Archives of Virology (2021) 166:3301–3310
https://doi.org/10.1007/s00705-021-05247-z

ORIGINAL ARTICLE

Pro-inflammatory CXCL-10, TNF-α, IL-1β, and IL-6: biomarkers of SARS-CoV-2 infection

Anuradha S. Tripathy[1] · Siddhesh Vishwakarma[1] · Diptee Trimbake[1] · Yogesh K. Gurav[2] · Varsha A. Potdar[3] · Nitin D. Mokashi[4] · Sudhir D. Patsute[5] · Himanshu Kaushal[3] · Manohar L. Choudhary[3] · Bipin N. Tilekar[6] · Prakash Sarje[1] · Varsha S. Dange[7] · Priya Abraham[8]

Received: 1 June 2021 / Accepted: 1 August 2021 / Published online: 23 September 2021

Abstract

Currently, the world is witnessing the pandemic of COVID-19, a disease caused by the novel coronavirus SARS-CoV-2. Reported differences in clinical manifestations and outcomes in SARS-CoV-2 infection could be attributed to factors such as virus replication, infiltration of inflammatory cells, and altered cytokine production. Virus-induced aberrant and excessive cytokine production has been linked to the morbidity and mortality of several viral infections. Using a Luminex platform, we investigated plasma cytokine and chemokine levels of 27 analytes from hospitalized asymptomatic ($n = 39$) and mildly symptomatic ($n = 35$) SARS-CoV-2-infected patients (in the early phase of infection), recovered individuals (45-60 days postinfection) ($n = 40$), and uninfected controls ($n = 36$) from the city of Pune located in the state of Maharashtra in India. Levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α and the chemokine CXCL-10 were significantly higher, while those of the antiviral cytokines IFN-γ and IL-12 p70 were significantly lower in both asymptomatic and mildly symptomatic patients than in controls. Comparison among the patient categories revealed no difference in the levels of the cytokines/chemokines except for CXCL-10 being significantly higher and IL-17, IL-4, and VEGF being significantly lower in the mildly symptomatic patients. Interestingly, levels of all key analytes were significantly lower in recovered individuals than in those in both patient categories. Nevertheless, the level of CXCL10 was significantly higher in the recovered patients than in the controls, indicating that the immune system of SARS-CoV-2 patients may take a longer time to normalize. Our data suggest that IL-6, IL-1β, TNF-α, CXCL-10, and reduced antiviral cytokines could be used as biomarkers of SARS-CoV-2 infection.

Abbreviations

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
CRS	Cytokine release syndrome
MERS-CoV	Middle East respiratory syndrome coronavirus
ACE2	Angiotensin-converting enzyme 2
ARDS	Acute respiratory distress syndrome
RT-PCR	Reverse transcription polymerase chain reaction
COVID-19	Coronavirus disease 2019
ROC	Receiver operating characteristic curve

Handling Editor: Zhenhai Chen.

Siddhesh Vishwakarma and Diptee Trimbake contributed equally.

✉ Anuradha S. Tripathy
anuradhastripathy@hotmail.com

[1] Hepatitis Group, ICMR-National Institute of Virology, Pashan, Pune 411021, Maharashtra, India

[2] Epidemiology Group, ICMR-National Institute of Virology, Pashan, Pune 411021, Maharashtra, India

[3] Influenza Group, ICMR-National Institute of Virology, Pune 411001, Maharashtra, India

[4] Yashwantrao Chavan Memorial Hospital, Pune 411018, Maharashtra, India

[5] Naidu Hospital, Pune 411001, Maharashtra, India

[6] Diagnostic Virology Group, ICMR-National Institute of Virology, Pashan, Pune 411021, Maharashtra, India

[7] Pimpri Chinchwad Municipal Corporation, Pimpri, Pune 411018, Maharashtra, India

[8] ICMR-National Institute of Virology, Pune 411001, Maharashtra, India

Introduction

In early December 2019, several pneumonia cases of unknown origin were observed in the city of Wuhan in China. The causative pathogen was identified as a novel enveloped RNA virus belonging to the genus *Betacoronavirus* that was later named "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2).The virus showed genetic similarity to both SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) [1]. Subsequently, it has spread all over the world and was declared a pandemic by the World Health Organization on March 11, 2020 [2]. There are reports of patients with SARS-CoV-2 infections having mild to severe respiratory illness with fever, cough, and shortness of breath as the common symptoms 2–14 days after exposure. However, the majority of patients diagnosed as positive using an RT-PCR test are reported to be either asymptomatic or minimally symptomatic after an incubation period of approximately 5–6 days [3]. Fever, dry cough, shortness of breath, myalgia, fatigue, leucopenia, and radiological signs of progressive pneumonia are the similar clinical and laboratory findings of disease progression seen in SARS-CoV-2, SARS-CoV, and MERS-CoV infections [2].

Severe pneumonia caused by coronaviruses has often been associated with rapid virus replication, infiltration of inflammatory cells, and elevated cytokines, resulting in damage to internal organs and acute respiratory distress syndrome (ARDS) [4]. Virus-induced aberrant and excessive cytokine production has been linked to morbidity and mortality in several viral infections [5]. Cytokine release syndrome (CRS), a systemic inflammatory response, can be caused by an infection, some drugs, or cancers and is characterized by a major increase in the level of a large number of pro-inflammatory cytokines. CRS was found to be the major cause of morbidity in patients infected with SARS or MERS-CoV [6]. In SARS-CoV infection, ARDS is the ultimate result of a cytokine storm in which large amounts of pro-inflammatory cytokines and chemokines released by immune effector cells sustain an aberrant systemic inflammatory response [1]. The observed peculiar ability of SARS-CoV to counteract the antiviral IFN response suggests that the severity of disease might be due to immune dysregulation, which could be characterized by an insufficient type I interferon response, paralleled by aberrant secretion of pro-inflammatory chemokines by alveolar macrophages, dendritic cells, and pneumocytes [1]. Data suggest that MERS-CoV infection shares certain immunological aspects with SARS-CoV infection in terms of involvement of the chemokine system [1]. Estimation of innate immune cell counts has indicated that activated dendritic cells, activated mast cells, and neutrophils are more abundant in patients with viral pneumonia than in healthy controls. A recent study has suggested the neutrophil-to-lymphocyte (NLR) ratio to be associated with the disease severity of COVID-19. In patients with ARDS, the alveolar spaces are reported to be occupied by infiltrating neutrophils and monocytes, suggesting a pathogenic role of those cells. SARS-CoV-2 triggers a robust IFN response, hallmarked by the expression of numerous ??interferon-stimulated genes?? (ISGs), thus indicating a role of the innate immune response in COVID-19 infection. The protective potential of ISGs may account for the lower proportion of severe cases and the lower case-fatality rate in COVID-19 compared to SARS [7]. There has been documentation of IFN treatment as an antiviral therapy against SARS-CoV-2 infection (ChiCTR: ChiCTR2000029600). However, the timing and the dose required need careful consideration.

