



## Research paper

# Adjuvanted SARS-CoV-2 spike protein elicits neutralizing antibodies and CD4 T cell responses after a single immunization in mice



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## ABSTRACT

**Background:** SARS-CoV-2 has caused a global pandemic, infecting millions of people. A safe, effective vaccine is urgently needed and remains a global health priority. Subunit vaccines are used successfully against other viruses when administered in the presence of an effective adjuvant.

**Methods:** We evaluated three different clinically tested adjuvant systems in combination with the SARS-CoV-2 pre-fusion stabilized (S-2P) spike protein using a one-dose regimen in mice.

**Findings:** Whilst spike protein alone was only weakly immunogenic, the addition of either Aluminum hydroxide, a squalene based oil-in-water emulsion system (SE) or a cationic liposome-based adjuvant significantly enhanced antibody responses against the spike receptor binding domain (RBD). Kinetics of antibody responses differed, with SE providing the most rapid response. Neutralizing antibodies developed after a single immunization in all adjuvanted groups with ID<sub>50</sub> titers ranging from 86–4063. Spike-specific CD4 T helper responses were also elicited, comprising mainly of IFN- $\gamma$  and IL-17 producing cells in the cationic liposome adjuvanted group, and more IL-5- and IL-10-secreting cells in the AH group.

**Interpretation:** These results demonstrate that adjuvanted spike protein subunit vaccine is a viable strategy for rapidly eliciting SARS-CoV-2 neutralizing antibodies and CD4 T cell responses of various qualities depending on the adjuvant used, which can be explored in further vaccine development against COVID-19.

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## 1. Introduction

A safe and effective vaccine against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is urgently needed. Antibody responses are the best correlate of protection for most vaccines [1] and inducing such responses is a central goal for a SARS-CoV-2 vaccine. The receptor binding domain (RBD) of the spike protein is an attractive target for neutralization of coronaviruses [2,3], and neutralizing monoclonal antibodies (mAbs) targeting the RBD and blocking receptor binding have been isolated from COVID-19 convalescent patients [4,5] and offer protection in animal studies [6–8]. Several of these are now under evaluation as prophylactic or therapeutic antibodies to protect against clinical COVID-19 disease. Acute SARS

CoV-2 infection also induced CD4 T cell responses directed against the membrane (M), nucleocapsid (N) and/or spike proteins in 100% of COVID-19 convalescent cases [9,10]. CD4 T cells shape the overall immune response, including antibody profiles and sustained humoral immunity [11], but may also reduce viral load by direct killing of infected cells [12]. Although the optimal vaccine profile remains unclear, a vaccine inducing both potent neutralizing antibody and CD4 T cell responses is likely to be protective.

The most progressed SARS-CoV-2 vaccine candidates rely on relatively novel vaccine technologies, including RNA- [13,14], DNA- [15,16], or adenovirus-vectored trimer proteins [17]. These strategies appear promising and have generated neutralizing antibodies in clinical trials [13,17] and protection against SARS-CoV-2 in preclinical models [15,18]. Preliminary data also demonstrate high efficacy for the RNA- and adenovirus vectored trimer proteins in clinical trials. However, the concurrent development of vaccines based on established vaccine platforms is prudent and particularly vaccines that show protection after a single immunization are desired. While

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## Research in context

### Evidence before this study

A safe and effective vaccine against the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) causing COVID-19 disease is urgently needed. A number of SARS-CoV-2 vaccine candidates are currently evaluated. Many of these rely on novel vaccine technologies, e.g. RNA-, DNA- or adenovirus-vectors. Subunit vaccines composed of purified recombinant proteins are used successfully in licensed vaccines against Hepatitis virus B (HBV) and human papilloma virus (HPV) and a SARS-CoV-2 subunit vaccine could therefore be a safe and effective alternative. However, as subunit vaccines are poorly immunogenic on their own, adjuvants are required to boost vaccine immune responses. Adjuvants may differentially affect antibody secreting B cell response magnitude and breadth as well as CD4 T cell profiles. The optimal adjuvant for a SARS-CoV-2 subunit vaccine is currently unclear.

### Added value of this study

Our study demonstrates that adjuvanted SARS-CoV-2 spike protein subunit vaccine can elicit neutralizing antibody responses after a single immunization and that the elicited T cell response profile depends on the type of adjuvant.

### Implications of all the available evidence

This study supports the use of subunit vaccines containing adjuvanted SARS-CoV-2 spike protein for rapidly inducing neutralizing antibodies and T cell responses against SARS-CoV-2 and warrants further studies for determining the optimal adjuvant in animal challenge models.

is in several licensed vaccines and is known to promote an antigen depot at the site of injection [28], an oil-in-water squalene emulsion (SE) adjuvant resembling MF59<sup>TM</sup>, which is licensed in a seasonal influenza vaccine, and a cationic liposome-based adjuvant (CAF<sup>®</sup>01), which has been tested in four phase 1 clinical trials and has been shown to induce strong CD4 T cell responses when tested with protein-based antigens [33,34].

## 2. Methods

### 2.1. Antigens and adjuvants

Both the SARS-CoV-2 S-2P stabilized spike trimer [32] antigen, and the RBD domain (RVQ-VNF) were produced by transient expression in freestyle 293-F cells, and purified first by affinity and then by size exclusion chromatography, as reported previously [24]. CAF<sup>®</sup>01 (DDA/TDB) was produced in house (Statens Serum Institut, Copenhagen, Denmark) [35], the AddaVax<sup>TM</sup> oil-in-water squalene emulsion (SE, vac-adx-10) was from Invivogen (Toulouse, France) and aluminium oxyhydroxide (AH) (2% Alhydrogel<sup>®</sup>) was from Croda (Frederiksund, Denmark).

