Dendritic Cell Mediated Delivery of Plasmid DNA Encoding LAMP/HIV-1 Gag Fusion Immunogen Enhances T Cell Epitope Responses in HLA DR4 Transgenic Mice

Gregory G. Simon1*, Yongli Hu2, Asif M. Khan2, Jingshi Zhou1, Jerome Salmon1, Priya R. Chikhlikar1, Keun-Ok Jung1, Ernesto T. A. Marques1,3, J. Thomas August1,3

1 Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America; 2 Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore; 3 Department of Medicine, Division of Infectious Diseases, The Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America

Abstract

This report describes the identification and bioinformatics analysis of HLA-DR4-restricted HIV-1 Gag epitope peptides, and the application of dendritic cell mediated immunization of DNA plasmid constructs. BALB/c (H-2d) and HLA-DR4 (DRA1*0101, DRB1*0401) transgenic mice were immunized with immature dendritic cells transfected by a recombinant DNA plasmid encoding the lysosome-associated membrane protein-1/HIV-1 Gag (pLAMP/gag) chimera antigen. Three immunization protocols were compared: 1) primary subcutaneous immunization with 1 x 10^7 immature dendritic cells transfected by electroporation with the pLAMP/gag DNA plasmid, and a second subcutaneous immunization with the naked pLAMP/gag DNA plasmid; 2) primary immunization as above, and a second subcutaneous immunization with a pool of overlapping peptides spanning the HIV-1 Gag sequence; and 3) immunization twice by subcutaneous injection of the pLAMP/gag DNA plasmid. Primary immunization with pLAMP/gag-transfected dendritic cells elicited the greatest number of peptide specific T-cell responses, as measured by ex vivo IFN-γ ELISPOT assay, both in BALB/c and HLA-DR4 transgenic mice.

Introduction

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that play a critical role in the generation of cellular immune responses. DCs acquire antigen (Ag) in the periphery and, upon activation, migrate to lymph nodes where they orchestrate the generation of Ag-specific CD4+ and CD8+ T-cell responses [1–3]. They are being used extensively as Ag carriers in candidate epitope-based vaccine strategies, and c) the application of variability analysis across reported sequences in public databases for selection of historically conserved HIV epitopes as vaccine targets.
immunogen. The BALB/c mice provided a comparison with the previous pLAMP/gag naked DNA immunization. The HLA DR4 Tg mice were used to compare the DR4-restricted T-cell peptide determinants identified in this study with those of infected humans. Both immunization protocols included different prime-boost formulations with transfected DCs as well as naked plasmid DNA. Additionally, a bioinformatics approach was used to study the representation and variability of the identified epitopes of all database recorded HIV-1 clade B gag T-cell epitopes. These data provide insight into the efficacy of different vaccine formulations and delivery, the diversity of Gag HLA DR4 T-cell epitopes, and criteria for selection of Gag sequences as vaccine targets.

Results

Immunization with pLAMP/Gag-Electroporated Immature DC Induces Enhanced T-Cell Epitope Repertoire and Avidity in BALB/c and HLA-DR4 Tg Mice

We previously have shown that immunization of BALB/c (H-2d) mice with naked DNA encoding pLAMP/gag elicited a broader repertoire and more robust Gag-specific T-cell responses than did immunization with a plasmid expressing the native Gag protein p55gag [16–20]. In this study, we first compared the T-cell responses of BALB/c mice immunized with immature DCs transfected by pLAMP/gag DNA in comparison to those immunized with naked pLAMP/gag DNA. The peptide-specific T-cell responses were measured by ex vivo IFN-γ ELISpot assay with a library of 123 overlapping peptides encompassing the entire p55gag sequence. The relative avidity of the epitope peptides was also evaluated using peptide concentrations varying by two orders of magnitude in the ELISpot assays (10, 1, and 0.1 μg/mL). Immunization with pLAMP/gag-transfected DCs followed by a naked pLAMP/gag boost elicited enhanced IFN-γ secreting T-cell responses with a greater repertoire and apparent avidity than did the naked pLAMP/gag DNA prime and boost immunization, particularly at the lower and intermediate peptide concentrations in the ELISpot assay (Figure 1; Table 1).

The efficacy of immunization with transfected DCs was further evaluated by immunization of HLA-DR4 Tg mice [21–23] with the same immunization protocol as applied to the BALB/c mice. This study had the additional advantage of allowing a comparison of the data from the transgenic animal model to that obtained from studies of infected humans and available in public HIV databases. Splenocytes from the immunized Tg mice were depleted of CD8+ T cells and IFN-γ secreting T-cell responses were measured by ELISpot assay as described above. A total of 25 epitope peptides were identified. Similar to the BALB/c study described herein, immunization with pLAMP/gag-transfected DCs prime followed by naked pLAMP/gag boost, or alternatively, pLAMP/gag-transfected DCs prime/Gag peptide boost, elicited a larger number of T-cell responses with greater avidity than did immunization with pLAMP/gag prime/boost (Figure 2; Table 2).

Localization and Clustering of the H-2d and HLA-DR4 Epitope Peptides Elicited by pLAMP/Gag Immunization

The ELISpot positive HIV-1 Gag epitope peptides elicited after immunization with pLAMP/gag by the various formulations were present as clusters of overlapping peptides at defined sequence localizations, with the majority of the epitope peptides detected in assays at 10 μg/mL peptide (Figure 2). The T-cell epitope peptides of BALB/c mice were present in four regions of 3 to 5 overlapping sequences comprising 16 of the 19 total epitope peptides (Figure 1; Table 1). The CD4+ T-cell epitope peptides of the HLA-DR4 Tg mice were also clustered in 4 to 5 regions detected with ELISpot assays containing 10 μg/mL peptide (Figure 2; Table 2). Results of the different immunization formulations indicated that despite differences between the immunogen delivery as DC, peptides, or naked DNA, there was little difference in the localization of the epitope peptides. DC-mediated immunization enhanced the breath and strength of the T-cell responses to a group of immunogenic peptides that included those elicited by immunization with naked pLAMP/gag, and did not primarily affect the selection of the major immunogenic regions.

