haematologica
Journal of the Ferrata Storti Foundation

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Received: November 4, 2023.
Accepted: April 11, 2024.

Citation: Gordon J. Ruan, Xiaosheng Wu, Kimberly A. Gwin, Michelle K. Manske, Jithma P. Abeykoon, Vaishali Bhardwaj, Taylor L. Witter, Matthew J. Schellenberg, Kari G. Rabe, Neil E. Kay, Sameer A. Parikh, and Thomas E. Witzig. Monocyte response to SARS-CoV-2 protein ORF8 is associated with severe COVID-19 infection in patients with chronic lymphocytic leukemia. Haematologica. 2024 Apr 24. doi: 10.3324/haematol.2023.284617 [Epub ahead of print]

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

Authors: Gordon J. Ruan[1*], Xiaosheng Wu[1], Kimberly A. Gwin[1], Michelle K Manske[1], Jithma P. Abeykoon[1], Vaishali Bhardwaj[1], Taylor L. Witter[2], Matthew J. Schellenberg[2], Kari G Rabe[3], Neil E. Kay[1,4], Sameer A. Parikh[1], Thomas E. Witzig[1]

Affiliations: [1]Division of Hematology, Department of Medicine, [2]Department of Biochemistry and Molecular Biology, [3]Department of Quantitative Health Sciences, [4]Department of Immunology, Mayo Clinic Rochester, MN, USA

Author contributions: G.J.R., X.W., K.A.G., and T.E.W. conceptually designed experiments. G.J.R., J.P.A., X.W., K.A.G., K.G.R., V.B., M.K.M., acquired, analyzed, and interpeted the data. G.J.R. and J.P.A. performed the statistical analyses. T.L.W. and M.J.S. provided the ORF8. K.G.R., N.E.K., and S.A.P. provided the CLL samples. X.W. and T.E.W. supervised the study. All authors participated in writing of the paper.

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Corresponding author: Thomas E. Witzig, M.D, Tel: 507-266-3427, Fax: 507-266-4972
Email: Witzig.thomas@mayo.edu

Data-sharing statement: For original data, please contact witzig.thomas@mayo.edu

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.

Keywords: Monocytes, COVID-19, chronic lymphocytic leukemia, CLL, interleukin-1β, cytokines, chemokines, ORF8

Conflicts of interest: The authors declare no potential conflicts of interest.

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 COVID-19. We tested ORF8 *ex vivo* on peripheral blood mononuclear cells (PBMCs) from 26 anonymous healthy blood donors and measured intracellular cytokine/chemokine levels in monocytes by flow cytometry. The % monocytes staining positive 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 PBMC samples from 60 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 vs 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 $\geq$ 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% CI: 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. To gain insight into how blood cells from 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, Minnesota, 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 a) diagnosis of CLL confirmed in the Mayo Clinic CLL database; b) a pre-COVID-19 PBMC sample available in the Predolin Biobank at Mayo Clinic; and c) 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 WHO (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

PBMCs 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 celsius 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 PBMCs 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 PBMCs 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 celsius. A dose of ORF8 200mg/nl was used because this was shown previously to be the optimal dose to activate the monocytes for cytokine production.[4]

Cryopreserved healthy donor PBMCs were thawed and washed with 10ml of pre-warmed RPMI. We used a 24-well plate and resuspended $1x10^6$ healthy donor PBMCs in 0.5ml RPMI 1640 (Gibco) containing 10% fetal calf serum (FCS). The cells were cultured at 37°C in a 5% CO_2 atmosphere in the absence or presence of ORF8 200ng/ml for a total of 24 hours. The cells that were cultured in the absence of ORF8 were considered the "matched basal unstimulated controls". $ORF8^{wt}$ 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 PBMCs 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 PBMCs were thawed and washed with 10ml pre-warmed RPMI and 0.01 ml of benzonase. We used a 12-well plate and resuspended $2x10^6$ PBMCs in 0.8ml 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 200ng/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 PBMCs 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 100uL 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 100uL 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 **Figure S1,** was applied to identify the monocytic population for intracellular cytokine analysis: 1. Gate on singlets, 2. Gate on fixed viability dye negative cells, 3. Gate on monocytes based on forward scatter and size scatter, 4. Gate on CD3/CD19 negative cells, 5. 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. 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) **(Figure S1-S3)**.[8] The MFI of the isotype control was calculated by gating on all of the isotype cells and the $\Delta MFI = MFI\ of\ positively\ staining\ cells - MFI\ of\ isotype\ control.$

Because an individual responding cell can be present in more than one of the total cytokine gates, 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 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. To do this, we measured the MFI per positively staining monocyte. To estimate the MFI per positively staining monocyte, we utilized the following formula:

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

Statistical analyses

Statistical analyses were performed using BlueSky Statistics version 7.40 or JMP 16. Descriptive analysis included medians, interquartile ranges, frequencies, and percentages. Fischer'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 cut-off because the monocytes from a CLL patient had different baseline characteristics compared to a normal patient and CLL patients had a higher IL-1β expression compared to healthy blood donors **(Supplemental Table 1).** 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 vs. non-reactive OMST are provided in **Supplementary Figure 4**. ROC curve analysis and AUC results for adjusted $MFI_{IL\text{-}6}$, $MFI_{IL\text{-}8}$, and $MFI_{CCL\text{-}2}$ are provided in **Supplementary Figure 5**.

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 87-418), 13 (5-27), 80 (58-100), and 269 (198-359), respectively **(Table 1)**. The median percentage of monocytes staining positive for IL-1β, IL-6, IL-8, and CCL-2 were 31% (12 - 58), 5% (1-21), 30% (5-45), 48% (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 vs. 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 the magnitude of this response was associated with subsequent severe COVID-19 infection. Testing this hypothesis required a dataset where patients had PBMCs cryopreserved prior to the pandemic coupled with extensive follow-up information on subsequent COVID-19 infection outcome.

CLL Patient Characteristics at Time of CLL 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 9 (15%), intermediate-risk in 23 (38%), low-risk in 23 (38%), and unknown in 5 (8%). 23 (38%) had unmutated IGHV genes, 26 (43%) had 13q deletion, 11 (18%) had trisomy 12, 4 (7%) had 11q deletion, 2 (3%) had 17p deletion, and 15 (25%) had no known cytogenetic abnormalities on fluorescence in situ hybridization. 21 (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 (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 + anti-CD20 + venetoclax (n=4), anti-CD20 therapy alone (n=3), chimeric antigen receptor T-cell therapy (n=1), and other (n=1) **(Supplemental Table 2)**. 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 8 were hospitalized in the intensive care unit (ICU). Six died at the date of last known follow up, with 4 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, gender, *IGHV* mutation status, CLL-IPI risk, weighted Charlson Comorbidity score, smoking status, 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 CLL 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 PBMCs 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 PBMCs 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 (0.06-1.38), IL-6 was 1.1 (0.25-5.7), IL-8 was 0.28 (0.08-1.9), and CCL-2 was 0.42 (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) **[Supplemental Table 1]**. 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^{+} (2237 vs. 1191, p=0.0002) and IL-1β+/CCL-2-/IL-6+/IL8+ subsets (2382 vs. 1103, p=0.0009) [**Supplemental Table 1]**. 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 CLL 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 cut-off 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 IL-1β.[4] By ROC analysis, the AUC for the IL-1β adjusted MFI was 0.68 **[Figure 3]**. The cut-off 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) versus not reached (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 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 ΔMFIs of the combinations are listed in the **Supplemental Table 3.**

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 **(Supplemental Table 4)**.

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 system.[14] However, since the ORF8 monocyte response measures all 4 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 PBMCs 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 vs 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 timepoints, 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 it is performed on blood, serial studies to monitor treatment effects are feasible to select patients for therapy and determine the duration of treatment.

