

Tenofovir Selectively Regulates Production of Inflammatory Cytokines and Shifts the IL-12/IL-10 Balance in Human Primary Cells

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Objectives: In this study, we aimed to investigate the possible immune modulatory effects of HIV nucleoside reverse transcriptase inhibitors during secondary infections and inflammation, focusing on inflammatory cytokine responses and the interleukin (IL)-12/IL-10 balance.

Methods: We investigated the in vitro effect of tenofovir and zidovudine (AZT) on production of proinflammatory cytokines in monocytes and human peripheral blood mononuclear cells (PBMCs). Stimulation panels included Toll-Like receptor (TLR) ligands; the inflammation mediator tumor necrosis factor- α ; and the pathogens cytomegalovirus, *Neisseria meningitidis*, *Escherichia coli*, and *Streptococcus pneumoniae*. Cytokine levels were measured using enzyme-linked immunosorbent assay and luminex technology. RNA levels were assessed using real-time polymerase chain reaction. Activity of mitogen-activated protein kinase and NF- κ B signaling was evaluated using flow cytometry and multispectral imaging cytometry, respectively.

Results: Tenofovir decreased and AZT increased both IL-8 and CCL3 production from monocytes after stimulation with TLR ligands, tumor necrosis factor- α , or live pathogens. Similarly, tenofovir decreased CCL3 levels in human PBMCs. Furthermore, tenofovir strongly decreased induction of IL-10 but increased levels of IL-12. AZT did not affect IL-12 or IL-10 levels. The observed drug-induced changes in cytokine production were independent from transcriptional regulation through the mitogen-activated protein kinase and nuclear factor kappa B pathways.

Conclusions: Our data suggest divergent effects of tenofovir and AZT on proinflammatory responses in monocytes (CCL3 and IL-8) and PBMCs (CCL3). Moreover, tenofovir shifts the IL-10/IL-12 balance after cell stimulation with TLR ligands or infection with live bacteria, thus suggesting that the choice of nucleoside reverse transcriptase inhibitor affects overall inflammation and early immune responses against secondary pathogens.

Key Words: tenofovir, pathogen, IL-12, IL-10, inflammation, microbicide

(*J Acquir Immune Defic Syndr* 2011;57:265–275)

Received for publication January 17, 2011; accepted March 2, 2011.

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Supported by grants from the Danish AIDS Foundation, the Scandinavian Society of Antimicrobial Chemotherapy (SSAC), the Aase and Ejnar Danielsen Foundation, Region Midts Forskningsfond, the Augustinus Foundation, the Dagmar Marshall Foundation, Fonden til Lægevidenskabens Fremme, and the King Christian X Foundation.

Part of the article's data was presented in a poster at the 27th Annual Meeting of the Scandinavian Society of Antimicrobial Chemotherapy (SSAC); September 30, 2010, to November 3, 2010; Stockholm, Sweden.

The authors declare that they do not have any competing financial interests.

M. W. Risør is recipient of a scholarship from the Danish Cancer Foundation.

O. S. Søgaard is recipient of a fellowship from University of Aarhus.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jaids.com).

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INTRODUCTION

The introduction of highly active antiretroviral therapy has substantially decreased the risk of infections in persons with HIV through repression of viral replication and normalization of the CD4⁺ count.^{1,2} Nevertheless, patients with HIV on treatment still show increased susceptibility to secondary infections, such as pneumonia and bacterial meningitis, compared with persons without HIV.^{2,3} Possible mechanisms could be loss of immunological and epithelial integrity, fibrosis of lymphatic tissue, and loss of interleukin (IL)-17 expressing CD4⁺ T lymphocytes from the gastrointestinal tract, leading to microbial translocation and generalized immune activation.^{4,5} Furthermore, the imbalance in immune function could be related to immune modulatory properties of the antiretroviral drugs, as particular treatments regimes have been shown to affect cytokine networks regulating immunity.^{6–10} Nucleoside reverse transcriptase

inhibitors (NRTIs) have not been investigated with regard to immune responses to secondary infections.

Microbial invasions, including infections in patients with HIV-1, are sensed by germ line-encoded pathogen recognition receptors, including Toll-Like receptors (TLRs). TLR triggering induces the production of proinflammatory cytokines. Regulated cytokine responses are important for immune responses, and a dysregulated cytokine response may be detrimental for the host.¹¹

Acyclic nucleoside phosphonates, including tenofovir, have been shown to induce cytokine mRNA in murine macrophages,¹² accumulation of cytokines [IL-1 β , tumor necrosis factor (TNF- α), CCL3, and IL-10] in murine peritoneal cell culture,¹³ and CCL3 and CCL5 in human peripheral blood mononuclear cells (PBMCs).⁷ Abacavir induces early changes in cells, including redistribution of heat-shock protein 70.¹⁴ Zidovudine (AZT) reportedly affects intracellular signaling differently in different cell types. For instance, AZT has been shown to activate nuclear factor kappa B (NF- κ B) in monocyte-like U937 cells, the promyelocytic leukemia cell line HL60, and the T-lymphoblastic cell line MOLT.¹⁵ In contrast, AZT has been reported to inhibit NF- κ B activation in Burkitt lymphoma B cells.¹⁶

IL-8 and CCL3 (macrophage inflammatory protein 1 α [MIP-1 α]) are inflammatory mediators broadly induced by infection with pathogens and via inflammatory signals. IL-8 is an important neutrophil and CD4⁺ T-cell chemoattractant and thus plays a pivotal role in the early innate response against invading pathogens as a regulator of adaptive responses.^{17,18} IL-8 affects HIV-1 pathogenesis in many ways. IL-8 stimulates HIV replication in both monocyte-derived macrophages and T cells, increases transmission of HIV-1 in cervical transplants, and is associated with inflammation.^{17–20} Furthermore, IL-8 enhances replication of cytomegalovirus (CMV) in human fibroblasts²¹ and reduces antiviral effects of IFN- α .²² CCL3 is an inflammatory chemokine produced by a variety of cells. CCL3 inhibits infection with M-tropic HIV, via binding to the chemokine receptor CCR5,²³ and is an important factor for host defense against HIV, evidenced by the finding that genetic deficiency in CCL3 is associated with increased risk of perinatal HIV-1 transmission in humans and faster progression to simian AIDS in macaques.^{24–27} However, CCL3 may also play a negative role in HIV pathogenesis because CCL3 is associated with inflammation-mediated diseases and tissue injury, including gastrointestinal and respiratory diseases.²⁸

