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Development of a Recombinant Yellow Fever Vector Expressing a HIV Clade C Founder Envelope gp120

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Abstract

Development of a HIV-1 vaccine is a major global priority. The yellow fever virus (YFV) attenuated vaccine 17D is among the most effective of currently used vaccines. However, the stability of the YFV17D vector when carrying non-flavivirus genes has been problematic. We have constructed and expressed HIV-1 Env in YFV17D with either single transmembrane (STM) or double transmembrane (DTM) YFV E protein domains for the development of anti-HIV antibodies. Here we describe modifications of the YFV17D vector such that HIV-1 Env gp120 is expressed in up to 5 passages in Vero cells. Immunization with recombinant YFV17D vector prime followed by HIV-1 CH505 TF gp120 protein boosts were able to induce neutralizing antibodies for a HIV-1 tier 1 isolate in mice. This modified YFV vector may be a starting point for constructing HIV-1 vaccine candidate priming vectors.

Keywords

HIV; HIV envelope; YFV17D; Antibodies; Neutralization; Transmitter/Founder

1. Introduction

The yellow fever virus (YFV) is the type member of the *Flavivirus* genus, a group of RNA viruses in the family *Flaviviridae* (Lindenbach and Rice, 2001). The YFV vaccine strain 17D

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live attenuated vaccine is a highly immunogenic and effective vaccine for protection against wild type strains of YFV (Monath, 2005; Pulendran, 2009). YFV17D was derived from the wild type Asibi strain and modified by 31 amino acid mutations with 12 amino acid changes in the envelope p protein (Galler et al., 1998; Hahn et al., 1987). Key features of the YFV17D vaccine vector are *in vivo* replication, induction of robust memory immune responses, a genome capable of tolerating insertion of foreign genes, and expression of foreign insert genes in forms that are both immunogenic and protective (Bonaldo, Sequeira, and Galler, 2014). The YFV17D vaccine has been used safely and induces rapid appearance of neutralizing antibodies in vaccinees worldwide. In large vaccine trials, 99% of 1440 adults (Monath et al., 2002) and 93% of 1107 children (Belmusto-Worn et al., 2005) developed neutralizing antibody responses (Monath, 2005).

The development of a safe and effective HIV-1 vaccine is a global priority. Anti-retroviral treatment (ART) has dramatically prolonged the lives of HIV-infected individuals, but ART is only one component of a HIV-1 treatment and preventive program (Gilks et al., 2006). If an HIV-1 vaccine could be developed, it would hasten the end of the global AIDS epidemic. One of the needs for HIV-1 vaccine development is a mode of administration of Env in a live and highly immunogenic vector (Nabel, 2001; Parks, Picker, and King, 2013). However, the studies of insert-specific antibody responses using YFV17D platforms carrying non-flavivirus genes have not been particularly successful, and many immunogenicity studies with YFV17D vectored immunogens have focused on T cell responses because inserted genes were fragmented, rather than whole genes (Bonaldo et al., 2010; Bonaldo et al., 2014; Martins et al., 2013; Martins et al., 2014). A study performed by Franco et al showed that recombinant YFV17D could induce both anti-p24 antibodies and T cell responses in mice (Franco et al., 2010).

In this report, we demonstrate the development of a recombinant modified YFV17D-based vector carrying HIV-1 transmitted/founder (TF) envelope (Env) gp120 protein. Also we generated tier 1 virus neutralizing antibodies against HIV-1 using recombinant YFV17D vector prime and a TF Env boost. Thus, we have constructed and expressed a HIV-1 transmitted/founder (TF) Env gene insert in the 17D vector that was stable for 5 passages in cell culture *in vitro*, and demonstrated YFV17D-Env vector immunogenicity in mice.

2. Materials and Methods

2.1. Plasmid construction

Plasmid pACNR-FLYF-17Dx (Bredenbeek et al., 2003) was used for generation of recombinant yellow fever viral vector expressing HIV-1 CH505 TF gp120 (Liao et al., 2013). The plasmid pENTR4 Dual Selection Vector (Invitrogen, Carlsberg, CA) was used to construct an intermediate vector to subclone HIV-1 Env genes in YFV17D. Library Efficiency DH5α Competent Cells (Invitrogen Carlsberg, CA) were used to transform vectors for generating plasmids. The resulting intermediate vector was pENTR4_YFV_Env that carried a kanamycin resistance gene and pUC origin from pENTR4_Dual vector, and, as well, contained the YF viral C-prM-E-NS1 gene from pACNR-FLYF-17Dx. For construction of pENTR4_YFV_Env, the pENTR4_Dual vector was cut by NheI and PvuII,

and pACNR-FLYF-17Dx by *NheI* and *PvuII*. The vector and insert were ligated and transformed into *E. coli* DH5 α .

