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Novel promiscuous HLA-DQ HIV Nef peptide that induces IFN-γ-producing memory CD4+ T cells

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SUMMARY

We describe the highly conserved sequence 56–68 of the HIV Nef protein as the first promiscuous HLA-DQ HIV-derived peptide. The Nef peptide exhibits an albeit rare capacity to bind 6 different HLA-DQ molecules whereas no binding is observed with the 10 HLA-DR molecules tested. In agreement with these data, after immunization with the Nef peptide, HLA-DQ transgenic Aβ^{o} mice display a vigorous cellular and humoral response while the specific immune response of HLA-DR expressing mice is minimal. The promiscuous potentiality of the Nef 56–68 peptide in humans has been confirmed by *ex vivo* immunization experiments with CD4+ T cells from 14 healthy donors expressing different HLA genotypes. Nef 56–68 specific CD4+ T cells rapidly acquire a memory cell phenotype and are characterized by the preferential usage of the TCR Vβ 6·1 gene segment and predominant production of IFN-γ. Taken together, these data indicate that the Nef 56–68 peptide constitutes an attractive component of vaccines aiming at inducing or enhancing HIV-specific T cell immunity.

Keywords epitope HLA-transgenic mice human Th1 cells T cell receptor

INTRODUCTION

Combination chemotherapy referred to as highly active antiretroviral treatment (HAART) dramatically suppresses human immunodeficiency virus type 1 replication and has thereby contributed substantially to reducing AIDS-related opportunistic infections and death [1]. However, recent studies have shown that, even in the presence of optimal HAART, when the virus is undetectable for several years in plasma, replication-competent provirus persists in 'resting 'memory T cells, with continuous transcription and evolution of HIV [2–5]. Moreover, since HAART regimens are complex and frequently associated with side-effects, long-term compliance with treatment is difficult, increasing the risk of development of drug-resistant virus. So, effective cellular and/or humoral immune responses against HIV-1 need to be actively maintained as a form of adjunctive therapy to HAART.

Protective immunity will probably depend upon both the production of neutralizing antibodies and the generation of HIV-specific cytotoxic T cells. In HIV-infected patients, however, CTL function is lost as the disease progresses, possibly because of the absence of HIV-specific CD4+ T cell help [6]. In the rare cases in which the CD4+ response to HIV antigens is persistently detectable, strong anti-HIV CTL responses are maintained and are associated with the control of viral replication and a good prognosis [7,8]. Although the majority of HIV+ patients progress towards AIDS, a minority does not show signs of disease and maintains stable CD4 counts despite a long-lasting infection [9]. The observation of relatively strong HIV-specific CD4+ T cell responses in these long-term nonprogressors compared with responses in progressive disease [8] has confirmed the idea that such a response may be the critical missing link in progressive infection. Thus, induction or restoration of HIV-specific CD4+ T cell responses in infected patients might be important for both treatment and prevention of HIV disease.

Much effort has been put into the identification of universal T helper (Th) epitopes capable of enhancing the induction of T cell immunity in a wide variety of subjects displaying different HLA types [10–12]. But the advantage in using epitopes from HIV itself is that natural boosting can occur early after exposure, whereas this might not occur if the helper epitopes are not virus-specific. Several conserved HIV-specific HLA-DR-restricted T helper epitopes exhibiting both high affinity and immunogenicity have been identified [13–15]. However, the targeting of HLA-DQ-restricted response also represents an interesting complementary approach in the identification of vaccine candidates. Regarding the Nef regulatory protein, T and B cell epitopes have previously been described particularly in the region 56–68 [16,17].

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The ability of this peptide to bind HLA class II DQ and DR molecules and to recruit naïve T cells by immunization *in vivo* in HLA-DQ and -DR transgenic Aβ° mice and *ex vivo* using CD4$^+$ T cells from HLA-typed healthy donors was evaluated. We describe a new HIV-derived peptide, presented by at least six different HLA-DQ molecules, that is capable of priming an HIV-specific Th1 response. Moreover, our *ex vivo* immunization protocol allowed us to rapidly induce memory, IFN-γ producing, CD4$^+$ T helper cells. Consequently, our search for Nef-derived Th epitopes resulted in the identification of an interesting candidate for vaccine strategy and/or cellular immunotherapy in HIV-infected patients.

MATERIALS AND METHODS

Peptides

Nef 56–68 (AWLEAQEEEEVGF), TT 830–846 (QYIKANSK FIGITELKK), MHC I α 46–63 (EPRAPWIEQEGPEYWDQE), DQB 45–57 (ADVEVYRAVTPLGPPD), Ig 44–60 (DTLRSYY ADWYQQKPG), INS 1–15 A (FVNQHLAGSHLVEAL), B7 150–164 (LNEDLRSWTAADTAA) peptides were synthesized on an Advanced ChemTech model 357 MPS Synthesizer (Advanced Chemtech Europe, Brussels, Belgium) as previously described [18]. Homogeneity was confirmed by analytical HPLC.

