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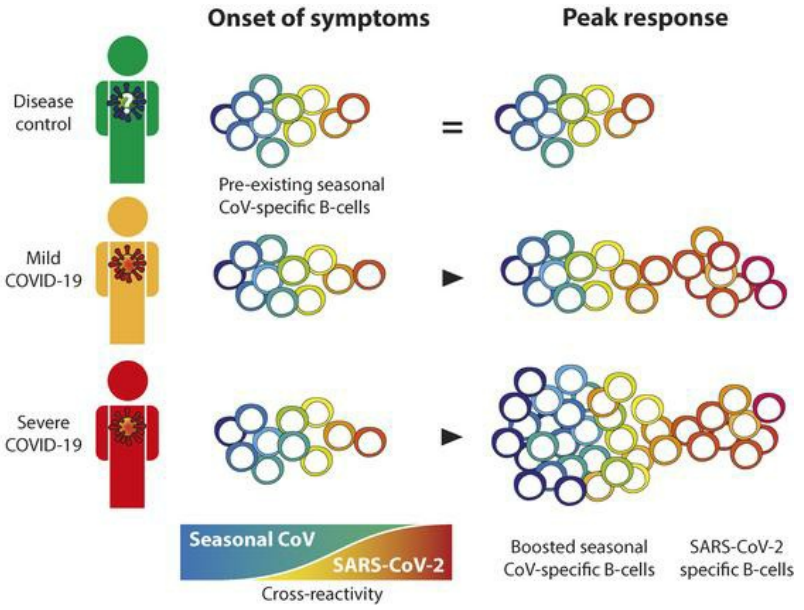
Graphical abstract



Seasonal coronavirus-specific B-cells with limited SARS-CoV-2 cross-reactivity dominate the IgG response in severe COVID-19 patients

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ABSTRACT

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the cause of coronavirus disease 2019 (COVID-19). Little is known about the interplay between pre-existing immunity towards endemic seasonal coronaviruses and the development of a SARS-CoV-2-specific IgG response. We investigated the kinetics, breadth, magnitude and level of cross-reactivity of IgG antibodies against SARS-CoV-2 and heterologous seasonal and epidemic coronaviruses at the clonal level in mild and severe COVID-19 patients and disease control patients. Antibody reactivity towards nucleocapsid and spike antigens was assessed and correlated to SARS-CoV-2 neutralization. COVID-19 patients mounted a mostly type-specific SARS-CoV-2 response. Additionally, IgG clones directed against seasonal coronavirus were boosted in patients with severe COVID-19. These boosted clones showed limited cross-reactivity and did not neutralize SARS-CoV-2. These findings support a boost of poorly protective coronavirus-specific antibodies in COVID-19 patients that correlates with disease severity, revealing original antigenic sin.

INTRODUCTION

The introduction of the zoonotic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to a pandemic of coronavirus disease 2019 (COVID-19) (1). The majority of COVID-19 patients experience mild symptoms including fever, cough, and myalgia, none of which can be considered specific to SARS-CoV-2 infection (2). Some COVID-19 patients develop acute respiratory distress syndrome (ARDS), which requires treatment at an intensive care unit (ICU) and results in a high mortality rate (2–4).

Although correlates of protection for severe COVID-19 are not fully defined in humans, SARS-CoV-2 neutralizing antibodies are considered a hallmark of immune protection (5–7). The kinetics of pre-existing and newly induced antibodies upon SARS-CoV-2 infection are expected to be important. Pre-existing memory B-cells that were once primed by antigenically related seasonal common cold coronaviruses (CCC) may provide fast protection from SARS-CoV-2 infection by rapid production of cross-reactive antibodies from memory recall, e.g. cross-neutralizing antibodies (5, 8). However, pre-existing immunity may also promote pathology (9). A lack of knowledge regarding specific effector mechanisms associated with protection against SARS-CoV-2 in COVID-19 hampers the development of targeted immune modulators to prevent or overcome severe disease (10). Therefore, there is an urgent need for detailed insight into the SARS-CoV-2 immune response in the context of a CCC-experienced immune system.

Immunity to antigenically related pathogens affects the development of a new immune response and is a key factor in the clinical outcome of infection (11). Memory recall of B-cells has been related to both positive and negative outcomes of heterologous virus infections. As an example, Fonville and others observed that influenza virus infections do not only induce new antibodies targeting the current infection or vaccination strain but also boost antibody titers towards a broad range of preceding heterologous influenza virus infections and vaccinations (12, 13). Here, the authors argue that this “backboost” has a positive contribution to vaccine efficacy by helping maintain immunity towards a broad range of influenza viruses. Consequently, they argue that backboosting offers a prospect on preemptive vaccination

updates (12, 14). Contrastingly, the B-cell clones that were primed to target a specific viral antigen may be boosted and dominate the IgG response to target a new infection where a related antigen is present. The antibodies that are boosted may have reduced affinity and functionality, e.g. poor neutralizing potential, towards the new infection and negatively affect the clinical outcome of infection (15–18). This mechanism, termed “original antigenic sin” (OAS), has been described for immunity to different viruses, including influenza and dengue virus (15–18).

Structural homology between the ectodomain of the beta-CoV SARS-CoV-2 spike (S, SARS2-S_{ECTO}) or nucleocapsid protein (N, SARS2-N) with those of other beta-CoV epidemic strains (SARS-CoV and MERS-CoV) and alpha-CCC (HCoV-229E and HCoV-NL63) and beta-CCC (HCoV-HKU1 and HCoV-OC43) suggests that memory B-cells capable of expressing cross-reactive antibodies may pre-exist in COVID-19 patients (19, 20). This is exemplified by the highly cross-reactive antibody response towards SARS-CoV-2 in individuals that were previously infected with SARS-CoV, likely due to their high sequence homology (88.6% shared amino acids in N and 69.2% in S) (19, 21, 22). The level of sequence homology between SARS-CoV-2 and other beta-CoV is lower (34-49% for N and 32-33% for S), and even less for alpha-CoV (28-29% for N and 28-30% for S) (23). However, in contrast to SARS-CoV, CCC cause millions of infections worldwide every year (24). Repeated exposure to CCC may therefore strongly affect the development of a SARS-CoV-2-specific response (25). For example, high serum OC43-S_{ECTO} IgG titers are associated with COVID-19 disease severity (23, 24). However, a limitation of the majority of polyclonal serological studies is that IgG cross-reactivity is rarely investigated. Therefore, the functional contribution of CCC-specific IgG clones to immune protection or immunopathogenesis remains largely unknown. Although monoclonal antibodies targeting shared epitopes in the stalk domain of S (S_2) of SARS-CoV-2 and HCoV-HKU1 or HCoV-OC43 have been identified (26, 27), little is known about CCC IgG cross-reactivity patterns with SARS-CoV-2 and how this affects the immune response towards SARS-CoV-2.

In this study, we longitudinally enumerated and categorized type-specific and cross-reactive circulating B-cell clones targeting a broad array of N and S antigens from all known human CoV. Subsequently, we determined the functional contribution of these clones to serum SARS-CoV-2 neutralization. In order to correlate these findings to disease severity, we compared severe ICU-admitted COVID-19 patients with ambulant mild cases and disease controls. The aim was to gain insight in antibody kinetics, magnitude, breadth and function towards SARS-CoV-2 in mild and severe COVID-19 patients. Here, we found evidence of a boost of CCC-specific IgG clones in severe COVID-19 patients that show limited cross-reactivity to SARS-CoV-2. These boosted clones did not contribute to SARS-CoV-2 neutralization which endorses original antigenic sin.

