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Characterizing the immune responses of those who survived or succumbed to COVID-19: Can immunological signatures predict outcome?

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ABSTRACT

Background: Immunodeficiency has pivotal role in the pathogenesis of coronavirus disease 2019 (COVID-19). Several studies have indicated defects in the immune system of COVID-19 patients at different disease stages. Therefore, this study investigated whether alters in immune responses of COVID-19 patients may be considered as predicting factors for disease outcome.

Methods: The percentages of innate and adoptive immune cells in the recovered and dead patients with COVID-19, and healthy subjects were determined by flow cytometry. The levels of pro- and anti-inflammatory cytokines and other immune factors were also measured by enzyme-linked immunosorbent assay.

Results: At the first day of hospitalization, the frequencies of $CD56^{dim}$ $CD16^{+}$ NK cells and $CD56^{bright}$ $CD16^{dim/-}$ NK cells in patients who died during treatment were significantly increased compared to recovered and healthy individuals ($P < 0.0001$). The recovered and dead patients had a significant increase in monocyte number in comparison with healthy subjects ($P < 0.05$). No significant change was observed in Th1 cell numbers between the recovered and dead patients while Th2, Th17 cell, and Treg percentages in death cases were significantly lower than healthy control and those recovered, unlike exhausted CD4 + and CD8 + T cells and activated CD4 + T cells ($P < 0.0001$–0.05). The activated CD8 + T cell was significantly higher in the recovered patients than healthy individuals ($P < 0.0001$–0.05). IL-1α, IL-1β, IL-6, and TNF-α levels in patients were significantly increased ($P < 0.0001$–0.01). However, there were no differences in TNF-α and IL-1β levels between dead and recovered patients. Unlike TGF-β1 level, IL-10 was significantly increased in recovered patients ($P < 0.05$). Lymphocyte numbers in recovered patients were significantly increased compared to dead patients, unlike ESR value ($P < 0.001$–0.01). CRP value in recovered patients significantly differed from dead patients ($P < 0.001$).

Conclusion: Changes in frequencies of some immune cells and levels of some immune factors may be considered as predictors of mortality in COVID-19 patients.

1. Introduction

In December 2019, Wuhan city, China, experienced the prevalence of novel viral pneumonia with unknown reasons. This disease was then recognized as a zoonotic disorder like Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS) coronaviruses and called COVID-19 which is caused by a severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [1–4]. Coronavirus is one of the enveloped RNA viruses with a diameter of 60–140 nm which are crone-shape with having spike-shape projections [5]. Coronavirus

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can be categorized into the kinds of alpha, beta, delta, and gamma. This virus has the ability to infect various species and leads to serious disorders such as septic shock and acute respiratory distress syndrome (ARDS) [5,6]. COVID-19 can appear with fever, dry cough, fatigue, myalgia, sore throat, conjunctivitis, headache, smell and taste impairments [7,8]. It also can spread in many ways, including mouth mucus membranes, feces, aerosols, and droplets [6]. Old individuals with critical conditions such as cerebrovascular disorder, cardiovascular disorder, hypertension, and diabetes are more vulnerable to COVID-19 compared to other populations [9]. Although, there is no particular antiviral treatment or certain vaccine for the disease, comprehending the pathogenesis of COVID-19 can be helpful in the management of this viral condition [10]. The pathogenesis of this viral agent is not fully explained yet, but there is evidence that a failure in the immune system is present in these patients such as inflammatory cytokine storm and lymphocytopenia, and probably can be lead to death cases because of SARS-COV2 [11–13]. Viral agents can activate immune reactions via the host immune system. Several respiratory viruses inhibit innate immune reactions which create a chance for effective viral replication and infection enhancement. As a result of an interaction between the cells and COVID-19, affected cells elevate the releasing a great number of cytokines and chemokines, like IFN-I [14,15]. But the exact role of IFN-I in destructing or protecting immune system reactions in coronavirus infections is a challenge [16]. It is reported that in the great numbers of severe cases, there were the increased productions of some immune factors such as TNFα, IL-2R, IL-6, and IL-10 [14]. Moreover, it is documented that the increased levels of C-reactive protein (CRP), IL-1α, IL-1β, IL-6, and TNF-α can participate in ARDS in these patients [11,17]. Previous studies have revealed that peripheral level of the natural killer cell (NK cell) was significantly decreased in the severe stage of the disease compared to the mild disease stage, but it is unknown whether this reduction was due to cell death and/or cell accumulation in the infected tissues [18,19]. In a study on patients in severe and nonsevere stages of COVID-19, it was revealed that severe COVID-19 patients had statistically significant reductions in the percentages of basophils (0.1 versus 0.2%), eosinophils (0.0 versus 0.2%), and monocytes (6.6 versus 8.4%) in comparison with nonsevere stages [20].

When a virus arrives at the cell, its antigens can be subjected to the antigen presentation cells (APC). The presentation of antigen induces adaptive immune system through involving T and B cells [21]. In infectious conditions, virus-specific CD8 + and CD4 + T cells are activated. In addition, T regulatory cells (Tregs) are able to decrease immune reactions and thereby reduce tissue injuries [22–26]. In this line, the reduced numbers of CD4 + and CD8 + T cells, and Tregs contribute to the exacerbation of tissue injuries [20]. The CD4 + T helper (Th) cells have a remark role in the function of coronaviruses through the interaction with Tregs [27]. Previous studies on MERS-CoV patients, a disease with similar pathogenesis to COVID-19, have shown that the number of Th1 was associated with the good prognosis of the disease, but Th2 cytokines were related to the high rate of death [17,28]. Furthermore, Th17, as another subset of CD4 + T helper cells, can participate in the cytokine storm in ARS-CoV-2 and probably enhance pulmonary edema [29]. Despite known roles of Th cells, the functional roles and numbers of other T cells in COVID-19 are not understood so far [30]. There is not enough information about the immune reactions in COVID-19 patients [20]. Our previous study indicated that changes in the immune system of patients with COVID-19 during a recovery period have critical role in determining disease severity [31]. This study was therefore focused on clarifying whether changes in immune cell numbers and immune factor values may be considered as a predicting factor to determine the outcome of COVID-19. Hence, the frequencies of innate immune cells, for instance, $CD56^{bright}CD16^{dim/-}$ NK cells and $CD56^{dim}CD16^{+}$ NK cells, and monocytes, and adaptive immune cells, such as B cells, Tregs, Th1 cells, Th2 cells, Th17 cells, activated CD4 + and CD8 + T cells, exhausted CD4 + and CD4 + T cells at the first day of hospitalization in recovered patients and death cases due to COVID-19 up to ten days were compared to healthy subjects. Moreover, the plasma levels of pro- and anti-inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-10, TNF-α, and TGF-β1 and other immune factors were measured.

