

Supplementary Materials

Phase 1 Safety and Immunogenicity Evaluation of a Next-Generation DNA-Based Yellow Fever Vaccine

Table of Contents

Supplementary Table 1. Overview of Vaccine-Related Solicited Adverse Events 2

Supplementary Table 2. Overview of Unsolicited Adverse Events 3

Supplementary Table 3. Overview of Vaccine-Related Unsolicited Adverse Events 5

Supplementary Table 4. Inclusion -and Exclusion Criteria (Part 1 of the SAFYR study) 6

Additional Information on the Study Design 8

Grading of Adverse Events..... 10

Evaluation of Cell-Mediated Immune Responses 11

Supplementary Table 1. Overview of Vaccine-Related Solicited Adverse Events

		30 µg (3-s) (N = 12)	100 µg (3-s) (N = 12)	100 µg (1-s) (N = 12)	300 µg (3-s) (N = 12)	Placebo (3-s) (N = 12)	Placebo (1-s) (N = 4)
Event	Grade	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Local							
Pain	Any	2 (17)	1 (8)	0	3 (25)	0	0
	Grade 1	2 (17)	1 (8)	0	3 (25)	0	0
Redness	Any	0	0	0	0	0	0
Swelling	Any	0	0	0	0	0	0
General							
Fatigue	Any	5 (42)	4 (33)	2 (17)	4 (33)	4 (33)	2 (50)
	Grade 1	4 (33)	4 (33)	2 (17)	3 (25)	4 (33)	2 (50)
	Grade 2	1 (8)	0	0	1 (8)	0	0
Fever	Any	0	0	0	0	0	0
Headache	Any	3 (25)	3 (25)	2 (17)	4 (33)	2 (17)	0
	Grade 1	2 (17)	2 (17)	2 (17)	4 (33)	2 (17)	0
	Grade 2	1 (8)	1 (8)	0	0	0	0
Myalgia	Any	1 (8)	2 (17)	1 (8)	2 (17)	1 (8)	1 (25)
	Grade 1	1 (8)	1 (8)	1 (8)	2 (17)	1 (8)	1 (25)
	Grade 2	0	1 (8)	0	0	0	0

Number (n) and percentage (%) of participants reporting the indicated event. 1-s, groups vaccinated through 1-site intradermal delivery; 3-s, groups vaccinated through 3-site intradermal delivery

Supplementary Table 2. Overview of Unsolicited Adverse Events

System Organ Class	30 µg (3-s) (N = 12)	100 µg (3-s) (N = 12)	100 µg (1-s) (N = 12)	300 µg (3-s) (N = 12)	Placebo (3-s) (N = 12)	Placebo (1-s) (N = 4)
Preferred Term	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Blood and Lymphatic System Disorders						
Leukopenia	0	0	1 (8)	0	0	0
Neutropenia	0	2 (17)	1 (8)	0	0	0
Ear and labyrinth disorders						
Ear pain	0	0	0	0	1 (8)	0
Eye disorders						
Conjunctival hemorrhage	0	0	0	1 (8)	0	0
Eye pain	1 (8)	0	0	0	0	0
Gastrointestinal disorders						
Abdominal pain	2 (17)	1 (8)	0	1 (8)	1 (8)	0
Aphthous ulcer	1 (8)	0	0	0	0	0
Dysphagia	0	0	1 (8)	0	0	0
Gastrointestinal pain	0	0	0	0	0	2 (50)
Hyperchlorhydria	0	0	0	0	0	1 (25)
Nausea	1 (8)	1 (8)	0	0	1 (8)	1 (25)
Rectal discharge	1 (8)	0	0	0	0	0
Toothache	0	0	1 (8)	0	0	0
Vomiting	0	1 (8)	1 (8)	0	0	0
General disorders and administration site conditions						
Chest discomfort	0	0	1 (8)	0	0	0
Chest pain	0	0	0	0	1 (8)	0
Feeling hot	0	0	1 (8)	0	0	0
Hangover	0	0	1 (8)	0	0	0
Injection site hemorrhage	1 (8)	0	0	0	1 (8)	0
Malaise	0	1 (8)	0	1 (8)	0	0
Peripheral swelling	0	0	1 (8)	0	0	0
Pyrexia	0	0	2 (17)	0	0	0
Injury, poisoning and procedural complications						
Joint dislocation	0	0	1 (8)	0	0	0
Post-traumatic pain	0	0	0	1 (8)	0	0
Infections and infestations						
Gastroenteritis	0	1 (8)	0	0	0	0
Nasopharyngitis	0	1 (8)	0	2 (17)	1 (8)	1 (25)
Oral herpes	1 (8)	0	0	0	0	0
Peritonsillar abscess	0	1 (8)	0	0	0	0
Pharyngitis	0	1 (8)	1 (8)	0	0	0
Respiratory tract infection	0	0	0	1 (8)	0	0
Sinusitis	0	0	0	0	1 (8)	0
Upper respiratory tract infection	0	1 (8)	0	0	1 (8)	0

