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Hierarchy of CD4 T Cell Epitopes of the ANRS Lipo5 Synthetic Vaccine Relies on the Frequencies of Pre-Existing Peptide-Specific T Cells in Healthy Donors

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The Agence National de Recherche sur le SIDA et les hepatitis Lipo5 vaccine is composed by five long fragments of HIV proteins and was recently shown to induce in seronegative volunteers a CD4 T cell response largely dominated by the G2 fragment. To understand this response profile, we submitted the five HIV fragments to HLA-DR–binding assays and evaluated the frequency of naive Lipo5-specific CD4 T lymphocytes in the blood of 22 healthy individuals. We enumerated the Lipo5-specific T cell lines induced in vitro by weekly rounds of specific stimulation. Four peptides and hence not only G2 exhibited a broad specificity for HLA-DR molecules. In contrast, most of the T cell lines specific for Lipo5 reacted with G2, revealing a G2-specific T cell repertoire superior to 2 cells per million, whereas it is close to 0.4 for the other peptides. We also found good cross-reactivity of all the peptides with clade B and C variants and that G2 and P1 are able to recruit T cells that recognize HIV-infected cells. We therefore mainly observed very good concordance between the frequency to individual Lipo5 peptides among vaccinees in a large-scale vaccine trial and the distribution of peptide specificity of the in vitro induced T cell lines. These findings underline the role of the size of the epitope-specific naive repertoire in shaping the CD4 T cell response after vaccination and highlight the value of evaluating the naive repertoire to predict vaccine immunogenicity. ***The Journal of Immunology*****, 2013, 190: 5757–5763.**

Despite more than 25 years of intensive research on HIV vaccines, the ideal viral components to be included in a vaccine are still unknown. To date, no vaccine candidates have been able to reproduce the spontaneous immunity developed by elite controllers. Only a combination of vaccines provides limited protection (1). Although neutralizing Abs protect HIV-infected macaques (2), immunogens designed to elicit the production of neutralizing Abs afford no protection (3, 4). Other vaccines do not induce appropriate cellular responses specific for HIV (5), although a large body of evidence suggests that HIV-specific cellular responses contribute to control HIV viremia (6–8). In elite controllers, both CD4 and CD8 T lymphocytes react very efficiently with HIV Ags and exhibit a polyfunctional profile (9–13). Association of resistance to HIV infection with HLA class I (11, 14, 15) and class II (13, 16, 17) molecules also confirms the key role of the cellular response in immunity to HIV. Accordingly, HLA class I and class II alleles associated with slower HIV-1 progression contribute to a strong HIV-1–specific CD8 and CD4 T cell response, respectively (13, 14, 18). The protective effect of HLA-B57 and HLA-B27 is mainly supported by a reduced number of epitopes, but these epitopes generate a response of large amplitude that dominates the HIV-specific CTL response (14, 18). The CD4 T cell response to HIV is mainly dominated by T lymphocytes specific for the Gag protein (6, 19–21). CD4 T lymphocytes target the Gag protein more than the Env protein in HIV controllers in comparison with progressors (22). The reason for this immunodominance of Gag is unclear and may be due to its expression during the HIV cycle or to intrinsic immunological properties of Gag T cell epitopes, such as affinity for HLA class II molecules, size of the specific naive T cell repertoire, or successful Ag processing (23, 24). Large differences therefore exist between HIV-specific CD8 and CD4 T cell epitopes in their capacity to generate strong responses, but the origin of these differences remains unclear, although they impact on immunity to HIV.

The Agence National de Recherche sur le SIDA et les hepatitis (ANRS) Lipo5 vaccine contains five HIV-1 long fragments deriving from Gag (G1 and G2) Pol (P1) and Nef (N1 and N2) proteins. They have been selected on the basis of their content of CD8 T cell epitopes and their conservation among HIV clades. All fragments are linked with a palmitoyl-lysylamide moiety to facilitate peptide entry into dendritic cells and their presentation to T cells. Lipopeptide vaccines were found to elicit in humans not only CTL, but also CD4 responses in seronegative (25–27) and HIV-infected volunteers (28–30). In a large vaccine study including 131 healthy volunteers, the CD4 T cell response to the Lipo5 vaccine was mainly raised against the peptide G2 (27). The question arose as to why this particular Gag peptide was strongly T cell stimulating in vaccinated individuals and in particular why it dominated the T cell response specific for the other Lipo5 peptides. Moreover, we also wished to evaluate whether Lipo5-specific CD4 T cells cross-react with clade B and C sequences

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Abbreviations used in this article: ANRS, Agence National de Recherche sur le SIDA et les hepatitis; VSV, vesicular stomatitis virus G protein.

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and recognize HIV-infected cells. We therefore performed a comprehensive analysis of the CD4 T cell response to Lipo5 vaccine by deriving Lipo5-specific T cell lines in multiple healthy donors. Our results mainly showed that immunodominance of G2 was not related to a better HLA-binding capacity, but to a higher frequency of $CD4^+$ naive T cell precursors in healthy donors.

