HIV-1 gp120 induces anergy in naive T lymphocytes through CD4-independent protein kinase-A-mediated signaling

Anna Maria Masci,* Mario Galgani,* Silvana Cassano,† Salvatore De Simone,† Adriana Gallo,† Veronica De Rosa,* Serafino Zappacosta,* and Luigi Racioppi*,[1]

*Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli Federico II, Naples, Italy; and †Istituto di Endocrinologia ed Oncologia Sperimentale, Napoli, Italy

Abstract: The ability of the envelope glycoprotein gp120 [human immunodeficiency virus (HIV) *env*] to induce intracellular signals is thought to contribute to HIV-1 pathogenesis. In the present study, we found that the exposure of CD4+ CD45RA+ naive T cells to HIV*env* results in a long-lasting hyporesponsiveness to antigen stimulation. This phenomenon is not dependent on CD4-mediated signals and also can be generated by the exposure of naive T cell to soluble CD4-HIV*env* complexes. The analysis of the proximal signaling reveals that HIV*env* does not activate Lck as well as the mitogen-activated protein kinase intermediate cascade. Conversely, the envelope glycoprotein stimulates the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) activity and induces the progressive accumulation of the phosphorylated form of the cAMP-responsive element binding. Of note, the ligation of CXCR4 by stromal cell-derived factor-1α but not the engagement of CD4 by monoclonal antibody stimulates the PKA activity and induces a long-lasting hyporesponsivity state in naive CD4+ lymphocytes. The pretreatment of lymphocytes with H89, a cell-permeable PKA inhibitor, prevents the induction of anergy. These findings reveal a novel mechanism by which HIV*env* may modulate the processes of clonal expansion, homeostatic proliferation, and terminal differentiation of the naive T lymphocyte subset. *J. Leukoc. Biol.* 74: 1117–1124; 2003.

Key Words: *CXCR4 · HIV*env *· cAMP · CREB*

INTRODUCTION

Human immunodeficiency virus (HIV) infection results in a functional impairment of CD4 T cells long before a quantitative decline of this lymphocyte subset becomes evident. This defect has been documented in a high percentage of HIV-infected individuals whose CD4 lymphocytes fail to proliferate in response to antigenic stimulation and may have relevant consequences on the capacity of the immune system to mount an effective response to HIV or to other pathogens [1–5]. The inability of HIV-exposed T cells to generate a vigorous response to antigens has been associated with an alteration in interleukin (IL)-2 production and an enhanced susceptibility to apoptosis of the lymphocytes [6–8]. Several reports have investigated the molecular bases of T cell abnormalities in HIV infection providing evidences for multiple defects in the early T cell receptor (TCR)-mediated signaling pathways. CD4 and CD8 T lymphocytes from AIDS patients showed a significant decrease in CD3-ζ expression, which appeared to be reversible upon reduction of the virus load with highly active antiretroviral treatment [9]. A marked decrease in the enzymatic activity of CD45 and altered levels of Lck and Fyn have been observed in lymphocytes isolated from HIV-infected individuals [10–16]. Most of these defects can be reproduced in vitro by exposure of normal T lymphocytes to inactivated HIV virions or purified HIV components. In fact, soluble (s)gp120 is able to inhibit IL-2 mRNA expression [17] and the proliferative response of T lymphocytes when stimulated through the TCR [18, 19]. The ability of envelope glycoprotein gp120 (HIV*env*) to interfere with TCR-mediated activation of the src-family tyrosine kinases also has been documented [20–22]. We proposed that the effects of gp120 on antigen-dependent proliferation of T lymphocytes are not homogenous and may vary according to their state of activation and differentiation [23]. Naive and memory CD4 subsets show differential susceptibility, although the exposure of CD4 naive T cells to the envelope glycoprotein results in a marked inhibition of the mitogenic responses, the growth of the memory subset is less severely restrained by gp120 [23]. This inhibitory effect is associated with alterations in the TCR-mediated signaling such as inhibition of the extracellular-regulated kinase (ERK) and stimulation of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA).

PKA is a serine/threonine kinase that regulates a number of cellular processes important for immune activation [24]. In lymphocytes, PKA regulates antigen receptor-induced signaling by altering protein–protein interactions and by changing the enzymatic activity of target protein [25]. Stimulation of the cAMP/PKA pathway may also increase the level of inducible cAMP early repressor and cAMP-responsive element modulator, two nuclear inhibitory factors involved in modulation of T

[1] Correspondence: Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli Federico II, 5 via S. Pansini, I-80131 Napoli, Italy. E-mail: racioppi@unina.it

Received May 22, 2003; revised August 11, 2003; accepted August 12, 2003; doi: 10.1189/jlb.0503239.

cell responsiveness by their capacity to transcriptionally attenuate IL-2 gene expression [26, 27]. Hofmann et al. [28] suggested the involvement of PKA in the mechanism leading to the HIV-induced defect in T cell function. More recently, this hypothesis has been corroborated by direct measurements of the cAMP intracellular levels in T lymphocytes derived from HIV+ individuals and by the ability of PKA type I antagonist to restore the responsiveness of T cells from HIV-infected patients [29, 30]. Although these reports revealed an involvement of the PKA pathway in the mechanism leading to T cell abnormalities in HIV infection, the molecular base of this phenomenon is still unclear. Here, we provide novel evidences on the ability of the soluble envelope glycoprotein to induce long-lasting effects on naive lymphocytes through a CD4-independent, PKA-mediated pathway. This phenomenon may contribute to the development of immune defects in HIV-1 infected individuals.

MATERIALS AND METHODS

Cell culture and reagents

Complete medium was RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% sodium piruvate, 1% nonessential amino acids (all from Life Technologies, Rockville, MD), and 10% heat-inactivated fetal calf serum (FCS; Hyclone laboratories, Logan, UT). H89 (Sigma Chemical Co., Milan, Italy) and PP1 (Alexis, Lausen, Switzerland) were dissolved in dimethyl sulfoxide and added at the final concentration of 10 μM. The anti-CD4 antibody Leu3a was from Becton Dickinson (San Jose, CA). Human stromal cell-derived factor-1 (hSDF-1)α was from Alexis. sh-recombinant CD4 (shrCD4), rgp120 IIIB (HIV*env*), and biotinylated rgp120 were from Intracell Corp. (London, UK). sCD4-HIV*env* complexes were generated by incubating sCD4 and HIV*env* in serum-free medium at a 1:1 ratio for 1 h at 4°C before being added to cells at the final concentration of 2 μg/ml.

