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HIV-1 envelope glycoprotein gp120 down-regulates CD4 expression in primary human macrophages through induction of endogenous tumour necrosis factor-α

V. KARSTEN, S. GORDON,* A. KIRN & G. HERBEIN *Virology Institute of the Faculty of Medicine and I.N.S.E.R.M. Unit 74, Louis Pasteur University, Strasbourg, France, and *Sir William Dunn School of Pathology, University of Oxford, UK*

SUMMARY

Among immunological abnormalities present in human immunodeficiency virus type 1 (HIV-1)-infected individuals are dysregulation of cytokine production and CD4 down-regulation in both T-helper cells and monocytes/macrophages. The HIV-1 envelope glycoprotein 120 (gp120) has the ability to induce different cytokines in peripheral blood mononuclear cells and in monocytes/macrophages *in vitro* which in some instances have been reported to down-regulate macrophage CD4 expression. This study provides evidence that HIV-1 recombinant gp120 (rgp120) down-regulates both surface and total CD4 expression in primary tissue culture-differentiated macrophages (TCDM) at the level of transcription. The CD4 down-regulation observed in TCDM occurred between 6 and 12 hr after rgp120 treatment preceded by a peak of endogenous tumour necrosis factor-α (TNF-α) observed at 3–6 hr post-treatment. We demonstrate that the TCDM CD4 down-regulation observed after rgp120 treatment was inhibited by the use of an anti-huTNF-α monoclonal antibody (mAb), but not by mAb directed against other cytokines induced by rgp120, such as interleukin-1β (IL-1β) and interferon-α (IFN-α). The present findings roughly parallel those observed both in the sera of patients and in the monocytes/macrophages isolated from HIV-positive individuals, suggesting that gp120 by stimulating endogenous TNF-α production could be a good candidate for the CD4 down-regulation observed in the monocytes/macrophages of HIV-1-infected individuals. In contrast to CD4 down-regulation in HIV-infected lymphocytes, which results from a direct effect of viral genes on CD4 expression, soluble factors such as cytokines induced during HIV infection might explain the monocyte/macrophage CD4 dysregulation observed in acquired immune deficiency syndrome.

INTRODUCTION

Progression of acquired immune deficiency syndrome (AIDS) is characterized by a dramatic fall in $CD4^+$ T cells concomitant with the development of host immunosuppression. In parallel, monocytes and macrophages, which also express the human immunodeficiency virus (HIV) receptor, the CD4 molecule, on their surface, play a key role in pathogenesis of the disease by acting as a reservoir of virions and by spreading the infection to different organs. An immunological dysregulation consisting of a Th1/Th2 cytokine shift[1] and hyperactivation of the immune system[2] could account for the progression of the disease via stimulation of viral replication, B-cell activation and apoptosis.[3]

In vivo the envelope glycoprotein gp120 is detected in the serum of AIDS patients[4] and *in vitro* recombinant gp120 (rgp120) has the ability to induce cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-α (IFN-α) from monocytes/macrophages.[5–10] These cytokines are also detected in peripheral blood and tissues of HIV-infected patients.[11,12] Surface CD4 expression on human monocytes/macrophages is down-regulated by rgp120 *in vitro*[13,14] and *in vivo* decreased surface CD4 expression has been described on blood monocytes isolated from AIDS patients.[15] Decreased surface CD4 expression has been reported in HIV-inoculated tissue culture-differentiated macrophages (TCDM) versus uninfected control cells *in vitro*. However among the HIV-inoculated TCDM, cells defined as virus-negative or virus-positive for HIV by flow cytometry analysis both display surface CD4 down-regulation,[16] suggesting that soluble factors induced by HIV or rgp120, such as cytokines, rather than viral products, might be involved in

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Abbreviations: IFN-α, interferon-α; IL-1β, interleukin-1β; mAb, monoclonal antibody; rgp120, recombinant envelope glycoprotein 120; TCDM, tissue culture-differentiated macrophages; TNF-α, tumour necrosis factor-α.

