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The yellow fever 17D virus as a platform for new live attenuated vaccines

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The live-attenuated yellow fever 17D virus is one of the most outstanding human vaccines ever developed. It induces efficacious immune responses at a low production cost with a well-established manufacture process. These advantages make the YF17D virus attractive as a vector for the development of new vaccines. At the beginning of vector development studies, YF17D was genetically manipulated to express other flavivirus prM and E proteins, components of the viral envelope. While these 17D recombinants are based on the substitution of equivalent YF17D genes, other antigens from unrelated pathogens have also been successfully expressed and delivered by recombinant YF17D viruses employing alternative strategies for genetic manipulation of the YF17D genome. Herein, we discuss these strategies in terms of possibilities of single epitope or larger sequence expression and the main properties of these replication-competent viral platforms.

The Virus and the Vaccine

The yellow fever virus is the prototype of the *Flavivirus* genus within the family *Flaviviridae*, which includes about 70 viruses. The flaviviruses are small (40–60 nm), spherical, enveloped, with a single-stranded RNA genome. The majority of these viruses is transmitted by arthropodes, as mosquitoes or ticks, and is therefore called arboviruses ("arthropod-borne viruses") causing important diseases in man and animals.

In 1927, the yellow fever virus was isolated in the Rhesus monkey (*Macaca mulatta*), through the inoculation of blood from an African patient named Asibi.[1] When subcutaneously inoculated in monkeys, it results in death in 95% of the animals after 4 to 7 d with high viremia rates in the blood of these animals. The serial passage of the Asibi YF virus strain in different types of cultivated tissues led to the identification of the 17D strain at passage 176, to 17DD at passage 195 and to 17D-204 at passage 204. The 17DD strain was cultivated afterwards until passage 243 and underwent 43 extra passages in chicken embryos (vaccine at passage 287). The 17D-204 strain, in turn, gave rise to the different seed viruses used in several countries. The 17D-204 and 17DD viruses are the 2 substrains of the 17D virus nowadays adopted to produce the world's YF vaccine. These 2 strains have accumulated genotypic and phenotypic differences due to the independent serial passages.[2,3] However, both are equally immunogenic and safe for man.[4]

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Immunological Aspects of Vaccination with the Yellow Fever 17D Virus

The attenuated Yellow Fever 17D (YF17D) vaccine has been widely adopted for the immunization of man for over 70 y.[5] A single primary dose of YF17D not only can promote effective and safe seroconversion in more than 90% of the vaccinees but can also provide immunity for over 40 y.[6,7] Although YF17D represents an enormous success in terms of human protection against yellow fever, the cellular and molecular mechanisms by which it can activate a polyvalent and long-lived immune response still remains to be fully elucidated.

General immune response to YF17D is mainly characterized by the stimulation of a broad range of innate immune responses, which will ultimately result in the production of high titers of neutralizing antibodies and robust YF-specific human effector T cells and memory $CD8^+$ T cell responses.[8] In addition, YF17D vaccination induces lymphocytes that produce a balanced mix of both T_H1 and T_H2 cytokines.[9,10]

Dendritic cells (DCs) have been shown to play a key role in the initiation of an innate immune response to YF17D.[10,11] The YF17D virus infects and activates multiple subsets of DCs, via Toll-like receptors (TLRs), including TLRs 7, 8, and 9, to elicit proinflammatory cytokines interleukin (IL)-12p40, IL-6, and interferon (IFN)-α.[10] Additionally, myeloid DCs also express the cytosolic nucleic acid-sensing receptors retinoic acid-inducible gene (RIG)-I-like (RIG-I) and melanoma differentiation associated gene-5 (MDA5) which will activate signal transduction pathways that trigger the production of type I IFNs.[12] Moreover, the mammalian target of the rapamycin (mTOR) signaling pathway was also implicated in regulating TLR7/9-dependent IFN-α/β production by plasmacytoid DCs.[13]

The role of other cell types, like Natural Killer (NK) cells and monocytes, in the YF17D innate immune response has been suggested by systems biology studies.[14] It was demonstrated that a subset of genes involved in NK cells and monocytes are

upregulated in the peripheral blood of vaccinated individuals. These cells might play a role in virus clearance. The activation of innate immunity cell types, such as NK and gamma-delta T cells, results in the early production of gamma interferon in man, monkeys, and mice which may be of importance to the overall adaptive immune response.[15-17]

The activation and maturation of different subsets of DCs is likely to contribute to the marked adaptive immune response after vaccination with the YF17D virus.[18] Analyzing microarrays on PBMCs of vaccinated humans, the most frequently activated gene was EIF2AK4 (GCN2) which is known to be related to the formation of stress granules.[19,20] Besides, upregulation of TNFRSF17, a key gene whose activation is predictive for the magnitude of neutralizing antibody response, has also been identified.