Purification of naive CD4 T lymphocytes

T lymphocytes were isolated from peripheral blood of healthy donors by Lymphoprep (Nyegaard and Co., Oslo, Norway) density-gradient centrifugation at 2500 rpm for 30 min and subsequently, by incubating mononuclear cells for 1 h at 37°C in plastic petri dishes. Resting T cells were then purified from nonadherent cells by nylon wool (Robbins Scientific, Sunnyvale, CA) adherence to remove B cells, followed by a two-step (40% and 50%, respectively) Percoll gradient (Pharmacia Biotech, Uppsala, Sweden). Resting lymphocytes were recovered from the high-density cell fraction. The CD4+ and CD8+ CD45RA cells were separated using negative selection with subset-specific monoclonal antibodies (mAb; anti-CD8, -CD19, -DR, -CD45RO, CD16, -CD56, -γδ TCR, all from Becton Dickinson) and anti-mouse immunoglobulin-coated magnetic beads (Dynabeads, Dynal, Oslo, Norwey). The purity of lymphoid populations was tested by flow cytometry (FACScan, Becton Dickinson) using appropriate mAb (PharMingen, San Diego, CA). Purified, naive lymphocytes contained 89–93% of CD3+ CD45 RA+. Contaminating cells were CD8+ (4–7%), CD45 RO+ (3–5%), and CD3– CD56+ (3–10%). The purity of naive lymphocytes was also tested by functional assay: Stimulation with an optimal dose of phytohemagglutinin (PHA; 2 μg/ml) did not induce a detectable mitogenic response. Conversely, an optimal response was observed upon addition to cultures of 10% of dendritic cells (DC) or anti-CD28 antibody (Becton Dickinson; data not shown).

Generation of monocyte-derived DC (Mo-DC)

Mo-DC were obtained according to protocol by Sallusto et al. [31]. Briefly, monocytes were isolated from peripheral blood mononuclear cells by adherence on plastic dishes coated with 2% gelatin. Adherent cells were removed by incubation with phosphate-buffered saline–EDTA and were cultured for 5 days at a density of 3×10^6 cells/ml in RPMI 1640 containing 10% FCS supplemented with 50 ng/ml granulocyte macrophage-colony stimulating factor and 1000 U/ml IL-4 (Sigma Chemical Co.). The purity of isolated populations was tested by flow cytometry using the appropriate mAb.

Anergy assay

To test the capability of HIV*env* to induce a prolonged hyporesponsivity state, namely anergy, naive lymphocytes were incubated with viral glycoprotein, rested in fresh medium, and stimulated with mitogens or allogenic Mo-DC. During the incubation step, $3–5 \times 10^6$ purified T cells were cultured in the presence or absence of 1–2 μg/ml rgp120 for 12–18 h. At the end of this incubation, lymphocytes were tested for viability, washed extensively, and rested in fresh medium for further 3–8 days (resting step). At the end of the resting step, Lymphoprep (Nyegaard and Co.) density gradient removed death cells, and living cells were tested for responsiveness to mitogens or allogenic Mo-DC (stimulation step). As tested in additional experiments, the purification step with Lymphoprep did not interfere with responsiveness and survival of lymphocytes. Proliferation assays were performed by culturing 10^5 lymphocytes/wells for 72 h in round-bottom 96 wells/plate with increasing doses of PHA (Sigma Chemical Co.) plus accessory cells (5% of plastic-adherent, allogenic cells). Mitogenic response to Mo-DC was tested by stimulation of T cells with increasing amounts of allogenic Mo-DC. ^{3}H-Thymidine (Amersham International, Buckinghamshire, UK) was added to cultures during the last 12 h of incubation.

Immunoblotting and immunocomplex kinase assays

Purified CD4 CD45 RA cells (5×10^6) were preincubated for 30 min on ice in the presence or absence of rgp120 (2 μg/ml) and then incubated at 37°C for different periods of time. Lymphocytes were lysed in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml tosylphenylalanine chloromethyl ketone, 1 μg/ml leupeptin, 0.83 μg/ml chymostatin, 10 μg/ml soybean trypsin inhibitor, 1 mM sodium orthovanadate, and 0.5 mM dithiothreitol. The lysates were boiled for 5 min and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was performed using mAb to Lck, phospho-ERK, ERK-2, cAMP response element-binding protein (CREB), and phospho-CREB (Santa Cruz Biotechnology, Santa Cruz, CA) and was developed using the enhanced chemiluminescence Western blotting kit (Amersham International).

PKA assays were performed in a final volume of 25 μl at 30°C for 10 min. The kinase reaction mixture contained 100 μM adenosine 5′-triphosphate (ATP), [γ^{32}P] ATP 10 μCi/100 ml, 10 mM $MgCl_2$, 20 mM Hepes (pH 7.4), and 100 μM Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide; Sigma Chemical Co.). Specificity of reaction was verified by adding 100 μM protein kinase inhibitor (PKI; P0300) synthetic rabbit PKA-specific inhibitor pseudo-substrate (Sigma Chemical Co.) to a duplicate of each experimental point. Kemptide phosphorylation was monitored by spotting 20 μl reaction mixture on phosphocellulode filters (Whatman, P81). After washing with phosphoric acid, 75 mM, radioactivity was determined by counting the filters in 4 ml scintillation liquid (Ecolite, ICN, Costa Mesa, CA). Free catalitic-PKA (C-PKA) activity was determined as PKI-inhibitable filter-bound radioactivity. Control assay indicated that 100 μM PKI used did not inhibit the binding of phosphorylated Kemptide to phosphocellulose filters. Data were expressed as picomoles of [γ^{32}P] phosphate transferred to the peptide substrate during 10 min incubation.

Statistical analysis

Data were analyzed by Wilcoxon test for paired comparison by using the StatView SE+ Graphic program for Macintosh (Abacus Concepts, Cary, NC).

RESULTS

HIV*env* induces anergy in naive CD4$^+$ T cells

To evaluate the ability of the soluble envelope glycoprotein to induce long-lasting, biological effects in naive T lymphocytes,