2. Materials and methods

2.1. Study subjects

The study was performed in Khorshid hospital, Isfahan, Iran from March 18 to May 24, 2020. The diagnosis of COVID-19 was approved by the specialist according to clinical and laboratory criteria including: 1) clinical evaluation; 2) chest CT scan imaging; 3) real time-polymerase chain reaction (RT-PCR) assay. Patients were negative for health problems, autoimmunity, infectious diseases, and malignancy. Pulmonary involvement was observed in the results of chest CT scan imaging of all patients as previously described [32]. Nasopharyngeal swab samples were collected from all participants and COVID-19 were confirmed at the time of admission by RT-PCR assay. The patients did not receive any immunosuppressive agents before entering the study. All patients had clinical signs and symptoms of COVID-19 at least 3–5 days prior to refer to Khorshid hospital to initiate disease treatments. The patients had age range from 30 to 92 years, while it was from 58 to 81 years in healthy individuals. Healthy subjects were recruited among those referred to a health screening center. The voluntaries had no signs and symptoms of respiratory infections, autoimmune and immunodeficiency syndromes according to laboratory and clinical evaluations. They had negative results of RT-PCR assay. The informed consent was obtained from participants and all experimental protocols were approved by the Ethics Committee of Isfahan University of Medical Sciences (IR.MUI.MED.REC.1399.750).

2.2. Sample collection and cell counting

Heparinized blood samples (10 ml) were obtained from patient and control groups. The blood sampling from patients was performed at first day of hospitalization. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Paque centrifugation according to the manufacturer's instructions (Lymphodex, Germany). The isolated cells were washed twice with phosphate buffered saline (PBS) at 300*g* for 10 min. The viability of the cell was determined by trypan blue dye exclusion.

Lymphocyte numbers in peripheral blood of COVID-19 and healthy subjects were determined using an automated cell counter system UF-100® (Sysmex, Kobe, Japan) within two hours after collecting heparinized blood samples.

2.3. Flow cytometry

To assess the percentages of innate and adoptive immune cells of patient (at first day of hospitalization) and healthy groups, PBMCs were stained with different monoclonal antibodies or matched to isotype control IgG for 30 min at 4 °C. Isotype-matched control antibodies served as negative controls. The staining of some intracellular molecules with different antibodies was carried out after the fixation and permebilization of the cells according to the manufacturer's guideline (eBiosciences, USA). The stained cells were washed several times with PBS and then centrifuged at 300*g* for 10 min at room temperature. The

Table 1
Primary and isotype control antibodies used for comparing the situation of the immune cells of COVID-19 patients who recovered and died by Flow cytometry.

Fluorochrome/Antibody	Isotype	Company (All from USA)
CD3-FITC antibody	Mouse IgG2a, κ	BioLegend
CD4-PE/CY5 antibody	Rat IgG2a, κ	BioLegend
CD4-FITC antibody	Rat IgG2b, κ	BioLegend
CD14-FITC antibody	Mouse IgG1, κ	BioLegend
CD16-PE antibody	Mouse IgG1, κ	BioLegend
CD19- PE/CY5 antibody	Mouse IgG1, κ	BioLegend
CD56-PE/CY5 antibody	Mouse IgG1, κ	BioLegend
CD8-PE/CY5 antibody	Mouse IgG1, κ	BioLegend
CD25-FITC antibody	Mouse IgG1, κ	BioLegend
CD69-PE antibody	Mouse IgG1, κ	BioLegend
PD1-PE antibody	Mouse IgG2b, κ	BioLegend
CD127-FITC antibody	Mouse IgG1, κ	BioLegend
CD22-PE antibody	Mouse IgG1, κ	BioLegend
IFNg-PE antibody	Hamster IgG	BioLegend
GATA3-PE antibody	Mouse IgG2b, κ	BioLegend
IL4- PerCP/Cyanine5.5 antibody	Rat IgG1, κ	BioLegend
Tbet-FITC antibody	Mouse IgG1, κ	BioLegend
IL17-PE antibody	Mouse IgG1, κ	BioLegend
Foxp3-PE antibody	Mouse IgG1, κ	BioLegend
RORγt-PE antibody	Mouse IgG1, κ	BioLegend

percentages of the stained cells were assessed by a FACSCalibur system (Becton Dickinson, San Jose, CA). Firstly, lymphocyte population was isolated from debris or dead cells using forward and side scatter. The gating strategy was done using FlowJo software (v10.1, FlowJo, Ashland, OR, USA). Afterwards, the gated lymphocytes were analyzed to determine the frequencies of the CD3 + cells and CD4 + cells which were used to measure the percentages of CD4 + T-bet + IFN-γ + Th1, CD4 + IL-4 + GATA3 + Th2, CD4 + IL-17α + RORγt + Th17 cells, CD4 + $CD127^{low}$ FoxP3 + Tregs, CD4 + CD25 + CD69 + activated T cells, CD3- CD19 + CD22 + B cells, CD3 + CD4 + PD-1 + exhausted T cells, CD3 + CD8 + PD-1 + exhausted T cells, CD3- $CD56^{dim}$ $CD16^{+}$ NK cells, and CD3- $CD56^{bright}$ $CD16^{dim/-}$ NK cells. In this study, the CD8 + activated T cells and monocytes were, respectively, considered as CD8 + CD25 + CD69 + cells and CD14 + CD16 + CD11b + cells. The monoclonal and their isotype control antibodies used in the study are indicated in Table. 1.