T CD8$^+$ lymphocytes are important components for the control of viral infections. The YF17D-specific effector CD8$^+$ T cell response is broad and complex. In 1998, Reinhardt and colleagues[21] first demonstrated that YF17D-vaccinated patients presented high levels of T CD8$^+$ lymphocytes in the peripheral blood. In the following years, CD8$^+$ T cell-specific epitopes were identified in mice, rhesus monkeys, and man, implicating the E and NS3 proteins as the most important targets for cytotoxic response to the YF17D virus.[22-24]

After human immunization with YF17D, it was also possible to detect effector CD8$^+$ T cells in peripheral blood, as demonstrated by the upregulation of CD38, HLA-DR, and Ki67, as well as by the downregulation of Bcl-2 in these cells.[8] The presence of effector CD8$^+$ cells dramatically increases, reaching a peak at day 15-post vaccination. After this period of time, the gradual differentiation of these effector cells into memory cells starts. These observations were further confirmed in a complete study of activation and differentiation kinetics of various cell types of both the innate and adaptive immune systems implicated in the immune response to YF17D[25]. A more detailed phenotypic analysis provided further evidence for YF17D-driven activation and differentiation of CD8$^+$ T cells, demonstrating the functional profile alteration of these lymphocytes in composition as these cell types mature from effector to memory. During the course of this transition, this cell type becomes less polyfunctional, correlating with the profile of a memory cell type.[26] The same work showed that CD4$^+$ and FoxP3$^+$ regulatory T cell (Treg) compartments all respond to the YF17D virus, displaying a kinetic profile of activation which precedes the CD8$^+$ T cell response. This observation suggests that the fast CD4$^+$ T cell response might be related to the pronounced ability of DCs to present the YF17D virus antigens. The presence of CD4$^+$ T cells secreting Th1 type cytokines has been related to higher titers of neutralizing antibodies.[18] It is possible that the efficient CD4$^+$ T cell response is crucial for the development of a high-quality humoral response to the vaccine. Concordantly, the generation of neutralizing antibodies is a hallmark of protective antiviral immunity against the YF17D virus.[27,28] Neutralizing antibodies directed against structural protein E is believed to be the most critical in terms of protection.[29,30] These neutralizing antibodies may persist for 40 y post vaccination, which exhibits the ability of the virus to elicit an efficient immune response that will result in a long-lived humoral response.

Development of the Live-Attenuated YF17D Virus as an Expression Vector

Live-attenuated viral vaccines, like yellow fever, polio, measles, mumps, and rubella are very efficient immunogens as they promote durable immunity in man. In fact, they are very appropriate for a large-scale production having a well-established production methodology, quality control procedures, and low cost. Thus, adoption as a replication competent vector to express heterologous antigens has been attempted with the purpose of developing new recombinant human vaccines. Since live-attenuated vaccine viruses are proliferation-competent vectors, it is expected that immunization results in viral replication in the host, spreading the viral antigen mass throughout many cells and tissues usually leading to a long-lasting and efficient protection based on a polyvalent immunity.[8,31]

The efficacious immune responses spurred the interest in the manipulation of the YF17D virus as a vector for the development of new human vaccines. Translation of the positive-stranded viral genomic RNA in infected cells results in the synthesis of a polyprotein precursor of 3411 amino acids, which when proteolytically processed, generates 10 viral proteins (**Fig. 1**) from the amino- to carboxi-terminus of the precursosr polyprotein: C; prM/M; E; NS1; NS2A; NS2B; NS3; NS4A; NS4B and NS5. The first 3 are viral structural proteins that form the virus particle together with the RNA molecule, denominated capsid (C, 12–14kDa), membrane (M of 8 kDa, its precursor prM of 18–22 kDa), and envelope (E, 52–54 kDa). These 3 genes are encoded in the first quarter of the genome. The remainder of the genome encodes the non-structural proteins (NS), numbered from 1 to 5 (NS1 to NS5) in accordance with the order of synthesis. This knowledge formed the basis for the development of an infectious cDNA clone from which the YF17D virus is regenerated through in vitro transcription and RNA transfection technologies.[32]

