Virology 428 (2012) 121–127

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Breadth of cellular and humoral immune responses elicited in rhesus monkeys by multi-valent mosaic and consensus immunogens

Sampa Santra [a], Mark Muldoon [b], Sydeaka Watson [c], Adam Buzby [a], Harikrishnan Balachandran [a], Kevin R. Carlson [a], Linh Mach [a], Wing-Pui Kong [d], Krisha McKee [d], Zhi-Yong Yang [d], Srinivas S. Rao [d], John R. Mascola [d], Gary J. Nabel [d], Bette T. Korber [e,f], Norman L. Letvin [a,*]

[a] Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA
[b] University of Manchester School of Mathematics, Manchester M60 1QD, UK
[c] The University of Chicago, Chicago, IL, USA
[d] Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA
[e] Los Alamos National Laboratory, Los Alamos, NM, USA
[f] Santa Fe Institute, Santa Fe, NM, USA

ARTICLE INFO

Article history:
Received 4 January 2012
Returned to author for revisions
12 March 2012
Accepted 22 March 2012
Available online 21 April 2012

Keywords:
HIV-1 vaccine
Mosaic immunogen
T cell

ABSTRACT

To create an HIV-1 vaccine that generates sufficient breadth of immune recognition to protect against the genetically diverse forms of the circulating virus, we have been exploring vaccines based on consensus and mosaic protein designs. Increasing the valency of a mosaic immunogen cocktail increases epitope coverage but with diminishing returns, as increasingly rare epitopes are incorporated into the mosaic proteins. In this study we compared the immunogenicity of 2-valent and 3-valent HIV-1 envelope mosaic immunogens in rhesus monkeys. Immunizations with the 3-valent mosaic immunogens resulted in a modest increase in the breadth of vaccine-elicited T lymphocyte responses compared to the 2-valent mosaic immunogens. However, the 3-valent mosaic immunogens elicited significantly higher neutralizing responses to Tier 1 viruses than the 2-valent mosaic immunogens. These findings underscore the potential utility of polyvalent mosaic immunogens for eliciting both cellular and humoral immune responses to HIV-1.

Introduction

One of the major obstacles preventing the development of an effective vaccine against HIV-1 is the extraordinary genetic diversity of the virus. Immunogens derived from single HIV-1 sequences, when evaluated in nonhuman primate studies and in human clinical trials, have elicited immune responses with limited cross-reactivity (McElrath et al., 2008). In contrast, immunogens created using group M consensus sequences have generated cellular immune responses in nonhuman primates with improved cross-clade breadth (Santra et al., 2008). Recent studies in mice and nonhuman primates have shown that polyvalent mosaic immunogens elicited cellular immune responses with even greater breadth and depth than group M consensus immunogens (Barouch et al., 2010; Kong et al., 2009; Santra et al., 2008).

Polyvalent mosaic immunogen sequences are derived from the *in silico* recombination of sequences of natural strains of HIV-1. This *in silico* recombination is done using a machine learning algorithm that selects a set of protein sequences that resemble natural proteins, but in combination optimize the coverage of the diversity of epitope sequences that exist worldwide in HIV-1 strains (Barouch et al., 2010; Fischer et al., 2007; Thurmond et al., 2008). The potential utility of these mosaic proteins for immunogens in humans was recently demonstrated when Ndhlovu et al. showed that processing of mosaic proteins generates epitope peptides that are recognized by human CD8+ T cells (Ndhlovu et al., 2011). Moreover, this strategy is also being explored for immunization against other pathogens, including Chlamydia (Nunes et al., 2010), Hepatitis C (Yusim et al., 2010), Ebola (Grard et al., 2011), and type 2 porcine reproductive and respiratory syndrome viruses (Shi et al., 2010).

During the optimization process, if 2 mosaic immunogens are generated, the mosaic design program assembles a pair of full-length mosaic proteins that incorporate the 2 most common forms of each potential epitope (where all 9-amino acid fragments are considered potential epitopes). If 3 mosaic immunogens are generated, a third variant of each potential epitope is

* Corresponding author. Mailing address: Harvard Medical School, Division of Viral Pathogenesis, Department of Medicine, Beth Israel Deaconess Medical Center, E/CLS-1043, 3 Blackfan Circle, Boston, MA 02215, USA.
Fax: +617 735 4527.
E-mail address: nletvin@bidmc.harvard.edu (N.L. Letvin).

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http://dx.doi.org/10.1016/j.virol.2012.03.012

included. Thus, increasing the number of mosaic proteins in a vaccine cocktail increases the coverage of potential T cell epitopes. However, this increased coverage comes with diminishing returns, as increasingly rare epitopes are incorporated into the mosaic proteins as the number of proteins in the vaccine cocktail is increased. In addition, as more variants are added, each one becomes more dilute in the vaccine cocktail. Thus the selection of the number of variant sequences for inclusion in a mosaic vaccine cocktail must be balanced by weighing increased coverage against the diminished epitope peptide representation as well as the increased cost and complexity associated with increasing the number of variant sequences incorporated into a vaccine (Fischer et al., 2008, 2007; Korber et al., 2009).

