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A model for testing the immunogenicity of simian immunodeficiency virus (SIV) and simian-human immunodeficiency virus (SHIV) vaccine candidates in mice

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Abstract

HIV-1 Gag protein represents a promising target of cellular immunity-based vaccines due to its immunogenicity and high conservation among diverse viral subtypes. Development of novel and effective Gag-targeted vaccine candidates inducing CD8⁺ and CD4⁺ T cell responses requires large scale pre-clinical testing in a small animal model. In this report, the MHC class I and II-restricted epitopes in the SIV Gag protein recognized in C57Bl/6 and Balb/c mice were determined and characterized. In addition, using the newly defined epitopes, the relationship is described between the amount of plasmid DNA, volume of inoculate, and the extent of ensuing immune responses following intramuscular DNA immunization.

Keywords

HIV-1; SIV; SHIV; vaccine; DNA vaccine; recombinant viral vaccine; CD4⁺ T cell; CD8⁺ T cell; T cell epitope

1. Introduction

Cytotoxic T lymphocytes (CTLs) play a pivotal role in the control of HIV-1 infection in humans and SIV infection in macaques (Jin et al., 1999; Matano et al., 1998; Goulder and Watkins, 2008). Although the vaccines based on the induction of cellular immune responses do not protect against the acquisition of the virus, the presence and rapid expansion of virus-specific CTLs at the time of infection results in a state of immune control restricting viral proliferation and decelerating progression to disease (Hel et al., 2006a; Hel et al., 2002; Hel et al., 2006b). Despite the recent setbacks in the HIV-1 vaccine field, results obtained using highly pathogenic SIV infection in macaques suggest that vaccine-induced cellular immunity can exert considerable control over replication of an immunodeficiency virus in a complete absence of Env-specific neutralizing antibodies (Watkins et al., 2008). Elicitation and maintenance of both CTL and humoral responses is dependent on help from CD4⁺ helper T cells (Bevan, 2004). CD4⁺ helper T cells support a prompt response to acute infection as well long-term control of chronic infection. We and others have demonstrated a key role of vaccine-induced CD4⁺ T cells in conferring protection against

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Competing interest statement

The authors declare no competing interests.

immunodeficiency viruses (Hel et al., 2000; Hel et al., 2002; Hel et al., 2006b; Tryniszewska et al., 2002; Letvin et al., 2006; Villinger et al., 2002). However, migration of activated virus-specific CD4⁺ T cells to the site of infection may fuel viral proliferation by providing highly susceptible target cell population (Staprans et al., 2004). Therefore, the net effect of vaccine-induced CD4⁺ T cells on HIV-1/SIV infection depends on their tissue distribution and functional characteristics.

A number of approaches aiming to enhance the frequency of CD4⁺ and CD8⁺ T cells elicited by DNA or recombinant viral vaccines and modulate their functional properties have been devised, such as strategies based on co-expression of cytokine and chemokine factors, costimulatory molecules, linkage to molecular adjuvants and antigen presenting cell-targeting ligands, modifications of protein sequences enhancing their immunogenicity, and design of poly-epitope constructs (Liu et al., 2006b). Infection of new world non-human primates with pathogenic SIV isolates such as SIV_{MAC239} or SIV_{MAC251} is the only available model for the testing of protective efficacy of vaccine candidates relevant to HIV-1 infection in humans. However, high cost and ethical issues bar large scale pre-testing of vaccines' immunogenicity in primates. Every effort should be taken to limit the size of experiments in non-human primates to the necessary minimum. Thus, small animal model is needed to test the immunogenicity and select the most promising SIV vaccine candidates in pre-clinical trials. Although several CD8⁺ T cell epitopes in SIV Env and Gag protein recognized in mice have been defined, no study has systematically mapped CD4⁺ T cell epitopes in mice immunized with SIV Gag, a basic component of most SIV and SHIV vaccine candidates. In this report, a detailed screening strategy is employed to map the available CD4⁺ and CD8⁺ T cell epitopes recognized in the two most common mouse strains, C57Bl/6 (B6) and Balb/c.