### 2.2. Characterization of prefusion-stabilized SARS-CoV-2 (S-2P) trimer and adjuvant formulations

A compatibility study of the spike trimer and adjuvant combinations was performed at room temperature (RT). Formulations were first analyzed visually for potential flocculation. Spike protein-adjuvant formulations were characterized for particle size and polydispersity index (PDI) by dynamic light scattering, by using the photon correlation spectroscopy technique. The surface charge of the particles was analyzed by measuring the zeta potential (laser-Doppler electrophoresis). Adjuvants alone or mixed with equal volumes of spike protein solutions were mixed and left to equilibrate for at least 10 min prior to the size and zeta potential measurements. Measurements were repeated after 24 h. For the size measurements, the samples were diluted 10 times, whereas for the zeta potential measurements, the samples were diluted 100 times in milli-Q water. The measurements were performed at 25°C by using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. Malvern DTS v.6.20 software was used for data acquisition and analysis. Particle size distribution was reflected in the PDI, which ranges from 0 for a monodisperse to 1.0 for a heterodisperse formulation.

### 2.3. Cryo-TEM

Formulations were prepared with CAF01 (1250 µg/ml DDA and 250 µg/ml TDB), SE (undiluted) and AH (2500 µg aluminium content/ml). The samples were analyzed directly or mixed 1:1 with antigen (25 µg/ml spike protein). Formulations with AH were further diluted 1:100 before analysis. Samples were prepared under controlled temperature (4 °C) and humidity conditions (100%) within an environmental vitrification system using a Vitrobot Mark IV (ThermoFisher). A sample droplet of 3 µL of formulation was deposited on a glow-discharged 300 mesh holey carbon grid. Excess liquid was blotted away for 3 s. The sample was immediately submerged into liquid ethane, resulting in the formation of a thin (10–500 nm) vitrified film. Samples were stored in liquid nitrogen and transferred to a Gatan 626 cryo-holder for imaging by cryo-TEM, using a Tecnai G2 T20 TWIN transmission electron microscope (ThermoFisher, USA). The sample temperature was constantly kept below –170°C. All observations were made in the bright field mode at an acceleration voltage of 200 kV. Images were recorded using a 4 × 4 CCD Eagle camera (ThermoFisher).

inactivated virus vaccines are a well-established technology, and are being explored for SARS-CoV-2 [19], particular care must be paid to the potential exacerbation of lung immunopathology following viral infection, possibly mediated by T helper type 2 (Th2) responses, as has been reported for other inactivated vaccines e.g. against RSV [20] and in mouse models for SARS-CoV [21]. Another concern with inactivated vaccines is elicitation of antibodies against non-neutralizing antigen targets, which may exacerbate viral infection through antibody-dependent enhancement (ADE) [22]. Studies in animal challenge models and clinical trials will reveal whether there are any concerns with using inactivated vaccines against SARS-CoV-2. Another approach is to use purified recombinant proteins (subunit vaccines), which can effectively elicit high titers of neutralizing antibodies against SARS-CoV-2 [23,24] and have demonstrated protective immunity for the highly successful hepatitis virus B (HBV) and human papilloma virus (HPV) vaccines, when administered in the presence of adjuvant.

Adjuvants are used to augment and orchestrate immune responses and influence affinity, specificity, magnitude and functional profile of B and T cell responses [25,26]. Although mechanisms of adjuvants may to some extent be translated across antigens and disease targets, antigen-specific responses have also been demonstrated, e.g. depending on antigen physicochemical properties, such as size and charge [27–29]. Adjuvants may also influence stability and integrity of antigens [28,30]. Importantly, to speed up SARS-CoV-2 vaccine development, a desired adjuvant should not only be safe and effective but also approved for clinical use or far in clinical development. In this study we tested prefusion-stabilized (S-2P) spike trimer [31,32] formulated in three different clinically tested adjuvant systems with diverse properties; Aluminium hydroxide (AH), which

## 2.4. Antigen adsorption

Spike trimer was mixed with AH, using similar antigen and adjuvant concentrations as used for immunizations. In addition, formulations were prepared containing two-fold dilutions of antigen, whilst keeping the adjuvant concentration constant. Samples were centrifuged using an Optima™ MAX-XP ultra-centrifuge (Beckman Coulter, Ramcon, Copenhagen, Denmark) at 135,700 g for 30 min. After centrifugation, the supernatant was quantified for non-adsorbed antigen by Micro BCA (Thermo Scientific, Wohlen, Switzerland). The antigen adsorption rates were extrapolated from results obtained when measuring antigen alone (in the absence of adjuvant).

## 2.5. Mice

Female C57Bl/6 wild type mice, 6–8 weeks old, were ordered from Harlan Laboratories (The Netherlands) and housed in the animal facilities at Statens Serum Institut, Denmark. Mice were housed with up to eight mice/cage.

## 2.6. Ethics

Mouse studies were conducted in accordance with the regulations set forth by the Danish National Committee for the Protection of Animals used for Scientific Purposes and in accordance with European Community Directive 2010/63/EU. The experiments have been approved by, and conducted in compliance with, the governmental Animal Experiments Inspectorate under license 2017-15-0201-01363.

## 2.7. Immunizations

Mice were randomized to different groups and given a single immunization subcutaneously (s.c.) at the base of the tail with 5  $\mu$ g recombinant SARS-CoV-2 trimer in a volume of 200  $\mu$ l TRIS buffer (pH 7.4) per immunization. Adjuvant doses were according to manufacturer's instructions: CAF01 (dose 250  $\mu$ g/50  $\mu$ g (DDA/TDB)), SE (dose of 100  $\mu$ l 4.3% w/v squalene, 0.5% w/v Tween 80, 0.5% w/v Span 85 mixed 1:1 with antigen/PBS) and AH (dose of 500  $\mu$ g aluminium content). The immunization studies were nonblinded.

## 2.8. Organ preparation

Mice were euthanized by CO<sub>2</sub> (80%)/O<sub>2</sub> (20%), using a flow rate of 3 l/min, followed by cervical dislocation. Inguinal lymph nodes (LNs) and spleens were filtered through a 70  $\mu$ m nylon mesh (BD Biosciences). Lungs were dissociated via AutoMACS (C-tubes, Miltenyi). The cells were washed and prepared as previously described [36] and resuspended in cell culture medium (RPMI-1640 supplemented with 5  $\times$  10<sup>-5</sup> M 2-mercaptoethanol, 1% pyruvate, 1% HEPES, 1% (v/v) premixed penicillin-streptomycin solution (Invitrogen Life Technologies), 1 mM glutamine, and 10% (v/v) fetal calve serum (FCS)). Cell numbers were 2  $\times$  10<sup>5</sup> cells/well for MSD cytokine profiling or 1  $\times$  10<sup>6</sup> cells/well for flow cytometry (added in 200  $\mu$ l of cell culture medium).

