SARS-CoV-2 S1 Protein Persistence in SARS-CoV-2 Negative Post-Vaccination Individuals with Long COVID/ PASC-Like Symptoms

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Short Report

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Abstract

Background

We sought to determine the immunologic abnormalities in patients following SARS-CoV-2 vaccines who experience post-acute sequelae of COVID-19 (PASC)-like symptoms > 4 weeks post vaccination. In addition, we investigated whether the potential etiology was similar to PASC.

Design:

We enrolled 50 post-vaccination individuals who experience PASC-like symptoms, 10 healthy individuals, and 35 individuals post-vaccination without symptoms. We performed multiplex cytokine/chemokine profiling with machine learning as well as SARS-CoV-2 S1 protein detection on monocyte subsets using flow cytometry and mass spectrometry.

Results

We determined that post-vaccination individuals with PASC-like symptoms had similar symptoms to PASC patients. When analyzing their immune profile, post-vaccination individuals had statistically significant elevations of sCD40L, CCL5, IL-6, and IL-8. SARS-CoV-2 S1 and S2 protein were detected in CD16 + monocytes using flow cytometry and mass spectrometry on sorted cells.

Conclusions

Post-vaccination individuals with PASC-like symptoms exhibit markers of platelet activation and pro-inflammatory cytokine production which may be driven by the persistence of SARS-CoV-2 S1 protein persistence in intermediate and non-classical monocytes.

Introduction

Over 1.5 billion doses of SARS-CoV-2 vaccine have been delivered to individuals worldwide. Vaccines exhibit a > 80% protection against infection by the original alpha variant with some vaccines offering > 90% protection[1]. Most importantly, vaccines have protected against serious disease and death[2].

Since the launch of global vaccination programs, side effects of the vaccine have been reported including increased thrombosis, cardiomyopathy, and other vascular events[3–5]. The possibility that long hauler symptoms could occur following a breakthrough infection was published by the Center for Disease Control (CDC). This publication did not discuss the possibility that long hauler symptoms could occur in vaccinated individuals in the absence of infection[6]. Here, we report on 50 individuals with symptoms > 4 weeks following vaccination that resemble the spectrum of symptoms reported in long COVID or PASC[7, 8].

We used machine learning to analyze the immune profiles in these individuals. Further, we used high parameter, single cell analysis to determine if S1 protein generated by vaccination persisted in immune cells as previously described[9].

Materials/methods

Patients

Fifty patients were enrolled following written informed consent. None of the patients reported any clinical signs and symptoms of previous SARS-CoV-2 infection, with some also having documented multiple negative polymerase chain reaction (PCR) tests. Lack of prior infection was determined using anti-nucelocapsid antibody testing or Food and Drug Administration (FDA) Emergency Use Authorization (EUA) T-Detect Test. Representative patients vaccinated with all four approved vaccines (Pfizer-BioNTech, Moderna, Johnson and Johnson, and Oxford/AstraZeneca), were included in the study as well as the duration of the symptoms post-vaccination. Thirty-five healthy controls all 90 days post-vaccination were enrolled as the control group following informed consent.

Multiplex Cytokine Quantification

Fresh plasma was used for cytokine quantification using a customized 14-plex bead based flow cytometric assay (IncellKINE, IncellDx, Inc) on a CytoFlex flow cytometer as previously described[8] using the following analytes: TNF-α, IL-4, IL-13,IL-2, GM-CSF, sCD40L, CCL5 (RANTES), CCL3 (MIP-1α),IL-6, IL-10, IFN-γ, VEGF, IL-8, and CCL4 (MIP-1β). For each patient sample, 25 µL of plasma was used in each well of a 96-well plate.

Flow Cytometric S1 Quantification in Monocyte Lineage Cells

Peripheral blood mononuclear cells were collected in EDTA anticoagulated whole blood collection tubes. Whole blood (200 uL) was lysed using 4 mL of 1X Red Blood Cell (RBC) Lysis Buffer (Abcam Part #ab204733) for 10 min. and centrifuged at 400x g for 5 min. Cells were resuspended in 1 mL 2%BSA/D-PBS and incubated for 5 min at room Temperature. The cells were then pelleted at 300x g for 6 min and the supernatant was aspirated leaving 50–100 µL residual liquid. An antibody cocktail with the following reagents: 5 µL True-Stain Monocyte Blocker, 5 µL CD45 FITC, 3 µL CD14 PE, 5 µL CD3 PC5.5, 1.5 µL CD19 PC7,1.5 µL CD16 AF700, 5 µL COVIDS1-AF647, 74 µL 2%BSA/D-PBS. (100uL) was added to each tube and the mixture was incubated in the dark for 25 min. 37° C. Cells were washed in 1 mL 2% BSA, centrifuged at 300x g for 6 min and re-suspended in 200 µL D-PBS for analysis. Single cells were analyzed on a Beckman Coulter Cytoflex flow cytometer and gated as previously described[8].

