Adenovirus Vectors Block Human Immunodeficiency Virus–1 Replication in Human Alveolar Macrophages by Inhibition of the Long Terminal Repeat

Robert J. Kaner[1,2], Francisco Santiago[2], Franck Rahaghi[1], Elizabeth Michaels[3], John P. Moore[3], and Ronald G. Crystal[1,2]

[1]Division of Pulmonary and Critical Care Medicine, [2]Department of Genetic Medicine and [3]Department of Microbiology and Immunology Weill Cornell Medical College New York, New York

Heterologous viruses may transactivate or suppress human immunodeficiency virus (HIV)–1 replication. An adenovirus type 5 gene transfer vector (Ad5) HIV-1 vaccine was recently evaluated in a clinical trial, without efficacy. In this context, it is relevant to ask what effect Ad vectors have on HIV-1 replication, particularly in cells that are part of the innate immune system. Infection of HIV-1–infected human alveolar macrophages (AMs) obtained from HIV-1$^+$ individuals with an Ad vector containing no transgene (AdNull) resulted in dose-responsive inhibition of endogenous HIV-1 replication. HIV-1 replication in normal AMs infected with HIV-1 *in vitro* was inhibited by AdNull with a similar dose response. Ad reduced AM HIV-1 replication up to 14 days after HIV-1 infection. Fully HIV-1–infected AMs were treated with 3′-azido-3′-deoxythymidine, after which Ad infection still inhibited HIV-1 replication, suggesting a postentry step was affected. Substantial HIV-1 DNA was still produced after Ad infection, as quantified by TaqMan real-time PCR, suggesting that the replication block occurred after reverse transcription. AdNull blocked HIV-1 long terminal repeat (LTR) transcription, as assessed by an vesicular stomatitis virus G protein pseudotyped HIV-1 LTR luciferase construct. The formation of HIV-1 DNA integrated into the host chromosome was not inhibited by Ad, as quantified by a two-step TaqMan real-time PCR assay, implying a postintegration block to HIV-1 replication. These data indicate that Ad vectors are inhibitory to HIV-1 replication in human AMs based, in part, on their ability to inhibit LTR-driven transcription.

Keywords: adenovirus; HIV-1 replication; human alveolar macrophage

Alveolar macrophages (AMs) are important reservoirs of human immunodeficiency virus (HIV)–1 infection in the lung (1, 2). In spite of highly active antiretroviral therapy (HAART) suppression of HIV-1 replication in T cells, various reservoirs, including AMs, dendritic cells, and other monocyte-derived tissue macrophages continue to harbor the virus (3, 4). We have previously shown that if AMs are infected with an E1$^-$E3$^-$ adenovirus (Ad) gene transfer vector before *in vitro* HIV-1 infection, subsequent replication of HIV-1 by the AM is markedly suppressed independently of the transgene in the Ad vector (5). In the context that heterologous viruses have disparate effects on HIV-1 replication, and clinical trials are now underway with another HIV-1 vaccine based on a type 5 Ad gene transfer vector, the focus of this study is to further characterize the effect of Ad vectors on HIV-1 replication in human AMs with regard to the HIV-1 life cycle (6–18). Specifically, we assessed the effect of Ad vectors on HIV-1 replication in AMs obtained from HIV-1$^+$ individuals, established the relevance of the model of HIV-1 infection of normal AMs *in vitro* with respect to the effects of Ad on HIV-1 replication, and characterized the step(s) in the HIV-1 life cycle during which AdNull suppresses HIV-1 replication. The data demonstrate that: (*1*) HIV-1 replication by endogenously HIV-1–infected AMs is blocked by Ad; (*2*) *in vitro* HIV-1 infection of normal AMs *in vitro* is a valid model with which to study the effects of Ad on HIV-1 replication; (*3*) the major effect of AdNull in causing inhibition of HIV-1 replication is at a step subsequent to reverse transcription and integration of the HIV-1 DNA into the human chromosome; (*4*) the effect of AdNull inhibiting HIV-1 replication is as a result of suppression of long terminal repeat (LTR)-dependent transcription; and (*5*) the suppression of LTR-dependent transcription by AdNull is due, in part, to activation of intracellular IFN-related signaling via phospho–signal transducer and activator of transcription (STAT)–1 and IFN regulatory factor (IRF)–8.

Some of the results of these studies have been previously reported in the form of an abstract (19–23).

CLINICAL RELEVANCE

A recent adenovirus type 5 gene transfer vector (Ad5)-based human immunodeficiency virus (HIV)–1 vaccine trial was stopped due to a higher incidence of HIV-1 infection. It is timely to ask what effect Ad vectors have on HIV-1 replication in cells that support active replication of HIV-1 such as human alveolar macrophages.

MATERIALS AND METHODS

Cells

Human AMs were obtained by bronchoalveolar lavage (BAL) from healthy volunteers, as previously described, after obtaining written informed consent under a protocol approved by the Committee for Human Rights in Research of the Weill Medical College of Cornell University (24, 25). AMs were obtained from HIV-1$^+$ individuals with lung infections who were not receiving highly active antiretroviral therapy because they were previously unaware of their HIV-1 status, noncompliant, or refused therapy. The AMs were purified from BAL fluid obtained from diagnostic bronchoscopies when the fluid recovered was in excess of that required for diagnostic studies under a separately approved protocol. AMs from HIV-1$^+$ individuals were used for this study if spontaneous p24 elaboration was detectable. The lavage fluid was filtered through gauze to remove debris. The cells were pelleted, washed with PBS (pH 7.4), and resuspended in RPMI 1,640 medium containing 10% FBS, 2 mM glutamine, 100 U of penicillin/ml, and 10 μg/ml of streptomycin (GIBCO BRL, Gaithersburg, MD) at a final concentration of 2×10^5 AMs/ml. AMs were purified by adherence to

(*Received in original form February 7, 2008 and in final form August 10, 2010*)

This work was supported in part by National Institutes of Health grants R01 HL59861, AI45463, and the Will Rogers Memorial Fund (Los Angeles, CA).

Correspondence and requests for reprints should be addressed to Ronald G. Crystal, M.D., Department of Genetic Medicine, Weill Cornell Medical College, 1,300 York Avenue, Box 96, New York, New York 10021. E-mail: geneticmedicine@med.cornell.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 43. pp 234–242, 2010
Originally Published in Press as DOI: 10.1165/rcmb.2008-0063OC on October 5, 2009
Internet address: www.atsjournals.org

plastic (2 h, 37°C) on a 24-well culture plate (COSTAR, Cambridge, MA). A 1-ml aliquot of the cell suspension (2×10^5 cells) was added per well. HeLa-CD4$^+$/CCR5$^+$ cells were infected with a strain of HIV-1 isolated from the frontal lobe of subject JR who died of AIDS dementia (JRFL-1) for 7 days and cultured in Dulbecco's modified Eagle medium with 10%FBS, 1% penicillin/streptomycin, and 0.1% Fungizone.

