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DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to CD8⁺ T cells in a spectrum of human MHC I haplotypes

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Optimal HIV vaccines should elicit CD8⁺ T cells specific for HIV proteins presented on MHC class I products, because these T cells contribute to host resistance to viruses. We had previously found that the targeting of antigen to dendritic cells (DCs) in mice efficiently induces CD8⁺ T cell responses. To extend this finding to humans, we introduced the HIV p24 gag protein into a mAb that targets DEC-205/CD205, an endocytic receptor of DCs. We then assessed cross-presentation, which is the processing of nonreplicating internalized antigen onto MHC class I for recognition by CD8⁺ T cells. Low doses of α DEC-gag, but not control Ig-gag, stimulated proliferation and IFN- γ production by CD8⁺ T cells isolated from the blood of HIV-infected donors. α CD205 fusion mAb was more effective for cross-presentation than α CD209/DC-SIGN, another abundant DC uptake receptor. Presentation was diverse, because we identified eight different gag peptides that were recognized via DEC-205 in 11 individuals studied consecutively. Our results, based on humans with highly polymorphic MHC products, reveal that DCs and DEC-205 can cross-present several different peptides from a single protein. Because of the consistency in eliciting CD8⁺ T cell responses, these data support the testing of α DEC-205 fusion mAb as a protein-based vaccine.

CD205, CD209 | cross-presentation | DC-SIGN | vaccine

Resistance to HIV is in part mediated by CD8⁺ T cells (1, 2), which recognize fragments of viral antigens presented on MHC class I products (3). HIV-specific, CD8⁺ T cells kill virus-infected targets in culture (4, 5) and produce antiviral chemokines (6, 7). CD8⁺ T cells also resist immunodeficiency viruses *in vivo*. In SIV-infected rhesus macaques, depletion of CD8⁺ T cells increases plasma viremia (8), including viremia due to attenuated vaccines (9). HIV mutants that escape recognition by CD8⁺ T cells *in vivo* also become more pathogenic (10–12). Therefore, effective protection against HIV will likely require vaccines that elicit strong and broad CD8⁺ T cell immunity.

Dendritic cells (DCs) are specialized antigen-presenting cells that capture infectious agents and tumors and initiate CD8⁺ T cell immunity (13, 14). DCs express a number of cytokines and membrane costimulators that drive the T cell response, and DCs “cross-present” antigens on MHC class I (15, 16). The cell biology underlying cross-presentation is not yet fully defined (17–19), but it allows DCs to extract peptides from nonreplicating internalized antigens for presentation to CD8⁺ T cells. Such peptides do not need to be synthesized in the DCs, but instead “cross” to their MHC I products from another source, e.g., from select proteins (20–22), tumor cells (23–25), inactivated virus or dying infected cells (26–28), immune complexes (29–31), and self-tissues (32). In contrast, the classical pathway for presentation on MHC I is to generate peptides from proteins produced during infection by replicating viruses (33, 34). The newly synthesized proteins, probably made as defective ribosomal

initiation products (35), are degraded in the proteasome before transport into the rough endoplasmic reticulum, where there is binding of peptides to newly synthesized MHC I. In mice, DCs are the major cell type capable of cross-presentation *in vivo* (36–40). However, it has yet to be shown that DCs can cross-present peptides across a spectrum of MHC haplotypes, an essential requirement for protein-based vaccines in humans who are highly polymorphic at the MHC or HLA locus.

A recent strategy to explore and harness DC biology for vaccination is to target antigens to DCs in intact lymphoid organs by incorporating specific antigens into anti-DC mAbs (41). Among other advantages, the targeting of antigens in this way enhances the efficiency of antigen presentation to CD4⁺ and CD8⁺ T cells *in vivo* by 100-fold or more (41–44).

