Efficient CD4 binding and immunosuppressive properties of the 13B8.2 monoclonal antibody are displayed by its CDR-H1-derived peptide CB1[1]

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Abstract A systematic exploration of the $V_H2/V_\kappa12–13$ variable domains of the anti-CD4 monoclonal antibody (mAb) 13B8.2 was performed by the Spot method to screen for paratope-derived peptides (PDPs) demonstrating CD4 binding ability. Nine peptides, named CB1 to CB9, were identified, synthesized in a cyclic and soluble form and tested for binding to recombinant soluble CD4. Among them, CB1, CB2 and CB8 showed high anti-CD4 activity. Competition studies for CD4 binding indicated that PDPs CB1, CB8, and the parental mAb 13B8.2 recognized the same complementarity determining region (CDR)3-*like* loop region. PDP CB1 was shown to mimic the biological properties of 13B8.2 mAb in two independent cellular assays, demonstrating inhibitory activities in the micromolar range on antigen presentation and human immunodeficiency virus promoter activation. Our results indicate that the bioactive CDR-H1 PDP CB1 has retained a significant part of the parental 13B8.2 mAb properties and might be a lead for the design of anti-CD4 peptidomimetics of clinical interest.

Key words: CD4; Complementarity determining region 3-*like* loop; Antibody; Paratope-derived peptide; Human immunodeficiency virus; T cell activation

1. Introduction

The CD4 molecule is a transmembrane glycoprotein of 58 kDa mainly expressed on the surface of mature T cells [1,2]. CD4 is composed of four extracellular domains (D1–D4), which share homology with the immunoglobulin V_κ region [3,4], a transmembrane portion and a cytoplasmic tail non-covalently associated with the protein tyrosine kinase $p56^{lck}$ [5]. The CD4 molecule acts as co-receptor for the major histocompatibility complex (MHC) class II and is a molecular partner for the T cell receptor (TcR) [6–9]. This trimolecular complex is critical for optimal activation of T cells [10–12]. Besides this physiological function, CD4 serves as a receptor for envelope glycoprotein of the human immunodeficiency virus (HIV), contributing to virus entry into cells [13].

Interactions with both MHC class II and gp120 involve residues of the complementarity determining region (CDR)2-*like* loop in D1 of CD4 [14–18]. On the opposite side of the D1 domain of CD4, the CDR3-*like* loop displays biological activities by acting as a target for molecules that inhibit immune response and HIV replication [19–22]. This latter role in a cascade of postbinding events has been demonstrated both by CDR3-*like* peptide analogs [23–27] and by anti-CDR3-*like* monoclonal antibodies (mAbs) such as 13B8.2 mAb [28–30]. The biological properties of 13B8.2 mAb have lead to its inclusion in phase I/II trials of HIV-infected patients [31–33].

Clinical applications of full-length murine mAbs may be limited by their high immunogenicity, their inability to cross the blood/brain barrier, and their limited ability to penetrate cells and tissues [34]. To overcome such problems, we have developed the concept of paratope-derived peptides (PDPs) which correspond to short amino acid sequences derived from antibody variable regions and which display antigen binding and biological activities [35–38]. These small molecules are screened from a systematic exploration of antibody variable domain sequences by the Spot method [39,40]. Given the pharmaceutical interest of 13B8.2 mAb, it appeared to us attractive to design such anti-CD4 PDPs.

To this end, we have identified nine PDPs from the 13B8.2 variable regions by using the Spot method. All the selected PDPs, prepared in a soluble cyclic form, were able to bind histidine-tagged recombinant CD4 (His_6-sCD4) expressed in baculovirus. MAb 13B8.2 specifically displaced the binding of His_6-sCD4 to PDPs CB1 and CB8, indicating that anti-CD4 PDPs recognize an epitope on the CD4 molecule closely related or similar to that identified for the 13B8.2 parental mAb. PDP CB1 displayed biological properties very similar to those of the parental 13B8.2 mAb, inhibiting in vitro antigen presentation and HIV-1 promoter activation. Taken together, our results indicate that the bioactive PDP CB1, derived from the CDR-H1 region of the anti-CD4 13B8.2 mAb, could be a valuable tool for the design of anti-CD4 peptidomimetics.

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[1] V_H and V_L sequences from 13B8.2 mAb have been submitted to the EMBL database under the accession numbers AJ279001 and AJ27900, respectively.

Abbreviations: mAb, monoclonal antibody; CDR, complementarity determining region; PDP, paratope-derived peptide; V_H, variable region of the heavy chain; V_κ, variable region of the κ light chain

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2. Materials and methods

2.1. Reagents, cell lines and vectors

Recombinant human soluble CD4 (rhCD4) was obtained from Repligen (Needham, MA, USA). rhCD4 was biotinylated using a commercial reagent (Amersham Pharmacia Biotech, Cleveland, OH, USA) according to the manufacturer's instructions and stored in PBS at −20°C until use. 13B8.2 mAb [19,31] was obtained from Immunotech-Coulter (Marseille, France). The murine hybridoma cell line that produces 13B8.2 mAb (IgG1/κ [19]) was kindly provided by Dr. D. Olive and Dr. C. Mawas (INSERM U119, Marseille, France). The pMV7-T4 plasmid, encoding the full-length CD4-cDNA sequence [41,42], was a kind gift from Dr. Q.J. Sattentau (Centre d'Immunologie de Marseille-Luminy, Marseille, France). The human lymphoblastoid B cell line EBV-Lu, expressing the HLA DR5,6, DRB52, DQ6,7, and A2 molecules, and the murine T cell pdb10F, expressing human CD4 and pep24 (PAGFAILKCNNKTFNY)-specific chimeric TcR, have been previously characterized [43,44]. The HeLa P4 HIV-1 LTR β-galactosidase indicator cell line [45] was provided by Dr. O. Schwartz (Institut Pasteur, Paris, France).

