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Optimisation of SARS-CoV-2 peptide stimulation and measurement of cytokine output by intracellular flow cytometry and bio-plex analysis

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ABSTRACT

Our study was conducted to optimise a peptide stimulation and an intracellular cytokine staining protocol, alongside Bio-Plex supernatant analysis, for use in patients who had previously contracted SARS-CoV-2 or received vaccination against this virus in a clinical laboratory setting. Peripheral Blood Mononuclear Cell extraction and cryopreservation allowed for cells to be stored long term and enhanced logistical processing of samples. Viability and functionality of cells were analysed by flow cytometric methodology using viability staining monoclonal antibodies conjugated to fluorochromes. Antibiotics and Benzonase Nuclease did not impact lymphocyte viability and so cell culture conditions were optimised in terms of retaining viability and functionality. Optimisation of peptide stimulation with Influenza and SARS-CoV-2 peptide pools was conducted through stimulation experiments assessing peptide concentration, peptide stimulation time and enrichment studies to increase precursor frequency. Cytokine output was measured by flow cytometry and Bio-Plex methodologies, with positive cytokine readings predominantly detected in the cell culture supernatant. Analysis of both intracellular and extracellular compartments allowed for detection of cytokines and established the retained cellular functionality post cryopreservation. These results also indicated that our peptide stimulation method can generate antigen-specific T lymphocytes upon exposure to SARS-CoV-2 peptide pools. Moreover, the measurement of specific cytokines could be applied to an array of conditions, such as chronic inflammatory diseases, but to also offer an alternative method of measuring vaccine responses. This platform is easily adaptable and can remain relevant alongside changing vaccine composition, thus ensuring its applicability to future vaccination programmes.

1. Introduction

In late December 2019, 41 cases of pneumonia with an unknown cause were reported in the city of Wuhan (Lu et al., 2020a). The World Health Organisation (WHO) announced that a novel Coronavirus had been identified and was to be known as 2019-nCoV/SARS-CoV-2 (SARS-CoV-2) and was the causative agent for coronavirus disease (COVID-19) (World Health Organization, 2020). On the 11th March 2020, WHO declared the SARS-CoV-2 outbreak to be a global pandemic. As of the 10th January 2022, there have so far been over 307 million cases confirmed and over 5.4 million deaths globally from COVID-19 (John Hopkins University of Medicine, 2022).

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and SARS-CoV-2 are the only known coronaviruses to possess the ability to replicate in the lower respiratory tract and inflict pneumonia (Tay et al., 2020). SARS-CoV-2 belongs to the betacoronavirus genus and shares genetic homology to both MERS-CoV and SARS-CoV at ~50% and 79% respectively (Lu et al., 2020b). The structure of SARS-CoV-2, and coronaviruses in general, consists of large spikes protruding from the viral envelope (Azkur et al., 2020). The viral envelope is constructed by a lipid bilayer from the infected host and contains four structural proteins known as Spike (S), Envelope (E), Membrane (M) and Nucleoprotein (N) (Azkur et al., 2020). Within the literature it is reported that entry of

Abbreviations: SARS-CoV-2, 2019-nCoV/SARS-CoV-2; APCs, Antigen Presenting Cells; CAR, Chimeric Antigen Receptor; COVID-19, coronavirus disease; CRS, Cytokine Release Syndrome; IFN, Interferon; ICS, Intracellular Cytokine Staining; MERS-CoV, Middle East Respiratory Syndrome Coronavirus; PBMC, Peripheral Blood Mononuclear Cell; SARS-CoV, Severe Acute Respiratory Syndrome Coronavirus; SLE, Systemic Lupus Erythematosus; TNF-α, Tumour Necrosis Factor Alpha; WHO, World Health Organisation.

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SARS-CoV-2 into host cells is facilitated by S-protein engagement with angiotensin-converting enzyme 2 (ACE2) and Transmembrane Serine Protease 2 (TMPRSS2) (Hoffmann et al., 2020). The majority of patients with SARS-CoV-2 infection display respiratory symptoms as expression of ACE2 receptor is present in vascular endothelial cells of the lower respiratory tract (Guo et al., 2020). Upon entry into the host cell, the innate immune response is initiated through pattern recognition receptors, including Toll-like Receptor TLR7 and TLR8, via recognition of single-stranded RNA (Moreno-Eutimio et al., 2020). Following viral recognition, inflammatory cytokines and chemokines, including IFNs, are secreted and recruit more immune cells to the site of infection. The increased infiltration of immune cells induces a pro-inflammatory environment (Hojyo et al., 2020). In severe and critical cases of COVID-19, post-mortem pathology has shown there is extensive immune cell infiltration in the lung which is predominated by lymphocytes alongside severe lymphopenia within the peripheral blood (Xu et al., 2020).

Within COVID-19 patients, an increase of pro-inflammatory cytokines has been reported. In the majority of severe COVID-19 cases cytokines such as Interleukin (IL)-1, IL-2, IL-6, IL-7, IL-10, Granulocyte-Colony Stimulating Factor (G-CSF), and Tumour Necrosis Factor Alpha (TNF-α) were significantly increased (Chen et al., 2020). This dysregulated and exacerbated production of cytokines is referred to as Cytokine Release Syndrome (CRS) (Huang et al., 2020). CRS within SARS-CoV-2 infection was associated alongside other severe clinical manifestations including life-threatening pneumonia and multi-organ failure. This correlated with SARS-CoV infection from the mid-2000's as research into this infection also reported CRS and was associated with poorer outcomes (Huang et al., 2005).

The cytokine profile of CRS in COVID-19 differs to that of other CRS triggers including Primary Hemophagocytic Lymphohistiocytosis (HLH) and Chimeric Antigen Receptor (CAR) T cell therapy. Interferon (IFN) Gamma expression is one major difference noted between the triggers of CRS. In CAR-T cell therapy and Primary HLH, median concentrations of 1000 pg/mL (Maude et al., 2014) and 901 pg/mL (Tang et al., 2008) have been reported. The concentration of IFN-γ is significantly increased in these forms of CRS in comparison to even the most severe COVID-19 cases which have reported a median peak of 10 pg/mL (Huang et al., 2020).

Immunological memory post SARS-CoV and MERS-CoV infection has been reported by several studies, which have identified CD4+ and CD8+ memory T-lymphocytes years after initial infection. Ng et al., identified memory CD8+ T-lymphocytes within a patient 11 years after initial SARS-CoV infection (Ng et al., 2016). MERS-CoV specific CD4+ and CD8+ lymphocytes were detected within all MERS survivors and were shown to retain their functionality through cytokine production after specific peptide pulsing (Zhao et al., 2017).

Initial evidence into early convalescent participants has indicated the presence of SARS-CoV-2 specific memory T-lymphocytes after COVID-19 recovery. Various groups have investigated and published work regarding this cellular presence in the early convalescent phase (Grifoni et al., 2020; Le Bert et al., 2020; Peng et al., 2020; Sekine et al., 2020; Rodda et al., 2021).

As the world has now lived with SARS-CoV-2 for over two years, data is beginning to emerge concerning the long-term effects of this infectious disease. A considerable number of cases have been reported which state that individuals who were infected with SARS-CoV-2 are still experiencing symptoms even though viral infection is no longer present. This condition is known as Post COVID-19 condition but has more commonly been referred to as "Long Covid". The most common symptoms which have been described by individuals suffering from Long Covid include fatigue, post-exertional malaise and cognitive dysfunction (Davis et al., 2021).

Cytokine dysregulation has been observed in symptomatic long covid patients 8 months post initial infection (Phetsouphanh et al., 2022). Reports of persistently increased expression of IFN-β and IFN-λ1 was present in symptomatic long covid patients and absent in the recovered cohort. The long covid cohort also displayed a lack of naïve T and B lymphocytes, indicating continual activation of these cellular populations. The observations within this study indicate the presence of an abnormal immune profile in symptomatic long covid patients. Long term immune dysregulation could provide an explanation for symptoms experienced post 12 weeks infection. A sustained pro-inflammatory environment may potentially provide a platform for other chronic conditions to arise such as autoimmune disease.

Furthermore, case study reports have also been published regarding the suspected development of a variety of autoimmune diseases post SARS-CoV-2 infection. There have been multiple reports of individuals developing demyelinating disease of the peripheral and central nervous system (Palao et al., 2020; Moore et al., 2021; Ismail et al., 2021). Additionally, there have also been cases of new-onset Systemic Lupus Erythematosus (SLE) post SARS-CoV-2 infection reported in the literature (Gracia-Ramos and Saavedra-Salinas, 2021; Bonometti et al., 2020; Mantovani Cardoso et al., 2020).

Patients with pre-existing comorbidities, including cardiovascular disease, cancer and diabetes, have been shown to have an increased mortality in comparison to healthy cohorts due to modulation of host immune responses and viral interactions (Bigdelou et al., 2022). In silico analysis has been utilised to predict whether any ACE2 mutations within cancer patients may present a level of resistance against SARS-CoV-2 infection (Raghav et al., 2023).

Multiple therapeutic options have been explored with regards to treatment of severe COVID-19 infection. These include human target based therapies such as ACE2 inhibitors (Monteil et al., 2020), TMPRSS2 serine protease inhibitors (Uno, 2020; McKee et al., 2020) and JAK inhibitors (Favalli et al., 2020). The use of immunomodulator proteins encapsulated nanoparticles have previously been used in the eradication of SARS-CoV (Wohlford-Lenane et al., 2009; Wiley et al., 2009). Assessment and application of this technology to SARS-CoV-2 has recently been suggested as a novel methodology of blocking S-protein binding (Kumar Raghav and Mohanty, 2020).