RESULTS

Clinical characteristics of study participants

Twenty SARS-CoV-2 RT-PCR-confirmed patients with severe COVID-19 that were admitted to the ICU with ARDS were included in this study. Additionally, twelve SARS-CoV-2 RT-PCR-confirmed COVID-19 patients with only mild coronavirus infection related symptoms and six RT-PCR-negative disease controls with similar mild symptoms were included. None of these patients with mild disease were admitted to the hospital. There was no significant difference in gender ($p \geq 0.28$) but patients with severe COVID-19 were older (average 63 years, range 29-75) than those with mild COVID-19 (50 years, range 33-66, $p=0.0028$) and controls (52 years, range 38-62, $p=0.023$). A selection of six patients with severe COVID-19 (patient 3, 4, 5 ,12 ,14 and 15, Table 1) were tested negative for NL63, OC43 and 229E at two time points by RT-PCR, ruling out CCC co-infections (data not shown). Six patients with severe COVID-19 were included in the first week post onset of symptoms, nine additional patients were included in the second week, three additional patients in the third week and two additional patients in the fifth week. Longitudinal samples were available from 15/20 patients with severe

COVID-19. From all patients with mild COVID-19 and disease controls, samples were collected in the first- (at nasopharyngeal swab sampling for RT-PCR) and third-week post onset of symptoms. Patient characteristics and information of blood collection time points are described in Table 1.

Serum IgG titers towards a range of coronavirus N and S antigens increase during SARS-CoV-2 infection in severe COVID-19 patients

Serum titers to CCC and epidemic CoV antigens were simultaneously determined by protein microarray (PMA), including N, S_{ECTO}, the outer head domain of S (S_1) and the receptor binding domain of S (S_{RBD}) antigens. Serum IgG responses specific for SARS2-N and influenza virus hemagglutinin H1N1(2009) (HA), included as a respiratory infection control virus, were determined separately by enzyme-linked immunosorbent assay (ELISA). Mild and severe COVID-19 patients mounted a SARS-CoV-2 immune response as shown by the induction of serum IgG titers against SARS2-N, SARS2-S_{ECTO}, SARS2-S_1 and SARS2-S_{RBD} (Figure 1). SARS-CoV-2 IgG titers were higher in patients with severe compared to mild COVID-19 and titers increased over the course of the infection. SARS-CoV-2 negative disease controls remained IgG seronegative for all SARS-CoV-2 antigens (Figure 1) and SARS-CoV-2 virus neutralization (data not shown). Influenza virus HA IgG titers remained stable over time in all patient groups. Patients recruited for this study were likely never exposed to SARS-CoV and MERS-CoV as seroprevalence in the general population is very low (<0.2%) (28, 29). Nevertheless, there was an IgG response towards all SARS-CoV antigens and MERS-S_{ECTO} in all patients with severe and the majority of patients with mild disease. Although the SARS-CoV N (SARS-N) IgG titers appeared higher than the SARS2-N, this likely reflects a difference in sensitivity of PMA versus ELISA. Given the high structural similarities between the respective structural proteins of SARS-CoV and SARS-CoV-2 and between MERS-S_2 and SARS2-S_2 (19, 24), it is plausible that a cross-reactive response was mounted. Close relatedness between SARS-CoV and SARS-CoV-2 likely also explains the strong correlation between SARS-N and SARS2-N IgG titers ($R=0.93$, $p<0.0001$, Supplementary Figure 1A).

All patients with mild and severe disease exhibited substantial IgG reactivity towards at least one of the 229E, NL63, HKU1 or OC43 antigens, suggesting they were seropositive for all CCC. Besides the increasing SARS-CoV-2 IgG response, serum IgG reactivity towards various CCC antigens increased in patients with mild and severe COVID-19. This increment was only significant in patients with severe COVID-19 for 229E-N, NL63-N and OC43-S_{ECTO}. OC43-S_{ECTO} IgG titers were already immunodominant at inclusion for all patient groups. In patients with severe COVID-19, these titers increased significantly more over time compared to mild cases in week three post onset of symptoms (Figure 1).

Frequencies of circulating B-cells targeting N and S of SARS-CoV-2 and CCC increase following SARS-CoV-2 infection

To analyze the kinetics of the CoV-specific B-cell response, we longitudinally enumerated circulating B-cell clones specific for CoV antigens using B-cell profiling (Figure 2). To that aim, we isolated CD19+ B-cells from peripheral blood mononuclear cells (PBMC) of a control partient, six patients with mild and seventeen patients with severe COVID-19, which were paired to the serum samples analyzed in the previous section (Table 1). CD19+ B-cells were stimulated in an antigen and B-cell receptor independent manner in oligoclonal cultures at a limiting dilution. This culture system ensured an unbiased clonal analysis of the CoV-specific response. Supernatants were individually screened for IgG reactivity towards all available CoV antigens using PMA. The frequency of reactive wells was 15%±14 for N and 22%±15 for S (average±SD). The number of reactive B-cell supernatants was normalized for the number of screened B-cells per patient in order to compare frequencies between different samples.

Overall, the frequency of in vitro stimulated peripheral blood-derived B-cells reflected total serum IgG reactivity, with a few exceptions. Potentially, differences in B-cell counts and serum IgG titers reflect in vitro and in vivo differences in B-cell activation or regulation. Similar to serum IgG reactivity, COVID-19 patients showed a strong expansion in SARS-CoV-2 reactive B-cells, which was most prominent in patients with severe disease. Moreover, the

number of B-cell clones reactive towards various CCC and epidemic CoV antigens expanded upon SARS-CoV-2 infection. This increase in CCC-specific B-cells was most striking for 229E-N, NL63-N, HKU1-N, OC43-S_{ECTO} and 229E-S_1. The frequency of HKU1-N reactive B-cells was higher than expected based on the low serum reactivity. The OC43-S_{ECTO} B-cell response was immunodominant in patients with severe COVID-19 and increased significantly in the following weeks. This response was significantly higher in severe COVID-19 compared to mild COVID-19 in the third week post onset of symptoms. Contrastingly, the frequency of 229E-S_1 reactive B-cells was immunodominant in patients with mild COVID-19 and increased significantly over time (Figure 2). However, similar to HKU1-N-specific B-cells, these differences were not observed in serum IgG titers (Figure 1).

B-cells reactive to heterologous coronaviruses are differentially boosted in mild and severe COVID-19 patients

The outgrowth of B-cells that target heterologous CoV strains is potentially driven by cross-reactivity to SARS-CoV-2. Therefore, we analyzed the multiplex PMA profiles of in vitro stimulated B-cell cultures to address the level of cross-reactivity of monoclonal IgG. A longitudinal profile of CoV antigen-reactive cultures that represents the observed kinetics in the pooled analysis (Figure 2) is shown for each patient group (Figure 3).

N-reactive IgG clones from the three representative donors are shown at different time points. In the case of the control patient, the number of detected N-reactive clones decreased, but for COVID-19 patients the number of N-specific B-cell clones increased in the patient with mild disease (n=20 in week one and n=42 in week three) and severe disease (n=17 at day 9, n=42 at day 16 and n=66 at day 23). For all patients, a substantial number of clones cross-reacted between 229E-N, NL63-N and HKU1-N. In contrast, the IgG clones reacting to OC43-N, MERS-N and SARS-N showed minor to no binding to CCC N antigens on PMA (Figure 3A). Analysis of selected SARS-N binding clones on SARS-N and SARS2-N ELISA showed significant cross-reactivity between both antigens (R=0.49, p=0.0034) (Supplementary Figure

1B), which confirmed that the serum IgG response to SARS-N is due to cross-reactivity with SARS2-N.

The control patient showed stable S-reactivity over time. Some SARS-CoV-2 reactive B-cells were detected and most cross-reacted with CCC. These likely represent pre-existing CCC-specific clones or naïve B-cells. In contrast, both COVID-19 patients showed an expanding, mostly type-specific SARS-CoV-2 spike response with increasing numbers of SARS2-S_{ECTO} and SARS2-S_1 reactive B-cell clones. In the case of the patient with mild COVID-19, no SARS2-S_{ECTO}-specific IgG clones were detected in the first week. In week three, 8/14 (57%) SARS2-S_{ECTO}-specific clones also reacted with SARS2-S_1. In the case of the patient with severe COVID-19, 0/3 SARS2-S_{ECTO}-specific IgG clones reacted with SARS2-S_1 at day 9, 10/13=77% at day 16 and 22/30 (73%) at day 23 (Figure 3B). The relative increasing number of SARS2-S_{ECTO} and SARS2-S_1 reactive clones indicated that over time, S_1 reactive clones are positively selected *in vivo* in both the mild and severe COVID-19 patients This domain is the target of most potent neutralizing antibodies (5). Therefore, this indicates maturation of a SARS-CoV-2-specific IgG response.

