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# HIV-1 induces tumour necrosis factor and IL-1 gene expression in primary human macrophages independent of productive infection

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## SUMMARY

Cytokines such as tumour necrosis factor-alpha (TNF-α) and IL-1β may play a role in immunopathogenesis of AIDS. We studied early effects (0·5–48 h) of monocytotropic (ADA) or lymphotropic (IIIB) strains of HIV-1 on TNF-α and IL-1β mRNA expression in primary human macrophages by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Three-day-old monocyte-derived macrophages were exposed either to tissue culture supernatants containing virus (at multiplicity of infection (m.o.i.) of 0·05) or to control supernatants free of virions and gp120. ADA strain, but not IIIB, replicated in primary tissue culture-differentiated macrophages (TCDM). Soluble CD4 (sCD4) was used to inhibit binding of both strains to macrophages. We found that TNF-α and IL-1β gene expression was induced by both strains 0·5–3 h after addition of virus, and that enhanced expression of both cytokines was inhibited by sCD4. We conclude that CD4-dependent binding to the cell surface is sufficient to enhance TNF-α and IL-1β mRNA, whereas productive viral replication in primary human macrophages is not required. Therefore, similar pathways regulate gene expression of TNF-α and IL-1β by macrophages during initial infection by HIV-1 *in vitro*.

**Keywords** HIV macrophages tumour necrosis factor-alpha reverse transcriptase-polymerase chain reaction IL-1β

## INTRODUCTION

The macrophage plays a central role in AIDS pathogenesis, as target cell for HIV infection and indirectly following CD4 T cell infection and dysregulation [1–3]. Macrophages are known to be versatile secretory cells able to release a great variety of high and low molecular weight products that contribute substantially to host defence and inflammation as well as tissue injury, by acting on systemic and local cells in their environment [4]. Cytokines represent a heterogeneous group of mediators acting on and produced by macrophages, and have been implicated in many aspects of HIV–host interactions. Although it is likely that macrophages contribute substantially to cytokine release in different stages of the disease after HIV infection, the pathways by which cytokine gene expression is activated are obscure, nor is it known how coordinately different cytokines are regulated. Cytokine production in macrophages is controlled by complex transcriptional and translational mechanisms influenced by the state of cell priming, e.g. by interferon-gamma (IFN-γ) as well as by triggers acting via selected plasma membrane receptors, e.g. for Fc portions of IgG. The best characterized stimuli that trigger cytokine release by primed macrophages include lipopolysaccharide (LPS), microorganisms and particulate agents such as mycobacteria and zymosan, and immune complexes [5]. Direct action of viruses as triggering agents is less well characterized, nor have effects of different viruses on macrophage priming been clearly defined [6]. Proinflammatory cytokines tumour necrosis factor-alpha (TNF-α) and IL-1β have been reported to increase HIV-1 replication in monocyte-derived macrophages through activation of NF-kB [7]. High levels of TNF-α and IL-1β reported to be present in sera or peripheral blood mononuclear cell (PBMC) culture supernatants of HIV-infected individuals [8–12] can cause fever, loss of appetite and cachexia [13,14], and may therefore contribute to the catabolic state observed in AIDS patients.

The development of the reverse transcriptase-polymerase chain reaction (RT-PCR) has made it possible to examine expression of several cytokine genes at the same time, using relatively small amounts of material from human macrophages in culture. We selected TNF-α and IL-1β as well characterized proinflammatory cytokines for study. A semi-quantitative RT-PCR was used to define the role of HIV-1 strain tropism and viral binding to CD4 in expression of TNF-α and IL-1β mRNA

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by primary macrophages. We show here that TNF-α and IL-1β mRNA are closely regulated during acute HIV-1 infection of primary tissue culture-differentiated macrophages (TCDM) *in vitro*, depending on virus binding to cell surface and irrespective of viral replication within macrophages.

## MATERIALS AND METHODS

*Isolation and cultivation of TCDM*

PBMC were obtained by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) separation of buffy coats from HIV⁻ volunteers (John Radcliffe Hospital, Oxford, UK). PBMC were washed three times in PBS, resuspended in 20 ml serum-free medium (X-Vivo, Whittaker M.A. Bioproducts, Walkersville, MD), and placed in 75-cm² tissue culture flasks (Costar, Cambridge, MA) previously coated with 2% gelatin [15]. After incubation for 1 h at 37°C, non-adherent cells, >90% of which were peripheral blood-derived lymphocytes (PBL), were removed and cultivated in RPMI 1640 medium (GIBCO, Paisley, UK) with 10% v/v pooled human serum. The adherent cells were washed three times with PBS and then incubated in serum-free medium at 37°C. After 48 h, the adherent cells were detached by gentle agitation, resuspended in serum-free medium and cultivated for further assays in replicate 35-mm Petri dishes (Nunc, Paisley, UK) at a concentration of $2 \times 10^6$ cells/ml for 1 day at 37°C. After initial purification, adherent cells were >95% monocytes as determined by surface markers [15].

