Immunogenicity, Safety, and Efficacy of a Standalone Universal Influenza Vaccine, FLU-v, in Healthy Adults
A Randomized Clinical Trial

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Background: FLU-v is a broad-spectrum influenza vaccine that induces antibodies and cell-mediated immunity.

Objective: To compare the safety, immunogenicity, and exploratory efficacy of different formulations and dosing regimens of FLU-v versus placebo.

Design: Randomized, double-blind, placebo-controlled, single-center phase 2b clinical trial. (ClinicalTrials.gov: NCT02962908; EudraCT: 2015-001932-38)

Setting: The Netherlands.

Participants: 175 healthy adults aged 18 to 60 years.

Intervention: 0.5-mL subcutaneous injection of 500 μg of adjuvanted (1 dose) or nonadjuvanted (2 doses) FLU-v (A-FLU-v or NA-FLU-v) or adjuvanted or nonadjuvanted placebo (A-placebo or NA-placebo) (2:2:1:1 ratio).

Measurements: Vaccine-specific cellular responses at days 0, 42, and 180 were assessed via flow cytometry and enzyme-linked immunosorbent assay. Solicited information on adverse events (AEs) was collected for 21 days after vaccination. Unsolicited information on AEs was collected throughout the study.

Results: The AEs with the highest incidence were mild to moderate injection site reactions. The difference between A-FLU-v and A-placebo in the median fold increase in secreted interferon-γ (IFN-γ) was 38.2-fold (95% CI, 4.7- to 69.7-fold; *P* = 0.001) at day 42 and 25.0-fold (CI, 5.7- to 50.9-fold; *P* < 0.001) at day 180. The differences between A-FLU-v and A-placebo in median fold increase at day 42 were 4.5-fold (CI, 2.3- to 9.8-fold; *P* < 0.001) for IFN-γ–producing CD4$^+$ T cells, 4.9-fold (CI, 1.3- to 40.0-fold; *P* < 0.001) for tumor necrosis factor-α (TNF-α), 7.0-fold (CI, 3.5- to 18.0-fold; *P* < 0.001) for interleukin-2 (IL-2), and 1.7-fold (CI, 0.1- to 4.0-fold; *P* = 0.004) for CD107a. At day 180, differences were 2.1-fold (CI, 0.0- to 6.0-fold; *P* = 0.030) for IFN-γ and 5.7-fold (CI, 2.0- to 15.0-fold; *P* < 0.001) for IL-2, with no difference for TNF-α or CD107a. No differences were seen between NA-FLU-v and NA-placebo.

Limitation: The study was not powered to evaluate vaccine efficacy against influenza infection.

Conclusion: Adjuvanted FLU-v is immunogenic and merits phase 3 development to explore efficacy.

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Influenza is a severe respiratory disease that affects 3 to 5 million people worldwide each year, leading to an estimated 650 000 deaths (1). Currently, the best available prophylactic treatment is annual vaccination with inactivated or attenuated influenza virus. However, availability of doses is limited, and these vaccines have little capacity for cross-protection (2), which affects efficacy when mismatching occurs or new pandemic strains emerge (3). FLU-v is a mix of 4 synthetic peptides that originate from conserved regions of the matrix-1, matrix-2, and nucleoprotein influenza proteins. It is designed to provide cross-protection against A and B influenza strains by inducing humoral and cell-mediated immunity (4–6).

The objective of this study was to investigate the safety, cellular and humoral immunogenicity, and exploratory efficacy of different formulations and dosing regimens of FLU-v in healthy adults in a phase 2b setting.

METHODS
Trial Design
The study was a randomized, placebo-controlled, double-blind, single-center clinical trial. Healthy adults aged 18 to 60 years who met the eligibility criteria reported previously (7) were recruited at Isala Hospital in Zwolle, the Netherlands. Study approval was obtained after ethical review by the Central Committee on Research Involving Human Subjects (reference NL55061.000.15), followed by review by the competent authority (the Dutch Ministry of Health, Welfare and Sport). Informed consent was obtained from each participant before the trial interventions.

Interventions
FLU-v is a sterile 500-μg equimolar mix of 4 lyophilized synthetic peptides (5) (**Appendix Table 1**, available at Annals.org). Montanide ISA-51 (mineral oil/mannide monooleate) (SEPPIC) was used as an adjuvant to enhance immune responses to FLU-v antigens.

See also:

Web-Only
Supplement

A volume of 0.5 mL of the following formulations was administered subcutaneously: nonadjuvanted FLU-v (NA-FLU-v) (FLU-v as neutral HCl/NaOH suspension on days 0 and 21), adjuvanted FLU-v (A-FLU-v) (FLU-v in water-in-oil emulsion [water: Montanide ISA-51] on day 0 and saline on day 21), nonadjuvanted placebo (NA-placebo) (saline on days 0 and 21), and adjuvanted placebo (A-placebo) (water: Montanide ISA-51 on day 0 and saline on day 21).

Outcomes
Safety
Solicited information on adverse events (AEs) was collected via diary card for 21 days after each vaccination. Unsolicited information on AEs and serious AEs (SAEs) was collected throughout the study. The AE severity, seriousness, and relationship with the intervention were assessed by the investigator (Appendix, available at Annals.org). Adverse events were coded using the Medical Dictionary for Regulatory Activities (version 17.1) terminology for System Organ Class and Preferred Terms.

Primary Immunogenicity
Changes in cellular immune responses on days 42 and 180 compared with day 0 were evaluated as the change in Th1 cytokine production after vaccination with FLU-v (given as a suspension or an emulsion [adjuvanted]) compared with placebo. Cellular immunogenicity data were assessed on days 0, 42, and 180 by using enzyme-linked immunosorbent assay (ELISA) to measure the amount of interferon-γ (IFN-γ) secreted by peripheral blood mononuclear cells in vitro in response to FLU-v antigens, and by using multiparametric flow cytometry (MFC) to measure the number of CD4+ and CD8+ T cells producing IFN-γ, tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), and CD107a.

Secondary Immunogenicity
Geometric mean titers of FLU-v–specific IgG and IgM were measured on days 0, 42, and 180 via ELISA.

Exploratory Efficacy
The number of influenza infections between 1 December 2016 and 31 March 2017 was assessed by reverse transcriptase polymerase chain reaction (RT-PCR) of viral RNA extracted from nasopharyngeal nasal and tonsil swabs. An additional analysis not mentioned in the protocol (Supplement, available at Annals.org) evaluated the proportion of responders and is described in the Statistical Analysis section.

Randomization
The randomization list was created with ALEA by the Trial Coordination Centre at University Medical Centre Groningen. Participants were assigned in a 2:2:1:1 ratio (A-FLU-v: NA-FLU-v: A-placebo: NA-placebo) and with stratification by age group (18 to 40 years and 41 to 60 years) to ensure similar age distribution among study groups. The randomization codes were

sent to the unblinded study pharmacist at Isala Hospital and were stored in a locked cabinet at the pharmacy that was accessible only to the pharmacist. Eligible participants were allocated the next number in sequence within the appropriate age group stratum by the unblinded onsite pharmacist who prepared the appropriate treatment. The syringe with the participant identifier was collected by the blinded clinical staff member and administered to the blinded participant.

Blinding
Clinical personnel were blinded to the presence of FLU-v antigens in the vaccine but not to the presence of adjuvant because of its different appearance and the injection site reactions it causes (Appendix).

Multiparametric Flow Cytometry
Peripheral blood mononuclear cells were stimulated for 24 hours with FLU-v antigen (1 and 2 μM of each peptide), staphylococcal enterotoxin B (positive control), or medium (negative control). Cytotoxicity was detected using anti-CD107a antibodies (eBioscience). Cells were stained for viability; fixed; permeabilized; and stained for CD3, CD4, CD8, intracellular IFN-γ, TNF-α, and IL-2 (eBioscience). After acquisition of samples by flow cytometry (LSR II and FACSDiva [BD Biosciences]), cells were gated (lymphocytes, viable cells, CD3+, CD4+, or CD8+). The number of cells in each population staining positive for each of the immune markers was determined by using the unstimulated cells to set gates for positive cells.

IFN-γ ELISA
Peripheral blood mononuclear cells were stimulated in vitro as was done for MFC. Secreted IFN-γ was measured with the IFN-γ Human ELISA Kit (Thermo Scientific).

IgG and IgM Antibody Responses by ELISA
Plates were coated with FLU-v antigen at 2 μM or human IgG/IgM standards (Sigma). Participants' sera were added at a 1:50 dilution followed by alkaline phosphatase–conjugated detection antibody (Sigma). Plates were developed and absorbance was measured at 450 and 405 nm (BioTek ELx808). A standard curve, plotted using Gen5, version 2.04 (BioTek Instruments), was used to interpolate the concentration of antibodies in the serum samples.

Hemagglutination Inhibition Assay
To assess the level of protection in the population at screening as part of the evaluation of demographic characteristics, antibodies against the circulating influenza strains in the 2016–2017 season (A/H3N2/Hong Kong/5738/2014 [clade 3C.2a], A/H1N1/pdm09 A/Michigan/45/2015 [subgroup 6B.1], B/Brisbane/60/2008 [Victoria lineage clade 1A], and B/Phuket/3073/2013 [Yamagata lineage clade 3]) were measured in serum samples. Hemagglutination inhibition (HAI) tests were performed in a single laboratory (8). The antibody titer was equal to the denominator of the highest serum dilution that fully or mostly inhibited agglutination. A cutoff of 40 was used as the protective titer for influenza A (9). For influenza B, the

cutoff was 80 because the sensitivity was enhanced through ether treatment of the antigen (10).

