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ORIGINAL ARTICLE

Severe SARS-CoV-2 patients develop a higher specific T-cell response

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Abstract

Objectives. Assessment of the adaptive immune response against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is crucial for studying long-term immunity and vaccine strategies. We quantified IFN γ -secreting T cells reactive against the main viral SARS-CoV-2 antigens using a standardised enzyme-linked immunospot assay (ELISpot). **Methods.** Overlapping peptide pools built from the sequences of M, N and S viral proteins and a mix (MNS) were used as antigens. Using IFN γ T-CoV-Spot assay, we assessed T-cell and antibody responses in mild, moderate and severe SARS-CoV-2 patients and in control samples collected before the outbreak. **Results.** Specific T cells were assessed in 60 consecutive patients (mild, $n = 26$; moderate, $n = 10$; and severe patients, $n = 24$) during their follow-up (median time from symptom onset [interquartile range]: 36 days [28;53]). T cells against M, N and S peptide pools were detected in $n = 60$ (100%), $n = 56$ (93.3%), $n = 55$ patients (91.7%), respectively. Using the MNS mix, IFN γ T-CoV-Spot assay showed a specificity of 96.7%

(95% CI, 88.5–99.6%) and a specificity of 90.3% (75.2–98.0%). The frequency of reactive T cells observed with M, S and MNS mix pools correlated with severity and with levels of anti-S1 and anti-RBD serum antibodies. **Conclusion.** IFN γ T-CoV-Spot assay is a reliable method to explore specific T cells in large cohorts of patients. This test may become a useful tool to assess the long-lived memory T-cell response after vaccination. Our study demonstrates that SARS-CoV-2 patients developing a severe disease achieve a higher adaptive immune response.

Keywords: ELISpot, SARS-CoV-2, T cells

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread rapidly worldwide. A large majority of SARS-CoV-2 patients, reportedly up to 60% in a highly confined population, remain asymptomatic.¹ A few patients will need hospitalisation for moderate symptoms related to pneumonia but their requirement for oxygen therapy is low.² The most severely affected patients, who require intensive care unit admission, develop acute respiratory distress syndrome at about 7–10 days after symptom onset and multiple organ failure.³ Severe lung damage can be induced by heterogeneous patterns ranging from diffuse alveolar damage to acute fibrinous and organising pneumonia, with frequent pulmonary thrombosis.^{4–6} Several convergent reports describe an immune dysregulation in these severe patients, associating an impaired type I interferon production,^{7,8} and a dramatic increase in plasma levels of other cytokines, such as interleukin-6 (IL-6), TNF α , IL-2 and the soluble IL-2 receptor (sIL-2R), IFN γ and IL-10.^{2,9–12} Peripheral lymphopenia is also reported in more than 80% of patients, and severe patients presented the lowest lymphocyte counts,^{9,13–15} and exhausted and reduced functional T cells.^{14,16} Blood lymphopenia was also associated with lymphocyte depletion in spleen and lymph nodes, decreased or absent germinal centres,^{5,17} and with aggressive migration to the lungs.¹⁴ All these features could affect specific immunisation in these patients. Earlier seroconversion and/or higher titres of specific antibodies were detected in the patients with a severe outcome,^{18–20} suggesting that an adaptive response may be involved in severe patients. Some patients also remain RT-PCR-positive for weeks despite the presence of antibodies, and, conversely, some patients recover without generating high

titres of specific antibodies: these discordant data suggest that cellular-specific immunity may be critical for clinical resolution of SARS-CoV-2 infection.^{21,22} Given that SARS-CoV-2 is an emerging virus potentially responsible for severe disease with a high mortality rate, it is crucial to understand the role of the immune adaptive response, that is after primo-infection, and at the earliest opportunity, to explore the immune response after induced immunisation, that is after vaccination. Among the major viral structural proteins, spike (S), nucleocapsid (N) and membrane (M) proteins bear the immunodominant B-cell epitopes, but also T-cell epitopes.²³ Currently, the anti-S adaptive response is the one mainly being investigated, but anti-M and anti-N responses may be of interest to better apprehend naturally acquired specific immunity. Understanding the cellular response may also be important regarding the suspected cross-reactivity with human coronaviruses that cause the common cold: indeed, anti-SARS-CoV-2-reactive CD4⁺ T cells were found in 34% to 60% of unexposed subjects.^{24,25}

To address the major issue of the cellular response in SARS-CoV-2 patients, and especially in severe patients, we quantified IFN γ -secreting T cells reactive against the main viral SARS-CoV-2 antigens using a standardised enzyme-linked immunospot (ELISpot) assay (IFN γ T-CoV-Spot assay) and compared T-cell and antibody responses in mild, moderate and severe SARS-CoV-2 patients.

RESULTS

Quantification of M, N, S and MNS mix-specific reactive T cells in SARS-CoV-2 patients

IFN γ T-CoV-Spot assay was first performed in 60 consecutive SARS-CoV-2 patients using the M, N, S

peptide pools and the MNS mix. Main characteristics of the patients are detailed in Table 1. The median (range) time from symptom onset (day 0, D0) to sampling was 36 (10–70) days and only 5 patients were sampled before D21. The patients were classified as mild ($n = 26$), moderate ($n = 10$) or severe ($n = 24$). Severe patients were older, more likely to be male and were sampled later compared to mild and moderate patients (Table 1). Variations in numbers of IFN γ -Spot-forming cells (SFCs) were observed from one peptide pool to another in a given patient (Figure 1a), a higher magnitude of response was detected with M peptide pool, and the MNS mix induced the highest *in vitro* T-cell response compared with each pool of peptides alone (Figure 1b). Despite these differences in reactivity from one protein to another, there was a large correlation between the numbers of IFN γ -SFCs detected with M, N and S pools in this population of patients ($r > 0.6$ for each M/N, M/S and N/S correlation), suggesting that a given patient could have a high frequency of reactive T cells against the three proteins or, conversely, a low frequency of reactive T cells against the three proteins (Figure 1c–e).

Assessment of sensitivity and specificity of the IFN γ T-CoV-Spot assay

Among the 60 patients, reactive T cells were detected against M, N, S and the MNS mix of peptides in $n = 60$ (100%), $n = 56$ (93.3%), $n = 55$ (91.7%) and $n = 58$ (96.7%), respectively. Considering the high correlations between IFN γ -SFCs detected with each of M, N and S proteins and with the MNS mix ($r \geq 0.7$) (Figure 2a), we tested 31 cryopreserved pairs of sera and PBMCs collected before the outbreak (control samples) using the MNS mix (Figure 2b). Three of the PBMC samples (9.7%) tested positive for reactive T cells against the MNS mix. From the ROC curve, we estimated that a number of IFN γ -SFCs ≥ 4 (optimal cut-off value) had a sensitivity (95% CI) of 96.7% (88.5–99.6%) and a specificity of 90.3% (95% CI, 75.2–98.0%) (Figure 2c). An additional cohort of 112 SARS-CoV-2 patients was tested with the MNS mix using an entirely automated procedure including PBMC isolation and spot detection. Detailed characteristics of both cohorts of patients are shown in Table 2. ROC curve applied to this confirmation cohort gave a similar sensitivity of 95.5% (95% CI, 89.9–98.5%) for a cut-off ≥ 4 IFN γ -SFCs.

