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Mycoplasma genitalium promotes epithelial crossing and peripheral blood mononuclear cell infection by HIV-1

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SUMMARY

Background: *Mycoplasma genitalium* co-infection in HIV-infected individuals has been reported to increase the shedding of HIV in the urogenital region of females. To better understand this relationship, we investigated the influence of *M. genitalium* on the transmission and replication of HIV using an in vitro model.
Methods: The Transwell co-culture system was employed to assess the crossing of an endocervical cell barrier by HIV-1. Immunocytochemistry and confocal microscopy were used to assess the distribution of the nectin-1 molecule on *M. genitalium*-infected epithelial cells of the End1/E6E7 endocervical cell line, grown as monolayers in the insert wells. Peripheral blood mononuclear cells (PBMC) were cultured in the bottom wells to assess the effects of *M. genitalium*, passing through the semipermeable culturing membrane, on subsequent HIV infection of susceptible target cells.
Results: Infection of the endocervical cells with the adhesion-positive *M. genitalium* G37 strain (wild-type) significantly elevated the passage of HIV across the epithelial cell barrier relative to HIV transfer across endocervical cells infected with the adhesion-negative *M. genitalium* JB1 strain. Immunostaining of the *M. genitalium*-G37-infected epithelial cells disclosed capping and internalization of the junctional regulatory protein nectin-1, in association with reduced transepithelial resistance (TER) in the cell monolayer. When PBMC were cultured beneath insert wells containing *M. genitalium*-G37-infected epithelial cell monolayers, we observed significantly enhanced infectivity and replication of HIV added afterward to the cultures.
Conclusions: *M. genitalium* influences events on both sides of a cultured mucosal epithelial monolayer: (1) by infecting the epithelial cells and reducing the integrity of the barrier itself, and (2) by activating HIV target cells below it, thereby promoting HIV infection and progeny virus production.

1. Introduction

Women are highly susceptible to heterosexual transmission of HIV due to biological and socio-economic reasons. Epidemiological studies indicate that susceptibility to HIV infection is further promoted by existing infections with sexually transmitted pathogens in women, particularly *Chlamydia trachomatis* and type II herpesvirus.[1–3] It is believed that these pathogens compromise mucosal epithelial barriers in the female reproductive tract and activate local subepithelial T cells and dendritic cells to spread HIV. Recently, *Mycoplasma genitalium* has been identified as another pathogen that increases a woman's susceptibility to HIV infection.[4] Women concurrently infected with this pathogen and HIV have been shown to shed increased numbers of HIV particles in the cervical mucosae.[5] A meta-analysis of the reports describing this phenomenon supports an association between *M. genitalium* and HIV, thus suggesting that *M. genitalium* is an important cofactor for HIV transmission.[6]

M. genitalium causes non-gonococcal urethritis in men and cervicitis in women,[4] and both diseases cause extensive inflammation in the urogenital system. It primarily infects epithelial cells of the urogenital tracts by attaching to surface receptors[7] for cell

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entry and replication inside the cells.[8] An organelle on the tip of the flask-like bacterium, which contains the adhesion molecule P140, is required for cell entry or infection, and *M. genitalium* strains lacking the attachment organelle are avirulent.[9–11] Other *M. genitalium* surface lipid-associated membrane proteins, called LAMPs, can bind to surface molecules on vascular endothelial cells and macrophage-lineage cells through TLR receptors.[12,13] Although these binding events do not result in infection of the cells expressing the receptors, they can have significant effects on target cell physiology by promoting differentiation, activating cell division, and causing the cells to produce cytokines that affect nearby lymphoid cells and epithelial cells.

We undertook this study to investigate the relationship between endocervical *M. genitalium* infection and HIV transmission. We used the dual chamber Transwell culturing format for in vitro modeling of female genital–mucosal tissues, with the lumen represented by the insert well chamber, the epithelium by epithelial monolayers grown on the semipermeable bottoms of the insert wells, and the bottom wells supplying a microenvironment similar to the subepithelial lamina propria.[14–16] Using this layered cell culturing format, we asked (1) whether *M. genitalium* infection of the epithelium amplifies the movement of HIV through the epithelium, and (2) whether the presence of *M. genitalium*-infected epithelial cells amplifies the infectivity and replication of HIV in peripheral blood mononuclear cells (PBMC).

2. Materials and methods

2.1. Bacterial and viral strains

Two strains of *M. genitalium* were used: the wild-type, adherence-intact strain G37, and the mutant non-adherent strain JB1.[9] Both strains were grown in 150-cm^2 tissue culture flasks (Corning, NY, USA) containing 100 ml of SP-4 medium and were maintained at 37 °C; flasks containing JB1 were further supplemented with 50 μg/ml gentamicin. The bacteria were prepared for infection as described.[17] The macrophage-tropic HIV-1 strain HIV-1$_{BA-L}$ (Cat. No. 510) was obtained from the NIH-AIDS Research and Reference Reagent Program and from the HIV Core Laboratory, Baylor College of Medicine, Houston, Texas, USA.