System Organ Class	30 µg (3-s) (N = 12)	100 µg (3-s) (N = 12)	100 µg (1-s) (N = 12)	300 µg (3-s) (N = 12)	Placebo (3-s) (N = 12)	Placebo (1-s) (N = 4)
Preferred Term	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Investigations						
Alanine aminotransferase increased	0	0	2 (17)	0	0	0
Aspartate aminotransferase increased	0	0	1 (8)	0	0	0
Lymphocyte count decreased	0	0	1 (8)	0	0	0
Neutrophil count decreased	0	0	2 (17)	0	0	1 (25)
Musculoskeletal and connective tissue disorders						
Arthralgia	1 (8)	0	0	0	0	0
Back pain	0	2 (17)	0	0	2 (17)	1 (25)
Myalgia	0	0	1 (8)	1 (8)	0	0
Neck pain	0	0	0	1 (8)	0	0
Pain in extremity	0	0	1 (8)	0	0	0
Nervous system disorders						
Dizziness	0	0	0	1 (8)	0	0
Headache	0	0	1 (8)	3 (25)	1 (8)	1 (25)
Hypoesthesia	1 (8)	0	0	0	0	0
Reproductive system and breast disorders						
Dysmenorrhea	0	0	0	0	0	1 (25)
Respiratory, thoracic and mediastinal disorders						
Oropharyngeal pain	1 (8)	0	0	0	0	0
Rhinorrhea	0	0	0	0	1 (8)	0
Sneezing	0	0	0	0	0	1 (25)
Skin and Subcutaneous Tissue Disorders						
Pruritus	0	0	0	0	1 (8)	0
Sensitive Skin	0	0	1 (8)	0	0	0
Skin Irritation	0	0	1 (8)	0	0	1 (25)

Number (n) and percentage (%) of participants reporting the indicated event. 1-s, groups vaccinated through 1-site intradermal administration; 3-s, groups vaccinated through 3-site intradermal administration

Supplementary Table 3. Overview of Vaccine-Related Unsolicited Adverse Events

System Organ Class	30 µg (3-s) (N = 12)	100 µg (3-s) (N = 12)	100 µg (1-s) (N = 12)	300 µg (3-s) (N = 12)	Placebo (3-s) (N = 12)	Placebo (1-s) (N = 4)
Preferred Term	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Blood and Lymphatic System Disorders						
Leukopenia	0	0	1 (8)	0	0	0
Neutropenia	0	2 (17)	1 (8)	0	0	0
Gastrointestinal Disorders						
Abdominal pain	0	1 (8)	0	0	0	0
Nausea	0	1 (8)	0	0	0	0
Vomiting	0	0	1 (8)	0	0	0
General Disorders and Administration Site Conditions						
Chest discomfort	0	0	1 (8)	0	0	0
Feeling hot	0	0	1 (8)	0	0	0
Injection site hemorrhage	1 (8)	0	0	0	1 (8)	0
Peripheral swelling	0	0	1 (8)	0	0	0
Pyrexia	0	0	2 (17)	0	0	0
Investigations						
Alanine aminotransferase increased	0	0	2 (17)	0	0	0
Aspartate aminotransferase increased	0	0	1 (8)	0	0	0
Lymphocyte count decreased	0	0	1 (8)	0	0	0
Neutrophil count decreased	0	0	2 (17)	0	0	1 (25)
Musculoskeletal and connective tissue disorders						
Myalgia	0	0	1 (8)	0	0	0
Pain in extremity	0	0	1 (8)	0	0	0
Nervous System Disorders						
Dizziness	0	0	0	1 (8)	0	0
Hypoesthesia	1 (8)	0	0	0	0	0
Skin and Subcutaneous Tissue Disorders						
Pruritus	0	0	0	0	1 (8)	0
Sensitive skin	0	0	1 (8)	0	0	0
Skin irritation	0	0	1 (8)	0	0	0

