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Safety, tolerability, and immunogenicity of an aerosolised adenovirus type-5 vector-based COVID-19 vaccine (Ad5-nCoV) in adults: preliminary report of an open-label and randomised phase 1 clinical trial

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For a Chinese translation of the Summary see **Online** for appendix 1

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Summary

Background SARS-CoV-2 has caused millions of deaths, and, since Aug 11, 2020, 20 intramuscular COVID-19 vaccines have been approved for use. We aimed to evaluate the safety and immunogenicity of an aerosolised adenovirus type-5 vector-based COVID-19 vaccine (Ad5-nCoV) in adults without COVID-19 from China.

Method This was a randomised, single-centre, open-label, phase 1 trial done in Zhongnan Hospital (Wuhan, China), to evaluate the safety and immunogenicity of the Ad5-nCoV vaccine by aerosol inhalation in adults (≥18 years) seronegative for SARS-CoV-2. Breastfeeding or pregnant women and people with major chronic illnesses or history of allergies were excluded. Participants were enrolled and randomly assigned (1:1:1:1:1) into five groups to be vaccinated via intramuscular injection, aerosol inhalation, or both. Randomisation was stratified by sex and age (18–55 years or ≥56 years) using computer-generated randomisation sequences (block sizes of five). Only laboratory staff were masked to group assignment. The participants in the two aerosol groups received an initial high dose (2×10^{10} viral particles; HDmu group) or low dose (1×10^{10} viral particles; LDmu group) of Ad5-nCoV vaccine on day 0, followed by a booster on day 28. The mixed vaccination group received an initial intramuscular (5×10^{10} viral particles) vaccine on day 0, followed by an aerosolised booster (2×10^{10} viral particles) vaccine on day 28 (MIX group). The intramuscular groups received one dose (5×10^{10} viral particles; 1Dim group) or two doses (10×10^{10} viral particles; 2Dim group) of Ad5-nCoV on day 0. The primary safety outcome was adverse events 7 days after each vaccination, and the primary immunogenicity outcome was anti-SARS-CoV-2 spike receptor IgG antibody and SARS-CoV-2 neutralising antibody geometric mean titres at day 28 after last vaccination. This trial is registered with ClinicalTrials.gov, number NCT04552366.

Findings Between Sept 28, 2020, and Sept 30, 2020, 230 individuals were screened for inclusion, of whom 130 (56%) participants were enrolled into the trial and randomly assigned into one of the five groups (26 participants per group). Within 7 days after vaccination, adverse events occurred in 18 (69%) in the HDmu group, 19 (73%) in the LDmu group, 19 (73%) in the MIX group, 19 (73%) in the 1Dim group, and 15 (58%) in the 2Dim group. The most common adverse events reported 7 days after the first or booster vaccine were fever (62 [48%] of 130 participants), fatigue (40 [31%] participants), and headache (46 [35%] participants). More adverse events were reported in participants who received intramuscular vaccination, including participants in the MIX group (49 [63%] of 78 participants), than those who received aerosol vaccine (13 [25%] of 52 participants) after the first vaccine vaccination. No serious adverse events were noted within 56 days after the first vaccine. At days 28 after last vaccination, geometric mean titres of SARS-CoV-2 neutralising antibody was 107 (95% CI 47–245) in the HDmu group, 105 (47–232) in the LDmu group, 396 (207–758) in the MIX group, 95 (61–147) in the 1Dim group, and 180 (113–288) in the 2Dim group. The geometric mean concentrations of receptor binding domain-binding IgG was 261 EU/mL (95% CI 121–563) in the HDmu group, 289 EU/mL (138–606) in the LDmu group, 2013 EU/mL (1180–3435) in the MIX group, 915 EU/mL (588–1423) in the 1Dim group, and 1190 EU/mL (776–1824) in the 2Dim group.

Interpretation Aerosolised Ad5-nCoV is well tolerated, and two doses of aerosolised Ad5-nCoV elicited neutralising antibody responses, similar to one dose of intramuscular injection. An aerosolised booster vaccination at 28 days after first intramuscular injection induced strong IgG and neutralising antibody responses. The efficacy and cost-effectiveness of aerosol vaccination should be evaluated in future studies.

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Research in context

Evidence before this study

We searched PubMed for clinical trials published from the inception of the database to June 3, 2021, with the search terms "COVID-19" or "SARS-CoV-2", "vaccine", "mucosal vaccination", and "clinical trial"; no language restrictions were applied. To our knowledge, no data from human clinical trials of COVID-19 vaccine by mucosal route had been reported at the time of the search. We also searched ClinicalTrials.gov and the draft landscape and tracker of COVID-19 candidate vaccines (developed by WHO) from the inception of both databases to June 3, 2021, for unpublished trials of non-injection COVID-19 vaccine. Not including our study, there were 13 clinical trials investigating non-injection COVID-19 vaccines, including oral and intranasal vaccinations. Six of the oral and intranasal COVID-19 vaccines used adenovirus vectors, two used protein subunits, two used live attenuated viruses, one used influenza virus vector, one used DNA plasmids, and one used inactivated virus. The adenovirus type-5 COVID-19 vaccine expressing the spike of the SARS-CoV-2 (Ad5-nCoV) was assessed in a phase 1 and phase 2 clinical trial in China, the safety and immunogenicity data up to day 28 after one injection for which were published in a preliminary report. Interim analysis of data from the multicountry trial, in February, 2021, showed that Ad5-nCoV had an acceptable efficacy rate at preventing all symptomatic COVID-19 cases.

Added value of this study

To our knowledge, this study is the first to report clinical data for an aerosol COVID-19 vaccine. We evaluated the safety and immunogenicity of Ad5-nCoV by two-dose aerosol inhalation with a 28-day interval in an open-label, randomised, phase 1 clinical trial. Two doses of aerosol Ad5-nCoV was well tolerated, without causing any vaccine-related serious adverse events. One-dose of aerosolised Ad5-nCoV, equal to a fifth of a intramuscular dose, could induce strong cellular response and a two-dose aerosolised Ad5-nCoV could produce similar SARS-CoV-2 neutralising antibody titres as one dose of intramuscular vaccination. Furthermore, an aerosolised booster vaccination at 28 days after first intramuscular injection induced strong IgG and neutralising antibody responses.