2.2. Cloning and sequencing of 13B8.2 mAb V_H and V_L genes

All the general procedures concerning the cloning and sequencing of 13B8.2 mAb variable regions have been described [46].

2.3. Peptide synthesis on cellulose membranes

202 overlapping dodecapeptides frameshifted by one residue representing the V_H and V_L sequences of 13B8.2 mAb on a cellulose membrane were synthesized according to previously described protocols [37].

2.4. Assay for sCD4 interaction with cellulose-bound peptides

The saturated membrane was incubated with a 20 nM solution of biotinylated-rhCD4 for 2 h at 37°C. Bound biotinylated-rhCD4 was detected by incubating the membrane for 1 h at 37°C with a 1:3000 solution of alkaline phosphatase-conjugated streptavidin (Sigma, St. Louis, MO, USA) and subsequent addition of 5-bromo-4-chloro-3-indolyl phosphate substrate. Inhibition of biotinylated-rhCD4 binding was performed as described above, except that biotinylated-rhCD4 (20 nM) was pre-incubated for 18 h at 4°C with 13B8.2 mAb (6.25 μM). In all cases, the reactivity of the spots was evaluated by scanning the membrane and measuring the intensities of the spots with the NIH image 1.61 software [39].

2.5. Synthesis of soluble peptides

The nine selected PDPs, named CB1 to CB9 (see Fig. 2, right panel for sequences), a scrambled form of PDP CM9 (ScCM9: GSDQWNKMQYYP) [35], a scrambled form of the CD4-derived CDR3-*like* peptide (ScCDR3-*like*: KEEICEVEDQTY), and an unrelated peptide (Dig97c: FGDYYCLQYASS, derived from the CDR-L3 region of the anti-digoxin 1C10 mAb), with Lys–Cys residues added to both carboxyl- and amino-termini of all peptides, were synthesized by Fmoc solid-phase synthesis on an AMS422 robot (Abimed, Langelfeld, Germany), cyclized and purified as described previously [35]. Lys and Cys residues were added, respectively, to improve the solubility and to permit the cyclization of the peptides. All peptides showed homogeneity in high performance liquid chromatography at the expected monomeric molecular weight. Thereafter, all peptides were resuspended in deionized water, except for the scCDR3-*like* peptide which was suspended in a 10% acetonitrile solution and the CB8 PDP in a 20% acetonitrile solution.

2.6. Baculovirus expression of recombinant His_6-sCD4

The nucleotide sequence of soluble CD4 (D1–D4) was sorted by polymerase chain reaction from the pMV7-T4 plasmid by using the sense primer SCD4FB (5′-GAAGATCTATGAACCGGGGAGTCC), which matches codons 1 to 6, and the anti-sense primer SCD4RTB (5′-GAAGATCTTCAATGGTGATGGTGGTGGTGACCTAATGCGGCCATTGGCTGCACCGGG), which contains the reverse complement of codons 367 to 372 of CD4 and encodes the His_6-tag and a *Bgl*II restriction site. Following sub-cloning into the pGEM-T vector (Promega, Madison, WI, USA), the sCD4 sequence was verified by using the dideoxy termination method with the T7 sequencing kit (Pharmacia, Uppsala, Sweden). The *Bgl*II-linearized His_6-sCD4 fragment was cloned into the p119L baculovirus transfer vector to allow the expression of His_6-sCD4 under the P10 promoter. After transfection of *Spodoptera frugiperda* Sf9 cells (ATCC CRL 1711), recombinant baculoviruses were further purified by using a plaque assay and propagated in Sf9 cells [47,48]. Supernatant of Sf9 cells infected with the His_6-sCD4 recombinant baculovirus in a spinner culture (10^6 cells/ml) were harvested 6 days post-infection and clarified by centrifugation at $1000\times g$ for 5 min.

2.7. Purification and characterization of His_6-sCD4

Purification of the His_6-sCD4 product was carried out by using Ni-NTA agarose beads (Qiagen, Chatsworth, CA, USA) according to the manufacturer's procedure with minor modifications. Briefly, the clarified baculovirus supernatant was dialyzed against washing buffer (50 mM NaH_2PO_4, 500 mM NaCl, 5 mM imidazole) for 24 h at 4°C. Ni-NTA agarose beads were then added to a final concentration of 5%, and the binding of His_6-sCD4 was performed for 18 h at 4°C. Beads were washed with 8 volumes of washing buffer and His_6-sCD4 was eluted as 1 ml fractions with 3 volumes of elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 300 mM imidazole). The Ni-NTA agarose beads were regenerated with 1 M imidazole. All purification fractions were checked for the presence of His_6-sCD4 by enzyme-linked immunosorbent assay (ELISA) and Western blot using the anti-CD4 13B8.2 mAb as detection reagent.