Number (n) and percentage (%) of participants reporting the indicated event. 1-s, groups vaccinated through 1-site intradermal administration; 3-s, groups vaccinated through 3-site intradermal administration

Supplementary Table 4. Inclusion -and Exclusion Criteria (Part 1 of the SAFYR study)

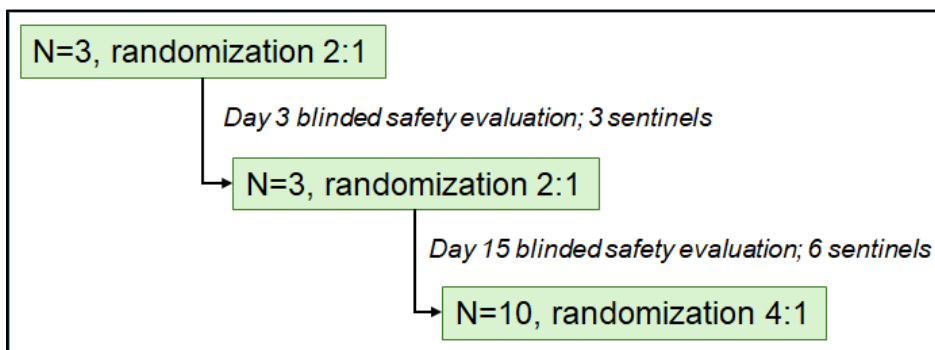
Inclusion Criteria	
1	Male or female between, and including, 18 and 40 years of age on the day of the Screening Visit
2	Healthy individual, as established by the investigator through medical history, physical examination and laboratory assessment (absence of clinically significant hematological [hemoglobin level, platelet count, white blood cell (WBC) count and WBC differential count] or biochemical [alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine] abnormalities, in the opinion of the investigator)
3	Able to read and understand the ICF, and written informed consent obtained from the participant
4	Participants who, in the opinion of the investigator, can and will comply with the requirements of the protocol (e.g. completion of the electronic diary [eDiary], return for follow-up visits)
Exclusion Criteria	
1	Body Mass Index <18.0 or >32.0 kg/m ²
2	Use of any investigational or non-registered product (vaccine, drug, medical device or medical procedure) other than the study intervention within 1 month preceding study vaccination, or planned use during the study period
3	Concurrently participating in another clinical study, at any time during the study period, in which the participant has been or will be exposed to an investigational or non-registered product (vaccine, drug, medical device or medical procedure)
4a	Administration / planned administration of any vaccine not foreseen by the study protocol within 1 month preceding study vaccination and up to Day 31.
4b	Previous vaccination (at any time) against a flavivirus disease (including yellow fever), as well as planned vaccination during the study period (e.g. due to travel to countries for which a yellow fever vaccination is required).
5	History (confirmed or suspected) of any flavivirus infection (including Yellow Fever virus, Dengue virus, Zika virus, West Nile virus, Japanese Encephalitis virus, Tick-Borne Encephalitis virus)
6	Administration of any anti-viral within 2 months preceding study vaccination and planned administration up to 6 weeks following the study vaccination. Topical anti-virals are allowed.
7	Chronic administration (defined as more than 14 consecutive days in total) of immunosuppressants or other immune-modifying drugs within 6 months preceding study vaccination, or planned chronic administration at any time during the study period (for corticosteroids, this will mean prednisone ≥ 20 mg/day, or equivalent). Inhaled and topical steroids are allowed.
8	Planned administration of long-acting immune-modifying drugs at any time during the study period
9	Administration of immunoglobulins and / or any blood or blood-derived products within 3 months preceding study vaccination, or planned administration during the study period
10	Any confirmed or suspected immunosuppressive or immunodeficient condition, based on medical history or physical examination (no laboratory testing required), including HIV infection, hepatitis B infection and hepatitis C infection
11	Family history of congenital or hereditary immunodeficiency
12	History of or current autoimmune disease
13	Personal or family history of thymic pathology (thymoma, thymectomy, or myasthenia gravis)
14	History of any neurological disorders or seizures including Guillain-Barré syndrome, with the exception of febrile seizures during childhood
15	History of any reaction or hypersensitivity likely to be exacerbated by any component of the investigational vaccines
16	Malignancies or lymphoproliferative disorders within previous 5 years (excluding effectively treated non-melanoma skin cancer)
17	Moderate or severe acute disease in the opinion of the investigator, and / or fever at the time of study intervention • Fever is defined as a temperature of ≥38.0 °C for oral route of measurement.
18	Alcohol, prescription drug, or substance (ab)use that, in the opinion of the investigator, might interfere with the study conduct or completion
19	Pregnant or lactating woman
20a	Woman of childbearing potential (including the partner of a man of reproductive potential who participates in the study) who is not utilizing a highly effective birth control method for at least 1 month preceding study vaccination, or planning to discontinue highly effective birth control methods during the study period.