## 2.9. Cytokine profiling

The Mouse U-plex (IFN- $\gamma$ , IL-17, IL-5, IL-13 and IL-10) was performed according to the manufacturer's instructions (Meso Scale Discovery) to measure CD4 T cell profiles after *ex vivo* re-stimulation of splenocytes with SARS-CoV-2 trimer antigen (2  $\mu$ g/ml cell culture medium incubated for 72 or 96 hours at 37 °C and 5% CO<sub>2</sub>) [37]. The plates were analyzed on a Sector Imager 2400 system (Meso Scale Discovery) and calculation of cytokine concentrations was performed

by 4-parameter logistic non-linear regression analysis of the standard curve.

## 2.10. ELISA for antibody responses

Maxisorp Plates (Nunc) were coated overnight with 0.05  $\mu$ g/well SARS-CoV-2 trimer antigen (4 °C). After blocking, serum was added in PBS with 2% BSA, starting with a 30-fold dilution for antigen-specific IgG or IgG subclasses. Polyclonal HRP-conjugated secondary antibody (rabbit anti-mouse IgG (ThermoFisher, RRID:AB\_138451), Goat anti-mouse IgG1 (Southern Biotech, 1070-05), IgG2c (Invitrogen, RRID:AB\_10983148), IgG3 (ThermoFisher, RRID:AB\_2536652) or rabbit anti mouse IgG2b (Invitrogen, RRID:AB\_2533920) was diluted in PBS with 1% BSA. After 1 h of incubation, antigen-specific antibodies were detected using TMB substrate as described by the manufacturer (Kem-En-Tec Diagnostics), and the reaction was stopped with H<sub>2</sub>SO<sub>4</sub>. Antibody titers were determined as the highest serum dilution corresponding to a cut-off of  $\geq 0.2$  OD<sub>450</sub>.

## 2.11. Neutralization assay

Neutralization of SARS-CoV-2 was evaluated using a pseudotyped lentivirus neutralization assay. The assay used the same format as an assay validated for the evaluation of HIV neutralization [38], but with the use of SARS-CoV-2 spike and HEK293T cells engineered to express human ACE2, as previously described [39]. ID50 values were estimated by fitting a logistic curve in Prism 5 (Graphpad Software), bounded between 0% and 100%, and interpolating the dilution at which luciferase expression was reduced by 50% relative to wells in the absence of serum. The investigator performing the neutralization assay was blinded to the experimental groups.

## 2.12. Flow cytometry

One million cells were stained in PBS+1% FBS and FcBlock (BD) was added to block unspecific binding. Cells were stained with a live/dead marker (Fixable viability dye EF780, eBioscience) and a cocktail of antibodies against the following surface proteins: B220 PerCP-Cy5.5 (RA3-6B2, RRID:AB\_394457) GL7 BV421 (GL7, 562967), GL7 FITC (GL7, 553666), IgD BV786 (11-26c.2a, RRID:AB\_2738322) (All BD), CD38 PE-Cy7 (90, RRID:AB\_11051806), CXCR5 BV421 (2G8, RRID:AB\_2562128), CD4 APC (RM4-5, RRID:AB\_469323) (eBioscience), PD-1 BV605 (29F.1A12, RRID:AB\_11125371) (Biolegend). Cells were analysed on a BD Fortessa flow cytometer.

## 2.13. Statistical analysis

Differences between groups were analyzed by Kruskal-Wallis test, using Dunn's multiple comparisons test with the unadjuvanted SARS-CoV-2 spike protein group as reference. Prism 8 software (GraphPad v8.2.1) was used for all statistical analyses.

## 2.14. Role of the funding source

The funding source had no role in study design, collection, analysis or interpretation of data or in the writing of the publication.

## 3. Results

### 3.1. Physicochemical characterization of adjuvanted spike trimer formulations

The SARS-CoV-2 spike trimer (spike protein) is the main determinant for viral entry into host cells [40]. The trimer protein is intrinsically metastable, but adding two proline mutations in the C-terminal S2 fusion domain resulted in a prefusion-stabilized spike antigen

[32], which is very similar in structure to functional SARS-CoV-2 spike proteins on intact virions [41]. Highly homogenous spike glycoprotein trimers were produced and verified to maintain a trimeric pre-fusion conformation [39]. In order to evaluate compatibility of the prefusion-stabilized SARS-CoV-2 spike trimer protein (spike protein) with different adjuvant systems, we initially performed physicochemical characterization of spike protein formulated with CAF01, SE and AH. The full spike is a 139.125 kDa protein predicted to have an isoelectric point of 6.24 [42]. Based on the net charge of the spike protein, it may adsorb to the CAF01 and AH adjuvants via electrostatic and hydrophobic interactions and, additionally to AH via ligand exchange. When mixing CAF01 with spike protein, nanoparticle size increased from approx. 210 nm to 500 nm, suggesting that CAF01 interacted with spike protein. The PDI did not change upon mixture and the particles remained highly cationic ( $z_p = +40$  mV) (Fig 1a). Mixing trimer with SE adjuvant did not influence particle size (150 nm) and emulsion droplets remained largely monodisperse with no change in PDI. The SE adjuvant also remained net negatively charged in presence of spike protein. Addition of spike protein to AH particles increased size from approx. 1200  $\mu\text{m}$  to 1800  $\mu\text{m}$ , suggesting interaction between spike protein and AH, whilst the charge of the AH particles was unaffected.

