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Broad and Cross-Clade CD4+ T-Cell Responses Elicited by a DNA Vaccine Encoding Highly Conserved and Promiscuous HIV-1 M-Group Consensus Peptides

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Abstract

T-cell based vaccine approaches have emerged to counteract HIV-1/AIDS. Broad, polyfunctional and cytotoxic CD4+ T-cell responses have been associated with control of HIV-1 replication, which supports the inclusion of CD4+ T-cell epitopes in vaccines. A successful HIV-1 vaccine should also be designed to overcome viral genetic diversity and be able to confer immunity in a high proportion of immunized individuals from a diverse HLA-bearing population. In this study, we rationally designed a multiepitopic DNA vaccine in order to elicit broad and cross-clade CD4+ T-cell responses against highly conserved and promiscuous peptides from the HIV-1 M-group consensus sequence. We identified 27 conserved, multiple HLA-DR-binding peptides in the HIV-1 M-group consensus sequences of Gag, Pol, Nef, Vif, Vpr, Rev and Vpu using the TEPITOPE algorithm. The peptides bound in vitro to an average of 12 out of the 17 tested HLA-DR molecules and also to several molecules such as HLA-DP, -DQ and murine IA^b and IA^d. Sixteen out of the 27 peptides were recognized by PBMC from patients infected with different HIV-1 variants and 72% of such patients recognized at least 1 peptide. Immunization with a DNA vaccine (HIVBr27) encoding the identified peptides elicited IFN-γ secretion against 11 out of the 27 peptides in BALB/c mice; CD4+ and CD8+ T-cell proliferation was observed against 8 and 6 peptides, respectively. HIVBr27 immunization elicited cross-clade T-cell responses against several HIV-1 peptide variants. Polyfunctional CD4+ and CD8+ T cells, able to simultaneously proliferate and produce IFN-γ and TNF-α, were also observed. This vaccine concept may cope with HIV-1 genetic diversity as well as provide increased population coverage, which are desirable features for an efficacious strategy against HIV-1/AIDS.

Introduction

The development of an efficacious vaccine against human immunodeficiency virus 1 (HIV-1) still remains as the best long-term approach to control the acquired immunodeficiency syndrome (AIDS) pandemic since resource-poor endemic regions are not able to afford sustained antiretroviral therapy (ART). Clinically tested HIV-1 vaccines have shown no or modest efficacy so far [1,2]. No vaccine strategy was able to induce broadly neutralizing antibodies and T-cell based vaccines have thus emerged as an alternative to counteract AIDS by limiting both viral transmission and disease progression [3]. Indeed, a recent study using non-human primates (NHP) has demonstrated that vaccine-induced virus-specific effector memory T-cell (TEM) responses can exert a profound early control on highly pathogenic simian immunodeficiency virus (SIV) infection after mucosal

challenge, which has given more hope for the development of new T-cell based vaccines against HIV-1 [4].

The breadth of T-cell responses induced against HIV-1 has become a central goal in AIDS vaccine development after the STEP trial failure [1,5]. In fact, different groups have shown that protection against SIV challenge is strongly associated with induction of either CD4+ or CD8+ T cells against multiple targets [6–9]. Thus, it is important to design novel vaccine platforms in order to broaden T-cell responses against HIV-1.

T-cell based vaccines against HIV-1 are frequently focused on the induction of CD8+ T-cell responses, which are known to be responsible for killing virus-infected targets [6,10–12]. However, mounting evidence suggests that CD4+ T-cell responses may be important for controlling HIV-1 replication [13]. Although HIV-specific CD4+ T cells are preferentially targeted by the virus, the vast majority of these cells remains virus-free at any time in vivo

[14], which may allow for their antiviral function. In fact, strong virus-specific CD4^{+} T-cell responses have been associated with natural control of HIV-1 infection [15,16] and cytotoxic CD4^{+} T cells were shown to suppress viral replication in both SIV and HIV-1-infected cells [17,18]. While the clinical associations of CD4^{+} T-cell responses with HIV-1 control must be carefully interpreted, due to a possible cause-effect issue, the finding that CD4^{+} T-cell depletion reduced vaccine-mediated protection [19] supports a direct role of such cells in HIV-1 immunity. Moreover, some groups have observed the association of vaccine-induced virus-specific CD4^{+} T-cell responses with protection against SIV challenge [7,20], which further supports a protective role of CD4^{+} T cells. Therefore, it is important to explore the anti-viral immunity exerted by CD4^{+} T cells in order to develop novel vaccines against HIV-1/AIDS. It is possible that the induction of CD4^{+} T cells will be beneficent both due to the help provided to B cells and CD8^{+} T cells as well as due to direct effects on HIV-1-infected targets.

An important concern regarding AIDS vaccine development is how to elicit cellular immune responses to cover multiple HIV-1 circulating variants, which can differ by up to 20% within a subtype and show up to 35% of amino acid divergences between subtypes [21]. Artificially designed M-group consensus sequences display average distances to HIV-1 variants similar to those found intra-subtype and have been considered a potential alternative to circumvent the barrier posed by viral genetic diversity [22]. Indeed, studies have demonstrated that immunogens based on HIV-1 M-group consensus Env were able to provide broad cross-clade T-cell responses in both mice and macaques [23,24], which suggests an important role for this strategy in HIV-1 vaccines.

The high polymorphism of human leukocyte antigens (HLA), which are responsible for determining the onset of T-cell responses, is also a challenge for vaccine development. It is expected that different HLA-bearing populations respond differently to the same immunogen and this may be decisive for the vaccine success. Vaccines encoding promiscuous peptides, each binding to multiple HLA molecules, may be a solution to this problem by allowing that multiple HLA molecules spread among the population contribute to the induction of broad T-cell responses in most of the immunized individuals. This would confer broader population coverage and enhance vaccine efficacy [25–27]. Thus, novel AIDS vaccines should be rationally designed to address both viral and host genetic diversity in order to confer immunity against multiple HIV-1 circulating variants in a population with diverse HLA alleles.

The inclusion of appropriate proteins in HIV-1 vaccines may be crucial for eliciting protective responses. While broad Gag- and Vif-specific responses have been correlated to vaccine-induced protection in SIV-challenged macaques [28], induction of Env-specific CD4^{+} T-cell responses contributed to enhanced SIV replication and accelerated progression to AIDS [29]. Env-specific CD8^{+} T-cell responses were also shown to be a strong predictor for disease progression in HIV-1-infected patients [30]. Furthermore, CD4^{+} T-cell responses targeting Gag and Env-specific epitopes were associated with spontaneous control of viral replication and progression to AIDS, respectively [31].

Recently, our group has designed a DNA vaccine encoding 18 conserved, multiple HLA-DR-binding epitopes from HIV-1 subtype B consensus sequence. This vaccine elicited broad, polyfunctional and long-lasting CD4^{+} T-cell responses in BALB/c and HLA class II transgenic mice [32,33]. In this work we sought to develop a DNA vaccine that would be able to provide broad CD4^{+} T-cell immunity in a diverse HLA-bearing population, now targeting multiple HIV-1 M-group consensus peptides, potentially cross-reactive to a high proportion of circulating HIV-1 variants. In addition, we excluded Env peptides from our novel vaccine based on the evidence that Env-specific T-cell responses are frequently related to disease progression.

To accomplish our goals, we used the TEPITOPE algorithm [34], which has been successfully applied for *in silico* identification of promiscuous T-cell epitopes in the context of oncology, allergy, autoimmunity and infectious diseases [35–40], to scan the HIV-1 M-group consensus sequence. We identified 27 peptides from 7 different HIV-1 proteins (Gag, Pol, Nef, Vif, Vpr, Rev and Vpu), predicted to bind to multiple HLA-DR molecules and conserved among all M-group subtypes. The identified peptides bound *in vitro* to several HLA-DR, -DP and -DQ molecules and also to murine IA[b] and IA[d] molecules. The peptides were antigenic in natural infection, being recognized by peripheral blood mononuclear cells (PBMC) from HIV-1-infected patients. Finally, we designed a DNA vaccine (HIVBr27) encoding the 27 peptides *in tandem* and immunized BALB/c mice. HIVBr27 immunization elicited broad, cross-clade and polyfunctional CD4^{+} and CD8^{+} T-cell responses.

