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Repeated COVID-19 mRNA vaccination results in IgG4 class switching and decreased NK cell activation by S1-specific antibodies in older adults

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Abstract

Background Previous research has shown that repeated COVID-19 mRNA vaccination leads to a marked increase of SARS-CoV-2 spike-specific serum antibodies of the IgG4 subclass, indicating far-reaching immunoglobulin class switching after booster immunization. Considering that repeated vaccination has been recommended especially for older adults, the aim of this study was to investigate IgG subclass responses in the ageing population and assess their relation with Fc-mediated antibody effector functionality.

Results Spike S1-specific IgG subclass concentrations (expressed in arbitrary units per mL), antibody-dependent NK cell activation, complement deposition and monocyte phagocytosis were quantified in serum from older adults ($n = 38–50$, 65–83 years) at one month post-second, -third and -fifth vaccination. Subclass distribution in serum was compared to that in younger adults ($n = 64$, 18–47 years) at one month post-second and -third vaccination.

Compared to younger individuals, older adults showed increased levels of IgG2 and IgG4 at one month post-third vaccination (possibly related to factors other than age) and a further increase following a fifth dose. The capacity of specific serum antibodies to mediate NK cell activation and complement deposition relative to S1-specific total IgG concentrations decreased upon repeated vaccination. This decrease associated with an increased IgG4/IgG1 ratio.

Conclusions In conclusion, these findings show that, like younger individuals, older adults produce antibodies with reduced functional capacity upon repeated COVID-19 mRNA vaccination. Additional research is needed to better understand the mechanisms underlying these responses and their potential implications for vaccine effectiveness. Such knowledge is vital for the future design of optimal vaccination strategies in the ageing population.

Keywords Fc-effector functions, Complement, Ageing, IgG subclasses, Booster

Background

To ensure prolonged protection against severe disease outcomes in the face of continued circulation of SARS-CoV-2 variants, public health authorities have recommended a repeated booster vaccination regimen, especially in older adults [1]. Previous research has shown that repeated COVID-19 mRNA vaccination results in a continued increase in post-vaccination

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antibody binding concentrations and neutralization titers [2–5]. Interestingly, however, it has recently been found that repeated COVID-19 mRNA vaccination results in the prominent induction of IgG4 antibodies in healthy adults [6–12]. This IgG subclass is expressed from the most distal constant gamma (Cγ) region gene in the immunoglobulin heavy chain locus (ordered Cγ3-Cγ1-Cγ2-Cγ4) through a process called class switch recombination (CSR). Regulation of CSR to IgG4 is incompletely understood, but there is evidence that IL-4 and IL-10 are involved [13]. Considering that it has been suggested that CSR might decrease with age and that reduced frequencies of specific memory B cells have been observed in older compared to younger adults upon COVID-19 mRNA vaccination [14–16], it is currently unclear whether older adults will also show changes in IgG4 expression upon repeated COVID-19 mRNA vaccination.

In humans, IgG4 is the only immunoglobulin that can undergo Fab-arm exchange, rendering it bispecific and thereby reducing its ability for antigen cross-linking [17]. Importantly, structural features of the IgG4 (and IgG2) Fc-tail also result in poor binding to activating Fc receptors (FcR) and complement, resulting in a very limited capacity to engage in Fc-mediated effector functions [18, 19]. In contrast, especially IgG3 and to a slightly lesser extent IgG1 are much more proficient in engaging with activating Fc-receptors and complement. Increasing evidence suggests that Fc-mediated antibody effector functions contribute to immunological protection from disease upon SARS-CoV-2 and other viral infections [20–28]. For this reason, knowledge on their development following repeated vaccine administration is important for the design of the most optimal vaccination policies for endemic viruses as well as during future pandemic outbreaks with extended circulation of the emerging pathogen.

Well known Fc-mediated effector functions are antibody-dependent cellular phagocytosis (ADCP), complement deposition (ADCD), and cellular cytotoxicity (ADCC) by natural killer (NK) cells [29, 30]. During ADCP, antibody-opsonized (viral) particles or infected cells are internalized and degraded by FcR-expressing phagocytes such as monocytes and macrophages. Antibody-opsonization can also result in deposition of complement molecules on the surface of viral particles or infected cells, which can in turn result in enhanced phagocytosis or the formation of a cytotoxic membrane-attack complex. Finally, antibody-dependent NK cell activation (ADNKA) resulting from FcR interaction is characterized by NK cell degranulation and the release of cytotoxic molecules that will ultimately kill the infected target cell, i.e. ADCC.

In the current analysis, we assessed the development of SARS-CoV-2 spike S1-specific IgG subclass levels in older adults ($n=50$, 65–83 years of age) up to one month after the fifth vaccination. We then compared the patterns observed in older adults with a younger adult reference group ($n=64$, 18–47 years of age) for the timepoints up to one month following the third vaccination. Finally, we focused on the older adult participants and assessed the capacity of serum antibodies to mediate spike S1-specific ADCP, ADCD, and ADNKA up to one month after the fifth vaccination. Together, these data provide novel insights into the development of antibody quality beyond neutralization upon repeated vaccination in the older adult population.

Methods

Sample collection

Serum samples were selected from participants enrolled in two previously described prospective COVID-19 vaccination studies in (older) adults in the Netherlands [16, 31]. All participants received their COVID-19 immunizations via the standard national vaccination campaign starting February 2021. Blood samples were obtained by vena punction during a scheduled visit at various timepoints surrounding vaccination until December 2022. For the current analysis, samples were selected that were obtained approximately 1 month (post-second dose) and 5–7 months (post-second dose follow-up) after receiving the second dose of the primary series, 1 month after receiving the third dose, 5 months after receiving the fourth vaccination (post-fourth dose follow-up) and approximately one month after receiving the fifth dose. For an overview of sampling intervals (median and range), see Table 1. Participants that were previously infected or experienced a breakthrough infection before completing the primary series (based on assessment of nucleoprotein seropositivity) were excluded from the overall analysis. Only participants who had received the Pfizer-BioNTech COVID-19 mRNA vaccine (BNT162b2) during the primary series were selected. Additional booster doses were either the Pfizer-BioNTech or Moderna COVID-19 mRNA vaccine. For the first to fourth vaccination the original monovalent vaccine was used and for the fifth vaccination the bivalent vaccine was used.

Cell culture conditions

THP-1 cells (ATCC TIB-202, RRID: CVCL_0006) were cultured in ATCC modified RPMI1640 medium (A1049101, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (hiFBS), 1×pen/strep and 0.05 mM 2-mercaptoethanol. NK-92/CD16+cells (ATCC, RRID: CVCL_V429) were cultured in MEM Eagle,

