CONTRIBUTION TO THE REGULATION OF VIRUS REPLICATION IN CELLS LATENTLY INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS 1

S. OWATARI, J. ZHANG, M. GAO, A. TANABE-TOCHIKURA, N. WAKAMIYA, H. TSUCHIE, T. KURIMURA*

Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan

Received December 17, 1996; revised January 27, 1997

Summary. – Monocytes/macrophages have been known to play an important role in the initiation and propagation of human immunodeficiency virus 1 (HIV-1) infection. To analyze the function of these cells during the clinical asymptomatic period of infection, we examined the effect of murine peritoneal macrophages and human peripheral blood macrophages on two cell lines latently infected with HIV-1, a promonocytic cell line, U1, and a T-cell line, ACH-2. Monokines of the murine peritoneal macrophages induced significant viral expression in U1, but not in ACH-2 cells. Experiments employing transient transfection of U937 and CEM cells with HIV long terminal repeat (LTR)-chloramphenicol acetyl transferase (CAT) plasmids indicated that the effect of these monokines was due to specific activation of the HIV LTR. In contrast, supernatants of human macrophages induced viral expression in both ACH-2 and U1 cells. These results suggest that several monokines are active in regulating the transition from the clinical asymptomatic period of HIV infection to progression to acquired immunodeficiency syndrome (AIDS).

Key words: monocyte/macrophage; monokines; ACH-2 cells; U1 cells

Introduction

HIV is the causative agent of AIDS which is characterized by a long clinical asymptomatic period of a decade or more. During this stage, $CD4^+$ T-lymphocytes form the primary viral reservoir in the periphery, whereas monocytes/macrophages constitute the main solid tissue reservoir which is relatively resistant to HIV-1-induced cytopathological changes. To analyze mechanism(s) of activation or replication of HIV in the persistent state, U1 and ACH-2 cells were used to study the cellular and viral factors involved in these processes. Each U1 cell harbors 2 copies of the HIV provirus, whereas an ACH-2 cell harbors only one. These cells are of monocyte/macrophage and T-cell lineages, respectively. They constitutively produce progeny virions at a very low level. It has been reported that a productive replication of HIV in these cells can be triggered by a variety of activators, such as the phorbol-12-myristate-13 acetate (PMA) (Folks *et al.*, 1988; Biswas *et al.*, 1992) as well as by several cytokines, such as tumour necrosis factor alpha (TNF-α) (Duh *et al.*, 1989; Folks *et al.*, 1989; Poli *et al.*, 1990; Bressler *et al.*, 1993; Szabo *et al.*, 1993). Several studies have reported that the mechanisms of HIV replication in these two cell lines are different (Chen *et al.*, 1994; Fujinaga *et al.*, 1995). For instance, viral replication is enhanced in U1 cells by their incubation with exogenous Tat protein whereas that in ACH-2 cells does not increase with the addition of this protein (Cannon *et al.*, 1994). Lipopolysaccharide (LPS)

*Corresponding author.

Abbreviations: AIDS = acquired immunodeficiency syndrome; CAT = chloramphenicol acetyl transferase; ELISA = enzyme-linked immunosorbent assay; FBS = foetal bovine serum; GM-CSF = granulocyte/macrophage-colony stimulating factor; HIV-1 = human immunodeficiency virus 1; LPS = lipopolysaccharide; LTR = long terminal repeat; PBL = peripheral blood leukocytes; PBMC = peripheral blood mononuclear cells; PBS = phosphate-buffered saline; PMA = phorbol-12-myristate-13-acetate; TNF-α = tumour necrosis factor alpha

plus granulocyte/macrophage colony-stimulating factor (GM-CSF) dramatically induced HIV-1 production in U1 but not in cloned T cell lines (Pomerantz *et al.*, 1990). This study was undertaken to investigate the influence of monokines on latently infected monocytic and T-lymphocytic cells using murine or human monocytes/macrophages.

Materials and Methods

Cells. A subclone of HIV-1-infected U937 cells, U1 (Folks *et al.*, 1988), and a subclone of the CEM T-lymphocytic line, ACH-2 (Clouse *et al.*, 1989), were kindly supplied by Dr. T.M. Folks, Centers for Disease Control and Prevention, Atlanta, GA. U937 (Sundstrom *et al.*, 1976) and CEM (Foley *et al.*, 1965) cells are described elsewhere. All cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and kanamycin (60 mg/ml) at 37°C in 5% CO_2.