2.2. Cell lines and PBMC culture conditions

The human endocervical cell line End1/E6E7 (CRL-2615)[18] was purchased from the American Type Culture Collection (ATCC). The cells were grown in keratinocyte serum-free medium (K-SFM), supplemented with bovine pituitary extract and human recombinant epidermal growth factor (Gibco, NY, USA), and grown at 37 °C in a humid chamber with 5% CO_2.

Blood for PBMC preparation was purchased from the Gulf Coast Regional Blood Center, Houston, Texas, USA. PBMC were separated from the erythrocytes using BD Vacutainer CPT Cell Preparation tubes (Becton-Dickinson, NJ), washed twice with sterile phosphate-buffered saline (PBS, pH 7.2), and re-suspended in RPMI medium containing 20% fetal bovine serum (FBS).

2.3. Crossing of the epithelial monolayer by HIV-1

End1/E6E7 endocervical cells (2.5×10^5) were seeded in Transwell insert wells (top wells) whose bottoms were 0.4-μm pore size semipermeable membranes (12 mm insert on each of 12 wells; Cat. No. 3460, Corning, NY, USA). The top wells containing cells and the empty bottom wells were filled with 500 μl and 1000 μl, respectively, of K-SFM medium. The plates were incubated for 48 h to obtain a confluent monolayer. At 48 h, *M. genitalium* (either the G37 or JB1 strain) was added to the top chambers, and the plates incubated for 6 h. The cells were then washed with PBS three times, and their medium was replaced with 500 μl and 1000 μl RPMI medium (with 20% FBS) in the upper and lower chambers, respectively. This was followed by the addition of HIV-1$_{BA-L}$ (amount of virus added is given in the Figure legends) and incubation for 12 h. The lower chamber medium was then removed and amounts of p24 (p24 is the HIV nucleocapsid protein; this is a proxy value for the presence of HIV) were quantitated using RETROtek HIV-1 p24 Antigen ELISA kits (ZeptoMetrix, NY, USA), following the manufacturer's instructions.

2.4. M. genitalium *ability to cross the epithelial cell monolayer*

Transwell permeable (0.4 μm) supports containing K-SFM medium were either seeded (or not) with End1/E6E7 cells (2.5×10^5) for 48 h. Before infection with *M. genitalium* G37 or JB1, the K-SFM medium was replaced with 500 μl of SP-4 medium in both the upper and lower chambers. *M. genitalium* G37 or JB1 was added to the insert well medium over the monolayers, at an multiplicity of infection (MOI) of 1:5. Control wells received only PBS. Twelve hours later, Mycoplasma growth in the lower chamber was assessed visually by orange-to-yellow color change in the SP-4 culturing medium (redox conditions causing culturing medium to become acidic and turn yellow). The experiment was also done with serial doubling dilutions of G37 added to the top wells, followed by spectrophotometric quantitation of *M. genitalium* present in the upper and lower chambers using MTT reagent (M5655, Sigma-Aldrich, MO, USA).

2.5. Measurement of transepithelial electrical resistance (TER)

The TER of End1/E6E7 cells exposed either to medium or to the two *M. genitalium* strains was assessed using an equivalent series resistance (ESR) meter, AC ohm meter, with 1 mm-separated electrodes. Briefly, End1/E6E7 (1×10^5) cells were plated in 96-well plates for 48 h and then exposed to *M. genitalium* G37 or JB1 (MOI 1:5) for 4–6 h. Control wells received an equal volume of PBS. Following infection, the cells were washed with PBS, and the TER was recorded for three monolayers for each of the three treatment conditions (control, or infection with G37 or JB1). TER values were averaged from recordings taken from five different positions in each monolayer.

2.6. Immunohistochemistry

Whole-mount immunofluorescence assays were performed to detect changes in surface expression and location of cytoskeletal and intercellular adhesion-related proteins following *M. genitalium* infection of the End1/E6E7 cells. G37 or JB1 (MOI 1:5) bacteria were added to End1/E6E7cell monolayers (5×10^5) that had been grown on glass coverslips placed on the bottom of 6-well plates and incubated for 4–6 h. The monolayers were then washed twice with PBS, fixed with 2% paraformaldehyde for 30 min, and permeabilized with 0.1% Triton X-100 and 0.1% Tween-20 in PBS for 30 s. For staining, the monolayers were first pre-incubated in a blocking solution composed of PBS containing 5% bovine serum albumin (BSA) and 5% normal donkey serum. They were then washed and incubated with rabbit antisera against human actin (Sigma-Aldrich, MO, USA), or nectin-1 (R&D systems, MN, USA), or occludin (Abnova, Taiwan) (all at 1:1000 dilution). After overnight incubation at 4 °C, the monolayers were washed three times with PBS, followed by staining with fluorescein-tagged anti-rabbit IgG (Vector, CA, USA) for 2 h at room temperature. After this final incubation, the monolayers were again washed three times with sterile PBS, and the coverslips carrying them were inverted onto mounting media containing DAPI to stain cell nuclei (Vector, CA,

