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## THE ROLE OF MUCOSAL EPITHELIAL CELLS IN HIV-1 INFECTION

Danielle Fontenot

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# THE ROLE OF MUCOSAL EPITHELIAL CELLS IN HIV-1 INFECTION

by

Danielle Fontenot, BS, MS

Approved:

---

Dr. K. Jagannadha Sastry, PhD, Supervisory Professor

---

Dr. Rakesh Kumar, PhD

---

Dr. Wei Cao, PhD

---

Dr. Michel Gilliet, MD

---

Dr. Pramod N. Nehete, PhD

Approved:

---

Dr. George M. Stancel, PhD  
Dean, The University of Texas Health Science Center at Houston  
Graduate School of Biomedical Sciences

# THE ROLE OF MUCOSAL EPITHELIAL CELLS IN HIV-1 INFECTION

A

DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Danielle R. Fontenot, BS, MS

Houston, Texas

May, 2010

Dedicated to my parents  
Jerold and Gail Fontenot  
Who have been the true inspirations behind my desire to succeed

## ACKNOWLEDGEMENTS

I would first and foremost like to thank God for granting me the ability and the strength to complete this project. I would also like to thank my parents Jerold and Gail and my brother Jerold Jr. for their continual support. I also want to thank my husband Richard for his support throughout my research and this dissertation writing process.

I would like to express thanks to my advisor Dr. K. Jagannadha Sastry for his continued support and advice in my development as a scientist. He has been a very supportive mentor who has pushed me to heights that I never thought I could experience as a graduate student. In addition to having such a wonderful mentor, there have been a number of individuals whom I must acknowledge. I would like to thank all my committee members in my graduate studies. I would like to thank Drs. Pramod Nehete, Rakesh Kumar, Wei Cao Michel Gillet who were part of all of my academic committees including my advisory, exam, and supervisory committees. In addition, I would like to thank members of my other committees which include Drs. Frank Marini and Bingling Fang.

I am extremely thankful to past and current members of Dr. Sastry's lab. Members of this laboratory have played an essential part in my advancement and made my experience as a graduate student a rewarding experience. I would also like to thank past and current members of Dr. Yong-Jun Liu's lab in particular Dr. Liu and Dr. Shino Hanabuchi who have been supportive in providing advice and direction in various scientific projects.

# THE ROLE OF MUCOSAL EPITHELIAL CELLS IN HIV-1 INFECTION

Publication No. \_\_\_\_\_

Danielle R. Fontenot, BS, MS

Supervisory Professor: K. Jagannadha Sastry, PhD

The predominant route of human immunodeficiency virus type 1 (HIV-1) transmission is infection across the vaginal mucosa. Epithelial cells, which form the primary barrier of protection against pathogens, are the first cell type at these mucosal tissues to encounter the virus but their role in HIV infection has not been clearly elucidated. Although mucosal epithelial cells express only low levels of the receptors required for successful HIV infection, productive infection does occur at these sites. The present work provides evidence to show that HIV exposure, without the need for productive infection, induces human cervical epithelial cells to produce Thymic Stromal Lymphopoietin (TSLP), an IL7-like cytokine, which potently activated human myeloid dendritic cells (mDC) to cause the homeostatic proliferation of autologous CD4<sup>+</sup> T cells that serve as targets for HIV infection. Rhesus macaques inoculated with simian immunodeficiency virus (SIV) or with the simian-human immunodeficiency virus (SHIV) by the vaginal, oral or rectal route exhibited dramatic increases in: TSLP expression, DC and CD4<sup>+</sup> T cell numbers, and viral replication, in the vaginal, oral, and rectal tissues, respectively within the first 2 weeks after virus exposure. Evidence obtained showed that HIV-mediated TSLP production by cervical cells is dependent upon the expression of the cell surface salivary agglutinin (SAG) protein gp340. Epithelial cells expressing gp340 exhibited HIV endocytosis and TSLP expression and genetic knockdown of gp340 or use of a gp340-blocking antibody inhibited TSLP expression by HIV. On the other hand, gp340-null epithelial cells failed to endocytose HIV and produce TSLP, but transfection of gp340 resulted in HIV-induced TSLP expression. Finally, HIV-induced TSLP expression was found to be mediated

by TLR7/8 signaling and NF- $\kappa$ B activity because silencing these pathways or use of specific inhibitors abrogated TSLP expression in gp340-positive but not in gp340-null epithelial cells.

Overall these studies identify TSLP as a key player in the acute phase of HIV-1 infection in permitting HIV to successfully maneuver the hostile vaginal mucosal microenvironment by creating a conducive environment for sustaining the small amount of virus that initially crosses the mucosal barrier allowing it to successfully cause infection and spread to distal compartments of the body

# TABLE OF CONTENTS

	Page
Title Page.....	ii
Dedication.....	iii
Acknowledgements.....	iv
Abstract.....	v
Table of Contents.....	vii
List of Figures.....	ix
List of Tables.....	x
List of Abbreviations.....	xi
CHAPTER	
1 Introduction.....	1
Background on HIV Infection.....	1
HIV Transmission through Mucosal Tissues and the Mucosal Microenvironment.....	9
Mucosal Epithelial Cells and Receptors: DMBT1.....	14
Immune Mediators Produced by Epithelial Cells: Thymic Stromal Lymphopoietin (TSLP).....	18
2 Statement of Objectives.....	23
3 Results: Genital epithelial cells produce TSLP in response to immunodeficiency virus and trigger dendritic cell-mediated expansion and infection of CD4 <sup>+</sup> T cells.....	26
HIV induces production of TSLP in human epithelial cell lines and and primary human keratinocytes in vitro.....	28



TSLP is expressed in vivo within the mucosal tissues of rhesus macaques infected with simian immunodeficiency virus.....	37
TSLP is expressed in the vaginal mucosal tissues infected with human immunodeficiency virus ex vivo.....	46
TSLP from HIV exposed epithelial cells activates human CD11c <sup>+</sup> myeloid dendritic cells.....	51
Myeloid DC activated by TSLP from HIV exposed epithelial cells induce CD4 <sup>+</sup> T cell expansion and HIV infection in vitro.....	56
Higher expression of HIV co-receptors on CD4 <sup>+</sup> T cells co-cultured with myeloid DC activated by TSLP from HIV exposed epithelial cells.....	68
Transcriptional activation of the human TSLP promoter by HIV includes NFkB activation.....	71
Gp340 expressing human epithelial cells produce TSLP in response to HIV.....	75
Binding of gp120 to gp340 is necessary but not sufficient for HIV-mediated TSLP expression in epithelial cells .....	81
Endocytosis of HIV-1 mediated by gp340 on epithelial cells induces TSLP expression.....	85
HIV-1 RNA is responsible for inducing TSLP expression in gp340 <sup>+</sup> cells.....	92
TLR activation as potential mechanism for HIV-induced TSLP expression.....	95
Discussion.....	99
4 Future Directions.....	103
5 Materials and Methods.....	108
6 References.....	123
7 Vita.....	137

## LIST OF FIGURES

Figure		Page
1.1	Cartoon representing the process of HIV-1 entry into human cells .....	3
1.2	Model of delayed systemic SIV replication.....	7
1.3	Potential routes of HIV transmission.....	12
1.4	Model depicting interaction of gp340 SRCR domain with gp120.....	16
1.5	Pathology of TSLP in allergic inflammation.....	21
3.1	HIV induces TSLP expression in cervical epithelial cells.....	30
3.2	HIV induces TSLP expression in a variety of epithelial cells but not non-epithelial cells .....	33
3.3	Productive HIV infection is not necessary for TSLP induction .....	35
3.4	Increased expression of TSLP along with higher numbers of CD11c <sup>+</sup> mDC and CD4 <sup>+</sup> T cells in vaginal mucosal tissues from rhesus macaques after vaginal SIV infection.....	39
3.5	Expression of TSLP by epithelial cells in oral mucosal tissues collected from SHIV infected rhesus macaques .....	42
3.6	Expression of TSLP by epithelial cells in rectal mucosal tissues collected from SHIV infected rhesus macaques .....	44
3.7	Expression of TSLP by epithelial cells in cervical explant tissues .....	47
3.8	Increased expression of TSLP in human cervical explant tissues after HIV infection .....	49
3.9	TSLP within the supernatant from HIV exposed epithelial cells potently activates human CD11c <sup>+</sup> DCs .....	52
3.10	Chemokine production by epithelial cell supernatant treated DCs is specific for TSLP .....	54

3.11	mDC activated by HIV-induced TSLP from epithelial cells promote naïve autologous CD4 <sup>+</sup> T cell proliferation and increased HIV infection.....	58
3.12	mDC activated by HIV-induced TSLP from epithelial cells promotes the generation of Th2 cells .....	62
3.13	mDC activated by HIV-induced TSLP from epithelial cells promote increased HIV infection .....	64
3.14	mDC activated by HIV-induced TSLP from epithelial cells promotes higher expression of chemokine co-receptors on CD4 <sup>+</sup> T cells.....	69
3.15	Transcriptional Activation of the Human TSLP promoter by HIV involves NFκB signaling .....	74
3.16	Gp340 <sup>+</sup> cells produce TSLP in response to HIV. ....	77
3.17	Gp340 cross linking does not signal TSLP expression in the epithelial cells.....	83
3.18	Endocytosis of HIV into epithelial cells .....	87
3.19	Gp340 receptor mediated endocytosis of HIV triggers HIV induced TSLP expression .....	89
3.20	HIV RNA stimulates TSLP production in epithelial cells .....	93
3.21	TLR activation involved in HIV induced TSLP expression .....	96
4.1	9-cis-RA inhibition of TSLP expression .....	106

## LIST OF TABLES

1	SIV and TSLP mRNA levels in the vaginal tissues of rhesus macaques prior to and at sequential time points after vaginal infection with SIV <sub>mac251</sub> .....	41
2	Well studied HIV-1 binding molecules expression on different cell lines.....	82

## LIST OF ABBREVIATIONS

4mBr-5 .....	Rhesus Macaque epithelial cells
9-cis-RA .....	9 cis Retinoic Acid
AT-2 .....	Aldrithiol-2
BFLA-1 .....	Bafilomycin A1
C33A .....	Cervical epithelial cells
CCD .....	Cytochalasin D
cDNA .....	Complimentary Deoxyribonucleic acid
DC .....	Dendritic Cells
DMA .....	Dimethyl amiloride
DMBT1 .....	Deleted in Malignant Brain Tumors
DMEM.....	Dubulcco Modified Eagle Medium
DNA.....	Deoxyribonucleic acid
DOTAP .....	Liposomal Transfection Reagent
ELISA.....	Enzyme-Linked Immunosorbent Assay
EMEM .....	Eagle's Minimum Essential Medium
Fadu .....	Pharyngeal Epithelial cells
FBS.....	Fetal Bovine Serum
GALT.....	Gut Associated Lymphoid Tissue
GHOST.....	GFP-expressing Human Osteosarcoma Tumor Cells
Gp120.....	HIV Envelope surface glycoprotein 120
Gp340... ..	Cell surface glycoprotein 340
H9 .....	Human T-lymphoblastoid cells
HIV-1.....	Human Immunodeficiency Virus type 1

HT29 .....	Human Intestinal Epithelial cells
I $\kappa$ Ba ...	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, Alpha
IL-1 $\beta$ .....	Interleukin 1 $\beta$
IL-4 .....	Interleukin 4
IL-5 .....	Interleukin 5
IL-7 .....	Interleukin 7
IL-10 .....	Interleukin 10
IL-13 .....	Interleukin 13
Inf $\gamma$ .....	Interferon gamma
ISH .....	In situ hybridization
KDa.....	Kilodalton
LC .....	Langherans cells
LPS .....	Lipopolysaccharide
MAGI cells.....	Multinucleate Activation of Galactosidase Indicator Cells
MDC.....	Macrophage Derived Chemokine
mDC .....	Myeloid Dendritic Cells
NF $\kappa$ B.....	Nuclear Factor $\kappa$ B
NHEK .....	Normal Human Epidermal Kidney cells
PBMCs.....	Primary human peripheral blood mononuclear cells
PCR.....	Polymerase Chain Reaction
PolyI:C.....	polyinosinic:polycytidylic acid
RNA.....	Ribonucleic acid
RPMI 1640.....	Roswell Park Memorial Institute 1640
RT .....	Reverse Transcriptase
RXR.....	Retinoid X Receptors

SAG .....	Salivary agglutinin
SHIV .....	Simian Immunodeficiency Virus
SIV .....	Simian Human Immunodeficiency Virus
siRNA .....	Small interfering RNA
SKMN .....	Neuronal epithelial cells
Sn .....	Supernatant
ssRNA .....	Single Stranded RNA
TARC .....	Thymus and Activation Regulated Chemokine
TLR .....	Toll Like Receptor
TNF .....	Tumor Necrosis Factor
TNFR .....	Tumor Necrosis Factor Receptor
TSLP .....	Thymic Stromal Lymphopoietin
VSV .....	Vesicular Stomatitis Virus

# CHAPTER 1

## Introduction

### Background on HIV Infection

The human immunodeficiency virus (HIV) is a double stranded RNA virus belonging to the Lentivirus genus and the Retroviridae family [1-4]. The viral genome contains 2 identical (+) RNA strands that are non-covalently bound and are approximately 9.5 kb in size [5]. Upon entry of the target cell the single-stranded RNA is reverse transcribed by the viral RNA-dependent DNA polymerase, also referred to as the reverse transcriptase [3, 5]. Two distinct sub-types of HIV, HIV-1 and HIV-2, are identified based on their introduction into human population as well as by their differential virulence to humans [1-4]. The HIV type 1, HIV-1, is the descendent of chimpanzee derived simian immunodeficiency virus (SIVcpz), and it is the more virulent of the two forms of HIV. HIV-1 accounts for the majority of human infections worldwide [6]. The HIV type 2, HIV-2, is a descendent of SIV isolated from sooty mangabey monkeys (SIVsmm), and is generally found in West Africa. In humans, HIV-2 is less virulent and less transmissible as compared to HIV-1 [7] and also exhibits a longer clinical latency period and a lower rate of morbidity of the resulting disease. Nevertheless, infection by either HIV-1 or HIV-2 ultimately leads to the development of AIDS in humans.

The HIV-1 infection process begins with the binding of the viral envelope surface protein, a 120 kd glycoprotein, to the CD4 receptor on the target cell [8](Fig. 1.1). Subsequently, the gp120 protein on the virion undergoes a series of conformational changes required for binding to a chemokine receptor, termed co-receptor, on the host cell [8]. Different strains of HIV-1 are classified based on their usage of the chemokine co-receptor: the T-cell-tropic viruses use the CXCR4 chemokine receptor and are referred to as X4 viruses while the macrophage-tropic viruses use the CCR5 chemokine receptor and are referred to as

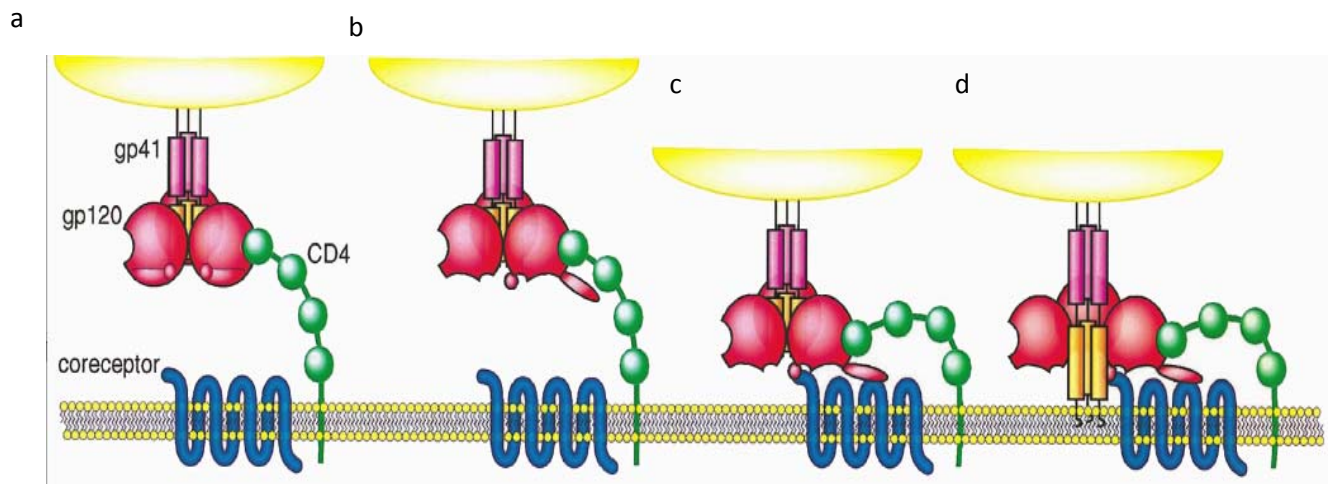
R5 viruses [9-11]. Strains of HIV-1 that use both types of chemokine co-receptors are referred to as dual-tropic viruses [8]. Even though infected individuals harbor a mixture of these different types of viral strains, it is generally believed that HIV-1 transmission in humans is initiated with the infection by the macrophage-tropic HIV-1 strains but during progressive infection, the virus shifts towards T-cell tropism. This shift in viral tropism usually occurs late in the course of disease and marks an accelerated phase in the clinical disease progression [12]. Once the gp120-CD4 complex is bound to the appropriate co-receptor, additional conformational changes expose the transmembrane glycoprotein gp41 which gets inserted into the membrane of the target cell for subsequent fusion of the viral and target cell membranes (Fig. 1.1). One consequence of HIV infection is the decline in the numbers of memory subsets of CD4<sup>+</sup> T cells and when this decline reaches below a critical level, HIV infected individuals develop susceptibility to opportunistic infections that eventually culminate into the acquired immunodeficiency syndrome (AIDS). In 2007, there were an estimated 33.2 million cases of HIV infection worldwide with oral and genital mucosal infections accounting for the majority of all reported cases of HIV-1 infection and transmission [13].



**Fig. 1.1: Cartoon representing the process of HIV-1 entry into human cells:** The surface envelope glycoprotein gp120 of the virus binds to the CD4 receptor on the host cell membrane resulting in conformational changes in gp120 (a) that enable co-receptor binding (b) followed by the exposure of the gp41 transmembrane glycoprotein (c) for fusion of the viral and host cell membranes (d). Reprinted from Cell, Vol 85, Doranz, B. et al., *A dual-tropic primary HIV-1 isolate that uses CXCR4 and the b-chemokine receptor CKR-5, CKR-3, and CKR2b as fusion cofactors*, Pages 1149-1158, with permission from Elsevier.

Source: Doranz et al. [8]

**Fig. 1.1:**



**Animal model for genital HIV transmission:**

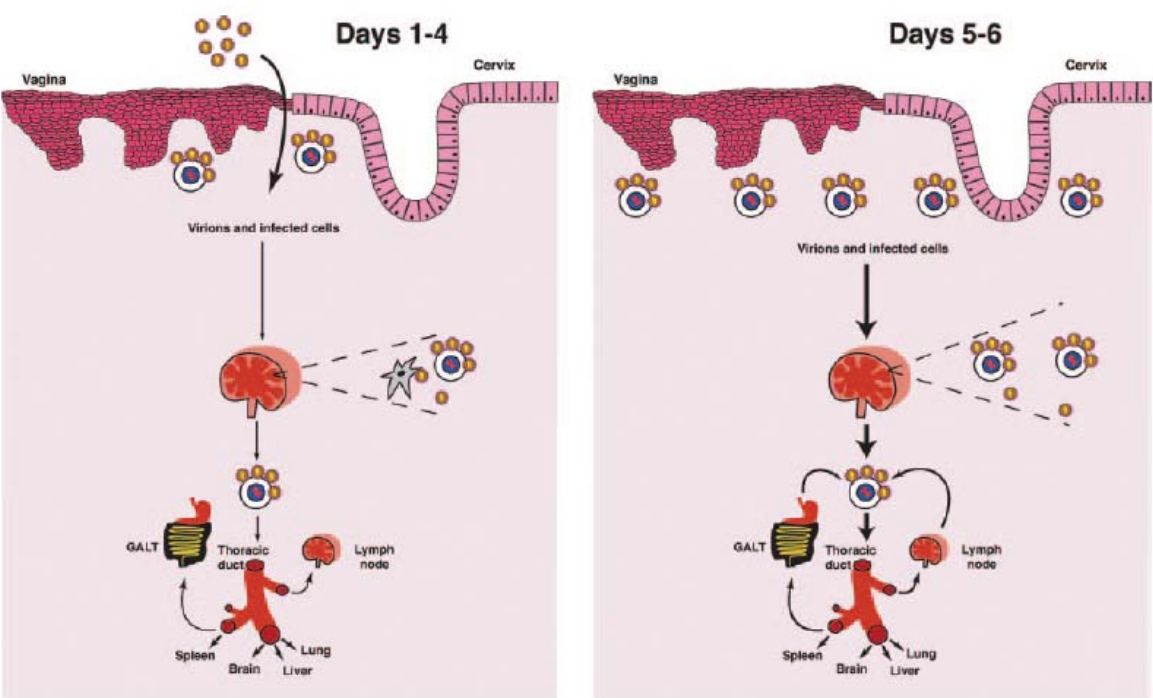
The rhesus macaque (*Macaca mulatta*) model is widely used to extensively study HIV transmission. This model uses either the simian immunodeficiency virus (SIV), a primate lentivirus closely related to HIV, or a laboratory-created hybrid virus called simian-human immunodeficiency virus (SHIV) consisting of genes that encode the HIV envelope and the SIV core. Both of these viruses can be easily transmitted to macaques by vaginal inoculation and cause AIDS-like illness characterized by severe loss of CD4<sup>+</sup> T cells and opportunistic infections. This model of SIV/SHIV infection of macaques is similar to that of HIV in humans in that the target cells, physiology, and immunology of the genital tract are comparable. Using this model, Miller et. al. [14] described the propagation and dissemination of SIV after vaginal transmission (Fig. 1.2). This study showed that between days 1-4 post-infection only a few infected cells could be found in the vaginal tissue, the original site of inoculation, as well as at distant gut-associated lymphoid tissues (GALT). However, beyond day 4 post-infection, there was a substantial increase in infected cells at the local vaginal tissue and a concurrent increase over days 10-14 at the GALT. These observations correlate well with later reports describing mucosal SIV infection in rhesus macaques and HIV infection in humans, both of which result in rapid depletion of intestinal CD4<sup>+</sup> T cells by day 14 post-exposure [15]. This extreme level of depletion of CD4<sup>+</sup> T cells occurs primarily in the mucosal tissues of the gastrointestinal (GI) tract where more than 60% of T lymphocytes reside [15-19]. It is unclear how the virus effectively manipulates the mucosal microenvironment during and soon after exposure such that the initial founder population of infected cells in the vaginal mucosa not only survives but also leads to the systemic spread of infection. The role of the mucosal microenvironment, specifically the mucosal epithelium in sensing HIV infection, triggering immune activation, and promoting HIV spread and amplification remains unknown. A better understanding of the

detailed steps during the mucosal infection process should provide a framework for understanding the propagation, dissemination, and establishment of infection during the acute stage of infection.

**Fig. 1.2: Model of delayed systemic SIV replication.** Study from Miller et al. showing that between days 1-4 post-infection only a few infected cells could be found in the vaginal tissue, the original site of inoculation, as well as at distant lymphoid tissues. However, beyond day 4 post-infection, there is a substantial increase in infected cells at the local vaginal tissue and a concurrent increase over days 10-14 at the distant lymphoid tissues. Reprinted from the Journal of Virology, Vol 79, Miller, C.J. et al., *Propagation and Dissemination of Infection after Vaginal Transmission of Simian Immunodeficiency Virus*, Pages 9217-9227, with permission from the American Society for Microbiology.

Source: Miller et al.[14]

Fig. 1.2:



## **HIV Transmission through Mucosal Tissues and the Mucosal Microenvironment**

The majority of all new HIV transmissions occur in women through vaginal intercourse, a route that carries the lowest HIV transmission probability rate suggesting that the genital epithelium fails to serve as a major barrier against HIV entry [20]. For successful virus transmission, HIV must be efficient in devising strategies to cross the mucosal barrier of the genital tract to infect CD4<sup>+</sup>T cells. Since oral and genital mucosal epithelial cells express low to negligible levels of the receptors for HIV, in particular CD4, it is suggested that the virus utilizes unconventional mechanisms to cross the genital epithelial cell layers [20, 21]. Studies in the rhesus macaque model with SIV suggested that the initial targets of infection may include Langerhans Cells (LCs), subepithelial T-cells and dendritic cells (DCs) within the genital mucosa [14, 20, 22-29]. In order for HIV to reach these targets during mucosal transmission, it must successfully penetrate the epithelial barrier. There are several proposed mechanisms for how HIV-1 may cross the mucosal epithelium (Fig. 1.3). The susceptibility of epithelial cells to HIV infection is controversial with some studies reporting productive HIV infection in epithelial cell lines of gut origin based on the expression of certain glycosphingolipids such as galactosylceramide (GalCer) on the epithelial cells [30-34]. Other studies have suggested that genital epithelial cells can bind, transport, harbor, and transmit virus to additional targets, but cannot be productively infected [21, 34-36]. Yet another proposed mechanism for HIV transmission across the mucosal epithelium is by transmigration of the virus and infected cells through the mucosal epithelial cells. The infected cells can include CD4<sup>+</sup> T cells and monocytes found in semen [20, 37, 38]. Langerhans cells (LC) constitute another vehicle by which virus may traverse the mucosal epithelium. Since LC express CD4, CCR5, and CXCR4 and can be found within the genital tract it has been suggested that LC at these mucosal sites can capture HIV and migrate to the draining lymph

nodes where the virus is transmitted to CD4<sup>+</sup> T cells, the main targets of HIV replication and dissemination[39-41]. Whether or not the LC can be infected after exposure to virus inoculation is still controversial. Some studies of vaginal mucosal transmission of SIV in rhesus macaques demonstrated that submucosal LCs rapidly became infected after virus inoculation while other studies suggest that the CD4<sup>+</sup> T cells are the cells infected and that they are the major source of infectious virus during the acute stages of infection [29, 42]. Ex vivo studies have demonstrated that LCs are not required for, but may aid in, viral transmission [20]. It is also possible that HIV can be transmitted across the mucosal epithelium through breaches in the epithelial layer resulting from trauma associated with sexual intercourse or as a result of co-infection with other sexually transmitted diseases that can weaken the mucosal barrier [43-45]. These breaks in the epithelial barrier allow the virus access to susceptible targets of infection in the underlying tissues but studies in the rhesus macaque model have revealed that barrier disruption is not necessary for successful viral transmission [27]. Finally, it is also proposed that HIV can be transmitted across the mucosal epithelium by transcytosis / endocytosis through the mucosal epithelium involving interactions with epithelial cell surface molecules such as heparin sulfate, enabling infection of the nearby DC and CD4<sup>+</sup> T cells [34-36]. This pathway was initially proposed after virus was observed being transported into endosome like structures after coming into contact with the surface of epithelial cells [20]. Ex vivo studies using the cervical explant model have also shown that HIV-1 virions can be found in endocytic compartments as well as in the cytosol of epithelial cells [20]. However, elimination of heparin sulfate did not abrogate viral transcytosis suggesting that other host cell factors may also be involved in viral transcytosis in epithelial cells [46, 47]. Several recent reports have demonstrated a specific interaction of HIV with epithelial cells through the binding of the HIV surface envelope protein gp120 to the cell surface salivary agglutinin



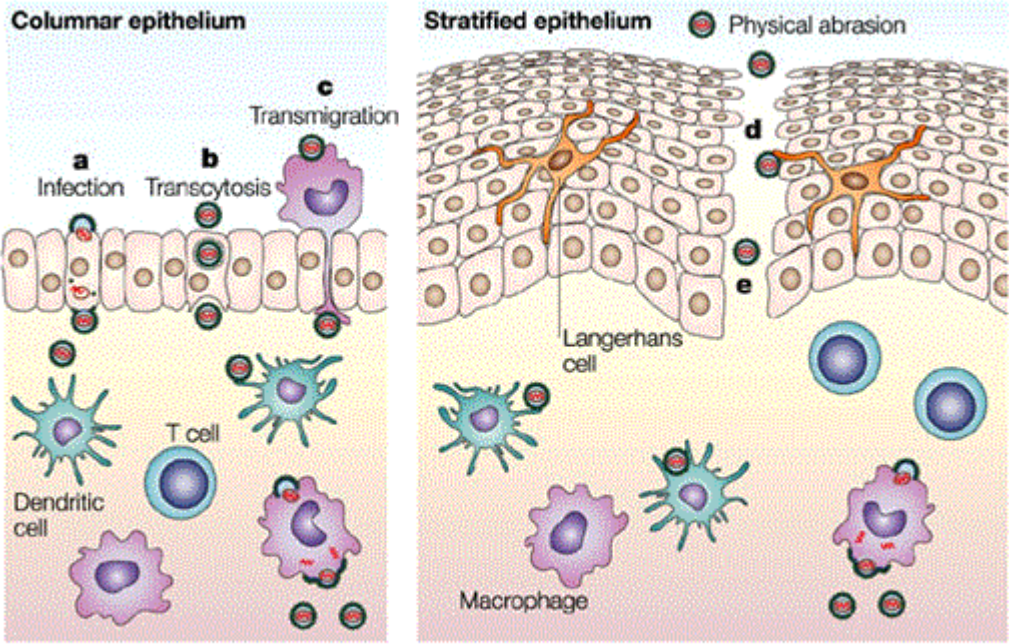
(SAG) glycoprotein gp340, a member of the scavenger receptor cysteine-rich (SRCR) superfamily of proteins and protein splice variant of the Deleted in Malignant Brain Tumors I (DMBT1) that can be either secreted or membrane-bound [21, 48, 49].

**Fig.1.3:**

**Depiction of Potential routes of HIV transmission across the mucosal epithelial layers:** a) direct infection of epithelial cells; b) transcytosis through epithelial cells; c) transmigration of infected donor cells; d) uptake by migratory Langerhans cells; e) direct entry through breaches in the epithelial layer. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology 2003.

Source: Shattock & Moore 2003 [44]

Fig.1.3:



## **Mucosal Epithelial Cells and Receptors: DMBT1**

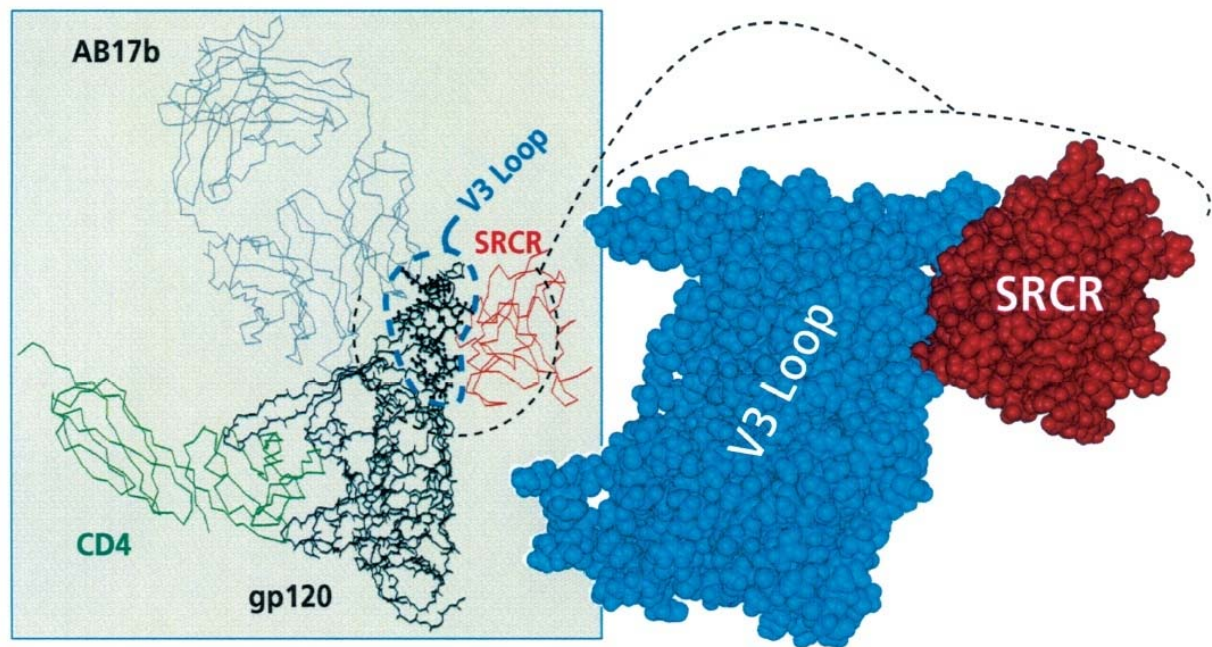
Glycoprotein-340 (gp340) is expressed as a splice variant of the Deleted in Malignant Brain Tumors I (DMBT1), which has been identified as a member of the scavenger receptor cysteine-rich (SRCR) family of proteins. These proteins are known for their role as pattern recognition receptors for interactions with specific pathogen motifs that drive signaling events [21, 48-50]. Differentially spliced variants of DMBT1 can encode both secreted as well as membrane associated proteins [21, 48-50]. Abundant expression has been found in tissues such as the lung, trachea, salivary glands, stomach and small intestine while minor expression has been found in the testis, mammary glands, uterus and brain [50]. The gp340 protein, a cell membrane associated splice variant of DMBT1 has been found to be expressed at high levels in the lung and upon recognition of specific pathogen motifs has been shown to stimulate the migration of alveolar macrophages [50]. The gp340 protein, as the salivary agglutinin (SAG) protein, is the secreted splice variant of DMBT1 found in saliva and was initially identified based on its role in the clearance of bacterial pathogens including *S. mutans* [39, 50, 51]. SAG was the first DMBT1 splice variant to be identified to play a role in HIV infection [50]. Compared to the vaginal route, HIV transmission within the oral cavity is of low frequency despite the presence of similar cell types in both oral and vaginal tissues. Fractionation of human saliva led to the identification of SAG as an inhibitor of HIV infection in the oral cavity [50, 48, 52]. It was shown that SAG interaction with HIV leads to viral agglutination [53]. Immunoprecipitation assays showed that SAG binds to gp120 in an area different from the CD4 binding site, and it was reported that pretreatment of HIV with soluble CD4 resulted in enhanced SAG-gp120 binding [39, 53-55]. Further studies revealed that SAG interacts with HIV within the base of the V3 loop, an area that is well conserved within the viral genome ([48, 49, 52] Fig. 1.4). While the role of SAG in HIV biology has been studied in great detail, the

role of epithelial cell surface gp340 in HIV transmission across genital mucosa has only recently begun to be characterized. Stoddard et al reported that gp340 is expressed on human vaginal and cervical tissues and that the cells expressing the cell surface associated form of gp340 are capable of binding and facilitating HIV transmission [21]. Based on these reports showing that HIV is capable of interacting with epithelial cells surface gp340, I conducted experiments in this investigation to determine the potential consequences of viral transmission through the mucosal epithelial cells in terms of signaling processes for the production of soluble immune mediators that can influence viral survival and/or infection in situ.

