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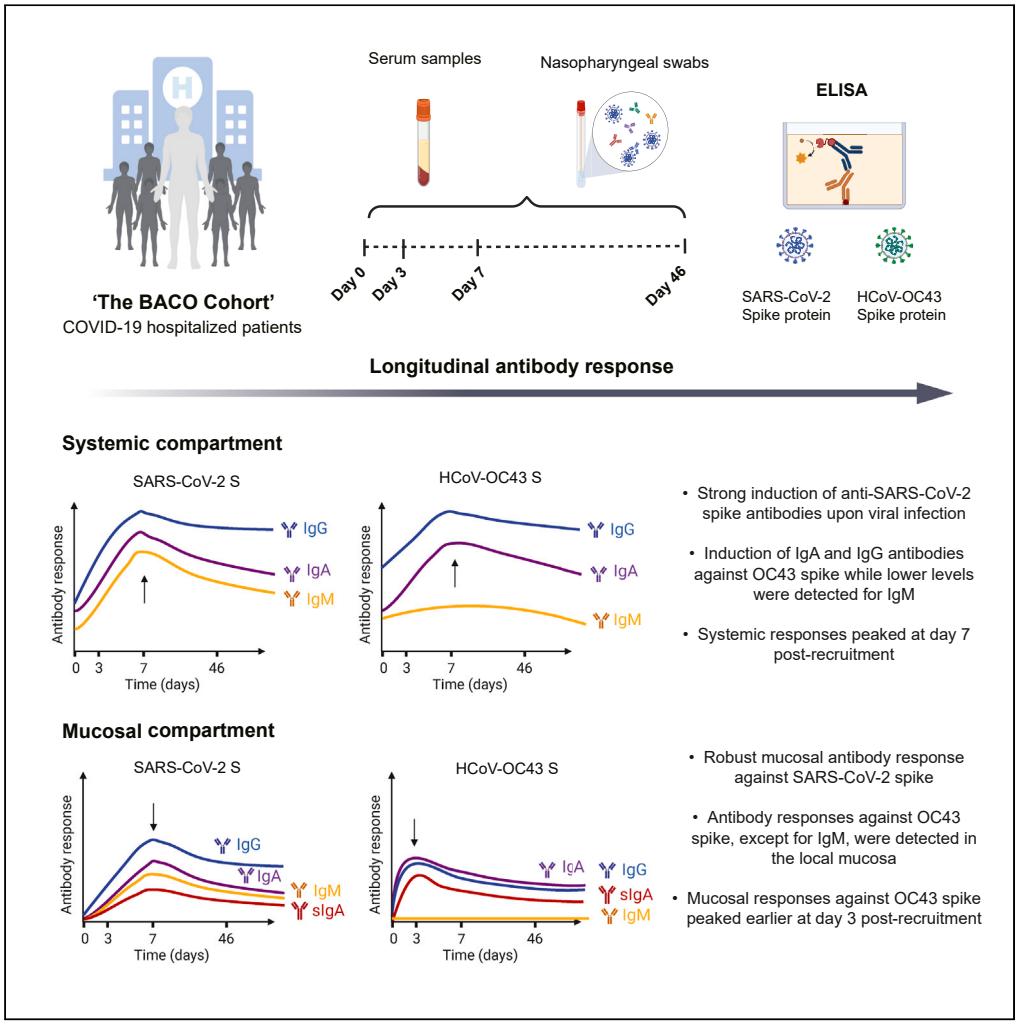

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Highlights

COVID-19 patients mounted robust mucosal antibody responses against SARS-CoV-2 S

COVID-19 patients showed IgG, sIgA, and IgA, but not IgM, responses against HCoV-OC43 S

Mucosal antibody titers against SARS-CoV-2 and OC43 peaked at day 7 and 3, respectively

Our data suggest an immune memory recall toward conserved beta-HCoVs epitopes

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SARS-CoV-2 infection induces robust mucosal antibody responses in the upper respiratory tract

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SUMMARY

Despite multiple research efforts to characterize coronavirus disease 2019 (COVID-19) in humans, there is no clear data on the specific role of mucosal immunity on COVID-19 disease. Here, we longitudinally profile the antibody response against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and seasonal HCoV-OC43 S proteins in serum and nasopharyngeal swabs from COVID-19 patients. Results showed that specific antibody responses against SARS-CoV-2 and HCoV-OC43 S proteins can be detected in the upper respiratory tract. We found that COVID-19 patients mounted a robust mucosal antibody response against SARS-CoV-2 S with specific secretory immunoglobulin A (sIgA), IgA, IgG, and IgM antibody subtypes detected in the nasal swabs. Additionally, COVID-19 patients showed IgG, IgA, and sIgA responses against HCoV-OC43 S in the local mucosa, whereas no specific IgM was detected. Interestingly, mucosal antibody titers against SARS-CoV-2 peaked at day 7, whereas HCoV-OC43 titers peaked earlier at day 3 post-recruitment, suggesting an immune memory recall to conserved epitopes of beta-HCoVs in the upper respiratory tract.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19), a respiratory illness that has affected more than 770 million people worldwide (https://covid19.who.int/, as September 2023). Since the emergence of this novel betacoronavirus in late 2019,[1] research efforts have been focused on understanding the nature and dynamics of the systemic immune responses against SARS-CoV-2 virus. Upon infection, COVID-19 patients rapidly produce immunoglobulin M (IgM), IgG, and IgA antibodies that predominantly target the spike (S) protein, the main surface glycoprotein that binds to the human angiotensin-converting enzyme 2 (ACE2) receptor and mediates viral entry into the host cell. Additionally, antibodies directed against the viral nucleocapsid protein have also been detected.[2–4] These antibodies are present in serum within the first week of symptom onset and have been shown to exert different properties such as binding, neutralizing, and Fc-mediated effector functions.[5,6] Although levels of these serum antibodies tend to decay, they can remain stable for months, especially in the case of IgG antibodies.[7–9] Additionally, local immune responses are also expected to be induced in the respiratory tract upon SARS-CoV-2 infection due to ACE2 receptor expression in the human airway epithelia and lung parenchyma.[10,11] Humoral responses in the mucosal compartment are mainly characterized by the production of secretory IgA (sIgA) antibodies.[12,13] These antibodies are generated through a complex multi-step process in which dimeric IgA antibodies secreted by local plasma cells are covalently linked by a protein component known as the joining (J) chain. Then, this complex migrates to the mucosal lumen where a proteolytic cleavage occurs, resulting in the attachment of dimeric IgA to the secretory component (SC). This SC is one of the main features of sIgA and protects the complex from proteolysis. Importantly, mucosal sIgA and IgA antibodies serve as the first line of defense[14,15] against respiratory pathogens such as influenza virus and can effectively block infection.[16,17] Similarly, some studies have reported the presence of virus-specific IgG and IgA in saliva and nasal secretions of patients with COVID-19 disease.[18–20] However, the specific role of local immune responses in mucosal surfaces upon SARS-CoV-2 infection is unclear. On the other hand, there is now evidence that pre-existing immunity against other seasonal human coronaviruses (HCoV) can modulate *de novo* immune responses against SARS-CoV-2 virus.[2,21–25] The authors[2]

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and others[24–26] have shown that antibody cross-reactivity between conserved epitopes from SARS-CoV-2 proteins and seasonal HCoVs may occur upon SARS-CoV-2 infection.[2] This effect led to an imprinted antibody response on the systemic responses to SARS-CoV-2 antigens. However, our knowledge about the consequences of pre-existing immunity and cross-reactivity to HCoVs on COVID-19 disease outcomes when considering mucosal immune memory is limited. Whether cross-reactive antibodies in respiratory secretions are protective or not against SARS-CoV-2 transmission or severe disease outcomes is not known.

