





ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

Anti-TNF therapy impairs both short- and long-term IgG responses after repeated vaccination

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Abstract

Background: Recently, it has been questioned whether vaccination of patients with inflammatory (auto)immune diseases under anti-tumor necrosis factor (TNF) treatment leads to impaired vaccine-induced immune responses and protection against breakthrough infections. However, the effects of TNF blockade on short- and long-term immune responses after repeated vaccination remain unclear. Vaccination studies have shown that initial short-term IgG antibodies (Abs) carry highly galactosylated and sialylated Fc glycans, whilst long-term IgG Abs have low levels of galactosylation

Abbreviations: Ab, antibody; COVID-19, coronavirus disease 2019; DMARD, disease-modifying anti-rheumatic drug; GC, germinal center; GlcNAc, N-acetylglucosamine; PC, plasma cell; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TNF, tumor necrosis factor.

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and sialylation and are most likely generated by long-lived plasma cells (PCs) derived primarily from the germinal center (GC) response. Thus, IgG Fc glycosylation patterns may be applicable to distinguish short- and long-term vaccine responses after repeated vaccination under the influence of anti-TNF treatment.

Methods: We used COVID-19 vaccination as a model to investigate vaccine-induced IgG subclass levels and Fc glycosylation patterns, B cell subsets, and effector functions of short- and long-term Ab responses after up to three vaccinations in patients on anti-TNF or other immunosuppressive treatments and in healthy individuals. Using TriNetX, a global healthcare database, we determined the risk of SARS-CoV-2 breakthrough infections in vaccinated patients treated with anti-TNF or other immunosuppressive drugs.

Results: Anti-TNF treatment reduced the long-term abundance of all anti-S IgG subclasses with low levels of galactosylation and sialylation. Re-activation of potential memory B cells initially generated highly galactosylated and sialylated IgG antibodies, which were progressively reduced after each booster dose in anti-TNF-treated patients, especially in the elderly. The reduced short- and long-term IgG (1) levels in anti-TNF-treated patients correlated with diminished functional activity and an increased risk for the development of COVID-19.

Conclusions: The data suggest that anti-TNF treatment reduces both GC-dependent long-lived PCs and GC-dependent memory B cell-derived short-lived PCs, hence both the long- and short-term IgG subclass responses, respectively, after repeated vaccination. We propose that anti-TNF therapy, especially in the elderly, reduces the benefit of booster vaccination.

KEYWORDS

antibody, anti-TNF treatment, COVID-19, germinal center, IgG, IgG glycosylation, IgG4, inflammatory diseases, long-lived plasma cell, memory B cell, mRNA vaccine, SARS-CoV-2, short-lived plasma cell, TriNetX, vaccination

1 | INTRODUCTION

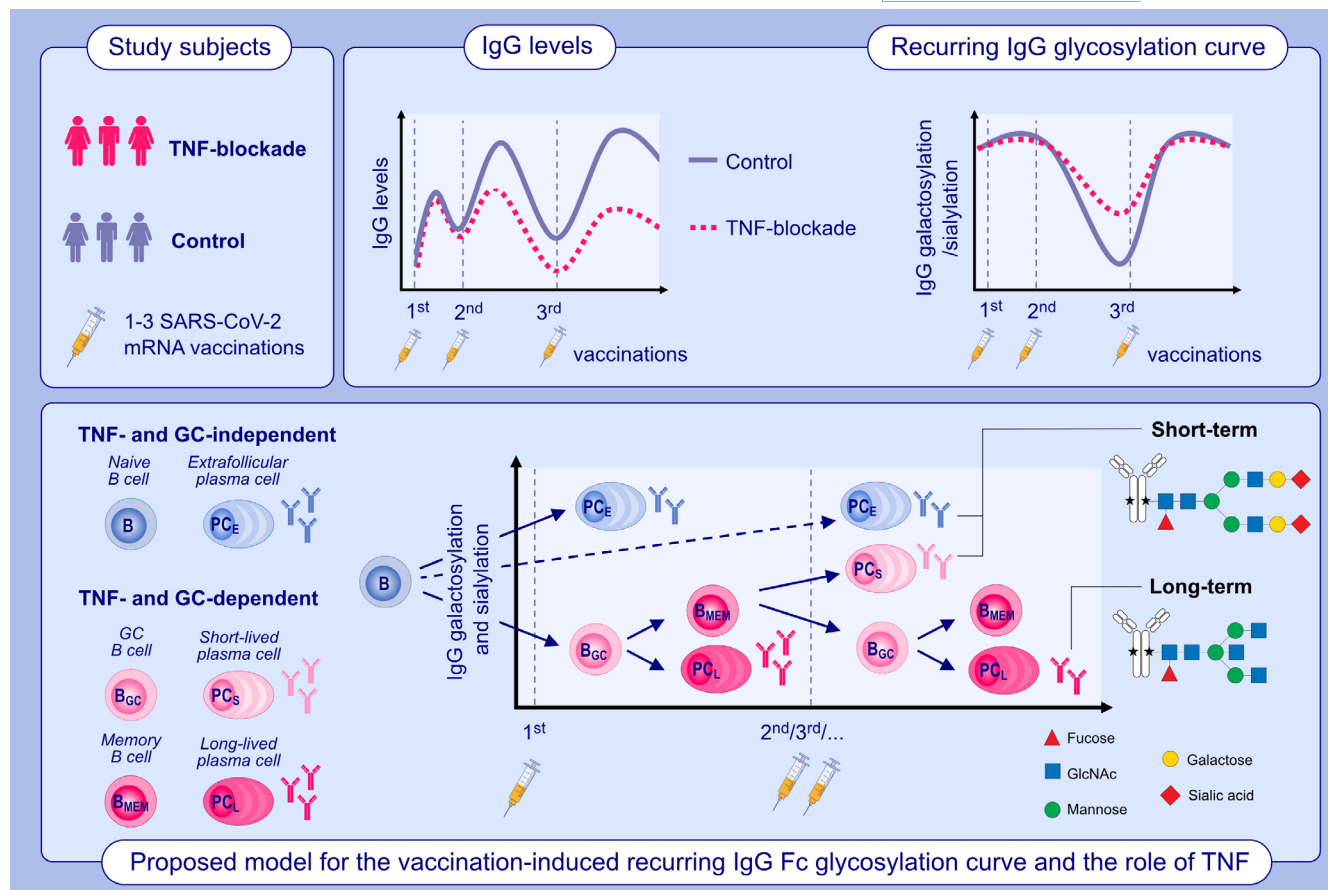
A recent study suggests that treatment of patients with inflammatory (auto)immune diseases with tumor necrosis factor (TNF)-blocking biologics may potentiate breakthrough infections following vaccination against pathogens.¹ A key question is how TNF blockade affects the outcome of vaccine-induced immune responses and consequently, the incidence of breakthrough infections.

The general idea of T cell-dependent immune responses to infection and vaccination is the induction of an initial adequate short-term IgG antibody (Ab) response that declines over time to a more stable long-term response.^{2–9} The rapid short-term IgG response is mainly produced by extrafollicular-derived short-lived plasma cells (PCs). In contrast, the long-term response is increasingly characterized by high-affinity IgG Abs originating from germinal center (GC)-derived long-lived PCs. GCs are the hotspots of B cell somatic hypermutation and affinity maturation and are the main route for the generation of memory B cells and long-lived PCs.^{9–14} After booster vaccination, a more potent short-term IgG⁺ PC response is induced, peaking at day 7, mainly by re-activated GC-derived memory B cells, which again declines over time. Notably, re-activation of extrafollicular-derived memory B cells may also occur after booster vaccination.¹⁵

Our recent studies have shown that blocking TNF in inflammatory (auto)immune patients reduces the level, avidity and neutralizing capacity of long-term antiviral spike (S) IgG Abs after two BNT162b2 (BioNTech/Pfizer) mRNA vaccinations¹⁶ against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).^{17–19} The data suggest that blocking of TNF reduces the long-term IgG response generated by GC-derived long-lived PCs,¹⁹ a finding previously reported in other murine and human studies.^{20–23} In addition, anti-TNF treatment has been shown to reduce the generation of GC-derived memory B cells in patients with rheumatoid arthritis.²⁴ Notably, anti-TNF treatment had only modest effects on T cell independent vaccination.²³ However, the effects of anti-TNF treatment on the generation of T cell-dependent extrafollicular PC and Ab responses, memory B cells, and memory B cell-derived PC and Ab responses after vaccination are less clear.

Whether the long-term IgG deficit under anti-TNF treatment can be compensated by increasing the number of booster vaccinations is a matter of debate; therefore, it is important to explore how anti-TNF treatment affects both the long- as well as short-term IgG responses after booster vaccinations.

Recently, we and others have shown that vaccine-induced initial short-term and long-term IgG Ab responses exhibit distinct Fc



GRAPHICAL ABSTRACT

Human vaccination study discovers that booster vaccinations induce a recurring IgG antibody Fc glycosylation curve across all IgG subclasses. Proposed model: initial extrafollicular B cell activation as well as re-activation of GC-derived IgG⁺ memory B cells generate short-lived PCs that produce highly galactosylated and sialylated short-term IgG, whereas GC-derived long-lived PCs produce low galactosylated and sialylated long-term IgG. Anti-TNF therapy reduces both GC-derived pathways, thereby reducing the benefit of repeated booster vaccinations in both the short and long-term.