20b	Man of reproductive potential unwilling to use a barrier method of contraception (preferably male condom) when engaging in sexual intercourse with a women of childbearing potential, from study vaccination up to Day 61.
21	Woman planning to do egg / ovum donation, or man planning to donate sperm, from study vaccination up to Day 61
22	Close contact (<i>i.e.</i> living under the same roof, caregiver, involved in clinical care) for 2 months after study vaccination with an immunocompromised person, an infant < 6 months of age, or any individual that, in the judgement of the investigator, may be at increased risk
23	Planning to donate blood or organs from study vaccination up to 3 months after study vaccination
24	Participants employed by the Sponsor, by the vendors working on behalf of the Sponsor, by the investigator or the study site, or close relatives of research staff working on this study
25	Medical condition or use of medication(s) that can increase the risk of bleeding or hematoma from the blood sampling
26	Any other condition or finding that the investigator judges may interfere with study procedures (<i>e.g.</i> drawing blood) or study results (<i>e.g.</i> large tattoo on the location[s] for the intradermal injection)

Additional Information on the Study Design

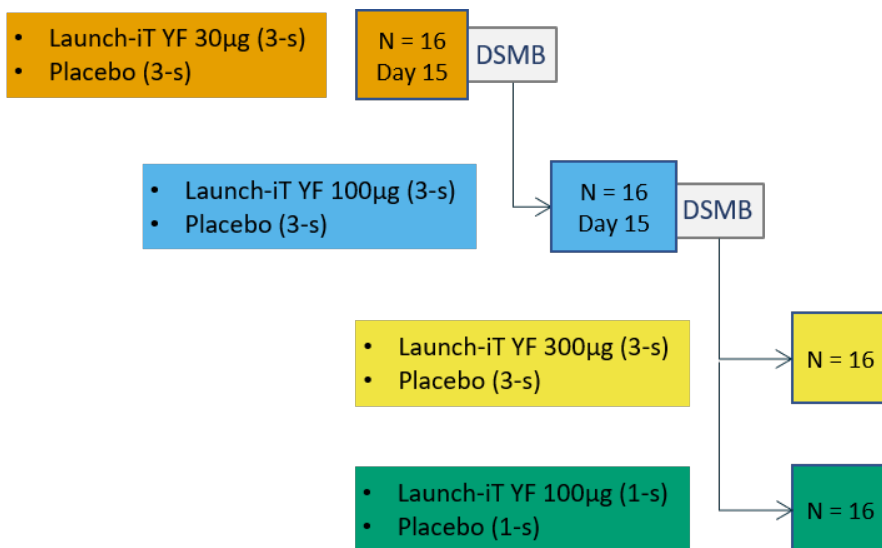
Staggered Design within Each Dose Group

Within each dose group, 16 participants were randomized to receive Launch-iT YF or placebo. Vaccination within each dose group followed a sentinel design, starting with the vaccination of three sentinel participants, who were randomized in a 2:1 ratio to Launch-iT YF or to placebo, and vaccinated at least one hour apart. The safety of the first three sentinel participants was monitored by the investigator in a blinded manner up to the Day 3 visit. If no safety concerns were identified, three additional sentinel participants were randomized and vaccinated under the same conditions as the first sentinels. After that, the safety of all sentinel participants was monitored by the investigator in a blinded manner up to at least the Day 15 visit. If no safety concerns were identified, the remaining 10 participants were vaccinated. These participants were randomized in a 4:1 ratio to Launch-iT YF or to placebo. Refer to Supplementary Figure S.1 for a schematic overview of the staggered design within each dose group.



Supplementary Figure 1. Staggered design within each dose group.

Dose Escalation Schema



Supplementary Figure 2. Dose Escalation Schema. DSMB, data safety monitoring board

Data Safety Monitoring Board

A data safety monitoring board (DSMB) was established. The DSMB was composed of 3 physicians (who were the DSMB voting members) and a statistician, all of whom were independent and external to the study. They were supported by an unblinded DSMB manager who was not a DSMB member as such.

