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Patients Hospitalized with COVID-19 Demonstrate Distinct Plasma Cytokine and Chemokine Concentrations in vivo and TLR-Mediated Cytokine and Chemokine Production in Whole Blood in vitro

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COVID-19 · SARS-CoV-2 · Innate immunity · Toll-like receptors

Abstract
Introduction: SARS-CoV-2's continued global health impact underscores the importance of ongoing pathogenesis research. Insights into the host's first line of defense against severe COVID-19 identify actionable biomarkers, informing disease management or therapeutics. Yet, the innate immune response, including cytokines, chemokines, adenosine deaminases (ADAs) and Toll-like receptors (TLRs), relevant to COVID-19 remain incompletely characterized. ***Methods:*** Peripheral blood was longitudinally collected between May 2020 and March 2021 from COVID-19 hospitalized adults ($N = 79$) and healthy controls (HCs) ($N = 14$; not tested, assumed COVID-negative, no viral exposure or symptoms). Heparinized blood was fractionated for plasma cryopreservation and in vitro whole blood TLR-stimulation employing TLR-3, -4, and -7/8 agonists. Post-stimulation culture supernatants were analyzed using multiplex and enzymatic assays. ***Results:*** Upon hospitalization, plasma concentrations of IFNγ, IL-6, CXCL10, and ADAs were significantly upregulated compared to convalescent time points and HCs. Participants with fatal COVID-19 exhibited higher IL-27, CXCL10, and ADAs concentrations upon admission. Plasma cytokines, chemokines, and ADAs were positively correlated and associated with distinct temporal patterns. TLR-stimulated cell cultures from patients produced reduced IFNα2, IFNγ, IL-12p40, and IL-12p70 compared to HCs or later time points. ***Conclusion:*** Higher

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plasma concentrations of IL-27, CXCL10, and ADAs at admission were associated with severe COVID-19 and mortality. Reduced TLR-mediated IFNα2, IFNγ, and IL-12p70 production suggests COVID dampens Th1-polarizing innate immune responses, providing insight into immunological sequelae of SARS-CoV-2 infection.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic has had a devastating impact on global health. While the pandemic has ended, SARS-CoV-2 has become endemic and continues to cause morbidity and mortality worldwide [1]. Moreover, the threat of future pandemics persists, highlighting the importance of understanding disease pathogenesis. Acute responses to SARS-CoV-2 infection include hyperinflammation, cytokine storm, thrombosis [2], and long-term morbidity, including fatigue, myocardial damage, and lung injury [3–5]. Further characterization of the longitudinal immunological effects of SARS-CoV-2 is important for identifying actionable biomarkers and developing innovative strategies for the prevention and treatment of COVID-19.

The innate immune system is the first line of defense against pathogens, generating an inflammatory response via production of mediators such as cytokines and chemokines [6–8]. Characterizing the innate immune response to SARS-CoV-2 infection is crucial for assessing disease severity, analyzing individual responses over time, and identifying relevant biomarkers. Since the onset of the pandemic, immunologic studies have defined key events post SARS-CoV-2 infection, including the mechanism of viral entry into host cells [9], the role of cytokine storm [10], and complement dysregulation in patients with acute and long COVID-19 [11–14].

In patients with COVID-19, plasma concentrations of IL-6, IL-18, and TNFα are elevated [15, 16]. These acutely regulated cytokines have been implicated as potential biomarkers of disease severity [17, 18]. Chemokines are also implicated in COVID-19 pathogenesis as CXCL8 contributes to inflammation in viral infections [19, 20], and CXCL10 correlates with disease severity [21]. Adenosine deaminases (ADAs) are enzymes involved in immunoregulation and exist in two distinct isoforms in humans [22, 23]. ADA1 is intracellular and converts adenosine, which inhibits inflammation, to the immunologically inert inosine, thereby enhancing inflammation [24, 25]. ADA2 is extracellular and is produced by M2-type macrophages and monocytes, enhancing Th2-polarized responses while inhibiting inflammation [26, 27]. Studies examining ADA in the context of COVID-19 suggest its use as a biomarker of SARS-CoV-2 infection [28], due to elevated total ADA mRNA levels in nasopharyngeal samples of infected Hong Kong patients compared to healthy controls (HCs), while past studies suggest the use of ADA inhibitors as possible therapeutic agents [29, 30].

Toll-like receptors (TLRs), pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), are crucial for pathogen detection and initiating host response to infection. Infection and inflammation can alter the immunologic set point for TLR signaling via epigenetic modifications [31]. This is important because TLR signaling mediates robust innate immune responses, in part via NF-kB pathways, which subsequently lead to the production of cytokines and chemokines [32, 33]. While the expression of TLRs during COVID-19 has been explored [32, 33], there is limited information on the impact of COVID-19 on TLR-mediated cytokine production. A few studies describe impaired cytokine production upon TLR agonist stimulation of whole blood from COVID-19 patients [34, 35] and peripheral blood mononuclear cells (PBMCs) [35–37]. While these studies suggested a dampening of the innate immune response following in vitro TLR stimulation, they were limited in the TLR agonists tested, time points assessed, and cytokines analyzed post-stimulation. Our study provides a comprehensive analysis of TLR-mediated cytokine and chemokine production in COVID-19, comparing response during and post-hospitalization to HCs.

To better understand the initial innate immune response to SARS-CoV-2 infection, we measured in vivo cytokine and chemokine concentrations, in vivo ADA activities, and in vitro TLR-mediated cytokine and chemokine production. Immunological responses of 79 study participants hospitalized with COVID-19 were profiled over 1 year following hospital admission. HCs with no history of SARS-CoV-2 infection ($N = 14$), collected prior to the emergence of the virus in the USA, served as a comparison group. Our study increases the depth of innate immune phenotyping in individuals with COVID-19 by interrogating the functionality of the cells in response to common stimuli [38] that activate specific TLRs as we highlight a set of cytokines and chemokines associated with severe disease. Modifications in these downstream processes reveal how infection may dampen pro-inflammatory innate immune responses, and the longitudinal study design enables assessment of TLR-

