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Persistent humoral and CD4$^+$ T_H cell immunity after mild SARS-COV-2 infection—The CoNAN long-term study

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Understanding persistent cellular and humoral immune responses to SARS-CoV-2 will be of major importance to terminate the ongoing pandemic. Here, we assessed long-term immunity in individuals with mild COVID-19 up to 1 year after a localized SARS-CoV-2 outbreak. CoNAN was a longitudinal population-based cohort study performed 1.5 months, 6 months, and 12 months after a SARS-CoV-2 outbreak in a rural German community. We performed a time series of five different IgG immunoassays assessing SARS-CoV-2 antibody responses on serum samples from individuals that had been tested positive after a SARS-CoV-2 outbreak and in control individuals who had a negative PCR result. These analyses were complemented with the determination of spike-antigen specific TH cell responses in the same individuals. All infected participants were presented as asymptomatic or mild cases. Participants initially tested positive for SARS-CoV-2 infection either with PCR, antibody testing, or both had a rapid initial decline in the serum antibody levels in all serological tests but showed a persisting T_H cell immunity as assessed by the detection of SARS-CoV-2 specificity of T_H cells for up to 1 year after infection. Our data support the notion of a persistent T-cell immunity in mild and asymptomatic cases of SARS-CoV-2 up to 1 year after infection. We show that antibody titers decline over 1 year, but considering several test results, complete seroreversion is rare.

Trial registration: German Clinical Trials Register DRKS00022416.

KEYWORDS

antibody response, immunity, SARS – CoV – 2, quarantine, T cell response

Introduction

Understanding immunity to SARS-CoV-2 will be of major importance to terminate the ongoing pandemic (1, 2). A growing body of evidence shows that SARS-CoV-2 infections lead to the induction of a broad humoral and cellular immune response that correlate with disease severity (1, 3, 4). This immune response is affected by individual host factors such as age, sex, and comorbidities similar to other infectious disease (5–7).

After infection, seroconversion, that is, the development of antibodies against structural proteins of the virus such as spike protein including the receptor-binding domain (RBD) or the nucleocapsid protein of the virus has been demonstrated in 50 to 100% of patients. However, depending on the studied population, its utility for the assessment of immunity has been questioned (3, 8–11). In contrast, neutralizing antibodies that are not measured routinely have been show to persist for up to 1 year (12, 13).

Rapidly after infection, also a T cell–mediated immunity is mounted that directly controls disease severity (3, 14, 15). Higher numbers of antigen-specific $CD4^+$ and $CD8^+$ T cells were associated with a milder course of disease (16, 17). In line with this, a higher degree of T cell activation with concomitant decreased numbers of T cells was correlated with an increased disease severity (17–19). Furthermore, COVID-19 severity was associated with a stronger inflammatory T cell–mediated cytokine response against S, M, or N proteins early after infection (20, 21). Additionally, infection by SARS-CoV-2 also provokes a specific memory T_H cell response that has shown to be stable at least for several months (15, 19, 22–24). Surprisingly, only few studies report follow-ups up to 1 year after infection (25, 26). Notably, the huge majority of studies over a time period beyond 6 months follows hospitalized cases of COVID-19 (27), leading to an overrepresentation of medium or severe cases of COVID-19. Only a few studies report antibody or T-cell responses after mild or even asymptomatic cases after more than 6 months after infection (14, 28, 29).

While disease severity correlates with levels of SARS-CoV-2–specific T cells and serum antibodies early after infection (30), in mild cases, a stable T cell response appears to be preserved as well up to 1 year after infection (26, 31). It appears that these cases are most important to understand the role of antibody and T cell–mediated herd immunity (32) and protection from death and severe disease after vaccination (33). Due to the global vaccination campaign that started in 2021 and multiple SARS-CoV-2 infection waves, it becomes increasingly challenging to enroll and follow up infected subjects in the Western World without vaccination or re-infection, which allows to assess the natural long-term course of infection. Thus, long-term data on the natural course of immunity after a single SARS-CoV-2 infection are scarce.

The CoNAN study was a prospective longitudinal population-based study enrolling participants living in the small rural German community of Neustadt-am-Rennsteig, Germany starting in May 2020. After a local SARS-CoV-2 outbreak in the community and a 14-day quarantine of the entire village, a field study was performed (8). This included sampling 1.5; 6 and 12 months after the outbreak. The combination of an isolated location and the well documented and controlled SARS-CoV-2 outbreak are unique features of this study allowing to assess the long-term immunity of SARS-CoV-2 infections without major biases. Here we report the long-term immunity in previously SARS-CoV-2 infected participants and healthy controls from the CoNAN study.

Materials and methods

Study design and enrollment

The CoNAN study (*COVID-19 Outbreak in Neustadt-am-Rennsteig*) was a prospective longitudinal population-based cohort study in Neustadt am Rennsteig a village in the Ilm-district in central Thuringia, Germany with 883 inhabitants in which a SARS-CoV-2 outbreak had occurred in Spring 2020. Due to the isolated location of the village, the extensive testing of the population, and the clear and controlled outbreak, Neustadt am Rennsteig is well suited to study the seroprevalence and potential development of immunity of SARS-CoV-2 infections. On March 22, 11 confirmed COVID-19 cases had been diagnosed in the district of which six (55%) were Neustadt residents with further 69 residents classified as contact persons. As a consequence, local public health authorities declared 14-day quarantine on the entire village. With support of the local family physician, an outbreak containment team of the public health department conducted a mandatory mass screening using nasopharyngeal swabs starting on April 2, in which 865 SARS-CoV-2 PCR tests were performed. Health authorities reported 51 SARS-CoV-2 infections and three SARS-CoV-2–associated deaths in the community during the outbreak. All persons with positive PCR results were defined as COVID-19 cases. The initiated containment measured controlled the outbreak; the spread to neighboring villages was prevented. Quarantine on the village was lifted on 5 April 2020. For the CoNAN study, samples were taken at three defined time points. The first sampling was performed from 13 to 16 May 2020. A total of 626 participants were included. The results of the antibody testing have been published (8). The second sampling was performed from 7 to 9 October 2020 and included the participants of the first round who had shown antibodies in at least two different IgG antibody assays and a control group matched after sex, age, and comorbidities, 145 participants in total. The third sampling was performed from 13 to 15 April 2021, with the participants of round 2 along with some new participants, 224 in total (Figure 1). Participation in the study was voluntary and could be withdrawn at any time. Refusal to participate had no consequences. Participants were enrolled at a central study site that was set up in the villages' town hall or in rare cases if requested by home visits. After informed consent, questionnaires and blood samples were directly taken at the study site. All inhabitants of the community of Neustadt am Rennsteig regardless of age, gender, or infection status were eligible for participation in the first phase. Individuals who do not reside in Neustadt am Rennsteig or who live in the adjacent community of Kahlert were not eligible for inclusion. Informed consent was provided by the participants or the parents/legal representatives. In the second and third phase of the study, participants who had a proven infection with SARS either by SARS-CoV-2 PCR or antibody positivity in the first phase were invited along with an age, sex, and

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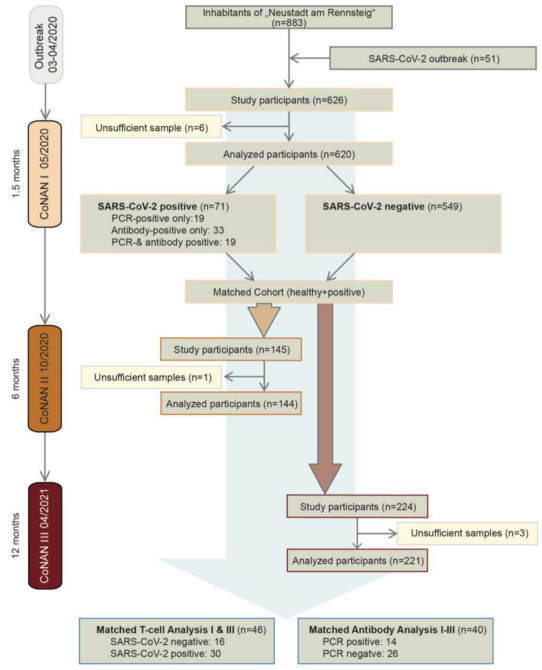

FIGURE 1
Flow chart of the CoNAN long-term study.

comorbidity matched control group. Inhabitants of the village who were not invited, however, could also perform antibody testing.

