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Costimulatory Properties of the Human CD4 Molecule: Enhancement of CD3-Induced T Cell Activation by Human Immunodeficiency Virus Type 1 through Viral Envelope Glycoprotein gp120

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ABSTRACT

This study was designed to investigate the T cell costimulatory activity of ligands binding to different regions on the human CD4 molecule. We assayed the costimulatory properties of a panel of CD4 MAbs, intact HIV, and viral envelope glycoproteins in CD3-induced activation of resting T cell subpopulations. Our data using MAbs reveal epitope-specific variations in the functional activities of CD4 MAbs under specific conditions in which CD3 and CD4 molecules are co-cross-linked. We show that both naive and memory $CD4^+$ T cell subsets are susceptible to CD4-mediated costimulation, which overcomes the functional differences between the two cell populations in responsiveness to CD3 MAbs. We show for the first time that, analogous to CD4 MAbs, preparations of HIV and viral envelope glycoprotein gp120 are also potent costimulators of T cell proliferation and IL-2 production. On the basis of these results we propose possible mechanisms for polyclonal cell activation in the course of HIV infection and suggest that viral inhibitory and costimulatory effects may together disrupt the normal balanced function of the immune system, leading to AIDS.

INTRODUCTION

THE CD4 PROTEIN, expressed on a subset of T lymphocytes and antigen-presenting cells (APCs), has the capacity to alter various aspects of T cell function. It facilitates cell-to-cell adhesion by interacting directly with major histocompatibility complex (MHC) class II molecules on APCs, thus promoting activation of the T cell by specific peptide–MHC complexes (for a review, see Ref. 1). On activated MHC class II-restricted T lymphocytes CD4 molecules are also involved in direct cis-type interactions with the T cell receptor (TCR)–CD3 complex and may modulate or determine the fidelity of TCR specificity.[2]

On its cytoplasmic domain, CD4 is associated with the Src-family tyrosine kinase $p56^{lck}$ and as a signal-transducing molecule, CD4 is directly capable of regulating T cell activation.[3] Physical association of the CD4–$p56^{lck}$ complex with the TCR is required for optimal T cell stimulation.[4–6] Coaggregation of TCR–CD3 and CD4 in multimeric clusters, referred to as co-cross-linking, potentiates a variety of biochemical and cellular responses such as protein tyrosine phosphorylation,[7] production of cytoplasmic inositol triphosphate and Ca^{2+},[8] interleukin 2 (IL-2) secretion, and proliferation of the responding cells.[9] Experimental co-cross-linking can be achieved by using CD3 and CD4 MAbs immobilized on the same solid surface or aggregated with a secondary antibody, to activate T lymphocytes. However, nonaggregated CD4 MAbs presented in solution appear to be directly capable of downregulating T cell responsiveness, even in the absence of APCs.[8,10] Binding of bivalent antibodies may hinder sterically the formation of functional TCR–CD3–CD4 complexes and/or could lead to dissociation of $p56^{lck}$ from the cytoplasmic domain of CD4,[11] resulting in inhibition of T cell activation,[8] T cell anergy, and apoptosis.[12]

The CD4 protein also binds HIV via the viral envelope glycoprotein gp120 (for a review, see Ref. 13). In patients infected with HIV, there is a diverse range of progressive

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immunological abnormalities and clinical symptoms characteristic of AIDS. These include early depression of T helper cell functional responses in the presence of normal numbers of $CD4^+$ cells. Besides direct viral cytotoxic effects, the binding of HIV to CD4 through gp120 may prevent function of cells bearing this molecule by a mechanism similar to the effect of bivalent CD4 antibodies.[12,14,15]

A number of clinical and immunological findings also show markers of general activation of the immune system after HIV infection. Manifestations of immune activation include elevated cytotoxic T lymphocyte (CTL) activity, increased numbers of $CD8^+$ T cells bearing activation markers,[16,17] polyclonal activation of B cells,[18] and increased levels of β_2-microglobulin and IL-2 receptors in the serum of patients infected with HIV.[19] Both the initial integration of the virus into the host cell DNA and HIV-1 gene expression require T cell stimulation,[20] hence widespread T cell activation may play a major role in increasing virus replication and viral protein production.

In the present study we have investigated the costimulatory activity of CD4 in CD3-induced activation of T lymphocytes. We have addressed the functional importance of different extracellular CD4 epitopes, using a panel of MAbs to CD4, and have assessed HIV and viral envelope proteins for costimulatory activity. We describe efficient costimulation of T cell proliferation and IL-2 production with a variety of CD4 MAbs, using different cross-linking conditions. We also present novel observations showing costimulatory activity of virus and recombinant envelope preparations. On the basis of these results we propose possible mechanisms for polyclonal cell activation in the course of HIV infection.

TABLE 1. SUMMARY OF ANTIBODIES USED IN THIS STUDY

MAb name	*Specificity*	*Isotype*	*Refs.*
Anti-Leu-3a[a]	CD4/D1, loop 2	IgG_1	21, 22
L77[b]	CD4/D1, loop 2	IgG_1	21
L200[b]	CD4/D1	IgG_1	23
OKT4A[c]	CD4/D1, loop 3	IgG_{2a}	22
SIM.4[d]	CD4/D1	IgG_1	
L68[b]	CD4/D1, loop 4	IgG_1	21
L71[b]	CD4/D1, loop 4	IgG_1	21, 24
L104[b]	CD4/D1, loop 4	IgG_{2a}	21, 22
Q428[d]	CD4/D3	IgG_1	25
Q425[d]	CD4/D3	IgG_1	25
L83[b]	CD4/D1 + D3	IgG_1	24
L120[b]	CD4/D4	IgG_1	21, 24, 25
SIM.2[d]	CD4/ND	IgG_{2b}	
59.1[e]	gp120/V3	IgG_1	
OKT8[c]	CD8	IgG_2	
10F7MN[f]	Anti-glycophorin A	IgG_1	
Anti-Leu-M3[a]	CD14	IgG_{2b}	
Anti-Leu-16[a]	CD20	IgG_1	
Anti-Leu-45RO[a]	CD45RO	IgG_{2a}	
KD-3[g]	CD45RA	IgG_1	26

[a] Purchased from Becton Dickinson (San Jose, CA).
[b] Donated by D. Buck and S. Maine (Becton Dickinson).
[c] Obtained from Ortho Diagnostic Systems, Inc. (Raritan, NJ).
[d] Obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH).
[e] Purchased from Repligen Corporation (Cambridge, MA).
[f] Hybridoma obtained from American Type Culture Collection (ATCC HB 8162) (Rockville, MD).
[g] Provided by I. Andó and É. Monostori (BRC, Szeged, Hungary).