2. Materials and methods

2.1. Mice

Female C57Bl/6 and Balb/c mice, 6–8 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal care and procedures conformed to the UAB Institutional Animals Care and Use Committee guidelines and requirements. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

2.2. Reagents

Gene encoding SIV Gag based on the sequence of SIV_{MAC239} isolate (accession nos. [M19499](#), [M15897](#), [M16125](#), [M24614](#), and [AAB59905](#)) was generously provided by Dr. G. Pavlakis. To optimize for mammalian expression, the previously identified *gag* inhibitory sequences (INS) were mutated by introducing multiple silent point mutations not affecting the encoded protein precursor, as previously described for HIV-1 *gag* (Qiu et al., 1999; Hel et al., 2001). DNA fragments encoding SIV Gag and sequence-optimized luciferase gene were cloned into an expression vector pCMV-1 encoding kanamycin resistance (Gene Therapy Systems, San Diego, CA). A set of 123 peptides (15-mers overlapping by 11 amino-acids) covering the entire SIV Gag protein sequence was obtained from the NIH AIDS Research & Reference Reagents program. All other peptides were synthesized by Genemed Synthesis, Inc. (South Francisco, CA). Peptides were dissolved in DMSO (Sigma) at a final concentration 100 mg/ml. Gag 312_{AL11}-specific tetramer was prepared in the NIH Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). Cells were incubated in complete RPMI medium (RPMI-1640, 10% FBS, 100 U penicillin, 100 U streptomycin, 50 µM 2-mercaptoethanol; Invitrogen, Carlsbad, CA).

2.3. Immunizations

Unless otherwise specified, mice were immunized by a single injection of 50 µg of SIV Gag DNA in 50 µl PBS into the anterior tibialis muscle using an insulin syringe equipped with 27 G needle (BD). For the studies on the effect of the volume of inoculate on ensuing immune responses, DNA was injected into surgically exposed quadriceps or anterior tibialis muscles to ensure precise delivery. For the delivery of 5 µl of inoculate, high pressure syringe (Hamilton, Reno, NE) equipped with custom-made 27 G needle was used.

2.4. Intracellular cytokine staining (ICS) and tetramer staining

10^6 splenocytes were incubated with specific peptides at indicated concentrations in complete RPMI medium for 6 hrs at 37°C. GolgiStop (BD Pharmingen, San Diego, CA) was added for the final 4 hrs of stimulation. The cells were blocked with CD16 antibody, stained with CD4-PE and CD8-PerCP (BD), permeabilized in the Cytofix/Cytoperm buffer (BD), and stained intracellularly with IFN-γ-FITC antibody (BD). Cells were washed, fixed in 1 % paraformaldehyde in PBS, and analyzed by flow cytometry (FACSCalibur, BD) as described previously (Hel et al., 2001; Hel et al., 2002).

To detect Gag 312_AL11-specific T cells, the splenocytes or peripheral blood mononuclear cells (PBMCs) were incubated with PE-conjugated 312_AL11-specific tetramer for 20 min at 4°C, CD8-PerCP antibody (BD) was added and the cells were stained for additional 20 min prior to the washing and analysis (Hel et al., 2001; Hel et al., 2002).