Implications of all the available evidence

The aerosolised Ad5-nCoV was well tolerated and induced similar humoral and cellular immune responses to intramuscular vaccination, and it would be valuable in restricting the COVID-19 pandemic. The efficacy and cost-effectiveness of aerosol vaccination should be evaluated in future studies

Introduction

The development of COVID-19 vaccines has achieved breakthroughs at an unprecedented speed. Some COVID-19 vaccines, including mRNA-1273, BNT162b2, and AZD1222, were shown to have protective efficacy in phase 3 clinical trials,[1–3] and 20 intramusuclar COVID-19 vaccines have been approved for use as of July, 2021.[4] Previous work in animal models has shown that a single mucosal vaccination with an adenovirus-vectored COVID-19 vaccine (Ad5-nCoV; developed by the Beijing Institute of Biotechnology, Beijing, China, and CanSino Biologics, Tianjin, China) protects from wild-type SARS-CoV-2 replication in the upper respiratory tract.[5–8] Compared with conventional parenteral immunity, mucosal immunity has potential benefits in triggering mucosal and systemic immune defence, thereby preventing pathogens from invading the mucosal surface.[9]

Ad5-nCoV is a replication-defective adenovirus type-5 vectored vaccine that encodes the SARS-CoV-2 spike protein. Ad5-nCoV had good safety and immunogenicity profiles in phase 1/2 clinical trials.[10,11] Interim analysis of data from a multicountry trial showed that Ad5-nCoV has an acceptable efficacy for preventing symptomatic COVID-19 cases.[12] We aimed to evaluate the safety, tolerability, and immunogenicity of aerosolised Ad5-nCoV administered via nebulisation inhalation 28 days after the last vaccination in adults aged 18 years and older.

Methods

Study design and participants

We did a single-centre, open-label, randomised phase 1 trial in Zhongnan Hospital (Wuhan, China). Participants were recruited through the trial recruitment advertisement on the official WeChat (Tencent, Shenzhen, China) account of the clinical trial centre of Zhongnan Hospital. Investigators analysed the volunteers' personal characteristics after an individual expressed an interest in participating, contacted them one by one via telephone, and confirmed whether they could participate in trial screening. Before recruitment onto the trial, all the volunteers were tested for SARS-CoV-2-specific antibodies (IgG and IgM) with the 2019-nCoV IgG/IgM detection kit (colloidal gold-based, Nanjing Vazyme, Nanjing, China). Participants who were seronegative for SARS-CoV-2 were randomly assigned to receive aerosol or intramuscular vaccination with Ad5-nCoV. Eligible participants were aged 18 years and older, were HIV-negative, and had not had previous SARS-CoV-2 infection (determined by the detection of SARS-CoV-2-specific IgG and IgM antibodies). Recruited participants who had an axillary temperature of 37·0°C or less and were in general good health—as established by medical history and physical examination—were eligible for inclusion. Pregnant or breastfeeding women were excluded. People with psychiatric disorders, history of any allergies, serious cardiovascular disease, other major chronic illnesses, or abnormal laboratory tests (blood counts, alanine aminotransferase, aspartate

aminotransferase, total bilirubin, fasting blood glucose, and creatinine were measured before each vaccination [appendix 2 p 7]), or with clinically significant abnormalities (as judged by the investigators) were also excluded. Participants with severe allergic reactions or other severe adverse events related to the first vaccination were excluded before booster vaccination. A complete list of the inclusion and exclusion criteria is provided in the protocol (appendix 2 pp 22–108).

See Online for appendix 2

The protocol and informed consent were approved by the medical ethics committee of Zhongnan Hospital. All participants provided written informed consent and completed an assessment of understanding before screening for eligibility. An independent data and safety monitoring board was established before the trial started to monitor the safety data and evaluate the risks in the participants during the trial.

Randomisation and masking

Eligible participants were randomly assigned (1:1:1:1:1) to one of five groups by computer-generated randomisation sequences. Randomisation was stratified by age (18–55 years or ≥56 years) and sex (female and male). We used the PLAN procedure of SAS software (version 9.4) for the randomisation, and the block size was five. Patients were assigned to one of the following groups: HDmu group (first and booster mucosal vaccination at a 28-day interval with two doses of aerosolised Ad5-nCoV [2×10^{10} viral particles]), LDmu group (first and booster mucosal vaccination at a 28-day interval with two doses of aerosolised Ad5-nCoV [1×10^{10} viral particles]), MIX group (mixed vaccination at a 28-day interval [first an intramuscular vaccination with 5×10^{10} viral particles and an aerosolised booster vaccination with 2×10^{10} viral particles]), 1Dim group (single intramuscular vaccination with 5×10^{10} viral particles; no booster vaccine), or 2Dim group (two single doses of 5×10^{10} viral particles, one in the right and one in the left deltoid; no booster vaccine). Participants could not be masked to group assignment because of the nature of the treatment. Laboratory staff who analysed immunological outcomes were masked to treatment allocation during the whole study; they identified samples by serial numbers.

A group of participants who received two doses of Ad5-nCoV intramuscularly with a 56 day interval was included in the protocol. Participants in this group were recruited from people who had received a dose of Ad5-nCoV vaccine outside of this study. The participants in this group were not randomly assigned with the participants in the other five groups. The results of this group are not reported in this study, but will be published when the trial is complete.

Procedures

Ad5-nCoV contains replication-defective Ad5 vectors expressing the full length spike gene of wild-type SARS-CoV-2, Wuhan-Hu-1, stored at 2–8°C. Ad5-nCoV was supplied at a concentration of 1×10^{11} viral particles per mL as a liquid formulation in prefilled syringes, with an extractable volume of 0·5 mL. The same vaccine was used for intramuscular and aerosol administration.

The first vaccinations were administered on study day 0, with boosters administered at day 28 for the HDmu, LDmu, and MIX groups. For aerosolised vaccination, the vaccine was administered for 30–60 s using nebulisation inhalation (Aerogen Ultra device, Aerogen, Galway, Ireland), during which time 0·2 mL or 0·1 mL (depending on group allocation) of the vaccine was nebulised and delivered into a disposable mouthpiece. The nebuliser generated aerosol with a volume median diameter of 5·4 μm, as determined by means of laser diffraction (appendix 2 p 3). Each nebuliser freshly charged with the vaccine could be used to vaccinate one person. For intramuscular injection in the 1Dim group, 0·5 mL of Ad5-nCoV vaccine was administered into the deltoid muscle of one arm. In the 2Dim group 1·0 mL Ad5-nCoV vaccine was administered (0·5 mL into each arm). All participants were monitored for 60 min after aerosolised vaccination and 30 min after intramuscular injection for immediate adverse reactions.

Baseline characteristics (including age, sex, body-mass index, and laboratory safety test) of all participants were recorded within 3 days before enrolment. Blood samples were taken from participants for immunogenicity assessment before vaccination and at days 14 and 28 after each vaccination. We used ELISA kits (Beijing Kewei, Beijing, China) to measure serum IgG and IgA specific to the receptor-binding domain (RBD) of the SARS-CoV-2 spike glycoprotein. The titre of neutralising antibodies was measured by a 50% plaque reduction neutralisation test using SARS-CoV-2/human/CHN/Wuhan_IME-BJ01/2020 (GenBank number MT291831.1). Cellular immune responses were measured at days 0 and 14 after each vaccination by intracellular cytokine staining of CD4 and CD8 T cells by flow cytometry and by quantification of interferon-γ (IFN-γ) release using fresh peripheral blood mononuclear cells stimulated with overlapping spike glycoprotein peptide pool. The cytokine profiling from the supernatants of the stimulated peripheral blood mononuclear cells was analysed. The before and after vaccination anti-Ad5 neutralising antibody titres were detected with a serum neutralisation assay. Full details on the assays are reported in appendix 2 (pp 1–2).

