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An adaptable *in vitro* cytokine release assay (CRA): Susceptibility to cytokine storm in COVID-19 as a model

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ABSTRACT

Translational *in vitro* models such as cytokine release assay (CRA) are essential to assess the susceptibility to cytokine storm or CRS in a non-interventional manner in a human *in vitro* laboratory setting. Such models are also helpful to unravel disease mechanisms, to study the effects of new therapeutics and vaccines thereon and to diagnose or monitor diseases. Such assay will be important in predicting, planning and preparing for hospital intensive care units that are needed during the course of a pandemic. We present a CRA that can be adapted for assessing acute cytokine release risk against viral antigens, and potentially be used for cytokine storm simulation in viral infection outbreaks. We have used SARS-CoV-2 antigens and COVID-19 as a model. The assay can be challenged by changed or mutated forms of a virus in follow on waves of the epidemic and it can easily be modified for other future pandemics. We show that the membrane protein of SARS-CoV-2 is playing a major role in cytokine release (CR), mainly that of IL-6, IFNγ, TNFα and IL-8, that may be associated with COVID-19. These results are in agreement with recent clinical findings and new vaccine designs.

1. Introduction

Cytokine storm (CS) is a severe to fatal condition caused by infectious pathogens or drug compounds mediated by immune response, the mechanism of which is not yet fully understood and which was recently seen in COVID-19 patients ((Moore and June, 2020; Ragab et al., 2020). The underlying pathophysiology and clinical readings of CS or cytokine release syndrome (CRS) in COVID-19 is not yet well characterised (Mahmudpour et al., 2020; Shah et al., 2020). During the COVID-19 pandemic there has been a lack of safe and translational experimental models to predict a tolerance or progression into CS or CRS in asymptomatic or mildly symptomatic patients. Such models could aid in a better understanding of the disease mechanism, and in the development of new therapeutics and vaccines. *In vitro* models are essential to support risk-free research and testing of contagious agents like SARS-CoV-2. Our objective was to test the adaptability of an in-house *in vitro* CRA into assessing CR with reference to individuals being low or high responders using SARS-CoV-2 antigens as a model, with the possibility of developing a prototype for a CS susceptibility screening assay. The aim is to develop a simplified and safe non-interventional *in vitro* human assay that can be instrumental in diagnosis and research of serious infectious disease and vaccine development. CRA is widely applied in preclinical drug research but, if modified, may also have the potential to assess the risk of viral antigens to trigger excessive levels of cytokine release *in vivo* (Finco et al., 2014).

We present *in vitro* data of cytokine release against SARS-CoV-2 antigens of healthy donors compared with a COVID-19 CS survivor (post-recovery). This assay is predictive of CR-associated risk in individuals who may be susceptible to CS or CRS, and can be challenged by mutated viral segments or peptides or drug compounds and also adapted for mechanistic studies in a cell line model.

2. Materials and methods

2.1. In vitro study

An in-house (proprietary) liquid-phase CRA based on cell lines (consisting of TALL-1, THP-1, BALL-1, KHYG-1 and Jurkat cells) developed by Immundnz and a PBMC-based CRA (methods adapted from protocols described in Vessillier et al. (2015) and Eastwood et al. (2010)) were used to test cytokine release (CR) against SARS-CoV-2 antigens. The cell line CRA has the advantage of using isolated cell

Abbreviations: CRA, Cytokine release assay; CS, Cytokine storm; PBMC, Peripheral blood mononuclear cells.

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populations and combinations of selective leukocytes at selective stage of differentiation or maturity to assess cell-specific functions in target immune pathways. Human PBMCs from healthy volunteers (N = 35) were used of which frozen PBMCs (No. 1–11, unvaccinated against COVID-19, male = 5, female = 6) were purchased from BioIVT (UK) and PBMCs (No. 12–35, male = 13, female = 11) were isolated and frozen from blood of COVID-19-vaccinated internal staff and volunteers at ages between 18 and 61. Blood was collected with the help of a certified phlebotomist and with signed donor consent. Different fragments of the S (spike protein) and M (membrane) regions of the SARS-CoV-2 virus in the form of full length (f) or pooled peptides (pp) were applied (1 μg/ml). Phytohaemagglutinin (PHA, 10 μg/ml) was used as a positive control for CRA. Established cells or PBMCs were cultured in a CRA at 1 × 10^6/ml density for 18–24 h followed by analyses of supernatants and cells by ELISA and flow cytometry (FC) respectively. Cytokines known to be associated with CS/CRS such as IL-6, IFNγ, TNFα and IL-8 were measured. Flow cytometry was performed using a Guava system (Luminex, USA) and cells were gated on forward and side scatter and cell markers CD3, CD56, CD11c or CD19.