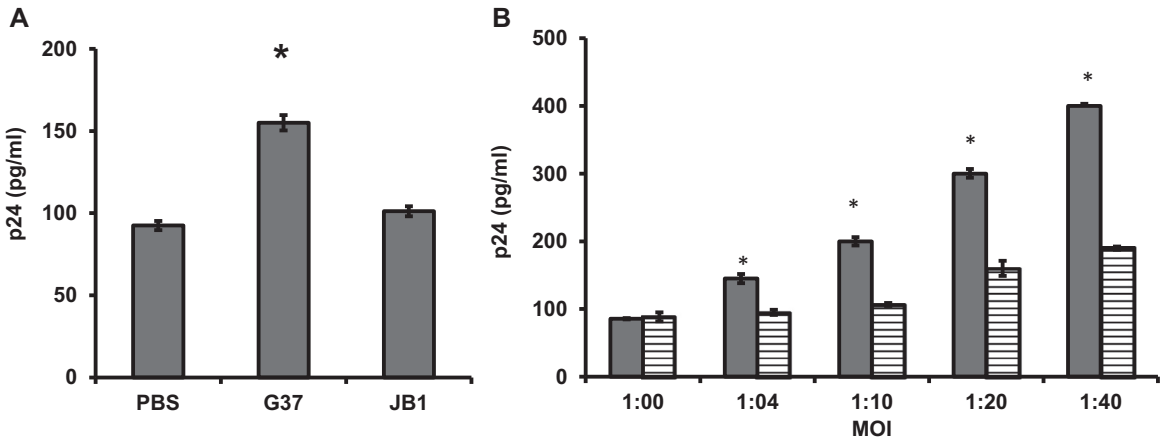

Figure 1. *Mycoplasma genitalium* enhances HIV passage across End1/E6E7. (A) With a single MOI (1:5) of *M. genitalium*. Monolayers of End1/E6E7 were infected with either G37 or JB1 strains *of M. genitalium* for 6 h, in duplicate Transwell inserts. The cells were washed twice with 1× sterile PBS and infected with HIV-1_{BA-L} (1015 ± 105 pg/ml or 50 μl from a stock of virus of 1×10^6 $TCID_{50}$/ml) for 12 h. Medium in the lower chamber was then aspirated and its total p24 was quantified using ELISA kits. The data shown are representative of three independent experiments. $^*p \leq 0.05$ in comparison to JB1. (B) With serially diluted MOI of *M. genitalium*. Monolayers of End1/E6E7were infected with either G37 or JB1 strains *of M. genitalium* at a various MOI for 6 h, in duplicate Transwell inserts. The cells were washed twice with 1× sterile PBS and infected with 100 μl HIV-1_{BA-L} (100 μl of input virus ($TCID_{50}$ = 261 300/ml) corresponding to p24 of 642.97 ± 18.31 pg/ml in ELISA) for 12 h. Medium in the lower chamber was then aspirated and its total p24 was quantified using ELISA kits. Solid and striped bars denote infection with wild-type (G37) and mutant (JB1) *M. genitalium*, respectively. The data shown are representative of three independent experiments. $^*p \leq 0.05$ in comparison to JB1.

USA). Images were captured with an Olympus FV1000 laser scanning confocal microscope, using a 20 × objective. Parallel experiments were done with secondary antibody only, to rule out nonspecific staining.

2.7. *Effect of* M. genitalium*-infected epithelial cells on HIV production by PBMC*

End1/E6E7 cells (2.5×10^5) were plated on 0.4-μm Transwell permeable supports, allowed to form monolayers, and infected with *M. genitalium* G37 or JB1 (MOI 1:5). The lower chamber contained PBMC in 1 ml RPMI medium (with 20% FBS). After 4 h of co-incubation with monolayer-containing inserts inside the lower wells, the inserts were removed and equal numbers of R5 HIV-1_{Ba-L} virus particles were added (the amount of HIV inoculum added is given in the Figure legend) to the lower chamber for 6 h to allow viral attachment. These cells were then washed three times with sterile PBS, to remove any unattached virus, and incubated for 5 days in 2 ml of RPMI medium. At the end of this final incubation, the cells were lysed, and total progeny virus p24 was determined using an ELISA, as described above.

2.8. *CFSE labeling of PBMC*

Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling of PBMC was done by adding 1 μM CFSE to 10^6 cells/ml in PBS, and incubating the cell suspension at room temperature for 10 min. The suspension was then resuspended in RPMI with 5% FBS, and washed three times with PBS containing 1% FBS. Co-culturing of the labeled PBMC with *M. genitalium* strains and preparation of slides for confocal observation of glass-adherent cells were done as described previously.[17] The cells were imaged using an Olympus FV1000 laser scanning inverted confocal microscope with a 10× objective.