2.4. Assessment of pro- and anti-inflammatory cytokines and other immune agents

To determine cytokine profiles of alive and death cases due to COVID-19 up to ten days of hospitalization, the plasma samples were isolated from whole blood of patients (at the first day of hospitalization) and healthy subjects. The levels of IL-1α, IL-1β, TNF-α, IL-6, TGF-β1, and IL-10 cytokines were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mabtech, Sweden) according the manufacturer's instructions. Turbidimetric immunoassay CRP of COVID-19 patients was performed by the Mindray BS-800 automated biochemistry analyzer (Shenzhen Mindray Bio-Medical Electronics, China). The level of erythrocyte sediment rate (ESR) of blood samples was measured using the erythrocyte sedimentation rate (ESR) analyzer (Parsian Teb, Iran).

3. Statistical analysis

The results were analyzed by GraphPad Prism 6 (GraphPad software, San Diego, CA) and are represented as the mean ± standard error of the mean (SEM). Comparisons of the groups with normal distributions were performed using One-way analysis of variance (ANOVA) and unpaired t-tests, while those with non-normal distributions were compared by Kruskal–Wallis and Mann–Whitney tests. Pearson's and Spearman's tests were used to determine the correlation coefficients of the results with normal and non-normal distributions, respectively. p value < 0.05 was considered statistically significant.

4. Results

4.1. Subject descriptions

A total of 71 subjects with COVID-19, who recovered (60 cases) or died (11 cases) up to ten days of hospitalization, and 50 healthy individuals were participated in the study (Table. 2). The mean age of subjects and number of lymphocytes were significantly higher in recovered patients than those who died during the disease recovery, unlike ESR value ($P < 0.001$–0.01, Table. 2). The frequencies of background diseases and the value of CRP in recovered patients significantly differed from dead patients ($P < 0.001$, Table. 2). Table. 2 shows the demographic and other information of healthy individuals and COVID-19 patients.

4.2. Investigations of innate immune cells of COVID-19 death cases with recovered patients

At the first day of hospitalization, the frequencies of $CD56^{dim}$ $CD16^{+}$ NK cells and $CD56^{bright}$ $CD16^{dim/-}$ NK cells in death cases up to ten days of hospitalization were significantly increased compared to alive patients and healthy individuals (Fig. 1A, C, and D, $P < 0.0001$). Furthermore, the numbers of these cells were significantly higher in alive patients than control group (Fig. 1A, C, and D, $P < 0.0001$). Our results indicated that death cases had a significant increase in the number of monocyte compared to healthy subjects (Fig. 1B and E, $P < 0.05$). The same trend was also observed in alive patients (Fig. 1B and E, $P < 0.05$).

4.3. Assessments of adaptive immune cells of COVID-19 death cases with recovered patients

To compare the situations of adaptive immunity in the recovered patients and those who died up to ten days of hospitalization, adoptive immune cell frequencies of patients were assessed at the first day of hospitalization. In the early recovery stage, no significant change was observed in Th1 cell numbers between the recovered patients and death cases due to COVID-19 up to ten days (Fig. 2A and I), while the percentages of Th2, Th17 cells, and Tregs in death cases were significantly lower than healthy control and those recovered (Fig. 2B-D and J-L, $P < 0.0001$–0.05). Interestingly, the numbers of exhausted CD4 + T cells, exhausted CD8 + T cells, and activated CD4 + T cells in death cases were significantly increased compared to those recovered and healthy subjects, however the percentage of activated CD8 + T cell was significantly higher in the recovered patients than healthy individuals (Fig. 2E-H and M-P, $P < 0.0001$–0.05).

4.4. The comparisons of cytokine profiles of COVID-19 death cases with recovered patients

Regarding the fact that cytokine storming plays an indispensable role in pathogenesis of COVID-19, cytokine profiles were studied in COVID-19 patients who recovered and dead during hospitalization. As shown in Fig. 3A-D, statistically significant increases in the levels of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, and TNF-α) in patients were observed compared to healthy individuals ($P < 0.0001$–0.01). However, these significant increases in TNF-α and IL-1β levels were not found in the cases who died up to ten days of hospitalization in

Table 2
The demographic and clinical characteristics of COVID-19 and healthy subjects.