Materials and Methods

Peptides

Lipo5 peptide Lai sequences were provided by Activotec (Cambridge, U.K.) or Intavis (Koeln, Germany). Nef 66-97 (N1), VGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGL; Nef 116-145 (N2), HTQGYFPDWQNYTPGPGVRYPLTFGWCYKL; Gag 17-35 (G1), EKIRLRPGGKKKYKLKHIV; Gag 253-284 (G2), NPPIPVGEIYKRWIILGLNKIVRMYSPTSILD; Pol 325-355 (P1), AIFQSSMTKILEPFRKQNPDIVIYQYMDDLY. Lipo5 consensus sequences were provided by Intavis (Koeln, Germany). N1 Cons B, VGFPVRPQVPLRPMTYKGALDLSHFLKEKGGL; N1 Cons C, VGFPVRPQVPLRPMTYKAAFDLSFFLKEKGGL; N2 Cons B, HTQGYFPDWQNYTPGPGIRYPLTFGWCFKL; N2 Cons C, HTQGYFPDWQNYTPGPGVRYPLTFGWCFKL; G1 Cons C, EKIRLRPGGKKHYMLKHLV; G2 Cons C, NPPIPVGDIYKRWIILGLNKIVRMYSPVSILD; P1 Cons C, AIFQSSMTKILEPFRAQNPEIVIYQYMDDLY. The 15-mer peptides of G2 were synthesized using standard Fmoc chemistry on an Advanced ChemTech model 357 MPS synthesizer (Advanced ChemTech Europe, Brussels, Belgium), cleaved from the resin by 95% trifluoroacetic acid and purified by reverse-phase HPLC on a C_{18} Vydac column (Interchim, Montluçon, France). The sequence of each peptide was assessed by mass spectroscopy.

HLA II peptide-binding assays

HLA-DR molecules were purified from homozygous EBV cell lines by affinity chromatography using the monomorphic mAb L243 (31, 32). The binding to HLA-DR molecules was assessed by competitive ELISA, as previously reported (31, 32). The peptide concentration that prevented binding of 50% of the labeled peptide (IC_{50}) was evaluated. Data were expressed as relative affinity: ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide, which is a high binder to the HLA-DR molecule. Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment. Their sequences and mean IC_{50} values were the following: hemagglutinin 306-318 (PKYVKQNTLKLAT) for DRB1*0101 (5 nM), DRB1*0401 (39 nM), DRB1*1101 (21 nM), and DRB5*0101 (11 nM); YKL (AAYAAAKAAALAA) for DRB1*0701 (33 nM); A3 152-166 (EAEQLRAYLDGTGVE) for DRB1*1501 (33 nM); MT 2-16 (AKTIAYDEEARRGLE) for DRB1*0301 (180 nM); B1 21-36 (TERVRLVTRHIYNREE) for DRB1*1301 (600 nM); LOL 191-210 (ESWGAVWRIDTPDKLTGPFT) for DRB3*0101 (13 nM); and E2/E168 (AGDLLAIETDKATI) for DRB4*0101 (24 nM).

Blood samples and HLA-DR genotyping

Blood cells were collected at the Etablissement Français du Sang (Rungis, France) as buffy-coat platelet preparations from anonymous healthy donors after informed consent and following the Etablissement Français du Sang guidelines. PBMCs were isolated by density centrifugation on Ficoll-Hyperpaque gradients (Sigma-Aldrich, St. Quentin Fallavier, France), followed by three centrifugations at 150 × *g* to remove platelets. HLA-DR genotyping was performed by using the Gold SSP DRB1 typing kit (Invitrogen, Cergy, France).

Generation and specificity of Ag-specific T cell lines

Monocyte-derived dendritic cells (immature and mature) were generated from plastic-adherent monocytes by a 5-d culture in AIM-V medium supplemented with 1000 U/ml human rGM-CSF (Miltenyi Biotec, Paris, France) and 1000 U/ml human rIL-4 (R&D Systems). LPS (Sigma-Aldrich) (1 μg/ml) was used as maturation agent. $CD4^+$ T lymphocytes were isolated by positive selection using a $CD4^+$ T cell magnetic isolation kit, as recommended by the manufacturer (Miltenyi Biotec). Their purity was assessed by flow cytometry. They were diluted in IMDM (Invitrogen) supplemented with 0.24 mM glutamine, 0.55 mM asparagine, 1.5 mM arginine (all amino acids from Sigma-Aldrich), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen), and 10% human serum (hereafter referred to as complete IMDM). Mature dendritic cells (5×10^5) were incubated at 37°C, 5% CO_2, for 4 h in 1 ml complete IMDM containing a mixture of peptides, each peptide being at a concentration of 10 μg/ml. Pulsed cells were added at 10^4 per round-bottom microwell to 1×10^5 to 3×10^5 autologous $CD4^+$ lymphocytes in 200 μl complete IMDM with 1000 U/ml IL-6 and 10 ng/ml IL-12 (R&D Systems, Abingdon, U.K.). Twenty to 100 wells per donor were seeded with the coculture of dendritic cells and $CD4^+$ T lymphocytes. The $CD4^+$ T lymphocytes were restimulated on days 7, 14, and 21 with autologous dendritic cells freshly loaded with the HIV peptide mixture and were grown in complete IMDM supplemented with 10 U/ml IL-2 and 5 ng/ml IL-7 (R&D Systems). The stimulated $CD4^+$ T cells were investigated for their peptide specificity by IFN-γ ELISPOT assays at least 5 d after the last stimulation.

