

A Vaccine Encoding Conserved Promiscuous HIV CD4 Epitopes Induces Broad T Cell Responses in Mice Transgenic to Multiple Common HLA Class II Molecules

Susan Pereira Ribeiro^{1,3,9}, Daniela Santoro Rosa^{1,3,9}, Simone Gonçalves Fonseca^{1,2}, Eliane Conti Mairena^{2,3}, Edilberto Postól^{2,3}, Sergio Costa Oliveira⁴, Luiza Guilherme^{2,3}, Jorge Kalil^{1,2,3}, Edecio Cunha-Neto^{1,2,3*}

1 Laboratory of Clinical Immunology and Allergy-LIM60, Division of Clinical Immunology and Allergy, Department of Medicine, University of São Paulo School of Medicine, São Paulo, Brazil, **2** Heart Institute (InCor), University of São Paulo School of Medicine, São Paulo, Brazil, **3** Institute for Investigation in Immunology-INCT, São Paulo, Brazil, **4** Department of Biochemistry and Immunology, Federal University of Minas Gerais, Belo Horizonte, Brazil

Abstract

Current HIV vaccine approaches are focused on immunogens encoding whole HIV antigenic proteins that mainly elicit cytotoxic CD8+ responses. Mounting evidence points toward a critical role for CD4+ T cells in the control of immunodeficiency virus replication, probably due to cognate help. Vaccine-induced CD4+ T cell responses might, therefore, have a protective effect in HIV replication. In addition, successful vaccines may have to elicit responses to multiple epitopes in a high proportion of vaccinees, to match the highly variable circulating strains of HIV. Using rational vaccine design, we developed a DNA vaccine encoding 18 algorithm-selected conserved, “promiscuous” (multiple HLA-DR-binding) B-subtype HIV CD4 epitopes - previously found to be frequently recognized by HIV-infected patients. We assessed the ability of the vaccine to induce broad T cell responses in the context of multiple HLA class II molecules using different strains of HLA class II-transgenic mice (-DR2, -DR4, -DQ6 and -DQ8). Mice displayed CD4+ and CD8+ T cell responses of significant breadth and magnitude, and 16 out of the 18 encoded epitopes were recognized. By virtue of inducing broad responses against conserved CD4+ T cell epitopes that can be recognized in the context of widely diverse, common HLA class II alleles, this vaccine concept may cope both with HIV genetic variability and increased population coverage. The vaccine may thus be a source of cognate help for HIV-specific CD8+ T cells elicited by conventional immunogens, in a wide proportion of vaccinees.

Citation: Ribeiro SP, Rosa DS, Fonseca SG, Mairena EC, Postól E, et al. (2010) A Vaccine Encoding Conserved Promiscuous HIV CD4 Epitopes Induces Broad T Cell Responses in Mice Transgenic to Multiple Common HLA Class II Molecules. PLoS ONE 5(6): e11072. doi:10.1371/journal.pone.0011072

Editor: Mario A. Ostrowski, University of Toronto, Canada

Received: March 18, 2010; **Accepted:** May 19, 2010; **Published:** June 11, 2010

Copyright: © 2010 Ribeiro et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was supported by the Brazilian National Research Council (CNPq), São Paulo State Research Funding Agency (FAPESP), International Centre of Genetic Engineering and Biotechnology (ICGEB) and from the Brazilian Ministry of Health (Brazil). S. P. Ribeiro and D. S. Rosa are recipients of a São Paulo State Research Funding Agency (FAPESP) fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist. The International Centre of Genetic Engineering and Biotechnology (ICGEB) is a non-profit organization related to the UNESCO which funds basic research in developing and middle-income countries. There are no links (employment, consultancy, patents, products in development or marketed products) between the authors and the ICGEB that may alter the authors' adherence to all the PLoS ONE policies on sharing data and materials. The use of the peptide combination for vaccination purposes, among others, has been patented (international application number PCT/BR2006/000175).

* E-mail: edecunha@gmail.com

9 These authors contributed equally to this work.

Introduction

Given the proportions of the AIDS pandemic, development of an effective vaccine against human immunodeficiency virus type 1 (HIV-1) remains one of the most important biomedical research priorities. Only three vaccine concepts have completed clinical efficacy studies so far, with two negative results (Env gp 120 vaccine -AIDSVAX; recombinant Adenovirus 5 HIV-1 gag/pol/nef trivalent vaccine -STEP trial [1]), and one showing borderline efficacy, with no effect on HIV-1 viral load (recombinant canarypox ALVAC (gp120/Gag/Protease) prime - gp120 protein boost - ALVAC-AIDSVAX) [2].

Vaccination regimens that induced cell-mediated immunity were shown to significantly reduce simian immunodeficiency virus

(SIV) replication in non-human primates [3], [4], [5]. Therefore, most of the HIV-1 vaccine field is currently focused on development of vaccines that elicit potent cytotoxic CD8+ responses. The lack of efficacy of the recently studied STEP trial of the Adenovirus 5 HIV-1 gag/pol/nef trivalent vaccine [6] may have been related to the narrowness of the induced T cell response – on average, only one epitope was recognized per HIV-1 gene product in each vaccinee [7]. Recent evidence from the SIV infected macaque model show that heterozygote animals, which carry more MHC molecules and present a broader T cell response, display better control of viral load than their homozygote counterparts [8]. It has thus been argued that the development of novel vaccine strategies that elicit a greater epitope breadth, matching T cell responses to circulating HIV strains, is a

critical step to improve effectiveness of a vaccine against the highly variable HIV-1 [7]. In addition, 30% and 60% of vaccinees in the STEP trial failed to display CD8+ and CD4+ T cell responses to HIV epitopes, respectively [9]. Regarding the ALVAC-AIDSVAX trial, no CD8+ T cell responses were detected among the vaccinees, and 66% of them failed to display CD4+ T cell responses to gp120 [2]. Thus, vaccines tested in recent efficacy trials failed to induce CD8+ and especially CD4+ T cell responses in a significant proportion of vaccinees. The lack of population coverage may thus have been another cause of the insufficient results of the trials. Thus, innovative vaccine antigen design and immunogen formulation is needed in order to develop a vaccine able to induce broad immune responses in the majority of vaccinees. One possible way to increase the breadth of the response would be to include most or all of the HIV-1 proteome into a viral vector [5]. However, most viral vectors pose constraints on insert size, and developing such a vaccine for large-scale use could be technically challenging or impractical. On the other hand, epitope-based vaccines combine multiple T cell epitopes assembled in tandem, and can focus the immune response on any chosen group of epitopes (e. g. conserved and highly antigenic), generating much smaller insert sizes. Every single epitope can be immunogenic in multiple epitope-based vaccines; in addition, they have been reported to generate broad and potent immune responses [10], [11], [12], [13], [14]. By eliminating epitope flanking regions, epitope vaccines are also devoid of mutations that impair antigen processing and presentation, which may have accumulated in the flanking regions along viral evolution [15].