Since then, various recombinant live attenuated YF viruses have been developed by different approaches. Indeed, there are various possibilities to express heterologous antigens by the YF17D vaccine virus. First, the use of YF as an expression platform consists of the creation of chimeric flaviviruses in which the genes coding for prM/M and E proteins of the attenuated Yellow Fever 17D virus are substituted by the corresponding genes of the Japanese Encephalitis (JE), West Nile (WN), and dengue (DEN) viruses. This approach was feasible, in part, due to the similarity of the flavivirus genes and also because these proteins are functionally essential for virus proliferation. The second approach is the insertion of short sequences coding for antigenic epitopes into the YF17D genome having been employed into different intragenic as well as intergenic sites (**Fig. 1**). The YF17D vector system was also developed for the expression of larger gene fragments (**Fig. 1**). In this regard, 3 main alternatives of expression of larger polypeptides were tentatively established: (1) the insertion of heterologous genes into the E/NS1 intergenic region,

(2) insertion into the C gene, and (3) the creation of bicistronic viral RNA molecules.

Replacement of YF17D Structural Genes with those of other Flaviviruses

Based on the original description of chimeric dengue viruses by Bray and Lai,[33] new YF17D recombinant viruses have been constructed in which the prM-E genes of YF were substituted for the equivalent genes of other flaviviruses including Japanese encephalitis,[34] West Nile,[35] and the 4 dengue serotypes.[36] Although the prM-E genes may vary among diverse flaviviruses by over 40% of the encoded amino acids, chimeric 17D viruses are still viable. The gene fusion is made at the signal peptidase cleavage site between C and prM and the signal peptidase site at the E and NS1 junction.[37] Despite the high-fidelity of YF17D virus RNA polymerase during viral RNA replication, the introduction of mutations can be detected.[38] Mutations noted at the structural protein level would be related to adaptation of the flavivirus envelope and prM-M protein to the YF17D capsid protein toward virus assembly. In addition, mutations observed elsewhere in the genome could be due to adaptation to growth in cell culture. This underlying genetic variation may be the basis for the observed variability of these chimeric YF17D viruses with regard to phenotypic properties such as growth rates in animals and cultured cells, attenuation to experimental animals (mice and monkeys), and immunogenicity.[39]

For more than 10 years, YF17D has been developed as a viral vector to express other flavivirus proteins.[39] These recombinant live attenuated chimeric viruses have been tested in man to different extents, and due to the inexistence of any alternative, particular attention has been devoted to the immunogenicity and safety of the YF17D-DV tetravalent vaccine.[40-46] The live attenuated chimeric 17D-DEN tetravalent vaccine candidate from Sanofi-Pasteur constitutes the most tested dengue vaccine possibility. The chimeric viruses proved to be genetically and phenotypically stable,[47] less virulent when compared with the yellow fever vaccine 17D and unable to infect mosquitoes orally.[39] It has displayed adequate safety and immunogenicity in clinical studies in flavivirus naive and immune subjects. Early in 2011, the vaccine was administered to over 6000 children and adults from endemic and non-endemic areas with no questions regarding its safety.[41,42,46]

A phase IIb clinical trial in 2669 Thai schoolchildren with the tetravalent vaccine showed poor efficacy (30.3%) of protection against febrile DEN infections.[45] While vaccination reduced incidence of DEN1, DEN3, and DEN4 by 90%, the protection against DEN2 was low (9.2%), downgrading overall protection. There is a need for monitoring longer-term duration of immunity and protection. Data from an efficacy (phase III) trial of the YF17D-DEN-based tetravalent candidate vaccine should serve for the utility of such technological platform.

