Blood **2008**; 111:4660–4663.

179. Chen P, Hübner W, Spinelli MA, Chen BK. Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses. Journal of Virology **2007**; 81:12582–12595.

180. Shapiro RL, Hughes MD, Ogwu A, et al. Antiretroviral regimens in pregnancy and breast-feeding in Botswana. N. Engl. J. Med. **2010**; 362:2282–2294.

181. Kilewo C, Karlsson K, Massawe A, et al. Prevention of mother-to-child transmission of HIV-1 through breast-feeding by treating infants prophylactically with lamivudine in Dar es Salaam, Tanzania: the Mitra Study. J. Acquir. Immune Defic. Syndr. **2008**; 48:315–323.

182. Koulinska IN, Villamor E, Chaplin B, et al. Transmission of cell-free and cell-associated HIV-1 through breast-feeding. J. Acquir. Immune Defic. Syndr. **2006**;

41:93–99.

183. Nakakeeto ON, umaranayake L. The global strategy to eliminate HIV infection in infants and young children: a seven-country assessment of costs and feasibility. AIDS **2009**; 23:987–995.

184. Hassiotou F, Geddes D. Anatomy of the human mammary gland: Current status of knowledge. Clin Anat **2013**; 26:29–48.

185. Coovadia HM, Coutsoudis A. HIV, infant feeding, and survival: old wine in new bottles, but brimming with promise. AIDS **2007**; 21:1837–1840.

186. Grulee CG, Sanford HN, Schwartz H. Breast and Artificially Fed Infants. Jour. A. M. A. **1935**; :1–3.

187. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. Annu. Rev. Nutr. **2005**; 25:37–58.

188. Newburg DS, Walker WA. Protection of the neonate by the innate immune system of developing gut and of human milk. Pediatr. Res. **2007**; 61:2–8.

189. Lönnerdal B. Bioactive Proteins in Human Milk: Mechanisms of Action. J. Pediatr. **2010**; 156:S26–S30.

190. Lönnerdal B. Nutritional and physiologic significance of human milk proteins. Am. J. Clin. Nutr. **2003**; 77:1537S–1543S.

191. Davidson LA, Lönnerdal B. Persistence of human milk proteins in the breast-fed infant. Acta Paediatr Scand **1987**; 76:733–740.

192. van der Strate BW, Beljaars L, Molema G, Harmsen MC, Meijer DK. Antiviral activities of lactoferrin. Antiviral Res. **2001**; 52:225–239.

193. Field CJ. The immunological components of human milk and their effect on immune development in infants. J. Nutr. **2005**; 135:1–4.

194. Aldrovandi GM, Kuhn L. What infants and breasts can teach us about natural protection from HIV infection. The Journal of Infectious Diseases **2010**; 202 Suppl 3:S366–70.

195. Kuhn L, Trabattoni D, Kankasa C, et al. Hiv-specific secretory IgA in breast milk of HIV-positive mothers is not associated with protection against HIV transmission among breast-fed infants. J. Pediatr. **2006**; 149:611–616.

196. Hocini H, Belec L, Iscaki S, et al. High-level ability of secretory IgA to block HIV type 1 transcytosis: contrasting secretory IgA and IgG responses to

glycoprotein 160. AIDS Res. Hum. Retroviruses **1997**; 13:1179–1185.

197. Saeland E, de Jong MAWP, Nabatov AA, Kalay H, Geijtenbeek TBH, van Kooyk Y. MUC1 in human milk blocks transmission of human immunodeficiency virus from dendritic cells to T cells☆. Molecular Immunology **2009**; 46:2309–2316.

198. Groot F, Geijtenbeek TBH, Sanders RW, et al. Lactoferrin prevents dendritic cell-mediated human immunodeficiency virus type 1 transmission by blocking the DC-SIGN--gp120 interaction. Journal of Virology **2005**; 79:3009–3015.

199. McNeely TB, Dealy M, Dripps DJ, Orenstein JM, Eisenberg SP, Wahl SM. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. J. Clin. Invest. **1995**; 96:456–464.

200. Semba RD, Miotti PG, Lan Y, et al. Maternal serum lactoferrin and vertical transmission of HIV. AIDS **1998**; 12:331–332.

201. Bernt KM, Walker WA. Human milk as a carrier of biochemical messages. Acta Paediatr Suppl **1999**; 88:27–41.

202. Gomez HF, Ochoa TJ, Carlin LG, Cleary TG. Human lactoferrin impairs virulence of Shigella flexneri. The Journal of Infectious Diseases **2003**; 187:87–95.

