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Unique autoantibody prevalence in long-term recovered SARS-CoV-2-infected individuals

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ABSTRACT

The variability in resolution of SARS-CoV-2-infections between individuals neither is comprehended, nor are the long-term immunological consequences. To assess the long-term impact of a SARS-CoV-2-infection on the immune system, we conducted a prospective study of 80 acute and former SARS-CoV-2 infected individuals and 39 unexposed donors to evaluate autoantibody responses and immune composition. Autoantibody levels against cyclic citrullinated peptide (CCP), a specific predictor for rheumatoid arthritis (RA), were significantly ($p = 0.035$) elevated in convalescents only, whereas both acute COVID-19 patients and long-term convalescents showed critically increased levels of anti-tissue transglutaminase (TG), a specific predictor of celiac disease (CD) ($p = 0.002$). Both, anti-CCP and anti-TG antibody levels were still detectable after 4–8 months post infection. Anti-TG antibodies occurred predominantly in aged patients in a context of a post-SARS-CoV-2-specific immune composition ($R^2 = 0.31$; $p = 0.044$). This study shows that increased anti-CCP and anti-TG autoantibody levels can remain long-term after recovering even from mildly experienced COVID-19. The inter-relationship of the lung as viral entry side and RA- and CD-associated autoimmunity indicates that a SARS-CoV-2-infection could be a relevant environmental factor in their pathogenesis.

1. Introduction

COVID-19 is caused by severe acute respiratory syndrome-like coronavirus 2 (SARS-CoV-2) [1,2] that has so far claimed over 3 million lives worldwide and 142.5 million confirmed SARS-CoV-2 infected persons [3]. The disease course of COVID-19 is extremely variable among acutely infected individuals as well as convalescents and remains poorly understood. Although most of the infected individuals show mild symptoms, some convalescents develop long-lasting symptoms such as fatigue, loss of smell, and chronic muscle pain. As autoantibodies against IFNs [4], anticoagulants [5] and phospholipids have been observed in severely ill COVID-19 patients [6], and loss of smell as well as fatigue is a typical symptom in some of the autoimmune diseases [7], the question arises whether SARS-CoV-2 infections could induce and/or contribute to autoimmune pathologies [8], e.g. rheumatic diseases, such as rheumatoid arthritis (RA), Kawasaki-like disease (KD) [9], or coeliac disease (CD) [4,10]. Due to the long-term effects and the limited number of currently available treatment options for the therapy of COVID-19 and the chronic nature of autoimmune diseases, it seems reasonable to clarify this relationship.

The control of SARS-CoV-2 infection is decisively determined by the host's immune response. During acute severe COVID-19, lymphopenia and exhaustion of T-cells occurs, but also life-threatening hyper-reactivity of the immune response known as "cytokine storm" [11,12]. SARS-CoV-2 initiates intense CD4 and CD8 T-cell responses and induces antibodies to SARS-CoV-2 proteins such as Spike (S), membrane, and nucleocapside protein (N) [13,14]. Indeed, there appears to be a strong correlation between levels of antibodies against the S- and N-proteins

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Abbreviations

ACE2	Angiotensin-converting enzyme 2
ANA	Antinuclear antibodies
aPL	Anti-phospholipid antibodies
BMI	Body mass index
CCP	Cyclic citrullinated peptide
CD	Celiac disease
CoV-2	Coronavirus 2
COVID-19	Coronavirus disease 2019
HD	Healthy unexposed donors
ICU	Intensive care unit
IFN	Interferon
MC	Mild COVID-19 convalescents
MCV	Mutated citrullinated vimentin
N	Nucleocapside
RA	Rheumatoid arthritis
RF	Rheumatoid factor
S	Spike
SARS	Severe acute respiratory
TG	Tissue transglutaminase

and T cell responses specific for these proteins [13] and disease severity in COVID-19 patients [15]. Three weeks post onset of infection, CD4 central memory T-cells (T_{CM}) have been suggested to be the major responding population [14] and accumulated evidence indicates that the composition of T-cell subpopulations of convalescents are still altered months after resolving a SARS-CoV-2 infection [16].

Autoimmune diseases have a worldwide prevalence of approximately 4% [17]. For several entities, such as RA and CD, women show increased susceptibility. Accumulated evidence indicates that autoantibodies contribute or even initiate autoimmune pathology [18,19]. In genetically prone patients with RA, a specific autoimmune response occurs against post-translationally modified antigens e.g. by citrullination. The physiologic process of citrullination occurs at sites of inflammation and is driven by peptidyl-arginine-deiminase (PAD) enzymes, which are inducible by environmental factors such as smoking or viral infections. The current widely accepted model of anti-citrullinated peptide/protein antibody (ACPA) induction locates this process to mucosal tissues like the lungs [18,20]. Similarly to the occurrence of ACPAs in RA, specific autoantibodies are used as surrogate markers of pathologic processes e.g. in systemic lupus erythematosus (SLE), Scleroderma, anti-phospholipid syndrome (APLS), and also for CD [10]. In addition, while elevated levels of IgA specific antibodies against transglutaminase (TG) are shown to be associated with CD, they also appear to be associated with a higher mortality risk in males of >50 years of age whose death is due to respiratory failure [21].

It is of utmost importance to diagnose the onset of autoimmune processes as early as possible as advances have been made by using the "window-of-opportunity" for treatment [22,23]. In the case of rheumatic diseases, it is known that the first immunological signs of the disease, namely induction of autoantibodies such as ACPA, can be observed several years before the onset of clinical symptoms [24]. In this context, viral infections have been proposed as one triggering factor. In this prospective study in convalescent adults having recovered from mild SARS-CoV-2 infections, we provide first evidence for specific and long-lasting autoimmune responses.

