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Ancestral and consensus envelope immunogens for HIV-1 subtype C

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Abstract

Immunogens based on "centralized" (ancestral or consensus) HIV-1 sequences minimize the genetic distance between vaccine strains and contemporary viruses and should thus elicit immune responses that recognize a broader spectrum of viral variants. However, the biologic, antigenic and immunogenic properties of such inferred gene products have to be validated experimentally. Here, we report the construction and characterization of the first full-length ancestral (AncC) and consensus (ConC) *env* genes of HIV-1 (group M) subtype C. The codon-usage-optimized genes expressed high levels of envelope glycoproteins that were incorporated into HIV-1 virions, mediated infection via the CCR5 co-receptor and retained neutralizing epitopes as recognized by plasma from patients with chronic HIV-1 subtype C infection. Guinea pigs immunized with AncC and ConC *env* DNA developed high titer binding, but no appreciable homologous or heterologous neutralizing antibodies. When tested by immunoblot analysis, sera from AncC and ConC *env* immunized guinea pigs recognized a greater number of primary subtype C envelope glycoproteins than sera from guinea pigs immunized with a contemporary subtype C *env* control. Mice immunized with AncC and ConC *env* DNA developed gamma interferon T cell responses that recognized overlapping peptides from the cognate ConC and a heterologous subtype C Env control. Thus, both AncC and ConC *env* genes expressed functional envelope glycoproteins that were immunogenic in laboratory animals and elicited humoral and cellular immune responses of comparable breadth and magnitude. These results establish the utility of centralized HIV-1 subtype C Env immunogens and warrant their continued evaluation as potential components of future AIDS vaccines.

Keywords: HIV-1 genetic variation; Centralized HIV-1 immunogens; HIV-1 envelope glycoprotein; Subtype C

Introduction

Globally circulating strains of HIV-1 exhibit an extraordinary degree of genetic diversity, which poses a formidable challenge for AIDS vaccine development (Douek et al., 2006; Garber et al., 2004; Gaschen et al., 2002; Joseph et al., 2005). Members of the pandemic-associated main group of HIV-1 (group M) can differ in up to 35% of envelope amino acid residues, and even within individual group M subtypes, envelope protein sequence variation can be as high as 20% (Gaschen et al., 2002). Despite this extent of diversity, only few AIDS vaccine strategies are addressing HIV-1 genetic variation directly. The great majority of HIV-1 immunogens in preclinical and clinical evaluation are derived from contemporary viruses, frequently selected based on availability and geographic representation, and in some instances envelope co-receptor preference or preservation of cross-reactive epitopes (IAVI, 2006; Douek et al., 2006; HVTN, 2006; Nkolola and Essex, 2006). Several studies are also aiming to improve cross-reactivity by including immunogens from more than one HIV-1 subtype (Chakrabarti et al., 2005; Kong et al., 2003; Seaman et

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al., 2005), and in one instance a vaccine strain was selected based on its genetic relatedness to the consensus sequence of the locally circulating subtype (Burgers et al., 2006). However, the most direct approach to minimize HIV-1 genetic diversity is the use of "centralized" immunogens (Gaschen et al., 2002; Novitsky et al., 2002; Ellenberger et al., 2002; Nickle et al., 2003; Mullins et al., 2004). This concept is based on the fact that reconstructed ancestral or consensus sequences are only about half as distant from contemporary HIV-1 strains as these are to each other. Thus, centralized immunogens should elicit more cross-reactive immune responses than immunogens derived from any single contemporary HIV-1 strain. However, this prediction remains to be tested in vivo, by rigorous comparative testing in appropriate animal models and ultimately human vaccine trials.

Three centralized *env* genes have thus far been generated and investigated as DNA and/or protein immunogens. Two comprise group M consensus *env* genes (CON6 and CONS) (Gao et al., 2005; Weaver et al., in press; Liao et al., in press), whereas the third represents a reconstructed ancestral subtype B *env* gene (An1-EnvB) (Doria-Rose et al., 2005). All three centralized *env* genes were shown to express functional envelope glycoproteins that were incorporated into virus particles and mediated cell fusion, although infectivity was markedly reduced for the two group M consensus *env* genes (Gao et al., 2005; Doria-Rose et al., 2005; Liao et al., in press). Mice vaccinated with CON6 *env* DNA developed potent and broadly cross-reactive (cross-clade) T cell responses (Gao et al., 2005; Weaver et al., in press), whereas guinea pigs immunized with CON6 gp120 or a cleavage site and fusion peptide deleted gp140ΔCF protein developed only low titer neutralizing antibody responses (Gao et al., 2005). Rabbits immunized with full-length (non-modified) gp160 An1-EnvB DNA and boosted with the cognate gp120 protein also developed neutralizing antibody responses, but again breadth and potency were limited (Doria-Rose et al., 2005). In contrast, guinea pigs immunized with gp140 oligomers of a cleavage site, fusion peptide and gp41 immunodominant region deleted ConS glycoprotein (ConS gp140ΔCFI) elicited cross-reactive antibodies that neutralized primary Env containing viruses from three different clades (Liao et al., in press). Together, these results provided proof of concept for the utility of centralized gene products as components of an AIDS vaccine. However, studies of additional centralized immunogens, including consensus and ancestral genes from the same subtype, are necessary to examine the influence of the reconstruction process on their immunogenic properties.

Among the many subtypes and circulating recombinant forms of HIV-1 group M, subtype C is the most prevalent. This subtype is responsible for more than half of all infections worldwide, dominates the epidemic in Southern Africa (the region with the highest HIV-1 prevalence globally) and fuels the rapidly expanding epidemics in India and China (Osmanov et al., 2002; Nkolola and Essex, 2006; Novitsky et al., 2002). Subtype C viruses also appear to share unique biological properties. Most notably, the V3 region and adjacent *env* domains of subtype C strains are under markedly different evolutionary pressures than the corresponding *env* regions in other subtypes, suggesting differences in epitope presentation and/or accessibility (Gaschen et al., 2002). In addition, recently transmitted subtype C viruses have shorter hypervariable regions and individuals infected with these viruses develop higher titer autologous neutralizing antibodies than individuals acutely infected with subtype B (Derdeyn et al., 2004; Chohan et al., 2005; Li et al., 2006). Finally, subtype C strains use the CCR5 co-receptor almost exclusively (Morris et al., 2001; Peeters et al., 1999). Together, these findings suggest subtype-specific differences in envelope antigenicity and possibly also immunogenicity, which represents a rationale for exploring subtype C specific vaccines (Nkolola and Essex, 2006). Here, we describe the first centralized subtype C Env immunogens, including both ancestral (AncC) and consensus (ConC) *env* sequences, and compare their antigenic and immunogenic properties to those of a wild-type subtype C *env* control.

