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Original Article

Biodistribution and environmental safety of a live-attenuated YF17D-vectored SARS-CoV-2 vaccine candidate

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New platforms are needed for the design of novel prophylactic vaccines and advanced immune therapies. Live-attenuated yellow fever vaccine YF17D serves as a vector for several licensed vaccines and platform for novel candidates. On the basis of YF17D, we developed an exceptionally potent COVID-19 vaccine candidate called YF-S0. However, use of such live RNA viruses raises safety concerns, such as adverse events linked to original YF17D (yellow fever vaccine-associated neurotropic disease [YEL-AND] and yellow fever vaccine-associated viscerotropic disease [YEL-AVD]). In this study, we investigated the biodistribution and shedding of YF-S0 in hamsters. Likewise, we introduced hamsters deficient in signal transducer and activator of transcription 2 (STAT2) signaling as a new preclinical model of YEL-AND/AVD. Compared with YF17D, YF-S0 showed improved safety with limited dissemination to brain and visceral tissues, absent or low viremia, and no shedding of infectious virus. Considering that yellow fever virus is transmitted by *Aedes* mosquitoes, any inadvertent exposure to the live recombinant vector via mosquito bites is to be excluded. The transmission risk of YF-S0 was hence compared with readily transmitting YF-Asibi strain and non-transmitting YF17D vaccine, with no evidence for productive infection of mosquitoes. The overall favorable safety profile of YF-S0 is expected to translate to other vaccines based on the same YF17D platform.

INTRODUCTION

Roughly 2 years after its first emergence in 2019–2020, more than 5 million people have succumbed to coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (https://coronavirus.jhu.edu/map.html). Mass immunization is key to mitigating the expanding pandemic.[1] A set of rapidly developed prophylactic vaccines plays a crucial role in global immunization against SARS-CoV-2. Several of these vaccines are first in class, based on novel platforms, including game-changer mRNA vaccines and viral vector vaccines that are unprecedented in both their high clinical efficacy and the incremental advance in breakthrough innovation.[2–4] However, a global vaccine supply shortage, the dependence on an ultra-cold chain system in case of mRNA vaccines, and the continuous emergence of virus variants pose unmet challenges.[5,6] Unfortunately, the long-term effectiveness of current SARS-CoV-2 vaccines is waning because of the combined effect of (1) a rapid decay of virus-neutralizing antibodies (nAbs) over time and (2) emergence of new variants escaping vaccine-induced immunity.[7–9] Furthermore, several first-generation COVID-19 vaccines have a rather high reactogenicity. With the growing number of vaccinated people, more cases and a wider spectrum of adverse effects following immunization (AEFI), including severe adverse effects (SAEs) such as myocarditis and life-threatening deep-venous thrombosis, are described.[10–15] In summary, there is an urgent to develop new and improved second-generation COVID-19 vaccines to eliminate the pandemic.

Recently, we used an alternative vaccine platform that uses the fully replication-competent live-attenuated yellow fever (YF) vaccine YF17D as a vector[16] and developed a virus-vectored SARS-CoV-2

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vaccine candidate (YF-S0) that expresses a stabilized prefusion form of SARS-CoV-2 spike protein (S0).[17] YF-S0 was shown to induce vigorous humoral and cellular immune responses in hamsters (*Mesocricetus auratus*), mice (*Mus musculus*), and cynomolgus macaques (*Macaca fascicularis*) and was able to prevent COVID-19-like disease after single-dose vaccination in a stringent hamster model. Because of its YF17D backbone, YF-S0 could serve as dual vaccine to also prevent yellow fever virus (YFV) infections, which should provide an added benefit for populations living in regions at risk for YFV outbreaks.[18]

In addition to preclinical efficacy, development of such a new vaccine requires in-depth evaluations of its safety to support progression from preclinical study to clinical trials. In particular for live-attenuated viral vaccines such as YF-S0, the biodistribution of the vaccine virus after administration needs to be assessed[19] to understand the viral organ tropism and hence to exclude potential direct harm to specific tissues. Our vaccine candidate, YF-S0, showed an excellent safety profile in multiple preclinical models, including in non-human primates (NHPs) and interferon-deficient mice and hamsters.[17] However, use of such a recombinant YF17D vaccine entails some potential concerns.[19] In particular, replication and persistence of YF-S0 in tissues and body fluids pose a theoretical risk for YF vaccine-associated viscerotropic disease (YEL-AVD) and YF vaccine-associated neurotropic disease (YEL-AND), which are originally linked to parental YF17D.[20] With this regard, parental YF17D vaccine is commonly used as benchmark for direct comparison in safety assessment.[19]

Here, we investigated the biodistribution and shedding of YF-S0 following vaccination in hamsters, with the following aims: (1) to understand to what extent YF-S0 causes viremia resulting in virus dissemination to vital organs; (2) to evaluate the risks of YF-S0 for YEL-AVD/AND by confirming its transient and self-limited replication *in vivo*,[17] restricting the risks for YEL-AVD/AND; and (3) to determine to what extent viral RNA remains detectable in body secretions and, if so, (4) whether this poses any environment risks for shedding of recombinant infectious virus. Furthermore, YFV is a mosquito-borne virus, and YF-S0 uses YFV-derived YF17D as a vector. Recombinant YF-S0 might hence, though highly attenuated, pose an environmental risk due to unforeseen phenotypical changes. Taking this theoretical consideration into account, we tested the infectivity of YF-S0 on *Aedes aegypti (Ae. aegypti)* mosquitoes to assess its transmission potential. *Ae. aegypti* was selected as target mosquito species because of its well-known high vector competence for YFV.[21] It is well documented that wild-type YF-Asibi can infect and disseminate in *Ae. aegypti*, while YF17D only occasionally infects the midgut and is unable to disseminate to secondary organs.[22,23] Therefore, these two YFV strains were used as controls to assess transmission of YF-S0 by a competent vector.

