Maximal HIV-1 Replication in Alveolar Macrophages during Tuberculosis Requires both Lymphocyte Contact and Cytokines

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Abstract

HIV-1 replication is markedly upregulated in alveolar macrophages (AM) during pulmonary tuberculosis (TB). This is associated with loss of an inhibitory CCAAT enhancer binding protein β (C/EBPβ) transcription factor and activation of nuclear factor (NF)-κB. Since the cellular immune response in pulmonary TB requires lymphocyte–macrophage interaction, a model system was developed in which lymphocytes were added to AM. Contact between lymphocytes and AM reduced inhibitory C/EBPβ, activated NF-κB, and enhanced HIV-1 replication. If contact between lymphocytes and macrophages was prevented, inhibitory C/EBPβ expression was maintained and the HIV-1 long terminal repeat (LTR) was not maximally stimulated although NF-κB was activated. Antibodies that cross-linked macrophage expressed B-7, and vascular cell adhesion molecule and CD40 were used to mimic lymphocyte contact. All three cross-linking antibodies were required to abolish inhibitory C/EBPβ expression. However, the HIV-1 LTR was not maximally stimulated and NF-κB was not activated. Maximal HIV-1–LTR stimulation required both lymphocyte-derived soluble factors, and cross-linking of macrophage expressed costimulatory molecules. High level HIV-1–LTR stimulation was also achieved when IL-1β, IL-6, and TNF-β were added to macrophages with cross-linked costimulatory molecules. Contact between activated lymphocytes and macrophages is necessary to down-regulate inhibitory C/EBPβ, thereby derepressing the HIV-1 LTR. Lymphocyte-derived cytokines activate NF-κB, further enhancing the HIV-1 LTR.

Key words: infection • cellular immunity • costimulatory molecules • transcription factors • derepression

Introduction

Worldwide, 1.86 billion persons are infected with *Mycobacterium tuberculosis* and 8% of all tuberculosis (TB)* cases occur in persons coinfected with HIV. There is a synergistic interaction between HIV-1 and *M. tuberculosis*. HIV-1 infection predisposes to activation of latent TB and accelerates the clinical course of the disease. Conversely, recent studies also demonstrate that TB accelerates the course of AIDS. In

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**Abbreviations used in this paper:* AM, alveolar macrophages; BAL, bronchoalveolar lavage; CAT, chloramphenicol acetyltransferase; C/EBPβ, CCAAT enhancer binding protein β; EMSA, electrophoretic mobility shift assay; LTR, long terminal repeat; NF, nuclear factor; NRE, negative regulatory element; TB, tuberculosis; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

the absence of an opportunistic infection, there is little or no viral replication in the lung even in patients with advanced AIDS (1). TB markedly increases HIV-1 replication and mutation in involved lung segments (2). Macrophages are the major cell type in which HIV-1 replication occurs in patients with opportunistic infections including TB (3). Activation of HIV-1 replication during opportunistic infection may underlie the increased mortality observed in patients coinfected with HIV-1 and TB (4).

The CCAAT enhancer binding protein β (C/EBPβ) gene is the predominant C/EBP isoform expressed in alveolar macrophages (AM) (5). C/EBPβ has a stimulatory 37-kD isoform and an inhibitory 16-kD isoform. The inhibitory isoform is dominant–negative, repressing promoters with C/EBP sites when expressed at 20% of the level of the stimulatory 37-kD isoform (6). Multiple regulators of inflammation such as TNF-α have C/EBP sites in their promoters (7). The serum response factor, a global activator of inflammation, is also suppressed by inhibitory C/EBPβ (8), which leads to the hypothesis that this dominant–negative transcription factor is responsible for maintaining AM in their baseline quiescent state.

The C/EBP family of transcription factors is essential for HIV-1 replication in macrophages but not in lymphocytes (9). There are three C/EBP binding sites present in the negative regulatory element (NRE) of the HIV-1 long terminal repeat (LTR) (10). AM from normal lung strongly express an inhibitory 16-kD C/EBPβ transcription factor that represses the HIV-1–LTR activity in model systems (11). AM from lung segments involved with TB lose expression of inhibitory 16-kD C/EBPβ, which raises the possibility that derepression is needed before the HIV-1 LTR can be maximally stimulated. Activation of the 5′ HIV-1–LTR promoter is an essential step in the viral life cycle. The nuclear factor (NF)-κB binding site in the HIV-1 LTR is essential for promoter activity and leads to transcriptional induction of viral replication in both lymphocytes and macrophages (12, 13).

In vitro infection of macrophages with *M. tuberculosis* fails to reproduce loss of the inhibitory 16-kD C/EBPβ isoform, or the increase in HIV-1 replication, observed in involved lungs of AIDS patients with TB (14). Allogeneic lymphocytes are able to increase HIV-1 replication in macrophages (15). Further, isolated membranes from activated lymphocytes enhance HIV-1 replication in macrophages (16). Because cell-mediated immunity requires interaction between lymphocytes and macrophages, we hypothesized that activated lymphocytes were essential to reproduce macrophage activation observed in vivo. We found that lymphocyte contact was required to down-regulate inhibitory C/EBPβ, and that soluble factors activated NF-κB. Both contact and soluble factors were required for maximal HIV-1–LTR induction.

Materials and Methods

Study Population. We performed bronchoscopy on two patients with stable HIV infection without pulmonary disease (see Fig. 2, Patients 6 and 7) and 1 HIV-1–infected patient with active pulmonary TB (see Fig. 2, Patient 5). The TB patient had unilateral segmental infiltrates. Radiographically uninvolved lobes were identified and a separate bronchoalveolar lavage (BAL) was performed and processed from these segments. The BAL protocol was approved by the Human Subjects Review Committees of New York University Medical Center and Bellevue Hospital Center, and was performed as described (2). BAL cells were centrifuged, resuspended in RPMI 1640 (BioWhittaker) with 10% FCS (Life Technologies), and allowed to adhere to plastic plates for 3 h. Cells were recovered by gentle scraping with a rubber policeman. AM were 95% pure by morphology and nonspecific esterase staining. HIV-1 viral loads were quantitated by RT-PCR assay (AMPLICOR HIV-1 Monitor™ Test; Roche Molecular Systems).

Immunohistochemistry. We used autopsy (see Fig. 1, Patients 1, 2, and 4) or transbronchial lung biopsy samples (see Fig. 1, Patient 3) from HIV patients coinfected with TB. Patient 1 was dead of miliary TB, acute renal failure, and pulmonary edema. Patient 2 was dead of miliary TB, and hilar and mediastinum lymph node TB. Specimens were fixed in periodate/lysine/paraformaldehyde at 4°C for 10 h. Fixed specimens were dehydrated with sucrose gradient and embedded in Histofine Simplestain (Nichirei), frozen in liquid nitrogen, and sectioned (5 μm thickness) using a cryostat. Nonspecific staining was blocked with 10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween 20, and pH 8.0, for 30 min at room temperature. After washing with TBS, one of the following primary antibodies was applied: anti–C/EBPβ rabbit and anti–NF-κB polyclonal antibody (1:100; Santa Cruz Biotechnology, Inc.) or anti-CD68 monoclonal (1:100; Dako). After incubating for 1 h at room temperature, sections were washed in TBS four times and incubated with peroxidase-labeled anti–rabbit or anti–mouse antibody (Histofine Simplestain Max PO; Nichirei) for 30 min at room temperature. Peroxidase activity was detected with diaminobenzidine (DAB; Sigma-Aldrich). Sections were counterstained with hematoxylin and dehydrated.

Purification and Activation of T Lymphocytes. PBMC were purified over Ficoll-Hypaque (Amersham Pharmacia Biotech) sedimentation. T lymphocytes were separated by nylon wool (Robbins Scientific Corp.). The purity of T lymphocytes was more than 90% by flow cytometry (FACScan®; Becton Dickinson) using FITC–anti-CD3 antibodies (Becton Dickinson). To obtain $CD4^+$ or $CD8^+$ T lymphocytes, the Rosettesep (StemCell Technologies Inc.) was used for negative selection. The purity of $CD4^+$ or $CD8^+$ lymphocytes was more than 95% by flow cytometry (FACScan; Becton Dickinson) using PE–anti-CD4 or PE–anti-CD8 antibodies (Becton Dickinson). T lymphocytes were activated by Con A (Amersham Pharmacia Biotech), 2.5 μg/ml, or anti–human CD3ε antibodies (R&D Systems), 5 μg/ml, for 48 h as described.