Here we experimentally evaluate the benefit of increasing the number of mosaic HIV Envelope (Env) proteins in a vaccine cocktail from 2 to 3. We also compare the immunogenicity of these vaccine constructs to a natural HIV immunogen and a M group consensus immunogen, making use of data generated in previous studies (Fischer et al., 2007, 2008; Korber et al., 2009). To determine the relative benefits of these types of immunogens, we determine how many immune responses each construct elicits that cross-react with representative circulating strains of HIV-1. We assess this cross-reactivity by evaluating vaccine-elicited cellular immune responses for both breadth, the number of distinct targeted epitopes that are recognized, and depth, the number of variants recognized per targeted epitope. We used Env vaccines in this study so that we could study both T cell and B cell responses.

Results

We monitored immune responses using peptide sets that fully span diverse natural Env proteins, so we could directly determine the number responses we generate to actual HIV-1 Env proteins (Santra et al., 2008). In the past we have used 10 proteins for these purposes that were selected to be representative of the global diversity of HIV-1 (Santra et al., 2008). To determine if a subset of 5 of these 10 proteins would be adequate for evaluating immune breadth in this and future studies, we compared the potential T cell epitope (PTE) or 9-mer coverage of a series of Env immunogen sequences to different sets of envelopes. The series of Env immunogen sequences in this comparison included the single strains HXB2 and MN, the M group Consensus (CON-S) sequence, and finally the 2-valent and 3-valent mosaic sequences (Fig. 1). For each immunogen sequence, we calculated the coverage of all 9-mers in the collection of global envelope sequences in the current Los Alamos database (Database), in a set of 10 divergent natural envelope sequences used to estimate the breadth of vaccine coverage in a previous nonhuman primate study (Santra et al., 2008), and a subset of 5 of those 10 natural divergent envelope sequences. The sets of 5 and 10 Env sequences were comparably representative of 9-mer coverage based on the global HIV database, suggesting that peptides derived from this set of 5 Env sequences would provide a reasonable framework for estimating the breadth and depth of vaccine coverage of circulating HIV-1 isolates worldwide (Fig. 1). We therefore decided to use these 5 Env peptide series to monitor T cell responses in the present study. These 5 diverse HIV Env protein sequences included one each from clade A, B and G and two from clade C (GenBank accession numbers: A1, AY371163; B1, AY561237; G1, AY371121; C1, AY563169, and C3, AF457054, respectively (see Santra 2008) for details).

Fig. 1 reveals important differences in the potential coverage that might be induced by these various immunogens. Natural proteins had very low levels of coverage of potential T cell epitopes (PTEs), with MN matching only approximately 17% of the PTE sequences found in the global database of circulating strain M group strains (Fig. 1, blue bar). The CON-S immunogen provided 26% coverage of PTEs, or a 1.6-fold increase relative to MN (Fig. 1, blue bar). In contrast, a 3-valent mosaic Env immunogen increased the coverage of PTE sequences to 44% (Fig. 1, blue bar).

A vaccination study in nonhuman primates was initiated to evaluate the breadth and depth of cellular and humoral immune responses elicited by 2-valent and 3-valent HIV-1 Env mosaic immunogens. Thirty *Mamu-A*01*-negative Indian-origin rhesus monkeys were divided into three groups: two experimental groups, each consisting of 12 monkeys, and a control group consisting of 6 monkeys. One experimental group received 2-valent mosaic Env immunogens, the other experimental group received 3-valent mosaic Env immunogens, and the control group received empty vectors. Monkeys received priming immunizations by the intramuscular route at weeks 0 and 4 with a total of 4 mg plasmid DNA expressing 2-valent mosaic Env, 3-valent mosaic Env, or a sham construct. The monkeys that received mosaic Env plasmid DNA immunogens also received a single clade B Gag plasmid DNA immunogen. At week 8, monkeys were boosted by intramuscular immunizations with 10^{11} particles of recombinant adenovirus serotype 5 (rAd5) expressing the same mosaic Env and single clade B Gag genes. The vaccination strategy was adapted