2.2. Reagents

Full length or large protein sequences and peptide forms of S protein and M protein were sourced: CovS 819–919 (S-819), CovS 679–833 (S-679), SARS-CoV-2 Spike full length G614 (S-G614) and M protein from Proteogenix (France) and Peptivator Prot_S+ (aa689-895) and Peptivator Prot_M (pooled peptides of full length M) from Miltenyi Biotec (UK). PHA, cell culture medium and reagents were acquired from Sigma

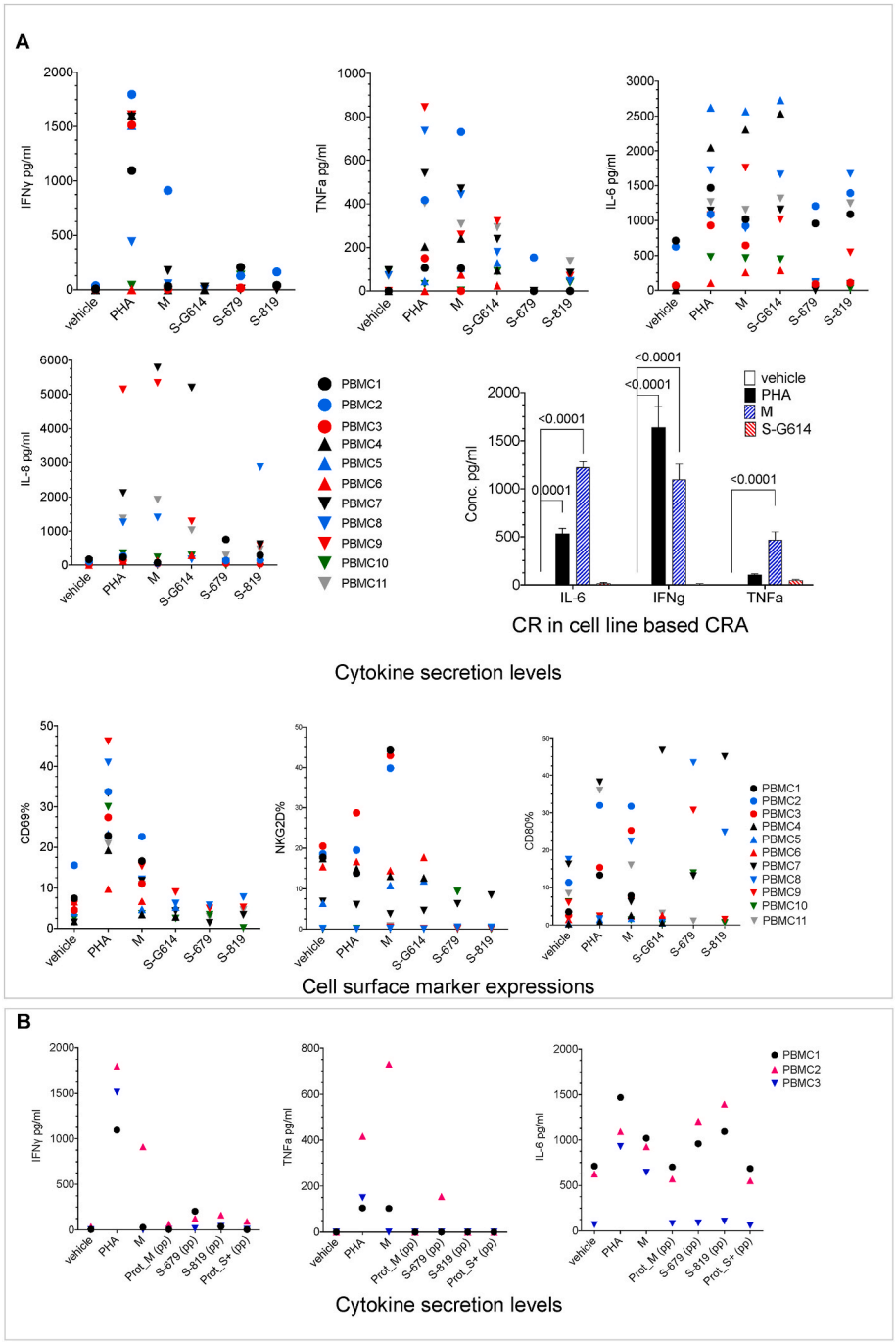

Fig. 1. SARS-CoV-2 antigens can induce CR in an *in vitro* human PBMC or cell line based CRA model (combination of different immune cell lines). (A) Secretion levels of IFNγ, TNFα, IL-6 and IL-8 and cell surface marker expression of CD69, NKG2D and CD80 against treatment with vehicle (cell culture medium with water), PHA, full length or large sequence of S and M proteins in PBMCs and human cell line CRA model; (B) Secretion levels of IFNγ, TNFα and IL-6 against full length or pooled peptide sequences of S and M proteins in PBMCs, pp refers to peptide/pooled peptide or large protein sequence. Bars represent Mean ± SE.

Aldrich (UK). Antibodies for ELISA and FC were acquired from Bio-Legend (UK). For more details, please contact the authors.

2.3. Data analysis

To perform statistical analysis on the collected data, the results were combined in Prism 8 (GraphPad) containing all samples and variables. These variables consist of all readouts and treatments. 2-way ANOVA and Tukey's multiple comparison test were performed to identify significant differences between groups.

3. Results

3.1. SARS-CoV-2 antigens can induce CR in an in vitro PBMC model

An in-house cell line based liquid-phase CRA responded against the M protein but not the S protein of SARS-CoV-2. In the model adapted to PBMCs, healthy donors (PBMCs 1–11, frozen) responded with varying degrees of secretion of IFNγ, TNFα, IL-6 and IL-8, indicating to high and low responders. There was more secretion of IFNγ, IL-6 and IL-8 and the levels varied among the PBMCs (donors) that responded for CR (Fig. 1A). IL-6 and IL-8 were more abundant in majority of the responding PBMCs. The PBMCs were exposed to different regions of the SARS-CoV-2 sequence, to asses which region was effective in stimulation. The M protein was more potent than the S protein. S-G614, the larger sequence of S protein (with D614G mutation, important variant during the COVID-19 outbreak), was more potent than the smaller S-679 and S-819 sequences. The CR pattern was different from the PHA-induced pattern in some responders, implicating that SARS-CoV-2 antigens possibly activate a different pathway.