It is believed that a dysregulated host immune response and production of inflammatory cytokines, known as a "cytokine storm", correlate with disease severity and poor prognosis during both SARS-CoV and MERS-CoV infections [8]. Angiotensin-converting enzyme 2 (ACE2), an essential regulatory enzyme of cardiovascular function, has become of extreme interest during the SARS-CoV-2 pandemic, as reduction in ACE2 expression leads to the release of pro-inflammatory cytokines, ARDS, myocarditis, and hypercoagulability, with the possibility of exacerbation of acute coronary syndrome and induction of pulmonary embolism [9]. During SARS-CoV-2 infection, many patients develop ARDS, leading to pulmonary edema and respiratory failure, and these symptoms are associated with a cytokine storm [10]. The cytokine and chemokine profiles in SARS-CoV-2-infected patients with different clinical manifestations remain unclear. In the present study, we have investigated the plasma cytokine and chemokine levels in hospitalized asymptomatic ($n = 39$) and mildly symptomatic ($n = 35$) SARS-CoV-2-infected patients, recovered individuals 45-60 days after infection with SARS-CoV-2 ($n = 40$), and healthy control individuals ($n = 36$) from Pune, Maharashtra, India.

Materials and methods

Study population

The current study was carried out between March 2020 and June 2020. The prevention and control plan for the management of close contacts of individuals with COVID-19 have evolved, keeping the focus on emphasizing identification and quarantine of asymptomatic patients. To identify asymptomatic patients, reverse transcription polymerase chain reaction (RT-PCR) screening of throat and

nasal swabs for SARS-CoV-2 for close contacts of index cases was done, and positive individuals were transferred to designated hospitals for centralized isolation [11]. Blood samples from asymptomatic (n = 39) and mildly symptomatic (n = 35) patients with SARS-CoV-2 infection were collected in the acute phase during hospitalization (≤6 days). The current study population consisted of a subset of the patients whose nasal/throat swabs tested positive for SARS-CoV-2 by RT-PCR to ICMR-NIV, Pune. They were admitted to the state-run Naidu Hospital and the YCM Hospital, which are tertiary-care hospitals in Pune, Maharashtra. Asymptomatic patients who developed symptoms within 6 days after admission were excluded from the study. Finally, 39 asymptomatic cases, defined by a SARS-CoV-2-positive nucleic acid test result but without any relevant clinical symptoms during hospitalization, were included in the current study. The symptoms of mildly symptomatic COVID-19 patients were cough, fever, fatigue, headache, myalgias, and diarrhoea. A total of 35 mildly symptomatic patients were selected for comparison with the asymptomatic individuals. All patients from both categories recovered. The recovered individuals (n = 40) included in the study had a previous history of symptomatic/asymptomatic SARS-CoV-2 infection and had become negative for SARS-CoV-2 by RT-PCR. Blood samples from recovered individuals were collected between 45 and 60 days post-diagnosis. The recovered individuals and the patient groups (both asymptomatic and mild symptomatic) were all distinct.

Thirty-six apparently healthy individuals from blood donation camps organized by Sassoon General Hospital, Pune, in December 2019, were recruited as healthy controls for cytokine comparison (Table 1). The healthy controls were screened for SARS-CoV-2 by ELISA. Plasma samples were used to detect the presence of IgG antibodies using a commercial ELISA (COVID Kavach-Anti-SARS-CoV-2 IgG Antibody Detection ELISA, M/s Cadila Healthcare Limited, Ahmedabad). Only those who were negative for anti-SARS-CoV-2 IgG antibody were included in the study.

The study was approved by the Institutional Ethical Committee for Research on Humans, based on the guidelines set by the Indian Council of Medical Research, New Delhi. Informed written consent was obtained from all study participants.

Plasma separation and storage

About 3 to 4 ml of blood from each study participant was collected in a K3 EDTA tube, and the plasma was separated within 4 hours of collection by the Ficoll-Hypaque (Sigma, USA) density gradient centrifugation method and stored at −80 °C. Plasma samples were thawed at the time of estimation of cytokine, chemokine, and growth factors levels.

Estimation of cytokine, chemokine, and growth factor levels

Plasma concentrations of cytokines, chemokines, and growth factors were determined using a Bio-plex Multiplex Immunoassay System (Bio-Rad, Hercules, CA, USA) using a Bio-plex Pro^{TM} Human Cytokine 27-plex assay kit as reported previously [12, 13] as per the manufacturer's instructions. Levels of 15 cytokines, including the pro-inflammatory (IL-1β, IL-5, IL-6, IL-7, IL-9, IL-15, IL-17, TNF-α), anti-inflammatory (IL1-RA, IL-4, IL-10, IL-13), and Th1 (IL-2, IFN-γ, IL-12 p70) cytokines along with seven chemokines (eotaxin, CCL-2,CCL-3,CCL-4,CCL-5, IL-8, CXCL-10) and five growth factors (basic fibroblast growth factor [FGF], G-CSF, GM-CSF, vascular endothelial growth factor [VEGF], platelet-derived growth factor-bb [PDGF-bb]), were estimated.

Statistical analysis

Levels of all analytes were analysed after $\log_{10}$ transforming the observed concentrations of individual cytokines/chemokines/growth factors. A value of 0.1 pg/ml was used in cases where the concentrations of the cytokines, chemokines, and growth factors in the tested samples were too low to be detected. The Mann–Whitney U test was used for numerical data for comparisons among the study groups. A p-value of <0.05 was considered significant. Statistical analysis was performed using IBM SPSS Statistics

Table 1 Characteristics of the study population

Parameter	Patient group			
	Asymptomatic	Mildly symptomatic	Recovered	Healthy controls
Study population	n = 39	n = 35	n = 40	n = 36
Gender ratio (male: female)	1.05	2.5	1.35	2.6
Median age (years), (range)	31.5 (16–79)	38 (15–75)	37.5 (18–70)	20 (18–26)
Post-onset days of illness (POD)	NA	0–17 days	45–60 days[a]	NA

NA not applicable

[a]Recovered from symptomatic infection

25 software (SPSS Inc., Chicago, IL, USA). A global-view heat map was generated using the heatmap.2 package in R. Receiver operating characteristic analysis (ROC) was performed using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA).