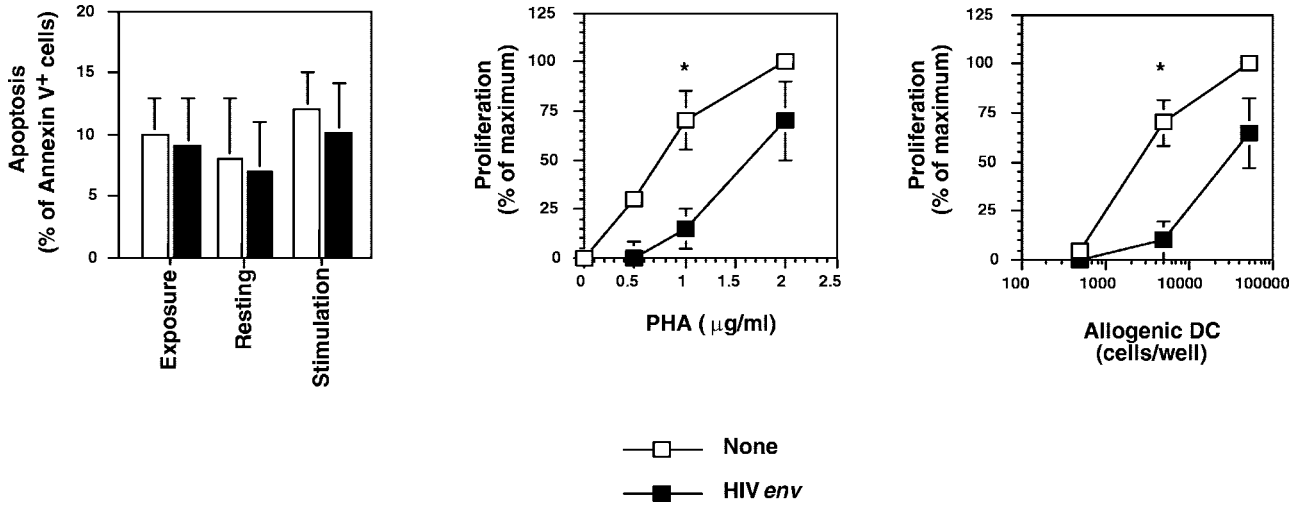

Fig. 1. Effects induced by transient exposure of naive T cells to purified HIV*env*. Purified CD4+ CD45 RA lymphocytes were exposed for 12 h to 2 μg/ml rgp120, washed, and rested for 72 h in fresh medium. At different times, aliquots of T cells were stained with Annexin V and analyzed by flow cytometry. The left panel reports the percentage of Annexin V-positive cells upon 12 h of exposure to rgp120 at the end of the resting step and 72 h from addition of PHA 2 μg/ml (stimulation step). Responsiveness of T cells to PHA or allogenic DC is shown in the middle and right panels, respectively. The results of the proliferation assays are expressed as the percent of the maximal response, assuming 100% the response of HIV*env*-untreated lymphocytes to an optimal dose of PHA (2 μg/ml; 75,000±12,000 cpm of ^{3}H-thymidine incorporation) or to 50,000 allogenic DC/well (35,000±5400 cpm). Each data point represents the mean of three independent experiments ± SD. *, Statistical significant differences ($P<0.001$) were observed between the proliferation values measured in HIV*env*-treated and untreated samples stimulated with 1 μg/ml PHA or 5000 allogenic DC/well, respectively (center and right panels).

purified CD4 CD45RA lymphocytes were exposed to rHIV*env* for a prolonged time (12–18 h). At the end of this incubation, T cells were tested for viability, washed extensively, and rested in fresh medium for an additional 3–8 days (resting step). Finally, viable cells were isolated by density gradient, and their responsiveness to polyclonal mitogens and allogenic DC was tested. The exposure of naive T cells to HIV*env* did not induce a significant increase in T cell death at any step of the experimental procedure (**Fig. 1**). However, lymphocytes cultured with the viral glycoprotein showed a marked decrease in the mitogenic response to PHA and allogenic DC stimulation (Fig. 1). This effect was dependent on an increase in the activation threshold as revealed by statistical analysis, showing that the exposure to rgp120 reduced significantly ($P<0.001$) the response of naive T cells to suboptimal stimuli (PHA or allogenic DC) but was less effective at higher doses of mitogens.

Anergy-induced gp120 may be dependent on the prolonged down-regulation of CD4 from cell surface, a receptor able to stabilize major histocompatibility complex:peptide/TCR complexes and to transduce activating signals in T cells. To verify this hypothesis, we compared kinetics of HIV*env*-induced CD4 down-regulation with the persistence of hyporesponsiveness. In parallel, cell-surface expression of CXCR4 and CD3-ε was also evaluated. After 12 h of incubation with HIV*env*, 80% of CD4 and 40% CXCR4 molecules were removed from the cell surface, and no change in CD3-ε expression was observed (**Fig. 2**). During the resting step, CD4 and CXCR4 surface levels progressively recovered until at later time points, no difference was found between HIV*env*-treated and untreated lymphocytes (Fig. 2). These data indicate that the effects induced by HIV*env* on CD4 expression were transient and required the continuous presence of the HIV*env* in the culture medium. Conversely, a

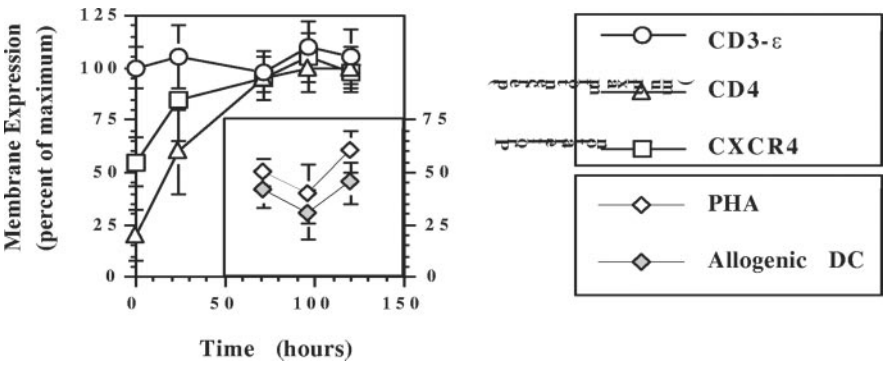

Fig. 2. Kinetic analysis of the effects induced by HIV*env* on naive T cells. CD4+ naive lymphocytes were exposed to HIV*env* for 12 h, washed, and rested in fresh medium. The expression of CD4, CXCR4, and CD3-ε has been tested by flow cytometry at different time points. The results have been reported as the percentage of the maximum corresponding to the cell-surface expression levels measured in untreated lymphocytes. Inset reports the results of the anergy assay. Briefly, upon exposure to HIV*env*, T cells were washed and rested for 72 h in fresh medium before being stimulated with T cell responsiveness to PHA (1 μg/ml) or allogenic DC (5000 cell/well). The results have been reported as the percentage of the maximum proliferation measured in HIV*env*-unexposed lymphocytes stimulated with the optimal doses of PHA or allogenic DC. (^{3}H-Thymidine incorporation was 41,000±7000 and 25,000±3500 cpm, respectively.) Each data point reports the mean of three independent experiments ± SD.

pulsed exposure to HIV*env* was able and sufficient to induce a prolonged hyporesponsiveness in naive T lymphocytes to PHA and allogenic DC stimulation (Fig. 2, inset).

Analysis of early biochemical changes induced by HIV*env* in CD4+ naive T cells

To identify the biochemical pathway responsible for the induction of anergy, we analyzed the activation status of several tyrosine kinases in HIV*env*-exposed and unexposed, naive lymphocytes. As the viral glycoprotein binds to CD4, we should expect that the incubation with HIV*env* should stimulate Lck activity. Of note, we found no difference in Lck autophosphorylation activity between HIV*env*-treated and untreated lymphocytes (**Fig. 3**). These data were confirmed by Western blot analysis showing no shift-up of the Lck band (Fig. 3). To further analyze the proximal signal pathway triggered by HIV*env*, we measured the level of the phosphorylated form of ERK. These experiments showed that HIV*env* was unable to stimulate the mitogen-activated protein kinase (MAPK) pathway in naive T lymphocytes (Fig. 3). Finally, we measured the effects induced by HIV*env* treatment on PKA and on the phosphorylation status of CREB, a putative PKA substrate. PKA phosphotransferase activity was analyzed in naive T cells exposed to HIV*env* for short periods of time (5–30 min) by an in vitro kinase assay on a synthetic peptide. Western blotting revealed the phosphorylated and unphosphorylated forms of CREB by using appropiate commercial antibodies (see Materials and Methods for details). As shown in Figure 3, HIV*env* stimulated the PKA activity and induced a progressive phospho-CREB accumulation.

