



Micropatterned co-cultures of T-lymphocytes and epithelial cells as a model of mucosal immune system

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ABSTRACT

Gut-associated lymphoid tissue is a major target and reservoir of human immunodeficiency virus (HIV)-infected T-cells. Our studies seek to recapitulate, *in vitro*, interactions between HIV-infected T-lymphocytes and intestinal epithelial cells in order to investigate the mechanisms underlying the disruption of normal epithelial cell and barrier function. Here, we describe a novel approach for creating co-cultures of healthy or HIV-infected T-lymphocytes (Jurkat) and human intestinal epithelial (HT-29) cells where both cell types are positioned on the same surface in a precise spatial configuration (micropattern). This co-culture method simplified observation/monitoring of the two cell types and was particularly suited for laser microdissection-based retrieval of the desired cells for downstream gene expressions studies. DNA microarray analysis of epithelial cells retrieved from co-cultures with HIV-1-infected vs. uninfected Jurkat cells revealed that epithelial cells from HIV-infected co-cultures exhibited gene expression patterns consistent with disruption of epithelial barrier formation. Overall, the micropatterned co-culture system described here is envisioned as a valuable new tool for delineating how HIV and other infections contribute to dysfunction of mucosal epithelium.

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Introduction

Recent studies of the immunology and pathogenesis of HIV infection have revealed that the gastrointestinal tract is a major site of viral replication and CD4⁺ T-cell loss throughout all stages of infection [1,2], and may be the major viral reservoir [3], even in patients receiving anti-retroviral therapy (ART) who display undetectable viral burden in the peripheral blood [4]. Additional investigations have demonstrated structural and functional abnormalities within the HIV-infected GI tract that correlate with increased levels of inflammation, immune activation, and decreased levels of mucosal repair and regeneration [5]. However, despite the wealth of descriptive information, the molecular mechanisms that drive enteropathogenesis remain unclear.

Previous transcriptional studies of the intestinal mucosa in HIV-infected patients have utilized microarray analysis of mucosal tissue obtained from endoscopic biopsy to show dysregulation of host genes involved in mucosal epithelial growth. This profile of impaired growth and regeneration initiates in acute stage and persists during chronic infection in the absence of antiviral therapy

[6,7] as well as HIV-1-infected patients on ART who display high viral loads [8]. However, endoscopic biopsy techniques are invasive and limited in their practical use for detailed mechanistic studies. There is considerable need for effective *in vitro* cell culture systems with which to study intestinal epithelial growth in the context of HIV infection.

Co-culture models are particularly useful for investigating heterotypic interactions and have previously been used extensively for the investigation of mucosal barrier and cell function. Traditionally, these co-culture systems employ transwell configuration where the two cell types lack direct contact and can only engage in endocrine communication [9]. Alternatively, epithelial and immune cells have been co-cultured on the same surface but in random configuration. Such random co-cultures do not permit precise control over the extent of contacts between the cell types of the co-culture.

A number of microtechnology-based approaches have been proposed to enable placement of the cell types of the co-culture in a precise spatial pattern (micropattern) [10]. These approaches included photolithography [11], removable stencils [12], and robotic microarraying [13–15]. Interestingly, robotically printed arrays of antibodies (Abs) have also been employed to capture leukocytes on substrates for immunophenotyping applications [16–18].

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The goal of this study was to construct micropatterned co-cultures of intestinal epithelial cells and T-lymphocytes in order to establish a new *in vitro* model of HIV pathogenesis of the gastrointestinal mucosa. This co-culture was created by capturing T-cells on printed arrays of anti-CD3 Ab spots and then cultivating mucosal epithelial cells alongside the surface-bound immune cells on the same culture substrate. Laser microdissection was used to retrieve epithelial cells after 3 days of cultivation with HIV-infected or uninfected T-cells (Jurkats). DNA microarray analysis of the epithelial cells removed from HIV-infected co-cultures revealed marked differences in expression of genes controlling epithelial differentiation, cytoskeletal functions, and tight junction formation compared to uninfected co-cultures. These results suggested that epithelial barrier formation was less effective when HIV-infected Jurkat cells were present alongside mucosal epithelial cells. This is one of the first reports describing the use of microfabrication approaches for orchestrating interactions of cells relevant to the mucosal immune response. We envision this cell culture system as an important tool for investigating pathophysiology of HIV or other infections in the context of mucosal epithelium.