**Fig.1.4: Model depicting interaction of gp340 SRCR domain with HIV gp120:** a) Model of the quaternary complex between the HIV surface envelope protein gp120, human cell surface receptor CD4, and the antibody AB17b, which binds to the conserved conformation dependent epitope of gp120 that is exposed after gp120 is bound to CD4 (referred to as CD4 induced epitope), and the SRCR domain on gp340 on epithelial cells. b) Space filling model of interaction between gp120 and SRCR domain on gp340.

Source: Wu et al 2004 [49]

**Fig. 1.4:**



## **Immune Mediators Produced by Epithelial Cells: Thymic Stromal Lymphopoietin (TSLP)**

Epithelial cells in the skin and respiratory tract secrete thymic stromal lymphopoietin (TSLP) in response to allergens and bacteria [56]. TSLP is an IL-7 like cytokine that was originally identified in the supernatants of the mouse thymic stromal cell line, Z210R.1 and was reported to display a remarkable ability to support the long-term growth of a pre-B cell line as well as promote proliferation of thymocytes [57-61]. Isolation of a cDNA clone encoding human TSLP revealed that it is structurally similar to murine TSLP but shares only 43% amino acid homology [59, 62, 63]. The TSLP receptor complex is a heterodimer consisting of a TSLP receptor (TSLPR) binding chain and the interleukin 7 receptor- $\alpha$  (IL-7R $\alpha$ ) chain, which together bind TSLP with a high affinity resulting in Signal Transducers and Activator of Transcription-5 (STAT-5) activation and cell proliferation [58, 59]. Early studies demonstrated that unlike murine TSLP, the human TSLP does not promote the differentiation and growth of B cells nor does it have a direct effect to support the activation of T cells [59].

In humans, TSLP is mainly expressed by skin keratinocytes, epithelial cells, smooth muscle cells, lung fibroblasts, or IgE stimulated mast cells. Microbial infection or allergen exposure triggers mucosal epithelial cells to produce TSLP [56, 58] (Fig.1.5). TSLP up-regulates activation markers such as CD54, CD80, CD83, CD86, DC-SIGN, and HLA-DR on myeloid dendritic cells (mDC) and activates immature DC to produce the neutrophil and eosinophil attractant chemokines Interleukin-8 (IL-8) and eotaxin-2 as well as the T helper 2 (Th2) attractant chemokines thymus and activation regulated chemokine (TARC or CCL17) and macrophage-derived chemokine (MDC or CCL22) [58, 59, 64]. Unlike the DC activated by a variety of factors, such as CD40-L, LPS and poly I:C, TSLP-activated DC do not produce IL-12 the pro-inflammatory cytokines TNF, IL-1 $\beta$ , and IL-6, or induce TH1 responses [59,



64]. Instead, mature TSLP-activated DC migrate to the draining lymph node, express high levels of OX40L, trigger homeostatic proliferation of naïve CD4<sup>+</sup> T cells and induce their differentiation into a unique type of inflammatory Th2 cells that produce Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-13 (IL-13), and Tumor Necrosis Factor (TNF) and not the classic Th2 cells that produce Interleukin-10 (IL-10) [58, 65]. These inflammatory Th2 cells then migrate back to the site of inflammation due to the local production of TARC and MDC by the TSLP-activated DC. These studies suggest that TSLP produced by epithelial cells plays an important role in inducing allergic inflammatory responses [59]. This hypothesis is supported by studies showing that the airway epithelium of asthma patients expresses increased levels of TSLP and also that infection by rhinoviruses or exposure to pro-inflammatory mediators such as IL-1 $\beta$ , TNF- $\alpha$  and some TLR agonists induce TSLP production in human airway epithelial cells causing the exacerbation of asthma [66, 67]. Furthermore, TSLP is highly expressed in patients with acute and chronic atopic dermatitis lesions and this increased TSLP expression is associated with Langerhan cell migration and activation suggesting that TSLP is a critical link between epithelial cells and the DC of the immune system at a molecular level [64, 68]. The fact that TSLP produced by epithelial cells exposed to allergens and microbes induces DC-mediated expansion of CD4<sup>+</sup> T cells formed the basis for the central hypothesis of this dissertation research that: **HIV exposure at the mucosal tissues stimulates epithelial cells to produce TSLP for DC-mediated expansion of susceptible target CD4 T cells.**

In 2007, Lee and Ziegler identified an NF $\kappa$ B site within the human TSLP gene promoter that was critical for IL-1 $\beta$  and TNF- $\alpha$  induced TSLP expression [69]. This provided a link for the mechanism by which TSLP expression is induced by ligands for TLR2, TLR8, and TLR9. Since NF $\kappa$ B has such a broad range of activation signals, several labs explored the

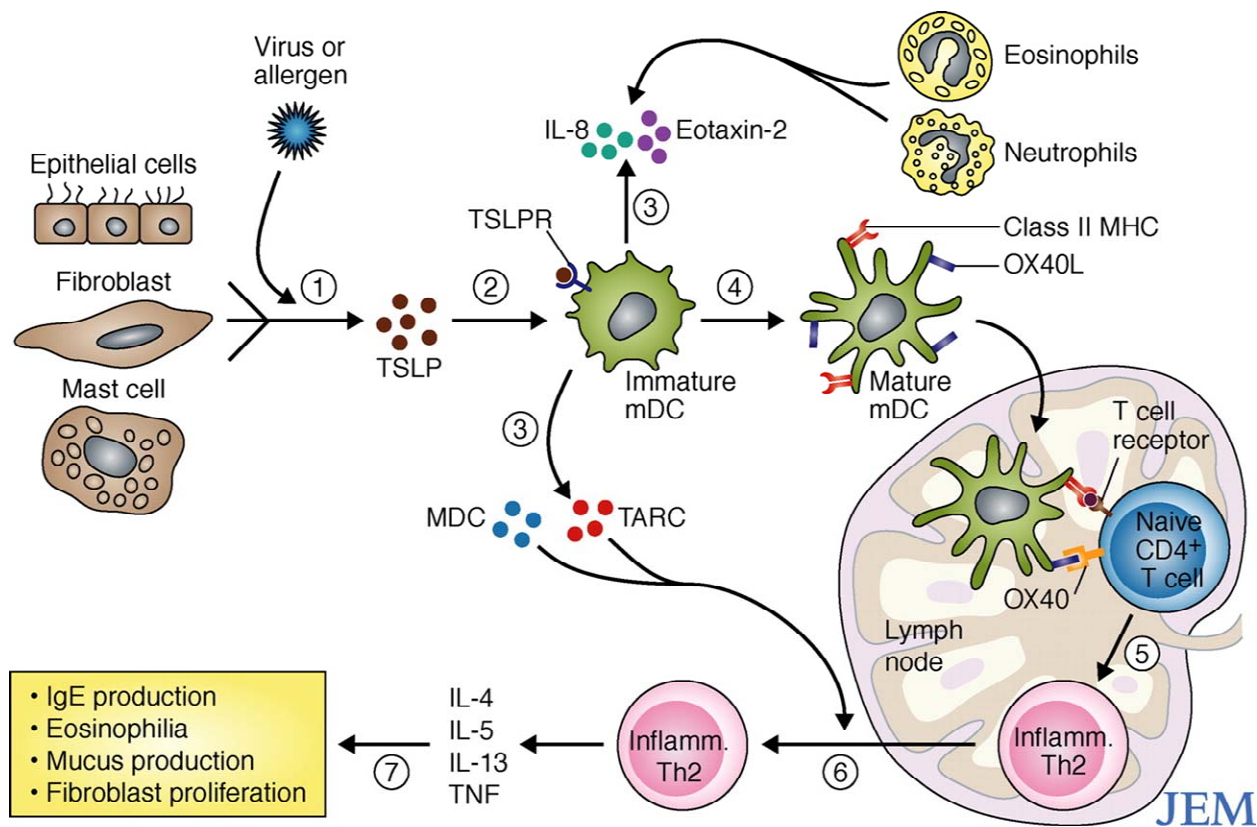
potential involvement of other factors for regulating TSLP expression. Li et al. identified two nuclear receptor binding sites in both the human and mouse TSLP gene promoters, in particular the retinoid X receptors (RXR)  $\alpha$  and  $\beta$  for which 9-cis- retinoic acid (9-cis-RA) serves as a high-affinity ligand [70]. Studies showed that keratinocytes with selective ablation of RXR $\alpha$  and RXR $\beta$  display an inflammatory response similar to that of atopic dermatitis in humans [70]. High levels of TSLP expression were also found in these cells. In 2008, Lee et al. demonstrated that 9-cis-RA represses IL-1 $\beta$  mediated TSLP gene expression through direct inhibition of NF $\kappa$ B binding and signaling [71]. These studies suggested that RXR $\alpha$  and RXR $\beta$ , when bound to ligand, are involved in regulating TSLP expression and therefore, could potentially be exploited for developing novel therapeutic strategies for asthma and atopic dermatitis. Results from the current investigation point to similar application of the RXR ligands for inhibiting HIV-induced TSLP expression by genital mucosal epithelial cells as a strategy in the fight against the transmission of HIV.

**Fig.1.5: Pathology of TSLP-mediated inflammation**

Microbial infection or allergen exposure triggers mucosal epithelial cells to produce TSLP. TSLP activates immature DC to produce the neutrophil and eosinophil attractant chemokines, interleukin-8 (IL-8) and eotaxin-2 as well as the T helper 2 (Th2) attractant chemokines, thymus and activation regulated chemokine (TARC or CCL17) and macrophage-derived chemokine (MDC or CCL22). Mature TSLP-activated DC migrate to the draining lymph node, express high levels of OX40L, and trigger homeostatic proliferation and differentiation of naïve CD4<sup>+</sup> T cells into inflammatory Th2 cells that produce Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-13 (IL-13), and Tumor Necrosis Factor (TNF). These inflammatory Th2 cells then migrate back to the site of inflammation due to the local production of TARC and MDC.

Source: Y.J. Liu, 2006 [58] (© **Y.J. Liu, 2006. *JEM*, 203: 269-273**)

**Fig.1.5:**



## CHAPTER 2

### Statement of Objectives

Genital mucosal infections account for the majority of the reported cases of human immunodeficiency virus (HIV) transmission worldwide. Ironically, mucosal epithelial cells are resistant to HIV infection because they express low to negligible levels of the receptors necessary for productive HIV infection and also because the mucosal tissue microenvironment is laden with numerous soluble antiviral factors and exhibits low pH. For these reasons, the outcome of HIV exposure is dependent on the extent to which the virus can manipulate the mucosal tissue microenvironment, specifically the epithelial cells. In this regard, it is known that airway epithelial cells respond to exposure to allergens and bacteria by producing thymic stromal lymphopoietin (TSLP), an IL7-like cytokine that can mediate allergic and inflammatory T cell responses by acting directly on dendritic cells (DC) resulting in homeostatic proliferation of T cells. Since T cells are the major targets for HIV infection and their accessibility at the site of virus exposure could positively influence the successful invasion by HIV, **I hypothesized that HIV exposure at the mucosal tissues stimulates epithelial cells to produce TSLP for DC-mediated expansion of susceptible target CD4 T cells.** I obtained evidence to show that HIV exposure, without the need for productive infection, induced human cervical epithelial cells to produce TSLP, which potently activated human myeloid DC (mDC) to cause the homeostatic expansion of autologous CD4<sup>+</sup> T cells that served as targets for HIV infection. The following three specific aims describe the comprehensive approach and experimental design I adopted to test the hypothesis and understand the potential role of epithelial cells for ensuing mucosal HIV infection through TSLP production.

## **Specific Aims:**

**1. Determine whether HIV-induces TSLP expression in epithelial cells.** Various human epithelial cell lines and primary keratinocytes were cultured with different laboratory adapted as well as patient derived (primary) strains of HIV-1 and TSLP expression was monitored at the RNA level by quantitative real time RT-PCR and at the protein level by ELISA and western blot analyses. In order to determine the in vivo relevance, tissues or biopsies collected from vaginal, oral, and the rectal mucosa of monkeys prior to and after infection with SIV or SHIV by the respective route were analyzed for changes in TSLP expression by employing immunohistochemistry and mRNA analyses. Collaborative studies using the human cervical explant model further confirmed HIV-induced TSLP expression in the epithelial cells.

**2. Determine the potential impact of HIV-induced TSLP expression by epithelial cells on HIV infection of CD4 T cells.** Since TSLP activated DC can promote expansion of CD4<sup>+</sup> T cells, purified DC from normal human PBMC were cultured with supernatants collected from HIV exposed epithelial cells and analyzed for the expression of DC activation markers and for the expression of the TH2 attractant chemokines CCL17 and CCL22. Subsequently, DC treated with supernatants collected from HIV exposed epithelial cells were co-cultured with purified autologous naïve CD4<sup>+</sup> T-cells and T cell expansion was determined. The phenotype of the resulting expanded cultures of T cells was determined in terms of cytokine mRNA analyses by RT-PCR. The susceptibility of these CD4<sup>+</sup> T cells resulting from co-culturing with the TSLP-activated DC to HIV infection was assessed by RNA analyses for monitoring the expression of the different HIV receptors. The degree of HIV infection of these CD4<sup>+</sup> T cells resulting from co-culture with TSLP-activated DC was measured by determining reverse transcriptase activity in the culture supernatants. Biopsies collected from both vaginal and

rectal tissues of monkeys infected with SIV or SHIV were tested for the correlation of increased TSLP expression with increased viral infection in vivo.

**3. Investigate the mechanism of HIV-mediated induction of TSLP expression in epithelial cells.** Epithelial cells have been shown to not express the typical receptors required for HIV infection. One potential possibility for specific interaction of HIV with epithelial cells is through the reported binding of the HIV envelope protein gp120 to the epithelial cell surface salivary agglutinin (SAG) protein gp340 which has been proposed to be involved in HIV transcytosis/endocytosis. **I conducted experiments to test the hypothesis that HIV binding to the epithelial cell receptor gp340 induces TSLP expression.** Different epithelial cells were screened for the expression of gp340 both by flow cytometry for cell surface expression and by analyzing gp340 RNA expression. A series of experiments were conducted to determine the importance of gp340 for HIV-induced TSLP expression: by incubating gp340<sup>+</sup> epithelial cells with HIV-1 in the presence or absence of a specific gp340 antibody; silencing gp340 expression in gp340<sup>+</sup> epithelial cells; and introducing gp340 into a gp340-null epithelial cell line. HIV binding to gp340 alone was observed to be not sufficient for inducing the signals needed for TSLP expression in experiments involving cross linking of immobilized HIV to gp340<sup>+</sup> C33A cervical epithelial cells. The potential role for gp340-mediated endocytosis of HIV in inducing TSLP expression was tested by exposing epithelial cells to HIV in the presence of a variety of inhibitors of endocytosis and measuring TSLP expression. The downstream TLR7/8-mediated signaling pathway of endocytosed HIV for inducing TSLP expression was also investigated.

## CHAPTER 3

### **Genital epithelial cells produce TSLP in response to immunodeficiency virus and trigger dendritic cell-mediated expansion and infection of CD4<sup>+</sup> T cells**

**Rationale:** Genital mucosal epithelial cells express low to negligible levels of the receptors for HIV and the genital mucosal microenvironment exhibits low pH and contains numerous antiviral compounds. Thus, a major portion of the viral inoculum deposited at these locations has little chance to survive and cause infection. Yet, genital transmission, specifically intravaginal exposure, accounts for majority of HIV infections acquired by women worldwide [13, 72-75]. Therefore, it is important to understand the changes within the genital mucosal microenvironment subsequent to viral exposure that enable the limited infectious virus and/or the small founder population of infected cells within the genital mucosa to prevail and successfully invade local as well as systemic tissues. Thymic stromal lymphopoietin (TSLP) is an epithelial cell derived interleukin 7 (IL-7) like cytokine expressed in response to microbial infection or allergen exposure [58, 76]. It has been shown that dendritic cells (DC) as well as the Langerhan's cell (LC), which reside within the genital mucosal tissues, are activated by TSLP and exhibit unique phenotypic and functional characteristics, specifically the capacity to induce homeostatic expansion of naïve CD4<sup>+</sup> T cells [58, 76, 77].

**Hypothesis:** In this investigation, I tested the hypothesis that HIV entry at the mucosal tissues stimulates epithelial cells to produce TSLP, which may activate DC leading to recruitment and expansion of CD4<sup>+</sup> T-cells, the ideal targets for enhanced HIV infection.

**Experimental Design:** To test the hypothesis, the following series of experiments were conducted:



- A number of human epithelial cell lines and primary human keratinocytes were exposed in vitro to a variety of HIV-1 strains to determine TSLP expression and production.
- In rhesus macaques, the well-established nonhuman primate model for HIV infection, the in vivo TSLP expression was determined in the vaginal, rectal and oral mucosa after exposure to simian immunodeficiency virus (SIV) or the simian human immunodeficiency virus (SHIV), by the respective route.
- TSLP expression in vaginal tissue explants was investigated after ex vivo exposure to HIV.
- Analyzed the effects of TSLP produced by HIV-exposed epithelial cells for activating DC that in turn were tested for promoting the expansion of naïve autologous CD4<sup>+</sup> T cells and subsequent HIV infection.
- Determined the phenotype and functional properties of CD4<sup>+</sup> T cells resulting from co-culturing with DC activated by TSLP from HIV-exposed epithelial cells.
- Investigated the mechanism of HIV-mediated induction of TSLP expression in epithelial cells and determined the potential role of the epithelial cells surface molecules such as the gp340 scavenger receptor for interaction with HIV as well as the signaling molecules for TSLP expression within the epithelial cells.

## **Specific Aim 1: Determine whether HIV-induces TSLP expression in epithelial cells**

### **Results**

#### **HIV induces production of TSLP in human epithelial cell lines and primary human keratinocytes in vitro**

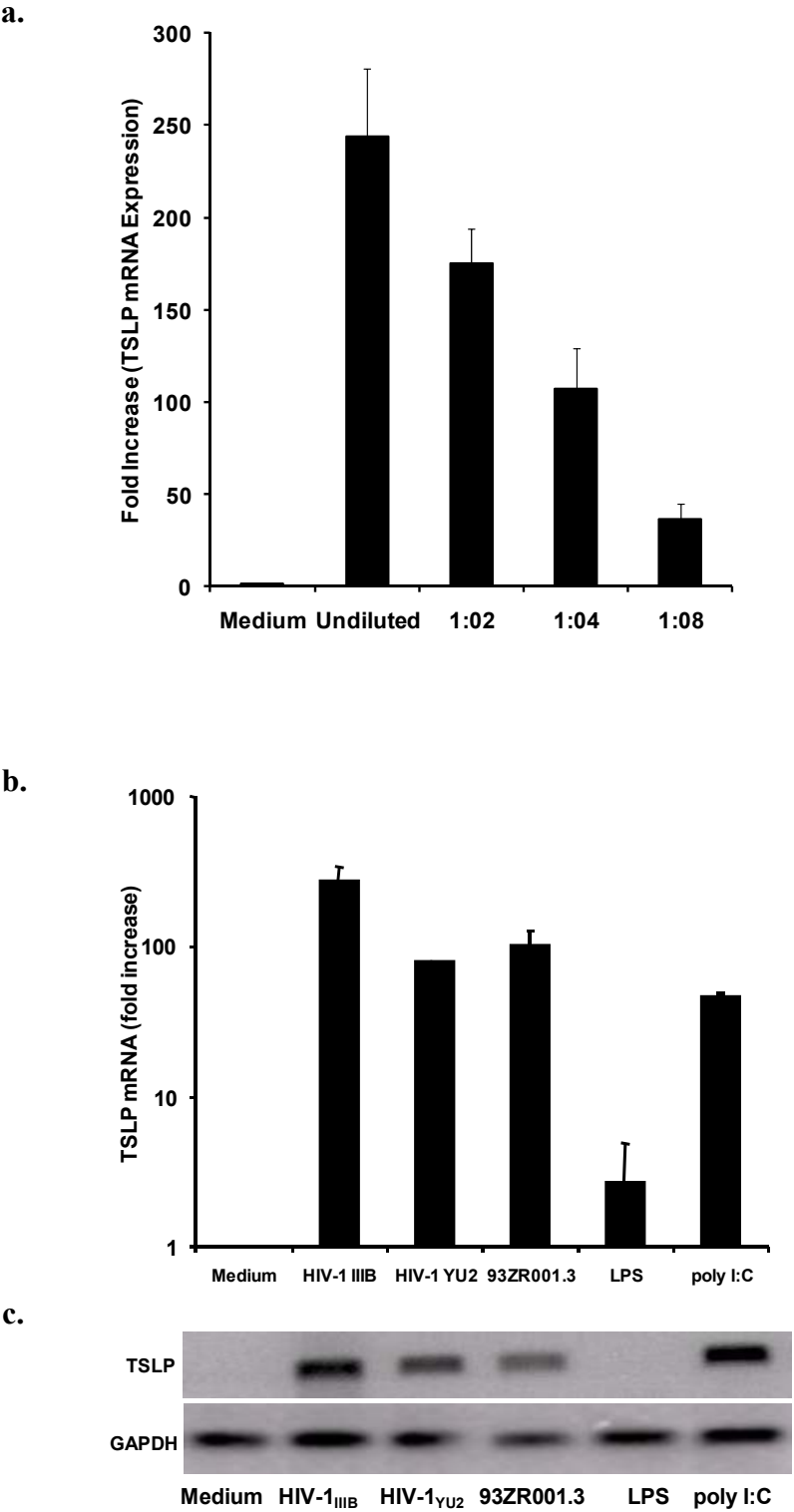
To investigate whether HIV induces TSLP production in epithelial cells, human cervical epithelial cells (C33A) were cultured with HIV-1 for 12-18 h at 37°C and the cell extracts as well as the culture supernatants were analyzed for TSLP expression. When compared with the medium control treatment, HIV-1<sub>IIIB</sub>, in a concentration dependent manner induced an increase in the levels of TSLP expression (Fig. 3.1a). Capacity to enhance expression of TSLP was not restricted to the laboratory-adopted and T-cell tropic virus HIV-1<sub>IIIB</sub>, since exposure of the epithelial cells to the macrophage tropic virus HIV-1<sub>YU2</sub> as well as a primary HIV-1 isolate 93ZR001.3 resulted in increased TSLP RNA expression (Fig. 3.1b). Analysis of cDNA by RT-PCR confirmed enhanced TSLP expression in these cells (Fig. 3.1c). Furthermore, culture supernatants as well as cell lysates from the epithelial cells exposed to HIV-1<sub>IIIB</sub> showed increased levels of TSLP protein as determined by ELISA and western blot analyses (Fig. 3.1d-f). Secreted TSLP protein could only be detected in the cell supernatants of  $3 \times 10^6$  cells or above (Fig. 3.1d). As described in the literature, TSLP expression at the RNA and protein levels was also observed with poly I:C but not lipopolysaccharide (LPS), which served as positive and negative controls, respectively in these experiments [66]. HIV-induced TSLP expression was also observed in several different human epithelial cell lines including human neuroblastoma cells (SKNM), intestinal epithelial cells (HT29), and primary human keratinocytes exposed to HIV-1<sub>IIIB</sub>, as well as in macaque epithelial cells (4MBr-5) incubated with SHIV<sub>89,6P</sub> (Fig. 3.1F). However, TSLP expression was not observed in other cell types

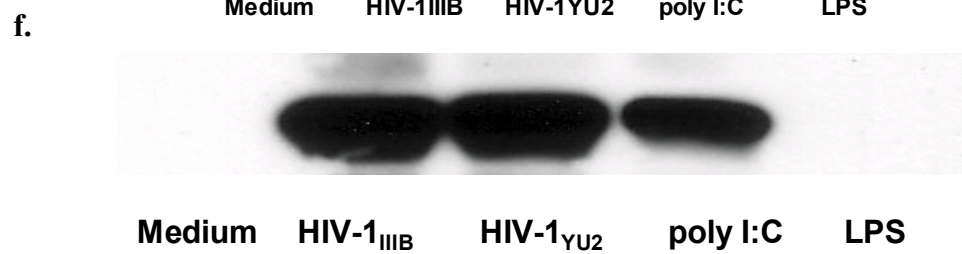
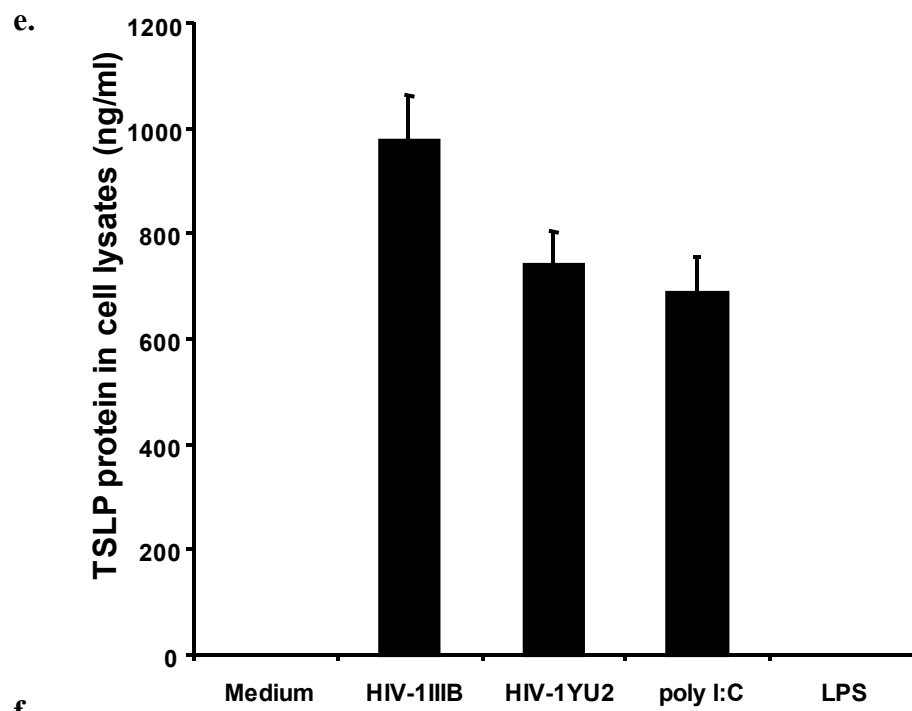
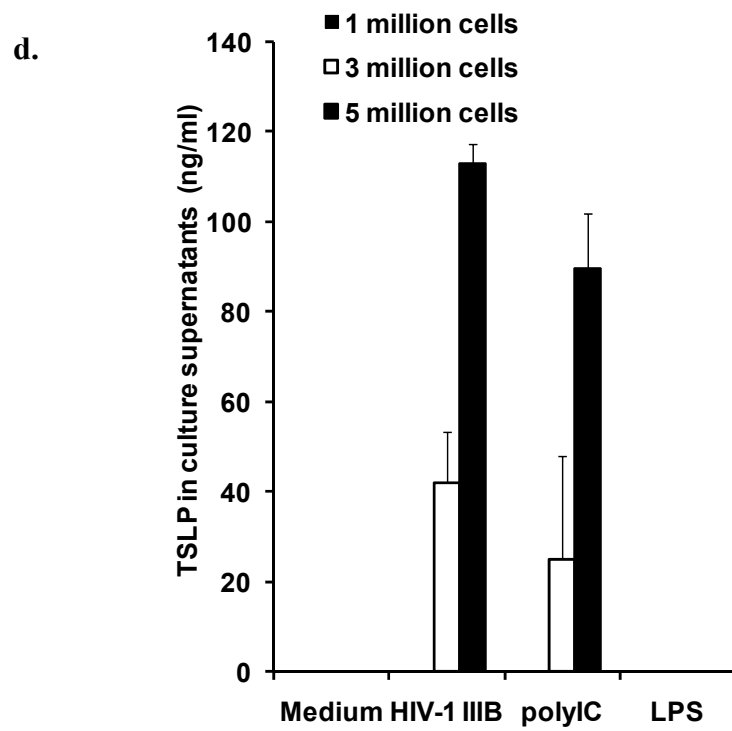
such as the fibroblast-like human osteosarcoma (GHOST X4) or lymphocytes such as the human T-lymphoblastoid cells (H9 cells) exposed to HIV-1<sub>IIIB</sub> (Fig. 3.2).

It is known that epithelial cells corresponding to various anatomical locations in general do not permit productive infection by HIV, even though infection and/or transcytosis of HIV through certain epithelial cells is documented [34-36]. To test whether productive HIV infection is required for the observed TSLP expression in epithelial cells, C33A cells were cultured with HIV-1 that is pseudotyped with Vesicular stomatitis virus (VSV) envelope (VSV-HIV) or inactivated by treatment with aldrithiol-2 (AT-2), a reagent shown to covalently modify the essential zinc fingers in the nucleocapsid (NC) protein of HIV-1 thereby arresting the viral life cycle before initiation of reverse transcription [78, 79]. As shown in Fig. 3.3, both infectious as well as AT-2 inactivated X4 and R5 strains of HIV-1 (HIV-1<sub>MN</sub> and HIV-1<sub>ADA</sub>, respectively), but not VSV-HIV, induced significantly increased levels of TSLP mRNA in the epithelial cells. These results demonstrate that diverse HIV-1 strains, both infectious and noninfectious, can efficiently induce TSLP expression in a variety of epithelial cells including primary human keratinocytes.

**Fig. 3.1: HIV induces TSLP expression in cervical epithelial cells.** Increased levels of TSLP expression were dependent on the amount of HIV used to culture the C33A cervical epithelial cells (a). Increased levels of TSLP mRNA were detected in cervical epithelial cells (C33A cells exposed to HIV-1<sub>IIIB</sub>, HIV-1<sub>YU2</sub>, the primary clade D HIV isolate 93ZR001.3, or polyI:C (5 ug/ml) when compared to medium alone or in cells cultured with LPS (10 ug/ml), as assessed by quantitative real time PCR (b) and RT PCR (c). Increased levels of TSLP protein were detected in the supernatants as well as the lysates of the C33A cells exposed to HIV-1<sub>IIIB</sub>, HIV-1<sub>YU2</sub> or polyI:C by ELISA (d and e) and also by western blot analysis (f). Data represents average values along with standard deviation from three independent experiments. Fold increase in TSLP mRNA expression was shown for various HIV strains and poly I:C as compared to medium control treatment.

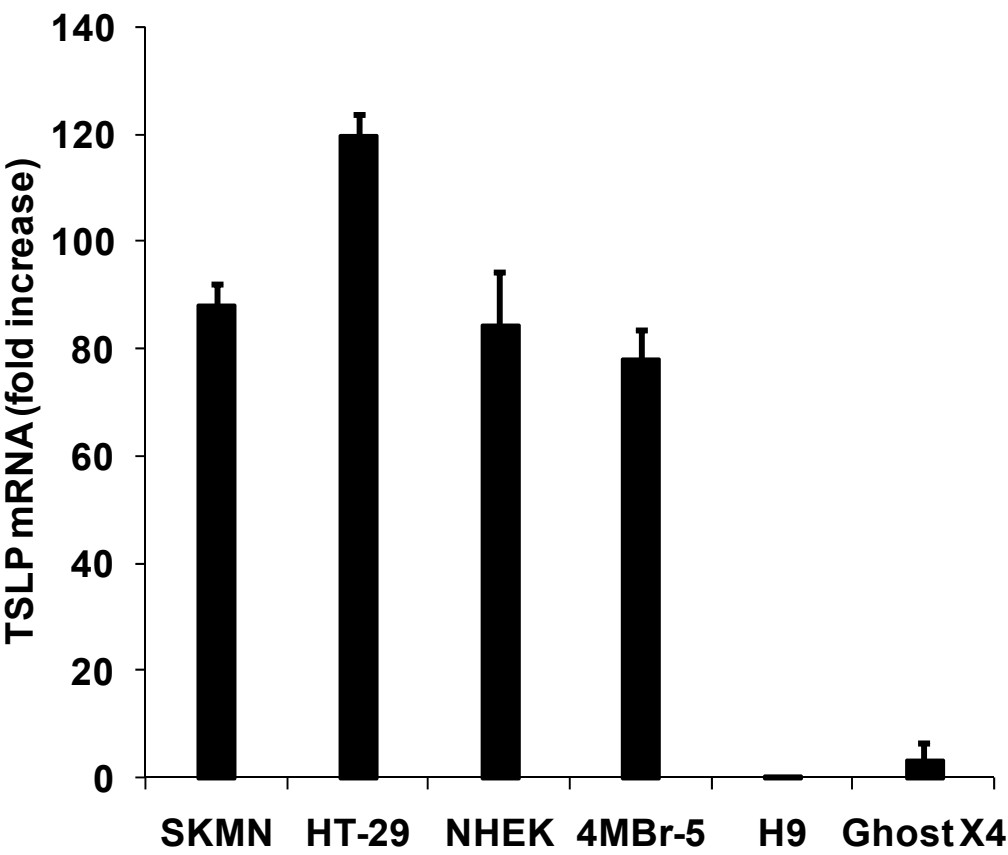
**Figure 3.1:**





**Fig.3.2: HIV induces TSLP expression in a variety of epithelial cells but not non-epithelial cells.** As determined by quantitative real time PCR, increased levels of TSLP mRNA were detected in neuronal (SKMN) and intestinal (HT29) epithelial cells, primary human keratinocytes (Normal Human Epidermal Keratinocytes, NHEK), and monkey epithelial cells exposed to immunodeficiency viruses but not in non-epithelial cells (human osteosarcoma or T lymphoblastoid cells). Data represents average values along with standard deviation from three independent experiments. Fold increase in TSLP mRNA expression was shown for various HIV strains and poly I:C as compared to medium control treatment.

