Differential alterations in peripheral lymphocyte subsets in COVID-19 patients: upregulation of double-positive and double-negative T cells

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Background: Viral infections cause alteration in the total number of lymphocytes and their subset distribution. We aimed to study peripheral blood lymphocyte subsets in COVID-19 patients and to correlate these subsets with clinical and laboratory data, which may help in clarifying the pathogenesis to develop novel diagnostic and prognostic biomarkers for COVID-19.

Methods: Twenty-six reverse-transcription polymerase chain reaction (RT-PCR) confirmed COVID-19 patients were subjected to medical history-taking and a thorough clinical examination. Laboratory tests included complete blood count, D dimer, ferritin, and C-reactive protein (CRP). Chest CT was used to diagnose COVID-19 pneumonia. Lymphocyte subsets were compared with those in 20 healthy controls using flow cytometry.

Results: Leucopenia, relative neutrophilia, lymphopenia, eosinopenia together with marked elevation in neutrophil/lymphocyte ratio were observed in our COVID-19 patients. A marked reduction was observed in T cells, including both CD4 and CD8 cells, natural killer (NK), and natural killer T cells (NKT). Double-positive T (DPT) cells, double-negative T (DNT) cells, and B cells were elevated in the patients relative to the other lymphocyte subsets.

Conclusion: Immune-inflammatory parameters are of utmost importance in understanding the pathogenesis and in the provisional diagnosis of COVID-19. Yet, adequate care must be taken during their interpretation because of the vast discrepancies observed between studies even in the same locality. Further studies are needed to clarify the role of B cells, DPT, and DNT cells in the pathogenesis and control of COVID-19.

Key words: COVID-19; SARS-CoV-2; lymphocyte subsets; double-positive T cells; double-negative T cells.

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Introduction

Coronaviruses (CoVs) are well-known causes of severe infections; respiratory, enteric, and systemic, in humans and numerous animal hosts. In December 2019, an increasing number of cases of pneumonia of unknown etiology was reported in Wuhan, Hubei Province, China. Several studies suggested that the disease was caused by a novel coronavirus that has been transferred from animals [1]. The virus was termed SARS-CoV-2, and the disease was designated coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO) [2]. Patients with COVID-19 have several symptoms such as fever, dry cough, bone pain, and fatigue in the early stage. Later, patients may develop acute respiratory distress syndrome (ARDS) that eventually evolves into shock, respiratory failure, serious multiorgan failure, or even death [3]. In Egypt, according to the Egyptian Ministry of Health’s daily reports, SARS-CoV-2 has infected 185,587 patients and has caused 10,871 deaths (as of March 4th, 2021).

Infection by SARS-CoV-2 can result in excessive production of cytokines and chemokines (namely a cytokine storm), resulting in immune dysregulation and patient mortality [4]. Viral infections are known to cause alteration in the total number of lymphocytes, and their subsets vary with the different virus types. Lymphocyte subsets play a crucial role in the preservation of immune system function. This indicates the potential association between lymphocyte subset alterations and the mechanism of viral pathogenicity [5].

Recent studies indicated a clear decrease in peripheral lymphocytes in COVID-19 patients [6]. Also, there is a growing list of publications reporting that the assessment of lymphocyte subset counts can provide prognostic information for COVID-19 disease severity and convalescence when these findings are correlated with other clinical data [7,8].

Little is known about theses lymphocyte subset counts in Egyptian COVID-19 patients. Thus, we aimed to study peripheral blood lymphocyte subsets in COVID-19 patients and to correlate these subsets with clinical and laboratory data, which may help in clarifying the pathogenesis to develop novel diagnostic and prognostic biomarkers for COVID-19.