3. Results

3.1. M. genitalium *enhances HIV passage across End1/E6E7 monolayers*

To test the hypothesis that *M. genitalium* infection of endocervical epithelial cells would allow HIV to cross the cells, monolayer cultures of End1/E6E7 cells growing on the upper chamber of Transwell culture plates were infected with virulent vs. avirulent *M. genitalium* strains and then with HIV-1_{BA-L} (see **Supplementary Material** Figure S1 for an illustration). After 12 h, the lower chamber medium was collected and the amounts of the HIV p24 determined. The bar graph in Figure 1A shows that the bottom wells from empty inserts and from JB-1 mutant-exposed End1/E6E7 cells, contained small amounts of p24. By contrast, bottom well medium from cells infected with *M. genitalium* wild-type G37 contained significantly more HIV p24, indicating that virulent *M. genitalium* promotes HIV movement across the epithelium, while the avirulent JB-1 strain does not. To further clarify this, we performed experiments using serial doubling of MOI of *M. genitalium* G37 (Figure 1B). The data show that HIV movement across the epithelium is positively related to amounts of *M. genitalium* added to the top insert wells. Although the background level of HIV crossing through the monolayers not infected with *M. genitalium* indicates that monolayers are not very tight, the significant difference in HIV crossing of uninfected or JB-1-infected vs. G37-infected cells clearly identifies that *M. genitalium* infection compromises the integrity of mucosal tissues.

3.2. M. genitalium *G37 crosses the Transwell membrane*

Although *M. genitalium* may help in the passage of HIV particles through the epithelial monolayer as noted above, whether it can get into HIV target cells below the epithelial barrier, particularly in the lamina propria region of the urogenital tract, is unknown. To determine this, we assembled the Transwell chambers with and without monolayers of End1/E6E7 cells growing on the insert well bottom membranes (see **Supplementary Material** Figure S2 for a graphic illustration). These wells were then filled with medium containing (1) adhesion-positive *M. genitalium* G37, (2) adhesion-negative (mutant) *M. genitalium* JB1, or (3) PBS with no bacteria. We hypothesized that if the *M. genitalium* crossed the membrane/monolayer, it would grow in the SP-4 medium of the bottom wells, which would turn the color of the medium from orange to yellow (because of the release of metabolites). The plate photographed (Figure 2A) after 12 h infection revealed the expected color change in wells filled with only *M. genitalium* G37, indicating that only this strain was able to cross the membrane or the cell monolayer. The

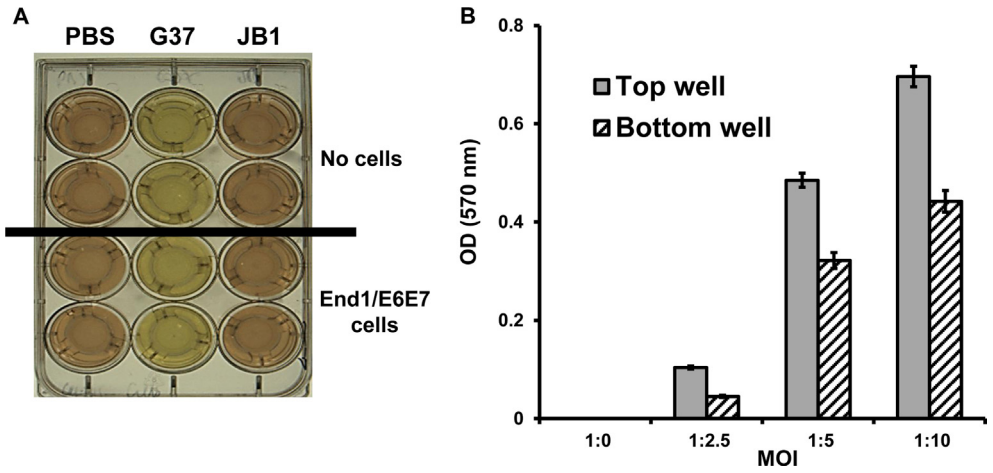

Figure 2. *Mycoplasma genitalium* crosses Transwell membranes with and without End1/E6E7 monolayers. (A) Color change on culture plates due to *M. genitalium* growth. Transwell permeable supports (0.4 μm) were seeded with or without End1/E6E7 cells for 48 h. The K-SFM medium was replaced with 500 μl of SP-4 medium in both chambers and the layers were then infected with *M. genitalium* G37 or JB1, at an MOI of 1:5, for 12 h; the insert wells were then removed and crossing of Mycoplasma was assessed visually by orange-to-yellow color change of the SP-4 medium in the bottom wells. Infection was done in duplicate wells, and 'No cells' in the diagram indicates the absence of End1/E6E7 cells on the Transwell permeable supports. The data shown are representative of three independent experiments. (B) Quantification of *M. genitalium* passage across End1/E6E7 cell monolayers. End1/E6E7 cell layers were infected with G37 with increasing amounts of bacteria (MOI 1:0–1:10). After 12 h, the numbers of bacteria present in the upper and lower chambers were assessed colorimetrically using MTT reagent. Solid bars represent bacteria present in the upper chamber; bars with horizontal stripes represent bacteria present in the lower chamber. The data shown are representative of three independent experiments.

absence of color change in wells infected or filled with JB1 could be due to its inability to reach the bottom wells, possibly because of the absence of motility in this strain. To further confirm these results, we conducted experiments in which monolayers were challenged with serially diluted (different MOI) *M. genitalium* G37 followed by quantification of the amounts of *M. genitalium* G37 in the top vs. bottom wells after 12 h of incubation (Figure 2B). This revealed that the amount of *M. genitalium* crossing to the bottom wells increased with the increase in MOI. Overall, these results suggest that virulent *M. genitalium* has the ability to penetrate epithelial cells, which may allow it to reach the cells underneath it and interact with HIV target cells.