Plasmids. To construct the HIV-LTR-CAT reporter plasmid, proviral HIV-LTR of ACH-2 or U1 cells was amplified by PCR with an upstream sense primer containing a *Pst*I site and a downstream antisense primer containing a *Xba*I site. The upstream primer was ACGTACTGAAGC$^{(8753)}$GCGCAAGGCTACTTCC$^{(8737)}$ and the downstimer was TCGTATCTAGA$^{(131)}$CCAGAGTCACACAACAGACGGG$^{(109)}$(restriction sites are underlined), and these mapped to positions 8753 to 8737 and 131 to 109, respectively, on the HIV Bru sequence (Delassus *et al.*, 1991). The PCR products were digested with *Pst*I and *Xba*I and ligated to the basic CAT vector (Promega). Therefore, the expression vector pACH-LTR-CAT contained ACH-2 proviral LTR and pU1-LTR-CAT contained U1 proviral LTR.

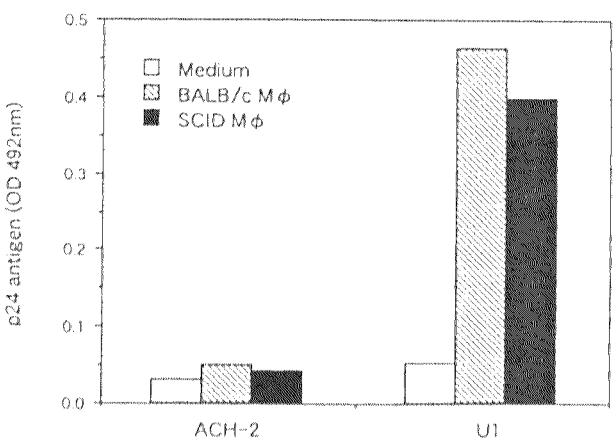

Fig. 1
Effect of BALB/c and SCID mouse peritoneal macrophages (Mϕ) on ACH-2 and U1 cells

ACH-2 or U1 cells (2 x 10^5/ml) were cultured with or without murine peritoneal macrophages (5 x 10^5/ml) for 3 days. Culture supernatants were collected and assayed for HIV p24 antigen. The results are expressed as the means of duplicate samples for each set of culture conditions.

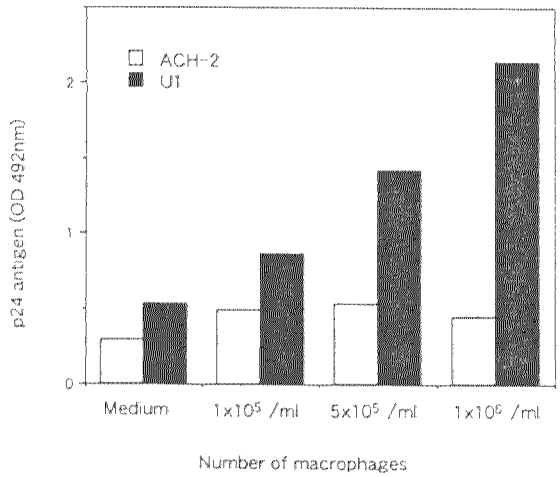

Fig. 2
Effect of culture supernatants of BALB/c mouse peritoneal macrophages on ACH-2 and U1 cells

The supernatants of cultures in which various number of BALB/c mouse peritoneal macrophages were incubated for 48 hrs were added to ACH-2 or U1 cells (2.5 x 10^5/ml). After incubation, the culture supernatants were collected and assayed for HIV p24 antigen. The results are expressed as the means of duplicate samples for each set of culture conditions.

CAT assay. CEM and U937 cells were transfected with the HIV-LTR-CAT reporter plasmids by DEAE-dextran co-precipitation. Then, 1 x 10^7 cells were washed with phosphate-buffered saline (PBS) and incubated for 30 mins with 10 μg of plasmid DNA in 1 ml of DEAE-dextran (Pharmacia) solution (300 mg/ml). Five hours after transfection, the cells were co-cultured with peritoneal macrophages of BALB/c or SCID mice. After 48 hrs of incubation, cell extracts were prepared and CAT activity was measured as previously described (Lifson *et al.*, 1986).