There is currently no clinical assay available to identify or predict whether a patient is at higher risk, or is definitively presenting with, long covid. It is important to establish whether these patients do have a sustained immune dysregulation, the duration of dysregulation and subsequent consequences. The development of a clinical assay to assess whether cellular responses are dysregulated in comparison to healthy cohorts could be useful in defining a diagnosis of long covid and identifying those patients at risk of potential chronic inflammatory conditions.

One candidate platform which could be utilised for the assessment of immune dysregulation is Intracellular Cytokine Staining (ICS). ICS is a method often used within research laboratories to evaluate cellular responses by allowing the visualisation of any response generated by specific cellular populations. This is through the use of fluorescently labelled monoclonal antibodies and flow cytometric technology.

For visualisation of intracellular cytokines, cells usually require stimulation for an active response to be induced. ICS can be applied to assess the magnitude and characteristics of antigen-specific T-lymphocytes within a Peripheral Blood Mononuclear Cell (PBMC) population. To determine whether antigen-specific T-lymphocytes are present, overlapping peptide pools containing the protein of interest can be used as a stimulus in this protocol (Maecker et al., 2001). This paper will establish a protocol and assess the utility of ICS in detecting cytokine output from SARS-CoV-2 stimulated PBMCs.

1.1. Materials and methodologies

1.1.1. Sample collection

Heparinised peripheral blood was collected from volunteers registered to the EXCOVIR clinical trial. In accordance with the Health Research Authority, patient samples were only obtained after written

consent was collected prior to inclusion into the study (IRAS283457). Samples for this paper consisted of 4.5 mL Lithium Heparin anti-coagulated peripheral whole blood. PBMCs were separated from anti-coagulant. Patient groups included within this paper were unvaccinated healthy, vaccinated healthy, mild-asymptomatic convalescent and recovered critically ill in-patients. For SARS-CoV-2 infected cohorts, peripheral blood samples were obtained 28 days post infection and cryopreserved in liquid nitrogen. (See Table 1.)

1.1.2. PBMC extraction, counting, cryopreservation and thawing

PBMCs were extracted from heparinised peripheral blood using Lymphoprep density gradient as per previous protocols (Maneta et al., 2022).

PBMCs were then washed in RPMI 1640 cell culture medium containing 10% Heat Inactivated Foetal-Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Sigma-Aldrich), also referred to as Complete Medium, at 200 x g. Cells were then re-suspended in sterile red blood cell lysis buffer (Beckman Coulter) for 15 min at room temperature. Cells were re-suspended in 2 mL of Complete Medium before counting by the Z1-particle counter (Beckman Coulter).

Post PBMC counting, cells were cryopreserved at a maximum concentration of 4 × 10 (Azkur et al., 2020). Cryopreservation and thawing of PBMCs was conducted according to ProImmune Pro5 MHC Pentamer Handbook protocols. Post thawing, PBMCs were transferred to a T-25 cell suspension flask and rested in an incubator at 37 °C 5% CO_2. Samples were thawed only when required.

1.1.3. Viability analysis using 7-AAD

Viability of PBMCs within initial experiments used the intracellular marker 7-AAD (Beckman Coulter) PBMCs were incubated with 7-AAD and subsequently washed as per manufacturer guidelines.

1.1.4. Peptide stimulation

Antigen-specific stimulation of PBMCs was conducted using SARS-CoV-2 PepTivators from Miltenyi Biotech. The following PepTivators were used for the SARS-CoV-2 stimulation condition:

- PepTivator® SARS-CoV-2 Prot_M (6 nmol/peptide).
- PepTivator® SARS-CoV-2 Prot_S (6 nmol/peptide).
- PepTivator® SARS-CoV-2 Prot_N (6 nmol/peptide).

Table 1
Patient demographics of COVID-19 infection and vaccination status.

Patient Identifier	Patient COVID Group	SARS-CoV-2 Infection Status	SARS-CoV-2 Vaccination Status	Additional information
Patient A	Mild-asymptomatic convalescent	Infection (Sept 2020)	Negative	
Patient B	Vaccinated healthy control	Negative	Two doses of BNT162b2 Pfizer-BioNTech	
Patient C	Mild-asymptomatic convalescent	Infection with suspected B.1.1.529 Omicron variant (Dec 2021)	Three doses of BNT162b2 Pfizer-BioNTech	Infection occurred <1 month post third vaccination
Patient D	Critically ill convalescent	Infection (May 2021)	One dose of BNT162b2 Pfizer-BioNTech at time of admission	Admitted as in-patient to hospital for COVID-19
Healthy Control	Unvaccinated healthy control	Negative	Negative	

Peptide pools were used both individually and as a combination master mix for the stimulation of PBMCs. Stimulation of PBMCs with SARS-CoV-2 PepTivators was conducted in duplicate.

Positive controls consisted of PMA + BrefA (Thermo Fisher) PMA (Sigma-Aldrich) and PepTivator® Influenza A (H1N1) HA (Miltenyi Biotech). The concentration of each stimulant was conducted at manufacturer guidelines unless otherwise stated.

PBMCs were seeded at a concentration of 1×10^6 cells per 100 μL into a 96-well plate. Either PepTivator or positive control was added to corresponding wells and mixed thoroughly. A negative control was conducted for each experiment by plating cells at the same concentration without addition of a peptide stimulus. Samples were incubated at 37 °C and 5% CO2 for 2 h. After 2 h, 1 μg/mL Brefeldin A was added to each well apart from the PMA + BrefA condition. Cells were then incubated at 37 °C and 5% CO_2 for a further 4–16 h dependent upon experiment.

1.1.5. IL-2 enrichment

PBMCs were enriched with IL-2 (Stem Cell Technologies) for up to 72 h at a concentration of 10 IU/mL. PBMCs were plated at 2×10^5 cells per 100 μL in a 96-well plate. IL-2 and the corresponding stimulant (PMA, Influenza or SARS-CoV-2) were added to their designated wells. PBMCs were then incubated at 37 °C 5% CO_2 for 72 h unless otherwise stated.

1.1.6. Intracellular cytokine staining

ICS was conducted using the PerFix-nc Kit and high fixation protocol (Beckman Coulter). Samples were analysed using the Navios flow cytometer. (See Table 2.)

1.1.7. PBMC fixation and staining

PBMCs were fixed onto clear glass slides using 100% ethanol and left to dry overnight. Post fixation, cell slides were stained with May Grunwald and Giemsa. PBMCs were reviewed and photographs under a light microscope at a x40 magnification.

1.1.8. Bio-Plex cytokine analysis

Supernatant samples required for Bio-Plex (Bio-Rad) multi-plex analysis were collected and stored at -80 °C until required. Analysis of supernatant was conducted for the following time points:

- Post 72-h IL-2 Enrichment
- Post second stimulation with corresponding stimulant (16 h)

Analysis of supernatant cytokine concentration was conducted using the 17-Plex (Bio-Rad) pre-coated plate multi-plex assay. The protocol followed was per manufacturer guidelines and detailed within the Bio-Plex Pro Cytokine, Chemokine, and Growth Factor Assays instruction manual.

1.1.9. Statistical analyses

Statistical analysis was carried out where sufficient data was

Table 2
Monoclonal Antibody Panel for ICS.

Monoclonal Antibody	Fluorochrome	Manufacturer	Product Code
ViaKrome405	Pacific Blue	Beckman Coulter	C36614
CD3	APC-AF750	Beckman Coulter	A94680
CD4	Krome Orange	Beckman Coulter	A96417
CD8	PC7	Beckman Coulter	6,607,102
CD137	ECD	Beckman Coulter	B76262
IFN-γ	PE	Beckman Coulter	IM2717U
TNF-α	AF700	Beckman Coulter	B76295
IL-17A	PerCP-Cy5.5	BioLegend	512,314
IL-10	AF488	BioLegend	501,411
Granzyme B	APC	BioLegend	372,204

collected using the GraphPad Prism 9.0 software. Statistically significant results are indicated, and the statistical test performed is stated. Statistical tests used include one way ANOVA, Kruskal-Wallis, Dunn's multiple comparisons test, Holm-Šídák's multiple comparisons test, Tukey's multiple comparison test and Mann-Whitney.

1.2. Results

1.2.1. Establishment of PBMC extraction and cryopreservation protocols

All results generated in 1.3.1 were from healthy control samples. To establish whether PBMCs could be successfully extracted via our proposed method, analysis of the extracts by the Cellular Immunology departments Immunophenotyping protocol was conducted (Fig. 1A).

Viability of PBMCs post thawing was observed with the percentage of 7-AAD negative cells. Viability assessment was conducted to optimise the time post thawing required and cell culture medium conditions (Fig. 1B). Lymphocyte viability was maintained across all conditions and was recorded between 83.55% and 99.80% post liquid nitrogen thawing. No statistical significance was detected between the viability of cells in differing post thaw timings and cell culture medium conditions. This allowed for the use of antibiotics to prevent cell culture medium contamination and the use of Benzonase Nuclease when thawing cells to prevent clumping.

