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Transcriptional analysis of cervical epithelial cell responses to HIV-1

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Human Immunodeficiency Virus type 1 (HIV-1) infection causes a growing pandemic throughout the world, of which women comprise 51% of people who live with HIV-1, more than 60% in sub-Saharan Africa. HIV-1 infections of women are mainly acquired through female reproductive tract where cervical and vaginal epithelial cells are the first line of defense. Although HIV-1 does not directly infect epithelial cells, HIV-1 obligatorily interacts with and crosses over epithelial layer to infect susceptible target cells, mainly CD4+ T cells, in the lamina propria to initiate an infection. However, the mechanism and ramification of the interaction of HIV-1 and epithelial cells in vaginal transmission of HIV-1 remain to be elucidated. We hypothesized that cervical epithelial cells are not a passive barrier, but actively respond to HIV-1 to modulate the mucosal milieu and facilitate HIV-1 transmission. We tested this hypothesis by studying the responses of cervical epithelial cells to HIV-1 through profiling genome-wide transcription, analyzing of cytokine and chemokine proteins, and confirming some differentially expressed key genes in rhesus macaques model of SIV infection. We found: 1) cervical epithelial cells actively respond to HIV-1. Five hundred forty-three transcripts/genes in cervical epithelial cells were significantly altered in expression at four hours post exposure to HIV-1, of which many relate to important signaling pathways, such as innate immune responses, pattern recognition receptors, apoptosis, biosynthesis, and energy production, 2) HIV-1 increases the expression of CXC Chemokines (IL-8, CXCL1 and CXCL3) in cervical epithelial cells. IL-8 and CXCL1 are potent chemotactic for multinuclear neutrophils (MNP), monocytes and a minority of lymphocytes, and CXCL3 is predominant chemotactic for monocytes, 3) HIV-1 increases the expression of

key inflammatory enzymesCOX-1 and COX-2. COX-1 is responsible for the production of prostaglandins that are important for homeostasisi, and COX-2 is a key enzyme to convert arachidonic acid to prostaglandins, key inflammatory mediators, and 4) the increased expression of IL-8 and COX-2 revealed using microarraywas mapped to the endocervical epithelial cells of the macaques intravaginally inoculated with SIV *in vivo*. Our date lead to a role model of epithelial cells in HIV-1 vaginal transmission, that is the axis of HIV-1, epithelial cells, proinflammatory molecules (IL-8, CXCL1, CXCL3, COX-1 and COX-2), cell recruitment (MNP, monocytes and T cells), and inflammation. This model implies that moderating epithelial proinflammatory response to HIV-1 may be a utilityto prevent of HIV-1 vaginal transmission.

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CHAPTER 1

Transcriptional analysis of cervical epithelial cell responses to HIV-1

Introduction

Human immunodeficiency virus type 1 (HIV-1) is a global pandemic that disproportionally infects women. Early events of HIV-1 transmission are not well understood, but dictate the course of infection. The female reproductive tract (FRT) - more specifically cervical epithelial cells - is the first barrier to HIV infection. The overarching goal of this research is to investigate cervical epithelial cells and HIV-1 interaction and better understand the role of cervical epithelial cells in HIV-1 vaginal transmission. The introduction reviews the background of HIV-1 including the progression to the acquired immune deficiency syndrome (AIDS), the genetic bottleneck, and the role of the female reproductive tract in HIV-1 transmission.

Section I: Background of HIV-1, transmission and pathogenesis

Human immunodeficiency virus (HIV) is classified in the *Retroviridae* family and evolved from primates. HIV includes type 1 (HIV-1) and type 2 (HIV-2). *Lentiviriae* - 'slow viruses' - are a member of the *Retroviridae* family and cause slow immunodeficiency diseases (Chiu et al.). *Lentiviriae* infects a range of mammals including ovines, bovines, equines, felines, and primates. *Lentiviriae* infecting primates are more closely related to each other than to those of other mammals (Myers, MacInnes, and Korber 1992). HIV evolved from Simian Immunodeficiency Virus (SIV). Primate Lentiviriae fall into five groups: (1) HIV-1 & Chimpanzees, (2) HIV-2, Sooty mangabeys & Macaques, (3) African green moneys, (4) Mandrills and (5) Sykes' monkeys (Myers, MacInnes, and Korber 1992; Emau et al. 1991). The evolution of the five groups of SIV/HIV is not completely understood.

HIV contains two strains of positive single-stranded RNA encoding nine genes surrounded by the capsid and a plasma membrane from the host-cell. HIV attaches to the cell CD4 using the Env glycoprotein gp120. CD4 is found on T helper cells, regulatory T cells, monocytes, macrophages, and dendritic cells. The virus also has two co-receptors: C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4). CCR5 normally interacts as a receptor for RANTES, MIP-1β, and MIP-1α. CXCR4 normally interacts as a receptor for stromal-derived factor-1. Once HIV enters the cell, the reverse transcriptase transcribes the RNA to DNA in the cytoplasm. Host factors transport the DNA to the nucleus. The DNA inserts itself into the host genome in random locations by integrase. The integrated copies of DNA serve as templates for RNA synthesis. The virion particles form and bud from the cell.

HIV has three major genetic factors that drive evolution: inefficient coping, recombination, and modular evolution. Reverse transcriptase (RT) is responsible for RNA-dependent DNA synthesis and has inefficient proof-reading (Bebenek et al. 1993). Single nucleotide polymorphics (SNIPs) from template-primer misalignment, base miscoding, or frame shift errors causes variations within HIV-1. The most variable region of HIV is the envelope gene, specifically the V3-V5 region (Lemey, Rambaut, and Pybus). The rate of nucleotide substitution per silent site per year is $10x10^{-3}$ for HIV-1 compared to $10x10^{-9}$ in a range of mammals including humans (Wolfe, Sharp, and Li 1989). HIV's evolution is about a million times faster than the human genome and at a similar rate as influenza A virus (Buonagurio et al. 1986). HIV-1 is able to go through high levels of recombination during replication due to the enzyme dissociation-re-

association (Zhuang et al. 2002). Infected cells can have more than one copy of viral genome integrated with the host, the average is two, and allowing for more recombination (Jung et al. 2002). Recombination can also occur in the virion, due to the two copies carried within the virion. Modular evolution, the ability to change packages, sets of genes, lead to the transformation and evolution of the *Lentiviriae* (McClure 1996).

HIV-1 is transmitted through the exchange of body fluids by sexual, parenteral, and mother-to-child transmission. Transmission by sexual contact varies from 1 in 10 to 1 in 3,000 exposures, while mother-to-child is 1 in 4 exposures (Galvin and Cohen 2004). Women comprise 51% of people who live with HIV-1 globally, and comprise more than 60% in sub-Saharan Africa (Sidibé 2010). Women mainly acquired HIV-1 through mucosal surfaces of female reproductive tract (FRT) (Shattock et al. 2008; Brenchley and Douek 2008; Hladik and Hope 2009; Hladik, Florian and McElrath 2008). Paradoxically, vaginal transmission of HIV-1 is not efficient and the estimated transmission rate is about 0.0005 to 0.004 per coital contact (Gray et al. 2001; Wawer et al. 2005). The CCR5-tropic HIV-1 strain is more prevalent in North America (Wainberg 2004).

HIV-1 once set a foot in mucosa rapidly spreads throughout the body leaving a limited time frame to stop the infection locally. In *ex vivo* models and simian immunodeficiency virus (SIV) - rhesus macaque models, the virus is able to cross the epithelial mucosal in hours (Hu, Gardner, and Miller 2000; Bomsel 1997). Two to three days later, HIV is found in the portal of entry or blood of macaques (C. Miller et al. 2005). During transmission, the viruses go through a genomic bottleneck and reduce the number of viruses as founder and transmitted viruses. However, the location and the

mechanism of genomic bottleneck has not been fully elucidated. CCR5 tropism HIV-1 is the major viruses establishing a new infection. The infection becomes entrenched in the body 10-14 days later. The virus reaches peak viral replication around 25 days. The host experiences acute HIV-1 syndrome. The viral load decreases over time and several months later, HIV reaches a steady level of replication or set point. The long-term repercussion of a HIV-1 infection is acquired immune deficiency syndrome (AIDS). The targeted cells, such as CD4+ T cells, are lysed or made nonfunctional and no longer respond to foreign pathogens. People usually die from an opportunistic pathogen infection that would be harmless to a healthy immune system or tumor such as Kaposi's sarcoma.

Section II: Genetic bottleneck during HIV-1 transmission

Inter-host transmission poses a genetic bottleneck to Human immunodeficiency virus type 1 (HIV-1) viruses (Rambaut et al. 2004). During the bottleneck, HIV-1 regains a homogeneous viral population that resets the evolutionary clock back to the original starting point (Stilianakis and Schenzle 2006). We would expect the virulence of HIV to increase overtime without the bottleneck. The founder population has several characteristics which show the evolution of HIV-1 during transmission. CCR5-tropic virus variants dominate early HIV-1 infections (Tersmette et al. 1989). The phenotype of CCR5 virus comes from the V3 domain (De Jong et al. 1992; Fouchier et al. 1992). CXC4 tropic virus variants are identified between 24 and 30 months post infection (Kuiken et al. 1992). Viral pathogenesis also relates to the type of co-receptor used by the virus. Early T-cell tropic viruses contain both CXCR4 and CCR5 receptors (Doranz

et al. 1996; Dragic et al. 1996). Evidence suggests CCR5 is the only co-receptor used by HIV-1 during entry, although other transmembrane proteins have been shown to play a role in the entry of HIV-1 (Alfsen et al. 2005; Arthos et al. 2008; Bergelson 2009; de Witte et al. 2007; Liu, Lingwood, and Ray 1999). Cytotoxic T-lymphocyte (CTL) immune response also shapes intra-host HIV-1 evolution, but does not affect the population level (Leslie et al. 2004). The overall mechanism of inter-host transmission is a complicated interaction between sub-types of the virus and individual immune system.

The evolution sub-subtype mosaic forms of HIV-1 can be classified as quasispecies. When individual variants gain the ability to outcompete the population, the virus is driven to extinction by the immune system. The high turn over of HIV-1 can lead to mutations and phenotype changes. The different quasispecies also lead to parallel evolution, allowing for more successful variants to dominate at one time . The intra-host evolution is based on the community, not just the individual virion.