Results

Design of subtype C consensus and ancestral env sequences

Both ancestral (AncC) and consensus (ConC) *env* sequences were reconstructed from alignments of full-length subtype C *env* sequences available in the 2001 Los Alamos HIV Sequence Database. The ancestral *env* sequence was inferred from maximum likelihood phylogenetic analyses and represents the most likely sequence at the interior node of the subtype C cluster (Gaschen et al., 2002). The consensus *env* sequence was generated by selecting the most common amino acid at each position in the protein alignment. Hypervariable regions of both centralized *env* genes were aligned by anchoring on common glycosylation sites, and only minimal common elements spanning the region were retained. Fig. 1 depicts the deduced amino acid sequences of the AncC and ConC *env* genes. Overall, the two protein sequences are 95.5% identical and share all major known functional domains. The AncC *env* gene encodes one additional N-linked glycosylation site near the base of the V1/V2 loop (boxed); all other 25 N-linked glycosylation sites are conserved between the two proteins.

Expression and virion incorporation of AncC and ConC Env glycoproteins

Codon usage optimization is known to increase HIV/SIV protein expression in vitro as well as in vivo (Andre et al., 1998; Haas et al., 1996; Schneider et al., 1997; Schwartz et al., 1992). We therefore synthesized the full-length AncC and ConC *env* genes as codon-usage-optimized versions of their amino acid sequences (Andre et al., 1998). We also constructed truncated gp145 and gp120 *env* genes by introducing premature stop codons immediately following the membrane-spanning domain and the gp120/gp41 cleavage site, respectively. As shown previously for a codon-usage-optimized *env* gene of a wild-type subtype C strain (Gao et al., 2003), full-length and truncated AncC and ConC genes expressed high levels of glycoprotein upon transfection into

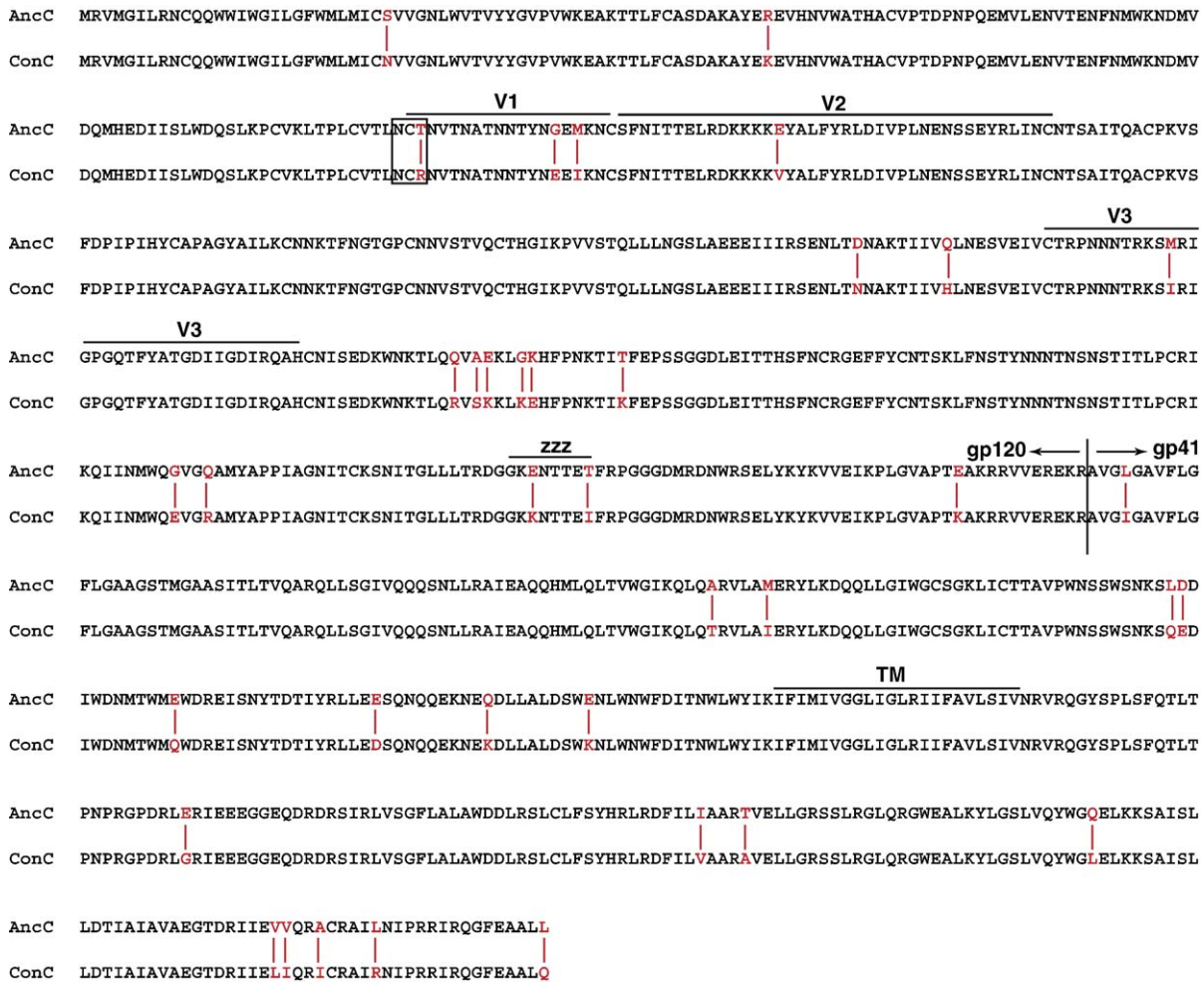

Fig. 1. Alignment of ancestral (AncC) and consensus (ConC) subtype C Env protein sequences. Amino acid sequence differences are highlighted in red. A potential N-linked glycosylation site present in AncC but absent in ConC is boxed. Variable regions (V1–V5) were reconstructed using a minimal loop approach, reasoning that shorter loops may be of advantage for exposure of neutralizing epitopes (Srivastava et al., 2003). The gp120/gp41 cleavage site and the transmembrane domain (TM) are indicated.

293T cells (Fig. 2). Optimized gp160 and gp145 Env proteins were processed, albeit incompletely; this was most likely due to the saturation of cellular proteases involved in the cleavage of the gp160 Env precursor (Binley et al., 2002). Cleavage products were seen for both gp160 and gp145 constructs upon longer exposure (not shown).

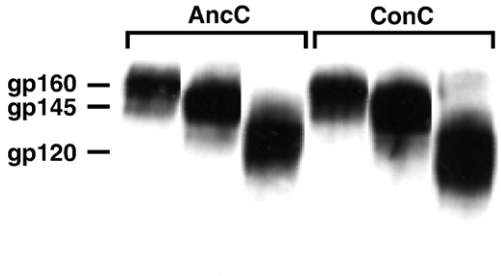

Fig. 2. Expression of AncC and ConC envelope glycoproteins. Full-length and truncated *env* genes were transfected into 293T cells and lysates examined by Western blot analysis using plasma from a patient infected with HIV-1 subtype C. The sizes of the gp160, gp145 and gp120 expression products are indicated.

