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Mechanisms for Macrophage-Mediated HIV-1 Induction

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Viral latency is a long-term pathogenic condition in patients infected with HIV-1. Low but sustained virus replication in chronically infected cells can be activated by stimulation with proinflammatory cytokines such as TNF-α, IL-1 β, or other host factors. However, the precise mechanism by which cellular activation induces latently infected cells to produce virions has remained unclear. In the present report, we present evidence that activation of HIV-1 replication in latently infected U1 or ACH2 cells by human macrophages is mediated by a rapid nuclear localization of NF-κB p50/p65 dimer with concomitant increased expression of proinflammatory cytokines. Multiplexed RT-PCR amplification of mRNA isolated from cocultures of macrophages and U1 and ACH2 cells showed significant induction of IL-1β, IL-6, IL-8, TNF-α, and TGF-β expression within 3 h of coincubation. Fixation of macrophages, U-1, or ACH2 cells with paraformaldehyde before coculture completely abrogated the induction of NF-κB subunits and HIV-1 replication, suggesting that cooperative interaction between the two cell types is an essential process for cellular activation. Pretreatment of macrophage-U1 or macrophage-ACH2 cocultures with neutralizing anti-TNF-α Ab downregulated the replication of HIV-1. In addition, pretreatment of macrophage-U1 or macrophage-ACH2 cocultures with the NF-κB inhibitor (*E*)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile (BAY 11-7082) prevented the induction of cytokine expression, indicating a pivotal role of NF-κB-mediated signaling in the reactivation of HIV-1 in latently infected cells by macrophages. These results provide a mechanism by which macrophages induce HIV-1 replication in latently infected cells. ***The Journal of Immunology,*** **2004, 173: 6735–6744.**

Infection with HIV-1, the causative agent of AIDS, is characterized clinically by a long asymptomatic period of latency preceding the development of AIDS. Even during this period of clinical latency, the virus is continuously replicating and causing new rounds of infection. Recent studies using combination antiviral therapy have revealed a population of latently infected cells that are refractory to antiviral therapy, which is believed to be the leading cause of the persistence of infection seen in HIV-1 disease (1). Furthermore, there is evidence of ongoing virus replication even in patients on highly active antiretroviral therapy in whom viremia is suppressed to undetectable levels (2). Thus, a detailed study of the mechanisms that modulate viral replication during this clinically latent stage would help to further understand the pathogenesis of this disease and to develop targeted therapies for eradicating latently infected cells or preventing the activation of viral replication in these cells.

$CD4^+$ T cells are thought to be the major reservoirs of both actively replicating and latent HIV-1 throughout the course of disease. Several in vitro studies have identified potentially stable reservoirs of inducible latently infected $CD4^+$ cells carrying an integrated form of the viral genome (3–5). Recently, it was shown that integrated proviral DNA is present at low frequency in resting, memory $CD4^+$ ($CD45RO^+$, $HLA\text{-}DR^-$) cells (6). Because memory $CD4^+$ T cells can persist for months to years, these resting memory $CD4^+$ cells carrying replication-competent viral genomes may represent an important long-term viral reservoir in patients (7). This population of latently infected cells has been clearly identified even in patients who have been on triple therapy for long periods (>30 mo) of time (8, 9), suggesting that these resting T cells provide a stable reservoir of HIV-1 for extended periods during the course of HIV-1 disease.

In addition to $CD4^+$ T cells, monocytes are a major reservoir for HIV-1 in vivo, since a number of blood monocytes are maintained in HIV-1-infected patients even during late-stage disease when T cells may be practically undetectable (10–12). Unlike $CD4^+$ T cells, monocytes are nonlytically infected. These cells serve as viral reservoirs in infected tissues, vectors for virus transmission to target cells, and sources of potent cytokines that can affect cell function and virus replication, and they thereby play a key role in the pathogenesis of HIV-1 disease. Peripheral blood monocytes that are latently infected in vivo can be activated by a number of agents to produce large quantities of virus (13). These observations suggest that infected macrophages (Mϕ)[2] may serve as a major reservoir for HIV-1, causing the persistence and spread of the virus.

The chronically infected, promonocytic cell line U1 harbors two copies of the HIV-1 provirus and under unstimulated conditions expresses low levels of viral transcripts encoding Tat, Rev, and Nef, but little or no full-length viral RNA (14–16). In a similar manner, the chronically infected T cell line ACH2 contains a single copy of the integrated HIV-1 provirus (16). Viral replication is enhanced in U1 cells following treatment with exogenous Tat, Nef,

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[2] Abbreviations used in this paper: Mϕ, macrophage; BAY 11-7082, (*E*)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile; LTR, long terminal repeat.

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LPS, GM-CSF, or a variety of other factors (16–19). The progeny viruses from U1 cells are defective and cannot infect susceptible cells. In contrast, ACH2 cells can produce viral particles that can infect susceptible T cells. Several studies have reported the activation of HIV-1 replication in these cells when cocultured with several other cell types, including endothelial cells (19–26). However, conditioned medium from endothelial cells failed to up-regulate HIV-1 replication. Thus, the activation of HIV-1 replication by coculture required cell–cell contact. The activation of HIV-1 replication was preceded by the activation of NF-κB and cytokine secretion (27). It is probable that the activation of NF-κB by coculture could involve the induction of transcription factors such as C/EBP and other cellular factors (28). NF-κB plays a critical role in the activation of HIV-1 gene expression by cytokines and other stimuli, but the pathways that regulate the switch from latency to the infectious phase have not been elucidated.

Immune activation as a result of the host response to HIV-1 infection itself or the presence of exogenous stimuli has a profound effect on the life cycle of HIV-1. Persistent immune activation caused by the release of endogenous cytokines can result in increased HIV-1 replication and lead to phenotypic and genotypic changes in the virus. HIV-1 replication is dependent on the host transcriptional machinery and is closely regulated by a complex pathway of proinflammatory and immunoregulatory cytokines (29, 30). Some of these cytokines are known to activate NF-κB, which in turn can further induce long terminal repeat $(LTR)^2$-directed gene expression (31, 32). For example, TNF-α secretion induced by the virus can up-regulate HIV-1 replication in an autocrine and paracrine manner in chronically infected cells (33, 34).

In this report, we have examined the factors associated with activation of HIV-1 replication in latently infected cells by cultured Mϕ. The coculture of ACH2 and U1 cells with uninfected Mϕ resulted in activation of HIV-1 replication. Cell–cell contact caused the rapid nuclear localization of NF-κB p65/p50 dimers with a concomitant increase in the expression of proinflammatory cytokines, suggesting a crucial role for Mϕ in the reactivation of HIV-1 in latently infected cells.

Materials and Methods

M-CSF, anti-TNF-α Ab, anti-TRAIL Ab, and goat anti-rabbit IgG were purchased from R&D Systems (Minneapolis, MN). CytoXpress multiplex PCR primer pairs and TNF-α and GAPDH PCR primer pairs were obtained from BioSource International (Camarillo, CA). NF-κB inhibitor (*E*)3-[4-methylphenyl)sulfonyl]-2-propenenitrile (BAY 11-7082) was purchased from Calbiochem (San Diego, CA). Proteinase K and Pronase E were purchased from Sigma-Aldrich (St. Louis, MO).

