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R5 HIV-1 PREFERENTIALLY TRANSLOCATES ACROSS GENITAL MUCOSA WHILE X4 VIRUS IS SELECTIVELY SEQUESTERED INSIDE GENITAL EPITHELIAL CELLS AND ACTIVATES TYPE I IFN SIGNALING

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INTRODUCTION

Currently there are 36.9 million people of all ages living with HIV/AIDS worldwide. Women are disproportionately affected with HIV-1 and constitute more than 50% of the population currently living with human immunodeficiency virus (HIV-1) worldwide. Approximately 40% of global HIV-1 transmission occurs in the female genital tract (FGT) through heterosexual transmission. Epithelial cells lining the genital tract are the first line of defense to encounter the pathogens entering through FGT.

Although both R5 and X4 HIV-1 variants are present in seminal and other body fluids, R5 HIV-1 appears to transmit infection and dominates early stages of HIV disease. The mechanism underlying this preferential selection of R5 HIV-1 during transmission is not understood completely. Most previous studies have examined the R5 selection in immune cells. However, during primary transmission, HIV-1 has to first cross the epithelial barrier lining the mucosa before it can infect target cells. In this study we examined the interactions between X4 and R5 strains of HIV-1 with genital epithelial cells (GECs) that result in preferential selection of R5 strains for mucosal transmission. This indicates the existence of a natural "gate keeper" phenomenon in the genital mucosa.

Results from this study indicate that epithelial cell innate immune responses are biased towards X4 virus and there is a robust protective response which sequesters X4 strain of HIV-1 and does not allow it to cross the genital epithelium. Minimal levels of innate immune responses were observed against HIV-1 R5 strain and majority of the HIV-1 R5 virus crossed the epithelial monolayer into the basolateral compartment where it would have easy access to target cells *in vivo*. Understanding this gatekeeper function may enable us to enhance existing barriers against R5 transmission and to develop new strategies to prevent HIV-1 infection.

MATERIALS AND METHODS

1. Viruses were propagated either in primary macrophages (for R5 strains) or in H9 /Jurkat T-cell lines (for X4 strains) or transfected into HEK293 cell lines as molecular clones. Viral titration and concentrations were assessed in TZMbl cell lines or by P24 ELISA.

2. Primary endometrial epithelial cells were isolated and grown in transwell inserts. Method of isolation have been published in detail. Kaushic C, Nazli A, Ferreira VH, Kafka JK. Methods. 2011 Oct;55(2):114-21.

3. Immunofluorescent staining was performed by using standard sandwich antibody technique. After treatment epithelial monolayers were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), permeabilized, and stained for CD4 receptor, CCR5, CXCR4 coreceptors, Early endosomal protein (EEA1), TLR2 and HIV-1. Images were acquired using an inverted laser-scanning microscope (Nikon Eclipse T*i*2). An x63 oil immersion objective lens with 3X zoom was used. For each experiment, image acquisition (i.e., confocal microscope settings) and processing was identical between controls and treated cells.

4. Quantitative Real Time PCR was performed using total RNA isolated extracted from treated cells using the RNAeasy kit (Qiagen) as described by manufacturer. Quantitative real-time PCR (qRT-PCR) was performed using the gene specific primers for the select genes (ISG15, BST1, MX1, OAS1, OAS2, OAS3, RSAD2, IFIT1, IFIT3, IFFI44L, CXCR4, CCR5). The reaction was performed with RT² SYBR® Green qPCR Mastermix according to the manufacturer's manual (Qiagen) using the StepOne Plus™ Real-Time PCR System (Thermo Fisher, Canada).

RESULTS: R5 PREFERENTIALLY TRANSLOCATES THROUGH ENDOMETRIAL EPITHELIAL MONOLAYERS WHILE X4 IS SELECTIVELY SEQUESTERED INSIDE GENITAL EPITHELIAL CELLS

