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Role of tumour necrosis factor-alpha (TNF-α) in the induction of HIV-1 gp120-mediated CD4$^+$ T cell anergy

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SUMMARY

The HIV-1 envelope glycoprotein (gp120) is known to induce antigen-specific and non-specific CD4$^+$ T cell anergy. We found that early T cell activation, as indicated by HLA-DP expression in the early G_1 (G_{1A}) phase of the cell cycle, and the inhibition of mitogen-mediated IL-2 production induced by gp120, required TNF-α produced by gp120-stimulated macrophages. In the presence of an antibody to TNF-α, these changes induced by gp120 were inhibited, while recombinant TNF-α induced similar abnormalities of CD4$^+$ T cells, even in the absence of gp120. On the other hand, inhibition of the mixed lymphocyte reaction (MLR) in CD4$^+$ T cells by gp120, which may be related to gp120-mediated down-regulation of CD4 expression on T cells and activation of protein tyrosine kinase p56^{lck} in CD4$^+$ T cells, was observed even in the absence of macrophage-derived TNF-α induced by gp120. These observations indicate that both TNF-α-dependent and independent events contribute to gp120-mediated CD4$^+$ T cell anergy, and TNF-α appears to play an important role in inducing CD4$^+$ T cell anergy in HIV-1 infection.

Keywords HIV-1 gp120 T cell anergy TNF-α

INTRODUCTION

AIDS is characterized by a decrease in the number and function of CD4$^+$ T cells. Even in the asymptomatic phase of HIV-1 infection, when CD4$^+$ T cell numbers are still within the normal range, several immunological abnormalities of CD4$^+$ T cells have been reported [1–6]. CD4$^+$ T cell function may be altered as a consequence of the interaction between the CD4 receptor and the envelope glycoprotein of HIV-1 (gp120), which is shed from HIV-infected cells (both *in vivo* and *in vitro*), and is detected in the serum of AIDS patients at a high concentration [7–9]. Several studies have shown that gp120 can induce T cell abnormalities, such as CD4$^+$ T cell anergy [10–15] or apoptotic CD4$^+$ T cell death *in vitro* [16–18], and can promote the production of several cytokines by monocytes/macrophages, including TNF-α [19–24]. TNF-α has been reported to play an important role in viral replication [25–27] and immunodysregulation related to HIV infection, including polyclonal B cell activation [28], down-regulation of CD4 expression on macrophages [24], and apoptotic death of CD4$^+$ T cells *in vitro* [17,18], as well as in the development of AIDS-associated cachexia *in vivo* [29].

The precise mechanisms of CD4$^+$ T cell dysfunction are still being debated, and several different factors may contribute to the loss of CD4$^+$ T cell function in HIV-1 infection. Differences in the results reported so far may be due to the differences between the experimental systems used. In this study, we examined the effect of gp120 on the induction of CD4$^+$ T cell dysfunction using mitogen-mediated IL-2 production and the mixed lymphocyte reaction (MLR) in healthy human peripheral blood mononuclear cells (PBMC). We also investigated the role of TNF-α in the induction of gp120-mediated CD4$^+$ T cell anergy.

MATERIALS AND METHODS

Cells and reagents

PBMC were separated from normal human blood by centrifugation on a Ficoll–Paque cushion, after which CD4$^+$ T cells were obtained by negative selection using an immunoabsorption column (Cellect; Biotex Labs Inc., Alberta, Canada). Macrophages adherent to Petri dishes (Corning Glass Works, Corning, NY) were collected by incubating PBMC for 2 h at 37°C. The CD4$^+$ T cells and CD14$^+$ macrophages prepared by these procedures showed