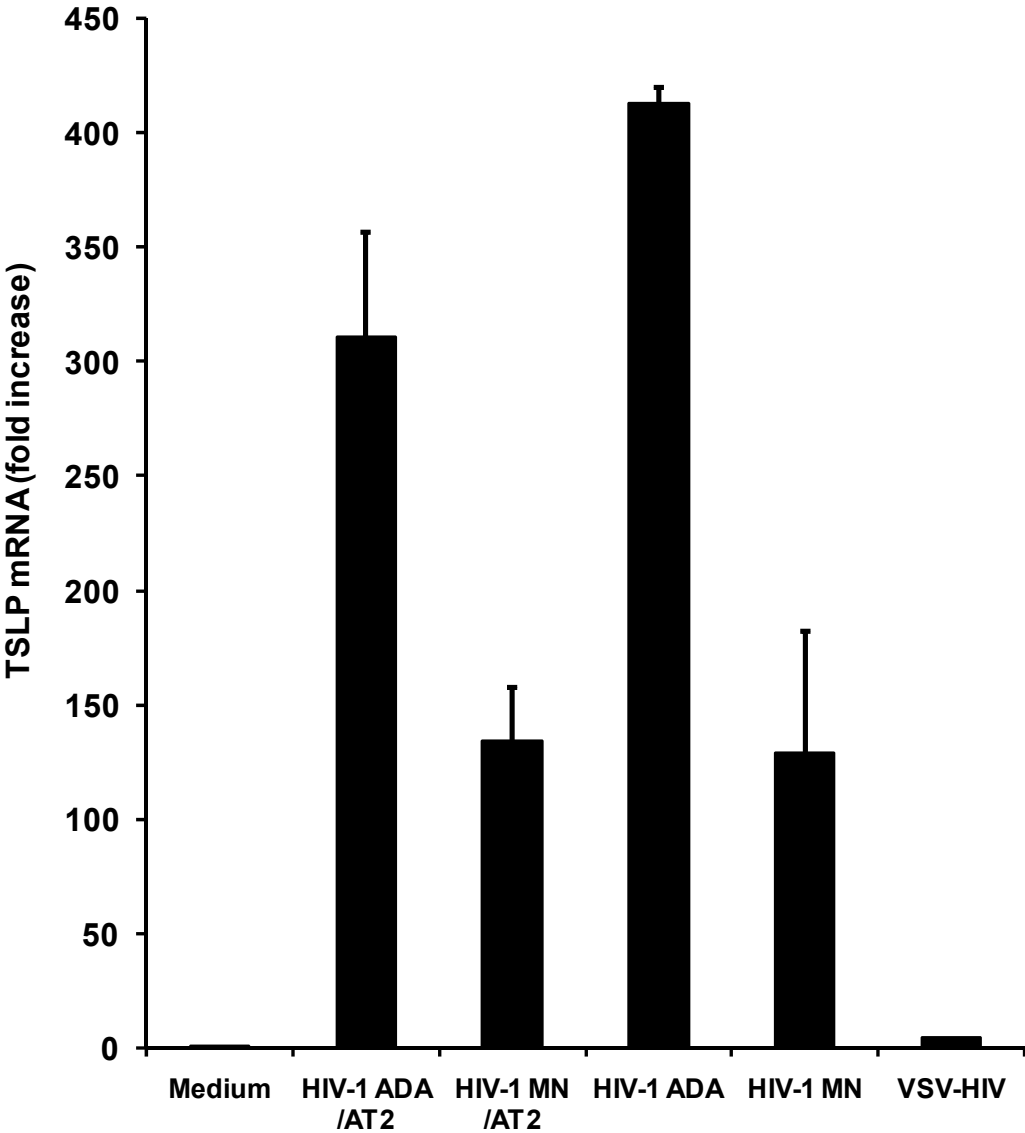
Figure 3.2:





**Fig.3.3: Productive HIV infection is not necessary for TSLP induction.** Increased levels of TSLP mRNA were detected in C33A cervical epithelial cells cultured with infectious as well as AT-2 treated non-infectious HIV-1<sub>ADA</sub> and HIV-1<sub>MN</sub> strains but not in medium alone or cells cultured with VSV pseudotyped HIV. Data represents average values along with standard deviation from three independent experiments. Fold increase in TSLP mRNA expression was shown for various HIV strains and poly I:C as compared to medium control treatment.

Figure 3.3:



## **TSLP is expressed in vivo within the mucosal tissues of rhesus macaques infected with simian immunodeficiency virus**

To investigate whether the HIV-induced TSLP expression observed in the in vitro studies with human epithelial cells also occurs after in vivo infection within the mucosal sites of viral entry in the nonhuman primate model, vaginal tissues from rhesus macaques intravaginally inoculated with the simian immunodeficiency virus, SIVmac251, were analyzed. For this, we used rhesus monkey vaginal tissue samples available from an earlier study by our collaborators from the California Primate Center, Davis, CA, where the animals were infected by the vaginal route with SIVmac251 and vaginal samples were obtained after necropsy at different time-points post-challenge [14]. Relative to before SIV infection (Fig. 3.4a), immunohistochemistry analyses demonstrated high levels of TSLP in the vaginal mucosa starting as early as day 1 post-inoculation (post-SIV) and was significantly higher by day 14 (Fig. 3.4b and c). The levels of TSLP expression correlated with a 41 and 99-fold increase in TSLP expression at days 1 and 14 respectively, as determined by quantitative real time PCR analyses of the mRNA isolated from the tissue samples (Table 1). This increase in TSLP expression coincided with an increase in viral RNA positive cells between days 1 and 14 in these tissues as determined by in situ hybridization (ISH) analyses at our collaborators' laboratory in Davis, CA (Fig. 3.4d-f). Thus, there was a rapid and sustained increase in TSLP expression in the vaginal mucosa of rhesus macaques after SIV inoculation. Similarly, mRNA analyses of tonsil and rectal biopsies collected from rhesus macaques (at the UTMD Anderson Cancer Center primate facility in Bastrop, TX) after atraumatic application of the chimeric simian human immunodeficiency virus (SHIV) by the oral and rectal routes, respectively, showed increased TSLP levels between days 4 and 7 post-infection (Figs. 3.5 and 3.6). Concurrent with the increased TSLP expression in the tonsil tissues, we observed increased

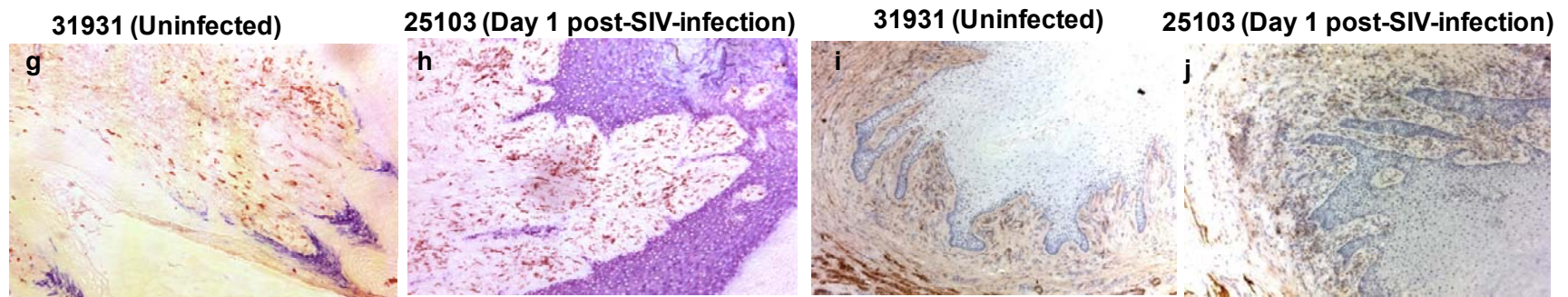
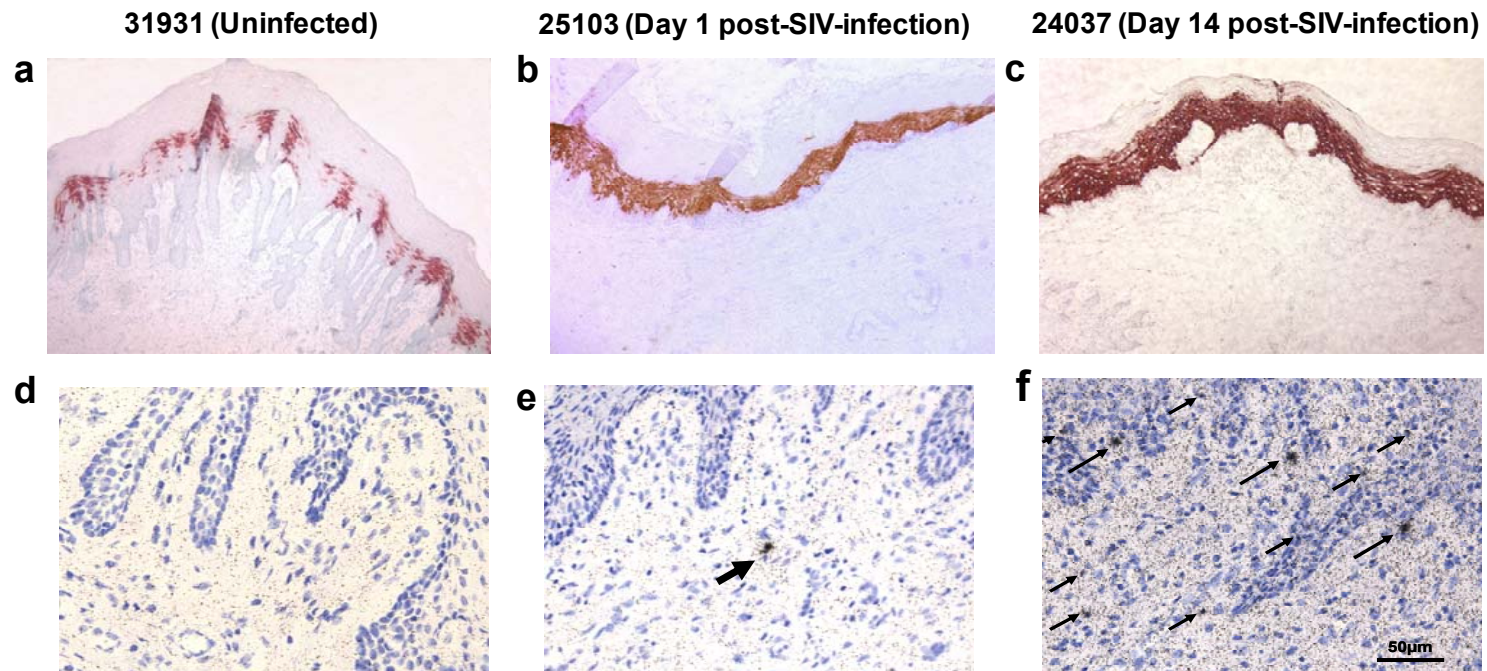
levels of thymus and activation regulated chemokine (TARC also known as CCL17), a TH2 cell attracting chemokine known to be expressed in TSLP-activated DC (Fig. 3.5b) [76, 80]. Coinciding with the increased TSLP expression in the rectal tissues, there was also an increase in plasma viral RNA levels (Fig. 3.6). Furthermore, vaginal tissues with the high levels of TSLP expression on day 1 post-infection exhibited significantly increased numbers of CD11C<sup>+</sup> mDC and CD4<sup>+</sup> T cells when compared to that from the vaginal tissues of an uninfected animal (Fig. 3.4h and g; and Fig. 3.4j and i, respectively). These results support our in vitro data and show that during the early stages of infection the mucosal microenvironment favors virus expansion by recruiting large numbers of CD4<sup>+</sup> T cell targets through DC that are activated in response to TSLP from HIV-exposed epithelial cells.

**Fig. 3.4: Increased expression of TSLP along with higher numbers of CD11c<sup>+</sup> mDC and CD4<sup>+</sup> T cells in vaginal mucosal tissues from rhesus macaques after vaginal SIV infection.**

Samples collected from the vaginal tissues of rhesus macaques prior to (a, uninfected) and days 1 (b) and 14 (c) post-infection with SIV<sub>mac251</sub> were used for immunohistochemical staining for TSLP showed low levels of TSLP in the normal vaginal tissues, but higher levels after 1 and 14 days post-SIV exposure (red staining indicates TSLP protein expression). The sequential samples were also subjected to in situ hybridization (ISH) analyses using radiolabeled riboprobes. A low number of SIV RNA<sup>+</sup> cells were observed at day 1 which increased substantially at day 14 post-infection (shown with black arrows in panels e and f), relative to tissue from an uninfected monkey (d). Double staining for TSLP (purple) and CD11c<sup>+</sup> dendritic cells (brown) showed higher numbers of mDC in the vicinity of TSLP staining in the vaginal epithelium at one day after vaginal SIV infection (h) compared to that in uninfected sample (g). Increased numbers of CD4<sup>+</sup> cells (brown) were also observed in the vaginal tissue at one day after SIV infection (i) compared to that from an uninfected animal (h).

Figure

3.4:



**Table 1: SIV and TSLP RNA levels in the vaginal tissues of rhesus macaques prior to and at sequential time points after vaginal infection with SIVmac251**

Days post infection	vRNA* (copies/ug tissue RNA)	Fold increase in TSLP mRNA expression	Quantification of IH staining <sup>e</sup>		
			TSLP	CD11c	CD4
0	-		+	+	+
1	3.6x10 <sup>4a</sup>	41	++	++	++
9	6.1x10 <sup>5b</sup>	425	++	++	++
10	5.1x10 <sup>5c</sup>	719	++		++
14	5.6x10 <sup>5d</sup>	99	++	+	++

\* The vRNA data was previously published (19)

<sup>a</sup> vRNA level is representative of one monkey from a group of 6 necropsied 1 day post infection. vRNA levels range from 4x10<sup>2</sup> to 3.6x10<sup>4</sup>

<sup>b</sup> vRNA level is representative of one of two samples taken from animal 28103 at 9 days p.i. vRNA levels were determined to be 6.0x10<sup>5</sup> by repeat analysis.

<sup>c</sup> vRNA level is representative of one of two samples taken from animal 24818 at 10 days p.i. vRNA levels were determined to be 5.0x10<sup>5</sup> by repeat analysis.

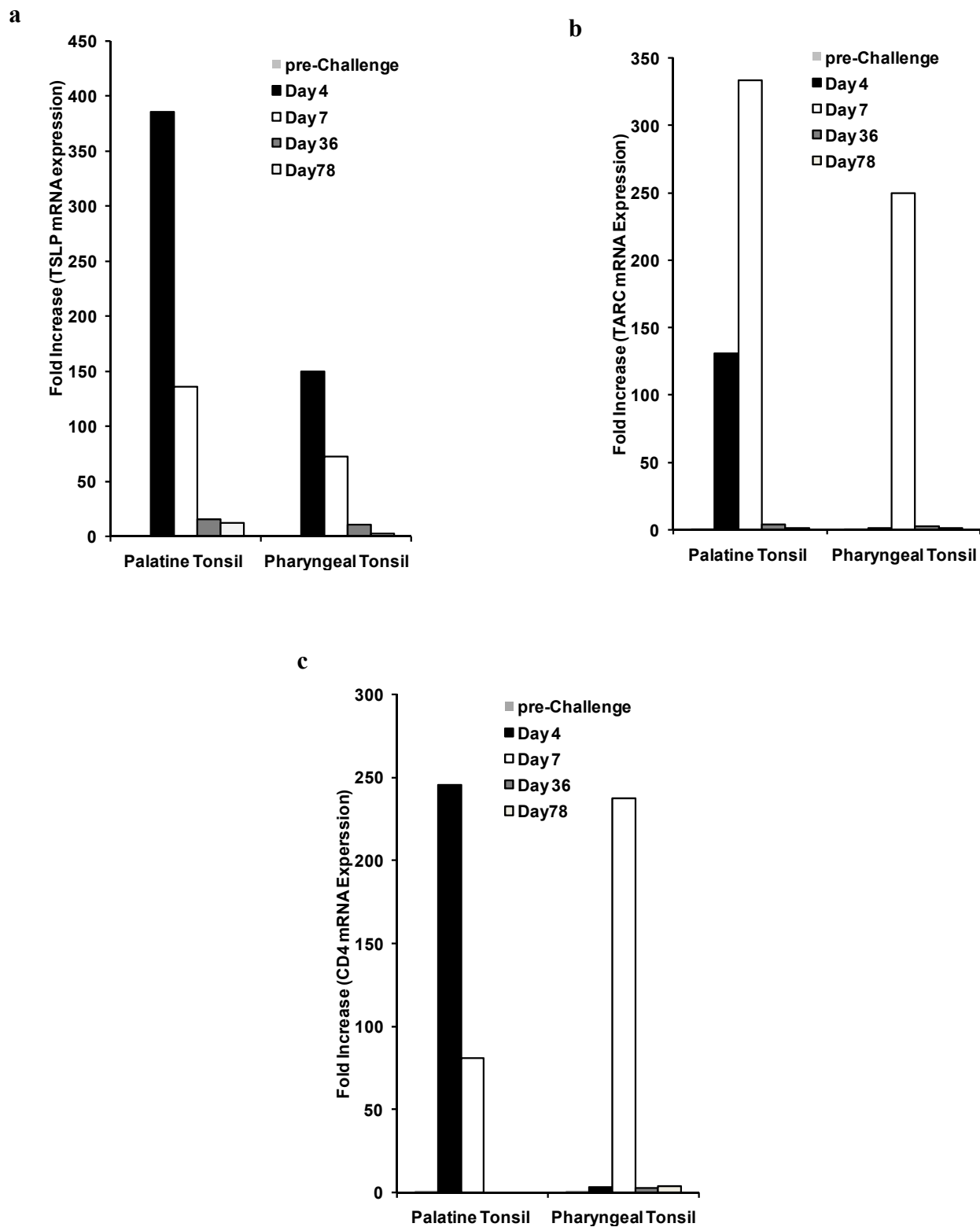
<sup>d</sup> vRNA level is representative of one of two samples taken from animal 24037 at 14 days p.i. vRNA levels were determined to be 5.6x10<sup>5</sup> by repeat analysis.

<sup>e</sup> Quantification of IH staining is based on visual observation

**Fig. 3.5: Expression of TSLP in the oral mucosal tissues collected from SHIV infected rhesus macaques.** (a) Increased levels of TSLP mRNA in SHIV infected tissues showing the highest levels on days 4 and 7 in the palatine tonsils and pharyngeal tonsils. (b, c) Increased levels of TARC and CD4 mRNA were also detected in the SHIV infected tissues collected with the highest levels detected on days 4 and 7 in the palatine and pharyngeal tonsil tissues

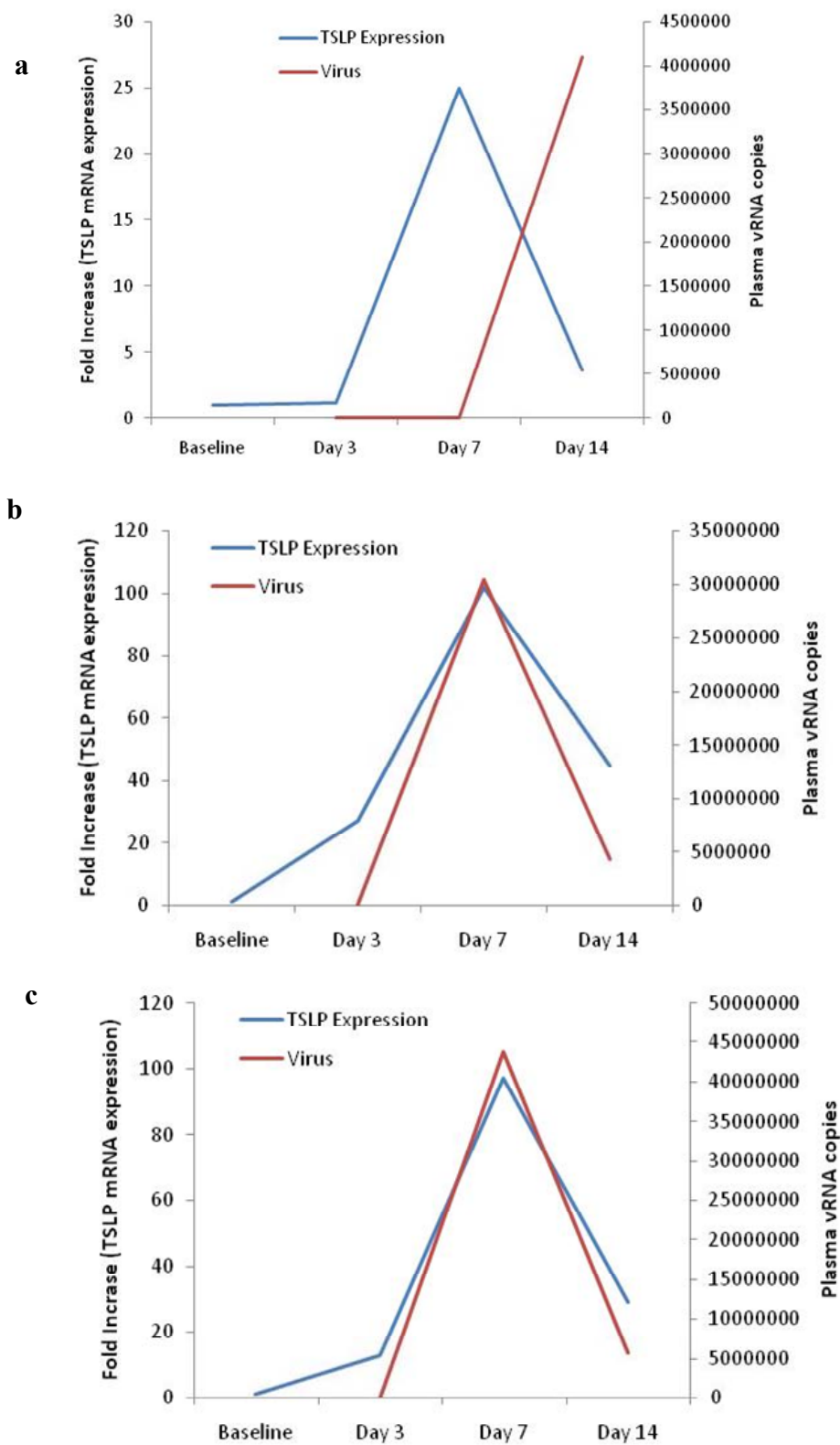


Figure 3.5:



**Fig. 3.6: Expression of TSLP in the rectal mucosal tissues collected from SHIV infected rhesus macaques.** Increased levels of TSLP mRNA were detected after rectal inoculation with SHIV<sub>162P3</sub> within the rectal tissue biopsies collected at different time points post-infection from animals Rh 57 (a), Rh 65 (b), and Rh H196 (c). Increased TSLP levels coincided with increased plasma viremia levels shown on the Y-2 axis in each panel.

Figure 3.6:



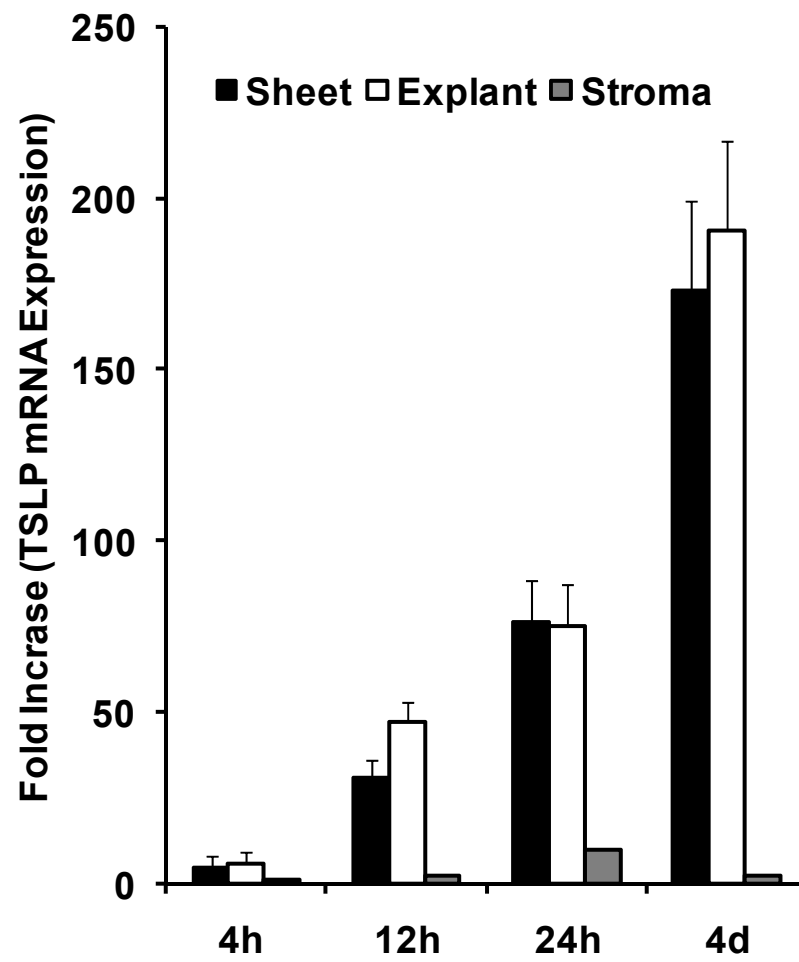
## **TSLP is expressed in the vaginal mucosal tissue explants infected with human immunodeficiency virus ex vivo**

Because in vitro models are based on cells lines which do not provide the natural cellular structures of female genital mucosal tissues that may be important in viral transmission, we examined the HIV-induced TSLP expression in a cervical explant model in collaboration with our colleagues from the Fred Hutchinson Cancer Center, Seattle, WA [29]. This model uses vaginal tissues rather than cell lines which provides the natural structure, including epithelial cells, dendritic cells (Langerhan's cells, LC) and T cells to allow observation of how HIV targets cells of the vaginal mucosal tissue [29]. The squamous vaginal epithelium, the initial barrier to infection, is separated from the underlying stroma and the tissues are exposed to HIV. Within four hours of HIV exposure, TSLP expression could be detected by mRNA analysis in the epithelial sheet and the explant tissues which continued to significantly increase up to 4 days post exposure (Fig. 3.7). This was further confirmed by confocal microscopy. Relative to before HIV infection (Fig. 3.8a), confocal microscopy demonstrated high levels of TSLP in the vaginal mucosa (green color staining) starting as early as day 1 post-infection that significantly increased by day 5 (Fig. 3.8b and c). TSLP expression, assessed by real-time RT-PCR analyses, was only detected in the epithelial sheet and explant tissues and not the separated underlying stroma further confirming the mucosal epithelial cells as the source of TSLP production (Fig. 3.7).

**Fig. 3.7: Expression of TSLP by epithelial cells in cervical explant tissues.**

Samples collected from human cervical explant tissues exposed to HIV at different time points used for mRNA analysis of TSLP expression showed an increase in TSLP production over time in the epithelial sheet and whole cervical explant tissue, but not the separated underlying stroma tissue. The fold increase shown was calculated using the control tissue from each time point as the background value for comparing TSLP expression in the infected tissues.

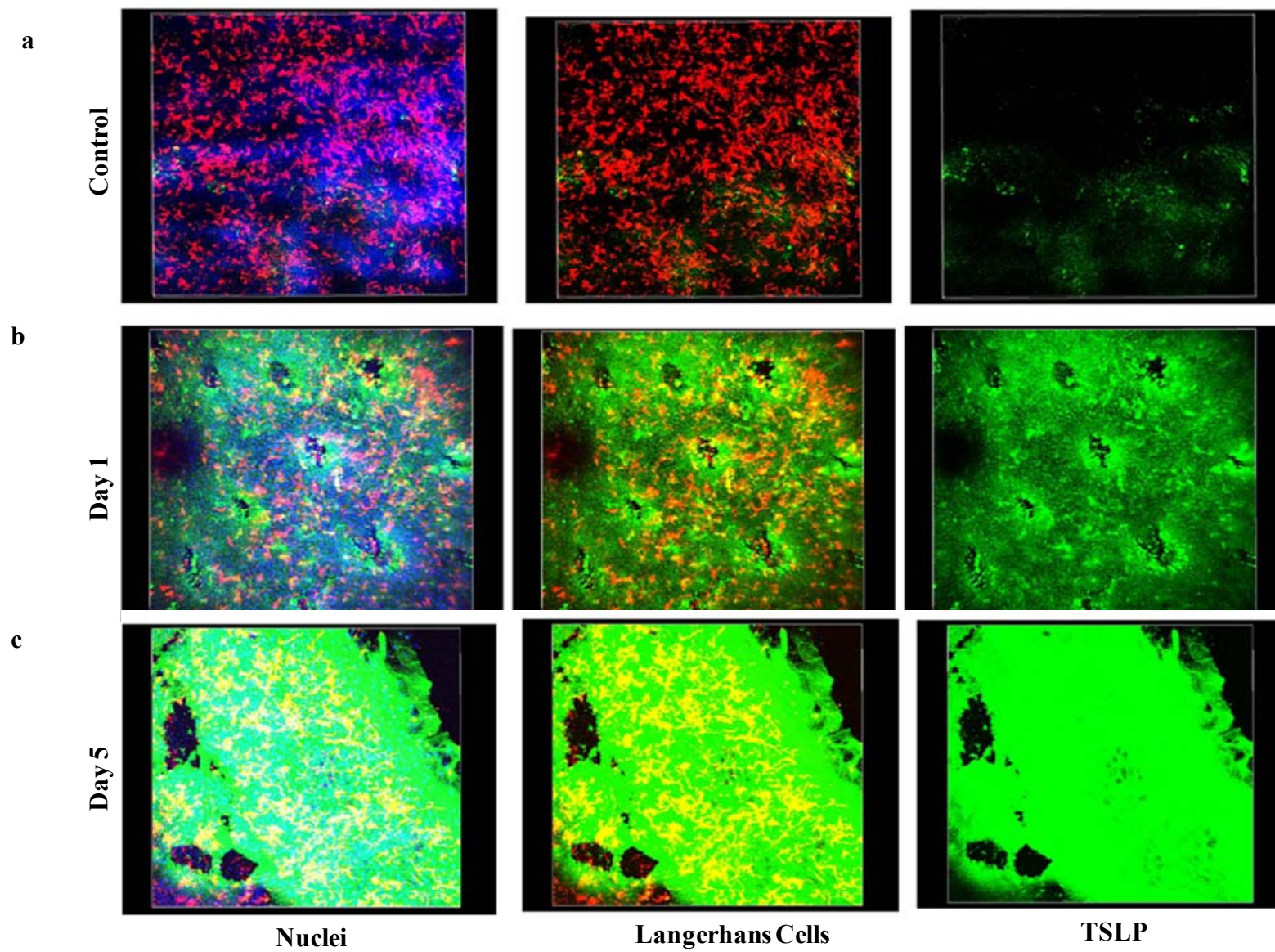
Figure 3.7:



**Fig. 3.8: Increased expression of TSLP in human cervical explant tissues after HIV infection.** Samples collected from human cervical explant tissues exposed to 100ng/ml HIV at different time points were used for confocal analysis of TSLP expression. Tissues showed an increase in TSLP production over time. The TSLP levels are higher on day 5 compared to day 1. Green color represents TSLP staining, red represents Langerhans cells, and blue represents nuclei.