Materials and methods

Printing antibody microarrays. Regular microscope slides (25 × 75 × 1 mm, Fisher Scientific, Pittsburgh, PA) were cleaned with Piranha solution (1:1 v/v mixture of 98% sulfuric acid and 37% hydrogen peroxide) in a quartz container for 15 min and thoroughly rinsed with de-ionized (DI) water. The slides were then silanized according to procedures described earlier [17,18]. Briefly, microscope slides, after Piranha cleaning, were soaked in 40 mL of toluene containing 0.05% (v/v) 3-acryloxypropyl-trichloro-silane (Gelest, Inc., Morrisville, PA) for 1 h and then dried at 100 °C for no <2 h. The silanized slides were stored in a desiccator prior to microarray printing. A robotic microarrayer (GMS 417, Affymetrix, Santa Clara, CA) was involved in creating multiplexed Ab microarrays. Monoclonal mouse anti-human CD3 Ab (Beckman Coulter, Inc., Fullerton, CA) was adjusted to a concentration of 0.2 mg/mL in 1 × PBS containing 0.005% (v/v) Tween-20. The working Ab solution was loaded into a 384-well micro-plate and the robotic microarrayer was used to dispense 1–2 nL of each solution onto a silanized surface forming 150–300 μm circular spots. The slides with Ab spots were air dried and stored in a sealed box at 4 °C prior to use. Characterization of morphology and quality of printed Ab spots was accomplished with 0.01 mg/mL anti-mouse IgG-FITC by fluorescence microscopy.

Cultivation of cells and construction of micropatterned co-cultures. Jurkat, clone E6-1, (TIB-152™, ATCC) was grown in 75-cm² flasks (Costar) in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), sterile filtered, with 100 U/mL penicillin/streptomycin, L-glutamine (Invitrogen). The human colorectal adenocarcinoma cells HT-29 were routinely cultured in McCoy's 5A media (Sigma) and completed with 10% FBS, 2 mM glutamine and 100 U/mL of penicillin-streptomycin. All cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere. Cells were harvested from the culture flasks by a 10-min treatment with trypsin-EDTA (modified Puck's saline solution containing 0.5 g of trypsin and 0.2 g of EDTA per liter, Gibco). The cell suspension was then added to an equal volume of culture medium, centrifuged, and resuspended in fresh medium.

Glass slide with printed antibody microarray was sterilized with 70% ethanol, and washed twice with 1 × PBS. The process of cell patterning is shown in Fig. 1. Prior to seeding the Jurkat T-cells, HT-29 epithelial cells were seeded around the Ab spots at concentration of 1–2 × 10⁶ cells/mL and incubated for 2 h at 37 °C. The epithelial cells were grown until they reached about 75% conflu-