MATERIALS AND METHODS

Reagents and cells

The MAbs used in this study were of mouse origin and their source and characteristics are given in Table 1.[21–26] $F(ab')_2$ fragments of polyclonal rabbit anti-mouse IgG, IgG_1, IgG_{2a}, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig) were obtained from Organon Teknika (Durham, NC). Immunoglobulins were extensively dialyzed against phosphate-buffered saline (PBS), pH 7.2, in order to remove NaN_3 prior to use in functional assays.

Single-density gradient-purified HTLV IIIB[27] (HIV sdIIIB, 1.3×10^{11} virus particles [vp]/ml) was purchased from Pharmacia Diagnostics, Inc. (Columbia, MD). Direct pelleted HTLV IIIB (HIV dpIIIB, 1.14×10^{11} vp/ml) and HIV Bal[28] (2.06×10^{11} vp/ml) were obtained from Advanced Biotechnologies, Inc. (Columbia, MD). HIV-1 recombinant gp120 was obtained from Celltech, Inc., through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) (Catalog No. 534). Viral envelope glycoproteins gp120 and gp140 (strain HIV BH8) were expressed in BSC-1 cells infected with vaccinia vectors carrying DNA inserts of gp120 and cleavable gp140 sequences, respectively (generous gift of P. L. Earl and C. Broder, NIH, Bethesda, MD[29]). The estimated concentration of gp120 and gp140 in the supernatants of BSC-1 cells was 1–5 μg/ml as determined by Western blotting, using serial dilutions of standard gp120 preparations as a reference.

Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors by leukapheresis and separation on Ficoll-Hypaque (Pharmacia Diagnostics, Inc.). Monocytes were isolated by countercurrent centrifugal elutriation[30] and T cell subsets were purified from the lymphocyte fraction as follows. Contaminating monocytes were removed with adherence to plastic petri dish for 2 hr at 37°C at a cell concentration of 10^6/ml. Nonadherent cells were treated with a 10-μg/ml concentration of anti-Leu-M3, anti-Leu-16 MAb, and a mixture of OKT8 and KD-3 (for isolating $CD4^+CD45RA^-$ cells) or OKT8 and anti-Leu-45RO (for $CD4^+CD45RO^-$ cells) or with SIM.2 MAb (for $CD8^+$ cells). Cells were washed extensively and magnetic beads (Dynal, Inc., Great Neck, NY) coated with anti-mouse Ig were added at a 3:1 ratio of beads to target cells for negative selection of resting T cells. After 30 min at 4°C cells attached to magnetic beads were removed, using a magnetic particle concentrator (Dynal, Inc.). Remaining cells were mixed with 10% monocytes and used for functional assays. Purity of the different T cell subsets was greater than 95% as determined by immunofluorescence analysis.

The IL-2-dependent murine T cell line CTLL[31] was cultured in RPMI-1640 tissue culture medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (GIBCO Laboratories), 10^{-5} M 2-mercaptoethanol (2-

ME), and human rIL-2 (10 IU/ml) (Genzyme Corporation, Cambridge, MA).

Indirect immunofluorescence

For titrations of CD4 MAb, aliquots (5×10^4) of $CD4^+$ cells were incubated with serial dilutions of the MAb listed in Fig. 1B, washed with ice-cold PBS containing 0.2% bovine serum albumin (BSA) and 0.1% azide, and stained with FITC-conjugated anti-mouse Ig for 30 min on ice. Live cells, 10^4/sample, were collected on a FACScan (Becton Dickinson) and analyzed with FACScan and Lysys software (Becton Dickinson).

Surface coupling of reagents

Coupling of reagents to plastic plates was optimized and carried out as follows. Antibodies, HIV, and viral envelope gp120 were directly coupled to the surface of flat-bottomed 96-well tissue culture plates (Costar, Cambridge, MA) at concentrations indicated in Figures 1–5, in 100 μl of PBS for 6 hr at 37°C, followed by washing of the plates three times with PBS. For indirect immobilization of antibodies, the wells were first coupled with 100 μl of rabbit anti-mouse IgG $F(ab')_2$ at 10-μg/ml concentration. After incubation, the wells were washed extensively with PBS. Thereafter OKT3 MAb was added in 100 μl of RPMI-1640 tissue culture medium supplemented with 10% heat-inactivated AB^+ human serum (ICN Biomedicals, Costa Mesa, CA) (complete medium), and incubated for 2 hr at 37°C. The plates were washed and CD4 or control MAb was added in complete medium at a 1-μg/ml concentration and incubated as OKT3. Unbound MAb was removed by washing the plates in PBS. For indirect immobilization of gp120 and gp140, plates were directly coupled first with OKT3, and then with anti-V3 or control MAb. Viral envelope glycoproteins were captured on the layer of anti-V3 MAb by incubating the wells with 100-μl supernatants of BSC-1 cells infected with HIV envelope-expressing vaccinia vectors.

Cell proliferation and interleukin 2 assay

Proliferation assays were carried out in flat-bottomed 96-well tissue culture plates in complete medium. The rate of T cell proliferation was monitored by the incorporation of tritiated thymidine ([^{3}H]TdR) into the DNA of the cells in triplicate samples. Cells were seeded at 10^5 cells/well on plates coated by antibodies or with antibodies added in solution at a final volume of 200 μl and were incubated for 72 hr. Cells were pulsed with 1 μCi/well (37 kBq/well of [^{3}H]TdR, (or 185 GBq/mmol; Amersham, Arlington Heights, IL) in 50 μl of complete medium for the last 16 hr of culture and then harvested for scintillation counting.

For measurement of IL-2 production supernatants were harvested at 36 hr of culture from the individual wells of proliferation assays. For IL-2 assays, 5× diluted supernatants were cultured in triplicate with CTLL cells (6×10^3/well) for 36 hr in a total volume of 200 μl. The rate of proliferation of CTLL in response to IL-2 was measured by [^{3}H]TdR incorporation. The cpm values were fitted to a calibration curve obtained with serial dilutions of human rIL-2 and the amount of IL-2 released into the culture supernatants was calculated and expressed as IU/ml.