Adverse events were self-reported by the participants and were verified by investigators every day during the first 7 days after each vaccination via a diary card issued at day 0. From day 8 to 28 after each vaccination, adverse events were recorded by the participants on contact cards that were retrospectively verified by the investigators on day 28. Local or systemic adverse events were not differentiated because of the influence of aerosolised vaccination on the respiratory system. All solicited adverse events are listed in the protocol (appendix 2) and the unsolicited adverse events were reported by participants

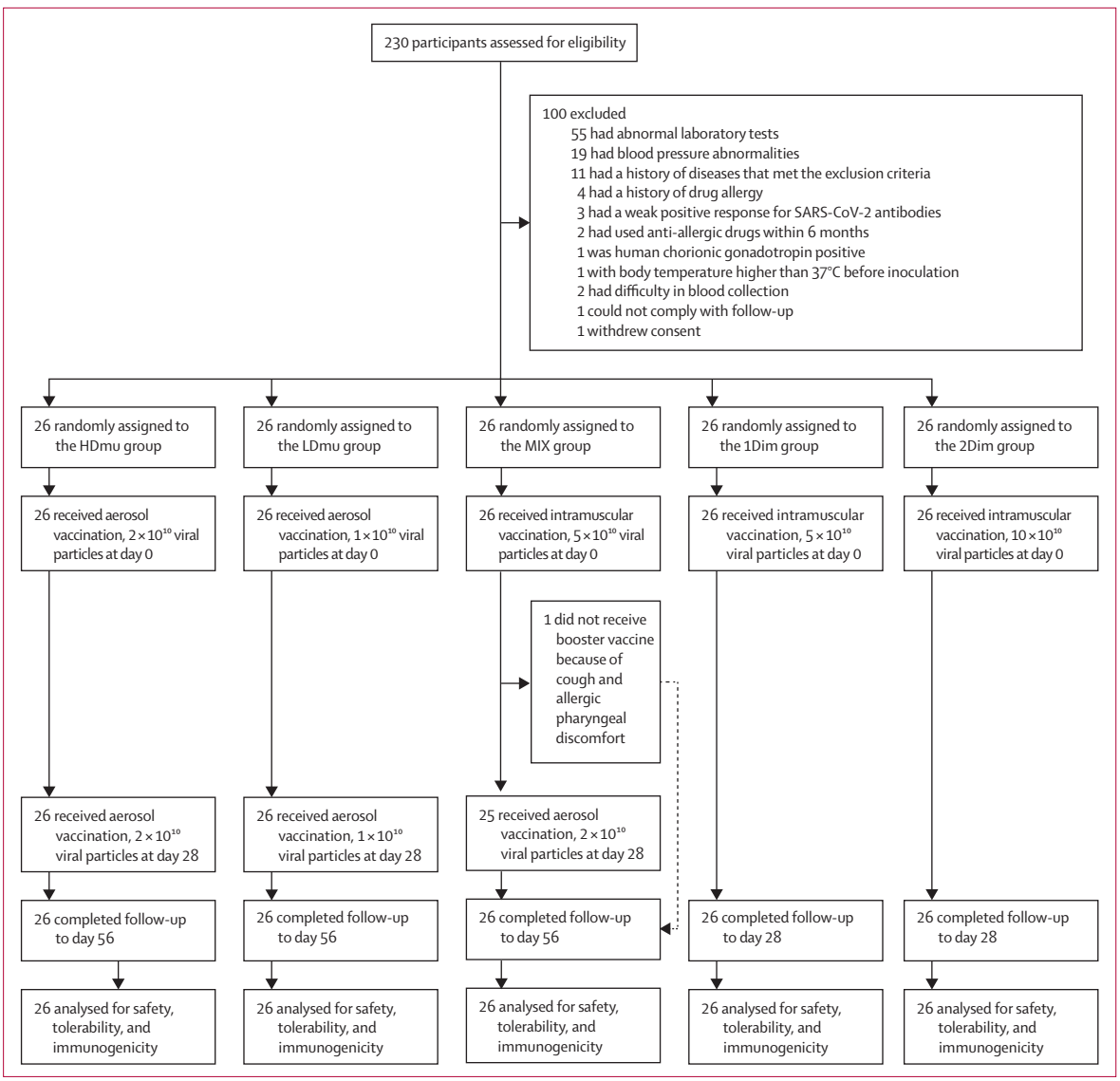

Figure 1: **Trial profile**
1Dim=low-dose intramuscular injection. 2Dim=high-dose intramuscular injection. Ad5-nCoV=recombinant adenovirus type 5 carrying full-length SARS-CoV-2 spike gene. HDmu=high-dose aerosol vaccine. LDmu=low-dose aerosol vaccine. MIX=intramuscular and aerosol vaccine.

throughout the study. Laboratory safety tests including blood counts, alanine aminotransferase, aspartate aminotransferase, total bilirubin, fasting blood glucose, and creatinine were measured before each vaccination and at day 8 after each vaccination to assess any toxic effects after vaccination. The adverse events and abnormal changes in laboratory tests were graded according to the scale issued by the China National Medical Products Administration (version 2019; appendix 2 p 7). Serious adverse events will be recorded up to 6 months after last vaccination.

Outcomes

The primary endpoint for safety was the occurrence of adverse events within 7 days after each vaccination. The primary immunogenicity outcome was anti-SARS-CoV-2 spike receptor IgG antibody and SARS-CoV-2 neutralising antibody geometric mean concentrations and titres at day 28 after last vaccination. The secondary safety endpoints were the occurrence of adverse events within 30 min after intramuscular vaccination and 60 min after aerosol vaccination, adverse events from day 8 to 28 after each vaccination, and serious adverse events reported from first dose until 6 months after full vaccination (not reported in this study). The secondary endpoints for immunogenicity were RBD-specific IgG response, SARS-CoV-2 neutralising antibody response, SARS-CoV-2 spike protein-specific IFN-γ ELISpot response, and Ad5 neutralising antibody response at baseline and day 14

	HDmu group (n=26)	LDmu group (n=26)	MIX group (n=26)	1Dim group (n=26)	2Dim group (n=26)
Age					
18–55 years	20 (77%)	20 (77%)	20 (77%)	20 (77%)	20 (77%)
≥56 years	6 (23%)	6 (23%)	6 (23%)	6 (23%)	6 (23%)
Median age	36·0 (25·0–54·0)	29·0 (26·0–52·0)	27·5 (23·0–55·0)	27·5 (24·0–44·0)	29·5 (25·0–53·0)
Sex					
Male	13 (50%)	13 (50%)	13 (50%)	13 (50%)	13 (50%)
Female	13 (50%)	13 (50%)	13 (50%)	13 (50%)	13 (50%)
Mean BMI, kg/m^2	23·7 (3·5)	21·9 (2·1)	23·3 (3·5)	23·7 (3·7)	23·6 (2·7)
Pre-existing Ad5 neutralising antibody titres					
Geometric mean titre (95% CI)	148 (54–406)	154 (59–410)	131 (48–359)	123 (41–369)	231 (78–689)
Median geometric mean titre	345 (6–1602)	239 (13–1061)	244 (6–1024)	154 (6–1364)	484 (11–2810)
Participants with geometric mean titre ≤200	12 (46%)	11 (42%)	12 (46%)	13 (50%)	11 (42%)
Participants with geometric mean titre >200	14 (54%)	15 (58%)	14 (54%)	13 (50%)	15 (58%)

Data are n (%), median (IQR), or mean (SD), unless otherwise stated. Ad5=adenovirus type 5. BMI=body-mass index.