RT-PCR for Influenza Infection

Participants completed a daily symptom questionnaire during the influenza season. Nasal and tonsil swabs were collected after sudden onset of at least 1 respiratory and 1 systemic symptom (7). RNA was isolated using NucliSENS easyMAG (bioMérieux) followed by RT-PCR to detect influenza RNA from A or B strains using the matrix gene as the target. The results were analyzed using FlowG MiddleWare solution and FlowG 2010 software (Labhelp International). Samples that tested positive for influenza A were typed for H1 or H3.

Statistical Analysis

The sample size was based on the postvaccination increase in secretion of the main Th1 cytokine (IFN-γ), measured by ELISA. Power calculations were performed to determine the sample size necessary to detect a treatment effect of each active vaccine compared with its respective placebo. The estimation assumed a 2-fold difference between NA-FLU-v and NA-placebo and a 5-fold difference between A-FLU-v and A-placebo. A 2-sided type I error (α) with a 95% CI, a target power of 80%, and an SD of 1 were used as inputs, and calculations were done for 2:1 allocation (active vs. placebo). For this assessment, 150 participants were required. Enrolling 222 participants allowed dropout of 32% (7). Assessment of the interaction between treatment effects and the addition of adjuvant would have required 180 participants, but we did not aim to test this.

An electronic database was created with Oracle Clinical (version 4.6.6). Manipulation of data, tabulation of descriptive statistics, calculation of inferential statistics, and creation of graphical representations were done using Microsoft Office; SAS, version 9.4 (SAS Institute); SPSS, version 23 (IBM); and R, version 3.4.2 (11).

Safety was assessed in all participants who received at least 1 vaccine dose. Formal hypothesis testing was not done on the AE data because the study was not powered to detect differences in AE incidence. Cumulative incidence was calculated for each group. For the analysis of immunogenicity and efficacy end points, participants were included if they had received 2 doses of the vaccine and had data from before vaccination and at least 1 time point after. Differences in IgG and IgM geometric means and the ratio of geometric means were analyzed using the *t* test. Differences between groups in the median ratio from day 0 to days 42 and 180 for levels of secreted IFN-γ and the number of CD4$^+$/CD8$^+$ T cells producing the different markers were analyzed using the Mann–Whitney U test. Confidence intervals were calculated using the "wilcox.test" command in R.

Two approaches were used to define responders. Participants were considered responders if the immune parameter (cytokine-specific CD4$^+$/CD8$^+$ T cells measured via MFC, IFN-γ measured via ELISA, and IgM/IgG measured via ELISA) increased by at least 2-fold between day 0 and days 42 and 180 after vaccination. In addition, a mixture models for single-cell assays (MIMOSA) analysis (12–14) (**Appendix**) was done to detect true responders in flow cytometry for a marker at a time point if the false discovery rate–derived *P* value was less than 0.05. Differences in the percentages of responders between groups were compared using the χ^2 test or the Fisher exact test for each time point. The CIs for the proportions and the difference in proportions between groups were calculated using the Wilson score interval.

Vaccine efficacy was calculated as (1 − relative risk) × 100, where relative risk equaled the risk for influenza in the active treatment group divided by the risk in the combined placebo group. Calculation of the CI for vaccine efficacy was based on the CI for the relative risk. *P* values were calculated using the Fisher exact test. A 2-sided 5% level of significance was used throughout.

Role of the Funding Source

Authors affiliated with SEEK and Groningen Research Institute of Pharmacy contributed to the conception and design of the study. Authors affiliated with Isala Hospital collected participant data, authors affiliated with the Norwegian Institute of Public Health acquired antibody and HAI data, and authors affiliated with University Medical Centre Groningen collected data from swabs. All data were analyzed by Groningen Research Institute of Pharmacy and interpreted by all authors on the basis of their area of expertise. Authors affiliated with SEEK wrote the manuscript, and all authors provided critical feedback. The European Commission Directorate-General for Research and Innovation had no role in the study design, data analysis, writing of the manuscript, or the decision to submit the manuscript for publication. SEEK played a major role in designing the study, analyzing the data before unblinding, and writing and submission of the manuscript.

RESULTS

Demographic Characteristics and Study Populations

A total of 195 adults were screened starting in August 2016. Of these, 20 were not randomly assigned (3 were lost to follow-up, 9 withdrew consent, and 8 did not meet inclusion or exclusion criteria) (**Figure**). Recruitment was stopped in January 2017 when 175 participants had been vaccinated. A total of 165 participants attended the 5 scheduled visits (6% dropout). One participant in the A-FLU-v group was excluded from the analysis because of receipt of a single dose of NA-FLU-v, which did not fit into any of the treatment groups. Ninety-seven percent of participants were white, 44% were men, and the average age was 40 years (30% were aged ≥50 years). Twenty-nine percent of participants had received an influenza vaccination within the previous 2 years, 14% had received one more than 2 years previously, and 57% had never received one. The HAI titer was greater than 80 for

Figure. Study flow diagram.
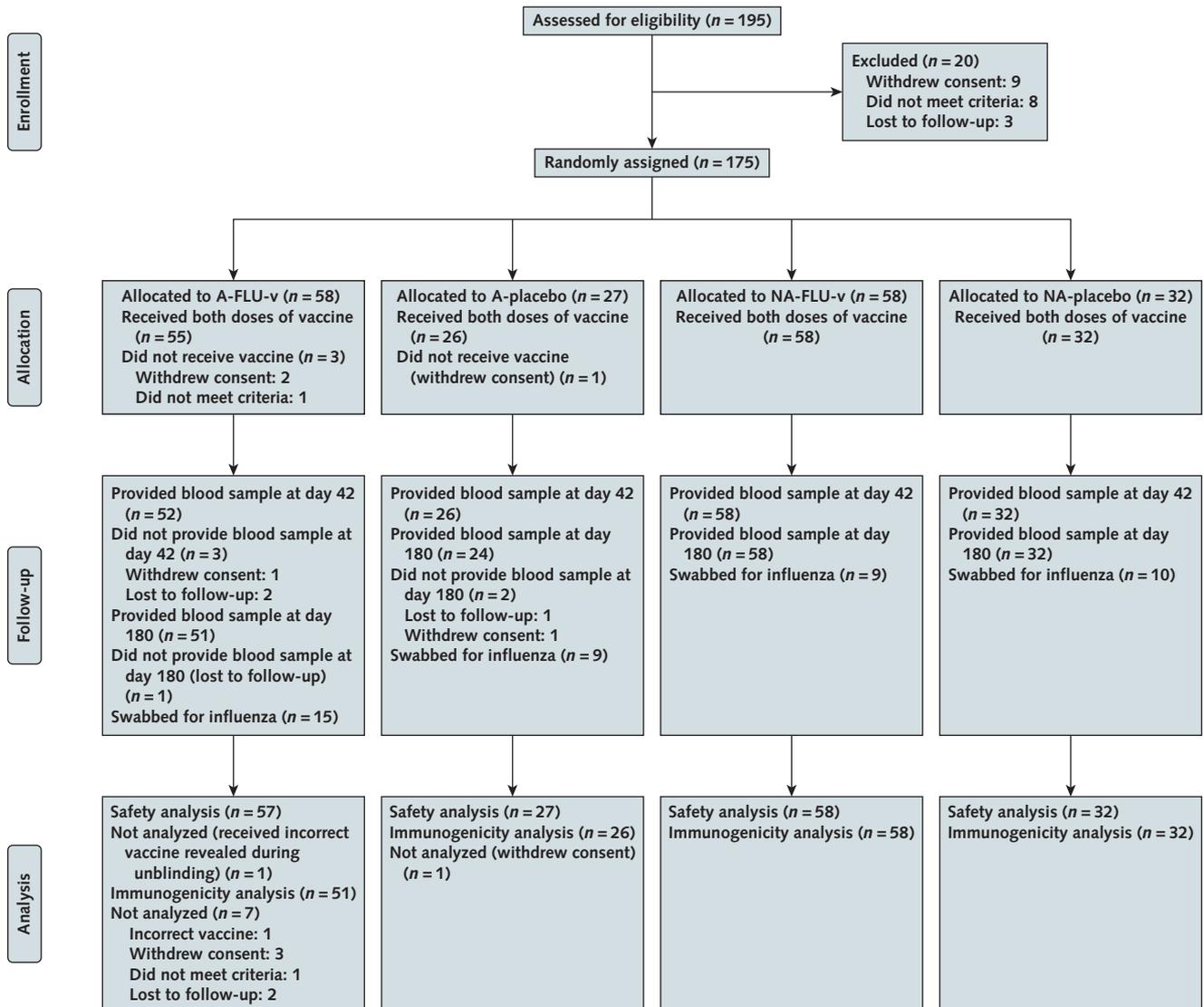
The diagram shows the number of adults who were screened and allocated to each treatment group. The number who completed the scheduled interventions and follow-ups and the number included in the safety and immunogenicity analyses are also presented. All randomly assigned participants who received their allocated intervention were included in safety analyses. Participants who were vaccinated and provided blood samples for immunogenicity on days 0 and 42 and/or 180 were included in the immunogenicity analyses (the participant who was lost to follow-up on day 180 had attended the day-42 visit and was included in the immunogenicity analysis). A-FLU-v = adjuvanted FLU-v; A-placebo = adjuvanted placebo; NA-FLU-v = nonadjuvanted FLU-v; NA-placebo = nonadjuvanted placebo.

