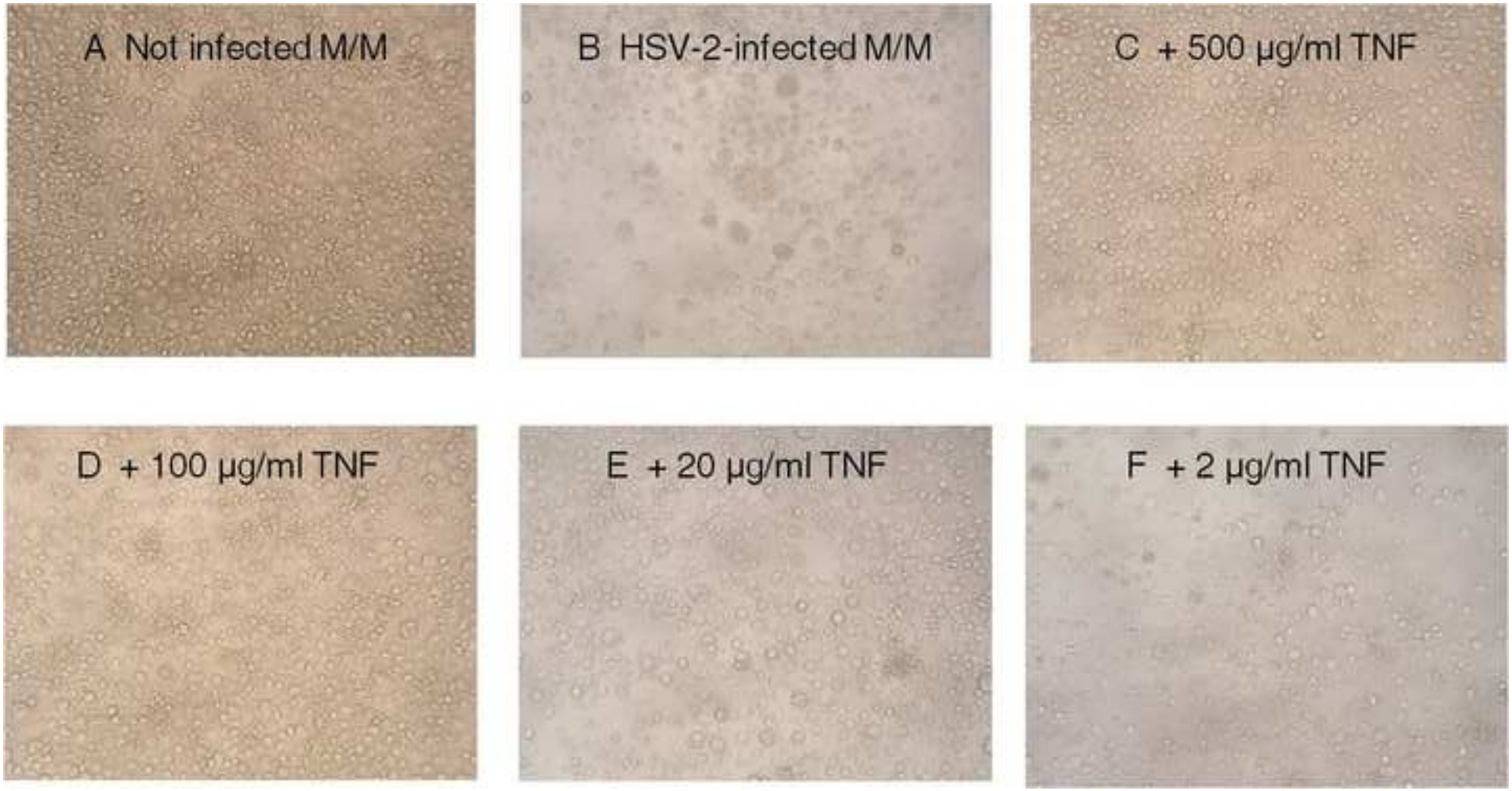
Figure S1


A Not infected M/M
B HSV-2-infected M/M
C + 500 µg/ml TNF
D + 100 µg/ml TNF
E + 20 µg/ml TNF
F + 2 µg/ml TNF

SUPPLEMENTAL DATA

Herpes virus infection of primary monocyte/macrophage cell cultures

Human peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy seronegative donors by Ficoll–Hypaque density gradient centrifugation. PBMCs were resuspended in RPMI 1640 medium supplemented with 20% heat-inactivated (56°C, 30 min) fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 mg/L) and L-glutamine (2 mM), then seeded into 48-well plates ($1.8x10^6$ cells/well). M/M were separated by adherence onto plastic. After 5 days, non-adherent cells were carefully removed by repeated gentle washings with warm medium, and adherent monocyte/macrophages (M/M) were cultured for an additional 3 days to mature and to form a monolayer. The number of cells present in each well was assessed by counting nuclei extracted from M/M with lysing buffer in a cell counting chamber under a phase contrast microscope, according to a previously published procedure (Bergamini et al., 1994). About 10^5 cells/well were present at the time of infection. African green monkey fibroblastoid kidney (Vero) cells were grown in RPMI medium supplemented with 10% heat inactivated FCS and were used in infectious virus titration assays. This cell line is highly sensitive to the cytopathic effect of HSV-2.