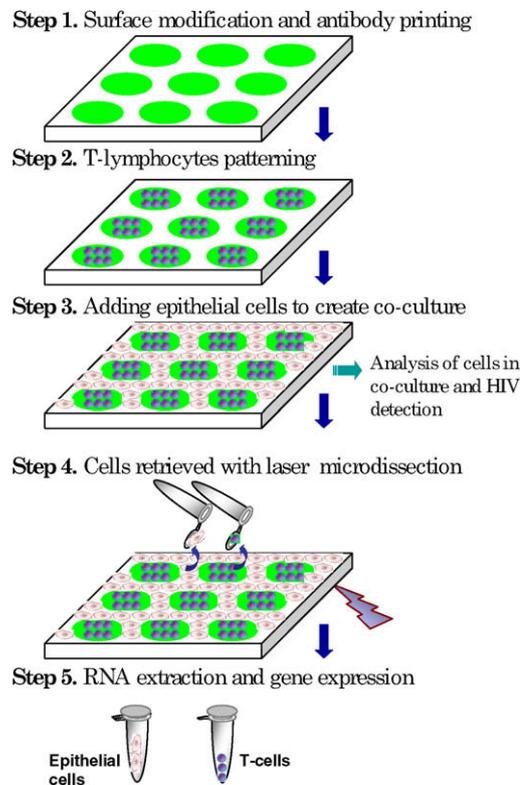


Fig. 1. Schematic diagram of experiments aimed at capturing T-cells on Ab microarrays and co-cultivation with epithelial cells. *Step 1:* Arrays consisting of anti-CD3-Abs were printed onto silane modified glass slide. *Step 2:* T-cells (Jurkat) were incubated with the Ab microarrays. *Step 3:* The glass surface containing arrays of T-cells was then incubated with mucosal epithelial cells (HT-29 cell line) which adhered and spread around T-lymphocyte clusters. Over the course of 10–12 days in culture cells were analyzed for viability and tight junction protein expression. Both uninfected and HIV-1-infected Jurkat cells were cultivated with epithelial cells. *Step 4:* Arrangement of the two cell types in distinct locations of the surface enabled retrieval of the desired cells from the co-culture for downstream gene expression analysis. *Step 5:* Gene expression profiles of epithelial cells extracted from normal and HIV-infected co-cultures were analyzed using DNA microarrays.

ence (up to 3 days). The antibody microarrays were then incubated with Jurkat cell suspensions in RPMI 1640 media at 37 °C for 30 min at a concentration of 1 × 10⁶ cells/mL. Unattached cells were removed by washing twice with 1 × PBS. Characterization of the co-culture revealed that both cell types remained viable after 12 days in culture (data not shown).

HIV infection of Jurkat cells. Jurkat cells were infected with HIV-1 subtype B laboratory strain LAI and maintained 3 days at 37 °C before using for co-culture with epithelial cells. Expression of HIV gag was determined and compared between infected and uninfected Jurkat T cell cultures by RT-PCR as previously described [19]. Probes were tagged with a fluorescent dye (FAM) at the 5' end and a quencher dye at the 3' end. The reaction was carried out using one tube RT-PCR master mix (PE Applied Biosystems, CA) on the ABI Prism 7900 sequence detector (PE Applied Biosystems, CA). The data were analyzed with Sequence Detector Software (SDS) and quantitated using the relative computational method. Viral RNA was extracted with the QIAmp Viral RNA mini kit (Qiagen). TaqMan Universal PCR Master Mix (Roche Molecular Systems, Inc.) was used to generate cDNA templates used in the RT-PCR-based HIV assay.

Immunocytochemistry. Immunofluorescent staining was performed according to standard protocols [10]. Primary antibodies were used at the following dilutions: mouse anti-human ZO-1 (BD Transduction Laboratories™) – 20 μg/mL, rabbit anti-human

Occludin (Zymed® Lab, Invitrogen Immunodetection) – 20 µg/mL, and phalloidin-FITC (Sigma–Aldrich, France) – 10–25 µg/mL. The proper secondary antibodies were used: goat anti-rabbit IgG conjugated with Cy™3 or FITC (Zymed® Lab, Invitrogen Immunodetection) at 1:50 dilution, and goat anti-mouse IgG FITC or Texas red conjugated (Santa Cruz Biotechnology, Inc.) at a dilution of 1:100. Cells were exposed to DAPI at concentration of 0.1 µg/mL in 1 × PBS to stain the nuclei.

Imaging of cell cultures. Cell arrays formed on the glass slide were imaged using a brightfield microscope (Carl Zeiss Inc.). For immunofluorescence imaging, the glass substrates containing cells arrays were observed using confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss, Inc.). High-resolution images of the captured cell patterns were obtained using Hitachi S3500N scanning electron microscope (SEM) operating at 5 kV of accelerating voltage. For SEM imaging cells on microarrays were fixed with 4% paraformaldehyde and sputter-coated with ~10 nm of Au–Pd.

Laser capture microdissection and DNA microarray analysis. The microdissection apparatus PALM LMPC system (PALM Microlaser Technologies) allowed to select cells of interest from specific locations within the micropatterned co-cultures. Prior to laser catapulting, glass slides with cellular micropatterns were dehydrated, fixed with ice-cold 70% ethanol, and dried under nitrogen. These samples were stored in an airtight container at –80 °C prior to catapulting. Under direct visual control, selected cell compartments were isolated from the surrounding cells by the focused nitrogen laser beam. The detached cell samples were then collected in a microfuge cap coated with 1 × Applied Biosystems (AB) lysis buffer and mounted above the object slide. The cap containing the catapulted sample was placed on a microfuge tube and the sample mixed with 500 µL of the lysis buffer and stored at –20 °C until RNA extraction. Total RNA was extracted from the cell lysates using a 6700 automated nucleic acid workstation (Applied Biosystems) according to the manufacturer's instructions.