HIV-1 p24 gag antigen assay. In each experiment, the cell-free culture supernatant was collected after incubation and the p24 antigen level was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit with an anti-p24 gag monoclonal antibody, as described in the manufacturer's instructions (Abbott Laboratories).

Macrophages. C.B-17 scid/scid (SCID) and BALB/c mice were kept under sterile conditions in an animal house. Murine peritoneal cells were collected by washing intraperitoneal cavities of unstimulated SCID and BALB/c mice with PBS. Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donor blood by Ficoll-Hypaque gradient centrifugation, suspended in serum-free RPMI 1640 medium in a 75 cm^2 flask (Iwaki Pyrex) and incubated for 1 hr in a humidified CO_2 incubator at 37°C. After washing off non-adherent cells, the adherent ones were collected and resuspended in RPMI 1640 medium with 10% FBS.

Substances. TNF-α (Australia Biologicals), PMA (Promega); recombinant human GM-CSF and recombinant murine GM-CSF (Genzyme); recombinant human TNF-α and recombinant murine TNF-α (Pepro Tech EC Ltd); polyclonal anti-human TNF-α antibody, polyclonal anti-murine TNF-α antibody and polyclonal anti-murine GM-CSF antibody (R & D Systems) were used.

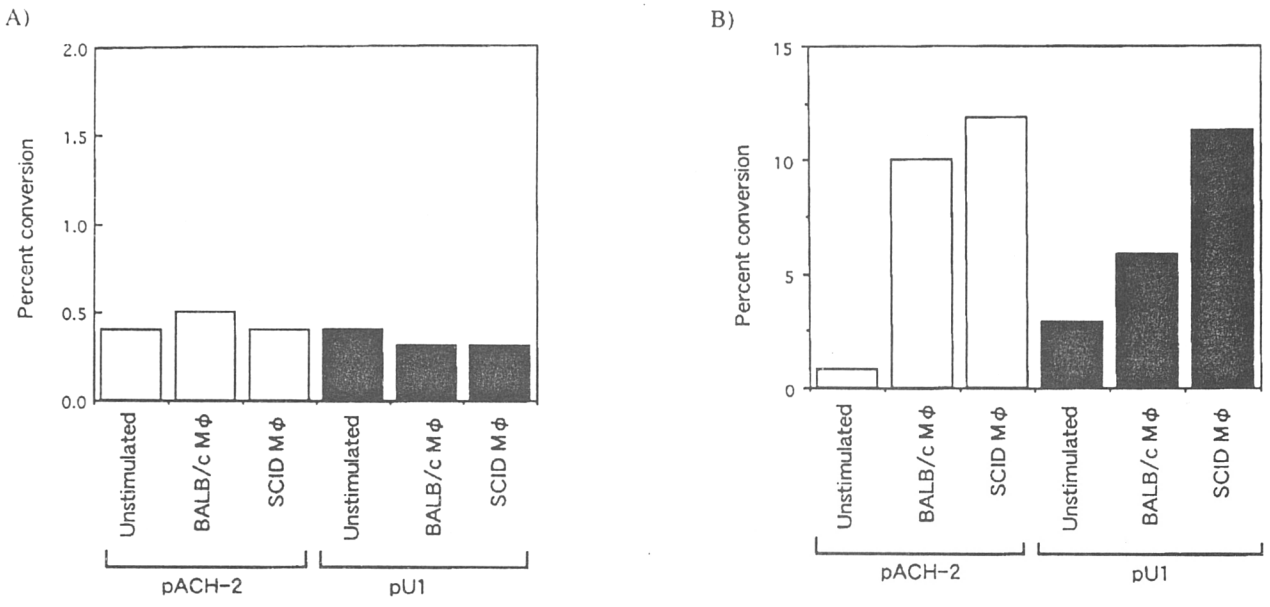

Fig. 3

Effect of murine peritoneal macrophages (Mϕ) on CEM and U937 cells transfected with HIV-LTR-CAT plasmid

CEM (A, 1 x 10^7 cells) or U937 (B, 1 x 10^7 cells) were transfected with either pACH-LTR-CAT (empty columns) or pU1-LTR-CAT plasmid (full columns). Five hours after transfection, the cells were co-cultured with peritoneal cells (5 x 10^5/ml) of BALB/c or SCID mice. After an incubation for 48 hrs, the cell extracts were assayed for CAT activity. The results are expressed as the means of duplicate samples for each set of culture conditions.