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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-value	Basal Unstimulated (n=60)	ORF8 stimulated (n=60)	p-value
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
% 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

ΔMFI: delta median fluorescence intensity

Table 2. Characteristics of chronic lymphocytic leukemia patients

Characteristics	N=60
Sex	
Male	46 (76)
Age at time of CLL diagnosis, y	64 (54-74)
Mutation Status at time of CLL diagnosis	
IGHV unmutated	23 (38)
Del(17p) and/or TP53 mutation status	2 (3)
CLL-IPI Risk Category	
High	9 (15)
Intermediate	23 (38)
Low	23 (38)
Unknown	5 (8)
CCS, median (range)	3 (2-5)
BMI, median (range)	27.1 (24.5-31.9)
Smoking status	
Current	1 (2)
Former	20 (33)
Never	39 (65)
On Treatment at time of COVID-19 diagnosis	25 (42)
BTK-inhibitor based therapy	22 (37)
Other	3 (5)
CAR-T cell recipient	2 (3)
Autoimmune disease	4 (7)
Number of COVID-19 vaccinations before COVID-19 diagnosis	0 (0-3)
Age at time of COVID-19 diagnosis, y	74 (63-80)
COVID-19 Symptoms	
Non-productive cough	34 (57)
Fever	21 (35)
Hypoxia (SpO2 <90%)	17 (28)
Received monoclonal antibody	17 (28)
Hospitalized	22 (37)
Elevated D-dimer (ref: ≤500 ng/ml)	14/18 (78)
Elevated ferritin (ref: 24-336 mcg/L)	21/21 (100)
Elevated C-reactive protein (ref: ≤8.0 mg/L)	13/16 (81)
Median hospital-stay	8 (2-53)
Developed VTE	4 (18)
ICU status	8 (36)
Alive	54
Deaths due to COVID-19	4

Data are presented as n (%), n/N (%), or median (25-75% interquartile range). BMI, body mass index; CAR-T, chimeric antigen receptor; CCS, Charlson Comorbidity score (1 year before COVID-19 diagnosis), CLL, chronic lymphocytic leukemia; COVID-19, coronavirus disease-19; del, deletion; FISH, fluorescence in-situ hybridization;

ICU, intensive care unit; IGHV, immunoglobulin heavy chain gene; IPI, international prognostic index; intravenous immune globulin; IVIG; ref, reference range; VTE, venous thromboembolism; WBC, white blood cell count.

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

Parameters	Hazard Ratio (95% CI)	p-value
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

BMI, body mass index; BTK, Bruton-tyrosine kinase; CLL, Chronic lymphocytic leukemia; IGHV, immunoglobulin heavy chain gene; IPI, international prognostic index

Figure Legends

Figure 1. Peripheral blood mononuclear cells from 26 healthy donors display a heterogeneous response to open reading frame 8 (ORF8). A) Delta 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.

Figure 2. Peripheral blood mononuclear cells from 60 patients with chronic lymphocytic leukemia display a heterogeneous response to open reading frame 8 (ORF8). A) Delta 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.

Figure 3. Receiver-operating curve analysis to establish a reactive vs. non-reactive open reading frame 8 (ORF8) monocyte response**.**

Figure 4. A reactive open reading frame 8 (ORF8) monocyte response based on the interleukein-1β adjusted median fluorescence intensity is associated with severe coronavirus disease 2019.

Figure 1

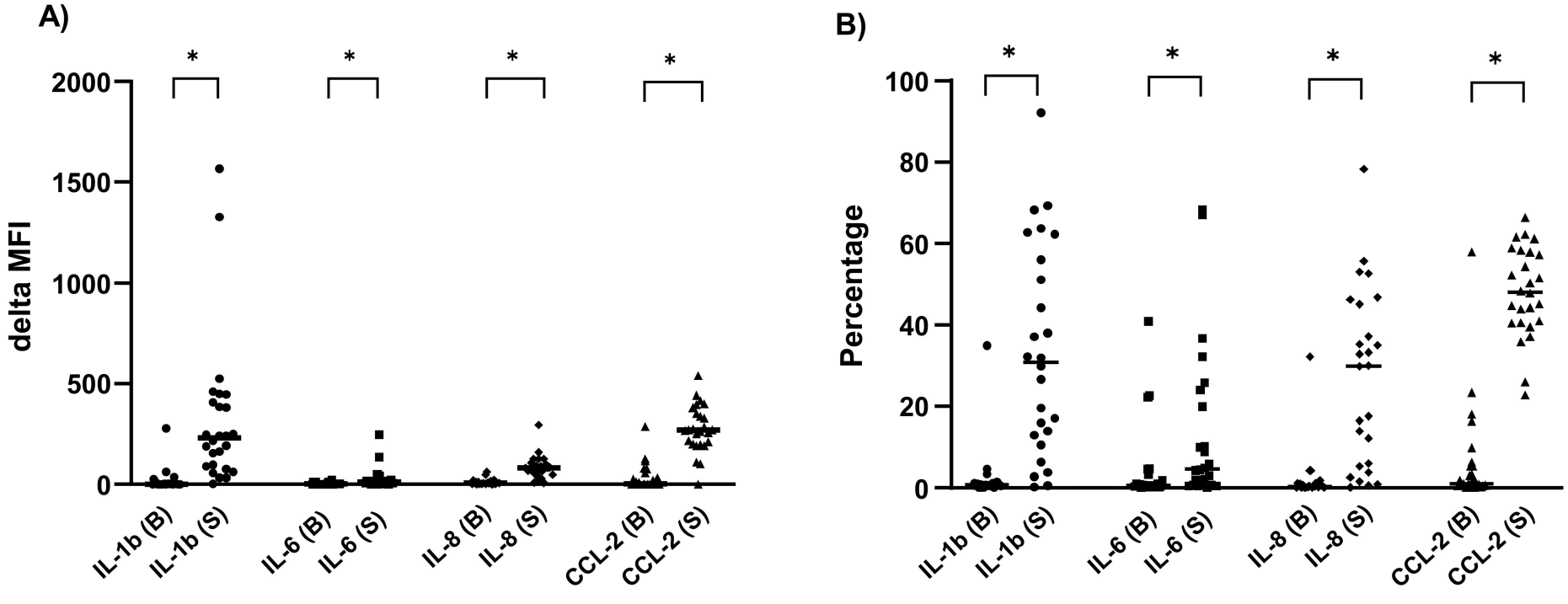

A)
delta MFI
2000
1500
1000
500
0
*
*
*
*
IL-1b (B)
IL-1b (S)
IL-6 (B)
IL-6 (S)
IL-8 (B)
IL-8 (S)
CCL-2 (B)
CCL-2 (S)
B)
Percentage
100
80
60
40
20
0
*
*
*
*
IL-1b (B)
IL-1b (S)
IL-6 (B)
IL-6 (S)
IL-8 (B)
IL-8 (S)
CCL-2 (B)
CCL-2 (S)