Table: **Baseline characteristics**

and day 28 after each vaccination. The exploratory endpoints for immunogenicity were RBD-specific IgA response, RBD-specific IgG subclass, and SARS-CoV-2 spike protein-specific cellular response, detected by intracellular cytokine staining and cytokine profiling.

Seroconversion of IgG, IgA, and neutralising antibody were defined as a four-times increase in post-vaccination titre compared with baseline. ELISpot IFN-γ positive response and CD4 and CD8 T-cell responses were compared across the groups as endpoints for cell-mediated responses. Stratified analyses of the immune responses were done on the basis of sex, age (18–55 years, >55 years), and pre-existing Ad5 neutralising antibody titres in the participants categorised as low or negative (≤200) or high (>200).

Statistical analysis

The sample size for the trial was based on clinical and practical considerations, not on a formal statistical power calculation. Safety data and immunogenicity data were analysed descriptively using SAS (version 9.4). χ^2 test and Fisher's exact test were used to analyse categorical data. Fisher's exact test was used when the actual number of cases was less than 40 or the theoretical frequency was less than five. We assessed the number and proportion of participants with adverse events after vaccination and compared safety profiles across groups. Antibodies against SARS-CoV-2 were presented as geometric mean titres with 95% CIs, and the cellular responses were shown as a proportion of positive responders. The 95% CIs for geometric means were calculated by transforming the original antibody data by logarithmic function; then the two-sided 95% CI ([×1, ×2]) of logarithmic data was calculated through proc means CLM; and then the two-sided 95% CI of geometric mean [exp (×1), exp (×2)] was calculated. The increase in antibody response after booster vaccination was expressed as the geometric mean increase of the antibody concentrations or titres from day 28 to day 56. Two-sided p values of less than 0·05 were considered significant. This study reports an interim analysis that includes data until day 56 after the first vaccine.

Safety data for the first 7 days after prime vaccination were assessed and reviewed by the data safety monitoring committee to ensure that it was safe to administer booster vaccination. This study is registered with ClinicalTrials.gov, NCT04552366.

Role of the funding source

The funder had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between Sept 28 and Sept 30, 2020, 230 individuals were screened for eligibility; 130 (56%) participants were randomly assigned, with 26 participants in each group (figure 1). One participant in the MIX group had an allergic pharyngeal reaction to the first dose and was excluded from booster vaccination, but they were followed-up and included in the final analyses. In each group 20 (77%) volunteers were 18–55 years old and six (23%) volunteers were older than 55 years; 13 (50%) participants in each group were men and 13 (50%) were women (table). All the participants were Chinese.

Within 7 days after the first or booster vaccinations, adverse events occurred in 18 (69%) of 26 participants in the HDmu group, 19 (73%) in the LDmu group, 19 (73%) in the MIX group, 19 (73%) in the 1Dim group, and 15 (58%) in the 2Dim group. The two vaccination routes showed different safety profiles, but there was no significant difference in the incidence of any solicited adverse events within 7 days after the first vaccination or booster vaccination between all the groups (χ^2=2·17, df=4·00, p=0·71).