Table 1. Clinical characteristics of the Boston study site cohort

	Death (*N* = 9)	Recovered (*N* = 70)	HC (*N* = 14)
Sex			
Female	3 (33.3%)	27 (38.6%)	5 (35.7%)
Male	6 (66.7%)	43 (61.4%)	9 (64.3%)
Age, years			
Median [IQR]	72.0 [64.0, 84.0]	63.0 [52.3, 70.0]	66.5 [53.0, 70.8]
Age by category, years			
<65	3 (33.3%)	39 (55.7%)	5 (35.7%)
>65	6 (66.7%)	31 (44.4%)	9 (64.3%)
BMI[a], kg/m^2			
Median [IQR]	24.9 [24.2, 27.0]	28.8 [25.7, 32.6]	–
BMI by category, kg/m^2			
Underweight (<18.5)	0 (0%)	0 (0%)	–
Healthy weight (>18.5 and <25)	5 (55.6%)	8 (11.4%)	–
Overweight (>25.5 and <30)	3 (33.3%)	33 (47.1%)	–
Obese (>30)	1 (11.1%)	28 (40.0%)	–

[a]BMI of healthy controls (HC) was not recorded.

mediated responses over time. Our findings show higher plasma concentrations of IL-27, CXCL10, and ADAs at admission in those who succumb to COVID-19, as well as impaired IL-12p40 production after in vitro stimulation of TLR-2, -3, -4, and -7/8, indicating COVID-19-associated dysregulation of the immune system.

Methods

Study Design and Sample Collection

This study was part of the IMmunoPhenotyping Assessment in a COVID-19 Cohort (IMPACC), a prospective study examining hospitalized patients with COVID-19 [39]. Our primary objective was to define biomarkers of disease severity and outcome in adults hospitalized with COVID-19 at Brigham and Women's Hospital (BWH), the Local Assay Site (LAS) in Boston. Participants aged ≥18 years who were hospitalized between May 2020 and March 2021 and agreed to study procedures were enrolled within 72 h of admission. HCs (*N* = 14), who were not part of the IMPACC study and were recruited with sample collection prior to the widespread emergence of the virus in the USA, were used for comparison. These HCs were not tested (as testing was not accessible to non-hospitalized patients at the start of the pandemic) and assumed COVID negative due to a lack of symptoms and no known viral exposure. Demographic information, medical history, and laboratory findings were collected upon recruitment and are briefly summarized in Table 1. Confirmation of SARS-CoV-2 infection was based on a clinical presentation with COVID-19 symptoms and a positive polymerase chain reaction (PCR) test. In the case of an initial negative PCR test, participants could remain enrolled based on a clinical presentation consistent with COVID-19 and a positive follow-up SARS-CoV-2 PCR test. If the second PCR test was negative, participants were removed from the study and classified accordingly.

Biological Samples

Peripheral blood samples were collected according to the IMPACC standardized operating procedure [34] using EDTA (BD) vacutainer tubes on the first or second day of admission (V1), between 3 and 6 days (V2), 6 and 9 days (V3), 12 and 17 days (V4), 19 and 22 days (V5), and 23 and 27 days (V6) of hospitalization, and during follow-up visits at 3 months (V7), 6 months (V8), 9 months (V9), and 12 months (V10) post discharge (Fig. 1a). Blood samples from participants and HCs were processed within 6 h of collection and samples were stored at −80°C for downstream analysis or used for the TLR stimulation whole blood assay as described below. Out of the samples collected at a single time point from 14 HCs, 11 were included in TLR stimulation assay, nine in the cytokine and chemokine multiplex assay, and 10 in the ADA assay.