Figure 2

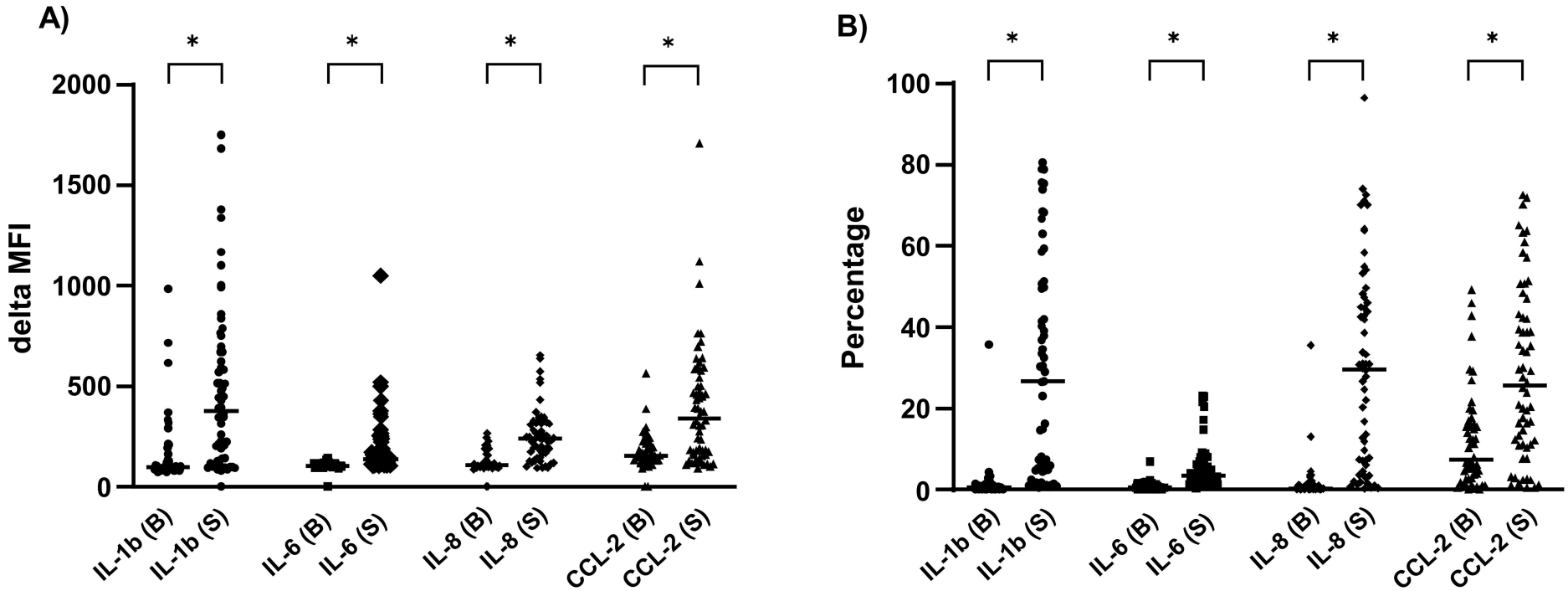
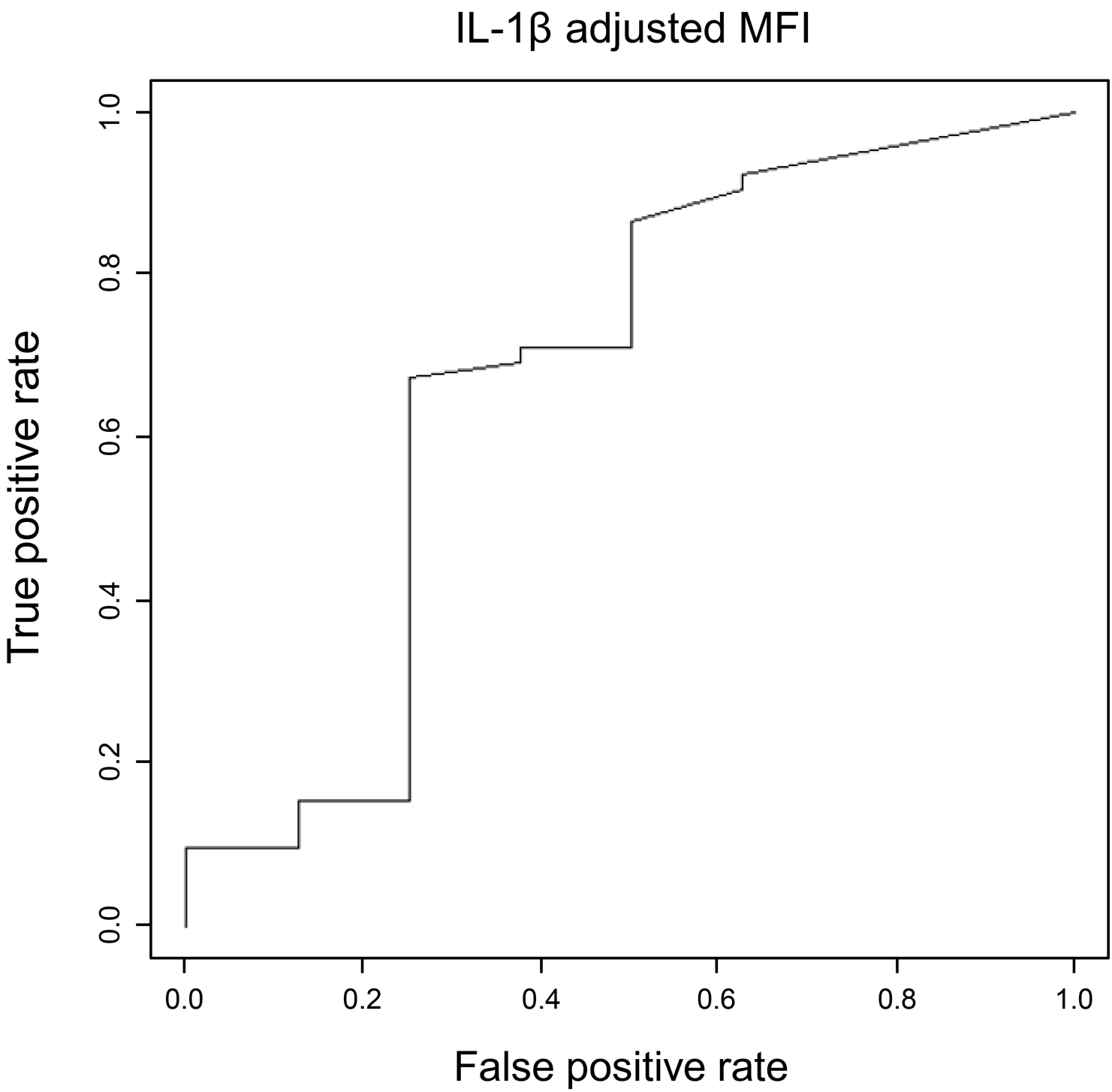
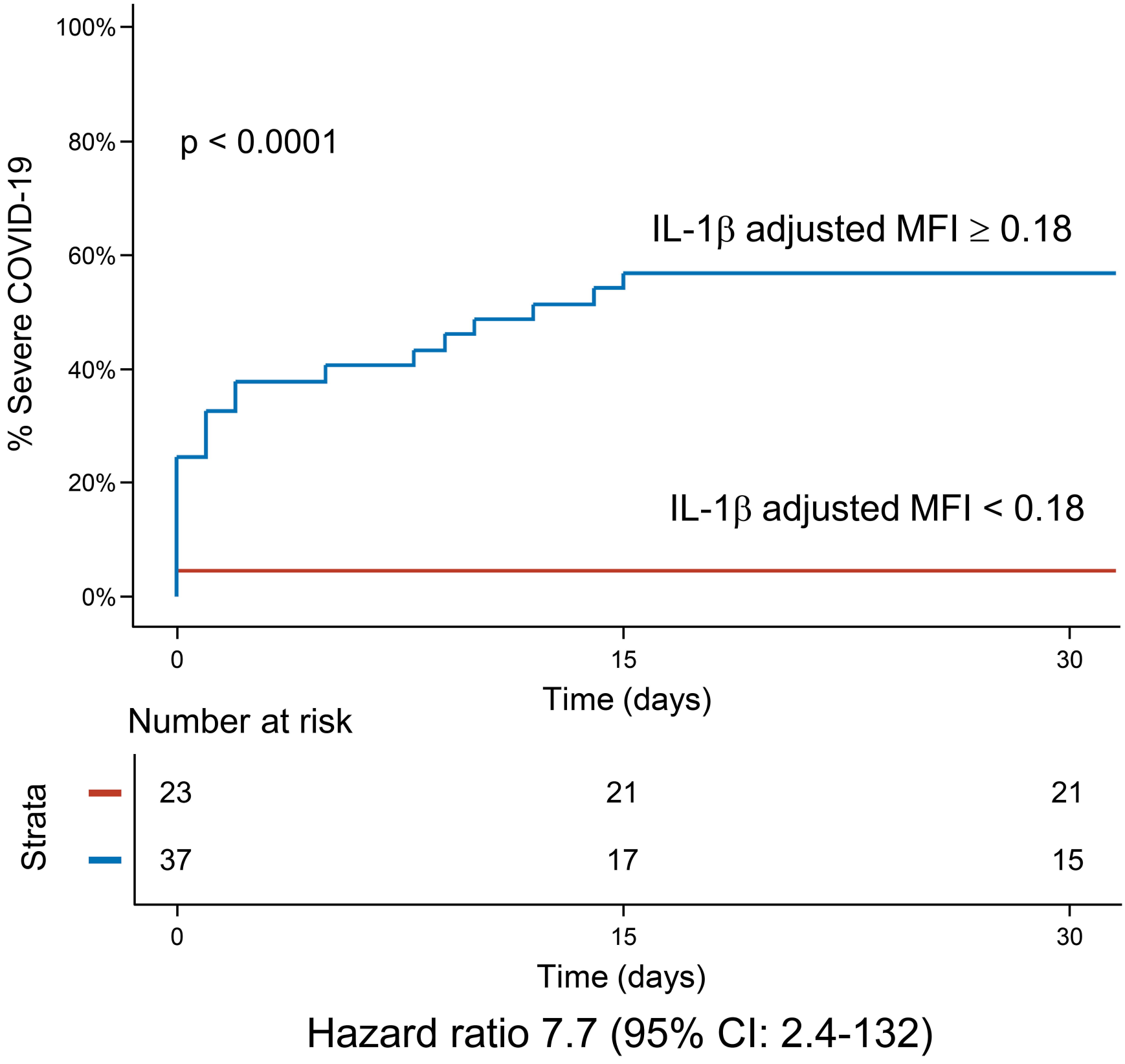