To investigate in more detail how spike protein affected the morphology of the different adjuvants, we performed cryo-TEM. CAF01 contained both multilamellar and unilamellar spherical liposomes but also more elongated liposomes (Fig 1b). This was largely similar when spike protein was added, although we noticed more clustering of liposomes and the appearance of rod-like structures, which could be collapsed liposomes binding the protein. As expected from the dynamic light scattering studies, we did not see noticeable changes in the SE droplets in the presence of spike protein. In contrast, with AH we observed larger aggregates in the formulation when spike protein was added compared to for AH alone. Thus, CAF01 and AH interacted with spike and formed aggregates as expected from the presence of electrostatic attractive forces, whereas SE with neutral surface charge did not interact with the protein.

Traditionally, the WHO has recommended that at least 80% of antigen is associated with AH, e.g. for diphtheria and pertussis vaccines [43]. The degree of antigen adsorption may affect both stability and structural integrity of antigens [30] and influence immune responses. Modifying antigens to increase adsorption to AH has led to increased antibody titers [29,44,45], illustrating the importance of interactions between antigens and AH. However, a very tight antigen-AH binding may compromise antibody responses [27,46]. Thus a certain degree of adsorption to AH seems beneficial. We tested adsorption capability of AH by measuring spike protein in the supernatant following centrifugation of the antigen-adjuvant mixture. Spike protein levels were below detection limit (2  $\mu\text{g}/\text{ml}$ ) in the supernatant following centrifugation of the AH-spike protein formulation, demonstrating that at least at the concentrations used for immunization, most if not all spike protein was adsorbed to AH (Fig 1c). We thus continued to evaluate spike trimer in combination with the different adjuvants *in vivo*.

### 3.2. Adjuvanted spike trimer induces neutralizing antibody responses after a single immunization

A pandemic vaccine against SARS-CoV-2 should preferably be effective after only a single immunization. To compare different adjuvant systems for stimulating immune responses to a single dose of SARS-CoV-2 spike trimer protein, we immunized mice subcutaneously with spike protein (5  $\mu\text{g}$ ) given alone or formulated in CAF01, SE or AH. To investigate if the formulations differed in their capacities to induce a rapid antibody response, we examined the kinetics of IgG antibody responses against the total spike protein and the RBD, which is the most important determinant for neutralization. Notably,

spike-specific IgG antibody responses could be observed already at seven days post immunization in the SE adjuvanted group (significantly higher than in the spike alone group ( $p < 0.05$ , Kruskal-Wallis test)) (Fig 2a). All the adjuvants enhanced both total spike protein-specific and RBD-specific (Fig 2b) antibody responses, although AH induced a delayed antibody response compared to the CAF01 and SE adjuvants. At 45 days post immunization, when the experiment was terminated, the antibody titers were similar in all the adjuvanted groups (Fig. 2a and b). The anti-RBD IgG response consisted of IgG1 and IgG2b in all groups, whilst IgG2c was only detected in the CAF01 and SE adjuvanted groups and IgG3 was not detected in any of the groups (sFig1).

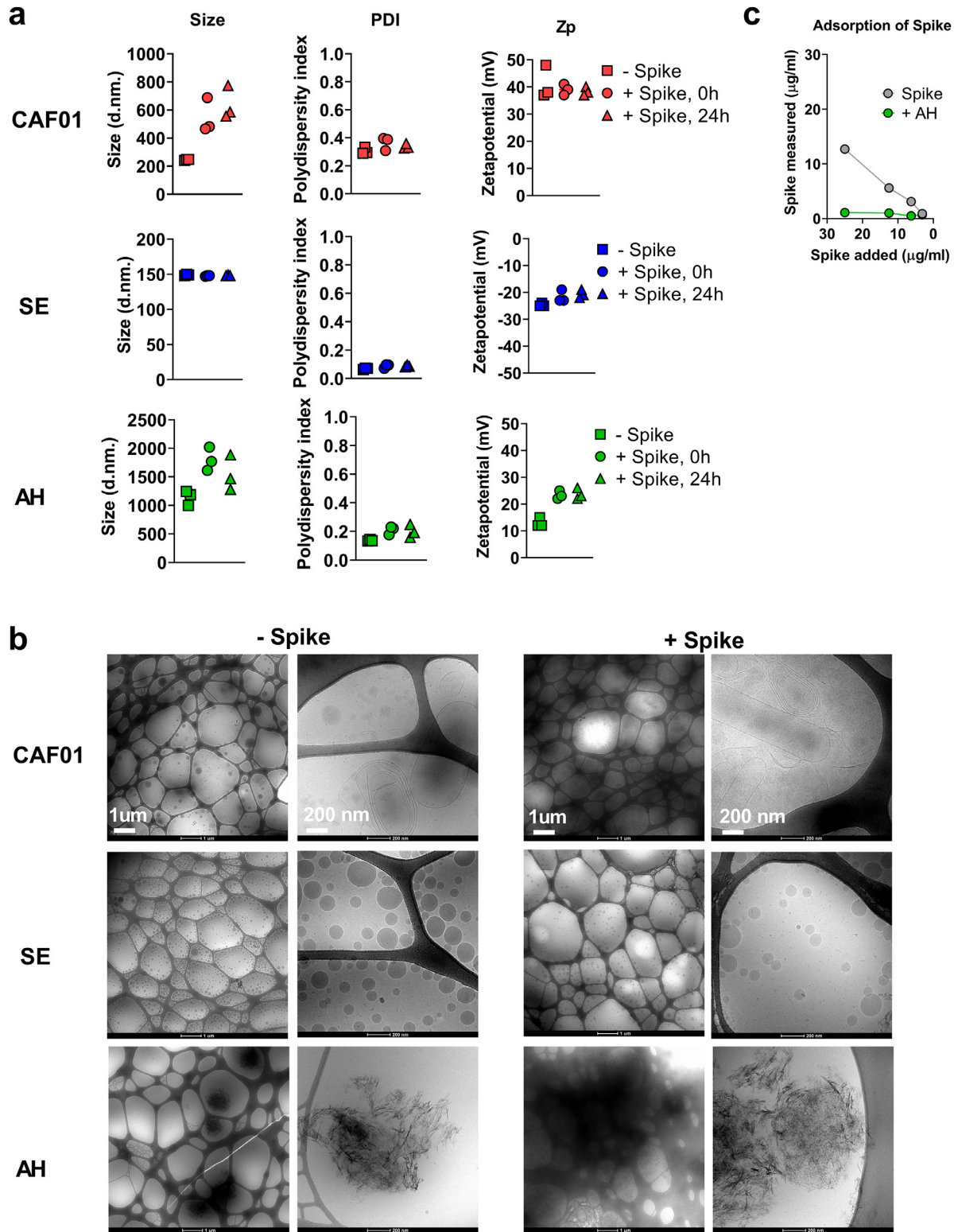
To investigate if the vaccine-elicited antibodies were capable of neutralizing SARS-CoV-2, we performed a homotypic SARS-CoV-2 pseudovirus neutralization assay. Little neutralizing activity was detected at seven days post immunization and only in the SE group. However, at day 45, neutralizing antibodies were found in all the adjuvanted groups, but not in the unadjuvanted spike protein group (Fig 2c). Neutralizing antibody responses in mice immunized with the CAF01 and AH adjuvanted formulations were significantly higher than in the spike protein alone group ( $p < 0.05$ , Kruskal-Wallis test).  $\text{ID}_{50}$  titers in the adjuvanted groups ranged from 86–4063 and there was a significant correlation between anti-RBD IgG antibody and neutralization titers (spearman  $R = 0.77$ ,  $p = 0.0002$ ) (Fig 2d). Thus, a single immunization with spike trimer in adjuvant was sufficient to induce neutralizing antibody responses.

