# UCSF UC San Francisco Previously Published Works

## Title

HIV-1 proteins gp120 and tat induce the epithelial-mesenchymal transition in oral and genital mucosal epithelial cells.

## Permalink

https://escholarship.org/uc/item/0rf683k4

Journal PLoS ONE, 14(12)

## Authors

Lien, Kathy Mayer, Wasima Herrera, Rossana <u>et al.</u>

Publication Date 2019

## DOI

10.1371/journal.pone.0226343

## **Copyright Information**

This work is made available under the terms of a Creative Commons Attribution License, available at <u>https://creativecommons.org/licenses/by/4.0/</u>

Peer reviewed



# GOPEN ACCESS

**Citation:** Lien K, Mayer W, Herrera R, Rosbe K, Tugizov SM (2019) HIV-1 proteins gp120 and tat induce the epithelial–mesenchymal transition in oral and genital mucosal epithelial cells. PLoS ONE 14(12): e0226343. https://doi.org/10.1371/journal. pone.0226343

Editor: Guido Silvestri, Emory University, UNITED STATES

Received: August 28, 2019

Accepted: November 19, 2019

Published: December 23, 2019

**Copyright:** © 2019 Lien et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was supported by R01CA232887, NATIONAL CANCER INSTITUTE, and NATIONAL INSTITUTE OF DENTAL & CRANIOFACIAL RESEARCH, R01DE028129, to ST. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. **RESEARCH ARTICLE** 

# HIV-1 proteins gp120 and tat induce the epithelial–mesenchymal transition in oral and genital mucosal epithelial cells

# Kathy Lien<sup>1</sup>, Wasima Mayer<sup>1</sup>, Rossana Herrera<sup>1</sup>, Kristina Rosbe<sup>2,3</sup>, Sharof M. Tugizov<sup>1</sup>\*

1 Department of Medicine, University of California–San Francisco, San Francisco, CA, United States of America, 2 Department of Otolaryngology, University of California–San Francisco, San Francisco, CA, United States of America, 3 Department of Obstetrics, Gynecology & Reproductive Sciences, University of California–San Francisco, San Francisco, CA, United States of America

\* sharof.tugizov@ucsf.edu

# Abstract

The oral, cervical, and genital mucosa, covered by stratified squamous epithelia with polarized organization and strong tight and adherens junctions, play a critical role in preventing transmission of viral pathogens, including human immunodeficiency virus (HIV). HIV-1 interaction with mucosal epithelial cells may depolarize epithelia and disrupt their tight and adherens junctions; however, the molecular mechanism of HIV-induced epithelial disruption has not been completely understood. We showed that prolonged interaction of cell-free HIV-1 virions, and viral envelope and transactivator proteins gp120 and tat, respectively, with tonsil, cervical, and foreskin epithelial cells induces an epithelial-mesenchymal transition (EMT). EMT is an epigenetic process leading to the disruption of mucosal epithelia and allowing the paracellular spread of viral and other pathogens. Interaction of cell-free virions and gp120 and tat proteins with epithelial cells substantially reduced E-cadherin expression and activated vimentin and N-cadherin expression, which are well-known mesenchymal markers. HIV gp120- and tat-induced EMT was mediated by SMAD2 phosphorylation and activation of transcription factors Slug, Snail, Twist1 and ZEB1. Activation of TGF-β and MAPK signaling by gp120, tat, and cell-free HIV virions revealed the critical roles of these signaling pathways in EMT induction. gp120- and tat-induced EMT cells were highly migratory via collagen-coated membranes, which is one of the main features of mesenchymal cells. Inhibitors of TGF-β1 and MAPK signaling reduced HIV-induced EMT, suggesting that inactivation of these signaling pathways may restore the normal barrier function of mucosal epithelia.

### Introduction

The oropharyngeal, ectocervical, vaginal, and foreskin epithelia consist of a multilayered, stratified squamous epithelium supported by an underlying layer of fibrous connective tissue, the lamina propria. The endocervical and intestinal mucosa are covered with monostratified simple epithelium. All mucosal epithelia form multiple intercellular junctions, including tight and **Competing interests:** The authors have declared that no competing interests exist.

adherens junctions [1-10], which are critical for maintaining the morphologic and physiologic features of mucosal epithelia, including their barrier functions. Tight junctions of mucosal epithelium form the physical tissue barrier between epithelial cells that protects the internal body from the penetration of external infectious agents [11], including pathogenic viruses.

In individuals with HIV-caused acquired immunodeficiency syndrome (AIDS), tight junctions in oral, intestinal, and genital mucosal epithelia are disrupted, leading to impairment of mucosal functions [7, 12–18]. In vitro studies show that the interaction of HIV proteins gp120 and tat with mucosal epithelia may disrupt tight and adherens junctions of epithelial cells, reducing their barrier functions [7, 19–26].

We have shown that prolonged interaction of HIV envelope protein gp120 and transactivator protein tat with oral and genital epithelia reduces the expression of tight junction proteins occludin and zonula occludens-1, claudin-1, and adherens junction protein E-cadherin, leading to depolarization of epithelial cells [7, 19, 21, 22]. Downregulation of proteins of adherence and tight junctions of epithelial cells and their depolarization may lead to an epithelial–mesenchymal transition (EMT) [27–29].

EMT is a normal multistep epigenetic process in embryonic development that regulates the differentiation of cell lineage identity [30-32]. However, the EMT phenotype also plays an important role in neoplastic processes, facilitating growth, migration and metastasis of tumor cells [30, 33–39]. During cancer-associated EMT, epithelial cells lose cell-cell junctions and become proliferative and invasive [40]. The TGF- $\beta$  signaling pathway is the dominant canonical regulatory network for this process [41, 42]. Binding of mature TGF-β to TGF-β1 R2 activates TGF-β signaling, leading to activation of downstream molecules, including Smad family transcription factor complexes [43]. These complexes activate the transcriptional regulators Snail, Slug, and Twist1. Activation of Snail and Twist1 may lead to activation of other transcription factors, ZEB1 and ZEB2 [44]. Cooperation between these transcription factors leads to downregulation of E-cadherin and cytokeratin and upregulation of vimentin, fibronectin, and N-cadherin expression [45–49]. Expression of fibronectin is critical for invasion of cancer cells [50–52]. N-cadherin expression plays an important role in the transmigration of cancer cells via endothelial cells, promoting spread and metastasis of neoplastic cells via blood circulation [53–55]. Overexpression of Snail also represses expression of tight junction proteins claudins and occludin-1, leading to depolarization of epithelial cells and EMT [27]. TGF- $\beta$  may activate Ras-MAPK signaling pathways, which also play a critical role in EMT induction by phosphorylation of Smad2/3 and TWIST1 [56-63]. Crosstalk between TGF-β and MAPK signaling is highly critical for induction and maintenance of the EMT phenotype [64].

The incidence of HPV-associated oropharyngeal cancer is increased in HIV-infected individuals [65–74]. HIV-positive individuals have about a sixfold greater risk for oropharyngeal and tonsillar cancers [75–79] than do uninfected individuals. In addition to oral cancer, the incidence of HPV-associated anal and cervical cancer is 80 and 22 times higher, respectively, in HIV-infected individuals than in uninfected individuals [80–84]. Thus, in HIV- and HPVcoinfected individuals, HIV-induced EMT may accelerate the HPV neoplastic process by increasing the paracellular spread of HPV and the invasion of HPV-infected malignant cells.

The primary goal of this study was to investigate the role of HIV proteins gp120 and tat in the induction of EMT in tonsil, cervical, and foreskin epithelial cells. We show here that exposure of normal tonsil, cervical, and foreskin keratinocytes to tat and gp120 and to cell-free virions for several days leads to the development of the EMT phenotype in these cells, including activation of TGF- $\beta$ 1 and vimentin expression and reduction of E-cadherin expression. HIV-induced EMT of oral and genital epithelium may play a critical role in reducing the barrier function of these epithelia, which may allow the penetration of various viral, bacterial, and fungal pathogens through oral and genital mucosal epithelia.

#### Results

#### HIV-associated EMT in oral mucosal epithelial cells of HIV/AIDS patents

We have shown that the oral epithelia of HIV-infected individuals have disrupted tight junctions [7] and that HIV tat/gp120 induces disruption of tight and adherens junctions of keratinocytes in vitro [7, 21]. Since disruption of epithelial junctions is one of the critical features of EMT [31, 35, 85], we immunostained buccal tissues from 10 HIV-infected and 3 uninfected individuals for E-cadherin, pancytokeratin, and vimentin. Four HIV-infected individuals were receiving antiretroviral treatment (ART) (#1- #4), and 6 were not receiving ART treatment (#5 - #10) (Figs 1 and 2).

Three buccal epithelia of uninfected donors showed expression of E-cadherin and pancytokeratin, but not vimentin, in all epithelial cells (Figs 1 and 2), which is characteristic of normal mucosal epithelia. E-cadherin expression in buccal epithelia of HIV-infected individuals receiving ART was reduced by 20–50% compared to uninfected epithelia. A significant reduction of pancytokeratin expression was not detected. However, these tissues showed induction of vimentin expression in 10–40% of epithelial cells (Fig 2). Buccal tissues from HIV-infected individuals not receiving ART also showed a reduction of E-cadherin and pancytokeratin expression. A substantial reduction of pancytokeratin expression was detected in 3 of 6 tissues (50%) from HIV-infected individuals without ART. In these 3 tissues (#5, #9 and #10), 60– 80% of cells lost E-cadherin and pancytokeratin expression. This was well correlated with a substantial induction of vimentin expression conforming to EMT phenotype in these epithelia. Moreover, EMT induction of all 3 tissues was correlated with higher viral load (S1 Table) and in 2 of them (#9 and #10) also was correlated with low CD4 count.

To confirm the HIV-induced EMT phenotype of oral mucosal epithelial cells, we isolated buccal keratinocytes from 3 uninfected and 3 HIV-infected individuals not receiving ART. Phase-contrast microscopy of oral keratinocytes showed that cells from uninfected individuals had cobblestone-like morphology of epithelial cell sheets with tightly connected cell borders. In contrast, keratinocytes from HIV-infected individuals had a spindle-like shape with weakly connected cell borders, which is typical of the EMT phenotype (Fig 3A).

Immunostaining of keratinocytes for E-cadherin and vimentin showed that oral keratinocytes from uninfected individuals had E-cadherin expression but no vimentin expression (Fig 3A). In contrast, oral keratinocytes from HIV-infected individuals showed a loss of E-cadherin and pancytokeratin and upregulation of vimentin (Fig 3A). These results were confirmed by Western blot assay (Fig 3B).

# HIV proteins tat and gp120 induce the EMT phenotype in oral and genital epithelial cells

To determine the role of HIV-1 in EMT induction, we cultured normal tonsil keratinocytes from uninfected individuals with HIV tat or gp120 proteins at their physiologically relevant concentrations (10 ng/ml each) [86–89], as described in our previous work [7, 21]. In parallel experiments, cells were treated with biologically inactive mutant tat lacking its basic arginine-rich domain and RGD motif [90–92]. HIV gp120 was heat-inactivated. Keratinocytes were maintained for 5 days with viral proteins, and culture medium was changed daily to add fresh virus or proteins.

This treatment should reflect the in vivo prolonged HIV interaction with cells/tissues, because in HIV/AIDS disease, virus progeny are detected in the blood/plasma, saliva, and cervicovaginal secretions; i.e., virions may interact with cells/tissues for days, months, or years. Phase-contrast microscopy of tonsil epithelial cells showed that untreated cells and cells treated



Fig 1. Oral epithelium of HIV-positive individuals has the EMT phenotype. Buccal tissues from HIV-infected and uninfected individuals were immunostained for Ecadherin, vimentin, and pancytokeratin expression (red). Nuclei are stained in blue. GR, granulosum; SP, spinosum; BL, basal; LP, lamina propria. Original magnification: x400. Representative immunofluorescence images are shown.

https://doi.org/10.1371/journal.pone.0226343.g001



#### **HIV** infected

**Fig 2. Quantitative analysis of EMT markers in oral epithelium of HIV-infected and uninfected individuals.** For quantitative evaluation of EMT marker expression in oral tissues of HIV-infected and uninfected individuals, epithelial cells expressing E-cadherin, vimentin, and pancytokeratin were counted from 10 randomly selected regions of mucosal epithelia. Results are presented as a percentage of epithelial cells expressing E-cadherin, vimentin, and pancytokeratin. Data are shown as the mean  $\pm$  SD (n = 10). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. E-cadherin, pancytokeratin and vimentin expression were compared in ART-treated or untreated samples from HIV-infected individuals with HIV uninfected (control) samples.

https://doi.org/10.1371/journal.pone.0226343.g002

with control tat and gp120 had normal epithelial morphology (Fig 4A). In contrast, tonsil keratinocytes treated with active tat and gp120 showed extensive changes in cell morphology, with a spindle-like shape.

To examine the expression of epithelial and mesenchymal markers, at day 5 after treatment we immunostained tonsil cells for E-cadherin, pancytokeratin, and vimentin. Immunofluorescence microscopy showed that untreated tonsil cells or tonsil cells treated with inactive tat and gp120 showed expression of E-cadherin and pancytokeratin, but not vimentin (Fig 4B). However, cells treated with active tat and gp120 inhibited E-cadherin and pancytokeratin expression but upregulated vimentin expression (Fig 4B). Quantitative analysis of E-cadherin and pancytokeratin expression showed that treatment of cells with tat and gp120 led to reduction of E-cadherin and pancytokeratin expression in 70–90% of treated cells compared to control cells (Fig 4C). Induction of vimentin expression in cells treated with active tat and gp120 reached 60–70%. Inhibition of E-cadherin and pancytokeratin and induction of vimentin expression by a combination of tat and gp120 reached ~90%.

Western blot analysis of EMT markers in tonsil epithelial cells treated with gp120 and tat proteins and their inactive controls showed that gp120 and tat substantially reduced E-cadherin expression and induced vimentin and N-cadherin expression (Fig 5). In contrast, inactive controls of gp120 and tat did not reduce E-cadherin and did not activate vimentin and Ncadherin expression. Furthermore, HIV-1 gp120 and tat activated phosphorylation of SMAD2



Fig 3. Oral keratinocytes isolated from HIV-infected individuals have an EMT phenotype. (A) Buccal keratinocytes propagated from HIV-infected and uninfected individuals were subjected to phase-contrast microscopy (upper panel) and immunostained for E-cadherin, vimentin, and pancytokeratin (lower panels). (B) Oral keratinocytes from 3 HIV-negative and 3 HIV-infected individuals were examined for E-cadherin, vimentin, and pancytokeratin by Western blot assay.

https://doi.org/10.1371/journal.pone.0226343.g003

and most of the critical transcription factors, including Slug, Snail, Twist1, and ZEB1, which play critical roles in EMT induction (Fig 5).