The transmission of HIV-1 from donor to recipient causes evolutionary changes in the viral variant depending on the transmission method. The first transmission method is a direct passage of variants (Takahashi et al. 1989; Siliciano and Guthrie 1988; Palker et al. 1988; Looney et al. 1988). Donor variants escape immune surveillance, and when transmitted, the variants have survival advantages. Another transmission method is when a limited number of the majority variants in the donor are transmitted and within-host selection causes the majority to become the minority (Wolinsky et al. 1992; Mano and Chermann; Courgnaud et al. 1991). Third, the donor may have a minority variant that has selective advantages in cell tropism, co-receptors, or replication capacities and allow

the variant to become the majority in the recipient (Zhu et al. 1993; Connor and Ho 1994). Each of these methods supports the selection of a particular variation based on phenotype for intra-host evolution.

Section III: The role of the female reproductive tract in HIV-1 transmission

The female reproductive tract (FRT) can be divided into two major compartments (Hladik, Florian and McElrath 2008). The lower tract consists of the vaginal and ectocervix and the upper track consist of endocervix, uterus, and fallopian tubes. The mucosal membranes are divided into two different types: type I and type II (Iwasaki 2007). The lower tract consists of multi-layered squamous epithelium, type II; whereas the upper tract contains a single layer columnar epithelium, type I. The multiple layers of epithelial cells in the ectocervix and vagina provide better mechanical protection than that of the single layer in endocervix, although the vaginal wall and ectocervix has a greater surface area compared to the endocervix. Several lines of evidence indicate Human immunodeficiency virus type 1 (HIV-1) preferentially gains entry of FRT through the endocervix (C. Miller et al. 2005; Zhang et al. 1999; Q. Li, Estes, et al. 2009).

The low efficiency of HIV-1 vaginal transmission indicates that cervicovaginal mucosal tissue including epithelial cell lining provides a robust barrier to HIV-1 infections. Thus HIV-1 vaginal transmission is a complex process of HIV-1 overcoming host defenses. Mucus is secreted into the lumen of the FRT to trap or delay HIV-1 and other microorganisms from gaining access to the epithelial cells (Lai et al. 2009). Anti-HIV-1 proteins secreted by epithelial cells into the lumen include Beta-defensins, Trappin-2/Elafin, CCL20/MIP3α, Serine Protease Inhibitor Secretory Leukocyte Protease

Inhibitor (SLPI), and LL-37 (Sun et al. 2005; Zapata et al. 2008; Ghosh et al. 2010; Levinson et al. 2009; McNeely et al. 1997; Wahl et al. 1997; Bergman et al. 2007). Since the epithelial cells of cervix and vagina are the first line of defense, HIV-1 obligatorily interacts with and crosses over in order to gain access to submucosal target cells to initiate an infection. Once in the laminar propria, HIV-1 has to find a small set of CD4+ T cells to initiate an infection, but the precise role of dendritic cells (DCs) and macrophages in vaginal transmission remains controversial (Shen, Richter, and Smith 2011; Haase 2010).

Despite recent efforts and progress made in understanding the acute events following HIV-1 vaginal transmission, how HIV-1 interacts with epithelial cells, and what role this interaction may play in HIV-1, vaginal transmission remain incompletely understood. Further, the mechanisms of how HIV-1 crosses the epithelial barrier remain undefined (Shattock and Moore 2003). Four plausible mechanisms are proposed to explain how HIV-1 crosses epithelial cells. First, HIV-1 gains access to susceptible target cells in mucosa via a damaged epithelial barrier. Second, HIV-1 is transported through the mucosal barrier by dendritic cells (de Witte et al. 2007). Lawrence *et al* suggested monocytes preferential transmit CCR5-tropisms (Lawrence et al. 2012). Both of these models are difficult to test and does not explain results described below. Third, HIV-1 could contact epithelial cells, causing changes within the epithelial cells. HIV-1 interacts by some unknown mechanism with those cells to down regulate tight junction proteins allowing HIV-1 and other microorganisms to pass through the submucosa (Nazli et al. 2010). Fourth, HIV-1 is transcytosis - the process by which HIV-1 is transported across

the interior of a epithelial cell by endosomes, and is released on basolateral side. Both e*x vivo* cervico-vaginal culture model and transformed epithelial cells in transwells have been used to study transcytosis (Bomsel 1997; de Witte et al. 2007; Maher et al. 2005; Collins et al. 2000). Intestinal epithelial cells have also been shown to transcytosis HIV-1 indicating a common mechanism (Meng et al. 2002).

Conversely, some *in vitro* studies showed HIV-1 could productively infect epithelial cells, but there is no convincing *in vivo* evidence to support that (Tan, Pearce-Pratt, and Phillips 1993). Many different surface proteins are suggested in HIV-1 and epithelial cell interactions: salivary agglutinin (SAG) glycoprotein gp340, beta 1 integrin, epithelial cell sulfated lactosylceramide, integrin alpha4 beta7, syndecans and intercellular junctions (Alfsen et al. 2005; Arthos et al. 2008; Bergelson 2009; Bobardt et al. 2007; Stoddard et al. 2007b). HIV-1 may interact with several of these proteins at the same time or one of these proteins and/or other an unidentified protein.

Studies of Rhesus macaque (*Macacca mulatta*)/ Simian Immunodeficiency Virus (SIV) model of HIV-1 vaginal transmission suggested that HIV-1 may interact with cervical epithelial cells to trigger an "outside-in" chemokine signaling cascade to recruit CD4+ T cells into submucosa and facilitate HIV-1 infection (Q. Li, Estes, et al. 2009). However, this study has not directly evaluated the interaction of HIV-1 and epithelial cells. Research shows Interleukin 6 (IL-6), IL-8, IL-1Ra, MIP-1α, CCL20/MIP3α, MCP-1, RANTES, TNF-α, INF-α, and INF-γ can be induced in the cervix from 3 to 10 days post SIV infection. Nazli *et al* used mono-layer of epithelial cells, but only tested six different cytokines (Nazli et al. 2010). Katsikis *et al* and Abel *et al* infected rhesus

macaques and isolated mRNA from tissue samples to identify changes in cytokines, but homogenized complex mucosal tissues cannot discern altered genes in expression (Katsikis, Mueller, and Villinger 2011; Abel et al. 2005). Jespers *et al* used cervicovaginal lavage samples from highly exposed, limited exposure or no exposure to HIV-1 to identify changes in cytokines and chemokines (Jespers et al. 2011). Jespers' study is limited by not knowing the direct cause of the changes in expression. Overall, these studies are limited in numbers of cytokines and chemokines tested and where the cytokines were derived. Additionally, conflicting results have been found on the different expression levels and time post HIV-1/SIV infection.

Section IV: Hypothesis and goals

HIV-1 transmission in women is a major problem worldwide. During transmission, HIV-1 interacts with epithelial cells lining cervicovaginal tract and crosses this first line of defense. We hypothesize cervicovaginal epithelial cells actively respond to the presence of HIV-1 during HIV-1 vaginal transmission. We tested the hypothesis by (1) measuring cytokines and chemokines proteins levels, (2) profiling genome-wide transcription and (3) confirming some differentially expressed key genes in rhesus macaques model of SIV infection. This study underscores the importance of epithelial cells in HIV-1 vaginal transmission and suggests that modulating epithelial cell responses to HIV-1 may be a new target for preventing HIV-1 vaginal transmission.

Methods and Materials

Cervical Epithelial Cells and HIV-1

Human endocervical epithelial cells, CRL-2615, were obtained from ATCC and maintained in keratinocyte-serum free ATCC complete media. The cells were cultured in six well plates and incubated over 48 hours to ensure attachment to the plate, and then the media was removed and fresh media containing CCR5 tropism HIV-1_{ME1} at 0.2 $TCID_{50}$ per cell were added. HIV-1_{ME1} was obtained from Dr. Phalguni Gupta through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Chen et al. 1997). Cells in fresh media without adding HIV-1 were used as a control. The cells and supernatant from both HIV-1 treated and control cultures were collected at 0, 4, 6, 12 and 24 hours post HIV-1 exposure. The supernatant was centrifuged at 1000 rpm for 6 minutes and the total volume was measured and frozen at -80°C until analysis. The cells were rinsed with 0.25% trypsin, 0.53 mM EDTA solution, and detached by incubating at 37°C with 0.1M trypsin-EDTA solution. The trypsin was neutralized by adding a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% fetal bovine serum. The cell pellet was collected after centrifugation at 1000 rpm for 6 minutes and placed at -80°C until use. The viral stock suspension was separated from the viral stock by centrifugation at 10,000 rpm for 60 minutes (Iordanskiy and Bukrinsky 2009). The suspension was added and processed similar to the viral stock.

Cytokine, Chemokine and growth factor analysis

Cytokine Human 30-Plex Panel (Catalog number: LHC6003, Invitrogen) quantified thirty cytokine, chemokine and growth factor proteins, following the

manufacturer's instructions. Briefly, samples were diluted using a 1:1 mixture of assay dilutant and media. Each sample contained three replicates with one technique replicate. The panel was read on a Bio-Plex 200 System using Bio-Plex Manager software version 4.0 (Bio-Rad, Hercules, CA). The calibration curves were generated using the kit standards. IL-6, IL-8, IL-1Ra, and MIP3α were quantified using Quantikine human Enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Statistical analysis was conducted with SigmaPlot (San Jose, CA). The samples were normalized to the amount of supernatant collected for each sample. Any measured level below the sensitivity of the individual cytokine detection was considered as a zero.

Quantitative real-time reverse transcriptase polymerase chain reaction

Gene expression quantifications were performed using qRT-PCR and reported according to MIQE guidelines (Bustin et al. 2009). Qiagen mRNA purification kit (Valencia, CA) extracted and purified mRNA. Melting curve analysis was performed at the end of each run to check for primer dimers. Four target genes were selected from the microarray data, and the primers were selected from the RTPrimer database (table 1) (Birkenkamp-Demtroder et al. 2002; Johnson et al. 2002; Lefever et al. 2009; Vandesompele et al. 2002). The cDNA was synthesized and quantified was conducted with iScript™ One-step RT-PCR kit with SYBR® Green (Bio-Rad) with the program: 50°C 10 min, 95°C 5min, and 40 cycles of 95°C 10 sec, and 55°C 30sec. Data analyses were conducted using Biogazelle qbase^{PLUS} version 2.3 (Hellemans et al. 2007). Briefly,

quantification cycle (Cq) values were converted into relative expression values taking into account amplification efficiency, and the relative expression values were normalized using GAPDH as a reference gene. Calibrated Normalized Relative Quantity values were exported from the qbase^{PLUS} software and statistically analyzed using SigmaPlot.

