The Comparison of Monocyte Human Leukocyte Antigen-D-Related (mHLA-DR) Expression Levels Between Corona Virus Disease 2019 (COVID-19) Patients and Healthy Subjects

Musholli Himmatun Nabilah[1,2], Puspa Wardhani[3,4,5], Aryati[3,4], Yetti Hernaningsih[3], Bambang Pujo Semedi[6]

[1]Laboratory Medicine Study Interest, Master Program of Basic Medical Science, Faculty of Medicine Universitas Airlangga, Surabaya, Indonesia.
[2]Department of Medical Laboratory Technology, Health Polytechnique, Ministry of Health Republic Indonesia, Surabaya, Indonesia.
[3]Department of Clinical Pathology, Faculty of Medicine Universitas Airlangga - Dr. Soetomo General Academic Teaching Hospital, Surabaya, Indonesia.
[4]Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.
[5]Postgraduate School of Universitas Airlangga, Surabaya, Indonesia.
[6]Department of Anesthesiology and Reanimation, Faculty of Medicine Universitas Airlangga - Dr. Soetomo General Academic Teaching Hospital, Surabaya, Indonesia.

Corresponding Author:
Puspa Wardhani, MD. Department of Clinichal Pathology, Faculty of Medicine Universitas Airlangga. Jl. Mayjen. Prof. Dr. Moestopo no. 47, Surabaya 60131, Indonesia. Email: puspa-w-2@fk.unair.ac.id.

ABSTRACT

***Background**: SARS-CoV-2 can trigger a dysfunctional immune response in COVID-19 patients and lead to immunosuppression. HLA-DR molecule expressed on the surface of monocytes, known as mHLA-DR, has been widely used as a reliable marker of immunosuppression. Downregulation of mHLA-DR reflects an immunosuppressed state. This study aimed to compare the expression level of mHLA-DR between COVID-19 patients and healthy subjects concerning immune system dysregulation that can be triggered by SARS-CoV-2 and lead to immunosuppression. **Methods**: This was an analytic observational study with a cross-sectional design that measured the mHLA-DR expression in EDTA blood samples from 34 COVID-19 patients and 15 healthy subjects using the BD FACSLyric™ Flow Cytometry System. The mHLA-DR examination results were expressed in AB/C (antibodies bound per cell) that were quantified using a standard curve constructed with Quantibrite phycoerythrin beads (BD Biosciences). **Results**: Expression of mHLA-DR in COVID-19 patients (n = 34) were 21,201 [2,646-92,384] AB/C, with 40,543.5 [9,797-92,384] AB/C mild cases (n = 22), 21,201 [9,831-31,930] AB/C moderate cases (n = 6), and 7,496 [2,646-13,674] AB/C severe to critical cases (n= 6). Expression of mHLA-DR in healthy subjects (n = 15) was 43,161 [25,147-89,846] AB/C. Based on the Mann-Whitney U test, the mHLA-DR expression in COVID-19 patients significantly differed from the mHLA-DR expression in healthy subjects (p = 0.010). **Conclusion**: The level of mHLA-DR expression in COVID-19 patients was lower and significantly different from healthy subjects. Moreover, immunosuppression could be indicated by the decrease of mHLA-DR expression, which was below the reference range found in severe to critically ill COVID-19 patients.*

***Keywords**: COVID-19, Monocyte Human Leukocyte Antigen-D-Related (mHLA-DR), Immunosuppression.*

INTRODUCTION

Coronavirus disease (COVID-19) is caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Clinical manifestations in COVID-19 patients range from asymptomatic, mild, moderate, and severe to critical symptoms.[1] Viral and host factors play a role in SARS-CoV-2 infection. The cytopathic effect of the virus and its ability to defeat immune response determine the severity of infection in COVID-19 patients.[2] Several studies have shown that infection with SARS-CoV-2 could trigger immune dysregulation and immunosuppression that is associated with disease severity in COVID-19 patients.[3-5] Some dysregulation of immune response in COVID-19 patients can be triggered by excessive production of proinflammatory cytokines.[3] Excessive production of these proinflammatory cytokines lead to impaired antigen presentation by inhibiting HLA-DR molecule expression.[6] The HLA-DR molecule is a transmembrane glycoprotein that plays a role in antigen presentation and T-cell activation.[5] The stage of antigen presentation to lymphocytes is critical for activating an adaptive immune response and a sustained immune response to clear pathogens.[7] Zmijewski & Pittet (2020) mentioned that HLA-DR was a class II human leukocyte antigen (HLA) expressed on the surface of antigen-presenting cells (APCs), such as monocytes, differentiated macrophages, dendritic cells, and B lymphocyte cells.[8]