Materials and Methods

Our pilot study included a cohort of 26 reverse-transcription polymerase chain reaction (RT-PCR) confirmed COVID-19 patients, and 20 age and sex-matched healthy volunteers. All data and laboratory investigation were obtained at day one hospital admission and before the start of any medication for treatment of COVID-19 symptoms. Patients with known hematomal disorders or other chronic diseases were excluded from our study. Patients were subjected to medical history-taking and a thorough physical examination including oxygen saturation and measurement of PaO2/FiO2 ratio. Baseline laboratory tests included complete blood count and levels of hemoglobin (Hb), D dimer, ferritin, and C-reactive protein (CRP). Neutrophil to lymphocyte ratio (NLR) was calculated by dividing the absolute neutrophil count by absolute lymphocyte count.

The diagnosis of COVID-19 pneumonia was established considering the following chest CT patterns: ground-glass opacity (GGO), crazy-paving, and consolidation [9,10]. The CT findings were in accordance with the standard glossary for thoracic imaging reported by the Fleischner Society [11]. A semi-quantitative CT severity scoring was calculated per each of the five lobes, as was reported by the Fleischner Society [11]. A semi-quantitative CT severity scoring was calculated per each of the five lobes, as was reported by the Fleischner Society [11]. A semi-quantitative CT severity scoring was calculated per each of the five lobes, as was reported by the Fleischner Society [11]. A semi-quantitative CT severity scoring was calculated per each of the five lobes, as was reported by the Fleischner Society [11]. A semi-quantitative CT severity scoring was calculated per each of the five lobes, as was reported by the Fleischner Society [11]. A semi-quantitative CT severity scoring was calculated per each of the five lobes, as was reported by the Fleischner Society [11]. A semi-quantitative CT severity scoring was calculated per each of the five lobes, as was reported by the Fleischner Society [11]. A semi-quantitative CT severity scoring was calculated per each of the five lobes, as was reported by the Fleischner Society [11].

Flow cytometric detection of lymphocyte subsets

Whole blood samples collected in EDTA tubes were analysed by flow cytometry. For the detection of lymphocytes, 50 µl of a whole blood sample was stained with 10 µl of fluorescein isothiocyanate (FITC)-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-CD16/56, pyridinium-chlorophyll-protein (Per-CP)-conjugated anti-CD19 in one tube. For lymphocyte subset analysis, a second tube was stained with FITC conjugated anti-CD4, PE-conjugated anti-CD8, and Per-Cp conjugated anti-CD3. All previously mentioned monoclonal antibodies were purchased from Becton Dickinson (BD) Biosciences (San Jose, CA, USA). After incubation for 15 minutes, red blood cell lysis and washing were done, and the cells were resuspended in phosphate-buffered saline (PBS). 20,000 cells were acquired by FACS Calibur flow cytometer, and analysis was carried out using BD Cell Quest Pro software.

Statistical analysis

Statistical analysis was done using the IBM Statistical Package for the Social Sciences (IBM SPSS statistics, version 20). Categorical data were displayed as numbers and percentages, while continuous data were presented as the mean and standard error. Mann-Whitney U test was used to compare groups, and Spearman’s rank correlation was employed to evaluate the strength of association between different variables.

Results

Demographic characteristics, clinical presentation, and some laboratory features of COVID-19 patients

The mean age of the patients’ group was 32.3±15 years. Out of these 26 patients, 20 (77%) were males and 6 (23%) were females. Table 1 presents some clinical and laboratory features of COVID-19 patients. Twenty-three patients (88.5%) had fever, 18 (69.2%) had cough, 13 (50%) had dyspnea, 6 (23%) had anemia and 4 (15.4%) had diarrhea. Regarding chest CT findings, 13 (50%) were mild cases (7 patients with the score “1” and 6 with score “2”); seven (26.9%) were moderate cases (3 patients with the score “3” and 4 with the score “4”) and six (23%) were severe cases (score 5), regarding PaO2/FiO2, 10 patients (38.5%) had values >300; 9 (34.6%) had values ≤300 ≥200 and 7 (26.9%) <200.