Blocking of T Lymphocytes and Cross-linking of Antibodies. Where indicated, Con A– (or anti-CD3–) activated T cells were pretreated with culture medium containing anti-CD28 antibodies (25 μg/ml), anti-CD40 ligand (CD154) antibodies (25 μg/ml), and anti–very late antigen (VLA)-4 (CD49δ) antibodies (25 μg/ml) (R&D Systems) for 60 min at 4°C before coculture with THP-1 cells or AM. For cross-linking assays, protein A/G agarose beads (100 μl; Santa Cruz Biotechnology, Inc.) were mixed with anti–B7-1 (CD80) antibodies (25 μg/ml), anti–B7-2 (CD86) antibodies (25 μg/ml), anti-CD40 antibodies (25 μg/ml), and anti–vascular cell adhesion molecule (VCAM)-1 (CD106) antibodies (25 μg/ml) (R&D Systems), or control goat serum (Santa Cruz

Biotechnology, Inc.) for 60 min at 4°C. These were then added to culture medium and incubated for 48 h.

Cell Culture and Cytokines. THP-1 cells (TIB-202; American Type Culture Collection) or BF24 cells (AIDS Research and Reference Reagent Program #1296) were cultured in RPMI 1640 with 10% FCS. Cells were differentiated with 20 ng/ml PMA (Sigma-Aldrich) for 24 h and incubated with IFN-β (Biosource International) at 1 U/ml for 48 h after PMA treatment. Where noted, activated T lymphocytes and AM or THP-1 cells were separated by 0.4 μm pore cell culture insert (Millipore). IL-1β, IL-6, and TNF-β in cell culture supernatants from insert experiments were measured by ELISA (R&D Systems). Cloned and purified IL-1β, IL-6, and TNF-β (R&D Systems) were added to BF-24 cells for 48 h before chloramphenicol acetyltransferase (CAT) measurement.

Cell Extract Preparation. Cells were washed twice in PBS (Bio-Whittaker). Whole cell extracts were prepared for immunoblot analysis by incubation in NP-40 buffer (0.5% NP-40, 10% glycerol, 0.1 mM EDTA, 20 mM Hepes [pH 7.9], 10 mM NaF 10 mM NaPpi, 300 mM NaCl, 3 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM DTT, 1 mM PMSF, and 1 mM Na_3VO_4) for 30 min on ice with vigorous shaking. Nuclear extracts were prepared by NP-40 lysis (buffer A: 10 mM Hepes-KOH [pH 7.8], 10 mM KCl, 0.1 mM EDTA [pH 8.0], 0.1% NP-40, 3 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM DTT, 1 mM PMSF, and 1 mM Na_3VO_4) and incubation of recovered nuclei in high salt buffer (buffer C: 10 mM Hepes-KOH [pH 7.8], 420 mM KCl, 0.1 mM EDTA [pH 8.0], 5 mM $MgCl_2$, 2% glycerol, 3 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM DTT, 1 mM PMSF, and 1 mM Na_3VO_4). Where indicated, we added 20 μg/ml calpain inhibitor (Sigma-Aldrich) into NP-40 buffer, buffer A, or buffer C before cell extraction. Pierce BCA reagents were used to determine extract protein concentrations. Protein extracts for chloramphenicol acetyl transferase CAT ELISA (Roche Molecular Biochemicals) were processed according to manufacturer's instructions.

Immunoblots. Proteins were separated by SDS-PAGE (Bio-Rad Laboratories) as described previously (11), and then probed with antibodies against anti-C/EBPβ, followed by visualization with anti–rabbit HRP antibodies (Santa Cruz Biotechnology, Inc.) and ECL plus (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assays (EMSA). The DNA probe used for C/EBP EMSA is the HIV-1 LTR NRE (11). The NF-κB probe was TGGGCT**GGGGAATCCC**GCTAA with bold letters denoting the NF-κB binding domain. The DNA probe was labeled with [γ^{32}P]ATP using T4 polynucleotide kinase in an end-labeling reaction. Full-length reaction products were isolated and 10^5 cpm-labeled DNA mixed with 10 μg of protein extract, 2.5 μg poly dI/dC, and gel mobility shift buffer. For supershift experiments, 1–2 μg of antibody was added to the reaction (anti-C/EBPβ, anti–NF-κB p65, or anti–NF-κB p50 antibodies; Santa Cruz Biotechnology, Inc.). Within the experiments, each binding reaction included a constant amount of extract protein. The DNA–protein complexes were electrophoresed on a 6% polyacrylamide (Bio-Rad Laboratories) gel at 4°C with 20 mM Tris-borate, pH 8.3, and 0.4 mM EDTA buffer. Images were produced by a PhosphorImager (Molecular Dynamics).

Results

Transcription Factor Expression and HIV-1 Replication in Pulmonary TB. To test if BAL accurately reflects what is occurring in the lung, immunohistochemistry using antibodies to C/EBPβ, NF-κB p65, and HIV-1 p24 antigen was performed on lung sections obtained from normal and TB patients, with and without HIV-1 infection. Lung sections from TB patients included regions involved and uninvolved with TB.

C/EBPβ was strongly expressed in the nucleus and cytoplasm of macrophages of normal lung, as demonstrated by immunoperoxidase staining of macrophages (Fig. 1 A). Scoring of 154 AM from normal lung demonstrated 64 (41%) C/EBPβ with strong nuclear staining. The uninvolved lung segments of an HIV-1–TB-coinfected patient also showed strong C/EBPβ expression in AM (Fig. 1 B). Scoring of 116 AM from uninvolved lung demonstrated 39 (33%) C/EBPβ with strong nuclear staining. Near sites of granulomatus inflammation, AM lost nuclear expression of C/EBPβ expression. Only 3 out of 116 (3%) of AM from patient 1 (Fig. 1 C), and 1 out of 21 (5%) AM from patient 2 (Fig. 1 D), expressed nuclear C/EBPβ. There was cytoplasmic C/EBPβ staining in AM from the involved lung segment (Fig. 2 C) and in type II pneumocytes (unpublished data).

NF-κB p65 had a markedly different pattern of expression. In normal and uninvolved lung segments of patients with TB, there was little or no binding of antibody to the lung (Fig. 1 E). In normal lung, 0 out of 43 (0%) AM expressed NF-κB, whereas only 3 out of 104 (3%) AM from an uninvolved lung expressed NF-κB. Lung segments involved with TB had increased nuclear NF-κB p65 staining in both lymphocytes and macrophages (Fig. 1 F). In involved lung segments, 105 out of 192 cells (55%) from patient 1, and 123 out of 181 cells (68%) from patient 2, expressed NF-κB. The identity of AM was confirmed by anti-CD 68 staining in serial sections. The presence of mycobacteria was confirmed by numerous acid-fast bacilli in the alveolar space (unpublished data).

HIV-1 p24 was expressed in and around areas of granulomatous inflammation (Fig. 1 G). Only epithelioid macrophages (Fig. 1 H, arrows) demonstrated positive immunostaining. Multinucleated cells, dendritic cells, and lymphocytes, had no detectable staining for p24 (unpublished data). The blood lymphocytes in areas of pulmonary hemorrhage and normal pulmonary parenchyma from two patients did not stain with anti-p24 antibody. The distribution of anti-p24 immunostaining was markedly different in the lymph node samples where dendritic cells were the predominant cell type, staining with anti-p24 antibody (unpublished data). There was no background staining in either the lung or lymph node.

Lymphocyte Contact Is Required for Loss of Inhibitory C/EBPβ in AM. Similar to our previously reported results (11), BAL cells obtained from an uninvolved lobe of an HIV-1–infected patient with TB, strongly expressed inhibitory 16-kD C/EBPβ (Fig. 2 A, lane 1), whereas BAL cells from involved lung segments did not have inhibitory 16-kD C/EBPβ expression (Fig. 2 A, lane 7). The addition of allogenic lymphocytes stimulated with Con A to AM preparations from the uninvolved lung, abolished inhibitory C/EBPβ expression over 4 d (Fig. 2 A, lane 3).

