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Human Immunodeficiency Virus Type 1 gp120 Induces Anergy in Human Peripheral Blood Lymphocytes by Inducing Interleukin-10 Production

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The effects of recombinant gp120 on the proliferative responses and cytokine production by normal peripheral blood mononuclear cells (PBMC) were investigated. gp120 inhibited in a dose-dependent fashion the anti-CD3 monoclonal antibody (MAb)- and concanavalin A-induced proliferative responses. The production of interleukin-2 (IL-2) and IL-4 was diminished by gp120 in the anti-CD3- and concanavalin A-stimulated cultures. In unstimulated PBMC, gp120 induced the production of considerable amounts of IL-10, gamma interferon, and tumor necrosis factor alpha. The gp120-induced reduction in the proliferative responses of PBMC was at least partially reversed by the addition of IL-2, anti-CD28 MAb, or transfectants expressing CD80, CD86, or CD40 but not with exogenous IL-4. Also, a neutralizing anti-IL-10 MAb reversed the inhibitory effect of gp120 on the proliferative responses whereas exogenous IL-10 further enhanced this inhibitory effect. These findings indicate that IL-10 plays an important role in the inhibitory effect of gp120 on PBMC proliferation. The ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ T cells was the same in gp120-treated and untreated cell cultures. No apoptosis in these two T-cell populations was observed. However, the number of activated $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells, as judged by CD25, CD69, and HLA-DR expression, was consistently reduced. gp120 induced the expression of IL-10 in the monocyte/macrophage population, and therefore gp120 also reduced the proliferative responses of $CD4^+$ T-cell-depleted PBMC. Taken together, our observations point to the importance of the cytokine pattern changes and, in particular, the role of IL-10 (produced by the monocytes) in the inhibitory effect of gp120. This mechanism of gp120-induced immunosuppression, if operative in vivo, could contribute to the depressed immune responses associated with human immunodeficiency virus infection and thus have important implications for immunotherapeutic strategies to slow down disease progression in AIDS.

AIDS has generally been attributed to the destruction of $CD4^+$ lymphocytes, which are infected by human immunodeficiency virus (HIV) (17, 29). However, already in the asymptomatic phase of HIV type 1 (HIV-1) infection, when $CD4^+$ T-cell numbers are still within the normal range, several immunological abnormalities have been reported (40). In particular, $CD4^+$ lymphocytes isolated from HIV-infected persons are less reactive to soluble and viral antigens. Several groups demonstrated that in a high percentage of healthy HIV-infected persons, stimulation of peripheral blood mononuclear cells (PBMC) with anti-CD3 monoclonal antibody (MAb) is decreased (41), levels of proliferation and interleukin-2 (IL-2) production in response to anti-CD3 MAb and recall antigens are lower (15, 41, 49, 50), the level of helper activity induced by pokeweed mitogen is low (26), and an increased number of T cells are programmed for cell death (39). Proliferative responses of both naive and memory $CD4^+$ T cells to recall antigens are progressively lost (38), and later in the course of infection the responses to strong stimuli, such as lectins, are also lost (49). The functions of the $CD4^+$ T cells may be altered as the consequence of the interaction between the CD4 receptor and soluble gp120. It has been shown that gp120 inhibits IL-2 mRNA expression in $CD4^+$ lymphocytes (45) and the proliferative responses of PBMC when stimulated through the T-cell receptor (TCR) (13, 33). The addition of IL-2 was able to restore the proliferation of PBMC in the presence of gp120 (33). Very little is known about the mechanism of this suppression and the role of cytokines other than IL-2. In addition, cross-linking of the CD4 receptor by gp120 has also been reported to induce apoptosis in $CD4^+$ T cells (5), although these findings could not be confirmed by others (33). Several reports have shown that monocytes/macrophages can produce different cytokines such as interferon (IFN), tumor necrosis factor (TNF), IL-1, and IL-6 in response to HIV-1 infection or even after exposure to the gp120 protein (9, 16, 23, 36, 43, 53). However, Molina et al. demonstrated that HIV-1 did not induce IL-1, IL-6, or TNF production in PBMC in the absence of endotoxin (42). Three recent studies described the inhibitory effects of IL-10 on HIV replication in monocytes/macrophages (7, 30, 46) and that HIV-1 possibly through its gp120 protein up-regulated IL-10 expression in monocytes/macrophages (7).

In the study reported here, we examined the effects of recombinant gp120 on TCR- and mitogenic-driven proliferation of both unfractionated PBMC and $CD4^+$ and $CD8^+$ T-cell-depleted PBMC. We also evaluated the ability of gp120 to induce or inhibit the production of cytokines in normal PBMC cultures and determined whether the effects of gp120 could be reversed by the addition of cytokines, neutralizing MAbs, or transfectants expressing costimulatory molecules important for T-cell activation.

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MATERIALS AND METHODS

Isolation of lymphocytes. Buffy coat preparations from healthy donors were obtained from the Leuven Blood Bank and kindly provided by O. Souw. PBMC

were isolated by density gradient centrifugation over Lymphoprep (density, 1.077 g/ml; Nycomed, Oslo, Norway). PBMC were depleted of $CD4^+$ T cells or $CD8^+$ T cells with Dynabeads M-450 CD4 or Dynabeads M-450 CD8 as described by the manufacturer (Dynal, Oslo, Norway).