The IL-10 and IL-12 balance is dysregulated in patients with HIV. IL-12 plays a pivotal role in regulating cell-mediated immunity essential for control of HIV and secondary infections.^{29,30} Although IL-12 levels are elevated during acute HIV infection,³¹ an impaired IL-12 response is observed later in infection. The hampered IL-12 response is associated with increased HIV disease severity and faster progression to AIDS.^{32–34} At the cellular level, PBMCs from patients with HIV are impaired in their IL-12 production in response to bacterial and mitogen stimuli.³⁵ IL-10 is a key molecule in immune suppression, terminating inflammatory responses and inhibiting activation and effector functions of T cells, macrophages, and monocytes.^{36,37} IL-10 is a main inhibitor of IL-12 production,³⁸ and the presence of IL-10 can result in anergy and

nonresponsiveness to infections and antigens, including HIV-derived antigens.^{36,37} HIV-1 induces IL-10 in monocytes, CD4⁺ and CD8⁺ T cells,^{39–42} and IL-10 induces expression of the HIV-1 coreceptor CCR5 in human monocytes and neutrophils.⁴³ Further suggesting an important role for IL-10 during HIV progression, a higher frequency of IL-10-producing CD4⁺ T cells, is seen in HIV-1-infected individuals with progressive HIV-1 compared with cells from nonprogressors.⁴⁰

In this work, we investigated the effect of tenofovir and AZT both on the proinflammatory responses focusing on the cytokines IL-8 and CCL3, due to their induction by several pathways, thus allowing delineation of specific effects of tenofovir and zidovudine on pathogen-activated, single TLR-activated pathways and after inflammatory signal by TNF- α . Furthermore, we investigated the IL-10/IL-12 balance in human primary cells after challenge with live pathogens, TLR stimulation, and stimulation with the inflammatory mediator TNF- α .

MATERIALS AND METHODS

HIV Drugs

Pure compound AZT, the prodrug tenofovir disoproxil fumarate (tenofovir DF), tenofovir, and abacavir were obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, and National Institutes of Health. Five millimolar stocks of synthetic NRTIs tenofovir DF, tenofovir, and AZT were made in sterile water. Abacavir was solubilized in dimethyl sulfoxide (DMSO), and subsequently, all stocks were stored at -20°C . Tablets containing tenofovir DF (Viread; Gilead, Foster City, California) abacavir (Ziagen; GlaxoSmithKline, Brentford, United Kingdom) and AZT (Retrovir; GlaxoSmithKline) were solubilized in DMSO for stock concentration 20 mM. Stock solutions were sterile filtered and stored at -20°C .

Cells

Monocytic U937 cells, THP-1 cells, and PBMCs were cultured in RPMI 1640 supplemented with L-glutamine (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]), penicillin, and streptomycin and 10% heat-inactivated fetal bovine serum (Invitrogen). PBMCs were isolated from blood obtained from healthy donors by Isopaque-Ficoll separation. MRC-5 fibroblasts were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). PBMCs were seeded in 96-well culture plates at a density of 2×10^5 cells per well or 1×10^6 cells per 6-well plate and cultured overnight before further treatment. U937 cells and THP-1 cells were seeded at a density of 2×10^5 cells per 96-well plate or 1×10^6 cells per 6-well plate 3 hours before further treatment. For ImageStream experiments, U937 cells were used at a concentration of 3×10^6 cells per milliliter.

Pathogens

Clinical bacterial isolates of *Escherichia coli*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* were grown overnight in brain heart infusion broth with 10% Levinthal broth. CMV strain AD169 was grown in fibroblasts MRC-5

cells until full cytopathic effect was seen (7–9 days). Cell culture media containing virus and control media from mock-infected cells were cleared from cell debris by centrifugation and stored at -80°C until experiments. The virus titer was approximately 4×10^6 plaque-forming units per milliliter determined by titration on MRC-5 cells. Briefly, confluent MRC-5 cells were infected with 10-fold dilutions of CMV. After 3 days of infection, cells were washed with PBS and fixed with 80% acetone for 10 minutes. After rinsing, the cells were incubated with 1:10 diluted monoclonal anti-CMV antibody (Dako, Glostrup, Denmark) for 30 minutes and subsequently incubated 30 minutes with fluorescein isothiocyanate-conjugated goat anti-murine F(ab)2 antibody (Dako). CMV-positive cells were visualized by fluorescence microscopy.

Stimulation Experiments for Protein and RNA

NRTIs were diluted in growth media before use. Similar volume of vehicle (DMSO or water) was used as control. Cells were pretreated with indicated concentrations of drug for the indicated time before stimulation with TLR2 ligands Pam3CSK4 (200 ng/mL; InvivoGen, Toulouse, France), TLR4 ligand lipopolysaccharide (LPS) (100 ng/mL; Sigma-Aldrich, Copenhagen, Denmark), TLR7/8 ligand CL097 (1 $\mu\text{g/mL}$; InvivoGen), or TNF- α (25 ng/mL; R&D Systems, Abingdon, United Kingdom). Freshly grown *E. coli* was added to cell culture in multiplicity of infection (MOI) of 10. *Neisseria meningitidis* and *S. pneumoniae* were added to the cell culture in MOI of 4. CMV was used in MOI of 0.5. As control for bacterial and CMV infection, uninfected broth or mock cell culture media was used, respectively. At indicated time, cell culture was harvested for detection of cytokine levels or total RNA was harvested for reverse transcriptase–polymerase chain reaction analysis.

Enzyme-Linked Immunosorbent Assay and Luminex

Harvested cell culture supernatants were assayed using DuoSet enzyme-linked immunosorbent assays (ELISAs) for IL-8, CCL3 (MIP-1 α), and IL-6 (R&D Systems) or Cytoset ELISAs for IL-1 β , IFN- γ , IL-10, or IL-12p40/p70 (Invitrogen). For some experiments, IL-8 levels were determined by multiplex bead array (Luminex) using IL-8-specific kits (Invitrogen). ELISAs and bead arrays were performed as specified by the manufacturers.