HLA class II/peptide binding assays

EBV homozygous cell lines were used as source of human HLA class II molecules [19]. As previously described [20], purified HLA-DR and HLA-DQ molecules were incubated with a referenced biotinylated peptide in the presence of serial dilutions of Nef 56–68 competitor peptide. Data are expressed as the peptide concentration that prevented binding of 50% of the labelled peptide (IC_{50}). Average and SE values were deduced from at least three independent experiments.

HLA-transgenic Aβ° *mice*

Mice expressing different HLA alleles (HLA-DR2, HLA-DQ6 and HLA-DQ8) and deficient in murine class II molecules (Aβ°) were a kind gift of Dr Ch. David (Mayo Clinic Rochester, MI, USA) [21]. Mice expressing the HLA-DR1 transgene on an FVB/N background were kindly provided by Dr D. Altmann (Hammersmith Hospital, London, UK) [22] and backcrossed with Aβ° mice [23]. HLA-transgenic Aβ° mice were immunized s.c. with Nef 56–68 peptide (50 μg) in CFA (Sigma-Aldrich, Saint Quentin Fallavier, France) and two booster injections with peptide (25 μg) in IFA (Sigma-Aldrich) at 2 weekly intervals were performed. The proliferative response was measured as previously described [24] by incubating 5×10^5 splenic or lymph node cells, removed seven days after the last injection, with an optimal concentration of Nef 56–68 peptide (25 μg/ml) and testing the cell culture supernatants for cytokine release.

Antibody and cytokine detection

The quantification was performed by ELISA as previously described [24]. Mouse sera were diluted 1/100 for IgG1 and 1/10 for IgG2a and IgG2b detection and peroxidase labelled anti-mouse IgG1(dilution 1/3000) or IgG2a (dilution 1/2000) were provided by Diagnostic Pasteur (Marnes-la-Coquette, France). IL-4 and IFN-γ in the sera (dilution 1/10) and IL-2, IL-4, IL-5, IL-10 and IFN-γ in the supernatants were detected using sandwich ELISA. The antibody pairs used for the detection of mouse and human IL-2, IL-4, IL-5, IL-10 and IFN-γ were provided by BD PharMingen (San Diego, CA, USA). Absorbances at 492 nm were measured using a multichannel spectrophotometer (Titertek Multiskan MCC 1340). Results were expressed as the mean of duplicate wells after subtraction of the background.

Blood donors

Blood was collected from healthy, adult HIV-uninfected individuals. Donors were informed of the details of the study and signed an appropriate consent form according to the guidelines for research volunteers. HLA typing was performed by E.T.S. (Lille, France) using standard serotyping assays.

Dendritic cell generation

PBMCs were isolated from heparinized whole blood, CD14$^+$ cells separated by high gradient magnetic sorting (VARIOMACS, Miltenyi Biotech GmbH, Bergish Gladbach, Germany) [25] and cultured for 5 days at a cell density of 1×10^6 cells/ml in a cell culture bag (AFC, Columbia, MA, USA) in RPMI 1640 supplemented with 10% human AB$^+$ serum (pool of 3 sera, E.T.S). Differentiation into dendritic cells (DC) was obtained by addition of rhIL-4 (1000 U/ml) and rhGM-CSF (800 U/ml) (Peprotech Inc, Rocky Hill, NJ, USA). After 5 days, the cells expressed a typical dendritic cell phenotype with high levels of CD11c (99%), HLA class II (99%), CD86 (99%) and CD40 (76%) molecules. They showed very limited expression of CD1a (2%), CD80 (10%) and the CD83 maturation marker (6%) and no longer expressed the monocyte lineage marker CD14 (0·5%).

Ex vivo *immunization of CD4$^+$ T cells*

CD4$^+$ T cells were separated by negative selection using the VARIOMACS technique (Miltenyi) from CD14$^-$ cells cultured for 5 days in RPMI 1640 supplemented with 10% human AB$^+$ serum. 1×10^6 cells were immunized *ex vivo* with Nef 56–68 peptide (50 μg/ml) in the presence of DCs (1×10^6). After 15 days of culture in RPMI 1640 supplemented with 10% human AB$^+$ serum, 15 day cycles of restimulation with the peptide were performed using as APCs, first DCs and then B cells.

Naive (CD45RA) and memory (CD45RO) T cells were isolated from 10 day peptide-immunized CD4$^+$ T cells by depletion using the VARIOMACS technique (Miltenyi), stimulated with Nef 56–68 peptide (50 μg/ml) for 48 h and the culture supernatants were tested for cytokine production.

Cytofluorimetric cell-surface phenotyping

Cell staining was performed using FITC- or PE-conjugated and affinity purified mouse moAb for direct labelling. For DC phenotyping, moAb anti-CD1a, anti-CD11c, anti-CD14, anti-CD40, anti-CD80, anti-CD83, anti-CD86 and anti-HLA-DR were purchased from BD Pharmingen. For T cell phenotyping, moAb anti-CD3, anti-CD25, anti-CD28, anti-CD30, antiCD40L, anti-CD45RA, anti-CD45RO, anti-CD62L and anti-CD69 were obtained from Caltag (Burlingame, CA, USA).