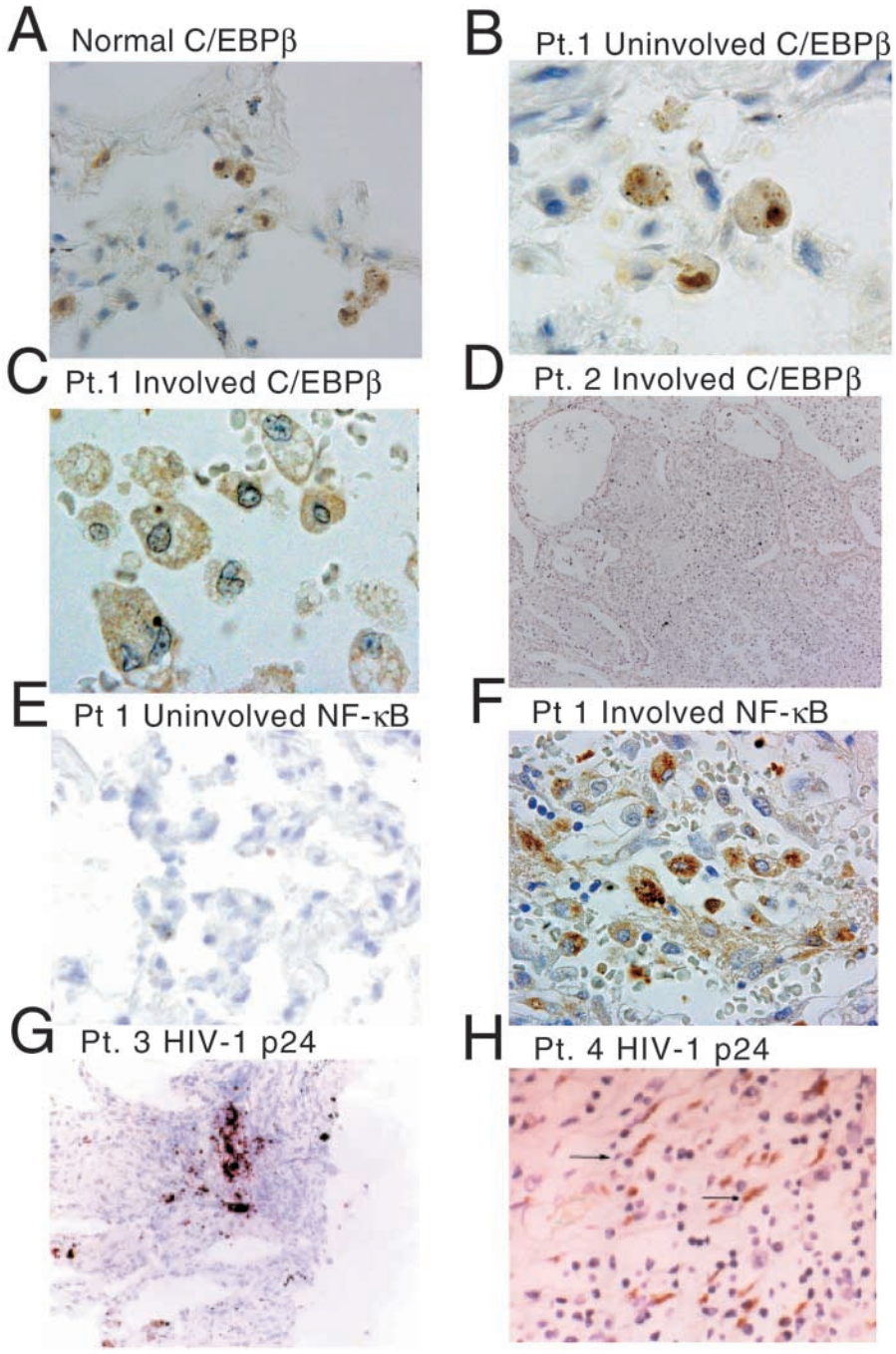

Figure 1. Transcription factor and HIV-1 p24 expression in the lung using immunohistochemistry. (A) Immunoperoxidase staining of normal lung with C/EBPβ polyclonal antibody. (B) Immunoperoxidase staining of uninvolved lung of an HIV-1–TB-coinfected patient with C/EBPβ polyclonal antibody (1,000×). (C) Involved lung segment of the same HIV-1–TB patient with C/EBPβ polyclonal antibody (1,000×). (D) Involved lung segment from a second HIV-1–TB patient with C/EBPβ polyclonal antibody (200×). (E) Uninvolved lung segment of an HIV-1–TB coinfected patient with p65 NF-κB immunoperoxidase staining (1,000×). (F) Involved lung segment of an HIV-1–TB coinfected patient with p65 NF-κB immunoperoxidase staining (1,000×). (G) HIV-1 p24 immunoperoxidase staining of a transbronchial lung biopsy (400×). (H) HIV-1 p24 immunoperoxidase staining of a lung section of HIV-1– TB involved lung (1,000×).

When resting syngenic blood lymphocytes were added to the AM, there was stable expression of inhibitory C/EBPβ after 4 d in culture (Fig. 2 A, lanes 4 and 5). This demonstrates that alteration of C/EBPβ expression was not an artifact of cell culture. Therefore, with regard to C/EBPβ expression, the addition of activated lymphocytes to AM from uninvolved lung, reproduced the state of activation found in AM from lung segments involved with TB.

To test if lymphocyte contact was required to produce loss of inhibitory C/EBPβ, lymphocytes and AM were separated by a porous 0.4-μm insert. In the absence of direct contact, Con A–activated lymphocytes did not reduce inhibitory C/EBPβ expression in AM (Fig. 2 A, lane 6). Similar results were obtained when allogenic lymphocytes, stimulated to produce soluble factors by MHC incompatibility, were added to the upper chamber of the insert and macrophages were cocultured in the lower chamber (unpublished data). These data suggest that the loss of inhibitory C/EBPβ expression in AM requires lymphocyte contact.

We next tested if activated lymphocytes could stimulate HIV-1 replication in AM isolated from AIDS patients with no lung disease. These cells had been provirally infected in

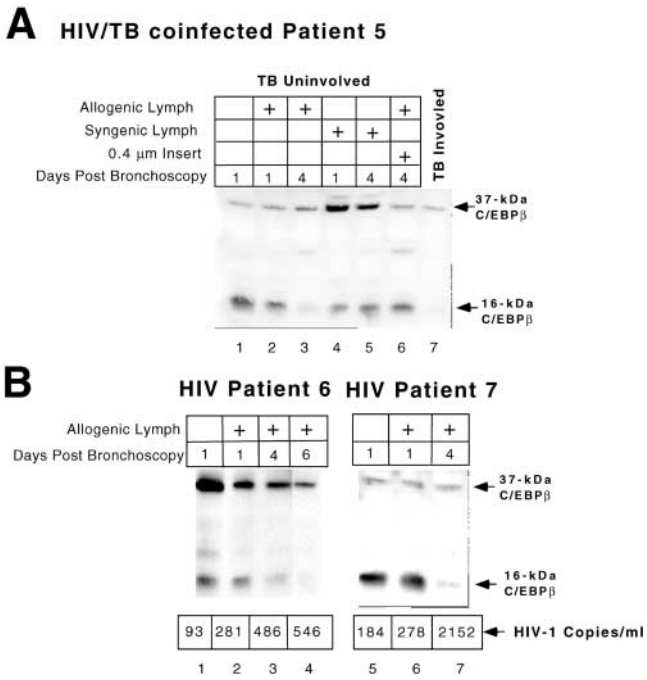

Figure 2. C/EBPβ expression and HIV-1 viral loads in AM after lymphocyte addition. (A) Western blot of whole cell extract probed with C/EBPβ polyclonal antibody. AM from an uninvolved lung segment of an HIV-1–TB-coinfected patient (lane 1). Con A–stimulated allogenic lymphocytes were added to the AM for 1 (lane 2) or 4 d of coculture (lane 3). Resting syngenic lymphocytes were added for 1 (lane 4) or 4 d (lane 5). Con A–activated lymphocytes are separated from AM by a 0.4-μm filter insert (lane 6). AM from a lung segment involved with TB (lane 7). (B) AM from two HIV-1–infected patients with normal chest radiographs after 1 d of culture (lanes 1 and 5). Activated allogenic lymphocytes were added for 1 (lanes 2 and 6), 4 (lanes 3 and 7), or 6 d (lane 4). The HIV-1 viral copy number released into the cell culture supernatant for each condition is shown in the boxes below the Western lanes.

vivo and therefore contained variable amounts of HIV-1. The addition of allogenic lymphocytes from an HIV-1–negative donor enhanced HIV-1 replication in AM preparations from two HIV-1–infected patients (Fig. 2 B, compare lane 1 with lanes 2–4, and lane 5 with lanes 6 and 7). Increasing levels of HIV-1 production correlated with the loss of inhibitory C/EBPβ expression in this system.

