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To cite this article: Bruce K. Patterson , Ram Yogendra, Edgar B. Francisco , Jose Guevara-Coto , Emily Long, Amruta Pise, Eric Osgood, John Bream, Mark Kreimer, Devon Jeffers, Christopher Beaty, Richard Vander Heide & Rodrigo A. Mora-Rodríguez (2025) Detection of S1 spike protein in CD16+ monocytes up to 245 days in SARS-CoV-2-negative post-COVID-19 vaccine syndrome (PCVS) individuals, Human Vaccines & Immunotherapeutics, 21:1, 2494934, DOI: [10.1080/21645515.2025.2494934](https://doi.org/10.1080/21645515.2025.2494934)

To link to this article: <https://doi.org/10.1080/21645515.2025.2494934>



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Published online: 13 May 2025.



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RESEARCH ARTICLE



Detection of S1 spike protein in CD16+ monocytes up to 245 days in SARS-CoV-2-negative post-COVID-19 vaccine syndrome (PCVS) individuals

Bruce K. Patterson^a, Ram Yogendra^{ib*}, Edgar B. Francisco^a, Jose Guevara-Coto^c, Emily Long^a, Amruta Pise^a, Eric Osgood^d, John Bream^e, Mark Kreimer^f, Devon Jeffers^g, Christopher Beaty^a, Richard Vander Heide^h, and Rodrigo A. Mora-Rodríguez^c

^aResearch and Development Department, IncellDx Inc, Hayward, CA, USA; ^bDepartment of Anesthesiology, Lawrence General Hospital, Lawrence, MA, USA; ^cLab of Tumor Chemosensitivity, CIET/DC Lab, Faculty of Microbiology, Universidad de Costa Rica, San Jose, Costa Rica; ^dDepartment of Medicine, St. Francis Medical Center, Trenton, NJ, USA; ^eDepartment of Emergency Medicine, Novant Health Kernersville Medical Center, Kernersville, NC, USA; ^fDepartment of Emergency Medicine, New York Presbyterian Hospital, Brooklyn, NY, USA; ^gDepartment of Anesthesiology, Stamford Hospital, Stamford, CT, USA; ^hDepartment of Pathology, Marshfield Medical Center, Marshfield, WI, USA

ABSTRACT

Despite over 13 billion SARS-CoV-2 vaccine doses administered globally, persistent post-vaccination symptoms, termed post-COVID-19 vaccine syndrome (PCVS), resemble post-acute sequelae of COVID-19 (PASC). Symptoms like cardiac, vascular, and neurological issues often emerge shortly after vaccination and persist for months to years, mirroring PASC. We previously showed the S1 subunit of the SARS-CoV-2 spike protein persists in CD16+ monocytes after infection, potentially driving PASC. Approved vaccines (Pfizer, Moderna, Janssen, AstraZeneca) deliver synthetic S1 to elicit immunity, suggesting a shared mechanism. We hypothesized that vaccine-derived S1 persistence in CD16+ monocytes sustains inflammation akin to PASC, contributing to PCVS. We studied 50 individuals with PCVS symptoms lasting over 30 days post-vaccination and 26 asymptomatic controls, using (1) machine learning-based immune profiling to compare cytokine signatures with PASC, (2) flow cytometry to detect S1 in CD16+ monocytes, and (3) LC-MS to confirm S1 across vaccine types. We correlated S1 persistence with symptom duration and inflammation. Prior infection was excluded via clinical history, anti-nucleocapsid antibody tests, and T-detect assays, though definitive tests are lacking. Preliminary findings suggest S1 persistence in CD16+ monocytes and an associated inflammatory profile may contribute to PCVS. Further studies are needed to confirm causality and prevalence.

SUMMARY

SARS CoV-2 S1 Protein in CD16+ Monocytes in Post-COVID-19 Vaccine Syndrome (PCVS).

ARTICLE HISTORY

Received 12 November 2024
Revised 2 April 2025
Accepted 15 April 2025

KEYWORDS

COVID-19; PASC; SARS CoV-2 S1 protein; non-classical monocytes; CCR5; fractalkine

Introduction

Despite the global administration of over 13 billion doses of SARS-CoV-2 vaccines, reports of persistent post-vaccination symptoms, commonly referred to as Post-COVID-19 Vaccine Syndrome (PCVS), have sparked growing concern.¹ These symptoms, which resemble Post-Acute Sequelae of COVID-19 (PASC, or Long COVID), include new-onset cardiac, vascular, and neurological issues that can manifest within minutes to hours of vaccination and persist for months or even years in some individuals.^{2–6} Notably, the symptomatology of PCVS closely mirrors that of PASC,^{7,8} pointing to potential shared pathophysiological mechanisms. Our prior research demonstrated that the S1 subunit of the SARS-CoV-2 spike protein is detected in non-classical (CD14–CD16+) and intermediate (CD14+CD16+) monocytes for months to years following acute infection, a phenomenon that may drive PASC.⁹

Currently approved vaccines, such as BNT162b2 (Pfizer), mRNA-1273 (Moderna), Ad26.COV2.S (Janssen), and

ChAdOx1 nCoV-19 (AstraZeneca), introduce a synthetic S1 subunit into muscle cells to trigger an immune response.¹⁰ Given the overlap in symptoms and the role of S1 in both PASC and vaccine mechanisms, we hypothesized that vaccine-derived S1 persistence in CD16+ monocytes may sustain an inflammatory cytokine profile similar to that seen in PASC, thereby contributing to PCVS in individuals without prior SARS-CoV-2 infection.

To investigate this hypothesis, we conducted a study involving 50 individuals with new-onset PCVS symptoms lasting beyond 30 days post-vaccination (across Pfizer, Moderna, Janssen, and AstraZeneca vaccines) and compared them to 26 asymptomatic vaccinated controls. We employed three complementary analytical approaches: (1) machine learning-based immune profiling to compare cytokine signatures of PCVS with those of PASC, (2) flow cytometry to detect S1 persistence in CD16+ monocyte subsets, and (3) liquid chromatography–mass spectrometry (LC–MS) to confirm the presence of S1 and related peptides across different vaccine types. Additionally, we assessed correlations between S1 persistence,

CONTACT Ram Yogendra  ryogendra@geisinger.edu  Department of Anesthesiology, Geisinger Medical Center, 100 North Academy Avenue, Danville, PA 17822, USA.

*Current address: Department of Anesthesiology, Geisinger Medical Center, Danville, PA, USA

 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/21645515.2025.2494934>

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symptom duration, and inflammatory markers to establish a potential link to clinical outcomes.

A significant challenge in this study was ruling out prior SARS-CoV-2 infection, as no definitive gold standard test exists to confirm this history. To address this, we screened participants using a combination of clinical history, negative anti-nucleocapsid antibody tests, and T-Detect assays (FDA EUA Adaptive Technologies) to minimize the likelihood of prior exposure. Our findings provide preliminary evidence that S1 persistence in CD16+ monocytes may contribute to PCVS, highlighting a potential mechanism for post-vaccination symptoms. However, we underscore the need for larger-scale studies to confirm causality and determine the broader prevalence of these effects.