In this regard, it is noteworthy that YF17D-JE chimeric virus containing the prM-E genes of the SA14-14-2 virus strain has been developed. This strain of JE virus is used in some Asian countries as live attenuated vaccine and the use of its structural genes in the construction of the YF17D recombinant virus was expected to yield a bona-fide vaccine candidate. Indeed, clinical trials have shown that YF17D-JE is safe and immunogenic with a single dose providing long-lasting immunity to wild-type strains of JE virus.[48-51] This recombinant technology-based vaccine was licensed for human use in Australia (Imojev—Sanofi-Pasteur). Accordingly, YF17D-WN chimeric viruses have also undergone clinical tests. Studies on human subjects based on age and dose demonstrated that the vaccine was well tolerated and immunogenic in younger adults (18–40 y) as well as patients 50 y of age or older.[52] This YF17D-WN chimeric vaccine denominated ChimeriVax-WN02 displayed a highly attenuated phenotype associated with the presence of 3 mutations into the E protein of WN strain NY99 causing a reduction of neurovirulence. However, the equine WNV vaccine based on the ChimeriVax technology, expressing a wild type WN, was licensed by the USDA in 2006 and recalled in 2010 due to acute side effects including fatality among vaccinated horses.[53]

Insertion of Foreign Epitope Coding Sequences into the YF Genome

The first approach describing the expression of short epitopes by a replication-competent YF17D virus was conceived by McAllister et al. (2000)[54] (**Table 1**). In this study, a short ovalbumin immunodominant cytotoxic T cell epitope (SIINFEKL) was expressed at the junction of the NS2B and NS3 genes.[54] The rational of this methodology was based on endowing both sides of the heterologous sequence with the viral protease recognition motif. Consequently, the insertion was cleaved off the polyprotein due to the duplication of the viral protease NS2B/3 cleavage site. The expression of small epitopes by this methodology seems to not considerably interfere with the maturation of viral proteins, since the viral proliferation properties of this kind of recombinant viruses are similar to the vaccine counterpart.[54-57] However, the foreign sequence cloning into the NS2A-NS2B, NS3-NS4A, and NS4A-NS4B junctions did not allow the recovery of a viable virus. Interestingly, the same YF cloning strategy was employed in a subsequent work to express a *Plasmodium yoelli* $CD8^+$ T cell epitope.[57] Both of these studies demonstrated that YF17D recombinant viruses do protect vaccinated mice against lethal challenge with malignant melanoma cells or the malaria parasite. However, in recent studies for evaluating the capacity of distinct recombinant YF17D viral vectors to induce a protective immune response against the *Trypanosoma cruzi* protozoan parasite, the immunodominant $CD8^+$ T cell epitope TEWETGQI of the ASP-2 (Amastigote Surface Protein -2) cloned and expressed between NS2B and NS3 led only to marginal protection after the *T.cruzi* challenge.[56,58]

Another approach for the expression of defined epitopes by the YF17D recombinant virus was based on the use of the major envelope protein of flaviviruses, the E protein. Since this protein is the major target of the humoral immune response presenting 180 monomers at the surface of mature virions, the expression of