RNA Extraction, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Total RNA was extracted using Trizol as described by the manufacturer (Invitrogen) and stored at -80°C . cDNA synthesis was done using the moloney murine leukemia virus reverse transcriptase (MMLV-RT), according to the manufacturer's instructions (Invitrogen). Gene expression was determined using SYBR Green qPCR mix (Qiagen, Copenhagen, Denmark). Gene-specific primers were designed using Primer-blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). To avoid amplification of chromosomal DNA, primers were designed to span an exon–exon junction or be separated by one intron. RNA not subjected to reverse transcription was used as a negative control for polymerase chain reaction (PCR)

amplification. The gene-specific primers were as follows: GAPDH forward, 5'-CGACCACTTTGTCAAGCTCA-3'; GAPDH reverse, 5'-GGTGGTCCAGGGGTCTTACT-3'; IL-8 forward, 5'-CTGCGCCAACACAGAAATTA-3'; IL-8 reverse, 5'-TGAATTCTCAGCCCTCTTCAA-3'; IL-12p40 forward, 5'-GCAGCTTCTTCATCAGGGAC-3'; IL-12p40 reverse, 5'-AGGGTACTCCCAGCTGACCT-3'; IL-10 forward, 5'-CCAGACCCAGACATCAAGGC-3'; IL-10 reverse, 5'-GGCCTTGCTCTTGTGTTTCAC-3'. qPCR was performed on a LightCycler 2.0 instrument (Roche, Hvidovre, Denmark), and crossing point values were determined using the LightCycler software v. 4.05 (Roche). Relative gene expression levels were calculated as previously described using GAPDH as the housekeeping reference gene.⁴⁴

Whole Blood Stimulation and Flow Cytometry

Freshly drawn whole blood was pretreated with tenofovir DF (250 μM), the mitogen-activated protein kinase (MAPK) inhibitor U0126 (100 μM ; Promega, Madison, Wisconsin), or vehicle as control (15 minutes at 37°C). Subsequently, the cells were incubated with LPS (1 $\mu\text{g/mL}$) for 30 minutes at 37°C and cells were stained, as previously described,⁴⁵ using anti-CD45-KO, anti-CD14-PC7, anti-phospho-extracellular signal-regulated kinase (ERK)Alexa647, and anti-phospho-p38MAPK-Alexa488 (all from Beckman Coulter, Birkeroed, Denmark). Flow analysis was performed on a Gallios flow cytometer (Beckman Coulter) and data analysis using Kaluza software (Beckman Coulter).

Multispectral Flow Cytometry Experiments

U937 or PBMCs were incubated with tenofovir DF (250 μM) or saquinavir (100 μM) for 15 minutes before subsequent stimulation with TNF- α (100 ng/mL) or LPS (1000 ng/mL) for 30 minutes. Cells were harvested and then fixed using 4% formaldehyde (Polysciences, Warrington, Pennsylvania) for 10 minutes after which the cells were permeabilized using 0.1% triton X-100 and stained with 1:20-diluted rabbit anti-p65 antibody (Santa Cruz) at room temperature for 30 minutes, followed by incubation for 30 minutes at room temperature with fluorescein isothiocyanate-conjugated F(ab)₂ donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, Pennsylvania). Before analysis, nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI). Samples were analyzed on an ImageStream^x imaging Cytometer (Amnis, Seattle, Washington), which simultaneously acquires spectrally separated spatially correlated images from each individual cell. The nuclear translocation of NF- κB (p65), quantitatively determined as the so-called similarity score between the nuclear (DAPI) and NF- κB (p65) images of each cell, was used as a parameter of NF- κB signaling activity as described previously.⁴⁶

RESULTS

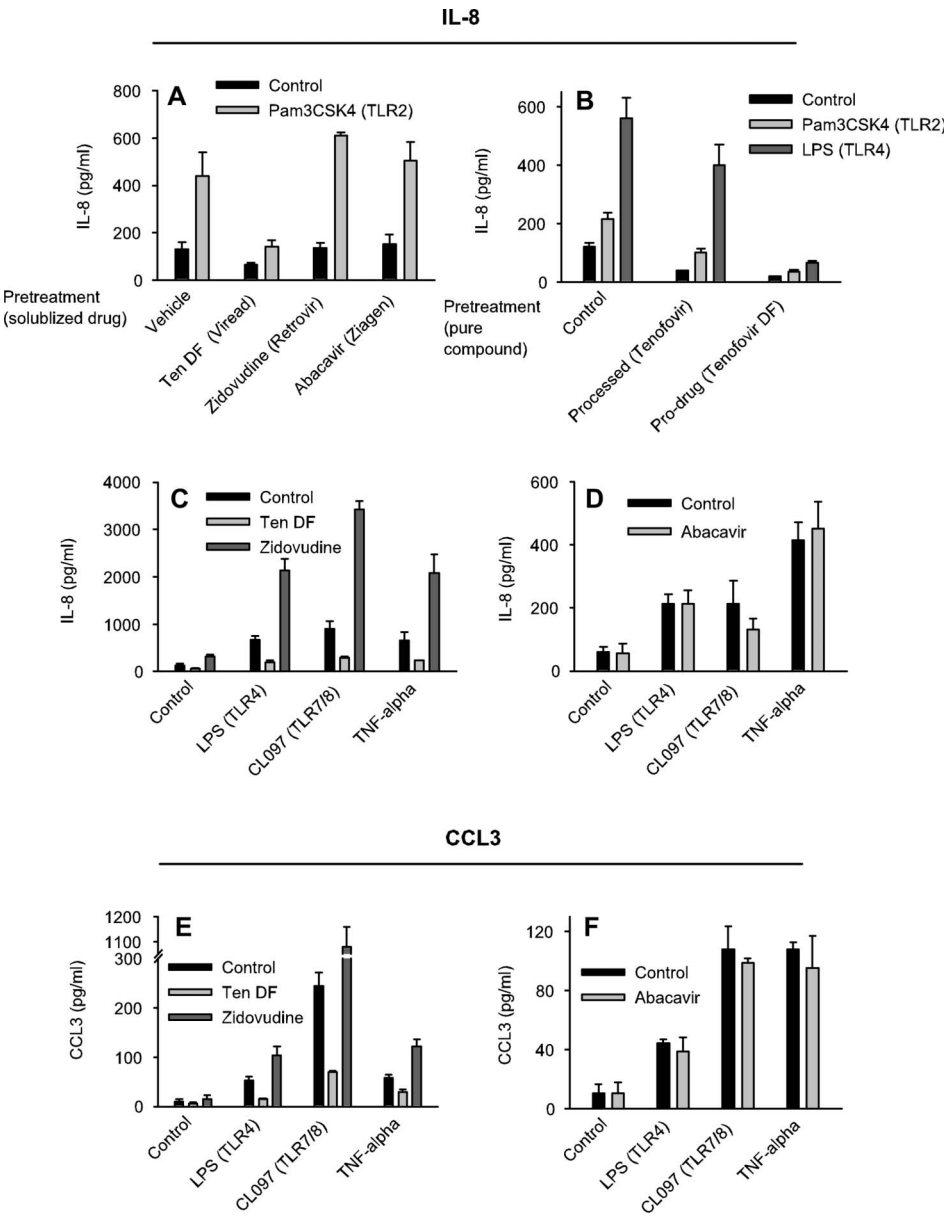
Tenofovir and AZT Differentially Affect TLR- and TNF- α -Induced Cytokine Production in U937 Cells

