Monocyte response to SARS-CoV-2 protein ORF8 is associated with severe COVID-19 infection in patients with chronic lymphocytic leukemia

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Received: November 4, 2023.
Accepted: April 11, 2024.
Early view: April 24, 2024.

https://doi.org/10.3324/haematol.2023.284617

Abstract

The open reading frame 8 (ORF8) protein, encoded by the SARS-CoV-2 virus after infection, stimulates monocytes/macrophages to produce pro-inflammatory cytokines. We hypothesized that a positive *ex vivo* monocyte response to ORF8 protein pre-COVID-19 would be associated with subsequent severe Coronavirus disease 2019 (COVID-19). We tested ORF8 *ex vivo* on peripheral blood mononuclear cells from 26 anonymous healthy blood donors and measured intracellular cytokine/chemokine levels in monocytes by flow cytometry. The percentage of positive monocyte staining in the sample and change in mean fluorescence intensity (ΔMFI) after ORF8 were used to calculate the adjusted MFI for each cytokine. We then tested pre-COVID-19 peripheral blood mononuclear cell samples from 60 chronic lymphocytic leukemia (CLL) patients who subsequently developed COVID-19 infection. Severe COVID-19 was defined as hospitalization due to COVID-19. In the 26 normal donor samples, the adjusted MFI for interleukin (IL)-1β, IL-6, IL-8, and CCL-2 were significantly different with ORF8 stimulation *versus* controls. We next analyzed monocytes from pre-COVID-19 PBMC samples from 60 CLL patients. The adjusted MFI to ORF8 stimulation of monocyte intracellular IL-1β was associated with severe COVID-19 and a reactive ORF8 monocyte response was defined as an IL-1β adjusted MFI ≥0.18 (sensitivity 67%, specificity 75%). The median time to hospitalization after infection in CLL patients with a reactive ORF8 response was 12 days *versus* not reached for patients with a non-reactive ORF8 response with a hazard ratio of 7.7 (95% confidence interval: 2.4-132; *P*=0.005). These results provide new insight on the monocyte inflammatory response to virus with implications in a broad range of disorders involving monocytes.

Introduction

The severity of coronavirus disease 2019 (COVID-19) and survival outcomes have been linked to whether a patient develops an inflammatory cytokine storm.[1] We and others have found that classical monocytes ($CD14^+/CD16^-$) and activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome of these cells play a significant role in the development of COVID-19-related cytokine storm.[2-4] Activation of the NLRP3 inflammasome leads to production of interleukin (IL)-1β, a key pro-inflammatory cytokine that is a mediator of fever and in excessive quantities has been shown to cause tissue site injury.[5]

The SARS-CoV-2 virus produces up to 29 possible viral proteins and the RNA genome for the virus replication after entering the host. We previously reported that open reading frame 8 (ORF8), a SARS-CoV-2 protein, when glycosylated, stimulates monocytes to produce pro-inflammatory cytokines/chemokines through the NLRP3 pathway.[4] Human blood monocytes are divided into three major populations, classical monocytes ($CD14^+/CD16^-$), intermediate monocytes ($CD14^+/CD16^+$), and non-classical monocytes ($CD14^{low}/CD16^+$). Our previous data show that ORF8 only stimulates $CD14^+$ monocytes (classical and intermediate) and that patients with high levels of ORF8 glycoprotein in the blood had inferior outcomes from the infection compared to patients with low levels of ORF8 glycoprotein.[4]

Given that only a subset of people develops massive inflammation responses leading to severe COVID-19 after contracting the virus, understanding who may develop severe

disease is of clinical importance. Knowing that ORF8 directly induces CD14+ monocytes to produce pro-inflammatory cytokines/chemokines including IL1-β, IL-8 and CCL-2, we hypothesized that the functional characteristics of blood monocytes at baseline pre-infection may predispose certain patients to develop severe COVID-19, if infected. In order to gain insight into how blood cells from the general population would respond to ORF8 stimulation, we first assessed the monocyte response to ORF8 on healthy blood donors using flow cytometry analysis of intracellular cytokine staining. We observed a heterogeneous response, consistent with the fact that different individuals respond to SARS-CoV-2 infection very differently. Because granular clinical data was not available for the anonymous healthy blood donors, we subsequently used pre-COVID-19 samples from our Predolin Biobank from CLL patients with known COVID-19 infection outcomes. We show that monocytes from normal controls as well as monocytes from patients with CLL have a measurable and variable cytokine secretion response to ORF8 *ex vivo* and that this pre-infection response to ORF8 stimulation was associated with subsequent severe COVID-19 infection in patients with CLL. These results provide new insight on the monocyte response to viral infection and a potential new target for treatment.

Methods

Study population

The study was approved by the Institutional Review Board of Mayo Clinic and was conducted in accordance with the principles of the Declaration of Helsinki. Healthy volunteers consented and donated blood at the Division of Transfusion Medicine, Mayo Clinic, Rochester, MN, in accord with the current regulations by the US Food and Drug Administration. Initial samples were taken from these healthy blood donors; however, no retrospective chart review was available for these patients since they were anonymous. For the validation cohort, we tested monocytes from patients with CLL. Eligibility criteria were: i) diagnosis of CLL confirmed in the Mayo Clinic CLL database; ii) a pre-COVID-19 peripheral blood mononuclear cell (PBMC) sample available in the Predolin Biobank at Mayo Clinic; and iii) the patient subsequently developed COVID-19 infection, based on an ICD-10-code of coronavirus disease 2019 (COVID-19) from July 2020 to February 2022. All patients with a diagnosis of COVID-19 were required to have the presence of SARS-CoV-2-RNA confirmed by a reverse transcriptase quantitative polymerase chain reaction test. We defined patients to have "severe" COVID-19 if they were hospitalized due to COVID-19, which is different from the definition of severe COVID-19 from the World Health Organization (https://www.who.int/teams/health-care-readiness/covid-19). Retrospective chart review was performed on all patients to assess clinical outcomes.

