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BRIEF CONCLUSIVE REPORT

Mismatch between circulating cytokines and spontaneous cytokine production by leukocytes in hyperinflammatory COVID-19

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Abstract

The disease COVID-19 has developed into a worldwide pandemic. Hyperinflammation and high levels of several cytokines, for example, IL-6, are observed in severe COVID-19 cases. However, little is known about the cellular origin of these cytokines. Here, we investigated whether circulating leukocytes from patients with COVID-19 had spontaneous cytokine production. Patients with hyperinflammatory COVID-19 ($n = 6$) and sepsis ($n = 3$) were included at Skåne University Hospital, Sweden. Healthy controls were also recruited ($n = 5$). Cytokines were measured in COVID-19 and sepsis patients using an Immulite immunoassay system. PBMCs were cultured with brefeldin A to allow cytokine accumulation. In parallel, LPS was used as an activator. Cells were analyzed for cytokines and surface markers by flow cytometry. High levels of IL-6 and measurable levels of IL-8 and TNF, but not IL-1β, were observed in COVID-19 patients. Monocytes from COVID-19 patients had spontaneous production of IL-1β and IL-8 ($P = 0.0043$), but not of TNF and IL-6, compared to controls. No spontaneous cytokine production was seen in lymphocytes from either patients or controls. Activation with LPS resulted in massive cytokine production by monocytes from COVID-19 patients and healthy controls, but not from sepsis patients. Finally, monocytes from COVID-19 patients produced more IL-1β than from healthy controls ($P = 0.0087$) when activated. In conclusion, monocytes contribute partly to the ongoing hyperinflammation by production of IL-1β and IL-8. Additionally, they are responsive to further activation. This data supports the notion of IL-1β blockade in treatment of COVID-19. However, the source of the high levels of IL-6 remains to be determined.

KEYWORDS
cytokine storm, monocytes

1 | INTRODUCTION

The emerging coronavirus SARS-CoV-2 and the subsequent disease COVID-19 (coronavirus disease 2019) has had severe impact worldwide. Clinical manifestations range from asymptomatic diseases to severe disease and death. Hyperinflammation and subsequent cytokine storm, with high systemic levels of proinflammatory cytokines (e.g., IL-6 and TNF) are observed in patients with severe COVID-19.[1] In addition, these patients display some clinical and laboratory signs resembling macrophage activation syndrome (MAS).[2] Hence, treatments for MAS with IL-6 or IL-1β blockade have been suggested for COVID-19 patients[2] and several clinical trials are ongoing

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(www.clinicaltrials.gov). However, the primary cellular source of the circulating cytokines is unknown. Circulating immune cells, primarily monocytes, are generally considered as potent cytokine producers, including cytokines such as IL-1β, IL-6, and TNF. In sepsis, high levels of proinflammatory cytokines are observed in plasma but circulating leukocytes do not seem to be the major cellular source.[3] In addition, monocytes from septic patients seem unresponsive to further activation of cytokine production using LPS.[3] This so called immunoparalysis is thought to contribute to the secondary infections and complications after sepsis.[4] In hyperinflammatory COVID-19 patients, high levels of circulating cytokines are measured but little is known concerning the contribution of circulating leukocytes to cytokine production. Thus, we investigated spontaneous cytokine production by monocytes, T and B cells from COVID-19 patients with hyperinflammation.

2 | RESULTS AND DISCUSSION

Six patients with severe COVID-19 with signs of hyperinflammation (C-reactive protein [CRP] >15 mg/dL) and no confirmed or suspected superinfection treated at Skåne University Hospital were enrolled in the study. Three patients with blood-culture confirmed sepsis were enrolled as sepsis controls. Five healthy volunteers served as healthy controls. The healthy controls were without any history or symptoms resembling COVID-19 and negative for COVID-19 antibodies when resampled approximately 8 wk after the inclusion in the study (one control had antibodies controlled when included in the study and was tested negative and was not retested after 8 wk). Patient characteristics are summarized in Table 1. Ethical approval was obtained from the Swedish Ethical Review Authority (2019-05146).