Results

Characteristics of the study population

The characteristics of the study groups are presented in Table 1. The patients investigated in this study included (a) 74 patients with SARS-CoV-2 infection (45 males, 29 females) with age ranging from 15 to 79 years, which included 39 asymptomatic and 35 mildly symptomatic patients, (b) 40 recovered individuals, and (c) 36 healthy controls. The patients did not have any coinfections with other respiratory infections and had an uneventful recovery. The patients in the acute phase of infection were positive for SARS-CoV-2 by qRT-PCR [11, 14], while the recovered individuals had a reported history of SARS-CoV-2 infection. The clinical features of the mildly symptomatic patients are described in Table 2.

Peripheral cytokines and chemokines in asymptomatic and mildly symptomatic patients with SARS-CoV-2 infection

IL-1β, IL-6, IL-7, IL-9, TNF-α, IL1-RA, IL-4, IL-13, CCL-4 (MIP-1α), and CXCL-10 (IP-10) were significantly elevated in both asymptomatic and mildly symptomatic patients compared to controls ($p < 0.05$). Importantly, the levels of IL-17, eotaxin, and PDGF-BB were significantly elevated in asymptomatic patients only, when compared to controls. IL-5, IFN-γ, IL-12 p70, CCL-2, CCL-3, IL-8, and VEGF were significantly lower in both asymptomatic and mildly symptomatic patients than in controls ($p < 0.05$) (Table 3). Importantly, the level of G-CSF was significantly lower only in mildly symptomatic patients when compared to controls. Levels of IL-15, IL-10, IL-2, CCL-5 (RANTES), FGF, and GM-CSF were comparable among all groups (Table 3 and Figs. 1 and 2).

Comparison among the disease categories revealed no difference in the levels of the chemokines/cytokines except for (a) CXCL-10 being significantly higher and (b) IL-17, IL-4, and VEGF being lower in mildly symptomatic patients ($p < 0.01$) (Table 3 and Figs. 1 and 2).

Table 2 Clinical features of mildly symptomatic patients

Parameter	Positive/total number of cases	95% CI (range)
Cough	22/35	63% (47–79%)
Fever	21/35	60% (44–76%)
Sore throat	13/35	37% (21–53%)
Nasal discharge	9/35	26% (11–40%)
Breathlessness	7/35	20% (7–33%)
Body ache	6/35	17% (5–30%)
Headache	1/35	3% (0–8%)
Diarrhoea	1/35	3% (0-8%)

Peripheral cytokines and chemokines, post-recovery

Among the patient groups

IL-1β, IL-6, IL-7, IL-9, IL-17, TNF-α, IL1-RA, IL-4, IL-10, IL-13, IFN-γ, eotaxin, CCL-2, CCL-3, CCL-4, CCL-5, IL-8, CXCL-10, G-CSF, and PDGF-BB were significantly lower in recovered individuals than in both asymptomatic and mildly symptomatic patients. However, Basic FGF was significantly lower only in the recovered group than in the asymptomatic group, while VEGF was significantly lower only in the recovered group than in the mildly symptomatic group. Levels of IL-5, IL-15, IL-2, IL-12 p70, and GM-CSF were comparable among the recovered, asymptomatic, and mildly symptomatic groups (Table 3).

Recovered vs. control groups

IL-5, IL-9, IL-17, IL1-RA, IL-10, IFN-γ, IL-12p70, eotaxin, CCL-2, CCL-3, CCL-4, CCL-5, IL-8, basic FGF, G-CSF, VEGF, and PDGF-BB were significantly lower in the recovered group than in the control group. Levels of IL-1β, IL-6, IL-7, IL-15, TNF-α, IL-4, IL-2, and GM-CSF were comparable among the recovered and control groups. Importantly, the level of CXCL-10 was significantly higher in the recovered group than in the control group (Table 3 and Fig. 2).

ROC analysis of cytokines

To generate a cutoff for the analytes among the patient categories, ROC curve analysis was performed (Fig. 3 and Table 4). The ROC analysis generated cutoffs for individual cytokines/chemokines that could be used to distinguish healthy controls from patients with SARS-CoV-2 infection and might have future diagnostic importance.

The ROC characteristics of CXCL-10 included a cutoff value of 124.9 pg/mL (sensitivity, 97.30%; specificity, 91.67%). Similarly, the ROC characteristics of TNF-α, IL-1β, and IL-4 showed cutoff values of 29.19 pg/mL, 0.545 pg/mL, and 1.395 pg/mL, respectively (sensitivity, 98.65%, 91.89%, and 97.30%, respectively; specificity, 83.33%, 86.11%, and 66.67%, respectively). The AUC values of the above analytes were greater than 0.9 ($p < 0.0001$), which is

Table 3 Levels of cytokines/chemokines in patients with recent SARS-CoV-2 infection and in healthy controls