A)
B)
delta MFI
Percentage
2000
1500
1000
500
0
100
80
60
40
20
0
*
IL-1b (B)
IL-1b (S)
IL-6 (B)
IL-6 (S)
IL-8 (B)
IL-8 (S)
CCL-2 (B)
CCL-2 (S)

Figure 3



Cut-off: IL-1β adjusted MFI ≥ 0.18
AUC 0.68
Sensitivity 67%
Specificity 75%

Figure 4


100%
80%
60%
40%
20%
0%
% Severe COVID-19
p < 0.0001
IL-1β adjusted MFI ≥ 0.18
IL-1β adjusted MFI < 0.18
0
15
30
Time (days)
Number at risk
Strata
23
21
21
37
17
15
0
15
30
Time (days)
Hazard ratio 7.7 (95% CI: 2.4-132)

Supplementary Figure Legends

Supplementary Figure 1: Multi-color flow cytometry gating strategy for intracellular cytokine staining on PBMCs from a patient with chronic lymphocytic leukemia. The gating is used to analyze cytokine expression on monocytes, while CD14-/CD16- cells are excluded. The lower right panel shows the increased level of IL-1β that is stimulated with open reading frame 8. This patient developed severe coronavirus disease 2019 (COVID-19). patient that required hospital admission due to COVID-19.

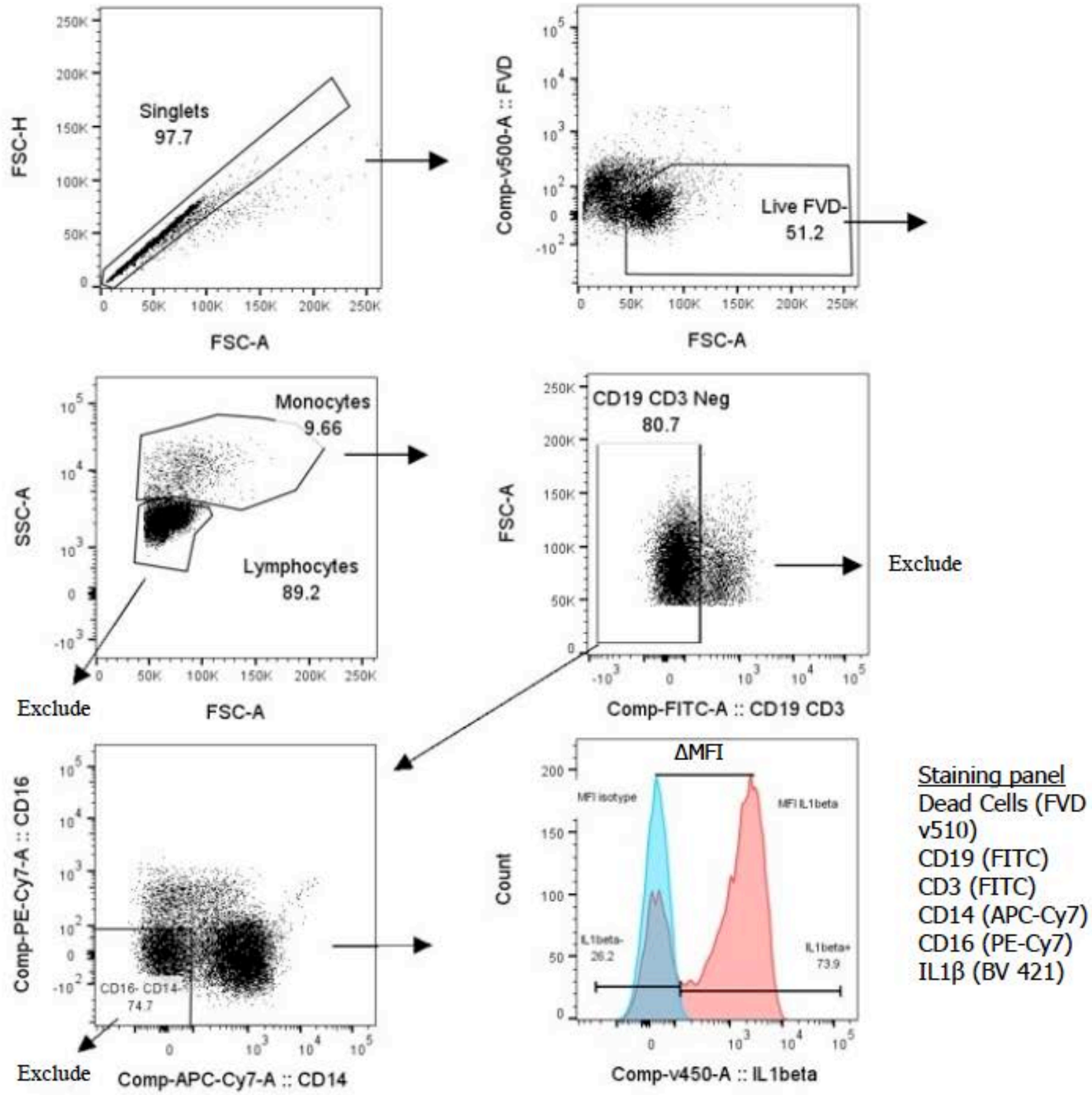
Supplementary Figure 2: Multi-color flow cytometry gating strategy for intracellular cytokine staining. This patient did not develop severe coronavirus disease 2019.

Supplementary Figure 3: Multi-color flow cytometry gating strategy for intracellular cytokine staining with Boolean gating strategy.

