Scand. J. Immunol. **53**, 259–267, 2001

Modulation of CD8 and CD3 by HIV or HIV Antigens

J. JASON & K. L. INGE

Immunology Branch, Division of AIDS, Sexually Transmitted Diseases, and Tuberculosis, Laboratory Research (DASTLR), National Center for Infectious Diseases, Centers for Disease, Control and Prevention (CDC), Department of Health and Human Services, Public Health Service, Atlanta, GA, USA

(Received 14 September 2000; Accepted in revised form 27 November 2000)

Jason J, Inge KL. Modulation of CD8 and CD3 by HIV or HIV Antigens. Scand J Immunol 2001;53:259−267

To investigate whether human immunodeficiency virus (HIV)-1 and HIV-1 antigens modulate surface and cytoplasmic CD8 or CD3, as well as CD4, we used cell permeabilization reagents, surface/cytoplasmic fluorescent staining, multiparameter flow cytometric techniques and an *in vitro* culture system in which relatively few lymphocytes are actively infected with HIV. Human peripheral blood lymphocytes were: not stimulated, not stimulated but HIV-inoculated, phytohaemagglutinin (PHA)-stimulated, PHA/HIV-inoculated (PHA/HIV), or placed into media with soluble gp120, Rev or Nef. HIV inoculation and Nef had striking modulatory effects on CD8. The cytoplasmic CD8 median fluorescent intensity (MFI) of positive lymphocytes was lower for cells in unstimulated/HIV-infected cultures than unstimulated cultures (44 versus 62% of *ex vivo* value, $P = 0.032$) and lower for cells in PHA/HIV cultures than in PHA cultures (56 versus 100% of *ex vivo*, $P = 0.041$). The surface CD8 MFI values for Nef were significantly lower than the *ex vivo* value (75% of *ex vivo*, $P = 0.006$). At days 2–7 of culture, Rev was associated with slight reductions in surface CD4 MFI (58% of *ex vivo* versus 78% of *ex vivo* for unstimulated cultures, $P = 0.047$) and greater effects on cytoplasmic CD3 MFI (131 versus 179% of *ex vivo* for unstimulated cultures, $P = 0.035$), and surface CD8 MFI (70% of *ex vivo*, $P = 0.006$ versus *ex vivo* value). The globality of Rev's effects suggests these are related to a shared processing pathway, i.e. not due to direct interaction with CD3, CD4 and CD8; the effects of HIV inoculation and Nef on CD8 expression appear to be more CD8 specific. Because CD8 is essential for cytotoxic T-cell function, its down-modulation could inhibit this activity, including anti-HIV cytotoxicity. Given the critical roles of CD3 and CD8 in T-lymphocyte signal transduction and antigen responsiveness, the effects of HIV, Rev and Nef on these molecules have clinically significant implications concerning the pathogenesis and treatment of HIV.

Dr J. Jason, Mailstop A-25, Immunology Branch, DASTLR, NCID, CDC, 1600 Clifton Rd NE, Atlanta, GA 30333, USA. E-mail: JMJ1@cdc.gov

INTRODUCTION

CD4 is synthesized in the endoplasmic reticulum and transported to the cell surface via the Golgi complex [1, 2]. It is a co-receptor for the human immunodeficiency virus (HIV) gp120/160; the direct interaction between CD4 and the HIV envelope proteins has been examined in depth. Gp120 and human leukocyte antigen (HLA) class II antigens competitively bind CD4 [3, 4]. Cell-surface expression of CD4 is masked by both HIV infection and gp120/160 [5–7]. One report has suggested that HIV-2, but not HIV-1, envelope glycoprotein binds to human CD8, as well as CD4 [8]. The binding of gp120 to CD4 not only masks the CD4 molecule, but also induces down-modulation of surface CD4 [9]. This modulation is not due solely to cell-surface interactions. In a transfection system, it was shown that gp160 complexed with CD4 in the endoplasmic reticulum, leading to decreased transport to the cell surface [10]. CD4 may also associate with $p56^{lck}$ in the endoplasmic reticulum, forming a ternary complex with gp160, possibly further decreasing transport of both CD4 and $p56^{lck}$ to the cell surface [11]. Other HIV antigens appear to also be involved in CD4 modulation. In CEM cells and human T-cell lines, HIV Nef, localized to the cytoplasmic surfaces of cell membranes, may interact directly with [12, 13], and/or induce the downregulation of, cell surface CD4 [14–17], as well as rapid internalization and subsequent degradation of surface CD4 [18–22]; others have suggested that its action is indirect [23]. Several recent reports have shown additional effects of soluble

Nef, including leukocyte recruitment, cytokine induction and apoptotic cytolysis of uninfected cells [24–26]. These effects were seen when Nef was used in soluble form, not through expression, and given to animals or used in *in vitro* systems.

CD4/gp120 interaction interferes with antigen responsiveness [27]. Whether this is due solely to the CD4/gp120 interaction or also involves signal transduction through CD3/Ti, the T-cell receptor (TCR), is unclear [28–34]. Similarly, it has been reported that Nef affects CD3 signalling [15], as well as CD4 responsiveness to phorbol esters [33] and transport activities in the Golgi pathway [19, 34]. Surface CD3 expression in normal human peripheral blood lymphocytes is reported to be unaffected by gp120 [9]. *In vitro* HIV infection has been reported to lead to loss of surface CD4 but not CD3 or CD8 [5, 31]. Contrary findings have been reported with HIV-infected CEM cells [35].

We used cell permeabilization methods, surface/cytoplasmic fluorescent staining and multiparameter flow cytometric techniques to investigate how HIV and HIV antigens affect modulation of surface and cytoplasmic CD3 and CD8, as well as CD4. We inoculated unstimulated and phytohaemagglutin (PHA)-stimulated human peripheral blood mononuclear cells (PBMCs) with HIV-1, to examine the effects of cell activation status and HIV inoculation itself on the expression and intensity of expression of these key receptor molecules. We then examined the effects of the HIV-1 antigens gp120, Rev and Nef. Our results suggest that the effects of HIV and HIV antigens are not limited to the CD4 antigen; there are also subtle effects on CD3 and striking effects on CD8. Given the critical roles of these receptor molecules, these effects may be of physiological importance.