Peripheral blood mononuclear cell sample processing

PBMC were isolated from leukocyte reduction system chambers obtained from healthy blood donors through the Mayo Clinic Blood Bank using Ficoll density centrifugation.[6] For cryopreservation, 1 mL of freezing media (50% RPMI with glutamine, 40% fetal bovine serum with 10% dimethyl sulfoxide [DMSO]) was added to the cells, followed by the cells being stored at -80°C for 1 day, and then transferred into liquid nitrogen. CLL blood samples were provided after written informed consent according to the Declaration of Helsinki and the Mayo Clinic Institutional Review Board. The PBMC from the CLL patients were isolated by Ficoll density centrifugation, then stored immediately in liquid nitrogen in the Mayo Clinic Predolin Biobank. All blood samples were acquired from CLL patients who provided written informed consent according to the Declaration of Helsinki and the Mayo Clinic Institutional Review Board.

Stimulation of peripheral blood mononuclear cells with ORF8, intracellular cytokine staining, and detection by flow cytometry

The purification process of ORF8 is previously described in Wu *et al.*[4] The ORF8 protein was stored in aliquots at -20°C. A dose of ORF8 200 mg/nL was used because this was shown previously to be the optimal dose to activate the monocytes for cytokine production.[4] Cryopreserved healthy donor PBMC were thawed and washed with 10 mL of prewarmed RPMI. We used a 24-well plate and resuspended $1x10^6$ healthy donor PBMC in 0.5 mL RPMI 1640 (Gibco) containing 10% fetal calf serum. The cells were cultured at 37°C in a 5% CO_2 atmosphere in the absence or presence of ORF8 200 ng/mL for a total of 24 hours. The cells that were cultured in the absence of ORF8 were considered the "matched basal unstimulated controls". ORF8 was purified from HEK293F cell supernatant as previously described.[4] Our rationale for a 24-hour stimulation test is based on previous data showing that ORF8-stimulated PBMC from healthy donors have the highest IL-1β gene expression at 24 hours.[4] We have also shown previously that monocytes secrete IL-1β, IL-8, CCL-2, and also IL-6 but to a lesser extent.[4]

Subsequently, cryopreserved CLL PBMC were thawed and washed with 10 mL pre-warmed RPMI and 0.01 mL of benzonase. We used a 12-well plate and resuspended $2x10^6$ PBMC in 0.8 mL RPMI 1640 (Gibco) containing 10% fetal calf serum. The cells were cultured at 37°C in a 5% CO_2 atmosphere in the absence or presence of ORF8 200 ng/mL for a total of 24 hours. The cells that were cultured in the absence of ORF8 were considered the "matched basal unstimulated controls". At the 18.5-hour point, the ORF8-stimulated CLL PBMC were treated with BD Golgi Plug containing Brefeldin A (1 uL/1 mL of cell culture) for 5.5 hours. Following isolation, the cells were washed with MACS buffer and then blocked with 100 uL of diluted human FcR (Miltenyi Biotec, San Diego, CA, USA) for

10 minutes at 4°C. The cells were then washed with and resuspended in phosphate-buffered saline (PBS), and then aliquoted into separate wells of a 96-well round bottom plate. Surface staining with monoclonal antibodies and fixable viability dye was performed on ice in PBS for 30 minutes. Following washing, the cells were fixed and permeabilized for 20 minutes according to manufacturer's directions (cells mixed with 100 uL of the fixation/permeabilization solution) to allow for intracellular staining. Cells were then stained for 30 minutes at 4°C with monoclonal antibodies against intracellular targets or appropriate isotype controls. Following staining, cells were washed with 1X perm/wash buffer and then fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for acquisition. Flow cytometry data was collected on a FACS-Canto (Becton Dickinson, Franklin Lakes, NJ, USA) that is standardized daily and calibrated with Spherotech beads to allow for direct comparisons of median fluorescent intensity (MFI) across separate experiments.[7] Antibodies were purchased from BD Biosciences except for CCL-2, IL-8, and IL-8 isotype, which were purchased from Invitrogen.

Flow cytometry analysis

The following gating strategy, depicted in *Online Supplementary Figure S1*, was applied to identify the monocytic population for intracellular cytokine analysis: i) gate on singlets, ii) gate on fixed viability dye negative cells, iii) gate on monocytes based on forward scatter and side scatter, iv) gate on CD3/CD19-negative cells, v) exclude CD14/CD16 double-negative cells.

The gated monocyte population exhibited a bimodal staining pattern for IL-1β, IL-6, IL-8, and CCL-2. In order to determine the intracellular cytokine expression level, we first gated on the cytokine-positive cells as set by the isotype controls and calculated the median fluorescence intensity (MFI) (*Online Supplementary Figures S1-S3*).[8] The MFI of the isotype control was calculated by gating on all of the isotype cells and the ΔMFI=MFI of positively staining cells - MFI of isotype control As an individual responding cell can be present in more than one of the total cytokine gates, in order to assess for the total cytokine response of a given cell, we used Boolean combinations of the four total cytokine gates (IL-1β, IL-6, IL-8, CCL-2) to therefore assess whether there were monocytes that could produce more than one cytokine. Flow cytometry analysis was performed with FlowJo version 10.5.3.