Table 1 Participant characteristics

	Younger adults	Older adults (all)	Older adults (Fc-functionality subgroup)
Size, *n*	64	50	38
Age in years, median (range, SD)	33 (18–47, 7.0)	70 (65–83, 3.8)	69 (65–77, 3.3)
Female sex, *n* (%)	42 (66)	23 (46)	18 (47)
Confirmed SARS-CoV-2 infection			
After second dose, before post-third sample, *n* (%)	4 (6.3)	2 (4.2)	1 (2.8)
After second dose, before post-fifth sample, *n* (%)	NA	20 (41)	13 (35)
Vaccine type[a]			
First dose Pfizer-BioNTech, *n* (%)	64 (100)	50 (100)	38 (100)
Second dose Pfizer-BioNTech, *n* (%)	64 (100)	50 (100)	38 (100)
Third dose Moderna, *n* (%)	1 (1.6)	46 (92)	35 (92)
Third dose Pfizer-BioNTech, n (%)	63 (98)	4 (8)[b]	3 (8)
Fourth dose Moderna, *n* (%)	NA	35 (70)	26 (68)
Fourth dose Pfizer-BioNTech, n (%)	NA	15 (30)	12 (32)
Fifth dose Moderna (bivalent), *n* (%)	NA	48 (98)[c]	36 (97)[c]
Fifth dose Pfizer-BioNTech (bivalent), n (%)	NA	1 (2)[c]	1 (3)[c]
Vaccination interval in days			
First to second dose, median (range, SD)	35 (32–39, 1.0)	35 (31–36, 0.7)	35 (35–36, 0.3)
Second to third dose, median (range, SD)	162 (140–310, 21)	208 (160–295, 21)	208 (160–295, 21)
Third to fourth dose, median (range, SD)	NA	103 (90–204, 36)	105 (90–204, 38)
Fourth to fifth dose, median (range, SD)	NA	182 (102–221, 37)[c]	182 (105–221, 38)[c]
Sampling interval in days			
Post-second dose, median (range, SD)	30 (28–44, 2.5)	32 (28–40, 3.1)	32 (28–40, 3.2)
Post-second dose FU, median (range, SD)	164 (141–310, 21)	200 (169–289, 20)	197 (169–289, 21)
Post-third dose, median (range, SD)	31 (25–39, 2.7)	30 (23–46, 4.8)	30 (23–46, 4.9)
Post-fourth dose FU, median (range, SD)	NA	166 (75–216, 34)	166 (75–216, 35)
Post-fifth dose, median (range, SD)	NA	30 (23–42, 4.8)[c]	30 (23–42, 4.9)[c]

Abbreviations: *FU* Follow-up, *n* Number, *NA* Not applicable, *SD* Standard deviation

[a] Participants received either the Pfizer-BioNTech Comirnaty or Moderna Spikevax mRNA vaccine, of which the first to fourth dose were the original monovalent vaccines and the fifth vaccine dose was bivalent (original/BA.1)

[b] Serum for one Pfizer-BioNTech-vaccinated individual is unavailable at one month post-third vaccination

[c] One participant dropped out of the study after the fourth vaccine dose

Alpha modification medium (M0644, Sigma-Aldrich) supplemented with 2.2 g/L sodium bicarbonate, 0.2 mM myo-inositol (I5125, Sigma-Aldrich), 10% hiFBS, 10% heat-inactivated Fetal Horse Serum (16050122, Gibco), 2.5 µM folic acid, 1×non-essential amino acids (11140050, Gibco), 1 mM sodium pyruvate (11360070, Gibco), 1×pen/strep/glut (Gibco), 200 IU/ml recombinant human IL-2 (78220.1, Stemcell Technologies) and 1 mM 2-mercaptoethanol. Both cell lines were cultured in a standard 37°C, 5% CO_2 incubator.

Determination of SARS-CoV-2 spike S1-specific IgG binding concentration

A previously validated multiplex bead-based immunoassay (MIA) was employed to quantify SARS-CoV-2 spike S1-specific IgG in serum samples [32]. Briefly, microplex fluorescent beads were coated with SARS-CoV-2 monomeric spike S1 protein derived from the original Wuhan strain (40591-V08H, Sino Biologicals), matching the vaccine immunogen, and incubated with diluted serum samples. Following incubation and washing steps, captured antibodies were detected with a 1:400 dilution of phycoerythrin (PE)-conjugated goat anti-human IgG (109–116-098, Jackson ImmunoResearch Laboratories) for 30 min, followed by additional washing.

Samples were acquired on an FM3D instrument (Luminex). The median fluorescence intensity (MFI) values were converted into binding arbitrary units (BAU/mL) by interpolation from a 5-parameter logistic standard curve using Bioplex Manager 6.2 software

(Bio-Rad Laboratories). The data was then exported to Microsoft Excel for further analysis.

Measurement of SARS-CoV-2 spike S1-specific IgG subclasses

For measurement of SARS-CoV-2 spike S1-specific IgG subclasses, the validated MIA described above was used with some modifications. Following incubation and washing steps, captured antibodies were labeled by adding 50 µl of a 1:400 dilution of monoclonal mouse anti-human IgG1, IgG2, IgG3 or IgG4 (A-10630/05–3500/MH1532/A-10651, all from Invitrogen, CA) to each well for 30 min, followed by the addition of 50 µl of a 1:800 dilution of R-Phycoerythrin-conjugated goat anti-mouse IgG (115–116-071, Jackson immunoresearch, UK). Eight-fold serial dilutions of an in-house reference serum were included on each plate and for each participant all subclasses were measured in one experiment. Reference curves were parallel between the assays. Since the concentrations of the four IgG subclasses against the spike protein are unknown in both the FDA and the in-house reference serum, we have set the measured MFI concentrations in arbitrary units (AU) of this positive control at 500 AU for each subclass. For the spike S1 protein, cut-offs for seropositivity were set at 1, 16, 16 and 4 AU per mL for IgG1, IgG2, IgG3 and IgG4, respectively, based on the median value of various negative control sera measured in 25 independent experiments.

Assessment of SARS-CoV-2 spike S1-specific IgG avidity

To determine the S1-specific IgG avidity index, serum samples were diluted 400- and 4,000-fold and incubated for 45 min with S1-conjugated beads and then washed. Subsequently, the samples were incubated for 10 min at RT with either 2M NH_4SCN (Sigma-Aldrich) or phosphate-buffered saline (PBS) with 1% BSA. Following washing steps and incubation with PE-conjugated goat anti-human IgG, the MFI values were acquired using an FM3D instrument. The avidity index (AI) was calculated using the formula:

$$\text{AI (\%)} = ((\text{MFI NH4SCN}) / (\text{MFI PBS})) * 100\%$$

To ensure accurate determination, the avidity index was calculated only when the MFIs of the PBS-incubated samples fell within the limits of linearity (LOL) of the reference serum sample. A lower limit of 1,000 MFI was applied to ensure accurate determination of low-concentration samples. Samples outside these LOL were retested in a different dilution.

Antigen-coating of microspheres for ADCP and ADCD assays

Sulfo-NHS-SS-Biotin (EZ-Link™ Micro Sulfo-NHS-SS-Biotinylation Kit, 21945, Thermo Scientific) was added to recombinant SARS-CoV-2 monomeric Spike S1 derived from the original Wuhan strain (40591-V08H, Sino Biologicals) in a 50:1 molar ratio and incubated at RT for 60 min. Excess biotin was removed using a Zeba Spin Desalting Column (89889, Thermo Scientific) according to manufacturer's instructions and biotinylated protein was stored at -80°C until further use. Spike S1-biotin was added to either red or green fluorescent microbeads (Fluospheres NeutrAvidin-Labeled Microspheres, F8775 or F8776 respectively, Invitrogen) in a 1:1 (w/v) ratio and incubated o/n at 4 °C. The beads were then washed with a 10×volume of PBS, centrifuged at 5,000 xg for 20 min and blocked in PBS/2%BSA for at least 1 h. Beads were centrifuged again and stored in PBS/0.1%BSA at 4 °C for a maximum of 1 week.