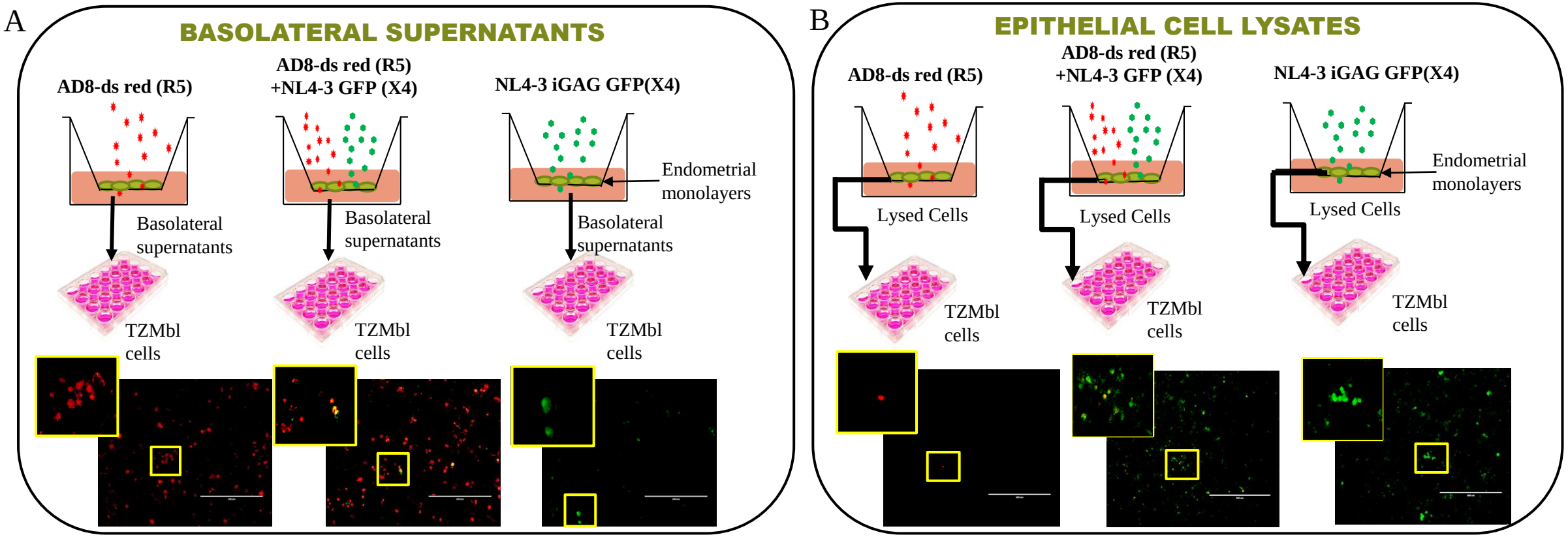


Figure 1: Confluent primary endometrial monolayers were exposed to 10^5 IU/ml HIV-1 X4 and R5 strains individually or in combination for 24 hours. After 24 hours basal supernatants were collected and added to TZMbl cells (1A). After 24 hours of exposure endometrial cells were also collected, lysed by freeze thawing and cell lysate was added to TZMbl cells (1B). The infected TZMbl cells were fixed and imaged under EVOS fluorescent microscope for fluorescently tagged virus. Experiment was repeated multiple times using cells for 3 different donors. Results indicated that HIV-1 R5 translocated through monolayers within 24 hours of exposure while X4 strain was less frequently transcytose and trapped inside the cells.

Primary Endometrial Epithelial cells respond differentially to HIV-1 X4 and R5 strains and the anti-viral innate responses are more robust against X4 than R5