RNA extraction and microarray

The genome-wide transcriptional responses in epithelial cells exposed to HIV-1 were analyzed using human microarrays (Human Genome U133 Plus 2.0 Array, Affymatrix, Santa Clara, CA). Cells at four hours post HIV-1 exposure and the un-exposed control, in duplication, were analyzed. mRNA was extracted and purified using the Qiagen mRNA purification kit (Valencia, CA). The mRNA (15ng) was amplified and labeled with biotin using Ovation WGA System and Ovation Pico WTA System (NuGEN, San Carlos, CA). The Genomics Core Research Facility of the University of Nebraska –Lincoln labeled and hybridized the cDNA to microarray per the manufacturer's instructions. The signals on the chips were scanned with the Affymetrix GCS 3000 7G scanner and GeneChip Operating Software.

Data normalization and statistical analysis

Data normalization and statistical analysis were based on published methods (Gillespie et al. 2010). Briefly, raw microarray data were processed and analyzed using Affy and Lumma packages of Bioconductor, an R package (http://www.bioconductor.org/, http://www.r-project.org/). The backgrounds were corrected with Robust Multiple-array Average (RMA). Significance of differential

expressed genes in controls and HIV-1 exposed group were compared with a moderated t-statistic. Significantly altered genes in expression were defined as a log 2 fold change of > 1 or < 1 and P <0.05, and were annotated and assigned biological function using the Database of Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp) and Ingenuity Pathways Analysis (Ingenuity Systems, http://www.ingenuity.com/). All microarray data has been deposited in the NCBI's Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/gds; accession number GSE42291).

Mapping IL-8 and COX-2 to the cervical epithelial cells of Indian rhesus macaques

Five adult female Indian rhesus macaques (*Macaca mulatta*) were intravaginally inoculated with SIV_{smB7}, a non-infectious virus-like particle (VLP), twice daily for 3 days. The macaques were euthanized on the 4th day post inoculation. The cervix was collected and fixed in 4% paraformaldehyde and embedded in paraffin for sectioning at 6 microns (Kraiselburd and Torres 1995). Immunohistochemical staining of IL-8 and COX-2 were conducted using previously published protocol (Q. Li, Smith, et al. 2009). Antibodies against IL-8 (clone Ab7747, 1:50, Abcam) and COX-2 (clone CX-294, 1:25, Dako) were used and with an isotope control IgG as the negative control. Staining was detected and visualized using the Dako Envision Polymer kit and 3, 3-Diaminobenzidine (DAB) as substrate.

Results

Measurement of cytokine, chemokine and growth factor proteins

We measured the interactions of HIV-1 with cervical epithelial cells at protein level. Cervical epithelial cells, CRL-2615, were exposed to HIV-$1_{ME1,}$ and the supernatant of cultures was collected at 0, 4, 6, 12, and 24 hours post HIV-1 exposure. Out of the 30-Plex panel, eight proteins (IL-6, IL-8, IL-1Ra, RANTES, IL-13, IP-10, VEGF and MIL-1α) from HIV-1 exposed cervical epithelial cells changed expression over the time course in comparison with that of control epithelial cells (Figure 1). We used ELISA to: 1) confirm the Bio-plex results and 2) measure CCL20/MIP3α, which was not included in the 30-Plex panel. There were more prominent changes at four hours post exposure than at other time points. Interleukin 6 was significantly up regulated at four hours, but not at any other time points ($t = -3.648$, d.f. = 4, $p = 0.022$, Figure 2a). Three proteins increased at four hours, but not significantly: IL-8 ($t = -1.997$, d.f. = 4, $p = 0.116$, Figure 2b), IL-1Ra ($t = -2.535$, d.f. = 3, $p = 0.056$, Figure 2c), and CCL20/MIP3α ($t = -1.158$, d.f. = 4, $p = 0.311$, Figure 2d).

Global gene expression measurement using human microarray

The epithelial cells at four hours post HIV-1 exposure changed more than at any of the other time points based upon the results of human 30-Plex and ELISA. We selected four hours to conduct genome-wide transcriptional analysis of epithelial cells response to HIV-1 using the Affymetrix human microarray. The gene expression in HIV-1 exposed epithelial cells was compared to that of epithelial cells without HIV-1 exposure. The microarray showed 574 altered expression out of 54,675 transcripts/genes (213 up

regulated and 361 down regulated). Based on functional annotation from DAVID and extensive examination of published literature, we were able to classify ~55% (314) of the altered genes in expression (Figure 3 and 4, Table 2). The microarray results were validated using qRT-PCR (Figure 5). We exclude the possibility that the altered genes in expression in epithelial cells exposed to HIV-1 were caused by other factors, such as growth factors in culture media used in virus stock preparation rather than HIV-1. The viral stock supernatant was separated from virus using centrifugation. The analysis of the supernatant for its effects on the epithelial cells detected no significant difference in gene expression compared to that of controls (Figure 5).

Of the classifiable altered genes in expression, ~7% (23) are related to innate immune function. Proinflammatory chemokine IL-8 - one of the major mediators of the inflammatory response and a chemoattractant for MNP, monocytes and T cells - was significantly upregulated, which is in concurs with the increase at protein level revealed by Bio-Plex assay (Figure 1b). Other proinflammatory chemokine, CXCL1 and CXCL3, were also significantly upregulated. CXCL1 and CXCL3 are chemoattractants for MNP and monocytes respectively. COX-1 and 2 -rate-limiting enzymes for prostaglandins production and key mediators for inflammation- were upregulated. Another inflammation related molecule CD55, a complement pathway regulator, was also upregulated. Furthermore, genes encoding toll-like receptor regulators were downregulated (TIRAP, IL1-RL1 & IRAK2). Toll-interleukin 1 receptor (TIR) domain containing adapter protein (TIRAP) is involved in TLR2 and TLR4 signaling pathways in innate immune response. Interleukin 1 receptor-like 1 (IL1-RL1) is a member of the

Toll-like receptor superfamily and receptor for IL-33. Interleukin-1 receptor-associated kinase 2 (IRAK2) binds to the IL-1 type I receptor following IL-1 engagement to trigger intracellular signaling cascades. TRIM6 (Tripartite motif-containing protein 6) and S100A8, known antiviral peptides, were significantly down regulated. Four transcripts of genes related to phagocytosis were significantly altered in expression (three upregulated and one downregulated). Of note, RAB7 (Ras-related protein Rab-7a), an important molecule in the late endocytic pathway, was upregulated; ELMO3 (engulfment and cell motility 3), involving in cytoskeletal rearrangements required for phagocytosis, was downregulated. In concurrency with innate immune genes alteration in expression, Ingenuity Pathway Analysis revealed the activation of Jak/Stat canonical pathway, known for regulating interferon, interleukin, growth factors, or other chemical messengers (Figure 6b).

DAVID indicated ~4% (13) of classifiable altered genes in expression relate to epithelial cells (Figure 4), including epithelial membrane protein 1, keratinocyte growth factor-like protein 2, epithelial membrane protein 1 and endothelin 1. IRF6 (interferon regulatory factor 6), a key regulator for the keratinocyte proliferation-differentiation, was upregulated. The genes (19, 8 up regulated) related to cytoskeleton organization were altered in expression. The genes encoding membrane bound proteins were downregulated and genes encoding cell-to-cell adhesion proteins (11, 7 up regulated) were altered in expression. This indicates epithelial cells changing their internal and external structure in the presence of HIV-1. Most ubiquitin genes are downregulated, but genes encoding SMAD specific E3 ubiquitin protein ligase 2 and calcyclin binding protein were

upregulated (11, 4 up regulated). In addition, genes related to cell cycle (~11%), apoptosis (~3%) and some transcription factors (~16%) were altered in expression (Figure 6c).

Biosynthesis was the largest group of altered genes in expression (~24%, 74) (Figure 4), a process of synthesis of tRNAs and the production of and regulation of energy within the cell. Major categories with biosynthesis are GTP production, glucose metabolic process, ion binding, membrane lipid biosynthetic process, sulfur metabolism and sphingoid metabolic process. The genes of sphingoid metabolic process were upregulated, including sphingomyelin synthase 1, sphingomyelin synthase 2, UDP-glucose ceramide glucosyltransferase and sialidase 3.

Mapping IL-8 and COX-2 proteins to the endocervical epithelial cells of rhesus macaques

We tested whether the altered IL-8 and COX-2 genes expression are relevant in vivo using immunohistochemical staining. Adult female Indian rhesus macaques were intravaginally inoculated with inactivated SIV. We found that both IL-8 and COX-2 were expressed in the cervical epithelial cells in addition to the cells in the lamina propria, (Figure 7).

Discussion

Women, especially in sub-Saharan Africa, are disproportionally impacted by HIV-1 infections, which are mainly acquired through vaginal transmission. The interplay between HIV-1 and its host at cervicovaginal mucosal surfaces, where epithelial cells are the first line of defense, ultimately determine the outcomes of infection or protection. Although epithelial cells are not directly infected by HIV-1, its interactions with HIV-1 are prerequisite for HIV-1 to establish vaginal transmission. However, the interaction of epithelial cells and HIV-1 remains incompletely understood and the epithelial cells are thought to function only as a passive barrier in HIV-1 infection *(Stoddard et al. 2007a; Bouschbacher et al. 2008; C. J. Miller and Shattock 2003)*. To better understand the interaction of cervical epithelial cells and HIV-1 and its role in HIV-1 vaginal transmission, we studied genome-wide transcriptional responses of cervical epithelial cells to HIV-1.

We found that cervical epithelial cells actively respond to HIV-1. We found 574 transcripts/genes (213 upregulated and 361 downregulated) were altered in expression in the epithelial cells at 4 hours post HIV-1 exposure (Figure 3) indicating cervical epithelial cells are not a passive barrier, but play an active role in HIV-1 vaginal transmission.

Strikingly, ~7% (23 transcripts/genes) of classifiable, altered genes are related to innate inflammatory immune response (Figure 4). Our results demonstrated that HIV-1 can increase the expression of IL-8 , CXCL1 and CXCL3 in cervical epithelial cells. IL-8 and CXCL1 are potent chemotactic for MNP, monocytes and a minority of lymphocytes (Bouschbacher et al. 2008). CXCL3 is predominant chemotactic for monocytes (Smith et

al. 2005). It has been shown that IL-8 enhances HIV-1 replication in macrophage and T cells and increase susceptibility of cervical tissue to HIV-1 infection (Lane et al. 2001; Narimatsu, Wolday, and Patterson 2005). Elevated cervical IL-8 correlated with increased HIV-1 shedding in female reproductive tract (Gumbi et al. 2008). Furthermore, it has been shown that mononuclear phagocytes (MNP) transepithelial migration mediates epithelial injury, comprises barrier function and enhances luminal pathogen such as HIV-1 to cross epithelial barrier (Chin and Parkos 2007). IL-6 was upregulated within the microarray data, but not significantly. The difference between the results is currently not known. Our data suggest the recruitments of cells (MNP, monocytes and T cells) through the upregulation of CXC Chemokines by cervical epithelial cells that is triggered by HIV-1, may play a key role in HIV-1 vaginal transmission.