The mHLA-DR is an HLA-DR expressed on the surface of monocytes. Monocytes derived from myeloid bone marrow precursors are considered as the key immune cell that takes part in the infection. Furthermore, it is a first-line cellular response that initiates and promotes an adaptive immune response.[9] The mHLA-DR expressed on $CD14^+$ monocytes plays an important role in synapsing innate immunity with adaptive immunity in infectious diseases.[10]

Downregulation of mHLA-DR expression on $CD14^+$ monocytes represents a state of immunosuppression, also refers to an injury-associated immunosuppression.[9] As in the sepsis case, the expression of HLA-DR in monocytes is expected to be a reliable marker for evaluating immune dysfunction in COVID-19 patients.[11,9] Recent studies confirmed that immune dysregulation and immunosuppression in COVID-19 patients with respiratory failure were associated significantly with downregulation of mHLA-DR.[3]

Monitoring immune dysfunction in COVID-19 patients is still not a complete concern because it is generally not monitored in clinical routines during patient care, especially in Indonesia. Understanding the role of immune cells (mHLA-DR) in the various clinical symptoms of COVID-19 is critical to develop the effective treatment strategies. Accordingly, data regarding the measurement of mHLA-DR expression in COVID-19 in Indonesia needs to be made available. Studies on the measurement of mHLA-DR expression in the COVID-19 population in Indonesia and healthy subjects as a comparison have never been carried out until now. From measurements in healthy subjects, normal values of mHLA-DR expression can be obtained in the population of Indonesia. This study uses the AB/C (antibodies bound per cell) unit of measurement as the result of mHLA-DR expression using the flow cytometry method, in which AB/C describes the quantitative amount of the targeted molecule through its binding to specific fluorescently labeled antibodies.[13] The unit is different from the MFI (*mean fluorescent intensity*) which is often used in the measurement results of flow cytometry method, which can only describe the fluorescence intensity of the targeted cell or molecule. This study aimed to analyze the level of mHLA-DR expression in COVID-19 patients concerning the incidence of immunosuppression by comparing the mHLA-DR expression in COVID-19 patients with healthy subjects.

METHODS

Study Design, Participant, and Data Collection

This cross-sectional study analyzed the differences of mHLA-DR expression in COVID-19 patients and healthy subjects. This study was conducted from June to September 2022 at Dr. Soetomo General Hospital, Surabaya, Indonesia. Inclusion criteria included COVID-19 patients (aged >18 years), confirmed by nucleic acid amplification test (NAAT) from nasopharyngeal swab specimens using real-time PCR (RT-PCR)

and was hospitalized, also healthy subjects (not having an acute illness or comorbidities) aged >18 years who have been confirmed negative through a rapid diagnostic test for SARS-CoV-2 antigen. We excluded COVID-19 patients who receive immunosuppressive drugs, human immunodeficiency virus (HIV) patients or other diseases related to immune system disorders, patients with bacterial sepsis, or critical patients who were not accompanied by COVID-19 pneumonia with ARDS despite showing positive PCR results. The Hematology laboratory examination of the research subjects was evaluated on the blood specimens collected simultaneously for measurement of mHLA-DR expression.

Ethics

This study received approval from the Health Research Ethics Committee of Dr. Soetomo General Academic Teaching Hospital, Surabaya, Indonesia (reference no. 0382/KEPK/III/2022, on March 8, 2022).

Sample Size and Sampling Technique

The method used in collecting samples in this study was consecutive sampling. The sample estimate was calculated using the "compare two means" formula, with $\alpha = 0.05$, $\beta = 0.2$, and ratio groups 1 and 2 (r) = 1. The minimum number of samples required for each group was 13.