Isolation and culture of monocyte-derived Mϕ

Monocytes were isolated from PBMC of donors seronegative for HIV-1 and hepatitis B after leukophoresis and were purified by countercurrent centrifugal elutriation (35). Cell suspensions contained >95% monocytes by criteria of cell morphology on Wright-stained cytosmears, by granular peroxidase and by nonspecific esterase. The cells were cultured for 5 days in DMEM supplemented with 10% FBS, 20 μg/ml gentamicin, and 1000 U/ml M-CSF. After 5 days, culture medium was completely removed, and the cells were washed three times with PBS and transferred to RPMI 1640 medium supplemented with 10% FBS and 20 μg/ml gentamicin, and the resultant Mϕ were cultured for 2 additional days without M-CSF. All cell culture reagents were tested using the *Limulus* lysate assay (Associates of Cape Cod, Cape Cod, MA) for endotoxin contamination and the levels were found to be <0.06 endotoxin units/ml.

Culture of chronically infected U1 and ACH2 cells

U1 cells, a subclone of HIV-1-infected U937 cells (36), and ACH2 cells, a subclone of HIV-1 infected A301 cells (37), were cultured in RPMI 1640 supplemented with 10% FBS and 20 μg/ml gentamicin at 37°C in 5% CO_2.

Coculture of Mϕ and U1 or ACH2 cells

Mϕ (1×10^6) and U1 or ACH2 cells (1×10^5) were cocultured in 24-well plates in 2 ml of RPMI 1640 medium supplemented with 10% FBS and 20 μg/ml gentamicin. For Transwell experiments, Mϕ were plated on the plastic substrate of 24-well plates, and U1 or ACH2 cells were plated in the Transwell insert. Supernatants were collected at 24 and 48 h, respectively.

Coculture of monocytes and U1 or ACH2 cells

Freshly isolated monocytes (1×10^6) and U1 or ACH2 cells (1×10^5) were cocultured in 24-well plates in 2 ml of RPMI 1640 medium supplemented with 10% FBS and 20 μg/ml gentamicin. Supernatants were collected at 24 and 48 h, respectively.

Coculture of PBMC and U1 or ACH2 cells

Freshly isolated PBMC (5×10^6) were washed once in RPMI 1640 medium and cocultured with U1 or ACH2 cells (5×10^5) in RPMI 1640 medium supplemented with 10% FBS and 20 μg/ml gentamicin. Supernatants were collected at 24 and 48 h, respectively.

Quantitation of HIV-1 replication

Culture supernatants were assayed for HIV-p24 using a NEN/DuPont ELISA analysis kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instructions. Assays were performed in triplicate.

Treatment of cells with paraformaldehyde

Mϕ, U1 cells, and ACH2 cells were washed three times in PBS (pH 7.2) and incubated at 4°C for 2 h with 10% paraformaldehyde in PBS. After 2 h the cells were washed three times with PBS and then twice with RPMI 1640 medium before placing them in coculture.

Treatment of cells with proteinase K and Pronase E

Adherent Mϕ were treated overnight with 100 μg/ml proteinase K in serum-free medium for 18 h at 37°C or with 200 μg/ml Pronase E for 4 h in serum-free medium at 37°C. After incubation the cells were washed six times with PBS and placed in coculture.

Isolation of RNA from cells

Cocultures of Mϕ and U1 or ACH2 cells, grown at a ratio of 2:1, were harvested and washed in ice-cold PBS three times. Total RNA was extracted using Qiagen RNAeasy columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometry at A_{260} and A_{280}. RNA samples were aliquoted and stored at −70°C until further use.

Isolation of nuclear extract

Cocultures of Mϕ and U1 or ACH2 cells, grown at a ratio of 2:1, were harvested and washed three times in ice-cold PBS. Nuclear extracts were prepared as previously reported (38). Briefly, 1×10^3 cells were allowed to swell in 400 μl of ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) for 15 min on ice, after which 25 μl of 0.5% Nonidet P-40 was added and the tubes were thoroughly mixed for 10 s. The homogenate was centrifuged for 30 s and the pellet was saved. The nuclear pellet was resuspended in 50 μl of ice-cold buffer C (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF) and incubated on ice for 15 min. The nuclear extract was centrifuged at $1100 \times g$ for 5 min, and the supernatant containing the nuclear proteins was transferred to a fresh tube. Protein concentration was determined using a Pierce BCA protein assay kit (Pierce, Rockford, IL), and the concentration was adjusted to 1 mg/ml protein. The nuclear extracts were aliquoted and stored at −70°C until use.

NF-κB analysis

NF-κB p50 and p65 activities were determined using the BD Mercury transfactor kit (BD Clontech, Palo Alto, CA) according to the manufacturer's instructions. The assay was performed in triplicate using 15 μg of nuclear extract in each well.

RT-PCR

First-strand cDNA synthesis was conducted using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The first-strand cDNA was synthesized using 1–2 μg of total cell RNA. The cDNA was subsequently analyzed by PCR using an AccuPrime *Taq*DNA Polymerase System (Invitrogen Life Technologies) and CytoXpress multiplex

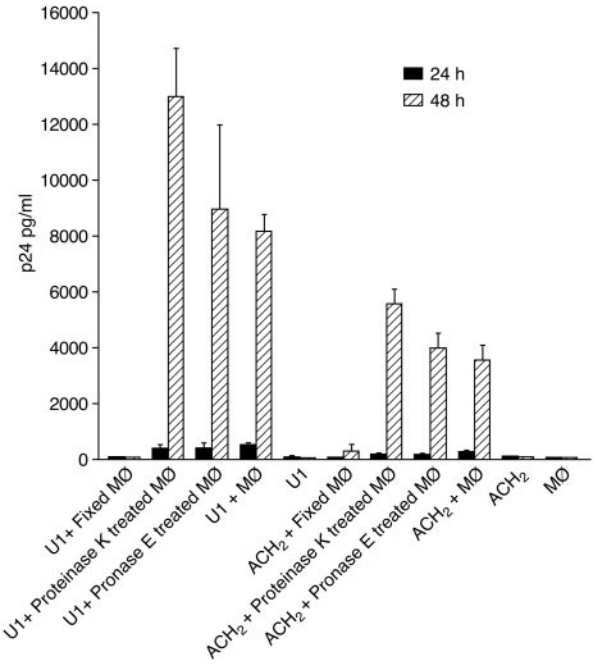


FIGURE 1. Determination of HIV-1 p24 production in culture supernatants by ELISA. Briefly, 1×10^5 U1 and 1×10^6 Mϕ or 1×10^5 ACH2 and 1×10^6 Mϕ were cocultured at a ratio of 1:10, and culture supernatants were collected at 24 and 48 h. Culture supernatants were analyzed in triplicate. Results expressed as mean ± SEM are representative of three independent experiments.

PCR human IFN cytokine set 1 primers according to the manufacturer's instructions. The PCR products were separated on a 2% agarose gel using a Tris-borate buffer and visualized by ethidium bromide staining.

TNF-α immunoassay

Supernatants from cocultures of Mϕ and U1 or ACH2 cells, grown at a ratio of 2:1, were collected at 24 and 48 h, respectively, and TNF-α concentrations were determined using the Quantikine kit (R&D Systems) according to the manufacturer's instructions. Assays were performed in triplicate.

