DEMONSTRATION OF SARS-COV-2 IN BONE MARROW CELLS AND CHANGES IN THE HEMATOPOIETIC CELL LINES

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ABSTRACT

Introduction*: SARS-CoV-2 binds to angiotension coverting enzyme-2 (ACE-2) receptors on the surface of the the host cells. ACE receptors are found in bone marrow (BM). SARS-CoV-2 can reduce hematopoiesis in all cell lines by infecting the BM cells directly and by changing the local RAS in addition to the suppression by cytokines during the course of COVID-19. However, there has been no study that could demonstrate the presence of SARS-CoV-2 in BM. Therefore in this study, we aim to demonstrate SARS-CoV-2 in the BM and investigate the changes in the BM of critically ill COVID-19 patients.*

Materials and method*: This study is single center research and six critically ill COVID-19 patients were included in the study. Flowcytometry and RT-PCR were studied in BM aspiration samples. Histopathological evaluation of bone marrow biopsy materials was performed.*

Results*: The most striking finding in BM was reactive plasmacytosis. CD4 / CD8 ratio of 3 patients was reversed (<1). There was an average of 90% CD14+ CD16- classical monocyte. In 1 patient SARS-CoV-2 was demonstrated in BM cells by RT-PCR.*

Conclusion*: To the best of our knowledge, this is the first study which demonstrated SARS-CoV-2 in the BM. In our study revealed an increase in polyclonal plasma cells, CD14+ CD64+ CD68+ active monocytes in BM and demonstrated SARS-CoV-2 in the BM by RT-PCR analysis in 1 patient. Evaluation of flowcytometric analysis of the BM in COVID-19 patients may help the scientists to understand the pathogenesis of SARS-CoV-2 and its effects on hematopoietic cells.*

Keywords: *COVID-19, RT-PCR, Bone Marrow, Flowcytometry.*

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Introduction

SARS-CoV-2 was first detected in China in December 2019 and spread rapidly in all over the world[(1)]. It was declared a pandemic by the World Health Organization (WHO) on 11 March 2020[(2)]. The disease caused by SARS-CoV-2 was officially named as COVID-19. As of 5 June 2020, in the worldwide, there have been 6.485.526 confirmed cases of COVID-19, including 386.704 deaths, as reported to WHO[(3)].

Recent studies revealed that the S spike of SARS-CoV-2 binds to angiotension coverting enzyme-2 (ACE-2) receptors on the surface of the the host cells and this binding leads to the entry of the virus into the host cells[(4)]. Alveolar epithelial type II cells in lung highly express ACE-2 and are the main target of SARS-CoV-2[(5)].

ACE-2 receptor is also expressed in many extrapulmonary tissues including heart, kidney, endothelium, and intestine[6]. In addition to these tissues, studies have shown that ACE receptors are also found in the bone marrow (BM)[7].

SARS-CoV-2 causes hypercytokinemia in the body and some of these cytokines suppress the function of BM. Leukopenia and lymphopenia are the two important hematological findings observed during the couse of COVID-19. Furthermore, lymphopenia has been reported to be associated with disease severity, especially in critically ill COVID-19 patients[8]. The existence of a local renin-angiotensin system (RAS) specific to the BM microenvironment had been shown and it regulates hematopoiesis via autocrine, paracrine, and intracrine pathways[9, 10]. Angiotensin II (Ang-II) is the main RAS effector mediator, and it demonstrates its hematopoietic effects by stimulating angiotensin receptor type 1 (AT1) and type 2 (AT2) in the BM microenvironment. ACE triggers primitive stem cells into S phase and Ang-II activates AT1 to stimulate the JAK-STAT pathway promoting hematopoiesis[11, 12]. In addition, CD34+ hematopoietic cells contain AT1 receptors that augment production of hematopoietic progenitors of BM[13]). ACE-2 is expressed in CD34+ cells and Ang-(1-7) stimulate proliferation of CD34+ cells[14, 15].