To extend these ideas to humans, we have selected a mAb to human DEC-205/CD205 (45). In mice, DEC-205 mediates cross-presentation (42–44). The receptor is also expressed on human monocyte-derived DCs along with other endocytic receptors (reviewed in ref. 46), such as the mannose receptor/CD206 and DC-SIGN/CD209 (47, 48). A potential advantage of CD205 over these other receptors is its high expression by DCs in the T cell areas of lymph nodes in the steady state, whereas CD206 and CD209 are abundant in macrophages in the medullary region of lymph nodes (49). This finding means that α CD205 mAb might provide superior targeting of vaccine antigens to DCs in lymphoid tissues, where the DCs are ideally positioned to select specific T cell clones from the repertoire. We now find that a fusion α CD205 mAb targets HIV gag for broad and efficient cross-presentation in HIV-infected individuals. The data provide a rationale for further testing of this vaccine approach in humans.

Results

Characterization of HIV gag Fusion mAbs. To deliver HIV antigens to human DCs, we cloned HIV gag p24 protein in frame into the carboxyl terminus of the heavy chain of mAbs to DEC-205, DC-SIGN and MMR, which are endocytic receptors expressed on monocyte-derived DCs; the heavy chain of an isotype-matched control Ig was also engineered as a negative control

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The authors declare no conflict of interest.

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; PBMCs, peripheral blood mononuclear cells.

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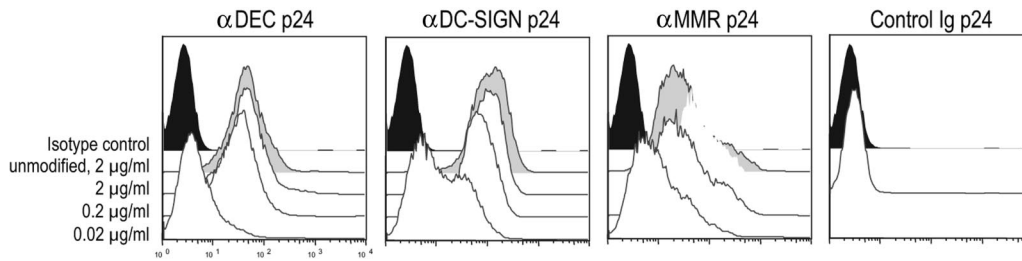


Fig. 1. Binding of HIV gag p24 fusion mAbs to monocyte-derived DCs. Immature DCs were treated with 0.02, 0.2, and 2 $\mu\text{g/ml}$ of $\alpha\text{DEC p24}$, $\alpha\text{DC-SIGN p24}$, and $\alpha\text{MMR p24}$ and with 2 $\mu\text{g/ml}$ control Ig p24. DCs were also treated with supernatant from each mAb clone as positive control and with nonreactive mAb as negative control, followed by incubation with an $\alpha\text{-mIgG}$ phycoerythrin-conjugated antibody.

[supporting information (SI) Fig. 5A]. The fusion mAbs were produced by transient transfection in 293 T cells ($\approx 1\text{--}2\text{ mg/liter}$) and purified from culture supernatants by protein G-affinity chromatography. Purity was assessed after SDS/PAGE under reducing conditions, by both Coomassie staining and Western blot with a HRP-conjugated $\alpha\text{-mouse IgG}$. The fusion mAbs were composed of a 75-kD heavy chain, as opposed to $\approx 50\text{-kD}$ heavy chain of an unconjugated mouse IgG2b, the same isotype as the $\alpha\text{DEC-205}$ and $\alpha\text{DC-SIGN}$ mAbs (SI Fig. 5B). We also verified the functional integrity of the mAbs by binding to immature DCs, similar to that of the original unmodified mAbs (Fig. 1).

Cross-Presentation of gag p24 Protein by $\alpha\text{DEC-205}$ Fusion mAb. To study the ability of $\alpha\text{DEC p24}$ to mediate antigen presentation, we added fusion mAb to blood cells from HIV-infected individuals and measured proliferation and $\text{IFN}\gamma$ production by bulk peripheral blood mononuclear cells (PBMCs) as well as cocultures of monocyte-derived DCs and T cells. We examined cells from treated chronically infected individuals as well as untreated long-term nonprogressors. All were clinically stable and had CD4^+ T cell counts of >400 per microliter. The T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) to follow their proliferation by successive halving of the amount of CFSE per cell with each division. Although we did not detect responses by CD4^+ cells to gag antigen, the CD8^+ T cells proliferated actively after stimulation with a pool of 55 peptides spanning the HIV gag sequence and to a low dose of $\alpha\text{DEC p24}$