To evaluate how HIV NRTIs affect immune cells, we pretreated monocytic U937 cells with NRTIs tenofovir DF

(Viread), AZT (Retrovir), or abacavir (Ziagen) for 1 hour and subsequently stimulated the cells with the TLR2 or TLR4 agonists (Pam3CSK4 and LPS, respectively). The concentrations used did not affect cell viability within the duration of the studies (not shown), and concentrations were in line with concentrations used by others for in vitro studies.^{7,12,13,16,47} After 20 hours, cell supernatants were harvested and assayed for IL-8 using Luminex. AZT or abacavir did not significantly affect TLR2-induced IL-8, whereas tenofovir reduced levels of IL-8 (Fig. 1A). Tenofovir in its processed form, however, did not induce IL-8 and compared with the prodrug tenofovir DF only very modestly affected levels of TLR-induced IL-8 (Fig. 1B). To evaluate whether the effect seen was directly linked to the active components of the drugs, similar

experiments were done using pure components. AZT in pure form induced IL-8 and CCL3 and increased TLR- and TNF- α -induced IL-8 and CCL3 secretion (Figs. 1C, E). IL-1 β and IL-6 were investigated, but because both were not induced in U937 cells (not shown), only CCL3 and IL-8 were assessed further. In accordance with results gained using solubilized tablets (Fig. 1A), pure synthetic prodrug tenofovir DF decreased IL-8 and CCL3 levels induced by TLR agonists and by TNF- α stimulation (Figs. 1B, C, E). Abacavir did not affect TLR4-, TLR7/8-, or TNF- α -activated IL-8 or CCL3 or stimulation (Figs. 1D, F). Thus, tenofovir DF and AZT differentially affect TLR- and TNF- α -induced cytokine production in monocyte U937 cells, whereas abacavir does not affect proinflammatory cytokine production.

FIGURE 1. HIV drugs differently affect TLR- and TNF- α -induced cytokine production. Monocyte U937 cells were pretreated with NRTIs abacavir, tenofovir DF, or AZT (50 μ M) from solubilized drugs (A) or NRTIs abacavir, tenofovir, tenofovir DF, and AZT (50 μ M) as pure chemical (B–F). After 1 hour, cells were stimulated with TLR2 ligand Pam3CSK4 (200 ng/mL), TLR4 ligand LPS (100 ng/mL), TLR7/8 ligand CL097 (1 μ g/mL), or TNF- α (25 ng/mL). After 20 hours, cell supernatants were harvested and analyzed for levels of IL-8 or CCL3 by ELISA or Luminex. The figures represent average from 1 of 2 experiments performed in triplicate culture (A, B, D, F) or 3 experiments performed in triplicate culture (C, E) with similar results \pm SD.



Characterization of Tenofovir and AZT Effects in Monocytes

To evaluate dose-dependency, we pretreated U937 cells with increasing concentrations of tenofovir DF or AZT before stimulation with ligands for TLR2, TLR4, and TLR7/8. AZT dose dependently increased secretion of IL-8 and CCL3, whereas levels of these cytokines were decreased by tenofovir

DF (Figs. 2A, B). To assess whether tenofovir DF and AZT directly affect IL-8 transcription or RNA stability, we pretreated cells with tenofovir DF or AZT for 1 hour before stimulation with TNF- α . After 5 hours, total RNA was harvested and IL-8 mRNA was determined using real-time PCR. AZT did not affect IL-8 mRNA levels (Fig. 2C). In contrast, tenofovir DF decreased IL-8 mRNA levels after TNF- α