2. Materials and methods

2.1. Subject details of patients and donors

This study was formerly approved by the Ethics Board of the University of Magdeburg (certificate 159/18). All patients, healthy donors or relatives of severe COVID-19 patients provided written informed consent in accordance with the declaration of Helsinki.

In our study, 68 COVID-19 convalescent individuals with a history of mild disease and 39 volunteers considered as being unexposed due to being negative for anti-SARS-CoV-2 S- and N-antibodies, devoid of COVID-19 specific symptoms and acquired in a region and time with less than 10 infected persons per 100,000 inhabitants were examined from April to November 2020, as well as 12 acute severely ill patients from December 2020 to January 2021 (see Table 1). Different study group sizes were processed, as healthy individuals were recruited within a limited time window with less than 10 infected persons per 100,000 inhabitants during spring 2020.68 convalescent COVID-19 patients were tested positive for SARS-CoV-2 RNA and/or for anti-SARS-CoV-2 Spike antibodies. 19 of these patients that showed anti-S antibody levels below 12 ng/ml were tested as anti-S antibody negative and vice versa 49 positive. All healthy donors were tested negative for anti-SARS-CoV-2 antibodies. None of the convalescents or healthy subjects had a history of autoimmune disease.

2.2. Preparation of blood samples and flow cytometric analysis

Serum separation was performed from blood samples by centrifugation (5 min at 3500 rpm) and Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient sedimentation using Pancoll (PAN Biotech) as previously described [25]. 1.5×10^5 PBMCs were stained using specific antibodies against following molecules (clone names in parentheses): CD4 (RPA-T4), CD3 (SK7), CD8 (HIT8a), CD45RO (UCHL1), CCR7 (G043H7), KLRG1 (1D11) and CTLA-4 (L3D10) (all Biolegend). Cell samples were analyzed by polychromatic flow cytometry using the FACS Canto II and FACSDiva software (all from BD Biosciences) and the data analyzed using Flow Jo (FlowJo).

2.3. ELISA, immunoblotting and HLA-DRB-1 genotyping

Serum levels of calprotectin, of anti-SARS-CoV-2, and of autoantibodies were determined by ELISA using commercially available kits according to manufacturer's instructions. Concentration of anti-SARS-CoV-2 Spike S1 protein IgG antibody (ELISA Genie, Dublin, Ireland); anti-SARS-CoV-2 N protein Human IgG (Proteintech, Manchester, United Kingdom) and SARS-CoV-2 neutralizing antibodies (AdipoGen Life Sciences, Liestal Switzerland) were ascertained with the indicated ELISA-kits. Duplicate measurements were performed for each sample. The samples along with the standards provided for each kit were analyzed using an ELISA plate reader (MULTISKAN FC, Thermo Scientific). To monitor inflammation, calprotectin concentration were analyzed using a sandwich ELISA-kit according to manufacturer's instructions (Orgentec Diagnostika, Mainz, Germany) with a detection range of 0–27 μg/ml. Final absorbance measurement were conducted at 450 nm using an ELISA reader (Sunrise, Tecan, Switzerland). Anti-CCP IgG (Medipan, Dahlewitz, Germany) and anti-TG IgA (Generic Assays, Dahlewitz, Germany) were analyzed by ELISA. Serum samples were diluted 1:100 in sample buffer and transferred in duplicate into the respective microtiter plate together with standards and controls. Final analysis was performed using a Tecan Sunrise Microplate reader (Tecan, Männedorf, Switzerland). Values were calculated according to linearity scale. Autoantibodies against Cardiolipin, β-2-Glycoprotein, MCV, dsDNA, and rheumatoid factor (RF) were determined by ELISA using the random-access analyzer Alegria (Orgentec, Mainz, Germany) (test strips: ORG 215S, ORG 215G, ORG 215 M, ORG 221S, ORG 248, ORG 204G, ORG 222S). The detection of IgG and IgM autoantibodies against phospholipids (Fig. S1A) was carried out by immunoblot analysis (Generic Assays, Dahlewitz, Germany). The presence of autoantibodies against DFS70 was evaluated by immunoblot using the EUROLINE Anti-DFS70 (IgG) system (Euroimmun, Germany). Samples were diluted 1:100 in sample buffer prior to analysis. Final staining of test strips was evaluated densitometrically using a scanner with a software scaling

Table 1
Characteristics of research subjects.