In the next experiments, we compared HIV-1 tat- and gp120-induced EMT in tonsil, cervical, and foreskin primary epithelial cells by prolonged treatment of cells with HIV proteins tat and gp120. Quantitative analysis of E-cadherin, pancytokeratin, and vimentin expression showed that tat and gp120 proteins inhibited E-cadherin and pancytokeratin and upregulated vimentin expression in all 3 epithelial cell cultures compared to controls (Fig 6). In cervical epithelial cells, reduction of E-cadherin and pancytokeratin by tat and gp120 was ~50%. Interestingly, in foreskin epithelial cells, tat and gp120 led to complete inhibition of E-cadherin expression but did not drastically affect pancytokeratin expression. Nevertheless, induction of vimentin expression was detected in ~70% of foreskin cells. These data indicate that HIV tatand gp120-induced EMT may occur in oral, cervical, and foreskin epithelial cells.



**Fig 4.** HIV proteins tat and gp120 induce the EMT phenotype in normal oral keratinocytes isolated from uninfected individuals. (A) Normal tonsil keratinocytes isolated from HIV-negative donors were untreated or treated with HIV tat and gp120; their inactive

forms were tested independently. After 5 days the live cells were examined using phase-contrast microscopy. (B) Tonsil keratinocytes treated as described in panel A were immunostained for E-cadherin, vimentin, and pancytokeratin. (C) Tonsil keratinocytes untreated or treated with HIV-1 gp120 and/or tat, and cells expressing E-cadherin, vimentin, and pancytokeratin were counted and presented as a percentage of cells expressing E-cadherin, vimentin, and pancytokeratin were counted and presented as a percentage of cells expressing E-cadherin, vimentin, and pancytokeratin of 3 independent experiments using tonsil epithelial cells derived from three donors and are shown as the mean  $\pm$  SD (n = 10). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. E-cadherin and pancytokeratin expression were compared with the gp120- and tat-treated and untreated (control) groups.

https://doi.org/10.1371/journal.pone.0226343.g004

# HIV cell-free virions via envelope gp120 induce EMT in oral and genital epithelial cells

The experiments described above showed that HIV protein gp120 induced EMT, suggesting that HIV cell-free virions may also induce EMT. To test this hypothesis, we treated tonsil, cervical, and foreskin keratinocytes with cell-free dual-tropic HIV- $1_{SF33}$ , R5-tropic HIV- $1_{SF170}$ , and X4-tropic HIV- $1_{92UG029}$  strains for 5 days. All three viruses reduced expression of E-cadherin and pancytokeratin and induced expression of vimentin (Fig 7).

To further confirm the role of HIV-1 gp120 in induction of EMT, HIV-1<sub>BAL</sub> gp120 protein were preincubated with a pool of 5 neutralizing antibodies (b12, 2G12, F105, 39F and ID6) or their isotype controls. Then tonsil cells were untreated or treated with gp120, gp120+isotype antibodies, or gp120+ neutralizing antibodies. Cell medium was changed every day, and EMT induction was evaluated after 5 days by quantitation of cells expressing E-cadherin, pancytokeratin, and vimentin. Results revealed that gp120 completely inhibited E-cadherin expression and reduced pancytokeratin expression by ~50% (Fig 8A). Vimentin induction by gp120 was in 50% of cells. In contrast, anti-gp120 antibodies prevented gp120-induced EMT in tonsil epithelial cells; i.e., E-cadherin and pancytokeratin expression were reduced by ~20% and induction of vimentin expression was eliminated. The isotype control antibodies did not affect gp120-mediated induction of EMT in tonsil cells.

To examine if gp120 from different viral strains induce EMT, gp120 proteins from 4 HIV-1 strains—HIV-1<sub>BAL</sub>, HIV-1<sub>IIIB</sub>, HIV-1<sub>CN-54</sub>, and HIV-1<sub>96ZM651</sub>—were preincubated with a pool of 5 neutralizing antibodies or their isotype controls and then added to the tonsil epithelial cells. Quantitation of cells expressing E-cadherin, pancytokeratin, and vimentin showed that gp120 proteins from 3 HIV-1 strains induced EMT, and anti-gp120 antibodies protected cells from gp120-induced EMT (Fig 8B). However, gp120 from HIV-1<sub>CN-54</sub> did not induce EMT in tonsil epithelial cells.

The role of HIV gp120 in induction of EMT was examined by using HIV-1  $\Delta$ env-NL4.3 and HIV-1 NL4.3 viruses. Analysis of EMT of tonsil epithelial cells incubated with these viruses showed that HIV-1  $\Delta$ env-NL4.3 failed to induce EMT in contrast to HIV-1 NL4.3, which inhibited E-cadherin and pancytokeratin expression and upregulated vimentin expression (Fig 8C). These findings clearly indicate that envelope protein of gp120 of cell-free HIV-1 induces EMT in tonsil epithelial cells.

To determine if HIV-induced EMT is a common phenomenon in mucosal oral epithelium, we incubated tonsil epithelial cells from 14 independent donors with  $HIV_{SF33}$  cell-free virions for 12 days. Expression of E-cadherin and vimentin showed cell-free HIV-induced downregulation of E-cadherin and upregulation of vimentin in tonsil cells from 8 of 14 donors (57%) (Fig 9), suggesting that HIV-induced EMT may develop in oral mucosal epithelia in 50% of HIV-infected individuals.

#### Activation of TGF-β1 and MAPK signaling in oral epithelia of HIVinfected individuals

To examine the status of TGF- $\beta$ 1 and MAPK signaling in oral epithelia of HIV-infected and uninfected individuals, we immunostained tissue sections of buccal biopsy samples from 3



**Fig 5. HIV gp120 and tat induce activation of EMT markers.** Tonsil epithelial cells isolated from uninfected individuals were treated for 7 days with gp120 and tat; their inactive controls were treated independently. Then cells

were examined for expression of E-cadherin, vimentin, N-cadherin, SMAD2, phosphorylated SMAD2, Slug, Snail, Twist1, and ZEB1 by Western blotting. Similar data were obtained in two independent experiments. Immunoblots were performed at least twice, and representative results are shown.

https://doi.org/10.1371/journal.pone.0226343.g005

HIV-infected and 3 uninfected individuals for TGF- $\beta$ 1 and for phosphorylated and total ERK1//2. Immunofluorescence analysis showed substantially higher levels of TGF- $\beta$ 1 expression in buccal epithelium of HIV-infected individuals than that in uninfected individuals (Fig 10A). Furthermore, ERK1/2 was highly phosphorylated in the oral epithelia of HIV-infected individuals but not in that of uninfected individuals (Fig 10B). Our recent work showed that HIV-tat/gp120-induced activation of MAPK in tonsil epithelial cells leads to reduction of E-cadherin expression, suggesting the role of HIV-associated MAPK activation in EMT [21, 22]. A similar trend was observed in all 3 biopsy tissues of HIV-infected individuals. These data indicate that TGF- $\beta$ 1 and MAPK signaling in the oral epithelia of HIV-infected individuals are activated, consistent with their potential roles in the induction of EMT.

HIV-induced activation of TGF-β1 and ERK1/2 signaling was also examined in tonsil keratinocytes in the in vitro experiments. Tonsil keratinocytes were treated with gp120 and tat proteins and their inactive forms, as well as with cell-free virions of dual-tropic HIV-1<sub>SF33</sub>, R5-tropic HIV-1<sub>SF170</sub>, and X4-tropic HIV-1<sub>92UG029</sub> strains for 10 days. After confirmation of EMT induction by light microscopy, cells were examined for TGF-β1 and ERK1/2 activation by Western blot assay. Data showed that tonsil keratinocytes incubated with active gp120 and tat as well as cell-free virions of HIV-1<sub>SF33</sub>, HIV-1<sub>SF170</sub>, and HIV-1<sub>92UG029</sub> strains induce activation of TGF-β1 by the resulting formation of cleaved mature TGF-β1 protein (Fig 11). In contrast, cells treated with inactive gp120 and tat proteins showed only the inactive form of TGF-β1. Phosphorylated ERK1/2 was also detected, mostly in cells treated with gp120, tat, and cell-free virions. These data clearly demonstrated that HIV proteins gp120 and tat are responsible for the activation of TGF-β1 and MAPK signaling, which are critical for induction of the EMT phenotype.

To confirm the functional roles of TGF- $\beta$ 1 and ERK1/2 signaling in HIV-induced EMT, we treated tonsil, cervical, and foreskin keratinocytes with nontoxic concentrations of inhibitors of MAPK UO126, TGF- $\beta$ 1 SB431542, and their combination. Drug-treated and untreated cells were incubated with cell-free HIV-1<sub>SF33</sub> virions. At 5 days after treatment, cells were immunostained for E-cadherin, pancytokeratin, and vimentin. Quantitative analysis showed that cells incubated with only HIV virions led to complete inhibition of E-cadherin and pancyto-keratin expression and significant induction of vimentin expression; i.e., ~75–80% of cells expressed vimentin (Fig 12). In contrast, cells incubated with HIV virions and inhibitors of TGF- $\beta$ 1 or/and MAPK show reduction of E-cadherin and pancytokeratin expression in ~20–40% of cells. Moreover, reduction of vimentin expression was highly significant, i.e., 50–80%. Thus, inhibition of TGF- $\beta$ 1 and ERK1/2 signaling significantly prevented EMT induction in tonsil, cervical, and foreskin epithelial cells by cell-free virions, indicating the critical roles of TGF- $\beta$ 1 and ERK1/2 signaling in HIV-induced EMT.

#### Transmigration of HIV-induced EMT cells

It is well known that EMT cells are highly motile and have intensive migratory activity [36–39]. To determine if tonsil, cervical, and foreskin cells incubated with HIV have migratory activity, we incubated cells with gp120 and tat proteins and their inactive forms for 5 days. Then, cells were seeded in Transwell inserts coated with collagen for 2 days. KGM medium was added to the lower chamber with 10% serum as a chemoattractant. Transmigration of cells via collagen-coated membrane pores was visualized by Giemsa staining (Fig 13A), and the rate



Fig 6. HIV gp120 and tat proteins induce the EMT phenotype in tonsil, cervical, and foreskin epithelial cells. Normal tonsil, cervical, and foreskin keratinocytes were isolated from HIV-negative donors and untreated or treated with HIV tat and gp120; their

inactive forms were treated independently. After 7 days, cells were fixed and immunostained for E-cadherin, pancytokeratin, and vimentin. Cells expressing E-cadherin, vimentin, and pancytokeratin were counted in 10 randomly selected regions. Results are presented as a percentage of epithelial cells expressing E-cadherin, vimentin, and pancytokeratin. Data are representative of 2 independent experiments using tonsil, cervical, and foreskin epithelial cells derived from two donors and are shown as the mean  $\pm$  SD (n = 10). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. E-cadherin and pancytokeratin expression were compared with the gp120- and tat-treated and untreated (control) groups.

https://doi.org/10.1371/journal.pone.0226343.g006

of transmigration and invasion was measured after 24 h by counting cells in the lower side of the membranes. Quantitative analysis showed that gp120 and tat proteins increased transmigration of cells by four- to fivefold compared to untreated cells or cells treated with inactive gp120 or tat (Fig 13B). These data indicate that HIV gp120- and tat-induced EMT cells transmigrated via collagen-coated membranes; i.e., they are highly motile and invasive, which are typical features of the EMT phenotype.

#### Discussion

Our findings clearly demonstrate that prolonged interaction of HIV proteins gp120 and tat and cell-free HIV virions with tonsil, cervical, and foreskin epithelial cells leads to development of the EMT phenotype in these cells. Loss of E-cadherin and upregulation of vimentin expression in the buccal epithelial tissues and their in vitro isolated keratinocytes from HIVinfected individuals suggest that HIV infection may play a critical role in EMT induction in oral epithelium in vivo. This idea is fully supported by the induction of EMT by HIV gp120 and tat and cell-free virions in vitro in the tonsil, cervical, and foreskin keratinocytes isolated from HIV-negative individuals.

A substantial reduction of E-cadherin and pancytokeratin expression and induction of vimentin in oral biopsy samples from ART-untreated individuals with higher viral load suggest that HIV infection may play a direct role in EMT induction. However, the moderate level of EMT induction also was observed in oral mucosal epithelium of ART-treated individuals, suggesting that oral intramucosal Langerhans cells, macrophages, and CD4+ T cells in the ART-treated patients may still have replicating virus, as shown in our previous work [7]. It is possible that ART may not completely eliminate HIV from intramucosal immune cells [7] due to a lower level of penetration of drugs into solid tissues [93–96]. We also cannot completely rule out the possible contribution of HIV-associated systemic elevation of proinflammatory cytokines in EMT induction [97–100].

If HIV infection is indeed a biologically relevant contributor to EMT, then HIV proteins should be present in the mucosal environment. Indeed, cell-free HIV-1 virions and viral DNA/RNA can be isolated from oral and genital mucosal epithelium, as well as the saliva and cervicovaginal secretions of HIV-positive individuals [7, 101–114]. HIV-infected lymphocytes, macrophages, and Langerhans cells were detected in the oral and genital mucosal epithelia of HIV-infected individuals, including those receiving ART [7, 101, 102, 107, 108, 112–115]. Secretion of HIV tat into blood has been shown [86, 87, 89]. HIV-1 gp120 and tat have also been detected in blood, saliva and lymphoid tissues [7, 88, 116–120]. Mucosal epithelium may therefore be exposed to cell-free HIV and tat and gp120 from multiple sources, including saliva, cervicovaginal secretions, and circulating immune cells (Fig 14). Mucosal epithelium may also serve as an HIV reservoir [113], as well as a source of proteins that induce EMT in the setting of HIV infection.