B/Phuket/3073/2013 in 32% of participants and for B/Brisbane/60/2008 in 32%. The HAI titer was greater than 40 for A/Michigan/45/15(H1N1) in 45% of participants and for A/HongKong/5738/2014(H3N2) in 45% (Table 1).

Safety

There were no early withdrawals due to vaccine-related AEs. Adverse events were mainly mild to moderate and were related to reactions at the injection site, with the highest incidence observed in the A-FLU-v group followed by the A-placebo group (Table 2). A small number of participants had severe reactions at the injection site; all were resolved with over-the-

counter anti-inflammatory drugs and required no additional follow-up (Table 2). The only severe AE that occurred in more than 5% of participants was pain at the injection site in the single-dose A-FLU-v group (n = 3). Five SAEs were recorded, 2 of which were in the same participant and none of which were considered by the medical study team to be related to the vaccine. These included an upper limb fracture, alcohol abuse, and depression in the A-FLU-v group and a myocardial infarction 2 months after the second vaccination and a hernia repair in the NA-FLU-v group. A female participant in the A-FLU-v group became pregnant during the study. She was monitored throughout her

pregnancy and delivered a healthy baby before the study terminated.

Cell-Mediated Immunity by MFC and IFN-γ ELISA

Cellular responses increased after vaccination with A-FLU-v compared with A-placebo. No differences were observed between NA-FLU-v and NA-placebo. In the A-FLU-v group, the median fold increases from day 0 to day 42 in the number of CD4$^+$ T cells producing IFN-γ (5.9-fold [95% CI, 1.0- to 10.9-fold]), TNF-α (5.0-fold [CI, −10.9- to 20.9-fold]), IL-2 (8.2-fold [CI, −0.4- to 16.7-fold]), and CD107a (2.0-fold [CI, 0.3- to 3.7-fold]) were higher than in the A-placebo group (IFN-γ: 0.2-fold [CI, −0.6- to 1.0-fold]; TNF-α: 0.0-fold [CI, −0.3- to 0.3-fold]; IL-2: 0.0-fold [CI, −0.2- to 0.2-fold]; CD107a: 0.0-fold [CI, −0.2- to 0.2-fold]) (Table 3). This difference was still apparent on day 180 in the A-FLU-v group compared with A-placebo for CD4$^+$ T cells producing IFN-γ (4.4-fold [CI, 1.0- to 7.8-fold] vs. 0.9-fold [CI, −1.4- to 3.2-fold]) and IL-2 (6.5-fold [CI, 2.1- to 10.9-fold] vs. 0.0-fold [CI, −0.1- to 0.1-fold]) (Table 3). No differences were detected in the median fold increases between the vaccinated and placebo groups for CD8$^+$ T cells producing any of the markers tested.

Higher percentages of participants had at least a 2-fold increase in response at day 42 in the A-FLU-v group compared with the A-placebo group for CD4$^+$ T cells producing IFN-γ (difference, 49 percentage points [CI, 25 to 70 percentage points]; $P < 0.001$), TNF-α (dif-

ference, 25 percentage points [CI, −1 to 50 percentage points]; $P = 0.056$), IL-2 (difference, 60 percentage points [CI, 35 to 78 percentage points]; $P < 0.001$), and CD107a (difference, 39 percentage points [CI, 9 to 63 percentage points]; $P = 0.010$). At day 180, there were more responders in the A-FLU-v group than the A-placebo group for IFN-γ (difference, 26 percentage points [CI, 3 to 50 percentage points]; $P = 0.049$) and IL-2 (difference, 42 percentage points [CI, 17 to 63 percentage points]; $P < 0.001$) but not for TNF-α (difference, 12 percentage points [CI, −15 to 37 percentage points]; $P = 0.39$) or CD107a (difference, 0 percentage point [CI, −28 to 29 percentage points]; $P = 0.98$). The only marker in CD4$^+$ T cells with a 2-fold or greater increase after vaccination with NA-FLU-v was CD107a on day 180, with 48% of participants having an increase compared with 23% in the NA-placebo group (difference, 25 percentage points [CI, 1 to 45 percentage points]; $P = 0.044$) (Table 3). Little CD8$^+$ T-cell activation was observed, apart from 44% of participants in the A-FLU-v group and 45% in the NA-FLU-v group having a 2-fold or greater increase in the number of CD8$^+$ T cells stained with CD107a on day 42 compared with 12% and 18% in the respective placebo groups (differences, 32 percentage points [CI, 6 to 50 percentage points] [$P = 0.015$] and 27 percentage points [CI, 2 to 43 percentage points] [$P = 0.023$], respectively) (Appendix Table 2, available at Annals.org).

Table 1. Demographic and Baseline Characteristics*

Characteristic	A-FLU-v (n = 57)	A-Placebo (n = 27)	NA-FLU-v (n = 58)	NA-Placebo (n = 32)
Age				
Mean (SD), y	40.1 (12.2)	39.1 (13.1)	40.0 (13.7)	41.2 (12.5)
<50 y, n (%)	40 (70)	17 (63)	41 (71)	23 (72)
≥50 y, n (%)	17 (30)	10 (37)	17 (29)	9 (28)
Sex, n (%)				
Female	30 (53)	13 (48)	36 (62)	18 (56)
Male	27 (47)	14 (52)	22 (38)	14 (44)
Race/ethnicity, n (%)				
Asian	1 (2)	0 (0)	1 (2)	0 (0)
Black	0 (0)	0 (0)	0 (0)	0 (0)
White	54 (95)	27 (100)	56 (97)	32 (100)
Other	2 (4)	0 (0)	1 (2)	0 (0)
Influenza vaccination, n (%)				
≤2 y previously	20 (35)	5 (19)	14 (24)	12 (38)
>2 y previously	8 (14)	4 (15)	7 (12)	5 (16)
Never	29 (51)	18 (67)	37 (64)	15 (47)
Positive prevaccination HAI titer, n (%)†				
Phuket	16 (28)	7 (26)	21 (36)	12 (38)
Brisbane	19 (33)	7 (26)	18 (31)	12 (38)
Michigan	26 (46)	10 (37)	25 (43)	18 (56)
Hong Kong	29 (51)	11 (41)	23 (40)	16 (50)
Any	40 (71)	17 (63)	39 (67)	26 (81)

A-FLU-v = adjuvanted FLU-v; A-placebo = adjuvanted placebo; HAI = hemagglutination inhibition; NA-FLU-v = nonadjuvanted FLU-v; NA-placebo = nonadjuvanted placebo.
* Analysis includes all participants who received ≥1 vaccination.
† Detection of protective prevaccination HAI titers against influenza strains that circulated in the Netherlands during the 2016–2017 season. The protective threshold was ≥80 for B strains (Phuket and Brisbane) and ≥40 for A strains (Michigan and Hong Kong). Brisbane = B/Brisbane/60/2008; Hong Kong = A/Hong Kong/5738/2014 (H3N2); Michigan = A/Michigan/45/15(H1N1)pdm09; Phuket = B/Phuket/3073/2013.

Table 2. Summary of Treatment-Emergent AEs and Injection Site Reactions*

Variable	A-FLU-v (n = 57)	A-Placebo (n = 27)	NA-FLU-v (n = 58)	NA-Placebo (n = 32)
≥1 treatment-emergent AE	54 (95)	27 (100)	52 (90)	28 (88)
Severe treatment-emergent AE	10 (18)	2 (7)	5 (9)	2 (6)
Treatment-emergent AE leading to early termination	0 (0)	0 (0)	0 (0)	0 (0)
SAE†	2 (4)	0 (0)	2 (3)	0 (0)
Death	0 (0)	0 (0)	0 (0)	0 (0)
Treatment-emergent AE definitely related to vaccine‡	53 (93)	20 (74)	20 (34)	6 (19)
Myalgia				
Mild	1 (2)	0 (0)	0 (0)	1 (3)
Moderate	0 (0)	0 (0)	0 (0)	0 (0)
Severe	1 (2)	0 (0)	0 (0)	0 (0)
Pain in extremity				
Mild	3 (5)	1 (4)	1 (2)	0 (0)
Moderate	0 (0)	0 (0)	0 (0)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)
Sensory disturbance				
Mild	0 (0)	0 (0)	1 (2)	0 (0)
Moderate	0 (0)	0 (0)	0 (0)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)
Hematoma at injection site				
Mild	17 (30)	5 (19)	4 (7)	0 (0)
Moderate	2 (4)	0 (0)	0 (0)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)
Erythema at injection site				
Mild	27 (47)	6 (22)	12 (21)	0 (0)
Moderate	10 (18)	0 (0)	1 (2)	0 (0)
Severe	1 (2)	0 (0)	0 (0)	0 (0)
Induration at injection site				
Mild	29 (51)	10 (37)	16 (28)	0 (0)
Moderate	18 (32)	0 (0)	1 (2)	0 (0)
Severe	2 (4)	0 (0)	0 (0)	0 (0)
Pain at injection site				
Mild	28 (49)	8 (30)	3 (5)	4 (13)
Moderate	12 (21)	3 (11)	1 (2)	0 (0)
Severe	3 (5)	1 (4)	1 (2)	0 (0)
Pruritus at injection site				
Mild	17 (30)	10 (37)	8 (14)	0 (0)
Moderate	6 (11)	0 (0)	1 (2)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)
Swelling at injection site				
Mild	31 (54)	7 (26)	5 (9)	0 (0)
Moderate	7 (12)	0 (0)	1 (2)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)
Warmth at injection site				
Mild	21 (37)	6 (22)	5 (9)	1 (3)
Moderate	8 (14)	0 (0)	1 (2)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)
Rash at injection site				
Mild	0 (0)	0 (0)	0 (0)	0 (0)
Moderate	1 (2)	0 (0)	0 (0)	0 (0)
Severe	0 (0)	0 (0)	0 (0)	0 (0)