RESULTS

Functional epitope analysis of the CD4 molecule

Our initial experiments were carried out to correlate CD4 functional aspects with structural features of the molecule by testing a panel of MAbs to CD4 for functional effects on CD3-mediated T cell proliferation. The MAbs were directed to a variety of CD4 epitopes, including sites on domains associated with binding of gp120 and subsequent fusion events induced by HIV. Purified $CD4^+$ T lymphocytes were incubated in microtiter plates coated with either CD3 MAb alone or CD3 together with CD4 MAbs (Fig. 1A). None of the CD4 MAbs were mitogenic alone (as shown in Fig. 1A for L83). However, 9 of 13 CD4 MAbs tested enhanced the rate of cell proliferation by 3- to 7-fold compared to the [^{3}H]TdR uptake induced by CD3

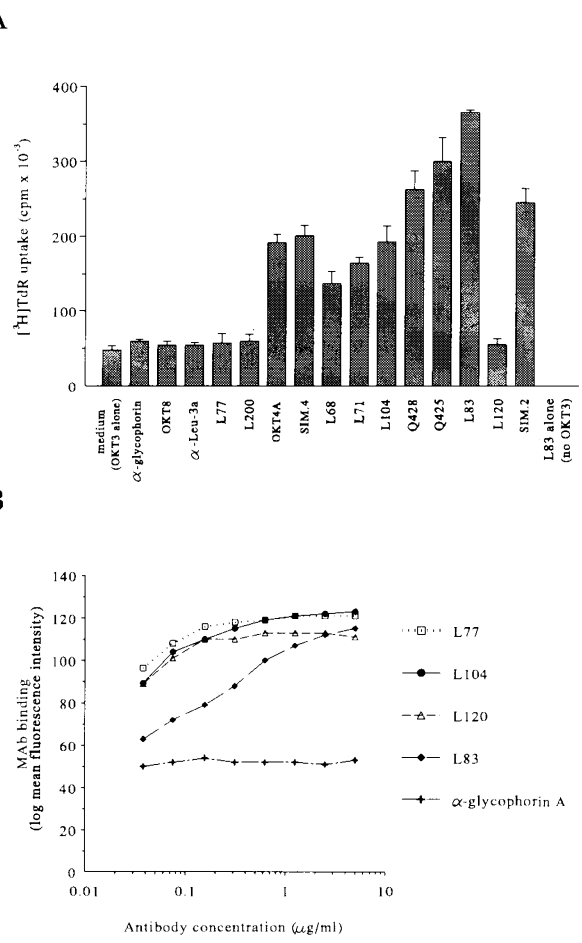

FIG. 1. Effect of MAbs directed to various CD4 epitopes on CD3-induced proliferation of $CD4^+$ T lymphocytes. (A) Purified $CD4^+$ T cells were incubated with CD3 MAb (1 ng/well) and with other antibodies indicated on the horizontal axis (100 ng/well) coimmobilized on the surface of microtiter plates. Data represent mean values ± SD of [^{3}H]TdR incorporation of triplicate samples. (B) Titration of MAbs by indirect immunofluorescence staining of $CD4^+$ T cells over the range of Ig concentrations indicated on the horizontal axis. The vertical axis shows mean fluorescence intensity in arbitrary logarithmic units.

alone. All the MAbs specific for loop 4 of D1 (corresponding to CDR3 of antibody molecules) (L68, L71, and L104) stimulated CD3-induced T cell activation. In contrast, from the five MAbs that bind inside a 20-amino acid (aa) long region (aa 40–60) of D1, three (anti-Leu-3a, L77, and L200) have not affected, whereas two others (OKT4A and SIM.4) stimulated, the rate of T cell proliferation. Of the MAbs directed to non-D1 sites, one MAb (L120) also did not stimulate T cell reponses to CD3 under these experimental conditions. The recognition sequence for this MAb is between aa 306 and 370 on D4 of CD4. The other four MAbs that have enhanced T cell activation include two MAbs (Q425 and Q428) reacting with aa 249–252 on D3, and two MAbs to incompletely characterized determinants (L83 and SIM.2).

To determine if the differences observed in the functional activity of CD4 antibodies could be accounted for by different affinities of the MAb to CD4, antibodies were titrated using indirect immunofluorescence staining. All but one (L83) MAb exhibited similar titration curves in immunofluorescence staining (as shown in Fig. 1B for L77, L120, L104, and L83), suggesting that the antibodies were of similar binding affinity. We also did not find differences in the binding characteristics of the MAbs to the plastic surface as determined by enzyme-linked immunosorbent assay (ELISA), using peroxidase-conjugated anti-mouse IgG (data not shown). Furthermore, as low as 10 ng/well of MAb L104 appeared to be sufficient for costimulation, whereas the inactive MAbs L77 and L120 did not show activity even at a 10-μg/well concentration (data not shown).

Effect of CD3–CD4 co-cross-linking on the activation of naive and memory T cells

The peripheral T lymphocyte compartment has been divided into reciprocal subpopulations by the differential expression of the CD45RA and CD45RO epitopes.[32] To test the responsiveness of the T cell subsets to CD4-mediated costimulation, we isolated naive ($CD45RA^{high}CD45RO^{low/-}$) and memory ($CD45RO^{high}CD45RA^{low/-}$) $CD4^+$ T cells by negative selection and then activated the purified cells with plastic coated CD3 and CD4 antibodies (Fig. 2). Memory cells showed a higher capacity for proliferation in response to the same amount of CD3 MAb in comparison to naive cells. However, introduction of CD4 MAb L104 along with CD3 overcame the differences observed between the $CD4^+$ cell subsets by costimulating both cell types, while having no effect on the $CD8^+$ cells. The two other CD4 MAbs tested (L77 and L120) and the control MAb did not stimulate either subpopulation.

Requirements for efficient costimulation via the CD4 molecule

Several MAbs to CD4 are not capable of stimulating T cell proliferation when immobilized directly to the plastic surface. This could be because the epitopes recognized by these MAbs have no direct functional importance in positive signaling via CD4 or that the method used was not sufficient for effective cross-linking of CD4 to CD3 through these particular epitopes. Two representatives of these MAbs (L77 and L120), and the costimulator MAb L104, were presented to purified $CD4^+$ cells in two different ways together with the CD3 MAb: (1) in solution, aggregated by secondary antibody anti-mouse IgG (Table 2A), or (2) immobilized indirectly on a layer of anti-mouse IgG coupled to the plastic surface of microtiter plates (Table 2B). We applied optimal (10 ng/well) and suboptimal (1 ng/well) concentrations of CD3 MAb as defined by the induction of maximal and submaximal [^{3}H]TdR incorporation of T cells, respectively. At suboptimal CD3 concentrations, both L120 and L104 MAbs costimulated, independent of the method used for cross-linking by secondary antibody. However, MAb L77 directed to the CD4/D1, loop 2 required immobilization by the secondary antibody for costimulation to occur. Slight or no