Here, we expand on our previous study[2] and add data on the immune profile in the systemic and mucosal compartments by using our previously published clinical cohort study of SARS-CoV-2-infected individuals—the BACO cohort.[2] We longitudinally characterized the early antibody response and immunoglobulin repertoire against SARS-CoV-2 and seasonal HCoV-OC43 S proteins in the serum and nasopharyngeal (NP) swabs of COVID-19 patients. We found that specific antibody responses against SARS-CoV-2 S protein can be detected in nasal swabs early upon SARS-CoV-2 infection. Half of the patients showed detectable levels of IgG and IgM at baseline, whereas IgA and sIgA were found in 80% and 44% of infected patients, respectively. Moreover, COVID-19 patients showed an induction of IgG and IgA against HCoV-OC43, whereas no specific IgM levels were detected, suggesting a memory recall of pre-existing immune cells targeting conserved S epitopes shared between SARS-CoV-2 and HCoV-OC43 virus. Interestingly, mucosal antibody titers against SARS-CoV-2 peaked at day 7, similar to systemic responses, whereas specific antibodies against HCoV-OC43 showed a higher increase at day 3 post-recruitment. Despite intense efforts to monitor specific immune responses after SARS-CoV-2 infection, we still have limited knowledge about the role of mucosal immunity in COVID-19 disease. This study shows that SARS-CoV-2 infection induces robust mucosal immunity. Additionally, a back-boosting effect of human beta-HCoVs on the mucosal respiratory compartment was present upon SARS-CoV-2 infection.

RESULTS

Systemic antibody responses against SARS-CoV-2 and beta-HCoV-OC43

We used serum samples from a previously published longitudinal cohort of hospitalized COVID-19 patients from Barcelona, Spain—the BACO cohort—[2]to first expand on the systemic immunoglobulin profile against SARS-CoV-2 virus and HCoV-OC43 and second to characterize the immune responses to both SARS-CoV-2 virus and HCoV-OC43 in the local upper respiratory mucosa of COVID-19 patients. A detailed description of clinical characteristics and serum IgG responses against SARS-CoV-2 and other HCoVs antigens in the BACO cohort can be found in Aydillo et al.[2] and Table S1. Briefly, this clinical cohort was composed of a total of 37 COVID-19 patients who were hospitalized at the University Hospital of Bellvitge during the first pandemic wave of SARS-CoV-2 in Barcelona, Spain (March-May 2020). Study participants had a mean age of 67 years and 67% were male. Blood samples were collected longitudinally upon hospital admission (day 0, baseline) and at days 3 and 7 in 33 (89.1%) and 22 (59.4%) patients, respectively. An additional sample was collected at the convalescence period (mean time of 46 days, range 30–56 days) in 28 patients (75.7%). For the present study, we quantified the levels of IgA and IgM antibodies against SARS-CoV-2 and HCoV-OC43 full-length spike (S) protein in the serum samples using enzyme-linked immunosorbent assay (ELISA) and used these data to complement the IgG responses described in Aydillo et al.[2] In general, we observed a strong induction of anti- SARS-CoV-2 S IgA, IgG, and IgM antibodies upon viral infection (Figures 1A and S1A). Antibody titers significantly increased up to day 7 and started waning during the convalesce phase in the case of IgM and IgA. As expected, we found that SARS-CoV-2 infection strongly boosted long-lasting IgG responses as compared with other immunoglobulin isotypes. Consistently, we observed that fold-increase peaked at day 7 post-recruitment, and only IgA and IgM responses decreased at the convalescent time point reaching titers below those detected at day 3 (Figure 1B, and Tables S2 and S3).

We previously showed that the COVID-19 patients from The BACO cohort developed a strong IgG response against the conserved S2 domain of the beta-HCoVs S protein upon SARS-CoV-2 infection. This back-boosting effect related to residual effects from past virus exposures to the antigenically related beta-HCoV-OC43 and HCoV-HKU1.[2] Therefore, we next characterized IgA and IgM against the S protein of HCoVs-OC43 in serum. All patients mounted a strong IgA and IgG response against HCoV-OC43 S, whereas lower levels were detected for IgM antibodies (Figures 1C and S1B). Similar to SARS-CoV-2 responses, IgG titers against HCoV-OC43 S were strongly induced, and levels were higher compared with the other immunoglobulin subtypes. Besides, all immunoglobulins followed a similar induction pattern than antibodies directed against SARS-CoV-2, with peak titers at day 7 post-recruitment (Figure 1D, and Tables S2 and S3). Finally, and to understand whether differences on immunoglobulin levels could influence disease trajectory, we compared the serum antibody responses in patients according to disease severity. For this, COVID-19 patients were classified into mild/moderate (N = 26, 70.3%) or severe/severe end-of-organ disease (EOD, N = 11, 29.7%) based on a previously described severity scale.[27] Figure 2 shows the antibody responses against SARS-CoV-2 and HCoV-OC43 S antigens according to disease phenotype. Data showed no significant differences in the humoral immune response against SARS-CoV-2 nor HCoV-OC43 S protein in mild/moderate versus severe/severe EOD patients. However, severe patients seemed to have lower baseline antibody levels, suggesting a delay in mounting humoral responses against SARS-CoV-2 S as compared with moderate patients.