Analytes	Asymptomatic COVID19 (*n*=39)	*p* value[a]	Mild symptomatic (*n*=35)	*p* value[b]	*p* value[c]	Healthy controls (*n*=36)	*p* value[d]	Recovered (*n*=40)	*p* value[e]	*p* value[f]
Pro-inflammatory cytokines										
IL-I	0.42 (−0.44–1.08)	NS	0.31 (−0.44–1.17)	<0.05	<0.05	−0.3 (−1.09–1.52)	NS	−0.1 (−1.09–0.62)	<0.05	<0.05
IL-5	0.1 (0.1–1.63)	NS	0.1 (0.1–1.53)	<0.05	<0.05	1.04 (0.1–2.31)	<0.05	0.1 (0.1–1.80)	NS	NS
IL-6	0.17 (−0.3–1.17)	NS	0.1 (−0.63–1.74)	0.04 1	0.01	0.082 (−0.67–1.17)	NS	0.1 (−0.67–0.68)	0.016	0.047
IL-7	1.0 (0.1–1.4 1)	NS	1.0 (0.1–1.4 1)	<0.05	<0.05	0.1 (0.1–1.94)	NS	0.1 (0.1–0.81)	<0.05	<0.05
IL-9	2.41 (1.92–2.61)	NS	2.36 (2.05–2.56)	<0.05	<0.05	2.23 (0.68–2.37)	<0.05	2.03 (1.17–2.35)	<0.05	<0.05
IL-15	0.1 (0.1–2.58)	NS	0.1 (0.1–2.36)	NS	NS	0.1 (0.1–3.11)	NS	0.1 (0.1–0.1)	NS	NS
IL-17	0.84 (0.09–1.25)	0.023	0.7 (−0.2–1.46)	NS	<0.05	0.56 (−0.74–1.9)	<0.05	0.39 (−0.74–0.85)	<0.05	<0.05
TNF-α	1.65 (1.46–1.88)	NS	1.62 (136–1.78)	<0.05	<0.05	1.32 (0.1–1.95)	NS	1.28 (0.76–1.64)	<0.05	<0.05
Anti-inflammatory cytokines										
IL1-RA	2.59 (0.1–3.55)	NS	2.65 (0.1–3.26)	<0.05	<0.05	1.82 (0.1–3.32)	0.037	0.1 (0.1–3.19)	<0.05	<0.05
IL-4	0.64 (0.08–0.95)	0.037	0.5 (0.12–0.9)	<0.05	<0.05	0.11 (−0.69–0.71)	NS	0.03 (−0.45–0.65)	<0.05	<0.05
IL-10	0.45 (−0.48–1.15)	NS	0.34 (−0.48–0.96)	NS	NS	0.32 (−0.49–1.61)	<0.05	0.1 (0.02–0.65)	<0.05	<0.05
IL-13	0.41 (−0.1–0.98)	NS	0.24 (−0.3–1.14)	<0.05	<0.05	−0.45 (−0.92–1.13)	0.001	−0.14 (−1.69–0.67)	<0.05	<0.05
TH1 cytokines										
IL-2	0.1 (0.07–0.46)	NS	0.1 (0.07–0.1)	NS	NS	0.1 (−0.4–1.89)	NS	0.1 (0.1–0.37)	NS	NS
IFN-y	0.52 (−0.03−1.15)	NS	0.52 (−0.03–1.37)	0.001	0.043	0.69 (−0.1–1.43)	<0.05	0.017 (−1.69–0.86)	<0.05	<0.05
IL-12 p70	0.1 (0.08–1.07)	NS	0.1 (−0.27–127)	0.005	0.009	0.34 (−1.69–1.81)	0.007	0.1 (−1.69–1.01)	NS	NS
Chemokines										
Eotaxin	1.77 (1.25–2.25)	NS	1.62 (1.27–2.08)	NS	0.002	1.57 (−0.69–1.97)	<0.05	1.27 (0.8–1.65)	<0.05	<0.05
CCL2	1.6 (1.13–2.1)	NS	1.56 (0.72–2.09)	0.003	0.003	1.71 (0.04–2.19)	<0.05	1.37 (0.73–1.65)	<0.05	<0.05
CCL3	0.17 (−0.36–0.67)	NS	0.17 (−0.36–0.62)	0.004	0.007	0.46 (−1.09–1.27)	<0.05	–0.09 (−1.09–0.26)	<0.05	<0.05
CCL 4	2.28 (1.83–2.46)	NS	2.65 (1.93–2.42)	<0.05	<0.05	2.15 (0.1–2.33)	<0.05	1.94 (1.13–2.21)	<0.05	<0.05
CCL5	3.61 (2.73–5.74)	NS	3.47 (2.88–4.49)	NS	NS	3.52 (0.86–4.02)	<0.05	2.87 (2.17–3.7)	<0.05	<0.05
IL-8	0.65 (−0.61–1.54)	NS	0.82 (−0.26–1.6)	<0.05	<0.05	1.27 (−0.52–2.03)	<0.05	0.05 (−0.92–1.05)	<0.05	<0.05
CXCL-10	2.48 (2.02–3.81)	0.032	2.63 (2.16–3.61)	<0.05	<0.05	1.88 (0.1–2.22)	<0.05	2.11 (1.69–2.65)	<0.05	<0.05
Growth factors										
Basic FGF	2 (0.1–1.64)	NS	0.95 (0.1–1.81)	NS	NS	1.18 (0.1–2.4)	<0.05	0.6 (0.1–1.45)	0.013	NS
G-CSF	1.87 (1.42–238)	NS	1.77 (133–2.17)	0.002	NS	2.0 (1.39–2.61)	<0.05	1.48 (0.95–1.91)	<0.05	<0.05
GM-CSF	0.1 (−0.26–0.71)	NS	0.1 (−0.26–0.21)	NS	NS	0.1 (−0.88–1.12)	NS	0.1 (−0.88–1.09)	NS	NS
VEGF	0.1 (0.1–2.59)	0.023	0.1 (0.1–2.44)	<0.05	<0.05	2.38 (1.83–2.68)	<0.05	0.1 (0.1–2.28)	NS	<0.05
PDGF-BB	2.78 (0.1–3.58)	NS	2.53 (0.16–3.63)	NS	0.004	2.27 (1.02–2.96)	<0.05	0.75 (−0.58–3.09)	<0.05	<0.05

*Values for cytokines and chemokines are presented as median Log_{10} pg/ml (range); *p*-values: [a]asymptomatic vs. mildly symptomatic, [b]control vs. mildly symptomatic, [c]control vs. asymptomatic, [d]control vs. recovered, [e]asymptomatic vs. recovered, [f]mildly symptomatic vs. recovered, NS, non-significant