but not control Ig p24 (SI Fig. 6). The proliferating CD8^+ T cells produced $\text{IFN}\gamma$ if the cultures were rechallenged with p24 peptides for 6 h at the end of the 6- to 7-d expansion culture (SI Fig. 6 and Fig. 2). Results from five individuals are shown in Fig. 2 and indicate that the frequency of $\text{IFN}\gamma^+ \text{CD8}^+ \text{CFSE}^{\text{low}}$ T cells was greater after addition of the pool of gag peptides relative to $\alpha\text{DEC p24}$. However, $\alpha\text{DEC p24}$ was reliably a more efficient form of antigen than control Ig p24, which was comparable to the no-antigen or medium control. In each case, it was necessary to restimulate the proliferated T cells with gag peptides to detect their production of $\text{IFN}\gamma$ (compare filled vs. open symbols in Fig. 2). Higher frequencies of responding T cells were noted in the DC-T cell cocultures than in bulk PBMCs (Fig. 2), which may reflect either the greater numbers or improved maturation of monocyte-derived DCs. Two seronegative donors did not show responses to $\alpha\text{DEC p24}$ in these assays (data not shown). Together, these results demonstrate that low concentrations of $\alpha\text{DEC p24}$ fusion mAb lead to antigen presentation on MHC I, inducing consistent expansion of CD8^+ T cells capable of producing $\text{IFN}\gamma$ from HIV-infected donors.

$\alpha\text{DEC-205}$ Fusion mAb is Superior to $\alpha\text{DC-SIGN}$ mAb for CD8^+ T Cell Responses. To assess the consequences of targeting different receptors, we compared αDEC , αMMR , and $\alpha\text{DC-SIGN p24}$ fusion mAbs. We found that $\alpha\text{DEC p24}$ was reliably more effective than the other fusion mAbs in inducing proliferation and $\text{IFN}\gamma$ production from $\text{CD3}^+ \text{CD8}^+$ cells in PBMCs (Fig. 3 and SI Fig. 7A). We made similar findings in a limited study of