Also, in the patients’ group, the mean value of Hb concentration was 12±2 g/dL, C-reactive protein (CRP) was 37±4 mg/dL, ferritin was 552±88 ng/mL, D-dimer was 389.8±54 µg/L. Levels of ferritin increased markedly with disease severity (mild: 276.5±45; moderate: 563.7±133; and severe: 1137.5±178). The same was observed in the levels of D dimer (mild: 289.2±50; moderate: 417.2±112; and severe:575.4±142) and CRP (mild: 22.6±7; moderate: 52.9±29; and severe: 51.5±27), yet with no many differences between moderate and severe cases.
Immune cells in COVID-19 patients and the control group

As presented in Table 2, the mean leukocyte count for the patient group (5.7±0.5 x 10⁹/L) was lower than that for the control group (7.4±0.4 x 10⁹/L), with a statistically significant difference between the two groups (p<0.008). Initially, total leucocyte count (TLC) was low in the mild cases then increased, inconsistently, in some moderate and severe cases. Both relative and absolute eosinophil counts were obviously lower in COVID-19 patients than controls (2.4±0.4 vs 4±0.4, p=0.003 and 0.1±0.02 vs 0.3±0.03, respectively, p<0.0001) and to some extent lower in severe cases.

The relative neutrophil count was 68±2% for the patient group and 46.4±2% for the control group with a highly significant difference between both groups (p<0.0001). Eleven patients (52%) had lymphopenia <1 x 10⁹/L and both the mean relative and absolute lymphocyte counts were significantly reduced in patients than in the controls (21±2 x 10⁹/L vs 42±2 x 10⁹/L, p<0.0001 and 1± 0.1 x 10⁹/L vs 3±0.3 x 10⁹/L, respectively, p<0.0001). The NLR was markedly elevated in the COVID patients (2.3±0.2) than in the healthy controls (0.9±0.08), p<0.0001, but did not show significant changes with disease severity.

A significant drop was observed in both the mean relative and absolute counts of T-cells in the patient group compared with the control group (61.4±2% vs 69.9±3%, p=0.003 and 0.7±0.01 x 10⁹/L vs 2.3±0.2 x 10⁹/L, p<0.0001, respectively). Similarly, lower relative and absolute counts of CD4+ T-cells (49±2% vs 55.9±2%, p=0.005 and 0.3±0.03 x 10⁹/L vs 1.3±0.1 x 10⁹/L, p=0.0001, respectively) and CD8+ T-cells (29±1% vs 34.3±2%, p=0.01 and

Table 1. Clinical presentation and some laboratory features of COVID-19 patients.

| Outcome                      | Patients (n=26) |
|------------------------------|-----------------|
| Fever                        | 23 (88.5%)      |
| Cough                        | 18 (69.2%)      |
| Dyspnea                      | 13 (50%)        |
| Anosmia                      | 6 (23%)         |
| Diarrhea                     | 4 (15.4%)       |
| Chest CT scoring             |                 |
| Mild                         |                 |
| score 1                      | 7 (26.9%)       |
| score 2                      | 6 (23%)         |
| Moderate                     |                 |
| score 3                      | 3 (11.5%)       |
| score 4                      | 4 (15.4%)       |
| Severe                       |                 |
| score 5                      | 6 (23%)         |
| PaO₂/FiO₂                     |                 |
| >300                         | 10 (38.5%)      |
| ≤300 ≤200                    | 9 (34.6%)       |
| <200                         | 7 (26.9%)       |
| CRP (mg/dL)*                 | 37.4±10 (normal: up to 1) |
| Ferritin (ng/mL)*            | 552.5±88 (normal: 22-322) |
| D dimer (µg/L)*              | 389.7±54 (normal: up to 550) |

Results expressed as number (percent) or *mean ±SE (normal range). GIT, gastrointestinal tract; CT, computed tomography; PaO₂, arterial O₂ tension; FiO₂, fraction inspired oxygen; CRP, C-reactive protein.