Materials and Methods

Ethics Statement

The research involving human participants reported in this study was approved by the institutional review board of the University of São Paulo under protocol number 0458/08. Written informed consent was obtained from all subjects.

Mice were housed and manipulated under SPF conditions in the animal care facilities of the Institute of Tropical Medicine, University of São Paulo (IMT/FMUSP). Experiments were performed in accordance to the guidelines of the Ethics committee of University of São Paulo (CAPPesq- HCFMUSP). This study was approved by CAPPesq- HCFMUSP under protocol number 0653/09.

Identification of HIV-1 M-group Consensus Peptides

We scanned the HIV-1 M-group proteome consensus sequence available at http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html with the TEPITOPE algorithm to identify multiple HLA-DR-binding peptides [34]. The TEPITOPE algorithm predicts binding of peptides to 25 distinct HLA-DR molecules based on results from *in vitro* HLA-peptide binding assays. We selected the peptides predicted to bind to at least 18 out of the 25 HLA-DR molecules in the TEPITOPE matrix, using a threshold of 5%. The selected peptides were further analyzed regarding the conservation status when compared to consensus sequences of the HIV-1 subtypes A, B, C, D, F1, F2, G and H. We ended up with 27 peptides (14–24 aa in length) that had each amino acid conserved among at least 50% of the consensus sequences from the HIV-1 subtypes.

Peptide Synthesis

Peptides were synthesized by solid phase technology using 9-fluorenylmethoxycarbonyl (Fmoc) strategy, with the C terminal carboxyl group in amide form (GL Biochem). Peptide purity and quality were assessed by reverse-phase high performance liquid chromatography and mass spectrometry, and was routinely above 90%.

HLA Class II and Murine MHC Class II Peptide-binding Assays

Peptide binding assays were performed by incubating purified HLA class II molecules (HLA-DR, -DP and -DQ) or murine IA[b] and IA[d] molecules (5–500 nM) with different concentrations of

unlabeled peptide inhibitors and 1–10 nM ^{125}I-radiolabeled probe peptides for 48 h as previously described [41].

Construction of a DNA Plasmid Encoding Multiple HIV-1 Peptides

We designed a multiepitopic construct encoding the 27 HIV-1 peptides: protease (53–75), protease (79–95), RT (343–357), RT (354–368), RT (369–391), RT (413–427), RT (431–445), RT (528–546), integrase (28–43), integrase (69–85), integrase (96–113), integrase (216–235), integrase (249–268), p17 (72–90), p17(131–132)/p24(1–18), p24 (33–48), p24 (127–145), p24 (138–153), p24 (182–201), vif (1–15), vif (142–158), rev (9–27), vpr (29–42), vpr (58–80), vpu (13–26), nef (67–87) and nef (133–156). Peptide sequences were codon optimized for mammalian expression and assembled in tandem in the above mentioned order with glycine-proline (GPGPG) spacers at C and N termini to avoid the creation of junctional peptides, which can interfere on processing and presentation [42]. The artificial gene (EZBiolab) was subcloned into pVAX1 vector (Invitrogen) using EcoRI/XhoI sites to generate the HIVBr27 plasmid, which was purified using the Endofree Plasmid Giga Kit (Qiagen) according to manufacturer's instructions.

Selection of Variants from HIV-1 M-group Consensus Peptides

The peptides p24(127–145), RT(413–427), RT(528–546) and int(216–235) were aligned with all HIV-1 circulating variants available at http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGN/QuickAlign.html. Sequences from the most frequent variants of each peptide were synthesized to perform immunological analysis.

Subjects

Cryopreserved peripheral-blood mononuclear cells (PBMC) were obtained from a cohort of HIV-1-infected individuals (n = 25) [43,44], as well as from healthy volunteers (n = 5), and were used for IFN-γ ELISPOT assay. Infecting viral strains were characterized as previously described [45,46]. Clinical characteristics of enrolled patients are summarized in the table S1.

Mice and Immunizations

Six to eight week-old female BALB/c mice were used in this study. Six mice per group were injected intramuscularly, at weeks 0, 2 and 4, with HIVBr27 plasmid or empty pVAX1. Each quadriceps was injected with 50 μL of DNA at a concentration of 1 μg/μL in saline such that each animal received a total of 100 μg of plasmid DNA per immunization. Two weeks after the last DNA immunization, mice were euthanized with CO_2.

Spleen Cell Isolation for Immune Assays

Two weeks after the last immunization, mice were euthanized and spleens were removed aseptically. After obtaining single cell suspensions, cells were washed in 10 mL of RPMI 1640. Cells were then resuspended in R-10 (RPMI supplemented with 10% of fetal bovine serum (GIBCO), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 1% vol/vol non-essential amino acids solution (Life Technologies), 1% vol/vol vitamin solution (Life Technologies), 40 mg/mL of Gentamicin, 20 mg/mL of Peflacin and 5×10^{-5} M 2β-mercaptoetanol (Life Technologies). The viability of cells was evaluated using 0.2% Trypan Blue exclusion dye to discriminate between live and dead cells. Cell concentration was estimated with the aid of a Neubauer chamber and adjusted in cell culture medium.

Detection of IFN-γ Producing Human and Murine Cells by ELISPOT Assay

PBMC (1×10^5 cells/well) from HIV-1-infected patients and splenocytes (3×10^5 cells/well) from HIVBr27 or pVAX1 immunized mice were tested for their ability to secrete IFN-γ after in vitro stimulation with 5 μM of individual or pooled HIV-1 peptides using ELISPOT assay. The ELISPOT assay was performed using human or murine IFN-γ Becton Dickinson kit according to manufacturer's instructions. Spots were counted using an AID ELISPOT reader (Autoimmun Diagnostika GmbH). The number of antigen-specific T cells, expressed as spot-forming units (SFU)/10^6 PMBC or SFU/10^6 splenocytes, was calculated after subtracting negative control values (medium only). Responses in human ELISPOT assay for each patient were considered positive when ≥ 50 SFU/10^6 PBMC [47]. Responses in murine ELISPOT were considered positive when > 15 SFU/10^6 splenocytes, which was calculated as the mean response +3 standard deviations (SD) of splenocytes from pVAX1 immunized mice, stimulated with each peptide.

CFSE-based Proliferation Assay

To monitor the expansion and proliferation of HIV-1-specific T cells, splenocytes from HIVBr27 or pVAX1 immunized mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) [48]. Briefly, freshly isolated splenocytes were resuspended (50×10^6/mL) in PBS and labeled with 1.25 μM of CFSE (Molecular Probes) at 37°C for 10 minutes. The reaction was quenched with RPMI 1640 supplemented with 10% FBS and cells were washed before resuspending in RPMI 1640 at a density of 1.5×10^6/mL. Cells were cultured in 96 well round-bottomed plates (3×10^5/well in triplicate) for 5 days at 37°C and 5% CO_2 with medium only or 5 μM of HIV peptides. Positive controls were stimulated with 2.5 μg/mL of Concanavalin A (Sigma). Cells were then harvested, washed with 100 μL of FACS buffer (PBS with 0.5% BSA and 2 mM EDTA) and stained with anti-mouse CD3 phycoerythrin (PE), anti-mouse CD4 peridinin chlorophyll protein (PerCP) and anti-mouse CD8 allophycocyanin (APC) monoclonal antibodies (BD Pharmingen) for 45 minutes at 4°C. Samples were acquired on a FACSCanto flow cytometer (BD Biosciences) and then analyzed using FlowJo software (version 9.0.2, Tree Star). Fifty thousand events were acquired in a live lymphocyte gate. The percent of proliferating CD4$^+$ and CD8$^+$ CFSE^{low} cells was determined in the CD3$^+$ T-cell population. Positive proliferation of T cells was determined as CFSE^{low} T cells > cutoff, which was calculated as median +3 SD of unspecific proliferative responses obtained with splenocytes from pVAX1 immunized mice stimulated with HIV-1 peptides.