To examine whether the AncC and ConC *env* expressed glycoproteins could be packaged into HIV-1 particles, gp160 and gp145 constructs were co-transfected with an *env*-deficient HIV-1 (SG3Δenv) backbone vector in 293T cells. Codon-usage-optimized and *rev*-dependent *env* constructs of a wild-type subtype C strain (96ZM651.8), along with the *env*-minus backbone vector, were analyzed in parallel as controls. Pseudovirion preparations were purified by centrifugation through a 20% sucrose cushion, normalized by p24 Gag content, and examined by Western blot analysis using plasma from an HIV-1 subtype C infected individual. Both AncC and ConC *env*-derived gp160 and gp145 glycoproteins were incorporated into virus particles (Fig. 3). Indeed, AncC and ConC gp160 containing pseudovirions (lanes 1 and 3) contained at least ten-fold more envelope glycoprotein than pseudovirions generated with the *rev*-dependent 96ZM651.8 subtype C *env* control (Fig. 3, lane 6). Again, a large fraction of particle associated envelope glycoprotein remained uncleaved, although full-length and truncated gp41 proteins were visible in the AncC and ConC Env containing pseudovirion preparations (Fig. 3). Taken together, both full-length and truncated centralized Envs were

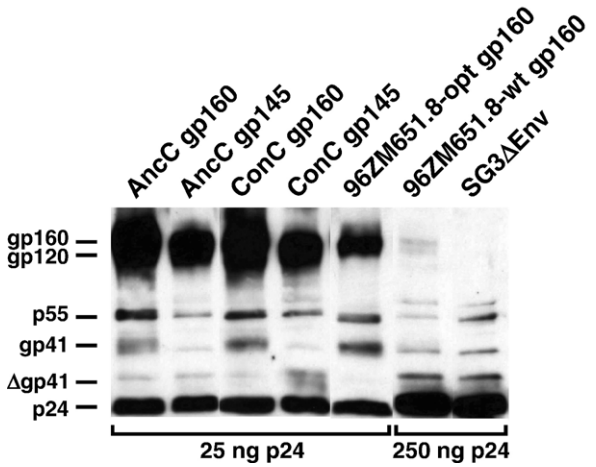

Fig. 3. Protein profiles of AncC and ConC Env containing pseudovirions. Full-length (gp160) and truncated (gp145) AncC and ConC as well as codon-usage-optimized (opt) and wild-type (wt) 96ZM651.8 *env* genes were co-transfected with the HIV-1/SG3Δenv backbone vector and subjected to Western blot analysis using plasma from a patient infected with HIV-1 subtype C. The *env*-minus SG3 backbone vector (SG3Δenv) was included for control. Molecular weights of major viral proteins are indicated. The truncated gp41 cleavage product (Δgp41) of the membrane anchored gp145 protein is also shown. Pseudovirions derived from optimized and wild-type *env* genes were loaded at 25 and 250 ng of p24 per lane, respectively.

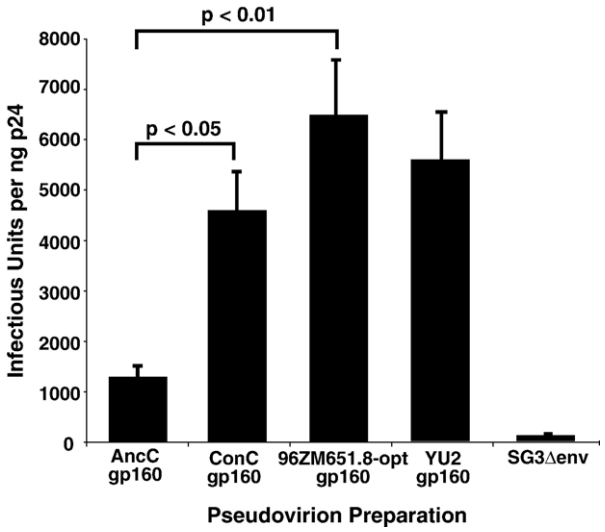

Fig. 4. Infectivity of AncC and ConC Env containing pseudovirions. Virus stocks were generated by co-transfection of centralized (AncC and ConC) and wild-type (96ZM651.8-opt) *env* genes (gp160) with the HIV-1/SG3Δenv backbone vector. Infectivity was determined in JC53-BL cells by counting the number of blue cells (infectious units, IU) per nanogram of p24 pseudovirion stock (IU/ng p24). Bars indicate standard errors (values are averaged from five independent experiments). *P* values are indicated. The infectivity of pseudovirions generated by co-transfection of the HIV-1/SG3Δenv backbone with a non-codon-optimized standard envelope (YU2 gp160) is included for control.

packaged efficiently into HIV-1 virions and are thus suitable for inclusion into virus-like particle based vaccines.

Infectivity and co-receptor usage of AncC and ConC Env containing pseudovirions

To determine whether AncC and ConC envelope glycoproteins were capable of mediating fusion and entry into appropriate target cells, purified pseudovirion preparations were analyzed for infectivity on JC53-BL cells (Derdeyn et al., 2000; Platt et al., 1998; Wei et al., 2002). JC53-BL cells express high levels of CD4, CCR5 and CXCR4 receptor molecules and are stably transfected with beta-galactosidase and luciferase reporter cassettes under the control of the HIV-1 long terminal repeat (LTR). These cells can thus be used to determine the infectious titer of pseudovirion preparations by determining luciferase activity or by staining cultures for beta-galactosidase expression and counting the number of blue cells. As shown in Fig. 4, both AncC and ConC Env glycoproteins conferred infectivity to HIV-1/SG3Δenv when complemented *in trans*; however, in five independent experiments, the relative infectivity of the AncC Env containing particles was approximately 4-fold lower than that of ConC Env containing pseudovirions and approximately 6-fold lower than that of wild-type 96ZM651.8 Env containing pseudovirions. These differences were statistically significant.

Using antagonists of the CXCR4 (AMD3100) and CCR5 (TAK779) co-receptors, the JC53-BL cell assay was also used to determine the co-receptor usage of the AncC and ConC *env* gene products. As shown in Fig. 5, both centralized glycoproteins required CCR5 as the co-receptor for entry (pseudovirions containing the CXCR4 tropic NL4.3 Env and the CCR5-tropic YU2 Env are shown for control). Thus, the centralized subtype C envelope glycoproteins resemble primary subtype C Envs in their co-receptor preference.