AdNull Vector

The AdNull vector is an E1a$^-$, partial E1b$^-$, partial E3$^-$ based on the Ad5 backbone (26, 27). The AdNull vector contains the cytomegalovirus immediate-early promoter/enhancer in the expression cassette in the E1 position, but no transgene. Empty Ad capsids were isolated as a separate band during ultracentrifugation by CsCl density gradient purification of the AdNull preparation.

Endogenous HIV-1 Replication by Human AMs *In Vitro*

AMs obtained from HIV-1$^+$ individuals undergoing diagnostic bronchoscopy were purified by adherence, as described subsequently here. The following organisms were isolated from these bronchoscopies ($n = 5$ individuals): *Pneumocystis jirovecii* ($n = 2$); *Mycobacterium avium* intracellulare with methicillin-resistant *Staphylococcus aureus* ($n = 1$); *Aspergillus fumigatus* ($n = 1$); *Rhizopus* and *Candida* ($n = 1$). None of the individuals had viral pneumonia. After washing, the media were replaced with fresh media, and 50-μl aliquots were removed and replaced with 50 μl of fresh media to maintain constant volume in the well each time the media were assayed for viral replication (*see below*).

Ad Infection of Normal AMs

At 24 hours after plating AMs obtained from normal individuals, the cells were washed twice with medium (RPMI 1,640 medium containing 2% FBS, 2 mM glutamine, 100 U penicillin/ml, and 10 μg/ml streptomycin [GIBCO BRL]) to remove red blood cells and other nonadherent cells. Infections were based on particle units (pu) of Ad (28). AMs were infected with Ad at doses of 10^2 to 2.5×10^4 pu/cell or empty Ad capsids 2.5×10^4 pu/cell. Dilutions of thawed Ad stock were added to wells with a minimal volume of media (250 μl/well) to maximize contact of particles with AMs. Infections were initiated by placing the plates on a rocker for 1.5 hour at 37°C. The cells were then washed three times with culture medium to remove any residual Ad, and replaced in the incubator with 1 ml culture medium per well.

HIV-1 Infection of Normal AMs

At 2 or 72 hours after Ad infection, the normal AMs (2×10^5/well) were infected with the HIV-1 laboratory strain, JRFL, at 10^3 median tissue culture infectious doses ($TCID_{50}$) per well, a dose known to infect cells of monocytic origin (29, 30). For some experiments, HIV-1 infection was performed before Ad infection, as indicated. A 10-μg/ml aliquot of 3′-azido-3′-deoxythymidine (AZT; Sigma, St. Louis, MO) was added daily to some wells as a control to inhibit HIV-1 replication. For this experiment, the media were left unchanged until 12 days after the Ad infection. After 12 days of incubation at 37°C, the cells were washed, and 1 ml fresh culture media was added.

Quantification of HIV-1 Replication

Productive HIV-1 infection for both JRFL and endogenous HIV-1 was determined by quantifying p24 antigen in the media supernatant by ELISA (Beckman Coulter, Miami, FL) at 0–28 days after HIV-1 JRFL infection. The effects of Ad infection of AMs on subsequent HIV-1 infection and replication were compared on Days 1–38.

Assessment of HIV-1 LTR Transcription

Vesicular stomatitis virus G protein (VSV-G)–1 is an HIV-1 VSV-G pseudotyped virus containing the firefly luciferase gene cloned downstream of the full-length HIV-1 LTR promoter (31, 32). AMs were plated at 2×10^5 and subsequently infected with AdNull at 2.5×10^4 pu/cell or left uninfected. After 72 hours, 150 μl of the VSV-G-1 preparation was added to the AM media. Alternatively, cells were infected with VSV-G-1 first, followed by subsequent AdNull infection after an additional 4 hours. After 72 hours, the media were removed, AMs were lysed with cell lysis buffer (Promega, Madison, WI), and luciferase activity was quantified by luminometry, and normalized to protein concentration, as determined by the bicinchoninic acid method (Pierce, Rockford, IL). Additional experiments were performed with HIV-1/LTR-Luc, a nonpseudotyped fully HIV-1 envelope virus containing the luciferase reporter downstream of the HIV-1 LTR promotor, derived from the pNL4–3 plasmid.

TaqMan Real-Time PCR Analysis of HIV-1 DNA Synthesis in AMs

The HIV-1 inoculum was pretreated with RNase-Free DNAase (Qiagen, Valencia, CA) for 1 hour at 37°C to degrade any HIV-1 DNA present in the viral preparation (33). AMs were infected with HIV-1 JRFL, as described previously here, and DNA was extracted from the AMs with the Dneasy Tissue Kit (Qiagen, Valencia, CA). Triplicate PCR reactions (50 μl each) were established with 5 μl of both 1:10 and 1:100 dilutions of the DNA in water. Primers and probes were developed to quantify the JRFL envelope gene. Amplification reactions were prepared with reagents from Perkin Elmer (Foster City, CA) and contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 μm ethylenediaminetetraacetate acid, 60 nM 5-carboxyrhodamine, 3 mM $MgCl_2$, 200 nM each of dCTP, dGTP, and dATP, 400 nM dUTP, 0.5 U urical N-glycosylase, 0.25 U AmpliTaq Gold DNA polymerase, 200 nM forward primer (5′-TCCTTTGAGCCAATTCCCAT), 200 nM reverse primer (5′-TCCATTGAACGTCTTATCATTACACTT), and 100 nM TaqMan central probe (5′-ATTATTGTGCCCCGGCTGGTTTTGC). The TaqMan probe was labeled with the reporter group, FAM (6-carboxy fluorescein) on the 5′ end, and the quencher, 6-carboxy-N,N,N,N-tetramethylrhodamine on the 3′ end. The primer and probe combination was designed with the PrimerExpress software (Perkin Elmer). Samples (standard or unknown) were amplified for 40 cycles in a Perkin Elmer 7700 sequence detection system after 10 minutes at 50°C for urical N-glycosylase to degrade carryover contamination, and 10 minutes at 94°C to activate the AmpliTaq Gold. Cycling conditions were 15 seconds at 94°C, followed by 1 minute at 60°C with continuous monitoring of the fluorescence. Data were processed with SDS 1.6 software (Perkin Elmer) to generate standard curves and to determine the concentration of target in the unknowns by interpolation. A 25% difference in the vector amount adjusted for dilution in the 1:10 and 1:100 dilutions of the DNA was considered acceptable. Total DNA concentration was determined by measuring the A260 of dilutions of the DNA stocks.