Correspondence: Dr G. Herbein, The Picower Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, USA.

TCDM CD4 down-regulation. In order to assess this hypothesis we tested the ability of rgp120 to down-regulate CD4 expression in primary TCDM. Then we determined the role of cytokines produced by rgp120-treated TCDM (TNF-α, IL-1β, IFN-α) in regard to TCDM CD4 down-regulation.

MATERIALS AND METHODS

Cell line

The CEM-SS lymphoid cell line was obtained from Dr P. Nara (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Washington, D.C.). CEM-SS were maintained in RPMI-1640 (Gibco-BRL, Paisley, UK) with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Isolation and culture of TCDM

Human monocytes were isolated from healthy donors as described;[17] in short, Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) isolated peripheral blood mononuclear cells (PBMC) were incubated for 1 hr on 2% gelatin-coated plates. Adherent TCDM, >94% $CD14^+$ by fluorescence-activated cell sorter (Becton Dickinson & Co., Mountain View, CA), were cultivated in RPMI supplemented with 10% v/v pooled AB human serum for 48 hr before transfer to six-well plates (Falcon, Lincoln Park, NJ) at a density of 4×10^6 cells in 3 ml total volume.

rgp120 treatment

TCDM were untreated or treated with 1 μg/ml recombinant HIV-1 IIIB gp120. This preparation was purchased from ABT (Cambridge, MA) and was produced in a baculovirus expression system. The presence of endotoxin in the rgp120 preparation was ruled out by using the Limulus E-Toxate assay (Limulus ameobocyte lysate; Sigma, Poole, UK). At times 1, 3, 6, 12 and 24 hr after rgp120 treatment, the supernatants were harvested in order to quantify TNF-α, IL-1β and IFN-α levels and the cells were collected for flow cytometry analysis and Western blotting.

To block the endogenous secretion of different cytokines induced by rgp120-treated TCDM, 2·6 μg/mL mouse/human (hu) chimeric anti-huTNF-α monoclonal antibody (mAb), cA2, (kindly provided by Dr M. Feldmann, Kennedy Institute of Rheumatology, London, UK), 3·3 μg/ml mouse anti-huIL-1β mAb (Genzyme, Cambridge, MA) or 660 IU/ml sheep anti-huIFN-α mAb (Sigma) were added to 4×10^6 TCDM treated with 1 μg/ml rgp120 in 3 ml final volume.

Detection of TNF-α, IL-1β and IFN-α in culture supernatants

Supernatants of primary human TCDM cultures were harvested from untreated cells or after 1 μg/ml rgp120 treatment for 1, 3, 6, 12 and 24 hr, and frozen at −20°. The levels of TNF-α and IL-1β were determined using an enzyme-linked immunosorbent assay (ELISA; Quantikine, R & D Systems, Abingdon, UK). The IL-1β ELISA allowed us to detect 0·3 pg/ml of IL-1β in culture supernatants. As a positive control for IL-1β secretion we treated for 12 hr primary human TCDM with 100 ng/ml of lipopolysaccharide (LPS) (phenol extract from *Escherichia coli*, Sigma); LPS-induced IL-1β levels ranged from 100 to 200 pg/ml (data not shown). IFN titration was performed on MDBK cells by inhibition of vesicular stomatitis virus growth as previously described.[18]

Flow cytometry

TCDM fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) were quenched with 10% v/v goat serum in PBS, labelled, and analysed by flow cytometry. Surface CD4 was detected by using mouse anti-huCD4 (mAb L120.3, ADP359, provided by the MRC AIDS Directed Programme, Herts, UK) followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Fab-specific) (Sigma Immunochemicals, Poole, UK). Specific fluorescence was assessed by comparison with irrelevant isotype-matched control mAb (Serotec, Oxford, UK). Intracellular staining was performed as above, but reagents were supplemented with 0·1% saponin during washing and with 0·3% saponin during labelling. Relative antigen density was calculated as follows:

$$\frac{\text{geometric mean (rgp120)} - \text{geometric mean (isotype control)}}{\text{geometric mean (mock)} - \text{geometric mean (isotype control)}}$$

The characterization of the TCDM population was confirmed by using mouse anti-huCD68 IgG_1 mAb (Dako Ltd, Cambridge, UK).