Table 1. Main characteristics of included SARS-CoV-2 patients (at the time of IFN γ T-CoV-Spot assay)

	All patients ($n = 60$)	Mild disease ($n = 26$)	Moderate disease ($n = 10$)	Severe disease ($n = 24$)	<i>P</i> -value
Age, years	49 (20;92)	35 (28.7;47)	53 (37;64)	63 (51;72)	< 0.0001
Sex (male)	29 (48.3)	5 (19.2)	3 (30)	21 (87.5)	< 0.0001
Time since symptom onset, days					
Median (IQR)	36 (28;53)	32 (25;37)	34 (28;57)	52 (36;56)	0.001
Range	10–70	10–59	15–70	13–64	
Time since diagnosis by RT-PCR, days					
Median (IQR)	31 (20;41)	26 (8;31)	35 (24;53)	40 (28;48)	0.003
Range	3–64	5–57	3–64	8–52	
Patient management					
Outpatients	25 (41.7)	25 (96.1)	0	0	na
Hospitalised patients					
Discharged at the time of sample	28 (46.7)	1 (3.8)	10 (100)	17 (70.8)	na
Still hospitalised at the time of sample	7 (11.7)	0	0	7 (29.2)	na
T-cell counts ^a					
CD3 ⁺ T cells ($\times 10^9$ L ⁻¹)	1.2 (0.6;2.7)	1.2 (0.8;1.8)	1.0 (0.7;1.4)	1.3 (1.0;1.8)	0.4
CD3 ⁺ CD4 ⁺ T cells ($\times 10^9$ L ⁻¹)	0.8 (0.2;1.5)	0.7 (0.4;1.0)	0.7 (0.4;0.9)	0.7 (0.6;1.1)	0.64
CD3 ⁺ CD8 ⁺ T cells ($\times 10^9$ L ⁻¹)	0.5 (0.1;1.2)	0.5 (0.3;0.7)	0.3 (0.2;0.5)	0.5 (0.4;0.6)	0.35

Data are median (IQR) or n (%). *P*-values were calculated using the Kruskal–Wallis test or chi-square test, as appropriate. IQR, interquartile range; na, not applicable; RT-PCR, real-time reverse transcription PCR assay; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

^aT-cell counts were available in 38 of 60 patients and in 17, 6 and 15 mild, moderate and severe patients, respectively.

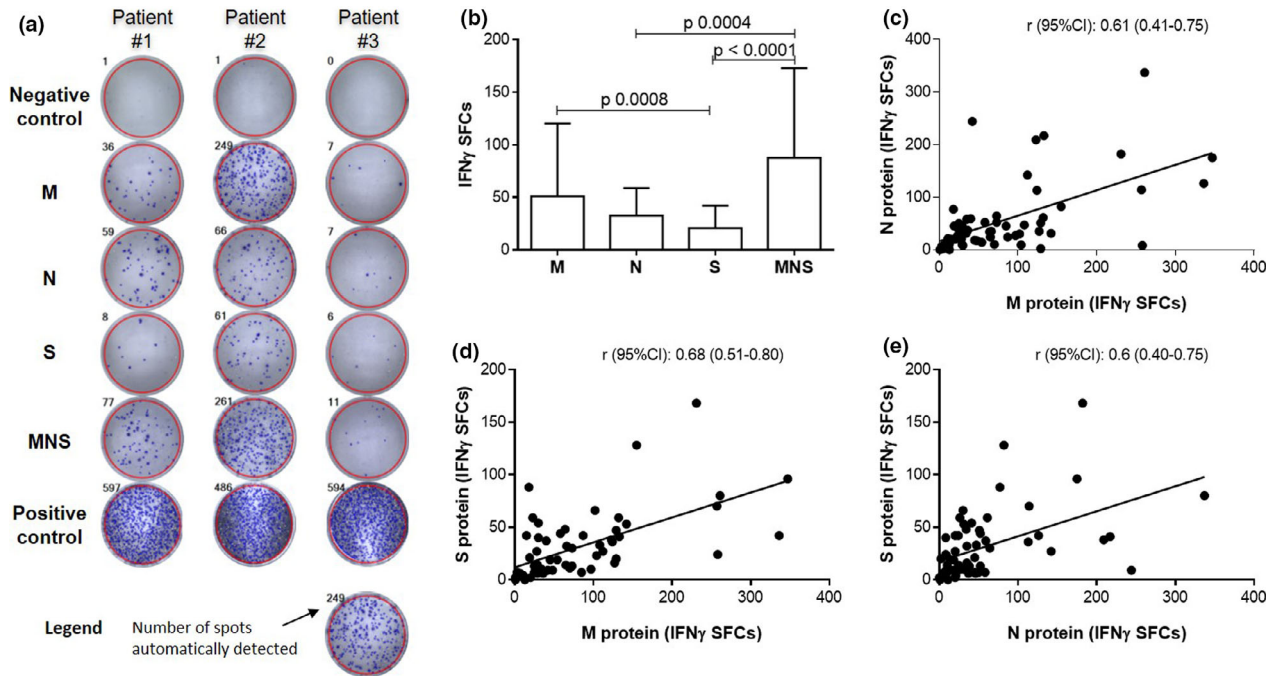


Figure 1. M, N, S and MNS mix-reactive T cells in 60 SARS-CoV-2 patients. **(a)** Automated detection of IFN γ -SFCs in wells after 16–20 h of stimulation. Negative and positive control wells allow quality control assessment; reactive T cells are counted in ‘M’, ‘N’, ‘S’ and ‘MNS’ wells. Three representative patterns of response are illustrated. Negative control: well without antigen/mitogen. Positive control: well with phytohaemagglutinin (PHA). **(b)** Comparisons of IFN γ -SFCs according to the tested antigens. Data are presented as the median + IQR and P -values comparing the tested antigens from Dunn’s tests (*post hoc* for the Kruskal–Wallis test). **(c–e)** Comparisons of IFN γ -SFCs according to the tested antigens. Correlations were assessed using Spearman’s rank correlation coefficients (r).