	Patients (n: 71)		Control [c] (n: 50)	P value
	Recovered patients [a] 60 (84.5%)	Death [b] 11 (15.5%)		
Sex	Male: 23 (55%) Female: 27 (45%)	Male: 6 (54.54%) Female: 5 (45.46%)	Male: 26 (52%) Female: 24 (48%)	a versus (vs) b: 0.61 a vs c: 0.45 b vs c: 0.57
Age year (range of age)	65 ± 14.82 (30–92)	81.9 ± 6.72(68–91)	64.48 ± 4.95(58–81)	a vs b: <0.001 a vs c: 0.81 b vs c: <0.001
*RT-PCR	Positive: 60 (100%)	Positive: 11 (100%)	Negative: 50 (100%)	
GGO	Yes: 44 (73.33%)	Yes: 7 (63.63%)	–	a vs b: 0.37
Hemoglobin	11.33 ± 2.26	10.64 ± 1.62	14.5 ± 0.86	a vs b: 0.33 a vs c: <0.001 b vs c: <0.001
Lymphocyte count	842.73 ± 139.62	671.81 ± 136.34	3363 ± 979.65	a vs b: <0.001 a vs c: <0.001 b vs c: <0.001
CRP	Positive: 58 (96.08%)+1: 14 (23.34%)+2: 22 (36.65%)+3: 19 (31.66%)+4: 3 (5%)	Positive: 11 (100%)+1: 1 (9.09%)+2: 1 (9.09%)+3: 1 (9.09%)+4: 8 (72.72%)	Negative: 50 (100%)	a vs b: <0.001
ESR	35.75 ± 23.24	54.90 ± 20.7	7.92 ± 4.09	a vs b: <0.01 a vs c: <0.001 b vs c: <0.001
Background diseases	27 (45.1%)Diabetes: 7 (25.92%)Kidney disease (ESRD & CKD): 9 (33.33%)Lung disease (COPD, asthma): 6 (22.22%)Hypertension: 19 (70.37%)Hypothyroidism: 1 (3.7%)Colon cancer: 1 (3.7%)IHD: 6 (22.22%)CVA: 1 (3.7%)RA: 1 (3.7%)	8 (72.73%)Diabetes: 1 (9.09%)Kidney disease (ESRD & CKD): 3 (27.27%)Lung disease (COPD, asthma): 5 (45.46%)Hypertension: 7 (63.63%)Hypothyroidism: 0 (0.0%)Colon cancer: 0 (0.0%)IHD: 0 (0.0%)CVA: 0 (0.0%)RA: 0 (0.0%)	0 (0.0%)	a vs b: <0.001
Anorexia	15 (25%)	Yes: 3 (27.27%)	0 (0.0%)	a vs b: 0.56
Fever	31 (51.66%)	8 (72.71%)	0 (0.0%)	a vs b: 0.16
Temperature	37.54 ± 0.99	38.27 ± 0.84	37.01 ± 0.1	a vs b: 0.02
Headache	41 (68.33%)	7 (63.63%)	0 (0.0%)	a vs b: 0.5
Dyspnea	40 (66.66%)	8 (72.71%)	0 (0.0%)	a vs b: 0.49
Cough	43 (71.66%)	10 (81.82%)	0 (0.0%)	a vs b: 0.38
Sore throat	41 (68.33%)	6 (90.9%)	0 (0.0%)	a vs b: 0.11
Diarrhea	17 (28.34%)	3 (27.27%)	0 (0.0%)	a vs b: 0.62
Vomiting	16 (26.66%)	3 (27.27%)	0 (0.0%)	a vs b: 0.61
Smoking history	17 (28.34%)	5 (45.46%)	15 (30)	a vs b: 0.21 a vs c: 0.5 b vs c: 0.25
O2 saturation	89.75 ± 7.19	88.9 ± 3.2	98 ± 1.2	a vs b: 0.01
Window period	7.9 ± 6.37	8.18 ± 8.43	–	a vs b: 0.89
Treatment	26 (43.34%)Anti-viral: 21 (80.76%)Anti-inflammatory: 8 (30.76%)Antibiotic: 25 (96.15%)	7 (63.64%)Anti-viral: 5 (45.45%)Anti-inflammatory: 1 (9.09%)Antibiotic: 7 (100%)	–	

[*] RT-PCR: Real time-polymerase chain reaction, GGO: Ground-glass opacity, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, ESRD: End stage renal disease, CKD: Chronic kidney disease, COPD: Chronic obstructive pulmonary disease, IHD: ischemic heart disease, CVA: Cerebrovascular accident, RA: Rheumatoid arthritis.

comparison with those recovered. The level of IL-10 was significantly higher in the recovered patients than healthy group, unlike death cases (Figure. E, $P < 0.05$). However, there is no significant change in TGF-β1 level among the recovered patients, death cases, and healthy subjects (Figure. F).

5. Discussion

COVID-19 originated from SARS-COV2, is a prominent public health problem around the world [33]. The pathogenesis of this disease is not exactly declared yet, but according to the recent documents, a defect in the function and/or modulation of the immune system can be involved in the pathogenic mechanisms of COVID-19 [34,35]. Hereby, the present study was accomplished for investigating some innate and adaptive immune cells and immune agents of COVID-19 patients in the course of ten days after hospitalization, which is approximately a period that the disease is worsen and may lead to death or recovery from COVID-19 [12,36]. Having considered that the innate immune system is related to the early reactions versus viral infections, some agents of innate immunity were investigated. At the first day of hospitalization, we observed significant increases in the frequencies of $CD56^{dim}$ $CD16^{+}$ NK cells and $CD56^{bright}$ $CD16^{dim/-}$ NK cells in patients who died up to ten days than the recovered patients and healthy subjects. Unlike our findings, Wang et al. declared that the amount of NK cell was decreased in COVID-19 patients, and in the severe stage of the disease, these cells are lower than the mild stage [37]. Regarding the expressions of CD16 and CD56, NK cells are categorized into various populations, and two main types of them are $CD56^{dim}$ $CD16^{+}$ NK cells and $CD56^{bright}$ $CD16^{dim/-}$ NK cells [38]. $CD56^{dim}$ $CD16^{+}$ NK cells can express high levels of the maturation marker CD57 and killer inhibitory receptors (KIR)[39].