For introduction of the desired cloning sites in pENTR4_YFV_Env vector, DNA fragments were generated by PCR using primers containing sequences of corresponding restriction enzyme sites. Specifically, for *BglII* and *KasI* recognition sites, DNAs were amplified by PCR using Platinum *pf*x polymerase (Invitrogen, CA) using primer A and primer B_*BglII* (for amplifying PCR AB_*BglII*). For amplification of PCR CD_*KasI*, DNAs were amplified using primer C_*KasI* and primer D. (Table 1) For construction of an intermediate vector, pENTR4_YFV_BK, we performed overlapping PCR using mixed templates of PCR AB_*BglII* and PCR CD_*KasI* in the same molar ratio as used with primer A and primer D sets. We constructed a series of intermediate vectors introducing pENTR4_YFV_AX (*AgeI*/*XhoI*), pENTR4_YFV_AK (*AgeI*/*KasI*), pENTR4_YFV_BX (*BglII*/*XhoI*), and pENTR4_YFV_BK (*BglII*/*KasI*).

To clone the HIV-1 CH505 TF gp120 gene into pENTR4_YFV_BX, DNA fragments were generated by PCR using primers containing sequences of restriction enzyme sites CH505TF_*BglII* and CH505TF_*XhoI*, then subcloned into pENTR4_YFV_BX resulting in the construct pENTR4_YFV_CH505TF_STM. For construction of pENTR4_YFV_CH505_DTM, PCR reaction #1 was performed using primers DTM_1A and DTM_1B and pENTR4_YFV_BX served as a template (Table 1). PCR reaction #2 generated CH505 TF gp120 gene using DTM_CH505_2A and DTM_2B primer set while pENTR4_YFV_CH505_STM served as a template. In order to generate pENTR_YFV_CH505_DTM, we performed overlapping PCR reaction #3 using mixed templates of PCR reaction #1 and PCR reaction #2 in the same molar ratio, with the DTM_1A and DTM_2B primer sets. All *XhoI* sites in pENTR_YFV_CH505_DTM and pENTR_YFV_CH505_STM were replaced to *KasI* using YFV_CH505DTM_*KasI* insertion primers (Table 1). We used *NsiI* and *MluI* restriction endonuclease sites of pENTR_YFV_CH505_DTM for directional cloning back into pACNR-FLYF-17Dx; thus the resulting vector was pFLYF17Dx_CH505_DTM (rYFV DTM) (Figure 1). Another vector pFLYF17Dx_CH505_STM (rYFV STM) was generated with the same method (Figure 1). All final rYFV plasmids were confirmed by DNA sequencing using sequencing primers shown in Table 2.

2.2. Cell culture, *in vitro* transcription, transfection and viral stocks

BHK21 [C-13], Vero E6, and SW-13 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 5 % fetal bovine serum (FBS). Full-length pACNR-FLYF-17Dx (WT), pFLYF17Dx_CH505_DTM, and pFLYF17Dx_CH505_STM vectors were linearized by *XhoI* and capped transcripts were generated using SP6 RNA polymerase (Life Technologies, Cat # AM2071, Carlsbad, CA). RNA was purified using phenol/chloroform/isoamyl alcohol extraction and precipitated by ethanol. The integrity of *in vitro* transcribed RNA was monitored by running RNA gel electrophoresis under denaturing conditions in 2.2M formaldehyde using the MOPS buffer system (Sambrook, Fritsch, and Maniatis, 1989). Full-length RNA transcripts YFV17D (wild type), rYFV DTM, and rYFV STM were transfected into BHK21 or Vero E6 cells via

electroporation (BioRad Gene Pulser Xcell Electroporation Systems, Hercules CA). Virus stocks were obtained by harvesting the supernatant of cells transfected with *in vitro*-transcribed RNA of linearized plasmid DNA of wild-type or recombinant YFV17D at 72 hours post-transfection.

Virus stocks were titrated by plaque assays on SW13 cells as described (Barba-Spaeth et al., 2005). Serial 10-fold dilutions were used to infect monolayers of SW13 cells (5 h at 37°C). After infection, cells were washed with PBS and overlaid with 0.6% agarose-containing medium, and plaques developed at 37°C 4 days. Plaques were fixed in 7% formaldehyde for 1 h and stained with crystal violet (1.25% in 20% ethanol).

2.3. Expression of HIV-1 CH505 TF envelope in rYFV vectors

Purified RNA was transfected into BHK21 cells via electroporation, 3 µg per 200 µl of 10⁶ BHK21 cells. Fourteen µl of clarified supernatant was fractionated on 4–12% SDS-PAGE and blotted onto nitrocellulose filters (Invitrogen, CA). HIV Env bands were detected by monoclonal antibody (mAb) 16H3 (Gao et al., 2009) followed by goat anti-mouse IgG-HRP (Sigma, Cat# A8924-.5ML). Env bands were developed with Pierce ECL Western Blotting Substrate (Thermo Scientific, Cat# 32106). Yellow fever viral envelopes were fractionized in SDS-PAGE and detected by Dengue mAb 4G2 (Henchal et al., 1982) under non-reducing conditions and then conjugated with goat anti-mouse IgG-AP (Sigma, Cat # A3652-.5ML). Yellow fever viral envelope bands were developed with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega Cat# S3841).