Finally, we corroborate the favorable safety profile of YF-S0 by reporting limited dissemination and shedding in vaccinated hamsters and no risk for mosquito-borne transmission.

RESULTS

Tissue distribution of YF-S0 and parental YF17D in hamsters

For our assessment, we chose wild-type (WT) Syrian golden hamsters as a preferred small animal model of YFV infection[24] and injected them with a high dose (10^4 plaque-forming units [PFU]) of either YF17D (n = 6) or YF-S0 (n = 6) via the intraperitoneal (i.p.) route to achieve maximal exposure; primary pharmacodynamics have been previously documented[17] and are confirmed here by consistently high seroconversion rates (at least 80%) to YFV-specific nAbs (Figure S1). Likewise, a 10^4 PFU i.p. dose was shown to elicit saturating levels of SARS-CoV-2 nAbs in the hamster model, similar to a two-dose regimen using a 10-fold lower inoculum.[17] As methods control, we inoculated STAT2 knockout ($STAT2^{-/-}$) hamsters with 10^4 PFU YF17D (n = 2). $STAT2^{-/-}$ hamsters are deficient in antiviral type I and type III interferon responses[25] and therefore prone to uncontrolled flavivirus replication.[26] Tissues sampled for analysis were chosen on the basis of biodistribution data available from non-human primates and humans. In macaques, detection of YF17D RNA has been reported in lymph nodes, spleen, and liver at 7 days after subcutaneous inoculation.[27] Likewise, viral RNA is widespread and abundantly found in spleen, liver, brain, kidney, and other organs in patients who developed YEL-AVD.[20,28] On the basis of this knowledge, we collected spleen, liver, brain, and kidney as the most common target organs to assess the risks for YEL-AVD and YEL-AND. Ileum and parotid gland were collected as additional excretory tissues and lung as the main target of COVID-19 (Figure 1A). From our previous experience,[17] we observed that the replication of YF17D or YF-S0 is transient and well tolerated in WT hamsters. Tissue analysis in hamsters was thus performed 7 days post-inoculation (dpi) (i.e., a few days after peak of viremia), in line with similar studies performed for chimeric YF17D vaccine in macaques before[27] and at a time point at which $STAT2^{-/-}$ hamsters needed to be euthanized for humane reasons.[17]

Viral RNA above detection limits in YF17D-vaccinated WT hamsters was mostly limited to spleen (RNA detected in 4 of 6 animals), with the exception of a single hamster in which viral RNA was widespread to the brain, parotid gland, and lung (Figure 1B; Table S1). Detection of YF-S0 was markedly less frequent and restricted to only kidney (2 of 6) and lung (1 of 6) (Figure 1B). Overall, in either group RNA level was low and barely detectable by sensitive RT-qPCR, indicative for limited replication in WT hamsters. In contrast, unrestricted replication of virus to high viral loads was observed in $STAT2^{-/-}$ hamsters (Figures 1B and 1C). Importantly, no viral RNA nor infectious virus could be detected in the brains of YF-S0-vaccinated hamsters, suggesting a low associated YEL-AND risk (Figures 1D and 1E).

Viremia is considered a key indicator for the risk for developing YEL-AVD. Thus, longitudinal blood sampling was conducted as shown in Figure 2A. Kinetics of viral RNA in serum as proxy for viremia have been reported earlier for WT hamsters vaccinated with YF17D or YF-S0[17] and are discussed here in comparison with respective data from $STAT2^{-/-}$ controls (Figure 2B). Viremia