Antibody responses to T-dependent antigens, including antibody affinity maturation and class-switching, depend on germinal center (GC) formation. We evaluated GC responses by staining for GC B cells (B220+IgD-CD38-GL7+) and T follicular helper cells (Tfh) (B220-CD4+PD-1+CxCR5+) [47] at seven days post immunization with a single dose of spike trimer antigen alone or formulated in adjuvant. Only the SE adjuvant significantly boosted GC B cell responses compared to unadjuvanted trimer protein ( $p < 0.01$ , Kruskal-Wallis test) at seven days post immunization (Fig 3a). Tfh responses on the other hand were robustly induced in both the CAF01 and SE groups (significantly different from unadjuvanted trimer protein,  $p < 0.01$ , Kruskal-Wallis test) (Fig 3b). Notably, GL7 expression amongst Tfh cells was highest in the CAF01 group (sFig2). DC priming is sufficient to induce early Tfh differentiation and expression of CxCR5 and GL7 even without cognate B cell help [48], which may explain why GC B cell and Tfh responses did not correlate. Since adjuvants may differentially affect kinetics of GC responses [49], it is possible that GC initiation was delayed when spike protein was formulated in CAF01 and AH adjuvants compared to SE. To investigate GC persistence, we evaluated GC B cells at day 45, at which, there was still a tendency towards higher GC B cell responses in the adjuvanted groups, compared to trimer protein alone (only significant for the AH group,  $p < 0.05$ , Kruskal-Wallis test), indicating that the adjuvants promoted long-lived GCs (sFig3). Overall, the tested adjuvants differentially influenced timing of GC initiation in response to spike protein, however the antibody responses, including capacity to neutralize SARS-CoV-2, were largely similar at 45 days post immunization.

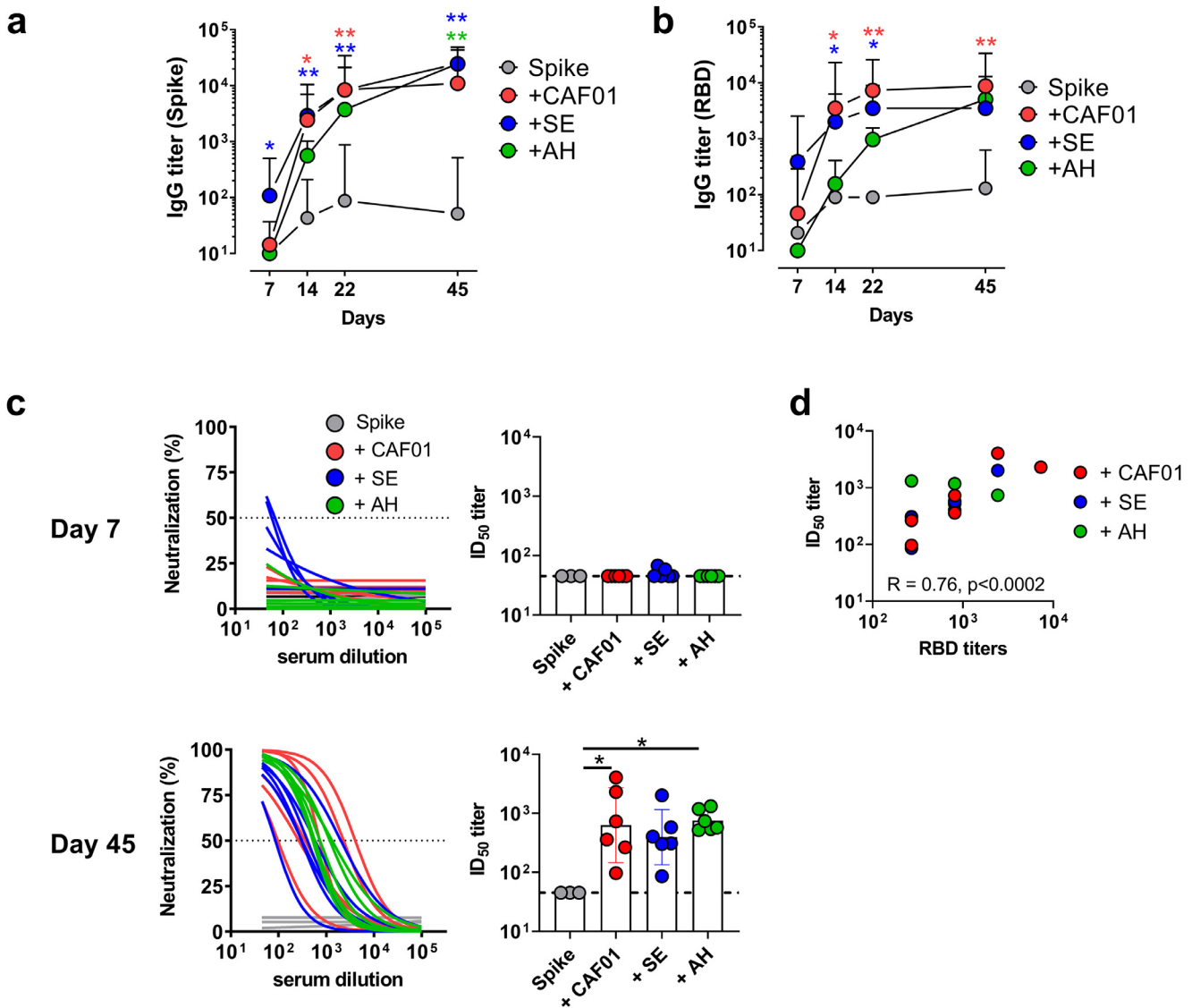
### 3.3. T cell profile in response to spike trimer protein is dependent on the adjuvant