Results and Discussion

The mechanisms by which a low-productive HIV infection is converted into a productive one are still unclear. Latently infected cell lines have been intensively investigated to elucidate the kinetics of HIV expression in persistent infection models of HIV *in vivo*. Different responses of ACH-2 and U1 cells to several stimulators have been reported.

To investigate the role of the monocyte/macrophage in virus expression in latently infected cells, we initially used peritoneal macrophages of BALB/c or SCID mice as the stimulators. The SCID mouse in particular is characterized by the absence of functional T- and B-lymphocytes (Schuler *et al.*, 1986), therefore, it was useful to examine the macrophage function in a T-cell-independent manner. ACH-2 or U1 cells (2 x 10^4/ml) were co-cultured for 3 days with peritoneal macrophages of BALB/c or SCID mice (5 x 10^4 cells/ml), and supernatants were collected to measure the HIV p24 antigen production. In this experiment, peritoneal macrophages from both types of mice had the ability to induce virus expression in U1 cells, but neither had any effect on virus expression in ACH-2 cells (Fig. 1).

These results suggested that certain substances released from these macrophages were selectively activating provirus in the latently infected monocytic U1 cells. The released substances might account for the discordant results obtained from a study using the SCID mouse transplanted with human PBL model in which the degree of $CD4^+$ depletion following HIV infection did not correlate with the *in vitro* cytopathicity of the viral strain (Mosier *et al.*, 1993).

To examine whether the substances released from the murine macrophages could be found as soluble factors in the culture supernatants, various numbers of BALB/c peritoneal macrophages were incubated for 48 hrs and the supernatants were added to ACH-2 or U1 cells (2.5 x 10^5/ml). After incubation, the p24 production was calculated. In this experiment, the supernatants selectively activated virus expression in U1 cells in a dose-dependent manner (Fig. 2). In contrast, ACH-2 cells were not affected by the culture supernatants and therefore we presumed that the active substances in these supernatants were soluble and were most likely cytokines that specifically affected the monocytic line, and that ACH-2 cells could be deficient in their receptors. A high cell density of latently infected cells or murine peritoneal macrophages used in this experiment apparently accounted for a high level of the p24 antigen production as compared to the experiment in Fig. 1.

To examine whether the activation of the provirus contributes to the direct stimulation of the LTR region, U937 and CEM cells were transfected with either pACH-LTR-CAT or pU1-LTR-CAT using the DEAE-dextran co-precipitation procedure. After transfection, peritoneal macrophages of SCID or BALB/c mice were co-cultured with transfected cells and incubated at 37°C for 48 hrs. The cells were then collected and their extracts were assayed for CAT ac-

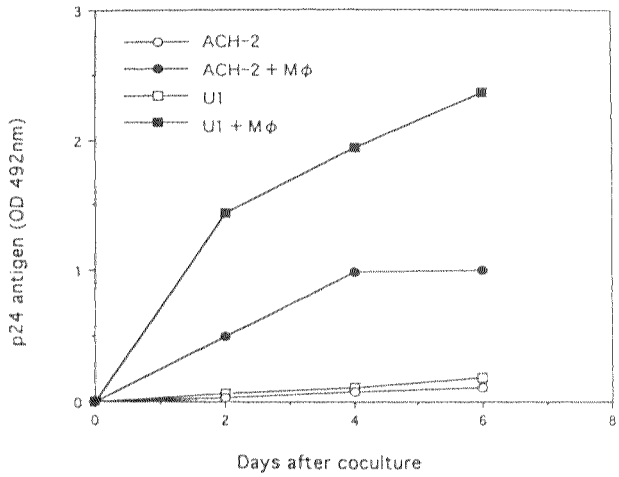

Fig. 4
Effect of human macrophages (MΦ) on ACH-2 and U1 cells
ACH-2 or U1 cells (2.5 x 10^5/ml) were co-cultured with or without human macrophages (5 x 10^5/ml). The culture supernatants were collected on days 2, 4, and 6 and measured for HIV p24 antigen. The results are expressed as the means of duplicate samples for each set of culture conditions.