Sensitivity of AncC and ConC Env containing pseudovirions to neutralization by plasma from individuals chronically infected with HIV-1 subtype C

To determine whether AncC and ConC envelope glycoproteins retained neutralizing epitopes also found in contemporary subtype C viruses, we tested pseudovirion preparations for

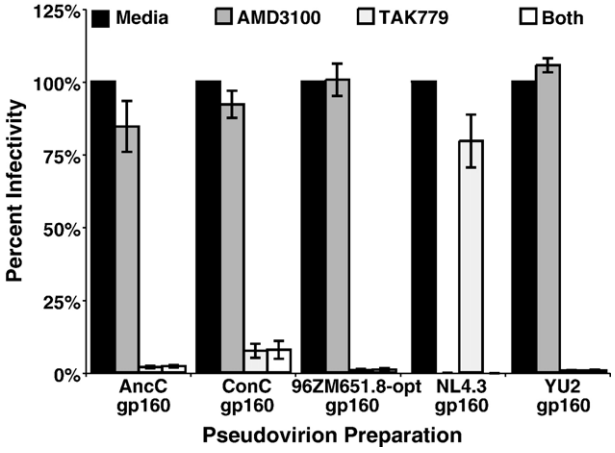

Fig. 5. Co-receptor usage of AncC and ConC Env containing pseudovirions. JC53-BL cells were pretreated with AMD3100 (inhibitor of CXCR4), TAK779 (inhibitor of CCR5), both or neither (media) before being infected by AncC, ConC, 96ZM651.8-opt, NL4.3 and YU2 Env containing pseudovirions. Virus infectivity, as a percentage of the untreated control, is plotted on the vertical axis.

sensitivity to neutralization by plasma from nine individuals chronically infected with subtype C strains. The results are summarized in Fig. 6. Interestingly, AncC and ConC Envs were equally sensitive to neutralization by patient plasma, although IC_{50} titers varied widely from 1:170 to 1:5450. Moreover, these titers were very similar to those obtained for 96ZM651.8 Env pseudovirions, indicating that both centralized and contemporary subtype C envelope glycoproteins contain neutralizing epitopes that are accessible to patient antibodies on the surface of virus particles. Thus, the overall structure and function of centralized Envs appear to resemble those of contemporary subtype C strains.

Humoral immune responses in AncC and ConC env DNA immunized guinea pigs

To examine whether centralized subtype C immunogens were capable of eliciting humoral immune responses in a small animal model, we immunized guinea pigs (three animals per group) three times at three-week intervals with 400 μg of AncC and ConC *env* DNA. Plasmids containing the codon-optimized 96ZM651.8 *env* gene as well as an empty vector were tested in parallel as positive and negative controls. Two weeks following the third immunization, sera were collected from each animal and assayed for the presence of binding antibodies to purified 96ZM651.8 (Fig. 7) and ConC (data not shown) gp120 glycoprotein using an ELISA assay. All DNA vaccines elicited *env*-specific binding antibodies, which were measurable after the second injection, but reached endpoint titers of up to 1:500,000 after the third immunization. Moreover, centralized *env* vaccines elicited binding antibody responses that were slightly higher than those induced by the contemporary control, but these differences were not statistically significant.

We next asked whether antibodies in sera from guinea pigs immunized with the AncC and ConC DNA vaccines were

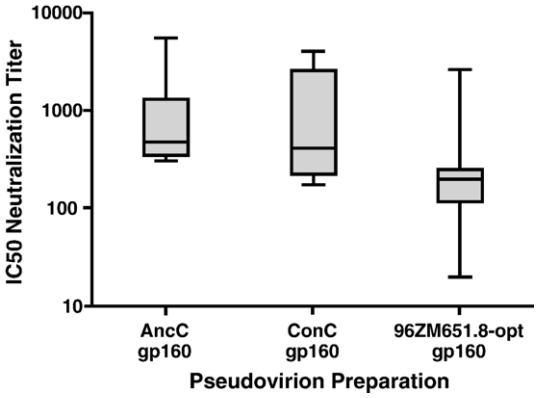

Fig. 6. Sensitivity of AncC and ConC Env containing pseudovirions to neutralization by patient plasma. Plasma from nine HIV-1 subtype C infected individuals were tested for their ability to neutralize AncC, ConC and 96ZM651.8-opt Env containing pseudovirions. Neutralization was scored as the plasma dilution required to reduce virus infectivity by 50% (IC_{50}). Vertical boxes represent the 25th to 75th percentiles of the IC_{50} values, the line in the box the median and the lines emerging from the box the highest and lowest serum dilutions observed for the group, respectively.

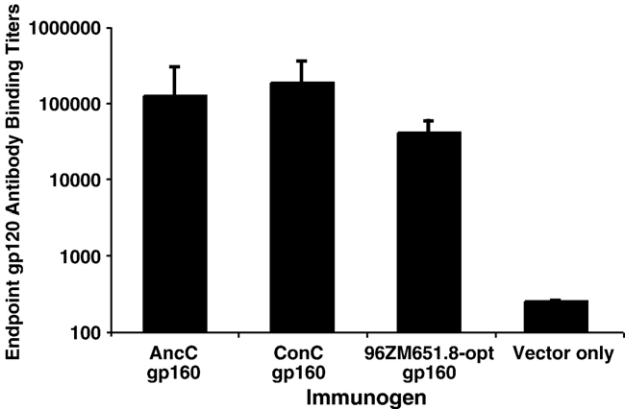

Fig. 7. Humoral immune responses in AncC and ConC *env* DNA vaccinated guinea pigs. Female Hartley guinea pigs (three per group) were vaccinated three times at three-week intervals with 400 μg of AncC, ConC, 96ZM651.8-opt and empty vector DNA. Animals were bled two weeks following the third vaccination and assayed for the presence of binding antibodies to 96ZM651.8 gp120 protein. Sera were serially diluted and the last dilution giving absorbance values greater than twice the OD value of the negative control was identified as the endpoint titer. Vertical boxes indicate the mean endpoint titer ±SD for each of the groups indicated.

capable of binding to a greater number of contemporary subtype C envelopes than antibodies in sera from animals immunized with the wild-type control. Fourteen expression plasmids containing primary (non-codon-optimized) *env* genes of divergent subtype C viruses were transfected into 293T cells, cell lysates harvested and tested for reactivity with individual guinea pig sera from each immunization group by Western blot analysis (Fig. 8A). Codon-optimized AncC and ConC *env* genes as well as the wild-type 96ZM651.8-opt *env* were included for control. This analysis revealed that sera from guinea pigs immunized with the centralized *env* vaccines were more cross-reactive than sera from animals immunized with the 96ZM651.8 wild-type control; however, the observed differences were only minor. As shown in Fig. 8A, AncC and ConC immunized animals recognized up to nine of the fourteen subtype C envelopes (the most cross-reactive serum of each group is shown). In contrast, one of three 96ZM651.8 immunized guinea pigs recognized seven (Fig. 8A), whereas the other two reacted with less than five primary subtype C envelopes. Fig. 8B shows that the selected primary envelopes are representative of the full spectrum of subtype C diversity. Thus, both centralized *env* genes induced binding antibodies that recognized a slightly greater number of linear Env epitopes than those induced by a wild-type subtype C *env* vaccine.