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>95% purity. Recombinant glycosylated HIV-1_{IIIB} protein gp120 (rgp120 produced in a baculovirus expression system >95% pure as estimated by analysis of coomassie blue-stained SDS–PAGE gels; Intracel Co., Issaquah, WA) was used in this experiment. All cells were cultured at 37°C, 5% CO_2, in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C, 40 min) selected non-mitogenic fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all reagents from Gibco Europe, Basel, Switzerland). In some experiments, adequate concentrations of anti-human TNF-α neutralizing antibody (anti-TNF-α antibody; R&D Systems Inc., Minneapolis, MN), anti-human IL-6 neutralizing antibody (anti-IL-6 antibody; R&D Systems Inc.), or recombinant TNF-α (rTNF-α; R&D Systems Inc.), recombinant IL-6 (rIL-6; R&D Systems Inc.) were added to cell cultures (1×10^6 cells/well) at the beginning of cultures.

Cytokine assay
Cells (1×10^6 cells/well) were cultured for 3 days with or without rgp120 and then cytokine levels in the culture supernatant were measured. The assay system of TNF-α is a solid-phase ELISA system (Amersham International plc., Aylesbury, UK), which utilizes a highly specific MoAb for cytokines bound to the wells of a microtitre plate, together with a polyclonal antibody to cytokines conjugated to horseradish peroxidase. IL-6 and IL-2 levels in culture supernatants were detected by bioassay using an IL-6- and IL-2-dependent cell line, respectively. Culture supernatant was incubated with an IL-6-dependent cell line (MH-60; 5×10^4 cells/well) and IL-6 activity was determined by a proliferation assay using MTT (3-(4,5-dimetylthiazole-2-yl)-2,5-diphenyltetrozolium bromide; Sigma Chemical Co., St Louis, MO). To examine the production of IL-2, cells (1×10^6 cells/well) were cultured with or without rgp120 for 1 day, then 10 μg/ml phytohaemagglutinin (PHA; Sigma Chemical Co.) were added to the culture. After 2 days, culture supernatant was incubated with IL-2-dependent cell line (CTLL; 5×10^4 cells/well) for 3 days and IL-2 activity was determined by the proliferation assay using MTT, as described above. The same procedure was applied to formulate a reference curve using human recombinant IL-6 and IL-2 (Shionogi Pharmaceutical Inc., Osaka, Japan). Based on this reference curve, the units of IL-6 and IL-2 were determined.

Flow cytometry
To investigate the binding of gp120 to normal human PBMC, two-colour flow cytometry was performed. Briefly, cells were exposed to rgp120 labelled by FITC (Intracel Co.) at various concentrations for 1 day. The cells were then stained with PE-labelled OKT4 (anti-CD4; Ortho Diagnostic Systems Inc., Raritan, NJ), Leu-2a (anti-CD8), and Leu-M3 (anti-CD14) (Becton Dickinson Immunocytometry Systems, San Jose, CA), after which they were analysed by using a FACStar (Becton Dickinson Immunocytometry Systems). To investigate the expression of HLA-DP antigen on T cells, which is known to appear at an earlier T cell activation phase than other HLA molecules (DR and DQ) [30], cells were cultured with or without rgp120 for 3 days, and they were then stained with anti-HLA-DP antibody (Becton Dickinson Immunocytometry Systems) and FITC-labelled goat F(ab$'$)$_2$ anti-mouse immunoglobulins (Tago Inc., Burlingame, CA). After blocking of non-specific binding with whole mouse serum (Cappel Research Products, Durham, NC), the cells were stained with PE-labelled OKT4, after which two-colour flow cytometry analysis was carried out using FACStar.