3.3. M. genitalium *G37 infection reduces the transepithelial electrical resistance (TER) of End1/E6E7 monolayers*

In polarized epithelia, the junctional complexes between the cells create a barrier to the passage of specific solutes, particles (e.g., pathogens), and cells (e.g., infiltrating lymphoid cells). These adhesion molecules confer a measurable property upon polarized monolayers, called the transepithelial electrical resistance (TER). For a given cell type, the TER is a global indicator of the integrity of the intercellular junctional complexes under different disease or treatment conditions.[19] Figure 3 shows that the TER of monolayers infected with *M. genitalium* G37 was significantly reduced relative to untreated monolayers or monolayers exposed to the avirulent JB1 strain bacteria.

3.4. M. genitalium *affects nectin-1 localization in End1/E6E7 cells*

Because End1/E6E7 integrity is compromised by *M. genitalium* G37 infection, we then asked whether their cytoskeletal or intracellular junctional complexes are affected under these conditions. For this, we chose to immunostain some selected proteins: the cytoskeletal protein actin, the regulatory adherens junction membrane protein nectin-1, and the tight junction protein occludin.[20,21] We exposed End1/E6E7 monolayers to PBS or G37 or JB1 and immunostained the monolayers with rabbit antibodies to all three, followed by fluorescein-tagged anti-rabbit IgG. Figure 4 shows that neither PBS nor the addition of *M. genitalium* JB1 affected the localization of any of these three proteins, while *M. genitalium* G37-infected cells dramatically altered surface nectin-1 localization, with some patches of bound antibody which may be lipid rafts on cells. Immunostaining of the cells infected with *M. genitalium* strains with secondary antibody alone showed no fluorescence (see **Supplementary Material** Figure S3), thus confirming that the results in Figure 4 are due to reactivity of the antibodies with their cognate epitopes. The results are significant in this study because degradation of nectin-1 causes breakdown of tight junctions in many epithelia.[20]

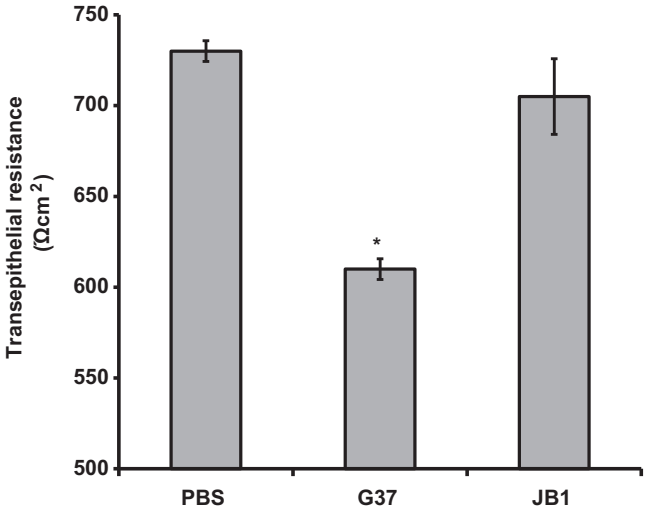

Figure 3. Transepithelial resistance (TER) of End1/E6E7 cells following infection with *Mycoplasma genitalium*. End1/E6E7 monolayers were grown in 96-well plates for 48 h, followed by addition of *M. genitalium* strains G37 or JB1, or heat-killed G37, at an MOI of 1:5 for 4 h. The monolayers were then washed and their TER values recorded with an ohm meter, as described in the Materials and methods section. Each value represents the mean ± standard deviation of TER measurements recorded from five different positions for each of three monolayers in triplicate wells. $^{*}p \leq 0.05$ for G37 vs. JB1 and PBS. The data shown are representative of three independent experiments.