2.8. Binding studies of PDPs to CD4

Three replicates corresponding to 10-fold serial dilutions of the nine cyclic PDPs (CB1–CB9) were coated overnight at 4°C onto 96-well ELISA plates (Nunc, Paisley, UK) with an initial peptide concentration of 50 μM. Four washes in 160 mM PBS, pH 7.2, containing 0.1% Tween 20 (PBS-T) were performed before and after saturating the wells with 1% non-fat dry milk in PBS-T for 1 h at 37°C. Thereafter, 100 μl of 20 nM His_6-sCD4 was added to each well. Following a 2 h incubation and four washes in PBS-T, bound His_6-sCD4 was detected by addition of 100 μl of a 1:2000 solution of peroxidase-conjugated anti-His_6 mAb (Sigma) and subsequent addition of peroxidase substrate. Absorbance was measured at 490 nm.

2.9. Binding specificity of PDPs CB1 and CB8 to CD4

Inhibition of His_6-sCD4 binding to PDPs was performed by an ELISA method with PDPs CB1 and CB8 coated at 12.5 and 2.5 μM, respectively, as capture reagent. A 20 nM solution of His_6-sCD4 showing an absorbance at 490 nm of 1.0 was co-incubated with two-fold serial dilutions of 13B8.2 mAb. Three replicates were tested for each dilution with an initial mAb concentration of 3 μM. His_6-sCD4 binding was evaluated as described above.

2.10. Interleukin-2 (IL-2) secretion assay following antigen presentation

EBV-Lu antigen-presenting cells (10^6 cells/ml), overnight pulsed with the pep24 stimulator peptide (75 μM) from HIV gp120 [44], were co-cultured with pdb10f responder cells (4×10^5 cells/ml) in the presence or absence of inhibitor PDPs or mAbs for 24 h. Thereafter, 100 μl of supernatant was harvested and tested for IL-2 secretion using an ELISA commercial kit (Pharmingen, San Diego, CA, USA). A positive control for IL-2 secretion was performed as described above except that activation of pdb10F cells was done using a murine anti-CD3 antibody at a concentration of 0.6 nM (Pharmingen) [49].

2.11. HIV-1 promoter activation assay

HeLa P4 indicator cells (8×10^4 cells/ml) were cultured in medium supplemented or not with 1000 $TCID_{50}$ of infectious HIV-1_{Lai} in the presence or absence of peptides or mAb for 3 days, harvested and lysed. The β-galactosidase activities were then determined as previously described [45], by measuring the absorbance at 410 nm.

3. Results

3.1. Characterization of nine peptides from 13B8.2 mAb demonstrating CD4 binding ability

The nucleotide sequences of the V_H and V_L domains of 13B8.2 mAb were established according to the general procedure described by Chardès et al. [46] and made available from

VH domain

	1	10	20	30
VH 13B8.2	QVQLKQSGPG		LVQPSQSLSI	TCTVSGFSLT
Ox2 germline				

	CDR-H1	40	50	*CDR-H2*	60
VH 13B8.2	**TFGVH**WVRQS		PGKGLEWLG**V**	**IWRSGITDYN**	
Ox2 germline	**SY**			**SG S**	

	70	80	87
VH 13B8.2	**VPFMS**RLSIT	KDNSKSQVFF	KLNSLQPDDT
Ox2 germline	**AA I** S		M AN

	97	*CDR-H3*	106	113
VH 13B8.2	AIYYCAK**NDP**	**GTGFAY**WGQG		TLVTVSA
Ox2 germline	R			

VL domain

	1	10	20	*CDR-L1*	30
VL 13B8.2	DIQMTQSPAS		LSASVGETVT	FTC**RASENIY**	
k2 germline				**G H**	

	40	50	*CDR-L2*	60
VL 13B8.2	**SYLA**WYQQKQ	GKSPQLLVH**D**	**AKTLAE**GVPS	
k2 germline	**N**	Y**N**	**D**	

	70	80	90
VL 13B8.2	RFSGGGSGTQ	FSLKINTLQP	EDFGTYYC**QH**
k2 germline	S	Y S	S

	CDR-L3	100	107
VL 13B8.2	**HYGNPPT**FGG	GTKLEIK	
k2 germline	**FW T Y**		

Fig. 1. Amino acid sequences of V_H and V_L domains of the anti-CD4 mAb 13B8.2. The sequences were deduced from nucleotide sequences of both regions (submitted to the EMBL database under the accession numbers AJ279001 and AJ279000, respectively). Somatic mutations between 13B8.2 V_H versus Ox2 germline V_H and 13B8.2 V_L versus k2 germline V_L, respectively, are indicated. Numbering of the amino acids is that of Kabat et al. [73]. Boldfaced residues belong to the CDRs.

the EMBL database under the accession numbers AJ279001 and AJ27900, respectively. The complete amino acid sequences of both chains are given in Fig. 1 with somatic mutations indicated. Genetic analysis of these sequences showed that the V_H region of 13B8.2 mAb resulted from the rearrangement of V_H2-DQ52-J_H3 genes and that the V_L region resulted from a $V_\kappa12/13$-$J_\kappa2$ gene rearrangement. More precisely, computer-assisted comparison of these sequences showed that the V_H and V_L genes of 13B8.2 displayed significant homologies with Ox2 [50] and k2 [51] germline genes, respectively (Fig. 1). It is worth noting that no significant homology was found between 13B8.2 mAb variable sequences and other anti-CD4 variable domains.