Figure 1. Flow cytometric analysis of lymphocyte subsets in COVID-19 patients. A) Forward and side scatter dot plot was used to define the lymphocyte population (R1). B) The expression of CD16/56 and CD3 was assessed on the lymphocyte population to detect NK and NKT cells. C) The expression of CD3 and CD19 was calculated on lymphocytes to detect B and T lymphocytes. D) CD3+ T-lymphocytes were then gated for further analysis of CD4 and CD8. E) The expression of CD4 and CD8 on the CD3+ T-lymphocytes was evaluated to detect CD4+ (T helper), CD8+ (T cytotoxic), double positive T-lymphocytes (CD4+CD8+), and double negative T-lymphocytes (CD4CD8).
Double negative T-lymphocytes (CD4-/CD8-)

Table 2. Alterations in immune cells in COVID-19 patients.

| Immune cells | Patients (n=26) | Controls (n=20) | p* |
|--------------|----------------|----------------|----|
| TLC (x 10^9/L) | 5.7±0.5        | 7.4±0.4        | 0.008 |
| Eosinophils  |                |                |    |
| Relative to lymphocyte count (%) | 2.4±0.4 | 4±0.4 | <0.0001 |
| Absolute count (x 10^9/L) | 0.1±0.02 | 0.3±0.03 | <0.0001 |
| Neutrophils  |                |                |    |
| Relative to lymphocyte count (%) | 68±2 | 46.4±2 | <0.0001 |
| Absolute count (x 10^9/L) | 4±0.4 | 3.4±0.2 | 0.1 |
| Lymphocyte   |                |                |    |
| Relative to lymphocyte count (%) | 21±2 | 42.4±2 | <0.0001 |
| Absolute count (x 10^9/L) | 1±0.1 | 3±0.3 | <0.0001 |
| Neutrophil/lymphocyte ratio | 2.3±0.2 | 0.9±0.08 | <0.0001 |
| T-lymphocytes (CD3+) |                |                |    |
| Relative to lymphocyte count (%) | 61.4±2 | 69.9±3 | 0.003 |
| Absolute count (x 10^9/L) | 0.7±0.01 | 2.3±0.2 | <0.0001 |
| CD4+ T-lymphocytes |                |                |    |
| Relative to CD3-T-lymphocyte count (%) | 49±2 | 55.9±2 | 0.005 |
| Absolute count (x 10^9/L) | 0.3±0.03 | 1.3±0.1 | <0.0001 |
| CD8+ T-lymphocytes |                |                |    |
| Relative to CD3-T-lymphocyte count (%) | 29±1 | 34.3±2 | 0.01 |
| Absolute count (x 10^9/L) | 0.2±0.02 | 0.8±0.09 | <0.0001 |
| CD4/CD8 ratio | 1.8±0.2 | 1.8±0.1 | 0.8 |
| Double positive T-lymphocytes (CD4+/CD8+) |                |                |    |
| Relative to CD3-T-lymphocyte count (%) | 6.2±0.3 | 1.2±0.04 | 0.001 |
| Absolute count (x 10^9/L) | 0.05±0.004 | 0.03±0.003 | <0.0001 |
| Double negative T-lymphocytes (CD4-/CD8-) |                |                |    |
| Relative to CD3-T-lymphocyte count (%) | 14.7±0.8 | 8.6±0.6 | <0.0001 |
| Absolute count (x 10^9/L) | 0.1±0.009 | 0.2±0.03 | 0.001 |
| B-lymphocytes (CD19+) |                |                |    |
| Relative to lymphocyte count (%) | 19.2±0.9 | 11.6±0.4 | <0.0001 |
| Absolute count (x 10^9/L) | 0.2±0.02 | 0.4±0.03 | <0.0001 |
| Natural killer (CD16+CD56-) |                |                |    |
| Relative to lymphocyte count (%) | 8.9±0.3 | 10.4±0.6 | 0.02 |
| Absolute count (x 10^9/L) | 0.09±0.007 | 0.3±0.03 | <0.0001 |
| NKT cells (CD3+CD16+CD56+) |                |                |    |
| Relative to lymphocyte count (%) | 6.2±0.4 | 8.5±0.4 | <0.0001 |
| Absolute count (x 10^9/L) | 0.06±0.006 | 0.3±0.02 | <0.0001 |