Figure

3.8:





**Specific Aim 2: Determine the potential impact of HIV-induced TSLP expression by epithelial cells on HIV infection of CD4 T cells**

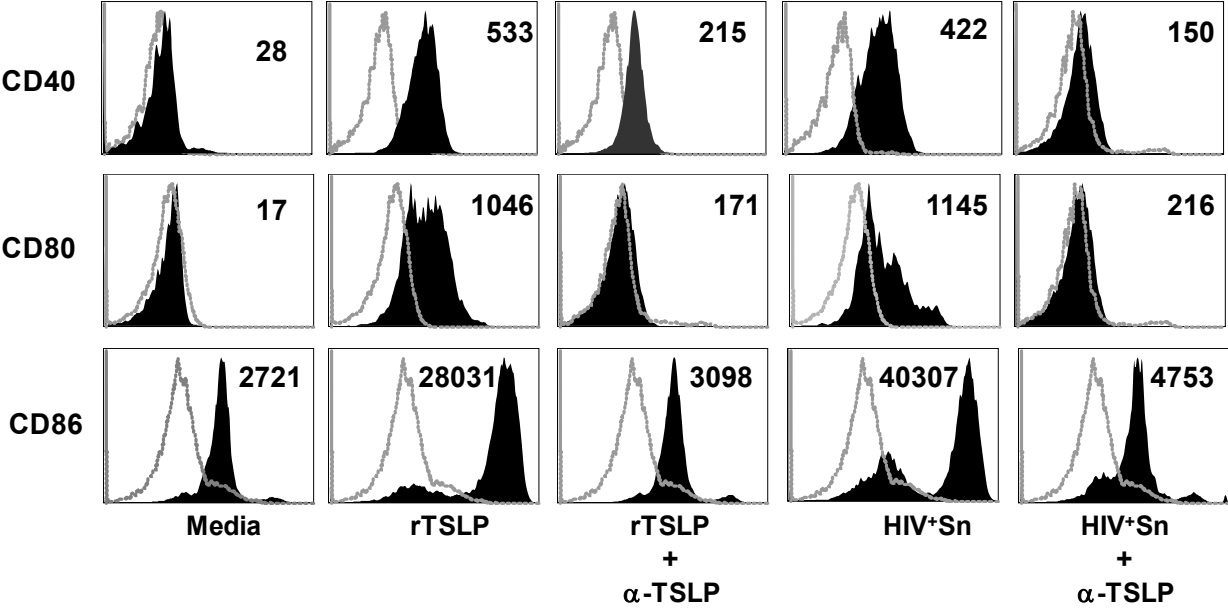
**Results**

**TSLP from HIV exposed epithelial cells activates human CD11c<sup>+</sup> myeloid dendritic cells**

Human myeloid DC (mDC) exposed to TSLP exhibit up-regulation of the activation markers CD40, CD80 and CD86, along with induction of the production of the chemokines CCL17 and CCL22 [76, 80]. To determine the biological activity of TSLP produced in the supernatants of HIV exposed epithelial cells, highly purified CD11c<sup>+</sup> mDC isolated from human peripheral blood mononuclear cells (PBMC) by fluorescence activated cell sorting were incubated with supernatants of epithelial cells cultured with HIV (HIV<sup>+</sup>Sn), recombinant TSLP (rTSLP) at 100ng/ml, or culture medium (medium) for 24h and the expression of activation markers on DC and production of chemokines were assessed (Fig. 3.9). In a manner similar to rTSLP, HIV<sup>+</sup>Sn dramatically up-regulated CD80, CD86, and CD40 expression on mDC after 24 hours of culture (Fig. 3.9). The ability of HIV<sup>+</sup>Sn and rTSLP to up-regulate CD40, CD80 and CD86 expression could be abrogated by neutralizing TSLP antibodies (Fig. 3.9). Both rTSLP and HIV<sup>+</sup>Sn stimulated mDC to produce high levels of thymus and activation regulated chemokine (TARC or CCL17) and macrophage-derived chemokine (MDC or CCL22) at both the mRNA and protein levels (Fig. 3.10). The activity of TSLP within the HIV<sup>+</sup>Sn, along with that of rTSLP for inducing chemokine production could also be blocked by neutralizing TSLP antibodies (Fig. 3.10).

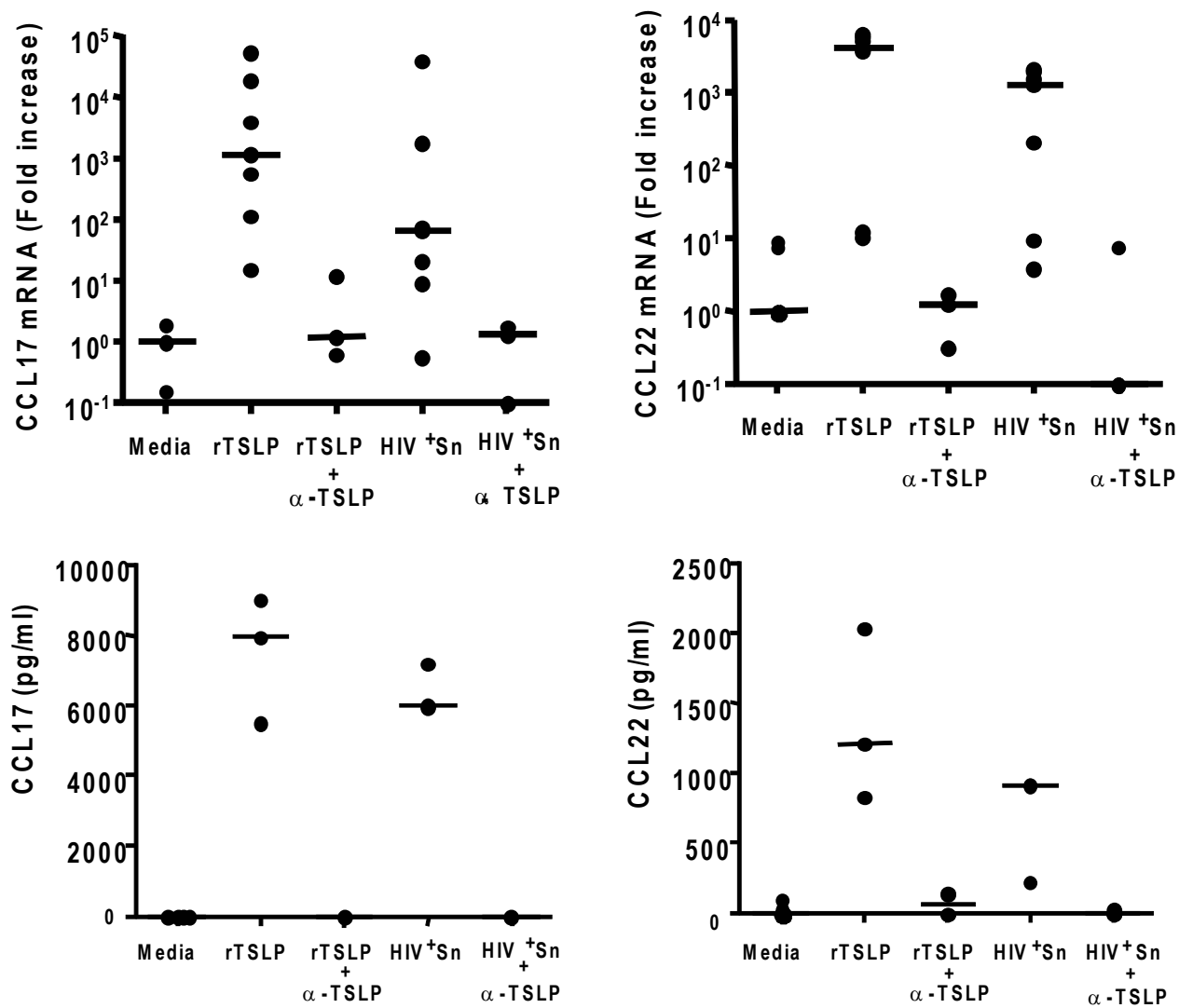
**Fig. 3.9: TSLP within the supernatant from HIV exposed epithelial cells potently activates human CD11c<sup>+</sup>DCs.** Supernatant from HIV exposed C33A cells (HIV<sup>+</sup>Sn) and recombinant TSLP (rTSLP) potently activate mDC in terms of up-regulation of CD40, CD80 and CD86 expression on CD11c<sup>+</sup>DC as compared to cells cultured with medium. The ability of HIV<sup>+</sup>Sn and rTSLP to up-regulate CD80 and CD86 expression could be abrogated by neutralizing TSLP antibodies. The open histograms represent the isotype control and the filled histograms represent staining of DC activation markers. Numbers in each panel indicate the mean fluorescence intensity (MFI) values.

Figure 3.9:



**Fig. 3.10: TSLP within the supernatant from HIV exposed epithelial cells induces expression of chemokines by DC.** DCs stimulated with supernatant collected from HIV exposed C33A cells (HIV<sup>+</sup>Sn) and recombinant TSLP (rTSLP) produced high levels of the chemokines CCL17 and CCL20 as determined by mRNA (upper panels) and protein analysis (lower panels). Production of the chemokines was blocked in mDC that were pretreated with a TSLP-specific antibody ( $\alpha$ -TSLP). Data represents values from one of three independent experiments.

Figure 3.10:



## **Myeloid DC activated by TSLP from HIV exposed epithelial cells induce CD4<sup>+</sup> T cell expansion and HIV infection in vitro**

To further delineate the biological activity of the HIV-induced TSLP, mDC activated by the culture supernatants of HIV-exposed epithelial cells (HIV<sup>+</sup>Sn/DC) were co-cultured at a 1:1 ratio with autologous, naïve CD4<sup>+</sup> T cells for 7 days. Since HIV preferentially infects the memory subset of CD4<sup>+</sup> T cells and because these cells are lost earlier in HIV infection than their naïve counterparts, we also separately co-cultured memory CD4<sup>+</sup> T cells with HIV<sup>+</sup>Sn/DC [81]. Relative to mDC cultured either with medium (medium/DC), supernatant from HIV untreated epithelial cells, HIV<sup>-</sup>Sn, (HIV<sup>-</sup>Sn/DC) or polyI:C (PolyI:C/DC), both rTSLP-treated DC (rTSLP/DC) and HIV<sup>+</sup>Sn/DC induced significant expansion of both naïve and memory CD4<sup>+</sup> T cells by day 7 (Fig. 3.11a and b). Increases in the numbers of CD4<sup>+</sup> T cells on day 7, as determined by enumerating the total viable cells using the Trypan-blue dye exclusion method, were 1.8-fold for naïve CD4<sup>+</sup> T cell co-cultures with HIV<sup>+</sup>Sn/DC and 3.04-fold for those with rTSLP/DC. We observed a 4-fold increase for memory CD4<sup>+</sup> T cells co-cultured with HIV<sup>+</sup>Sn/DC and 4.5-fold for those with rTSLP/DC. To rule out any activity from trace amounts of HIV that may be present within the HIV<sup>+</sup>Sn despite thorough washing of HIV-exposed epithelial cells, we treated mDC with supernatants from epithelial cells exposed to AT2-treated virus (AT-2<sup>+</sup>Sn) in parallel with untreated virus (AT-2<sup>-</sup>Sn) and observed that AT-2<sup>+</sup>Sn/DC as well as AT-2<sup>-</sup>Sn/DC induced expansion of naïve CD4<sup>+</sup>T cells by 3.9 and 4 fold, respectively by day 7 (Fig. 3.11d). Additionally, we confirmed the significant level of CD4<sup>+</sup> T cell proliferation in co-cultures with rTSLP/DC and HIV<sup>+</sup>Sn/DC, compared to that with medium/DC and HIV<sup>-</sup>Sn/DC, respectively by employing the [<sup>3</sup>H]thymidine incorporation assay (Fig 3.11c). A key feature of TSLP-DC is their ability to not only promote homeostatic proliferation of the autologous CD4<sup>+</sup> T cells, but also to induce their differentiation to be

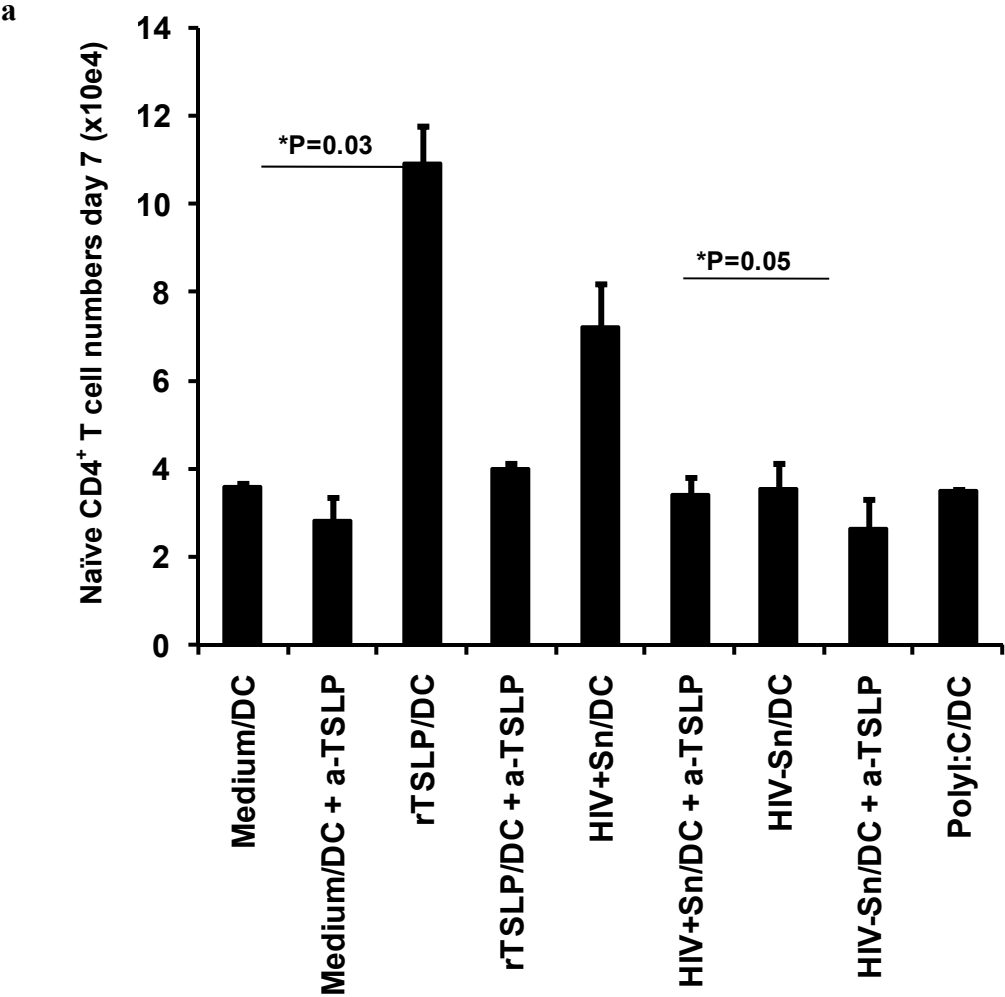
inflammatory Th2 cells that produce the inflammatory cytokine TNF- $\alpha$  in addition to the traditional Th2 cytokines IL-4, IL-5, IL-13 [80]. We observed high levels of expression of each of these cytokines, but not IL-10 or IFN- $\gamma$  in the CD4<sup>+</sup> T cells co-cultured for 7 days with HIV<sup>+</sup>Sn/DC or rTSLP/DC (Fig. 3.12). Addition of HIV-1<sub>IIIB</sub> to the naïve and memory co-cultures at the beginning of the culture period resulted in significant increases in infection, as determined by the analysis of the reverse transcriptase (RT) activity, with HIV<sup>+</sup>Sn/DC and rTSLP/DC when compared to respective control co-cultures of HIV<sup>-</sup>Sn/DC, PolyI:C/DC and medium/DC (Fig. 3.13a and b). The relative levels of increased HIV infections amounted to 3.2- and 3.4-fold by day 7 in the co-cultures of naïve T cells with HIV<sup>+</sup>Sn/DC and rTSLP/DC and 14 and 43-fold in the co-cultures of memory T cells with HIV<sup>+</sup>Sn/DC and rTSLP/DC, respectively compared to that with HIV<sup>-</sup>Sn/DC or medium/DC. In each case, the effects of TSLP activated DC on expansion as well as HIV infection of the naïve autologous CD4<sup>+</sup> T cells could be blocked by including neutralizing TSLP antibodies during the co-culturing. Similar results showing increased infection of co-cultured T cells were observed when HIV-1<sub>YU2</sub>, an R5 strain of HIV-1 was used (Fig. 3.13c).

**Fig. 3.11: mDC activated by HIV-induced TSLP from epithelial cells promote autologous CD4<sup>+</sup> T cell proliferation.**

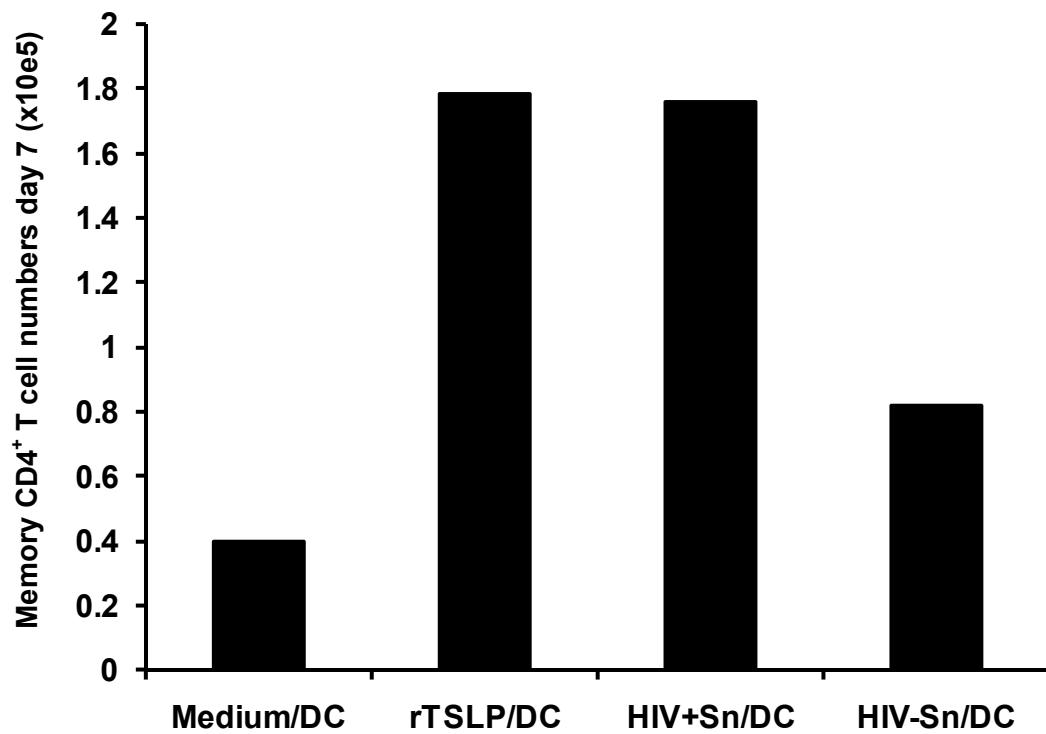
Highly purified autologous naïve or memory CD4<sup>+</sup> T cells were co-cultured with mDC that were activated by pre-incubating with the culture medium (Medium/DC), rTSLP (rTSLP/DC), or supernatants collected from C33A cells treated with or without HIV-1<sub>IIIB</sub> (HIV<sup>+</sup>Sn/DC and HIV<sup>-</sup>Sn/DC, respectively). Proliferation in terms of increased numbers of viable cells on day 7 were determined by Trypan blue dye-exclusion for the naïve and memory CD4<sup>+</sup> T cells (panels a, and b, respectively). Proliferation of naïve CD4<sup>+</sup> T cells was also observed as determined by the standard [<sup>3</sup>H]thymidine incorporation assay (c). Naïve autologous CD4<sup>+</sup> T cells co-cultured with mDC that were activated by pre-incubating with supernatants of epithelial cells exposed to AT2-treated noninfectious HIV (AT2<sup>+</sup>sup/DC) or infectious HIV AT2<sup>-</sup>sup/DC) also demonstrated increased numbers of viable cells on day 7 as determined by Trypan blue dye-exclusion (d). Error bars represent standard deviation values for triplicate cultures, and data shown are representative of multiple experiments.



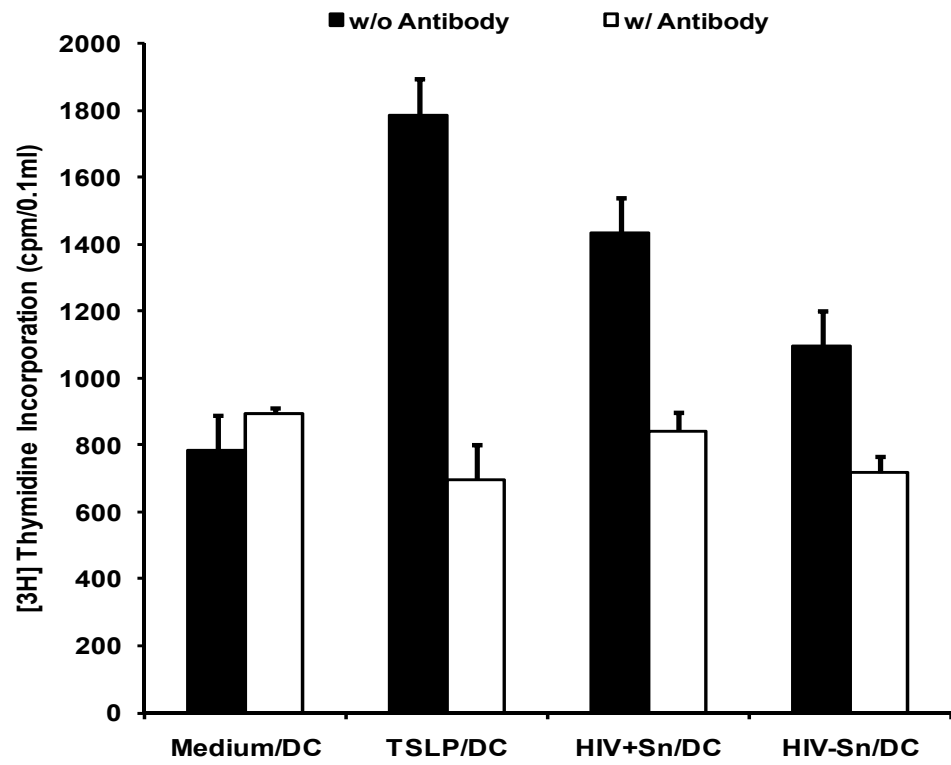
Figure 3.11:



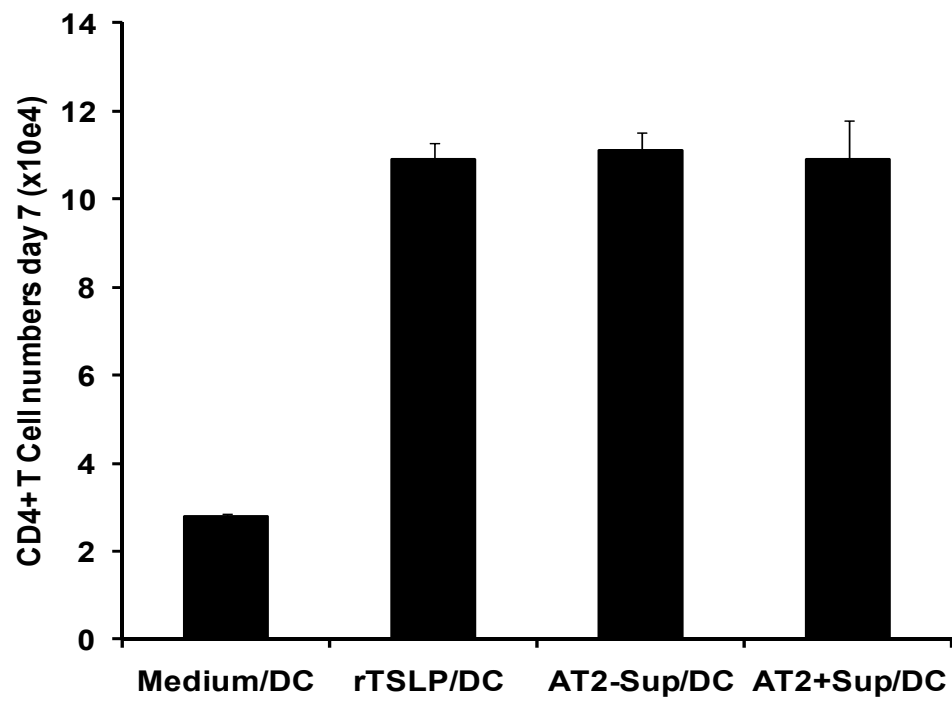
**b**



c

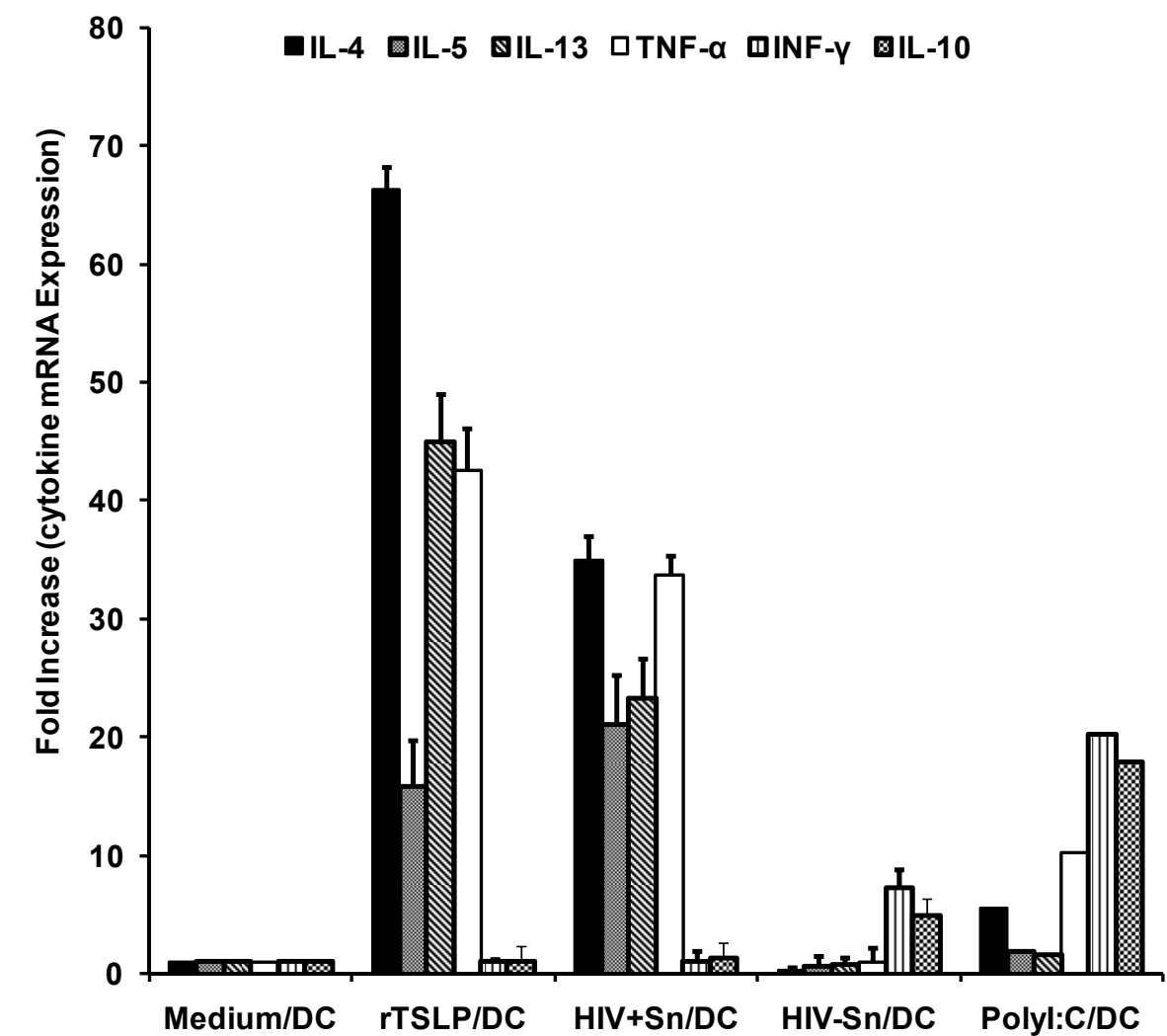


d



**Fig. 3.12: mDC activated by HIV-induced TSLP from epithelial cells promotes differentiation of the naïve CD4<sup>+</sup> T cells to inflammatory Th2 cells.** Naïve autologous CD4<sup>+</sup> T cells were co-cultured with mDC that were activated by pre-incubating with the culture medium (Medium/DC), rTSLP (TSLP/DC), polyI:C (PolyI:C/DC) or supernatants collected from C33A cells treated with or without HIV-1<sub>IIIIB</sub> (HIV<sup>+</sup>Sn/DC and HIV<sup>-</sup>Sn/DC, respectively). After 7 days, the T cells were analyzed for the induction of different cytokines by real-time quantitative RT-PCR and the fold increase with each treatment, relative to that in medium only control cultures is shown as average with standard deviation of triplicate values.

Figure 3.12:

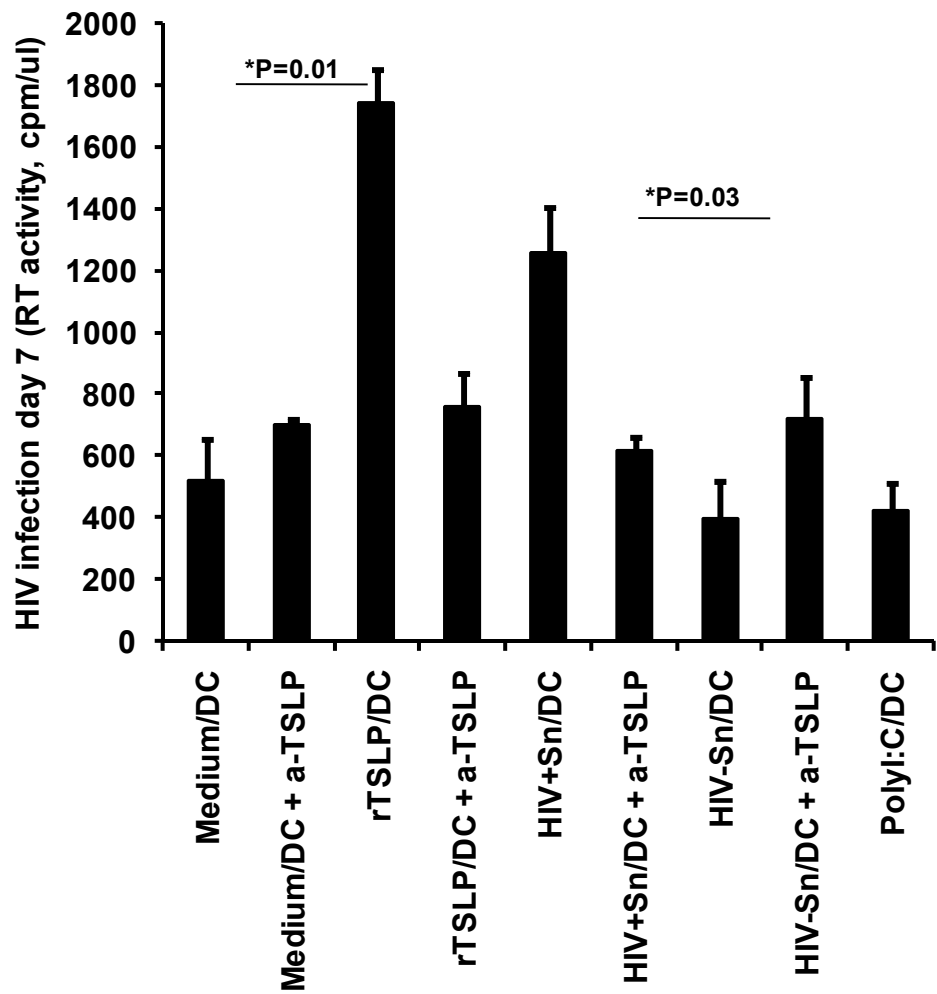


**Fig. 3.13: mDC activated by HIV-induced TSLP from epithelial cells promote increased HIV infection of co-cultured CD4<sup>+</sup> T cells.**

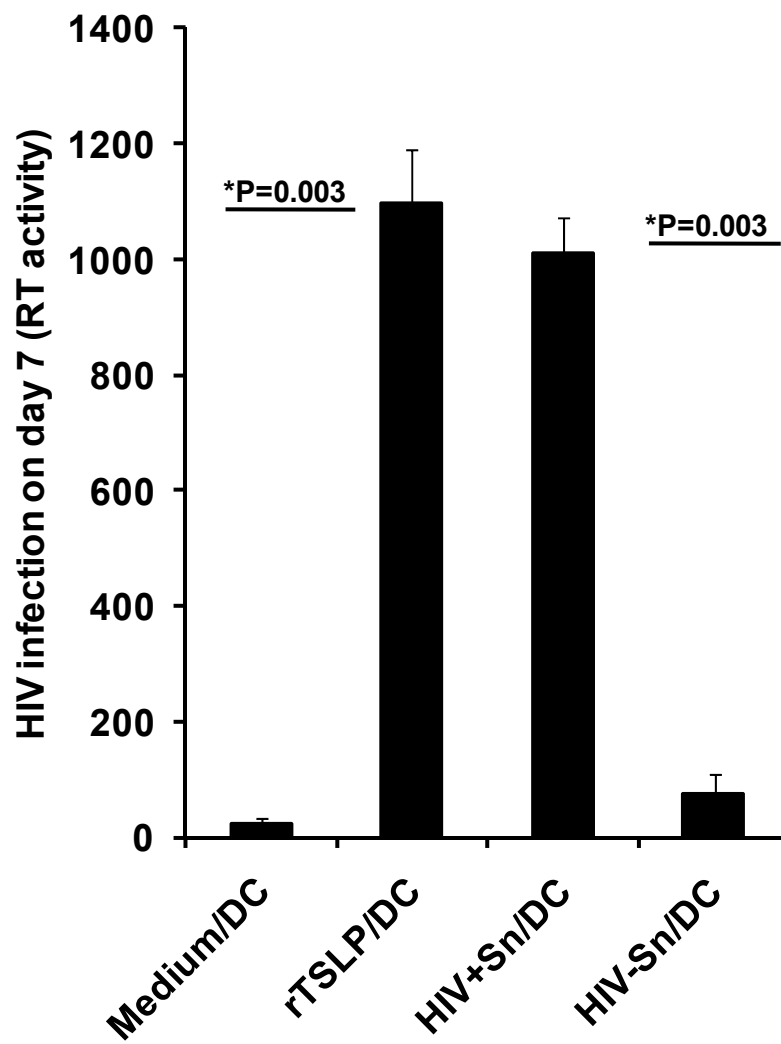
Autologous naïve or memory CD4<sup>+</sup> T cells were co-cultured with mDC that were activated by pre-incubating with the culture medium (Medium/DC), rTSLP rTSLP/DC), or supernatants collected from C33A cells cultured with or without HIV-1<sub>IIIIB</sub> (HIV<sup>+</sup>Sn/DC and HIV<sup>-</sup>Sn/DC, respectively). Co-cultures were also incubated with the X4 viral strain, HIV-1<sub>IIIIB</sub> for seven days and the amount of virus produced in to the supernatants was measured by estimating the reverse transcriptase (RT) activity on day 7 in naïve CD4<sup>+</sup> T cells (a) and memory CD4<sup>+</sup> T cells (b). Similar increases in infection were observed in co-cultures infected with the R5 viral strain, HIV-1<sub>YU2</sub> (c). Error bars represent standard deviation values for triplicate cultures, and data shown are representative of multiple experiments.