In spite of the abundant evidence that cytotoxic CD8+ T lymphocytes are the primary anti-HIV-1 effectors [16], several studies have shown that a strong specific CD4+ T-cell response is associated with control of viral replication and long-term nonprogression to AIDS [17], [18], [19], [20]. It has been shown that specific CD4+ T cells play a major role in the generation of a CD8+ cytotoxic T cell response and neutralizing antibodies, able to control viral replication [21], [22], [23]. Furthermore, CD4+ T cells contribute to T cell vaccine-induced protection in SIV-infected primates [24], [25]. Protective mechanisms include cognate help for functional CD8+ T cell memory [26], [27], mobilization of effector CD8+ T cells to peripheral sites of infection [28], and inhibition of SIV replication in infected macrophages [29]. In addition, virus-specific CD4+ T cells may be able to tolerate more sequence diversity in their target epitopes than CD8+ T cells, therefore being more resistant to mutational escape [30]. It follows that deliberate inclusion of HIV-1 CD4+ T cell epitopes in HIV-1 immunogens, to provide cognate help and boost CD8+ T cell responses, might be a desirable approach for prophylactic T cell vaccines.

With the aid of the TEPITOPE algorithm [31], our group has previously identified a set of 18 conserved CD4+ T-cell epitopes, derived from HIV-1 subtype B consensus whole proteome, capable of binding to multiple HLA-DR molecules [32]. The 18 peptides were recognized, and PBMC (peripheral blood mononuclear cells) from over 90% of HIV-1-infected patients displayed IFN- γ ELISPOT responses to the peptides; each patient recognized on average 5 peptides, including both CD4+ and CD8+ T cell responses [32]. A vaccine encoding multiple conserved epitopes may increase crossreactivity between different HIV strains, possibly circumventing HIV-1 genetic variability [1]. Furthermore, one would expect that a vaccine built with multiple “promiscuous” peptides, capable to bind to several HLA class II molecules could lead to an increased coverage of the genetically heterozygous human population. Since essentially all HLA class II molecules tested were shown to bind to multiple promiscuous

HIV-1 epitopes [32], it is expected that most individuals could develop broad T cell responses. We thus hypothesized that an immunogen containing such a set of 18 conserved, highly promiscuous, immunodominant epitopes from 8 different HIV-1 proteins could potentially elicit a broad T cell response in a high proportion of individuals bearing distinct HLA class II molecules. In order to test our hypothesis, we designed a DNA vaccine encoding the 18 described HIV-1 epitopes. To assess its immunogenicity, we used four mouse strains transgenic to common HLA class II molecules (HLA-DR2, -DR4, -DQ6, -DQ8 present in 35–50% of the population), as a preclinical rodent model of the HLA class II diversity found in humans [33], [34], [35]. It has been reported that HLA class II-transgenic mice are able to develop CD4+ T cell responses to the same HLA-restricted epitopes recognized by humans carrying the same HLA class II molecule [36], [37]. Broad T cell responses were observed in all strains, covering 16 out of the 18 epitopes encoded by the DNA vaccine. We observed multiple CD4+ T cell responses restricted by several HLA-DR and -HLA-DQ molecules, as well as CD8+ T cell responses restricted to murine class I MHC. We believe this vaccine design may be potentially useful as a source of cognate T cell help to CD8+ T cell responses in novel HIV-1 vaccine candidates.

Results

The HIVBr18 vaccine is immunogenic and induces significant HIV-specific cytokine and proliferative T cell responses in HLA class II transgenic strains of mice

To assess the magnitude and coverage of the peptide-specific response induced by the HIVBr18 vaccine in mouse strains transgenic to HLA-DR2, -DR4, -DQ6 and -DQ8, we analyzed T cell proliferation and cytokine production against pooled HIV-1 peptides. Using the CFSE (carboxyfluorescein diacetate succinimidyl ester, Molecular Probes) - based proliferation assay, we observed significant peptide-specific proliferation of both CD4+ and CD8+ splenic T cells derived from HIVBr18-immunized mice. Figure 1 depicts a representative experiment, using the DR4-transgenic strain. All four HLA class II-transgenic strains of mice presented positive proliferative responses against pooled HIV-1 peptides in splenic CD4+ T cells (Figure 2A), while strong specific CD8+ T cell responses were essentially detected only in -DR2- and DR4-Tg mice (Figure 2B). Mice from all four HLA class II-transgenic strains were able to secrete IFN- γ against pooled HIV-1 peptides as measured by ELISPOT (Figure 2C). In contrast, splenocytes from mice immunized with pVAX1 presented negligible numbers of IFN- γ secreting cells.

Broad specific CD4+ and CD8+ T cell responses following immunization HIVBr18 in HLA class II transgenic mice

To determine whether this vaccine concept would be able to induce a broad specific immune response, splenocytes from immunized mice were incubated with each of the 18 individual HIV-1 peptides encoded by the DNA vaccine (Table S1). Immunization with HIVBr18 elicited significant numbers of IFN- γ secreting cells directed to different vaccine-encoded epitopes in HLA-DR2, -DR4, -DQ6 and -DQ8 transgenic mice, which recognized 5, 4, 10 and 1 peptides, respectively (Figures 3 A, B, C and D, respectively). Of note, the epitope p17 (73–89) induced IFN- γ secretion by spleen cells from all transgenic strains. We also evaluated the proliferative T cell responses of immunized HLA class II transgenic mice against the 18 individual peptides. Splenocytes from immunized HLA-DR2, -DR4, -DQ6 and -DQ8 transgenic mice also displayed CD4+ T cell responses to different