Finally, sera from immunized guinea pigs were tested individually for the presence of neutralizing antibodies. This was done in a single round neutralization assay using pseudovirions containing the cognate AncC, ConC and 96ZM651.8-opt Env proteins, as well as two neutralization sensitive envelopes representing subtypes B (SF162) and C (TV-1), respectively (Li et al., 2005). Each serum was analyzed at a 1:10 dilution and its neutralization activity determined relative to the baseline activity of the pre-immune serum from the same animal (Grundner et al., 2005). Sera that

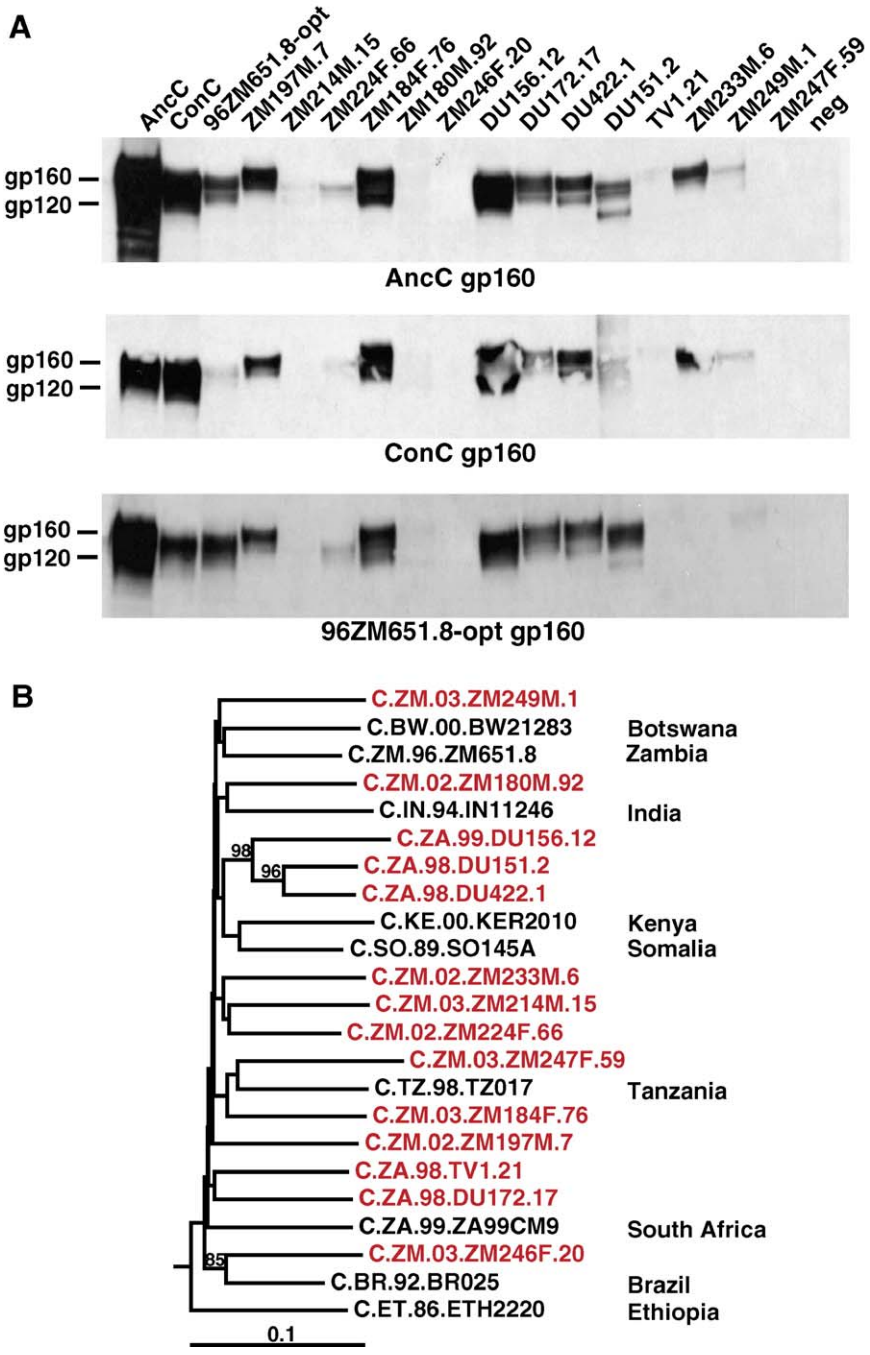

Fig. 8. Cross-reactivity of AncC and ConC induced Env binding antibodies. (A) Sera from guinea pigs vaccinated with AncC, ConC or 96ZM651.8-opt *env* DNA were tested for cross-reactivity with Env glycoproteins from divergent subtype C strains (as shown in B). Western blot strips containing equal amounts of *env* transfected 293T cell lysates were tested. The most cross-reactive serum per immunization group is shown. Expressed glycoproteins (gp160 and gp120) are indicated. (B) Phylogenetic relationship of divergent subtype C *env* sequences. Sequences used for Env protein expression (as shown in A) are highlighted in red; reference strains from the database (and their country of origin) are shown in black. The tree was inferred by the neighbor joining method and rooted using the subtype B strain YU2;

reduced viral infectivity by less than 50% were considered neutralizing antibody negative. Despite high titer cross-reactive binding antibodies, only one ConC immunized guinea pig serum neutralized the homologous ConC Env, and then only by 51% (not shown). All other eight sera failed to neutralize both their autologous and the other two heterologous envelope glycoproteins. Sera from one AncC and two ConC immunized animals exhibited weak neutralizing activity against SF162, but in each case the observed reduction in viral infectivity was less than 70%. No neutralizing activity was observed against the second isolate, TV-1. Thus, despite the fact that AncC, ConC and 96ZM651.8 envelopes were quite sensitive to neutralization by patient plasma (Fig. 6), they failed to elicit appreciable homologous and heterologous neutralizing antibodies in guinea pigs when delivered in the form of a DNA vaccine.

Cellular immune responses in AncC and ConC env DNA immunized mice

BALB/c mice (four animals per group) were immunized intramuscularly four times at three-week intervals with 50 μg of AncC, ConC, 96ZM651.8-opt *env* (and vector control) plasmid DNA. Two weeks after the last DNA immunization, all mice were sacrificed, their splenocytes isolated and tested in a gamma-interferon (INF-γ) ELISpot assay for the number of spot forming cells (SFCs). Env peptides (15-mers overlapping by 11) of the cognate ConC Env as well as a wild-type subtype C (Chn19) Env control (20-mers overlapping by 10) (Chen et al., 2000) were used to stimulate T cell responses. All *env* expressing DNA vaccines elicited T cell responses, whereas splenocytes of mice immunized with the empty vector control developed no appreciable Env-specific SFC responses (Fig. 9). When tested with ConC overlapping peptides, 841 ± 102 and 785 ± 215 SFCs were detected in splenocytes of AncC and ConC *env* immunized mice, respectively. 96ZM651.8 immunized mice had slightly lower responses (636 ± 99 SFCs), but this difference was not statistically significant. As expected, responses to Chn19 wild-type peptides were lower in all groups.