T cell clones

A cloning feeder cell mixture was prepared by adding together 5×10^5 cells/ml of irradiated (4000 rad) PBMC from any healthy donor, 5×10^4 cells/ml of an irradiated (5000 rad) EBV-LCL, and 50 ng/ml PHA and used for serial dilutions of Nef 56–68 specific T cells, recovered 10 days after the first restimulation with the

peptide, in 96-well round-bottom plates as previously described [26]. Culture medium containing rIL-2 (20 U/ml)(Peprotech Inc) was added after 5 days of culture. After identification of growing T-cell clones, usually after 10–14 days of culture, the cells were transferred to a 24-well plate in the presence of fresh culture medium and with a 2× feeder cell mixture to expand the cells.

TCR analysis

Total cellular RNA was isolated from $CD4^+$ T cell lines or clones and then reverse transcribed to cDNA by MMLV reverse transcriptase (Gibco BRL, France). PCR analysis of the TCR Vβ repertoire was accomplished as previously described by Genevée *et al*. [27].

RESULTS

Conservation of the Nef 56–68 sequence

As shown in Table 1, the 56–68 region of the HIV Nef protein corresponds to a region conserved among various HIV isolates referred to in the Data Bank (GenBank). Indeed, the native sequence is represented at a frequency of about 55% in the 620 sequences of Nef evaluated and in the other cases only single amino acid mutations were observed.

High binding capacity of Nef 56–68 peptide to HLA-DQ alleles

To first evaluate the potency of the peptide 56–68 to stimulate T cells in humans, we investigated its capacity to bind a large number of HLA class II molecules. More precisely, it was submitted to binding assays specific for 10 HLA-DR and 7 HLA-DQ alleles which are all frequently encountered in the Caucasian population (Table 2). Data were expressed as IC_{50} but also as relative activity. The latter is the ratio between the peptide and a highly active peptide (reference peptide). It therefore allows a comparison between the binding activity to relevant peptides, such as T cell epitopes, and naturally processed peptides. The lower the ratio is, the closer the Nef peptide is to the reference peptide and hence the higher is its affinity. In this context, we observed that the Nef 56–68 peptide exhibited good binding to 6/7 HLA-DQ alleles. It was especially active towards DQ2 and DQ8 since it was more active for these alleles than the corresponding reference peptide and presented low IC50, which are close to or below 100 nM. Only the DQ7 allele bound at a very high concentration and hence with low efficiency. Low binding efficiency was also observed for the HLA-DR molecules. IC50 were mostly greater than 10 000 nM and the relative activities were at least higher by a factor of 77. The Nef peptide was only moderately active on HLA-DR11, its mid-activity being in the

Table 1. Sequences of Nef 56–68 peptide published in databases*

A	W	L	E	A	Q	E	E	–	E	E	V	G	F	55.3%
								X						9.2%
							D	–						7%
								–		D				1.8%
							D	–		D				1.4%
Others														each < 1.4%

*research of homologies on 620 sequences published in PubMed

Table 2. Binding capacities of Nef 56–68 peptide to HLA class II molecules

	Alleles	Allelic frequencies (%)	Referenced peptide	Referenced peptide IC_{50} (nM)	Nef 56–68 peptide IC_{50} (nM)	Ratio	Binding
DQ2	DQA1*0201/DQB1*0201	12·4/23·1	MHC I α_{46-63}	620 (±150)	1100 (±188)	1·8	+
DQ2	DQA1*0501/DQB1*0201	27·4/23·1	MHC I α_{46-63}	340 (±160)	180 (±30)	0·5	++
DQ7	DQA1*0501/DQB1*0301	27·4/18·5	DQB_{45-57}	60 (±20)	31000 (±18000)	517	–
DQ8	DQA1*0301/DQB1*0302	14·2/8·1	DQB_{45-57}	1150 (±350)	44 (±9)	0·04	++
DQ5	DQA1*0101/DQB1*0505	17/14·9	Ig_{44-60}	620 (±150)	3500 (±1800)	5·6	+
DQ6	DQA1*0102/DQB1*0602	15·8/9·8	INS_{1-15A}	170 (±35)	3100 (±760)	18	+
DQ6	DQA1*0103/DQB1*0603	6·2/5·8	$B7_{150-164}$	2200 (±760)	24000 (±8660)	11	+
DR1	DRB1*0101	9·3	$HA_{306-318}$ [20]	6 (±1)	>10000	>1666	–
DR3	DRB1*0301	10·9	MT_{2-16} [20]	120 (±40)	>10000	>84	–
DR4	DRB1*0401	5·6	$HA_{306-318}$ [20]	30 (±30)	>10000	>333	–
DR7	DRB1*0701	14·0	YKL [20]	130 (±40)	>10000	>77	–
DR11	DRB1*1101	9·2	$HA_{306-318}$ [20]	20 (±4)	2200 (±350)	110	–
DR13	DRB1*1301	6·0	$B1_{21-36}$ [20]	1000 (±0)	>100000	>100	–
DR15	DRB1*1501	8·0	$A3_{152-166}$ [20]	30 (±17)	>10000	>333	–
DR51	DRB5*0101	7·9	$HA_{306-318}$ [20]	10 (±0)	>10000	>1000	–
DR52	DRB3*0101	9·2	14·166 [20]	10 (±0)	>10000	>1000	–
DR53	DRB4*0101	28·4	E2/E7 [20]	4 (±1)	>10000	>2500	–

micromolar range and one hundred fold less active than the reference peptide. Taken together, the results suggest that Nef 56–68 peptide appears to be a promiscuous ligand for HLA-DQ molecules preferentially.