TaqMan Real-Time PCR Analysis of Chromosomally Integrated HIV-1 DNA in AMs

The HIV-1 inoculum was pretreated with RNase-Free DNAase (Qiagen, Valencia, CA) for 1 hour at 37°C to degrade any HIV-1 DNA present in the viral preparation (33). For an integrated HIV-1 standard, HeLa-CD4$^+$/CCR5$^+$ cells were infected with JRFL. After 7 days of infection at 37°C, cells were trypsinized and DNA was purified with the Dneasy Tissue Kit (Qiagen, Valencia, CA). AMs were infected with HIV-1 JRFL, as described previously here, and DNA was extracted from the AMs after 72 hours with the Dneasy Tissue Kit (Qiagen). A two-step nested amplification was performed to initially amplify sequences exclusively containing only human 300–base pair repetitive DNA sequences (Alu) adjacent to HIV-1 envelope, based on a recently published method (34). Primers and probes were developed to quantify human Alu sequences and the JRFL envelope gene with the PrimerExpress software (Perkin Elmer). During the first-round PCR, integrated HIV-1 sequences in the samples (standard or unknown) were amplified with a JRFL-specific primer JRFL-R1: ctcgatgtcagcagtccttg tagtac) and *Alu*-targeting primers (*Alu-f1* gcactttgggaggccgaggcg and *Alu-r1* acagagcgagactccgtctcaaaaa) that annealed to conserved regions of the *Alu* repeat element. During the first-round PCR, *Alu*-JRFL sequences were amplified from 2.5 μl of purified cellular DNA in a 25-μl reaction mixture comprised of 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 10 μM primers, and DEPC-Treated Water (Ambion). The first-round PCR cycle conditions were as follows: An initial denaturing of 95°C for 10 minutes, followed by 35 repetition cycles of: 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3 minutes.

The second-round TaqMan PCR was performed with 2.5 μl of the first-round PCR product in a mixture comprised of: 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 10 μM primers Inner JRFL-L2:gctacttccctgattggcagaa, Inner JRFL-R2:gaagcaccatc caaaggtcaa, and 10 μM probe and DEPC-Treated Water (Ambion). The second-round PCR cycle began with a DNA-denaturing and polymerase-activation step (95°C for 10 min), followed by 45 cycles of amplification (95°C for 15 s, 60°C for 60 s) with an ABI 7,700 sequence detector system (Applied Biosystems). The TaqMan probe, cacaccagggccaggaatcagatttc, internal to the sequence spanning the Inner JRFL-L2 and Inner JRFL-R2, was labeled with the reporter group, FAM (6-carboxy fluorescein), on the 5′ end and the quencher, 6-carboxy-N,N,N,N-tetramethylrhodamine, on the 3′ end. Data were processed by the SDS 1.6 software (Perkin Elmer) to generate standard curves based on serial 10-fold dilutions of DNA from JRFL-infected JC-48 cells, and to determine the concentration of target in the unknowns by interpolation.

Western Analysis

To assess intracellular levels of phosphorylated STAT-1 and IRF-1, NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) were used for electrophoresis, with equal amounts of AM cell lysate protein, 15 μg/lane added to each lane. Proteins were transferred to polyvinylidene flouride (PVDF) membrane (0.45-μm pore size; Invitrogen), washed, and blocked with 5% nonfat milk for 1 hour. The membrane was incubated with anti–phospho–STAT-1 rabbit immunoaffinity-purified IgG (Upstate, Lake Placid, NY) or anti–IRF-8 mouse monoclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) 1:500–1:750 in 5% nonfat milk (16 h, 4°C). After washing and reblocking, the secondary horseradish peroxidase–labeled goat anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) 1:8,000–1:10,000 was applied (2 h, 23°C). Detection was performed with enhanced chemiluminescence Western analysis detection reagents (Amersham, Piscataway, NJ) and used BioMax Light Film (Kodak, Rochester, NY).

Statistical Analysis

Data are expressed as means (±SEM). Treatment group means were compared by ANOVA. A P value less than 0.05 was considered statistically significant. The Newman-Keuls *post hoc* test was performed when the ANOVA indicated significance. Statistics were compared with the Number Cruncher Statistical System software (NCSS, Kaysville, UT).

RESULTS

Effect of Ad on Endogenous HIV-1 Replication by Human AMs

Although cultured AMs obtained from most HIV-1$^+$ individuals exhibit no spontaneous HIV-1 replication (35), we were able to identify several individuals who, for various reasons, were not on HAART, whose CD4$^+$ cell counts were less than 200×10^6/L, and whose AMs spontaneously replicated HIV-1 in the setting of acute lung infection. In the absence of Ad, spontaneous HIV-1 replication was observed as the level of p24 increased steadily over the first 12 days of culture *in vitro* (Figure 1). The HIV-1 replication was suppressed by 10 μg/ml AZT added daily to the culture media. Infection with AdNull (2.5×10^4 pu/cell) produced nearly complete inhibition of HIV-1 replication comparable to the effect of AZT ($P < 0.05$, ANOVA). Similar results were observed with AMs from four other HIV-1$^+$ individuals (data not shown).

Dose–Response Effect of Ad Vectors on HIV-1 Replication in Endogenously Infected Human AMs

AMs were isolated from BAL fluid of an HIV-1$^+$ individual (different from Figure 1) not on HAART in the setting of acute lung infection. In the absence of Ad, spontaneous HIV-1 replication was observed as the level of p24 increased linearly over the first 4 days of *in vitro* culture. The HIV-1 replication was suppressed by AZT added daily to the culture media (Figure 2A). Infection with AdNull (2.5×10^4 pu/cell) produced substantial inhibition of HIV-1 replication. This inhibition was dose dependent over a range of input Ad of 10^2–2.5×10^4 pu/cell. For Day 4, the correlation between percent inhibition versus log[AdNull] was significant ($r^2 = 0.96$). These data indicate that infection with Ad vectors, in the absence of any transgene, block HIV-1 replication in endogenously infected AMs in a dose-responsive fashion.