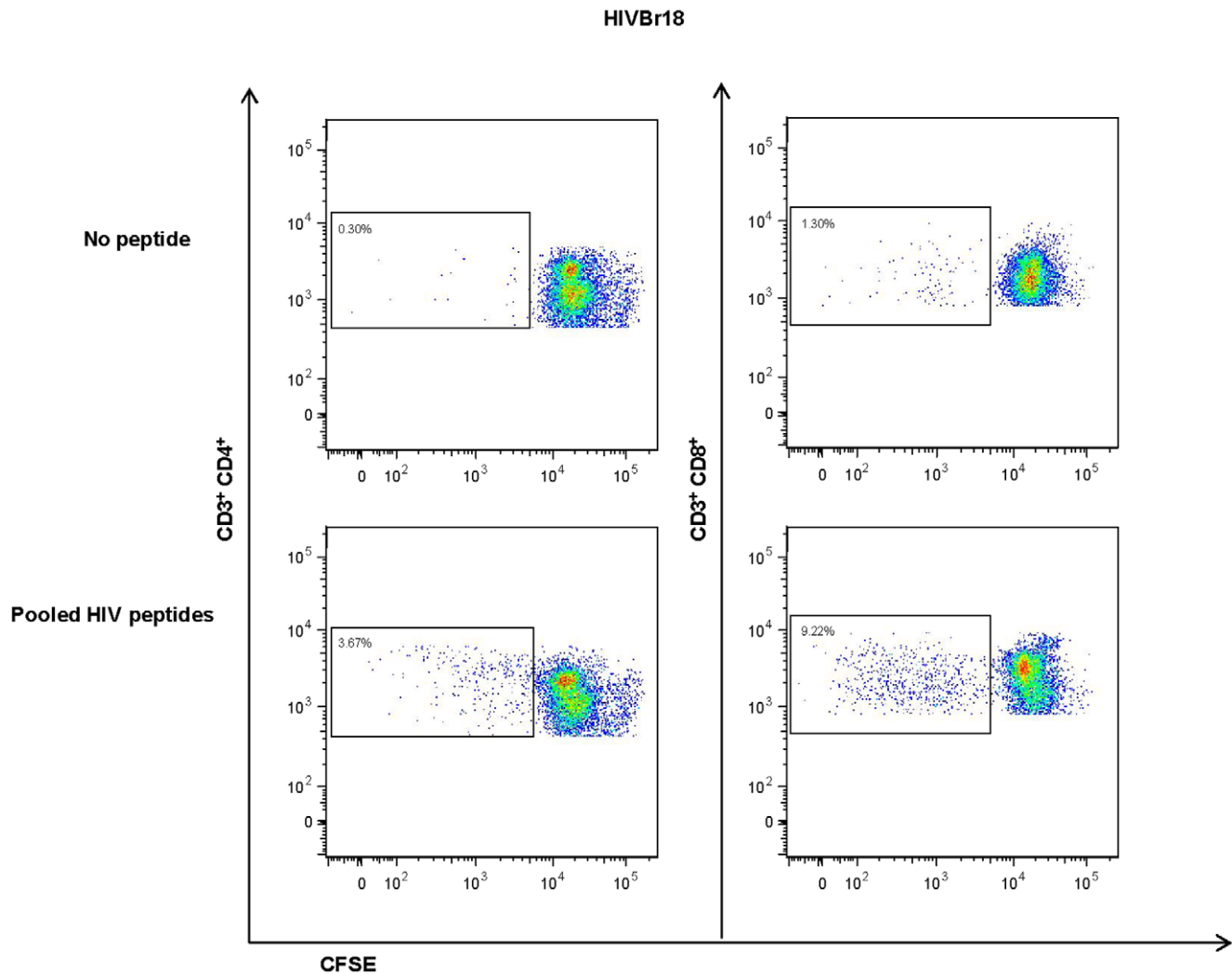


Figure 1. Immunization with HIVBr18 induces significant CD4+ and CD8+ T cell proliferation against pooled HIV peptides. Two weeks after the last immunization with HIVBr18, spleen cells from a HLA-DR4 mouse were labeled with CFSE (1.25 μ M) and cultured for 5 days in the presence of 5 μ M of 18 pooled HIV-1 peptides. Cells were analyzed by flow cytometry and CFSE^{low} cells on gated CD3+CD4+ or CD3+CD8+ was used as a readout for antigen-specific proliferation. Representative dot plots of CD4+ (left) and CD8+ (right) T cell proliferation (%CFSE^{low} cells) of splenocytes stimulated with medium or pooled peptides from HIVBr18 immunized mice. doi:10.1371/journal.pone.0011072.g001

vaccine-encoded epitopes (1, 6, 11 and 0 peptides were recognized, respectively) (Table 1). Several such responses were shared between different HLA class II-transgenic mouse strains. CD8+ T cell responses, on the other hand, were less frequent among immunized HLA-DR2, -DR4, -DQ6 and DQ8 transgenic mice (4, 2, 0 and 1 peptides, respectively) (Table 2). Taken together, HLA class II transgenic mice displayed CD4+ and CD8+ T cell responses to 11 and 6 epitopes, respectively.

In summary, immunization with HIVBr18 was able to induce multiple T cell responses in all 4 HLA class II-transgenic mouse strains tested, against 16 out of the 18 epitopes encoded by the vaccine (Figure 4). Significantly, 11 peptides were recognized by proliferating CD4+ T cells, some of them being recognized by several HLA class II transgenic strains of mice.

Discussion

In this report, we have developed a DNA vaccine encoding 18 conserved, multiple HLA-DR-binding HIV-1 CD4+ T cell

epitopes frequently recognized by HIV-1-infected patients. The HIVBr18 vaccine was immunogenic in four mouse strains transgenic to the common HLA class II molecules HLA-DR2, -DR4, -DQ6 and -DQ8. Moreover, vaccination with HIVBr18 induced broad MHC class II-restricted T cell responses in the HLA class II-transgenic strains of mice, and 16 out of the 18 encoded epitopes could be recognized. Indeed, recent clinical trials of T cell-based HIV vaccines [9] strongly suggested that induction of broad immune responses towards conserved epitopes, in the majority of the genetically heterogeneous vaccinees, may be an essential pre-requisite for novel vaccine candidates [38], [39]. Vaccination of non-human primates with an Adenovirus 5 vaccine encoding 8 SIV proteins and devoid of Env caused a significant reduction in viral load after heterologous challenge [5], and induced broad CD4+ and CD8+ T cell responses. The breadth of pre-challenge SIV-specific T-cell responses correlated with lower viral loads and higher CD4+ lymphocyte counts [40].

An effective T cell vaccine should also have significant population coverage, given the extensive HLA polymorphism

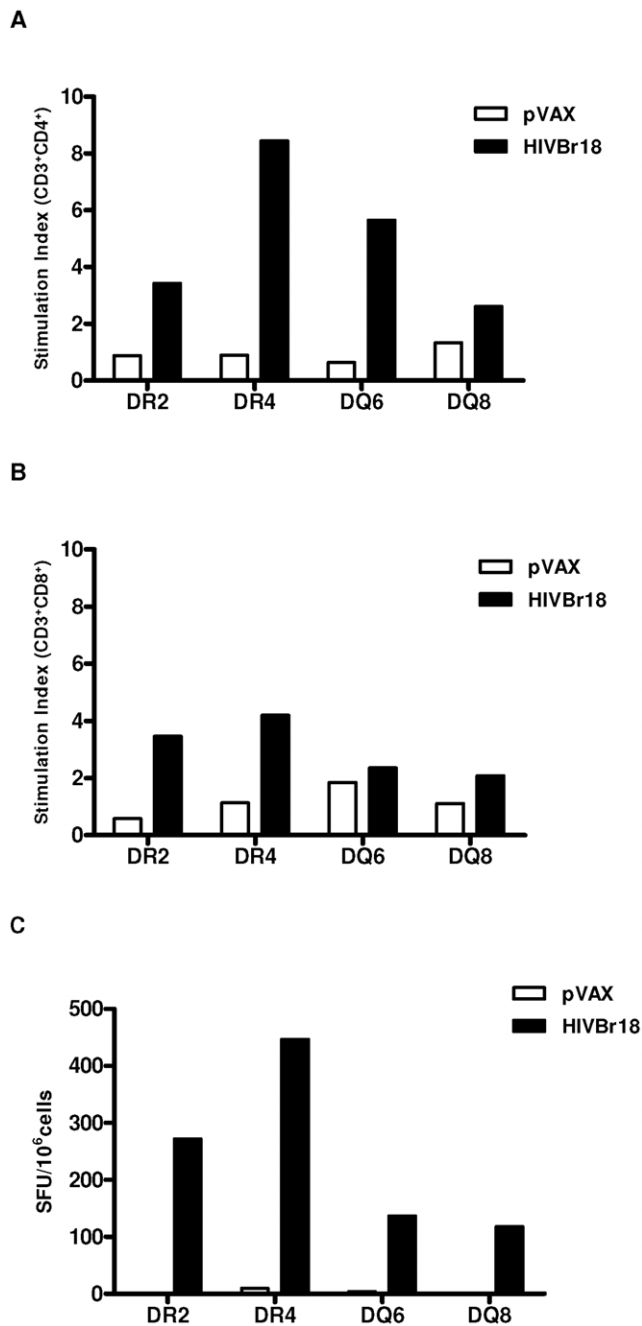


Figure 2. Immunization with HIVBr18 induces responses in human HLA- class II transgenic mice. Splenocytes derived from -DR2, -DR4, -DQ6 and -DQ8 transgenic mice immunized with HIVBr18 or the pVAX1 vector alone were cultured with pooled HIV-1 peptides. Proliferation of CFSE- labeled CD3⁺CD4⁺ (A) and CD3⁺ CD8⁺ (B) T cells from transgenic mice. The stimulation index was calculated by the fold increase between stimulated versus unstimulated cell cultures. (C) IFN-γ production by T cells from HIVBr18- (black bars) or pVAX1- (white bars) immunized mice. HLA class II-transgenic mouse strains are indicated in X axis.
doi:10.1371/journal.pone.0011072.g002

