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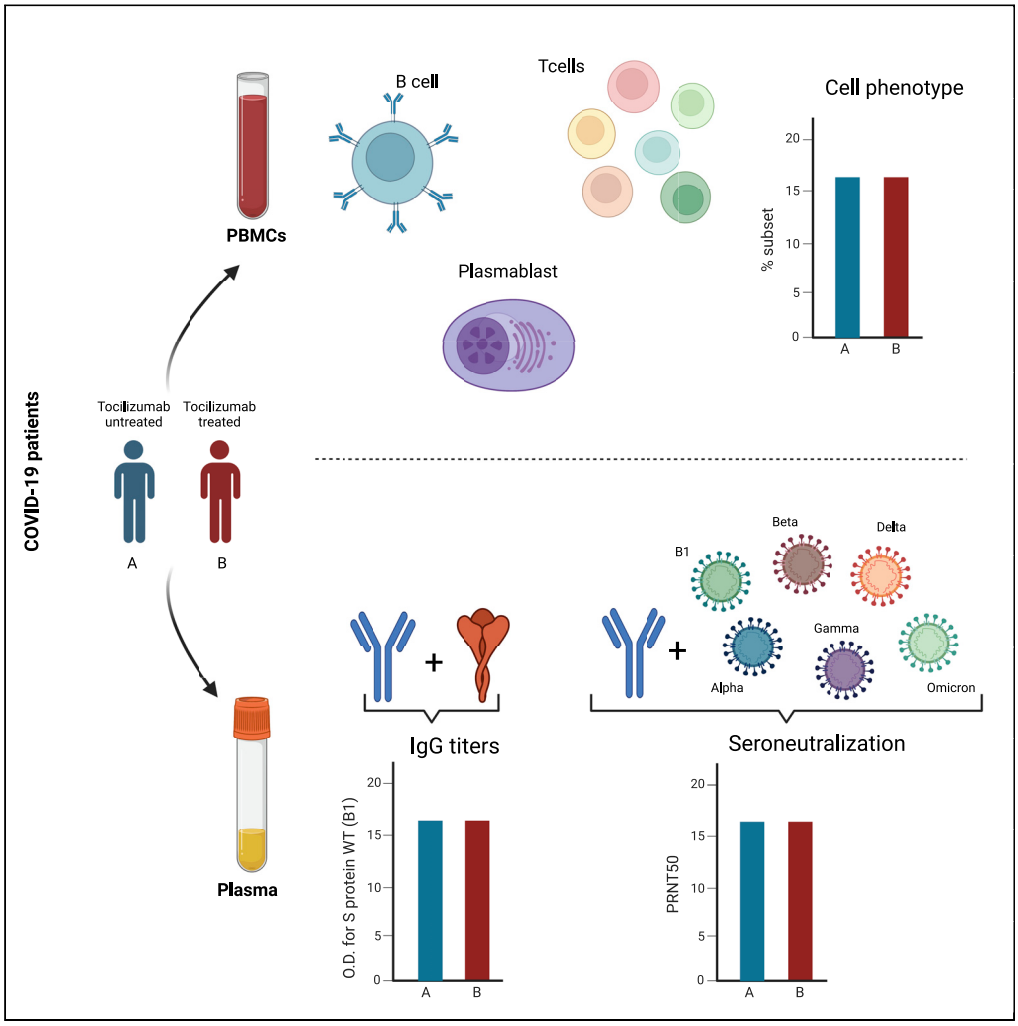

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Highlights

SARS-CoV-2-specific IgG1 titers depended on disease severity but not on tocilizumab

No major impact of tocilizumab on the B cell subsets and SARS-CoV-2-specific IgG1

Tocilizumab does not alter plasma virus neutralization capacity for SARS-CoV-2 VOCs

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Tocilizumab-treated convalescent COVID-19 patients retain the cross-neutralization potential against SARS-CoV-2 variants

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SUMMARY

Although tocilizumab treatment in severe and critical coronavirus disease 2019 (COVID-19) patients has proven its efficacy at the clinical level, there is little evidence supporting the effect of short-term use of interleukin-6 receptor blocking therapy on the B cell sub-populations and the cross-neutralization of SARS-CoV-2 variants in convalescent COVID-19 patients. We performed immunological profiling of 69 tocilizumab-treated and non-treated convalescent COVID-19 patients in total. We observed that SARS-CoV-2-specific IgG1 titers depended on disease severity but not on tocilizumab treatment. The plasma of both treated and non-treated patients infected with the ancestral variant exhibit strong neutralizing activity against the ancestral virus and the Alpha, Beta, and Delta variants of SARS-CoV-2, whereas the Gamma and Omicron viruses were less sensitive to seroneutralization. Overall, we observed that, despite the clinical benefits of short-term tocilizumab therapy in modifying the cytokine storm associated with COVID-19 infections, there were no modifications in the robustness of B cell and IgG responses to Spike antigens.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the coronavirus disease 2019 (COVID-19), has spread worldwide over the past 2 years, with more than 6 million deaths.[1] Convalescent individuals have been shown to benefit from a natural protection against reinfection, whereas vaccination reduces the risk of reinfection from 50% to 95% to wild-type virus.[2–4] COVID-19 disease severity varies from asymptomatic form to life-threatening acute respiratory distress syndrome (ARDS). Increased SARS-CoV-2-specific antibodies and neutralizing titers have been reported in subjects with more severe symptoms.[5] After October 2020 when the new variants of concern (VOCs) emerged, the sensitivity to antibody neutralization in convalescent patient (infected before October 2020) sera has been reported to fluctuate according to the viral variants. An impaired sensitivity of the Beta, the Delta, and the Omicron variants and other variants to neutralizing antibodies was reported in convalescent and vaccinated sera when compared with the ancestral virus.[6–8]

The pathogenesis of COVID-19 is mediated by a hyperactivated immune system following SARS-CoV-2 infection, with the release of a large amount of pro-inflammatory cytokines. Several immunotherapies that target inflammation have been investigated for the management of severe and critical COVID-19 patients. Many reports have shown that interleukin-6 (IL-6) is one of the major inflammatory cytokines involved in the pathogenesis of COVID-19.[9] IL-6 is a multipotent inflammatory cytokine that regulates various functions of both innate and adaptive immune cells and hence contributes to inflammation.[10] It plays a vital role in mounting protective antibody responses, and in particular by stimulating B cell proliferation and plasma cell maturation.[11]

Continued

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Tocilizumab, a humanized monoclonal antibody that targets the α-chain of IL-6 receptor, has been used in severe and critical COVID-19 patients. A beneficial effect including a survival benefit in severe or critical COVID-19 patients has been observed after tocilizumab treatment in a clinical trial involving a large number of patients.[12–14] However, some conflicting data did not reveal a beneficial role of tocilizumab in COVID-19 patients.[15] Previous data in autoimmune patients treated with tocilizumab have shown that blocking IL-6 signaling declines IgG level, autoantibodies, memory B cells, and circulating plasma cells.[10,16–18] These reports thus suggest that tocilizumab therapy in non-vaccinated COVID-19 patients might interfere with the protective antibody response to SARS-CoV-2. Therefore, in this study, we compared neutralizing antibodies and B cell population profile in 69 convalescent tocilizumab treated and non-treated COVID-19 patients admitted to four hospitals around Paris area, France, from June 2020 to April 2021 (COVIMUNE cohort). Our study shows that short-term IL-6 blockade via tocilizumab treatment does not impair the specific antibody response against SARS-CoV-2 and the cross-neutralization of VOCs. This observational study shows that tocilizumab, in addition to improving the clinical outcome of severe and critical COVID-19 patients, does not compromise the immune response of convalescent patients against potential reinfection with variants.