Effects of anti-coagulant on cytokine output was examined by ICS post PMA + BrefA stimulation for 6 h. We compared cytokine detection in PBMCs extracted from either EDTA or Lithium Heparinised blood (Fig. 1C). Lithium Heparinised anti-coagulant blood was chosen as the anti-coagulant of choice due to a generally increased cytokine readout was recorded. Lithium Heparin was also chosen due to EDTA's chelation properties and the ability to diminish cytokine response if PBMCs are left in the anti-coagulant for >4 h.

1.2.2. PMA stimulation is effective in producing cytokine response

Upon successful optimisation of PBMC extraction and cryopreservation protocols, the establishment of a suitable positive control was conducted. All results generated from 1.3.2 were from healthy control samples.

PBMC PMA + BrefA stimulation was conducted for three different

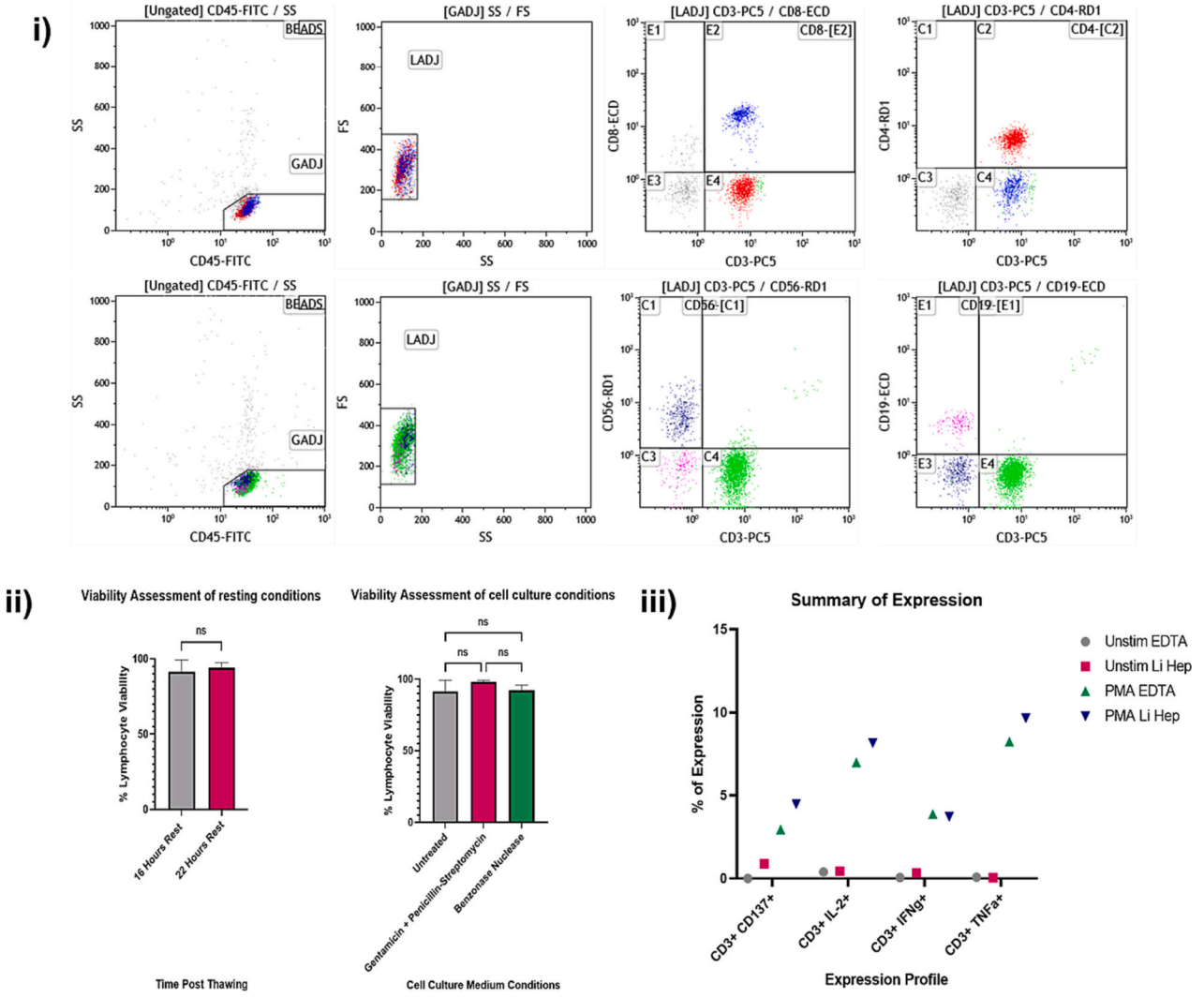

Fig. 1. (i) Detection of PBMCs from extraction protocol using flow cytometry lymphocyte immunophenotyping panel. All expected populations were detected including CD4+ T-Helper, CD8+ -Cytotoxic, CD56+ -NK and CD19+ —B lymphocytes, proving the successful extraction of PBMCs. (ii) Viability assessment of PBMCs thawed from liquid nitrogen storage by the use of the intracellular marker 7-AAD ($n = 8$). Cells which were dead would have an increase fluorescence of 7-AAD. The time post thawing was assessed and no significant difference in lymphocyte viability was observed. The cell culture conditions of antibiotics and addition of benzonase nuclease in washing post-thawing was assessed and found no statistically significant differences in lymphocyte viability by Kruskal-Wallis. (iii) Cytokine production analysis of differing blood anti-coagulant from which PBMCs were extracted showed a generalised increase in the activation marker CD137 and cytokine production within cells extracted from Lithium Heparin anti-coagulated blood. PBMCs were stimulated with PMA + BrefA.

concentrations, with 1× concentration being adopted from the manufacturers recommended protocol. Measurement of response was conducted via the percentage expression of CD137 and the expression of intracellular cytokines (Fig. 2A). Samples were stimulated in triplicate and pooled together for analysis to minimise error with regards to small stimulant volumes. An increased response in both CD137 and cytokine expression was observed with increased concentration of PMA + BrefA (Fig. 2B). The manufacturer recommended concentration was chosen as

