ORIGINAL ARTICLE

Whole-blood cytokine secretion assay as a high-throughput alternative for assessing the cell-mediated immunity profile after two doses of an adjuvanted SARS-CoV-2 recombinant protein vaccine candidate

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Abstract

Objectives. We previously described the Phase I-II evaluation of SARS-CoV-2 recombinant protein candidate vaccine, CoV2-PreS-dTM, with AF03- or AS03-adjuvant systems (ClinicalTrials.gov, NCT04537208). Here, we further characterise the cellular immunogenicity profile of this vaccine candidate using a whole-blood secretion assay in parallel to intracellular cytokine staining (ICS) of cryopreserved peripheral blood mononuclear cells (PBMCs). **Methods.** A randomly allocated subset of 90 healthy, SARS-CoV-2-seronegative adults aged $\geq$ 18 years who had received (random allocation) one or two separate injections (on study day [D]1 and D22) of saline placebo or CoV2-PreS-dTM formulated with AS03 or AF03 were included. Cytokine secretion was assessed using a TruCulture® whole-blood stimulation system in combination with multiplex bead array, and intracellular cytokine profiles were evaluated on thawed PBMCs following *ex vivo* stimulation with recombinant S protein at pre-vaccination (D1), post-dose 1 (D22) and post-dose 2 (D36). **Results.** Both methods detected similar vaccine-induced responses after the first and second doses. We observed a Th1 bias (Th1/Th2 ratio > 1.0) for most treatment groups when analysed in whole blood, mainly characterised by increased IFN-γ, IL-2 and TNF-α secretion. Among participants aged $\geq$ 50 years, the Th1/Th2 ratio was higher for those who received vaccine candidate with AS03 versus AF03 adjuvant. ICS revealed that this higher Th1/Th2 ratio resulted from higher levels of IFN-γ expression and that the vaccine induced polyfunctional

CD4$^+$ T cells. **Conclusions.** The whole-blood cytokine secretion assay is a high-throughput alternative for assessing the quantity and character of vaccine-induced cellular responses.

Keywords: adjuvant, AF03, AS03, cell-mediated immunity, CoV2-PreS-dTM, intracellular cytokine staining, multiplex bead array, SARS-CoV-2 recombinant protein candidate vaccine, whole-blood cytokine secretion assay

INTRODUCTION

Over the past 18 months, since the coronavirus disease 2019 (COVID-19) pandemic was declared, unprecedented efforts in vaccine development have led to the approval, or conditional or emergency use authorisation, for almost 20 COVID-19 vaccines worldwide.[1] With the continued spread of COVID-19 and the emergence of new variants, sustained efforts are needed to ensure sufficient numbers of doses and an adequate range of vaccines are available to offer the breadth and quality of protection needed to contain the pandemic at a global level.[2]

Although the humoral immune response tends to be the critical immunological parameter considered for vaccine licensure,[3,4] cell-mediated immunity (CMI) has gained increased recognition for its role in the host immune response to vaccination and protection against intracellular infections, including virus-related illness. CMI analyses, as part of the clinical development of a novel vaccine candidate, would help fully characterise immune responses and further guide identification of appropriate lead formulations, as well as provide insights into potential safety concerns such as vaccine-enhanced disease.

Enhanced respiratory disease upon challenge with SARS-CoV or other coronaviruses has been observed in a number of,[5,6] but not all,[7,8] animal models following prior natural exposure or vaccination. Strong Th1 T-cell responses (in particular, T cells producing interferon-γ [IFN-γ]) have been suggested to potentially reduce the risk of disease enhancement in animals receiving SARS-CoV vaccine candidates.[8-10] Currently, there is no clear evidence from clinical or epidemiological data of enhanced respiratory disease related to coronavirus vaccines in humans. High-frequency T-cell responses targeting the SARS-CoV-2 spike (S) protein have been detected in patients who recover from COVID-19, and have been associated with mild or asymptomatic

disease in those with low antibody levels.[11-14] Of note, SARS-CoV-1 cellular immunity was shown to persist up to 11 years post-infection in a SARS-recovered patient.[15]

The evaluation of CMI in large-scale clinical trials is challenging.[16] While antibody assays use serum or plasma samples that are relatively easy to extract from blood and to cryopreserve, prototypical CMI assays such as intracellular cytokine staining (ICS) by flow cytometry or IFN-γ ELISpot require specialised staining of peripheral blood mononuclear cells (PBMCs) or whole blood in laboratories located at or near the clinical site. The preparation of PBMCs requires considerable expertise. In particular, PBMCs should be cryopreserved within hours of venepuncture to maintain high recovery and viability of the cells and to ensure the quality of the data, with harmonisation of logistics both within and between clinical sites.[17,18] In the context of a large clinical trial, the necessary quality control required to perform this process across a network of laboratories can be costly and time-consuming. Alternatively, whole blood can be used to assess CMI in a cytokine secretion assay that is initiated in a simple procedure at the clinical site or local laboratory and then shipped cryopreserved to a central location.[19] Such stimulated whole-blood cytokine secretion assays are similar to the IFN-γ release assays used to detect cellular responses to tuberculosis infection, and may include multiplexed measurement of multiple cytokines.[20-22]

Sanofi Pasteur has developed an adjuvanted SARS-CoV-2 recombinant protein candidate vaccine, CoV2-PreS-dTM. The safety and immunogenicity of one or two doses of this vaccine were recently evaluated in SARS-CoV-2-seronegative adults in a Phase I–II clinical trial.[23] No major safety concerns were demonstrated and robust neutralising antibody titres were elicited after two doses of adjuvanted vaccine in participants aged 18–49 years, with weaker antibody responses in those 50 years or older. Initial analysis of specific cytokine production

indicated a Th1-biased response. Here, we describe in detail the *ex vivo* CMI responses after two doses of CoV2-PreS-dTM formulations containing oil-in-water based adjuvants, AF03 (Sanofi Pasteur, Marcy l'Etoile, France)[24] or AS03 (GlaxoSmithKline, Rixensart, Belgium) adjuvant systems,[25] in a subset of participants from the Phase I–II study using a whole-blood cytokine secretion assay and ICS of PBMCs.

RESULTS

Cell-mediated immunity was measured in a randomly allocated subset of 90 participants; of these, 88 received at least one study injection and 68 met criteria for inclusion in the per-protocol subset for CMI analysis.[23] Given the small number of participants included in each treatment group (i.e. those receiving formulations containing high or low antigen dose, with or without AF03 or AS03 adjuvant) and per age stratum, we assessed the feasibility of combining high and low antigen dose groups for those receiving AF03-adjuvanted vaccine (AF03 group) and those receiving AS03-adjuvanted vaccine (AS03 group). Linear regression models examining the effect of antigen dose on the log-fold change over baseline for whole blood and ICS responses indicated only a minor dose effect, that is $P < 0.05$ for IFN-γ (post-first injection, ICS only) and for interleukin (IL)-2 (post-first injection, whole blood and ICS assays) (Supplementary table 1). As this effect would not be considered significant after adjusting for multiple comparisons, high and low antigen dose groups were combined: AF03 group, $n = 15$; AS03 group, $n = 41$; no adjuvant, $n = 5$; placebo, $n = 7$. The numbers of participants with data available at each time point for each combined group, per assay method, are shown in Supplementary table 2.
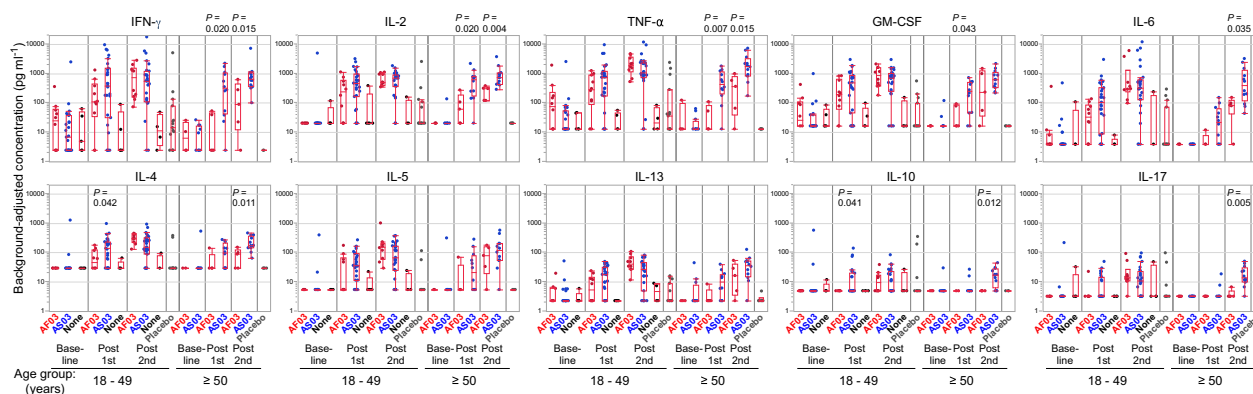
Cytokine secretion in whole-blood supernatants