RESULTS

Patients' characteristics

We studied 69 vaccine-naive COVID-19 patients admitted to four different hospitals located near Paris, France, from May 2020 to April 2021 (Tables 1 and S1). None of the individuals included in the study were vaccinated. We divided the patients into three groups according to disease severity grade. There were 11 mild/moderate (7 mild and 4 moderate), 21 severe, and 23 critical patients recruited during the first wave of COVID-19, and 10 mild/moderate (8 mild and 2 moderate), 3 severe, and 1 critical patient recruited during the second/third wave of COVID-19. Nine of 24 (37.5%) patients from the severe group and 11 of 24 (45.8%) from the critical group received tocilizumab therapy either once or twice at a dose of 8 mg/kg body weight. All treated patients in the severe group and 3 patients (27%) in the critical group had a positive outcome after tocilizumab.[14]

Fifteen of the tocilizumab-treated patients were sampled twice giving us paired samples at two different timings. The first time (defined as 3 months; 3M) was at a median of 75 days post-symptom onset (range 67–105 days) for the severe group and at a median of 92 days (range 33–113 days) for the critical tocilizumab-treated group. No significant difference was observed in the blood sampling time at 3M between the treated and untreated severe ($p = 0.509$) and critical groups ($p = 0.285$) (Table S2).

The second blood sampling (defined as 6 months; 6M) was carried out at a median of 181 days post-symptom onset (range 163–223 days) for the severe group, and at a median of 172 days (range 111–184 days) for the critical group.

Of note, although the recruitment of the patients was carried out at the beginning of the COVID-19 pandemic, only 22% of the patients of the severe and 55% of the critical treated group received corticosteroids. The cohort represents the natural history of COVID-19 without conflicting effect of steroids.

We conducted an analysis of the antibody response and seroneutralization only on samples from patients recruited until October 2020 (first wave) ($n = 58$). For logistical reasons, B cell phenotyping was carried out on samples of patients from the first ($n = 15$) and second wave of COVID-19 ($n = 9$) of the 69 samples.

Tocilizumab does not decrease antibody titers in convalescent patients at 3M

To assess the influence of tocilizumab on anti-SARS-CoV-2 antibody response, we measured the total anti-S IgG and IgG1 titers in the plasma of mild/moderate, severe, and critical patients according to their blood sampling time post-symptom onset (Figures 1 and S1). The total anti-S IgG showed a positive correlation with the IgG1 ($r = 0.849$) (Figure S1D). Anti-S IgG1 titers followed a normal distribution (Shapiro-Wilk, $p = 0.161$); however, total IgG titers deviated from a normal distribution (Shapiro-Wilk, $p < 0.001$). There was no significant difference in the level of anti-S total IgG antibodies between tocilizumab-treated and non-treated severe and critical patients at 3M considering disease severity and variation of day of sampling after symptom onset (severity: $p = 0.887$; treated: $p = 0.297$; Generalized Linear Model [GLM] Poisson regression). However, anti-S IgG1 titers were significantly higher in the treated individuals at

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Table 1. Patients' characteristics between tocilizumab- and non-tocilizumab-treated groups

	Mild/moderate untreated	Severe untreated	Critical untreated	Severe tocilizumab-treated	Critical tocilizumab-treated
Number of patients	21	15	13	9	11
Gender (n males (%))	13 (62%)	9 (60%)	11 (85%)	9 (100%)	8 (72%)
Age at diagnostics (median (IQR))[a]	34 (29-46)	64 (46-69)	62 (56-65)	57 (45-66)	58 (52-68)
Sampling date (interval)	May 2020-March 2021	June 2020-April 2021	May 2020-August 2020	June 2020-October 2020	July 2020-April 2021
Sampling time (median (IQR))	40 (32-86)	3M[b] : 85 (78-91)	3M : 94 (82-116)	3M :75(72-91) 6M : 181 (174-185)	3M : 92 (73-96) 6M : 172 (165-181)
Diabetes, n (%)	5 (24%)	8 (53%)	4 (31%)	2 (22%)	6 (55%)
Hypertension, n (%)	5 (24%)	7 (47%)	5 (38%)	2 (22%)	3 (27%)
Obesity, n (%)	2 (10%)	4 (27%)	3 (23%)	3 (33%)	3 (27%)
Asthma, n (%)	2 (10%)	2 (13%)	1 (8%)	1 (11%)	0
More than 1 comorbidity, n (%)	5 (24%)	10 (67%)	6 (46%)	2 (22%)	3 (27%)
BMI (median (IQR))	24.6 (22-29)	28 (22-30)	28.1 (26-30)	28 (27-30)	27 (27-30)
CRP(mg/ml) (median (IQR))	0.9 (0-4)	4 (4-8)	1.45 (1-2)	2 (1-4)	4 (2-7)
Gammaglobulin (g/L) (median (IQR))	10.1 (10-12)	11.7 (9-14)	11.5 (10-12)	10 (9-11)	12 (9-13)
Steroids treatment, n (%)	2 (10%)	5 (33%)	2 (15%)	2 (22%)	6 (55%)
Hydroxychloroquine, n (%)	1 (5%)	0	11 (85%)	4 (44%)	2 (18%)
Tocilizumab treatment	0	0	0	9 (5=1 dose; 4=2 doses)	11 (8=1 dose; 3=2 doses)
Response to tocilizumab, n (%)	NA	NA	NA	9 (100%)	3 (27%)

BMI, body mass index; CRP, C-reactive protein; IQR, interquartile range.
Patients were segregated according to disease severity (mild/moderate, severe, critical; with 9 severe and 11 critical patients having been treated by tocilizumab).
Variables are presented as median (IQR) or number of patients (%).
[a]Variables presented as median (IQR) or number of patients (%).
[b]3M: 3 months samples; 6M: 6 months samples.

3M (untreated: mean titers 1.47 [SEM 0.11] vs. treated: 1.87 [SEM 0.13], $p = 0.024$; GLM Poisson regression). There was no significant difference in the severity classes ($p = 0.419$). We then compared the IgG titers over time in the treated groups (severe and critical); we could not compare IgG titers in the untreated vs. treated group at 6M as we did not have plasma samples from the non-treated group at 6M. Both total IgG and IgG1 titers followed a normal distribution (Shapiro-Wilk $p > 0.05$). Among tocilizumab-treated patients, anti-S total IgG and IgG1 levels significantly decreased at 6M (Figures S1E and S1F) ($p < 0.001$ for total IgG and $p = 0.002$ for IgG1). There was no significant difference between the severity groups ($p = 0.08$ for total IgG and $p = 0.099$ for IgG1). These results suggest that tocilizumab treatment does not compromise the titers of anti-SARS-CoV-2 total IgG and IgG1 in the severe and critical patients of the cohort.

Neutralization of SARS-CoV-2 ancestral strain and VOCs is not impaired by tocilizumab treatment

To assess the potential protective effect of antibodies of convalescent patients infected with the wild-type strain against SARS-CoV2 VOCs, we performed neutralization assays using live virus on Vero E6 cells. We studied the ability of plasma to neutralize five different SARS-CoV-2 isolates including the ancestral strain (termed WT as it was the main lineage circulating in France at the beginning of the pandemic), a B.1.1.7 (Alpha), a B.1.351 (Beta), a P.1 (Gamma), a B.1.617.2.2 (Delta), and a B1.1.529 (Omicron) virus. The plaque reducing neutralizing titer 50 (PRNT50) values of the convalescent plasma samples of 41 non-treated COVID-19 patients sampled before the emergence of VOCs were determined on the samples harvested 1 to 3 months after symptom onset for mild/moderate and severe and critical groups (Table 1; Figure 2A). We observed that whereas the WT, Alpha, Beta, and Delta isolates were similarly sensitive, the Gamma and Omicron virus were less sensitive to convalescent plasma leading to potential sub-protective neutralizing responses against these VOCs.