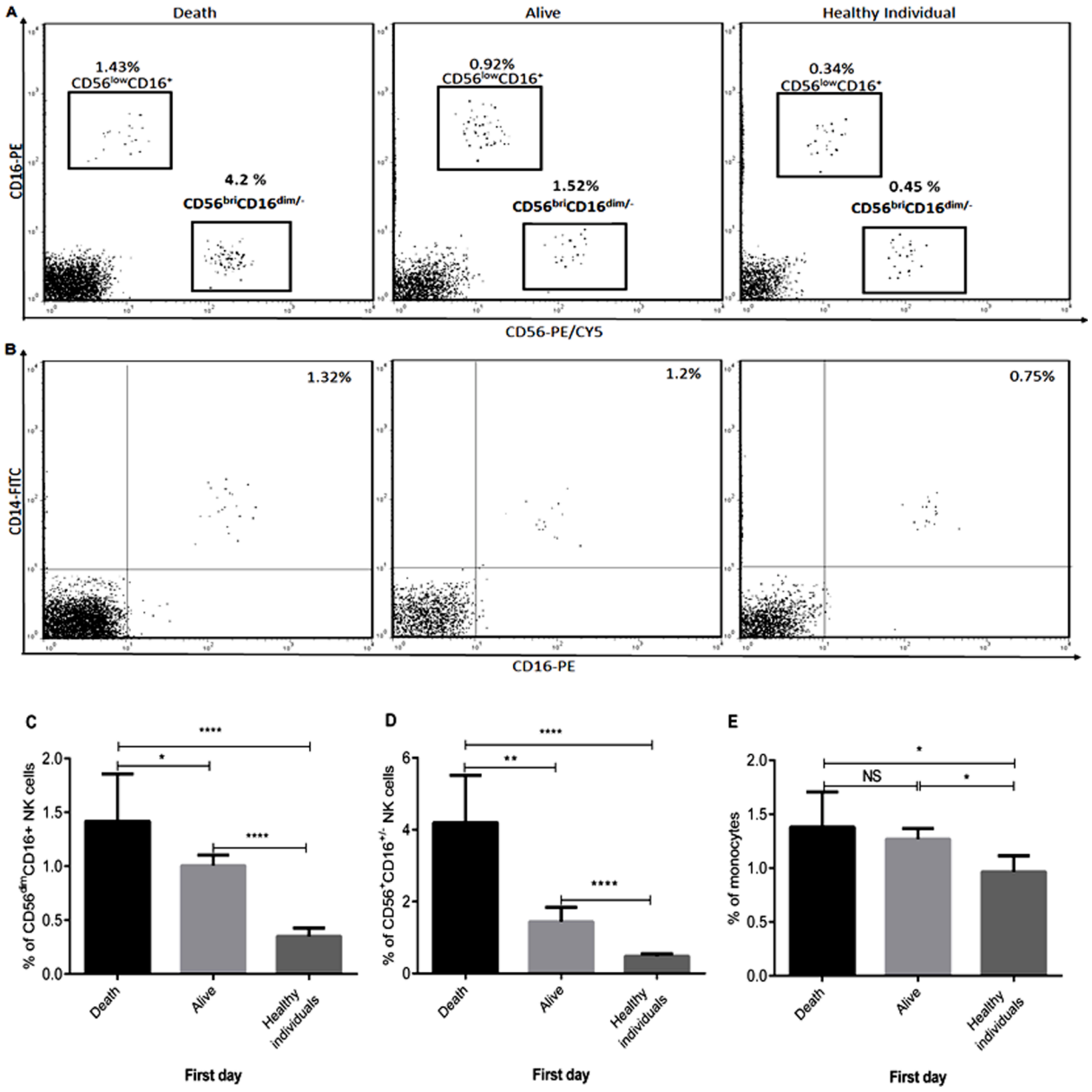


Fig. 1. The numbers of innate immune cells in control individuals and COVID-19 subjects who died or recovered. The percentages of $CD56^{dim}$ $CD16^{+}$ NK cells, $CD56^{bright}$ $CD16^{dim/-}$ cells, and monocytes were assessed by flow cytometry (**A and B**) and then analyzed (**C-E**). The data are representative of 71 independent experiments for COVID-19 patients (60 recovered and 11 dead individuals) at the first day of hospitalization and 50 independent experiments for healthy individuals. Each bar in Fig. 1(**C-E**) indicates mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001.

These cells are involved in natural and antibody-dependent cellular cytotoxicity through producing high values of perforin and enhanced killing. $CD56^{bright}$ $CD16^{dim/-}$ NK cells can be characterized by the expression of NKG2A, low level of perforin, and high productions of cytokines [40]. Thus, this cell population may be involved in the mortality and pathogenic processes of this novel coronavirus. Our other findings about the role of the innate immune system showed that the number of monocytes in patients who died up to ten days of hospitalization was dramatically increased compared to these cells of the recovered patients and healthy individuals. Inconsistent with our results, Zhang et al. by investigating clinical features of 82 dead patients with COVID-19 showed that the number of monocytes was in a normal range [41]. This difference in the results may be attributed to the disease stage when the patients were studied.

In the investigation of the numbers of adaptive immune cells, we observed that Th2, Th17 cells, and Tregs in dead patients were significantly lower than healthy and recovered patients. However, there was no remark change in Th1 cell frequency between recovered and dead patients at the first day of recovery. In this respect, a study by Qin et al. manifested that the percentages of suppressor and helper T cells in the patients were lower than healthy individuals. Moreover, in their study on the severe stage of the disease, naive Th cell frequency were increased, but memory Th cells and Tregs were decreased [20]. In our study, the number of Tregs in the early stage of recovery was significantly higher in dead and recovered patients than the control group. Similar to this, the results of Tan et al. implicated that Treg number was elevated in the mild stage of COVID-19 in comparison with healthy subjects [42]. We also indicated that the numbers of the exhausted CD4 + and CD8 + T cells and activated CD4 + T cells at the first day of recovery were significantly higher than recovered and control groups,