Materials and methods

Patient population characteristics and inclusion criteria

We enrolled participants who received at least one dose of an approved SARS-CoV-2 vaccine, BNT162b2 (Pfizer), mRNA-1273 (Moderna), Janssen (Johnson & Johnson), or ChAdOx1 nCoV-19 (AstraZeneca), between November 2020 and August 2021 and reported new-onset symptoms lasting >30 days post-vaccination. A total of 50 symptomatic individuals (aged 13–65 years; mean age 42 years; 36 females, 14 males) and 26 asymptomatic vaccinated controls (aged 20–70 years; mean age 40 years; 16 females, 10 males) were included after providing written informed consent. 32 out of the 50 symptomatic patients received only 1 COVID vaccine dose and 18 out of the 50 received 2. 23 out of 26 control individuals received two doses (BNT162b2 (Pfizer) or mRNA-1273 (Moderna)), and the other three received 1 Janssen dose. This range was selected to capture persistent symptoms beyond the expected 30-day S1 clearance window.¹¹

Blood draws in the study occurred between 38–245 days from last COVID-19 vaccination (mean: 105 days for study group and 97 days for control group), with specific timing detailed in the Results section (Table 1).

Participants were identified through the Chronic COVID Treatment Center (CCTC), a private telehealth clinic, via patient intake forms documenting persistent post-vaccination symptoms, and through physician referrals across the United States. Most symptomatic participants were managed as outpatients in ambulatory care settings; however, two individuals had brief emergency department visits for initial symptom evaluation without requiring hospitalization. Vaccination dates and batch numbers were verified using CDC-issued vaccine cards, ensuring accurate documentation of vaccine administration.

Exclusion criteria

Participants were excluded if they had a prior history of conditions that could confound symptom attribution, including seizure disorders, migraines, neuropathy, inflammatory bowel disease, depression, anxiety disorders, chronic fatigue syndrome, Lyme disease, fibromyalgia, arthritis, chronic obstructive pulmonary disease (COPD), asthma, diabetes, chronic

kidney disease, chronic heart failure (CHF), arrhythmias, bleeding disorders, or were on anticoagulation therapy. To minimize the inclusion of individuals with prior SARS-CoV-2 infection, we also excluded those with a documented history of positive polymerase chain reaction (PCR) tests, positive anti-nucleocapsid antibody tests (indicating past natural infection), or positive T-detect test (indicating SARS-CoV-2-specific T-cell responses). These criteria were assessed through patient clinical history and laboratory testing conducted prior to enrollment. While these measures aimed to rule out prior infection, the absence of a universally validated test for asymptomatic cases was a noted limitation.

High parameter immune profiling/flow cytometry

Whole blood was collected in a 10 ml EDTA tube and a 5 ml plasma preparation tube (PPT). Peripheral blood mononuclear cells were isolated from peripheral blood using Lymphoprep density gradient (STEMCELL Technologies, Vancouver, Canada). Aliquots of 500,000–10⁶ cells were frozen in media that contained 90% fetal bovine serum (HyClone, Logan, UT) and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and stored at –70°C for <1 week to preserve viability and run as a single batch to avoid intrarun variability. Cells were quick-thawed for 1 min at 37°C, washed with D-PBS, then blocked with 2% BSA solution (Sigma-Aldrich, St. Louis, MO) for 5 min at RT. A cocktail was added that contained the following antibodies and reagents: Brilliant Stain Buffer (Cat. #563794, BD Biosciences, San Jose, CA), True-Stain Monocyte Blocker (Cat. #426103, BioLegend, San Diego, CA), anti-CD19-PE-Dazzle594 (Cat. #302252, BioLegend), anti-CTLA-4 PE-Cy7 (Cat. #369614, BioLegend), anti-CD3-APC (Cat. #300412, BioLegend), anti-CD16-Alexa Fluor 700 (Cat. #302026, BioLegend), and anti-SARS-CoV-2 Spike S1 Subunit-Alexa Fluor 405 (Cat. #FAB105403V, R&D Systems, Minneapolis, MN). The following antibodies were then added individually: anti-CD8 BUV496 (Cat. #612942, BD Biosciences), anti-CD4-BUV661 (Cat. #612963, BD Biosciences), anti-CD45-BUV805 (Cat. #612891, BD Biosciences), anti-PD-1-BB700 (Cat. #566460, BD Biosciences), anti-CD56-BV650 (Cat. #318344, BioLegend), anti-LAG-3 BV711 (Cat. #369320, BioLegend), and anti-CD14 BV785 (Cat. #301840, BioLegend). Cells were stained for 30 min at RT and then washed twice with 2% BSA. Cells were fixed for 1 h at RT in 1 mL incellMAX (IncellDx, Hayward, CA), and then incubated with anti-FoxP3-PE antibody (Cat. #560082, BD Biosciences, San Jose, CA) for 30 min. Cells were washed twice with 2% BSA and then acquired on a 5-laser CytoFLEX LX (Beckman Coulter, Brea, CA). Cells were gated on viable, single cells based on forward and side-scatter properties and with positive CD45 staining. Samples were analyzed using Kaluza Analysis software Version 2.3 (Beckman Coulter, Brea, CA).

Flow cytometric cell sorting

Cryopreserved PBMCs were quick-thawed, centrifuged, and washed in 2% BSA solution in D-PBS. Cells were blocked for 5 min in 2% BSA and then incubated at room temperature for 30 min with Alexa Fluor® 488 Anti-CD45 antibody (IncellDx, 1/100 dilution), 2.5 ug of Alexa Fluor® 647 Anti-CD16 antibody

Table 1. Demographic and vaccination details of 50 post-COVID-19 vaccine syndrome individuals and 26 asymptomatic COVID-19 vaccine control group.

Age	Sex	Vaccine	Days from Last Vaccine to Blood Draw	Control Group				
				Age	Sex	Vaccine	Days from Last Vaccine to Blood Draw	
46	F	PFE	75	P1	39	M	PFE	47
28	M	MRNA	76	P2	29	F	MRNA	103
40	F	MRNA	69	P3	52	F	PFE	86
34	F	MRNA	128	P4	33	M	MRNA	139
44	F	PFE	97	P5	41	M	PFE	91
55	M	PFE	45	P6	46	F	JNJ	144
39	F	PFE	63	P7	31	M	PFE	162
46	F	MRNA	125	P8	36	F	MRNA	67
40	F	MRNA	142	P9	27	F	JNJ	50
44	M	PFE	97	P10	20	F	PFE	61
22	F	PFE	97	P11	34	F	MRNA	106
47	F	PFE	83	P12	49	F	MRNA	73
50	M	MRNA	85	P13	54	F	JNJ	89
32	F	PFE	80	P14	61	F	PFE	132
54	F	PFE	106	P15	44	M	PFE	156
46	M	MRNA	79	P16	24	M	MRNA	89
56	M	MRNA	38	P17	30	F	PFE	83
64	M	MRNA	129	P18	70	M	PFE	114
40	F	AZN	245	P19	41	F	MRNA	98
45	M	JNJ	93	P20	39	F	MRNA	68
44	F	PFE	100	P21	43	F	PFE	83
13	F	PFE	162	P22	52	M	PFE	93
37	F	MRNA	118	P23	33	F	MRNA	125
51	F	PFE	149	P24	41	M	PFE	57
58	F	MRNA	62	P25	45	F	PFE	66
36	F	PFE	61	P26	38	M	MRNA	134
45	F	PFE	163					
37	M	JNJ	83					
37	F	MRNA	42					
65	F	JNJ	168					
45	F	PFE	183					
47	F	PFE	82					
34	F	PFE	90					
42	F	PFE	70					
44	M	JNJ	89					
47	M	PFE	87					
42	F	JNJ	91					
45	F	PFE	71					
38	M	PFE	88					
61	M	PFE	121					
36	F	PFE	63					
35	F	PFE	58					
38	F	JNJ	144					
25	F	MRNA	39					
33	F	PFE	178					
24	F	MRNA	155					
43	F	MRNA	179					
45	M	PFE	116					
24	F	JNJ	146					
46	F	PFE	153					