Questionnaire

Participants completed a pseudonymized questionnaire directly at the study site during all three rounds. After re-assessing the original case report forms on paper, obvious errors were corrected, and duplicated entries were deleted. Plausibility checks of demographic data were performed. Symptoms were noted if reported. Strength and duration of symptoms was not weighted in the analysis of this manuscript. Self-reported information on a positive SARS-CoV-2 PCR test at the time point of the outbreak/quarantine initiation was double checked with the information by the health department of the Ilm-district if the participants gave their permission on the consent form.

SARS-CoV-2 antibody testing

Five serological tests were performed in all three rounds. Characteristics of the tests are provided in Supplementary Table S1. Two of the tests detected antibodies recognizing the S-antigen, one recognized the 2019-nCoV recombinant antigen and two tests recognized the N-antigen of SARS-CoV-2. Detection of SARS-CoV-2 IgG antibodies was performed with five different quantification methods, of which two were enzyme-linked immunosorbent assays (ELISA) and three were chemiluminescence-based immunoassays (CLIA/CMIA). All tests were carried out according to manufacturers' instructions. For detailed information on assay characteristics and instruments used, see Supplementary Table S1. Sensitivities and specificities are shown as provided by the manufacturer. The following assays were used; EDI Novel Coronavirus SARS-CoV-2 IgG ELISA kit (Epitope Diagnostics Inc., San Diego, CA, USA), SARS-CoV-2 IgG ELISA kit (Euroimmun, Lübeck, Germany), SARS-CoV-2 S1/S2 IgG CLIA kit (DiaSorin, Saluggia, Italy), 2019-nCoV IgG kit (Snibe Co., Ltd., Shenzhen, China) and Elecsys Anti–SARS-CoV-2 kit (Roche, Basel, Switzerland).

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) from 56 inhabitants of the village Neustadt am Rennsteig were analyzed for S-Protein-specific T_H cell response. DMSO as solvent of S-peptide mixes was used as control. S-peptide mixes 1 and 2 represent the S-Protein N-terminal part and C-terminal part, respectively. General reactivity was controlled by SEB/TSST1 stimulation. There were no non-responders. PBMCs were isolated by gradient density centrifugation on Biocoll solution (Bio&SELL GmbH, Feucht, Germany) at 800*g* at room temperature (RT) for 20 min without brakes. PBMCs were washed with PBS and subsequently cryoconserved in medium containing penicillin/streptomycin, 10% DMSO and 50% FCS (all Sigma-Aldrich, St. Louis, MO, USA). For analysis, PBMCs were thawed and immediately washed with cell culture medium (supplemented with 10% human AB serum [PAN Biotech, Aidenbach, Germany], penicillin/streptomycin). Upon recovery at 37°C for 2h, a maximum number of 5×10^6 PBMCs were restimulated in cell culture medium containing 1 µg/ml recombinant anti-human CD28 antibody (clone CD28.2, BioLegend, San Diego, CA, USA, RRID : AB_314303) and either 0.2% DMSO (negative control), SARS-CoV-2 Spike glycoprotein PepMix 1 (S1, N-terminal coverage) or 2 (S2, C-terminal coverage) (both JPT Peptide Technologies GmbH, Berlin, Germany). As high controls 10^6 PBMCs were restimulated with 1 µg/ml TSST1 and 1 µg/ml SEB (both Sigma-Aldrich) in presence of 1 µg/ml recombinant anti-human CD28, or with anti-human CD3/CD28 beads (Gibco/Thermo Fisher Scientific, Lithuania) at a ratio of 1 bead/PBMC. After stimulation for 2h, Brefeldin A (BioLegend, San Diego, CA, USA) was added for another 14h of incubation. Cells were shortly incubated with 1 mg/ml beriglobin followed by staining with anti-human CD3 Pacific Blue (clone UCHT1, BioLegend, RRID : AB_1595437) and anti-human CD4 Brilliant Violet 605 (clone RPA-T4, BioLegend RRID : AB_2564391). After 5 min at 4°C in the dark, Zombie Aqua fixable dead cells stain (BioLegend) was added and samples were mixed and incubated for another 10 min at 4°C in the dark. Incubation was stopped with PBA/E and the cells were fixed in 2% Formaldehyde/PBS at RT for 20 min, blocked with 1 mg/ml beriglobin/0.5% Saponin and intracellularly stained with anti-human CD154 APC (clone 24-31, BioLegend, RRID : AB_314832), anti-human CD137 PE/Cy7 (clone 4B4-1, BioLegend, RRID : AB_2207741), anti-human IFNγ APC/Cy7 (clone 4S.B3, BioLegend, RRID: AB_10663412), anti-human TNFα PerCP/Cy5.5 (clone MAb11, BioLegend, RRID: AB_2204081), anti-human IL-4 PE (clone MP4-25D2, BioLegend, RRID: AB_315129), and anti-human IL-17A FITC (clone BL168, BioLegend, RRID: AB_961390) in 0.5% Saponin (Sigma-Aldrich) in PBA/E at 4°C for 20 min. Samples were analyzed on a FACS-Canto-Plus (BD), and data were analyzed with FlowJo V10.7 (BD, Ashland,

OR, USA). S-protein–specific $CD3^+CD4^+$ T helper (TH) cells are depicted as $CD137^+CD154^+$ among living $CD4^+CD3^+$. Representative FACS plots are shown in (Supplementary Figure S2).

Multi-dimensional flow cytometry analyses

The Uniform Manifold Approximation and Projection (UMAP) algorithm and the FlowSOM algorithm were used for unsupervised high dimensional flow cytometric analyses of the entire dataset with FlowJo version 10.8.1. Proportionally downsampled single cells/live/$CD3^+$/$CD4^+$ populations for each FCS file were concatenated in one single FCS file. UMAP was used for dimensionality reduction by using Euclidean as distance function with 15 Nearest Neighbors and 0.5 minimum distance. The following markers were used for building UMAP maps: 4-1BB, CD154, TNF, IFNγ, IL-4, and IL-17a. Resulting UMAP maps were fed into the FlowSOM (34). To identify clusters, heatmaps were built with median fluorescence intensity (MFI) values from each marker.