In the cell line model, an in-house assay that exhibits consistent IL-6 and IFNγ secretion against PHA, the M protein induced high levels of IL-6, IFNγ and TNFα, while the S protein generated no such response. This model also shows a $CD8^+$-dependent pathway and NK cell involvement for the CR ($CD8^+$, $CD4^+$, $CD56^+$, $CD19^+$, $CD11c^+$, $CD11b^+$, $CD14^+$ cells used in combinations, data not shown).

3.2. SARS-CoV-2 M protein induces CR more potently than S protein

The interaction of proteins with and the impact on immune cells can vary significantly depending on whether the protein is in whole/large form or small peptide form. The binding or internalisation of viral fragments by immune cells initiating intracellular signalling can be very different with different downstream effect depending on protein size. PBMCs of donors (PBMC 1–3, frozen) were exposed to full length, large fragment, peptide or pooled peptide sequences of S and M proteins. While IL-6 release was more generally stimulated by all antigens, IFNγ and TNFα were only stimulated by full length M protein, but not by full length pooled peptides (M-f-pp) of M protein (Fig. 1B). Some donors responded to the M protein with an increased percentage of $CD69^+$ and/or $NKG2D^+$ cells (similar in cell lines, data not shown), indicating a role of T cells and/or NK cells, while a larger number of donors responding to M protein and other antigens showed an increase in $CD80^+$ percentage, a possible role of macrophages (Fig. 1A). We found the smaller fragments or peptide forms of the sequences to be more inducive for certain cell surface marker expression or cytokines, such as CD80 (Fig, 1A) or IL-6 (Fig. 1B), than the full-length protein sequences. This may be important in understanding how immunogenic particles differentially impact cytokine release and cell surface expression in CS or CRS by binding and initiating pathways differently and to what extent the two responses may be correlated. It is not clear whether such extracellular binding of peptides may be associated with downstream signalling that affects cytokine release.