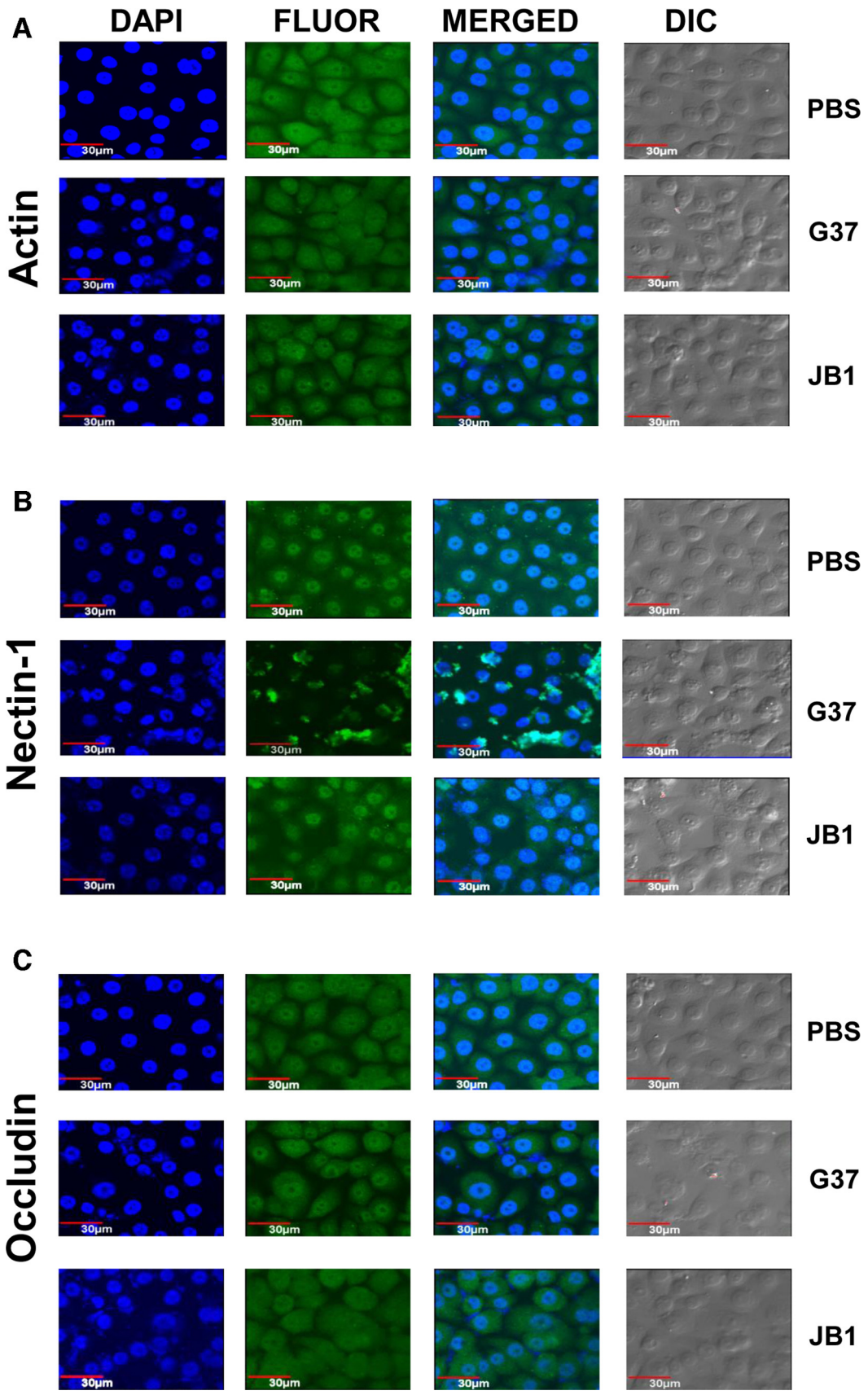

Figure 4. Immunocytochemical analysis of End1/E6E7 cells following *Mycoplasma genitalium* infection. End1/E6E7 monolayers were grown on glass coverslips and *M. genitalium* G37 or JB1 (MOI 1:5) were added for 4–6 h. The monolayers were then washed, fixed, permeabilized, and then incubated overnight with antibodies against (A) actin, or (B) nectin, or (C) occludin. After washing, the monolayers were incubated with secondary antibody (FITC-conjugated anti-rabbit IgG) and washed. The coverslips were then inverted onto mounting medium containing DAPI. Images were captured with an Olympus FV1000 laser scanning confocal microscope, using a 20× objective. The data shown are representative of three independent experiments.