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 (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. Six 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. 10 ug of anti-S1 mAb were used to immunoprecipitate the S1 Spike protein from cell lysate of each patient. 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 (3cm x 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). 1µL of TCEP (100 mM) was added and the samples were incubated at 37ºC for 30 min. 1 µL of chloroacetamide (500 mM) was added to the samples and incubated at room temperature for 30 min. 1 µL rAspN (Promega 0.5 µg/µL) and 1 µL of LysC (Pierce, 1 µg/µL) were added and the samples incubated at 37ºC 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 µm) 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 H_2O) and a solvent B (0.1% formic acid in 90% CH_3CN). 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

Results

Vaccine-Specific PASC-Like Symptoms

We investigated 50 individuals that exhibited PASC-like symptoms similar to those previously published[7,8]. On average these symptoms were reported after 105 days post-vaccination (Table 1) and previously published in post-vaccination individuals[10]. There was variability in symptoms according to the vaccine administered, however, the predominant symptoms were fatigue, neuropathy, brain fog and headache. All of these symptoms were reported in all four of the vaccines administered in this cohort. Notably infrequent in the post-vaccination individuals with PASC-like symptoms compared to PASC itself were shortness of breath and loss of taste/smell.

Immune Profile Determined using a 14-Plex Cytokine/Chemokine Panel.

To determine whether the PASC-like symptoms may be associated with similar cytokine/chemokine abnormalities found in PASC[8], we performed statistical analysis on the 50 post-vaccination patients with PASC-like symptoms and compared the cytokine expression levels to individuals post-vaccination without PASC-like symptoms. Post-vaccination patients with symptoms had statistically significant elevation in CCL5 ($p=0.006$), sCD40L ($P<0.001$), IL-8 ($P<0.001$), and IL-6 ($P=0.04$) (Fig. 3). Vaccinated individuals without PASC-like symptoms had elevated IL-2 ($P=0.007$) and reduced CCL4 ($P=0.001$) compared to the group of vaccinated individuals with PASC-like symptoms. Elevated IL-8 is a key difference compared to the elevated cytokines in PASC.

Analysis of S1 Protein Persistence

Similar to our published findings of S1 persistence in non-classical and intermediate monocytes in PASC[9], we investigated whether a similar mechanism could account for the PASC-like symptoms in post-vaccination individuals with PASC-like symptoms. We used flow cytometry to screen patients for S1 protein in their monocyte subsets. We screened 14 post-vaccination individuals with simultaneously collected PBMCs and with PACS-like symptoms and 10 normal controls. As demonstrated in figure 4, there was statistically significant elevation of S1 containing non-classical monocytes (13 of 14, $P<0.001$) and S1 containing intermediate monocytes (9/14, $P=0.006$).

Of the S1 positive post-vaccination patients, we sorted the CD16+ cells from six patients as previously performed for PASC patients[9]. Upon isolation of the protein, we demonstrated by LC-MS that these CD16+ cells from post-vaccination patients also contained S1 protein months after vaccination (Fig 5A). Further analysis revealed that these S1 positive, CD16+ cells also contained peptide sequences of S2, and mutant S1 peptides (Fig 5B).

Discussion

To date, clinical manifestations of post-vaccination side effects or injuries have been described but very few mechanisms have been offered to explain these findings. In the present study, we investigated whether an S1 protein mechanism of inflammation similar to what we published in PASC might underlie the persistent, PASC-like symptoms that remain for months following vaccination with currently available vaccines in the US.