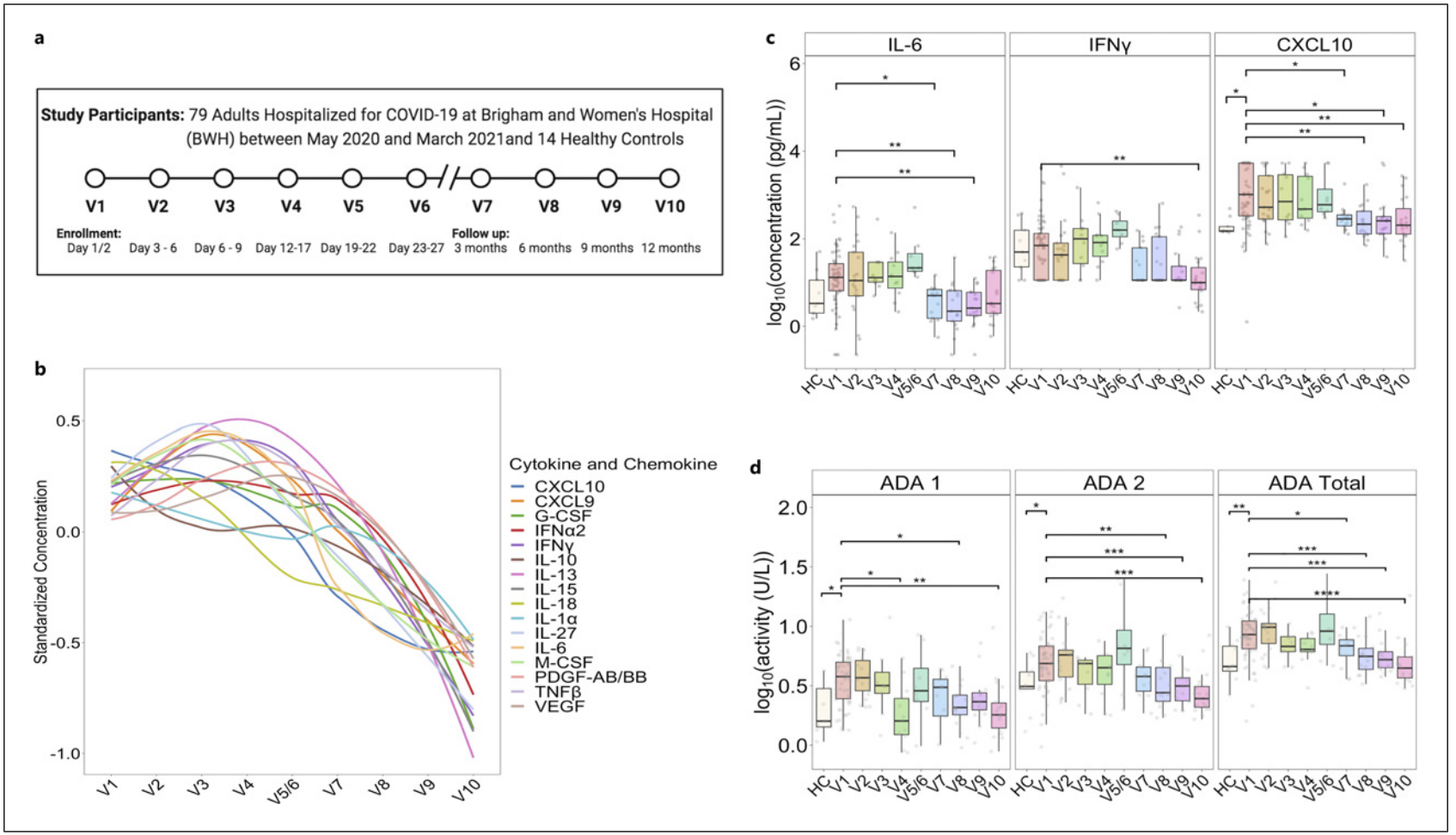

Fig. 1. Longitudinal analysis of COVID-19 hospitalized patients show a dynamic innate immune profile of cytokines, chemokines, and ADA levels compared to HCs. **a** Boston study site visit overview. **b** LOESS regression of standardized cytokine and chemokine concentrations present at a significantly higher level in hospitalized COVID-19 patients upon admission compared to later time points. **c** IL-6, IFNγ, and CXCL10 show significant changes in plasma concentrations over time. **d** ADA1, ADA2, and total ADA activities show significant changes in plasma over time and compared to the HCs. The cytokine and chemokine concentrations (in pg/mL) and ADA activities (in U/L) are log-10 transformed, and the adjusted *p* values are calculated using Benjamini-Hochberg (BH) procedure to correct for the false discovery rate. **p*.adj <0.05, ***p*.adj <0.01, ****p*.adj <0.001, and *****p.adj* <0.0001.

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TLR Stimulations

A previously published polychromatic flow cytometric high-throughput assay [40] was adapted to measure analyte production in response to TLR stimulation from participants at V1 and V7, and HCs. Ninety-six-well plates were coated with TLR agonists PAM3CSK4 (PAM; TLR-1/2 agonist) (Sigma Aldrich, cat. # tlrl-pms), PGN-SA (PGN; TLR-2 agonist) (Invivogen, cat. # tlrl-pgnsa), Poly(I:C) (TLR-3 agonist; Invivogen, cat. # tlrl-picw), LPS (TLR-4 agonist; Invivogen, cat. # tlrl-pelps), R848 (TLR-7/8 agonist; Invivogen, cat. # tlrl-r848-5), and CpG-ODN2395 (CpG; TLR-9 agonist) (Invivogen, cat. # tlrl-2395), as well as RPMI 1640 (Gibco) as a vehicle stimulation. These agonists stimulate TLR-1/2, TLR-2, TLR-3, TLR-4, TLR-7/8, and TLR-9, respectively. Heparinized whole blood samples were mixed at a 1:1 ratio with warmed RPMI 1640 (Gibco) and were pipetted onto the pre-prepared plates, ensuring mixing. Blood samples were stimulated with the TLR agonists for 6 h along with Brefeldin A (Sigma Aldrich). Culture cell supernatants were then collected for further analysis.

Cytokine and Chemokine Multiplex Assay

A bead-based multiplex assay was utilized to determine the concentrations of 48 cytokines and chemokines in the plasma and supernatants. Plasma samples, manufacturer's standards, and controls were loaded into a Corning CellBIND® 384-well plate (cat. #CLS3764) with manufacturer's reagents and Dulbecco's phosphate-buffered saline (DPBS, cat. #14190) in accordance with the manufacturer's instructions included in the 48-plex Millipore Milliplex Map Kit (cat. #HCYTMAG-60K-PX48). A FlexMAP 3D system and Luminex xPONENT software (both from Luminex-Corp.; Austin, TX, USA) were used to determine the best fits of four-parameter logistic, five-parameter logistic, or exponential functions to a dilution series standard curve. This software calculated the concentrations of CCL2, CCL3, CCL4, CCL11, CCL22, CXCL1, CX3CL1, CXCL8, CXCL9, CXCL10, EGF, FGF-2, FLT-3L, G-CSF, IFNα2, IFNγ, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IL-27, CCL7 (MCP-3), M-CSF, PDGF-AA, PDGF-AB/BB, TGFα, TNFα, TNFβ, VEGF in our samples in pg/mL. Lower and upper limits of detection were defined by the software, and any concentrations of aforementioned analytes outside this range were listed as the upper or lower limits of detection.

ADA Assay

An ADA Assay Kit (cat. #DZ117A) (Diazyme Laboratories, Poway, CA, USA) was utilized to determine ADA1, ADA2, and total ADA concentrations in the plasma samples. Duplicates of samples treated with Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (cat. #1261) (Tocris Bioscience, Bristol, UK) (20 μM), in addition to duplicates of samples treated with DPBS, were loaded onto a Corning CellBIND® 384-well plate to determine ADA2 and total ADA activity. A dilution series of the ADA calibrator (cat. #DZ117A-Cal) and quality controls (cat. #DZ117A-Con) were also loaded onto the plate to calculate the activity within each well with a log-standard curve. Following addition of the Assay Kit Reagents, the absorbance of each well within the 384-well plate was read at 500 nm at five-minute intervals for 1 h using an Infinite M Plex (Tecan, Mannedorf, Switzerland). ADA2 and total ADA were defined as the average of the duplicates for a given sample, and ADA1 was defined as the difference between total ADA and ADA2.

Statistical Analysis

ADA, cytokine, and chemokine concentrations (in vivo and in vitro) were log10-transformed. Analysis was performed using R version 4.2.1. To assess the differences in cytokines, chemokines, and ADAs over time, unpaired Wilcoxon Rank-sum Test (ggpubr_0.4.0 package) was used with Benjamini-Hochberg (BH) adjustment to control the false discovery rate. Accordingly, reported *p* values were adjusted. The trajectories of cytokines, chemokines, and ADAs were generated using the locally estimated scatterplot smoothing (LOESS) method (ggplot2_3.4.0 package) and the hierarchical clusters were determined using a p-heatmap (pheatmap_1.0.12 package). The correlation table was generated using Spearman's rank correlation coefficients (sjPlot_2.8.12). Principal component analysis (PCA) was performed using factoextra_1.0.7. Additional packages used for data assessment and formatting included tidyverse_1.3.2, dplyr_1.0.10, knitr_1.40, rstatix_0.7.0, patchwork_1.1.2, alpacage_0.1.0, scales_1.2.1, FactoMineR_2.6, ggrepel_0.9.1, table1_1.4.3, forcats_0.5.2, ggsci_2.9, and splitstackshape_1.4.8.