Whole blood from participants pre-vaccination (study day [D]1), post-dose 1 (D22) and post-dose 2 (D36) was stimulated *ex vivo* for 48 h with recombinant full-length S protein and cytokine secretion assessed by multiplex bead array. Increased secretion of IFN-γ, IL-2, tumor necrosis factor (TNF)-α, IL-4, IL-5, IL-6, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) was detected after one and two doses of the vaccine candidate formulations in the AS03- and AF03-adjuvanted groups, among both younger (18–49 years) and older ($\geq$ 50 years) adults, compared to pre-vaccination samples. Notably, minimal secretion of IL-10 and IL-17 was detected with limited, if any, increase after vaccination (Figure 1). Increases in concentration, from pre-vaccination to post-vaccination, were particularly high for IFN-γ, IL-2, TNF-α and GM-CSF after just one dose for both age groups in the AS03 group, and among younger adults in the AF03-adjuvanted group; for older adults in the AF03-adjuvanted group, the magnitude of response tended to be lower after the first vaccination, but reached similar levels to those of the other groups after the second vaccination. No or very little increased secretion above baseline was observed for those who received the non-adjuvanted candidate vaccine formulation (younger adults only) and for placebo recipients (Figure 1). In exploratory analyses, these differences between the adjuvant groups were found to be statistically different for several cytokines with significantly higher responses in the AS03 group than in the AF03 group among participants aged $\geq$ 50 years ($P \leq 0.02$ for IFN-γ, IL-2 and TNF-α, post-first and post-second vaccination). Due to the relatively small sample sizes and the exploratory nature of these analyses, these *P*-values mainly identify potential differences to be confirmed in further studies. No differences were observed between the adjuvanted groups following a 48-h stimulation with staphylococcal enterotoxin B (SEB) as positive control (Supplementary figure 1).

To assess the balance between Th1 and Th2 responses, the ratios of background-subtracted prototype Th1 cytokine concentrations (IFN-γ) to Th2 cytokine concentrations (IL-4, IL-5 and IL-13) were determined. Ratios above 1 indicated a predominant Th1 response among both age groups in the AS03 group and the younger adults in the AF03 group (Figure 2). The Th1/Th2 ratio was more balanced among the older adults in the AF03-adjuvanted group, driven by the lower IFN-γ concentrations in these participants (Figure 1).

In exploratory analyses, we analysed response rates using cytokine secretion thresholds that were determined empirically for each cytokine, based on fold increases over the negative control than those in the placebo group at any timepoint and in the treatment groups at baseline (Supplementary figure 2).[26] Most ($\geq$ 70%) participants showed positive responses for at least

Figure 1. Vaccine-induced cytokine concentrations in whole-blood supernatant after S protein stimulation: whole-blood cytokine secretion assay. Data show background-subtracted specific cytokine concentrations in pg mL^{-1} (log scale) in participants receiving the placebo, the non-adjuvanted SARS-CoV2 recombinant protein candidate vaccine (None) or SARS-CoV2 recombinant protein candidate vaccine adjuvanted with AF03 (red dots) or AS03 (blue dots), by age groups (18–49 or ≥ 50 years) at baseline, 21 days after dose 1 (Post 1st) and 14 days after dose 2 (Post 2nd). For the placebo group, data for all three time points are combined. Box and whisker plots represent median (mid-line of the box) and 25th and 75th percentiles (bottom and top lines, respectively); the whiskers that extend from the top and bottom of the box extend to the most extreme data points that are no more than 1.5 times the interquartile range or if no value meets this criterion, to the data extremes. Wilcoxon rank *P*-values < 0.05 are shown (not adjusted for multiplicity) (exploratory analyses).
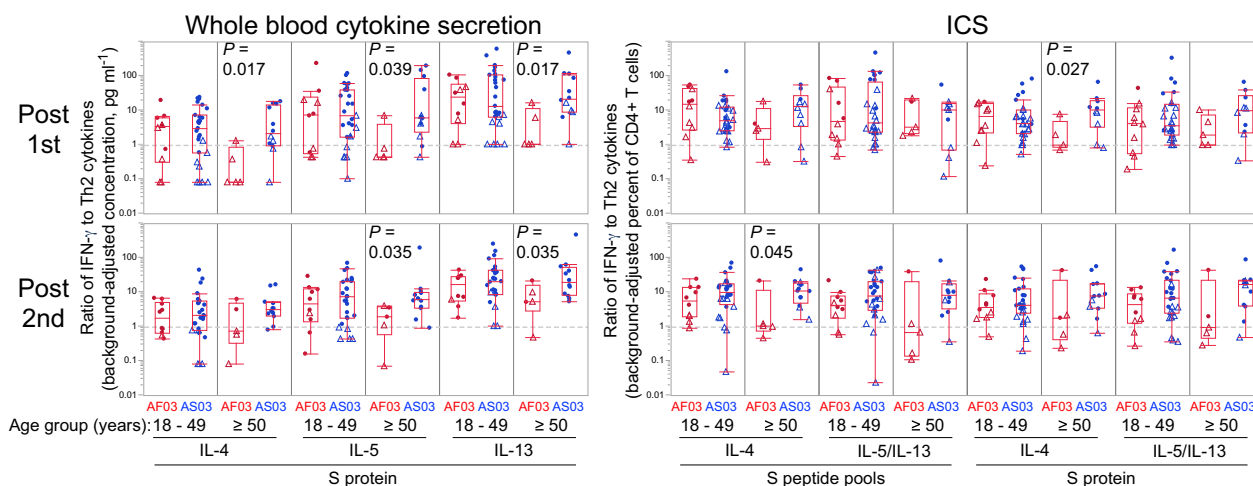
Figure 2. Ratios of Th1 (IFN-γ) to Th2 (IL-4, IL-5, IL-13) responses after one or two doses of SARS-CoV2 protein adjuvanted with AF03 or AS03 (whole-blood cytokine secretion assay or intracellular staining of PBMCs). Whole-blood cell assay: ratios based on background-adjusted concentrations (values below LLOQ set to LLOQ/2); Intracellular staining (ICS): ratios based on background-adjusted percentage of CD4$^+$ T cells expressing IFN-γ to those expressing Th2 cytokines. Data points where the response for IFN-γ was negative are identified as triangles. Wilcoxon rank *P*-values < 0.05 are shown (not adjusted for multiplicity) (exploratory analyses).

one of the cytokines measured (IFN-γ, IL-2 or TNF-α, IL-4, IL-5 and IL-13) after two vaccinations for both age groups in the AS03-adjuvanted group, and for younger adults in the AF03-adjuvanted group (Table 1). The older adults in the AF03-adjuvanted group tended to have lower response rates for any cytokine measured after the second dose (≤ 60% for IFN-γ, IL-2, TNF-α, IL-4 and IL-13; 80% for IL-5) (Table 1).

