

Immunodeficiency and other clinical immunology

Replication of human immunodeficiency virus-1 in primary human T cells is dependent on the autocrine secretion of tumor necrosis factor through the control of nuclear factor- κ B activation

M. Angeles Muñoz-Fernández, PhD,^{a, b} Joaquín Navarro, PhD,^a
Angel García, MSc,^b Carmen Punzón, MSc,^b Eduardo Fernández-Cruz, MD,^a and
Manuel Fresno, PhD^b *Madrid, Spain*

Tumor necrosis factor (TNF)- α controls T-cell activation and is a major inducer of human immunodeficiency virus (HIV)-1 replication in chronically infected cells. Therefore, we have investigated its role in primary cultures of HIV-infected human T lymphocytes by using neutralizing anti-TNF- α antibodies or TNF- α . Primary resting T lymphocytes produced TNF- α and supported HIV replication after T-cell receptor activation. Addition of neutralizing anti-TNF- α antibodies drastically reduced p24 antigen release and prevented CD4⁺ cell depletion associated with infection. Anti-TNF- α also prevented nuclear factor- κ B (NF- κ B) activation, and a good correlation between this inhibition and inhibition of HIV replication was observed. Moreover, supplementing the cultures with high doses of IL-2 reverted anti-TNF- α inhibition of cell proliferation but did not affect the inhibition of HIV p24 antigen release or NF- κ B activation in the same cultures. Moreover, anti-TNF- α inhibited HIV-1 long terminal repeat (LTR)-driven transcription of a reporter gene in primary T cells in response to activation, either in the presence or the absence of HIV-1 Tat. Our results support an important role for autocrine TNF- α secretion in controlling HIV replication in primary T cells because of its ability to maintain NF- κ B elevated in the nucleus of T cells. (*J Allergy Clin Immunol* 1997;100:838-45.)

Key words: Acute infection, activation, human immunodeficiency virus, nuclear factor- κ B, primary T lymphocytes, tumor necrosis factor, cytokines

The pathogenic mechanisms underlying human immunodeficiency virus (HIV)-1 infection and disease are extremely complex.^{1,2} Among the various pathogenic

Abbreviations used

HIV:	Human immunodeficiency virus
LTR:	Long terminal repeat
mAb:	Monoclonal antibody
NF- κ B:	Nuclear factor- κ B
PBMC:	Peripheral blood mononuclear cells
PKC:	Protein kinase C
PMA:	Phorbol myristate acetate
TNF:	Tumor necrosis factor

mechanisms, altered production of certain cytokines that may be involved either in the induction of virus replication or in the pathogenesis of the immune dysregulation associated with disease progression have been suggested to play an important role in HIV-1 disease progression.^{3,4}

It appears that at any given stage of the disease, the majority of HIV integrated provirus are silent in most of the T CD4⁺ cells.^{5,6} Thus 10-fold more CD4⁺ T cells contain HIV DNA than HIV RNA,⁷ which suggests that HIV replication is not taking place in resting T cells. Activation of infected T cells is thought to be required for efficient reverse transcription, production of infectious virus particles, and virus spreading.⁸⁻¹⁰

HIV transcription is dependent on the long terminal repeat (LTR) promoter that contains several binding sites for several mammalian transcription factors.^{11,12} Among those, the main inducible element is the core enhancer element, which binds to nuclear factor- κ B (NF- κ B).¹³ NF- κ B is composed of heterodimers and homodimers of a family of proteins involved in gene regulation.^{14,15} T-cell activation by protein kinase C (PKC), through the T-cell receptor (TcR) or by tumor necrosis factor (TNF)- α activates NF- κ B by inducing this nuclear translocation.¹⁶⁻¹⁹

On the other hand, several proinflammatory cytokines have been shown to affect HIV replication, as shown by their ability to trigger HIV expression in chronically

From the ^aDivision of Immunology, Hospital Universitario Gregorio Marañón, Madrid, and ^bCentro de Biología Molecular, CSIC-UAM Universidad Autónoma de Madrid.

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Reprint requests: Manuel Fresno, Centro de Biología Molecular, CSIC-UAM, Universidad Autónoma de Madrid, E-28049 Madrid, Spain.