Role of CD4 in HIV*env*-induced anergy

CD4 plays a key role in the pathogenesis of HIV infection by its ability to bind the envelope glycoprotein and to transduce inhibitory signals responsible for apoptosis or anergy [32–35]. In this set of experiments, we analyzed the effects of Leu3a, an anti-CD4 antibody, and SDF-1, the natural ligand of CXCR4 on anergy, and binding of HIV*env* to the cell surface. The presence of Leu3a but not SDF-1 during the incubation step with rgp120 was effective in preventing the long-lasting, inhibitory effects exerted by the viral glycoprotein (**Fig. 4**). In addition, treatment with Leu3a but not SDF-1 was effective in decreasing the binding of biotinylated HIV*env* to the T cell surface (Fig. 4). These findings indicate that CD4 but not CXCR4 is crucial to recruit HIV*env* to the cell surface but not provide direct information regarding the ability of these receptors to transduce inhibitory signals. To investigate this point, we analyzed the effect induced by the prolonged exposure of naive CD4 T cells to Leu3a and SDF-1. As expected, exposure to Leu3a resulted in a marked down-regulation of CD4 from the cell surface; however, this phenomenon was not associated with the induction of anergy. Of note, Leu3a did not stimulate the PKA activity (**Fig. 5**). The prolonged exposure of naive lymphocytes to SDF-1 resulted in a long-lasting hyporesponsiveness to antigen stimulation. This phenomenon was associated with marked down-regulation of CXCR4 from cell surface and with a detectable stimulation of the PKA activity (Fig. 5). These findings indicated that although CD4 was responsible for binding of HIV*env* to the cell surface, its ligation did not activate the inhibitory cascade in naive T lymphocytes.

The binding of sCD4 to HIV*env* induces conformational changes in the virus glycoprotein: sCD4/HIV*env* complex will be expected to bind CXCR4 but not the membrane CD4 receptor [36]. Therefore, this complex is a useful tool to investigate further the role of CD4 and CXCR4 molecules in the induction of anergy. In fact, we found that the preincubation of HIV*env* with sCD4 prevents the ability of gp120 to down-regulate the expression of CD4 from the cell surface without affecting its capability to induce a decrease in the level of CXCR4 expression (Fig. 5). Of note, the pretreatment of HIV*env* with sCD4 did not prevent the anergizing activity of the envelope glycoprotein and did not abrogate the ability of gp120

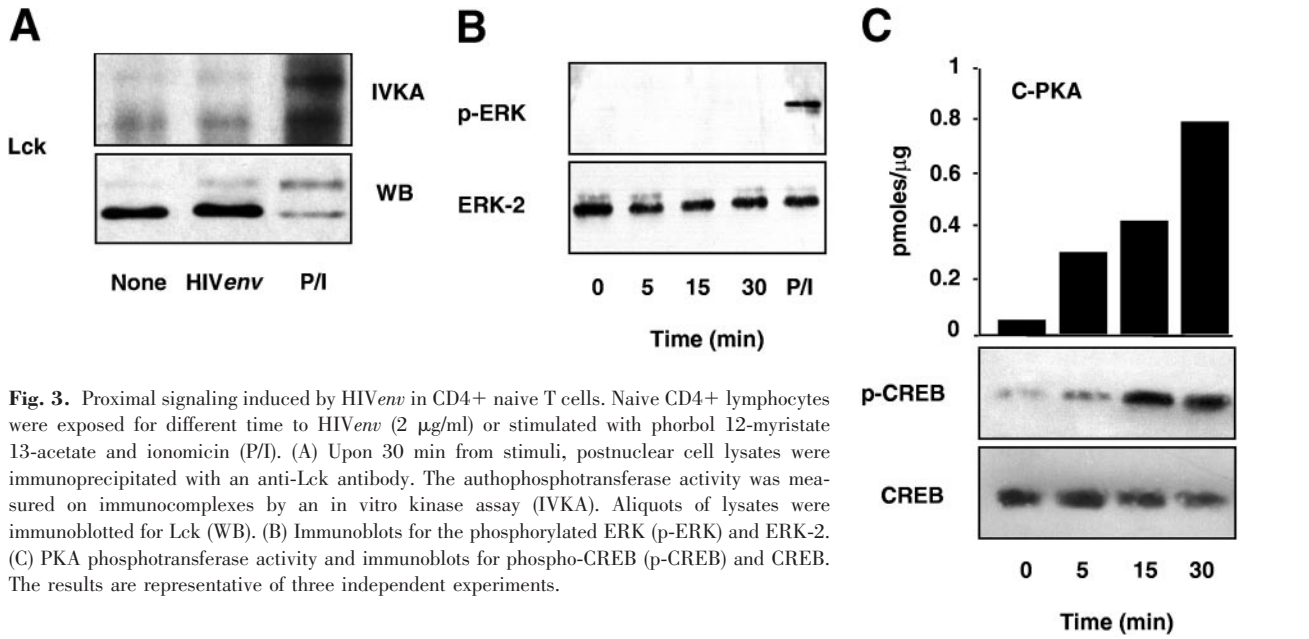

Fig. 3. Proximal signaling induced by HIV*env* in CD4+ naive T cells. Naive CD4+ lymphocytes were exposed for different time to HIV*env* (2 μg/ml) or stimulated with phorbol 12-myristate 13-acetate and ionomicin (P/I). (A) Upon 30 min from stimuli, postnuclear cell lysates were immunoprecipitated with an anti-Lck antibody. The authophosphotransferase activity was measured on immunocomplexes by an in vitro kinase assay (IVKA). Aliquots of lysates were immunoblotted for Lck (WB). (B) Immunoblots for the phosphorylated ERK (p-ERK) and ERK-2. (C) PKA phosphotransferase activity and immunoblots for phospho-CREB (p-CREB) and CREB. The results are representative of three independent experiments.