*U937 monocytoid cells*

U937 cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Sera Lab, Crawley Down, UK), 1% non-essential amino acids (GIBCO), and 1 mM sodium pyruvate (GIBCO).

*HIV-containing and virus-free preparations*

TCDM were isolated from the peripheral blood of a healthy adult. One half of the TCDM was infected with an HIV-1 strain ADA originally isolated and passaged in monocytes [16]. After 2 weeks of infection with the ADA strain in RPMI 1640 and 10% pooled human serum, the TCDM were recovered by scraping with a rubber policeman, spun at 800 ***g*** for 10 min at 4°C, and the supernatant harvested to yield a stock preparation of ADA. The other half of the TCDM was handled in the same way without infection, and was used to provide supernatants for 'mock-infected' controls.

The HIV-1 lymphotropic strain HIV-IIIB [17] was grown in the C8166 cell line in RPMI 1640 supplemented with 10% FCS. After 2 weeks the cells were spun at 800 ***g*** for 10 min at 4°C and the supernatant harvested to provide a IIIB viral stock. ADA and IIIB preparations were titrated by end point dilution on primary TCDM and C8166 cells, respectively. The multiplicity of infection (m.o.i.) used was 0·05 for both ADA and IIIB strains.

Supernatants free of infectious virus and of gp120 were obtained by spin-filtration of the ADA and IIIB preparations through a 100-μm filter (Millipore, Watford, UK) at 2750 ***g*** for 30 min. Amounts of virus-free fluid used in our experiments were comparable to those used with the whole viral preparations.

*HIV infection of TCDM*

For each virus strain, cells were harvested at 0·5, 1, 2, 3, 4, 6, 12, 24, and 48 h after infection or exposure to virus-free fluid. Cells harvested at 0·5 h were not washed; all other preparations were washed 1 h after infection and cultivated with fresh serum-free medium.

*RNA extraction and reverse transcription*

Total cellular RNA was extracted at different times after viral or mock treatment. The TCDM of each Petri dish were recovered by scraping with a rubber policeman, spun for 10 min at 4°C and the pellet washed twice with PBS at 4°C. RNAzol* (Cinna/Biotex Lab, Houston, TX) was added to the pellet, 1 ml per $10^7$ cells, together with 20 μg of *Escherichia coli* ribosomal RNA as carrier (Boehringer, Mannheim, Germany). RNA was recovered by chloroform/ethanol precipitation [18]. The reverse transcription reaction was performed in a final volume of 50 μl using 800 U M-MLV reverse transcriptase (GIBCO-BRL, Paisley, UK), 2·5 mM dNTP (Pharmacia), 31 U RNAse inhibitor (Pharmacia), and 100 ng/μl oligo dT12–18 at 37°C for 1 h. The cDNA obtained was stored at −20°C.

*Amplification of cDNA by the PCR*

PCR primer pairs were synthesized by Dr K. Gould at the Dunn School of Pathology (Oxford, UK) as follows: hu β actin (5′ primer: 5′-GATGCAGAAGGAGATCACTG-3′; 3′ primer: 5′-AGTCATAGTCCGCCTAGAAG-3′; size of amplified fragment: 205 bp), hu IL-1β (5′ primer: 5′-CAGAGAGTCCTGTGCTGAAT-3′; 3′ primer: 5′-GTAGGAGAGGTCAGAGAGGC-3′; size of amplified fragment: 235 bp), hu TNF-α (5′ primer: 5′-TCTCGAACCCCGAGTGACAA-3′; 3′ primer: 5′-TATCTCTCAGCTCCACACCA-3′; size of amplified fragment: 124 bp). cDNA (1 μl) was amplified in a final volume of 50 μl in the presence of 7·5 μM of each 5′ and 3′ primer and PCR master mix (0·25 mM of each dNTP (Pharmacia), 1 U of Taq polymerase (Boehringer), 50 mM KCl, 10 mM Tris-HCl (pH 8·4), 2·5 mM $MgCl_2$). The reaction mixture was overlaid with a drop of sterile light mineral oil (Sigma, Poole, UK), and the PCR performed using an MJ Research programmable thermal controller (Genetic Research Instrumentation Ltd, Felsted, UK) operating the following temperature steps: 60 s at 94°C, then 30 cycles (30 s denaturation at 94°C; 30 s annealing at 60°C; 60 s extension time at 72°C). Forty microlitres of the reaction mix were removed and separated at 150 V for 1 h in 2% agarose in 1 × Tris-buffer-EDTA (TBE) containing 1 μg/ml ethidium bromide (Sigma). A 123-bp DNA ladder (GIBCO-BRL) was run in parallel to provide molecular weight markers. A UV transilluminator was used to view and photograph the gels. Specificity of the amplified bands was validated by their predicted size. Differences in intensity of PCR bands were confirmed by further dilution of cDNA samples. All results shown are representative of three to five independent experiments.