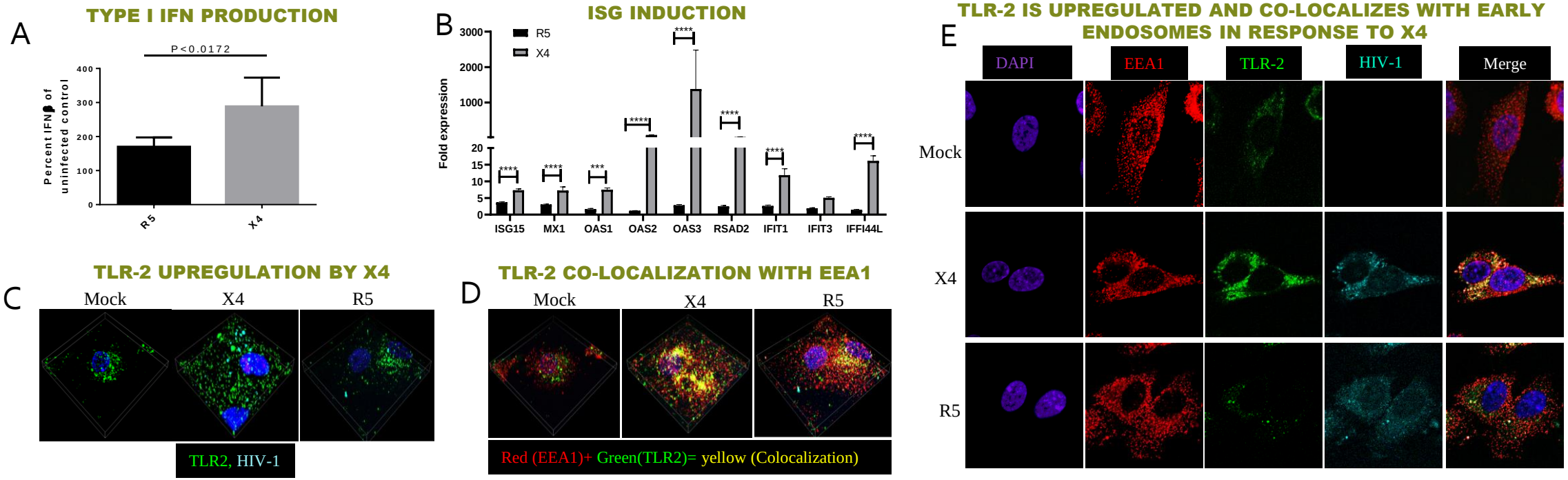


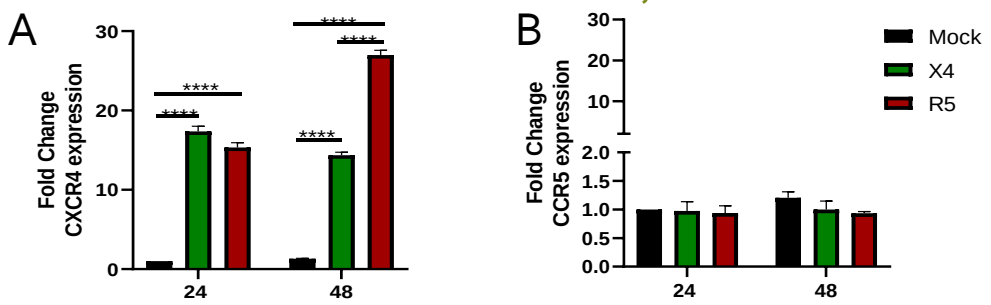
Figure 2: Confluent primary endometrial monolayers were exposed to 10^5 IU/ml HIV-1 X4 and R5 strains individually for 24 hours. After 24 hours supernatants were collected and subjected to Interferon-β ELISA and the results are presented Figure 2A. Results showed that although both HIV-1 X4 and R5 strains induced upregulation of IFN- β production as compared to mock controls but the amount of IFN-β produced after exposure to X4 was significantly more than the IFN-β produced in response to R5 strain.

Primary endometrial cells were collected after HIV-1 X4 and R5 exposure and RNA was extracted and subjected to cDNA synthesis. These cDNA samples were used as templates to amplify 9 different Interferon stimulatory genes (ISGs; ISG15, MX1, OAS1, OAS2, OAS3, RSAD2, IFIT1, IFIT3, IFFI44L) by specific primer sequences by quantitative PCR. Gene expression of different ISGs showed that X4 exposure upregulated many folds higher induction of all 9 different ISGs when compared to the ISGs gene expression induced by R5 exposure (2B).

Primary endometrial epithelial monolayers were stained for TLR2 and HIV-1 after 2 hours of X4 and R5 HIV-1 exposure. In images we observed that TLR2 expression is upregulated in endometrial epithelial cells in response to only HIV-1 X4 Exposure (2C). Also monolayers were stained for TLR2, HIV-1 and early endosomal maker (EEA1) after 4 hours of X4 and R5 exposure. Colocalization of EEA1 and TLR2 (shown in yellow) indicated endosomal uptake of TLR2 after HIV-1 exposure which is more pronounced after X4 exposure (2D and 2E).