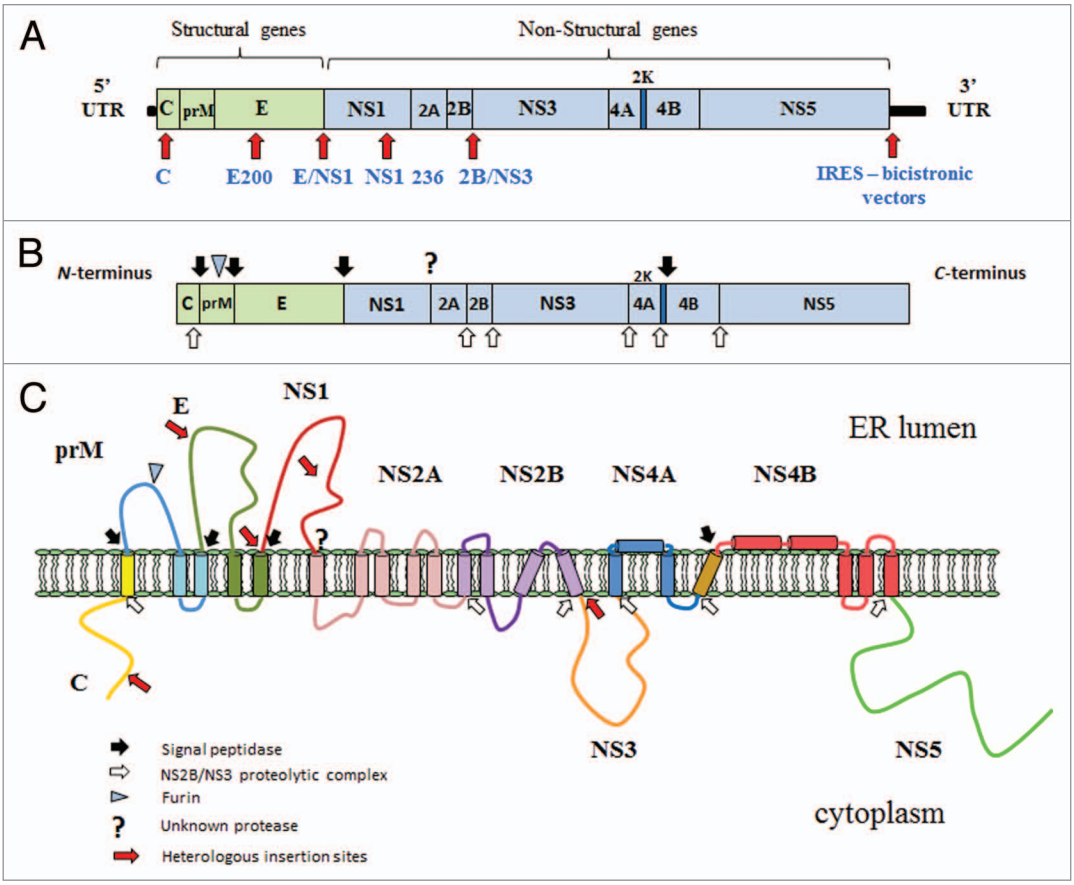

Figure 1. Insertion of heterologous sequences into the YF 17D genome. (**A**) YF genome is composed of a single positive single-stranded RNA containing 10 862 nucleotides. The major portion of the genome consists of an open reading frame flanked by a 5` and 3` untranslated region. The heterologous sequence insertion sites that have been described so far for recovering recombinant YF virus are indicated by a red arrow; (**B**) The translation of this large ORF produces a precursor polyprotein of 3411 amino acid residues, which are mainly co- and post-translationally processed at specific sites by the viral NS2B-NS3 proteolytic complex (white arrows) and signal peptidase (black arrows) producing the virion (structural proteins) and replicase elements (non-structural proteins). (**C**) Topological arrangement of YF proteins and the regions in which were possible to express heterologous sequences, indicated by red arrows. On the cytoplasmatic side, the viral proteins are processed by the viral NS2B/NS3 proteolytic complex (white arrows) whereas on the ER side by signal peptidases, furin, and an unknown protease that cleaves the carboxi-terminus of NS1.

heterologous humoral epitopes in this context would be appropriate to elicit antibodies specific to the foreign epitope. Additionally, several epitopes related to T-cell response have also been mapped onto the E protein.[24,59] Thus, based on three-dimensional structure modeling of the YF E protein it was possible to identify 2 expression sites permissive to heterologous insertions localized at the viral envelope surface at amino acid positions 154 and 200.[60,61] A number of recombinant YF17D viruses that express T cell and humoral epitopes of the *Plasmodium spp* CS protein inserted at the fg loop (site at amino acid 200 of the E protein) have been constructed. These viruses were stable through serial passages in cultured vertebrate cells and also retained attenuation for mice and monkeys.[58,60,62] In addition, the recombinant virus expressing a B-cell epitope from the malarial parasite *Plasmodium falciparum* induced antibodies against the CS protein after mouse and monkey vaccination.[60,62] The epitope expression in the E protein is an interesting approach, since it was recently reported that this protein elicits dominant immune responses corresponding either to MHC class I or class II antigens.[8,22,59]

Another possibility for epitope expression was developed by means of a transposon-mediated technique to randomly insert and express influenza A virus epitopes into the yellow fever virus NS1 protein.[63] Intriguingly, by selecting recombinant viruses with a methodology combining plaque purification with immunostaining, only one single insertion site was obtained at NS1–236 in which the influenza A epitope was expressed, strongly suggesting that NS1 is not tolerant in terms of alternative insertion points. Immunization of mice with this recombinant virus was not sufficient to afford protection against challenge with the mouse- adapted influenza A/H1N1 virus.