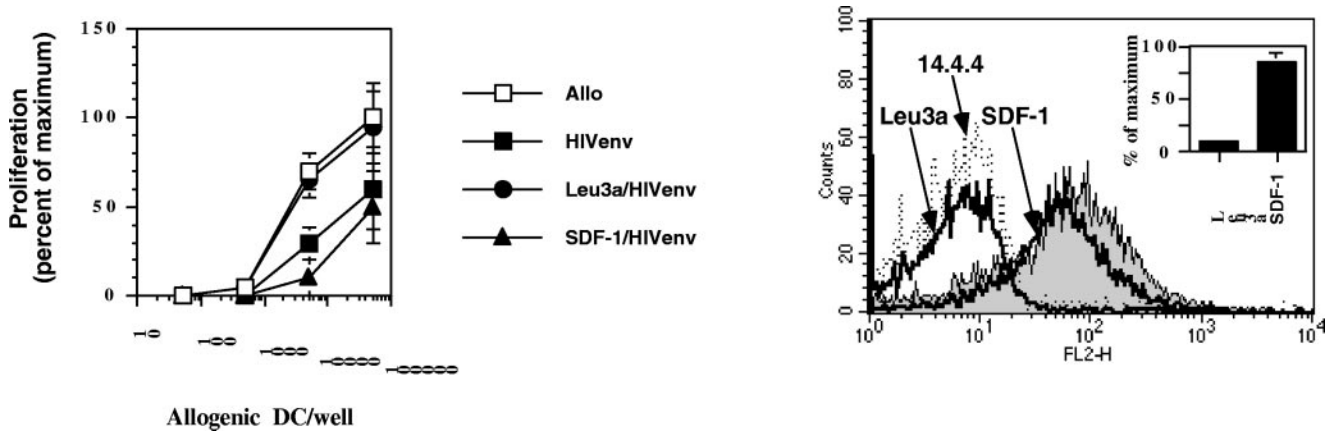

Fig. 4. Effects of anti-CD4 antibody and SDF-1 on HIV*env*-induced anergy. Naive CD4+ lymphocytes were exposed to HIV*env* (2 μg/ml) in the presence or absence of Leu3a (0.5 μg/ml) or SDF-1 (100 nM). (Left) Results of the anergy assay (see Materials and Methods). T cell responsiveness to allogenic (Allo) DC has been expressed as the percentage of the maximum proliferation measured in HIV-untreated T lymphocytes exposed to 50,000 allogenic DC/well corresponding to 35,000 ± 8000 cpm ^{3}H-thymidine incorporation. Each data point reports the mean of three independent experiments ± SD. (Right) Ability of Leu3a and SDF-1 to prevent the binding of HIV*env* to the cell surface. In this set of experiments, naive CD4+ lymphocytes were exposed for 2 h to biotinylated HIV*env* (250 ng/ml) in the presence or absence of Leu3a (0.5 μg/ml) or SDF-1 (100 nM). Phycoerythrin (PE)-conjugated avidin was used to detect the binding of biotinylated HIV*env*. The filled, gray profile refers to PE-avidin staining, measured on T cells preincubated with biotinylated HIV*env* (maximal staining). Open, thick profiles refer to PE-avidin staining measured in cells preincubated with biotinylated HIV*env* in the presence of Leu3a or SDF-1. Open, dotted profile refers to PE-avidin staining measured in lymphocytes not exposed to biotinylated HIV*env* (negative control). The result is representative of three independent experiments.

to trigger the PKA activity (Fig. 5). Taken together, these findings confirmed the inability of CD4 to stimulate the PKA pathway and to transduce negative signals in naive T cells.

The pharmacological block of PKA prevented the HIV*env*-induced anergy

HIV*env* and sCD4-HIV*env* complexes share a comparable ability to induce anergy in naive T cells, a phenomenon associated with their capability to stimulate the PKA pathway. This association strongly suggested an involvement of PKA in the mechanism of anergy. Therefore, we pretreated lymphocytes with H89 and PP1, selective inhibitors of PKA and tyrosine kinases, before being exposed to this moiety. This set of experiments showed that the pharmacological block of PKA but not of the tyrosine kinase activity prevented the hyporesponsiveness induced by lymphocyte exposure to the viral glycoprotein. These data provided direct evidence about the involvement of the PKA pathway in the mechanism of anergy induction (**Fig. 6**).

DISCUSSION

Signal cascades mediated by the HIV-1 envelope glycoprotein gp120 have been proposed to influence HIV-1 replication as well as viral-associated cytopathicity and apoptosis [37]. The data presented here demonstrate the ability of HIV*env* to induce a long-lasting, hyporesponsive state in CD4+ naive T cells through a mechanism that does not require a signaling mediated by the CD4 receptors. The analysis of the proximal signaling reveals that this phenomenon is associated with stimulation of the PKA/CREB pathway and is prevented by H89, a pharmacological inhibitor of PKA.

The ability of gp120 to modulate the biological response of lymphoid cells via a CD4-independent mechanism has been proposed by several authors. Fantuzzi and colleagues [38] have recently shown that the treatment of freshly isolated monocytes with rgp120-IIIB resulted in a dose-dependent enhancement of secretion of monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, and regulated on activation, normal T expressed and secreted. This phenomenon is independent by interaction of gp120 with CD4 but relates to the specific interaction of the viral glycoprotein with CCR5 and CXCR4 coreceptors. Other authors have found that in contrast to the CD4-dependent gp120 signaling via CCR5, the envelope-mediated CXCR4 signaling is CD4-independent and can induce chemotaxis of CD4 and CD8 T cells [39]. In our experimental model, treatment with Leu3a, a gp120 mimicking anti-CD4 antibody, induces a marked CD4 down-regulation but was unable to stimulate the PKA pathway as well as to induce anergy in naive T cells. Conversely, sCD4-HIV*env* complexes, which are able to bind CXCR4 but not CD4, stimulate the PKA pathway and induce anergy in naive lymphocytes. These findings strongly support the hypothesis that CD4 molecules are required for the recruitment of the envelope glycoprotein to the T cell surface but are unable to mediate the negative signaling responsible for the induction of the prolonged hyporesponsiveness to mitogens. These data are in contrast with a number of reports showing that the engagement of CD4 by gp120 may induce pleiotropic effects and interfere with the activation process of T cells [13–16, 20–22]. Other studies have also documented that gp120 can itself transduce intracellular signals via CD4 and CXCR4/CCR5 receptors, including the activation of the MAPK pathway [40, 41]. The majority of these studies have been performed on transformed T cell lines or on preactivated T lymphocytes. When signaling has been investigated on normal, quiescent T cells, it has been observed that