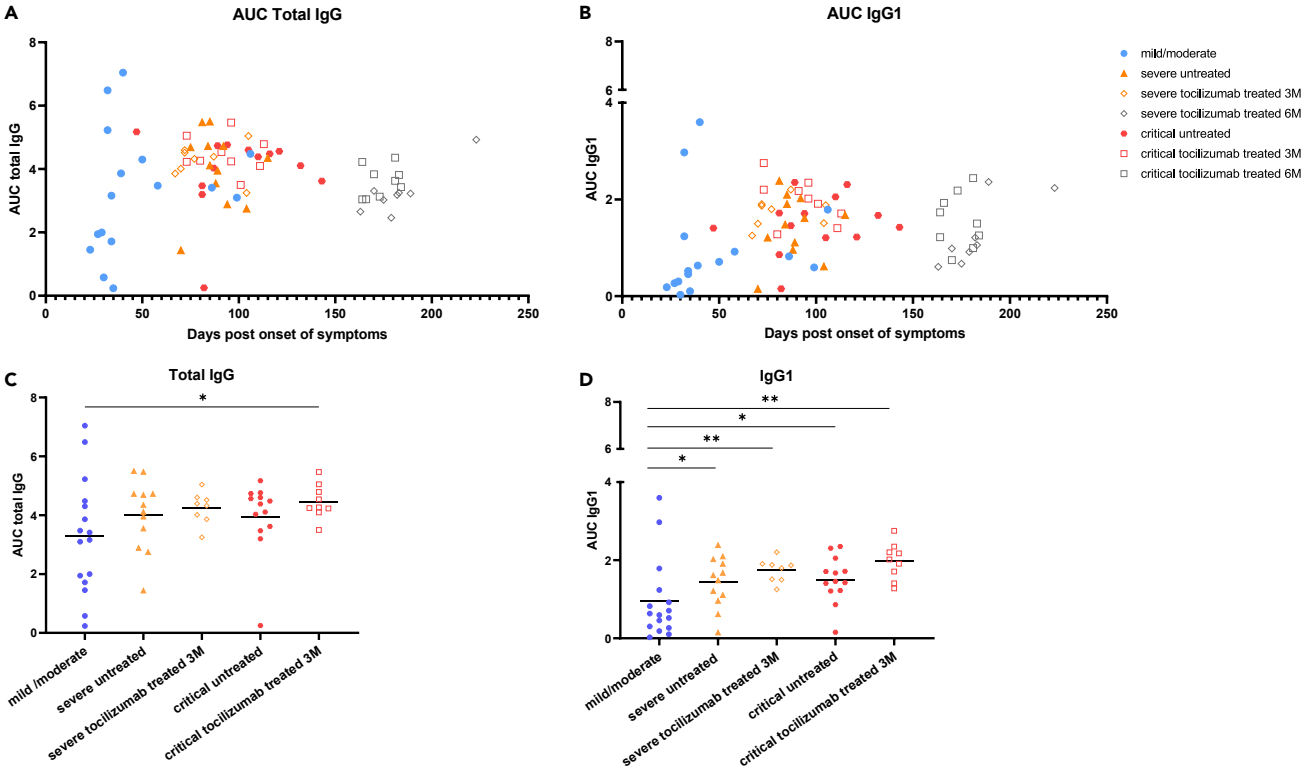

Figure 1. Impact of tocilizumab treatment on anti-S total IgG and IgG1 titers

(A–D) (A) Anti-S total IgG and IgG1 were measured by ELISA in the plasma of non-treated and tocilizumab-treated COVID-19 patients recruited until October 2020. The titers of mild/moderate (n = 16), severe (n = 12), and critical (n = 13) untreated patients, and of severe (n = 8) and critical (n = 9) tocilizumab-treated patients sampled 3 and 6 months after symptoms, were analyzed. The area under the curve (AUC) is represented according to the day post-symptoms onset. See also Figure S1. (B) IgG1 titers were measured as previously. (C) Anti-S total IgG was analyzed 1M–3M after symptoms. The AUC is represented according to disease severity. (D) IgG1 titers were measured as previously. Data are represented as mean ± SEM. The data were analyzed by an unpaired nonparametric Mann-Whitney test. *$p < 0.05$, **$p < 0.01$.

As IL-6 has also been shown to play a major role in viral infection[19] being involved in B cell proliferation, antibodies production, and survival of plasma cells,[20] which are critical in clearing viral infection, we aimed to determine whether IL-6 blockade with tocilizumab could impact the development of neutralizing anti-SARS-Cov-2 antibodies in treated patients. To assess the effect of tocilizumab treatment on variant neutralization, the PRNT50 values were determined on the plasma of 20 tocilizumab-treated severe and critical patients at 3M and 6M after disease onset (Table 1). Only 15 of 20 of the patients had paired samples at 3M and 6M. Similarly, 3M (Figure 2B) and 6M (Figure 2C) plasma of tocilizumab-treated severe and critical patients appeared less efficient at neutralizing the Gamma and Omicron variants. As observed with non-treated patients, no significant difference was observed with the WT, Alpha, Beta, and Delta VOCs. These results suggest that despite an impact on the clinical response, tocilizumab treatment does not affect the neutralization response of convalescent patients. This study highlights a possible escape of the Gamma and Omicron variants in the convalescent patients previously infected with the ancestral virus, and even in tocilizumab-treated individuals.

Dissecting the prolonged effect of tocilizumab treatment on effector B cells

The anti-SARS-CoV-2 antibody response, commonly used as a biomarker of the response to the virus and of the protection against the virus, has certain limitations, as the neutralizing antibody titers tend to decline over time.[4] We therefore investigated the long-term effect of tocilizumab on the effector B cell subsets. A previous report showed that severe COVID-19 patients exhibit an expansion of class-switched antibody-secreting cells (ASCs), and effector B cells lacking both naive and memory markers, i.e., "double-negative" (DN) in severe COVID-19 patients similar to an autoimmune setting.[21] Other studies noted alterations in