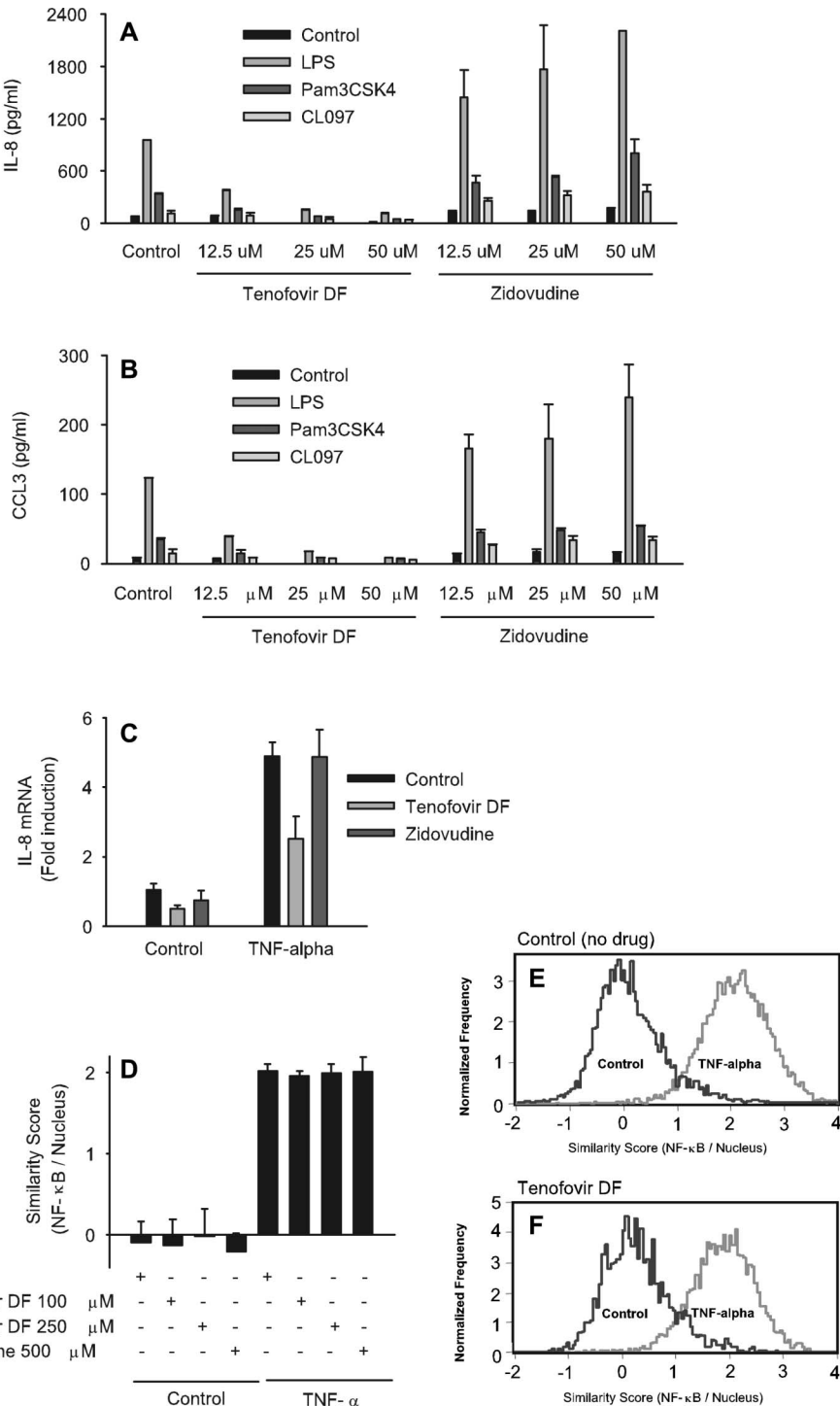


FIGURE 2. Characterization of tenofovir and AZT effects on cytokine expression in monocytes. U937 were pretreated with increasing concentrations of tenofovir DF or AZT for 1 hour before stimulating the cells with Pam3CSK4 (200 ng/mL), LPS (100 ng/mL), or CL097 (1 μ g/mL). (A, B) After 20 hours, cell culture media were harvested and assayed for IL-8 and CCL3 by ELISA. C, Total RNA was harvested after 5 hours and accumulation of IL-8 mRNA quantified using real-time RT-PCR. Figures (A–C) represent average \pm SD of 1 of 2 independent experiments with similar results. D–F, U937 cells were pretreated for 15 minutes with tenofovir DF or AZT at indicated concentration before stimulation with TNF- α (100 ng/mL) for 30 minutes. Cells were stained for p65 (NF- κ B) and the nucleus (DAPI) and analyzed by ImageStream cytometry. D, Mean NF- κ B/nucleus similarity scores from 3 independent experiments \pm SD. E, F, Similarity score plot from 1 of the 3 experiments. RT-PCR, reverse transcriptase–polymerase chain reaction.

stimulation, suggesting that tenofovir DF may exert its suppression partly at regulating RNA levels. Because IL-8 is transcriptionally regulated by the transcription factor NF- κ B,⁴⁸ we assessed whether NF- κ B activation is directly affected by tenofovir DF. As illustrated in Figures 2D–F, neither AZT nor tenofovir DF directly affected NF- κ B activation after TNF- α stimulation, thus suggesting that tenofovir's regulatory role is independent of interference with signaling, leading to cytokine production.

Early Pathogen-Induced Cytokine Responses in Monocytes and PBMCs Are Differently Affected by Tenofovir and AZT

To assay the effect of tenofovir DF and AZT on early cytokine responses during secondary infections, cells were infected with *S. pneumoniae*, *N. meningitidis*, *E. Coli*, or CMV in the presence or absence of tenofovir DF or AZT. As observed in Figures 3A–C, AZT enhanced IL-8 and CCL3 responses, whereas tenofovir DF reduced pathogen-induced cytokine secretion. Similar results were obtained in the monocytic cell line THP-1 (see **Figure, Supplemental Digital Content 1**, <http://links.lww.com/QAI/A158>). We next performed similar experiments in human primary PBMCs. Like U937 cells and THP-1 cells, PBMCs were inhibited in their ability to produce CCL3 in the presence of tenofovir DF (Figs. 3D, E). In contrast, TLR-induced and pathogen-induced IL-6 levels were only slightly affected or unaffected by the presence of tenofovir DF (Figs. 3F, G). In conclusion, early innate immune response to bacterial and virus infections is inversely affected by tenofovir DF and AZT in monocytes, whereas tenofovir DF affects cytokine production selectively in human PBMCs.

Tenofovir Primes Human PBMCs for Enhanced IL-12 and Decreased IL-10 Expression

Because IL-12 is an important regulator of cell-mediated immunity controlling infections, and the fact that HIV-1 infection reduces levels of IL-12 and upregulates levels of IL-10,^{35,39,41} we evaluated the effect of tenofovir DF and AZT on IL-10 and IL-12 in human cells. After infections with live bacteria, TLR stimulation or stimulation with TNF- α , IL-10 levels were strongly reduced in the presence of tenofovir DF, whereas AZT did not affect IL-10 secretion (Figs. 4A–C). In contrast, tenofovir DF strongly enhanced IL-12 secretion after pathogen infection and TLR stimulation (Figs. 4E–G). Similarly, tenofovir DF decreased IL-10 mRNA accumulation and increased IL-12p40 mRNA accumulation after TLR triggering (Figs. 4D, H, respectively), thus suggesting a regulatory role at the level of RNA regulation. In conclusion, tenofovir DF affects the IL-12/IL-10 balance during infection and inflammation.

No Significant Effect of Tenofovir on MAPK or NF- κ B Signaling in Human PBMCs

Because mRNA accumulation of IL-10 and IL-12 was affected by tenofovir DF (Figs. 4D, E), we evaluated the role of tenofovir DF on signaling pathways regulating cytokine production. Tenofovir DF did not affect NF- κ B signaling after TNF- α stimulation and only slightly affected LPS-induced

NF- κ B activation (Fig. 5A). The protease inhibitor saquinavir, known to interfere with the proteasome and NF- κ B activation after LPS stimulation,¹⁰ inhibited LPS-induced NF- κ B modestly. To determine whether tenofovir DF affects MAPK signaling, we assayed whole blood stimulated with LPS in the presence or absence of tenofovir DF or the MAPK inhibitor U0126. As illustrated in Figure 5B, no change in activation profiles for ERK or p38 MAPK was seen in the presence of tenofovir DF, whereas the inhibitor U0126 strongly inhibited ERK phosphorylation after LPS stimulation. In conclusion, tenofovir DF does not strongly affect MAPK or NF- κ B activation in human primary cells.

