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MINIREVIEW

Evolving T-cell Vaccine Strategies for HIV, the Virus with a Thousand Faces

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HIV's rapid global spread and the human suffering it has left in its wake have made AIDS a global heath priority for the 25 years since its discovery. Yet its capacity to rapidly evolve has made combating this virus a tremendous challenge. The obstacles to creating an effective HIV vaccine are formidable, but there are advances in the field on many fronts, in terms of novel vectors, adjuvants, and antigen design strategies. SIV live attenuated vaccine models are able to confer protection against heterologous challenge, and this continues to provide opportunities to explore the biological underpinnings of a protective effect (9). More indirect, but equally important, is new understanding regarding the biology of acute infection (43), the role of immune response in long-term non-progression (6, 62, 81), and defining characteristics of broadly neutralizing antibodies (4). In this review we will focus on summarizing strategies directed towards a single issue, that of contending with HIV variation in terms of designing a T-cell vaccine. The strategies that prove most effective in this area can ultimately be combined with the best strategies under development in other areas, with the hope of ultimately converging on a viable vaccine candidate.

Only two large HIV vaccine efficacy trials have been completed and both have failed to prevent infection or confer a benefit to infected individual (23, 34), but there is ample reason to continue our efforts. A historic breakthrough came in 1996, when it was realized that although the virus could escape from a single antiretroviral (ARV) therapy, it could be thwarted by a combination of medications that simultaneously targeted different parts of the virus (HAART) (38). This revelation came after 15 years of research, thought, and clinical testing; to enable that vital progress the research and clinical communities had to first define and understand, then develop a strategy to counter, the remarkable evolutionary potential of the virus. HAART, for the first time, provided an effective treatment to help those with living with HIV stay healthy. Nonetheless, the treatment has limitations. People with HIV face a lifetime of expensive daily multi-drug regimens, often with side effects; drug resistance at the individual and population level are issues (56); and universal access, despite substantial progress, is a dream not yet realized for many of the millions of the world's poor who are living with HIV (68). These issues, combined with the growing numbers of people infected globally and impact of HIV on society, make the development of an HIV vaccine or a prophylactic prevention strategy a crucial if elusive goal. In some ways, the history of HIV vaccine development has paralleled the early stages of designing effective therapy. We had to test the simple strategies first, but meanwhile the story of the impact of

diversity from an immunological perspective is still unfolding, and novel ideas countermeasures are being explored.

TOWARDS A HIV T-CELL VACCINE

The STEP vaccine trial was designed to test whether an HIV vaccine could reduce infection rates or viral load upon infection through eliciting T-cell responses to HIV Gag, Pol and Nef expressed from genes in an Ad5 vector. The trial was stopped for lack of efficacy, and the outcome sent a wave of concern through the community. Not only did the vaccine not confer a benefit, the vaccine-treated group with pre-existing adenovirus serotype 5 (Ad5) immunity had a higher incidence of HIV infection than the placebo treated group (23, 60). Despite this disappointing outcome, the STEP vaccine is informative in that it provides a baseline that we know we have to improve upon for success. There are many innovations already being explored that have the potential to offer improvements. The motivation for the STEP trial in the first place was the history of vaccine experiments in animal models, as well as the many experiments that have helped define a positive role of T-cells in the course of natural HIV infection. This history remains intact, and the depth of understanding in these areas continues to grow.

First, there is the litany of evidence demonstrating the value of the CD8+ T cell responses in HIV-infected people. Here are some highlights: i) CTL escape is a major force driving of HIV evolution at the population level (11, 15, 73) and within individuals (13, 14); this observation is in itself a testament to the value of CTL responses, as HIV changes profoundly to escape them. ii) Highly functional CD8+ T cell responses are correlated with HIV non-progression (10). iii) The most profoundly significant genetic marker associated with host control of viral load in a genome wide study were HLA B*5701, and a site near the HLA C gene (26). iv) Responses to specific epitopes may be associated with a the good outcome observed in HLA B*5701(62) and HLA B*2701 (6) infected people.

Second is the body of work in macaques demonstrating the value of a vaccine-induced T-cell response in conferring protection against progression upon challenge virus infection. Infection with live attenuated virus as a vaccine provides an interesting model. Although it is not directly applicable for human use due to safety concerns, it provides the greatest degree of protection in challenge experiments and hence provides basis that allows investigators to systematically explore what works in monkeys. Heterologous challenge (i.e. challenging with a virus that is at least as different from the vaccine as the viruses that are found in different individuals in the human population) of a pathogenic virus in animals vaccinated with live attenuated virus can give variable results, but generally live attenuated viral vaccination lowers levels of viremia, improves CD4 T cells counts, and slows progression to disease (2, 66, 87). In a recent study, a live attenuated SIV vaccine (SIVmac239Dnef) was shown to confer a high degree of protective immunity against a pathogenic heterologous challenge (SIVsmE660), with a 2 log decrease in viral replication over 32 week period (69). In this case, two lines of evidence supported the notion that positive impact was mediated by CD8+ T cells: the most extreme reduction in peak viremia was in animals with beneficial MHC types (Mamu B*08 and B*17), and a CD8+ T cell depletion experiment in 4 of the animals

precipitated a 1-3 log increase in viral load.

Another interest recent vaccine studies in non-human primates was a T cell depletion study, that showed while depletion of CD8+ T-cells can abrogate vaccine-induced protection, depletion of CD4+ T-cells diminished vaccine induced CD8+ T cell functionality and protective capacity, thus both CD4+ and CD8+ T cell responses contributed to vaccine elicitation of a protective responses (79). In a study of the impact of using different recombinant adenovirus vectors for in a prime boost regimen (57), Liu et al. demonstrated that improving delivery protocols for a T-vell vaccine could dramatically improve the outcome: a gag gene delivered in rAd26/rAd5 prime boost regimen gave T-lymphocyte responses that had greater magnitude, breadth and polyfunctionality than a rAd5/rAd5 prime boost vaccine, as was used in the STEP trial. Animals given the rAd26/rAd5 prime boost regimen had decreased peak viremia, lower set points, and delayed progression to disease (79).

In contrast, the role for CD8+ T cells is less clear in other recent studies and either undefined factors, or T cells that are either rare in the periphery or difficult to measure by standard assays, are conferring a protective effect. In a study of heterologous virus superinfection, SIVsmE660 and SIVmac251 were used to serially infect rhesus monkeys, and it was found that either strain, when used as the second superinfecting strain, was be held in check and was only transiently detected at low levels (89). In this case the mechanism for the control of the superinfecting virus was not clearly attributable to classical T or B cell immunity. In another live attenuated vaccine study (59), in 5 macaques given attenuated SIV239DeltaV1-V2 and challenged with homologous SIV239 a protective effect was observed, but in 2 of the 5 cases both T-cell and B-cell immunity was low, leaving the mechanism of protection unresolved.