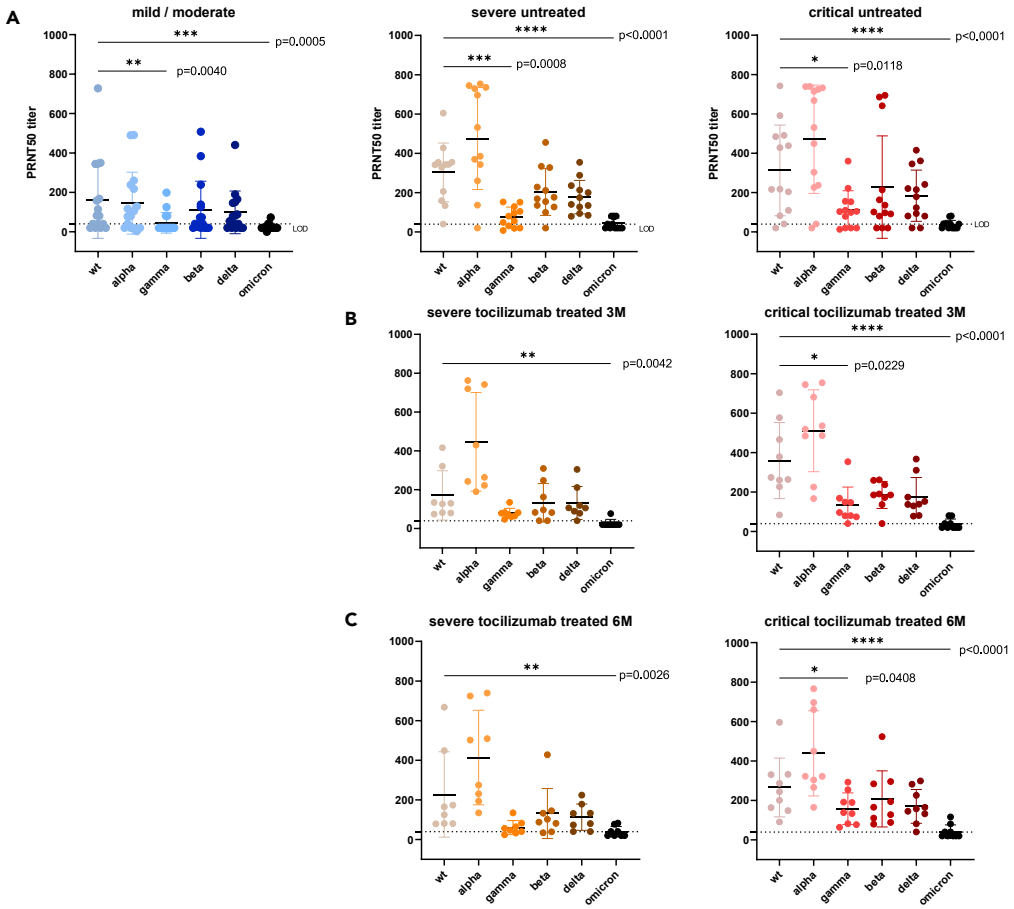

Figure 2. Sensitivity of SARS-CoV-2 variants to neutralization by plasma from convalescent patients with different disease severity and treatment status

The neutralizing activities of the plasma from COVIMUNE cohort of convalescent individuals from the first wave of COVID-19 (infected with the wild-type strain) against the indicated viral isolates are expressed as PRNT50 titers.

(A–C) (A) Neutralizing activity of plasma from non-treated individuals with mild/moderate (n = 16), severe (n = 12), or critical (n = 13) COVID-19 disease. (B) Neutralizing activity of plasma from tocilizumab-treated individuals with severe (left panel, n = 8) and critical (right panel, n = 9) disease 3 months after symptoms. (C) Neutralizing activity of plasma from tocilizumab-treated individuals with severe (left panel, n = 8) and critical (right panel, n = 9) disease 6 months after symptoms. Data are representative of two independent experiments. Two-sided Friedman test with Dunn's multiple comparison was performed between each viral strain. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. LOD, limit of detection. The threshold of detection was at a value of 40. The sera that did not neutralize at the dilution of 1/40 were set under the limit of detection.

transitional B cells, terminally differentiated memory B cells, and ASC compartments, a few days after symptom onset.[22]

We performed flow cytometry analyses using four B cell markers to identify B cell sub-populations and explore the effect of tocilizumab on B cell populations in convalescent patients. The peripheral blood mononuclear cell samples of 25 patients sampled before October 2020 (non-treated mild/moderate [n = 4] and severe/critical [n = 6] or tocilizumab-treated [n = 6]) and 9 patients sampled after October 2020 (mild/moderate [n = 5], severe [n = 3], and tocilizumab-treated [n = 1]) were analyzed 1 to 6 months after disease onset to investigate whether tocilizumab treatment modified the distribution of B cell populations. The data were compared with B cell phenotype of healthy donors (n = 4) sampled before COVID-19 pandemic. $CD19^+$ total B cells were first subdivided into unswitched memory IgD^+ (USM) B cells, switched memory IgD^-(SWM) B cells, naive ($CD27^-IgD^+$) B cells, and DN ($CD27^-IgD^-$) B cells based on the expression of the markers CD27 and IgD (Figures 3A and 3B). The ASCs/plasmablasts were identified as $CD27^+CD38^{hi}$ cells (Figures 3A and 3C).

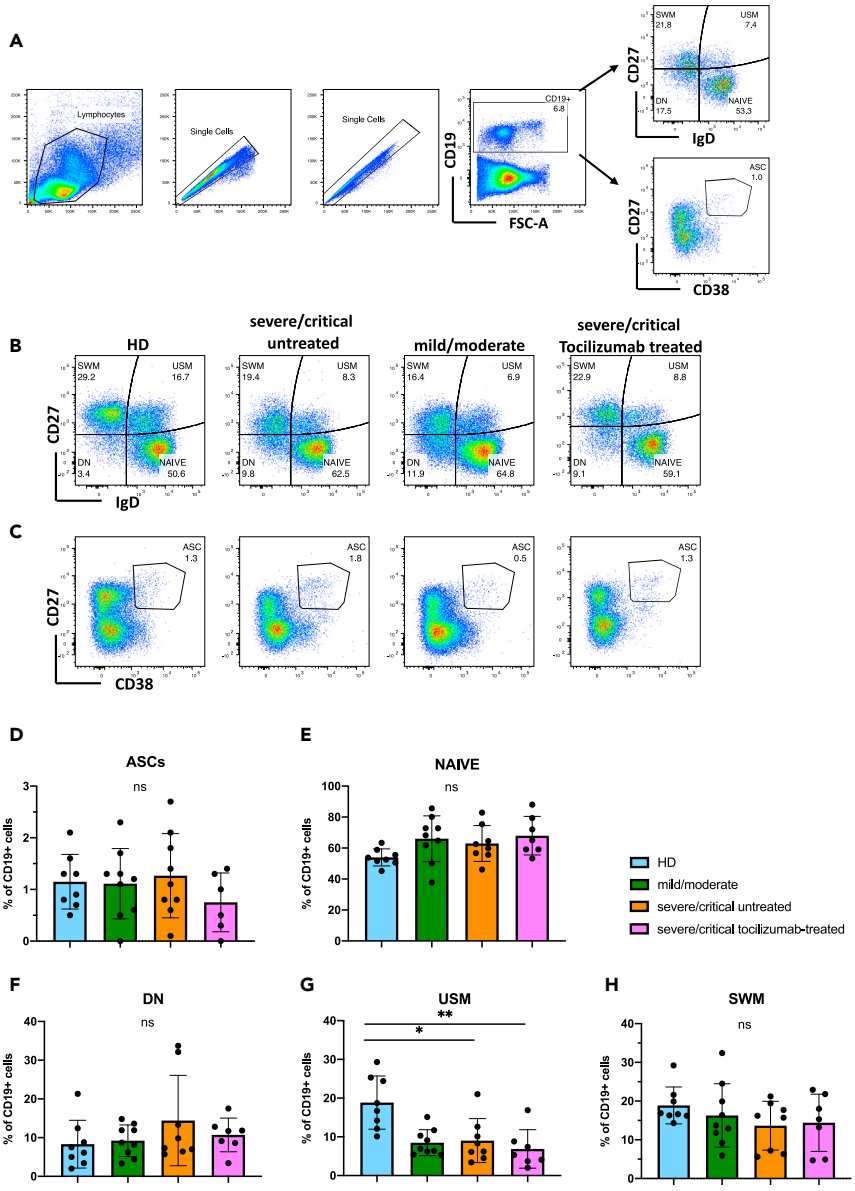

Figure 3. Tocilizumab does not affect the B cell phenotype of COVID-19 patients

B cell characterization of peripheral blood mononuclear cell of healthy donors (HD) (n = 4) and mild/moderate (n = 9), non-treated severe/critical (n = 9), and tocilizumab-treated severe/critical patients (n = 6) was analyzed by flow cytometry. Patients recruited from May 2020 to April 2021 were included. Representative patient samples of the different groups are displayed.