DISCUSSION

In this study, we have investigated the effects of the often used NRTIs tenofovir and AZT with respect to their ability to affect cytokine production from human cells during infections, after inflammatory stimuli and TLR stimulation. Tenofovir decreased and AZT increased both IL-8 and CCL3 production from monocytes after stimulation with TLR ligands, TNF- α , or live pathogens (Fig. 1–3). Similarly, tenofovir decreased CCL3 levels in human PBMCs (Figs. 3D, E). AZT did not affect cytokine induction in PBMCs (Figs. 3, 4). Although the presence of tenofovir DF reduced levels of TNF- α -induced IL-8 mRNA (Fig. 2C), the inhibition of mRNA accumulation was not linked to direct interference with signaling essential for cytokine transcription because neither tenofovir DF nor AZT affected NF- κ B activation in U937 cells (Fig. 2D). For AZT, our findings are in agreement with the report from Kurata¹⁵ observing no significant NF- κ B activation in U937 cells at early time points 3–5 hours after AZT treatment.

In contrast to our results, previous studies have shown that tenofovir directly promotes expression of cytokines in murine (peritoneal macrophages) and human cells (U937 cells and PBMCs).^{7,12,13} Based on the previous findings, we were surprised not to observe any tenofovir-induced cytokine expression (IL-8, CCL3, IL-12, IL-10, or IL-6) neither in U937 cells nor in human PBMCs (Figs. 1–4). To make sure that the preparation of drug did not affect the outcome, we included tenofovir DF from solubilized drug (Viread) and tenofovir in the processed form, which was used in the studies showing cytokine induction by tenofovir. No inducing effect was observed (Figs. 1A, B). We cannot explain the discrepancy in results gained. However, having tested 2 sources of prodrug tenofovir (tenofovir DF) obtaining similar results, our data strongly suggest that, at least in the model systems used in our experiments, tenofovir is not by itself an efficient cytokine inducer. Rather, tenofovir DF is a selective negative regulator of inflammatory cytokines.

IL-10 and IL-12 are pivotal cytokines in regulating immune responses. IL-10 is a potent inhibitor of inflammation and immune responses to infections and antigens.^{36,37} HIV infection results in increased levels of IL-10 in monocytes and CD4⁺ and CD8⁺ T cells.^{39,41} Tenofovir DF pretreatment of human PBMCs reduced levels of IL-10 when challenged with the inflammatory cytokine TNF- α , TLR ligands, or live bacteria (Figs. 4A–D). Investigation of human PBMCs and whole blood confirmed that tenofovir DF is neither a strong

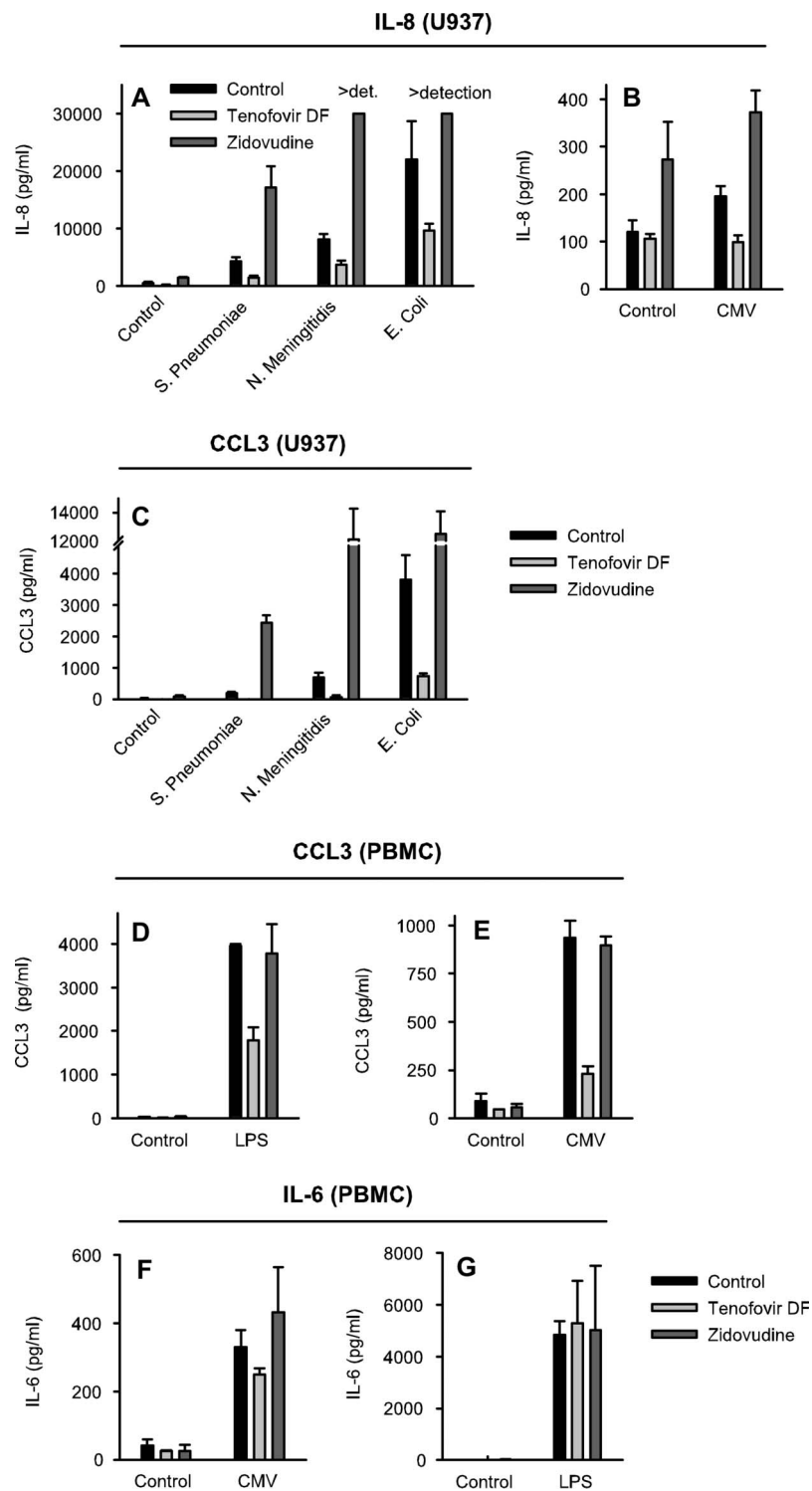


FIGURE 3. Pathogen-induced cytokine production is differential affected by tenofovir and AZT. A–C, U937 cells were pretreated with either tenofovir DF or AZT (50 μ M) for 1 hour before stimulation with clinical isolates of *E. coli* (MOI of 10), *Neisseria meningitidis* (MOI of 4), or *Streptococcus pneumoniae* (MOI of 4) or infected with CMV (MOI of 0.5). After 20 hours, cell media were harvested and analyzed by ELISA. D–G, Human PBMCs were treated with LPS (100 ng/mL) or infected with CMV (MOI 0.5) in the presence or absence of tenofovir DF or AZT (50 μ M). After 20 hours, cell supernatants were harvested and analyzed for levels of IL-6 or CCL3 by ELISA. The figure depicts average levels of IL-6, IL-8, or CCL3 \pm SD from 1 of 2 independent experiments with corresponding results. Det, detection range.