Statistical analysis

All statistical analyses were performed in the analysis population as indicated and stratified by age, PCR status, symptomatic disease, sex or sero-status from the serological assays if applicable. Descriptive analyses included the calculation of mean with standard deviation (SD) and medians with minimum and maximum or interquartile range (IQR) values for continuous variables, and absolute counts (*n*, with percentages) for categorical variables. Owing to the great data completeness, we performed no data imputations. All reported *p*-values are unadjusted and two sided. Time course experiments were analyzed with the Friedman test and Dunn's post-hoc test for multiple comparisons. Antibody ratios were compared with the Student's *t*-test with Welch correction. Non-parametric estimation of Spearman's rank correlation was performed with the following strength cutoffs ≥ 0.8 as very strong; ≥ 0.6–0.8 as moderately strong; ≥ 0.3–0.6 as fair and < 0.3 as poor (adapted from (35)). For comparison of two paired samples, the Wilcoxon-matched-pairs signed-rank test as implemented in GraphPAD PRISM 9 was used. When more than two paired groups were compared, non-parametric Kruskal–Wallis with the Dunns *post-hoc* test was used.

Study approval

The study was conducted according to the current version of the Declaration of Helsinki and has been approved by the institutional ethics committees of the Jena University Hospital and the respective data protection commissioner (approval number 2020-1776) and the ethics committee of the Thuringian chamber of physicians. All data were collected with unique pseudonyms on paper case report forms. These identifiers were later used to merge the questionnaire information with the laboratory information in an electronic study database. The study is registered at the German Clinical Trials Register: DRKS00022416.

Results

Participant characteristics

The study flow chart of all three visits of the CoNAN study is shown in Figure 1. A total of 626 of the 883 community inhabitants (71%) participated in the 1st round of the study in April 2020. Of those, individuals with a prior positive SARS-CoV-2 PCR and/or antibody titer ("infected") and age and sex matched non-infected controls were invited *via* mail to participate in the 2nd and 3rd visit. Villagers that were not invited but nevertheless wanted to take part additionally in the 2nd and 3rd visit, for example, to be informed about their antibody status or to contribute to the scientific project, could also partake. Thus, of the initial 626 participants, 146 individuals took part in the 2nd visit in October 2020 and 224 in the 3rd visit in April 2021. There were 132 individuals that participated in all three rounds. Antibody levels were determined in all of these participants. T-cell analysis was performed in all previously infected participants as well as in randomly chosen previously non-infected individuals. The detailed characteristics of the subjects are given in Tables 1 and 2 and Supplementary Material Table S1.

A matched analysis of antibody concentrations in 40 participants was conducted from all three time points. These were defined as being "seropositive" if at least two of five performed serological tests. In a matched analysis of T_H cell immunity, we investigated 30 previously infected participants and 16 non-infected controls and excluded 10 participants that had been vaccinated or infected during the survey period. A comparison of the test performance between the five serological IgG assays in the participants is shown in Supplementary Table S1.

Long-term antibody responses to SARS-CoV-2

In the population-wide CoNAN 1 study—in this manuscript referred to as 1.5-month time point—52 participants were anti–SARS-CoV-2 antibody seropositive (AB+) (reported in 8). Of these, 44 individuals participated in the 2nd and 46 individuals in the 3rd round of the study, respectively. Four participants had been vaccinated during the course of the study and were excluded. The remaining 40 participants were assessed for antibody course and are referred to as the "infected" group.

From the participants of the 3rd visit, 161 had initially been tested negative (AB-) of which 40 (24, 8%) participants became AB+. Of these, 18 persons had been vaccinated against SARS-CoV-2. For one participant, the information on vaccination history was missing. Nine participants had a PCR-confirmed SARS-CoV-2 infection between the 2nd and 3rd visit. Furthermore, 21 participants had not been vaccinated against SARS-CoV-2.

The remaining 40 participants (median age 60.5 years [range 5–83, IQR 51,75–71], male *n* = 24 [57, 43%], female 18 [42, 86%]) were antibody positive in the first round and had no re-infection or vaccination against SARS-CoV-2. These were assessed in the longitudinal serology study. Participants' characteristics are provided in Table 1.

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TABLE 1 Characteristics of the analyzed seropositive participants with matched samples from all three time points.

Characteristics	All antibody positive, (*n* = 40)	PCR positive (*n* = 14)	PCR negative (*n* = 26)
Age [mean, SD]	61,55 (12,05)	66,93 (13,04)	58,65 (10,64)
Median [min, max]	62 (36, 83)	68 (39, 83)	57 (36, 76)
Male	22, (55,0%)	7, (50,0%)	15, (57,69%)
Female	18, (45,0%)	7, (50,0%)	11, (42,31%)
Chronic disease category (no, [%])			
BMI (mean, SD)	27.4 (4,43)	27,61 (4,52)	27,29 (4,47)
Arterial hypertension	20, (50,0%)	6, (42,86%)	14, (53,85%)
Myocardial infarction	3, (7,5%)	2, (14,29%)	1, (3,85%)
Congestive heart failure, CHD	4, (1,0%)	3, (21,43%)	1, (3,85%)
pAVK	1, (2,5%)	0, (0%)	1, (3,85%)
Stroke	1, (2,5%)	0, (0%)	1, (3,85%)
Chronic lung disease	3, (7,5%)	2, (14,29%)	1, (3,85%)
Autoimmune disease/immunodeficiancy	2, (5,0%)	1, (7,14%)	1, (3,85%)
Liver disease	0, (0%)	0, (0%)	0, (0%)
Diabetes mellitus	3, (7,5%)	2, (14,29%)	1, (3,85%)
Chronic renal disease	4, (1,0%)	2, (14,29%)	2, (7,69%)
Tumor	0, (0%)	0, (0%)	0, (0%)
Chronic wounds, eczema	0, (0%)	0, (0%)	0, (0%)
Chronic viral infection	0, (0%)	0, (0%)	0, (0%)
Other disease	7, (17,5%)	1, (7,14%)	6, (23,08%)
Smoker	5, (12,5%)	0, (0%)	5, (19,23%)
Former smoker	3, (7,5%)	1, (7,14%)	2, (7,69%)
Number of performed antibody tests			
CoNAN 1 (1.5 months) (median, IQR)	6 (6, 6)	6 (6, 6)	6 (5, 6)
CoNAN 2 (6 months) (median, IQR)	4 (3, 5)	4,5 (3,75; 5)	3 (2, 4)
CoNAN 3 (12 months) (median, IQR)	4 (3, 4)	4 (4; 4,25)	3,5 (3; 4,25)
Clinical symptoms (outbreak)			
Fever	9, (22.5%)	4, (28.57%)	5, (19.23%)
Cough	18, (45.0%)	10, (71.43%)	8, (30.77%)
Nose congestion	7, (17.5%)	3, (21.43%)	4, (15.39%)
Dyspnoe	7, (17.5%)	4, (28.57%)	3, (11.54%)
Fatigue	18, (45.0%)	9, (64.29%)	9, (34.62%)
Joint pain	14, (35.0%)	8, (57.14%)	6, (23.08%)
Sweating/chills	10, (25.0%)	5, (35.71%)	5, (19.23%)
Taste disorder	16, (40.0%)	9, (64.29%)	7, (26.92%)
Smell disorder	9, (22.5%)	6, (42.86%)	3, (11.54%)
Diarrhea, vomiting, abdominal Pain	6, (15.0%)	4, (28.57%)	2, (7.69%)
Admitted to hospital for COVID	8, (20.0%)	6, (42.86%)	2, (7,69%)
Admitted to intensive care unit for COVID	1, (2.5%)	0, (0%)	1, (3.85%)

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TABLE 2 Participants characteristics of the T_H cell longitudinal cohorts.