COVID-19 patients mount robust mucosal antibody responses against SARS-CoV-2 and HCoVs-OC43 in the upper respiratory tract

Immune responses in the mucosal compartment are largely mediated by IgA and secretory IgA (sIgA) antibodies, which have been shown to provide protection against some respiratory infections.[16,17] However, there are no clear data on the role of mucosal immunity on COVID-19 disease. Besides, it is not known whether pre-existing immunity against seasonal HCoVs in the upper respiratory tract could also mediate protection against SARS-CoV-2 infection. We used NP swabs from COVID-19 patients from the BACO cohort to investigate the nature

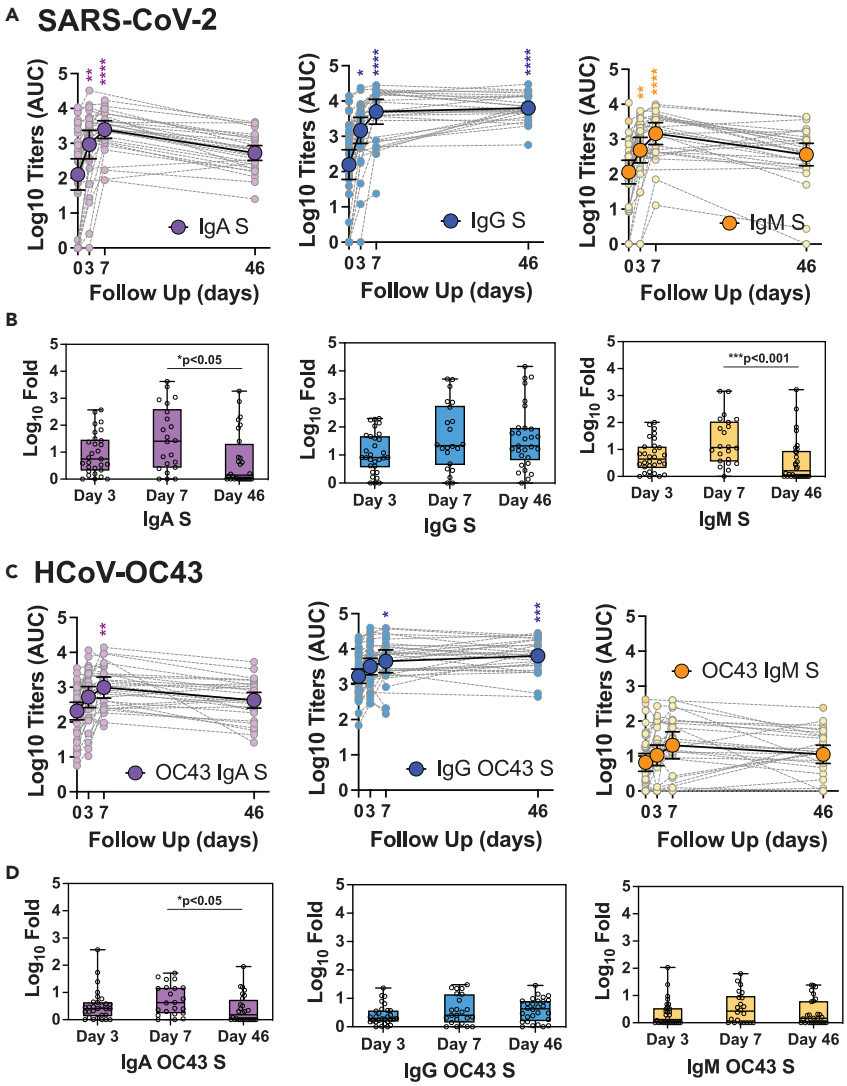

Figure 1. Longitudinal antibody profile against SARS-CoV-2 and seasonal beta-HCoV-OC43 spike proteins in serum

Serum samples from hospitalized COVID-19 patients were collected upon hospital admission (baseline, day 0) and days 3 and 7. A convalescence sample was collected from survivors after recovery with a mean time of 46 days (range, 30–56 days). IgA, IgG, and IgM antibody titers against SARS-CoV-2 full-length S protein (A) and OC43 full-length S protein (C). Antibody titers were calculated and represented as area under the curve (AUC). Small dots with dotted lines represent the antibody response of each individual over time. Geometric mean titer (GMT, big dots) and confidence interval (CI 95%) are also shown. Kruskal-Wallis test was performed to compare differences at each time point over baseline. Statistical significance was considered when $p \leq 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, not significant). Fold change antibody titers against SARS-CoV-2 full-length S protein (B) and HCoV-OC43 full-length S protein (D) represented as box-and-whisker diagrams. Box indicates interquartile range (IQR, Q1–Q3) with horizontal line showing the median and vertical lines indicating minimum and maximum. All individual values are represented as small dots. Kruskal-Wallis test was performed, and significant adjusted p values after pairwise comparisons are shown for each comparison. A total of 116 biologically independent serum samples (day 0 = 37, day 3 = 29, day 7 = 22, day 46 = 28) were run against SARS-CoV-2 and HCoV-OC43 S antigens to examine the three different immunoglobulin isotypes using ELISA. ELISAs for each antigen and isotype were performed once due to the limited amount of serum samples. A value of 1 was assigned to the samples with no detectable antibodies.

and dynamics of immune responses in the local immune compartment against both SARS-CoV-2 and HCoV-OC43 S antigens. For this, NP swabs were collected at the same time points than serum samples in 36 out of the 37 hospitalized COVID-19 patients. Acute NP specimens were taken longitudinally upon hospital admission (day 0, baseline) in 34 patients (94.4%) and at days 3 and 7 in 23 (63.9%) and 20 (55.6%) patients, respectively. A follow-up sample during the convalescence phase with a mean time of 46 days after hospital admission (range, 30–56 days) was also collected in four (11.1%) patients. To inactivate any potentially infectious SARS-CoV-2 virus in these samples, NP swabs were treated with Triton X-100 prior to performing any antibody quantification. Importantly, because it has been shown that total IgA antibodies in the saliva can vary between individuals and between samples from the same individual due to factors such as stress,[12,28] we first

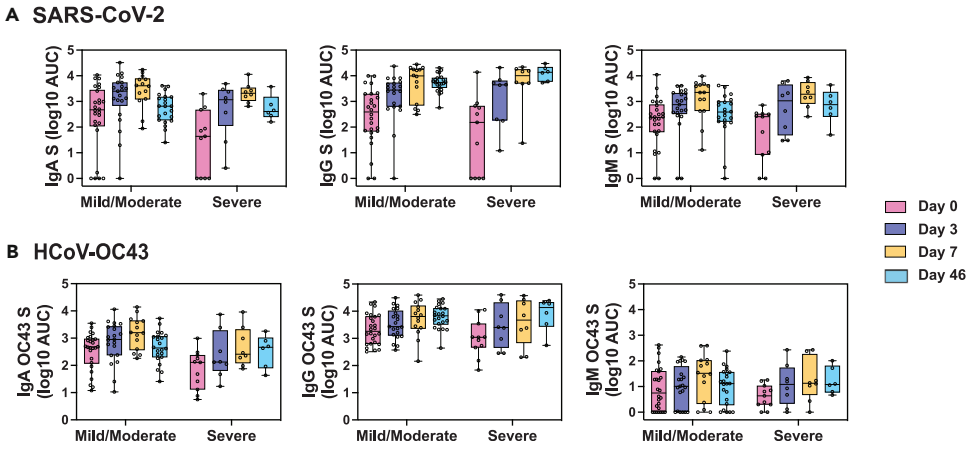

Figure 2. Systemic antibody response according to disease severity in the BACO cohort