2.5. In vivo cytotoxicity assay

14 days prior the experiment, mice were immunized once with 50 µg of SIV Gag DNA in 50 µl PBS injected into the anterior tibialis muscle. Purified splenocytes were obtained from syngeneic donor mice and divided into two groups. One group of splenocytes was incubated in complete RPMI-1640 medium containing 10 µM specific peptide for 1 hr and washed twice (target population). Both groups were labeled with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). Peptide-pulsed cells were incubated in pre-warmed PBS containing 3 µM CFSE at 10^7 cells per ml at 37°C for 9 min with regular agitation and washed twice. Unpulsed splenocytes were labeled with 0.3 µM CFSE (control population). Immunized and control mice were injected intravenously with 10^7 splenocytes labeled with 0.3 µM CFSE (control population) and 10^7 splenocytes labeled with 3 µM CFSE and pulsed with specific peptide (target population). The two populations were mixed immediately prior the injection to minimize the time during which a transfer of antigenic peptide to unpulsed population might occur. Cells were injected into lateral tail vein. Only the peptide-pulsed population serves as a target of peptide-specific CTLs in vivo. The spleens and lymph nodes (LNs) of immunized and control animals were harvested 20 hrs later and the relative killing of target cells was determined as the ratio between the target and control populations. Naïve mice served a negative control.

2.6. Lymphoproliferation assay

2×10^5 splenocytes per well were incubated in 96-well flat bottom plates in complete RPMI medium alone, with specific peptides, or with Concanavalin A as a positive control (10 µg/ml, Sigma) for 3 days. ^3H -thymidine (1 µCi / well) was added for the last 24 hrs and the rate of proliferation was determined by ^3H incorporation into DNA as described (Hel et al., 2001; Hel et al., 2002).

2.7. Luciferase assay

The indicated amounts of plasmid DNA encoding luciferase were injected in indicated volumes of PBS into surgically exposed muscles. The muscle tissue was harvested 3 days

later and homogenized in a lysis buffer using a tissue homogenizer (Fisher Scientific). The concentration of luciferase was determined using a Luciferase Reporter Assay Kit (BD Biosciences Clontech) according to manufacturer's instructions and the emitted fluorescence was quantified on a fluorometer (Turner Biosystems, Sunnyvale, CA). Protein concentration was determined using a Bradford-based assay (Promega, Madison, WI) and the concentration of luciferase was expressed as a percentage of relative light units (RLUs) per mg of protein.

2.8. Statistical analysis

All reported P values are two-sided. Group comparisons were performed using the Mann-Whitney rank sum test. The Sigmasat (version 3.1, SPSS, Chicago, Illinois) statistical software package was used for the analysis.

3. Results

3.1. Definition of H-2^b-restricted SIV Gag CD8⁺ and CD4⁺ T cell epitopes in C57Bl/6 (B6) mice

In order to map comprehensively the T cell epitopes recognized in SIV Gag-immunized B6 mice, a set of 123 15-meric peptides overlapping by 11 amino acids and encompassing the entire SIV Gag protein was employed. To limit the number of samples for initial screening, peptides were arranged in peptide pool arrays as depicted in an example in Table 1 (Tobery et al., 2001). Each peptide mix tested contained 6–8 peptides thus reducing the number of samples in the initial screening from 123 to 32. Each individual 15-meric peptide was present in exactly one horizontal and one vertical peptide pool, allowing for an easy identification of candidate epitopes. Candidate epitopes were identified by their ability to induce intracellular IFN- γ production in CD8⁺ or CD4⁺ T cell population in splenocytes from DNA-immunized mice. Direct screening method was combined with computer-aided prediction of epitopes using SYFPEITHI and BIMAS programs predicting potential epitopes based on the homology to known epitopes, presence of known anchor residues, and estimated affinity of MHC-peptide interaction. IFN- γ production induced by two overlapping peptides indicated a presence of a single dominant CD8⁺ T cell epitope at a position 309 – 328 (Fig. 1A). Since the amino acid sequence did not conform to the known D^b-restricted consensus sequence (Asn at position 5 and Met, Leu, or Ile at position 9 (Falk et al., 1991)), precise mapping was performed. A non-traditional 11 amino acids epitope 312_AL11 was identified with predicted anchor residues Asn at position 5 and a hydrophobic C-terminal Leu at position 11 (AAVKNWMTQTL; Fig. 1 B and C). 312_AL11 epitope exerts exceptionally high affinity and activates T cells at concentrations as low as 10⁻¹⁰ (Fig. 1D). In contrast, truncation of the peptide by a single amino acid on either side results in about 1,000-fold decrease of affinity. The correlation between the in silico prediction of the potential epitopes in B6 mice and the actual epitope identified was limited, primarily due to the unusual structure of this epitope. To facilitate further studies, a H-2^b-312_AL11 tetramer was constructed. Tetramer staining of lymphocytes from a B6 mouse immunized with a single dose of DNA plasmid encoding SIV Gag resulted in an elicitation of 312_AL11-specific cells at 1.7 % of total CD8⁺ T cells. To confirm that 312_AL11-specific T cells exert CTL activity, an in vivo CTL assay was performed. The results indicated 91 % killing of 312_AL11-coated target cells in spleen and 68 % killing in lymph nodes of DNA-immunized animals 20 hrs following injection (Fig. 2B).