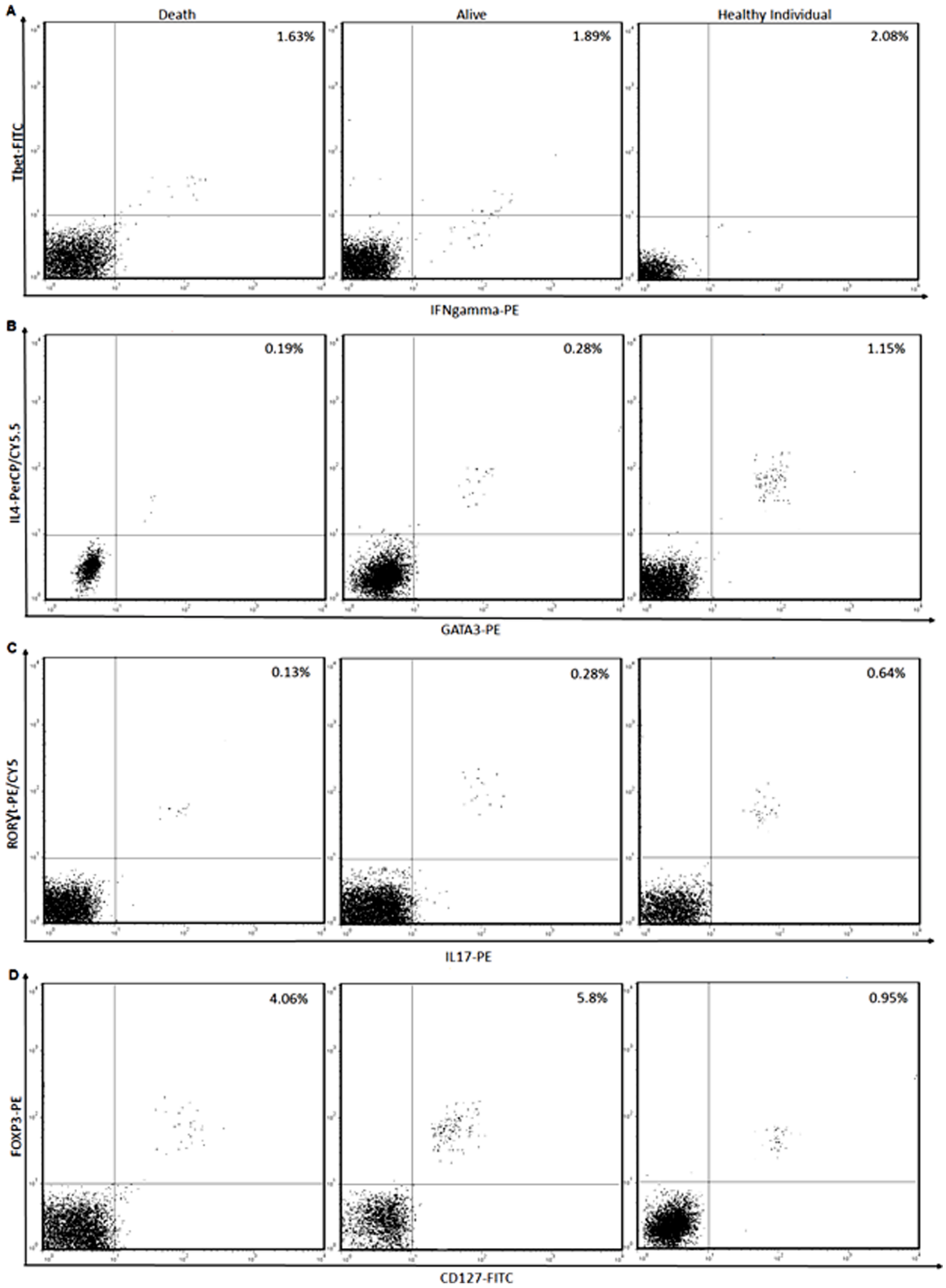
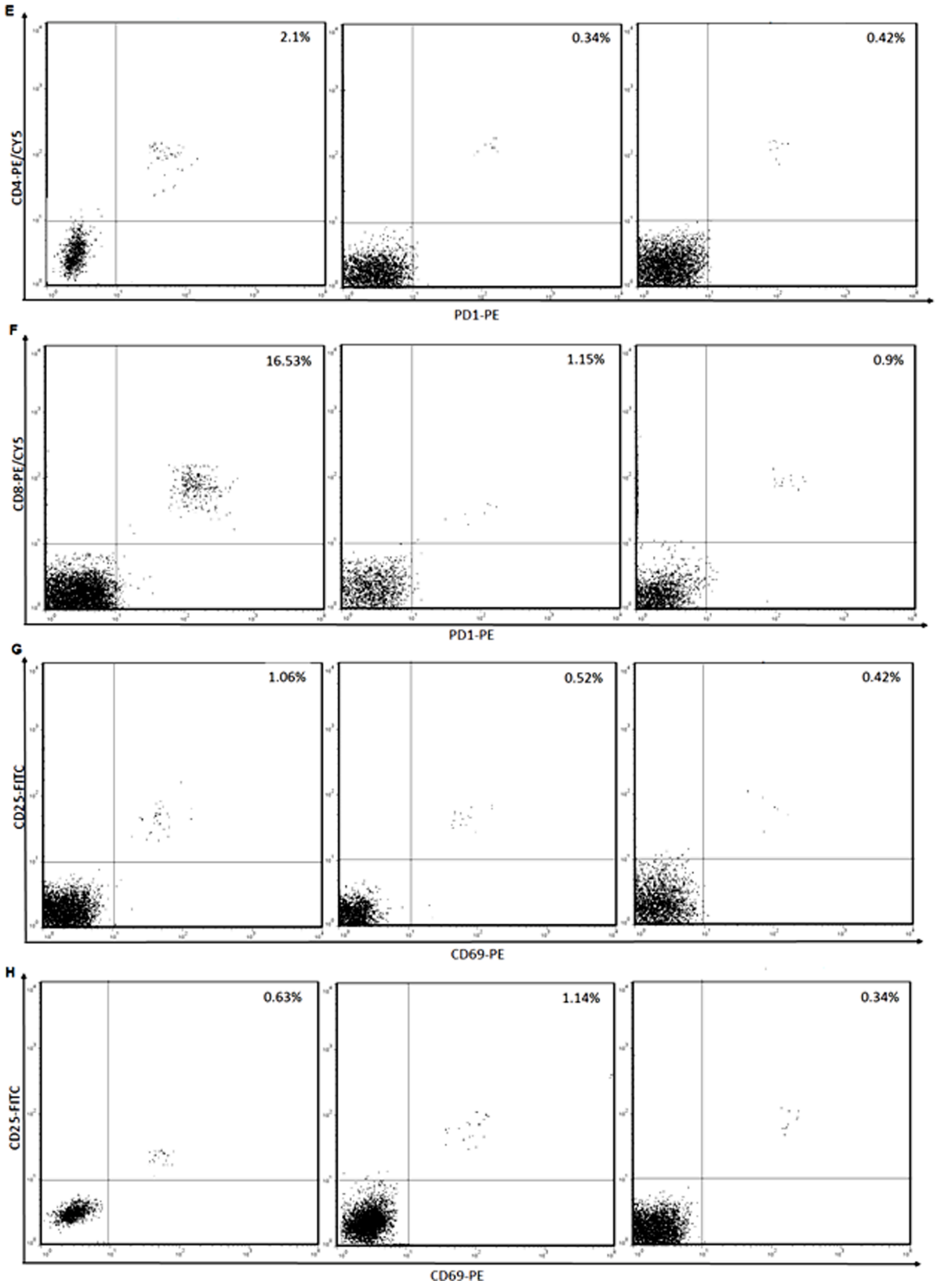