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infected cells or to activate viral LTR promoter in transient transfection assays.²⁰⁻²² Among these, TNF- α appears to be one of the most important. Thus induction of HIV expression from chronically infected monocytic cells has been associated with endogenous TNF- α production.²⁰⁻²⁴ TNF- α induces HIV-1 expression through activation of the transcription factor NF- κ B, increasing HIV transcription.^{25, 26}

TNF- α is a cytokine mainly produced by activated macrophages,²⁷ although it is also produced by activated T cells.^{28, 29} In addition, recent results from our laboratory indicate that the autocrine secretion of TNF- α plays a very important role in quantitatively controlling T-cell activation.³⁰ The induction of the synthesis of this cytokine by HIV infection has been documented in vitro,³¹ an action that has been ascribed to the transactivating activity of HIV-Tat protein.³² Moreover, TNF- α levels are elevated in many patients in vivo.³³

Establishing an experimental system that mimics or serves as surrogate of a physiological system, similar to those that HIV replicates in vivo, is of great importance for understanding the pathogenesis of AIDS. Recent results indicate that requirements for HIV replication in normal and transformed cell lines are quite different,³⁴ emphasizing the need to analyze the mechanism of viral activation in a more physiologic model. However, most of the studies were carried out in already activated T cells such as transformed T-cell lines or T-cell clones,^{17, 22, 24} and very little was known about the mechanism of HIV activation in normal resting T cells.

Because TNF- α controls T-cell activation and is a major inducer of HIV replication in chronically infected cells, we decided to further investigate the effects of TNF- α in primary cultures of HIV-infected human T lymphocytes. Our results suggest that autocrine TNF- α plays a role in controlling HIV replication. This effect is due to the ability of this cytokine to maintain elevated levels of NF- κ B in the nucleus of activated T cells.

METHODS

Reagents

Recombinant IL-2 and recombinant TNF- α (10^7 U/mg) were a generous gift from EURO CETUS and Antibioticos Farma, respectively. The neutralizing anti-TNF- α (IgG1) monoclonal antibody (mAb) B13.2 was developed in our laboratory.³⁰ It was used in purified form obtained from ascitic fluid; $10 \mu\text{g/ml}$ was able to completely neutralize the biological activity of 300 ng/ml of TNF- α in a cytotoxic assay. The X63 IgG1 mAb, purified in the same manner, was used as negative control. The SPV 3Tb anti-CD3 mAb, a generous gift of Dr. Jan de Vries, was purified from ascitic fluid.

Cell cultures

Human peripheral blood mononuclear cells (PBMCs) were obtained from whole blood from healthy HIV-1-seronegative donors as described.³⁵ PBMCs were depleted from adherent cells by incubation in plastic dishes at 37°C for 2 hours, and purified T cells (greater than 95% CD3⁺ cells) were obtained by passing the nonadherent population through a nylon fiber wool column.^{30, 35}

For in vitro infections, triplicate cultures of purified T cells ($10^6/\text{ml}$ in Dulbecco's modified Eagle's medium containing 5% FCS) were activated with immobilized ($1 \mu\text{g/ml}$ coated wells) anti-CD3 antibody and infected or mock-infected with 5000 HIV-1 (strain SP61 kindly provided by Dr. C. Lopez Galindez, Instituto Carlos III; Madrid, Spain) infectious particles per milliliter.³⁴ Different concentrations of human recombinant cytokines and/or purified anti-TNF- α mAb were added to the cultures. At the third and sixth days after infection, 50% of the culture supernatants were harvested and the wells replenished with an equivalent volume of fresh medium containing 5 U/ml of recombinant human IL-2 to maintain a viable culture, together with the same concentration of respective reagents (TNF- α or anti-TNF- α mAb).³⁵

Proliferation assay

Three, 6, or 9 days after infection, cell proliferation was evaluated by incorporation of tritiated thymidine (New England Nuclear; Boston, Mass.) into DNA during the last 16 hours of each culture as described.^{30, 35} The assays were carried out in triplicate cultures.

HIV-1 p24 antigen and TNF assays

Three, 6, and 9 days after infection, culture supernatants were harvested and p24 antigen and TNF contents were quantified with the use of an antigen capture immunoassay (ELAVIA Ag, Pasteur Diagnostics; Paris, France) and specific ELISA (Innogenetics; Zwijnaarde, Belgium), respectively.