Box-and-whisker diagrams of area under the curve (AUC) IgA, IgG, and IgM titers against SARS-CoV-2 S (A) and seasonal HCoV-OC43 S (B) in mild/moderate and severe COVID-19 patients from the BACO cohort. Severity of COVID-19 was assigned following a previously described severity scale.[27] Box indicates interquartile range (IQR, Q1–Q3), with horizontal line showing the median and vertical lines indicating minimum and maximum. All individual values are represented as small dots and each time point is shown in different colors. A total of 116 biologically independent serum samples (day 0 = 37, day 3 = 29, day 7 = 22, day 46 = 28) were run against SARS-CoV-2 and HCoV-OC43 S antigens to assess the antibody isotype profile of these patients using ELISA. A value of 1 was assigned to the samples with no detectable antibodies.

tested whether total IgA titers in the upper respiratory tract were different among the COVID-19 patients from the BACO cohort. Our results showed that COVID-19 patients had comparable concentrations of nasal IgA antibodies (Figure S2A), limiting any potential bias on the quantification of anti- SARS-CoV-2 sIgA antibodies in the nasal cavity. Next, we measured the levels of sIgA, IgA, IgG, and IgM against SARS-CoV-2 full-length S protein in these samples using ELISA assays. Results showed that COVID-19 patients from the BACO cohort developed detectable mucosal immune responses in the upper respiratory tract against SARS-CoV-2 S protein for all the immunoglobulin isotypes tested (Figures 3A and S3A). Additionally, antibody quantification showed higher antibody levels for IgA, IgG, and IgM subtypes compared with antigen-specific sIgA. Interestingly, antibody profiles showed similar kinetics than systemic responses, and peak titers were observed at day 7 post-hospitalization for all the immunoglobulin subtypes tested (Figure 3B and Tables S4 and S5). Next, we characterized and profiled the mucosal immune responses against seasonal HCoV-OC43 full-length S protein in the NP swabs (Figures 3C and S3B). Similar to our previous data on serum, a high percentage of COVID-19 patients showed induction of IgG (N = 15, 41.7%), IgA (N = 28, 77.8%), and sIgA (N = 15, 41.7%) antibodies against HCoV-OC43 S protein in the upper respiratory mucosa. These cross-reactive immune responses, probably directed against conserved epitopes of human beta-HCoVs, showed some degree of maturity as none of the patients showed detectable levels of IgM antibodies. Moreover, anti-OC43 S sIgA, IgA, and IgG titers peaked earlier at day 3 (Figure 3D; Tables S4 and S5), in contrast to the SARS-CoV-2 mucosal responses, suggesting a back-boosting effect upon SARS-CoV-2 infection. These data support our conclusion of this effect being a result of a recall of pre-existing immune memory cells toward conserved beta-HCoVs epitopes.

Next, and to investigate the relationship between systemic and mucosal immune compartments, we performed a correlation analysis of antibody titers in the paired serum samples and NP swabs. Interestingly, a strong correlation between serum and mucosal IgA, IgG, and IgM titers against SARS-CoV-2 S protein was found in the aggregate of samples (Spearman correlation coefficients: 0.54 (IgA), 0.64 (IgG), and 0.62 (IgM); p value <0.0001, respectively) (Figure 4A). On the contrary, serum and nasal anti-OC43 S protein IgA and IgG titers correlated poorly with Spearman correlation coefficients ranging from 0.17 for IgA and 0.34 for IgG levels (p value = 0.19 and 0.01, respectively) (Figure 4B). No correlation analysis was performed for IgM responses against OC43 S as no IgM titers were detected in the mucosal compartment. Additional correlation analysis according to time point of collection between serum and mucosal antibody titers against both SARS-CoV-2 and OC43 S was also performed (Figure S4). To understand whether a correlation was also found between different immunoglobulin isotypes, we performed additional correlation analysis between IgG and IgA serum and mucosal compartments. As expected, a significant positive correlation between both immunoglobulins was found (Figure S5).

Finally, we tested whether humoral immune responses against SARS-CoV-2 and HCoV-OC43 S antigens in the mucosal compartment correlated with disease outcomes. Similar to earlier discussion, we compared antibody responses in the mild/moderate (N = 25, 69.4%) versus severe/severe end-of-organ disease (EOD, N = 11, 30.6%) patients. Mann-Whitney test showed no significant differences in the antibody response against both SARS-CoV-2 and OC43 S antigens between mild/moderate and severe groups in the upper respiratory tract (Figures 5A and 5B). Nonetheless, severe patients tended to have lower early antibody levels similar to systemic responses.

DISCUSSION

The COVID-19 pandemic has highlighted the importance of understanding host immune responses against emerging pathogens. Many advances have been made in characterizing the immunopathogenesis of COVID-19. However, most of the effort has been focused on