Expression of Larger Fragments by the YF17D Virus

Despite the importance of epitope insertion in establishing a number of methodologies to manipulate the YF genome and accessing the immunological potential of these recombinant viruses, it was of interest to improve the vectorial capacity of the

Table 1. Expression of small heterologous sequences by the YF viral platform

Insertion: viral genomic region	Heterologous sequence (origin)	Regimen of immunization, if any	References
NS2B/NS3	SIINFEKL (chicken ovalbumin)	2 doses (mice)	MacAllister et al., 2000.[54]
	SYVPSAEQIL (CSP of *Plasmodium yoelii*)	1 dose or with boost with irradiaded sporozoite (mice)	Tao et al., 2005.[57]
	TEWETGQI (ASP-2 of *Trypanosoma cruzi*)	2 doses (mice)	Nogueira et al., 2011.[58]
	GILGFVFTL (influenza matrix protein)	Human Dendritic cells	Barba-Spaeth et al., 2005.[55]
E protein at *fg loop*	TEWETGQI(ASP-2 of *Trypanosoma cruzi*)	2 doses (mice)	Nogueira et al., 2011.[58]
	DYENDIEKKI, (NANP)3, and SYVPSAEQI (CS proteins from *P. falciparum* and *P.yoelii*)	2 doses (mouse and rhesus monkeys)	Bonaldo et al., 2005[62] and 2006.[61]
NS1 at 236 residue	modified influenza A M2e epitope (SLLTEVETPIRNEWGSRSNDSSD)	2 doses (mice)	Rumyantsev et al., 2010.[63]

YF17D virus, since the expression of larger gene fragments could allow the expression of a more complex antigenic repertoire and, consequently, increase the chances to induce a more appropriate and polyvalent immune response. Basically, 3 different methodologies approached the expression of proteins or fragments thereof.

One of the possibilities to express heterologous sequences, in general up to 2.0 kb in length, into the YF17D genome consists of the insertion between the genes that code for the E and NS1 proteins. This is mainly based on the fact that in the flavivirus genomic organization, the intergenic E/NS1 region represents a functional shift from the structural to non-structural genes. So, larger heterologous insertions might be better accommodated in this region inducing fewer disturbances in the virus cycle as compared with other sites. This method is similar, theoretically, to the insertion between genes that encode proteins cleaved by viral protease. However, the cleavage between E and NS1 is accomplished by a cellular enzyme (signal peptidase, ref. 64) present in the ER membrane. Consequently, the cleavage sites and other structural elements needed for viral viability are different, and their presence should be taken into account when designing the expression cassette at the E and NS1 intergenic region. In most of the recombinant YF17D viruses bearing insertions at the E-NS1 intergenic region (**Table 2**), the recombinant proteins were fused at their amino-termini with a duplication of the codons for the first 9 amino acids of YF NS1 and at their carboxi-termini with whole or part of the YF E protein stem-anchor domain, or an equivalent sequence from another flavivirus.[56,65,66] A similar approach was employed to improve the genetic stability of Lassa virus glycoproteins expressed by recombinant YF17D viruses in this genomic site.[67,68] Interestingly, a variant of this general methodology was described in the expression of the HIV clade B consensus Gag p24 sequence by the YF17D virus. In this case, the heterologous protein was directly expressed in frame with the YF E protein and fused to the single transmembrane anchoring segment of the Sindbis virus E2 protein. Hence, this motif is expected to display a role as a signal peptide to the NS1 translocation to the ER lumen. Even though significant humoral and cellular immune responses against p24 were elicited by the 17D recombinant virus, this construct was genetically unstable, resulting in the specific deletion of the p24 gene.[9] The viral instability might be related to a decrease of viral fitness imposed by some constraints on either the viral genome or the precursor polyprotein that could have been provoked by the construct design. However, it is reasonable to suppose that in some instances the origin of the viral instability could be related to the primary function of the heterologous sequences cloned into the viral genome, such as the presence of secondary structural RNA elements that might interact with viral RNA or the expression of heterologous proteins that might disturb processes involved in viral infection. Actually, we attributed the genetic instability of a recombinant YF virus expressing a fragment of the SIV Gag precursor protein to the presence of an IRES element localized at the 3′end of gene encoding the SIV Matrix protein.[69] We managed to substantially improve the genetic stability of this recombinant YF17D virus by introducing mutations in the IRES element.[70]

The foreign antigens expressed by the recombinant YF17D virus in the E/NS1 region appear to remain cell-associated as described elsewhere.[56,65,66] The heterologous proteins are indeed retained in the ER compartment due to the presence of anchor transmembrane motifs at their carboxi-terminus. Although these recombinant viruses elicited humoral immune responses against the foreign antigens, hypothetically, the accumulation of antigens inside the infected cells could drive the immune response to be mainly cellular.