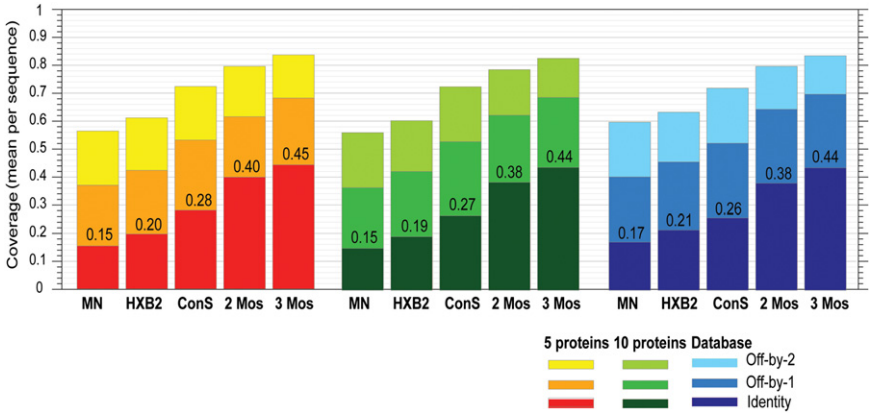

Fig. 1. The coverage of potential T cell epitopes (PTEs) by different vaccine candidates. Shown is the coverage of PTEs by different vaccine candidates as would be detected by the 5 Env proteins used to monitor this study (red), the 10 proteins used in a previous study (green) (Santra et al., 2008) and the 2010 Los Alamos database of HIV-1 sequences (blue). All proteins in each set were broken down into all possible PTEs (9 amino acid fragments) and each of these PTEs was considered. The fraction of perfect matches (dark shades), the fraction that differed by one amino acid (lighter shades) and the fraction that differed by two amino acids (light shades) from the 9-mers found in the immunogen proteins were calculated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from our earlier study of the immunogenicity of a natural strain and an M group consensus Env immunogen (Santra et al., 2008); we did this to facilitate a direct comparison of the data generated in the two studies. These studies did, however, differ in that Gag immunogens were included in the current study, but not in the prior experiment comparing HXB2 and the M group consensus Env immunization. Gag immunogens were added to the current study because they would also be administered if mosaic immunogens were to be evaluated in human populations. It should be noted, however, that if strong T cell responses were made to Gag, these responses might divert some of the T cell responses away from Env (Ferre et al., 2010).

The breadth of the vaccine-elicited cellular immune responses was evaluated by assessing peripheral blood T lymphocyte recognition of the set of 5 different natural Env sequences discussed above, using a peptide/IFN-γ ELISpot assay and matrix epitope mapping. Peripheral blood lymphocytes (PBL) from each vaccinated monkey were evaluated for IFN-γ ELISpot responses to pools of 15-mers overlapping by 11 amino acids spanning each of the 5 envelope sequences. Epitopes of each of the 5 indicator envelope proteins recognized by PBL sampled from each of the experimentally vaccinated monkeys were enumerated as previously described (Santra et al., 2008). To determine if there were differences in the epitope recognition by PBL of monkeys vaccinated with HXB2, the M group consensus, or the 2-valent and 3-valent mosaic immunogens, we evaluated the number of epitope peptides recognized per natural Env sequence for all immunized animals (Fig. 2A). To compare the immunogenicity of each of the four vaccines, we used a quasi-binomial regression model to study the number of vaccine-elicited positive responses per animal per peptide series, using a strategy similar to analyses described in previous studies (Santra et al., 2008). No immunogen elicited a response that was biased toward a particular clade or strain of virus. The HXB2 vaccine elicited significantly fewer epitope peptide responses per peptide series than the other three immunogens. CON-S had an odds ratio (OR) of 3.09, $p=8.3\times10^{-7}$ relative to HXB2; the 2-valent mosaic, an OR of 3.95, $p=6.4\times10^{-10}$; and the 3-valent mosaic, an OR of 4.12, $p=2.0\times10^{-10}$. The immunogens can be rank ordered on the basis of odds ratio of the number of epitope responses they elicited from lowest to highest: HXB2 $\ll$ CON-S $<$ 2-valent mosaic $<$ 3-valent mosaic, and this ordering is consistent with the median number of epitope-specific responses per strain elicited by each vaccine; 1 for HXB2, 2 for CON-S, 3 for 2-valent mosaic, and 4 for the 3-valent mosaic (Fig. 2B). Further statistical comparisons were consistent with our previous findings (Santra et al., 2010) that the CON-S immunogens elicited fewer epitope peptide-specific responses than the mosaic vaccines, but in this study the p-values showed only a trend: 2-valent mosaic compared to CON-S had an OR 1.28 ($p=0.08$), and 3-valent mosaic compared to CON-S had an OR of 1.33 ($p=0.04$). Of note, in this analysis we are comparing results from two different studies (the HXB2 and CON-S data was from Santra et al., 2008, the 2 and 3 valent mosaics from the present study). The numbers of epitope peptide-specific responses elicited by the 2- and 3-valent mosaics were not statistically distinguishable.