Virus production and cell infection

Virus stocks were prepared by transfection of 293T cells, as previously described (33). To allow efficient infection of target B-EBV cells, which express low or undetectable levels of CD4, viruses were pseudotyped with the vesicular stomatitis virus G protein (VSV). Homozygous Hom-2 (HLA-DR1) or HHKB (HLA-DR13) B-EBV cell lines were infected for 4 h with 300 ng/ml virus. Cells were cultured for 30–36 h before use as APCs in IFN-γ ELISPOT. Infection efficiency was assessed by measuring the p24 concentration in the supernatant using HIV-1 p24 mAb (183-H12-5C) catalog 3537 from the National Institutes of Health AIDS Reagent Program and by intracellular staining of HIV Gag using the anti-p24 clone KC-57 from Beckman Coulter.

IFN-γ ELISPOT

Multiscreen hemagglutinin plates (Millipore, St. Quentin en Yvelines, France) were coated overnight with 2.5 μg/ml mAb anti-human IFN-γ (1-D1K; Mabtech, Stockholm, Sweden) in PBS (Invitrogen) and saturated for at least 1 h at 37°C with complete IMDM. APCs were autologous PBMCs (5×10^4 /well), HLA-DR–transfected L cells (5×10^4/ well), or B-EBV–infected cells. Peptides were directly added to the Multiscreen plates at the indicated concentration. $CD4^+$ T cells were seeded at 5×10^3/well. After overnight incubation at 37°C, captured IFN-γ was detected by subsequent addition of biotinylated mAb anti-human IFN-γ (7-B6-1; Mabtech) (0.25 μg/ml), extravidin-phosphatase (Sigma-Aldrich), and NBT/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich). Spot number was automatically determined by the AID ELISPOT Reader System (AID, Strassberg, Germany). T cell lines were considered as peptide specific when their production of spots in the presence of Ags was at least two times higher than in their absence with a minimum of 20 spots. Positivity in a statistical unpaired Student *t* test ($p < 0.05$) was also evaluated. CD4 T cell precursor frequencies were analyzed statistically using the nonparametric Wilcoxon signed-rank test.

Results

HIV Lipo5 fragments exhibited a broad specificity for HLA-DR molecules

To characterize the $CD4^+$ T cell response to HIV Lipo5 lipopeptides, we first submitted the corresponding peptide fragments to HLA-DR–specific binding assays (Table I). HLA-DR molecules introduced in these assays are present at an allelic frequency of >5% in the European and North American populations and together are carried by >85% of these populations. The data were presented as relative affinities to compare easily their binding properties to high binder peptides, which we used as references. Lipo5 peptides bound between 2 and 8 HLA-DR molecules. The peptide P1 showed the best binding capacity with 8 of 10 bound HLA-DR molecules. G2 and N1 bound to 6 HLA-DR molecules, whereas N2 bound to 5 of 10 HLA-DR molecules and G1 to 2 of 10 HLA-DR molecules. Only two molecules (HLA-DR3 and HLA-DRB4) did not accommodate good binders from the Lipo5. Four of the Lipo5 fragments (N1, N2, G2, and P1) bound to at least half of the investigated HLA-DR molecules.

The G2 peptide is recognized by numerous pre-existing T cells in healthy donors

To quantify the Lipo5-specific repertoire of pre-existing CD4 T lymphocytes in normal donors, the five Lipo5 peptides were tested for their capacity to prime in vitro human $CD4^+$ T lymphocytes, harvested from 22 HIV-seronegative donors. These donors exhibited a diversity of HLA-DR molecules that included all the HLA-DR molecules introduced in the binding experiments. Purified $CD4^+$ T lymphocytes were seeded in 20–100 wells per donor and

Table I. Relative affinity of Lipo5 peptides for HLA-DR molecules

Peptides	Relative Affinity										Bound HLA-DR
	DR1	DR3	DR4	DR7	DR11	DR13	DR15	DRB3	DRB5	DRB4	
N1	4	>149	1	35	35	>111	7	>1111	2	154	6
N2	**1**	>149	258	**44**	**58**	>111	**27**	**31**	444	3077	5
G1	3873	>149	>222	>1091	219	**16**	4615	>577	**4**	1637	2
G2	**14**	>149	**8**	**35**	**6**	**6**	**2**	>1111	139	2308	6
P1	**13**	>149	**33**	**3**	**24**	**3**	**2**	**87**	**10**	218	8