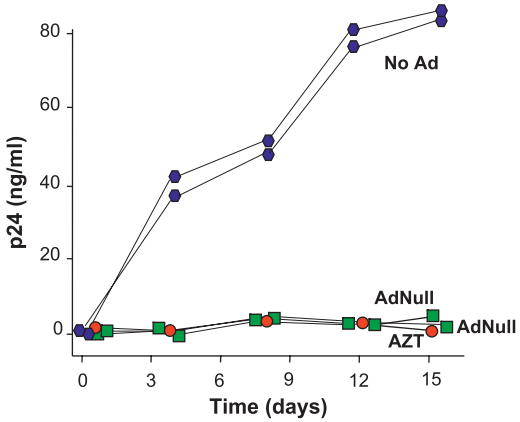

Figure 1. Effect of an adenovirus vector with no transgene (AdNull) on endogenous human immunodeficiency virus (HIV)–1 replication by human alveolar macrophages (AMs). AMs were isolated from an HIV-1$^+$ individual not on treatment with highly active antiretroviral therapy (HAART). The AMs (2×10^5/well) were infected with AdNull 2.5×10^4 particle units (pu)/cell, treated daily with 10 μg/ml 3′-azido-3′-deoxythymidine (AZT), or left untreated. Aliquots of media were collected every 4 days and assayed for HIV-1 p24 protein to quantify viral replication. Each *symbol* represents the measurement from a single well. Ordinate, p24 (ng/ml); abscissa, time (d). The results were similar in AMs obtained from five different individuals.

Effect of Ad Vectors on HIV-1 Replication in Normal Human AMs Infected with HIV-1 *In Vitro*

To model the effect of Ad on HIV-1 replication, normal AMs were infected with AdNull for 2 hours *in vitro*. After washing the AMs, they were infected with HIV-1 10^3 $TCID_{50}$/well. The media were changed after 12 days, and p24 levels were monitored daily thereafter. To assess the fidelity of the *in vitro* HIV-1 infection of AMs as a model for endogenously infected AMs, the dose–response curves for Ad inhibition of endogenous HIV-1 replication in human AMs were compared with *in vitro* infection. The results indicate that, in the absence of Ad, robust HIV-1 replication was obtained in AMs under these conditions (Figure 2B). The HIV-1 replication was suppressed by AZT. Infection with AdNull (10^2–2.5×10^4 pu/cell) produced a dose-dependent inhibition of HIV-1 replication. These data demonstrate that the *in vitro* HIV-1 infection of normal AMs is a reasonable model for the effect of Ad on endogenously HIV-1–infected AMs.

Effect of Ad Vectors on HIV-1 Replication when AMs are First Infected with HIV-1

To begin to assess the steps in the HIV-1 life cycle where Ad exerts an inhibitory effect, we evaluated the ability of Ad vectors to inhibit HIV-1 replication after HIV-1 infection had already occurred. We compared four different scenarios for infection: (*1*) Ad infection followed by HIV-1 infection 3 days later; (*2*)

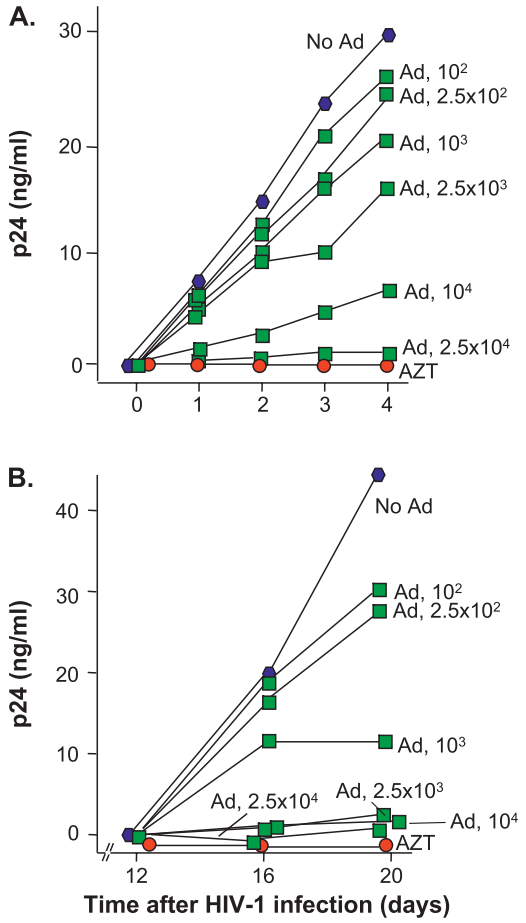

Figure 2. Dose- and time-dependent effect of AdNull on endogenous and laboratory-infected HIV-1 replication by human AMs. (*A*) Time dependency of HIV-1 replication in naive and AdNull-treated AMs. AMs were isolated from an HIV-1$^+$ individual not on treatment with HAART. The AMs (2×10^5/well) were infected with AdNull 10^2 - 2.5×10^4 pu/cell or treated daily with AZT, 10 μg/ml or left untreated. Aliquots of media were collected and assayed for HIV-1 p24 protein to quantify viral replication. Each *symbol* represents the measurement from a single well. Ordinate, p24 (ng/ml); abscissa, time (d). The results were similar in AMs obtained from five different individuals. (*B*) Effect of time after HIV-1 infection on HIV-1 replication with laboratory infection of AMs with HIV-1. AMs were isolated from a normal volunteer. The AMs (2×10^5/well) were infected with 10^2 to 2.5×10^4 pu/cell AdNull, treated daily with 10 μg/ml AZT, or left untreated. After 2 hours, all of the wells were washed and infected with an HIV-1 strain isolated from the frontal lobe of subject JR who died of AIDS demetia (JRFL) 10^3 median tissue culture infectious dose ($TCID_{50}$)/well. The media were replaced at Day 12. Aliquots of media were collected at Days 12, 16, and 20, and assayed for HIV-1 p24 protein to quantify viral replication. Ordinate, p24 (ng/ml); abscissa, time (d). The results were similar in AMs obtained from eight different individuals.

Ad infection followed by HIV-1 infection 2 hours later; (*3*) HIV-1 infection followed by Ad infection 7 days later; and (*4*) HIV-1 infection followed by Ad infection 14 days later. Consistent with the previous results, Ad significantly reduced the levels of p24 when the AMs were infected 3 days or 2 hours before HIV-1 infections (Figures 3A and 3B). To a lesser degree, p24 production was reduced even when the AMs were infected with Ad 7 or 14 days after HIV-1 infection (Figures 3C and 3D). These data suggest that Ad could be affecting a step in the HIV-1 life cycle after HIV-1 cellular entry, but are not definitive.