*HIV-1 detection by ELISA for p24 antigen*

Viral replication was detected by assay of p24 levels in culture supernatants as described [19].

*Soluble CD4*

Soluble CD4 (40 μg/ml; MRC-AIDS Directed Programme, Potters Bar, UK) was incubated with either whole viral preparations (m.o.i. = 0·05) or virus-free fluids for 1 h at 37°C

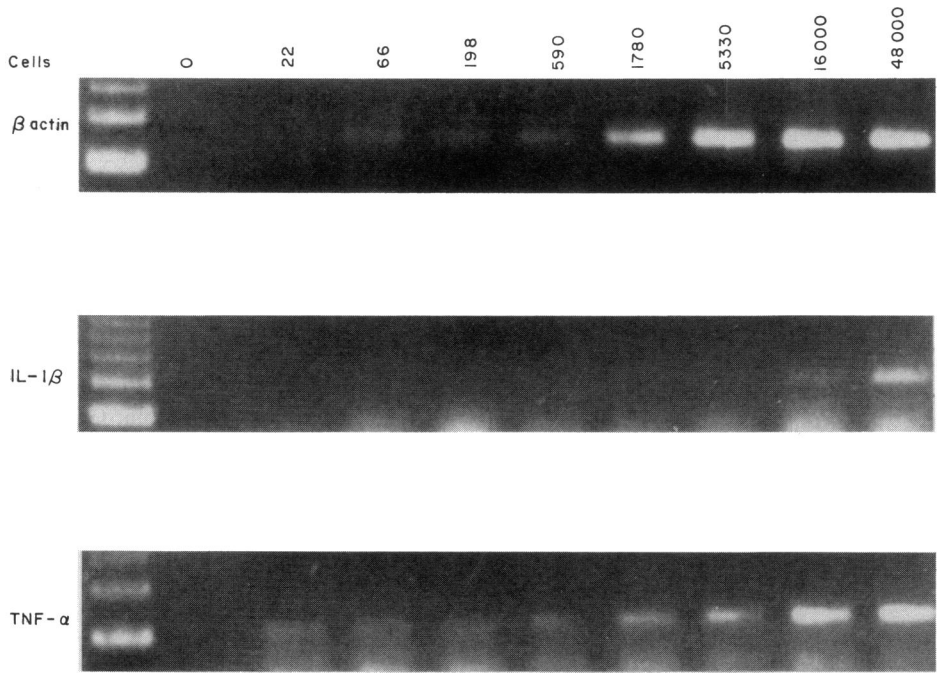


**Fig. 1.** Cytokine mRNA detection in unstimulated U937 cells is proportional to cell input. A 1:3 sequential dilution was performed on unstimulated U937 cells. A reverse transcriptase-polymerase chain reaction (RT-PCR) assay was used. Total cellular RNA was extracted from $3 \times 10^6$ to 1370 cells with 20 μg *Escherichia coli* ribosomal RNA carrier and reverse transcribed using oligo(dT) as a primer. cDNA was PCR amplified for 30 cycles with specific primer pairs. Numbers shown represent actual cellular input analysed per lane. The limit of detection of IL-1β mRNA was approximately 16 000 cells per assay and fewer than 22 cells for tumour necrosis factor-alpha (TNF-α). A 123-bp DNA ladder was run in parallel as molecular weight marker.

before addition to the 3-day-old TCDM and left throughout the period of incubation.

*Morphology*

Three-day-old TCDM were cultivated in serum-free medium. TCDM were either uninfected or exposed to ADA or IIIB whole viral preparations or virus-free fluids for 10 days. At day 10, supernatants were collected to determine p24 levels and cell monolayers were observed and photographed by phase contrast microscopy.