2.4. Mouse immunizations

Female BALB/c mice (Charles River Laboratory, Raleigh, NC) 6 to 8 weeks of age were used for immunogenicity studies. For wild type YFV or rYFV vector, mice were immunized subcutaneous (s.c.) at the base of the tail with 10⁴ pfu of viral stocks P2. For recombinant Env protein, 20 µg of Env gp120 was injected intraperitoneally (i.p.). Viral stocks and proteins were clarified by 0.22 µm filtration before mice immunization. Mice were immunized in five different groups for prime-boost regimen: YFV17D-YFV17D (group 1), YFV17D-protein (group 2), rYFV DTM-rYFV DTM (group 3), rYFV DTM-protein (group 4), and 1× PBS-protein (control group). Then, mice were immunized with three additional protein immunizations. Wild type vector, pACNR-FLYF-17D_X (YFV17D), served as an empty vector control (Figure 2). Prior to necropsy, mice were boosted with CH505 TF gp120 proteins. There were no adjuvants used until the final protein boosts with GLA/SE (IDRI) at week 35. Injection route was intraperitoneal (i.p.) for proteins and subcutaneous (s.c.) for YFV17D and rYFV vectors. Mouse serum was collected 2 weeks after immunization. Serum antibody titers against HIV-1 gp120 were determined in a standard ELISA. The plate coated antigens for ELISA were either CH505 TF gp120 or CH505 TF 371I gp120. For isotype analysis of serum antibodies, we used goat-anti mouse IgG, IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM (Southern Biotechnology, AL) as HRP-conjugated antibodies in ELISA. Plasma samples in serial threefold dilutions starting at 1:30 to 1:5,314,410 diluted in PBS were assayed for binding to HIV-1 Env proteins. Data are reported as log area under the curve (AUC). Animals were housed under AALAC guidelines

with animal use protocols approved by the Duke University Animal Use and Care Committee and the Duke University Institutional Biosafety Committee.

2.5. Flow cytometry

Forty-two hours after transfection, BHK21 cells were harvested, washed with two to four volumes of staining buffer (1% BSA in PBS), and centrifuged (500 ×g, 5 min, 4°C). Five hundred thousand cells were resuspended in 80 µl of staining buffer and 20 µl of diluted antibodies added (5 µg per mL, 1 hour, 4°C). Cells were washed and incubated in 80 µl of staining buffer with 20 µl isotype-APC control (BD cat #555751) or 20 µl anti-Human IgG-APC (BD cat #550931) for 30–45 minutes at 4°C. Cells were washed and fixed with 250 µl of BD Cytofix Buffer (Cat #554655) for 30 minutes at 4°C, and were analyzed on a BD LSRFortessa flow cytometer.

2.6. Neutralization assay of mouse serum

Neutralizing antibody assays with TZM-bl cells were performed as previously described (Montefiori, 2005). Mice sera were tested starting at 1:20 dilutions and titrated by using serial 3-fold dilutions. Pseudotyped lentiviruses were added to the antibody dilutions at a predetermined titer to produce measurable infection and incubated for 1 h. TZM-bl cells were added and incubated for 48 hours before lysis, after which the supernatant was measured for firefly luciferase (Britelite Plus; Perkin-Elmer, Waltham, MA) activity by use of a luminometer. The data were calculated as a reduction in luminescence compared with control wells and are reported as the serum 50% inhibitory dose (ID₅₀) in dilution serum titers.

2.7. Statistical analysis

Comparisons were made between groups after the second and the final vaccination. All comparisons were made using Wilcoxon-Mann-Whitney non-parametric tests with Benjamini-Hochberg (1995) false discovery rate correction for multiple comparisons in SAS v9.4 (SAS Institute, Inc. Cary, NC).

3. Results

3.1. Expression of HIV-1 CH505 TF Env gene in recombinant YFV17D

Previous work with foreign protein expression YFV17D has generated an in-frame fusion protein between the genes that code YFV E and NS1 proteins (Bonaldo et al., 2014). Here we have expressed HIV-1 Env in YFV17D with either single transmembrane (STM) or double transmembrane (DTM) YFV E protein domains (Figure 1). In the STM design, the Env insert was directly fused to the YFV E gene-YFV transmembrane gene. In the DTM construct, we introduced one YFV transmembrane gene and 7 amino acids of NS1 at the N-terminus between YFV E gene and CH505 TF gp120 gene, and another YFV E transmembrane gene in between CH505 TF gp120 C-terminus and the YFV NS1 gene (Figure 1). Therefore, proteins generated in the rYFV DTM construct were designed to be smaller than proteins from rYFV STM because a cellular signal peptidase cleaves between YFV E and NS1 protein in the DTM vector. (Auclair, Bhanu, and Kendall, 2012; Bonaldo et al., 2014) (Figure 2A). Thus, the rYFV DTM construct generated 110 kDa CH505 TF gp120

proteins, while rYFV STM generated 150 kDa proteins due to in-frame fusion to YFV E protein (Figure 2A). We found that rYFV DTM had greater stability for expression of HIV-1 Env than rYFV STM vector over 5 passages in Vero cells (Figure 2A). While the expression level of HIV CH505 proteins in rYFV diminished from passage 2 to passage 5, the viral titers of YFV17D or rYFV were maintained at similar levels from passage 2 through passage 5 (Figure 2B). In addition, the expression of yellow fever viral envelope proteins were detected in wild-type vectors as well as recombinant yellow fever vectors under non-reducing conditions using anti-dengue mAb 4G2, known to cross-react with yellow fever viral envelop (Figure 2C) (Henchal et al., 1982).