Ad Inhibits HIV-1 Replication by AMs under Conditions where Reverse Transcription Is Blocked

To establish that Ad is acting at a post–viral entry step, AMs were infected with HIV-1 *in vitro* and cultured for an additional 9 days to allow a near-maximal number of HIV-1–infected AMs. AZT was then added to all wells and maintained in the culture media to prevent any productive infection by blocking reverse transcription in previously uninfected cells. After an additional 2 days, some wells were infected with Ad (2.5×10^4 pu/cell). The media were changed at Day 14, and levels of p24 were measured to quantify HIV-1 replication. Under these conditions, all HIV-1 replication must occur from cells that had been previously infected, and the HIV-1 RNA reverse transcribed to DNA. In the absence of Ad, robust p24 production occurred (Figure 4). With Ad infection, the amount of p24 was reduced, even though HIV-1 was prevented from productively infecting new cells. The difference in p24 production between the Ad-infected and uninfected AMs continuously increased, up to nearly a 100-fold reduction by Day 45 (overall ANOVA, $P < 0.01$). These data indicate that Ad is acting at least in part at a post–reverse transcription step to inhibit HIV-1 replication.

Effect of Ad on the Formation of HIV-1–Specific DNA in AMs

To confirm the effect of Ad on reverse transcription of HIV-1 RNA to DNA, AMs were infected with AdNull (2.5×10^4 pu/cell), treated with AZT, or left untreated. After 72 hours, the AMs were infected with HIV-1 JRFL (10^3 $TCID_{50}$/well). DNA was then isolated at 0, 24, 48, and 120 hours, and HIV-1 DNA was quantified by TaqMan real-time PCR. On Days 2 and 5, untreated cells accumulated significant amounts of HIV-1 DNA, which was inhibited by AZT ($P < 0.05$, ANOVA; Figure 5). Although there was a numerical reduction in the number of HIV-1 DNA copies per cell, Ad-infected AMs still accumulated significant amounts of HIV-1 DNA. These data indicate that the block to HIV-1 replication by Ad is not at the level of reverse transcription from HIV-1 RNA to DNA, suggesting that the suppressive effect of AdNull occurs at a later stage of the HIV-1 life cycle.

Effect of Ad on HIV-1 LTR Transcription in AMs

To determine whether AdNull suppresses HIV-1 replication via modulation of LTR-dependent transcription, a VSV-G pseudotyped HIV-1 LTR-luciferase construct (VSV-G-1) was used (31, 32). AMs were infected with AdNull 2.5×10^4 pu/cell for 2 hours, followed by washing and addition of fresh media, or were left uninfected. After an additional 3 days, the AMs were infected with VSV-G-1. In some wells, the cells were infected with VSV-G-1 first, followed by AdNull infection 4 hours later. At 72 hours later, the AMs were harvested, and luciferase expression was measured. The LTR expression, as quantified by luciferase expression, decreased from 3×10^4 relative light units (RLU)/mg to 3×10^3 RLU/mg ($P < 0.05$, ANOVA; Table 1). This represents a greater than 1 log inhibition of LTR transcription. Moreover, the inhibition was similar if the Ad infection occurred 4 hours after VSV-G-1 infection, at a time when all of the VSV-G-1 was inside the cell, eliminating the possibility that Ad had blocked VSV-G-1 entry ($P < 0.05$, ANOVA; Table 1; [36]). To further ascertain that the VSV-G pseudotyped virus was not merely interfering with HIV-1 entry into the cell by overwhelming the endocytotic pathway, the experiment was repeated with an HIV-1 LTR-luciferase constructed on a purely HIV-1 envelope, with similar results ($P < 0.05$, ANOVA; Table 1). These data indicate that Ad infection of AMs suppresses transcription from the HIV-1 LTR.

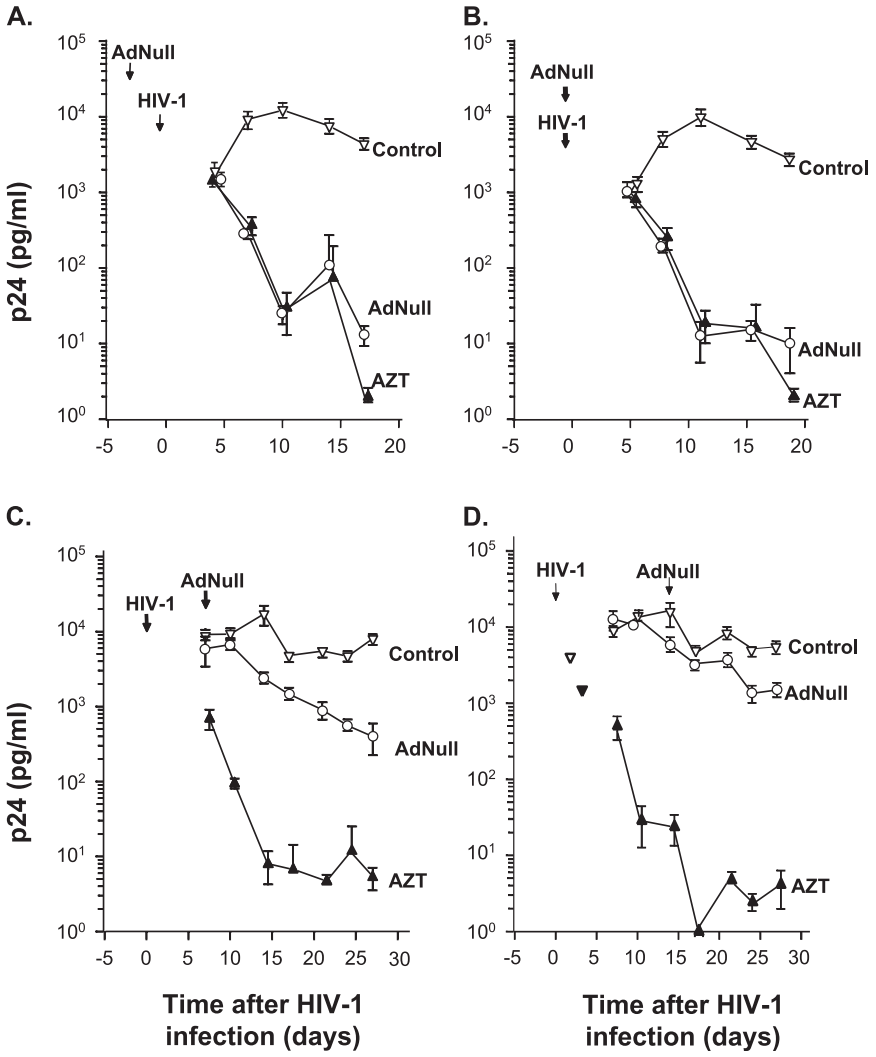

Figure 3. Effect of timing of Ad infection on HIV-1 replication by human AMs infected *in vitro* with HIV-1. AMs were isolated from a normal volunteer. The AMs (2×10^5/well) were infected with 10^2 to 2.5×10^4 pu/cell AdNull at Days −3 (before HIV-1 infection), 0, 7, or 14 after HIV-1 infection, treated daily 10 μg/ml with AZT, or left untreated. (*A*) Ad infection 3 days before HIV-1 infection. (*B*) Ad infection 2 hours before HIV-1 infection. (*C*) Ad infection 7 days after HIV-1 infection. (*D*) Ad infection 14 days after HIV-1 infection. Ordinate, p24 (pg/ml); abscissa, time (d). The results were similar in AMs obtained from three different individuals.

