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# Frequency, activation status, and functionality of circulating T follicular helper cells differ across disease severity in COVID-19 patients

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## Abstract

Coronavirus disease 2019 (COVID-19) is an emerging respiratory disease caused by SARS-CoV-2, giving rise to a deadly pandemic since 2019. The interaction and dynamics between different immune cells, especially those contributing to antibody generation toward this virus during natural infection, have yet to be explored. Here, we characterized the phenotype and functional capacity of circulating T follicular helper cells (cTfh) in acute COVID-19 patients and 3-month convalescence individuals. A total of 46 COVID-19 patients (of whom 43 were recruited 3 months later as convalescence individuals) were recruited during the first wave of COVID-19 pandemic in Sweden (March-May 2021). Ten blood samples from healthy donors were collected in pre-pandemic years were included as healthy controls. The frequency and phenotypes of cTfh cells were analyzed by flow cytometry, whereas SARS-CoV-2 specific cTfh cells were identified using in-vitro stimulation assay followed by flow cytometry. The supernatants from in-vitro stimulation assay were evaluated for cTfh-related cytokine levels (IL-21, IFN-gamma, IL-4, and IL-17A) using Luminex multiplex assay. First, cTfh cells (especially cTfh1 and cTfh17 cells) were decreased in frequencies in severe patients than mild and moderate patients. Circulating Tfh cells expressed a higher level of activation markers (ICOS and CD38) and migration marker (CCR7) in patients exhibiting severe symptoms than mild and moderate symptoms. Second, SARS-CoV-2 specific cTfh cells were detected in acute patients' samples (with patients with more severe symptoms showed higher frequency than those having mild symptoms). The specific cTfh cells were still detected in 3-month convalescence, especially from severe patients. Third, positive correlations between activated cTfh and SARS-CoV-2 specific cTfh cells towards SARS-CoV-2 antibodies were found in the acute period but not convalescence. Finally, in-vitro stimulation with specific SARS-CoV-2 proteins showed higher production of IL-21 (hallmark cytokine produced by cTfh cells) in severe patients than in mild patients. In conclusion, the frequency, phenotype, and functional capacity of cTfh cells were differed in severe compared to mild patients' group, and these might be related to different disease severity states during COVID-19 infection.

## Key Words

Circulating T follicular helper cells, IL-21, SARS-CoV-2 infection, COVID-19, disease severity.

## Popular Scientific Summary

In the 21st century, several viruses from the coronavirus family caused massive outbreaks in the world. The first outbreak happened in China, 2003 by Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), while the second outbreak was found in Middle East in 2012 by Middle East Respiratory Syndrome (MERS-CoV). Recently, the coronavirus disease 2019 (COVID-19) outbreak followed by a global pandemic that occurs until now results in millions of infections and death cases. COVID-19 is the third outbreak disease caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), closely related to SARS-CoV and MERS-CoV. As human populations have been hit hardly by the third coronavirus outbreak in this century, many questions need to be dug out. Do we know in-depth mechanisms about why these viruses could cause sickness? Why some infected people only showed very mild symptoms while others are severely ill (or even die)? We have "military forces" called immune systems to tackle infectious agents that enter our bodies. The ability of the immune cells to combat those agents is essential to clear the infections. While too little potency of immune cells could not kill the microbes, hyperactive immunity is also harmful. Therefore, the immune system is one of the critical factors determining the severity of infection.

We investigated one type of cells named circulating T follicular helper (cTfh) cells from the blood. These cells are the represented population of T follicular helper (Tfh) cells, which are located in the lymph nodes (the organs where our immune cells reside and activate when the infection or inflammation happens). The task of Tfh cells is mainly helping another type of immune cells called B cells to develop and produce potent antibodies, which ultimately could kill the infectious agents. As we would like to know the profiles of cTfh cells across different disease severity, we collected the blood samples from various patients. The peripheral blood mononuclear cells (PBMCs) from mild, moderate, and severe patients during their illness and 3 months after they recovered were examined based on cTfh frequencies and activation status. We also stimulated the cells with various SARS-CoV-2 proteins (spike, receptor binding domain, and nucleoprotein) to detect the presence of SARS-CoV-2 specific cTfh cells. In addition, we examined the cytokines (proteins secreted by the cells) profile of these stimulated cells to see if the disease severity is also associated with these aspects. In this study, we used flow-cytometer techniques to examines the cells' profiles and Luminex kit to study the cytokine profiles.

The results showed that the frequency of cTfh cells declined as the disease severity progress during infection. Severe patients had significantly less amount of cTfh cells compared to mild patients in the acute phase. The frequency of cTfh in severe patients during 3-month post-recovery normalized into similar levels of other groups and healthy controls. When we explored the activation and migration status, cTfh of severe patients were more active and exhibited more migration markers than mild patients during acute infections. The severe patients exhibited more SARS-CoV-2 specific cTfh cells than mild patients in acute infection compared to mild, and this trend persisted in 3-month post-recovery. Furthermore, higher levels of IL-21 (a cytokine secreted mainly by cTfh cells) were produced in severe compared to mild patients during acute infections.

In conclusion, we managed to show that cTfh cells' profiles were greatly varied between COVID-19 patients' severity status. As interactions between immune cells and the infectious agents are complex, this study provided additional insights into our "military forces" profiles during SARS-CoV-2 infection. These results would also be helpful for other studies to generate the vaccines and treatments of COVID-19 and similar diseases that might be happened in the future.

## Introduction

Coronavirus disease 2019 (COVID-19) is a newly emerging disease that occurs at the end of 2019 and still causing pandemics in the world. Until the end of March 2021, there are over 120 million cumulative cases of COVID-19, with around 2.7 million deaths reported to the World Health Organization (WHO).[1] This disease is caused by a novel type of beta-coronavirus that was never identified previously, named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The origin of SARS-CoV-2 is suspected from animals (bats or pangolins), as they share significant similarities from the genome and amino acid sequencing.[2] After SARS-CoV and MERS-CoV, SARS-CoV-2 is the third virus from the coronavirus family that accounts for global outbreaks in the twenty-first century.[2]

SARS-CoV-2 genome is a single-stranded, positive-sense RNA genome, and its size is approximately 29.9 kb.[3] This genome constitutes cap regions at 5' end, poly-A tail at 3' end, and several open reading frames (ORFs) that contain structural and nonstructural genes. The nucleocapsid (N) proteins bind to the RNA genome, and this structure is enveloped further by lipid bilayer and three other structural proteins: membrane (M) protein, envelope (E) protein, and spike (S) protein (Figure 2a).[3,4] The S protein is essential for the virus to enter host cells by interacting with angiotensin-receptor enzyme 2 (ACE) receptor on the host cells membrane.[3,4] This protein is composed of two subunits (S1 and S2) (Figure 2b). Subunit S1 contains the domain for recognizing the ACE2 receptor, also known as receptor binding domain (RBD), while S2 functions as mediator for fusing the viral and host cells' membrane.[3] Therefore, understanding SARS-CoV-2 structure is essential for developing this virus's potential preventive and therapeutic target.

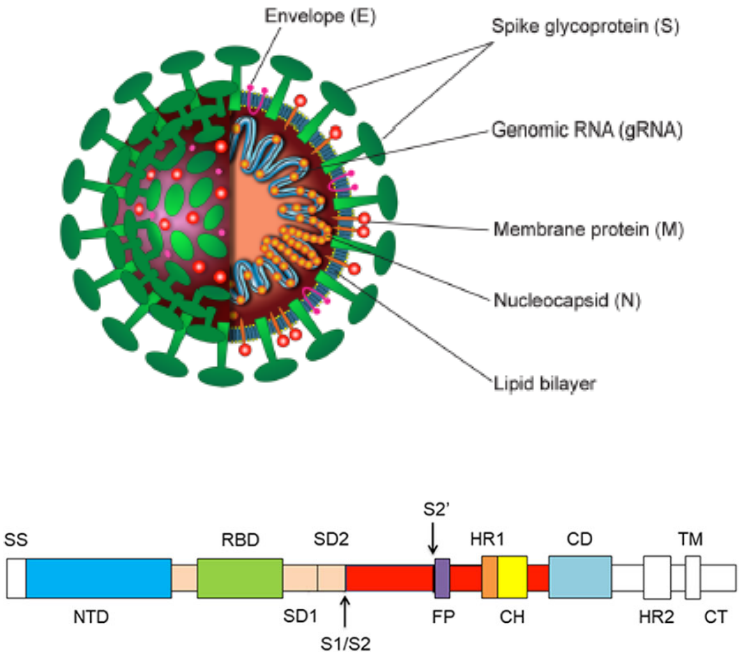



**Figure 1.** Schematic figure of SARS-CoV-2 structure (a) and the primary structure of spike protein, showing S1 and S2 subunit as well as RBD domain (b). Figure adapted by Parikhani et al. and Wang et al.

Transmission of SARS-CoV-2 viruses mainly occurs via respiratory routes (droplets and aerosols). Upon the virus entry, the incubation time of this virus ranges from 1- 14 days (with median of 5 days).[2]

The clinical presentations and disease progression of COVID-19 vary between individuals from different age groups and comorbidities. Asymptomatic cases and mild presentations are often found in young people infected with SARS-CoV-2, whereas severe and fatal cases mainly occur in older patients with comorbidities.[5,6] The clinical presentation in the early course and mild severity are mainly malaise, myalgia, fever, and upper respiratory tracts related symptoms (cough, sore throat, rhinorrhea, reduced sense of taste and smells).[5,7] Disturbance of lower respiratory organ functions (e.g., pneumonia and acute respiratory distress syndrome / ARDS), as well as systemic involvements of this disease (e.g., coagulopathy, septic shock, acute kidney, and cardiac injury), are more pronounced in severely ill patients.[8] A study from 72,314 cases from China showed the case-fatality rate was 49.0% among patients who experience respiratory failure, septic shock, and multiple organ dysfunction or failure.[9]

Immune response towards SARS-CoV-2 infections is a critical factor determining the disease severity and outcome of COVID-19. Although this disease pandemic occurs less than two years, there are abundant papers published to reveals the complex characteristic and mechanism of immune systems (both innate and adaptive) towards this infection. Innate immunity is the front line of defense towards pathogen infection, and remarkable characteristics of these components were described towards SARS-CoV-2 interactions in several papers. Several studies suggested that type I interferon system is downregulated during early COVID-19 infections, and in vitro studies show that some SARS-CoV-2 non-structural proteins directly interfere production of these essential mediators.[10–12] When the disease progress to become more severe, the pro-inflammatory cytokines were elevated in circulation and tissues. These hyperinflammatory states during the late stages of the disease are strongly correlated with poor outcomes.[12–14] Like the soluble marker profiles, the innate immune cells compartment also showed dysregulation during severe disease states.[15] During the early phase of infections, innate immune cells (mainly neutrophils and monocytes) are recruited to the nasopharyngeal mucosal in response to infected epithelial cells.[15] However, severe patients exhibit neutrophils that are overly activated and exhibit neutrophil extracellular traps (NETs) features in both airways and circulations.[16] Monocyte subsets of severe patients showed a decline of non-classical, CD16+ monocytes while the dysfunctional monocytes were increased in circulation.[17] Macrophage/monocyte cells in bronchoalveolar lavage also upregulate genes related to fibrosis in severe patients.[15] Therefore, innate immune systems seem to be dysregulated during disease progression and might contribute to the pathological features seen in severe patients. However, details of these interactions need to be further investigated in in-vitro and clinical settings.