## RESULTS

The RT-PCR assay provides a potentially powerful tool to study effects of HIV-1 infection on cytokine gene expression in mononuclear phagocytes. It is possible to measure mRNA levels and kinetics of expression for a range of cytokines in replicate cells derived from a single blood donor under controlled conditions. Proinflammatory cytokines, TNF-α and IL-1β, were selected for study in view of their potential importance in relation to HIV–macrophage interactions.

Figure 1 shows that specific RT-PCR signals could be detected for TNF-α and IL-1β in U937 monocytoid cells even in the absence of an inducing stimulus such as LPS. As shown in serial dilution assays, the level of detection for each set of cytokine probes chosen varied, and could be related to the cell concentration used to prepare cDNA by reverse transcription. Approximately 16 000 U937 cells yielded a reproducible signal for IL-1β, whereas TNF-α mRNA could be detected in fewer than 22 cells, very similar to the sensitivity of the control β actin probe. Similar results were obtained with another monocytoid cell line, THP-1 (data not shown). Routinely, RNA extractions of primary TCDM were performed with $2 \times 10^6$ total cells (equivalent to 32 000 cells per lane analysed), an amount of cell extract cDNA corresponding to the exponential part of the PCR amplification curve. TNF-α and IL-1β mRNAs were detectable from $2 \times 10^6$ total cells in unstimulated or HIV-infected TCDM as described below. Taken together, these results suggest that

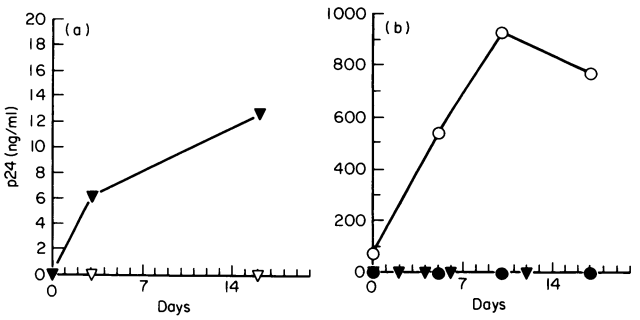


**Fig. 2.** Growth properties of HIV-1 and virus-free/gp120-free fluids in primary blood mononuclear cells. p24 was measured by ELISA (see Materials and Methods). (a) Three-day-old tissue culture-differentiated macrophages (TCDM) were exposed to the ADA whole viral preparation (▼) or to ADA-free fluid (▽). (b) Peripheral blood lymphocytes (PBL) were stimulated for 48 h with phytohaemagglutinin A (5 μg/ml; Sigma) and maintained in the presence of IL-2 (50 NIH/BRMP U/ml; Pharmacia) before exposure to IIIB whole viral preparation (○) or to IIIB-free fluid (●). When 3-day-old TCDM were exposed to IIIB whole viral preparation (▼), no productive infection was detected. A multiplicity of infection (m.o.i.) of 0·05 was used.