Cytokine concentrations of IL-1β, IL-6, IL-8, and TNF were measured in plasma and serum samples from COVID-19 and sepsis patients. Detectable levels (median pg/ml) of circulating IL-6 (158.0), IL-8 (29.1), and TNF (18.4), but not IL-1β were seen in all COVID-19 patients (Fig. 1A). In the three sepsis patients, we observed measurable levels of all cytokines: IL-1β (7.7), IL-6 (264.4), IL-8 (14.9) and, TNF (55.80).

Spontaneous cytokine production by specific leukocyte populations was analyzed using flow cytometry following culturing of PBMCs with brefeldin A. CD14 was used to identify monocytes, CD3 for T cells and CD19 for B cells. Gating strategy can be found in Supporting Information Fig. S1. CD14$^+$ monocytes from COVID-19 patients had increased spontaneous production (median percentage positive cells) of IL-1β (11.4 vs. 0.5, $P = 0.0043$) and IL-8 (33.5 vs. 9.2, $P = 0.0043$) compared to healthy controls. To our surprise there was no increased production of either IL-6 (1.2 vs. 0.84, $P \geq 0.99$) or TNF (4.9 vs. 1.2, $P = 0.66$) compared to healthy controls despite elevated levels of IL-6 in the circulation (Fig. 1B). The total median fluorescence intensity (MFI) values of each cytokine and the MFI of the positive populations are found in Supporting Information Fig. S2. There was no measurable spontaneous production of any cytokine in CD3$^+$ T cells or CD19$^+$ B cells in any patient group (Supporting Information Fig. S3A and C).

Next we investigated the cytokine production in response to stimulation. Upon activation with LPS, a massive production of all measured cytokines was observed in both COVID-19 patients as well as in healthy controls, but not in septic patients (Fig. 1C). Interestingly enough, the production of IL-1β was specifically increased in COVID-19 patients compared to healthy controls (median percentage positive cells: 76.32 vs. 42.41, $P = 0.0087$). There was no significant difference in production of IL-6, IL-8, and TNF between COVID-19 patients and healthy controls. Monocytes from patients with sepsis did not respond to LPS activation to the extent of monocytes from COVID-19 patients or from healthy controls. This diminished response to LPS in sepsis is well known and called immunotolerance or immunoparalysis although the mechanisms have still not been fully elucidated.[5-9] Studies have implicated various cytokines and intracellular proteins/messengers, for example, IL-10, IRAK4, IRAK-M, TREM-1, miRNA, SHIP-1, A20, IL-27 but also down-regulation of HLA-DR.[7,10-15] Further studies have also indicated that defects in energy metabolism may contribute to the paralysis[16,17] and a very recent study demonstrated that loss of metabolic plasticity and antimicrobial efficacy including oxidative burst characterized monocytes of the immunotolerant phase.[18] However, there are also reports that very low doses of LPS may have the opposite effect and prime the response to LPS.[19-21] Various drugs (e.g., IFNγ and acetylsalicylic acid) have been tested to revert the immunoparalysis in sepsis.[14,22-24] Furthermore, sepsis also induces a shift in the monocyte subsets favoring the expression of monocytes resembling the M2 subtype. It is, however, still debated if endotoxin-tolerant macrophages are of the M2 phenotype or if they are from a more complex phenotype.[25-28]

The response of T cells was also studied. Activation using PMA and ionomycin resulted in production (median percentage positive cells) of IL-1β (6.9) and TNF (59.3) by T cells in COVID-19 patients ($n = 4$), but neither IL-6 nor IL-8 was produced (Supporting Information Fig. S3B and D). PMA and ionomycin activation could not be performed in two patients with COVID-19.

We also investigated the contribution of cells negative for the cell markers CD3, CD14, and CD19, to cytokine production (Supporting Information Fig. S4). We did not find any noticeable cytokine production by this population, either spontaneous or upon LPS activation. This population consists of several cell types, for example, NK cells and basophils. However, this population of heterogeneous cells do not account for the observed high circulating levels of IL-6 in COVID-19 patients.