202 overlapping dodecapeptides frameshifted by one residue, corresponding to the deduced amino acid sequence of V_H and V_L from 13B8.2 mAb, were synthesized on a cellulose membrane by using the Spot method. The anti-CD4 immunoreactivity of these peptides was assessed by incubating the membrane with biotinylated-rhCD4. The results are quantitatively expressed in Fig. 2 (left panel) in which the reactivity of peptides that comprise at least one residue from the CDRs are boxed. Anti-CD4 reactivity was observed for peptides including amino acids from five of the six CDRs of 13B8.2 mAb (peptides 20, 22, 28–35; 46, 48–52, and 93–97 for CDR-H1, CDR-H2, and CDR-H3, respectively, and 22, 23, 29–34; 83–89, and 91 for CDR-L1 and CDR-L3, respectively). Anti-CD4 activity was also obtained for peptides containing residues from the framework, mainly flanking the CDRs (peptides 18, 36–38 and 66–71 for V_H, and 35–38 and 57–61 for V_L) but the majority of peptides comprising only framework residues did not display any significant binding activity. This reactivity was drastically decreased when biotinylated-rhCD4 was pre-incubated with an excess of 13B8.2 parental mAb (data not shown). Nine peptides (29, 30, 48, 52, 90 and 94 for V_H and 22, 29 and 86 for V_L), named CB1 to CB9 (Fig. 2, right panel), were selected for further study in a soluble form. These peptides, except for 22 and 29 from CDR-L1, showed the highest anti-CD4 activity and comprised at least 50% of residues belonging to CDRs. Since the most reactive peptides derived from the CDR-L1 showed less than 50% of residues from the CDR (peptides 31 and 34), we selected two adjacent reactive peptides, namely, 22 and 29. Except for PDP CB4, that exclusively comprised amino acids from the CDR-H2, all selected PDPs comprised amino acids from both CDR and framework sequences.

3.2. Soluble cyclized selected PDPs demonstrate CD4 binding activity

Peptides selected according to the Spot results were synthesized and N- to C-terminus cyclized through cysteine oxidation. Cyclization has already been demonstrated as a useful tool to improve antigen binding [36]. The ability of recombinant His_6-sCD4 expressed in baculovirus to specifically bind cyclic PDPs was assessed by ELISA (Fig. 3). All the selected PDPs, except CB3 from the CDR-H2, showed a dose-dependent CD4 binding activity. Three of them, PDPs CB1 and CB2 derived from the 13B8.2 CDR-H1 region, and PDP CB8 derived from the 13B8.2 CDR-L1 region, displayed high binding activity in a 0.5–50 μM concentration range. The non-reactivity of irrelevant ScCDR3-*like* and ScCM9 peptides indicated that the addition of lysine and cysteine residues for solubilization and cyclization of peptides had no effect on CD4 binding. Since PDPs CB1 and CB2 only differ by one amino acid residue, we focused our attention on PDP CB1, derived from the CDR-H1, and PDP CB8, derived from the CDR-L1 of 13B8.2 mAb for further specificity studies.

3.3. PDPs CB1 and CB8 specifically bind to CD4 on the same region as that of parental 13B8.2 mAb

The ability of the parental mAb 13B8.2 to displace the binding of PDPs CB1 and CB8 to His_6-sCD4 was studied by using an ELISA inhibition assay. The absorbance of residual His_6-sCD4 binding to PDPs was measured at 490 nm and expressed as percent inhibition of the binding (Fig. 4). We found that 13B8.2 mAb was able to displace the binding of His_6-sCD4 (20 nM), in a dose-dependent manner, to both coated PDPs CB1 (12.5 μM) and CB8 (2.5 μM) with similar efficiencies. A 50% inhibition of binding of PDPs CB1 and CB8 to CD4 was obtained for 13B8.2 mAb concentrations of 20 and 8 nM, respectively. No inhibition was found when using the IgG1 isotype-unrelated anti-digoxin mAb 1C10, demonstrating the specificity of the competition studies.

Similar results were obtained in a symmetric experiment in which the binding of His_6-sCD4 (20 nM) to 13B8.2 mAb (0.3 nM) was inhibited by various concentrations of PDPs CB1