Bead-based monocyte ADCP assay

Antigen-coated green fluorescent microbeads were diluted to 728×original bead volume in PBS/0.1%BSA and 20 ul (500,000 beads) was added per well in 96-well V-bottom plates. Heat-inactivated (HI) serum (20 µl, 8,000×diluted in PBS) was added and incubated for 2 h at 37 °C, after which the beads were washed twice with PBS/0.1%BSA. PBS and serum from a healthy adult volunteer obtained before the SARS-CoV-2 pandemic were included as negative controls, giving similar results. THP-1 cells (20,000) in RPMI/10%hiFBS were added per well and incubated for 1 h at 37 °C. Cells were washed with cold PBS and fixated in 100 ul 1% formaldehyde. Following centrifugation, cells were resuspended in PBS/0.5% hiFBS with 2 mM EDTA and data was acquired on a BD FACSCanto II. Data analysis was performed in FlowJo and the integrated mean fluorescence intensity (iMFI) or phagocytic score was determined by multiplying the percentage FITC-positive cells with their MFI and dividing the result by 1000, which accounts for both the fraction of phagocytosing cells and the number of internalized beads per cell. The gating strategy is depicted in Figure S1A.

Bead-based ADCD assay

Antigen-coated red fluorescent microbeads were incubated with serum at a final dilution of 2,000x, as described for ADCP. PBS and serum from a healthy adult volunteer obtained before the SARS-CoV-2 pandemic were included as negative controls, giving similar results. Low-Tox® Guinea Pig Complement (CL4051, Cedarlane) was reconstituted in 1 ml dH_2O, diluted 25×in HBSS and 100 ul was added per well. Plates were incubated at 37 °C

for 15 min and washed twice with cold PBS/0.5%hiFBS with 2 mM EDTA. Beads were stained with 100×diluted FITC conjugated goat IgG anti-Guinea Pig Complement C3 (11499934, MP Biomedicals) and acquired on a BD LSRFortessa. Analysis was performed in FlowJo. Single beads were selected based on FSC/SSC plots and PE signal. The FITC median of this population was determined as a measure of C3b deposition. The gating strategy is depicted in Figure S1B.

Plate-based ADNKA assay

High-binding 96-well plates were coated with recombinant S1 protein derived from the original Wuhan strain at a concentration of 1 ug/ml by overnight incubation at 4°C. Plates were washed with PBS and blocked with PBS/2%BSA for 30 min. After removal of blocking buffer, HI serum samples (50 µl, 2,000×diluted in PBS) were added and incubated for 2h at 37°C. PBS and serum from a healthy adult volunteer obtained before the SARS-CoV-2 pandemic were included as negative controls, giving similar results. Following washing, 25,000 NK-92/CD16+cells in medium containing GolgiPlug (51-2301KZ, BD) and CD107a-PerCP-Cy5.5 (328616, BioLegend) were added per well and incubated for 4h. Cells were then stained with anti-CD56-PE (318306, BioLegend) and Fixable Viability dye eFluor^{TM}780 (65–0865-14, ThermoFisher) prior to fixation. Data was acquired on a BD LSRFortessa and analyzed in Flowjo. The gating strategy is depicted in Figure S1C.

Statistics

Box plots show individual data points with medians and quartiles. Medians were chosen as the most appropriate summary statistic because a considerable number of individuals had values below the limit of quantification, especially for the subclass measurements. Samples below the limit of quantification were set to half the lowest measured value for visualization purposes. Correlation plots show lines indicating either linear regression (straight lines) or locally estimated scatterplot smoothing (LOESS). Comparison between timepoints in older adults was performed using the non-parametric Wilcoxon signed rank test with Bonferroni's correction for multiple testing. For clarity, statistical significance is only indicated for comparisons between the one month post-vaccination timepoints, not the follow-up timepoints. For comparisons between younger and older adults an unpaired Wilcoxon test with Bonferroni's correction for multiple testing was used. For clarity, statistical significance is only indicated for comparisons within the same sampling timepoint. Spearman's rank-order correlation was used to assess the correlations between IgG subclass concentrations and age. Statistical analysis and graph design were performed using R version 4.4.0.

Results

Median spike S1-specific total IgG concentration increases upon repeated mRNA vaccination in older adults

As previous research has shown that repeated COVID-19 mRNA vaccination leads to the induction of virus-specific IgG4 antibodies in adult vaccinees [6–9], we asked whether a similar response would be observed in older adults (≥65 years of age). To investigate this, we selected samples from our ongoing vaccination studies of older adult participants ($n=50$, 65–83 years of age) up to one month after receiving the fifth vaccine dose. For comparison, we included samples from younger adult participants ($n=64$, 18–47 years of age) up to one month following the third vaccine dose, as vaccination schedules for younger and older adults started to diverge considerably starting from the fourth vaccine dose. Participants that had been infected with SARS-CoV-2 before completing the primary vaccination series (vaccine dose 1 and 2) were excluded from the overall analysis to avoid an effect of the infection on IgG concentrations and isotype usage. A schematic overview of the sampling schedule is depicted in Fig. 1A and a detailed overview of participant characteristics and vaccination timelines can be found in Table 1.

To first obtain a general overview of the vaccine-induced antibody response in these participants, we measured SARS-CoV-2 spike S1-specific total IgG concentrations using a bead-based multiplex immunoassay. These data confirm that older adults show an increase in median S1-specific total IgG concentrations upon repeated booster vaccinations (Fig. 1B). Median total IgG levels have increased 3.3-fold when comparing post-second to post-third vaccination levels ($P<0.001$) and 2.8-fold when comparing post-third to post-fifth vaccination levels ($P<0.001$). Furthermore, the second vaccination results in lower median antibody levels in older compared to younger adults (Fig. 1C, $P<0.001$). This difference in median S1-specific IgG levels increases over time ($P<0.001$), but is largely overcome following the third vaccination.

Repeated mRNA vaccination results in a more pronounced increase in median IgG2 and IgG4 concentrations than IgG1 and IgG3 in older adults

Next, we assessed the SARS-CoV-2 spike S1-specific concentrations of IgG1, IgG2, IgG3 and IgG4 using a bead-based multiplex immunoassay (Fig. 2). Median IgG1 levels show a clear 2.4-fold increase when comparing post-second to post-third vaccination levels ($P<0.001$), whereas only a 1.6-fold increase is seen between