FACS analysis

Mϕ (1×10^6), cocultured with U1 or ACH2 cells (5×10^5), were treated with 100 μg/ml anti-TNF-α Ab or 100 μg/ml goat anti-rabbit IgG for 48 h. After 48 h the cells were harvested and washed three times in PBS. After centrifugation, the cell pellets were permeabilized using 0.5% saponin, and intracellular p24 was labeled with FITC-labeled mAb to HIV-1 core protein p24 (Coulter Immunology, Hialeah, FL) or control IgG as described previously (39) and acquired for FACS analysis.

Treatment with anti-TNF-α Ab

U1 or ACH2 cells (5×10^5) and Mϕ (1×10^6) were treated for 2 h with 100 μg/ml anti-TNF-α Ab or 100 μg/ml anti-TRAIL Ab and placed in coculture. As a control, cells were treated with 100 μg/ml goat anti-rabbit IgG for 2 h and placed in coculture. Supernatants were collected at 24 and 48 h, respectively. Culture supernatants were assayed for HIV-p24. Viable cell numbers were determined using trypan blue dye and counting in a hemocytometer.

Treatment with NF-κB inhibitors

To study the possible down-regulation of cytokine expression and HIV-1 replication by the NF-κB inhibitor BAY 11-7082 (Calbiochem, San Diego, CA), ACH2, U1 and Mϕ were pretreated with 30 μM BAY 11-7082 for 30 min and placed in coculture along with the inhibitor for the specified durations. Culture supernatants were collected at specified times, and TNF-α levels and HIV-1 p24 levels were determined. No evidence of cell toxicity was detected at the concentrations of BAY 11-7082 used.

Results

Induction of HIV-1 replication in U1 and ACH2 cells by coculture with Mϕ

To determine the effect of coculture on the induction of HIV-1 replication in chronically infected U1 cells, Mϕ and U1 cells were cultured together at a ratio of 10:1, and culture supernatants were collected after 24 and 48 h and assayed for HIV-1 p24 by ELISA. Coculture of Mϕ with U1 cells at a ratio of 10:1 induced HIV-1 replication within 24 h. HIV-1 p24 levels reached greater than 8000 pg/ml in culture supernatants after 48 h in coculture (Fig. 1). Neither Mϕ nor U1 cells alone showed any appreciable amounts of HIV-1 p24. In addition, the supernatants from coculture of U1 cells with proteinase K- and Pronase E-treated Mϕ showed no significant inhibition of HIV-1 induction (Fig. 1). However, supernatants from coculture of U1 cells with paraformaldehyde-fixed Mϕ showed no significant increase of HIV-1 p24 levels even after 48 h of culture (Fig. 1). In a similar manner, coculture of ACH2

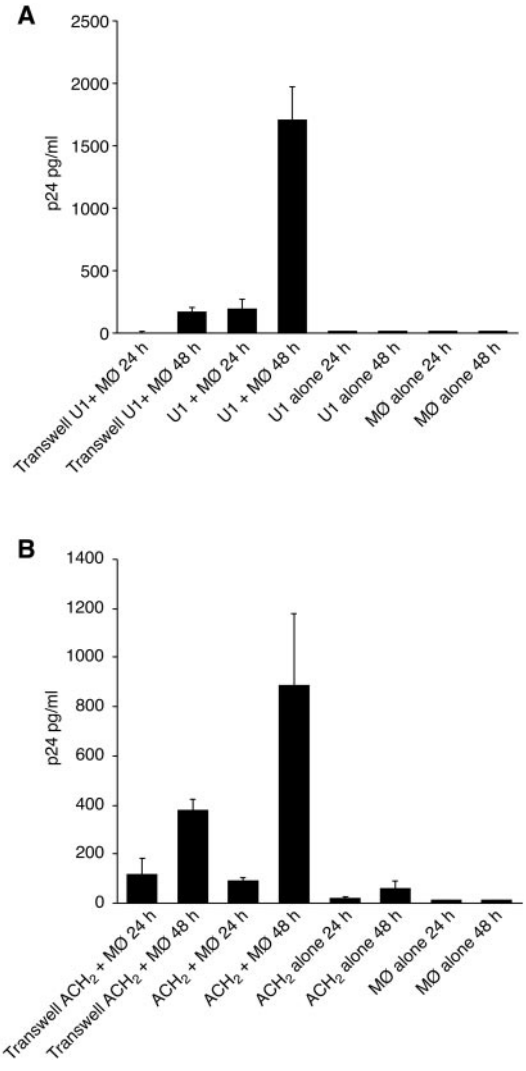


FIGURE 2. Comparison of HIV-1 production in supernatants derived from cells grown in a Transwell system and in coculture. *A*, One $\times 10^5$ U1 cells or 1×10^6 Mϕ were plated in Transwell plates and in coculture in 24-well plates. *B,* One $\times 10^5$ ACH2 cells or 1×10^6 Mϕ were plated in Transwell plates and in coculture in 24-well plates. After 24 and 48 h, culture supernatants were collected and the production of HIV-1 p24 was determined by ELISA. Culture supernatants were analyzed in triplicate. Results expressed as mean ± SEM are representative of two independent experiments.

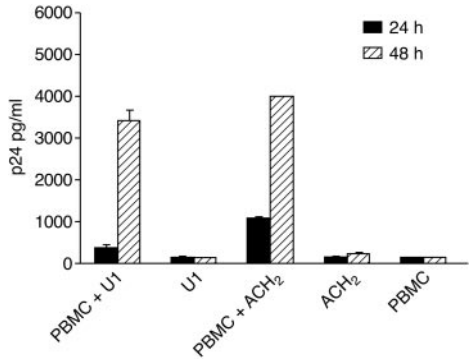


FIGURE 3. Induction of HIV-1 replication by PBMC. Briefly, 1×10^5 U1 and 1×10^6 PBMC or 1×10^5 ACH2 and 1×10^6 PBMC were cocultured at a ratio of 1:10, and culture supernatants were collected at 24 and 48 h. Culture supernatants were analyzed in triplicate. Results expressed as mean ± SEM are representative of three independent experiments.

and Mϕ at a ratio of 1:10 induced viral replication within 24 h and resulted in 3500 pg/ml HIV-1 p24 in culture supernatants after 48 h (Fig. 1). The Mϕ or ACH2 cells not in coculture produced a low amount of viral p24 even after 48 h in culture. In addition, proteinase K- and Pronase E-treated Mϕ did not inhibit HIV-1 induction in ACH2 cells. However, paraformaldehyde-fixed Mϕ failed to induce viral replication (Fig. 1). Furthermore, when a permeable membrane physically separated Mϕ and U1 cells, a 10-fold reduction of HIV-1 induction was seen (Fig. 2*A*). Likewise, when ACH2 cells and Mϕ were grown in Transwell inserts that prevented cell–cell contact, viral replication was significantly lower (Fig. 2*B*). In addition, induction of HIV-1 replication was also observed when latently infected U1 and ACH2 cells were cocultured with PBMC (Fig. 3) and freshly isolated monocytes (Fig. 4).