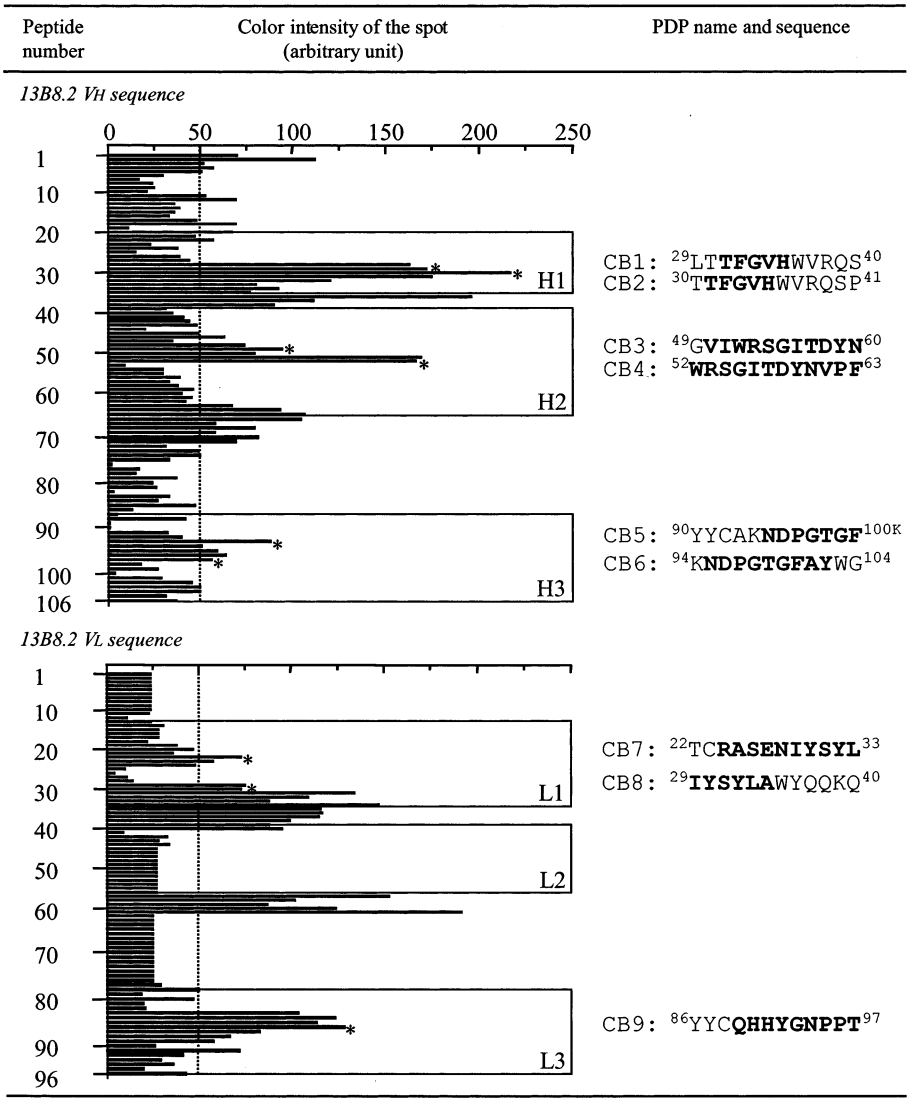

Fig. 2. Quantitative analysis of the binding of biotinylated-rhCD4 to overlapping dodecapeptides derived from the variable sequences of anti-CD4 13B8.2 mAb. On the left, the reactivity of peptides derived from the V_H sequence (numbered 1 to 106) and the V_L sequence (numbered 1 to 96) was evaluated after incubation of the membrane with 20 nM biotinylated-rhCD4 (cutoff taken at 50 arbitrary units, dotted line). Peptides comprising at least one residue from the CDRs sequences are boxed (H1, H2 and H3 and L1, L2 and L3 correspond to CDR1, CDR2, and CDR3 of the heavy and light chains, respectively). On the right, sequences of the nine PDPs selected for further functional characterization, indicated by asterisks, are shown. Numbering of the amino acids is that of Kabat et al. [73]. Boldfaced residues belong to the CDRs.

and CB8 (data not shown). A 50% inhibition of binding of His_6-sCD4 to 13B8.2 mAb were obtained for concentrations of PDPs CB1 and CB8 up to 75 and 125 μM, respectively. Taken together, these data are consistent with the hypothesis that 13B8.2 mAb, CB1, and CB8 peptides recognized the same antigenic region on the CD4 molecule.

3.4. PDP CB1 is able to inhibit IL-2 secretion following antigen presentation

The stimulation of pdb10f responder T cells by pep24-pulsed EBV-Lu APC leads to the lymphocyte secretion of IL2 [43,44]. This T cell activation model is specific since no IL-2 secretion occurs when EBV-Lu antigen-presenting cells are pulsed with a non-stimulator pep23 antigen. We checked the viability of the pdb10f responder cell line by activating cells with a murine anti-CD3 mAb which induced IL-2 secretion (data not shown). As shown in Table 1, the irrelevant anti-digoxin mAb 1C10 showed no inhibitory activity of IL2 secretion in contrast to the anti-CD4 13B8.2 mAb which blocked the IL2 production (99.6 ± 0.2% inhibition). As compared to the irrelevant ScCM9 peptide showing no inhibition, PDP CB1 displayed inhibitory activity of IL2 secretion in a 125–250 μM concentration range. The biological activity of PDP CB2 was found to be very moderate since no activity was found at a concentration lower than 250 μM.

The lack of activity for PDP CB8 was found to be consecutive to cell death, probably due to the presence of 20% acetonitrile in the buffer used to solubilize the peptide. The other selected PDPs demonstrated no or extremely low blocking of IL2 secretion. Taken together, these results indicate that, as already demonstrated for other anti-CD4 mAbs [52,53], the CDR-H1-derived PDP CB1 is able to inhibit the antigen-presenting function, a biological property also demonstrated for the 13B8.2 parental mAb.