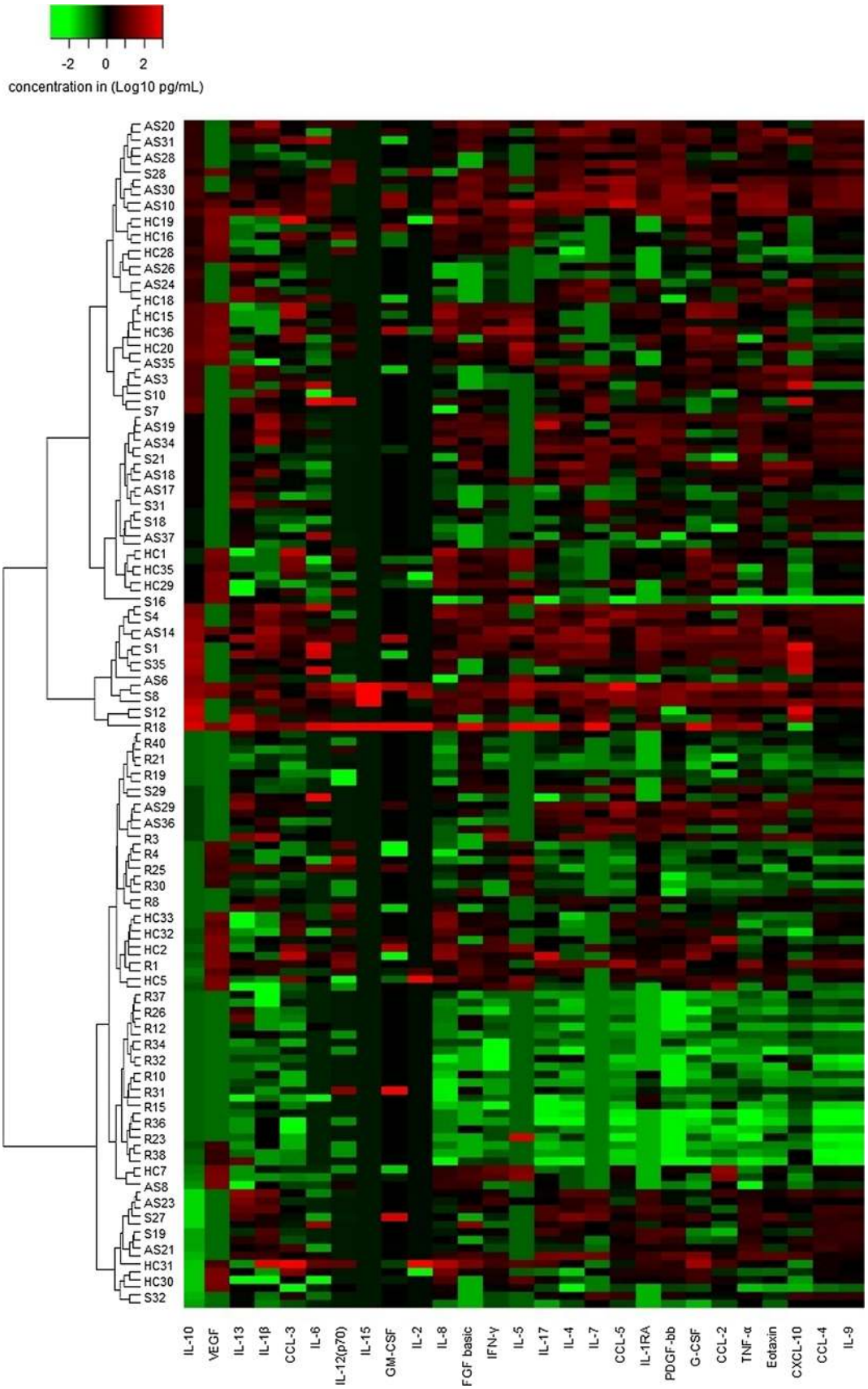

Fig. 1 Global heat map of levels of cytokines/chemokines in SARS-CoV-2-infected patients, recovered individuals, and healthy controls