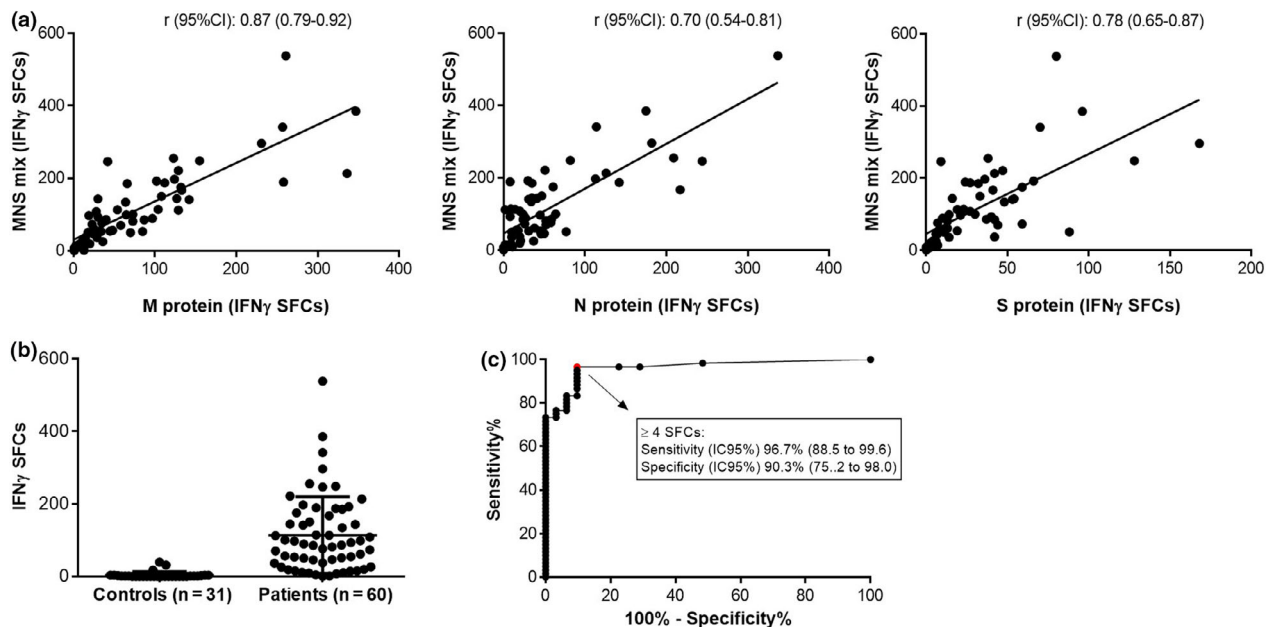


Figure 2. Characteristics of IFN γ T-CoV-Spot assay using the MNS mix as antigenic preparation. **(a)** Correlation (Spearman’s rank correlation) between IFN γ -SFC counts with MNS and with M, N and S alone in the first 60 patients. **(b)** IFN γ -SFC counts with MNS in 31 controls and 60 SARS-CoV-2 patients. **(c)** ROC curve for T-CoV-Spot using the MNS mix with an optimal cut-off value at 4 IFN γ -SFCs.

Table 2. Detailed characteristics of SARS-CoV-2 patients

	Patients tested with M, N, S peptide pools and MNS mix (n = 60)	Patients tested with MNS mix only, for sensitivity confirmation (n = 112)	Total, n = 172
Age, years	49 (20;92)	57 (19;94)	55.5 (19;94)
Sex (male)	29 (48%)	71 (63%)	100 (58%)
Comorbidities			
Hypertension	17 (28%)	43 (38%)	60 (35%)
Chronic heart disease	4 (7%)	23 (21%)	27 (16%)
Diabetes	9 (15%)	28 (25%)	37 (22%)
Malignancy	7 (12%)	8 (7%)	15 (9%)
Chronic lung disease (COPD, asthma and/or sleep apnoea syndrome)	3 (5%) (0/1/2)	22 (20%) (3/6/15)	25 (15%) (3/7/17)
Chronic kidney disease	2 (3%)	7 (6%)	9 (5%)
Chronic liver disease	3 (5%)	2 (2%)	5 (3%)
HIV infection	0	0	0
Overweight/obesity	15/58 (26%)	50/94 (53%)	65/152 (43%)
Smokers	0/56 (0%)	5/84 (6%)	5 (4%)
Systemic steroids	4 (7%)	4 (4%)	8 (5%)
Chemotherapy and/or immunosuppressants	3 (5%)	8 (7%)	11 (6%)
Signs and symptoms			
General symptoms			
Fever	48 (80%)	92 (82%)	140 (81%)
Fatigue	50 (83%)	86 (77%)	136 (79%)
Myalgia	26 (43%)	56 (50%)	82 (48%)
Respiratory symptoms			
Cough	41 (68%)	86 (77%)	127 (74%)
Dyspnoea	39 (65%)	86 (77%)	125 (73%)
Pneumonia	36 (60%)	86 (77%)	122 (71%)
Ear, nose and throat symptoms			
Rhinorrhoea, nasal obstruction	13 (22%)	22 (20%)	35 (20%)
Anosmia and/or dysgeusia	18 (30%)	39 (35%)	57 (33%)
Digestive symptoms			
Diarrhoea	11 (18%)	25 (22%)	36 (21%)
Nausea/vomiting	6 (10%)	10 (9%)	16 (9%)
Abdominal pain	10 (17%)	9 (8%)	19 (11%)
Headaches	18 (30%)	26 (23%)	44 (26%)
Skin symptoms			
Chilblains	0	0	0
Rash	3 (5%)	2 (2%)	5 (3%)
Positive PCR at diagnosis			
Deep nasal	46 (63%)	71 (63%)	117 (68%)
Tracheal	34 (57%)	78 (70%)	112 (65%)
Baseline blood tests			
Total white blood cells ($\times 10^9 \text{ L}^{-1}$)	6.39 (2.6;17.9) (n = 40)	6.23 (1.4;22.0) (n = 74)	6.37 (1.4;22.0) (n = 114)
Neutrophils ($\times 10^9 \text{ L}^{-1}$)	4.6 (1.8;9.7) (n = 34)	4.3 (0.64;18) (n = 59)	4.44 (0.64;18) (n = 93)
Lymphocytes ($\times 10^9 \text{ L}^{-1}$)	0.92 (0.05;1.4) (n = 34)	0.90 (0.25;3) (n = 59)	0.9 (0.05;3) (n = 93)
Eosinophils ($\times 10^9 \text{ L}^{-1}$)	0 (0;0.3) (n = 34)	0 (0.0.4) (n = 59)	0 (0;0.4)
Creatinine ($\mu\text{mol L}^{-1}$)	70 (44;160) (n = 39)	79 (44;336) (n = 73)	71 (44;336) (n = 112)
C-reactive protein (mg L^{-1})	58.5 (7;254) (n = 38)	60 (0;400) (n = 71)	60 (0;400)
Onset of symptoms to T-CoV-Spot assay (days)	36 (10;70)	38 (8;92)	37 (8;92)
Patient management			
Outpatients	25 (42%)	24 (21%)	49 (28%)
Hospitalised, discharged at the time of sample	28 (47%)	79 (71%)	107 (62%)
Hospitalised, still hospitalised	7 (12%)	9 (8%)	17 (10%)
Severity status (since diagnosis)			
Mild	25 (42%)	24 (21%)	49 (28%)

(Continued)

Table 2. Continued.