Nef 56–68 peptide is highly immunogenic in HLA-DQ transgenic Aβ° mice

In agreement with the binding data, HLA-DQ (in particular DQ8) transgenic (Tg) Aβ° mice showed vigorous cellular and humoral responses after immunization with the Nef 56–68 peptide while HLA-DR Aβ° mice exhibited only a very limited responses (Fig. 1).

So, Nef 56–68 immunized-DQ6 and -DQ8 Tg mice responded with the predominant production of IgG2b Abs (Fig. 1a), no antibody production being observed in the control non-immunized mice. The cells from immunized DQ Tg mice showed a significant proliferative response associated with predominant IFN-γ secretion after *in vitro* specific restimulation with the Nef 56–68 peptide (Fig. 1b,c) whereas cells from control non-immunized mice did not (data not shown). The presence of IFN-γ was also detected in the sera of Nef-peptide-immunized DQ8 and DQ6 Tg mice (Fig. 1c).

In contrast, after immunization with the Nef 56–68 peptide, only small amounts of IgG2b Abs were detected in the sera of DR2 Tg mice (Fig. 1a) and a weak peptide-specific proliferative response, associated with minimal production of IL-2, was observed in DR1 Tg mice (Fig. 1b).

HLA-DQ dependent induction of memory CD4 Th1 cells

As a preclinical study, we performed *ex vivo* immunizations of $CD4^+$ T cells from a cohort of 14 HLA-typed healthy donors with Nef 56–68 peptide in the presence of autologous DCs as antigen presenting cells and cytokine production was evaluated.

All the donors, expressing various HLA genotypes, showed a response to the Nef 56–68 peptide demonstrating its promiscuous potential in humans. Thus, 48 h after immunization with the peptide, $CD4^+$ T cells displayed a Th1 phenotype with a predominant production of IFN-γ associated either with or without IL2 production. IL-4 and IL-5 secretions were never observed (Table 3). This profile was conserved after specific restimulation of $CD4^+$