Induction of NF-κB

Several studies have demonstrated that coculture of latently infected cells with Mϕ and other cell types induced the nuclear localization of NF-κB (20, 25–28). To determine the kinetics of the activation of NF-κB by coculture, nuclear proteins were isolated from cocultures of U1 or ACH2 and Mϕ at 0, 3, 6, and 24 h, and the amounts of NF-κB p50 and p65 in the nuclear extracts were determined by ELISA. The results (Fig. 5*A*) demonstrated a rapid

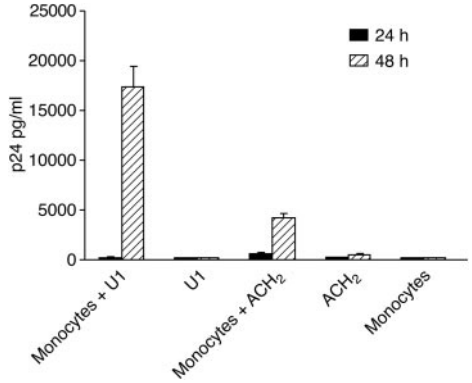


FIGURE 4. Induction of HIV-1 replication by freshly isolated monocytes. Briefly, 1×10^5 U1 and 1×10^6 monocytes or 1×10^5 ACH2 and 1×10^6 monocytes were cocultured at a ratio of 1:10, and culture supernatants were collected at 24 and 48 h. Culture supernatants were analyzed in triplicate. Results expressed as mean ± SEM are representative of three independent experiments.

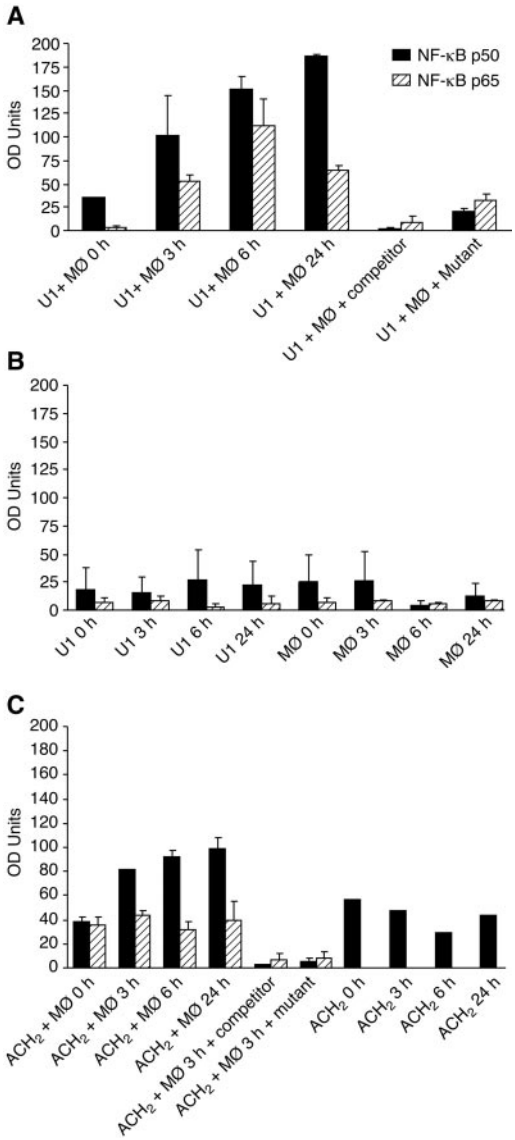


FIGURE 5. Determination of NF-κB in cocultures. NF-κB p50 and p65 activity was determined using the BD Mercury transfactor kit. Fifteen micrograms of nuclear proteins isolated from Mϕ (*A*) cocultured with U1 cells, U1 cells or Mϕ alone (*B*), or Mϕ (*C*) cocultured with ACH2 cells were used in each well. A 100-fold excess of competitor oligonucleotide was used where indicated. The assay was performed in triplicate. Results expressed as mean ± SEM are representative of three independent experiments.

nuclear accumulation of NF-κB p50 among the nuclear proteins isolated from U1 and Mϕ cocultures within 3 h, followed by a continuous increase to the 24-h time point. The kinetics of nuclear localization of NF-κB p65 were slightly different from that of NF-κB p50. NF-κB p65 induction was slower than that of NF-κB p50 and showed maximum activity at 6 h followed by a decrease in activity after 24 h. Neither the Mϕ nor the U1 cells alone demonstrated nuclear localization of NF-κB p50 or p65 subunits at the different time points tested (Fig. 5*B*). In a similar manner, activation of the NF-κB p50 subunit was observed in nuclear proteins isolated from ACH2 and Mϕ cocultures (Fig. 5*C*). The kinetics of activation indicated a rapid nuclear accumulation at 3 h that continued to the 24-h time point. In nuclear proteins isolated from ACH2 and Mϕ coculture, no activation of the NF-κB p65 subunit was observed. The inclusion of a large excess of competitor oligomers or mutant oligomers greatly reduced the binding affinity,

demonstrating that the binding assays were specific for NF-κB p50 and NF-κB p65, respectively. Treatment of U1 cells, ACH2 cells, and Mϕ with paraformaldehyde before coculture abolished activation of both NF-κB subunits (Fig. 6). Thus, the activation of NF-κB p50 and p65 subunits required the interaction of viable Mϕ and U1 cells.

Induction of cytokines by coculture

The effect of coculture of U1 or ACH2 cells with Mϕ on the induction of cytokine mRNA was examined by RT-PCR. The U1 or ACH2 cells and Mϕ were cocultured, and total RNA was isolated at 0, 3, 6,, and 24 h and subjected to RT-PCR analysis. The RT-PCR results from U1 coculture clearly demonstrated that the mRNAs of the proinflammatory cytokines IL-1β, IL-8, and TNF-α were up-regulated, in comparison to GAPDH mRNA (Fig. 7*A*). The mRNAs for IL-1β and IL-8 were up-regulated at the 3-h time point and continued to persist even after the 24-h time point. In contrast, TNF-α transcripts showed up-regulation only at 3 h, after which the mRNA returned to basal levels (Fig. 7*A*). In contrast, the levels of IL-6, TGF-β, and GM-CSF remained unchanged at all of the time points tested (Fig. 7*A*).

Likewise, the RT-PCR results from ACH2 and Mϕ coculture demonstrated up-regulation of TNF-α, IL-1β, IL-6, and IL-8 (Fig. 7*B*). Analogous to the results obtained with U1 and Mϕ coculture, increased transcription of IL-1β and IL-8 was also observed. In addition, increased IL-6 mRNA expression was detected as early as 3 h and remained constant up to 24 h. In contrast, only a 2-fold induction of TNF-α was seen at the 3-h time point. The levels of TGF-β and GM-CSF mRNAs remained unchanged at all time