Abbreviations: GC, germinal center; GlcNAc, N-acetylglucosamine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TNF, tumor necrosis factor.

glycosylation phenotypes at the N-glycosylation site at Asn 297.^{25–27} This highly conserved site carries a complex type N-glycan characterized by a pentasaccharide core structure, that can be further modified with a core fucose, a bisecting N-acetylglucosamine (GlcNAc) and one or two galactose residues, each of which can be further capped by a sialic acid (Figure 1A).^{28,29}

Studies in mice and humans have shown that T cell-dependent vaccine-induced short-term antigen-specific IgG Abs are highly galactosylated and sialylated and are associated with short-lived extrafollicular-derived PC responses. In contrast, long-term IgG Abs are low in galactosylation and sialylation and are linked to GC-derived long-lived PCs.^{25–27} Consequently, IgG Fc glycosylation patterns may reflect the development of short- and long-term IgG responses.

We propose that anti-TNF therapy reduces the generation of long-term anti-S IgG Abs with low galactosylation and sialylation, potentially produced by GC-dependent long-lived PCs, across all IgG subclasses. In addition, anti-TNF treatment may reduce initial IgG subclass responses following booster vaccinations generated by GC-derived memory B cells. Accordingly, the degree of galactosylation and sialylation of anti-S IgG subclass Abs shortly after repeated

vaccination, high or low, may indicate whether initial memory B cell-derived PCs are short- or long-lived, respectively.

To address these ambiguities, we performed a holistic, time-resolved analysis including anti-S IgG subclass levels, Fc glycosylation patterns, and corresponding Fc-mediated functional activities after up to three vaccinations against SARS-CoV-2 in healthy individuals and in patients receiving anti-TNF or other immunosuppressive therapies.

2 | MATERIALS AND METHODS

2.1 | Study cohorts

2.1.1 | Cohort of patients treated with anti-TNF or other DMARDs and healthy controls, all vaccinated up to three times with SARS-CoV-2 mRNA vaccines

Recruitment of patients with chronic inflammatory immune diseases (Systemic Lupus Erythematosus (SLE), Rheumatoid arthritis (RA), Psoriasis (Pso), Psoriasis arthritis (PsoA), Spondyloarthritis

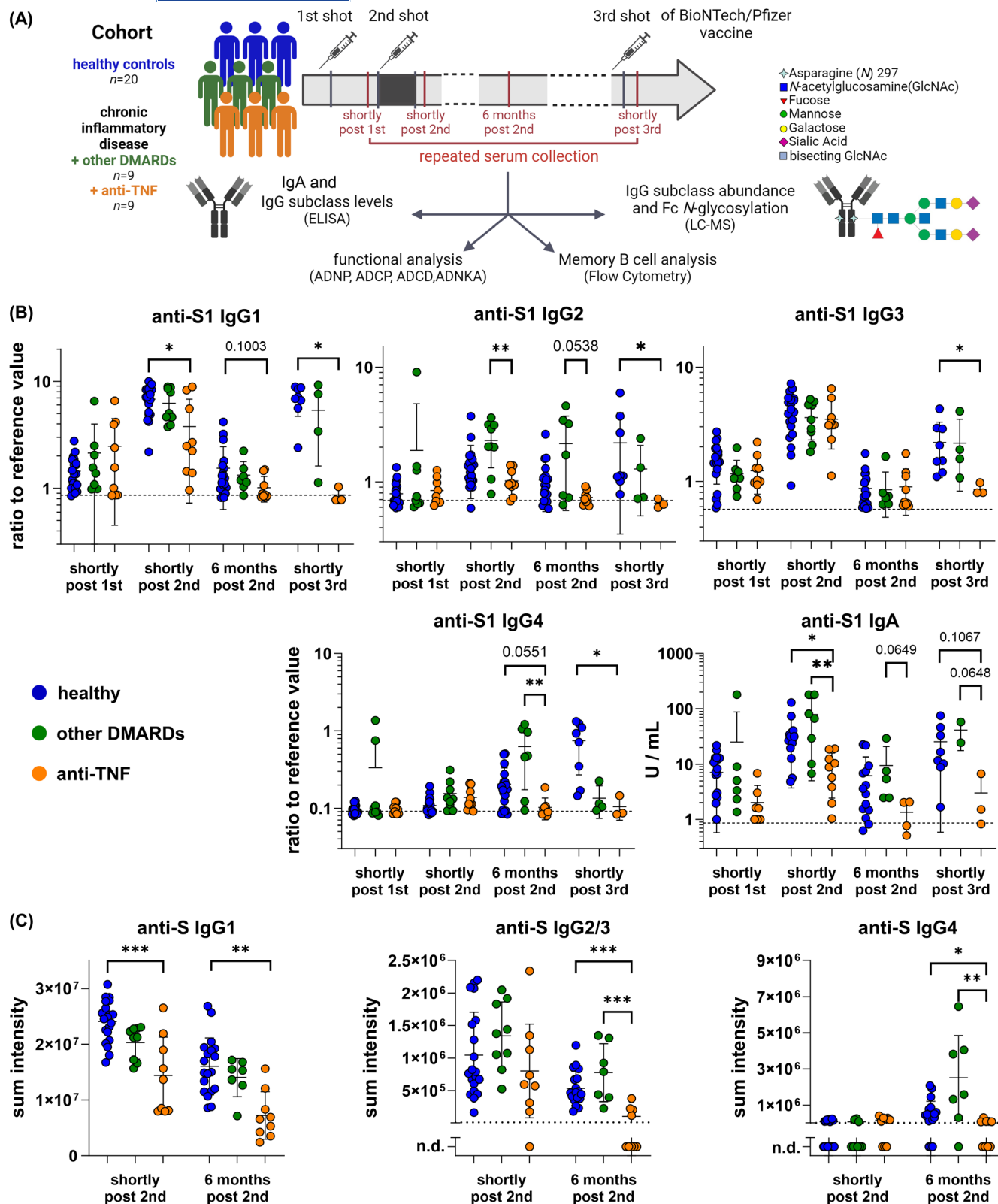


FIGURE 1 Anti-S(1) serum IgG subclass and IgA levels in anti-TNF-treated vaccinees. (A) Cohort description (created with biorender.com). (B) Vaccination-induced anti-S1 serum IgG1-4 and IgA levels detected by ELISA of the three groups indicated. Four different time points were analyzed: (i) shortly (3–5 weeks) post first, (ii) shortly (1 week) post second, (iii) long (6 months) post second, and (iv) shortly (1 week) post third mRNA vaccination. Dashed lines: Anti-S1 IgG subclass levels of unvaccinated healthy (negative) controls. (C) Summed anti-S serum glycopeptide intensities of the IgG subclasses as detected by LC-MS (IgG2 and IgG3 glycopeptides were not distinguished by our method). Dotted line: Cut-off threshold. Statistics: Kruskal-Wallis test for each time point, * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

(SpA), Crohn's disease (Crohn's), Sarcoidosis, Mixed Connective Tissue Disease (MCTD/Sharp) treated with TNF inhibitors or other DMARDs (disease-modifying anti-rheumatic drugs: anti- $\alpha\beta 7$ integrin, anti-BlyS, anti-IL-17, hydroxychloroquine (HCQ), sulfasalazine (Sulfa), or steroids) and healthy subjects, as well as sampling, and immunologic and clinical characterization have been described previously.^{17–19} The slightly modified cohort consists of 9 patients receiving TNF inhibitors, 9 patients receiving other DMARDs (one of these patients was untreated), and 20 healthy individuals, all between the ages of 24 and 57 years (Table S1). SARS-CoV-2 pre-infection prior to the first vaccination was excluded by serology (anti-SARS-CoV-2 nucleocapsid protein (NCP) IgG enzyme-linked immunosorbent assay (ELISA)). The three groups were matched for age resulting in median ages of 43 (TNF inhibitors, range from 33 to 54), 46 (other DMARDs, range from 24 to 53), and 33.5 (healthy controls, range 24–57) years. All subjects received the mRNA vaccine BNT162b2 for the first two vaccinations. The second vaccination was administered 21 or 35 days after the first vaccination. Fifteen individuals received a third dose of BNT162b2, the timing of which was more variable. Samples were collected (i) before the first vaccination, (ii) shortly (3 to 5 weeks) after the first vaccination (just before the second dose), (iii) shortly (1 week), (iv) 6 weeks, and (v) long (6 months) after the second vaccination, and (vi) shortly (1 week) after the third vaccination.

2.1.2 | Healthy cohort to investigate short-term anti-S IgG subclass responses following third SARS-CoV-2 vaccination

We analyzed 32 blood serum samples from 22 SARS-CoV-2 naïve vaccinees (no history of SARS-CoV-2 infection prior to first vaccination (excluded by anti-SARS-CoV-2 NCG IgG ELISA (data not shown))) and 4 SARS-CoV-2 pre-infected vaccinees (Table S2). The 26 subjects (18 females, 8 males; median age 31 (21–63)) were vaccinated with different vaccine combinations against SARS-CoV-2 including the mRNA vaccines BNT162b2 and mRNA-1273, and the adenovirus-based vaccine AZD1222. Out of the 32 samples, 20 samples were collected long after the second vaccination and before the third vaccination and 12 samples were collected shortly after the third vaccination. Six of these subjects were analyzed both before and after the third vaccination. Those who received the mRNA vaccine first received their second dose between days 21 and 45, and those who received the adenovirus-based vaccine first received their second dose between days 70 and 84. The timing of the third dose was more variable. No other selection criteria were used, and both participants and samples were randomly selected based on availability. All subjects were recruited at the University of Lübeck and the University Medical Center Schleswig-Holstein (Lübeck, Northern Germany).