Concomitantly, cyclooxygenase (COX)- 1 and -2 genes were upregulated in expression. COX-1 is responsible for the production of prostaglandins (PG) that are important for homeostatic functions (Crofford 1997). COX-2 is a key enzyme to convert arachidonic acid to prostaglandins, key inflammatory mediators. It has been demonstrated that COX-2 is upregulated during various inflammatory conditions (Martel-Pelletier, Pelletier, and Fahmi 2003; Chang et al. 2003; Morton and Dongari-Bagtzoglou 2001; Tsujii and DuBois 1995). It was previously demonstrated that COX-2 was upregulated in the presence of vaginal topic contraceptive microbicide, Nonoxynol-9, a well-known agent inducing cervicovaginal mucosal inflammation and damage (Zalenskaya et al. 2011). The clinic trials of Nonoxynol-9 as vaginal topical microbicide showed it

increased HIV-1 vaginal transmission (Pettersen et al. 2011). Furthermore, increased prostaglandins from epithelial cells may activate adjacent T cells and monocytes in submucosa, since it has been demonstrated that COX-2 contributes to immune activation during HIV-1 infection (Pettersen et al. 2011). Chronic HIV-1 infection is associated with significantly increased COX-2 in cervical cells collected using cytobrush compared that of HIV-1 uninfected women (Fitzgerald et al. 2012). Our data extended these results and unambiguously showed that cervical epithelial cells increase COX-2 expression after exposure to HIV-1. COX-1 and 2 are key in initiating and amplify mucosal inflammation, thus moderating mucosal inflammation by selectively inhibiting COX-2 using non-steroidal anti-inflammatory drugs is worthy to further explore.

Ingenuity Pathway Analysis revealed that Jak/Stat canonical pathway, known for regulating interferon, interleukin, growth factors, or other chemical messengers, was up regulated (Fig 6b). The Jak/Stat pathway has been shown to be important in HIV infections (Wang et al. 2010). Concurrently, with increased expression of proinflammatory innate immune genes, four genes related to phagocytosis were significantly altered in expression, of note, RAB7 (Ras-related protein Rab-7a), an important molecule in the late endocytic pathway, was upregulated; and ELMO3 (engulfment and cell motility 3), involving in cytoskeletal rearrangements required for phagocytosis, were downregulated.

The transcriptome analysis also showed alternations of genes related to biosynthesis and life cycle of the epithelial cells. Biosynthesis is an important part of the interactions of HIV-1 and epithelial cells. Sphingoid metabolic process is the synthesis of

lipids and other compounds associated with lipid rafts. Recent studies have shown that lipid rafts are important to the entry and budding of HIV-1 *(Fantini et al. 2004; Clayton et al. 2001)*. Our results showed an increase in transcripts in sphingoid metabolic process, but the role in HIV-1 transmission is unknown.

The internal and external structure of epithelial cells creates a barrier to pathogens. Our results show a loss of alterations in the structure of the epithelial cells. The loss of receptors and alterations of the internal and external structure may indicate significant tissue rearrangement. The lost of tight junction proteins may allow gaps in the epithelial barrier allowing HIV-1 to past the barrier without infecting the cells *(Nazli et al. 2010)*. Actin and cytoskeleton play an important part in the assembly and transmission of HIV-1 *(Matarrese and Malorni 2005)*. Alterations in the cytoskeleton can lead to apoptosis. Although our results do not indicate apoptosis; further study needs to be done on the effects the alteration of the internal and external structure has on the entrance of HIV-1.

Some anti-HIV-1 molecules, such as TRIM5α, Tetherin, LL-37, trappin-2, and CCL20/MIP3α, are naturally expressed by epithelial cells and may increase in expression in the presence of HIV-1 *(Ghosh et al. 2010; Neil, Zang, and Bieniasz 2008; Perez-Caballero et al. 2009; Bergman et al. 2007; Levinson et al. 2009; Ghosh et al. 2009)*. CCL20/MIP3α has also been shown as anti-HIV-1 peptide secreted into the lumen of the cervix *(Ghosh et al. 2009)*. Our results showed the down regulation of TRIM6 and S100A8, known antiviral peptides. TRIM6 is associates with HIV-1 virion, but does not show inhibition like TRIM5α *(X. Li et al. 2007; X. Li et al. 2006)*. S100A8 has not been

shown to effect HIV-1, but has been shown to be important to Human Papillomaviruses 18 *(Lo et al. 2007)*. Drannik et al. suggested trappin-2 has an inhibitory effect on HIV-1 by altering epithelial cell surface proteins (Drannik et al. 2012). Trappin-2 reduce activation of NF-kB, AP-1, RIG-I, and MDA5 (Henriksen et al. 2004; Drannik, Henrick, and Rosenthal 2011). Our results show an increase in , indicating trappin-2 is not functioning with the epithelial cells. We speculate increasing the expression of the anti-HIV-1 molecules or stopping the downregulation will help prevent HIV-1 transmission.

Our results lacked some genes that have been previously found. Trappin-2 downregulates activation of NF-kB, AP-1, Retinoic acid-inducible gene 1 (RIG-I), Melanoma differentiation-Associated protein 5 (MDA5) (Henriksen et al. 2004; Drannik, Henrick, and Rosenthal 2011). Our results matched an increases in trappin-2, but we did not find changes in any of the other proteins. Past studies used HeLa cells or TZM-bl cells; theses cells are a cancer line and do not represent cervical epithelial cells. We used a cell line created by inoculating the cells with viral oncogenes. The cells are closer to an accurate representation of cervical epithelial cells, and are easy to culture and manipulate. We did not test if the cells could be infected by HIV-1. Our intentions were to study the effect of HIV-1 on epithelial cells - not HIV-1 transmission by the epithelial cells.

Our data and previously published works provides a model for the interactions between epithelial cells and HIV-1 in vaginal transmission. HIV-1 interacts with the epithelial cells by some unknown surface protein and is recognized as a pathogen. The epithelial cells induce pro-inflammatory molecules (IL-8, CXCL1, CXCL3, COX-1 and COX-2) triggering cell recruitment (MNP, monocytes and T cells) and inflammation.

The inflammation and alterations within the epithelial cells causes changes in the cell and tissue structure possibly allowing for HIV-1 access to the sub-mucosa.

In summary, our study has gained new insights into the interaction of HIV-1 and cervical epithelial cells. We found 1) cervical epithelial cell actively respond to HIV-1, 2) HIV-1 increases the expression of CXC Chemokines (IL-8, CXCL1 and CXCL3) in cervical epithelial cells, 3) HIV-1 increases the expression of key inflammatory enzymes-COX-1 and COX-2, and 4) the increased expression of IL-8 and COX-2 revealed using microarray analysis was mapped into the endocervical epithelial cells of macaques inoculated with inactivated SIV *in vivo* (Figure 6). Our data lead to a role model of epithelial cells in HIV-1 vaginal transmission, that is an axis of HIV-1, epithelial cells, proinflammatory molecules (IL-8, CXCL1, CXCL3, COX-1 and COX-2), cell recruitment (MNP, monocytes and T cells), and inflammation. This model implies that moderating epithelial proinflammatory response to HIV-1 may be utilized in prevention of HIV vaginal transmission.

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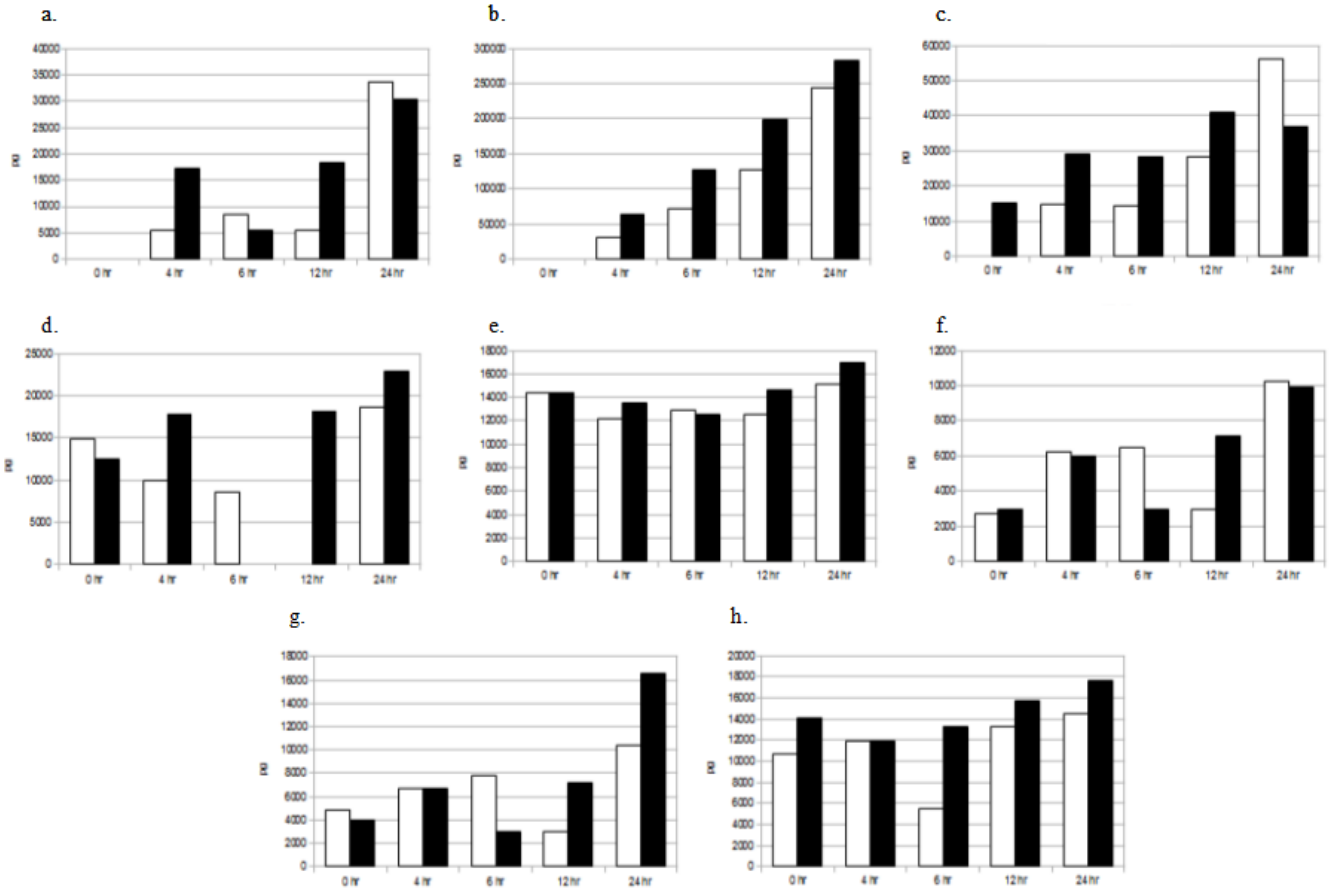