Figure 3.13:

a

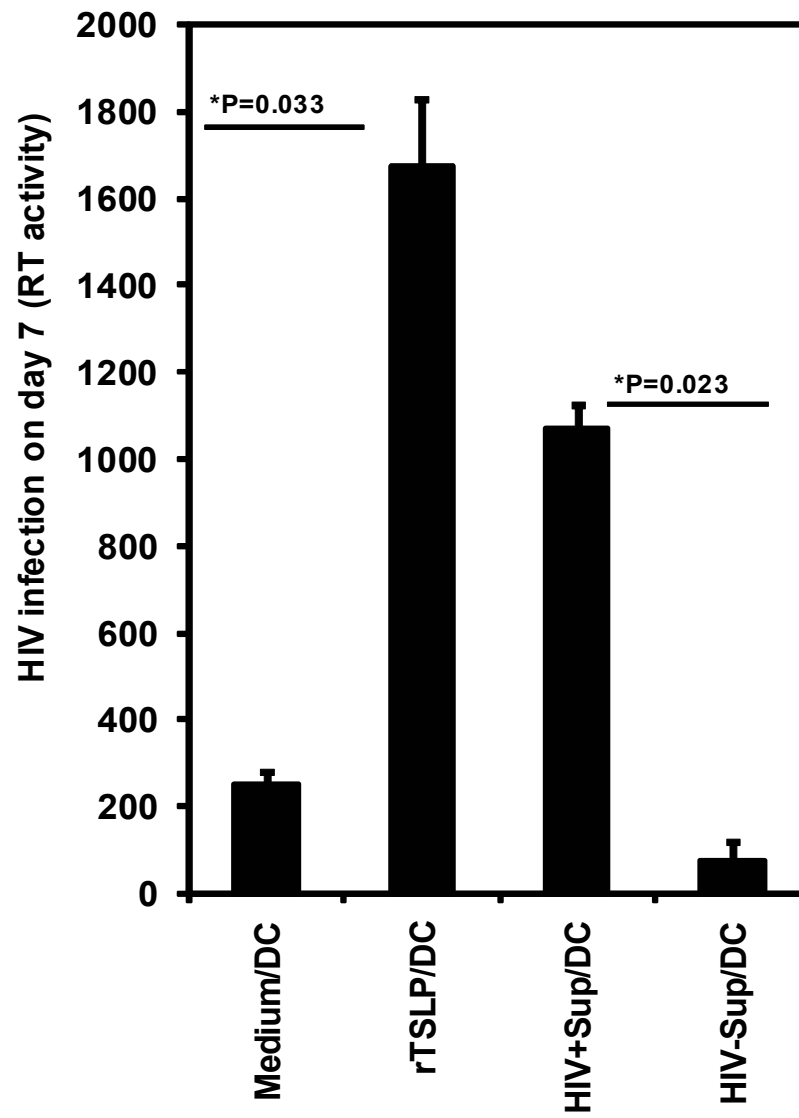


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c

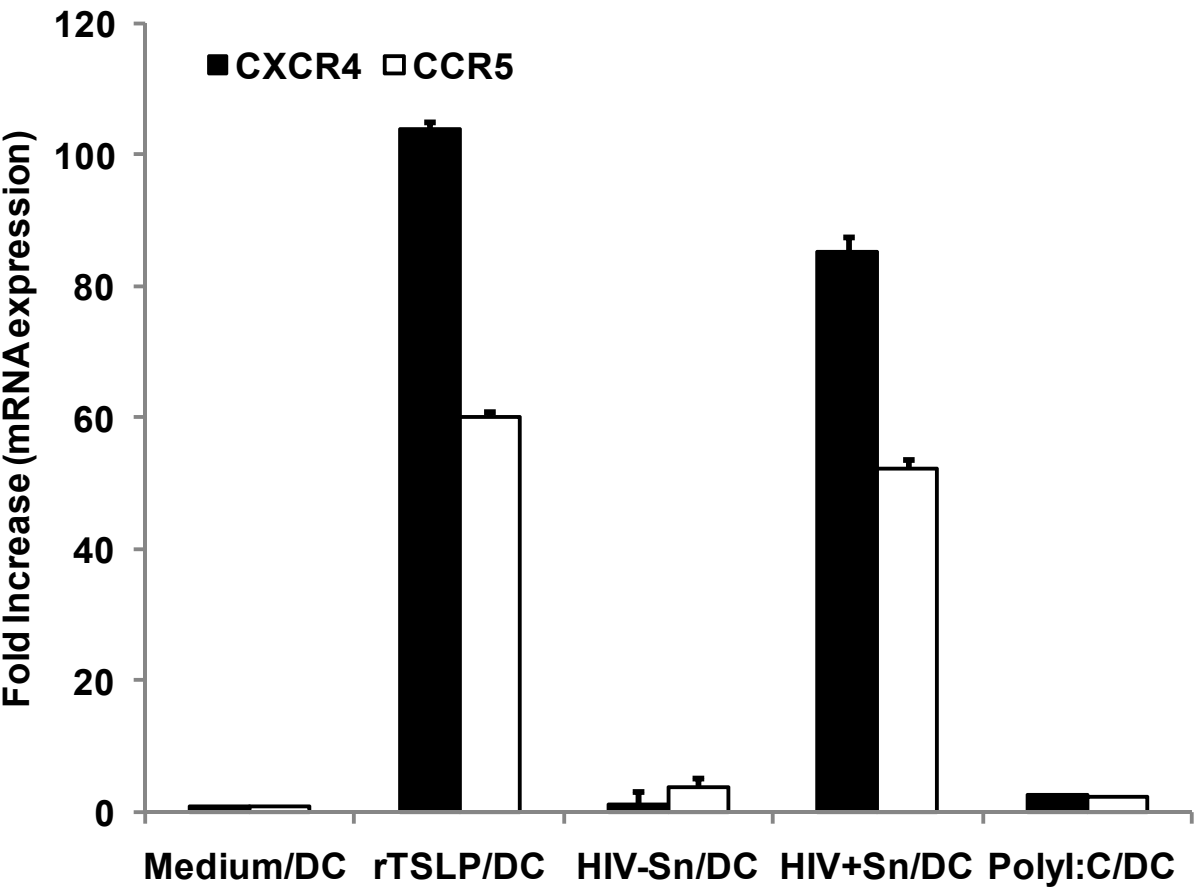


### **Higher expression of HIV co-receptors on CD4<sup>+</sup> T cells co-cultured with myeloid DC activated by TSLP from HIV exposed epithelial cells**

It has previously been reported that Th2 polarized CD4<sup>+</sup> T cells express more chemokine receptors resulting in enhanced HIV-1 replication [82]. Since there was an observed increase in viral replication in CD4<sup>+</sup> T cells co-cultured with mDC activated by TSLP from HIV exposed epithelial cells, the cells were examined for chemokine receptor expression. As shown by mRNA analysis, relative to that of CD4<sup>+</sup> T cells co-cultured with DC treated either with medium (medium/DC), HIV<sup>-</sup>Sn (HIV<sup>-</sup>Sn/DC) or polyI:C (polyI:C/DC), both rTSLP-treated DC (rTSLP/DC) and HIV<sup>+</sup>Sn/DC induced a 108 and 85 fold increase in the expression of CXCR4 as well as 60 and 52 fold increase in the expression of the CCR5 chemokine co-receptor of HIV (Fig. 3.14).

**Fig. 3.14: mDC activated by HIV-induced TSLP from epithelial cells promotes higher expression of chemokine co-receptors on co-cultured CD4<sup>+</sup> T cells.** Naïve CD4<sup>+</sup> T cells were co-cultured with mDC that were activated by pre-incubating with the culture medium (Medium/DC), rTSLP (TSLP/DC), polyI:C (PolyI:C/DC) or supernatants collected from C33A cells cultured with or without HIV-1<sub>IIIIB</sub> (HIV<sup>+</sup>Sn/DC and HIV<sup>-</sup>Sn/DC, respectively). After 7 days, the T cells were analyzed for chemokine co-receptor expression by real-time quantitative RT-PCR. Error bars represent standard deviation values for triplicate cultures, and data shown are representative of multiple experiments.

Figure 3.14:



**Specific Aim 3: Investigate the mechanism of HIV-mediated induction of TSLP expression in epithelial cells.**

## **Results**

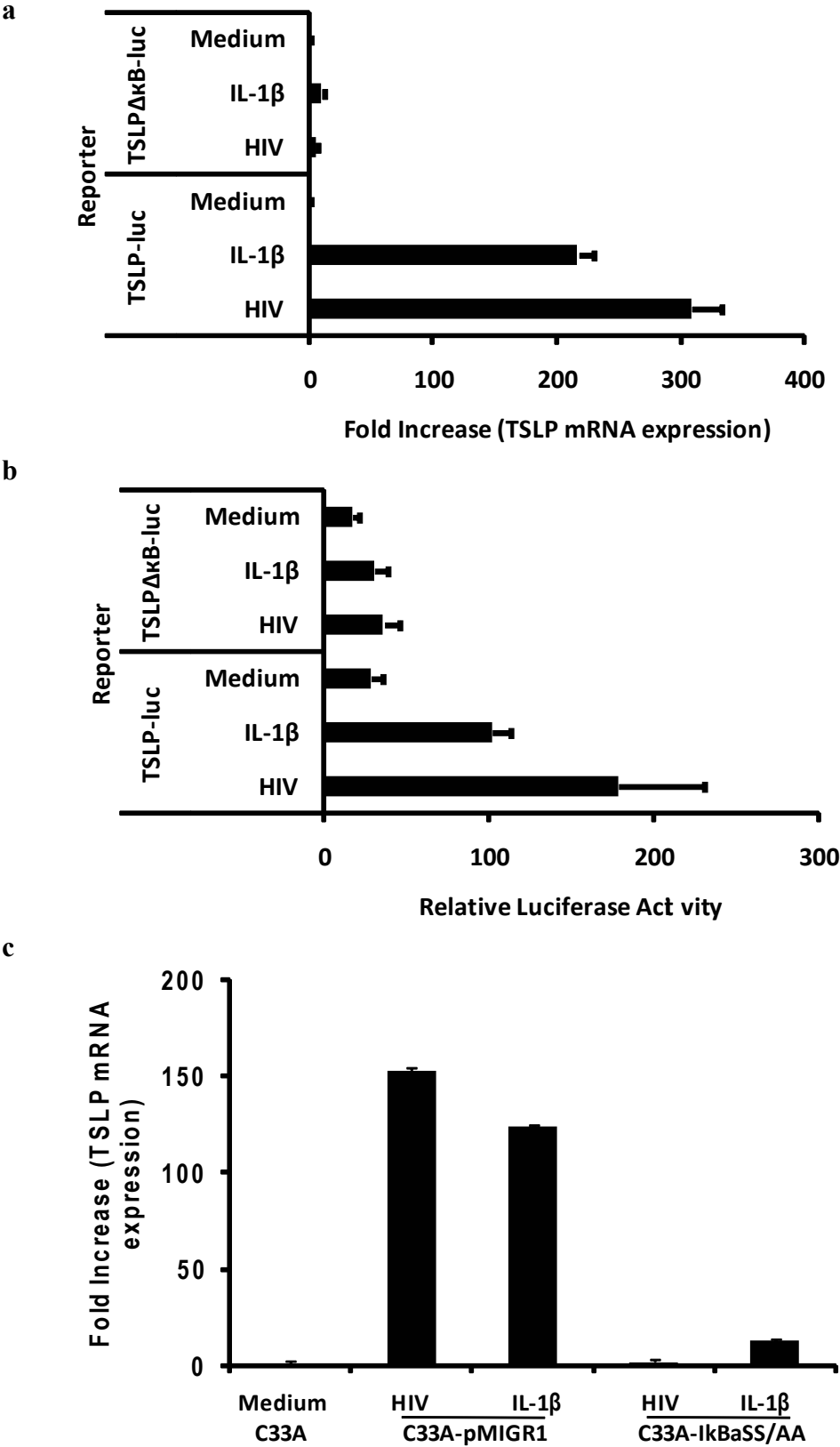
### **Transcriptional activation of the human TSLP promoter by HIV includes NF $\kappa$ B activation**

It has recently been shown that TSLP expression in the airway epithelial cells can be induced by pro-inflammatory mediators (IL-1 $\beta$ , TNF- $\alpha$ ) and agonists for toll-like receptors 2, 8 and 9 (TLR2, TLR8, and TLR9, respectively), via the induction of NF $\kappa$ B through an NF $\kappa$ B binding site identified within the human TSLP (hTSLP) promoter [69]. In collaboration with Dr. Shao-Cong Sun's laboratory in our department of immunology at the UT MD Anderson Cancer Center, Houston, TX, I investigated whether the mechanism by which HIV induces TSLP expression in epithelial cells involves NF $\kappa$ B signaling. The cervical epithelial cells (C33A) were transfected with a luciferase reporter plasmid encoding the full length hTSLP promoter which contains a wild-type NF $\kappa$ B binding site 3.7 kb upstream of the start of transcription (TSLP-luc), or a mutated NF $\kappa$ B binding motif (TSLP $\Delta$  $\kappa$ B-luc) constructed by site-directed mutagenesis as described previously [69[83]]. The transfected cells were incubated with HIV or IL-1 $\beta$  and TSLP mRNA expression and luciferase activity were determined. When compared to medium alone, HIV was able to induce an increase in TSLP mRNA expression (309-fold) as well as the hTSLP promoter activity (178-fold) at levels similar to those of the cells stimulated with the positive control IL-1 $\beta$  (Fig. 3.15a and b). On the other hand, both TSLP mRNA expression and the luciferase activity were unchanged in C33A cells transfected with the mutated NF $\kappa$ B binding site construct suggesting that HIV induced TSLP expression involves the NF $\kappa$ B signaling (Fig. 3.15a and b). Additionally, retroviral vectors expressing an NF- $\kappa$ B super-repressor, I $\kappa$ B $\alpha$ SS/AA, or a control sequence

pMIGR1 were constructed and transfected into C33A cells to derive C33A-I $\kappa$ B $\alpha$ SS/AA and C33A-pMIGR1 cell lines, respectively [83]. Expression of I $\kappa$ B $\alpha$ SS/AA, but not pMIGR1, resulted in the abrogation of TSLP expression in response to HIV as well as IL-1 $\beta$  in these cells (Fig. 3.15c). Together, these results confirm the role of the NF $\kappa$ B signaling pathway in the transcriptional activation of HIV induced TSLP expression.

**Fig. 3.15: Transcriptional Activation of the Human TSLP promoter by HIV involves NFκB signaling.** The cervical epithelial cells (C33A) were transfected with a Luciferase reporter plasmid encoding human TSLP promoter harboring either the wild-type NFκB binding site (hTSLP-luc) or the mutated NFκB binding site (TSLPΔκB-luc) and exposed to HIV-1<sub>IIIB</sub> and IL-1β. Significantly increased levels of TSLP mRNA, assessed by quantitative real time PCR (a), and Luciferase activity (b) were detected after exposure to HIV-1<sub>IIIB</sub> and IL-1β when compared to untreated cells (medium) in cells transfected with hTSLP-luc plasmid but not TSLPΔκB-luc. Panel c shows TSLP expression, as determined by real-time PCR, in response to HIV-1<sub>IIIB</sub> or IL-1β was significantly reduced in C33A cells transfected with a retroviral vector expressing an NFκB super-repressor, IκBαSS/AA, (C33A-IκBαSS/AA), but not in those transfected with a control vector, pMIGR1, (C33A-pMIGR1) or untreated control cultures (medium). Error bars represent standard deviation values for triplicate cultures, and data shown are representative of multiple experiments.

Figure 3.15:





## **The scavenger receptor gp340 expressed on epithelial cells is necessary for HIV-induced TSLP expression**

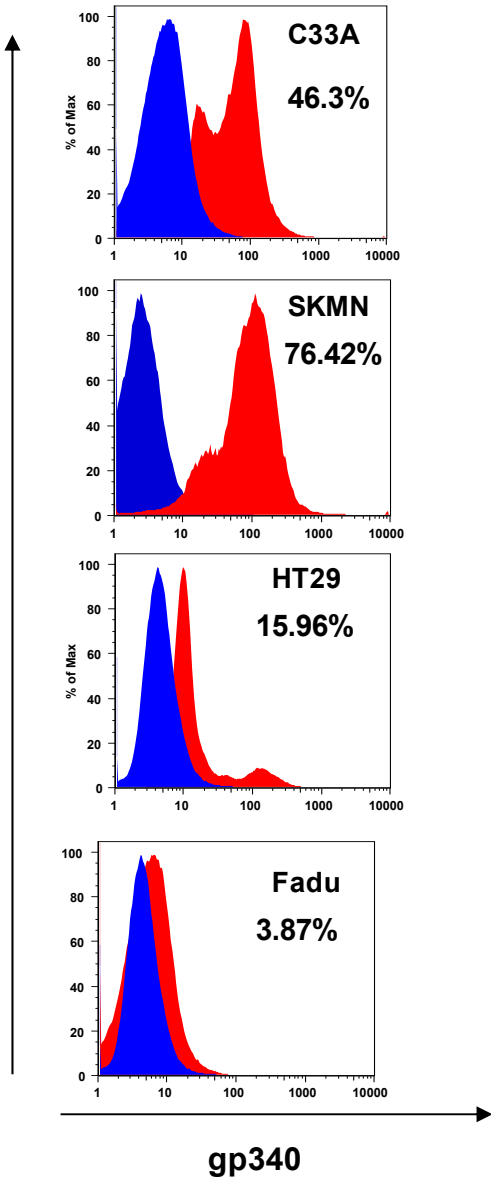
We investigated the mechanism underlying the HIV interaction with epithelial cells to induce TSLP expression. Specifically, we determined the epithelial cells surface molecules necessary for interaction with HIV. Recent reports implicated the epithelial cell surface scavenger receptor gp340, a splice variant of the human protein, the Deleted in Malignant Brain Tumors I (DMBT1), to bind to the HIV envelope protein gp120 and facilitate transcytosis/endocytosis of the virus [21, 84]. We reasoned that such interaction with the virus may be necessary for TSLP expression by the epithelial cells. First, a variety of epithelial cell lines were screened for cell surface expression of gp340 by flow cytometry. Cervical (C33A), intestinal (HT-29), and neuronal (SKNMC) epithelial cells, which we observed earlier to secrete TSLP in response to HIV exposure, showed detectable levels of cell surface gp340 (Fig. 3.16a), with one exception, the pharyngeal epithelial cells (Fadu), which did not express gp340 (Fig. 3.16a). To determine the role played by the epithelial cell surface protein gp340 in TSLP induction by HIV, gp340 expressing human cervical epithelial cells (C33A) and gp340- null pharyngeal epithelial cells (Fadu) were cultured with HIV and the cell extracts analyzed for TSLP expression. High levels of TSLP mRNA as measured by quantitative RT-PCR were detected in the C33A cells but not the Fadu cells (Fig. 3.16b). Furthermore, the HIV-induced TSLP expression in C33A cells could be blocked with an antibody to gp340 (Fig. 3.16b). Both Fadu cells and C33A cells produced TSLP mRNA in response to the positive controls PolyI:C, TNF- $\alpha$ , and IL-1 $\beta$ , suggesting the specificity of gp340 for HIV-induced TSLP expression (Fig. 3.16b). To begin elucidating the signaling for HIV-induced TSLP expression, the C33A cells were transfected with a reporter plasmid expressing luciferase under the control of the human TSLP(hTSLP) promoter followed by incubation with HIV, which resulted in high levels of

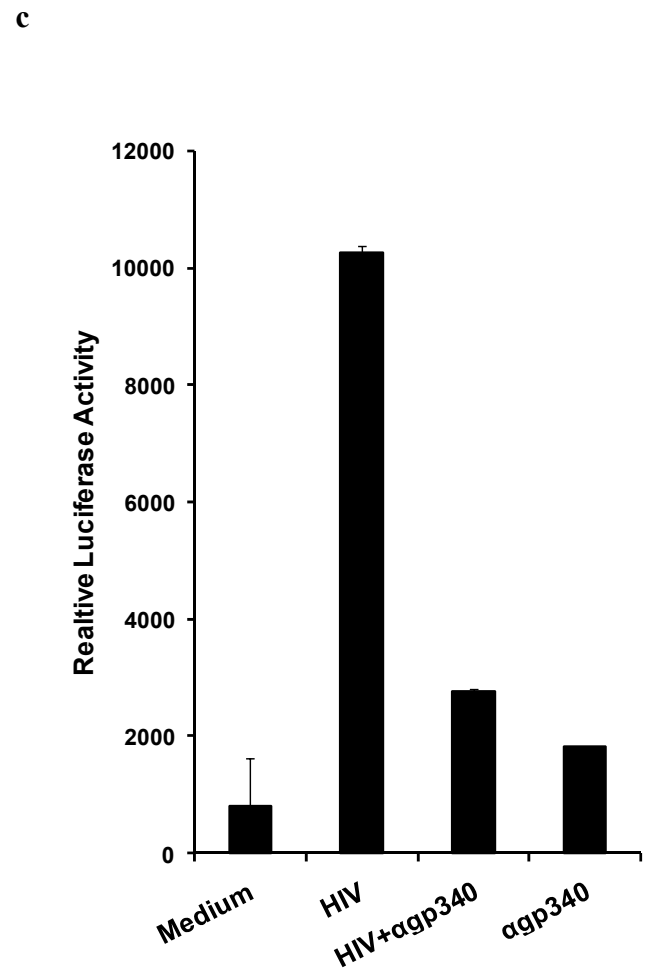
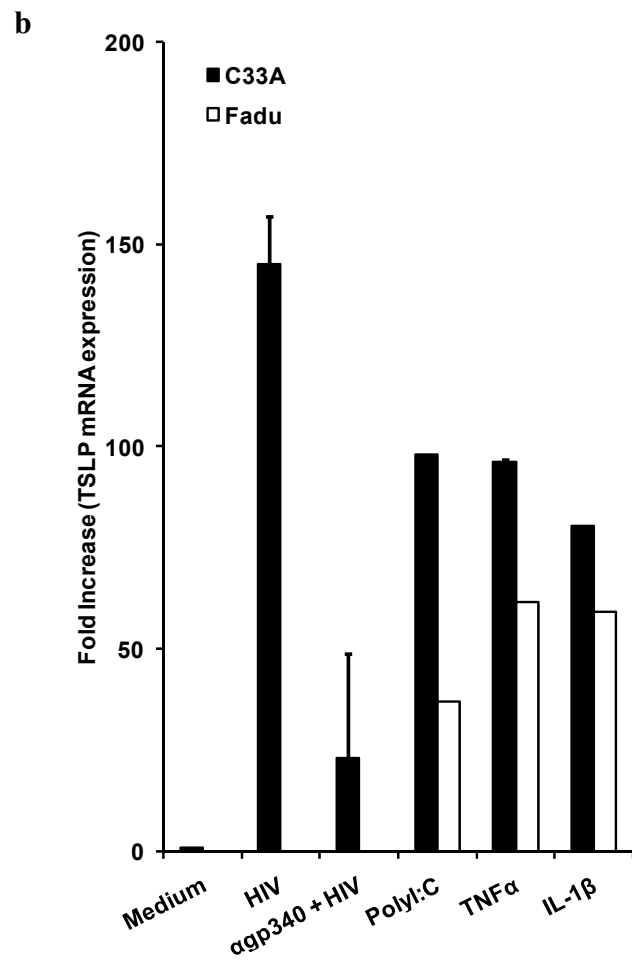
luciferase activity that were blocked when the transfected cells were pre-incubated with gp340 antibody prior to HIV exposure (fig. 3.16c). To further confirm the role of gp340 in HIV induced TSLP expression, we established a variant of the Fadu cell line that expresses a cell-surface associated form of gp340 by transfecting cells with a plasmid encoding the full length form of DMBT1 (Fig. 3.16d). Gp340 expression in the transfected Fadu cells was confirmed by flow cytometry and RNA analyses, and when these cells were cultured with HIV, TSLP expression was detected as measured by quantitative RT-PCR (Fig. 3.16e). The effect of gp340 silencing on HIV-induced TSLP production was also determined by knocking down gp340 expression in C33A cells with siRNA specific to gp340, which resulted in negligible levels of gp340 detectable in the knock down cells measured in terms of quantitative RT-PCR as well as flow cytometry (Fig. 3.16 d and e). The C33A cells with the gp340 knocked down, when cultured with HIV showed significantly reduced levels of TSLP expression as measured by quantitative RT-PCR (Fig. 3.16f).

**Fig. 3.16: Gp340<sup>+</sup> epithelial cells produce TSLP in response to HIV.** Surface expression of gp340 was observed on the cervical epithelial cells (C33A), neuronal epithelial cells (SKMN), and intestinal epithelial cells (HT-29) but not pharynx-derived epithelial cell line (Fadu) assessed by flow cytometry using a mouse gp340-specific antibody (A). The red histograms represent specific staining for gp340 and the blue histograms represent the isotype control staining, and the numbers in each panel indicate the mean fluorescence intensity (MFI). High levels of TSLP mRNA were detected in the cervical epithelial cells (C33A) cultured with HIV-1<sub>IIIB</sub> which was specifically blocked by anti-gp340 antibody, while the Fadu epithelial cell line exposed to HIV did not show TSLP expression. Both C33A and Fadu cell lines produce TSLP in response to polyI:C, TNF- $\alpha$  and IL-1 $\beta$  (B). Increased levels of luciferase activity were detected in C33A cells transfected with a wild-type human TSLP promoter luciferase-reporter plasmid and exposed to HIV-1<sub>IIIB</sub>, which was specifically blocked by anti-gp340 antibody, when compared to medium alone or with cells treated with the gp340-specific antibody and not exposed to HIV (C). Surface expression of gp340 was observed on wild type C33A and Fadu cells transfected with gp340 but not in wild type Fadu or C33A cells where gp340 was knocked down as assessed by flow cytometry using a gp340-specific antibody (D). The red histograms represent specific staining for gp340 and the blue histograms represent the isotype control staining, and the numbers in the panels indicate the MFI values. Expression of gp340 and TLR3 in wild type C33A and gp340 knockdown C33A cells and wild type and gp340-transfected Fadu cells was assessed by RT-PCR (E). High levels of TSLP mRNA were detected in gp340 expressing C33A and Fadu cells cultured with HIV. All cell types expressed TSLP in response to polyI:C (F). Data shown are averages with standard deviation values of a representative of multiple experiments with similar results.

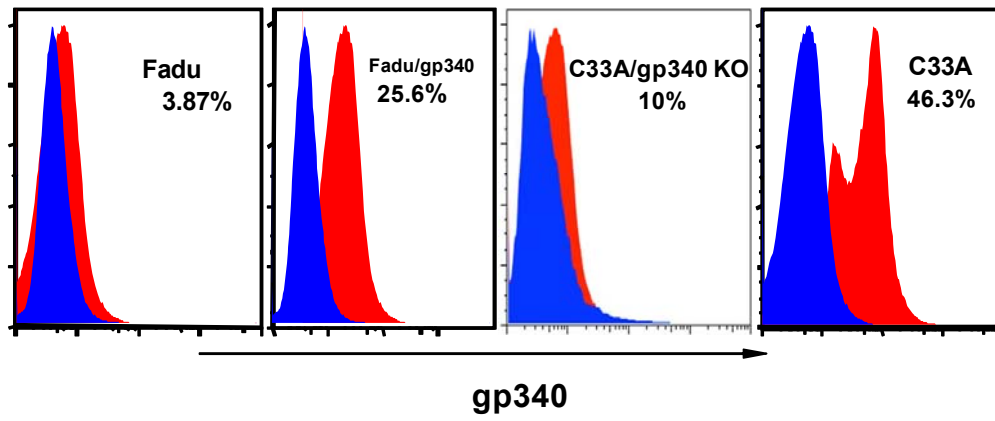
Figure 3.16:

a

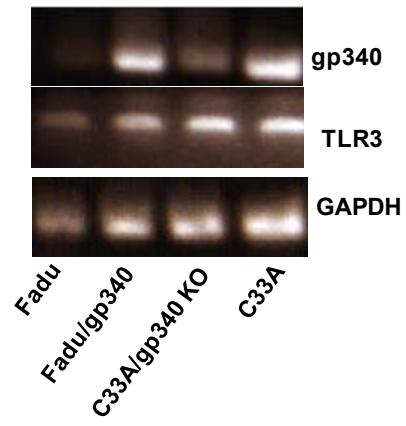




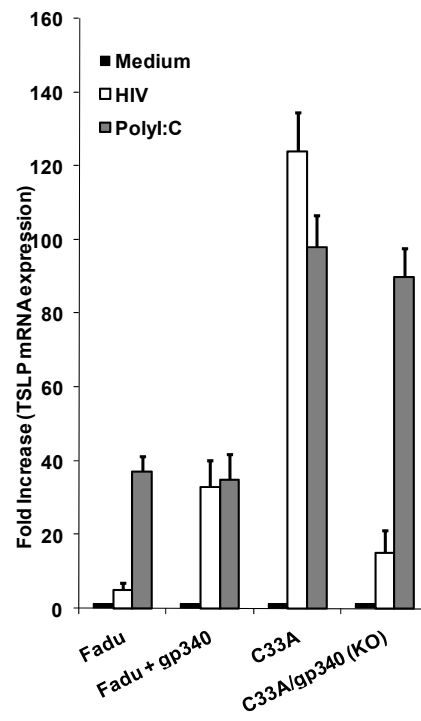
d



e



f



### **Binding of HIV gp120 to gp340 in epithelial cells is necessary but not sufficient for HIV-mediated TSLP expression in epithelial cells**

Since we and others have shown that the HIV receptors CD4, CXCR4, and CCR5 are not expressed on human genital epithelial cells [21, 35] (Table 2), we investigated whether gp340 on the epithelial cell surface would be necessary and sufficient for inducing TSLP expression in response to HIV. In 2007, Stoddard et al. showed that gp340 expressed on human genital epithelial cells binds HIV-1 envelope protein, specifically involving the V3 loop region for potential viral endocytosis and/or transcytosis in the epithelial cells [21]. To evaluate the potential role of gp340 interaction in HIV induced TSLP expression, we examined binding of gp340 on the C33A cells with the envelope protein on HIV-1 virions or the recombinant HIV-1 envelope protein gp120. Specific wells within the 96-well plates were coated with either HIV-1, recombinant gp120, anti-gp340, anti-TNFR antibody, or the appropriate isotype control antibodies (the latter two reagents as positive and negative control reagents, respectively, because TNFa can induce TSLP expression in epithelial cells, [69]). The C33A cells were seeded in the coated wells and after overnight incubation the cell extracts were prepared and analyzed for TSLP expression, in comparison to cross linking TNF on these cells with TNF-receptor antibody. Neither HIV, recombinant gp120, nor anti-gp340, used for gp340 cross linking, induced TSLP expression as compared to the positive control treatment (Fig. 3.17). These data, combined with our earlier results showing TSLP expression in gp340 containing but not gp340-null epithelial cells, suggest that HIV binding to the gp340 receptor is necessary but not sufficient to produce the signal needed to induce TSLP expression.

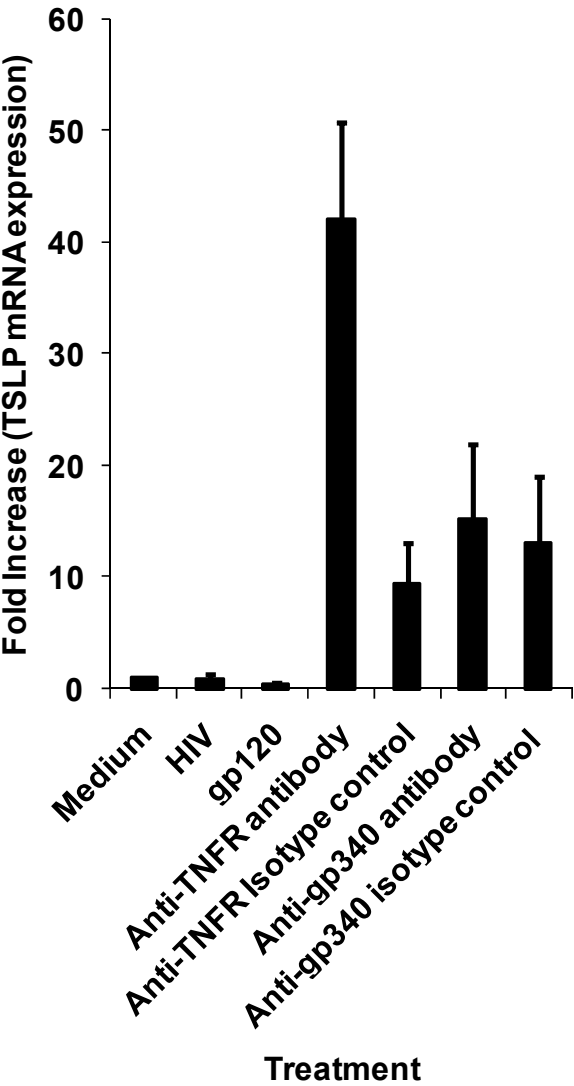
**Table 2: Flow cytometry analyses of HIV binding and entry receptors on different cells**

<b>Cell line</b>	<b>CD4 Expression (MFI)</b>	<b>CXCR4 Expression (MFI)</b>	<b>CCR5 Expression (MFI)</b>
<b>C33A</b>	<b>0.14</b>	<b>0.92</b>	<b>0.22</b>
<b>Ghost X4</b>	<b>91.58</b>	<b>89.81</b>	<b>-</b>
<b>Ghost Hi5</b>	<b>38.92</b>	<b>3.6</b>	<b>53.6</b>
<b>SKMN</b>	<b>0.44</b>	<b>0.11</b>	<b>0.05</b>
<b>PBMC</b>	<b>42</b>	<b>41.2</b>	<b>52.30</b>



**Fig. 3.17: Gp340 binding does not signal TSLP expression in the epithelial cells.** TSLP production was detected in cervical epithelial cells (C33A) after cross linking TNF receptor with an antibody to TNF-R when compared to medium alone or cells where the surface gp340 was bound to with immobilized HIV, recombinant gp120, or cross-linked to an antibody to gp340. Data shown are average with standard deviation values of a representative of three separate experiments.