MATERIALS AND METHODS

Cells and virus. PBMCs were obtained from normal human donors seronegative for HIV and hepatitis viruses. These were gradient-separated and cultured in RPMI-1640 containing antibiotics and 10% heat-inactivated (56 °C for 45 min) donor serum (unstimulated cultures) or 10% heat-inactivated (56 °C for 45 min) fetal calf serum (all other cultures), at 37 °C in 5% CO_2 in a humidified incubator, at a concentration of 2×10^6 cells/ml. Cells were either stimulated for 1.5 days with 5 μg/ml PHA (Difco Laboratories, Detroit, MI, USA; the use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the US Department of Health and Human Services) or retained in the above media without stimulation; cells with these culture conditions will be referred to herein as PHA or unstimulated, respectively. One aliquot of each donor's unstimulated or PHA-stimulated PBMCs was then inoculated with the T-cell tropic HIV-1 isolate LAI [5]. PHA-stimulated cultures had 10% interleukin (IL)-2 (Advanced Biotechnologies, Columbia, MD, USA) added to the media and the PHA removed at the time of inoculation; these cultures will be referred to as PHA/HIV herein. Unstimulated cultures had their media changed at the time of inoculation, IL-2 was not added; these are referred to as unstimulated/HIV herein. Uninoculated PHA cultures had the PHA removed after 1.5 days, and 10% IL-2 was added. The unstimulated culture conditions used here have been shown by others to be associated with a lack of DNA synthesis for up to 2 weeks, while $>90\%$ of PHA-stimulated cells proceed through the S phase within 24 h [36]. Cultures of stimulated cells were harvested at day 4 or 5 of culture, on the day following the decrease in the per cent of $CD4^+$ cells in the PHA/HIV culture [37]; parallel unstimulated cultures were harvested the following day. Some cultures were maintained for an additional week and harvested a second time; these data are consistent with the results at earlier times and, for the sake of conciseness, are only mentioned here. In this culture system, it has been shown that very few $CD4^+$ cells are productively infected with HIV at any given time. Rare, if any, $CD8^+$ cells are infected [5, 37]; therefore, effects reported herein were due to nonproductive infection or to contact with HIV-infected cells or antigens.

HIV antigens. For some experiments, PBMCs were divided into cultures with media and one of the following HIV-1 antigens: baculovirus-derived recombinant gp120 (0.1 or 1 μg/ml, Microgenesis Corp., West Haven, CT, USA and Protein Sciences Corp., Meriden, CT, USA), vector-expressed Rev (0.1 or 1.0 μg/ml, NIH AIDS Research and Reference Program, Rockville, MD, USA) and vector-expressed LAV Nef (0.1 or 1.0 μg/ml, NIH AIDS Research and Reference Program). (Lower doses used in one experiment.) These cultures were harvested at 16–32 h *ex vivo* (five experiments with surface staining alone, four for surface and cytoplasmic reactivity), 2–7 days *ex vivo* (six experiments for surface gp120 and five for surface and cytoplasmic reactivity, five experiments for surface and cytoplasmic reactivity with Rev and Nef), and 8–14 days *ex vivo* (two experiments for gp120 and one experiment for the rest).

Reagents. These included fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MoAbs) and phycoerythrin (PE)-conjugated MoAb reagents to: CD4 (clone SK3, reactive with the V1 region of the extracellular portion of CD4, Becton-Dickinson Immunochemistry Systems, San Jose, CA, USA), CD8 (clone SK1, reactive with the α chain, Becton-Dickinson) and CD3 (clone SK7, reactive with the ε chain, Becton-Dickinson). PE-indodicarbocyanine 5 (PE-Cy5) tandem conjugated reagents included MoAbs to CD4 (clone RPA-T4, Pharmingen, San Diego, CA, USA), CD8 (clone RPA-T8, Pharmingen) and CD3 (clone UCHT1, Pharmingen). Peridinin chlorophyll protein (PerCP)-conjugated MoAb to CD8 was used (clone SK1, Becton-Dickinson). Allophycocyanin (APC)-conjugated MoAbs were to CD4 (clone SK3, Becton-Dickinson). Isotype controls included mouse immunoglobulin (Ig)G2a FITC (clone X39, Becton-Dickinson), mouse IgG2b PE (clone 27–35, Pharmingen), mouse IgG1 PerCP (clone X40, Becton-Dickinson), mouse IgG1 PE-Cy5 (clone MOPC-21, Pharmingen) and mouse IgG1 APC (clone X40, Becton-Dickinson). Unconjugated MoAbs to CD4 (clone SK3, Becton-Dickinson), CD8 (clone SK1, Becton-Dickinson or clone RPA-T8, Pharmingen) and CD3 (clone SK7, Becton-Dickinson) were used to block surface staining. Permeabilization was done with ORTHO PermeaFix™ (Ortho Diagnostics, Inc., Raritan, NJ, USA). Other reagents included 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co., St. Louis, MO, USA) and 10 μg/ml brefeldin-A (BFA, Sigma) (both for 4–5.5 h at 37 °C).

Blocking of surface antigens. For experiments in which cytoplasmic antigen expression and intensity were assessed in parallel with surface assessments, blocking of surface staining was performed as follows. Cells were incubated with unconjugated MoAbs for 15 min at room temperature in the dark, centrifuged and washed with a buffered saline solution. They were then permeabilized/fixed with Ortho Permeafix and stained with identical, conjugated MoAbs. With this protocol, $<1\%$ of cells were positive when incubated with the corresponding MoAbs post-blocking but pre-permeabilization [38].

PMA ± BFA. To examine potential intracellular and transduction mechanisms for CD8 and CD3 modulation by HIV inoculation and HIV antigens, we examined the effects of adding PMA, BFA or both for the final 4–5.5 h of culture (at days 4–7 of culture for unstimulated, unstimulated HIV/PHA and PHA/HIV cultures, $n = 3$ experiments and at days 2–7 of culture for HIV antigens, $n = 1$ experiment).

Flow cytofluorometry. Multiparameter cytofluorometry was performed using a FACSort (Becton-Dickinson) and LYSIS II or CELLQUEST software. As negative controls, cell aliquots were stained with the isotype controls above. Staining for surface antigens was carried out prior to permeabilization, for 15 min at room temperature in the dark. Staining for cytoplasmic antigens was performed post blocking (above), post permeabilization for 30 min at room temperature in the dark. At least 25 000 ungated events were collected for each tube; lymphocytes were defined by their forward- and side-scatter properties. Duplicate or triplicate tubes were run for each sample. Cell collection was performed on a single FACSort (Becton-Dickinson), by a single operator. The flow cytometer was standardized daily, using chicken erythrocytes. Voltage was adjusted to maintain the standard in the same target channel throughout any given experiment. The same MoAbs were used throughout any given experiment; saturating amounts of antibodies were used. The fluorochrome to which a MoAb was conjugated was not changed within an experiment but did differ between experiments, as described above. All analyses for a given experiment were performed by a single individual; gates and margins were consistent throughout any given experiment.