Clonal S-specific IgG cross-reactivity patterns were similar between the control patient and week one of the mild COVID-19 case. However, patterns were strikingly different between the two representative COVID-19 patients. A broadly cross-reactive S_1 response was present in the patient with mild COVID-19 in the first week of symptoms (8/22=36% of S_1-specific clones cross-reacted) and 229E-S_1 was immunodominant (11/27=41% of total S-specific clones). Three weeks after onset of symptoms, more clones were detected, the S-IgG cross-reactivity was reduced (11/40=28% of S_1-specific clones cross-reacted with heterologous strains) and 229E-S_1 remained immunodominant (24/68=35% of total S-specific clones). The patient with severe COVID-19 displayed a stronger type specific SARS-CoV-2 response. This patient had less cross-reactivity between S_1 antigens compared to the patient with mild COVID-19 (1/19=5% of S_1 clones cross-reacted at day 9, 4/21=19% at day 16 and 5/45=11% at day 23). OC43-S_{ECTO} clones dominated the coronavirus-specific IgG response on day 9 (29/57=51% of total S-specific clones) and these were prominently boosted on day 16

(149/170=88%) and day 23 (119/159=75%). Although many OC43-S_{ECTO} clones cross-reacted with SARS-CoV S_{ECTO} (SARS-S_{ECTO}) (101/297=34% on all three days combined), a limited number showed binding to SARS-CoV-2 antigens on PMA (18/297=6%) (Figure 3B).

N-specific IgG have similar cross-reactive patterns in mild and severe COVID-19, while S-specific IgG show differential cross-reactivity patterns in both patient groups

In order to confirm our previous findings and identify additional differences in the IgG cross-reactivity patterns between patients with mild and severe COVID-19, we analyzed all coronavirus-specific IgG clones that were detected at available overlapping time points. Specifically, week one (n=6 mild and n=3 severe COVID-19) and week three after onset of clinical symptoms (n=6 mild and n=11 severe COVID-19) were compared. In order to quantify cross-reactivity between antigens we used Pearson regression analysis.

For N, this analysis validated that 229E-N, NL63-N and HKU1-N IgG clones cross-reacted in patients with mild and severe COVID-19 during the first week of SARS-CoV-2 infection. Three weeks after onset of symptoms, the cross-reactivity patterns that were observed in week one became more robust in both COVID-19 patient groups. Additionally, weak yet highly significant correlations between OC43-N and the other CCC strains were observed. However, no significant cross-reactivity was detected between CCC and epidemic CoV strains indicating that different epitopes are involved (Figure 4A).

The combined analysis of all S-IgG clones showed complex cross-reactivity patterns including CCC and epidemic strains. In patients with mild disease sampled in the first week after onset of symptoms we confirmed the significant cross-reactivity between S_1 of CCC and epidemic strains (Figure 3 and 4B). Contrastingly, patients with severe disease sampled in the first week displayed a weak but broadly S-cross-reactive response, including S_{ECTO} and S_1 antigens. Moreover, matched S_1 and S_{ECTO} antigens of each CoV strains significantly correlated, indicating substantial reactivity towards an S_1 epitope (Figure 4B). Three weeks after onset of symptoms, cross-reactivity patterns of both COVID-19 groups differed from the ones observed in week one. For patients with mild disease, we confirmed the cross-reactivity

of the S_1 response was greatly reduced. Conversely, strain matched S_1 and S_{ECTO} cross-reactivity increased. In contrast, patients with severe disease had a similar but more pronounced cross-reactive pattern in the third week compared to first week post onset of symptoms. Reactivity with shared epitopes in all SARS-CoV-2 antigens increased. However, cross-reactivity between CCC and epidemic CoV on S_1 antigens disappeared almost completely. Strikingly, patients with severe disease displayed a significant negative correlation between the immunodominant OC43-S_{ECTO} IgG response and all SARS-CoV-2 antigens (Figure 4B). This confirms that OC43-S_{ECTO} reactive clones are overall unlikely to show detectable cross-reactivity with SARS-CoV-2 on PMA in severe COVID-19 patients.

The majority of boosted OC43-S_{ECTO} reactive clones did not react with OC43-S_1. To determine if they were reactive to S_2, we analyzed OC43-S_2 reactivity of randomly selected OC43-S_{ECTO}-reactive clones on ELISA. Out data confirms that all selected OC43-S_{ECTO} clones bind to OC43-S_2 (Supplementary Figure 2).

IgG cross-reactivity between OC43-S_{ECTO} and SARS-CoV-2 S antigens remains limited over time in severe COVID-19

In depth analysis of the boosted immunodominant OC43-S_{ECTO} response in all patients with severe COVID-19 where PBMC were available (n=17, Table 1) confirmed that the majority of OC43-S_{ECTO} clones (752/920=82% of clones) did not cross-react with SARS-CoV-2. However, the relative number of OC43-S_{ECTO} clones that cross-reacted with SARS-CoV-2 S antigens moderately increased over time (from 13% to 21%, grouped per week after onset of symptoms). Strikingly, this minority of OC43-S_{ECTO}-reactive clones that cross-reacted with SARS-CoV-2 increasingly recognized epitopes in SARS2-S_1 and SARS2-S_{RBD} (Figure 5). This suggests that there is incremental recognition of epitopes with high neutralization potential in a minority of OC43-S_{ECTO} clones (5, 21).

No evidence for functional contribution of seasonal CoV-specific antibodies to SARS-CoV-2 neutralization

To determine what CoV-reactive IgG functionally contribute to SARS-CoV-2 neutralization we correlated serum S reactive IgG titers to the 50% plaque reduction neutralization test ($PRNT_{50}$) for SARS-CoV-2. Serum SARS2-S_{ECTO}, SARS2-S_1 and SARS2-S_{RBD}-specific IgG titers selectively correlated with serum SARS-CoV-2 $PRNT_{50}$ titers. All other S-specific IgG titers did not correlate with neutralization (Figure 6A).

To determine which circulating CoV S-specific B-cells contribute to SARS-CoV-2 neutralization, the normalized S-reactive B-cell counts were correlated with paired serum SARS-CoV-2 $PRNT_{50}$. Similar to serum IgG titers, SARS2-S_{ECTO}, SARS2-S_1 and SARS2-S_{RBD} specific B-cells correlated with serum $PRNT_{50}$ titers. Additionally, the frequency of OC43-S_1 and SARS-S_1-specific B-cells showed a positive correlation with $PRNT_{50}$. Notably, the frequency of OC43-S_1-reactive B-cells is low (<0.01% of screened B-cells) and therefore likely has limited contribution to the total SARS-CoV-2 virus neutralization response (Figure 6B).

DISCUSSION

Multiplex IgG analysis of paired serum and culture supernatants of in vitro stimulated B-cells from patients with mild and severe COVID-19 and disease controls allowed us to *i)* show the kinetics and magnitude of the SARS-CoV-2 IgG response in the context of the CCC-specific immunological background, *ii)* perform in-depth analysis of a large number of IgG B-cell clones (in total 2420 N-specific and 3261 S-specific B-cell clones) that are representative of the complete CoV immunological breadth, *iii)* distinguish de novo induced strain-specific SARS-CoV-2-specific IgG clones from pre-existing CCC strain-specific IgG clones and CCC/SARS-CoV-2 cross-reactive IgG clones and *iv)* correlate our findings to disease severity.

We show that all COVID-19 patients displayed an evolving mostly type-specific SARS-CoV-2 specific IgG response. Cross-reactivity patterns differed between severe and mild patients. The magnitude of the SARS-CoV-2 response in patients with severe disease was greater than with mild disease. Notably, those with severe disease also displayed a strong rise of CCC-specific IgG and B-cell clones. Strikingly, this phenomenon did not correlate with detectable cross-reactivity towards SARS-CoV-2. Serum SARS-CoV-2 S-specific IgG and the frequency of the respective circulating B-cell clones correlated with SARS-CoV-2 neutralization titers. However, except for a minor fraction of OC43-S_1-specific B-cells, the boosted CCC-specific IgG response did not correlate with neutralization titers. These findings indicate that the boost of CCC-specific IgG in patients with severe COVID-19 does not contribute to SARS-CoV-2 neutralization. Notably, we do not observe a limited or delayed elicitation of a SARS-CoV-2 response in severe COVID-19 patients which may be a feature of OAS. However, the strong association with severe disease suggests a negative impact on clinical outcome of infection, which corroborates OAS (14–18, 30).