Lipo5 fragments were submitted to competitive ELISA specific for HLA-DR molecules. Data were expressed as relative activity (ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide, which is a high binder to the HLA-DR molecule). Relative activities <100 are in bold and correspond to good binders. Means were calculated from at least two independent experiments.

stimulated weekly by autologous mature dendritic cells, the Lipo5 fragments, and appropriate cytokines to enrich the coculture in Lipo5-specific CD4 T lymphocytes. After four stimulation rounds, each independent T cell line (CD4 T cells present in a single well) was evaluated for its specificity for individual Lipo5 peptides by IFN-γ ELISPOT assay (Fig. 1). Because of the low frequency of Ag-specific T cells in healthy donors, only part of the T cell lines contained Lipo5-specific CD4 T lymphocytes and reacted specifically with Lipo5 peptides in the ELISPOT assay. Indeed, 648 of 6640 T cell lines tested were specific for individual Lipo5 peptides. Four representative Lipo5-specific T cell lines are presented in Fig. 1A. G2 and P1 stimulated T cells in all the donors and gave rise to 281 and 94 specific T cell lines, respectively. N1, N2, and G1 generated fewer peptide-specific T cell lines in ~80% of the donors. On the basis of the proportion of Lipo5-specific T cell lines, we calculated the frequencies of pre-existing Lipo5-specific $CD4^+$ T cells in the blood of the donors by considering that their distribution in the wells at the initiation of the culture followed a Poisson distribution (34, 35). A mean of 2.79 precursors per million was found for G2, 0.61 precursors per million for P1, and, respectively, 0.37, 0.25, and 0.31 for G1, N1, and N2. We showed therefore that the G2-specific CD4 T cell repertoire is greater than the T cell repertoire specific for the other peptides.

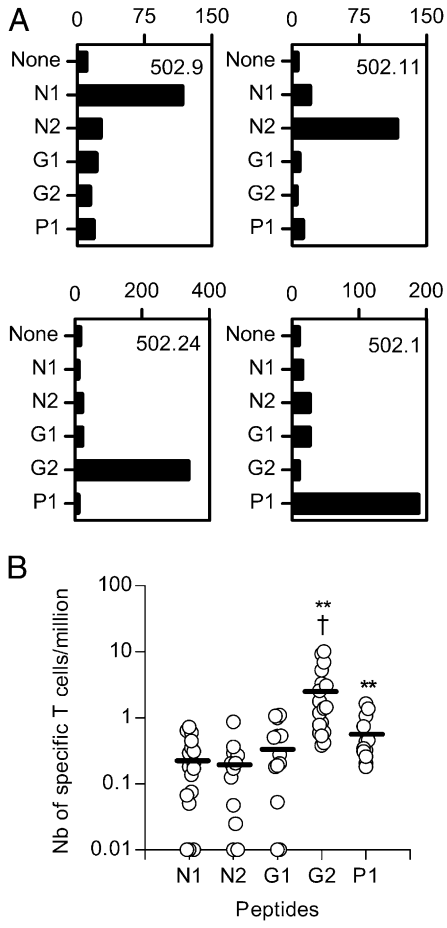
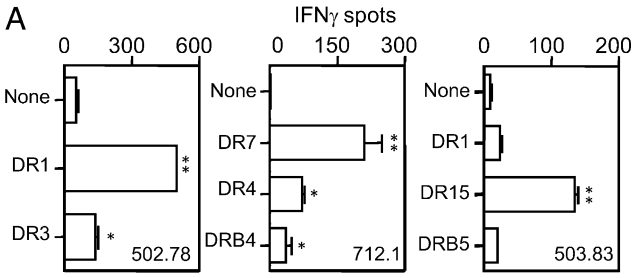


FIGURE 1. Frequencies of Lipo5-specific CD4 T cells in the blood of normal donors. (**A**) Lipo5-specific $CD4^+$ T cell lines were obtained after weekly stimulations by autologous mature dendritic cells loaded with the Lipo5 peptide mixture (10 μg/ml of each peptide). T cell lines (~5×10^3 cells/well) were submitted to IFN-γ ELISPOT assays using autologous PBMCs (5×10^4 /well) as APCs. In the ELISPOT assay, the peptides were tested individually and used at 10 μg/ml. (**B**) Lipo5 peptide-specific T cell precursors were estimated on the basis of the percentage of negative wells and the number of distributed CD4 T cells according to Poisson's law: frequency = −Ln ([number of negative wells/total number of wells tested]/[number of $CD4^+$ T cells/well]). The nonparametric Wilcoxon signed-rank test was used to evaluate the statistical differences of the mean frequencies. Statistical differences with N1 or N2: $^{**}p < 0.01$. Difference with P1: $^{\dagger}p < 0.05$.