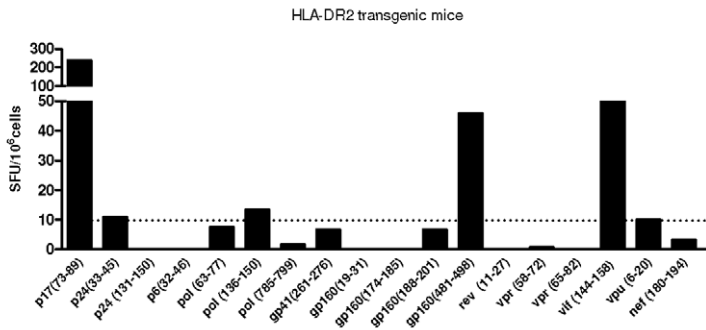
observed in human populations. In an epitope-based vaccine, one way to ensure maximum coverage is to include multiple epitopes where each one could bind to multiple HLA molecules. The TEPITOPE prediction algorithm has been successfully applied to the identification of multiple HLA-DR-binding, “promiscuous” T

cell epitopes in the context of several human infectious, allergic and autoimmune diseases [41], [42], [43], [44]. A significant correlation was observed between the TEPITOPE-predicted promiscuity (ie the number of HLA-DR molecules predicted to bind to a certain peptide) and the number of HLA-DR molecules that could actually bind the peptide in biochemical assays [45]. In the conserved HIV-1 peptide set identified by our group and encoded by the HIVBr18 vaccine, each peptide could bind on average to 50% of the 9 common HLA-DR specificities tested. Conversely, most HLA-DR molecules bound to at least 10 of the peptides, indicating that an individual bearing at least one such HLA-DR molecule could develop broad CD4⁺ T cell responses against the HIV-1 peptide set [32]. Concerning the 6 peptides that elicited IFN-γ secretion or CD4⁺ T cell proliferative responses in HLA-DR2 (DRB1*1501) transgenic mice, we observed that all of them were also recognized by one or more of the 5 HLA-matched HIV-1-infected individuals tested; all 6 peptides presented binding capacity to the HLA DRB1*1501 molecule (data not shown [32]) (Table S2). Regarding the 7 peptides that elicited IFN-γ secretion or CD4⁺ T cell proliferative responses in HLA-DR4 transgenic mice, we observed that 3 were also recognized by the single HLA-DR4 HIV-1-infected individual tested [32] (Table S3).

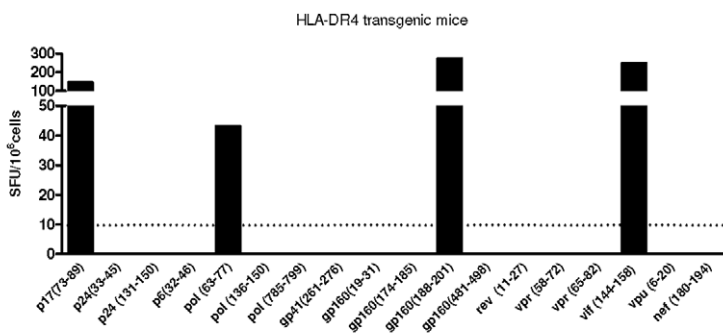
The promiscuity of HLA class II binding of TEPITOPE-selected peptides may extend beyond the limited number of molecules in the prediction matrix. This is suggested by the cross-species recognition of TEPITOPE-selected peptides [45], indicating that the algorithm may select for peptides that share MHC class II binding motifs similar to many other human and non-human MHC class II molecules [31]. This may explain why so many HIV-1 infected patients and, in the present case, mice transgenic to HLA-DQ6 and -DQ8, HLA class II alleles whose binding is not predicted by the TEPITOPE algorithm, also displayed broad T cell responses to the selected HIV-1 peptide set. In addition, all TEPITOPE-selected HIV-1 peptides were predicted to bind to H-2^d class II molecules, and immunization of BALB/c (H-2^d) mice with HIVBr18 also induced broad specific T cell responses to predicted peptides (unpublished observations).

The fact that each HLA class II transgenic strain recognized a different set of CD4 epitopes after immunization with HIVBr18 is in line with MHC class II-restricted recognition. Further in support of this, the fact that all peptides recognized by HLA-DR2(DRB1*1501)-transgenic mice were also recognized by at least one of the 5 tested HLA-DR15 HIV-1 infected patients [32] indicates that findings in HLA class II-transgenic mice accurately translates human responses (Table S2). The recognition of additional epitopes by some patients could possibly be explained by the expression of other HLA-DR, -DQ and -DP molecules in these individuals, contrasting to transgenic mice that carry only one HLA class II allele. In the case of HLA-DR4-transgenic mice, the fact that only 3 out of 7 recognized epitopes matched human responses is probably due to the fact that only a single HLA-DR4 HIV-1 infected patient was tested [32] (Table S3). Our finding that HLA class II transgenic strains could recognize up to 11 CD4⁺ T cell epitopes further indicates the ability of HIVBr18 to induce broad responses in the context of multiple HLA class II molecules. Considering that each HLA class II-transgenic strain only expresses one, rather than the 3–8 distinct class II specificities found in different HLA-DR, -DQ, and -DP haplotypes carried by humans; and the inefficient binding of HLA class II molecules with the murine CD4 molecule [46], our results indicate that the breadth and magnitude of the CD4⁺ T cell response to the HIVBr18 immunogen may be even higher in humans. We observed that approximately 80% of mice from HLA-DR2, -DR4 and -DQ6 transgenic strains responded to the vaccination regimen, while