Characteristics of subjects	Unexposed healthy donors (HD) (n = 39)	Convalescent, mild COVID-19 (MC) (n = 68)	Acute, severe COVID-19 (ICU) (n = 12)	Reference Range
Age (range) – yr.	45 ± 13 (17–79)	48 ± 12 (15–71)	68 ± 10 (59–85)	–
Gender – no. (%)				
Male	12 (31)	28 (41)	8 (66.7)	–
Female	27 (69)	40 (59)	4 (33.3)	
Smoker – no. (%)				
Non smoker	27 (69)	45 (66.2)	N/A	
Smoker	9 (23)	13 (19.1)	N/A	
Former smoker	3 (8)	10 (14.7)	N/A	
Personal history of autoimmune disease – no. (%)	2 (5)	5 (7)	4 (33.3)	
Co-morbidities such as – no. (%)				
Hypertension	5 (12.8)	13 (19)	7 (58)	
Diabetes (D)	0 (0)	4 (5.9)	3 (25)	
Allergy	13 (33.3)	29 (42.6)	1 (8.3)	
Autoimmune disease (no D)	0 (0)	0 (0)	5 (41.7)	
NIH COVID-19 Severity Scale – no. (%)				
Asymptomatic				
Mild	–	8 (11.7)		
Moderate		60 (88.3)		
Severe/critically ill at ICU			12 (100)	
COVID-19 Symptoms - no. (%)				
Fever	2 (5)	34 (50)		
Cough	5 (12.8)	48 (71)		
Shortness of breath	2 (5)	27 (40)		
Loss of smell and taste	0 (0)	47 (69)		
Antibody Test Results (U/ml)				
IgG – S	0 (0)	49 (71)	12 (100)	<12 ng/ml
IgG – N	0 (0)	44 (63.8)	12 (100)	<12 ng/ml

samples from (-) to (+++). Autoantibodies against nuclear antigens (ANA) were detected by immunofluorescence analysis using the ANA HEp-2 plus kit (Generic Assays, Dahlewitz, Germany). Serum was diluted in sample buffer 1:80 to 1:2560. Final evaluation was done using a fluorescence microscope (Olympus, Japan).

DRB-1*04 genotyping of study subjects was conducted on DNA extracted from EDTA blood by DKMS Life Science Lab, Dresden, Germany. DRB-1*04 alleles 04:01, 04:04, 04:05, and 04:08 were classified as shared epitopes.

2.4. Statistical analyses

Statistical analyses was performed with Prism 9 (GraphPad). Outliers were excluded by Grubbs test. Normal distribution of data sets was tested with Shapiro-Wilk test and significance analysis were performed using two-tailed Student's *t*-test, Mann-Whitney-U-Test, or ANOVA test. Contingencies of distributions were analyzed using Fisher exact test. Multiple regression analysis including assumption diagnosis was performed using jamovi software version 1.2.

3. Results

3.1. Characteristics of study subjects

To evaluate long lasting autoimmune responses initiated by a SARS-CoV-2 infection, we recruited 68 convalescent individuals and 39 presumed unexposed controls selected randomly in a less-burdened region (greater area of Magdeburg, Germany, with less than 10 infections per 100,000 inhabitants at the time) (see Table 1 for patient characteristics). Serum and PBMCs were collected (mean: 101d, SD 37d) after positive SARS-CoV-2 PCR test from nasopharyngeal swaps and/or at time of IgG antibody testing >12 ng/ml antibodies against S-protein of SARS-CoV-2. All convalescents (MC) had mild symptoms during acute infection with SARS-CoV-2 (Table 1). In addition, twelve acute severe COVID-19 patients (ICU) were enrolled from ICU and 10 of them required ventilation-aided breathing. None of convalescent or unexposed donors had a history of rheumatic diseases or CD.

3.2. Elevated anti-CCP autoantibodies in convalescents

So far, in severely COVID-19 patients autoantibodies against multiple targets have been correlated with inflammation and/or mortality [5,6]. To assess the relation between SARS-CoV-2 infections and the occurrence of autoantibodies in 68 mild COVID-19 convalescents against mucosal-associated autoantibodies, we monitored specific predictors for RA such as rheumatoid factor (RF) and anti-CCP autoantibodies (Fig. 1A and B). The serum levels of RF in specimens from the convalescent patients (MC) did not show any significant difference than those found in healthy donors (HD) or those with acute disease (ICU) (Fig. 1A left). The levels were also similar to those noted in autoimmune-prone donors who were >50 years of age (data not shown). Anti-CCP autoantibodies that were not increased in acute severe COVID-19 patients [26], however, were elevated specifically ($p = 0.0357$) in convalescents when compared to unexposed donors (Fig. 1A right). These anti-CCP antibodies remained elevated in convalescents even after 8 months post infection ($p = 0.0165$) (Fig. 1B). Anti-CCP antibody levels did not result from smoking in this particular patient group (Fig. 1C left). Interestingly, together with elevated anti-CCP antibody levels in the sera from the convalescents these individuals showed a significant higher frequency of anti-CCP-associated HLA-DRB1*04 shared epitope alleles in comparison to the anti-CCP negative donors [27] (10/55 vs. 6/13, $p = 0.043$) that could further contribute the development of RA [28] (Fig. 1C right). To exclude inflammation causing high antibody levels in convalescents [29], we confirmed resolved inflammatory processes by low calprotectin concentrations in these sera (Fig. 1D). In conclusion, these data reveal a link between SARS-CoV-2 infections and anti-CCP specific autoantibody levels that become and remain detectable during convalescence phase.