Gene expression profiles in co-culture samples were determined as previously described [7]. Briefly, labeling of samples, hybridization to human U133 Plus2 GeneChips (Affymetrix, Santa Clara, CA, USA), staining, and scanning were performed as described in the Affymetrix Expression Analysis Technical Manual. mRNA extracted from catapulted cells was amplified and labeled utilizing protocols of the Affymetrix Gene Expression Analysis Technical Manual. Labeling, hybridization, staining and scanning were performed as described previously [7]. Labeled cRNA was hybridized for 16 h at 45 °C.

Stringent statistical criteria were applied to the analysis of microarray data using robust model-based (dChip) algorithms. A minimum 2-fold difference between mean levels of transcription in epithelial and Jurkat cells in the presence or absence of HIV infection was used as criteria for determining changes in transcription. Fold differences were calculated from comparison of the transcription levels using at least 11 independent oligonucleotide probes for each gene or predicted gene with 95% confidence (P -value ≤ 0.05) using standard student t -tests. The Affymetrix GeneChip Operating System data files (*.cel and *.chp) have been deposited in and made available to the public at the Gene Expression Omnibus database (Accession Number: GSE10629).

Results and discussion

Using Ab microarrays to construct T-lymphocyte-epithelial cell co-cultures

Ab microarrays have previously been applied to the quantification of proteins in a complex biological milieu. Beyond detection of protein–protein interactions, arrays of Abs specific to cell surface antigens have been used to capture distinct subpopulations of leu-

kocytes from whole blood or other heterogeneous cell suspensions [16]. Our lab has previously employed Ab arrays to capture pure T-cell subsets from whole blood [17] and to detect T-cell secreted cytokines [20]. In this study, we sought to utilize the ability to capture T-cells on the Ab spots (300 or 500 µm diameter) in order to cultivate these cells in the immediate proximity of the intestinal epithelial cells. Selective placement of the T-cells on the Ab domains and attachment of epithelial cells around these domains allowed to create micropatterned co-cultures with distinct interface between immune and mucosal cells.

To create the T-cell adhesive regions, anti-CD3 Abs were printed onto silane-coated glass slides into microarrays comprised of either 300 or 500 µm diameter spots. Printed Ab arrays were comprised of seven by five spots (500 µm diameter) or 20 × 4 spots of 300 µm diameter. Modification of glass slides with silane was found to be useful for improving quality of the printed microarrays and for diminishing non-specific binding of T-cells. At the same time, these glass substrates supported attachment and growth of mucosal epithelial cells. Immobilization of Ab molecules on the silane-modified glass surfaces was robust and no smearing was observed during sterilization and washing steps. Quality of the printed spots was verified by immunofluorescent staining of printed spots of mouse anti-human CD3 Abs with anti-mouse IgG-FITC (Fig. 2A and B).

Construction of micropatterned co-cultures could be initiated by seeding either T-lymphocytes or mucosal epithelial cells. As shown in Fig. 2C and D, incubation of anti-CD3 microarrays with Jurkat cells followed by careful washing resulted in capture of the T-cells on Ab spots with minimal non-specific binding occurring silane-modified glass. Incubation of human mucosal epithelial