HIV-1 gp120- and tat-induced EMT cells express most critical transcription factors, including Slug, Snail, Twist1 and ZEB1, which downregulate epithelial markers and upregulate mesenchymal markers. Expression of these transcription factors was correlated with expression of the mature form of TGF- $\beta$ 1; i.e., gp120- and tat-induced EMT is due to activation of TGF- $\beta$ 1



Fig 7. Cell-free HIV virions induce the EMT phenotype in tonsil, cervical, and foreskin epithelial cells. Normal tonsil, cervical, and foreskin keratinocytes isolated from HIV-negative donors were untreated or treated for 7 days with cell-free dual-tropic HIV-1<sub>SF33</sub>, R5-tropic HIV-1<sub>SF170</sub>, and

X4-tropic HIV-1<sub>92UG029</sub> strains. Cells were fixed and immunostained for E-cadherin, pancytokeratin, and vimentin, and cells expressing these proteins were quantitatively evaluated. Results are presented as a percentage of epithelial cells expressing E-cadherin, vimentin, and pancytokeratin. Data are representative of 2 independent experiments using tonsil, cervical, and foreskin epithelial cells derived from two independent donors and are shown as the mean  $\pm$  SD (n = 10). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. E-cadherin and pancytokeratin expression were compared with the gp120- and tat-treated and untreated (control) groups. #, not detected.

https://doi.org/10.1371/journal.pone.0226343.g007

signaling. Blood levels of TGF- $\beta$  are elevated in HIV-positive individuals [121–124]. HIV-1 gp120 and tat induce TGF- $\beta$  expression in macrophages, CD4 and CD8 lymphocytes, and natural killer cells [89, 125–127]. HIV-associated elevation of TGF- $\beta$  expression within the mucosal environment may play a critical role in induction of EMT in mucosal epithelia. We have shown that HIV-1 gp120 and tat activate expression of matrix metallopeptidase 9 through MAPK and NF- $\kappa$ B signaling [22]. It is possible that HIV-1 gp120- and tat-activated matrix metallopeptidase 9 may contribute to cleavage of the TGF- $\beta$  precursor protein, generating formation of its C-terminal mature fragment, which binds to receptors and activates TGF- $\beta$  signaling [128, 129].

Both gp120 and tat activate MAPK signaling, which also plays a critical role in EMT induction. TGF- $\beta$  expression is activated by the AP-1 transcription factor [89, 130], which is induced by MAPK signaling [89, 131, 132], and this may lead to elevation of TGF- $\beta$  expression. HIV-1 gp120 binds to the chemokine receptors CXCR4 and CCR5 and *galactosylceramide* (GalCer), inducing MAPK activation [133–135] [24, 133, 134, 136]. HIV gp120 also binds to heparan sulfate proteoglycans (HSPG) [8, 9, 137–140], which bind TGF- $\beta$  superfamily proteins leading to activation of TGF- $\beta$  signaling [141]. Reduction of gp120-mediated EMT by anti-gp120 antibodies suggested that gp120 interaction with one or more receptors on the epithelial surface could be critical for the induction of EMT.

HIV tat binds to  $\alpha$ 5 $\beta$ 1,  $\alpha$ 5 $\beta$ 3, and  $\alpha$ v $\beta$ 3 integrins [142–146] and induces ras-dependent activation of MAPK [91]. We and others have shown that tonsil and genital epithelia express receptors CXCR4 and CCR5, GalCer, and  $\beta$ 1 and  $\alpha$ v integrins [8, 9, 137–140, 147].

The lack of difference between X4, R5, and dual-tropic HIV-1 viruses in EMT induction in tonsil, cervical, and foreskin cells suggests that gp120 interaction with these epithelial cells is not dependent on viral tropism. However, the lack of gp120-induced EMT induction by one of 4 HIV-1 strains suggests that some HIV-1 strains may have altered gp120, which may not bind its epithelial receptors and induce EMT.

MAPK signaling may also play a direct role in EMT induction by phosphorylation of Smad2/3 and TWIST1 [56–63].

HIV-1 tat may penetrate into cells and tissues through its protein transduction domain, based on the amino acids arginine and lysine, which facilitate protein internalization into cells and tissues by multiple mechanisms, including endocytosis and macropinocytosis [148–152]. Indeed, we have shown that recombinant HIV-1 tat is internalized into stratified oral epithe-lium [7]. Intracellular tat may increase TGF- $\beta$  expression by interacting with AP-1 [153], suggesting that tat may activate TGF- $\beta$  signaling by multiple mechanisms.

HIV proteins may also induce EMT through TGF-β- and MAPK- independent mechanisms. HIV tat binding to integrins facilitates activation of epidermal growth factor receptor (EGFR) [91]. HIV gp120 binding to GalCer of epithelial cells induces intracellular calcium elevation, which leads to the activation of protein kinase C [24, 154, 155] and subsequent activation of EGFR [156, 157]. Both tat- and gp120-induced activation of EGFR upregulate expression of the transcription factor STAT3, which subsequently activates Twist1 and downregulates E-cadherin expression, initiating EMT.

HIV-1 gp120 and tat activate expression of proinflammatory cytokines, including tumor necrosis factor alpha [158, 159], which may stimulate EMT [97–100].



Fig 8. HIV-1 gp120 is critical for EMT induction. (A) gp120 from HIV-1<sub>BAL</sub> was preincubated with a pool of 4 anti-gp120 antibodies or their isotope controls. Normal tonsil keratinocytes isolated from uninfected donors were untreated or treated with gp120 with neutralizing antibodies

or isotype controls. Culture medium was changed every day and after 5 days, cells were quantitatively analyzed for expression of E-cadherin, pancytokeratin, and vimentin. (B) Tonsil keratinocytes were untreated or treated with gp120 from HIV-1<sub>BAL</sub>, HIV-1<sub>IIIB</sub>, HIV-1<sub>CN-54</sub>, and HIV-1<sub>IR-CSF</sub> strains with a pool of neutralizing antibodies or their isotype controls. At day 5, cells were quantitatively analyzed for expression of E-cadherin, pancytokeratin, and vimentin. (C) Tonsil epithelial cells were incubated with HIV-1  $\Delta$ env-NL4.3 and HIV-1 NL4.3 viruses, and cells were maintained for 5 days; medium was changed with fresh viruses. After 5 days cells were quantitatively examined for expression of E-cadherin, pancytokeratin, and vimentin. Results are presented as a percentage of E-cadherin-, pancytokeratin-, or vimentin-positive cells. Data are representative of 3 independent experiments using tonsil epithelial cells derived from three donors and are shown as the mean  $\pm$  SD (n = 10). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. E-cadherin and pancytokeratin expression were compared in gp120-treated and untreated control cells. #, not detected.

https://doi.org/10.1371/journal.pone.0226343.g008

HIV-1 gp120- and tat-induced EMT in tonsil, cervical, and foreskin epithelial cells indicates that HIV-induced EMT could be common in oral and genital epithelia. Induction of EMT by HIV-1 recombinant gp120 and cell-free HIV virions revealed that the initial interaction of the cell-free virions envelope with mucosal epithelium from uninfected individuals may lead to



**Fig 9. HIV-induced EMT in tonsil epithelial cells isolated from multiple donors.** Normal tonsil keratinocytes isolated from 14 uninfected individuals were treated with cell-free HIV-1<sub>SF33</sub> or untreated for 10 days. Cells were immunostained, and expression of E-cadherin and vimentin were quantitatively evaluated. Data represent the mean  $\pm$  SD (n = 10). #, not detected. \*P<0.05. E-cadherin expression was compared with that of the virus-treated and untreated control cells.

https://doi.org/10.1371/journal.pone.0226343.g009





**Fig 10.** Analysis of MAPK and TGF-β1 signaling in the oral epithelia of HIV-infected and uninfected individuals. Tissue sections from HIV-infected and uninfected individuals were immunostained with antibodies against TGF-β1 (A) or

phosphorylated and total ERK1/2 (B) (both in green). Nuclei are stained in blue. EP, epithelium; LP, lamina propria; GR, granulosum; SP, spinosum; BL, basal. Representative images of three independent biological replicates are shown.

https://doi.org/10.1371/journal.pone.0226343.g010

the loss of epithelial junctions and paracellular penetration by HIV. However, the lack of HIV- $1_{SF33}$  cell-free virion-induced EMT in 45% of tonsil keratinocytes isolated from independent donors suggests that not all mucosal epithelial cells have critical epithelial receptors or signaling molecules for gp120.

HIV-1 gp120- and tat-induced EMT during HIV/AIDS disease may reduce the barrier functions of oral and genital mucosal epithelium, leading to the spread of viral, bacterial, fungal, and other pathogens. In the intact epithelium the assembled epithelial junction may sequester receptors for viruses, reducing their infection and mucosal transmission. HIV-induced EMT may liberate hidden receptors owing to the disassembly of epithelial junctions. Indeed, we reported that nectin-1, a receptor for herpes simplex virus-1, is hidden within the



**Fig 11.** Activation of TGF-β1 and MAPK signaling by HIV-1 tat, gp120, and cell-free virions in tonsil cells with the EMT phenotype. Normal tonsil keratinocytes isolated from uninfected individuals were treated for 7 days with gp120, tat, and their inactive forms, and cell-free HIV-1<sub>SF33</sub>, HIV-1<sub>SF170</sub>, and HIV-1<sub>92UG029</sub> strains. Cells were lysed and used to detect TGF-β1, phosphorylated and total ERK1/2, and actin by Western blotting using specific antibodies. \*Precursor; \*\*mature active form. A representative Western blot is shown from two independent experiments.

https://doi.org/10.1371/journal.pone.0226343.g011



Fig 12. Inhibition of TGF-β1 and MAPK signaling reduces HIV-induced EMT phenotype in tonsil, cervical, and foreskin epithelial cells. Normal tonsil, cervical, and foreskin keratinocytes isolated from uninfected individuals were incubated for 5 days with cell-free HIV-

 $1_{SF33}$  in the presence or absence of MAPK UO126 or TGF- $\beta$ 1 SB431542 inhibitors. Cells were immunostained for E-cadherin, pancytokeratin, and vimentin, and their expression was quantitatively analyzed. Results are presented as a percentage of E-cadherin-, pancytokeratin-, or vimentin-positive cells. Data are representative of 2 independent experiments using tonsil, cervical, and foreskin epithelial cells derived from two donors and are shown as the mean  $\pm$  SD (n = 10). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. Vimentin expression were compared in cells incubated with HIV or HIV plus drugs. #, not detected.

https://doi.org/10.1371/journal.pone.0226343.g012

adherens and tight junctions of oral epithelium, and HIV-induced disruption of epithelial junctions exposes nectin-1 to herpes simplex virus-1, facilitating rapid viral infection and spread [21].

HIV-induced disruption of epithelial junctions via EMT facilitates paracellular penetration of oncogenic human papillomavirus (HPV) [7]. Moreover, HIV gp120- and tat-induced transmigration of EMT cells suggests that, if this occurs within the HPV-infected environment, it may significantly accelerate invasion of HPV-infected malignant cells, leading to the progression of HPV neoplasia. Indeed, our ongoing studies show that HIV gp120 and tat substantially increase migration and invasion of HPV-infected cervical cancer cells through EMT mechanisms (manuscript in preparation).

HIV has been shown to cause EMT in renal epithelium. HIV infection is associated with kidney failure due to severe nephropathy, characterized by the loss of the renal epithelial phenotype and acquisition of mesenchymal features, including dedifferentiation, depolarization, and proliferation [160–167]. Accumulating evidence indicates that HIV-associated nephropathy is caused by EMT [166] and that HIV infection may play a critical role in the induction of EMT [160–165, 168, 169]. HIV replication has been observed in renal epithelial cells [169, 170]. Studies using HIV transgenic mice have shown that renal epithelial cells express HIV mRNA and develop the EMT phenotype with decreasing E-cadherin expression [160, 171, 172]. Transgenic mice expressing the HIV proteins nef or tat show EMT-like changes in the renal epithelium [173]. An HIV nef-induced EMT-like phenotype has been seen in experiments with renal epithelial cells in vitro [173–176].

HIV-associated reduction of E-cadherin expression have been shown in lung and gut epithelia [177, 178] suggesting HIV-induced EMT may also take place in other epithelial organs in HIV/AIDS disease, such as skin, nasopharyngeal mucosal epithelium, and liver. HIVinduced EMT may accelerate the epithelial neoplasia associated with other oncogenic viruses, such as Epstein Barr virus (EBV), Kaposi sarcoma-associated herpesvirus, and hepatitis C (HCV) and B (HBV) viruses. It is well known that HIV coinfection is common with EBV, Kaposi sarcoma-associated herpesvirus, HCV, and HBV with the development of mutual pathogenesis for these copathogens [179–181].

In summary (Fig 14), we have shown that HIV-1 gp120 and tat proteins induce EMT in oral and genital mucosal epithelia via activation of TGF- $\beta$  and MAPK signaling. EMT-associated changes to the integrity of mucosal epithelia may reduce a wide spectrum of physiologic functions of mucosa, including barrier, transport, secretion, and maintenance of the innate and adaptive immune response by recruiting monocyte/macrophages, Langerhans cells, and T and B lymphocytes [6, 182, 183]. Importantly, HIV-induced EMT may occur within premalignant cells, which may accelerate the progression of neoplastic processes. Inhibition of TGF- $\beta$  and MAPK signaling pathways may inhibit development of HIV-induced EMT of oral and genital mucosal epithelia, preserving their normal barrier and other functions.

#### Materials and methods

#### **Ethics statement**

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Committee on Human Research of the University of California–San

# **PLOS** ONE



Fig 13. Transmigration and invasion of HIV gp120- and tat-induced EMT cells. Normal tonsil, cervical, and foreskin keratinocytes were isolated from uninfected donors and treated for 5 days with gp120 and/or tat and their inactive forms. Untreated cells served as a control. Transmigration of cells was

examined in collagen-coated Transwell inserts. Cells were stained (A) and quantitatively evaluated (B). Transmigrated invasive cells were counted in 10 randomly selected regions. Results are presented as average number of cells per field. Data are representative of 2 independent experiments using tonsil, cervical, and foreskin epithelial cells derived from two donors and are shown as the mean  $\pm$  SD (n = 10). \*\*\*P<0.001. Cell numbers were compared with those of the gp120- and tat-treated cells and their inactive forms (control).

https://doi.org/10.1371/journal.pone.0226343.g013

Francisco (IRB approval #s 10–03277 and 19–27275). All human subjects gave informed written consent for the collection of tissue samples. The parents provided informed consent for all minors.

#### Collection of buccal tissues

Buccal biopsy samples from HIV-uninfected and HIV-infected individuals were collected by the UCSF Oral AIDS Center. The main criteria of tissue collection were the lack of clinically detectable HPV-, human cytomegalovirus (HCMV)-, HSV- and EBV-specific lesions and inflammation.