Results

The Boston study LAS participants consisted of adults enrolled at BWH between May 2020 and March 2021 as part of the IMPACC study ($N = 79$) as well as HCs ($N = 14$) that were recruited for comparison (Fig. 1a; Table 1).

Of the 79 hospitalized participants, most of whom were male (62.0%), nine died of COVID-19 (66.7% males in the deceased group) while 70 recovered (61.4% males in the recovered group). The median age of deceased participants was 72 years and that of those who recovered was 63 years. The median age of HCs was 66.5 years.

Distinct Trajectories of Plasma Cytokines, Chemokines, and ADAs in Patients Hospitalized with COVID-19

Patients hospitalized with COVID-19 demonstrated a distinct innate immune profile compared to HCs. The p-heatmap analysis revealed that mean plasma cytokine and chemokine concentrations exhibited dynamic trajectories during hospitalization and after discharge (Fig. 1b; online suppl. Fig. 1; for all online suppl. material, see https://doi.org/10.1159/000545432). Hierarchical clustering identified five clusters of cytokines/chemokines with similar profiles. Clusters 3 and 5 demonstrated an increase in concentration throughout the first 2 weeks of hospitalization followed by a decrease as participants recovered, while Cluster 4 showed a constant decrease over time (online suppl. Fig. 1). Clusters 1 and 2 demonstrated minimal concentration change over the year. Of the 48 cytokines measured, 16 (CXCL9, CXCL10, G-CSF, IFNα2, IFNγ, IL-1α, IL-6, IL-10, IL-13, IL-15, IL-18, IL-27, M-CSF, PDGF-AB/BB, TNFβ, and VEGF) were significantly higher on the day of admission (V1) compared to the convalescent time points (V7-V10) (online suppl. Table 1). Their individual trajectories are depicted in Figure 1b. Most of the cytokines and chemokines that were upregulated at admission compared to convalescent time points and HC are in Clusters −3, −4, and −5 (except IL-1α and IL-10, which are in Cluster 1). Plasma concentrations of pro-inflammatory cytokines and chemokines such as IFNγ, IL-6, and CXCL10 were high during the acute infection period, then decreased in concentration during the recovery phase (Fig. 1c; online suppl. Fig. 2).

A similar pattern is also visualized in plasma ADA isotype activity, which was significantly greater at V1 compared to later convalescent time points (Fig. 1d; online suppl. Table 2). Furthermore, plasma ADA1 (1.9-fold), ADA2 (1.6-fold), and total ADA (1.7-fold) activities at V1 were significantly greater in infected individuals than in HCs (Fig. 1d; online suppl. Fig. 2). This coincides with the initial pro-inflammatory response observed among participants following hospital admission with COVID-19, which also decreased longitudinally with recovery. We correlated activities of ADA1, ADA2, and total ADA with cytokine and chemokine concentrations

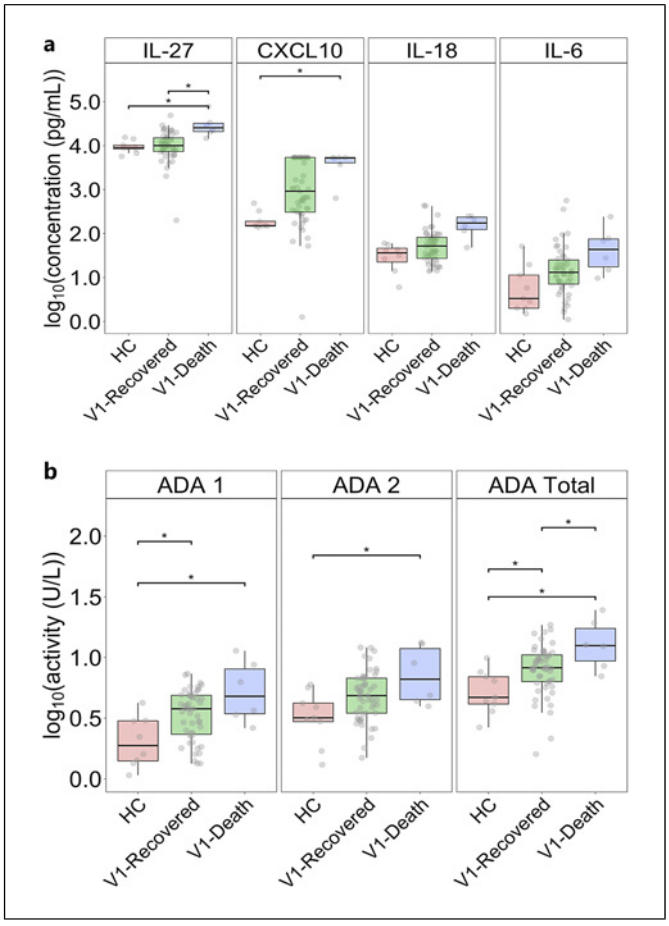

Fig. 2. Inflammatory immune profile of deceased patients at the time of hospital admission compared to patients who recovered and HCs. **a** Upon hospitalization, patients who succumbed to infection had significantly higher levels of IL-27 and CXCL10, as well as elevated levels of IL-18 and IL-6 compared to HCs and patients who recovered from COVID-19. **b** Upon hospitalization, patients who succumbed to infection had significantly higher ADA1, ADA2, and total ADA activities compared to HCs and higher total ADA activity compared to patients who recover. Compared to HCs, recovered patients had higher ADA1 and total ADA activities at V1. The cytokine and chemokine concentrations (in pg/mL) and ADA activities (in U/L) are log-10 transformed, and the adjusted *p* values are calculated using BH procedure to correct for the false discovery rate. **p*.adj <0.05.

at V1. Only the cytokines and chemokines with significant adjusted *p* values at V1 vs V7-V10 were selected for Spearman correlation analysis (online suppl. Table 1). Plasma ADA1, ADA2, and total ADA positively correlated (*p* value <0.05) with CXCL9, CXL10, G-CSF, IL-6, IL-10, IL-18, and IL-27 at V1 (online suppl. Fig. 3). In addition, even when corrected for multiple comparisons, ADA1 positively correlated with TNFβ and VEGF, while

ADA2 positively correlated with IFNγ and M-CSF (online suppl. Fig. 3). ADA1 positively correlated with ADA2 at V1. No significant correlations were observed between ADA and IFNα2, IL-1α, IL-13, and PDGF-AB/BB (online suppl. Fig. 3).