3.2. Cell surface expression of Env following recombinant YFV17D infection of BHK21 cells

We have tested binding of HIV-1 monoclonal antibodies to surface-expressed CH505 TF gp120 expressed in rYFV DTM on BHK21 cells. We used mAbs to bind the Env CD4 binding site (VRC01 and DH522) and the CCR5 binding site (17b) in surface staining assays. Human and rhesus mAbs reacted better to rYFV DTM/BHK21 (orange lines) than YFV17D/BHK21 (light green lines) with surface-expressed HIV Env gp120 (Figure 3). A negative control antibody, palivizumab (RSV Ab), did not bind either rYFV DTM or YFV WT in transfected BHK21 cells. Gray area indicates cells reacted with isotype control and blue lines indicate non-transfected BHK21 cells.

3.3. Recombinant YFV17D-HIV-1 Env vector primed for a protein boost of anti-Env antibody

Mice were immunized with either wild-type pANCR-FLYF17Dx (YFV17D), or rYFV DTM followed by a boost with recombinant HIV-1 Env gp120. We monitored anti-HIV polyclonal antibodies against CH505 TF gp120 proteins via ELISA. Table 3 shows the immunization protocol, and Figure 4 shows the binding data of mouse sera to CH505 TF gp120 protein. Group 1 and group 2 were primed with YFV17D (wild type), 1.0×10^4 pfu per mouse and boosted with YFV17D (group 1) or protein (group 2, HIV Env CH505 TF gp120, 20 μ g per mouse). Similarly, group 3 and group 4 were primed and boosted with rYFV DTM $\times 2$ or rYFV DTM–protein, respectively. rYFV DTM was the final construct (pFLYF17Dx_CH505_DTM) and the immunization route was subcutaneous (s.c.) with 1.0×10^4 pfu per mouse. Finally, we boosted all mice with CH505 TF gp120 protein at weeks 10 and 34 without adjuvant (to allow for observation of effects of the prime), and at week 35 with the TLR4-agonist GLA/SE (Arias et al., 2012) (Figure 4A). Wild-type YFV17D or rYFV DTM prime and gp120 protein boost showed that rYFV DTM prime enhanced the level of gp120 antibody boosted by Env protein (group 2 vs group 4), ($p=0.03$) (Figure 4B). There were no detectable antibody responses after 2 immunizations with rYFV DTM-rYFV DTM alone (group 3), YFV17D alone (group 1), YFV17D-protein (group 2), and PBS-protein (group control), except antibody seen in the rYFV DTM-protein group (group 4) (Figure 4B).

After the third immunization (week 10), experimental animals had high HIV-1 specific antibody responses in YFV17D-protein-protein (group 2), PBS-protein-protein (group control), rYFV DTM-rYFV DTM-protein (group 3), and rYFV DTM-protein-protein (group 4), while YFV17D-YFV17D-protein (group 1) showed low HIV specific antibody responses (Figure 4C and 4D). Antibody titers of rYFV DTM-protein-protein (group 4) showed

statistical significance compared to YFV17D-protein-protein (group 2) ($p=0.03$) but was not statistically significant compared to PBS-protein-protein (group control) ($p=0.09$) (Figure 4C and 4D). Terminal bleed mouse sera of groups 3 and 4 primed with rYFV DTM showed higher binding reactivity to CH505 TF gp120 than groups 1 and 2 with either no priming, or primed with the YFV17D control vector prime (pre-correction $p=0.024$). However, after Benjamini-Hochberg (1995) false discovery rate correction for multiple tests (all pairwise comparisons, 98 in total) this finding only demonstrated a trend (Figure 4E, $p=0.089$). We used CH505 TF 371I gp120 for testing immunogens that could prime/induce CD4 binding antibodies in mice. Mutations at 371Ile in HIV-1 Env reduce the binding of most CD4-binding site broad neutralizing antibodies such as mAb b12 (Wu et al., 2010). Groups 3 and 4 had higher antibody titers than groups 1 and 2 when using CH505 TF 371I gp120 as an antigen indicating no CD4 binding site broad neutralizing reactivity (Figure 4F, pre-correction $p=0.01$).

Next, HIV-1 specific IgG subclass responses were assayed by ELISA using sera from mice immunized with YFV17D or rYFV DTM. Binding data of groups 3 and 4 primed with rYFV DTM showed higher binding titers to CH505 TF gp120 than groups 1 and 2 with the YFV17D empty vector prime in IgG subclass analysis with IgG1 (Figure 4G, $p=0.004$) and IgG2b (Figure 4I, $p=0.03$), but there was no statistically significant difference in IgG2a (Figure 4H, $p=0.265$) and IgG3 (Figure 4J, $p=0.064$). Interestingly, mice immunized with rYFV DTM followed by protein boosts (group 3 and 4) showed statistically significant higher IgG2b responses than IgG2a responses (Figure 4H and 4I) ($p=0.019$, Wilcoxon-Mann-Whitney non-parametric test). Mice group immunized with YFV17D and boosted with protein (groups 1 and 2) showed similar IgG2b responses compared to IgG2a responses (Figure 4H and 4I) ($p=0.738$).