(BD, Cat. # 55710) and 1 ug of PerCP/Cy5.5 Anti-human CD14 antibody (Biolegend, Cat. #325622). Cells were washed twice with 2% BSA/D-PBS, filtered, and kept on ice for the duration of the cell sort. Data was acquired on a Sony SH800, and only CD45 + cells staining positive for both CD14+ and CD16+ were sorted into test tubes with 100 uL 2% BSA solution. Sort purity of control PBMCs was confirmed to be >99% by re-analyzing sorted PBMCs using the same template and gating strategy.

Single cell protein identification

Patient cells were sorted based on phenotypic markers (as above) and frozen at -80°C . Five patient samples with positive flow cytometry signal and sufficient cell counts were chosen for LCMS confirmation. Frozen cells were lysed with the IP

Lysis/Wash Buffer from the kit according to the manufacturer's protocol. To immunoprecipitate the S1 Spike protein from cell lysate of each patient, 10 ug of anti-S1 mAb were used. After overnight incubation with end-over-end rotation at 4°C and then three washes with IP Lysis/Wash Buffer, bound S1 Spike protein was eluted with the elution buffer from the kit. IP elution fractions were dried in vacuo, resuspended in 20 uL of water, pooled, and purified by Agilent 1290 UPLC Infinity II on a Discovery C8 (3 cm \times 2.1 mm, 5 μm , Sigma-Aldrich, room temperature) using mobile-phase solvents of 0.1% trifluoroacetic acid (TFA) in water or acetonitrile. The gradient is as follows: 5–75% acetonitrile (0.1% TFA) in 4.5 min (0.8 mL/min), with an initial hold at 5% acetonitrile (0.1% TFA) for 0.5 min (0.8 mL/min). The purified protein was dried in vacuo and resuspended in 50 μL of 100 mm

HEPES, pH 8.0 (20% Acetonitrile). One microliter of TCEP (100 mM) was added and the samples were incubated at 37°C for 30 min. One microliter of chloroacetamide (500 mM) was added to the samples and incubated at room temperature for 30 min. One microliter of rAspN (Promega 0.5 µg/µL) and 1 µL of LysC (Pierce, 1 µg/µL) were added and the samples incubated at 37°C for 16 h, prior to LCMS analysis.

Liquid chromatography/mass spectroscopy (LC-MS) analysis

Digested recombinant SARS-CoV-2 Spike S1 protein was analyzed by a high accuracy mass spectrometer to generate a list of detectable peptides with retention time and accurate masses. An Agilent 1290 Infinity II high pressure liquid chromatography (HPLC) system and an AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 mm) were used for peptide separation prior to mass analysis. The mobile phase used for peptide separation consists of a solvent A (0.1% formic acid in H₂O) and a solvent B (0.1% formic acid in 90% CH₃CN). The gradient was as follows: 0–1 min, 3% B; 1–30 min, to 40% B; 30–33 min, to 90% B; 33–35 min, 90% B; 37–39 min, 3% B. Eluted peptides were electrosprayed using a Dual JetStream ESI source coupled with the Agilent 6550 iFunnel time-of-flight MS analyzer. Data was acquired using the MS method in 2 GHz (extended dynamic range) mode over a mass/charge range of 50–1700 Daltons and an auto MS/MS method. Acquired data were saved in both centroid and profile mode using Agilent Masshunter Workstation B09 Data acquisition Software. The same analytical method was applied to immunoprecipitated samples from sorted patient cells except no ms/ms was acquired.

Machine learning

We deployed machine learning to the acute COVID, PASC, and post-vaccination datasets as previously described¹⁰ (Figure 2). Immunologic severity scores were generated using the algorithm $SC = (IL-6 + sCD40L/1000 + VEGF/10 + 10 \cdot IL-10)/(IL-2 + IL-8)$ and the long hauler index (PASC) was generated using the algorithm $LHI = (IFN-\gamma + IL-2)/CCL4-MIP-1\beta$.

Results

Clinical presentation and vaccine distribution

We investigated 50 individuals (mean age 41.8 years, 36 females, 14 males) who developed PASC-like symptoms post-vaccination (Pfizer: 27, Moderna: 15, JNJ: 7, AstraZeneca: 1), with an average of 105 days (range 38–245) from last inoculation to blood draw. Controls ($n = 26$, mean age 41 years, 16 females, 10 males) were asymptomatic post-vaccination (mean 97 days, range 47–162) (Table 1). Predominant symptoms included fatigue (27/50), neuropathy (27/50), brain fog (22/50), and headaches (23/50), varying by vaccine type (Figure 1).

Cytokine profile and machine learning classification

To assess whether these symptoms were associated with an immune profile similar to PASC, we performed machine learning on a 14-plex cytokine/chemokine panel (selected from 158 biomarkers based on prior PASC studies¹² across the 50 symptomatic patients, and 26 healthy vaccinated controls. The single classifier algorithm classified the symptomatic group's immune profile as PASC-like, though a dual classifier