Cytofluorometric analysis

The percent of the CD4⁺ T-lymphocyte subset present in T-cell cultures was evaluated by direct flow cytometry as previously described.³⁵ Briefly, 3, 6, and 9 days after infection, the cells (1 to 2×10^5) were harvested and centrifuged three times in phosphate-buffered saline containing 2% BSA (Sigma) and 0.1% sodium azide. They were subsequently incubated with fluorescein isothiocyanate-labeled anti-CD4 mAb (Becton Dickinson) or a fluorescein isothiocyanate-labeled irrelevant mAb as negative control for 30 minutes at 4°C . Surface fluorescence was determined in an FACS spectrofluorometer (Becton Dickinson).

Electrophoretic mobility shift assays

Nuclear extracts were obtained from T cells essentially as previously described.³⁰ The binding assays were performed with $5 \mu\text{g}$ of nuclear protein in presence of $5 \mu\text{g}$ poly dIdC, 6 mmol/L Cl_2Mg , $0.5 \times$ buffer C, and 0.8 ng of labeled of the κB enhancer of the HIV-LTR ($5' \text{ G C A G G G G A A T C T C C C T C T } 3'$). The binding complexes were separated in a 5% acrylamide gel, and their specificity was determined by competition with $50\times$ molar excess of the same unlabeled oligonucleotide.³⁰

Transfection assays

The LTRWT-luc expression vector plasmid was a generous gift of Dr. J. L. Virelizier and has been described previously.³⁶ They carry U3⁺R of the LTR of the LAI strain of HIV-1 from nucleotide -644 to $+78$. The CMV-Tat was a gift of Dr. Jose Alcamí and contains full-length HIV-Tat under control of cytomegalovirus immediate early promoter.³⁴

Cells were resuspended in RPMI supplemented with 10% FCS and electroporated at 320 V , $1500 \mu\text{F}$, with a BIO-RAD Gene Pulser II with $1 \mu\text{g}/10^6$ cell of purified plasmid(s). After transfection, the cells were cultured at 37°C for 14 hours before being activated with phorbol myristate acetate (PMA) (10

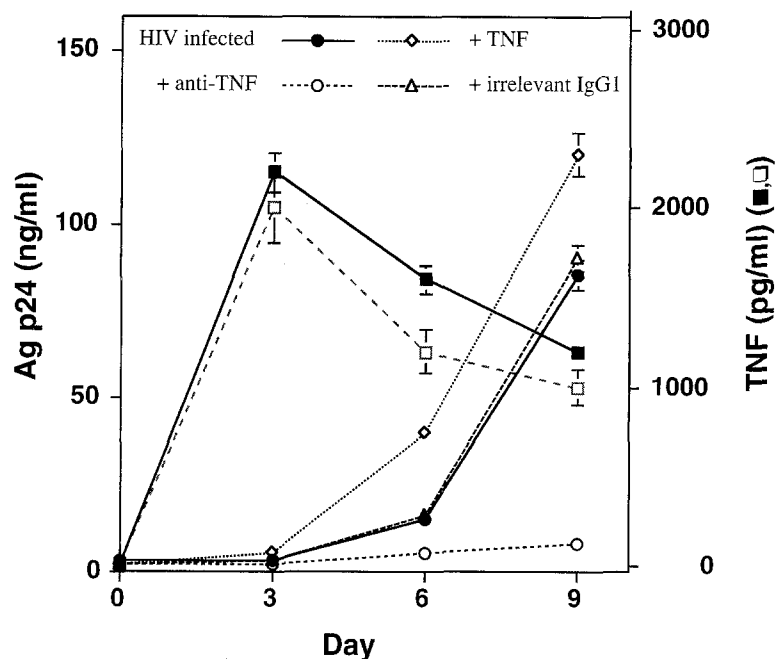


FIG. 1. Role of endogenous TNF- α on HIV-1 replication in cultures of activated human T cells. Human T cells were stimulated with immobilized anti-CD3 antibody and infected with HIV-1. Anti-TNF- α mAb (20 μ g/ml), irrelevant P3 mAb (20 μ g/ml), or TNF- α (10 ng/ml) was added at the initiation of the cultures where indicated. Mean antigen p24 $\pm$ SEM of triplicate culture supernatants 3, 6, and 9 days after infection are shown. TNF production (mean $\pm$ SEM) in mock-infected (*open squares*) or HIV-infected (*filled squares*) anti-CD3 stimulates cultures. TNF production did not reach values above 30 pg/ml in unstimulated cells, either infected or not infected. Ag, Antigen.