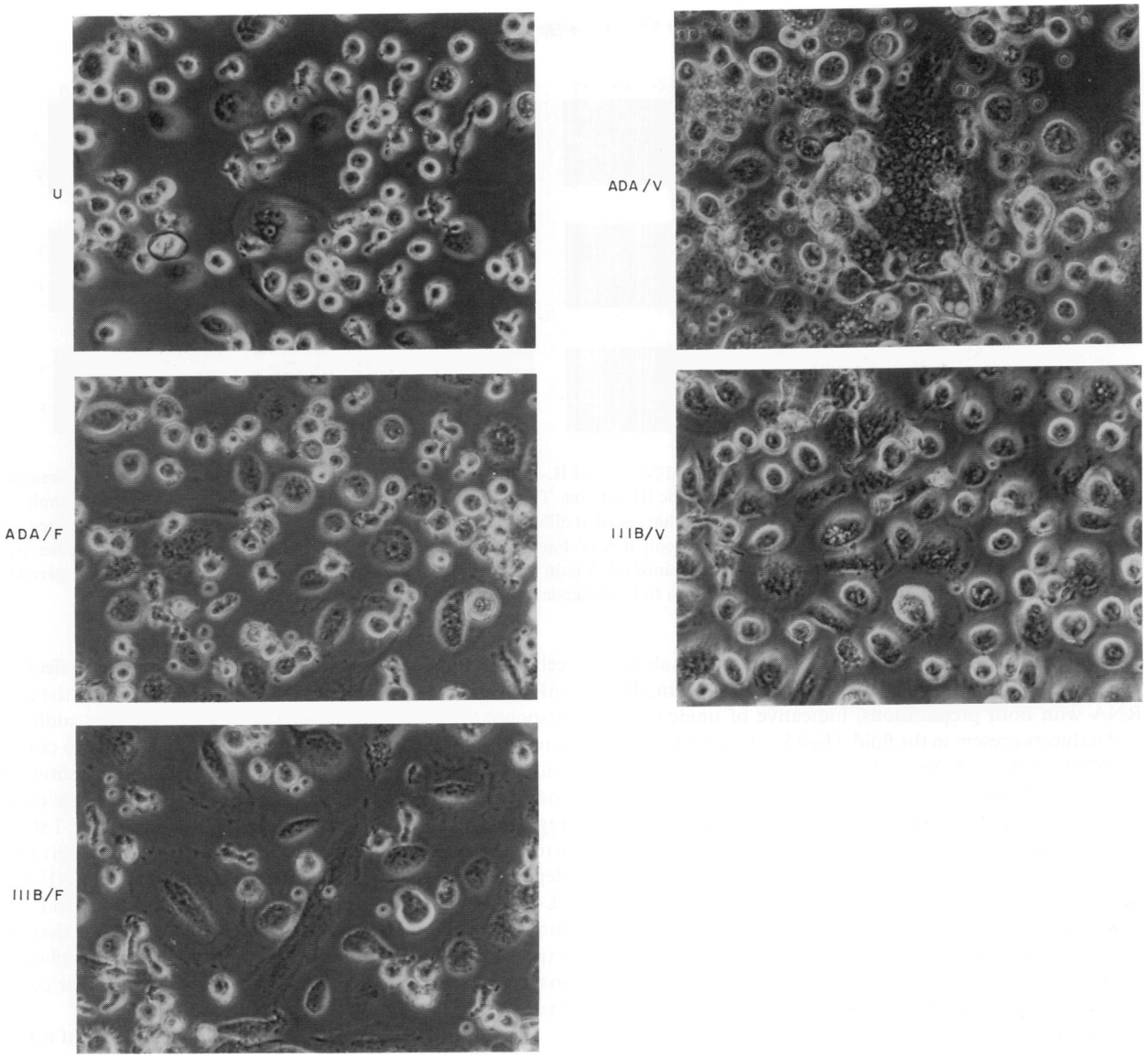


**Fig. 3.** Syncytia formation in primary tissue culture-differentiated macrophages (TCDM) depends on tropism and whole virus preparation. Three-day-old TCDM were cultivated in serum-free medium. TCDM were either uninfected (U) or exposed for 10 days to ADA whole viral preparation (ADA/V), ADA-free fluid (ADA/F), IIIB whole viral preparation (IIIB/V), or IIIB-free fluid (IIIB/F) at a multiplicity of infection (m.o.i.) of 0·05. Multinucleated giant cells were observed only in TCDM infected with the ADA total preparation. Phase contrast microscopy, × 70.

RT-PCR provides a semi-quantitative assay with high sensitivity as a powerful tool to study cytokine gene expression in HIV-1-infected human TCDM.

To determine the pathway involved in TNF-α and IL-1β gene expression after acute infection of primary TCDM, we used two HIV-1 strains with different tropism. In our study the ADA monocytotropic and IIIB lymphotropic HIV-1 strains productively infected TCDM and peripheral blood lymphocytes respectively, whereas IIIB virus did not productively infect human primary TCDM (Fig. 2). To determine the direct effect of the virus on cytokine gene expression it was essential to obtain a control without infectious virus or gp120. Spin-filtration of whole viral preparations (respectively ADA and IIIB) through a 100-μm filter removed infectious virus from the filtrate (Figs 2a,b). Moreover, the gp120 content in both virus-free fluids was below the level of detection (10 ng/ml) in an ELISA assay (data not shown). Syncytia formation occurred only with ADA whole viral preparation (ADA/V), but neither with IIIB whole viral preparation (IIIB/V) nor with virus-free fluids (ADA/F, IIIB/F) (Fig. 3). High levels of p24 were detected in culture supernatants of TCDM infected with ADA whole viral preparation, but were never present in supernatants from TCDM exposed to IIIB whole viral preparation and virus-free fluids (data not shown).