We also evaluated the depth of T lymphocyte responses, the diversity of sequences of a given epitope that might be recognized by T lymphocytes of the vaccinated monkeys (Fig. 3). This was estimated by counting the responses made to all variant peptides from every epitopic region of the Env protein for each vaccinated monkey. It was very common for the vaccine-elicited responses to be strain specific, i.e., only 1 of 5 variant peptides was recognized by the vaccine-elicited T cell population (Fig. 3). However, the number of T cell responses that recognized an epitope in two or more divergent strains was increased in the monkeys that received polyvalent mosaic immunogens. We first did a Fisher's exact test comparison of a 2×4 contingency table to compare the number of epitope-specific responses that recognized only 1 of the 5 variant strains to the number that recognized 2 or more strains; the p-value was 0.0006, indicating there were vaccine-associated differences in the depth of the elicited responses. As shown in Fig. 3, more than 60% of the responses to the single valent CON-S and HXB2 vaccines recognized only 1 of the 5 strains. In contrast, the polyvalent mosaic vaccines elicited more cross-reactive responses (Fisher's exact $p=4\times10^{-5}$). At the other end of the spectrum, there was a 4-fold greater representation of responses that recognized epitopes derived from all 5 evaluated virus strains in mosaic-vaccinated animals than in animals vaccinated with the single antigens CON-S or MN. (12% compared to 3%). There was no clear difference in the depth of T cell responses elicited by the 2- and 3-valent mosaic immunogens.

Finally, we assessed the effect of increasing the valency of the mosaic immunogens on humoral responses. Sera sampled from immunized monkeys at week 13 were assessed for antibody binding to purified BaL.01 and Du156.12 gp120 and 93TH057 gp120 core proteins by ELISA as previously described (Wu et al., 2010) Both the 2- and 3-valent mosaic immunogens elicited comparable titers of binding antibody responses to the Env proteins used in the assays (Fig. 4A). We also assessed the neutralizing antibody responses in the sera of immunized monkeys to a limited number of Tier 1 (viruses that are highly sensitive to antibody-mediated neutralization) and Tier 2 (viruses that are moderately sensitive to antibody-mediated neutralization) viruses (Seaman et al., 2010). Interestingly, the 3-valent mosaic immunogens elicited significantly higher titer neutralizing antibody responses to tier 1 viruses than the 2-valent mosaic immunogens (Fig. 4B). However, the 2-valent and 3-valent mosaic immunogens elicited comparable, very weak or undetectable neutralizing antibody responses to tier 2 viruses (Fig. 4B). These findings are comparable to the antibody responses elicited by natural strain and consensus Env immunogens (data not shown).

Discussion

An effective HIV-1 vaccine should elicit robust effector CD8+ T cell responses that can contain virus replication and CD4+ T cell responses that can potentiate both antibody and effector CD8+ T cell responses. The ELISpot assay employed in the present study does not differentiate between CD8+ and CD4+ T cell populations. However, based on previous studies in mice, monkeys and humans to evaluate plasmid DNA prime/recombinant adenovirus serotype 5 boost immunizations, it is likely that the vaccine-elicited cells in the present study were predominantly CD8+ T cells (Santra et al., 2005; Shiver et al., 2002; Sumida et al., 2004). It is encouraging that the breadth and depth of the vaccine elicited cellular immune responses induced in the present study by the 2- and 3-valent mosaic immunogens were statistically comparable, since it would be easier and less expensive to deploy a 2-valent immunogen. We were limited, however, by practical constraints in the number of animals that could be evaluated; thus the present study was underpowered (data not shown) for demonstrating the small increase in coverage that was predicted in increasing from 2-valent to 3-valent Env mosaics immunogens (Fig. 1). Since the monkeys that received the 3-valent mosaic immunogens developed T cell responses with greater breadth and depth than monkeys that received the 2-valent mosaic immunogens, it is possible that a larger study may have shown a statistically significant benefit associated with receiving the 3-valent mosaic vaccines.

The depth of the cellular immune responses elicited by the polyvalent mosaic immunogens was greater than cellular immune responses elicited by the natural and consensus immunogens. Responses with greater depth may mediate greater