Given that ongoing viral replication may not be required for prolonged symptoms[9] and given the overlap in symptomatology, we applied machine learning to a panel of previously published immune biomarkers to determine if an immune signature for post-vaccination PASC-like symptoms might exist. Using two algorithms (severity score and long hauler index) previously derived from these biomarkers[8], we found that post-vaccination PASC-like symptoms were associated with an inflammatory profile with statistically significant elevations in CCL5, sCD40L, IL-6, and IL-8. Further these patients were classified as PASC using a single classifier and PASC with inflammation using a dual classifier. Elevated IL-8 was a unique marker relative to PASC in post-vaccination individuals with PASC-like symptoms. We recently found a statistically significant correlation between decreased IL-8 and improvement in the NYHA cardiac symptom score in PASC following treatment with a CCR5 antagonist and statin[11].

Because of the similarities between PASC and patients with post-vaccination PASC-like symptoms, we examined whether S1 protein persistence might also occur in patients with post-vaccination PASC-like symptoms. We demonstrated a statistically significant elevation of S1 protein containing non-classical monocytes (NCM) and in S1-containing intermediate monocytes (IM) in post-vaccination PASC-like patients compared to normal controls. We sorted these CD16 + monocytes as previously performed and used mass spectroscopy to interrogate whether S proteins (S1 and or S2) were present in these highly mobile cells. We confirmed the presence of S1 sequences as well as S2 sequences in these monocytic subsetss. Further, we found amino acid mutations in all six patients analyzed; the impact of which remains to be elucidated.

NCM bind to fractalkine expressed on vascular endothelial cells through the expression of the fractalkine receptor CX3CR1 on the surface of NCM[11]. Fractalkine is also upregulated by IL-1, IFN-γ, and TNF-α[12], cytokines that we have reported to be elevated in PASC[9]. CX3CR1 also provides a survival signal to non-classical monocytes through CX3CR1-dependent expression of the anti-apoptotic protein BCL2[13, 14]. Stress and exercise mobilize non-classical monocytes including up to 4-fold with exercise[15, 16]. The interaction between fractalkine and CX3CR1 has been reported to be involved in the pathogenesis of atherosclerosis, vasculitis, vasculopathies, and inflammatory brain disorders[17] and could also be contributing to a vascular endotheliitis in post-vaccination individuals with PASC-like symptoms. Vascular inflammation has been shown to expose the collagen surface and platelet activation/adherence by way of glycoprotein 1b-IX-V-receptor (GPIb-IX-V) with collagen-bound von Willebrand factor (vWF)[18,19]. Activated platelets also release soluble CD40 ligand (sCD40L) which leads to recruitment of both

neutrophils and monocytes to the sites of vascular inflammation, thus activating the coagulation cascade[20]. Activated platelets also release CCL5/RANTES which binds to endothelial cells, promoting monocyte adhesion to inflamed endothelial tissues[21] and acting as a chemoattractant for inflammatory cells. Studies in atherosclerosis have shown that CCR5 antagonists reduce non-classical monocyte recruitment to sites of atherosclerosis[21,22]. In addition, accumulation of non-classical monocytes can be reduced by statin treatment through reduction in fractalkine expression[23,24]. Interfering with these pathways may hold potential therapeutic targets for PASC and post-vaccination individuals with PASC-like symptoms[10].

Further, activated platelets and endothelial cells also secrete (VEGF) which induces angiogenesis and microvascular hyperpermeability. VEGF contributes to vasculitic neuropathy and also promotes a pro-inflammatory-prothrombotic environment[25]. Given the elevated levels of sCD40L and the frequent co-expression of VEGF, this pathway may also contribute to the thrombosis seen in some individuals post-vaccination.

Taken together, these findings suggest a possible mechanism for the debilitating symptoms found in some patients weeks and months following vaccination. The findings that the immune profile and persistent S1 protein in CD16 + monocytes suggest that S1 protein persistence is a major contributor not only of symptoms in post-vaccination individuals with PASC-like symptoms but also may be a major contributor of PASC itself given that S1 alone delivered by vaccination can cause similar pathologic features.

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Declarations

Ethics:

All the patients/participants provided their written informed consent to participate in this study. The study was approved by the IRB or the Chronic COVID Treatment Center.

Data and materials availability:

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

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Competing interests:

B.K.P, A.P., E.L, and EBF are employees of IncellDx, Inc

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Author contributions:

R.Y., J.B, M.K. E.O. organized the clinical study and actively recruited patients.

B.K.P, A.P., E.L., E.B.F, performed experiments and analyzed the data.