For both study cohorts, blood samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki and a protocol approved by the local ethics committee (cohort 1: Ethics Committee of the Christian-Albrecht

University Kiel, Germany (D409/21); cohort 2: Ethics Committee of the University of Lübeck, Germany (20–123)). The vaccinations were performed by local physicians or vaccination centers independently of the studies. The anti-TNF study is registered at DRKS (DRKS00024214).

2.2 | Serum antibody detection (ELISA)

Serum samples were collected and the levels of anti-S1 (the extracellular portion of S containing the angiotensin-converting enzyme (ACE) 2 receptor binding domain)³⁰ IgG subclasses were analyzed by ELISA (enzyme-linked immunosorbent assay) protocols established in our laboratory; anti-S1 IgG1–4 HL (Hansestadt Lübeck)-1 ELISA protocols.^{27,31} Briefly, 96-well ELISA plates were coated with 4 μ g/mL of SARS-CoV-2-S1 antigen (ACROBiosystems, Newark, DE 19711, USA; #S1N-C52H3). Coated plates were washed with 0.05% Tween 20 in PBS to remove unbound antigen and incubated with serum (diluted 1/1000 with PBS for IgG1 and 1/100 or 1/1000 for IgG2–4 detection). Bound Abs were detected with horseradish peroxidase (HRP)-coupled monoclonal anti-human IgG1 (clone HP-6001), IgG2 (clone HP-6014), IgG3 (clone HP-6050), or IgG4 (clone HP-6025)-specific Abs purchased from Southern Biotech (Birmingham, AL, USA) and 3,3',5,5'-tetramethylbenzidine substrate (BD Biosciences, San Diego, CA, USA). Secondary Ab specificity has recently been demonstrated.³¹ The ratio to a reference value was calculated by dividing the sample OD value by the OD value of an internal reference sample. Anti-S1 IgA serum levels were determined by ELISA using a standard curve according to the manufacturer's instructions (Aeskulisa, Aeskulap) and are expressed in units per mL (U/mL). Some of the data have been published recently.¹⁸

2.3 | Antibody Fc glycosylation analysis by mass spectrometry

Serum IgG subclass Fc glycosylation analysis was performed as recently described.²⁶ Briefly, anti-S Abs were affinity-captured from sera using recombinant, in-house produced trimerized spike protein-coated microtitration plates (Thermo Fisher Scientific, Roskilde, Denmark).²⁶ Ab elution was performed using 100 mM formic acid.²⁶ Eluates from anti-S Ab affinity purification were dried by vacuum centrifugation and subjected to tryptic cleavage followed by nano liquid chromatography (LC)-mass spectrometry (MS) analysis.²⁶

2.4 | LC-MS data processing

IgG subclass-specific glycopeptides were identified based on their mass-to-charge ratio and specific migration position in nanoLC-MS³² and assigned to IgG1, IgG2/3, and IgG4 Fc glycosylation sites. Trypsinized IgG2 and IgG3 Fc glycopeptides were not distinguishable by our method.^{32,33} Raw LC-MS spectra were converted to mzXML files.

LaCyTools, an in-house developed software, was used for the alignment and targeted extraction of raw data.³⁴ Alignment was performed based on the average retention time of at least three highly abundant glycoforms. Inclusion of analytes for targeted extraction of the 2⁺ and 3⁺ charge states was based on manual annotation and on literature reports. Inclusion of an analyte in the final data analysis was further based on quality criteria including signal-to-noise (>9), isotope pattern quality (less than 25% deviation from the theoretical isotope pattern), and mass error (within ± 20 ppm range), resulting in a final list of 14 analytes for IgG1 and 7 glycopeptides for both IgG2/3 and IgG4 (Table S3). The (absolute) sum intensity of each IgG subclass was determined by summing the corresponding glycopeptide intensities. The relative intensity of each glycopeptide in the final analyte list was calculated by normalizing the glycopeptide intensity to the summed intensity of all glycopeptides of the corresponding subclass (total area normalization). The normalized intensities were used to calculate the glycosylation traits fucosylation, bisection, galactosylation, and sialylation of each subclass (Table S4). Serum samples with low levels of anti-S IgG2/3 and/or IgG4 did not always result in sufficient MS signal intensity and were then excluded from the analysis or set "0." We were unable to detect any afucosylated anti-S IgG2/3 or anti-S IgG4 glycopeptides above the detection threshold. Data preprocessing and curation was performed in a dedicated in-house developed software called GlycoDash (<https://github.com/Center-for-Proteomics-and-Metabolomics/glycodash>).

2.5 | Functional assays

The following functional assays were recently described³⁵:

2.5.1 | Antibody-dependent neutrophil phagocytosis (ADNP)

To determine the phagocytosis score of primary human neutrophils, SARS-CoV-2 Spike S1 + S2 (D614G) trimer protein (Sino Biologicals) was biotinylated using an NHS-Sulfo-LC-LC kit according to the manufacturer's instructions (Thermo Fisher). Biotinylated antigens were coupled to fluorescent neutravidin beads (Thermo Fisher) and incubated with 1:50 diluted serum to allow immune complex formation. Primary cells were isolated from whole blood lysed in ammonium chloride-potassium buffer from healthy donors and incubated with immune complexes for 1 h at 37°C. Neutrophils were stained for the surface expression marker CD66b (BioLegend Cat# 305112, RRID:AB_2563294) and fixed in 4% paraformaldehyde. Samples (technical duplicates) were analyzed using an ID7000 Spectral Cell Analyzer (Sony).

2.5.2 | Antibody-dependent cell phagocytosis (ADCP)

Biotinylated S-antigen (Sino Biologicals) was coupled to FluoSphere NeutrAvidin beads (Thermo Fisher Scientific) and incubated with

10 μ L of 1:100 diluted serum for 2 h at 37°C to form immune complexes. THP-1 monocytes (American Type Culture Collection) were added to the beads, incubated for 16 h at 37°C, washed, and fixed with 4% paraformaldehyde. Samples (technical duplicates) were analyzed using an ID7000 Spectral Cell Analyzer (Sony).

2.5.3 | Antibody-dependent complement deposition (ADCD)

For the complement deposition assay, biotinylated S-antigen (Sino Biologicals) was coupled to FluoSphere NeutrAvidin beads (Thermo Fisher Scientific) and incubated with 10 μ L of 1:10 diluted serum samples for 2 h at 37°C. The washed immune complexes were incubated with guinea pig complement in GVB++ buffer (Hözel) for 20 min at 37°C. The complement reaction was stopped with EDTA-containing phosphate-buffered saline, and C3 deposition on the beads was stained with a 1:100 diluted anti-guinea pig C3-FITC Ab (Th Geyer, Cat# 10823719, RRID:AB_2334913). Samples (technical duplicates) were analyzed using an ID7000 Spectral Cell Analyzer (Sony).

2.5.4 | Antibody-dependent NK cell activation (ADNKA)

To determine Ab-dependent NK cell activation, ELISA plates (high binding; Greiner) were coated with S-antigen (Sino Biological) and incubated overnight at 4°C with 50 μ L of 1:30 diluted serum samples. NK cells were isolated from buffy coats of healthy donors using the RosetteSep NK cell enrichment kit and SepMate50 tubes (STEMCELL Technologies). The isolated NK cells were stimulated with recombinant human interleukin-15 (1 ng/mL, STEMCELL Technologies) at 37°C overnight. The next day, the NK cells were added to the coated wells and incubated together with anti-human CD107a BV605 (BioLegend Cat# 328634, RRID:AB_2563851), brefeldin A (Sigma-Aldrich), and monensin (BD Biosciences) for 5 h at 37°C. Cells were then surface stained with anti-CD3-APC-Cy7 (BioLegend Cat# 300426, RRID:AB_830755) and anti-CD56-PE-Cy7 (BD Biosciences Cat# 335791, RRID:AB_399970). Cells were fixed and permeabilized with FIX & PERM Cell Permeabilization Kit (Thermo Fisher Scientific) and stained for intracellular markers using anti-human MIP-1 β -BV421 (BD Biosciences Cat# 562900, RRID:AB_2737877) and anti-human IFN γ -PE (BD Biosciences Cat# 554701, RRID:AB_395518). NK cells were defined as CD3⁺CD56⁺, and the frequencies of degranulated (CD107a⁺), IFN γ ⁺, and MIP-1 β ⁺ NK cells were determined using an ID7000 Spectral Cell Analyzer (Sony). Each sample was tested with NK cells from two different donors (biological duplicates) and the values reported are the average of the two measurements.

The functional data were additionally normalized to the summed intensity levels of anti-S IgG (IgG1 + IgG2/3 + IgG4) determined by LC-MS.

2.5.5 | Fc-mediated virus reduction assay

To test the contribution of Fc biology to antiviral activity, we adapted a previously published virus neutralization assay.³⁶ The day before the assay, African green monkey kidney Vero-B4 cells expressing the ACE 2 receptor were plated in 96-well plates at a density of 20,000 cells/well, resulting in a confluent monolayer on the day of the assay. Diluted serum samples of healthy or anti-TNF-treated subjects 1 week or 6 months after the second vaccination or PBS controls were mixed with recombinant vesicular stomatitis virus (VSV) pseudotypes encoding green fluorescent protein (GFP) and expressing SARS-CoV-2 spike proteins (D614G) in equal volumes (330 ffu/well) and incubated at 37°C for 1 h. During incubation, neutrophils were prepared as described for the ADNP assay. Neutrophils (50,000 cells/well) or medium (controls) were then added to the antibody-virus mixture for an additional hour at 37°C, and all were transferred to pre-plated Vero monolayers and incubated at 37°C for an additional 24 h. The number of infected cells (GFP+) were identified by full-well wide-field fluorescence microscopy using an Olympus FV3000 microscope and quantified using ImageJ. All serum samples were tested in triplicate with or without the addition of neutrophils. In addition, the neutrophil assay was repeated with cells from two independent blood donors. Mean values were calculated from the number of GFP+ cells. The area under the curve (AUC) of the number of infected cells versus dilution factor was calculated for each antibody sample in the presence or absence of neutrophils.