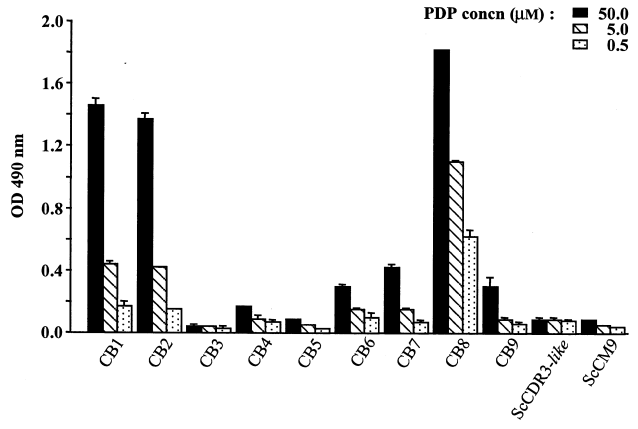

Fig. 3. ELISA binding assay of His_6-sCD4 (20 nM) onto adsorbed cyclic peptides derived from the sequence of 13B8.2. Each value represents the mean ± S.E.M. of triplicate determinations. Irrelevant peptides (ScCDR3-*like* and ScCM9) were used as negative controls.

3.5. PDP CB1 displays a strong capacity to inhibit HIV-1_{Lai} LTR-driven β-galactosidase reporter gene expression

The parental mAb 13B8.2 has been previously demonstrated to be an inhibitor of viral particle production by cells infected with HIV-1_{Lai}, HIV-1_{Eli}, HIV-1_{Sf2}, HIV-1_{Ger} and HIV-2_{Rod} strains [13,54]. In addition, viremia negativation has been observed for HIV-infected patients treated with 13B8.2 antibody, demonstrating its efficiency towards primary clinical isolates [31,32]. In order to assess the ability of selected 13B8.2 PDPs to inhibit HIV-1 promoter activity, we measured the β-galactosidase reporter gene expression after infection of the indicator cell line HeLa P4 cultured for 3 days in the presence of peptides. As shown in Fig. 5A, the 1C10 mAb did not display any inhibitory activity in contrast to the parental 13B8.2 mAb which inhibited HIV promoter activation. Culturing infected HeLa P4 cells with irrelevant Dig97c or ScCM9 peptides did not affect β-galactosidase expression, whereas a significant inhibition was found when the cells were cultured with a 50 μM solution of 13B8.2 PDPs CB1, CB2 and CB5.

The lack of activity for PDP CB8 was found, in this assay, also to be consecutive to cell death caused by the presence of 20% acetonitrile in the buffer used to solubilize the peptide since no inhibition was observed with the PDP CB1 diluted in the same 20% acetonitrile buffer (data not shown). In Fig. 5B, we demonstrate that the inhibitory effect of CB1 is dose-dependent, with an IC_{50} for CB1 of about 15 μM, whereas the IC_{50} of the parental mAb was found to be 5 nM. These results indicate that the CDR-H1-derived dodecapeptide CB1 has anti-viral activity, as already demonstrated for the parental anti-CD4 13B8.2 mAb.

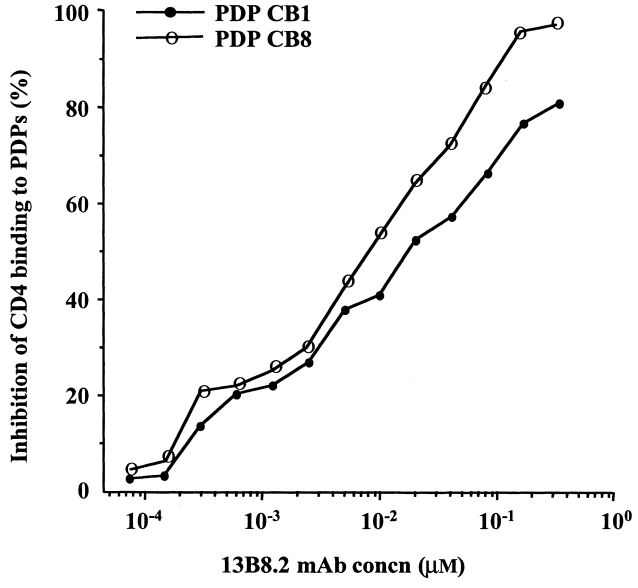

Fig. 4. Epitope specificity of the binding of PDPs CB1 and CB8 to sCD4. An ELISA was performed by coating CB1 (12.5 μM) (●) and CB8 (2.5 μM) (○) onto plates. Binding of His_6-sCD4 (20 nM) to PDPs was then inhibited by various dilutions of 13B8.2 mAb. Percent inhibition of the binding was calculated with reference to the His_6-sCD4 binding to PDPs in the absence of 13B8.2 mAb. Inhibition values were calculated from the mean 490 nm absorbances of triplicate determinations. Mean absorbance at 490 nm ranged from 0.20 (background level) to 1.25 (in absence of inhibitor).