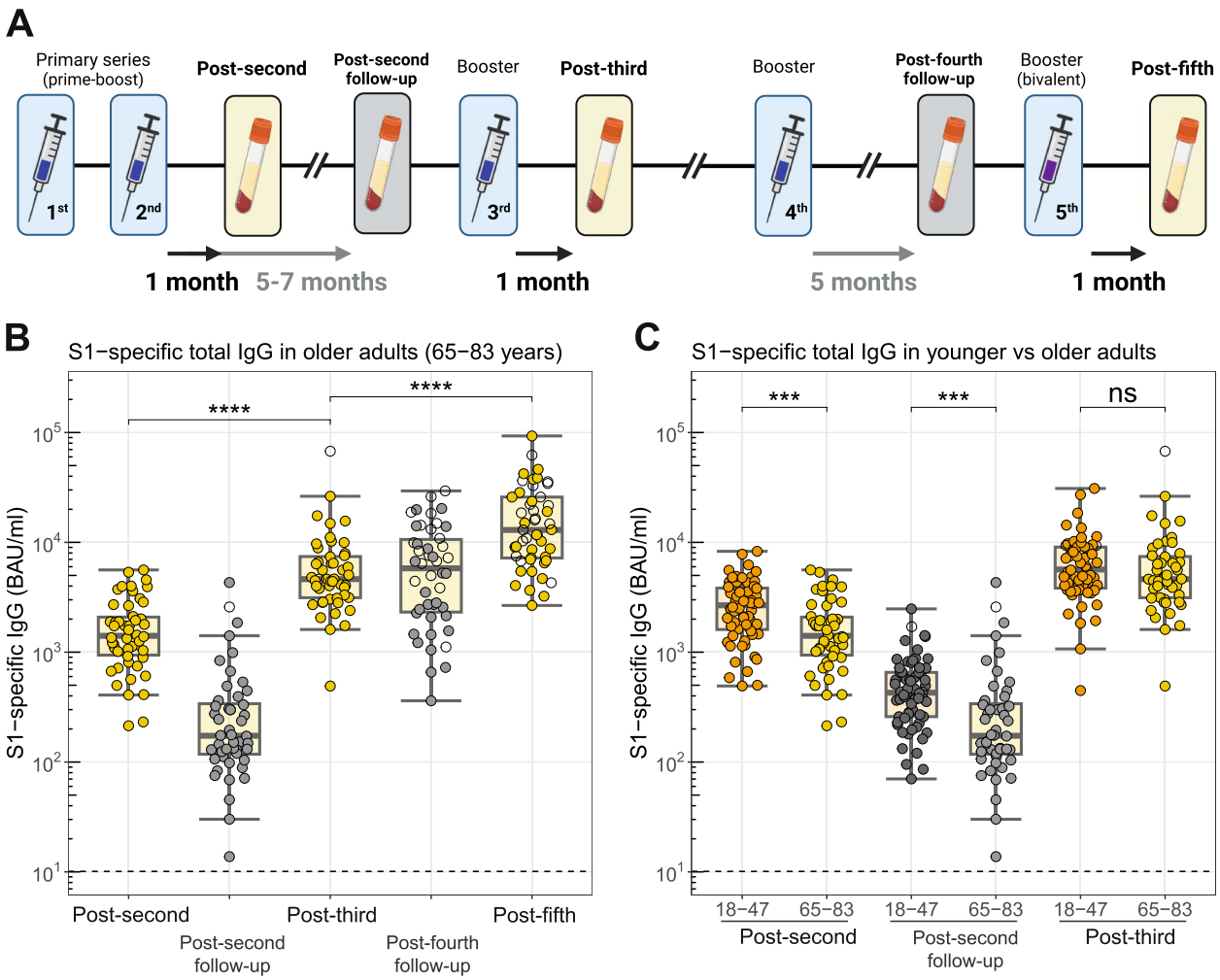

Fig. 1 SARS-CoV-2 spike S1-specific total IgG levels in younger and older adults following mRNA vaccination. **A** Schematic overview of the vaccination and sampling scheme in which participants received original monovalent (1st to 4th dose) and bivalent (5th dose) mRNA vaccines. SARS-CoV-2 spike S1-specific total IgG concentrations (BAU/mL) following mRNA vaccination were measured in serum by multiplex immunoassay for **B** older adults ($n=50$) up to one month after the fifth vaccination and for **C** younger ($n=64$) and older ($n=50$) adults up to approximately one month after the third vaccination. Dotted lines indicate the cut-off for seropositivity. SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. Differences between groups were assessed using a non-parametric (unpaired) Wilcoxon signed rank test with Bonferroni's correction for multiple testing. *** $P<0.001$; **** $P<0.0001$. BAU, binding antibody units; N, nucleoprotein; ns, not significant

post-third and post-fifth vaccination levels ($P<0.01$). For median IgG3 concentrations, an almost twofold decrease was observed between post-second and post-third vaccination levels ($P<0.001$). Although the median IgG3 levels increase again from post-third to post-fifth vaccination ($P<0.001$), the median post-fifth vaccination IgG3 level remains similar to the post-second vaccination level. In contrast, median concentrations for IgG2 show a very strong increase from post-second to post-third vaccination levels ($P<0.001$) with a further increase from post-third to post-fifth vaccination levels ($P<0.001$). Median IgG4 levels show a similar pattern as seen for IgG2, with a strong increase from post-second to post-third vaccination levels ($P<0.001$) and a continued 4.8-fold increase from post-third to post-fifth vaccination levels ($P<0.001$). Whereas only a (small) proportion of participants has detectable levels of IgG2 and IgG4 post-second vaccination (58% and 2.1%, respectively), virtually all participants have detectable levels of these subclasses after the third vaccination (100% and 94%, respectively). Despite the very limited group size ($n=4$), we observed that those older individuals that were excluded from the overall analysis because they had been infected prior to receiving their first COVID-19 vaccination displayed