Fig 14. Model of HIV-induced EMT. In HIV-infected individuals, HIV-infected CD4 lymphocytes, monocyte/macrophages, and Langerhans cells migrate into oropharyngeal, cervical, and foreskin epithelia and produce cell-free virions and secrete HIV proteins tat and gp120 in the mucosal environment (A). Interaction of cell-free virions and viral proteins with epithelial cells activates MAPK and TGF- $\beta$ , leading to induction of the EMT phenotype: Epithelial cells lose their cobblestone-like morphology and acquire a spindle-like shape (B). Epithelial markers, including E-cadherin expression, are lost and the mesenchymal marker vimentin expression is induced. EMT cells lose adherens and tight junctions and cell polarity, leading to impairment of the epithelial barrier. The lack of epithelial junctions causes the opening of the paracellular space for penetration of viral, bacterial, fungal, and other pathogens. HIV-induced EMT cells of mucosal epithelia are highly motile and may migrate through basement membranes. If HIV-induced EMT occurs in the premalignant or malignant mucosal epithelia, it may accelerate the neoplastic process, leading to more migration and invasion of cancer cells.

https://doi.org/10.1371/journal.pone.0226343.g014

#### Viruses, viral proteins, and cells

Laboratory-adapted dual-tropic (X4-R5) HIV- $1_{SF33}$  and the primary isolates R5-tropic HIV- $1_{SF170}$  and X4-tropic HIV- $1_{92UG029}$  were grown in peripheral blood mononuclear cells, which were isolated from heparinized blood using a Ficoll-Paque Plus density gradient (Sigma). Cells were activated with 2.5 µg/ml phytohemagglutinin (Sigma) and 1 µg/ml interleukin-2 (BD Biosciences) for 3 days. Viral stocks were purified using the Amicon Ultra-15 ultracentrifugation filtration system (Millipore). Viral stocks were titered by p24 concentration using HIV-1 p24 ELISA (PerkinElmer) according to the manufacturer's instructions.

Recombinant HIV-1<sub>Bal</sub> tat and its inactive form were purchased from ImmunoDX, LLC (Woburn, MA). Inactive tat was created through substitution of its basic arginine-rich domain at the 49–57 aa and the integrin-binding RGD motif in the C terminus with alanines [90–92]. Recombinant gp120 proteins from HIV-1<sub>BAL</sub>, HIV-1<sub>IIIB</sub>, HIV-1<sub>CN-54</sub>, and HIV-1<sub>96ZM651</sub> strains were provided by the NIH AIDS Reagent Program. gp120 was inactivated by incubation at 85°C for 30 min [184–186]. All HIV proteins were stored at –80°C in the dark before use.

Wild-type and HIV-1 NL4.3 and HIV-1 NL4.3-E (HIV-1  $\Delta$ env) lacking envelope protein were obtained from the NIH AIDS Reagent Program. HIV-1 NL4.3 and HIV-1 NL4.3-E  $\Delta$ env were propagated in HEK293 cells, purified using the Amicon Ultra-15 ultracentrifugation filtration system (Millipore), and titered by p24 concentration using HIV-1 p24 ELISA.

Primary tonsil epithelial keratinocytes were established from tonsil tissue from 20 HIV-negative children <5 years of age after routine tonsillectomy. Primary cervical keratinocytes were established from ectocervical tissue specimens from 4 HIV-negative donors. Foreskin keratinocytes from three independent donors were obtained from Lonza (Hayward, CA). Keratinocytes were grown in keratinocyte growth medium (KGM gold) (Lonza). Epithelial cell purity and absence of mesenchymal cells were determined with a cocktail of antikeratin antibodies containing Ab-1 and Ab-2 (Thermo Fisher Scientific). Only epithelial cell populations with 100% keratin were used. Keratinocytes were used at early passages and frozen in liquid nitrogen.

# Treatment of tonsil, foreskin, and cervical epithelial cells with cell-free HIV virions and HIV proteins gp120 and tat

Cervical, foreskin, and tonsil epithelial cells were treated with active tat and gp120, inactive mutant tat, and heat-inactivated gp120 at a concentration of 10 ng/ml (each) for 5–12 days. Cells were exposed to dual-tropic HIV- $1_{SF33}$ , R5-tropic HIV- $1_{SF170}$ , and X4-tropic HIV- $1_{92UG029}$  at a concentration of 10 ng/ml of p24. Culture medium was changed daily to add fresh virus or proteins. One set of cells was treated with MAPK inhibitor U0126 (Sigma) or with TGF- $\beta$ 1 inhibitor SB431542 (Tocris Bioscience) at 10  $\mu$ M each. The absence of a toxic effect by virus, tat, gp120, U0126, or SB431542 was confirmed by the MTT cell viability assay (Biotium). Cervical, foreskin, and tonsil epithelial cells were also exposed to HIV-1 NL4.3 and NL4.3-E  $\Delta$ env viruses at 10 ng/ml.

To neutralize the EMT induction effect of gp120 in tonsil epithelial cells, gp120 from HIV- $1_{BAL}$ , HIV- $1_{IIIB}$ , HIV- $1_{CN-54}$ , and HIV- $1_{96ZM651}$  were incubated with a pool of 5 neutralizing antibodies—b12, 2G12, F105, 39F and ID6—or pool of their isotype controls for 1 h at 37°C at 1 µg/ml of each. Then gp120 with antibodies were added to the tonsil epithelial cells. Cell culture medium was changed every day with fresh proteins and antibodies, and after 5 days, cells were quantitatively analyzed for expression of E-cadherin, pancytokeratin, and vimentin. gp120 and antibodies were obtained from NIH AIDS Reagent Program.

#### Immunofluorescence assay

For immunofluorescence assays, cells or tissue sections were fixed with 4% paraformaldehyde and 2% sucrose in PBS for 5 min, and then permeabilized with 0.01% Triton X-100 in 4% paraformaldehyde for 5 min. Normal donkey serum (5%) in PBS was used to prevent nonspecific binding. E-cadherin was detected using either rabbit or goat antibodies (Vector Laboratories and R&D Systems, respectively). Vimentin was detected using goat antibodies (Millipore), and pankeratin was detected using rabbit antibodies (Life Technologies). Primary isotype control antibodies were used as a negative control to confirm the specificity of each antibody. Primary antibodies were incubated for 1.5 h. Secondary antibodies used in this assay include Dylight 488, Dylight 594 (Vector Laboratories), and Alexa Fluor 594 (Jackson ImmunoResearch Laboratories). Cell nuclei were counterstained with DAPI (Molecular Probes). Images were captured with a Nikon Eclipse E400 fluorescence microscope (Nikon) at magnification 200x. Quantitative analysis of EMT was undertaken by counting E-cadherin-, vimentin-, and pancytokeratin-expressing cells relative to the total number of cells per image in 10 separate, randomly chosen fields on each slide (n = 10). Cell counting was performed independently by 2 investigators (KL, WM).

#### Western blot assay

Cells were extracted with 1.0% Triton X-100 buffer (150 mM NaCl, 10 mM Tris/HCl, pH 8.0, and a cocktail of protease inhibitors). Proteins were separated on an SDS-polyacrylamide gel with a 4–20% gradient. The following antibodies were used: rabbit antibody to E-cadherin (R&D); rabbit antibodies against N-cadherin, vimentin, SMAD2, Slug, Snail, and ZEB1 (Cell Signaling); rabbit antibodies against phosphorylated SMAD2 (Abcam); mouse monoclonal antibodies against TGF- $\beta$ 1 (Thermo Fisher Scientific and Abcam); rabbit antibodies against ERK1/2 total and ERK1/2 phosphorylated. An equal protein load was confirmed by the use of beta-actin (Ambio).

#### **Transmigration assay**

In vitro transmigration and invasion assays were performed using the Collagen Cell Invasion Assay-Colorimetric (8  $\mu$ M) (EMD Millipore) according to the manufacturer's protocol. Cells were treated with HIV-1 gp120 and tat proteins or their inactive controls for 5–7 days, and  $5 \times 10^4$  cells/insert were seeded in the collagen-coated inserts in basal KBM medium without supplements. KMB alone or KGM containing 10% fetal bovine serum as chemoattractant was added to the lower chamber. Cell migration and invasion were evaluated 24 h later using light microscopy to count individual cells that invaded the collagen inserts. To quantify cell migration/invasion, cell numbers on 10 randomly selected fields were counted under various experimental conditions; data are presented as average number of cells per field.

#### Statistical analysis

Statistical comparisons were made by a two-tailed Student's t-test. A p value of <0.05 was considered significant. Results are expressed as mean  $\pm$  SD.

#### Supporting information

**S1** Table. Antiretroviral therapy status, viral load and CD4 counts of oral biopsy of donors. (DOCX)

#### Acknowledgments

This project was supported by the NIDCR R01DE028129 and NCI R01CA232887 grants (to SMT). We thank the NIH AIDS Reagent Program for providing HIV-1 viral strains, gp120 proteins, and anti-gp120 neutralizing antibodies. We also thank Drs. Deborah Greenspan and Piri Veluppillai for providing biopsy samples and Dr. Joel Palefsky for discussion.

#### **Author Contributions**

Conceptualization: Sharof M. Tugizov.

Data curation: Sharof M. Tugizov.

Formal analysis: Kathy Lien, Wasima Mayer.

Funding acquisition: Sharof M. Tugizov.

Investigation: Kathy Lien, Wasima Mayer, Rossana Herrera, Sharof M. Tugizov.

Methodology: Kathy Lien, Wasima Mayer, Rossana Herrera, Kristina Rosbe, Sharof M. Tugizov.

Project administration: Sharof M. Tugizov.

Resources: Kristina Rosbe, Sharof M. Tugizov.

Supervision: Sharof M. Tugizov.

Validation: Kathy Lien, Wasima Mayer, Rossana Herrera, Sharof M. Tugizov.

Writing – original draft: Kathy Lien, Sharof M. Tugizov.

#### References

- Schluter H, Wepf R, Moll I, Franke WW. Sealing the live part of the skin: the integrated meshwork of desmosomes, tight junctions and curvilinear ridge structures in the cells of the uppermost granular layer of the human epidermis. Eur J Cell Biol. 2004; 83(11–12):655–65. https://doi.org/10.1078/0171-9335-00434 PMID: 15679110.
- Langbein L, Pape UF, Grund C, Kuhn C, Praetzel S, Moll I, et al. Tight junction-related structures in the absence of a lumen: occludin, claudins and tight junction plaque proteins in densely packed cell formations of stratified epithelia and squamous cell carcinomas. Eur J Cell Biol. 2003; 82(8):385–400. https://doi.org/10.1078/0171-9335-00330 PMID: 14533737.
- Langbein L, Grund C, Kuhn C, Praetzel S, Kartenbeck J, Brandner JM, et al. Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derived therefrom. Eur J Cell Biol. 2002; 81(8):419–35. https://doi.org/10.1078/0171-9335-00270 PMID: 12234014.
- Brandner JM, Kief S, Grund C, Rendl M, Houdek P, Kuhn C, et al. Organization and formation of the tight junction system in human epidermis and cultured keratinocytes. Eur J Cell Biol. 2002; 81(5):253– 63. https://doi.org/10.1078/0171-9335-00244 PMID: 12067061.
- Takano K, Kojima T, Go M, Murata M, Ichimiya S, Himi T, et al. HLA-DR- and CD11c-positive dendritic cells penetrate beyond well-developed epithelial tight junctions in human nasal mucosa of allergic rhinitis. J Histochem Cytochem. 2005; 53(5):611–9. https://doi.org/10.1369/jhc.4A6539.2005 PMID: 15872054.
- Blaskewicz CD, Pudney J, Anderson DJ. Structure and function of intercellular junctions in human cervical and vaginal mucosal epithelia. Biology of reproduction. 2011; 85(1):97–104. Epub 2011/04/08. https://doi.org/10.1095/biolreprod.110.090423 PMID: 21471299; PubMed Central PMCID: PMC3123383.
- Tugizov SM, Herrera R, Chin-Hong P, Veluppillai P, Greenspan D, Michael Berry J, et al. HIV-associated disruption of mucosal epithelium facilitates paracellular penetration by human papillomavirus. Virology. 2013; 446(1–2):378–88. Epub 2013/10/01. https://doi.org/10.1016/j.virol.2013.08.018 PMID: 24074602.