By using RT-PCR, we studied expression of TNF-α and IL-1β genes in human primary TCDM infected with either the ADA monocytotropic or the IIIB lymphotropic HIV-1 strain. Both ADA and IIIB HIV-1 strains up-regulated TNF-α and IL-1β gene expression in human TCDM (Fig. 4), irrespective of their ability to infect these macrophages productively or not. In uninfected (U) human primary TCDM only low steady-state levels of TNF-α and IL-1β mRNA were detected. The virus-free

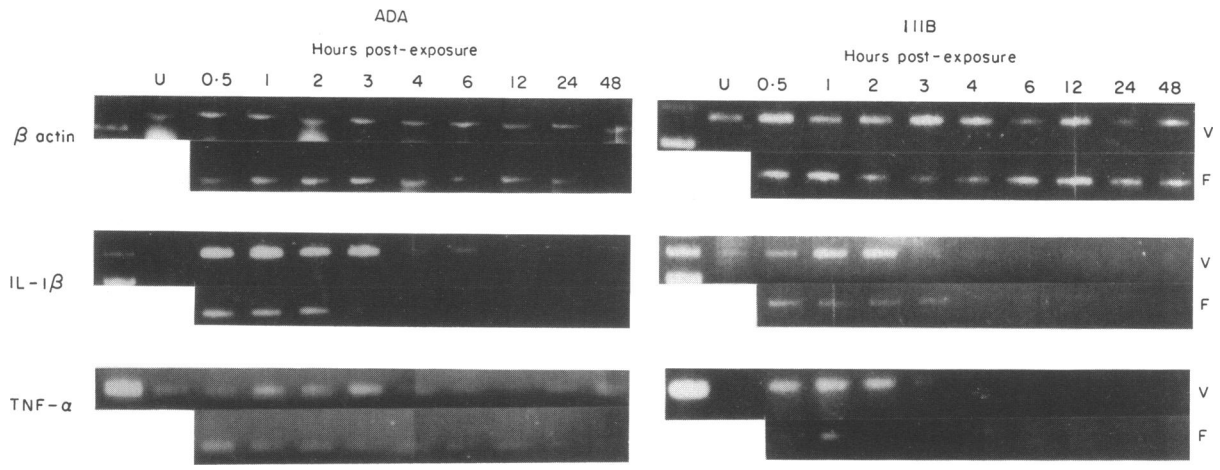


**Fig. 4.** Kinetics of induction of tumour necrosis factor-alpha (TNF-α) and IL-1β mRNAs after exposure of 3-day-old tissue culture-differentiated macrophages (TCDM) to monocytotropic ADA or lymphotropic IIIB strains. Three-day-old TCDM were uninfected (U), infected by the whole viral preparation (V), or exposed to virus-free fluid (F). TCDM were harvested at different times after exposure and total cellular RNA was extracted from $2 \times 10^6$ cells and reverse transcribed as described. cDNA was polymerase chain reaction (PCR) amplified for 30 cycles with specific primer pairs. Amplified fragments were visualized on an ethidium bromide-stained gel. β actin was used as an internal control. A 123-bp ladder was run in parallel as molecular weight marker. Comparable results were obtained in five independent experiments.

fluids (F), prepared and characterized as described above, induced only a slight and transient expression of TNF-α and IL-1β mRNA with both preparations, indicative of unidentified non-viral inducers present in the fluid. High levels of TNF-α and IL-1β mRNA were induced by both monocytotropic and lymphotropic HIV-1 whole viral preparations (V) from 0·5 h to 3 h after the input, and then decreased to control levels. β actin was used as an internal standard. Exposure of TCDM to mock-infected preparations did not show any difference in TNF-α and IL-1β mRNA expression during the period post-exposure (data not shown). By comparison, exposure of TCDM to recombinant glycosylated HIV-1 gp120 enhanced TNF-α and IL-1β mRNA expression at 1 h after the input (data not shown).

To determine more precisely the pathway implicated in cytokine gene expression, soluble CD4 (sCD4) was used to block the induction of TNF-α and IL-1β mRNAs (Fig. 5). β actin was used as an internal standard. For both HIV-1 strains the effect of sCD4 was investigated at 2 h and 12 h after infection. As described above, TNF-α and IL-1β mRNAs were up-regulated at 2 h after infection and decreased by 12 h after infection. Addition of sCD4 to the whole viral preparation, either to the monocytotropic ADA or to the lymphotropic IIIB strains, blocked induction of TNF-α and IL-1β mRNAs observed at 2 h post-infection. Soluble CD4 totally blocked TNF-α mRNA expression. Approximately two-thirds of the enhanced IL-1β mRNA induced at 2 h after infection were inhibited by sCD4 as demonstrated by comparing three-fold dilution titrations of cDNA (data not shown). These results demonstrated that enhanced TNF-α and IL-1β gene expression is substantially CD4-dependent in HIV-1-infected human primary TCDM.