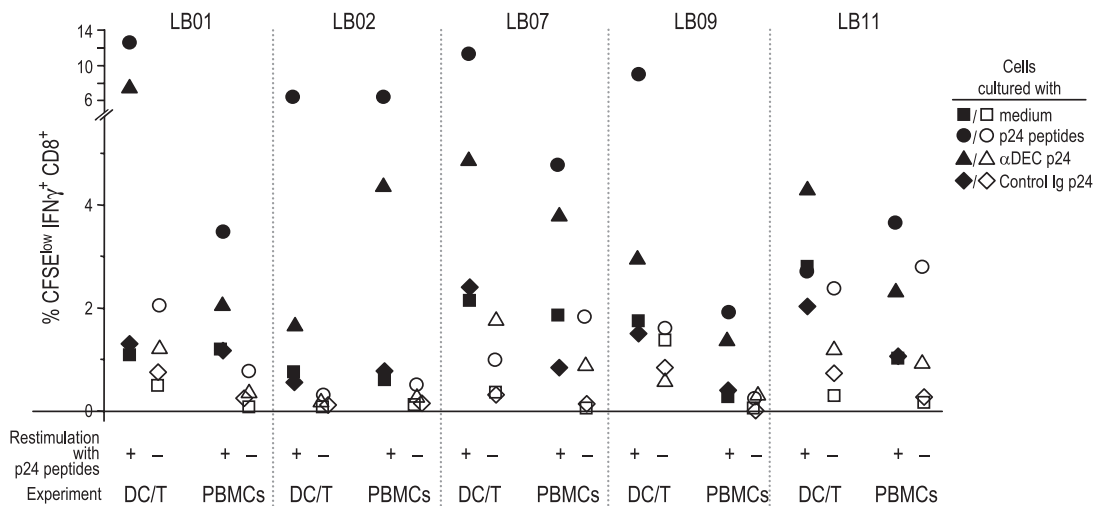


Fig. 2. HIV gag p24-specific CD8^+ T cell responses to αDECp24 . Summary of the frequencies of $\text{IFN}\gamma$ -producing, proliferating, $\text{CD3}^+ \text{CD8}^+$ T cells in response to medium, p24 peptides, $\alpha\text{DEC p24}$, and control Ig p24 (1 $\mu\text{g/ml}$), with or without restimulation at the end of the 6- to 7-day culture with p24 peptides. Frequencies are shown for both PBMCs and cocultures of antigen-pulsed DCs and CFSE-labeled T cells.

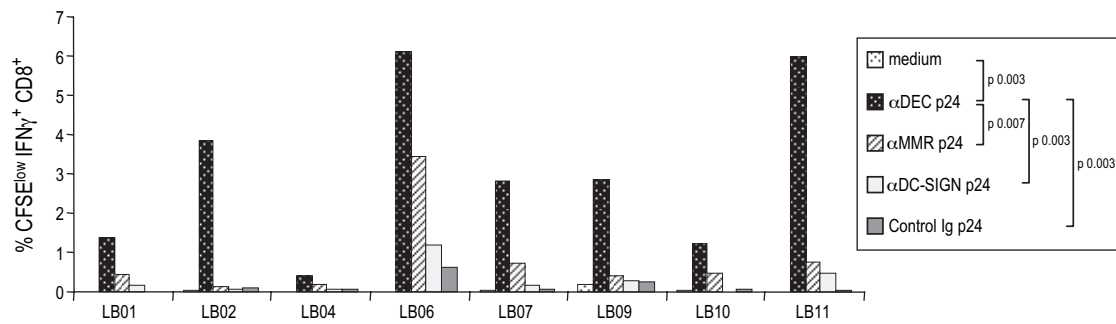


Fig. 3. Comparison of CD8⁺ T cell response after targeting of gag through different endocytic receptors. Frequency of IFN γ ⁺ proliferating CD3⁺ CD8⁺ T cells measured in CFSE-labeled PBMCs stimulated for 6 days with medium, p24 peptides at 2 μ g/ml and α DEC p24, α DC-SIGN p24, α MMR p24, and control Ig p24, all at 1 μ g/ml. All samples were restimulated for the last 6 h with p24 peptides.

DC-T cell cocultures from four individuals (e.g., SI Fig. 7B). These findings suggest that antigen delivery via DEC-205 is superior to the other endocytic receptors for expanding gag-specific CD8⁺ T cells.

Broad CD8⁺ T Cell Responses After DEC-205 Targeting. To document which gag peptides were presented by DEC-205 targeting, we studied 11 consecutive patients. All of them showed CD8⁺ T cell proliferation to the pool of HIV gag peptides and to the fusion α DEC p24 fusion mAb. Following expansion of the CFSE-labeled T cells in response to α DEC p24, we rechallenged the cultures for 8 h with five pools of 15-mer overlapping peptides that spanned the gag p24 sequence. In each donor, 1 or 2 pools of peptides were recognized, but when different donors were compared, all five pools could be recognized by one individual or another (Table 1). By further breaking down the reactive peptide pools, we found eight different “mimotope” peptides, i.e., the 15-mer peptides that mimic the actual peptide naturally processed by the DCs. Fig. 4 illustrates and Table 2 summarizes the identification of diverse peptides that could be presented from gag p24 by DEC-205 on DCs. The upper rows in Fig. 4A–C

show the identification of the active peptide pool, and the lower rows show the identification of the best peptide mimotope in the pool. After HLA typing and consultation with the Los Alamos database on known HIV gag peptides that are presented on specific MHC I products, we were able to identify the likely peptide sequences that were being presented after uptake, processing, and cross-presentation of α DEC p24. The data in Tables 1 and 2 indicate that the targeting of gag within α DEC-205 mAb allows DCs from highly polymorphic human MHC I products to cross-present at least 1 or 2 different peptides from this small protein.