Characteristics	All (*n* = 46)	SARS-CoV-2 positive (antibody and/or PCR positive) (*n* = 30)*	SARS-CoV-2 Antibody-positive only (*n* = 14)	SARS-CoV-2 PCR-positive only (*n* = 6)	SARS-CoV-2 negative (n=16)
Age [mean, SD]	58,78 (13,93)	63,93 (13,26)	59,07 (10,86)	68,50 (18,60)	51,88 (11,72)
Median [min, max]	61,00 (32, 85)	68 (32, 85)	59 (41, 73)	73,50 (32, 85)	54 (34, 70)
Male	23 (50%)	18 (60%)	9 (64,29%)	4 (66,67%)	6 (37,5%)
Female	23 (50%)	12 (40%)	5 (35,71%)	2 (33,33%)	10 (62,5%)
Chronic disease category (no, [%])					
BMI (mean, SD)	28,43 (5,112)	28,10 (4,574)	27,84 (5,593)	27,92 (3,904)	28,80 (6,172)
Arterial hypertension	21 (45,65%)	16 (53,33%)	8 (57,14%)	4 (66,67%)	4 (25%)
Myocardial infarction	5 (10,87%)	4 (13,33%)	1 (7,14%)	1 (16,67%)	1 (6,25%)
CHF, CHD	8 (17,39%)	6 (20%)	1 (7,14%)	2 (33,33%)	1 (6,25%)
pAVK	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Stroke	1 (2,17%)	1 (3,33%)	1 (7,14%)	0 (0%)	0 (0%)
Chronic lung disease	1 (2,17%)	1 (3,33%)	0 (0%)	0 (0%)	0 (0%)
Autoimmune Disease/ immunodeficiancy	3 (6,52%)	1 (3,33%)	1 (7,14%)	0 (0%)	2 (12,5%)
Liver disease	0 (0%)	0 (0%)	0 (0%)	1 (16,67%)	0 (0%)
Diabetes mellitus	4 (8,70%)	3 (10%)	1 (7,14%)	1 (16,67%)	0 (0%)
Chronic renal disease	4 (8,70%)	3 (10%)	1 (7,14%)	0 (0%)	1 (6,25%)
Tumor	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Chronic wounds, eczema	1 (2,17%)	0 (0%)	0 (0%)	0 (0%)	1 (6,25%)
Chronic viral infektion	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Other disease	10 (21,74%)	7 (15,22%)	4 (28,57%)	2 (33,33%)	4 (25%)
Smoker	8 (17,39%)	2 (6,67%)	1 (7,14%)	1 (16,67%)	6 (37,5%)
Former smoker	8 (17,39%)	4 (13,33%)	2 (11,76%)	1 (16,67%)	4 (25%)
Number of performed antibody tests					
CoNAN 1 (1.5 months) (Median, IQR)	3 (0, 6)	6 (3, 6)	6 (5,75; 6)	0 (0, 1)	0 (0, 0)
CoNAN 2 (6 months) (Median, IQR)	3 (0,75; 4,25)	4 (2, 5)	4 (3; 4,25)	0,5 (0; 1,25)	0 (0; 0,5)
CoNAN 3 (12 months) (Median, IQR)	3 (0, 4)	4 (2,5; 4)	4 (3, 5)	0 (0, 1)	0 (0, 0)
Clinical symptoms					

(Continued)

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TABLE 2 Continued

Characteristics	All (*n* = 46)	SARS-CoV-2 positive (antibody and/or PCR positive) (*n* = 30)*	SARS-CoV-2 Antibody-positive only (*n* = 14)	SARS-CoV-2 PCR-positive only (*n* = 6)	SARS-CoV-2 negative (n=16)
Fever	9 (19.57%)	6 (20%)	2 (11,76%)	1 (16,67%)	3 (18,75%)
Cough	21 (45.65%)	16 (66,67%)	6 (42,86%)	2 (33,33%)	6 (37,5%)
Nose congestion	10 (21.74%)	6 (20%)	2 (11,76%)	2 (33,33%)	5 (31,25%)
Dyspnoe	6 (13.04%)	5 (16,67%)	2 (11,76%)	0 (0%)	1 (6,25%)
Fatigue	20 (43,48%)	16 (66,67%	6 (42,86%)	2 (33,33%)	4 (25%)
Arthralgy	14 (30,44%)	11 (36,67%)	2 (11,76%)	2 (33,33%)	3 (18,75%)
Sweating/chills	13 (28,26%)	10 (33,33%)	5 (35,71%)	2 (33,33%)	3 (18,75%)
Taste disorder	11 (23,91%)	10 (33,33%	3 (21,43%)	1 (16,67%)	1 (6,25%)
Smell disorder	7 (15,22%)	6 (20%)	1 (7,14%)	1 (16,67%)	1 (6,25%)
Diarrhea, vomiting, abdominal pain	8 (17,39%)	5 (16,67%)	0 (0%)	1 (16,67%)	3 (18,75%)
Admitted to hospital for COVID	7 (15,22%)	6 (20%)	1 (7,14%)	0 (0%)	0 (0%)
Admitted to intensive care unit for COVID	1 (2,17%)	1 (3,33%)	1 (7,14%)	0 (0%)	0 (0%)

*10 participants were PCR and antibody positive
no, number; SD, standard deviation.

In a first step, we assessed the course of the serum-antibody concentrations over 1 year after SARS-CoV-2 infection. Three of five tests revealed discrete results normalized to a standard for all three time points (1). *EDI*, recognizing anti-nucleocapsid antibodies; (2). *Liason Diasorin*, recognizing anti-spike antibodies and (3). *Maglumi Snibe*, recognizing anti-spike and anti-nucleocapsid antibodies), whereas one test (Euroimmune; recognizing anti-spike antibodies) provided an OD and semi-quantitative data and one test (Roche, recognizing anti-nucleocapsid antibodies) provided qualitative results only. As the missing standardization of the Roche test is a possible bias for tests performed at different time points, the course of the Roche test was not assessed further. During the 1-year observation period, the four quantitive tests showed a significant decline of the serum antibody concentrations (Figure 2A). The extent of the decline varied between the individual tests and time points (shown in detail in Figure 2A and Supplementary Figure S1A). Other authors had shown that anti-nucleocapsid antibodies become undetectable as early as already 8 months infection (36), suggestive of a shorter half-life of this antibody subset (37, 38). However, in our study, the opposite was the case. When comparing the decline of antibody concentrations between the EDI (N) test and the Euroimmune (S) test between 1.5 and 6 months, the decrease in anti-nucleocapsid antibodies was less pronounced ($mean_{EDI}$ = 0.84; SD_{EDI} = 0.37 *vs.* $mean_{EU}$ = 0.21; SD_{EU} = 0.10; *t*-test $p < 0.001$). This effect persisted after 12 months ($mean_{EDI}$ = 0.31; SD_{EDI} = 0.11 *vs.* $mean_{EU}$ = 0.21; SD_{EU} = 0.12; *t*-test $p < 0.001$) (Supplementary Figure S2A). For the early time points, this was confirmed in the comparison of the EDI test with the Diasorin (S1/S2) test ($mean_{DS}$ = 0.65; SD_{EDI} = 0.46; *t*-test p = 0.05). Yet, after 12 months, the Diasorin assay had a less pronounced decline ($mean_{DS}$ = 0.76; SD_{EDI} = 0.62; *t*-test $p < 0.001$). The data suggest a rapid waning of serum antibodies detected in some but not all tests during the first 6 months and a less rapid waning and preservation of antibodies within one year after SARS-CoV-2 infection.