- Tugizov SM, Herrera R, Veluppillai P, Greenspan D, Soros V, Greene WC, et al. HIV is inactivated after transepithelial migration via adult oral epithelial cells but not fetal epithelial cells. Virology. 2011; 409(2):211–22. Epub 2010/11/09. https://doi.org/10.1016/j.virol.2010.10.004 PMID: 21056450; PubMed Central PMCID: PMC3034249.
- Tugizov SM, Herrera R, Veluppillai P, Greenspan D, Soros V, Greene WC, et al. Differential transmission of HIV traversing fetal oral/intestinal epithelia and adult oral epithelia. Journal of Virology. 2012; 86(5):2556–70. Epub 2011/12/30. https://doi.org/10.1128/JVI.06578-11 PMID: 22205732; PubMed Central PMCID: PMC3302289.
- Go M, Kojima T, Takano K, Murata M, Ichimiya S, Tsubota H, et al. Expression and function of tight junctions in the crypt epithelium of human palatine tonsils. J Histochem Cytochem. 2004; 52 (12):1627–38. https://doi.org/10.1369/jhc.4A6339.2004 PMID: 15557217.
- Sawada N, Murata M, Kikuchi K, Osanai M, Tobioka H, Kojima T, et al. Tight junctions and human diseases. Med Electron Microsc. 2003; 36(3):147–56. Epub 2003/09/25. https://doi.org/10.1007/s00795-003-0219-y PMID: 14505058.
- Epple HJ, Allers K, Troger H, Kuhl A, Erben U, Fromm M, et al. Acute HIV infection induces mucosal infiltration with CD4+ and CD8+ T cells, epithelial apoptosis, and a mucosal barrier defect. Gastroenterology. 2010; 139(4):1289–300. Epub 2010/07/06. https://doi.org/10.1053/j.gastro.2010.06.065 PMID: 20600014.
- Epple HJ, Schneider T, Troeger H, Kunkel D, Allers K, Moos V, et al. Impairment of the intestinal barrier is evident in untreated but absent in suppressively treated HIV-infected patients. Gut. 2009; 58 (2):220–7. Epub 2008/10/22. https://doi.org/10.1136/gut.2008.150425 PMID: 18936106.
- Assimakopoulos SF, Dimitropoulou D, Marangos M, Gogos CA. Intestinal barrier dysfunction in HIV infection: pathophysiology, clinical implications and potential therapies. Infection. 2014; 42(6):951–9. Epub 2014/07/30. https://doi.org/10.1007/s15010-014-0666-5 PMID: 25070877.
- Kapembwa MS, Fleming SC, Orr M, Wells C, Bland M, Back D, et al. Impaired absorption of zidovudine in patients with AIDS-related small intestinal disease. Aids. 1996; 10(13):1509–14. <u>https://doi.org/ 10.1097/00002030-199611000-00008 PMID: 8931785</u>.
- Obinna FC, Cook G, Beale T, Dave S, Cunningham D, Fleming SC, et al. Comparative assessment of small intestinal and colonic permeability in HIV-infected homosexual men. Aids. 1995; 9(9):1009–16. https://doi.org/10.1097/00002030-199509000-00005 PMID: 8527072.
- Kapembwa MS, Fleming SC, Sewankambo N, Serwadda D, Lucas S, Moody A, et al. Altered smallintestinal permeability associated with diarrhoea in human-immunodeficiency-virus-infected Caucasian and African subjects. Clin Sci (Lond). 1991; 81(3):327–34. <u>https://doi.org/10.1042/cs0810327</u> PMID: 1655333.
- Stockmann M, Fromm M, Schmitz H, Schmidt W, Riecken EO, Schulzke JD. Duodenal biopsies of HIV-infected patients with diarrhoea exhibit epithelial barrier defects but no active secretion. Aids. 1998; 12(1):43–51. https://doi.org/10.1097/00002030-199801000-00006 PMID: 9456254.
- Tugizov S. Human immunodeficiency virus-associated disruption of mucosal barriers and its role in HIV transmission and pathogenesis of HIV/AIDS disease. Tissue Barriers. 2016; 4(3):e1159276. https://doi.org/10.1080/21688370.2016.1159276 PMID: 27583187; PubMed Central PMCID: PMC4993574.
- Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, Gray-Owen SD, et al. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. PLoS pathogens. 2010; 6(4):e1000852. Epub 2010/04/14. https://doi.org/10.1371/journal.ppat.1000852 PMID: 20386714; PubMed Central PMCID: PMC2851733.
- Sufiawati I, Tugizov SM. HIV-Associated Disruption of Tight and Adherens Junctions of Oral Epithelial Cells Facilitates HSV-1 Infection and Spread. PIoS one. 2014; 9(2):e88803. Epub 2014/03/04. https:// doi.org/10.1371/journal.pone.0088803 PMID: 24586397; PubMed Central PMCID: PMC3931628.
- Sufiawati I, Tugizov SM. HIV-induced matrix metalloproteinase-9 activation through mitogen-activated protein kinase signalling promotes HSV-1 cell-to-cell spread in oral epithelial cells. Journal of General Virology. 2018; In press.
- Pope M, Haase AT. Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. Nat Med. 2003; 9(7):847–52. Epub 2003/07/02. <u>https://doi.org/10.1038/nm0703-847</u> [pii]. PMID: 12835704.
- Dayanithi G, Yahi N, Baghdiguian S, Fantini J. Intracellular calcium release induced by human immunodeficiency virus type 1 (HIV-1) surface envelope glycoprotein in human intestinal epithelial cells: a putative mechanism for HIV-1 enteropathy. Cell Calcium. 1995; 18(1):9–18. Epub 1995/07/01. <u>https:// doi.org/10.1016/0143-4160(95)90041-1</u> PMID: 7585886.

- Pu H, Tian J, Andras IE, Hayashi K, Flora G, Hennig B, et al. HIV-1 Tat protein-induced alterations of ZO-1 expression are mediated by redox-regulated ERK 1/2 activation. J Cereb Blood Flow Metab. 2005; 25(10):1325–35. https://doi.org/10.1038/sj.jcbfm.9600125 PMID: 15829913.
- Pu H, Tian J, Flora G, Lee YW, Nath A, Hennig B, et al. HIV-1 Tat protein upregulates inflammatory mediators and induces monocyte invasion into the brain. Mol Cell Neurosci. 2003; 24(1):224–37. https://doi.org/10.1016/s1044-7431(03)00171-4 PMID: 14550782.
- Ikenouchi J, Matsuda M, Furuse M, Tsukita S. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. J Cell Sci. 2003; 116(Pt 10):1959–67. Epub 2003/04/02. https://doi.org/10.1242/jcs.00389 PMID: 12668723.
- Antony J, Thiery JP, Huang RY. Epithelial-to-mesenchymal transition: lessons from development, insights into cancer and the potential of EMT-subtype based therapeutic intervention. Phys Biol. 2019; 16(4):041004. https://doi.org/10.1088/1478-3975/ab157a PMID: 30939460.
- Lim J, Thiery JP. Epithelial-mesenchymal transitions: insights from development. Development. 2012; 139(19):3471–86. https://doi.org/10.1242/dev.071209 PMID: 22949611.
- Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci. 2007; 98(10):1512–20. Epub 2007/07/25. https://doi.org/10.1111/j.1349-7006.2007.00550.x PMID: 17645776.
- Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009; 139(5):871–90. https://doi.org/10.1016/j.cell.2009.11.007 PMID: 19945376.
- **32.** Ocana OH, Nieto MA. Epithelial plasticity, stemness and pluripotency. Cell Res. 2010; 20(10):1086–8. Epub 2010/09/08. https://doi.org/10.1038/cr.2010.127 PMID: 20820188.
- Nieto MA, Cano A. The epithelial-mesenchymal transition under control: global programs to regulate epithelial plasticity. Semin Cancer Biol. 2012; 22(5–6):361–8. Epub 2012/05/23. <u>https://doi.org/10.1016/j.semcancer.2012.05.003</u> PMID: 22613485.
- Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. The Journal of clinical investigation. 2009; 119(6):1420–8. Epub 2009/06/03. <u>https://doi.org/10.1172/JCI39104</u> PMID: <u>19487818</u>; PubMed Central PMCID: PMC2689101.
- Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nature reviews Molecular cell biology. 2014; 15(3):178–96. Epub 2014/02/22. https://doi.org/10.1038/ nrm3758 PMID: 24556840; PubMed Central PMCID: PMC4240281.
- Moustakas A, Heldin CH. Mechanisms of TGFbeta-Induced Epithelial-Mesenchymal Transition. J Clin Med. 2016; 5(7). Epub 2016/07/02. <u>https://doi.org/10.3390/jcm5070063</u> PMID: <u>27367735</u>; PubMed Central PMCID: PMC4961994.
- 37. Wang H, Zhang G, Zhang H, Zhang F, Zhou B, Ning F, et al. Acquisition of epithelial-mesenchymal transition phenotype and cancer stem cell-like properties in cisplatin-resistant lung cancer cells through AKT/beta-catenin/Snail signaling pathway. Eur J Pharmacol. 2014; 723:156–66. Epub 2013/ 12/18. https://doi.org/10.1016/j.ejphar.2013.12.004 PMID: 24333218.
- Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. Sci Signal. 2014; 7(344):re8. Epub 2014/09/25. https://doi.org/10.1126/scisignal.2005189 PMID: 25249658; PubMed Central PMCID: PMC4372086.
- Dongre A, Weinberg RA. New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol. 2019; 20(2):69–84. Epub 2018/11/22. <u>https://doi.org/10. 1038/s41580-018-0080-4 PMID: 30459476.</u>
- 40. Talbot LJ, Bhattacharya SD, Kuo PC. Epithelial-mesenchymal transition, the tumor microenvironment, and metastatic behavior of epithelial malignancies. Int J Biochem Mol Biol. 2012; 3(2):117–36. Epub 2012/07/10. PMID: 22773954; PubMed Central PMCID: PMC3388731.
- Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. The New England journal of medicine. 2000; 342(18):1350–8. Epub 2000/05/04. https://doi.org/10.1056/ NEJM200005043421807 PMID: 10793168.
- Gordon KJ, Blobe GC. Role of transforming growth factor-beta superfamily signaling pathways in human disease. Biochimica et Biophysica Acta. 2008; 1782(4):197–228. Epub 2008/03/04. <u>https://doi.org/10.1016/j.bbadis.2008.01.006 PMID: 18313409.</u>
- Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014; 15(3):178–96. Epub 2014/02/22. <u>https://doi.org/10.1038/nrm3758</u> PMID: 24556840; PubMed Central PMCID: PMC4240281.
- Dave N, Guaita-Esteruelas S, Gutarra S, Frias A, Beltran M, Peiro S, et al. Functional cooperation between Snail1 and twist in the regulation of ZEB1 expression during epithelial to mesenchymal transition. J Biol Chem. 2011; 286(14):12024–32. Epub 2011/02/15. https://doi.org/10.1074/jbc.M110. 168625 PMID: 21317430; PubMed Central PMCID: PMC3069405.

- Meulmeester E, Ten Dijke P. The dynamic roles of TGF-beta in cancer. The Journal of pathology. 2011; 223(2):205–18. Epub 2010/10/20. https://doi.org/10.1002/path.2785 PMID: 20957627.
- 46. Wendt MK, Tian M, Schiemann WP. Deconstructing the mechanisms and consequences of TGFbeta-induced EMT during cancer progression. Cell and tissue research. 2012; 347(1):85–101. Epub 2011/06/22. https://doi.org/10.1007/s00441-011-1199-1 PMID: 21691718.
- Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development. 2005; 132(14):3151–61. Epub 2005/06/29. <u>https://doi.org/ 10.1242/dev.01907</u> PMID: 15983400.
- Peinado H, Marin F, Cubillo E, Stark HJ, Fusenig N, Nieto MA, et al. Snail and E47 repressors of Ecadherin induce distinct invasive and angiogenic properties in vivo. J Cell Sci. 2004;117(Pt 13):2827– 39. Epub 2004/06/01. https://doi.org/10.1242/jcs.01145 PMID: 15169839.
- **49.** Hennig G, Behrens J, Truss M, Frisch S, Reichmann E, Birchmeier W. Progression of carcinoma cells is associated with alterations in chromatin structure and factor binding at the E-cadherin promoter in vivo. Oncogene. 1995; 11(3):475–84. Epub 1995/08/03. PMID: 7630631.
- Stivarou T, Patsavoudi E. Extracellular molecules involved in cancer cell invasion. Cancers (Basel). 2015; 7(1):238–65. https://doi.org/10.3390/cancers7010238 PMID: 25629807; PubMed Central PMCID: PMC4381257.
- Xia S, Wang C, Postma EL, Yang Y, Ni X, Zhan W. Fibronectin 1 promotes migration and invasion of papillary thyroid cancer and predicts papillary thyroid cancer lymph node metastasis. Onco Targets Ther. 2017; 10:1743–55. https://doi.org/10.2147/OTT.S122009 PMID: 28367057; PubMed Central PMCID: PMC5370387.
- Ohnishi T, Hiraga S, Izumoto S, Matsumura H, Kanemura Y, Arita N, et al. Role of fibronectin-stimulated tumor cell migration in glioma invasion in vivo: clinical significance of fibronectin and fibronectin receptor expressed in human glioma tissues. Clin Exp Metastasis. 1998; 16(8):729–41. <u>https://doi.org/ 10.1023/a:1006532812408 PMID: 10211986</u>.
- van Zijl F, Krupitza G, Mikulits W. Initial steps of metastasis: cell invasion and endothelial transmigration. Mutat Res. 2011; 728(1–2):23–34. https://doi.org/10.1016/j.mrrev.2011.05.002 PMID: 21605699; PubMed Central PMCID: PMC4028085.
- Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol. 2000; 148(4):779–90. https://doi.org/10.1083/jcb.148.4.779 PMID: 10684258; PubMed Central PMCID: PMC2169367.
- Sandig M, Voura EB, Kalnins VI, Siu CH. Role of cadherins in the transendothelial migration of melanoma cells in culture. Cell Motil Cytoskeleton. 1997; 38(4):351–64. https://doi.org/10.1002/(SICI) 1097-0169(1997)38:4<351::AID-CM5>3.0.CO;2-6 PMID: 9415377.
- 56. Moustakas A, Heldin CH. Non-Smad TGF-beta signals. J Cell Sci. 2005; 118(Pt 16):3573–84. Epub 2005/08/18. https://doi.org/10.1242/jcs.02554 PMID: 16105881.
- 57. Mu Y, Gudey SK, Landstrom M. Non-Smad signaling pathways. Cell Tissue Res. 2012; 347(1):11–20. Epub 2011/06/28. https://doi.org/10.1007/s00441-011-1201-y PMID: 21701805.
- Lee MK, Pardoux C, Hall MC, Lee PS, Warburton D, Qing J, et al. TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. EMBO J. 2007; 26(17):3957–67. Epub 2007/08/04. https://doi.org/10.1038/sj.emboj.7601818 PMID: 17673906; PubMed Central PMCID: PMC1994119.
- Cordenonsi M, Montagner M, Adorno M, Zacchigna L, Martello G, Mamidi A, et al. Integration of TGFbeta and Ras/MAPK signaling through p53 phosphorylation. Science. 2007; 315(5813):840–3. Epub 2007/01/20. https://doi.org/10.1126/science.1135961 PMID: 17234915.
- Matsuura I, Wang G, He D, Liu F. Identification and characterization of ERK MAP kinase phosphorylation sites in Smad3. Biochemistry. 2005; 44(37):12546–53. Epub 2005/09/15. <u>https://doi.org/10.1021/</u> bi050560g PMID: 16156666.
- Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, et al. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol. 2002; 156(2):299–313. Epub 2002/01/16. https://doi.org/10.1083/jcb.200109037 PMID: 11790801; PubMed Central PMCID: PMC2199233.
- **62.** Mulder KM. Role of Ras and Mapks in TGFbeta signaling. Cytokine Growth Factor Rev. 2000; 11(1–2):23–35. Epub 2000/03/10. https://doi.org/10.1016/s1359-6101(99)00026-x PMID: 10708950.
- Hong J, Zhou J, Fu J, He T, Qin J, Wang L, et al. Phosphorylation of serine 68 of Twist1 by MAPKs stabilizes Twist1 protein and promotes breast cancer cell invasiveness. Cancer Res. 2011; 71(11):3980–90. Epub 2011/04/20. https://doi.org/10.1158/0008-5472.CAN-10-2914 PMID: 21502402; PubMed Central PMCID: PMC3107354.