Analysis of Polyfunctional HIV-1-specific T-cell Responses

Splenocytes from immunized mice were labeled with CFSE as described above. CFSE-labeled cells were cultured in 96 well round-bottomed plates (5×10^5/well in triplicate) for 4 days at 37°C and 5% CO_2 in the presence of medium only or pooled HIV-1 peptides (5 μM). After incubation, cells were restimulated with 2 μg/mL anti-CD28 (BD Pharmingen), 5 μM of pooled HIV-1 peptides and Brefeldin A- GolgiPlugTM (BD Pharmingen) for the last 12 hours. After this period, cells were washed with FACS buffer and surface stained using the monoclonal antibodies anti-CD8-Alexa700 and anti-CD4-PerCP for 30 minutes at 4°C. Cells were then fixed and permeabilized using the Cytofix/CytopermTM kit (BD Pharmingen). Permeabilized cells were washed with Perm/Wash buffer (BD Biosciences) and stained with the monoclonal antibodies anti-CD3-APCCy7, anti-IL-2-PE, anti-

Peptide	Binding capacity/IC$_{50}$ (nM)																	HLA-DR binding	
	DRB1*01:01	DRB1*03:01	DRB1*04:01	DRB1*04:05	DRB1*07:01	DRB1*08:02	DRB1*09:01	DRB1*10:01	DRB1*11:01	DRB1*12:01	DRB1*13:02	DRB1*15:01	DRB1*16:02	DRB3*01:01	DRB3*02:02	DRB4*01:01	DRB5*01:01	Bound molecules	Frequency (%)
protease(53-75)	29	347	675	282	1769	325	234	62	1358	77	4080	111	388	545	9692	302	93	13	76.5
protease(79-95)	161	7977	146	824	9072	1594	2730	1131	7218	6118	253	165	5694	668	5173	2816	2042	6	35.3
RT(343-357)	81	23283	591	277	6212	947	335	19	428	239	24950	47	464	5610	8614	2921	1.6	11	64.7
RT(354-368)	44	7102	79	926	120	2.1	66	16	1761	4843	74	773	58	2846	1443	80	369	12	70.6
RT(369-391)	65	287	146	2631	20	24	310	24	329	471	1291	447	2133	6234	8841	20	68	12	70.6
RT(413-427)	26	469	42	78	39	58	57	12	312	260	2224	329	10	2394	4.4	1419	14	14	82.4
RT(431-445)	5.1	107	23	50	75	19	23	4.4	3188	293	266	230	80	13	611	243	148	16	94.1
RT(528-546)	16	612	19	68	41	6.3	56	33	27	3152	2104	97	346	576	82	57	16	15	88.2
integrase(28-43)	531	14823	161	1998	1711	119	1558	23	960	22667	10140	5335	3889	6755	5955	23	731	7	41.2
integrase(69-85)	0.56	7945	7.6	430	70	11	58	4.8	318	2937	16951	146	167	624	7192	56	523	13	76.5
integrase(96-113)	4.3	224	1769	629	1383	365	390	13	27	1507	4412	284	106	583	7475	3079	4.4	11	64.7
integrase(216-235)	0.7	11	203	179	63	8.8	542	45	288	6.6	1933	5.3	7.1	39	414	2078	9.5	15	88.2
integrase(249-268)	110	90	2866	6077	5714	89	7042	913	28	2277	5421	139	500	2651	4531	847	46	9	52.9
p17(72-90)	20	652	279	421	661	73	217	39	1072	1676	1050	143	140	907	6290	1098	435	12	70.6
p17(131-132)/p24(1-18)	4.4	3350	83	428	5417	71	248	59	496	1125	940	211	68	517	2880	131	547	13	76.5
p24(33-48)	1.1	12854	5.7	49	2627	0.73	74	3.6	57	172	23102	18	4.9	8782	3915	58	112	12	70.6
p24(127-145)	10	91	66	286	2184	35	151	52	88	170	66	17	59	333	321	137	172	16	94.1
p24(138-153)	2.6	3909	12	49	9.4	3.1	36	6.6	79	49	156	72	5.9	1042	1048	25	24	14	82.4
p24(182-201)	10	4274	157	114	407	31	1291	19	336	738	1297	767	51	535	250	94	933	14	82.4
vif(1-15)	74	-	907	389	3810	1176	287	62	19450	9036	-	6915	1126	423	27233	1669	6583	6	35.3
vif(142-158)	0.35	4513	6.4	2.3	4.1	25	64	1.3	27	48	1666	7.1	3.8	942	577	78	2.4	15	88.2
rev(9-27)	7.4	632	13	64	41	9.5	124	3.9	68	55	2121	66	74	457	978	4.4	133	16	94.1
vpr(29-42)	71	-	427	1289	1386	130	1266	311	793	1248	-	112	367	7870	9487	278	731	9	52.9
vpr(58-80)	305	14386	361	294	885	61	477	170	724	845	5011	149	588	11559	4090	475	443	13	76.5
vpu(13-26)	261	-	19598	14939	-	765	6636	1780	12745	12673	-	2030	4359	11824	-	22922	1814	2	11.8
nef(67-87)	341	4805	98	1323	4027	46	205	105	681	150	3410	303	1720	6291	15080	22	191	10	58.8
nef(133-156)	315	704	747	398	472	2158	360	9.7	4543	4428	4290	718	2745	3438	-	1644	317	9	52.9
Peptides bound to HLA-DR (n)	27	12	24	21	14	24	21	25	19	14	6	24	20	14	8	18	24		

Figure 1. HLA-DR binding assay for M-group consensus peptides. Peptide binding assays were performed by incubating purified HLA-DR molecules (5–500 nM) with different concentrations of unlabeled peptide inhibitors and 1–10 nM ^{125}I-radiolabeled probe peptides for 48 h. Significant affinity threshold <1000 nM are shown in gray. A dash represents 50% inhibitory concentration (IC50) >30000 nM.
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TNFα-PECY7 and anti-IFNγ-APC for 30 minutes at 4°C. Following staining, cells were washed twice and resuspended in FACS buffer. All antibodies were from BD Pharmingen. Samples were acquired on a FACSCanto flow cytometer (BD Biosciences) and then analyzed using FlowJo software (version 9.0.2, Tree Star, San Carlo, CA). Cells were gated on forward scatter (FSC)/side scatter (SSC) gate (500,000 events) followed by CD3$^+$ gate and subsequent gates on CD4$^+$ or CD8$^+$ populations. After identification of CD4$^+$ and CD8$^+$ populations, we designed a gate in each positive population for IFN-γ, TNF-α and IL-2 expression within or not the CFSE^{low} gate. In addition, we used the Boolean gate (FlowJo software version 9.0.2, Tree Star) platform to create several combinations of the three cytokines (IFN-γ, TNF-α and IL-2) within CFSE^{low} population resulting in seven distinct patterns. The percentages of cytokine producing cells within CFSE^{low} population or not were calculated by subtracting background values. For each flow cytometry experiment performed in this paper, unstained and all single-color controls were processed to allow proper compensation.

Statistical Analysis

Statistical significance (p-values) was calculated by using Student's T test or Two-way ANOVA followed by Bonferroni post test. Statistical analysis and graphical representation of data was performed using GraphPad Prism version 5.0 software.