Statistical analyses. Data were collected in terms of both per cent positive and median fluorescence intensity (MFI) of the positive cells; mean values of duplicate or triplicate tubes were used in all analyses. Matched comparisons were made only between identical MoAbs conjugated to identical fluorochromes. Per cent positivity and

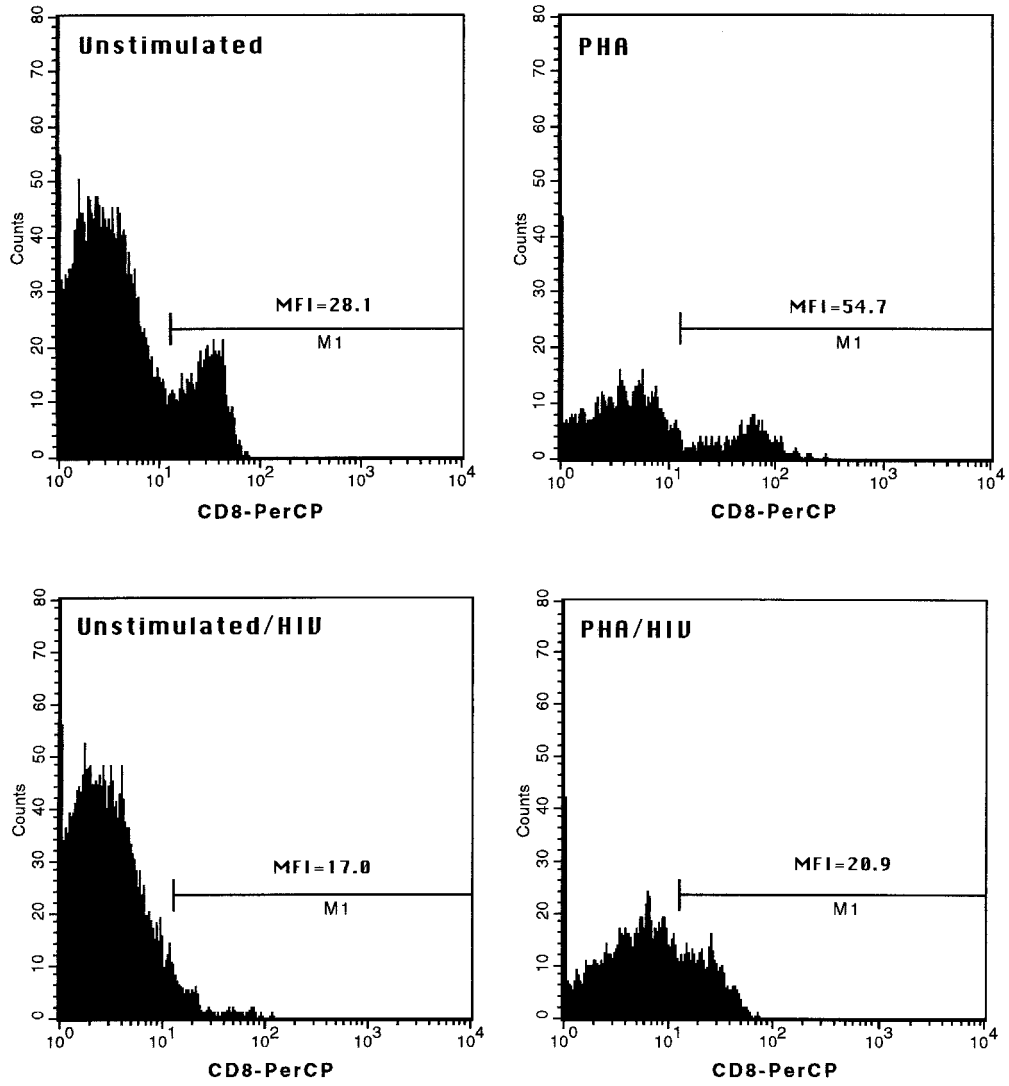

Fig. 1. Histograms of cytoplasmic CD8 staining at day 5 and 7, by culture condition. Unstimulated human peripheral lymphocytes (PBL), by scatter gating, day 7 of culture; unstimulated PBLs inoculated with HIV, day 7 of culture (unstimulated/HIV); PHA-stimulated PBLs, day 5 of culture (PHA); and PHA-stimulated PBLs inoculated with HIV, day 5 of culture (PHA/HIV). Median fluorescent intensities (MFI) of positive cells, with margins set at identical positions.

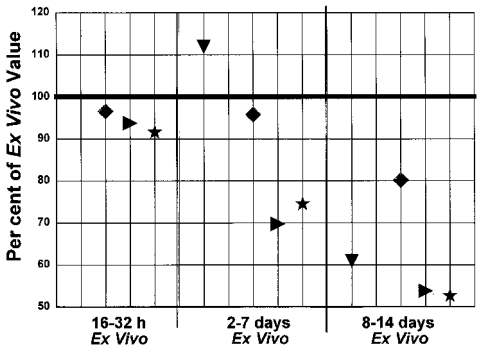

Fig. 2. Mean relative surface CD8 MFI by culture condition and time. Median fluorescence intensity of cells expressing CD8 (MFI). Fluorochromes used varied between experiments; therefore, normalized values were used in all analyses (see Materials and methods). (▼) Unstimulated human peripheral blood lymphocytes (PBL), by scatter gating ($n = 6$ at 2–7 days *ex vivo* and $n = 2$ at 8–14 days *ex vivo*), PBLs cultured with (◆) HIV-1 gp120 ($n = 5$ at 16–32 h *ex vivo*, $n = 6$ at 2–7 days, and $n = 2$ at 8–14 days), (►) Rev or (★) Nef ($n = 5$, 5 and 1 for each). *P*-values for Rev and Nef, respectively, at 2–7 days were 0.004 and 0.006 versus *ex vivo* and 0.030 and 0.015 versus their 16–32 h values.