Furthermore, immunization of mice with YFV17D (groups 1 and 2) induced predominantly IgG1 HIV-1 specific antibodies with an IgG1:IgG2a ratio of 3.87 (Figure 5A), that is characteristic of a Th2-like response, while Th1 biased responses have dominant IgG2a responses (Marinaro et al., 1995; VanCott et al., 1996). HIV-1 Env specific antibody responses primed by immunizations with rYFV DTM and boosted with protein (group 3 and 4) showed a dominance of IgG1 over IgG2a antibodies (IgG1:IgG2a ratio of 5.7, Figure 5B). In addition, HIV-1 specific antibody responses primed by immunizations with wild type YFV17D or rYFV DTM showed dominance of IgG1 over IgG2a antibodies with adjuvant (Figure 5A and 5B, IgG1:IgG2a ratio of 3.87 and 5.7, respectively) and without adjuvant (Figure 5C and 5D, IgG1:IgG2a ratio of 7.18 and 17.8, respectively). Thus, priming immunization with CH505 TF Env in the form of rYFV DTM followed by gp120 proteins induced Th2-like HIV-1 specific antibody responses.

3.4. Neutralization profile of mice sera

The neutralizing activity of mouse sera, pre-bleed 2 and terminal bleed, was tested on a panel of three pseudotyped viruses, comprised of the tier 2 (difficult-to-neutralize) CH505 TF virus, the tier 1 (easy-to-neutralize) CH505.4.3 strain, as well as murine leukemia virus (MuLV) as a negative control (Table 4) (Liao et al., 2013; Seaman et al., 2010). Results are shown as inhibitory dose (ID_{50}) - neutralization titers of sera of mouse serum and

demonstrated that rYFV DTM primed for a protein boost for induction of tier 1 neutralizing antibodies compared to no prime or compared to control prime with the YFV17D vector, groups 1 and 2 vs groups 3 and 4 ($p = 0.00018$, Exact Wilcoxon test, Table 4).

4. Discussion

In this paper, we have developed a strategy for expression of HIV-1 gp120 in the attenuated YFV17D vector in a double transmembrane domain construct. Previous work has reported expression of small inserts by the YF viral platform bearing exogenous gene insertions at the E-NS1 intergenic region (Bonaldo et al., 2007; Bonaldo et al., 2014).

Another strategy to overcome the size limitation of YFV17D inserts is the “minigene” strategy (Martins et al., 2013; Martins et al., 2014). Martins et al. described the generation of seven new rYFV17D viruses expressing fragments of simian immunodeficiency virus (SIV)mac239 *gag*, *nef*, and *vif* between YFV17D E gene and NS1 gene (Martins et al., 2013). The largest inserts were 165 amino acids, *gag* 250–415 and *nef* 45–210. Priming with rYFV17D/SIV elicited low levels of SIV-specific T-cell response after rAd5 boosting. A later report of Martins et al. demonstrated that the minigene approach improved the breadth of a T-cell-based SIV vaccine by using nine minigenes (Martins et al., 2014). Improved genetic stability of rYFV17D is also important to express large inserts in rYFV17D. Others have demonstrated that an insertion region RNA was detected up to twenty serial passages in Vero cells (de Santana et al., 2014). In spite of increase in viral genetic stability, these changes did not lead to an increase in immunogenicity in a mouse model by modifying IRES elements at the 5' end of the SIV *gag* gene (de Santana et al., 2014). The inserts of EGFP (green fluorescent protein variant 238 amino acids) (Bonaldo et al., 2007) and HIV p24 capsid protein (231 amino acids) (Franco et al., 2010) have been studied by others, while here, the CH505TF gp120 has an insert size encoding 458 amino acids (Liao et al., 2013). We have shown preliminary success in modifying the YFV17D vector by expressing gp120 of HIV Env that was detectable for 5 passages. While not a final product, this design is an improvement over prior efforts to stabilize a large insert in this vector.

Vaccination of live attenuated YFV17D induces polyclonal adaptive immune responses including robust T lymphocyte responses with mixed T helper 1 (Th1) and Th2 and neutralizing antibodies that persist for years (Pulendran, 2009). Recent studies performed in rhesus macaques showed that recombinant YFV17D viruses expressing fragments of SIV proteins induced cytotoxic T cell responses specific to SIV antigens in YFV17D vector platform (Bonaldo et al., 2010; Martins et al., 2013). A comparison study of recombinant YFV17D vector to the recombinant Adenovirus 5 and Modified Vaccinia Ankara viruses, all of them expressing HIV p24 protein, showed that recombinant YFV17D viruses elicited better balanced CD8+ and CD4+ T cell responses in mouse model (Franco et al., 2010). However, in this study, we have focused the ability of various YFV constructs to induce HIV-1-specific antibodies.

The CH505 TF gp120 can bind to the unmutated common ancestor (UCA) of the CH103 CD4 binding site broadly neutralizing antibody (BnAb) lineage (Liao et al., 2013). We have previously demonstrated that the binding of a transmitted/founder Env to a UCA B-cell

receptor of a BnAb lineage was responsible for the induction of broad neutralizing antibodies (Liao et al., 2013). Thus, a combined live virus that express CH505 transmitted/founder Env and followed by subsequently evolved CH505 Env variants could initiate activation of a naïve B-cell of a BnAb lineage (Haynes et al., 2012; Liao et al., 2013; Zhang et al., 2016).