MAbs. In proliferation assays, the following MAbs were used: OKT-3 (anti-CD3) (American Type Culture Collection); anti-gp120 (AMAC, Westbrook, Maine); 19F1 (anti-IL-10), kindly provided by J. Abrams (DNAX Research Institute, Palo Alto, Calif.); L293 (anti-CD28), kindly provided by L. Lanier (DNAX); and RPA-T4 (anti-CD4), kindly provided by G. Aversa (DNAX). For flow cytometric staining, MAbs OKT4 (Ortho Diagnostics) and Leu-3a (CD4), Leu-2a (CD8), Leu-4 (CD3), anti-IL-2R (CD25), Leu-M3 (CD14), Leu-28 (CD28), Leu-23 (CD69), and anti-HLA-DR and isotype-matched control MAbs were purchased from Becton Dickinson Immunocytometry Systems, San Jose, Calif.

Cell lines. P815, a murine mastocytoma cell line, was transfected with human B7 (CD80) ($CD80^+$P815) (3), human B70 (CD86) ($CD86^+$P815) (4), or human CD40 ($CD40^+$P815) (10) and kindly provided by L. Lanier.

Thymidine incorporation. The proliferation of total peripheral blood lymphocytes was tested by culturing 2×10^5 cells per well in a final volume of 200 μl of modified Eagle medium (Life Technologies Ltd., Paisley, Scotland) in 96-well plates (Costar, Cambridge, Mass.). Cells were stimulated with anti-CD3 (1 μg/ml), concanavalin A (ConA) (1 μg/ml; Sigma, Bornem, Belgium), phytohemagglutinin (1 μg/ml; Wellcome Diagnostics, Kent, England), and staphylococcal enterotoxin B (0.2 μg/ml; Sigma) in the absence or presence of recombinant gp120 (HIV-1, strain IIIB) (0.2 to 10 μg/ml) (Intracel, London, England), anti-CD28 MAb (1 μg/ml), mitomycin (Sigma)-treated P815 transfectants (2×10^4 cells per well), anti-CD4 MAb (1 μg/ml), IL-2 (1 to 25 U/ml), IL-4 (25 to 100 U/ml), IL-10 (25 to 100 U/ml), or anti-IL-10 MAb (5 μg/ml). The gp120 was >95% pure as estimated by analysis of Coomassie blue stained sodium dodecyl sulfate-polyacrylamide gels and Western blots (immunoblots). The stock solution of gp120 at 100 μg/ml showed an endotoxin concentration of 80 pg/ml as tested by the *Limulus* amebocyte lysate assay (Coatest endotoxin; limit of detection of endotoxin, 10 to 25 pg/ml; Chromogenix AB, Mölndal, Sweden). Cultures were incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2 and then pulsed with 1 μCi of [^{3}H]thymidine (Amersham, Little Chalfont, England) for an additional 16 h. Subsequently, the cells were harvested onto fiberglass filters and [^{3}H]thymidine incorporation, expressed in counts per minute, was measured by liquid scintillation spectroscopy.

Flow cytometric analyses. Single-cell suspensions of 2×10^5 PBMC were preincubated with 1% normal mouse serum in phosphate-buffered saline (PBS) with 1% bovine serum albumin and 0.01% NaN_3 for 10 min at 4°C. Cells were then washed, and 10 μl of MAbs directly labeled with fluorescein isothiocyanate, phycoerythrin (PE), and peridinin chlorophyll protein was added for 20 min at 4°C in a 200-μl volume of PBS. PBMC were analyzed by three-color analysis with a FACScan (Becton Dickinson) flow cytometer. Data were acquired and analyzed with CellQuest software (Becton Dickinson) on an Apple Macintosh Quadra computer. Data were displayed as dot plots to measure the proportion of lymphocyte subsets that expressed the activation markers as CD25, CD69, and HLA-DR. For staining of apoptotic and dead cells, the method described by Schmid et al. (47) with 7-amino-actinomycin D (Euro Biochem, Bièrges, Belgium) was used. Briefly, 2×10^5 PBMC were incubated for different time periods (24 h, 48 h, and 3 days) with various concentrations of gp120 (5 to 1 μg/ml), anti-CD3 (1 μg/ml), or ConA (1 μg/ml). As a positive control for the induction of apoptosis, PBMC were treated with staurosporine (1 to 0.01 μg/ml; Sigma). Cells were washed once in PBS, incubated with 7-amino-actinomycin D (20 μg/ml) in PBS for 20 min at 4°C protected from light, and analyzed on the flow cytometer in their staining solution.

Cytokine production by peripheral blood lymphocytes. Freshly isolated peripheral blood lymphocytes (10^6) were stimulated with anti-CD3 (1 μg/ml) or ConA (1 μg/ml) in the absence or presence of gp120 (0.2 to 5 μg/ml). In addition, lymphocytes were stimulated with phytohemagglutinin (1 μg/ml) and staphylococcal enterotoxin B (0.2 μg/ml). All stimulations were carried out in a volume of 1 ml of modified Eagle medium in 24-well plates (Costar). Cell-free supernatants were collected after 24 and 48 h and stored at −20°C before their cytokine content was tested.

Cytokine measurements. The production of IL-2, IL-4, IL-10, gamma IFN (IFN-γ), and TNF alpha (TNF-α) was measured by cytokine-specific enzyme-linked immunosorbent assay (ELISA) (BioSource International, Camarillo, Calif.). The ELISA sensitivity was as follows: 10 pg/ml for IL-2, 20 pg/ml for IL-4, 5 pg/ml for IL-10 and for IFN-γ, and 1 pg/ml for TNF-α. The plates were read with a THERMOmax microplate reader (Molecular Devices, Menlo Park, Calif.) and analyzed with the SOFTmax program (Molecular Devices).