THE IMPLICATIONS OF GLOBAL HIV DIVERSITY FOR T-CELL RESPONSES

Eliciting an immune response that can protect against the extraordinary diversity found naturally circulating HIV is a formidable challenge (33). Fig. 1 is an illustration of a two phylogenies of global sampling of HIV M group variants based on the Gag and Env genes. The M group, or main group, of HIV-1 viruses encompasses the viruses of pandemic. Subtypes A-J within the M group form relatively clear genetic groups that consistently show the same clustering pattern through out different regions in the HIV genome (70) (Fig. 1); some of these distinct lineages may have evolved different phenotypic characteristics relating to transmission and progression (39, 40, 45). The trees model evolution by base substitutions, and branch lengths reflect the number of mutations estimated to have occurred between the sequences used to construct the tree. But HIV-1 has many other mechanisms to generate diversity: recombination, insertion and deletions, and gain and loss of glycosylation sites (92). Recombination occurs at high rates, and intersubtype recombination is rampant in populations where multiple subtypes co-circulate and incidence is high (7, 44). Recombination obscures phylogenetic relationships and violates the assumptions that are used to infer phylogenetic trees (48), and recombination events play a major role in HIV evolution. So trees like the ones shown in Fig. 1, that excludes intersubtype recombinants, should be

interpreted with the awareness that the clades as shown can be considered as basic building blocks that contribute sections to the intersubtype recombinant viruses, and that co-circulate with them. The within clade phylogenetic structure will be also muddled by recombination patterns that are not readily resolved. Despite their limitations, phylogenetic trees group viruses according to genetic relatedness and are very useful. Phylogenetically defined lineages can have different phenotypes, as discussed above. Different subtypes have different immunological cross-potential patterns (9, 12). Phylogenies also provide a model for studying the timing of epidemic expansion (47, 86, 91) and evolutionary dynamics and immune presssure in infected individuals (43, 49). As a cautionary aside, while gentoypic based phylogenetic patterns are associated with patterns ing neutralizing antibody profiles, there can be dramatic exceptions. For example the C clade viruses from India tend to form a realtively tight genetic cluster within the C clade, but Envelopes from this group tend have extremely diverse patterns of neturalization suceptibility when tested with a large panel of sera (Montifiori et al., Virology, in press).

The basic structure of the phylogenetic tree provides a framework for people to think about vaccine design to contend with HIV diversity at different levels. For example, a clade-specific vaccine like a B clade vaccine might be particular useful in the US which has a B clade epidemic, but the two failed efficacy trials completed to date were done essentially in this model, as a B clade vaccines was tested in a clade B setting. Similarly a C clade vaccine might be helpful in Southern Africa, and a circulating recombinant form CRF01 vaccine in pars of Asia (Fig. 2). Other vaccine designs tailor their vaccine antigens to forms found in particular geographic regions or nations(16, 21), and some not only consider the regionally circulating viral forms but the regional human popluation HLA allele frequences as well (42). The trees in Figure 1 were based a selected subset of sequences that include only the most recently sampled viruses from the most heavily sampled subtypes, B and C, and the available sequences from the other subtypes. The terminal branches that correspond to US sequences are labeled in blue, and they have a narrower diversity pattern compared to the full set of B viruses including those from Asia, South America and Europe. South African sequences are shown in red and are somewhat more dispersed throughout the international C clade sequences. A few non-C subtype full length gene sequences were available form South African, the red lines in the A and D clades. The red and blue lines give a sense of the limited advantage trying to make a national vaccine. But given that we have no successful vaccine candidate in humans yet at all, regional and subtype specific vaccine strategies are worth exploring; if successful they would be of immediate value to the traget populations, and could lead the way for more comprehensive strategies. In parallel to vaccines projects that focus on a subtype, others groups, including our own, have chosen to empahsize using rational design srategies to attempt to acheive a global vaccine (33). These strategies will be discussed in greater detail in subsequent sections of this review. The goal of a global vaccine, though a daunting prospect, is motivated by several factors. First, even in a regional epidemic dominanted by a single subtype, such as the US B clade epidemic, pockets of HIV with great genetic diversity can be found (55, 78). Second, most nations have complex epidemics and would not benefit by a single clade vaccine scenario (Fig. 2). Finally, the cost of human testing and production of multiple tracks of regional and national vaccines would be extraordinarily high and resource intensive.

The genetic distances shown in Fig. 1 reflect enormous changes at the amino acid level when considered from a T cell receptors point of view. HLA class I and class II molecules present epitopes that are contiguous peptides fragments of a various of lengths; 9-amino acids long the most common length of optimal class I epitopes, although optimal epitopes range between 8-12 amino acids long; optimal class II epitopes have greater diversity in terms of lengths. Thus a sensible way of describing HIV diversity from a vaccine perspective is not just in terms of single amino acids, but in how much variation there is in potential T-cell epitopes (53). Human T cell epitopes have been found virutually everywhere in the HIV proteome (http://www.hiv.lanl.gov/content/immunology/maps/maps.html), and the same short stretch can be embedded in many epitopes with different overlapping boundaries presented by different HLAs. Fig. 3a shows the distribution of human T-cell epitopes that have been described in the literature and assembled in the HIV immunology database. This system is biased towards the regions that have undergone the most intensive study, still the ridge line of peaks and valleys of all known HIV CD8+ T cell eptiopes (Fig. 3a) parallels the frequency of epitope responses at the population level when spanning the full proteome (3, 29). The number of perfect 9/9 potential epitope matches between a single natural putative vaccine protein and the aligned translation of the current full genome sequence M group alignment at the Los Alamos data base is shown in Fig. 3b. The full length M group alignment contained 1206 sequences, one per person, and includes hundreds of recombinants as well as pure subtypes from throughout the world. The natural protein used for this comparison was selected by virtue of providing the most perfect 9/9 matches with the M group set; the overall fraction of identical 9 mers matches between the best natural protien and the M group set is shown Fig. 3d. As a sliding window of 9 amino acids that progresses position by position through the proteome you can see the covereage of each 9 mer section of the alignment, shown in Fig. 3b. The relative frequency of perfect matches with the puative vaccine is by definition essentially as good as coverage can get for a single natural strain.

What is immediately evident in Fig. 3 is that not all regions of all proteins are equivalent. While people generally think of Gag as conserved, and Env as variable, not Gag is not all conserved (only p24 is well conserved throughout), and Env is not all variable. Pol is the most conserved protein over all, but it is spanned by alternating stripes of near identity and poor coverage. It is the only protein with more than half (56%) of the 9 mers in the global collection matching the best natural strain. Env and the regulatory proteins typically only match about one quarter of the 9 mers. This means a vaccine elicited response to a typical epitope in a single strain is more likely than not to have mismatch in the corresponding epitope region in an infecting strain. While numerous studies suggest that single mismatches can often be tolerated, a recent detailed examination of cross-reactivity of T-cell response in 3 eptiopes and natural variants found in different clades suggests that cross-reactivity in traditional EliSpot and chromium release assays may over-estimate true cross-reactivity of relevant responses(9). CD8+ T cells against particlar epitopes that appeared to be highly promiscuous and allow recognition of multipe pepide variants when using assays that depend on exogneous loaded peptide, did not allow cross-recognition when expressed from within an infected cell using an HIV-1 inhibition assay, which was much more sentitive to eitope variation(9).

