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Research Article

Severe SARS-CoV-2 infected patients are associated with up-regulation of CCL3 and down-regulation of CCL2

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ABSTRACT

The diverse range of clinical manifestations of SARS-CoV-2 infection indicates that individual immune responses to the virus are pivotal in shaping the subsequent clinical trajectory post-initial exposure. Research in immunology has primarily concentrated on individuals with moderate to severe illness, revealing heightened inflammation in tissues and resultant organ impairment. Chemokines play key roles in the regulation of immune responses against viral infections. The current study was aimed to evaluate the CC ligand 2 (CCL2) and CCL3 in the SARS-CoV-2 infected patients with severe inflammation. The patients tested positive for SARS-CoV-2 RNA through Real-Time PCR analysis of the nasopharyngeal sample obtained at the onset of hospitalization and prior to any therapeutic interventions. Serum levels of CCL2 and CCL3 were evaluated in 69 Iranian SARS-CoV-2 infected patients with severe inflammation and 65 healthy subjects using ELISA technique. The results showed that serum levels of CCL2 and CCL3 were significantly decreased and increased, respectively in the Iranian SARS-CoV-2 infected patients with severe inflammation when compared to healthy controls. Based on the results, it may be concluded that CCL3, but not CCL2, significantly participates in the induction of severe inflammation during severe coronavirus disease 2019 (COVID-19).

1. Introduction

SARS-CoV-2 is a newly identified coronavirus, previously unknown to humans. Consequently, all individuals are vulnerable to infection as the virus swiftly disseminates during the ongoing COVID-19 pandemic (Guo et al., 2020). The clinical manifestations of SARS-CoV-2 infection vary widely, encompassing asymptomatic cases, mild upper respiratory tract symptoms, as well as moderate to severe illness characterized by respiratory complications and multi-organ dysfunction necessitating intensive medical intervention and organ support (Carsetti et al., 2020). Immune cells, including the cells of innate and adaptive immune system, play key roles in the induction of severe inflammation in the SARS-CoV-2 infected patients via direct interactions in the infected tissues (García, 2020; Nahavandi-Parizi et al., 2022). The excess directly immune responses to the virus can be associated with increased chances of morbidity and mortality (Buicu et al., 2021). The roles played by chemokines in activation and recruitment of the immune cells to the SARS-CoV-2 infected tissues have been demonstrated

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previously (Hsu et al., 2022). Chemokines are the small molecules with conserved cysteine, a non-essential amino acid, at the carboxyl terminal end (Gilchrist, 2020; Mohammadi and Kariminik, 2021). Chemokines use several receptors to induce the intracellular pathways in the immune cells (Ulvmar et al., 2011). Although some of chemokines use common receptors on an immune cell, they induce different outcomes in some cases (Ulvmar et al., 2011). Therefore, they may play various roles during viral infections. Monocyte chemoattractant protein 1 (MCP1), which is known as CC ligand 2 (CCL2), is a main factor for recruitment of monocytes, memory T cells, and dendritic cells to the inflammation sites (Deshmane et al., 2009; Wang et al., 2008). However, it is unable to recruit neutrophils, as the main cells participate in induction of inflammation (Deshmane et al., 2009). Additionally, macrophage inflammatory protein 1-alpha (MIP-1-α) that is known as CCL3, is a main recruitment factor for polymorphonuclear leukocytes (De Buck et al., 2018). However, CCL2 and CCL3 can play some redundancy roles via interaction with a common receptor, CC receptor 4 (CCR4), on the immune cells (De Buck et al., 2018; Deshmane et al., 2009). Additionally, CCL2 and CCL3 chemokines are crucial in the development of COVID-19, primarily by orchestrating inflammatory responses and attracting immune cells to infection sites (Majumdar and Murphy, 2020). Increased concentrations of CCL2 (also known as monocyte chemoattractant protein-1) and CCL3 (macrophage inflammatory protein-1α) have been linked to severe cases of the disease, as they exacerbate the hyper-inflammatory conditions seen in critically ill patients, contributing to cytokine storms and worsening lung damage (Santos et al., 2024). Additionally, these chemokines promote the migration of monocytes and macrophages into lung tissues, which can intensify inflammation and lead to acute respiratory distress syndrome (ARDS) in those affected by COVID-19 (Lamers and Haagmans, 2022). Due to the fact that the main roles played by chemokines in the pathogenesis of severe COVID-19 needs more investigations, this project was aimed to evaluate the serum levels of CCL2 and CCL3 in the Iranian SARS-CoV-2 infected patients with severe inflammation. Additionally, due to the roles of age and sex on the immune responses (Moulton, 2018; Müller et al., 2019), the serum levels of the chemokines were also compared in male and female patients and in various ages.