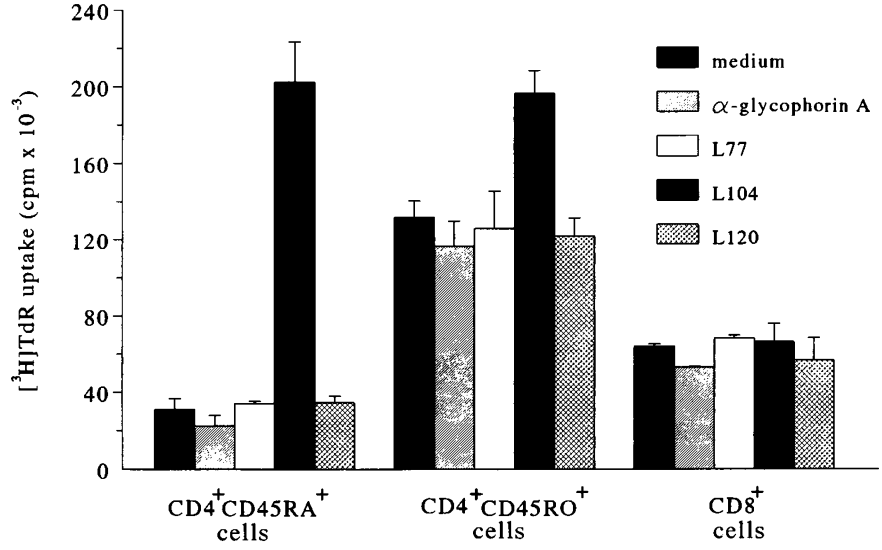

FIG. 2. Proliferative response of isolated T cell subsets to immobilized CD3 and CD4 antibodies. Designated populations of T cells were coincubated with 1 ng of OKT3 MAb per well and with 100-ng/well concentrations of other antibodies as indicated. Data represent mean values ± SD of [^{3}H]TdR incorporation of triplicate samples.

TABLE 2. PROLIFERATIVE RESPONSE OF $CD4^+$ T CELLS TO CD3 AND CD4 MONOCLONAL ANTIBODIES AGGREGATED BY SECONDARY ANTIBODY[a]

Monoclonal antibody	*[^{3}H]TdR incorporation (cpm)*		
	1 ng/well OKT3	*10 ng/well OKT3*	*100 ng/well OKT3*
A. Aggregated in Solution			
None	2,974 ± 911	81,462 ± 4,837	91,421 ± 4,746
Anti-glycophorin A	2,899 ± 193	79,765 ± 6,545	89,777 ± 6,798
L77	2,741 ± 715	74,682 ± 4,774	91,057 ± 5,925
L104	77,927 ± 9,811	82,022 ± 5,050	90,528 ± 6,110
L120	117,789 ± 7,500	110,534 ± 4,590	102,159 ± 8,013
B. Aggregated on the Surface of Microtiter Plates			
None	6,892 ± 456	69,009 ± 2,301	
Anti-glycophorin A	6,576 ± 698	66,131 ± 1,567	
L77	43,309 ± 3,706	80,021 ± 1,603	
L104	50,828 ± 2,166	75,830 ± 1,619	
L120	39,745 ± 2,548	95,085 ± 2,884	

[a]CD3 MAbs and other antibodies indicated were coaggregated by secondary antibody in solution (A) or on the surface of microtiter plates (B), as described in Materials and Methods, using the following final concentrations of antibodies: anti-mouse IgG, 500 ng/well; CD3 MAb, as indicated in the table; other MAbs, 100 ng/well. Data represent mean values ± SD of [^{3}H]TdR incorporation of triplicate samples.

costimulation was observed with CD4 MAbs at the optimum concentration of CD3 MAb or with the control anti-glycophorin A MAb.

We also examined whether the epitopes recognized by CD4 antibodies had a functional role in T cell activation when CD4 molecules are not cross-linked (Table 3A) or cross-linked separately from the TCR–CD3 complex (Table 3B). When CD4 MAbs were presented in solution with CD3 MAb coupled directly to the plastic surface, all the antibodies to CD4 inhibited CD3-induced T cell proliferation. The inhibition ranged between 50 and 70% at a 1-μg/ml concentration of CD4 MAb. Inhibition was also observed, although to a lesser extent, using a

TABLE 3. INHIBITION OF T CELL ACTIVATION BY CD4 MONOCLONAL ANTIBODIES PRESENTED IN SOLUTION OR AGGREGATED SEPARATELY FROM CD3 MONOCLONAL ANTIBODY[a]

Monoclonal antibody	*[^{3}H]TdR incorporation (cpm)*		
	1 ng/well OKT3	*10 ng/well OKT3*	*100 ng/well OKT3*
A. In Solution (non-aggregated)			
None		83,671 ± 6,021	
Anti-glycophorin A		95,491 ± 12,473	
OKT8		92,533 ± 2,036	
L77		42,688 ± 7,762	
L104		29,543 ± 2,821	
L120		40,234 ± 1,381	
B. Aggregated Separately			
None	6,952 ± 701	32,987 ± 2,477	82,548 ± 4,979
Anti-glycophorin A	7,123 ± 657	31,425 ± 2,432	81,234 ± 6,754
L77	3,982 ± 523	19,942 ± 803	59,368 ± 1,201
L104	79,380 ± 1,586	95,085 ± 8,501	73,984 ± 5,673
L120	2,253 ± 140	9,888 ± 1,285	55,902 ± 9,536

[a]Part A: $CD4^+$ T cells were stimulated with immobilized CD3 MAb (10 ng/well). At the initiation of the culture, the soluble MAbs indicated were added at a concentration of 100 ng/well. Part B: Monoclonal antibodies to CD3 (IgG_{2a}) and other antibodies (100 ng/well) listed were presented in solution and aggregated using a mixture of soluble anti-mouse IgG_1 (500 ng/well) and anti-mouse IgG_{2a} (500 ng/well) antibodies. Isotypes of antibodies are as follows: OKT3, IgG_{2a}; anti-glycophorin A, IgG_1; L77, IgG_1; L104, IgG_{2a}; L120, IgG_1. Data represent mean values ± SD of [^{3}H]TdR incorporation of triplicate samples.

mixture of isotype-specific anti-mouse immunoglobulin antibodies against the IgG_1 MAbs L77 and L120, and the IgG_{2a} MAb, OKT3, for separate cross-linking of CD4 and CD3. Since L104 is an IgG_{2a} MAb, it showed a costimulatory effect under the later experimental conditions. Similar results were observed when cells were preincubated with the CD4 MAb (up to 24 hr) prior to activation via CD3 in either assay system (data not shown). The control MAb anti-glycophorin A and OKT8 did not affect the rate of proliferation of $CD4^+$ cells under any circumstances.