## DISCUSSION

We have used a semi-quantitative RT-PCR assay to compare expression of proinflammatory cytokines TNF-α and IL-1β by primary TCDM from healthy donors following treatment with cell-free HIV-1 of defined tropism. We examined effects on mRNA, rather than protein, to obtain kinetic data on the direct response of the cells to virus. HIV was used without additional stimuli such as IFN-γ, LPS or phagocytosis, and we concentrated on initial (0–48 h) responses to binding and infection. Our main findings were: (i) TNF-α and IL-1β mRNAs were closely regulated, induced early and transiently by both HIV-1 strains irrespective of viral replication within macrophages; (ii) CD4-dependent binding to the cell surface played a role in TNF-α and IL-1β induction by both monocytotropic (ADA) and non-monocytotropic (IIIB) strains. These results suggest that cells exposed to HIV-1 strains of different tropism, able to replicate in macrophages or not, can influence properties of other cells in their vicinity, including their response to HIV.

The RT-PCR assay provided a sensitive measure of mRNA in macrophages, and was ideal for kinetic analysis and comparison of matched cell preparations from a single donor. To our knowledge this is the first PCR-based kinetic study of TNF-α and IL-1β mRNA expression in macrophages in relation to acute HIV-1 infection. It should be noted that different primer pairs may vary in efficiency of amplification, so that detection of various cytokines at different threshold levels may not reflect absolute differences in the mRNA content for each cytokine. In serum-free medium the background for cytokine mRNA detection by RT-PCR was lower than in serum supplemented cultures (data not shown). The serum-free medium (Fig. 3) consistently yielded fully viable monocytes which adhered, spread and differentiated into macrophages, without lipid accumulation, a hallmark of cultivation in human serum. HIV-1 growth and virus-induced syncytium formation were reduced under serum-free conditions (data not shown), perhaps because of slower differentiation [20,21] or the absence of serum-factors that might potentiate HIV replication [21,22]. Finally, our cultures contained >95% macrophages, with virtually no T and B lymphocytes present. In order to control for other non-viral modulatory factors in the viral stocks, possibly macrophages or lymphocyte-derived cytokines, we devised a spin-filtration