Western blot

Adherent TCDM were untreated or incubated with 1 μg/ml rgp120 for 3, 6, 12 and 24 hr, then washed and lysed. Protein concentrations in cell lysates were determined by a Bradford assay. Cell lysates were prepared in 150 mM NaCl, 10 mM EDTA, 10 mM Tris pH 8, 10 mM NaN_3, 1 mM phenylmethylsulphonylfluoride, 5 mM iodoacetamide, and 1% v/v Nonidet P-40. Cell lysates, heated at 56° for 30 min to dissociate gp120 binding to CD4, were electrophoresed in a 10% sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE) under non-reducing conditions and then electroblotted onto nitrocellulose (Amersham, Bucks, UK). Before probing, blots were blocked in PBS with 3% w/v dried milk, and 0·1% v/v Tween 20. Binding of IgG1 mouse anti-huCD4 mAb (Q4120) (Sigma Immunochemicals) was determined by incubation with a peroxidase-labelled anti-mouse IgG1 (Sigma Immunochemicals) and by chemiluminescence (ECL, Amersham). Incubations with both anti-huCD4 and peroxidase-labelled anti-mouse IgG1 were usually performed in the presence of 10% v/v pooled AB human serum as described previously.[19]

RNA extraction, reverse transcription and amplification of cDNA by polymerase chain reaction (RT–PCR)

Total cellular RNA was extracted from 4×10^6 TCDM using 1 ml of RNAzol (Cinna-Biotex, Houston, TX) with 20 μg *E. coli* rRNA (Boehringer, Mannheim, Germany) as carrier. Reverse transcription was performed in a volume of 50 μl containing 800 U of Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Gibco-BRL), 2·5 mM dNTP, 5 μg oligo-dT (12–18 mer) and 31 U RNAase inhibitor (all from Pharmacia) for 1 hr at 37°. PCR amplification was performed using a programmable thermal controller (Perkin Elmer Cetus, Bucks, UK) operating the following temperature steps: 60 s at 94°, then 30 cycles (30 s denaturation at 94°; 30 s annealing at 60°; 60 s extension time at 72°) as reported previously.[20] Magnesium chloride concentrations used for hu CD4 and hu

β-actin cDNA amplifications were 2·0 and 2·5 mM respectively. The following primer pairs were used: huCD4 (5′ primer: GCTAGGCATCTTCTTCTGTG; 3′ primer: CTGCTACATTCATCTGGTCC; size of amplified fragment: 223 bp); huβ-actin (5′ primer: GATGCAGAAGGAGATCACTG; 3′ primer: AGTCATAGTCCGCCTAGAAG; size of amplified fragment: 205 bp), all synthesized by Eurogentec (Seraing, Belgium). To test equal amount of cellular extract for each sample we performed one-third sequential dilution of each RNA extract. After reverse transcription we adjusted β-actin cDNA for one sample to another and measured CD4 mRNA time–course after rgp120 treatment. The detection of both β-actin and CD4 cDNA was performed in the linear part of the amplification curve. PCR products were separated by 2% agarose electrophoresis and visualized by ethidium bromide staining. A DNA ladder (type VI, Boehringer) was used for molecular weight determination.

Statistical analysis

Figures show the means of at least three independent experiments and standard deviations.