Initial screening revealed a presence of three CD4⁺ T cell epitopes at positions 13–28, 57–76, and 297–316 (Fig. 3A). Since MHC-II can present peptides up to 20 amino acids long, 19-meric peptides combining the two neighboring peptides were tested alongside the 15-mers (Fig. 3B). While the ability of 19-mers to induce intracellular IFN- γ production fell

between the individual 15-mers, they exerted higher ability to induce *ex vivo* lymphoproliferation. Altogether, responses to epitope 297_YK19 were stronger than those to epitope 57_CL19 and 13_AK15 (Fig. 3A, C, D).

3.2. Definition of H-2^d-restricted SIV Gag CD8⁺ and CD4⁺ T cell epitopes in Balb/c mice

Using the techniques described above, a single SIV Gag H-2^d-restricted CD8⁺ T cell epitope (426_KF9) recognized in DNA-immunized Balb/c mice was mapped (Fig. 4A). Pro, Arg, and Phe at positions 3, 5, and 9, respectively, of the D^d consensus sequence are conserved in the 426_KF9 epitope (KCPDRQAGF). H-2^d-restricted epitope 426_KF9 was among the top candidate epitopes predicted by *in silico* modeling. Furthermore, four potential CD4⁺ epitopes were identified (5_NI15, 101_KA15, 109_VR15, and 297_YD15). It remains to be determined whether the epitopes at positions 101–116 and 109–124 represent one overlapping or two independent epitopes. Interestingly, epitope 297_YD15 is recognized in both H-2^b and H-2^d-restricted animals.

3.3. The volume of DNA vaccine inoculate affects its immunogenicity

Using the newly defined SIV Gag epitopes, the relationship between the amount of DNA, volume of inoculate, and the ensuing immunogenicity of a DNA vaccine was addressed. As shown on Fig. 5A, large volume of inoculate is critical for the induction of CD8⁺ and CD4⁺ T cell responses by intramuscular DNA injection. Identical amount of injected DNA (20 µg) induced 10 to 20-fold higher frequencies of antigen-specific CD8⁺ and CD4⁺ T cells when injected in 50 µl of inoculate than when injected in 5 µl ($P < 0.03$). To determine the effect of volume on intramuscular production of the transgene-encoded protein, the activity of luciferase protein in extracts from muscle tissues from animals injected with luciferase-encoding plasmid into the quadriceps and anterior tibialis muscles was determined. Larger volume of inoculate (50 µl) resulted in 5 to 10-fold higher production of the enzyme compared to lower volume (5 µl; $P < 0.01$) (Fig. 5B).