Median fluorescence intensity per positively staining monocyte

Measurement of the ORF8 monocyte response was done by measuring the total expression of cytokines/chemokines by considering the total monocyte count, the percentage of monocytes staining positive for respective cytokines, and the intracellular expression level of respective cytokines in response to ORF8 stimulation. In order to do this, we measured the MFI per positively staining monocyte. In order to estimate the MFI per positively staining monocyte, we utilized the following formula:

$$\text{Adjusted MFI} = \frac{\Delta\text{MFI for given cytokine}}{(\text{Total monocyte count}*\%\text{ staining positive for cytokine})}$$

Statistical analyses

Statistical analyses were performed using BlueSky Statistics version 7.40 or JMP 16. Descriptive analysis included medians, interquartile ranges, frequencies, and percentages. Fisher's exact test was used to compare categorical parameters. The Wilcoxon rank sum test was used to compare continuous variables. A paired *t* test analysis was used to compare results between matched ORF8-stimulated samples and unstimulated controls. Because some matched unstimulated controls had a "basal" level of intracellular cytokine production, the matched unstimulated controls are henceforth referred to here as "matched basal unstimulated controls". A value of $P<0.05$ was considered statistically significant.

Receiver-operating curves (ROC) were used to calculate the area under the curve (AUC), sensitivity, specificity, and optimal cutoff levels to determine what would be considered a reactive or non-reactive ORF8 monocyte response. Although we measured IL-1β, IL-6, IL-8, and CCL-2 on all cases, we set the dependent variable as the monocyte production of IL-1β in response to ORF8. The rationale for this selection was based on our previous work with ORF8[4] and that IL-1β regulates secretion of the IL-6, IL-8, and CCL-2. Monocytes were considered responsive to ORF8 if the ORF8-stimulated sample had a higher ΔMFI compared to unstimulated controls. The CLL control was used to set the cutoff because the monocytes from a CLL patient had different baseline characteristics compared to healthy blood donors and CLL patients had a higher IL-1β expression compared to healthy blood donors (*Online Supplementary Table S1*). The adjusted $MFI_{IL\text{-}1\beta}$ was used as the independent variable. The optimal cutoff point was chosen as the point with the highest Youden index (sensitivity + specificity - 1).[9] Visual examples of a reactive OMST *versus* non-reactive OMST are provided in *Online Supplementary Figure S4*. ROC curve analysis and AUC results for adjusted $MFI_{IL\text{-}6}$, $MFI_{IL\text{-}8}$, and $MFI_{CCL\text{-}2}$ are provided in *Online Supplementary Figure S5*.

Time to severe COVID-19 infection was defined as the date of COVID-19 diagnosis until the date of hospitalization and analyzed using the Kaplan-Meier method. The event was defined as the patient being admitted to the hospital due to COVID-19, and patients who were alive were censored. Cox proportional hazard models were utilized to test for associations between time to severe COVID-19 infection and different factors.

Results

Monocytes from healthy blood donors have a heterogeneous response to ORF8

Previously, we have shown that ORF8-stimulated blood monocytes secrete pro-inflammatory cytokine/chemokines including IL-1β, IL-6, IL-8, and CCL-2.[4] We hypothesized that the measured monocyte response to ORF8 *ex vivo* could be associated with severe COVID-19 experienced in the patient. We first studied blood monocytes from 26 healthy blood donors with a median age of 63 years (range, 31-80) and found a heterogeneous cytokine expression response with ORF8-stimulation. The ΔMFI for IL-1β, IL-6, IL-8, and CCL-2 were 230 (interquartile range [IQR], 87-418), 13 (IQR, 5-27), 80 (IQR, 58-100), and 269 (IQR, 198-359), respectively (Table 1). The median percentage of monocytes staining positive for IL-1β, IL-6, IL-8, and CCL-2 were 31% (IQR, 12-58), 5% (IQR, 1-21), 30% (IQR, 5-45), 48% (IQR, 40-58), respectively, and the adjusted MFI for IL-1β, IL-6, IL-8, and CCL-2 were all less than 0.01 respectively. Comparing ORF8-stimulated samples *versus* unstimulated samples, the percentage of monocytes staining positive and ΔMFI for IL-1β, IL-6, IL-8, and CCL-2 were significantly different compared to matched basal unstimulated controls (Figure 1; Table 1). These data demonstrate that monocytes from presumed healthy individuals have a variable response to ORF8 *ex vivo*.

We next investigated whether blood monocytes from CLL patients would also respond to an ORF8 challenge and whether