3.3. Multiple cytokines are secreted by COVID-19 CS recovery donor

Fig. 2 shows the cytokine secretion profile of PBMCs against the M protein. The PBMCs of a severe COVID-19 surviving patient (PBMC-34) who was diagnosed with and had recovered from CS several months prior to blood collection and was COVID-19-negative at the time of the sample collection, produced very high levels of all 4 cytokines when stimulated with the full length M protein (Fig. 2B). We have considered this donor as a positive control for CS. Among the healthy donors (non-COVID, vaccinated), only 2 (PBMC-17, -24) out of 28 (note: PBMCs of 6 donors did not respond to CR in the assay) showed high secretion of IFNγ, TNFα, IL-6 and IL-8 at the same level. The overall secretion profiles of the cytokines were similar to secretion profiles of PHA-induced stimulation (Fig. 2A) and did not vary between unvaccinated (PBMC 1–11) and vaccinated (PBMC 12–35) donors (Fig. 2B).

4. Discussion

The cell line CRA is a potential model to test general and cell-specific response against proteins and particles that can be immunogenic, which could prove useful in mechanistic studies. Our preliminary data show that the *in vitro* CRA is able to test human PBMCs for cytokine release against various forms of SARS-CoV-2 antigens, as a working model, and can differentiate high, low and non-responders. If the high responders and their secretion pattern correspond with individuals who are prone to a CS or CRS in the actual event of infection then this *in vitro* model could prove to be a potential simulation or pre-screening method in a category 2 biosafety level laboratory for highly infectious agents such SARS-CoV-2 and their mutant forms. Cytokine pathways will vary among individuals depending on T cells, macrophages or NK cells being involved. The low- or non-respondent group may correlate with individuals who have a high tolerogenic or a very weak immune profile, the latter also being susceptible to acquiring a high infection by the agent.

The donors included in this study were COVID-19 negative, with no known case of previous CS or CRS. We assume that the PBMCs of the responders were naïve to the SARS-CoV-2 M protein and that any response may not have resulted from immunologic memory specific to this region. The exception was the donor (PBMC-34) who had previously suffered COVID-19 cytokine storm diagnosed with very high levels of IL-6, CRP and ferritin as well as other inflammatory markers, and was rescued from cytokine storm with anti-IL-6R monoclonal antibody treatment. The current *in vitro* test showed very high levels of all four cytokines in response to the M protein. Given the extreme difficulty in getting a COVID-19 CS patient or survivor, we have considered this PBMC as the positive control for CS in our study. Noteworthy are two donors (PBMC-17 and PBMC-24) who showed very high secretion of all four cytokines at the same threshold. It was not in the scope of this study to test whether the responders would respond positively for CR when and if they acquired a COVID-19 infection. The fact that all donors, including those vaccinated (immunised against the S protein), have shown secretions of cytokines in response to the M protein, with 3 vaccinated donors secreting very high levels of all four cytokines, suggests that vaccination was not a factor in the cytokine levels. This assay can be extended to measure a larger panel of cytokines to determine the cytokinetic profile of a responder and response of patients who have recovered from a severe COVID-19 infection. In addition, the assay performs very well with frozen cells making it suitable for cryo-preserved specimens, a convenient feature in experimentation and surveillance studies.