As ACE receptors are found in the BM, SARS-CoV-2 can reduce hematopoiesis in all cell lines by infecting the BM cells directly and by changing the local RAS in addition to the suppression by cytokines during the course of COVID-19. However, there has been no study that could demonstrate the presence of SARS-CoV-2 in BM. Therefore in this study, we aim to demonstrate SARS-CoV-2 in the BM and investigate the changes in the BM of critically ill COVID-19 patients.

Materials and methods

Patients

Six critically ill, laboratory confirmed COVID-19 patients who were admitted to intensive care unit of Sakarya University Medical Faculty between 1-10 May 2020, were included in the study. Critical COVID-19 was defined as the presence of respiratory failure, septic shock and/or multiple organ dysfunctions.

Bone Marrow Biopsy

BM biopsy was performed to all patients in the study. Flowcytometry and reverse transcription polymerase chain reaction (RT-PCR) were studied simultaneously in the BM aspiration samples. Trucut biopsy samples of the patients were embedded in paraffin blocks after decalcification and routine tissue processing. Sections 4 μm in thickness made from paraffin blocks histochemically with Haematoxylin-Eosine (H&E), Masson's trichrome (MT), reticulin stain and Prussian blue, and also immunohistochemically with CD3, CD4, CD8, CD 20, CD33, CD 34, CD56, CD117, CD 138, Kappa, Lambda, MPO, lysozyme and GFA It was stained with and then evaluated under a light microscope.

Flowcytometry

Samples of EDTA anticoagulated BM aspiration (2 mL) were collected and all samples were tested within 6 hours. Lymphocyte and monocyte populations were analyzed by multiparameter flowcytometry with 6-color analyses. CD3+/CD4+/CD8+ T-cells and CD14+/CD16+/CD64+/68+ monocytes examined. T cells subsets were determined using antibodies as; CD3 (FITC) / CD4 (PeCY7) / CD8 (APC Cy7) / CD45ROPE / CD45RA (APC / CD197 (PerCpCy5,5) / CD25 (APC Cy7) (BD Biosciences, AB) Monocytes subsets were determined using antibodies as; CD14 (PerCpCy5, 5) / CD16 (FITC) / CD64 (PE) / CD68 (PeCY7) / CD45 (APC) (BD Biosciences, EU) and monocyte subsets are identified according to the surface expression of CD14++ CD16– (monocyte 1), CD14+ CD16+ (monocyte 2) and CD14-CD16++ (monocyte 3). The data were obtained and analyzed with Cell Quest software (Becton Dickinson).

Nucleic Acid Isolation and Reversed Transcriptase Polymerase Chain Reaction (RT-PCR)

BM aspiration and simultaneous peripheral blood samples were taken into EDTA tubes for nucleic acid isolation and RT-PCR. All samples were kept in the refrigerator at 2-8 °C and quickly transported to the laboratory. The samples were sent to the laboratory in accordance with the cold chain rules with the triple transport system, following the infection prevention and control procedures. All samples were centrifuged at 4500 g for 4 minutes. 400ul of the supernatant was taken and loaded on the BioRobot EZ1 (Qiagen, Germany) device and 60ul solution was taken. The peripheral blood samples were separated from the plasma part after centrifugation and made from isolation plasma. Total nucleic acid isolation was performed with

EZ1 Virus Mini Kit v2.0 (Qiagen, Germany) in accordance with the company's recommendations. For RT-PCR study, a total of 20 μl reaction volume was generated by genesig RT- PCR COVID-19 (Primer Design, UK) kit with 10 μl master mix, 2 μl primerand 8 μl mold RNA persample. Curves with a Cycle Threshold (CT) value of less than 45 at the end of there action and sigmoidal observed curves were interpreted as positive for SARS-CoV-2 RNA.

Results

Patients

The demographic characteristics of the patients are given in Table 1. At least one chronic disease was present in 5 patients. Day of illness was defined as the time from hospitalization to the day of BM biopsy and it's mean value was 27 day. At the time of BM biopsy, 4 patients were PCR negative and 2 patients were still PCR positive. All patients received hydroxychloroquine, oseltamivir, favipiravir, low molecular weight heparin as COVID-19 treatment.