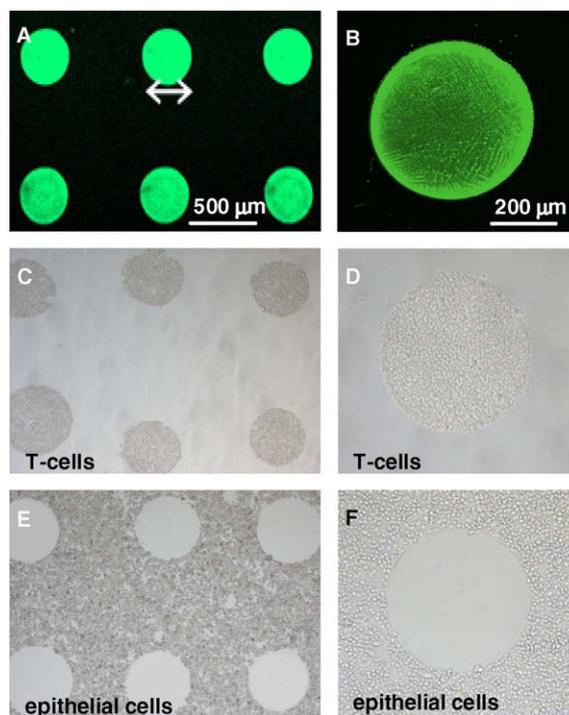


Fig. 2. Printing Ab microarrays and micropatterning of cells. (A,B) Immunofluorescent staining shows printed Ab domains (500 µm diameter) with well-demarcated boundaries. Spots of mouse anti-human CD3 Ab molecules were stained with anti-mouse IgG-FITC (green). (C,D) Jurkat cells adherent on anti-CD3 Ab spots (500 µm diameter). Note limited non-specific attachment on silane-modified glass substrate. (E,F) Attachment of HT-29 epithelial cells on the glass substrate around Ab spots. Epithelial cells neither attached on anti-CD3 Ab spots nor invaded these domains over time (5 days in culture). (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

cells (HT-29) with Ab microarrays led to the attachment of these cells on the silane-modified glass regions and exclusion of epithelial cells from Ab domains. As shown in Fig. 2E and F, epithelial cells formed a confluent layer but did not invade regions protected by the Ab molecules, allowing for addition of T-cells as the second step.

Images in Fig. 2C–F highlight distinct and complementary binding preferences of epithelial cells and T-lymphocytes. This complementarity allowed us to assemble both cell types on the same surface as shown in Fig. 3A–F. In a typical experiment, T-cells were seeded first followed by the epithelial cells, however, as discussed above the sequence of seeding steps could be reversed. Observing co-cultures over the period of two weeks (Fig. 3A–F) revealed that both cell types proliferated on their distinct regions of the surface with the boundary remaining clearly demarcated even after prolonged cultivation time (see Fig. 3E and F). This point was particularly advantageous for laser-mediated retrieval of lymphocytes and epithelial cells from uninfected or HIV-infected co-cultures.

Tight junction formation contributes to the barrier function of the mucosal epithelium and is therefore commonly used to characterize differentiation and growth of epithelial cells *in vitro*. We readily detected both zona occludins (ZO) and occludin by immunohistochemistry in the co-culture system after 5 days in growth (Fig. 3G–I). Fig. 3I depicts the interface region between T-cells and epithelial cells, and shows that tight junction staining was observed only in the area occupied by the epithelial cells.

Micropatterned co-cultures of HIV-infected T-cells and epithelial cells

In order to investigate HIV pathogenesis in the gastrointestinal tract, mucosal epithelial cells were co-cultured with HIV-infected

Jurkats (model T-cells). Presence of HIV in the Jurkat cultures was verified by RT-PCR analysis (data not shown). After confirming presence of infection, Jurkat cells were captured on the anti-CD3 Ab arrays as described in the previous section. HT-29 intestinal epithelial cells were added onto the same surface, completing the co-culture.

By creating micropatterned co-cultures we wanted to investigate whether cultivation of HIV-infected Jurkat cells in the proximity of HT-29 intestinal epithelial cells will affect epithelial function. The micropatterned format of the HT-29/Jurkat co-culture was particularly well suited for rapid laser capture microdissection-mediated retrieval of the desired cells for downstream gene expression studies. Fig. 4A highlights that both T-cells and epithelial cells could be retrieved from specific locations within the co-culture ensuring that the subsequent gene expression analysis reflected the local microenvironment context of the co-culture.