While innate immune components are crucial to combat the pathogens upon their entry, adaptive immunity provides specific and compelling protections to clear the infectious agents. The main actors of adaptive immunity are T and B lymphocytes, and their team works with other immune cells to eliminate the pathogens. The roles of T cells during adaptive immunity are diverse, including orchestrating innate immune cells response, directly killing infected cells, and helping B cells in producing neutralizing antibodies.[18,19] Both CD4 and CD8 T cells in circulation are drastically reduced in longer duration compared to other respiratory virus infections, and this phenomenon is more pronounced in severe patients.[18,19] The reasons behind reduced quantities of these cells remain elucidated, but several mechanisms are being proposed. The influence of rising inflammatory cytokines and suppressor cells that dampen T cell proliferation accelerated cell death in lymph nodes, and redistributions of lymphocytes to affected tissues might contribute to the overall reduction of these cells in peripheral blood.[18,19] Several studies showed that these cells are more activated (marked increase of CD38, ICOS, and PD1) and showed signs of exhaustion during more severe COVID-19 diseases.[18,19] These suggested that T cells also experience dysfunctions during acute SARS-CoV-2 infections and might influence their disease progression.

Humoral adaptive immune response during SARS-CoV-2 infections has become an interesting topic as antibodies blocking spike (S) protein could prevent virus attachment to the host cells. B cells

lymphocytes are known to generate the antibodies with the help of other cells, notably T follicular helper (Tfh) cells.[20] The generation process of antibodies mainly occurs in germinal centers (GCs) of secondary lymphoid organs. In this location, Tfh cells mediate the development and differentiation of naïve or memory B cells to become plasmablast and plasma cells (which are capable of secreting antibodies) and memory B cells (reserved cells for future infections). The cells are a subset of CD4 T cells that express different transcription factors, surface markers, and cytokines (i.e., BCL6, CXCR5, and IL-21), which separated themselves from other CD4 T cell subsets (T helper / Th1, Th2, and Th17 that do not express this marker).[20]

The characteristics of Tfh cells were mainly described in animal models but poorly in human studies since these cells reside in lymph nodes that are hard to be accessed.[20] However, recent studies found a counterpart of Tfh cells that express CXCR5 in peripheral blood compartments, known as circulating T follicular helper (cTfh) cells.[21] The origin of these cells is still under investigation, but studies found that these cells could promote B cell differentiation to plasmablasts and plasma cells.[22,23] Therefore, cTfh cells could become reflections on the generation of antibody-secreting plasmablasts and plasma cells during acute disease settings and vaccination studies in humans. Several published studies describe the frequency and role of cTfh cells during viral infections (dengue, hepatitis B, and C) as well as influenza vaccination. However, the characteristic and functional role studies of cTfh during the COVID-19 acute phase remain to be elucidated. Therefore, this study aims to explore characteristics of cTfh cells, B cells, and different SARS-CoV-2 antibodies and their correlations during different disease severity in COVID-19 disease.

## Aim

The aims of this study are:

- Characterize the frequency of total cTfh cells and the subsets (cTfh1, 2, and 17) as well as their activation and migration marker expressions in COVID-19 patients during their disease state and after recovery.
- Characterize the frequency of SARS-CoV-2 specific cTfh cells during disease state of COVID-19.
- Explore how cTfh cells correlate with disease severity in COVID-19 patients during acute infection.

## Materials and Methods

### Patient recruitment and sample processing

This project enrolled adult patients' cohort that was recruited from March-May 2020 at Karolinska University Hospital (Solna and Huddinge) and Haga Outpatient Clinic (Haga Närakut), Stockholm. Inclusion criteria for this project were age more than 18 years old and positive confirmations of SARS-CoV-2 by PCR. To include more individuals with mild symptoms and asymptomatic cases, household contacts of patients were screened to examine if they had SARS-CoV-2 infection by PCR, and those with positive results were enrolled for this study. For asymptomatic and mild patients who did not required hospitalization, samples were taken once during acute infection. Multiple samplings were done for patients who required hospitalization during their disease course. Several samples were taken up for immunological studies: blood samples, nasal swabs, nasopharyngeal aspirates, and endotracheal aspirates (for those who require mechanical ventilation). Convalescence samples were taken twice after

patients' recovery and/or hospitalization discharge. The first follow up was approximately 3 months at April – September 2020 (referred as 3-month follow up) and the second follow up was approximately taken November 2020 – February 2021. Samples collected from people who had no symptoms and at the timing outside of flu season, and confirmed IAV-negative by PCR for during 2016-2018 were served as pre-pandemic healthy controls.

The severity of COVID-19 patients during their disease course was determined by the respiratory domain of the sequential organ failure assessment score (SOFA) with some modifications.[24] This scoring system was calculated by dividing the arterial partial pressure of oxygen ($PaO_2$) to the fraction of inspired oxygen ($FiO_2$, determined by $O_2$ flow estimation during hospitalization from the Swedish Intensive Care Registry). Peripheral transcutaneous hemoglobin saturation ($SpO_2$) values were used instead if PaO2 was not available, then modified SOFA (mSOFA) was calculated. These SOFA or mSOFA values were used as daily severity scores for these patients. Patients were also categorized by their peak respiratory SOFA or mSOFA values. The 5-point SOFA/mSOFA scores were then extended in the bottom end (to differentiate between hospitalized vs non-hospitalized mild patients) and the upper end (to include patients who had death outcome). Full severity scoring criteria is shown in table 1.

**Table 1.** Disease severity scoring of COVID-19 patients

| Severity Category | Disease Severity Score | Hospital Admission (Y/N) | Description |
|---|---|---|---|
| Mild | 1 | N | Do not seek healthcare |
| | 2 | Y | $PaO_2/FiO_2 > 53$ or $SpO_2/FiO_2 > 400$ |
| Moderate | 3 | Y | $PaO_2/FiO_2 < 53$ or $SpO_2/FiO_2 < 400$ |
| | 4 | Y | $PaO_2/FiO_2 < 40$ or $SpO_2/FiO_2 < 315$ |
| Severe | 5 | Y | $PaO_2/FiO_2 < 27$ or $SpO_2/FiO_2 < 235$ and mechanical ventilation needed |
| | 6 | Y | $PaO_2/FiO_2 < 13$ or $SpO_2/FiO_2 < 150$ and mechanical ventilation needed |
| Fatal | 7 | Y | Fatal outcome |

Both blood and respiratory samples were taken by trained medical students and nurses. The nasal swab samples were taken by Floqswabs then they were and they were put in two tubes containing two different solutions: phosphate-buffered saline (PBS) and RNA-later solutions. The nasopharyngeal and endotracheal tube aspirates were taken by using surgical suction pump with respiratory suction catheter. The blood samples were processed by separating the plasma components and blood cell components by centrifugation at 800 g/8 min/room temperature. The plasma samples then were aliquoted and stored in -80$^{o}$C Biobank Refrigerator, while the blood cell components were further processed using Ficoll separation techniques to isolate blood mononuclear cells. In brief, the blood cell components were topped up with 35 ml sterile PBS, and these diluted blood cells were overlaid on Ficoll-Paque Plus solution (GE Healthcare) in 50 ml sterile falcon tubes. These samples were then centrifuged at 900 g/Acc 0/Dec 1/ for 25 minutes in room temperature. After centrifugation, the cloudy layer that contained peripheral blood mononuclear cells were collected, and then washed with PBS. The pellet was then incubated with red blood cell (RBC) lysis solution to remove the remaining RBCs, and after another washing, the cell number and viability of PBMCs were counted using automatic cell counter after

staining with trypan blue. After counting and last washing with PBS, the cells were resuspended in freezing medium (anhydrous dimethyl sulfoxide/DMSO with filtered heat-inactivated fetal bovine serum/FBS in 1:10 dilution) Around 10 million cells were stored in each vial, and the PBMCs-contained vials were stored in -80$^{o}$C refrigerator overnight in Mr. Frosty before transferred into liquid nitrogen tank for long-term storage.

**Immunophenotyping of cTfh cells from patients' PBMCs**

The frozen PBMCs were thawed in 37$^{o}$C waterbath and washed with R10 to remove the freezing medium. The pellet then was diluted with 3 ml of PBS and counted with automatic cell counter (Countess$^{TM}$ Cell Counter, Invitrogen) to ensure that the cell number were enough before specific cell staining. Around 2 million cells from each patient were aliquoted into FACS tubes. The cells were stained using Live/Dead Blue (Invitrogen) in 4$^{o}$C in 5 minutes, then incubated with human FcR blocking reagent (Miltenyl Biotec) for another 5 minutes in 4$^{o}$C. The cells were then stained with antibodies: CD3 (SK7; BD Biosciences), CD4 (OKT4; Biolegend), CD45RA (HI100; BD Biosciences), CXCR5 (MU5UBEE; Thermofischer), CXCR3 (G025H7; Biolegend), CCR6 (11A9; Biosciences), CCR7 (G043H7; Biolegend), CD38 (HIT2; Biolegend), CD40L (24-31; Biolegend), CD62L (DREF-56; Biolegend); PD-1 (EH12.1; BD Biosciences) and ICOS (C398.4A; Biolegend). The stained cells were incubated at 4$^{o}$C in 20 minutes, then washed and fixated with 1% paraformaldehyde (4$^{o}$C, 1 hour). After washing, the samples were run for flow cytometry on LSR Fortessa$^{TM}$ Flow cytometer. The data analysis acquired in flow cytometry was analyzed further in FlowJo$^{TM}$ software.

**In-vitro stimulation assay and identification of SARS-CoV-2 specific cTfh cells**

Frozen PBMCs are thawed, washed, and the pellet was suspended with 1 mL of R10 media (RPMI 1640 medium (MilliporeSigma) supplemented with 10% fetal calf serum (FCS), 5 mM L-glutamine and 100 U/mL penicillin and streptomycin (Invitrogen, Thermofisher Scientific). The cells were counted to ensure the counts were enough for the experiments. After counting, the cell suspensions were divided into five equal volumes (100 µL each) in five different wells of 96-wells plate. The cell suspensions in each well were stimulated under five different conditions by added different proteins solutions in R10 media (100 µL each): S, RBD, and N recombinant proteins of SARS-CoV-2 (received through the global health-vaccine accelerator platforms (GH-VAP) funded by Bill & Melinda Gates Foundation), staphylococcal enterotoxin B (SEB, as positive controls, Sigma-Aldrich), and bovine serum albumin (BSA, as negative controls, Thermofisher Scientific). In total, each well consisted of 200 µL cell suspension with the cell concentration was 0,5 x $10^{6}$ cells / mL and the protein concentration was 0.5 µg/mL. The cells then were incubated at 37$^{o}$C cell incubator for 22 hours. After incubation, the 96-wells plate was centrifuged at 2000 rpm for 3 minutes in room temperature to separate the cells and supernatants. The supernatants were collected to measures different cytokines, and the cells were stained to determine activated CD25+, CD134+ cTfh cells (which represent specific cTfh cells). The surface staining for specific cTfh cells used several antibodies: CD3 (SK7, BD Biosciences), CD4(L200, BD Biosciences), CD45RA (HI100; BD Biosciences), CXCR5 (MU5UBEE; Thermofischer), CXCR3 (G025H7; Biolegend), CCR6 (11A9; Biosciences), CD25 (BC96, Biolegend) and CD134 (ACT35, BD Biosciences). The flow of in-vitro stimulation assay could be seen in figure 2.