	Patient 1	2	3	4	5	6	Mean-value
Sex	M	M	M	M	F	F	
Age	75	58	77	91	82	82	77.5
Coexisting chronic diseases	N	HT M	ALZ	HT, İHD, CHF	DM	ALZ	
Blood Group	B	B	A	A	AB	A	
Status	İCU/ İ	İCU/ HFNO	İCU/ HFNO	İCU/ HFNO	İCU/ İ	İCU/ İ	
Day of Illness	37	15	33	20	42	19	27
Nasal Swab PCR	Day1 + Day 4 + Day 10 + Day 32 – (BAL)	Day 1 + Day 8 +	Day 1 + Day 30 – Day 33 +	Day 1 + Day 12 –	Day 1 + Day 13 – Day 20 –	Day 1 + Day 11 – Day 17 –	
Threatments	Hydroxychloroquine Oseltamivir Favipiravir Prednisolone İVİG Dipyridamole LMWH CP (400ml)	Hydroxychloroquine Oseltamivir Favipiravir Azithromycin LMWH CP (400ml)	Hydroxychloroquine Oseltamivir Favipiravir Azithromycin LMWH	Hydroxychloroquine Oseltamivir Favipiravir Azithromycin LMWH	Hydroxychloroquine Oseltamivir Favipiravir Azithromycin LMWH Prednisolone Kaletra Plasmapheresis	Hydroxychlo-roquine Oseltamivir Favipiravir LMWH	
Current status as of May31, 2020	Exitus	Discharged home	Discharged home	Discharged home	Exitus	Exitus	

Table 1: Clinical characteristics of patients.
İCU: İntensive care unit, HFNO: High Flow Nazal Oxygen, İ: intubated, M: Male, F: Female, N: Not, HT: Hypertension, ALZ: Alzheimer, İHD: İschemic Heart Disease, CHF: Congestive Heart Failure, DM: Diabetes Mellitus, BAL: Broncho Alveolar Lavage, İVİG: İntravenous immuneglobulin, LMWH: Low Molecular Weight Heparin, CP: Convelesan Plasma

Laboratory

Laboratory results of the patients are given in Table 2. At the time of admission, the lymphocyte countsof 5 patients were below 1.0×10^9/L and mean lymphocyte count were below 0.5×10^9/L. After an average of 27 days of hospitalization, the mean lymphocyte count increased to 1.02×10^9/L Although hemoglobin levels at the time of admission were normal, it was observed that mean hemoglobin levels decreased to 9g/dl in the following period. The platelet counts were normal in all patients.

Patient	1		2	3			4		5	6			Mean values	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
WBC ($\times10^3$/L) (3.5-9.5x10^3/L)	6.7	17.4	9.5	15.2	4.9	7.2	10.5	9.02	10.1	11.4	9.02	8.6	8.45	11.47
Neutrophils (1.8-6.3x10^3/L)	5.5	12.2	7.5	13.2	3.8	5.4	9.8	7.01	8.8	10.6	8.3	7.8	7.2	9.3
Lymphocytes (1.1-3.2 x10^3/L)	0.6	1.94	1.1	1.28	0.5	1.15	0.4	1.0	0.48	0.3	0.2	0.5	0.54	1.02
Monocyte (0-0.8x10^3/L)	0.5	0.75	0.4	0.66	0.5	0.6	0.1	0.89	0.6	0.28	0.4	0.1	0.41	0.54
Eosinophils (0-0.45x10^3/L)	0.003	2.48	0.07	0.006	0.01	0.07	0.003	0.02	0.09	0.08	0.001	0.06	0..2	0.43
Basophils (0-0.2x10^3/L)	0.02	0.09	0.04	0.062	0.02	0.05	0.02	0.09	0.04	0.01	0.02	0.02	0.02	0.05
Hemoglobin (13-17.5g/dl)	15.4	8.7	14.9	13.5	11.1	9.3	15	10.4	13	6.1	11.6	8.4	13.5	9.4
Platelets (125-350 x10^3/L)	218	298	118	308	181	395	152	203	216	35	142	142	171	230
C-ReactiveProtein (0-5 mg/L)	182	66	190	77	13.9	67.3	354	224	215	177	44	174	166	130
Sedimentation rate (0-15)(1.hour)	107	111	23	61	94	115	41	104	72	60	8	9	57	76
Procalcitonin (0-5 ng/ml)	0.5	0.5	0.14	0.09	0.2	0.14	1.7	1.78	1.15	18.5	12.5	0.06	2.6	3.5
Ferritin (21-274 mg/L)	2754	2130	408	372	231	336	>2000	1353	927	3373	114	868	1072	1405
Lactatedehydrogenase (120-250 U/L)	537	335	718	484	211	216	516	360	327	501	410	306	453	367
D-DIMER (0-500 mg/L)	490	9360	3820	1180	2510	1730	6560	2180	3400	6170	9920	3760	4450	4063
Fibrinogen (mg/dL)	452	409	360	434	434	472	380	697	394	452	221	348	373	468
INR	1.4	1.4	1.1	1.19	1.1	1.2	1.2	2.9	1.05	1.19	1.29	1.24	1.19	1.52
Lactate (mmol/L)	2.3	1.2	1.8	1.5	2.1	4.2	5.1	2.1	2.3	2.4	3.1	3	3.6	2.4
Troponin-I (ng/L)	13.4	25.8	13.9	11.7	22.2	19	60	49.6	3563	330	2384	3521	1009	659
Bone Marrow PCR		+		-		-		-		-		-		