(A) Gating strategy of the B cell characterization of the convalescent patients.

(B) Unswitched memory IgD^{+} (USM; CD27^{+}IgD^{+}), switched memory IgD^{-}(SWM; CD27^{+}IgD^{-}), naive (CD27^{-}IgD^{+}), and double-negative (DN) (CD27^{-}IgD^{-}) B cells.

(C) The ASCs/plasmablasts were identified as CD27^{+}CD38^{hi} cells.

(D–H) The following subset frequencies were analyzed: (D) ASCs, (E) naive B cells, (F) double-negative B cells (DN), (G) memory B cells (USM, unswitched memory), and (H) memory B cells (SWM, switched memory). The data were analyzed by a multi-comparison Kruskal-Wallis test followed by a Dunn's post-hoc test. *$p < 0.05$, **$p < 0.01$. Data are represented as mean ± SEM.

We found a similar distribution of all B cell sub-populations when the tocilizumab-treated and non-treated patients were compared (Figures 3D and 3H). However, all COVID-19 patients, irrespective of their severity or treatment status, had significantly lower proportions of USM subset, which correspond to the natural memory, compared with the healthy controls. In addition, no significant changes were observed in the

frequencies of various subsets of T helper cells, T follicular helper cells, or regulatory T cells (Tregs) in the tocilizumab versus non-treated patients (Figure S2).

DISCUSSION

We report that IL-6 blockade via tocilizumab treatment does not alter the specific antibody response against SARS-CoV-2 and the capacity for seroneutralization of VOCs. Although in tocilizumab-treated autoimmune patients IgG levels were reduced,[23] in COVID-19 patients, tocilizumab treatment did not hinder the anti-SARS-CoV-2 antibody response. On the contrary, we observed that the antibody-neutralizing capacity was maintained in tocilizumab-treated patients. Possible reasons could be that in addition to IL-6, various cytokines, including IL-4, IL-21, B-cell activating factor (BAFF), interferons, tumor necrosis factor, and also co-stimulatory molecules, have the capacity to promote B cell growth, proliferation, differentiation, immunoglobulin class switch, plasma cell differentiation, and immunoglobulin secretion.[11]

A previous report based on influenza virus-stimulated human tonsillar mononuclear cells has shown that IL-6 is mandatory for antigen-specific antibody responses by B cells.[24] Moreover, IL-2 but not IL-6 could restore antibody production by B cells under T-depleted conditions. In view of the enhanced production of various cytokines, including IL-2, in COVID-19 patients,[25] blocking IL-6 signaling might have an insignificant role in the evolution of anti-SARS-CoV-2 antibody responses. An alternative explanation could be that IL-6 signaling might not be inhibited in secondary lymphoid organs where antibody response is initiated. It is important to note that tocilizumab therapy in rheumatoid arthritis patients did not inhibit antibody responses to influenza vaccine[26] and that influenza-vaccinated patients maintained protective antibody responses up to 22 months post-vaccination.[27] Moreover, tocilizumab is chronically administered to autoimmune patients, whereas COVID-19 patients received only one or two doses of tocilizumab to counteract the cytokine overproduction. Therefore, we did not expect to detect a dramatic effect of tocilizumab therapy in COVID-19 patients. Long-term treatment of autoimmune diseases with tocilizumab has been reported to alter antibody titers[28] or B cell subsets.[17,29] Nonetheless, B cell and plasmablast changes seen during acute COVID-19 were largely restored in convalescence.[30] However, chronic tocilizumab therapy in kidney transplant patients with chronic antibody-mediated rejection slightly reduced the total IgG and IgG_{1-3} after 6 months therapy.[31] The reduction in total IgG titers became significant 12 months after treatment suggesting a delayed long-term effect of tocilizumab on IgG levels.

In line with our data, a recent prospective cohort study has concluded that tocilizumab therapy in COVID-19 patients did not prevent anti-SARS-CoV-2 antibody responses.[32] In our cohort, no significant difference but only a trend was observed for the levels of anti-S antibodies between treated and non-treated patients in the severe and critical groups. This discrepancy could be explained by the larger cohort size of the previous study. A large multi-database cohort study found that chronically tocilizumab-treated rheumatoid arthritis patients had a higher risk of serious bacterial infections.[33] Therefore, long-term follow-up of tocilizumab-treated COVID-19 patients is required for the incidence of opportunistic infections.

Interestingly, Garcia-Beltran et al. described that tocilizumab-treated patients had a significant increase in the neutralization potency index.[34] These results suggest that despite the decreased titers of anti-SARS-CoV2 antibodies, their overall neutralizing capacity does not decrease. We observed a preserved neutralizing capacity in the tocilizumab-treated severe/critical patients. However, our results show that this neutralizing effect is mainly dependent on the maintained PRNT50 because tocilizumab did not seem to affect antibodies titers in these patients.

Concerning the B cell compartment, ASCs have been associated with an early production of anti-SARS-CoV-2 antibodies. However, we did not observe an expansion of ASCs during convalescent stage as previously described in acute phase[21,34,35] or at the beginning of the remission phase.[36] Even if their response is typically transient, some ASCs could still be detected until 45 days in some respiratory syncytial virus infection.[37] The decrease in USM B cells when compared with healthy donors during the convalescent stage of COVID-19 patients, irrespective of severity or treatment, has been previously observed at an earlier time point of the disease.[22] The decrease in USM cells could be due to activation of pre-existing memory cells specific for previous coronavirus infections other than SARS-CoV-2, which differentiated into "atypical" ($CD27^{-}$) and/or ASC. Similarly, we did not observe a modulation of the T cell compartment including Tregs by tocilizumab treatment.

In conclusion, we show that the IgG titers, neutralization responses to variants of SARS-CoV-2, and B and T cell subsets were not significantly modified by tocilizumab. Surprisingly, we demonstrated that whereas no significant differences were observed in the neutralization of WT and Alpha, Beta, and Delta strains, the Gamma and Omicron variants were less neutralized by convalescent plasma of individuals infected with the ancestral strain, irrespective of the disease severity. This may be explained by a cross-reactivity of the neutralizing antibodies more efficiently for some of the variants but weaker for the Gamma and Omicron variants. On the other hand, the neutralizing efficacy against the Omicron variant is strongly reduced in the sera from vaccinated individuals[8] despite the presence of neutralizing memory B cells clones.[38] The intense circulation and the rise of infections and re-infections observed in non-immune and vaccinated populations could be justified by the low neutralizing antibody potential against Omicron.

This study gives clear evidence that, even if limited by sample size, tocilizumab, in addition to its improvement of the clinical outcome in COVID-19 patients, does not affect the long-term immunological responses to SARS-CoV-2 and reinfection with variants during the convalescent period.

Limitations of the study

This is a pilot study with small sample size, and therefore making robust conclusions is not possible and will need further confirmation with a higher number of patients. Also, the patients were not randomly chosen, but selected on clinical features that could introduce potential bias. However, no significant differences in the patient's characteristics in both treated and untreated groups were noted. Our study evaluated non-vaccinated COVID-19 convalescent patients up to six months after disease onset. The patients analyzed for antibody response and seroneutralization were only infected with an ancestral SARS-CoV-2 strain. However, we could perform assays for cross-neutralization of several SARS-CoV-2 VOCs by using convalescent plasma of the patients. Also, we could not compare IgG titers at 6M between treated and non-treated patients as we did not have plasma samples from non-treated group at this time point. Finally, the blood sampling time between mild/moderate and the other groups was different due to the outcome of the symptoms.

