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Immunology

CD14 is involved in control of human immunodeficiency virus type 1 expression in latently infected cells by lipopolysaccharide

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ABSTRACT **Lipopolysaccharide (LPS) potently stimulates human immunodeficiency virus type 1 (HIV-1) long terminal repeat-directed transcription in transfected monocyte–macrophage cell lines and dramatically increases HIV-1 production in the latently infected monocyte–macrophage-like cell line U1. This response to LPS, however, can only be observed after pretreatment of the U1 cells with granulocyte–macrophage colony-stimulating factor (GM-CSF). CD14, the differentiation antigen that acts as a receptor for complexes of LPS and LPS-binding protein, is now demonstrated to be involved in LPS-induced stimulation of HIV-1 replication. CD14 is shown to be expressed on a subpopulation of U1 cells only after treatment with GM-CSF and correlates with HIV-1 production stimulated by LPS. Importantly, only those U1 cells that express CD14 can be induced by LPS to upregulate HIV-1 production. In addition, a monoclonal antibody directed against CD14 can block LPS-induced stimulation of HIV-1 production from these latently infected cells.**

Human immunodeficiency virus type 1 (HIV-1) replicates by using various virally encoded and cell-specific proteins (1–4) and infects a variety of cell types in cell cultures and *in vivo* (5–7). Exogenous factors have been demonstrated to critically affect HIV-1 production (4). Certain stimuli, such as tumor necrosis factor α (TNF-α) and phorbol esters, stimulate HIV-1 expression in many cell types (1–4). Other agents, though, appear to be cell-specific stimulators of HIV-1 replication (8). We have recently demonstrated that lipopolysaccharide (LPS) or endotoxin, which is a major component of the envelopes of Gram-negative bacteria, is an extremely potent stimulator of HIV-1 replication in certain monocyte–macrophage-like cells but is not a stimulator of HIV-1 expression in T-lymphocyte-like cell lines (8).

The two cell types that constitute the predominant, but not the sole, reservoirs for HIV-1 *in vivo* are the CD4$^+$ T-lymphocyte and the monocyte–macrophage (7, 9, 10). The CD4$^+$ lymphocyte appears to be the primary viral reservoir in the peripheral bloodstream, while the monocyte–macrophage constitutes the main solid tissue reservoir, is relatively resistant to HIV-1-induced cytopathic effects, and may be the major cell type infected by HIV-1 in the central nervous system (5–7, 9, 10). As such, understanding the molecular events involved with HIV-1 expression in the monocyte–macrophage lineage may hold significant clinical importance. As HIV-1-infected individuals appear to undergo a variable and often lengthy period of relatively quiescent infection (11), factors that affect HIV-1 production in specific cell types in culture may provide clues to the events that control HIV-1 replication *in vivo*.

Using the latently infected subclone U1 (2) of the monoblastoid cell line U937, which constitutively produces very low levels of HIV-1, we have previously shown that LPS stimulates HIV-1 replication in these cells only after pretreatment with granulocyte–macrophage colony-stimulating factor (GM-CSF) (8). This was hypothesized to be due to the immature baseline state of the U1 cells, with the absence of a putative LPS receptor (8). We have also recently shown, using U1 cells, that HIV-1 proviral latency is characterized by an aberrant pattern of HIV-1 RNA expression (12).

Recent work has defined molecules involved in the interaction of low concentrations of LPS with leukocytes (13, 14). LPS is first bound by serum proteins, such as LPS-binding protein (LBP). The resulting LPS–LBP complex is then bound by CD14, a 55-kDa glycoprotein expressed on monocytes, macrophages, and polymorphonuclear leukocytes (15, 16). Synthesis of TNF-α by monocytes or macrophages is dramatically enhanced by LPS bound to LBP and can be blocked by anti-CD14 monoclonal antibodies (14). Here we show that CD14 plays a crucial role in replication of HIV-1 in the U1 model of proviral latency.

MATERIALS AND METHODS

Cells. The monocytoid cell line U937 and the T-lymphocytic cell line Jurkat were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. A subclone of HIV-1-infected U937 cells, U1 (2), and a subclone of the CEM T-lymphocytic line, ACH-2, exhibit minimal amounts of constitutive expression of HIV-1 (3).