The Procedure of mHLA-DR Measurement with Flow Cytometry

The instrument used for measuring mHLA-DR was the BD FACSLyric™ Flow Cytometry. The reagents needed in this measurement were BD Quantibrite™ HLA-DR PE/Monocyte PerCP-Cy5.5 (catalog numb: 340827), BD Anti-Human CD45 FITC (catalog numb: 347463), BD Quantibrite™ Beads PE (catalog numb: 340495), BD FACS Lysing Solution (catalog numb: 349202), and BD FACSFlow™ (catalog numb: 342003). The compensation was done before acquiring the samples using BD CS&T beads (Lot ID: 2031932). The instrument acquisition was set at 10.000 events at high speed.

Sample Preparation

We collected blood samples from COVID-19 patients and healthy subjects with 3 mL volume in EDTA tubes. The blood was stored at 4-8 ^{0}C and immediately processed within 4 hours of withdrawal.[12] Before measuring with flow cytometry, whole blood EDTA (50 µl) was added into a falcon test tube, then stained with the addition of BD Quantibrite HLA-DR PE/Monocyte PerCP-Cy5.5 (20 µl) and Anti-Human CD45 FITC (20 µl). The mixtures were incubated in a dark chamber at room temperature for 25 minutes. The sample was lysed with the addition of BD FACS Lysing Solution (450 µl), homogenized by vortex, and incubated at room temperature in a dark chamber for 10 minutes. Next, Centrifugation at 500 g (0.5 rcf) for 5 minutes after the incubation process was completed before discarding the supernatant. It was then rinsed with 1 mL BD FACSFlow™, homogenized with a vortex and centrifugation again at 500 g (0.5 rcf). After centrifugation, the supernatant was discarded, and BD FACSFlow™ (400 µl) was added, which then was homogenized with a vortex. The sample was ready to run on BDFACSLyric™ Flow Cytometry.

Beads PE Measurement to Calculate AB/C

The number of antibodies bound per cell (AB/C) was quantified by calibration with a standard curve, determined with BD Quantibrite™ Beads PE (Phycoerythrin). One tube of BD Quantibrite™ Beads PE was removed from the foil punch just before they were used. Then it was reconstituted with 0.5 mL BD FACSFlow™, homogenized with vortex, and run on BD FACSLyric™ Flow Cytometry. Each BD Quantibrite™ Beads PE tube contained lyophilized pelletized beads conjugated with four phycoerythrin (PE) grades. Standard curves and linear regression equations were made from Log10 of PE molecules per bead (Low, Med-Low, Med- High, and High) from insert kit (x) against Log10 of PE geo means of 4 populations of PE beads results from running with flow cytometry (y).[13]

mHLA-DR Expression Measurement

The same instrument was used to measure mHLA-DR in the prepared sample. Monocytes were first gated out from other leukocytes expressing CD45 (detected with BD Anti-Human CD45 FITC) based on their CD14 expression (detected with anti-CD14 conjugated with PerCP-Cy5.5 in BD Quantibrite™ HLA-DR

PE/Monocyte PerCP-Cy5.5, anti-CD14 PerCP-Cy5.5 could detect all monocytes [CD14 brightly positive and weakly positive]). The mHLA-DR expression was then measured on their surface (detected by anti-HLA-DR conjugated with PE (phycoerythrin) in BD Quantibrite HLA-DR PE/Monocyte PerCP-Cy5.5) as the median fluorescence intensity (MFI) which was associated with the entire population of monocytes.[12,14,15] A linear regression line equation was used to quantify AB/C (figure 1). Log10 of geo means of the sample measurement results were entered in the linear regression line equation as the "y" value. The equation was solved to find the "x" value as log AB/C, and then the antilog of "x" was determined to get the number of AB/C.[13] The normal values of mHLA-DR expression were >15,000 AB/C.[12]

Statistical Methods

The data obtained in this study were presented in tables and graphs. Data were analyzed by univariate and bivariate analysis using SPSS software (IBM Statistical Package for Social Sciences, version 26.0, Chicago, Illinois). In bivariate analysis, the data obtained were tested for normality with the Shapiro-Wilk test, and then tested for homogeneity with Lavene's test. We used independent T-test to find out whether there was a difference in the data on COVID-19 patients with the healthy group, if the data were normally distributed. In contrast, the Mann- Whitney U was used if the data were not normally distributed. In categorical data, the Chi-Square test was used to see whether there were differences between groups. We considered difference between groups to be statistically significant if p value < 0.05. We presented normally distributed data in the form of mean ± SD, whereas skewed data were described with median and range as a median with the minimum-maximum value (median [min-max]).