In a subgroup analysis, we also stratified the serum antibody concentrations with respect to age, PCR-positive *versus* PCR-negative participants, the presence or absence of symptoms or the sex of the individuals. For all groups, the time course results remained unaffected by this stratification (Figures 2B–D; Supplementary Figures S1B and C). Notably, except three, all of the participants that initially had been tested seropositive—defined as at least two different positive assays—remained seropositive after one year.

Long-term t-cell immunity to SARS-CoV-2

To assess SARS-CoV-2 T cell–mediated immunity, we then analyzed S-protein–specific $CD154^+4\text{-}1BB^+$ cells among peripheral blood $CD3^+CD4^+$ T helper (TH) cells. This T cell–mediated

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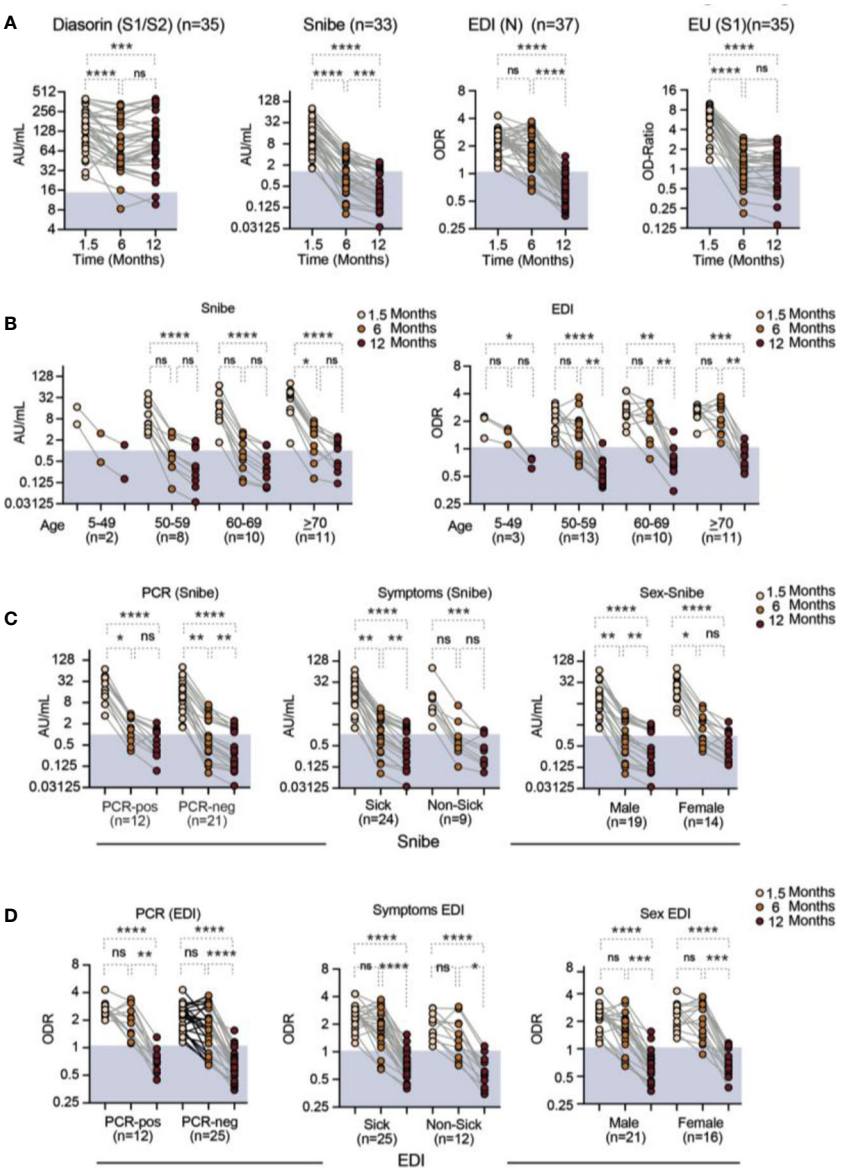

FIGURE 2
Anti–SARS-CoV-2 antibody levels over time as assessed with the three quantitative and one semiquantitative (Euroimmune; EU) antibody tests as indicated **(A)** all participants. Stratified by **(B)** age for the Snibe (left panel) and EDI test (right panel). **(C)** Results from the Diasorin Snibe antibody test stratified for PCR status (left panel), asymptomatic *versus* symptomatic disease (middle panel) and sex (right panel). **(D)** Same as **(C)** for EDI test. *N* = number of individuals per group. Friedman test with Dunns *post-hoc* analysis. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. AU, arbitrary units; ns, non-significant ($p > 0.05$); ED, EDI Novel Coronavirus SARS-CoV-2 IgG ELISA kit (Epitope Diagnostics Inc., San Diego, CA, USA); EU, Euroimmune (anti–SARS-CoV-2-ELISA (IgG); SN, 2019-nCoV IgG kit (Snibe Co., Ltd., Shenzhen, China).

immunity was determined in a matched cohort of 46 study individuals that participated in the first and third round of the study (Figure 1). Of these, 16 individuals were non-infected and considered as control cohort. Thirty individuals constituted the infected cohort. Of which, six were PCR-positive only, 10 were PCR-positive and antibody-positive after 1.5 months, and 14 were antibody-positive only.

Characteristics of the participants in the longitudinal T-cell study are provided in Table 2. To identify the spike-reactive T_H cells, we compared T_H cells restimulated with mixes of peptides covering the N-terminal part (*S.Pep1 [N]*) or the C-terminal part (*S.Pep2* [*C*]) of the SARS-CoV-2 spike protein to T_H cells responding in presence of DMSO alone (Figure 3). As shown in Figures 3A, B, we could detect the presence of spike-specific $CD154^+4\text{-}1BB^+$ T_H cells among all TH cells at 1.5 months and still 12 months after infection (Figures 3A, B). Notably, there was a slight, but significant reduction in the frequency of spike-specific T_H cells in the previously infected cohort over time (Figures 3C, D). In only two participants (6.7%), the spike-specific T_H cell response had vanished after 1 year (Figures 3C, D). When compared with SARS-CoV-2–negative participants, previously SARS-CoV-2–infected participants clearly showed an overall higher frequency of SARS-CoV-2 specific T cells at all time points that were investigated (Figures 3E, F). In addition, when assessing the subgroup of antibody-positive (and PCR-negative) participants only, this trend persisted (Figures 3G, H). Despite a persistent presence of spike-reactive T_H cells 12 months after infection in this group, a slight