Here, we describe a mismatch between circulating levels of cytokines and spontaneous cytokine production by leukocytes from COVID-19 patients. We specifically analyzed monocytes, T and B cells, and found that monocytes spontaneously produced IL-1β and IL-8, but to our surprise, only minimal amounts of IL-6 or TNF were produced, although the patients display elevated levels of circulating IL-6. As the elevated level of IL-6 in the circulation from patients with highly inflammatory COVID-19 disease does not seem to be of leukocyte origin, we speculate that it might be of endothelial or alveolar macrophage origin. High levels of circulating cytokines have been described in patients with COVID-19 and hence the term viral sepsis

TABLE 1 Clinical data of the included patient

	COVID-19 patients						Sepsis patients		
Patient number	1	2	3	4	5	6	1	2	3
Age	82	74	64	75	73	64	79	67	62
Sex	M	M	M	M	M	M	M	F	M
Disease duration at sampling (days)	10	14	14	13	3	17	3	4	7
Days in hospital before sampling	4	2	5	4	2	3	2	3	4
Oxygen delivery	Oxymask (5 l/min)	Oxymask (8 l/min)	HFNC (55 l, 77%)	Ventilator FiO2 35%	Ventilator FiO2 30%	Ventilator FiO2 40%	Mask (4 l/min)	Mask (1 l/min)	Ventilator (FiO2 35%)
Mortality (days past sampling)	1	–	–	–	–	8	–	–	–
COVID-19-treatment (days started before sampling)	Chloroquine (2 d) Prednisolone 7.5 mg (due to polymyalgia rheumatica),	–	Chloroquine (4 d)	–	–	–	–	–	–
Steroids for sepsis treatment	–	–	–	–	–	–	–	Hydrocortisone 50 mg TID started 24 hrs before sampling	–
Blood culture	Negative at admission	Negative at admission	Negative at admission	Negative at admission	Negative at admission	Negative at admission	E. coli	Klebsiella variicola	S. aureus and S. dysgalactiae
Other microbiology	Negative for RSV, influenza and metapneumo-virus at admission	Negative for RSV, influenza and metapneumo-virus at admission	Negative for RSV, influenza and metapneumo-virus at admission	Negative for RSV, influenza and metapneumo-virus at admission	Negative for RSV, influenza and metapneumo-virus at admission	Negative for RSV, influenza and metapneumo-virus at admission	ND	ND	ND
CRP (mg/dL)	17.5	19.2	26.1	24.6	19.7	22.5	29.1	30.1	29.2
PCT (μg/l)	0.72	3.5	ND	6.6	0.53	0.33	ND	ND	ND
Lymphocytes ×10⁹/l	0.4	1.2	0.8	ND	1.5	N/A	ND	ND	ND
LD (IU/l)	500	765	318	376	647	353	ND	ND	ND
D-dimer (mg/l FEU)	2.9	3	0.87	1	2.4	3.2	ND	ND	ND
Ferritin (μg/l)	1171	3949	1973	2093	1907	3314	ND	ND	ND