NATURAL PROTEINS AS VACCINE ANTIGENS

Much of the HIV vaccine effort to date has focused on either the use of natural proteins as vaccines, or essentially natural proteins but with engineered deletions for safety or to try to enhance immunogenicity (17, 20). A natural strain can be deliberately selected to be the natural strain that is closest to a consensus sequence (17), or be selected to provide the best coverage of potential epitopes in a target population (28) (Fig. 3c and 4a). Fig. 4a illustrates a comparison of the coverage of all Gag found in the full genome alignment of 1206 M group sequences from the Los Alamos database by different vaccine antigen design concepts, including the coverage by Cam.1 and HXB2, two natural B clade strains that have been used in vaccine trials, to the natural strain selected to give the best coverage of the database collection. The two first HIV vaccine efficacy trials to be completed to date that have failed, the VaxGen trial (34) and the STEP trial (23) used essentially natural Env genes/proteins as vaccine antigens. B clade antigens were used in this first attempt to protect against infection in an essentially B clade infected trial populations. In terms of antigen design, this was the simplest model and in many ways the obvious thing to try first: deliver some part HIV, either as natural protein or gene that can be expressed upon vaccination, and hope it elicits immune responses with enough potency, long-term immune memory, and cross-reactivity to confer protection from infection or else better viral control if infected. This is basic kind of vaccine approach worked for other viruses, for example Hepatitis B (93) and influenza (76). The intent of VaxGen trial was to determine if vaccination with a recombinant protein rgp120 vaccine would elicit beneficial neutralizing antibodies (neutralizing antibodies can block viral entry into host cells and target the viral Envelope glycoprotein). The potential for this vaccine to produce cross-reactive neutralizing antibodies was much debated even prior to the initiation of the Phase III trial (18, 22), and although anti-HIV antibodies were induced the vaccine did not reduce the incidence of HIV-1 infection (34). The STEP trial, on the other hand, was based on eliciting T-cell responses to Gag, Pol and Nef expressed from genes in an Ad5 vector. 77% percentage of the individuals that received this vaccine mounted an HIV-specific T cell response (60). However, many individuals only responded to 1 or 2 of the proteins. If there was generally only a single response per protein (23) it is plausible that in the many cases there were either mismatches between the vaccine epitope and the infecting strain, or that naturally infected cells did not present the vaccine epitope. The experimental and analysis teams for the STEP trial are in the process of sequencing the transmitted virus and defining the reactive epitopes to resolve this question.

Another basic strategy employing natural antigens is to use a polyvalent vaccine that incorporates natural antigens from multiple clades. While there is reasonable concern about the complexity and expense of generating a polyvalent vaccines, polyvalent vaccines been successfully used. In the case of influenza, even in the context of the ongoing need to make new vaccines that match the current epidemic strains (76), a polyvalent vaccine is successfully produced, and the current pneumococcal vaccine includes 23 capsular serotypes, although its efficacy has be questioned (41). One group is using an HIV polyvalent natural strain approach in a DNA prime, protein boost vaccine strategy that includes 1 A, 2 B, 2 C and 1 E envelope proteins and a monovalent Gag, and

this study has now completed a phase 1 safety and immunogenicity study (80). The vaccine group wth highest dose for the DNA prime in this study yielded CD4+ T cells with greater polyyfunctionality and some CD8+ T cells against Env and Gag, although overall this vaccine regiment was skewed toward the induction of CD4+ T cell responses (8). The T-cell responses were elicited against peptides from the diverse vaccines strains (80). Another group investigating this appoach designed a polyvalent vaccine including 3 Envelope proteins, 1 A, 1 B and 1 C, and a single Gag, Pol and Nef protein in a DNA prime/rAd5 boost regimen (75). This the polyvalent Env gave T-cell responses and nautralizing antibody responses with greater breadth than a did monovalent Envs, with no evidence of antigenic interference (75). In this study, SHIV-89.6P peak viremia, set point, were lower for all vaccinated animals, with a delayed decline in CD4+ T cells. These promising results in macaque led to a successful Phase I trial (19), and the vaccine was slated for an efficacy trial, but this was halted due to concerns abou the rAd5 vector in the aftermath of the STEP trial (http://www.nih.gov/news/health/jul2008/niaid-17.htm).

POLY-EPITOPE VACCINES

The poly-epitope strategy involves investigator designed artificial mini-genes, expressed in either DNA or a viral vector, comprised of a string of epitopes line up in a single artificial vaccine construct. The hope is that if the protein can be expressed and is immunogenic in animal studies, this will translate to eliciting appropriate T-cell responses in humans. This is conceptually elegant, as it has the virtue of allowing the investigator the freedom to select epitopes that are deemed most desirable. For example the most conserved epitopes, or epitopes that are most frequently presented by the most common HLAs, could be the direct focus of the vaccine. This concept had encouraging precedent; early studies using the "beads on a string" poly-epitope approach gave protection in a lethal dose challenge of the arenavirus lymphocytic choriomeningitis virus LCMV in mouse (67, 84). A priori, the potential of this concept for an HIV vaccine merited serious exploration, although the initial results in HIV vaccine human immunogenicity trials with these approaches have been disappointing.

The first attempt at this kind of vaccine for HIV was initiated by Andrew McMichael and colleagues (37). They developed a vaccine targeting the Kenyan epidemic, including a clade A Gag p24 and p17, linked to a string of 22 epitopes selected by virtue of being presented by the most common HLA types in Kenya and eliciting immunodominant responses. The HIV construct was expressed from DNA or in a modified vaccinia virus Ankara (MVA), and given as DNA, MVA or DNA/MVA prime boost combinations. The vaccines were immunogenic in mice and macaques (83), and so it progressed through a human safety and immunogenicity trial first in England, where promising immunogenicity was observed (63). A study which specifically broke down the responses to the Gag and epitope string portions of the construct (35) found most of the observed responses were to CD4+ T cell epitopes in the Gag portion. 5/16 individuals had responses to the epitope string, and 3 of these were mapped to the same epitope, indicating very few of the epitopes in the string elicited CD8-T responses. Recently completed trials in Kenya and Uganda had a disappointing outcome, as the majority of individuals did not make a detectable Eli Spot response to the vaccines (for example, a

DNA prime/MVA boost elicited EliSpot responses in only 5/38, or 13%, of volunteers), and those that did respond tended to have weak and transient responses (42). This series of studies, although disappointing for these particular vector/insert combinations, had several strengths: they tested an important and reasonable concept, they demonstrated safety of DNA/MVA vaccine combinations, and the team successfully performed several small human trails in sub-Saharan Africa.