Effect of HIV and the virus envelope protein gp120 on the activation of T cell subpopulations

The above experiments showed that MAbs to CD4 have potent costimulatory activity on $CD4^+$ cells. We thought that, analogous to binding of MAb, engagement of the CD4 molecule by HIV through the envelope gp120 may result in costimulation of CD3-induced T cell activation. To test HIV and gp120 for costimulatory activity, we incubated $CD4^+$ T lymphocytes and $CD8^+$ cells in microtiter plates coated with both CD3 MAb and cell-free virus preparations of HIV IIIB or monocytotropic HIV Bal (Fig. 3) or affinity-purified gp120 (Fig. 4A and B). While neither the virus preparations tested nor gp120 was mitogenic alone (data not shown), both augmented the CD3-induced proliferation of $CD4^+$ T cells. In addition to their effect on naive and memory $CD4^+$ cells, HIV preparations also showed a costimulatory effect on $CD8^+$ lymphocytes (Fig. 3A). This costimulatory effect could not be accounted for by contamination of $CD8^+$ cells with $CD4^+$ lymphocytes, because the CD4 MAb L104 costimulated exclusively the $CD4^+$ cell population. The IIIB virus showed more potent costimulatory activity compared to the Bal strain (Fig. 3B). The minimum sufficient amount of virus that enhanced the CD3-induced response was 9×10^5 vp/well for IIIB and 8×10^6 vp/well for Bal strain of HIV. Significant costimulation was observed within a range of 1 to 100 ng/well of recombinant gp120 with suboptimal concentrations of CD3 MAb, while only slight effects were observed on optimal CD3 activation (Fig. 4B).

Next, we coupled the plastic surface of microtiter plates with CD3 MAb alone or together with MAb reacting with the V3 loop

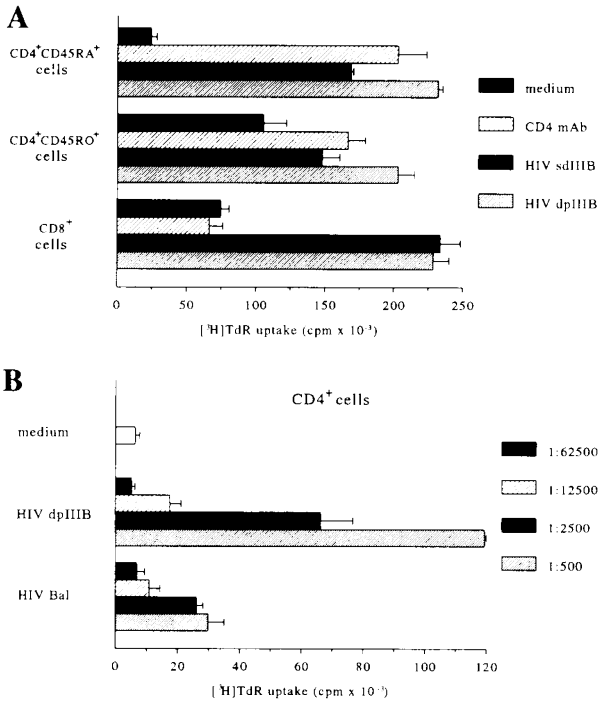

FIG. 3. Costimulatory activity of cell-free HIV preparations on CD3-induced proliferation of purified T cell subsets. Lymphocyte subpopulations were stimulated with CD3 MAb and with indicated reagents coimmobilized on the surface of microtiter plates. Final concentrations of reagents were as follows. (A) MAb OKT3 (CD3), 1 ng/well; MAb L104 (CD4), 100 ng/well; direct pelleted (dp) and single-density gradient-purified (sd) HIV IIIB, 1:5000 dilution of stock virus. (B) Serial dilutions of virus strains are indicated; CD3 and CD4 MAbs were used at concentrations of 1 and 100 ng/well, respectively. Data presented on the horizontal axis show mean values ± SD of [^{3}H]TdR incorporation of triplicate samples.

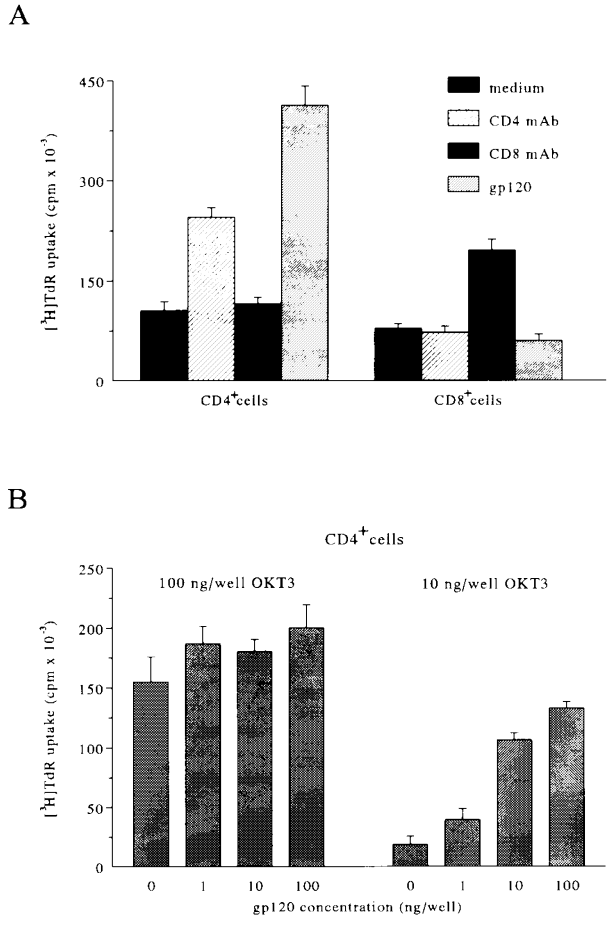

FIG. 4. HIV envelope gp120 augments proliferation of $CD4^+$ T cells activated through CD3. Antibody to CD3 and designated anti-CD4 reagents were coimmobilized on the surface of microtiter plates and indicated cell populations were incubated on the plates. Final concentrations of reagents were as follows. (A) MAb OKT3 (CD3), 1 ng/well; MAbs L104 (CD4) and OKT8 (CD8), 100 ng/well; gp120, 100 ng/ml. (B) Serial dilutions of gp120 are indicated on the horizontal axis, and MAb OKT3 was added at concentrations of 10 and 100 ng/well. Data represent mean values ± SD of [^{3}H]TdR incorporation of triplicate samples.

of gp120 to capture envelope. Recombinant gp120 and gp140 (present in supernatants of BSC-1 cells infected with HIV envelope-expressing vaccinia vectors) were immobilized on the layer of anti-V3 MAb and plates were incubated with $CD4^+$ and $CD8^+$ lymphocytes (Fig. 5). Both gp120 and gp140 specifically increased the rate of CD3-induced proliferation of $CD4^+$ T cells, having no effect on $CD8^+$ lymphocytes. The MAbs to CD4 and CD8, used as a control, exclusively costimulated the $CD4^+$ and $CD8^+$ cells, respectively. No costimulation was observed when envelope proteins were incubated on a layer of irrelevant MAb reacting with glycophorin A.