CD14 and HIV-1 Assays. Cell lines were plated at 5×10^5 cells per ml in 24-well plates (Costar). In some experiments, cells were pretreated with GM-CSF (Genetics Institute, Cambridge, MA) at 500 units/ml for 48 hr before harvest. In other cultures, cells were treated with GM-CSF for 24 hr before addition of LPS from *Escherichia coli* 0127:B8 (Difco) (10 μg/ml) and were harvested after an additional 24 hr. Cells were harvested for analysis by indirect immunofluorescence and fluorescence-activated cell sorting (FACS). Supernatants were harvested for HIV-1 p24 antigen determination by using a sensitive ELISA (DuPont).

Indirect Immunofluorescence. As described (17), cells were harvested for single and dual staining immunofluorescence. For CD14 staining, a murine monoclonal antibody, 3C10 (18), at 20 μg/ml was used; for HIV-1 staining, an anti-HIV-1 polyclonal antiserum (17) at 1:40 dilution was used. The 3C10 monoclonal antibody has been demonstrated to efficiently bind to CD14 moieties and block LPS-induced TNF-α secretion from monocyte–macrophages (14).

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Abbreviations: HIV-1, human immunodeficiency virus type 1; TNF-α, tumor necrosis factor α; LPS, lipopolysaccharide; GM-CSF, granulocyte–macrophage colony-stimulating factor; LBP, LPS-binding protein; FACS, fluorescence-activated cell sorting; CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; EMSA, electrophoretic mobility-shift assay; NF, nuclear factor.

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FACS. For FACS analyses, cells were treated with either 3C10 or anti-HIV-1 polyclonal antiserum, followed by fluorescein isothiocyanate-conjugated or rhodamine-conjugated antibodies. The secondary antibodies, goat anti-human and goat anti-mouse, without the primary antibodies were used as negative controls.

Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. Cell lines were transfected, with a plasmid (HIV–CAT) containing the HIV-1 long terminal repeat (LTR) upstream of the CAT gene (1), using DEAE-dextran, as described (8). At 24 hr posttransfection, cells were either treated with LPS alone, anti-CD14 alone, both LPS and anti-CD14, or were left untreated. After 2 hr of incubation at 37°C, cells were thoroughly washed with fresh medium. Forty-four hours after transfection, cell extracts were prepared for CAT assay and the quantity of protein per assay was normalized (70 μg for U937). CAT assays were performed according to the method of Gorman *et al.* (19).

Nuclear Extracts and Electrophoretic Mobility-Shift Assays (EMSAs). Nuclear extracts of U937 cells were prepared 2 hr posttreatment by a rapid method (20) modified from Dignam *et al.* (21). Proteins were measured and 8 μg of nuclear extract protein was used in each reaction. EMSAs to detect nuclear factor kappa B (NF-κB) binding were carried out as described (8). The ^{32}P-labeled DNA probe was a 44-base-pair *Hin*dIII/*Sal* I fragment cut from a plasmid (J10) containing a single κB enhancer site (22).

Blocking HIV-1 Production by Using Anti-CD14 Antibodies. U1 and ACH-2 cells, both untreated and prestimulated for 24 hr with GM-CSF (500 units/ml), were incubated with 3C10 (20 μg/ml) for 10 min at 37°C. At that point, LPS (10 μg/ml) was added to the medium. After an additional 24 hr in culture, the peak of HIV-1 stimulation (8), supernatants were harvested for HIV-1 p24 antigen ELISA. For a control, an unrelated murine monoclonal antibody, anti-CD8 (IOT8, 0450; AMAC, Westbrook, ME), was used.

RESULTS

CD14 Expression on U1 Cells Is Induced by GM-CSF. To determine the levels of surface expression of CD14 on the U1 cell line before and after maturation with GM-CSF, U1 cells were harvested for immunofluorescence microscopy. As illustrated in Fig. 1, only 0.1% of unstimulated U1 cells expressed the CD14 antigen on their surfaces. After 24 hr of treatment with GM-CSF, 27% of U1 cells (270-fold increase) were demonstrated to express CD14. As shown in Fig. 2, FACS analyses confirmed these findings. As shown in Fig. 1, little or no CD14 was detected on the Jurkat or the ACH-2