For N, serum IgG levels towards the alpha-CoV 229E and NL63 were immunodominant at inclusion of the study in all patients, yet they were selectively boosted in patients with severe COVID-19. The outgrowth of CCC N-specific B-cells was most striking for 229E, NL63 and the beta-CoV HKU1 in patients with mild and severe COVID-19. Very limited cross-reactivity was observed between CCC and epidemic CoV strain for this antigen.

We could not confirm this for all SARS2-N-reactive clones due to lack of the antigen on protein microarray. However, the SARS-N and SARS2-N-specific serum IgG titers (Supplementary Figure 1A) and SARS2-N reactivity of SARS-N-specific B-cell clones strongly correlated (Supplementary Figure 1B). These data, together with the high level of structural and sequence homology between both antigens (88.6%) (23, 24), suggests the SARS-N antigen can be used as a proxy for SARS2-N in our analysis.

For S, serum IgG levels towards the beta-CoV OC43 were immunodominant in all patients at inclusion and selectively boosted in severe COVID-19 cases, similar to immunodominant alpha-CoV N responses. This suggests that pre-existing immunodominant serum IgG responses towards heterologous strains are preferentially boosted by SARS-CoV-2, even though amino acid identity with other strains is higher (23). This is a key feature of OAS (12, 13, 18).

In the first week after onset of symptoms, significant cross-reactivity was observed between S_1 of SARS-CoV-2 and alpha and beta CoV. These correlations were strongest in patients with mild COVID-19. Potentially, an early S_1 cross-reactive response sets the stage for rapid development of an antibody response that protects against severe disease. However, the breadth of S_1 cross-reactivity declines over time.

The outgrowth of CCC S-reactive B-cell clones was most striking for OC43-S_{ECTO} in severe COVID-19 cases. Of OC43-S_{ECTO} reactive clones only a minor fraction reacted with a shared epitope in OC43-S_1 (66/920=7% of OC43-S_{ECTO} clones) and the majority recognized OC43-S_2, which corresponds with previous studies (24, 26, 31). The fraction of the immunodominant OC43-S_{ECTO}-specific clones that showed detectable cross-reactivity with SARS-CoV-2 antigens on PMA was limited. Of these cross-reactive clones, reactivity was increasingly directed towards SARS2-S_1 and SARS2-S_{RBD}. Of interest, IgG targeting S_1 and S_{RBD} confer the strongest neutralization potential (5, 20). Indeed, the minority of cross-reactive OC43-S_1 clones contributed to SARS-CoV-2 neutralization but the dominant OC43-S_2 IgG response did not correlate with SARS-CoV-2 neutralization. The lack of detectable cross-reactivity between CCC S-specific IgG and SARS2-S_{ECTO} is congruent with their lack of SARS-

CoV-2 neutralization potential. While it is possible that CCC-specific IgG restrict viral replication via Fc-mediated mechanisms, they may also play no role, or even have a detrimental effect by delaying the development of a type-specific response or by enhancing immune pathology (11, 32).

Likely, the avidity of boosted IgG is below the detection limit of PMA, yet is sufficient to drive expansion of the respective CCC-specific B-cells in vivo. As the clonal selection of B-cells is driven by the affinity of the B-cell antigen receptor, one would expect that clones with detectable high-affinity cross-reactivity would overgrow low-affinity clones. Indeed, the fraction of OC43-S_{ECTO}-specific clones that cross-reacts with SARS-CoV-2 antigens marginally increases over time (13% in week 1 to 21% after 4 weeks). Nevertheless, this process is too slow to substantially contribute to the SARS-CoV-2 neutralizing response. Alternatively, boosted OC43-S_2 clones target cryptic epitopes that are not exposed in the stabilized trimeric conformation of SARS2-S_{ECTO} on PMA. Potentially, the respective SARS2-S_2 epitope is only available after conformational changes that occur after ACE-2 binding or in linear epitopes that are only available in degraded or denatured proteins in vivo. The stable influenza-specific IgG responses over time in all patient groups suggests this SARS-CoV-2 induced back boost is CoV-specific. This argues against a general inflammatory state to drive IgG production in a fully antigen-independent manner. The mechanism underlying the outgrowth of CCC-specific B-cells remains to be determined.

A limitation of our study is that clonality was not confirmed. However, on average, less than 22% of oligoclonal cultures showed reactivity towards at least one antigen which suggests the majority of cultures contained only a single reactive clone. Furthermore, to correct for potential dual reactive cultures, we performed Pearson regression analysis to ensures that only robust and significant cross-reactivity patterns are identified that are not influenced by rare artifactual cross-reactive events. A second limitation is that due to the limited amount of culture supernatant, we were not able to confirm the neutralization potential of individual S-specific IgG clones.

In conclusion, we find robust OAS in patients with severe COVID-19. Boosted CCC-specific IgG do not substantially contribute to SARS-CoV-2 neutralization, which is considered key in immune protection. Moreover, recent studies showed that the rise in CCC-specific IgG titers does not contribute to CCC neutralization and are not associated with protection (33, 34). These findings mitigate any positive effect of CCC antibody backboost in maintaining broad immunity towards preceding infections as was described for influenza virus (12). The detailed insights in kinetics and cross-reactivity patterns of N and S-reactive IgG presented in this study will aid in the interpretation of serological studies and further expands our understanding of how the CCC-experienced humoral immune system responds towards SARS-CoV-2. Our study underscores that the immunological background of individuals needs to be considered as an important factor in assessing the quality and quantity of a newly initiated response towards SARS-CoV-2, either by infection or vaccination.

MATERIALS AND METHODS

Study design, patient characteristics and clinical specimen

Twelve PCR-confirmed SARS-CoV-2 infected healthcare workers of the Erasmus Medical Center (EMC) with mild COVID-19 symptoms, including fever, cough or myalgia, and six PCR negative mild disease control patients with similar clinical symptoms were included in the COVID-19 EMC health care worker study (MEC-2020-0264). Twenty PCR-confirmed patients with severe COVID-19 (35) that were admitted to the ICU of the EMC with ARDS were included in a biorepository study (MEC-2017-417). From patients with mild disease (health care workers), serum and PBMC were collected in the first and third week after onset of clinical symptoms. From patients with severe COVID-19, serum and PBMC were collected weekly with the initial sample taken/collected within two days post-admittance to the ICU, until patients were released from ICU. Additional serum samples from patients with severe disease were included in the study when available (Table 1). All samples were analyzed according to the SARS-CoV-2 protocol (MEC-2020-0222). Peripheral blood was collected in EDTA tubes for PBMC and SST-II tubes for serum (both BD Biosciences). PBMC were isolated using Lymphoprep (GE Healthcare) density gradient centrifugation and cryopreserved at -196 °C (36). Serum was isolated according to manufacturers' instructions and aliquots were stored at -80˚C.

B-cell profiling

Short-term B-cells were cultured essentially as described elsewhere (37). In brief, B-cells were isolated from cryopreserved PBMC using the EasySep human CD19 positive selection kit (Stem Cell technologies) according to manufacturer's instructions. Oligoclonal cultures of 100-300 CD19+ B-cells per well were seeded in 96-wells U-bottom plates in AIM-V AlbuMAX medium supplemented with 10% fetal bovine serum, penicillin-streptomycin (all Invitrogen) and beta-mercaptoethanol (Sigma) (B-cell medium). Oligoclonal B-cell cultures were stimulated in an antigen and B-cell receptor independent manner. Each culture was stimulated for 48 hours with 50 U/ml interleukin 2 (IL-2, Novartis), 10 ng/ml IL-10 (Peprotech), 25 ng/ml

IL-21 (Peprotech), 1 µg/ml resiquimod (Invivogen), and 1000 L-CD40L cells (kindly provided by Dr. J Banchereau, Lyon, France) that were growth arrested by 40 Gray γ-irradiation. CD40L expression and absence of mycoplasma was confirmed for L-CD40L cells. B-cells were subsequently cultured for 12 days in B-cell medium supplemented with 25ng/ml IL-21. Culture supernatants were harvested and reactivity of secreted IgG was determined using protein microarray analysis.