In a second HIV study of this kind, a more bioinformatically intensive approach was applied to the poly-epitope design problem (58). The design focused on the selection of epitopes that bind multiple HLA allelic products that have the potential for presentation by many HLA proteins. 21 conserved epitopes that could be presented by HLA-A2, A3 and B7 supertypes were linked, and 85% of individuals globally were predicted to be able to present these epitopes. Rather than just linking epitopes directly, the vaccine was specifically engineered to enhance correct processing, and to minimize unnatural junctional epitopes (58). De Groot and colleagues have proposed a similar design strategy, focusing on deriving consensus epitopes and computationally attempting to minimize junctional epitopes (24). Initial studies in HLA transgenic mice with the polyepitope vaccine indicted that multiple epitopes within the construct could elicit a response (85). Despite meticulous planning, rigorous logic, and encouraging preliminary results, again the vaccine had disappointing results in human immunogenicity studies; only 1/42 uninfected vaccinated adults made a detectable gamma interferon EliSpot response, and 3 had a response that could be detected by a chromium-release CD8+ T cell assay (36).

For both of the vaccine constructs discussed here it is possible that the particular delivery strategies and vaccination protocols could be altered to achieve better outcomes. To date, however, an interesting concept and encouraging results in animal studies using poly-epitope HIV vaccines has not translated well into human immunogenicity studies.

FOCUSING ON THE MOST CONSERVED REGIONS OF HIV

The notion of focusing the vaccine response on conserved epitopes is part of what motivated the poly-epitope vaccines described in the previous section, but in those cases precisely defined epitopes were combined into a mini-gene, whereas in the strategy described in this section, longer sections of proteins spanning only the most conserved regions of the proteome are linked in a chimeric protein with the intention of capturing all of the CD4+ and CD8+ T-cell epitopes harbored within these regions. Vaccine induced T-cell responses would thus have a higher probability of interacting with the spectrum of circulating viruses at the population level. At the level of the infected individual with prior vaccination, the induced response may have the potential to shift the immunodominance profile and focus the initial immune response in a newly infected individual on conserved regions, where mutations that elicit immune escape would be more likely to have a high fitness cost (5, 72, 77, 81, 88). The underlying hypothesis is that at least part of the benefit conferred by HLA-B*5701 and other protective HLA alleles is realized through high fitness costs of escape mutations (62), and that this type of a vaccine might be able extend this kind benefit to infected individuals who do not carry one of the few HLA alleles associated with a good outcome.

Epitope processing and T-cell elicitation remains an important issue to resolve experimentally in this kind of approach; while including long fragments in a chimeric protein may allow for more natural processing and presentation than a beads-on-a-string approach, the basic strategy may still have issues with processing and presentation. Bioinformatic approaches such as those used to attempt to minimize junctional epitopes for the poly-epitope vaccines (58) could also applied to the design of conserved chimeric proteins (72). The most extremely conserve regions of HIV may not be very immunogenic in naturally infected cells. For example, the most conserved regions in the proteome are found in Pol but relative few CD8-T responses recognize Pol epitopes (Fig. 3a). Rolland et al. suggest this may be a consequence of a lower ratio of Pol protein expression relative to Gag (72), which could be an issue for Pol vaccine candidates. Furthermore, some of the most highly conserved domains may have evolved strategies to avoid immune pressure long ago in evolutionary history of these viruses (90). If the epitopes in a natural context are not presented, the vaccine will not confer the desired protective effect.

Regardless of the reservations expressed above, the first mouse study based on the conserved-region approach was encouraging (52). The vaccine design included the 14 most conserved regions of the HIV proteome linked in a chimeric protein. The sequence used to represent each region was selected from one of 4 subtype consensus sequences, but these regions were globally very conserved so that the M group coverage of potential epitopes was good throughout the vaccine construct. BALB/c and HLA-A*0201 transgenic mice were able to generate T cells responses to this vaccine antigen expressed as DNA, in a MVA construct, and in a human adenovirus serotype 5 (Ad5) construct (52). 13/13 HIV infected people had memory T cell responses to epitopes carried in the chimeric protein, and many known epitopes in the Los Alamos HIV database are harbored in these regions (52). Based on this promising start, this vaccine antigen is going forward into a small human safety and immunogenicity trial (T. Hanke and A. McMichael, personal communication).

CENTRAL VACCINES

One way to begin to address the diversity of the HIV, particularly if one is aiming for the simplicity and cost-effectiveness of monovalent vaccine, is to design an antigen that is central to the circulating strains the vaccine is targeting. This can be attempted at a within subtype or a global level. Three different strategies for accomplishing this have been suggested. The first is based on a phylogenetically reconstructed ancestral sequence (25, 33, 51), which is the string of bases that represents the most likely base in each position in an alignment at an ancestral node in a phylogenetic tree, for example a node at or near the based of a clade, or the node that is the ancestral sequence for the entire M group. The second strategy is to take the most common amino acid at every position in an alignment and concatenate those together; this is called a consensus or CON sequence (32, 33, 51). A third strategy finds the point in a phylogenetic tree that minimizes sum of the distances to all branch tips, the "center of tree" or COT sequences (64, 71). Central sequences are all similar to each other, and the differences between them are in the same range as the expected number of errors inherent in phylogenetic based ancestral reconstruction methods (31). They will change slightly if re-estimated based on newer input data, as the

global sampling increases, but are basically robust. Subtype-specific centralize protein vaccines can reduce the distance between a vaccine and contemporary circulating strains by roughly half, relative to a typical natural vaccine strain (33). This can be visualized in the phylogenetic trees (Fig. 1); if the vaccine is based on the model sequence near the root of clade, you only have to traverse one branch into ancestor of the clade, and you don't have to add in the distance from the root back out to the natural strain selected for a vaccine. M group central sequences essentially bring vaccine distances to all circulating sequences to the level of within-clade distances. Another way to consider this in terms of 9-mer coverage; two B clade strains which have been developed as vaccine candidates (HXB2 and CAM.1) do not match as many 9-mers in the M group an M ancestral or consensus strain do, and M group ancestral and consensus sequences are roughly comparable (Fig. 4a).

When such strategies were still untested, there was serious skepticism regarding synthesizing proteins based on these artificial protein designs - would they fold well, be recognized by conformational antibodies, be functional, and be immunogenic? We started with the synthesis of a reconstructed M group Consensus/Ancestral Env (we have several generations of this concept now tested, most of our work has been done with a version called Con S (32, 33, 82)). This was the most challenging central protein, and a priori the least likely to succeed, because the M group consensus/ancestral Env was the farthest of the synthetic protein designs from a real HIV protein. There are two reasons for this. First, Env is the most variable protein in HIV, and second, by we had to go all the back to the center of the entire M group rather than just to the root of a single subtype (Fig. 1). Con S, however, was also the most intriguing in terms of striving for a global vaccine and testing both B cell and T cell responses. Despite its distance from a natural strain and inherent uncertainty in modeling, we found it to be weakly functional in a pseudotyping assay, and when expressed it bound key conformation antibodies, and most critically, it was immunogenic for both T cells and B cells when tested in small animal studies (32, 54, 82). Similarly, all other Consensus, Ancestral and COT sequences for M group and subtypes B and C that have been tested to date are well expressed and immunogenic in small animals (32, 50, 51, 54, 71). COT sequences for B clade Gag, Nef and Tat all retained biological function (71). The M group Con S and a Con B Env protein (50, 54) elicited antibodies with good titers and breadth against tier 1 viruses, those that readily neutralized, but not against more difficult to neutralize isolates. One possible application for central sequences in terms of a B cell vaccine would be to use them as the foundation for rational strategies to specific modify the Envelope to better expose useful neutralization epitopes (50).