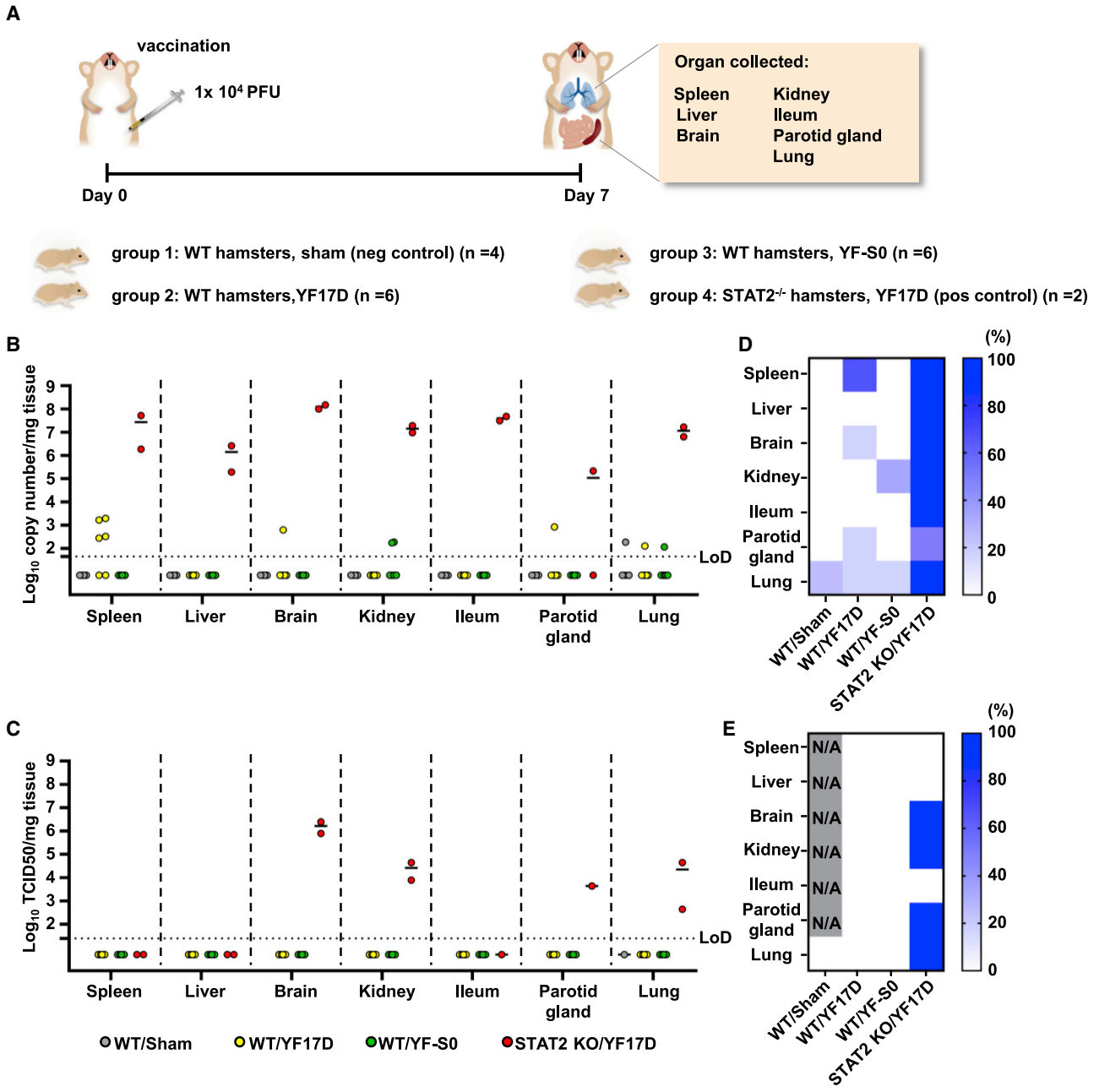

Figure 1. Biodistribution of YF-S0 in hamsters

(A) Schematic of hamster vaccination and organ collection. Hamsters were inoculated i.p. with 10^4 PFU/mL of either YF17D or YF-S0 and sacrificed 7 days later. Organs from 4 different experimental groups, including sham-vaccinated wild-type (WT) hamsters and YF17D-vaccinated STAT2$^{-/-}$ (knockout [KO]) hamsters as respective negative and positive controls, were collected and divided for RNA extraction and virus isolation. (B) Viral RNA load by RT-qPCR. (C) Virus isolation by $TCID_{50}$ assay. For sham and STAT2 KO, only PCR-positive samples were analyzed. (D and E) Heatmap representing positivity rates by organ and experimental group on the basis of the results of RT-qPCR (D) or $TCID_{50}$ assay (E). Bars in (B) and (C) represent median values. N/A, not applicable.

could be detected consistently in all YF17D-vaccinated WT hamsters (6 of 6) starting at 1 dpi and lasting for a median of 2.5 days (95% confidence interval, 1–4 days); in contrast, viral RNA was detected only once at 3 dpi in a single YF-S0-vaccinated hamster (1 of 6) (Figure 2B; Table S2). In STAT2$^{-/-}$ hamsters, YF17D grew unrestrictedly to markedly increased viral RNA levels (Figure 2B), readily detectable by virus isolation (Figure S2). Integration of data over the course of immunization (area under the