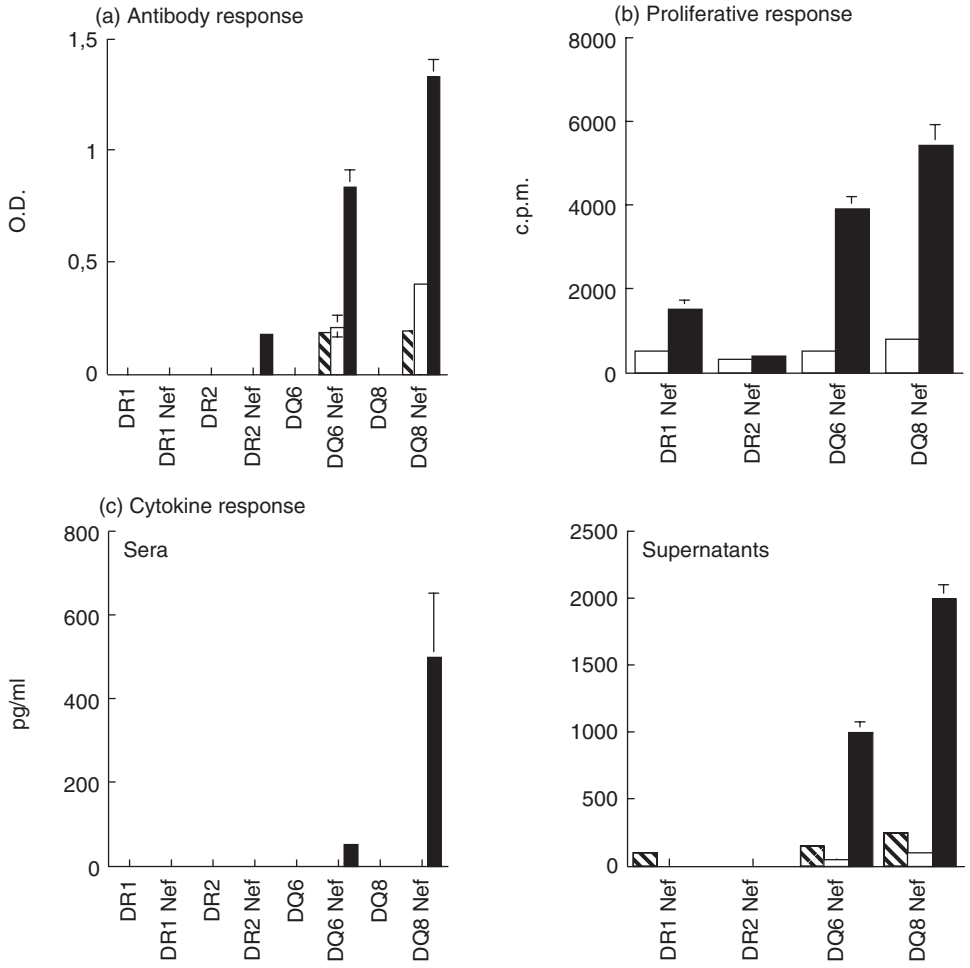

Fig. 1. Response of HLA-transgenic Aβ° mice to Nef 56–68 peptide. (a) Antibody isotypes were determined in pools of mouse sera ($n = 5$) 35 days after immunization with or without 50 μg of Nef 56–68 peptide. ▨ IgG1, □ IgG2a, ■ IgG2b. (b) Proliferative response of splenic cells recovered 35 days after immunization with 50 μg of Nef 56–68 peptide and stimulated *in vitro* with 25 μg/ml of Nef 56–68 peptide (■), control without *in vitro* stimulation with Nef peptide (□). (c) Cytokine (▨ IL-2; □ IL-4; ■ IFN-γ) secretion was evaluated in pools of mouse sera ($n = 5$) 35 days after immunization with or without 50 μg of Nef 56–68 peptide and in 48 h culture supernatants of splenic cells recovered 35 days after immunization and stimulated *in vitro* with 25 μg/ml of Nef 56–68 peptide. $n = 3$ series of experiments.

Table 3. Type 1 cytokine secretion after *ex vivo* immunization with Nef 56–68 peptide

	Cytokines (pg/ml)							
	IL-2		IL-4		IL-5		IFN-γ	
Donor	–	Peptide	–	Peptide	–	Peptide	–	Peptide
1. DR1/DR1; DQ6/DQ3	<250	1000	<250	<250	<250	<250	<250	2300
2. DR1/DR3; DQ5/DQ2	<250	<250	<250	<250	<250	<250	<250	2500
3. DR3/DR4; DQ2/DQ3	<250	2400	<250	<250	<250	<250	<250	<250
4. DR3/DR11; DQ2/DQ3	<250	<250	<250	<250	<250	<250	<250	7500
5. DR3/DR11; DQ2/DQ3	<250	<250	<250	<250	<250	<250	<250	2500
6. DR4/DR7; DQ2/DQ5	<250	<250	<250	<250	<250	<250	<250	2000
7. DR4/DR13; DQ3/DQ6	<250	2400	<250	<250	<250	<250	<250	1800
8. DR4/DR13; DQ3/DQ3	<250	7500	<250	<250	<250	<250	<250	10000
9. DR7/DR9; DQ2/DQ3	<250	5000	<250	<250	<250	<250	<250	20000
10. DR13/DR8; DQ6/DQ4	<250	8500	<250	<250	<250	<250	<250	2500
11. DR13/DR11; DQ3/DQ3	<250	<250	<250	<250	<250	<250	<250	2600
12. DR15/DR3; DQ6/DQ2	<250	<250	<250	<250	<250	<250	<250	3100
13. DR15/DR4; DQ6/DQ3	<250	7000	<250	<250	<250	<250	<250	10000
14. DR15/DR13; DQ6/DQ6	<250	<250	<250	<250	<250	<250	<250	8000

IL-2, IL-4, IL-5 and IFN-γ productions were evaluated in 48 h-culture supernatant of $CD4^+$ T cells immunized or not *ex vivo* with 50 μg/ml of Nef 56–68 peptide in the presence of autologous DCs as APCs (n = 14 donors).

T cells with Nef 56–68 peptide 15 days later (data not shown). Confirming in the human model the DQ dependence of the response to the Nef 56–68 peptide which was suspected in the mouse model, this production of Th1 cytokines was inhibited by about 80% when *ex vivo* immunizations with peptide were performed in the presence of an anti HLA-DQ antibody (20 μg/ml) whereas an anti HLA-DR antibody was without effect (data not shown).

Three days after *ex vivo* immunization with the Nef 56–68 peptide, $CD4^+$ T cells from naïve donors expressed the CD69, CD28 and CD40L activation markers (Fig. 2a). From day 6 after activation, we observed a strong decrease in CD62L expression and the induction of the CD45RO molecule, which was expressed on 36% of $CD4^+$ T cells at day 10 (Fig. 2a,b), whereas the ratio CD45RA/CD45RO was not modified in unstimulated cells (data not shown). So, a memory T cell response was rapidly (under 10 days) induced after stimulation with the Nef 56–68 peptide. At this time of culture, after separation of $CD45RA^+$ and $CD45RO^+$ T cells, production of IFN-γ was observed only in the memory population and amplified after specific restimulation with the Nef 56–68 peptide (Fig. 2c).

Preferential usage of Vβ6·1 by Nef 56–68 specific T cell clones

Finally, we derived Nef 56–68 specific T cell clones from 6 of the 14 HLA-typed healthy donors. These T cell clones exhibited the same characteristic phenotype and Th1 cytokine profile described above (data not shown). They strikingly expressed the same TCR Vβ6·1 gene segment although they were derived from donors expressing different HLA genotypes (Fig. 3a). This Vβ6·1 recruitment was, however, specific for the Nef 56–68 peptide since using the same experimental procedure, Th1 clones specific for two other promiscuous peptides (HA 307–319 and TT 830–846) expressed different TCR Vβ usage (Vβ2 and Vβ5, respectively) (Fig. 3b).