2. Materials and Methods

In this cross-sectional study, 65 healthy non-SARS-CoV-2 infected Iranian men and women, as controls, and 69 hospitalized SARS-CoV-2 infected patients were explored regarding the serum levels of CCL2 and CCL3. Sample size was calculated using our previous investigations on SARS-CoV-2 infected patients (Asadpour-Behzadi et al., 2023). Table 1 illustrates the similarities of sex and age distribution in the patients and healthy controls. The patients were SARS-CoV-2 infected patients and suffered from severe COVID-19 and were hospitalized in Afzalipour Hospital, Kerman, Iran. The patients underwent Real-Time PCR testing to verify their SARS-CoV-2 infection, and a specialist in infectious diseases conducted a medical examination to assess their symptoms. The healthy control group exhibited no symptoms of bacterial or viral respiratory illnesses, including COVID-19, and all tested negative for SARS-CoV-2 through Real-Time PCR analysis. Along with the previously mentioned criteria, being of Iranian ethnicity was also included as an additional criterion for inclusion. The patients and healthy controls had no history of cardiovascular diseases, cancers, respiratory illnesses in the past six months, autoimmune disorders, or the use of immunosuppressive medications. Neither the patients nor the control subjects had used anti-inflammatory medications in the three months prior to the study. Accordingly, the patients were positive for SARS-CoV-2 RNA using a Real-Time PCR test on a nasopharyngeal and oropharyngeal specimen, which were taken at the at the beginning of hospitalization and before any treatment. The severities of the disease were confirmed by an expert physician in infection and paraclinical data, such as Sonography and chest ray. To evaluate the serum levels of CCL2 and CCL3, the blood samples were taken from both severe SARS-CoV-2 infected patients and healthy controls. The Ethical Committee of the Islamic Azad University, Kerman Branch, evaluated and approved the project protocol (Ethical code: IR.IAU.Kerman.REC.1400.028).

Before blood sampling, the participants filled out a written informed consent.

2.1. SARS-CoV-2 detection

To investigate SARS-CoV-2 infection, three swabs were taken from the nasopharyngeal and oropharyngeal regions of the participants and preserved in viral transport media. After transferring to the virus laboratory, SARS-CoV-2 RNA was extracted using CinnaPure RNA kit (Cinnaclone, Iran). The extracted RNA was used to be converted to cDNA and tested by kit (KPG, Iran). The kit detects either N or RDRP genes of SARS-CoV-2. Additionally, RNase P was detected, as the internal control, in this kit.

2.2. Evaluation of CCL2 and CCL3

Whole blood samples were collected in the tubes without anti-coagulant and after 1 hour, the serum was collected by centrifuging in 5000 RPM for 5 minutes. ELISA kits (KPG, Iran), were used for evaluation of CCL2 and CCL3. Briefly, 50 µL serum and standards were added to the wells of the ELISA plate and incubated for 1 hour. After washing for three times, Biotin-conjugated antibodies were added and after 1 hour incubation, were washed using washing buffer. 50 µL HRP-Avidin was added and after 30 minutes incubation and washing, 50 µL substrate was added and incubated for 15 minutes. The reactions were stopped using stopping buffer and the yellow stain was measured at 450 nm using BMB ELISA reader (China).

2.3. Statistical analysis

Kolmogorov Smirnov test, under SPSS software version 16, was applied to examine the normality of data distribution. Due to the normal distribution of the data, the differences between the groups and also between male and female participants in each group were analyzed using student t test. The data are presented as Mean ± standard error (SE). To analyze the correlations, Pearson correlation test was used.