Discussion

Improved presentation of antigens by DCs offers the potential of increased vaccine efficacy. DCs are potent inducers of T cell-mediated immunity and memory and have the potential to cross-present antigens from safe forms of vaccines to generate protective CD8⁺ T cells. Nevertheless, the prior literature on cross-presentation has emphasized the study of single peptides presented on single MHC I proteins. In mice, research is dominated by the presentation of one peptide from ovalbumin

Table 1. IFN γ ⁺ CFSE^{low} CD8⁺ T cell frequencies after stimulation with gag peptides

Patient	6-d culture in medium	6-d culture in peptides	6-d culture in α hDEC-p24					
		6-h restim in p24 peptides	6-h restim in peptides	6-h restim in pool I	6-h restim in pool II	6-h restim in pool III	6-h restim in pool IV	6-h restim in pool V
LTNP								
LB01 PBMC	0.02	3.1	1.4	0.25	0.34	1	0.38	0.29
LB01 DC/T	0.12	12	6.3	0.97	0.92	6.1	ND	ND
LB02 PBMC	0.04	6.3	3.8	3.2	0.07	0.14	0.64	0.11
LB03 DC/T	0.04	17	2.6	0.54	0	2.0	0.88	0.03
LB04 PBMC	0.02	16	2.1	1.5	0.13	0.27	0.36	0.37
LB04 DC/T	1.52	34.7	43.3	33.7	1.51	5.87	1.58	4.02
LB05 DC/T	0.55	37.6	26.4	1.27	0.45	16.3	19.1	0.44
LB06 PBMC	0	9.73	6.09	1.8	1.38	4.94	1.8	1.58
Chronic								
LB07exp1 PBMC	0.02	4.6	2.8	0.28	0.83	1.2	0.54	3.7
LB07 DC/T	0.23	11	3.4	1.2	1.5	3.9	1.4	4.4
LB07exp2 PBMC	0.17	3.9	1.7	0.25	0.14	0.61	0.36	1.3
LB08 DC/T	0.18	4.1	1.5	ND	ND	0.21	0.46	1.7
LB09 PBMC	0.19	4.9	2.8	0.47	1.4	0.39	1.4	0.45
LB10 PBMC	0.022	1.28	1.22	0.09	1.42	0.09	0.09	0.05
LB10 DC/T	1.04	13.6	1.63	0.41	1.32	0.3	0.28	0.21
LB11 PBMC	0.006	10.6	5.96	0.39	5.22	0.55	1.06	0.57

Summary of the frequencies of IFN γ ⁺ CFSE^{low} CD8⁺ cells obtained from either PBMCs or DC/T cell cocultures stimulated for 6–7 days with medium, p24 peptides, and α DEC p24. The samples were then restimulated for 6–8 h with p24 peptides or with the individual peptide pools (see *Materials and Methods*). The bold data represent pools of peptides that yield responses that are three times more than the background of nonstimulated cells. LTNP, long-term nonprogressor.