Patients with Fatal COVID-19 had Distinct Plasma Cytokine, Chemokine and ADA Concentrations Compared to Convalescent and HCs

Analyzing plasma cytokine, chemokine, and ADA levels at V1 demonstrated that individuals with different clinical outcomes displayed distinct immune profiles at the onset of infection. Notably, plasma IL-27 and CXCL10 were significantly higher at V1 in participants who succumbed to COVID-19 compared to HCs (3.4-fold for IL-27 and 20.2-fold for CXCL10, see online suppl. Table 3 for adjusted *p* values). Moreover, plasma IL-27 was significantly higher at V1 among those who later died compared to those who recovered (2.7-fold, online suppl. Table 3). IL-18 and IL-6 levels were higher in participants with fatal COVID-19 compared to HCs and patients who recovered, although not statistically significant (*p*.adj <0.08 for IL-18, *p*.adj <0.23 for IL-6 Fig. 2a; online suppl. Fig. 4). Upon admission, ADA1 (3.0-fold), ADA2 (2.3-fold), and total ADA (2.5-fold) were consistently higher at V1 in the deceased group compared to HCs, and total ADA (1.6-fold) activity was significantly higher in participants who died compared to those who recovered. Furthermore, participants who recovered presented a significantly higher activity of ADA1 (1.7-fold) and total ADA (1.6-fold), but not of ADA2, compared to HCs at the time of admission (Fig. 2b; online suppl. Fig. 4, online suppl. Table 4).

Upon Hospital Admission, Patients with COVID-19 Demonstrated Impaired TLR-Mediated Whole Blood Cytokine Response in vitro

To characterize TLR function, whole blood was stimulated in vitro with PAM (TLR-1/2), PGN (TLR-2), Poly(I:C) (TLR3), LPS (TLR-4), R848 (TLR-7/8), or CpG (TLR-9). Here, we focused on the stimulation of TLR-3, -4, -7/8, and -9 given their association with SARS-CoV-2 infection [41–43]. PCA of TLR-mediated cytokines and chemokines produced at V1, V7, and among HCs (Fig. 3a) demonstrated clustering by TLR agonist, with consistent effects across study participants (Fig. 3a; online suppl. Fig. 5a).

TLR-mediated production of cytokines and chemokines were distinct at admission compared to HCs (Fig. 3b, c). We noted increased concentrations of CCL2, EGF, IL-4, IL-10, IL-15, IL-18, M-CSF, PDGF-AA, and VEGF in individuals at V1 compared to HCs when stimulated with the vehicle condition (PBS) (Fig. 3b); however, there was no decreased concentrations of cytokine or chemokine during V1 compared to HC. In contrast, post-stimulation via TLR agonists resulted in either upregulation or downregulation of several cytokines and chemokines in hospitalized participants when compared to HCs (online suppl. Fig. 7). Specifically, upon TLR-3 stimulation (Poly(I:C)), we observed an upregulation of CXCL8 and CCL5, and a downregulation of CCL4, CXCL10, IFNγ, IL-1RA, IL-12p40 in participants at V1 compared to HCs. Upon TLR-4 stimulation (LPS), we observed lower production of IFNγ, IL-1β, IL-12p40, IL-12p70, and TNFα in participants at V1 compared to HCs. Finally, upon TLR-7/8 stimulation (R848), we observed an upregulation of CCL7 and G-CSF, but lower production of IFNα2, IFNγ, IL-12p40, and IL-12p70 in participants with COVID-19 at V1 as compared to HCs (Fig. 3b). Volcano plots demonstrating differences in TLR-mediated cytokine and chemokine production, including CXCL10, IFNγ, IL-6, IL-10, IL-12p40, IL-12p70, IL-1RA, or TNFα in patients at V1 vs. V7 and in HCs vs. patients at V7 are shown in online supplementary Figure 5b, c, with *p* values in online supplementary Table 5. Furthermore, after controlling for the vehicle condition in patients at V1 vs. V7, our results demonstrate global impairment in TLR-mediated production of cytokines and chemokines, including CXCL10, IFNγ, IL-12p40, IL-12p70, IL-1RA, TNFβ (online suppl. Fig. 6).

Our whole blood assay revealed that at V1, TLR-mediated production of several cytokines and chemokines was impaired in infected participants compared to at V7 or in HCs. Following stimulation with Poly(I:C) (TLR-3), LPS (TLR-4), or R848 (TLR-7/8), production of IFNα2, IFNγ, and IL-12p40 was induced at significantly lower concentrations in participants at V1 compared to either at V7 or in HCs (Fig. 3c). Furthermore, TLR-mediated production of IFNα2, IFNγ, and IL-12p40 3 months after admission was closer to that observed in HCs. No such differences were seen in IFNα2, IFNγ, and IL-12p40 production in the vehicle condition; however, differences were observed in IL-6 concentration, which was significantly higher at V1 compared to V7 (119-fold, *p*.adj = 0.017). This difference in IL-6 production observed in the vehicle condition was not observed following TLR stimulation (Fig. 3c). Stimulation with PAM (TLR-1/2), PGN (TLR-2), and CpG (TLR-9) revealed fewer significant differences in the production of cytokines and chemokines at V1 compared to V7 and HCs (online suppl. Fig. 7). Finally, we performed a literature review to compare and summarize our findings