Discussion

In this study, we compared the function, antigenicity and immunogenicity of reconstructed subtype C ancestral and consensus *env* gene products. A central question regarding the use of such immunogens is whether the expressed glycoproteins are functional and whether the algorithms used for their reconstruction affect their antigenicity and immunogenicity. An ancestral sequence is an attempt to re-create the sequence of a parental strain for a particular lineage. However, this reconstruction is dependent on the particular phylogenetic model used, tends to generate sequences artificially enriched for adenine (A), and does not include recently fixed escape mutations (Gaschen et al., 2002). Consensus sequences tend to represent fixed escape mutations but have the potential of linking polymorphisms not found in natural infections (Doria-Rose et al., 2005). Finally, both ancestral and consensus sequences are vulnerable to sampling bias. Thus, both consensus and ancestral sequences represent only imperfect approximations of the evolutionary history of the virus. This is particularly true in hypervariable regions, which evolve by insertion and deletion. As a consequence, consensus or ancestral genes constructed for any one HIV-1 group or subtype have to be tested individually to examine their function and utility as immunogens.

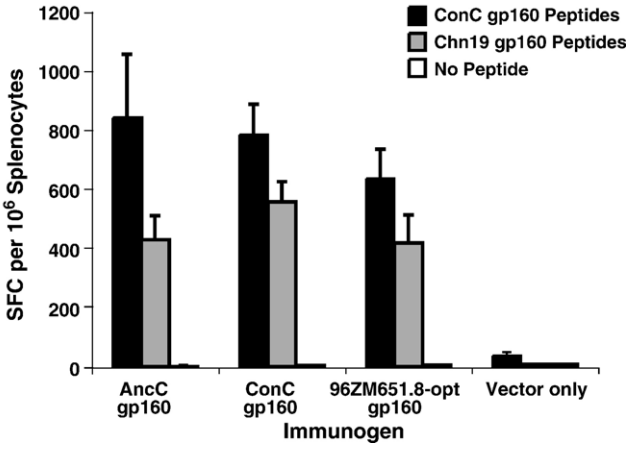

Fig. 9. T cell immune responses in AncC and ConC *env* DNA vaccinated mice. Balb/c mice (four per group) were vaccinated four times at three-week intervals with 50 μg of AncC, ConC, 96ZM651.8-opt or empty vector DNA. Two weeks following the last vaccination, splenocytes were isolated from individual mice and stimulated with consensus and wild-type (Chn19) subtype C overlapping Env (or no) peptide pools. Total responses are expressed as spot forming cells (SFC) per million splenocytes. The values for each column are the mean ± SEM of IFN-γ SFCs.

We characterized ancestral and consensus *env* genes of HIV-1 subtype C and show that both centralized constructs express functional envelope glycoproteins. AncC and ConC Envs incorporated efficiently into virus particles (Fig. 3), mediated virus entry via the CCR5 co-receptor (Figs. 4 and 5), and were sensitive to neutralization by plasma from individuals with chronic HIV-1 subtype C infection (Fig. 6). Moreover, AncC and ConC *env* expressing DNA vaccines elicited humoral and cellular immune responses in guinea pigs and mice, respectively, indicating that they were immunogenic in vivo (Figs. 7 and 9). Thus, both ancestral and consensus reconstruction approaches yielded biologically active subtype C Env proteins. Using a similar approach, Lian and colleagues recently constructed a consensus *env* gene from published subtype C sequences from Botswana (Lian et al., 2005). However, this centralized Env failed to bind CD4 and to mediate fusion, indicating that not all re-constructed consensus sequences express functional gene products. In our study, we found no evidence of functional impairment of the consensus *env* glycoprotein. ConC Env containing pseudovirion preparations were significantly more infectious than pseudovirions containing the AncC Env (Fig. 4), and ConC *env* DNA vaccine induced higher titer binding antibodies (Fig. 7). Moreover, ConC and AncC immunized guinea pig sera recognized the same number of divergent Env proteins on immunoblots (Fig. 8). Thus, at least for the subtype C *env* consensus gene, previously noted theoretical concerns (Doria-Rose et al., 2005) and functional limitations (Lian et al., 2005) were not substantiated.

A major goal of AIDS vaccine development is the elicitation of broadly cross-reactive neutralizing antibodies against HIV-1 envelope glycoproteins. Fig. 6 shows that both the AncC and ConC *env* gene products retained neutralizing epitopes present in contemporary subtype C viruses, perhaps because they contain very short hypervariable loops that minimize camouflage and glycosylation (Srivastava et al., 2003). However, neither DNA immunogen (nor the wild-type control) was capable of eliciting appreciable neutralizing antibodies against their homologous as well as heterologous Env glycoproteins. Given that non-modified (full-length) *env* DNA vaccines rarely elicit neutralizing antibodies, these results are not unexpected. Rasmussen et al. (2006) immunized mice with a codon-optimized *env* gene from a contemporary subtype C isolate and did not detect neutralizing antibodies in pooled sera against the

homologous strain. Similarly, no appreciable neutralizing antibody titers against SF162 were observed in rabbits immunized five times with the full-length AN1 Env B gene (Doria-Rose et al., 2005). Gao et al. (2005) were able to detect neutralizing antibodies to the subtype B strain SF162 in CON6 immunized guinea pigs; however, in this study, the centralized immunogen was delivered as a secreted Env protein (gp120 or gp140ΔCF) rather than plasmid DNA. This was also the case for the group M consensus CONS Env which induced high titer cross-clade neutralizing antibody responses when delivered as gp140ΔCFI glycoprotein oligomers (Liao et al., in press). Thus, additional modes of delivery will need to be tested to examine the full neutralizing antibody induction potential of AncC and ConC Env immunogens.

In addition to suboptimal vaccine delivery, there may be an inherent, as yet unexplained inability of subtype C *env* immunogens to induce neutralizing antibodies. Seaman et al. (2005) found in primates that among contemporary subtype A, B and C *env* immunogens in a DNA prime/recombinant adenovirus (rAd) boost regimen, wild-type subtype C *env* was by far the weakest inducer of neutralizing antibodies, and this was also observed when these same *env* immunogens were delivered in the form of gp140 protein oligomers and studied in guinea pigs (Liao et al., in press). Also, subtype B, but not subtype C, V3 peptides induced neutralizing antibodies against Tier 1 subtype B and C primary isolates, again suggesting subtype-C-specific differences in V3 loop presentation (Haynes et al., 2006; B. Haynes and B. T. K Korber, unpublished). Atomic level understanding of susceptible Env epitopes and their interaction with neutralizing antibodies will likely be necessary to design novel immunogens that are capable of inducing cross-reactive neutralizing antibodies (Burton et al., 2004; Huang et al., 2005; Pancera et al., 2005). In this regard, a better understanding of the structural basis of subtype-C-specific neutralizing antibody responses will be important. Once this is accomplished, the theoretical advantages of centralized envelope glycoprotein backbones will have to be revisited.