In addition to the above experiments, in which HIV and envelope were coimmobilized with CD3 MAb, these preparations were added to cultures in solution to test for inhibitory activities. In contrast to the stimulatory effect of immobilized virus and gp120, both preparations inhibited CD3 MAb-induced T cell proliferation when presented in solution (Table 4). The degree of inhibition (35%) was comparable to the effect of CD4 MAb in the same experiment.

HIV envelope costimulation induces interleukin 2 production

We also investigated whether HIV envelope-mediated enhancement of proliferation was accompanied by IL-2 production from the T cells. We measured the IL-2 content of the individual cell culture supernatants obtained from an experiment in which anti-V3 MAb was used to immobilize envelope proteins (Fig. 6). Costimulation by gp120 and gp140 resulted in a 10- to 30-fold increase (1.5 and 3.5 IU/ml, respectively) in IL-2 secreted into the culture supernatants compared to the effect of CD3 MAb alone (0.15 IU/ml).

DISCUSSION

We have investigated how engagement of CD4 through different epitopes of the molecule affects CD3-induced T cell activation. We have used a variety of experimental methods for CD3–CD4 cross-linking to assess the requirements for costimulation, using a panel of MAbs to CD4, HIV, and viral envelope preparations. These methods include (1) direct immobilization of the stimulating reagents on the plastic surface of microtiter plates (Figs. 1–4), (2) aggregation of cell surface-bound MAbs by secondary antibody in solution (Table 2A), and (3) indirect immobilization on a layer of secondary antibody coupled to the plastic surface (Table 2B).

Our data, using MAbs, demonstrates that fine specificity of the CD4 MAb determines the amount of costimulation observed under certain cross-linking conditions. Of eight MAbs binding to different regions on D1 of the CD4 molecule, five MAbs (OKT4A, SIM.4, L68, L71, and L104) costimulated, whereas three others (anti-Leu-3a, L77, and L200) had no effect on cell proliferation when immobilized directly on the plate. The epitopes recognized by six of these MAbs have been mapped to specific sequences in D1.[21,22,24] Two of the nonstimulatory MAbs, anti-Leu-3a and L77, react with the gp120-binding loop 2 of CD4, whereas the stimulatory MAb OKT4A detects residues on loop 3, which also interacts with gp120. This indicates that the gp120-binding site on CD4 may overlap CD4 epitopes with different functional properties. Other stimulatory MAbs L68, L71, and L104 bind to loop 4, the Ig-CDR3-like region of CD4, which is near the gp120-binding site and appears to be involved in postbinding fusion events.[22] We have also found differences in the functional properties of MAb binding outside D1, using direct antibody coupling to the solid phase. One MAb directed to D4 did not stimulate T cell responses to CD3, whereas three MAbs to D3 were costimulatory. All the MAbs tested have similar binding characteristics to cells and to the microtiter plates as determined by indirect immunofluorescence staining and ELISA, respectively. Thus the inability of particular MAbs to costimulate under direct coupling conditions cannot be explained by different antibody affinity to CD4 or to the plastic surface. Although it is not clear how the MAbs to various CD4 epitopes mediate costimulatory activity, the different functional activities of the CD4-directed antibodies probably reflect the differences in their fine specificities.

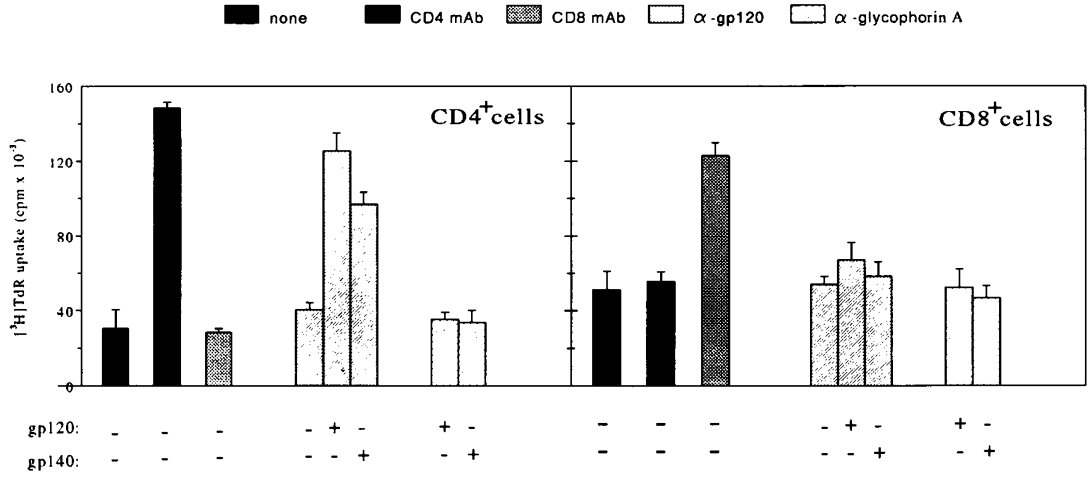

FIG. 5. Costimulatory effect of HIV envelope glycoproteins bound by anti-gp120 V3 MAb. $CD4^+$ and $CD8^+$ T lymphocytes were incubated on microtiter plates coupled with MAb to CD3 (1 ng/well) and with other antibodies (100 ng/ml) as shown. Recombinant gp120 or gp140, as indicated, was immobilized on the plate attached layer of anti-gp120 MAb. Data represent mean values ± SD of [^{3}H]TdR incorporation of triplicate samples.