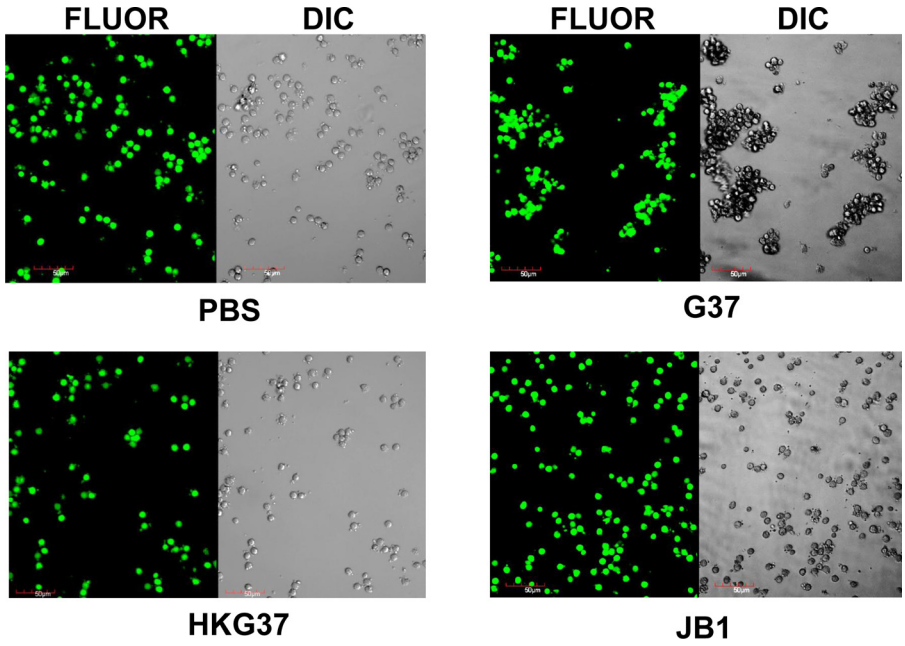

Figure 5. Effect of *Mycoplasma genitalium* on PBMC. CFSE- labeled PBMC were overlaid with *M. genitalium* strains G37 or JB1, heat-killed G37, or PBS alone (bacterial MOI 1:5) for 1 h. The monolayers were then washed and rinsed with PBS and images of adherent cells were acquired using an Olympus FV1000 laser scanning inverted confocal microscope. PBS, phosphate-buffered saline control; G37, wild-type *M. genitalium*; JB1, adhesion-deficient mutant *M. genitalium*; FLUOR and DIC identify fluorescence and differential interference contrast images of the same field. The data shown are representative of three independent experiments.

3.5. M. genitalium *G37 promotes aggregation of PBMC*

Previously published studies from this laboratory have shown that the monocytoid cell line THP-1 forms aggregates and differentiates (giving rise to adherent macrophages) when its cells are grown with *M. genitalium* G37.[17] To determine whether cells in human PBMC cultures also respond to *M. genitalium* by aggregation and adherence, we stained PBMC with CFSE and incubated with either PBS, *M. genitalium* G37, heat-killed *M. genitalium* G37, or *M. genitalium* JB1. The confocal photomicrographs in Figure 5 show that PBMC incubated with live *M. genitalium* G37 had large, firmly attached CFSE-stained aggregates, while PBMC incubated with JB-1 or with PBS did not. To determine whether the attached aggregates were monocytes/macrophages, we exposed monocyte-depleted PBMC to either *M. genitalium* G37 or JB1 strains. Figure S4 (**Supplementary Material**) shows that neither *M. genitalium* nor JB1 caused aggregation of cells, suggesting that the *M. genitalium*-induced aggregates formed by PBMC are macrophages derived from differentiated monocytes from the PBMC cell mixture. Since macrophages are target cells of HIV, aggregation of these cells by *M. genitalium* may enhance transmission of HIV because infected cells can spread virus efficiently, from cell to cell, via transinfection.

3.6. M. genitalium *G37 promotes HIV infection and progeny virus production*

Once we had determined that *M. genitalium* was capable of moving across the Transwell membranes in our co-cultures, we asked whether its presence in PBMC cultured below the membrane might affect the replication of HIV in these cells. To test this, we conducted experiments, as described in the Materials and methods section, in which the lower well PBMC were pre-exposed to upper well End1/E6E7 monolayers infected with *M. genitalium* G37 or JB1 and then infected with $HIV_{BA\text{-}L}$ (an R5 HIV strain). Figure 6A shows that the HIV-infected PBMC exposed to G37-infected monolayers produced significantly more progeny virus by 5 days post infection than PBMC exposed for the same amount of time to PBS or JB1-infected monolayers. This G37-specific elevation in virus production was associated with marked aggregation and adherence of PBMC, as shown in the inverted photomicrographs in Figure 6B.

4. Discussion

During male-to-female sexual contact, HIV transmission occurs when virus in the semen crosses genital–mucosal barriers in the female reproductive tract and infects target hematolymphoid cells in the subepithelial lamina propria. This is a complex microenvironment composed of sperm and seminal lymphocytes (both of which can bind and carry HIV[22–24]), seminal fluid components,[25,26] and non-HIV sexually transmitted disease pathogens (in the semen donor or the female genital–mucosal tissues[1,27–30]). All of these affect HIV passage across the epithelium and then influence its infectivity and replication in its target cells beneath the epithelium. Only a few of these components are represented in our Transwell system, but our findings are important in improving our understanding of the role of *M. genitalium* in HIV transmission. Also, it is important to note that all experiments were conducted with $HIV\text{-}1_{BA\text{-}L}$, which is an R5-tropic laboratory strain of HIV.[31,32]

We have shown here that *M. genitalium* infection of epithelial monolayers enhances the crossing of HIV. This is consistent with the prediction that HIV transmission may be increased in individuals with inflammatory lesions.[33] In the urogenital tract, inflammation is partly due to infection by sexually transmitted pathogens, which may include *M. genitalium*. Infection by these pathogens induces the secretion of proinflammatory cytokines, which lead to conditions like cervicitis and vaginosis.[33,34] These conditions may compromise the epithelial barrier in the urogenital tract and allow the HIV-1 to reach target cells beneath it. Consistent with this, we and others have previously reported that *M. genitalium* is capable of inducing the secretion of both proinflammatory and anti-inflammatory cytokines in epithelial cells.[17,35] It is reasonable to assume, therefore, that *M. genitalium*-infected End1/E6E7 cells have reduced barrier integrity. We have