The M group Con S Envelope vaccine has been shown to induce T cells with enriched cross-reactive potential in both mouse (82) and in rhesus monkeys (74). In Santra et al., a Con S vaccine was compared with a natural by modified B subtype Env. Both vaccines made strong autologous responses as determined by reactions with pooled peptides designed to match the vaccine protein sequences, but the responses to Cons S had much greater breadth. The number of responses to a peptide series spanning10 different natural proteins including representatives of 4 different clades was tested, and the Con S vaccine yielded 3-4 fold more detected responses per protein than did a natural B clade vaccine. This indicates that the cross-reactive potential of T cell responses to the Con S protein was greatly enhanced relative to the cross-reactive potential of T cell

responses elicited by a single natural protein vaccine, and so by extension, these responses would be better able to interact with naturally infecting strains in the global population (74).

Another encouraging result is the based on the use of centralized proteins as a foundation for designing peptides as reagents for EliSpot (30). The cross-reactive potential of natural T cell responses in human HIV infection with regard to different epitopes variants provides indirect evidence regarding whether or not a particular epitope variant is likely to stimulate a response that can cross-react. When within-subtype and M group central sequences were used to design peptides, the three strategies (Consensus, ancestral and COT) were found to be comparable in terms of enabling detection T cell responses. As anticipated, responses were better detected with subtype-matched reagents (more T cell responses from a B subtype infection were detected with B subtype peptides than C subtype peptides, and vice versa). The M group based reagents, as hoped, performed as well was within-subtype based reagents for response detection (30).

POLYVALENT MOSAIC VACCINES

Given the emerging evidence that central strain computer model-based proteins were well expressed an immunogenic, and had desirable properties in terms of inducing T-cell responses with improved cross-reactive potential, we decided to build on this concept and design polyvalent protein cocktails that could in combination provide the maximum coverage of potential T cell epitopes (28). This computational polyvalent approach was further motivated by the promising results observed with polyvalent natural immunogens (75). We utilized a machine learning strategy called a genetic algorithm to computationally design optimized sets of protein sequences that in combination maximize the coverage of potential epitopes in the population. The resulting mosaic sequences are derived from *in silico* recombinants of natural strains, and are constrained to be "like" natural sequences: boundary regions spanning breakpoints are required to be found recurring among the natural sequences, and mosaic proteins align readily to a sequence alignment of natural proteins. This strategy attempts to maximize the coverage of natural variation in all potential epitopes, and so to improve the chances of benefitting a vaccinee with any combination of HLAs. Given the extensive overlap between known epitopes (Fig 3a) (http://www.hiv.lanl.gov/content/immunology/maps/maps.html), this seems desirable. Furthermore, by mimicking natural proteins, we hope to mimic natural processing so that epitopes that stimulate a response in a vaccinee will be the same epitopes that are processed and presented in a natural infection. Mosaic polyvalent vaccines can significantly improve the population coverage of potential epitopes for every protein in HIV (Fig. 3b-d). Mosaics designed to optimize coverage of a single subtype do very well in terms of coverage of that subtype, however there is a big drop off in terms of coverage of other subtypes. In contrast, mosaics designed to optimize over the full M group, not only do almost as well as within-subtype mosaics, but cover all subtypes at this high level (28), giving these vaccines potential to serve as a global vaccine. By design, mosaics minimize the inclusion of rare and unique potential epitopes, and do not contain unnatural junctional epitopes. Another vaccine design strategy, COT+, also uses computational tools to maximize 9-mer coverage (65), but this strategy does not attempt to reconstruct proteins, rather produces a set of protein fragments which

can be assembled into a poly-fragment protein (27, 65). The mosaic strategy gave somewhat enhanced coverage of potential epitopes over the COT+ strategy when the algorithms were applied to the same data set (27).

There are several common questions about mosaics that are addressed in Fig. 4 using Gag vaccine designs compared to the M group database as an example. Fig 4a simply shows the incrementally improved coverage of 9-mers in Gag in the global M group alignment using different strategies. Fig. 4b. shows that the coverage of the sequences is robust over time. The M group input data used to generate the mosaic vaccine designs for figures for this review was based on the 2008 database, with 2 additional years of sequence acquisition relative to the Fischer et al study (28). The current data set has more than doubled in size relative to the data set used for mosaic design in Fischer et al. But the vaccine designed two years ago in the Fisher study provided comparable coverage of potential epitopes in the more recent data set to the vaccine designed using the more recent data set as input (Fig. 4b). Figure 4c shows that the M group 9-mer coverage of a combination of 4 different clade consensus sequences (A, B, C and D) is roughly comparable with a selection of the 4 best natural strains, and that the coverage by 4 mosaics is substantially better than either of the other two designs. Also, random selection of 4 natural strains gives a distribution of coverage, and most combinations are far from optimal. Fig. 4d shows that as more sequences are included in either an optimal natural set or a mosaic vaccine design set, population coverage of potential epitopes increases, but with diminishing returns. Fig. 4e resolves a very important point. We generally optimize for coverage of 9-mers, as that is the most common light of CD8+ T cell epitopes. But the solution based on 9-mers is also a very good solution for epitopes of similar lengths, and 8-mers, 10-mers... are nearly optimally covered.

Several vaccines based on the mosaic approach are currently in the pipeline. The trajectory for such new reagents is to first design the mosaics as protein sequences, then design a gene that encodes them that optimizes expression (61), then synthesize and express the genes, and then study their protein properties such as binding to relevant antibodies. After this is accomplished, the immunogenicity in mice is tested. Intact Gag, Nef, Pol, and Env M group mosaics have all been synthesized, and all are well expressed and immunogenic in mice (BH and NL, data not shown). In contrast, a GAG-partial Nef fusion protein mosaic that was immunogenic in the Gag portion was not immunogenic in the Nef portion in mice, so the fusion protein strategy we originally proposed from mosaics (28) was dropped and the two genes were expressed separately (27). The first study in mice to test whether the breadth of the response to mosaic vaccines was enhanced showed a marked increase in breadth of response for both CD4+ and CD8+ T cells, but the increase was most pronounced in for CD8+ T cells (46). In the most striking comparison in this study, a DNA vaccine based on 3 natural Envs, one each from subtypes A, B and C, was compared with a 3 mosaic vaccine. The trivalent vaccine based on 3 natural strains elicited only 2 positive CD8+ T cell responses to a series of peptides pools representing M group diversity (53), while the trivalent mosaic vaccine elicited CD8+ T cell responses to 10 peptides pools. Two macaque trials comparing different mosaic designs to natural strains and consensus vaccines are currently underway.