3.3. Age-related anti-SARS-CoV-2 antibody reactions in convalescents

We next examined the potential role of the immune response to SARS-CoV-2 to the presence of autoimmune antibodies in the sera of the convalescent and the acutely ill patients. According to the cutoff value these sera contained no (less than 12 ng/ml) or positive concentrations

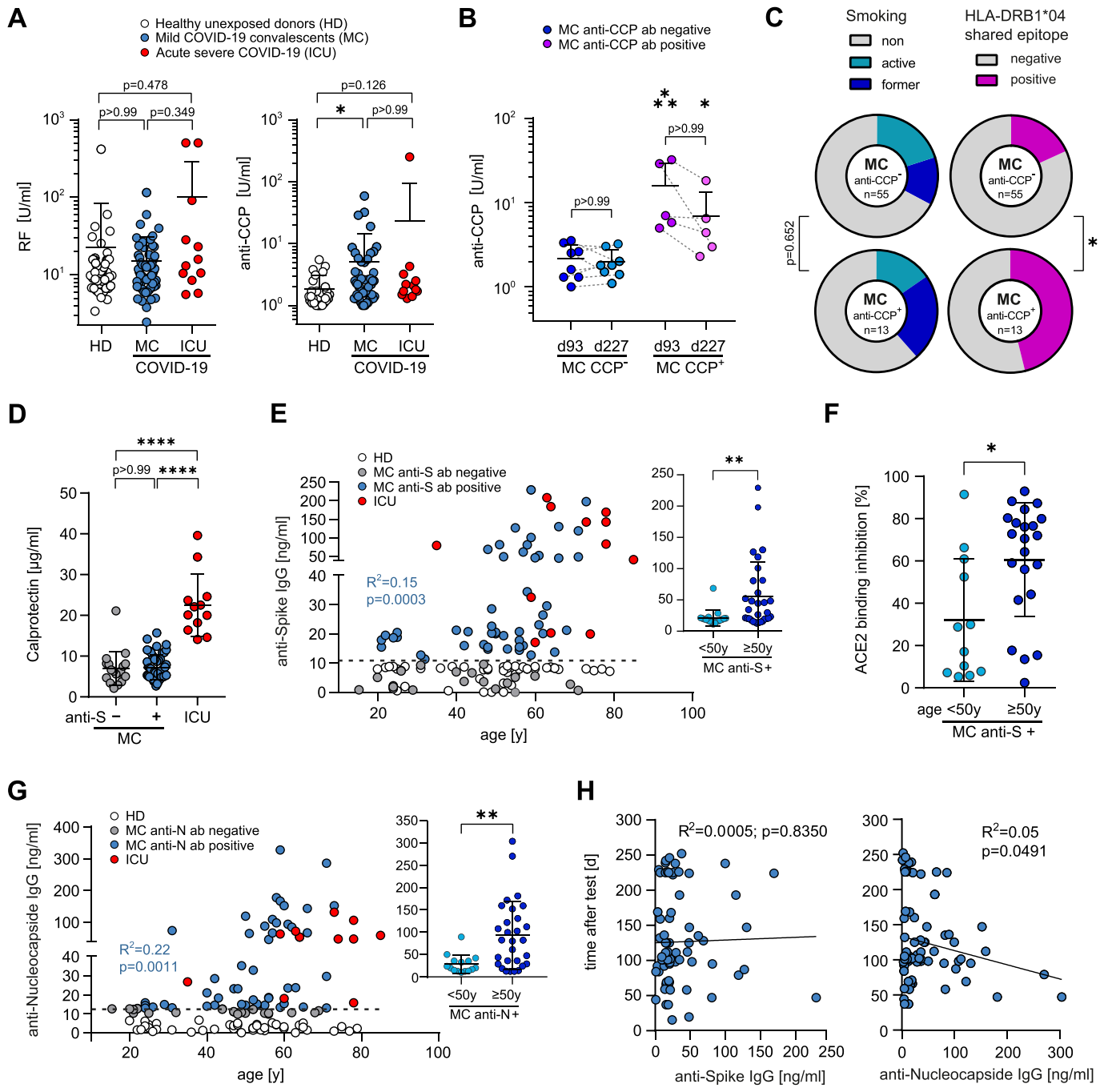

Fig. 1. Anti-SARS-CoV-2 and autoantibodies of acute and convalescent COVID-19 patients. A, Levels of RF (left) and anti-CCP autoantibodies (right) in sera of healthy unexposed donors (HD, open), convalescent (MC, blue), and acute severe COVID-19 patients (ICU, red). B, Anti-CCP antibody levels of negative (blue) and positive (violet) MC on mean day 93 after SARS-CoV-2 positive test and their anti-CCP levels on mean day 227 (light blue and violet, respectively). Asterisks show significances to healthy unexposed donor group. C, Distribution of smoking (left) and HLA-DRB1*04 alleles of shared epitopes (right) among anti-CCP negative and positive MC. D, Calprotectin levels of anti-Spike negative (gray) or positive (blue) MC, and ICU (red). E, Anti-Spike antibody levels of HD (open), anti-S antibody negative (gray) or positive MC (blue), and ICU (red) in correlation against age. Correlation coefficients and significance are shown for anti-Spike positive MC. Insert showing anti-S antibody levels of positive MC of less than 50 years of age and 50 years or older. F, ACE2 binding inhibition of anti-Spike positive MC of less than 50 years of age and 50 years or older. G, Anti-Nucleocapside antibody levels and age correlation of donors analog to (E). H, Correlation of anti-S (left) and anti-N (right) antibody levels against time elapsed since SARS-CoV-2 positive test. Data points represent donors with mean and SD (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

with more than 12 ng/ml of antibodies against S-protein of SARS-CoV-2 (Fig. 1E, left), whereas acute severe COVID-19 patients (ICU) had 3 times higher levels (mean 31.44 ng/ml SD 42.97 ng/ml; mean 94.88 ng/ml, SD 70.83 ng/ml, p = 0.0072) (data not shown).