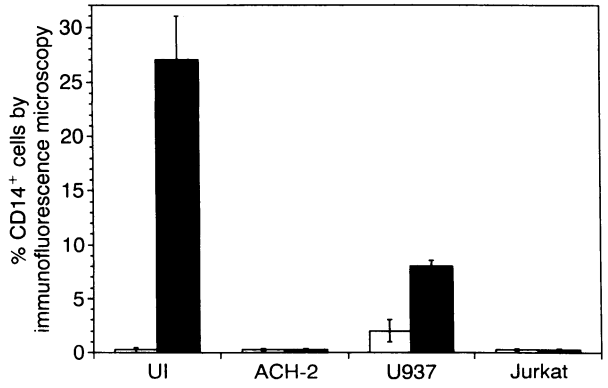

FIG. 1. Immunofluorescence microscopy for surface CD14 expression on various cell lines. Cells were either left untreated (□) or were harvested after 24 hr of treatment with GM-CSF (500 units/ml) (■). Arithmetic means ± SD of four independent experiments are illustrated.

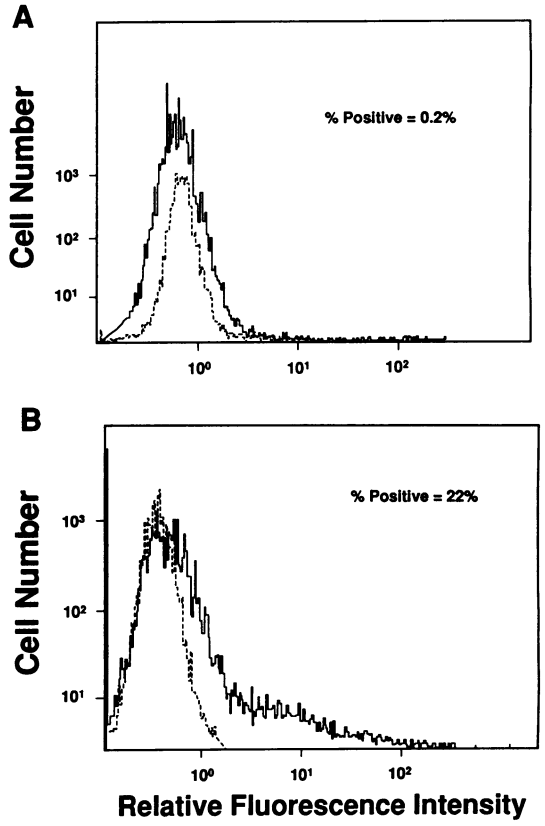

FIG. 2. FACS analyses of surface CD14 expression on untreated and GM-CSF-treated U1 cells. Fluorescence distributions for untreated U1 cells (*A*) and GM-CSF-stimulated U1 cells (*B*), using the anti-CD14 antibody (solid line) 3C10, are shown. Mean fluorescence of cells, stained with a negative control antibody, goat anti-mouse, is shown as a dotted line.

cells both before and after treatment with GM-CSF. Thus, the U1 cell line at baseline does not express significant levels of surface-bound CD14, but this is dramatically upregulated by treatment with GM-CSF.

Only CD14$^+$ U1 Cells Produce HIV-1 in Response to LPS. To determine whether those U1 cells that have increased surface expression of CD14 after GM-CSF treatment were the same cells from which increased HIV-1 expression occurs after subsequent LPS treatment (8), double-labeling immunofluorescence was undertaken. As illustrated in Table 1, >99% of GM-CSF-pretreated U1 cells that expressed HIV-1-specific surface antigens after LPS stimulation were also the cells that exhibited coexpression of surface CD14 antigen. In addition, Table 1 documents the fact that the relatively small increase in HIV-1 expression in GM-CSF-treated U1 cells occurs via increased viral production in those cells that concomitantly express CD14. Fig. 3 is a photomicrograph that illustrates representative fields of untreated U1 cells and GM-CSF-pretreated U1 cells after stimulation with LPS by double-

Table 1. Double-labeling immunofluorescence microscopy for CD14 and HIV-1 expression

	Cells singly staining for CD14, %	Cells singly staining for HIV-1, %	Cells dually staining for CD14 and HIV-1, %
No treatment	≤0.1	≤0.1	≤0.1
GM-CSF	21 ± 3	≤0.1	4 ± 1
GM-CSF plus LPS	≤0.1	≤0.1	24 ± 2

Data represent arithmetic means ± SD of three separate experiments performed in triplicate on U1 cells.