	Patients tested with M, N, S peptide pools and MNS mix (<i>n</i> = 60)	Patients tested with MNS mix only, for sensitivity confirmation (<i>n</i> = 112)	Total, <i>n</i> = 172
Moderate	11 (18%)	49 (44%)	60 (35%)
Severe	24 (40%)	39 (35%)	63 (37%)
T-cell counts (at the time of T-CoV-Spot assay)			
CD3 ⁺ T cells ($\times 10^9$ L ⁻¹)	1.2 (0.6;2.7) (<i>n</i> = 39)	1.2 (0.5;2.2) (<i>n</i> = 72)	1.4 (0.55;2.7) (<i>n</i> = 111)
CD3 ⁺ CD4 ⁺ T cells ($\times 10^9$ L ⁻¹)	0.75 (0.25;1.5) (<i>n</i> = 39)	0.7 (0.3;1.8) (<i>n</i> = 72)	0.7 (0.25;1.8) (<i>n</i> = 111)
CD3 ⁺ CD8 ⁺ T cells ($\times 10^9$ L ⁻¹)	0.5 (0.08;1.2) (<i>n</i> = 39)	0.4 (0.1;1.2) (<i>n</i> = 72)	0.4 (0.08;1.2) (<i>n</i> = 111)

[†]Data are median (range), *n* (%), or *n/N* (%), where *N* is the total number of patients with available data.

Frequency of specific reactive T cells according to disease severity

Using this validated standardised approach, we then compared the number of IFN γ -SFCs according to severity status in the cohort of 60 patients. The frequency of reactive T cells observed with M, S and MNS mix pools correlated with severity (after adjustment for time from symptom onset to follow-up with IFN γ T-CoV-Spot assay) (Figure 3). We also tested the other secreted cytokines in the well containing the MNS mix (*n* = 38 available samples, median [IQR] time from symptom onset: 36 [28;53] days) (Figure 4). We detected IL-2, IL-4, IL-5, IL-13 and IL-17A secretion in supernatants of stimulated cells in a large majority of patients, with high concentrations for IL-2 (Figure 4a). Despite the fact that higher levels of IL-2, IL-5, IL-13 and IL-17A were detected in supernatants of stimulated PBMCs from severe patients, only IL-2 levels correlated with severity (Figure 4b).

Outcome of immunological features from diagnosis to follow-up, according to disease severity

To explain the higher cellular immune response in severe patients, we retrospectively assessed the main relevant immune characteristics previously reported in severe patients. Data were reported when available among the 172 patients tested by IFN γ T-CoV-Spot assay. At SARS-CoV-2 diagnosis, lower lymphocyte counts and higher plasma levels of IL-6, TNF α , sIL-2Rs and IL-10 correlated with severity (Figure 5a and b). At follow-up, lymphocyte counts for each subset returned to normal values and correlation with severity had disappeared (Figure 5a). However, plasma levels of IL-1 β , TNF α , sIL-2R, IFN γ and IL-10 remained

increased in moderate-to-severe patients and, interestingly, IL-6, TNF α and sIL-2R levels still correlated with severity (Figure 5b).

Specific antibody levels and frequency of reactive T cells according to disease severity

We also studied the anti-N, anti-S1 and anti-RBD antibodies in the 49 of the 60 patients for whom a serum sample was available on the same day as the blood sample for IFN γ T-CoV-Spot assay. In the whole population, anti-RBD IgM was detected in 40 (82%) patients and anti-S1 IgA, IgG and anti-RBD total antibodies (IgG, IgA, IgM) were detected in 46 (94%), 47 (96%) and 46 (94%) patients, respectively. The antibody ratios of anti-S1 IgA, anti-S1 IgG, anti-RBD IgM and anti-RBD total antibodies correlated with severity (Figure 6) (no correlation was observed for anti-N antibodies, data not shown). We then compared the calculated ratio for anti-S antibody ELISA response with the number of corresponding S-reactive T cells (IFN γ -SFCs). A small (anti-S1 IgG), moderate (anti-S1 IgA, anti-RBD IgM) and large correlation (anti-RBD IgG/IgA/IgM) was observed, respectively (Figure 7) (no correlation was observed for N protein, data not shown).

DISCUSSION

To improve our understanding of SARS-CoV-2 pathophysiology, it is important to comprehend how the T-cell response develops in relation to disease severity. To this end, we prospectively explored a large group of patients with various levels of severity status. By combining antibody and T-cell responses, our study demonstrates that SARS-CoV-2 patients achieve a higher adaptive immune response after developing a more severe disease.

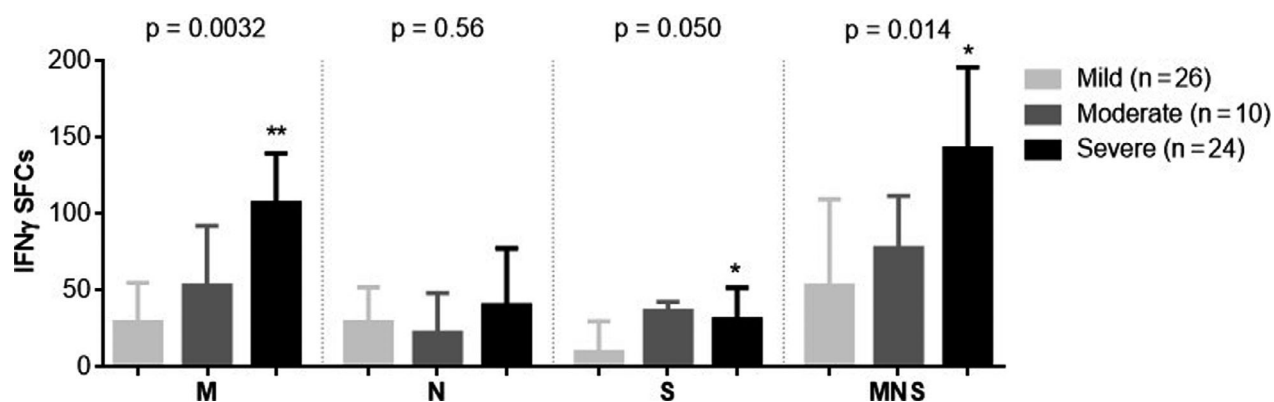


Figure 3. Assessment of IFN γ -secreting SARS-CoV-2-reactive T cells according to patients' severity status ($n = 60$). P -values are calculated using partial Spearman's rank correlation coefficients with severity status, adjusted for time from symptom onset to sampling. Comparisons between severe and mild patients using Dunn's tests (post hoc for the Kruskal–Wallis test) are indicated as $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ or $****P \leq 0.0001$.