As autoimmune diseases such as RA commonly show peak manifestation after the age of 50 [30], we grouped donors accordingly. Intriguingly, concentrations of anti-S IgG were positively associated with age (R^2 = 0.15; p = 0.0003) in anti-S antibody positive convalescents (Fig. 1E, left) indicating a possible association like autoimmune diseases to age. When grouping convalescents with anti-S IgG responses by age, the mean concentration was more than doubled in the adults being 50 years and older (mean 55.10 ng/ml, SD 54.98 ng/ml) compared with being below 50 years (mean 21.25 ng/ml, SD 12.43 ng/ml) (p = 0.0067) (Fig. 1E right). Additionally, anti-N IgG titers even

showed a stronger age-correlation (Fig. 1G). Furthermore, the capacity of the serum of anti-S IgG positive convalescents to inhibit viral binding to ACE2 was significantly enhanced in those above 50 years of age (mean (<50) 31.96% SD 29.03%; mean (>50) 60.52%, SD 26.87%, p = 0.01) (Fig. 1F). Of note, neither anti-S nor anti-N IgG levels of acute severe patients (ICU) correlated with age (data not shown) and the variety of anti-SARS-CoV-2 antibodies was not explainable by decay, while anti-S antibody levels remained stable and anti-N antibodies vanished over time (Fig. 1H). Taken together, antibodies against SARS-CoV-2 structures especially were induced for long-term in convalescents being 50 years and older and prone to autoimmune diseases.

3.4. Post-COVID-19 specific T-cell subsets

To analyze age dependent COVID-19 effects on T-cell subsets during convalescent phase, we determined absolute numbers of peripheral $CD4^+$ naive T-cells (T_N, $CD4^+CD45RA^+CCR7^+$), regulatory T-cells (T_{reg}, $CD4^+CD127^{low}CD25^{high}$, data not shown), central memory T-cells (T_{CM}, $CD4^+CD45RO^+CCR7^+$) [14] *ex vivo* from samples of former SARS-CoV-2-infected individuals that suffered mild symptoms (Fig. 2A and B). No significant correlation of T_{CM} or naïve T cells appeared with age (Fig. 2 A and B, left), and neither of them correlated with anti-S antibody titers (Fig. 2A and B, right), respectively. To assess the post-infectious impact of SARS-CoV-2 on the responsiveness of $CD4^+$ T-cell subpopulations, we conducted analysis of the T-cell surface surrogate markers KLRG1 for terminal differentiation and CTLA-4 for T-cell exhaustion [31]. In contrast to healthy unexposed donors that showed a correlation between age and $CD4^+$ T-cells expressing KLRG1 ($R^2 = 0.10$; p = 0.0455, data not shown) no age association of KLRG1 expression could be detected in the convalescents group (Fig. 2C). However, $CD4^+$ T cells of convalescents that expressed CTLA-4 on the surface, significantly increased by the age and were significantly higher in donors of 50 years and older when compared to younger ones (Fig. 2D). Thus, even months after recovery, changes in frequencies of a T cell subset of convalescent SARS-CoV-2 infected individuals showed age associations compared to the healthy controls. This could indicate systemic post-infectious COVID-19 effects on the immune system, especially in autoimmune prone individuals above 50 years of age.

3.5. Prevalence of autoantibodies linked to mild SARS-CoV-2 infections

To determine whether beside anti-CCP antibodies additional autoantibodies were prevalent in convalescents, we monitored antibodies to a number of autoantigens that included cardiolipin, ANAs (anti-nuclear antibodies), TG etc. In the sera samples of the convalescent and for comparison of healthy controls (Fig. 3A–C, sFig. 1C). The previously reported elevated anti-prothrombin antibody titers of ICU patients were rare, but twice as many in convalescents than in unexposed [5,6,19] (2/39 versus 7/68) (sFig. 1C). Autoantibody testing with HEp-2 cells (ANA) revealed unusually high titers and patterns showing spindle fibers and centrosomes in convalescents (3 of 68 versus 0 of 39) with all positive ones being women (Fig. 3A, sFig. 1D). Levels of anti-β2-GPI and anti-cardiolipin remained low in both unexposed donors and COVID-19 convalescents (Fig. 3A, sFig. 1C). However, mildly experienced convalescent and acute severe COVID-19 patients showed significantly increased levels of (p = 0.002) autoantibodies against tissue transglutaminase (TG) (Fig. 3A and B). In contrast to RF or anti-CCP antibody levels that showed no association to detected anti-SARS-CoV-2 antibodies, anti-TG titers were significantly increased in anti-S IgG positive convalescents (sFig. 1A, B left). Intriguingly, anti-TG antibody levels were furthermore significantly higher in convalescents aged 50 years and older when compared to unexposed donors of this age group (sFig. 1B right) in a rather gender balanced appearance (sFig. 1F). Most importantly, anti-TG antibody levels remained elevated even after 8 months post SARS-CoV-2 infection (Fig. 3C). In general, the convalescent group showed significantly more frequent co-occurring autoantibodies when compared to unexposed donors (p = 0.031) (Fig. 3D). Strikingly, 85% of all anti-CCP positive convalescents expressed also elevated anti-TG antibody levels, compared to 0% in unexposed donors (Fig. 3E). Notably, positive anti-CCP and TG antibody levels in convalescents appeared to be independently from the symptom loss of smell and further these patients showed no different distribution of gender and BMI compared to healthy unexposed controls (sFig. 1E-G).