2.6 | S1-specific memory B cell and DN2 B cell analysis

Flow cytometry analysis of S1-specific B cell subsets was recently described (Geisen et al, 2022). Briefly, PBMCs were isolated from EDTA blood by density gradient centrifugation within 3 h after blood collection (Biocoll, Bio & SELL GmbH). Subsequently, 4×10^6 PBMCs were incubated with his-tagged S1 protein (kind gift from Michael Hust, Technical University Braunschweig, Germany or from Euroimmun, Germany) and then stained with pre-mixed Abs (anti-CD19-PerCP-Vio-700 (REA657, Miltenyi Biotec), anti-CD20-PE-Vio770 (REA780, Miltenyi Biotec), anti-CD3-PacificBlue (OKT3, Biolegend), anti-CD14-PacificBlue (M5E2, Biolegend), anti-CD27-APC (M-T271, Biolegend), anti-HIS-PE (JO95-G46, Biolegend), anti-IgD-APC-Vio770 (REA740, Miltenyi Biotec), anti-IgM-BV570 (MHM-88, Biolegend), anti-IgA-FITC (M24A, Merck/Millipore)), and analyzed using a MACSQuant 16 cytometer (Miltenyi Biotec). CD14⁺ and CD3⁺ cells were excluded from the analysis. S1-specific memory B cells were identified as CD19⁺ CD20⁺ CD27⁺. DN2 B cells were identified as CD19⁺ CD20⁺ CD27⁻ IgD⁻. IgG⁺ memory B cells were identified as IgD⁻ IgM⁻ IgA⁻. A second staining was performed to calculate immune cells per unit blood volume. 50 µL of whole blood was stained (anti-CD3-Pacific Blue (Biolegend), anti-CD14-FITC (REA599, Miltenyi Biotec), anti-CD4-PE (Vit4, MiltenyiBiotec), anti-CD19-PerCP-Vio700 and anti-CD45-APC-Vio770 (H130,

Biolegend)), lysed (Red Blood Lysis, BD) and measured on the MACSQuant 16. Cell counts per 50 µL of blood for each sample were used to calculate all other cell counts from the PBMC staining. Flow Cytometry analysis was performed using FlowJo v10.9.0 (BD Biosciences) and Cytolution Platform v1.1.0 (Cytolytics).

2.7 | TriNetX database analysis

2.7.1 | Study design and database

A population-based retrospective cohort study with propensity score matching was conducted according to previously published protocols.^{37–39} Specifically, data from electronic medical records (EMRs) were retrieved (using natural language processing) from the TriNetX Global Collaborative Network, which at the time of analysis in 2023 included over 127 million EMRs from 106 health care organizations (HCOs). The following two groups were retrieved: (i) EMRs with a diagnosis of Crohn's disease (CD), ulcerative colitis (UC), rheumatoid arthritis (RA), or psoriasis (Pso) and two vaccinations with a SARS-CoV-2 mRNA vaccine treated with a TNF inhibitor but not treated with an $\alpha 4\beta 7$ integrin inhibitor or methotrexate (MTX), or, (ii) CD, UC, RA, Pso EMRs with a second SARS-CoV-2 mRNA vaccination treated with an $\alpha 4\beta 7$ integrin inhibitor (vedolizumab) or methotrexate (MTX) but not treated with a TNF inhibitor. Further details of this study are described in the Supplementary Methods and Tables S6 and S7.

2.8 | Statistical analysis

Statistical analyses were performed using GraphPad Prism v9.0 and v10.0 (GraphPad, La Jolla, CA). Differences between groups at a given time point were assessed by the Mann–Whitney or the Kruskal–Wallis tests. Confidence intervals were presented at the 95% confidence level. Spearman or Pearson correlations with a 95% confidence interval were used to assess the relationship between variables. *p*-values were considered significant as follows: *, **, ***, ****: *p*-value < .05, .01, .001, and .0001, respectively.

3 | RESULTS

3.1 | Anti-TNF treatment weakens long- and short-term anti-S IgG subclass responses following booster vaccination

Recent studies have provided details on IgG and IgA immune responses following SARS-CoV-2 vaccination. The new mRNA vaccines BNT162b2 (BioNTech/Pfizer)¹⁶ and mRNA-1273 (Moderna)⁴⁰ induce a strong initial anti-S IgG1 Ab response, accompanied by lower IgG3 and IgA responses, all of which decline over time.^{14,26,27,31,41} The mRNA vaccines also induce a low but persistent anti-S IgG2

response.^{14,27} In addition, unlike AstraZeneca's adenovirus-based vaccine ChAdOx1 nCoV-19 (AZD1222), the mRNA vaccines induce late-emerging anti-S IgG4 Abs after two vaccinations (including heterologous vaccinations starting with AZD1222), which further increase upon a third mRNA booster.^{14,27,42}

All three vaccines have been shown to induce substantial GC responses, GC-derived memory B cells, and long-lived PCs.^{4,6-8,13,14} In addition, it has been suggested that the late-emerging anti-S IgG4 Abs arise upon isotype/subclass switching in persistent GC responses.¹⁴

To study the effect of anti-TNF treatment, we compared the anti-S1 IgG subclass levels between anti-TNF-treated patients with inflammatory diseases, patients treated with other disease-modifying anti-rheumatic drugs (DMARDs), and healthy individuals, all of whom received up to three doses of the mRNA vaccine BNT162b2 (Figure 1A).¹⁷⁻¹⁹

Shortly after the first vaccination, when activation of naïve B cells determines the short-term antibody response, anti-S IgG subclass and IgA levels were comparable among the three groups

(Figure 1B), suggesting that the initial extrafollicular PC response was not diminished in the anti-TNF-treated patients. However, the short-term antibody response in the anti-TNF-treated group was significantly reduced, or at least trended to be reduced, with each additional vaccination compared to both the healthy group and the group treated with other DMARDs. Of note, the anti-TNF-treated group had only three samples shortly after the third vaccination.

The 6-month long-term anti-S IgG1, IgG2, IgG4, and IgA levels after the second vaccination in the anti-TNF-treated vaccinees were also significantly or trending reduced compared to the other two groups.

Anti-S IgG subclass levels and Fc N-glycosylation patterns 1 week and 6 months after two vaccinations were then quantified by nanoLC-MS (Table S1). The resulting summed glycopeptide intensities of each IgG subclass were used to quantify IgG subclass levels, which were comparable to the ELISA results (Figure 1C).

When the short-term anti-S IgG subclass and IgA levels after the second vaccination were stratified by age (all vaccinees were