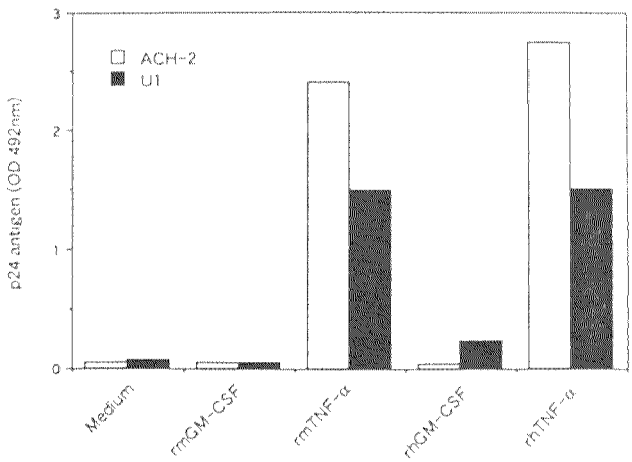

Fig. 5
Effect of recombinant murine (rm) or human (rh) cytokines on HIV-1 expression in latently infected cells
rmGM-CSF, rmTNF-α, rhGM-CSF and rhTNF-α were added to ACH-2 or U1 cells (2 x 10^5/ml) to a final concentration of 100 ng/ml. After 48 hrs, the supernatants were collected and assayed for HIV p24 antigen. The results are expressed as the means of duplicate samples for each set of culture conditions.

tivity. In this experiment, murine peritoneal macrophages did not enhance CAT activity in CEM cells transfected with either pACH-LTR-CAT or pU1-LTR-CAT (Fig. 3A). In contrast, these macrophages significantly enhanced CAT activity in U937 cells transfected with HIV-LTR-CAT

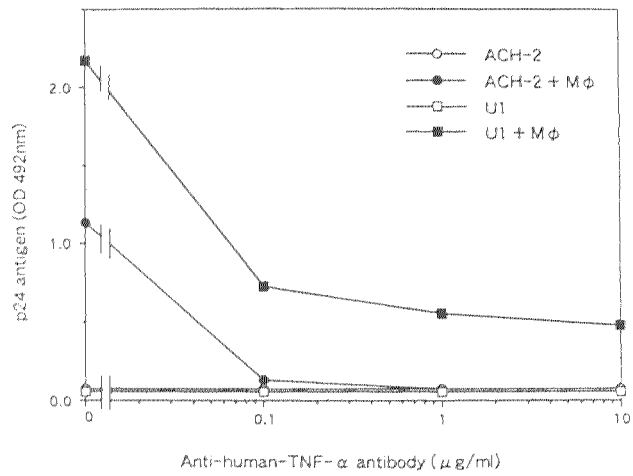

Fig. 6
Neutralization of HIV-1 expression in latently infected cells stimulated with human macrophages (MΦ) or non-stimulated by anti-human TNF-α antibody
Human macrophages (2 x 10^5/ml) were cultured for 48 hrs and the culture supernatants were collected. Various concentrations of anti-human TNF-α antibody were added to the supernatants, incubated for 1 hr at 37°C and then further incubated with ACH-2 or U1 cells (2 x 10^5/ml) for 48 hrs. After incubation, the culture supernatants were collected and assayed for HIV p24 antigen. The results are expressed as the means of duplicate samples for each set of culture conditions.

(Fig. 3B). To investigate whether the difference in the character of the provirus latency in ACH-2 and U1 cells might be correlated with the LTR promoter activity, we compared the activity of pACH-LTR-CAT and pU1-LTR-CAT stimulated with TNF-α and PMA. In this experiment, there was no difference in the CAT activity of pACH-LTR-CAT and pU1-LTR-CAT (data not shown). This suggests that the difference in the response to macrophages of these two cell lines is not related to the LTR promoter activity.