Anti-HSV-2 activity assay in human macrophage cell cultures

To evaluate the anti-HSV-2 activity of tenofovir on human macrophages, the compound was added to macrophage cell cultures at a variety of concentrations (0.04, 0.2, 1, 5, 20, 100 or 500 µg/ml) 1 hour before infection. Similarly, macrophage cultures were treated

with different concentrations of adefovir (0.04, 0.2, 1, 5, 20, 100 or 500 µg/ml) used as a control drug. Macrophage cultures were then infected with HSV-2 (100 $TCID_{50}$) in the presence of the compounds. After 2 hrs adsorbtion, the cultures were extensively washed to remove any residual virus particles. Fresh complete medium and compounds, at the established concentrations, were then added to the cultures. Tenofovir and adefovir were maintained throughout the experiment. Appropriate positive (infected but not treated M/M) and mock-infected negative (uninfected and untreated M/M) controls were run for each experiment as well. All assays were performed in triplicate. The cytopathic effect on macrophages was daily monitored by microscopic observation. HSV-2 induced on infected but not treated M/M a cytopathic effect (CPE) already detectable 48 h after virus challenge and the CPE was found complete after 120 h. The assessment of virus production was performed in the supernatants of M/M at 3 and 6 days after infection. Data given refer to virus production at 6 days after infection. Results at 3 days after virus challenge were superimposable with those obtained at 6 day.

The amount of infectious virus in the supernatants was determined by a CPE reduction assay. Ten-fold serially diluted supernatants were added to confluent monolayers of Vero cells in 96-well plates (100 µl/well, 6 parallel wells). The plates were incubated at 37°C for 4 to 5 days, when full cytopathic effect was evident. The titers of produced virus were calculated according to the Reed and Muench method and expressed as 50% tissue culture infective dose per mL ($TCID_{50}$/mL).The quantification of infectious viral particles was also confirmed by a standard PFU reduction assay. Serial dilutions of the M/M supernatants were added to confluent monolayers of Vero cells plated in 24-well. After 1h, the inoculum was replaced by complete medium containing 0.8% (wt/vol)

methylcellulose (Sigma) and incubated for an additional 3 days. The plates were then stained with 1% (wt/vol) crystal violet in 20% methanol– 80% H_2O (vol/vol), and the numbers of plaques were counted. Titers were calculated as PFU per milliliter of virus suspension. The inhibition capacity was expressed in % and calculated considering as 100% for the virus production in HSV-2-infected untreated cultures.

Human *ex vivo* tissues. Human tonsillar tissues were obtained from patients undergoing routine tonsillectomy at the Children's National Medical Center (Washington, DC) under an IRB-approved protocol. Cervical tissues were obtained through the National Disease Research Interchange (NDRI, Philadelphia, PA) also under an IRB-approved protocol. Tissues were dissected into ~2 mm blocks and cultured as described earlier (Grivel & Margolis, 2009). Briefly, tissue blocks were placed onto collagen sponge gels in culture medium at the air-liquid interface, and cultured in RPMI 1640 (GIBCO BRL, Grand Island, NY) medium containing 15% heat-inactivated fetal calf serum (FCS; Gemini Bio-Products, Woodland, CA).

Tonsillar tissue: For each experimental condition 27 tissue blocks (9 blocks/well/3 ml of complete medium) were inoculated with 5 µL of viral stock of HSV-1 (strain F) or HSV-2 (strains G and MS) (ATCC) placed on top of each block. Coinfection experiments were performed by inoculating tissue blocks with subsequently 5 µL of $HSV\text{-}2_G$ and 5 µL (0.5 ng of p24) of $HIV_{LAI.}$ (obtained from the Rush University Virology Quality Assurance Laboratory (Chicago, IL)). In all experiments using tonsillar tissues, tenofovir was added to the culture medium 12h prior to viral infection and again every 3 days at each culture medium change.

Cervico-vaginal tissue: For each experimental condition 16 tissue blocks were soaked with HSV-2G in 500 µl of viral suspension for 2 hours at 37ºC, washed three times with PBS and then placed on the gelfoam rafts. Tenofovir was added during the infection and added again at each culture medium change.

Viral replication: Herpes simplex viral replication was evaluated by the release of viral DNA into the culture medium as measured with real-time PCR (Lisco et al., 2008). Briefly, DNA from 200 µL of culture medium was purified with the QIAamp kit according to the manufacturer's instructions. Measurements were performed with an ABI Prism 7700 sequence detector (PE Applied Biosystems, Foster City, CA) using a real-time PCR TaqMan assay. A reference standard curve using serially diluted plasmids containing the target genes or a commercially available reference standard (Advanced Biotechnologies, Columbia, MD) was then created. Primers and probe used in the quantification of HSV-2 UL27 gene were as follows: forward primer**:** cgcatcaagaccacctcctc / reverse primer: gctcgcaccacgcga / Probe: cggcgatgcgccccag). HIV-1 replication was evaluated by the release of p24 gag antigen using a bead-based assay as described (Biancotto et al., 2009).

Figure S1 (related to table 2). Inhibitory activity of tenofovir against HSV-2-induced cytopathicity in primary monocyte/macrophage (M/M) cell cultures.

M/M, separated by adherence onto plastic, were infected and treated as described under Material and Methods. HSV-2 induced a cytopathic effect (CPE) on infected, not-treated M/M already detectable at 48 h after virus challenge and the CPE was found to be complete after 120 h. Infectious virus produced in the supernatants at this time point was measured by a CPE reduction assay and a plaque formings units (PFU) reduction assay as described under Materials and Methods. The inhibition of cytopathicity by tenofovir visible by microscopical inspection was in agreement with a dose-dependent reduction of virus production as measured by cytopathogenicity measurements of the M/M supernatants in Vero cell cultures as well as in a Vero cell virus plaque assay. The figure shows M/M cultures after 6 days from infection. Panel A: mock-infected cell culture; Panel B: HSV-2-infected cell culture; Panels C, D, E and F: HSV-2-infected cell cultures exposed to tenofovir at 500, 100, 20 and 2 μg/ml, respectively.