Extraction and gene expression analysis of the two cell types after 3 days of co-cultivation revealed significant increases in the transcription of genes regulating T-cell activation, antigen presentation, and antiviral response in HIV-infected Jurkat cells as compared to uninfected controls (results not shown). Gene expression analysis of the epithelial cells pointed to increases in inflammation-associated gene transcription, juxtaposed by marked decreases in genes mediating epithelial cell adhesion, growth, and development, including several involved in Wnt signaling (Fig. 4B). Epithelial barrier functions also appeared to have been compromised in the HIV-infected microenvironment, as evidenced by >10-fold down regulation of zona occludin-2, E-cadherin, and F11 receptor. These data are consistent with our previous findings of impairment in protective and regenerative capacity in intestinal epithelium in HIV-infected patients [19] that were based on the

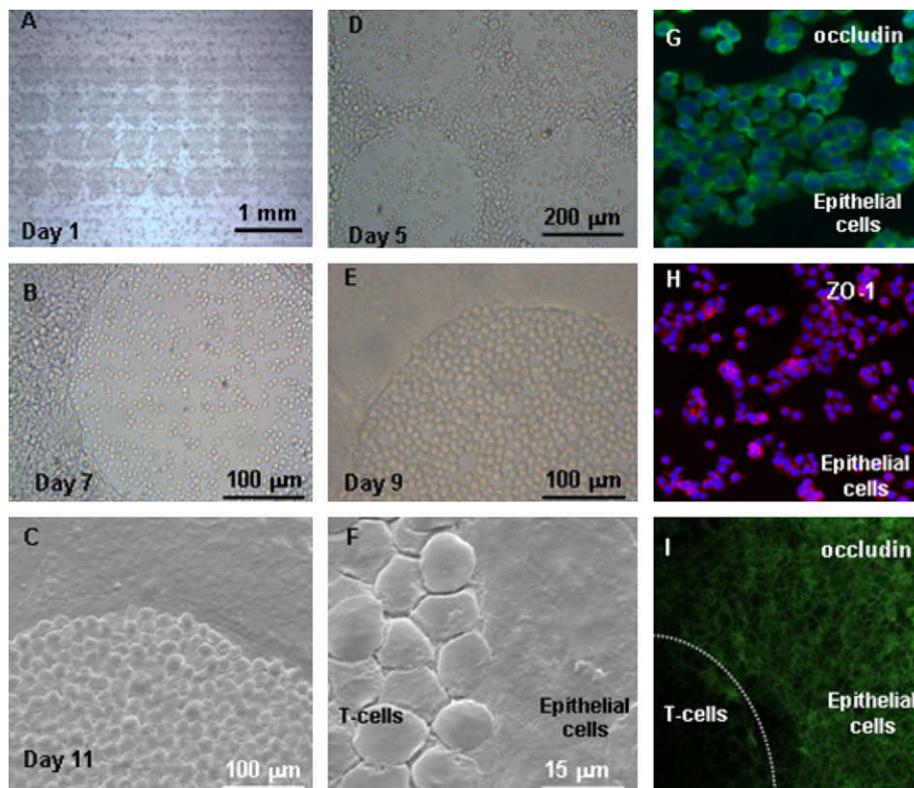


Fig. 3. Micropatterned co-cultures. (A) Jurkat cells adherent on 300 μm diameter Ab spots and surrounded by HT-29 cells – one day after seeding (magnification $\times 40$). (B–D) Bright-field images taken at day 5 (B), day 7 (C) and day 9 (D) demonstrate proliferation of both cell types within the co-culture. (E,F) SEM images collected after 11 days in culture. (G,H) Immuno-fluorescent staining of HT-29 cells shows presence of tight junction protein occludin (G) and ZO-1 (H) suggesting formation of a normal epithelial cell monolayer. (I) Immunostained region at the interface of T-cells and epithelial cells shows that tight junction formation (occludin – green) is associated exclusively with epithelial cells. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