We found that mice primed with rYFV DTM expressing HIV-1 CH505 Env induced higher antibody titers than the groups primed with the wild-type YFV17D vector followed by CH505 TF gp120 protein immunization (Figure 4). Even though rYFV DTM primed detectable levels of binding antibody upon protein boosting, anti-HIV-1 Env antibody was not induced by rYFV DTM alone (Figure 4B). However, immunizations with rYFV DTM prime/CH505 TF gp120 protein boosts in mice were able to prime for induction of neutralizing antibodies for the autologous HIV-1 tier 1 isolate, CH505.4.3 (Table 4). Thus, our data are proof-of-concept that rYFV vectors can prime for Env protein boosts that elicit HIV-1 neutralizing antibodies. By optimizing rYFV's ability to cross-prime for HIV CD8⁺ T cell Gag responses, altering the insert to encode for Env trimers, and optimizing the vector for increased levels of insert expression, rYFV could be a candidate to prime for heterologous vector boost in an HIV-1 candidate vaccine formulation. Moreover, the rYFV DTM vector approach may present a viable strategy for co-expressing other heterologous flavivirus inserts such as Zika prM-E genes (Cugola et al., 2016; Fauci and Morens, 2016; Li et al., 2016; Miner et al., 2016).

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Highlights

- Construction of YFV17D vector with double transmembrane YFV17D E domain.
- Modified YFV17D vector could express full-length HIV-1 gp120 in mammalian cells.
- rYFV17D vector as a priming immunogen for inducing anti-HIV antibody responses.

Figure 1. Genomic schematic of recombinant YF17D vaccine vector

Construction of HIV-1 Env gene with single (STM) or double (DTM) YFV transmembrane domain.

Figure 2. Vector stability test of HIV-1 CH505 TF envelope expression in attenuated YFV vector
 Serial passages of yellow fever viral vector expressing CH505 TF Env in Vero E6. Passage experiments were performed using a fixed volume of supernatant in each passage. Supernatant was cleared by sequential centrifugation. (A) This figure shows that rYFV DTM expressed at least transfer after 5 passages. It showed rYFV DTM expressed HIV-1 CH505 TF gp120 as its' intrinsic protein size about 110 kDa while rYFV STM expressed larger protein than HIV-1 gp120. Western blot was blotted with VRC 16H10 under reducing conditions. (B) Recombinant virus titration by plaque forming assay using harvested supernatant from passage 2 to passage 5. The error bar represents the standard error of the mean (SEM). (C) Blotting passage 5 supernatants with Dengue monoclonal antibody 4G2 under non-reducing conditions. MW: protein molecular weight standard, mock: mock transfection, YFV17D: transfected with full-length RNA transcribed using linearized wild type pANCR-FLYF17Dx vector, rYFV DTM: transfected with full-length RNA transcribed using linearized pFLYF17Dx_CH505_DTM vector, and rYFV STM: transfected with full-length RNA transcribed using linearized pFLYF17Dx_CH505_STM vector.

Figure 3. Binding of anti-HIV-1 monoclonal antibodies to HIV Env in transfected BHK21 cells with recombinant YFV17D

Monoclonal antibodies that bind the Env CD4 binding site (VRC01 and DH522) and the CCR5 binding site (17b) were tested in this study. Palivizumab (RSV Ab) was used as a negative IgG1 control and transfected BHK21 cells with YFV17D (WT) were used negative expressed cells. Orange lines indicate BHK21 cells were transfected with rYFV DTM while light green lines indicate BHK21 cells with YFV17D (wild type). Gray area indicates cells reacted with isotype control and blue lines indicate non-transfected BHK21 cells.

Figure 4. Mouse immunization study

For mouse immunizations, we used P2 viral stocks. The route of immunization was subcutaneous (s.c.) at the base of the tail for wild type YFV or rYFV vector. The intraperitoneal (i.p) route was used with protein immunization. Panel A shows the immunization regimen with immunizations and bleeding time points indicated. PI indicates post-immune bleed. Panel B shows that the binding data of mice sera to CH505 TF gp120 protein after prime-boost. Prime-boost immunization data clearly showed that rYFV DTM prime was able to induce protein boost but WT prime did not. Mice were immunized in five different groups for prime-boost regimen such as YFV17D-YFV17D (group 1), YFV17D-protein (group 2), PBS-protein (group control), rYFV DTM-rYFV DTM (group 3), and rYFV DTM-protein (group 4). Wild type vector, pACNR-FLYF-17D_X (YFV17D), served as an empty vector control. The scale of y axis in panel B is lower than other panels. Panels C and D show that the binding data of mice sera to CH505 TF gp120 protein at PI 3 and PI 3.1. Panels E to J show the IgG isotype binding data of terminal bleeding sera against CH505 TF gp120 except panel F which used CH505 TF 371IgP120 Env protein on the ELISA plate.