Figure 3.17:



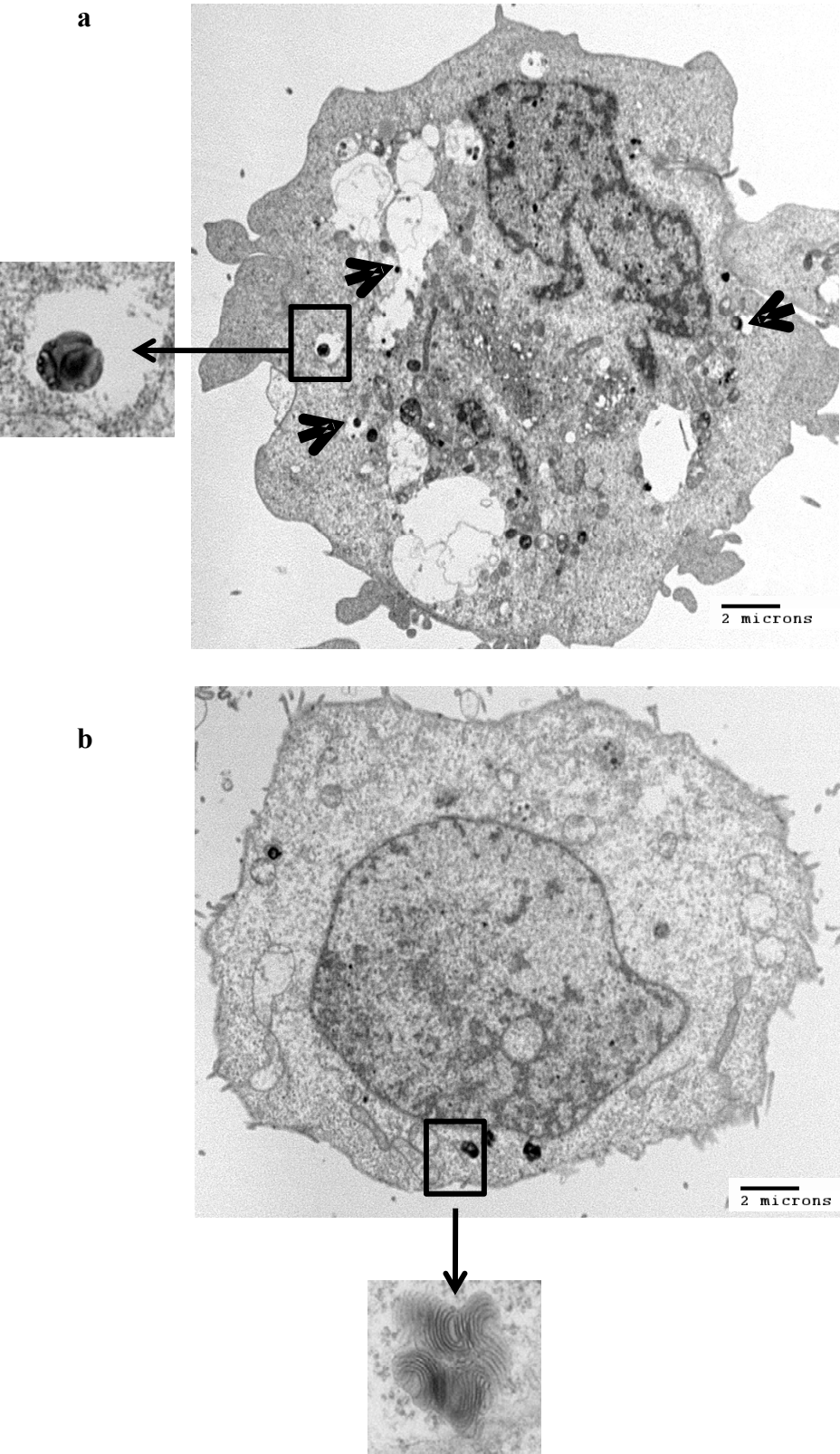
## **Endocytosis of HIV-1 mediated by gp340 on epithelial cells induces TSLP expression**

The gp340 on the epithelial cell surface was also identified in 2002 by Cannon et al to be endosome-associated leading to the suggestion that gp340 may be capable of trafficking to intracellular compartments [84]. Based on our results showing that gp340 on the epithelial cells is necessary but not sufficient for HIV-induced TSLP expression, we investigated by transmission electron microscopy whether gp340 expressed on the C33A cells could be involved in the endocytosis of HIV-1. Cells and virus were pre-incubated at 4°C for two hours and then transferred to 37°C for various time intervals. Samples collected at the different time point were processed and examined using electron microscopy. Fig. 3.18, shows a selection of the C33A cell culture incubated with HIV and a single cell with an enlarged subsection containing the virus particles inside the endosomes. A similar analysis showed no virus particles within endosomal compartments in the gp340 null Fadu cell line. This data suggests that virus is internalized into the endosomes of the gp340 positive cells even though these cells do not show CD4, CXCR4, and CCR5 surface expression required for productive infection by HIV (Table 2). To further confirm the requirement for endocytosis of HIV to induce TSLP expression, the gp340-positive C33A cells were cultured with HIV in the presence or absence of several different types of inhibitors of endocytosis: Dimethyl amiloride (DMA), which inhibits macropinocytosis; Cytochalasin D (CCD), which blocks endocytic trafficking including clathrin-mediated entry; Chlorpromazine, which blocks clathrin-coated pit-mediated endocytosis [85]. Cells were cultured with HIV in the presence of these various endocytosis inhibitors and the cell extracts were analyzed for TSLP. Treatment with DMA, CCD, and chlorpromazine reduced TSLP expression as measured by quantitative RT-PCR analysis of the mRNA (Fig. 3.19a) and by ELISA for the protein in the cell extract (Fig. 3.19b) suggesting that endocytosis of HIV into the C33A cells leads to induction of TSLP expression.

The toll-like receptors (TLRs) 3, 7, 8, and 9, which sense pathogens and initiate signaling, have been shown to localize within the endosomal compartment [85-87]. For single-stranded RNA viruses, a class to which HIV belongs to, TLRs 7 and 8 are important mediators of pathogen-specific RNA recognition and immune activation [86, 87]. Reduction of TSLP expression in response to the TLR7/8 ligands CL097 and CL075 was observed in both C33A and Fadu cells, and in response to HIV in the C33A cells, when treated with DMA, CCD, and chlorpromazine suggesting that activation of the intracellularly located toll like receptors TLR7 or TLR8 may be involved in HIV-mediated TSLP expression in the gp340+ C33A cells (Fig 3.19a). Therefore, we examined whether acidification of the endosomes is necessary for HIV RNA within the endosomes to interact with TLR7/8 and induce TSLP expression. Specifically, we tested two weak bases, chloroquine and quinacrine, that neutralize the acidic environment of endocytic vesicles along with another endosomal acidification inhibitor bafilomycin A1 (BFLA-1) which acts on vacuolar H<sup>+</sup>-ATPases [85]. We observed that all three inhibitors were able to reduce TSLP expression in response to HIV as well as the specific ligands CL097 and CL075 to TLR7 and 8, respectively (Fig. 3.19a) suggesting that endocytosis of HIV-1 followed by acidification of the endosome is involved in HIV induced TSLP expression in these gp340+ cervical epithelial cells.

**Fig. 3.18: Endocytosis of HIV into epithelial cells.** Transmission electron microscopy (TEM) analyses of gp340+ C33A (a) and gp340- Fadu (b) cells exposed to HIV. Enlargement of the framed area from within the C33A cell exposed to HIV for 20 minutes at 37° C shows a virus particle with electron dense core structure typical of HIV within an endosome of the cell (a) while a similar analyses of an area with a dark spot in the HIV-exposed Fadu cells showed nonspecific particles that exhibited no organized structure (b).

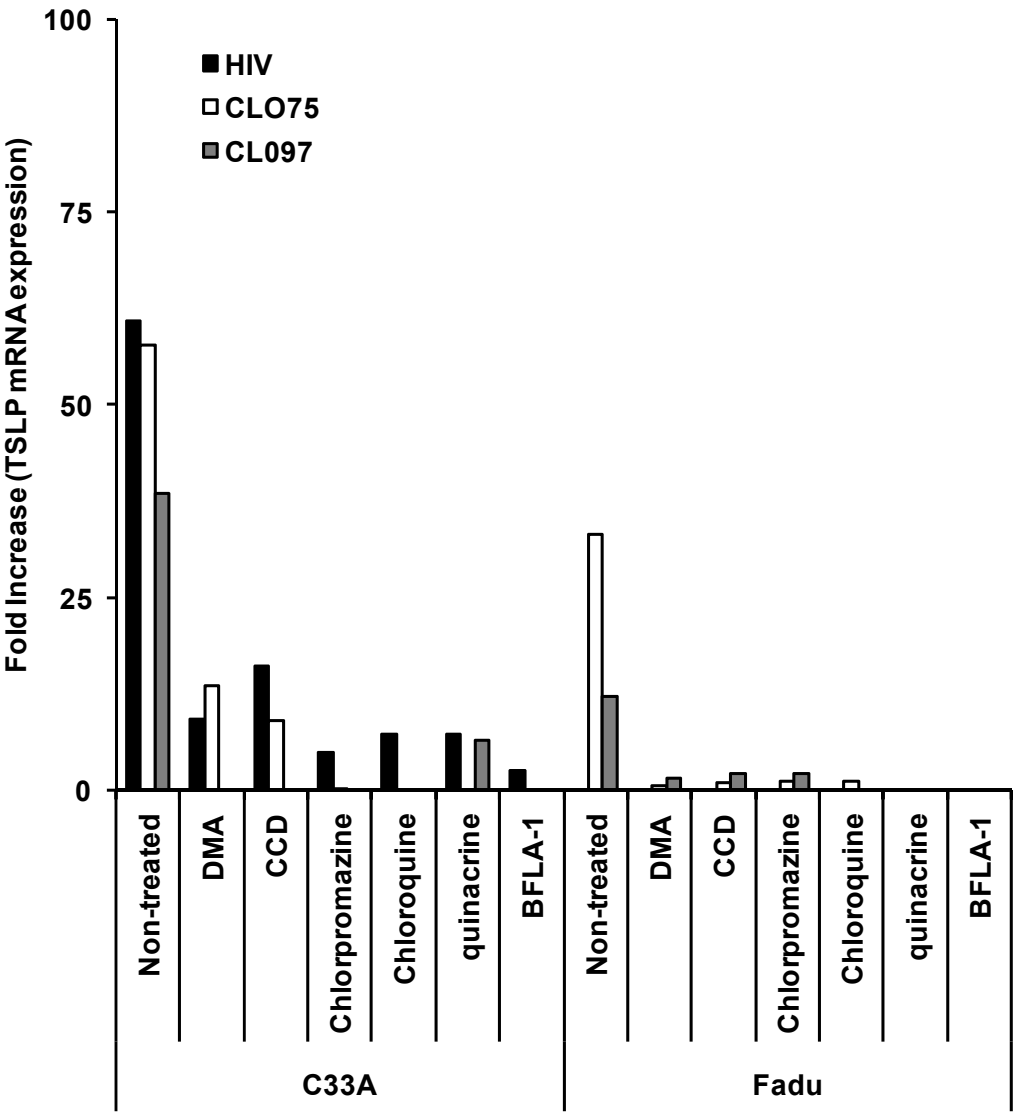
Figure 3.18:



**Fig. 3.19: Gp340 receptor mediated endocytosis of HIV triggers HIV induced TSLP expression.** Pretreatment of gp340+ cervical epithelial cells (C33A) with various inhibitors of endocytosis (shown on the x-axis) reduced HIV induced TSLP expression (a). Both gp340+ C33A and gp340- Fadu cells produced TSLP in response to the TLR7/8 ligands CL075 and CL097 which was reduced in the presence of the various inhibitors of endocytosis (a). Protein analysis by ELISA in the cell extracts or culture supernatants of cells treated with various endocytosis inhibitors and exposed to HIV or TLR7/8 ligands confirmed the reduction of TSLP production in the C33A cells (b).

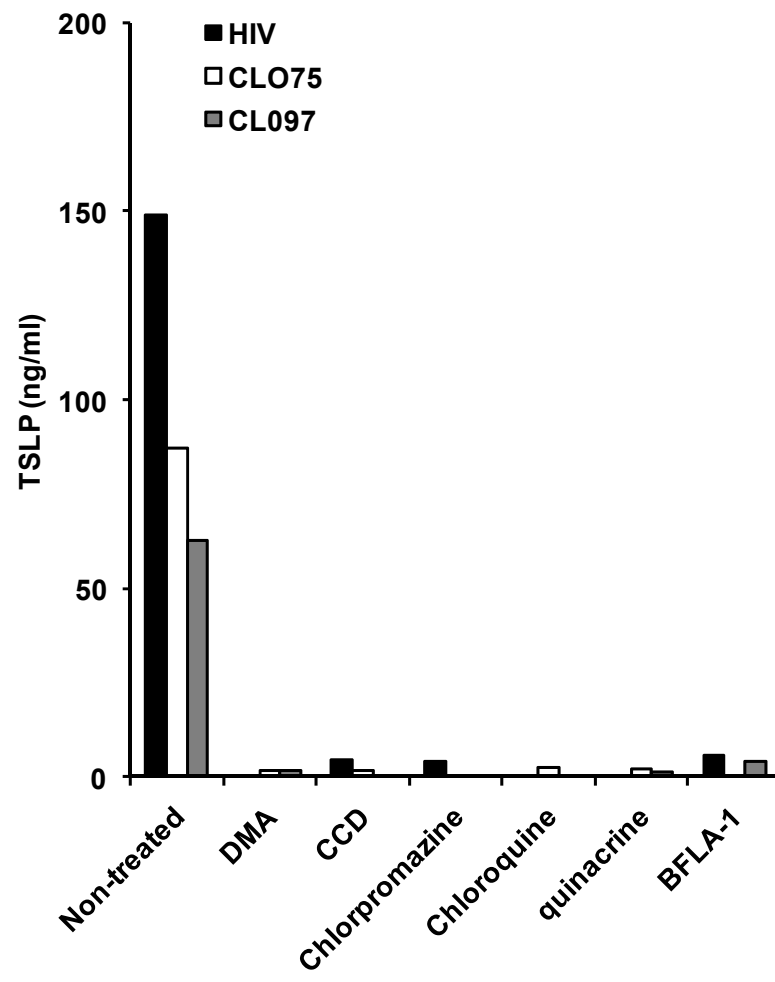
Figure 3.19:

a





**b**

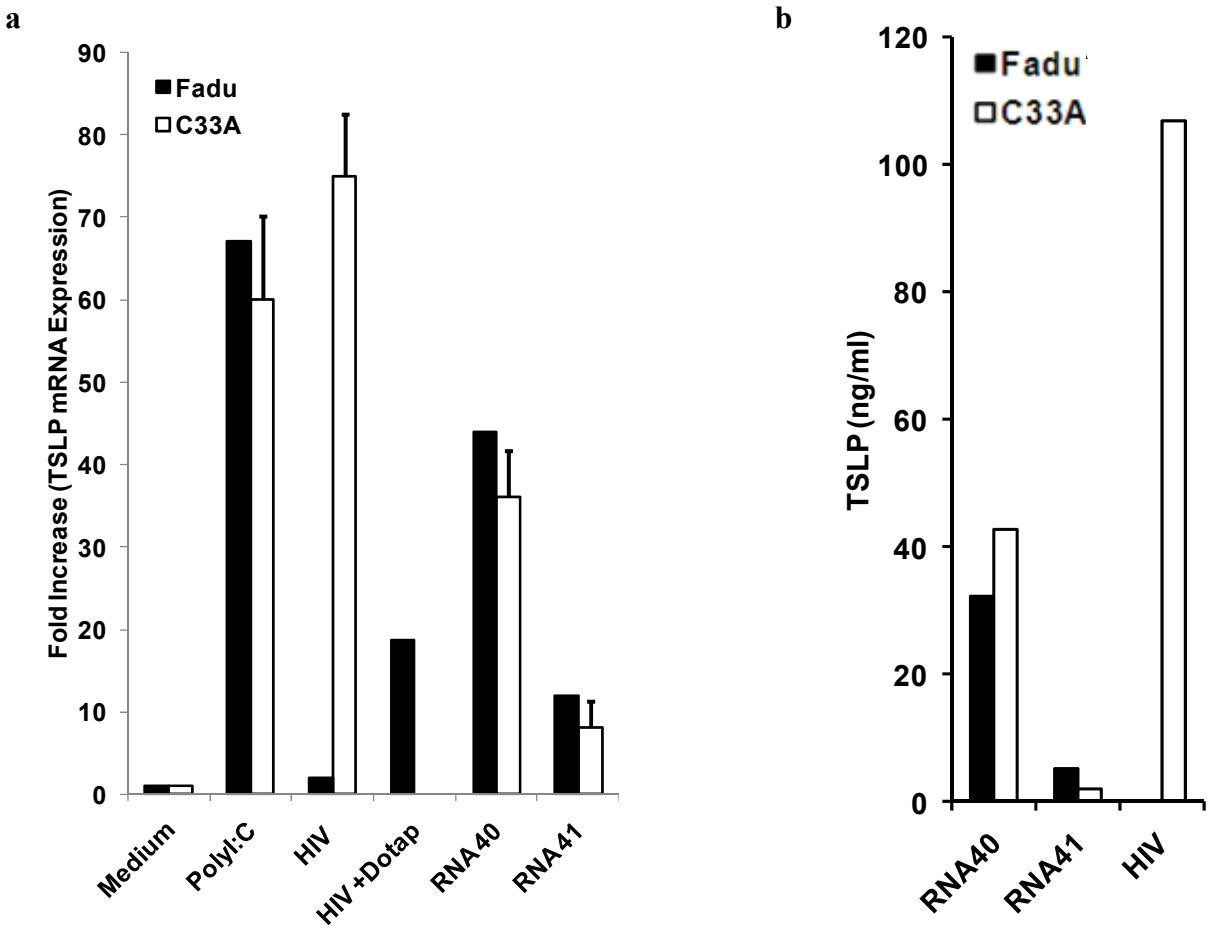


### **HIV-1 RNA is responsible for inducing TSLP expression in gp340<sup>+</sup> cells**

Both TLR7 and TLR8 are localized in the endosomes within the cell cytoplasm and endosomal acidification is required for ligand recognition and signaling by these TLRs [86, 87]. Single stranded RNA (ssRNA) containing viruses that signal through these TLRs enter cells through endocytosis and are held in the endosomal compartment where pH changes induce degradation of the viral particles allowing the viral nucleic acid to come into contact with TLR7 or TLR8 [91]. The structural and functional features of TLR7 and 8 show a high degree of phylogenetic similarity with both receptors capable of recognizing the guanosine and uridine rich ssRNAs derived from HIV [91]. Since we observed endosomal acidification to be involved in HIV induced TSLP expression we tested whether a synthetic oligonucleotide corresponding to the HIV-1 RNA would induce TSLP expression. We cultured the gp340<sup>+</sup> C33A and gp340<sup>-</sup> Fadu epithelial cells with RNA40 (a synthetic [G+U] rich short oligoribonucleotide corresponding to the U5 region of HIV-1). To facilitate uptake into the cells, RNA40 was pre-incubated with DOTAP (liposomal transfection delivery system) prior to adding to the cells, and TSLP expression was observed, in terms of RNA as well as protein, in both C33A and Fadu cells but not when the negative control RNA41 was introduced with DOTAP (Fig. 3.20a and b). Furthermore, the gp340<sup>-</sup> Fadu cells that do not express TSLP in response to HIV, when cultured with HIV pre-incubated with DOTAP, to bypass the receptor mediated endocytosis, exhibited TSLP expression (Fig. 3.20a) suggesting that gp340-mediated entry of HIV into the endosomes for HIV-induced TSLP expression.

**Fig. 3.20: HIV RNA stimulates TSLP production in epithelial cells.** C33A and Fadu cells were cultured with HIV, RNA 40 and RNA 41, each mixed with DOTAP, and the positive control Poly I:C followed by the analyses of TSLP mRNA expression by qPCR (a). Both C33A and Fadu cells cultured with HIV-1 mixed with DOTAP showed TSLP expression (a). Protein analysis (ELISA) of C33A and Fadu cells cultured with HIV and RNA40 and RNA41 mixed with DOTAP showed TSLP production only with RNA40 (b).

Figure 3.20:

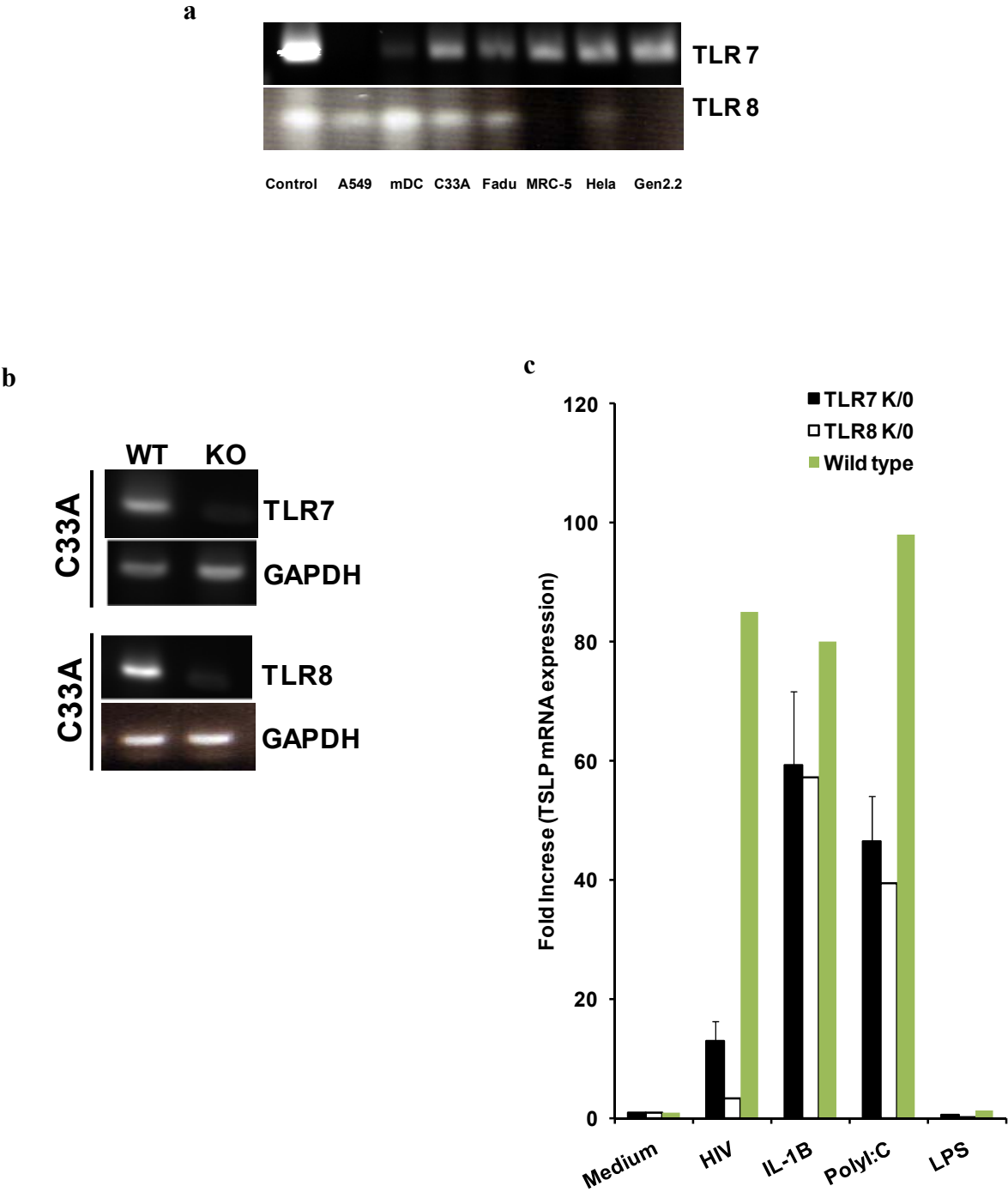


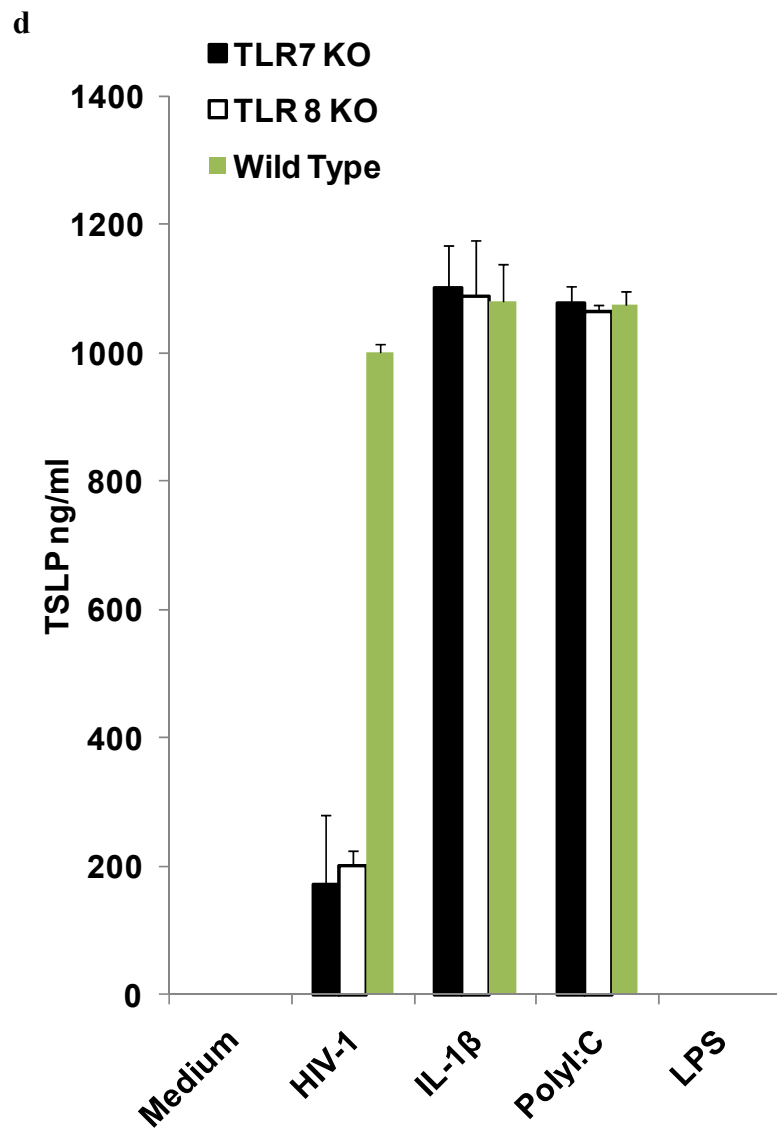
## **TLR activation as potential mechanism for HIV-induced TSLP expression**

To more directly investigate the role of TLR7/8 in signaling for HIV-induced TSLP expression in epithelial cells we first determined whether C33A and Fadu cells express TLR7 and TLR8 by RT-PCR analysis (Fig 3.21a). Both C33A and Fadu cells were found to express TLR7 and TLR8 in comparison to the TLR7 positive control pDC cell line Gen 2.2 and the TLR8 positive control mDC [87]. We employed siRNA mediated knock down of either TLR7 or TLR8 in C33A cells by transecting plasmid constructs corresponding to either the human TLR 7 or 8 genes, and after 36 hours assessed by RT-PCR the levels of TLR7 and TLR8 expression (fig. 321b). Next we exposed these transfectants, with TLR7/8 genes knocked down, to HIV along with control reagents and analyzed the cell extracts for TSLP mRNA and protein. Relative to the control reagents polyI:C and IL-1 $\beta$ , which signal TSLP expression through TLR3 and IL-1b receptor, respectively and the wild type C33A cells, the C33A transfectants with TLR7 or TLR8 knocked down showed significantly reduced of TSLP mRNA as measured by quantitative PCR (Fig. 3.21c) and TSLP protein measured by ELISA (Fig. 3.21d). These results strongly support the involvement of TLR7/8-mediated signaling by HIV to induce TSLP expression in the gp340-positive genital epithelial cells.

**Fig. 3.21: TLR activation is involved in HIV induced TSLP expression in the epithelial cells.** RT-PCR analysis of TLR7 or TLR8 expression in plasmacytoid dendritic cells (pDC), myeloid dendritic cells (mDC), and a variety of epithelial cells (a). RT-PCR analysis of TLR7 or TLR8 expression in wild type or knockdown C33A cells (b). TSLP mRNA analysis in wild type or TLR7 or TLR8 knockdown C33A cells (c). TSLP protein analysis in wild type or TLR7 or TLR8 knockdown C33A cells.

Figure 3.21:







## Discussion

Genital and oral mucosal tissues are the primary sites of entry and infection by HIV, but the mechanisms by which the virus crosses the naturally protective barriers of the lower female genital tract are not clear. Predicted mechanisms for at least trace amounts of virus to enter the mucosal microenvironment, based on some experimental evidence, include breaches in the epithelial layers, virus capture by the Langerhan's cells beneath the epithelium, or endocytosis of the virus through the epithelium. Nevertheless, a major portion of even the limited amount of virus that penetrated the epithelial barrier will perish in the harsh mucosal microenvironment filled with antiviral factors and low pH. In fact, studies from the nonhuman primate model showed that within the first 3-4 days of oral or vaginal exposure only a small number of infected cells can be detected in the tonsil and vaginal mucosal tissues, respectively, but the infection increases in situ as well as spreads rapidly to the distal lymphoid tissues leading to CD4 cell loss first detectable in the gut at around week 2 post-challenge [14, 19, 88]. These data suggest that the virus manipulates the tissue microenvironment to not only ensure survival and infection in situ, but also successfully spread the infection to distal compartments.

Results from this investigation provide strong support for the hypothesis that one way HIV maneuvers the hostile oro-genital mucosal microenvironment is by increasing the population of CD4<sup>+</sup> T cell targets at the site of viral entry by inducing TSLP production from the epithelial cells that can activate the surrounding DC/LC for the expansion of the CD4<sup>+</sup> T cells. Supporting evidence was obtained from in vitro studies that showed HIV exposure induces TSLP production in a variety of epithelial cell lines corresponding to different anatomical locations as well as primary keratinocytes. Also, consistent with the prevailing notion that genital epithelial cells cannot be infected by HIV we observed that infectious as well as non-infectious HIV-1 strains were equally effective to induce TSLP production.

Results from the present investigation also demonstrated that the TSLP produced by the epithelial cells is biologically functional in terms of activating myeloid DC characterized by the expression of the chemokine receptors TARC and MDC that are shown to be important for the DC to recruit inflammatory Th2 CD4<sup>+</sup> T cells to the site of inflammation [58, 80]. Furthermore, evidence was obtained that these CD4<sup>+</sup> T cells serve as additional targets for HIV infection where co-culturing with DC activated by TSLP within the HIV-exposed culture supernatants induced expansion and differentiation of inflammatory Th2 CD4<sup>+</sup> T cells as well as increased HIV infection which could be blocked in the presence of a neutralizing TSLP antibody.

In support of the results from in vitro studies showing HIV-induced TSLP production by epithelial cells, evidence was obtained from the nonhuman primate model where immunohistochemistry and RNA analyses clearly demonstrated increased production of TSLP in the vaginal, oral, and rectal tissues of monkeys exposed to SIV and SHIV, respectively. Furthermore, in situ hybridization analysis of SIV positive cells as well as the measurement of plasma viremia levels provided correlation between increased TSLP expression and viral infection within the vaginal mucosa. Finally, studies employing the human cervical explant model further corroborated the critical role of HIV-induced TSLP where RNA and confocal microscopy analyses showed increased production of TSLP in epithelial tissue after HIV exposure. It is believed that once DC are activated, they migrate to the draining lymph node where they trigger the proliferation of naïve CD4<sup>+</sup> T cells. Previous studies performed in the vaginal explant model have demonstrated that langerhans cells and CD4<sup>+</sup> T cells can interact in the vaginal epithelium providing a means for T cell expansion to occur directly at the site of infection [20].