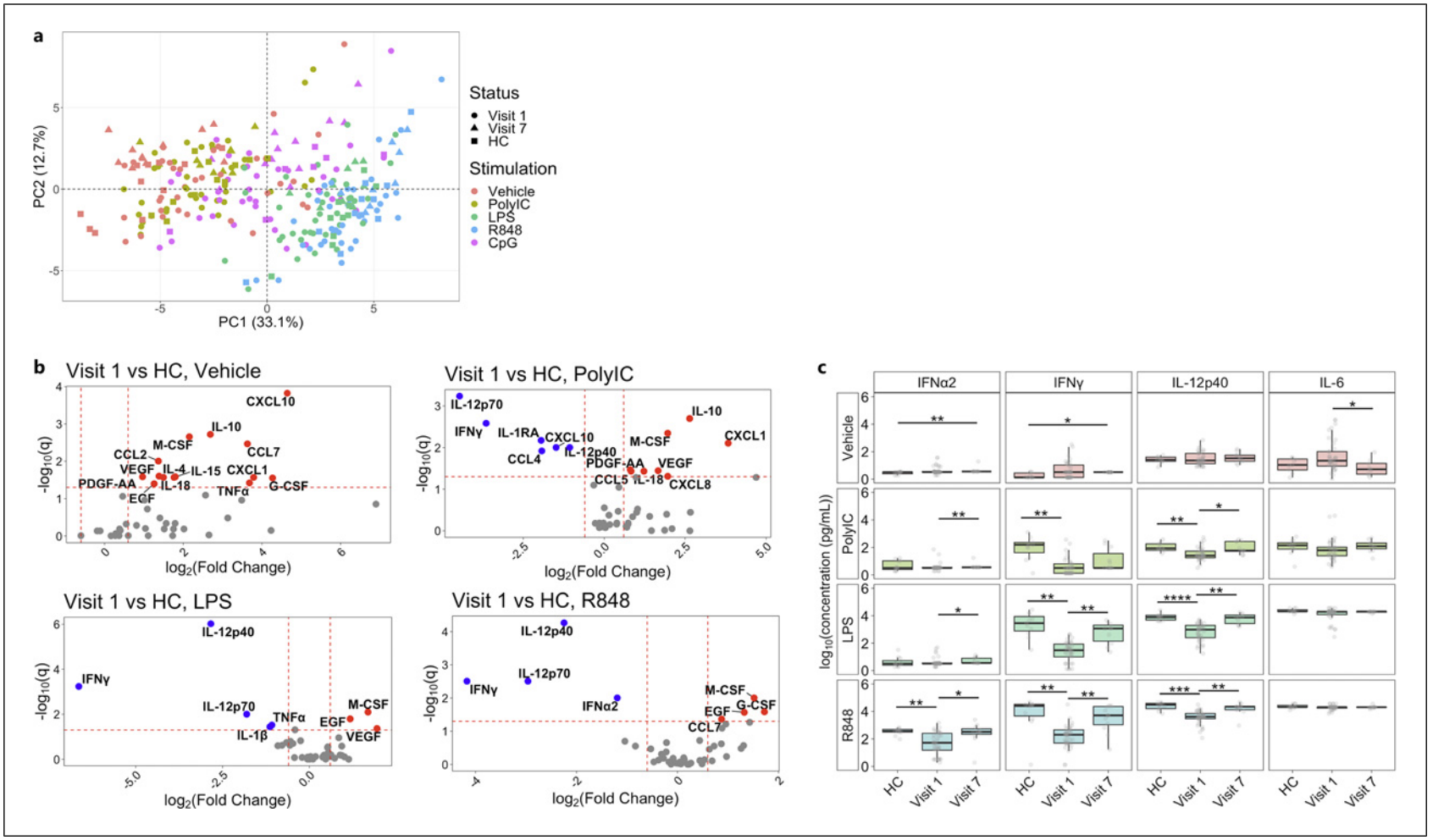

Fig. 3. Whole blood TLR stimulation revealed a distinct activation pattern of individual cytokine and chemokine responses at time of hospital admission. **a** PCA shows the difference in cytokine and chemokine production based on the TLR agonist used for stimulation. The samples primarily cluster by TLR agonist. PC1 and PC2 account for a total of 45.8% of variability. **b** Volcano plots showing the up- and downregulation of specific cytokines and chemokines at Visit 1 compared to HCs. The red dots show upregulation while the blue dots show downregulation of the cytokines and chemokines. **c** IFNα2, IFNγ, and IL-12p40 production upon TLR stimulation shows altered magnitude of the response upon admission in patients hospitalized with COVID-19 (Visit 1), with the response being mostly resolved at Visit 7. IL-6 shows no differences upon TLR stimulation but is increased in participants at Visit 1 compared to Visit 7 in the vehicle condition. The cytokine and chemokine concentrations (in pg/mL) are log10-transformed and the adjusted *p* and *q* values are calculated using BH procedure to correct for the false discovery rate. **p*.adj <0.05, ***p*.adj <0.01, ****p*.adj <0.001, *****p*.adj <0.0001.

on TLR-mediated cytokine production in adults hospitalized with COVID-19 to previously published studies (online suppl. Table 6). Several novel findings highlighted include the upregulation of TLR3-mediated CCL5 and CXCL8, and the downregulation of TLR2/3/4/7/8-regulated IL-12p40, as discussed below.

Discussion

Here, we report the modified regulation of specific cytokines and chemokines in SARS-CoV-2 infected participants following in vitro stimulation of TLRs. A longitudinal analysis of 79 adults hospitalized with COVID-19 followed over 1 year reveals unique immune profiles compared to convalescent time points and HCs. Our findings show a consistent decrease in IL-12p40 production after stimulation of TLR-2, -3, -4, and -7/8 in participants hospitalized with COVID-19 compared to HCs. We report additional impairments in the production of cytokines and chemokines that occur only after stimulation of certain TLRs, such as CXCL10 and IFNγ, following TLR-3 and TLR-9 activation, respectively. We also observed novel increases in CCL5 and CXCL8 concentrations after in vitro stimulation of TLR-3 in whole blood of hospitalized participants compared to HCs, indicating that an active SARS-CoV-2 infection modulates cytokine and chemokine production.

Our extensive TLR agonists' panel combined with a large number of infected participants allows for thorough characterization of TLRs' intricate role following the initial innate immune response to SARS-CoV-2 infection. We complemented our in vitro findings with in vivo cytokine, chemokine, and ADA levels in response to SARS-CoV-2 in hospitalized participants. We report plasma IL-27, CXCL10, ADA1, ADA2, and total ADA as potential biomarkers for assessing disease severity and plasma levels of IL-27, ADA1, ADA2, and total ADA at admission to the hospital as potential predictors of mortality. Given that our study participants were enrolled in the early phase of the pandemic (May 2020 to March 2021), our findings provide a unique perspective on the innate immune response to COVID-19 in individuals' naïve to SARS-CoV-2.