Genital epithelial cells differentiate between HIV-1 X4 and R5 strains through the presence of coreceptors CXCR4 and CCR5 on cell surface

CXCR4 is upegulated by X4 and R5, but not CCR5



HIV-1 X4 and R5 entry after blocking CXCR4 and CCR5 coreceptors

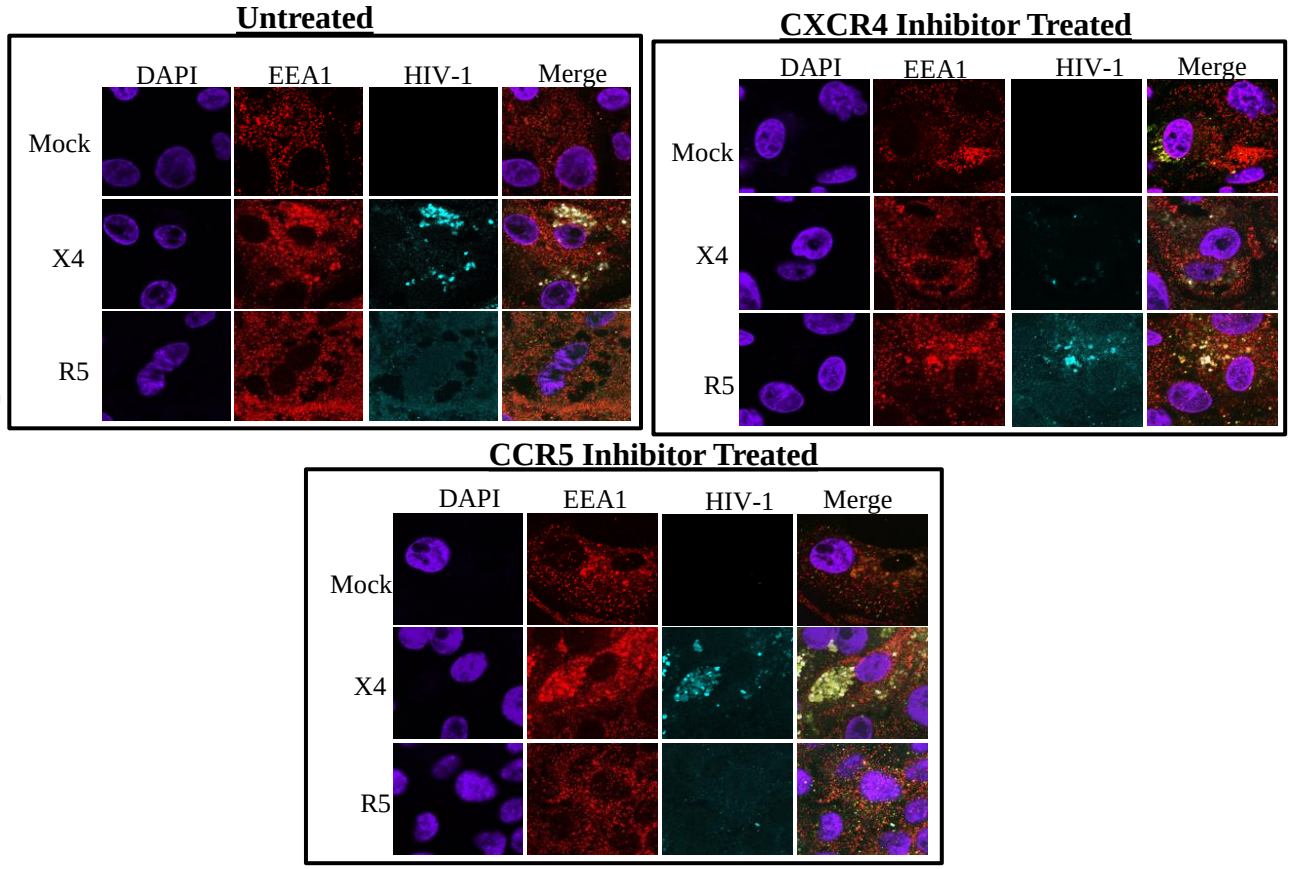


TABLE 1

ISG upegulation by X4 is inhibited by only by CXCR4 blocking

ISGs	R5	R5+ CXCR4 inhibitor	R5+ CCR5 inhibitor	X4	X4 +CXCR4 inhibitor	X4+CCR5 inhibitor
ISG15	3.75	2.5	1.22	38	0.95	58
OAS1	1.66	1.74	0.779	12	0.84	11.5
OAS2	3.1	2.4	1.01	7.0	0.71	4.6
OAS3	3.8	4.0	0.99	6.6	1.08	8.0