Intracellular cytokine staining of peripheral blood mononuclear cells

Cryopreserved PBMCs obtained from participants pre-vaccination (D1), post-dose 1 (D22) and post-dose 2 (D36) were stimulated with recombinant full-length S protein (9-h incubation) or pools of overlapping peptides covering the S protein (S peptide pools; 6 h) and their intracellular cytokine

Table 1. Response rate for specific cytokine secretion after the first and second dose of SARS-CoV2 protein adjuvanted with either AF03 or AS03 measured in whole blood stimulated with spike protein

Cytokine	Time point	Age group, years	Participants with positive response, n/M (%)			
			AF03 (N = 15)	AS03 (N = 41)	No adjuvant (N = 5)	Placebo (N = 7)
IFN-γ	Baseline	18–49	0/10 (0%)	1/27 (3.7%)	0/5 (0%)	1/5 (20%)
		≥ 50	0/4 (0%)	0/12 (0%)		0/2 (0%)
	Post 1st	18–49	5/10 (50%)	19/27 (70.4%)	0/5 (0%)	1/5 (20%)
		≥ 50	0/5 (0%)	8/12 (66.7%)		0/2 (0%)
	Post 2nd	18–49	9/10 (90%)	22/27 (81.5%)	0/4 (0%)	0/5 (0%)
		≥ 50	2/5 (40%)	12/12 (100%)		0/2 (0%)
IL-2	Baseline	18–49	0/10 (0%)	1/27 (3.7%)	0/5 (0%)	0/5 (0%)
		≥ 50	0/4 (0%)	0/12 (0%)		0/2 (0%)
	Post 1st	18–49	5/10 (50%)	18/27 (66.7%)	1/5 (20%)	1/5 (20%)
		≥ 50	1/5 (20%)	9/12 (75%)		0/2 (0%)
	Post 2nd	18–49	9/10 (90%)	21/27 (77.8%)	0/4 (0%)	0/5 (0%)
		≥ 50	3/5 (60%)	12/12 (100%)		0/2 (0%)
TNF-α	Baseline	18–49	1/10 (10%)	1/27 (3.7%)	0/5 (0%)	1/5 (20%)
		≥ 50	0/4 (0%)	0/12 (0%)		0/2 (0%)
	Post 1st	18–49	5/10 (50%)	17/27 (63%)	0/5 (0%)	2/5 (40%)
		≥ 50	0/5 (0%)	9/12 (75%)		0/2 (0%)
	Post 2nd	18–49	10/10 (100%)	23/27 (85.2%)	0/4 (0%)	1/5 (20%)
		≥ 50	3/5 (60%)	11/12 (91.7%)		0/2 (0%)
IL-4	Baseline	18–49	0/10 (0%)	2/27 (7.4%)	0/5 (0%)	1/5 (20%)
		≥ 50	0/4 (0%)	1/12 (8.3%)		0/2 (0%)
	Post 1st	18–49	5/10 (50%)	18/27 (66.7%)	1/5 (20%)	1/5 (20%)
		≥ 50	1/5 (20%)	7/12 (58.3%)		0/2 (0%)
	Post 2nd	18–49	10/10 (100%)	22/27 (81.5%)	1/4 (25%)	0/5 (0%)
		≥ 50	3/5 (60%)	12/12 (100%)		0/2 (0%)
IL-5	Baseline	18–49	0/10 (0%)	1/27 (3.7%)	0/5 (0%)	1/5 (20%)
		≥ 50	0/4 (0%)	1/12 (8.3%)		0/2 (0%)
	Post 1st	18–49	4/10 (40%)	18/27 (66.7%)	1/5 (20%)	1/5 (20%)
		≥ 50	1/5 (20%)	6/12 (50%)		0/2 (0%)
	Post 2nd	18–49	10/10 (100%)	21/27 (77.8%)	1/4 (25%)	0/5 (0%)
		≥ 50	4/5 (80%)	11/12 (91.7%)		0/2 (0%)
IL-13	Baseline	18–49	1/10 (10%)	2/27 (7.4%)	0/5 (0%)	0/5 (0%)
		≥ 50	0/4 (0%)	2/12 (16.7%)		0/2 (0%)
	Post 1st	18–49	0/10 (0%)	10/27 (37%)	0/5 (0%)	0/5 (0%)
		≥ 50	0/5 (0%)	0/12 (0%)		0/2 (0%)
	Post 2nd	18–49	7/10 (70%)	14/27 (51.9%)	1/4 (25%)	1/5 (20%)
		≥ 50	1/5 (20%)	6/12 (50%)		0/2 (0%)

Data show the number of participants who tested positive, the total number examined (per-protocol cohort) and the percentage who tested positive. The two dose groups for AF03 and AS03 are combined. A positive response is based on fold cytokine level over the negative controls with the threshold empirically determined for each cytokine (Supplementary figure 2).

profiles evaluated. The primary responses of interest were CD4^{+} and CD8^{+} cells expressing IFN-γ and/or IL-2. T-cell responses were also measured separately for IFN-γ, IL-2, IL-4 and IL-5/IL-13 cytokines (reagents for IL-5 and IL-13 were combined for one detector).

The frequency of CD4^{+} T cells expressing IFN-γ or IL-2 following stimulation with recombinant full-length S protein increased substantially from baseline after the first vaccination in both the AS03- or AF03-adjuvanted groups, and was further boosted after the second vaccination (e.g. for IFN-γ,