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
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Sample collection from patients with COVID-19
  - Cell line
  - Viral isolates
- METHOD DETAILS
  - Peripheral blood mononuclear cells isolation and plasma collection
  - Flow cytometry analysis
  - Virus production
  - Seroneutralization assay
  - ELISA isotypes
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Data and code availability

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106124.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.S., L.G., J.B., C.R., E.S.-L., and B.P.; formal analysis, C.C., L.L., and R.P.; data curation, L.G.; funding acquisition, A.S., J.B., L.G., C.R., E.S.-L., and B.P.; investigation, C.C., L.L., A.K., and S.R.B.; methodology, C.C., L.L., A.K., and S.R.B.; resources, M.R., H.N., A. Bourgarit, C.D., A. Bousquet, S.L.B., R.M., D.S., J.G., M.V., M.G., M.L.M., C.V.-D., J.-F.G., N.P.-T., C.V., C.P., and H.M.; writing – original draft, C.C., L.L., J.B., and A.S.; writing – review & editing, all the authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Brilliant Violet 421(TM) anti-human CD19 antibody (Clone HIB19)	BioLegend	Cat# 302233; RRID: AB_10897802
FITC Mouse anti-human CD27 (Clone M-T271)	BD Pharmingen	Cat# 555440; RRID: AB_395833
PE Mouse anti-human CD38 (Clone HIT2)	BD Pharmingen	Cat# 560981; RRID: AB_10563932
BV510 Mouse anti-human IgD (Clone IA6.2)	BioLegend	Cat# 348219; RRID:AB_2561386
Peroxidase AffiniPure Goat Anti-Human IgG, Fcγ fragment specific	Jackson ImmunoResearch	Cat# 109-035-098; RRID:AB_2337586
IgG1 Fc Mouse anti-Human, HRP	Invitrogen	Cat# 10070738; RRID:AB_2534051
PE Mouse anti-human CD25 (Clone M-A251)	Biolegend	Cat# 356104; RRID: AB_2561861
BV421 Mouse Anti-Human CD127 (Clone HIL-7R-M21)	BD Biosciences	Cat# 562437; RRID:AB_11151911
APC-H7 Mouse Anti-Human CD4 (Clone RPA-T4)	BD Biosciences	Cat# 560158; RRID:AB_1645478
Alexa Fluor® 700 Mouse Anti-Human CD8 (Clone RPA-T8)	BD Biosciences	Cat# 557945; RRID:AB_396953
FITC Mouse Anti-Human CD3 (Clone UCHT1)	BD Biosciences	Cat# 561806; RRID:AB_11154397
APC Mouse anti-human FoxP3 (Clone 236A/E7)	Thermo Fisher Scientific	Cat# 17-4777-42; RRID:AB_10804651
PE-Cy™5 Mouse anti-human CD45RO (Clone UCHL1)	Thermo Fisher Scientific	Cat# 15-0457-42; RRID:AB_11043685
Brilliant Violet 510™ anti-human CD183 (CXCR3) (Clone G025H7)	BioLegend	Cat# 353725; RRID:AB_2562066
FITC Mouse anti-human CD185 (CXCR5) (Clone MU5UBEE)	Thermo Fisher Scientific	Cat# 11-9185-42; RRID:AB_2572526
PE-Cy™7 Mouse Anti-Human CD196 (CCR6) (Clone 11A9)	BD Biosciences	Cat# 560620; RRID:AB_1727440
APC Mouse Anti-Human CD4 (Clone RPA-T4)	BD Biosciences	Cat# 555349; RRID:AB_398593
APC-eFluor™ 780 Mouse anti-human CD8a (Clone OKT8)	Thermo Fisher Scientific	Cat# 47-0086-42; RRID:AB_2573945
Bacterial and virus strains		
BetaCoV/France/IDF0372/2020 (wild-type strain) (GISAID ID: EPI_ISL_410720)	Institut Pasteur, CNR Respiratory Viruses (S. Van der Werf)	N/A
hCoV-19/France/IDF-IPP11324i/2020 (variant alpha; α) (GISAID ID: EPI_ISL_1138411)	Institut Pasteur, CNR Respiratory Viruses (S. Van der Werf)	N/A
hCoV-19/France/PDL-IPP01065i/2021 (variant Beta; β) (GISAID ID: EPI_ISL_1138745)	Institut Pasteur, CNR Respiratory Viruses (S. Van der Werf)	N/A
hCoV-19/French Guiana/IPP03772i/2021 (variant Gamma; γ)	Institut Pasteur, CNR Respiratory Viruses (S. Van der Werf)	N/A
hCoV-19/France/HDF-IPP11602i/2021 (variant Delta; δ)	Institut Pasteur, CNR Respiratory Viruses (S. Van der Werf)	N/A
hCoV-19/France/PDL-IPP46934/2021 (VOC Omicron)	Institut Pasteur, CNR Respiratory Viruses (S. Van der Werf)	N/A
Biological samples		
Cryopreserved PBMCs from mild, moderate, severe, critical COVID-19 patients	COVIMUNE cohort: Bégin hospital, Kremlin-Bicêtre hospital, Lariboisière hospital, Foch hospital; ethical committee (N°2020-A00761-38)	N/A
Cryopreserved PBMCs from naive patients	Etablissement Français du Sang, Rungis, France	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plasma of COVID-19 patients	COVIMUNE cohort: Bégin hospital, Kremlin-Bicêtre hospital, Lariboisière hospital, Foch hospital; ethical committee (N°2020-A00761-38)	N/A
Plasma of naive patient	Institut Pasteur, GFMI (M. Nguyen-De Bernon)	N/A
Chemicals, peptides, and recombinant proteins		
hexapro stabilized recombinant protein of the SARS-CoV-2 S protein	Institut Pasteur, Immunologie Humorale (H. Mouquet)	N/A
FICOLL PAQUE PLUS	fisher scientific	11778538
DMEM medium	fisher scientific	11594446
PENICILLINE STREPTOMICINE LIQUIDE (10.000 U/10.000 µg/mL)	life technologies	11548876
Foetal Bovine Serum, qualified, E.U.-approved, South America origin	life technologies	10270106
CMC medium viscosity	vwr	22525.296
Cristal Violet Oxalate	sigma-aldrich	94448-2,5ML-F
Ethanol, absolute (200 Proof), molecular biology	fisher scientific	10644795
Formaldehyde solution	sigma-aldrich	15512-1L-R
Paraformaldehyde solution 4% in PBS	santa cruz biotechnology	sc-281692
PBS,D'BECCOS W/OCA	fisher scientific	11530486
Tween-20	sigma-aldrich	P7949-500ML
BSA	Miltenyi Biotech	130-091-376
HRP chromogenic substrate ABTS solution	INTERCHIM	ZE8210
eBioscience™ Foxp3/Transcription Factor Staining Buffer Set	ThermoFisher Scientific	00-5523-00
Critical commercial assays		
High-binding ELISA plates	Maxisorp, Nunc	Cat# 44-2404-21
Experimental models: Cell lines		
Vero-E6	ATCC	RRID:CVCL_A7UJ
Software and algorithms		
GraphPad Prism software (v9.0.1)	GraphPad Prism Inc.	RRID:SCR_002798
FlowJo V10 – Version 10.8.0	FlowJo	RRID:SCR_008520
Biorender	Biorender	RRID:SCR_018361
Genstat Version 20 software	GenStat for Windows	RRID:SCR_014595

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Anavaj Sakuntabhai (anavaj.sakuntabhai@pasteur.fr).