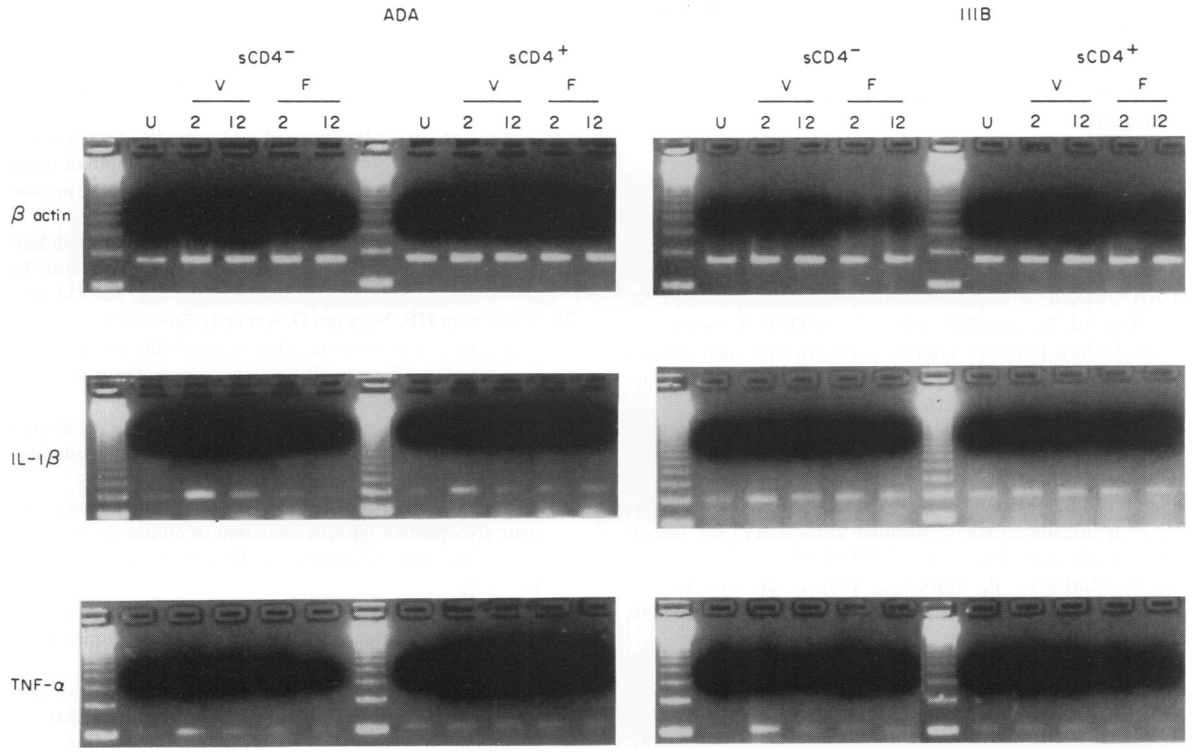


**Fig. 5.** Soluble CD4 (sCD4) prevents induction of tumour necrosis factor-alpha (TNF-α) and IL-1β mRNAs in HIV-1 exposed tissue culture-differentiated macrophages (TCDM). Three-day-old TCDM were uninfected (U) or exposed either to monocytotropic ADA or lymphotropic IIIB strains (V), or corresponding viral-free fluids (F). For each strain, two different times after exposure (2 h and 12 h) were assayed in serum-free medium at 37°C. Soluble CD4 (40 μg/ml) was incubated with either the whole viral preparation (V) or the virus-free fluid (F) at 37°C for 1 h before addition to the TCDM for the remainder of the incubation period. At each time total cellular RNA was extracted from $2 \times 10^6$ cells with 20 μg *Escherichia coli* ribosomal RNA as carrier and reverse transcribed using oligo(dT) as a primer. cDNA was polymerase chain reaction (PCR) amplified for 30 cycles with specific primer pairs and amplified fragments were visualized on an ethidium bromide-stained gel. β actin was used as an internal standard, and a 123-bp DNA ladder was run in parallel as molecular weight marker.

method to separate virions from virus-free supernatants, and these were shown also to be free of gp120 antigen, a potential trigger for cytokine induction in primary TCDM [23,24].

Once the above variables had been defined, it became apparent that both the ADA and IIIB strains of HIV-1 induced early and transient (0·5–3 h) expression of TNF-α and IL-1β mRNAs. Previous studies [25] showed enhanced TNF-α and IL-1β proteins 2–12 h and 2–6 h, respectively, after exposing TCDM to monocytotropic HIV-$1_{JR\text{-}FL}$ strain. The kinetics of up-regulation of TNF-α and IL-1β mRNAs by HIV-1 are similar to those observed with other stimuli, such as LPS, phorbol myristate acetate (PMA), or Sendai virus [5,6,26–28].

Control experiments, also reported elsewhere [29,30], have analysed in detail the kinetics of provirus formation in TCDM during the first cycle of infection up to 36 h post-infection. HIV-LTR detection started at 8 h after infection of primary TCDM with the ADA monocytotropic strain [31]; in contrast, no HIV-LTR was detected in the TCDM after exposure to IIIB lymphotropic strain [32]. The lymphotropic IIIB strain which does not enter TCDM [32] and does not replicate in TCDM as shown above, was able to up-regulate both TNF-α and IL-1β mRNAs at 0·5–3 h after infection. Taken together these data suggest that TNF-α and IL-1β mRNA induction is independent of HIV-1 entry and replication in TCDM.

Recombinant HIV-1 gp120, but not mock-infected preparations, induced the expression of TNF-α and IL-1β mRNAs in TCDM, underlining the role of virus–cell surface interactions in TNF-α and IL-1β gene expression. To test the hypothesis that viral binding to the TCDM surface is sufficient to account for a signal transduction pathway resulting in TNF-α and IL-1β gene expression, we used sCD4 which blocks HIV-1 entry into TCDM [29,33]. Soluble CD4 had no effect on its own, but prevented a substantial component of TNF-α and IL-1β mRNA transcription induced by both strains of HIV-1. These results ruled out contaminant LPS in the viral stocks as a possible inducer of cytokine mRNA. Others have also used sCD4 to inhibit TNF-α and IL-1β protein production in HIV-infected monocytes [25,34]. Further studies are needed to define the contribution of CD4 to virus binding and the role of CD4 in signal transduction of enhanced transcription of proinflammatory cytokine mRNA. Binding of MoAbs to other surface glycoproteins (LFA-3, CD44, CD45) has also been reported to induce TNF-α and IL-1β in primary human monocytes [35].

Some other studies have not observed TNF-α and IL-1β induction after HIV infection of peripheral blood monocytes or monocytic cell lines [36–39]. Differences in cell preparations and experimental conditions may account for discrepant results. Regulation of proinflammatory cytokines by HIV-1 interaction with macrophages may be modified profoundly by further exposure to activating (e.g. IFN-γ) or deactivating (e.g. IL-10) lymphokines [40], to LPS, phagocytic and other infectious agents. The present study has analysed *in vitro* some of the initial

responses of unstimulated primary macrophages to two HIV-1 strains with distinct tropism. The effects of chronic infection on expression of TNF-α and IL-1β genes in primary macrophages also need further study, to delineate more accurately their role in AIDS pathogenesis.

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