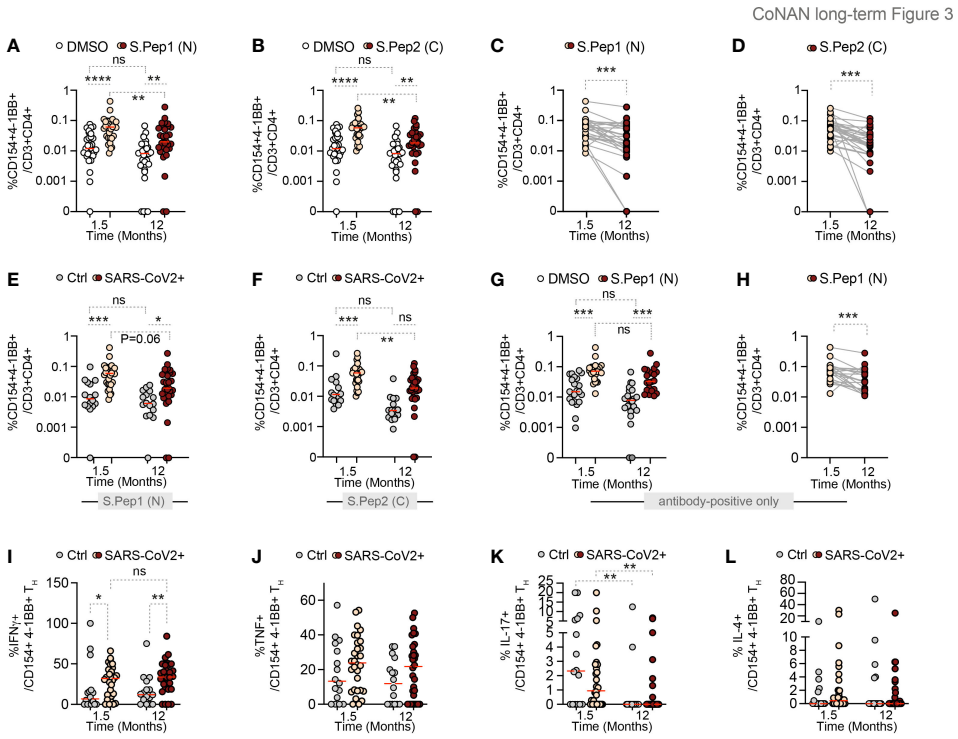

FIGURE 3
Longitudinal SARS-CoV-2–specific T_H cell response. **(A)** % $CD154^+4\text{-}1BB^+$ T_H cells from previously infected participants stimulated with DMSO or S.Pep.1 (N-terminal). **(B)** % of 154 + 4-1BB+ T_H cells stimulated with DMSO or S.Pep.2 (C-terminal). **(C, D)** Time course of SARS-CoV-2–specific T_H cells stimulated with **(C)** S.Pep.1 (N) and **(D)** S.Pep.2 **(C)**. **(E, F)** % $CD154^+4\text{-}1BB^+$ T_H cells stimulated with **(E)** S.Pep.1 (N) and **(F)** S.Pep.2 **(C)** in SARS-CoV-2 negative (Ctrl) *versus* positive (SARS-CoV-2+) participants. **(G, H)** % $CD154^+4\text{-}1BB^+$ T_H cells in a subset of antibody positive participants only. **(I–L)** Positive $CD154^+4\text{-}1BB^+$ T_H after stimulation with S.Pep.1 for the cytokines **(I)** IFNγ, **(J)** TNF, **(K)** IL -17, and **(L)** IL-4. Dots represent individual participants. Red line indicates median. Time indicates months after the SARS-CoV-2 outbreak. Wilcoxon-matched-pairs test for two matched groups and Kruskal–Wallis with Dunns *post-hoc* for other analysis with more than two groups.). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. AB, antibody; Ctrl, controls (non-infected participants); DMSO, dimethyl sulfoxide; IL, Interleukin; IFN, Interferon; ns, non-significant ($p \geq 0.05$).

decrease in the frequency of these antigen-specific T_H cells among all T_H cells was also detectable (Figure 3H). Interestingly, a significantly higher frequency of spike-specific T_H cells was detected in this antibody-only group, when compared with individuals with an initially positive PCR status without measurable antibody titers (Supplementary Figure S3). Notably, several healthy subjects showed a T_H cell response against SARS-CoV-2 already at the beginning of the study, which was maintained over time.

In conclusion, individuals with initially detectable antibody levels showed a higher T_H cell response after 12 months than individuals with an antibody titer below detection threshold despite a PCR-confirmed infection. When we assessed intracellular cytokine expression of INFγ, TNF, IL-4, and IL-17A (Figures 3I–L; Supplementary Figure S3), we observed that significantly elevated initial levels of INFγ expressing SARS-CoV-2 specific T_H cells in inpreviously infected patients remained elevated up to one year after infection (Figure 3I and Supplementary Figure S3), whereas the expression of TNF, IL-4, or IL-17A was not increased at any time point (Figures 3J–L and Supplementary Figure S3). Overall, despite the slight decrease in particular subgroups, the data suggest that a T_H cell–mediated immunity after SARS-CoV-2 infection prevails for at least 1 year and contains a robust and maintained specific T_{H1} cell immunity.

To gain a further unbiased perspective on the immunity toward the spike proteins as represented by the S.Pep.1 (N) or S.Pep.2 (C)–specific T_H response, we performed multi-dimensional flow cytometry analyses. Representative Uniform Manifold Approximation and Projection (UMAP) maps were color coded according to the resulting clusters using the FlowSOM algorithm (34) (Figure 4 and Supplementary Figure S4). With the limitation that only small populations could be analyzed, the global high-dimensional analyses revealed diverse T-cell activation status of SARS-CoV2–infected individuals in time (Figures 4A, B). For both S1(N) and S2(C), we observed populations that resembles different phenotypes: (i) resting cells (cluster 1: negative for activation markers and cytokines), (ii) $4\text{-}1BB^+$ activated $CD4^+$ T cells (cluster 2: $4\text{-}1BB^+IFN\gamma^{+}TNF^{low}$), and (iii) $CD154^+4\text{-}1BB^+$ activated $CD4^+$ T cells (cluster 3 for S.Pep1 and cluster 4 for S.Pep2: $CD154^+4\text{-}1BB^+IFN\gamma^{high}TNF^{low}$). Moreover, a S2(C)-specific population depicting a $CD154^{low}4\text{-}1BB^{high}$ phenotype was observed ($CD154^{low}4\text{-}1BB^{high}IFN\gamma^{high}TNF^{high}$) (Figure 4B). For both S1(N) and S2(C), we only observed a marginal contribution of IL-4 and IL-17A. Despite obtaining these clusters, we did not detect any significant differences in infected individuals between 1.5 months and 12 months. This unbiased result supports our notion that the T_H-specific response is maintained over time after SARS-CoV-2 infection.

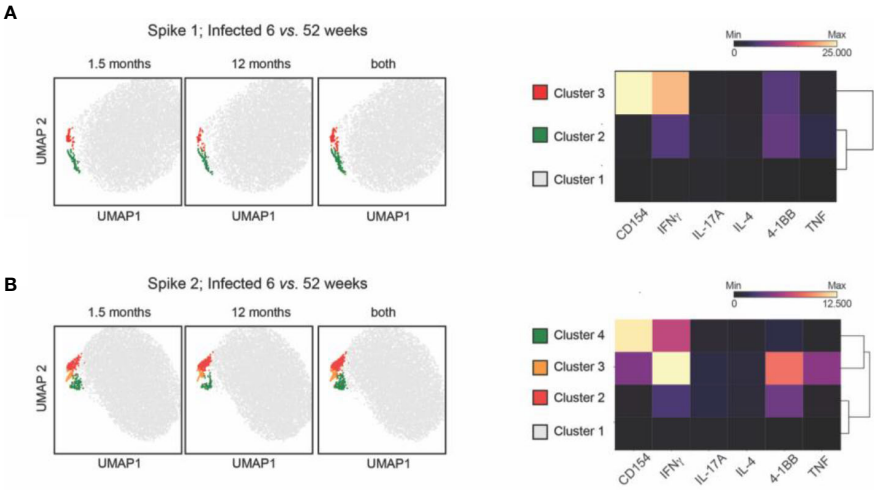

FIGURE 4
Multi-dimensional flow cytometry analyses using UMAP and FlowSOM clustering. Dot plots depict global UMAP projection pooled from $CD3^+CD4^+$ T cells from the study participants. For the first two maps, dot plots from each group are shown followed by a third dot plot with clusters identified with FlowSOM clustering using pooled $CD3^+CD4^+$ T cells from the compared groups. Heatmaps depict Median Fluorescence Intensity (MFI) values as indicated by clusters and markers. **(A)** S.1 and **(B)** S.2-specific T_H response in infected individuals for 1.5 *versus* 12 months. CD: cluster of differentiation; IL: interleukin; INF: interferon; TNF: tumor necrosis factor; UMAP: Uniform Manifold Approximation and Projection.