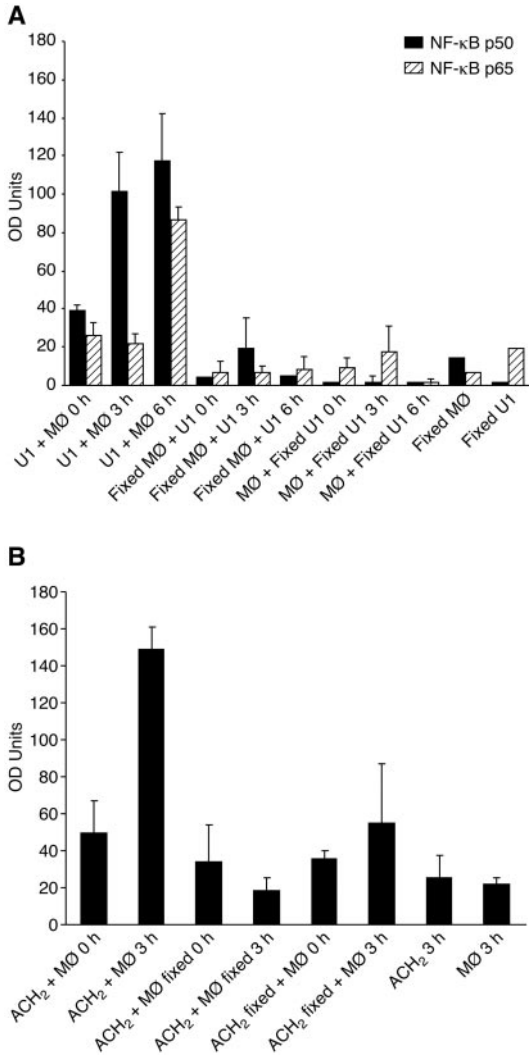
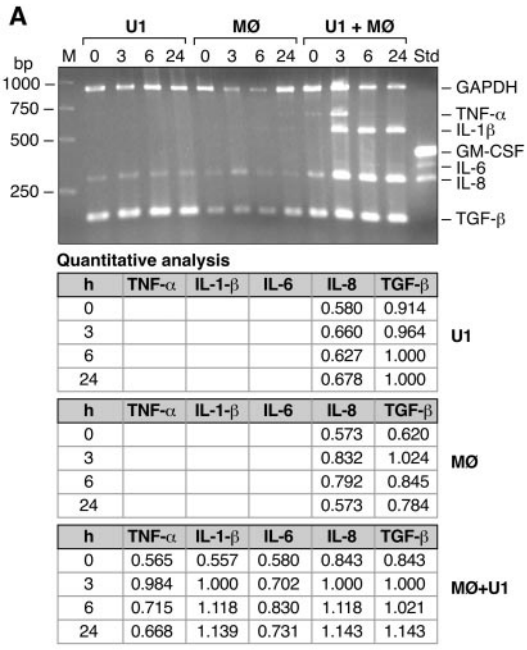


FIGURE 6. Determination of NF-κB in cocultures treated with paraformaldehyde. NF-κB p50 and p65 activities were determined using the BD Mercury transfactor kit. *A,* Fifteen micrograms of nuclear proteins isolated from Mϕ cocultured with U1 cells, Mϕ fixed with paraformaldehyde and cocultured with U1 cells, Mϕ cocultured with U1 cells fixed with paraformaldehyde, Mϕ fixed in paraformaldehyde alone, and U1 cells fixed in paraformaldehyde alone was used in each well. *B,* Fifteen micrograms of nuclear proteins isolated from Mϕ cocultured with ACH2 cells, Mϕ fixed with paraformaldehyde and cocultured with ACH2 cells, Mϕ cocultured with ACH2 cells fixed with paraformaldehyde, Mϕ fixed in paraformaldehyde alone, and ACH2 cells fixed in paraformaldehyde alone was used in each well. The assay was done in triplicate. Results expressed as mean ± SEM are representative of two independent experiments.

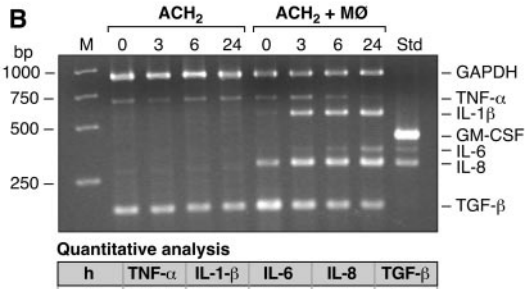


Quantitative analysis

h	TNF-α	IL-1-β	IL-6	IL-8	TGF-β	
0				0.580	0.914	
3				0.660	0.964	U1
6				0.627	1.000	
24				0.678	1.000	

h	TNF-α	IL-1-β	IL-6	IL-8	TGF-β	
0				0.573	0.620	
3				0.832	1.024	MØ
6				0.792	0.845	
24				0.573	0.784	

h	TNF-α	IL-1-β	IL-6	IL-8	TGF-β	
0	0.565	0.557	0.580	0.843	0.843	
3	0.984	1.000	0.702	1.000	1.000	MØ+U1
6	0.715	1.118	0.830	1.118	1.021	
24	0.668	1.139	0.731	1.143	1.143	



Quantitative analysis

h	TNF-α	IL-1-β	IL-6	IL-8	TGF-β	
0	0.524				0.955	
3	0.537				1.000	ACH2
6	0.574				1.054	
24	0.584				1.078	

h	TNF-α	IL-1-β	IL-6	IL-8	TGF-β	
0	0.564			0.868	1.123	
3	0.752	1.164	0.687	1.177	1.192	MØ
6	0.670	1.184	0.791	1.233	1.126	+
24	0.556	1.190	0.941	1.239	1.094	ACH2

FIGURE 7. Induction of proinflammatory cytokines. PCR products from amplification of cDNA derived from U1 cells alone, Mϕ alone, and U1 and macrophage coculture (*A*) and ACH2 cells alone and ACH2 and macrophage coculture (*B)*were separated on a 2% agarose gel and stained with ethidium bromide. M denotes molecular markers and Std denotes a positive control for the PCR. The data are representative of three independent experiments.

points tested (Fig. 7*B*). Neither the Mϕ alone nor the U1 and ACH2 cells alone showed any up-regulation of mRNAs for these cytokines (Fig. 7).

The effect of coculture of U1 or ACH2 cells with Mϕ on the induction of TNF-α was examined by ELISA. The results demonstrated that there was an increase in TNF-α levels in supernatants collected at 24 h which declined after 48 h (Fig. 8). Neither Mϕ alone nor the U1 and ACH2 cells alone showed any elevation of TNF-α levels at 24 h (Fig. 8)

Role of TNF-α in induction of HIV-1 replication

The RT-PCR results from the cocultures of U1 or ACH2 and Mϕ clearly demonstrated an up-regulation of mRNAs of the proinflammatory cytokines TNF-α, IL-1β, and IL-8. TNF-α is known to induce HIV-1 replication in chronically infected U1 cells (33). Thus, to study the role TNF-α in the induction of HIV-1 replication by coculture, U1 or ACH2 and Mϕ cocultures were treated with anti-TNF-α Abs or goat anti-rabbit IgG for 48 h, and the production of nascent cell-associated HIV-1 was determined by FACS analysis using Abs to HIV-1 p24. The FACS results confirmed that when U1 and ACH2 were cocultured, induction of HIV-1 replication occurred (Fig. 9, *A* and *C*). However, treatment with anti-TNF-α Ab considerably blocked the induction of HIV-1 replication as seen in Fig. 9 (*B* and *D*). Isotype-matched control IgG did not produce any shift in the relative fluorescence (Fig. 9) indicating the specificity of anti-p24 Ab.