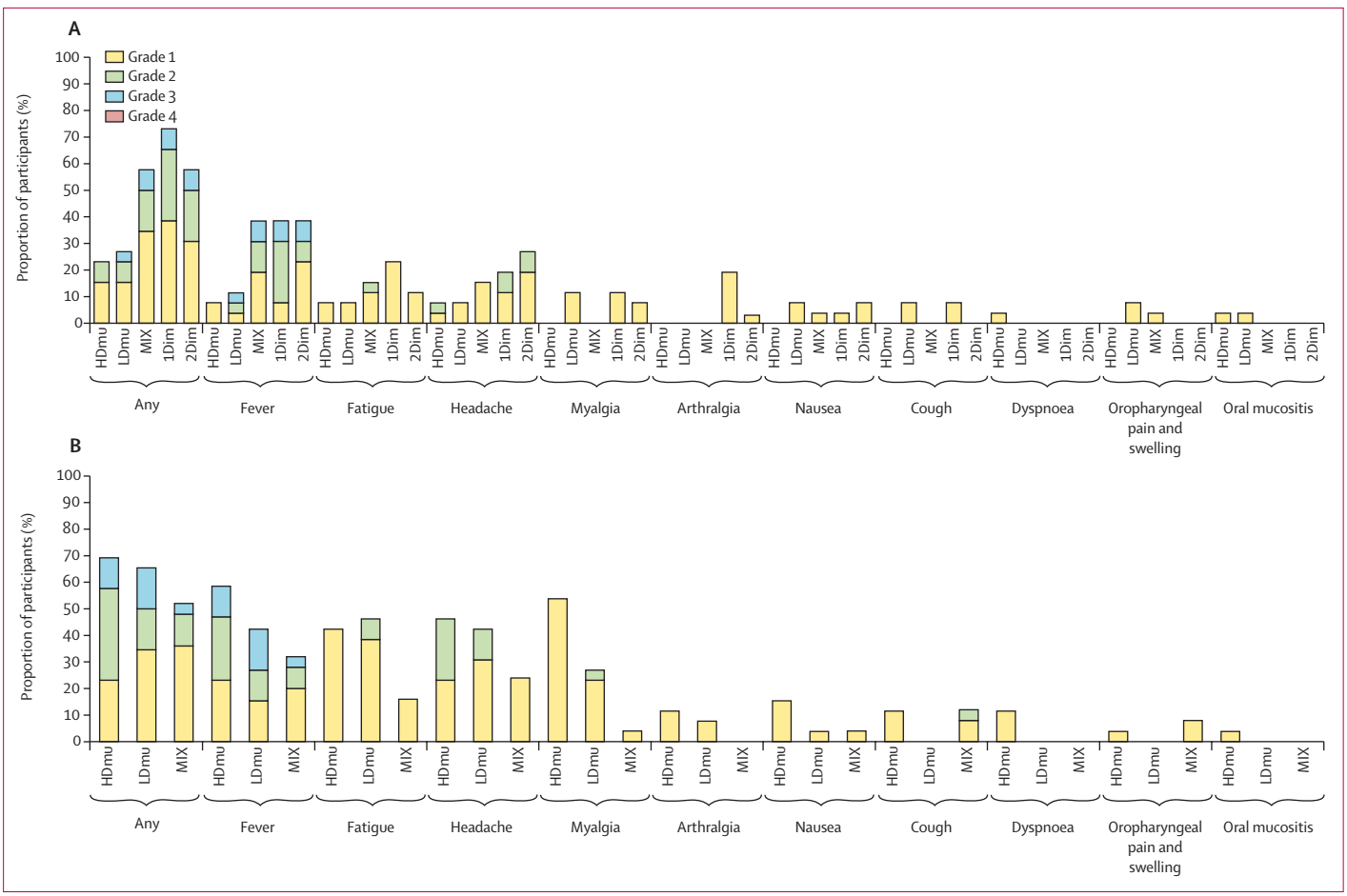

Figure 2: **Solicited adverse events following Ad5-nCoV vaccination**
Solicited adverse events reported within 7 days after prime (A) and booster (B) vaccination with Ad5-nCoV. The severity of solicited adverse events was graded as grade 1, grade 2, grade 3, or grade 4 according to the scale issued by the China National Medical Products of Administration (appendix 2 p 7). Participants in the 1Dim and 2Dim groups did not receive a booster vaccine. 1Dim=low-dose intramuscular injection. 2Dim=high-dose intramuscular injection. Ad5-nCoV=recombinant adenovirus type 5 carrying full-length SARS-CoV-2 spike gene. HDmu=high-dose aerosol vaccine. LDmu=low-dose aerosol vaccine. MIX=intramuscular and aerosol vaccine.

Within 7 days after the first vaccination, 62 (48%) of 130 participants reported at least one adverse event: six (23%) in the HDmu group, seven (27%) in the LDmu group, 15 (58%) in the MIX group, 19 (73%) in the 1Dim group, and 15 (58%) in the 2Dim group (figure 2; appendix 2 pp 4–6). There were significant differences in the overall number of adverse events between the groups (χ^2=19·61, df=4·00, p=0·0006). There were more adverse events in the participants who received an intramuscular injection compared with those who received aerosol vaccination (χ^2=17·89, df=1·00, p<0·0001). Overall, the most common systematic adverse events were fever (≥37·3°C; 35 [27%] of 130 participants), headache (20 [15%]), and fatigue (17 [13%]). No significant differences in respiratory adverse events, such as cough and oropharyngeal pain and swelling, were observed between participants who received aerosol and those who received an intramuscular vaccination (p=0·68).

The participants in the HDmu, LDmu, and MIX groups received booster aerosol vaccination at day 28, and 48 (62%) of 78 participants reported at least one adverse event within the first 7 days after booster vaccination: 18 (69%) in the HDmu group, 17 (65%) in the LDmu group, and 13 (50%) in the MIX group (figure 2; appendix 2 pp 4–6). There was no significant difference in the number of adverse events between the groups (χ^2=2·28, df=2·00, p=0·32).

Within the first 7 days after the first vaccination, fever (≥37·3°C) was reported in two (8%) participants in the HDmu group, three (12%) in the LDmu group, ten (38%) in the MIX group, ten (38%) in the 1Dim group, and ten (38%) in the 2Dim group. Most of the grade 3 fevers (≥38·5°C) were reported after booster vaccination. High pre-existing Ad5 neutralisation antibody titre (>200 *vs* ≤200) was associated with significantly fewer occurrences of grade 3 fever in participants who received intramuscular injection (p=0·0076) but not in those who received aerosol

(p=0·70; appendix 2 p 8). No serious adverse events were reported within 56 days after the first vaccination for any cohort.

Some non-clinically significant changes in routine clinical laboratory values, such as increased alanine aminotransferase, aspartate aminotransferase, or bilirubin concentrations, were reported at day 8 after prime or booster vaccination in ten (8%) participants (appendix 2 p 9).

Concentrations of RBD-binding IgG, IgA, and SARS-CoV-2 neutralising antibodies were assessed at baseline and at 14 and 28 days after each vaccination with Ad5-nCoV. Intramuscular vaccination produced higher concentrations of RBD-binding IgG, RBD-binding IgA, and SARS-CoV-2 neutralising antibody than did the aerosol vaccination at day 28 after the first vaccination. By 28 days after the first vaccination, geometric mean concentrations of RBD-binding IgG were 124 EU/mL (95% CI 83–186) in the HDmu group, 63 EU/mL (40–99) in the LDmu group, 960 EU/mL (603–1531) in the MIX group, 915 EU/mL (588–1423) in the 1Dim group, and 1190 EU/mL (776–1824) in the 2Dim group (p<0·0001; figure 3; appendix 2 pp 10–11). Geometric mean concentration of RBD-binding IgA in serum was 148 EU/mL (95% CI 104–211) in the HDmu group and 95 EU/mL (55–165) in the LDmu group, 475 EU/mL (281–803) in the MIX group, 425 EU/mL (231–784) in the 1Dim group, and 521 EU/mL (272–997) in the 2Dim group; with a significant difference between the groups (p<0·0001; figure 3; appendix 2 pp 10–11). The SARS-CoV-2 neutralising antibody geometric mean titres was 40 (95% CI 22–73) in the HDmu group, 27 (15–51) in the LDmu group, 73 (46-117) in the MIX group, 95 (61–147) in the 1Dim group, and 180 (113–288) in the 2Dim groups (p<0·0001). 17 (65%) participants in the HDmu group, 14 (54%) in the LDmu group, 24 (92%) in the MIX group, 24 (92%) in the 1Dim group, and 26 (100%) in the 2Dim group seroconverted with neutralising antibodies at day 28 after the first vaccination (figure 3; appendix 2 pp 10–11).

At day 28 after booster aerosol vaccination, RBD-binding IgG concentrations increased to 261 EU/mL (95% CI 121–563; geometric mean increase of 2·1 times [95% CI 1·2–3·7]) in the HDmu group, 289 EU/mL (95% CI 138–606; increase of 4·6 times [95% CI 2·7–7·6]) in the LDmu group, and 2013 EU/mL (95% CI 1180–3435; increase of 2·2 times [95% CI 1·4–3·4]) in the MIX group (p=0·0002). Additionally, RBD-binding IgA concentrations increased to 312 EU/mL (95% CI 153–634; a geometric mean increase of 2·1 times [95% CI 1·1–4·0]) in the HDmu group, 297 EU/mL (95% CI 132–665; increase of 3·1 [95% CI 1·9–5·2]) in the LDmu group, and 777 EU/mL (95% CI 378–1601; increase of 1·7 [95% CI 1·0–2·8]) in the MIX group (p=0·22) compared with concentrations at day 28 after prime vaccination. Furthermore, SARS-CoV-2 neutralising antibody titres increased to 107 (95% CI 47–245; a geometric mean increase of 2·7 [95% CI 1·4–5·2]) in the HDmu group, 105 (95% CI 47–232; increase of 3·9 [95% CI 1·9–8·0]) in the LDmu group, and 396 (95% CI 207–758; increase of 5·5 [95% CI 3·2–9·3]) in the MIX groups (p=0·019), compared with titres at day 28 after prime vaccination. 22 (85%) of 26 participants in the HDmu group, 21 (81%) of participants in the LDmu group, and 25 (100%) participants in the MIX group seroconverted with neutralising antibodies at 28 days after the booster vaccination (figure 3; appendix 2 pp 10–11).