RESULTS

Characteristics and Laboratory Data of Research Subject

This study involved 49 subjects consisting of 34 COVID-19 patients and 15 healthy subjects. The characteristics and laboratory data of COVID-19 patients and healthy subjects are presented in **Table 1**.

Table 1. Characteristics and Laboratory Data of Research Subjects.

	Characteristics	Research subjects		p-value
		COVID-19	Healthy	
	n	34	15	
Gender n (%)	Male	20 (58.8)	8 (53.34)	0.633[a]
	Female	16 (41.2)	7 (46.66)	
Age (years) n (%)	18-30	2 (5.89)	6 (40)	<0.001*[b]
	31-40	2 (5.89)	3 (20)	
	41-50	4 (11.76)	4 (26.67)	
	51-60	8 (23.53)	2 (13.33)	
	61-70	7 (20.59)	-	
	71-80	5 (14.70)	-	
	>80	6 (17.64)	-	
	Mean ± SD	62.12 ± 18.35	36.67 ± 9.75	
Conditions n (%)	With Comorbidities	25 (73.53)	-	<0.001*[a]
	Diabetes	9 (26.47)	-	
	Hypertension	7 (20.58)	-	
	Kidney disease	9 (26.47)	-	
	Pulmonary disease	5 (14.7)	-	
	Malignancy	6 (17.64)	-	
	Without Comorbidities	9 (26.47)	15 (100%)	
Laboratory Data (Median [min-max])	Total leukocyte counts (10^3/uL)[#]	9.02 [3.39-26.05]	7.89 [5.4-14.1]	0.288[c]
	Monocytes (%)[#]	7.4 [1.3-15.3]	6.1 [4-12]	0.079[c]
	Lymphocytes (%)[#]	12.95 [1.2-65]	28.3 [9-43]	<0.001*[c]
	Neutrophils (%)[#]	71.1 [4.8-89.4]	60 [48-82]	0.008*[c]

Notes: The results were expressed in mean ± SD, median [min-max], or n (%). Data analysis using Chi-square test (a), Independent T-test (b), and Mann-Whitney U test (c). Significant p- value <0.05. Data is significantly different*. Reference values of total leukocytes: 3.37 – 10.0[#]; monocytes: 4.3 – 10.10[#]; lymphocytes: 23.1 – 49.9[#]; and neutrophils: 39.80 – 70.50[#].

The Results of mHLA-DR Expression Measurement with Flow Cytometry

Based on the normality test using the Shapiro-Wilk test, the data obtained from the mHLA-DR expression in the two study groups were not normally distributed. Hence, the data were presented as median [min-max] and *p*-value was obtained using Mann Whitney U. COVID- 19 patients as the subject of this research consisted of 22 people with mild clinical manifestations (64.7%), six people with moderate clinical manifestations (17.65%), and six people with severe- clinical manifestations (17.65%). In this study, the mHLA-DR expression in COVID-19 patients was 21,201 [2,646-92,384] AB/C, mild clinical manifestations were 40,543.5 [9,797-92,384] AB/C, moderate clinical manifestations were 21,201 [9,831-3,930] AB/C, and severe-critical clinical manifestations were 7,496 [2,646-13,674] AB/C. Healthy subjects in this study consisted of 15 people with 43,161 [25,147-89,846] AB/C mHLA-DR expression. Expression of mHLA- DR in COVID-19 patients was lower and significantly different from the healthy subjects (p = 0.010) (**Figure 2**). The gating strategy of FACSLyric™ Flow Cytometry in measuring the mHLA DR expression of COVID-19 patients with mild, moderate, and severe-critical clinical manifestations, also healthy subjects, can be seen in **Figure 3**.

Table 2. The results of Log PE molecule/beads on BD Quantibrite™ Beads PE insert kit (catalog numb: 340495) and PE geometric means of four populations of PE beads runned by flow cytometry.