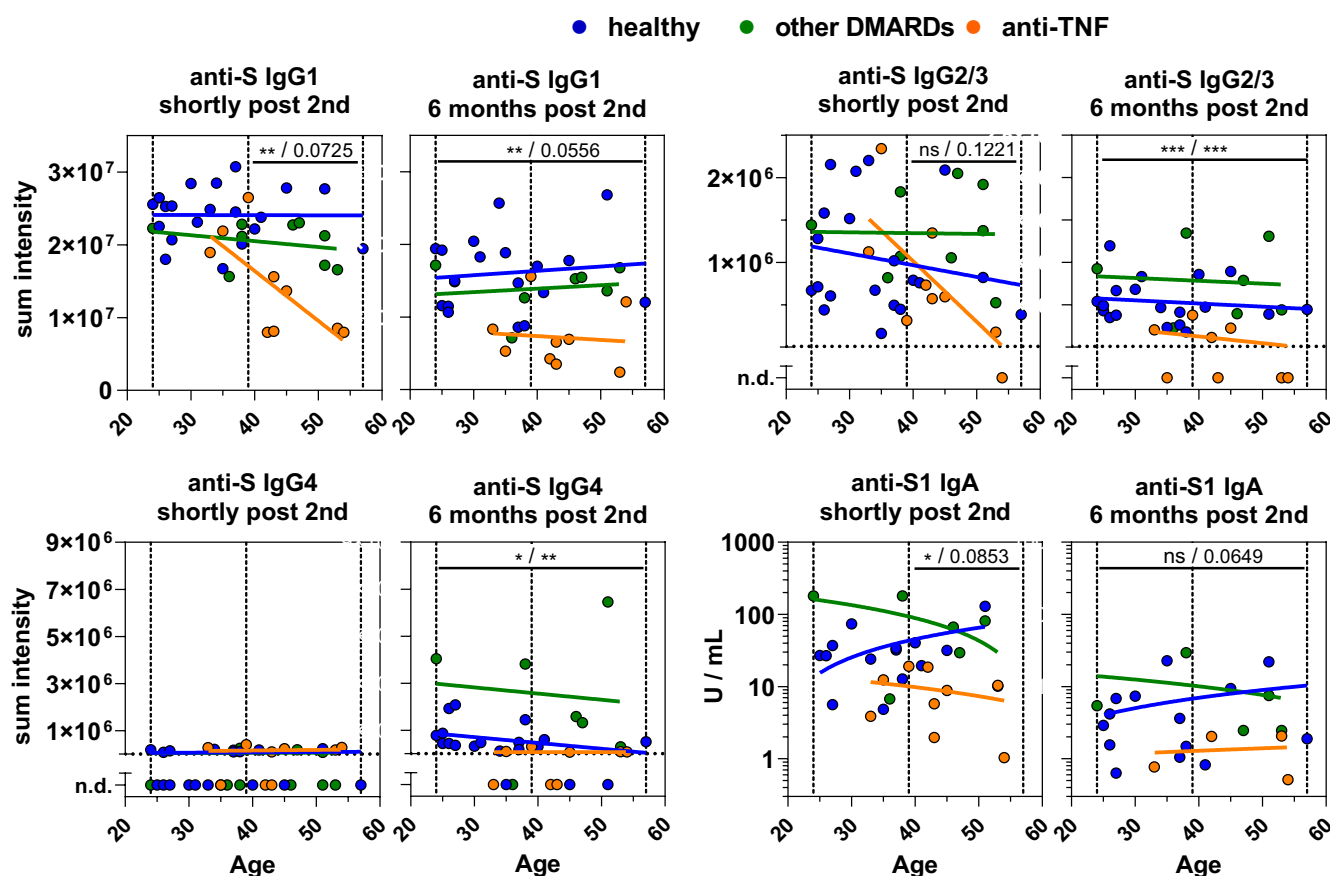


FIGURE 2 Anti-S(1) serum IgG subclass and IgA levels in anti-TNF-treated vaccinees stratified by age. Summed anti-S serum IgG subclass glycopeptide intensities as detected by LC-MS and anti-S1 IgA levels detected by ELISA shortly (1 week) post second and long (6 months) post second mRNA vaccination of the three groups are shown. Antibody levels were plotted against age and two different age groups were additionally examined: (i) 24–39 years and (ii) 40–57 years, indicated by the vertical dashed lines. Dotted line: Cut-off threshold. Statistics: Kruskal-Wallis test for the 40–57 years subgroup or total group as indicated, * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. The first value compares the healthy group with the anti-TNF-treated group; the second value compares the other DMARD-treated group with the anti-TNF-treated group. Colored lines indicate simple linear regression.

24–57 years old), we observed the reduced levels in the anti-TNF-treated group particularly in the older 40–57-year-old subgroup (Figure 2; Figure S1). In this older subgroup of anti-TNF-treated vaccinees, antibody levels were further reduced shortly after the third vaccination (Figure S1). When long-term antibody levels after the second vaccination were instead stratified by age, we observed reduced levels in the anti-TNF-treated group across all ages (Figure 2; Figure S1).

These data raise the likelihood that fewer GC-derived memory B cells were generated after the first vaccination and, accordingly, re-activated and differentiated into PCs shortly after the second and third vaccinations in older (40–57 years) anti-TNF-treated vaccinees across all IgG subclasses and IgA. Young anti-TNF-treated vaccinees (24–39 years) may partially compensate for these reduced short-term levels by alternative pathways, such as increased activation of naïve B cells. However, the anti-TNF-treated vaccinees of all ages most likely generated fewer GC-derived long-lived PCs, resulting in reduced long-term antibody levels after the second vaccination.

3.2 | Anti-TNF treatment abolishes long-term low galactosylated and sialylated IgG responses after second vaccination

Next, we evaluated the subclass-specific anti-S IgG Fc glycosylation patterns 1 week and 6 months after two vaccinations in the three treatment groups (Tables S1, S3, and S4).

The anti-S IgG subclass-specific glycosylation patterns of the healthy subjects confirmed previous reports of short- and long-term anti-S IgG1 responses after two SARS-CoV-2 mRNA vaccinations^{26,27,43,44} and showed comparable courses for short- and long-term anti-S IgG2/3 and IgG4 glycosylation patterns (Figure 3). The aforementioned studies had shown that initial short-term anti-S IgG1 Abs after a first mRNA vaccination, most likely generated by short-lived extrafollicular PCs, were low in fucosylation but high in galactosylation and sialylation (not re-analyzed here). Shortly after the second mRNA vaccination, as verified in this study, fucosylation levels were highly increased (approximately 98%–99%), but importantly, galactosylation and sialylation levels were still high and bisection levels were low, across all IgG subclasses (Figure 3). In contrast, in the long-term after the second dose, when anti-S IgG from the long-lasting PC response became visible, anti-S IgG from healthy subjects still showed high levels of fucosylation but low levels of galactosylation and sialylation and higher levels of bisection, across all IgG subclasses (Figure 3).

In the anti-TNF-treated patients, the reduced short-term anti-S IgG1 and IgG2/3 subclass Abs after the second mRNA vaccination were glycosylated similarly to those in the other two groups; only anti-S IgG4 showed significantly or trending higher galactosylation and sialylation and reduced bisection levels in the anti-TNF-treated group (Figure 3). In contrast, the reduced long-term anti-S IgG subclass Abs after the second vaccination in the anti-TNF-treated group were all significantly or trending to be more galactosylated and

sialylated and less bisected compared to the healthy group as well as the group treated with other DMARDs (Figure 3).

When the determined anti-S IgG subclass glycosylation patterns were stratified by age, we observed increased galactosylation and sialylation and decreased bisection for both the short-term IgG4 and all long-term IgG subclasses across all ages (Figure S2).

This supports the hypothesis that the vaccine-induced long-term IgG subclass Abs with low levels of galactosylation and sialylation and high levels of bisection, most likely generated by GC-derived long-lived PCs, are TNF-dependent across all investigated ages. The reduced levels of long-term anti-S IgG subclass Abs in the anti-TNF-treated vaccinees may contain a higher proportion of short-term anti-S IgG subclass Abs.

3.3 | Anti-TNF treatment reduces both short- and long-term Fc-mediated functional activities of anti-S Abs after second vaccination

In addition to Fab-mediated target binding and neutralization, Abs contribute to infection control and clearance through Fc-mediated effector mechanisms.⁴⁵ In particular, while the emergence of SARS-CoV-2 variants of concern, such as the Omicron variant, was associated with loss of neutralizing activity of vaccine-induced Abs, Fc-dependent recruitment was maintained and may partly explain the sustained vaccine-mediated protection against severe disease by Omicron.^{45,46} To investigate the changes induced by anti-TNF treatment, we examined serum Ab Fc-mediated functional activities in various cell and complement activation assays: (i) ADNP (Ab-dependent neutrophil phagocytosis), (ii) APCP (Ab-dependent cellular phagocytosis by the monocyte cell line THP-1), (iii) ADCD (Ab-dependent complement (C3) deposition), and (iv) ADNKA (Ab-dependent NK cell activation (with three different readouts)) (Figure 4).

As expected, we found that anti-TNF treatment significantly reduced the functional activity of the samples in all assays compared to the healthy group, both in the short-term at 1 week and in the long-term at 6 months after the second vaccination (Figure 4A–D). The functional differences between the anti-TNF-treated group and the other DMARD-treated group were also significant or trending to be significant for the long-term samples; the functional activities of the short-term samples from the other DMARD-treated group were more intermediate between the other two groups (Figure 4A–D).

When the functional data were stratified by age, very similar observations were made as after stratification of the anti-S(1) antibody levels as described above further demonstrating the strong dependence of the functional assays on the anti-S antibody titers (Figure S3).

Sera from both time points of anti-TNF-treated subjects also showed a significantly or trending reduced neutralizing activity in a pseudotyped recombinant vesicular stomatitis virus (VSV)-based target cell infection assay compared to healthy subjects (Figure S4A). The addition of neutrophils alone (without Abs) to this assay already reduced the number of infected target cells,