*p Mann-Whitney U test (p-value is significant if <0.05). Results are expressed as mean ±SE. TLC, total leucocytic count; NKT, natural killer T cells.
Discussion

Hematological changes are known to occur in SARS-CoV-2 virus infection and are often useful in the monitoring of the infection course or to indicate the severity of COVID-19 [13]. As well-known markers of systemic inflammation and infection, CRP and ferritin have been studied as predictors of bacterial infections, including pneumonia [14], and together with D dimer may be used to predict severe and fatal COVID-19 in hospitalized patients [15,16]. Our patients had more elevated CRP, ferritin, and D dimer than the normal reference ranges. Also, their levels increased with disease progression, especially, ferritin and D dimer.

In line with Qin et al. [14], most of our patients presented with significant leucopenia, absolute lymphopenia, and relative neutrophilia. Moreover, in previous studies, marked leucopenia, a higher number of neutrophils and a lower number of lymphocytes were more prominent in severe cases [6,14,17,18], signifying an impairment of the immune system during SARS-CoV-2 infection and the serious disruption of internal environment in those severely infected cases. In our patients TLC decreased in mild cases, but tended to increase, inconsistently, in some moderate and severe cases. This increase could be due to co-infections [19], medications as prednisone [20], or inconsistent immune response [19].

Neutrophilia may be initiated by virus-related inflammatory cytokines produced by lymphocytes and endothelial cells [21] to control the virus infection by producing reactive oxygen species (ROS) and other cytotoxic mediators [22] and by the release of neutrophil extracellular traps (NETs), to capture and destroy the virus [23-25]. On the other hand, the decrease or exhaustion of lymphocytes may be because the viral infection causes immune cells to enter an activated state and share in the anti-viral processes, causing profound damage and apoptosis [26].

The NLR is another marker of systemic inflammation and COVID-19 progression that considers both lymphocytes and neutrophils levels [26]. The NLR in another study [27] was increased in patients with severe COVID-19 and had a prognostic value. NLR was markedly elevated in our COVID patients and showed positive correlations with CRP and anemia but did not show significant changes with disease progression.

Consistent with earlier studies [14,28], both relative and absolute eosinophil counts were reduced in COVID patients and, to some extent, lower in severe cases. Du et al. observed eosinopenia in almost every COVID patient who died [29]. Others deduced that eosinopenia could be used as a reliable diagnostic factor when combined with lymphopenia [30].

Eosinophils have a potent proinflammatory function, and are equipped with molecules that allow them to recognize and respond to respiratory viruses [31]. Eosinopenia takes place in response to factors inducing acute inflammation [32]. The pathophysiology of eosinopenia in COVID-19 is so far not clear but is most probably multifactorial, including inhibition of eosinophils or eosinophil egress from the bone marrow, downregulation of chemokine receptors/adhesion molecules [32,33] or eosinophil apoptosis triggered by type I-interferons produced during acute infection [34].

Lymphocytes are involved in the humoral and cytotoxic immunity against viral infection. As with immune diseases and other infectious diseases, virus infection can also lead to dysregulation in the levels of lymphocyte subsets [35, 6]. In our study, the absolute count of T-cells (CD3+), which are the most important immune cells for protection against viral infections [37-39], was significantly decreased in patients. This finding was similar to that reported earlier [15,40].