fluorescence intensity varied from donor to donor and fluorochrome to fluorochrome. Therefore, for statistical analyses and graphing of results, summary results for each culture condition or antigen were standardized in two ways. First, results were expressed as ratios of the pertinent value to the value for that donor at the first assessment, prior to inoculation or culture. For example, if, for a given donor, the CD4 MFI was 600 *ex vivo* and 300 at the time of harvest from a certain culture condition, the harvest value was recorded as being 50% of the *ex vivo* value. Second, for experiments in which PMA and/or BFA were used, the effects of these were expressed as the per cent change from nontreatment, i.e. (treated value – nontreated value) ÷ (nontreated value) × 100. This standardization approach has been used by others to compare relative fluorescence intensities [39, 40]. Statistical analyses were performed using paired Student's *t*-tests, the latter providing comparisons between culture conditions and time points that take into account the parallel cultures/shared donor nature of the study design. Results of significance testing will be provided if the *P*-value associated with the two-sided paired *t*-test was < 0.05 and unstimulated/HIV, PHA/HIV or HIV antigen results differ from the parallel unstimulated or PHA cultures. Data on the effects of unstimulated and PHA cultures per se are dealt with in a separate publication [38].

RESULTS

CD4

CD4 findings were as expected and thus support that our approach is valid. By days 4–7, the mean relative percentage of lymphocytes expressing surface CD4 had decreased markedly in PHA/HIV cultures, to 47% of the *ex vivo* values (i.e. preculture), but not in cultures with PHA alone (100% of the *ex vivo* value). For unstimulated, unstimulated/HIV and PHA/HIV cultures, the mean relative surface CD4 MFIs decreased significantly compared with the *ex vivo* value; the decrease was greatest for PHA/HIV cultures (unstimulated: 74% of the *ex vivo* value, $P = 0.015$; unstimulated/HIV: 72%, $P = 0.003$; PHA/HIV: 49%, $P < 0.001$). Unlike the surface CD4 MFI, the

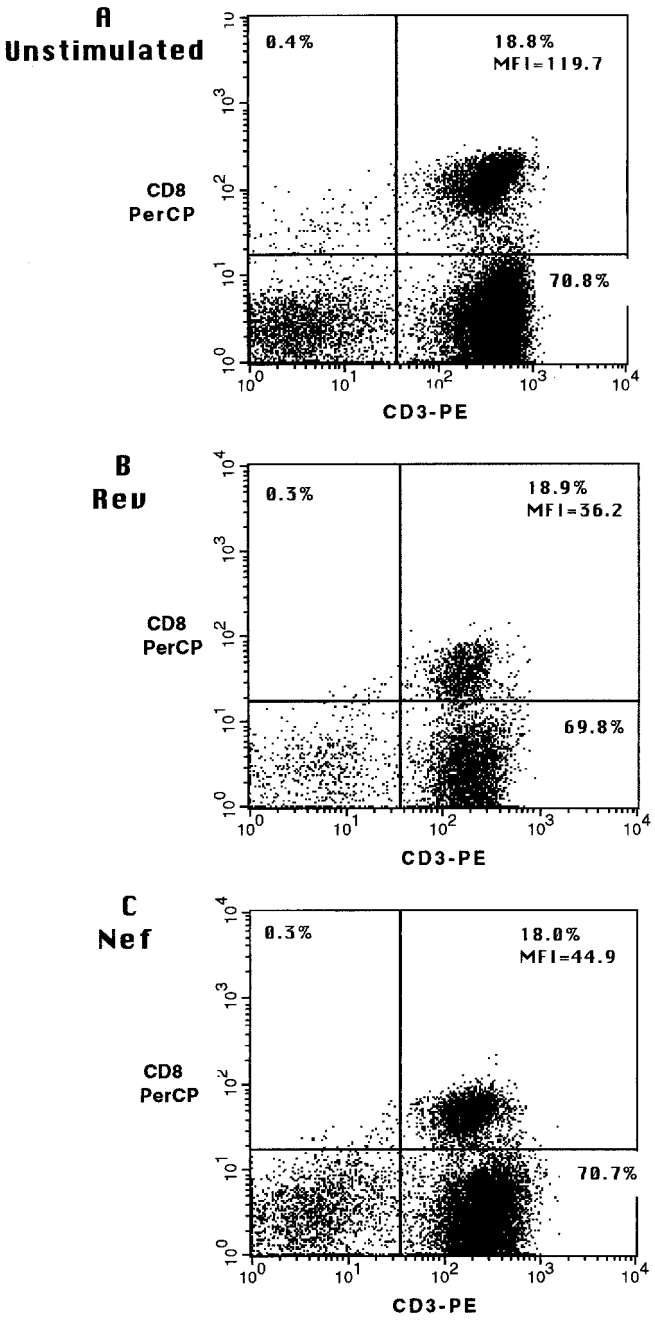

Fig. 3. Surface staining for CD8 and CD3 at day 5 of culture, by culture condition. (A) Unstimulated human peripheral blood lymphocytes (PBL), by scatter gating. (B) PBLs cultured with HIV-1 Rev. (C) PBLs cultured with HIV-1 Nef. Cells were incubated for 15–30 min at room temperature in the dark with saturating amounts of directly conjugated monoclonal antibodies to CD3 (Fl2, PE) and CD8 (Fl3, PerCP) prior to permeabilization. Median fluorescent intensities of positive cells (MFI).