ng/ml) or TNF- α (10 ng/ml). Anti-TNF- α mAb was added at the same time as PMA. Cells were incubated by an additional 12-hour period, harvested, and lysed. Luciferase activity was measured in a luminometer and expressed as relative luciferase units, calculated as light emission from experimental sample light emission from untransfected cells/ 10^6 cells.

RESULTS

Effect of TNF- α on HIV-infected T cells

Purified T cells, depleted of the great majority of monocytes, were infected with HIV-1, activated through the TcR with immobilized anti-CD3, and restimulated every 3 days with IL-2. Activation of T cells induced a secretion of TNF- α in those cultures that was maximal 3 days after infection and decreased thereafter (Fig. 1). No significant differences were observed in the absolute levels or in the kinetics of TNF- α secretion between infected or uninfected anti-CD3-activated T cells, in agreement with previous results.³⁵ Activated and infected T cells showed very little HIV-1 p24 release up to day 6 after infection; this release was much more evident after 9 days. A drastic reduction of the HIV-1 p24 antigen present in the supernatant was observed in T cells cultured in the continuous presence of neutralizing anti-TNF- α mAb (Fig. 1). The average inhibition with a high dose of anti-TNF- α was 85% (ranging from 70% to 98%) with T cells from five different donors. Moreover,

addition of TNF- α (10 ng/ml) to the cultures had a significant enhancing effect on HIV-1 replication (ranging from 120% to 180%).

The proportion of CD4⁺ cells from HIV-1-infected and activated T-cell cultures decreased over time (Fig. 2), paralleling the increase of HIV-1 replication in those same cultures (Fig. 1), and was significantly lower than that from the uninfected controls at day 9 after infection. We found it interesting that anti-TNF- α mAb prevented the CD4⁺ cell depletion associated with HIV infection and the percent of CD4⁺ cells remained similar to the one observed in uninfected control. Exogenous TNF- α slightly increased the CD4⁺ cell depletion rate, although this effect was not statistically significant (Fig. 2).

Because HIV replication is dependent on T-cell activation, and we have reported previously an inhibitory effect of anti-TNF- α in T-cell proliferation,³⁰ the observed inhibition by anti-TNF- α could be an indirect consequence of its ability to inhibit cell proliferation. To verify this hypothesis, we measured T-cell proliferation in HIV-infected cultures. The proliferation of HIV-1-infected T cells was inhibited by anti-TNF- α mAb to some extent but always to a lesser degree than HIV replication (Fig. 3). The inhibition was more pronounced (range from 30% to 65%, three donors) early after activation (day 3), before cultures were supplemented with exogenous IL-2 (Fig. 3B). The proliferation