Protein microarray analysis

Serum IgG reactivity and reactivity of secreted IgG in oligoclonal culture supernatants towards an array of CoV N and S proteins was analyzed using protein microarray analysis (PMA) as described elsewhere (38). All S_{ECTO} antigens were kindly provided by Dr. Berend Jan Bosch, Utrecht University, the Netherlands (39–43). All S_1 antigens were produced in house at the Erasmus MC, the Netherlands (23). N antigens were derived from commercial sources. Details on the antigens used are shown in Supplementary Table 1. Using a standard panel of control sera, the minimal amount of antigen required to reach a plateau in serum titration assays was determined for each antigen. These standard sera were also used to validate consistency of results by including them on each ELISA plate and testing each PMA batch that was used. MERS-S_{ECTO} reactivity was not determined in the control patient on PMA because this antigen batch did not pass quality control. Instead, MERS-S_{ECTO} IgG cross-reactivity for the control patients was determined using ELISA (Supplementary figure 3).

PMA slides were scanned using a Powerscanner (Tecan, Switzerland). Background was determined for each spot and mean fluorescence intensity signal (MFI, range 0 – 65,535) of two spots was calculated for each serum or culture supernatant. For B-cell culture supernatants, a cut-off was set at the average plus three times the standard deviation of 20 non-reactive cultures with a minimum MFI of 1,000. Serum IgG titers were calculated using 4-parameter logistic regression with inflection point as titer (25).

SARS-N, SARS2-N and influenza virus HA H1N1(2009) ELISA

Serum IgG titers or culture supernatant IgG reactivities towards SARS-N, SARS2-N, OC43-S_{ECTO}, OC43-S_2, MERS-S_{ECTO}, SARS2-S_{ECTO} and influenza virus HA of H1N1 2009 were separately determined by ELISA as described elsewhere (37). Antigens are specified in Supplementary table 1. In brief, high-binding Corning™ Costar™ 96-Well EIA/RIA Plates were coated overnight at 4°C with a titrated concentration of antigen that was required to reach a plateau in titration of standard sera. Plates were blocked with 1% BSA, 0.05% Tween-20 phosphate buffered saline (PBS) blocking buffer (BB) for 1h at 37°C. A dilution series of serum or a fixed dilution of B-cell supernatant were prepared in BB and incubated on the plates for 1h at 37°C. ELISA plates were washed with 0.05% Tween-20 PBS and incubated with goat anti-human IgG polyclonal antibody conjugated with horseradish peroxidase (Sigma A8667) in BB. Plates were washed with 0.05% Tween-20 PBS and incubated with 3,3',5,5'-Tetramethylbenzidine (Invitrogen). The peroxidase reaction was stopped by the addition of 0.5N sulfuric acid and optical density at 450 nm wavelength OD_{450} signal was analyzed on a Tecan Infinite F200 reader.

SARS-CoV-2 biosafety level 3 plaque reduction neutralization tests

SARS-CoV-2 neutralization titers were determined at biosafety level 3 facilities by plaque reduction neutralization tests ($PRNT_{50}$) as previously described (23).

Statistical analysis

Serum IgG and $PRNT_{50}$ titers and MFI values of PMA were ^{2}Log transformed for analysis. Two-way ANOVA with Tukey's multiple comparison test was used to assess differences in longitudinal serum IgG responses and B-cell clone counts within each patient group and of similar time points between patient groups. Cross-reactivity of clones was assessed using Pearson regression. Simple linear regression was used to correlate serum IgG, $PRNT_{50}$ and B-cell clone counts. Significant P-values, below 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****), are indicated throughout the manuscript. All analyses were performed using Graphpad Prism 9.

Study approval

Personal or deferred informed written consent was obtained from all study participants or from a legal representative, partner or family member of the participant, respectively. All studies were approved by the medical ethical committee of the Erasmus MC and performed in compliance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

Conceptualization: GPvN, MAB, BMW and MPGK. Methodology: MAB, BMW, EdB, and GPvN. Investigation: MAB, BMW, FDC, NMAO and GPvN. Visualization: GPvN. Funding acquisition: BLH, MPGK. Clinical sample acquisition: MPR, TL, HE, JPCvdA, DAMPJG, ECMvG, RDdV and CHGvK. Writing – original draft: GPvN and MAB. Writing – review & editing: BMW, RDdV, TL, RAMF, BHGR, MPGK

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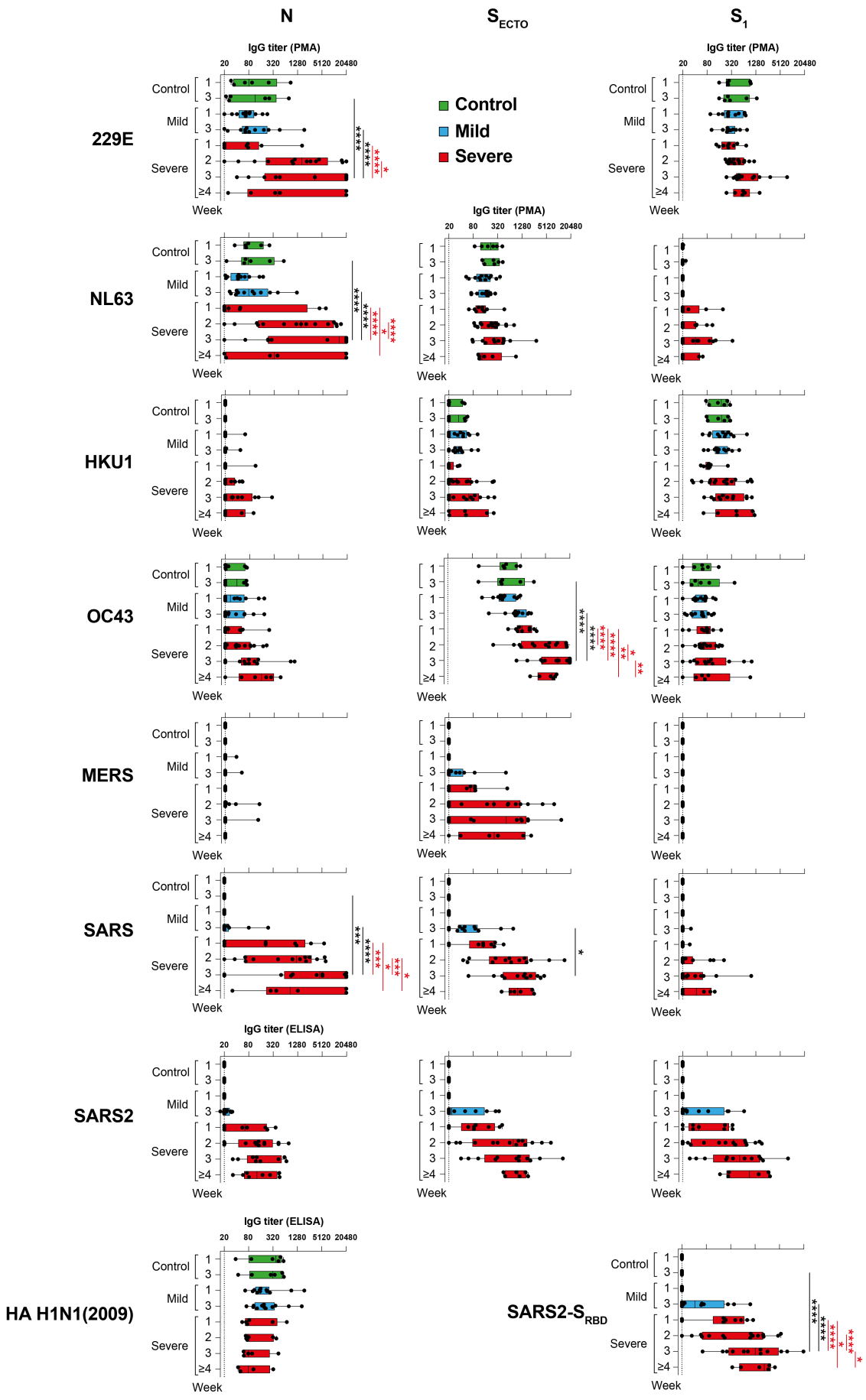
N
S_ECTO
S_1
Control
Mild
Severe
229E
NL63
HKU1
OC43
MERS
SARS
SARS2
HA H1N1(2009)
SARS2-S_RBD
IgG titer (PMA)
IgG titer (ELISA)
20 80 320 1280 5120 20480
Control
Mild
Severe
1 3 1 3 1 2 3 ≥4
Week