Although previous reports have indicated a correlation between the

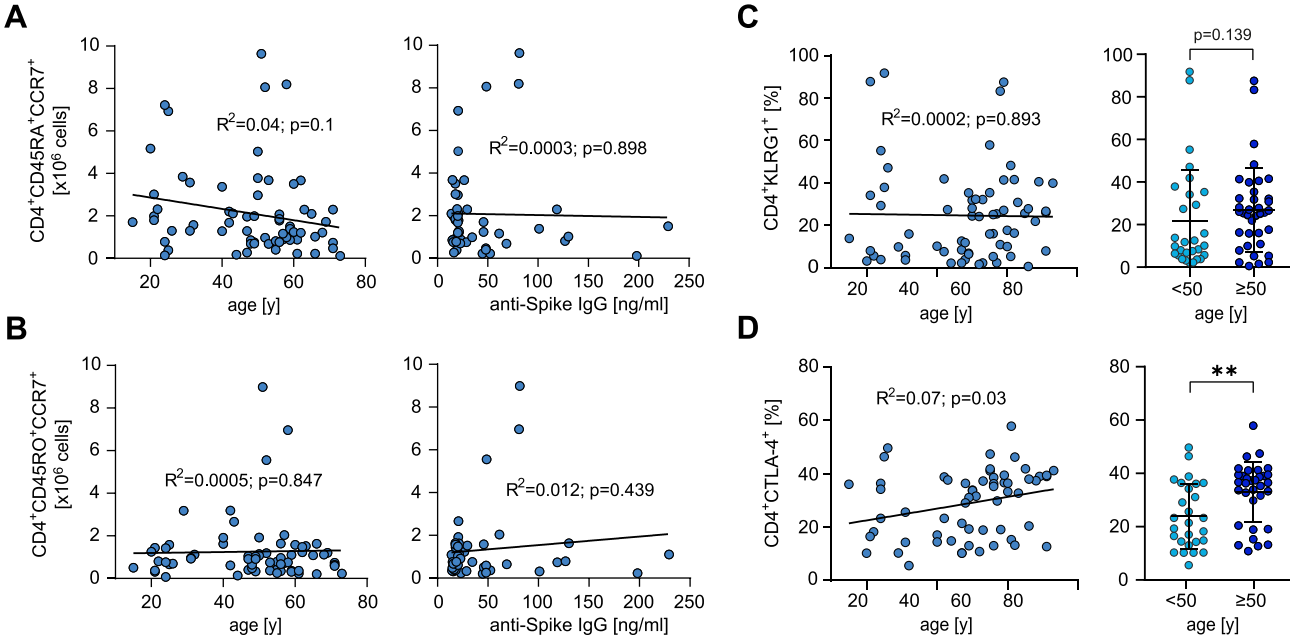

Fig. 2. T-cell subsets of convalescents 3–6 months after mildly experienced COVID-19. A,B, Panels showing correlation of naïve (T_N, $CD4^+CD45RA^+CCR7^+$) T cells (A) and central memory (T_{CM}, $CD4^+CD45RO^+CCR7^+$) T cells (B) of convalescents against age (left) and anti-S antibody levels of positive convalescents (right). C,D, Panels showing correlation of $CD4^+KLRG1^+$ (C) and $CD4^+CTLA\text{-}4^+$ T cells (D) of convalescents against age (left) and difference in $CD4^+KLRG1^+$ (C) and $CD4^+CTLA\text{-}4^+$ T cells (D) grouped by age of less than 50 years and 50 years or older (right). Data points represent donors with mean and SD. Numbers indicate correlation coefficients or p values (**p < 0.01).