Table 2: Laboratory features of patients.
A: Day of hospitalization, B: Day of bone marrow biopsy

Detection of SARS-CoV-2 in Bone Marrow

In 1 patientSARS-CoV-2 was demonstrated in BM cells byRT-PCR. The other 5 patients' BM samples were RT-PCR negative.

Pathology

Histopathological examination of BMsamples were evaluated (Table 3).

	Age/ Sex B.C	QUA	CEL	MD	RD	CF	ID	E/M	CD 15E	Kp / Lm	CD 20	CD 3	CD 4	CD 8	CD 3/20	CD 4/8	CD 56	CD 34	CD 33	MPO	LYZ	GPA	CD 117
Patient 1	75 M ANM	PE	NC	N	2	-	Gr4	1/10	15%	P	0.57	3.1	1.91	4.65	5.4	0.4	-	2%	25%	25%	80%	15%	
Patient 2	58 M ANM	E	NC	N	2	-	ND	1/10	7%	P	2.2	9.1	3.8	5.21	4.1	0.7	-	1%	35%	15%	80%	18%	
Patient 3	77 M ANM	PE	NC	N	2	-	ND	1/5	30%	P	1.66	6.5	10	4.1	4	2.4	-	2%	30%	35%	55%	20%	
Patient 4	91 M ANM	E	SHC	N	2	-	ND	1/7	20%	P	10.3	38.1	6.8	9.4	1.6	0.7	-	1%	35%	32%	40%	22%	
Patient 5	82 F ANM	PE	SHC	SI	1	-	Gr2	1/4	13%	P	2.85	15.5	6.3	10	5.4	0.6	-	1%	35%	35%	50%	20%	
Patient 6	82 F ANM	E	NS	N	2	-	ND	1/6	16%	P	1	9.2	5	4.2	9.2	1.2	-	1%	20%	60%	50%	25%	

Table 3: Distribution of histopathological and immunohistochemical findings.
ANM: Anemia, CEL: Cellularity, CF: Collagen fibrosis, B.C: Blood Count, E: Enough, E/M: Erythroid myeloid ratio, GPA: Glycophorin A percentage, Gr: Grade,
ID: Iron deposits, Kp/Lm: Kappa/Lambda clonality, LYZ: Lysozyme percentage, M: Monoclonal, MD: Megakaryocyte density, N: Normal, ND: No deposit,
QUA: Qualification, P: Polyclonal, PE: Partly enough, RD: Reticulin density, NC: Normocellular, SHC: Slightly hypercellular, SI: Slightly increased