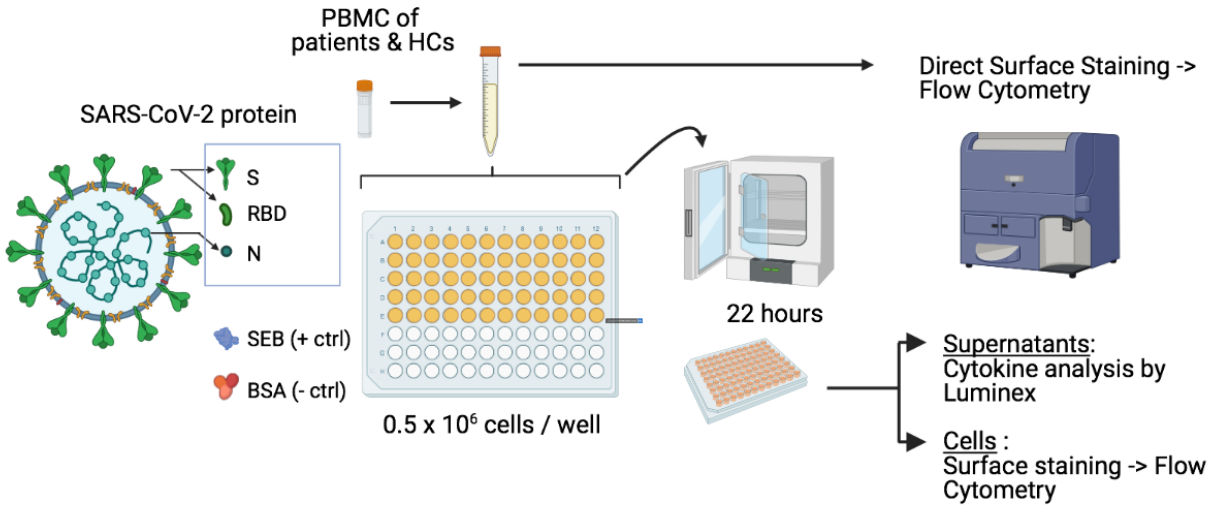



**Figure 2.** Workflow of in-vitro stimulation assay using different SARS-CoV-2 proteins (created by Biorender.com).

## Cytokine analysis of PBMC from in-vitro stimulation

Supernatants from stimulated PBMCs were analyzed by Luminex® Assay for their cytokine secretions. Five cytokines were selected for the analysis: IL-21 (mainly secreted from cTfh cells), IFN-gamma (mainly secreted by Th1 and cTfh1), IL-4 (mainly secreted from Th2 and cTfh2), IL-17 (mainly secreted from Th17 and cTfh17), and TNF-alpha (inflammatory cytokines secreted mainly from macrophage and monocytes). In brief, 50 µL of standards and samples as well as 50 µL of diluted Human Magnetic Premixed Microparticle Cocktail were added to each well of 96-well plate (Step 1). After 2 hours incubation (room temperature on shaker at 800 rpm), the plate was washed three times (using magnetic device holder). After washing, 50 µL of diluted Human Premixed Biotin-Antibody Cocktail was added to each well followed by 1-hour incubation (room temperature on shaker at 800 rpm) (Step 2). The plate then washed, and 50 µL of Streptavidine-PE was added to each well followed by 30 minutes incubation (room temperature on shaker at 800 rpm) (Step 2). After the third washing step, 100 µL of Wash Buffer was added to the well, and the plate was read using Biorad Analyzer (Step 3). The results yielded from analyzer were then normalized by multiplying the values with number of initial cells aliquoted in each well before stimulation (500,000 cells) and divided by the live events recorded in flow cytometry after stimulations. The workflow of Luminex® Assay could be seen in figure 3.

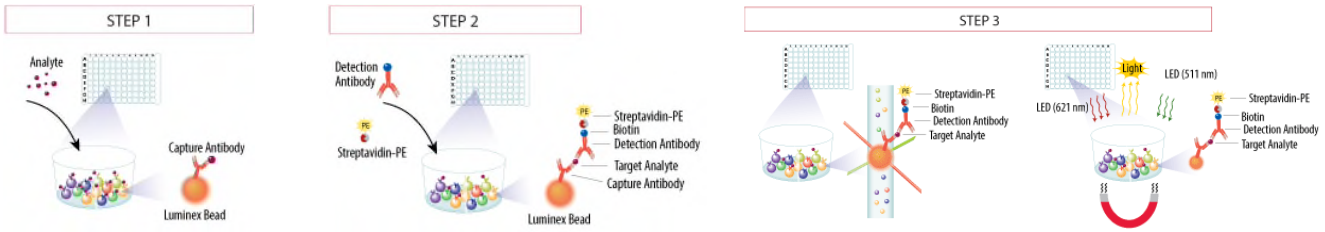



**Figure 3.** Workflow of multiple cytokines detection and analysis using Luminex® Assay (source: luminexcorp.com)

### Data analysis and statistics

Data analysis was conducted in Graphpad Prism version 8.0 (Graphpad Software Inc., San Diego, CA) and Microsoft Excel (Microsoft Corp., Redmond, WA). The median values of the datasets were compared using two-tailed non-parametric tests (Mann-U Whitney for unpaired data and Wilcoxon test for paired data, respectively). The correlation analysis was performed using Spearmann's Rho test. The significance level of 95% was used for this study, and significant p-values were marked with star symbols (* for ≤ 0.05, ** for ≤ 0.01, and *** for ≤ 0.001).

### Ethical Statement

The ethical statements required for this study were granted by the Swedish Ethical Review Authority in accordance to the Declaration of Helsinki (Dnr: 2015/1949-31/4, 2017/253-32, 2018/382-32, 2018/2360-32, 2019/061-09, 2020/005-50, 2020/017-57, 2020/033-244). All patients (and/or their relatives) and healthy controls participating in this study are given oral informed consent and signed the written consent forms.

## Results

### Study subject characteristics

In total, there were 46 adult COVID-19 patients as well as 10 pre-pandemic healthy adults (as controls) included in this study. Of those patients, 43 individuals participated in the 3-month convalescent (recovered) period. From 46 patients that were included in this project, 11 patients were categorized as mild, 15 patients were moderate, and 20 patients were severe. Demographic and clinical characteristics of these subjects were described in Table 1. The age and sex were matched between patients and healthy controls. The age and body-mass index (BMI) in COVID-19 patients tended to be higher and males were predominant in severe compared to mild groups. Clinical laboratory indices (including C-reactive protein (CRP), white blood cell count and neutrophils counts) were also elevated significantly as severity progresses, while lymphocyte counts tended to decline. However, most of these indices were back to normal ranges after three-month follow up in all severity groups.

**Table 1. Demographic and clinical characteristic of study subjects.**

| Demographic Characteristics | Total Patients | Mild | Moderate | Severe | Healthy Controls | Significance[A] |
|---|---|---|---|---|---|---|
| n | 46 | 11 | 15 | 20 | 10 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Age in year, mean (SD) | 54 (13) | 45 (14) | 55 (13) | 59 (12) | 54 (23) | P = 0.029 |
| Male, n (%) | 29 (63) | 3 (27) | 9 (60) | 17 (85) | 5 (50) | P = 0.006 |
| Female, n (%) | 17 (37) | 8 (73) | 6 (40) | 3 (15) | 5 (50) | |
| **Severity and Comorbidity Characteristics** | | | | | | |
| Earliest sampling from onset of symptoms in days, mean (SD) | 18.8 (10.6) | 14 (11.2) | 16 (7.4) | 23.6 (10.8) | n/a | P = 0.007 |
| Hospitalization duration in days, median (min-max) | 16 (6-109) | n/a | 12 (7-33) | 20.5 (6-109) | n/a | P = 0.009 |
| Bacterial superinfection, n (%) | 5 (11) | 0 (0) | 0 (0) | 5 (25) | n/a | NS |
| BMI in kg/m2, mean (SD) | 28.7 (4.6) | 24.7 (4.2) | 30.3 (3.8) | 29.8 (4.1) | n/a | P = 0.002 |
| CCI score, median (min-max) | 1 (0-8) | 0 (0-5) | 1 (0-8) | 2 (0-7) | n/a | NS |
| Hypertension, n (%) | 14 (30) | 1 (9) | 6 (40) | 7 (35) | 1 (10) | NS |
| Diabetes, n (%) | 14 (30) | 2 (18) | 6 (40) | 6 (30) | 0 (0) | NS |
| Current smoker, n (%) | 2 (4) | 0 (0) | 2 (13) | 0 (0) | 0 (0) | NS |
| Steroid use during hospitalization, n (%) | 6 (13) | 0 (0) | 2 (13) | 4 (20) | n/a | NS |
| **Laboratory characteristic during infection** | | | | | | |
| CRP (mg/L), median (min-max) | 148 (1-457) | 1 (1-30) | 138 (73-346) | 252.5 (65-457) | 1 (1-3) | P < 0.0001 |
| WBC (x$10^9$/L), mean (min-max) | 10.3 (3.5-41.2) | 4.3 (3.5-7.8) | 10.5 (4.6-14.6) | 14.9 (5.7-41.2) | 6.9 (4.5-8) | P < 0.0001 |