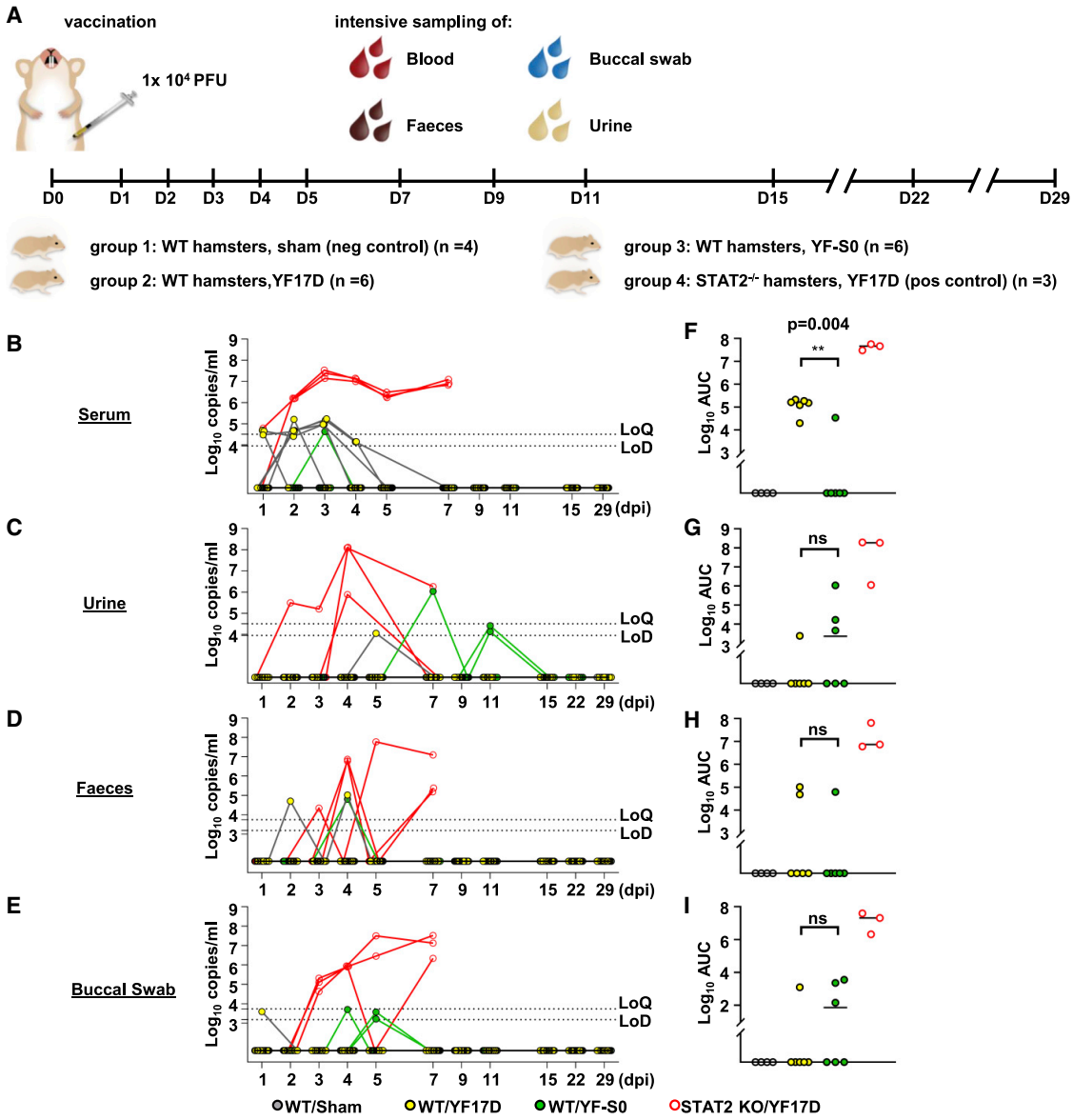

Figure 2. Shedding of YF-S0 by vaccinated hamsters

(A) Schematic of vaccination and specimen collection. Hamsters were inoculated as in Figure 1A, and serum, urine, feces, and buccal swabs were serially sampled at indicated time points. (B–E) Viral RNA load by RT-qPCR. (F–I) Area under curve (AUC; copies × day) calculated in GraphPad Prism 8; the Mann-Whitney test was used for the statistical analysis, with $p > 0.05$ marked as non-significant (ns); and $^{**}p \leq 0.01$. Serum RNA data for YF17D and YF-S0-vaccinated WT hamsters as previously published. LoQ, limit of quantification; LoD, limit of detection; dpi, days post-inoculation.

curve [AUC]) indicated a significant reduced overall serum virus load in YF-S0-vaccinated animals (Figure 2F).

Limited shedding of YF-S0 and parental YF17D RNA

As shedding of viral RNA in urine after YF17D vaccination has been reported,[29] we sampled different body fluids to investigate respective virus levels (Figure 2A). Within all longitudinally sampled specimens, viral RNA was detected only sporadically in urine (1 of 56 and 3 of 58), feces (2 of 65 and 1 of 66), and buccal swabs (1 of 66 and 3 of 66) of both YF17D and YF-S0-vaccinated hamsters, respectively, mostly at very low copy numbers and not linked to viremia (Figures 2C–2E and S3; Table S3–S5). Noteworthy, viral RNA could be detected, if at all, only within the first 11 dpi, clearly indicating that viral replication was self-limiting, leading to the final elimination of the live viral vector from all tissues. Also, there was no significant difference in AUC between both groups (Figures 2G–2I). The potential risk that YF-S0 could be spread through excrement of vaccinated individuals should hence be as low as for YF17D. In addition, no viable

virus could be isolated from urine samples, with RNA counts as high as 10^8 copies/mL (not shown), in line with no clinical evidence for secondary spread in urine, matching long-standing field experience for YF17D.