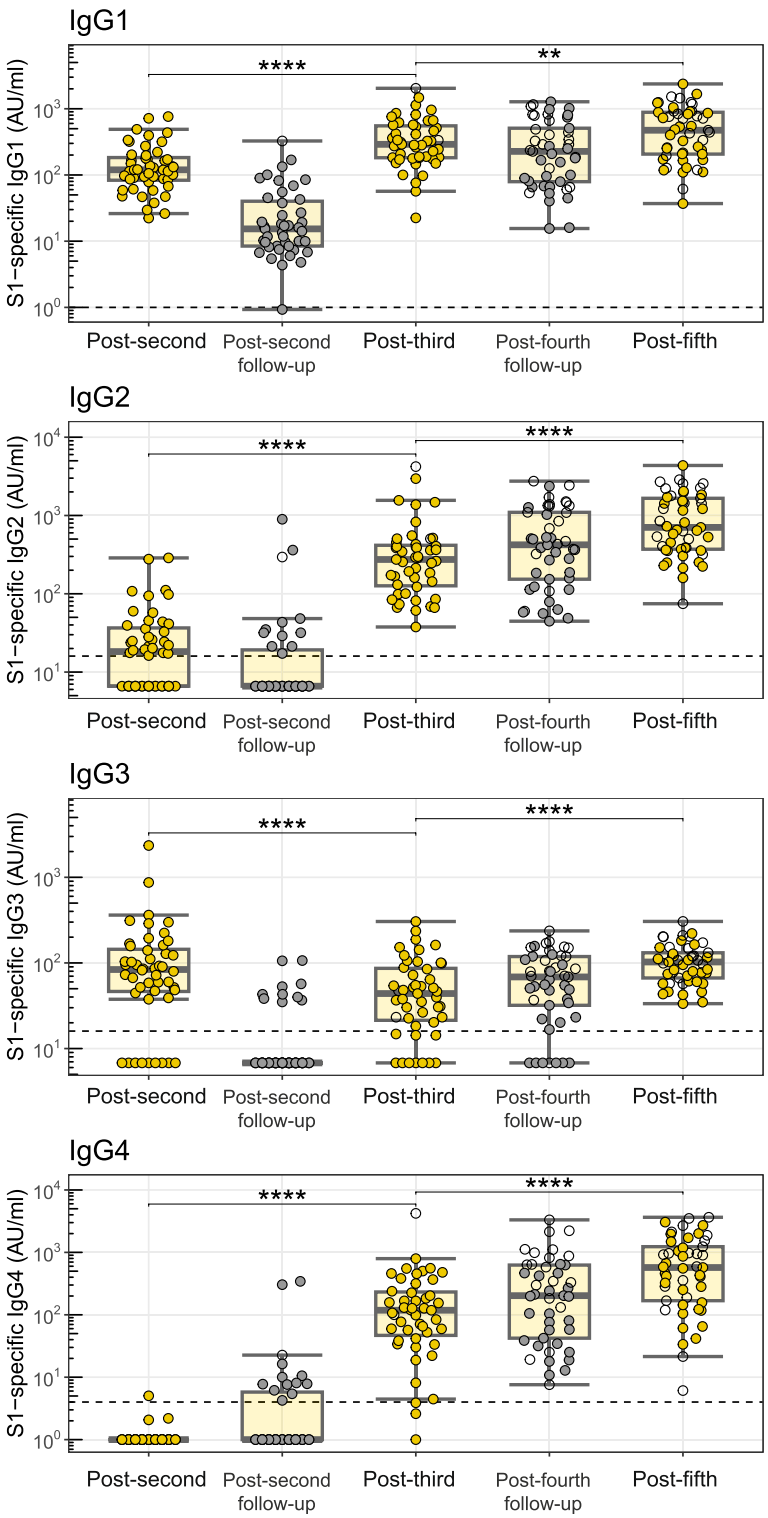

Fig. 2 SARS-CoV-2 spike S1-specific IgG subclasses in older adults following repeated mRNA vaccinations. SARS-CoV-2 spike S1-specific IgG1, IgG2, IgG3 and IgG4 concentrations (AU/mL) following mRNA vaccination were measured in serum by multiplex immunoassay for older adults ($n = 50$) up to one month after the fifth vaccination. Dotted lines indicate the cut-off for seropositivity. SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. Differences between groups were assessed using a non-parametric Wilcoxon signed rank test with Bonferroni's correction for multiple testing. ** $P < 0.01$, **** $P < 0.0001$. AU, arbitrary units; N, nucleoprotein

remarkably reduced levels of IgG4 and IgG2 following repeated vaccination (Fig. 3 and Supplementary Figure S2). We did not observe notable differences in IgG subclass concentrations between female and male participants (Supplementary Figure S3).

Repeated mRNA vaccination of older adults results in a more pronounced increase of IgG2 and IgG4 compared to younger adults

After having established that antibodies in older adults do undergo class switching to IgG2 and IgG4 upon repeated mRNA vaccination, we asked how the established levels compared between younger and older adults (Fig. 4). Following the second vaccine dose, IgG subclass concentrations were largely similar between younger and older adults, with a trend towards lower median IgG1 and IgG3 levels in older compared to younger individuals. Following the third vaccination, however, older adults showed markedly increased median concentrations of both IgG2 and IgG4 compared to younger adults ($P < 0.001$ for both), while median IgG1 and IgG3 levels remained similar, showing that older individuals are at least as capable as younger individuals to perform class switching. It is important to note, however, that although all participants received Pfizer-BioNTech for vaccination one and two, all but one of the younger adults had also received the Pfizer-BioNTech vaccine as a third dose at a median of 162 days following the primary series, whereas in contrast all but four of the older adults in our study had received the Moderna vaccine as a third dose at a median of 208 days after receiving the primary series (Table 1). Of note, no clear difference was observed in older adults between Moderna- and Pfizer-BioNTech-vaccinees following the third vaccination (Supplementary Figure S4), but numbers were very low for the latter group.

Repeated mRNA vaccination leads to a decrease in serum S1-specific antibody-dependent NK cell activation and complement deposition relative to antibody concentration in older adults

Compared to younger individuals, older adults have received more numerous booster vaccinations and showed the highest levels of IgG2 and IgG4. We therefore expect the most pronounced effect on Fc-mediated effector functions in this age group. For this reason, we focused our analysis of SARS-CoV-2 spike S1-specific ADNKA, ADCD and ADCP on a representative subgroup of older adult participants ($n = 38$, 65–77 years of age, Table 1), including samples that were collected one month post-second, post-third and post-fifth vaccination dose. In line with the increase in spike-specific total IgG concentration, the various Fc-effector functions also increased following repeated vaccinations (Fig. 5A). However, the capacity of S1-specific serum antibodies to mediate NK cell activation and, to a lesser extent, complement deposition relative to S1-specific total IgG concentrations appeared to decrease after the fifth to the third vaccination (Fig. 5B). This relative decrease can be deducted from the observed overall rightward shift after the fifth dose compared to earlier

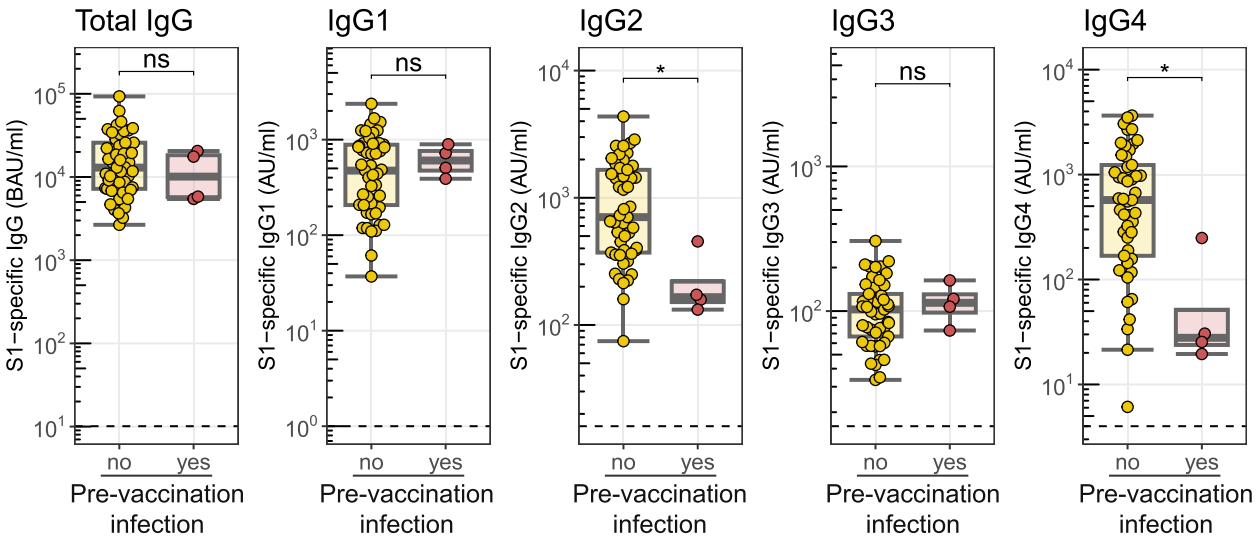

Fig. 3 S1-specific total IgG and IgG subclasses in older adults with and without SARS-CoV-2 infection prior to the start of COVID-19 vaccination. SARS-CoV-2 spike S1-specific total IgG, IgG1, IgG2, IgG3 and IgG4 concentrations following the fifth mRNA vaccination were measured in serum by multiplex immunoassay for older adults with ($n = 4$) and without ($n = 50$) SARS-CoV-2 infection prior to the start of vaccination. Dotted lines indicate the cut-off for seropositivity. Differences between groups were assessed using a non-parametric Wilcoxon signed rank test with Bonferroni's correction for multiple testing. * $P < 0.05$. AU, arbitrary units; BAU, binding antibody units