Anti-phosphotyrosine immunoblot analysis
Phosphorylation of protein tyrosine kinase $p56^{lck}$ was detected by the method described by Freedman *et al.* [31], with slight modification. Briefly, $CD4^+$ T cells (1×10^6 cells/100 μl per well) were incubated with 1 μg/ml of phorbol myristate acetate (PMA; Sigma Chemical Co.), 5 μg/ml anti-CD3 antibody (OKT3; Ortho Diagnostic Systems Inc.), anti-CD4 antibody (Leu-3a; Becton Dickinson Immunocytometry Systems), and rgp120 (1 μg/ml) at 37°C for 30 min, and then cells were washed by stop solution (PBS containing 5 mm EDTA, 10 mm NaF, 10 mm sodium pyrophosphate, 0·4 mm sodium vanadate) and solubilized in lysis buffer (1% Nonidet P-40, 150 mm NaCl, 50 mm Tris–HCl pH 8·0, 5 mm EDTA, 1 mm PMSF, 10 mm iodoacetamide, 10 mm NaF, 10 mm sodium pyrophosphate, 0·4 mm sodium vanadate) for 15 min on ice. After removal of insoluble material, sample buffer (0·1 m Tris pH 6·8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0·02% bromphenol blue) was added. Equal amounts of protein were loaded on 5–20% gradient polyacylamide gel (Funakoshi easy-gel II; Funakoshi Co., Tokyo, Japan), and then electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF; Millipore Co., Bedford, MA). Blots were incubated with 1 μg/ml horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine MoAbs (4G10; Upstate Biotechnology Inc., Lake Placid, NY) in blocking solution (pH 7·2, 0·9% NaCl, 10 mm Tris, 0·01% NaN_3, 5% bovine serum albumin (BSA), 1% ovalbumin) for 2 h at room temperature. Reaction was revealed with an enhanced chemiluminescence system (ECL; Amersham International plc), and autoradiographed. Molecular weight markers (BioRad Labs, Richmond, CA) were included on a gel as a standard.

Mixed lymphocyte reaction
Proliferation assay was carried out in 200 μl of culture medium, with 96-well round-bottomed microtitre plate (Corning Glass Works). After incubation of purified $CD4^+$ T cells (responder

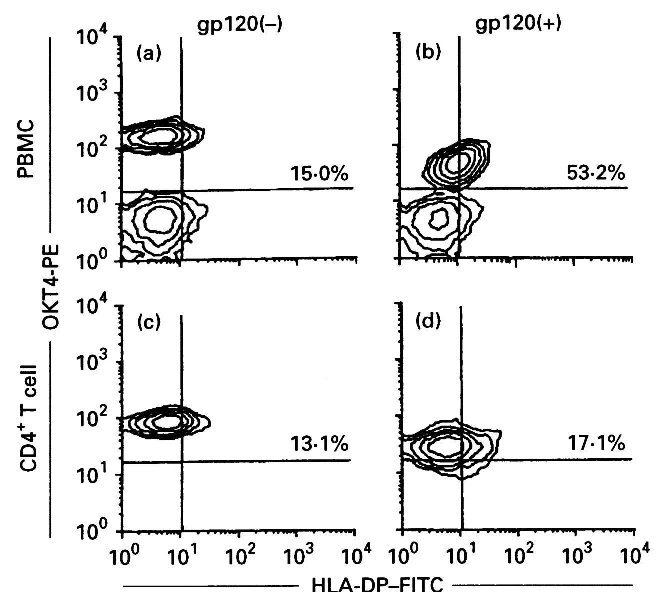

Fig. 1. Effect of gp120 on HLA-DP antigen expression by $CD4^+$ T cells. The percentages of HLA-DP^+ cells among $OKT4^+$ T cells are shown. T cell expression of CD4 molecules (detected by OKT4 antibody) decreased in cultures of peripheral blood mononuclear cells (PBMC) as well as purified $CD4^+$ T cells in the presence of gp120.