Immunofluorescent staining for intracellular cytokines. Total PBMC (10^6 cells per ml) were incubated in medium alone, in medium with lipopolysaccharide (LPS) (2 μg/ml) (Sigma), or in medium with gp120 (5 μg/ml) in the presence of 2 μM monensin (Sigma). Six hours later the cells were washed with PBS, stained with fluorescein isothiocyanate-conjugated anti-CD14 (Becton Dickinson), and fixed in 4% paraformaldehyde. The cells were then stained with anti-IL-10–PE (Pharmingen, San Diego, Calif.) according to the protocol of the manufacturer. LPS stimulation was used to obtain the optimal production of IL-10. The specificity of the staining was verified by preincubation of the conjugated MAb with an excess of IL-10 (1,000 U/ml).

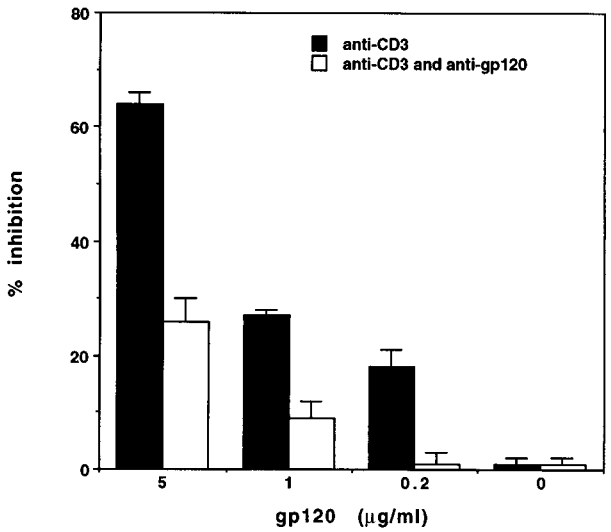

FIG. 1. Inhibitory effect of gp120 on anti-CD3 MAb-induced proliferation of PBMC. Cells were stimulated with anti-CD3 (1 μg/ml). gp120 was used at concentrations of 0.2, 1, and 5 μg/ml, and anti-gp120 MAb was used at a concentration of 5 μg/ml. The percentage of inhibition was calculated from the corresponding (counts per minute) values. Data represent the means (error bars, standard deviations) of triplicate samples.

RESULTS

gp120 inhibits proliferative responses in PBMC. To determine the effects of gp120 on the proliferative responses in PBMC, the cells were stimulated with anti-CD3 MAb in the presence of different concentrations of gp120. As shown in Fig. 1, gp120 inhibited the proliferative responses in a dose-dependent fashion. Significant inhibitory effects were already observed at gp120 concentrations as low as 0.4 μg/ml. The 50% inhibitory concentration of gp120 was 2.0 ± 0.5 μg/ml ($n = 5$) with anti-CD3 stimulation and 0.7 ± 0.2 μg/ml ($n = 5$) with ConA stimulation. As shown in Fig. 1, anti-gp120 MAb strongly reversed the gp120-induced inhibition of the cells' proliferative response. The proliferative responses to other mitogenic stimuli (i.e., phytohemagglutinin) or bacterial superantigens (i.e., staphylococcal enterotoxin B) were not inhibited by gp120 (50% inhibitory concentration, >5 μg/ml) (data not shown). The anti-CD4 MAb, RPA-T4 (2), had no inhibitory effect on the proliferative responses (data not shown).

gp120 does not induce apoptosis in PBMC. These inhibitory effects of gp120 were not due to cell death, since comparable numbers of viable cells and total numbers of cells were obtained with the trypan blue exclusion test when the PBMC were cultured in the presence or absence of gp120 (data not shown). To address the question of whether gp120 caused apoptosis, further experiments were performed by the technique described by Schmid et al. (47). A representative experiment is shown in Fig. 2. PBMC were incubated for 3 days with cell culture medium (Fig. 2A) or medium containing gp120 (5 μg/ml) (Fig. 2B), ConA (1 μg/ml) (Fig. 2C), or staurosporine (1 μg/ml) (Fig. 2D). PBMC cultured in medium alone did not show apoptosis (Fig. 2A), and also the addition of gp120 did not cause apoptosis (Fig. 2B). Also, the addition of ConA did not increase the percentage of apoptotic cells (Fig. 2C) whereas the addition of staurosporine clearly induced apoptosis in PBMC (Fig. 2D) (47). Also, no apoptosis was noted when PBMC were stimulated with ConA or anti-CD3 MAb in combination with different concentrations of gp120. Comparable results were obtained after 24 or 48 h of incubation of the cell