Table 1. Distribution of age and sex in the patients with severe SARS-CoV-2 infection and healthy controls

	Sex		Age (Years)
	Male	**Female**	
Severe SARS-CoV-2 infected patients	19	50	40.74 ± 2.07
Healthy controls	27	38	38.70 ± 1.02
P value	0.402		0.099

The analysis showed that the groups were similar regarding the ethnic variables.

3. Results

3.1. SARS-CoV-2 detection

The examination of patients and control subjects utilizing a Real-Time PCR kit revealed that every patient tested positive for SARS-CoV-2, while all control subjects were found to be negative for the virus.

3.2. Serum levels of CCL2 and CCL3 in the patients and controls

The analysis using the student t-test revealed significant differences in serum levels of CCL2 and CCL3 among severe SARS-CoV-2 infected patients compared to healthy controls. Specifically, CCL2 levels were significantly lower in patients (25.79 ± 0.82 pg/mL) than in healthy individuals (42.78 ± 7.38 pg/mL), with a p-value of 0.037. Conversely, CCL3 levels were higher in the infected group (23.11 ± 1.43 pg/mL) compared to the controls (15.53 ± 0.98 pg/mL), with a p-value of less than 0.001 (Figure 1).

3.3. Sex has no effects on the serum levels of CCL2 and CCL3

The serum levels of CCL2 and CCL3 were analyzed separately for males and females within each group. As shown in Figure 2, there were no significant differences in serum CCL2 levels between males and females among either the severe SARS-CoV-2 infected patients (P = 0.058) or the control group (P = 0.414). Similarly, CCL3 serum levels did not differ between genders in both the severe SARS-CoV-2 infected patients (P = 0.796) and the controls (P = 0.265).

Table 2 presents the correlations between CCL2 and CCL3 levels and age, analyzed using the Pearson correlation coefficient for both severely infected SARS-CoV-2 patients and control subjects. The analysis indicated that in healthy controls, CCL3 exhibited a significant negative correlation with age (rs: -0.665, P = 0.036). In contrast, among patients with severe SARS-CoV-2 infection, CCL3 showed a positive correlation with CCL2 (rs: 0.496, P < 0.001).

3.4. Correlation CCL2, CCL3 and age

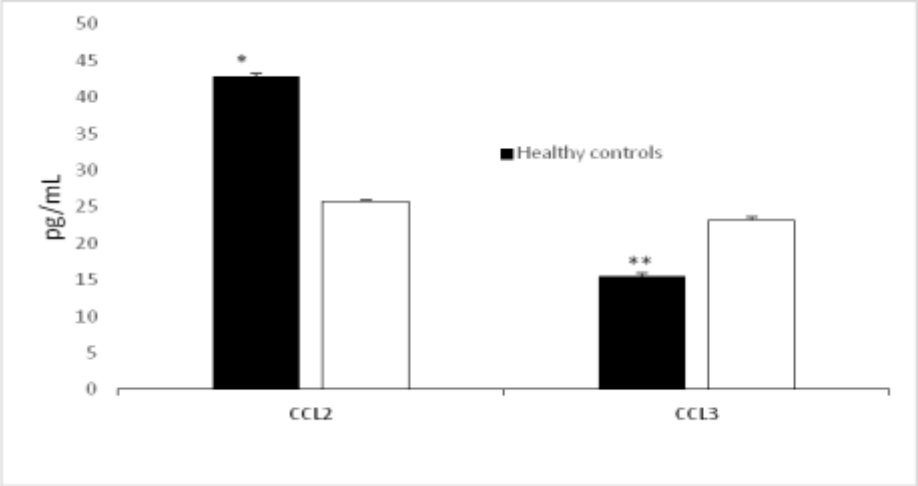

Figure 1. Serum levels of CCL2 and CCL3 in the healthy controls and the hospitalized COVID-19 infected patients. Serum levels of CCL2 (*P= 0.037) and CCL3 (**P< 0.001) significantly down and up-regulated, respectively, in the hospitalized COVID-19 infected patients.