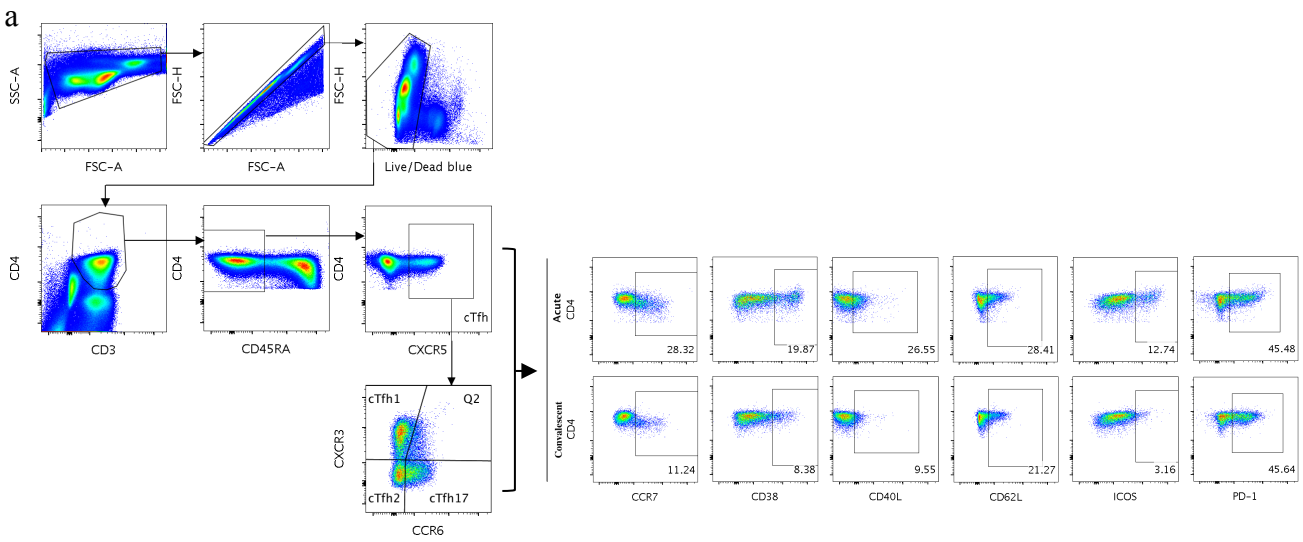
| | | | | | | |
|---|---|---|---|---|---|---|
| Neutrophils ($x10^9$/L), median (min-max) | 7.1 (1.5-28) | 2.1 (1.5-5.3) | 6.6 (3.4-10.4) | 11.0 (3.7-28) | n/a | P < 0.0001 |
| Lymphocytes ($x10^9$/L), median (min-max) | 0.9 (0.2-3.5) | 1.6 (0.4-2.6) | 0.9 (0.4-3.5) | 0.8 (0.2-2,7) | n/a | P = 0.0021 |
| NLR, median (min-max) | 7.2 (1-43.5) | 1.3 (1-6.5) | 6.3 (2.5-15.3) | 11.6 (2.6-43.5) | n/a | P < 0.0001 |
| **Laboratory characteristic in 3-month convalescence** | | | | | | |
| CRP (mg/L), median (min-max) | 1 (1-13) | 1 (1-2) | 2 (1-10) | 2 (1-13) | n/a | P = 0.0111 |
| WBC ($x10^9$/L), median (min-max) | 6.3 (3.9-12) | 5.3 (3.9-7.3) | 6.4 (4.4-10.5) | 6.4 (4-12 | n/a | NS |
| Neutrophils ($x10^9$/L), median (min-max) | 3.5 (1.7-6.6) | 3.0 (1.8-4.3) | 3.9 (2.2-6.6) | 3.8 (1.7-5.7) | n/a | NS |
| Lymphocytes ($x10^9$/L), median (min-max) | 1.8 (0.8-4.2) | 1.6 (1.3-2.7) | 2 (0.8-3.9) | 1.9 (1.2-4.2) | n/a | NS |
| NLR, median (min-max) | 1.8 (0.8-5.6) | 1.7 (1.2-2.7) | 1.9 (0.8-5.6) | 1.9 (0.8-2.7) | n/a | NS |

[A] Statistical significance were between disease severity groups (mild vs moderate vs severe), determined by 1-way ANOVA, Kruskall-Wallis, or Pearson's chi square test based on type and distribution normality of the data. BMI = body mass index, CRP = C-reactive protein, NLR = neutrophils-to-lymphocytes ratio, WBC = white blood cells. Normal range: CRP < 3 mg/L, WBC 3.5 – 8.8 $x10^9$/L, lymphocytes 1.1 – 3.5 $x10^9$/L, neutrophils 1.6 – 5.9 $x10^9$/L.

**The frequency of total cTfh, cTfh1 and cTfh17 was lower in severe compared to mild and moderate COVID-19 patients.**

Circulating T follicular helper cells frequencies were often change in patients with different diseases compared to healthy individuals. In order to explore the frequency of cTfh cells among COVID-19 patients, surface staining for circulating Tfh was done in subset of patients (n = 28, composed of 11 mild, 8 moderate, and 9 severe) and healthy controls (n=6). Circulating Tfh cell population was defined as single, live cells, CD3+CD4+CD45RA-CXCR5+. (Figure 4a). The total cTfh frequency decreased significantly between mild, moderate, and severe COVID-19 patients' groups (severe vs mild, p = 0.0002; severe vs moderate, p = 0.015; moderate vs mild, p = 0.0274). The decreased frequencies were not seen among individuals recovered from COVID-19 and no significant differences were found compared to healthy controls. The frequency of cTfh in severe patients was back to the level that are similar to healthy controls in 3 months follow-up (p = 0.0046) (Figure 4b).

Total cTfh cells can be dissected into different subpopulations based on their expression of CXCR3 and CCR6: cTfh1 (CXCR3+CCR6-), cTfh2 (CXCR3-CCR6-), and cTfh17 (CXCR3-CCR6+). Acute severe patients have lower cTfh1 cells frequencies compared to acute moderate and mild patients (severe vs mild, p = 0.0004; severe vs moderate, p = 0.0280), and this decrease was disappeared after 3 months recovery (severe acute vs severe 3-month convalescent, p = 0.0058). The cTfh17 cells frequencies were lower in severe and moderate acute patients compared to mild acute patients (severe vs mild, p = 0.0063; moderate vs mild, p = 0.0144). The cTfh17 frequency decrease in severe patients was also disappear after 3 months recovery (severe acute vs severe 3-month convalescent, p = 0.0004). The differences among patients' group were not observed in cTfh2 cells frequency, however their frequencies in severe patients are significantly lower than healthy controls (severe acute vs healthy controls, p =0.0052). Based on this, severity status of COVID-19 patients during disease states are associated with total cTfh cell frequencies and their subpopulations, especially cTfh1 and cTfh17 cells.



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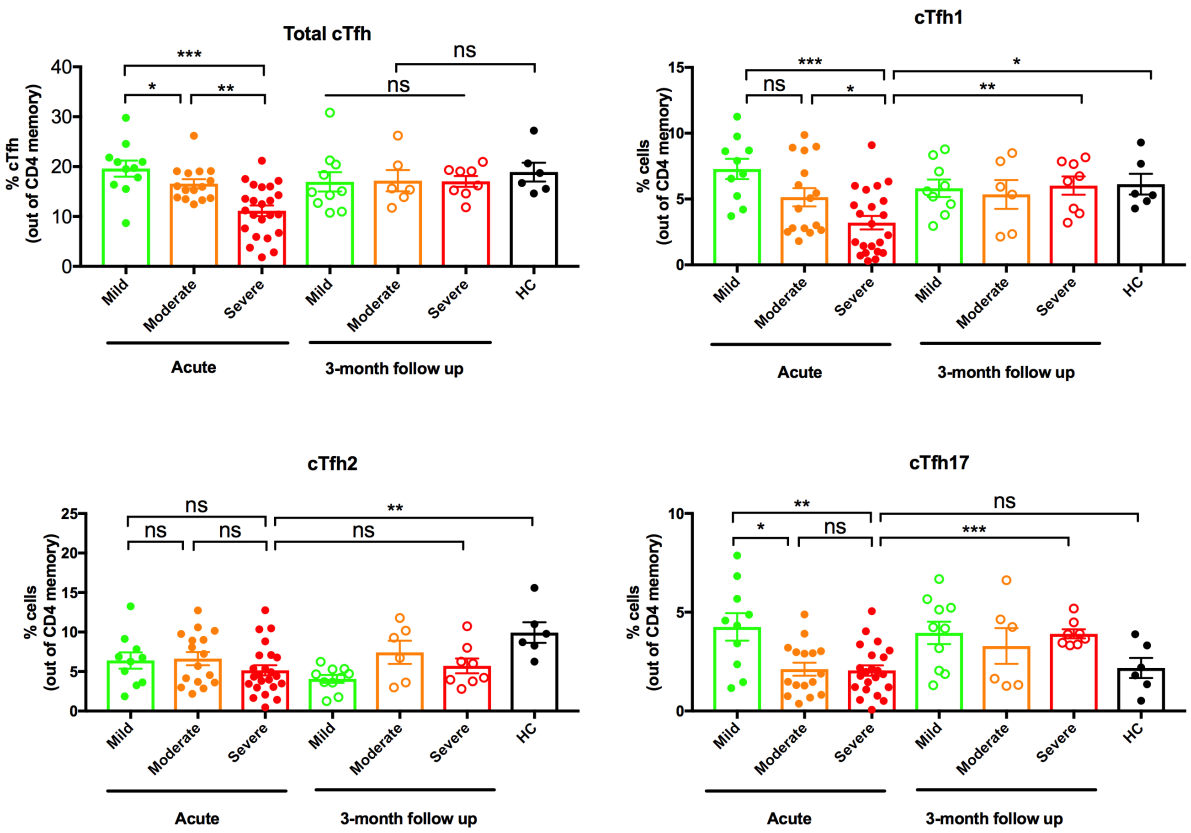


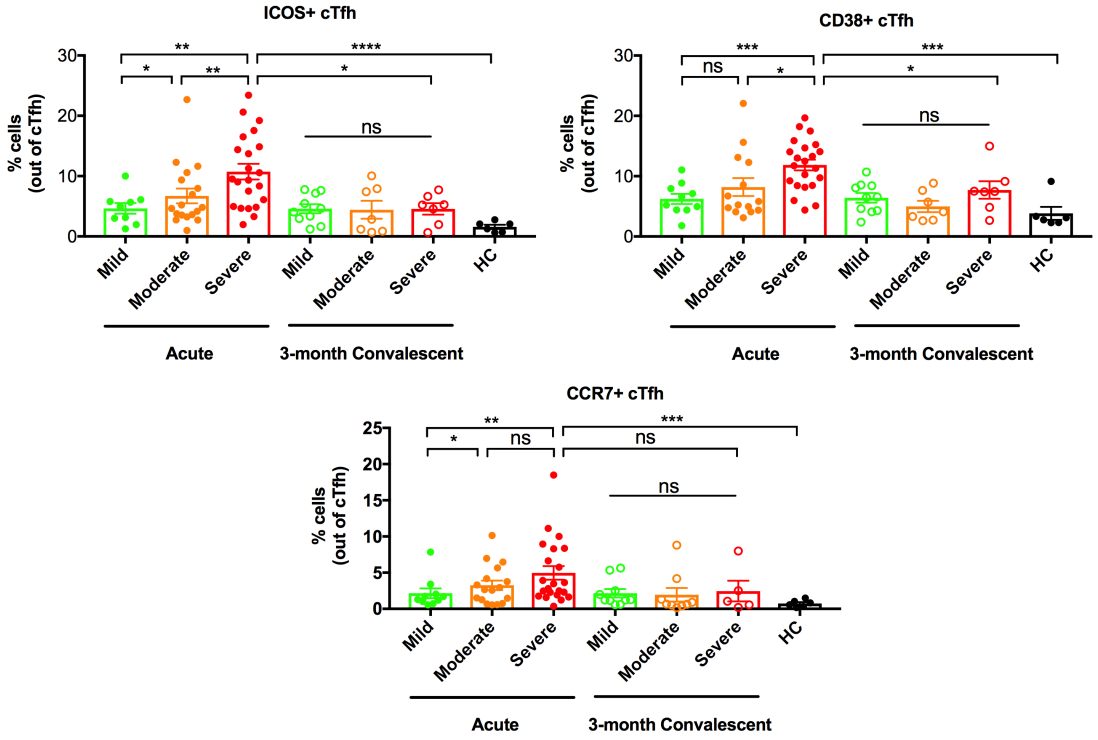

**Figure 4.** (a) Gating strategy to identify circulating circulating T follicular helper (cTfh) cells and three different subsets: cTfh1, cTfh2, and cTfh17 cells, as well as several activation and migration markers (ICOS, PD-1, CD38, CD40L, CCR7 and CD62L). (b) Frequency of total cTfh, cTfh1, cTfh2, and cTfh17 cells in acutely-ill patients and 3-month convalescence individuals (green: mild severity (n=11 patients), orange: moderate severity (n=8 patients), red: severe severity (n=9 patients)), and healthy controls (black, n=6).