- Chapnick DA, Warner L, Bernet J, Rao T, Liu X. Partners in crime: the TGFbeta and MAPK pathways in cancer progression. Cell Biosci. 2011; 1:42. Epub 2011/12/30. https://doi.org/10.1186/2045-3701-1-42 PMID: 22204556; PubMed Central PMCID: PMC3275500.
- 65. Beachler DC, Weber KM, Margolick JB, Strickler HD, Cranston RD, Burk RD, et al. Risk Factors for Oral HPV Infection among a High Prevalence Population of HIV-Positive and At-Risk HIV-Negative Adults. Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2011. Epub 2011/ 11/03. https://doi.org/10.1158/1055-9965.EPI-11-0734 PMID: 22045700.
- 66. Agrawal Y, Koch WM, Xiao W, Westra WH, Trivett AL, Symer DE, et al. Oral human papillomavirus infection before and after treatment for human papillomavirus 16-positive and human papillomavirus 16-negative head and neck squamous cell carcinoma. Clin Cancer Res. 2008; 14(21):7143–50. Epub 2008/11/05. https://doi.org/10.1158/1078-0432.CCR-08-0498 PMID: 18981014; PubMed Central PMCID: PMC2598779.
- Chaturvedi AK, Engels EA, Anderson WF, Gillison ML. Incidence trends for human papillomavirusrelated and -unrelated oral squamous cell carcinomas in the United States. J Clin Oncol. 2008; 26 (4):612–9. Epub 2008/02/01. https://doi.org/10.1200/JCO.2007.14.1713 PMID: 18235120.
- D'Souza G, Agrawal Y, Halpern J, Bodison S, Gillison ML. Oral sexual behaviors associated with prevalent oral human papillomavirus infection. The Journal of infectious diseases. 2009; 199(9):1263–9. Epub 2009/03/27. https://doi.org/10.1086/597755 PMID: 19320589.
- 69. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, et al. Case-control study of human papillomavirus and oropharyngeal cancer. The New England journal of medicine. 2007; 356 (19):1944–56. Epub 2007/05/15. https://doi.org/10.1056/NEJMoa065497 PMID: 17494927.
- Gillison ML. Human papillomavirus-related diseases: oropharynx cancers and potential implications for adolescent HPV vaccination. J Adolesc Health. 2008; 43(4 Suppl):S52–60. Epub 2008/10/01. https://doi.org/10.1016/j.jadohealth.2008.07.002 PMID: 18809146; PubMed Central PMCID: PMC3037092.
- Kreimer AR, Alberg AJ, Daniel R, Gravitt PE, Viscidi R, Garrett ES, et al. Oral human papillomavirus infection in adults is associated with sexual behavior and HIV serostatus. The Journal of infectious diseases. 2004; 189(4):686–98. Epub 2004/02/10. https://doi.org/10.1086/381504 PMID: 14767823.
- Gillison ML. Oropharyngeal cancer: a potential consequence of concomitant HPV and HIV infection. Current opinion in oncology. 2009; 21(5):439–44. Epub 2009/07/10. <u>https://doi.org/10.1097/CCO.</u> 0b013e32832f3e1b PMID: 19587593.
- 73. Beachler DC, D'Souza G. Oral human papillomavirus infection and head and neck cancers in HIVinfected individuals. Current opinion in oncology. 2013; 25(5):503–10. Epub 2013/07/16. <u>https://doi.org/10.1097/CCO.0b013e32836242b4</u> PMID: 23852381; PubMed Central PMCID: PMC3896303.
- 74. Beachler DC, D'Souza G, Sugar EA, Xiao W, Gillison ML. Natural history of anal vs oral HPV infection in HIV-infected men and women. The Journal of infectious diseases. 2013; 208(2):330–9. Epub 2013/ 04/19. https://doi.org/10.1093/infdis/jit170 PMID: 23596319; PubMed Central PMCID: PMC3685232.
- 75. Frisch M, Biggar RJ, Goedert JJ. Human papillomavirus-associated cancers in patients with human immunodeficiency virus infection and acquired immunodeficiency syndrome. Journal of the National Cancer Institute. 2000; 92(18):1500–10. Epub 2000/09/21. https://doi.org/10.1093/jnci/92.18.1500 PMID: 10995805.
- 76. Clifford GM, Polesel J, Rickenbach M, Dal Maso L, Keiser O, Kofler A, et al. Cancer risk in the Swiss HIV Cohort Study: associations with immunodeficiency, smoking, and highly active antiretroviral therapy. Journal of the National Cancer Institute. 2005; 97(6):425–32. Epub 2005/03/17. <u>https://doi.org/</u> 10.1093/jnci/dji072 PMID: 15770006.
- 77. Engels EA, Biggar RJ, Hall HI, Cross H, Crutchfield A, Finch JL, et al. Cancer risk in people infected with human immunodeficiency virus in the United States. International journal of cancer Journal international du cancer. 2008; 123(1):187–94. Epub 2008/04/26. https://doi.org/10.1002/ijc.23487 PMID: 18435450.
- Powles T, Robinson D, Stebbing J, Shamash J, Nelson M, Gazzard B, et al. Highly active antiretroviral therapy and the incidence of non-AIDS-defining cancers in people with HIV infection. J Clin Oncol. 2009; 27(6):884–90. Epub 2008/12/31. https://doi.org/10.1200/JCO.2008.19.6626 PMID: 19114688.
- Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. Lancet. 2007; 370 (9581):59–67. Epub 2007/07/10. https://doi.org/10.1016/S0140-6736(07)61050-2 PMID: 17617273.
- Machalek DA, Poynten M, Jin F, Fairley CK, Farnsworth A, Garland SM, et al. Anal human papillomavirus infection and associated neoplastic lesions in men who have sex with men: a systematic review and meta-analysis. The lancet oncology. 2012; 13(5):487–500. Epub 2012/03/27. <u>https://doi.org/10. 1016/S1470-2045(12)70080-3 PMID: 22445259</u>.

- Palefsky JM. Anal cancer prevention in HIV-positive men and women. Current opinion in oncology. 2009; 21(5):433–8. Epub 2009/07/10. <u>https://doi.org/10.1097/CCO.0b013e32832f511a</u> PMID: 19587592.
- Palefsky JM. Antiretroviral therapy and anal cancer: the good, the bad, and the unknown. Sexually transmitted diseases. 2012; 39(7):501–3. Epub 2012/06/15. https://doi.org/10.1097/OLQ. 0b013e31825f7921 PMID: 22695317.
- Mallari AO, Schwartz TM, Luque AE, Polashenski PS, Rauh SM, Corales RB. Anal cancer screening in HIV-infected patients: is it time to screen them all? Dis Colon Rectum. 2012; 55(12):1244–50. Epub 2012/11/09. https://doi.org/10.1097/DCR.0b013e31826ab4fb PMID: 23135582.
- Denny LA, Franceschi S, de Sanjose S, Heard I, Moscicki AB, Palefsky J. Human papillomavirus, human immunodeficiency virus and immunosuppression. Vaccine. 2012; 30 Suppl 5:F168–74. Epub 2012/12/05. https://doi.org/10.1016/j.vaccine.2012.06.045 PMID: 23199960.
- Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol. 2006; 7(2):131–42. https://doi.org/10.1038/nrm1835 PMID: 16493418.
- Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, et al. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature. 1995; 375(6531):497–500. <u>https:// doi.org/10.1038/375497a0 PMID: 7539892</u>.
- Xiao H, Neuveut C, Tiffany HL, Benkirane M, Rich EA, Murphy PM, et al. Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. Proc Natl Acad Sci U S A. 2000; 97(21):11466–71. https://doi.org/10.1073/pnas.97.21.11466 PMID: 11027346.
- Rychert J, Strick D, Bazner S, Robinson J, Rosenberg E. Detection of HIV gp120 in plasma during early HIV infection is associated with increased proinflammatory and immunoregulatory cytokines. AIDS research and human retroviruses. 2010; 26(10):1139–45. Epub 2010/08/21. <u>https://doi.org/10. 1089/aid.2009.0290 PMID: 20722464; PubMed Central PMCID: PMC2982714.</u>
- Poggi A, Zocchi MR. HIV-1 Tat triggers TGF-beta production and NK cell apoptosis that is prevented by pertussis toxin B. Clin Dev Immunol. 2006; 13(2–4):369–72. Epub 2006/12/13. https://doi.org/10. 1080/17402520600645712 PMID: 17162379; PubMed Central PMCID: PMC2270756.
- Ulich C, Dunne A, Parry E, Hooker CW, Gaynor RB, Harrich D. Functional domains of Tat required for efficient human immunodeficiency virus type 1 reverse transcription. Journal of Virology. 1999; 73 (3):2499–508. Epub 1999/02/11. PMID: 9971835; PubMed Central PMCID: PMC104497.
- Toschi E, Bacigalupo I, Strippoli R, Chiozzini C, Cereseto A, Falchi M, et al. HIV-1 Tat regulates endothelial cell cycle progression via activation of the Ras/ERK MAPK signaling pathway. Mol Biol Cell. 2006; 17(4):1985–94. https://doi.org/10.1091/mbc.E05-08-0717 PMID: 16436505.
- Modesti N, Garcia J, Debouck C, Peterlin M, Gaynor R. Trans-dominant Tat mutants with alterations in the basic domain inhibit HIV-1 gene expression. The New biologist. 1991; 3(8):759–68. Epub 1991/ 08/01. PMID: 1931822.
- 93. Fletcher CV, Staskus K, Wietgrefe SW, Rothenberger M, Reilly C, Chipman JG, et al. Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. Proc Natl Acad Sci U S A. 2014; 111(6):2307–12. https://doi.org/10.1073/pnas.1318249111 PMID: 24469825; PubMed Central PMCID: PMC3926074.
- 94. Huang Y, Hoque MT, Jenabian MA, Vyboh K, Whyte SK, Sheehan NL, et al. Antiretroviral drug transporters and metabolic enzymes in human testicular tissue: potential contribution to HIV-1 sanctuary site. J Antimicrob Chemother. 2016; 71(7):1954–65. https://doi.org/10.1093/jac/dkw046 PMID: 27076103; PubMed Central PMCID: PMC4896405.
- 95. Lorenzo-Redondo R, Fryer HR, Bedford T, Kim EY, Archer J, Kosakovsky Pond SL, et al. Persistent HIV-1 replication maintains the tissue reservoir during therapy. Nature. 2016; 530(7588):51–6. https:// doi.org/10.1038/nature16933 PMID: 26814962; PubMed Central PMCID: PMC4865637.
- 96. Di Mascio M, Srinivasula S, Bhattacharjee A, Cheng L, Martiniova L, Herscovitch P, et al. Antiretroviral tissue kinetics: in vivo imaging using positron emission tomography. Antimicrob Agents Chemother. 2009; 53(10):4086–95. https://doi.org/10.1128/AAC.00419-09 PMID: 19667288; PubMed Central PMCID: PMC2764156.
- Bates RC, Mercurio AM. Tumor necrosis factor-alpha stimulates the epithelial-to-mesenchymal transition of human colonic organoids. Mol Biol Cell. 2003; 14(5):1790–800. Epub 2003/06/13. https://doi. org/10.1091/mbc.E02-09-0583 PMID: 12802055; PubMed Central PMCID: PMC165077.
- 98. Wang H, Wang HS, Zhou BH, Li CL, Zhang F, Wang XF, et al. Epithelial-mesenchymal transition (EMT) induced by TNF-alpha requires AKT/GSK-3beta-mediated stabilization of snail in colorectal cancer. PLoS One. 2013; 8(2):e56664. Epub 2013/02/23. https://doi.org/10.1371/journal.pone. 0056664 PMID: 23431386; PubMed Central PMCID: PMC3576347.
- Ho MY, Tang SJ, Chuang MJ, Cha TL, Li JY, Sun GH, et al. TNF-alpha induces epithelial-mesenchymal transition of renal cell carcinoma cells via a GSK3beta-dependent mechanism. Mol Cancer Res.

2012; 10(8):1109–19. Epub 2012/06/19. https://doi.org/10.1158/1541-7786.MCR-12-0160 PMID: 22707636.