We have observed that THP-1 cells differentiated with PMA and treated with IFN-β are similar to AM, and we have used this model system to investigate the mechanisms controlling HIV-1 replication in macrophages (11, 14). We used EMSA with the HIV-1 LTR NRE to measure C/EBP binding activity in the THP-1 extracts. In differentiated THP-1 cells there is a single DNA protein complex that competes with excess unlabeled NRE oligonucleotide (Fig. 3 A, compare lanes 1 and 2). The addition of IFN-β leads to the induction of another specific, rapidly migrating NRE–protein complex (Fig. 3 A, lanes 3 and 4). Con A–activated lymphocytes produce loss of the rapidly migrating NRE–protein complex over 2 d (Fig. 3 A, lanes 5–8). Supershift with antibody to C/EBPβ demonstrates that both the NRE–protein complexes contain C/EBPβ (Fig. 3 A, lane 10). A minor rapidly migrating NRE–protein complex is unmasked by the supershift reaction, whereas C/EBPβ contributes over 90% of the NRE binding activity in this system.

Western blotting assessed expression of both C/EBPβ isoforms. Activated lymphocytes down-regulate inhibitory C/EBPβ expression in THP-1 macrophages (Fig. 3 B, lanes 1–3). When the activated lymphocytes and macrophages are separated by a 0.4-μm insert, inhibitory C/EBPβ expression is increased (Fig. 3 B, lanes 4–6). These data support using THP-1 cells treated with PMA and IFN-β as a model for AM, and demonstrate that contact between lymphocytes and macrophages is required for loss of inhibitory C/EBPβ in this model system. We then tested the ability of purified CD4 and CD8 lymphocyte subsets to down-regulate inhibitory C/EBPβ. Surprisingly, both activated $CD4^+$ and $CD8^+$ lymphocytes were able to down-regulate inhibitory C/EBPβ (Fig. 3 C, lanes 2 and 4). In addition, the CD 8^+ lymphocytes abolished stimulatory C/EBPβ expression (Fig. 3 C, lane 4). To address the possibility that production of the inhibitory 16 kD is due to calpain-mediated proteolytic cleavage (17), we repeated these experiments with a calpain inhibitor (18). The addition of calpain inhibitor did not lead to significant change in the expression of 16-kD C/EBPβ in whole cell extracts (Fig. 3 D, compare lanes 1 and 2) or nuclear extracts (Fig. 3 D, compare lanes 5 and 6). Ca^{2+}, a known stimulator of calpain-mediated proteolysis, increased the amount of 16-kD C/EBPβ (Fig. 3 D, compare lanes 2 and 3); calpain inhibitor blocked this increase (Fig. 3 D, lanes 3 and 4). The 16-kD C/EBPβ measured by Western blot analysis was present in the nuclear fraction (Fig. 3 D, lane 5), whereas a majority of the 37-kD C/EBPβ was in the cytoplasmic fraction (Fig. 3 D, lane 7). As a result, the ratio of inhibitory to stimulatory C/EBPβ in whole cell extracts is an underestimate of the state of transcriptional inhibition present in the nucleus.

The Effect of Lymphocyte Contact is Mimicked by Cross-linking Costimulatory Molecules CD-40, VCAM, and B7. Because macrophage-expressed costimulatory molecules CD40, VCAM, and B7 are important mediators of lymphocyte–macrophage interaction, we tested whether or not antibodies against CD-40, VCAM, and B7 would alter C/EBPβ expression. There was no change in C/EBPβ expression when a combination of antibodies against CD-40, VCAM, and B7 (stimulating antibodies) were added in the absence of protein A/G beads (Fig. 4 A, lanes 1, 3, and 5). When a combination of stimulating antibodies were attached to a solid substrate by incubating them with agarose protein A/G beads, macrophages markedly down-regulate C/EBPβ expression after 2 d (Fig. 4 A, lanes 4 and 6). Goat IgG isotype control did not alter C/EBPβ expression with or without protein A/G beads (Fig. 4 A, lanes 1 and 2). The beads by themselves did not alter C/EBPβ expression (unpublished data). The expression of inhibitory C/EBPβ was not significantly changed when only two of these antibodies were used in combination (Fig. 4 B, compare lane 1 with lanes 3, 5, and 7). The addition of protein A/G beads did, however, lead to the downregulation of

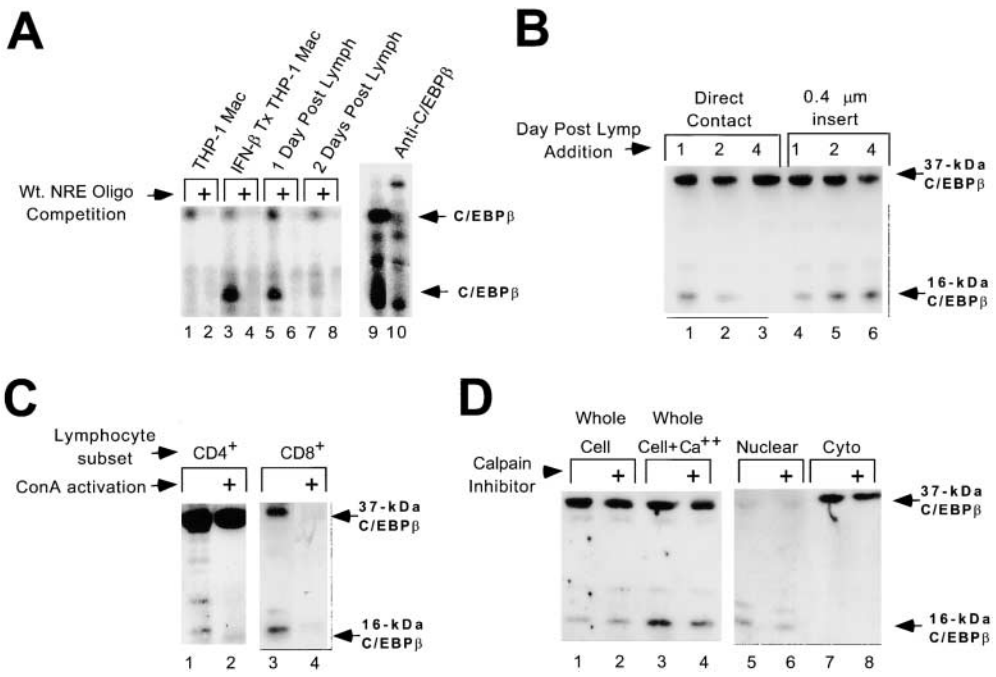

Figure 3. C/EBPβ expression in macrophages with and without lymphocyte contact. (A) EMSA of whole cell THP-1 cell extracts with the HIV-1–LTR NRE that contains a C/EBP binding site. THP-1 cells after PMA differentiation (lane 1) and the same extract with excess-unlabeled NRE probe (lane 2). IFN-β–treated THP-1 macrophages (lane 3) followed by coculture with activated lymphocytes for 1 (lane 5) and 2 d (lane 7). IFN-β–treated macrophages (lane 9) and supershifting with antibody to C/EBPβ (lane 10). (B) A Western blot of whole cell extracts of IFN-β–treated THP-1 macrophages 1 (lane 1), 2 (lane 2), and 4 d (lane 3) after coculture of Con A–stimulated allogenic lymphocytes. Separation of Con A–activated lymphocytes from IFN-β–treated THP-1 macrophages by a 0.4-μm filter for 1 (lane 4), 2 (lane 5), and 4 d (lane 6). (C) A Western blot of TNP-1 macrophage before (lane 1) and 2 d after the addition of purified $CD4^+$ lymphocytes (lane 2). A nuclear extract before (lane 3) and 2 d after the addition of purified $CD8^+$ lymphocytes (lane 4). (D) NP-40 whole cell extracts made without (lane 1) and with (lane 2) calpain inhibitor. Ca^{2+} is added to the extract without (lane 3) and with (lane 4) calpain inhibitor. Nuclear extracts made with NP-40 without (lane 5) and with (lane 6) calpain inhibitor. Cytoplasmic extracts without (lane 7) and with (lane 8) calpain inhibitor. All experiments were repeated three or more times with similar results.