Fig. 2. The frequencies of adoptive immune cells in control individuals and COVID-19 subjects who died or recovered. PBMCs were isolated from healthy subjects and COVID-19 patients who died or recovered and then stained with different monoclonal antibodies. The percentages of Th1, Th2, Th17, Tregs, exhausted CD4 + and CD8 + T cells, and activated CD4 + and CD8 + T cells were measured using flow cytometry (**A-H**) and then analyzed (**I-P**). The depicted results are representative of 71 independent experiments for COVID-19 patients (11 dead and 60 recovered subjects) at the first day of hospitalization and 50 independent experiments for healthy groups. Data reveal mean ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.



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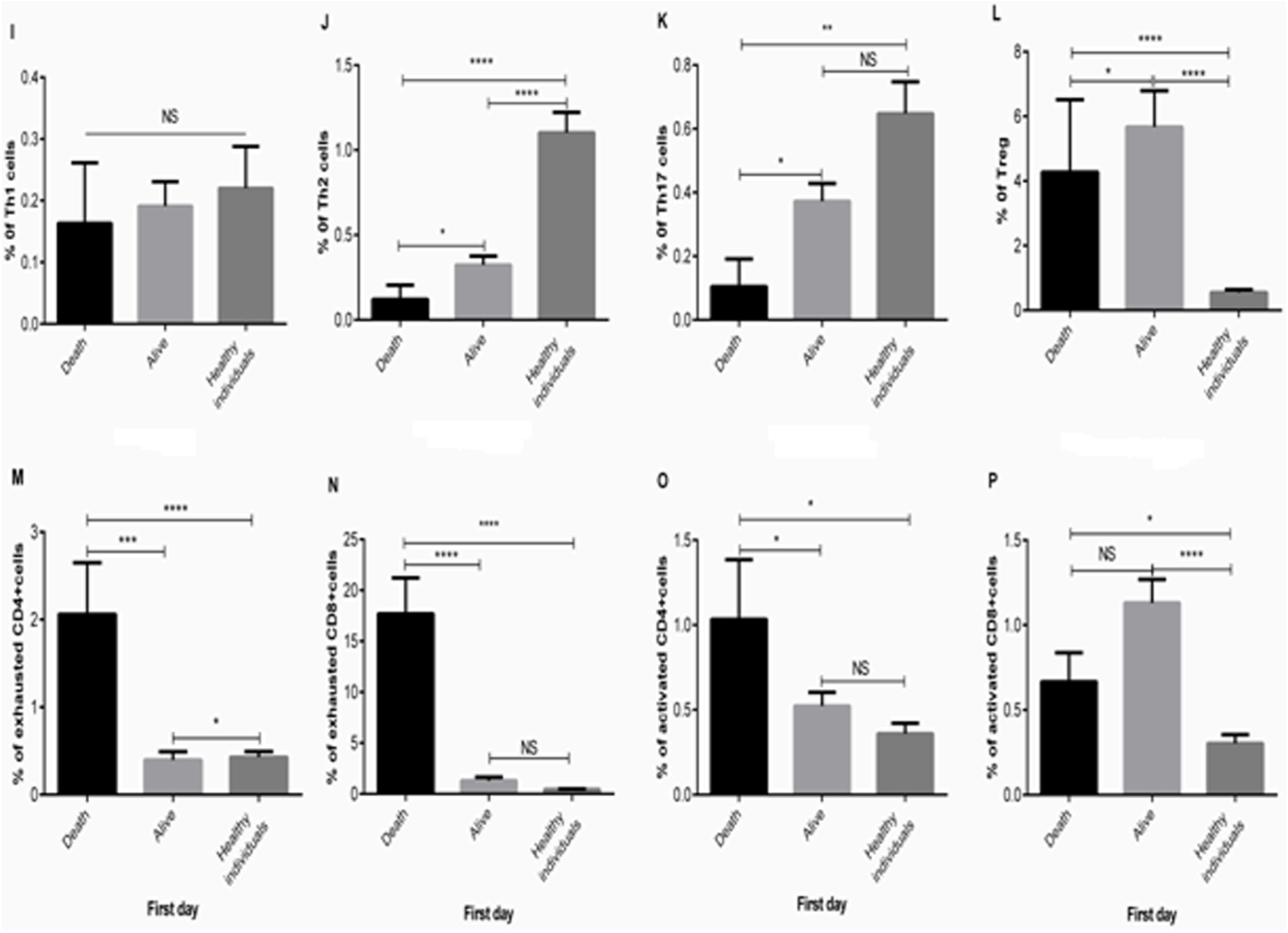

Fig. 2. (*continued*).

which is consistent with previous studies showing higher percentages of senescent/exhausted cells (PD1 + CD57 +) in COVID-19 patients [43]. Furthermore, the percentage of activated CD8 + T cells was dramatically higher in the recovered group than dead and healthy individuals, and totally, the percentage of this cell in patients (recovered and dead patients) were higher than the healthy group. In contrast with our findings, Wang et al. by analyzing subsets of peripheral lymphocyte from 60 patients with COVID-19 before and after treatment indicated that total lymphocytes, CD4 + T cells, CD8 + T cells were reduced in patients, and these cells were lower in cases with the severe disease stage than those with the mild disease stage [37]. This discrepancy may be pertinent to different therapeutic methods employed in diverse stages of the disease.