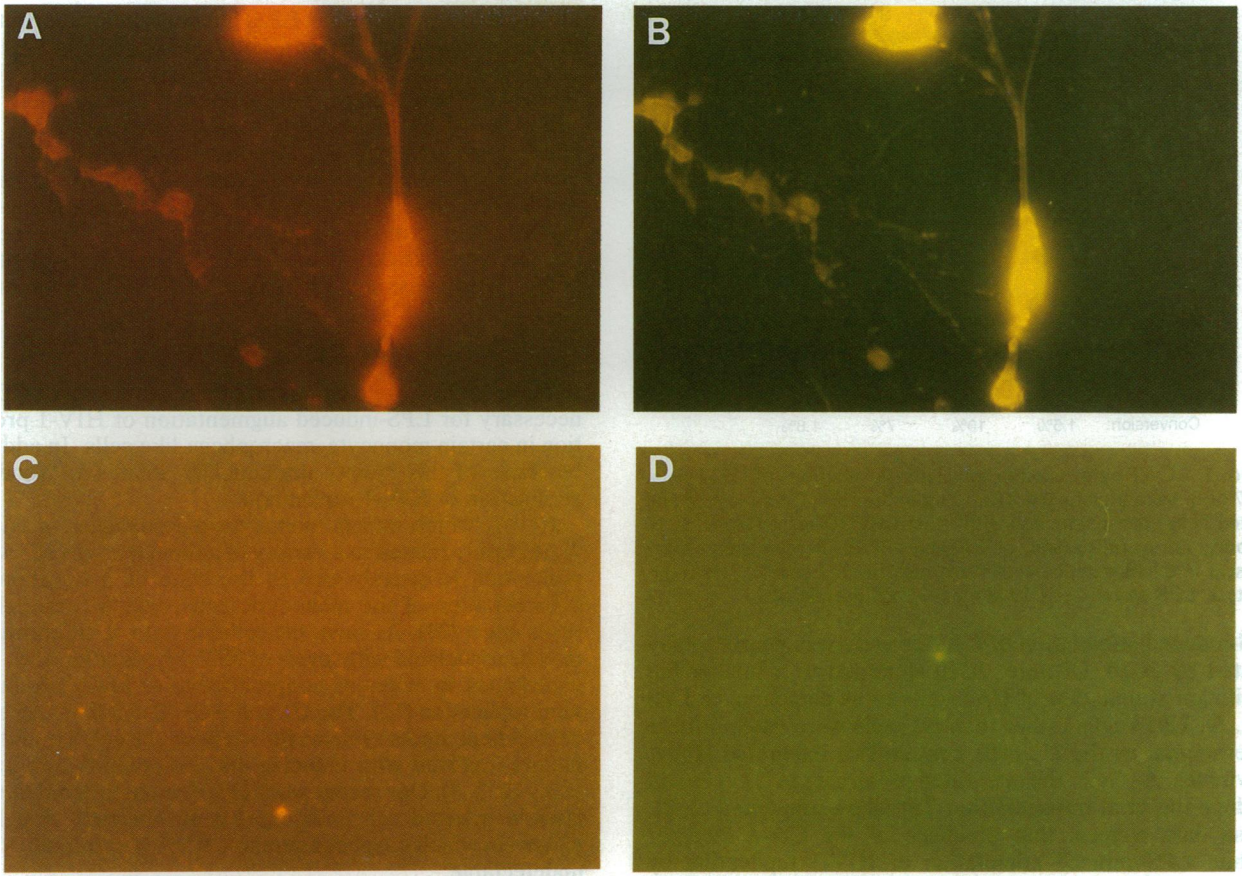

FIG. 3. Double-labeling immunofluorescence microscopy for CD14 and HIV-1 surface antigens. Representative photomicrographs of GM-CSF-pretreated U1 cell cultures stimulated with LPS and then stained for CD14 and HIV-1 antigens 24 hr after LPS treatment using 3C10 and a human polyclonal HIV-1-positive serum. (*A* and *B*) The same field of a GM-CSF plus LPS-stimulated U1 cell culture stained for HIV-1 and CD14, respectively. A rhodamine-conjugated secondary antibody was used for staining HIV-1 antigens (*A*), while a fluorescein isothiocyanate-conjugated secondary antibody was used for staining CD14 (*B*). (*C* and *D*) Representative fields of untreated U1 cells stained for HIV-1 antigens and CD14, respectively. (×400.)

labeling immunofluorescence. We thus demonstrate that levels of $CD14^+$ U1 cells, after GM-CSF plus LPS stimulation, correlate with the percentage of HIV-1-positive U1 cells. Furthermore, we show that only those U1 cells that express the CD14 antigen can be stimulated by LPS to productively express HIV-1.