Abortive infection of YF-S0 on yellow fever virus-competent vector *Ae. aegypti*

YF-S0 is derived from mosquito-borne YFV, and human-to-human transmission by a competent mosquito vector could theoretically lead to unintentional exposure to the vaccine, including in immune-compromised people.[30] Thus, the transmission risk of YF-S0 should be excluded regarding main indicators of mosquito vector competence[21,31,32] (Figure 3A): (1) sufficient virus ingestion from infectious blood meal, (2) productive infection of virus in mosquito midgut (midgut infection barrier [MIB]), and (3) virus escapes from midgut barrier (MEB) (i.e., dissemination to parenteral tissues to establish sufficiently high virus loads in salivary glands to enable transmission). To this end, *Ae. aegypti* mosquitoes, as the species of YFV-competent vector,[21] were given infectious blood meals with no virus, YF17D, YF-S0, or wild-type YF-Asibi strain as positive control.[22,23] Samples as collected on day 0 in whole mosquitoes (ingestion step), on day 14 in thorax and abdomen (virus infection and replication in mosquito midgut; marked as main body), and on day 14 in head, legs, and wings (dissemination) were determined using both RT-qPCR and a CPE-based assay (50% tissue culture infective dose [$TCID_{50}$]) for virus infection and replication (Figure 3A). Considering the relatively long extrinsic incubation period of YFV[21] in mosquitoes and particularly that of YF17D,[23] earlier time points were not considered for analysis.

Experimental feeding was equally efficient for all three virus groups regarding both viral RNA and infectious virus recovered (Figures 3B and 3C). However, 14 days after feeding, viral RNA was detected exclusively in specimens from the YF17D group (8 of 15) and YF-Asibi group (8 of 23) but none from the YF-S0 group. Importantly, infectious viral particles were detectable only in the YF-Asibi group, with virus loads as high as about 10^6 $TCID_{50}$/body on average (Figure 3C). For dissemination beyond the MEB, the remaining head, legs, and wings of each six virus-positive mosquitoes with highest body virus loads from the YF17D and YF-Asibi groups, respectively, and six randomly chosen specimens from the YF-S0 group were evaluated. All these specimens from the YF-Asibi group (6 of 6) scored positive for dissemination, compared with none from the YF-S0 or YF17D groups (Figures 3B and 3C). These results suggest that YF-S0 is able neither to pass the MIB for midgut infection nor to escape from the midgut (MEB) for dissemination (Figures 3D and 3E).

DISCUSSION

The live-attenuated YF17D vaccine is considered one of the most powerful and successful vaccines and has been used in humans for decades.[33] Its well-known characteristics of stimulating both vigorous humoral and cellular immune responses, as well as favorable innate responses, are of interest for other vaccine targets using the YF17D genome as a backbone.[16] We recently generated a particularly potent YF17D-vectored vaccine candidate, YF-S0, against SARS-CoV-2 infection, inserting the non-cleavable spike protein of SARS-CoV-2 (S0) between the E and NS1 region of YF17D.[17] This construct serves as antigens to induce vigorous immune responses against both SARS-CoV-2 and YFV infections.[17]

Apart from YF-S0, YF17D is currently the only fully replication-competent viral vector that is part of any licensed recombinant live viral vaccine in wide use for human medicine, that is, in the two licensed human vaccines, JE-CV (against Japanese encephalitis; Imojev[34]) and CYD-TDV (against all four serotypes of dengue virus; Dengvaxia[35]). Additional YF17D-based vaccine candidates are in different stages of (pre)clinical development, including vaccines against other flaviviruses (West Nile virus: ChimeriVax-WN02[36]; Zika virus: YF-ZIKprM/E[37]) or non-flaviviruses (HIV: rYF17D/SIV-Gag45-269[38]; Lassa virus: YFV17D/LASV-GPC[39]; chronic hepatitis B virus: YF17D/HBc-C[40]). Like other YF17D-vectored vaccines, YF-S0 triggers vigorous protective immune responses, including high levels of SARS-CoV-2 neutralizing antibodies after single-dose vaccination in hamsters, mice, and cynomolgus macaques.[17] However, all these YF17D-vectored vaccines share the theoretical concerns of SAEs associated with the parental YF17D vaccine, such as YEL-AVD (0.4 per 100,000) and YEL-AND (0.8 per 100,000),[19,20,41,42] notably despite little (pre)clinical evidence or reports from post-marketing surveillance.[43] In contrast, though originally contraindicated in more vulnerable people, such as children, pregnant women, people living with HIV or diabetes, and older persons, growing evidence now suggests increased risk from YF17D.[44,45]

To temper the safety concerns, the viscerotropism and neurovirulence of YF-S0 were compared head-to-head with parental YF17D virus by investigating the biodistribution and viremia following administration of either vaccine virus in hamsters. We demonstrate that parental YF17D can spread systemically and viral RNA can be detected in the spleen, brain, parotid gland, and lung in YF17D-vaccinated WT hamsters. However, replication of YF17D remains restricted, resulting in infectious virus loads below detection limits. Compared with YF17D, detection of YF-S0 was further limited, with minute amounts of viral RNA in the kidney and lung. Unrestricted virus replication to high viral loads as cause of viscerotropic or neurotropic disease was observed only in $STAT2^{-/-}$ hamsters, in line with the essential role innate interferon signaling plays in live vaccines[30,46] and control of viral infections in general.[47] In addition, in YF-S0-vaccinated WT hamsters, detection of viremia was rare (Figure 2B) and, importantly, less frequent (1 of 6) and markedly lower in magnitude (AUC) and duration (1 day) compared with parental YF17D (6 of 6 for >2 days). A limitation of our study may be the relatively small number of animals enrolled per group and the finite number of time points selected for analysis. However, taken together, the overall limited tissue distribution of YF-S0 as well as the low abundance of its RNA in blood, below detection limits for infectious virus, suggest a further lowered risk for YEL-AVD/AND for YF-S0 than that reported parental YF17D. It is thus to be decided to what extent such highly