## Expression of ICOS, CD38, CD40L and CCR7 in cTfh cells population were higher in severe group compared to mild and moderate COVID-19

During acute infections, immune cells might express different activation and migration markers in responding to the pathogens. Several activation markers (ICOS, PD-1, CD38, and CD40L) and migration markers (CCR7 and CD62L) were examined in cTfh cells to see if these populations were more in the activated state during COVID-19 disease. Severe COVID-19 patients during acute illness exhibited increase expression of activation markers (ICOS and CD38) in cTfh cells population compared to mild and/or moderate COVID-19 patients and healthy controls (ICOS+ cTfh in severe vs mild patients, p = 0.0069; ICOS+ cTfh severe vs moderate patients, p = 0,0189; CD38+ cTfh in severe vs mild patients, p = 0.0004; CD38+ cTfh in severe vs moderate patients, p = 0.0142). The migration marker CCR7 was also higher in severe patients compared to mild patients (p = 0.0093). In severe patients, ICOS and CD38 expressions decreased significantly in 3-month convalescent period compared to acute infection period (ICOS+ cTfh in severe acute vs 3-month convalescence, p = 0.0132; CD38+ cTfh in severe acute vs 3 month convalescence, p = 0.0241). Examinations of cTfh subpopulations revealed that cTfh1 and cTfh2 are more activated (increased in ICOS expression) in severe patients compared to mild and moderate patients (ICOS+ cTfh1 in severe vs mild, p = 0.0002; ICOS+ cTfh1 in severe vs moderate, p = 0.0011; ICOS+ cTfh2 in severe vs mild, p = 0.0046; ICOS+ cTfh1 in severe vs moderate, p = 0.0134). All of

cTfh subpopulations (cTfh1, cTfh2 and cTfh17) in severe patients are more activated compared to healthy controls (ICOS+ cTfh1 in severe patients vs healthy controls, p = < 0.0001; ICOS+ cTfh2 in severe patients vs healthy controls, p = < 0.0001; ICOS+ cTfh17 in severe patients vs healthy controls, p = 0.0034). These results suggest that cTfh cells are more activated during COVID-19 infections compared to 3-month convalescent period and healthy controls, and the degree of activation markers upregulations are associated with disease severity. The contributions of each subpopulation of activated cTfh cells were varied during acute disease state (especially in severe patients), and these phenomena might reflect each subset's specific function during SARS-CoV-2 infections.

a



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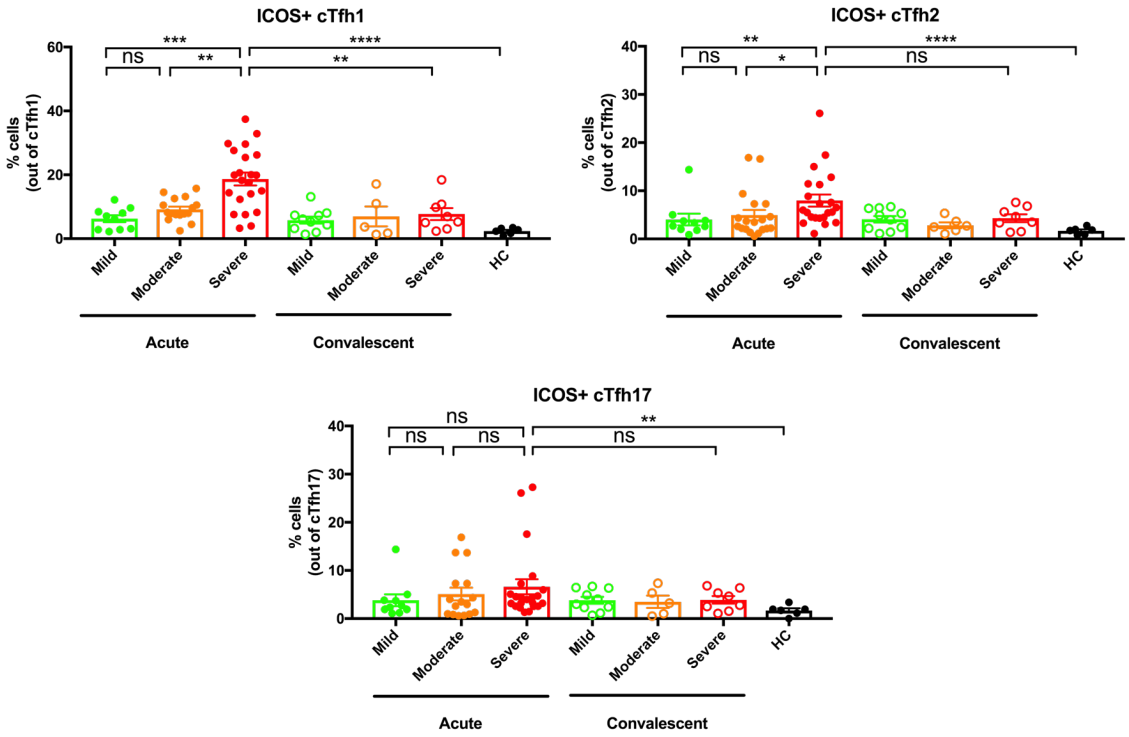



**Figure 5.** (a) Frequency of ICOS+, CD38+,and CCR7+ cTfh cells in different patients' groups (b) Frequency of ICOS+ cTfh1, cTfh2, and cTfh17 cells in different patients' groups (green: mild severity (n=11 patients), orange: moderate severity (n=8 patients), red: severe severity (n=9 patients)), and healthy controls (black, n=6).

## Severe patients exhibited higher SARS-CoV-2 specific cTfh cells frequency than mild and moderate patients, and the SARS-CoV-2 specific cTfh cells were still observed in 3-month convalescent period

The hallmark of adaptive immune cells are their abilities to generate pathogen specific cells, and these were also observed in cTfh cells population. The specific cTfh cells could be identified by their response to various SARS-CoV-2 proteins, including spike protein (S), receptor binding domain (RBD), and nucleoprotein (N). In-vitro stimulation assay with various SARS-CoV-2 proteins during 22 hours were conducted to identify activated (CD134+CD25+) cTfh cells during stimulation, thus these population represented SARS-CoV-2 specific cTfh cells in peripheral blood. In this experiment, 46 COVID-19 patients and 10 heathy donors were included for SARS-CoV-2 specific cTfh analysis. SARS-CoV-2 specific (S-specific, RBD-specific, and N-specific) cTfh cells could be detected in acute patients, and these specific cTfh cells frequencies were higher in severe compared to mild patients (S-specific cTfh cells in acute, severe vs mild, $p = 0.0002$; RBD-specific cTfh cells in acute, severe vs mild, $p = 0{,}0039$; N-specific cTfh cells in acute, severe vs mild, $p = 0.0238$). Interestingly, SARS-CoV-2 specific cTfh cells could be still observed in 3-month convalescent individuals (although the frequencies were lower compared acute infection period). During convalescence period, SARS-CoV-2 cTfh cells were higher in severe groups compared to mild groups (S-specific cTfh cells in 3-month follow up, severe vs mild, $p = 0.0014$; RBD-specific cTfh cells in 3-month follow up, severe vs mild, $p = 0.0007$; N-specific cTfh cells in 3-month follow up, severe vs mild, $p = 0.0005$).

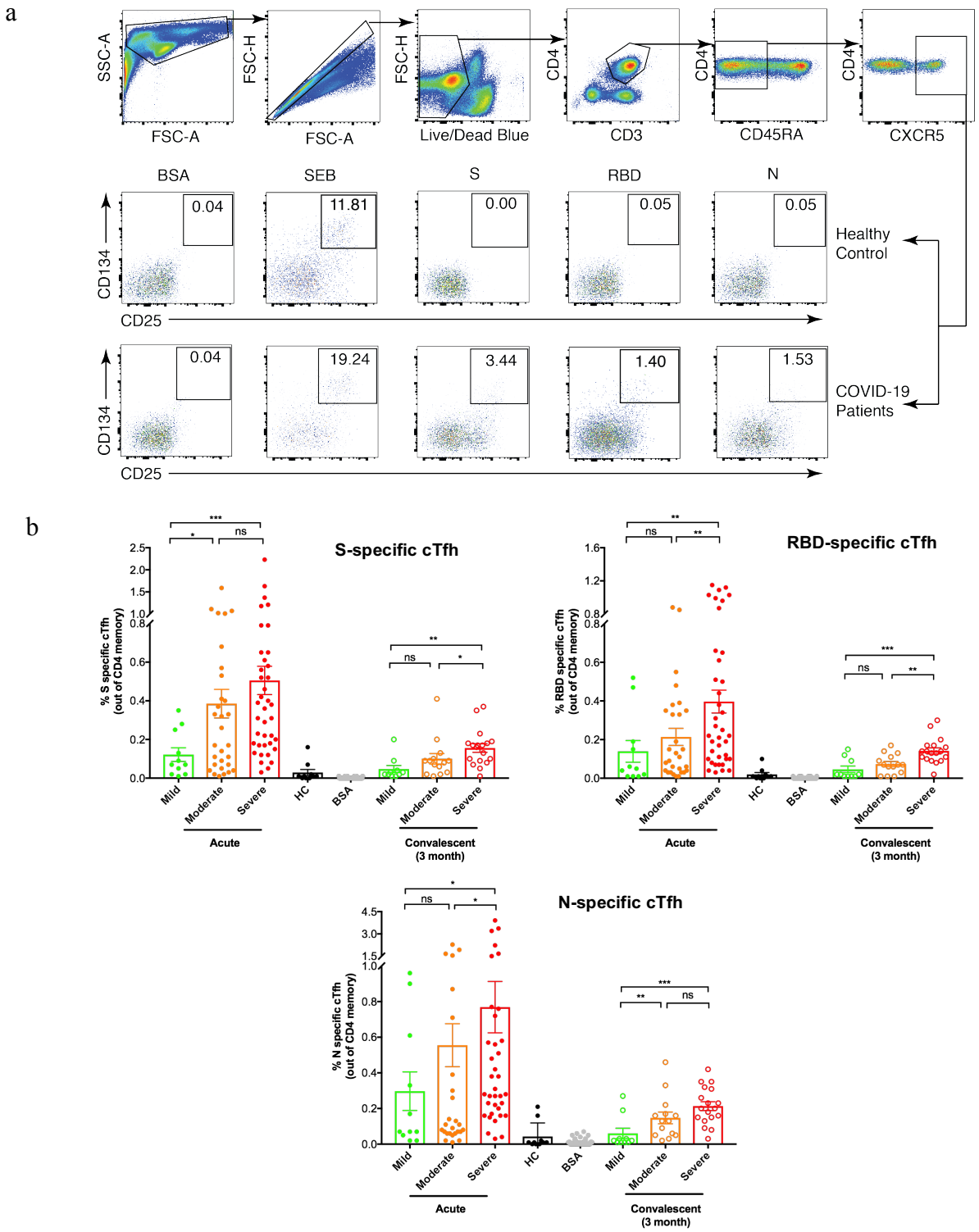



**Figure 6.** (a) Gating strategy for SARS-CoV-2 specific (including S-, RBD-, and N- specific) cTfh cells (b) Frequency of S-, RBD-, and N- SARS-CoV-2 specifc cTfh cells in different patients' group during acute disease and 3-month follow up