Anti-CD14 Antibodies Block LPS-Induced HIV-1 Expression. To determine whether anti-CD14 antibody could be used to block LPS-stimulated HIV-1 expression from GM-CSF-pretreated U1 cells, these cells were treated with 3C10 before treatment with LPS. Using 3C10, the LPS-induced stimulation of HIV-1 expression in GM-CSF-pretreated U1 cells could be fully blocked (Fig. 4). LPS alone does not significantly stimulate HIV-1 replication in U1 cells (Fig. 4). As a negative control, an unrelated murine monoclonal antibody, anti-CD8, did not affect LPS-induced HIV-1 replication in GM-CSF-treated U1 cells (Fig. 4).

Interestingly, 3C10 alone stimulated HIV-1 replication from GM-CSF-pretreated U1 cells to a significant degree (Fig. 4). Untreated U1 cells were not stimulated by 3C10 (Fig. 4). We thus show that an anti-CD14 monoclonal antibody can dramatically block LPS-induced HIV-1 replication in U1 cells after prior differentiation by GM-CSF. Moreover, this same anti-CD14 monoclonal antibody less potently mimics LPS when added alone to GM-CSF-stimulated U1 cells.

Anti-CD14 Monoclonal Antibodies Block LPS-Induced Stimulation of HIV-1 LTR-Directed Transcription. As LPS has been demonstrated to stimulate HIV-1 replication in certain monocyte-like cells, at least in part, by enhancing HIV-1 LTR-directed transcription (8), we sought to evaluate the ability of this anti-CD14 monoclonal antibody to block LPS stimulation of the HIV-1 LTR. By using a HIV-1–LTR–CAT construct (HIV–CAT), U937 cells were transiently transfected and treated 24 hr posttransfection with either LPS alone, anti-CD14 antibody alone (3C10), LPS plus anti-CD14 antibody, or were left untreated. As shown in Fig. 5, the

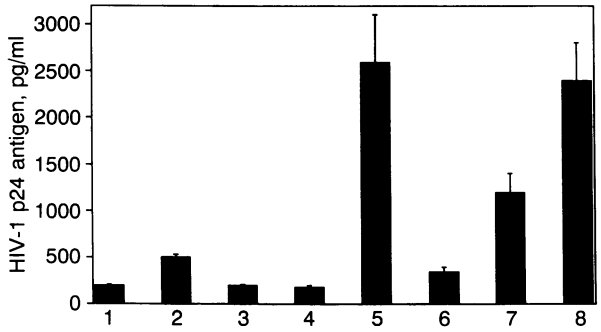

FIG. 4. Blockage of HIV-1 expression from LPS-stimulated GM-CSF-pretreated U1 cells by an anti-CD14 monoclonal antibody. Levels of HIV-1 production, measured using a HIV-1 p24 antigen ELISA, are illustrated for untreated U1 cells and for U1 cells treated with a variety of agents: 1, no treatment; 2, GM-CSF (500 units/ml) alone for 48 hr; 3, anti-CD14 monoclonal antibody 3C10 (20 μg/ml) alone for 48 hr; 4, LPS (10 μg/ml) alone for 48 hr; 5, GM-CSF for 24 hr followed by LPS for 24 hr; 6, GM-CSF for 24 hr followed by LPS plus anti-CD14 antibody for 24 hr; 7, GM-CSF for 24 hr followed by anti-CD14 antibody for 24 hr; 8, GM-CSF for 24 hr followed by LPS plus anti-CD8 antibody for 24 hr.

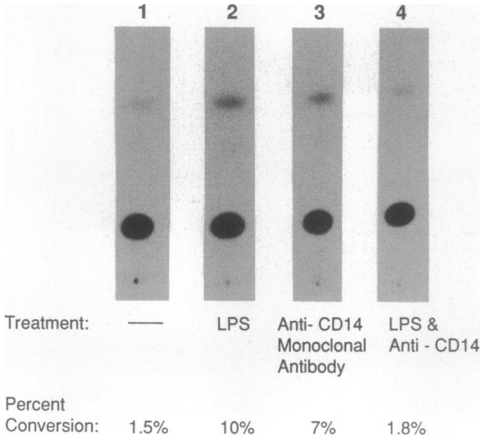

FIG. 5. CAT expression in transient transfections of U937 cells. U937 cells were transfected with 10 μg of the HIV–CAT plasmid and treated 24 hr posttransfection with either LPS (10 μg/ml) alone, 3C10 antibody (20 μg/ml) alone, or LPS plus 3C10. Percentage conversions of [^{14}C]chloramphenicol were measured. Lanes: 1, no treatment; 2, LPS alone; 3, 3C10 alone; 4, LPS plus 3C10.