Each cytokine is associated with a particular pathway and a number of cytokines may be involved in an immune response resulting from, or causing an overlap of, a combination of pathways. The type of immune cells activated for the secretion of cytokines may vary from one individual to another depending on their genetic make-up and immune profile. This is evident in our model as we find responding individuals exhibiting different patterns of cytokine secretion when exposed to the

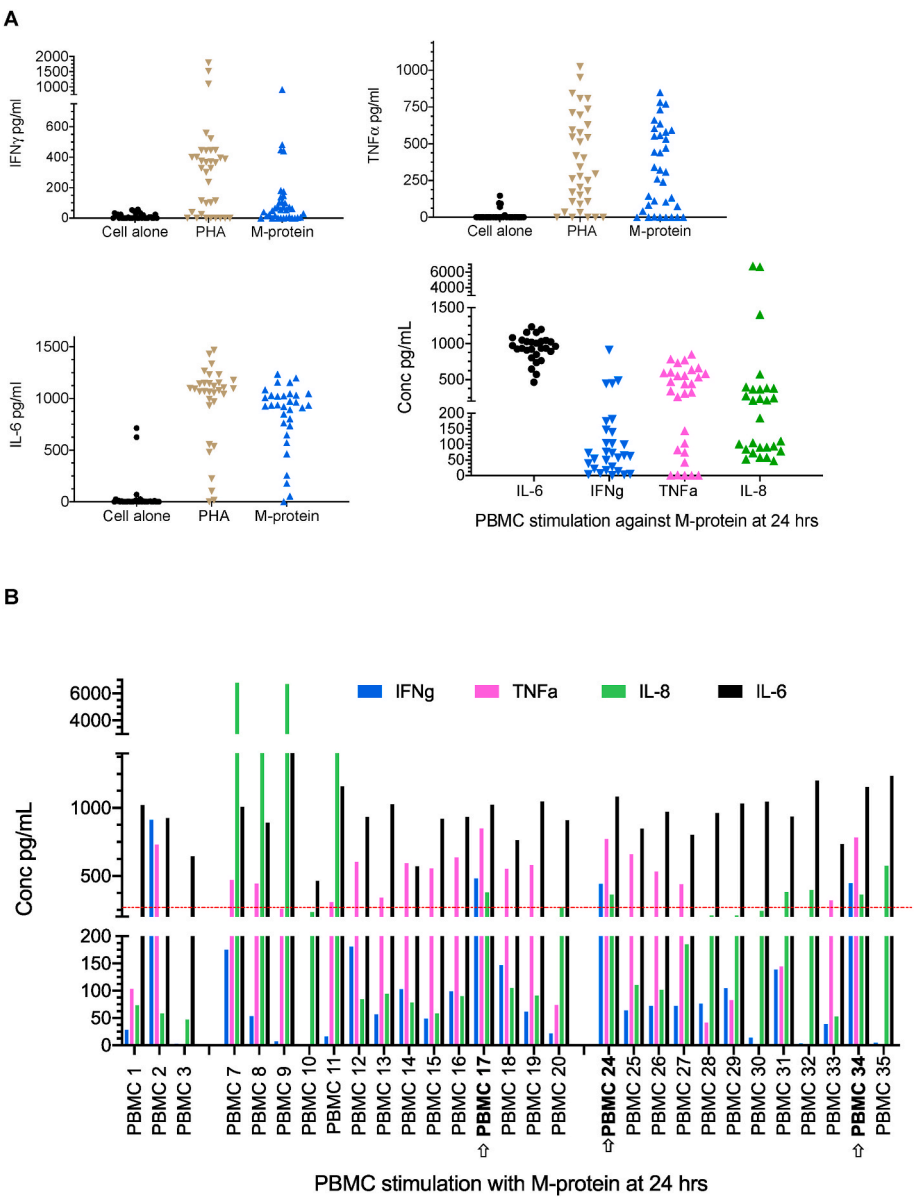

Fig. 2. Cytokine release profile of PBMCs from COVID-19 negative healthy donors on stimulation with M protein of SARS-CoV-2. (A) Secretion of IFNγ, TNFα, IL-6 and IL-8 in response to stimulation by PHA and M protein; lower right panel showing dot graph of cytokine secretion of all donor PBMCs against stimulation by M protein; (B) Bar graph of comparative cytokine secretion profile of all donor PBMCs against stimulation by M protein, cytokine storm control donor PBMC-34, red line marked as threshold based on control donor. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

same viral antigen. The response also varies among individuals factored by which region of the virus sequence is involved, hence in most responders the M protein is immunogenic and in only few the S protein causes a strong stimulation for cytokine release. The assay reveals that in a SARS-CoV-2 *in vitro* model the M protein is most potent in stimulating a CR and may be the major region involved in cytokine storms of COVID-19. Globally, people have been vaccinated against COVID-19 with formulations involving the S protein, and there have been little or no known adverse cytokine release reactions among recipients which is in line with our findings.