203. Farquhar C, VanCott TC, Mbori-Ngacha DA, et al. Salivary secretory leukocyte protease inhibitor is associated with reduced transmission of human immunodeficiency virus type 1 through breast milk. The Journal of Infectious Diseases **2002**; 186:1173–1176.

204. McNeely TB, Shugars DC, Rosendahl M, Tucker C, Eisenberg SP, Wahl SM. Inhibition of human immunodeficiency virus type 1 infectivity by secretory leukocyte protease inhibitor occurs prior to viral reverse transcription. Blood **1997**; 90:1141–1149.

205. Bode L, Kuhn L, Kim H-Y, et al. Human milk oligosaccharide concentration and risk of postnatal transmission of HIV through breastfeeding. Am. J. Clin. Nutr. **2012**; 96:831–839.

206. Dragic T, Litwin V, Allaway GP, et al. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature **1996**; 381:667–673.

207. Bosire R, Guthrie BL, Lohman-Payne B, et al. Longitudinal comparison of chemokines in breastmilk early postpartum among HIV-1-infected and

uninfected Kenyan women. Breastfeed Med **2007**; 2:129–138.

208. Walter J, Ghosh MK, Kuhn L, et al. High Concentrations of Interleukin 15 in Breast Milk Are Associated with Protection against Postnatal HIV Transmission. The Journal of Infectious Diseases **2009**; 200:1498–1502.

209. Lohman-Payne B, Slyker JA, Moore S, et al. Breast milk cellular HIV-specific interferon γ responses are associated with protection from peripartum HIV transmission. AIDS **2012**; 26:2007–2016.

210. Hughes A, Brock JH, Parrott DM, Cockburn F. The interaction of infant formula with macrophages: effect on phagocytic activity, relationship to expression of class II MHC antigen and survival of orally administered macrophages in the neonatal gut. Immunology **1988**; 64:213–218.

211. Michie CA, Tantscher E, Schall T, Rot A. Physiological secretion of chemokines in human breast milk. Eur. Cytokine Netw. **1998**; 9:123–129.

212. Goldman AS. Human milk, leukocytes, and immunity. J. Pediatr. **1977**; 90:167–168.

213. Goldman AS. The immune system of human milk: antimicrobial, antiinflammatory and immunomodulating properties. Pediatr. Infect. Dis. J. **1993**; 12:664–671.

214. Jain N, Mathur NB, Sharma VK, Dwarkadas AM. Cellular composition including lymphocyte subsets in preterm and full term human colostrum and milk. Acta Paediatr Scand **1991**; 80:395–399.

215. Hassiotou F, Geddes DT, Hartmann PE. Cells in Human Milk: State of the Science. Journal of Human Lactation **2013**; 29:171–182.

216. Dorosko SM, Connor RI. Primary human mammary epithelial cells endocytose HIV-1 and facilitate viral infection of CD4+ T lymphocytes. J. Virol. **2010**; 84:10533–10542.

217. Kramer MS. 'Breast is best': The evidence. Early Hum. Dev. **2010**; 86:729–732.

218. Morrow AL, Rangel JM. Human milk protection against infectious diarrhea: implications for prevention and clinical care. Semin Pediatr Infect Dis **2004**; 15:221–228.

219. LeBouder E, Rey-Nores JE, Raby A-C, et al. Modulation of neonatal microbial recognition: TLR-mediated innate immune responses are specifically and differentially modulated by human milk. J. Immunol. **2006**; 176:3742–3752.

220. Thacker TC, Johnson FB. Binding of bovine parvovirus to erythrocyte membrane sialylglycoproteins. J. Gen. Virol. **1998**; 79 (Pt 9):2163–2169.

221. Yang RB, Mark MR, Gray A, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature **1998**; 395:284–288.

222. Kirschning CJ, Wesche H, Merrill Ayres T, Rothe M. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J. Exp. Med. **1998**; 188:2091–2097.

223. Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J. Immunol. **2000**; 165:618–622.

224. Texereau J, Chiche J-D, Taylor W, Choukroun G, Comba B, Mira J-P. The importance of Toll-like receptor 2 polymorphisms in severe infections. Clin. Infect. Dis. **2005**; 41 Suppl 7:S408–15.

225. Montefiori DC. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol. Biol. **2009**; 485:395–405.

226. Semba RD, Kumwenda N, Taha TE, et al. Mastitis and immunological factors in breast milk of human immunodeficiency virus-infected women. J Hum Lact **1999**; 15:301–306.