CONCLUSIONS

This all leave the field with several promising paths to follow. Vaccines that focus on conserved regions have the potential to focus the immune response on regions where escape is disadvantageous, and where a vaccine elicited T-cell response is mostly to cross-recognize circulating viruses. The first study of this approach in mouse is encouraging (52), and the strategy merits further exploration. The limited human responses to poly-epitope vaccines (35, 36), however, suggests that conserved vaccine candidates should carefully address the number of responses in the poly-fragment proteins, and determine if junctional unnatural epitopes are an issue – i.e. peptide sets used to explore the response should span junctions as well as test the natural HIV sequences within the constructs. By including whole conserved regions rather than just specific epitopes, CD4+ T-cell responses and additional CD8+ T-cell response, which both are important, may elicited by these vaccines.

In a monkey models, live attenuated vaccines in the context of a heterologous challenge can, at least in some cases, achieve what we hope to achieve in humans with a T-cell vaccine – better control of the virus, and a better clinical outcome (69). Similarly, pre-existing infection can result in control of superinfection (89). What are distinctive about live attenuated models? These are nearly complete viruses that contain almost the full proteome, hence enable many responses, and attenuated viruses also provide long-term low-level stimulation (Norm is this correct – and should I be folding in other protection models in SIV here too?.) These aspects can be adapted to the scenario of a human vaccine, even if live attenuated viral vaccines are not possible. The notion of attempting to maximize the number of cross-reactive responses offers possibilities that are philosophically essentially the opposite of a conserved region approach – broadening the cross-reactive responses, rather than focusing the responses. Both strategies are well reasoned, however, with a valid experimental underpinning and motivation, and whether either or both will confer a benefit will require experimental resolution. Polyclonal vaccines, central vaccines, and mosaics each offer different ways to increase the number of cross-reactive responses in the context of a single protein, and each of these strategies are proving to be better than a single natural protein in animal studies of immunogenicity and breadth of response, particularly in the context of a global vaccine (46, 74, 80). The vaccine has to stimulate responses that will recognize the epitopes presented in an infected cell, and a closer matching epitope sequences to the circulating population and improved mimicking natural processing could both contribute to enhancing the frequency of such events. Gag and Pol, despite being relatively conserved for an HIV protein, are none-the-less quite variable in terms of recognition by a T cell receptor, and could also benefit from these strategies (Fig. 3).

Polyclonal and mosaic vaccines each offer another potential benefit: the common variant forms of an epitope at a population level are probably most often a fit route to escape from T-cell responses that target the region. One person's escape form can be another person's susceptible form (11). If the most common forms of an epitopes are simultaneously presented in a vaccine, T-cell responses may, at least in some cases, effectively block the natural and most common escape routes in an individual, forcing the virus to either remain susceptible to the CTL response, or find a less fit way out. These strategies, particularly in combination with better delivery strategies and adjuvants, have

the potential to significantly improve the vaccine induced T-cell response relative to what was used in the STEP trial. The STEP trial has set a bar, and future trials will need to surmount it to succeed. There are many promising strategies and experiments currently underway (94).

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FIG. 1. Phylogenetic trees of the major subtypes found in the HIV-1 M group Gag and Env. These trees are based on the Los Alamos database currated global alignment, as of Dec 2008, and contain one full length gene per person. The trees show the relationships between the major HIV subtypes; intersubtype recombinants were excluded. It represent global studies, but not systematic sampling, as whatever sequences were studied and published in GeneBank are included. Only representative subsets of B clade from the US or C clade from South Africa were included in these trees, as these are the most heavily studied epidemics and dominate the global sampling. To illustrate how these regional single-subtype epidemics fit into the global picture, South African sequences are have terminal lines colored in red, US sequences in blue, all other nations in black. The green circles show the approximate region the node selected for most recent common ancestor generation – generally we do not include the sequences that are outliers relative to the rest of the clade in these models. The trees are maximum likelihood trees uisng a GTR model with site rate variation estimated with a gamma distribution and were generated using PhyML (PMID: 14530136).

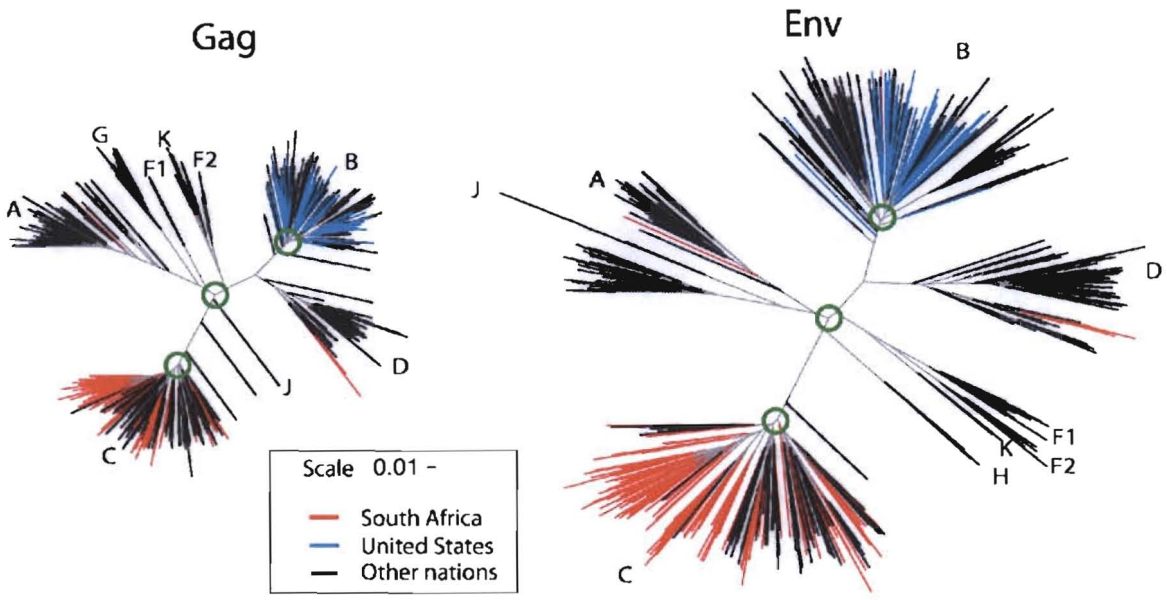
FIG. 2. This map shows the subtype designation of all seuqences in the Los Alamos database as of Jan. 1, 2009. These sequences are often single genes and fragments, so intersubtype recombination will be underestimated. The are also not sampled randomly, but are the product of all HIV studies with sequences submitted to GenBank. Despite this limitation, the maps largely reflect what we know about global distributions of subtypes, but the details should be interpreted with caution. The figures were made using the HIV geography tool at the Los Alamos database.