It has been reported that antiviral CD8 T cell responses were generated within the vaginal mucosal tissues, but at a slower rate and to a much lower level compared to the peak viral loads and that this trend was even more pronounced in the gut [89]. Based on these observations, it has been suggested that the antiviral immunity, as measured by analyzing for CD8 T cell response is “too little and too late” to provide protection in terms of clearance of infection and prevention of CD4<sup>+</sup> T cell loss. However, it is not clear what changes within the mucosal microenvironment subsequent to viral exposure facilitate kinetics of virus infection over that of antiviral host immune responses. Data from the present investigation showed that the kinetics of virus-induced TSLP expression paralleled SIV infection within the vaginal tissues over the first two weeks post-infection and also coincided with an increase in the numbers of CD11c<sup>+</sup> mDC and CD4<sup>+</sup> T cells. These data, together with the results from in vitro studies support HIV-induced TSLP production by the mucosal epithelial cells as a critical event for DC-mediated expansion and HIV infection of CD4<sup>+</sup> T cells, which secrete inflammatory cytokines and avoid/delay generation of appropriate host antiviral responses necessary for protection during vaginal HIV transmission.

The significance of the present investigation lies not only in providing evidence for HIV-mediated TSLP production by the genital epithelial cells as an important means for the virus to successfully manipulate the mucosal microenvironment, but also uncovered the underlying mechanism by which HIV-1 transmits signals in the epithelial cells for TSLP expression. Multiple lines of evidence were obtained in support of HIV interaction with gp340 on human cervical epithelial cells to be important for TSLP expression, including gp340-blocking antibody as well as gp340 gene knock down to inhibit TSLP production as well as the inability of HIV to induce TSLP expression in gp340-null cell line Fadu that was corrected by gp340 gene transfection[21]. Furthermore, HIV RNA endocytosed into gp340<sup>+</sup> epithelial cells

was shown to induce TSLP expression through TLR7/8-mediated signaling and NFkB transcription factor activity. The possibility that gp340 could facilitate endocytosis of HIV was explored in studies employing transmission electron microscopy where gp340 expressing C33A cells exposed to HIV exhibited endosomes with electron dense particles resembling viral core structure while the gp340 null cell line (Fadu) did not show endosomal HIV particles. It is known that TLR7 and TLR8 recognize ssRNA viruses [86, 87] and are located within the endosome. Knockdown of TLR7 or TLR8 in the gp340+ C33A cervical epithelial cells resulted in a reduction in HIV-induced TSLP expression. Both TLR 7 and TLR 8 receptors have been shown to be upstream activators of the NF-kB signaling pathway [69] and HIV-mediated TSLP expression was abrogated when mutations were introduced into the NF-kB sequence in the human TSLP promoter or in the presence of a dominant repressor of NF-kB.

Historically, the role of TSLP has been documented in the study of allergic diseases, but this investigation showed that TSLP also plays an important role in the acute phase of HIV-1 infection by creating conducive environment for the virus in sustaining the small dose of the initial virus inoculum that crosses the mucosal barrier. This raises the possibility of targeting TSLP as a novel strategy to block mucosal HIV-1 transmission.

## CHAPTER 4

### Future Directions

Sexual transmission is the major route of human immunodeficiency virus type 1 (HIV-1) infection worldwide, most occurring in developing countries [13, 72-75]. Unless preventative measures are taken, the number of infected cases will continue to grow. While vaccination is the most effective tool in preventing the spread of HIV, no suitable vaccine candidate is currently available. Since women acquire the majority of all new HIV infections, it is imperative to prevent or substantially reduce virus transmission by developing methods that are safe, effective, economical, and more importantly can be controlled by women. One such method is the use of topical microbicides that would be able to prevent HIV-1 entry through the genital mucosa. Methods of delivery could be as a gel, cream, sponge, film, suppository, ring or diaphragm. Such treatment could be applied before sexual intercourse to prevent HIV transmission. An important aspect of the preclinical path to developing anti-HIV therapeutic/vaccine/microbicide candidates is the use of suitable animal models where safety as well as efficacy can be demonstrated. Among the strategies for HIV microbicide development currently being pursued by many groups around the globe, are inhibitors of virus replication targeting the virus-encoded reverse transcriptase and/or the protease enzymes or virus entry inhibitors targeting the binding and/or fusion of HIV-1 with the host cell membrane once it crosses the mucosal epithelium and comes in contact with CD4<sup>+</sup> target cells. The present investigation identified a novel target that could prevent the recruitment of target cells to the site of infection, thereby letting the natural protective barrier of the mucosal microenvironment curtail virus survival and viral spread to distant compartments. This study has shown the importance of TSLP produced by genital epithelial cells in response to HIV exposure and how TSLP helps HIV-1 to overcome the barriers of the mucosal microenvironment to successfully

invade its host. TSLP should therefore be considered as a target for intervention in the prevention of HIV-1 infection. It has been established that TSLP is expressed in a “steady-state” by epithelial cells [90]. Using immunohistochemical analysis in the rhesus macaque model, this study demonstrated that the over expression of TSLP after viral exposure leads to inflammatory responses resulting in increased CD4<sup>+</sup> T cell targets available for HIV to not only ensure its survival in the local hostile environment but also aid in its spread to distal compartments. Therefore any intervention strategy should be aimed at the prevention of the over expression of TSLP in response to HIV exposure. Since HIV induced TSLP expression signals through NF $\kappa$ B, it would seem likely to target NF $\kappa$ B to inhibit HIV-induced signaling for TSLP expression but NF $\kappa$ B can be activated by exposure of cells to a variety of outside stimulants such as lipopolysaccharide (LPS) via toll like receptor (TLR) activation, or inflammatory cytokines such as TNF or Interleukin-1 (IL-1), growth factors, lymphokines, oxidant-free radicals, inhaled particles, viral infection, including HIV-1, B or T-Cell activation, and by other non-specific stimuli [91, 92]. Since NF $\kappa$ B activation includes such a broad range of stimulants, a more specific target would be the nuclear receptors retinoid x receptors (RXR)  $\alpha$  and  $\beta$  since their binding sites have been identified within the human TSLP gene promoter. In this regard, it has recently been reported that selective ablation of retinoid X receptors alpha and beta (RXR $\alpha$  and RXR $\beta$ , respectively) in mouse epidermal keratinocytes results in the development of chronic dermatitis similar to that observed in human atopic dermatitis (AD) patients, and this is associated with significant increases in the TSLP expression levels [70]. This suggests that these nuclear hormone receptors function as negative regulators of TSLP raising the interesting prospect of using RXR agonists as a unique strategy for targeting HIV-induced TSLP production. All-trans retinoic acid (ATRA) serves as the major ligand for the retinoid X receptors (RXRs), in particular the isomer 9-cis-retinoic acid (9-cis-RA) has been

shown to be a high affinity ligand for this receptor [93, 94]. Therefore, 9-cis-RA treatment of mucosal vaginal tissues could be a potential strategy against HIV-induced TSLP production. Preliminary data revealed strong inhibitory effects of 9-cis-RA on HIV induced TSLP expression (fig. 4.1).

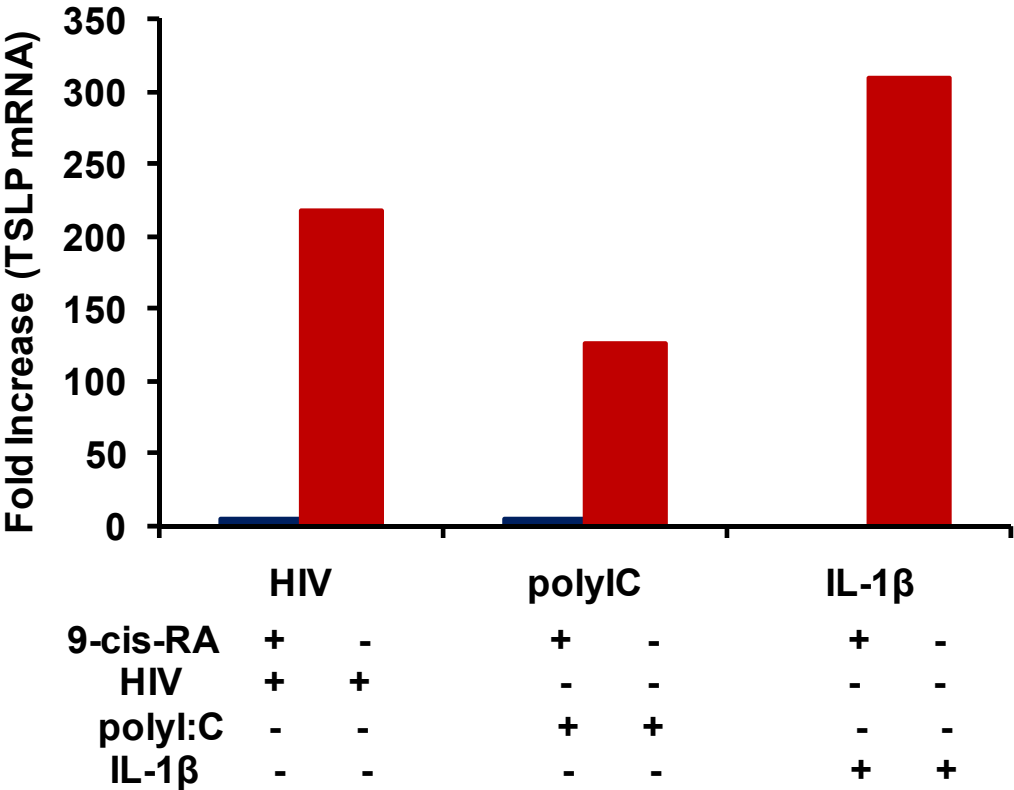
Another potential therapeutic approach specifically targeting TSLP induction by HIV in genital epithelial cells is the designing of microbicides to block the epithelial cell receptor gp340 necessary for HIV-induced TSLP expression. A potential candidate for this method would be the gp340 antibody. Expression of gp340 has been detected in vaginal tissues and data from this study revealed that pretreatment of epithelial cells with  $\alpha$ gp30 prior to HIV exposure resulted in a reduction in HIV induced TSLP expression (Fig. 3.16a). Vaginal delivery of this antibody could block viral binding thereby preventing an exacerbation of TSLP expression-mediated recruitment and infection of CD4<sup>+</sup> T cells. Continued use of the antibody could also potentially reduce female to male transmission of HIV-1 since  $\alpha$ gp340 could bind to a variety of HIV-1 strains found in vaginal secretions. Since this antibody binds to a well conserved portion of the viral envelope, the possibility for the development of resistant strains of HIV-1 is diminished.

Studies of the effectiveness of 9-cis-RA and  $\alpha$ gp340 in both the cervical explant and rhesus macaque models could provide a framework for the clinical development of suitable inhibitors of HIV-induced TSLP expression.

**Fig. 4.1: 9-cis-RA inhibition of TSLP expression.** Cervical epithelial cells (C33A) were incubated in the presence or absence of 9-cisRA with either HIV-<sub>1III</sub>B, polyI:C, or IL-1 $\beta$  for 12-18 hr. TSLP expression, measured in terms of mRNA analyses by RT-PCR, was completely abrogated in the presence of 9-cisRA.



Figure 4.1:



## CHAPTER 5

### Materials and Methods

**Cell Culture and Production of Virus Stock:** Epithelial cell lines representing cervical (C33A), neuronal (SKMN), and intestinal (HT29) origin were obtained from ATCC and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and the appropriate antibiotics. Rhesus macaque epithelial cells (4mBr-5) were obtained from ATCC and maintained in Hams F12 medium (F12K) supplemented with 2mM L-glutamine, 1.5gm/liter sodium bicarbonate, 30ng/mouse epidermal growth factor and 10% fetal bovine serum. Pharyngeal epithelial cells (Fadu) were obtained from ATCC and maintained in EMEM supplemented with 10% fetal bovine serum and the appropriate antibiotics. Adult normal human epidermal keratinocytes (NHEK) were obtained from Lonza Biosciences (Walkersville, MD) and maintained in KGM-2 media. The Ghost X4 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and the appropriate antibiotics. Chronically HIV-infected H9 cells (H9/HIV-1<sub>IIIB</sub>) were maintained in RPMI-1640 medium supplemented with 10% FBS. Primary human peripheral blood mononuclear cells (PBMC) were isolated by the standard Ficoll-Hypaque density gradient separation method from blood samples purchased from Gulf Coast Blood Center (Houston, Texas). Gen 2.2 (pDC cell line) and MDC were gifts from Dr. Yong-Jun Liu. A plasmid encoding the HIV-1 proviral DNA with a deletion in the envelope region, pMenv<sup>(-)</sup>, and another plasmid encoding HIV-1 env sequence representing clade D virus 93ZR001.3 and the plasmid encoding the full-length genome of the R5 virus YU2, were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The plasmid MD.6 encoding the VSV envelope glycoprotein was obtained from Dr. Inder Verma, Salk Institute, San Diego, CA. The

AT-2 treated and untreated HIV-1 strains ADA and MN were obtained from Dr. Jeff Lifson, Laboratory of Retroviral Pathogenesis, AIDS Vaccine Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD.

### **Production of virus stock**

Infectious virus stock was prepared from each of the different molecular clones of HIV-1 strains used in the study by transfecting 293T cells with 10 µg of plasmid DNA using the calcium phosphate method. Briefly, cells were grown to 70-90% confluency in 100mm tissue culture plates. For preparing the envelope-pseudotyped viruses, plasmid DNA encoding the env-defective HIV-1 (pMenv-) and the envelope sequences from the VSV or the HIV strain 93ZR001.3 were co-transfected into 293T cells. The infectious HIV-1<sub>IIIB</sub> was collected from the supernatants of chronically infected H9/HIV<sub>IIIB</sub> cells. The culture supernatants containing infectious virus were collected 48 hours after transfection of the 293 T cells or culturing of the chronically infected H9/HIV-1<sub>IIIB</sub>, quantitated by determining the reverse transcriptase activity or the Multinucleate Activation of Galactosidase Indicator Cells (MAGI) assay and either used immediately or stored at -80 °C.

**Induction of TSLP expression in epithelial cells:** Various epithelial cells were seeded at a density of  $1.2 \times 10^6$  cells per well in 12-well plates. In cases where ELISA analysis was performed, cells were seeded at a density of  $5.0 \times 10^6$ . Cells were incubated overnight with HIV-1<sub>IIIB</sub>, HIV-1<sub>YU2</sub>, HIV-1<sub>93ZR001.3</sub>, 10µg/ml LPS (Invivogen, San Diego, CA), 5 µg/ml PolyI:C (Invivogen, San Diego, CA), 1 ng/ml IL-1β or 10 ng/ml TNFα (R&D systems, Minneapolis, MN), anti-TNFR (Santa Cruz Biotechnology, Santa Cruz, CA), rgp120 (Immunodiagnostics, Woburn, Ma), CL075 or CL097 (Invivogen), 10 µg/ml RNA40 (ORN derived from HIV-1 U5 region nt 108–127, GCCCGUCUGUUGUGUGACUC); 10 µg/ml

RNA41 as a negative control (U to A replacement, in RNA40) (Dharmacon Inc.) formulated with 12.5 µg/ml DOTAP Liposomal Transfection Reagent (Roche Diagnostics Corp.).

**Cross linking of gp340 on epithelial cells for determining the effect on TSLP expression:**

Specific wells within the 96-well tissue culture plates were coated with either TNF-R antibody, its isotype control antibody (Santa Cruz Biotechnology, Santa Cruz, CA), HIV-1<sub>IIIB</sub> or recombinant gp120 (Immunodiagnosics, Woburn, MA). The plates were sealed with parafilm and placed at 4°C overnight. The following day, the plates were washed and blocked with 200µl of DMEM supplemented with 10% FBS for 30 min at 37°C. C33A cells were seeded at a density of  $2 \times 10^4$  per well. The plates were incubated for 12-18 hr at 37°C and cells lysed the following day for RNA analysis.

**Determination of HIV Endocytosis into epithelial cells.** The gp340+ C33A cervical epithelial cells as well as the gp340- Fadu epithelial cells were incubated with HIV-1<sub>IIIB</sub> or the TLR7/8 ligands CL075 and CL097 (Invivogen, San Diego, CA) in the presence or absence of various reagents that are known to inhibit endocytosis and endosomal maturation/acidification: DMA (50 µM); CCD (10 µM); chlorpromazine (6.25 µg/ml); chloroquine (5 µM); quinacrine (5 µM); NH<sub>4</sub>Cl (1 mM); and BFLA-1 (50 nM), all from Sigma-Aldrich (St. Louis, MO). Following incubation, the cell extracts were prepared and analyzed for TSLP expression by real-time RT-PCR analyses.

**Isolation and culturing of myeloid DC:** Buffy coats of blood samples from healthy adult donors were purchased from Gulf Coast Blood Center (Houston, TX) and the peripheral blood mononuclear cells (PBMC) were separated by the standard ficoll-centrifugation methodology to isolate the CD11c<sup>+</sup> myeloid DC as previously described[80]. In brief, the DC-enriched population (lineage<sup>-</sup>cells) was obtained from PBMC by negative selection using a mixture of

monoclonal antibodies (mAbs) against the lineage markers, CD3 (OKT3), CD14 (M5E2), CD16 (HB78), CD20 (L27), CD56 (B159), and CD235a (10F7MN) (BD Biosciences), followed by the use of goat anti-mouse IgG-coated magnetic beads (M-450; Dynal and Miltenyi Biotec). The CD11c<sup>+</sup> and lineage<sup>-</sup> DC and CD4<sup>+</sup> T cells were separately isolated by a FACS Aria sorter (BD Biosciences) using APC-labeled anti-CD11c (B-ly6), a mixture of FITC-labeled mAbs against lineage markers including CD3 (SK7), CD14 (MØP9), CD16 (3G8), CD19 (HIB19) and CD56 (NCAM16.2) (BD Biosciences); and Pacific Blue-labeled CD4 (OKT4; eBioscience) to reach >99% purity. The CD11c<sup>+</sup> DC were cultured in RPMI containing 10% FBS by seeding at a density of 1-3×10<sup>5</sup> cells/well in 200 µl volume in a flat-bottom 96-well plate in the presence of culture medium alone, 100 ng/ml rTSLP, prepared in-house using an adenovirus vector system as described previously[64], or supernatants collected from epithelial cells previously cultured without or with HIV (at an MOI of 0.5 or 600 TCID<sub>50</sub> based on MAGI assay and equivalent to 60,705 cpm of RT activity) and designated as HIV<sup>-</sup>Sn and HIV<sup>+</sup>Sn, respectively. The HIV<sup>+</sup>Sn contained 100 ng/ml of TSLP protein, as determined by ELISA. For determining TSLP-specificity of DC activation, a mixture of two neutralizing antibodies (including one TSLP monoclonal antibody and one rabbit anti-human TSLP polyclonal antibody) were added to mDC for 30 minutes prior to culturing the cells with culture medium, rTSLP, HIV<sup>+</sup>Sn, or HIV<sup>-</sup>Sn to block the TSLP activity. The neutralizing monoclonal anti-TSLP antibody IgG1 (R&D Systems) and the polyclonal rabbit anti-TSLP IgG were used at concentrations 10 and 20 µg/ml, respectively along with the corresponding isotype control monoclonal and polyclonal antibodies at matching concentrations.

**DC activation and viability:** The CD11c<sup>+</sup> myeloid DC cultured with medium, rTSLP, HIV<sup>-</sup>Sn or HIV<sup>+</sup>Sn for 24 h were collected and resuspended in EDTA-containing medium. Viability of the DC was determined by the standard Trypan blue dye-exclusion method. To determine the

activation status, the DC were stained with PE-conjugated mouse anti-human mAbs to CD40, CD80 and CD86 and an IgG1 isotype control (all from BD Biosciences), and analyzed on an LSR II flow cytometer (BD Biosciences) using flowjo software (version 8.3.4, Tree Star). The dead cells were excluded from the analyses by using the violet Live/Dead stain kit (Invitrogen). The DC culture supernatants were collected at 24 h, frozen at -80 °C and analyzed within 3 months with protein ELISA kits for TARC and MDC (R&D Systems, Minneapolis, MN). In some experiments, RNA samples prepared from the cultured mDC were used for gene expression analyses.

**Isolation of naïve CD4<sup>+</sup> T cells:** The naïve and central memory CD4<sup>+</sup> T cells were isolated from PBMC on the basis of CD45RA<sup>+</sup>, CD45RO<sup>-</sup>, and lineage<sup>-</sup> phenotype by using a human CD4<sup>+</sup> T cell enrichment cocktail (Stem Cell Technologies) followed by the use of a biotin labeled CD45RA, an APC-labeled anti-biotin antibody, a mixture of FITC-labeled mAbs against the lineage markers CD14(MØP9), CD16 (3G8), CD19 (HIB19), CD56 (NCAM16.2), CD11C (HL3), TCRγδ (V65), PE-labeled mAbs against CD45RO (UCHL1), (all from BD Biosciences), FITC-labeled BDCA2 (AC144), PE-labeled BDCA4 (AD5-17F6), and PE-labeled CD25(4E3) (all from Milteny Biotech) and pacific blue labeled CD4 (OKT4) (eBioscience) in a FACS Aria sorter (BD Biosciences). The final CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD45RA<sup>-</sup>CD45RO<sup>+</sup>CD25<sup>-</sup> lineage<sup>-</sup> fraction isolated was determined to be > 99% pure.

**mDC-CD4<sup>+</sup> T cell co-cultures:** Activated mDC were co-cultured with 2.5 to 5.0×10<sup>4</sup> purified autologous or allogeneic naïve CD4<sup>+</sup> T cells (DC:T cell ratio, 1:1 or 1:2) in round bottomed 96-well culture plates for 7 days. In some experiments HIV-1<sub>IIB</sub> or HIV-1<sub>YU2</sub> were added to the co-cultures overnight, and the cells were washed three times to remove any free viral particles. The co-cultures were resuspended in RPMI medium containing 10% FBS. The mixture was

then incubated for 5 minutes in 37°C in 10% FBS-RPMI 1640 and the cells were pelleted by centrifugation. The cells were subsequently washed three times with 10% FBS-RPMI 1640. On days 4 and 7, the viable cell counts were determined by the standard Trypan blue dye-exclusion method. In some cases the co-cultures were pulsed for 16-18 h with 1  $\mu$ Ci [<sup>3</sup>H]thymidine on day 6 and then the cells were collected to assess for proliferation in terms of [<sup>3</sup>H]thymidine incorporation. In the co-cultures where HIV is added, the relative levels of infection were determined by assaying for reverse transcriptase (RT) activity in the culture supernatants.

**Animals:** Vaginal tissue samples were obtained from rhesus macaques (*Macaca mulatta*) infected with SIV<sub>mac251</sub> by the vaginal route in a previous study at the California Primate Center [14]. The animal facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and the study was conducted according to National Institute of Health Guidelines on Care and Use of Laboratory Animals. Oral and rectal samples were obtained from rhesus macaques infected with SHIV89.6P and SIV<sub>mac251</sub>, by the respective routes from animals housed at the Michael Keeling Center for Comparative Medicine and Research of The University of Texas MD Anderson Cancer Center, Bastrop TX. All of the experiments were conducted according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, and the principles of the NIH Guide for the Care and Use of Laboratory Animals, and the policies and procedures of the University of Texas MD Anderson Cancer Center.

**Tissue collection in the macaque studies and RNA isolation:** Collection of vaginal tissues from animals euthanized at different days post-infection with SIV<sub>mac251</sub> was described earlier [14]. The tonsil and rectal biopsy samples were obtained by standard fenestrated cup (1.8mmx190 cm) endoscope from animals infected by the oral and rectal routes with

SHIV89.6P and SIV<sub>mac251</sub>, respectively. All the tissue samples from the macaques for RNA analyses were collected and stored in RNAlater at -20°C. At the time of the assay, the tissue samples were homogenized, and the total RNA was isolated by using the RNeasy Qiagen kit (Qiagen, Valencia, CA). The purified RNA was used to determine tissue TSLP RNA levels by real-time quantitative RT-PCR using the rhesus macaque-specific TSLP primers: 5'-CGACAGCATGGTTCTTCTCA-3' and 5'-CGATTTGCTCGAACTTAGCC-3' and the rhesus macaque specific MDC and TARC primers (Applied Biosystems, Foster City, CA).

**In situ hybridization for detection of SIV RNA-positive cells:** The in situ hybridization analysis (ISH) was performed by our collaborators from the California Primate Center, Davis, CA, on the vaginal tissue sections using <sup>35</sup>S-labeled SIV riboprobes as described [95], with modifications. Radioactive probes had a specific activity of > 3.5 x 10<sup>8</sup> cpm/ug as determined by the in-vitro transcription labeling of the SIV gag and env genes. The hybridization solution contained radiolabeled SIV probes at a total concentration of 1.5 x 10<sup>6</sup> cpm/100 ul. The riboprobe cocktail in the hybridization buffer was layered over each tissue section. The slides were coated with LM-1 autoradiographic emulsion (Amersham) and allowed to develop at 4° C for 14 days. Controls for ISH included: 1) matched tissues from SIV-uninfected rhesus monkeys; 2) tissues from SIV-infected rhesus monkeys with high virus loads (positive control); 3) serial tissue sections hybridized with SIV sense riboprobes and; 4) omission of the probe in the hybridization mixture.

**SIV RNA measurement:** Tissue RNA samples were analyzed by our collaborators from the California Primate Center, Davis, CA, for viral RNA (vRNA) using a quantitative branched DNA (bDNA) assay and reported as viral RNA copy numbers per ug of total tissue RNA [96]. The detection limit of this assay is 125 copies of vRNA. To evaluate the specificity of the tissue assay, samples were collected from 3 animals that had not been exposed to SIV [97].



Excluding one spurious result, average values for the bDNA assay were 113 copies/ug tissues RNA in the uninfected animals. The cut-off for the assay was 200 copies/ug tissue RNA average + 2 standard deviation (SD) values. Tissue sample with less than 200 copies of vRNA/ug total tissue RNA were reported as negative.

**Immunohistochemistry:** The vaginal tissue sections were analyzed for the expression of TSLP as well as presence of CD11c<sup>+</sup> DC and CD4<sup>+</sup> T cells by the personnel in our departmental immunohistochemistry core facility. The tissue slides were incubated with either rat anti-human TSLP (mAb 12F3, DNAX, Palo Alto, CA), mouse anti-human CD4 (mAb M-T477, BD PharMingen) or mouse anti-human CD11c (mAb AHS1153, Biosource) at room temperature for 1 h in PBS. The slides were washed with PBS twice and incubated with biotinylated secondary antibody for 30 min (PK-4004, Vector Laboratories, Burlingame, CA) before washing and treatment with avidin-peroxidase complex reagents for 30 min (PK-4004, Vector Laboratories). Subsequently, the slides were washed and incubated with the substrate SK-4200, which stained red or SK-4100, which stained brown (Vector, Laboratories).

**Real-time quantitative RT-PCR:** Epithelial cells exposed to different HIV-1 strains or mDC subjected to various treatments were lysed and mRNA was extracted with an RNeasy kit (Qiagen, Valencia, CA). Reverse transcription was done with SuperScript II (Invitrogen) and the cDNA samples were analyzed by real-time quantitative PCR assay with an ABI Prism 7500 Sequence Detection system (Applied Biosystems). Reactions were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and subjected to 40 two-step amplification cycles with annealing-extension for 60°C for 1 min followed by denaturation at 95°C for 15s. For the analysis of human TSLP, human TNF $\alpha$ , human GAPDH, human CXCR4, and human CCR5 real time PCR probes were purchased directly from the manufacturer (SuperArray). For the

analysis of human TARC and MDC the primer sequences were as follows: TARC: 5'-CATGGCCCCACTGAAGATG-3' and 5'-CCTGGAGCAGTCCTCAGATGTC-3' and; MDC: 5'-GCATGGCTCGCCTACAGACT-3' and 5'-CAGACGGTAACGGACGTAATCA-3'. For the analysis of human IL-4, IL-5, IL-10, IL-13, TNF- $\alpha$  and INF- $\gamma$ , the primer sequences were as follows: IL-4: 5'-CACCGAGTTGACCGTAACAG-3' and 5'-GCCCTGCAGAAGGTTTCC-3'; IL-5: 5'-AGCTGCCTACGTGTATGCCA-3' and 5'-GCAGTGCCAAGGTCTCTTTCA-3'; IL-10: 5'-GAGATCTCCGAGATGCCTTCA-3' and 5'-CAAGGACTCCTTTAACAACAAGTTGT-3'; IL-13: 5'-ACAGCCCTCAGGGAGCTCAT-3' and 5'-TCAGGTTGATGCTCCATACCAT-3' and INF- $\gamma$ : 5'-GTTTTGGGTTCTCTTGGCTGTTA-3' and 5'-AAAAGAGTTCCATTATCCGCTACATC-3'. Fold increase in the expression of various genes was calculated by normalizing to medium control.

**Ex vivo vaginal tissue explant model for HIV-induced TSLP expression:** These studies were done in collaboration with colleagues from the Fred Hutchinson's Cancer Center (FHCC), Seattle, WA where the tissues were collected and exposed to HIV for various times before shipping the tissue and culture supernatant samples to Houston, TX. Study details at our colleagues from Seattle include the following: Tissues were collected from patients in operating room and placed into specimen containers holding cold RPMI or PBS and transported to lab on ice within 1 hour [29] following institutional guidelines for collecting and handling the human samples at FHCC, Seattle, WA. The tissues were then processed by removing the excess stroma by gross dissection followed by washing in PBS to remove excess blood from surface. Forceps were used to pull any residual clots off. Tissues were cut into strips (5mm x 10mm) and placed in PBS. Tissues were washed 3 times with 50 ml PBS by filling 50 ml conical tube with PBS and inverting tube several times. Tissue pieces were allowed to settle before pouring

PBS off carefully. PBS + 5mM EDTA was added to tissue strips. Tissues were incubated over night at 4°C with shaking. The next day the epithelium was peeled carefully from the stroma using a dissecting microscope. The sheets were washed in HBSS + 5mM CaCl<sub>2</sub> and incubated in Hank's Buffered Saline Solution (HBSS) + 5mM CaCl<sub>2</sub> for 30 min on ice. The sheets were then placed in 6 well plates with the final volume/well equaling 3ml. The sheets were spinoculated with virus (p24 Dosage ~0.05ug/ml), at 2300 RPM for 2 hours at 37°C. Post spinoculation the sheets were extensively washed in PBS (~10 times). Fresh RPMI supplemented with 10% fetal bovine serum (FBS) and the appropriate antibiotics was added and the sheets were carefully placed in CO<sub>2</sub> incubators at 37°C for up to 4 days. For confocal microscopy virus-challenged epithelial sheets were incubated in PBS containing 1% bovine serum albumin and 0.05% sodium azide for 1 hr at RT antibodies to CD1a (NA1/34; Dako, Carpinteria, CA) or TSLP (MD Anderson core Facility). Sheets were washed in PBS containing 1% bovine serum albumin and 0.05% sodium azide and incubated for 30 min with the appropriate secondary antibodies, washed again, and fixed overnight in 4% paraformaldehyde. Nuclei were counterstained with TOPRO3 (Molecular Probes), and the sheets were embedded in Mowiol 40-88 containing 2.5% w/v DABCO (Aldrich, Milwaukee, WI). Cellular staining was visualized with a Leica TCS SP spectral confocal microscope.

**HIV Infection Assay:** Virus production in HIV-infected cells was analyzed by determining the reverse transcriptase (RT) activity as reported earlier [98]. Viral particles from the infected culture supernatants were precipitated with 30% polyethylene glycol (50% volume/volume) and pelleted by centrifugation at 3000 rpm for 30 min at 4°C. The viral pellets were resuspended in 100 µl of RT buffer (glycerol, 25mM Tris-HCl, pH 7.5, 5mM dithiothreitol, 150mM KCl and 0.025% Triton X-100) followed by lysing with the addition of 100µl of 0.9%

Triton X-100 in 1.5 M KCl. A 10 $\mu$ l aliquot of this preparation was mixed with 90 $\mu$ l of the RT cocktail (50 mM Tris-HCl, pH 7.8, 63 mM KCl, 4.2mM MgCl<sub>2</sub>, 0.08% Nonidet P-40, 0.85mM EDTA, 4.2  $\mu$ g/ml poly(A), and 0.13  $\mu$ g/ml of oligo(dT). 8  $\mu$ l of 0.5 M DTT and 10  $\mu$ l of [Methyl-<sup>3</sup>H] (ICN Pharmaceuticals Inc., Irvine, California, U.S.A.) and incubated at 37°C for 1.5 hr. The reaction mix was precipitated with 10% trichloroacetic acid (TCA) and 1% sodium pyrophosphate. The TCA precipitable raw counts per minute (cpm) were determined on a Wallace 1409 liquid beta-scentillation counter (Wallace, Gaththersburg, MD). The mean radioactivity for the duplicate infections was determined and the mean background cpm values from duplicate samples of uninfected control cultures run in parallel in each assay were subtracted to calculate the specific RT activity in the infected cell cultures.

**ELISA.** Concentrations of the TSLP, MDC or TARC protein in cell-free supernatants and cell lysates were measured using respective specific ELISA kits (R&D Systems). The minimal detection limit for TSLP is set to 31.25 pg/ml, MDC is set to 62.5 pg/ml, and TARC is set to 7 pg/ml.

**Western Blot Analysis HIV-induced TSLP production:** Aliquots of 2 x 10<sup>7</sup> epithelial cells exposed to HIV were subjected to lysis in 2x Laemmli buffer. The lysates were then mixed with 4x loading buffer (4mL glycerol, 0.4g SDS, 2.5 ml 1M Tris-HCl, pH 6.8, 80  $\mu$ l bromophenol blue, H<sub>2</sub>O to 8 ml, 20%  $\beta$ Me) and resolved on a 17.5% SDS polyacrylamide gel followed by western blot analyses using rat anti-TSLP (mAb 12F3, DNAX, Palo Alto, CA) at a dilution of 1:1000 as the primary antibody and horseradish peroxidase labeled donkey anti-rat IgG H&L secondary antibody (diluted 1:10,000,) and the femto fluorometric system (Pierce Biotechnology).