J.G-C., R.A.M., J.M., C.B. performed the statistics and bioinformatics

B.K.P., J.M., E.B.F, J.G-C., R.A.M., R.V. wrote and edited the draft of the manuscript and all authors contributed to revising the manuscript prior to submission.

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Tables

Table 1. Demographics of post-vaccination individuals with PASC-like symptoms.

Patient Demographics	
Gender-M	14
F	36
Average age	41.8 (range 13-65)
Time since last vaccine (mean)	105 days (range 38-245 days

Figures

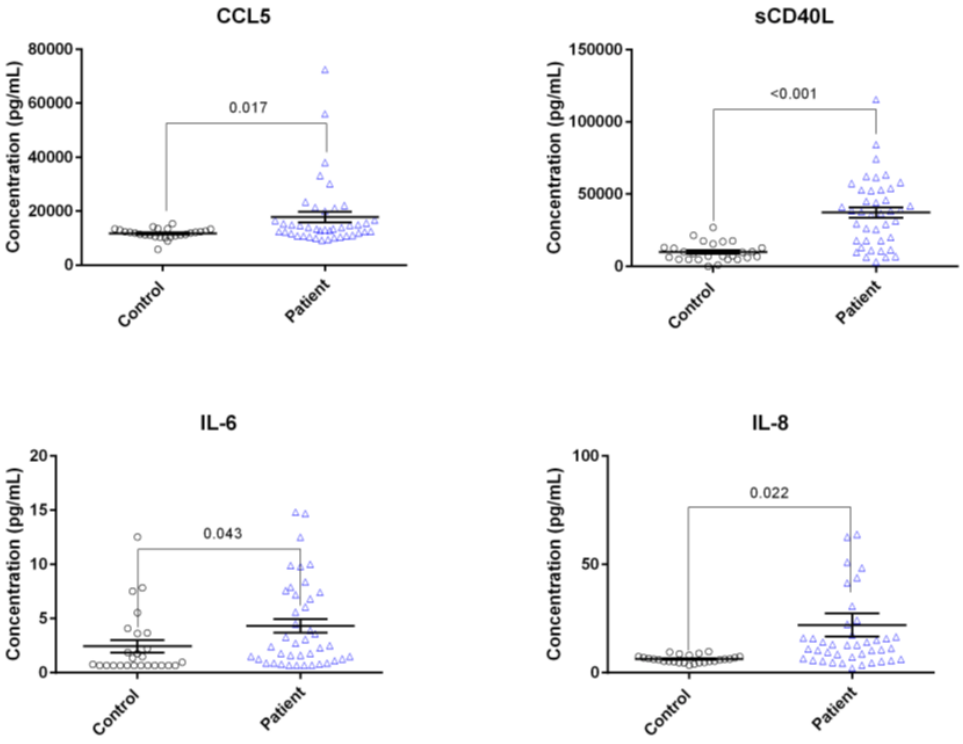


Figure 1

Statistically significant elevation of cytokines in post-vaccination individuals with PASC-like symptoms compared to vaccinated, healthy individuals.