Four of the cases were normocellular and two were mildly hypercellular. No pathology was detected in megakaryocytes, except for a slight increase in number in one case. None of the cases had reticulin fiber increase and collagen fibrosis. Grade 4 iron accumulation was observed in one case. Iron accumulation was not detected in other patients' biopsies. Erythroid/myeloid ratio in patients ranged from ¼ to 1/10. The most striking finding in BM was reactive

plasmacytosis, at rates ranging from 13 to 30% in five patients (Figure 1A). The lymphoid cells evaluated by CD3, CD4, CD8 and CD 20 had a CD3 / CD20 ratio of 5.4 /4.1/4/1.6/ 5.4/9.2, respectively (Figure 1B and Figure 1C). Also CD4 / CD8 ratio was calculated as 0.4 /0.7/2.4//0.7/0.6/1.2.No staining in favor of NK cell was detected in any case with CD56.

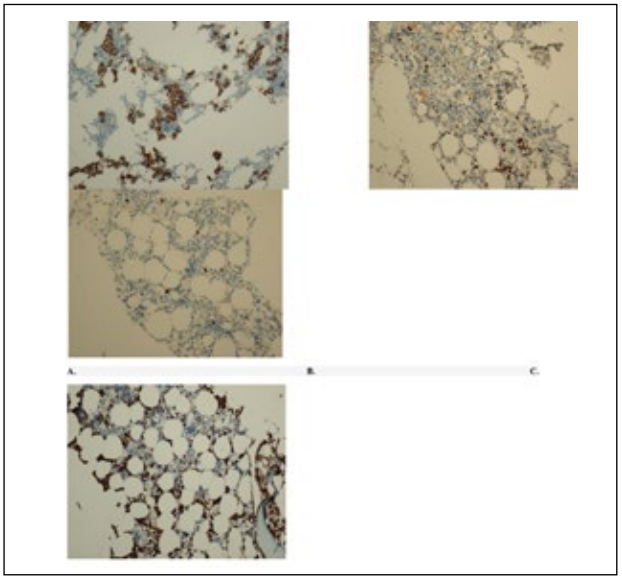

Fig. 1: Bone marrow biopsy.
A: Wide spread staining with CD138, B: CD3 positive T lymphocytes, C: CD20 positive B lymphocytes
D: MPO positivity in myeloid serial cells, E: Glycophorin A positive erythroid serial cells.

The ratio of CD 34+ cells ranged from 1% to 2%. When myeloid cells were evaluated immunohistochemically, staining was observed between 15-60% with MPO (Figure 1D), 20-35% with CD33, 40-80% with lysosim, 15-25% with glycophorin A (Figure 1E). No staining was detected with CD 117.

Flowcytometry

Patient	1	2	3	4	5	6	Mean values
Lymphocytes%	6.4	5.1	4.9	6.1	10.4	12.2	7.5
CD4+%	18.4	34.9	16.8	23.4	6.9	4.9	17.2
CD8 +%	33.5	15	6.6	21.9	13.6	7.3	16.3
CD4 +CD8 +%	0.2	0.9	0.2	1.2	0.1	0	0.43
CD4 / CD8	0.54	2.3	2.24	1.06	0.5	0.67	1.21
CD 45Ro+ CD4 +% *(CD4 memory)*	0.2	36.2	70.9	63.4	86	61.4	53.01
CD 45Ro+CD8 +% *(CD8 memory)*	49.2	32.5	61.4	45.4	42.1	38	44.7
CD45Ra+CD197 + *(T-naive CD4)*	39.2	66.8	27.6	23.5	52.8	33.3	40.5
CD45Ra- CD197 + *(Central memory-CD4)*	60.8	33.2	71.7	76.5	37.5	66.7	57.7
CD45Ra-CD197 - *(Effectorememory CD4)*	0	0	0.7	0	8.3	0	1.5
CD3+ CD4+ CD25+	39.6	65.5	57	24.1	0	29.4	35.9
CD14++ CD16- *(Monocyte 1)*	98.7	90.7	79.2	98.7	93.3	79.5	90
CD14 + CD16- *(Monocyte 2)*	1.3	0.6	0.3	1.3	6.7	7.7	2.98
CD14- CD16++ *(Monocyte 3)*	0	0	0	0	0	2.6	0.43
CD14 +CD64+ CD68+ *(Active monocyte)*	99.6	99.7	83.3	98.9	98.8	72.3	92.1

Table 4: Flowcytometric findings.
A: Wide spread staining with CD138, B: CD3 positive T lymphocytes, C: CD20 positive B lymphocytes
D: MPO positivity in myeloid serial cells, E: Glycophorin A positive erythroid serial cells.