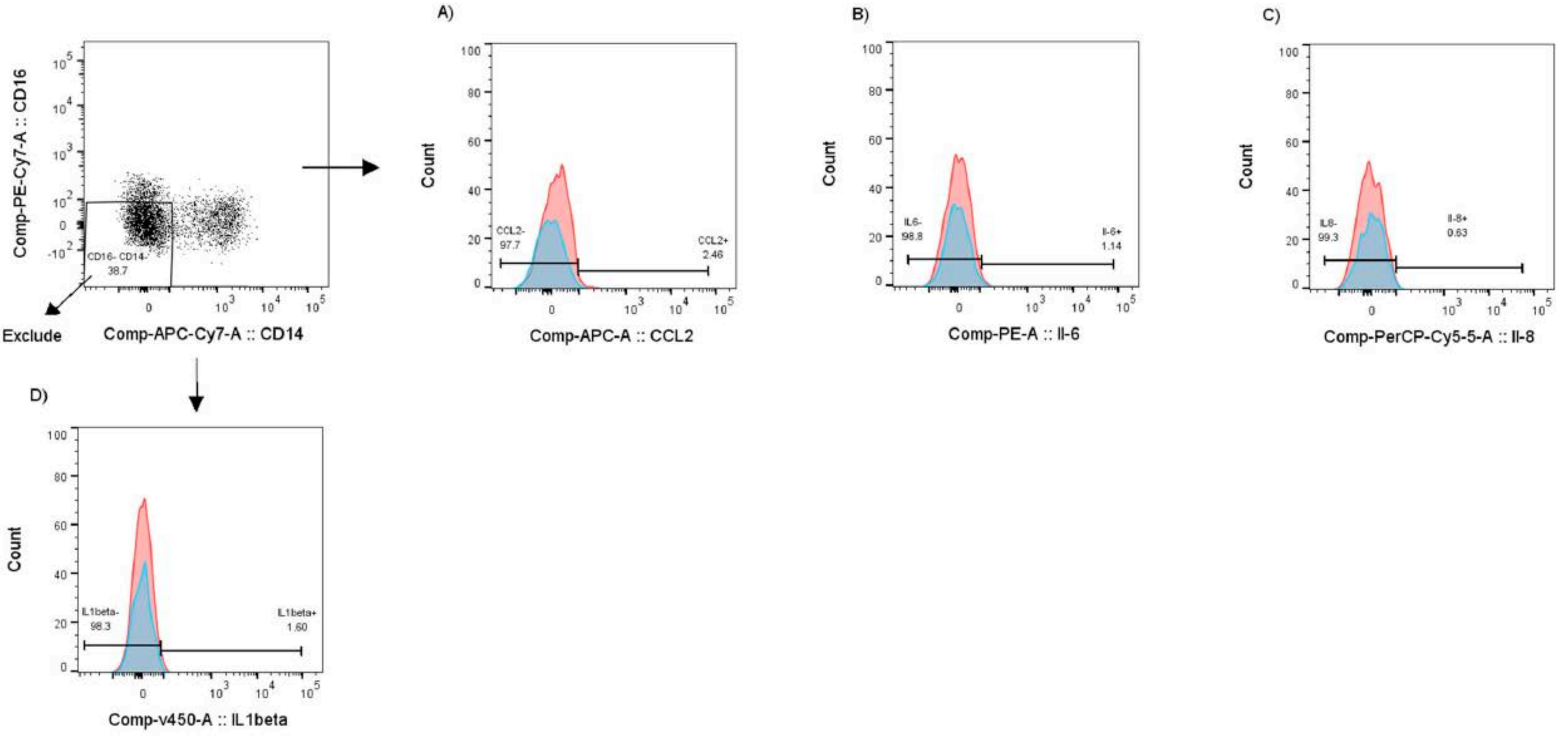
Supplementary Figure 4: Patterns of reactivity among ORF8-stimulated CLL sample

Supplementary Figure 5: Receiver operating curve analysis for adjusted MFI_{IL-6}, MFI_{IL-8}, and MFI_{CCL-2}

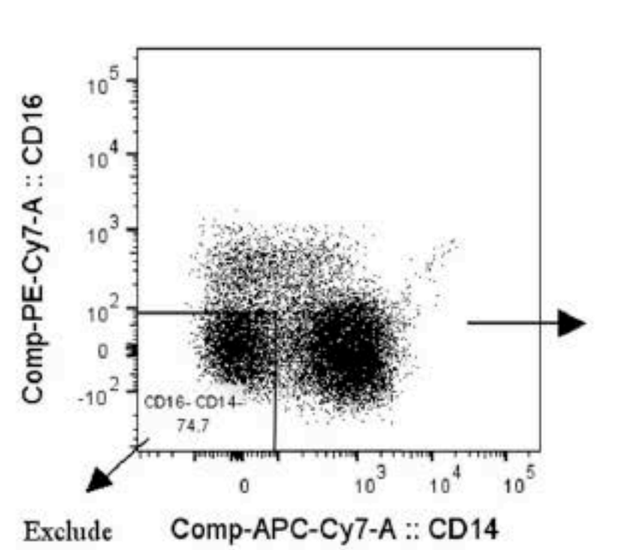
Supplementary Figure 1

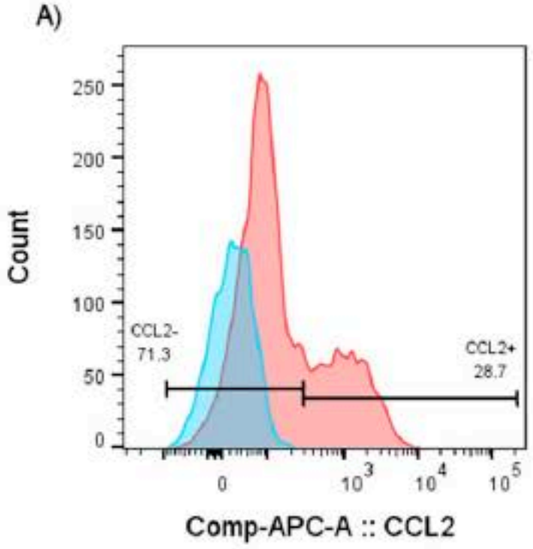


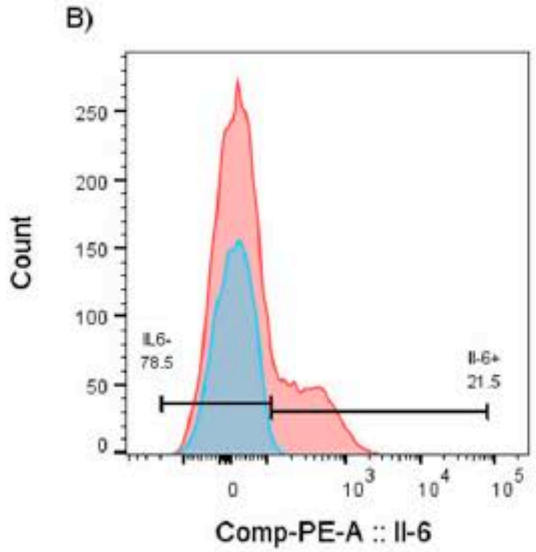
Staining panel
Dead Cells (FVD v510)
CD19 (FITC)
CD3 (FITC)
CD14 (APC-Cy7)
CD16 (PE-Cy7)
IL1β (BV 421)