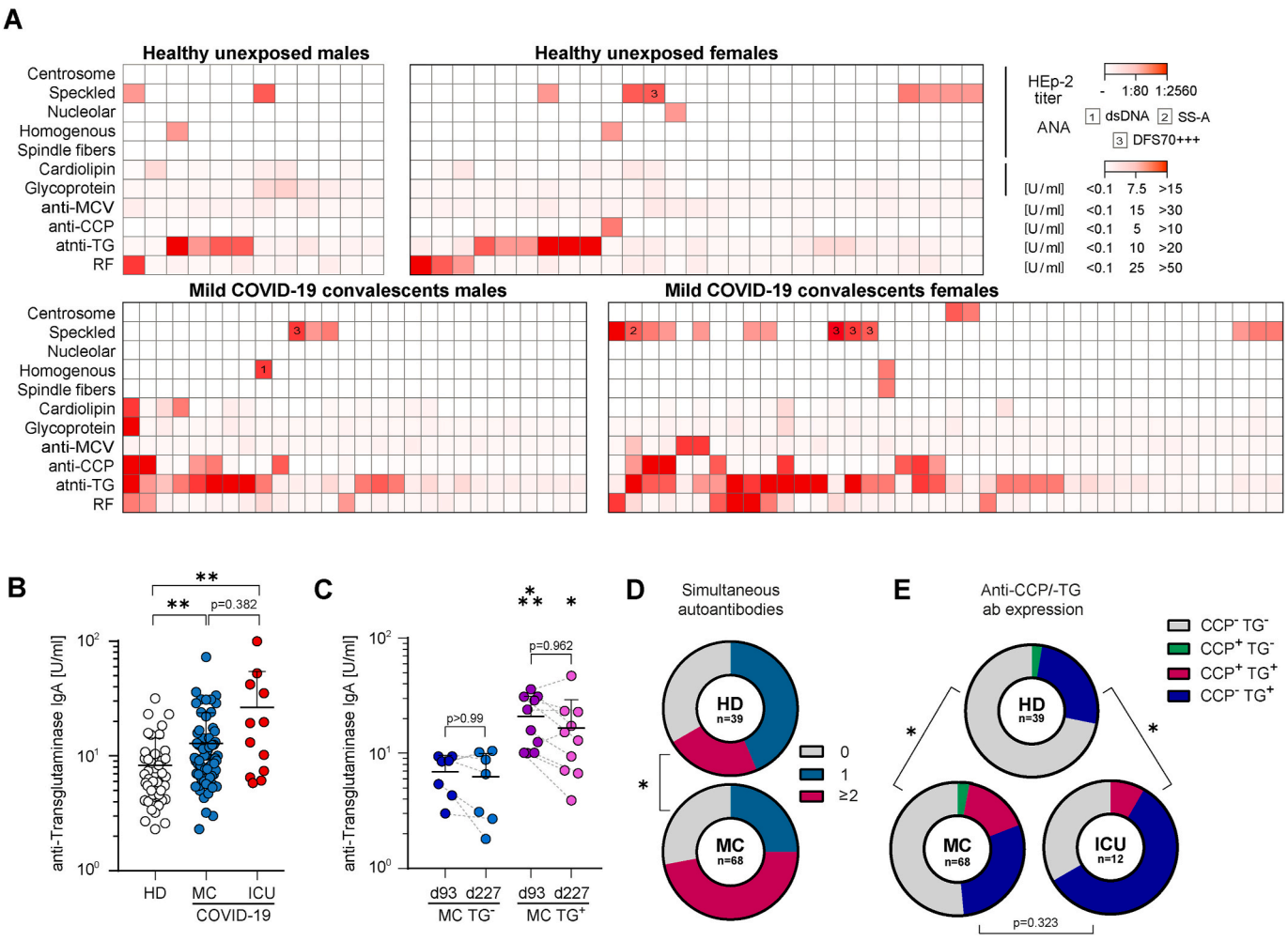

Fig. 3. Anti-tissue transglutaminase autoantibody levels are increased in COVID-19 patients. A, Heatmap of antibodies against autoimmune antigens in mildly experienced COVID-19 convalescent males and females (lower panels) in comparison to healthy unexposed ones (upper panels). Columns represent donors and color intensities the level of the indicated autoantibodies. Donors were aligned according average linkage clustering method. B, Anti-tissue transglutaminase (TG) autoantibody levels of healthy unexposed donors (HD, open), convalescent (MC, blue), and acute severe (ICU, red) COVID-19 patients. C, Anti-TG antibody levels of negative (blue) and positive (violet) MC on mean day 93 after SARS-CoV-2 positive test and their anti-TG levels on mean day 227 (light blue and violet, respectively). Asterisks show significances to healthy unexposed donor group. D,E, Distribution of simultaneously occurring autoantibodies (D) and expression patterns of anti-CCP and anti-TG antibodies (E) among donor groups as indicated. Data points represent donors with mean and SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

presence of anti-phospholipid antibodies (aPL) and severity of COVID-19 disease [32], the present studies failed to note such a correlation including a lack of correlation of such autoantibodies in sera from convalescent as compared with healthy controls (sFig. 1C). Of note, none of the autoimmune antibody titers correlated by themselves with anti-SARS-CoV-2 antibody levels (data not shown). Together, these data identify a co-occurrence of autoantibodies in COVID-19 patients and convalescents especially for anti-CCP and anti-TG antibodies that raised concerns of selective induction of long lasting autoimmune reactions resulting from SARS-CoV-2 infections.

3.6. *Anti-TG autoantibodies are connected to anti-SARS-CoV-2 immune responses*

As anti-TG antibody levels were detected to be significantly enhanced in convalescents being anti-S IgG positive or aged 50 years and older we sought to assess a relationship of an anti-TG response with further SARS-CoV-2 immune related parameters in these donors by using linear multi regression modeling (Table 2). The analysis resulted in the inclusion of the *ex vivo* ratio of $CD4^+$ to $CD8^+$ T-cells, indicating that upon SARS-CoV-2 infection peripheral CD4 and CD8 compartments may have been differently replenished. Further, CTLA-4 expression on the surface of $CD4^+$ T cells was a relevant parameter, as CTLA-4 expression implies immune exhaustion with age of convalescents (Fig. 2D) and CTLA-4^+ T cells persist at the site of chronic inflammation [33]. Using multiple regression, coefficients were $p = 0.045$, $p = 0.017$, $p = 0.065$, respectively. The overall model fit was $R^2 = 0.314$ with $p = 0.046$ showing that in convalescents aged 50 years or older the presence of anti-S antibodies together with frequencies of CTLA-4 expressing $CD4^+$ T-cells and the ratio of peripheral $CD4^+$ to $CD8^+$ T-cells determined the levels of anti-TG antibodies. Neither clinical symptoms, BMI, gender, anti-N protein antibodies, nor frequencies of other $CD4^+$ or $CD8^+$ T-cell subpopulations such as T_N, T_{CM} or T_{reg} further improved this model (data not shown). In conclusion, the induction of anti-TG immune responses upon a SARS-CoV-2 infection can be estimated by only few parameters.