AE = adverse event; A-FLU-v = adjuvanted FLU-v; A-placebo = adjuvanted placebo; NA-FLU-v = nonadjuvanted FLU-v; NA-placebo = nonadjuvanted placebo; SAE = serious adverse event.
* Data are numbers (percentages) of participants who reported an AE at any time after receiving the first vaccination that may or may not have been related to the intervention.
† Defined as any untoward medical occurrence that resulted in death, hospitalization or prolongation of a hospitalization, or disability/incapacity; was life-threatening; or was a congenital abnormality.
‡ Those that were clearly a consequence of administration of the drug. Mild AEs were easily tolerated, caused minimal discomfort, and did not interfere with everyday activities. Moderate AEs caused enough discomfort to interfere with normal everyday activities. Severe AEs prevented normal everyday activities.

Similarly, MIMOSA assessment showed that the percentages of responders were higher in the A-FLU-v group than the A-placebo group at day 42 for IFN-γ (difference, 67 percentage points [CI, 46 to 80 percentage points]; *P* < 0.001), TNF-α (difference, 44 percentage points [CI, 27 to 58 percentage points]; *P* < 0.001), and IL-2 (difference, 56 percentage points [CI, 40 to 69 percentage points]; *P* < 0.001), and these increases persisted at day 180 (IFN-γ: difference, 59 percentage points [CI, 37 to 72 percentage points] [*P* < 0.001]; TNF-α: difference, 24 percentage points [CI, 8 to 38 percentage points] [*P* = 0.013]; IL-2: difference, 57 percentage points [CI, 40 to 70 percentage points] [*P* < 0.001]) (**Table 3**). The MIMOSA analysis did not detect

any differences between A-FLU-v and A-placebo for CD107a or between NA-FLU-v and NA-placebo for any markers.

Similar results were seen for responders based on secretion of IFN-γ measured by ELISA, with a higher proportion of responders in the A-FLU-v group compared with the A-placebo group from day 0 to day 42 (difference, 50 percentage points [CI, 28 to 71 percentage points]; $P < 0.001$) and day 180 (difference, 38 percentage points [CI, 16 to 60 percentage points]; $P < 0.001$) (Table 4). In the A-FLU-v group, the fold increase in median secretion of IFN-γ compared with A-placebo was 59.0-fold versus 1.0-fold (difference, 38.2-fold [CI, 4.7- to 69.7-fold]; $P = 0.001$) between days 0 and 42 and 27.3-fold versus 0.9-fold (difference, 25.0-fold [CI, 5.7- to 50.9-fold]; $P < 0.001$) between days 0 and 180. There were no differences in the NA-FLU-v group compared with NA-placebo (Table 4). The proportion of re-

sponders based on a more than 2-fold increase in IFN-γ secretion on days 42 and 180 in the A-FLU-v group remained higher than in the combined placebo group for participants who had never received annual influenza vaccination (day 42: difference, 58 percentage points [CI, 39 to 76 percentage points] [$P < 0.001$]; day 180: difference, 40 percentage points [CI, 22 to 59 percentage points] [$P < 0.001$]) or had received it within 2 years of enrolling in this study (day 42: difference, 41 percentage points [CI, 5 to 68 percentage points] [$P = 0.042$]; day 180: difference, 25 percentage points [CI, −8 to 55 percentage points] [$P = 0.198$]) (Appendix Table 3, available at Annals.org).

FLU-v–Specific Antibody Responses

All participants showed response to FLU-v–specific IgG on day 42 (CI, 93% to 100%) and day 180 (CI, 93% to 100%) in the A-FLU-v group, whereas 72.4% (CI, 60%

Table 3. Median Fold Increase From Day 0 to Days 42 and 180 in Number of CD4$^+$ T Cells Positive for IFN-γ, TNF-α, IL-2, and CD107a, and Number of Responders Based on Fold Increase and MIMOSA Analysis*

Variable	A-FLU-v (n = 51)	A-Placebo (n = 26)	Difference (95% CI)	P Value	NA-FLU-v (n = 58)	NA-Placebo (n = 32)	Difference (95% CI)	P Value
Day 42								
IFN-γ								
Median fold increase (IQR) (95% CI)	5.9 (21.0) (1.0 to 10.9)	0.2 (2.3) (−0.6 to 1.0)	4.5 (2.3 to 9.8)	<0.001	0.5 (3.2) (−0.2 to 1.1)	1.0 (5.5) (−0.7 to 2.6)	0.0 (−1.0 to 0.2)	0.45
R/A, n/N; RFI (95% CI), %	40/44; 91 (79 to 96)	8/19; 42 (23 to 64)	49 (25 to 70)	<0.001	23/51; 45 (32 to 59)	13/27; 48 (31 to 66)	−3 (−26 to 20)	0.80
R/A, n/N; RM (95% CI), %	38/50; 76 (63 to 86)	2/23; 9 (2 to 27)	67 (46 to 80)	<0.001	7/54; 13 (6 to 24)	1/30; 3 (1 to 17)	10 (−5 to 22)	0.25
TNF-α								
Median fold increase (IQR) (95% CI)	5.0 (66.1) (−10.9 to 20.9)	0.0 (0.7) (−0.3 to 0.3)	4.9 (1.3 to 40.0)	<0.001	0.0 (0.6) (−0.1 to 0.1)	0.0 (1.8) (−0.5 to 0.5)	0.0 (0.0 to 0.0)	0.88
R/A, n/N; RFI (95% CI), %	33/44; 75 (61 to 85)	9/18; 50 (29 to 70)	25 (−1 to 50)	0.056	16/52; 31 (20 to 44)	12/27; 44 (28 to 63)	−13 (−35 to 8)	0.23
R/A, n/N; RM (95% CI), %	22/50; 44 (31 to 58)	0/22; 0 (0 to 15)	44 (27 to 58)	<0.001	3/55; 5 (2 to 15)	1/30; 3 (1 to 17)	2 (−12 to 12)	1.00
IL-2								
Median fold increase (IQR) (95% CI)	8.2 (36.3) (−0.4 to 16.7)	0.0 (0.5) (−0.2 to 0.2)	7.0 (3.5 to 18.0)	<0.001	0.0 (5.3) (−1.1 to 1.1)	0.0 (1.3) (−0.4 to 0.4)	0.0 (0.0 to 0.7)	0.21
R/A, n/N; RFI (95% CI), %	36/44; 82 (68 to 90)	4/19; 21 (8 to 43)	60 (35 to 78)	<0.001	23/52; 44 (32 to 58)	13/27; 48 (31 to 66)	−4 (−26 to 19)	0.74
R/A, n/N; RM (95% CI), %	28/50; 56 (42 to 69)	0/23; 0 (0 to 14)	56 (40 to 69)	<0.001	1/55; 2 (0 to 10)	1/30; 3 (1 to 17)	−2 (−15 to 7)	1.00
CD107a								
Median fold increase (IQR) (95% CI)	2.0 (6.6) (0.3 to 3.7)	0.0 (0.4) (−0.2 to 0.2)	1.7 (0.1 to 4.0)	0.004	0.0 (1.8) (−0.4 to 0.4)	0.0 (1.4) (−0.5 to 0.5)	0.0 (0.0 to 0.1)	0.77
R/A, n/N; RFI (95% CI), %	26/38; 68 (53 to 81)	4/14; 29 (12 to 55)	39 (9 to 63)	0.010	22/47; 47 (33 to 61)	8/23; 35 (19 to 55)	12 (−13 to 34)	0.34
R/A, n/N; RM (95% CI), %	5/48; 10 (5 to 22)	0/21; 0 (0 to 15)	10 (−6 to 22)	0.31	1/51; 2 (0 to 10)	1/29; 3 (1 to 17)	−1 (−16 to 7)	1.00
Day 180								
IFN-γ								
Median fold increase (IQR) (95% CI)	4.4 (14.3) (1.0 to 7.8)	0.9 (6.4) (−1.4 to 3.2)	2.1 (0.0 to 6.0)	0.030	0.5 (3.0) (0.2 to 1.2)	0.1 (1.5) (−0.4 to 0.6)	0.0 (0.0 to 0.5)	0.62
R/A, n/N; RFI (95% CI), %	37/44; 84 (71 to 92)	11/19; 58 (36 to 77)	26 (3 to 50)	0.049	26/53; 49 (35 to 62)	8/27; 30 (16 to 48)	19 (−3 to 39)	0.096
R/A, n/N; RM (95% CI), %	31/49; 63 (49 to 75)	1/21; 5 (1 to 23)	59 (37 to 72)	<0.001	8/56; 14 (7 to 26)	2/30; 7 (2 to 21)	8 (−9 to 21)	0.48
TNF-α								
Median fold increase (IQR) (95% CI)	0.6 (17.5) (−3.5 to 4.8)	0.0 (3.4) (−1.2 to 1.2)	0.0 (0.0 to 3.0)	0.075	0.0 (0.0) (0.0 to 0.0)	0.0 (0.0) (0.0 to 0.0)	0.0 (0.0 to 0.0)	0.87
R/A, n/N; RFI (95% CI), %	26/44; 59 (44 to 72)	9/19; 47 (27 to 68)	12 (−15 to 37)	0.39	17/53; 32 (21 to 45)	9/27; 33 (19 to 52)	−1 (−24 to 19)	0.91
R/A, n/N; RM (95% CI), %	12/49; 24 (15 to 38)	0/21; 0 (0 to 15)	24 (8 to 38)	0.013	2/56; 4 (1 to 12)	1/30; 3 (1 to 17)	0 (−14 to 9)	1.00
IL-2								
Median fold increase (IQR) (95% CI)	6.5 (18.6) (2.1 to 10.9)	0.0 (0.3) (−0.1 to 0.1)	5.7 (2.0 to 15.0)	<0.001	0.2 (4.0) (−0.6 to 1.1)	0.0 (0.6) (−0.2 to 0.2)	0.0 (0.0 to 1.1)	0.076
R/A, n/N; RFI (95% CI), %	39/44; 89 (76 to 95)	9/19; 47 (27 to 68)	42 (17 to 63)	<0.001	26/53; 49 (36 to 62)	13/27; 48 (30 to 66)	1 (−22 to 23)	0.94
R/A, n/N; RM (95% CI), %	28/49; 57 (43 to 70)	0/21; 0 (0 to 15)	57 (40 to 70)	<0.001	1/56; 2 (0 to 9)	1/30; 3 (1 to 17)	−2 (−15 to 7)	1.00
CD107a								
Median fold increase (IQR) (95% CI)	1.0 (3.0) (0.2 to 1.8)	0.6 (3.0) (−0.7 to 1.9)	0.0 (−0.8 to 1.0)	0.91	0.6 (3.0) (−0.1 to 1.3)	0.0 (0.2) (−0.1 to 0.1)	0.2 (0.0 to 1.1)	0.91
R/A, n/N; RFI (95% CI), %	21/37; 57 (41 to 71)	8/14; 57 (33 to 79)	0 (−28 to 29)	0.98	24/50; 48 (35 to 61)	5/22; 23 (10 to 43)	25 (1 to 45)	0.044
R/A, n/N; RM (95% CI), %	4/44; 9 (4 to 21)	1/19; 5 (1 to 25)	4 (−17 to 17)	1.00	2/53; 4 (1 to 13)	2/25; 8 (2 to 25)	−4 (−22 to 7)	0.59