Figure 1. Protein measurements in the supernatant of cultured cervical epithelial cells at different time points post HIV-1 exposure using Cytokine Human 30-Plex Panel. Attached human endocervical epithelial cells-CRL-2615 were cultured and exposed to R-5 HIV-1_{ME1} at 0.2 $TCID_{50}$ per cell. The supernatant from both HIV-1 treated and control cultures were collected at 0, 4, 6, 12 and 24 hours post HIV-1 exposure. Thirty cytokine, chemokine and growth factor proteins were quantified using Cytokine Human 30-Plex Panel. Eight proteins (IL-6, IL-8, IL-1Ra, RANTES, IL-13, IP-10, VEGF and MIL-1α) from HIV-1 exposed cervical epithelial cells showed alteration over the time course in comparison with that of control epithelial cells. a) IL-6, b) IL-8, c) Il-1Ra, d) RANTES, e) IL-13, f) IP-10, g) VEGF, and h) MIL-1α

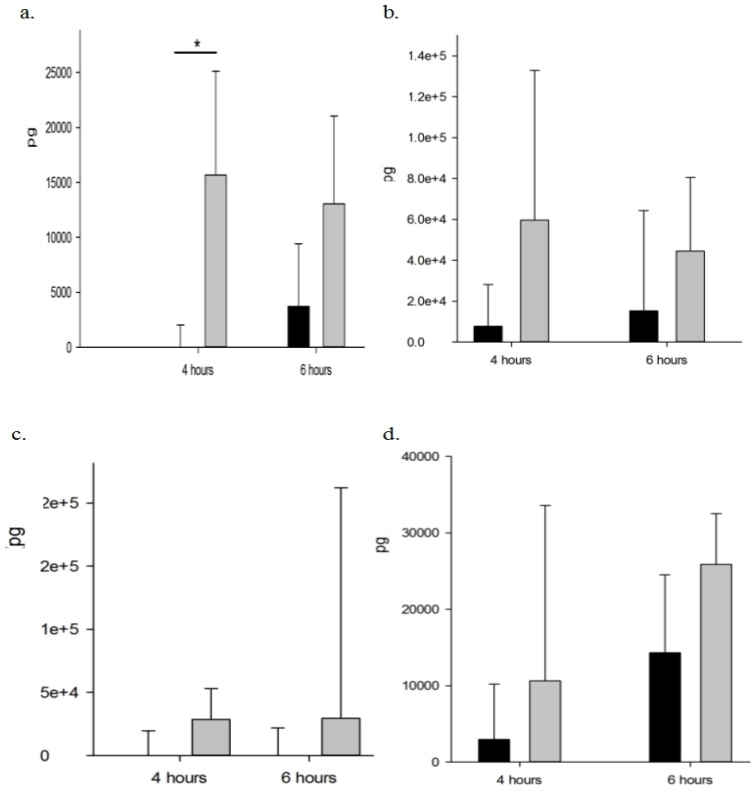

Figure 2. Quantified Proteins in the supernatant of cultured cervical epithelial cells at 4 and 6 hours post HIV-1 exposure using ELISA. IL-6 was significantly upregulated at four hours post HIV exposure ($t = -3.648$, d.f. = 4, $p = 0.022$, part a). IL-8 ($t = -1.997$, d.f. = 4, $p = 0.116$, part b), IL-1Ra ($t = -2.535$, d.f. = 3, $p = 0.056$, part c) and CCL20/MIP3α ($t = -1.158$, d.f. = 4, $p = 0.311$, part d) increased, but not significantly. The black bars represent the control and the gray bars represent epithelial cells exposed to HIV-1.

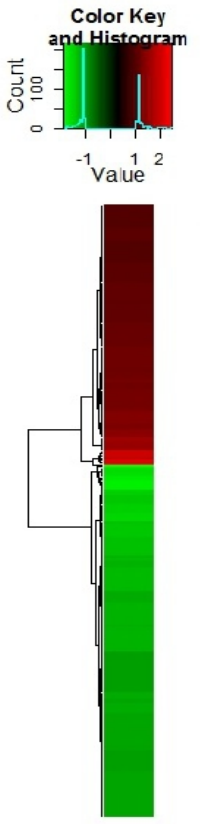

Figure 3. Heatmap of significantly altered 574 transcripts/genes in expression from the cervical epithelial cells at four hours post HIV-1 exposure using Affymetrix Human Genome microarray.

mRNA was extracted from the cultured cervical epithelial cells at 4 hours post R-5 HIV-1_{ME1} at 0.2 $TCID_{50}$ per cell, amplified and labeled with biotin. The labeled cRNA was hybridized to microarray chip data normalization and statistical analysis was based on published methods .

Significantly altered genes in expression were defined as a log 2 fold change of > 1 or < 1 and P <0.05 in comparison with that of control epithelial cells.

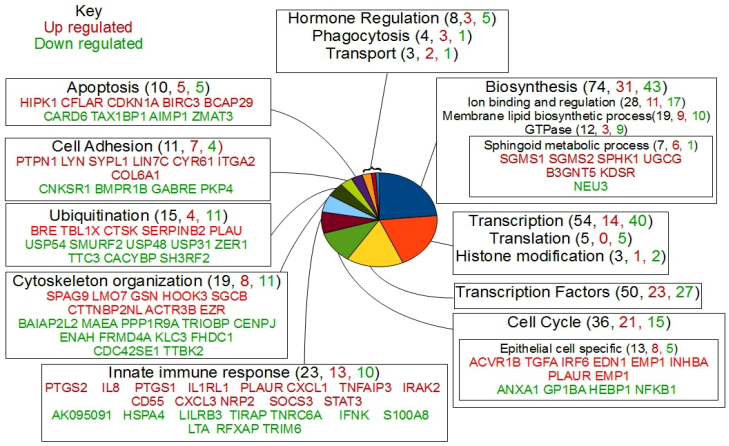

Figure 4. Functional classification of some significantly altered genes in expression from epithelial cells at 4 hours post HIV-1 exposure. A total of 574 transcripts/genes were significantly altered in expression, of which 314 genes can be classified (lfc = 1, p value = 0.05). The size of each sector in the pie diagram is proportional to the number of genes in the corresponding category. The numbers of altered genes and upregulated genes in expression for each category are shown in parentheses. All the gene names, abbreviations, log-fold change and p-values can be found in Table 2.

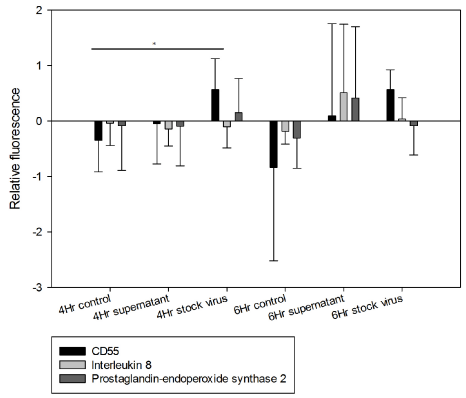

Figure 5. Detection of select genes in cervicovaginal epithelial cells inoculated with HIV-1. Data expressed as fold-change of cellular gene expression based on GAPDH gene with standard deviation based on three replicates with a technical replicate. Asterisks indicate statistically significant differences using an one way ANOVA and Holm-Sidak post-hoc analysis ($p < 0.05$).

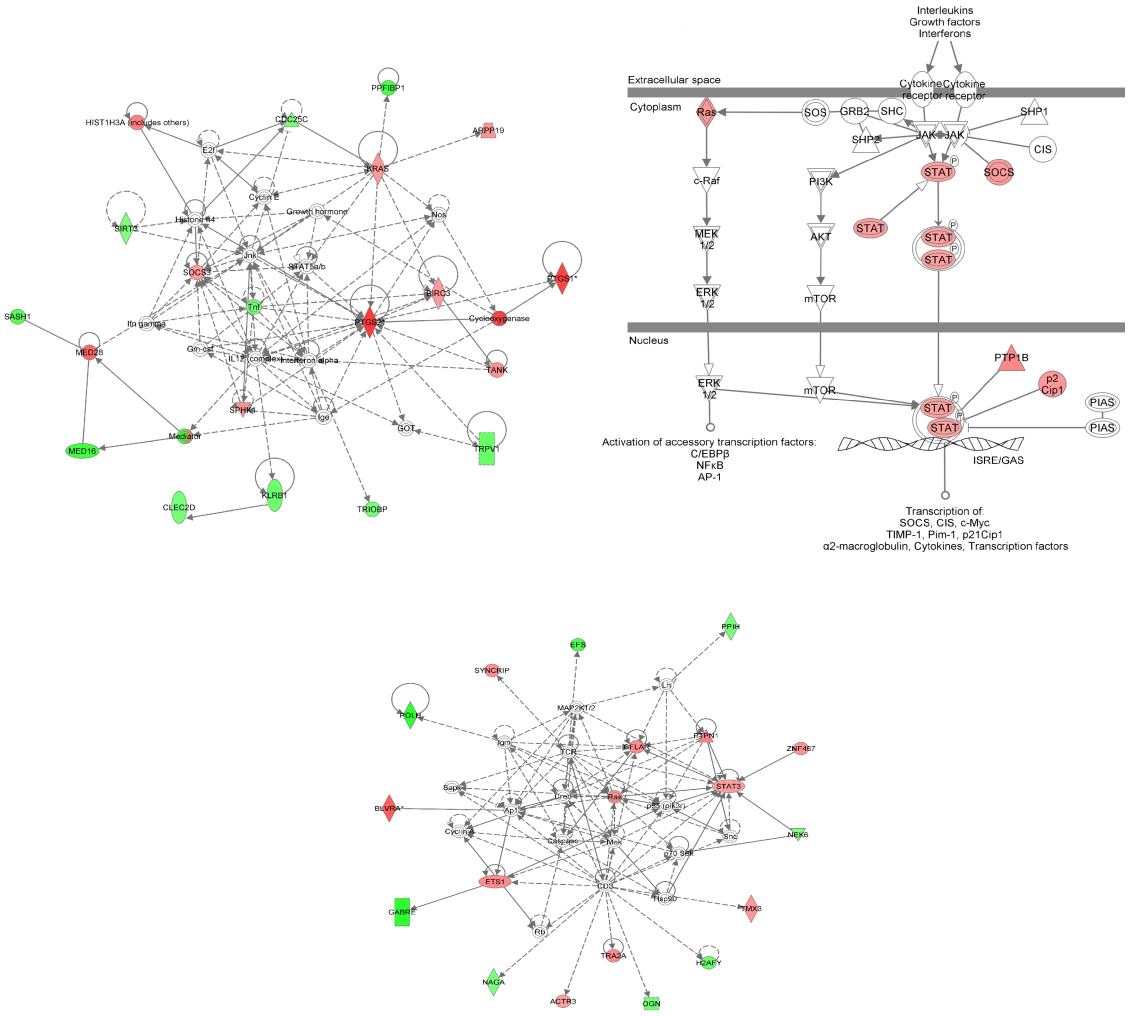

Figure 6. Significantly activated signaling pathway and networks. Pathway and networks of significantly altered genes in expression were generated using the Ingenuity Pathways Analysis software. a) the network of inflammatory response, b) Jak/Stat canonical pathway and c) the network of cellular development, proliferation & death. Red indicates genes significantly increased in expression, green indicates genes significantly decreased in expression, and black indicates no significant change in gene expression.