median fold increases for each vaccinated group ranged up to 17 after the first vaccination and up to 4.8 from the first to the second vaccination) (Figure 3). The frequency of CD4^{+} T cells expressing IL-2 was higher than those expressing IFN-γ, indicating a potential predominance of IL-2-secreting CD4^{+} T cells. Both the IFN-γ and the IL-2 T-cell responses tended to be of a higher magnitude in the AS03-adjuvanted group than the AF03-adjuvanted group among the older adults (in exploratory analyses, $P = 0.014$ for IFN-γ and $P = 0.028$ for IL-2 [post-first dose]). Th2-type
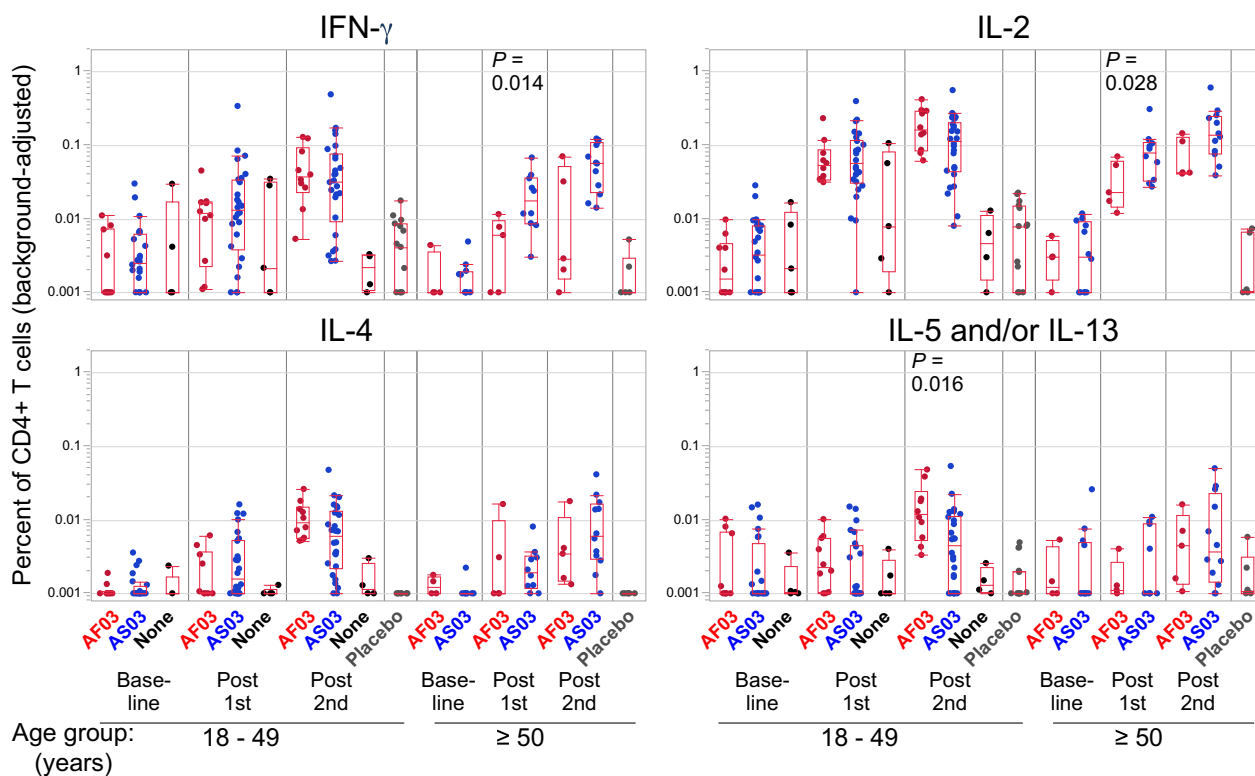
Figure 3. Frequencies of CD4^{+} T cells expressing different cytokines after stimulation with recombinant full-length S protein: ICS assay. Data show background-subtracted CD4^{+} T-cell frequencies (percentage of CD4^{+} T cells expressing the indicated cytokine; log scale) for participants receiving the placebo, the non-adjuvanted SARS- CoV2 recombinant protein candidate vaccine (None) or SARS-CoV2 recombinant protein candidate vaccine adjuvanted with AF03 (red dots) or AS03 (blue dots), by age groups (18–49 or ≥ 50 years) at baseline, 21 days after dose 1 (Post 1st) and 14 days after dose 2 (Post 2nd). For the placebo group, data for all three time points are combined. The *y*-axis is truncated at 0.001% and any values below this level are censored. The mid-line of the box denotes the median, and the ends of the box denote the 25th and 75th percentiles. The whiskers that extend from the top and bottom of the box extend to the most extreme data points that are no more than 1.5 times the interquartile range or if no value meets this criterion, to the data extremes. The Th2 cytokines include co-expression of CD40L since this reduces the background. Reagents for IL-5 and IL-13 were conjugated to the same fluorochrome and were therefore measured as IL-5 and/or IL-13. Wilcoxon rank *P*-values < 0.05 are shown (not adjusted for multiplicity).

cytokine responses (as measured by IL-4 and IL-5 and/or IL-13) also increased post-vaccination compared to baseline, although to a lesser extent (fold increases of up to 7.6, from baseline to post-second vaccination for each vaccinated group; Figure 3). No or very little increase above baseline was observed for those who received the non-adjuvanted candidate vaccine formulation (younger adults only) and for placebo recipients. The ratios of Th1 (IFN-γ) to Th2, in terms of cell frequency, were not as markedly different between the AS03 and AF03 groups as those determined for whole-blood cytokine secretion; however, there was a trend towards higher ratios for older participants in the AS03 group than those in the AF03 group (Figure 2). Similar results were obtained after stimulation with the S peptide pools (Supplementary figure 3).

We also assessed the response rates for CD4^{+} T cells expressing these cytokines using the validated Fisher's exact method by comparing the detection of cytokine-expressing cells following S protein stimulation with the negative controls. The rate of response to full-length S protein stimulation for CD4^{+} T cells expressing IFN-γ and/or IL-2 was high in the AS03-adjuvanted group in both age groups and for younger adults in the AF03-adjuvanted group after the first vaccination (D22) (≥ 69.2%), increasing to ≥ 80.8% on D36 (Table 2). The response rate for older adults in the AF03-adjuvanted group was 40% after the first and second vaccinations. In contrast, the response rates for CD4^{+} T cells expressing IL-4 or IL-5 and/or IL-13 were low across both adjuvanted groups and both age groups (Table 2). The rates of S-specific CD4^{+} T-cell responses were similar when

Table 2. CD4^{+} and CD8^{+} T-cell response rates after stimulation with recombinant full-length S protein as determined using the ICS assay

CD4^{+} or CD8^{+} T-cell subset	Time point	Age group	Participants with positive response, n/M (%)			
			AF03 (N = 15)	AS03 (N = 41)	No adjuvant (N = 5)	Placebo (N = 7)
IFN-γ and/or IL-2*						
	Baseline	18–49	0/10 (0%)	0/24 (0%)	0/5 (0%)	0/5 (0%)
		≥ 50	0/4 (0%)	0/12 (0%)		0/2 (0%)
	Post 1st	18–49	9/10 (90%)	18/26 (69.2%)	2/5 (40%)	0/5 (0%)
		≥ 50	2/5 (40%)	7/10 (70%)		0/2 (0%)
	Post 2nd	18–49	10/10 (100%)	21/26 (80.8%)	0/4 (0%)	0/5 (0%)
		≥ 50	2/5 (40%)	12/12 (100%)		0/2 (0%)
IL-4*						
	Post 1st	18–49	0/10 (0%)	0/26 (0%)	0/5 (0%)	0/5 (0%)
		≥ 50	1/5 (20%)	0/10 (0%)		0/2 (0%)
	Post 2nd	18–49	1/10 (10%)	2/26 (7.7%)	0/4 (0%)	0/5 (0%)
		≥ 50	1/5 (20%)	2/12 (16.7%)		0/2 (0%)
IL-5 and/or IL-13*						
	Post 1st	18–49	0/10 (0%)	0/26 (0%)	0/5 (0%)	0/5 (0%)
		≥ 50	0/5 (0%)	1/10 (10%)		0/2 (0%)
	Post 2nd	18–49	3/10 (30%)	1/26 (3.8%)	0/4 (0%)	0/5 (0%)
		≥ 50	0/5 (0%)	3/12 (25%)		0/2 (0%)
CD8^{+} T cells (IFN-γ and/or IL-2)						
	Baseline	18–49	0/10 (0%)	0/24 (0%)	0/5 (0%)	0/5 (0%)
		≥ 50	0/4 (0%)	0/12 (0%)		0/2 (0%)
	Post 1st	18–49	0/10 (0%)	0/26 (0%)	0/5 (0%)	0/5 (0%)
		≥ 50	0/5 (0%)	0/10 (0%)		0/2 (0%)
	Post 2nd	18–49	0/10 (0%)	0/26 (0%)	0/4 (0%)	0/5 (0%)
		≥ 50	0/5 (0%)	0/12 (0%)		0/2 (0%)

Data show the number of participants with a positive response (n)/the number of participants with available data (M), and the percentage (%) who were positive for the CD4^{+} (*) or CD8^{+} T-cell subset indicated at baseline or after the first (Post 1st) or second (Post 2nd) vaccine dose, in the AF03- and AS03-adjuvant groups, the non-adjuvant groups and placebo group (per-protocol CMI subset). Positive responses were determined using the Fisher's exact test to compare stimulated sample to the negative controls (see Methods).

stimulated with S peptide pools and across the adjuvant and age groups (Supplementary table 3).

There were no IFN-γ and/or IL-2 CD8^{+} T-cell responses detected following S protein stimulation and only two participants had CD8^{+} T-cell responses following stimulation with S peptide pools among younger adults in the AS03 group (Table 2; Supplementary table 3).