Isotype data were the followings: panel E – IgG binding data, panel F – IgG binding data to CH505 TF 371Igp120 (pre-correction p value), panel G – IgG1 binding data, panel H – IgG2a binding data, panel I – IgG2b binding data, and panel J – IgG3 binding data. Immunization regiments were shown in Table.3. Immunization doses of YFV17D or rYFV DTM were 1.0×10^4 pfu per mouse and doses of proteins, HIV Env CH505 TF gp120, were 20 µg per mouse. The code for the figure legends are the followings: filled blue circles – group 1, filled red squares – group 2, filled black triangle – group ctl (control), filled violet reverse triangles – group 3, and filled orange diamonds - group 4.

Figure 5. HIV envelope specific isotype IgG1 induction was dominant over IgG2a by recombinant YFV17D vector

The ratio of IgG1:IgG2a was calculated by IgG1 titers versus IgG2a titers of combined group 3 and 4 or group 1 and 2. There were no adjuvants used until the final protein boosts when GLA/SE (IDRI) was used. Therefore, terminal bleed samples were immunized with adjuvant while PI 3 bleed samples immunized with no adjuvant. IgG1:IgG2a ratios were following: panel A – terminal bleed of group 1 and 2, panel B – terminal bleed of group 3 and 4, panel C – PI 3 bleed of group 1 and 2, and panel D – PI 3 bleed of group 3 and 4. Immunization regimens were shown in Table.3. Immunization doses of YFV17D or rYFV DTM were 1.0×10^4 pfu per mouse and doses of proteins, HIV Env CH505 TF gp120, were 20 μ g per mouse.

Table 1

Lists of primers for vector construction

<u>Primers for RE generation in YFV</u>	<u>Oligo sequence (5' to 3')</u>
Primer A	CAGCGGCAATCAATAAAGGC
Primer B_AgeI	GTATGAATTCCTTTCCCAACCGGTGTGAAGAAC
Primer B_BglII	GTATGAATTCCTTTCCCAACCAATGTGAAGATCTCTCC
Primer C_KasI	GGGAAAGGAATTCATACGGTGTGTTGGCGCCGCCTTC
Primer C_XhoI	GGGAAAGGAATTCATACGGTGTGTTGGCTCGAGCTTTC
Primer D	TTTGGATCCTGCACGACAAC
<u>Primers for CH0505 TF D7gp120</u>	
CH505TF_BglII	GATGCTATAGATCTTCGTGCCCGTGTGGAAGGAG
CH505TF_XhoI	TATATCTCGAGCCCTCCTTCTCGCGCTCCACCACG
<u>Primers for DTM Construction</u>	
DTM_1A	ACACCGCCTGGGATTTCAGC
DTM_1B	GTTGATGGCGCATCCTTGATC
DTM_2A_CH505TF	GATCAAGGATGCGCCATCAACGTGCCCGTGTGGAAGGAGGCCAAG
DTM_2A_A244_V1V2	GATCAAGGATGCGCCATCAACGACCAGGTGAAGCTGACCCCCC
DTM_2A_qODwV3	GATCAAGGATGCGCCATCAACTCCACCCAGCTGCTGCTGAACGGC
DTM_2B	TACCATCTCCGCACCTTGAGC
<u>YFV_CH505DTM KasI Insertion</u>	
CH505DTMKasI_1A	CGCACCATCACCATCCACTG
CH505DTMKasI_1B	TGAAAGCTGGCGCCCTCCTTCTC
CH505DTMKasI_2A	AGAAGGAGGGCGCCAGCTTTCAG
CH505DTMKasI_2B	AGATACCATCTCCGCACTTG

Table 2

YFV sequencing primers

HIV Insert Sequencing	Oligo sequence (5' to 3')
FLYF17DII-F1532	GCGGTGGACTTTGGTAACAG
FLYF17Dx-R2541	TTCAGCCAGTCATCAGAGTC
FLYF17DII-F1995	CAGCGCAATCAATAAAGGC
FLYF17Dx-R3012	GCTGCACCCAAGATAGATCC
CH505DTM-F2769	TGGAAGGAGGCCAAGACCAC
Qodwv3DTM-F2803	TCATCATCCGCTCCGAGAAC
Full YFV sequencing	
FLYF17DII-F82	TCGTTCGTTGAGCGATTAGC
FLYF17DII-R544	TTCCCGAGGTCCTCAGATG
FLYF17DII-F35	CTGCAAATCGAGTTGCTAGG
FLYF17DII-F561	CAGGCAACTGCACAACAAAC
FLYF17DII-F1014	GGGTGCATGGAGGAACTTGG
FLYF17DII-F2523	ACTCTGATGACTGGCTGAAC
FLYF17DII-F3000	TCTTGGGTGCAGCGGTGAAC
FLYF17DII-F3345	CCAGATCCACCACGGATAGC
FLYF17DII-R3861	ACGCCACCCAAGGCAATCTC
FLYF17DII-F3767	CTCATCGGCTTTGGGCTCAG
FLYF17DII-R4234	TGCCAGCACTCCCACTAGAC
FLYF17DII-F4259	AACTTCCTTGGTCCGATTGC
FLYF17DII-F4795	GGAAGACCTTGTCGCCTATG
FLYF17DII-F5252	CCCACCAGGGTTGTTCTTTC
FLYF17DII-F5724	AGAGTGTGGTGGTCCTGAAC
FLYF17DII-F6238	GGCTGGTTTGAAGACGAATG
FLYF17DII-F6723	TCCTTGAGGCGTCAAACCC
FLYF17DII-F7266	TCAAAGCGCAGCAGTCAAAG
FLYF17DII-F7756	ACGCAGGCATTGGCCGAAG
FLYF17DII-F8241	TTGGCGGAACAGTGATCAGG
FLYF17DII-F8733	ATCCACCAGCGGGAAGTAGG
FLYF17DII-F9271	GGCAGACCTTGATGATGAAC
FLYF17DII-F9775	TTCCCACCACTTCCATGAAC
FLYF17DII-F10227	CCTCCCACATCCATTAGTC
FLYF17DII-F10754	ATTGACGCCAGGAAAGACC
FLYF17DII-F11204	CTTCACGAGGCAGACCTCAG
FLYF17DII-F11717	TGGACTGTATGCACGAACCC
FLYF17DII-F12223	ACCTATCTCAGCGATCTGTC
FLYF17DII-F12710	ACTGTCATGCCATCCGTAAG
FLYF17DII-F13257	GACGGCGTTACCAGAACTC