- 100. Lv N, Gao Y, Guan H, Wu D, Ding S, Teng W, et al. Inflammatory mediators, tumor necrosis factoralpha and interferon-gamma, induce EMT in human PTC cell lines. Oncol Lett. 2015; 10(4):2591–7. Epub 2015/12/02. <u>https://doi.org/10.3892/ol.2015.3518</u> PMID: <u>26622895</u>; PubMed Central PMCID: PMC4580000.
- Rodriguez-Inigo E, Jimenez E, Bartolome J, Ortiz-Movilla N, Bartolome Villar B, Jose Arrieta J, et al. Detection of human immunodeficiency virus type 1 RNA by in situ hybridization in oral mucosa epithelial cells from anti-HIV-1 positive patients. J Med Virol. 2005; 77(1):17–22. <u>https://doi.org/10.1002/jmv.</u> 20409 PMID: 16032727.
- 102. Chou LL, Epstein J, Cassol SA, West DM, He W, Firth JD. Oral mucosal Langerhans' cells as target, effector and vector in HIV infection. J Oral Pathol Med. 2000; 29(8):394–402. https://doi.org/10.1034/j. 1600-0714.2000.290805.x PMID: 10972348
- 103. Goto Y, Yeh CK, Notkins AL, Prabhakar BS. Detection of proviral sequences in saliva of patients infected with human immunodeficiency virus type 1. AIDS Res Hum Retroviruses. 1991; 7(3):343–7. https://doi.org/10.1089/aid.1991.7.343 PMID: 2064831
- 104. Kakizawa J, Ushijima H, Oka S, Ikeda Y, Schroder HC, Muller WE. Detection of human immunodeficiency virus-1 DNA, RNA and antibody, and occult blood in inactivated saliva: availability of the filter paper disk method. Acta Paediatr Jpn. 1996; 38(3):218–23. https://doi.org/10.1111/j.1442-200x.1996.tb03473.x PMID: 8741309
- 105. Liuzzi G, Chirianni A, Clementi M, Bagnarelli P, Valenza A, Cataldo PT, et al. Analysis of HIV-1 load in blood, semen and saliva: evidence for different viral compartments in a cross-sectional and longitudinal study. Aids. 1996; 10(14):F51–6. <u>https://doi.org/10.1097/00002030-199612000-00001</u> PMID: 8970677
- 106. Maticic M, Poljak M, Kramar B, Tomazic J, Vidmar L, Zakotnik B, et al. Proviral HIV-1 DNA in gingival crevicular fluid of HIV-1-infected patients in various stages of HIV disease. J Dent Res. 2000; 79 (7):1496–501. https://doi.org/10.1177/00220345000790071101 PMID: 11005734
- Qureshi MN, Barr CE, Hewlitt I, Boorstein R, Kong F, Bagasra O, et al. Detection of HIV in oral mucosal cells. Oral Dis. 1997;3 Suppl 1:S73-8.
- Qureshi MN, Barr CE, Seshamma T, Reidy J, Pomerantz RJ, Bagasra O. Infection of oral mucosal cells by human immunodeficiency virus type 1 in seropositive persons. J Infect Dis. 1995; 171(1):190– 3. https://doi.org/10.1093/infdis/171.1.190 PMID: 7798662
- 109. Zuckerman RA, Whittington WL, Celum CL, Collis T, Lucchetti A, Sanchez JL, et al. Factors associated with oropharyngeal human immunodeficiency virus shedding. J Infect Dis. 2003; 188(1):142–5. https://doi.org/10.1086/375741 PMID: 12825183
- Nuovo GJ, Forde A, MacConnell P, Fahrenwald R. In situ detection of PCR-amplified HIV-1 nucleic acids and tumor necrosis factor cDNA in cervical tissues. Am J Pathol. 1993; 143(1):40–8. PMID: 8317555
- 111. Clemetson DB, Moss GB, Willerford DM, Hensel M, Emonyi W, Holmes KK, et al. Detection of HIV DNA in cervical and vaginal secretions. Prevalence and correlates among women in Nairobi, Kenya. JAMA: the journal of the American Medical Association. 1993; 269(22):2860–4. Epub 1993/06/09. PMID: 8497089.
- Sonza S, Mutimer HP, Oelrichs R, Jardine D, Harvey K, Dunne A, et al. Monocytes harbour replication-competent, non-latent HIV-1 in patients on highly active antiretroviral therapy. Aids. 2001; 15 (1):17–22. https://doi.org/10.1097/00002030-200101050-00005 PMID: 11192864.
- 113. Henning TR, Kissinger P, Lacour N, Meyaski-Schluter M, Clark R, Amedee AM. Elevated cervical white blood cell infiltrate is associated with genital HIV detection in a longitudinal cohort of antiretroviral therapy-adherent women. The Journal of infectious diseases. 2010; 202(10):1543–52. Epub 2010/10/ 12. https://doi.org/10.1086/656720 PMID: 20925530.
- 114. Crowe SM, Sonza S. HIV-1 can be recovered from a variety of cells including peripheral blood monocytes of patients receiving highly active antiretroviral therapy: a further obstacle to eradication. Journal of leukocyte biology. 2000; 68(3):345–50. Epub 2000/09/14. PMID: 10985250.
- 115. Jayakumar P, Berger I, Autschbach F, Weinstein M, Funke B, Verdin E, et al. Tissue-resident macrophages are productively infected ex vivo by primary X4 isolates of human immunodeficiency virus type 1. J Virol. 2005; 79(8):5220–6. https://doi.org/10.1128/JVI.79.8.5220-5226.2005 PMID: 15795306.
- 116. Santosuosso M, Righi E, Lindstrom V, Leblanc PR, Poznansky MC. HIV-1 envelope protein gp120 is present at high concentrations in secondary lymphoid organs of individuals with chronic HIV-1 infection. The Journal of infectious diseases. 2009; 200(7):1050–3. Epub 2009/08/25. https://doi.org/10. 1086/605695 PMID: 19698075.

- 117. Oh SK, Cruikshank WW, Raina J, Blanchard GC, Adler WH, Walker J, et al. Identification of HIV-1 envelope glycoprotein in the serum of AIDS and ARC patients. Journal of acquired immune deficiency syndromes. 1992; 5(3):251–6. Epub 1992/01/01. PMID: 1740750.
- Montagnier L, Clavel F, Krust B, Chamaret S, Rey F, Barre-Sinoussi F, et al. Identification and antigenicity of the major envelope glycoprotein of lymphadenopathy-associated virus. Virology. 1985; 144 (1):283–9. Epub 1985/07/15. https://doi.org/10.1016/0042-6822(85)90326-5 PMID: 2414918.
- Fujii Y, Otake K, Tashiro M, Adachi A. Soluble Nef antigen of HIV-1 is cytotoxic for human CD4+ T cells. FEBS letters. 1996; 393(1):93–6. Epub 1996/09/09. https://doi.org/10.1016/0014-5793(96) 00859-9 PMID: 8804432.
- Ali SA, Huang MB, Campbell PE, Roth WW, Campbell T, Khan M, et al. Genetic characterization of HIV type 1 Nef-induced vesicle secretion. AIDS research and human retroviruses. 2010; 26(2):173– 92. Epub 2010/02/17. https://doi.org/10.1089/aid.2009.0068 PMID: 20156100; PubMed Central PMCID: PMC2835390.
- 121. Elrefaei M, Burke CM, Baker CA, Jones NG, Bousheri S, Bangsberg DR, et al. HIV-specific TGF-beta-positive CD4+ T cells do not express regulatory surface markers and are regulated by CTLA-4. AIDS research and human retroviruses. 2010; 26(3):329–37. Epub 2010/05/04. https://doi.org/10.1089/aid. 2009.0149 PMID: 20433405; PubMed Central PMCID: PMC2933167.
- 122. Elrefaei M, Burke CM, Baker CA, Jones NG, Bousheri S, Bangsberg DR, et al. TGF-beta and IL-10 production by HIV-specific CD8+ T cells is regulated by CTLA-4 signaling on CD4+ T cells. PloS one. 2009; 4(12):e8194. Epub 2009/12/18. https://doi.org/10.1371/journal.pone.0008194 PMID: 20016783; PubMed Central PMCID: PMC2791208.
- Kekow J, Wachsman W, McCutchan JA, Cronin M, Carson DA, Lotz M. Transforming growth factor beta and noncytopathic mechanisms of immunodeficiency in human immunodeficiency virus infection. Proceedings of the National Academy of Sciences of the United States of America. 1990; 87 (21):8321–5. Epub 1990/11/01. https://doi.org/10.1073/pnas.87.21.8321 PMID: 1700428; PubMed Central PMCID: PMC54947.
- 124. Amarnath S, Dong L, Li J, Wu Y, Chen W. Endogenous TGF-beta activation by reactive oxygen species is key to Foxp3 induction in TCR-stimulated and HIV-1-infected human CD4+CD25- T cells. Retrovirology. 2007; 4:57. Epub 2007/08/11. https://doi.org/10.1186/1742-4690-4-57 PMID: 17688698; PubMed Central PMCID: PMC2096626.
- 125. Garba ML, Pilcher CD, Bingham AL, Eron J, Frelinger JA. HIV antigens can induce TGF-beta(1)-producing immunoregulatory CD8+ T cells. Journal of Immunology. 2002; 168(5):2247–54. Epub 2002/ 02/23. https://doi.org/10.4049/jimmunol.168.5.2247 PMID: 11859112.
- 126. Zocchi MR, Contini P, Alfano M, Poggi A. Pertussis toxin (PTX) B subunit and the nontoxic PTX mutant PT9K/129G inhibit Tat-induced TGF-beta production by NK cells and TGF-beta-mediated NK cell apoptosis. Journal of Immunology. 2005; 174(10):6054–61. Epub 2005/05/10. <u>https://doi.org/10.4049/ jimmunol.174.10.6054</u> PMID: 15879099.
- 127. Hu R, Oyaizu N, Than S, Kalyanaraman VS, Wang XP, Pahwa S. HIV-1 gp160 induces transforming growth factor-beta production in human PBMC. Clin Immunol Immunopathol. 1996; 80(3 Pt 1):283–9. Epub 1996/09/01. https://doi.org/10.1006/clin.1996.0125 PMID: 8811049.
- 128. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGFbeta and promotes tumor invasion and angiogenesis. Genes Dev. 2000; 14(2):163–76. Epub 2000/02/ 01. PMID: 10652271; PubMed Central PMCID: PMC316345.
- 129. Kobayashi T, Kim H, Liu X, Sugiura H, Kohyama T, Fang Q, et al. Matrix metalloproteinase-9 activates TGF-beta and stimulates fibroblast contraction of collagen gels. Am J Physiol Lung Cell Mol Physiol. 2014; 306(11):L1006–15. Epub 2014/04/08. https://doi.org/10.1152/ajplung.00015.2014 PMID: 24705725; PubMed Central PMCID: PMC4042193.
- 130. Birchenall-Roberts MC, Ruscetti FW, Kasper J, Lee HD, Friedman R, Geiser A, et al. Transcriptional regulation of the transforming growth factor beta 1 promoter by v-src gene products is mediated through the AP-1 complex. Molecular and Cellular Biology. 1990; 10(9):4978–83. Epub 1990/09/01. https://doi.org/10.1128/mcb.10.9.4978 PMID: 2117705; PubMed Central PMCID: PMC361127.
- 131. Glauser DA, Schlegel W. Sequential actions of ERK1/2 on the AP-1 transcription factor allow temporal integration of metabolic signals in pancreatic beta cells. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2007; 21(12):3240–9. Epub 2007/05/17. https://doi.org/10.1096/fj.06-7798com PMID: 17504975.
- Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. The Journal of biological chemistry. 1995; 270(28):16483–6. Epub 1995/07/14. https://doi.org/10.1074/jbc.270.28.16483 PMID: 7622446.

- 133. Lee C, Liu QH, Tomkowicz B, Yi Y, Freedman BD, Collman RG. Macrophage activation through CCR5- and CXCR4-mediated gp120-elicited signaling pathways. Journal of leukocyte biology. 2003; 74(5):676–82. Epub 2003/09/10. https://doi.org/10.1189/jlb.0503206 PMID: 12960231.
- 134. Del Corno M, Liu QH, Schols D, de Clercq E, Gessani S, Freedman BD, et al. HIV-1 gp120 and chemokine activation of Pyk2 and mitogen-activated protein kinases in primary macrophages mediated by calcium-dependent, pertussis toxin-insensitive chemokine receptor signaling. Blood. 2001; 98 (10):2909–16. Epub 2001/11/08. https://doi.org/10.1182/blood.v98.10.2909 PMID: 11698270.
- 135. Freedman BD, Liu QH, Del Corno M, Collman RG. HIV-1 gp120 chemokine receptor-mediated signaling in human macrophages. Immunologic research. 2003; 27(2–3):261–76. Epub 2003/07/15. <u>https:// doi.org/10.1385/IR:27:2-3:261</u> PMID: 12857973.
- 136. Maresca M, Mahfoud R, Garmy N, Kotler DP, Fantini J, Clayton F. The virotoxin model of HIV-1 enteropathy: involvement of GPR15/Bob and galactosylceramide in the cytopathic effects induced by HIV-1 gp120 in the HT-29-D4 intestinal cell line. J Biomed Sci. 2003; 10(1):156–66. <u>https://doi.org/10.1007/</u> bf02256007 PMID: 12566994.
- Bobardt MD, Chatterji U, Selvarajah S, Van der Schueren B, David G, Kahn B, et al. Cell-free human immunodeficiency virus type 1 transcytosis through primary genital epithelial cells. J Virol. 2007; 81 (1):395–405. https://doi.org/10.1128/JVI.01303-06 PMID: 17050597.
- 138. Howell AL, Asin SN, Yeaman GR, Wira CR. HIV-1 infection of the female reproductive tract. Current HIV/AIDS reports. 2005; 2(1):35–8. Epub 2005/08/11. https://doi.org/10.1007/s11904-996-0007-0 PMID: 16091247.
- 139. Liu X, Zha J, Chen H, Nishitani J, Camargo P, Cole SW, et al. Human immunodeficiency virus type 1 infection and replication in normal human oral keratinocytes. J Virol. 2003; 77(6):3470–6. https://doi.org/10.1128/JVI.77.6.3470-3476.2003 PMID: 12610122
- 140. Herrera R, Morris M, Rosbe K, Feng Z, Weinberg A, Tugizov S. Human beta-defensins 2 and -3 cointernalize with human immunodeficiency virus via heparan sulfate proteoglycans and reduce infectivity of intracellular virions in tonsil epithelial cells. Virology. 2016; 487:172–87. https://doi.org/10.1016/j.virol.2015.09.025 PMID: 26539799; PubMed Central PMCID: PMC4679645.
- Rider CC, Mulloy B. Heparin, Heparan Sulphate and the TGF-beta Cytokine Superfamily. Molecules. 2017; 22(5). Epub 2017/05/05. https://doi.org/10.3390/molecules22050713 PMID: 28468283; PubMed Central PMCID: PMC6154108.
- 142. Barillari G, Sgadari C, Fiorelli V, Samaniego F, Colombini S, Manzari V, et al. The Tat protein of human immunodeficiency virus type-1 promotes vascular cell growth and locomotion by engaging the alpha5beta1 and alphavbeta3 integrins and by mobilizing sequestered basic fibroblast growth factor. Blood. 1999; 94(2):663–72. PMID: 10397733.
- 143. Watson K, Edwards RJ. HIV-1-trans-activating (Tat) protein: both a target and a tool in therapeutic approaches. Biochem Pharmacol. 1999; 58(10):1521–8. <u>https://doi.org/10.1016/s0006-2952(99)</u> 00209-9 PMID: 10535742.
- 144. Barillari G, Sgadari C, Palladino C, Gendelman R, Caputo A, Morris CB, et al. Inflammatory cytokines synergize with the HIV-1 Tat protein to promote angiogenesis and Kaposi's sarcoma via induction of basic fibroblast growth factor and the alpha v beta 3 integrin. J Immunol. 1999; 163(4):1929–35. PMID: 10438928.
- 145. Urbinati C, Mitola S, Tanghetti E, Kumar C, Waltenberger J, Ribatti D, et al. Integrin alphavbeta3 as a target for blocking HIV-1 Tat-induced endothelial cell activation in vitro and angiogenesis in vivo. Arter-ioscler Thromb Vasc Biol. 2005; 25(11):2315–20. https://doi.org/10.1161/01.ATV.0000186182.14908. 7b PMID: 16166568.
- 146. Vogel BE, Lee SJ, Hildebrand A, Craig W, Pierschbacher MD, Wong-Staal F, et al. A novel integrin specificity exemplified by binding of the alpha v beta 5 integrin to the basic domain of the HIV Tat protein and vitronectin. J Cell Biol. 1993; 121(2):461–8. https://doi.org/10.1083/jcb.121.2.461 PMID: 7682219.
- 147. Dwinell MB, Eckmann L, Leopard JD, Varki NM, Kagnoff MF. Chemokine receptor expression by human intestinal epithelial cells. Gastroenterology. 1999; 117(2):359–67. <u>https://doi.org/10.1053/gast. 1999.0029900359</u> PMID: 10419917.
- Kaplan IM, Wadia JS, Dowdy SF. Cationic TAT peptide transduction domain enters cells by macropinocytosis. Journal of controlled release: official journal of the Controlled Release Society. 2005; 102 (1):247–53. Epub 2005/01/18. https://doi.org/10.1016/j.jconrel.2004.10.018 PMID: 15653149.
- 149. Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TATfusion proteins after lipid raft macropinocytosis. Nat Med. 2004; 10(3):310–5. Epub 2004/02/11. https://doi.org/10.1038/nm996 PMID: 14770178.
- **150.** Mann DA, Frankel AD. Endocytosis and targeting of exogenous HIV-1 Tat protein. The EMBO journal. 1991; 10(7):1733–9. Epub 1991/07/01. PMID: 2050110; PubMed Central PMCID: PMC452844.