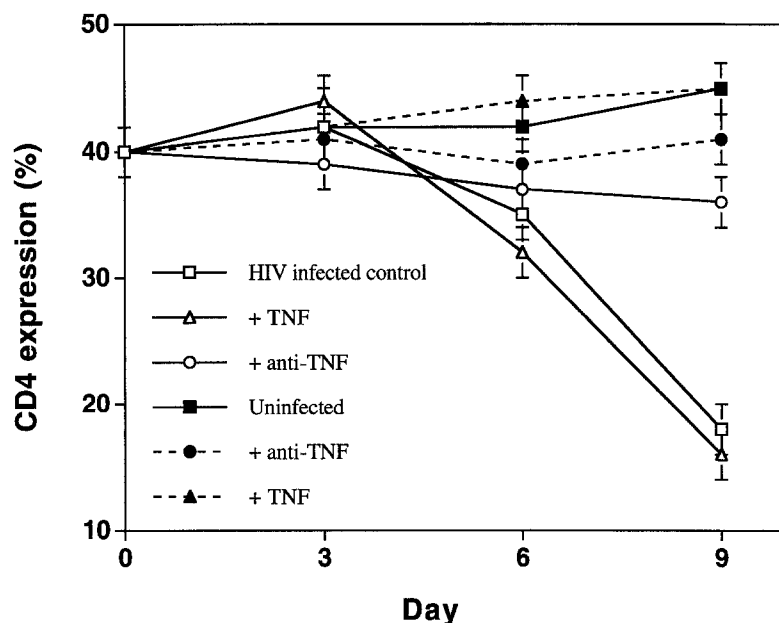


FIG. 2. Prevention of HIV-1-induced CD4⁺ cell depletion by anti-TNF- α . Human T cells were stimulated with anti-CD3 antibody and infected or mock-infected with HIV-1. Anti-TNF- α mAb (20 μ g/ml) or TNF- α (10 ng/ml) was added to the cultures where indicated. Three, 6, and 9 days after infection, the percentage of CD4⁺ cells was evaluated by direct flow cytometry. Data (mean $\pm$ SEM of triplicate cultures) correspond to the same experiment of Fig. 1. Control irrelevant P3 mAb had no effect (not shown).

of uninfected cells was similarly inhibited by anti-TNF- α treatment, although it did not decay after 6 days of activation, as in infected cells, because of the lack of cell destruction associated with HIV replication (Fig. 3C).

Although these results appear to suggest that inhibition of T-cell proliferation by anti-TNF- α was not responsible for the block of HIV replication, they cannot completely discard it. On the other hand, supplementing the cultures with higher doses of IL-2 (50 U/ml instead of 5 U/ml, added from the initiation of the cultures) increased T-cell proliferation and reverted the observed inhibition of anti-TNF- α mAb in cell proliferation at all time points (Fig. 3B) in all of the five donors tested. This treatment did not affect the inhibition of HIV-1 p24 antigen release by anti-TNF- α in the same cultures (Fig. 3A).

Control of NF- κ B activation by endogenous TNF- α

TNF- α is a major inducer of the activation of NF- κ B that has been recently shown to be essential for HIV-LTR transcription in primary T cells.³⁴ Therefore, anti-TNF- α could inhibit HIV replication by preventing the activation of NF- κ B endogenously secreted TNF- α . Two NF- κ B-DNA complexes (specifically competed by an oligonucleotide corresponding to the κ B sequence of the HIV-LTR) were detected by electrophoretic mobility shift assays in the nucleus of HIV-infected and activated T cells. The lower band has been shown previously to correspond to inactive p50 NF- κ B homodimers, whereas the upper one contains the p50/c-rel and p50/p65 heterodimers of the NF- κ B family,³⁰ which have strong transactivating activity.^{14,15} In

contrast, resting T cells only expressed detectable levels of p50 NF- κ B homodimers in the nucleus. In agreement with the above-mentioned hypothesis, we found that continuous presence of anti-TNF- α mAb in the culture almost completely prevented the appearance of the upper NF- κ B binding activity in the nucleus of T cells induced by the primary stimulus (anti-CD3 antibodies) either 12 hours after infection (Fig. 4) or at any time after activation (data not shown). Furthermore, exogenous TNF- α addition potentiated NF- κ B over the levels already induced by anti-CD3. Supplementation of the cultures with high doses of IL-2 (50 U/ml), which completely prevented inhibition of anti-TNF- α in T-cell proliferation, had no effect on NF- κ B activation and did not recover the NF- κ B inhibition observed in presence of anti-TNF- α (Fig. 4). The inhibition that we observed with different doses of anti-TNF- α in NF- κ B activation significantly correlated with a similar percent inhibition of HIV-1 replication for each of the three donors tested (Fig. 5).