B

HLA-DR	Nb of T cell lines				
	N1	N2	G1	G2	P1
DR1	1	2	7	2	3
DR3	0	1	-	1	1
DR4	4	-	-	2	-
DR7	3	1	-	-	-
DR11	6	-	-	6	3
DR15	-	0	8	3	3
DRB4	9	1	-	2	-
DRB5	-	1	9	2	3

FIGURE 2. HLA-DR restriction of the Lipo5-specific T cell lines. (**A**) Peptide-specific $CD4^+$ T cell lines were incubated with the appropriate peptide and L cells (5×10^4 cells/well) transfected by one HLA-DR molecule (DR1, DR3, DR4, DR7, DR15, DRB4, or DRB5) and submitted to IFN-γ ELISPOT assays. Each bar represents the mean spot number of duplicates. Statistical differences from negative control (no peptide): $^{*}p < 0.05$, $^{**}p < 0.01$. (**B**) The table includes the number of Lipo5-specific T cell lines and their HLA-DR restriction. "-", No peptide-specific T cell line was found for this combination of peptide and restriction element.

Table II. Cross-reactivity of the Lipo5-specific T cell lines

	No. of T Cell Lines	Cross-reactivity	
		Consensus B	Consensus C (%)
N1	20	55%	50
N2	9	89%	55
G1	18	id	39
G2	24	id	100
P1	18	id	94

The Lipo5 sequence has an LAI origin. Lipo5-specific T cell lines were incubated with autologous APCs and the original peptides or the consensus B or the consensus C corresponding peptide. T cell activation was revealed by IFN-γ ELISPOT. Data were expressed as a percentage of T cell lines reacting with the consensus peptides. id, Identical to the LAI sequence.

The T cell response to Lipo5 peptides is restricted to multiple HLA-DR molecules

To characterize the HLA-DR molecules involved in the Lipo5 peptide presentation, we introduced L cells transfected with HLA-DR molecules corresponding to the typing of the donor, as APCs in the ELISPOT assay. Forty-six peptide-specific T cell lines were tested for their potential restriction to HLA-DR molecules. Representative data are presented in Fig. 2A. From left to right, the T cell line 502.78 recognized N2 restricted by HLA-DR1 and, to a lesser extent by HLA-DR3, N1 was mainly presented by HLA-DR7 to the 712.1 specific T cell line, and the P1-specific response of T cell line 503.83 was restricted to HLA-DR15. The overall results of this study are presented in Fig. 2B. The CD4 T cell response to G2 was restricted to seven different HLA-DR allotypes in agreement with the broad specificity of G2 for HLA-DR molecules. N1, N2, and P1 elicited CD4 T cells restricted to five different HLA-DR allotypes, whereas only three HLA-DR molecules were involved in the CD4 T cell response to G1.

Consensus B and C sequences and G2 clade B circulating variants were recognized by the Lipo5 peptide-specific T cell lines (LAI/IIIB sequence)

The Lipo5 vaccine was designed on the basis of the LAI isolate. We therefore addressed the question whether Lipo5-specific T cells recognized other HIV variants, including clades B and C. We estimated the cross-reactivity of each peptide by the percentage of 89 Lipo5-specific T cell lines that were stimulated by the clade B and C consensus variants (Table II). For G1, G2, and P1, the LAI sequence corresponds to the consensus clade B sequence, but not to clade C. G2- and P1-specific T cell lines showed very good reactivity for consensus clade C with percentages of 100 and 94%, respectively. The clade C variant of the G1 peptide was less recognized (39%). For the N1 and N2 peptides, the percentage response was ~50% for clade C and 55 and 89%, respectively, for clade B.

Because of the pre-eminence of G2 in the Lipo5-specific CD4 response, we also evaluated the cross-reactivity of G2 with the most frequent clade B circulating variants. We first determined the minimal epitope recognized by the G2-specific T cell lines using three 15-mer peptides (Gag 253-267, Gag 259-273, and Gag 270-284) encompassing the entire sequence of G2 (data not shown). Of 21 T cell lines tested, we observed that 24% were specific for Gag 253-267, 9% for Gag 259-273, and 76% for Gag 270-284, two T cell lines being polyspecific. As the G2 sequence is identical to the clade B consensus sequence, G2 is the most represented variant (~60–70%). However, many clade B variants circulate in the population with significant frequencies ranging from ~3 to 20% (Fig. 3). We therefore tested the capacity of the G2-specific T cell lines to recognize these clade B variants. The data obtained from three T cell lines, which were stimulated by all the variants, are presented in Fig. 3A. Overall, the cross-reactivity of the natural variants varied from 50 to 93% (Fig. 3B). The G2 sequence contains five variable positions. Substitutions P255A, I256V, L268M, T280S, and T280I