In a similar manner, treatment of cocultures with anti-TNF-α Ab greatly reduced the amount of HIV-1 p24 detected in culture supernatants even after 48 h (Fig. 10*A*). However, treatment with anti-TRAIL Ab or normal goat anti-rabbit IgG did not have any effect on HIV-1 induction (Fig. 10*A*). Analysis of viable U1 and ACH2 cells from coculture with Mϕ revealed that the anti-TNF-α Ab treatment protected U1 and ACH2 cells from cytopathic effects caused by coculture (Fig. 10*B*). In contrast, considerable reduction in viable cells was detected in U1 or ACH2 cocultures treated with normal goat anti-rabbit IgG or anti-TRAIL Ab (Fig. 10*B*). Treatment of cocultures with neutralizing Abs against IL-1β, IL-6, IL-8, and M-CSF had no significant effect on the induction of HIV-1 replication (data not shown).

Effect of NF-κB inhibitors

Treatment of Mϕ and U1 or Mϕ and ACH2 cocultures with the NF-κB inhibitor BAY 11-0782 at 30 μM (40, 41) completely blocked the induction of NF-κB (Fig. 11). In addition, PCR analysis of cDNA derived from Mϕ and U1 or Mϕ and ACH2 cocultures treated with the inhibitor demonstrated a marked decrease in the induction of IL-1β (Fig. 12). A modest decrease in the induction of TNF-α and TGF-β was observed in U1 and Mϕ cocultures (Fig. 12*A*). However, only a marginal inhibition of TGF-β was observed with no change in the ongoing expression of TNF-α in Mϕ and ACH2 cocultures (Fig. 12*B*). Nevertheless, the induction of IL-8 expression was unaffected in Mϕ and U1 or Mϕ and ACH2 coculture (Fig. 12). Although only a modest decrease in the induction of TNF-α mRNA was observed when Mϕ and U1 or ACH2 cocultures were treated with NF-κB inhibitor BAY 11-7082, ELISA results of coculture supernatants treated with BAY 11-7082 demonstrated a profound decrease in TNF-α (Fig. 13). In a similar manner, treatment of Mϕ and U1 or Mϕ-ACH2 cocultures with this inhibitor substantially inhibited viral induction (Fig. 14).

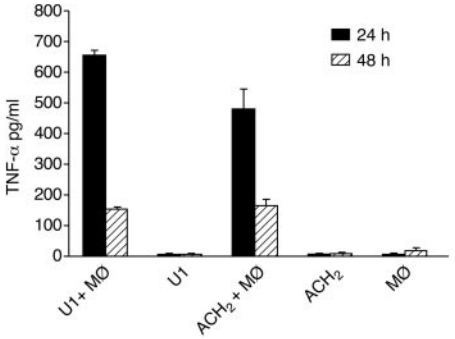


FIGURE 8. Induction of TNF-α. Briefly, 5×10^5 U1 and 1×10^6 monocytes or 5×10^5 ACH2 and 1×10^6 monocytes were cocultured at a ratio of 1:2, and culture supernatants were collected at 24 and 48 h. TNF-α levels in the culture supernatants were determined by ELISA. Culture supernatants were analyzed in triplicate. Results expressed as mean $\pm$ SEM are representative of three independent experiments.

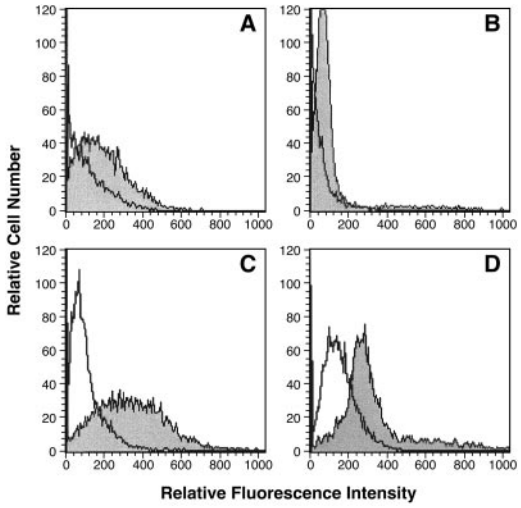


FIGURE 9. FACS analysis of nascent HIV-1 particles. *A,* Coculture of U1 and Mϕ treated with 100 μg/ml goat anti-rabbit IgG. *B,* Coculture of U1 cells and Mϕ treated with 100 μg/ml anti-TNF-α Ab. *C,* Coculture of ACH2 cells and Mϕ treated with 100 μg/ml goat anti-rabbit IgG. *D,* Coculture of ACH2 and Mϕ treated with 100 μg/ml anti-TNF-α Ab. The results are representative of three independent experiments.

Discussion

We have demonstrated that HIV-1 expression in U1 and ACH2 cells is markedly induced by coculture with PBMC, monocytes, and Mϕ. Several studies have documented the secretion of cytokines and other soluble factors by Mϕ in response to viral infection (42–46). To determine whether soluble factors were a source of the inductive stimuli, we cultured Mϕ and U1 or ACH2 cells in Transwell inserts separated by a permeable membrane. The results from these experiments clearly demonstrated a substantial but not complete reduction of HIV-1 replication when the two cell types were physically separated. Our data indicate that even though some interactions by soluble factors (e.g., cytokines) can occur, close proximity or contact of cells is required for full induction. These results are consistent with other investigations demonstrating that conditioned medium from Mϕ and other cell types did not induce viral replication in U1 and ACH2 cells to the same magnitude as that obtained by cell–cell contact (20, 27). The inductive effect required the presence of viable Mϕ, since we found that paraformaldehyde-fixed Mϕ did not induce viral replication. The failure of fixed Mϕ to induce HIV-1 replication may be due to the denaturation of membrane-bound TNF-α (47) or a ligand or cellular receptor interacting with a cognate receptor or ligand on the

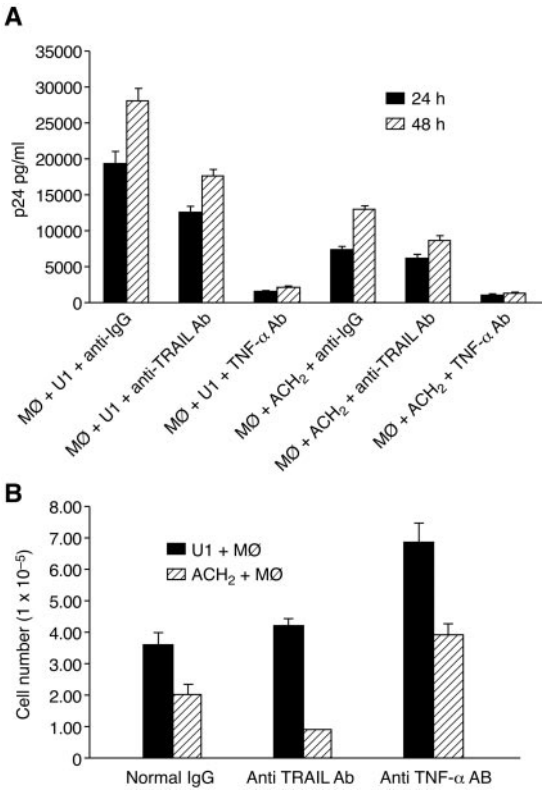


FIGURE 10. Effect of anti-TNF-α Ab treatment. U1 or ACH2 cells and Mϕ treated with 100 μg/ml goat anti-rabbit IgG, U1 or ACH2 cells and Mϕ treated with 100 μg/ml anti-TRAIL Ab, or U1 or ACH2 cells and Mϕ treated with 100 μg/ml anti-TNF-α Ab were cocultured at a ratio of 1:2, and culture supernatants were collected at 24 and 48 h. *A,* HIV-1 replication was determined by measuring HIV-1 p24 in culture supernatants by ELISA. Culture supernatants were analyzed in triplicate. *B,* The number of viable cells was determined by trypan blue staining and counting in a hemocytometer. Each sample was assayed in triplicate. Results expressed as mean $\pm$ SEM are representative of two independent experiments.