**Transfection and Luciferase Assay:** Cervical epithelial cells ( $3 \times 10^5$ ) were transfected, using the calcium phosphate method, with 1 $\mu$ g of luciferase reporter plasmid driven by the TSLP promoter encoding either wild-type or mutated NF $\kappa$ B element and 15ng of control renilla luciferase reporter driven by a constitutive thymidine kinase promoter (pRL-tk-luc) (Promega). The TSLP promoter plasmids were obtained from Dr. Steven F. Ziegler at the University Of Washington School Of Medicine. The cells were cultured for 48 hours and then exposed to IL-1 $\beta$  and HIV overnight. Cells were harvested and lysed in 100ul of lysis buffer. Luciferase activity was measured using a Lumat LB9507 luminometer to determine whether HIV exposure of epithelial cells resulted in an induction of luciferase expression as robust as that previously seen with IL-1 $\beta$ . Relative Luciferase activity was calculated as a ratio of relative light units to relative Renilla Luciferase units. In each experiment, samples were analyzed in triplicate and each experiment was repeated at least three times.

**Generation of C33A cells expressing an NF- $\kappa$ B super-repressor:** An I $\kappa$ Ba mutant harboring mutations in its phosphorylation sites, serines 32 and 36 (named I $\kappa$ BaSS/AA,[99]), was cloned into the retroviral vector pMIGR1 (provided by Warren Pear, Abramson Family Cancer Research Institute). Since the I $\kappa$ BaSS/AA is resistant to inducible degradation (34), it functions as a super-repressor of NF- $\kappa$ B. To produce recombinant retroviruses, pMIGR1-I $\kappa$ BaSS/AA or pMIGR1 vector control was transiently transfected into 293 cells along with the packaging plasmid pCL-Ampho and the VSV-G plasmid, as previously described [100]. The recombinant viruses were used to infect C33A cells, which were used as bulk of cells in the experiments.

**Analyses of surface gp340 expression:** Various epithelial cell lines employed in the investigation were grown in 12 well plates and harvested with PBS containing 5mM EDTA. Fluorescence staining of the cells was performed in the presence of 4mM Ca $^{2+}$ . Mouse anti-gp340 (abcam, Cambridge, MA) in PBS (1:100 dilution), 2% FBS, 4mM Ca $^{2+}$  was added to the

cells for 30 min on ice. Next, cells were incubated with a FITC labeled goat anti-mouse secondary antibody (Abcam) (1:200 dilution) for 30 minutes at 4°C and protected from light. The cells were then washed 3x with PBS and fixed with 1.0% paraformaldehyde. Stained and fixed cells were analyzed by a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA) using Flowjo software (Ashland, OR).

**Transfection of gp340 into gp340-null Fadu cells:** Fadu cells were transfected with an expression vector containing gp340 cDNA (OriGene) using the calcium phosphate method. The expression of gp340 was confirmed by FACS analysis and RT-PCR. For the analysis of human gp340 and TLR3 expression (as internal control gene expression), the primer sequences used were as follows: TLR3: 5'-AGCCTTCAACGACTGATGCT-3', and 5'-TTTCCAGAGCCCTGCTAAGT-3', for gp340: 5'-AAATTCATCCTATGGTCTA-3', and 5'-GAGAGGGGAACTCCGGTAG-3'.

**Knock down of gp340 knock in gp340+ C33A cells:** The C33A cervical epithelial cells were seeded at  $3 \times 10^4$  cells per well in 24-well culture plates and incubated for 2 days (to reach about 40–60% confluence) before transfecting with siRNA against gp340 or non-target control RNA at 5 nM (Dharmacon) using LipofectAmine 2000 transfection reagent (Invitrogen) following the manufacturer's instructions. Subsequently, the cells were cultured for another 48 h and the level of gp340 knockdown was confirmed by FACS analysis and RT-PCR.

**Transmission Electron Microscopy for detection of HIV endocytosis:** The C33A and Fadu human epithelial cells were grown to 50-60% confluency in 12 well plates. Cells were incubated with HIV for 2 hrs at 4°C and then moved to 37°C for 30min. Samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1M cacodylate buffer, pH 7.3 for 1 hour. Further processing of the samples was performed by personnel in our

institutional electron microscopy core facility as follows: the samples were washed and treated with 0.1% Millipore-filtered cacodylate buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 30 min, and stained en bloc with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in LX-112 medium. The samples were polymerized in a 70°C oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc, Peabody, MA) at an accelerating voltage of 80kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).

**Knock down of TLR7 and TLR8 expression C33A cells:** The C33A cervical epithelial cells were seeded at  $3 \times 10^4$  cells per well in 24-well culture plates and incubated for 2 days (to reach about 40–60% confluence) before transfecting with siRNA against TLR7, TLR8 or non-target control RNA at 5 nM (Invitrogen) using DOTAP liposomal transfection reagent (Roche Diagnostics Corp) following the manufacturer's instructions. Subsequently, the cells were cultured for another 48 h and the level of TLR7 or TLR 8 knockdown were confirmed by FACS analysis and RT-PCR. For the analysis of human TSLP human TLR7 or human TLR8, RT-PCR primers were purchased directly from the manufacturer (Invivogen).

**Determination of the involvement of RXR in HIV induced TSLP Expression:** Cervical epithelial cells co-treat with 9-cis-RA in the presence of HIV, IL-1 $\beta$ , or polyI:C overnight and analyzed for TSLP expression by both mRNA analysis. 9-cis-RA was a gift from Dr. Kapil Mehta at UT MD Anderson Cancer Center (Houston, TX)

**Statistical analyses:** Using the Student *t*-test the *P* values were calculated to determine the significance of fold increase in CD4<sup>+</sup>T cells and HIV infection (RT activity).



## CHAPTER 6

### References

1. Clavel, F., D. Guetard, F. Brun-Vezinet, S. Chamaret, M. Rey, M. Santos-Ferreira, A. Laurent, C. Dauguet, C. Katlama, C. Rouzioux, D. Klatzmann, J.L. Champalimaud, and L. Montagnier, *Isolation of a new human retrovirus from West African patients with AIDS*. Science, 1986. **233**: p. 343-346.
2. Coffin, J., *Gentic variation in AIDS viruses*. Cell, 1986. **46**: p. 1-4.
3. Coffin, J., A. Haase, J. Levy, L. Montagnier, S. Oroszlan, N. Teich, H. Temin, K. Toyoshima, H. Varmus, P. Vogt, and R. Weiss , *Human Immunodeficiency Viruses*. Science, 1986. **232**: p. 697.
4. Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon. , *Nucleotide sequence of the AIDS virus, LAV*. Cell, 1985. **40**: p. 9-17.
5. Levy, J.A., *HIV and the Pathogenesis of AIDS*. ASM Press, Washington, D.C., 1994.
6. Nathanson, N., R. Admed, M. A. Brinton, F. Gonzalez-Scarano, L. T. Chow, D. E. Griffin, K. V. Holmes, F. A. Murphy, J. Overbaugh, and H. L. Robinson. , *Viral Pathogenesis and Immunity*. Lippincott Williams & Wilkins, Philadelphia., 2002.
7. Reeves, J.D., and R. W. Doms. , *Human immunodeficiency virus type 2*. J Gen Virol 2002. **83**: p. 1253-1265.
8. Doranz, B., J. Rucker, Y. Yi, R. Smyth, M. Samson, S. Peiper, M. Parmentier, R. Collman, and R. Doms. , *A dual-tropic primary HIV-1 isolate that uses CXCR4 and the b-chemokine receptor CKR-5, CKR-3, and CKR2b as fusion cofactors*. . Cell, 1996. **85**: p. 1149-1158.

9. Flint, S.J., L. W. Enquist, R. M. Krug, V. R. Racaniello, and A. M. Skalka, *Principles of Virology; Molecular Biology, Pathogenesis, and Control*. ASM Press, Washington, D. C., 2000.
10. Zhu, T., H. Mo, N. Wang, D. S. Nam, Y. Cao, R. A. Koup, and D. D. Ho. , *Genotypic and phenotypic characterization of HIV-1 patients with primary infection*. Science, 1993. **261**: p. 1179-1181.
11. Zhang, L., P. Mackenzie, A. Cleland, E. C. Holms, A. J. Brown, and P. Simmonds, *Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 1 upon primary infection*. . J. Virol, 1993. **67**: p. 3345-3356.
12. Tersmette, M., R. Gruters, F. de Wolf, R. de Goede, J. Lange, P. Schellekens, J. Goudsmit, H. Huisman, and F. Miedema, *Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: Studies on sequential HIV isolates*. . Journal of Virology 1989. **63**: p. 2118-2125.
13. World Health Organization, *HIV AIDS epidemic update*. World Health Organization, Geneva, Switzerland, 2007.
14. Miller, C.J., Q. Li, K. Abel, E. Kim, Z.Ma, S. Wietgreffe, L. La Franco-Scheuch, L. Compton, L. Duan, M. Shore, M. Zupancic, M. Busch, J. Carlis, S. Wolinsky, and A. Hasse, *Propagation and Dissemination of Infection after Vaginal Transmission of Simian Immunodeficiency Virus*. Journal of Virology, 2005. **79**(14): p. 9217-9227.
15. Li Q., L. Duan , J.D. Estes , Z.M. Ma , T. Rourke , Y. Wang , C. Reilly , J Carlis , C.J. Miller, A. T. Haase, *Peak SIV replicatiion in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells* Nature, 2005. **435**: p. 1148-1152.

16. Guadalupe, M., E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. McNeil, and S. Dandekar, *Severe CD4<sup>+</sup> T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy*. Journal of Virology, 2003. **2007**: p. 11708-11717.
17. Brenchley, J.M., D. A. Price, and D. C. Douek, *HIV disease: fallout from a mucosal catastrophe?* Nature Immunology, 2006. **7**: p. 235-239.
18. Sankaran S, E Reay, M Guadalupe, J Flamm, T Prindiville, S Dandekar, *Rapid onset of intestinal epithelial barrier dysfunction in primary human immunodeficiency virus infection is driven by an imbalance between immune response and mucosal repair and regeneration*. Journal of Virology, 2008. **82**: p. 538-45.
19. Veazy, R., A.Lackner, *HIV swiftly guts the immune system*. Nature Medicine, 2005. **11**(5): p. 469-470.
20. Hladik F, M.McElrath, *Setting the stage: host invasion by HIV*. Nat Rev Immunol., 2008. **8**: p. 447-457.
21. Stoddard, E., G. Cannon , H. Ni , K. Karikó , J. Capodici , D. Malamud , D. Weissman, *gp340 expressed on human genital epithelia binds HIV-1 envelope protein and facilitates viral transmission*. The Journal of Immunology, 2007. **179**(5): p. 3126-3132.
22. Spira, A.I., P. A. Marx, B. K. Patterson, J. Mahoney, R. A. Koup, S. M. Wolinsky, and D. D. Ho, *Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques*. . J. Exp. Med, 1996. **183**: p. 215-225.
23. Harouse, J.M., A. Gettie, T. Eshetu, R. C. H. Tan, R. Bohm, J. Blanchard, G. Baskin, and C. Cheng-Mayer, *Mucosal Transmission and Induction of Simian AIDS by CCR5-*

- specific Simian/ Human Immunodeficiency Virus SHIVSF162P3*. . J. Virol. , 2001. **75**: p. 1990-1995.
24. Hu, J., M. B. Gardner, and C. J. Miller, *Simian Immunodeficiency Virus Rapidly Penetrates the Cervicovaginal Mucosa after Intravaginal Inoculation and Infects Intraepithelial Dendritic cells*. . J. Virol, 2000. **74**: p. 6087-6095.
  25. Jameson, B., F. Baribaud, S. Pohlmann, D. Ghavimi, F. Mortari, R. W. Doms, and A. Iwasaki, *Expression of DC-SIGN by Dendritic cells of Intestinal and Genital Mucosae in Humans and Rhesus Macaques*. J. Virol, 2002. **76**: p. 1866-1875.
  26. Lederman, M.M., R. S. Veazey, R. Offord, D. E. Mosier, J. Dufour, M. Mefford, M. Piatak, Jr., J. D. Lifson, J. R. Salkowitz, B. Rodriguez, A. Blauvelt, and O. Hartley, *Prevention of Vaginal SHIV Transmission in Rhesus Macaques Through Inhibition of CCR5*. . Science, 2004. **306**: p. 485-487.
  27. Miller, C.J., M. Marthas, J. Torten, N. J. Alexander, J. P. Moore, G. F. Doncel, and A. G. Hendrickx, *Intravaginal inoculation of rhesus macaques with cell-free simian immunodeficiency virus results in persistent or transient viremia*. . J. Virol, 1994. **68**: p. 6391-6400.
  28. Miller, C.J. and R.J. Shattock, *Target cells in vaginal HIV transmission* Microbes and Infection, 2003. **5**(1): p. 59-67.
  29. Hladik F, S.P., L. Ballweber, G. Lentz, M. Fialkow, D. Eschenbach, and M.J. McElrath, *Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1*. Immunity, 2007. **26**: p. 257-70.
  30. Yahi, N., S. Baghdiguian, H. Moreau, and J. Fantini, *Galactosyl ceramide (or a closely related molecule) is the receptor for human immunodeficiency virus type 1 on human colon epithelial HT29 cells*. Journal of Virology, 1992. **66**: p. 4848-4854.

31. Clapham, P.R., A. McKnight, S. Talbot, and D. Wilkinson, *HIV entry into cells by CD4-independent mechanisms*. Perspect. Drug Discov. Des., 1996. **5**: p. 83-92.
32. Harouse, J.M., S. Bhat, S. L. Spitalnik, M. Laughlin, K. Stefano, D. H. Silberberg, and F. Gonzalez-Scarano, *Inhibition of entry of HIV-1 in neural cell lines by antibodies against galactosyl ceramide*. Science, 1991. **253**: p. 320-323.
33. Harouse, J.M., R. G. Collman, and F. Gonzalez-Scarano., *Human immunodeficiency virus type 1 infection of SK-N-MC cells: domains of gp120 involved in entry into a CD4-negative, galactosyl ceramide/3' sulfo-galactosyl ceramide-positive cell line*. Journal of Virology, 1995. **69**: p. 7383-7390.
34. Argyris, E.G., E. Acheampong, G. Nunnari, M. Mukhtar, J. K. Williams, and R. J. Pomerantz, *Human immunodeficiency virus type 1 enters primary human brain microvascular endothelial cells by a mechanism involving cell surface proteoglycans independent of lipid rafts*. Journal of Virology, 2003. **77**: p. 12140-12151.
35. Bobardt, M., A. Saphire, H. Hung, X. Yu, B. Van der Schueren, Z. Zhang, G. David, P. Gallay, *Syndecan captures, protects, and transmits HIV to T lymphocytes*. Immunity, 2003. **18**: p. 27-39.
36. Mondor, I., S. Ugolini, and Q. J. Sattentau., *Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans*. Journal of Virology, 1998. **72**: p. 3623-3634.
37. Bouhlal, B., N. Chomont, N. Haeffner-Cavaillon, M. D. Kazatchkine, L. Belec, and H. Hocini, *Opsonization of HIV-1 by semen complement enhances infection of human epithelial cells*. . J. Immunol, 2002. **169**: p. 3301-3306.
38. Davidson, J.B., and G. C. Douglas, *Modulation of intergin inhibits HIV transmission to epithelial cells ad fertilization*. Journal of Reproductive Immunology 1998. **41**: p. 10.

39. Nagashunmugam, T., H. M. Friedman, C. Davis, S. Kennedy, L. T. Goldstein, and D. Malamud, *Human submandibular saliva specifically inhibits HIV type 1*. AIDS Res Human Retroviruses 1997. **13**: p. 371-376.
40. Lore, K., M. Larsson, *The role of dendritic cells in the pathogenesis of HIV-1 infection*. APMIS, 2003. **111**: p. 776-788.
41. Wu, L., and V.N. KewalRamani, *Dendritic-cell interactions with HIV: infection and viral dissemination*. Nature Reviews Immunology, 2006. **6**: p. 859-898.
42. Miller, C.J., and R. J. Shattock, *Target cells in vaginal HIV transmission*. Microbes and Infection 2003. **5**: p. 59-67.
43. Van Der Pol, B., C. Kwok, B. Pierre-Louis, A. Rinaldi, R. A. Salata, P. L. Chen, J. van de Wijgert, F. Mmiro, R. Mugerwa, T. Chipato, and C. S. Morrison, *Trichomonas vaginalis infection and human immunodeficiency virus acquisition in African women*. J Infect Dis 197, 2008. **197**: p. 548-554.
44. Shattock RJ and J.P. Moore, *Inhibiting sexual transmission of HIV-1 infection*. Nat Rev Microbiol. , 2003. **1**: p. 25-34.
45. Mbizvo, E.M., S. E. Msuya, A. Hussan, M. Z. Chirenje, and B. Stray-Pedersen, *HIV prevalence in Zimbabwean women: 54-67% knowledge and perceived risk*. Int J STD AIDS 2003. **14**: p. 202-207.
46. Bobardt MD, C.U., Selvarajah S, Van der Schueren B, David G, Kahn B, Gallay PA., *Cell-free human immunodeficiency virus type 1 transcytosis through primary genital epithelial cells*. Journal of Virology, 2007. **81**(1): p. 395-405

47. Saidi, H., G. Magri, N. Nasreddine, M. Requena, and L. Belec, *R5- and X4- HIV-1 use differentially the endometrial epithelial cells HEC-1A to ensure their own spread: Implication for mechanisms of sexual transmission*. Virology, 2007. **358**: p. 55-68.
48. Wu Z. , S.L., W.Abrams, D. Weissman, and D. Malamud, *The N-terminal SRCR-SID domain of gp340 interacts with HIV type 1 gp120 sequences and inhibits viral infection*. Aids Research and Human Retroviruses, 2006. **22**(6): p. 508-515.
49. Wu Z., E.G., W. R. Abrams, and D. Malamud, *gp340 (SAG) binds to the V3 sequence of gp120 important for chemokine receptor interaction*. Aids Research and Human Retroviruses, 2004. **20**(6): p. 600-607.
50. Ligtenberg, A.J., E. C. Veerman, A. V. Nieuw Amerongen, and J. Mollenhauer, *Salivary agglutinin/glycoprotein-340/DMBT1: a single molecule with variable composition and with different functions in infection, inflammation and cancer*. . Biol Chem 2007. **388**: p. 1275-1289.
51. Bikker FJ, AJ Lightenberg, JE van der Wal, PA van den Keijbus, U Holmskov, EC Veerman, AV Nieuw Amerongen., *Immunohistochemical detection of salivary agglutinin/gp-340 in human parotid, submandibular, and labial salivary glands*. J Dent Res. , 2002. **81**: p. 134-9.
52. Wu Z., D. VanRyke., C. Davis, W. R. Abrams, I.Chaiken, J. Magnani, and D. Malamud, *Salivary Agglutinin Inhibits HIV Type 1 Infectivity through Interaction with Viral Glycoprotein 120*. Aids Research and Human Retroviruses, 2003. **19**(3): p. 201-209.
53. Nagashunmugam, T., D. Malamud, C. Davis, W. R. Abrams, and H. M. Friedman, *Human submandibular saliva inhibits human immunodeficiency virus type 1 infection*

- by displacing envelope glycoprotein gp120 from the virus.* . J. Infect Dis. , 1998. **178**: p. 1635-1641.
54. Malamud D, and H. Freidman, *HIV in the oral cavity: virus, viral inhibitory activity, and antiviral antibodies: a review.* Crit Rev Oral Biol Med. , 1993. **4**: p. 461-6.
  55. Malamud D, C. Davis, P. Berthold, E. Roth, and H.Friedman, *Human submandibular saliva aggregates HIV.* AIDS Res Hum Retroviruses, 1993. **9**: p. 633-7.
  56. Allakhverdi Z, M.Comeau, H.K. Jessup, B.R.,Yoon A Brewer, S. Chartier, N. Paquette, S.F. Ziegler, M. Sarfati, and G. Delespesse., *Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells.* Journal of Experimental Medicine, 2007. **204**(2): p. 253-258.
  57. Levin, S.D., R. M. Koelling, S. L. Friend, D. E. Isaksen, S. F. Ziegler, R. M. Perlmutter, and A. G. Farr, *Thymic stromal lymphopoietin: a cytokine that promotes the development of IgM<sup>+</sup> B cells in vitro and signals via a novel mechanism.* . J Immunol 1999. **162**: p. 677-83.
  58. Liu, Y.J., *Thymic stomal lymphopoietin: master switch for allergic inflammation.* The Journal of Experimental Medicine, 2006. **203**(2): p. 269-273.
  59. LiuYJ,, *TSLP in epithelial cell and dendritic cell cross talk.* Adv Immunol, 2009(101): p. 1-25.
  60. Sims, J.E., D. E. Williams, P. J. Morrissey, K. Garka, D. Foxworthe, V. Price, S. L. Friend, A. Farr, M. A. Bedell, N. A. Jenkins, N. G. Copeland, K. Grabstein, and R. J. Paxton, *Molecular cloning and biological characterization of a novel murine lymphoid growth factor.* . J Exp Med, 2000. **192**: p. 671-80.



61. Ray, R.J., C. Furlonger, D. E. Williams, and C. J. Paige, *Characterization of thymic stromal-derived lymphopoietin (TSLP) in murine B cell development in vitro.* . Eur J Immunol 1996. **26**: p. 10-6.
62. Quentmeier H, D.H., Fleckenstein D, Zaborski M, Armstrong A, Sims JE, Lyman SD, *Cloning of human thymic stromal lymphopoietin (TSLP) and signaling mechanisms leading to proliferation.* . Leukemia., 2001. **15**: p. 1286-92.
63. Reche, P.A., V. Soumelis, D. M. Gorman, T. Clifford, M. Liu, M. Travis, S. M. Zurawski, J. Johnston, Y. J. Liu, H. Spits, R. de Waal Malefyt, R. A. Kastelein, and J. F. Bazan, *Human thymic stromal lymphopoietin preferentially stimulates myeloid cells.* . J Immunol 2001. **167**: p. 336-43.
64. Soumelis V., P.A.R., H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S. Ho, S. Antonenko, A. Lauerma, K. Smith, D. Gorman, S. Zurawski, J. Abrams, S. Menon, T. McClanahan, R. Waal-Malefyt, F. Bazan, R. A. Kastelein, and Y.J. Liu, *Human epithelial cells trigger dendritic cell-mediated allergic inflammation by producing TSLP.* Nature Immunology, 2002. **3**(7): p. 673-680.
65. Wang YH, and Y.Liu, *OX40-OX40L interactions: a promising therapeutic target for allergic diseases?* J Clin Invest. , 2007. **117**: p. 3655-7.
66. Kato, A., S. Favoreto, P.C. Avila, and R. P. Schleimer, *TLR3- and Th2 Cytokine-Dependent Production of Thymic Stromal Lymphopoietin in Human Airway Epithelial Cells.* The Journal of Immunology, 2007. **179**: p. 1080-1087.
67. Ying, S. B. O'Connor , J. Ratoff , Q. Meng , K. Mallett , D. Cousins , D. Robinson , G. Zhang , J. Zhao , T.H. Lee , C. Corrigan, *Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity.* J. Immunol, 2005. **174**: p. 8181-8190.

68. Ito, T., Y. Wang, O. Duramad, T. Hori, G. J. Delespesse, N. Wantanabe, F. Qin, Z. Yao, W. Cao, and Y.J. Liu, *TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand*. The Journal of Experimental Medicine, 2005. **202**(9): p. 1213-1223.
69. Lee, H. C. and S. F. Ziegler, *Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NFkB*. PNAS, 2007. **104**: p. 914-919.
70. Mei Li, N.M., M. Teletin, J. Pasquali, D. Metzger, and P. Chambon, *Retinoid X receptor ablation in adult mouse keratinocytes generates an atopic dermatitis triggered by thymic stromal lymphopoietin*. . PNAS, 2005. **102**: p. 14795-14800.
71. Lee HC, M. Headley, M. Iseki, K. Ikuta, S.F. Ziegler, *Cutting edge: Inhibition of NF-kappaB-mediated TSLP expression by retinoid X receptor*. J. Immunol, 2006. **176**: p. 5161-5171.
72. Pope, M., and A. T. Haase, *Transmission, acute HIV-1 infection and the quest for strategies to prevent infection*. Nat. Med., 2003. **9**(847-852).
73. Galvin, S.R., and M. S. Cohen, *The role of sexually transmitted diseases in HIV transmission*. Nat. Rev. Microbiol., 2004. **2**: p. 33-42.
74. Quinn, T.C., M. J. Wawer, N. Sewankambo, D. Serwadda, C. Li, F. Wabwire-Mangen, M. O. Meehan, T. Lutalo, R. H. Gray, and the Rakai Project Study Group, *Viral load and heterosexual transmission of human immunodeficiency virus type 1*. N. Engl. J. Med., 2000. **342**: p. 921-929.
75. Røttingen, J.A., D. W. Cameron, and G. P. Garnett., *A systematic review of the epidemiologic interactions between classic sexually transmitted diseases and HIV: how much really is known?* . Sex. Transm. Dis., 2001. **28**: p. 579-597.

76. S. F. Ziegler and Y. J. Liu, *Thymic stromal lymphopoietin in normal and pathogenic T cell development and function*. Nature Immunology, 2006. **7**(7): p. 709-714.
77. Ebner S., V. A. Nguyen, M. Forstner, Y. H. Wang, D. Wolfram, Y. J. Liu, N. Romani, *Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen-presenting cells that induce proallergic T cells*. J Allergy Clin Immunol., 2007. **119**: p. 982-990.
78. Frank I., M. Piatak, Jr., H. Stoessel, N. Romani, D. Bonnyay, J. D. Lifson, and M. Pope, *Infectious and whole inactivated Simian Immunodeficiency viruses interact similarly with primate dendritic cells (DCs): Differential intracellular fate of virions in mature and immature DCs*. Journal of Virology, 2002. **76**(6): p. 2936-2951.
79. Rossio, J. L., M. T. Esser, K. Suryanarayana, D. K. Schneider, J. W. Bess, Jr., G. M. Vasquez, T. A. Wiltout, E. Chertova, M. K. Grimes, Q. Sattentau, L. O. Arthur, L. E. Henderson, and J. D. Lifson, *Inactivation of Human Immunodeficiency Virus Type 1 Infectivity with Preservation of Conformational and Functional Integrity of the Virion Surface Proteins*. Journal of Virology, 1998. **72**(10): p. 7992-8001.
80. Watanabe N., S. Hanabuchi, Vassili Soumelis, Wei Yuan, Stephen Ho, Rene de Waal Malefyt, and Yong-Jun Liu, *Human thymic stromal lymphopoietin promotes dendritic cell-mediated CD4<sup>+</sup> T cell homeostatic expansion*. Nature Immunology, 2004. **5**(4): p. 426-434.
81. Schnittman S. M., H. C. Lane, J. Greenhouse, J. S. Justement, M. Baseler, and A. S. Fauci, *Preferential infection of CD4<sup>+</sup> memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals* PNAS 1990. **87**: p. 6058-6062.

82. Wang J, H.A., Matsushita S, Matsumi S, Zhang Y, Shioda T, Nagai Y, Matsushima K, *IL-4 and a glucocorticoid up-regulate CXCR4 expression on human CD4+ T lymphocytes and enhance HIV-1 replication.* J Leukoc Biol. , 1998. **64**: p. 642-9.
83. Fontenot D, H.H., Hanabuchi S, Nehete PN, Zhang M, Chang M, Nehete B, Wang YH, Wang YH, Ma ZM, Lee HC, Ziegler SF, Courtney AN, Miller CJ, Sun SC, Liu YJ, Sastry KJ, *TSLP production by epithelial cells exposed to immunodeficiency virus triggers DC-mediated mucosal infection of CD4+ T cells.* PNAS, 2009. **106**: p. 16776-16781.
84. Cannon G, Y.Yi, H Ni, E Stoddard, DA Scales, DI Van Ryk, I Chaiken, D Malamud, D Weissman., *HIV envelope binding by macrophage-expressed gp340 promotes HIV-1 infection.* J Immunol., 2008. **181**: p. 2065-70.
85. Beignon AS, M.K., Skoberne M, Manches O, DaSilva I, Kavanagh DG, Larsson M, Gorelick RJ, Lifson JD, Bhardwaj N., *Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions.* J Clin Invest. , 2005. **115**: p. 3265-75.
86. Lund JM, L Alexopoulou., A Sato, M Karow, NC Adams, NW Gale, A Iwasaki, RA.Flavell, *Recognition of single-stranded RNA viruses by Toll-like receptor 7.* PNAS, 2004. **101**: p. 5598-5603.
87. Kawai T, S.Akira, *Toll-like receptor and RIG-I-like receptor signaling.* Ann N Y Acad Sci. , 2008. **1143**: p. 1-20.
88. Stahl-Hennig, C., R M. Steinman, K Tenner-Racz, M Pope, NStolte, K Matz-Rensing, G Grobschupff, B Raschdorff, G Hunsmann, and P Racz, *Rapid infection of oral mucosal-associated lymphoid tissue with Simian Immunodeficiency Virus.* Science, 1999. **285**: p. 1261-1265.

89. Reynolds, M., E. Rakasz , P.J. Skinner , C. White , K. Abel , Z.M. Ma , L. Compton , G. Napoé , N. Wilson , C.J. Miller , A. Haase , D.I. Watkins, *CD8+ T-Lymphocyte Response to Major Immunodominant Epitopes after Vaginal Exposure to Simian Immunodeficiency Virus: Too Late and Too Little*. J. Virol, 2005. **79**: p. 9228-9235.
90. Comeau MR, S. Ziegler, *The influence of TSLP on the allergic response*. Mucosal Immunol. , 2009: p. 1-10.
91. Wahl, S.M., T. Greenwell-Wild , N. Vázquez, *HIV accomplices and adversaries in macrophage infection*. J Leukoc Biol., 2006. **80**: p. 973-983.
92. Hiscott, J., H. Kwon , P. Génin, *Hostile takeovers: viral appropriation of the NF- $\kappa$ B pathway*. J Clin Invest., 2001. **107**: p. 143-151.
93. Kastner, P., M. Mark , P. Chambon, *Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life?* Cell, 1995. **83**: p. 859–869.
94. Germain P, C.P., Eichele G, Evans RM, Lazar MA, Leid M, De Lera AR, Lotan R, Mangelsdorf DJ, Gronemeyer H., *International Union of Pharmacology. LXIII. Retinoid X receptors*. Pharmacol Rev. , 2006. **58**: p. 760-772.
95. Haase, A.T., *Detection of viral nucleic acids by in situ hybridization*. Methods in Virology. Edited by K Maramorosch, H Koprowski. New York: Academic Press, 1984: p. 189-226.
96. Dailey, P.J., *Quantitation of simian immunodeficiency virus (SIV) RNA in plasma of acute and chronically infected macaques using a branched DNA (bDNA) signal amplification assay*. 13th Annu.Symp.Nonhum. Primate Models of AIDS, Monterey, CA., 1995.
97. Abel, K., D M. Rocke, B Chohan, L Fritts, and C J. Miller, *Temporal and anatomic relationship between virus replication and cytokine gene expression after vaginal*

- simian immunodeficiency virus infection*. Journal of Virology, 2005. **79**(19): p. 12164-12172.
98. Nehete, P., E.M. Vela , M.M. Hossain , A.K. Sarkar , N. Yahia , J. Fantini ,and K.J. Sastry, *A post-CD4-binding step involving interaction of the V3 region of viral gp120 with host cell surface glycosphingolipids is common to entry and infection by diverse HIV-1 strains*. . Antiviral Res, 2002. **56**: p. 233-251.
  99. Good, L, S.B. Maggirwar , A. Kealiher , M. Uhlik , and S.C. Sun, *Multiple Structural Domains within I $\kappa$ B $\alpha$  Are Required for Its Inducible Degradation by both Cytokines and Phosphatase Inhibitors*. Biochem Biophys Res Commun. , 1996. **223**: p. 123-128.
  100. Cvijic, M.E., G. Xiao , and S.C. Sun, *Study of T-cell signaling by somatic cell mutagenesis and complementation cloning*. J Immunol, 2003. **278**: p. 293-304.

## **CHAPTER 7**

### **VITA**

Danielle Renee Fontenot was born in Baton Rouge, LA on March 29, 1982 to Jerold and Gail Fontenot. After graduating from Belaire High School in 2000, she entered Louisiana State University where she received a Bachelor of Science Degree in Biological Sciences in 2004. In August 2004, she entered the master's degree program at The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences where she worked with Dr. Jagannadha Sastry at the University of Texas-MD Anderson Cancer Center, Department of Immunology, as a Graduate Research Assistant. In 2006, she completed her requirements for her master's degree in biomedical research and later that year re-enrolled at the University of Texas Houston Health Science Center, Graduate School of Biomedical Science where she conducted her Ph.D. work under the supervision and guidance of Dr. Sastry.

## **Publications**

**Fontenot, D.,** He, H., Hanabuchi, S., Nehete, P., Zhang, M., Chang, M., Nehete, B., Wang, Y.H., Wang, Y.H., Ma, Z., Lee, H.C., Ziegler, S.F., Courtney, A., Miller, C.J., Sun, S.C., Liu, Y.J., and Sastry, K. J. TSLP production by epithelial cells exposed to immunodeficiency virus triggers DC-mediated mucosal infection of CD4<sup>+</sup> T cells. (PNAS,2009).

**Fontenot DR.,** Jones JK, Vela EM, Hossain M, Nehete P, Dwyer V, and Sastry KJ. Critical role of Arg<sup>59</sup> in the high-affinity gp120-binding region of CD4 for human immunodeficiency virus type 1 infection. (Virology; 2007).

**Fontenot DR.,** den Hollander, P., Vela EM, Yang Zhibo, Vadlamudi RN, Sastry KJ, and Kumar R. Dynein Light Chain Peptide Inhibits Human Immunodeficiency Virus Infection in Mammalian Cells. (Biochem Biophys Res Commun., 2007).