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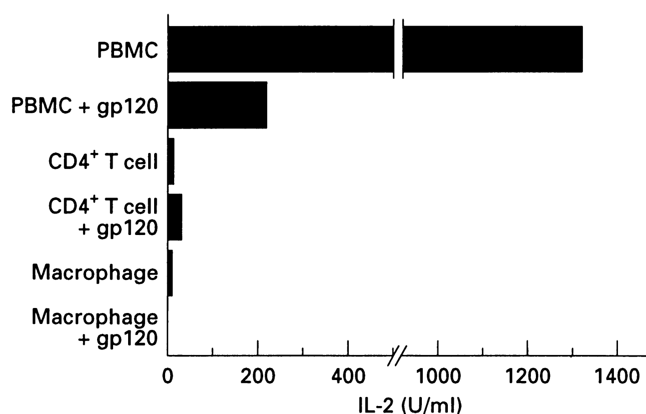

Fig. 2. Effect of gp120 on IL-2 production by phytohaemagglutinin (PHA)-stimulated cells. PBMC, Peripheral blood mononuclear cells.

cell) (2×10^5 cells/well) with rgp120 (1 μg/ml) for 2 days, responder cells were cultured with irradiated (30 Gy) macrophage-enriched PBMC (stimulator cell) (2×10^5 cells/well). Cells were pulsed with 1 μCi/well tritiated thymidine (^{3}H-TdR; New England Nuclear, Boston, MA) at 1, 3, and 5 days of incubation, and then cells were harvested onto glassfibre filters and washed using a cell harvester (Micro 96 Harvester; Skatron, Lier, Norway). ^{3}H-TdR; incorporation was measured by scintillation counter (Micro Beta Plus; Wallac, Turku, Finland). Background cellular proliferation in medium alone was <100 ct/min.

Results are shown as representative data for some experiments (Figs 1 and 6) or as mean with s.d. <15% obtained in three separate experiments (Figs 2–5).

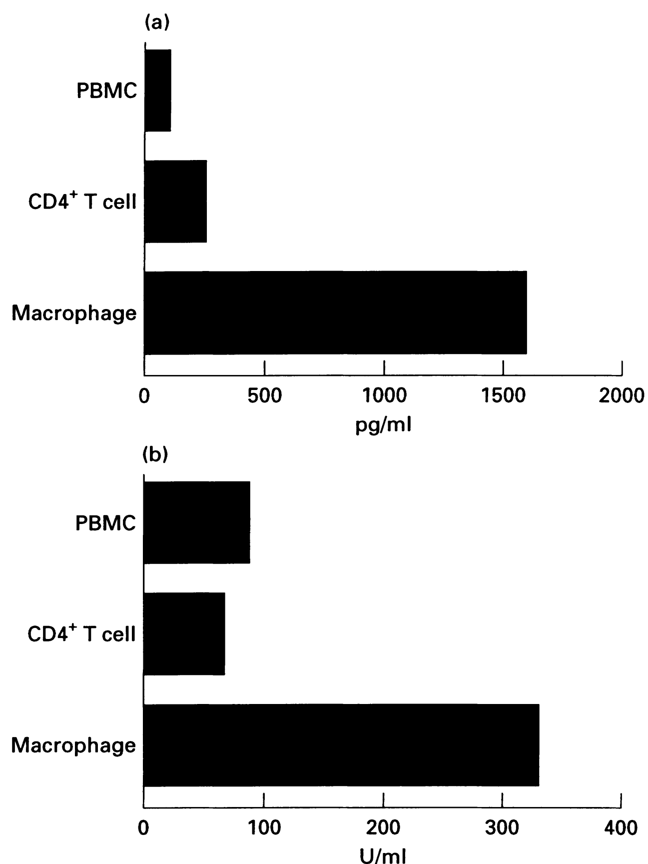

Fig. 3. TNF-α (a) and IL-6 (b) production from gp120-stimulated human cells. PBMC, Peripheral blood mononuclear cells.