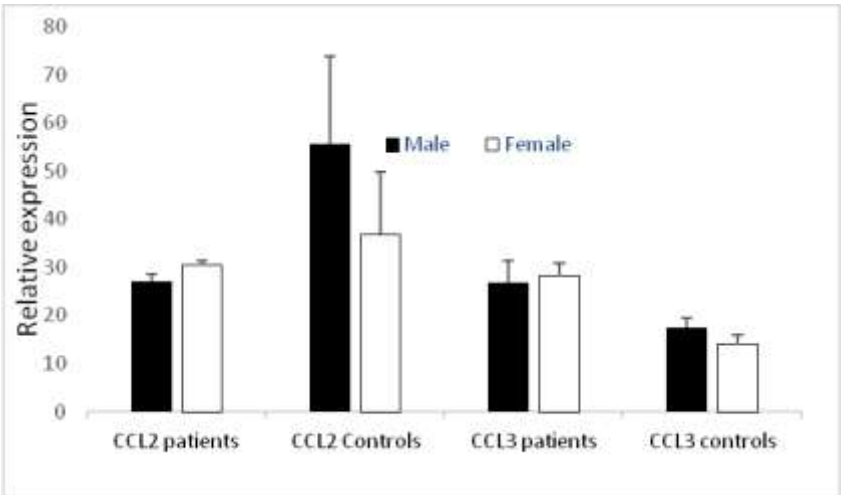

Figure 2. Serum levels of CCL2 and CCL3 in the male and female in both healthy controls and the severe COVID-19 infected patients. Serum levels of CCL2 and CCL3 were not changed significantly in the male versus female patients and controls.

Table 2. Correlation among CCL2, CCL3, and age among the participants

			CCL2	CCL3	Age
Severe SARS-CoV-2 infected patients	CCL2	Correlation Coefficient	1	**0.496**	-0.106
		P value	-	**<0.001**	0.522
	CCL3	Correlation Coefficient	**0.496**	1	0.198
		P value	**<0.001**	-	0.227
Healthy controls	CCL2	Correlation Coefficient	1	0.073	0.511
		P value	-	0.796	0.131
	CCL3	Correlation Coefficient	0.073	1	**-0.665**
		P value	0.796	-	**0.036**

The Pearson test revealed that CCL3 had a significant negative correlation with age in the healthy controls and positive correlation with CCL2 in the severe SARS-CoV-2 infected patients.

4. Discussion

Cytokine storm, also known as cytokine release syndrome, occurs when activated immune cells or other body cells produce an overwhelming number of inflammatory cytokines (Jarczak and Nierhaus, 2022). This immune system dysfunction can result in widespread inflammatory responses and may lead to serious complications, including multiorgan failure (Qudus et al., 2023). Immune cells are highly responsive to cytokines and can transform into inflammatory immune cells in the bloodstream during a cytokine storm. These cells persistently migrate into inflamed tissues, contributing to organ damage (Hu et al., 2021). Our results demonstrated that CCL2 serum levels were significantly decreased in the severe SARS-CoV-2 infected patients. As mentioned in the introduction CCL2 can regulate migration of memory T-cells, macrophages and dendritic cells to the inflammation sites. However, it is unable to affect migration of neutrophils, a main cell participates in the inflammatory responses during COVID-19 (Deshmane et al., 2009). Additionally, the results showed that CCL3 serum levels were significantly higher in the patients when compared to healthy controls. In contrast to CCL2, CCL3 can recruit neutrophils to the inflammation sites. Therefore, it may be hypothesized that CCL3 is a main factor to induce inflammation in the patients suffering from severe COVID-19, maybe via infiltration of neutrophils. However, CCL3 can increase the recruitment of other immune cells, including macrophages (Gibaldi et al., 2020), dendritic cells (Kang et al., 2021), T cells (Kang et al., 2021). Therefore, it seems that CCL3, but not CCL2, can be considered as an important factor that participates in the induction of severe inflammation in the SARS-CoV-2 infected patients. The differing roles of CCL2 and CCL3 illustrate a nuanced interaction within the immune response during severe SARS-CoV-2 infections. Reduced levels of CCL2 may impair the effective recruitment and functioning of immune cells, while increased levels of CCL3 might lead to heightened inflammation through the activation of neutrophils. Grasping these interactions is vital for creating targeted treatments that can adjust the effects of these chemokines, potentially enhancing patient outcomes in severe cases of COVID-19. More research is required to elucidate these mechanisms and their relevance for therapeutic strategies. In parallel with our results, several investigations proved the roles played by CCL3 in the induction of inflammation in the severe SARS-CoV-2 infected patients. For instance, Chi et al., reported that serum levels of CCL3 were significantly higher in the patients suffering from severe COVID-19 when compared to the patients with mild symptoms (Chi et al., 2020). Xiong and colleagues revealed that mRNA levels of CCL3 in the bronchoalveolar lavage fluid (BALF) and peripheral blood mononuclear cells (PBMC) of the severe SARS-CoV-2 infected patients were significantly increased when compared to healthy controls (Xiong et al., 2020). Therefore, it seems that CCL3 is a main molecule that participates in the induction of inflammation-