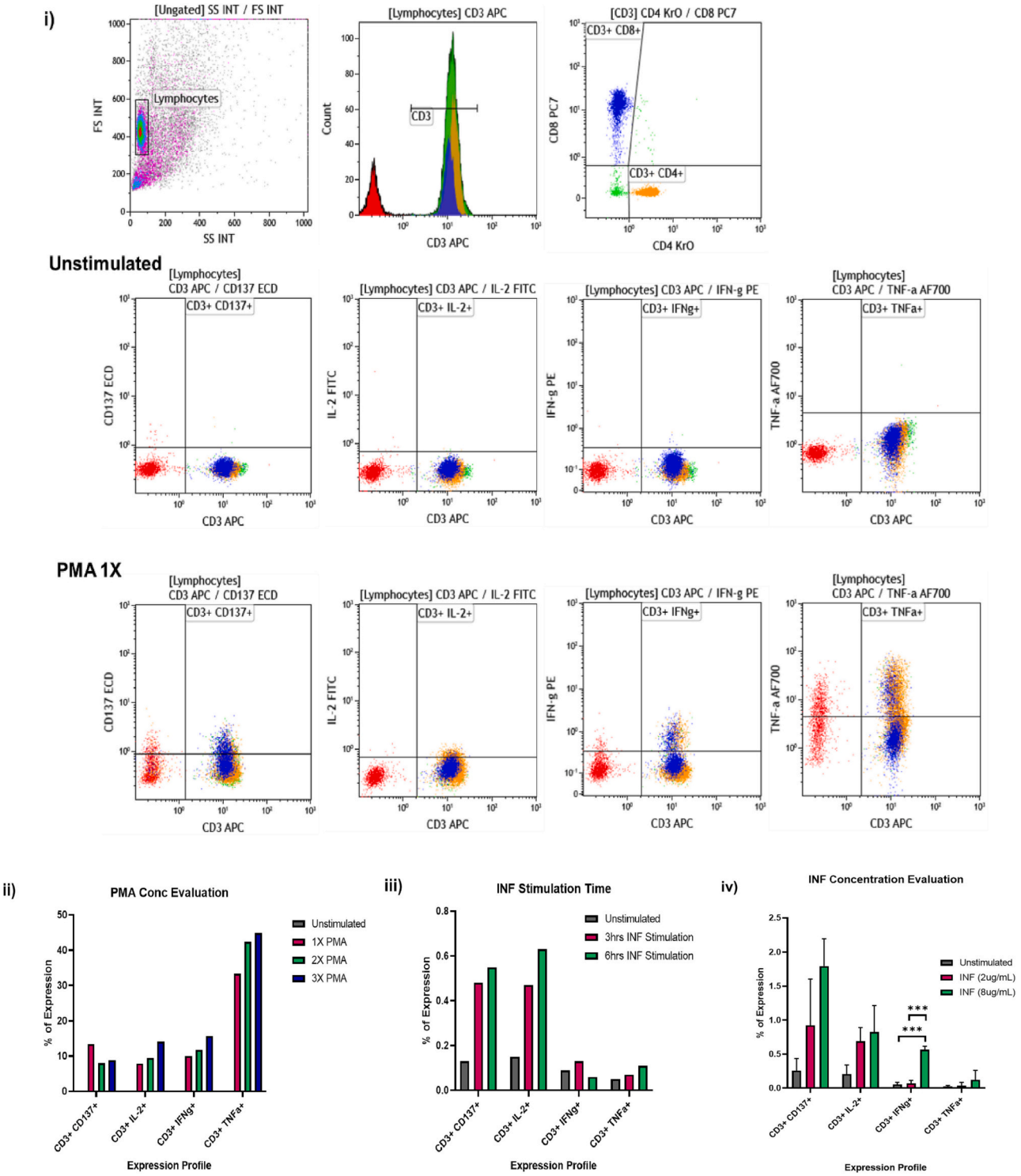

Fig. 2. (i) Flow cytometry gating strategy for observing lymphocyte cytokine production post PMA stimulation. Comparison plots between the negative unstimulated control and PMA 1× concentration are shown. An upregulation in CD137, IL-2, IFN-γ and TNF-α are observed in the PMA 1× condition. (ii) Concentration evaluation of PMA ($n = 1$). Increasing PMA concentration correlates with increasing cytokine detection in comparison to unstimulated control. CD137 is raised in all PMA conditions but appears highest in the PMA 1× condition. (iii) Evaluation of INF stimulation time (n = 1). PBMCs were stimulated with INF for either 3 or 6 h, with the greatest increase of CD137, IL-2 and TNFα observed at 6 h. IFN-γ production was highest at 3 h. (iv) Evaluation of INF stimulation concentration ($n = 3$). PBMCs were stimulated with INF at either 2 μg/mL or 8 μg/mL concentration for 6 h. An increase in CD137 expression and all cytokines was detected in the 8 μg/mL condition. Statistical significance by one way ANOVA followed by Holm-Šídák's multiple comparisons test was found for the IFN-γ condition ($p = 0.0001$).

an optimal response due to potential overstimulation and exhaustion of cells within higher concentration conditions.

Optimisation of responses to a viral positive control were assessed. The first optimisation experiment of responses to the Influenza PepTivator was in relation to stimulation time. PBMCs were stimulated with 2 μg/mL of Influenza PepTivator as per manufacturer guidelines. PBMCs were stimulated for either 3 or 6 h. PBMC responses again were measured by the expression of CD137 and intracellular cytokine expression (Fig. 2C). An increase in CD137 expression and cytokine production was generally observed in the 6-h stimulation condition, however only a very small percentage of cells recorded any expression. Following this, the concentration of Influenza PepTivator was evaluated. PBMCs were stimulated with either 2 μg/mL or 8 μg/mL of Influenza PepTivator for a total of 6 h (Fig. 2D). An increased response was observed in the 8 μg/mL stimulation condition, however the percentage of cells expressing CD137 and intracellular cytokines is still small. Statistical significance was detected by one way ANOVA analysis followed by Holm-Šídák's multiple comparisons test for the percentage of IFN-γ expressing cells in the 8μg/mL condition ($p = 0.0001$). A literature review was conducted for usage of Miltenyi PepTivators and it was noted that other groups had increased the stimulation time from 6 h to between 16 and 20 h, whilst using the recommended manufacturer concentration (Thieme et al., 2020; Anft et al., 2021; Demaret et al., 2020; Geers et al., 2021).

1.2.3. Stimulation of PBMCs with influenza and SARS-CoV-2 PepTivators changes the expression profile of patients

PBMC stimulation period was increased to 16 h for all conditions, following multiple published reports (Thieme et al., 2020; Anft et al., 2021; Demaret et al., 2020; Geers et al., 2021). The concentration of the PepTivators was returned to manufacturer recommendation to prevent excessive and overstimulation of the PBMCs. The gating strategy for ICS can be seen in Fig. 3A, which uses the PMA + BrefA stimulated cells as a strong positive control. Samples were stimulated in triplicate and pooled together for analysis to minimise error with regards to small stimulant volumes. This gating strategy was based off unstimulated negative control samples (Supplementary Fig. 1).

Stimulation with the Influenza PepTivator showed increased expression of CD137, IFN-γ and TNF-α in comparison to the unstimulated negative control (Fig. 3B). No statistical significance was detected by Mann-Whitney tests between these two conditions.

Stimulation with SARS-CoV-2 PepTivators was conducted for the Spike (S), Membrane (M) and Nucleocapsid (N) proteins on three different patients. Patient A had previous SARS-CoV-2 infection with the original strain seen in the UK (Fig. 3C). Patient B had received two doses of the BNT162b2 Pfizer-BioNTech COVID-19 vaccine. (Fig. 3D) Patient C had received three doses of the BNT162b2 Pfizer-BioNTech COVID-19 vaccine and had recent infection with SARS-CoV-2, with the variant being suspected as the B.1.1.529 Omicron variant. (Fig. 3E) A summary of the increases in expression can be seen in Table 3.

1.2.4. Enrichment of PBMCs with IL-2 increases CD137 expression

Despite increases in some cytokines observed in Fig. 3C, D and E, there was no substantial cytokine production recorded. This brought into question the precursor frequency of SARS-CoV-2 specific cells and whether peptide was being presented correctly via Antigen Presenting Cells (APCs). To increase precursor frequency of virally specific cells and potential cytokine responses, PBMCs were enriched with IL-2 and the peptide of the named condition for 72 h. A second stimulation with the named peptide was then conducted for a further 16 h without the presence of IL-2. SARS-CoV-2 peptides were combined into one stimulation condition to maximise the potential response (Fig. 4). Concentrations of 2 μg/mL and 20 μg/mL were used in both Influenza and SARS-CoV-2 stimulation conditions. Stimulation was conducted on PBMCs from a donor who had received two doses of BNT162b2 Pfizer-BioNTech COVID-19 vaccine and had previous SARS-CoV-2 infection. Within this experiment, the measurement of CD137 and IFN-γ expression was carried out via ICS (Fig. 4A and B). Expression of CD137 was induced in the PMA, Influenza and SARS-CoV-2 stimulation conditions. Alongside this, the expression of IFN-γ was induced in the PMA and Influenza conditions. SARS-CoV-2 expression of IFN-γ was negligible in comparison to the unstimulated control. The sample was stimulated in triplicate and pooled together for analysis to minimise error with regards to small stimulant volumes.

Total and lymphocyte viability analysis demonstrated a clear decrease in viability within the PMA condition and less pronounced decreases in all other conditions in comparison to the unstimulated negative control (Fig. 4C and D). The PMA condition uses a pre-made stimulation cocktail which also contains Brefeldin A and Monensin. It is possible the decrease in viability within this condition is due to Brefeldin A as within the literature it is not recommended that Brefeldin A is present in cell culture for >24 h due to cellular toxicity.

1.2.5. Extended Brefeldin A exposure negatively impacted cellular viability

To assess the potential effects of Brefeldin A upon the PMA + BrefA stimulation condition, a new condition of PMA without Brefeldin A was included in the stimulation protocol. PBMCs again were enriched with IL-2 and the corresponding peptide for the named condition for 72 h. A second stimulation of the corresponding peptide alone was conducted for 16 h post enrichment (Fig. 5). A clear decrease in both total (Fig. 5A) and lymphocyte (Fig. 5B) viability was observed in the PMA + BrefA condition in comparison to all other conditions.

Microscopic analysis of cells was also conducted (Fig. 5C). Monocytes and lymphocytes are present in the unstimulated, PMA and SARS-CoV-2 (2 μg/mL) conditions. In the PMA + BrefA condition cells appear much more sparse and cellular membranes less defined, which could indicate cell lysis and death.

1.2.6. Cytokine production increased at 72-h time point but still lacked TNF-α production

In light of a reduction in cellular viability, PMA + BrefA was removed as a condition of stimulation in the experiment. To assess the peak stimulation time for conducting cytokine detection, a three time-point stimulation experiment was conducted (Fig. 6). The initial enrichment of PBMCs with IL-2 was conducted at 24-h, 48-h, and 72-h. Following initial enrichment, PBMCs were exposed to their relative stimulant for a further 16 h without the addition of IL-2. The PBMCs used within this experiment were from Patient D, who was a critically ill COVID-19 in-patient 28 days post recovery.

Total leucocyte and lymphocyte viability were analysed using ViaKrome405 (Fig. 6A). Within the 48-h enrichment condition, there was a statistically significant total and lymphocyte viability decrease for all stimulation conditions detected by one-way ANOVA. However, both total and lymphocyte viability recovered within the 72-h enrichment condition. A decrease in total and lymphocyte viability for the PMA condition, statistically significant in the 48-h and 72-h conditions, was also observed within all three enrichment time conditions thus suggesting possible overstimulation of the cells with PMA.

With regards to cytokine production, again there was minimal detection by ICS. However, an increase within IFN-γ is observed within the 72-h enrichment condition and is characterised by a shift in the entire population within only this specific plot (Fig. 6B). No other increases of cytokine were recorded to this magnitude at any of the enrichment time points for SARS-CoV-2 stimulation (Fig. 6C). Statistical significance was detected in the 24-h enrichment condition for CD137, IFN-γ and TNF-α measurements for the PMA positive control condition as expected. An increase in CD137 expression was also observed within the 48-h enrichment condition, however no statistical significance was detected, and no obvious cytokine detection observed. Despite an increase in IFN-γ within the 72-h enrichment condition, unexpectedly there was still no significant intracellular TNF-α detection.