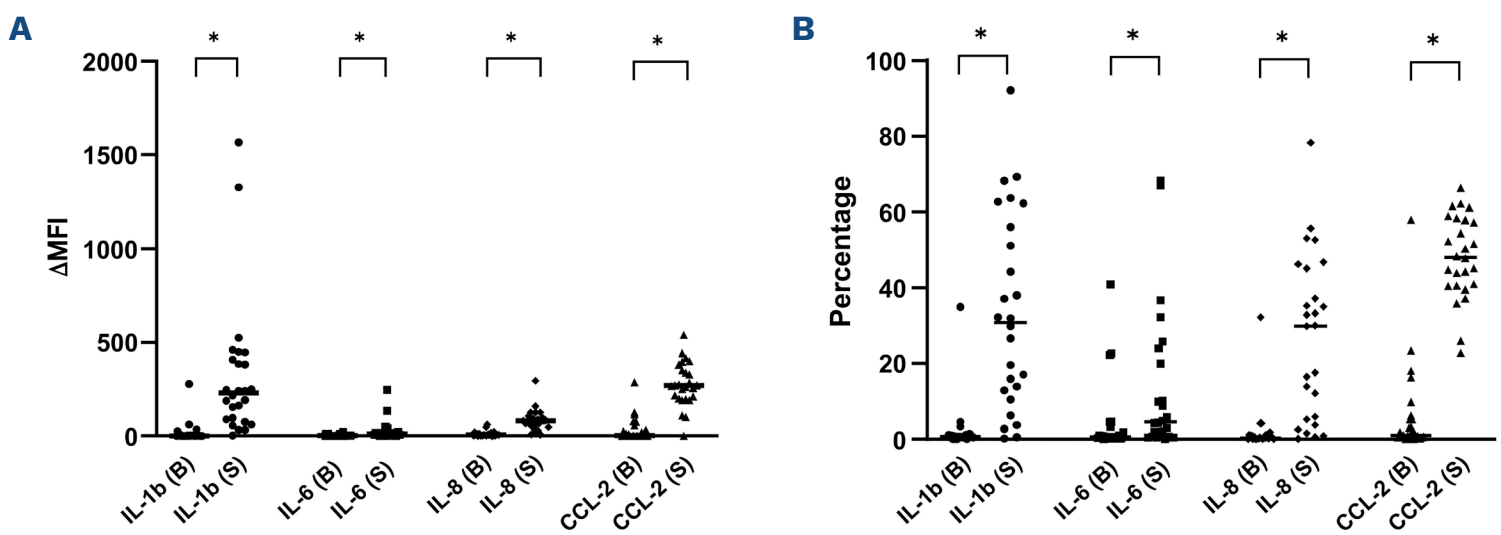

Figure 1. Peripheral blood mononuclear cells from 26 healthy donors display a heterogeneous response to open reading frame 8 (ORF8). (A) Δ median fluorescence intensity (ΔMFI) of each cytokine, measured by flow cytometry. (B) Percentage of monocytes staining positive for cytokine/chemokine, measured by flow cytometry. S: stimulated; B: matched unstimulated control; *: statistically significant *P* value.

Table 1. Cytokine and chemokine expression levels between paired basal unstimulated and ORF8-stimulated blood samples from normal donors and patients with chronic lymphocytic leukemia.

Parameters	Normal donor			CLL patients		
	Basal unstimulated N=26	ORF8 stimulated N=26	*P*	Basal unstimulated N=60	ORF8 stimulated N=60	*P*
ΔMFI	Median (25-75% IQR)	Median (25-75% IQR)		Median (25-75% IQR)	Median (25-75% IQR)	
IL-1β	1.2 (0.7-4)	230 (87-418)	0.0002	97 (85-122)	378 (127-669)	<0.0001
IL-6	2 (0-3.5)	13 (5-27)	0.02	105 (102-110)	136 (111-247)	<0.0001
IL-8	6.5 (3-13)	80 (58-100)	<0.0001	106 (96-157)	238 (165-320)	<0.0001
CCL-2	4 (0-36)	269 (198-359)	<0.0001	154 (130-203)	339 (162-533)	<0.0001
Percentage of monocytes staining positive						
IL-1β	0.7 (0.4-1)	31 (12-58)	<0.0001	0.4 (0-0.9)	27 (5-50)	<0.0001
IL-6	0.6 (0.4-2.6)	5 (1-21)	0.01	0.4 (0-0.8)	4 (2-6)	<0.0001
IL-8	0.3 (0-1)	30 (5-45)	<0.0001	0.2 (0-0.9)	30 (6-47)	<0.0001
CCL-2	1 (0.5-6)	48 (40-58)	<0.0001	7 (3-16)	25 (11-43)	<0.0001

CLL: chronic lymphocytic leukemia; ORF4: open reading frame 8; ΔMFI: Δ median fluorescence intensity;IQR: interquartile range.

the magnitude of this response was associated with subsequent severe COVID-19 infection. Testing this hypothesis required a dataset where patients had PBMC cryopreserved prior to the pandemic coupled with extensive follow-up information on subsequent COVID-19 infection outcome.

Chronic lymphocytic leukemia patient characteristics at time of chronic lymphocytic leukemia diagnosis

Blood samples from 60 patients with CLL met the study criteria and were tested for the ORF8 monocyte response (Table 2). The CLL International Prognostic Index (CLL-IPI) risk score was very high-risk in 0, high-risk in nine (15%), intermediate-risk in 23 (38%), low-risk in 23 (38%), and unknown in five (8%). Twenty-three (38%) had unmutated *IGHV* genes, 26 (43%) had 13q deletion, 11 (18%) had trisomy 12, four (7%) had 11q deletion, two (3%) had 17p deletion, and 15 (25%) had no known cytogenetic abnormalities on fluorescence *in situ* hybridization. Twenty-one (35%) patients had received CLL-directed therapy at the time of sample collection. The median time interval between collection of the PBMC sample and development of COVID-19 infection was 0.9 years (IQR, 0.4-2.0).

COVID-19-related patient characteristics

Twenty-eight patients had received at least one vaccination against SARS-CoV-2 and the median number of vaccinations before COVID-19 diagnosis was 0 (range, 0-3). Seventeen patients received the BNT162b2 mRNA vaccination (Pfizer) while 11 patients received the mRNA-1273 SARS-CoV-2 vaccine (Moderna). The median time interval between first vaccination and infection was 106 days (range, 2-322). Seventeen (28%) patients received monoclonal antibody therapy for COVID-19 (sotrovimab, casirivimab-imdevimab, bamlanivimab, or bamlanivimab-etesvimab). Before the diagnosis of COVID-19, 30 patients (50%) had received at least one type of CLL-directed therapy, and 25 (42%) patients were on CLL treatment at the time of COVID-19 diagnosis. Therapies patients received included BTK inhibitor with (N=2) or without anti-CD20 (N=17), BTK inhibitor and anti-CD20 and venetoclax (N=4), anti-CD20 therapy alone (N=3), chimeric antigen receptor T-cell therapy (N=2), and other (N=2) (*Online Supplementary Table S2*). Fifty (83%) were symptomatic and 22 (37%) developed severe COVID-19. Among the 22 patients who developed severe COVID-19, 17 (77%) were admitted due to hypoxia. D-dimer, C-reactive protein (CRP), and ferritin data are reported in Table 2. The median hospital stay was 8 days (range, 2-53). Four patients developed a venous thromboembolism and eight were hospitalized in the intensive care unit (ICU). Six died at the date of last known follow-up, with four having died from COVID-19 in the hospital due to respiratory failure, one patient died of pulmonary embolism in the hospital not related to COVID-19 and one patient died in the hospital from complications related to ovarian cancer.

In univariate Cox proportional hazards regression analyses, the following variables were not associated with severe COVID-19: age, sex, *IGHV* mutation status, CLL-IPI risk, weighted Charlson comorbidity score, smoking status,

Table 2. Characteristics of chronic lymphocytic leukemia patients.