agonist nor antagonist of MAPK or NF- κ B signaling (Figs. 5A, B). Collectively, the data suggest that the tenofovir DF-mediated changes in cytokine profiles are not mediated through changes of intracellular signaling activity via NF- κ B or MAPK pathways.

Whether decreased production of IL-10 is linked to an improved clinical prognosis remains to be determined. IL-12

plays an essential role in regulating cell-mediated immunity controlling both HIV and secondary infections.^{29,30} Decreased levels of IL-12 have been associated with increasing HIV disease severity and faster progression to AIDS.^{32,33} We found that tenofovir DF strongly enhanced PBMC's ability to respond with IL-12 after infection with live bacteria or stimulation with TLR ligands (Figs. 4E–H). Our findings are

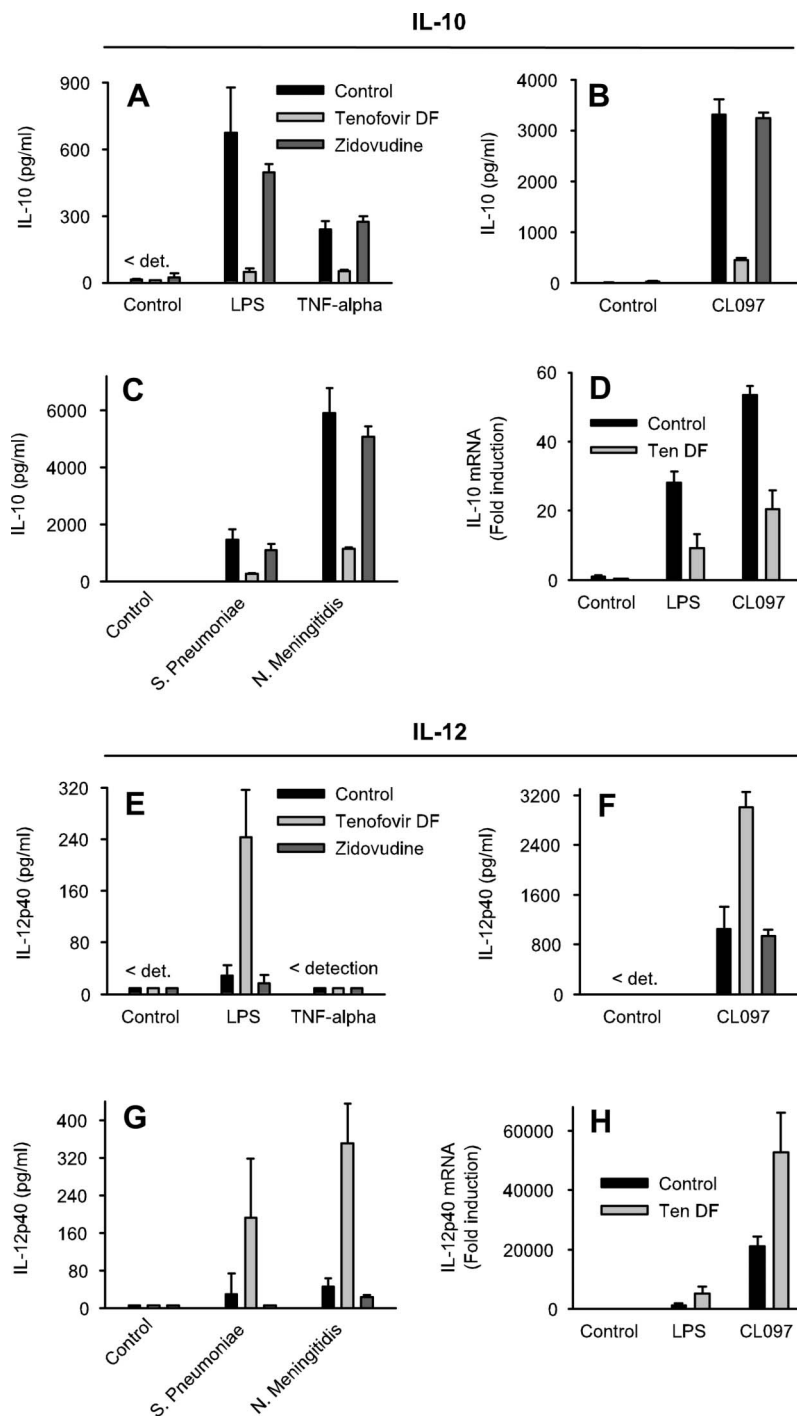


FIGURE 4. Tenofovir primes human PBMCs for enhanced IL-12 and decreased IL-10 expression. Human PBMCs were pretreated with tenofovir DF or AZT (50 μ M) for 1 hour before stimulation with LPS (100 ng/mL), CL097 (1 μ g/mL), TNF- α (25 ng/mL), or infection with pathogens *Streptococcus pneumoniae* (MOI of 4) or *Neisseria meningitidis* (MOI of 4). A–C and E–G, After 20 hours, cell supernatants were harvested and analyzed for levels of IL-10 or IL-12p40/p70 by ELISA. (D, H) Total RNA harvested at 5 hours after stimulation was assayed for IL-10 and IL-12p40 mRNAs using real-time RT-PCR. The figure represents average from 1 of 3 (A–C, E–G) or 2 experiments (D, H) in triplicate culture with similar results \pm SD. Det, detection range; RT-PCR, reverse transcriptase–polymerase chain reaction.

in line with studies from macaques showing that tenofovir primes PBMCs for IL-12 secretion.⁴⁹ Knowing that IL-12 administration to PBMCs from patients with HIV restores the cell's immune capability,^{50–53} it is tempting to speculate that tenofovir could improve the general immune response to secondary infection and possibly also prime the immune response against HIV antigens.