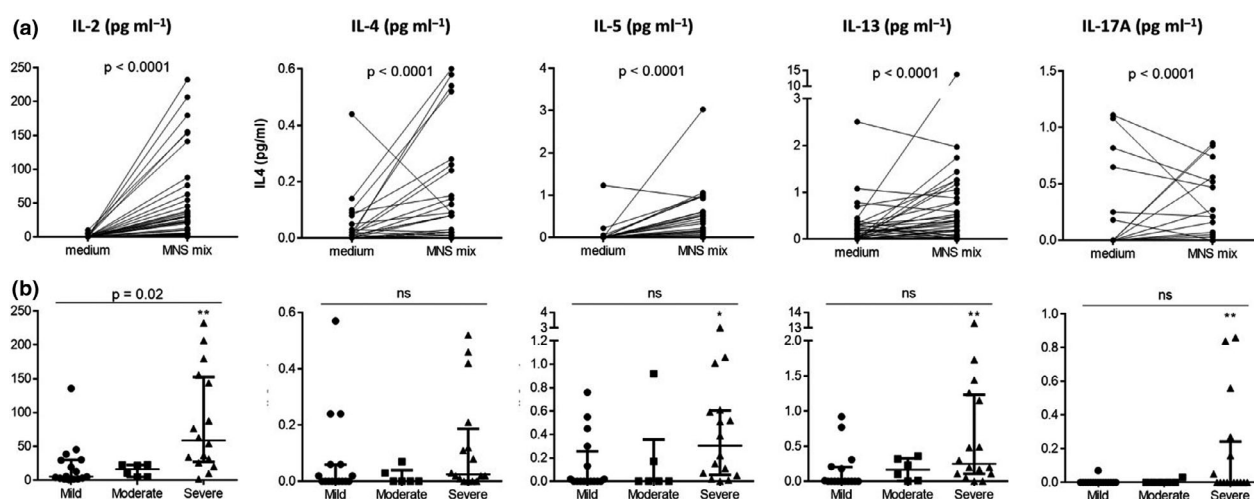


Figure 4. Cytokine levels in the supernatants of PBMCs from SARS-CoV-2 patients. **(a)** Cytokine levels detected after stimulation by the MNS mix or in the negative control (medium). P -values are from the Wilcoxon test. **(b)** Cytokine levels detected after stimulation by the MNS mix, according to the severity of the disease in mild ($n = 16$), moderate ($n = 6$) and severe patients ($n = 16$). P -values are calculated using partial Spearman's rank correlation coefficients with severity status, adjusted for time from symptom onset to sampling. Comparisons between severe and mild patients using Dunn's tests (post hoc for the Kruskal–Wallis test) are indicated as $*P \leq 0.05$ or $**P \leq 0.01$.

Among the available methods to quantify specific T-cell response, we chose to develop an ELISpot assay, a very sensitive technique to detect low-frequency antigen-specific T cells that secrete effector molecules.²⁶ Therefore, measuring T-cell IFN γ release after stimulation with SARS-CoV-2-specific antigens is an important tool to assess both T CD4⁺ and T CD8⁺ responses.

We first set up our method using the main SARS-CoV-2 antigens, and we identified some variations in the frequency of reactivity against M, N and S peptide pools. This difference could be

mainly explained by the different nature of the selected antigens: complete sequence of the protein for M and N overlapping peptide pools, selected domains for the S peptide pool. Distinct *in vivo* antigen uptake and presentation by dendritic cells (DCs) could also explain these variations, considering the nature of post-translational modifications, including phosphorylation (N protein) or glycosylation (M and S proteins).

Despite the disparity in terms of frequencies of reactive T cells in response to SARS-CoV-2 peptide

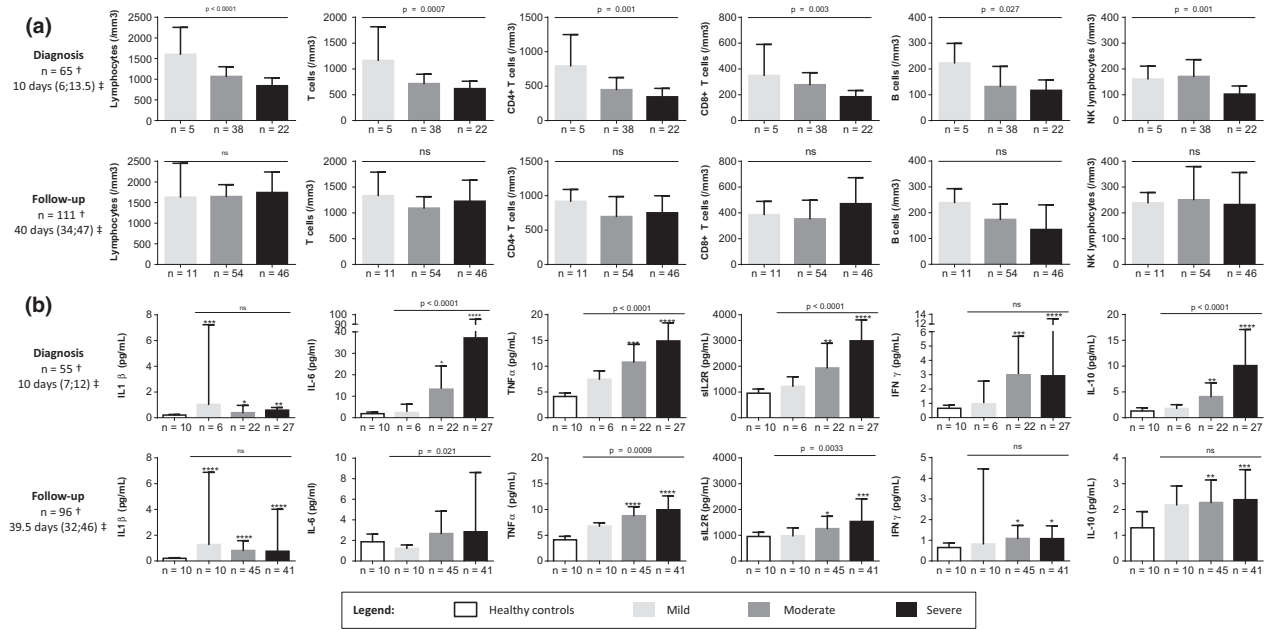


Figure 5. Outcome of main immunological characteristics in mild, moderate and severe SARS-CoV-2 patients from diagnosis to follow-up. **(a)** Lymphocyte subset counts at diagnosis and during follow-up. **(b)** Plasma cytokine levels at diagnosis and at follow-up (T-CoV-Spot assay). For the correlations with severity, *P*-values are calculated using partial Spearman’s rank correlation coefficients, adjusted for time from symptom onset to sampling. For comparisons of plasma cytokine levels between plasma controls (*n* = 10) and SARS-CoV-2 patients classified according to severity using Dunn’s tests (*post hoc* for the Kruskal–Wallis test) are indicated as **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001 or *****P* ≤ 0.0001. †, number of patients with available data or sample, ‡ time from symptom onset to follow-up, in days (median [IQR]).