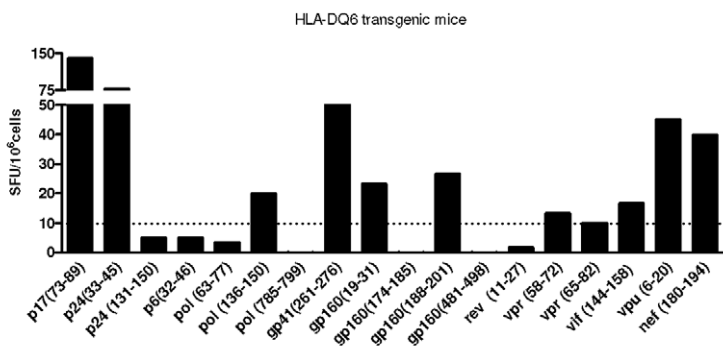
A



B



C



D

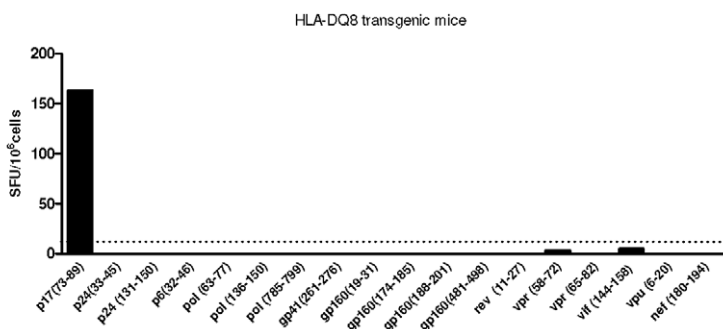


Figure 3. Immunization with HIVBr18 induces IFN- γ secretion against multiple epitopes in human HLA class II transgenic mice. Two weeks after the last immunization with HIVBr18 or the empty pVAX1 vector, splenocytes derived from individual HLA-DR2 (A), -DR4 (B), -DQ6 (C) and -DQ8 (D) transgenic mice (6 per group) were cultured with individual HIV-1 peptides overnight. IFN- γ production was measured by ELISPOT assay. HIV peptide-specific cellular immune responses from human HLA class II- transgenic mice that responded to the immunization are displayed. SFU, spot-forming units. Cutoff = 10 SFU/10⁶ cells and is represented by the dotted line. (SFU from pVAX1-immunized group were always below 5 SFU/10⁶ cells). doi:10.1371/journal.pone.0011072.g003

only 30% of vaccinated HLA-DQ8 transgenic mice presented any response (data not shown). This may be due to qualitatively different levels of HLA class II expression. Regarding CD8+ T cell recognition of murine MHC class I-restricted peptides, it should be noticed that each HLA class II transgenic strain was backcrossed to mouse strains with distinct H-2 class I backgrounds [47], [48], [49]. Our data confirmed the ability of the HIVBr18 immunization strategy in inducing broad HIV-1-specific proliferative and cytokine T cell responses in all HLA-DR and -DQ transgenic strains of mice tested. We observed T cell recognition of 16 out of the 18 epitopes encoded by the vaccine, thus showing that nearly all epitopes in HIVBr18 were adequately processed and presented. This implies that, on average, each strain of mice recognized epitopes corresponding to ca. 50% of the length of the insert encoded by HIVBr18. This is higher than the epitope coverage from conventional whole gene/protein HIV-1 vaccines. Although we used a CD4+ epitope-based DNA vaccine, we could also detect CD8+ T cell responses. This is not unexpected, since

78% of HIV-1 infected patients displayed CD8+ T cell responses to the pooled HIV-1 peptides, usually coexisting with CD4+ T cell responses [32]. Furthermore, 9 out of the 18 peptides contain known human or murine MHC class I-restricted CD8 epitopes [50]. To our knowledge, this is the first report of using multiple HLA class II transgenic strains of mice as a model to probe immunogenicity and HLA class II-restricted T cell responses elicited by a vaccine against an infectious disease. Data suggest that immunization with HIVBr18 might provide significant coverage of the genetically heterogeneous human population.

The significant HIV-1- specific proliferative CD4+ T cell responses in immunized mice was indicative of the magnitude of the T helper activity elicited by HIVBr18. In spite of the abundant evidence that cytotoxic CD8+ T lymphocytes (CTL) are the primary anti-HIV-1 effectors [16], a significant amount of information supports a protective role of specific CD4+ responses in HIV-1/SIV and other viral infections. Early HIV-1 specific CD4+ T cell responses were associated with slower progression in HIV-1 infection [19], [20]. Elite controller SIV-infected macaques mount broad CD4+-specific T cell responses, and certain macaque

Table 1. Immunization with HIVBr18 induces CD4+ T cell proliferation in human HLA- class II transgenic mice.

Stimulus	DR2	DR4	DQ6	DQ8
p17(73–89)		0.86	1.72	
p24(33–45)				
p24 (131–150)				
p6(32–46)			1.57	
pol (63–77)		0.40	1.64	
pol (136–150)		0.13	3.27	
pol (785–799)			2.54	
gp41(261–276)				
gp160(19–31)		0.35	1.42	
gp160(174–185)			1.35	
gp160(188–201)		0.31	4.33	
gp160(481–498)				
rev (11–27)			1.42	
vpr (58–72)				
vpr (65–82)				
vif (144–158)			3.24	
vpu (6–20)				
nef (180–194)	14.17	2.00	1.46	
cutoff	6.89	0.10	1.21	2.40
recognized peptides	1	6	11	0

Quantitative analysis of proliferating CD4+ T cells (gated on CD3+CD4+ CFSE^{low} cells) from HIVBr18 immunized transgenic mice. Proliferative responses measured 15 days after immunization. Only proliferative (%CFSE^{low}) responses above cutoff are shown. Cutoff values for each transgenic strain are shown in the table. % CFSE^{low} cell values were calculated after subtraction of % CFSE^{low} cells in unstimulated cultures. Since we observed different background values in the CFSE assay among each mouse strain, individual cutoffs were established to distinguish the random noise from the true proliferation values. The nonspecific proliferative response was found to be from 0.10% to 6.89%. doi:10.1371/journal.pone.0011072.t001

Table 2. Immunization with HIVBr18 induces CD8+ T cell proliferation in human HLA- class II transgenic mice.

Stimulus	DR2	DR4	DQ6	DQ8
p17(73–89)				
p24(33–45)				0.87
p24 (131–150)				
p6(32–46)				
pol (63–77)				
pol (136–150)	7.94	6.94		
pol (785–799)	8.82			
gp41(261–276)		2.4		
gp160(19–31)	8.33			
gp160(174–185)	12.42			
gp160(188–201)				
gp160(481–498)				
rev (11–27)				
vpr (58–72)				
vpr (65–82)				
vif (144–158)				
vpu (6–20)				
nef (180–194)				
cutoff	7.29	0.01	12.60	0.59
recognized peptides	4	2	0	1

Quantitative analysis of proliferating CD8+ T cells (gated on CD3+CD8+ CFSE^{low} cells) from HIVBr18 immunized transgenic mice. Proliferative responses measured 15 days after immunization. Only proliferative (%CFSE^{low}) responses above cutoff are shown. Cutoff values for each transgenic strain are shown in the table. For cutoff value calculation, see Table 1. The nonspecific proliferative response was found to be from 0.01% to 12.60%. doi:10.1371/journal.pone.0011072.t002

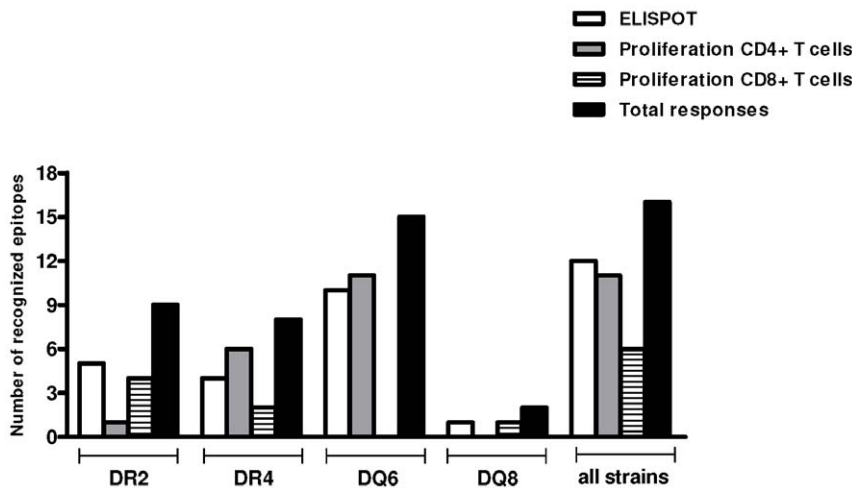


Figure 4. Number of recognized epitopes by spleen cells from HIVBr18 immunized HLA-DR2, -DR4, -DQ6 and -DQ8 transgenic mice. Overall peptide-specific responses observed with IFN- γ ELISPOT, CD4+ and CD8+ T cell proliferation assays in each HLA-transgenic mouse strain are depicted.