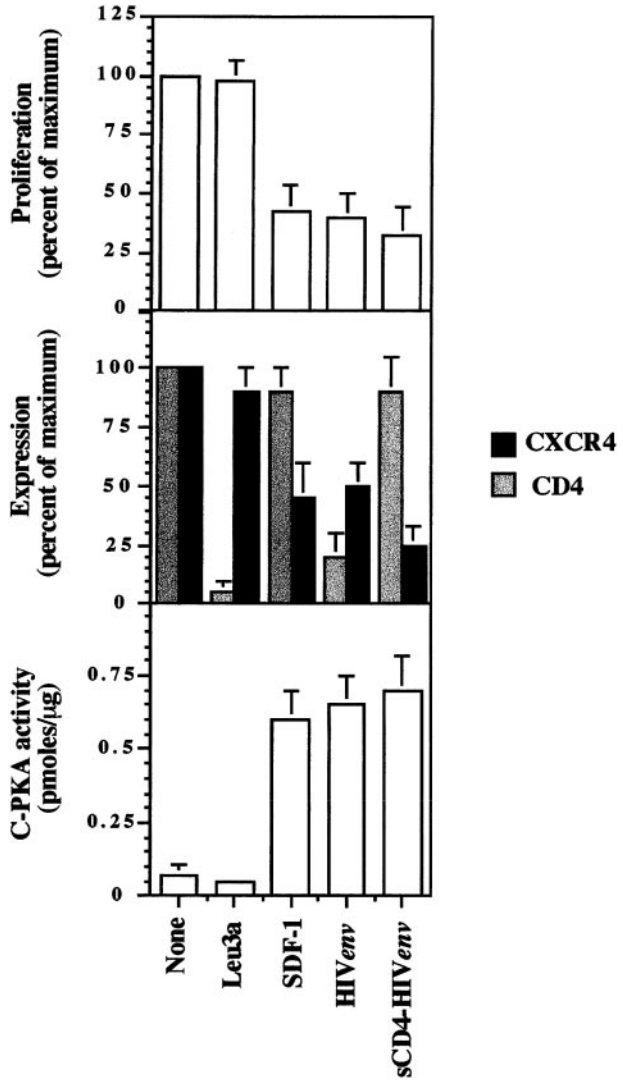

Fig. 5. CD4 engagement by HIV*env* or Leu3a is not required for the induction of T cell anergy in naive lymphocytes, which were cultured for 12 h with regular medium, Leu3a (0.5 μg/ml), SDF-1 (100 nM), HIV*env* (2 μg/ml), or sCD4-gp120 complexes (sCD4-HIV*env*; 2 μg/ml). To evaluate T cell responsiveness, lymphocytes were rested for 3 days in fresh medium before being stimulated with 10,000 cells/well allogenic DC (top). The results are expressed as the percentage of the maximal response measured in lymphocytes cultured in regular medium (38,000±2500 cpm). Aliquots of cells exposed for 12 h to the stimuli described previously were double-stained with CD4 and CXCR4 and analyzed by flow cytometry (middle). The results are expressed as the percentage of the maximum mean fluorescence intensity measured on lymphocytes cultured with regular medium alone. (Bottom) Phosphotransferase activity of the C-PKA measured in naive T cells exposed for 30 min to the reported stimuli (Leu3a, 0.5 μg/ml; SDF-1, 100 nM; HIV*env*, 2 μg/ml; sCD4-HIV*env*, 2 μg/ml). The results are representative of four independent experiments.

gp120 may induce differential effects on and activate different signal cascades in resting and preactivated lymphocytes [23, 41]. One apparent distinction between primary, quiescent T lymphocytes compared with preactivated lymphocytes or the transformed Jurkat cell line is that in the former, a significant proportion of CD4 is outside the lipid rafts [42, 43], whereas in the latter, it is located almost exclusively in raft structures [44, 45]. This localization may be one of the factors accounting for the HIV*env* signaling responses observed in the naive T lymphocytes and may explain the differential effects exerted by the envelope glycoprotein on the different T cell subsets.

The ability of HIV to stimulate PKA activity was originally shown a decade ago by Hofmann et al. [28] by exposing normal peripheral blood lymphocytes to inactivated virions. Here, we clarify the molecular bases of this phenomenon by identifying HIV*env* as a viral derivative responsible for PKA activation in naive T cells, showing for the first time that this biochemical change is not dependent on CD4-mediated signals. The coupling of the chemokine receptor CXCR4 to the PKA pathway has been documented recently in embryonic retinal ganglion cell neurons and in peripheral blood monocyte [46, 47]. Here, we confirm these findings, revealing for the first time the ability of SDF-1α to trigger the PKA pathway and to induce prolonged effects on the activation threshold in CD4+ naive T cells. Activation of the PKA pathway has a variety of transcriptional and post-transcriptional effects on different immune mediators. It has been reported that increased levels of cAMP have an important post-transcriptional effect on CD40 ligand expression, IL-10 secretion, and T helper cell type 2 (Th2) activation, and it inhibits T cell proliferation, IL-2 and IL-12 production, and Th1-mediated effector functions [48–53]. In addition, it has been shown that TCR stimulation of naive CD4 T cells in the presence of cAMP-elevating agents leads to a population of CD4 CD45RA T cells with functional and phenotypic features of effector Th2 cells [54]. Here, we found that HIV*env* glycoprotein can induce anergy in naive T cells by stimulating the PKA pathway, a phenomenon whose relevance remains to be determined in the context of the mechanisms affecting homeostatic proliferation, clonal expansion, and terminal differentiation of this subset of lymphocytes in AIDS. Despite this, our findings offer an ensemble of the complex and differential effects that HIV derivatives can exert on the various hematopoietic cell types.

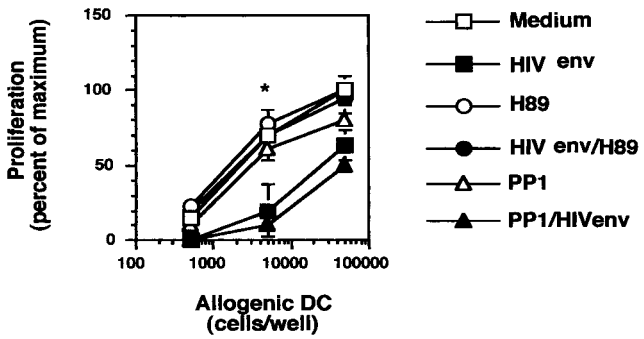

Fig. 6. Pharmacological block of PKA prevented *HIVenv*-induced anergy. Naive T lymphocytes were exposed for 12 h to rgp120 (2 μg/ml) in the presence or absence of PKA and tyrosine kinase inhibitors (H89 10 μM and PP1 10 μM, respectively). Lymphocytes were washed, rested for 96 h, and exposed for 72 h to an increasing number of allogenic DC. ^{3}H-Thymidine was added in the last 12 h of culture. The results are expressed as the percentage of the maximal response measured in HIV*env*-untreated lymphocytes stimulated with 50,000 allogenic DC (47,000+4000 cpm) and are calculated on three independent experiments. Statistical significant differences ($P<0.001$) were observed between the proliferation values measured in HIV*env*-treated lymphocytes cultured with or without PP1 and untreated lymphocytes stimulated with 5000 allogenic DC/well cultured in regular medium or in the presence of HIV*env* and H89.

ACKNOWLEDGMENTS

This work was supported by grants from Istituto Superiore di Sanità, Rome (ISS-AIDS Research Project No. 40C.78 and by MURST-PRINN 2001–2002 No. 2001065219). The contributions of A. M. M. and M. G. were equivalent, and their order should be considered arbitrary. We thank Dr. Ugo D'Oro and Dr. Albertina Fanelli for helpful suggestions and for the careful revision of the manuscript.