4. Discussion

The CD4 molecule plays a key role both in the MHC class II-restricted immune response and the human immunodeficiency virus infection process by acting as a receptor either for the TcR–antigen engagement complex or for the envelope glycoprotein gp120 of HIV [7,9,55,56]. In these two cases, CD4 has been demonstrated to induce signal transduction leading to T cell activation [12,19,21,22]. Both of these mechanisms can be inhibited by treatment with anti-CD4 mAbs including murine 13B8.2 mAb [28–30]. Such inhibitory properties have led to the inclusion of 13B8.2 mAb in phase I/II trials in HIV-infected patients [31–33]. To avoid problems encountered when using mAbs in therapeutic approaches, such as immunogenicity and low tissue diffusion, we designed and synthesized PDPs from the 13B8.2 anti-CD4 mAb.

Table 1
Inhibition of IL-2 secretion by pdb10f cells, sensitized with pep24-stimulated EBV-Lu presenting cells, and cocultured with cyclic PDPs

Inhibitor	Concentration (μM)	% Inhibition of IL-2 secretion[a]
13B8.02mAb	0.5	99.6 ± 0.2[b]
1C10mAb[c]	0.5	00.0
CB1	250	58.3 ± 1.9[b]
	125	20.0
	62.5	00.0
CB2	250	67.5 ± 12.0[b]
	125	00.0
	62.5	00.0
CB3	250	07.0
CB4	250	04.0
CB5	250	00.0
CB6	250	09.0
CB7	250	00.0
CB8	250	*NM*[d]
CB9	250	00.0
ScCM9[c]	250	00.0

[a]Calculated with respect to IL-2 secretion value in absence of inhibitor, taken as reference. Mean 490 nm absorbance values ranged from 0.05 for unactivated pdb10f cells to 1.40 for activated pdb10f cells in absence of inhibitors.
[b]Mean ± S.E.M. of three independent experiments.
[c]Negative controls.
[d]Not measurable.

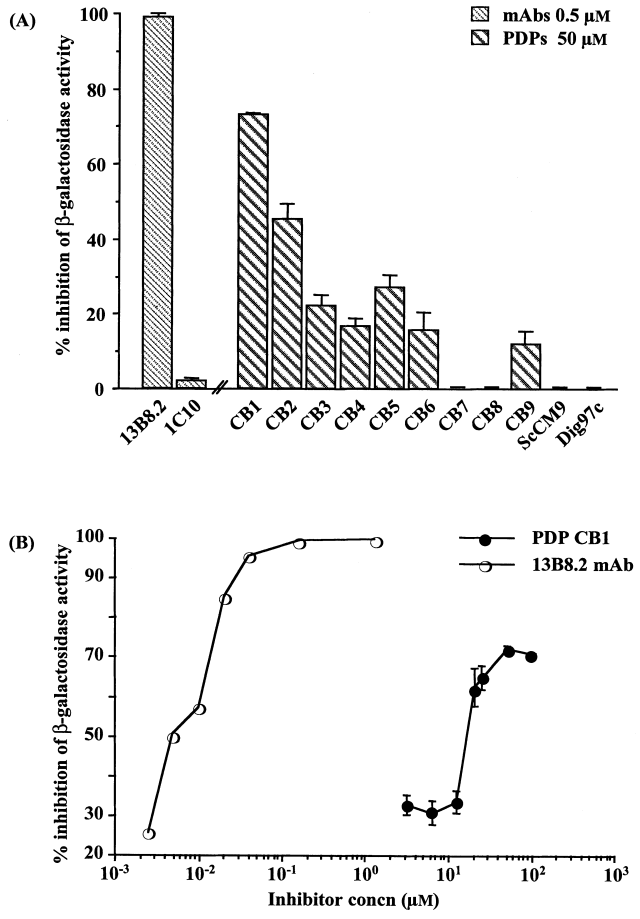

Fig. 5. Inhibition of LTR-driven β-galactosidase gene expression induced by HIV-1_{Lai} following incubation with cyclic PDPs. A: Percent inhibition of β-galactosidase expression in HIV-1_{Lai}-infected HeLa P4 cells cultured in the presence of 13B8.2 mAb and 1C10 mAb, an irrelevant anti-digoxin mAb, at 0.5 μM, and cyclic PDPs at 50 μM. ScCM9 or Dig97c peptides were used as negative controls. B: Dose–response curves for HIV-1_{Lai}-infected HeLa P4 cells cultured in the presence of various concentrations of 13B8.2 mAb (○) and PDP CB1 (●). In all cases, each value is the mean ± S.E.M. of at least three independent experiments. Mean absorbance at 410 nm ranged from 0.014 for uninfected HeLa P4 cells to 0.658 for HIV-1_{Lai}-infected cells.