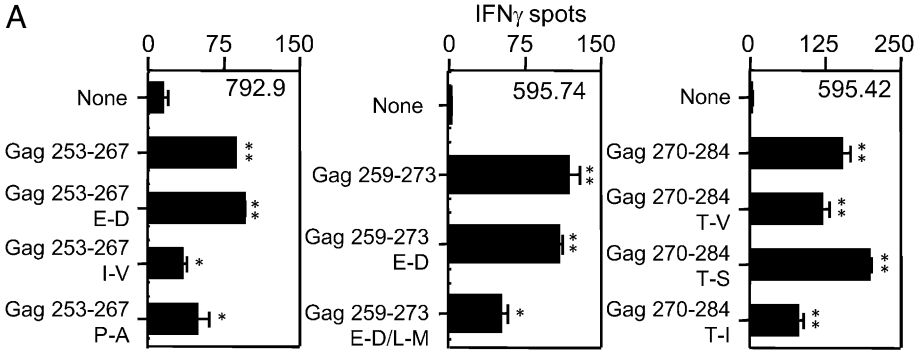


FIGURE 3. Recognition of Gag variants by peptide-specific T cell lines. (**A**) Recognition of natural variants by T cell lines specific for the three overlapping peptides (Gag 253-267, 259-273, and 270-284) of the peptide G2 (Gag 253-284). T cell lines were incubated with autologous PBMCs (5×10^4 cells/well) and peptide variants (10 μg/ml) in the IFN-γ ELISPOT assay. Each bar represents the mean spot number of duplicates. Statistical differences from the conditions without peptide: $*p < 0.05$, $**p < 0.01$. (**B**) Sequences in the G2 peptide of LAI and the natural variants and their frequency (% clade B) in the HIV clade B (Los Alamos HIV database, http://hiv-web.lanl.gov). Cross-reactivity indicates the number and percentage of T cell lines reacting with each variant.

B

	Sequences	% clade B	Cross-reactivity
Gag 253-267	**N P P I P V G E I Y K R W I I**	**69,47%**	
Gag 253-267 E-D	- - - - - - - D - - - - - - -	6,05%	3/6 (50%)
Gag 253-267 I-V	- - - V - - - - - - - - - - -	3,21%	5/6 (83%)
Gag 253-267 P-A	- - A - - - - - - - - - - - -	3,12%	5/6 (83%)
Gag 259-273	**G E I Y K R W I I L G L N K I**	**68,15%**	
Gag 259-273 E-D	- D - - - - - - - - - - - - -	4,06%	1/2 (50%)
Gag 259-273 E-D/L-M	- D - - - - - - - M - - - - -	1,51%	1/2 (50%)
Gag 270-284	**L N K I V R M Y S P T S I L D**	**59,83%**	
Gag 270-284 T-V	- - - - - - - - - - V - - - -	20,42%	8/15 (53%)
Gag 270-284 T-S	- - - - - - - - - - S - - - -	3,59%	14/15 (93%)
Gag 270-284 T-I	- - - - - - - - - - I - - - -	2,93%	12/15 (67%)

weakly affected the T cell activation of G2-specific T cell lines, as ~80% of them responded to the corresponding mutated peptides. Only substitutions E260D and T280V led to a reduction of cross-reaction of <50%.

Peptide-specific T cell lines recognized B-EBV infected with HIV

To evaluate the efficiency of Lipo5-specific T cells in recognizing HIV-infected cells, we used homozygous B-EBV cell lines with donor-matched HLA-DR typing. They were infected with X4-HIV-1 virions pseudotyped with VSV-G and introduced as APCs in the IFN-γ ELISPOT assay. We generated Lipo5-specific T cell lines in four donors and tested the response of 12 T cell lines specific for N1, G2, or P1 peptides. For 9 of the T cell lines, noninfected EBV cell lines gave rise to a strong allogenic response that obliterated the specific response. Three T cell lines specific for G2 or P1 were isolated from two different donors (900 and 940) and were significantly more stimulated with infected B-EBV cells than with noninfected B-EBV cells (Fig. 4, *left panels*). The three T cell lines differed strongly in avidity for their specific peptides (Fig. 4, *right panels*). Whereas the most avid T cell line 900.22 was the most sensitive in detecting HIV components, T cell lines 900.82 and 940.7 exhibited a moderate avidity apparently sufficient to be stimulated by HIV-infected cells. We did not evaluate the phenotype of the peptide-specific T cell lines as the T cells were voluntarily skewed by the culture conditions to a Th1 phenotype compatible with the IFN-γ ELISPOT assay.

Discussion

Initially designed on the basis of their content in CD8 T cell epitopes, the ANRS lipopeptide vaccines have been the subject of multiple vaccine trials (25–30), but their fine capacity to stimulate CD4 T cells has never been investigated. We therefore performed a comprehensive analysis of the in vitro CD4 T cell response induced by the Lipo5 vaccine and mainly showed that the hierarchy of the T cell epitopes is related to the size of the pre-existing peptide-specific CD4 T cell repertoire.