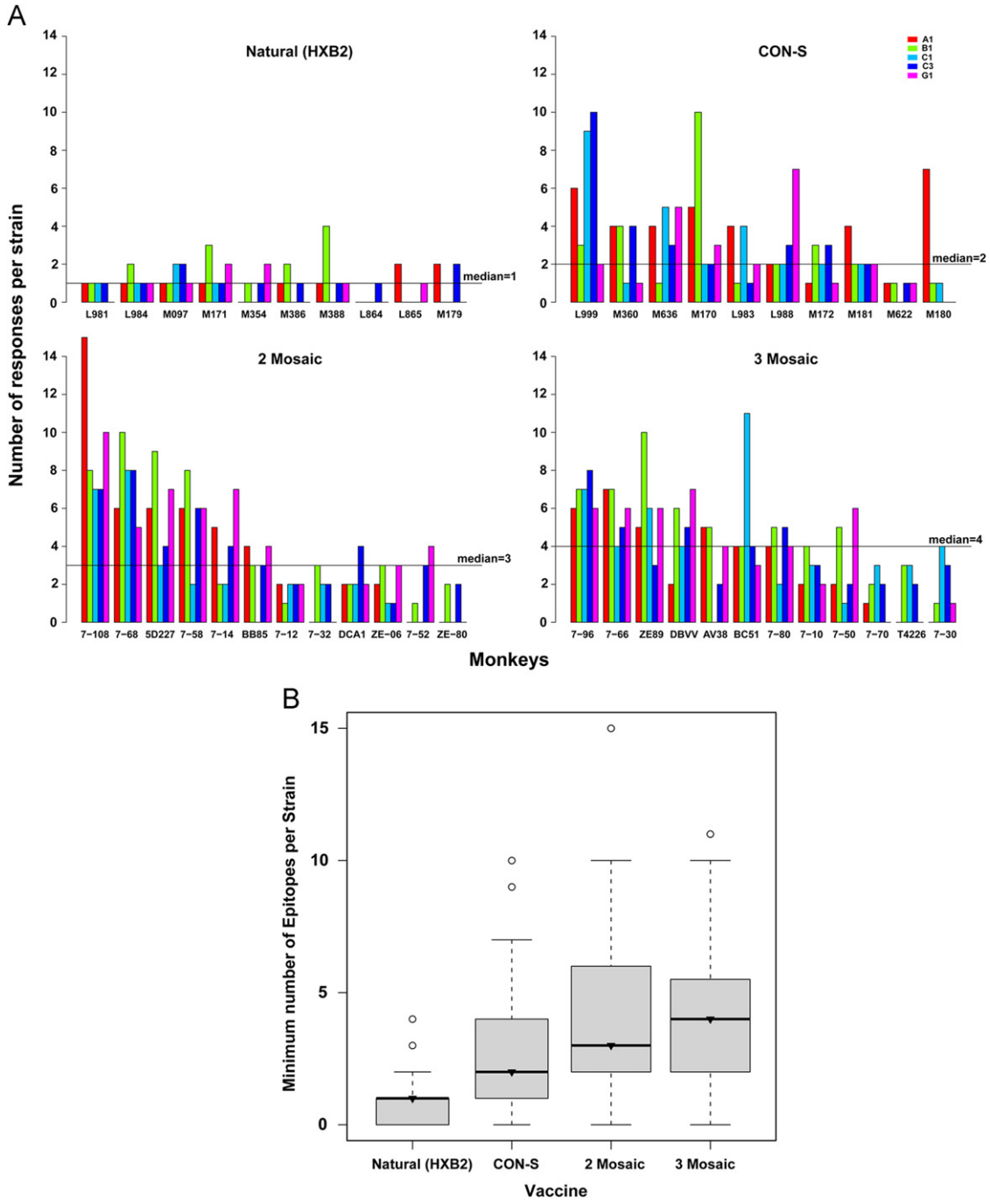

Fig. 2. Breadth of Env-specific T lymphocyte responses in the 12 individual monkeys from each experimental group, showing number of peptide epitopes recognized per protein. (A) The breadth of the vaccine-elicited cellular immune responses was determined by assessing peripheral blood T lymphocyte recognition of 5 different envelope sequences using a peptide/IFN-γ ELISpot assay. PBL from each vaccinated monkey were evaluated for IFN-γ ELISpot responses to pools of 15-mers overlapping by 11 amino acids spanning each of the 5 selected envelope sequences. Epitopes of each of the 5 indicator envelope proteins recognized by PBL of each of the experimentally vaccinated monkeys were enumerated by matrix mapping and confirmation with individual peptides. If two overlapping peptides gave a positive response, it was counted as a single response. The minimum numbers of possible T cell responses that can recognize each strain are shown. (B) A box plot showing the distribution of numbers of vaccine responses per strain elicited by the different vaccines.

cross-recognition of diverse circulating forms of HIV-1 at the time of initial exposure to the virus, and may be able to block common mutational immune escape routes for the virus if a vaccine recipient becomes infected. It is not clear if the mosaic immunogen-induced increase in cross-reactivity is the result of selection of new T cell clones that recognize the distinct epitope variants or selection of T cell clones that have a greater cross-reactive potential. Mosaic antigens are designed to include to the greatest extent possible the most common variants of each potential T cell epitope, and these common variants likely represent relatively the most fit immune escape variants.