RESULTS

TCDM were treated with 1 μg/ml HIV-1 rgp120 for 1–24 hr or left untreated and assessed for both surface and total CD4 expression. By using flow cytometry we showed that surface CD4 expression was not modulated during the first 3 hr post-treatment, but a 30% surface CD4 down-regulation occurred transiently at 6–12 hr post-treatment followed by recovery to steady-state levels at 24 hr (Fig. 1). To determine if both surface and total CD4 expression were modulated in parallel after rgp120 treatment, we studied the expression of total CD4 by performing flow cytometric analysis of saponin-permeabilized TCDM and Western blotting. Figure 2a demonstrates by flow cytometry analysis of saponin-permeabilized TCDM that total CD4 in TCDM was down-regulated with approximately 60% residual expression at 6–12 hr after rgp120 addition; a subsequent total CD4 increase was observed at 24 hr post-treatment. In agreement with these data a Western blot performed at different times after rgp120 treatment demonstrated that the total CD4 amount was not modified at 3 hr but decreased CD4 levels were observed at 6–12 hr post-treatment (Fig. 2b). At 24 hr post-treatment total CD4 expression started to increase to reach levels similar to that of untreated TCDM although the extent of up-regulation was inconsistent depending on donor variability (data not shown). At 24 hr post-treatment an additional band of 60 000 MW was observed (Fig. 2b) which disappeared when human serum was used during the labelling steps (data not shown) suggesting non-specific binding of the anti-huCD4 mAb to Fc receptor as reported previously.[19]

In order to determine if TCDM CD4 down-regulation occurred at the level of transcription we performed an RT–PCR assay (Fig. 3). An early CD4 mRNA decrease was observed between 1 and 3 hr post-treatment with rgp120 followed by a progressive increase over time with levels at 24 hr equal to or higher than those observed in an untreated TCDM. β-actin was used as an internal control. To perform a CD4 mRNA time–course after rgp120 treatment equal amounts of cellular extracts were used (data not shown). These results suggest