Materials availability

No unique reagents were generated in this study.

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.
- Additional Supplemental Items are available from Mendeley Data at https://doi.org/10.17632/krh7cy99wg.1.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Sample collection from patients with COVID-19

All study participants provided their written informed consent for the collection of blood samples and their subsequent analysis (COVIMUNE cohort see Table S1). The COVIMUNE study was approved by ethical committee (N°2020-A00761-38). The eligibility criteria were Adult Patients (≥18 years old) who have recovered from SARS-CoV-2 infection, confirmed with a positive SARS-CoV-2 RT-qPCR, that were free of clinical symptoms for more than 15 days and up to 6 months after onset of symptoms. Exclusion Criteria: Refusal of the patient to participate in the study and Patient under guardianship/curatorship.

Severity of the disease is defined as follows:

- Mild form or non-complicated is defined as no CT-scan signs.
- Moderate form is defined as pneumonia with clinical or CT-scan signs +/− fever
- Severe forms are defined as severe pneumonia with shortness of breath and/or requirement of oxygen therapy (respiratory rate >30/min, SaO2 >93%, PaO2/FiO2>300 mmHg) corresponding to WHO progression scale >4.
- Critical forms are defined as ARDS ($PaO_2/FiO_2 \leq 300$ mmHg; and when PaO_2 is not available, $SpO_2/FiO_2 \leq 315$ mmHg), septic shock or multiorgan acute dysfunction.

Treated patients received the first injection of tocilizumab at a median of 10 days after the onset of the symptoms (range 3–16 days). Seven patients received a second dose between two and four days after the first infusion. The outcome of tocilizumab treatment was evaluated regarding reduction of mechanical ventilation including high-flow nasal oxygen therapy and non-invasive and invasive mechanical ventilation according to the previous criteria.[14] Secondary outcomes (death from any cause, time to hospital discharge, modification of biological parameters) were also considered in the patient's response to treatment. No significant difference was observed between the patients infused once or twice with tocilizumab. The patients treated with tocilizumab were compared with a cohort of severe and critical COVID-19 untreated control individuals with the same selection criteria.

58 patient samples were collected before the emergence of variants tested in this study (between June and October 2020). Nine patient samples were collected after the emergence of the alpha variant (between February and April 2021) and were only used for B cell phenotyping: mild/moderate (n = 5), severe (n = 3), and tocilizumab-treated (n = 1).

Plasma and peripheral blood mononuclear cell (PBMC) specimens were collected from the patients. The plasmas of non-treated patients were sampled at a median of 34 (range 23–125 days), 85 (range 28–115 days), and 94 (range 47–143 days) days post-symptom onset for the mild/moderate, the severe and the critical groups respectively.

Disease severity was defined as mild, moderate, severe or critical based on a modified version of the WHO interim guidance.[1,39] In brief, mild disease (WHO scale 1–2) was defined as an uncomplicated upper respiratory tract infection (URI) that did not require hospitalization. Moderate disease (WHO scale 4) was defined as a lower respiratory tract infection without the need for oxygen therapy, without signs of severity (see further), or as a URI requiring hospitalization. Severe disease (WHO scale 5–6) was defined as a lower respiratory tract infection with fever plus any one of the following severity signs: tachypnea (respiratory rate >30 breaths per minute), respiratory distress, or oxygen saturation less than 93% on room air. Critical disease (WHO scale 6–8) was defined as the presence of acute respiratory distress syndrome (ARDS), or septic shock. A total of 54 out 69 were admitted to hospital: Mild patients were not admitted in hospital while moderate and severe patients were admitted in the ward; Critical patients were admitted in ICU.

The six moderate patients had documented chest CT-scan pneumonia without hospitalization requirement.

The median sampling time of plasma was 85 days (70–115) for the severe untreated group, 94 days (47–143) for the critical untreated group, 74.5 days (67–105) for the severe tocilizumab-treated group and 96 days (73–113) for the critical tocilizumab-treated group at 3M. 100% of the patients responded to tocilizumab in the severe group while only 27% of the critical patients responded to the treatment.

PBMCs of healthy donors sampled before COVID-19 pandemic (2013) were used as a control in some experiments (Etablissement Français du Sang, Rungis, France).

Cell line

Vero E6 (African green monkey kidney epithelial cells, ATCC, CRL-1586, RRID:CVCL_A7UJ) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 5 units/mL penicillin, and 5 μg/mL streptomycin (1% PS), and were cultured at 37°C 5% CO_2.

Viral isolates

All the virus isolates were supplied by the National Reference Center for Respiratory Viruses hosted at Institut Pasteur (Paris, France) and headed by Dr. Sylvie van der Werf. The human sample from which Beta-CoV/France/IDF0372/2020 (GISAID ID: EPI_ISL_410720) was isolated was kindly provided by Drs. Xavier Lescure, Yazdan Yazdanpanah from the Bichat Hospital, Paris. The human sample from which hCoV-19/France/IDF-IPP11324i/2020 (EPI_ISL_1138411; VOC Alpha) was isolated, has been provided by Dr. Vincent Foissaud, HIA Percy, Clamart, France. The human sample from which hCoV-19/France/PDL-IPP01065i/2021 (EPI_ISL_1138745; VOC Beta) was isolated has been provided by Dr. Besson J., Bioliance Laboratory, Saint-Herblain France. The human sample from which hCoV-19/French Guiana/IPP03772i/2021 (VOC Gamma) was isolated has been provided by Dr. Dominique Rousset, Institut Pasteur, Cayenne, Guyane. The human sample from which strain hCoV-19/France/HDF-IPP11602i/2021 (VOC Delta) was isolated has been provided by Dr Raphaël Guiheneuf, CH Simone Veil, Beauvais, France. The human sample from which strain hCoV-19/France/PDL-IPP46934/2021 (VOC Omicron) was isolated has been provided by Dr Axelle Paquin, CH de Laval, Laval, France.

METHOD DETAILS

Peripheral blood mononuclear cells isolation and plasma collection

Peripheral blood samples were collected in heparin sodium tubes and processed within 6 h of collection. PBMCs were isolated by density gradient centrifugation (Ficoll Paque Plus, GE Healthcare) at 1500 rpm for 25 min at room temperature. Aliquots from the plasma layer were collected and stored at −20°C until use. PBMCs were washed twice with RPMI at 1500 rpm for 5 min. Viability was assessed using trypan blue exclusion, and live cells were counted using an automated hemocytometer (Nexcelom, Ozyme).