1

Figure 1. Patients with severe COVID-19 generate a strong serum SARS-CoV-2 IgG response and display an increasing IgG response towards other coronaviruses. Longitudinal serum IgG titers (x-axis) of six disease control donors (green boxes), 12 patients with mild (blue boxes) and 20 with severe COVID-19 (red boxes) grouped per week number after onset of symptoms (y-axis), towards a panel of CoV nucleocapsid proteins (N, left column), the ecto- (S_{ECTO}, middle column) and head-domains (S_1, right column) of spike, the SARS-CoV-2 (SARS2) receptor binding domain (S_{RBD}) and hemagglutinin (HA) of 2009 H1N1 influenza virus (listed along the y-axis). A SARS-CoV-2-specific response is mounted in all patients with COVID-19, together with a boost of seasonal human 229E, NL63, HKU1, OC43 and epidemic MERS, SARS and SARS2 coronavirus antigens. Dotted line shows the assay background. Boxes represent median, upper and lower quartile. Whiskers show range. P values calculated with a two-way ANOVA with Tukey's multiple comparison test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) are shown in black (significant inter-group differences) in blue (mild COVID-19 intra-group significant differences) or in red (severe COVID-19 intra-group significant differences).

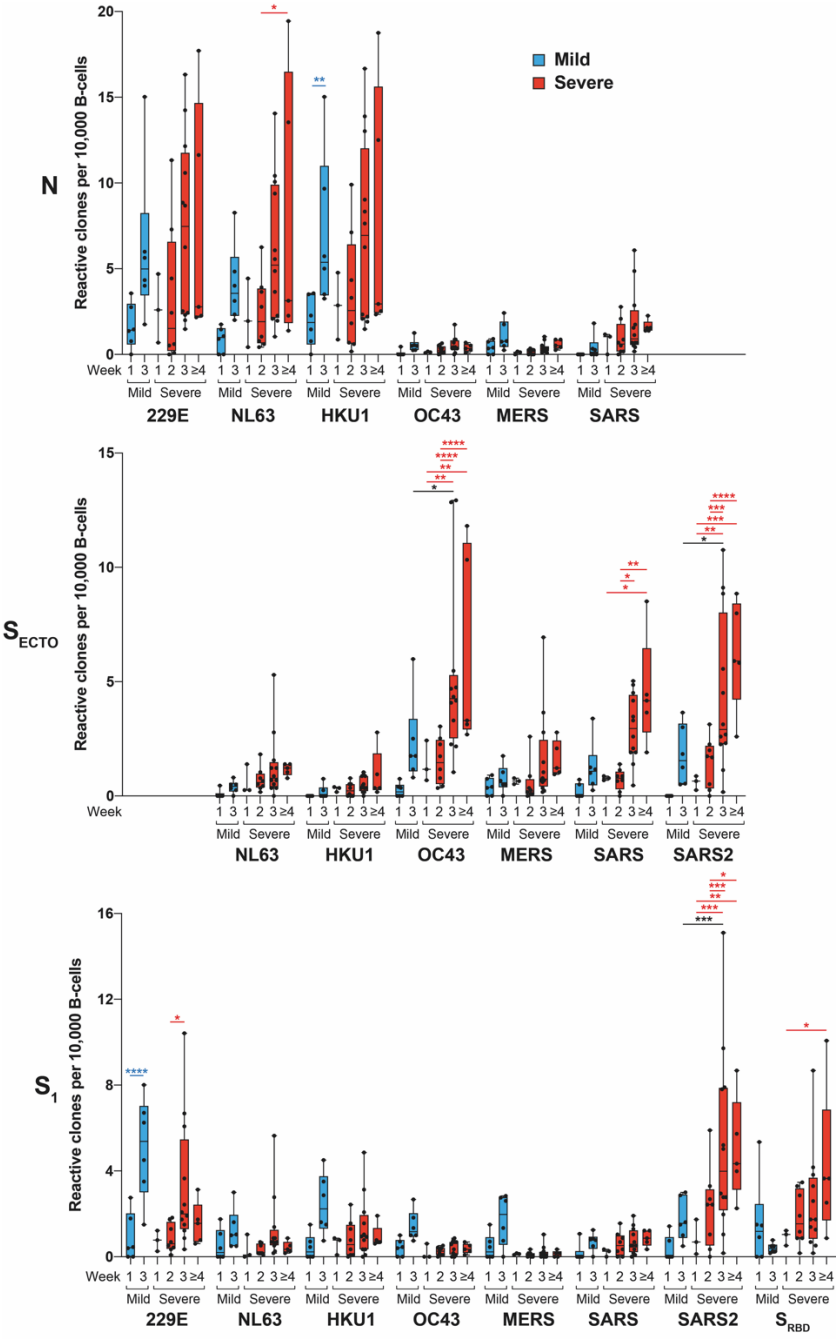

Figure 2. Patients with severe COVID-19 exhibit a prominent expansion of SARS-CoV-2 and OC43-S_{ECTO} specific IgG B-cells. Normalized enumeration of in vitro stimulated peripheral blood-derived IgG B-cells from patients with mild (blue boxes) and severe COVID-19 (red boxes) with reactivity towards N, S_{ECTO} and S_1 coronavirus antigens and SARS-CoV-2 S_{RBD}. Relative OC43-S_{ECTO}, SARS-S_{ECTO} SARS2-S_{ECTO}/S_1 and spike receptor binding domain (S_{RBD}) specific IgG B-cell clones show a highly significant outgrowth in the first four weeks after SARS-CoV-2 infection in patients with severe COVID-19. In patients with mild COVID-19, a highly significant increase is only seen for 229E-S_1-specific IgG clones three weeks after onset of symptoms. Boxes represent median, upper and lower quartile. Whiskers show range. P values calculated with a two-way ANOVA with Tukey's multiple comparison test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) are shown in black (inter-group significant differences) in blue (significant mild COVID-19 intra-group differences) or in red (severe COVID-19 intra-group significant differences).