Both male and female participants showed similar neutralising antibody responses at day 28 after prime and booster vaccination in each group, except for LDmu group at day 28 after booster vaccination (p=0·031; appendix 2 p 12). The stratified analysis based on age found that only participants older than 55 years in the MIX group were associated with relative low neutralising antibody responses at day 28 (p=0·040) and day 56 (p=0·0003) after vaccination, and no difference was reported in other groups (appendix 2 p 13).

High pre-existing Ad5 neutralising antibody titre (>200 *vs* ≤200) compromised the SARS-CoV-2 neutralising antibody titres in participants who received aerosol vaccination or intramuscular vaccination, except for the MIX group at day 28 after booster vaccination (appendix 2 p 14). The correlation between concentrations of RBD-binding IgG and neutralising antibody titres was better than the correlation between concentrations of RBD-binding IgA and neutralising antibody titres at day 28 and day 56 in the HDmu, LDmu, and MIX groups (appendix 2 pp 15–16). RBD-binding IgG2 was prominent in all groups at day 28 and day 56, RBD-binding IgG1 and IgG3 were detected in most participants who received intramuscular vaccination, but only in some of those who received aerosolised vaccination. No RBD-binding IgG4 was detected in any group (appendix 2 pp 17).

SARS-CoV-2 spike protein-specific IFN-γ ELISpot response was detected at day 0, 14, and 28 after prime vaccination in all five groups and at day 14 after the booster vaccine in the LDmu, HDmu, and MIX groups. IFN-γ responses were detected in participants who received intramuscular vaccination and in those who received aerosol vaccination, with the peak concentration reported at day 14 after initial vaccination (figure 4). IFN-γ response in the LDmu group was similar to that in the 1Dim group (p=0·66) and MIX group (p=0·55) at day 14 after initial vaccination, and a similar response was also reported between the HDmu group and the 2Dim group (p=0·35), which suggested that aerosol vaccination with a fifth of the intramuscular dose triggered similar IFN-γ response to that of the intramuscular injections. A booster vaccination at day 28 significantly enhance the IFN-γ ELISpot response in MIX group (p=0·015) and LDmu group (p=0·013), but not in HDmu group (p=0·72). Pre-existing Ad5 neutralising antibody significantly reduced the SARS-CoV-2 spike protein-specific IFN-γ response at day 14 after initial and booster

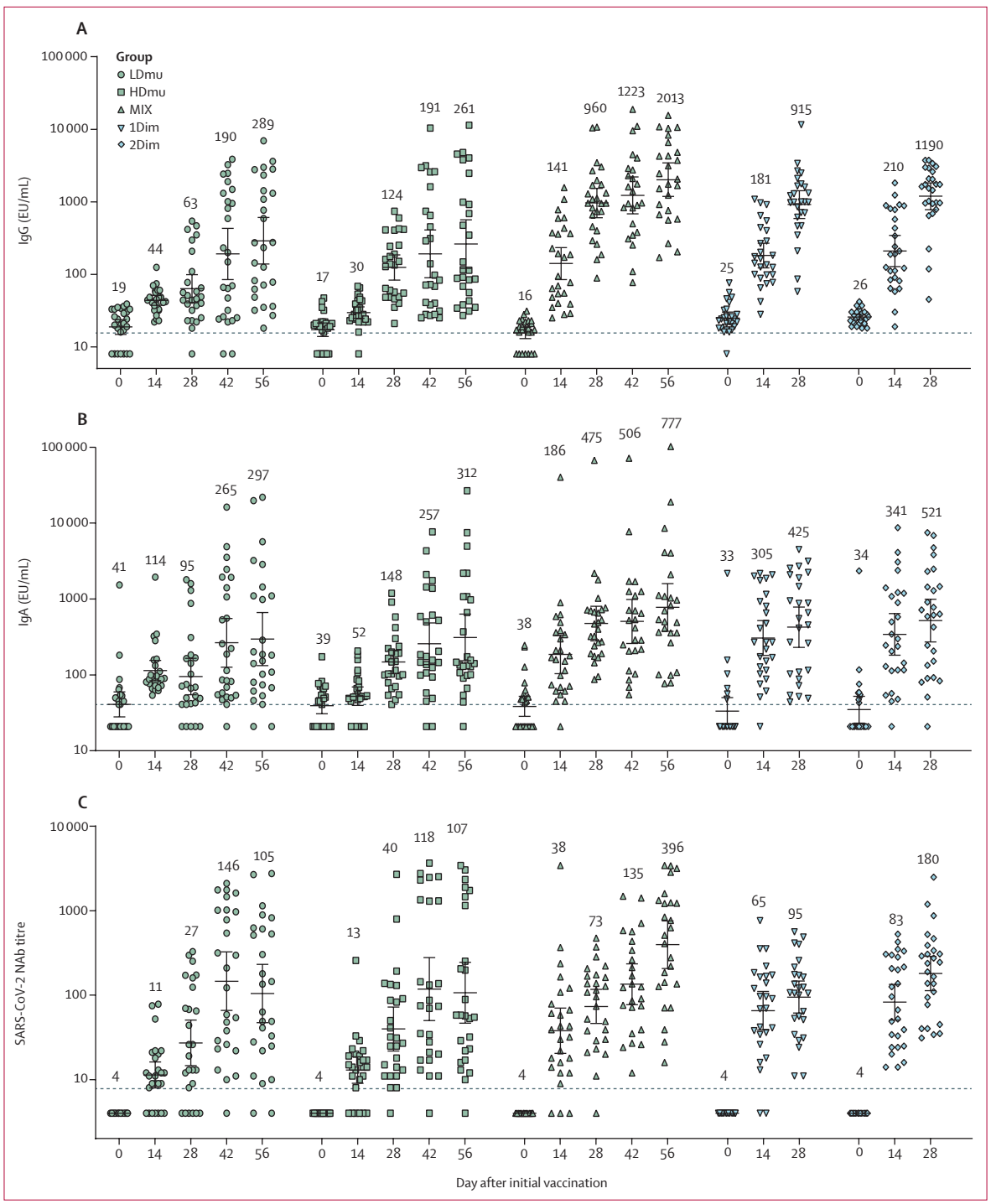

Figure 3: **Sera IgG, IgA, and neutralising antibody response following Ad5-nCoV vaccination**

Spike RBD-binding IgG (A) and IgA concentrations (B) by ELISA and SARS-CoV-2 neutralising antibody (C) following Ad5-nCoV vaccination. All groups received the first vaccine on day 0; the LDmu, HDmu, and MIX group received a booster vaccine on day 28. Each data point represents a serum sample. The error bars are geometric mean with 95% CI. Geometric mean concentration and geometric mean titre are reported. Dashed lines shows the lower limit of quantification. 1Dim=low-dose intramuscular injection. 2Dim=high-dose intramuscular injection. Ad5-nCoV=recombinant adenovirus type 5 carrying full-length SARS-CoV-2 spike gene. HDmu=high-dose aerosol vaccine. LDmu=low-dose aerosol vaccine. MIX=intramuscular and aerosol vaccine. RBD=receptor binding domain.