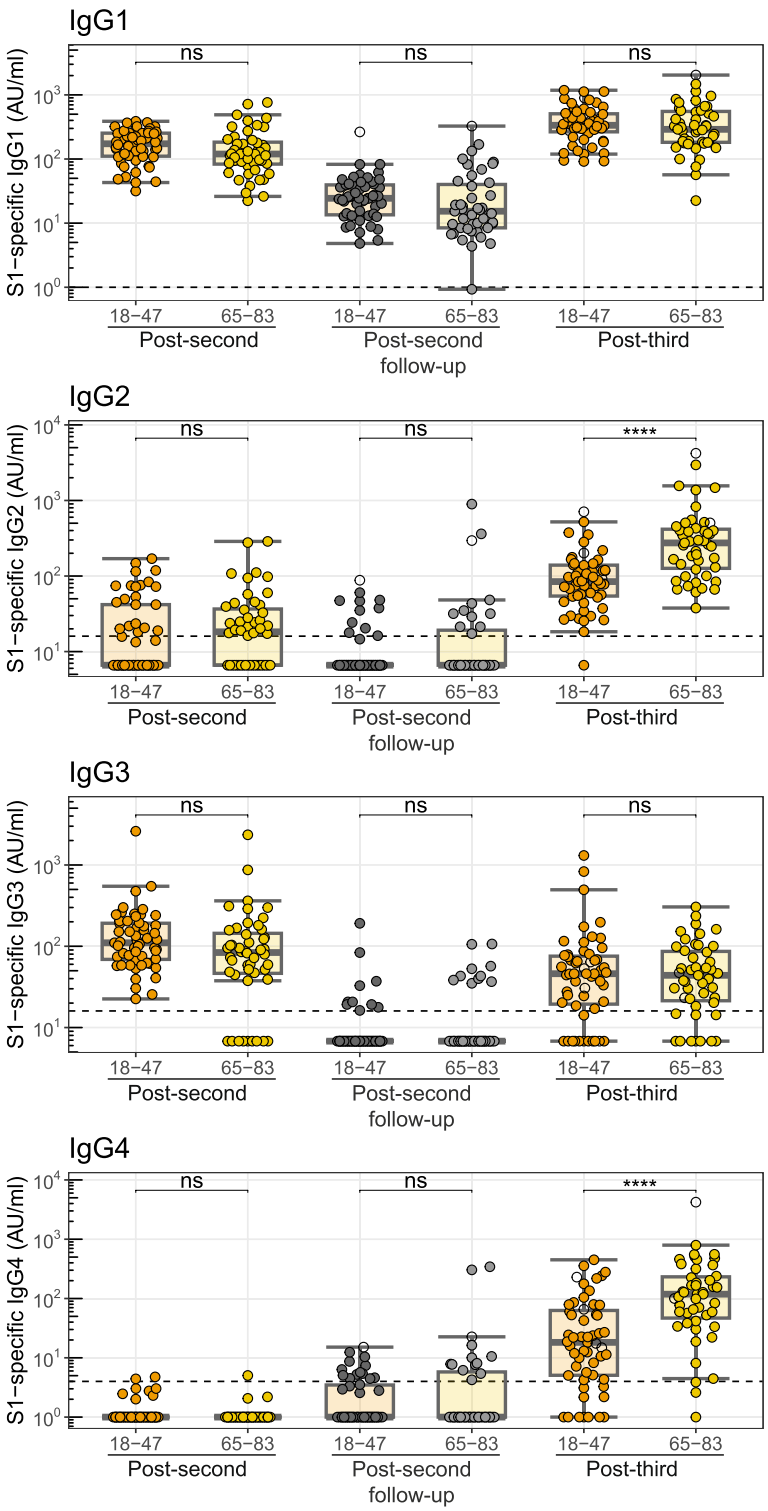

Fig. 4 Comparison of SARS-CoV-2 spike S1-specific IgG subclasses following mRNA vaccination between younger and older adults. SARS-CoV-2 spike S1-specific IgG1, IgG2, IgG3 and IgG4 concentrations (AU/mL) following mRNA vaccination were measured in serum by multiplex immunoassay for younger ($n = 64$) and older ($n = 50$) adults up to one month after the third vaccination. Dotted lines indicate the cut-off for seropositivity. SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. Differences between groups were assessed using a non-parametric unpaired Wilcoxon signed rank test with Bonferroni's correction for multiple testing. **** $P < 0.0001$. AU, arbitrary units; N, nucleoprotein; ns, not significant