Comparison of two methods (whole-blood cytokine assay and intracellular staining of PBMCs)

In an initial comparison of the two methods, the total numbers of samples testing positive or negative were tabulated for cytokines measured separately in both assays (IFN-γ, IL-2, TNF-α, IL-4 and IL-17), for the placebo group at any time point and any vaccine group after two doses. Among placebo samples, no positive responses were identified by ICS, whereas a few (≤ 4 for each cytokine) positive responses were identified by whole-blood assay

(Table 3). After the second vaccination, the most notable differences between the two methods were observed for detection of IL-4 (38 samples tested negative with ICS but positive in the whole-blood assay; Table 3). In a correlation analysis based on response magnitude for IFN-γ, IL-2, TNF-α and IL-4, IL-5 and/or IL-13, and IL-17 induced by full-length S protein stimulation, significant correlation was detected between the whole-blood and ICS assays overall, with the strongest correlation observed for the higher IFN-γ, IL-2 and TNF-α responses ($r = 0.58$–0.72) and the weakest correlation for IL-17 ($r = 0.23$; Figure 4). Similar correlations were observed with S protein peptide pool stimulation (Supplementary figure 4).

Polyfunctionality of CD4^{+} T cells

One of the critical advantages of ICS is that it allows simultaneous detection of multiple co-expressed cytokines in single cells or cell subsets. We examined the polyfunctionality of S-specific

Table 3. Assessment of the agreement between two methods: numbers (percentage) of samples testing positive or negative for cytokines measured separately in both whole-blood cell supernatant and in PBMCs by ICS after full-length S protein stimulation

	ICS−Whole blood−	ICS+Whole blood+	ICS−Whole blood+	ICS+Whole blood−
Placebo at any visit (*n* = 21)				
IFN-γ	19 (90.5%)	0 (0%)	2 (9.5%)	0 (0%)
IL-2	20 (95.2%)	0 (0%)	1 (4.8%)	0 (0%)
TNF-α	17 (81%)	0 (0%)	4 (19%)	0 (0%)
IL-4	19 (90.5%)	0 (0%)	2 (9.5%)	0 (0%)
IL-17	19 (90.5%)	0 (0%)	2 (9.5%)	0 (0%)
AF03 and AS03 post-dose 2 (*n* = 51)				
IFN-γ	6 (11.8%)	29 (56.9%)	13 (25.5%)	3 (5.9%)
IL-2	2 (3.9%)	36 (70.6%)	6 (11.8%)	7 (13.7%)
TNF-α	4 (7.8%)	34 (66.7%)	10 (19.6%)	3 (5.9%)
IL-4	7 (13.7%)	6 (11.8%)	38 (74.5%)	0 (0%)
IL-17	30 (58.8%)	0 (0%)	21 (41.2%)	0 (0%)

Data shown are numbers of participant samples testing negative (−) or positive (+) in whole-blood cell supernatant (as detailed in Table 1) or by ICS (as detailed in Table 2) in PBMCs after full-length S protein stimulation, for each cytokine indicated. Data for both age groups and for both the AF03 and the AS03 groups are combined. Percentages are the percentage of total.

CD4$^+$ T cells after the first and second vaccinations, as defined by their multiplicity of cytokine and/or cytotoxic effector expression induced by stimulation with recombinant full-length S protein (Figure 5). Most of the responding cells were highly polyfunctional, simultaneously expressing 3 or 4 functional markers. The dominant profiles comprised cells expressing IL-2, TNF-α and CD40L with or without IFN-γ. A small proportion of S-specific CD4$^+$ T cells also co-expressed granzyme B. Combinations co-expressing IL-4 or IL-5 and/or IL-13 were very rare, consistent with the dominant Th1 responses observed for the measurement of individual cytokines (Figure 3). Cells co-expressing IL-17 were not detected. Also consistent with the individual cytokine data, the frequency of polyfunctional CD4$^+$ T cells that included IFN-γ expression was higher for older adults in the AS03-adjuvanted group, especially after the second vaccination, contributing to the higher Th1 dominance in this group.

DISCUSSION

In this study, we describe the CMI responses induced by two adjuvanted formulations of SARS-CoV-2 recombinant protein vaccine as measured by two assays, one in which fresh whole-blood cells were directly stimulated *ex vivo* for 48 h with the recombinant full-length S protein and another in which cryopreserved PBMCs underwent a much shorter stimulation with either pools of peptides covering the S protein or with S protein (ICS). While the two assays do not measure exactly the same immune parameters, the observed cellular immunogenicity of the vaccine candidate was generally consistent between the two assays, characterised by a Th1 bias overall driven by a lower magnitude of Th2 cytokine response, and little or no Th17 response. The results showed similarly robust cellular immune responses for both adjuvanted formulations in the younger participants, but differences were observed in the older age group, with AS03 inducing stronger responses than AF03 for IFN-γ, IL-2, TNF-α and GM-CSF. A lower Th1 response contributed to a more balanced Th1/Th2 ratio among the older adults in the AF03-adjuvanted group and is also consistent with the lower antibody responses seen in this subgroup.[23] Based on prior findings, a new formulation has been developed with a view to improving the pre S vaccine antigen preparation and identifying the optimal antigen dose; this new formulation has shown stronger responses in adults aged ≥ 60 years equivalent to those observed in the younger age group.[27]

Some disparities were observed between the two methods, likely due to a number of technical differences. In particular, higher response rates for Th2 cytokines were observed using the whole-blood cytokine secretion assay. It is unclear whether this was due to high levels of Th2 secretion from a limited number of cells or potentially more limited secretion from a large number of cells, including from non-specific cell types. It is also notable that the amount of cytokine measured in the whole-blood assay reflects the net secretion (potentially
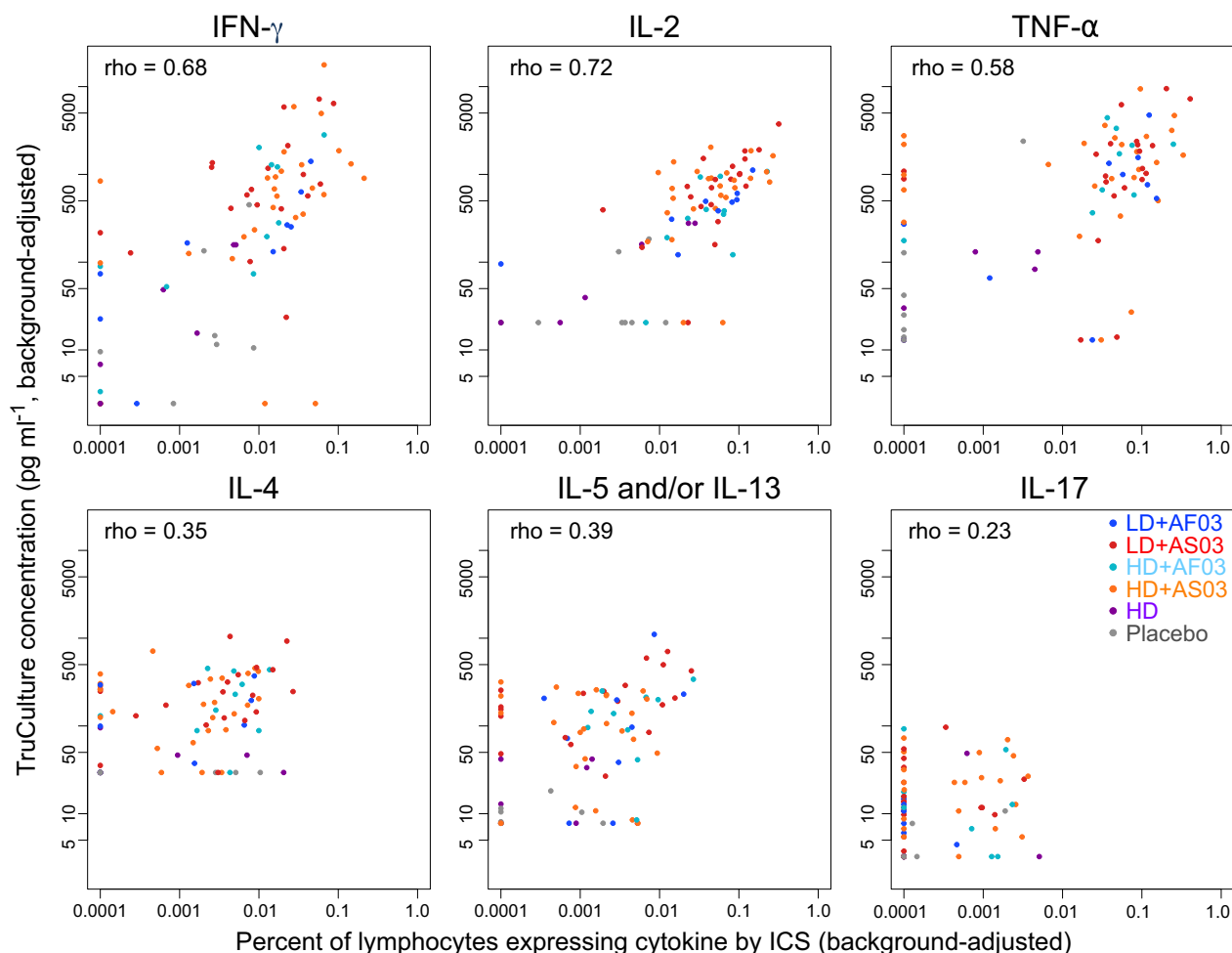
Figure 4. Correlation between cytokine profiles evaluated in the supernatants of whole blood or intracellular staining of PBMCs after the second vaccination (full-length S protein stimulation). ICS data (frequencies of CD4$^+$ T cells producing each cytokine for the recombinant full-length S protein stimulation) are expressed as percentage of lymphocytes rather than percentage of CD4$^+$ T cells in order to be more comparable to the measurement of concentrations in whole blood. Since IL-5 and IL-13 were not analysed separately by ICS, the data for those two cytokines were summed for the whole-blood cytokine secretion assay. Spearman's rank correlation coefficients (rho) are shown.