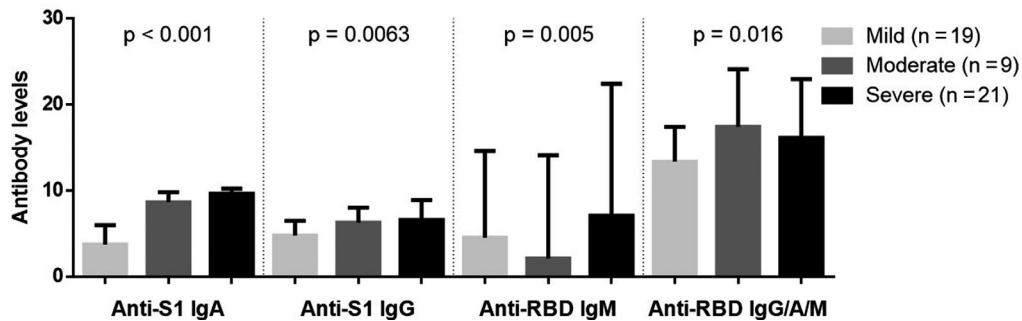


Figure 6. Anti-S1 and anti-RBD antibody levels in SARS-CoV-2 patients (*n* = 49), according to patients’ severity status. *P*-values were calculated using partial Spearman’s rank correlation coefficients with severity status, adjusted for time from symptom onset to sampling.

pools, we observed a large correlation between the three peptide pools. Similar results were published in a preliminary report on 57 samples collected in 28 patients within 21 days following SARS-CoV-2 diagnosis.²⁷ Combined with our data, these results suggest that a given patient may acquire and maintain a similar cellular *in vivo* response against the three proteins, at least during the first weeks following the infection (median [IQR] from symptoms onset: 36 [28;53] days in our study). In addition, our results

highlight that, even if S protein may be a major target for vaccine strategies, M and N are also of interest in anti-SARS-CoV-2 immunisation follow-up. Indeed, M and N-reactive T cells were detected in 100% and 93.3% of patients, respectively, versus 91.7% for S-reactive T cells. A large majority of our patients were convalescent when they were sampled (*n* = 53/60, 88%). Li *et al.*²⁸ observed that convalescent SARS-CoV patients sampled 12 months after diagnosis had developed a higher S-reactive T CD4⁺ response.

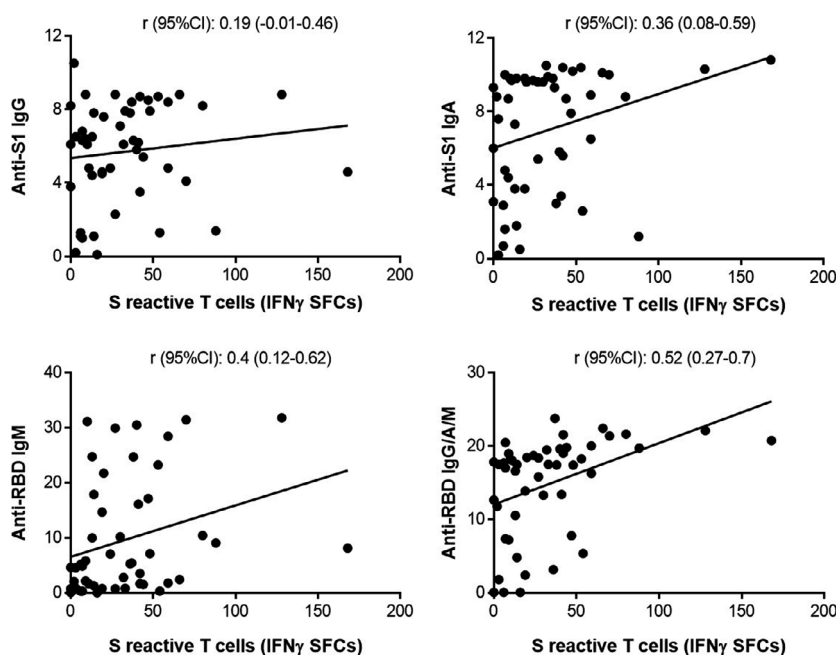


Figure 7. Correlation between anti-S1 and anti-RBD antibody ratios and S-reactive T cells in SARS-CoV-2 patients ($n = 49$). Correlation (Spearman's rank correlation) between IFN γ -SFCs obtained with S peptide pool, and anti-S1 or anti-RBD antibody index.

Considering the large correlations between IFN γ -SFCs detected in response to each M, N and S protein and with the MNS mix ($r \geq 0.7$), and the practical interest of using a single antigenic well (rather than three), assessment of sensitivity and specificity was performed with the MNS mix for the monitoring of SARS-CoV-2 patients cellular response in future routine practice.

The presence of pre-existing cross-reactive T cells in SARS-CoV-2 patients is reported in several studies.^{24,25,29,30} This cross-reactivity with other human coronaviruses could also help to protect individuals against SARS-CoV-2 infection. Braun *et al.*²⁴ reported the detection of S-reactive CD4⁺ T cells in 23 of 68 (35%) healthy donors and mostly against C-terminal epitopes. However, the cut-off defining reactive or unreactive T cells was not provided. These control subjects were sampled during the epidemic and were defined as asymptomatic seronegative subjects: SARS-CoV-2 infection was ruled out using RT-PCR in 10 healthy donors and by repeated serological testing at least 28 days after the first sample, which is important considering the low sensitivity of RT-PCR and delayed antibody detection in asymptomatic patients.^{19,31} Grifoni *et al.*²⁵ also detected S-, M- and ORF-reactive T cells in up to 60% of healthy donors. Cryopreserved PBMCs

collected before the epidemic were chosen as controls, as in our work and others,²⁹ considering that they may be more relevant and representative than asymptomatic seronegative subjects collected during the epidemic. According to the definition of detection threshold, up to two and up to four of 10 healthy donors had M- and S-reactive T cells, respectively, but no N-reactive T cells. Our data are quite in line with these results. We preferred to assess the presence of anti-SARS-CoV-2 in control samples using a mix of the three viral antigens. All these control subjects were seronegative for anti-S1, anti-N and anti-RBD antibodies. With this approach, we detected reactive T cells in only three of 31 control samples (9.7%) and a second analysis showed that T cells were reactive to M and S ($n = 3/3$) and to N ($n = 1/3$) with low spot counts in each case (data not shown). Finally, the interpretation of a positive or negative result, defining sensibility and specificity, must be discussed. As there are no consensual criteria to define a positive response for an ELISpot assay, we considered several methods to determine a cut-off. We first considered that a result was 'positive' when the number of IFN γ -SFCs in the stimulated well was at least twice the number of IFN γ -SFCs observed in the negative well

(unstimulated cells).^{29,32} We also considered a result as 'positive' when the number of IFN γ -SFCs in the stimulated well was greater than the mean of all negative controls + three standard deviations (data not shown). Lastly, we used an ROC curve to define the optimal cut-off for sensitivity and specificity. Based on those three methods, the specificity was comparable and only three control samples (9.7%) were considered as positive, with different reactive T-cell subsets in two samples. Considering these results and the absence of anti-N, anti-S1 and anti-RBD antibodies in control subjects, our data may also suggest a cross-reactivity with other coronaviruses. However, its potential protective properties remain to be demonstrated.

Preliminary data about the earlier stages of the disease suggest a peripheral and lymph node lymphocyte decrease,^{5,9,10,13,15,17} type I IFN deficiency⁷ and a diminished proportion of functional and reactive T cells in the most severe patients.¹⁶ When studying cellular response according to disease severity in our cohort of SARS-CoV-2 patients, the most severe patients were more likely to be male and more likely to be older (Table 1), and they had lower lymphocyte counts (Figure 7a) and higher plasma cytokine levels (Figure 7b).