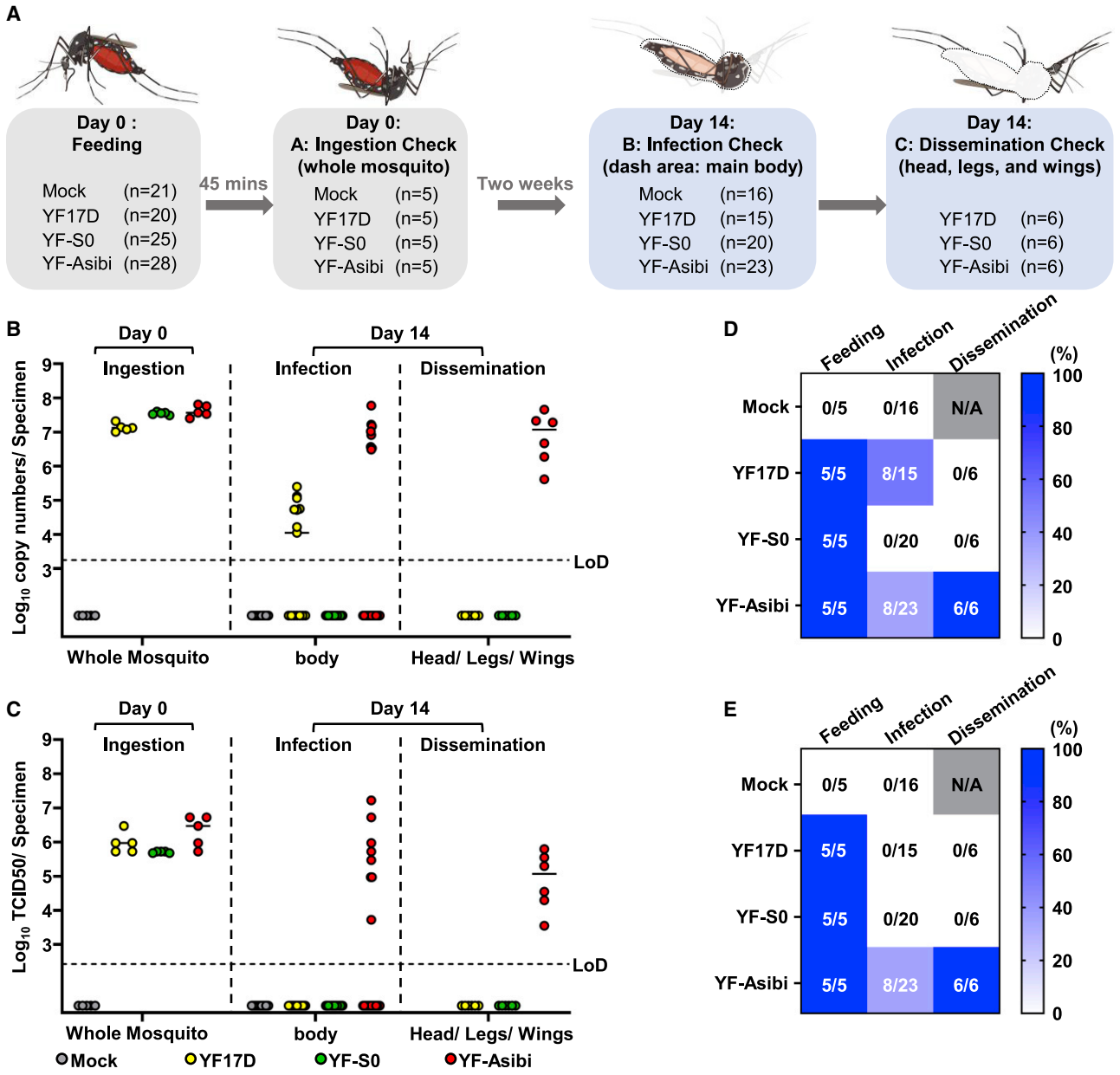

Figure 3. Assessment of YF-S0 transmission potential by *Aedes* mosquitoes

(A) Schematic of virus feeding of mosquitoes and specimen collection. Mosquitoes were fed with infectious blood meal containing YF17D, YF-S0, YF-Asibi, or mock. Five mosquitoes were collected each for ingestion assessment. At 14 days post-feeding (dpf), remaining mosquitoes were dissected into two parts, midgut (infection assessment) and head, legs, and wings (dissemination assessment). (B) Viral RNA load by RT-qPCR. (C) Virus isolation by $TCID_{50}$ assay. For assessment of ingestion and infection, RT-qPCR and $TCID_{50}$ were performed on all samples. For assessment of dissemination, only a selection of PCR-positive specimens from the YF17D and YF-Asibi groups (n = 6 each) were further analyzed using $TCID_{50}$ assay, plus 6 randomly chosen from the YF-S0 group. (D and E) Heatmap representing positivity rates per experiment group as scored by RT-qPCR (D) and $TCID_{50}$ assay (E). Bars in (B) and (C) represent median values. N/A, not applicable. Mosquito icons were adapted from BioRender.com.