Recently, studies performed in Indian rhesus macaques, in which recombinant YF17D viruses expressing fragments of

Table 2. Expression of heterologous proteins at the E-NS1 expression site of YF17D virus

Foreign gene		Immunization	Reference
reporter protein	EGFP	2 doses (mice)	Bonaldo and Galler, 2005[85]; Bonaldo et al., 2007.[65]
SIV	Gag 45–269	boost in rhesus monkeys primed with rBCG	Bonaldo et al., 2010[71]
	Gag 44–84	prime with rYF and boost with rAdenovirus 5 (rhesus monkey)	Martins et al., 2013.[72]
	Gag 76–123		
	Gag 142–186		
	Gag 250–415		
	Vif 1–110		
	Vif 102–214		
	Nef 45–210		
T.cruzi	ASP-2 261–380	2 doses (mice)	Nogueira et al., 2013.[56]
HIV	p24	2 doses (mice)	Franco et al., 2010.[9]
Lassa Virus	GPC	1 dose (guinea pigs)	Bredenbeek et al., 2006.[67]
	GP1	2 doses (mice and guinea pigs)	Jiang et al., 2011.[68]
	GP2	2 doses (mice and guinea pigs)	Jiang et al., 2011.[68]

SIV proteins, demonstrated the ability of YF17D vector to elicit cytotoxic T lymphocyte responses specific to recombinant antigens. .[71-73] In one of these experiments, animals expressing the *Mamu-B*08* allele, a rhesus model for an HLA-B*27 mediated elite controller, controlled the replication of SIV after vaccination with a heterologous immunization regimen with recombinant YF17D viruses as a prime dose and recombinant Adenoviruses 5 as a booster dose, both expressing fragments of Vif and Nef. These animals exhibited a high frequency of $CD8^+$ T cells detected in blood, lymph nodes, and colon that were specific to 3 different *Mamu-B*08* restricted epitopes. In order to better evaluate the efficacy of replicative-competent recombinant YF vectors, 7 YF17D viruses expressing fragments of the SIVmac239 Gag, Nef, and Vif proteins were utilized to vaccinate rhesus monkeys with different MHC haplotypes.[72] Despite the detection of replication of YF viruses in rhesus monkeys after vaccination, in general, they only elicited low levels of SIV- specific cellular immune responses, which considerably improved only after a recombinant Adenovirus type-5 vector booster. However, an increase of $CD4^+$ T cellular responses was only observed after heterologous YF17D/rAd5 immunization, but not after Ad5 vaccination only, advocating a role of recombinant YF17D viruses to prime virus-specific T-cell responses in heterologous prime-boost immunizations. Interestingly, the comparison of this YF17D vector to the recombinant Adenovirus 5 and Modified Vaccinia Ankara viruses, all of them expressing the HIV p24 protein, indicated that recombinant YF17D viruses induced better balanced $CD8^+$ and $CD4^+$ T cell immune responses in the mouse model.[9] Furthermore, in terms of cytokine release from splenocytes in these animals, a more polyfunctional pattern was detected. Other studies confirmed the capacity of this YF E/NS1 viral platform to elicit a T cell-specific response against other antigens.[31,56,67,68,74,75] These results point to a need of optimization of vaccination regimens as well as an improvement of the YF E/NS1 insertion platform immunogenicity, which should be considered since the yellow fever vaccine displays outstanding immunological properties as a human immunogen. Another possibility to exploit the expression of foreign proteins by the live-attenuated YF17D virus consists of the insertion of sequences into the C gene. This method takes into account the presence of a conserved hairpin RNA structure (CS) in the gene coding for the C protein that mediates the genome cyclization required for RNA replication.[76] Thus, in this model, the heterologous gene is flanked at its 5′ end by a truncated capsid sequence of 75 nucleotides containing the CS sequence and at its 3′ end with sequences coding for the foot-mouth disease virus (FMDV) 2A peptide and the mammalian ubiquitin to ensure efficient cleavage from the downstream YF. Initially, this platform was employed to express a fragment of the circumsporozoite protein (CS) from the murine malarial parasite *Plasmodium yoelii*.[77] A single immunization of naïve mice with recombinant YF17D resulted in robust production of IFN-γ by $CD8^+$ T-cells and IFN-γ and IL-2 by $CD4^+$ T-cells. When administered in a prime-boost regimen in conjunction with a boost dose of irradiated sporozoites, the vaccination protected mice from a challenge with sporozoites of *P.yoelii*. This YF vector was also utilized to promote the expression of the HIV p24 sequence.[9] Immunogenicity studies in mice indicated that this recombinant virus induced antibodies against p24