During follow-up (median [IQR] 36 [28;53] days) and despite normal absolute counts with similar T cell, CD4⁺ and CD8⁺ T-cell counts in the three groups, we observed a significant higher frequency of M- and S-reactive T cells according to severity, despite similar total lymphocyte counts in the groups (Figure 3). This correlation was also confirmed with the MNS mix in our cohort of 60 patients. A similar observation was suggested in a preliminary work by Thieme *et al.* with a live cell activation-marker technique in 28 recently diagnosed patients (median (range) time since diagnosis: 4 days (1–21) vs 31 days (3–64) in our study), but differences were not significant among hospitalised patients and no mild patients (i.e. outpatients) were included.²⁷ To better assess the functionality of specific reactive T cells, we further tested other cytokines in culture supernatants after antigenic stimulation. A strong IL-2 production suggests a primary response with high activation of naive T cells,³³ but low levels of IL-5, IL-13 and IL-17A were also detected in more severe patients. Another major concern is the type of T cells that we detected. Our results in SARS-CoV-2 patients in a median (IQR) interval

of 31 days (20;41) after diagnosis are consistent with other reports in which specific T cells were also detected up to 21 days following diagnosis with a trend for higher specific T-cell counts in severe patients.²⁷ To improve our understanding of the inflammatory status in SARS-CoV-2 patients, we also looked at the main cytokines in plasma, at diagnosis and during follow-up. Almost all the tested cytokine levels decreased over time, but a gradient remained between moderate and severe patients compared with mild patients (except for IL-6). A significant correlation with disease severity also persisted for sIL-2R, IL-6 and TNF α , illustrating both a higher and longer inflammatory response in moderate-to-severe patients. IL-1 β remained increased regardless of disease severity, suggesting a persistent activation of monocytes/macrophages.³⁴ Conversely, IL-6 dramatically decreased in moderate-to-severe patients after diagnosis, but a correlation persisted between plasma levels and severity (Figure 5). Elevated IL-6 has been shown to be associated with more severe symptoms, lower lymphocyte counts and poor prognosis.¹⁰ IL-6 may also play an important role in lung fibrosis, polarisation of naive CD4⁺ T cells into Th17 cells and an increase in B-cell IgG production. Its late decrease in surviving patients could explain the lymphocyte count adjustment and expansion of IFN γ -secreting specific T cells in severe patients. TNF α and sIL-2R remained increased and correlated with disease severity during follow-up (Figure 5). In addition, TNF α is reported to be related to inflammatory responses mediated by IL-1 β and IL-6.³⁵ The soluble sIL-2R can be released from activated T cells³⁶; therefore, elevated plasma levels at diagnosis and at follow-up may reflect a T-cell activation state and, presumably, a specific adaptive response against SARS-CoV-2 in this context. Several reports described a higher and/or longer viral load in severe patients^{37,38}; a tremendous antigenic stimulation could explain an elevated adaptive immune response; the latter may be less effective to clear virus in view of the higher senescence and exhaustion of activated T cells in severe patients.^{16,39,40}

Finally, we confirmed previous reports showing that antibody response was higher in patients with severe SARS-CoV-2. Consistent with the fact that specific T cells participate in the generation of specific antibodies, we observed that the numbers of reactive T cells and anti-S or RBD

antibodies were correlated. We can conclude that severe SARS-CoV-2 patients develop a globally higher adaptive immune response. Exploring specific T cells during the first days following SARS-CoV-2 infection in mild, moderate and severe patients may help us to better understand how the T-cell response becomes insufficient in the majority of severe patients.

Our study has some limitations. Our ELISpot assay did not allow us to discriminate between the CD4⁺ and CD8⁺ T-cell-specific responses, and we were not able to assess the *in vivo* activation state of immune cells. However, our priority was to study and follow up the cellular response in a large cohort of SARS-CoV-2 patients with a standardised method, using a reliable threshold for positive results. As a monitoring tool, this approach provides a clear and sensitive measure of immune responses in a short time frame and identifies responses that differ according to the severity of COVID-19 symptoms.

In conclusion, IFN γ ELISpot assay is a reliable method to explore large cohorts of patients and may be relevant for vaccine trials in healthy subjects and in at-risk groups of patients. It may also be useful in patients for whom specific antibody detection may be difficult (like in primary antibody deficiencies and/or in patients receiving IVIg). We also highlighted that severe patients develop a higher specific T-cell response against SARS-CoV-2 infection. In addition, our study brings some new insights in the adaptive response in SARS-CoV-2 patients and further studies are necessary to explain the importance of the adaptive response during the first days following infection and to assess whether a higher cellular immunity could be protective against new infections.

METHODS

Study participants

This study was conducted at Lille University Hospital, France, between 21 April and 20 May 2020. Consecutive patients with a recent symptomatic COVID-19 diagnosis confirmed by RT-PCR on a respiratory sample (SARS-CoV-2 patients) were sampled for the IFN γ T-CoV-Spot assay during hospitalisation and at a visit systematically planned 21–30 days after diagnosis (outpatients) or 21–30 days after hospitalisation discharge (follow-up visit). Epidemiological, clinical, laboratory and radiological characteristics were obtained from patients' electronic medical records. Patients were classified as follows: mild (outpatients with minimal

symptoms), moderate (SARS-CoV-2 symptoms requiring hospitalisation \geq 24 h with oxygen support (O_2) \leq 3 L min⁻¹) or severe (hospitalisation in intensive care unit and/or $O_2 > 3$ L min⁻¹).

Control samples

Cryopreserved peripheral blood mononuclear cells (PBMCs) collected before the outbreak (during 2018–2019) from heparinised blood samples from immunocompetent non-infectious subjects were used as negative controls. All serum samples were negative for anti-N, anti-S1 and anti-RBD antibodies. These samples were taken from the POMI-AF study, which included patients scheduled for cardiac surgery (coronary artery bypass graft surgery and/or valvular surgery) or transcatheter valve implantation (ClinicalTrials.gov Identifier: NCT03376165).

Cell preparation

Cell isolation and enumeration were performed using either a manual procedure (when reactivity against several peptide pools was assessed) or an automated procedure (when reactivity against only one peptide pool was assessed for future routine practice purposes). Briefly, PBMCs were isolated from 5 mL of freshly collected heparinised blood samples using Ficoll–Hypaque density gradient centrifugation (manual procedure) or using an automated magnetic bead-based system to isolate the cells using positive selection (automated procedure, T-Cell Select cell isolation, Oxford Immunotec, Abingdon, UK, on a Nimbus Presto platform system, Hamilton Robotics, Reno, Nev). Isolated cells were counted using the Guava ViaCount Reagent (Luminex, Austin, TX, USA) and analysed on a Guava easyCyte flow cytometer (Luminex). Cell suspension was normalised at a final concentration of 2.5×10^6 cells mL⁻¹, and plating with SARS-CoV-2 antigens was automatically performed on the Nimbus Presto platform system ($2\text{--}2.5 \times 10^5$ PBMCs added per well) or manually performed (2.5×10^5 PBMCs added per well).