The primary rationale of ancestral and consensus vaccines is the expectation that centralized immunogens induce broader, more cross-reactive T cell responses (Gaschen et al., 2002). We did not observe differences in the T cell response of ancestral, consensus and wild-type subtype C Env immunized mice when peptide pools were used for stimulation (Fig. 9); however, determining the breadth of T cell responses was not the purpose of the study. The mouse studies were intended to document basic in vivo immunogenicity of AncC and ConC *env* DNA vaccines. As shown in Fig. 9, this was accomplished because the observed T cell responses were at least as high as those elicited by the wild-type *env* control. Inbred mice are not an appropriate animal model to compare the breadth and quality of CD4+ and CD8+ T cell responses to centralized immunogens. This will have to be done in primates or humans with diverse MHC backgrounds.

Both ancestral and consensus *env* genes are a reflection of the particular HIV-1 sequences that were used for their reconstruction and consequently subject to change as additional sequences become available. The AncC and ConC *env* genes described here are representative of envelopes derived from chronically infected individuals. It is possible that centralized gene products derived from acute HIV-1 infection sequences would be of greater relevance to AIDS vaccine design. This is because HIV-1 strains from recently infected individuals may share biological properties that are distinct from those of viruses dominating in chronic infections. Studies are underway to compile an acute HIV-1 infection database and to generate such second generation centralized immunogens for comparative analyses.

In summary, we have shown here that ancestral and consensus subtype C envelope genes express functional envelope glycoproteins that are immunogenic in laboratory animals and induce humoral and cellular immune responses comparable to those of a contemporary HIV-1 subtype C DNA vaccine. As new avenues of vaccine design and delivery are uncovered, their application to centralized gene-derived immunogens will determine the full extent of B and T cell responses that can be elicited by these synthetic gene products. The results described here will serve as a baseline for future studies of modified and improved centralized immunogens. The codon-optimized AncC and ConC *env* genes have been submitted to the National institutes of Health Research and Reference Program (Rockville, MD) and are available to interested investigators.

Materials and methods

Gene synthesis

HIV-1 subtype C ancestral and consensus *env* gene sequences were codon-usage optimized as described (Gao et al., 2003) and are available at GenBank under accession numbers DQ401076 and DQ401075, respectively. The genes were cloned in pcDNA3.1.

Env gene expression

293T cells (5×10^5 cells/well) were seeded in 6-well plates 12–14 h prior to transfection. Monolayers were transfected with each *env* gene using FuGene 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN). Forty-eight hours post-transfection, cells were washed with Tris–saline (pH 7.5, 50 mM Tris–Cl and 150 mM NaCl) and resuspended in 150 μl of lysing buffer (20 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 2 mM EDTA, 2 μg/ml aprotinin, 1 mg/ml pepstatin A, 2 mg/ml leupeptin and 1 mM PMSF). The amount of total protein was quantified using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). For each expressed gene, 5 μg of cell lysate was boiled for 5 min in the presence of reducing buffer and separated on a 4–20% SDS–polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to a PVDF nitrocellulose membrane (GE Healthcare Life Sciences, Piscataway, NJ) by electroblotting and incubated with blocking buffer (5% nonfat dry milk in PBS) for 1 h. Western blots were probed with

plasma from an HIV-1 (subtype C)-infected individual and developed with HRP-labeled species-specific antibodies (SouthernBiotech, Birmingham, AL) using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Life Sciences, Piscataway, NJ).

Generation of pseudovirions

To test whether subtype C ancestral and consensus glycoproteins were capable of incorporating into virus particles, we transfected each gene with an *env*-minus HIV-1 backbone vector (pSG3Δenv). Plasmids were co-transfected into a 100-mm-diameter Petri dish containing a 60% confluent layer of 293T cells in complete Dulbecco's modified Eagle media (DMEM) using FuGene 6 (Roche Applied Science, Indianapolis, IN) at a 3:1 ratio as specified by the manufacturer. Forty-eight hours post-transfection, virus-containing supernatant was collected, clarified by low-speed centrifugation, passed through a 0.2-μm filter and pelleted through a 20% sucrose cushion. Pelleted virus was normalized by p24 for SDS–PAGE and Western blot analysis.

Infectivity and co-receptor usage determination

Infectivity assays were performed as described (Derdeyn et al., 2000). Briefly, JC53-BL cells were seeded in 24-well plates at 50,000 cells/well in DMEM supplemented with 10% FBS, L-glutamine and Pen/Strep and incubated overnight. Pseudotyped virus stocks containing AncC, ConC or 96ZM651.8 Env were added to each well in the presence of DEAE-Dextran hydrochloride (80 μg/ml) (Sigma-Aldrich, St. Louis, MO) in a final volume of 250 μl. Following a 48-h incubation, plates were washed, stained and the number of blue cells counted to determine the infectious virus titer.

A modification of the infectivity assay was used to determine co-receptor usage using antagonists to CXCR4 (AMD3100) and CCR5 (TAK-779) co-receptors (Zhang et al., 2000; Spenlehauer et al., 2001). Briefly, JC53-BL cells that had been seeded overnight were treated for 1 h with AMD3100 (1.2 μM/well), TAK-779 (10 μM/well) or a combination of these chemokine receptor blocking agents. Two thousand infectious units of pseudotyped virions were added to each well in the presence of 80 μg/ml DEAE-Dextran hydrochloride and incubated at 37 °C. Following a two-day incubation, supernatant was removed from the wells, and cells were lysed using a luciferase assay system kit (Promega, Madison, WI). The light intensity of each cell lysate was measured on a Tropix luminometer using Tropix WinGlow version 1.24 software. A reduction in infectivity in the presence of a specific inhibitor reflects a requirement of the targeted co-receptor for entry.

Neutralization assay

Pseudovirions containing the AncC, ConC or 96ZM651.8 envelope glycoproteins were probed for the presence of neutralizing epitopes using a sensitive, single round infectivity assay (Li et al., 2005). Briefly, JC53-BL cells were seeded at 8000 cells/well in a 96-well plate in 10% DMEM media overnight at 37 °C with 5% CO_2. Plasma from HIV-1 subtype C-infected individuals were serially diluted and incubated with 2000 units of infectious virus per well for 1 h at 37 °C. Pre-incubated virus/plasma dilutions were added to the cells in the presence of DEAE-Dextran and incubated for 2 days at 37 °C. Control wells containing pseudovirions that had not been pre-incubated with antibody were included for each virus tested. Additionally, cell-only wells were included on each plate as a measure of background. Cells were lysed and analyzed for luciferase production using the Luciferase Assay System (Promega, Madison, WI). Neutralization was measured as the percent reduction of viral infectivity in comparison to control wells infected with virus alone. The 50% inhibitory concentration (IC_{50}) was obtained by non-linear regression using Prism 4 (GraphPad Software, San Diego, CA).