Figure 3: Primary endometrial epithelial cells were exposed to medium (mock), X4 and R5 strain and total RNA was isolated, converted into cDNA and subjected to qPCR using primers against CXCR4 and CCR5 genes. The results indicated that exposure of either strain significantly increased CXCR4 gene expression (3A) while there was no difference CCR5 expression (3B). Monolayers were either treated with medium (mock) or with CXCR4 inhibitor (AMD3100; 200 nM) or CCR5 inhibitor (TAK779; 10 nM) an hour before exposure to X4 and R5 HIV-1 strains. Two hours after HIV exposure the treated monolayers were fixed and stained for early endosomes (EEA1) and HIV-1 using specific antibodies. As depicted in images, blocking CXCR4 coreceptor with AMD3100 significantly inhibit entry of X4 strain and blocking CCR5 significantly reduced R5 entry (3C).

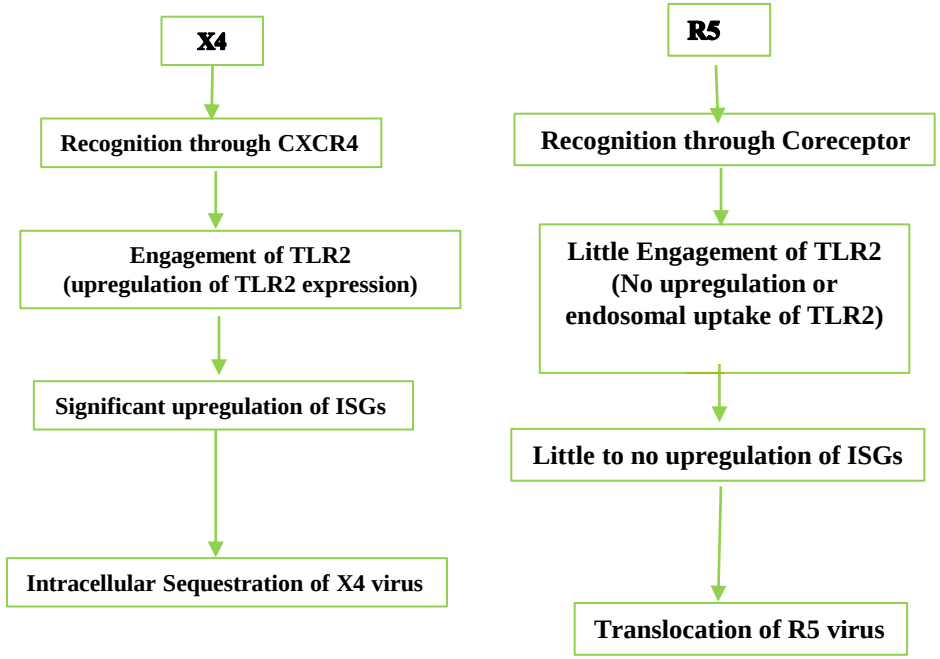
Table 1 showed gene expression of 4 different ISGs by qPCR in primary endometrial epithelial cells after 24 hour of exposure of X4 and R5 preceding treatment with either medium (mock), or CXCR4 inhibitor or CCR5 inhibitor. Inhibition of either coreceptor and further exposure to X4 and R5 inhibited ISGs upregulation which indicate cell recognition and strain specific responses are dependent on coreceptors.

Summary

To summarize these results:

- Endometrial epithelial cells selectively sequester X4 while R5 translocates easily through epithelial cells.

- **Epithelial cells induce differential antiviral response against X4 and R5 variants and anti-viral responses are more robust against X4 strain. Type I IFN and ISGs are significantly upregulated in epithelial cells in response to X4 strain compared to HIV-1 R5 exposure.**
- **The innate anti-viral responses are signalled through TLR2 and expression of TLR2 is significantly upregulated following exposure to X4 and colocalization with early endosomes marker indicated that uptake of TLR2 receptors in endosomes after HIV-1 exposure.**
- **Epithelial cells recognize and differentiate HIV variants through CXCR4 and CCR5 coreceptors.**



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