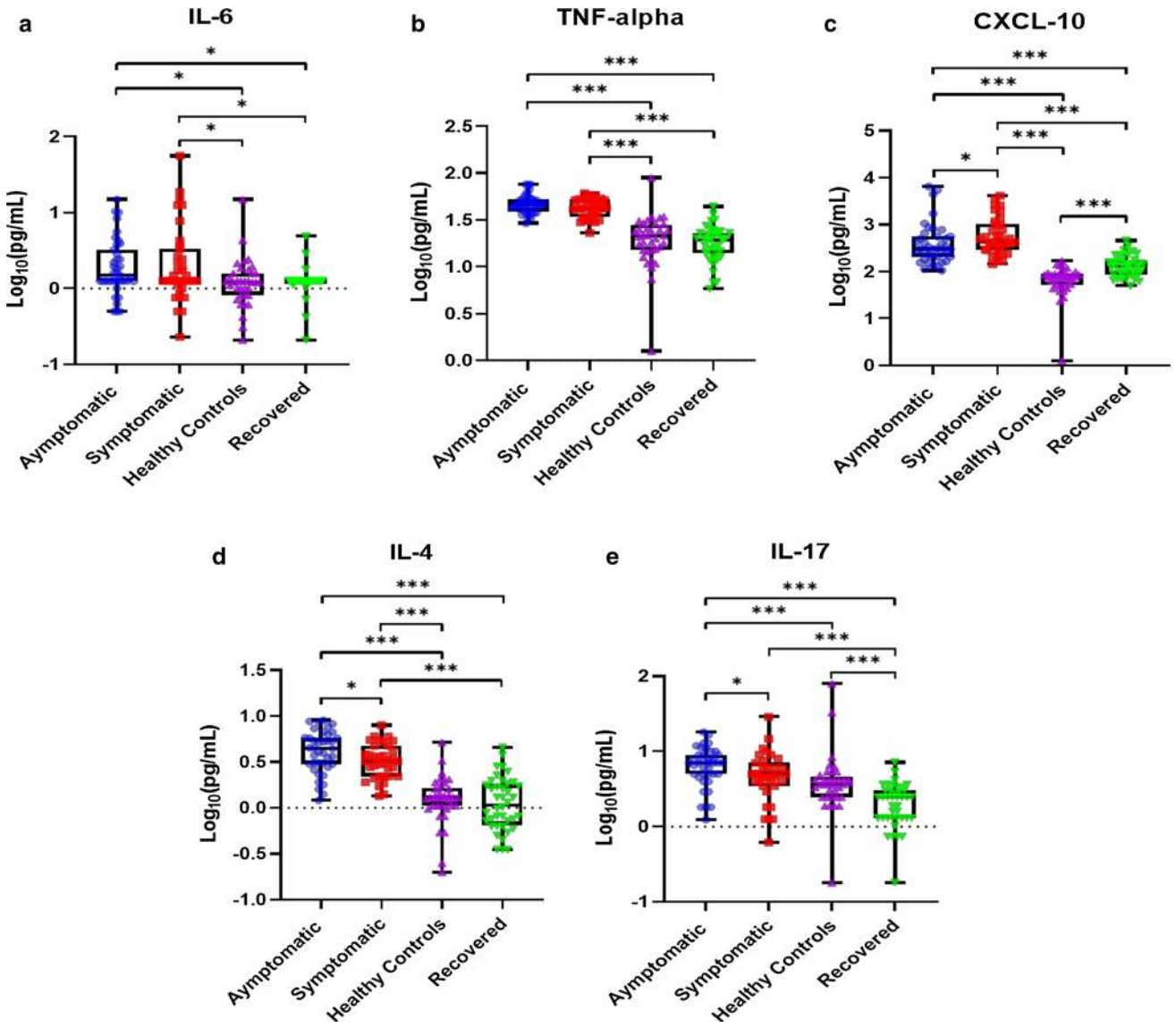

Fig. 2 Comparison of concentrations of cytokines and chemokines expressed in Log_{10}(pg/mL) in different study groups. **a** IL-6, **b** TNF-α, **c** CXCL-10, **d** IL-4, **e** IL-17. *p <0.05; **p < 0.005; ***p < 0.0001

indicative of higher diagnostic value. The cytokines IL-1β, IL-4, and TNF-α and the chemokine CXCL-10 were found to be predictive of early SARS-CoV-2 infection, which was confirmed by ROC analysis (AUC = 0.9917, 0.9202, 0.9336, and 0.9508, respectively). The high sensitivity and specificity of the analytes' cutoffs suggest that they can potentially act as a biomarker of SARS-CoV-2 infection.

Discussion

A good proportion of patients with SARS-CoV-2 infection are reported to have mild to moderate respiratory illness. However, in parallel, other patients (identified mostly through contact tracing) remain asymptomatic. Information such as epidemiological characteristics, virus levels, and immune responses in this subset of patients is scarce. To gain a better understanding of the host factors modulating the course of the infection in individuals with SARS-CoV-2 infection, we have compared the levels of plasma cytokines, chemokines, and growth factors in a group of hospitalized asymptomatic and mildly symptomatic patients with SARS-CoV-2 infection and in recovered individuals.

Comparative analysis of cytokine levels in MERS-CoV-infected patients and normal healthy controls had shown a Th2-skewed immune response in infected patients, which was somewhat unique for MERS-CoV infection when compared to SARS-CoV, which has been shown to induce

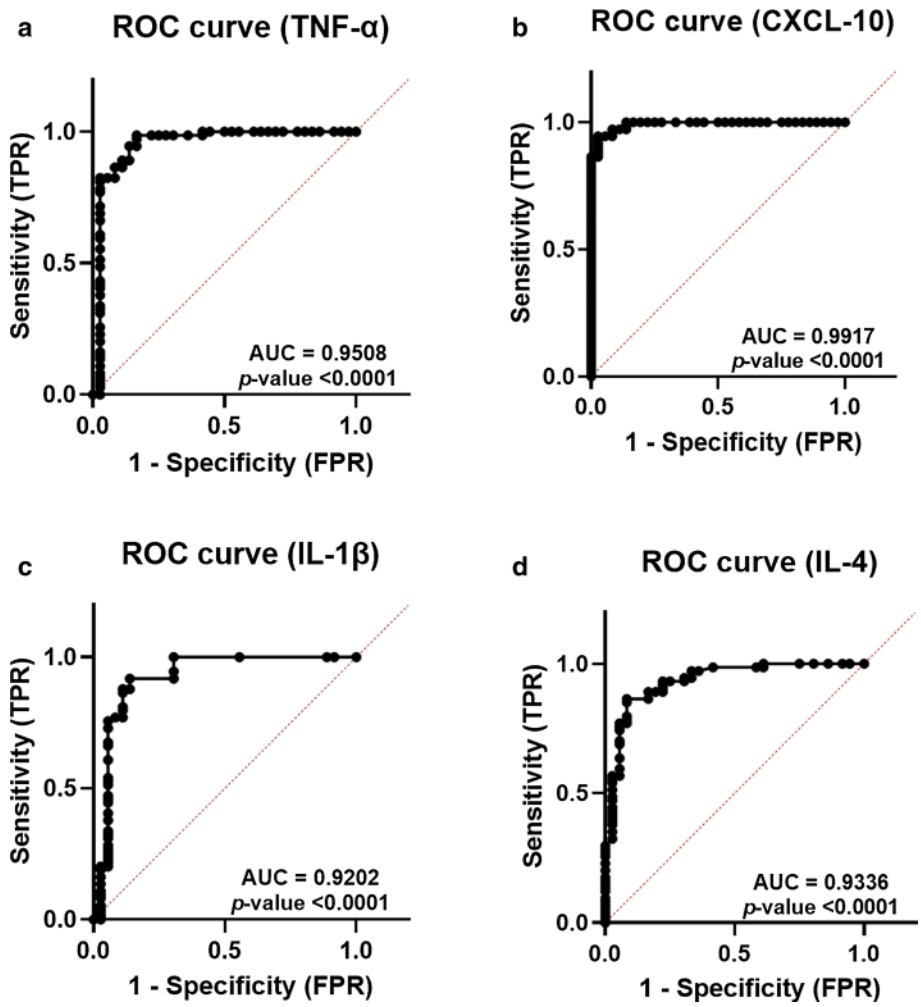

Fig. 3 Receiver operating characteristic (ROC) curves for IL-1β, TNF-α, CXCL-10, and IL-4, validating their applicability as biomarkers of recent infection. **a** ROC curve generated by comparing concentrations of TNF-α (pg/mL) in SARS-CoV-2-infected patients with their concentrations in healthy controls reveals the inefficiency of TNF-α as a biomarker (cutoff, 29.19; sensitivity, 98.65%; specificity, 83.33%), **b** ROC curve generated by comparing concentrations of CXCL-10 (pg/mL) in SARS-CoV-2-infected patients with their concentrations in healthy controls reveals the efficiency of CXCL-10 as a biomarker (cutoff, 124.9; sensitivity, 97.3%; specificity, 91.67%), **c** ROC curve generated by comparing concentrations of IL-1β (pg/mL) in SARS-CoV-2-infected patients with their concentrations in healthy controls reveals the efficiency of IL-1β as a biomarker of infection (cutoff, 0.545; sensitivity, 91.89%; specificity, 86.11%). **d** ROC curve generated by comparing concentrations of IL-4 (pg/mL) in SARS-CoV-2-infected patients with their concentrations in healthy controls reveals the efficiency of IL-4 as a biomarker of infection (cutoff, 1.395; sensitivity, 97.30%; specificity, 66.67%)