and p24-specific CD8+ IFN-T-cell responses. In both constructs the major concern was related to the viral genetic stability, since the heterologous genes coding for the CS protein and p24 were completely deleted in the fourth and sixth serial passages suggesting that this strategy deserves some improvement to stabilize the recombinant viruses.

The last method to express larger heterologous sequences by the YF17D virus was based on the natural length variation of the 3′ untranslated region of flaviviruses[78] that could accommodate the insertion of genetic cassettes containing internal ribosomal entry sites (IRES).[79] These bicistronic viruses were developed for other flaviviruses to express reporter proteins.[74,80-82] Nevertheless, these bicistronic flaviviruses were considered to be very unstable, since soon after few rounds of viral replication, the heterologous IRES-bearing cassettes suffered extensive deletions.[74,75]

Concluding Remarks

The insertion of heterologous sequences into the YF17D genome is not an obvious task, since heterologous inserts do not provide any selective advantage to the virus and in general, they indeed cause a reduction of viral fitness. This is due in part not only to the rather short YF genome size but also to interference in interactions among viral and cellular proteins, such as those that take place during the translation of the RNA, its replication and virus particle formation. Such interferences certainly contribute to the genetic instability for several foreign genes and poor immunogenicity of some recombinant YF17D viruses.

A very important aspect in establishing a strategy for heterologous expression specifically at intergenic sites is related to the topology of viral proteins in the endoplasmic reticulum membrane. It is well known that the polyprotein precursor traverses the ER membrane at various points being proteolytically processed in the ER lumen by cellular signal peptidases and in the cytoplasmic side by the viral NS2B/NS3 protease. Protein secretion and processing require the presence of functional motifs that allow the sorting to the appropriate compartment, the proteolytic processing to generate the mature viral proteins and their involvement in the replication complex. Therefore, the strategy to express a given antigen will depend upon its physical-chemical properties, cell trafficking, and the type of immune response expected against the antigen. It is conceivable that improving antigen processing will depend at least in part upon the appropriate cellular location.

One of the major concerns about this first generation of recombinant YF17D viruses is the low immunogenicity. Perhaps the increment in the ability to properly induce the immune system could be afforded by means of the introduction of mutations into the viral genome that promotes higher viral replication capacity. In this regard the NS4B (Leu52Phe) mutation introduced in a dengue virus 2 vector expressing different reporter proteins conferred a dramatic enhancement in virus infectivity and production in cell cultures and in mouse infectiousness that was consistent with the properties of the parental virus.[83,84]

Additionally, it was recently demonstrated that it is possible to increase the immunogenicity of recombinant YF vaccines through the use of formulations containing distinct platforms expressing heterologous antigens. Hence, a simultaneous administration of different recombinant YF viruses expressing a repertoire of antigens from the ASP-2 of *Trypanosoma cruzi* was considerably more immunogenic than the single administration of each virus promoting protection against a lethal challenge of the protozoan parasite in one of the mouse models.[56]

All in all, despite the initial results pointing to a need for improved vectorial properties, especially the genetic stability of the insertion, one must be aware that the 17D recombinant viruses generated so far are only the first generation. Considering that this vaccine virus has exceptional characteristics as a human immunogen, the improvement of such a platform is certainly warranted.

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Disclosure of Potential Conflicts of Interest

The authors have declared that the insertion methodology into the YF E/NS1 region is the subject of a patent application having as authors M.C.B. and R.G. and the Fundação Oswaldo Cruz.

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