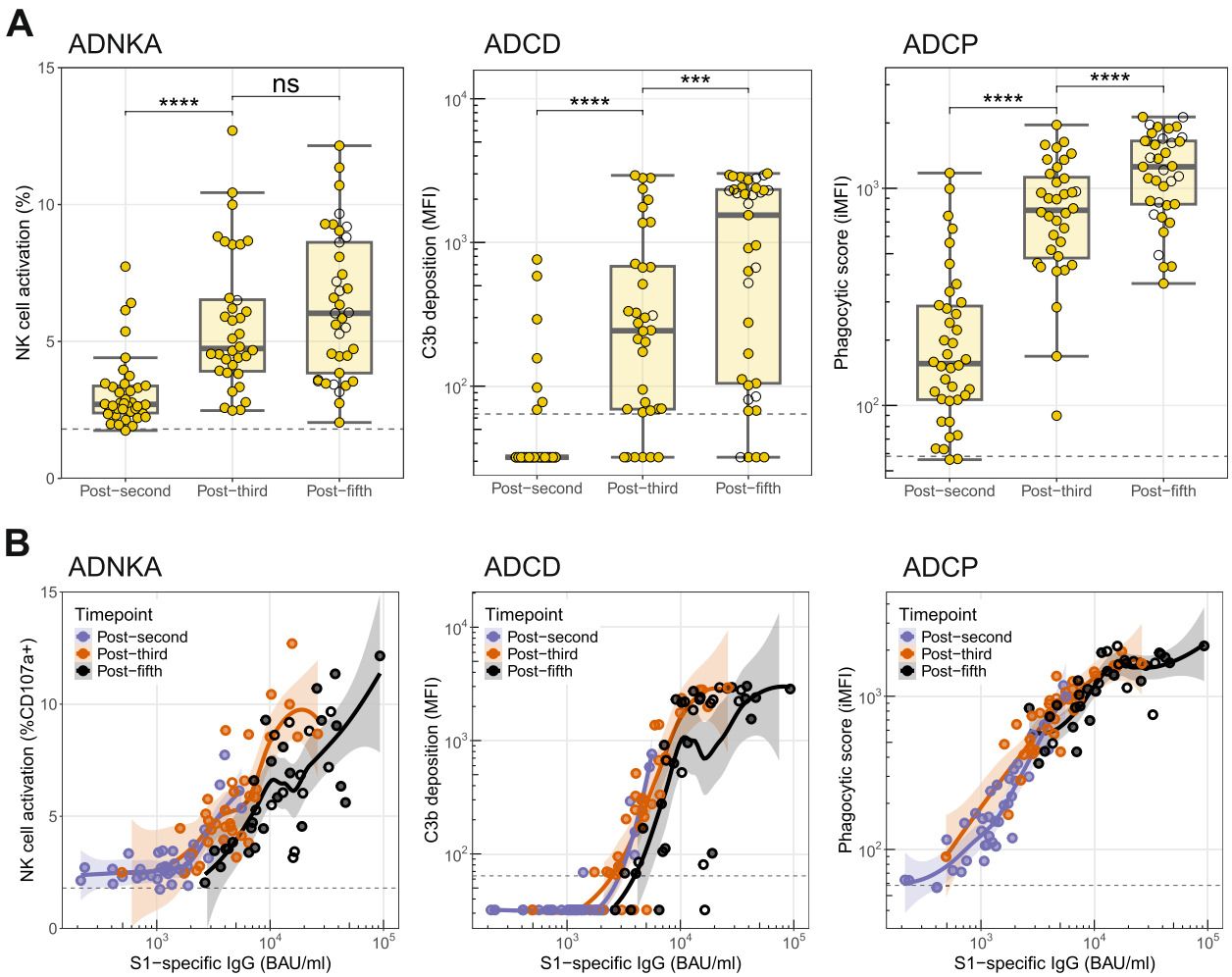

Fig. 5 SARS-CoV-2 spike S1-specific Fc-mediated effector functions upon repeated vaccination in older adults. The absolute capacity (**A**) and the capacity relative to S1-specific total IgG levels (**B**) of SARS-CoV-2 spike S1-specific serum antibodies for mediating ADNKA, ADCD and monocyte ADCP following mRNA vaccination in older adults ($n = 38$) up to one month after the fifth vaccination. Dotted lines indicate the level of the negative control (pre-pandemic serum sample). Solid lines were made using locally estimated scatterplot smoothing (LOESS). SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. Differences between groups were assessed using a non-parametric Wilcoxon signed rank test with Bonferroni's correction for multiple testing. *** $P < 0.001$; **** $P < 0.0001$. ADCD, antibody-dependent complement deposition; ADCP, antibody-dependent cellular phagocytosis; ADNKA, antibody-dependent natural killer cell activation; BAU, binding antibody units; iMFI, integrated median fluorescence intensity; N, nucleoprotein

post-vaccination timepoints, revealing similar levels of functionality at higher antibody concentrations. This effect was not clearly visible for S1-specific antibody-mediated phagocytosis.

The relative decrease in ADNKA and ADCD observed upon repeated mRNA vaccination in older adults associates with an increase in the ratio of IgG4/IgG1 concentration

Since we observed a striking induction of S1-specific IgG4 antibodies in older adults we wondered whether this could be associated with differences in the functional capacity of the antibodies relative to total S1-specific IgG concentration. Our data suggest that, in line with the known functional properties of IgG4, higher S1-specific IgG4/IgG1 ratios – i.e. relatively higher concentrations of IgG4 – indeed associate with a lower relative capacity to activate NK cells and complement deposition (Fig. 6A). From these graphs it is evident that the darker datapoints (lower IgG4/IgG1 ratios) tend to localize above the line representing the average correlation between functionality and concentration, while the lighter datapoints (higher IgG4/IgG1 ratios) tend to lie below this line, especially for ADNKA. The S1-specific phagocytosis capacity relative

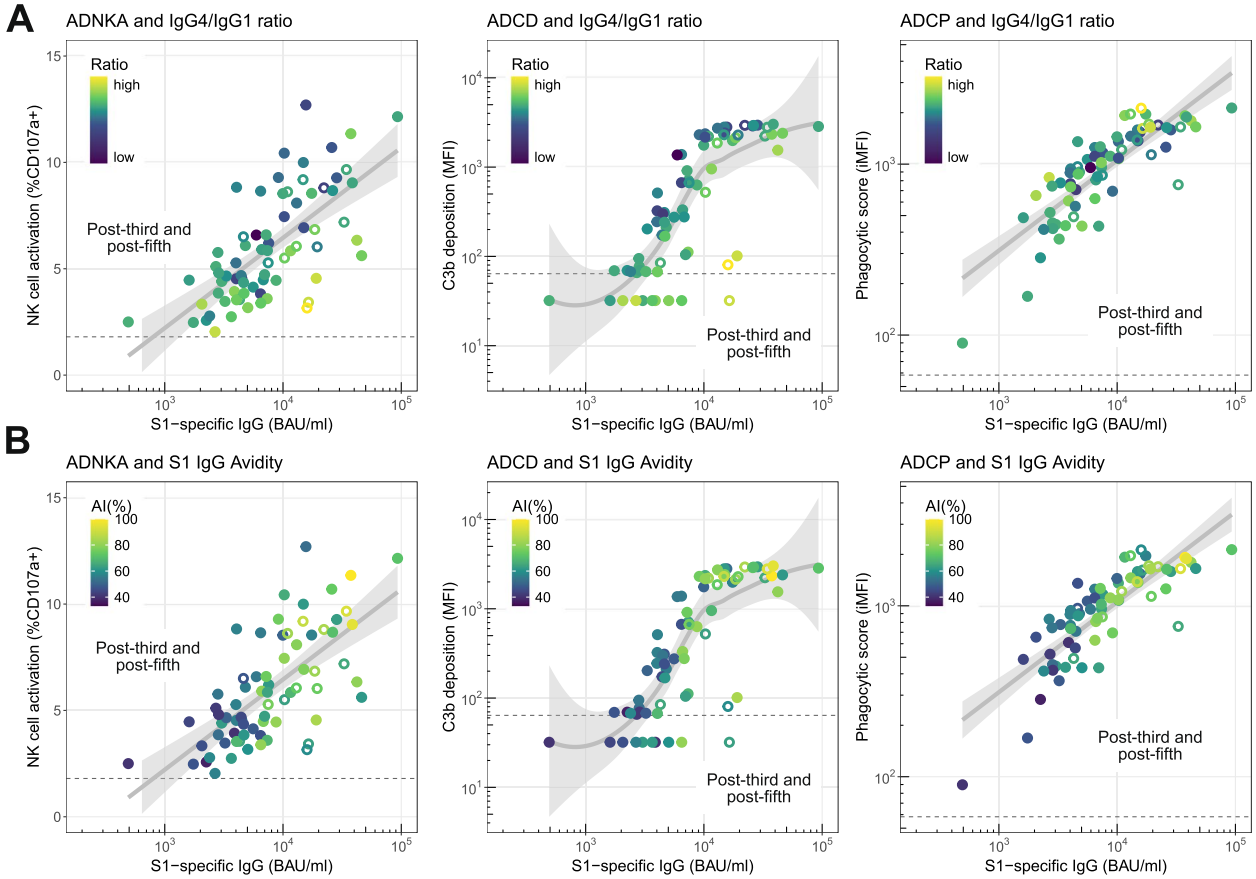

Fig. 6 Association between Fc-mediated effector functions and IgG4/IgG1 ratios or IgG avidity in older adults. The capacity of spike S1-specific serum antibodies to mediate ADNKA, ADCD and monocyte ADCP following mRNA vaccination in older adults ($n = 38$) at approximately 1 month after the third and fifth vaccinations relative to S1-specific total IgG levels at the same timepoint. Color scales indicate the IgG4/IgG1 ratio (**A**) or avidity index (**B**) of the SARS-CoV-2 spike S1-specific antibodies. Dotted lines indicate the level of the negative control (pre-pandemic serum sample). Solid grey lines were made using linear regression (straight lines) or locally estimated scatterplot smoothing (LOESS). SARS-CoV-2-infected individuals (based on N seropositivity) are indicated with open circles. ADCD, antibody-dependent complement deposition; ADCP, antibody-dependent cellular phagocytosis; ADNKA, antibody-dependent natural killer cell activation; BAU, binding antibody units; iMFI, integrated median fluorescence intensity; N, nucleoprotein

to total IgG concentration did not clearly associate with the IgG4/IgG1 ratio. For comparison, no obvious association between relative antibody functionality and avidity was observed, as higher avidity appeared mostly to associate with higher antibody concentrations (Fig. 6B).

Discussion

The induction of virus-specific IgG4 responses has previously been shown upon repeated COVID-19 mRNA vaccination in healthy younger adults [6–12], but it was unclear whether this would also occur in healthy older adults (≥65 years). In this study, we have shown that considerable class switching to IgG4 also occurs in older adults upon repeated mRNA vaccination and that IgG4 levels following the fifth vaccine dose even exceed those induced after the third dose. Furthermore, our data show that the increased ratio of IgG4/IgG1 following repeated vaccination associates with a reduced capacity of the virus-specific antibodies to mediate NK cell activation and complement deposition relative to total virus-specific IgG concentrations.