TABLE 4. INHIBITORY EFFECT OF SOLUBLE CELL-FREE VIRUS AND HIV ENVELOPE gp120 ON CD3-INDUCED T CELL ACTIVATION[a]

Reagent	*[^{3}H]TdR incorporation (cpm)*
None	252,787 ± 18,837
Anti-glycophorin A	255,682 ± 13,429
L104	166,802 ± 14,720
HIV IIIB	162,418 ± 3,716
gp120	173,740 ± 5,992

[a]$CD4^+$ T lymphocytes were stimulated with immobilized CD3 MAbs in the presence of the indicated soluble reagents. Final concentrations of reagents were as follows: CD3 MAb, 10 ng/well; other MAbs and gp120, 100 ng/well; HIV IIIB, 1:5000 dilution of stock virus preparation. Data represent mean values ± SD of [^{3}H]TdR incorporation of triplicate samples.

We have assumed that steric effects or conformational changes induced by the inactive antibodies may influence the precise orientation of CD4 to the TCR–CD3 complex. These antibodies may fail to facilitate the accumulation of functional CD3–CD4 multimers in the cell membrane or may require more extensive coupling of CD3 to CD4 to induce costimulation. Cross-linking of CD4 and CD3 MAbs, using secondary anti-mouse IgG, showed that MAbs L77 and L120, which did not costimulate when coupled directly, could enhance T cell proliferation.

Although the precise role of different epitopes of CD4 in the interaction with CD3 needs further investigation, the observed epitope specific variations in the functional activities of CD4 MAbs under various cross-linking conditions underline the requirement for precise spatial organization of CD3 and CD4 molecules at the cell surface in the course of T cell activation. Our data can partially explain the observed differences between the functional effect of some CD4 MAbs (i.e., OKT4 versus OKT4A) in other experimental systems[33] and emphasizes the requirement for careful methodological evaluation in studies in which functional properties of MAbs against cell surface determinants are under investigation.

In experiments designed to test the responsiveness of isolated T cell subsets to costimulation, we have not found differences between the functional capacity of the naive ($CD45RA^{high}$ $CD45RO^{low/-}$) and memory ($CD45RO^{high}CD45RA^{low-}$) $CD4^+$ T cell subsets to CD4-mediated costimulation of CD3-induced T cell proliferation (Fig. 2). In accordance with published data,[34] memory cells proliferated better than naive cells in response to CD3 MAb alone; however, costimulation via CD4 overcame this functional difference between the two cell populations. Our data raise the possibility that naive cells are more dependent on accessory signals through CD4 for efficient cell activation in comparison to competent memory lymphocytes.

Our results on CD4-mediated costimulation of cells with different CD45R phenotypes conflict with the data obtained by another group,[9] which observed preferential response of memory cells after activation of purified $CD4^+$ T cell subsets by CD3 and CD4 MAbs coupled to Sepharose beads. In their study, T cell subsets were purified by positive means, using sheep erythrocytes for T cell and CD45RA MAb for naive cell purification. Because signals through both the sheep erythrocyte receptor (CD2)[35] and CD45RA[26] have been shown to influence T cell activation, we chose to use negative selection in our experiments for naive and memory cell isolation. Thus the differences between the results may be explained by the different methods used for both presentation of antibodies and isolation of T cell subsets.

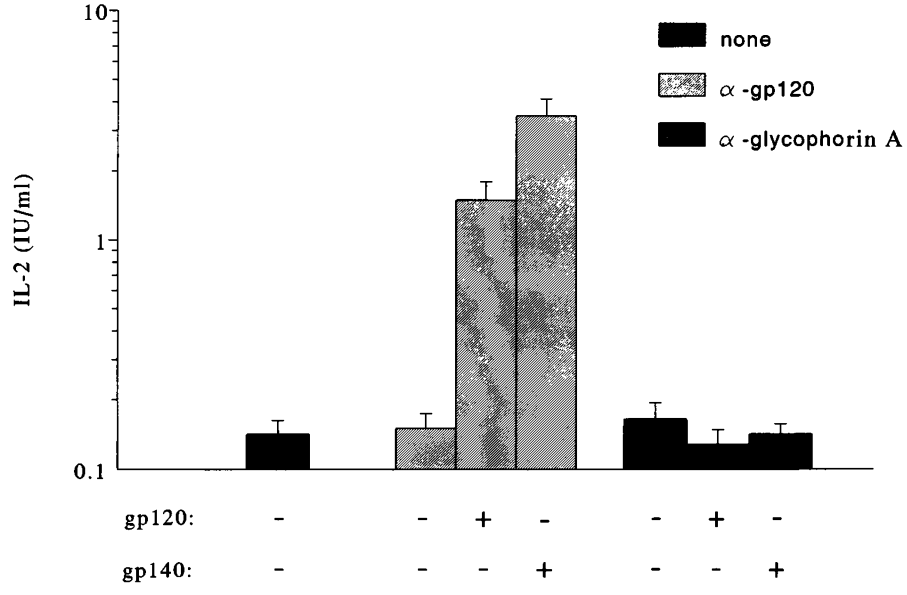

FIG. 6. Interleukin 2 production of $CD4^+$ T cells during gp120-mediated costimulation. Murine CTLL cells were cultured in the presence of diluted culture supernatants of T cell cultures from an experiment represented in Fig. 5. Reagents used for stimulation of $CD4^+$ T lymphocytes are indicated. Data presented on the vertical axis show mean values ± SD of IL-2 concentration measured in the culture supernatants.

In all the experiments presented, the costimulatory effect of CD4 MAb is more pronounced when cells are stimulated with suboptimal doses of CD3 MAb. These results are consistent with previous findings, that accessory molecules, including CD4, play a major role in facilitating weak antigen responses.[33]

In contrast to the potent costimulatory effects found with cross-linked MAbs, all MAbs to CD4, when presented in solution or aggregated separately from CD3 by isotype-specific antibodies, inhibited CD3-induced T cell activation (Table 3). We did not find differences in the inhibitory effect of MAbs recognizing different CD4 epitopes. These data extend the results obtained by others,[8,10] and show that ligand binding to the CD4–p56^{lck} complex without linkage to the TcR leads to either incomplete CD3 signaling or inhibitory signaling events.