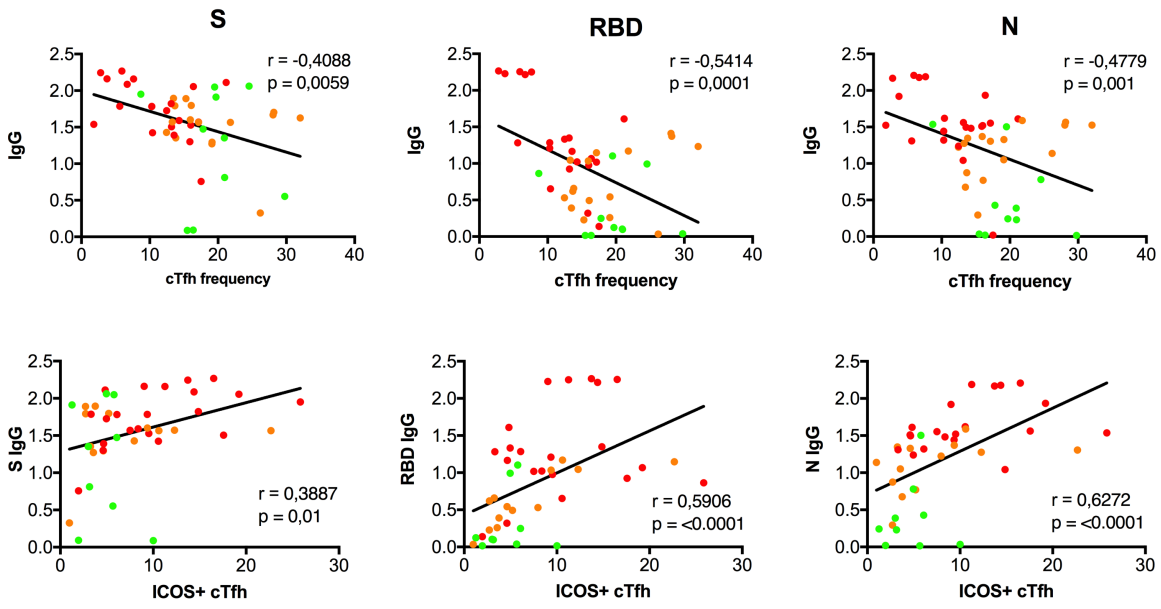
(green: mild severity (n=11 patients), orange: moderate severity (n=15 patients), red: severe severity (n=20 patients)), and healthy controls (black, n=10).

**Total and specific cTfh cells were correlated with SARS-CoV-2 antibody levels during acute infection, but not in convalescent period**

In order to explore the relationship between cTfh cells and humoral immunity responses, we did correlation analysis ocTfh frequencies and SARS-CoV-2 antibody levels data in these patients' cohort (which were generated by our coworker Cagigi et al., manuscript in preparation). There were negative correlations found between acute antibody levels (including IgG spike protein, RBD, and nucleoprotein) and total cTfh frequencies in acute infections, whereas positive correlations were found between antibody levels and activated (ICOS+) cTfh cells frequencies. These correlations could not be demonstrated between antibody levels and cTfh frequencies in 3-month convalescent individuals.

The S-, RBD-, and N- specific cTfh frequencies were also positively correlated with antibody levels (both IgG and IgA) during acute infection, except N-specific cTfh and IgG N. However, only RBD- and N-specific cTfh were positively corelated with their respective IgG antibodies during 3-month convalescence Therefore, these results might suggest that activated cTfh cells (including those SARS-CoV-2 specific cTfh cells) might support antibody productions during acute infection period.

a

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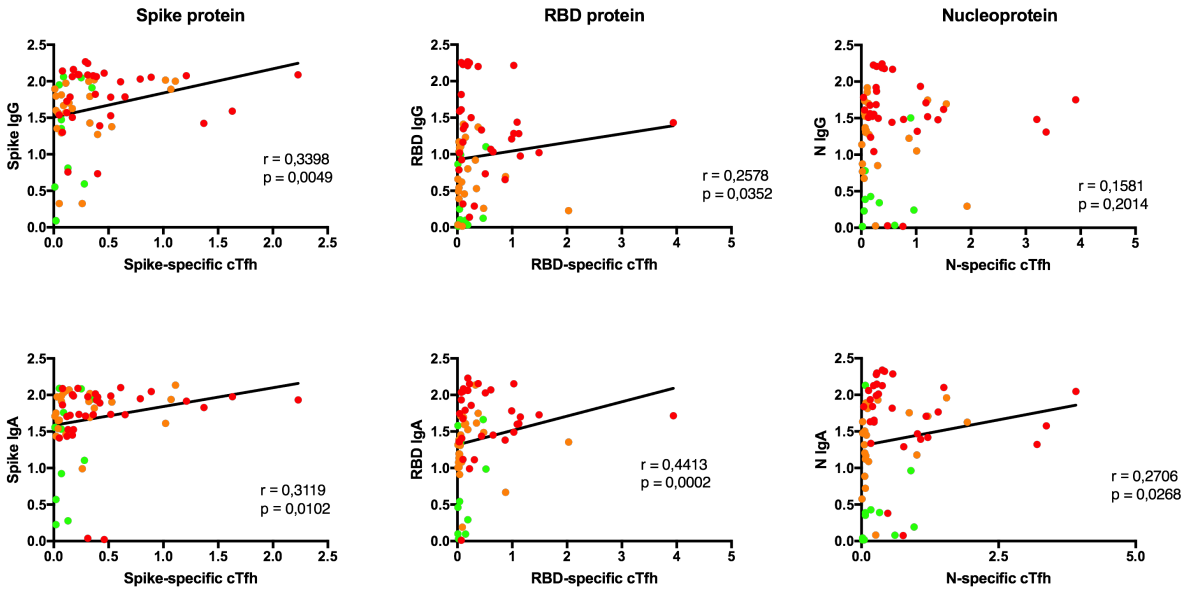
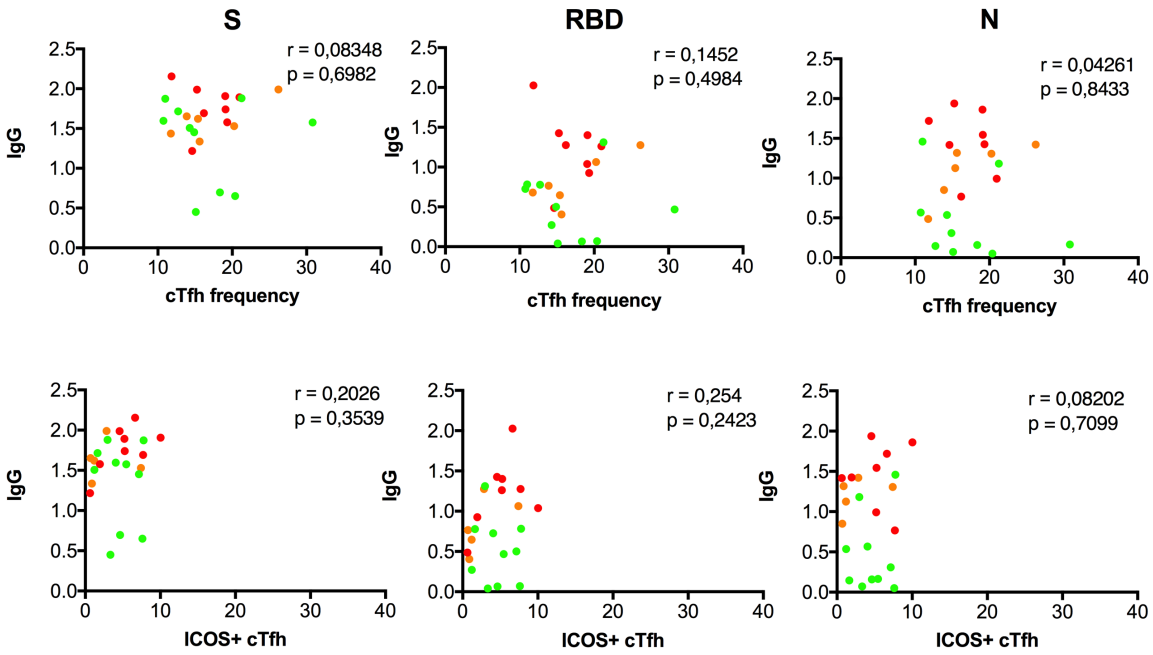

Spike protein
RBD protein
Nucleoprotein
Spike IgG
RBD IgG
N IgG
Spike IgA
RBD IgA
N IgA
Spike-specific cTfh
RBD-specific cTfh
N-specific cTfh
r = 0,3398
p = 0,0049
r = 0,2578
p = 0,0352
r = 0,1581
p = 0,2014
r = 0,3119
p = 0,0102
r = 0,4413
p = 0,0002
r = 0,2706
p = 0,0268

c


S
RBD
N
IgG
cTfh frequency
r = 0,08348
p = 0,6982
r = 0,1452
p = 0,4984
r = 0,04261
p = 0,8433
ICOS+ cTfh
r = 0,2026
p = 0,3539
r = 0,254
p = 0,2423
r = 0,08202
p = 0,7099