HFNC, high flow nasal cannula; CRP, C-reactive protein; PCT, procalcitonin; LD, lactate dehydrogenase; RSV, respiratory syncytial virus; and TID, 3 times daily.
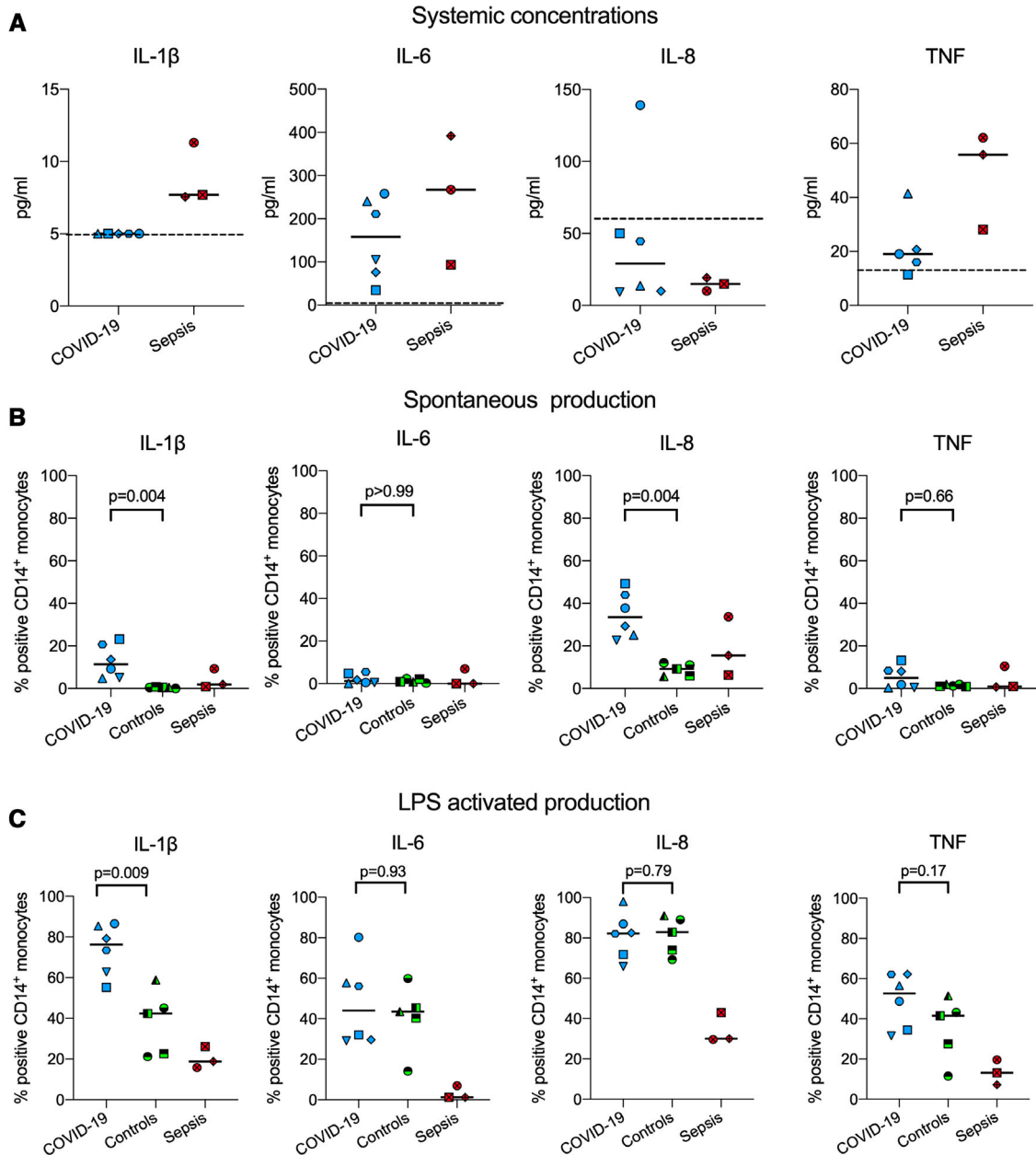
FIGURE 1 Cytokine pattern in circulation and production by monocytes from hyperinflammatory coronavirus disease 2019 (COVID-19) patients. (**A**) Systemic cytokine concentrations (IL-1β, IL-6, IL-8, and TNF) was measured in patients with COVID-19 and sepsis ($n = 3$) using the Immulite 1000 Immunoassay System (Siemens Healthineers, Erlangen, Germany) according to the manufacturer's instructions ($n = 6$ for IL-6 and IL-8, and $n = 5$ for IL-1β, and TNF for COVID-19). The dotted line represents the upper reference values of each cytokine in healthy controls. The detection limit for IL-1β is <5 pg/ml. (**B**) PBMCs from COVID-19 patients ($n = 6$), healthy controls ($n = 5$), and sepsis ($n = 3$) patients were isolated and cultured in the presence of brefeldin A for cytokine accumulation. The cells were stained to identify leukocyte populations and cytokine production. COVID-19 patients' monocytes (CD14$^+$) had a high ongoing production of IL-1β and IL-8 and low of TNF and IL-6, compared to controls. (**C**) Activation of cytokine production using LPS resulted in massive production of all cytokines by monocytes in COVID-19 and control PBMCs, but markedly less in monocytes from septic patients. Line at median. Statistical analysis between groups were tested with the Mann-Whitney U-test