Severe COVID-19 is associated with cytokine storm and hyperinflammation [2]. Plasma concentrations of inflammatory mediators such as IL-6, IL-18, and CXCL10 are increased during COVID-19 and are associated with disease severity [16, 18, 21, 44]. Our findings show that plasma CXCL10 concentrations are significantly higher in individuals with fatal COVID-19 compared to HCs, while similar trends for IL-6 and IL-18 did not achieve statistical significance. IL-6 induces IL-27 [45], which may be a predictor of mortality, as we observed significantly elevated levels of plasma IL-27 at admission in participants who succumbed to the disease compared to those who recovered and HCs. Further investigation is required to better characterize the role of IL-27 as a potential predictive biomarker for fatal outcome. This observation was contrary to another study in Valladolid, Spain, very early in the pandemic, from March to April 2020, in which low levels of total IL-27 were associated with increased odds of severe disease (odds ratio 0.58, $p < 0.005$) [46] but no significant differences were seen in IL-27 levels between mild disease and severe or critically ill patients. Given that this study did not collect plasma samples until the next morning after admission, the observed differences may be due to the likelihood that the data reflect the downward trajectory as shown in the Boston cohort after treatment was initiated. Furthermore, only one time point was collected in the Valladolid cohort, hence limiting the interpretation of detectable IL-27 levels in comparison to our longitudinal prospective cohort. Regulation of IL-27, a cytokine that promotes Th1 while suppressing Th2 immunity, is complex and time-sensitive [47, 48]. Differences in timing of recruitment, definition of disease severity, sample size, and sample collection between studies could account for apparent inconsistencies in the literature. For example, our time of sample collection from participants who died may not reflect the rapid changes in IL-27 concentrations that could have occurred thereafter.

We observed increased activity of plasma ADA1, ADA2, and total ADA in hospitalized participants at V1 compared to convalescent time points (V7-10) and HCs. The significantly higher levels of ADA1 and ADA2 in hospitalized participants at V1 may contribute to heightened inflammation during early COVID-19 compared to later time points (Fig. 1d). We detected increased plasma ADA1, ADA2, and total ADA activities among those who succumbed to the disease compared to HCs (Fig. 2b). In those who recovered, ADA1 and total ADA were also increased compared to HCs, but not to the level of those who died (Fig. 2b). These findings are consistent with previous studies [28, 49] while others have shown decreased plasma ADA activity during and after SARS-CoV-2 infection [50, 51], albeit under different conditions. Specifically, the former study [50] measured plasma ADA activity 8 weeks post-infection (~56 days later), a time point between our V6 and V7 time points, and ADA was measured via an ultra-high-performance liquid chromatography system (not via ELISA). Whereas the

latter study [51], in a smaller Maine, US adult cohort (N = 14–28), showed plasma ADA was lower in participants with COVID-19 versus HCs, contradictory to what was observed in our Boston cohort (N = 78). Hence, future investigations are needed to delineate the kinetics of plasma ADA during SARS-CoV-2 infection in a larger cohort.

In addition to the differences in plasma cytokines and chemokines at admission vs. convalescence, we report a difference in the immune responses of hospitalized, convalescent, and HC participants following in vitro TLR stimulation in a whole blood assay. While TLR-4 interacts with SARS-CoV-2 on the cell surface [43, 52], TLRs-3 and -7/8 are endosomal and recognize microbial RNAs that have entered the cell [53, 54]. We observed impaired in vitro TLR-mediated production of CCL4, CXCL10, IFNα2, IFNγ, IL-1β, IL-1RA, IL-12p40, IL-12p70, and TNFα in hospitalized participants compared to HCs. This downregulation suggests that SARS-CoV-2 infection may hinder activation of the TLR pathways following stimulation (Fig. 3). We noted a decrease in TLR3, -4 and 7/8-mediated production of IL-12p40 and IL-12p70. IL-12p70 is a Th1-polarizing cytokine [55] and inhibits early replication of viruses such as influenza [56]. Impaired production of IL-12p70, as well as TNFα, IFNα2, and IFNγ in those hospitalized with COVID-19 may suggest that their immune systems are less capable of mounting pro-inflammatory/Th1-polarizing responses important for inhibiting viral replication [57, 58]. We additionally noted a decrease in TLR3-mediated production of CXCL10, a biomarker for assessing COVID-19 disease severity [59, 60]. Our in vivo findings do show heightened plasma concentrations of CXCL10 at V1 in infected participants compared to HCs, supporting prior work, but our in vitro findings indicate that blood leukocytes of patients with COVID-19 display reduced TLR-mediated production of this chemokine. Impaired TLR-mediated cytokine production has been reported [36, 37], including decreased TLR-mediated pDC production of IFNα and TNFα in vitro in infected participants vs. healthy individuals [36], as well as IL-6, IFNγ, or TNFα in convalescent patients vs controls [61]. Our results demonstrate that reduced TLR-mediated cytokine and chemokine production is also noted in whole blood, rather than limited to a given cell type, validating previous work [34, 35]. TLR-mediated production of most of the cytokines and chemokine was similarly downregulated in participants at admission compared to convalescence when calculating the fold change for the vehicle condition over individuals at V1 and V7, further strengthening our results.

Our in vitro assay demonstrated reduced TLR-mediated cytokine and chemokine production, including lower IL-12p40 production after stimulation of TLR-2, -3, -4, and -7/8 in participants hospitalized with COVID-19 compared to HCs. We also observed that TLR-mediated production of other cytokines such as IL-10, M-CSF, and VEGF was upregulated in participants hospitalized with COVID-19 relative to HCs. TLR3-mediated IL-10 production was greater at V1 compared to HCs. This is also true in the vehicle condition (Fig. 3b). IL-10, an anti-inflammatory cytokine [62], is elevated in SARS-CoV-2-infected patients [63, 64] and may reflect a counter-regulatory effort in the setting of a hyper-inflammation and/or high concentrations of Th1-polarizing cytokines [65]. Our observations regarding M-CSF and VEGF are consistent with prior studies that noted higher plasma concentrations in hospitalized patients with COVID-19 [66, 67]. Plasma concentrations of pro-inflammatory cytokines are elevated in severe SARS-CoV-2 infection and can contribute to acute respiratory distress syndrome [68]. Our findings support studies of TLR antagonists to minimize excessive production of inflammatory cytokines during acute COVID-19 [54, 69]. Translation of TLR antagonists will need to address dose, timing and frequency as TLR activation is necessary to protect individuals from SARS-CoV-2, but excessive activation and resulting hyperinflammation may be harmful [70]. The complex nature of TLRs and their role in COVID-19 is further evinced by our demonstration of a possible modulation of TLR-mediated cytokine and chemokine production in infected individuals.