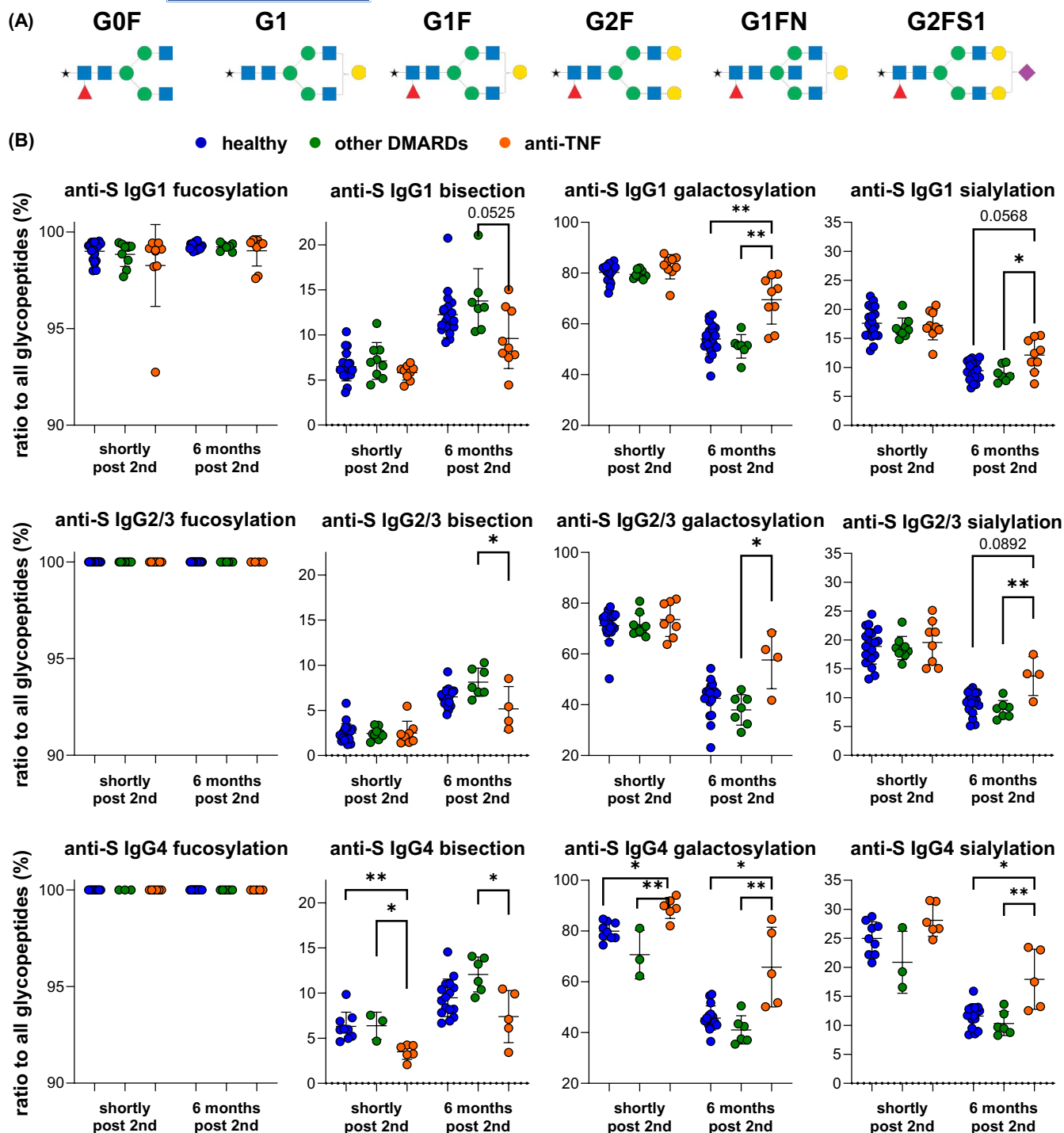


FIGURE 3 Glycosylation patterns of the anti-S IgG subclasses in anti-TNF-treated vaccinees. (A) The six major IgG Fc N-glycans attached to Asn 297 of IgG1: Galactose: G, yellow circle; sialic acid: S, purple diamond; fucose: F, red triangle; mannose: Green circle; N-acetylglucosamine: GlcNAc and bisecting GlcNAc: N, blue square. (B) Anti-S serum IgG subclass Fc glycosylation patterns: Fucosylation, bisection, galactosylation, and sialylation shortly post second and long post second vaccination of the indicated three groups. Statistics: Kruskal–Wallis test for each time point, * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

suggesting that the pseudovirus alone can activate neutrophils (Figure S4A). However, exclusion of this effect by normalization revealed Fc-mediated virus reduction effects by neutrophils (Figure S4B). Interestingly, Fc-mediated neutrophil effects were

particularly detectable in samples with low anti-S IgG levels and low neutralizing effects (Figure S4B,C).

To further approximate the Fc-mediated qualitative differences in anti-S Abs among the three groups, the functional data described in

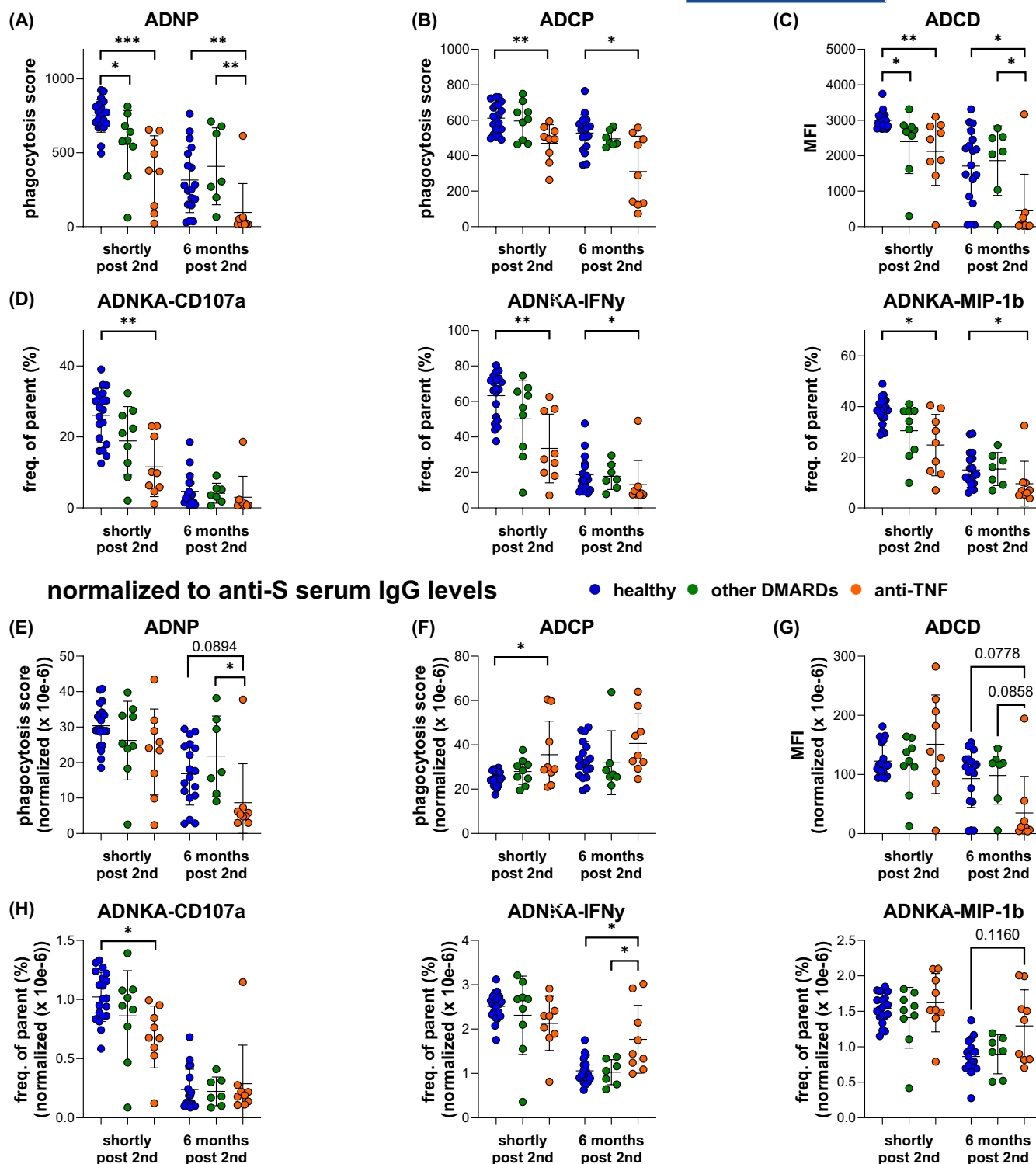


FIGURE 4 Functional activity of anti-S antibodies from anti-TNF-treated vaccinees. (A–D) Sera shortly (1 week) post second or long (6 months) post second vaccination of the three indicated groups were analyzed for their potential to activate cellular or complement assays: (A) antibody-dependent neutrophil phagocytosis (ADNP), (B) antibody-dependent cellular phagocytosis (ADCP), (C) antibody-dependent complement deposition (ADCD), and (D) antibody-dependent natural killer cell activation with three readouts (ADNKA-CD107⁺/IFN γ ⁺/MIP-1b⁺). (E–H) The functional data were normalized to the anti-S IgG (IgG1 + IgG2/3 + IgG4) summed intensities analyzed by LC–MS. Statistics: Kruskal–Wallis test for each time point, * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

Figure 4A–D were normalized to their anti-S IgG summed intensity levels determined by LC–MS (Table S1). No significant differences were observed between the healthy group and the other DMARD-treated

group (Figure 4E–H). In contrast, the anti-TNF-treated group showed a significantly or trending decreased activity in the long-term ADNP and ADCD assays and increased activity in the long-term ADNKA

(IFN γ ⁺ and MIP-1b⁺) assay compared to both groups (Figure 4E–H), which may additionally support our hypothesis that TNF depletion alters B cell priming in the GC and antibody quality.

Fc-dependent effector mechanisms depend on Ab levels, isotype and subclass distribution, binding geometry (i.e., epitope and avidity), and post-translational modifications. Given the heterogeneous Ab profile across the groups, we analyzed whether (i) the data containing non-normalized functional data or (ii) the data containing anti-S IgG level-normalized functional data separated the groups in an unsupervised principal component analysis (PCA) (Figure S5). At both time points and in both analyses (i and ii), healthy controls and other DMARD-treated subjects largely overlapped, whereas anti-TNF-treated individuals formed a separate cluster.

To explore this further, Spearman correlation analyses were performed for both data sets (i and ii) at both time points after the second vaccination to determine whether there was a relationship between the identified phenotypes (Figure S6–S9; Table S5A,B).

PCA and correlation analysis of the combined data of all three groups showed that the (i) non-normalized functional activities were

most highly correlated with anti-S IgG1 levels, both short- and long-term after the second vaccination, which were greatly reduced in the anti-TNF-treated vaccines (Figures S5, S6, S7, and S10).

Correlation analysis of the combined data of all three groups with (ii) anti-S IgG level-normalized functional data further suggested that ADNP and ADCD activities of the long-term samples were negatively correlated with anti-S IgG1 galactosylation, but not in short-term samples (Figures S8 and S9). Instead, ADNKA trended to be positively correlated with anti-S IgG galactosylation and sialylation (Figures S8 and S9).