There is an increasing appreciation for the importance of vaccine-induced anti-Env antibody responses for preventing HIV-1 acquisition (Rerks-Ngarm et al., 2009). While mosaic immunogens were designed to elicit cellular immune responses, it is interesting that these vaccine constructs also elicited neutralizing antibodies. This might have been due to improved cross-recognition of epitopes in the Envs tested due to simultaneous presentation of epitope

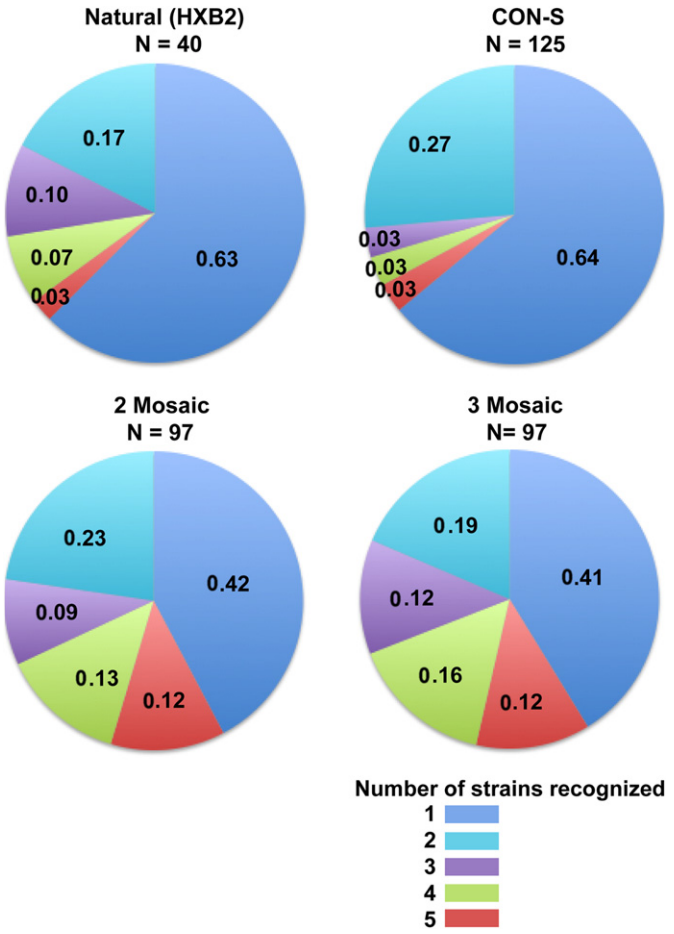

Fig. 3. Depth of cross-reactive T cell responses elicited by various vaccines. The peptides used for the ELISpot assays were designed to maintain their alignment, so they could be directly compared between strains. For each animal, if a vaccine response was found to a peptide, the cross-reactive potential of the response was assessed by counting how many of the 5 test strains were targeted by the response. For each vaccine, the PBL of a monkey that recognized only one strain (blue) were tallied for all animals in the group. Similarly, PBL populations that recognized 2 of the 5 strains (light blue), 3 of the 5 (purple), 4 of 5 (light green), and all 5 strains (pink) are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

variants during antibody selection and affinity maturation, or it might have been due to the third mosaic adding additional epitopes to the antibody response. However, these antibodies neutralized only the easy-to-neutralize tier 1 viruses, but not more difficult-to-neutralize tier 2 viruses. Since tier 2 viruses are representative of the transmitted viruses that are responsible for the world-wide AIDS epidemic (Mascola et al., 2005), it is likely that complementing immunogens will be needed to generate an effective anti-HIV-1 Env antibody response. The profound impact of the increase from single valent natural or consensus to 2 to 3 mosaic polyvalent vaccines on the strength of the Tier 1 antibody response to diverse strains is, however, intriguing. It is possible that exposure of antibodies to epitope variants during their clonal evolution and affinity maturation may improve their breadth.

Materials and methods

Experimental groups and immunization schedule

All animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care—accredited institution and with the approval of the Animal Care and Use Committees of the National Institutes of Health and Harvard Medical School. Thirty *Mamu-A*01*-negative Indian-origin rhesus monkeys were distributed into three groups—two experimental groups, each consisting of 12 monkeys, and a control group consisting of 6 monkeys. Immunizations were done as described in the Results section.