Autocrine effect of TNF- α in HIV-LTR-dependent transcription

To corroborate that the observed inhibition of NF- κ B activation in the presence of anti-TNF- α mAb could be responsible for the blocking of HIV replication, we tested the effect of those antibodies in LTR-dependent transcription. TcR triggering of resting human T cells induces activation that in turn activates NF- κ B.^{19,37} So, we transfected human resting T cells with a luciferase reporter gene under control of the HIV-LTR and stimulated the cells with PMA, a strong PKC activator. In

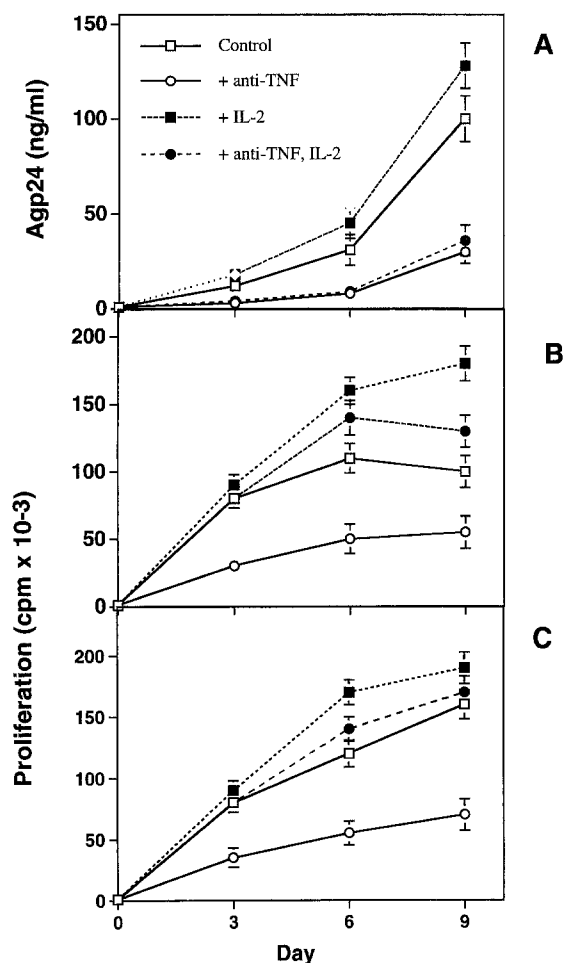


FIG. 3. Effect of exogenous IL-2 on the inhibitory effect of anti-TNF-α. Human T cells were stimulated with anti-CD3 antibody and infected with HIV-1 (A and B) or mock-infected (C). Anti-TNF-α mAb (20 μg/ml) and/or IL-2 (50 U/ml) was added to the cultures where indicated. A, Mean Ag p24 ± SEM in triplicate culture supernatants. B, C, Mean (³H) thymidine ± SEM incorporated of triplicate cultures. Control irrelevant P3 mAb had no effect (not shown).

agreement with previous results,³⁴ the activity of this construct was negligible in resting T cells. However, upon 12 hours of PMA activation, luciferase activity could be detected in those cells. We observed an almost complete inhibition (range of 85% to 95%, two donors) of the luciferase activity induced by anti-TNF-α (Fig. 6).

Some reports indicate that LTR-controlled transcription in primary T cells requires the presence of active Tat.^{34, 38} To study whether the presence of Tat may alter the anti-TNF-α effect, we cotransfected T cells with a CMV-Tat plasmid expressing HIV-1 Tat under the control of the CMV promoter, which is able to drive Tat synthesis in the absence of T-cell activation. In those conditions, some enhancing effect of Tat was seen, although this was not as great as previously reported.³⁶ Neutralizing anti-TNF-α mAb was also able to inhibit to a similar extent LTR-driven transcription in presence of