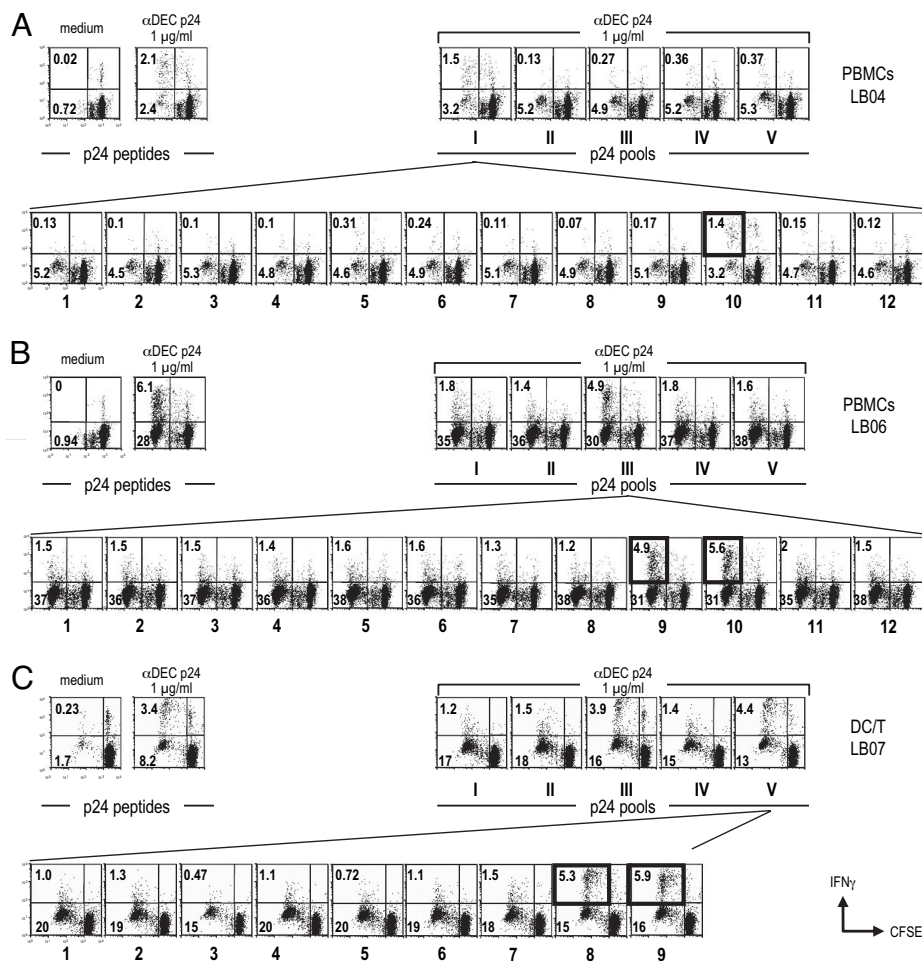


Fig. 4. Identification of mimetope peptides recognized by CD8⁺ T cells in response to α -DECp24. Results from patients LB04, LB06, and LB07 are shown in A–C, respectively. CFSE-labeled cells were stimulated with medium or α -DEC p24 at 1 μ g/ml for 6–7 d, whereupon the cells were restimulated with five different pools of gag peptides to detect IFN γ secretion from proliferated CFSE^{low}, CD3⁺CD8⁺ T cells (Upper). The next day, parallel cultures were used to identify the individual mimetope peptides from the reactive peptide pools (Lower).

on H-2K^b, in part because the binding of this peptide to MHC class I is of such high affinity that it becomes easier to detect cross-presentation. In humans, select peptides have been defined that can be presented on HLA-A2.1, the MHC I molecule that has dominated the literature.

Using the DEC-205/CD205 receptor to deliver antigens to monocyte-derived DCs *in vitro*, we established that numerous peptides from the HIV protein, gag p24, can be processed and presented by DCs on many allelic forms of human MHC I. Our data indicate that DEC-205 accesses the cross-presenting pathway for many human HLA haplotypes.

Interestingly, antibodies to other receptors expressed on the same DCs, DC-SIGN/CD209 or mannose receptor/CD206, were less effective than α CD205. All three mAbs bound comparably to monocyte-derived DCs and were internalized by DCs (data not shown), so we suspect that the DEC-205 receptor better allows access to the cross-presentation pathway. This pathway typically requires that portions of the gag protein gain access to the cytoplasm, followed by proteasome-mediated degradation and transport into the rough endoplasmic reticulum (reviewed in refs. 18, 50, and 51). Another advantage of CD205 targeting is that it is expressed on many DCs in the T cell areas of human lymphoid tissues, whereas CD206 and CD209 are abundant on macrophages in the lymph node medulla (49). This positioning would allow the CD205-targeted vaccine to efficiently select

specific clones of T cells that recirculate from the blood through lymphoid organs.

Although HIV-infected individuals exhibit CD4⁺ T cell responses that were too weak for us to study here, other research in mice shows that DEC-205 targeting is a powerful means for inducing CD4⁺ T cell immunity (43, 44) as well as antibody responses (43). Together, the results with α DEC-205 targeting provide preclinical support to develop this strategy as a means to induce strong T cell immunity in humans.