In this study, we demonstrated that the CDR-H1-derived PDP CB1 displays significant and specific biological properties mimicking those of the parental anti-CD4 13B8.2 mAb. Firstly, in an in vitro model of MHC class II-restricted immune response, we demonstrated that anti-CD4 PDP CB1, as well as parental mAb 13B8.2, inhibits IL2 secretion by activated T cells following antigen presentation; this inhibitory effect, classically observed for anti-CD4 mAbs [52,53], was dose-dependent. Since, anti-CD4 mAbs have been described to prevent T cells from IL2-induced proliferation and B cell adhesion through inhibition of Ca^{2+} and $p21^{ras}$ signaling pathways [57,58], it remains to be assessed whether our anti-CD4 PDPs could interfere with such mechanisms. The effect of CB1 in an in vivo model of immune disorder remains to be investigated, as it was done for anti-CD4 CDR3-*like*-derived analogs in a murine experimental allergic encephalomyelitis model [59]. Secondly, we found that the PDP CB1 inhibited HIV-1 promoter activation, as 13B8.2 mAb does [29]. The mechanism by which 13B8.2 mAb exerts this anti-HIV property has been related to inhibition of signal transduction, involving the extracellular regulated kinase/mitogen-activated protein kinase kinase signaling pathway [13]. We are currently investigating whether CB1 also acts by disrupting the same signal transduction machinery, thereby preventing HIV provirus transcription.

We found that peptides CB1 and CB8 displayed specific anti-CD4 binding activity. We recall, however, that a relatively high concentration of peptides was needed in our experiments to demonstrate efficient biological activity with regard to the efficient antibody dose. To explain this difference, we measured the binding of CD4 to PDP CB1 and observe a 50% decrease in binding when following pre-incubation of the peptide for 25 min at 37°C in a buffer containing 10% fetal calf serum (data not shown). This finding strongly suggests that the requirement for high concentrations of CB1 to observe a biological effect reflects degradation of the peptide in the culture medium. The use of D-amino acids for peptide synthesis could be of interest as a means of improving their metabolic stability [60].

Concerning PDP CB8, the biological properties of this peptide remain to be investigated in buffer solutions compatible with the cellular assays. A longer sequence of PDP CB8 including hydrophilic flanking residues is under analysis to improve the solubility of the peptide and to avoid the use of organic solvent. It would be of great interest to investigate this point because of a high sequence/position homology between this CDR-L1-derived PDP CB8 and a previously characterized CDR-L1-derived PDP, the CM9 peptide, derived from the anti-CD4 ST40 mAb that inhibits HIV transcription [35]. Comparison of alanine-scanning analysis of these two peptides indicated that similar residues located at the same positions, i.e. Tyr^{32}, Trp^{35}, Tyr^{36}, Lys^{39}, contribute to CD4 binding ([36] and unpublished data). This may help us understand the structure–function relationship in these series of anti-CD4 mAb-derived bioactive molecules.

The systematic exploration of the 13B8.2 mAb paratope has led us to the characterization of PDPs CB1 and CB8, displaying high anti-CD4 reactivity and including residues from the CDRs and from the framework flanking these CDRs. The role of residues outside the CDRs (i.e. Trp^{36} and Arg^{38} in the CB1 sequence, and Tyr^{36} in the CB8 sequence) has already been described as being important in structuring the active CDR loops in the full mAb paratopes [61,62]. Moreover, Park et al. [63] demonstrated the relevant importance of adding aromatic residues to improve the efficiency of their CDR-H3-derived anti-HER2/neu peptide mimetics. The addition of aromatic residues has been found to be a valuable strategy for enhancing stability, folding, and avidity of peptidomimetics [25,64–66]. This could explain the high reactivity obtained for PDP CB1, in which the natural Trp^{36} residue may have such function. We demonstrated differences, both in anti-CD4 binding activity and in biological properties, between PDPs CB1 and CB2, that are frameshifted by only one residue. Two key points may explain these differences. Firstly, PDP CB2 contains the Pro^{41} residue which may constrain the peptide in an unfavorable conformation. Secondly, in PDP CB2 the Leu^{29} residue is absent; the Leu^{29} residue has been demonstrated to be part of the Vernier zone [67], already described to stabilize Ag/Ab interactions. These two factors may contribute to twisting PDP CB2 into a less favorable conformation for CD4 binding, thereby explaining the decreased bio-

logical properties of CB2. Taken together, these data confirm the capacity of the Spot method in defining antigen-specific peptides derived from a mAb paratope that present paratope-derived residues in an environment compatible for antigen binding.

The CDR3-*like* loop of domain 1 of CD4, and more precisely the negatively charged residues Glu^{91} and Glu^{92}, has been shown to play a role in activating T cell signal transduction [20,21]; Glu^{91} and Glu^{92} are also involved in the 13B8.2 paratope [16]. Inhibition studies demonstrated that PDP CB1 and 13B8.2 mAb specifically compete for CD4 binding on the same region of the molecule. Alanine scanning of the CB1 sequence showed that the main contributor residues are positively charged (i.e. His^{35} and Arg^{38}, unpublished data), thereby possibly interacting with its negatively charged epitope on the CD4 molecule. The CDR3-*like* loop from the D1 domain of CD4 has been reported to be involved in one of the two potential CD4 dimerization sites [20,21,68,69]. The dimerization/oligomerization processes have been shown to be necessary for optimal activation of $CD4^+$ T lymphocytes [16,26,70–72]. It is therefore possible that the inhibitory effects of PDP CB1 on T cell stimulation and HIV-1 promoter activation could result from a CD4 dimerization/oligomerization disruption, thereby uncoupling the CD4 molecule from the signal transduction machinery. Even if this molecular mechanism remains to be established, our approach has led to the characterization of the anti-CD4 PDP CB1. This kind of small bioactive molecule could be a lead for the development of a second generation of more potent and stable molecules of pharmaceutical interest.

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