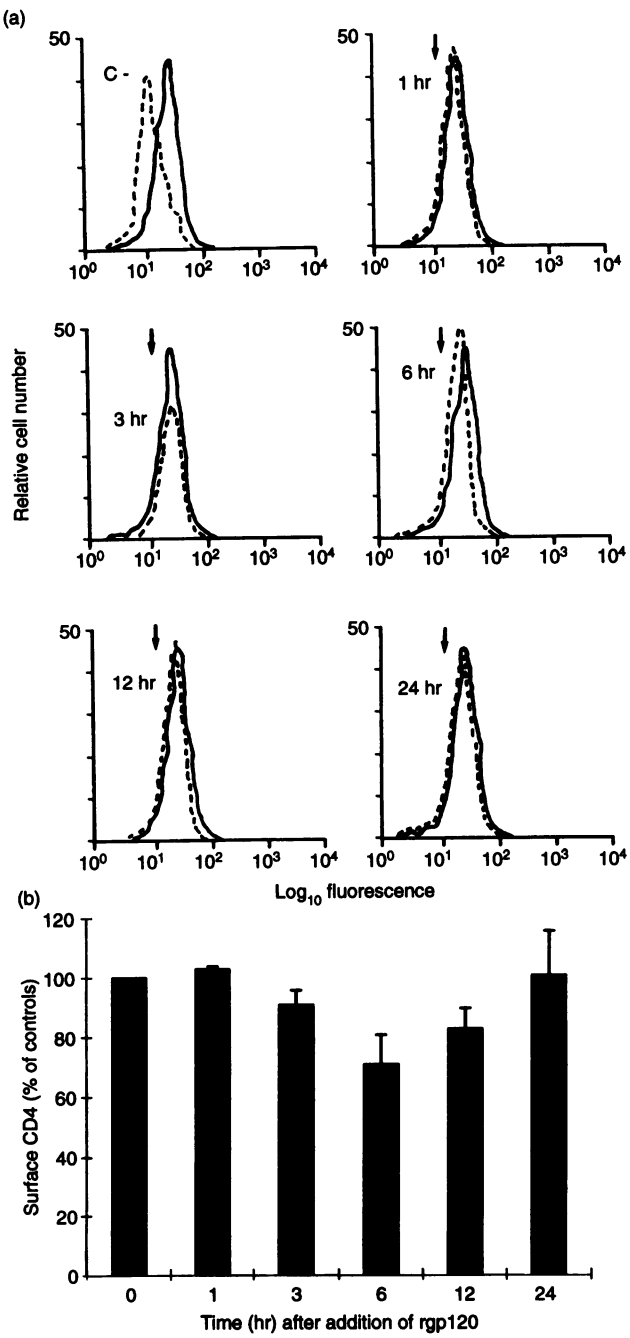

Figure 1. Time–course of surface CD4 expression in primary human TCDM treated with rgp120. Three-day-old TCDM were left untreated or treated with 1 μg/ml rgp120 for 1, 3, 6, 12 and 24 hr, then analysed for surface CD4 expression by flow cytometry as described in the Materials and Methods. Fixed TCDM were first gated on the basis of forward scatter and side scatter. All gated cells were TCDM as confirmed by anti-CD68 mAb labelling (data not shown). (a) Fluorescence profiles show surface CD4 levels at different times after addition of rgp120 (broken lines) versus untreated control cells (solid lines). Negative control (C−) and arrows indicate fluorescence of TCDM incubated with the irrelevant isotype-matched control mAb. (b) The histogram summarizes the results of three independent experiments. Bars show standard deviations.

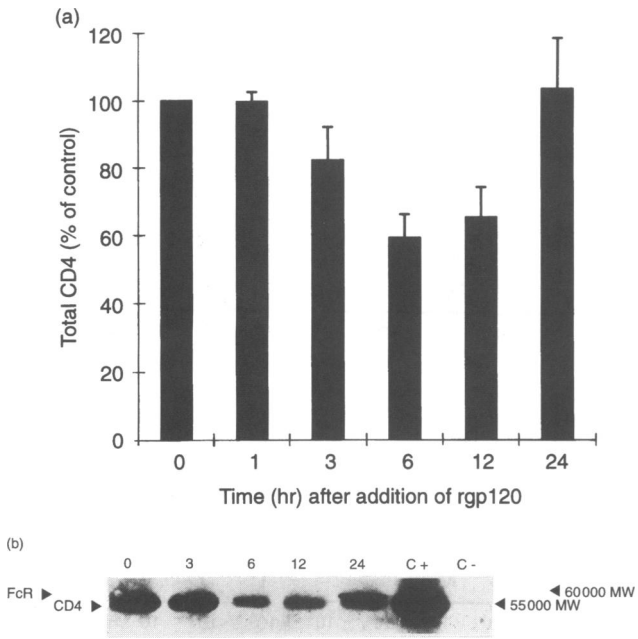

Figure 2. Time–course of total CD4 expression in primary human TCDM treated with rgp120. Three-day-old TCDM were left untreated or treated with 1 μg/ml rgp120 for 1, 3, 6, 12 and 24 hr, then analysed for total CD4 expression. (a) Total CD4 expression of saponin-permeabilized rgp120 treated TCDM analysed by flow cytometry. The histogram summarizes the results of three independent experiments. Bars show standard deviations. Fixed saponin-permeabilized rgp120-treated TCDM were gated on the basis of forward scatter and side scatter; all gated cells were TCDM as confirmed by anti-CD68 labelling (data not shown). (b) Total CD4 expression in TCDM either untreated or treated with rgp120 for different times was analysed by Western blot; 20 μg of cellular protein were loaded per lane, 20 μg of CEM-SS extract were used as positive control (C+), and water as negative control (C−). In this experiment the Western blot was performed without 10% v/v pooled AB human serum during labelling steps so that, in addition to the specific 55 000 MW CD4 band, a 60 000 MW band corresponding to the non-specific binding of anti-CD4 mAb to Fc receptor could be observed at 24 hr post-treatment.