Characteristics	N=60
Sex, N (%) Male	 46 (76)
Age in years at time of CLL diagnosis, median (IQR)	64 (54-74)
Mutation status at time of CLL diagnosis, N (%) IGHV unmutated Del(17p) and/or TP53 mutation status	 23 (38) 2 (3)
CLL-IPI risk category, N (%) High Intermediate Low Unknown	 9 (15) 23 (38) 23 (38) 5 (8)
CCS, median (IQR)	3 (2-5)
BMI, median (IQR)	27.1 (24.5-31.9)
Smoking status, N (%) Current Former Never	 1 (2) 20 (33) 39 (65)
On treatment at time of COVID-19 diagnosis, N (%) BTK-inhibitor based therapy Other	25 (42) 22 (37) 3 (5)
CAR T-cell recipient, N (%)	2 (3)
Autoimmune disease, N (%)	4 (7)
Number of COVID-19 vaccinations before COVID-19 diagnosis, median (IQR)	0 (0-3)
Age in years at time of COVID-19 diagnosis, median (IQR)	74 (63-80)
COVID-19 symptoms, N (%) Non-productive cough Fever Hypoxia (SpO^2 <90%)	 34 (57) 21 (35) 17 (28)
Received monoclonal antibody, N (%)	17 (28)
Hospitalized, N (%) Elevated D-dimer (ref: ≤500 ng/mL), 18 evaluated Elevated ferritin (ref: 24-336 mcg/L), 21 evaluated Elevated C-reactive protein (ref: ≤8.0 mg/L), 16 evaluated Median hospital-stay days Developed VTE ICU status (IQR)	22 (37) 14 (78) 21 (100) 13 (81) 8 (2-53) 4 (18) 8 (36)
Alive, N	54
Deaths due to COVID-19, N	4

IQR: 25-75% interquartile range; BMI: body mass index; BTK-inhibitor: Bruton's tyrosine kinase inhibitor; CAR T: chimeric antigen receptor T cell; CCS: Charlson comorbidity score (1 year before COVID-19 diagnosis); CLL: chronic lymphocytic leukemia; COVID-19: coronavirus disease 2019; del: deletion; FISH: fluorescence *in situ* hybridization; ICU: intensive care unit; IGHV: immunoglobulin heavy chain gene; IPI: International Prognostic Index; ref: reference range; VTE: venous thromboembolism.

number of mRNA COVID-19 vaccinations, or being on therapy for CLL. There was a trend to higher risk significance in patients who had a higher weighted Charlson comorbidity score, peripheral vascular disease, and chronic pulmonary disease (Table 3).

Monocytes from chronic lymphocytic leukemia patients have a heightened response to ORF8

We assessed the *ex vivo* monocyte response to ORF8 from the 60 pre-COVID-19 samples from our CLL patients. Compared to matched basal unstimulated controls, monocytes from ORF8-stimulated PBMC had a significantly higher ΔMFI for IL-1β (378 *vs*. 97; *P*<0.0001), IL-6 (136 *vs*. 105; *P*<0.0001), IL-8 (238 *vs*. 106; *P*<0.0001), and CCL-2 (339 *vs*. 154; *P*<0.0001) (Figure 2; Table 1). Fifty-two patients (87%) had a higher IL-1β ΔMFI compared to control. Compared to matched basal unstimulated controls, monocytes from ORF8-stimulated PBMC also had a significantly higher percentage of monocytes staining positive for IL-1β (27 *vs*. 0.4; *P*<0.0001), IL-6 (4 *vs*. 0.4; *P*<0.0001), IL-8 (30 *vs*. 0.2; *P*<0.0001), and CCL-2 (25 *vs*. 7; *P*<0.0001). The median IL-1β adjusted MFI for ORF8-stimulated samples was 0.25 (IQR, 0.06-1.38), IL-6 was 1.1 (IQR, 0.25-5.7), IL-8 was 0.28 (IQR, 0.08-1.9), and CCL-2 was 0.42 (IQR, 0.16-1.6).

Compared to healthy blood donors, CLL patient monocytes were more likely to have a significantly higher IL-6 ΔMFI (136 *vs*. 13; *P*<0.0001), IL-8 ΔMFI (238 *vs*. 80; *P*<0.0001), IL-8 adjusted MFI (0.28 *vs*. 0.004; *P*=0.005), and CCL-2 adjusted MFI (0.42 *vs*. 0.005; *P*=0.006) (*Online Supplementary Table S1*). CLL patient monocytes were also more likely to have a significantly higher IL-1β ΔMFI for the IL-1β$^+$/CCL-2$^+$/IL-6$^+$/IL-8$^+$ (2,237 *vs*. 1,191; *P*=0.0002) and IL-1β$^+$/CCL-2$^-$/IL-6$^+$/IL8$^+$ subsets (2,382 *vs*. 1,103; *P*=0.0009) (*Online Supplementary Table S1*). However, healthy blood donors were more likely to have a significantly higher percentage of monocytes staining positive for IL-6 (5% *vs*. 4%; *P*=0.02), CCL-2 (48% *vs*. 25%; *P*=0.0003), and IL-1β$^+$/CCL-2$^+$/IL-6$^-$/IL-8$^-$ (5% *vs*. 2%; *P*=0.002).

A reactive ORF8-monocyte response is associated with subsequent development of severe COVID-19 in chronic lymphocytic leukemia patients

Given the variability in monocyte responses to ORF8 *ex vivo*, we were next interested in assessing whether we could identify what level of response is associated with the development of severe COVID-19.

We used a ROC analysis on CLL patient data to establish a cutoff to distinguish between a reactive *versus* non-reactive monocyte response for CLL patients. We chose to focus on IL-1β because we previously showed that ORF8 stimulates monocytes to produce pro-inflammatory cytokine/chemokines through the NLRP3 pathway and activation of the NLRP3 pathway is known to mediate the secretion of

Table 3. Univariate analysis of baseline characteristics and association with severe COVID-19 (Cox proportional hazards regression model).