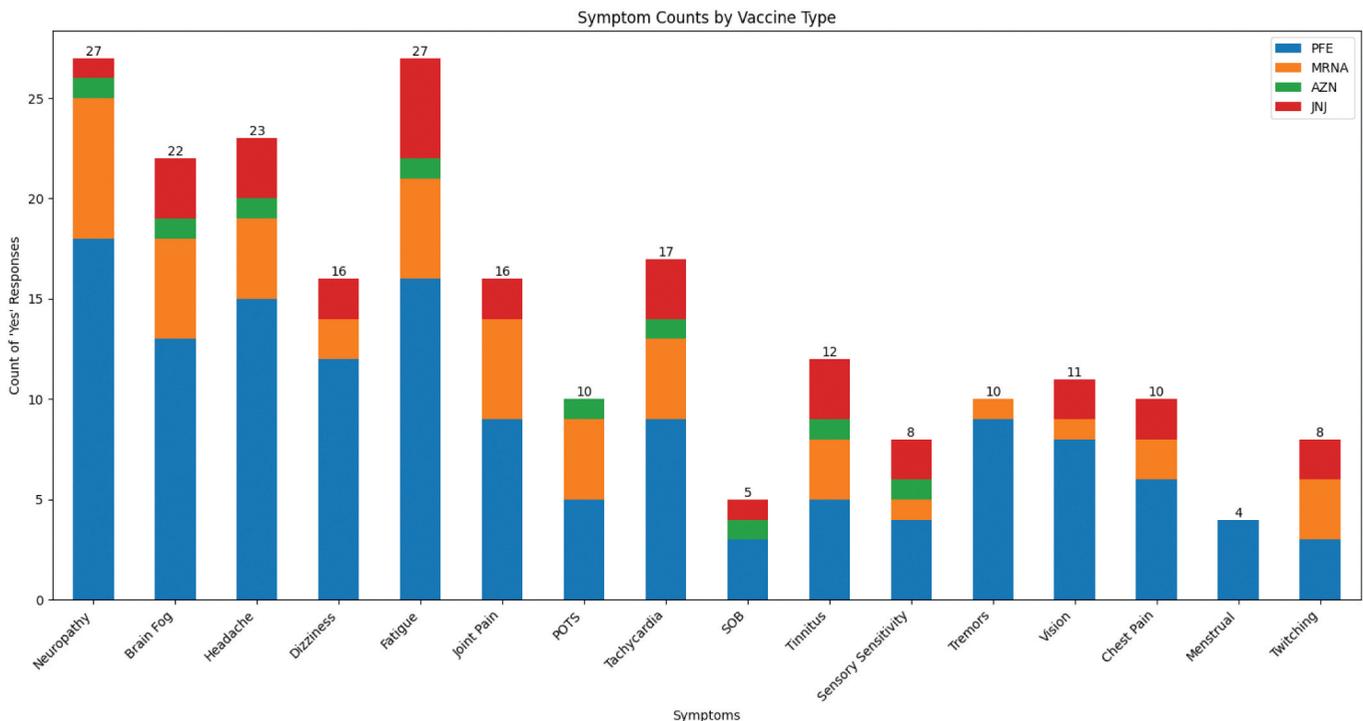


Figure 1. Frequency of symptoms in post-COVID-19 vaccine syndrome patient.

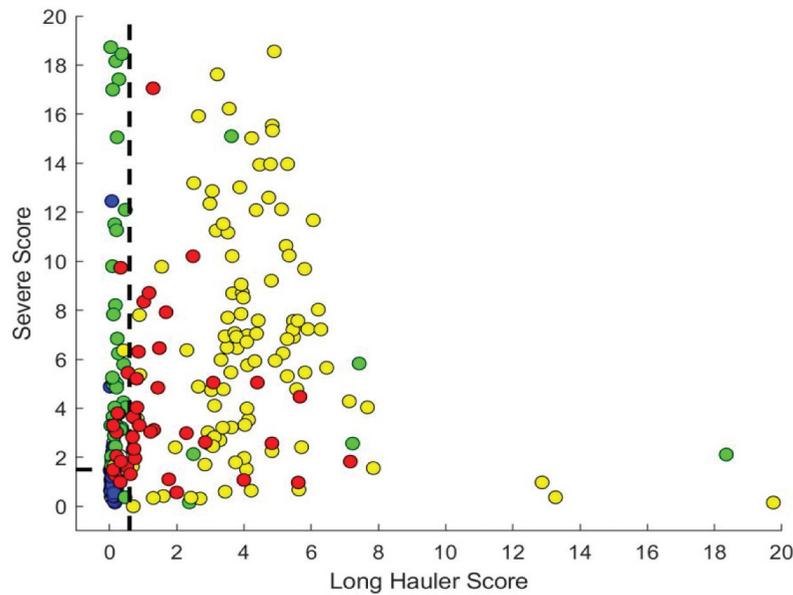


Figure 2. Machine learning classification of the immune profile seen in post-vaccination individuals with PASC-like symptoms (red) compared to the immune profile in individuals with PASC (yellow) due to SARS-CoV-2 infection, mild-moderate COVID infection (blue), and severe COVID (green).

indicated less inflammation than acute SARS-CoV-2 (Figure 2). Statistically significant elevations were observed in IL-4 ($p = .020$), CCL3 ($p = .0012$), CCL5 ($p = .0067$), sCD40L ($p < .0001$), IL-8 ($p < .0208$), and VEGF ($p = .0175$) in symptomatic patients compared to controls (Figure 3). Statistically significant downregulation was observed in TNF- α ($p = .0011$) and GM-CSF ($p = .0036$) (Supplementary Figure S2). Non-significant cytokines are reported in Supplementary Figure S1.

S1/S2 persistence and mechanistic insights

Building on our prior finding of S1 persistence in PASC,⁹ we hypothesized that a similar mechanism might underlie post-vaccination symptoms. We screened 12 of the 50 symptomatic patients and 10 controls via flow cytometry for S1 protein in monocyte subsets. Twelve out of fifty patients were screened due to sample availability and flow cytometry signal strength. Results showed a significant elevation of S1-containing non-