Table 4 ROC characteristics of the cytokines TNF-α, CXCL-10, IL-1β, and IL-4 in plasma of SARS-CoV-2-infected patients and healthy controls

Analyte	Cutoff (pg/mL)	Sensitivity (%)	Specificity (%)	AUC value	*p*-value
TNF-α	29.19	98.65	83.33	0.9508	<0.0001
CXCL-10	124.9	97.3	91.67	0.9917	<0.0001
IL-1β	0.545	91.89	86.11	0.9202	<0.0001
IL-4	1.395	97.30	66.67	0.9336	<0.0001

a Th1-type immune response [15]. The asymptomatic and mildly symptomatic patient groups from the current study did not exhibit any such specific pattern. The observations of the present study and published data on the cytokine profiles in acute MERS-CoV and SARS-CoV infections appear to be conflicting. It is possible that SARS-CoV-2 may have evolved a different mechanism to mitigate host defences.

Infection with coronaviruses results in the activation of monocytes, macrophages, and dendritic cells, leading to the secretion of prominent pro-inflammatory cytokines

such as IL-6, along with other inflammatory cytokines [16]. Elevated IL-6 can activate either classical *cis*-signaling or *trans*-signaling, leading to cytokine release syndrome/cytokine storm [16]. It is understood that the timing of IL-6 activation is critical, as it is necessary for the early immune response for fighting SARS-CoV-2 and other viral infections. Low IL-6 levels in the early stages of infection could result in uncontrolled viral replication. Thus, rather than blocking IL-6 release, regulating it would have therapeutic value [17]. Several studies have found elevated serum concentrations of inflammatory cytokines, including interleukin IL-6, in cases of severe COVID-19. However, these elevated levels of inflammatory cytokines, including IL-6, were much lower than those reported in patients with ARDS unrelated to COVID-19, such as sepsis and chimeric antigen receptor (CAR) T-cell-induced cytokine release syndrome [18] The dynamic role of IL-6 in the cytokine storm and CRS in the context of SARS-CoV-2 infection has been elucidated by Zhang et al. [19]. It is believed that IL-6 blockade is a promising strategy for treatment of COVID-induced CRS, as elevated IL-6 levels were consistently reported in several studies of COVID-19 and might serve as a predictive biomarker for disease severity [20]. Treatment of the cytokine storm has become an imperative part of rescuing severe COVID-19 patients, as mild or severe cytokine storms are reported in severe cases. For this purpose, the IL-6R antagonist tocilizumab has been suggested as an important drug to save patients' lives [19]. On similar lines, regulated higher levels of IL-6 in both asymptomatic and mildly symptomatic patients in the current study may be correlated with disease outcome. The lower IL-6 levels in the recovered individuals compared to both patient groups could be used as a marker of recovery, which goes hand in hand with the reported beneficial effects of IL-6 blockade therapy [8].

Chemokines are known to play a role in the cytokine storm related to coronavirus infection [1]. In an analysis of 23 patients with SARS infection, Jiang et al. showed that serum CXCL-10 was markedly elevated during the early stage of SARS and remained at a high level until convalescence [21]. Analysis of serum cytokines and chemokines in patients with MERS-CoV infection revealed that levels of IL-6 and CXCL-10 were significantly elevated in MERS-CoV patients who developed severe disease [22]. A recent report indicated that increased production of pro-inflammatory chemokines, mainly CXCL-10 and CXCL-8, is a characteristic of COVID-19. Interestingly, CXCL-8 transcription is upregulated by SARS-CoV infection but not by SARS-CoV-2 infection, while the opposite has been observed for CXCL-10 [1]. This coincides with the current study's finding of elevated CXCL-10 in both patient categories. The levels of CXCL-10 have been reported to be high in SARS-CoV-2-infected patients with severe disease compared to healthy controls, indicating that the levels of CXCL-10 increase as the disease progresses in severity [5]. CXCL10/IP10 and MCP-1 have also been reported as biomarkers associated with the severity of COVID-19 and can be related to the risk of death in COVID-19 patients. Studies have indicated CXCL10 to be associated with thrombosis and responsible for inhibition of endothelial healing. Since the serum CXCL10 level correlates directly with the severity of COVID-19, anti-CXCL10 antibody treatment might represent a new regimen for COVID-19 patients, especially those with thrombotic events. Anti-CXCL10 antibody is reported to be in a clinical trial to evaluate its ability to prevent cardiovascular events [23]. In the same line, the current study suggests that CXCL-10 could be associated with pathogenesis of SARS-CoV-2 infection. At the same time, recovered individuals at 45-60 days post-onset had higher levels of CXCL-10 than the controls, suggesting that the immune system of SARS-CoV-2 patients may take a longer time to normalize.

SARS CoV-2 infection is associated with the depletion of antiviral defences as well as enhanced production of pro-inflammatory cytokines [24]. Our patient population exhibited a similar scenario, with downregulation of antiviral cytokines and higher levels of pro-inflammatory cytokines. Lower levels of IFN-γ, the key antiviral cytokine, could be attributed to the observed lower levels of IL-12 p70, as reported elsewhere [25]. A study carried out in a Chinese population showed that IFN-γ, IL-12, and the inflammatory cytokines IL-1β and IL-6 can induce hyper-innate inflammatory response due to invasion of the respiratory tract by SARS-CoV, leading to the activation of Th1-cell-mediated immunity by the stimulation of NK and cytotoxic T lymphocytes (CTLs) [26]. Our study patients exhibited increased levels of the inflammatory cytokines IL-1β, IL-6, and TNF-α, which got normalized during the recovery phase with the activation of the CTL response (our unpublished data).

Comparison of the immunological profiles of children with symptomatic and asymptomatic SARS-CoV-2 infection indicated that those with symptomatic infection had higher IL-6, IL-10, TNF-α, and IFN-γ levels [27]. Comparison of serum cytokine and chemokine levels in asymptomatic, mild, moderate, severe, and convalescent SARS-CoV-2 infection cases in China showed that serum concentrations of IL-1RA, IL-1β, IL-6, and IP-10 were lower in asymptomatic cases than in symptomatic cases [5]. However, in the current study, comparison of asymptomatic patients and those with mild symptomatic disease revealed no difference in the levels of chemokines/cytokines, except for CXCL-10 being significantly higher and IL-17, IL-4, and VEGF being lower in mildly symptomatic patients, suggesting that these molecules could be used as biomarkers of the clinical severity of SARS-CoV-2 infections in Indian patients, which may help to evaluate the disease severity and outcome of COVID-19 patients. The overall variations in the cytokines

TNF-α, IL-1β, IL-4, and CXCL-10 among the patients and healthy controls in the current study suggest their applicability as biomarkers of recent SARS-CoV-2 infection (Fig. 3 and Tables 3 and 4).