Parameters	Hazard ratio (95% CI)	*P*
Age	1.02 (0.98-1.1)	0.29
Male	0.92 (0.3-2.5)	0.87
IGHV, unmutated	0.97 (0.4-2.4)	0.95
CLL-IPI risk	1.09 (0.6-2.0)	0.77
Charlson comorbidity score, weighted	1.2 (0.99-1.4)	0.052
Myocardial infarction	2.4 (0.8-7.2)	0.11
Congestive heart failure	1.4 (0.5-3.8)	0.50
Peripheral vascular disease	2.2 (0.9-5.2)	0.08
Cerebrovascular disease	2.5 (0.6-10.6)	0.22
Chronic pulmonary disease	2.2 (0.9-5.5)	0.08
Diabetes	1.5 (0.5-04.3)	0.5
BMI	0.99 (0.94-1.05)	0.78
Former smoker	1.02 (0.7-1.6)	0.91
Number of COVID-19 vaccinations before infection	0.90 (0.7-1.2)	0.53
Received monoclonal antibody	0.3 (0.09-1.1)	0.07
On CLL-directed therapy at time of COVID-19 diagnosis (%)	0.7 (0.3-1.7)	0.44
On BTK inhibitor at time of COVID-19 diagnosis	0.7 (0.3-1.7)	0.43

COVID-19: coronavirus disease 2019; BMI: body mass index; BTK: Bruton tyrosine kinase; CLL: chronic lymphocytic leukemia; *IGHV*: immunoglobulin heavy chain gene; IPI: International Prognostic Index ; CI: confidence interval.

IL-1β.[4] By ROC analysis, the AUC for the IL-1β adjusted MFI was 0.68 (Figure 3). The cutoff for IL-1β adjusted MFI was ≥0.18 (sensitivity 67%, specificity 75%). Thus, a reactive ORF8 monocyte response was defined as an IL-1β adjusted MFI ≥0.18, while a non-reactive OMST was defined as an IL-1β adjusted MFI <0.18. Thirty-seven CLL patients (62%) met these criteria for a reactive ORF8 monocyte response. We then assessed whether a reactive ORF8 monocyte response based on IL-1β adjusted MFI was associated with severe COVID-19. Patients with a reactive ORF8 monocyte response were significantly more likely to develop severe COVID-19 with a hazard ratio of 7.7 (95% CI: 2.4-132; *P*=0.005). The median time to hospitalization for patients with a reactive ORF8 monocyte response based on the IL-1β adjusted MFI was 12 days (95% CI: 2- not reached [NR]) *versus* NR for patients with non-reactive ORF8 monocyte response (95% CI: NR-NR); 54% of patients with a reactive ORF8 monocyte response developed severe COVID-19 by day 15 while 9% of patients with a non-reactive ORF8 monocyte response developed severe COVID-19 (Figure 4).

IL-1β expressing monocytes co-express IL-6, IL-8, and CCL-2

Since IL-1β in CLL patients was associated with severe

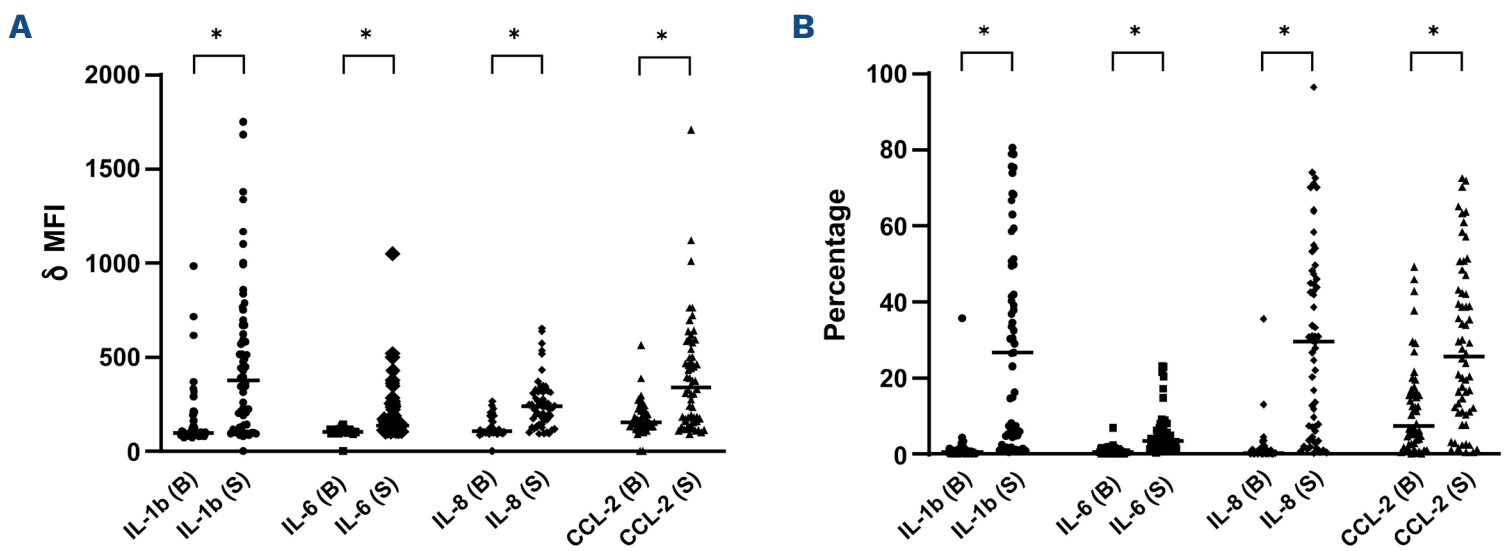

Figure 2. Peripheral blood mononuclear cells from 60 patients with chronic lymphocytic leukemia display a heterogeneous response to open reading frame 8 (ORF8). (A) Δ median fluorescence intensity (ΔMFI) of each cytokine, measured by flow cytometry. (B) Percentage of monocytes staining positive for cytokine/chemokine, measured by flow cytometry. S: stimulated; B: matched unstimulated control; *: statistically significant *P* value; IL: interleukin.