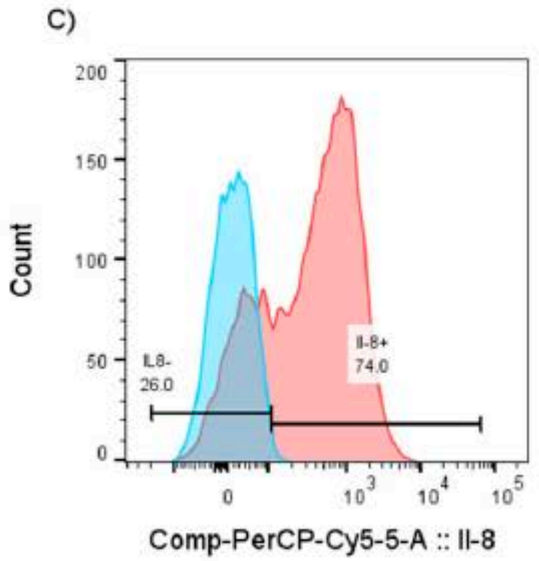


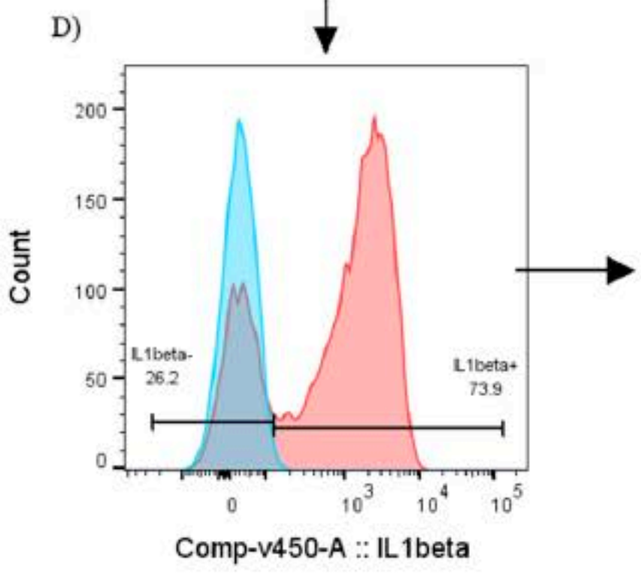
Supplementary Figure 2. Monocyte intracellular cytokine expression of A) CCL2; B) IL-6; C) IL-8; D) IL-1β





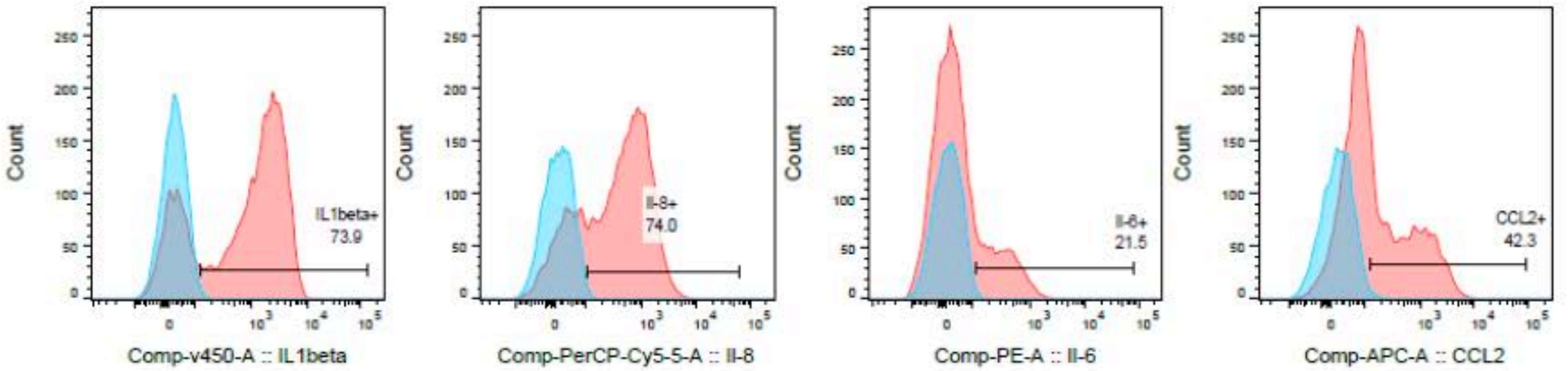




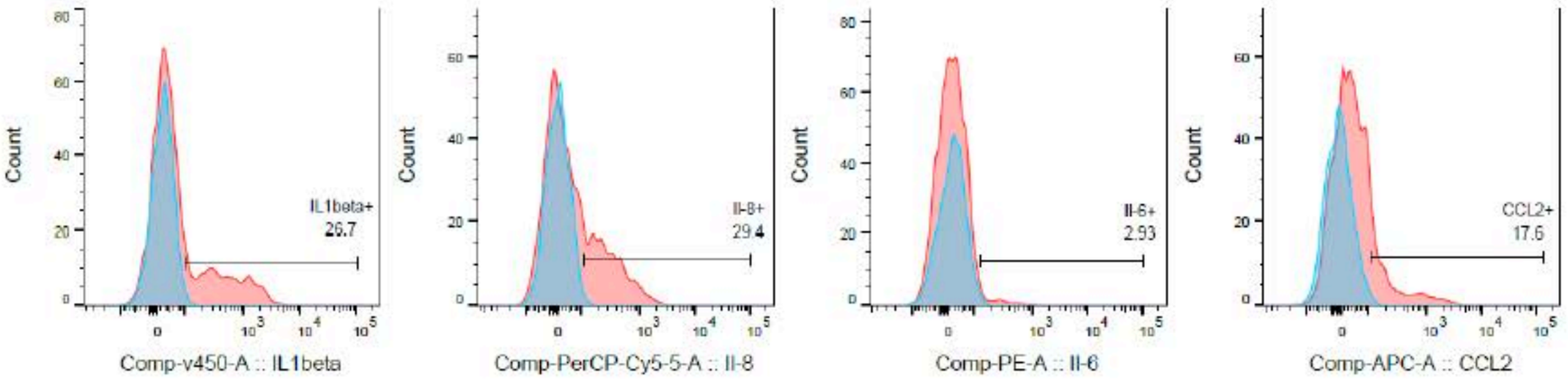


Boolean gating among IL1β+ staining cells
CCL2+/IL6+/IL8+
CCL2+/IL6-/IL8+
CCL2+/IL6+/IL8-
CCL2-/IL6+/IL8+
CCL2+/IL6-/IL8-
CCL2-/IL6+/IL8-
CCL2-/IL6-/IL8+
CCL2-/IL6-/IL8-

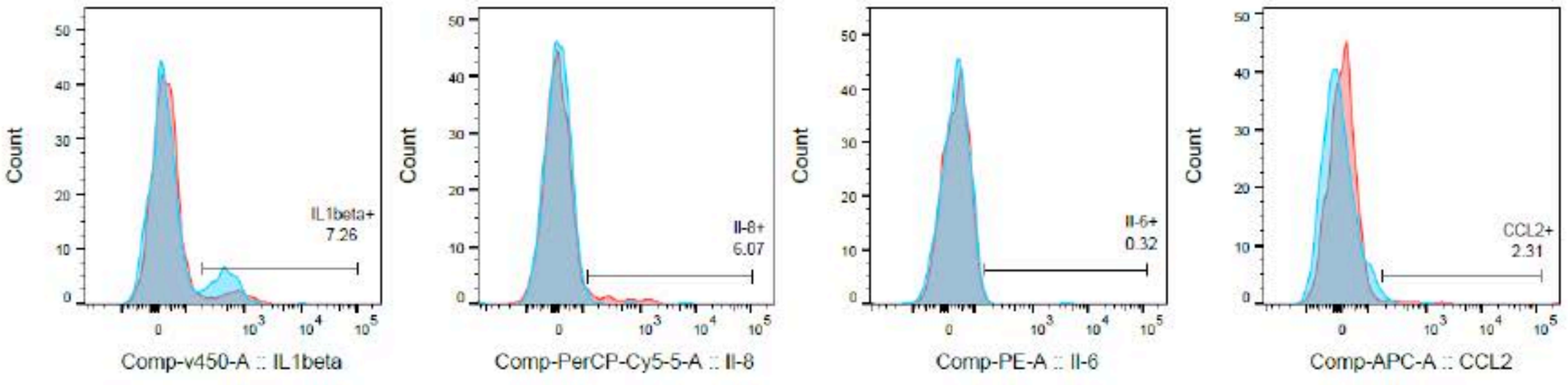
Supplementary Figure 3. Monocyte intracellular cytokine expression of A) CCL2; B) IL-6; C) IL-8; D) IL-1beta