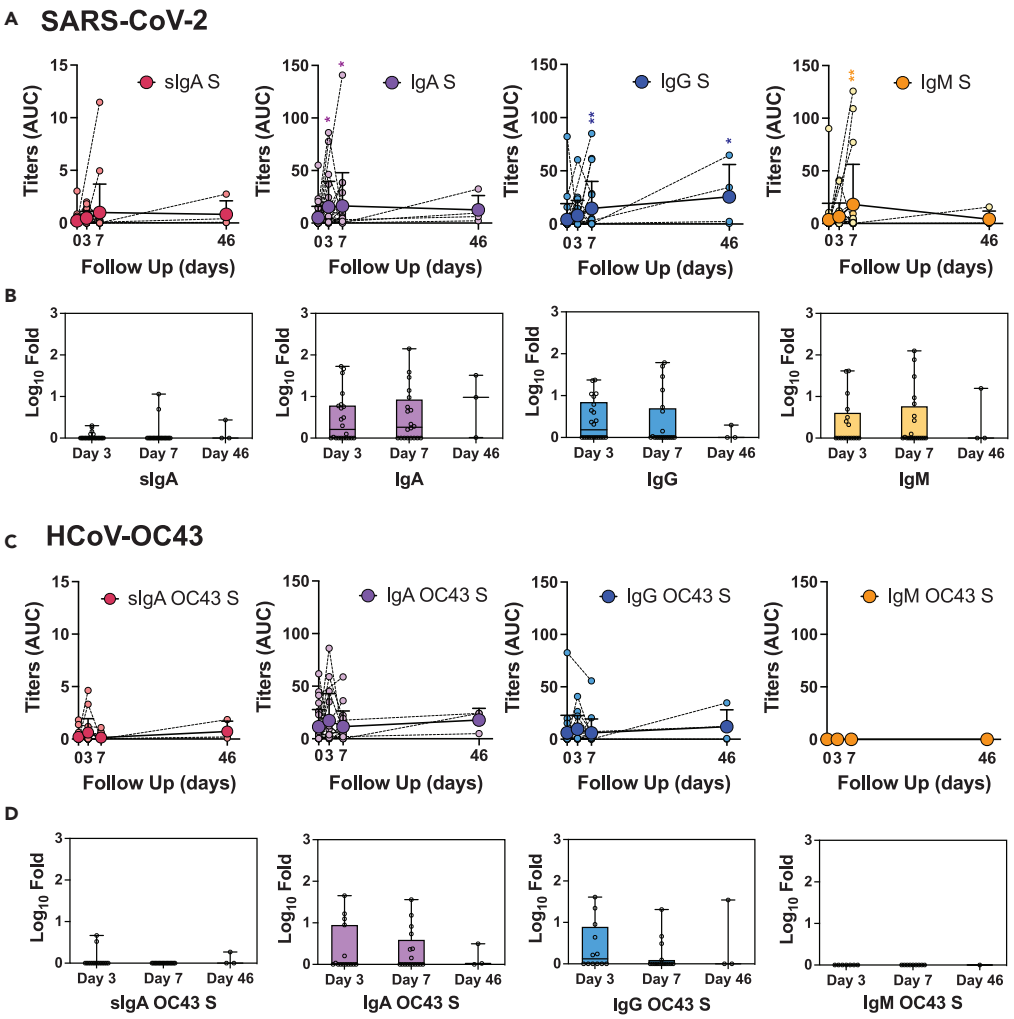

Figure 3. Longitudinal antibody profile against SARS-CoV-2 and seasonal beta-HCoV-OC43 spike proteins in upper respiratory tract

Nasopharyngeal (NP) swabs specimens from hospitalized COVID-19 patients were collected upon hospital admission (baseline, day 0), and days 3 and 7. A convalescence sample was collected from survivors after recovery with a mean time of 46 days (range, 30–56 days). Secretory IgA (sIgA), IgA, IgG, and IgM antibody titers against SARS-CoV-2 full-length S protein (A) and HCoV-OC43 full-length S protein (C). Antibody titers were calculated and represented as area under the curve (AUC). Small dots with dotted lines represent the longitudinal antibody profile of each individual. Mean titers (big dots) and standard deviation (SD) are also shown. Kruskal-Wallis test was performed to compare differences at each time point over baseline. Statistical significance was considered when $p \leq 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, not significant). Fold change antibody titers against SARS-CoV-2 full-length S protein (B) and HCoV-OC43 full-length S protein (D) represented as box-and-whisker diagrams. Box indicates interquartile range (IQR, Q1–Q3), with horizontal line showing the median and vertical lines indicating minimum and maximum. All individual values are represented as small dots. Kruskal-Wallis test was performed, and significant adjusted p values after pairwise comparisons are shown for each comparison. A total of 81 biologically independent NP swabs samples were collected. Number of NP samples run against each S antigen and immunoglobulin subtype and summarized in Table S6. A value of 0.001 was assigned to the samples with no detectable antibodies.

understanding systemic immune responses after COVID-19 infection and vaccination, and little attention has been given, so far, to the role of local immune responses in mucosal surfaces, like the upper respiratory tract. Although some studies have documented the presence of virus-specific IgG and IgA in saliva and NP samples of patients with COVID-19 infection or vaccination, these studies failed to address the role of adaptive immune mechanisms at mucosal sites in preventing transmission or severe outcomes. Here, we provide a dynamic and comprehensive characterization of the immunoglobulin repertoire elicited in the mucosal compartment upon SARS-CoV-2 infection. Additional studies should be conducted to analyze the impact of these responses in protection against SARS-CoV-2 infection.

sIgA and IgA antibodies are the predominant immunoglobulin isotypes at mucosal surfaces.[12,29] We found a strong induction of IgA antibodies against SARS-CoV-2 S protein in the upper respiratory tract. Moreover, our results showed robust mucosal sIgA production in the NP swabs from the BACO cohort by measuring the SC levels associated with SARS-CoV-2 S-specific antibodies (Figures 3A and 3B). Although some studies have found that IgA levels in the saliva can be very variable due to differences in the method of sample collection,[12,28] our results

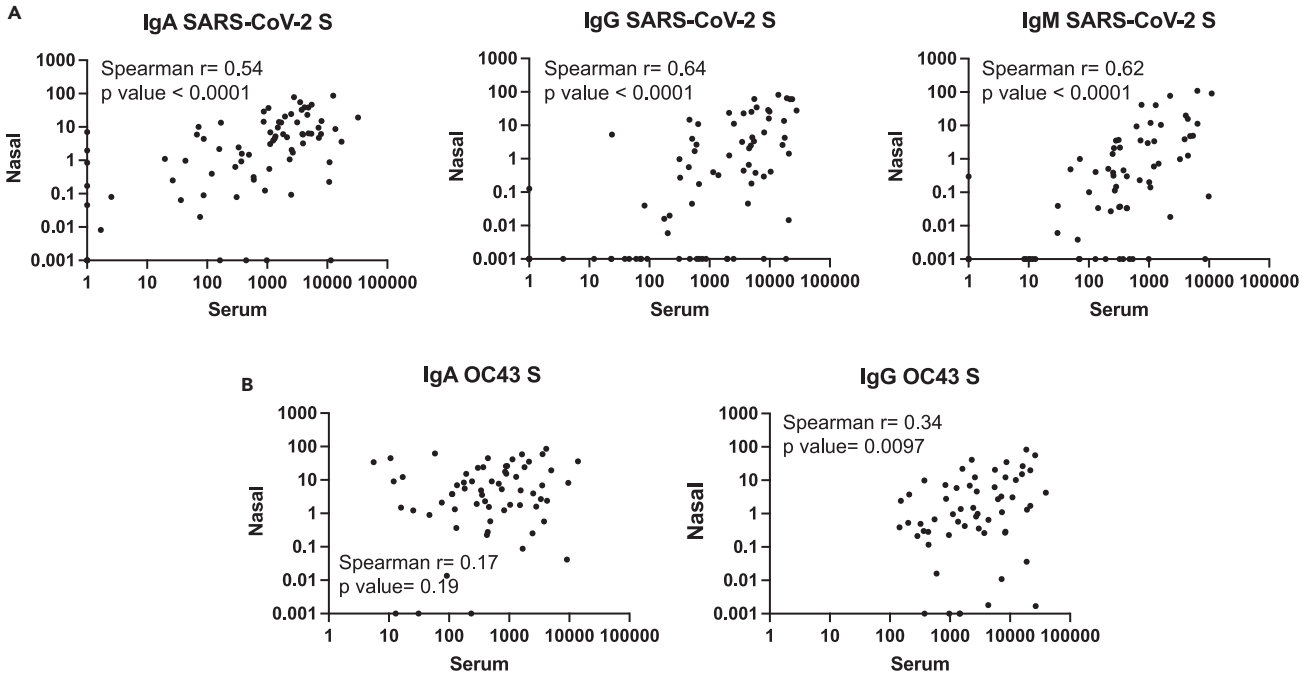

Figure 4. Correlation between antibody responses in serum and upper respiratory tract

(A) Correlation between anti-SARS-CoV-2 S IgA, IgG, and IgM antibody titers measured in serum and NP swabs.