Results

Identification of Conserved, Multiple HLA-DR-binding Peptides in the HIV-1 M-Group Consensus Sequence

The HIV-1 M-group consensus sequence was scanned with the TEPITOPE algorithm in order to identify multiple HLA-DR-binding (promiscuous) peptides. The identified peptides were aligned with consensus sequences from HIV-1 subtypes A1, B, C, D, F1, F2, G and H. Twenty-seven peptides, from 7 different HIV-1 proteins, showing each amino acid conserved among at least 50% of the aligned subtypes consensus sequences were selected (table 1). No Tat peptides were identified with the employed threshold. Env peptides were identified but excluded from further analysis due to the potential prejudice of T-cell responses targeting such protein. We also performed an alignment in order to evaluate whether the 27 peptides from M-group consensus sequence were highly conserved among HIV-1 circulating variants. According to the alignment, most of our peptides had 0–1 amino acid substitutions when compared to all sequences from HIV-1 circulating variants available at the Los Alamos HIV Database (table S2). We performed in vitro HLA-peptide binding assays to confirm the TEPITOPE accuracy and observed that each of the 27 peptides bound to an average of 12 out of the 17 tested HLA-DR molecules and that each HLA-DR molecule bound to an average of 19 out of the 27 peptides (figure 1). The peptides also bound to several HLA-DP and –DQ molecules (table S3 and S4). We also performed murine MHC class II-peptide binding assays and observed that 21 and 6 peptides bound to IA^d and IA^b molecules, respectively (table 2). Thus, the scanning of HIV-1 M-group consensus sequence with TEPITOPE allowed the identification of highly conserved and promiscuous peptides.

HIV-1 M-group Consensus Peptides are Antigenic in Natural HIV-1 Infection

To evaluate whether the 27 predicted promiscuous peptides would be antigenic during natural infection we performed an IFN-γ ELISPOT assay using PBMC from twenty-five patients infected with different HIV-1 variants (Table S1). We observed that 7 out of the 13 Pol peptides were recognized at least once (figure 2A). Four out of the 6 Gag peptides were also recognized at least once (figure 2B). Among Vif, Rev, Vpr, Vpu and Nef, we observed recognition of 5 out of the 8 peptides (figure 2C). Overall, we found that 72% of the patients recognized at least 1 peptide and that each patient recognized an average of 2 peptides. In addition, our data shows that 16 out of the 27 peptides (60%) were recognized by HIV-1-infected patients, while no responses were observed in PBMC from healthy individuals (data not shown). Therefore, most of the conserved and promiscuous HIV-1 M-group consensus peptides identified with the TEPITOPE algorithm were showed to be antigenic in natural HIV-1 infection.
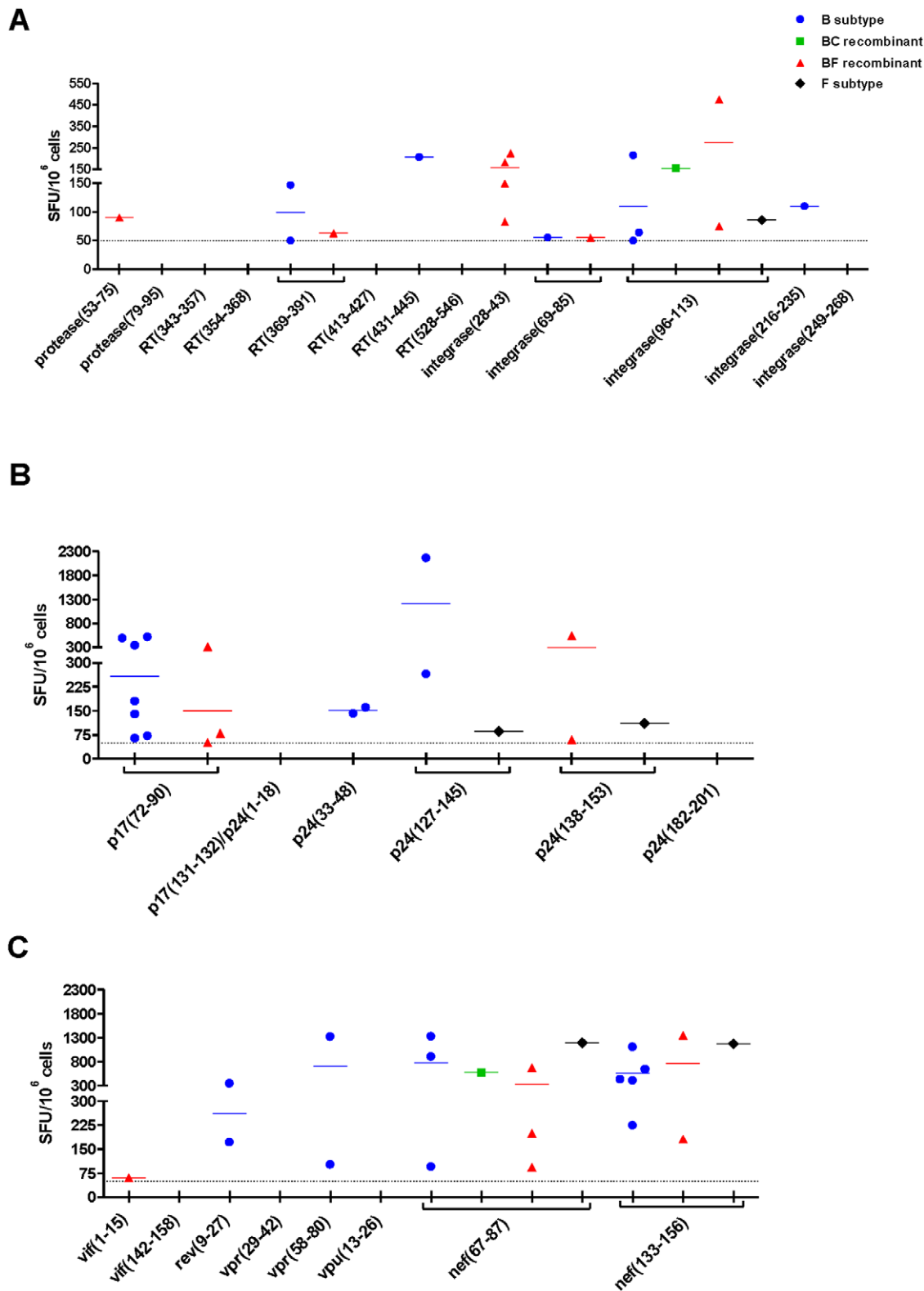
Figure 2. M-group consensus peptides are recognized by PBMC from HIV-1-infected patients. IFN-γ ELISPOT assay was performed to evaluate T-cell responses against the 27 M-group consensus peptides by stimulating PBMC from patients infected with different variants of HIV-1, which are represented by colored symbols. A) IFN-γ secretion against Pol peptides. B) IFN-γ secretion against Gag peptides. C) IFN-γ secretion against Vif, Rev, Vpr, Vpu and Nef peptides. B subtype-infected patients (n = 14), BC recombinant-infected patient (n = 1), BF recombinant-infected patients (n = 9), F subtype-infected patient (n = 1). Dotted lines represent IFN-γ ELISPOT cutoff, which is $\geq$50 spots/10[6] cells. Only positive responses are shown in the graphs.
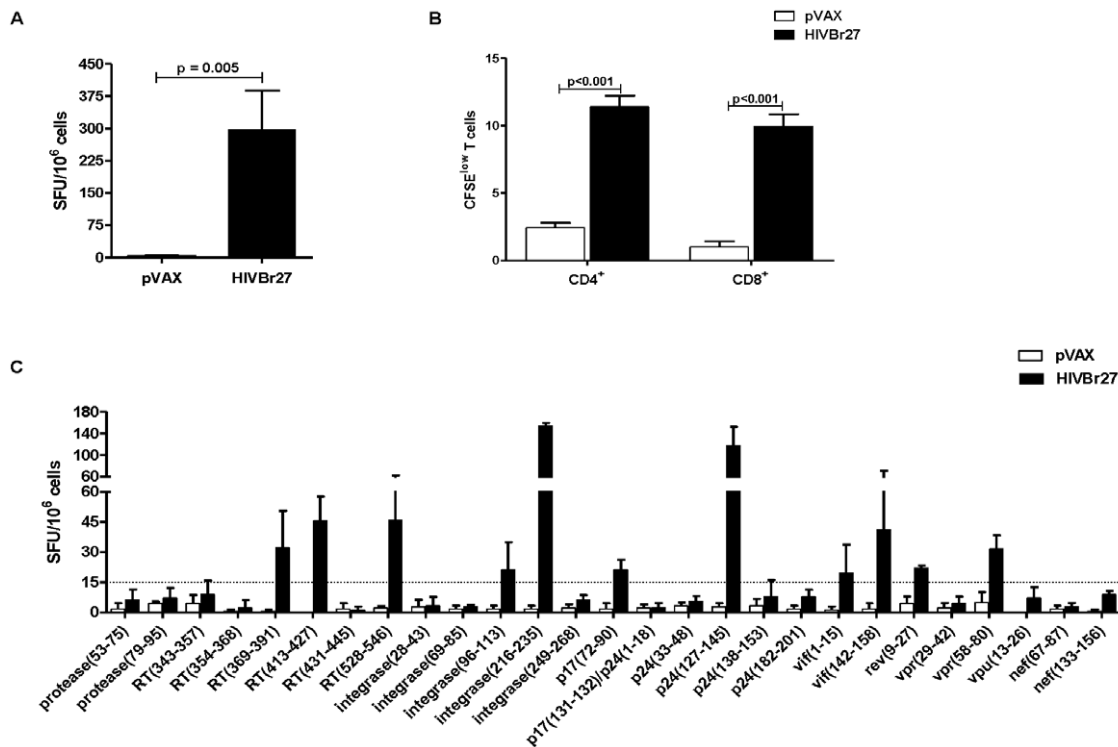doi:10.1371/journal.pone.0045267.g002