We also investigated the effect of human monocytes/macrophages obtained from healthy donor blood on cells latently infected with HIV. As shown in Fig. 4, both ACH-2 and U1 cells were activated by human monocytes/macrophages. TNF-α and GM-CSF (Folks *et al.*, 1987; Pomerantz *et al.*, 1990) were previously reported to induce virus production in latently infected cells. We tested virus induction in ACH-2 and U1 cells stimulated by recombinant human TNF-α and GM-CSF. TNF-α markedly activated both ACH-2 and U1 cells, but on the other hand, GM-CSF activated only U1, but not ACH-2 cells (Fig. 5). Moreover, the effect of human monocytes/macrophages was significantly neutralized by polyclonal anti-human TNF-α. Especially, proviral activation in ACH-2 cells was completely inhibited by this antibody (Fig. 6). These results indicate that TNF-α plays a major role in viral expression in latently infected cellls stimulated with human monocytes/macrophages.

In this study, we have shown that monokines of murine peritoneal macrophages have the ability to activate virus production in U1, but not in ACH-2 cells. As shown in Fig. 5, recombinant murine TNF-α stimulated both ACH-2 and U1 cells, although recombinant murine GM-CSF did not stimulate either cells. The activation of latently infected cells by murine peritoneal macrophages was not inhibited by 10 μg/ml anti-murine TNF-α antibody and anti-murine GM-CSF antibody, which were sufficient to neutralize 12.5 ng/ml TNF-α and GM-CSF, respectively. Therefore, we presume the existence of an active substance(s) other than murine TNF-α and murine GM-CSF in the culture supernatant of murine peritoneal macrophages. The substance(s) which has an ability to induce viral expression in monocytic cells specifically may affect only monocytes in SCID mice transplanted with human PBL. Therefore, this model may be not appropriate for studying the profile of the pathogenic potential of HIV infection in humans.

It has also been reported that several cytokines regulate virus expression *in vivo* in the clinical asymptomatic period of HIV infection. High levels of TNF-α (Lahdevirta *et al.*, 1988; Reddy *et al.*, 1988) and IL-6 (Gallo *et al.*, 1989; Breen *et al.*, 1990) in sera of AIDS patients have been reported and an *in vitro* study on the provirus has shown that several agents including cytokines modulate virus expression in various ways. Moreover, inappropriate secretion of a series of cytokines which are usually active in normal homeostatic control may lead to stimulation of the provirus in persistent infections. Further studies are needed to identify the monocyte/macrophage-derived factors and to analyze the regulation of the provirus in detail in order to clarify the transition from the clinical asymptomatic period of HIV infection to progression to AIDS.

Acknowledgement. We thank Dr. T. Folks of the Centers for Disease Control and Prevention (Atlanta, GA), for supplying ACH-2 and U1 cells through Dr. S. Kageyama of Toyama Medical and Pharmaceutical University, Toyama, Japan.

References

Biswas P, Poli G, Kinter A L, Justement J S, Stanley SK, Maury WJ, Bresler P, Orenstein JM, Fauci AS (1992): Interferon induces the expression of human immunodeficiency virus in persistently infected promonocytic cells (U1) and redirects the production of virions to intracytoplasmic vacuoles in phorbol myristate acetate-differentiated U1 cells. *J. Exp. Med.* **176**, 739–750.

Breen EC, Rezai AR, Nakajima K, Beall GN, Mitsuyasu RT, Hirano T, Kishimoto T, Martinez-Maza O (1990): Infection with HIV is associated with elevated IL-6 levels and production. *J. Immunol.* **144**, 480–484.

Bressler P, Poli G, Justement JS, Biswas P, Fauci AS (1993): Glucocorticoids synergize with tumor necrosis factor α in the induction of HIV expression from a chronically infected promonocytic cell line. *AIDS Res. Hum. Retroviruses* **9**, 547–551.

Cannon P, Kim SH, Ulich C, Kim S (1994): Analysis of Tat function in human immunodeficiency virus type 1-infected low-level-expression cell lines U1 and ACH-2. *J. Virol.* **68**, 1993–1997.