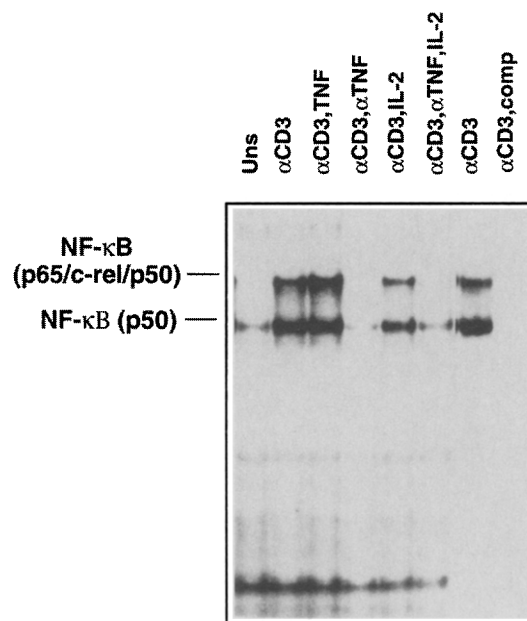


FIG. 4. Role of endogenous TNF-α in the activation of NF-κB. Human HIV-infected T cells were cultured in medium (Uns) or stimulated with anti-CD3 antibody in the presence of neutralizing anti-TNF-α mAb (10 μg/ml), IL-2 (50 U/ml), or TNF-α (20 ng/ml), alone or in combination, as indicated. The binding activity of NF-κB in the nucleus of T cells was assayed 12 hours later with a κB-HIV-labeled probe as described. Control specific binding was detected by using as competitor (comp) 50-fold excess of unlabeled κB oligonucleotide.

Tat (Fig. 6). On the other hand, TNF-α alone was insufficient to trigger LTR in resting T cells (data not shown), indicating that a previous activation is essential to trigger HIV expression in resting T cells. This effect probably is due to the fact that resting T cells do not express TNF-α receptors and need PKC activation to induce its expression.³⁰

DISCUSSION

Although there is considerable evidence that cytokine expression is altered in HIV-infected individuals, the precise role that endogenous cytokines play in modulating infection is not yet fully determined. Thus it has been proposed that several cytokines generated during the immune response play an important role in maintaining the continuous activation of HIV replication.³ Among those cytokines, TNF-α appears to be one of the most important. We have shown here that autocrine TNF-α production has an important role in controlling acute HIV-1 replication in primary T cells. This is indicated by the ability of neutralizing anti-TNF-α antibodies (1) to decrease p24 antigen release, (2) to prevent the depletion of CD4⁺ cells associated with HIV-1 infection, (3) to inhibit LTR driven transcription of a reporter gene in response to activation.

Previous reports have emphasized the importance of TNF-α in HIV replication.^{20, 22} Moreover, an autocrine role of TNF-α in HIV reactivation from latency has been

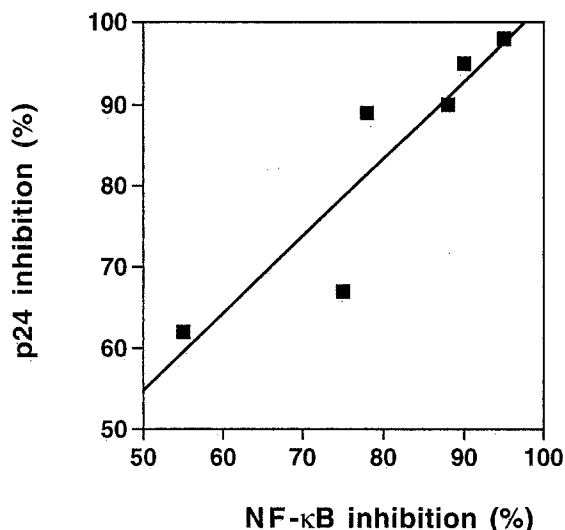


FIG. 5. Correlation between inhibition of NF- κ B activation and of HIV-1 replication by anti-TNF- α . The percentage of inhibition observed in three different donors with two doses of anti-TNF- α on NF- κ B activation as evaluated by densitometric scanning of the corresponding bands of the gels from electrophoretic mobility shift assays was plotted against the inhibition of p24 Ag production in the same cultures (correlation coefficient, $r = 0.9074$).

suggested through the use of chronically infected cells as a model.^{23,39} Evidence that autocrine/paracrine regulation of HIV replication also occurs in primary cells such as PHA-blasts and macrophages has also been obtained. Thus in acute *in vitro* infection of PBMCs, neutralizing anti-TNF- α blocked HIV replication.⁴⁰ Recently, Kinter et al. have demonstrated that in long-term cultures of PBMCs with IL-2, HIV replication is dependent on the autocrine secretion of various cytokines, including, TNF- α .⁴¹ Our results confirm and expand those results, providing an explanation about the molecular basis for this autocrine activity of TNF- α , which most likely takes place through the control of NF- κ B levels.

Although anti-TNF- α affects several other parameters of T-cell activation such as cell proliferation, its main antiretroviral effect appears to be related to the inhibition of NF- κ B activation because a good correlation between this inhibition and p24 inhibition was observed. Moreover, anti-TNF- α blockade of T-cell proliferation but not of HIV replication or NF- κ B activation could be bypassed by IL-2 addition.