Comparison of Identified HLA-DR4 Epitope Peptides with Reported Human DR4 Epitopes

The epitopes of the HLA DR4 Tg mice identified in this study were compared to the Gag DR4 epitopes of HIV-1 infected

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Peptide specificity and relative avidity of IFN-γ-secreting HIV-1 Gag-specific T-cell responses in BALB/c mice immunized with pLAMP/gag-based prime and boost formulations. Mice were immunized subcutaneously with naked pLAMP/gag plasmid 50 μg DNA or with 1×10⁷ pLAMP/gag-transfected immature DCs (DC-LAMP/gag), and boosted with naked pLAMP/gag plasmid (lower and upper lines) or Gag peptides (middle line). Gag peptide-specific T cell responses were measured by ex vivo IFN-γ ELISpot assay at peptide concentration of 10 μg/ml (top), 1 μg/ml (middle), and 0.1 μg/ml (bottom), as described in Materials and Methods. The domain structure of the unprocessed HIV-1 Gag protein, and the numbering of the corresponding peptides are indicated on the top. Colored boxes indicate peptides eliciting positive IFN-γ ELISpot responses in mice immunized with pLAMP/gag plasmid DNA prime/boost (yellow), DC-LAMP/gag prime and Gag peptide boost (red), and DC-LAMP/gag prime and pLAMP/gag plasmid DNA boost (black).

doi:10.1371/journal.pone.0008574.g001
humans reported in the HIV immunology database (Table 3). Strikingly, 21 of the 25 peptides that elicited CD4+ T-cell responses in DC immunized HLA-DR4 Tg mice overlapped at least nine consecutive amino acids in 18 of the 19 human HLA-DR4-restricted HIV-1 Gag epitopes (Figure 3). The reported human epitopes were detected by multiple methods, including

| BEI peptide | HIV Gag peptide positionsb | Amino acid sequencesb | IFN-γ SFC/ 10^6 total splenocytesc |
|-------------|---------------------------|----------------------|-----------------------------------|
|             |                           |                      | DC-LAMP/gag, pLAMP/gag            | DC-LAMP/gag, Gag peptides | pLAMP/gag, pLAMP/gag |
| 15          | 57–71                     | CRQILQOLQPSLQTG      | 57±12                             | 101±5                       | 40±4                  |
| 20          | 77–91                     | SLYNTVATLYCVHQVR     | 913±14                            | 248±18                      | 652±5                 |
| 49          | 193–207                   | GHQAAMQLMLKETINE     | 1078±25                           | 600±57                      | 748±20                |
| 50          | 197–211                   | AMQMLKETINAAEAE      | 929±39                            | 291±17                      | 785±6                 |
| 51          | 201–215                   | LKETINNAAAEWDRL      | 87±0                              | 158±11                      | -                     |
| 56          | 221–235                   | GPIAPGQMRPRGSD       | 62±1                              | -                           | -                     |
| 65          | 257–271                   | PVGEIYKRWILGLN       | 145±9                             | 54±3                        | 326±19                |
| 66          | 261–275                   | IYKRWIIGLNKIVR       | 124±4                             | 82±3                        | -                     |
| 67          | 265–279                   | WIIILNIVRMIPY      | 99±5                              | 106±19                      | 120±9                 |
| 74          | 293–307                   | FRDVDFRKYTLLRAE      | 166±8                             | 53±5                        | -                     |
| 75          | 297–311                   | VDRFYKTRLAEQASQ      | 233±9                             | 122±5                       | 262±11                |
| 76          | 301–315                   | YKTLAEQASQEVKN       | 138±6                             | 76±4                        | 132±6                 |
| 77          | 305–319                   | RAEQASQEVKNMTE       | 89±6                              | 45±3                        | 53±1                  |
| 78          | 309–323                   | ASQEVKNMTETLLV       | 46±4                              | -                           | -                     |
| 96          | 381–395                   | GNFRNQRTKVCFCN       | 245±7                             | 102±3                       | 183±15                |
| 97          | 385–399                   | NQRKRVKCPNCGEK       | 148±8                             | 81±5                        | 48±5                  |
| 98          | 389–403                   | TVKCPNCGKEGHIAK      | 166±4                             | 122±8                       | 60±6                  |
| 99          | 393–407                   | FNCGKEGHIANCRCA      | 128±2                             | 37±3                        | -                     |
| 100         | 397–411                   | KEGHIAKCRPAK         | 60±4                              | 31±3                        | -                     |

*a*As detected by ex vivo IFN-γ ELSpot assay.

*b*Referring to the HIV-1 HXB2 Gag sequence.

*c*SFC, spot-forming cells. ELSpot values ± SD were obtained from groups of 10 mice immunized with pLAMP/gag-transfected DCs prime and pLAMP/gag plasmid DNA boost (left column), pLAMP/gag-transfected DCs prime and Gag peptides boost (middle column), and pLAMP/gag plasmid DNA prime and boost (right column). Negative results are indicated by dashes.