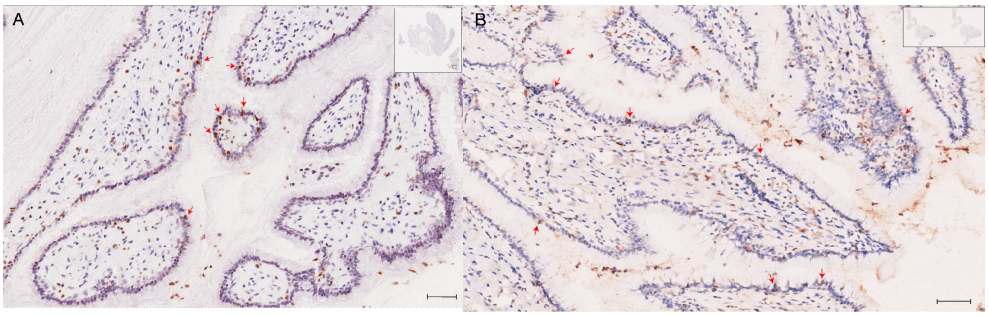

Figure 7. Photogram of IL-8 (A) and COX-2 (B) expression in the endocervical epithelial cells of Indian rhesus macaques (*Macaca mulatta*) after intravaginal inoculation of inactivated SIV. The enlarged photograms are from the rectangular boxes of whole cervical sections. Red arrows indicates detected positive signals in epithelial cells. Scale bar equals 50 microns.

Table 1. Primers used for Quantitative RT-PCR from the selected genes from microarray results

Gene abbreviation	Gene complete name	RTPrimerDB ID	Primer	Sequence (5′→3′)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	3	Forward	TGCACCACCAACTGCTTAGC
			Reverse	GGCATGGACTGTGGTCATGAG
PTGS2	prostaglandin-endoperoxide synthase 2	2456	Forward	GAATCATTCACCAGGCAAATTG
			Reverse	TCTGTACTGCGGGTGGAACA
IL-8	Interleukin *8*	3074	Forward	GAATGGGTTTGCTAGAATGTGATA
			Reverse	CAGACTAGGGTTGCCAGATTTAAC
CD55	CD55 molecule	273	Forward	GGTGCAACCATCTCCTTCTC
			Reverse	TGGTGGTGCTGGACAATAAA

Table 2. Complete list from the microarray experiment of known genes classified based on results from Database of Annotation, Visualization and Integrated Discovery (DAVID) and Ingenuity Pathways Analysis data.

Biosynthesis					
Id	Name	Abbreviation	Log	P-value	Notes
201805_AT	protein kinase, AMP-activated, gamma 1 non-catalytic subunit	PRKAG1	1.16	0.03	
232022_AT	T-cell lymphoma invasion and metastasis 2	TIAM2	-1.07	0.04	
1559204_X_AT	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	KRAS	1.14	0.03	
226392_AT	RAS p21 protein activator 2	RASA2	1.00	0.01	
221971_X_AT	ArfGAP with GTPase domain, ankyrin repeat and PH domain 10 /// ArfGAP with GTPase domain, ankyrin repeat and PH domain 4 /// ArfGAP with GTPase domain, ankyrin repeat and PH domain 5 /// ArfGAP with GTPase domain, ankyrin repeat and PH domain 9	AGAP8	-1.00	0.02	
216266_s_at	ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited)	Hs.411848	-1.01	0.01	
231087_AT	resistance to inhibitors of cholinesterase 8 homolog B (C. elegans)	Hs.666086	-1.08	0.04	
232223_AT	regulator of G-protein signaling 3	RGS3	-1.10	0.00	
242143_AT	RAN binding protein 9	Hs.611969	-1.20	0.02	
236041_AT	TBC1 domain family, member 9B (with GRAM domain)	TBC1D9B	-1.31	0.05	
1558473_AT	DEP domain containing 1B	Hs.560705	-1.36	0.01	
1555444_A_AT	protein phosphatase 1, regulatory (inhibitor) subunit 12B	PPP1R12B	-1.69	0.01	
212099_AT	ras homolog gene family, member B	RHOB	1.06	0.02	
240788_AT	malic enzyme 1, NADP(+)-dependent, cytosolic	ME1	-1.10	0.01	
219525_AT	solute carrier family 47, member 1	SLC47A1	-1.14	0.04	
201297_S_AT	MOB1, Mps One Binder kinase activator-like 1B (yeast)	MOBKL1B	1.26	0.04	
223527_S_AT	cytidine and dCMP deaminase domain containing 1	CDADC1	1.25	0.05	

Table 2. Continued

Id	Name	Abbreviation	Log	P-value	Notes
57588_AT	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	SLC24A3	1.23	0.03	
209274_S_AT	iron-sulfur cluster assembly 1 homolog (S. cerevisiae)	ISCA1	1.18	0.03	
203097_S_AT	Rap guanine nucleotide exchange factor (GEF) 2	RAPGEF2	1.16	0.02	
235899_AT	carbonic anhydrase XIII	CA13	1.11	0.05	
228181_AT	solute carrier family 30 (zinc transporter), member 1	SLC30A1	1.09	0.01	
206300_S_AT	parathyroid hormone-like hormone	PTHLH	1.08	0.03	
200059_S_AT	ras homolog gene family, member A	RHOA	1.04	0.03	
210854_X_AT	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	SLC6A8	1.01	0.02	
1555789_S_AT	PHD finger protein 23	PHF23	1.00	0.02	
212153_AT	pogo transposable element with ZNF domain	POGZ	-1.01	0.04	
1558748_AT	N-acyl phosphatidylethanolamine phospholipase D	Hs.660258	-1.02	0.02	
236475_AT	Microtubule associated monoxygenase, calponin and LIM domain containing 2	MICAL2	-1.02	0.05	
226435_AT	papilin, proteoglycan-like sulfated glycoprotein	PAPLN	-1.03	0.05	
218476_AT	protein-O-mannosyltransferase 1	POMT1	-1.07	0.02	
1552735_AT	protocadherin gamma subfamily A, 4	Hs.368160	-1.07	0.04	
202943_S_AT	N-acetylgalactosaminidase, alpha-	NAGA	-1.08	0.01	
221562_S_AT	sirtuin 3	SIRT3	-1.09	0.05	
1563203_AT	S-phase cyclin A-associated protein in the ER	Hs.684485	-1.10	0.01	
230885_AT	spastic paraplegia 7 (pure and complicated autosomal recessive)	SPG7	-1.17	0.01	
209381_X_AT	splicing factor 3a, subunit 2, 66kDa	SF3A2	-1.23	0.03	
235339_AT	SET domain, bifurcated 2	SETDB2	-1.27	0.01	

Table 2. Continued

Id	Name	Abbreviation	Log	P-value	Notes
1562495_AT	Ankyrin repeat and FYVE domain containing 1	Hs.696087	-1.27	0.00	
203865_S_AT	adenosine deaminase, RNA-specific, B1	ADARB1	-1.31	0.05	
223695_S_AT	arylsulfatase D	ARSD	-1.39	0.02	
242966_X_AT	acyl-CoA synthetase bubblegum family member 2	ACSBG2	-1.11	0.05	
232590_AT	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit	HADHA	-1.17	0.01	
205910_S_AT	carboxyl ester lipase (bile salt-stimulated lipase) /// bile salt-activated lipase-like	CEL	-1.35	0.01	
221553_AT	magnesium transporter 1	MAGT1	2.22	0.03	
203108_AT	G protein-coupled receptor, family C, group 5, member A	GPRC5A	1.32	0.01	
1554679_A_AT	lysosomal protein transmembrane 4 beta	LAPTM4B	1.23	0.01	
224949_AT	Yip1 domain family, member 5	YIPF5	1.19	0.05	
207791_S_AT	RAB1A, member RAS oncogene family	RAB1A	1.19	0.03	
243880_AT	Golgi SNAP receptor complex member 2	Hs.463278	1.10	0.02	
212218_S_AT	fatty acid synthase	FASN	1.03	0.02	
212040_AT	trans-golgi network protein 2	TGOLN2	1.02	0.05	
236470_AT	dehydrogenase/reductase (SDR family) member 1	Hs.606038	1.00	0.03	
214197_S_AT	SET domain, bifurcated 1	SETDB1	-1.04	0.03	
244511_AT	hypothetical protein LOC100134230; similar to KIAA0454 protein; similar to phosphodiesterase 4D interacting protein isoform 2; phosphodiesterase 4D interacting protein	Hs.669666	-1.07	0.01	
227014_AT	aspartate beta-hydroxylase domain containing 2	ASPHD2	-1.09	0.02	
1555385_AT	beta-1,4-N-acetyl-galactosaminyl transferase 1	B4GALNT1	-1.25	0.01	
238418_AT	solute carrier family 35, member B4	SLC35B4	-1.36	0.02	
212390_AT	phosphodiesterase 4D interacting protein	PDE4DIP	-1.49	0.02	