RESULTS

Effect of gp120 on T cell expression of HLA-DP and cytokine production

A rgp120 concentration of 1 μg/ml was used in this study, because it was sufficient to occupy all of the CD4 molecules on T cells and macrophages, as we reported previously [17,18,32]. PBMC cultured with gp120 showed an increase of HLA-DP$^+$ CD4$^+$ T cell populations, although this increase was not observed in the case of purified CD4$^+$ T cells (Fig. 1). Two-colour FACS analysis using PE-labelled Leu-M3 and anti-HLA-DP antibody followed by FITC-labelled goat F(ab$'$)$_2$ anti-mouse immunoglobulins indicated that there was no increase of HLA-DP expression on macrophages (data not shown). In the same experiment, gp120-mediated down-regulation of CD4 expression detected by an OKT4 antibody was observed to the same extent in both PBMC and purified CD4$^+$ T cells (Fig. 1).

To investigate the influence of gp120 on CD4$^+$ T cell function, IL-2 production by PHA-stimulated cells was examined in the presence of gp120. IL-2 production derived from CD4$^+$ T cells in PBMC by PHA stimulation was dependent on the presence of macrophages, because IL-2 production was not observed in purified CD4$^+$ T cells in the absence of macrophages, and gp120 inhibited PHA-mediated IL-2 production by PBMC (Fig. 2), as reported previously [10,11].

gp120 is known to promote production of several cytokines by

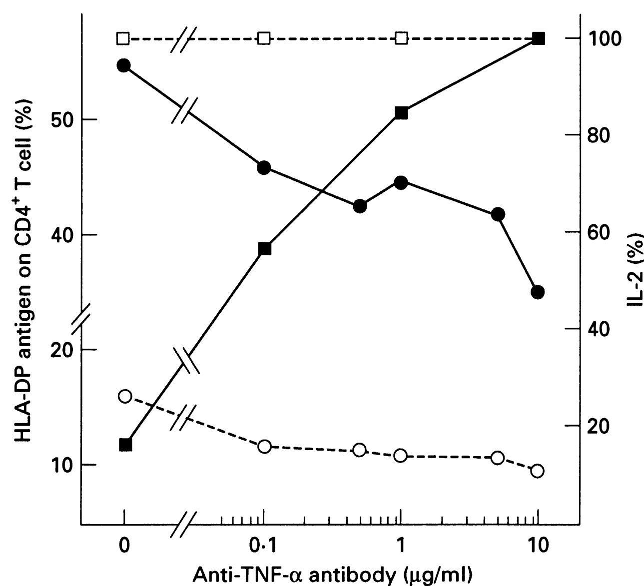

Fig. 4. Effects of anti-TNF-α antibodies on the expression of HLA-DP on CD4$^+$ T cells and the inhibition of IL-2 production induced by gp120. HLA-DP expression was detected by two-colour FACS analysis and the percentage of HLA-DP on CD4$^+$ T cells was examined. The increasing effect of HLA-DP expression on CD4$^+$ T cells by gp120 was inhibited by anti-TNF-α antibodies (●). Inhibition of IL-2 production from phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) by gp120 was restored by anti-TNF-α antibodies (■). IL-2 production in the absence of gp120 and anti-TNF-α antibodies is indicated as 100%. The influence of anti-TNF-α antibodies was not apparent both in the expression of HLA-DP on CD4$^+$ T cells (○) and in IL-2 production (□) in the absence of gp120.