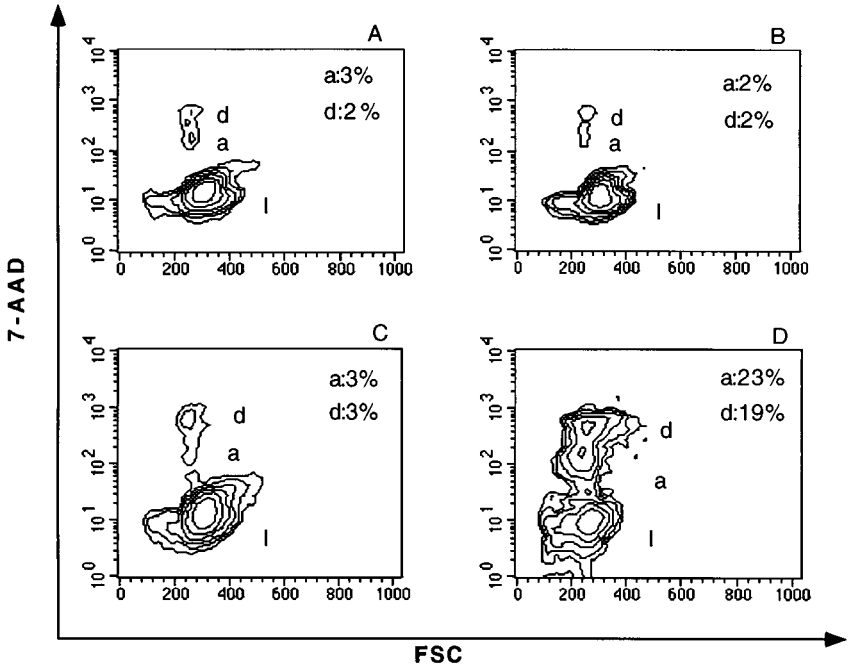

FIG. 2. PBMC were cultured in control medium (A) or medium with gp120 (5 μg/ml) (B), ConA (1 μg/ml) (C), or staurosporine (1 μg/ml) (D) for 3 days and then stained with 7-amino-actinomycin D (7-AAD) as described in Materials and Methods. The percentages of apoptotic (a) and dead (d) cells, relative to living (l) cells, are indicated. Contour plots were gated on forward scatter (FSC) and side scatter to exclude monocytes, cell debris, and cell clumps. The results of one representative experiment are shown. Similar results were obtained with samples from three different donors.

cultures (data not shown). This demonstrates that in our in vitro assay system the gp120-mediated suppression is not due to an increase in cell death but that the lymphocytes were unresponsive or anergic when stimulated with anti-CD3 MAb or ConA in the presence of gp120.

gp120 decreases the proportion of activated $CD4^+$ and $CD8^+$ T cells. The percentage of single $CD4^+$ and $CD8^+$ T cells remained the same in the cell cultures treated or not with gp120 (data not shown). However, the percentage of activated $CD4^+$ T cells expressing CD25, CD69, and HLA-DR was significantly reduced in the gp120-incubated cultures (Fig. 3). The percentage of activated $CD8^+$ T cells was reduced to the same extent as that of the $CD4^+$ T cells (Fig. 3). Flow cytometric analysis demonstrated binding of gp120 on the $CD4^+$ T cells but not on the $CD8^+$ T cells (data not shown). These data thus indicate that gp120 in total PBMC cultures affects not only the $CD4^+$ T cells but also, indirectly, the $CD8^+$ T cells.

Cytokine production of gp120-treated PBMC cultures. To investigate in detail the cytokine production profile, PBMC from normal donors were treated with anti-CD3 or ConA with or without gp120 at 5, 1, and 0.2 μg/ml and cytokines in the supernatants of the cell cultures were monitored after 24 and 48 h. Stimulation of PBMC with anti-CD3 or ConA resulted in a significant IL-2 and IL-4 production after 24 h. At 48 h these cytokines had disappeared from the supernatant. As expected (14), gp120 inhibited the production of IL-2 (Table 1), but in addition, gp120 also inhibited the production of IL-4 in a dose-dependent manner when the PBMC were stimulated with anti-CD3 or ConA (Table 1). IL-2 and IL-4 were not produced by the PBMC when these cells were incubated in control medium or medium containing different concentrations of gp120. In contrast, IL-10, IFN-γ, and TNF-α were produced by normal PBMC when incubated with gp120. The levels of production of these three cytokines were concentration dependent and reached their maximum at 24 h, although consumption of these cytokines at 48 h or even earlier could not be excluded. When the PBMC were stimulated with anti-CD3, IFN-γ and TNF-α titers remained high or increased even further when exposed to different concentrations of gp120. With ConA stimulation, the production of IFN-γ and TNF-α in the cultures

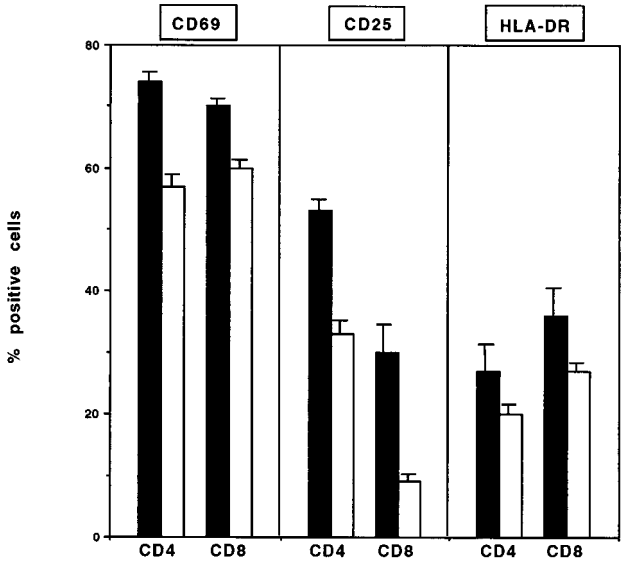

FIG. 3. Relative proportion of cells expressing CD25, CD69, and HLA-DR on $CD4^+$ and $CD8^+CD3^+$ T cells. PBMC were stimulated with ConA (1 μg/ml) (solid bars) or with ConA (1 μg/ml) in combination with gp120 (5 μg/ml) (open bars) and analyzed after 48 h. Cells were stained with fluorescein isothiocyanate-, PE-, and peridinin chlorophyll protein-labeled MAbs as described in Materials and Methods. Data represent the means (error bars, standard deviations) of three independent experiments.