3.4 | Reduced S-specific memory B cells in anti-TNF-treated patients

Reduced short-term anti-S1 IgG subclass and IgA levels in the anti-TNF-treated group after repeated vaccination may be due to reduced re-activation of TNF-dependent (GC-derived) memory B cells, as described in healthy subjects.¹³ Indeed, we found fewer

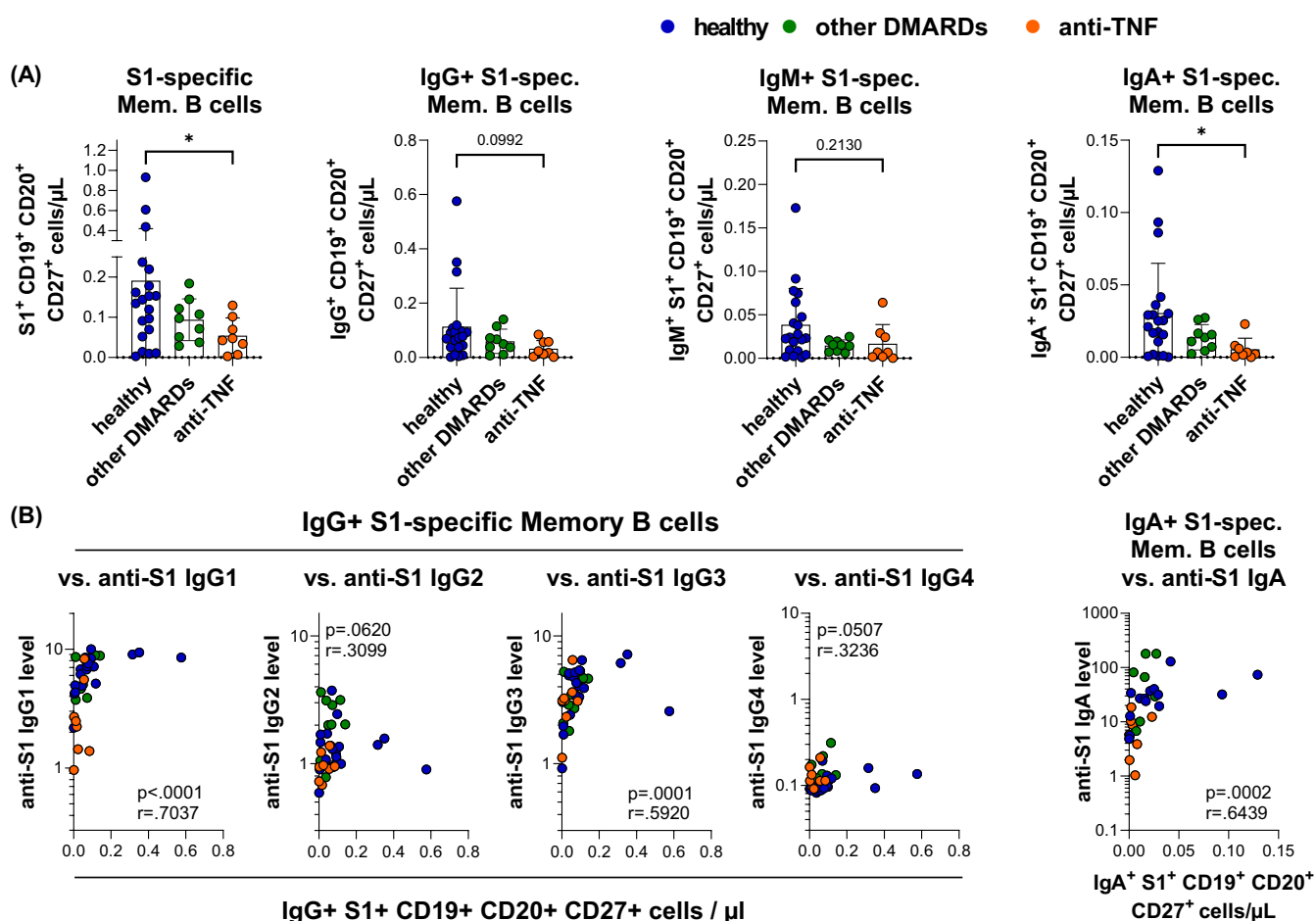


FIGURE 5 S1-specific memory B cells. (A) S1-specific total, IgG⁺ (IgM-IgA-IgD-), IgM⁺, and IgA⁺ memory B cells (CD19⁺ CD20⁺ CD27⁺), which were identified by flow cytometry as cells/ μ L blood shortly post second vaccination of the indicated groups. Statistics: Kruskal–Wallis test, * p < .05. (B) IgG⁺ and IgA⁺ S1-specific memory B cells shortly post second vaccination were correlated with anti-S1 IgG1–4 (ratio to reference values) or IgA (U/mL) levels, respectively, shortly post 2nd vaccination. Statistics: Spearman correlation, respective r - and p -values are shown.

S1-specific memory B cells in the anti-TNF-treated group compared to the healthy group 1 week after the second vaccination (Figure 5A; Figure S11). Furthermore, the number of S1-specific IgG⁺ and IgA⁺ memory B cells correlated with reduced short-term anti-S IgG1 and IgG3 or IgA levels, respectively, 1 week after the second vaccination across all three groups (Figure 5B). We found no differences in total memory B cells, total DN2 B cells, and S1-specific DN2 B cells between the three groups (Figure S11).

3.5 | Re-activation of potential memory B cells by a third vaccination initially induces highly galactosylated and sialylated anti-S IgG subclass antibodies in healthy individuals

To further interpret the above findings, and to address the question of whether re-activation of memory B cells after repeated vaccination initially induces (i) anti-S IgG Abs with high levels of galactosylation and sialylation, which would most likely be generated by short-lived PCs, or (ii) anti-S IgG Abs with low levels of galactosylation and sialylation, we examined anti-S IgG subclass levels and their glycosylation patterns just before and shortly after a third vaccination in another cohort of healthy subjects vaccinated with different SARS-CoV-2 vaccine combinations (Figure 6; Figure S12).

We focused specifically on anti-S IgG4 responses because recent data suggest that the late-emerging long-term anti-S IgG4 Abs are derived exclusively from persistent GC responses and that the initial short-term anti-S IgG4 Abs after repeated vaccination may be generated by GC-derived (IgG4⁺) memory B cells.^{14,47}

Our analysis showed that the Fc glycosylation patterns of anti-S IgG subclasses (including IgG4) were highly galactosylated and sialylated and low bisected shortly after the third vaccination (Figure 6; Figure S12), suggesting that re-activation of GC-derived (IgG4⁺ as well as other subclasses) memory B cells induces short-lived PCs that generate highly galactosylated and sialylated IgG Abs.

3.6 | More COVID-19 cases with anti-TNF treatment up to 18 months after a second vaccination

Next, we used the TriNetX database to determine the risk of developing COVID-19 during an 18-month follow-up period after a second mRNA vaccination in patients with inflammatory bowel disease, rheumatoid arthritis, or psoriasis treated with either (i) TNF inhibitors (cases) or (ii) the $\alpha 4\beta 7$ integrin inhibitor vedolizumab, which blocks intestinal but not systemic immunity, or methotrexate (MTX) (controls).

After propensity matching for demographic variables, we retrieved 4623 electronic medical records (EMRs) from patients treated with TNF inhibitors (cases) and 4623 EMRs from patients treated with $\alpha 4\beta 7$ integrin inhibitors or MTX (controls). No differences in age and ethnicity were found, except for a slightly increased proportion of females in

the controls (Table S6). The analysis showed a significantly increased risk of developing COVID-19 in anti-TNF-treated patients (cases) (Table S7; Figure 7). Specifically, 771 of 2742 (28.1%) cases developed COVID-19 during the 18-month follow-up period. In contrast, only 683 of 2783 (24.5%) controls developed COVID-19 during the same period.

4 | DISCUSSION

Recent studies show that vaccination-induced short-term IgG Ab responses are highly galactosylated and sialylated and are most likely generated by short-lived PCs. In contrast, T cell-dependent long-term IgG Ab responses are less galactosylated and sialylated and are largely derived from GC-dependent long-lived PCs in both humans and mice.²⁵⁻²⁷ This concept suggests that IgG glycosylation phenotypes can be used to trace the origin of short- and long-term IgG Ab responses.

Our findings support other studies showing that TNF is important for the generation of (cyclic) T follicular helper (T_{FH}) cells and GC responses and concluding that vaccination-induced long-term IgG Ab responses are mainly derived from TNF-dependent GC-derived long-lived PCs.^{19-21,24,48,49} Second, the data support recent suggestions that TNF is important for the generation of GC-dependent memory B cells.^{24,48,50} Third, the data suggest that re-activation of such memory B cells initially generates short-lived IgG⁺ PCs, characterized by high levels of IgG galactosylation and sialylation, rather than long-lived IgG⁺ PCs that would be characterized by low levels of IgG galactosylation and sialylation.

We propose that these memory B cells were already class-switched to IgG prior to re-activation, as evidenced by the rapid increase in initial anti-S IgG subclass Abs after repeated vaccination as early as 7 days post-vaccination. Accordingly, repeated SARS-CoV-2 mRNA vaccination has been described to progressively increase the proportion of the late-emerging anti-S IgG4 Abs in the short- and long-term responses.^{14,27,47,49} Corresponding anti-S IgG4⁺ long-lived PCs and memory B cells were therefore thought to be generated by GC responses.^{14,27} Re-activation of such GC-derived anti-S IgG4⁺ memory B cells may lead in part to the generation of short-lived PCs and in part to re-entry into the GC reaction. As we found comparable results for all anti-S IgG subclasses, the data suggest a similar scenario for all subclasses.

These findings suggest that programming of low galactosylated and sialylated IgG Abs in TNF-dependent GC-derived long-lived IgG⁺ PCs is not determined in corresponding TNF-dependent GC-derived IgG⁺ memory B cells. Accordingly, a GC-dependent determination of IgG Fc glycosylation in GC-derived long-lived PCs appears to be determined after the branching differentiation of GC-derived IgG⁺ memory B cells. This notion is supported by our recent finding that the determination of long-term IgG Fc glycosylation occurs late in the GC response.²⁵ In the future, sequencing of individual B cell subsets, including V(D)J sequencing, from different time points after repeated vaccination would allow verification of our observed results at single cell level.