A-FLU-v = adjuvanted FLU-v; A-placebo = adjuvanted placebo; IFN-γ = interferon-γ; IL-2 = interleukin-2; IQR = interquartile range; MIMOSA = mixture models for single-cell assays; NA-FLU-v = nonadjuvanted FLU-v; NA-placebo = nonadjuvanted placebo; R/A = number of responders divided by number of participants included in the analysis; RFI = responders based on fold increase; RM = responders according to MIMOSA analysis; TNF-α = tumor necrosis factor-α.

* The fold increase is based on the number of CD4$^+$ T cells that are positive for the different cytokines from day 0 (prevaccination) to days 42 and 180 (postvaccination). Medians and IQRs were calculated using the R package "doBy" ("summaryBy" command), and the CIs for the medians were calculated as the median ± 1.57 × IQR/($\sqrt{n}$). Between-group differences in median fold increases and their respective CIs were calculated using the Mann–Whitney U test ("wilcox.test" command in R). Participants with a ≥2-fold increase in response were considered to be RFIs. In a separate analysis, participants were determined to be RMs on the basis of a false discovery rate–derived P value <0.05. P values for the between-group differences in the percentage of responders were calculated using the χ^2 or Fisher exact test. CIs for the percentages were calculated using the Wilson score interval ("scoreci" command in the R package "PropCIs"), and CIs for the differences between percentages were calculated using the score interval ("diffscoreci" command in the R package "PropCIs").

Table 4. IFN-γ Responses, Measured by ELISA*

Variable	A-FLU-v	A-Placebo	Difference (95% CI)	P Value	NA-FLU-v	NA-Placebo	Difference (95% CI)	P Value
R/A, *n/N*; responders (95% CI), %								
Day 42	42/44; 95 (85 to 99)	9/20; 45 (26 to 66)	50 (28 to 71)	<0.001	28/47; 60 (45 to 72)	10/25; 40 (23 to 59)	20 (−5 to 42)	0.113
Day 180	40/43; 93 (81 to 98)	11/20; 55 (34 to 74)	38 (16 to 60)	<0.001	22/48; 46 (33 to 60)	13/23; 57 (37 to 74)	−11 (−34 to 14)	0.40
Median fold increase in IFN-γ secretion								
Day 42								
Participants analyzed, *n*	43	20	–	–	47	25	–	–
Median fold increase (IQR) (95% CI)	59.0 (107.5) (33.3 to 84.7)	1.0 (9.6) (−2.4 to 4.4)	38.2 (4.7 to 69.7)	0.001	0.8 (13.4) (−2.3 to 3.9)	1.0 (5.6) (−0.8 to 2.8)	0.04 (−0.5 to 1.6)	0.59
Day 180								
Participants analyzed, *n*	43	20	–	–	48	23	–	–
Median fold increase (IQR) (95% CI)	27.3 (92.3) (5.2 to 49.4)	0.9 (2.6) (−0.0 to 1.9)	25.0 (5.7 to 50.9)	<0.001	1.0 (1.16) (0.7 to 1.3)	1.0 (7.5) (−1.4 to 3.4)	−0.1 (−1.2 to 0.3)	0.61

A-FLU-v = adjuvanted FLU-v; A-placebo = adjuvanted placebo; ELISA = enzyme-linked immunosorbent assay; IFN-γ = interferon-γ; IQR = inter-quartile range; NA-FLU-v = nonadjuvanted FLU-v; NA-placebo = nonadjuvanted placebo; R/A = number of responders divided by number of participants included in the analysis.
* Response was defined as a ≥2-fold increase from prevaccination (day 0) to postvaccination (days 42 and 180) in the amount of IFN-γ secreted by peripheral blood mononuclear cells in response to FLU-v antigens in vitro, as measured by ELISA.

to 82%) and 51.7% (CI, 39% to 64%) in the NA-FLU-v group responded at these time points. No participants in either placebo group responded. The IgG geometric mean titers were 8740.5 ng/mL (CI, 6640.8 to 11 861.3 ng/mL) on day 42 and 4769.2 ng/mL (CI, 3456.6 to 6580.2 ng/mL) on day 180 for the A-FLU-v group and 2593.0 ng/mL (CI, 1767.2 to 3804.7 ng/mL) and 1276.3 ng/mL (CI, 948.0 to 1718.5 ng/mL), respectively, for NA-FLU-v (**Table 5**). The proportion of IgM responders after vaccination was low and was significant only on day 42 in the A-FLU-v group (20%) compared with the A-placebo group (0%) (CI, 6% to 33%; *P* = 0.014).

Efficacy

A total of 167 participants took part in follow-up. Among 47 who were swabbed, 17 were positive for influenza (9 in the combined placebo group, 5 in the A-FLU-v group, and 3 in the NA-FLU-v group). H3N2 was the dominant strain, with 14 total cases (7 in the combined placebo group, 4 in the A-FLU-v group, and 3 in the NA-FLU-v group), 4 of which had H3N2 HAI titers of 40 or higher at screening. Three participants were infected with B strains. Thus, overall vaccine efficacy was 37% (CI, −76% to 77%) for A-FLU-v and 67% (CI, −17% to 91%) for NA-FLU-v compared with the combined placebo group. Seven participants (1 in the A-FLU-v group, 2 in the NA-FLU-v group, and 4 in the combined placebo group) had severe infections (**Appendix Tables 4** and **5**, available at Annals.org).

DISCUSSION

Greater responses and higher percentages of responders for Th1 markers were observed in the A-FLU-v group than in the A-placebo group. A MIMOSA analysis and an assessment of responders based on a 2-fold or higher increase in immune parameters on MFC found similar results overall, with MIMOSA detecting fewer responders in the placebo groups, suggesting that the