No	Beads Population	Log PE/beads (x)	Log PE geo means (y)
1	Low	2.675778342	2.681241237
2	Med-Low	3.729083757	3.726156466
3	Med-High	4.377360899	4.362199639
4	High	4.794738931	4.801328234

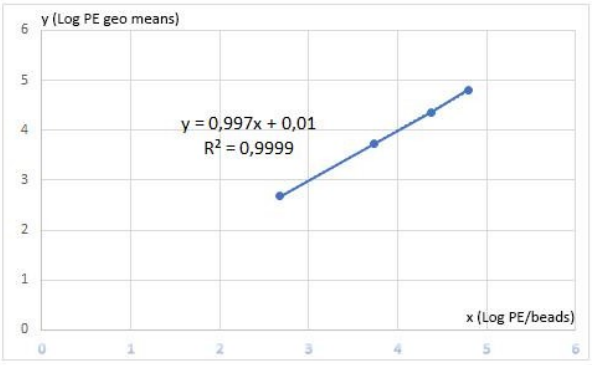

Figure 1. Standard curve and linear regression equation of Log PE/beads against Log PE geo means

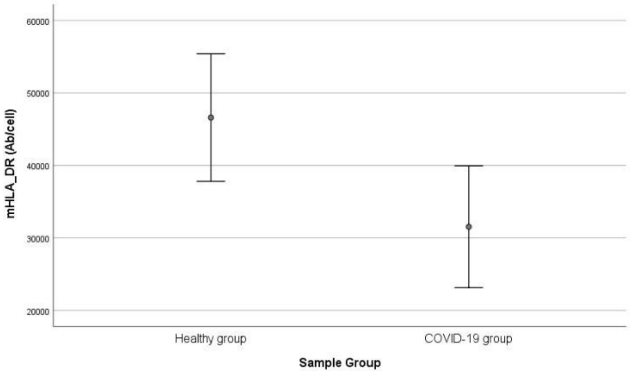

Figure 2. Differences in mHLA-DR expression between the COVID-19 group and thehealthy group