Figure 3. Immunization with HIVBr27 elicits T-cell responses in BALB/c mice. Two weeks after the last immunization with HIVBr27 or empty pVAX1, pooled spleen cells from 6 BALB/c mice were cultured in the presence of individual or pooled HIV-1 peptides (5 μM). Frequency of IFN-γ secreting cells (A) and proliferating CD4$^+$ and CD8$^+$ T-cells (B) against pooled HIV-1 peptides. C) Frequency of IFN-γ secreting cells against individual HIV-1 peptides. Dotted line represents ELISPOT cutoff values. Data are shown as mean of three independent experiments for ELISPOT assays. Data from proliferation assay are representative of three independent experiments.
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A DNA Vaccine Encoding 27 Conserved and Promiscuous HIV-1 M-group Consensus Peptides Elicits Broad T-cell Responses in BALB/c Mice

Given the fact that multiple peptides were able to bind to IA^d molecules, we chose the BALB/c strain to undergo immunization. To evaluate the immunogenicity of the 27 conserved and promiscuous HIV-1 M-group consensus peptides we designed a DNA vaccine (HIVBr27) encoding the peptides *in tandem*. We assessed the magnitude and breadth of vaccine-elicited HIV-1 specific immune response by IFN-γ ELISPOT and CFSE-based proliferation assays 2 weeks after the last immunization with HIVBr27 or empty pVAX1. HIVBr27 immunization elicited a significantly higher number of IFN-γ secreting cells when compared to empty pVAX1 (figure 3A) and elicited 10% of both CD4$^+$ and CD8$^+$ proliferating T cells against the pool of 27 HIV-1 peptides (figure 3B). To evaluate the breadth of T-cell responses elicited by HIVBr27, we used individual HIV-1 peptides to stimulate splenocytes *in vitro*. Immunization of BALB/c mice resulted in IFN-γ secretion against 11 peptides (figure 3C) and proliferation of CD4$^+$ and CD8$^+$ T-cells against 8 and 6 peptides, respectively (figure 4A and 4B). All peptides that stimulated T-cell proliferation also induced IFN-γ secretion. In contrast, pVAX1 immunized mice presented negligible numbers of IFN-γ secreting and proliferating CD4$^+$ and CD8$^+$ T cells after incubation with the same HIV-1 peptides in all performed experiments. Thus, a DNA vaccine encoding the 27 identified peptides was immunogenic in BALB/c mice, eliciting broad T-cell responses.

HIVBr27 Immunization Provides Cross-clade T-cell Immunity

To evaluate whether HIVBr27 would provide cross-clade immunity, we synthesized the most frequent variant sequences of the 4 most immunogenic HIVBr27 encoded peptides, which are RT(413–427), RT(528–546), int(216–235) and p24(127–145) (table S5), and used them to stimulate splenocytes from immunized BALB/c mice. HIVBr27 immunization elicited comparable frequencies of IFN-γ secreting cells against M-group peptides RT(528–546), int(216–235) and p24(127–145) when compared to most of their variants, while only IFN-γ secretion against RT(413–427) B and D variants was reduced (figure 5A). The same pattern was observed for CD4$^+$ and CD8$^+$ T-cell proliferation (figure 5B and 5C). We evaluated the avidity of T-cell responses against the 4 HIVBr27 encoded peptides and their variants in IFN-γ ELISPOT assay by serial peptide dilution and observed similar responses among all peptides in a concentration range from 5 μM to 5 pM (data not shown). Besides, most of the variant sequences kept the promiscuous profile observed in their parent peptides, according to the TEPITOPE algorithm (table S5). Taken together, these results suggest that HIVBr27 immunization elicits cross-clade T-cell responses of similar avidity, towards the most frequent variants of the M-group consensus peptides.