Altered TLR-mediated production of cytokines and chemokines may reflect innate memory or trained immunity [31]. COVID-19 can induce long-term changes in the innate immune system. Examples of this have been characterized as increased secretion of CXCL-8 and CCL2 for at least 3–4 months after infection [71]. SARS-CoV-2 can induce epigenetic innate memory in human bone marrow progenitors [72]. Our findings support SARS-CoV-2's potential to induce trained immunity as we report that at convalescent time points, individuals with COVID-19 still display distinct TLR-mediated production of certain cytokines and chemokines (e.g., CCL3, CCL7, CCL22, PDGF-AB/BB, TNFβ, VEGF; online suppl. Fig. 5c) compared to HCs following TLR stimulation and vehicle control stimulation. Although further studies are needed to characterize the nature by which SARS-CoV-2 induces these changes, there are lasting impacts on the innate immune system.

There are several strengths of our study including (1) enrollment early in the pandemic between May 2020 and

March 2021, a phase during which, at hospitalization, there was little natural immunity or immunization to SARS-CoV-2, enabling study of a naïve population; (2) robust clinical data capture and longitudinal sampling of infected participants up to 1 year post hospital discharge, which are previously described and show that high viral load at baseline is associated with disease severity [73]; and (3) inclusion of HCs, providing a robust reference to compare plasma cytokine and chemokine concentrations and ADA activities.

Along with multiple strengths, our research had some limitations including (1) moderate attrition rate, resulting in a limited number of participants for which we could complete longitudinal analysis across hospitalization and the entire year following hospital discharge; (2) collected data regarding treatment of infected participants, including steroid use, was limited due to rapidly evolving guidelines pertaining to COVID-19 treatment early in the pandemic; and (3) assessment of TLR function was restricted to in vitro assays, though studies by our group and others suggest such in vitro modeling is predictive of relevant biology in vivo [74, 75].

We observed impaired TLR-mediated cytokine and chemokine production in study participants with COVID-19 at the time of hospital admission as compared to HCs and later convalescent time points. This suggests that the host response to SARS-CoV-2 infection impacts TLR function at V1, but the impairment was largely resolved by V7 (3 months post discharge). Our study suggests that targeted and well-timed TLR signaling modulation may represent a novel approach to prevent and/or treat COVID-19. Future studies should characterize how TLR pathways are longitudinally impacted by infection and, in parallel, analyze B- and T-cell immunity to enable integrative analysis of innate and adaptive responses to SARS-CoV-2 in relation to severity of COVID-19. In summary, our study demonstrated distinct plasma cytokine and chemokine concentrations in vivo and distinct in vitro response to TLR simulation after COVID-19 disease, highlighting potential markers for disease severity and targets for immunomodulation.

Appendix

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Statement of Ethics

The National Institute of Allergy and Infectious Diseases (NIAID) staff conferred with the Department of Health and Human Services Office for Human Research Protections (OHRP) regarding potential applicability of the public health surveillance exception [45CFR46.102] to the IMPACC study protocol. OHRP concurred that the study satisfied criteria for the public health surveillance exception, and the IMPACC study team sent the study protocol, and participant information sheet for review, and assessment to Institutional Review Boards (IRBs) at participating institutions. Brigham and Women's Hospital (BWH) and Boston Children's Hospital (BCH) elected to conduct the study as public health surveillance. Each participant reviewed the information sheet and gave their written informed consent prior to enrollment in the study.

Conflict of Interest Statement

O.L. and S.H. are named inventors on patents held by Boston Children's Hospital related to vaccine adjuvants and human in vitro systems that model immune responses and have received a sponsored research award from GSK and Pfizer. O.L. is co-founder of and advisor to ARMR Sciences (formerly *Ovax Inc*), a company that advances vaccines against fentanyl overdose, and has served as a consultant to GlaxoSmithKline (GSK), Sanofi, and Hillevax. S.H. has received a sponsored research award from Sanofi. The

sponsors had no role in the design, execution, interpretation, or writing of the study. The other authors declare no competing interests.

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Author Contributions

Conceptualization O.L., L.R.B., S.D.H., O.A.O., K.K.S.; Investigation K.K.S.; Methodology A.N.N., A.L.P., O.A.O., S.D.H., K.K.S.; Data generation and curation A.N.N., T.S.K., K.R., A.L.P., S.D.-G., S.T., J.D.-A., K.K.S.; Visualization A.N.N., T.S.K., K.R., A.L.P., K.K.S.; Resources K.M., A.O., J.D.-A., L.R.B., K.K.S.; Supervision A.N.N., K.K.S.; Funding acquisition O.L., O.A.O., L.R.B., S.D.H., K.K.S.; Writing – original draft A.N.N., T.S.K., K.R., A.L.P., S.D.H., K.K.S. All authors reviewed and agreed with the information presented in the manuscript.

Data Availability Statement

The IMPACC Data Sharing Plan is designed to enable the widest dissemination of data, while also protecting the privacy of the participants and the utility of the data by de-identifying and masking potentially sensitive data elements. This approach is fully compliant with the NIH public data sharing policy. The study protocol and clinical dataset are deposited at Shared Data under the accession No. SDY1760 at https://www.immport.org/shared/search?text=SDY1760%20. After publication, it will be available to appropriate academic parties upon request and submission of a suitable study protocol, analysis plan, and signed data use agreement subject to NIAID approval via AccessClinicalData@NIAID (https://accessclinicaldata.niaid.nih.gov/study-viewer/clinical_trials). Please contact ImmPort_Helpdesk@immport.org to view data for review purposes.

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