4. Discussion

To answer the intriguing question whether mild SARS-CoV-2 infections could trigger long-term autoimmune events that can be detected during convalescent phase, our prospective study demonstrates that indeed in former SARS-CoV-2 infected individuals that suffered mild symptoms elevated autoantibody levels and rare diagnostic patterns on

Table 2
Context of anti-TG titers in > 50 year-old SARS-CoV-2 convalescents after 3–5 months of infection. Multiple regression analysis was applied to characterise the context of anti-TG concentrations in convalescent donors having recovered from mild or asymptomatic infection of SARS-CoV-2. The model relies on concentrations of anti-S protein of SARS-CoV-2, the $CD4^+$ T-cell to $CD8^+$ T-cell ratio and the $CD4^+$ CTLA-4^+ T-cell population. Grubb's test identified one outlier within the CD4/CD8 ratio, which was excluded from modeling. Assumption check of the model revealed normal distribution (Shapiro-Wilk test 0.0946) and multicollinearity was excluded as all predictors showed VIF values < 1.3. Dubin Watson test for autocorrelation equaled 2.25, p = 0.272.

Linear Regression						
Model Fit Measures						
Model	R	R^2	Overall Model Test			
			F	df1	df2	p
1	0.560	0.314	3.20	3	21	0.044
Model Coefficients – SARS-CoV-2 anti-Transglutaminase IgA [U/ml]						
Predictor		Estimate	SE	t	p	Stand. Estimate
Intercept		8.6005	4.9631	1.73	0.098	
anti-Spike IgG [ng/ml]		0.0725	0.0351	2.06	0.052	0.386
CD4/CD8 ratio *ex vivo*		2.8172	1.0929	2.58	0.018	0.523
$CD4^+$ CTLA-4+ [%]		−0.2937	0.1469	−2.00	0.059	−0.405

HEp 2 cells (ANA) actually persist months later. This long-lasting effects appear similar to those persistent antibody and T-cell responses observed in acute COVID-19 patients [34]. The induction of autoimmune events has been frequently monitored as a common event during acute viral infections and anti-viral antibodies have been shown to react against tissue antigens [35]. In case of SARS-CoV-2 infection with life-threatening COVID-19, acute autoimmunity against IFNs and phospholipids has been observed [5,6]. Here we report for the first time the unexpected finding of specifically enhanced autoimmune responses against CCP and TG even eight months after recovery from mild COVID-19, which could cause long-lasting autoimmune symptoms.

Anti-CCP antibodies that specifically predict the onset of RA occurred often in the group aged below 50 years. This age-relation was different from the common average age onset in the population that is 56 years in Germany [17]. Similarly, a shift in age incidence due to acute severe infections and in particular with SARS-CoV-2 has been suggested from another autoimmune disease that is KD in childhood [9,36]. Therefore, SARS-CoV-2 infections accompanied with mild symptoms likely could trigger anti-CCP antibody responses. The citrullination of proteins that is enhanced by inflammation could aim for marking abundant SARS-CoV-2 proteins, which in turn supports generation of anti-CCP antibodies. People who are already susceptible for developing RA probably release a rather pronounced inflammatory response against SARS-CoV-2 infections. This is in line with an associated susceptibility of RA patients to infections especially in the respiratory tract [37,38]. This would also be conform with enhanced autoantibodies to IFNs in acute COVID-19 patients, which likely have been preexisted already at disease onset [4]. Since RA is strongly associated with certain HLA loci, which we detected to be present in those individuals showing elevated anti-CCP antibody titers [27], it is tempting to speculate that especially those with genetic predisposition will be affected to develop overt disease. Nevertheless, the enhanced co-autoimmunity especially as 85% of anti-CCP antibody positive convalescents also express anti-TG antibodies, which is not observed in unexposed donors, indicate SARS-CoV-2 infections as a shared environmental factor in their induction. In addition, using regression analysis, we report that the appearance of anti-TG autoantibodies in convalescents >50 years of age is related to anti-S antibody levels (Table 2) pointing towards a specific feature of an overt SARS-CoV-2 infection. Furthermore, the increased CTLA-4 expression in these convalescents indicates a cellular exhaustion phenotype and therefore overcoming the SARS-CoV-2 infection preferentially depends on antibody-mediated mechanisms. The failure to detect antibodies in some convalescents could be explained as that these individuals likely resolved their SARS-CoV-2 infections by an effective cellular adaptive immune response instead.

Our data show that patients recovered from mild SARS-CoV-2 infections exhibit an increased persistence of anti-TG antibodies. Although only few donors showed antibody levels that indicate an immediate onset of clinical symptoms, it is known that autoimmune antibodies can already be elevated below the pathological range to inevitably signal a disease progression [39]. Besides gluten and α-interferon our data are supporting the idea that SARS-CoV-2 indeed may trigger anti-TG expression [40–42]. Nevertheless, transient occurrence of anti-TG antibodies has been described in patients with type 1 diabetes at onset [43].

Our data recommend to include the monitoring of serum antibodies against CCP and TG as biomarkers for the serological surveillance of SARS-CoV-2 convalescent individuals [44]. Interestingly, among all predictive biomarkers examined especially these two are co-expressed and both associated with inflammatory lung-involvement [18,21]. Since SARS-CoV-2 uses the respiratory tract as entry site, we speculate that especially in the lung autoimmune events may help to control potentially life-threatening SARS-CoV-2 infections. Therefore, as the first entry site of an antigen matters profoundly, vaccination against SARS-CoV-2 i. m [45]. may be of advantage to avoid elevated autoimmunity during a SARS-CoV-2 infection, thus, avoiding a potential development of RA or coeliac disease. Further longitudinal studies with anti-SARS-CoV-2 vaccinated individuals and the specific biomarkers anti-CCP and anti-TG are warranted.

Declaration of competing interest

DRo (shareholder and manager positions at Medipan and Generic Assays GmbH) provided valuable reagents. All other authors have no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2021.102682.

Author statement

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