HIVBr27 Immunization Elicits Polyfunctional CD4$^+$ and CD8$^+$ T-cell Responses

To assess the functional profile of both CD4$^+$ and CD8$^+$ T-cell vaccine-induced responses we used multiparameter flow cytometry. The gating strategy is outlined in figure 6A. HIVBr27
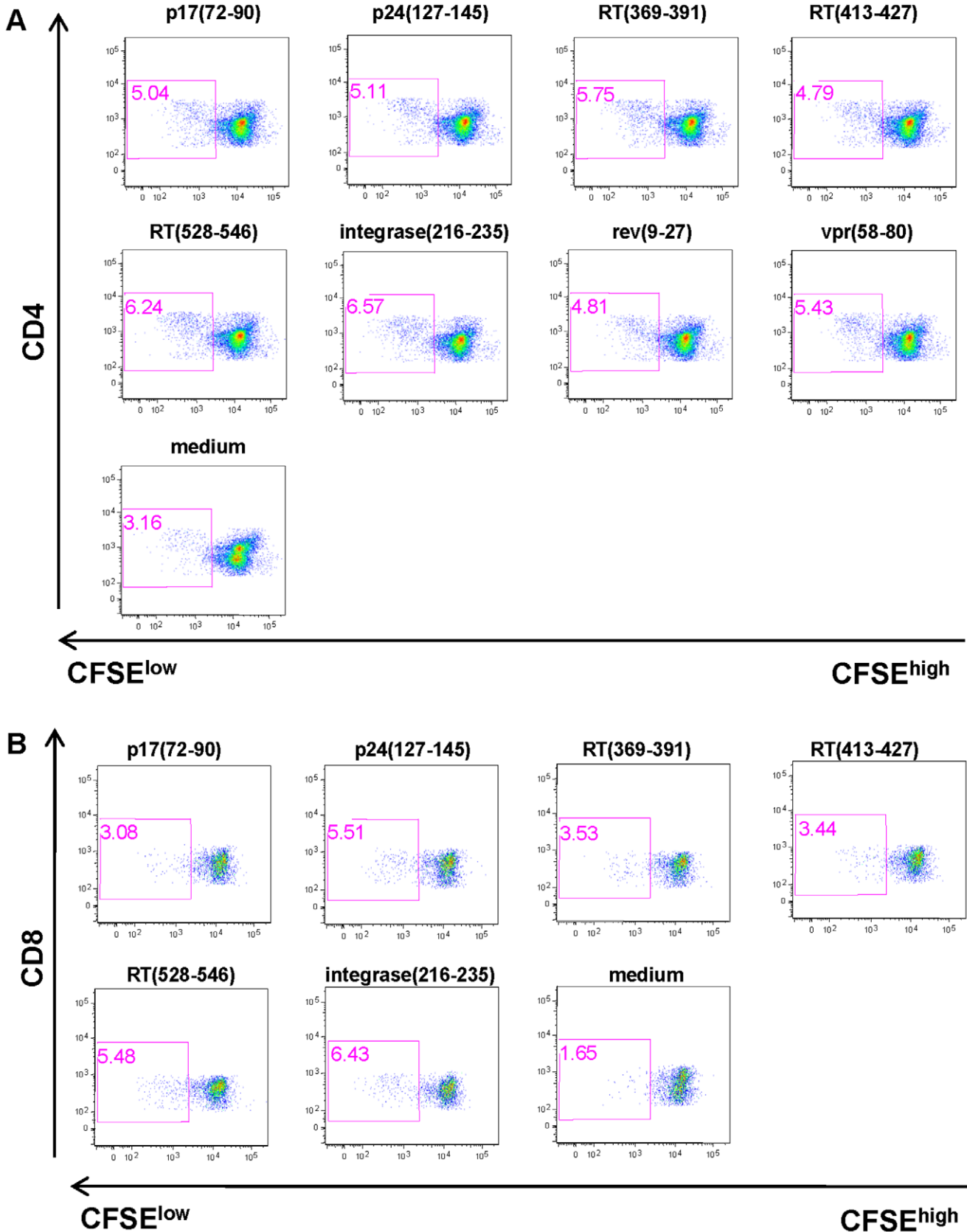
Figure 4. Immunization with HIVBr27 elicits CD4⁺ and CD8⁺ T-cell proliferation against multiple HIV-1 peptides. Two weeks after the last immunization with HIVBr27 or empty pVAX1, pooled spleen cells from 6 BALB/c mice were cultured in the presence of HIV-1 peptides (5 μM) or medium only. Splenocytes were labeled with CFSE (1.25 μM) and cultured for 5 days. After staining with fluorochrome-labeled anti-CD3, -CD4 and -

CD8 monoclonal antibodies, cells were analyzed by flow cytometry. CFSE dilution on gated CD3$^+$CD4$^+$ (A) or CD3$^+$CD8$^+$ (B) cells was used as readout for antigen-specific proliferation. Data are representative of three independent experiments.
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immunization elicited higher frequencies of IFN-γ and TNF-α producing CFSE^{low} CD4$^+$ T cells (4% and 3.4%, respectively) than CD8$^+$ T cells (2% and 1.8%, respectively) against pooled HIV-1 peptides (figure 6B). It is of note that there were lower frequencies of IL-2 producing CFSE^{low} CD4$^+$ and CD8$^+$ T-cells (0.34% and 0.36%, respectively) compared to other cytokine producing cells (figure 6B). To analyze whether HIVBr27 elicits polyfunctional T cells, Boolean combinations of cytokine-producing CFSE^{low} CD4$^+$ and CD8$^+$ T cells were generated. HIVBr27 immunization elicited about 0.2% of IFN-γ$^+$IL-2$^+$TNF-α$^+$ CFSE-^{low} CD4$^+$ T cells and less than 0.1% of IFN-γ$^+$IL-2$^+$TNF-α$^+$ CFSE^{low} CD8$^+$ T cells (figure 6C). We observed higher frequencies of both CFSE^{low} CD4$^+$ and CD8$^+$ T cells able to produce the combination of IFN-γ and TNF-α (2.5% and 1.12%, respectively) compared to triple cytokine producing CFSE^{low} T cells (figure 6C). Overall, these data showed that HIVBr27 immunization can elicit polyfunctional CD4$^+$ and CD8$^+$ T cells that simultaneously proliferate and produce effector cytokines.

Discussion

In the present study, we designed a DNA vaccine encoding 27 highly conserved and promiscuous peptides from the HIV-1 M-group consensus sequence. This vaccine (HIVBr27) elicited broad, cross-clade and polyfunctional CD4$^+$ and CD8$^+$ T-cell responses in BALB/c mice. The 27 encoded peptides bound *in vitro* to multiple HLA-DR, -DP, -DQ and to murine IA^b and IA^d molecules. Furthermore, the peptides were shown to be antigenic in natural infection, being recognized by PBMC from patients infected with different HIV-1 variants.