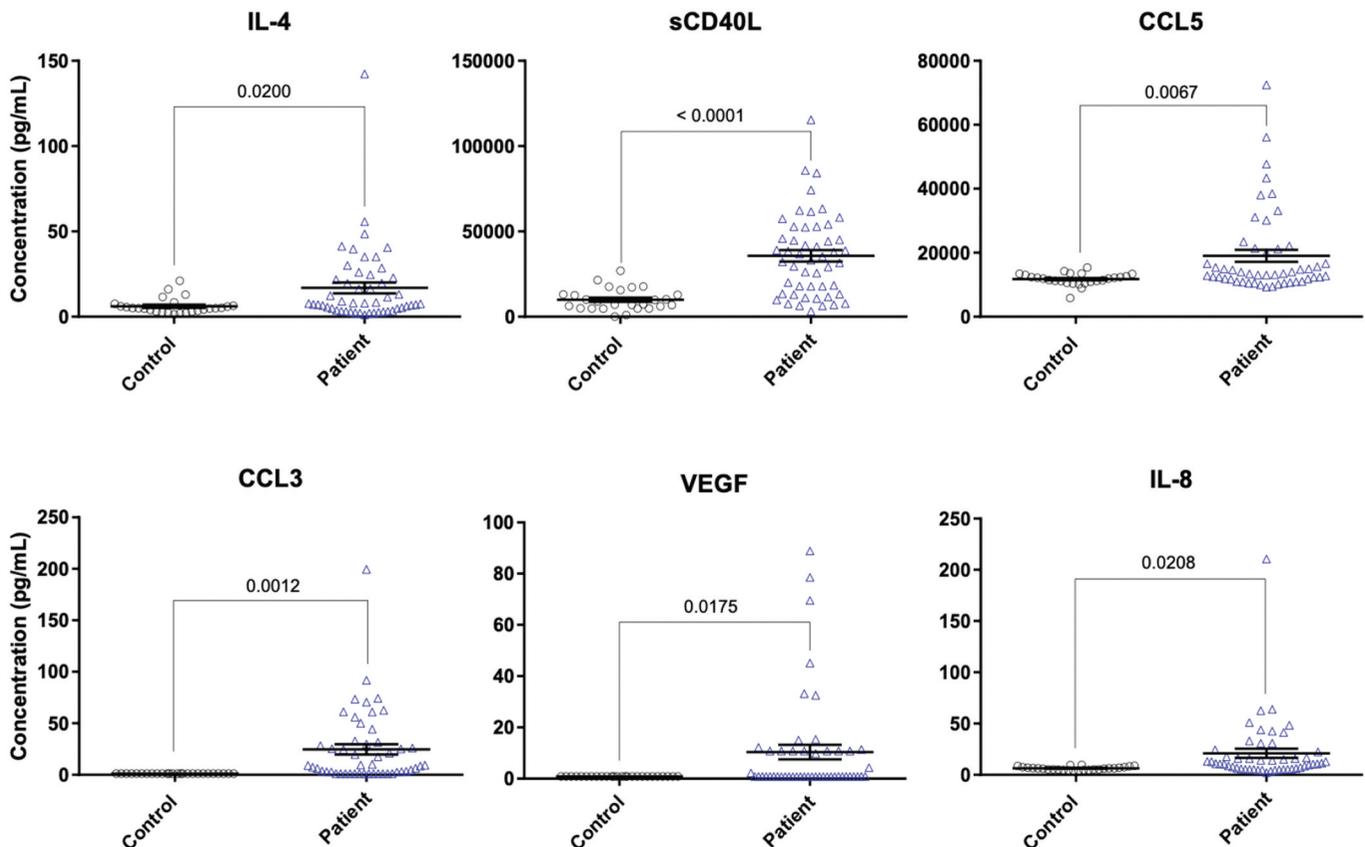


Figure 3. Statistically significant elevation of cytokines in PCVS individuals compared to vaccinated, healthy individuals.

classical monocytes (11/12, 92%, $p < .0007$) and intermediate monocytes (8/12, 67%, $p = .0234$) in symptomatic patients, with S1 detected in 1 control intermediate monocyte (1/10) (Figure 4). From the 12 S1-positive patients, we sorted CD16+ cells from 5 individuals and confirmed S1, S1 mutant, and S2 peptide sequences via LC-MS months post-vaccination (Figures 5 and 6).

Correlations between S1 persistence, inflammatory markers, and symptom profiles

The correlograms present Pearson correlation coefficients between symptoms and biological markers, where red shades indicate positive correlations and blue shades indicate negative correlations. The correlation analysis between cytokines and symptoms revealed several associations (Figure 7). Dizziness showed a strong positive correlation with IL-2, IL-4, and IL-6, while shortness of breath (SOB) is positively correlated with IL-2, CCL3, and VEGF. Tinnitus also exhibited moderate positive correlations with IL-4, CCL3, IFN-gamma, and VEGF. Interestingly, fatigue had a negative correlation with IL-10, GM-CSF, TNF-alpha, and VEGF indicating a potential inverse relationship. The correlation analysis between monocyte subsets and symptoms showed significant relationships (Figure 8). The CD16+ CD14- (nonclassical monocytes) subset displayed positive correlations with symptoms such as neuropathy, brain fog, headache, fatigue, tachycardia, POTS, shortness of breath, tinnitus, sensory sensitivity, tremors, chest pain, and menstrual symptoms. The CD14hiCD16+ (intermediate monocytes) subset exhibited negative correlations with all reported symptoms. Additionally, CD14hiCD16+ COVIDS1+ (intermediate monocytes containing S1) presented a combination of positively correlated symptoms (headache, POTS, tachycardia, tinnitus, shortness of breath, and sensitivity), with the remaining symptoms being either negatively correlated or having no significant correlation.

Assessing cytokine level <120 days and >120 days from last vaccination

We conducted a Mann–Whitney U test to compare cytokine levels between two groups: individuals assessed before 120 days from last vaccination and those at 120 days or beyond last vaccination (Figure 9). This non-parametric test was chosen to evaluate differences in distributions without assuming normality. The results indicated none of the biomarkers presented statistically significant differences between the two groups, as all p -values exceed 0.05. The lowest p -value observed was for TNF-alpha ($U = 237.5$, $p = .1507$), suggesting a potential trend toward a difference, though not reaching statistical significance. Similarly, CCL5 ($U = 253.0$, $p = .2560$) and sCD40L ($U = 262.0$, $p = .3364$) exhibited lower p values compared to other biomarkers, but still did not indicate a meaningful difference. These findings were indicative that cytokine levels remain relatively stable between the two time points, with no strong evidence supporting a shift in inflammatory marker distribution over time.

Discussion

Clinical reports of persistent post-vaccination symptoms have emerged, yet mechanistic explanations remain elusive. Building on our prior discovery of S1 protein persistence in CD16+ monocytes in PASC (Long COVID),⁹ we investigated whether a similar inflammatory mechanism underlies PASC-like symptoms following vaccination with approved COVID-19 vaccines (Pfizer, Moderna, Janssen, AstraZeneca). Our findings of an altered immune profile, prolonged S1 presence, and specific symptom associations suggest a potential shared pathophysiology driving these effects.

Given the symptomatic overlap with PASC and the absence of an ongoing replication competent virus,⁹ we applied machine learning to a 14-plex cytokine panel from 50 symptomatic individuals, uncovering significant elevations in IL-4, CCL3, CCL5 (RANTES), sCD40L, VEGF (Vascular endothelial growth factor), and IL-8 (Figure 3) along with significant