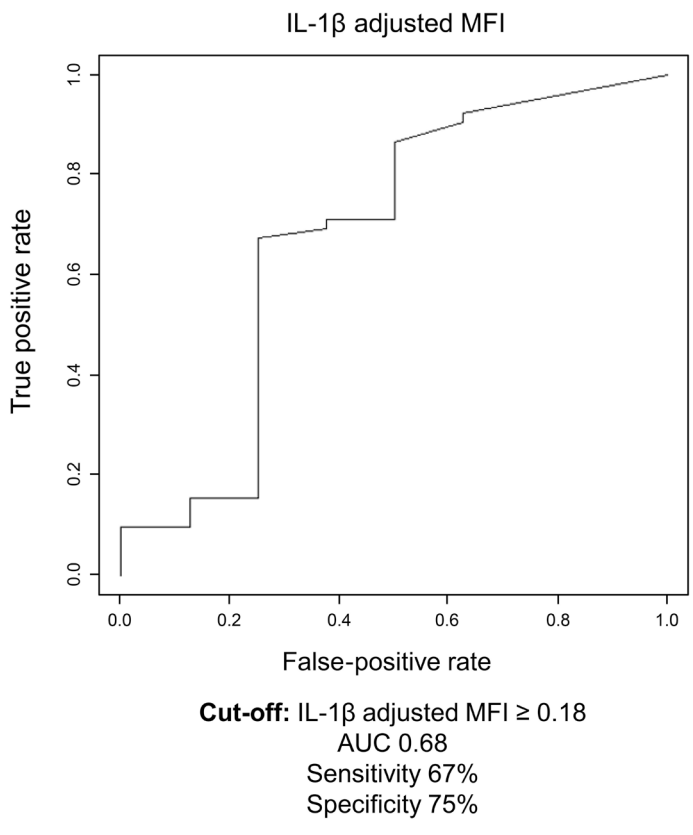

Figure 3. Receiver-operating curve analysis to establish a reactive *versus* non-reactive open reading frame 8 (ORF8) monocyte response. IL: interleukin; AUC: area under the curve; MFI: median fluorescence intensity.

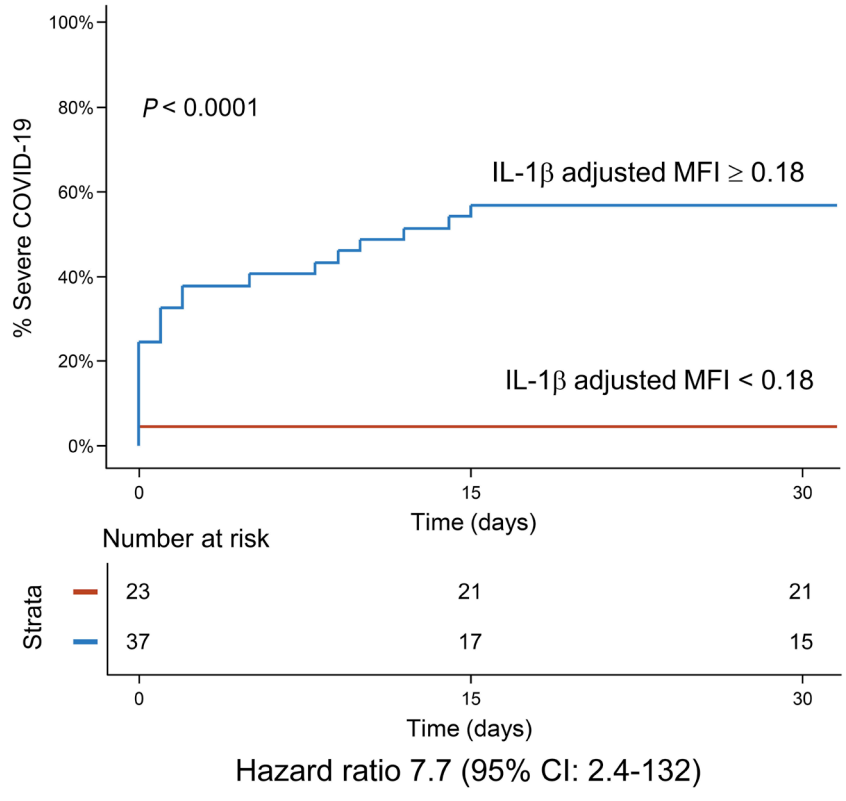

Figure 4. A reactive open reading frame 8 (ORF8) monocyte response based on the interleukin-1β adjusted median fluorescence intensity is associated with severe coronavirus disease 2019. COVID-19: coronavirus disease 2019; CI: confidence interval; IL: interleukin.

COVID-19, we were interested in whether IL-1β-expressing monocytes co-express IL-6, IL-8, and CCL-2 and if there are specific subsets. Using Boolean gating, distinct populations of cytokine-producing cells were delineated at the single-cell level based on any combination of IL-1β, IL-6, IL-8, or CCL-2. Frequencies and ΔMFI of the combinations are listed in the *Online Supplementary Table S3*.

On univariate Cox analysis, the IL-1β ΔMFI for IL-1β$^+$/CCL-2$^+$/IL-6$^+$/IL-8$^-$, IL-1β$^+$/CCL-2$^+$/IL-6$^-$/IL-8$^-$, and the IL-1β adjusted MFI for IL-1β$^+$/CCL-2$^-$/IL6$^+$/IL8$^-$ were associated with severe COVID-19 but the hazard ratio was not clinically significant. The frequency of IL-1β$^+$ subsets, other IL-1β$^+$ subsets for ΔMFI, and adjusted MFI were not associated with severe COVID-19 (*Online Supplementary Table S4*).