The 27 peptides identified with the TEPITOPE algorithm [34] bound *in vitro* to an average of 12 out of the 17 tested HLA-DR molecules and to several HLA-DP and -DQ molecules, showing that our peptides are highly promiscuous and that TEPITOPE is accurate in predicting promiscuous HLA-peptide binding. Moreover, each tested HLA-DR molecule bound on average to 19 out of the 27 peptides, which indicates that a vaccine encoding such peptides would have potential to induce T-cell responses against multiple targets in a wide proportion of the population. Notably, all peptides bound to at least one HLA class II molecule associated
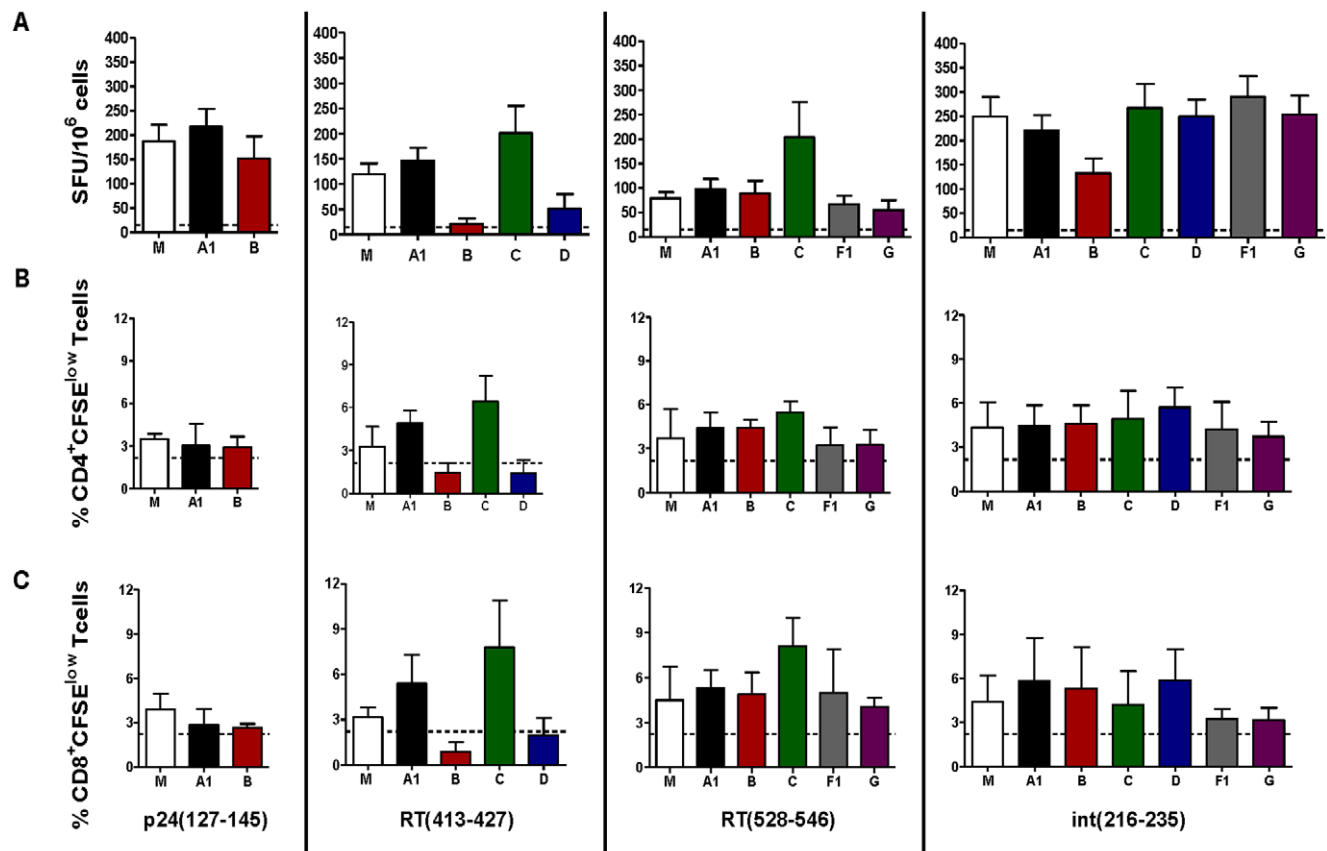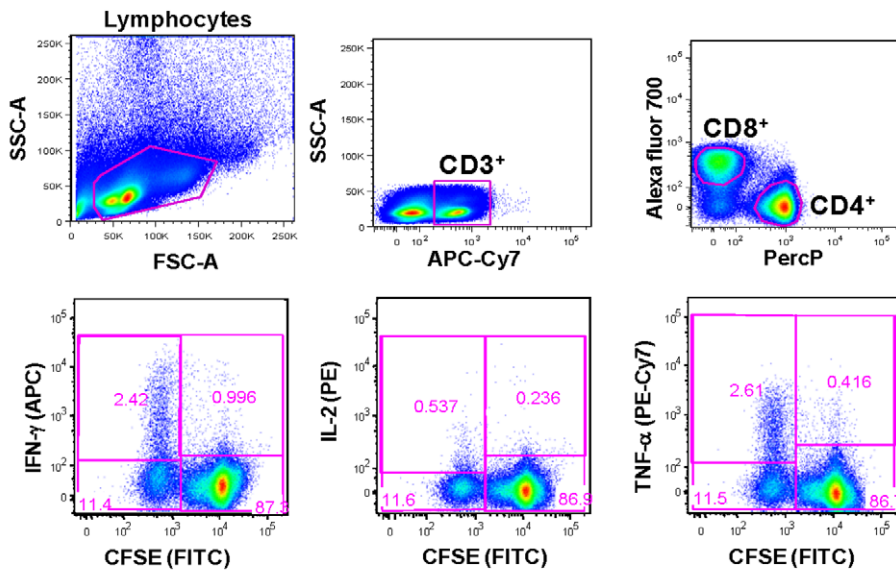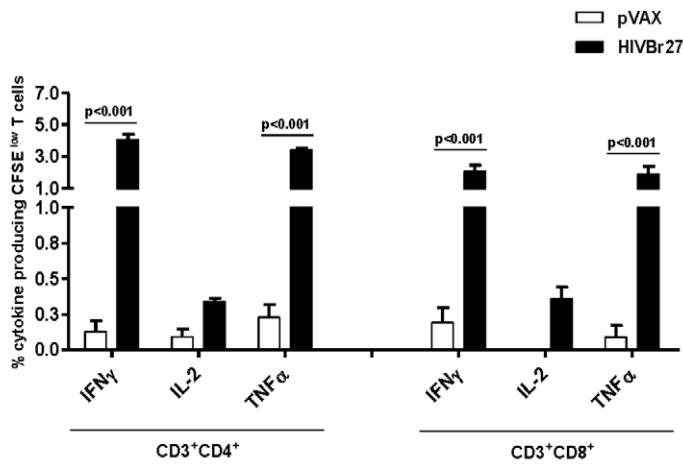

Figure 5. HIVBr27 immunization provides cross-clade immunity. Two weeks after the last immunization with HIVBr27 or empty pVAX1, pooled spleen cells from 6 BALB/c mice were cultured in the presence of HIV-1 M-group consensus peptides (5 µM) (white bars) or their variants (colored bars), from diverse HIV-1 subtypes. Frequency of IFN-γ secreting cells was assessed by ELISPOT assay (A) and proliferative CD4$^+$ (B) and CD8$^+$ (C) T-cell responses were assessed by CFSE dilution assay. Dotted lines represent ELISPOT or proliferation cutoff, which were calculated as median +3 SD of unspecific responses obtained with splenocytes from pVAX1 immunized mice stimulated with HIV-1 peptides. Data are representative of two independent experiments.
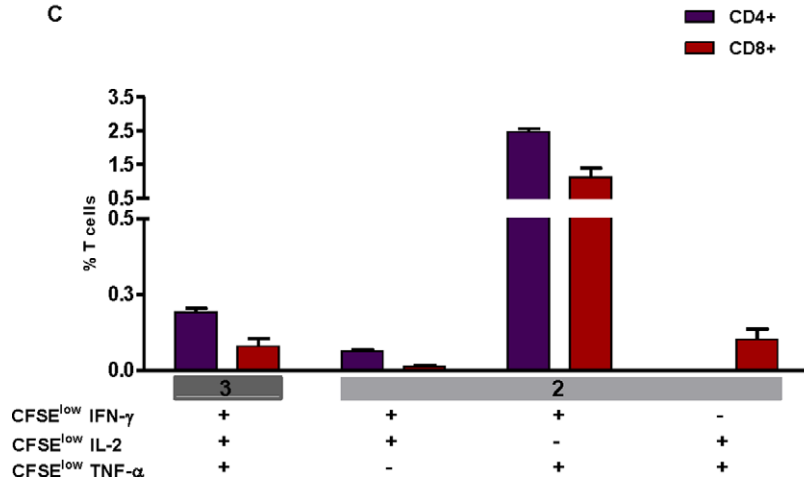doi:10.1371/journal.pone.0045267.g005

A

B

C

Figure 6. HIVBr27 immunization elicits polyfunctional CD4^{+} and CD8^{+} T cells. Two weeks after the last immunization with HIVBr27 or empty pVAX1, pooled spleen cells from 6 BALB/c mice were collected, labeled with CFSE (1.25 μM) and cultured for 4 days in the presence of pooled HIV-1 peptides (5 μM) or medium only. On day 4, cells were pulsed for 12 hours with pooled peptides in the presence of Brefeldin A and costimulatory antibody (anti-CD28). A) Multiparameter flow cytometry strategy used to determine the frequency of IFN-γ, IL-2 or TNF-α producing CFSE^{low} CD4^{+} and CD8^{+} T cells. B) Frequency of IFN-γ, IL-2 or TNF-α producing CFSE^{low} CD4^{+} (left) and CD8^{+} (right) T-cells. C) Boolean combinations of IFN-γ, IL-2 and TNF-α producing CFSE^{low} CD4^{+} and CD8^{+} T cells from HIVBr27 immunized mice. Background responses detected in negative control tubes (cells stimulated with medium and cells from pVAX1 immunized mice stimulated with pooled peptides) were subtracted from those detected in stimulated samples. Data are representative of three independent experiments.
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with AIDS protection, such as HLA-DRB1*0101, -DRB3*0202, -DRB1*13 and -DQB1*06 [49,50], which suggests that those peptides may be involved in protective immunity against HIV-1. The 27 peptides were also shown to be highly conserved when compared to sequences from all HIV-1 circulating variants available at the Los Alamos HIV Database, indicating that the identified peptides, or sequences highly homologous to them, are broadly represented across several HIV-1 subtypes.