Construction of the plasmid DNA vaccines

The 2-valent and 3-valent mosaic HIV Env proteins were designed using the methods described by Fischer et al. (Fischer et al., 2007). This strategy uses a genetic algorithm that maximizes the coverage of potential epitopes for a given vaccine valency *N* (where *N* is either 2 or 3 in this study) by selecting *in silico* recombined sequences that in combination essentially contain the *N* most common circulating epitope variants. The design of the mosaic proteins is alignment-independent. Thus the highly variable regions with many insertions and deletions, such as the V2 loop, are spanned by identifying the most common potential variants in the region in the same manner as the rest of the protein. Short stretches of these regions can be so variable, however, that they are essentially unique in every individual; in such cases the mosaic will represent a variant that exists at least once in nature. Although the proteins are artificial, they are intact HIV proteins; mosaic antigens have been shown to process human T cell epitopes naturally (Barouch et al., 2010). The clade B Gag plasmid DNA was based on HXB2 strain as described before (Wu et al., 2005). All the HIV immunogen genes were synthesized using human-preferred codons [GeneArt, Regensburg, Germany] (Huang et al., 2001). The cDNAs were cloned into a plasmid expression vector, pCMV/R, which mediates high-level expression and immunogenicity in vivo (Barouch et al., 2005; Fischer et al., 2008; Yang et al., 2007).

Construction of the recombinant adenoviruses

The construction and propagation of the rAd5 vectors expressing the same immunogens as in the plasmid DNA vaccines were previously described (Einfeld et al., 2001). The total particle unit {PU} titer was determined by absorbance (Mittereder et al., 1996).

HIV-1 Env peptide sets and design of peptide matrices

15-mer peptides overlapping by 11 amino acids, spanning the entire HIV-1 Env were used. Five sets of HIV-1 Env peptides from 4 different clades were synthesized, 1 from clade A, 1 from clade B, 2 from clade C and 1 from clade G.

Pooled peptide and peptide matrix IFN-γ ELISpot assays

Multiscreen ninety-six well plates were coated overnight with 100 μl per well of 5 μg/ml anti-human interferon-γ (IFN-γ) (B27; BD Pharmingen) in endotoxin-free Dulbecco's-phosphate buffered saline (D-PBS). The plates were then washed three times with D-PBS containing 0.1% Tween-20 and blocked for 2 h with RPMI-1640 containing 10% FBS. Then peptide pools and 2×10^5 PBMCs were added to each well in 100 μl reaction volumes in triplicate for pooled peptides assays and in duplicate for peptide matrix assays. Each peptide in a pool was present at a 1 μg/ml concentration. Following an 18 h incubation at 37 °C, the plates were washed nine times with D-PBS/Tween-20 and once with distilled water. The plates were then incubated with 2 μg/ml biotinylated rabbit anti-human IFN-γ (U-Cytech) for 2 h at room temperature, washed six times with D-PBS containing 0.1% Tween-20, and incubated for 2.5 h with a 1:500 dilution of streptavidin-AP (Southern Biotechnology Associates). After five washes with D-PBS/Tween-20 and one with D-PBS, the plates were developed with NBT/BCIP chromogen (Pierce), stopped by washing with tap water, air dried, and read with an