Table 3

Mice immunization protocol.

	Prime (wk 0)	Boost (wk 2)	Boost (wk 10)	Boost (wk 34)	Boost (wk 35)
Group 1	YFV17D	YFV17D	Protein	Protein	Protein/GLAse
Group 2	YFV17D	Protein	Protein	Protein	Protein/GLAse
Group, control	1× PBS	Protein	Protein	Protein	Protein/GLAse
Group 3	rYFV DTM	rYFV DTM	Protein	Protein	Protein/GLAse
Group 4	rYFV DTM	Protein	Protein	Protein	Protein/GLAse

Immunization dose of YFV17D or rYFV DTM: 1.0×10^4 pfu per mouse via subcutaneous route at the base of the tail, dose of proteins: 20 µg per mouse via intraperitoneal route.

Table 4

Neutralization profile of mice sera against tier1 and tier 2 isolates.

Group	Animal#	Prebleed 2			Terminal Bleed#		
		CH505.TF	CH505.w4.3	MuLv	CH505.TF	CH505.w4.3	MuLv
Group 1 (YFV17D - YFV17D -Protein -Protein/GLA/se)	Y270	< 20 *	< 20	< 20	< 20	< 20	< 20
	Y271	< 20	26	< 20	< 20	< 20	< 20
	Y272	< 20	< 20	< 20	< 20	< 20	< 20
	Y273	< 20	32	< 20	< 20	< 20	< 20
	Y274	< 20	22	< 20	< 20	< 20	< 20
	Y275	< 20	< 20	< 20	< 20	173	< 20
Group 2 (YFV17D - Protein -Protein -Protein/GLA/se)	Y276	< 20	36	< 20	< 20	< 20	< 20
	Y277	< 20	< 20	< 20	< 20	< 20	< 20
	Y278	< 20	< 20	< 20	< 20	< 20	< 20
	Y279	< 20	< 20	< 20	< 20	< 20	< 20
	Y280	< 20	< 20	< 20	< 20	< 20	< 20
	Y281	< 20	< 20	< 20	< 20	< 20	< 20
Group, control (1× PBS - Protein -Protein -Protein/GLA/se)	Y282	< 20	51	< 20	< 20	< 20	< 20
	Y283	< 20	54	< 20	< 20	< 20	< 20
	Y284	< 20	22	< 20	< 20	< 20	< 20
	Y285	< 20	< 20	< 20	< 20	2344	< 20
	Y286	< 20	< 20	< 20	< 20	24	< 20
	Y287	< 20	< 20	< 20	< 20	< 20	< 20
Group 3 (rYFV DTM - rYFV DTM - Protein - Protein - Protein/GLA/se)	Y289	< 20	< 20	< 20	< 20	610	< 20
	Y291	< 20	< 20	< 20	< 20	2662	< 20
	Y292	< 20	< 20	< 20	< 20	1683	< 20
	Y293	< 20	< 20	< 20	< 20	1192	< 20
	Y294	< 20	< 20	< 20	< 20	797	< 20
	Y295	< 20	< 20	< 20	< 20	3212	< 20
Group 4 (rYFV DTM - Protein - Protein - Protein/GLA/se)	Y296	< 20	27	< 20	< 20	< 20	< 20

Group	Animal#	Prebleed 2				Terminal Bleed#			
		CH505.TF	CH505.w4.3	MuLv		CH505.TF	CH505.w4.3	MuLv	
	Y297	< 20	< 20	< 20		< 20	1771	< 20	
	Y298	< 20	< 20	< 20		< 20	43	< 20	
	Y299	< 20	< 20	28		< 20	39359	< 20	

* Values are positive ID50 - neutralization titers of mouse serum.

* Lowest dilution tested was 1:20.

#Virus isolates - Tier 1 isolate: CH505.w4.3, tier 2 isolate: CH505.TF, a negative control: MuLV.