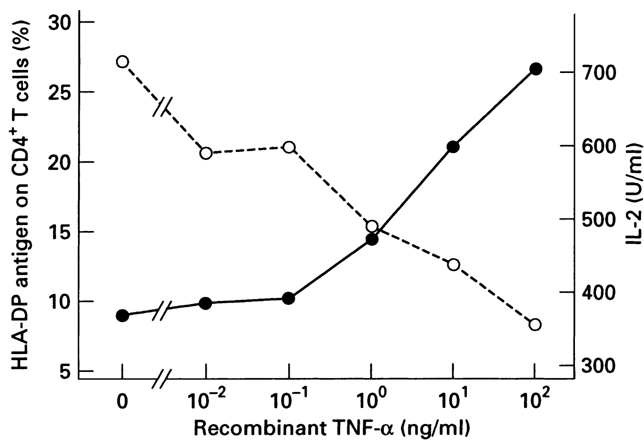

Fig. 5. Effect of rTNF-α on HLA-DP antigen expression and IL-2 production by CD4$^+$ T cells. The expression of HLA-DP on CD4$^+$ T cells was detected by two-colour FACS analysis. rTNF-α promoted the expression of HLA-DP on CD4$^+$ T cells in peripheral blood mononuclear cells (PBMC) (●), and inhibited production of IL-2 from phytohaemagglutinin (PHA)-stimulated PBMC (○) in a concentration-dependent manner. rTNF-α alone did not have any influence on cell growth and viability over the range of concentrations used in this culture system.

monocytes/macrophages. As shown in Fig. 3, TNF-α and IL-6 were detected in culture supernatants of gp120-stimulated cells at high concentrations. These cytokines were mainly produced by macrophages, but not by CD4$^+$ T cells.

Effect of TNF-α on gp120-mediated T cell abnormalities

Next we examined the effects of these cytokines on the gp120-mediated T cell abnormalities shown in Figs 1 and 2. Antibodies to TNF-α inhibited the increase of HLA-DP$^+$ CD4$^+$ T cells in gp120-stimulated PBMC and blocked the inhibition of IL-2 production induced by gp120 in a concentration-dependent fashion (Fig. 4). In addition, when rTNF-α was added to PBMC, it induced HLA-DP expression on CD4$^+$ T cells and inhibited PHA-mediated IL-2 production by PBMC, even in the absence of gp120 (Fig. 5). Although similar experiments were performed using an anti-IL-6 antibody and rIL-6, these agents did not have a similar effect to anti-TNF-α and rTNF-α (data not shown). These results indicate that TNF- α may play an important role in CD4$^+$ T cell activation, indicated by HLA-DP expression and the suppression of PHA-mediated IL-2 production from CD4$^+$ T cells.

Effect of gp120 on the phosphorylation of tyrosine kinase p56^{lck} in CD4$^+$ T cells and the MLR

Several reports have described a relationship between gp120 stimulation and the activity of protein tyrosine kinase p56^{lck} in CD4$^+$ T cells [33–36]. To investigate the effect of gp120 on signal transduction in CD4$^+$ T cells, we examined the phosphorylation of protein tyrosine kinase p56^{lck} in gp120-stimulated CD4$^+$ T cells purified from PBMC. As shown in Fig. 6, gp120 induced phosphorylation of p56^{lck} in purified CD4$^+$ T cells even in the absence of macrophages. In addition, the MLR was inhibited when purified CD4$^+$ T cells (responder cell) were stimulated by gp120, and this did not require the presence of macrophages in the responder cell (Table 1).

DISCUSSION

gp120 has been reported to inhibit T cell responses, including antigen-specific and non-specific reactions [10–15], although the mechanisms of gp120-mediated T cell dysfunction are still under debate. In this study, HLA-DP antigen was used as a marker of