In the recent vaccine trial, approximately half of the donors developed a sustained CD4 response specific for the Lipo5 vaccine (27). The G2 peptide induced the most frequent response as 75% of the responders to Lipo5 developed a response specific for the G2 peptide, the other peptides being less active (27) (Fig. 5A). To account for this particular epitope hierarchy, we evaluated the capacity of the Lipo5 peptides to bind to multiple HLA-DR molecules and to prime in vitro CD4 T cells. It is generally assumed that interindividual variations in the T cell response are due to HLA polymorphism and hence to the differential capacity of the peptides to be presented to T cells (36). Most of the immunodominant T cell epitopes correspond to good binders to HLA class II molecules (20, 37, 38). We found that four peptides (P1, G2, N1, and N2) bound to at least half of the common HLA-DR molecules introduced in the assay. N1 and N2 elicited a T cell response in a limited number of vaccinees, confirming that a broad binding specificity for HLA class II molecules is necessary, but not sufficient to mount a CD4 T cell response in a high percentage of donors (38, 39). The broad binding specificity of G2 and P1 allows them to be recognized by T cells from different donors, but does not explain the dominance of G2 over P1, P1 being more promiscuous for HLA-DR molecules than G2. In contrast, we clearly observed that the G2 peptide differed from the other Lipo5 peptides by its strong in vitro T cell properties. As previously published (34, 35, 40), the yield of peptide T cell lines isolated in vitro depends on the frequency of pre-existing peptide-specific T cells present in the blood donors. We therefore estimated the frequencies of pre-existing Lipo5-specific CD4 T cells, as

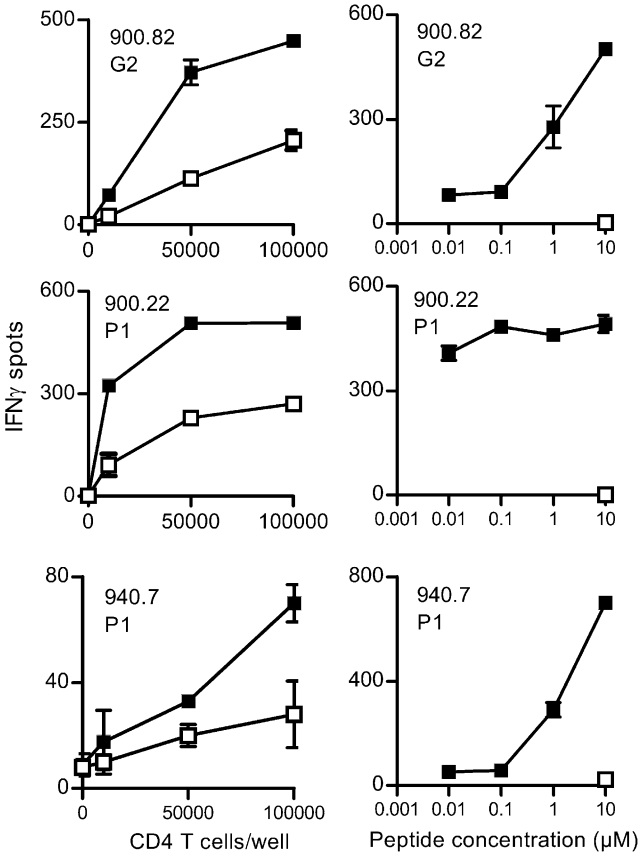


FIGURE 4. Recognition of HIV-infected B-EBV by Lipo5-specific T cell lines. *Left panels*, Peptide-specific $CD4^+$ T cells (1×10^4 to 1×10^5) were incubated with 8×10^4 B-EBV cells infected with VSV-G–pseudotyped HIV NL4-3 virions (black square) or noninfected cells (white square) and submitted to IFN-γ ELISPOT assays. *Right panels*, Peptide dose response of the T cell lines. The same T cell lines were incubated with a dose range of peptides and B-EBV cells. Each dot represents the mean spot number of duplicates.

previously described (34, 35, 40). A mean of 2.79 precursors per million was found for G2, whereas the frequency of precursors was below 1 cell per million for the other peptides. Another way to present these data is to distribute the T cell lines on the basis of their peptide specificity (Fig. 5B). The G2 peptide and to a lesser extent the P1 peptide are the source of a large part of the Lipo5-specific