Table 5. Geometric Mean Titers of FLU-v–Specific IgG and IgM in Serum

Variable	Mean Titer (SD) (95% CI), ng/mL		Between-Group Ratio of Mean Titer (95% CI)	P Value	Mean Titer (SD) (95% CI), ng/mL		Between-Group Ratio of Mean Titer (95% CI)	P Value
	A-FLU-v (*n* = 51)*	A-Placebo (*n* = 26)†			NA-FLU-v (*n* = 58)	NA-Placebo (*n* = 32)		
IgG								
Day 0	362.9 (592.3) (295.4–445.7)	371.9 (344.0) (292.2–473.3)	0.98 (0.72–1.33)	0.88	499.1 (785.8) (398.4–625.2)	331.2 (333.7) (264.5–414.6)	1.51 (1.10–2.06)	0.011
Day 42	8740.5 (17 374.4) (6440.8–11 861.3)	381.2 (312.0) (299.9–484.5)	22.93 (15.66–33.58)	<0.001	2593.0 (12 583.9) (1767.2–3804.7)	336.4 (333.3) (268.6–421.2)	7.71 (4.97–11.96)	<0.001
Day 180	4769.2 (8002.1) (3456.6–6580.2)	387.3 (301.2) (303.2–494.7)	12.32 (8.28–18.32)	<0.001	1276.3 (2623.8) (948.0–1718.5)	344.9 (376.6) (274.1–434.0)	3.70 (2.56–5.36)	<0.001
IgM								
Day 0	1152.8 (1523.6) (972.8–1366.2)	1198.8 (1637.2) (899.7–1597.4)	0.96 (0.69–1.34)	0.81	1236.2 (941.6) (1053.9–1450.1)	1408.5 (1185.0) (1115.2–1779.0)	0.88 (0.66–1.16)	0.35
Day 42	1689.7 (2036.8) (1397.7–2042.6)	1188.7 (1666.4) (888.7–1589.9)	1.42 (1.01–2.00)	0.044	1366.7 (1229.9) (1152.3–1621.1)	1359.4 (1150.2) (1076.5–1716.6)	1.01 (0.76–1.34)	0.97
Day 180	1303.7 (1660.4) (1080.9–1572.5)	1173.1 (1707.8) (856.2–1607.2)	1.11 (0.78–1.59)	0.56	1307.9 (2647.6) (1072.8–1594.4)	1419.0 (1183.6) (1120.2–1797.4)	0.92 (0.68–1.25)	0.59

A-FLU-v = adjuvanted FLU-v; A-placebo = adjuvanted placebo; NA-FLU-v = nonadjuvanted FLU-v; NA-placebo = nonadjuvanted placebo.
* *n* = 50 at day 180 due to missing samples.
† *n* = 24 at day 180 due to missing samples.

threshold for the fold increase could be more stringent in future studies.

Adjuvanted FLU-v elicited antigen-specific cellular responses characterized by activation of CD4+ T cells producing IFN-γ, TNF-α, IL-2, or CD107a. Administration of a single dose of A-FLU-v resulted in strong cellular responses, as shown by MFC and IFN-γ ELISA and consistent with previous studies (5, 15, 16). Peptide antigens are known to require adjuvants that enhance the immune responses by providing a depot effect, leading to more efficient cell recruitment and antigen presentation.

The Th1 cytokine IFN-γ is considered an important marker associated with protection against influenza disease (17). However, it has also been reported that CD4+ T cells producing multiple cytokines show antiviral efficacy superior to that of single-cytokine–producing T cells (18). A post hoc analysis of MFC data showed that 40% of participants in the A-FLU-v group were triple responders, having CD4+ T cells producing IFN-γ, TNF-α, or IL-2 in response to FLU-v, which indicates induction of protective multifunctional T-cell responses (data not shown).

Vaccination with FLU-v has previously been reported to induce CD8+ T-cell responses in animals and humans (4, 6). Splenocytes from vaccinated HLA*0201 transgenic mice secreted IFN-γ when exposed to HLA-syngeneic human cells transfected with the FLU-v antigens, but not when exposed to allogeneic transfected cells, demonstrating presentation of the antigens bound to MHC class I (4). Moreover, data from a phase 1b trial showed a reduction in IFN-γ secretion in participants vaccinated with FLU-v when CD8+ T cells were depleted from peripheral blood mononuclear cells exposed to the vaccine antigens (6). The low CD8+ T-cell responses observed in this study may be due to several factors, such as suboptimal timing of sample collection and assay conditions for efficient FLU-v antigen presentation by MHC class I molecules in vitro. Nevertheless, an increase in the number of participants with a 2-fold or greater increase in the number of CD8+ T cells expressing the cytotoxicity surface marker CD107a was seen in both FLU-v groups compared with their respective placebo groups.

Adjuvanted FLU-v and NA-FLU-v vaccination induced vaccine-specific IgG antibodies, which are the major effector molecules of the humoral immune response, have high affinity, and persist longer in the circulation. Although annual influenza vaccination elicits neutralizing IgG against variable viral surface proteins preventing cellular uptake, FLU-v–specific IgGs target conserved internal antigens and are therefore nonneutralizing, potentially contributing to protection against viral infection (19) through antibody-dependent (20) and complement-mediated cytolysis (21), increasing T-cell responses (22), and reducing viral replication (23).

The small number of participants in the study limited assessment of exploratory end points, such as vaccine efficacy and differences in responses to FLU-v based on influenza vaccine history or age of the participant. In addition, the low infection rate hinders determination of whether a vaccine-specific immune marker is associated with disease protection. Influenza efficacy field trials require thousands of participants to allow efficacy assessment. This study was not powered to assess efficacy but provided important information on disease and logistics to implement in future phase 3 trials of efficacy. During the 2016–2017 season in the Netherlands, H3N2 virus was dominant, with H1N1 pdm09 and B (Yamagata and Victoria lineage) viruses only sporadically detected, as was also the case in our study. In the 2016–2017 season, an estimated 2.9% of the Dutch population had symptoms of influenza virus infection (24), whereas our study detected confirmed infection in 15.5% of participants in the combined placebo group, indicating that the estimates may have underreported the infection levels. The seasonal vaccine effectiveness against H3N2 for persons younger than 60 years was 25%. Adjuvanted FLU-v and NA-FLU-v showed 35% (CI, −109% to 80%) and 57% (CI, −58% to 88%) efficacy, respectively, although the cohort studied was too small to make meaningful conclusions (**Appendix Tables 4** and **5**). In a recent H1N1 influenza challenge study, 1 dose of A-FLU-v was associated with a reduction in the number of participants with mild to moderate influenza disease and the number with more severe disease (25).

In conclusion, the data show that a single dose of A-FLU-v elicited cell-mediated and humoral immune responses to FLU-v antigens. Further development is warranted for a single dose of A-FLU-v to be tested in a phase 3 setting with a larger cohort of vaccinees, where efficacy and safety can be further explored as primary end points.

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APPENDIX: METHODS

Blinding

To maintain blinding, the investigational medicinal product and the placebo control were prepared by a qualified person other than the one administering the injection. The study was double-blinded, although it was not possible to completely mask the presence of adjuvant in the injections at day 0 because the solution and the emulsion were physically distinguishable. Because of the local injection site reactions that were likely caused by Montanide ISA-51 adjuvant, masking of syringe barrels was deemed superfluous. However, the blinded study personnel remained blinded to the presence or absence of FLU-v antigens in the vaccine.

Safety Definitions

Definitions of AE Causality

Causality was assessed by the investigator using the following definitions:

• *Unrelated:* The event was not considered to be related to the study drug.

• *Unlikely:* Although a relationship with the study drug could not be completely ruled out, the nature of the event, the underlying disease, concomitant medication, or the temporal relationship made other explanations more likely.

• *Possibly related:* The temporal relationship and the absence of a more likely explanation suggested that the event could be related to the study drug.

• *Probably related:* The known effects of the study drug or its therapeutic class or results of challenge testing suggested that the study drug was the most likely cause.

• *Definitely related:* The AE was clearly a consequence of administration of the drug. Such events are likely to be widely documented and generally accepted as being associated with the study medication.

Definitions of AE Severity

The assessment was based on the investigator's clinical judgment. The intensity of each AE and SAE recorded in the electronic case report form was assigned to 1 of the following categories:

• *Mild:* An event that is easily tolerated by the participant, causes minimal discomfort, and does not interfere with everyday activities.

• *Moderate:* An event that causes sufficient discomfort to interfere with normal everyday activities.

• *Severe:* An event that prevents normal everyday activities.

Definition of SAE

An SAE is any untoward medical occurrence that:

• Results in death.

• Is life-threatening. This refers to an event in which the participant was at risk for death at the time of the event. It does not refer to an event that might hypothetically have caused death if it were more severe.

• Requires or prolongs hospitalization. In general, hospitalization signifies that the participant has been detained (usually involving at least an overnight stay) at the hospital or emergency ward for observation and/or treatment that would not have been appropriate in the physician's office or outpatient setting. Complications that occur during hospitalization are AEs. If a complication prolongs hospitalization or fulfills any other serious criteria, the event is serious. When there is doubt about whether hospitalization occurred or was necessary, the AE should be considered serious. Hospitalization for

elective treatment of a preexisting condition that did not worsen from baseline is not considered an AE.

● Results in disability or incapacity. The term *disability* means a substantial disruption of a person's ability to conduct normal life functions. This definition is not intended to include experiences of relatively minor medical significance, such as uncomplicated headache, nausea, vomiting, diarrhea, influenza, and accidental trauma (for example, sprained ankle), which may interfere with or prevent everyday life functions but do not constitute a substantial disruption.

● Is a congenital anomaly.

The definition also included the following 2 provisos:

● Medical or scientific judgment should be exercised in deciding whether reporting is appropriate in other situations, such as important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the participant or may require medical or surgical intervention to prevent 1 of the other outcomes listed above. These should also be considered serious. Examples of such events are invasive or malignant tumors, intensive treatment in an emergency department or at home for allergic bronchospasm, blood dyscrasias or convulsions that do not result in hospitalization, or development of drug dependency or drug abuse.

● All pregnancies will be reported and followed up. The investigator will notify the medical monitor of any women who become pregnant during the study within 24 hours, who will then notify the sponsor within 1 business day. Any resulting offspring will be monitored for up to 6 months postpartum unless otherwise medically indicated.