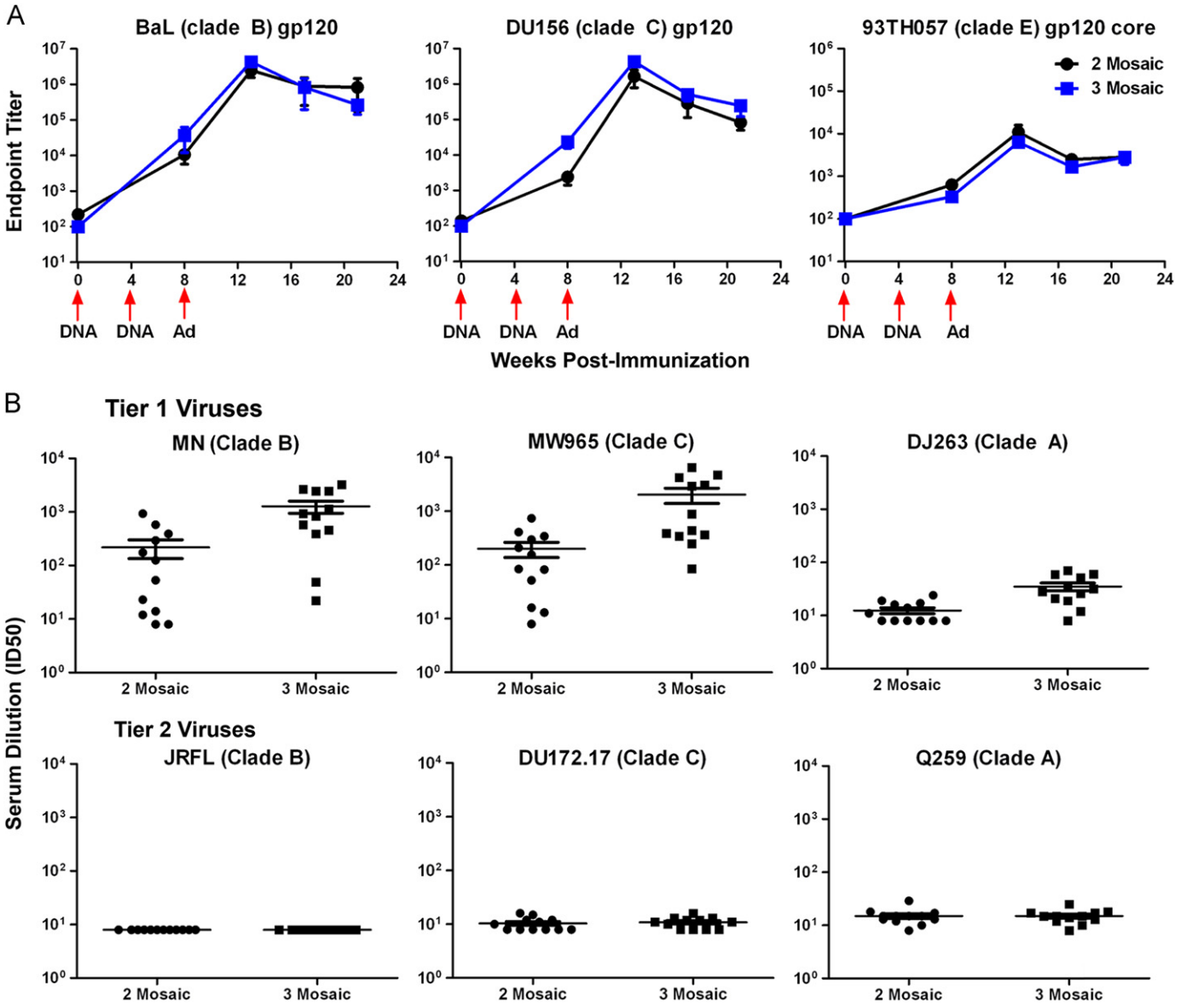

Fig. 4. Vaccine-elicited binding antibody and neutralizing antibody responses. (A) Five weeks post-rAd5 boost serum samples were collected from monkeys vaccinated with either 2-valent or 3-valent mosaic immunogens. Antibodies from these serum samples were assessed for binding to purified BaL.01 and Du156.12 gp120 and 93TH057 gp120 core envelope proteins by ELISA. (B) The sera were also assessed for the presence of neutralizing antibodies to a limited number of Tier 1 and Tier 2 viruses. There was a significant increase in the level of Tier 1 neutralization for each of the 3 strains tested in the 3-valent mosaic group (MN, p-value=0.003, using a non-parametric Wilcoxon rank sum test; MW965, p-value=0.0007; and DJ263, p-value=0.0004).

ELISpot reader (Cellular Technology Ltd.). The number of spot forming cells per 10^6 PBMCs was calculated.

HIV-1 neutralization assays

Neutralization was measured using single-round-of-infection HIV-1 Env-pseudoviruses and TZM-bl target cells, as described previously (Li et al., 2005; Seaman et al., 2010; Wu et al., 2009). Neutralization curves were fit by nonlinear regression using a 5-parameter hill slope equation as previously described (Seaman et al., 2010). The 50% and 80% inhibitory concentrations (IC50 and IC80) were reported as the antibody concentrations required to inhibit infection by 50% and 80% respectively.

gp120 ELISAs

Binding of antibodies to purified gp120s was assessed by ELISA as previously described (Wu et al., 2010). Briefly, 200 ng of purified recombinant gp120 was adsorbed onto Reacti-Bind 96-well plates (Pierce), followed by blocking and incubation of serially diluted antibodies. Bound antibody was detected using a horseradish peroxidaseconjugated goat anti-human IgG Fc antibody (Jackson ImmunoResearch Laboratories). Plates were developed using SureBlue 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories). Purified gp120 proteins were full-length gp120 BaL (Clade B), full-length gp120 DU156 (Clade C), or gp120 core 93TH057 (Clade E).

Statistics

All statistical analyses in this study were performed in R (www.r-project.org/). To predict the probability of a positive response as a function of vaccine group and strain, we fit the number of reactive peptides per strain as binomial counts in a generalized linear model. We used a quasi-binomial model to appropriately adjust the standard errors and p-values given evidence of slightly

overdispersed data (dispersion parameter=1.88). Vaccine effect did not significantly vary according to strain, nor did strain significantly impact the probability of eliciting a positive response. Thus, we did not find it necessary to adjust for these variables in the final model. All pairwise comparisons of the vaccine groups were considered, and the significance level ($\alpha=0.05$) was adjusted accordingly. We also assessed the relationship between depth of response and vaccine group via Fisher's exact test, and compared the magnitude of the Tier 1 antibody responses using a Wilcoxon rank statistic.

Acknowledgment

We thank Jason Gall of GenVec for providing reagents. This work was supported in part by funds from the intramural research program of the Vaccine Research Center, NIAID, NIH and by the Center for HIV/AIDS Vaccine Immunology NIAID grant U19 AI067854.

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