The peptides identified in this study showed to be antigenic during natural infection since we observed that 72% of the HIV-1-infected patients recognized at least one peptide and that 16 out of the 27 peptides were recognized. The responses were similar to those previously found by our group among clinically matched HIV-1-infected patients, towards HIV-1 B subtype consensus peptides [51]. It is of note that T-cell responses against M-group consensus Gag peptides were previously observed in HIV-1-infected patients [52]. In this study, 34% of the 15-mer M-group consensus peptides were recognized by HIV-1 B subtype-infected patients, while we observed recognition of 44% of our peptides by patients infected with the same subtype. Considering only our Gag peptides, we observed 50% of recognition by HIV-1 B subtype-infected patients, suggesting that our TEPITOPE-selected conserved and promiscuous HIV-1 M-group consensus peptides were more frequently recognized than the 15-mer peptides employed in the previous study.

HIVBr27 immunization led to broad T-cell responses in BALB/c mice, with recognition of 11 peptides in IFN-γ ELISPOT assay, which is a desirable result since broad T-cell responses have been associated with protective vaccines [6,7,20]. We extended our immunological analysis and also observed broad CD4^{+} and CD8^{+} T-cell proliferation, against 8 and 6 peptides, respectively. Elicitation of HIV-1-specific proliferating CD8^{+} T cells with this vaccine designed to elicit CD4^{+} T-cell responses may have occurred due to the fact that long peptides can harbor CD8^{+} T-cell targets [53]. In addition, 24 out of the 27 peptides were predicted to bind to MHC class I molecules from BALB/c mice, according to the Pred[Balb/c] algorithm [54], which could explain the potential of HIVBr27 to induce CD8^{+} T-cell responses. The broad T-cell response observed in BALB/c mice, despite the fact that the peptides were selected for promiscuous HLA-DR binding, is supported by results from the in vitro MHC-peptide binding assay, in which 21 out of the 27 peptides effectively bound to IA[d] molecules. Moreover, previous studies have already reported cross-species recognition of peptides identified with TEPITOPE, probably by the fact that such algorithm may select for promiscuous peptides that share MHC class II binding motifs similar to many other human and non-human MHC class II molecules [33,37,55].

HIVBr27 immunization elicited comparable T-cell responses against M-group consensus peptides and their respective high-frequency variants, providing evidence that HIVBr27 is able to elicit cross-clade immunity across several HIV-1 subtypes, which is thought to be essential to afford protection against the global diversity of HIV-1. Moreover, we believe that our approach has brought significant improvement on the HIV-1 M-group consen-

sus-based vaccine strategy, since we designed a vaccine encoding highly conserved and promiscuous CD4^{+} T-cell epitopes from 7 different viral genes, while no other study had addressed all these features together. Previous HIV-1 M-group consensus-based vaccines only focused on inducing cross-clade immune responses against Env [23,24,56]. These studies were able to elicit broader cross-subtype neutralizing antibodies and broader cross-clade T-cell responses when compared to immunogens based on single HIV-1 subtypes. However, their immunogens failed to include other HIV-1 proteins, as was done in HIVBr27. We conceive that multiepitopic approaches as HIVBr27 may facilitate the emergence of each peptide as individually immunogenic [25,42,57], being more attractive to broaden T-cell responses against HIV-1. Indeed, it has been shown that immunization with multiple peptides increased the breadth of the T-cell responses, as compared to those induced by whole proteins [58,59].

Given the frequent association of either Env-specific CD4^{+} or CD8^{+} T-cell responses with progression to AIDS [29–31,60,61], it is possible to speculate that an Env-based T-cell immunogen may not provide protection against AIDS progression. However, results from the RV144 trial showing predominant anti-Env CD4 responses [2], and the correlation between Env-specific antibodies and protection against HIV-1 infection [62] suggest that a vaccine able to elicit strong CD4^{+} T-cell responses against Env would be valuable in order to improve antibody-mediated protection. The higher risk of HIV-1 infection observed in vaccinated subjects from the Step study may raise questions about the association of T-cell responses specific to other viral proteins with susceptibility of infection. However, even after extended analysis, it still seems to be an event related to the prior adenovirus 5-specific immunity [63]. Thus, it is important to carefully evaluate the inclusion of Env in HIV-1 vaccine formulations.

Different approaches have been suggested to overcome HIV-1 genetic diversity. Ad26-vectored mosaic vaccines comprising Gag, Pol and Env were found to be more immunogenic than a similar vaccine based on M-group consensus sequence in rhesus macaques [64]. However, it is important to note that the M-group vaccine studied by the authors was based on whole HIV-1 proteins, unlike our approach. Another strategy proposed to elicit cross-clade T-cell responses was based on a chimaeric protein composed of conserved regions of Pol, Gag, Vif and Env from HIV-1 A, B, C and D subtypes [65]. This approach induced significant CD8^{+} T-cell responses in immunized mice, but limited CD4^{+} T-cell responses compared to those induced with HIVBr27 immunization. Indeed, HIVBr27 is the first vaccine specifically aimed to elicit broad and cross-clade CD4^{+} T-cell responses against HIV-1. Thus, we hypothesize that HIVBr27 may confer direct CD4^{+} T-cell-mediated antiviral immunity, as well as provide help for CD8^{+} T-cell-based vaccines, since CD4^{+} T cells are required for both enhancement of virus-specific CD8^{+} T-cell effector function and mobilization of these cells to infected tissues [66–68].

HIVBr27 immunization was also able to elicit high frequencies of both polyfunctional CD4^{+} and CD8^{+} T cells, which simultaneously proliferate and produce effector cytokines such as IFN-γ and TNF-α. We observed a low but detectable frequency of CD4^{+}

T cells able to proliferate and produce IFN-γ, IL-2 and TNF-α. These results indicate that HIVBr27 has potential to induce T-cell responses with a functional profile related to natural control of viral replication and non progression to AIDS, as previously observed in HIV-1-infected patients [50,69–71] and vaccine-mediated protection in NHP models [9,72].

We hereby demonstrate that immunization with the HIVBr27 vaccine encoding multiple conserved and promiscuous HIV-1 M-group consensus peptides is able to elicit broad, cross-clade and polyfunctional T-cell responses. This vaccine concept may cope with HIV-1 genetic diversity as well as provide increased population coverage, which are desirable features for an efficacious strategy against HIV-1/AIDS.

Supporting Information

Table S1 Clinical characteristics of HIV-1-infected patients.
(XLS)

Table S2 Frequency of HIV-1 circulating variants according to the number of amino acid substitutions compared to M-group consensus peptides. M-group consensus peptides were aligned with HIV-1 circulating variants using all available sequences at http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGN/QuickAlign.html. For each peptide, the highest frequency of variants is shown in bold.
(XLS)

Table S3 HLA-DP binding assay for M-group consensus peptides. Significant affinity threshold <1000 nM are shown in

bold. A dash represents 50% inhibitory concentration (IC50) >30000 nM.
(XLS)

Table S4 HLA-DQ binding assay for M-group consensus peptides. Significant affinity threshold <1000 nM are shown in bold. A dash represents 50% inhibitory concentration (IC50) >30000 nM.
(XLS)

Table S5 HIV-1 M-group consensus peptides and variant sequences. The most frequent variants of the 4 most immunogeic M-group consensus peptides were obtained at http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGN/QuickAlign.html. The percentage of HLA-DR molecules predicted to bind to each sequence according to TEPITOPE (threshold of 5%) is also shown.
(XLS)

Acknowledgments

Author Contributions

Conceived and designed the experiments: RRA EC-N. Performed the experiments: RRA DSR SPR VCS JS. Analyzed the data: RRA DSR SPR VCS EGK AS JK EC-N. Contributed reagents/materials/analysis tools: EGK JK EC-N. Wrote the paper: RRA DSR EC-N.

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