**Figure 2.** Peptide specificity and relative avidity of IFN-γ-secreting HIV-1 Gag-specific CD4 T-cell responses in HLA-DR4 (DRB1*0401) Tg mice immunized with pLAMP/gag-based formulations. Mice were immunized subcutaneously with 50 μg pLAMP/gag plasmid DNA or with 1 × 10^6 pLAMP-1/gag-transfected immature DCs (DC-LAMP/gag), and boosted with pLAMP/gag plasmid DNA (lower and upper lines) or Gag peptides (middle line). Gag peptide-specific CD4 T cell responses were measured by IFN-γ ELSpot assay using CD8-depleted splenocytes, at peptide concentration of 10 μg/ml (top), 1 μg/ml (middle), and 0.1 μg/ml (bottom). The domain structure of the HIV-1 Gag polyprotein is indicated on the top, and the corresponding overlapping peptides are numbered below. Peptides eliciting positive ELSpot responses in mice immunized with pLAMP/gag plasmid DNA prime/boost (yellow), DC-LAMP/gag prime and Gag peptide boost (red), and DC-LAMP/gag prime and plasmid DNA boost (black) are indicated below.

doi:10.1371/journal.pone.0008574.g002
| BEI peptide number | HIV-1 Gag peptide positions | Amino acid sequences | IFN-γ SFC ± SD |
|-------------------|-----------------------------|----------------------|-----------------|
|                   |                             |                      | DC-LAMP/gag     | DC-LAMP/gag   | pLAMP/gag      |
|                   |                             |                      | pLAMP/gag       | gag peptides  | pLAMP/gag      |
| 10                | 37–51                       | ASRELERFAVNPGLLL     | -               | 69±8          | -              |
| 11                | 41–55                       | LERFAVNPGLLETSE      | 125±3           | -             | -              |
| 19                | 73–87                       | EELRSLYNTVALYCC      | 165±3           | 78±16         | 35±8           |
| 20                | 77–91                       | SLNTVALYCVHQR        | 225±7           | 270±2         | 81±6           |
| 21                | 81–95                       | TVATLYCVHQRREVK      | 380±4           | 334±28        | 134±28         |
| 22                | 85–99                       | LKYCHQRIEVGDTKE      | 121±20          | 63±2          | 53±2           |
| 41                | 161–175                     | EKAFSPIVEPMFSAE      | -               | 40±11         | -              |
| 42                | 165–179                     | SPEIVPMFSALSEGA      | 169±7           | 117±18        | 62±15          |
| 43                | 169–183                     | IPMFSALSEGATPQD      | 120±1           | 96±1          | 30±1           |
| 59                | 233–247                     | GSQDAGGTTSLOQRI      | -               | 42±7          | -              |
| 61                | 241–255                     | STLOEQGWMTNNPP       | 130±3           | 107±3         | 47±6           |
| 62                | 245–259                     | EQIGWMTNNPPVPG       | 136±8           | 120±8         | 50±8           |
| 63                | 249–263                     | WMNPNPPPVGEYK        | 80±13           | 63±5          | 23±5           |
| 64                | 253–267                     | NPPPVGEYKRVWII       | 84±4            | 44±2          | 24±6           |
| 65                | 257–271                     | PVPGEYKRVWILGLN      | 112±4           | -             | -              |
| 66                | 261–275                     | IYRWMILGLMNKMR      | 89±2            | -             | -              |
| 67                | 265–279                     | WILGLMNKMMYP         | 302±9           | -             | -              |
| 68                | 269–283                     | GSNRVRMYSPSIL        | 99±1            | 141±2         | -              |
| 69                | 273–287                     | IYARMYSPTSLERQ       | 53±3            | 43±3          | -              |
| 70                | 277–291                     | YSPKSLERQGPKK       | 29±2            | -             | -              |
| 71                | 281–295                     | SILERQGPKKEFPRD      | 25±3            | -             | -              |
| 75                | 297–311                     | VDFKLYTREAYQASQ      | 198±8           | 182±9         | 82±18          |
| 76                | 301–315                     | YFRLREAYQSVKYN       | 30±8            | 58±21         | 58±21          |
| 77                | 305–319                     | RAEOAYQSVKNWMTE      | 60±2            | -             | -              |
| 78                | 313–327                     | VKNWMTEETLLVONAN     | -               | 55±1          | -              |

*As detected by ex vivo IFN-γ ELISpot assay.
*Referring to the HIV-1 HXB2 Gag sequence.
*SFC, spot-forming cells (10^6 CD8-depleted splenocytes). ELISpot values ± SD were obtained from groups of 10 mice immunized with pLAMP/gag-transfected DCs prime and pLAMP/gag plasmid DNA boost (left column), pLAMP/gag-transfected DCs prime and Gag peptides boost (middle column), and pLAMP/gag plasmid DNA prime and boost (right column). Negative results are indicated by dashes.

doi:10.1371/journal.pone.0008574.t002
chromium-release assay, proliferation assay, tetramer binding, intracellular cytokine staining, and IFN-γ ELISpot of CD4 T cells. In addition, the other epitope peptides of this study that are clustered but not coincident with the currently described human CD4 epitopes [peptides no. (position): 62 (245–259), 63 (249–263), 64 (253–267), and 79 (313–327)], suggest the possible presence of other CD4 epitopes. Only one of the reported human epitopes (accession no.: 201011), was not matched by those of the DR4 Tg mice.