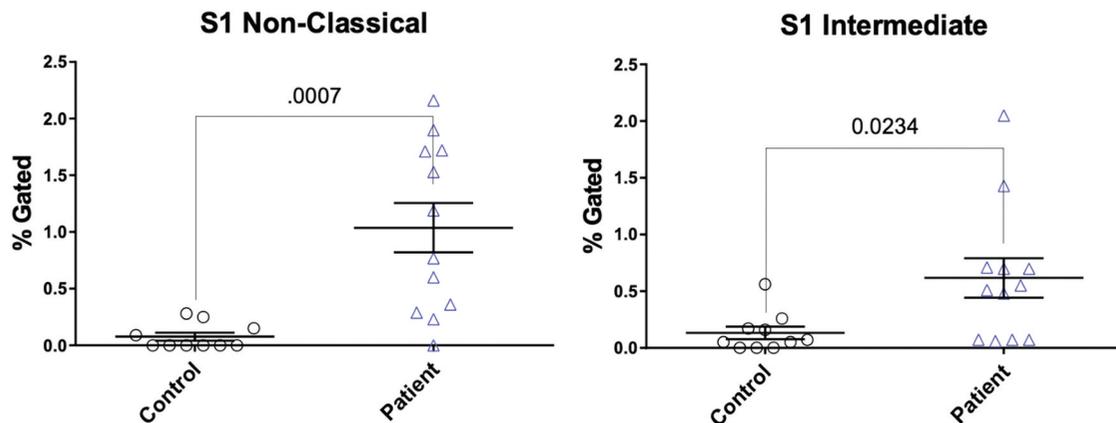
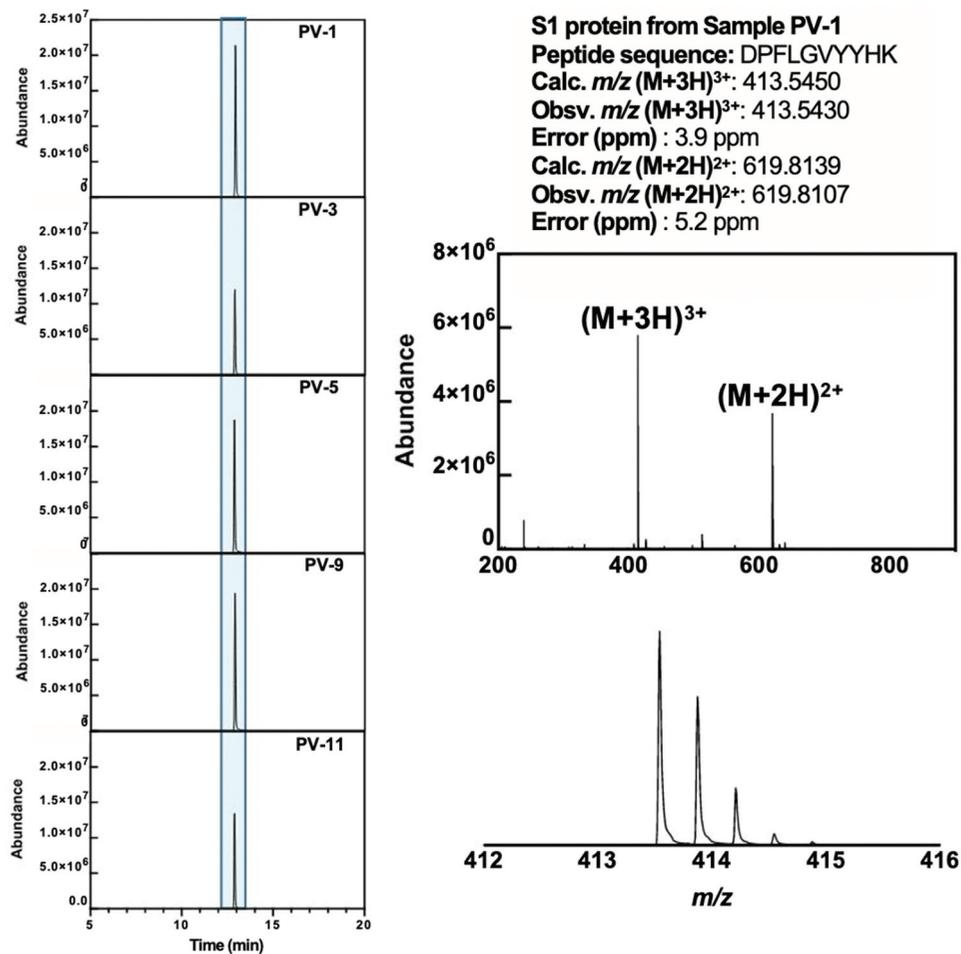


Figure 4. Flow cytometric quantification of S1-containing monocyte subsets as previously performed. Increased S1-containing intermediate and non-classical monocytes in patients (12) was statistically significant compared to healthy controls (10).



LCMS confirmation of the presence of S1 subunit in the samples. A. Extracted ion chromatogram (EIC) displaying the DPFLGVYYHK peptide. B. Mass Spectra of the DPFLGVYYHK from PV-1. The Spectrum shows the same mass, isotope distribution, and charge states.

Figure 5. Liquid chromatography/mass spectrometry confirmation of S1 and S2 protein in flow cytometrically isolated cells from five post-vaccination individuals with PASC-like symptoms.

downregulation of TNF- α and GM-CSF. These profiles aligned with PASC-like features based on single and dual classifiers (severity score and long hauler index) (Figure 2), though IL-8's prominence set this group apart from PASC.¹² Correlation analyses tied these cytokines to specific symptoms (Figure 7): IL-8 tracked with chest pain, joint pain, and neuropathy; sCD40L with twitching, tremors, fatigue, and brain fog; and CCL3 with a wide range, including chest pain, vision issues, sensory sensitivities, tinnitus, shortness of breath, headaches, brain fog, and neuropathy.

Interestingly, both CCL3 and CCL5 (RANTES), which are pro-inflammatory chemokines involved in recruiting monocytes and macrophages, have been linked to their interaction with CCR5 receptors, contributing to neuropathic pain, neuroinflammation, and migraine headaches.¹³ However, neuropathic pain and inflammation associated with CCL3/CCR5 upregulation have been reported to be mitigated by the anti-inflammatory effects of IL-4 expression. Thus, the statistically significant IL-4 elevation observed in this study may reflect an anti-inflammatory response to CCL3 and CCL5

(RANTES) expression.¹⁴ IL-4 is known to inhibit TNF- α production in monocytes, supporting the hypothesis that the observed significantly downregulated TNF- α was an anti-inflammatory counterbalance.¹⁵ Additionally, TNF- α can induce monocyte apoptosis via the TNFR1 death pathway, and the downregulated levels of TNF- α seen in this study may potentially explain the elevated CD16+ monocytes observed weeks and months in our patient cohort (Figure 4).¹⁶ GM-CSF plays a role in promoting monocyte maturation into macrophages and dendritic cells. A decrease in GM-CSF, as observed in this study (Supplementary Figure S2) could limit this process, potentially favoring the persistence of less differentiated monocytes, such as nonclassical monocytes (Figure 4).¹⁷ Though the pro-inflammatory IL-2 did not reach statistical significance (Supplementary Figure S1), it correlated with neuropathy, brain fog, headaches, dizziness, shortness of breath, POTS, and tachycardia. These patterns suggest certain cytokines may reflect symptom severity or immune dysregulation, offering clues to their roles in PCVS and potential therapeutic targets.

Detected S1 Peptides		Potential S1 Mutant Peptides		Detected S2 Peptides	
Sequence	Mass	Sequence	Mass	Sequence	Mass
CVNFNFNGLTGTGVLTESNK	2171.0317	DVYHK	660.3231	DAGFIK	649.3435
DAVR	459.2441	DVYYHK	823.3864	DCTMYICG	1034.3508
DFTGCVIAWNSNNL	1609.7195	PCNGVK	616.3003	DFGGFNFSQILP	1340.6401
DLCFTNVYA	1101.4801	RFANPVLPFN	1173.6294	DLLFNK	748.4119
DLPIGINITRFQTLALHRSYLTPG	2808.565	STYLVK	709.401	DNTFVSGNC	955.3705
DPFLGVYYHK	1237.6131	VCFEQFCNYPFLDVYHK	2265.0023	DPLQPEL	810.4123
FLPFQQFGR	1138.5923			DSFK	495.2329
HTPINLVR	948.5505			DVVIGIVNNTVY	1304.6976
LP	228.1474			LNDILARLDK	1191.6588
NLREFVFK	1051.5814			NTQEVFAQVK	1162.5982
RF	321.1801			RSFIE	650.3388
RISNCVA	818.4069				
STNLVK	660.3806				
VFRSSVLHSTQ	1259.6622				

Figure 6. Protein sequences found via liquid chromatography/mass spectrometry in CD16+ sorted monocytes.