from different cell types) during stimulation, that is regardless of differing rates of cytokine release versus uptake or destruction. The longer duration of antigen stimulation for the whole-blood assay (48 h) compared with the ICS assay (9 h with full-length S protein), as well as the absence of the protein transport inhibitor brefeldin A in the whole-blood assay, may have contributed to a greater accumulation of cytokines in whole-blood supernatant. It is also possible that the secretion of inflammatory cytokines in whole blood during the 48-h incubation may have caused indirect ('bystander') activation of other cells leading to non-specific secretion of cytokines (i.e. from sources other than CD4$^+$ T cells). In contrast, ICS detects

cytokines that are blocked within the cell cytoplasm, eliminating the possibility of a bystander effect. The high levels of Th2 cytokine secretion may also have been accentuated by the fact that granulocytes, which can secrete Th2 cytokines, are present in whole blood but are eliminated during the PBMC purification step.

There are critical advantages to the ICS assay in providing specific and precise identification and characterisation of vaccine-reactive T cells as well as high-dimensional single-cell functional and phenotypic data of responding cells. However, there are practical limitations in the use of ICS in large Phase III clinical trials due to the logistical complexity of PBMC processing from whole blood and cryopreservation across multiple clinical sites.
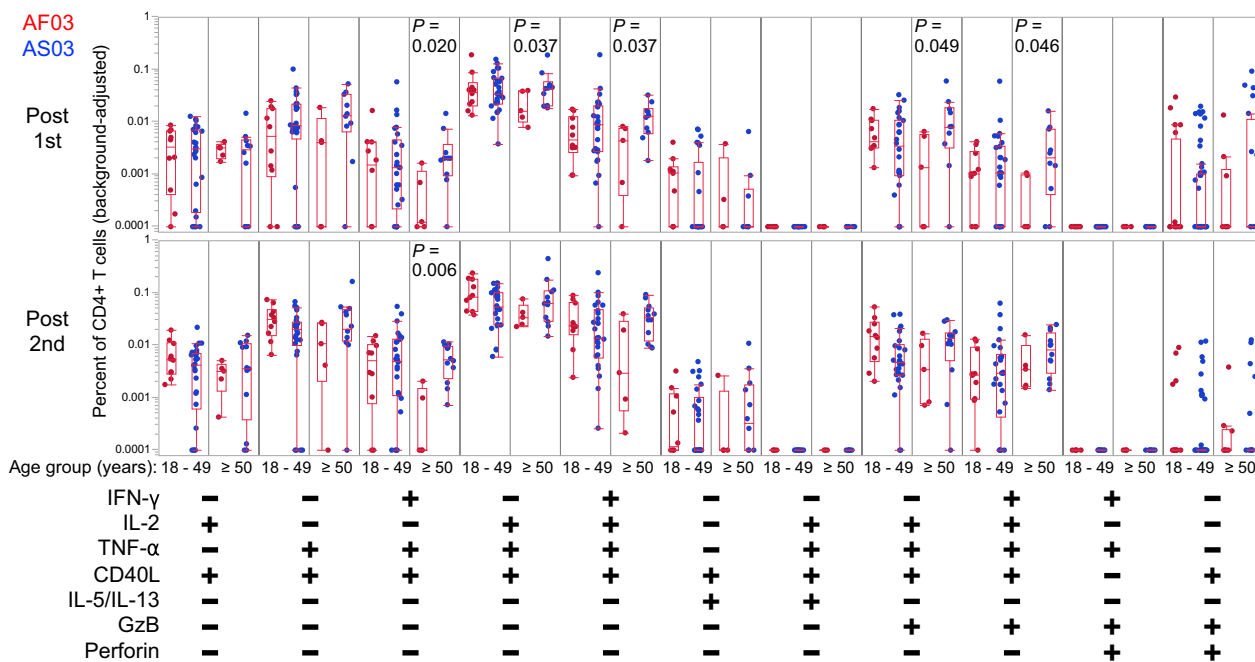
Figure 5. Polyfunctionality of CD4+ T-cell responses measured by ICS after stimulation with recombinant full-length S protein after one or two doses of SARS-CoV2 protein adjuvanted with AF03 or AS03. Data show background-subtracted CD4+ T-cell frequencies (percentage of CD4+ T cells expressing the indicated cytokine combination; log scale) for participants receiving the SARS-CoV2 recombinant protein candidate vaccine adjuvanted with AF03 (red dots) or AS03 (blue dots), by age groups (18–49 or ≥ 50 years) at baseline, 21 days after dose 1 (Post 1st) and 14 days after dose 2 (Post 2nd). The y-axis is truncated at 0.0001%, and any values below this level are censored. Each functional profile is shown in the key on the x-axis as determined by gating as positive or negative for each marker (see Supplementary figure 5 for the gating strategy and representative scatter plots). Because the focus of this figure was polyfunctional T cells, data for cells expressing only one cytokine are not shown. Also, to limit the size of the graph, cellular profiles for which the frequency of cells was very low are not included. Cells co-expressing IL-4 or IL-17 were either not detected or only present at low frequency; all profiles shown are negative for those cytokines and they are not included in the key on the x-axis. The mid-line of the box denotes the median and the ends of the box denote the 25th and 75th percentiles. The whiskers that extend from the top and bottom of the box extend to the most extreme data points that are no more than 1.5 times the interquartile range or if no value meets this criterion, to the data extremes. Wilcoxon rank P-values < 0.05 are shown (not adjusted for multiplicity).