TABLE 1. Effects of recombinant gp120 on cytokine production by PBMC[a]

Time (h)	Stimulus	Amt of cytokine (pg/ml) produced at gp120 concn (μg/ml)																			
		IL-2				IL-4				IL-10				IFN-γ				TNF-α			
		0	0.2	1	5	0	0.2	1	5	0	0.2	1	5	0	0.2	1	5	0	0.2	1	5
24	Medium	<	<	<	<	<	<	<	<	<	39	300	427	<	<	<	110	411	345	831	3,310
	Anti-CD3	165	88	16	<	163	128	22	<	723	650	531	477	954	1,024	1,161	2,206	824	934	1,871	4,206
	ConA	279	227	<	<	33	<	<	<	277	247	59	14	853	750	167	44	919	650	265	459
48	Medium	<	<	<	<	<	<	<	<	<	20	173	263	<	24	89	235	136	183	430	2,835
	Anti-CD3	<	<	<	<	<	<	<	<	705	588	429	265	2,088	1,905	2,044	2,351	710	691	862	3,434
	ConA	<	<	<	<	<	<	<	<	574	542	82	31	2,513	2,484	758	362	1,092	1,819	552	620

[a] PBMC were stimulated with anti-CD3 (1 μg/ml) or ConA (1 μg/ml) in combination with gp120 (0.2, 1, and 5 μg/ml). Supernatants were collected at 24 h and 48 h after stimulation. Results shown are in picograms per milliliter and are representative for and obtained from three different donors. <, below level of sensitivity of ELISA.

with increasing concentrations of gp120 decreased. With increasing concentrations of gp120, less IL-10 was produced by anti-CD3- or ConA-stimulated PBMC, suggesting consumption of IL-10 by the PBMC.

gp120-induced inhibition of the proliferative responses is reversed by IL-2 and anti-IL-10 MAb and enhanced with IL-10. Since exogenous IL-2 can reverse T-cell unresponsiveness in different cell culture models (21, 27, 48) and since gp120 reduces the expression of the IL-2 gene and IL-2 production in T-cell clones (45), exogenous IL-2 was added to the PBMC cultures when stimulated with anti-CD3 or ConA and gp120 (Fig. 4). As expected, IL-2 reversed, although not completely, the inhibitory effect of gp120. These results indicate that the lack of IL-2 is not the only limiting factor responsible for the reduced proliferative responses observed when PBMC were stimulated with anti-CD3 or ConA in the presence of gp120. In contrast to IL-2, addition of IL-4 (100 U/ml) did not show any reversal of the poor proliferative responses (data not shown). As IL-10 is produced by PBMC exposed to gp120 alone, neutralizing anti-IL-10 MAb was added at the onset of the culture. This MAb partially reversed the inhibitory effect of gp120 on the proliferative responses of PBMC (Fig. 4). Also, the addition of exogenous IL-10 (100 U/ml) further enhanced the inhibitory effect of gp120 (Fig. 4), indicating that IL-10 plays a major role in suppressing proliferation and IL-2 production of T cells. The combination of IL-2 and anti-IL-10 MAb completely reversed the inhibitory effects of gp120 at 1 μg/ml, but at 5 μg/ml some slight inhibition (±10%) was still noticed (data not shown). When the PBMC were stimulated with ConA and IL-10 (100 U/ml), a slight inhibition (~10%) was observed compared with that observed with ConA stimulation alone. IL-2 (25 U/ml) in combination with ConA had no effect, whereas compared with ConA alone, the anti-IL-10 MAb (5

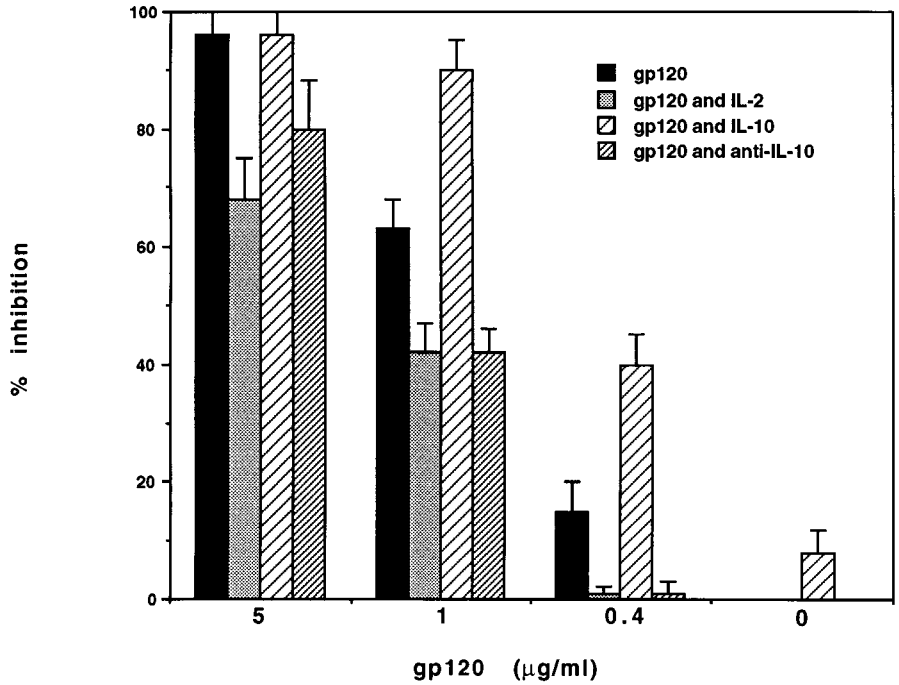

FIG. 4. Inhibitory effect of gp120 on ConA-induced proliferation of PBMC in combination with IL-2, IL-10, or anti-IL-10 MAb. Cells were stimulated with ConA (1 μg/ml) and gp120 (0.2, 1, and 5 μg/ml) without exogenous lymphokine or MAb or were stimulated with IL-2 (25 U/ml), IL-10 (100 U/ml), or anti-IL-10 MAb (5 μg/ml). The percentage of inhibition of gp120 was calculated from the corresponding (counts per minute) values. Data represent the means (error bars, standard deviations) of triplicate samples from two representative experiments.