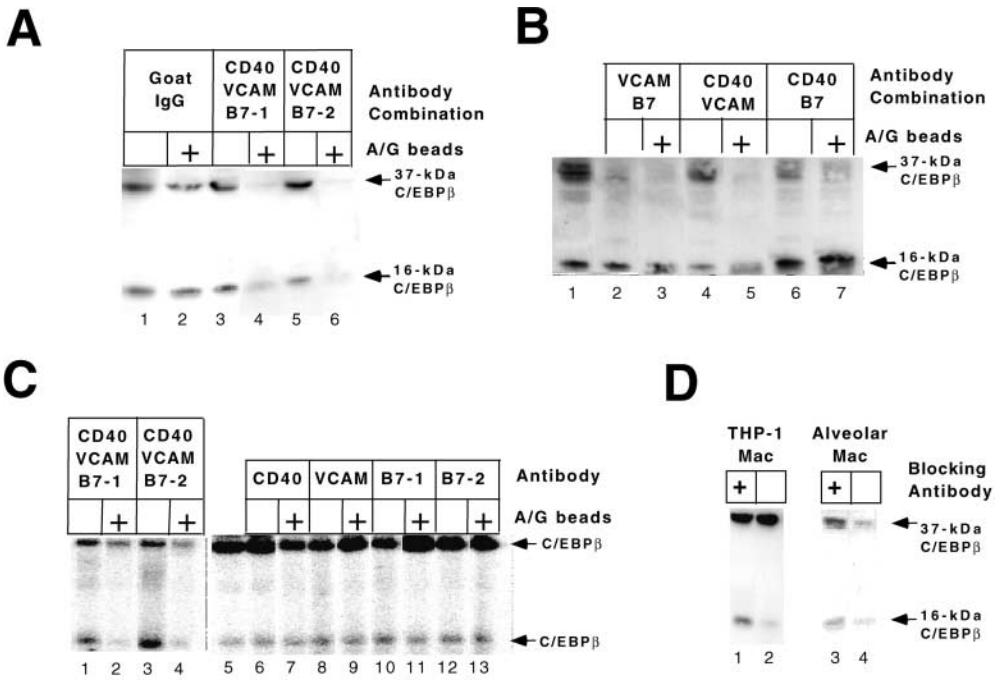

Figure 4. C/EBPβ expression in THP-1 macrophages after addition of antibodies. (A) Whole cell extract of IFN-β–treated THP-1 macrophages 2 d after goat IgG without (lane 1) or with (lane 2) protein A/G agarose. Affinity-purified goat IgG specific for CD40, VCAM, and B7-1 was added without (lane 3) and with (lane 4) protein A/G agarose beads for 2 d. Antibodies for B7-2 are substituted for antibodies of B7-1 without (lane 5) and with (lane 6) protein A/G beads. (B) C/EBPβ Western of nuclear extract of IFN-β–treated THP-1 macrophage (lane 1); 2 d after addition of antibodies to VCAN and B7-1 without (lane 2) and with (lane 3) protein A/G beads; or antibodies to CD40 and VCAM (lanes 4 and 5); or antibodies to CD40 and B7-1 (lanes 6 and 7). (C) EMSA with the HIV-1–LTR NRE 2 d after combination of antibodies IgG specific for CD40, VCAM, and B7-1 without and with protein A/G beads (lanes 1 and 2). Antibodies for B7-2 are substituted for antibodies of B7-1 (lanes 3 and 4). Single antibodies to CD40, VCAM, B7-1, or B7-2 with and without protein A/G beads (lanes 5–13). All experiments were done at least three times with similar results. (D) Coculture of IFN-β–treated THP-1 macrophages with Con A activate allogenic lymphocytes. C/EBPβ Western of whole cell extracts with the addition of blocking antibodies to lymphocyte expressed CD 40 ligand (CD 154), VLA-4 (CD 49δ), and CD 28 (lane 1). Lymphocyte addition without added antibodies (lane 2). Whole cell extracts of coculture experiments AM. Blocking added (lane 3) and no antibodies added (lane 4).

the 37-kD C/EBPβ in this set of experiments. EMSA with the HIV-1 LTR NRE shows similar results. Stimulating antibodies on a solid substrate markedly reduced NRE–protein complexes when compared with antibodies added in solution (Fig. 4 C, compare lanes 1 and 3 with lanes 2 and 4). When single antibodies to CD-40, VCAM, B7-1, or B7-2 were added to the THP-1 model, there was no change in NRE–protein complexes. This was true whether or not protein A/G beads were added (Fig. 4 C, compare lane 5 with lanes 6–13). These data suggest that the cross-linking of multiple macrophage-expressed costimulatory molecules is required to down-regulate inhibitory C/EBPβ expression.

CD40 ligand, VLA-4, and CD-28 are the lymphocyte-expressed ligands that activate macrophage-expressed CD-40, VCAM, and B7 (16, 17). FACS® analysis showed that CD-40 ligand, VLA-4, and CD-28 were strongly expressed on Con A–activated lymphocytes, and that both CD-40 ligand and CD-28 were upregulated by activation (unpublished data). A combination of antibodies to CD-40 ligand, VLA-4, and CD-28 blocked the downregulation of macrophage 16-kD C/EBPβ (Fig. 4 D, compare lane 3 with lanes 2 and 4). This was true in both the THP-1 model and in primary AM. Blocking occurred when lymphocytes were activated by MHC incompatibility (Fig. 4 D, lanes 1–4) or by antibody to CD-3 (unpublished data).

Maximal HIV-1–LTR Induction Requires both Lymphocyte–Macrophage Contact and Lymphocyte-derived Cytokines. To investigate the functional effects of macrophage–lymphocyte interaction on HIV-1 replication we used BF-24 cells, which are THP-1 cells with an integrated HIV-1 LTR CAT reporter construct. As shown in Fig. 5, LTR activity increased 12.5- ± 1.6-fold (mean ± SEM) when Con A–activated lymphocytes were mixed with BF-24 cells. LTR activity increased only 5.1- ± 1.5-fold when the activated lymphocytes were separated from the BF-24 cells with a 0.4-μm pore-size insert ($P < 0.01$ Student's *t* test when compared with contact with Con A–stimulated lymphocytes), in spite of a marked elevation of IL-1β (350 pg/ml), IL-6 (11,600 pg/ml), and TNF-β (1,000 pg/ml) in the cell culture supernatant. Similarly, LTR activity increased only 3.3- ± 1.1-fold when a combination of cross-linking antibodies to CD-40, VCAM, and B7 were added in the presence of protein A/G beads ($P < 0.01$ when compared with Con A–lymphocyte contact).

To combine the contribution of soluble factors and contact, activated lymphocytes were added to the upper chamber of an insert well to provide both lymphocyte-derived soluble factors, and stimulatory antibodies on a solid substrate were added to BF-24 cells in the lower chamber to provide contact-mediated stimuli. Maximal LTR activation was restored (14.6- ± 0.7-fold increase) when BF-24 cells were exposed to lymphocyte-soluble factors and contact with antibodies to CD40, VCAM, and B7 on protein A/G beads. When IL-1β (350 pg/ml), IL-6 (11,600 pg/ml), and TNF-β (1,000 pg/ml) were added to BF-24 cells with cross-linking antibodies to CD40, VCAM, and B7, HIV-1–LTR activity was markedly increased (8.1- ± 1-fold). This demonstrates that both contact and soluble factors are necessary and sufficient to produce the level of LTR stimulation observed when lymphocytes and macrophages are cocultured.

The NF-κB transcription factors are excellent candidates to mediate the effects of soluble factor(s) released by lymphocytes. We used EMSA to measure NF-κB DNA binding activity in cocultured cells. THP-1 cells that differentiated with PMA have no specific NF-κB DNA binding activity (Fig. 6 A, lane 1). The addition of IFN-β produces a slight increase in the amount of NF-κB DNA binding activity (Fig. 6 A, lane 3). The addition of activated lymphocytes produced a marked increase in NF-κB DNA binding activity after 1 or 2 d of coculture (Fig. 6 A, lanes 5 and 7). This DNA binding activity is specific for the NF-κB site because excess, unlabeled oligonucleotide competes with