Representation and Variability of the Identified DR4 Epitope Peptide Sequences

Sequences of the 25 DR4-restricted clade B Gag epitope peptides of this study were analyzed for their representation (frequency) and variability in all human HIV-1 clade B Gag sequences listed in the NCBI Entrez protein database [as of August 2008]. Many of the epitope peptides were evolutionarily highly variable in their representation in the recorded HIV-1 Gag sequences; 8 were present in 71–79%; 6 in 50 to 69%; and the remaining 11 in less than 50% (Table 3 and Table S1). The number of unique variants for each of the epitope peptides ranged from 51 to a remarkable 627, with as many as 15 amino acid differences per epitope peptide (Table 3). Many of the individual variants for each epitope peptide sequence represented a significant percentage of the total representation ranging, up to 37% (Table S1). However, only four epitopes (out of 25) had a mutant variant sequence as the predominant peptide [peptide no. (position): 21 (81–95), 22 (85–99), 78 (305–319), and 79 (313–327)]; in majority of the cases (21 out

### Table 3. Representation of identified DR4-restricted T-cell epitope peptides and their variants in reported human HIV-1 clade B Gag protein sequences, obtained from the NCBI Entrez protein database.

| HLA-DR4-restricted Gag epitope peptide | Total no. of corresponding sequencesb | Representation of T-cell epitope peptide | Variants of the T-cell epitope peptide | % maximum representation of individual variantsc | Maximum no. of amino acid variations |
|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| Representation of variant peptides     | No. of unique variants                 |                                        |                                        |                                        |                                        |
| 37-ASRELREFAVNPCGGLL-51                | 3282                                   | 2322 (−71%)                            | 960 (−29%)                             | 162                                    | 307 (−9%)                             | 15                                     |
| 41-LERFANPGLLETSE-55                   | 3479                                   | 1660 (−48%)                            | 1819 (−52%)                            | 266                                    | 235 (−7%)                             | 15                                     |
| 73-EELRSLYNTVAATLYC-87                 | 3655                                   | 559 (−15%)                             | 3096 (−85%)                            | 347                                    | 427 (−12%)                            | 9                                      |
| 77-SLYNTVATLYCVHQR-91                  | 3601                                   | 341 (−9%)                              | 3260 (−91%)                            | 415                                    | 459 (−13%)                            | 6                                      |
| 81-TVATLICYCVHRIEINK-95                | 3570                                   | 144 (−4%)                              | 3426 (−96%)                            | 627                                    | 311 (−9%)                             | 8                                      |
| 85-LYCVHRIEIKDTE-99                    | 3578                                   | 297 (−8%)                              | 3281 (−92%)                            | 466                                    | 426 (−12%)                            | 7                                      |
| 161-EKAFSEPVPFMSAL-175                 | 1792                                   | 1343 (−75%)                            | 4490 (−25%)                            | 72                                     | 248 (−14%)                            | 8                                      |
| 165-SEVPMPMSALSEGA-179                | 1839                                   | 1327 (−72%)                            | 512 (−28%)                             | 71                                     | 259 (−14%)                            | 6                                      |
| 169-IPNFSALSEGPCTPDQ-183               | 1913                                   | 1319 (−69%)                            | 594 (−31%)                             | 86                                     | 267 (−14%)                            | 8                                      |
| 233-GDIAAGTTSLQEHQI-247                | 1508                                   | 1196 (−79%)                            | 312 (−21%)                             | 63                                     | 140 (−9%)                             | 8                                      |
| 241-SLQEMQIQGWNTNNPP-255               | 1489                                   | 646 (−43%)                             | 843 (−57%)                             | 133                                    | 151 (−10%)                            | 10                                     |
| 245-EIQGWMGNPPQPG-259                  | 1489                                   | 671 (−45%)                             | 818 (−55%)                             | 126                                    | 165 (−11%)                            | 13                                     |
| 249-WMNNPPIPVGIEKYK-263                | 1585                                   | 787 (−50%)                             | 796 (−50%)                             | 105                                    | 228 (−14%)                            | 14                                     |
| 253-NPPIPVEGIYKRNW-267                 | 1471                                   | 1040 (−71%)                            | 431 (−29%)                             | 78                                     | 134 (−9%)                             | 6                                      |
| 257-PVGEIYKRNWILLGLN-271               | 1474                                   | 958 (−65%)                             | 516 (−35%)                             | 76                                     | 143 (−10%)                            | 5                                      |
| 261-1YKRNWILLGLNKIVR-275               | 1473                                   | 1014 (−69%)                            | 459 (−31%)                             | 66                                     | 142 (−10%)                            | 5                                      |
| 265-WILLNKLKIVRMYSSP-279               | 1421                                   | 1013 (−71%)                            | 408 (−29%)                             | 53                                     | 236 (−17%)                            | 5                                      |
| 269-GLNKIVRMYSPTIL-283                 | 1392                                   | 790 (−57%)                             | 602 (−43%)                             | 51                                     | 398 (−29%)                            | 5                                      |
| 273-IVRMSYPSITLDIQ-287                 | 1437                                   | 542 (−38%)                             | 895 (−62%)                             | 73                                     | 365 (−25%)                            | 5                                      |
| 277-YPSITSILDIRQPGE-291                | 1460                                   | 545 (−37%)                             | 915 (−63%)                             | 71                                     | 380 (−26%)                            | 4                                      |
| 281-SILDIRQPGEKIFPR-295                | 1455                                   | 1007 (−69%)                            | 448 (−31%)                             | 61                                     | 307 (−21%)                            | 5                                      |
| 297-VDRFYKTLRRAEQASQA-311              | 1221                                   | 883 (−72%)                             | 338 (−28%)                             | 66                                     | 118 (−10%)                            | 9                                      |
| 301-YKTLRRAEQASQEVEKN-315              | 1117                                   | 369 (−33%)                             | 748 (−67%)                             | 84                                     | 409 (−37%)                            | 13                                     |
| 305-RAEQASQEVEKNN-319                  | 1113                                   | 430 (−39%)                             | 683 (−61%)                             | 80                                     | 370 (−33%)                            | 15                                     |
| 313-VKNWMTETLLVQAN-327                 | 1095                                   | 851 (−78%)                             | 244 (−22%)                             | 64                                     | 68 (−6%)                             | 3                                      |

*Example interpretation of the table: The peptide epitope 37-ASRELREFAVNPCGGLL-51 was present in 2322 sequences (71%) of all 3282 sequences analyzed corresponding to the epitope. The remaining 960 sequences (29%) at that position were variants to the epitope, which were made up of 162 unique peptides with the maximum representation of any of these peptides at 9% (307 peptides) of all the sequences analyzed. The maximum number of amino acid variations between the epitope and the variants was 15 amino acids.