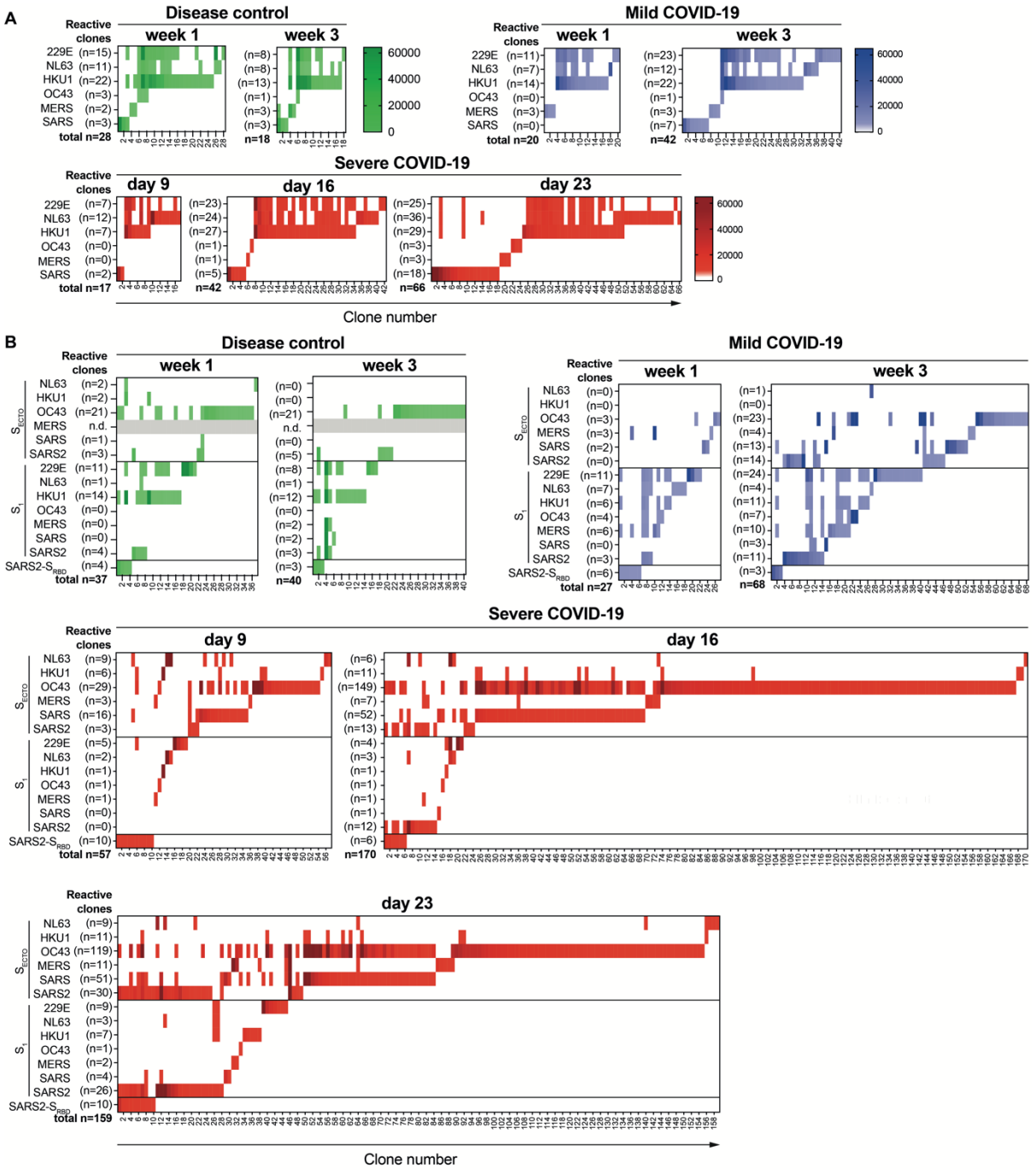

Figure 3. In vitro stimulated B-cells of a representative disease control, a mild and a severe COVID-19 case show clonal IgG cross-reactivity patterns towards different coronavirus strains. B-cells were isolated from peripheral blood samples and stimulated in vitro in oligoclonal cultures at limiting dilution to analyze IgG reactivity at the clonal level. The representative patients were analyzed at the indicated day/week post onset of symptoms. **A**) Heatmaps of the mean fluorescent intensity (MFI) of clonal IgG reactivity towards N of a panel of coronaviruses. The number of single- and cross-reactive N-specific B-cell clones (x-axis) remained stable in the disease control (green) but increased over time after SARS-CoV-2 infection in the mild (blue) and severe COVID-19 patient (red). **B**) Heatmaps of MFI of clonal IgG reactivity towards S_{ECTO}, S_1 and S_{RBD} antigens from the same representative patients. The disease control showed stable reactivity. Contrastingly, mostly the severe COVID-19 case displayed a strong boost of a OC43-S_{ECTO} clonal response that poorly cross-reacted with SARS-CoV-2 antigens.

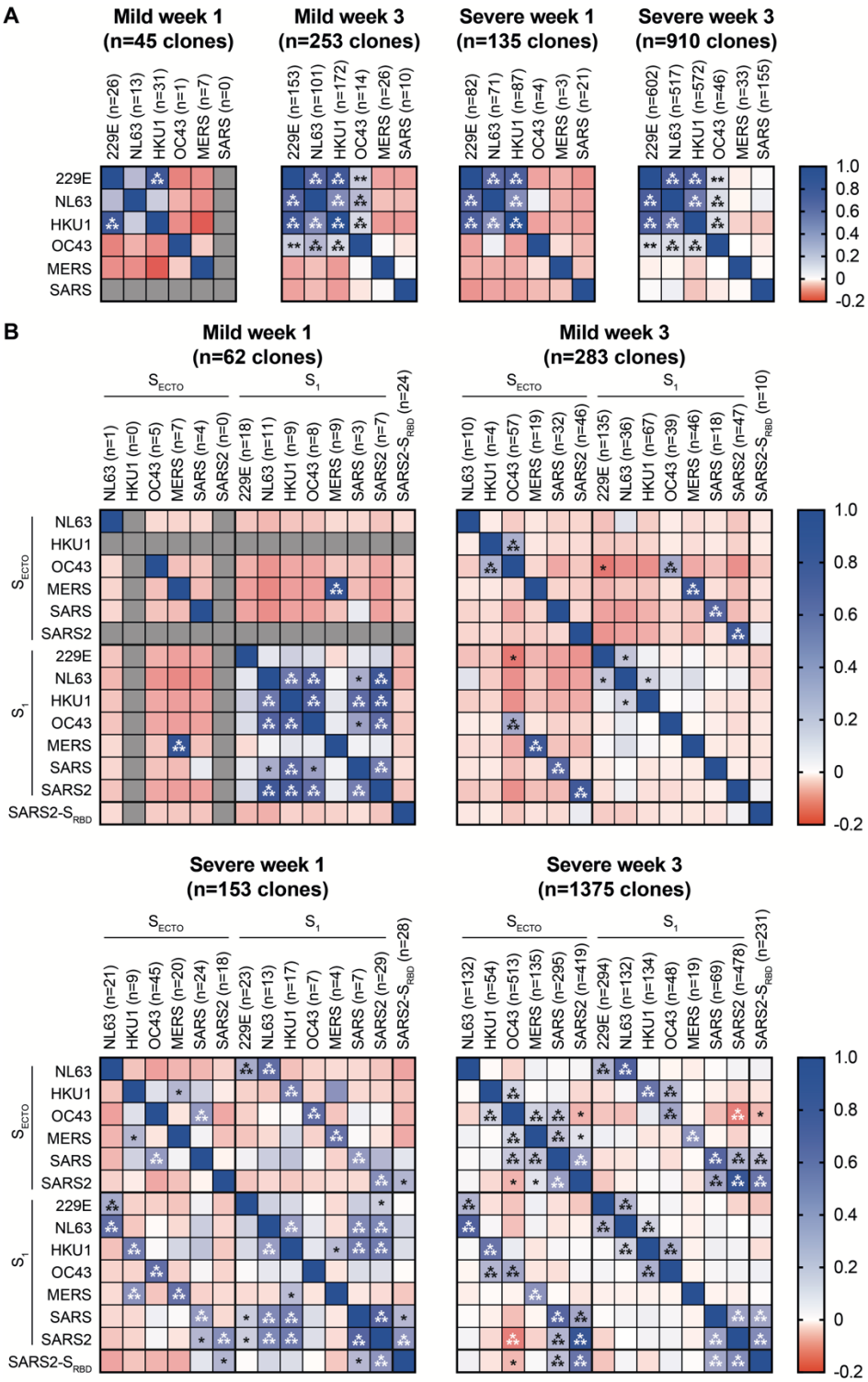

Figure 4. Combined analysis of reactive IgG clones from patients with mild or severe COVID-19 shows major differences in cross-reactivity patterns. Pearson correlation analysis of (**A**) N and (**B**) S-reactive IgG clones identified in mild COVID-19 patients (n=6) on week 1 and week 3 after onset of symptoms, and in severe COVID-19 patients on week 1 (n=3) and on week 3 (n=11) after onset of symptoms. Heatmaps show the R of Pearson regression (red to blue shades, range -0.2 to 1) and the significant p value (* p<0.05, **p<0.01, *** p<0.001) of these correlations.