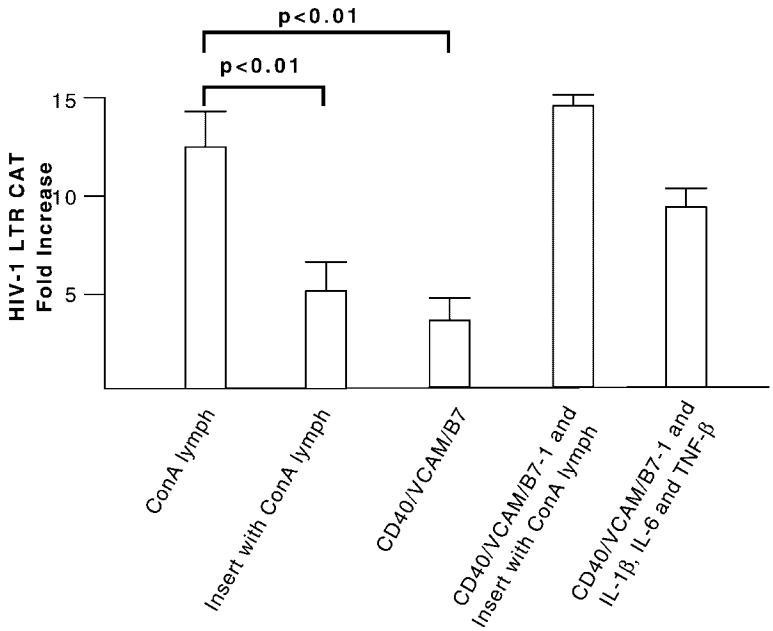

Figure 5. HIV-1 LTR promoter in IFN-β–treated THP-1 macrophages. From left to right, HIV-1–LTR CAT production 2 d after the addition of: Con A–activated allogenic lymphocytes; activated lymphocytes separated from macrophages with a 0.4-μm filter; antibodies to CD-40, VCAM, and B7 in the presence of protein A/G beads; activated lymphocytes added to the upper chamber of an insert well and stimulatory antibodies with protein A/G beads added to the lower chamber; and stimulatory antibodies attached to protein A/G beads in addition to IL-1β (350 pg/ml), IL-6 (11,600 pg/ml), and TNF-β (1,000 pg/ml).

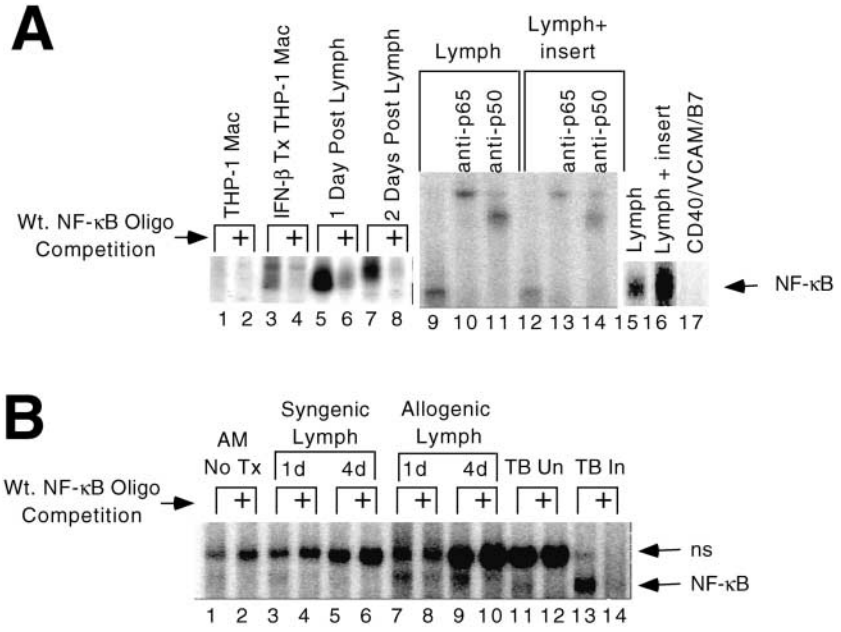

Figure 6. NF-κB DNA binding activity in THP-1 macrophages and AM after lymphocyte addition. (A) EMSA with an oligonucleotide containing an NF-κB binding site in whole cell, extracts THP-1 cells after PMA differentiation (lanes 1), the addition of IFN-β (lane 3), and the addition of lymphocytes for 1 (lane 5) and 2 d (lane 7). Excess unlabeled oligonucleotide was added to the extract in the preceding lane (lanes 2, 4, 6, and 8). EMSA with nuclear extracts from an independent experiment 2 d after coculture of THP-1 macrophages with stimulated allogeneic lymphocytes (lane 9) supershifted with anti-p65 NF-κB antibody (lane 10), or anti-p50 NF-κB antibody (lane 11). EMSA of nuclear extracts 2 d after separation of activated lymphocytes from macrophages with a 0.4-μm filter insert (lane 12), supershifted with anti-p65 NF-κB antibody (lane 13), or anti-p50 NF-κB antibody (lane 14). An independent experiment with whole cell extracts 2 d after addition of lymphocytes (lane 15) or separation of activated lymphocytes from macrophages by a 0.4-μm filter insert (lane 16), or 2 d after antibodies to CD-40, VCAM, and B7 in the presence of protein A/G beads (lane 17). Experiments were repeated three or more times with similar results. (B) EMSA of whole cell extracts from AM after 1 d of culture without treatment (lane 1). Extracts from the same patient treated with 1 (lane 3) and 4 d (lane 5) of coculture with resting syngenic lymphocytes, and 1 (lane 7) and 4 d (lane 9) of coculture with Con A–activated allogeneic lymphocytes. EMSA of BAL from an HIV-1–TB-coinfected patient with uninvolved lung segment (lane 11) and involved lung segment (lane 13). Excess unlabeled oligonulceotide was added to the extract (lanes 2, 4, 6, 8, 10, 12, and 14).

the complex (Fig. 6 A, lanes 4, 6, and 8). Antibody to the p50 or p65 isoform of NF-κB supershifts the complex (Fig. 6 A, compare lane 9 with lanes 10 and 11). When lymphocytes are separated from the THP-1 cells with an insert, there is an increase in NF-κB binding activity similar to direct contact of lymphocytes and macrophages (Fig. 6 A, lane 12). This complex also contains NF-κB p50 and p65 (Fig. 6 A, lanes 13 and 14). These data demonstrate that lymphocyte-derived soluble factors are capable of activating NF-κB in this system. In an independent experiment, the soluble factors were more effective than contact in inducing NF-κB (Fig. 6 A, compare lanes 15 and 16), whereas protein A/G beads with stimulatory antibodies to CD-40, VCAM, and B7, failed to induce NF-κB (Fig. 6 A, lane 17).

Coculture of primary AM of an HIV-1–infected patient without lung disease and without TB was used to validate THP-1 cells as a model system. In the absence of stimulation, NF-κB was not induced in AM (Fig. 6 B, lanes 1 and 2). The addition of resting syngenic lymphocytes did not induce NF-κB DNA binding activity at 1 or 4 d (Fig. 6 B, lanes 3–6). The addition of activated allogenic lymphocytes did lead to the induction of NF-κB (Fig. 6 B, lanes 7–10). The uninvolved lung segment of a patient with TB had little NF-κB (Fig. 6 B, lane 11), whereas there was marked upregulation of NF-κB in the involved lung segment from the same patient (Fig. 6 B, lane 13). Upregulation of NF-κB was observed in the involved lung segment of two additional patients (unpublished data). Competition with excess unlabeled oligonucleotide demonstrated a nonspecific band in the resting and uninvolved AM preparations. The similarity of these results with primary and transformed cells supports the use of THP-1 cells in this system.

Discussion

HIV-1 replication in AM is normally suppressed with fewer than 1 in 10,000 cells harboring provirus and little or no HIV-1 present in bronchoalveolar lining fluid (1). During TB there is a marked increase in HIV-1 replication in the lung with the AM as the major source (1–3). The likely source of this virus is latently infected cells that reside in the lung before the occurrence of TB (1, 2). TB accelerates AIDS mortality even when the TB is appropriately treated (4). The enhanced viral replication and increased viral mutation likely underlies the accelerated course of AIDS observed in TB-coinfected patients. High levels of HIV-1 replication in vivo are associated with the loss of an inhibitory C/EBPβ transcription factor and the increase of NF-κB (11). Attempts to model this state of macrophage activation by infecting cells in vitro with *M. tuberculosis* did not reproduce the changes in C/EBPβ expression nor viral replication observed in patients with pulmonary TB (14). We now report that the addition of activated lymphocytes to macrophages reproduces the state of activation observed during TB. There is a loss of inhibitory C/EBPβ, an activation of NF-κB, and an increase in HIV-1 replication. Investigation of this model further demonstrated that maximal transcriptional activation of the HIV-1 LTR is a two-step process that requires both contact and soluble factors. The contact leads to a loss of in-

hibitory C/EBPβ that derepress the HIV-1 LTR, whereas soluble factors produce NF-κB activation that enhances HIV-1–LTR transcription.