T and B cell immunity to SARS-CoV-2 correlate, but only weak

We then asked whether the 12-month antibody levels could be predicted by the T cell immune response mounted after 1.5 months and *vice versa*. Therefore, we correlated antibody concentrations and T cell immunity at 1.5 months and 12 months after infection. We assessed the Spearman correlation for the quantitative Diasorin, the Snibe, the EDI as well as for the semiquantitive Euroimmune assays with the percentage of the S1(N)-specific $CD154^+4\text{-}1BB^+$ T_H cells (Figures 5A–D) and the S2(C)-specific $CD154^+4\text{-}1BB^+$ T_H cells (Supplementary Figures S5A–D). None of the comparisons revealed a strong correlation. There was a moderate correlation (Spearman r = 0.6–0.8) of the serological tests at 1.5 months to T_H cell responses at 1.5 months and 12 months after infection (Figures 5A–D; Supplementary Table S3). When including all participants with T-cell data regardless of the antibody status, this prevailed for the 1.5 months correlation. However, S2(C)-specific T_H cells moderately correlated with antibody concentrations for the EDI test at 1.5. months (r_{EDI} = 0.61; $p < 0.0001$) (Supplementary Figure S5A) and the IgG index of the Euroimmune assay (r_{EU} = 0.62; $p < 0.0001$) (Supplementary Figure S5D). The correlation to S1(N)-specific T_H cells was only weak (r_{EDI} = 0.39, p = 0.05; r_{EU} = 0.48, p = 0.0079 (Figures 5A, D).

Discussion

The *COVID-19 Outbreak in Neustadt-am-Rennsteig* (CoNAN) study was a longitudinal cohort study after a localized SARS-CoV-2 outbreak in a rural community in the federal state of Thuringia, Germany. We followed previously infected patients with predominant mild disease and uninfected participants for one year after the outbreak. We provide evidence for a persistent T-cell immunity and a prevailing antibody response over a 1-year period. While the level of serum antibodies declined in a relevant manner during the first 6 months after infection, this decrease was slower during the subsequent 6 months.

To date, it is unclear, what determines protection against SARS-CoV-2 reinfection and which individuals are more likely to be persistently protected. In our initial study, only 50% of the previously infected individuals became seropositive (8). This assessment was performed approximately 1.5 months after infection and should represent the time period around the peak of an antibody response. Furthermore, it provides a good estimate about the humoral immune response directly after infection (37). Here, we show that serum antibody concentrations declined significantly over 1 year and thereby support previous data (29). Of note, combining the results of all five antibody tests, which included the non-quantitative Roche assay, a seroconversion to negative was only observed in three participants. All others remained seropositive. Antibody decay appeared not to be linear with a more rapid decline directly after infection and a subsequent less pronounced waning as previously shown (39, 40). Modeling of humoral immune response suggest that antibody-mediated protection against severe disease courses could be maintained for several years post-infection even after mild disease (37, 40), which is supported by our data. Also, neutralizing capacity of the serum antibodies seemed to be preserved to a certain extent (12, 23, 41–44). Our data suggest that seropositivity of SARS-CoV-2 antibodies could be of shorter duration when compared with other severe corona virus-mediated disease. Several authors reported cellular immune response in patients after infection with the Severe Acute Respiratory Syndrome Corona Virus (SARS-CoV) or the Middle East Respiratory Syndrome (MERS)–CoV during previous epidemics (45). Both diseases are associated with a much higher mortality than SARS-CoV-2 (1). Long-term immunity against the related pathogens has been suggested on the basis of detectable serum antibodies up to two years after MERS infection (46, 47). Herein,

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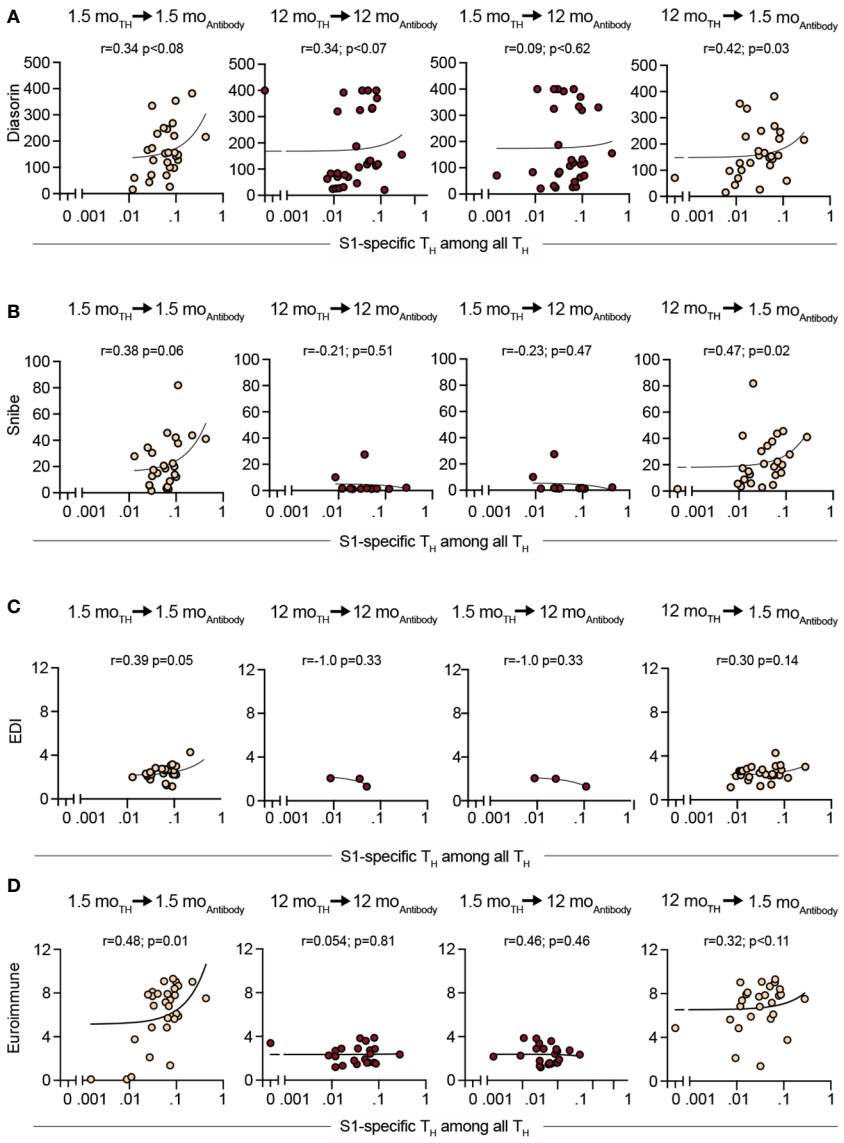