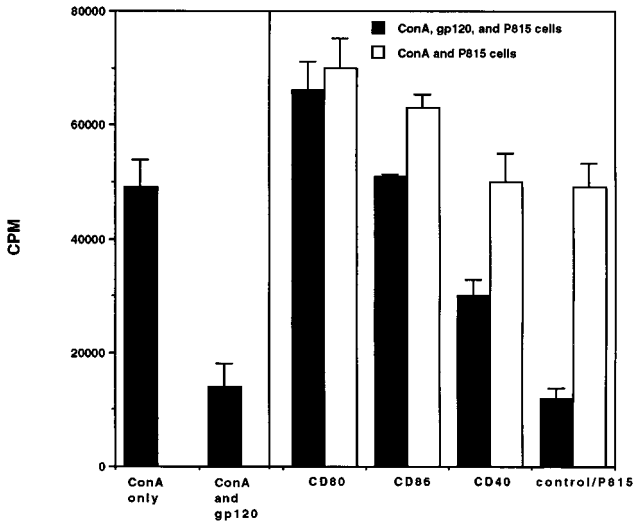

FIG. 5. Proliferation of PBMC stimulated with ConA (1 μg/ml) or with ConA (1 μg/ml) and gp120 (1 μg/ml) and in the presence of mitomycin-treated $CD80^+$P815, $CD86^+$P815, or $CD40^+$P815 cells or control transfectants. As controls, PBMC were stimulated with ConA in the presence of the different transfectants. Data represent the means (error bars, standard deviations) of triplicate samples. Similar results were collected from three different donors.

μg/ml) in combination with ConA slightly enhanced the proliferation (~15%) of the PBMC.

Reversal of proliferative response by triggering the CD28/cytotoxic T lymphocyte A-4 activation pathway. As triggering of the CD28/cytotoxic T lymphocyte A-4 activation pathway restores the proliferative responses of anergic T cells (28) mainly through their increase in IL-2 production (24, 34, 51), we investigated whether anti-CD28 MAb or transfectants expressing the CD28 ligand, i.e., CD80 or CD86, could restore the proliferative responses. ConA-stimulated PBMC incubated in the presence of anti-CD28 MAb (data not shown) or $CD80^+$P815 or $CD86^+$P815 cells regained their proliferative responses despite the presence of gp120 (Fig. 5). Both transfectants were equally effective in restoring the proliferative responses of the gp120-treated cultures and were demonstrated to be equally effective in generating IL-2 production by T cells (32). Although $CD80^+$P815 or $CD86^+$P815 cells somewhat enhanced the proliferation of ConA-stimulated PBMC cultures, the effect of gp120 was clearly diminished and reached almost the maximum level of proliferation. Interestingly, the $CD40^+$P815 transfectants partially reversed the low-level response whereas, as expected, the control transfectants were without effect (Fig. 5).

$CD4^+$ T cells are not necessary for the inhibitory effect of gp120 in PBMC cultures. When total PBMC were stimulated with ConA, gp120 at 0.4 μg/ml strongly inhibited the proliferative response (~40% inhibition) (Fig. 6). When the PBMC were depleted from $CD8^+$ T cells (from 21% $CD8^+$ T cells in the original PBMC to <1% after depletion) with CD8 magnetic beads, gp120 still strongly inhibited the response (~40% inhibition). Interestingly, when the PBMC were depleted of $CD4^+$ T cells with CD4 magnetic beads (from 52% $CD4^+$ T cells in the original PBMC to <1% after depletion), the response to ConA decreased but gp120 still had a strong inhibitory effect (~50% inhibition). Monocytes ($CD14^+$, weakly $CD4^+$) were not removed from the cultures (17% $CD14^+$ cells in the original PBMC, 24% in the $CD4^+$ T-cell-depleted PBMC, and 22% in the $CD8^+$ T-cell-depleted PBMC). The responses of the $CD4^+$ and $CD8^+$ T-cell-depleted PBMC were enhanced to normal control values, compared with the total PBMC (Fig. 3), following the addition of IL-2 (data not shown) or anti-IL-10 MAb (Fig. 6). The responses of the CD8-depleted PBMC were also enhanced with the $CD80^+$, $CD86^+$, and $CD40^+$P815 cells, whereas the responses of the $CD4^+$ T-cell-depleted PBMC were enhanced with the $CD80^+$ or $CD86^+$ transfectants but not with the $CD40^+$P815 cells (data not shown). This indicates that gp120 even suppresses the proliferation of the $CD8^+$ T cells in the absence of $CD4^+$ T cells.

gp120 induces IL-10 expression in monocytes/macrophages. Because gp120 induced inhibition of the proliferation of $CD4^+$