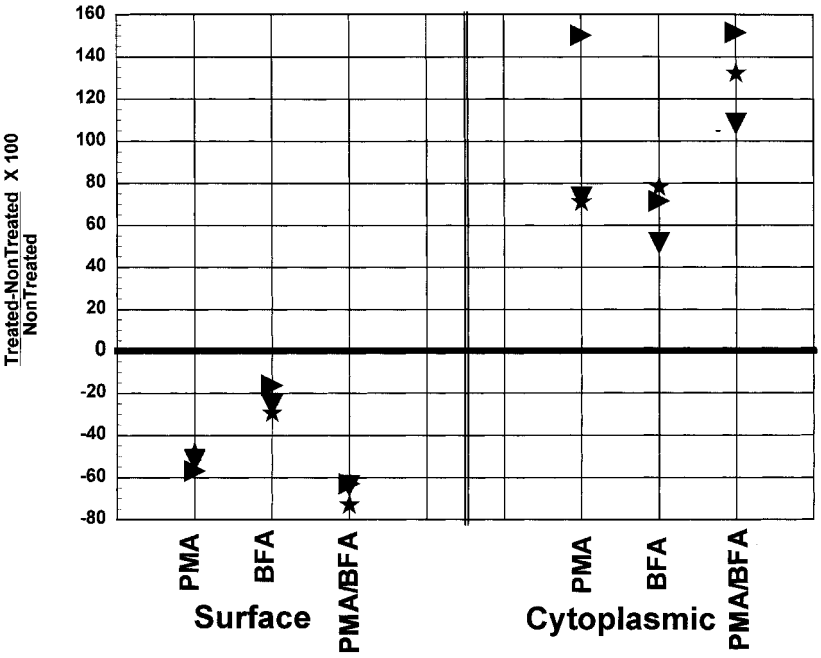

Fig. 4. Proportionate difference in surface and cytoplasmic CD3 MFI, by culture condition and treatment received. Median fluorescence intensity of cells expressing CD3 (MFI). (▼) Unstimulated human peripheral blood lymphocytes, (PBL) by scatter or PBLs cultured with HIV-1 gp120, (►) Rev or (★) Nef. Phorbol ester 12-myristate 13-acetate (PMA), Brefeldin A (BFA), both or neither added to cultures for the last 4–5.5 h of culture period. Values are expressed as the per cent change from nontreatment, i.e. (value for treated condition minus value for nontreated condition)÷(value for nontreated condition) × 100.

cytoplasmic CD4 MFI decreased only for PHA/HIV, which was 76% of the *ex vivo* value and 53% of the PHA value at days 4–7 ($P = 0.005$).

By days 2–7, the mean relative percentage of lymphocytes expressing surface CD4 had decreased with gp120 (to 44% of the unstimulated value at the same time point, $P = 0.030$ or 56% of the *ex vivo* value). The decrease with gp120 had become apparent but was not statistically significant at 16–32 h *ex vivo* (to 54% of the unstimulated value or 76% of *ex vivo* value). The per cent of lymphocytes expressing CD4 did not decrease with any other HIV antigen (data not shown). At days 2–7, the mean relative surface CD4 MFI of cells cultured with gp120 was significantly lower than that of unstimulated cells (28% of *ex vivo* versus 78% of *ex vivo*, $P = 0.018$). At days 2–7, the mean relative surface CD4 MFI with Rev was slightly lower than that for unstimulated cultures (58% of *ex vivo* versus 78% of *ex vivo*, $P = 0.047$) but was never significantly lower than the *ex vivo* value per se. No HIV antigen affected cytoplasmic CD4 significantly (data not shown).

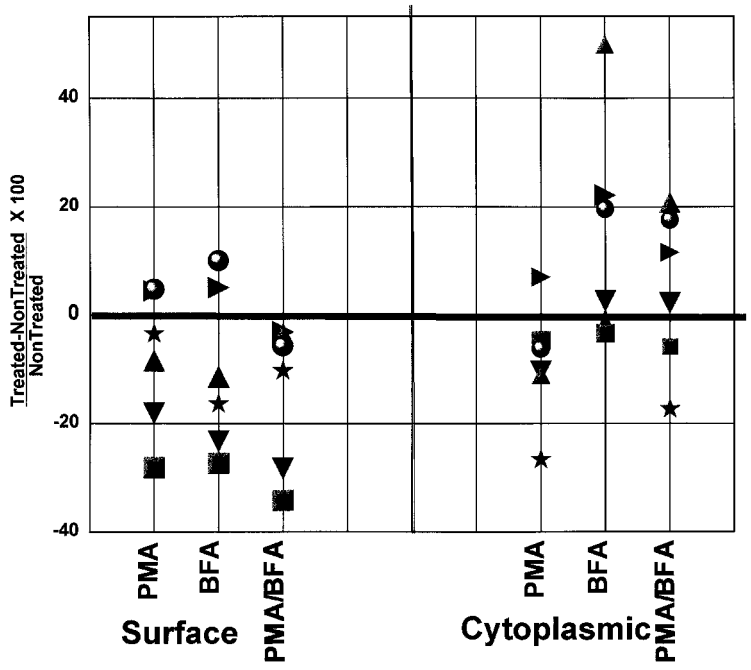

Fig. 5. Proportionate difference in surface and cytoplasmic CD8 MFI, by culture condition and treatment received. Median fluorescence intensity of cells expressing CD8 (MFI). (▼) Unstimulated human peripheral blood lymphocytes (PBL), by scatter ($n = 3$), PBLs stimulated with (●) PHA ($n = 3$), (■) PHA-stimulated PBLs inoculated with HIV-1 ($n = 3$), (▲) unstimulated PBLs inoculated with HIV-1 ($n = 3$), or unstimulated PBLs cultured with HIV-1 gp120, (►) Rev or (★) Nef ($n = 1$ for each). Phorbol ester 12-myristate 13-acetate (PMA), Brefeldin A (BFA), both or neither added to cultures for the last 4–5.5 h. Values are expressed as the per cent change from nontreatment, i.e. (value for treated condition minus value for nontreated condition)÷(value for nontreated condition) × 100.

CD3

At days 2–7, the mean relative cytoplasmic CD3 MFIs had increased for all culture conditions; however, the value for Rev was significantly lower than that for the unstimulated condition (132% of *ex vivo* versus 179% of *ex vivo*, $P = 0.035$). This difference was found for both $CD4^+$ and $CD8^+$ lymphocytes (data not shown).

CD8

As expected, and in compensation for the decrease in the proportion of lymphocytes expressing surface CD4, at days 4–7, the percentage of lymphocytes expressing surface CD8 was greater with PHA/HIV than with PHA (145% of *ex vivo* versus 100% of *ex vivo*, $P < 0.001$). At days 4–7, the mean relative cytoplasmic CD8 MFI for unstimulated/HIV cultures was significantly lower than that for unstimulated cultures (44% of *ex vivo* versus 62% of *ex vivo*, $P = 0.032$) and the value for PHA/HIV was lower than that for PHA (56% of *ex vivo* versus 100% of *ex vivo,* $P = 0.041$). For some experiments, this effect was marked (Fig. 1).