Table 2. Continued

Id	Name	Abbreviation	Log	P-value	Notes
203656_AT	FIG4 homolog, SAC1 lipid phosphatase domain containing (S. cerevisiae)	FIG4	-1.54	0.03	
239930_AT	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2)	GALNTL2	-1.03	0.01	
236075_S_AT	hypothetical LOC100506676	LOC100506676	-1.18	0.03	
1555282_A_AT	peroxisome proliferator-activated receptor gamma, coactivator 1 beta	PPARGC1B	-1.03	0.04	
1552829_AT	sphingomyelin synthase 1	SGMS1	1.24	0.01	
227038_AT	sphingomyelin synthase 2	SGMS2	2.16	0.00	
219257_S_AT	sphingosine kinase 1	SPHK1	1.23	0.03	
221765_AT	UDP-glucose ceramide glucosyltransferase	UGCG	1.14	0.01	
1554835_A_AT	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	B3GNT5	1.11	0.01	
229850_AT	3-ketodihydrosphingosine reductase	KDSR	1.05	0.03	
216083_S_AT	sialidase 3 (membrane sialidase)	NEU3	-1.03	0.04	
206504_AT	cytochrome P450, family 24, subfamily A, polypeptide 1	CYP24A1	1.05	0.00	
203615_X_AT	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	SULT1A1	-1.04	0.02	
1559916_A_AT	carbohydrate (chondroitin 4) sulfotransferase 12	BC029471	-1.29	0.01	
210070_S_AT	choline kinase-like, carnitine palmitoyltransferase 1B (muscle) transcription unit /// carnitine palmitoyltransferase 1B (muscle)	CPT1B	-1.57	0.01	

Table 2. Continued

Transcription, translation, and histone modification

Id	Name	Abbreviation	Log	P-value	Notes
236899_AT	interferon regulatory factor 2 binding protein 2	BC022885	1.30	0.04	Transcription
239788_AT	smu-1 suppressor of mec-8 and unc-52 homolog (C. elegans)	ATF7IP2	1.29	0.04	Transcription
224367_AT	brain expressed X-linked 2	AIMP1	1.24	0.01	Transcription
213575_AT	transformer 2 alpha homolog (Drosophila)	ABCA1	1.23	0.05	Transcription
207791_S_AT	RAB1A, member RAS oncogene family	AI076370	1.19	0.03	Transcription
236146_AT	synaptotagmin binding, cytoplasmic RNA interacting	BEX2	1.17	0.02	Transcription
214688_AT	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)	ARPP19	1.17	0.02	Transcription
239321_AT	prothymosin, alpha pseudogene	Hs.684898	1.16	0.04	Transcription
1567303_AT	CDC14 cell division cycle 14 homolog A (S. cerevisiae)	Hs.600876	1.14	0.02	Transcription
1570533_AT	cytidine monophosphate (UMP-CMP) kinase 1, cytosolic	CDKN1A	1.10	0.04	Transcription
201862_S_AT	leucine rich repeat (in FLII) interacting protein 1	CMPK1	1.10	0.02	Transcription
226952_AT	ELL associated factor 1	CYP24A1	1.08	0.00	Transcription
214245_AT	ribosomal protein S14	DLX6	1.08	0.02	Transcription
219294_AT	centromere protein Q	EAF1	1.00	0.01	Transcription
241092_AT	bobby sox homolog (Drosophila)	EID2B	-1.00	0.05	Transcription
227465_AT	MAU2 chromatid cohesion factor homolog (C. elegans)	EIF4EBP2	-1.01	0.01	Transcription
212153_AT	pogo transposable element with ZNF domain	AK024185	-1.01	0.04	Transcription
237508_AT	NHP2 ribonucleoprotein homolog (yeast)	AL049930	-1.02	0.04	Transcription
205522_AT	homeobox D3 /// homeobox D4 /// microRNA 10b	GEMIN8	-1.02	0.05	Transcription
240074_AT	Snf2-related CREBBP activator protein	H2AFY	-1.03	0.01	Transcription
214197_S_AT	SET domain, bifurcated 1	Hs.667420	-1.04	0.03	Transcription
228252_AT	PIF1 5'-to-3' DNA helicase homolog (S. cerevisiae)	Hs.596208	-1.05	0.04	Transcription

Table 2. Continued

Id	Name	Abbreviation	Log	P-value	Notes
223930_AT	torsin A interacting protein 1	HEXIM1	-1.05	0.04	Transcription
1569868_S_AT	essential meiotic endonuclease 1 homolog 2 (S. pombe)	HMG20A	-1.05	0.02	Transcription
1559044_AT	exosome component 1	HDAC9	-1.06	0.01	Transcription
244511_AT	hypothetical protein LOC100134230; similar to KIAA0454 protein; similar to phosphodiesterase 4D interacting protein isoform 2; phosphodiesterase 4D interacting protein	HOXA2	-1.07	0.01	Transcription
226900_AT	hypothetical LOC100129387	Hs.591609	-1.08	0.01	Transcription
232621_AT	ubiquitin specific peptidase 48	Hs.669666	-1.08	0.02	Transcription
239654_AT	Chromodomain helicase DNA binding protein 9	Hs.482077	-1.08	0.02	Transcription
221562_S_AT	sirtuin 3	BF445387	-1.09	0.05	Transcription
219658_AT	pentatricopeptide repeat domain 2	KLHL31	-1.10	0.04	Transcription
1563203_AT	S-phase cyclin A-associated protein in the ER	Hs.147710	-1.10	0.01	Transcription
242470_AT	EP300 interacting inhibitor of differentiation 2B	LRRFIP1	-1.10	0.03	Transcription
218152_AT	high-mobility group 20A	Hs.684041	-1.10	0.03	Transcription
239699_S_AT	postmeiotic segregation increased 2 pseudogene 1 /// postmeiotic segregation increased 2 pseudogene 5	MAU2	-1.11	0.03	Transcription
218860_AT	nucleolar complex associated 4 homolog (S. cerevisiae)	MBNL2	-1.13	0.04	Transcription
203117_S_AT	PAN2 poly(A) specific ribonuclease subunit homolog (S. cerevisiae)	MYH11	-1.19	0.02	Transcription
214457_AT	homeobox A2	Hs.197071	-1.20	0.03	Transcription
213851_AT	transmembrane protein 110	NOC4L	-1.20	0.01	Transcription
239197_S_AT	enhancer of zeste homolog 1 (Drosophila)	NOL12	-1.21	0.03	Transcription
222057_AT	nucleolar protein 12	PAN2	-1.25	0.00	Transcription
1564656_AT	5'-3' exoribonuclease 1	SMU1	-1.26	0.01	Transcription

Table 2. Continued

Id	Name	Abbreviation	Log	P-value	Notes
215545_AT	excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)	POGZ	-1.26	0.00	Transcription
235339_AT	SET domain, bifurcated 2	POLH	-1.27	0.01	Transcription
222139_AT	KIAA1466 gene	PMS2P5	-1.32	0.02	Transcription
219870_AT	activating transcription factor 7 interacting protein 2	Hs.653264	-1.35	0.02	Transcription
236699_AT	muscleblind-like 2 (Drosophila)	RAB1A	-1.38	0.00	Transcription
239398_AT	kelch-like 31 (Drosophila)	RPA4	-1.41	0.02	Transcription
212390_AT	phosphodiesterase 4D interacting protein	RC3H2	-1.49	0.02	Transcription
221143_AT	replication protein A4, 30kDa	SETDB1	-1.51	0.01	Transcription
228928_X_AT	BTG3 associated nuclear protein	SETDB2	-1.58	0.05	Transcription
222879_S_AT	polymerase (DNA directed), eta	Hs.673510	-1.70	0.02	Transcription
244381_AT	HEAT repeat containing 1	SPAG9	-1.88	0.00	Transcription
1568449_AT	ribosomal protein S6 kinase, 90kDa, polypeptide 3	SF3A2	-1.66	0.02	Transcription
232121_AT	tRNA aspartic acid methyltransferase 1		-1.05	0.04	Translation
228133_S_AT	myosin, heavy chain 11, smooth muscle	TAF13	-1.10	0.03	Translation
236142_AT	Peptidylprolyl isomerase H (cyclophilin H)	TAF9B	-1.11	0.01	Translation
230758_AT	gem (nuclear organelle) associated protein 8	Hs.644466	-1.45	0.02	Translation
239402_AT	COX10 homolog, cytochrome c oxidase assembly protein, heme A: farnesyltransferase (yeast)	TGFBR1	-1.47	0.00	Translation
214509_AT	histone cluster 1, H3i	POLH	1.40	0.04	Histone modificatio
1552760_AT	histone deacetylase 9	TRIOBP	1.08	0.01	Histone modificatio
229593_AT	H2A histone family, member Y	RPS6KA3	-1.25	0.02	Histone modificatio

Table 2. Continued

Cell cycle

Id	Name	Abbreviation	Log	P-value	Notes
208223_S_AT	activin A receptor, type IB	ACVR1B	1.59	0.00	epidermis
211258_S_AT	transforming growth factor, alpha	TGFA	1.32	0.00	epidermis
1552478_A_AT	interferon regulatory factor 6	IRF6	1.25	0.00	epidermis
1564630_AT	endothelin 1	EDN1	1.14	0.01	epidermis
201324_AT	epithelial membrane protein 1	EMP1	1.06	0.03	epidermis
210511_S_AT	inhibin, beta A	INHBA	1.03	0.01	epidermis
233011_AT	Annexin A1	ANXA1	-1.09	0.04	epidermis
207389_AT	glycoprotein Ib (platelet), alpha polypeptide	GP1BA	-1.12	0.01	epidermis
1559976_AT	heme binding protein 1	HEBP1	-1.19	0.03	epidermis
231031_AT	keratinocyte growth factor-like protein 2	Hs.536967	-1.39	0.02	epidermis
239876_AT	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	-1.44	0.02	epidermis
201324_AT	epithelial membrane protein 1	PLAUR	1.06	0.03	Tissue
210511_S_AT	inhibin, beta A	EMP1	1.03	0.01	Tissue
1555520_AT	patched 1	PTCH1	1.64	0.01	
223709_S_AT	wingless-type MMTV integration site family, member 10A	WNT10A	1.45	0.02	
232354_AT	vacuolar protein sorting 37 homolog B (S. cerevisiae)	AK022083	1.37	0.01	
243376_AT	TRAF family member-associated NFKB activator	TANK	1.23	0.02	
221482_S_AT	cAMP-regulated phosphoprotein, 19kDa	ARPP19	1.16	0.04	
231920_S_AT	casein kinase 1, gamma 1	CSNK1G1	1.13	0.02	
206943_AT	transforming growth factor, beta receptor 1	TGFBR1	1.11	0.03	
216040_X_AT	RAB11 family interacting protein 3 (class II)	RAB11FIP3	1.08	0.01	
203788_S_AT	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	1.06	0.01	