Materials and Methods

Patients. Peripheral blood (40–100 ml) was obtained from HIV-1 infected individuals who were recruited to the St. Vincent’s Hospital Comprehensive Clinic and The Rockefeller University Hospital according to institutional guidelines and after obtaining informed consent. Patient information is summarized in **SI Table 3**. Blood samples from two healthy seronegative subjects were analyzed as negative controls.

Preparation of Dendritic Cells and T Cells. PBMCs were isolated from heparinized blood on Ficoll density gradients. Monocytes were enriched by using CD14 microbeads (Miltenyi Biotec, Auburn, CA) and then cultured for 5 d with IL-4 (10 ng/ml) and GM-CSF (100 units/ml) in RPMI 5% human serum to generate immature DCs (52). The CD14[−] fraction was used as source of

Table 2. Identification of gag mimotope peptides presented via α hDECp24

Patient	Reactive p24 peptides pool	Active p24 peptide in pool		HLA class I					
		No.	Sequence	A1	A2	B1	B2	C1	C2
LB02	I	10	FVEK AFSPEVIPMFS AL	2010	3201	4001	5701	0304	0602
LB03	III	5	AGTT STLQEQIGWMT	0202	3601	5301	5701	0401	0401
		6	STLQEQIGWMT TNNPP						
LB04	I	10	EK AFSPEVIPMFS AL	0301	1101	3501	5701	0401	0401
LB05	III	5	AGTT STLQEQIGWMT	3101	3303	5801	7801	0701	1601
		6	STLQEQIGWMT TNNPP						
	IV	7	FRDYVDRFYKTLRAE						
LB06	III	8	VDRFYKTLRAEQASQ						
		9	NPPIPV GEIYKRWII	2301	7401	0801	1801	0304	0202
		10	PV GEIYKRWII LGLN						
LB07	III	11	IYKRWW IILGLNKIVR	0301	<u>1101</u>	0702	0733	0702	0704
		8	EMMTACQ QGVGGPGHK						
	V	9	ACQ QGVGGPGHK ARVL						
LB10	II	10	AAEWDRL HPVHAGPI	2402	6601	1503	3501	0401	0401
		11	DRL HPVHAGPI APGQ						
LB11	II	9	INEEAA AEWDRLHPVH	3201	3201	1402	4002	0802	0202
		10	AAEWDRL HPVHAGPI						

As in Table 1 and Fig. 4, gag peptides were identified that were recognized by CD8⁺ T cells after proliferation in response to α DEC p24. We first identified a pool of gag peptides (column 2), and then the peptide pool was broken down into individual peptides to identify the optimal mimotope (columns 3 and 4). After typing for HLA class I alleles at HLA-A, B, and C loci for each patient, we were able to identify from the Los Alamos database (www.hiv.lanl.gov/content/index) known peptide sequences that are presented on a corresponding HLA product in bold and, in one case (patient LB07), a second peptide (underlined).

bulk T cells and cryopreserved in freezing medium (GIBCO) before use.

Cloning and Production of Fusion HIV gag mAbs. mAbs for the human receptors DEC-205, DC-SIGN, and MMR as well as a control mAb to mouse I-Ak were cloned from total RNA from the MG38.2 (45), 25B9G8 (49), 3.29 (47) (kindly provided by A. Lanzavecchia, Bellinzona, Switzerland), and 10–2.16 (TIB93; American Type Culture Collection, Manassas, VA) hybridomas. The variable regions were produced with 5'-RACE PCR kit (GIBCO-BRL, Carlsbad, CA) by using primers for the 3' ends of mouse IgG2b (DEC-205 and DC-SIGN mAbs) or mouse IgG1 (MMR), and Ig λ (DEC-205) or Ig κ (DC-SIGN, MMR). To obtain full-length heavy and light chain Ig cDNA, the V regions were cloned in-frame with a signal peptide and the respective mouse Ig heavy and light constant domains (41 7753). DNA coding for the BH10 clade B HIV-1 gag p24 (NIH AIDS Reference Reagent), amino acid 133–363 was cloned in-frame into the carboxyl terminus of the heavy chains. Fusion HIV gag p24 mAbs were produced by transient transfection (calcium-phosphate) in 293 T cells in serum-free DMEM supplemented with Nutridoma SP (Roche, Indianapolis, IN), purified on protein G columns (GE Healthcare), and characterized by SDS/PAGE and Western blot analysis (41). The integrity of the mAbs was further characterized on immature DCs by FACS using a phycoerythrin-conjugated goat α -mouse IgG (Jackson ImmunoResearch, West Grove, PA).