attenuated recombinant YF17D-vectored vaccines need to fulfill all World Health Organization (WHO) requirements for original yellow fever vaccine,[48] including monkey neurovirulence test as previously endorsed.[19]

To further investigate the potential environment risk associated with shedding of recombinant virus, we collected urine, feces, and buccal swabs from vaccinated hamsters and checked for the presence of viral RNA for 29 days to determine how long YF-S0 would remain

detectable in body secretions compared with YF17D. No significant differences in vaccine RNA shedding were observed between YF17D and YF-S0 during the course of immunization (Figures 2G–2I, AUC). Importantly, no infectious virus could be isolated, suggesting the risk is very low, even if any inadvertent exposure by vaccinated individuals to their environment. In summary, these results obtained in a hamster model of YF vaccination clearly demonstrate that (1) the overall viral tissue burden for YF-S0 was considerably lower than for parental YF17D, and (2) presence of viral RNA in body secretions (urine, feces, and buccal swab) was equally low as for YF17D, mostly likely void of residual infectious virus particles. YF-S0 vaccine virus infection is transient and harbors minimal, if at all any, risk for shedding or evidence for environmental biosafety concern.

Last, though the chances of YF17D-vectored vaccines to be transmitted by arthropod vectors are minimal, we evaluated the replication competence of YF-S0 in yellow fever mosquito vector (i.e., *Ae. aegypti*). Although parental YF17D passed the MIB and was restricted at the MEB as previous documented,[22,23] YF-S0 was already blocked at the first barrier, with no remaining viral RNA or infectious virus detectable after an infectious bloodmeal. Hence, the transmissibility of YF-S0 by mosquitoes is to be considered neglectable.

Altogether, YF-S0 can be considered a safe and efficacious vaccine candidate for the prevention of COVID-19. A similar improved safety compared with parental YF17D can be expected for other vaccines following the same design principle (i.e., using transgenic, yet fully replication-competent YF17D as a vector).[16,40]

MATERIALS AND METHODS

Animal experiment

Hamsters

Wild-type outbred specific pathogen-free Syrian hamsters (*Mesocricetus auratus*) were purchased from Janvier Laboratories (France). The generation[49] and characterization[25] of STAT2$^{-/-}$ (gene identifier: 101830537) hamsters has been described elsewhere. STAT2$^{-/-}$ hamsters were bred in-house. Hamsters (maximum n = 2) were housed in individually ventilated cages (Sealsafe Plus, Tecniplast; cage type GR900), under standard conditions of 21°C, 55% humidity, and 12 :12 h light/dark cycles. Hamsters were provided food and water ad libitum, as well as extra bedding material and wooden gnawing blocks for enrichment as previously described. This project was approved by the KU Leuven ethics committee (P015-2020), following institutional guidelines approved by the Federation of European Laboratory Animal Science Associations (FELASA). Hamsters were euthanized by intraperitoneal administration of 500 µL (hamsters) Dolethal (200 mg/mL sodium pentobarbital; Vétoquinol SA).

Vaccine and virus stocks

Vaccine viruses used throughout this study have been described.[17] YF-S0 was derived from a cDNA clone of YF17D (GenBank: X03700) with an in-frame insertion of a non-cleavable version of the SARS-CoV-2 S protein (GenBank: MN908947.3) in the YFV E/NS1 intergenic region. YF-S0 vaccine stocks were grown on baby hamster kidney (BHK21) cells. The molecular and antigenic structure and replication of YF-S0 have been described in detail.[17] Original YF17D vaccine (Stamaril, Sanofi-Pasteur; lot number G5400) was purchased via the pharmacy of the University Hospital Leuven and passaged twice in Vero E6 cells prior to use.

For the construction of YF-Asibi, the cDNA of YF17D sequence in plasmid pShuttle/YF17D[50] was adjusted to match previously described molecular YF-Asibi clone Ap7M (GenBank: MF926243) by using standard recombinant DNA technology. The respective cDNA was custom synthesized (IDT, Belgium) as six partially overlapping fragments (averaging 2 kb in size) and assembled by homologous recombination in yeast, *S. cerevisiae*. The thus obtained plasmid pShuttle/Ap7M was transfected into BHK-21J cells for virus rescue. The virus stock was prepared by passaging twice in BHK-21J cells, and the genome of YF-Asibi virus was confirmed by direct sequencing. All virus titers were determined by plaque assay on BHK-21J cells as before.[17]

Biodistribution

WT hamsters (6–8 weeks old, female) were inoculated intraperitoneally with a 10^4 PFU/mL dose of YF17D (n = 6) or YF-S0 (n = 6). STAT2$^{-/-}$ hamsters (6–8 weeks old, female) were inoculated intraperitoneally with 10^4 PFU/mL of YF17D (n = 2). At 7 dpi, blood, spleen, liver, brain, kidney, ileum, parotid gland, and lung were collected.