THP-1 cells were used to investigate the mechanisms underlying the contact-mediated loss of inhibitory C/EBPβ. THP-1 cells are a human monocytic cell line that are similar to AM when they have been differentiated with PMA and stimulated with low-dose IFN-β; both express inhibitory C/EBPβ and repress HIV-1 replication. C/EBP sites in the HIV-1 LTR are required for HIV-1 replication in macrophages, but not in lymphocytes (9). C/EBPβ is the predominant C/EBP family member expressed in macrophages (5, 11), and overexpression of inhibitory 16-kD C/EBPβ strongly inhibits HIV-1–LTR transcription in model systems (12). TB leads to the loss of inhibitory C/EBPβ, which derepresses the HIV-1 LTR (11). Both Western blots of BAL cells and immunohistochemistry of lung sections confirm a significant reduction of C/EBPβ expression in lung segments involved with TB. This is particularly true of short-form C/EBPβ that is localized in the nucleus. The expression of cytoplamic long form is maintained in a number of conditions, raising the possibility of nonnuclear functions of this protein in macrophages. The regulation of short-form C/EBPβ is particularly important because it is a dominant-negative transcription factor (6) and a strong repressor of HIV-1 transcription in macrophages (12).

Mechanisms that produce inhibitory C/EBPβ are not fully understood. Genetic evidence using expression constructs suggests that inhibitory C/EBPβ is produced by translational initiation at an internal AUG start site (6, 19, 20). Proteolysis occurring in vivo has also been proposed as a mechanism for short-form production (21). Concern has been raised, however, that production of inhibitory C/EBPβ occurs during the extraction procedure and is mediated by a calpain protease (17). The protease is inhibited by NP-40 detergent and calpain inhibitor and is enhanced by Ca^{2+} (18). All of the cell extracts presented in this investigation were made with NP-40 to prevent a proteolytic artifact. The addition of calpain inhibitor to the NP-40 extraction buffer did not affect the amount of inhibitory C/EBPβ. The addition of Ca^{2+} to the extraction buffer increased the amount of inhibitory C/EBPβ, demonstrating that a Ca^{2+}-responsive protease was present. Calpain inhibitor was effective in blunting the increase of inhibitory C/EBPβ after the addition of Ca^{2+} to the extraction buffer, which suggests that although a calpain-like protease is present in the THP-1 macrophages extracts, it did not contribute to the amount of 16-kD C/EBPβ observed. These data strongly support the conclusion that under the conditions used in this investigation, the amount of 16-kD C/EBPβ provides a relevant measure of the inhibition of promoters with C/EBP binding sites.

Adding activated lymphocytes to macrophages overcomes the transcriptional repression of the HIV-1 LTR induced by low-dose IFN-β. This interaction is not MHC restricted and occurs with both $CD4^+$ and $CD8^+$ lymphocyte subsets. HIV-1–TB-coinfected patients have a $CD8^+$ lymphocytic alveolitis (22). Our finding that $CD8^+$ lymphocyte subsets are capable of downregulating both C/EBPβ isoforms fits with the observation that in some patients no C/EBPβ is expressed in involved lung segments (11). Lymphocytes activated by MHC incompatibility, Con A, or anti-CD3 antibody abolished the expression of inhibitory C/EBPβ in both AM and THP-1 macrophages. AM cocultured for 4 d with resting syngenic lymphocytes had stable expression of both stimulatory and inhibitory C/EBPβ, indicating that the changes in C/EBPβ produced by activated lymphocytes were not an artifact of ex vivo tissue culture. When allogenic lymphocytes from an HIV-1–negative donor are added to AM from HIV-1–infected patients, HIV-1 replication increases and inhibitory C/EBPβ is lost. The increased HIV-1 replication may be due in part to HIV-1 infection and replication in the added lymphocytes. However, the 12.5-fold increase in HIV-1–LTR transcriptional activity when lymphocytes are added to THP-1 macrophages, suggests that transcriptional activation of the LTR in macrophages significantly contributes to the increased viral replication observed in the ex vivo coculture experiments.

When the lymphocytes and macrophages were separated by a 0.4-μm insert, activated lymphocytes failed to reduce inhibitory C/EBPβ expression in either AM or the THP-1 cell model. Functionally, HIV-1–LTR activation in macrophages is reduced by 66% when macrophages are separated from activated lymphocytes. This demonstrates that direct contact between lymphocytes and macrophages is required for downregulation of inhibitory C/EBPβ and maximal induction of the HIV-1 LTR in macrophages. These findings are consistent with the observation that the membrane fraction of activated lymphocytes enhances HIV-1 replication in macrophages (16).

HIV-1 viral load and TNF-α production are strongly correlated ($r^2 > 0.95$) in involved lung segments of AIDS patients with pulmonary TB (2). One explanation of these findings is that the HIV-1 LTR and the TNF-α promoter are coordinately regulated in the lung during opportunistic infections. The TNF-α promoter, like the HIV-1 LTR, contains C/EBP sites. One of the consequences of expressing the inhibitory C/EBPβ in macrophages is that proinflammatory cytokine production is strongly suppressed (7). Similar to the observations that maximal induction of the HIV-1 LTR requires contact, maximal induction of TNF-α requires contact between activated lymphocytes and brain macrophages (23, 24). The stimulation of TNF-α is due in part to lymphocyte-expressed CD-40 ligand, VLA-4, and CD-28 binding to macrophage-expressed CD-40, VCAM, and B7.

Antibodies binding macrophage costimulatory receptors CD-40, VCAM, and B7 were used to mimic the effect of lymphocyte contact. To further enhance the cross-linking effect of the antibodies, the Fc portion of these antibodies was attached to protein A/G beads. Expression of inhibitory C/EBPβ is lost only when a combination of affinity-purified antibodies to CD-40, VCAM, and B7-1 or B7-2

are attached to agarose beads and presented to macrophages. The specific effect of the stimulating antibody–agarose bead combination is demonstrated by the stable expression of C/EBPβ after the addition of goat IgG isotype-control antibody, either in the presence or absence of protein A/G agarose beads. These findings show that lymphocyte-derived soluble factors are not required to abolish inhibitory C/EBPβ expression, and antibodies to CD-40, VCAM, and B7 on a solid substrate, are able to substitute for lymphocyte contact.

C/EBPβ expression is unchanged when single antibodies to CD-40, VCAM, and B7-1 or B7-2 are attached to agarose beads and presented to macrophages. This demonstrates that multiple costimulatory receptors must be cross-linked before the signal to reduce C/EBPβ expression is transduced. This suggests that when antibodies are oriented on a solid substrate they are more effective in producing signal transduction, possibly because all costimulatory molecules are cross-linked at the point of contact between the bead and the macrophage.

Lymphocyte-expressed CD-40 ligand, VLA-4, and CD-28 bind macrophage-expressed CD-40, VCAM, and B7. Activated lymphocytes expressed all three ligands. Antibodies directed against lymphocyte-expressed ligands were tested for the ability to block downregulation of inhibitory C/EBPβ expression. A mixture of antibodies to lymphocyte-expressed CD-40 ligand, VLA-4, and CD-28 blocked the downregulation of C/EBPβ in both AM and THP-1 macrophages. These antibodies were capable of blocking the effect of activated lymphocytes. These data support a model in which multiple lymphocyte-costimulatory molecules must interact with multiple macrophage-costimulatory molecules in order to down-regulate inhibitory C/EBPβ (Fig. 7).

Whereas lymphocyte-derived soluble factors are unable to down-regulate inhibitory C/EBPβ, they are able to partially activate the HIV-1 LTR. There are many cytokines released in this system including high levels of IL-1β, IL-6, and TNF-β. All of these cytokines are capable of activating NF-κB, and the binding of NF-κB to the HIV-1 LTR strongly stimulates the HIV-1 LTR (12, 13). EMSA demonstrated that NF-κB p50 and p65 were activated in coculture experiments. In addition to activation of NF-κB, lymphocyte-derived soluble factors alter macrophage morphology and activate other DNA binding activities (unpublished data). The activation of NF- κB, and possibly other transcription factors by soluble factors, accounts for the partial activation of the HIV-1 LTR observed in experiments in which activated lymphocytes are separated from macrophages by an insert.

The combination of lymphocyte-derived soluble factors with contact-mediated stimuli provided by antibodies to CD-40, VCAM, and B7-1 or B7-2, restored maximal HIV-1–LTR activity. The addition of IL-1β, IL-6, and TNF-β at concentrations found in coculture experiments to THP-1 macrophages with cross-linked CD-40, VCAM, and B7, also markedly increased HIV-1–LTR activity. This leads to a model in which two steps are required for full activation of the HIV-1 LTR. Contact leads to the loss of inhibitory C/EBPβ derepressing the HIV-1 LTR, whereas soluble factors leads to the activation of NF-κB. Neither stimulus is sufficient for full induction of the HIV-1 LTR.