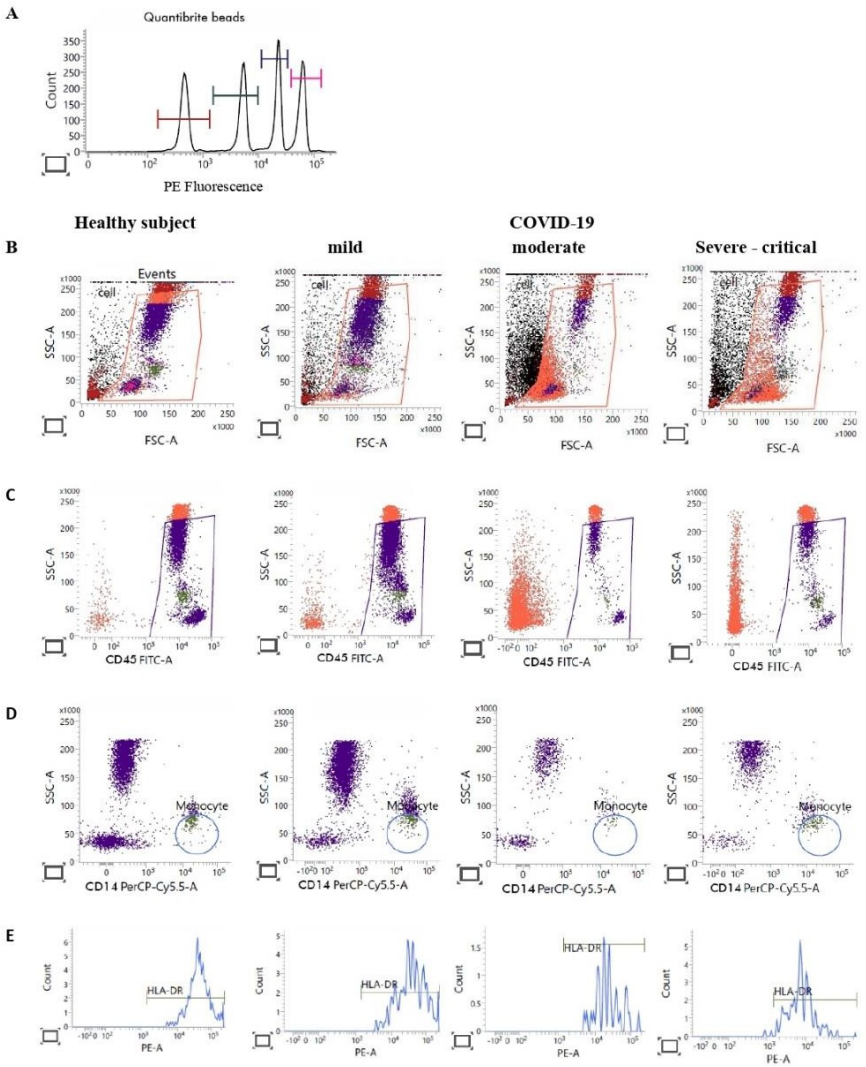

Figure 3. Gating strategy in FACSLyric™ Flow Cytometry on the analysis of mHLA-DR expression of COVID-19 patients with mild, moderate, severe-critical clinical manifestation, and healthy subjects. Beads were gated based on their SSC and FSC characteristics, and PE Fluorescence was plotted (A). Scatter graph of SSC and FSC (B). Patients' leukocytes were gated based on their binding to CD45 FITC-A and the characteristics of SSC and FSC on the scatter graph of SSC to CD45 (C). The patient's monocytes were gated based on their binding to CD14 PerCP-Cy5.5-A and the characteristics of SSC and FSC on the scatter graph of SSC against CD14 (D). The mHLA-DR expression was calculated based on the fluorescence of Anti- HLA-DR PE on monocytes (E). CD45 indicates a cluster of differentiation 45; CD14 indicates a cluster of differentiation 14; PE, phycoerythrin; COVID-19, coronavirus disease 2019; FSC, forward scatter; SSC, side scatter; HLA-DR, human leukocyte antigen-DR; mHLA-DR, monocyte human leukocyte antigen-DR.

DISCUSSION

In this study, the results of measuring the expressions of mHLA-DR in COVID-19 patients and the healthy groups showed a significant difference (p = 0.010), in which the expression of mHLA-DR in COVID-19 patients (21,201 [2,646-92,384] AB/C) were lower than in the healthy groups (43,161 [25,147-89,846] AB/C). This was in line with another study by Bonnet et al. (2021) which stated that the HLA-DR expressed in monocytes was significantly lower in the group of COVID-19 patients, in which the mild case (21,566 AB/C) and severe case (5,926 AB/C) were lower than the healthy subjects (44,544 AB/C)[12]. The expression of mHLA- DR in COVID-19 patients in this study, whose most proportion was the mild case (64.7%), was still at the reference values (>15,000 AB/C). It indicated that the overall mean of immune response in COVID-19 patients was normal and did not lead

to low mHLA-DR expressions in association with immunosuppression. The low expression of mHLA-DR in COVID-19 patients compared to healthy groups could be due to the release of various proinflammatory cytokines, some of which could trigger the low expression of HLA-DR molecules on monocytes through various signal transduction mechanisms.[3,6] Viral load could influence the immune response, including the number of proinflammatory cytokines released.

On the other hand, although the difference could not be seen statistically due to the proportion of the number between groups that did not meet the statistical requirements, the expressions of mHLA-DR in COVID-19 patients with the severe-critical case were lower than in the moderate case. The expressions of mHLA-DR in COVID-19 patients with mild cases were lower than in the healthy groups. Expressions of mHLA-DR in patients with severe-critical clinical manifestation showed the results below the reference values (7,496 [2,646-13,674] AB/C). This was in line with other studies by Spinetti et al. (2020), which stated that the mHLA- DR (AB/C) expression of COVID-19 patients treated in the ICU (severe to the critically ill patients) was significantly lower and below the reference values (9,280 AB/C), compared to COVID-19 patients that were not treated in the ICU (30,900 AB/C).[9] The expression of mHLA-DR which was below the reference values indicated a dysfunctional immune response and led to an immunosuppressed state.[9] SARS-CoV-2 infection, in addition to activating the immune response against the virus, could also cause immune system disorders in severe cases, such as hyperinflammation characterized by excessive release of proinflammatory cytokines resulting in a cytokine storm.[16] One factor that triggered hyperinflammation in COVID-19 patients was the excessive release of the proinflammatory cytokine IL-6.[17] This excessive release of IL-6 could further trigger the low expression of mHLA-DR through signal transduction mechanisms in the STAT3 (signal transducer and activator of transcription 3) signaling pathway.[3,12] Another study conducted by Giamarellos-Bourboulis et al. (2020) reported that high levels of IL-6 were negatively correlated with the levels of mHLA-DR expression in circulating CD14 monocytes.[3] Neutralizing IL-6 via tocilizumab, which could restore HLA-DR expression in monocytes, also supported this hypothesis.[3]