Previously, we have also observed a decline in antibody-dependent NK cell activation relative to antibody concentration over time following primary SARS-CoV-2 infection in children and adults [33]. Although we did not evaluate subclass levels in that study, the decline in that setting was likely due to waning of virus-specific IgG3 antibodies, which are known to be superior mediators of ADNKA but have a short half-life compared to other subclasses [19]. In addition, it is highly unlikely that significant amounts of IgG4 have been produced in response to a primary viral infection. In contrast, in the current study the decline in ADNKA relative to antibody concentration does not appear to relate to the presence of IgG3,

as the median levels for this subclass actually increase from the post-third to post-fifth vaccination timepoint. The potential role of IgG4 in the decline in ADNKA relative to antibody concentration is in line with the known functional characteristics of IgG4 [18, 19]. However, it is important to note that our data do not provide evidence for a causal relationship between changes in IgG4 levels and ADNKA. To address this remaining question, future studies should include IgG4 depletion experiments, the lack of which is a limitation of our study. It is furthermore possible that other antibody characteristics, for example differential glycosylation of the antibody Fc-tail or somatic hypermutation, have an important role in shaping the functional response upon repeated mRNA vaccination and these should be further investigated [8, 34–36].

The underlying factors that lead to IgG4 expression upon COVID-19 mRNA vaccination remain incompletely understood. There is increasing evidence that especially mRNA vaccines are prone to induce CSR to distal subclasses [6–9, 12], likely due to prolonged availability of the vaccine antigen as was shown in the past for IgG4 responses to honey bee venom in bee keepers [37]. IgG4 induction is however not limited to mRNA vaccination, as it has previously also been observed following four or five repeated diphtheria, tetanus, and acellular pertussis vaccinations (DTaP) in children [38, 39]. Interestingly, in line with wat has previously been observed in younger healthy adults [7, 8], our data suggest that infection prior to the start of vaccination reduces subsequent class switching to IgG4, although the group size is too small to draw any firm conclusions.

In our study populations, we observed that older adults show increased levels of virus-specific IgG4 compared to younger adults following the third vaccination. Since the participants of our studies received their vaccinations as part of the national COVID-19 vaccination campaign, we unfortunately could not control the type of vaccine or timing of administration. Consequently, for the third vaccination the vast majority of older adults received the Moderna vaccine at a median of 208 days after the second vaccination, while almost all younger adults were vaccinated with the Pfizer-BioNTech vaccine with a median vaccination interval of 162 days. The observed difference between younger and older adults might thus relate to differences in vaccine type, as it was previously found that the Moderna vaccine induces higher levels of IgG4 than the Pfizer-BioNTech vaccine, potentially due to its higher antigen dose [8]. Although we did not observe a clear separation between Moderna- and Pfizer-BioNTech-vaccinees after the third vaccination, the disbalance in group size makes it difficult to draw firm conclusions. In addition, the difference between younger and older adults might relate to discrepancies in the vaccination interval, as it has previously been suggested that IgG4 levels were increased with a longer interval between vaccination and breakthrough infection, allowing more time for ongoing B cell maturation and class switching [6]. Alternatively, older adults might actually be able to class switch more efficiently than younger adults, leading to an accumulation of IgG2 and IgG4 as was shown previously for adults compared to children [40]. Further research is needed to better understand the specific conditions leading to IgG4 switching.

Another question that remains unanswered concerns the origin of the IgG4-expressing B cells. Does class switching towards IgG4 occur immediately from IgM/IgD, or via for example IgG1- and IgG3-expressing B cells? As we and others observe a decrease in IgG3 levels following the third vaccination, it is tempting to speculate that this is the result of CSR of IgG3-expressing B cells to IgG4 [6]. More in-depth analysis including B cell receptor sequencing studies to determine the clonal origin of these virus-specific B cells is however required to prove this assumption. Another point regarding IgG3 (encoded by the most proximal Cγ region gene) is that following the decrease from post-second to post-third vaccination, we again observe an increase from post-third to post-fifth vaccination. Potentially, these increased IgG3 responses represent newly matured B cells responding to novel epitopes present in the bivalent booster vaccine (fifth vaccination), thereby providing a possible means of mitigating the induction of IgG4.

On a technical note, our data provide a clear representation of the development of each subclass individually, but concentrations cannot be compared directly between subclasses as no absolute concentrations were defined. For reference, previous research has shown that after three COVID-19 mRNA vaccinations in healthy adults, 19% to 45% of S1-specific IgGs are of the IgG4 subclass [6, 7]. Regarding the Fc-mediated effector function assays, we have used a single serum dilution that most optimally fits the window of quantification per assay. Because of the large difference in antibody concentration between the different timepoints and the inherently limited dynamic range of the assays however, some samples remain outside the upper and lower limits of quantification, especially for the ADCD assay. Despite this limitation, the presented data provide clear insights into the development of Fc-mediated effector functions upon repeated COVID-19 mRNA vaccination in older adults.

Conclusions

At present, it remains unclear to what extent (if any) the occurrence of virus-specific IgG4 will affect vaccine effectiveness, which thus far appears to remain

sufficient [41, 42]. As expected based on earlier work, our study confirms that increased levels of IgG4 associate with reduced Fc-mediated effector functionality [6, 19]. Considering that in addition to virus neutralization (which is not affected by IgG4 induction), there is increasing evidence suggesting that these Fc-mediated effector functions contribute to immunological protection from disease [20–28, 43], one might expect that IgG4 induction is not beneficial for vaccine effectiveness. Alternatively, IgG4 might play a beneficial role in reducing the inflammatory potential of continuously increasing IgG levels upon repeated vaccination [18]. Either way, it will be imperative to follow this development in larger population studies in which breakthrough infections and symptoms are duly recorded, especially in light of potential additional booster vaccinations.

In conclusion, we have shown that older adults, like younger individuals, are inclined to develop IgG4 responses upon repeated COVID-19 mRNA vaccination and that increased IgG4 levels associate with a relative reduction in Fc-mediated effector functionality. Additional research is needed to better understand the mechanisms underlying these class switch events and their potential implications for vaccine effectiveness. Such knowledge is vital for the future design of optimal vaccination strategies in the ageing population.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12979-024-00466-9.

Supplementary Material 1.

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Authors' contributions
A.G., Data curation, Formal Analysis, Investigation, Visualization, Writing – original draft; M.V., Data curation, Formal Analysis, Writing – review & editing; I.M., Investigation; M.Z.B., Data curation, Investigation, Writing – review & editing; R.B., Conceptualization, Formal Analysis, Methodology, Supervision, Writing – review & editing; A.B., Conceptualization, Project administration, Resources, Supervision, Writing – review & editing; P.K., Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The Medical Research Ethics Committee Utrecht approved of both studies. The studies were carried out in accordance with the declaration of Helsinki and written informed consent was obtained from all participants. For older adults, recruitment focused on participants who previously participated in the Immune System and Ageing substudy of the Doetinchem Cohort Study (EudraCT: 2021–002363-22) [44, 45]. Younger adult participants were enrolled as part of another prospective observational cohort study (EudraCT: 2021–001357-31) [31]. Both cohort studies were designed to follow immune responses to COVID-19 vaccination.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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