DISCUSSION

In this study, we gave particular interest to the sequence 56–68 of the Nef regulatory protein which exhibited both antigenic and immunogenic properties for T and B cells as described in rodents (mice and rat) and in nonhuman primates (chimpanzee) [16,17]. Interestingly, this sequence is a conserved region of the HIV Nef protein represented among various HIV strains. Our search for Nef-derived Th epitopes, identified the peptide 56–68 as the first promiscuous HLA-DQ HIV-derived peptide capable of priming an HIV-specific Th1 response. The promiscuous peptides described so far, such as TT 830–846, HA 307–319 or other HIV-derived peptides [13–15,28,29] are restricted to HLA-DR molecules. In fact a large number of HLA-DR molecules are known to share common criteria regard their interaction with antigenic peptides [30] which may account for the existence of promiscuous HLA-DR epitopes. Similarities have been already highlighted for the HLA-DQ molecules [31,32] but none of the previously described peptides bound as many HLA-DQ molecules as the Nef 56–68 peptide. In particular, this peptide is more active than the reference peptides in binding to HLA-DQ2 and HLA-DQ8 molecules, demonstrating a high affinity for these molecules.

This wide specificity for HLA-DQ molecules may result from the presence of four consecutive glutamic acid residues within the nef peptide sequence. Indeed, glutamic acid, situated at the C-terminal part of the peptide, may represent an advantageous anchor candidate for the P6/7 to P9 pocket of different HLA-DQ alleles [31,32]. Such a pattern strongly suggests the existence of an HLA-DQ supermotif. In contrast, the glutamic residue generally appears to be a deleterious residue for P6 to P9 HLA-DR contact and hence may dramatically diminish the capacity of Nef 56–68 to bind HLA-DR molecules [30].

As a result, this peptide led to a vigorous cellular and humoral response in HLA-DQ6 and DQ8 mice while the response was

(a)

	Day 3	Day 6	Day 10
CD25	1%	4%	7%
CD28	10%	17%	16%
CD69	74%	7%	2%
CD40L	10%	17%	12%
HLA-DR	5%	8%	8%
CD45RA	**92%**	**75%**	**44%**
CD45RO	**10%**	**26%**	**36%**
CD62L	**88%**	**70%**	**58%**