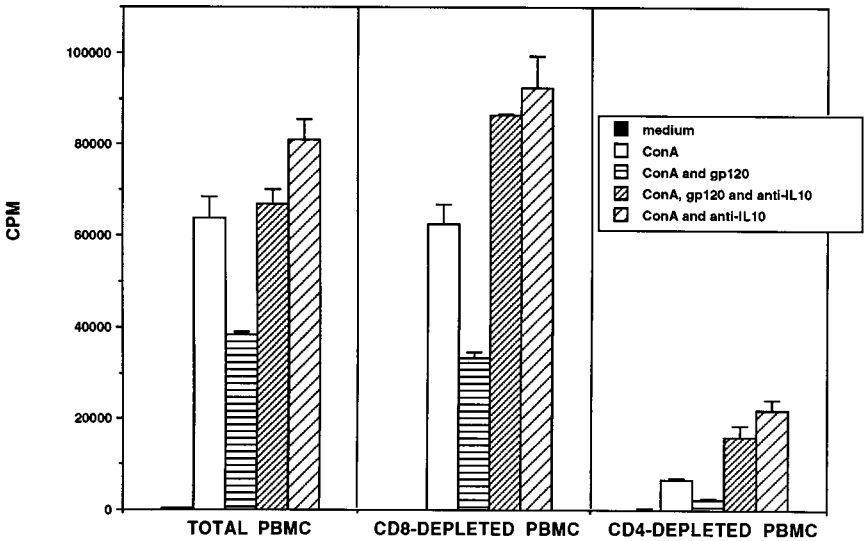

FIG. 6. Proliferation of total PBMC, $CD8^+$ T-cell-depleted PBMC, and $CD4^+$ T-cell-depleted PBMC stimulated with ConA; ConA and gp120 (0.4 μg/ml); ConA, gp120, and anti-IL-10 (5 μg/ml); or ConA and anti-IL-10. The percentages of $CD4^+$ and $CD8^+$ T cells in the total PBMC were 52 and 21%, respectively, those in the $CD8^+$ T-cell-depleted PBMC were 69 and <1%, respectively, and those in the $CD4^+$ T-cell-depleted PBMC were <1 and 48%, respectively. Data represent the means (error bars, standard deviations) of triplicate samples from two independent experiments.

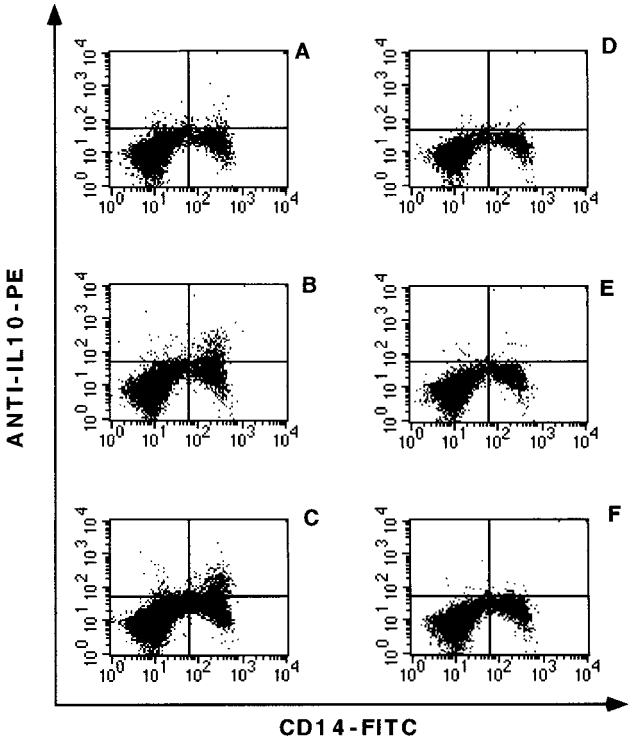

FIG. 7. Expression of IL-10 by monocytes/macrophages ($CD14^+$ cells) in total human PBMC. PBMC were incubated in medium (A and D) or were stimulated with LPS (2 μg/ml) (B and E) or gp120 (5 μg/ml) (C and F) for 6 h in the presence of 2 μM monensin and then were stained with fluorescein isothiocyanate-conjugated anti-CD14 (CD14-FITC). The cells were fixed, permeabilized, stained with anti-IL-10–PE, and analyzed by two-color flow cytometry. The binding of anti-IL-10–PE was blocked by preincubation of the conjugated MAb with an excess of IL-10 (1,000 U/ml) (D to F).

and $CD8^+$ T-cell-depleted cultures and was measured in the presence of monocytes, we investigated whether monocytes would be the main source of IL-10. As shown in Fig. 7, total PBMC incubated with LPS revealed the presence of IL-10 in the monocyte/macrophage ($CD14^+$) population (Fig. 7B). Also, gp120 was clearly able to induce IL-10 production in the monocyte population (Fig. 7C). The $CD14^-$ population (lymphocytes) was negative for IL-10. The specificity of the staining was attested by the addition of an excess of IL-10 (Fig. 7D to F). When purified $CD4^+$ T cells, $CD8^+$ T cells, or monocytes were incubated with gp120, only the monocytes produced considerable amounts of IL-10 as determined by the IL-10-specific ELISA (data not shown). This demonstrated that gp120 is capable of inducing IL-10 expression in monocytes/macrophages.

DISCUSSION

Our data imply that PBMC from apparently healthy donors incubated with gp120 demonstrate a less vigorous response when stimulated with anti-CD3 MAb or ConA. The production of IL-2 was diminished by gp120 in the cultures, and the addition of IL-2 partially restored this response. We found, in agreement with others (33), that gp120 did not induce significant cell death by apoptosis (Fig. 2). Relatively few studies on TCR-mediated signaling events in HIV infection have been reported, and the intrinsic defect in the signal transduction pathway is not clear yet. In HIV-infected persons, proliferation of PBMC is less vigorous when stimulated with anti-CD3 with a lower level of IL-2 production, and the addition of IL-2 restored the decreased proliferative responses (49). In a later study, anti-IL-10 MAb was also able to restore the proliferative responses of PBMC from HIV-infected persons (15).

Our data demonstrate that gp120 induced the production of considerable amounts of IL-10 in normal PBMC. In vitro studies have shown that IL-10 inhibited IFN-γ, granulocyte-macrophage colony-stimulating factor, TNF-α, and IL-3 production by PBMC activated with anti-CD3 MAb or mitogens (52). IL-10 also directly suppresses T-cell proliferation by specific inhibition of IL-2 production in T-cell clones and purified T cells (19). This inhibition of proliferation was easily overcome with exogenous IL-2, although in mixed lymphocyte cultures of total PBMC exogenous IL-2 only partially overcame the effects of IL-10 (6).