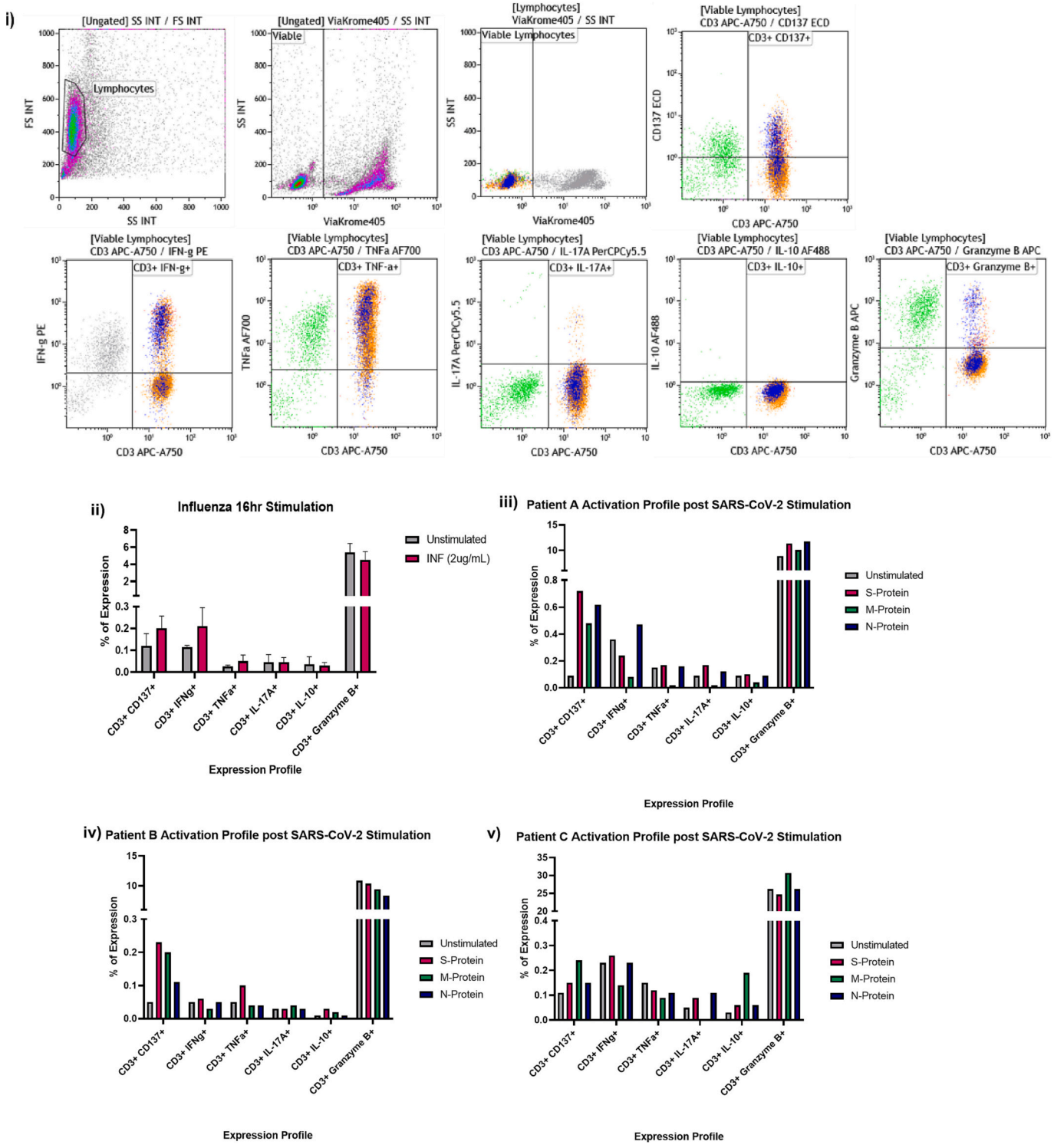

Fig. 3. (i) Gating strategy for ICS. Viable cells are distinguished through ViaKrome405 negative staining. Viable lymphocytes are distinguished through FS/SSC positioning and ViaKrome405 negativity. Condition shown in the gating strategy is PMA + BrefA stimulation positive control. (ii) INF 16-h stimulation appeared to show increase in CD137 and cytokine production besides Granzyme B, however results were not statistically significant by Mann-Whitney testing (n = 2). (iii) Patient A (natural infection, no vaccination) stimulation profile with S, M and N SARS-Cov-2 proteins (n = 1). Increases in CD137, IFN-γ and Granzyme B expression observed. (iv) Patient B (2× BNT162b2 Pfizer-BioNTech vaccination) stimulation profile with S, M and N SARS-CoV-2 proteins (n = 1). Increases in CD137 and TNF-α (S-protein only) observed. (v) Patient C (natural infection post 3× BNT162b2 Pfizer-BioNTech vaccination) stimulation profile with S, M and N SARS-CoV-2 proteins (n = 1). Increases in CD137, IL-10 (M-protein only) and IL-17A observed.

Table 3
Summary of the increased expression of patients within Fig. 3C, D and E.

Patient	SARS-CoV-2 Protein	Expression Increase
A	S-protein	CD137, Granzyme B (slight)
	M-protein	CD137, Granzyme B (slight)
	N-protein	CD137, Granzyme B (slight) and IFN-γ
B	S-protein	CD137, TNF-α
	M-protein	CD137
	N-protein	CD137
C	S-protein	CD137, IL-17A
	M-protein	CD137, IL-10
	N-protein	CD137, IL-17A

1.2.7. Bio-Plex analysis of supernatant reveals presence of cytokines

To investigate the immediate release or retention of cytokines post stimulation, analysis of cell culture supernatant was conducted using Bio-Plex technology. Analysis was conducted on the saved supernatant from the experiment in Section 1.2.5. Supernatant was saved from each sample post 72-h enrichment with IL-2 and post 16-h stimulation with relative stimulant. The cytokines present within the Bio-Plex and ICS panels were IFN-γ, IL-10, IL-17 and TNF-α (Fig. 7). All results were compared with age-matched healthy controls, for which results below the limit of detection were recorded for the majority of cytokines.

Detection of IFN-γ was observed in all conditions, with cytokine levels appearing to be increased within the Influenza and SARS-CoV-2 stimulation conditions in comparison to the unstimulated negative control. As expected, PMA and PMA + BrefA had a higher concentration of IFN-γ, with the latter recording the highest amount. (Fig. 7A).

IL-10 was detected within all stimulation and time conditions, however only the positive control wells appeared to have an increase in this cytokine in comparison to the unstimulated condition. Both Influenza and SARS-CoV-2 conditions appear similar in IL-10 concentration to the negative control. (Fig. 7B).

Positive detection of IL-17 was also observed within all conditions, however surprisingly cytokine levels within the PMA + BrefA, Influenza and SARS-CoV-2 conditions appeared similar or lower in comparison to the unstimulated control. The PMA control had a much greater concentration of IL-17 detected in comparison to all other conditions, which was consistent at both stimulation timepoints (Fig. 7C).

TNF-α supernatant detection was observed within all conditions, with the stimulation conditions, including Influenza and SARS-CoV-2 appearing to have a higher concentration in comparison to the unstimulated control. It also should be noted that the overall concentration detected in all conditions was lower post the 16-h stimulation in comparison to the initial 72-h IL-2 enrichment (Fig. 7D). This was largely observed in all cytokines detected, however most prominent in the