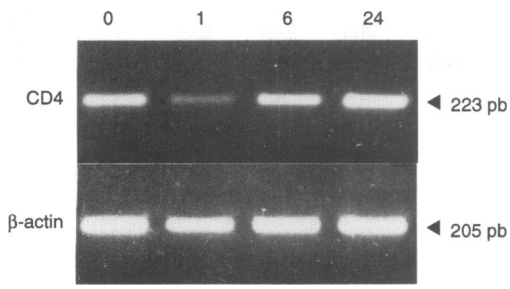

Figure 3. Recombinant gp120 modulates CD4 mRNA in primary human TCDM. Three-day-old TCDM were left untreated or treated for 1, 6 and 24 hr with 1 μg/ml rgp120. RNA was extracted from 4×10^6 cells with 20 μg *E. coli* rRNA as carrier, reverse transcribed and the cDNA amplified as described in the Materials and Methods. The PCR products for human CD4 (223 bp) and for control β-actin (205 bp) were visualized on a 2% agarose gel. The results shown are representative of three independent experiments.

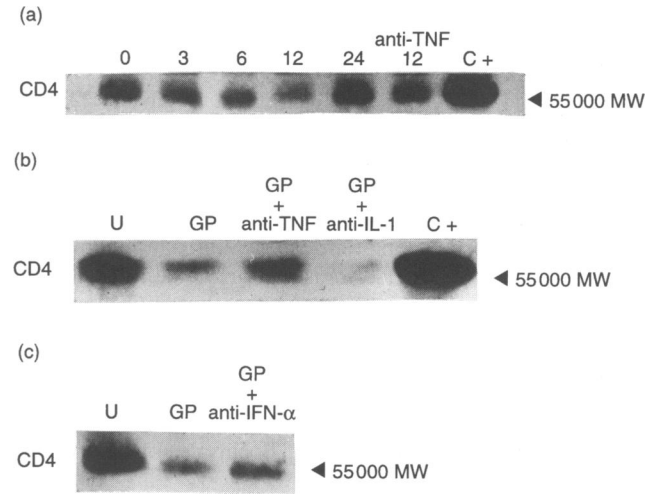

Figure 4. Neutralization of endogenous TNF-α, but not IL-1β and IFN-α, blocks TCDM down-regulation of CD4 by rgp120. Three-day-old-TCDM were untreated or treated for different times with rgp120 (1 μg/ml), rgp120 (1 μg/ml) mixed with either anti-huTNF-α mAb (2·6 μg/ml), anti-huIL-1β mAb (3·3 μg/ml) or anti-huIFN-α mAb (660 IU/ml). Then Western blotting was performed (in the presence of 10% v/v pooled AB human serum during labelling steps to avoid non-specific binding to Fc receptor) to detect total CD4 (55 000 MW) in TCDM; 20 μg of cellular protein were loaded per lane and 20 μg of CEM-SS extract were used as positive control (C+). (a) Time-course (0–24 hr) of total CD4 expression in TCDM treated with rgp120. Addition of anti-huTNF-α mAb to rgp120-treated TCDM restored CD4 expression at 12 hr post-treatment. (b) In contrast to anti-huTNF-α mAb, anti-huIL-1β mAb did not restore total CD4 expression in rgp120-treated TCDM at 12 hr post-treatment. Total CD4 expression was studied in untreated TCDM (U) versus TCDM treated for 12 hr with rgp120 alone (GP) or mixed with anti-huTNF-α (GP + aTNF) or anti-huIL-1β (GP + aIL-1) mAb. (c) Anti-hu IFN-α mAb did not neutralize significantly total CD4 down-regulation in rgp120-treated TCDM. Total CD4 expression was studied in untreated TCDM (U) versus TCDM treated for 12 hr with rgp120 alone (GP) or mixed with anti-huIFN-α mAb (GP + aIFN-α). For each mAb Western blot analyses were repeated in three independent experiments and showed the same trends.

that CD4 down-regulation in rgp120-treated TCDM might be regulated at the level of transcription.