REFERENCES

1. Miedema, F., Petit, A. J., Terpstra, F. G., Schattenkerk, J. K., de Wolf, F., Al, B. J., Roos, M., Lange, J. M., Danner, S. A., Goudsmit, J. (1988) Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men: HIV affects the immune system before CD4+ T helper cell depletion occurs. *J. Clin. Invest.* **82,** 1908–1914.
2. Clerici, M., Stocks, N. I., Zajac, R. A., Boswell, R. N., Lucey, D. R., Via, C. S., Shearer, G. M. (1989) Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. *J. Clin. Invest.* **84,** 1892–1899.
3. Gurley, R. J., Ikeuchi, K., Byrn, R. A., Anderson, K., Groopman, J. E. (1989) CD4+ lymphocyte function with early human immunodeficiency virus infection. *Proc. Natl. Acad. Sci. USA* **86,** 1993–1997.
4. Musey, L. K., Krieger, J. N., Hughes, J. P., Schacker, T. W., Corey, L., McElrath, M. J. (1999) Early and persistent human immunodeficiency virus type 1 (HIV-1)-specific T helper dysfunction in blood and lymph nodes following acute HIV-1 infection. *J. Infect. Dis.* **180,** 278–284.
5. Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A., Walker, B. D. (1997) Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278,** 1447–1450.
6. Roilides, E., Clerici, M., De Palma, L., Rubin, M., Pizzo, P. A., Shearer, G. M. (1991) Helper T-cell responses in children with human immunodeficiency virus type 1. *J. Pediatr.* **118,** 724–730.
7. Groux, H., Torpier, G., Monte, D., Mouton, Y., Capron, A., Ameisen, J. C. (1992) Activation-induced death by apoptosis in CD4+ T cells from HIV-1 infected asymptomatic individuals. *J. Exp. Med.* **175,** 331–340.
8. Meyaard, L., Otto, S. A., Jonker, R. R., Mijnster, M. J., Keet, R. P., Miedema, F. (1992) Programmed death of T cells in HIV-1 infection. *Science* **257,** 217–219.
9. Geertsma, M. F., van Wengen-Stevenhagen, A., van Dam, E. M., Risberg, K., Kroon, F. P., Groeneveld, P. H., Nibbering, P. H. (1999) Decreased expression of zeta molecules by T lymphocytes is correlated with disease progression in human immunodeficiency virus-infected persons. *J. Infect. Dis.* **180,** 649–658.
10. Giovannetti, A., Pierdominici, M., Mazzetta, F., Mazzone, A. M., Ricci, G., Prozzo, A., Pandolfi, F., Paganelli, R., Aiuti, F. (2000) HIV type 1-induced inhibition of CD45 tyrosine phosphatase activity correlates with disease progression and apoptosis, but not with anti-CD3-induced T cell proliferation. *AIDS Res. Hum. Retroviruses* **16,** 211–219.
11. Cayota, A., Vuillier, F., Gonzalez, G., Dighiero, G. (1996) CD4+ lymphocytes from HIV-infected patients display impaired CD45-associated tyrosine phosphatase activity which is enhanced by anti-oxidants. *Clin. Exp. Immunol.* **104,** 11–17.
12. Guntermann, C., Amft, N., Murphy, B. J., Nye, K. E. (1998) Impaired CD45-associated tyrosine phosphatase activity during HIV-1 infection: implications for CD3 and CD4 receptor signalling. *Biochem. Biophys. Res. Commun.* **252,** 69–77.
13. Guntermann, C., Dye, J., Nye, K. E. (1997) Human immunodeficiency virus infection abolishes CD4-dependent activation of ZAP-70 by inhibition of p56lck. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **14,** 204–212.
14. Goldman, F., Crabtree, J., Hollenback, C., Koretzky, G. (1997) Sequestration of p56 (lck) by gp120, a model for TCR desensitization. *J. Immunol.* **158,** 2017–2024.
15. Stefanova, I., Saville, M. W., Peters, C., Cleghorn, F. R., Schwartz, D., Venzon, D. J., Weinhold, K. J., Jack, N., Bartholomew, C., Blattner, W. A., Yarchoan, R., Bolen, J. B., Horak, I. D. (1996) HIV infection-induced posttranslational modification of T cell signaling molecules associated with disease progression. *J. Clin. Invest.* **98,** 1290–1297.
16. Cayota, A., Vuillier, F., Siciliano, J., Dighiero, G. (1994) Defective protein tyrosine phosphorylation and altered levels of p59fyn and p56lck in CD4 T cells from HIV-1 infected patients. *Int. Immunol.* **6,** 611–621.
17. Oyaizu, N., Chirmule, N., Kalyanaraman, V. S., Hall, W. W., Pahwa, R., Shuster, M., Pahwa, S. (1990) Human immunodeficiency virus type 1 envelope glycoprotein gp120 produces immune defects in CD4+ T lymphocytes by inhibiting interleukin 2 mRNA. *Proc. Natl. Acad. Sci. USA* **87,** 2379–2383.
18. Cefai, D., Debre, P., Kaczorek, M., Idziorek, T., Autran, B., Bismuth, G. (1990) Human immunodeficiency virus-1 glycoproteins gp120 and gp160 specifically inhibit the CD3/T cell-antigen receptor phosphoinositide transduction pathway. *J. Clin. Invest.* **86,** 2117–2124.
19. Chirmule, N., Kalyanaraman, V. S., Oyaizu, N., Slade, H. B., Pahwa, S. (1990) Inhibition of functional properties of tetanus antigen-specific T-cell clones by envelope glycoprotein GP120 of human immunodeficiency virus. *Blood* **75,** 152–159.
20. Hubert, P., Bismuth, G., Korner, M., Debre, P. (1995) HIV-1 glycoprotein gp120 disrupts CD4–p56lck/CD3-T cell receptor interactions and inhibits CD3 signaling. *Eur. J. Immunol.* **25,** 1417–1425.
21. Morio, T., Chatila, T., Geha, R. S. (1997) HIV glycoprotein gp120 inhibits TCR-CD3-mediated activation of fyn and lck. *Int. Immunol.* **9,** 53–64.
22. Goldman, F., Crabtree, J., Hollenback, C., Koretzky, G. (1997) Sequestration of p56(lck) by gp120, a model for TCR desensitization. *J. Immunol.* **158,** 2017–2024.
23. Masci, A. M., Lago Paz, F., Borriello, A., Della Pietra, V., Stoiber, H., Matarese, G., Della Ragione, F., Zappacosta, S., Racioppi, L. (1999) Effects of exposure to inactivated human immunodeficiency virus type 1 of T-cell subset activation. *Eur. J. Immunol.* **29,** 1879–1889.
24. Torgersen, K. M., Vang, T., Abrahamsen, H., Yaqub, S., Tasken, K. (2002) Molecular mechanisms for protein kinase A-mediated modulation of immune function. *Cell. Signal.* **14,** 1–9.
25. Vang, T., Torgersen, K. M., Sundvold, V., Saxena, M., Levy, F. O., Skalhegg, B. S., Hansson, V., Mustelin, T., Tasken, K. (2001) Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent protein kinase inhibits signaling through the T cell receptor. *J. Exp. Med.* **193,** 497–507.
26. Bodor, J., Bodorova, J., Gress, R. E. (2000) Suppression of T cell function: a potential role for transcriptional repressor ICER. *J. Leukoc. Biol.* **67,** 774–799.
27. Tenbrock, K., Juang, Y-T., Tolnay, M., Tsokos, G. C. (2003) The cyclic adenosine 5-monophosphate response element modulator suppresses IL-2 production in stimulated T cells by a chromatin-dependent mechanism. *J. Immunol.* **170,** 2971–2976.
28. Hofmann, B., Nishanian, P., Nguyen, T., Insixiengmay, P., Fahey, J. L. (1993) Human immunodeficiency virus proteins induce the inhibitory cAMP/protein kinase A pathway in normal lymphocytes. *Proc. Natl. Acad. Sci. USA* **90,** 6676–6680.
29. Hofmann, B., Nishanian, P., Nguyen, T., Liu, M., Fahey, J. L. (1993) Restoration of T-cell function in HIV infection by reduction of intracellular cAMP levels with adenosine analogues. *AIDS* **7,** 659–664.
30. Aandahl, E. M., Aukrust, P., Skalhegg, B. S., Muller, F., Froland, S. S., Hansson, V., Tasken, K. (1998) Protein kinase A type I antagonist restores immune responses of T cells from HIV-infected patients. *FASEB J.* **12,** 855–862.
31. Sallusto, F., Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179,** 1109–1118.
32. Juszczak, R. J., Turchin, H., Truneh, A., Culp, J., Kassis, S. (1991) Effect of human immunodeficiency virus gp120 glycoprotein on the association of the protein tyrosine kinase p56lck with CD4 in human T lymphocytes. *J. Biol. Chem.* **266,** 11176–11183.
33. Cefai, D., Ferrer, M., Serpente, N., Idziorek, T., Dautry-Varsat, A., Debre, P., Bismuth, G. (1992) Internalization of HIV glycoprotein gp120 is associated with down-modulation of membrane CD4 and p56lck together with impairment of T cell activation. *J. Immunol.* **149,** 285–294.
34. Banda, N. K., Bernier, J., Kurahara, D. K., Kurrle, R., Haigwood, N., Sekaly, R. P., Finkel, T. H. (1992) Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. *J. Exp. Med.* **176,** 1099–1106.
35. Tamma, S. M., Chirmule, N., McCloskey, T. W., Oyaizu, N., Kalyanaraman, V. S., Pahwa, S. (1997) Signals transduced through the CD4 molecule interfere with TCR/CD3-mediated ras activation leading to T cell anergy/apoptosis. *Clin. Immunol. Immunopathol.* **85,** 195–201.
36. Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., Hendrickson, W. (1998) Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393,** 648–659.