Table 2. Continued

Id	Name	Abbreviation	Log	P-value	Notes
201324_AT	epithelial membrane protein 1	EMP1	1.06	0.03	
228394_AT	serine/threonine kinase 10	Hs.519756	1.03	0.01	
210511_S_AT	inhibin, beta A	INHBA	1.03	0.01	
237761_AT	NIMA (never in mitosis gene a)-related kinase 6	NEK6	-1.03	0.05	
242581_AT	Mitogen-activated protein kinase kinase kinase 15	Hs.530331	-1.06	0.02	
233986_S_AT	pleckstrin homology domain containing, family G (with RhoGef domain) member 2	PLEKHG2	-1.07	0.04	
219407_S_AT	laminin, gamma 3	LAMC3	-1.09	0.04	
202453_S_AT	general transcription factor IIH, polypeptide 1, 62kDa	GTF2H1	-1.10	0.04	
226004_AT	Cdk5 and Abl enzyme substrate 2	CABLES2	-1.11	0.03	
205167_S_AT	cell division cycle 25 homolog C (S. pombe)	CDC25C	-1.17	0.02	
227330_X_AT	hypothetical protein LOC100132288 /// hypothetical LOC100233156 /// MAFF interacting protein	MAFIP	-1.22	0.04	
1559394_A_AT	receptor tyrosine kinase-like orphan receptor 1	AF086217	-1.23	0.02	
233118_AT	AT rich interactive domain 5B (MRF1-like)	ARID5B	-1.45	0.02	
1552478_A_AT	interferon regulatory factor 6	IRF6	1.25	0.00	

Innate immune response

Id	Name	Abbreviation	Log	P-value	Notes
1554997_A_AT	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	2.49	0.00	
202859_X_AT	interleukin 8	IL8	2.08	0.03	
215813_S_AT	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	2.02	0.02	
207526_S_AT	interleukin 1 receptor-like 1	IL1RL1	2.01	0.00	
211924_S_AT	plasminogen activator, urokinase receptor	PLAUR	1.95	0.01	

Table 2. Continued

Id	Name	Abbreviation	Log	P-value	Notes
204470_AT	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	CXCL1	1.47	0.01	
202644_S_AT	tumor necrosis factor, alpha-induced protein 3	TNFAIP3	1.45	0.00	
231779_AT	interleukin-1 receptor-associated kinase 2	IRAK2	1.31	0.01	
201926_S_AT	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	CD55	1.31	0.03	
207850_AT	chemokine (C-X-C motif) ligand 3	CXCL3	1.30	0.03	
228699_AT	Neuropilin 2	NRP2	1.19	0.02	
227697_AT	suppressor of cytokine signaling 3	SOCS3	1.11	0.01	
243213_AT	signal transducer and activator of transcription 3 (acute-phase response factor)	STAT3	1.05	0.03	
235531_AT	interleukin 17 receptor B	IL17RB	-1.01	0.01	
238099_AT	heat shock 70kDa protein 4	HSPA4	-1.06	0.03	
211133_X_AT	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 6 /// leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	LILRB3	-1.07	0.01	
1554091_A_AT	toll-interleukin 1 receptor (TIR) domain containing adaptor protein	TIRAP	-1.09	0.03	
241316_AT	trinucleotide repeat containing 6A	TNRC6A	-1.11	0.01	
224093_AT	interferon, kappa	IFNK	-1.12	0.04	
214370_AT	S100 calcium binding protein A8	S100A8	-1.16	0.03	
206975_AT	lymphotoxin alpha (TNF superfamily, member 1)	LTA	-1.18	0.00	
208492_AT	regulatory factor X-associated protein	RFXAP	-1.35	0.02	
223599_AT	tripartite motif-containing 6	TRIM6	-1.45	0.04	

Table 2. Continued

Cytoskeleton organization

Id	Name	Abbreviation	Log	P-value	Notes
212468_AT	sperm associated antigen 9	SPAG9	1.57	0.02	
202674_S_AT	LIM domain 7	LMO7	1.42	0.01	
234240_AT	gelsolin (amyloidosis, Finnish type)	GSN	1.22	0.02	
236192_AT	hook homolog 3 (Drosophila)	HOOK3	1.21	0.00	
205121_AT	sarcoglycan, beta (43kDa dystrophin-associated glycoprotein)	SGCB	1.12	0.04	
226000_AT	CTTNBP2 N-terminal like	CTTNBP2NL	1.06	0.03	
239170_AT	ARP3 actin-related protein 3 homolog (yeast)	ACTR3B	1.01	0.01	
208621_S_AT	ezrin	EZR	1.01	0.02	
228876_AT	BAI1-associated protein 2-like 2	BAIAP2L2	-1.05	0.03	
1555261_AT	macrophage erythroblast attacher	MAEA	-1.07	0.03	
242420_AT	protein phosphatase 1, regulatory (inhibitor) subunit 9A	PPP1R9A	-1.15	0.01	
243690_AT	TRIO and F-actin binding protein	TRIOBP	-1.18	0.03	
223513_AT	centromere protein J	CENPJ	-1.22	0.03	
228310_AT	enabled homolog (Drosophila)	ENAH	-1.27	0.02	
225168_AT	FERM domain containing 4A	FRMD4A	-1.30	0.00	
1552749_A_AT	kinesin light chain 3	KLC3	-1.30	0.01	
239023_AT	CDC42 small effector 1	FHDC1	-1.34	0.02	
244333_AT	FH2 domain containing 1	CDC42SE1	-1.86	0.00	
1554293_AT	tau tubulin kinase 2	TTBK2	-1.29	0.01	

Table 2. Continued

Ubiquitination

Id	Name	Abbreviation	Log	P-value	Notes
204614_AT	serpin peptidase inhibitor, clade B (ovalbumin), member 2	BRE	1.58	0.02	
232020_AT	SMAD specific E3 ubiquitin protein ligase 2	TBL1X	1.43	0.02	
1569306_AT	similar to calcyclin binding protein; calcyclin binding protein	CTSK	1.38	0.03	
1569472_S_AT	tetratricopeptide repeat domain 3	SERPINB2	1.26	0.05	
205479_S_AT	plasminogen activator, urokinase	PLAU	1.19	0.00	
227334_AT	ubiquitin specific peptidase 54	USP54	-1.04	0.03	
243554_AT	zer-1 homolog (C. elegans)	SMURF2	-1.07	0.02	
232621_AT	ubiquitin specific peptidase 48	USP48	-1.08	0.02	
1561370_AT	brain and reproductive organ-expressed (TNFRSF1A modulator)		-1.14	0.03	
236075_S_AT	hypothetical LOC100506676	USP31	-1.18	0.03	
239554_AT	ring finger protein 13	ZER1	-1.18	0.02	
202450_S_AT	cathepsin K	TTC3	-1.24	0.04	
239348_AT	ubiquitin specific peptidase 31	Hs.667512	-1.24	0.01	
235768_AT	SH3 domain containing ring finger 2	CACYBP	-1.26	0.04	
201869_S_AT	transducin (beta)-like 1X-linked	SH3RF2	-1.55	0.00	

Cell Adhesion

Id	Name	Abbreviation	Log	P-value	Notes
217689_AT	Protein tyrosine phosphatase, non-receptor type 1	PTPN1	1.33	0.04	
202933_S_AT	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	LYN	1.18	0.02	
201259_S_AT	synaptophysin-like 1	SYPL1	1.06	0.01	
219399_AT	lin-7 homolog C (C. elegans)	LIN7C	1.03	0.04	

Table 2. Continued

Id	Name	Abbreviation	Log	P-value	Notes
201289_AT	cysteine-rich, angiogenic inducer, 61	CYR61	1.02	0.01	
227314_AT	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	ITGA2	1.02	0.01	
212939_AT	collagen, type VI, alpha 1	COL6A1	1.00	0.03	
204740_AT	connector enhancer of kinase suppressor of Ras 1	CNKSR1	-1.08	0.01	
240331_AT	bone morphogenetic protein receptor, type IB	BMPR1B	-1.34	0.01	
204537_S_AT	gamma-aminobutyric acid (GABA) A receptor, epsilon	GABRE	-1.73	0.02	
240417_AT	plakophilin 4	PKP4	-1.74	0.00	

Apoptosis

Id	Name	Abbreviation	Log	P-value	Notes
224016_AT	homeodomain interacting protein kinase 2	HIPK1	1.28	0.01	
211317_S_AT	CASP8 and FADD-like apoptosis regulator	CFLAR	1.24	0.03	
202284_S_AT	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	1.14	0.01	
230499_AT	baculoviral IAP repeat-containing 3	BIRC3	1.11	0.01	
225674_AT	B-cell receptor-associated protein 29	BCAP29	1.03	0.05	
224414_S_AT	caspase recruitment domain family, member 6	CARD6	-1.01	0.03	
238888_AT	Tax1 (human T-cell leukemia virus type I) binding protein	TAX1BP1	-1.04	0.02	
235594_AT	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	AIMP1	-1.10	0.03	
204860_S_AT	baculoviral IAP repeat-containing protein 1-like	LOC100510700	-1.11	0.01	
219628_AT	zinc finger, matrin-type 3	ZMAT3	-1.23	0.04	

Table 2. Continued

Hormone Regulation

Id	Name	Abbreviation	Log	P-value	Notes
226847_AT	follistatin	FST	1.40	0.01	
1568874_AT	nuclear receptor coactivator 6	NCOA6	1.32	0.01	
209750_AT	nuclear receptor subfamily 1, group D, member 2	NR1D2	1.07	0.00	
232958_AT	phosphorylase, glycogen, liver	PYGL	-1.04	0.01	
1560259_AT	RAR-related orphan receptor A	RARA	-1.11	0.01	
224653_AT	eukaryotic translation initiation factor 4E binding protein 2	EIF4EBP2	-1.14	0.03	
1561872_AT	transforming growth factor, beta receptor II (70/80kDa)	TGFBR2	-1.14	0.01	
227432_S_AT	insulin receptor	INSR	-1.29	0.05	

Phagocytosis

Id	Name	Abbreviation	Log	P-value	Notes
211960_S_AT	RAB7A, member RAS oncogene family	RAB7A	1.48	0.04	
223763_AT	dystrobrevin binding protein 1	DTNBP1	1.21	0.02	
216066_AT	ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1	1.13	0.01	
219411_AT	engulfment and cell motility 3	ELMO3	-1.19	0.02	

Transport

Id	Name	Abbreviation	Log	P-value	Notes
235295_AT	pannexin 1	PANX1	1.11	0.04	
243880_AT	Golgi SNAP receptor complex member 2	Hs.463278	1.10	0.02	
1569713_AT	SEC24 family, member B (S. cerevisiae)	SEC24B	-1.17	0.02	