Single round neutralization assays were also conducted to examine sera from immunized guinea pigs for the presence of neutralizing antibodies. For these assays, guinea pig sera were incubated at a 1:10 dilution with pseudovirions containing the AncC, ConC and 96ZM651.8-opt Env proteins, as well as two neutralization sensitive envelopes representing subtypes B (SF162) and C (TV-1), respectively (Li et al., 2005). Neutralization was measured as the percent reduction of viral infectivity compared to control wells where virus was incubated with the same animal's pre-immune serum (Grundner et al., 2005). Sera that reduced viral infectivity by less than 50% were considered neutralizing antibody negative.

Guinea pig immunization and serum collection

Guinea pigs were housed in the University of Alabama at Birmingham (UAB) Animal Facility according to Accreditation of Laboratory Animal Care (AALAC) guidelines. Animals use protocols were approved by the UAB Institutional Animal Care and Use Committee. Female Hartley guinea pigs (Harlan Sprague, Indianapolis, IN) (n = 3/group) were immunized intramuscularly three times at 3-week intervals with 400 μg plasmid DNA. Two weeks following the last immunization, 5 ml of blood was collected from each animal via the cranial vena cava. Sera were obtained by tabletop centrifugation using Becton Dickinson SST Tubes (BD, Franklin Lakes, NJ) as directed by the manufacturer. Samples were stored at −20 °C until analysis.

Endpoint binding titer ELISA

Guinea pig sera were tested for binding antibodies to subtype C consensus and 96ZM651.8 recombinant gp120 glycoprotein by enzyme linked immunosorbent assay (ELISA). Recombinant 96ZM651.8 gp120 was obtained from the NIH AIDS Research and Reference Reagent Program (Bethesda, MD). Subtype C consensus recombinant gp120 was produced by transfecting 293T with the corresponding plasmid, harvesting culture supernatant 72 h post-transfection, clarifying the supernatant by tabletop centrifugation and passing through a 0.2-μM filter. Recombinant gp120 was purified using Galanthus Nivalis

Lectin (GNL) (Vector Laboratories, Burlingame, CA), eluted with 500 mM alpha-methyl mannoside (Vector Laboratories, Burlingame, CA), dialyzed overnight in PBS and quantified by BCA Protein Assay (Pierce Biotechnology, Rockford, IL). Microtiter plates were coated with recombinant gp120 (0.5 μg/ml in PBS), washed and blocked with 200 μl/well 5% nonfat milk in PBS-T. Serial five-fold dilutions were made of each guinea pig serum, added to individual wells and set to incubate for 1 h at 37 °C. Following a wash, 100 μl of HRP-conjugated goat anti-guinea pig antibody (ICN Pharmaceuticals, Costa Mesa, CA) diluted to 1:50,000 in blocking buffer was added to each well. After an additional 1-hour incubation at 37 °C, 100 μl of liquid TMB (3,3′,5,5′-tetramethylbenzidine) was added to each well. Reactions were stopped by the addition of 100 μl of 4N sulfuric acid. Absorbances were read at 405 nm on an MRX Microplate reader (DYNEX Technologies, West Sussex, UK). Endpoint titers were determined as the serum titer at which the absorbance value was 2× the mean OD of the negative serum control.

Immunization of mice and splenocyte isolation

Female BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Raleigh, NC) and housed in the Duke University Animal Facility under AALAC guidelines with animal use protocols approved by the Duke University Animal Use and Care Committee. Four mice per group were immunized intramuscularly in the quadriceps with AncC, ConC or 96ZM651.8-opt gp160 or empty vector plasmid DNA (50 μg) four times at 3-week intervals. Two weeks after the 4th DNA immunization, mice were euthanized and spleens were collected. Spleens from individual mice were minced and forced through a 70-μm Nylon cell strainer (BD Labware, Franklin Lakes, NJ). Splenocytes were then washed, treated with ACK lysis buffer and resuspended in HEPES-buffered complete RPMI medium with 10% fetal bovine serum, gentamicin (50 μg/ml), 10 mM non-essential amino acids and 0.053 mM β-mercaptoethanol.

Enzyme linked immune spot (ELISpot) assay

Overlapping Env peptides of ConC (subtype C consensus; 211 peptides, 15-mers overlapping by 10 amino acids) and Chn19 (subtype C; 87 peptides, 20-mers overlapping by 10 amino acids) were obtained from the NIH AIDS Research and Reference Reagent Program (Bethesda, MD) and used to stimulate mouse splenocytes. Single-cell suspensions of splenocytes were plated in 96-well polyvinylidene difluoride-backed plates (MultiScreen-IP, Millipore, Billerica, MA) coated with 50 μl of anti-mouse IFN-γ Mab AN18 (5 μg/ml; Mabtech, Stockholm, Sweden) overnight at 4 °C. The plates were blocked with HEPES-buffered complete RPMI medium at 37 °C for 2-h. Equal volumes (50 μl) of each peptide pool (one pool of 87 peptides for Chn19; 211 peptides for ConC divided into two pools) and splenocytes (10^7 cells/ml) were added to the wells in duplicate. Wells containing cells and complete RPMI medium served as negative controls, whereas wells containing cells and concanavalin A (5 μg/ml) (Sigma, St. Louis, MO) served as positive controls. Plates were incubated overnight (14–16 h) at 37 °C with 5% CO_2. After the plates were washed 6 times with phosphate buffered saline (PBS), 50 μl of 1:1000-diluted biotinylated anti-mouse IFN-γ mAb (Mabtech, Stockholm, Sweden) was added to each well. Plates were then incubated at room temperature for 2-h, washed 3 times with PBS, and 50 μl of streptavidin–alkaline phosphatase conjugate (1:1000 dilution; Mabtech, Stockholm, Sweden) was added to each well. After incubation for 1 h at room temperature, plates were washed 5 times with PBS-T, and 100 μl of BCIP/NBT (Plus) alkaline phosphatase substrate (Moss, Pasadena, MD) was added to each well. Following an incubation for 10 min at room temperature and a final wash with water, plates were air-dried. Spots were counted using an automated ELISpot plate reader (Immunospot counting system, CTL Analyzers, Cleveland, OH) and expressed as spot-forming cells (SFC) per 10^6 splenocytes. Responses were considered positive if the number of spots was four times greater than the negative control and at least 50 SFC/10^6 cells/well.

Phylogenetic analyses

Deduced Env protein sequences of HIV-1 subtype C strains used to evaluate binding antibody cross-reactivity were aligned with geographically diverse subtype C Env sequences from the database using CLUSTAL W (Thompson et al., 1994). The tree was inferred from a gap stripped alignment using the neighbor joining method as implemented in CLUSTAL W using Kimura's correction. Robustness of the branching order was evaluated using the bootstrap method (1000 replicates) as implemented in CLUSTAL W.

Statistical analyses

Analysis of variance (ANOVA) was used to compare infectivity, neutralization sensitivity and ELISPOT means across envelope comparison groups. Statistical tests were performed using Prism 4 (GraphPad Software, San Diego, CA). The F ratio was used to assess significance at the 5% level. If the F ratio was significant, pairwise comparisons using a modification of the t test were computed (Tukey–Kramer test). A result was considered significant if the P value <0.05 (5% level of significance). Two-tailed P values are cited.

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