LPS-induced stimulation of CAT activity was nearly totally ablated by 3C10. Of note, 3C10 alone led to a smaller but significant stimulation of CAT activity as compared to LPS (Fig. 5). U937 cells rather than U1 cells were used in these experiments, as the U1 cells contain two integrated HIV-1 proviruses. As such, the integrated proviruses in U1 cells will produce the viral transactivator Tat when stimulated (2, 8). These data suggest that blockage of the CD14 receptor inhibits LPS-induced stimulation of HIV-1 replication by blocking LPS-induced activation of cellular factors, such as NF-κB, which affect the HIV-1 LTR.

Anti-CD14 Antibodies Block LPS-Induced Activation of NF-κB. As LPS stimulates HIV-1 replication in monocyte-like cells through a process directly involving activation of NF-κB, we sought to demonstrate whether CD14 blockade with this monoclonal antibody would lead to a decrease in LPS-induced activation of NF-κB. As shown in Fig. 6 (lanes 2 and 3), EMSAs revealed a significant decrease in LPS-induced activation of NF-κB in U937 cells. In addition, 3C10

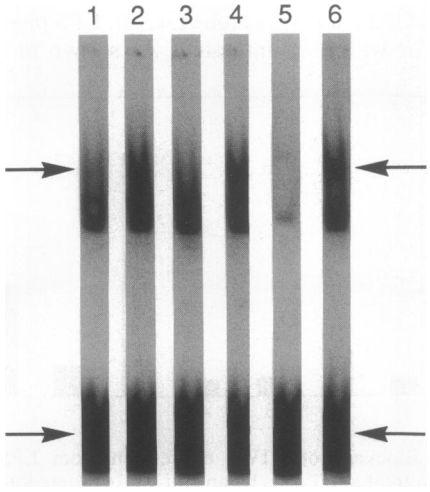

FIG. 6. EMSAs of NF-κB activation in U937 cells. Nuclear extracts of U937 cells were prepared and EMSAs were performed. Before harvest, U937 cells were either left untreated or were treated with LPS (10 μg/ml) alone for 2 hr, 3C10 alone (20 μg/ml) for 2 hr, or LPS plus 3C10 for 2 hr. Lanes: 1, no treatment; 2, LPS alone; 3, LPS plus 3C10; 4, 3C10 alone; 5, LPS alone plus 20 ng of unlabeled competitor; 6, LPS alone plus 20 ng of mutant competitor. Upper arrows, NF-κB band; lower arrows, unbound probe.

alone led to a smaller (than LPS) but consistent augmentation in NF-κB activation (lane 4). U937 cells rather than U1 cells were used in this model system, as we have previously shown that even after GM-CSF pretreatment only extremely small quantities of activated NF-κB can be detected in U1 cells, and this requires prolonged stimulation with LPS (8). Thus, quantitation of changes in activated NF-κB in U1 cells is technically difficult. In sum, based on these findings, we would propose a model whereby blocking the CD14 receptors on monocyte-like cells decreases LPS-induced augmentation of HIV-1 expression via inhibition of NF-κB activation.

DISCUSSION

In these studies, we demonstrate that expression of CD14 is necessary for LPS-induced augmentation of HIV-1 production in certain monocyte–macrophage-like cells. In addition, blockade of this moiety dramatically ablates LPS-induced stimulation of HIV-1 replication.

HIV-1, which infects monocyte–macrophages *in vivo* (5, 7), is closely related to a variety of animal lentiviruses whose replication is directly tied to the state of maturation and differentiation of the monocytic cells, which these lentiviruses infect (23). As such, the cellular state of differentiation may be associated with levels of HIV-1 replication and, thus, contributes to states of nonproductive or lowly productive viral replication (12). The U1 and ACH-2 cell lines appear to be excellent model systems for studying the cellular and viral factors involved with inducing and maintaining proviral latency (2, 3, 8). Our recent work (12) demonstrated that these latently infected cell lines express an aberrant viral RNA pattern in which unspliced genomic RNA is extremely low or undetectable.