Recently, the Centre for the AIDS Program of Research in South Africa (CAPRISA) 004 study showed that tenofovir as

a microbicide provides protection against HIV-1.⁵⁴ One of the pivotal characteristics of microbicides is their ability to preserve mucosal immune function and not induce inflammation.⁵⁵ The present study, describing tenofovir's ability to reduce releases of inflammatory mediators, including chemokines IL-8 and CCL3 (Figs. 1–3), may play a part in tenofovir microbicide gel's protective effect. Inhibited inflammation may reduce the influx of potential host cells for HIV-1 and reduce inflammatory tissue damage. Interestingly, the CAPRISA 004 study also found that

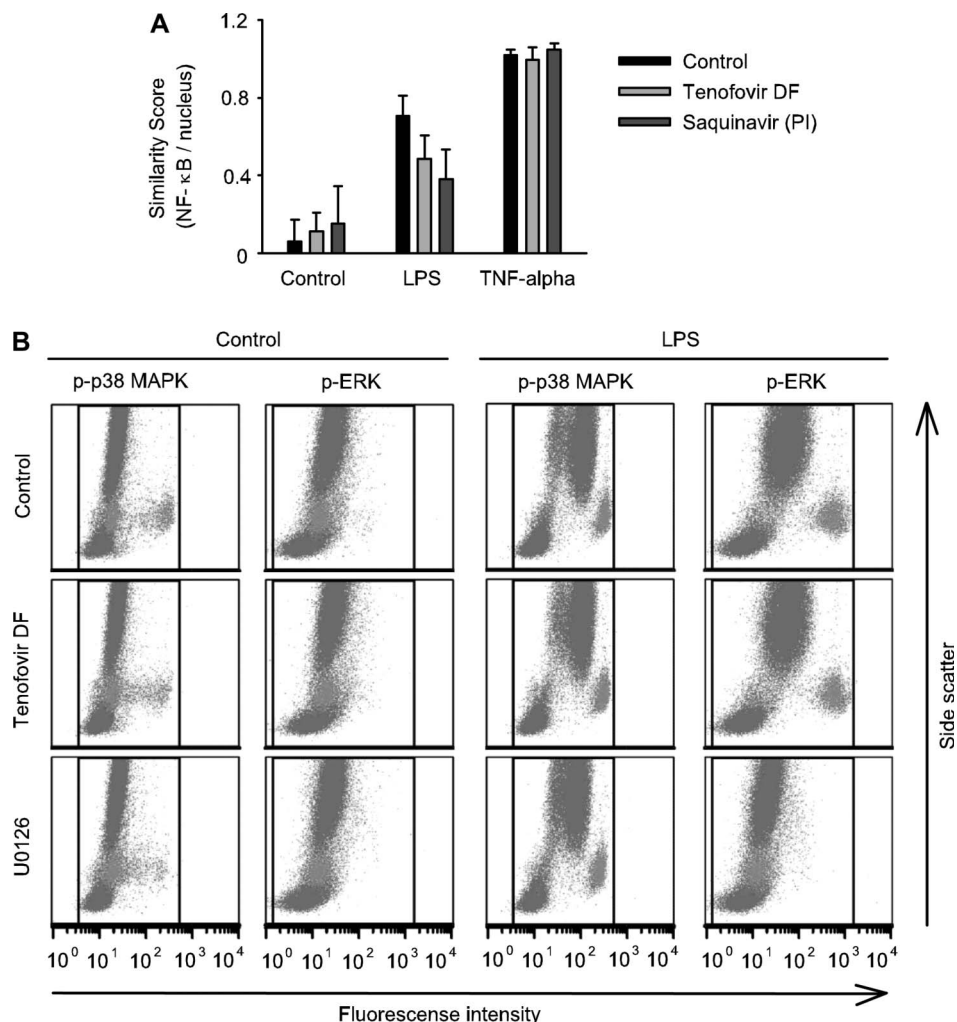


FIGURE 5. Tenofovir does not strongly affect MAPKs or NF-κB signaling pathways in human primary cells. **A**, PBMCs were pre-treated with tenofovir DF (250 μM) or the proteases inhibitor saquinavir (100 μM) for 15 minutes before stimulation with LPS (1 μg/mL) or TNF-α (100 ng/mL). After 30 minutes, cells were harvested and stained for intracellular p65 (NF-κB). Using image flow cytometry, nuclear localization of NF-κB was determined. **B**, Whole blood was pre-treated with tenofovir DF (250 μM) or ERK inhibitor U0126 (100 μM) for 10 minutes before activation with LPS (1 μg/mL) for 30 minutes. Cells were gated on CD45- and CD14-positive cells and analyzed for intracellular phospho-ERK and phospho-p38 MAPK using flow cytometry. Figures represent mean ± SD of 2 independent experiments for (A) and show 1 of 2 donors with similar results for (B).

tenofovir decreased acquisition of herpes simplex virus (HSV-2) but not via a direct antiviral effect.⁵⁶ Supporting the assumption that tenofovir does not possess strong direct antiviral effect against HSV-2, no decreases in HSV-2 shedding are observed in patients receiving oral tenofovir DF.⁵⁷ One might speculate that regulation of cytokine networks by tenofovir could prime the host for a better anti-HSV-2 response. Although we demonstrate that tenofovir primes human cells for decreased inflammatory responses and enhanced IL-12 secretion after a secondary stimulus, it is very difficult to extrapolate the effects to human diseases. One reason is the fact that the plasma drug concentration in patients is lower than the concentrations used in the in vitro studies (most often 1–100 μM). As an example, the maximum concentration of tenofovir in plasma after administration of 1 dose of tenofovir DF (Viread, 300 mg) is approximately 300 μg/L,⁵⁸ which corresponds to approximately 1 μM. Further studies will have to delineate the in vivo effect of tenofovir on inflammation and acquiring secondary/opportunistic infections.

In summary, our results provide data on tenofovir's immune modulatory effect. Tenofovir selectively affects levels of secreted inflammatory cytokines and shifts the IL-10/IL-12 balance after cell stimulation with TLR ligands or infection

with live bacteria. Collectively, our data suggest that the choice of NRTI may affect overall inflammation and early immune responses against secondary pathogens.

ACKNOWLEDGMENTS

Pure compounds of AZT, tenofovir, tenofovir DF, abacavir, and saquinavir were obtained from the AIDS Reagent and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, and National Institutes of Health.

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