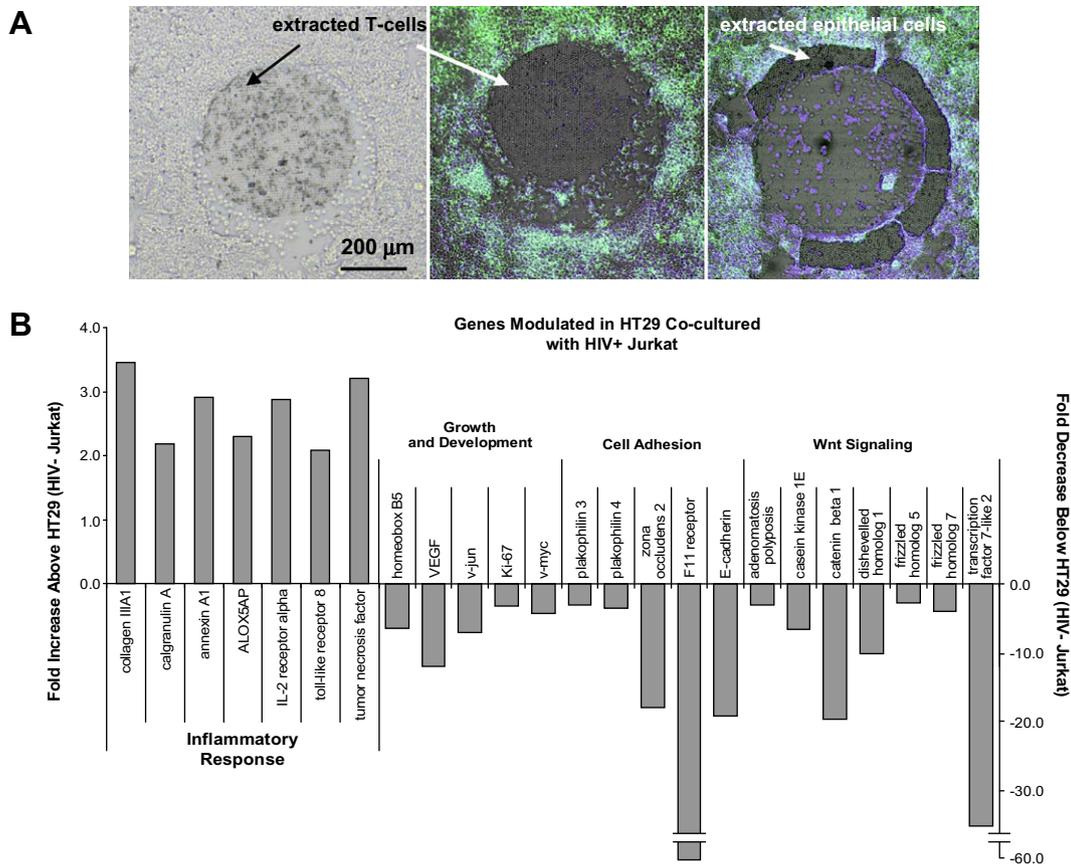


Fig. 4. Laser capture microdissection of cells and gene expression analysis. (A) Bright-field and fluorescence images demonstrating the site of Jurkat cell retrieval. Staining of the nuclei of cells with Dapi (blue) and actin with phalloidin (green) helped visualize location of cells in the co-cultures. Epithelial cells expressed actin while T-cells did not. Both T-cells and mucosal epithelial cells could be retrieved for gene expression analysis. (B) DNA microarray analysis of epithelial cells (HT-29) cultivated with infected and uninfected T-lymphocytes. Gene expression analysis of HT-29 cells pointed to the disruption of epithelial barrier function and upregulation of genes associated with inflammation in epithelial cells co-cultured with HIV-infected T-cells. Epithelial cells collected from co-cultures with uninfected Jurkat cells were used as controls for normalization of gene array data. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

endoscopic biopsies. Our results suggest that a disruption in Wnt signaling may be an underlying pathological mechanism and also highlight potential utility of the proposed micropatterned co-cultures as an *in vitro* model of intestinal epithelial response to HIV infection.

Conclusion

We have designed a novel, miniaturized *in vitro* model system of the human intestinal mucosa, consisting of spatially defined (micropatterned) co-cultures of human T-cells and intestinal epithelial cells. The conditions for assembly of the co-culture system and simultaneous growth of human HT-29 colonic epithelial cells and Jurkat T-lymphocytes were optimized, and the effects of HIV infection on epithelial growth and function were investigated. The novel configuration of the co-culture, whereby both cell types were localized to specific regions on the culturing substrate, uniquely suited our system for laser microdissection-mediated retrieval of cells and downstream high throughput gene expression analysis by DNA arrays. Further development of this cell culture model may lead to its establishment as a new investigative tool for exploring and testing diagnostic, treatment and prevention strategies of HIV/AIDS. Beyond HIV research, micropatterned virus-host co-cultures will be generally applicable for the study of biology of viral infections, pathogenesis of diseases, opportunistic complications and host defense immune responses.

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