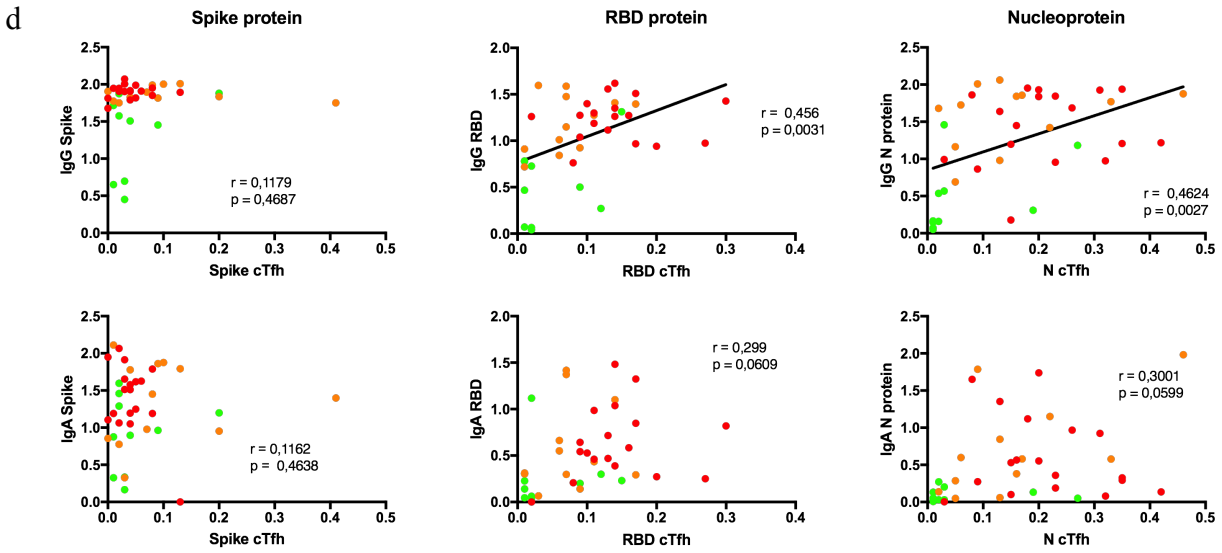
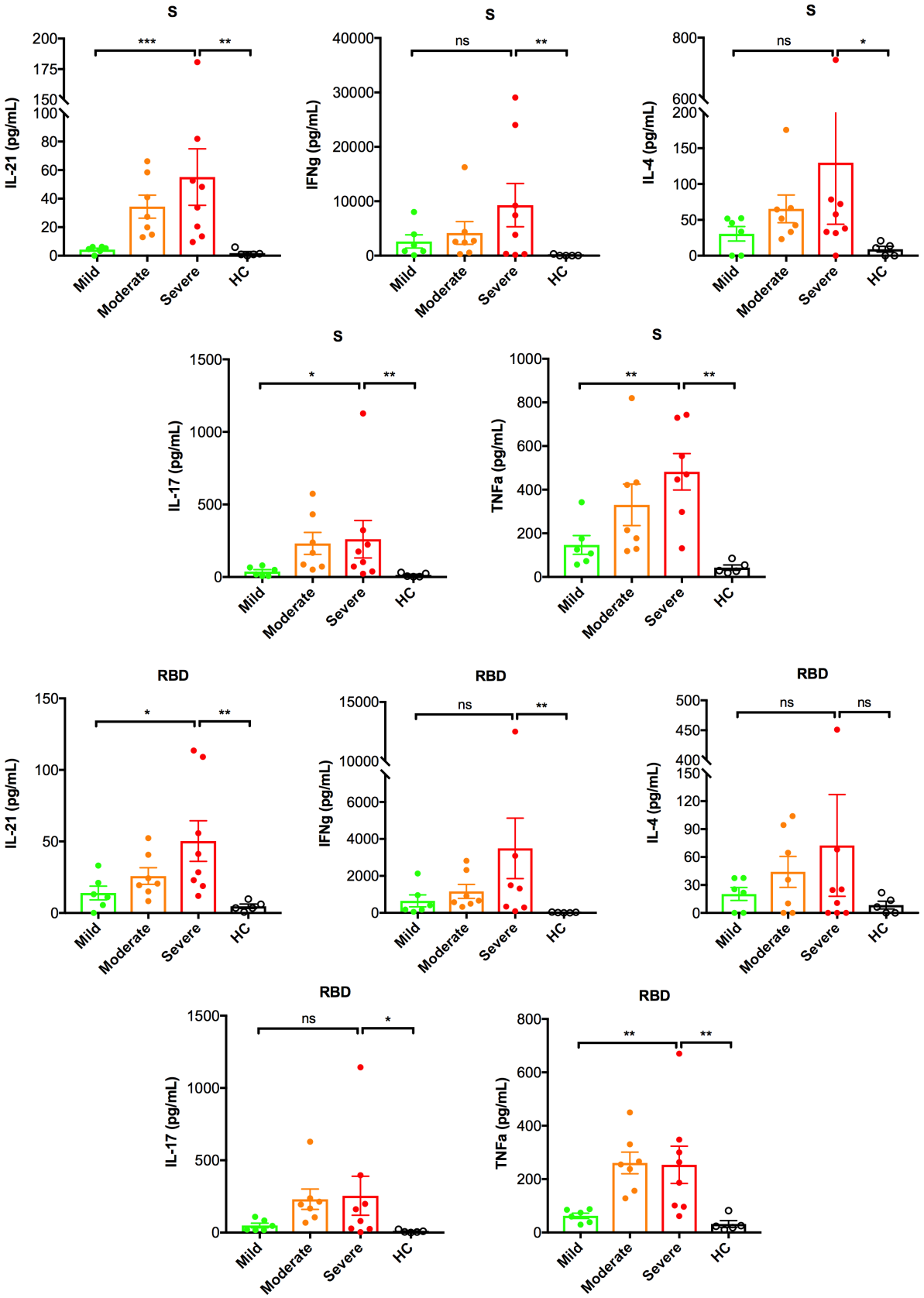



**Figure 7.** (a) Negative correlations were found between total cTfh frequencies and SARS-CoV-2 antibody levels; while positive correlations were found between ICOS+ cTfh frequencies and antibody levels during acute infection; (b) Positive correlations were found between S-, RBD, and N- specific cTfh frequencies and respective antibody levels (except N-specific cTfh and IgG N). (c) No correlation was found between cTfh (total and activated) and antibody levels in 3-month convalescent individuals. (b) Positive correlations were only found between RBD-, and N- specific cTfh frequencies and respective IgG antibody levels in 3-month convalescent individuals. Green dots corresponded to mild patients, orange dots corresponded to moderate patients, red dots corresponded to severe patients.

## PBMCs from severe patients during acute infection produce higher level of cytokines (IL-21, IFN-gamma, IL-4 and IL-17) when stimulated with SARS-CoV-2 specific proteins

Circulating T follicular helper cells are known producing several cytokines to mediate B cell differentiation, including IL-21 (hallmark cytokine of Tfh cells), IFN-gamma (secreted by Tfh-1 like, but also Th1 cells), IL-4 (secreted by Tfh-2 like, but also Th2 cells), and IL-17 (secreted by Tfh-17 like, but also Th17 cells). In order to explore further the functionality aspects of cTfh cells, supernatants from PBMCs from different patients that had been stimulated by SARS-CoV-2 proteins (S, RBD, and N) were analyzed using Luminex. This platform enabled us to analyze different cytokines concentration using limited amounts of samples. Overall, cytokines productions from PBMCs after various stimulation were increase in accordance to disease severity. Severe patients showed the significant higher production of IL-21 when stimulated by SARS-CoV-2 S (severe vs mild, $p = 0.0007$; severe vs HC, $p=0.016$) and RBD (severe vs mild, $p = 0.0293$; severe vs HC, $p=0.016$) during acute infection. This trend was also seen in TNF-alpha (produced by mononuclear phagocytes), and less pronounced in IFN-gamma, IL-4 and IL-17. Therefore, these findings strengthened that cTfh cells from severe patients were more responsive towards SARS-CoV-2 proteins (by secreting more cytokines) compared to mild patients' groups.


S
IL-21 (pg/mL)
IFNg (pg/mL)
IL-4 (pg/mL)
IL-17 (pg/mL)
TNFa (pg/mL)
RBD
Mild
Moderate
Severe
HC
ns
*
**
***

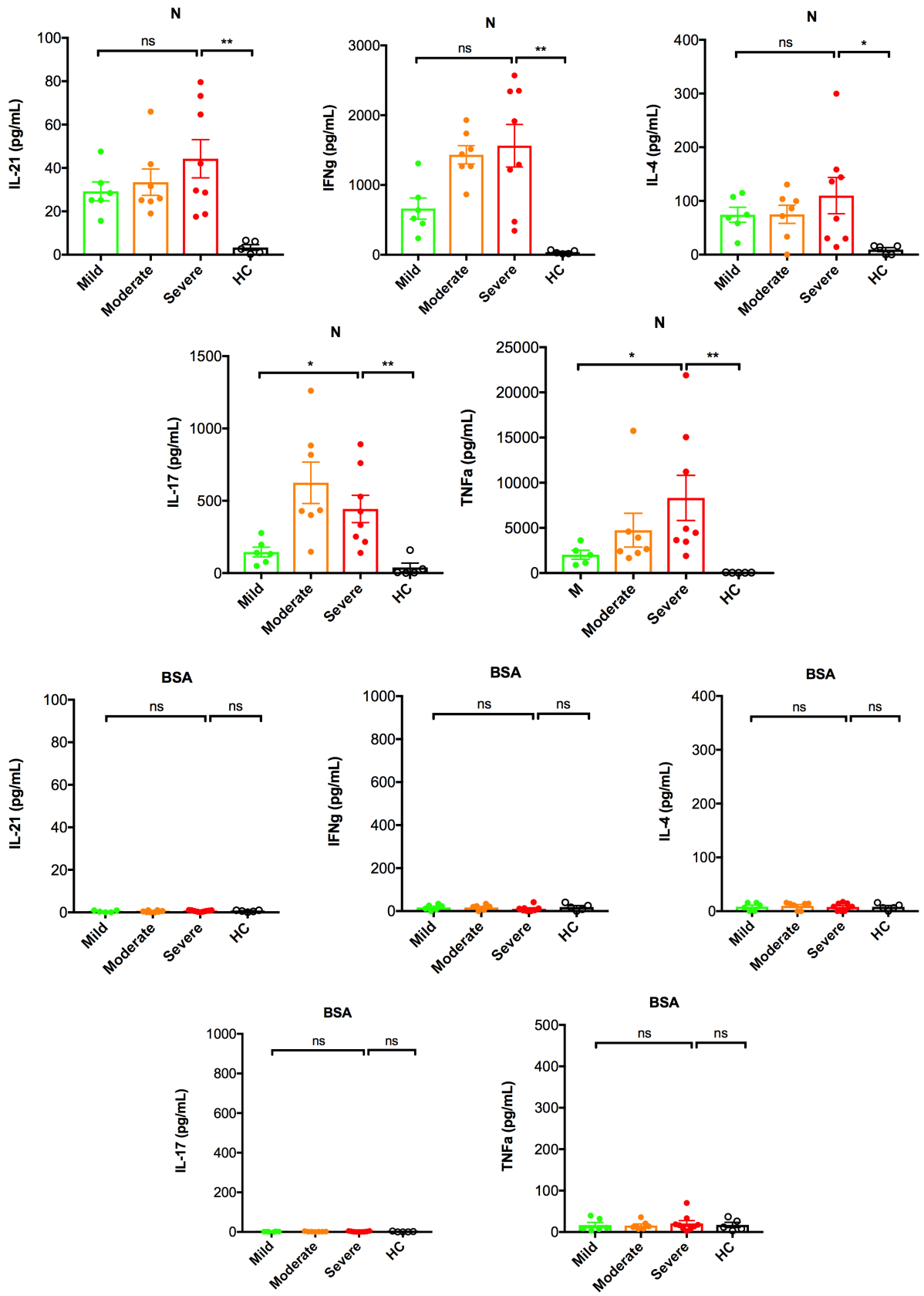



**Figure 8.** Comparison of IL-21, IFN-gamma, IL-4, IL-17, and TNF-alpha concentrations from acute PBMCs (from different patients' severity group) stimulated by SARS-CoV-2 proteins: S (a), RBD (b), or N (c), compared to negative control BSA (d).