Shedding

WT hamsters (6–8 weeks old, female) were inoculated intraperitoneally with 10^4 PFU/mL of YF17D (n = 6) or YF-S0 (n = 6). STAT2$^{-/-}$ hamsters (6–8 weeks old, male) were inoculated intraperitoneally with 10^4 PFU/mL of YF17D (n = 3). Blood, urine, faces, and buccal swab were collected daily for the first 5 dpi, then every other day until 11 dpi and 15, 22 (except for the blood) and 29 dpi, and afterward once a week until 29 dpi.

Mosquito experiment

Mosquito strain

Ae. aegypti Paea[51] were obtained via the Infravec2 consortium (https://infravec2.eu/product/live-eggs-or-adult-females-of-aedes-aegypti-strain-paea-2/) from Institute Pasteur of Paris. Mosquitoes were maintained at the insectary of Rega Institute, and the fourth generation was used for this study. In brief, larvae were fed with yeast tablets (Gayelord Hauser, France) until the pupae stage prior to transfer to cages for emergence. Adults were maintained with cotton soaked in 10% sucrose solution under standard conditions (28°C, 80% relative humidity, and 14 h:10 h light/dark cycle).

Oral infection and sample collection

Seven-day-old female mosquitoes were starved 24 h prior to infection. Infectious blood meals contained rabbit erythrocytes plus 5 mM adenosine triphosphate as phagostimulant, supplemented with virus stocks to final titers of 2×10^5 PFU/mL for both YF17D and YF-S0, and 5×10^6 PFU/mL for YF-Asibi, respectively.

After 45 min, five fully engorged females from each group were frozen for viral input assessment (ingestion check; Figure 3A), and the rest were kept with 10% of sugar solution under both controlled conditions (28 ± 1°C, relative humidity 80%, light/dark cycle 14 h:10 h, supplied with 10% sucrose solution) and BSL-3 containment conditions. At 14 dpi, mosquitoes were dissected into two parts; main body (thorax and abdomen) and remainder, collected individually in tubes containing PBS and 2.8 mm ceramic beads (Precellys). The samples were homogenized and pass through 0.8 μm column filters (Sartorius, Germany). Thus, cleared supernatants were used for $TCID_{50}$ assay or kept at −80°C for RNA extraction and subsequent RT-qPCR analysis.

RNA extraction

Solid tissues (organs), feces, and buccal swabs were homogenized in a bead mill (Precellys) in lysis buffer (catalog no. 740984.10; Macherey-Nagel). After homogenization, samples were centrifuged at 10,000 rpm for 5 min to remove cell debris, and total RNA was extracted by using NucleoSpin Plus RNA virus Kit (catalog no. 740984.10; Macherey-Nagel). For serum (50 μL), urine (50 μL) and homogenates of mosquito samples (150 μL), NucleoSpin RNA virus kit (catalog no. 740956.250; Macherey-Nagel) was used for RNA extraction.

RT-qPCR

RT-qPCR for YFV detection was performed as previously described[17] using primers and probe targeting the YFV NS3 gene[23] on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Absolute quantification was based on standard curves generated from 5-fold serial dilutions of YF17D cDNA with a known concentration.

$TCID_{50}$ assay

For virus isolation and quantification BHK21 cells were infected with 10-fold serial dilutions in 96-well plates and incubated at 37°C for 6 days using DMEM with 2% fetal bovine serum (Hyclone), 2 mM L-glutamine (Gibco), 1% sodium bicarbonate (Gibco), and 1% antibiotics (PenStrep) as assay medium. Solid tissues were homogenized in a bead mill (Precellys) in assay medium and centrifuged at 10,000 rpm for 5 min (4°C) to remove debris. Resulting viral titers were calculated using the Reed and Muench method.

Serum neutralization test

Titers of YFV-specific neutralizing antibodies were determined using BHK21 cells and a mCherry-tagged variant of YF17D virus (YFV-mCherry) as described.[17] In brief, YFV-mCherry was mixed and incubated with serial diluted of sera for 1 h at 37°C and subsequently transferred to BHK21 cells grown in 96-well plates for infection. At 3 days post-infection, the relative infection rate was quantified by counting mCherry-expressing cells versus total cells on a high-content screening platform (CX5, Thermo Fisher Scientific), normalizing the infection rate of untreated virus controls as 100%. Half-maximal serum neutralizing titers (SNT_{50}) were determined by curve fitting in GraphPad Prism 8.

Statistics

Data were analyzed using GraphPad Prism 8. Results are represented as individual values and median for summary statistics. Statistical significance was determined using non-parametric Mann-Whitney U test (*$p \leq 0.05$ and **$p \leq 0.01$; ns, not significant).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.03.010.

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AUTHOR CONTRIBUTIONS

Overall Conceptual Design, L.-H.L., L.L., D.T., and K.D.; Methodology: L.-H.L., L.L., L.W.; *In Vivo* Experiments (Hamsters), L.L. and M.L.; *In Vivo* Experiments (Mosquitoes), L.W. and A.L.R.R.; *In Vitro* Experiments, L.-H.L. and X.Z.; *In Vitro* Experiment (Serology), H.J.T.; Design and Generation of YF-Asibi, M.B.Y. and S.J.; Data Management and Analysis: L.-H.L. and L.L.; Writing – Original Draft, L.-H.L.; Writing – Review & Editing: L.-H.L., L.L., L.W., L.D., D.T., and K.D.; Supervision, J.N. and K.D.; Funding Acquisition, J.N. and K.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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