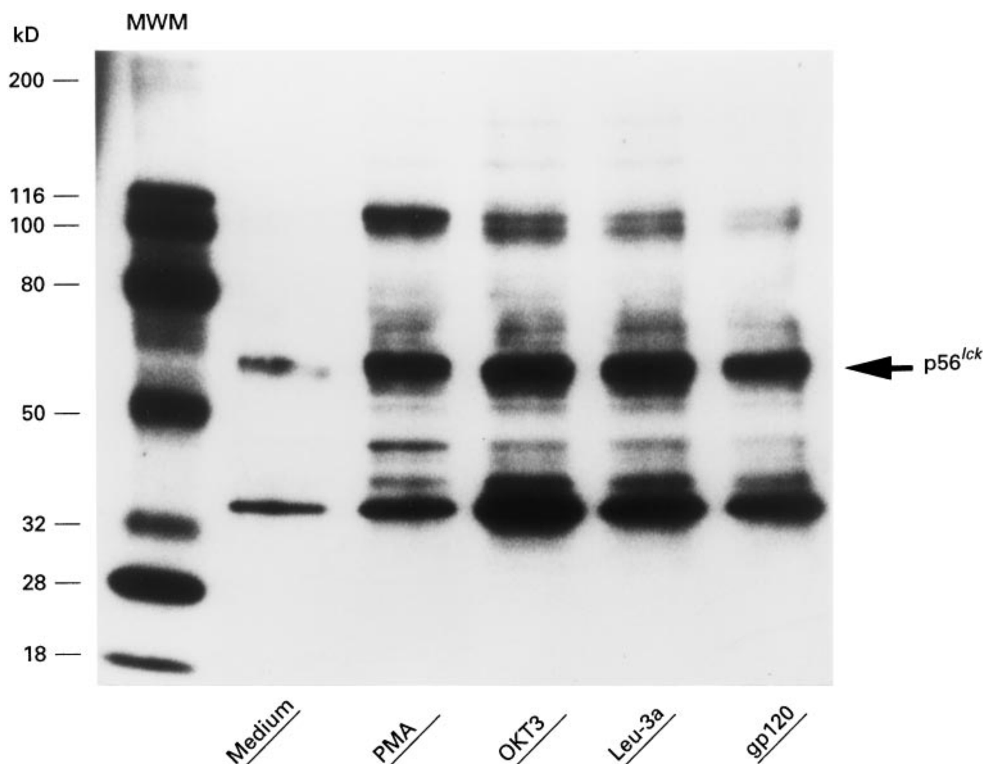

Fig. 6. Phosphorylation of protein tyrosine kinase p56^{lck} by gp120. PMA, Phorbol myristate acetate.

Table 1. Effect of gp120 on the response of the mixed lymphocyte reaction (MLR)*

	Day 1	Day 3	Day 5
gp120 (−)	17·5 ± 5·4	8449·8 ± 594·3	15 414·6 ± 1133·2
gp120 (+)	9·3 ± 17.0	4849·1 ± 570·7	8543·3 ± 706·9
P†	0·47	<0·005	<0·001

*Results expressed are the proliferative response in mean ct/min ± s.d. obtained in three separate experiments.

†*P* value was obtained by Student's *t*-test (with *versus* without gp120-stimulation).

activated T cells to detect gp120-induced early T cell activation in the cell cycle. This antigen is generally expressed on B cells, macrophages, null cells, an extremely small population of T cells, and a large population of activated T cells that are mainly in the G_{1A} (but not G_0) phase of the cell cycle [30]. Although HLA-DP expression on $CD4^+$ T cells in PBMC was increased by gp120 stimulation (Fig. 1), an increase of the IL-2 receptor or transferrin receptor, which are expressed on T cells in a later phase of the cell cycle than HLA-DP antigen, was not observed on $CD4^+$ T cells in PBMC stimulated by gp120 (data not shown), as reported by others [30,33]. We previously reported that IL-2 production by $CD4^+$ T cells decreased in association with the increase of HLA-DP^+ $CD4^+$ T cells and the expression of HLA-DP by $CD4^+$ T cells might be closely related to the suppression of IL-2 production [30]. The present study indicated that HLA-DP expression on $CD4^+$ T cells and the suppression of IL-2 production induced by gp120 stimulation were regulated by TNF-α derived from gp120-stimulated macrophages (Figs 4 and 5).