doi:10.1371/journal.pone.0011072.g004

class II alleles are associated with significantly decreased viral loads [51]. Vaccination with the attenuated virus SIVmac239 Δ Nef [52] or an 8-valent SIV Adenovirus 5 vaccine [5] both induce broad, high frequency CD4+ T cell responses and protect against pathogenic SIV challenge. Significantly, CD4+ T cell depletion in Rhesus macaques reduced the vaccine-induced protective effect against SIV challenge [53]. Some vaccine approaches have been especially designed to induce HIV-specific CD4+ T cells. An anti-DEC205-HIV-1 gag fusion mAb induced a CD4+ T cell response which conferred protection against challenge with recombinant vaccinia-HIV-1 gag [54]. It follows that novel immunization strategies should also aim to elicit strong CD4+ as well as CD8+ T cell responses, in order to confer long-term protective immunity [55].

We hereby demonstrate that immunization with HIVBr18, a DNA plasmid encoding a string of conserved multiple HLA class II-binding HIV-1 CD4+ T cell epitopes, can induce IFN- γ secretion, CD4+ and even some CD8+ T cell proliferation against multiple epitopes. Moreover, this T cell response was multiallelic, being able to elicit responses restricted to several distinct HLA class II molecules. Previous data from our group has shown that common HLA-DR molecules bind to multiple peptides encoded in the vaccine [32]. This indicates that T cells from individuals bearing such HLA molecules could potentially develop broad immune responses to vaccination with the conserved epitopes of HIVBr18. Thus, this vaccine concept may cope with HIV genetic variability by increasing breadth, as well as providing increased population coverage. We believe this insert design may be useful as a source of cognate T cell help in novel HIV-1 vaccine candidates.

Materials and Methods

Construction of DNA plasmid encoding multiple HIV-1 epitopes

We designed a multi-epitopic construct containing the nucleotide sequence encoding the 18 HIV-1 epitopes described by Fonseca et al. (2006): [32] p17(73–89), p24 (33–45), p24 (131–150), p6 (32–46), protease (7–21), protease (80–94), integrase (70–84), gp41(261–276), gp160 (19–31), gp160 (174–185), gp160 (188–201), gp160 (481–498), rev (11–27), vpr (58–72), vpr (65–82), vif

(144–158), vpu (6–20), nef (180–194). Epitope sequences, assembled *in tandem* in the above mentioned order, had GPGPG spacers at C and N termini, to avoid the creation of junctional epitopes and interference with processing and presentation [56]. The sequences of such epitopes are available in Supplementary Table S1. The nucleotide sequence was codon-optimized and a Kozak sequence was included at the 5' end to improve mammalian expression. The synthetic gene was built (EZBiolab, USA, <http://www.ezbiolab.com>) and subcloned using *HindIII* and *XhoI* sites of the expression vector pVAX-1 (Invitrogen) for production of the recombinant DNA plasmid HIVBr18. The presence and correct orientation of the gene encoding the selected epitopes was confirmed by direct sequencing using the T7 oligonucleotide. Large-scale preparations of plasmid DNA's HIVBr18 and the empty vector pVAX1 were prepared with the Endofree[®] Giga Plasmid Purification Kit from Qiagen according to manufacturer's instructions. The yield and quality of purified DNA was determined by spectrophotometry at 260 nm and confirmed by agarose gel electrophoresis with ethidium bromide staining.

Mice and Immunizations

Six to eight week-old female HLA-class II transgenic mice. DRB1*1502 (DR2), DRB1*0401 (DR4), DQB1*0601 (DQ6) and DQB1*0302 (DQ8) were used in this study [47], [48], [49]. All transgenic mice were kindly provided by Dr. Chella S. David (Department of Immunology, Mayo Clinic, Rochester). Mice used for transgene expression were made genetically deficient for the endogenous class II genes (I-A⁰, E⁰) by homologous recombination. The expression of human HLA class II molecules on antigen-presenting cells in the thymus and periphery of these transgenic mice has a similar distribution to that of endogenous mouse MHC class II molecules [46]. Mice were kept and manipulated in 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 Ethical committee of University of São Paulo. Six mice per group were injected with 10 mM cardiotoxin (Sigma) five days before vaccination. At weeks 0, 2 and 4, DNA plasmid HIVBr18 or empty vector pVAX1 was administered intramuscularly. Each quadriceps was injected with 50 μ l of DNA at a concentration of

1 $\mu\text{g}/\mu\text{l}$ in saline such that each animal received a total of 100 μg of plasmid DNA. Two weeks after the last DNA injection, mice were euthanized with CO_2 .

Peptides

The eighteen multiple HLA-DR binding, frequently recognized peptides, derived from the conserved regions of HIV-1 B-subtype consensus and selected from the whole proteome [32] (sequences in Supplementary Table S1) were synthesized in house by solid phase technology. The 9-fluorenylmethoxycarbonyl (Fmoc) strategy, with the C-terminal carboxyl group in amide form, was used for synthesis [57]. Peptide purity and quality were assessed by reverse-phase high performance liquid chromatography and mass spectrometry and was routinely above 90%.

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 (Sigma), 10 mM Hepes (Sigma), 1 mM sodium pyruvate, 1% vol/vol non-essential amino acid solution, 40 $\mu\text{g}/\text{ml}$ of Gentamicin, 20 $\mu\text{g}/\text{ml}$ of Peflacin and 5×10^{-5} M 2-mercaptoethanol (SIGMA). The viability of the 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.

IFN γ ELISPOT assays

Splenocytes from HIVBr18 or pVAX1 immunized mice were assayed for their ability to secrete IFN- γ after *in vitro* stimulation with 5 μM of individual or pooled HIV-1 peptides using an ELISPOT assay. The ELISPOT assay was performed using Becton Dickinson murine IFN- γ ELISPOT kit according to manufacturer's instructions. Spots were counted using an automated stereomicroscope (KS ELISPOT, Zeiss, Oberkochen, Germany). The number of antigen specific T cells, expressed as IFN- γ spot-forming units (SFU)/ 10^6 splenocytes was calculated after subtracting negative control values (wells with cells in the absence of peptide). The positivity cutoff was calculated as the mean ± 3 SD of splenocytes from pVAX1 immunized mice, stimulated with all peptides. The cutoff for IFN- γ was 10 SFU/ 10^6 splenocytes.