For the 3-s dose groups, dose escalation was only permitted following a favorable DSMB review of at least the Day 15 safety data from all participants in the previous dose group. In addition, the 1-s dose group was allowed to proceed in parallel with the vaccination of the highest 3-s dose group. A schematic presentation of the scheduled DSMB meetings can be found in **Supplementary Figure 2**. In addition, *ad hoc* DSMB meetings could be called at any time if a safety signal was observed outside the scheduled meetings.

During each DSMB data review meeting, the DSMB reviewed all available safety data. After each meeting, the DSMB provided their recommendations in a blinded manner to the Sponsor.

Randomization and Blinding

An unblinded statistician generated the fixed randomization list and the treatment kit allocation list, which uploaded into the electronic case report form system (Viedoc). Following confirmation of the participant's eligibility at Day 1, the investigator obtained the allocated kit number corresponding with the participant's randomization assignment through the system. To achieve blinding, Launch-iT YF and Placebo kits (including their contents) were visually indistinguishable. The study participants and site staff remained blinded to the participants' treatment assignments throughout the study.

Grading of Adverse Events

Solicited adverse events (AEs) were graded per the table below.

Adverse Event(s)	Grade	Parameter
Pain at the injection site	0	None
	1	Pain that is easily tolerated, causing minimal discomfort and not interfering with normal everyday activities
	2	Painful when limb is moved, and the pain is sufficiently discomforting to interfere to some extent with normal everyday activities
	3	Pain that interferes significantly with or prevents normal, everyday activities ¹
Redness at the injection site, Swelling at the injection site	0	< 25mm
	1	25mm to 50mm
	2	51mm to 100mm
	3	> 100mm
Fever ²	0	< 38.0°C
	1	38.0°C to 38.4°C
	2	38.5°C to 38.9°C
	3	≥ 39.0°C
Fatigue, Myalgia	0	None
	1	Easily tolerated, causing minimal discomfort and not interfering with normal everyday activities
	2	Sufficiently discomforting to interfere to some extent with normal everyday activities
	3	Interfering significantly with or prevents normal, everyday activities ¹
Headache	0	None
	1	Easily tolerated, causing minimal discomfort and not interfering with normal everyday activities
	2	Sufficiently discomforting to interfere to some extent with normal everyday activities, or repeated use of non-narcotic pain reliever > 24 hours
	3	Interfering significantly with or prevents normal, everyday activities ¹ , or any use of narcotic pain reliever

¹ This would for instance mean the prevention of going to work.