MIMOSA Analysis

One of the objectives of the study was to assess changes in cellular responses before and after vaccination with FLU-v. Multiparametric flow cytometry was one of the assays used to measure such responses. Blood is composed of many functionally distinct cell subsets, and this technique allows counting of the number of specific cell types of interest ($CD4^+$ and $CD8^+$ T cells) based on markers on their surface that also stain positive for a particular immune marker (IFN-γ, TNF-α, or IL-2). In immunologic studies, these can be measured accurately only by using single-cell assays. Characterization of these small cell subsets is crucial to decipher system-level biological changes. For this reason, an increasing number of studies rely on assays that pro-

vide single-cell measurements of proteins from bulk cell samples. A common problem in the analysis of such data is to identify biomarkers or combinations of biomarkers that are differentially expressed between 2 biological conditions (for example, before–after stimulation), where expression is defined as the proportion of cells expressing that biomarker (or biomarker combination) in the cell subsets of interest.

MIMOSA (12) is a Bayesian hierarchical framework based on a β-binomial Dirichlet-multinomial mixture model for testing for differential biomarker expression using single-cell assays. The model allows the inference to be participant-specific, as is typically required when assessing vaccine responses, while borrowing strength across participants through common prior distributions. An empirical Bayesian approach using an expectation-maximization algorithm and a fully Bayesian one based on a Markov-chain Monte Carlo algorithm are applied for parameter estimation. Cell counts are modeled by a binomial distribution, and information is shared across participants through a prior distribution on the unknown proportions of the binomial likelihood. To discriminate between responders and nonresponders, the prior distribution is written as a mixture of 2 β distributions where the hyperparameters for each mixture component are shared across participants. The 2 components characterize 2 hypotheses for the required data. Under the null hypothesis of nonresponse of the first component, the proportion of cytokine-expressing T cells in the stimulated sample is equal to the proportion in the unstimulated sample. The first component models this nonresponse. Under the null hypothesis of the response of the second component, both stimulated and unstimulated sample counts would be generated by the nonresponse component.

The expected proportion of positive cells arising from the responder distribution should be greater than that arising from the background/nonresponder distribution. MIMOSA provides separate results on whether a participant had a positive response for a particular Th1 cell activation marker (IFN-γ, TNF-α, IL-2, or CD107a) to a specific stimulant (FLU-v antigens) at a specific time point (day 0, day 42, and day 180). In these analyses, a positive responder for a specific marker under study at a certain time point (day 0, day 42, or day 180) is defined as a person who has a positive response for that marker to stimulation with FLU-v antigens based on the false discovery rate–derived *P* value (<0.05) at day 0, day 42, or day 180.

Appendix Table 1. Amino Acid Sequences of the Peptides Included in the FLU-v Vaccine and Their Proteins of Origin

Peptide	Protein of Origin	Amino Acid Sequence
FLU-5 acetate	M1 protein	L-Aspartyl-L-leucyl-L-glutamyl-L-alanyl-L-leucyl-L-methionyl-L-glutamyl-L-tryptophanyl-L-leucyl-L-lysyl-L-threonyl-L-arginyl-L-prolyl-L-isoleucyl-L-leucyl-L-seryl-L-prolyl-L-leucyl-L-threonyl-L-lysyl-glycyl-L-isoleucyl-L-leucyl-glycyl-L-phenylalanyl-L-valyl-L-phenylalanyl-L-threonyl-L-leucyl-L-threonyl-L-valyl-L-proline, acetate salt
FLU-7 acetate	NP protein from A strains	L-Aspartyl-L-leucyl-L-isoleucyl-L-phenylalanyl-L-leucyl-L-alanyl-L-arginyl-L-seryl-L-alanyl-L-leucyl-L-isoleucyl-L-leucyl-L-arginyl-glycyl-L-seryl-L-valyl-L-alanyl-L-histidyl-L-lysyl-L-seryl-Lcysteine, acetate salt
FLU-8N acetate	NP protein from B strains	L-Prolyl-glycyl-L-isoleucyl-L-alanyl-L-aspartyl-L-isoleucyl-L-glutamyl-L-aspartyl-L-leucyl-L-threonyl-L-leucyl-L-leucyl-L-alanyl-L-arginyl-L-seryl-L-methionyl-L-valyl-L-valyl-L-valyl-L-arginine, acetate salt
FLU-10 acetate	M2 protein	L-Isoleucyl-L-isoleucyl-glycyl-L-isoleucyl-L-leucyl-L-histidyl-L-leucyl-L-isoleucyl-L-leucyl-L-tryptophanyl-L-isoleucyl-L-leucyl-L-aspartyl-L-arginyl-L-leucyl-L-phenylalanyl-L-phenylalanyl-L-lysyl-L-cysteinyl-L-isoleucyl-L-tyrosinyl-L-arginyl-L-leucyl-L-phenylalanine, acetate salt

Appendix Table 2. Median Fold Increase From Day 0 to Days 42 and 180 in Number of CD8$^+$ T Cells Positive for IFN-γ, TNF-α, IL-2, and CD107a, and Number of Responders Based on Fold Increase and MIMOSA Analysis*

Variable	A-FLU-v (n = 51)	A-Placebo (n = 26)	Difference (95% CI)	P Value	NA-FLU-v (n = 58)	NA-Placebo (n = 32)	Difference (95% CI)	P Value
Day 42								
IFN-γ								
Median fold increase (IQR) (95% CI)	0.3 (2.0) (−0.2 to 0.7)	0.0 (0.9) (−0.3 to 0.3)	0.0 (0.0 to 0.1)	0.38	0.3 (1.7) (−0.1 to 0.6)	0.0 (0.4) (−0.1 to 0.1)	0.0 (0.0 to 0.1)	0.080
R/A, n/N; RFI (95% CI), %	16/44; 37 (35 to 64)	4/18; 22 (19 to 58)	15 (−13 to 36)	0.28	22/53; 42 (29 to 54)	9/27; 34 (18 to 52)	8 (−14 to 28)	0.48
R/A, n/N; RM (95% CI), %	3/50; 6 (2 to 16)	0/22; 0 (0 to 15)	6 (−9 to 16)	0.55	3/56; 5 (2 to 15)	0/30; 0 (0 to 11)	5 (−6 to 15)	0.55
TNF-α								
Median fold increase (IQR) (95% CI)	0.3 (4.0) (−0.6 to 1.3)	0.0 (0.3) (−0.1 to 0.1)	0.0 (0.0 to 0.1)	0.156	0.3 (1.4) (0.0 to 0.6)	0.0 (2.0) (−0.6 to 0.6)	0.0 (0.0 to 0.1)	0.49
R/A, n/N; RFI (95% CI), %	23/44; 53 (37 to 66)	6/18; 34 (16 to 56)	19 (−0.8 to 42)	0.175	22/53; 42 (29 to 54)	10/27; 43 (21 to 55)	−1 (−18 to 25)	0.70
R/A, n/N; RM (95% CI), %	1/50; 2 (0 to 11)	0/22; 0 (0 to 15)	2 (−13 to 11)	1.00	0/56; 0 (0 to 6)	0/30; 0 (0 to 11)	0 (−11 to 6)	NA
IL-2								
Median fold increase (IQR) (95% CI)	0.0 (1.6) (−0.4 to 0.4)	0.0 (2.0) (−0.7 to 0.7)	0.0 (0.0 to 0.0)	0.89	0.5 (2.0) (0.1 to 0.9)	0.0 (1.6) (−0.5 to 0.5)	0.0 (0.0 to 0.0)	0.125
R/A, n/N; RFI (95% CI), %	17/44; 39 (25 to 54)	8/19; 43 (27 to 68)	−4 (−34 to 17)	0.80	25/52; 48 (35 to 60)	9/27; 34 (18 to 52)	14 (−9 to 34)	0.24
R/A, n/N; RM (95% CI), %	0/50; 0 (0 to 7)	0/23; 0 (0 to 14)	0 (−14 to 7)	NA	0/56; 0 (0 to 6)	0/30; 0 (0 to 11)	0 (−11 to 6)	NA
CD107a								
Median fold increase (IQR) (95% CI)	0.0 (2.3) (−0.5 to 0.5)	0.0 (0.4) (−0.1 to 0.1)	0.0 (0.0 to 0.1)	0.34	0.0 (1.1) (−0.2 to 0.2)	0.0 (0.8) (−0.2 to 0.2)	0.0 (0.0 to 0.0)	0.72
R/A, n/N; RFI (95% CI), %	19/44; 44 (29 to 57)	2/18; 12 (3 to 32)	32 (6 to 50)	0.015	23/47; 45 (31 to 57)	5/27; 18 (8 to 36)	27 (2 to 43)	0.023
R/A, n/N; RM (95% CI), %	0/50; 0 (0 to 7)	0/22; 0 (0 to 15)	0 (−15 to 7)	NA	0/55; 0 (0 to 7)	0/30; 0 (0 to 11)	0 (−11 to 7)	NA
Day 180								
IFN-γ								
Median fold increase (IQR) (95% CI)	0.2 (2.5) (−0.4 to 0.7)	0.0 (1.3) (−0.5 to 0.5)	0.0 (0.0 to 0.1)	0.55	0.9 (2.0) (0.5 to 0.6)	0.0 (1.8) (−0.6 to 0.6)	0.0 (0.0 to 0.6)	0.55
R/A, n/N; RFI (95% CI), %	22/44; 50 (71 to 92)	7/19; 37 (36 to 77)	26 (3 to 50)	0.34	27/53; 51 (37 to 63)	13/27; 48 (31 to 66)	3 (−19 to 25)	0.81
R/A, n/N; RM (95% CI), %	3/49; 6 (2 to 17)	1/21; 5 (1 to 23)	1 (−17 to 13)	1.00	4/56; 7 (3 to 17)	0/30; 0 (0 to 11)	7 (−5 to 17)	0.29
TNF-α								
Median fold increase (IQR) (95% CI)	0.2 (1.7) (−0.2 to 0.6)	0.1 (5.0) (−1.8 to 1.85)	0.0 (−0.1 to 0.3)	0.91	0.1 (2.4) (−0.5 to 0.6)	0.1 (2.5) (−0.7 to 0.9)	0.0 (−0.1 to 0.1)	0.91
R/A, n/N; RFI (95% CI), %	18/44; 41 (27 to 55)	12/19; 53 (41 to 80)	−12 (−45 to 4)	0.105	21/53; 40 (27 to 53)	14/27; 52 (34 to 69)	−12 (−34 to 10)	0.30
R/A, n/N; RM (95% CI), %	1/49; 2 (0 to 11)	0/21; 0 (0 to 15)	2 (−14 to 11)	1.00	1/56; 2 (0 to 9)	0/30; 0 (0 to 11)	2 (−10 to 10)	1.00
IL-2								
Median fold increase (IQR) (95% CI)	0.0 (2.6) (−0.6 to 0.6)	0.0 (1.0) (−0.4 to 0.4)	0.0 (0.0 to 0.0)	0.48	0.3 (1.0) (−0.3 to 0.7)	0.0 (1.0) (−0.3 to 0.3)	0.0 (0.0 to 0.0)	0.21
R/A, n/N; RFI (95% CI), %	16/44; 37 (23 to 52)	5/19; 27 (12 to 48)	10 (−16 to 31)	0.44	11/53; 21 (12 to 34)	8/27; 30 (18 to 52)	−9 (−34 to 7)	0.38
R/A, n/N; RM (95% CI), %	0/49; 0 (0 to 7)	0/21; 0 (0 to 15)	0 (−16 to 7)	NA	0/56; 0 (0 to 6)	0/30; 0 (0 to 11)	0 (−11 to 6)	NA
CD107a								
Median fold increase (IQR) (95% CI)	0.0 (1.2) (0.9 to 1.5)	0.5 (5.0) (3.2 to 6.8)	0.0 (−0.9 to 0.0)	0.23	0.0 (1.8) (−0.4 to 0.4)	0.0 (1.8) (−0.5 to 0.5)	0.0 (0.0 to 0.0)	0.62
R/A, n/N; RFI (95% CI), %	24/44; 32 (40 to 68)	8/19; 42 (23 to 63)	−10 (−14 to 37)	0.43	19/53; 36 (24 to 49)	8/27; 30 (16 to 48)	6 (−16 to 26)	0.58
R/A, n/N; RM (95% CI), %	1/49; 2 (0 to 11)	0/21; 0 (0 to 15)	2 (−14 to 11)	1.00	0/56; 0 (0 to 6)	0/30; 0 (0 to 11)	0 (−11 to 6)	NA