(B) Correlation between anti-HCoV-OC43 S IgA and IgG antibody titers measured in serum and NP swabs. Number of NP swabs and paired serum samples for each correlation analysis is summarized in Table S7. Spearman correlation coefficient and p values (two-tailed) are shown above each graph. A value of 1 or 0.001 was assigned to serum or mucosal samples, respectively, when no antibodies were detected.

showed equivalent levels of non-specific IgA in the upper respiratory tract of these COVID-19 patients (Figure S2), and therefore, no normalization was required to assess antigen-specific sIgA responses. Importantly, our study shows that IgA and sIgA antibodies are robustly induced in the mucosal compartment upon SARS-CoV-2 infection. This is important because recent publications have also described the production of specific mucosal IgA responses during SARS-CoV-2 mRNA intramuscular vaccination.[28,30] Although it is still unclear the mechanism by which intramuscularly vaccines induced mucosal sIgA responses, there is a growing interest to develop next-generation COVID-19 vaccines that boost mucosal antibody responses in the nasal cavity to potentially reduce viral transmission and protect from severe disease.[31] In this scenario, mucosal vaccines delivered intranasally would be ideal.[32,33] However, many questions about the nature and durability of mucosal responses upon SARS-CoV-2 infection or vaccination remained opened. Some preliminary studies have suggested that mucosal immunity could last up to 7 months after SARS-CoV-2 infection,[34] whereas others have described low-level but durable (>6 months) sIgA response after COVID-19 mRNA vaccination.[35] Further research is needed to develop next-generation COVID-19 vaccine candidates that could provide broader and lasting immune protection against emerging SARS-CoV-2 variants in the systemic and mucosal compartments.

In this context, the potential effect of an imprinted immune response to human coronaviruses is of great importance. We have previously shown that systemic IgG responses against SARS-CoV-2 antigens are strongly induced in COVID-19 patients from the BACO cohort.[2] Additionally, when we quantified the levels of pre-existing immunity against seasonal HCoVs OC43, HKU1, and 229E in the serum samples from these patients, we found a strong back-boosting effect to conserved epitopes of the S protein from beta-HCoV OC43 and HKU1. Importantly, this memory recall to conserved S antigens of seasonal beta-HCoVs negatively correlated with *de novo* antibody responses to SARS-CoV-2 virus. Although our previous study provides evidence of immunological imprinting in the systemic compartment, it still remains unknown whether imprinting could also occur in the upper respiratory tract. In the present study, we detected IgG, sIgA, and IgA antibodies against HCoV-OC43 S protein in the local upper respiratory mucosa, whereas no IgM responses were observed (Figure 3C). Moreover, antibody responses against HCoV-OC43 S peaked earlier than SARS-CoV-2 S antibody titers in the local mucosa (Figure 3D, and Table S3), suggesting some maturity in the cross-reactive immune responses against conserved epitopes of beta-HCoVs in the upper respiratory tract. Further studies are needed to address the role of immune imprinting in the mucosal compartment during COVID-19 disease. However, our findings support the idea that upon SARS-CoV-2 infection, memory B cells generated from prior infections with antigenically related HCoVs will be rapidly activated, perhaps competing with the activation of naive B cells specific for SARS-CoV-2 novel epitopes.

Limitations of the study

There are some potential limitations in our study. The BACO cohort is composed of 37 hospitalized COVID-19 patients from Spain, a relatively small number of individuals in a study cohort. Still, this number of participants allowed us to perform a robust and unbiased descriptive

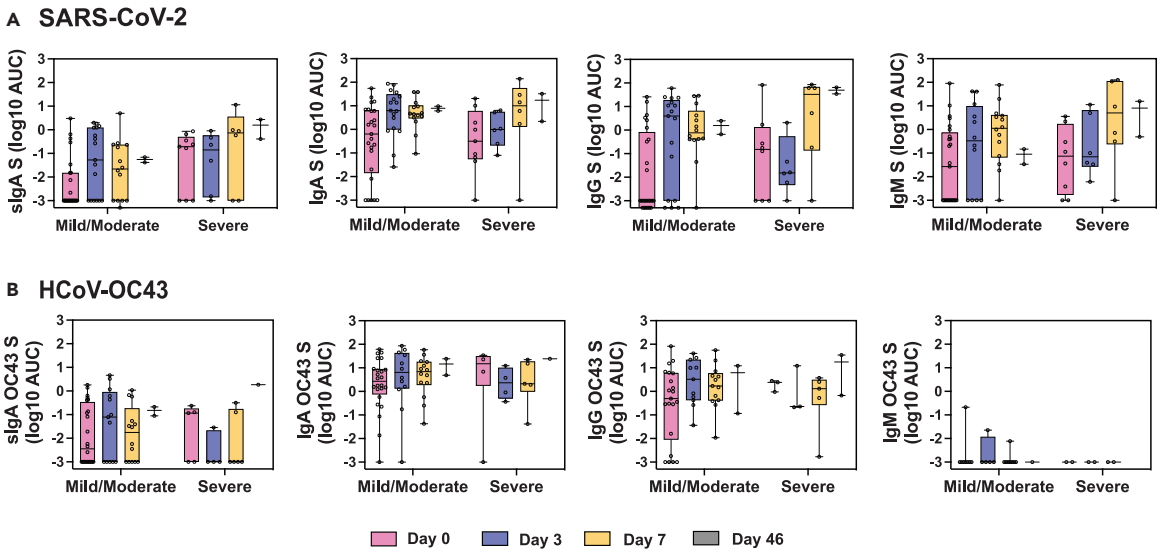

Figure 5. Antibody response in the upper respiratory tract according to disease severity in the BACO cohort

Box-and-whisker diagrams of area under the curve (AUC) ELISA IgA, IgG, and IgM titers against SARS-CoV-2 spike (A) and seasonal HCoV-OC43 spike (B) in mild/moderate and severe COVID-19 patients from the BACO cohort. Severity of COVID-19 was assigned following a previously described severity scale.[27] Box indicates interquartile range (IQR, Q1–Q3), with horizontal line showing the median and vertical lines indicating minimum and maximum. All individual values are represented as small dots, and each time point is shown in different color. A total of 81 biologically independent NP samples were collected. Number of NP samples run against each S antigen and immunoglobulin subtype and summarized in Table S6. A value of 0.001 was assigned to the samples with no detectable antibodies.

characterization of the immunoglobulin repertoire in the local mucosa. Although this number could limit some potential conclusions on the role of mucosal immune responses in disease outcome, our data allowed to detect specific trends, and severe patients showed a delay in antibody responses to SARS-CoV-2 antigens when compared with mild cases. Additionally, as found by the authors and others,[28] levels of mucosal sIgA were generally lower as compared with total specific IgA titers that could have also compromised the statistical power of the analysis. Moreover, no long-term samples were collected; therefore, we still do not know the durability of the immune response in the upper respiratory tract. In summary, our study provides a better understanding of the immune responses elicited in the upper respiratory tract upon SARS-CoV-2 infection as well as some evidence of pre-existing immunity and immune memory recall in the local respiratory mucosa in COVID-19 disease.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - The BACO cohort
  - Recombinant proteins
- METHOD DETAILS
  - Antigen-specific IgG, IgM and IgA ELISAs in serum
  - Antigen-specific sIgA, IgA, IgG and IgM ELISAs in nasopharyngeal swabs
  - Quantification of total IgA in nasopharyngeal swabs
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109210.