We have shown previously that the duration and the amount of the NF- κ B response are regulated by the amount and persistence of the local levels of TNF- α that maintains the continuous translocation of the factor and stimulates the synthesis of the NF- κ B proteins.^{30,42} Those results together with those reported here strongly suggest that endogenously produced TNF- α maintains the levels of NF- κ B elevated, which are also essential for LTR transactivation in primary T cells,³⁴ and pointed to an important role of TNF- α in the maintenance of viral replication. In this regard, it is worth mentioning that HIV infection of

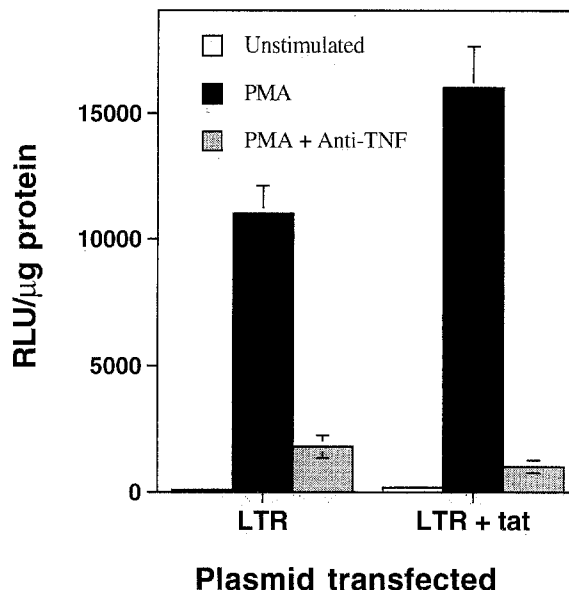


FIG. 6. Effect of anti-TNF- α on HIV-1 LTR activity. Human resting T cells were electroporated with the plasmids HIV-LTR-luc alone or together with CMV Tat. After 12 hours, the cells were stimulated with PMA (10 ng/ml) in the presence or absence of anti-TNF- α mAb (10 μ g/ml) or kept in culture medium unstimulated as indicated and the amount of luciferase in the cells estimated 12 hours later. Control irrelevant P3 mAb had no effect (not shown). RLU, Relative luciferase units.

mononuclear cells is associated with increased synthesis of several cytokines including TNF- α .^{31,32,43} and that B cells from HIV patients spontaneously secrete TNF- α .⁴⁴ In primary cell cultures, the continuous secretion of TNF- α by infected T cells³² and monocytes^{31,43} may provide the constant stimulus for NF- κ B activation potentiating HIV replication in neighboring activated T cells. Furthermore, the TNF- α produced after T-cell activation could be increased by HIV-1 Tat as a consequence of infection,³² resulting in further enhancement of HIV replication, therefore constituting a vicious circle. In support of this, it has been shown that TNF- α levels are higher in the lymph nodes, where viral burden and replication are higher.⁷

TNF- α has been shown to induce HIV in T-cell lines but not in T-cell clones despite inducing similar levels of NF- κ B. Furthermore, deletion of NF- κ B does not appear to affect anti-CD3-induced HIV replication in those T-cell clones.¹⁷ Those results are in apparent contradiction with ours. The reason for the discrepancies is not known, but it may lie in the different cell types used, since recent results indicate that requirements for HIV replication in primary and established cell lines are quite different.³⁴ T-cell clones are already activated, and restimulation with anti-CD3 may not lead to the same intracellular signals as in resting T cells.

Finally, our results indicate that HIV-1 replication can be separated from T-cell proliferation, which usually is taken as a marker of T-cell activation. This result opens

some new therapeutic possibilities because it indicates that it will be theoretically possible to inhibit NF- κ B and prevent HIV reactivation without affecting T-cell proliferation with a combination of NF- κ B inhibitor plus IL-2 mimetic drugs. Our results support the theory that the use of drugs for neutralizing inappropriately elevated production of NF- κ B or TNF activity, as is currently being carried out with thiocarbamate antioxidants⁴⁵ or pentoxifylline,⁴⁶ may be helpful as adjunctive therapy in AIDS and should be considered as an approach for management of HIV disease.

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