*Total No. of HIV-1 Gag sequences corresponding to the T-cell epitope peptide obtained from the NCBI Entrez protein database (as of August 2008). The number of corresponding sequences for each epitope varies because the database contained both partial and full-length sequences, the consequence of which is that some regions have more sequence information than others.

*For each one of the T-cell epitope peptides, the % minimum representation of individual variants was less than 1%.

doi:10.1371/journal.pone.0008574.t003
of 25), the epitope sequence was the peptide with the highest representation. There was no specific pattern for the distribution of the mutant amino acids in the variants (difference of 3 to 15 aa).

**Discussion**

DCs serve critical roles in facilitating immune responses to foreign Ag, in particular by promoting efficient Ag presentation and co-stimulation to CD4$^+$ T lymphocytes. Although several different bone marrow–derived cells can present Ag in vitro, the main APCs that do so in vivo are DCs [24–26]. DCs also engage in cross-presentation of peptide epitopes to CD8$^+$ T-cells and cross-priming of cells for MHC class I-dependent Ag presentation [27]. Because of these capacities, DC-based vaccines are widely considered to be promising strategies for cancer and persistent pathogen infections such as HIV. This may be compared to naked DNA vaccines which typically express limited amounts of Ag largely restricted to inoculation sites, have restricted access to professional APCs, and poorly activate inflammatory responses, unless combined with additional genetic adjuvants [28].

Here we have studied the immunization of BALB/c (H-2d) and HLA-DR4 Tg mice by autologous DCs transfected ex vivo by a DNA plasmid encoding HIV-1 Gag inserted into the LAMP-1 luminal domain, or by direct immunization with the naked DNA plasmid. Immunization by DCs transfected ex vivo is believed to deliver a large number of Ag-activated DCs to the immunized animal, with greater antigen presentation to T cells than would occur with direct immunization by the naked DNA vaccine plasmid. However, the transfected DCs as the primary immunogen would likely have limited delivery of antigen to any other cell in vivo, greatly reducing the transfection of non-professional APCs and MHC class I dependent presentation of peptide epitopes to CD8$^+$ cells [29]. This was likely offset, however, by the subsequent boost with naked DNA encoding LAMP/gag or with peptides, both of which provide Ag access to cells for MHC class I Ag presentation. In contrast to immunization with transfected DC, immunization with the naked DNA plasmid encoding LAMP/gag results in transfection at the site of immunization of large numbers of muscle and other non-professional MHC class I APCs and likely results in relatively large scale activation of CD8$^+$ T cells [30]. Targeting of the LAMP/gag chimera to lysosomes of professional APCs is known to result in marked co-localization of the fusion Ag with MHC class II molecules, and activation of both Ag-specific CD4$^+$ and CD8$^+$ T cells [31,32]. An additional factor could be the release of HIV Gag from cells as exosome-like vesicles [33] which has been shown to occur in animal model studies [34]. This peculiar property of HIV gag could represent a significant route for its acquisition by DCs in vivo in the absence of LAMP-1 targeting.

The epitope-specific T-cell responses to HIV-1 Gag generated by immunization with either the autologous immature DC transfected with pLAMP/gag or naked pLAMP/gag was measured by IFN-γ ELISpot assay using a panel of 123 overlapping Gag peptides encompassing the entire protein. Groups of mice were primed by sc immunization with LAMP/gag DNA-transfected DCs or naked LAMP/gag plasmid DNA, and then boosted with the LAMP/gag plasmid DNA or gag peptide pools. The consistency of T-cell epitope peptide responses elicited by the
three immunization protocols with both the BALB/c and HLA DR4 Tg mice is evident from the ELISpot assays with 10 μg/mL peptide. The major differences were the greater fraction of total peptide repertoire and greater apparent avidity of the peptides in the ELISpot assay for either MHC or T-cell receptor binding following the DC LAMP/gag prime-pLAMP/gag boost protocol. The stronger responses to the DC LAMP/gag-pLAMP/gag prime-boost can most simply be attributed to an increased number of antigen presenting DCs following in vitro transfection, or to an increased level of antigen presentation or access to MHC loading compartments. Some of the peptides were distinguished by strong T-cell activation at low peptide dose in all tested groups of either the BALB/c or HLA DR4 Tg mice at low peptide dose. Other peptides required higher concentration of peptides in the ELISpot assay and the response was enhanced in or unique to mice primed with plasmid DNA-transfected DCs and boosted with peptides or plasmid DNA. The biological correlates to the differences in the immune responses to the peptides are not known. However, it can be expected that specific epitopes may have unique properties. It has been reported that DCs transfected with interleukin-12 and tumor-associated Ag-encoding mRNA were found to induce high avidity cytotoxic T-lymphocytes (CTLs) [35], and that DCs transfected with RNA encoding tumor antigens resulted in prolonged Ag presentation and the generation of high-affinity tumor-reactive CTLs [36]. Other experiments have also suggested that epitopes that elicit extremely strong T-cell responses may in fact lead to immune tolerance [37].