Flowcytometric evaluation of the BM is given in Table 4. The mean lymphocyte ratio was found to be 7.5% and mean CD4 / CD8 ratio of the patients was 1.2. Three (50%) patient's (Patient no 1,5, 6) CD4 / CD8 ratio was reversed (<1). The majority of the CD4+ T cells (57.7%) were central memory CD4 cells. Considering the classification of monocytes, there was an average of 90% CD14+ CD16- (monocyte type 1) classical monocyte dominance. CD14+ CD64+ CD68+ monocytes were present at an average of 92%.

Discussion

Changes in hemogram parameters are common in espcecially severe and critically ill COVID-19 patients. Previous studies reported that lymphopenia can be observed in 50-80% of COVID-19 patients[16]. Liu et al. suggested that neutrophil count increases and the number of lymphocytes in peripheral blood decreases in critical patients, and the increase in neutrophil / lymphocyte ratio may have a prognostic value[17]. Fan et al. showed that intensive care patients have deeper anemia and lymphopenia and much more neutrophilia than non-intensive care patients[18]. In our study, in accordance with the literature, lymphopenia, neutrophilia and deep anemia were the most prominent hemogram changes in our intensive care patients.

In our study, the most striking finding in the histopathological examination of the critically ill COVID-19 patients's BM was a 15-30% increase in polyclonal plasma cells. IL-6 plays a critical role in B cell differentiation to plasma cells and elevated levels of IL-6 was shown in COVID-19 patients[19]. Therefore, the increase of polyclonal plasma cells in the BM may be attributed to the increased levels of proinflammatory cytokines especially IL-6 leading to proliferation and activation of plasma cells. In addition, the increase in the number of polyclonal plasma cells in the BM may be a sign of producing antibodies against SARS-CoV-2.

Previous studies revealed a suppression of T lymphocytes, including CD4+ T and CD8+ T lymphocytes, and this suppression was more pronounced in critically ill patients[20-23]. On the otherhand, Ganji et al. showed an increase in CD8 + T lymphocytes[24]). Similar to their result, we observed an increase in the number of CD8+ T lymphocytes. CD4 / CD8 ratio was <1 in 4 patientsand all patients with reversed CD4 / CD8 ratio died.

Wen et al. showed an increase in the number

of CD14+, CD16- classical type monocytes wheras Zhou et al. showed an increase in the number of CD14+, CD16+ inflammatory monocytes[25, 26]. In our study CD14+ CD64+ CD68+ active monocytes, which will serve as macrophages in peripheral blood, increase by an average of 92% in BM.

In a previous study, researchers evaluated the BM tissue in autopsies of COVID-19 patients and could not demonstrate SARS-CoV-2 in the BM[27]. In our study, in 1 patientSARS-CoV-2 was demonstrated in BM cells by RT-PCR.

Conclusion

Our study revealed an increase in polyclonal plasma cells, CD14+ CD64+ CD68+ active monocytes in BM and demonstrated SARS-CoV-2 in the BM by RT-PCR analysis in 1 patient. To the best of our knowledge, this is the first study which demonstrated SARS-CoV-2 in the BM. In conclusıon, evaluation of flowcytometric analysis of the BM in COVID-19 patients may help the scientists to understand the pathogenesis of SARS-CoV-2 and its effects on hematopoietic cells.

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Abbreviations: ACE-2, angiotension coverting enzyme-2; Ang-II, Angiotensin II; BM, bone marrow; RAS, renin-angiotensin system; RT-PCR, reverse transcription polymerase chain reaction

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