² Oral route of measurement.

Unsolicited AEs were graded as follows:

- Mild: usually transient and may require only minimal treatment or therapeutic intervention. The event does not generally interfere with normal everyday activities.
- Moderate: usually alleviated with additional specific therapeutic intervention. The event interferes to some extent with normal everyday activities, causing discomfort but poses no significant or permanent risk of harm to the participant.
- Severe: interrupts significantly with or prevents normal everyday activities, or significantly affects clinical status, or may require intensive therapeutic intervention.

Hematology and biochemistry results were evaluated against the local normal range. For parameters outside the normal range, the investigator used clinical judgement to determine whether the event was clinically significant. Clinically significantly abnormal laboratory parameters were recorded as unsolicited AEs and graded.

For each AE, the maximum severity that occurred for the duration of the event was assigned.

Evaluation of Cell-Mediated Immune Responses

Thirty (30) mL blood was collected by venous puncture in lithium heparin coated blood collection tubes (Becton Dickinson). Peripheral blood mononuclear cell (PBMC) isolation was performed within 24 hours of sample collection at the Center for Vaccinology (CEVAC), Ghent University (Ghent, Belgium), as described in detail elsewhere (Waerlop *et al.*, 2022). In brief, blood samples were diluted 1:2 in Hanks buffered salt solution (HBSS) and layered over a density gradient medium (Lymphoprep™). After centrifugation, the PBMC layer was collected. Cells were then washed twice in HBSS, counted and resuspended in freezing medium (10% dimethyl sulfoxide [DMSO] / 90% fetal bovine serum [FBS]). Cells were cryopreserved at a concentration of minimum 5×10^6 PBMC/vial and stored in liquid nitrogen until use.

Intracellular cytokine staining (ICS) was performed as described in detail elsewhere (Begue *et al.*, 2022). Briefly, cryopreserved PBMCs were thawed and suspended in RPMI 1640 medium supplemented with Minimum Essential Medium non-essential amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate, 2-mercapto-ethanol (all from Invitrogen), and fetal bovine serum (Seradigm). Then, PBMCs were stimulated *in vitro* with yellow fever peptide pool (PepMix Pan-Yellow Fever, JPT Peptide Technologies) or left unstimulated (background condition with 0.32% DMSO) and subsequently incubated at 37°C with 5% CO₂. After 2 hours, a protein transport inhibitor was added prior to overnight incubation. The following day, cells were stained for viability and surface markers (CD4/CD8), then fixed, permeabilized and stained intracellularly (CD3/CD40L/IL2/TNFα/IFNγ). Samples were acquired on a BD LSR FORTRESSA X-20 (Becton Dickinson). Data was analyzed using Diva.

All ICS measurements were background-corrected by subtracting the percentage of cytokine-producing CD4⁺ or CD8⁺ T cells in corresponding negative (DMSO) samples from the percentage in YFV peptide-stimulated samples. Negative values after background subtraction were set to 0.0001% for log-scale graphical displays and to 0% for linear-scale displays. Samples with elevated background responses, defined as DMSO control values exceeding a predefined acceptance threshold (>0.1% polyfunctional T cells), were excluded from the analysis.

References

- Begue, S. et al. FLUCOP Consortium Collaborators. Harmonization and qualification of intracellular cytokine staining to measure influenza-specific CD4⁺ T cell immunity within the FLUCOP consortium. *Front Immunol.* **13**; 10.3389/fimmu.2022.982887 (2022).
- Waerlop, G. et al. Harmonization and qualification of an IFN-γ Enzyme-Linked ImmunoSpot assay (ELISPOT) to measure influenza-specific cell-mediated immunity within the FLUCOP consortium. *Front Immunol.* **13**; 10.3389/fimmu.2022.984642 (2022).