Both the U1 and ACH-2 cells can be stimulated by a variety of agents to greatly augment HIV-1 production (2, 3). Many of these agents operate via activation of NF-κB, which stimulates HIV-1 LTR-directed transcription (4). LPS in picogram quantities stimulates HIV-1 replication in GM-CSF-pretreated U1 cells but not in the T-lymphocyte-derived ACH-2 cells (8). LPS-induced HIV-1 stimulation in U1 cells is correlated with NF-κB activation (8). Exquisitely tight control of NF-κB in U1 cells may be a key factor involved in maintaining proviral latency (8, 12). In our previous studies, we were intrigued by the lack of HIV-1 stimulation by LPS in U1 cells before pretreatment with GM-CSF (8). As GM-CSF is a monocytic differentiation and maturational agent (24), we suggested that GM-CSF may function in this context via surface expression of a functional LPS receptor, which would be lacking in untreated U1 cells.

Recently, CD14, a surface differentiation antigen, has been shown to be a functional receptor on monocyte–macrophages for LPS (14). After binding of the LPS–LBP complex to CD14 on the surface of monocytes, the monocyte is activated and produces TNF-α (14, 25). CD14, which has been cloned and sequenced (26), is a glycoprotein with a phosphatidylinositol anchor and does not express a transmembrane or cytoplasmic domain (27). This antigen has significant homology to a family of leucine-rich glycoproteins and is induced as pre-myeloid cells are differentiated toward mature monocyte–macrophages (28). Our studies demonstrate that CD14 is expressed on U1 cells only after GM-CSF treatment, and only those cells that express CD14 also express HIV-1 antigens when stimulated by LPS. A specific anti-CD14 monoclonal antibody dramatically blocks LPS-induced HIV-1 augmentation in GM-CSF-pretreated U1 cells. Furthermore, NF-κB activation by LPS in monocyte-like cells, as assessed by EMSAs, is ablated by this anti-CD14 antibody. Thus, blockade of LPS-induced NF-κB activation may be a primary factor in the mechanism(s) of anti-CD14 antibody suppression of HIV-1 induction in these cells.

By itself, the monoclonal anti-CD14 antibody used in this study stimulated HIV-1 replication, HIV-1 LTR-directed transcription, and NF-κB activation to a modest but significant degree. This suggests that certain anti-CD14 antibodies, as described (25), may activate the CD14 receptor and that activation of NF-κB is among the early events triggered by ligation of CD14. We suggest that these data support a model in which LPS and the monoclonal IgG 3C10 may bind to different but overlapping epitopes on the CD14 molecule. Thus, LPS and 3C10 would, upon binding to CD14, bidirectionally inhibit activation of this receptor molecule.

These findings are consistent with observations of Schutt *et al.* (25), who demonstrated interleukin 1 secretion in response to anti-CD14 antibodies, but differ from our previous findings that incubation of whole blood with anti-CD14 antibodies caused no activation of TNF-α secretion by monocytes (14). Preliminary data (unpublished) suggest that the ability of an anti-CD14 antibody to either activate a cell or to block activation depends on its ability to crosslink CD14. Our data also suggest that ligation of CD14 by antibody is sufficient to stimulate cellular responses and that LPS *per se* is not necessary for this stimulation.

If, as in many animal lentiviruses (23) and in certain human cell lines (12), HIV-1 is maintained in specific cells within the body in a latent state, then therapeutic modalities that help maintain HIV-1 in quiescent states may be clinically useful. As many patients infected with HIV-1 have Gram-negative bacterial infections and LPS, even in healthy HIV-1-seronegative persons (29), reaches significant levels in the portal venous system and liver, before being cleared by mononuclear phagocytes in the liver, the ability to block LPS-induced stimulation of certain monocytic cell populations may be of some therapeutic efficacy.

Primary well-differentiated human macrophages, obtained from peripheral blood, are protected from HIV-1 infection in cell cultures by pretreatment with LPS (30). This suggests that HIV-1 production *in vivo* may differ for various monocytic cell populations within the human body. As such, the interplay of LPS and CD14 on HIV-1 replication *in vivo* may be quite complex.

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