While the use of PBMCs provides an enriched population of leukocytes for analysis, operators need to be fully trained and isolation and sample preparation procedures need to be carefully followed to avoid substantial technical variability.[19,28] Additionally, ICS methodology is labour-intensive, costly, and requires specialised instruments, with complex staining panel optimisation and data analysis. In the current study, the analysis of a panel of specific cytokines in stimulated whole blood, although not providing information on cell type, led to similar conclusions overall for the vaccine-induced cell-mediated immunity and thus may provide an alternative to ICS for CMI analysis in large clinical trials.

The strong CD4+ T-cell responses seen in this study driven by high levels of IFN-γ and IL-2 are consistent with previous observations during the clinical development of other COVID vaccines, which have since been authorised for use.[29-32] Available data from a H5N1 influenza vaccine candidate indicate that the AS03 adjuvant may promote or modulate Th1-biased cellular responses.[33] Th1-biased CD4+ T-cell responses were also reported for the SARS-CoV-2 candidate vaccine SCB-2015 (S-Trimer protein) adjuvanted with AS03 or CpG/Alum.[34] Preliminary data for a recombinant virus-like particle vaccine candidate for COVID-19 with AS03-adjuvant showed that both IFN-γ and IL-4 increased to near equivalence after the second dose indicating a more balanced Th1/Th2 response overall.[35] These data are aligned with recent findings of a strong, balanced Th1/Th2 profile in non-human primates immunised with candidate subunit SARS-CoV-2 vaccine

formulated with AS03; those receiving AS03-adjuvanted formulations also showed a particularly strong IL-21^{+}CD154^{+} cellular response, indicative of a role of T follicular helper cells.[36]

In the current study, we also observed increased secretion of IL-6 and GM-CSF in post-vaccination samples, as measured in the whole-blood cytokine secretion assay. These cytokines may both play potential roles in the development of severe SARS-CoV-2 infection and clinical deterioration, and have thus attracted interest as potential therapeutic targets for COVID-19.[37-39] While a potential relationship between levels of IL-6 and GM-CSF and hyperactive immune responses would be of interest to explore in relation to vaccine reactogenicity, these would need to be assessed shortly after vaccination (i.e. at time of the adverse event) rather than after 2–3 weeks and thus were beyond the scope of the current substudy. We also assessed levels of IL-17, which plays a major role in neutrophil recruitment. Notably, neutrophilia is thought to be characteristic of the vaccine-enhanced respiratory syncytial virus (RSV) disease previously observed in children who received formalin-inactivated RSV vaccine and subsequently acquired natural RSV infection.[40,41] IL-17 secretion could therefore possibly signal an enhanced and potentially detrimental neutrophil response, but was detected only at low levels in the whole-blood assay and not detected in the ICS assay.

Previous analyses using rapid whole-blood stimulation overnight with peptide pools to evaluate SARS-CoV-2-specific memory T-cell immunity in 58 recipients 12–23 days after a single dose of AstraZeneca CHadOx1-S COVID-19 vaccine also showed significant increases in IFN-γ and IL-2 in the majority of vaccinated individuals (87.9% tested positive for IFN-γ or IL-2; 63.8% were positive for both).[42] There were no significant differences in the cytokine response when analysed by age or gender. Another study using whole-blood stimulation with peptide pools for 24 h in 44 convalescent and 21 unexposed individuals showed significantly elevated levels of IFN-γ, IL-2 and IL-8 in convalescent versus unexposed individuals, as we observed in our study.[43] Levels of TNF, IL-1β, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and IL-17a were unchanged. The differences we can see in the cytokines (except IFN-γ and IL-2) could be due to the different timing of the sampling (a median of 58 days post initial diagnosis in the previous study versus 2 weeks after 2nd injection in our study).

Together, using these two compendial methods, we have shown that adjuvanted SARS-CoV-2 recombinant protein vaccine candidate elicits, after the first vaccination, a robust Th1-biased cell response upon stimulation with recombinant S protein or S peptide pools, mainly characterised by increased IFN-γ, IL-2 and TNF-α secretion. IL-4 secretion was also detected but to a lesser extent. The AS03-adjuvanted formulation elicited responses with a higher Th1/Th2 ratio overall than the AF03-adjuvanted formulation, in both younger and older participants. These findings add to our understanding of cellular immune response elicited by this vaccine candidate and support further development of an AS03-adjuvanted SARS-CoV-2 recombinant protein vaccine. Moreover, our findings support the use of a whole-blood cytokine secretion assay as a suitable alternative to the single-cell flow cytometry assay for use in large, complex clinical trials.

METHODS

Study design

The study design for this phase I–II, randomised, modified double-blind, parallel-group, placebo-controlled, multicentre study has been previously described (ClinicalTrials.gov, NCT04537208).[23] Briefly, healthy adults aged ≥ 18 years who tested negative for SARS-CoV-2 antibodies (using COVID-19 IgG/IgM Rapid Test Cassette; Healgen Scientific, Houston, TX, USA) were enrolled across 10 centres in the USA. Participants were stratified by age (18–49 years and ≥ 50 years) and randomised using an interactive response technology system, with blocks of varying sizes (Calyx, Nottingham, UK), to receive one or two separate injections (on study day [D]1 and D22) of placebo (saline) or one of five candidate vaccine formulations: low-dose antigen plus AF03, high-dose antigen plus AF03, low-dose antigen plus AS03, high-dose antigen plus AS03 or high-dose antigen with no adjuvant.[23] Due to the erroneous characterisation of the final bulk drug substance, the concentration of functional SARS-CoV-2 pre-fusion S (pre S) antigen in the formulated vaccine product was substantially lower than planned with effective dose levels administered of 1.3 and 2.6 μg, respectively. A subset of participants was randomly selected from the two-dose cohort for evaluation of CMI. Here, we describe exploratory CMI analyses conducted on blood samples and isolated PBMCs obtained from this participant subset pre-vaccination (D1), 21 days after the first vaccination (D22) and 14 days after the second vaccination (D36).

The study was conducted in compliance with the International Conference on Harmonisation guidelines for Good Clinical Practice and the principles of the Declaration of Helsinki. The protocol and amendments were approved by applicable Independent Ethics Committees and

Institutional Review Boards (Advarra, Inc.) and the regulatory agency as per local regulations. Written informed consent was obtained from the participants before any study procedures were done.

Cytokine production in whole blood

Cytokine responses in whole blood following *ex vivo* stimulation with recombinant S protein were measured by multiplex bead array (TruCulture OptiMAP™ and CytokineMAP® A assay, Myriad Rules-Based Medicine [RBM]), as previously described.[23] TruCulture® tubes were batch prepared to give a final concentration of 6 μg mL^{-1} recombinant full-length SARS-CoV-2 S protein (S 2P-GCN4, GeneArt), or SEB plus anti-cluster of differentiation 28 (anti-CD28) (positive control; Myriad RBM) resuspended in 2 mL buffered media. Sodium heparin was added as anticoagulant, and the prepared tubes were maintained at −20°C until use. Then, 1 mL of blood was drawn directly into the thawed TruCulture® tubes (Myriad RBM) and inserted into a dry heat-block and maintained at 37°C (± 1°C) for 48 h. At the end of the incubation period, tubes were opened, and a valve was inserted to separate the sedimented cells from the supernatant and to stop the stimulation reaction. Tubes were frozen at −20°C until analysis. Two cytokine profiling panels, validated by Myriad RBM (according to Good Clinical Laboratory Standards), were used: Human CustomMAP, HMPCORE1, for the evaluation of IL-4 and IL-5 concentrations, and Truculture OptiMAP™ (HMPC122) for the evaluation of IFN-γ, TNF-α, IL-2, IL-6, IL-10, IL-13, IL-17 and GM-CSF in whole-blood cell supernatants. Panels were analysed on a Luminex platform (Myriad RBM).