Vaccine-associated enhanced respiratory disease (VAERD) has been associated with CD4 T cell responses biased towards a Th2 profile in the context of RSV [50] and SARS-CoV [21,51]. To evaluate polarization of the vaccine-induced T cell responses, we re-stimulated splenocytes with either the RBD or total spike protein early (day 7) and late (day 45) after immunization. Compared to the group having received unadjuvanted spike protein, there was a tendency for increased IFN- $\gamma$  production, suggesting Th1 responses, in the CAF01 group (not significant) at day 7 (Fig 4a). CAF01 also induced a





**Fig 1.** Characterization of vaccine formulations. Different adjuvant systems, cationic liposomes (CAF01), squalene emulsion (SE) and aluminium hydroxide (AH), were tested for compatibility with pre-fusion stabilized (S-2P) spike trimer protein. a) The particle size (left panels), polydispersity index (PDI, middle panels) were analyzed by dynamic light scattering and zeta potentials (Zp, right panels) of the adjuvant formulations were tested by laser-Doppler electrophoresis without the addition of spike protein (-Spike) and in the presence of spike protein, directly after mixture (+ Spike 0 h) and after 24 h (+ Spike, 24 h). Size was measured as diameter in nanometer (d.nm) and three replicates are shown. B) Adsorption of spike protein to aluminium hydroxide (AH). b) Cryo-TEM micrograph of the different adjuvant formulations in the absence or presence of spike protein (diluted 1:1 with the adjuvant formulation). Scale bar = 1 μm or 200 nm. C) Adsorbed spike retention onto AH was determined by measuring protein content recovered in the supernatant after ultracentrifugation.



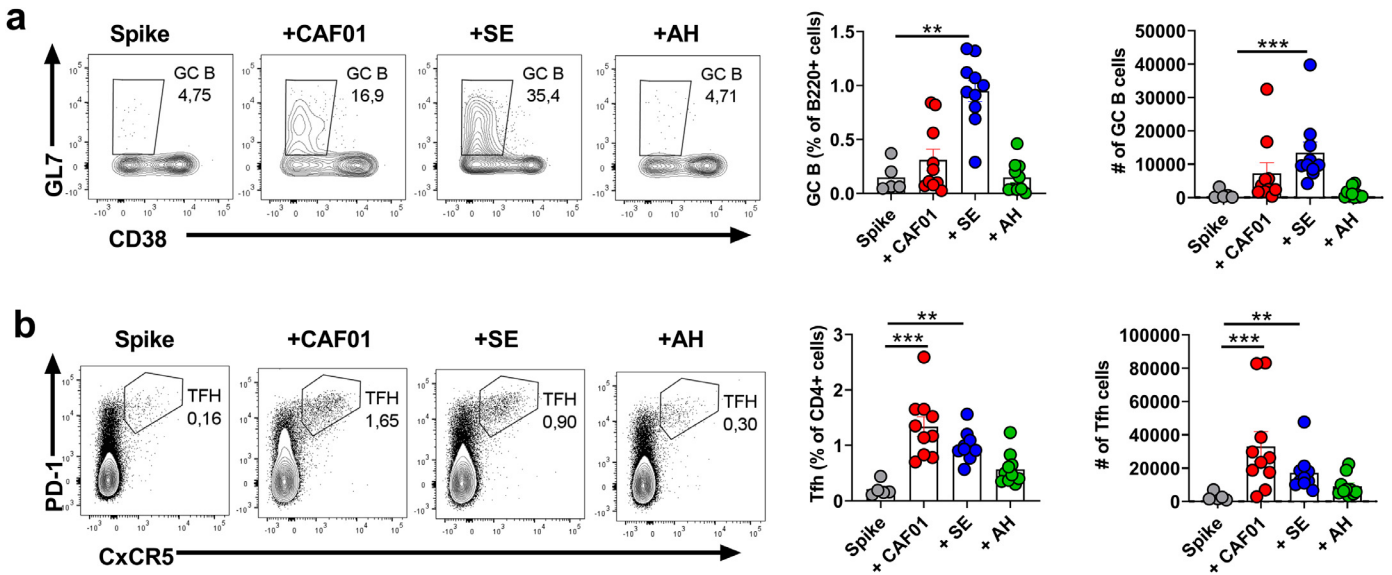
**Fig 2.** Adjuvanted pre-fusion stabilized (S-2P) spike protein elicits neutralizing antibody responses after a single dose. Mice were immunized with a single dose of spike trimer protein alone or formulated in CAF01, aluminium hydroxide (AH) or squalene emulsion (SE). Kinetics of IgG antibody responses against spike protein (a) and the receptor binding domain (RBD) (b) were measured by ELISA. c) Neutralization of SARS-CoV-2 by pseudovirus neutralization assay measured at day 7 (upper panels) and 45 (lower panels). d) Correlation between IgG titers against RBD and neutralization titers (Spearman R and p-value indicated) (right panel). Bars show geometric mean  $\pm$  95% CI. Statistically significant differences are indicated by \* and \*\* (Kruskal Wallis test, using the unadjuvanted spike trimer group as reference and significance levels of  $p < 0.05$  and  $p < 0.01$ , respectively). Figures represent three to six mice per group from one experiment.