IFN γ ELISpot assay – T-CoV-Spot assay

Overlapping peptide pools covering the complete sequences of SARS-CoV-2M and N proteins and of the immunodominant domain surface S protein and a mix of M, N and S peptides (MNS mix) were used as antigens (PepTivator[®] SARS-CoV-2 Prot_M [ref 130-126-703], PepTivator[®] SARS-CoV-2 Prot_N [ref 130-126-699], PepTivator[®] SARS-CoV-2 Prot_S [ref 130-126-701], Miltenyi Biotec, Bergisch Gladbach, Germany). Peptides consisted of 15-mer sequences with 11 amino acids overlap. Microtitre plates coated with anti-IFN γ antibodies (T-SPOT.TB, Oxford Immunotec) were used. SARS-CoV-2M-protein, N-protein and S-protein peptide pools were added at a concentration of $0.5 \mu\text{g mL}^{-1}$, and a mix of M, N, and S peptide pools (MNS mix) was used at the same concentration of $0.5 \mu\text{g mL}^{-1}$ for each pool, without any mitogenic stimulator. Reactivity against M, N, S proteins and the MNS mix was compared using the manual procedure for cell

preparation to obtain the exact same number of cells in each well. When the MNS mix was tested alone, the automated procedure for cell preparation was used. After incubation for 16–20 h at 37°C in a humidified atmosphere containing 5% CO₂, wells were washed and incubated with conjugate reagent for 1 h at 2–8°C. After a washing step, wells were developed for 7 min with substrate solution. The reaction was stopped with distilled water. Plates were allowed to dry in an oven at 37°C for 1 hour. Spot-forming cells (SFCs) were detected using the CTL ImmunoSpot plate reader. Quality controls included a negative control to assess the spontaneous IFN γ release (wells without antigen) and a positive control to assess cell functionality (wells with the mitogenic stimulator phytohaemagglutinin [PHA]). Samples with insufficient isolated PBMCs (less than 2.5×10^5 cells per well) were excluded. In the negative control, an excess of 10 SFCs should be considered as 'invalid'. For the positive control, the spot count should be ≥ 20 SFCs; a spot count < 20 SFCs was therefore considered 'invalid'. For frozen PBMC samples, a spot count < 40 SFCs was considered 'invalid'. A well with stimulated cells was considered as 'positive' if the number of SFCs was at least twice the number of SFCs observed in the negative well (unstimulated cells).^{29,32}

Cytokine measurements

Culture supernatants were collected in the negative control well and in the MNS mix well in a subgroup of patients. IL-2, IL-4, IL-5, IL-13 and IL-17A concentrations were assessed using a cytometric bead-based system according to the manufacturer's instructions (CBA Flex set, Becton Dickinson, Franklin Lakes, NJ, USA). Cytokine concentrations in supernatants were first assessed in the negative control well and in the MNS mix well. The final cytokine concentration was calculated as the concentration measured in the antigen well minus the concentration in the negative control well. Plasma IL-1 β , IL-6, TNF α , soluble sIL-2R, IFN γ and IL-10 concentrations were assessed using the Ella Automated Immunoassay System (ProteinSimple, San Jose, CA) following the manufacturer's recommendations.

Serological assays

Considering that the S protein may be the privileged target antigen in vaccine developments and considering the importance of analysing both antibody and cellular response,⁴¹ we focused our work on the comparison of both antibody and cellular response against S protein in SARS-CoV-2 patients. Anti-S1 domain IgA and IgG (Euroimmun Medizinische Labordiagnostika, Lübeck, Germany) and IgM and total antibody (IgG/A/M) antireceptor binding domain (RBD) (Beijing Wantai Biological Pharmacy Enterprise, Beijing, China) were assessed by ELISA assay, according to the manufacturer's instructions on a serum sampled on the same day as the heparinised blood for IFN γ T-CoV-Spot assay. Anti-N total antibody ELISA results (Elecsys Anti-SARS-CoV-2, Roche Diagnostics, Risch-Rotkreuz, Switzerland) were also compared with reactive anti-N protein-specific T-cell

results obtained with IFN γ T-CoV-Spot assay. For ELISA assays, a ratio between each sample and the calibrator was calculated and positive results were defined according to the manufacturer's recommendations.

Statistics

Continuous variables are expressed as median (interquartile range [IQR], or range) and categorical variables as numbers (percentage). Comparisons according to severity of SARS-CoV-2 patients were performed using the Kruskal–Wallis test for continuous variables (followed by post hoc Dunn's tests) and chi-squared test for categorical variables. We assessed the correlation between M-, N-, S- and MNS-reactive T cells (IFN γ -SFCs) by calculating Spearman's rank correlation (r) coefficients. We also assessed the association of the number of IFN γ -SFCs, the antibody ELISA results and the cytokine levels in supernatants and plasma with the severity of SARS-CoV-2 patients using Spearman's rank correlation coefficients. The strength of the correlation was classified as non-substantial ($r < 0.1$), small ($0.1 \leq r < 0.3$), moderate ($0.3 \leq r < 0.5$) or large ($r > 0.5$).⁴² Considering the difference in time from symptom onset to sampling between outpatients and hospitalised patients, associations with severity were adjusted for the time from symptom onset to sampling for IFN γ T-CoV-Spot assay by calculating partial Spearman's rank correlation. The optimal cut-off value for the number of SFCs in response to MNS mix to differentiate healthy controls from SARS-CoV-2 patients was determined using receiver operating characteristic (ROC) curve by maximising the Youden Index; sensitivity and specificity values of the optimal cut-off value were calculated with their 95% confidence intervals (95% CI) (estimated using the Clopper–Pearson exact method). Statistical testing was performed at the two-tailed α -level of 0.05. Data analyses and graphs were performed using GraphPad Prism software version 6.04 (GraphPad Software, La Jolla, CA, USA).

Study approval

This study was performed in accordance with the Declaration of Helsinki principles for ethical research. Blood and sera used for T-CoV-Spot and serological assays were sampled during routine blood tests (no specific sampling was performed for this study). The study was approved by the French data protection agency (CNIL: Commission Nationale de l'informatique et des libertés, registration no. DEC20-086) and by the local ethics committee (ID-CRB 2020-A00763-36).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

JD, GL, FV, PV and ML had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. JD and GL equally contributed to this work. JD, GL, FV, TJ, AD, JL, PV, JP, KF and ML involved in concept and design. JD, GL, FV, TJ, AD, JL, PV, AD, SS, BG, JB, BP, LB, EKA, ML, CY, BM, LD, DL, SD, DM, EW, FM, MB, IT, VE, EJ, AD, SS, TB, JP, KF and ML acquired, analysed or interpreted the data. JD, GL, AD, JL, PV and ML drafted the manuscript. PV, AD, SS, BG, JB, BP, LB, EKA, ML, CY, BM, LD, DL, SD, DM, EW, FM, MB, IT, VE, EJ, AD, SS, TB, JP and KF critically revised the manuscript for important intellectual content. AD and JL made statistical analysis. JT, AD and EW made administrative, technical or material support.

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