Discussion

CLL patients are at increased risk of morbidity and mortality from COVID-19 due to their impaired humoral response to COVID-19 vaccination, inherent immunocompromised status, and additional immunosuppression from CLL-directed therapy. The development of a cytokine storm has been associated with inferior outcomes and is also seen in patients with CLL.[2,10-12] Our previous study has shown that the SARS-CoV-2 encoded protein, ORF8, when glycosylated, plays a major role in the pathogenesis for severe COVID-19 by stimulating CD14$^+$ monocytes to produce pro-inflammatory cytokines/chemokines.[4] Among our 26 healthy blood donors, we observed that ORF8-stimulated monocytes had a variable response and had a significantly higher level of IL-1β, IL-6, IL-8, and CCL-2 when compared to matched basal unstimulated controls. The ORF8-stimulated monocytes from CLL patients had a more robust response of cytokine/chemokines compared to ORF8-stimulated monocytes from healthy blood donors. Our findings reveal that a reactive ORF8 monocyte response by IL-1β is associated with severe COVID-19 in CLL patients infected with SARS-CoV-2. Further studies are needed to investigate whether other cytokines/chemokines also play a major role and whether a reactive ORF8 monocyte response in a non-CLL patient by IL-1β would also be associated with severe COVID-19.

While ORF8-stimulated monocytes from CLL patients produced a significantly higher level of IL-1β, IL-6, IL-8, and CCL-2 when compared to matched basal unstimulated controls, only IL-1β was associated with severe COVID-19. One plausible explanation for this finding is that ORF8 activates the NLRP3 inflammasome to directly produce IL-1β,[4,13] while IL-6, IL-8, and CCL-2 may be produced as the result of IL-1β production. It is also possible that different cytokine/chemokine may have different expression kinetics in different diseases. In addition, IL-1β is primarily produced by monocytes while other cytokines such as IL-6 can be produced by other cell types such as epithelial cells that are absent in our *ex vivo* testing.[14] However, since the ORF8 monocyte response measures all four of these cytokines/chemokines, future studies in other macrophage-mediated diseases should evaluate all of them for relevance to disease activity.

Given that IL-1β was the primary cytokine associated with inferior outcomes, we investigated whether there were multifunctional monocytes that secreted more than one cytokine. Indeed, we identified that 1% of monocytes secreted all four cytokines. We also identified a subset of monocytes that secreted both IL-1β$^+$ and IL-8$^+$ but did not identify any monocytes that secreted an IL-1β$^+$/CCL-2$^+$/IL-6$^+$/IL-8$^-$ or IL-1β$^+$/CCL-2$^-$/IL-6$^+$/IL-8$^-$ pattern. Since the expression of cytokines are each driven by different gene expression programs, co-activation of these programs may be governed by different cell activation status. Further studies are needed to investigate what role these multifunctional monocytes play in protection against COVID-19 or contribution to the development of a cytokine storm.

Strengths of our study include our relatively large sample size of CLL patients who had pre-COVID-19 PBMC available for *ex vivo* testing and detailed follow-up regarding the outcome of the COVID-19 infection. Our study reports that BTK inhibitors at COVID-19 diagnosis did not impact outcomes, which is consistent with previously reported literature.[15] The need for samples pre-COVID-19 necessitated the use of cryopreserved samples; however, we have not observed any differences in ORF8 monocyte response when the sample was tested fresh *versus* cryopreserved (*data not shown*). Limitations of this study include the lack of granular data on the blood donors; however, these donors were required to be healthy enough to donate blood. Some of our pre-infection samples from our CLL patient population was long before the infection, so there are other potential factors that could have impacted monocyte response including CLL treatment that occurred between the time points, increases or decreases in quantity or reactivity of monocytes with CLL progression or improvement. These are difficult factors to control for and will require substantial extended studies of serial samples to determine. Previous studies have reported a significantly higher rate of hospitalizations due to COVID-19 and reported that age and certain comorbidities are associated with severe COVID-19 but these studies were retrospective and prone to bias.[15,16] Additional studies in larger and more defined CLL and healthy patient populations with different ages and ethnicities are needed to validate our results and better establish the normal range of the *ex vivo* ORF8 monocyte response. The effect of therapy on the monocyte will also be important to evaluate in prospective studies to learn if the ORF8 monocyte response predicts the risk of bacterial and fungal infections. In these situations, a very low ORF8 monocyte response might actually predict a higher risk of those infections.

The increased understanding of how ORF8 targets the

monocyte inflammasome opens the way to therapies targeting the NLRP3 pathway.[17] Future studies should investigate whether patients with a reactive ORF8 monocyte response would benefit from prophylactic use of NLRP3 inhibitors or other anti-inflammatory medications. Indeed, the ORF8 monocyte response has potential beyond COVID-19 infection for wider application to explore monocyte/macrophage function in a variety of infections or malignant diseases. Since the ORF8 monocyte response is an *ex vivo* test using peripheral blood, serial studies to monitor treatment effects are feasible to select patients for therapy and determine the duration of treatment.

Disclosures

No conflicts of interest to disclose.

Contributions

GJR, XW, KAG, and TEW conceptually designed experiments. GJR, JPA, XW, KAG, KGR, VB, MKM, acquired, analyzed, and interpreted the data. GJR and JPA performed the statistical analyses. TLW and MJS provided the ORF8. KGR, NEK, and SAP provided the CLL samples. XW and TEW supervised the study. All authors participated in writing of the paper.

Funding

This work was supported in part by the University of Iowa/Mayo Clinic SPORE (CA97274), the Predolin Foundation Biobank, and Mayo Clinic Center for Individualized Medicine.

Data-sharing statement

For original data, please contact the corresponding author.

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