Flow cytometric intracellular staining of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated and cryopreserved until analysis. Before testing, PBMC specimens were thawed, rested overnight at 37°C, and then stimulated with either recombinant full-length S protein (S 2P-GCN4, GeneArt) or pools of overlapping peptides covering the S protein. For the protein stimulation, cells were stimulated with 6 μg mL^{-1} (as determined by NanoDrop Spectrophotometer) full-length S protein for 3 h, and then, brefeldin A was added for an additional 6-hour incubation. The cells stimulated with S peptide pools were stimulated in the presence of brefeldin A for 6 h using methods that have been described previously.[44,45] An extra 3 h of stimulation with the full-length protein before addition of brefeldin A was required to enable processing of the recombinant protein and presentation at the surface; this is not required for stimulation with S peptide pools. The peptide pools encompassed 320 S peptides split into two pools to cover the S protein subunits S1 (173 peptides) and S2 (147 peptides). These include four overlapping peptides that cover the variants (D/G) at position 614. The peptides were synthesised by Bio-Synthesis Inc. (BSI) and used at a final concentration of 1 μg mL^{-1} (of each individual peptide) and diluted in dimethyl sulfoxide (DMSO, final concentration < 0.5%). As a negative control,

cells were incubated with 0.5% DMSO. As a positive control, cells were stimulated with a polyclonal stimulant, SEB, to assure that the PBMCs were still functional.

Only specimens with at least 66% viability, measured after thawing and overnight resting, were considered acceptable for testing.[45] Isolated PBMCs were stained using a validated 27-colour staining panel (Supplementary table 4).[46,47] Data were collected on BD FACSymphony A5 instruments and analysed using FlowJo software (version 9). See Supplementary figure 5 for the gating strategy and representative scatter plots.

Outcomes

For the whole-blood cytokine assay, concentrations for each cytokine were automatically calculated on D1, D22 and D36 using adapted software (Myriad RBM) with a standard curve; background levels (Truculture® tubes with medium alone) were subtracted from these values.

The frequencies of CD4$^+$ cells expressing IFN-γ, IL-2 or the combination, IFN-γ and/or IL-2, and CD4$^+$ cells expressing IL-4 with CD40L and IL-5 and/or IL-13 with CD40L were determined after stimulation with recombinant full-length S protein or S protein peptide pools in the total population of CD4$^+$ T helper (Th) cells, on D1, D22 and D36. Co-expression of the Th2 cytokines with CD40L was used because prior data showed that this increased the specificity for detection of these cytokines. Reagents for IL-5 and IL-13 were conjugated to the same fluorochrome and could not be separately analysed. The percentage of CD8$^+$ T cell expressing IFN-γ and/or IL-2 was also determined.

Statistical analysis

Cell-mediated immunity was analysed in a participant subset of the per-protocol analysis set (PPAS), defined as the set of randomised participants who received at least one injection, and who fulfilled all inclusion criteria as specified in the protocol. The per-protocol CMI participant subset additionally excluded those without results of CMI sample or for whom all post-dose CMI samples were obtained outside the pre-defined time window (pre-vaccination samples, on D1; post-vaccination, D22 + 7 days or D36 + 7 days). The per-protocol CMI subset was planned to include 87 participants and analysed according to the study group to which the participants were randomised.[23] To assess the feasibility of combining antigen dose groups for further analysis, we used linear regression models to examine the effect of antigen dose on the log-fold change over baseline for whole-blood cytokine secretion and ICS responses, while adjusting for age, adjuvant type and whether or not adjuvanted. For all functional subsets, all through stimulations and at each visit post baseline, linear regression models were fitted for log-fold change over baseline of background-adjusted response magnitude to per-protocol vaccines (Groups 6–10, not including placebo recipients), including main effects for age group (18–49 years, ≥ 50 years); adjuvant dose level (none, low, high); and adjuvant (AF03, AS03, none). The reference categories in the linear regression were 18–49 years old, low dose and with adjuvant AF03. The models included indicator

variables for age over 50, high dose, adjuvant AS03 and no adjuvant. The coefficient of each indicator variable had the interpretation of average log-fold change for the particular category compared to the reference category. To highlight differences between the adjuvants, statistical comparisons were performed using the Wilcoxon rank sum test without adjustment for multiple comparisons. Due to the relatively small sample sizes and the exploratory nature of these analyses, these *P*-values mainly identified potential differences for further study. For the whole-blood assay, the positivity of the cytokine responses was determined based on fold-change over the negative control using empirically determined thresholds (Supplementary figure 2). Since these thresholds were based on the data from this study and not pre-determined, the positivity results should be considered exploratory.

For ICS analyses, a positive or negative cytokine response for the T-cell subsets (CD4$^+$ or CD8$^+$) after stimulation with full-length S protein or S peptide pools was determined using a two-by-two contingency table comparing the stimulated and negative control data. The four entries in each table were the number of cells positive for the cytokine (s) and the number of cells negative for the cytokine(s), for both the stimulated and the negative control data. If both negative control replicates were included, then the average number of total cells and the average number of positive cells was used. A one-sided Fisher's exact test was applied to the table, testing whether the proportion of cytokine-producing cells for the stimulated data was equal to that for the negative control data.

Since multiple individual tests (for each of the two peptide pools) were conducted simultaneously, a multiplicity adjustment was made to the individual peptide pool *P*-values using the Bonferroni–Holm adjustment method. If the adjusted *P*-value for a peptide pool was ≤ 0.00001, the response to the peptide pool for the T-cell subset was considered positive. Because the sample sizes (i.e. total cell counts for the T-cell subset) were large, for example as high as 100,000 cells, the Fisher's exact test had high power to reject the null hypothesis for very small differences. Therefore, the adjusted *P*-value significance threshold was chosen stringently (≤ 0.00001). If at least one peptide pool for the spike protein (S1 or S2) for a T-cell subset was positive, then the overall response to spike protein was considered positive for that T-cell subset.

Responses to the recombinant full-length S protein stimulation were analysed separately and not combined with the S protein peptide pool responses. Corresponding 95% CIs were calculated by the score test method.[48]

The magnitude of the overall response to the S protein peptide pools was calculated as the sum of the individual S protein peptide pool magnitudes. Both pools were included regardless of positive or negative responder status, and negative magnitudes were not censored at 0.

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CONFLICTS OF INTEREST

AUTHOR CONTRIBUTION

Stephen C De Rosa: Conceptualization; Funding acquisition; Methodology; Writing – review & editing. **Kristen W Cohen:** Conceptualization; Formal analysis; Funding acquisition; Writing – review & editing. **Matthew Bonaparte:** Conceptualization; Methodology; Writing – review & editing. **Bo Fu:** Validation; Writing – review & editing. **Sanjay Garg:** Formal analysis; Validation; Writing – review & editing. **Catherine Gerard:** Methodology; Validation; Writing – review & editing. **Paul A Goepfert:** Methodology; Validation; Writing – review & editing. **Ying Huang:** Methodology; Writing – review & editing. **Daniel Larocque:** Methodology; Validation; Writing – review & editing. **M Juliana McElrath:** Methodology; Writing – review & editing. **Daryl Morris:** Methodology; Writing – review & editing. **Guy de Bruyn:** Conceptualization; Methodology; Writing – review & editing. **Anke Pagnon:** Methodology; Writing – review & editing.

DATA AVAILABILITY STATEMENT

Qualified researchers can request access to patient-level data and related study documents, including the clinical study report, study protocol with any amendments, blank case report form, statistical analysis plan and dataset specifications. Patient-level data will be anonymised and study documents will be redacted to protect the privacy of trial participants. Further details on Sanofi's data sharing criteria, eligible studies and process for requesting access can be found at https://www. clinicalstudydatarequest.com.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.