37. Popik, W., Pitha, P. M. (2000) Exploitation of cellular signaling by HIV-1: unwelcome guests with master keys that signal their entry. *Virology* **276,** 1–6.
38. Fantuzzi, L., Canini, I., Belardelli, F., Gessani, S. (2001) HIV-1 gp120 stimulates the production of b-chemokines in human peripheral blood monocytes through a CD4-independent mechanism. *J. Immunol.* **166,** 5381–5387.
39. Iyengar, U., Schwartz, D. H., Hildreth, J. E. K. (1999) T cell-tropic HIV gp120 mediates CD4 and CD8 cell chemotaxis through CXCR4 independent of CD4: implications for HIV pathogenesis. *J. Immunol.* **162,** 6263–6267.
40. Siliciano, R. F. (1996) The role of CD4 in HIV envelope-mediated pathogenesis. *Curr. Top. Microbiol. Immunol.* **205,** 159–179.
41. Kinet, S., Bernard, F., Mongellaz, C., Perreau, M., Goldman, F. D., Taylor, N. (2002) gp120-mediated induction of the MAPK cascade is dependent on the activation state of CD4(+) lymphocytes. *Blood* **100,** 2546–2553.
42. Kozak, S. L., Heard, J. M., Kabat, D. (2002) Segregation of CD4 and CXCR4 into distinct lipid microdomains in T lymphocytes suggests a mechanism for membrane destabilization by human immunodeficiency virus. *J. Virol.* **76,** 1802–1815.
43. Schade, A. E., Levine, A. D. (2002) Lipid raft heterogeneity in human peripheral blood T lymphoblasts: a mechanism for regulating the initiation of TCR signal transduction. *J. Immunol.* **168,** 2233–2239.
44. Rodgers, W., Rose, J. K. (1996) Exclusion of CD45 inhibits activity of p56lck associated with glycolipid-enriched membrane domains. *J. Cell Biol.* **135,** 1515–1523.
45. Xavier, R., Brennan, T., Li, Q., McCormack, C., Seed, B. (1998) Membrane compartmentation is required for efficient T cell activation. *Immunity* **8,** 723–773.
46. Chalasani, S. H., Baribaud, F., Coughlan, C. M., Sunshine, M. J., Lee, V. M., Doms, R. W., Littman, D. R., Raper, J. A. (2003) The chemokine stromal cell-derived factor-1 promotes the survival of embryonic retinal ganglion cells. *J. Neurosci.* **23,** 4601–4612.
47. Damas, J. K., Waehre, T., Yndestad, A., Ueland, T., Muller, F., Eiken, H. G., Holm, A. M., Halvorsen, B., Froland, S. S., Gullestad, L., Aukrust, P. (2002) Stromal cell-derived factor-1alpha in unstable angina: potential antiinflammatory and matrix-stabilizing effects. *Circulation* **106,** 36–42.
48. Suárez, A., Mozo, L., Gayo, A., Zamorano, J., Gutiérrez, C. (1997) Requirement of a second signal via protein kinase C or protein kinase A for maximal expression of CD40 ligand: involvement of transcriptional and posttranscriptional mechanisms. *Eur. J. Immunol.* **27,** 2822-2830.
49. Zhang, F., Rincon, M., Flavell, R. A., Aune, T. M. (2000) Defective Th function induced by a dominant-negative cAMP response element binding protein mutation is reversed by Bcl-2. *J. Immunol.* **165,** 1762–1767.
50. Snijdewint, F. G. M., Kalinski, P., Wierenga, E. A., Bos, J. D., Kapsenberg, L. (1993) Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J. Immunol.* **150,** 5321–5326.
51. Elenkov, I. J., Webster, E., Papanicolau, D. A., Fleisher, T. A., Chrousos, G. P., Wilder, R. L. (1998) Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. *J. Immunol.* **161,** 2586–2592.
52. Kolenko, V., Rayman, P., Roy, B., Cathcart, M. K., O'Shea, J., Tubbs, R., Rybicki, L., Bukowski, R., Finke, J. (1999) Downregulation of JACK3 protein levels in T lymphocytes by prostaglandin E2 and other cyclic adenosine monophosphate-elevating agents: impact on interleukin-2 receptor signalling pathway. *Blood* **93,** 2308–2314.
53. Chen, C., Zhang, D., LaPorte, J. M., Ray, A. (2000) Cyclic AMP activates p38 mitogen-activated protein kinase in Th2 cells: phosphorylation of GATA-3 and stimulation of Th2 cytokine gene expression. *J. Immunol.* **165,** 5597–5602.
54. Suarez, A., Mozo, L., Gutierrez, C. (2002) Generation of CD4(+)CD45RA(+) effector T cells by stimulation in the presence of cyclic adenosine 5′-monophosphate-elevating agents. *J. Immunol.* **169,** 1159–1167.