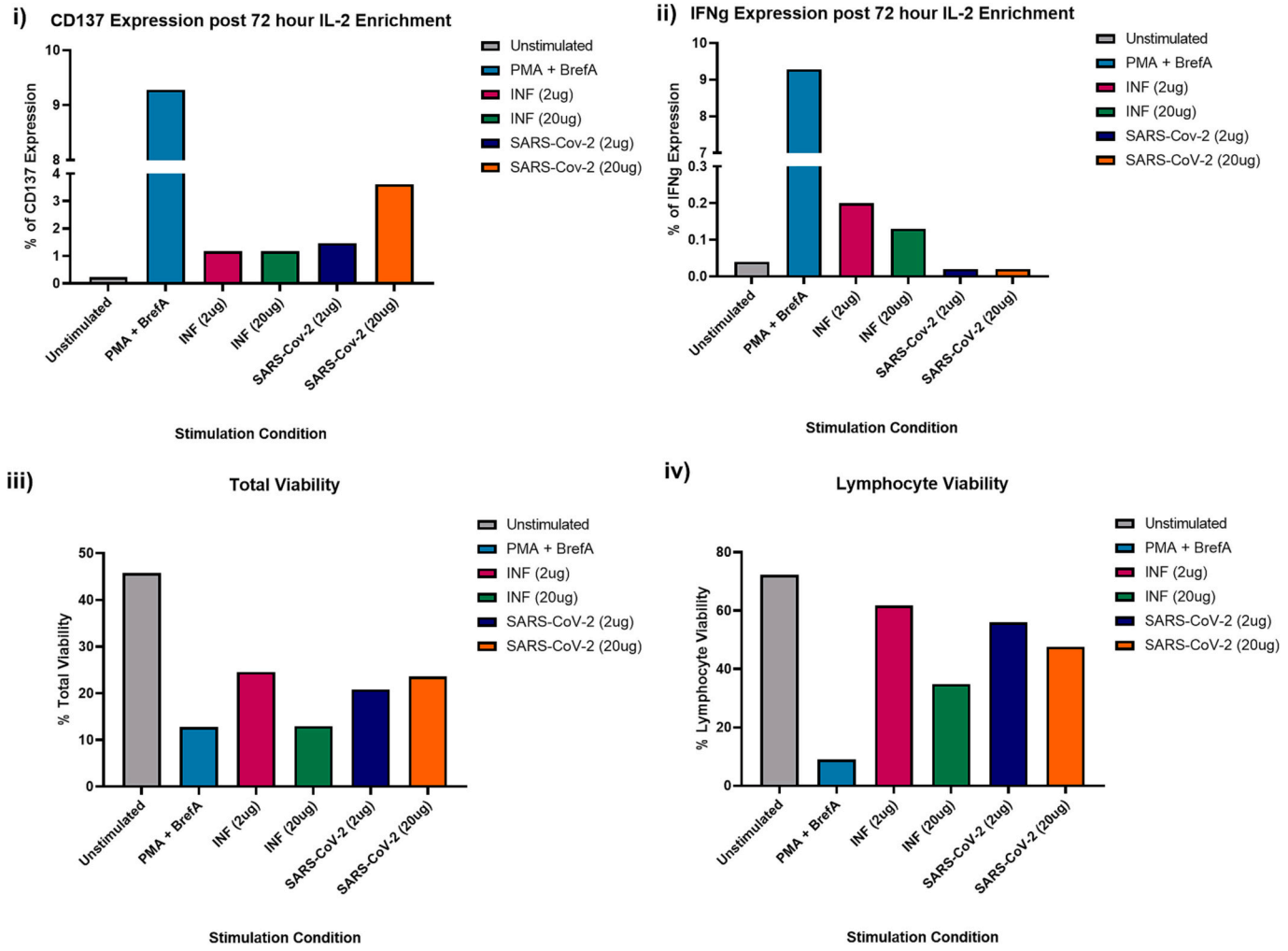

Fig. 4. (i) ICS analysis of CD137 expression post 72-h IL-2 enrichment and further 16-h stimulation ($n = 1$). An increase in CD137 was observed in all conditions in comparison to the unstimulated negative control. (ii) ICS analysis of IFN-γ expression post 72-h IL-2 enrichment and further 16-h stimulation (n = 1). An increase was observed in PMA and Influenza conditions. IFN-γ expression did not increase within the SARS-CoV-2 stimulation condition. (iii) Total viability assessed by ViaKrome405 fluorescence (n = 1). A decrease in total viability for all conditions observed, with PMA decreased the most. (iv) Lymphocyte viability assessed by ViaKrome405 fluorescence (n = 1). A clear decreased in PMA lymphocyte viability observed.

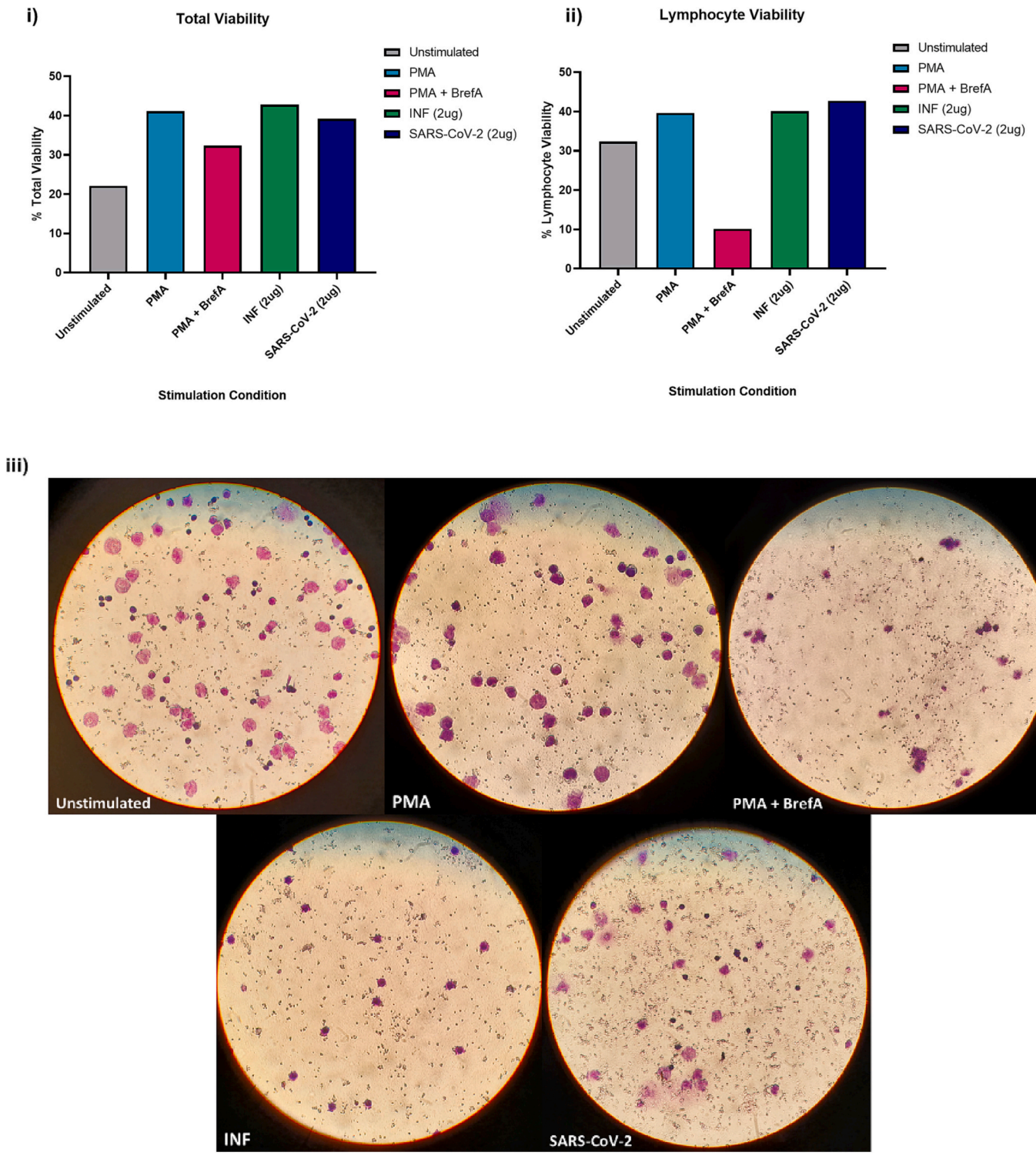

Fig. 5. (i) Total viability of cells assessed by ViaKrome405 ($n = 1$). All stimulation conditions appeared to have increased total viability in comparison to the unstimulated control. The PMA + BrefA condition had the lowest viability of the stimulation groups. (ii) Lymphocyte viability assessed by ViaKrome405 (n = 1). The PMA + BrefA condition lymphocyte viability was visibly much lower than all other conditions. (iii) Ethanol fixed cells were stained with May Grunwald and Giemsa stains and viewed at x40 magnification under a light microscope. PBMCs are much sparser within the PMA + BrefA condition and have disrupted cellular membranes in comparison to other conditions, indicating potential cellular death. Decrease in cellular volume could indicate apoptotic cellular death.

detection of TNF-α.

1.3. Discussion

Here we have reported a successful PBMC cryopreservation protocol which did not impact cellular viability and functionality post thawing. Within our data we have been able to show this through the successful stimulation of PBMCs with a variety of stimulants including PMA, Influenza and SARS-CoV-2 peptides. The monitoring and detection of cytokines is an underutilised technology; given the pivotal role of