227. Lee-Huang S, Maiorov V, Huang PL, et al. Structural and functional modeling of human lysozyme reveals a unique nonapeptide, HL9, with anti-HIV activity. Biochemistry **2005**; 44:4648–4655.

228. Chowanadisai W, Lönnerdal B. Alpha(1)-antitrypsin and antichymotrypsin in human milk: origin, concentrations, and stability. Am. J. Clin. Nutr. **2002**; 76:828–833.

229. Swart PJ, Kuipers ME, Smit C, et al. Antiviral effects of milk proteins: acylation results in polyanionic compounds with potent activity against human immunodeficiency virus types 1 and 2 in vitro. AIDS Res. Hum. Retroviruses **1996**; 12:769–775.

230. Taggart CC, Cryan S-A, Weldon S, et al. Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding. J. Exp. Med. **2005**; 202:1659–1668.

231. Legrand FA, Nixon DF, Loo CP, et al. Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants and neonates revealed after regulatory T cell removal. PLoS ONE **2006**; 1:e102.

232. Bosire R, John-Stewart GC, Mabuka JM, et al. Breast Milk α-Defensins Are Associated with HIV Type 1 RNA And CC Chemokines in Breast Milk But Not Vertical HIV Type 1 Transmission. AIDS Res. Hum. Retroviruses **2007**; 23:198–203.

233. Chitnis V, Pahwa R, Pahwa S. Determinants of HIV-specific CD8 T-cell responses in HIV-infected pediatric patients and enhancement of HIV-gag-specific responses with exogenous IL-15. Clin. Immunol. **2003**; 107:36–45.

234. Mueller YM, Petrovas C, Bojczuk PM, et al. Interleukin-15 increases effector memory CD8+ t cells and NK Cells in simian immunodeficiency virus-infected macaques. Journal of Virology **2005**; 79:4877–4885.

235. Dolganiuc A, Oak S, Kodys K, et al. Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. Gastroenterology **2004**; 127:1513–1524.

236. Fiorentini S, Giagulli C, Caccuri F, Magiera AK, Caruso A. HIV-1 matrix protein p17: a candidate antigen for therapeutic vaccines against AIDS. Pharmacol. Ther. **2010**; 128:433–444.

237. Becker PD, Fiorentini S, Link C, et al. The HIV-1 matrix protein p17 can be efficiently delivered by intranasal route in mice using the TLR 2/6 agonist MALP-2 as mucosal adjuvant. Vaccine **2006**; 24:5269–5276.

238. Postler TS, Desrosiers RC. The Cytoplasmic Domain of the HIV-1 Glycoprotein gp41 Induces NF-κB Activation through TGF-β-Activated Kinase 1. Cell Host and Microbe **2012**; 11:181–193.

239. Borghi P, Fantuzzi L, Varano B, et al. Induction of interleukin-10 by human immunodeficiency virus type 1 and its gp120 protein in human monocytes/macrophages. Journal of Virology **1995**; 69:1284–1287.

240. Schneider J, Kaaden O, Copeland TD, Oroszlan S, Hunsmann G. Shedding and interspecies type sero-reactivity of the envelope glycopolypeptide gp120 of the human immunodeficiency virus. J. Gen. Virol. **1986**; 67 (Pt 11):2533–2538.

241. Andrew A, Strebel K. HIV-1 Vpu targets cell surface markers CD4 and BST-2 through distinct mechanisms. Mol. Aspects Med. **2010**; 31:407–417.

242. Sheehy AM, Gaddis NC, Malim MH. The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. Nat. Med. **2003**; 9:1404–1407.

243. Dimitrov DS, Willey RL, Sato H, Chang LJ, Blumenthal R, Martin MA.

Quantitation of human immunodeficiency virus type 1 infection kinetics. Journal of Virology **1993**; 67:2182–2190.

244. Popovic M, Tenner-Racz K, Pelser C, et al. Persistence of HIV-1 structural proteins and glycoproteins in lymph nodes of patients under highly active antiretroviral therapy. Proc. Natl. Acad. Sci. U.S.A. **2005**; 102:14807–14812.

245. Ding J, Rapista A, Teleshova N, et al. Neisseria gonorrhoeae enhances HIV-1 infection of primary resting CD4+ T cells through TLR2 activation. J. Immunol. **2010**; 184:2814–2824.

246. Heggelund L, Damås JK, Yndestad A, et al. Stimulation of toll-like receptor 2 in mononuclear cells from HIV-infected patients induces chemokine responses: possible pathogenic consequences. Clin. Exp. Immunol. **2004**; 138:116–121.