Th17 response, which was significantly higher than in the unadjuvanted group ( $p < 0.01$ , Kruskal-Wallis test). Th1/Th17 cell responses were lower in the SE and AH groups and low IL-13, IL-5 and IL-10 responses were detected in all groups. Examining T cell responses in the lung (Fig 4b), CAF01 also induced a Th17 response, significantly higher than in the spike protein alone group ( $p < 0.01$ , Kruskal-Wallis test). We also detected IL-5 and IL-10 in the lungs of some of the mice in the AH group, although levels were not significantly different from in the unadjuvanted spike group. IFN- $\gamma$  and IL-17 responses remained detectable in the CAF01 group at day 45 post immunization and were significantly higher compared to in the unadjuvanted spike protein group ( $p < 0.05$ , Kruskal-Wallis test) (sFig 4). In contrast to when re-stimulating with intact spike protein, we failed to detect any T cell responses when re-stimulating with the RBD (data not shown). To investigate if the observed T cell responses were mice strain-dependent, we repeated the studies using BALB/c mice, which are more Th2-prone. Similarly to in C57Bl/6 mice, spleen and lung Th17 responses were significantly higher in mice that had received

spike protein formulated in CAF01 than when spike was given alone (sFig 5). In contrast, Th2 responses were generally low although there was a tendency for higher splenic Th2 responses in the AH group than in the other adjuvanted groups. Overall, the adjuvants differentially influenced the magnitude and profile of the elicited T helper response against the prefusion-stabilized SARS-CoV-2 spike protein.

#### 4. Discussion

SARS-CoV-2 has caused more than 1 300 000 deaths worldwide to date, and triggered the deepest global recession in decades. A vaccine against SARS-CoV-2 is thus urgently needed. The immune profile required for vaccine-mediated protection against SARS-CoV-2 is currently unknown. Vaccine-induced antibody responses are the best correlate of protection for many infectious agents [1] and several anti-SARS-CoV-2 antibodies can neutralize the virus *in vitro* [4,52] and afford protection against SARS-CoV-2 when passively transferred to Syrian hamsters [53]. Vaccine-induced CD4 T cell responses may

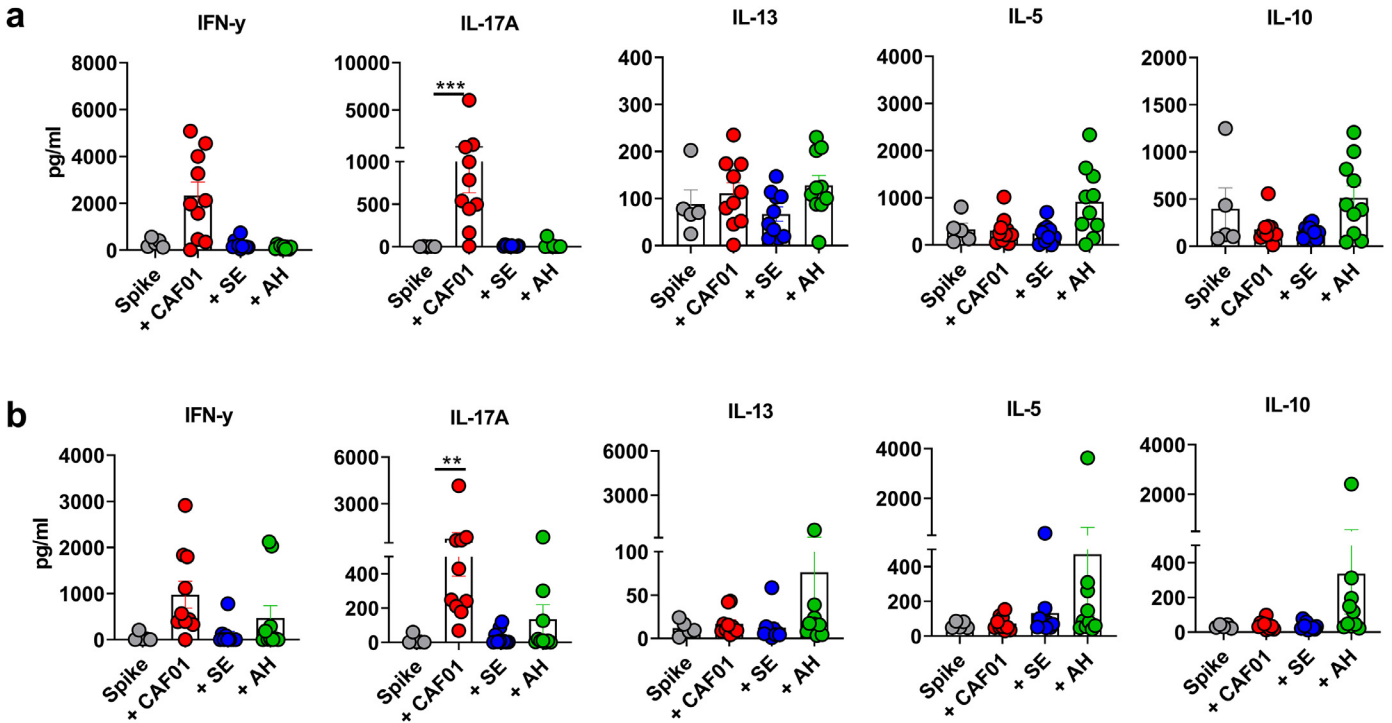


**Fig 3.** Squalene emulsion elicits a rapid germinal center (GC) B cell response when formulated with pre-fusion stabilized (S-2P) spike protein. Mice were immunized with a single dose of unadjuvanted spike trimer protein or formulated in CAF01, aluminium hydroxide (AH) or squalene emulsion (SE) and GC B cell responses measured in the draining lymph node at seven days post immunization. a) GC B cell responses measured by flow cytometry (gated on live B220<sup>+</sup>CD38<sup>-</sup>GL7<sup>+</sup> cells). b) T follicular helper (Tfh) cells (gated on live B220-CD4+PD-1+CxCR5+ cells). Bars show mean ± SEM. Statistically significant differences are indicated by \*, \*\* and \*\*\* (Kruskal Wallis test, using the spike trimer alone group as reference and significance levels of  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively). Figures represent five to ten mice per group from one experiment.