In the subsequent experiments, we have assessed HIV and its viral envelope gp120 for costimulatory activity in CD3-induced activation of T lymphocytes (Figs. 3–6). We have used a series of cell-free HIV preparations representing two strains of the virus, which vary in their capacity to infect cells of different origins. Both the T cell-tropic (IIIB) and the monocytotropic (Bal) viruses stimulated CD3-induced activation of T lymphocytes in a dose-dependent manner, when coupled to the surface of microtiter plates along with CD3 MAb. However, in addition to their effect on naive and memory CD4$^+$ cells, HIV preparations have also stimulated the response of CD8^{+2} lymphocytes. This costimulatory effect on CD8$^+$ cells may be mediated either by viral proteins or other virus-associated molecules. HIV is a membrane-budding virus and isolated virus preparations also include host cell membrane antigens.[36] We assume that membrane-associated cell surface proteins may potentiate CD3-induced T cell responses and contribute to CD8$^+$ T cell stimulation.

To study the effects of cell-free virus antigens, we have tested the CD4-binding envelope glycoprotein (gp120) of the virus for costimulatory activity. We find that as low as 1 ng/well of recombinant gp120 coupled directly to the solid phase along with CD3 MAb costimulated CD4$^+$ T lymphocyte responses, while having no effect on CD8$^+$ cells. This potent effect of gp120 resembles that of CD4 MAb, in preferentially costimulating T lymphocytes only under suboptimal conditions of cell activation.

We have demonstrated that viral envelope preparations of different types (gp120 and gp140) immobilized on a layer of MAb directed against the V3 loop of gp120 also specifically costimulated CD4$^+$ lymphocytes (Fig. 5). The V3 loop of the viral envelope glycoprotein gp120 plays a role in the fusion reaction[37] and controls cell tropism[15] of different HIV-1 isolates. This region of the protein, also called the principal neutralizing domain, serves as a target of the host immune response against HIV.[38] Using a MAb to V3 to immobilize HIV envelope shows that exposure of the V3 loop is not required for gp120-mediated costimulation of T cell activation. Furthermore, these data suggest that the presence of virus-neutralizing antibodies against V3 may not affect the costimulatory properties of HIV. Because the gp140 preparations also contain the peptide sequence of gp41 in addition to gp120, our finding that gp120 preparations show similar if not a higher degree of costimulation also rules out a direct requirement for gp41 in viral envelope-mediated T cell activation.

Abundant clinical and laboratory evidence supports an association between generalized immune activation and disease progression after HIV infection. There are several immunological features common to both AIDS and experimentally induced graft-versus-host disease in mice, which indicate that HIV may induce autoimmunity.[13] It has been proposed that the immune response against a potential MHC class II homolog in gp120 may destabilize the network of the immune system.[39] According to this concept, T cells positively selected in the thymus on the basis of the weak complementarity of their receptors to MHC class II molecules would be the main targets of an autoimmune response. Indeed, selective increases in expression of TCR V_β sequences,[40] common in autoimmune conditions,[41,42] have been described in HIV-1-seropositive individuals and there may be structural homology between the carboxy terminus of gp120 and MHC class II.[43] However, it is not clear how the gp120–CD4 interaction could enhance the presentation of small MHC-homologous regions in gp120 in a way that breaks self-tolerance, particularly if activation of responding cells is supposed to be blocked by gp120 binding. The same question arises if HIV encodes a superantigen, as suggested on the basis of the observation that selective elimination of T cells expressing a defined set of V_β sequences occurs in persons infected with HIV.[44] In this case HIV may suppress immune function by continually activating immune cells, thus making them less responsive to immune signals encountered during normal immune responses. Although direct lymphocyte activation by HIV-1 and its envelope glycoprotein has been described,[45] it has not been confirmed[14,46] and we have also not observed direct mitogenic effects of the HIV preparations tested.

From the results presented here, we suggest a model in which HIV particles presented on the surface of APCs interact with the TCR and CD4 on T cells, inducing CD3–CD4 cross-linking and subsequent T cell activation. HIV in this model possibly functions both as a polyclonal activator by mimicking alloantigens or superantigens and, additionally, may provide a costimulatory signal. However, the main consequence of the costimulatory activity of HIV on the autoimmune model is that it is not necessary to presume that gp120 behaves as an image of MHC class II. In our view, HIV may serve as an enhancer of weak cell-to-cell interactions between CD4$^+$ T lymphocytes and APCs, thereby augmenting antigenic responses that otherwise would not occur. When cellular elements of this system are infected with HIV, high-affinity costimulatory interactions between CD4 and gp120 may alone disrupt the equilibrium in the immune network.

Other evidence for immune dysregulation in AIDS includes elevated levels of IL-2 receptors in the serum of patients infected with HIV.[19] The data presented in this article (Fig. 6) show that gp120-mediated enhancement of proliferation is accompanied by an increase in IL-2 production. This is consistent with an autocrine mechanism of IL-2 receptor expression and release by cells costimulated with HIV.

HIV-mediated costimulatory mechanisms may also affect the outcome of negative selection. In contrast to the positive selection of thymocytes with low-affinity receptors to MHC, immature T lymphocytes that strongly interact with antigens of the thymic environment are deleted by negative selection.[47] T cell precursors at many stages of T cell ontogeny can be infected with HIV-1[48] and susceptibility of cells of the intrathymic microenvironment to HIV infection has also been shown.[49] We

assume that the presence of HIV in the thymus may strengthen the interaction between APCs and those T lymphocytes that otherwise have low-affinity receptors to MHC, thereby resulting in extensive thymic T cell depletion and reduced peripheral T cell pools. Moreover, because the costimulatory process results in enhanced activation of T lymphocytes, costimulation could facilitate initial integration of the virus into the host cell DNA and potentiate subsequent HIV-1 gene expression.[20] Thus, the costimulatory properties of HIV through a combination of enhancement of autoreactivity, thymic deletion, and T cell activation may contribute to loss of $CD4^+$ T cells in AIDS.

Decline of $CD4^+$ T cells may also result from direct inhibitory effects of HIV and gp120. There is evidence that gp120 is shed from the surface of HIV-infected cells *in vitro*, and gp120 has been detected in the serum of patients with AIDS.[50] Our data confirm previous observations,[12,14,15] that both intact virus and gp120 inhibit CD3 MAb-induced T cell proliferation, when presented in solution (Table 4). Thus, binding of HIV or gp120 to CD4 under appropriate conditions may prevent the signaling function of CD3–CD4–$p56^{lck}$ complexes in $CD4^+$ cells by a mechanism similar to the inhibition by bivalent CD4 antibodies, resulting in inhibition of T cell activation,[8] T cell anergy, and apoptosis.[12]

In summary, in this article we present novel observations showing potent costimulatory activity of HIV and envelope glycoproteins. On the basis of these results, we suggest that HIV infection results in the appearance of both viral inhibitory and costimulatory effects, which together disrupt the normal balanced function of the immune system, leading to AIDS.

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