CFSE-based proliferation assay

Splenocytes from HIVBr18 or pVAX1-immunized mice were assayed for their ability to proliferate *in vitro* after stimulation with HIV-1 peptides using the CFSE dilution based proliferation assay [58]. Freshly isolated splenocytes from immunized mice were resuspended ($50 \times 10^6/\text{ml}$) in PBS, labeled with 1.25 μM of CFSE 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 $1.5 \times 10^6/\text{ml}$. Cells were cultured in 96-well-round-bottomed plates ($3 \times 10^3/\text{well}$ in triplicate) for 5 days at 37°C and 5% CO_2 with medium alone or 5 μM of HIV peptides. Positive controls were stimulated with 2.5 $\mu\text{g}/\text{mL}$ 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) (BD Pharmingen, San Jose, CA) for 45 minutes at 4°C. Cells were then washed twice with FACS buffer, fixed with 4% paraformaldehyde, and resuspended in FACS buffer. Samples were acquired on a FACSCanto flow cytometer (BD Biosciences) and then analyzed using FlowJo software (version 8.7.1, Tree Star, San Carlo, CA). Fifty thousand events (proliferation evaluation) were acquired in a live lymphocyte gate. The percent of proliferating CD4 + and CD8 + T cells, i.e., CFSE-low cells, was determined in the CD3 + cell population. The criteria for scoring as positive the proliferating cell cultures included CFSE-low cells > cut off. The cutoff of unspecific proliferative response was determined based on the median percentage of proliferating cells (% of CD3+CD4+ or CD3+CD8+ CFSE low cells) on splenocytes from pVAX-immunized groups after stimulating with individual peptides +3 standard deviations; or stimulation index >2: the stimulation index was calculated by the following equation: %CFSE^{low} cells after stimulus / %CFSE^{low} unstimulated cell cultures.

Data Analysis

Statistical significance (p-values) was calculated by using a Student's T test or One-way ANOVA and Tukey's honestly significantly different (HSD). Statistical analysis was performed with GraphPad Prism version 4.0 software.

Supporting Information

Table S1 Peptide sequences derived from conserved regions of B-subtype HIV-1 consensus selected for multiple HLA-DR binding by the TEPITOPE algorithm and recognition by PBMC from HIV-1-infected patients.

Found at: doi:10.1371/journal.pone.0011072.s001 (0.04 MB DOC)

Table S2 Epitope recognition by HLA-DR2 transgenic mice and HIV-1-infected patients bearing the same haplotype.

Found at: doi:10.1371/journal.pone.0011072.s002 (0.06 MB DOC)

Table S3 Epitope recognition by HLA-DR4 transgenic mice and HIV-1-infected patient bearing the same haplotype.

Found at: doi:10.1371/journal.pone.0011072.s003 (0.04 MB DOC)

Acknowledgments

We thank Dr. Claudio Puschel and Mr. Washington Robert da Silva for peptide synthesis; Mr. Luis Roberto Mundel for assistance at the animal facility; and Dr Silvia Boscardin for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: SPR DSR SGF SCO LG JK ECN. Performed the experiments: SPR DSR ECM EP. Analyzed the data: SPR DSR SGF ECN. Contributed reagents/materials/analysis tools: SPR DSR JK ECN. Wrote the paper: SPR DSR ECN.

References

- Barouch DH, Korber B (2010) HIV-1 vaccine development after STEP. *Annu Rev Med* 61: 153–167.
- Rerks-Ngarm S, Pitisutithum P, Nitayaphan S, Kaewkungwal J, Chiu J, et al. (2009) Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* 361: 2209–2220.
- Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, et al. (2009) Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat Med* 15: 293–299.
- Liu J, O'Brien KL, Lynch DM, Simmons NL, La Porte A, et al. (2009) Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature* 457: 87–91.