Recombinant gp120 has been reported to induce the production of cytokines, such as TNF-α, IL-1β and IFN-α, in TCDM cultures. We showed that in contrast to anti-huIL-1β and anti-huIFN-α mAb, anti-huTNF-α mAb specifically inhibited the CD4 down-regulation observed in TCDM treated with rgp120 for 12 hr (Fig. 4). In order to determine the levels of endogenous TNF-α and IL-1β produced in gp120-treated TCDM compared to untreated control cells, we performed an ELISA. We showed that rgp120 induces a transient TNF-α peak in TCDM culture at 3–12 hr post-treatment (Fig. 5). TNF-α levels were maximal at 6 hr after rgp120 treatment with a 100-fold increase compared to untreated TCDM (Fig. 5). In contrast, throughout the time–course after addition of rgp120, IL-1β and IFN-α levels in culture supernatants were < 50 pg/ml (Fig. 5) and $< 0{\cdot}5$ IU/ml (data not shown) respectively. All these data taken together suggest that endogenous TNF-α induced by rgp120, rather than IL-1β or IFN-α, might be involved in TCDM CD4 down-regulation.

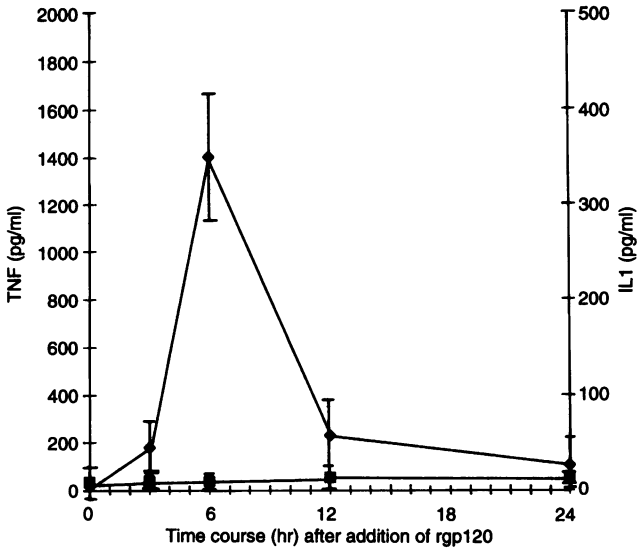

Figure 5. Time–course of TNF-α (diamonds) and IL-1β (squares) synthesis in rgp120 treated-TCDM. Supernatants of human TCDM were harvested at different times after addition of rgp120 and an ELISA was performed as described in the Materials and Methods.

DISCUSSION

Our results indicate that rgp120 transiently down-regulates both surface and total CD4 expression in human primary TCDM with a lowest level at 6–12 hr after treatment. Also, that the CD4 modulation induced by rgp120 in human primary TCDM might be regulated at a transcriptional level; and rgp120 down-regulates CD4 expression in primary TCDM through the induction of endogenous TNF-α. All these data taken together suggest that, rather than a direct effect of viral genes on CD4 expression, a main feature of CD4 down-regulation in HIV-infected lymphocytes, cytokines induced during HIV infection might down-regulate monocyte/macrophage CD4 expression in AIDS patients.

We studied the kinetics of CD4 modulation in primary human TCDM treated with 1 μg/ml rgp120. Both surface and total CD4 down-regulation occurred at 6–12 hr after addition of rgp120. As reported previously,[14] the increase of rgp120 concentrations up to 5 μg/ml did not modify significantly the intensity of TCDM CD4 down-regulation observed at 6–12 hr post-treatment (data not shown). At 24 hr post-treatment TCDM CD4 expression either remained low or recovered to similar levels to those observed in untreated TCDM. Differences in TCDM CD4 expression at 24 hr post-treatment might reflect variability among different donors. Western blot analyses performed in the absence of human pooled serum showed an increase of TCDM Fc receptor expression at 24 hr post-treatment, in agreement with previously reported up-regulation of surface Fc receptor expression by IL-10[21,22] which could also be induced in rgp120-treated PBMC.[23] Interestingly, both surface and total TCDM CD4 expression was modulated in parallel during the time after addition of rgp120 as shown by flow cytometry and Western blot analyses. This finding suggests that CD4 modulation in primary human TCDM treated with rgp120 might be regulated at the level of transcription. The study of CD4 mRNA expression in rgp120-treated primary TCDM demonstrates that as early as 1–3 hr after addition of rgp120, the amount of CD4 message was strongly diminished and that during the following 24 hr it increased, reaching levels at least as high as those observed in untreated TCDM. Our results suggest that rgp120 might down-regulate CD4 expression in primary TCDM at the transcriptional level. CD4 mRNA levels in rgp120-treated TCDM could be decreased as a result of either inhibition of gene expression or the lack of CD4 mRNA stability, although we did not attempt to distinguish between these possibilities. We reported previously that CD4 expression in human primary TCDM could be down-regulated *in vitro* by lipopolysaccharide, TNF-α and IL-1β at the level of transcription;[20] in contrast, both adherence and phorbol ester have been shown to down-regulate TCDM surface CD4 through an endocytic pathway.[24,25] All the preparations were tested and found free of endotoxin suggesting that the CD4 modulation observed in rgp120-treated TCDM was induced specifically by the viral envelope glycoprotein.