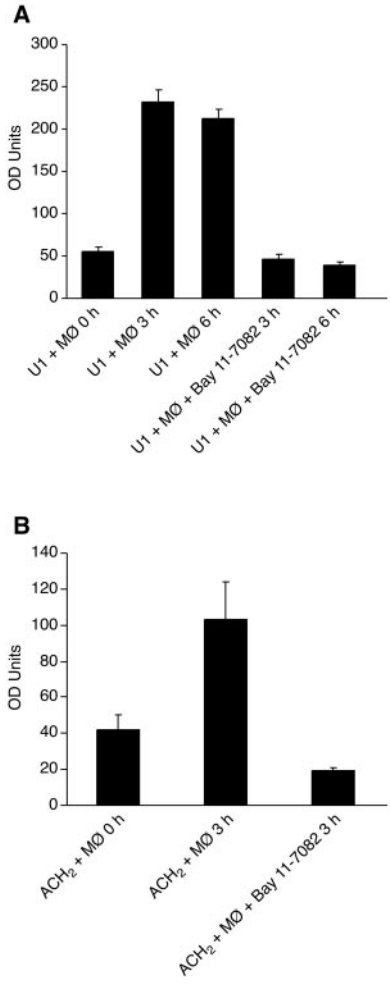
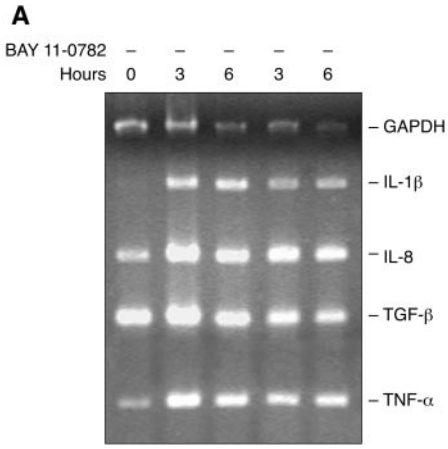


FIGURE 11. Determination of NF-κB in cocultures treated with BAY 11-0782. NF-κB p50 activity was determined using the BD Mercury transfactor kit. Fifteen micrograms of nuclear proteins per well isolated from U1 and Mϕ cocultures pretreated with 30 μM BAY 11-0782 (*A*) and ACH2 and Mϕ cocultures pretreated with 30 μM BAY 11-0782 (*B*). The assay was performed in triplicate. Results expressed as mean $\pm$ SEM are representative of two independent experiments.

U1 or ACH2 cells involved in triggering signals for activating HIV-1 replication.

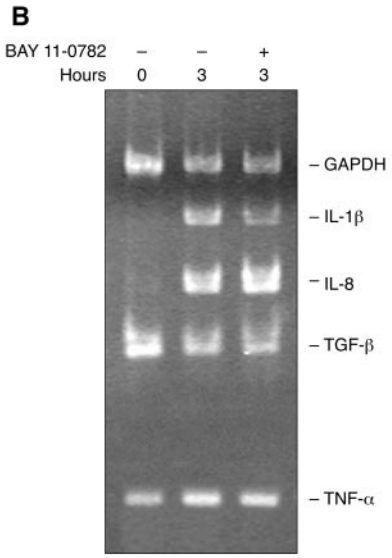
The life cycle of HIV-1 is closely associated with the activation state of its host cells. HIV-1 is dependent on a number of host factors for entry, transcription, and viral gene expression. The HIV-1 LTR contains at least six defined elements that bind cellular transcription factors (48). Among these, the best characterized is the NF-κB, which plays a central role in mediating inducible gene expression. Binding of the cellular transcription factor SP1 to three adjacent sites further enhances NF-κB activity (49). Apart from the activation of HIV-1, the family of NF-κB transcriptional activators is also involved in the transduction of immunological response, cellular differentiation, and cell growth.

The activation of NF-κB is a rapid immediate early event that occurs within minutes after exposure to relevant inducers, does not require de novo protein synthesis, and results in the strong transcriptional stimulation of several genes. Other factors, including cell type specificity, subcellular localization, differential regulation by IκB, and phosphorylation of Rel (50) play important roles in regulating gene expression. In most cell types, the NF-κB complex is sequestered in the cytoplasm by a family of inhibitory proteins (IκB). IκB binds to the NF-κB complex and masks the nuclear localization signal, thereby preventing the translocation of the NF-κB complex into the nucleus. Upon activation by various upstream signals, IκB is phosphorylated and degraded, releasing the NF-κB complex, which is then free to translocate to the nucleus where it can regulate gene expression. Cytokines such as IL-1 and TNF-α, phorbol esters, radical oxygen intermediates, and UV irradiation can rapidly stimulate NF-κB activation (50). In addition, NF-κB is also activated by viral infection. Many viral proteins like Tat and Tax are known to activate NF-κB. The activation of NF-κB mediates the immune response to the invading pathogens and in several instances promotes viral replication (51). The cell–cell contact of U1 or ACH2 cells with Mϕ in coculture may trigger transducing signals that result in the observed activation of the NF-κB p50/p65 subunits, which in turn can act on the NF-κB motif on the HIV-1 LTR for inducing viral replication. The persistent induction of NF-κB subunit p50 observed in this study indicates that there is continuous stimulation of the cells. Fixation of U1, ACH2, and Mϕ with paraformaldehyde disrupted this cooperative interaction and hence blocked activation of the NF-κB p50-p65 complex. Likewise, paraformaldehyde-fixed Mϕ failed to induce HIV-1 replication in U1 or ACH2 cells by coculture. Thus, taken together, our data suggest that cooperative interactions involving intimate cell association is essential for the activation of NF-κB and subsequent induction of HIV-1 replication.



Quantitative analysis

MØ + U1	IL-1-β	IL-8	TGF-β	TNF-α
0h	0.767	1.21	1.21	0.785
3h	1.24	1.33	1.33	1.33
6h	1.62	1.75	1.75	1.66
+ BAY 11-7802 3h	1.07	1.43	1.43	1.21
+ BAY 11-7802 6h	1.26	1.75	1.75	1.45



Quantitative analysis

MØ + ACH_2	IL-1-β	IL-8	TGF-β	TNF-α
0h			1.03	0.86
3h	1.13	1.29	1.16	1.26
+ BAY 11-7802 3h	0.98	1.31	1.18	1.26

FIGURE 12. Inhibition of cytokine induction by NF-κB inhibitors. PCR products from amplification of cDNA derived from U1 and Mϕ coculture pretreated with 30 μM BAY 11-0782 (*A*) and ACH2 and Mϕ coculture pretreated with 30 μM BAY 11-0782 (*B*) were separated on a 2% agarose gel and stained with ethidium bromide.