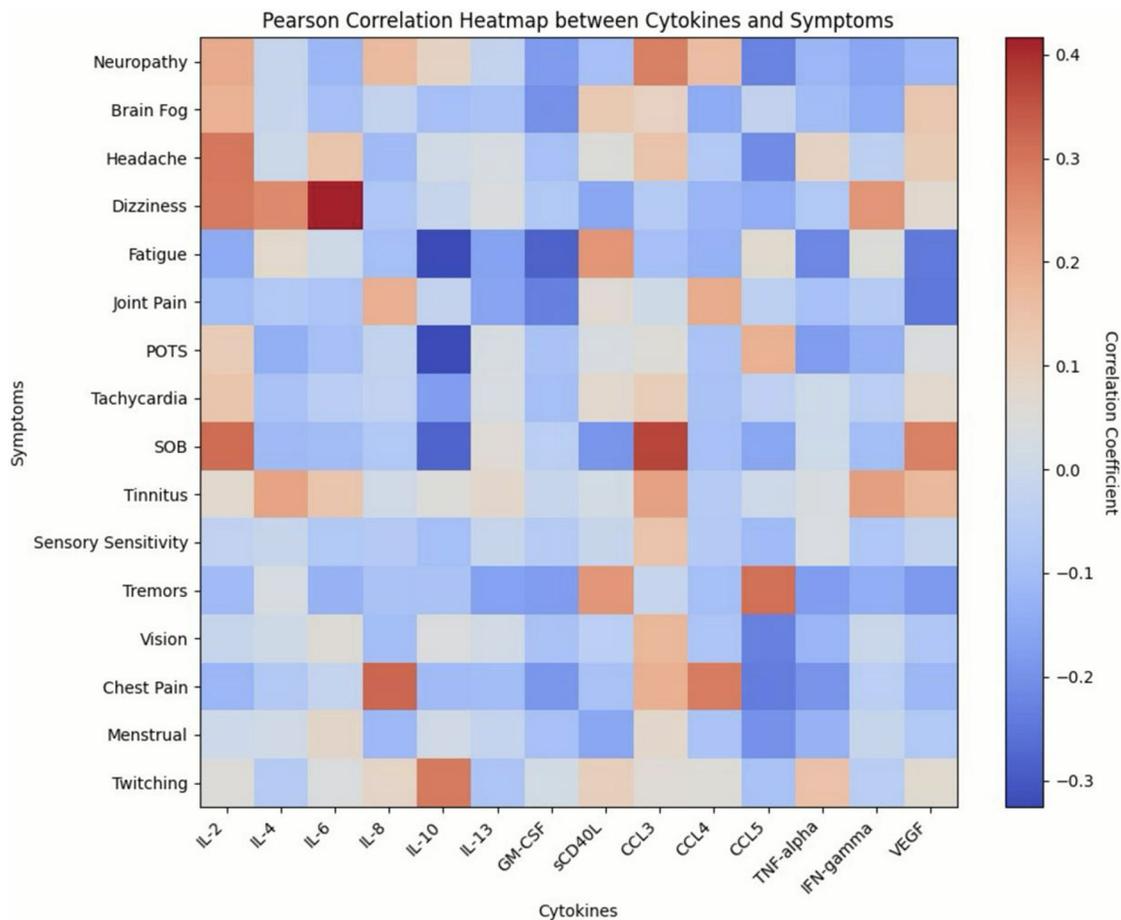


Figure 7. Pearson correlation coefficients between symptoms and biological markers, where red shades indicate positive correlations and blue shades indicate negative correlations.

To explore a cellular basis, we examined S1 protein detection in monocyte subsets. Flow cytometry detected S1 in non-classical monocytes (NCM: 11/12 patients) and intermediate monocytes (IM: 8/12) up to 245 days post-vaccination, with LC-MS confirming S1, S2, and mutant S1 peptides across all vaccine types – unlike PASC, where only S1 was detected.⁹ Symptom correlations showed S1 + IM associated with sensory hypersensitivity, tinnitus, SOB, tachycardia, POTS, and

headaches; S1 + NCM with vision disturbances, POTS, fatigue, and headaches; and S1 + classical monocytes with menstrual irregularities, sensory hypersensitivity, tachycardia, joint pain, and headaches. Elevated NCM (non-S1 +) correlated with menstrual irregularities, chest pain, tremors, sensory hypersensitivity, tinnitus, SOB, tachycardia, POTS, fatigue, and neuropathy, suggesting broader monocyte activation beyond S1 alone (Figure 8). Notably, a recent PCVS study also reported

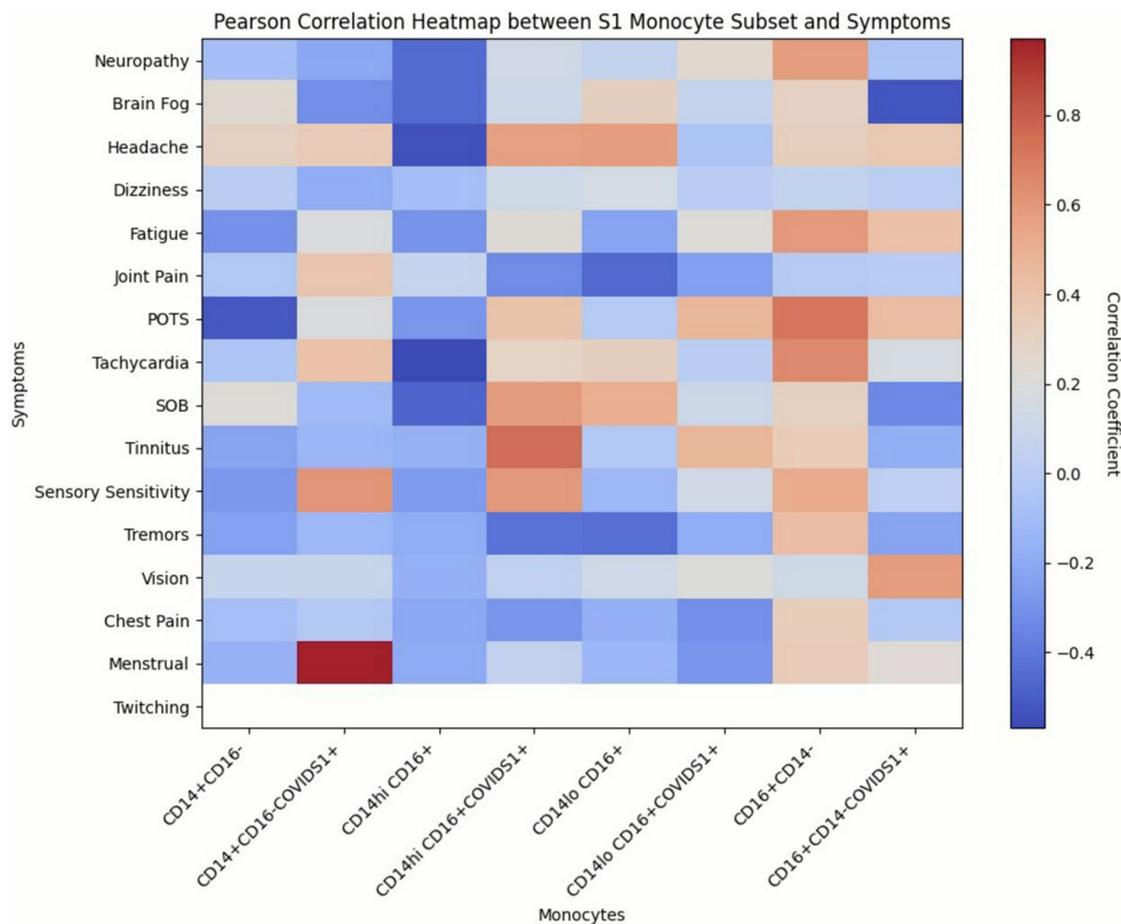


Figure 8. Pearson correlation coefficients between symptoms and S1 monocyte subsets, where red shades indicate positive correlations and blue shades indicate negative correlations.

significantly elevated NCM levels.¹⁸ These patterns suggest that specific monocyte populations could contribute to symptom severity, potentially through inflammatory pathways linked to COVID-related immune responses.