Reactive OMST
Severe COVID-19



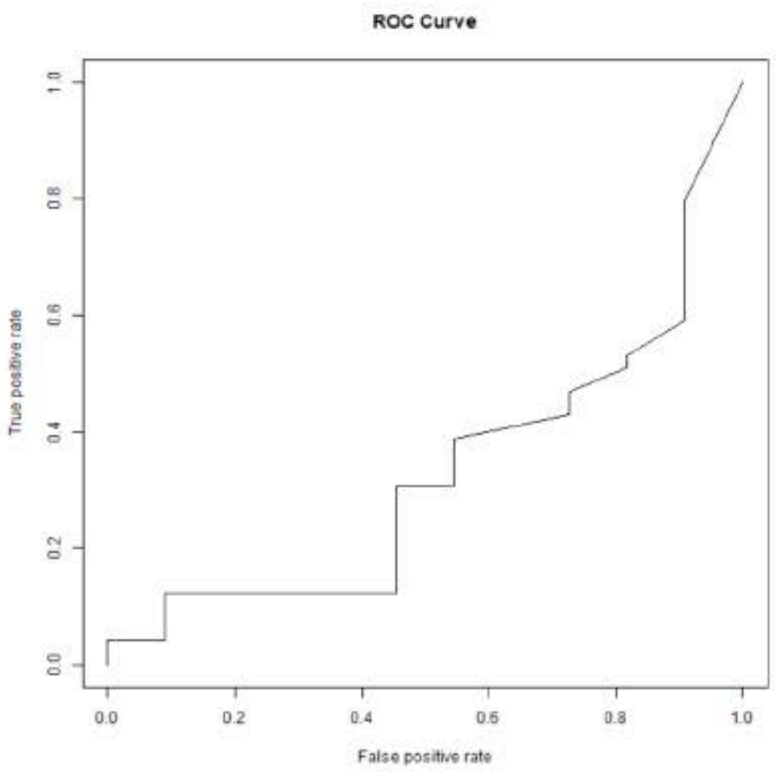
Reactive OMST
Severe COVID-19



Non-reactive OMST
Non-severe COVID-19

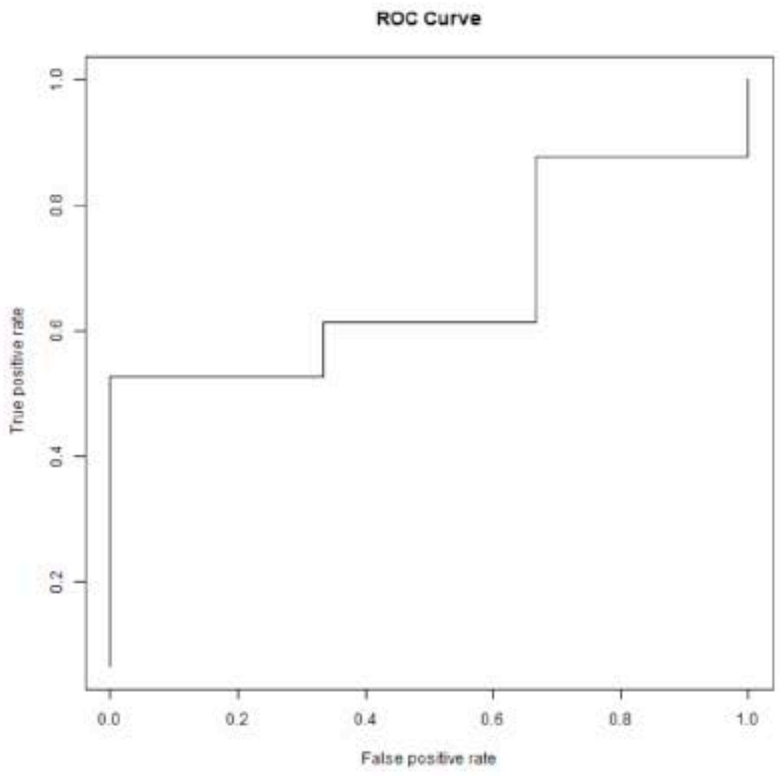
Supplementary Figure 4

IL-6 adjusted MFI



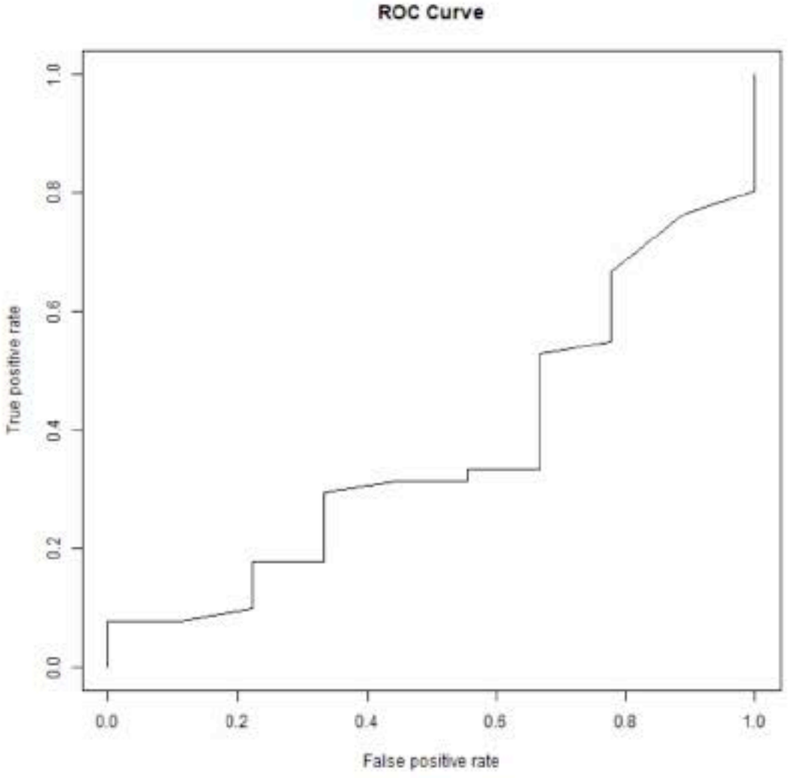
AUC 0.33

IL-8 adjusted MFI



AUC 0.67

CCL-2 adjusted MFI



AUC 0.37

Supplementary Figure 5

Supplemental Table 1. Comparisons of cytokine/chemokine expression levels between ORF8-stimulated monocytes from patients with chronic lymphocytic leukemia versus healthy controls.