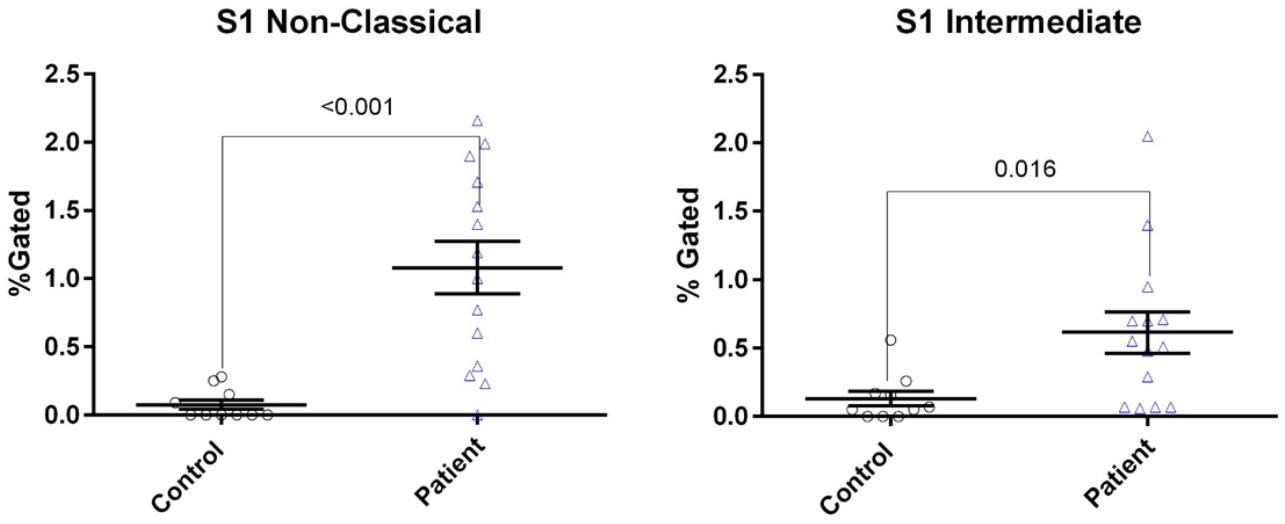


Figure 2

Flow cytometric quantification of S1-containing monocyte subsets as previously performed[9]. Increased S1-conatining intermediate and non-classical monocytes was statistically significant compared to vaccinated, healthy controls.

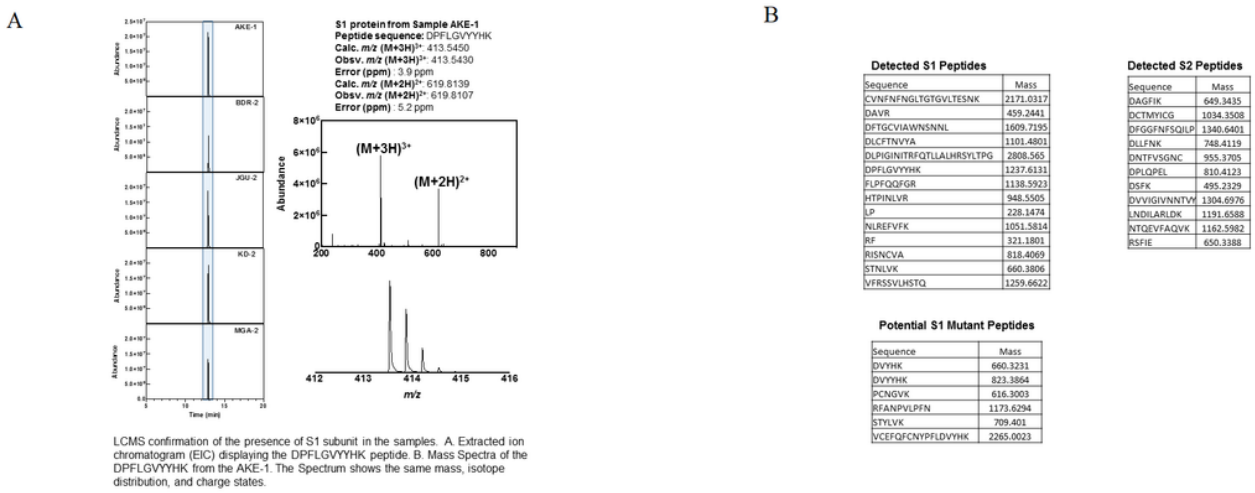


Figure 3

(A) Liquid chromatography/mass spectrometry confirmation of S1 and S2 protein in flow cytometrically isolated cells from post-vaccination individuals with PASC-like symptoms. (B) Protein sequences found in CD16+ sorted monocytes including S1-mutated sequences.