At days 2–7, the percentage of lymphocytes expressing surface CD8 decreased slightly for all HIV antigens (data not shown), but only the decrease for Nef was significant (70% of *ex vivo* versus 100% of *ex vivo*, $P = 0.032$). At days 2–7, the mean relative surface CD8 MFIs for Rev and Nef were significantly lower than the *ex vivo* value and than their own values at 16–32 h (from 94 to 70% of *ex vivo*, $P = 0.030$ and from 92 to 75% of *ex vivo*, $P = 0.015$, respectively; Figs 2 and 3A−C).

Effects of PMA ± BFA

CD4. For CD4, PMA treatment was associated with large decreases in surface and cytoplasmic CD4 MFIs of lymphocytes in unstimulated cultures (proportionate change from nontreatment cultures: −85% for surface CD4 MFI and −88% for cytoplasmic CD4 MFI, respectively), unstimulated/HIV cultures (−89 and −85%), and all HIV antigen cultures (−67 to −80% and −50 to −64%, respectively). PMA caused smaller decreases in CD4 MFIs of lymphocytes in PHA cultures (−48 and −32%, respectively) and PHA/HIV cultures (−19 and −24%, respectively). BFA was associated with minimal changes in surface and cytoplasmic CD4 MFIs of lymphocytes in unstimulated cultures (−2 and +7%), unstimulated/HIV cultures (−9 and −1%) and PHA/HIV cultures (−5 and −8%). BFA effects were somewhat larger for PHA (+17 and +21%), gp120 (+15 and +17%), Nef (+10 and +16%) and, especially, Rev (+32 and +46%). The combination of PMA and BFA was associated with lesser relative reductions in surface and cytoplasmic CD4 MFI than noted above for PMA alone.

CD3 (Fig. 4). PMA and BFA treatments were associated with decreases in surface CD3 MFIs and increases in cytoplasmic CD3 MFIs of lymphocytes in all the culture conditions assessed, i.e. unstimulated, Nef and Rev cultures. The PMA effect on cytoplasmic CD3 MFI was higher for the Rev culture than for unstimulated and Nef cultures, which were similar to each other. BFA did not alter the PMA effect on lymphocytes in Rev cultures.

CD8 (Fig. 5). As reported in the literature, the general effects of PMA and BFA were much less on CD8 than on CD3 or CD4 [40–44]. PMA and BFA added individually to PHA/HIV cultures both led to decreases in lymphocytes' surface CD8 MFIs, without concomitant increases in their cytoplasmic CD8 MFIs. These PMA and BFA effects on lymphocytes in PHA/HIV cultures appeared to be additive. The effects of PMA and BFA on the surface CD8 MFI were smaller on lymphocytes in unstimulated/HIV cultures than on unstimulated, non-inoculated cultures. However, BFA treatment was associated with a relatively large increase in the cytoplasmic CD8 MFIs of lymphocytes in unstimulated/HIV cultures. This BFA-associated cytoplasmic retention in unstimulated/HIV-inoculated cultures was modulated by the co-presence of PMA. With Nef, PMA was associated with a relatively large decrease in the cytoplasmic CD8 MFI and a minimal decrease in surface CD8 MFI. The effect of BFA alone was similar to its effect on unstimulated cultures but appeared to modify the BFA effect on cells in the Nef culture. With Rev, PMA was associated with a slight increase in both surface and cytoplasmic CD8 MFIs and BFA was associated with increased cytoplasmic CD8 MFI.

DISCUSSION

Because it has long been known that CD4 is a receptor for HIV, modulation of CD4 by HIV infection and HIV antigens has been investigated vigorously. Our findings concerning CD4 modulation by HIV inoculation and by gp120 are consistent with those reported by others and therefore support the soundness and relevance of our system and analytic approach to the assessments of effects on CD3 and CD8. The effects of HIV on the surface and cytoplasmic expression of CD3 and CD8 have not been studied in depth, in large part because HIV antigens are not thought to interact with these antigens and because any effects on their surface expression would be dwarfed by the dramatic effect of HIV on CD4. With the recent commercial availability of higher quality reagents for cell permeabilization and cytoplasmic staining, it has become feasible to assess less extreme effects, using flow cytometric techniques. We previously used an *in vitro* HIV-1 inoculation culture system to compare actively infected cells with noninfected cells and with cells without productive infection [37]. In this culture system, as in *in vivo* infection, relatively few $CD4^+$ cells are productively infected with HIV at any given time and rare, if any, $CD8^+$ cells are HIV-infected [5, 37]. This system thus allows an efficient examination of the effects of HIV and HIV antigens on noninfected or latently infected cells. We therefore applied flow cytometric techniques and this culture system to investigate the effects of HIV inoculation and

three HIV antigens on the expression and modulation of two critical T-lymphocyte receptors, CD3 and CD8.

We found no effects unique to CD3 but repeatedly found dramatic effects of HIV inoculation, Rev and Nef on CD8 expression. For PHA/HIV cultures, the proportionate increases in the percentages of lymphocytes expressing surface and cytoplasmic CD8 were expected, in mathematical compensation for the decreased proportion of cells expressing CD4. However, reductions in cytoplasmic CD8 MFIs were also found for lymphocytes in both unstimulated/HIV and PHA/HIV cultures, relative to their parallel unstimulated and PHA cultures. Obviously, this change in fluorescent intensity cannot be attributed to the effects of HIV on $CD4^+$ cells. In PHA/HIV cultures, PMA and BFA treatments were associated with decreases in surface CD8 MFI, without much alteration in cytoplasmic CD8. In unstimulated/HIV cultures, BFA was associated with very increased cytoplasmic CD8 MFI. This constellation of findings suggests that: (i) the CD8 effects did not occur by chance; (ii) CD8 is likely not being internalized and degraded but, rather, is being released; and (iii) CD8 production/turnover is increased, at least in unstimulated/HIV cultures.