Effect of Ad on the Formation of Integrated HIV-1 DNA in AMs

To quantify the effect of Ad on incorporation of HIV-1 DNA into the host chromosome, AMs were infected with AdNull (2.5×10^4 pu/cell), treated with AZT, or left untreated. After 1 hour, the AMs were infected with HIV-1 JRFL (10^3 $TCID_{50}$/well). DNA was then isolated at 72 hours and HIV-1 DNA was quantified by a two-step nested TaqMan real-time PCR. In the first step, template DNA was amplified with primers designed to amplify HIV-1 DNA exclusively adjacent to human Alu sequences. In the second step, HIV-1 DNA was amplified and detected with primers, and a probe within the predicted sequence of the first HIV-1/human Alu primer sequence was quantified by TaqMan. JC-48, a HeLa cell line transfected with CD4 and CCR5, was infected with HIV-1 JRFL for 7 days, and the DNA was used as a standard. At 72 hours, untreated AMs infected with JRFL accumulated significant amounts of HIV-1 DNA specifically integrated into chromosomal DNA, which was inhibited by AZT ($P < 0.05$, ANOVA; Figure 6). Ad coinfected AMs still accumulated HIV-1 DNA specifically integrated into chromosomal DNA at levels comparable to AMs infected with JRFL in the absence of Ad. To show the specificity of this assay for integrated HIV-1 DNA, the samples were also run directly in the second-round TaqMan without the first-round Alu-HIV PCR. Although HIV-1 DNA was detectable, the level was 100-fold less in all samples, indicating that the detection in the two-step procedure was greater than 99% due to integrated HIV-1 DNA (data not shown). These data indicate that the block to HIV-1 replication by Ad is not before HIV-1 nuclear import and integration into chromosomal DNA, suggesting that the suppressive effect of AdNull occurs at a later stage of the HIV-1 life cycle. Taken together with the inhibition of LTR-driven transcription, the effect of Ad can be localized to the LTR-transcription step itself.

Effect of Ad on IFN-Related Signal Transduction in AMs

To assess whether the Ad genome was necessary for the inhibition of HIV-1 replication in human AMs, we treated AMs *in vitro* with empty Ad capsids (2.5×10^4 pu/cell). At 5 and 10 days after infection with HIV-1, there was no inhibition of HIV-1 replication (Figure E1 in the online supplement). These results indicate that Ad DNA is absolutely required for the inhibitory effect of AdNull on HIV-1 replication. Because, previously, we were unable to identify a soluble factor(s) released by AMs that mediated the inhibitory effects of Ad on AMs HIV-1 replication, we focused on potential intracellular mediators of this effect. Because intracellular viral DNA is known to induce rapid activation of IFN-associated signaling pathways, we evaluated the effect of Ad infection of AMs on activation of STAT-1 by Western analysis. There was significant phosphorylation of STAT-1 induced by AdNull infection of AMs in the presence or absence of HIV-1 coinfection (Figure E2). These data indicate

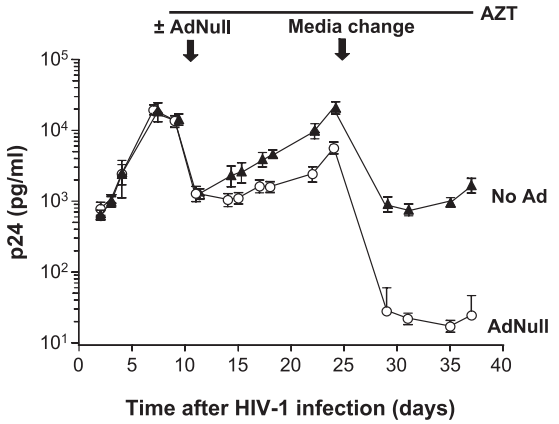

Figure 4. Effect of Ad on HIV-1 replication by human AMs under conditions where reverse transcription is blocked. AMs were isolated from a normal volunteer. The AMs (2×10^5/well) were infected with HIV-1 JRFL 10^3 $TCID_{50}$/well. At Day 12, the cells were treated with 10 μg/ml AZT. After an additional 4 days, the cells were infected with 2.5×10^4 pu/cell AdNull or left uninfected. AZT was added daily. Aliquots of media were collected and assayed for p24 protein, but the media were not changed until 14 days later, as indicated. Ordinate, p24 (pg/ml); abscissa, time (d). The results were similar in AMs obtained from three different individuals.

TABLE 1. EFFECT OF AD ON HUMAN IMMUNODEFICIENCY VIRUS–1 LTR TRANSCRIPTION IN HUMAN ALVEOLAR MACROPHAGES

	Luciferase Activity (*RLU/mg*)
VSV-G/HIV-1/LTR-Luc	3,940 ± 1,640
VSV-G/HIV-1/LTR-Luc + AdNull	365 ± 164
VSV-G/HIV-1/LTR-Luc + AdNull (4 h after HIV-1)	231 ± 138
Background	<10
HIV-1/LTR-Luc	1,307 ± 131
HIV-1/LTR-Luc + AdNull	76 ± 54

Definition of abbreviations: Ad, adenovirus vector; HIV, human immunodeficiency virus; LTR, long terminal repeat; Luc, luciferase; RLU, relative light units; VSV-G, vesicular stomatitis virus G protein.

AM were isolated from a normal volunteer. For the first two conditions, the alveolar macrophages (AMs) (2×10^5/well) were infected with AdNull 2.5×10^4 pu/cell or left uninfected. After 72 hours, the cells were infected with a VSV-G pseudotyped HIV-1 LTR-luciferase construct (VSV-G/HIV-1/LTR-Luc). As a third condition, the cells were infected with VSV-G/HIV-1/LTR-Luc first, followed by AdNull 4 hours later. After an additional 72 hours, cell lysates were collected and luciferase activity and protein were quantified. Data are presented as means (±SD) for triplicate detection. In a separate experiment, the AMs (2×10^5/well) were infected with AdNull 2.5×10^4 pu/cell or left uninfected. After 1 hour, the cells were infected with a nonpseudotyped HIV-1 LTR-luciferase construct (HIV-1/LTR-Luc). After an additional 72 hours, cell lysates were collected and luciferase activity and protein were quantified. This experiment was repeated with AMs from five separate volunteers with similar results.

that the STAT-1 pathway is activated by AdNull infection of AMs. To determine if downstream IFN-inducible proteins could be playing a role in the suppression of LTR expression, we evaluated the effect of AdNull on the expression of IRF-8 by Western analysis. Up-regulation of IRF-8 is known to interfere with the induction of HIV-1 LTR transcription by the IRF-1/tat complex (37). AdNull infection of AMs significantly up-regulated IRF-8 expression in the presence and absence of HIV-1 coinfection (Figure E3). These data suggest a potential mechanism for suppression of LTR transcription via increased IRF-8 expression mediated by activation of STAT-1.