The following limitations of the current study should be taken into consideration: A major limitation was that we could not analyse immunological responses in patients with severe COVID-19. It is possible that the differences between the asymptomatic and mildly symptomatic patients would have been more pronounced, as reported elsewhere [3, 5].

In conclusion, the immunological reaction triggered by infection with SARS-CoV-2 in Indian patients is mainly of pro-inflammatory character and could be due to a reduced antiviral cytokine response against a background of CXCL-10 chemokine upregulation.

Acknowledgment Special thanks are due to Mr. Vasant Walkoli, Mr. K.D. Ramaiah, and Mr. PB Jawalkar for their help in collection of clinical samples from the study participants. The graceful support and the friendly atmosphere of the NIC Team are gratefully acknowledged.

Funding This work was carried out with the internal financial support provided by the ICMR-NIV, Pune.

Availability of data All data generated and analysed during this study are included in this article.

Declarations

Conflicts of interest The authors declare no conflicts of interest.

Ethical approval This study was approved by the Institutional Ethical Committee for Research on Humans, based on the guidelines set by the Indian Council of Medical Research, New Delhi.

Consent to participate Written informed consent was obtained from the study participants.

References

1. Coperchini F et al (2020) The cytokine storm in COVID-19: an overview of the involvement of the chemokine/chemokine-receptor system. Cytokine Growth Factor Rev 53:25–32
2. Soy M et al (2020) Cytokine storm in COVID-19: pathogenesis and overview of anti-inflammatory agents used in treatment. Clin Rheumatol 39(7):2085–2094
3. Long QX et al (2020) Clinical and immunological assessment of asymptomatic SARS-CoV-2 infections. Nat Med 26(8):1200–1204
4. Conti P et al (2020) Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. J Biol Regul Homeost Agents 34(2):327–331
5. Chi Y et al (2020) Serum cytokine and chemokine profile in relation to the severity of coronavirus disease 2019 in China. J Infect Dis 222(5):746–754
6. Pons S et al (2020) The vascular endothelium: the cornerstone of organ dysfunction in severe SARS-CoV-2 infection. Crit Care 24(1):353
7. Zhou Z et al (2020) Heightened innate immune responses in the respiratory tract of COVID-19 patients. Cell Host Microbe 27(6):883–890
8. Xiong Y et al (2020) Transcriptomic characteristics of bronchoalveolar lavage fluid and peripheral blood mononuclear cells in COVID-19 patients. Emerg Microbes Infect 9(1):761–770
9. Barilla F et al (2020) Focus on clinical practice: angiotensin-converting enzyme 2 and corona virus disease 2019: pathophysiology and clinical implications. J Cardiovasc Med (Hagerstown) 21(9):630–633
10. Wu D, Yang XO (2020) TH17 responses in cytokine storm of COVID-19: an emerging target of JAK2 inhibitor fedratinib. J Microbiol Immunol Infect 53(3):368–370
11. Potdar V et al (2020) Genomic analysis of SARS-CoV-2 strains among Indians returning from Italy, Iran & China, & Italian tourists in India. Indian J Med Res 151(2 & 3):255–260
12. Arankalle VA et al (2010) Role of host immune response and viral load in the differential outcome of pandemic H1N1 influenza virus infection in Indian patients. PLoS One 5(10):e13099
13. Tripathy AS et al (2011) Epidemic of hepatitis B with high mortality in India: association of fulminant disease with lack of CCL4 and natural killer T cells. J Viral Hepat 18(10):e415–e422
14. Potdar V et al (2020) Respiratory virus detection among the overseas returnees during the early phase of COVID-19 pandemic in India. Indian J Med Res 151(5):486–489
15. Alhetheel A et al (2020) Assessment of Th1/Th2 cytokines among patients with Middle East respiratory syndrome coronavirus infection. Int Immunol 32(12):799–804
16. Chatterjee SK, Saha S, Munoz MNM (2020) Molecular pathogenesis, immunopathogenesis and novel therapeutic strategy against COVID-19. Front Mol Biosci 7:196
17. Shirazi J et al (2020) Significant unresolved questions and opportunities for bioengineering in understanding and treating COVID-19 disease progression. Cell Mol Bioeng 13(4):1–26
18. Leisman DE et al (2020) Cytokine elevation in severe and critical COVID-19: a rapid systematic review, meta-analysis, and comparison with other inflammatory syndromes. Lancet Respir Med 8(12):1233–1244
19. Zhang C et al (2020) Cytokine release syndrome in severe COVID-19: interleukin-6 receptor antagonist tocilizumab may be the key to reduce mortality. Int J Antimicrob Agents 55(5):105954
20. Liu B et al (2020) Can we use interleukin-6 (IL-6) blockade for coronavirus disease 2019 (COVID-19)-induced cytokine release syndrome (CRS)? J Autoimmun 22351–22356
21. Jiang Y et al (2005) Characterization of cytokine/chemokine profiles of severe acute respiratory syndrome. Am J Respir Crit Care Med 171(8):850–857
22. Kim ES et al (2016) Clinical progression and cytokine profiles of middle east respiratory syndrome coronavirus infection. J Korean Med Sci 31(11):1717–1725
23. Chen Y et al (2020) IP-10 and MCP-1 as biomarkers associated with disease severity of COVID-19. Mol Med 26(1):1–12
24. Costela-Ruiz VJ et al (2020) SARS-CoV-2 infection: the role of cytokines in COVID-19 disease. Cytokine Growth Factor Rev 54:62–75
25. Huang KJ et al (2005) An interferon-gamma-related cytokine storm in SARS patients. J Med Virol 75(2):185–194
26. Wong CK et al (2004) Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. Clin Exp Immunol 136(1):95–103
27. Xiong X et al (2020) Haematological and immunological data of Chinese children infected with coronavirus disease 2019. Data Brief 31:105953