Each of the three HIV antigens examined had very different effects on CD4, CD3 and CD8. Soluble gp120 had marked effects on CD4 but none on CD3 or CD8. Both Nef and Rev were associated with decreased surface CD8 MFIs; these effects persisted, and even increased, over the time of culture. However, only Nef had effects limited to CD8. In addition to Nef decreasing surface CD8 MFI, when PMA was added to a Nef culture, there was a striking decrease in cytoplasmic CD8 MFI, suggesting that soluble Nef has some impact on the protein kinase C transduction system. The effects of Rev were quite different from those of Nef. The MFIs of all three T-lymphocyte receptor molecules were reduced in some fashion by Rev. Rev was associated with a reduced surface CD4 MFI relative to unstimulated cultures, a reduced cytoplasmic CD3 MFI relative to unstimulated cultures and a reduced surface CD8 MFI relative to *ex vivo* values. BFA had a large, positive effect on surface and cytoplasmic CD4 MFI of lymphocytes in Rev cultures, little effect on CD3 expression and a moderately positive effect on cytoplasmic CD8 expression. PMA decreased CD4 expression in all cultures, including Rev. The effect on CD3 was very different: PMA added to a Rev culture was associated with a large increase in cytoplasmic CD3 MFI. This effect was not altered by the addition of BFA, suggesting that this CD3 redistribution did not involve the Golgi network. The effects of PMA and BFA on CD8 expression in Rev-cultured cells were slight, generally in a positive direction, and greater for BFA.

CD8 is largely restricted to the cell surface [41]. Consistent with this, we found cytoplasmic CD8 MFI values at all settings and culture conditions to be markedly lower than surface CD8 MFI values. However, both Nef and Rev had measurable effects on these small amounts of cytoplasmic, transiting CD8. A previous report suggested that Nef does not directly down-regulate human CD8; however, a Nef expression vector was used in that study, not soluble Nef [44]. Nef functions at the level of the Golgi complex, endoplasmic reticulum and plasma membranes [19, 34, 44]. In addition, exogenous Nef, used in the soluble form, has recently been shown to have several, physiologically significant immunoregulatory effects [24–26]. Nef's modulation of CD8 could be related to these or other mechanisms and likely results in CD8 release from the cell, rather than degradation, because CD8 metabolism differs from that of CD4 and CD3 [45, 46].

Rev has never been shown to interact with CD4 in any direct fashion. Furthermore, because Rev affects CD8 and CD3 expression as well as CD4, its effects are likely related to a shared processing system. Rev serves a transport function, although not necessarily for peptide antigens [45, 46]. Rev appears to be primarily nuclear or nucleolar in location, not surface or cytoplasmic, but Rev's association with membranes could affect activation, transduction or a cellular transport system involving these molecules.

In summary, we found that HIV inoculation and/or HIV antigens had effects on CD8 and CD3 expression, as well as the expected effects on CD4. HIV inoculation of unstimulated cells, HIV inoculation of PHA-stimulated cells, and soluble Nef all had marked modulatory effects limited to CD8. HIV Rev had modulatory effects on both CD3 and CD8. CD3 is a key molecule in signal transduction/activation following antigen recognition, thus a modulatory effect on this molecule has physiological implications. CD8 is essential for cytotoxic T-cell activity; its down-modulation may inhibit this activity, including anti-HIV cytotoxicity. Given the critical roles of these molecules in T-lymphocyte functions, these CD8 and CD3 effects have clinically relevant implications for the pathogenesis and treatment of HIV-related immune defects.

ACKNOWLEDGMENTS

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: LAV Nef and HIV-1 Rev (wild-type), the latter provided by Dr David Rekosh, Dr Marie-Louise Hammarskjöld and Mr Michael Orsini. Dr J. Steven McDougal graciously provided the LAI preparation and protocol used in inoculating these cells with HIV.

REFERENCES

1 Willey RL, Maldarelli F, Martin MA, Strebel K. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J Virol 1992;66:7193–200.

2 Willey RL, Maldarelli F, Martin MA, Strebel K. Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160−CD4 complexes. J Virol 1992;66:226–34.

3 Houlgatte R, Scarmato P, Marhomy SE *et al.* HLA class II antigens and the HIV envelope glycoprotein gp120 bind to the same face of CD4. J Immunol 1994;152:4475–88.

4 Rosenstein Y, Burakoff SJ, Herrmann SH. HIV-gp120 can block CD4-class II MHC-mediated adhesion. J Immunol 1990; 144:526–31.

5 McDougal JS, Mawle A, Cort SP *et al.* Cellular tropism of the human retrovirus HTLV-III/LAV. I. Role of T cell activation and expression of the T4 antigen. J Immunol 1985;135:3151–62.

6 Patterson BK, Till M, Otto P *et al.* Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven *in situ* hybridization and flow cytometry. Science 1993;260:976–9.

7 Carrière D, Vendrell J-P, Berthier A-M *et al.* CD4 masking during human immunodeficiency virus type 1 infection, quantified on peripheral blood lymphocytes, is a potential marker of disease progression. J Infect Dis 1996;173:565–73.

8 Kaneko H, Neoh LP, Takeda N *et al.* Human immunodeficiency virus type 2 envelope glycoprotein binds to CD8 as well as to CD4 molecules on human T cells. J Virol 1997;71:8918–22.

9 Theodore AC, Kornfeld H, Wallace RP, Cruikshank WW. CD4 modulation of noninfected human T lymphocytes by HIV-1 envelope glycoprotein gp120: contribution to the immunosuppression seen in HIV-1 infection by induction of CD4 and CD3 unresponsiveness. J Acquir Immune Defic Syn 1994;7:899–907.

10 Jabbar MA, Nayak DP. Intracellular interaction of human immunodeficiency virus type 1 (ARV-2) envelope glycoprotein gp160 with CD4 blocks the movement and maturation of CD4 to the plasma membrane. J Virol 1990;64:6297–304.

11 Crise B, Rose JK. Human immunodeficiency virus type 1 glycoprotein precursor retains a CD4−$p56^{lck}$ complex in the endoplasmic reticulum. J Virol 1992;66:2296–301.

12 Grzesiek S, Stahl SJ, Wingfield PT, Bax A. The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. Biochemistry 1996;35:10256–61.

13 Rossi F, Gallina A, Milanesi G. Nef–CD4 physical interaction sensed with the yeast two-hybrid system. Virology 1996;217:397–403.

14 Garcia JV, Miller AD. Serine phosphorylation-independent down-regulation of cell-surface CD4 by *nef*. Nature 1991;350:508–11.

15 Iafrate AJ, Bronson S, Skowronski J. Separable functions of Nef disrupt two aspects of T cell receptor machinery: CD4 expression and CD3 signaling EMBO J 1997;16:673–84.

16 Hua J, Cullen BR. Human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus Nef use distinct but overlapping target sites for downregulation of cell surface CD4. J Virol 1997;71:6742–8.

17 Goldsmith MA, Warmerdam MT, Atchison RE, Miller MD, Greene WC. Dissociation of the CD4 downregulation and viral infectivity enhancement functions of human immunodeficiency virus type 1 Nef. J Virol 1995;69:4112–21.