Recombinant gp120 has been reported to induce the production of TNF-α, IL-1β, and IFN-α in primary human TCDM *in vitro*.[5–10] Since both rgp120 and the proinflammatory cytokines TNF-α and IL-1β down-regulate CD4 expression in primary human TCDM[13,20] we were interested to see if rgp120 modulates TCDM CD4 expression through the induction of endogenous cytokines. The use of mAb directed against human TNF-α, IL-β or IFN-α allowed us to demonstrate that only the anti-TNF-α mAb inhibited the CD4 down-regulation observed in rgp120-treated TCDM. Moreover we were able to detect a transient peak of endogenous TNF-α in the culture supernatant of rgp120-treated TCDM as described previously;[6] this TNF-α peak preceded the observed TCDM-CD4 down-regulation. We have previously reported that, in contrast to IFN-α, IL-1β down-regulates CD4 expression in primary human TCDM, but to a lesser extent than TNF-α[20] and therefore could be a candidate for CD4 down-regulation in rgp120-treated TCDM. In contrast to other studies,[7–9] we detected only very low levels of both endogenous IL-1β and IFN-α in rgp120-treated TCDM. Different results with regard to cytokine induction in rgp120-treated TCDM might be explained by culture conditions, differences in glycosylation of rgp120 or the HIV-1 strain from which rgp120 originated.[26] Neither anti-huIL-1β nor anti-huIFN-α mAb restored CD4 expression in rgp120-treated TCDM. All these data taken together suggest that, *in vitro*, rgp120 down-regulates CD4 expression in primary human TCDM through the induction of endogenous TNF-α.

In vivo TNF-α, IL-1β and acid-labile IFN-α are found in both plasma and tissues of AIDS patients.[11,12,27] Moreover, monocytes isolated from HIV-infected patients produce TNF-α and IL-1β.[28] Envelope glycoprotein gp120 is present in serum of AIDS patients[4] and monocytes isolated from the peripheral blood of HIV-positive individuals display decreased surface CD4 expression[15] especially at the late stage of the disease when plasma TNF-α levels are high. Our results suggest that the down-regulation of monocyte surface CD4 expression observed in AIDS patients could be explained by the induction of endogenous TNF-α from monocytes/macrophages by the gp120 envelope glycoprotein or the whole virion which both are able to trigger TNF-α production.[6] Interestingly it has been shown that *in vitro*, infection of monocytes by the HIV_{Ba-L}

monocytotropic strain induces similar surface CD4 down-regulation in HIV-inoculated cells irrespective of whether cell-associated virus could be detected by reactivity with fluorescein isothiocyanate–anti-HIV or not.[16] These data suggest that, rather than a direct effect of viral genes on CD4 expression, a main feature of CD4 down-regulation in lymphocytes,[29,30] soluble factors such as cytokines induced during HIV infection might explain the monocyte/macrophage CD4 dysregulation observed in AIDS.

In conclusion the envelope glycoprotein gp120 down-regulates CD4 expression in primary human TCDM through the synthesis of endogenous TNF-α *in vitro*. Further studies have to be performed *in vivo* to specify the exact role of monocyte/macrophage CD4 modulation during the course of AIDS.

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