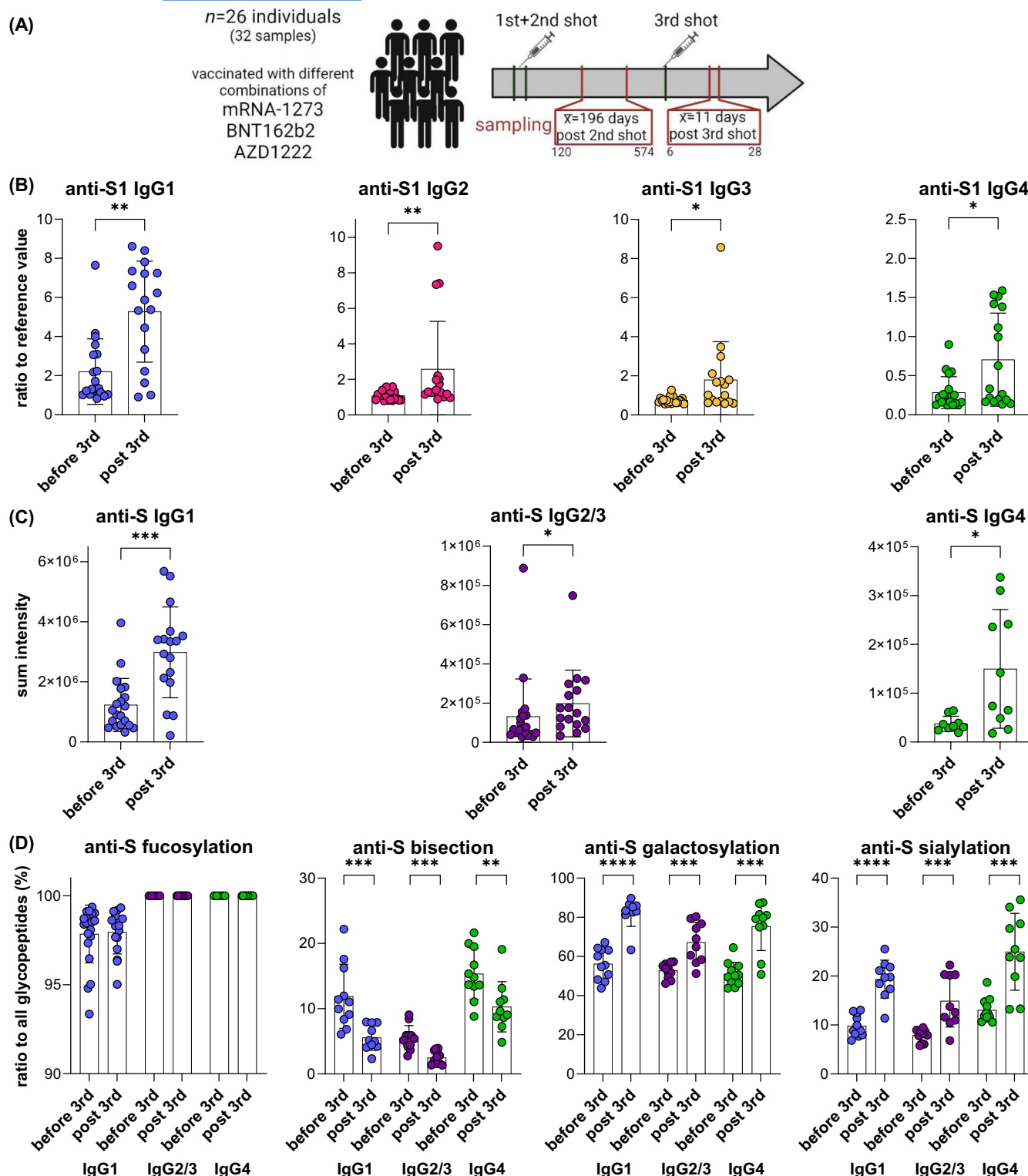


FIGURE 6 IgG subclass glycosylation patterns after re-activation of potential (GC-derived) IgG subclass⁺ memory B cells shortly post third vaccination. (A) Cohort description. (B) Anti-S1 IgG subclass levels identified by ELISA, (C) anti-S summed glycopeptide intensities of the Ig subclasses as detected by LC-MS, and (D) anti-S IgG subclass glycosylation before and shortly post third vaccination. Statistics: Mann-Whitney tests for the individual IgG subclasses, ** $p < .001$, *** $p < .0001$.

The data also suggest a model of a repeating glycosylation curve after each vaccination with high galactosylation and sialylation and low bisection in the short-term and low galactosylation and sialylation

and high bisection in the long-term across all IgG subclasses. These differently glycosylated short-term and long-term IgG Abs may have different roles. Short-term IgG Abs may mediate cytotoxic functions

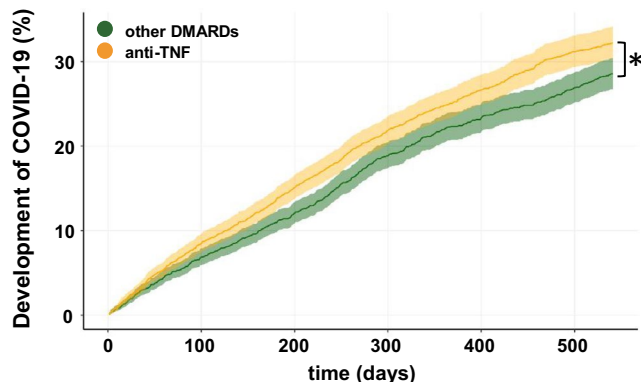


FIGURE 7 Meta-analysis of COVID-19 cases in patients with inflammatory (auto)immune diseases. Increased risk of developing COVID-19 observed in patients with inflammatory (auto)immune diseases treated with TNF inhibitors versus those receiving $\alpha 4\beta 7$ integrin inhibitors or MTX (other DMARDs). Line thickness represents the 95% confidence interval. Kaplan–Meier curves were compared using the log-rank test; $p = .0027$.

(ADCC) via NK cells, whereas long-term IgG Abs may be important for immune surveillance, for example by neutrophils, and for re-activation of memory B cells to subsequently induce a strong cytotoxic short-term Ab response. Accordingly, highly galactosylated and sialylated antigen-specific IgG1 Abs have a higher potential to activate NK cell cytotoxicity.^{51–53} In contrast, de-galactosylation of antigen-specific human IgG and murine IgG1 has been shown to enhance neutrophil activation.^{54,55}

Here, anti-S IgG normalization of the functional data showed that the more galactosylated and sialylated long-term samples of the anti-TNF-treated vaccinees had decreased ADNP but increased ADNKA activity. In addition, normalized long-term ADNP activity data of all three combined groups trended to be correlated negatively with anti-S IgG1 galactosylation, whereas normalized ADNKA data trended to be correlated positively with anti-S IgG1 galactosylation and sialylation. However, further studies with larger number of samples are needed to verify and further investigate these observations.

The TriNetX analysis showed that anti-TNF treatment increased the risk of developing COVID-19 0–18 months after a second mRNA vaccination in patients with various inflammatory (auto)immune diseases compared to correspondingly vaccinated patients treated with anti- $\alpha 4\beta 7$ integrin or MTX. The dependence of TNF on proper immune responses following vaccination was also suggested in another study.¹ The authors showed that psoriasis patients treated with TNF inhibitors had more infections induced by 26 different pathogens than psoriasis patients treated with IL-23 or anti-IL-17.¹ Collectively, these data suggest that patients with inflammatory (auto)immune diseases may benefit more from DMARDs other than TNF inhibitors during vaccination than from increasing vaccine doses, since not only long-term but also short-term IgG(1) responses with neutralizing and Fc-mediated effects are greatly reduced after vaccine boosters in anti-TNF-treated patients.

5 | CONCLUSION

Our data suggest that the different short- and long-term IgG Fc glycosylation phenotypes can be used to trace the origin of antigen-specific IgG subclass Abs after vaccination. The findings suggest that anti-TNF treatment reduces both the generation of long-lived GC-derived PCs, which produce IgG subclass Abs with low galactosylation and sialylation, as well as the generation of short-lived PCs from re-activated GC-derived memory B cells, which produce IgG subclass Abs with high galactosylation and sialylation. Hence, increased breakthrough infections following anti-TNF treatment may be dependent on both reduced short- and long-term anti-S IgG, particularly IgG1, responses.

AUTHOR CONTRIBUTIONS

Conceptualization: JSB, TP, UMG, YCB, BFH, MW, ME. Methodology: JSB, TP, UMG, MaS, WW, JN, UMG, SL, JR, RJL. Formal analysis: JSB, TP, UMG, MaS, RJL. Investigation: JSB, TP, UMG, MaS, WW, JN, MO, SL, JR, JP, UK, RJL. Resources: UMG, FT, SS, MS, DB, SG, JS, ACL, PH, BFH, JSB, ME. Data curation: JSB, TP, UMG, MaS, RJL. Writing—Original draft: JSB, ME. Writing—Review and editing: JSB, TP, UMG, MaS, RJL, BFH, MW, ME. Visualization: JSB, UMG, MaS, RJL. Supervision: RJL, YCB, BFH, MW, ME.

Verified underlying data: JSB, TP, UMG, MaS, RJL, YCB, BFH, MW, ME. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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