related severe symptoms in the Iranian SARS-CoV-2 infected patients.In contrast to our results, some investigations revealed that CCL2 serum levels increased in the severe SARS-CoV-2 infected patients (Chi et al., 2020; Kwon et al., 2020; Sierra et al., 2020; Xiong et al., 2020; Zhang et al., 2020; Zhou et al., 2020). However, declined serum levels of CCL2 were significant in our evaluated patients. Due to the fact that the investigations were performed on the different ethnic population and also different vaccinations are performed in the countries, hence, it may be hypothesized that Iranian patients with severe symptoms may suffer from increased expression of CCL3 rather than CCL2. Due to the results, it appears that immunotherapy against hypercytokinemia, which is prevalent among the severe SARS-CoV-2 infected patients, needs considering the ethnic and the kind of vaccination project. Collectively, our study offers a distinct viewpoint on the involvement of CCL2 and CCL3 in COVID-19. We observed lower levels of CCL2, which contrasts with numerous studies that indicate higher levels. Conversely, our results showing elevated CCL3 levels align with existing research. The discrepancies noted may stem from factors such as variations in population demographics and the impact of vaccination, highlighting the intricate nature of immune responses in COVID-19 across diverse populations. The results also demonstrated that sex did not affect CCL2 and CCL3 serum levels among the Iranian severe SARS-CoV-2 infected patients and also healthy controls. Previous investigation on the Iranian population regarding the immune system-related molecules, including chemokine and cytokines, confirmed our results and were not associated with different expression of the molecule between male and female (Abbasifard et al., 2022; Bagheri-Hosseinabadi et al., 2021; Bahramabadi et al., 2017). Therefore, it appears that sex cannot affect immune responses to the various inflammatory conditions in the Iranian population. One significant drawback of the research examining the roles of CCL2 and CCL3 in patients with severe inflammation due to SARS-CoV-2 is its limited sample size. While the study includes 69 patients suffering from severe inflammation and 65 healthy controls, this number may not be sufficient to apply the findings broadly across various populations or to identify subtle differences in immune responses among diverse demographic groups. Another limitation stems from the study's cross-sectional design, which collects data at a single time point. This approach limits the ability to monitor changes in chemokine levels and immune responses throughout the course of infection or recovery. Furthermore, the study's concentration on specific chemokines (CCL2 and CCL3) might neglect other important inflammatory mediators that could also significantly impact the pathophysiology of severe COVID-19. A more thorough analysis that includes a wider array of cytokines and chemokines could offer a more comprehensive view of the inflammatory environment associated with SARS-CoV-2 infection.

Conclusions

The result also demonstrated that CCL3 had a negative correlation with age in the healthy controls, but not in the Iranian severe SARS-CoV-2 infected patients. Due to the results, it seems that CCL3 is down-regulated in the older cases in the normal conditions. However, infection with SARS-CoV-2 affects the relation and led to inhibition of negative roles played by age on the serum levels of CCL3, which results in induction of inflammation. However, more investigations need to confirm the roles played by aging on the expression of CCL3 in normal and pathological conditions. Although the project is associated with valuable information, low sample size is the main limitation. Additionally, due to the network functions of the chemokines, more chemokines need to be explored simultaneously.

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Ethical Considerations

The Ethical Committee of the Islamic Azad University, Kerman Branch, evaluated and approved the project protocol (Ethical code: IR.IAU.Kerman.REC.1400.028).

Authors' Contribution

AA Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **AK** Formal analysis, Investigation, Writing – review and editing. **BK** Data curation, Methodology.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence.

Refereces

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