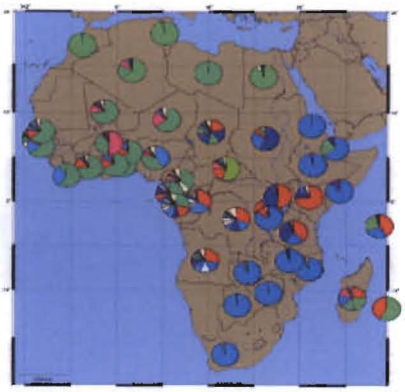
FIG. 3. A. This figure maps known and distinctive CD4+ and CD8+ epitopes described in the literature and included in the Los Alamos database. They reflect experimental maps of population responses (29), with the exception of under-representation of T-cell epitope mapping of regulatory proteins. These are somewhat under-represented in the literature, and so also under-represented in the database and hence in Fig. 3a. B. This shows the

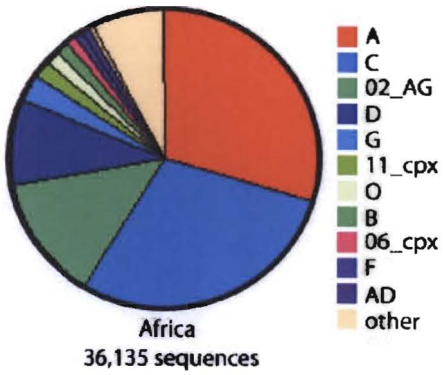
fraction of identical matches with a the single natural strain that provides the optimal coverage of the M group for each 9 mer (potential epitope) in the HIV proteome. The optimal natural strain turns out to be a C subtype sequence, C.ZA.99.DU422 accession number: AY043175. This is not surprising as C is the most common subtype in the full-length genome database. This is an alignment based figure; the grey background illustrates how many sequences have 9-mer in the alignment, such that a section in the alignment with an insertion in only 1 or a few sequences will appear as a white band. C. shows the increase in the fraction of perfectly matched 9-mers at each position when a 4 mosaic combination is used rather than a single natural strain. D. Shows the total percentage of total 9-mers covered for each protein, corresponding to the single natural strain coverage shown in B and the 4 mosaic coverage shown in C.

FIG. 4. Comparisons that address frequently asked questions about mosaic and central sequence vaccine designs. The comparisons in this figure are based on Gag, The relative ranking of the tests performed on Gag is consistent for all HIV proteins, but are set at higher or lower levels depending on the innate variability of the protein. A. This shows the incrementally improved coverage of 9-mers in Gag in the global M group alignment using different design strategies – two single natural strains which have been used for vaccines in the past, the single natural strain selected to provide the best 9-mer coverage, the M group ancestor, the M group consensus, a single mosaic, the 4 natural strains that in combination give the best coverage of Gag, and 4 mosaics. The single best natural turns out to be it turns out to be a C subtype sequence, which is not surprising as C is the most common subtype in the full length genome database: C.ZA.99.DU422 AY043175B. The 4 best naturals are a set that includes a C, a B, an AD recombinant, and a CRF01 sequence. This illustrates the robustness of these designs over time and with the acquisition of new data. The 2006 db were the 4 mosaic Gag sequences generated in the Fischer et al. study (28) based on the set of 551 M group sequences, one sequence per person, that were available for inclusion in the curated alignment at the end of 2005 for the 2006 Los Alamos Database. The current designs are based on the 2008 db using Gag proteins extracted from 1206 group M full length sequences that were available at the end of 2007 for inclusion in the 2008 database (db) curated alignments. The 2008 db set was the test set used in both cases, and the 2006 db mosaics provides almost the same coverage as the 2008 db mosaics; given the cost of develop and

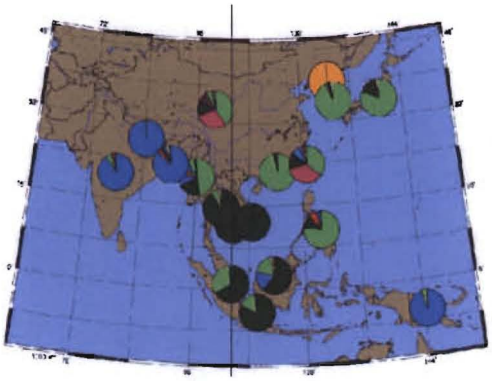
FIG. 1

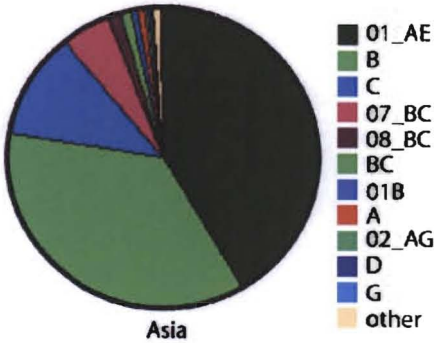

Gag
G
K
F1
F2
A
B
J
D
C
Scale 0.01 –
South Africa
United States
Other nations
Env
B
A
J
D
F1
K
F2
H
C