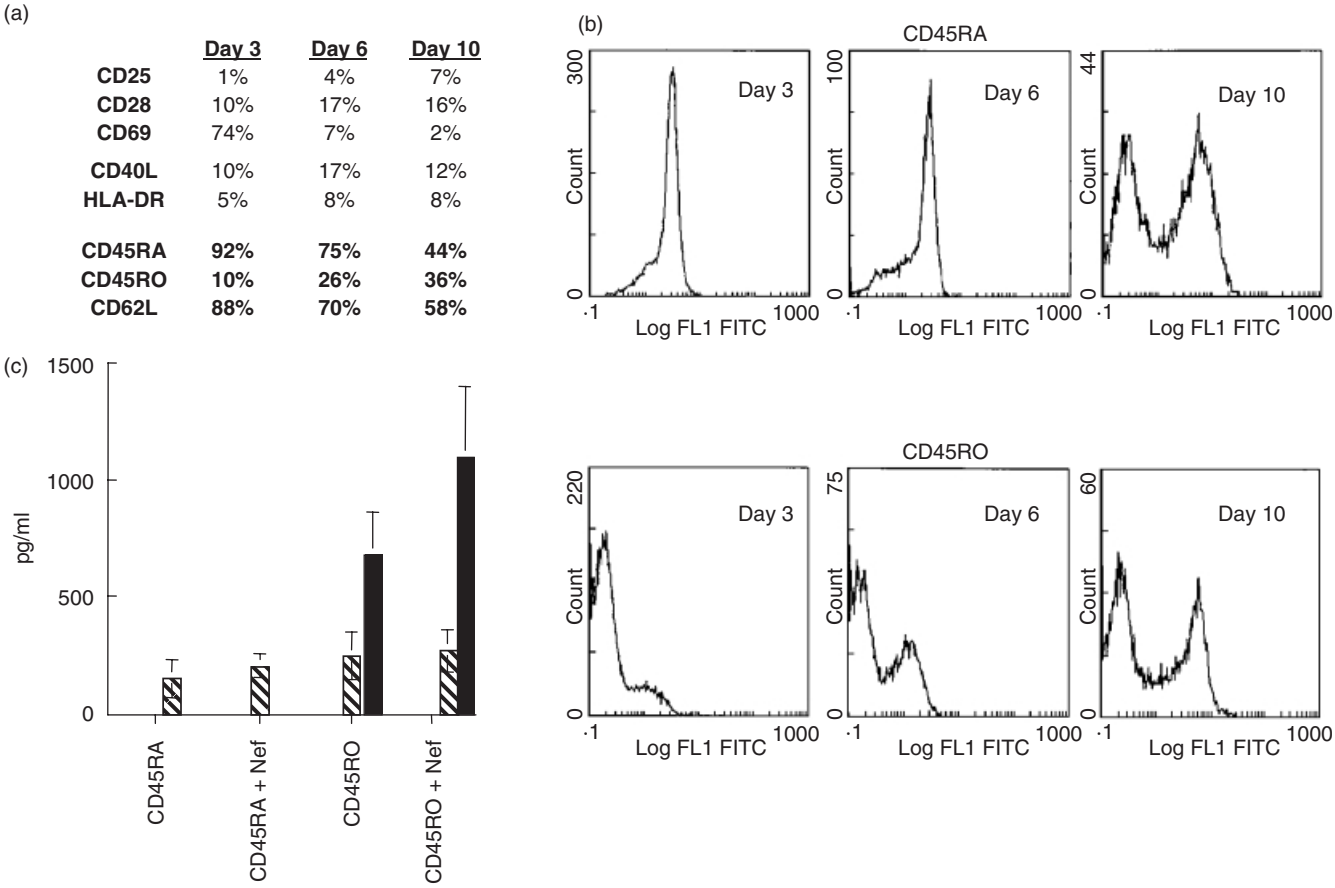

Fig. 2. Human memory CD4⁺ T cell specific for Nef 56–68 peptide. (a) Phenotypic characteristics of CD4⁺ T cells from HLA-typed healthy donors (n = 6) 3, 6 and 10 days after *ex vivo* immunization with 50 μg/ml of Nef 56–68 peptide in the presence of DCs as APCs. This phenotype is representative for the 6 donors. (b) Expression of CD45RA⁺ and CD45RO⁺ populations in Nef 56–68 specific CD4⁺ T cells from 3 to 10 days after *ex vivo* immunization. Representative profile for the 6 donors. (c) Cytokine secretion (▫ IL-2; □ IL-4; ▪ IL-5; ▨ IL-10; ■ IFN-γ) in Nef 56–68 specific CD45RA⁺ and CD45RO⁺ CD4⁺ T cells stimulated with or without 50 μg/ml of peptide 10 days after *ex vivo* immunization.

very weak in HLA-DR transgenic mice. Accordingly, similar conclusions emerged from *ex vivo* immunization with the Nef 56–68 peptide using cells from 14 healthy donors expressing different HLA genotypes. All immunizations led to proliferation mediated by HLA-DQ molecules. Interestingly, the Nef 56–68 specific T cells displayed a type 1 cytokine secretion phenotype. This type 1 profile, obtained systematically, was assigned to the usage of DCs derived from peripheral blood monocytes: in the human system, myeloid DCs have been shown to generate Th1 responses whereas lymphoid/plasmacytoid DCs generate Th2 responses [33]. However, it has recently been reported that, depending on the DC culture conditions and activation signals, DCs may acquire the capacity to induce either Th1, Th2 or Th0/Tr1 (T regulatory) T-cell responses [34,35]. This seems to indicate that the type of the T-cell response induced by DCs depends on the nature of the DC-activating stimulus, and less on their ontogeny. Consequently, this Th1-biased production was probably favoured by our experimental conditions and the use of immature DCs.

The Nef 56–68-specific cells rapidly display a memory T cell phenotype, quantified by a strong diminution of CD62L and the appearance of the CD45RO marker. They also produced IFN-γ after restimulation with Nef 56–68 and could be considered as 'effector memory' T cells [36]. These cells lack lymph node homing receptors but express receptors to enter into inflamed tissues; therefore, their function is to provide immediate protection to contain pathogens in peripheral tissues.

Surprisingly, all T cell clones obtained after *ex vivo* immunization with Nef 56–68 were found to express the same TCR Vβ6·1 gene segment, despite the fact that they were derived from donors expressing different HLA genotypes. This preferential usage was, however, specific for the Nef 56–68 peptide as T cell clones specific for two other peptides, also described as promiscuous (HA 307–319 and TT 830–846), expressed different TCR Vβ usage. Previous studies in both mice and humans have frequently shown a limited usage of V gene segments in TCR that recognize defined peptide/MHC complexes. In some cases usage was correlated with MHC restriction or antigen specificity [37–39]. It seemed difficult to associate the preferential Vβ6·1 expression of the Nef-specific clones with the DQ promiscuous property of the peptide since the 6 donors showed various DQ-genotypes. This biased TCR gene usage may reflect a relatively low precursor frequency of high avidity T cells [40]. Furthermore, in peptide-specific T cell responses, TCR have often been found with CDR3 loops that are similar in both length and amino acid