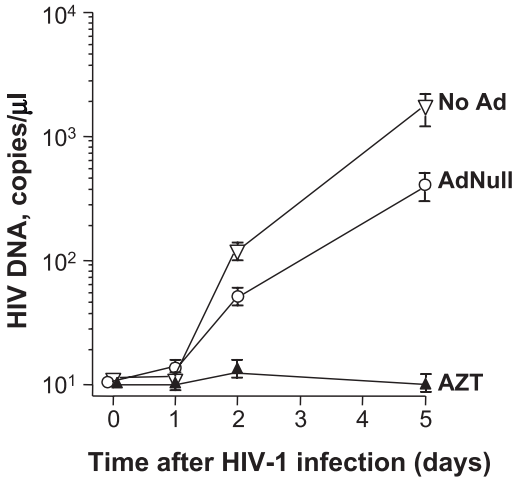

Figure 5. Effect of Ad on the transcription of HIV-1–specific DNA in AMs. To assess the effect of Ad on reverse transcription of HIV-1 RNA to DNA, AMs were infected with 2.5×10^4 pu/cell AdNull, treated with AZT, or left untreated. After 72 hours, the AMs were infected with HIV-1 JRFL, 10^3 $TCID_{50}$/well. DNA was then isolated at 0, 24, 48, and 120 hours, and HIV-1 DNA was quantified by TaqMan real-time PCR. Ordinate, HIV-1 DNA copies/μl; abscissa, time (d). The results were similar in AMs obtained from three different individuals.

DISCUSSION

Individuals infected with HIV-1 are frequently coinfected with other viruses (38–43). Individual cells recovered from HIV-1^+ individuals have been noted to be infected with HIV-1 and co-infected with a heterologous virus (44–47). These observations lead to the hypothesis that co-infections with heterologous viruses may accelerate the course of HIV-1 infection *in vivo* (48–50). For example, under some circumstances, HIV-1 replication is strongly stimulated in AMs by *M. tuberculosis* and by bacterial products (51, 52). HSV-1, CMV and HHV-8 gene products transactivate HIV-1 gene transcription *in vitro* (7, 8, 16). Depending upon the cell type and state of cellular activation and stage of HIV-1 infection, CMV and adeno-associated virus serotype 2 gene products inhibit HIV-1 transcription and replication (10, 11, 13, 14). This is consistent with the knowledge that viruses have evolved numerous methods for inhibiting replication of heterologous viruses within the same cell (12). These virally-induced blocks to HIV-1 replication are of interest since they may represent potential targets for therapeutic antiviral strategies.

The effect of type 5 Ad gene transfer vectors on HIV-1 replication is particularly relevant, since clinical trials are now underway with an HIV-1 vaccine based on type 5 Ad gene transfer vectors (17, 18). Wild-type adenovirus is known to block HIV-1 replication via the E1a region (9). We previously observed that replication deficient $E1^-$, partial $E3^-$ type 5 Ad gene transfer vectors inhibit HIV-1 replication in human alveolar macrophages independently of the transgene (5). Ad infection had no significant effect in the viability of HIV-1 infected AMs (5). Suppression of HIV-1 replication by $E1^-$ Ad vectors was a function of time and dose, was independent of the transgene in the Ad vector, and was not associated with the E4 genes in the Ad backbone. Inhibition did not appear to be due to a soluble factor in the AMs supernatant, and was not significantly overcome by addition of the CCR5 or CXCR4 coding sequences to the AMs.

AMs are an important element of pulmonary host defense including during HIV-1 infection (1, 2). They are also known to contribute to HIV-1 related lung diseases, and serve as a reservoir for HIV-1 infection despite HAART (1–4, 49). Inhibition of HIV

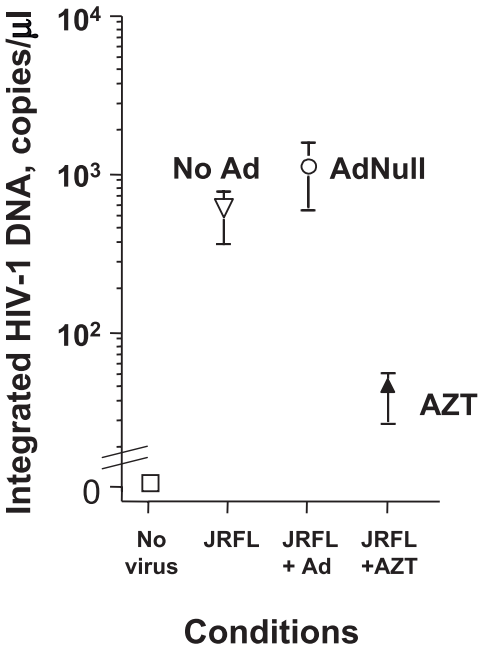

Figure 6. Effect of Ad on integrated HIV-1–specific DNA in AMs. To assess the effect of Ad on integration of HIV-1 DNA into the chromosomal DNA, AMs were infected with 2.5×10^4 pu/cell AdNull, treated with AZT, or left untreated. After 1 hour, the AMs were infected with HIV-1 JRFL, 10^3 $TCID_{50}$/well. DNA was isolated at 72 hours and integrated HIV-1 DNA was quantified by a two-step nested TaqMan real-time PCR. The initial PCR step specifically amplifies only sequences annealing to both human 300–base pair repetitive DNA sequences (Alu) primers and a JRFL env primer. In the second step, JRFL primers with an internal probe site quantify the Alu-JRFL amplification products with the first-round PCR products as a template. Ordinate, integrated HIV-1 DNA (copies/μl); abscissa, infection conditions (no virus, JRFL, JRFL + Ad, JRFL + AZT). The results were similar in AMs obtained from three different individuals.

replication in AMs by other micro-organisms has been previously observed in some circumstances. While *M. tuberculosis* can activate HIV-1^+ replication in AMs in some circumstances (51), in others it inhibits via an IFN-β–inducible inhibitory nuclear factor C/EBP-β which suppresses transcription from the HIV-1 LTR (53). A number of studies have examined the effect of co-infection of pathogens with HIV-1 in mononuclear phagocytes. Most *in vitro* studies reveal an upregulation of HIV-1 replication in AMs, mononuclear cell lines and peripheral blood mononuclear cells by co-pathogens, such as mycobacteria and cytomegalovirus (54, 55). *M. tuberculosis* and *M. avium* complex infection of blood monocytes *in vitro* both upregulate HIV-1 replication (56). *In vitro* studies of the effect of *M. tuberculosis* on HIV-1 replication in monocyte-derived macrophages have rendered conflicting results depending upon the experimental conditions (57). When inhibitory, the effect of *M. tuberculosis* is dependent on the state of differentiation of the monocytic cells (53).