— APPENDIX I —

Figure 7

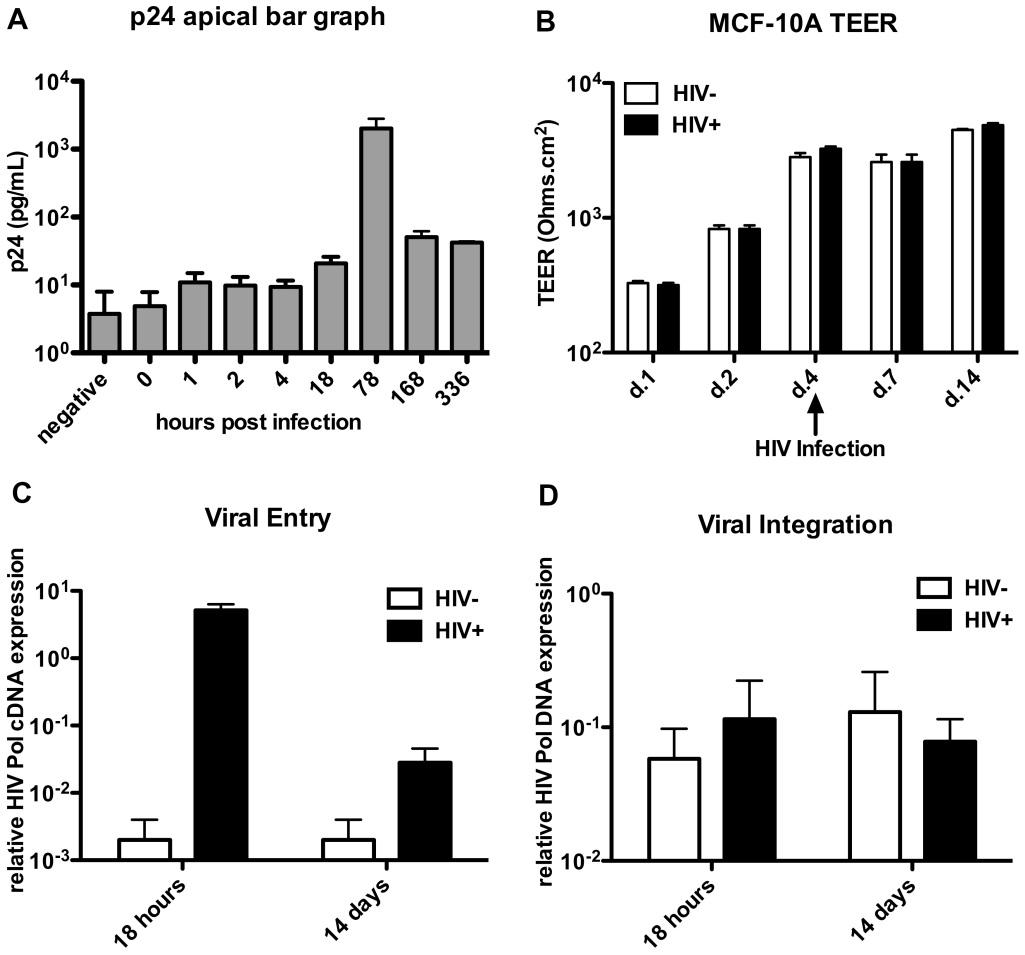


Figure 7. MCF-10A basolateral monolayer exposed to cell-free R5 HIV-1. (A) Apical p24 was quantified at various time points after a one-hour exposure of 100 $TCID_{50}$ R5 virus on the basolateral surface. (B) Transepithelial electrical resistance (TEER) was measured in HIV exposed and unexposed MCF-10A monolayers at various time points before and after infection. (C) MCF-10A cells were lysed and evaluated for presence of HIV-1 Pol cDNA at 18 hours and 14 days after basolateral HIV exposure. (D) MCF-10A cells were lysed and evaluated for presence of HIV-1 Pol DNA at 18 hours and 14 days after basolateral HIV exposure.