Flow cytometry analysis

Isolated PBMCs (5×10^6) were centrifuged and resuspended in FACS buffer (PBS +1% Human serum). Samples were stained with antibodies cocktail for 20 min at 4°C. The following antibodies were used to stain B cells: BV421 Mouse anti-human CD19 (Clone HIB19; Cat# 302233; BioLegend; RRID: AB_10897802), FITC Mouse anti-human CD27 (Clone M-T271; Cat# 555440; BD Pharmingen; RRID: AB_395833), PE Mouse anti-human CD38 (Clone HIT2; Cat# 560981; BD Pharmingen; RRID: AB_10563932), BV510 Mouse anti-human IgD (Clone IA6.2; Cat# 348219; BioLegend; RRID:AB_2561386). The following antibodies and reagent were used to stain T cells: eBioscience Fixable Viability Dye eFluor 506 (Cat# 65-0866-14; Thermo Fisher Scientific), PE Mouse anti-human CD25 (Clone M-A251; Cat# 356104; Biolegend; RRID: AB_2561861), BV421 Mouse Anti-Human CD127 (Clone HIL-7R-M21; Cat# 562437; BD Biosciences; RRID:AB_11151911), APC-H7 Mouse Anti-Human CD4 (Clone RPA-T4; Cat# 560158; BD Biosciences; RRID:AB_1645478), Alexa Fluor 700 Mouse Anti-Human CD8 (Clone RPA-T8; Cat# 557945; BD Biosciences; RRID:AB_396953), FITC Mouse Anti-Human CD3 (Clone UCHT1; Cat# 561806; BD Biosciences; RRID:AB_11154397), APC Mouse anti-human FoxP3 (Clone 236A/E7; Cat# 17-4777-42; Thermo Fisher Scientific; RRID:AB_10804651), PE-Cy5 Mouse anti-human CD45RO (Clone UCHL1; Cat# 15-0457-42; Thermo Fisher Scientific; RRID:AB_11043685), Brilliant Violet 510 anti-human CD183 (CXCR3) (Clone G025H7; Cat# 353725; BioLegend; RRID:AB_2562066), FITC Mouse anti-human CD185 (CXCR5) (Clone MU5UBEE; Cat# 11-9185-42; Thermo

Fisher Scientific; RRID:AB_2572526), PE-Cy7 Mouse Anti-Human CD196 (CCR6) (Clone 11A9; Cat# 560620; BD Biosciences; RRID:AB_1727440), APC Mouse Anti-Human CD4 (Clone RPA-T4; Cat# 555349; BD Biosciences; RRID:AB_398593), APC-eFluor 780 Mouse anti-human CD8a (Clone OKT8; Cat# 47-0086-42; Thermo Fisher Scientific; RRID:AB_2573945). After staining, cells were washed twice in FACS buffer and fixed at 2% paraformaldehyde (PFA) for 10 min at room temperature in the dark before a final wash and resuspension for analysis. The eBioscience Foxp3/Transcription Factor Staining Buffer Set was used for intracellular staining (Cat# 00-5523-00). Data were analyzed on a BD LSR II instrument (BD).

Virus production

Viral stocks were produced on Vero E6 cells, in DMEM 2% FBS, 1% PS viral titers were determined by plaque assay in 24-wells plates, seeded 24h prior with 1.5×10^5 cells per well in DMEM supplemented with 10% FBS and 1% PS. We used 10-fold serial dilution in DMEM 0% FBS 1% PS in triplicates. After 1 h of incubation at 37°C, 5% CO_2, the medium was replaced by overlaying carboxymethyl cellulose CMC/DMEM 2% FBS (v/v). After 72 h of incubation, the overlay was removed and a mixture of crystal violet/ethanol/formaldehyde was added, followed by incubation at room temperature for 30 min. All experiments with live SARS-CoV-2 were performed in compliance with Institut Pasteur guidelines for Biosafety Level 3 work.

Seroneutralization assay

Plasma from patients who recovered from COVID-19 was decomplemented for 30 min at 56°C. Vero E6 cells were seeded in a 24-wells plate at 1.5×10^5 cells per well in DMEM supplemented with 10% FBS and 1% PS. The following day, virus inoculum was prepared to obtain 70 plaques per well and incubated with serial two-fold dilutions of sera, starting from 1:20 to 1:320 (final 1:40 to 1:640 v/v with the virus) in 200 µL DMEM 0% FBS 1% PS during 1 h at 37°C, 5% CO_2. After 1 h, the medium was replaced onto the cells by the mix of plasma/virus and then incubated 2 h at 37°C 5% CO_2. The supernatant was discarded, the overlay CMC/DMEM 2% FBS (v/v) was added on the cells and incubated for 72 h at 37°C, 5% CO_2. Three days after, the overlay was removed and 200 µL mixture of crystal violet/ethanol/formaldehyde were added during 30 min before plaques counting. PRNT50 (plaque reducing neutralizing titer 50) were expressed as the plasma dilution in which 50% of the virus was neutralized.

ELISA isotypes

S protein was a kind gift from Cyril Planchais and Hugo Mouquet, Institut Pasteur. The S protein used is an hexapro stabilized recombinant protein of the SARS-CoV-2 S protein of the virus. All plasma samples were heat-inactivated at 56°C for 30 min before use. The plasma from a healthy donor sampled before COVID-19 pandemic was used as a naive control. High-binding ELISA plates (Maxisorp, Nunc; 44-2404-21) were coated with S recombinant protein at 2.5 µg/mL (50 µL per well) in PBS overnight at room temperature. Wells were washed with PBS-T (PBS with 0.05% Tween 20) and then blocked with 200 µL of 2% BSA/1 mM EDTA in PBS-T for 2 h at room temperature. After PBS-T washings, 1:100-diluted sera in PBS (50 µL per well) and four consecutive 1:4 dilutions were added and incubated for 2 h. A negative control plasma was included and diluted like the COVID samples. Wells were washed with PBS-T. Secondary antibody was diluted in 2% BSA/1 mM EDTA in PBS-T and incubated for 1 h at room temperature (50 µL; 1:1,000 dilution). Total IgG were detected using Peroxidase AffiniPure Goat Anti-Human IgG, Fcγ fragment specific (Jackson ImmunoResearch; 109-035-098). IgG1 was detected using IgG1 Fc Mouse anti-Human, HRP (Invitrogen; 10070738). Plates were revealed by adding 100 µL of HRP chromogenic substrate (ABTS solution, Euromedex) after PBS-T washings. ODs were measured at 405 nm (OD405nm) after a 30 min incubation on a Sunrise microplate absorbance reader (Tecan, Männedorf, Switzerland). Experiments were performed in duplicate at room temperature. The Area Under the Curve (AUC) values were calculated by plotting the log10 of the dilution factor values and the OD405nm values using GraphPad Prism software (v9.0.1, GraphPad Prism Inc.).

QUANTIFICATION AND STATISTICAL ANALYSIS

Nonparametric tests were used including the Kruskal-Wallis test, followed by Dunn's *post-hoc* test to compare multiple independent groups and the Wilcoxon t-test to compare paired values of two groups. Two-sided Friedman test with Dunn's test for multiple comparisons was performed between each viral strain. The Spearman correlation test was used to measure the correlation between two variables. For comparison of the delay to time of sampling post onset of symptoms among the severity classes (mild/moderate, severe and critical) at timepoints 3 or 6 months, a Generalized Linear Model (GLM) was

fitted. For comparison of total IgG and IgG1 titers in severity classes and treatment groups, a GLM was likewise fitted. For comparison of IgG titers over the two points in the same individuals, a Generalized Linear Mixed Model was used and Person fitted as the random factor. In all cases, the normality of the distribution of the dependent variable was assessed using the Shapiro-Wilks test for normality. When normal, a GLM with normal distribution was fitted. When found to deviate from a normal distribution a loglinear (i.e. Poisson) GLM (or GLMM) was fitted and a dispersion parameter estimated to account for any over-dispersion of the data.

Significant p values are indicated as described: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. ns is non-significant. Statistical analysis was performed using GraphPad Prism software, V6 (GraphPad, San Diego) and Genstat version 20 for the GLMM (GenStat for Windows 20th Edition, VSN International Ltd., Hemel Hempstead, UK).

Data and code availability

This paper does not report new datasets of a standardized datatype and does not report custom code. Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.