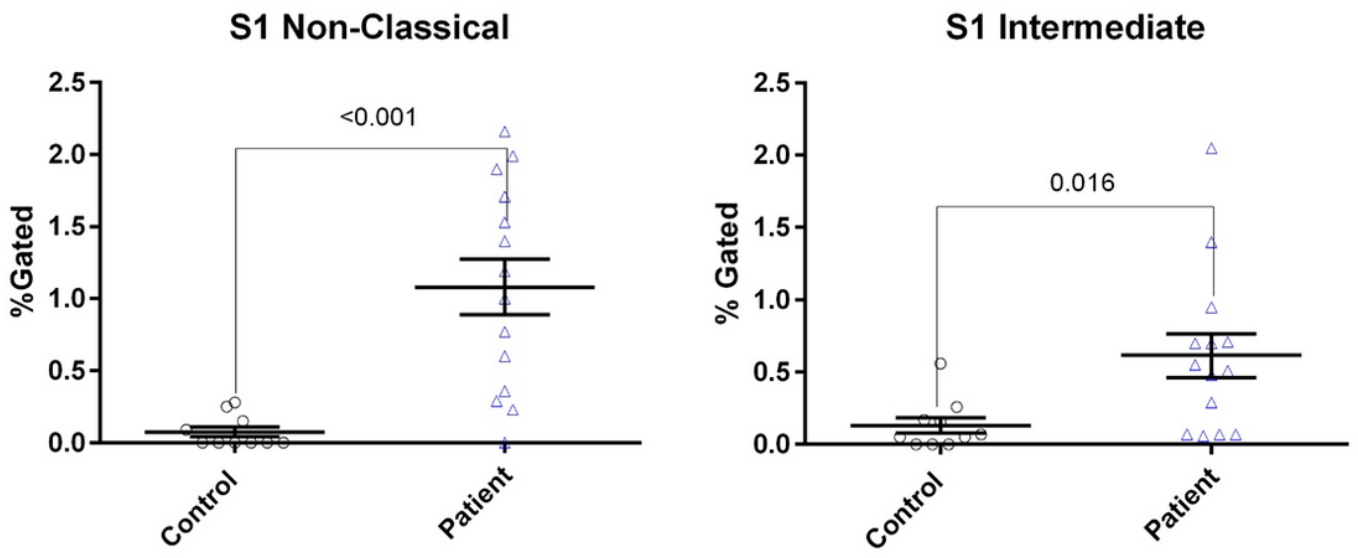
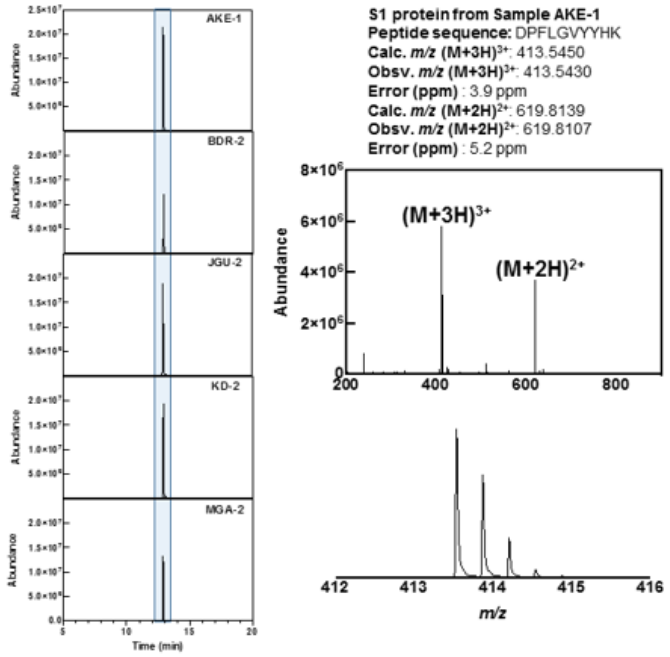


Figure 4

Figure 5.A



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 the AKE-1. The Spectrum shows the same mass, isotope distribution, and charge states.

Figure 5.B

Detected S1 Peptides

Sequence	Mass
CVNFNFNGLTGTGVLTESNK	2171.0317
DAVR	459.2441
DFTGCVIAWNSNNL	1609.7195
DLCFTNVYA	1101.4801
DLPIGINITRFQTLLALHRSYLTPG	2808.565
DPFLGVYYHK	1237.6131
FLPFQQFGR	1138.5923
HTPINLVR	948.5505
LP	228.1474
NLREFVFK	1051.5814
RF	321.1801
RISNCVA	818.4069
STNLVK	660.3806
VFRSSVLHSTQ	1259.6622

Detected S2 Peptides

Sequence	Mass
DAGFIK	649.3435
DCTMYICG	1034.3508
DFGGFNFSQILP	1340.6401
DLLFNK	748.4119
DNTFVSGNC	955.3705
DPLQPEL	810.4123
DSFK	495.2329
DVVIGIVNNTVY	1304.6976
LNDILARLDK	1191.6588
NTQEVFAQVK	1162.5982
RSFIE	650.3388

Potential S1 Mutant Peptides

Sequence	Mass
DVYHK	660.3231
DVYYHK	823.3864
PCNGVK	616.3003
RFANPVLPFN	1173.6294
STYLVK	709.401
VCEFQFCNYPFLDVYHK	2265.0023

Figure 5