Regarding the fact that one of the mechanisms involved in the pathogenesis of COVID-19 may be related to cytokine storm, as extreme and uncontrolled secretion of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1 and IL-6 which can lead to death [11,44,45], we measured some of pro- and anti-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-10, TNF-α, and TGF-β1. According to our observations, the plasma levels of IL-1α, IL-1β, IL-6, and TNF-α in patients at the first day of recovery were dramatically higher than the healthy group. In line with the role of inflammation in COVID-19 pathogenesis, it is reported that the increased level of CRP, as a marker of inflammation, was significantly correlated to mortality [46]. Other data demonstrated that the level of IL-10 was significantly higher in recovered patients than healthy subjects. However, no significant change was observed in the level of TGF-β1 among dead, recovered, and healthy subjects. Similarly, Henry et al. in a meta-analysis study demonstrated that in severe or fatal stages of the disease, there were high levels of IL-2, IL-8, IL-10 [39]. Others have revealed that patients in the severe stage of COVID-19 had the increased levels of IL-2, IL-6, and IL-10 [42]. These findings suggest further studies to clarify the particular roles of pro- and anti-inflammatory cytokines along with other immunological and non-immunological factors in the pathogenesis of COVID-19.

Taken together, our results can be beneficial to indicate that how a defect in the function and/or modulation of the immune system can participate in disease susceptibility and its pathogenesis through increasing the secretions of pro- and anti-inflammatory cytokines and inducing different immune responses. Furthermore, the results of the current study provide evidence to reveal that changes in immune cells and other immune agents may be considered as predicting factors for mortality of COVID-19. However, it should be noted that further studies are required to confirm our hypothesis and explain whether changes in other immune cells such as eosinophils and neutrophils may act as other predictors of disease outcome.

Author contribution

Ramin Sami: Carried out some of the experiments and participated in the design of the experiments. **Farshid Fathi:** Participated in visualization, methodology and preformed data analysis. **Nahid Eskandari:** Obtained funding for the work and participated in project administration. **Meysam Ahmadi:** Participated in conceptualization, data creation, and resources. **Reza Aref Nezhad:** Drafted the original manuscript and participated in investigation. **Hossein Motedayyen:** Participated in supervision, validation, and writing - review & editing.

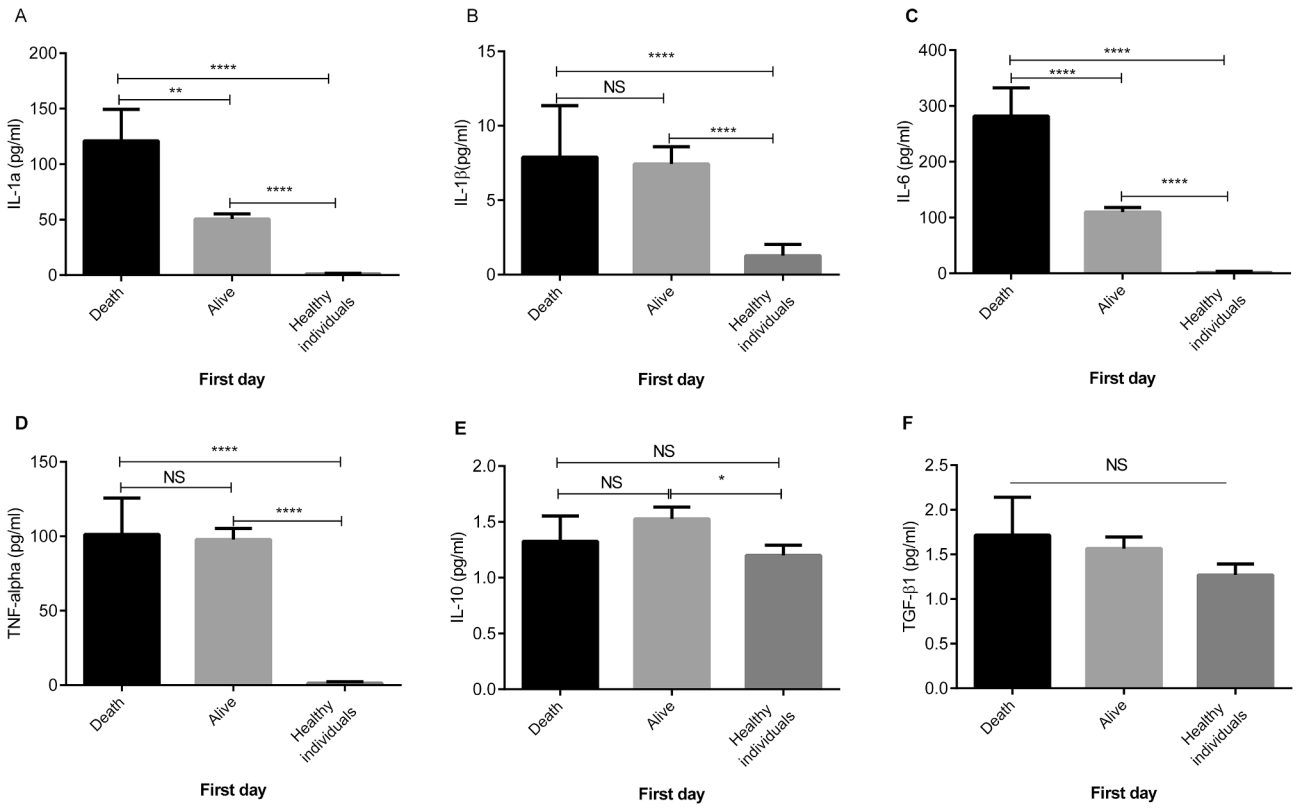

Fig. 3. The plasma values of pro-and anti-inflammatory cytokines in control group and COVID-19 patients who recovered or died. The levels of IL-1α, IL-1β, IL-6, TNF-α, IL-10, and TGF-β1 were studied by ELISA (**A-F**). The depicted results are representative of 71 independent experiments for patients with COVID-19 (70 recovered and 11 dead subjects) at the first day of hospitalization and 50 independent experiments for control group. All data show mean ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Finding

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