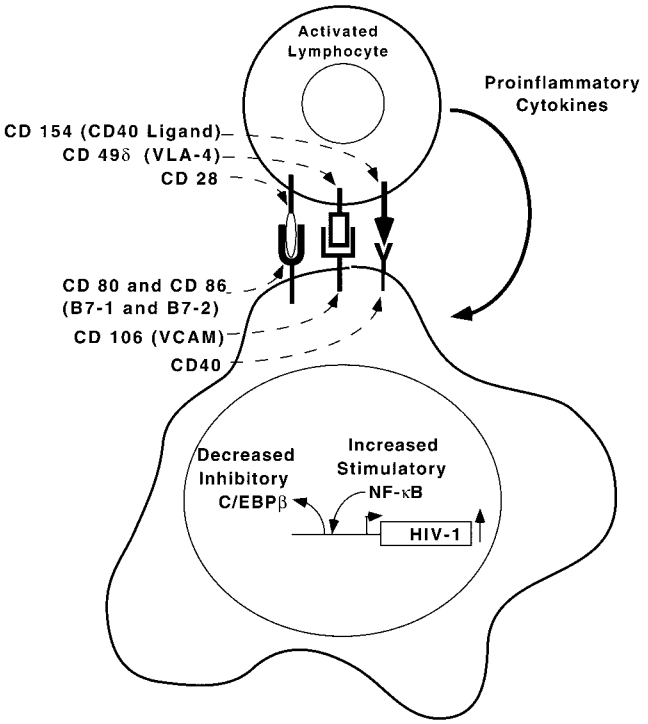

Figure 7. A model of lymphocyte–macrophage interaction during the cellular immune response leading to increased HIV-1 replication. Contact leads to the loss of inhibitory C/EBPβ, derepressing the 5′ HIV-1 LTR whereas soluble factors activate NF-κB, which enhances HIV-1 replication.

Upregulation of the HIV-1 LTR in macrophage by activated lymphocytes represents another example of HIV-1 usurping normal immune regulation in order to enhance its replication. Granulomatus inflammation is highly destructive to lung architecture. Many of the macrophage mediators of cellular immunity have C/EBP sites in their promoters, and inhibition of these proinflammatory pathways might be important for maintaining lung homeostasis in the absence of infection. The requirement for lymphocyte contact to produce the state of activation seen in TB, likely limits the tissue destruction observed in granulomatous inflammation to areas where lymphocytes are activated by the presence of antigen stimulation.

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References

1. Nakata, K., M. Weiden, T. Harkin, D. Ho, and W.N. Rom. 1995. Low copy number and limited variability of proviral DNA in alveolar macrophages from HIV-1 infected patients: evidence for genetic differences in HIV-1 between lung and blood macrophage populations. *Mol. Med.* 1:744–757.
2. Nakata, K., W. Rom, Y. Honda, R. Condos, S. Kanegasaki, Y. Cao, and M. Weiden. 1997. *M. tuberculosis* enhances human immunodeficiency virus-1 replication in the lung. *Am. J. Respir. Crit. Care Med.* 155:996–1003.
3. Orenstein, J.M., C. Fox, and S.M. Whal. 1997. Macrophages as a source of HIV during opportunistic infections. *Science.* 276:1857–1861.
4. Whalen, C., C.R. Horsburgh, D. Hom, C. Lahart, M. Simberkoff, and J. Ellner. 1995. Accelerated course of human immunodeficiency virus infection after tuberculosis. *Am. J. Respir. Crit. Care Med.* 151:129–135.
5. Natsuka, S., S. Akira, Y. Nishio, S. Hashimoto, T. Sugita, H. Isshiki, and T. Kishimoto. 1992. Macrophage differentiation-specific expression of NF-IL6, a transcription factor for Interleukin-6. *Blood.* 79:460–466.
6. Descombes, P., and U. Schibler. 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell.* 67:569–579.
7. Pope, R., A. Leutz, and S.A. Ness. 1994. C/EBP-beta regulation of the tumor-necrosis-factor-alpha gene. *J. Clin. Invest.* 94:1449–1455.
8. Hanlon, M., and L. Sealy. 1999. Ras regulates the association of serum response factor and CCAAT/enhancer-binding protein beta. *J. Biol. Chem.* 274:14224–14228.
9. Henderson, A.J., and K.L. Calame. 1997. CCAAT/enhancer binding protein (C/EBP) sites are required for HIV-1 replication in primary macrophages but not $CD4^+$ T cells. *Proc. Natl. Acad. Sci. USA.* 94:8714–8719.
10. Tesmer, V.M., A. Rajadhyaksha, J. Babin, and M. Bina. 1993. NF IL-6-mediated transcriptional activation of the long terminal repeat of the human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA.* 90:7298–7303.
11. Honda, Y., L. Rogers, K. Nakata, B. Zhao, R. Pine, Y. Nakai, K. Kurosu, W.N. Rom, and M. Weiden. 1998. Type I interferon induces inhibitory 16 kDa C/EBP-β repressing the HIV-1 LTR in macrophages: pulmonary tuberculosis alters C/EBP expression enhancing HIV-1 replication. *J. Exp. Med.* 188:1255–1265.
12. Henderson, A.J., X. Zou, and K. Calame. 1995. C/EBP proteins activate transcription from human immunodeficiency virus type 1 long terminal repeat in macrophages/monocytes. *J. Virol.* 69:5337–5344.
13. Zhang, Y., K. Nakata, M. Weiden, and W. Rom. 1995. *Mycobacterium tuberculosis* enhances HIV-1 replication by transcriptional activation at the long terminal repeat. *J. Clin. Invest.* 95:2324–2331.
14. Weiden, M., N. Tanaka, Y. Qiao, B.Y. Zhao, Y. Honda, K. Nakata, A. Canova, D.E. Levy, W.N. Rom, and R. Pine. 2000. Differentiation of monocytes to macrophages switches the *Mycobacterium tuberculosis* effect on HIV-1 replication from stimulation to inhibition: modulation of interferon response and C/EBPβ expression. *J. Immunol.* 165:2028–2039.
15. Moriuchi, H., M. Moriuchi, and A.S. Fauci. 1999. Induction of HIV-1 replication by allogeneic stimulation. *J. Immunol.* 162:7543–7548.
16. Mikovits, J., N. Lohrey, R. Schulof, J. Courtless, and F. Ruscetti. 1992. Activation of infectious virus from latent human-immunodeficiency-virus infection of monocytes *in vivo*. *J. Clin. Invest.* 90:1486–1491.
17. Baer, M., S.C. Williams, A. Dillner, R.C. Schwartz, and P.F. Johnson. 1998. Autocrine signals control CCAAT enhancer binding protein beta expression, localization, and activity in macrophages. *Blood.* 92:4353–4365.
18. Baer, M., and P.F. Johnson. 2000. Generation of truncated C/EBP beta isoforms by *in vitro* proteolysis. *J. Biol. Chem.* 275:26582–26590.
19. Calkhoven, C.F., C. Muller, and A. Leutz. 2000. Translational control of C/EBP alpha and C/EBP beta isoform expression. *Genes Dev.* 14:1920–1932.
20. Xiong, W., C.C. Hsieh, A.J. Kurtz, J.P. Rabek, and J. Papaconstantinou. 2001. Regulation of CCAAT/enhancer-binding protein-beta isoform synthesis by alternative translational initiation at multiple AUG start sites. *Nucleic Acids Res.* 29: 3087–3098.
21. Welm, A.L., N.A. Timchenko, and G.J. Darlington. 1999. C/EBP alpha regulates generation of C/EBP beta isoforms through activation of specific proteolytic cleavage. *Mol. Cell. Biol.* 19:1695–1704.
22. Law, K.F., J. Jagirdar, M. Weiden, M. Bodkin, and W.N. Rom. 1996. Tuberculosis in HIV-positive patients: cellular response and immune activation in the lung. *Am. J. Respir. Crit. Care Med.* 153:1377–1384.
23. Chabot, S., G. Williams, and V. Yong. 1997. Microglial production of TNF-alpha is induced by activated T Lymphocytes - involvement of VLA-4 and inhibition by Interferon beta-1b. *J. Clin. Invest.* 100:604–612.
24. Chabot, S., G. Williams, M. Hamilton, G. Sutherland, and V. Yong. 1999. Mechanisms of IL-10 production in human microglia-t cell interaction. *J. Immunol.* 162:6819–6828.