FIGURE 5
Spearman correlation analysis of SARS-CoV-2–specific T_H cells ($CD3^+CD4^+CD154^+4\text{-}1BB^+$) after S.Pep-1 (N) stimulation with initially (1.5 months) positive serological test only. Comparisons were the different combinations of the 1.5-month and 12-month time points after the SARS-CoV-2 outbreak in Neustadt-am-Rennsteig. **(A)** Liaison SARS-CoV-2 S1/S2 IgG CLIA DiaSorin, **(B)** Maglumi 2019-nCoV IgG CLIA *Snibe*, **(C)** EDI Novel Coronavirus COVID-19 IgG ELISA and **(D)** Euroimmune anti–SARS-CoV-2-ELISA (IgG). mo, month; T_H, T helper cells ($CD3^+CD4^+$). Dots indicate individual participants. Orange, 1.5-month antibody data; Dark red, 12-month antibody data.

memory T cell responses even persisted for several years (45, 48). Compared with MERS, SARS-CoV is associated with a longer memory response of specific T_H cells in two-thirds of SARS survivors up to 6 years (49) and persisting neutralizing antibodies up to 17 years after infection (45, 50). With our study, we contribute to the understanding of immune memory development in consequence of the related SARS-CoV-2 infection in mild and asymptomatic cases.

In this manuscript, we also assess the specific T_H cell memory response to SARS-CoV-2. SARS-CoV-2–specific memory T cells have been proposed to confer long-term protection against SARS-CoV-2 re-infections (51). Our findings expand the observation of previous data obtained 6 months after mild infection that showed a persistent T cell response against SARS-CoV-2 together with decreasing concentrations of spike- and nucleocapsid-specific antibodies (52). In general, strength and duration of an anti–SARS-CoV-2 T cell response depends on the severity of COVID-19 (53). Interestingly, the T cell response is directed against various epitopes (54, 55). The spike protein, a major cell entrance mediator of SARS-CoV-2 *via* ACE2 has been widely studied and used as targets for vaccination strategies, despite efficient responses were also induced against membrane and nucleocapsid proteins (56). While anti-nucleocapsid responses are dominated by $CD8^+$ cytotoxic T cells (23), the anti-spike responses evoked are mainly mediated by T_H cells leading to follicular T helper cell circulation and antibody-producing B-cell responses (56–58). However, others have shown that SARS-CoV-2 T_H cell responses start decreasing as early as after 6 months and then persist at a lower magnitude (23, 56). Independent from the initial disease severity, the

T_H cell-memory responses can mount protective T cell responses mediated by IFNγ at any investigated later time point (59). In our study, T cell responses were stable during the observation period. Surprisingly, especially in antibody-positive individuals that had no initial PCR confirmation of an infection, we observed a stable T_H cell response over 1 year. We can also show that there is an association between the early T cell response and the late antibody levels. However, the strength of this association was only weak. This is in accordance with data obtained at 6 months after infection (23).

Additionally, SARS-CoV-2–specific T_H cells were detected in non-infected control participants. This phenomenon has already been reported and suggested to represent T_H cells that are cross-reactive to seasonal human coronaviruses (HCoVs) (24, 60, 61). Relative to this, SARS-CoV-2–specific T_H cells were increased after SARS-CoV-2 infection when compared with cross-reactive T_H cells (62). Whether this can be explained by potentially different periods that have passed since infection and thereby, indicates a waning T_H cell response or a qualitative difference, is currently unknown. Interestingly, the presence of these cross-reactive T_H cells enhanced SARS-CoV-2 immunity and improved the vaccination response (60). It remains however speculative, whether such a trend could explain the increased antibody titers in the elderly *versus* the younger study participants, which during a lifetime likely have encountered several HCoVs and might have built a lasting T cell memory boosting antibody production (63, 64). Early studies about experimental infections of human volunteers with coronaviruses already showed that virus-induced antibody concentrations in the blood had been still increased after 1 year (65). While not completely preventing re-infection of such volunteers, the remaining immunity decreased the severity of the induced secondary infection (65). In how far the observed remaining antibody and T cell responses correlate to a protection against re-infection or less disease severity in case of reinfection in SARS-CoV-2, however, remains speculative.

While our study is special in the long follow-up period and in its observation of the waning of natural immunity not affected by any vaccination or re-infection, it has several limitations as well: (a) The CoNAN study was an initial population-based investigation of SARS-CoV-2 antibodies and T_H cell immunity (for further details see Weis et al. CMI 2021, PMID: 33221432). The cohort consisted of 626 individuals and 52 identified SARS-CoV-2-antibody positive participants and 19 participants with a previously PCR-confirmed SARS-CoV-2 infection but without detectable antibody titers. While this approach had the advantage of a very well-controlled longitudinal cohort, the sample size was rather small and did not include severe cases. (b) Since we had initially focussed on antibody detection and T_H cell immunity, no analysis of $CD8^+$ T_C-mediated immune responses was performed, which would have added additional information on immunity against SARS-CoV-2 infections. (c) There are no thresholds of antibody titres or T_H cell levels available that indicate a protective immunity. As such, we can only assume a persistence of immunity after infection from the presence of either parameter. (d) The infection was likely caused by the Wuhan or another early variant of SARS-CoV-2 in March 2020. It remains unclear, whether the observed long-term immunity would also be present upon infection with more evolved SARS-CoV-2 variants or in previously vaccinated individuals.

Collectively, our data indicate the persistence of a T_H cell immunity even after mild SARS-CoV-2 and asymptomatic infection, which is a better predictor of long-term immune memory than initially measurable antibody titers. While antibody responses potentially wane below a detection minimum beyond 1 year after infection, specific T_H cell responses remain at a detectable level.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the institutional ethics committees of the Jena University Hospital and the respective data protection commissioner (approval number 2020-1776) and the ethics committee of the Thuringian chamber of physicians. The study was conducted according to the current version of the Declaration of Helsinki. All data were collected with unique pseudonyms on paper case report forms. These identifiers were later used to merge the questionnaire information with the laboratory information in an electronic study database. The study is registered at the German Clinical Trials Register: DRKS00022416. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

CS, NA, MP and SW had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: MP, SW, TK. Acquisition of data: NA, MB, CS, SG, JG, MK, HP, SW, MP. Analysis and interpretation of data: All authors. Drafting of the manuscript: CS, NA, SW. Critical manuscript revision and additional important intellectual content, data interpretation: all authors. Statistical Analyses: CS, SW. Obtained funding: MP, SW. Administrative, technical, or material support: TK, SK, BL. Study supervision: MP, SW. All authors contributed to the article and approved the submitted version.

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Conflict of interest

SW received speaker fees from MSD and Infectopharm. SH received speaker fees from Pfizer, MSD and Astra Zeneca. TK received speaker fees from Roche. MP has participated in international advisory boards from Pfizer, Novartis, Basilea and Cubist and received speaker fees from the same companies. CB has participated in advisory boards from GSK and received speaking fees from Pfizer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1095129/full#supplementary-material

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