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AUTHOR CONTRIBUTIONS

T.A. and A.G.S. conceived and designed the study. T.A. supervised and provided training to A.E. and A.R.F. A.E. optimized and performed experiments. A.R.F. performed experiments. A.R., G.A.A., and J.C. collected samples and clinical data. T.A., A.G.S., and J.C. supervised the study. T.A. and A.E. analyzed data, prepared figures, and wrote the manuscript. All authors reviewed the manuscript.

DECLARATION OF INTERESTS

The A.G.-S. laboratory has received research support from GSK, Pfizer, Senhwa Biosciences, Kenall Manufacturing, Blade Therapeutics, Avimex, Johnson & Johnson, Dynavax, 7Hills Pharma, Pharmamar, ImmunityBio, Accurius, Nanocomposix, Hexamer, N-fold LLC, Model Medicines, Atea Pharma, Applied Biological Laboratories, and Merck, outside of the reported work. A.G.-S. has consulting agreements for the following companies involving cash and/or stock: Castlevax, Amovir, Vivaldi Biosciences, Contrafect, 7Hills Pharma, Avimex, Pagoda, Accurius, Esperovax, Farmak, Applied Biological Laboratories, Pharmamar, CureLab Oncology, CureLab Veterinary, Synairgen, Paratus, Pfizer, and Prosetta, outside of the reported work. A.G.-S. has been an invited speaker in meeting events organized by Seqirus, Janssen, Abbott, and AstraZeneca. A.G.-S. is inventor on patents and patent applications on the use of antivirals and vaccines for the treatment and prevention of virus infections and cancer, owned by the Icahn School of Medicine at Mount Sinai, New York, outside of the reported work.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human IgG-HRP antibody (Fc-specific)	Sigma-Aldrich	Cat. #A0170; RRID: AB_257868
Anti-human IgM-HRP antibody	Sigma-Aldrich	Cat. #A6907; RRID: AB_258318
Anti-human IgA-HRP antibody (α-chain)	Sigma-Aldrich	Cat. #A0295; RRID: AB_257876
Anti-human sIgA antibody	Millipore	Cat. #411423; RRID: AB_10681347
Anti-mouse IgG-HRP antibody	Abcam	Cat. #ab6823; RRID: AB_955395
Anti-human IgA antibody	Bethyl Laboratories	Cat. #A80-102A
Biological samples		
Human serum samples from COVID-19 infected individuals from 'The BACO Cohort'	University Hospital of Bellvitge, Barcelona (Spain)	*Aydillo* et al., *2021*[2]
Human nasopharyngeal swabs from COVID-19 infected individuals from 'The BACO Cohort'	University Hospital of Bellvitge, Barcelona	
Chemicals, peptides, and recombinant proteins		
SARS-CoV-2 full-length spike protein	Sino Biological	Cat. #40589-V08H4
OC43 full-length spike protein	Sino Biological	Cat. #40607-V08B
3,3′ ,5,5′ -Tetra- methylbenzidine (TMBE)	Rockland	Cat. #TMBE-1000
Sulfuric Acid 2M	Fisher Scientific	Cat. #S25898
Software and algorithms		
GraphPad Prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
Phosphate-buffered saline (PBS)	Corning	Cat. #21-040-CV
Tween-20	Fisher Scientific	Cat. #BP337-100
Non-fat milk powder	RPI	Cat. #M17200-500
Triton X-100	Fisher Scientific	Cat. #BP151-500
4 HBX 96-well microtiter plates	Thermo Fisher Scientific	Cat. #3855
405 TS microplate washer	BioTek	
Synergy 4 plate reader	BioTek	

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Teresa Aydillo (teresa.aydillo-gomez@mssm.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The BACO cohort

The BACO cohort is a prospective human cohort study of COVID-19 disease carried out during the first pandemic wave (March–May 2020) of SARS-CoV-2 in Barcelona (Spain). A positive COVID-19 case was defined according to international guidelines when a nasopharyngeal (NP) swab tested positive for SARS-CoV-2 by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) upon hospital admission. All patients or their legally authorized representatives provided informed consent prior to sample and data collection. Samples from patients including serum and NP swabs, were collected at the enrollment in the study (baseline), and at days 3 and 7 post enrollment. A convalescence sample was collected from survivors after recovery and hospital discharge with a mean time of 46 days (range, 30–56 days). The total number of serum samples and NP swabs was 116 and 81, respectively. All samples were stored at -80°C. Data on demographics, including age and sex, comorbidities, clinical signs and symptoms, interventions, and outcomes are described in Table S1. Severity of COVID-19 was assigned following a described severity scale based on oxygen saturation (SpO2), presence of pneumonia/imaging, oxygen support defined as use of high-flow nasal cannula (HFNC), non-rebreather mask (NRB), bilevel positive airway pressure (BIPAP) or mechanical ventilation (MV); and kidney (creatinine clearance, CrCl) and liver (alanine aminotransferase, ALT) function:[27] mild (SpO2 > 94% AND no pneumonia), moderate (SpO2 < 94% AND/OR pneumonia), severe (use of HFNC, NRB, BIPAP or MV AND no vasopressor use AND CrCl >30 AND ALT < 5x upper limit of normal) and severe with end-of organ disease (Use of HFNC, NRB, BIPAP or MV AND vasopressor use OR CrCl >30 or new HD OR ALT < 5x upper limit of normal). The study protocol was approved by the Institutional Review Board of University Hospital of Bellvitge, Barcelona, Spain, and by the Icahn School of Medicine at Mount Sinai, New York, US.

Recombinant proteins

The recombinant spike proteins from SARS-CoV-2 and beta-coronavirus OC43 were purchased from Sino Biological (Cat. #40589-V08H4 and #40607-V08B). Proteins were stored at -80°C until use.