4. Discussion

Using a combination of *in silico* prediction and direct screening methods, one CD8⁺ and three CD4⁺ H-2^b-restricted T cell epitopes recognized in SIV Gag-immunized B6 mice and one CD8⁺ and three CD4⁺ H-2^d-restricted T cell epitopes recognized in Balb/c mice were determined (Fig. 4B). 312_AL11 epitope represents a non-traditional, immunodominant, high affinity CTL epitope. This epitope has been independently reported during the course of these studies (Barouch et al., 2004). In addition, Liu et al. reported mapping of a CD4⁺ T cell epitope DD13 at position 299, confirming one of the three CD4⁺ T cell epitopes identified in this report (297_YK19) (Liu et al., 2006a). None of the other H-2^d and H-2^b-restricted epitopes defined hereby has been reported before. Careful mapping of the CD8⁺ and CD4⁺ epitopes facilitates efficient evaluation of immunogenicity of future SIV and SHIV vaccine candidates in pre-clinical studies prior the testing in non-human primates. Importantly, this model addresses concomitant induction of CTL and T helper responses. Since the responses to individual CD4⁺ T cell epitopes are of variable strength, vaccination strategies aiming at the enhancement of responses to subdominant epitopes may be tested using this model.

The newly defined CD4⁺ and CD8⁺ SIV Gag epitopes recognized in B6 mice were used to address whether the volume of inoculate affects the immunogenicity of a DNA vaccine. Administration of a larger volume of inoculum resulted in significantly higher responses compared to a smaller volume, both in the CD4⁺ and CD8⁺ T cell compartments. This can be explained either by an improved delivery of plasmid DNA into muscle cells due to higher hydrostatic pressure and extended distribution of the inoculum in the muscle or by ensuing

inflammatory reaction associated with partial destruction of muscle morphology. Using the luciferase reporter construct, it was demonstrated that the production of plasmid-encoded protein is increased following delivery in larger volume. This corresponds well with the observations of Doh et al. suggesting that high volume of inoculum may distend the extracellular space in the muscle longitudinally along paths of least resistance (Doh et al., 1997). Transfection occurs predominantly near the myotendinous junction where myofiber cells form invaginations and processes increasing sarcolemmal surface area. Increased diffusion rate of extracellular molecules at myotendinous junction may make this area particularly sensitive to the transfer of plasmid DNA. Although the precise mechanisms underlying the effect of vaccine inoculum on its immunogenicity remain to be determined, the data presented in this report provide hope that DNA vaccine efficacy might be significantly enhanced by increasing hydrostatic pressure via application of sufficient volume of inoculum to small or constricted muscle area.

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Figure 1. Precise mapping of the dominant H-2^b-restricted SIV Gag epitope recognized in B6 mice

A, B6 mice were immunized once with 50 μ g of plasmid expressing SIV Gag. Splenocytes were harvested and incubated with a pool of overlapping peptides covering the entire SIV Gag protein (123 peptides) or with individual peptides as indicated and the percentage of IFN- γ -producing cells of total CD8⁺ cells was determined. Each peptide is labeled using a position of the first amino acid in the Gag sequence, single-letter codes of the first and last amino acids, and the total number of amino acids it contains. B, Sequences of peptides used for the precise mapping of the dominant H-2^b-restricted epitope. Underlined amino acids represent putative anchor residues. C, Splenocytes from SIV Gag-immunized B6 mice were incubated with individual peptides at 1 μ M concentration and the percentage of IFN- γ -producing cells was determined. Representative data from 2 of 4 similar experiments are shown. D, Splenocytes from SIV Gag-immunized mice were incubated with individual peptides at increasing concentrations and the percentage of IFN- γ -producing cells was determined. Data from 1 of 2 similar experiments are shown.

Figure 2. Functional characterization of the dominant H-2^b-restricted SIV Gag epitope
A, Splenocytes from B6 mice immunized once with SIV Gag-encoding plasmid were incubated with H-2D^b-tetramer conjugated to peptide 312_AL11 and anti-CD8 antibody and the percentages of tetramer⁺ cells were determined. B, In vivo CTL assay. Naïve or SIV Gag-immunized B6 mice were injected with autologous splenocytes labeled with low concentration of CFSE (0.3 μ M) or splenocytes pulsed with 312_AL11 peptide and labeled with high concentration of CFSE (3 μ M). Lymphocytes isolated 20 hrs later from the spleens and lymph nodes were analyzed by flow cytometry. The numbers represent the percentages of surviving peptide-pulsed cells in immunized mice relative to control

populations in naive mice. 4 mice per group, representative data from 1 of 3 similar experiments are shown.