T cell maturity occurs in the thymus where CD4+CD8+ double-positive T cells mature into either CD4+ or CD8+ T cells that leave the thymus to secondary lymphoid organs and are responsible for the adaptive cellular immune responses to clear infections [41-43]. The relative and absolute counts of CD4+ and CD8+ T-cells were significantly decreased in our patients, as was previously reported [30], with no inversion in the CD4/CD8 ratio. While Jiang et al. [44] reported T lymphopenia, and in particular, a decrease of CD8+ T-cells in patients with COVID-19, Qin et al. [14] reported that the reduction of CD4+ T-cells was common with no significant change in the number of CD8+ cells.

Despite the negative correlation between the duration of fever and the relative count of CD4+ T lymphocytes in our patients, no apparent relations were detected between CD4+ and CD8+ T-cell counts and the CT severity scores. On the contrary, other studies reported an association between disease severity and the decrease in the count of lymphocyte subsets, especially CD3+, CD4+, and CD8+ T-cells [44-46].

In our study, DPT cell counts increased, pointing to the possible role of these cells in COVID-19 pathogenesis. Data regarding the functions of DPT cells seems to be very controversial, and very case specific. Some studies reported the cytotoxic potential in diseases, including viral infections as human immunodeficiency virus (HIV) and cancer. In contrast, others reported exhibiting a suppressive phenotype in cancer, systemic sclerosis, and inflammatory bowel disease [47].

Besides being a stage in T-cell development, mechanisms behind the peripheral expansion of DPT cells remain uncertain. CD8 expression could be acquired under continuous antigen stimulation of CD4+ T-cells [48]. Conversely, CD8+ T-cells were proposed to co-express CD4 after in vitro activation of human peripheral blood mononuclear cells [49], consequently rendering them susceptible to HIV infection [50]. Also, upregulation of FasL and IFN-γ, as hence increased antiviral effector functions, were observed after surface ligation of CD4 on CD8+ T-cells [51,52]. Furthermore, DPT cells in patients with urologic cancers were found to be high type-2 cytokine producers, and favor a Th helper (Th)-2 polarization of CD4 T cells in vitro, at the expense of the protective Th1 functional profile [53].

Likewise, several conflicting data had been published regarding the function of DNT cells. They contribute to inflammation and were found to act as regulatory T cells and/or cytotoxic T cells [54]. Their increase was noted in HIV infection, some lymphoproliferative disorders, graft-versus-host disease, and autoimmune diseases [43,55-57]. In this study, their relative count was increased in COVID-19 patients, despite the decreased absolute count. Additionally, a positive correlation was detected between DNT cells and the duration of fever. Thus, DNT cells might contribute to the pathogenesis of COVID through induction of proinflammatory responses or immune suppression with impaired immune responses to the SARS-CoV-2 virus.

Although in agreement with other studies [36,58,59], B cell absolute count was lower in COVID patients, while the relative count was elevated. A previous study found that patients with agammaglobulinemia had a mild course of COVID-19, suggesting a potential role of B lymphocytes in SARS-CoV-2-induced inflammation [60]. Inflammation is aggravating the COVID-19 clinical picture, as previously described, with a profound increase in the level of cytokines such as IL-6 [61]. Activated B cells produce IL-6 to stimulate germinal center formation. This IL-6 could increase the level of inflammation and contribute to the cytokine storm syndrome [60].

The NK cells play a vital role in the destruction of virus-infected cells and tumor cells [62,63]. NKT cells link the adaptive and innate immune system as they can produce large amounts of cytokines [64-66]. Agreeing with earlier studies [58,67], the NK and NKT cell counts decreased in our patients compared to the control group.
Conclusion

Immune-inflammatory parameters are of utmost importance in understanding the pathogenesis and in the provisional diagnosis of COVID-19. Yet, adequate care must be taken during their interpretation due to the vast discrepancies observed among studies even in the same locality. Further studies are needed to clarify the role of B cells, DPT and DNT cells in the pathogenesis and control of COVID-19.

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