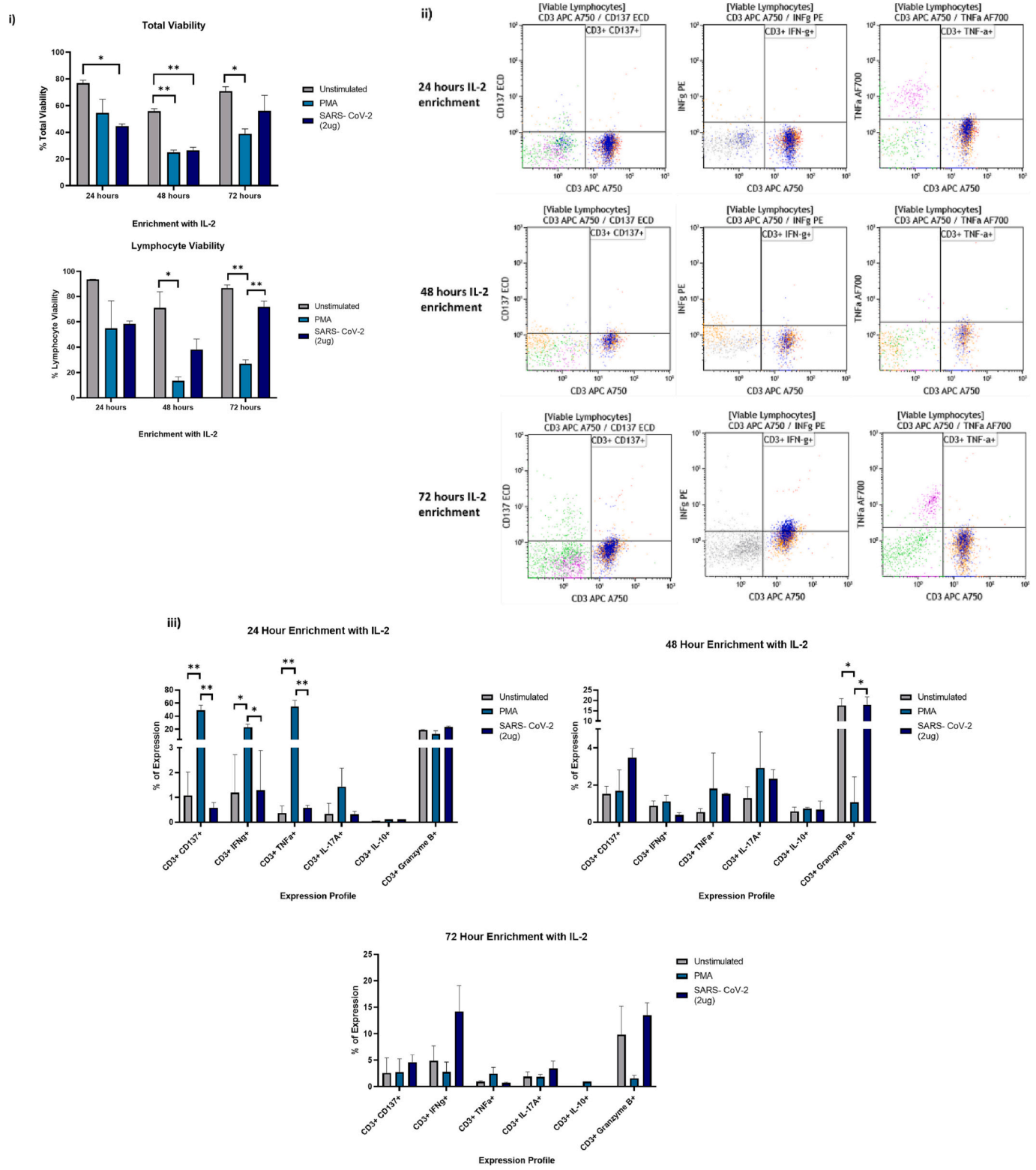

Fig. 6. (i) Total and lymphocyte viability assessment by ViaKrome405 for the IL-2 enrichment time points of 24-h, 48-h, and 72-h ($n = 2$). All statistically significant results were generated by ordinary ANOVA followed by Tukey's multiple comparison test. Decreased viability in comparison to unstimulated control was observed in all conditions and time-points. A generalised decrease in viability was observed at the 48-h enrichment time point for all conditions, including unstimulated. Statistically significant decreases in total viability (24-h $p = 0.0262$) (48-h $p = 0.0012$ and $p = 0.0014$) (72-h $p = 0.0435$). Statistically significant decreases in lymphocyte viability in PMA conditions (48-h $p = 0.0158$) (72-h $p < 0.005$). (ii) Flow cytometry plots of each enrichment time point for the markers CD137, IFN-γ and TNF-α (n = 2). A clear shift in the IFN-γ producing lymphocyte population is observed in the 72-h enrichment condition. (iii) Summary graphs of each enrichment time point condition and cytokine detection (n = 2). Statistically significant increase in CD137, IFN-γ and TNF-α were observed within the PMA positive control condition in the 24-h enrichment condition (p = 0.001 and $p = 0.0023$). Statistically significant decrease in Granzyme B expression was observed in the PMA condition at 48-h enrichment ($p = 0.0232$ and $p = 0.0218$). An increase in IFN-γ by SARS-CoV-2 stimulation occurred only within the 72-h enrichment time point condition.

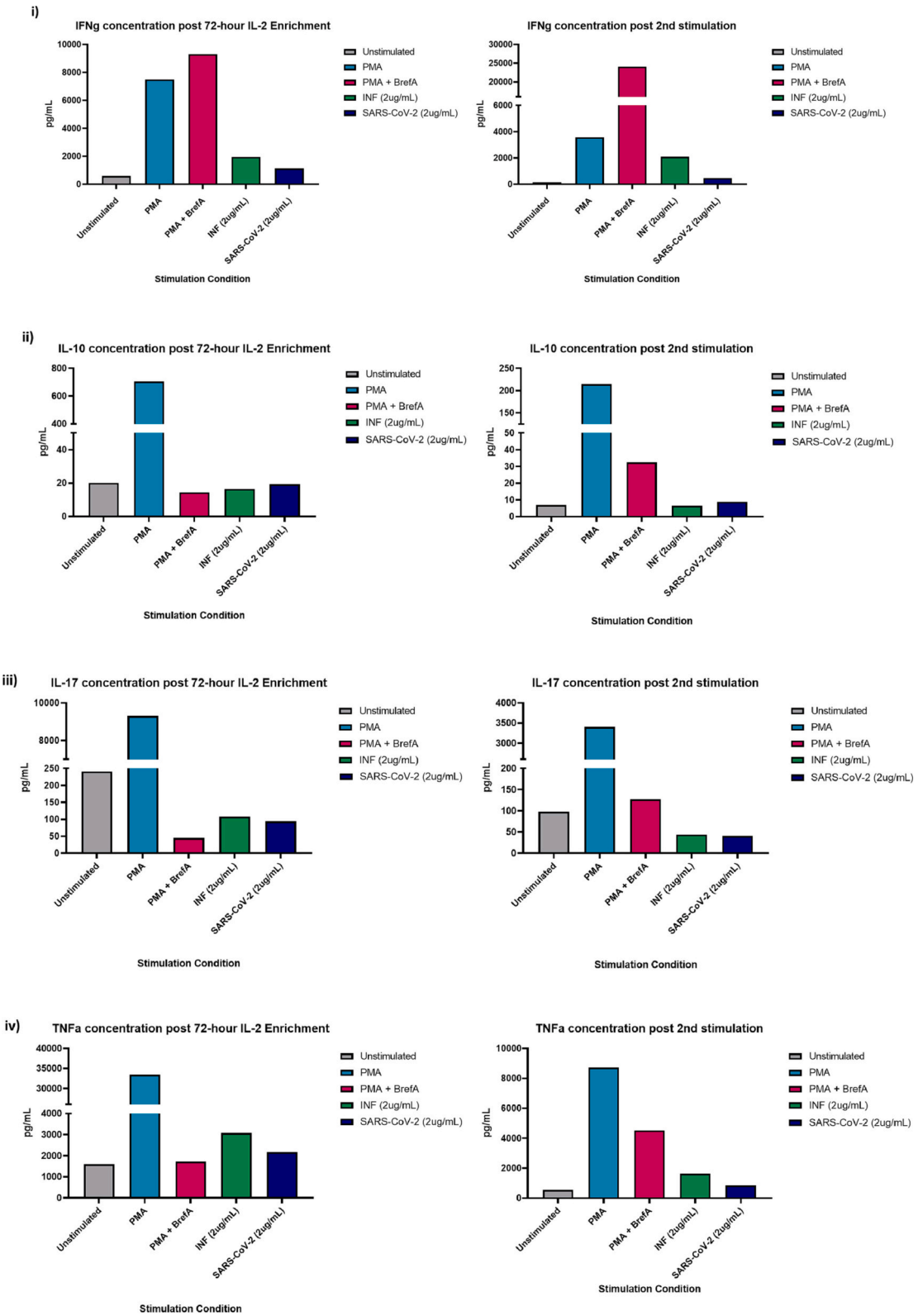

Fig. 7. Cytokine supernatant concentration analysed by Bio-Plex at post 72-h enrichment and post second stimulation. (i) IFN-γ supernatant concentration was increased in all stimulation conditions at both timepoints in comparison to unstimulated negative controls ($n = 1$). (ii) IL-10 supernatant concentration was increased in only the positive controls containing PMA in comparison to unstimulated negative controls at both time analyses (n = 1). (iii) IL-17 supernatant concentration was increased only in the positive controls containing PMA. The PMA condition had greatly increased IL-17 levels in comparison to all other stimulation conditions at both timepoints (n = 1). (iv) TNF-α supernatant concentration was increased in all stimulation conditions at both timepoints in comparison to the unstimulated negative controls. The concentration of TNF-α detected was higher in the post 72-h enrichment condition (n = 1).

cytokines in altering and controlling the magnitude of the immune response, the clinical impact of cytokine assessment could be far-reaching both in health and disease.

Cryopreservation of PBMCs was required to ensure that the protocol would be logistically manageable following implementation into the clinical setting. Establishment and optimisation of the PBMC extraction, cryopreservation and thawing protocols were successful. The optimal cell culture medium conditions included the use of the antibiotics penicillin-streptomycin to prevent bacterial contamination, and the use of Benzonase Nuclease to prevent clumping of cells whilst undergoing the thawing procedure. These additional factors were shown not to affect cellular viability and allowed for contamination and potential increased cell death through clumping to be avoided. Controlling these factors allowed us to minimise any non-specific activation when measuring cytokine output, thus strengthening our argument that responses generated were a result of antigen-specific cells.

Assessment of whether ICS could detect antigen-specific T-lymphocytes was conducted via altering the length of stimulation and concentration of Influenza PepTivator and measuring activation marker expression. The expression of CD137 and IL-2 increased in all conditions, whilst a statistically significant increase ($p = 0.0001$) in IFN-γ was detected in the 8 μg/mL stimulation condition only. Furthermore, minimal change was detected within the TNF-α measurements throughout all conditions despite increases in stimulant time and concentration. This prompted a wider literature review into the use of the Miltenyi PepTivators from published works. An updated stimulation protocol from Thieme et al., and Anft et al., extended the Miltenyi PepTivator stimulation time to 16 h (Thieme et al., 2020; Anft et al., 2021).