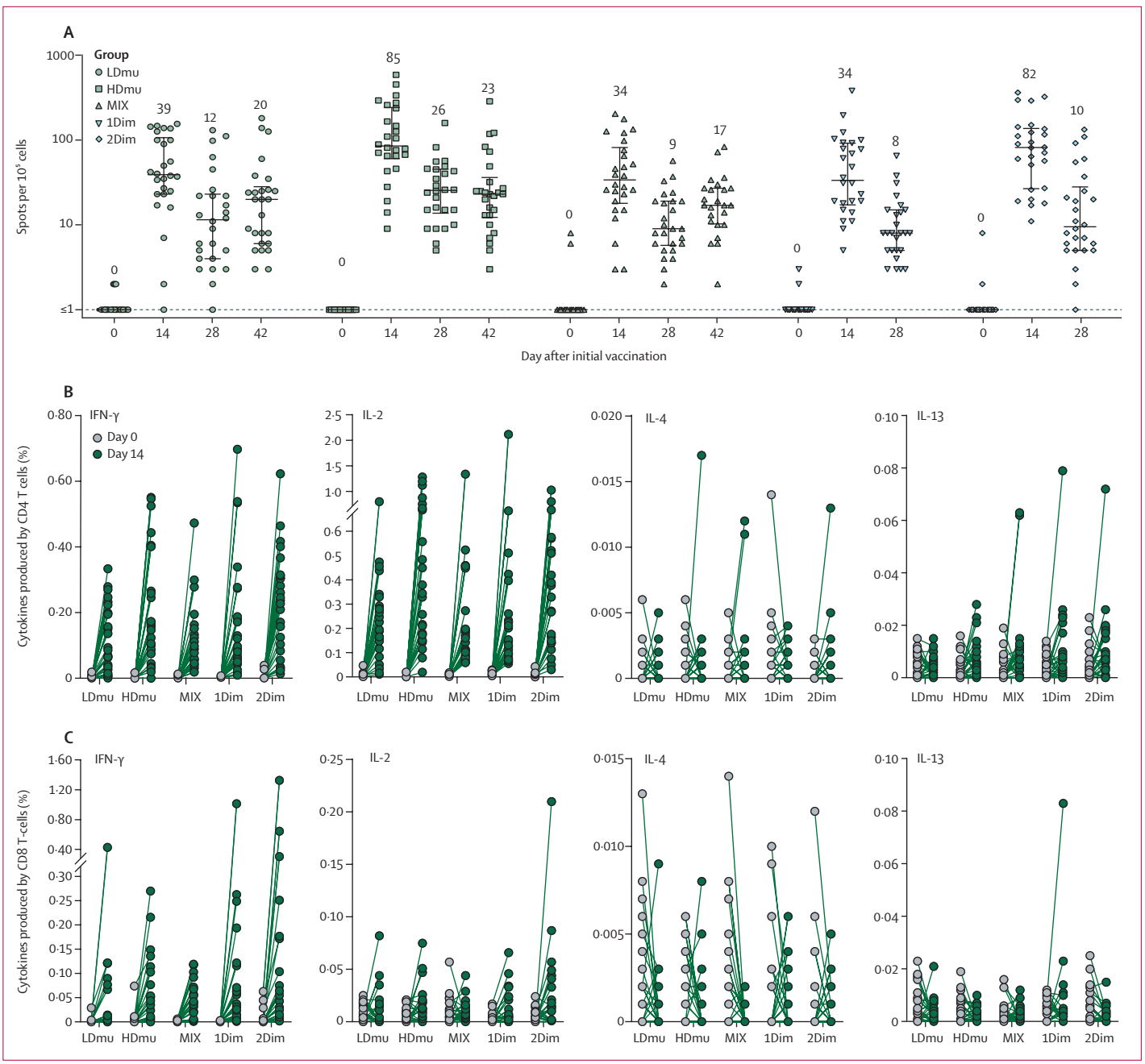

Figure 4: **SARS-CoV-2 spike protein-specific cellular immune response following Ad5-nCoV vaccination**

(A) SARS-CoV-2 spike-specific IFN-γ were detected by enzyme-linked immunospot following Ad5-nCoV vaccination. Each data point represents the mean number of spots from triplicate wells for one participant, after subtraction of the unstimulated control. Error bars show the interquartile range. Dashed line shows the lower limit of quantification. All groups received the first vaccine on day 0; the LDmu, HDmu, and MIX group received a booster vaccine on day 28. (B) Spike protein-specific IFN-γ, IL-2, IL-4, and IL-13 responses in memory CD4 T cells, measured at day 0 and day 14. (C) Spike protein-specific IFN-γ, IL-2, IL-4, and IL-13 responses in memory CD8 T cells, measured at day 0 and day 14. Responses were detected by intracellular cytokine staining; each data point represents the mean percentage of indicated cytokine in T cell subsets from duplicate tests, after subtraction of the unstimulated control. 1Dim=low-dose intramuscular injection. 2Dim=high-dose intramuscular injection. Ad5-nCoV=recombinant adenovirus type 5 carrying full-length SARS-CoV-2 spike gene. HDmu=high-dose aerosol vaccine. IFN=interferon. LDmu=low-dose aerosol vaccine. MIX=intramuscular and aerosol vaccine.

vaccination in both the LDmu and HDmu groups but not in the MIX group, and significantly reduced the IFN-γ response at day 14 and day 28 in the 2Dim group, but not in the 1Dim group (appendix 2 p 18).

Intracellular cytokine staining for IFN-γ, interleukin-2 (IL-2), IL-4, and IL-13 was done to assess the functionality and polarisation of spike protein-specific T cells. The specific memory CD4 T cells secreted IFN-γ and IL-2 but

not IL-4 and IL-13 in all groups at day 14 after the initial vaccination; similarly, the specific memory CD8 T cells secreted mainly IFN-γ and low concentrations of IL-2 (figure 4). The booster vaccination at day 28 only enhanced the specific IFN-γ (p=0·0086) and IL-2 (p=0·0008) response in CD4 T cells in the MIX group (appendix 2 p 19).