METHOD DETAILS

Antigen-specific IgG, IgM and IgA ELISAs in serum

Serum ELISAs against SARS-CoV-2 and OC43 spike proteins were performed as previously described.[2] Briefly, Immulon 4 HBX 96-well microtiter plates (Thermo Fisher Scientific) were coated overnight at 4°C with 50 μL recombinant protein (SARS-CoV-2 or OC43 full-length spike, respectively) at a concentration of 2 μg/mL. The next day, the plates were washed three times with phosphate-buffered saline (PBS; Corning) containing 0.1% Tween-20 (PBS-T, Fisher Scientific) using an automatic plate washer (BioTek). After washing, the plates were blocked for 1 h at room temperature (RT) with 200 μl/well of 3% (w/v) non-fat milk powder diluted in PBS-T. The blocking solution was removed, and 100 μL of serum samples diluted (starting concentration of 1:80 and serially diluted three-fold) in PBS-T containing 1% (w/v) non-fat milk was added to the wells and incubated for 1 h at RT. The plates were washed three times with PBS-T and 100 μl of anti-human IgG-HRP antibody (Fc-specific) (Sigma, Cat. A0170) at a dilution of 1:20,000; or anti-human IgM-HRP antibody at a dilution 1:3000 (Sigma, Cat. A6907); or anti-human IgA-HRP antibody (α-chain) at a dilution 1:3000 (Sigma, Cat. A0295) was added to the wells. All secondary antibodies were diluted in PBS-T containing 1% (w/v) non-fat milk and incubated for 1h at RT. The plates were washed four times with PBS-T with shaking using the plate washer and 100 μL of TMBE (Rockland) was added to all wells for 10 min. The reaction was stopped by the addition of 100 μL of sulfuric acid per well. Optical density (OD) at a wavelength of 450 nm was read using Synergy 4 (BioTek) plate reader. OD values of samples were adjusted by subtracting the average of the blank plus three times the standard deviation of the blank. Area under the curve (AUC) values were computed by plotting normalized OD values against the reciprocal serum sample dilutions in GraphPad Prism. The assay was done one per sample due to the limited amount of sample.

Antigen-specific sIgA, IgA, IgG and IgM ELISAs in nasopharyngeal swabs

All nasopharyngeal swabs were treated with 0.5% Triton X-100 (v/v) to inactivate SARS-CoV-2 virus prior performing any experiments. Sample inactivation was performed in an enhanced biosafety level 2 (BSL-2+) facility following Icahn School of Medicine biosafety guidelines. Immulon 4 HBX 96-well microtiter plates (Thermo Fisher Scientific) were coated overnight at 4°C with 50 μL recombinant protein (SARS-CoV-2 or OC43 full-length spike, respectively) at a concentration of 2 μg/mL. The next day, the plates were washed three times with PBS-T using an automatic plate washer (BioTek). After washing, the plates were blocked for 1 h at RT with 200 μl/well of 5% (w/v) non-fat milk powder diluted in PBS-T. The blocking solution was removed, and 50 μL of inactivated nasopharyngeal swab (starting undiluted and serially diluted two-fold with PBS-T containing 2.5% (w/v) non-fat milk) was added to the wells and incubated at 4°C overnight. The next day, plates were washed three times with PBS-T using the plate washer. For sIgA detection, 100 μl of anti-human sIgA antibody (Millipore, Cat. 411423) diluted to 5 μg/mL in PBS-T containing 2.5% (w/v) non-fat milk were added to the wells and incubated for 2 h at RT. Plates were washed again three times with PBS-T using the plate washer and secondary anti-mouse IgG-HRP antibody (Abcam, Cat. ab6823) diluted 1:5000 in 2.5% (w/v) non-fat milk PBS-T was added to the wells. For IgA, IgG and IgM measurement, incubation of samples with antigen was also performed overnight at 4°C. After washing with PBS-T three times, 100 μl of anti-human IgA-HRP antibody (α-chain) (Sigma, Cat. A0295) at a dilution of 1:3000; or anti-human IgG-HRP antibody (Fc-specific) (Sigma, Cat. A0170) at a dilution of 1:20,000; or anti-human IgM-HRP antibody at a dilution 1:3000 (Sigma, Cat. A6907) was added to the wells. All secondary antibodies were diluted in PBS-T containing 2.5% (w/v) non-fat milk and incubated for 1 h at RT. After incubation with the corresponding secondary HRP-conjugated antibodies, plates were washed four times with PBS-T with shaking using

the plate washer and 100 μL of TMBE (Rockland) was added to all wells for 10 min. The reaction was stopped by the addition of 100 μL of sulfuric acid per well. Optical density (OD) at a wavelength of 450 nm was read using Synergy 4 (BioTek) plate reader. OD values of samples were adjusted by subtracting the average of the blank plus three times the standard deviation of the blank. Area under the curve (AUC) values were computed by plotting normalized OD values against the reciprocal serum sample dilutions in GraphPad Prism. The assay was done one per sample due to the limited amount of sample.

Quantification of total IgA in nasopharyngeal swabs

Immulon 4 HBX 96-well microtiter plates (Thermo Fisher Scientific) were coated overnight at 4°C with 50 μL of goat anti-human IgA (Bethyl Laboratories #A80-102A) at a concentration of 250 ng/well. The next day, the plates were washed three times with PBS-T using an automatic plate washer (BioTek). After washing, the plates were blocked for 1 h at RT with 200 μl/well of 5% (w/v) non-fat milk powder diluted in PBS-T. The blocking solution was discarded, and 75 μL of inactivated nasopharyngeal swab (starting 1:40 and serially diluted three-fold) with 2.5% (w/v) non-fat milk PBS-T was added to the wells and incubated at 4°C for 2 h. The next day, plates were washed three times with PBS-T using the plate washer and anti-human IgA-HRP antibody (α-chain) (Sigma, Cat. A0295) diluted 1:1500 was added to the wells. After 1h incubation at RT, plates were washed four times with PBS-T with shaking using the plate washer and 100 μL of TMBE (Rockland) was added to all wells for 10 min. The reaction was stopped by the addition of 100 μL of sulfuric acid per well. Optical density (OD) at a wavelength of 450 nm was read using Synergy 4 (BioTek) plate reader. OD values of samples were adjusted by subtracting the average of the blank plus three times the standard deviation of the blank. Area under the curve (AUC) values were computed by plotting normalized OD values against the reciprocal serum sample dilutions in GraphPad Prism. The assay was done one per sample due to the limited amount of sample.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses and AUC calculation were performed using Graphpad Prism 9. Geometric mean titers (GMT) and 95% confidence intervals (CI 95%) were computed by taking the exponent (log10) of the mean and of the lower and upper limits of the 95% CI of the serum log10-transformed titers. Mean and standard deviation (SD) were calculated for mucosal antibody titers. Kruskal-Wallis test with Dunn's multiple comparison test, Mann-Whitney t test and non-parametric Spearman correlation were performed. Statistical significance was considered when $p \leq 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, not significant).