Here we show that there is a strong correlation between high-level IL-10 production and low-level IL-2 production. This demonstrates that the endogenous IL-10, induced by gp120, plays a role in suppressing IL-2 production. The addition of exogenous IL-10 further enhanced the effect of gp120, whereas the addition of an anti-IL-10 MAb partially neutralized the effect of gp120. However, IFN-γ and TNF-α, normally inhibited by IL-10, were also measured in the gp120-treated PBMC cultures, indicating a very complex cytokine network. The addition of neutralizing MAbs against IFN-γ and TNF-α did not influence the inhibitory effects of gp120 in the cell proliferation assay (data not shown), demonstrating again the important role of IL-10. Human monocytes are strong producers of IL-10, but B cells, $CD4^+$ T cells (not only Th2 but also Th1 and Th0 cells), and $CD8^+$ T cells can also produce it (20). Compared with in vivo data, one report demonstrated that during HIV infection levels of mRNA for IL-10, IFN-γ, and TNF-α increased in the $CD4^+$ T cells whereas the levels of mRNA for IL-2 and IL-4 did not change significantly (22).

Costimulation through CD28 via anti-CD28 MAb or with the natural ligand CD80 or CD86 resulted in a relative restoration of the impaired response of normal PBMC incubated with gp120 (Fig. 5). PBMC had no altered expression of CD28 when incubated with gp120 compared with PBMC not incubated with gp120 (data not shown), although in HIV-infected persons a significant proportion of T cells lose expression of CD28, especially on $CD8^+$ T cells (8). Recently, it was demonstrated that the impaired responses from HIV-infected individuals to CD3-TCR ligation were enhanced to normal values when costimulated by CD28 (37). Also, signaling through CD27 enhanced the response to normal values (37), which suggests that T cells from HIV-infected persons were defective in their response to signal one but are still able to respond to costimulation by CD28 and CD27. As CD28 and CD27 are expressed on the majority of both $CD4^+$ and $CD8^+$ peripheral blood T cells, interesting data were obtained with the $CD40^+$ transfectants. The CD40L is preferentially expressed on $CD4^+$ T cells, whereas the $CD8^+$ T cells express only low levels of CD40L after stimulation (31). $CD4^+$ T cells proliferate in culture with anti-CD3 and $CD40^+$ transfectants, whereas no proliferation is induced in the $CD8^+$ T-cell population (10). The partial reversal of the response with the $CD40^+$ transfectants (Fig. 5) suggests that only the $CD4^+$ T cells proliferate whereas the $CD8^+$ T cells are unresponsive. Also, CD4-depleted PBMC did not respond to anti-CD3 or ConA when incubated with gp120 and the $CD40^+$ transfectants but responded normally in combination with gp120 and $CD80^+$ or $CD86^+$ transfectants. Recently, it was shown that pretreatment of $CD4^+$ T-cell clones with gp120 inhibited anti-CD3 MAb-induced CD40L expression (13), although in our cell culture

system minor effects of gp120 on CD40L expression on T cells were noticed (data not shown).

That gp120 also had a strong suppressive effect on the CD4-depleted PBMC demonstrates that $CD4^+$ T cells do not need to be present for suppression of the proliferation of the $CD8^+$ T cells and that mainly IL-10 is responsible for the suppressive effect because also anti-IL-10 MAb neutralized the effect of gp120. As IL-10 is mainly produced by monocytes in total PBMC cultures, gp120 most likely binds to the CD4 receptor on the monocytes and upregulates their IL-10 production, as demonstrated in Fig. 7. Also, the level of expression of HLA-DR on monocytes, as judged by the mean fluorescence intensity, was reduced in the presence of gp120 (data not shown), a characteristic of the presence of considerable amounts of IL-10 in the culture (18). Borghi et al. demonstrated that HIV-1 infection induced IL-10 mRNA in monocytes/macrophages and in addition that recombinant gp120 by itself caused a marked increase in IL-10 secretion (7).

Thus, gp120 might directly influence monocytes in their antigen presentation by interference with expression of accessory molecules and cytokine release. So far, only a few studies on the antigen-presenting cell (APC) function of monocytes and dendritic cells in AIDS patients have been reported. Monocytes from HIV-infected persons have decreased accessory cell function (41), and dendritic cells from HIV-infected persons have decreased major histocompatibility complex class II expression and decreased accessory cell function (35). In addition, it has been recently demonstrated that the IL-10 levels in HIV-positive sera were significantly higher than those found in the controls (1). Therefore, the presence of IL-10 could disturb APC function and thus also induce T-cell unresponsiveness not only in the $CD4^+$ T-cell subset but also in the $CD8^+$ T-cell subset. Strong evidence for APC alteration stems from the diminished release of IL-12 by macrophages from HIV-infected persons (11) and, more importantly, from the fact that IL-12 can restore T-cell responses to recall antigens in HIV-infected persons (14). Recently, it was demonstrated that in the human leukemic cell line HPB, CD4 ligation by gp120 resulted in activation of the tyrosine kinase $p56^{lck}$ and inactivation of the TCR function (25). These in vitro findings may have direct relevance to impaired T-cell responses from HIV-infected persons since in some patients with AIDS concentrations of gp120 were reported to be even higher than p24 antigen levels (44).

Our in vitro data demonstrate that the inhibitory effect of gp120 on $CD4^+$ and $CD8^+$ T-cell proliferation is achieved mainly through IL-10. This inhibition is reversed with IL-2, costimulatory molecules, and especially anti-IL-10. Therapy, which is based on counteracting the effects of IL-10, might restore HIV-specific T-cell immunity, which should be the goal of immune-based therapy in AIDS patients.

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