The low representation of the epitope peptide sequences and their high number of variants represent a major issue for the development of an effective HIV vaccine. The DR4 epitope peptides exhibited variants with as little as 3 or as many as 15 amino acid differences. Vaccine strategies based on Gag or possibly any HIV-1 protein as immunogen may not be effective in preventing natural infection because of the large number of HIV-1 genomic variants as potential altered epitope peptides. Subtle amino acid mutational differences of HLA-restricted T-cell determinants between a vaccine formulation and a natural infection [38] can dramatically alter the magnitude of the T-cell response and result in a wide range of effects from increased immune activation to anergy [39–43]. This may possibly be in part overcome by selection of epitope peptides that have high conservation and representation, and where only a minority of subjects would be infected by a virus with a protein sequence that differed from the consensus sequence represented by the vaccine immunogen. It is evident, however, that the extremely high mutation rate of the HIV-1 virus represents a severe problem for the development of an effective vaccine. These issues may be elucidated by further use of HLA transgenic mice for analysis of T-cell activation with selected peptides and their variant forms.

In conclusion, the methodology described in this study, the use of LAMP/gag transfected immature DCs to stimulate an Ag-specific T-cell immune response in vivo, provides a broadly applicable strategy for the functional activation of T-cell antigens for HIV therapeutic vaccines that effectively induce HIV-gag specific T-cell responses, with increased avidity and repertoire. The identification of HIV epitopes that represent a large proportion of the circulating virus population must be included to develop a HIV vaccine that can stimulate a robust immune response.

Materials and Methods
Cloning of the pLAMP/Gag DNA Construct
The nucleotide sequences of the HIV-1 clade B HXB2 (NCBI Accession No. K03455, M38432) that codes for the p55gag Ag was cloned, with the addition of XhoI and EcoRI restriction sites, into the p43-LAMP-1 eukaryotic expression vector, between the LAMP-1 luminal and transmembrane sequences. The p43-LAMP-1 vector is a pcDNA3.1-based DNA plasmid containing a cytomegalovirus (CMV) promoter, the LAMP-1 luminal sequence, the XhoI and EcoRI cloning sites, the LAMP-1 vector is a pcDNA3.1-based DNA plasmid containing a cytomegalovirus (CMV) promoter, the LAMP-1 luminal sequence, the XhoI and EcoRI cloning sites, the LAMP-1 luminal and transmembrane sequences, and the bovine growth hormone polyadenylation sequence, flanked by two adeno-associated virus inverted terminal repeat sequences [44].

Mice
Murine H-2 class II-deficient, HLA-DR4 (DRA1*0101, DRB1*0401)/human CD4 (huCD4) Tg mice [45–47] were bred and maintained in the Johns Hopkins University School of Medicine Animal Facility. HLA-DR expression and CD4+ T-cell frequency in the experimental mice were evaluated by flow cytometry using HLA-DR-FITC and CD4-PE (BD Bioscience Pharmingen, San Diego, CA) conjugated antibodies, respectively. BALB/c mice were purchased from Charles River (Wilmington, MA). Mice were housed in specific pathogen-free colonies and maintained in a helicobacter-negative mice facility. All animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Experimental Animals and approved by Johns Hopkins Animal Care and Use Committee.

Bone Marrow Dendritic Cell Culture
Murine DCs were generated from the bone marrow using a protocol slightly modified from Inaba et al. [48]. Briefly, the donor mice were euthanized by CO2 asphyxiation followed by cervical dislocation, the femurs and tibias collected, and the muscle tissues removed with gauze. The bones were placed in a 60 mm dish with 70% ethyl alcohol for 1 min, washed twice with PBS, and transferred into a fresh dish with RPMI 1640. The bone marrows were then flushed with 3 ml RPMI 1640 per bone and the viable cells were counted using a Via-CELL Cell Viability Analyzer (Beckman Coulter, Fullerton, CA). The cells were centrifuged at 300g for 10 min, and the supernatant discarded. The cell pellet was resuspended in RPMI-1640 supplemented with 10% mouse serum, 2000 U/ml GM-CSF, 100 units/ml penicillin-streptomycin-c, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1 M HEPES buffer at 1×10⁶ cells/mL, transferred into a 24-well plate (1ml/well), and incubated in a 37°C incubator with a 5% CO2 atmosphere. Fresh medium containing GM-CSF was added every second day. On day seven the immature DCs were harvested by collecting non-adherent and loosely adherent cells.

Electroporation of Immature Dendritic Cells
After seven days culture, the immature DCs were harvested and centrifuged at 300 g for 10 min, resuspended in 100 μL of Nucleofector solution (Lonza Inc, Walkersville, MD) to a final concentration of 2.5×10⁷/100 μL, and 5 μg of the pLAMP/gag plasmid DNA was added to each microcentrifuge tube. The mixture was transferred to an Amaxa certified cuvette and electroporated with the preset Nucleofector program Y-001 for immature DCs. The transfected DCs were then suspended in 400 μl complete media and transferred onto a 48-well plate and incubated overnight in a humidified 37°C 5% CO2 incubator.

DC Immunization Protocol
Three experimental immunization groups of 10 mice (5 pools of 2 mice) was immunized and boosted three to four weeks later by subcutaneous injection at the base of the tail.
Group 1 Prime: 1 x 10^7 DCs transfected with p43LAMP/gag plasmid (5 μg); Boost: p43LAMP/gag plasmid (5 μg)
Group 2 Prime: same as Group 1; Boost: pooled peptide (1μg/peptide)
Group 3 Prime: p43LAMP/gag plasmid (50 μg); Boost: same as prime
Group 4 Prime: 1 x 10^7 DCs transfected with p43GFP plasmid (5 μg); Boost: p43GFP plasmid (5 μg)

Splenocyte Isolation
Ten days following the boost immunization, splenocytes were harvested from mice euthanized as previously described. Single cell suspensions were prepared by manual disruption and separation through a 70 μm cell strainer. The cells were washed with complete RPMI (1640 medium supplemented with 10% FBS, 100 units/mL penicillin-streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1 M HEPES buffer), centrifuged for 10 min at 300 g at 4°C, and resuspended in 5 ml ammonium chloride for 5 min at room temperature to lyse the red blood cells. Then 25 ml complete RPMI-1640 was added to stop the reaction. The cells were again centrifuged at 300 g at 4°C and the pellet was resuspended in 10 ml complete RPMI-1640. Cells were counted with the Vi-CELL Cell Viability Analyzer and adjusted to a concentration of 1 x 10^6/mL in complete RPMI.