18 Rhee SS, Marsh JW. Human immunodeficiency virus type 1 *nef*-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. J Virol 1994;68:5156–63.

19 Schwartz O, Dautry-Varsat A, Goud B *et al.* Human immunodeficiency virus type 1 Nef induces accumulation of CD4 in early endosomes. J Virol 1995;69:528–33.

20 Craig HM, Pandori MW, Guatelli JC. Interaction of HIV-2 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. Proc Natl Acad Sci USA 1998;95:11229–34.

21 Piguet V, Chen YL, Mangasarian A, Foti M, Carpentier JL, Trono D. Mechanism of *nef*-induced CD4 endocytosis: Nef connects CD4 with the mu chain of adaptor complexes. EMBO J 1998;17:2472–81.

22 Tokunaga K, Kojima A, Kurata T *et al.* Producer cell-dependent requirement of the Nef protein for efficient entry of HIV-1 into cells. Biochem Biophys Res Commun 1998;250:565–8.

23 Luo T, Anderson SJ, Garcia JV. Inhibition of Nef- and phorbol ester-induced CD4 degradation by macrolide antibiotics. J Virol 1996;70:1527–34.

24 Koedel U, Kohleisen B, Sporer B *et al.* HIV type 1 Nef protein is a viral factor for leukocyte recruitment into the central nervous system. J Immunol 1999;163:1237–45.

25 Quaranta MG, Camponeschi B, Straface E, Malorni W, Viora M. Induction of interleukin-15 production by HIV-1 nef protein: a role in the proliferation of uninfected cells. Exp Cell Res 1999;250:112–21.

26 Okada H, Takei R, Tashiro M. HIV-1 Nef protein-induced apoptotic cytolysis of a broad spectrum of uninfected human blood cells independently of CD95 (Fas). FEBS Lett 1997;414:603–6.

27 Diamond DC, Sleckman BP, Gregory T, Lasky LA, Greenstein JL, Burakoff SJ. Inhibition of CD4+ T cell function by the HIV envelope protein, gp120. J Immunol 1988;141:3715–17.

28 Chirmule N, Kalyanaraman V, Oyaizu N, Pahwa S. Inhibitory influences of envelope glycoproteins of HIV-1 on normal immune responses. J Acquir Immune Defic Syn 1988;1:425–30.

29 Chirmule N, Kalyanaraman VS, Oyaizu N, Slade HB, Pahwa S. Inhibition of functional properties of tetanus antigen-specific T-cell clones by envelope glycoprotein gp120 of human immunodeficiency virus. Blood 1990;75:152–9.

30 Weinhold KJ, Lyerly HK, Stanley SD, Austin AA, Matthews TJ, Bolognesi DP. HIV-1 gp120-mediated immune suppression and lymphocyte destruction in the absence of viral infection. J Immunol 1989;142:3091–7.

31 Linette GP, Hartzman RJ, Ledbetter JA, June CH. HIV-1-infected T cells show a selective signaling defect after perturbation of CD3/antigen receptor. Science 1988;241:573–6.

32 Hubert P, Bismuth G, Korner M, Debre P. HIV-1 glycoprotein gp120 disrupts CD4−p56lck/CD3−T cell receptor interactions and inhibits CD3 signaling. Eur J Immunol 1995;25:1417–25.

33 Luo T, Downing JR, Garcia JV. Induction of phosphorylation of human immunodeficiency virus type 1 Nef and enhancement of CD4 downregulation by phorbol mysitate acetate. J Virol 1997;71:2535–9.

34 Mangasarian A, Foti M, Aiken C, Chin D, Carpentier JL, Trono D. The HIV-1 *nef* protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane. Immunity 1997;6:67–77.

35 Stevenson M, Zhang X, Volsky DJ. Downregulation of cell surface molecules during noncytopathic infection of T cells with human immunodeficiency virus. J Virol 1987;61:3741–8.

36 Zack JA, Arrigo SJ, Weitsman SR, Go AS, Hasilip A, Chen ISY. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 1990;61:213–22.

37 Jason J, Inge KL. Increased expression of CD80 and CD86 in *in vitro*-infected CD3+ cells producing cytoplasmic HIV type 1 p24. AIDS Res Hum Retroviruses 1999;15:173–81.

38 Jason J, Inge KL. Mitogen-induced modulation of CD3, CD4, and CD8. Hum Immunol 2000;61:202–11.

39 Møller BK, Andresen BS, Christensen EI, Petersen CM. Surface membrane CD4 turnover in phorbol ester stimulated T-lymphocytes. FEBS Lett 1990;276:59–62.

40 Anderson SJ, Coleclough C. Regulation of CD4 and CD8 expression on mouse T cells. J Immunol 1993;151:5123–34.
41 Marsh M, Parsons IJ, Reid P, Pelchen-Matthews A. Endocytic regulation of the T lymphocyte co-receptor proteins CD4 and CD8. Biochem Soc Trans 1993;21:703–6.
42 Pascale MC, Erra MC, Malagolini N *et al.* Post-translational processing of an *O*-glycosylated protein, the human CD8 glycoprotein, during the intracellular transport to the plasma membrane. J Biol Chem 1992;267:25196–201.
43 Acres RB, Conlon PJ, Mochizuki DY, Gallis B. Phosphorylation of the CD8 antigen on cytotoxic human T cells in response to phorbol myristate acetate or antigen-presenting B cells. J Immunol 1987;139:2268–74.
44 Blue M-L, Hafler DA, Craig KA, Levine H, Schlossman SF. Phosphorylation of CD4 and CD8 molecules following T cell triggering. J Immunol 1987;139:3949–54.
45 Romanov VI, Zolotukhin AS, Aleksandroff NN, Pinto da Silva P, Felber BK. Posttranscriptional regulation by Rev protein of human immunodeficiency virus type 1 results in nonrandom nuclear localization of gag mRNA. Virology 1997;228:360–70.
46 Dundr M, Leno GH, Lewis N, Rekosh D, Hammarskjöld ML, Olson MOJ. Location of the HIV-1 Rev protein during mitosis: inactivation of the nuclear export signal alters the pathway for postmitotic re-entry into nucleoli. J Cell Sci 1996;109:2239–51.
47 Garcia JV, Alfano J, Miller AD. The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. J Virol 1993;67:1511–16.