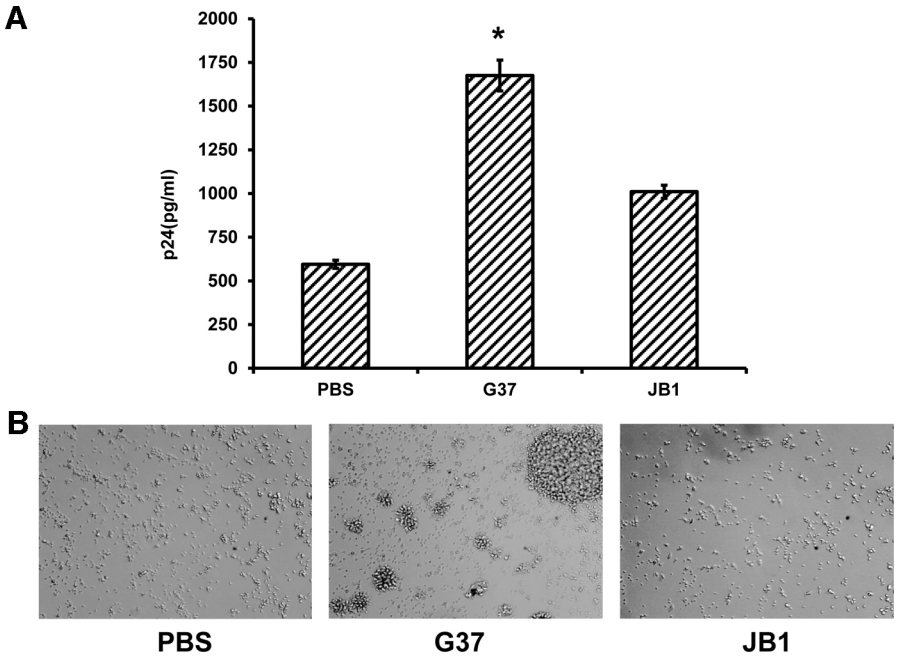

Figure 6. Effect of *Mycoplasma genitalium* on HIV-1 progeny virus production. (A) Replication of HIV in PBMC cultured in bottom wells under *M. genitalium*-infected End1/E6E7 cells. End1/E6E7 cell monolayers overlaid with either *M. genitalium* G37 or JB1 in duplicate transwells and these insert wells were then lowered into chambers containing PBMC for 4 h. After removing the insert wells, HIV-$1_{BA\text{-}L}$ (1489.8 ± 210.5 pg/ml or 200 μl of $TCID_{50}$/ml = 261 300) was added to the PBMC cultures for 6 h. Unattached HIV was removed by spinning and washing the PBMC three times with PBS. The PBMC were then resuspended in 2 ml of fresh RPMI medium (containing 20% FBS), followed by incubation at 37 °C for 5 days. The amount of progeny virus generated by the PBMC was measured using RETROtek HIV-1 p24 Antigen ELISA kits. The mean values represent ELISA measurements for six replicate wells. The data shown are representative of three independent experiments. $^{*}p \leq 0.05$ for G37 in comparison to JB1. (B) Images of HIV-infected PBMC cultured for 120 h in medium conditioned by *M. genitalium* G37 or JB-1. The plate was photographed using a Leica DMI 6000 inverted microscope with a 10× objective. The data shown are representative of three independent experiments.

obtained data that suggest how this may happen, by showing that nectin-1 placement and location are dysregulated on *M. genitalium*-infected cells. Nectin-1 must be present and properly located for the formation of adherens junctions between cells in polarized epithelial monolayers, and it has been reported that *C. trachomatis* post-translationally downregulates nectin-1 levels via the chlamydial protease-like activity factor (CPAF), thereby damaging the junctional complexes between the infected cells.[36,37] Disturbance of nectin-1 and loss of barrier integrity would produce areas of 'leaky' epithelium in the *M. genitalium*-infected female reproductive tract, allowing HIV to pass through *M. genitalium*-infected female mucosal epithelia even if open lesions that expose the lamina propria are not present.

It is interesting to note that exposure of PBMC to *M. genitalium*-infected epithelial cells (across the Transwell semipermeable barrier) increases infection/multiplication of HIV in the PBMC. It is possible that this may also be due to the influence of cytokines secreted by the interaction of *M. genitalium* with the host cells. One of the epithelium-derived factors that influences the multiplication of HIV in T-cells is thymic stromal lymphopoietin (TSLP), an interleukin 7 (IL-7)-related cytokine.[38] In epithelial cells, TSLP production can be induced by microbial infections that include HIV,[38,39] and some microbial pathogens or their products induce TSLP in epithelial cells by interacting with Toll-like receptors (TLR) of the innate immune system, particularly TLR3.[40] *M. genitalium* is well known for its interactions with TLR through surface lipoproteins called lipid-associated membrane proteins (LAMPs).[13] It is likely that HIV replication in the bottom wells of Transwell PBMC cultures is promoted by both cytokines released by *M. genitalium*-infected epithelial cells and by the bacterium itself, through its interactions with monocytes leading to their differentiation and substrate adhesion.

In conclusion, *M. genitalium* seems to have some of the attributes necessary to make it a potent cofactor for the promotion of sexual transmission of HIV. It can increase the crossing of HIV through the epithelial monolayer to reach the target cells, and it can promote multiplication of HIV on its target cells to some extent. Additional studies involving three-dimensional modeling of cervical epithelial tissues will shed more light on these phenomena.

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Conflict of interest: The authors have no conflict of interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2013.11.022.

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