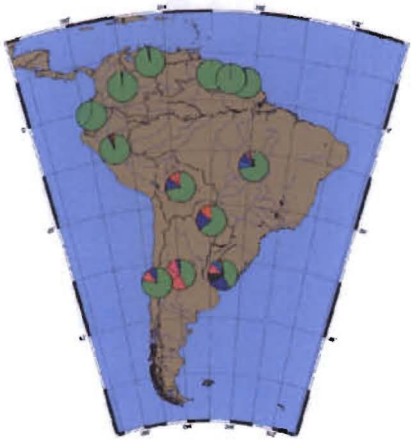


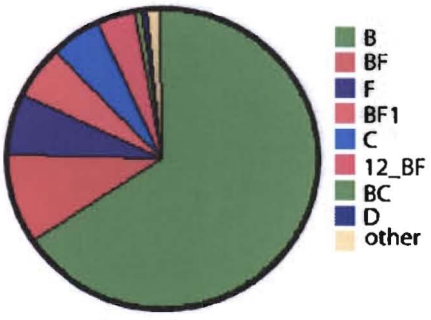
Africa
36,135 sequences



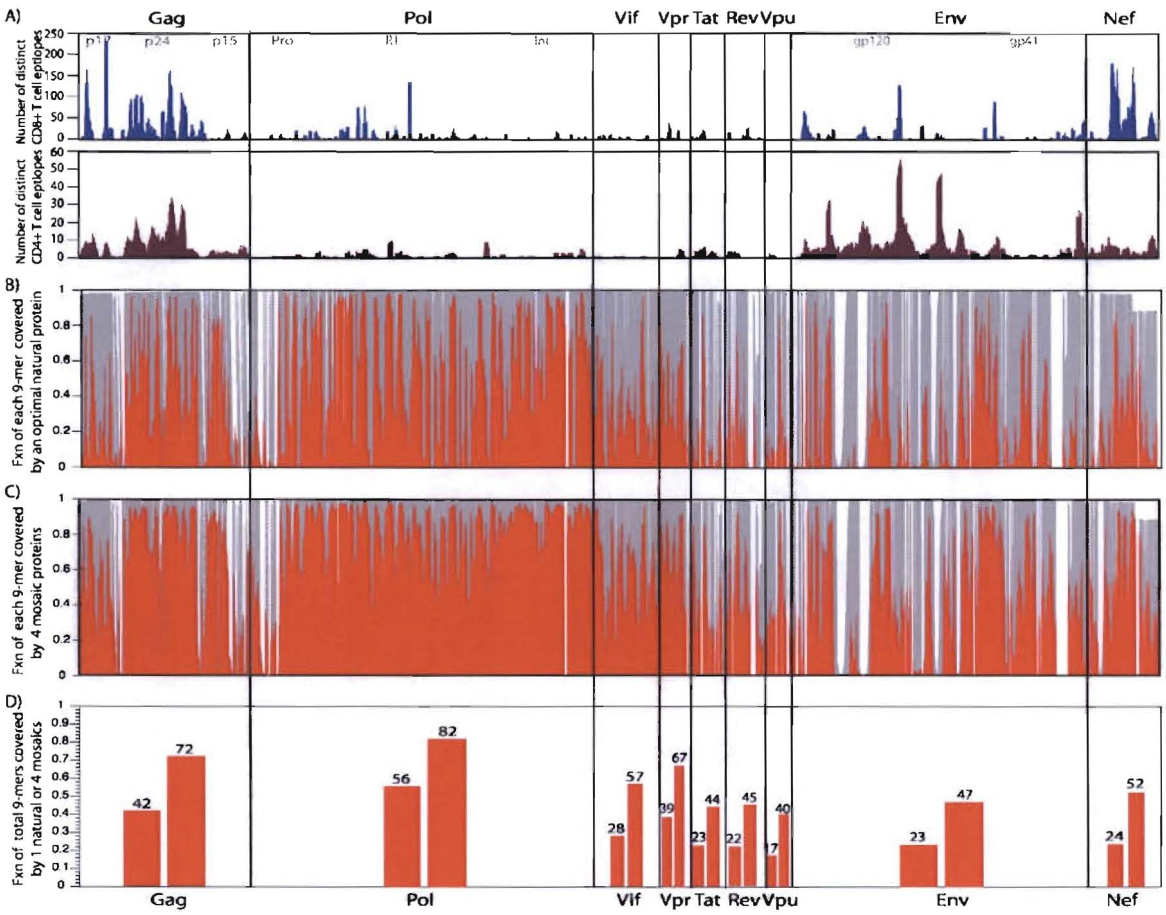


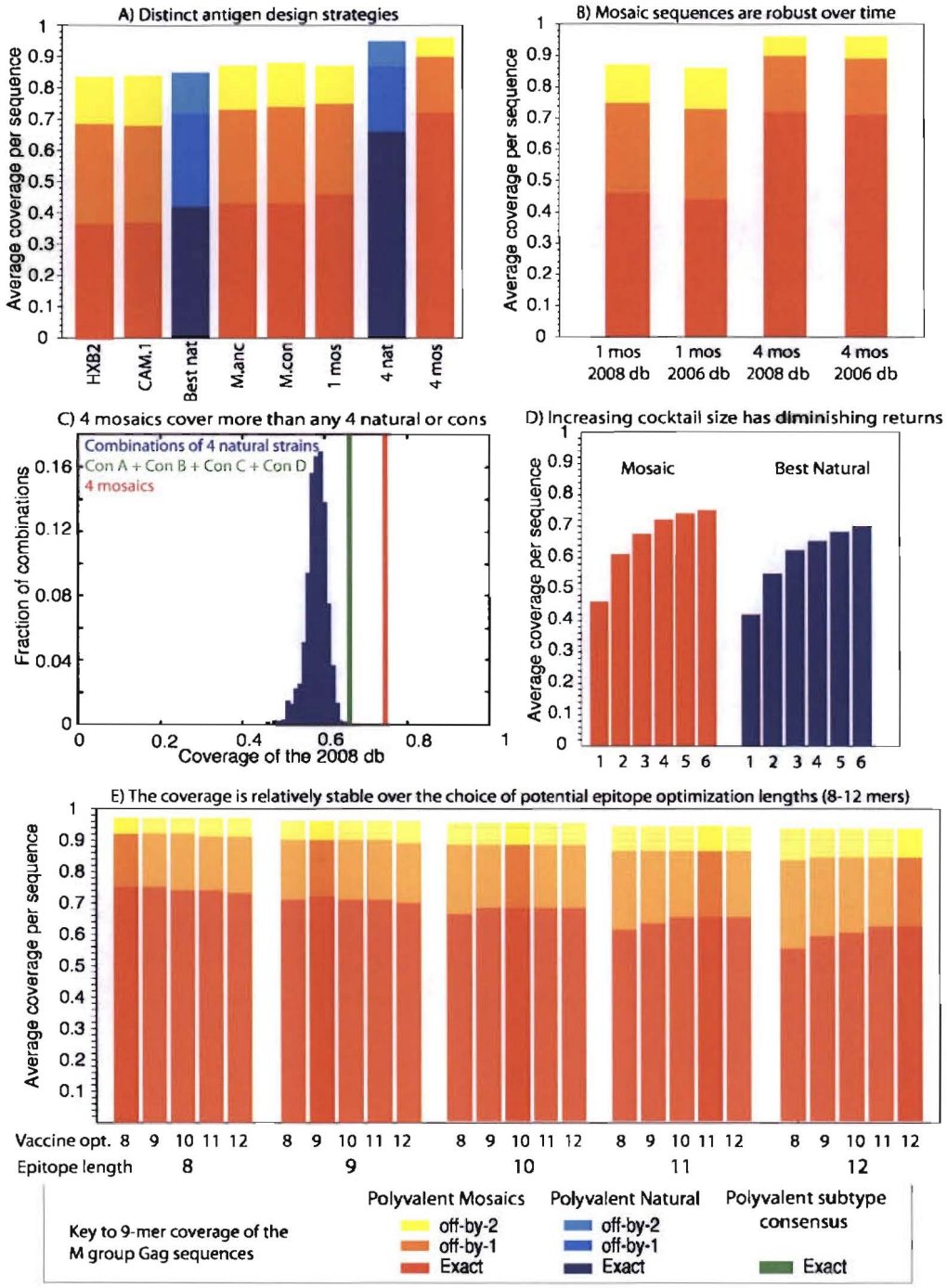
Asia
18,233 sequences





South America
8,160 sequences


A)
Gag
Pol
Vif
Vpr
Tat
Rev
Vpu
Env
Nef
p17
p24
p15
Pro
RT
Int
gp120
gp41
Number of distinct CD8+ T cell epitopes
Number of distinct CD4+ T cell epitopes
B)
Fxn of each 9-mer covered by an optimal natural protein
C)
Fxn of each 9-mer covered by 4 mosaic proteins
D)
Fxn of total 9-mers covered by 1 natural or 4 mosaics
42
72
56
82
28
57
39
67
23
44
22
45
17
40
23
47
24
52



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