## Discussion

The SARS-CoV-2 pandemic now occurs in the second year, and different countries faced the second and third waves of rising cases. Albeit numerous studies have been published, there were still many questions to be answered about how this infection exhibited different severity degrees in different individuals. This study explored the characteristic of circulating T follicular helper (cTfh) cells, which might involve determining severity in COVID-19. The T follicular helper (Tfh) cells were a subset of CD4+ T cells that mainly responsible for shaping B-cell differentiation in the germinal center.[20] Several features are identified as unique for Tfh cells, i.e., CXCR5 surface marker, gene expression profiles (BCL6, ASCL2, PD-1, and ICOS), and their IL-21 production capabilities.[20]

The development of Tfh cells in secondary lymphoid organs initiates when the naïve CD4 T cells are primed by follicular dendritic cells (FDCs). FDCs act as antigen-presenting cells (APCs) as they harbor MHC class II, and they also secrete several cytokines that support Tfh formations: TGF-beta, IL-12, and IL-23. After priming, pre-Tfh cells express more CXCR5 on their surface and upregulate BCL6 transcriptional factor; and they migrate to the germinal centers (GCs). In this location, they interact with B cells. GC B cells that have been activated (indicated by CD80, CD86, and ICOSL surface markers) support the pre-Tfh cells to become fully mature Tfh cells in the germinal centers. Thus, the interaction between Tfh cells and B cells in the GCs supports both cells' development. From B cell perspective, cTfh cells help their differentiation by responding to ICOS and CD40L from the Tfh surface as well as IL-21 that are secreted by Tfh cells. The cells could also secrete different cytokine by the stimulation type they receive. For example, Th-1 type stimuli could induce Tfh cells that secrete IFN-gamma, while Th-2 type stimuli resulted in Tfh cells producing IL-4.[20,25]

Circulating Tfh cells (cTfh) were found recently in peripheral blood, and several experiments in vivo support these cells are also capable of supporting B-cell differentiation.[21] Circulating Tfh cells also exhibit CXCR5 surface marker and produce IL-21 while stimulated, but they do not upregulate BCL-6 transcriptional factors (as opposed to Tfh cells in GCs).[21] Circulating Tfh cells could be grouped into three subsets based on CXCR3 and CCR6 expression (which associated with additional transcriptional factors and cytokines they produce)[21]:

- cTfh1 (CXCR3+ CCR6-, upregulate Th1-like T-bet transcription factor and secrete IFN-gamma),
- cTfh2 (CXCR3- CCR6-, upregulate Th2-like GATA-3 transcription factor and secrete IL-4),
- cTfh-17 (CXCR3- CCR6+, upregulate Th-17 like RORγT transcription factor and secrete IL-17A).

Some hypotheses are brought up for cTfh cell origins, i.e., cTfh cells are originated from GC Tfh cells after being activated and interact with GC B cells.[21,26] Vella et al. examined the properties of cTfh cells from blood, lymphocytes from thoracic duct lymph (TDL), and GC Tfh cells from mesenteric lymph nodes (LNs).[27] They found that TDL carries lymphocytes that exhibit similar phenotypes ($CXCR5^{bright}$ and $PD1^{bright}$) and epigenetic profiles with GC Tfh cells.[27] This study also showed that multiple sclerosis patients who consumed fingolimod (drugs that prevent cellular egress from lymphoid tissues by downregulating sphingosine-1-phosphate receptors (S1PRs)) show a reduced level of cTfh cells in peripheral blood.[27] Furthermore, the Tfh cells from TDLs showed intermediate profiles of ICOS, CD38 and CCD7 expression between GCs Tfh cells and cTfh cells in peripheral blood, suggesting that cTfh cells in peripheral blood are mainly supplied from GC Tfh in secondary lymphoid organs.[27] Therefore, examining cTfh cells in peripheral blood from patients could give some insight into different processes in GCs, since these regions are relatively difficult to access in humans.

This study showed that total cTfh cell frequencies decrease as disease severity progress in SARS-CoV-2 patients during acute infection, and these differences disappeared in the 3-month convalescence period. Interestingly, cTfh1 and cTfh17 frequencies were more strikingly reduced in severe patients than mild patients and the same individuals after 3-month follow-up. The frequency changes of cTfh cells have been demonstrated in previous studies in the context of infectious diseases, vaccination, and autoimmune diseases. Circulating Tfh cell frequencies (especially cTfh2 and cTfh17) have been reported to increase in autoimmune diseases, i.e., systemic lupus erythematosus, myasthenia gravis, allergic rhinitis and bronchial asthma, multiple sclerosis, atopic dermatitis, and immune thrombocytopenia.[28–32] The expansion of cTfh cells was also positively correlated with disease severity and outcomes in these diseases. In the perspective of infectious diseases, these cells frequencies in peripheral blood were also changed in dengue, hepatitis B, hepatitis C, and Trypanosoma cruzii infection cases in humans.[23,33–35] Circulating Tfh cell frequencies were also seen to be changed after influenza vaccine administration.[36,37] The decreases of cTfh frequencies in this study were also seen in our previous studies in Influenza A infection cohort and other studies of SARS-CoV-2 patients' cohort during acute infection compared to convalescences. This trend seemed different with other virus infections (i.e., dengue, hepatitis B and hepatitis C), where cTfh cells were increased during disease state compared to convalescence.[23,33,34] Several markers (i.e., ICOS, CD38 (activation markers), and CCR7) were also seen to be elevated in SARS-CoV-2 patients in this study, and these profiles were most prominently seen in more severe patients. CCR7 upregulation in T cells has been shown in different infection disease contexts.[38] (Yanyan et al., 2019). High levels of CCR7+ CD4+ T cells were also observed in lung draining lymph nodes during influenza infection mice.[39]

Diving into each subset, different trends of cTfh subsets frequencies and activation patterns were found between different patients' severity statuses. While cTfh1 and cTfh17 frequencies markedly decreased, cTfh1 and cTfh2 were more activated in severe patients. However, three of these subsets were consistently more activated (expressing more ICOS in their surface) in severe patients than healthy controls. In comparison, other virus infection cases in human studies showed predominant cTfh1 involvement during the disease state, for example, in acute (influenza and dengue) and chronic (hepatitis B and C) diseases. The other two subsets (cTfh2 and cTfh17) were observed an increase in frequency and activation state in many well-known autoimmune diseases. Recent reports showed that autoimmune and autoinflammatory processes were happened during and after SARS-CoV-2 infection in some patients and survivors. For instance, hospitalized acute COVID-19 patients developed higher titers of autoantibodies against nuclear antigens (ANA), anti-neutrophil cytoplasmic antibodies (ANCA), and Anti-Saccharomyces cerevisiae IgA antibodies (ASCA-IgA) than healthy controls.[40,41] Furthermore, there are emerging cases of multisystem inflammatory syndrome in children and adolescents (MIS-C) that temporally associated with SARS-CoV-2 infection.[42,43] Albeit the relationship of COVID-19 and autoinflammatory state has not been firmly concluded, increased cTfh2 and cTfh17 during severe COVID-19 disease state might be partly related to these phenomena. Further studies are needed to prove the relationship between these two cTfh subsets and autoinflammatory conditions in COVID-19.

While exploring cTfh frequencies and activation status with antibody data, we found that activated cTfh are positively correlated to IgG antibodies towards different antigens (S-, RBD, and N-) during acute infection. There are several publications up to the current date that explore cTfh activations and frequencies and their relationships with antibodies level in COVID-19. Koutsakos et al. and Sandberg et al. reported that activated cTfh cells (especially activated cTfh1) were significantly rose during acute infection and resolved in convalescence periods.[44,45] Positive correlations of activated cTfh and SARS-CoV-2 antibodies could also be seen in these studies.[44,45] Together with those papers, we strengthened the relationship between cTfh and antibody production in SARS-CoV-2 infection.

SARS-CoV-2 specific (S-, RBD-, and N-) cTfh were observed in this cohort during acute infection and 3-month convalescence. Severe patients exhibited more SARS-CoV-2 specific cTfh cells than those with milder diseases during acute infection, and in the 3-month convalescence period, this significant difference was persisted. As cTfh cells are parts of circulating memory CD4 T cells pool in blood, the generation of specific long-lasting memory T cells (including cTfh cells) is one of the interesting aspects of the immunological view infection studies. Sandberg et al. showed that polyfunctional memory T cells persisted and responded towards SARS-CoV-2 different peptides in 9-month convalescent individuals, indicating that the SARS-CoV-2 specific T cells were still present in circulation.[45] Regarding cTfh, different studies demonstrated the significant presence of S-specific cTfh cells in acute patients convalescent individuals (1-2 months, even up to 8-months convalescent after symptoms onset).[46–49] These results imply that memory cTfh cells could be detected in people recovered from COVID-19 patients. Our results demonstrate that SARS-CoV-2 specific cTfh frequencies in patients were positively correlated with corresponding antibodies during acute infection. Furthermore, some of the positive correlations were still observable in the 3-month convalescence period. This result is coherent with Juno et al.'s findings that neutralizing antibody activity was positively correlated with S-specific cTfh in individuals recovered from COVID-19.[47] Interestingly, they could divide the S-specific cTfh into three subsets (cTfh1, cTfh2, and cTfh17).[47] While S-specific cTfh1 and cTfh2 frequencies are positively correlated, S-specific cTFh17 were inversely correlated with neutralizing antibodies.[47]

Finally, we demonstrated that PBMCs from severe patients produce more IL-21 when stimulated with SARS-CoV-2 proteins than mild patients. IL-21 is one of the hallmark cytokines produced by Tfh cells to regulate B cell differentiation into plasmablasts and plasma cells.[50] The concentration of IL-21 in serum was higher in autoimmune diseases (i.e., diabetes type I, SLE, and rheumatoid arthritis) and viral infections (i.e., dengue) compared to healthy individuals.[51–55] Both IL-21 concentration in serum and IL-21+ cTfh cells were positively correlated in disease severity in SLE and dengue infection.[51,53] IL-21 productions in severe patients were higher in our cohort after stimulation, and these were in line with our results that show different cTfh activation status in patients with different disease severity.

Therefore, this study revealed different functional markers and the capacity of cTfh cells across disease severity in responding to SARS-CoV-2 infection. There were also relationships between frequencies and activation cTfh cells and antibody levels in serum, strengthening the importance of these cells population in shaping adaptive immunity in COVID-19 diseases. As this project is still ongoing, the in-vitro studies of cTfh and B cell coculture are under experiments at the moment to understand more about the roles of cTfh cells in helping humoral immune response across different diseases severity.

## Acknowledgements

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