Figure 3. Mapping of H-2^b-restricted SIV Gag CD4⁺ T cell epitopes

A, Splenocytes from SIV Gag-immunized B6 mice (single immunization) were incubated with total SIV Gag peptide pool (123 peptides) or individual peptides and the percentage of IFN- γ -secreting cells of total CD4⁺ cells was determined. B, Sequences of two dominant CD4⁺ T cell epitopes. C, Splenocytes from SIV Gag-immunized B6 mice were incubated with individual peptides at increasing concentrations and the percentage of IFN- γ -producing cells was determined. D, Lymphoproliferation assay at increasing concentrations of the peptides covering two putative CD4⁺ T cell epitopes.

Figure 4. Mapping of H-2^d-restricted SIV Gag CD8⁺ and CD4⁺ T cell epitopes in Balb/c mice
A, Splenocytes from SIV Gag-immunized Balb/c mice (single immunization) were incubated in the presence of peptides identified as putative epitopes by peptide library screening and the percentage of IFN- γ -secreting cells of total CD8⁺ and CD4⁺ cells was determined. B, Map of H-2^b and H-2^d-restricted CD8⁺ and CD4⁺ T cell epitopes recognized in SIV Gag-immunized B6 and Balb/c mice. MA, matrix; CA, capsid; NC, nucleocapsid.

Figure 5. The volume of a DNA vaccine inoculate affects its immunogenicity

A, Anterior tibialis muscles of B6 mice were injected once with 20 or 5 μg of plasmid encoding SIV Gag (pGag) resuspended in 50 or 5 μl of PBS and the resulting immune responses were determined as percentages of IFN-γ-secreting cells of total CD8⁺ or CD4⁺ T cells. Total SIV Gag peptide pool of 123 peptides was used to determine the immune response. 1 of 4 similar experiments is presented. B, 10 or 2 μg of plasmid encoding luciferase (pLuc) resuspended in 50 or 5 μl of PBS were injected into quadriceps or anterior tibialis muscles of B6 mice. 3 days later, harvested muscles were homogenized in lysis buffer and the concentration of luciferase in the lysate was expressed as percentage of relative light units (RLUs) per mg of protein. 1 of 2 similar experiments is presented.

Table 1

Example of a peptide pool array

Rows and columns labeled by bold numbers represent individual peptide pools; numbers in the table represent the sequential numbers of 15-meric peptides derived from SIV Gag protein included in each pool (6–8 peptides per pool, 123 peptides total). Each peptide is present in one horizontal and one vertical peptide pool. Numbers in italics on top and left side of the table represent the percentages of IFN- γ producing cells of total CD8⁺ cells determined in an assay using the corresponding peptide pool. Overlapping peptides 79 (308_QL15; present in pools R10 and C9) and 78 (313_AA15; present in pools R13 and C15) contain the immunodominant epitope 312_AL11 (see Fig. 1B).

		0.79	0.2	.02	.02	.04	0.07	1.2	0.03
		C9	C10	C11	C12	C13	C14	C15	C16
0.02	R9	63	65	67	69	71	73	75	77
1.16	R10	79	81	83	85	87	89	91	93
0.04	R11	95	97	99	101	103	105	107	109
0.06	R12	111	113	115	117	119	121	123	64
0.87	R13	66	68	70	72	74	76	78	80
0.09	R14	82	84	86	88	90	92	94	96
0.23	R15	98	100	102	104	106	108	110	112
0.04	R16	114	116	118	120	122	123		