has been proposed.[29] Many patients with severe COVID-19 develop thromboembolic events,[30] suggesting endothelial activation. Previous studies on cytokine production by circulating PBMCs have been conflicting both demonstrating both a lack of IL-6 as well as IL-8 production in PBMCs,[31] whereas another study, only investigating IL-6 and TNF found increased production of IL-6 by both monocytes and T cells.[32]

In addition, we found that monocytes from COVID-19 patients retain their capacity to produce IL-1β, IL-6, IL-8, and TNF when stimulated by LPS. In accordance to our results, a recently published article showed that PBMCs from COVID-19 patients retained their capacity to produce IL-1β, IL-6, and TNF when stimulated by LPS, in contrast to PBMCs from patients with bacterial pneumonia or H1N1.[33] However, neither the cellular origin of the cytokines

nor the spontaneous cytokine production was addressed in that study. Importantly, our results indicate a hyperresponsiveness with increased production of IL-1β when monocytes from COVID-19 patients are stimulated with LPS compared to monocytes from healthy controls.

Bearing our results in mind, that monocytes remain fully responsive to LPS, it would be interesting to further elucidate the mechanisms and characterize the functions of monocytes isolated from patients with COVID-19. Especially the HLA-DR expression and intracellular signaling molecules but also the metabolic pathways including the ability to undergo oxidative burst would be of interest and we are now pursuing such studies. The possible potentiating effect of very low concentrations of LPS would also be of further interest. Could the most severely ill COVID-19 patient have been exposed to very low levels of LPS, thus potentiating their monocyte response?

COVID-19 is a very heterogeneous disease ranging from asymptomatic individuals to death; thus a careful selection of patients with different phenotypes is necessary to obtain consistent results. The strength of the study is that only severe cases with signs of hyperinflammation and no concomitant infection was included, thus rendering a more homogenous subphenotype of severe COVID-19 patients. The presence of an infection may skew the results and thus it is important not to include patients with confirmed or suspected superinfection. The main limitation of the study is the small sample size. However, the results obtained were homogenous for the COVID-19 patients included although further studies are warranted in larger cohorts to confirm our observations as well as studying less severe cases of COVID-19. We are currently pursuing such studies. Nevertheless, the results of this study are important as the results could have impact on clinical trials and treatments currently given to patients with COVID-19. One limitation is also how to exclude any possible COVID-19-infection among the healthy controls. All healthy controls were serologically tested and proved negative for COVID-19. However, there have been discussions on the validity of such results.[34] Importantly, none of the healthy controls had any symptoms resembling COVID-19 and combined with a negative result this gives a low probability of a COVID-19 infection.

In conclusion, we show that monocytes, but not lymphocytes, have spontaneous production of IL-1β and IL-8, but not of IL-6 despite elevated levels of IL-6 in the circulation. In addition, monocytes do not display signs of exhaustion. The cellular origin of the ongoing hyperinflammation of patients with severe COVID-19 remains elusive. Others have proposed a mechanism were the endothelial-epithelial barrier in the lung is interrupted leading to extravasion of plasma components into the alveolar space and activation of alveolar macrophages and epithelial cells with production of proinflammatory cytokines and chemokines. Monocytes and neutrophils are then summoned and activated leading to an uncontrolled inflammation.[29] Such a mechanism could very well fit with our observation especially when the recruited monocytes as demonstrated by our study retain their capacity to produce cytokines. Based on our findings, we speculate that treatment strategies blocking IL-1β signaling might be more effective for targeting ongoing monocyte hyperinflammation.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.