Chen BK, Saksela K, Andino R, Baltimore D (1994): Distinct modes of human immunodeficiency virus type 1 proviral latency revealed by superinfection of nonproductively infected cell lines with recombinant luciferase-encoding viruses. *J. Virol.* **68**, 654–660.

Clouse KA, Powell D, Washington I, Poli G, Strebel K, Farrar W, Barsted P, Kovacs J, Fauci AS, Folks TM (1989): Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *J. Immunol.* **142**, 431–438.

Delassus S, Cheynier R, Wain-Hobson S (1991): Evolution of human immunodeficiency virus type 1 nef and long terminal repeat sequences over 4 years in vivo and in vitro. *J. Virol.* **65**, 225–231.

Duh EJ, Maury WJ, Fouks TM, Fauci AS, Rabson AB (1989): Tumor necrosis factor α activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-KB sites in the long terminal repeat. *Proc. Natl. Acad. Sci. USA* **86**, 5974–5978.

Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA, McCarthy RE (1965): Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer* **18**, 522–529.

Folks TM, Clouse KA, Justement J, Rabson A, Duh E, Kehal JH, Fauci AS (1989): Tumor necrosis factor α induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc. Natl. Acad. Sci. USA* **86**, 2365–2368.

Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS (1987): Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science* **238**, 800–802.

Folks TM, Justement J, Kinter A, Schnittman S, Orenstein J, Poli G, Fauci AS (1988): Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. *J. Immunol.* **140**, 1117–1122.

Fujinaga K, Zhong Q, Nakaya T, Kameoka M, Meguro T, Yamada K, Ikata K (1995): Extracellular nef protein regulates productive HIV-1 infection from latency. *J. Immunol.* **155**, 5289–5298.

Gallo P, Frei K, Rordorf C, Lazdins J, Tavolato B, Fontana A (1989): Human immunodeficiency virus type 1 (HIV-1) infection of central nervous system: an evaluation of cytokines in cerebrospinal fluid. *J. Neuroimmunol.* **23**, 109–116.

Lahdevirta J, Maury CPJ, Teppo AM, Repo H (1988): Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. *Am. J. Med.* **85**, 289–291.

Lifson JD, Feinberg MB, Reyes GR, Rabin L, Banapour B, Chakrabarti S, Moss B, Wong-Staal F, Steimer KS, Engleman EG (1986): Induction of CD4-dependent cell fusion by

the HTLV-III/LAV envelope glycoprotein. *Nature* **323**, 725–728.

Moiser DE, Gulizia RJ, MacIsaac PD, Torbett BE, Levy JA (1993): Rapid loss of CD4⁺T cells in human-PBL-SCID mice by noncytopathic HIV isolates. *Science* **260**, 689–691.

Poli G, Kinter A, Justement JS, Kehrl JH, Bressler P, Stanley S, Fauci AS (1990): Tumor necrosis factor α functions in an autocrine manner in the induction of human immunodeficiency virus expression. *Proc. Natl. Acad. Sci. USA* **87**, 782–785.

Pomerantz RJ, Feinberg MB, Trono D, Baltimore D (1990): Lipopolysaccharide is a potent monocyte/macrophage specific stimulator of human immunodeficiency virus type 1 expression. *J. Exp. Med.* **172**, 253–261.

Reddy MM, Sorrell SJ, Lange M, Grieco MH (1988): Tumor necrosis factor and HIV p24 antigen levels in serum of HIV-infected populations. *J. AIDS* **1**, 436–440.

Schuler W, Weiler IJ, Schuer A, Phillips RA, Rosenberg N, Mak TW, Kearney JF, Perry RP, Bosma MJ (1986): Rearrangement of antigen receptor genes is defective in mice with severe combined immune deficiency. *Cell* **46**, 963–972.

Sundstrom C, Nilsson K (1976): Establishment and characterzation of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* **17**, 565–577.

Szabo B, Locardi C, Presti EL, Belardelli F, Benedetto A (1993): Tumour necrosis factor-α increases the sensitivity of human immunodeficiency virus (HIV)-infected monocyic U937 cells to the complement-dependent cytotoxicity of sera from HIV type 1 infected individuals; role of the gp120 protein. *J. Gen. Virol.* **74**, 1271–1276.