A-FLU-v = adjuvanted FLU-v; A-placebo = adjuvanted placebo; IFN-γ = interferon-γ; IL-2 = interleukin-2; IQR = interquartile range; MIMOSA = mixture models for single-cell assays; NA = not applicable; NA-FLU-v = nonadjuvanted FLU-v; NA-placebo = nonadjuvanted placebo; R/A = number of responders divided by number of participants included in the analysis; RFI = responders based on fold increase; RM = responders according to MIMOSA analysis; TNF-α = tumor necrosis factor-α.
* The fold increase is based on the number of CD8$^+$ T cells that are positive for the different cytokines from day 0 (prevaccination) to days 42 and 180 (postvaccination). Medians and IQRs were calculated using the R package "doBy" ("summaryBy" command), and the CIs for the medians were calculated as the median ± 1.57 × IQR/($\sqrt{n}$). Between-group differences in median fold increases and their respective CIs were calculated using the Mann–Whitney U test ("wilcox.test" command in R). Participants with a ≥2-fold increase in response were considered to be RFIs. In a separate analysis, participants were determined to be RMs on the basis of a false discovery rate–derived P value <0.05. P values for the between-group differences in the percentage of responders were calculated using the χ^2 or Fisher exact test. CIs for the percentages were calculated using the Wilson score interval ("scoreci" command in the R package "PropCIs"), and CIs for the differences between percentages were calculated using the score interval ("diffscoreci" command in the R package "PropCIs").

Appendix Table 3. Effect of Influenza Vaccination in Previous 2 Years on Immunogenicity*

Variable	Vaccination ≤2 Years Previously								No Previous Vaccination							
	A-FLU-v (n = 18)	Combined Placebo (n = 16)	Difference (95% CI), percentage points	P Value	NA-FLU-v (n = 14)	Combined Placebo (n = 16)	Difference (95% CI), percentage points	P Value	A-FLU-v (n = 25)	Combined Placebo (n = 33)	Difference (95% CI), percentage points	P Value	NA-FLU-v (n = 37)	Combined Placebo (n = 33)	Difference (95% CI), percentage points	P Value
Day 42																
Participants analyzed, n	15	13	–	–	12	13	–	–	21	24	–	–	31	24	–	–
Responders, n (% [95% CI])	13 (87 [62 to 96])	6 (46 [23 to 71])	41 (5 to 68)	0.042†	6 (50 [25 to 75])	6 (46 [23 to 71])	4 (–34 to 40)	0.85‡	21 (100 [85 to 100])	10 (42 [24 to 61])	58 (39 to 76)	<0.001‡	20 (65 [47 to 79])	10 (42 [24 to 61])	23 (–4 to 47)	0.092‡
Day 180																
Participants analyzed, n	15	13	–	–	10	13	–	–	21	25	–	–	33	25	–	–
Responders, n (% [95% CI])	13 (87 [62 to 96])	8 (62 [36 to 82])	25 (–8 to 55)	0.198‡	5 (50 [24 to 76])	8 (62 [36 to 82])	–12 (–48 to 28)	0.69†	21 (100 [85 to 100])	15 (60 [41 to 77])	40 (22 to 59)	<0.001†	15 (45 [30 to 62])	15 (60 [41 to 77])	–15 (–39 to 11)	0.27‡

A-FLU-v = adjuvanted FLU-v; IFN-γ = interferon-γ; NA-FLU-v = nonadjuvanted FLU-v.
* Participants who received their last vaccination >2 years previously were excluded from the analysis. Responders were defined as those having a ≥2-fold increase in response in IFN-γ secretion by peripheral blood mononuclear cells from day 0 to day 42 or day 180, as measured by enzyme-linked immunosorbent assay. The combined placebo group includes participants randomly assigned to adjuvanted placebo and those assigned to nonadjuvanted placebo. CIs for percentages were calculated using the Wilson score interval ("scoreci" command in the R package "PropCIs"), and CIs for the differences between percentages were calculated using the score interval ("diffscoreci" command in the R package "PropCIs").
† Calculated using the Fisher exact test.
‡ Calculated using the χ² test.

Appendix Table 4. Confirmed Infection With Influenza A or B Strain During the 2016–2017 Influenza Season and Vaccine Efficacy

Strain	A-FLU-v (n = 51), n (%)	NA-FLU-v (n = 58), n (%)	Combined Placebo (n = 58), n (%)	Vaccine Efficacy (95% CI), %*	
				A-FLU-v vs. Combined Placebo	NA-FLU-v vs. Combined Placebo
Positive for influenza A (H1 or H3)	4 (8)	3 (5)	7 (12)	35 (−109 to 80)	57 (−58 to 88)
Positive for influenza B	1 (2)	0 (0)	2 (3)	43 (−509 to 95)	100 (NaN to 100)
Positive for influenza A or B	5 (10)	3 (5)	9 (15)	37 (−76 to 77)	67 (−17 to 91)

A-FLU-v = adjuvanted FLU-v; NA-FLU-v = nonadjuvanted FLU-v; NaN = not a number.
* Calculated as (1 − infection rate in vaccine group/infection rate in combined placebo group) × 100. The 95% CIs were calculated using the "riskratio" command in the R package "fmsb."

Appendix Table 5. Confirmed Infection With Influenza A or B Strain and Severe Symptoms* During the 2016–2017 Influenza Season and Vaccine Efficacy

Strain	A-FLU-v (n = 51), n (%)	NA-FLU-v (n = 58), n (%)	Combined Placebo (n = 58), n (%)	Vaccine Efficacy (95% CI), %†	
				A-FLU-v vs. Combined Placebo	NA-FLU-v vs. Combined Placebo
Positive for influenza A (H1 or H3)	1 (2)	2 (3)	3 (5)	62 (−253 to 96)	33 (−284 to 88)
Positive for influenza B	0 (0)	0 (0)	1 (2)	100 (NaN to 100)	100 (NaN to 100)
Positive for influenza A or B	1 (2)	2 (3)	4 (7)	72 (−146 to 97)	50 (−162 to 91)

A-FLU-v = adjuvanted FLU-v; NA-FLU-v = nonadjuvanted FLU-v; NaN = not a number.
* Defined as a total symptom score above the median for the overall set of participants (total symptom score >58).
† Calculated as (1 − infection rate in vaccine group/infection rate in combined placebo group) × 100. The 95% CIs were calculated using the "riskratio" command in the R package "fmsb."