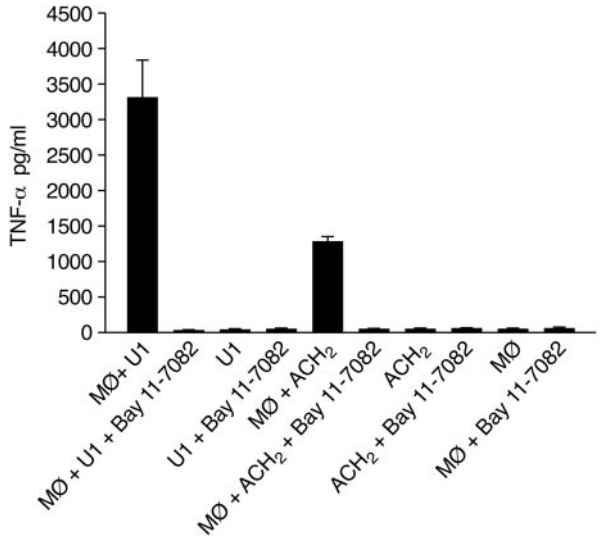


FIGURE 13. TNF-α levels in coculture supernatants treated with BAY 11-0782. TNF-α concentration was determined in culture supernatants derived from U1 and Mϕ coculture pretreated with 30 μM BAY 11-0782 and ACH2 and Mϕ coculture pretreated with 30 μM BAY 11-0782 by ELISA. The data are representative of three independent experiments.

Consistent with the activation of NF-κB, an induction of downstream proinflammatory cytokines was observed. RT-PCR results clearly demonstrated the induction of IL-1β, IL-6, IL-8, and TNF-α expression within 3 h of coculture. However, the expression of other cytokines like GM-CSF and TGF-β was not significantly induced. In addition, quantitation of TNF-α levels in culture supernatants clearly indicate high levels of TNF-α accumulation after 24 h. Several in vitro and in vivo studies have demonstrated that HIV-1 infection can induce the secretion and elevation of IL-1β, IL-6, and TNF-α (42–46, 52). Activated Mϕ secrete IL-1 β, IL-6, IL-8, and TNF-α during inflammatory reactions (9) that promote leukocyte recruitment and initiate the adaptive immune response. Consequently, the up-regulation of proinflammatory cytokines by both U1 and ACH2 when in contact with Mϕ may provide a mechanism for induction of HIV-1 replication in latently infected cells.

A direct correlation between elevated levels of these cytokines and viral load during the progression of HIV-1 infection indicate an important role of cytokines in the activation of NF-κB (53, 54). Up-regulated TNF-α can activate NF-κB within minutes and can induce apoptosis upon prolonged stimulation (55, 56). Soluble and the membrane-bound forms of TNF-α are known to enhance HIV-1 replication (57, 58) by the direct activation of NF-κB in T cells (18) and in Mϕ (43). Although IL-1β and IL-6 are also known to induce HIV-1 replication, their inductive capacity is much lower than that of TNF-α. Unlike TNF-α, the mechanism of action of IL-1β and IL-6 for the induction of HIV-1 replication is mediated by an alternative pathway independent of NF-κB activation (17). The role of IL-8 in HIV-1 pathogenesis is not clear. IL-8 does not induce HIV-1 replication in U1 cells (59), but primarily promotes the recruitment of leukocytes to areas of inflammation and chemotaxis.

The treatment of U1 or ACH2 and Mϕ cocultures with anti-TNF-α Ab had a profound effect on the production of nascent HIV-1 particles, indicating that TNF-α plays a major role in the induction of HIV-1 replication in U1 and ACH2 cells during coculture with Mϕ. Consistent with these findings, treatment of U1-Mϕ or ACH2-Mϕ cocultures with NF-κB inhibitor demonstrated a complete inhibition of the induction of NF-κB, with a concomitant down-regulation of the proinflammatory cytokines IL-1β and TNF-α. The inhibition of NF-κB activation and the down-regulation of NF-κB-induced cytokines TNF-α and/or IL-β resulted in complete inhibition of HIV-1 production (Fig. 14), indicating that NF-κB and cytokines play a pivotal role in the reactivation of HIV-1 in latently infected cells by Mϕ.

Some members of the TNFR superfamily are also known to play an important role in inducing the activation of HIV-1 replication. Recent studies have demonstrated that the activation of the TNFR superfamily of proteins, including TNFR1, TNFR11, CD30,

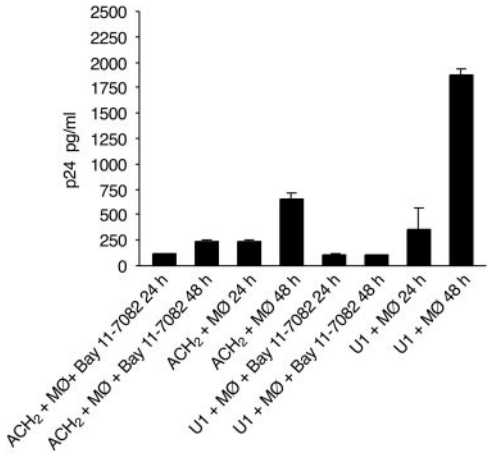


FIGURE 14. Effect of NF-κB inhibitors on HIV-1 induction. ACH2, U1, and Mϕ were pretreated with 30 μM BAY 11-0782 before placing them in coculture as described above. Culture supernatants were collected at 24 and 48 h, respectively. HIV-1 p24 production in culture supernatants was determined by ELISA. Culture supernatants were analyzed in triplicate. Results expressed as mean ± SEM are representative of two independent experiments.

4-1BB, CD40, and OX40, activate signal transducers that are responsible for the activation of NF-κB (46, 60–62). The triggering of CD30 can also induce HIV-1 replication through the NF-κB pathway in chronically infected T cell lines (63). Cross-linking of 4-1BB with agonistic mAbs significantly enhances HIV-1 replication in $CD4^+$ T cells (64). Similarly, the stimulation of CD40 in the presence of IL-4 and IL-2 up-regulates HIV-1 replication in B cells (65). Thus, TNF-α and its receptors play a central role in the induction of HIV-1 replication by the activation of NF-κB. Since the TNF-α promoter also contains NF-κB binding sites, TNF-α can positively up-regulate its own synthesis, leading to the persistence of infection (66).

In summary, our results indicate that in Mϕ grown in coculture, close cell interactions generate intracellular signals that activate the NF-κB p50-p65 complex. The active forms of the NF-κB subunits p50 and p65 are then translocated into the nucleus, where they can induce the expression of proinflammatory cytokines. When these cytokines are secreted into the culture medium, they can in turn act upon the U1 or ACH2 cells triggering the signal transduction cascade leading to the activation and translocation of NF-κB to the nucleus, where it can act upon the HIV-1 LTR inducing viral replication. In addition, the activated NF-κB can upregulate the transcription of proinflammatory cytokines which, when secreted into the culture medium, can act as an autocrine inducer of cell signaling in U1 or ACH2 cells. The persistent activation of NF-κB supports continuous activation of the cells in the coculture system. The exact mechanism of the induction of HIV-1 replication in U1 or ACH2 cells by Mϕ is not known at present. Future studies aimed at understanding the molecular mechanisms involved in the reactivation of HIV-1 from latently infected cells would help us to understand further the pathogenesis of this disease.

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