Decreased expression of mHLA-DR in CD14 monocytes indicated a decrease in antigen presentation capacity that caused impaired activation of $CD4^+$ T cells.[5] Decreased expression of HLA-DR led to increased surface expression of negative co-stimulator molecules such asprogrammed death 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), and B-and T- lymphocyte attenuator (BTLA), and their corresponding ligands, such as PD-1 ligand (PD-L1).[8] Increased surface expression of these negative co-stimulator molecules could disrupt innate and adaptive immune responses, such as impaired activation and induction of apoptosis in CD4 T cells that led to immune system dysregulation. Moreover, the presence or absence of immune system dysregulation in patients with COVID-19 also affected or related to the degree of disease severity.[8] Lymphopenia also occurred along with SARS-CoV-2 infection.[18] Decreased expression of mHLA-DR and lymphopenia are some indications of immunosuppressed status.[19] In this study, the results of lymphocyte measurements in COVID-19 patients showed a lymphopenia state and a significant difference with healthy subjects ($p = 0.002$). Lymphopenia in COVID-19 could be caused by several mechanisms, including increased levels of proinflammatory cytokines, which could cause a reduction in the lymphocyte population as the disease progresses[16]. SARS-CoV-2 could directly infect T lymphocyte cells through the ACE2 receptor, which was also expressed in T lymphocyte cells[16]. A damage to lymphatic organs by SARS-CoV-2 infection and an increase in lactic acid, especially in severe degrees, could also inhibit lymphocyte proliferation.[16]

CONCLUSION

The expression levels of mHLA-DR in COVID-19 patients were lower and showed a significant difference compared to the healthy groups. Although a significant difference could not be seen due to the limitation of subjects in this

study, immunosuppression could be indicated by the decrease in mHLA-DR expression below the reference value in severe to critical COVID-19 patients. The limitation of this study was that the clinical degrees of the COVID-19 patients involved were not proportionally (according to the number of statistics) collected due to limited samples collected. It was because the samples were collected when COVID-19 cases were declining in Indonesia. In future research, to see statistical differences in mHLA-DR expression between clinical manifestation groups in COVID-19 and the relationship between mHLA-DR expression and disease severity in COVID-19, it is suggested to group the COVID-19 samples based on their clinical manifestations according to statistical requirements.

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CONFLICT OF INTEREST

The authors ensure that there is no conflict of interest in this study.

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ampiran 1. Sertifikat Layak Etik

KOMITE ETIK PENELITIAN KESEHATAN
RSUD Dr. SOETOMO SURABAYA

KETERANGAN KELAIKAN ETIK
(" ETHICAL CLEARANCE ")

0382/KEPK/III/2022

KOMITE ETIK RSUD Dr. SOETOMO SURABAYA TELAH MEMPELAJARI SECARA SEKSAMA RANCANGAN PENELITIAN YANG DIUSULKAN, MAKA DENGAN INI MENYATAKAN BAHWA PENELITIAN DENGAN JUDUL :

" ANALISIS TINGKAT EKSPRESI MONOCYTE HUMAN LEUKOCYTE ANTIGEN D RELATE (mHLA-DR) PADA PENDERITA CORONAVIRUS DISEASE 2019 (COVID-19) "

PENELITI UTAMA : Dr. Puspa Wardhani, dr., Sp.PK (K)

PENELITI LAIN : 1. Prof. Dr. Aryati, dr., Sp.PK (K)
2. Bambang Pujo Semedi, dr., Sp.An., KIC
3. Musholli Himmatun Nabilah, S.Tr.Kes.
4. Dr. Yetti Hernaningsih, dr., Sp.PK (K)

UNIT / LEMBAGA / TEMPAT PENELITIAN : RSUD Dr. Soetomo

DINYATAKAN LAIK ETIK

Berlaku dari : 08/03/2022 s.d 08/03/2023
Surabaya, 8 Maret 2022
KETUA

(Prof. Dr. Hendy Hendarto, dr., SpOG (K))
NIP. 19610817 201601 6 101

**) Sertifikat ini dinyatakan sah apabila telah mendapatkan stempel asli dari Komite Etik Penelitian Kesehatan*

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