5. Wilson NA, Keele BF, Reed JS, Piaskowski SM, MacNair CE, et al. (2009) Vaccine-induced cellular responses control simian immunodeficiency virus replication after heterologous challenge. *J Virol* 83: 6508–6521.
6. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, et al. (2008) Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 372: 1881–1893.
7. Corey L, McElrath MJ, Kublin JG (2009) Post-step modifications for research on HIV vaccines. *AIDS* 23: 3–8.
8. O'Connor SL, Lhost JJ, Becker EA, Detmer AM, Johnson RC, et al. MHC heterozygote advantage in simian immunodeficiency virus-infected mauritian cynomolgus macaques. *Sci Transl Med* 2: 22ra18.
9. McElrath MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, et al. (2008) HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet* 372: 1894–1905.
10. Fuller DH, Shipley T, Allen TM, Fuller JT, Wu MS, et al. (2007) Immunogenicity of hybrid DNA vaccines expressing hepatitis B core particles carrying human and simian immunodeficiency virus epitopes in mice and rhesus macaques. *Virology* 364: 245–255.
11. Tenzer S, Wee E, Burgevin A, Stewart-Jones G, Friis L, et al. (2009) Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance. *Nat Immunol* 10: 636–646.
12. Suhbrier A (2002) Polytope vaccines for the codelivery of multiple CD8 T-cell epitopes. *Expert Rev Vaccines* 1: 207–213.
13. Ishioka GY, Fikes J, Hermanson G, Livingston B, Crimi C, et al. (1999) Utilization of MHC class I transgenic mice for development of minigene DNA vaccines encoding multiple HLA-restricted CTL epitopes. *J Immunol* 162: 3915–3925.
14. Restifo NP, Bacik I, Irvine KR, Yewdell JW, McCabe BJ, et al. (1995) Antigen processing in vivo and the elicitation of primary CTL responses. *J Immunol* 154: 4414–4422.
15. Allen TM, Altfeld M, Yu XG, O'Sullivan KM, Lichterfeld M, et al. (2004) Selection, transmission, and reversion of an antigen-processing cytotoxic T-lymphocyte escape mutation in human immunodeficiency virus type 1 infection. *J Virol* 78: 7069–7078.
16. Watkins DI, Burton DR, Kallas EG, Moore JP, Koff WC (2008) Nonhuman primate models and the failure of the Merck HIV-1 vaccine in humans. *Nat Med* 14: 617–621.
17. Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, et al. (1997) Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* 278: 1447–1450.
18. Gandhi RT, Walker BD (2002) Immunologic control of HIV-1. *Annu Rev Med* 53: 149–172.
19. Martinez V, Costagliola D, Bonduelle O, N'go N, Schnuriger A, et al. (2005) Combination of HIV-1-specific CD4 Th1 cell responses and IgG2 antibodies is the best predictor for persistence of long-term nonprogression. *J Infect Dis* 191: 2053–2063.
20. Pancre V, Delhem N, Yazdanpanah Y, Delanoye A, Delacore M, et al. (2007) Presence of HIV-1 Nef specific CD4 T cell response is associated with non-progression in HIV-1 infection. *Vaccine* 25: 5927–5937.
21. Matloubian M, Concepcion RJ, Ahmed R (1994) CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* 68: 8056–8063.
22. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, et al. (1998) Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 188: 2205–2213.
23. Bevan MJ (2004) Helping the CD8(+) T-cell response. *Nat Rev Immunol* 4: 595–602.
24. Vaccari M, Boasso A, Ma ZM, Cecchinato V, Venzon D, et al. (2008) CD4+ T-cell loss and delayed expression of modulators of immune responses at mucosal sites of vaccinated macaques following SIV(mac251) infection. *Mucosal Immunol* 1: 497–507.
25. Letvin NL (2006) Progress and obstacles in the development of an AIDS vaccine. *Nat Rev Immunol* 6: 930–939.
26. Shedlock DJ, Shen H (2003) Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 300: 337–339.
27. Lichterfeld M, Kaufmann DE, Yu XG, Mui SK, Addo MM, et al. (2004) Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells. *J Exp Med* 200: 701–712.
28. Nakanishi Y, Lu B, Gerard C, Iwasaki A (2009) CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature* 462: 510–513.
29. Sacha JB, Giraldo-Vela JP, Buechler MB, Martins MA, Maness NJ, et al. (2009) Gag- and Nef-specific CD4+ T cells recognize and inhibit SIV replication in infected macrophages early after infection. *Proc Natl Acad Sci U S A* 106: 9791–9796.
30. Wilson DB, Wilson DH, Schroder K, Pinilla C, Blondelle S, et al. (2004) Specificity and degeneracy of T cells. *Mol Immunol* 40: 1047–1055.
31. Sturmli T, Bono E, Ding J, Radrizzani L, Tuoreci O, et al. (1999) Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat Biotechnol* 17: 555–561.
32. Fonseca SG, Coutinho-Silva A, Fonseca LA, Segurado AC, Moraes SL, et al. (2006) Identification of novel consensus CD4 T-cell epitopes from clade B HIV-1 whole genome that are frequently recognized by HIV-1 infected patients. *AIDS* 20: 2263–2273.
33. Call MJ, Xing X, Cuny GD, Seth NP, Altmann DM, et al. (2009) In vivo enhancement of peptide display by MHC class II molecules with small molecule catalysts of peptide exchange. *J Immunol* 182: 6342–6352.
34. DaSilva L, Welcher BC, Ulrich RG, Aman MJ, David CS, et al. (2002) Humanlike immune response of human leukocyte antigen-DR3 transgenic mice to staphylococcal enterotoxins: a novel model for superantigen vaccines. *J Infect Dis* 185: 1754–1760.
35. Gregory SH, Mott S, Phung J, Lee J, Moise L, et al. (2009) Epitope-based vaccination against pneumonic tularemia. *Vaccine* 27: 5299–5306.
36. Geluk A, Taneja V, van Meijgaarden KE, Zanelli E, Abou-Zeid C, et al. (1998) Identification of HLA class II-restricted determinants of *Mycobacterium tuberculosis*-derived proteins by using HLA-transgenic, class II-deficient mice. *Proc Natl Acad Sci U S A* 95: 10797–10802.
37. Kawamura K, Yamamura T, Yokoyama K, Chui DH, Fukui Y, et al. (2000) Hla-DR2-restricted responses to proteolipid protein 95-116 peptide cause autoimmune encephalitis in transgenic mice. *J Clin Invest* 105: 977–984.
38. Sekaly RP (2008) The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? *J Exp Med* 205: 7–12.
39. Watkins DI (2008) The hope for an HIV vaccine based on induction of CD8+ T lymphocytes—a review. *Mem Inst Oswaldo Cruz* 103: 119–129.
40. Martins MA, Wilson NA, Reed JS, Ahn CD, Klimentidis YC, et al. (2010) T-cell correlates of vaccine efficacy after a heterologous simian immunodeficiency virus challenge. *J Virol* 84: 4352–4365.
41. Rosa DS, Iwai LK, Tzelepis F, Bargieri DY, Medeiros MA, et al. (2006) Immunogenicity of a recombinant protein containing the *Plasmodium vivax* vaccine candidate MSP1(19) and two human CD4+ T-cell epitopes administered to non-human primates (*Callithrix jacchus jacchus*). *Microbes Infect* 8: 2130–2137.
42. Garcia TC, Fonseca CT, Pacifico LG, Duraes Fdo V, Marinho FA, et al. (2008) Peptides containing T cell epitopes, derived from Sm14, but not from paramyosin, induce a Th1 type of immune response, reduction in liver pathology and partial protection against *Schistosoma mansoni* infection in mice. *Acta Trop* 106: 162–167.
43. Iwai LK, Yoshida M, Sidney J, Shikanai-Yasuda MA, Goldberg AC, et al. (2003) In silico prediction of peptides binding to multiple HLA-DR molecules accurately identifies immunodominant epitopes from gp43 of *Paracoccidioides brasiliensis* frequently recognized in primary peripheral blood mononuclear cell responses from sensitized individuals. *Mol Med* 9: 209–219.
44. Bian H, Hammer J (2004) Discovery of promiscuous HLA-II-restricted T cell epitopes with TEPITOPE. *Methods* 34: 468–475.
45. Rosa DS, Ribeiro SP, Cunha-Neto E (2010) CD4+ T cell epitope discovery and rational vaccine design. *Arch Immunol Ther Exp (Warsz)* 58: 121–130.
46. Wong FS, Wen L (2004) What can the HLA transgenic mouse tell us about autoimmune diabetes? *Diabetologia* 47: 1476–1487.
47. Gonzalez-Gay MA, Zanelli E, Khare SD, Krcro CJ, Zhou P, et al. (1996) Human leukocyte antigen-DRB1*1502 (DR2Dw12) transgene reduces incidence and severity of arthritis in mice. *Hum Immunol* 50: 54–60.
48. Pan S, Trejo T, Hansen J, Smart M, David CS (1998) HLA-DR4 (DRB1*0401) transgenic mice expressing an altered CD4-binding site: specificity and magnitude of DR4-restricted T cell response. *J Immunol* 161: 2925–2929.
49. Chapoval SP, Neeo T, Krcro CJ, Marietta EV, Harders J, et al. (1998) HLA-DQ6 and HLA-DQ8 transgenic mice respond to ragweed allergens and recognize a distinct set of epitopes on short and giant ragweed group 5 antigens. *J Immunol* 161: 2032–2037.
50. Korber BTM, Brander C, Haynes BF, Koup R, Moore JP, et al. (2006) In: HIV Molecular Immunology Los Alamos, New Mexico.: Los Alamos National Laboratory, Theoretical Biology and Biophysics.
51. Giraldo-Vela JP, Rudersdorf R, Chung C, Qi Y, Wallace LT, et al. (2008) The major histocompatibility complex class II alleles Mamu-DRB1*1003 and -DRB1*0306 are enriched in a cohort of simian immunodeficiency virus-infected rhesus macaque elite controllers. *J Virol* 82: 859–870.
52. Gauduin MC, Yu Y, Barabas A, Carville A, Piatak M, et al. (2006) Induction of a virus-specific effector-memory CD4+ T cell response by attenuated SIV infection. *J Exp Med* 203: 2661–2672.
53. Vaccari M, Mattapallil J, Song K, Tsai WP, Hryniewicz A, et al. (2008) Reduced protection from simian immunodeficiency virus SIVmac251 infection afforded by memory CD8+ T cells induced by vaccination during CD4+ T-cell deficiency. *J Virol* 82: 9629–9638.
54. Trumpfheller C, Finke JS, Lopez CB, Moran TM, Moltedo B, et al. (2006) Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *J Exp Med* 203: 607–617.
55. Khanolkar A, Badovinac VP, Hartly JT (2007) CD8 T cell memory development: CD4 T cell help is appreciated. *Immunol Res* 39: 94–104.
56. Livingston B, Crimi C, Newman M, Higashimoto Y, Appella E, et al. (2002) A rational strategy to design multi-epitope immunogens based on multiple Th lymphocyte epitopes. *J Immunol* 168: 5499–5506.
57. Atherton ND (1989) HPLC measurement of phenylalanine by direct injection of plasma onto an internal-surface reversed-phase silica support. *Clin Chem* 35: 975–978.
58. Quah BJ, Warren HS, Parish CR (2007) Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc* 2: 2049–2056.