HIV gag Peptides. A library of overlapping (staggered by 4 aa) 15-mer peptides was obtained from the NIH AIDS Reference Reagent Program. This library contained 55 peptides, covering the entire gag p24 region (amino acids 133–363), that were pooled and resuspended at 1 mg/ml of each peptide in 100% DMSO. The library was also divided into five pools of 9–12 single peptides, spanning amino acids 133–183 (pool I), amino acids 173–231 (pool II), amino acids 221–279 (pool III), amino acids 269–327 (pool IV), and amino acids 317–363 (pool V) of gag p24.

Expansion of Antigen-Specific T Cells in PBMCs. PBMCs were labeled with 1 μ M CFSE (Molecular Probes, Eugene, OR), and 10⁶ cells were plated in 96-well deep-well plates in 500 μ l of RPMI 5% HS. The cells were left unstimulated (negative control) or stimulated with SEB (Sigma, St. Louis, MO) at 20 ng/ml (positive control), HIV gag peptides at 2 μ g/ml, and with α DEC p24, α DC-SIGN p24, α MMR p24, and control Ig p24 at 0.1 to 10 μ g/ml. After 6–7 d of culture, samples were restimulated for 8 h with or without p24 peptides (2 μ g/ml), either all 55 peptides or individual pools of peptides, in the presence of 0.5 μ g/ml of α CD28 and α CD49d (clones L293 and L25; BD Biosciences), adding BFA (Sigma) at 10 μ g/ml for the last 6 h to block cytokine secretion. The cells were fixed, permeabilized and stained with a combination of fluorochrome-conjugated antibodies: perCP-CD3, APC-CD8, and PE-IFN γ or its respective isotype control. Cells were analyzed on a FACS-Calibur II using CELLQuest software, collecting 50,000–100,000 high-CD3⁺ events. Most of the data were displayed as two-color dot plots (FL1 vs. FL2) to measure CFSE dilution and IFN γ production in CD3⁺ CD8⁺ cells.

Expansion of Antigen-Specific T Cells in DC/T Cell Coculture. To directly assess the function of DCs in presenting the gag fusion mAbs, we used, in parallel to the PBMCs assay above, monocyte-derived DCs. Immature cells at day 5 of monocyte culture in GM-CSF and IL-4 were collected and pulsed overnight with medium, p24 peptides (2 μ g/ml), or α DEC p24, α DC-SIGN p24, α MMR p24, and control Ig p24 at 1 μ g/ml. The antigen-pulsed DCs were matured by adding γ -irradiated CD40L-expressing cells (kindly provided by J. Banchereau, Dallas, TX) at a ratio of 1:5 (CD40L:DC) for 48 h. CD40 ligation enhances cross-presentation by DCs (53). Syngeneic CD14⁺ cells were thawed, rested for 2 h at 37°C before CFSE labeling and plating in 48-well plates at 1.5 to 2 \times 10⁶ per ml and cultured with the antigen-pulsed mature DCs at a DC/T ratio 1:30. The cocultures were incubated at 37°C for 6–7 d. Each sample was then restimulated for the last 8 h with or without p24 peptides (2 μ g/ml) in the presence of costimulator mAbs (0.5 μ g/ml). Cells were analyzed for CFSE dilution and IFN γ secretion as above. Where necessary, data comparing the frequency of CFSE^{low} proliferating,

IFN γ secreting, CD3⁺ CD8⁺ T cells were compared by using a paired two-tail *t* test.

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