- 151. Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, Beltram F. Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. Molecular therapy: the journal of the American Society of Gene Therapy. 2003; 8(2):284–94. Epub 2003/08/09. https://doi.org/10.1016/ s1525-0016(03)00122-9 PMID: 12907151.
- 152. Fittipaldi A, Ferrari A, Zoppe M, Arcangeli C, Pellegrini V, Beltram F, et al. Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. The Journal of biological chemistry. 2003; 278(36):34141–9. Epub 2003/05/30. https://doi.org/10.1074/jbc.M303045200 PMID: 12773529.
- 153. Poggi A, Zocchi MR. HIV-1 Tat triggers TGF-beta production and NK cell apoptosis that is prevented by pertussis toxin B. Clin Dev Immunol. 2006; 13(2–4):369–72. Epub 2006/12/13. https://doi.org/10. 1080/17402520600645712 PMID: 17162379; PubMed Central PMCID: PMC2270756.
- 154. Fantini J, Maresca M, Hammache D, Yahi N, Delezay O. Glycosphingolipid (GSL) microdomains as attachment platforms for host pathogens and their toxins on intestinal epithelial cells: activation of signal transduction pathways and perturbations of intestinal absorption and secretion. Glycoconj J. 2000; 17(3–4):173–9. Epub 2001/02/24. https://doi.org/10.1023/a:1026580905156 PMID: 11201788.
- 155. Bozou JC, Rochet N, Magnaldo I, Vincent JP, Kitabgi P. Neurotensin stimulates inositol trisphosphatemediated calcium mobilization but not protein kinase C activation in HT29 cells. Involvement of a Gprotein. Biochem J. 1989; 264(3):871–8. Epub 1989/12/15. https://doi.org/10.1042/bj2640871 PMID: 2559720; PubMed Central PMCID: PMC1133666.
- 156. Stewart JR, O'Brian CA. Protein kinase C-{alpha} mediates epidermal growth factor receptor transactivation in human prostate cancer cells. Molecular cancer therapeutics. 2005; 4(5):726–32. Epub 2005/05/18. https://doi.org/10.1158/1535-7163.MCT-05-0013 PMID: 15897236.
- 157. Chen X, Zhou B, Yan J, Xu B, Tai P, Li J, et al. Epidermal growth factor receptor activation by protein kinase C is necessary for FSH-induced meiotic resumption in porcine cumulus-oocyte complexes. J Endocrinol. 2008; 197(2):409–19. Epub 2008/04/25. https://doi.org/10.1677/JOE-07-0592 PMID: 18434371.
- Planes R, Serrero M, Leghmari K, BenMohamed L, Bahraoui E. HIV-1 Envelope Glycoproteins Induce the Production of TNF-alpha and IL-10 in Human Monocytes by Activating Calcium Pathway. Sci Rep. 2018; 8(1):17215. Epub 2018/11/23. https://doi.org/10.1038/s41598-018-35478-1 PMID: <u>30464243</u>; PubMed Central PMCID: PMC6249280.
- 159. Leghmari K, Bennasser Y, Tkaczuk J, Bahraoui E. HIV-1 Tat protein induces IL-10 production by an alternative TNF-alpha-independent pathway in monocytes: role of PKC-delta and p38 MAP kinase. Cell Immunol. 2008; 253(1–2):45–53. Epub 2008/06/11. <u>https://doi.org/10.1016/j.cellimm.2008.04.015 PMID: 18541226.</u>
- Barisoni L, Bruggeman LA, Mundel P, D'Agati VD, Klotman PE. HIV-1 induces renal epithelial dedifferentiation in a transgenic model of HIV-associated nephropathy. Kidney international. 2000; 58(1):173– 81. Epub 2000/07/08. https://doi.org/10.1046/j.1523-1755.2000.00152.x PMID: 10886562.
- 161. Schwartz EJ, Cara A, Snoeck H, Ross MD, Sunamoto M, Reiser J, et al. Human immunodeficiency virus-1 induces loss of contact inhibition in podocytes. J Am Soc Nephrol. 2001; 12(8):1677–84. Epub 2001/07/20. PMID: 11461940.
- 162. Medapalli RK, He JC, Klotman PE. HIV-associated nephropathy: pathogenesis. Curr Opin Nephrol Hypertens. 2011; 20(3):306–11. Epub 2011/03/02. https://doi.org/10.1097/MNH.0b013e328345359a PMID: 21358326; PubMed Central PMCID: PMC3153858.
- 163. Bruggeman LA, Ross MD, Tanji N, Cara A, Dikman S, Gordon RE, et al. Renal epithelium is a previously unrecognized site of HIV-1 infection. J Am Soc Nephrol. 2000; 11(11):2079–87. Epub 2000/10/29. PMID: 11053484.
- Abd-El-Basset EM, Prashanth J, Ananth Lakshmi KV. Up-regulation of cytoskeletal proteins in activated microglia. Med Princ Pract. 2004; 13(6):325–33. https://doi.org/10.1159/000080469 PMID: 15467307.
- 165. Lu TC, He JC, Wang ZH, Feng X, Fukumi-Tominaga T, Chen N, et al. HIV-1 Nef disrupts the podocyte actin cytoskeleton by interacting with diaphanous interacting protein. The Journal of biological chemistry. 2008; 283(13):8173–82. Epub 2008/02/01. https://doi.org/10.1074/jbc.M708920200 PMID: 18234668; PubMed Central PMCID: PMC2276381.
- 166. Lan X, Wen H, Cheng K, Plagov A, Marashi Shoshtari SS, Malhotra A, et al. Hedgehog pathway plays a vital role in HIV-induced epithelial-mesenchymal transition of podocyte. Exp Cell Res. 2017; 352 (2):193–201. Epub 2017/02/06. https://doi.org/10.1016/j.yexcr.2017.01.019 PMID: 28159470.
- **167.** Rosenberg AZ, Naicker S, Winkler CA, Kopp JB. HIV-associated nephropathies: epidemiology, pathology, mechanisms and treatment. Nat Rev Nephrol. 2015; 11(3):150–60. Epub 2015/02/18. https://doi.org/10.1038/nrneph.2015.9 PMID: 25686569.
- 168. Kumar D, Konkimalla S, Yadav A, Sataranatarajan K, Kasinath BS, Chander PN, et al. HIV-associated nephropathy: role of mammalian target of rapamycin pathway. The American journal of pathology.

2010; 177(2):813–21. Epub 2010/06/29. https://doi.org/10.2353/ajpath.2010.100131 PMID: 20581056; PubMed Central PMCID: PMC2913356.

- 169. Marras D, Bruggeman LA, Gao F, Tanji N, Mansukhani MM, Cara A, et al. Replication and compartmentalization of HIV-1 in kidney epithelium of patients with HIV-associated nephropathy. Nat Med. 2002; 8(5):522–6. Epub 2002/05/02. https://doi.org/10.1038/nm0502-522 PMID: 11984599.
- Zerhouni-Layachi B, Husain M, Ross MJ, Marras D, Sunamoto M, Liu X, et al. Dual tropism of HIV-1 envelopes derived from renal tubular epithelial cells of patients with HIV-associated nephropathy. AIDS. 2006; 20(4):621–4. Epub 2006/02/14. https://doi.org/10.1097/01.aids.0000210618.68083.8e PMID: 16470129.
- 171. Yadav A, Vallabu S, Kumar D, Ding G, Charney DN, Chander PN, et al. HIVAN phenotype: consequence of epithelial mesenchymal transdifferentiation. American journal of physiology Renal physiology. 2010; 298(3):F734–44. Epub 2009/12/18. https://doi.org/10.1152/ajprenal.00415.2009 PMID: 20015943; PubMed Central PMCID: PMC2838599.
- 172. Mallipattu SK, Liu R, Zhong Y, Chen EY, D'Agati V, Kaufman L, et al. Expression of HIV transgene aggravates kidney injury in diabetic mice. Kidney international. 2013. Epub 2013/01/18. <u>https://doi.org/ 10.1038/ki.2012.445</u> PMID: 23325078.
- 173. Husain M, D'Agati VD, He JC, Klotman ME, Klotman PE. HIV-1 Nef induces dedifferentiation of podocytes in vivo: a characteristic feature of HIVAN. AIDS. 2005; 19(17):1975–80. Epub 2005/11/02. https://doi.org/10.1097/01.aids.0000191918.42110.27 PMID: 16260903.
- 174. Sunamoto M, Husain M, He JC, Schwartz EJ, Klotman PE. Critical role for Nef in HIV-1-induced podocyte dedifferentiation. Kidney international. 2003; 64(5):1695–701. Epub 2003/10/09. <u>https://doi.org/ 10.1046/j.1523-1755.2003.00283.x PMID: 14531802.</u>
- 175. Tan R, Patni H, Tandon P, Luan L, Sharma B, Salhan D, et al. Nef interaction with actin compromises human podocyte actin cytoskeletal integrity. Experimental and molecular pathology. 2012; 94(1):51–7. Epub 2012/06/23. https://doi.org/10.1016/j.yexmp.2012.06.001 PMID: 22721673; PubMed Central PMCID: PMC3463768.
- 176. He JC, Husain M, Sunamoto M, D'Agati VD, Klotman ME, Iyengar R, et al. Nef stimulates proliferation of glomerular podocytes through activation of Src-dependent Stat3 and MAPK1,2 pathways. The Journal of clinical investigation. 2004; 114(5):643–51. Epub 2004/09/03. https://doi.org/10.1172/JCl21004 PMID: 15343382; PubMed Central PMCID: PMC514582.
- Brune KA, Ferreira F, Mandke P, Chau E, Aggarwal NR, D'Alessio FR, et al. HIV Impairs Lung Epithelial Integrity and Enters the Epithelium to Promote Chronic Lung Inflammation. PLoS One. 2016; 11 (3):e0149679. Epub 2016/03/02. https://doi.org/10.1371/journal.pone.0149679 PMID: 26930653; PubMed Central PMCID: PMC4773117.
- 178. Chung CY, Alden SL, Funderburg NT, Fu P, Levine AD. Progressive proximal-to-distal reduction in expression of the tight junction complex in colonic epithelium of virally-suppressed HIV+ individuals. PLoS Pathog. 2014; 10(6):e1004198. Epub 2014/06/27. https://doi.org/10.1371/journal.ppat.1004198 PMID: 24968145; PubMed Central PMCID: PMC4072797.
- 179. Munawwar A, Singh S. Human Herpesviruses as Copathogens of HIV Infection, Their Role in HIV Transmission, and Disease Progression. J Lab Physicians. 2016; 8(1):5–18. Epub 2016/03/26. https:// doi.org/10.4103/0974-2727.176228 PMID: 27013807; PubMed Central PMCID: PMC4785766.
- Liberto MC, Zicca E, Pavia G, Quirino A, Marascio N, Torti C, et al. Virological Mechanisms in the Coinfection between HIV and HCV. Mediators Inflamm. 2015; 2015:320532. Epub 2015/10/27. <u>https://</u> doi.org/10.1155/2015/320532 PMID: 26494946; PubMed Central PMCID: PMC4606210.
- 181. Singh KP, Crane M, Audsley J, Avihingsanon A, Sasadeusz J, Lewin SR. HIV-hepatitis B virus coinfection: epidemiology, pathogenesis, and treatment. AIDS. 2017; 31(15):2035–52. Epub 2017/07/12. https://doi.org/10.1097/QAD.0000000001574 PMID: 28692539; PubMed Central PMCID: PMC5661989.
- Groeger S, Meyle J. Oral Mucosal Epithelial Cells. Front Immunol. 2019; 10:208. Epub 2019/03/07. https://doi.org/10.3389/fimmu.2019.00208 PMID: 30837987; PubMed Central PMCID: PMC6383680.
- 183. De Tomasi JB, Opata MM, Mowa CN. Immunity in the Cervix: Interphase between Immune and Cervical Epithelial Cells. J Immunol Res. 2019; 2019;7693183. Epub 2019/05/31. <u>https://doi.org/10.1155/2019/7693183</u> PMID: 31143785; PubMed Central PMCID: PMC6501150.
- 184. Berth S, Caicedo HH, Sarma T, Morfini G, Brady ST. Internalization and axonal transport of the HIV glycoprotein gp120. ASN Neuro. 2015; 7(1). Epub 2015/02/01. https://doi.org/10.1177/ 1759091414568186 PMID: 25636314; PubMed Central PMCID: PMC4720180.
- 185. Pandhare J, Dash S, Jones B, Villalta F, Dash C. A Novel Role of Proline Oxidase in HIV-1 Envelope Glycoprotein-induced Neuronal Autophagy. J Biol Chem. 2015; 290(42):25439–51. Epub 2015/09/04. https://doi.org/10.1074/jbc.M115.652776 PMID: 26330555; PubMed Central PMCID: PMC4646191.

**186.** Bai L, Zhang Z, Zhang H, Li X, Yu Q, Lin H, et al. HIV-1 Tat protein alter the tight junction integrity and function of retinal pigment epithelium: an in vitro study. BMC Infect Dis. 2008; 8:77. <u>https://doi.org/10.1186/1471-2334-8-77</u> PMID: 18538010.