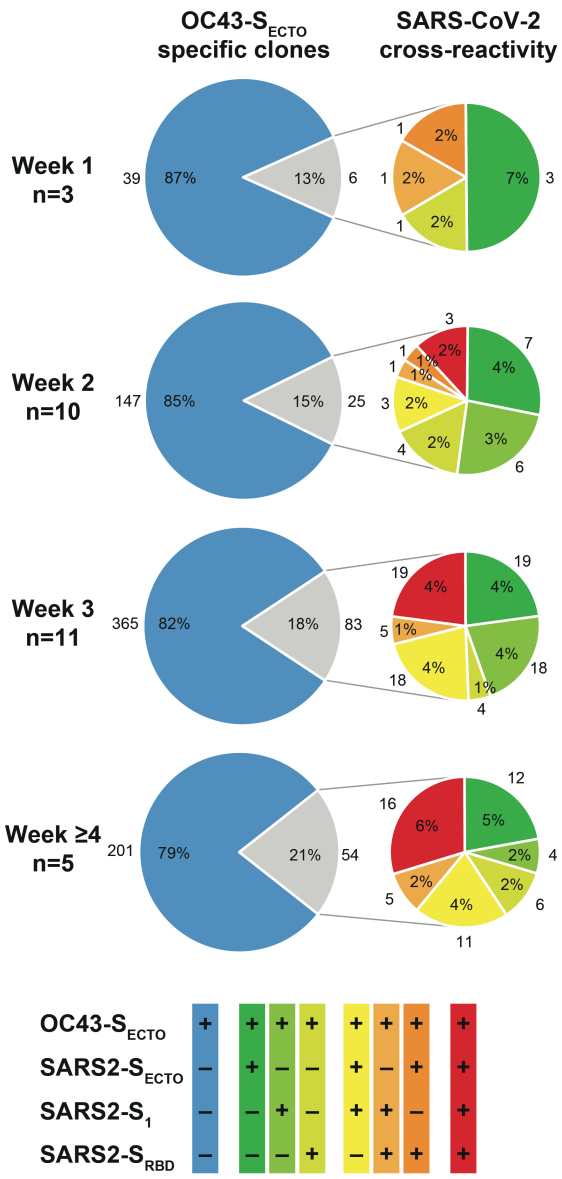

Figure 5. OC43-S$_{ECTO}$-reactive clones show limited yet antigenically evolving cross-reactivity towards SARS-CoV-2. All OC43-S$_{ECTO}$-reactive clones (n=920) from 17 patients with severe COVID-19 were stratified by week number post onset of clinical symptoms. The proportion of single OC43-S$_{ECTO}$ (blue) and SARS-CoV-2 cross-reactive clones (grey) is shown (left pie). The cross-reactivity towards a one (green colors), two (yellow and orange colors) and three SARS-CoV-2 antigens (red color) is depicted (right pie). The number of patients analyzed per week is indicated on the left. The frequency and number of contributing clones for each cluster is shown inside and adjacent to each part, respectively.

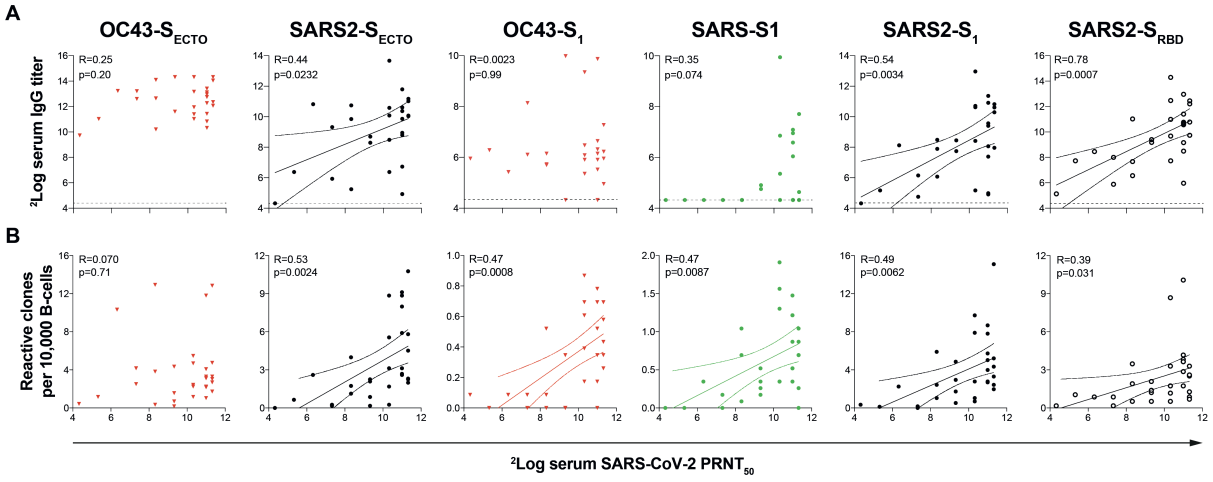

Figure 6. The IgG titers and normalized B-cell counts reactive towards SARS-CoV-2 S, but not OC43-S_{ECTO}, correlate with serum SARS-CoV-2 virus neutralization titers. A) Linear regression between ^{2}Log transformed serum IgG and SARS-CoV-2 virus neutralization ($PRNT_{50}$) titers shows that only SARS-CoV and SARS-CoV-2 spike-reactive IgG correlate with neutralization titers. Horizonal and vertical dotted line shows the background of the protein microarray assay. **B**) Normalized counts of B-cells specific for SARS-CoV-2 antigens, OC43-S_1 and SARS-S_1 selectively correlate with $PRNT_{50}$ titers. Serum samples of 20 patients and B-cell analysis of 17 patients with severe COVID-19 were included in these analyses**.** Solid line depicts the best-fit regression coefficient. Dashed line shows the 95% confidence of the best-fit line.

Table 1. Patient characteristics

Group	Gender (ratio)[a]	Average age (range)[b]	Average days at ICU (range)	Comorbidities, Complications, Clinical outcome	Patient Id[c]	Week 1[d]	Week 2	Week 3	Week ≥4
Severe (n=20)	13M, 7F (65:35)	63 (29–75)	20 (4–52)	Hypertension n=7 Type 2 diabetes n=5 Cardiac disease n=5 Lung disease n=3 Vascular disease n=2 Malignancy n=1 Neurological disease n=2, Thrombosis n=10, Recovered n=12 Deceased n=8	pt.1	d2			d23
					pt.2	d3/d4			
					pt.3	d4	d11	d18	
					pt.4	d5	d12	d19	
					pt.5	d5/d6	d12	d19	d26
					pt.6	d5		d19	
					pt.7		d8/d10		
					pt.8		d8/d10		
					pt.9		d8	d15	
					pt.10		d8	d15	
					pt.11		d9		
					pt.12		d9	d16	d23
					pt.13		d10/d12		
					pt.14		d10	d17	d24
					pt.15		d12	d19	d26
					pt.16			d16	
					pt.17			d18	
					pt.18			d20	
					pt.19				d29
					pt.20				d33
Mild (n=12)	5M, 7F (42:58) p=0.28	50 (33–66) p=0.0028	NA	ND, None n=12, Recovered n=12	Serum	n=12		n=12	
					B-cells	n=6		n=6	
Controls (n=6)	3M, 3F (50:50) p=0.64	52 (38–62) p=0.023	NA	ND, None n=6, Recovered n=6	Serum	n=6		n=6	
					B-cells	n=1			

ICU: intensive care unit, **M**: male, **F**: female, **ND**: not determined, **NA**: not applicable, [a]significant difference compared to patients with severe COVID-19 (Fisher's exact test), [b]significant difference compared to patients with severe COVID-19 (unpaired t test), [c]serum and paired peripheral blood B-cells samples collected from COVID-19 patients and disease controls, [d]sampled at week and day post onset of symptoms, underlined: paired B-cell analysis.

SUPPLEMENTARY MATERIAL:

Supplementary Figure 1. Serum IgG titers and IgG B-cell clone reactivity towards N of SARS-CoV and SARS-CoV-2 are strongly correlated

Supplementary figure 2. Boosted OC43-S_{ECTO} clones recognize epitopes in OC43-S_2

Supplementary figure 3. Clonal IgG response of a disease control patient towards OC43-, MERS- and SARS2-S_{ECTO}

Supplementary Table 1. Antigens used in this study, including a list of references