HIV p55gag Synthetic Peptides
A library of 123 overlapping 15 amino acid (aa) peptides spanning the entire HIV-1 HBX2 p55gag protein, more than 80% pure, were obtained from Biodefense & Emerging Infections Research Resources Repository (Manassas, VA). Lyophilized peptides were dissolved at 1 mg/mL in 20% DMSO and water, aliquoted, and stored at −20°C.

IFN-γ ELISPOT Assay
Ex vivo IFN-γ ELISPOT assays were performed by use of murine IFN-γ ELISPOT sets (BD Biosciences) according to manufacturer’s protocol. For Tg mice, single cell suspensions from freshly isolated splenocytes in complete RPMI-1640 were depleted of CD8 T-cells using antibody-coated magnetic beads (Miltenyi Biotec, Auburn, CA) according to manufacturer’s protocol. A total of 1 x 10^6 splenocytes diluted into 100 μl complete RPMI-1640 were added to wells containing individual p55gag peptides. Concanaavalin A (Sigma-Aldrich, St. Louis, MO), 5 μg/ml, and culture medium without peptide were used as positive and negative controls, respectively. Following assay protocol, the plates were air-dried, and the spots counted using a stereomicroscope at 40× magnification with the ImmunoSpot software (CTL analyzer, Cleveland, OH). The average number of spot forming cells (SFC) was adjusted for 1 x 10^6 splenocytes.

Evaluation Statistics
The average IFN-γ secreting T-cell responses against each peptide for vaccinated mice were compared by a nonparametric t test to the background values of control mice primed with a green fluorescent protein expressing p43 plasmid (p43GFP). The responses were considered positive when the p value was >0.05 and the average response was >10 IFN-γ SFC/10^6 splenocytes. Negative control assays for mice immunized with the p43-GFP plasmid DNA commonly gave blank values of 0–10 SFC per 10^6 splenocytes.

Comparison of Identified HLA-DR4 Epitope Peptides with Reported Human DR4 Epitopes
The epitopes of the HLA DR4 Tg mice identified in this study were compared to the reported epitopes of HIV-1 infected humans by performing blastp [49] search with an in house database (February 2009) of 388 T-helper/CD4+ epitope sequences, obtained from the HIV immunology database. BLAST hit sequences with at least nine consecutive amino acid matches to the query epitope were extracted for further analysis. All identified T-helper/CD4+ epitopes in the HIV immunology database were retrieved and recorded with HLA-DRB1*0401 or DR4 restrictions in the “Species (MHC/HLA)” field were selected.

Diversity Analysis of the Identified DR4 Epitope Peptide Sequences
A total of 6,405 complete and partial human HIV-1 clade B Gag protein sequences were retrieved from the NCBI Entrez protein database records as of August 2008. Multiple sequence alignment of the sequences was performed by use of Promals3D [50], which was then manually inspected and corrected for misalignments. The diversity of each of the identified HIV-1 Gag DR4 Tg epitope peptide sequence was studied by extracting all the sequences in the alignment corresponding to the position of the epitope, except those that contained gaps (-) or any one of the unresolved characters, including B (asparagine or aspartic acid), J (leucine or isoleucine), X (unspecified or unknown amino acid) and Z (glutamine or glutamic acid). All the extracted sequences were then analyzed for the representation (frequency) of the epitope sequence and its variants. Variants are defined as peptides with at least one amino acid difference from their corresponding identified epitope.

Supporting Information
Table S1 The representation of HLA-DR4 transgenic mice epitope peptides and their variants in clade B HIV Gag protein. Found at: doi:10.1371/journal.pone.0008574.s001 (0.35 MB DOC)

Acknowledgments
We thank Drs. Paul Tan and Eduardo Nascimento for critical reading of the manuscript and valuable discussions. We also thank Benjamin, Tan Yong Liang and Natascha May Thevasagayam for their technical help.

Author Contributions
Conceived and designed the experiments: GGS AMK ETMJ JTA. Performed the experiments: GGS AMK JZ PC KOJ. Analyzed the data: GGS YH AMK JTA. Contributed reagents/materials/analysis tools: GGS YH AMK JTA. Wrote the paper: GGS YH AMK JTA.

References
1. Casares S, Inaba K, Bruneau TD, Steinman RM, Bona CA (1997) Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. J Exp Med 186: 1401-1406.
2. Kurts C, Cannarile M, Klebba I, Broker T (2001) Dendritic cells are sufficient to cross-present self-antigens to CD8 T cells in vivo. J Immunol 166: 1439-1442.
3. Jung S, Unutmaz D, Wong P, Sano G, De los SK, et al. (2002) In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity 17: 211-220.
4. Barczaress J, Palucka AK (2005) Dendritic cells as therapeutic vaccines against cancer. Nat Rev Immunol 5: 296-306.
5. Nair SK, Heiser A, Boczkowski D, Majumdar A, Naoe M, et al. (2000) Induction of cytotoxic T cell responses and tumor immunity against unrelated...
tumors using telomerase reverse transcriptase DNA transfected dendritic cells. Nat Med 6: 1011–1017.

2. Liao X, Li Y, Bonini C, Nair S, Gilboa E, et al. (2004) Transfection of RNA into dendritic cells: efficient gene transfer by electroporation into human monocyte-derived dendritic cells. FEBS Lett 538: 149–154.