TNF-α is known to be produced by gp120-stimulated monocytes/macrophages, and has been reported to promote HIV-1 replication [25–27]. We previously reported that TNF-α production contributed to $CD4^+$ T cell death due to apoptosis induced by gp120 [17,18]. In this study, we demonstrated that TNF-α (but not IL-6) derived from gp120-stimulated macrophages could play an important role in T cell activation and the inhibition of IL-2 production. TNF-α is known to facilitate viral replication through the induction of a transcriptional factor (NF-κB, a DNA-binding protein which binds to viral enhancer sites) in HIV-1-infected cells [25]. Similar mechanisms may be related to gp120-induced TNF-α-dependent T cell dysfunction through T cell activation mediated by TNF-α, although the precise events are still unknown. Schols & de Clercq reported that IL-10 produced by gp120-stimulated monocytes had a significant role in the development of T cell anergy (shown as a low responsiveness to anti-CD3 antibody- and mitogen-stimulated proliferation) [15]. Thus, cytokines such as TNF-α and IL-10 produced by monocytes/macrophages after gp120 stimulation appear to be important in the induction of $CD4^+$ T cell anergy in HIV-1 infection. Recently, we reported that gp120 could induce several chemokines, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES (regulated-upon activation, normal T expressed and secreted) [32]. These chemokines and IL-16 have been reported to inhibit HIV-1 infection *in vitro* [37,38]. We are also investigating their roles in the induction of T cell dysfunction described here.

gp120-mediated down-regulation of CD4 expression and phosphorylation of tyrosine kinase $p56^{lck}$ in $CD4^+$ T cells were observed in purified $CD4^+$ T cells in the absence of macrophages (Figs 1 and 6). In addition, inhibition of the MLR of gp120-stimulated $CD4^+$ T cells did not require the presence of macrophages in responder cells (Table 1). These phenomena may be relatively uninfluenced by TNF-α, because gp120-mediated TNF-α production by purified $CD4^+$ T cells was very low in comparison with that by macrophages (Fig. 3), and irradiated macrophages in stimulator cells of the MLR could not have the ability to produce TNF-α (data not shown). Juszczak *et al.* reported that gp120 induced a rapid increase of CD4-associated $p56^{lck}$ activation, followed by CD4-$p56^{lck}$ dissociation and down-regulation of CD4 expression after long-term treatment [34]. Hivroz *et al.* also reported that gp120 could activate $p56^{lck}$ tyrosine kinase in $CD4^+$ T cells and suggested that this might be of significance with respect to $CD4^+$ T cell anergy in HIV-1 infection [35]. Furthermore, CD4 binding of gp120 to CD4 in a human cell line was demonstrated to result in not only activation of tyrosine kinase $p56^{lck}$ but also inactivation of the T cell receptor (TCR) [36]. These *in vitro* findings may also have relevance to the MLR impairment detected in the present study. The model of T cell activation suggests that induction of anergy in resting T cells may occur if primary proliferation is induced by high-density triggering of the TCR/CD3 complex in the absence of accessory signals such as antibodies to CD28 [39]. A recent study has indicated that gp120 can inhibit proliferation and cytokine production by T cell lines in response to anti-CD3 antibodies, while stimulation via the CD28 pathway partially restores these defective $CD4^+$ T cell responses [14]. We are now investigating whether anti-CD28 antibody stimulation can restore the MLR in our culture system after inhibition by gp120.

This study demonstrated that both TNF-α-dependent and independent events are involved in gp120-mediated T cell anergy. In HIV-1-infected patients, both mechanisms could play an important role in inducing T cell anergy. Because TNF-α can promote HIV-1 replication, treatment of HIV-1-infected patients using drugs having anti-TNF-α activity (such as vesnarinone; Otsuka Pharmaceutical Co., Osaka, Japan) has been evaluated in the USA with the permission of the Food and Drug Administration, based on the marked inhibitory effect of such agents on HIV-1 infection *in vitro* [40]. Furthermore, a recent study indicated that vesnarinone inhibited TNF-α-related apoptotic cell death [41]. Our results suggest that these agents are potentially useful for inhibition of not only viral replication and apoptosis but also T cell anergy in patients with HIV-1 infection.

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