As per updated literature, an extended stimulation protocol of 16-h was used for the stimulation of PBMCs with SARS-CoV-2 peptides. Patients used in this experiment had either had exposure to the original strain of SARS-CoV-2 seen in the UK, received two doses of the Pfizer-BioNTech COVID-19 vaccine, or had received three doses of the Pfizer-BioNTech COVID-19 vaccine and had recent infection with suspected Omicron variant. An increase in CD137 was observed in all patients with all SARS-CoV-2 proteins, which was unexpected for Patient B who had only received vaccination and no natural infection. Interestingly, Patient A and Patient C also differed in their expression of cytokines however they were infected with different variants including the Omicron variant of SARS-CoV-2, and Patient C had received two doses of the Pfizer-BioNTech COVID-19 vaccine. The differing expression in cytokines could indicate altered cellular responses in relation to SARS-CoV-2 variants exposure and vaccine induced responses. However, in comparison to the literature, the cytokine responses generated were less intense, specifically expression of IFN-γ and TNF-α which had previously been reported by multiple groups (Peng et al., 2020; Sekine et al., 2020; Rodda et al., 2021).

In this study we wanted to assess cytokine output from viable cells rather than a response to cellular death. It was observed that there was an evident decrease in viability in the PMA + BrefA condition in comparison to all other stimulation conditions. To confirm the toxic effects of Brefeldin A on PBMCs, PMA without Brefeldin A was used as a comparison and revealed a distinct difference in lymphocyte viability compared to the PMA + BrefA condition. This could indicate that cellular death has occurred through the disruption of cellular membranes and thus correlates with viability analysis conducted via flow cytometric methodology. The potential cell death could have occurred through apoptotic mechanisms, as Brefeldin A has previously been reported to induce apoptosis via mitochondrial and death receptor pathways (Lee et al., 2013). To investigate this further in future studies, Annexin-5 and Propidium Iodide staining could be utilised to establish by what mechanism cellular death is occurring, whether it be apoptosis or necrosis, to further optimise cell culture conditions. We predict that we will identify apoptotic cellular death as our microscopic analysis appeared to show decreased cellular volume alongside membrane disruption, which is associated with apoptosis rather than necrosis (Cummings et al., 2004).

Further optimisation of protocol was conducted by altering the initial enrichment time of cells to ascertain whether optimal cytokine production was being missed. Interestingly, it was observed that both total and lymphocyte viability for all stimulation conditions decreased at the 48-h enrichment timepoint but recovered by the 72-h time point. We believe that at the 48-h time point we may have captured the cells within the cellular replication cycle, which would account for the decrease and subsequent recovery of both cellular and lymphocyte viability. Within the 72-h enrichment condition an increase in IFN-γ was reported in comparison to the unstimulated control for SARS-CoV-2 stimulated PBMCs. A clear shift in the cellular population can be observed within the flow cytometric plots in comparison to the other enrichment time points, indicating 72-h was optimal for IFN-γ production. IL-17A and Granzyme B production also appeared to be increased within the 72-h enrichment condition, whereas TNF-α and IL-10 have been consistently low in detection. The low detection rate for IL-10 could be attributed to the fact that this cytokine is produced and peaks later than cytokines such as IFN-γ and IL-2.

Analysis of cytokine levels in both the intracellular compartment and cell culture supernatant allowed us to establish whether the cells predominantly released cytokines immediately before the addition of transport inhibitors, or whether cytokine production was delayed and could be retained within the cell. We noted a predominance of cytokines immediately released into the surrounding matrix, even in conditions where transport inhibitors were present from the outset of stimulation. The immediate release of these cytokines could suggest as to why detection intracellularly of some cytokines, such as TNF-α, was difficult to observe. However, detection of an increase in these cytokines within the cell culture supernatant confirms our ability to measure the cellular inflammatory cytokine profiles of patients with previous SARS-CoV-2 exposure, as well as the response to other infectious agents.

Further clinical application of this technology may include patients suffering from connective tissue diseases, such as SLE and Sjögren's Syndrome. The detection of persistent dysregulation in cellular responses and cytokine production could lead to the identification of new therapeutic targets for patients with this immune dysregulation, whilst also providing another analytical methodology in confirming disease flares.

Monitoring of cytokines can also be utilised in predicting and identifying patients in the early phases of Graft vs Host Disease as cytokines such as IFN-γ, TNF, IL-2, IL-17 are elevated in this dysregulated inflammatory response (Hill and Koyama, 2020). Studies into solid-organ transplant recipients have reported data which suggests a specific immune 'activation profile' can be identified through innate immune cytokines, such as IL-17, IL-6, TNF-α and IP-10 (Karaba et al., 2022). Monitoring this 'activation profile' could also be applied to assessment of cellular vaccination responses, particularly in patients who do not mount a substantial humoral response or are known to be immunocompromised, may be useful. Patients who had higher levels of these cytokines pre-3rd COVID-19 vaccine were shown to be more likely to develop a higher antibody titres in comparison to other initial poor responders. Further studies into COVID-19 vaccine responses of immunocompetent individuals also identified systemic increases in IL-15, IFN-γ and IP-10 post first vaccination, which was then enriched by IL-6 and TNF-α post second vaccination (Bergamaschi et al., 2021). These transient increases in cytokines correlated with Spike antibody levels, thus highlighting the association of cytokine generation with successful vaccine responses. Monitoring of these cytokines may help us establish differing immune profiles of patients who respond adequately to vaccination versus patients who will require more immunological support. As vaccines continue to be developed, additional peptides may be required for the generation of an optimal immunological response. This platform would allow for additional peptides not seen in the original vaccine to be added and tested easily, thus future proofing the

technology for continued use and applications beyond Covid-19.

The role of cytokines in the pathogenesis of disease is a research topic of great interest. Recent reports have indicated that alongside autoimmune disorders, cytokine-mediated inflammatory disorders such as secondary HLH and macrophage activation syndrome can be induced post COVID-19 infection (Toor et al., 2020; Giamarellos-Bourboulis et al., 2020; Gautam et al., 2022). This highlights another potential opportunity for developing clinical strategies of disease prediction and implementing faster diagnoses and treatment responses for patients before they develop severe disease. In line with this, the assessment of cytokines, such as IFN-λ1 and IFN-β, could be undertaken to aid our understanding of Long Covid and help to establish whether the immune profile of Long Covid patients differs to that of patients who have fully recovered form SARS-CoV-2 infection.

Interestingly, autoantibodies against cytokines, in particular type I IFNs, have recently been described to be present in 10.2% of severe COVID-19 patients and in 0% of non-severe COVID-19 cases (Bastard et al., 2020). Autoantibodies against other cytokines, including IL-17A/F and IL-6, have also been described in multiple infectious cases of Chronic mucocutaneous candidiasis and staphylococcal infection respectively (Puel et al., 2022). The identification and monitoring of anti-cytokine autoantibodies could be useful in highlighting patients who may be more susceptible to severe infectious diseases. In relation to COVID-19, clinical follow-up of patients positive for anti-cytokine antibodies may be an interesting avenue of research to establish whether there is any association between the presence of anti-cytokine autoantibodies and development of Long Covid observed. Research into the persistence of cytokines, and presence of anti-cytokine antibodies, could help establish whether differing immune profiles are associated with various inflammatory disease states. This assay platform offers a new angle for immune monitoring, focussing on T lymphocytes responses. The identification of differing immune profiles via T lymphocyte responses could determine underlying mechanisms of disease persistence in these patients and highlight potential therapeutic targets.

Commonly used anti-cytokine therapies for SLE in use include Anakinra (anti-IL-1) and Tocilizumab (anti-IL-6), however anti-TNF-α therapy has been controversial with regards to treatment for SLE due to this cytokine's dual role in the condition (Aringer and Smolen, 2012). TNF-α blockade has been shown to be effective in other chronic inflammatory conditions (Taylor and Feldmann, 2009) however this therapeutic strategy has previously induced increased titres of both antiphospholipid and ds-DNA autoantibodies in SLE patients (Aringer et al., 2004). The targeting of this cytokine is continually discussed with other literature reporting safety and efficacy of Infliximab within SLE patients (Uppal et al., 2009), further fuelling the debate of this controversial cytokine treatment. Further research using a variety of cohorts, such as a range of autoimmune patients, cytokine-mediated diseases, long covid patients and those undergoing CAR-T therapy should be conducted to evaluate the potential of the cytokine measurement platform as a diagnostic test.

1.4. Conclusions

In this study we have demonstrated the ability to measure cytokine output from peptide stimulated T cells. These results indicate that peptide stronger stimulation of PBMCs can successfully occur via the use of SARS-CoV-2 and Influenza Miltenyi PepTivators. This platform offers an alternative target population for monitoring responses to peptides, in the form of T lymphocytes and cytokines as opposed to traditional antibody measurement assays. Clinical scenarios which could benefit from this methodology include the assessment of long covid and continued monitoring of responses to vaccines. This platform can be easily adapted to changes in vaccine composition, thus ensuring its applicability to future vaccination programmes.

Author contributions

JVT generated the data, performed the analysis and wrote the manuscript. EC and AWR conceived and designed the project. All authors reviewed the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2023.113556.

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