	Normal Donors (n=26)	**CLL** (n=60)	p-value
ΔMFI			
IL-1β	230 (87-418)	378 (127-669)	0.11
IL-6	13 (5-27)	136 (111-247)	**<0.0001**
IL-8	80 (58-100)	238 (165-320)	**<0.0001**
CCL-2	269 (198-359)	339 (162-533)	0.05
% Monocytes staining positive			
IL-1β	31 (12-58)	27 (5-50)	0.46
IL-6	5 (1-21)	4 (2-6)	**0.02**
IL-8	30 (5-45)	30 (6-47)	0.56
CCL-2	48 (40-58)	25 (11-43)	**0.0003**
Adjusted MFI			
IL-1β	0.008 (0.004-0.01)	0.25 (0.06-1.38)	0.07
IL-6	0.001 (0.0006-0.003)	1.1 (0.2-5.9)	0.07
IL-8	0.004 (0.002-0.007)	0.28 (0.1-2.4)	**0.005**
CCL-2	0.005 (0.004-0.01)	0.42 (0.16-1.6)	**0.006**
Frequency	**% of Total Monocytes Median (25-75% IQR)**		
IL-1β+ cells (all)	18 (5-47)	27 (5-50)	0.89
IL-1β+/CCL2+/IL6+/IL8+	1 (0-4)	1 (0-3)	0.35
IL-1β+/CCL2+/Il6+/IL8-	0 (0-1)	0 (0-0.1)	0.3
IL-1β+/CCL2+/IL6-/IL8+	4 (1-15)	5 (0.3-16)	086
IL-1β+/CCL2+/IL6-/IL8-	**5 (2-9)**	**2 (0-4)**	**0.002**
IL-1β+/CCL2-/IL6+/IL8+	0.3 (0-1)	0.3 (0-2)	0.80
IL-1β+/CCL2-/IL6+/IL8-	0.1 (0-0.8)	0 (0-0.1)	0.20
IL-1β+/CCL2-/IL6-/IL8+	3 (0.4-7)	5 (0.3-14)	0.29
IL-1β+/CCL2-/IL6-/IL8-	4 (2-8)	3 (1-7)	0.51
MFI	**IL-1β (25-75% IQR)**		
IL-1β+ cells (all)	329 (198-512)	412 (153-700)	0.44
IL-β+/CCL2+/IL6+/IL8+	**1191 (670-1761)**	**2237 (1456-3024)**	**0.0002**
IL-1β+/CCL2+/Il6+/IL8-	223 (159-316)	307 (147-1273)	0.15
IL-1β+/CCL2+/IL6-/IL8+	429 (333-644)	484 (287-843)	0.35
IL-1β+/CCL2+/IL6-/IL8-	246 (182-342)	275 (154-479)	0.49
IL-1β+/CCL2-/IL6+/IL8+	**1103 (539-1825)**	**2382 (1300-3165)**	**0.0009**
IL-1β+/CCL2-/IL6+/IL8-	220 (152-290)	303 (146-710)	0.21
IL-1β+/CCL2-/IL6-/IL8+	421 (277-602)	532 (315-754)	0.28
IL-1β+/CCL2-/IL6-/IL8-	225 (176-293)	241 (125-395)	0.87

Supplemental Table 2. Previous CLL-directed therapy before COVID-19 diagnosis.

Treatment before COVID-19 diagnosis	N=60 (%)
Observation	30 (50)
BTK inhibitor (monotherapy)	17 (28)
Anti-CD20 (monotherapy)	3 (5)
BTK inhibitor and Anti-CD20	2 (3)
BTK inhibitor, anti-CD20, venetoclax	4 (7)
Chemotherapy, anti-CD20, BTK-inhibitor	1 (2)
Anti-CD20 and venetoclax	1 (2)
CAR-T	2 (3)

Supplemental Table 3. IL-1β^{+} producing cell subsets among ORF8-stimulated monocytes from patients with chronic lymphocytic leukemia

Parameters	**CLL** (n=60)
Frequency	**% of Total Monocytes Median (25-75% IQR)**
IL-1β+ cells (all)	27 (5-50)
IL-1β+/CCL2+/IL6+/IL8+	1 (0-3)
IL-1β+/CCL2+/Il6+/IL8-	0 (0-0)
IL-1β+/CCL2+/IL6-/IL8+	5 (0.3-16)
IL-1β+/CCL2+/IL6-/IL8-	**2 (0-4)**
IL-1β+/CCL2-/IL6+/IL8+	0.3 (0-2)
IL-1β+/CCL2-/IL6+/IL8-	0 (0-0)
IL-1β+/CCL2-/IL6-/IL8+	5 (0.3-14)
IL-1β+/CCL2-/IL6-/IL8-	3 (1-7)
ΔMFI	**IL-1β (25-75% IQR)**
IL-1β+ cells (all)	412 (153-700)
IL-β+/CCL2+/IL6+/IL8+	**2237 (1456-3024)**
IL-1β+/CCL2+/Il6+/IL8-	307 (147-1273)
IL-1β+/CCL2+/IL6-/IL8+	484 (287-843)
IL-1β+/CCL2+/IL6-/IL8-	275 (154-479)
IL-1β+/CCL2-/IL6+/IL8+	**2382 (1300-3165)**
IL-1β+/CCL2-/IL6+/IL8-	303 (146-710)
IL-1β+/CCL2-/IL6-/IL8+	532 (315-754)
IL-1β+/CCL2-/IL6-/IL8-	241 (125-395)

CLL, chronic lymphocytic leukemia; ΔMFI, delta median fluorescence intensity; IQR, interquartile range

Supplemental Table 4. Univariate Analysis of IL-1β subsets and association with severe COVID-19 (Cox proportional hazards regression model)

Parameters	HR (95% CI)	p-value
Frequency		
IL-1β+/CCL2+/IL6+/IL8+	1.1 (0.97-1.2)	0.17
IL-1β+/CCL2+/Il6+/IL8-	1.2 (0.7-2.2)	0.55
IL-1β+/CCL2+/IL6-/IL8+	0.99 (0.95-1.04)	0.75
IL-1β+/CCL2+/IL6-/IL8-	0.96 (0.86-1.06)	0.42
IL-1β+/CCL2-/IL6+/IL8+	1.3 (0.99-1.6)	0.05
IL-1β+/CCL2-/IL6+/IL8-	2.2 (0.3-16.6)	0.43
IL-1β+/CCL2-/IL6-/IL8+	1.0 (0.97-1.1)	0.62
IL-1β+/CCL2-/IL6-/IL8-	0.95 (0.85-1.1)	0.35
ΔMFI		
IL-1β+/CCL2+/IL6+/IL8+	1.0 (0.99-1.0)	0.47
IL-1β+/CCL2+/Il6+/IL8-	1.00 (1.00-1.0007)	**0.01**
IL-1β+/CCL2+/IL6-/IL8+	1.0 (0.99-1.0)	0.31
IL-1β+/CCL2+/IL6-/IL8-	1.00 (1.00-1.003)	**0.03**
IL-1β+/CCL2-/IL6+/IL8+	1.0 (0.99-1.0)	0.47
IL-1β+/CCL2-/IL6+/IL8-	1.0006 (0.99-1.00)	0.08
IL-1β+/CCL2-/IL6-/IL8+	1.0004 (0.99-1.00)	0.55
IL-1β+/CCL2-/IL6-/IL8-	0.9997 (0.997-1.00)	0.81
IL-1β Adjusted MFI		
IL-1β+/CCL2+/IL6+/IL8+	0.99 (0.98-1.01)	0.86
IL-1β+/CCL2+/Il6+/IL8-	1.00 (0.97-1.04)	0.75
IL-1β+/CCL2+/IL6-/IL8+	0.99 (0.99-1.00)	0.24
IL-1β+/CCL2+/IL6-/IL8-	1.00 (0.99-1.01)	0.86
IL-1β+/CCL2-/IL6+/IL8+	1.00 (1.00-1.007)	0.08
IL-1β+/CCL2-/IL6+/IL8-	1.00 (1.001-1.01)	**0.02**
IL-1β+/CCL2-/IL6-/IL8+	1.00 (0.99-1.004)	0.31
IL-1β+/CCL2-/IL6-/IL8-	0.99 (0.98-1.1)	0.53