also play a role in protection against SARS-CoV-2, as CD4 T cells shape various immune effector functions, including orchestration of innate immunity, cytotoxic T cells and B cell functionality (reviewed in [54]). CD4 T cells can be segregated into functional subsets based on their cytokine production. Skewing of T cell responses towards a predominant type 2 helper T-cell (Th2) cell profile (producing IL-4, IL-5 or IL-13) has been associated with vaccine-enhanced respiratory

disease, as seen in the context of RSV [20,50] and in a murine model of SARS-CoV [21,51,55]. A more balanced Th1/Th2 or a predominantly Th1-directed response on the other hand is generally considered favorable in anti-viral immunity [56].

Adjuvants can direct the T-helper response [36,57] and shape humoral immunity by influencing the magnitude, breadth and affinity of the antibody response [58] in addition to inducing specific



**Fig 4.** Adjuvants differentially influence CD4 T cell responses to pre-fusion stabilized (S-2P) spike protein. Mice were immunized with a single dose of unadjuvanted spike trimer protein or formulated in CAF01, aluminium hydroxide (AH) or squalene emulsion (SE). T cell responses were measured by stimulating a) splenocytes and b) lung cells with spike protein at seven days post immunization and measuring secreted cytokines in the supernatant. Bars show mean ± SEM. Statistically significant differences are indicated by \*\* and \*\*\* (Kruskal Wallis test, using the unadjuvanted spike trimer group as reference and significance levels of  $p < .$ , respectively). The figure represents five to ten mice per group from one experiment.



antibody subclasses with different Fc-based effector functions [59]. Here we tested three different adjuvant systems together with the pre-fusion stabilized [60] SARS-CoV-2 (S-2P) spike trimer protein [18]. Encouragingly, all the adjuvants boosted neutralizing antibody responses after only a single immunization, supporting the possibility of a one-dose vaccine regimen. In contrast, spike protein alone did not induce detectable neutralization. Neutralization titers in the adjuvanted groups were of similar magnitude to those seen in mice given a single dose of the mRNA-1273 vaccine (encoding the stabilized (S-2P) trimer) [31], and in convalescent SARS-CoV-2 cases [61], although it may be difficult to compare across different assays. The adjuvants induced different T helper cell profiles, with CAF01 promoting a mixed IFN- $\gamma$  (Th1)/IL-17 (Th17) response, whilst SE and AH displayed lower responses. Some of the mice that had received AH had lung T cells producing IL-5 and IL-10. Although Th2 responses in the SE and AH groups were low, possibly since the vaccine was given as a single dose, the adjuvant-directed T helper profile was similar to that described when tested with antigens from other disease targets [36]. However, the optimal T helper response in relation to COVID-19 is unclear. Convalescing COVID-19 cases had predominantly a Th1 response with little Th2 cytokines detected [9] and subjects with moderate COVID-19 disease had more IFN- $\gamma$ + Th1 cells than severe cases, which could imply a beneficial role of Th1 cells. The role for Th17 cells also remains elusive. High levels of peripheral blood CCR6 + CD4 T cells possibly representing Th17 cells were linked to severe disease in one case, although IL-17 expression was not directly examined [62]. IL-17 was identified amongst cytokines associated with SARS-CoV-2-induced pulmonary inflammation [63] and it has been proposed that Th17 cells may exacerbate lung immunopathology, possibly by facilitating eosinophil recruitment [64]. Th17 cells have also been described to regulate antibody glycosylation [65], which may influence Fc receptor binding and thus potentially antibody dependent enhancement (ADE). More studies focusing on the role of infection- and vaccine-induced T helper profiles in protection against SARS-CoV-2 are required to determine the optimal adjuvant-mediated T cell polarization.

DNA and RNA-based vaccine platforms are promising, due to their scalable and relatively rapid production, and preliminary data suggest that the SARS-CoV-2 mRNA based vaccines are highly effective for preventing COVID-19 infection. However, no nucleic-acid based vaccine has been approved for human use so far and these novel vaccine technology platforms require careful evaluation of efficacy and safety. Subunit vaccines based on proteins, are used successfully against other infections including HBV and HPV and should be considered as important components of the SARS-CoV-2 vaccine pipeline. Here we have demonstrated that three different clinically tested adjuvant systems could be formulated with the pre-fusion stabilized (S-2P) SARS-CoV-2 spike protein [31] to enhance neutralizing antibody titers after a single immunization in mice. Furthermore, the adjuvants differentially influenced T helper responses. Studies in animal challenge models are required to select the optimal adjuvanted SARS-CoV-2 subunit vaccine.

#### Declaration of Competing Interest

D.C. is co-inventor on patents on the cationic adjuvant formulations (CAF). All rights have been turned over to Statens Serum Institut, which is a non-profit government research facility. The rest of the authors declare that there are no competing interests.

#### Contributors

G.M., G.K.H., B.M., D.C. and G.K.P., designed research; D.S., L.H. J.Z and K.W. performed experiments; K.W., G.K.P., D.S., S.T.S., D.C. analyzed data; and G.M., G.K.H., B.M., D.C. and G.K.P. wrote the paper. All authors read and approved the final version of the manuscript, and

have had access to the raw data. Katharina Wörzner, Signe Tandrup Schmidt, Dennis Christensen and Gabriel Kristian Pedersen can verify the accuracy of the raw data for the study.

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#### Data sharing statement

Study protocol and all data collected for the study, including raw data and data analysis will be made available to others upon request. All data will be available upon publication of the manuscript, by contacting the corresponding author. Data will be made available after approval of a proposal and with a signed data access agreement.

#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ebiom.2020.103197](https://doi.org/10.1016/j.ebiom.2020.103197).

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