Possible Mechanisms of Ad Vector–Mediated Suppression of LTR-Driven HIV-1 Replication in AMs

HIV-1 virion attachment onto the cell occurs by interaction with cell surface CD4 acting in concert with co-receptors CCR5 and CXCR4 (25, 58–60). HIV-1 strains predominantly utilizing CCR5 as the co-receptor readily infect AMs (25). It has been shown that upregulation of expression of CCR5 or CXCR4 via gene transfer did not significantly abrogate Ad vector-mediated inhibition of HIV-1 replication, suggesting that the effect of Ad vectors is unlikely to involve depletion or blockade of cell surface co-receptors (5).

After HIV-1 virion entry, the viral RNA genome is reverse transcribed into double stranded DNA. The viral dsDNA is translocated to the nucleus, followed by the initiation of HIV-1 transcription leading to HIV-1 protein synthesis. Suppression of HIV-1 post-entry events might be due to either the direct effect of an Ad vector gene product or to the indirect effect of an inducible cellular factor(s) on LTR transcription. HIV-1 LTR possesses multiple nuclear factor binding sites used to modulate transcription including Nef, SP-1, NF-κB and C/EBP β (61, 62).

Upregulation of the inhibitory isoform of C/EBPβ modulation of HIV-1 LTR has been implicated as the mechanism through which *M. tuberculosis* exerts its suppressive effect on HIV-1 replication in AMs. An example of post-entry block is IFN-β which acts via repression of HIV-1 LTR transcription (51). In the Janus kinase–STAT IFN signaling pathway, phosphorylation of STAT-1 to p-STAT-1 causes dimerization, nuclear transport, and the induction of transcription of numerous genes, including the IRF genes (IRF1–9), which stimulate or inhibit a variety of cellular genes related to the differentiated state of macrophages, immune response, *et cetera*. In lymphocytes and monocytic cells, IRF-1 also stimulates HIV-1 replication via an interaction with the HIV-1 tat, which, together, bind the HIV-1 LTR and potentiate transcription (37). IRF-8 opposes the effect of IRF-1 by preventing the binding of IRF-1/tat to the HIV-1 LTR, and inhibits HIV-1 replication in lymphocytes and monocytic cells (37). We observed striking phosphorylation of STAT-1 as well as up-regulation of IRF-8 protein in Ad-infected AMs, suggesting that activation of an IFN signaling pathway may mediate the effect of AdNull on HIV-1 LTR transcription. This is consistent with a recent report of up-regulation of IFN-related gene expression by Ad infection of mouse macrophages (63). Other soluble cytokines, such as IL-4 and -10, transforming growth factor–β, $CD8^+$ T lymphocyte antiviral factor, and macrophage-derived chemokine, inhibit HIV-1 replication at one or more postentry levels (64–66). Influenza virus is known to inhibit subsequent HIV-1 replication via induction of release of soluble mediators (67). Previous studies have shown that passive transfer of media from Ad-infected AMs with conditioned media depleted of Ad vectors did not reveal a soluble inhibitor of HIV-1 replication (5).

Implications of Ad Vector–Mediated Suppression of LTR-Driven HIV-1 Replication in AMs

Monocyte-derived cells, such as alveolar macrophages remain a persistent source of transcriptionally activatable HIV-1, with the potential to initiate viral replication, even in the face of successful HAART where plasma levels of HIV-1 RNA become undetectable (3, 68). An Ad5 vector–based vaccine would be expected to transduce other types of cells of monocytic lineage when administered systemically. Our results do not directly address the question of whether a similar inhibitory effect of Ad on HIV-1 replication would be anticipated in other terminally differentiated tissue macrophages, such as Kupfer cells, as well as other cells of monocytic origin, such as dendritic cells, which may serve as reservoirs of HIV-1 and/or facilitate its transport *in vivo*. The inhibitory effect of Ad on HIV-1 replication observed here suggests that a suppressive effect in other monocytic cell types is plausible, but, until tested, remains a hypothesis. The observation that Ad mediates suppression of endogenous HIV-1 replication in AMs is reassuring in light of the ongoing clinical trial of an Ad-based HIV-1 vaccine (17, 18, 69; clinicaltrials.gov).

Author Disclosure: R.J.K. has received compensation for serving on the Board of Eli Lilly ($1,001–$5,000) and Centocor($1,001–$5,000) and has served as an expert witness for the U.S. District Attorney, NY ($1,001–$5,000) and for a private attorney ($1,001–$5,000) for review of Medical Malpractice defense; received Royalties from the commercial entity Uptodate ($1,001–$5,000), and received sponsored grants from the National Institutes of Health ($100,001 or more). F.R. has received reimbursement for consultancies to Gielad ($1,001–$5,000), Actelion ($1,001–$5,000), United Therapeutics ($1,001–$5,000), CSL Behring ($1,001–$5,000), and Baxter ($5,001–$10,000); has served on the Board/Advisory Board for Gielad ($1,001–$5,000), Actelion ($5,001–$10,000), United Therapeutics ($1,001–$5,000), CSL Behring ($1,001–$5,000), and Baxter ($1,001–$5,000); has received reimbursement for Lectures from Gielad ($1,001–$5,000), Actelion ($5,001–$10,000), United Therapeutics ($1,001–$5,000), CSL Behring ($1,001–$5,000), Baxter ($1,001–$5,000) and Pfizer ($1,001–$5,000); and has an Industry Sponsored Grant from Baxter ($10,000–$50,000) and United Therapeutics ($1,001–$5,000). None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Shawn Kuhman, Department of Microbiology, for the human immunodeficiency virus–1 vesicular stomatitis virus G protein–1 LTR-luciferase construct, B.-G. Harvey, Division of Pulmonary and Critical Care Medicine, Weill Cornell, for human alveolar macrophages, and N. Mohamed for help in preparing the manuscript.

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