Mechanistically, S1 persistence in CD16+ monocytes may drive inflammation through vascular pathways. Nonclassical monocytes (NCM), which express CX3CR1, bind fractalkine, upregulated by IL-1, IFN- γ , and TNF- α , on endothelial cells, boosting survival via the BCL2 protein and mobilization during stress or exercise.^{19–25} This interaction, implicated in atherosclerosis and vasculopathies,^{26,27} may spark endothelialitis, activating platelets through glycoprotein 1b-IX-V and von Willebrand factor.^{28,29} Activated platelets then release sCD40L and CCL5,³⁰ recruiting monocytes and neutrophils, consistent with symptoms like fatigue, tremors, and chest pain. Elevated VEGF, linked to tinnitus, shortness of breath, and headaches, may worsen microvascular permeability and thrombosis, mirroring post-vaccination coagulopathy reports.³¹ Therapies such as CCR5 antagonists or statins, which reduce monocyte recruitment and fractalkine expression, could hold promise, as seen in PASC, though randomized clinical trials are required to confirm their efficacy.^{32–34}

These findings suggest that the S1 detected in CD16+ monocytes, coupled with a PASC-like cytokine profile, may contribute to persistent post-vaccination symptoms. Interestingly, a similar mechanism of non-replicative

monocyte activation has been described in vitro with heat inactivated *Borrelia burgdorferi*, the causative agent in Lyme disease.^{35,36} The unique presence of S2 and mutant S1 peptides raises questions about vaccine-specific effects, though their clinical roles remain unclear and require further study. Symptom-specific correlations strengthen this hypothesis, yet causality is not established, as one patient lacked S1 in NCM and four patients lacked S1 in IM, and associations varied across subsets. Additionally, the detection of S1 in one control suggests it may not be exclusive to symptomatic PCVS, warranting further study of its pathogenic role (Figure 4).

Limitations include the inability to definitively exclude prior SARS-CoV-2 infection despite negative PCR, anti-nucleocapsid antibody, and T-Detect screening, suggesting some S1 might originate from undetected exposure. We conducted a Mann-Whitney U test to compare cytokine levels between two groups: individuals assessed before 120 days and those at 120 days or beyond (Figure 9). This non-parametric test was chosen to evaluate differences in distributions without assuming normality. The results indicated none of the biomarkers presented statistically significant differences between the two groups, as all p-values exceed 0.05. Although these findings do not definitively exclude confounding factors such as a subclinical

Biomarker	U-Statistic	P-Value
IL-2	316.5	0.9380
IL-4	323	0.8384
IL-6	374	0.2322
IL-8	355	0.4092
IL-10	288	0.6478
IL-13	323.5	0.8307
GM-CSF	276.5	0.4964
sCD40L	262	0.3364
CCL3	358.5	0.3705
CCL4	345	0.5279
CCL5	253	0.2560
TNF-alpha	237.5	0.1507
IFN-gamma	329	0.7486
VEGF	329	0.7436

Figure 9. Mann–Whitney U test between before 120 days and after 120 days post-vaccination groups.

infection or undetected post-vaccination SARS-CoV-2 exposure, they do indicate that cytokine levels remained relatively stable between the two time points, with no strong evidence of a shift in inflammatory marker distribution over time. Further studies with larger sample sizes and more subgroups may be needed to detect potential subtle changes. The small sample ($n = 50$, S1 analysis in 12 patients) and wide time range (38–245 days) limit associative robustness, while lacking longitudinal data hinders dynamic insights. Symptoms might also arise from unrelated factors, and prevalence cannot be inferred. Larger, longitudinal studies with advanced infection screening are needed to confirm these associations, explore S2/mutant S1 impacts, and test interventions targeting CCR5 or VEGF pathways.

Conclusion

This exploratory study suggests S1 persistence in CD16+ monocytes, coupled with a PASC-like cytokine profile, may contribute to PCVS symptoms. While S1 persistence and a PASC-like cytokine profile were detected, causality remains unproven due to limited sample size and variable S1 presence. Larger, longitudinal studies are essential to validate these associations and assess prevalence.

Author contributions

CreDiT: **Bruce K. Patterson:** Conceptualization, Formal analysis, Investigation, Resources, Visualization, Writing – original draft, Writing – review and editing; **Ram Yogendra:** Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Supervision, Visualization, writing – original draft, writing – review and editing; **Edgar B. Francisco:** Data curation, Formal Analysis, Software, Writing – original draft, Writing – review and editing; **Jose Guevara-Coto:** Formal Analysis,

Investigation, Methodology, Validation, Writing – original draft, Writing – review and editing. All authors contributed to revising the manuscript and approved submitted version.

Disclosure statement

Bruce Patterson, Edgar B. Francisco, Emily Long, Christopher Beaty and Amruta Pise are employees of IncellDX. Bruce Patterson is CEO of IncellDX.

Bruce Patterson, Ram Yogendra, John Bream, Eric Osgood, Devon Jeffers and Mark Kreimer are independent contractors of the Chronic COVID Treatment Center (CCTC). CCTC is a private physician practice group that is independent of and receives no funding or support from IncellDX or any other entity.

Funding

The author(s) reported there is no funding associated with the work featured in this article.

Notes on contributor

Ram Yogendra M.D., M.P.H. received his Bachelor of Arts in Biology from Rollins College, Winter Park, Florida, Master of Public Health at University of South Florida, Tampa, Florida, and Doctor of Medicine from St. George's University School of Medicine, Grenada. He completed an anesthesiology residency at SUNY Downstate, Brooklyn, New York and is board certified by the American Board of Anesthesiology. Dr. Yogendra is the medical director and co-founder of the Chronic Covid Treatment Center studying chronic inflammatory diseases including long COVID, Post-Treatment Lyme Disease, and ME-CFS.

ORCID

Ram Yogendra  <http://orcid.org/0000-0001-7966-4093>

Data and materials availability

All requests for materials and raw data should be addressed to the corresponding author

Ethics statement

The independent Chronic COVID Treatment Center (CCTC) Ethics and IRB group reviewed and approved the study (protocol CCTC 2021–005). All the patients/participants provided their written informed consent to participate in this study.

Abbreviations

PASC	post-acute sequelae of COVID-19
PACVS	post-vaccination individuals with PASC-like symptoms
NCM	non-classical monocytes
IM	intermediate monocytes
CX3CL1	C-X3-C motif chemokine ligand 1
CX3CR1	C-X3-C motif chemokine receptor 1
IL	interleukin
RANTES	regulation on activation, healthy control T-expressed and secreted
CCR	chemokine receptor
IFN	interferon
TNF	tumor necrosis factor
MIP	macrophage inflammatory protein
PBMCs	peripheral blood mononuclear cells
VEGF	vascular endothelial growth factor
LH	long hauler or PASC

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