3. Landi A, Babuik LA, van Drunen Littelvan den Hurk S (2007) High transfection efficiency, gene expression, and viability of monocyte-derived human dendritic cells after nonviral gene transfer. J Leukoc Biol 82: 189–190.

4. Artusio E, Hathaway B, Stansell J, Whiteside TL (2006) Transfer of human monocyte-derived dendritic cells with native tumor DNA DNA induces antigen-specific T-cell responses in vitro. Cancer Biol Ther 5: 1624–1631.

5. Nair SK, Boczkowski D, Morse M, Cumming RI, Lordy HK, et al. (1998) Induction of primary carinomycobacterial antigen (CEA) specific cytotoxic T lymphocytes in vitro using human dendritic cells transfected with RNA. Nat Biotechnol 16: 364–369.

6. Rosati M, Van Audenhove E, Jalal R, Patel V, von Gegerfelt A, et al. (2000) Increased immune responses in rhesus macaques by DNA vaccination combined with electroporation. Vaccine 26: 5223–5229.

7. Marques ET Jr, Chikhlikar P, de Arruda LB, Leao IC, Lu Y, et al. (2003) HIV-1 p55Gag encoded in the lysosome-associated membrane protein-1 as a DNA plasmid vaccine chimeric is highly expressed, traffic to the major histocompatibility class II compartment, and elicits enhanced long-term memory response. J Biol Chem 278: 37926–37936.

8. Chahal PK, Choudhury A, Agrawal S, Byrne B, Guggino W, et al. (2004) Inverted terminal repeat sequences of adenovirus-associated virus enhance the antibody and CD8+ responses to a HIV-1 p55Gag/LAMP DNA vaccine chimer. Virology 323: 220–232.

9. de Arruda LB, Chikhlikar PR, August JT, Marques ET (2004) DNA vaccine encoding human immunodeficiency virus-1 Gag, targeted to the major histocompatibility complex II compartment by lysosomal-associated membrane protein, elicits enhanced long-term memory response. Immunology 112: 126–133.

10. Chikhlikar P, de Arruda LB, Maciel M, Silveira P, Lewis MG, et al. (2006) DNA encoding an HIV-1 Gag/human lysosome-associated membrane protein-1 chimeric elicits a broad cellular and humoral immune response in Rhesus macaques. PLoS ONE 1: e135.

11. Arroja LB, Sim D, Chikhlikar PR, Maciel M Jr, Aksaki K, et al. (2006) Dendritic cell-lysosomal-associated membrane protein (LAMP)- and LAMP-1-HIV-1 gag chimeras have distinct cellular trafficking pathways and prime T and B cell responses to a diverse repertoire of epitopes. J Immunol 177: 2263–2275.

12. Fugger L, Michie SA, Rulifson I, Lock CB, McDevitt GS (1994) Expression of HLA-DR4 and human CD4 transgenes in mice determines the variable region beta-chain T-cell repertoire and mediates an HLA-DR-restricted immune response. Proc Natl Acad Sci U S A 91: 6151–6155.

13. Patel SD, Cope AP, Gongia M, Chen TT, Kim E, et al. (1997) Identification of immunodominant T cell epitopes of human glutamic acid decarboxylase 65 by using HLA-DR(alpha1*0101,beta1*0401) transgenic mice. Proc Natl Acad Sci U S A 94: 8082–8087.

14. Cope AP, Patel SD, Hall F, Gongia M, Hubers HA, et al. (1999) T cell responses to a human cartilage autoantigen in the context of rheumatoid arthritis-associated and nonassociated HLA-DR4 alleles. Arthritis Rheum 42: 1497–1507.

15. Kurts C, Cannarile M, Klebba I, Brocker T (2001) Dendritic cells are sufficient to present self-antigens to CD8 T cells in vivo. J Immunol 166: 1439–1442.

16. Jung S, Unutmaz D, Wong P, Sano G, Lee OS, et al. (2002) In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity 17: 211–220.

17. Bela GT, Behrens GM, Smith CM, Miller JP, Jous C, et al. (2002) The CD8 alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. J Exp Med 196: 1099–1104.

18. de Arruda LB, Chikhlikar P, de Arruda LB, Leao IC, Lu Y, et al. (2004) DNA vaccine encoding human immunodeficiency virus-1 Gag, targeted to the major histocompatibility complex II compartment by lysosomal-associated membrane protein, elicits enhanced long-term memory response. Immunity 17: 211–220.

19. Bedouin A, Heirman C, Tuyaerts S, Michiels A, Breckpot K, et al. (2004) Transfection of RNA encoding tumor antigens following maturation of dendritic cells leads to prolonged presentation of antigen and the generation of high-affinity tumor-reactive cytotoxic T lymphocytes. Mol Ther 9: 757–764.

20. Bonehill A, Heirman C, Tuyaerts S, Michiels A, Beekpoen K, et al. (2004) DNA vaccine encoding tumor antigens following maturation of dendritic cells leads to prolonged presentation of antigen and the generation of high-affinity tumor-reactive cytotoxic T lymphocytes. Mol Ther 9: 757–764.

21. Dell K, Klein G, Gissmann L (2008) Comparison of DNA- and mRNA-transfected monocyte-derived dendritic cells as potential vaccines against the human papillomavirus type 16 associated oncoprotein E7. Antivir Ther 13: 495–509.

22. Mitchell DA, Nair SK (2000) RNA-transfected dendritic cells in cancer immunotherapy. J Clin Invest 106: 1065–1069.

23. Marques ET Jr, Chikhlikar P, de Arruda LB, Leao IC, Lu Y, et al. (2003) HIV-1 p55Gag encoded in the lysosome-associated membrane protein-1 as a DNA plasmid virus chimeric is highly expressed, traffic to the major histocompatibility class II compartment, and elicits enhanced long-term memory response. J Biol Chem 278: 37926–37936.