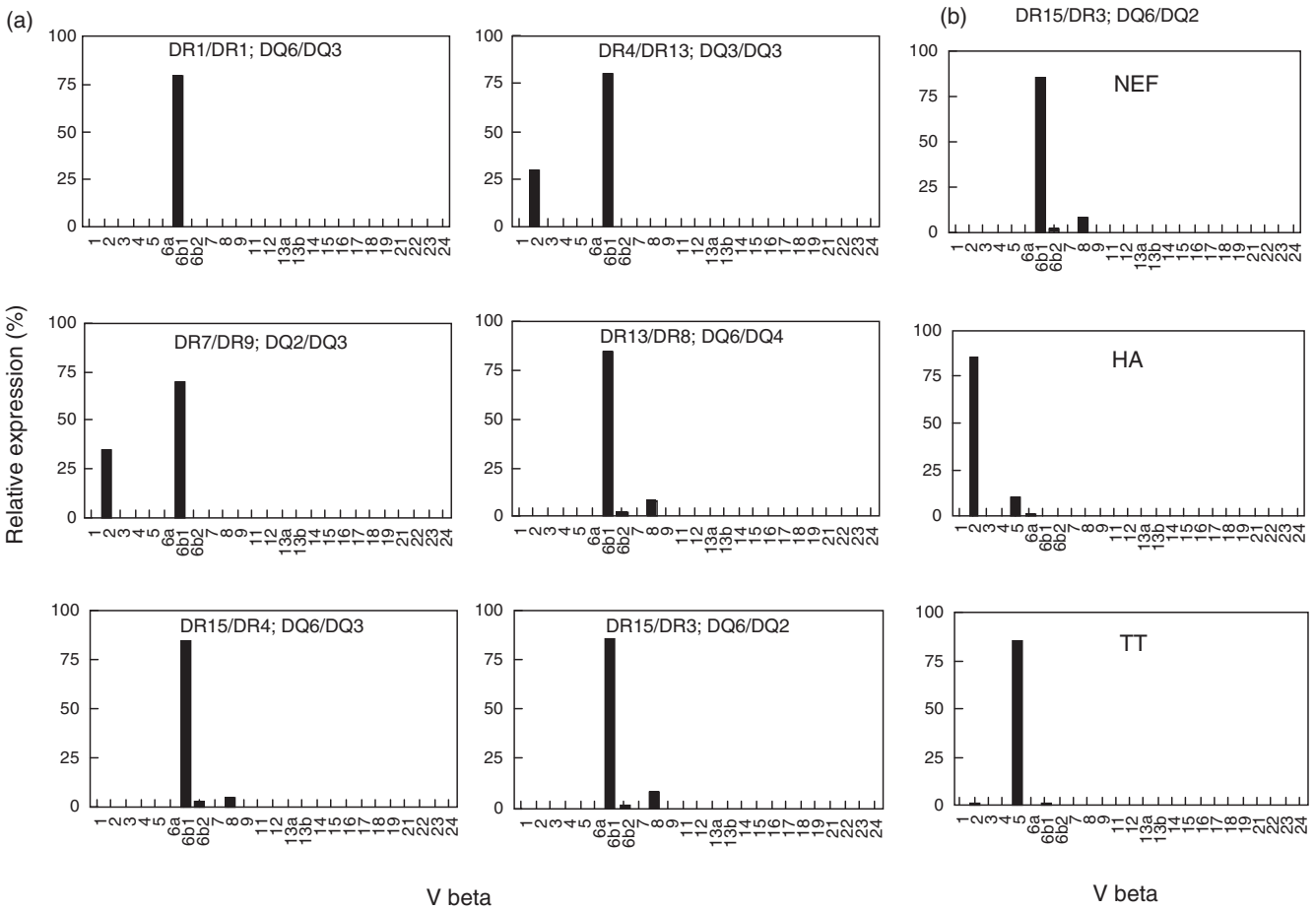

Fig. 3. Restricted usage of Vβ6·1 by Nef 56–68 T cell clones Amplification of specific Vβ gene segments was performed by PCR. (a) In Nef 56–68 specific CD4$^+$ clones derived from 6 different HLA-typed healthy donors. (b) In Nef 56–68, HA 307–319 and TT 830–846 specific CD4$^+$ clones derived from the same donor. The relative amount of TCR Vβ gene transcripts was described as the ratio (%) of each Vβ to total Vβ.

composition, suggesting that particular junctional sequences have an important role in determining the specificity for individual peptide/MHC complexes. It is possible that the peptide influences V gene usage, while the MHC may be flexible enough to interact with either the V and/or CDR3 region of the TCR.

The aim of this work was to identify an HIV-derived peptide able to react with several HLA molecules and therefore be potentially immunogenic in numerous individuals. We identified peptide 56–68 from the Nef protein as a promiscuous HLA-DQ peptide that could potentially be used as a component of a multiepitopic vaccine for the treatment of HIV infection, in addition to CTL epitopes for example. Moreover we were able to induce HIV-specific memory CD4$^+$ T cells producing IFN-γ in all the healthy donors tested and, in the context of HIV-infection, this appears to be of considerable interest. Although HAART appears to partially reconstitute immunity to microbial antigens no effect on enhancing immune responses to HIV antigens in chronic infection [41] has so far been demonstrated. Moreover a dominant Th1 cytokine profile is associated with a lack of progression in HIV infection [42]. We propose, using an *ex vivo* immunization protocol with the Nef 56–68 peptide, to induce/reconstitute an HIV-specific protective response in HIV-infected patients. Studies will be underway shortly with a cohort of HIV$^+$ patients who have stable CD4 counts and in whom the T cell response seems not to differ from that of healthy donors. Consequently, we could envisage the adoptive transfer of autologous CD4$^+$ T cells bearing the protective phenotype in addition (or alternatively) to HAART as an alternative strategy for treatment of HIV infection.

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