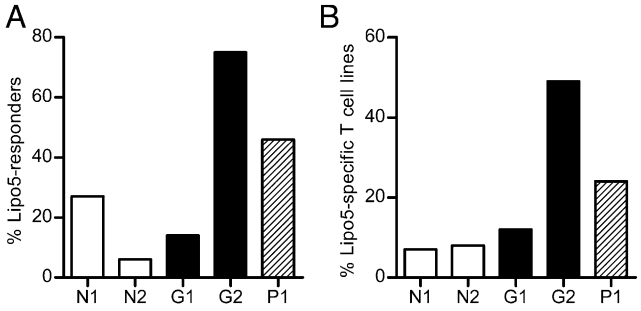


FIGURE 5. Distribution of the responding donors of the ANRS Vac18 vaccine trial and of the peptide-specific T cell lines raised against the Lipo5 vaccine in vitro. (**A**) Data were retrieved from Salmon-Céron et al. (27) and are the percentage of positive CD4 T cell responses classified by Lipo5 peptides in volunteers who received Lipo5. Ninety-seven volunteers received different doses of Lipo5 in four injections, whereas 34 volunteers received placebo. (**B**) The 543 Lipo5-specific T cell lines were distributed on the basis of their peptide specificity.

T cell response during the vaccine trial and in vitro (Fig. 5). We therefore strongly suggest that the hierarchy of Lipo5 T cell epitopes observed during the ANRS vaccine trial (27) is the result of the differences in size of the naive T cell repertoire specific for each individual Lipo5 peptide, in agreement with others (41). Accordingly, the size of the naive CD4 T cell repertoire from various T cell epitopes investigated in mice has been found to range from 0.8 to 15 cells per million (41, 42) and was correlated with the amplitude of the T cell response (42). More recently, analysis of the CD4 T cell repertoire of HLA-DRB*0101–restricted T cell epitopes of protective Ags of *Bacillus anthracis* before and after vaccination showed that the frequencies of epitope-specific memory $CD4^+$ T cells in vaccinees were directly correlated with the frequencies of precursors in the naive repertoire (43). Our data therefore extend these observations to promiscuous HIV-specific CD4 T cell epitopes. Moreover, G2 comprises two immunodominant CD4 T cell epitopes of Gag that were initially found by others in seropositive donors (19, 20). This suggests that the naive T cell frequencies could also shape the T cell response during the early phase of virus infection, as proposed for the HIV-specific CD8 T cell response (44). This is in agreement with observations we made for hepatitis C virus T cell epitopes (38, 45). Once sufficiently established, HIV infection disturbs the initial immunodominance by multiple mechanisms. Specific T cells are preferentially infected by HIV and hence more prone to senescence and cell death (46). Nonvital mutations located in T cell epitope modify the T cell specificity (47), whereas viral load alters activation of HIV-specific T cells (48). As a result, shift of the T cell repertoire is observed in infected donors.

Because of the large diversity of HIV strains, we also evaluated the cross-reactivity of the individual Lipo5 peptides with consensus sequences of clades B and C and natural variants for G2. The sequences included in the vaccine were selected from highly conserved HIV regions, but some positions are variable and can affect T cell recognition. Our data showed that the mutations were generally well tolerated, as the weakest level of cross-reactivity was 39%. In many cases, almost all the T cell lines were stimulated by the consensus or natural variants. We also showed that three T cell lines specific for G2 and P1 were specifically stimulated by HIV-infected cells, confirming at least for these peptides their relevance in immunity to HIV. These complementary results on cross-reactivity and HIV recognition confirm the value of the Lipo5 vaccine for further clinical investigations.

The role of HIV-specific CD4 T cells in the control of the infection remains unclear, especially as HIV-specific CD4 T cells are more prone than other CD4 T cells to be infected by HIV (46). Several properties dealing with their phenotypes (48–50), avidity (12), specificity (22), and localization (13) have been highlighted by comparative studies of seropositive subjects, progressors, and elite controllers. The challenge now is to find HIV vaccine candidates able to reproduce the HIV response observed in elite controllers. Animal models, ex vivo studies, and in vitro studies help to clarify the mechanisms involved in the qualitative and quantitative aspects of immune responses, but all have all their own limitations. The quantitative T cell assays we have developed using cells collected in naive donors mainly estimate the size of the Ag-specific repertoire in humans. Because a specific T cell line comprises between 250,000 and 10^6 CD4 T cells, this approach generates enough cells to enable characterization of their peptide and HLA specificity. We characterized 648 T cell lines from 22 donors with different HLA haplotypes, providing an important body of information on the Lipo5 vaccine. Concordance of our data with the ANRS vaccine trial data (27) with respect to the hierarchy of the Lipo5 T cell epitopes strongly endorses the utility of evaluating the specific naive T cell repertoire (41, 51) in predicting the immunogenicity of epitope-based vaccines in humans. This approach ranks the vaccine candidates on the basis of their expected T cell breadth in humans. We have also proposed using this approach to manage the immunogenicity risk of therapeutic proteins (35, 40). Our data help to clarify the CD4 T cell specificity of the Lipo5 vaccine and to improve experimental approaches to predicting vaccine immunogenicity in humans.

Disclosures

The authors have no financial conflicts of interest.

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