Figure 8

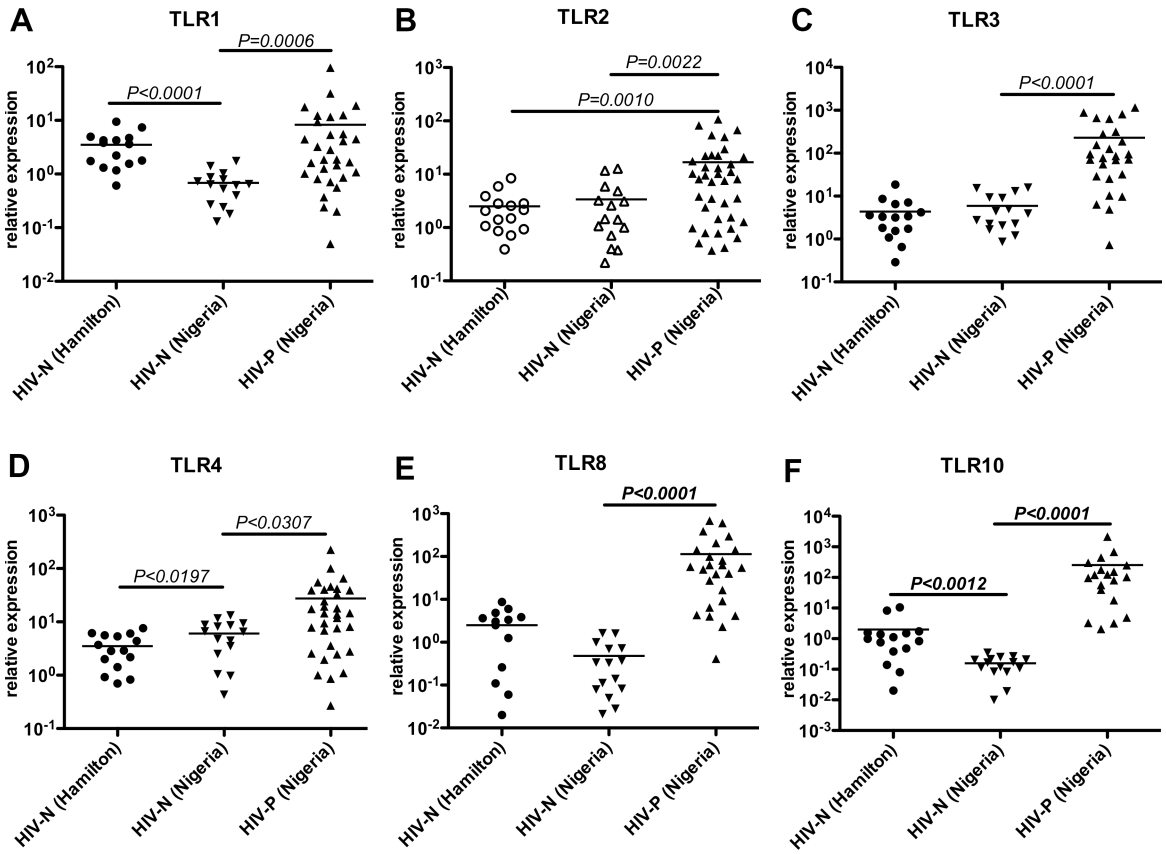


Figure 8. Breast milk cells from HIV-uninfected and HIV-infected women from the Hamilton Area and Nigeria were lysed and TLR mRNA was evaluated. TLR1, TLR2, TLR3, TLR4, TLR8, and TLR10 mRNA was significantly elevated in breast milk cells from HIV-infected compared to uninfected breast milk. Additionally, HIV-uninfected women from Nigeria had significantly reduced TLR1 and TLR10 mRNA expression, while TLR4 expression was significantly elevated compared to uninfected Hamilton women. Data considered significant if *P<0.05.*

Figure 9

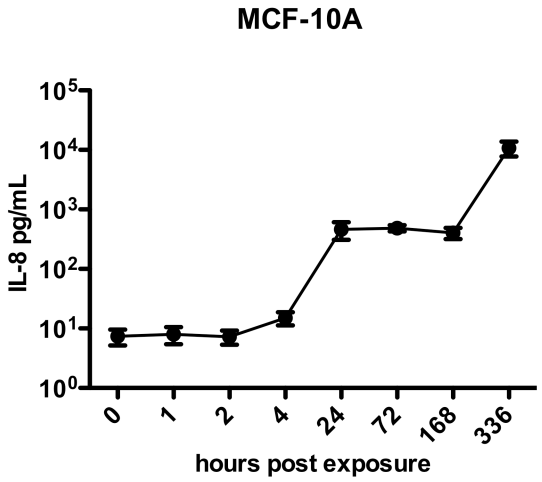


Figure 9. MCF-10A cell monolayers were exposed with 100TCID50 cell-free R5 HIV on the basolateral side for one hour before being washed twice with 1X PBS. Apical supernatants were collected at various time points and IL-8 production was quantified. Samples were tested in triplicate and the data is representative of two independent experiments.