At day 14 after initial vaccination, spike protein-specific IFN-γ, tumour necrosis factor α (TNFα), and IL-2 were produced in the supernatants of peripheral blood mononuclear cells, and low concentrations of specific IL-5, IL-9, IL-10, IL-13, and IL-22 were detected, but no IL-4 was reported. Furthermore, no significant difference in IL-6 concentration before and after vaccination was observed (appendix 2 p 20). These findings from intracellular cytokine staining and cytokine profiling suggest that Ad5-nCoV induced a T-helper-1 cell dominant response after both intramuscular injection and aerosol inhalation.

Discussion

No aerosol COVID-19 vaccines have been approved for use to date. In our study, a two-dose aerosolised Ad5-nCoV against COVID-19 equivalent to a fifth or two-fifths of an intramuscular dose was well tolerated and did not produce serious side-effects in healthy adults aged 18 years and older. Two doses of aerosol vaccination had a different safety profile to vaccination with two intramuscular injections, and the incidence of adverse events after a booster dose of intramuscular injection was lower than that after the first vaccination for the adenovirus-vectored COVID-19 vaccine.[13,14] The safety profile for aerosol vaccination is similar to that described in studies of mRNA COVID-19 vaccines and recombinant protein COVID-19 vaccines, which showed that adverse events tended to be more frequent and more severe after the booster vaccination.[15–17] Given the low power of this trial and the rule of three (a 95% upper limit of three of 130 patients with any serious adverse event being 2%), we cannot rule out serious adverse event risk, despite no such events being reported.[18]

In this trial, an aerosolised dose equal to a fifth of the usual injected dose induced antibody and cellular immune responses. Although RBD-binding IgG and IgA concentrations at day 28 after two aerosolised doses were lower than those at day 28 after one injected dose, SARS-CoV-2 neutralising antibody titres in the aerosol vaccination groups were similar to those in patients who received an intramuscular injection. This finding suggests that the different vaccination routes produce different antibody compositions, and that aerosol vaccination could trigger a higher ratio of neutralising antibodies to total antibodies than intramuscular vaccination. There was a moderate correlation between the RBD-binding IgG titres and the neutralising antibody titres in this trial. We know the SARS-CoV-2 neutralising antibodies not only came from the RBD, but also from the N-terminal domain of the spike protein. The antibodies against the N-terminal domain of the spike protein improve neutralising potency, which might partly explain the seroconversion rate difference between the RBD-binding IgG antibody and the neutralising antibody. The serological results of the systemic immune response indirectly indicated that aerosol vaccination brought about strong local mucosal immunity via inhalation of Ad5-nCoV. The vaccine efficacy of AZD-1222 tended to be higher when the interval between the two intramuscular doses was more than 12 weeks.[3,19] In this trial, the interval between the two vaccinations was 4 weeks, and future studies could explore whether increasing the interdose interval to 8–12 weeks is associated with higher antibody concentrations.

One dose of aerosolised Ad5-nCoV induces broad and polyfunctional T cell responses, peaking at day 14 after the first vaccination, which is similar to the phenotype observed with intramuscular Ad5-nCoV. CD4 T cells predominantly secrete T-helper-1 cell cytokines (IFN-γ and IL-2) rather than T-helper-2 cell cytokines (IL-4 and IL-13), as was found in individuals who received the other adenovirus-vectored or mRNA-based COVID-19 vaccines.[20,21] T-cell response is thought to have an important protective role in recognising and killing infected cells or secreting specific antiviral cytokines—several COVID-19 vaccines protected against symptomatic COVID-19 at day 14 after a single-dose vaccination, at which time the specific neutralising antibody titres are relatively low.[1,2]

For some vaccines, such as the nasal spray influenza vaccines, mucosal vaccinations provide similar protective efficacy even though lower antibody titres are reported than with intramuscular injections.[22,23] In a human challenge trial, an oral influenza adenovirus vector vaccine candidate induced lower haemagglutination inhibition titres and neutralising antibody titres than an intramuscular inactivated influenza vaccine, but generated similar protective efficacy.[24] The most relevant immune markers for predicting protection were the number of antibody-secreting cells that produce IgA in individuals who received oral influenza vaccine and the haemagglutination inhibition titres in individuals who received inactivated influenza vaccine. The data in this trial indicated that aerosolised Ad5-nCoV can induce antibody and T-helper-1 cell-bias T-cell responses and that a mixed vaccination strategy produces better responses than aerosol or muscular vaccination alone.

Our study has several limitations. The sample size was small, and no dose-dependent immune response was observed in participants who received aerosol vaccination, so a lower dose for aerosolised vaccination should be explored in future clinical trials. Another limitation is the absence of a sensitive assay to detect the secretory RBD-binding IgA concentration in participant throat swabs, which prevents us from determining the specific secretory IgA concentration in the mucosa after vaccination. We did not do any analyses of recirculating plasmablasts with mucosal homing patterns and tissue-resident memory CD8

T cells to help us understand the different delivery-mediated immune responses. In addition, we did not evaluate the immunogenicity of the double intramuscular immunisation at an interval of 1 month, thus cannot provide comparative data with those of double aerosol vaccination. However, because SARS-CoV-2 infection is initiated at the respiratory tract, it is important to develop strategies for targeting the virus at its entry port. The humoral and cellular immune response induced by aerosolised Ad5-nCoV and its dose-sparing potential show that aerosol vaccination is a promising for delivery of COVID-19 vaccines.

In conclusion, the aerosol inhalation of Ad5-nCoV is painless, simple, well tolerated, and immunogenic, and the current data support the evaluation of aerosolised Ad5-nCoV in ongoing phase 2 and 3 clinical trials.

Contributors

All authors had full access to all data in the studies and had final responsibility for the decision to submit for publication. SW, JH, and LH verified the data. XW is the principal investigator of this trial. JH and JW worked as coprincipal investigators of this trial. XW, WC, LH, JH, SW, and JW designed the trial and the study protocol. LH, SW, and JH drafted the manuscript. WC critically reviewed and revised the manuscript. SW, ZZ, JW, JiZ, JuZ, and HH interpreted the data and revised the manuscript. TS did the statistical analysis. JiZ, TZ, and LL supervised the study. JH, JW, HH, LL, and CC led and participated in the site work, including the recruitment, follow-up, and data collection. SW, ZZ, JiZ, JuZ, PF, BW, YC, XS, YW, and WS were responsible for laboratory analyses. JiZ and WS monitored the trial.

Declaration of interests

TZ, WS, JL are employees of CanSino Biologics. TZ has stock options in CanSino Biologics. All other authors declare no competing interests.

Data sharing

The study protocol and clinical study plan (appendix 2 pp 22–108) are available for review. Anonymised participant data that underlie the results reported in this Article will be made available when the trial is complete. The data will be accessible upon requests directed to the corresponding authors. All data will be made available for a minimum of 5 years from the end of the trial.

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