The CR patterns in this *in vitro* study showing increased levels of IFNγ, IL-8 and in particular, IL-6 are similar to cytokine data in clinical studies of COVID-19 (Chen et al., 2020; Paces et al., 2020; Varchetta et al., 2021; Del Valle et al., 2020). From the supporting cell line-based CRA data, we may interpret that the source of IL-6 secretion are monocytes, but also NK cells potentially, referring to an active role of these cells in responders. Importantly, IL-6 is a generally secreted inflammatory cytokine that might not be the best or minimal parameter to differentiate a patient's clinical data for cytokine storm. Noteworthy also, the assay shows that the cytokine secretion pattern among some of the responders may not correlate with the CR against PHA in some individuals, indicating that SARS-CoV-2 antigens and PHA invoke different pathways in these cases, the dynamics of which may be different. A screening study of a large number of donors with the inclusion of parameters such as age, sex, ethnicity and existing conditions, to unravel the patterns of these cytokine pathways among potential responders would be beneficial for future stratification and personalised medicine in COVID-19 or similar outbreaks.

The upregulation of cell surface receptors such as CD69, NKG2D and CD80, usually associated with activation of immune cells, by peptide or full length forms of SARS-CoV-2 sequence, were differentially associated with CR. This could be due to a different time-point of cytokine secretion, or that the full length or larger proteins that have caused CR are affecting the PBMCs in a mitogenic manner rather than immunogenic manner, similar to how PHA and anti-CD3 antibody (OKT3) induce PBMCs. The effect is most probably innate and less likely to be adaptive as there are no or inadequate antigen presenting cells (APCs) involved in the cultures that have caused CR. From our cell line based CRA using immune cell populations through exclusion strategy, CRA using frozen primary PBMCs and our experience with APCs we have learned that T cells, NK cells and monocytes are critically involved, and that active APCs are not involved in CRA. The increase of IFNγ, TNFα, IL-6 and IL-8

levels implicates a tendency toward a cytotoxic immune response and the involvement of T cells, NK cells and macrophages, which is also supported by a strong presence of $CD8^+$ T cells ($CD8^+$ and/or $CD4^+$ populations used in cell line CRA, flow cytometry data, not shown) in the elevated cytokine readings. Further mechanistic studies in conjunction with the current CRA model may be carried out to determine the interplay of different immune cells and cytokine signals involved in SARS-CoV-2 mediated CR. Unraveling whether the CR involved in COVID-19 is an IFNγ, TNFα or IL-6 or IL-8-mediated pathway or a combination of these, and that it may vary in patients, would be important to know to target drug treatments and develop vaccines. In most PBMCs there is an increase in IL-6 and IL-8 upon treatment with the SARS-CoV-2 antigens, especially the M protein. The differentiator seems to be IFNγ that was secreted in high amount in only a few samples that also co-secreted the other cytokines, suggesting that IFNγ could be most important in COVID-19 CS.

While the membrane region was most effective in inducing CR, the spike region was also able to generate a cytokine response in some donors. This may be useful in designing vaccines and in treating recipients of vaccines who may respond adversely. An important feature of the *in vitro* model is that any form of the SARS-CoV-2 sequence can be used to challenge donor PBMCs to test for a potential CR and this can include any mutated sequence of the virus. The assay also showed that PBMCs of some donors may not respond to control stimulations, a point to be noted when designing *in vitro* assays based exclusively on primary PBMCs.

This model can be applied generally as an *in vitro* susceptibility screening test for CRS and potentially, cytokine storm in asymptomatic and symptomatic subjects. In an ongoing COVID-19 and SARS-like infection surveillance, an ideal validation study for such application would be to screen asymptomatic and COVID-19-negative (tested by PCR) donors for CR and follow-up if any of the donors acquire severe COVID-19 infection with a cytokine storm. The assay can be adapted to other scenarios in which CS is involved, such as sepsis and other viral infections. A next step in our research is a validation study for the CRA in which a larger number of cryo-preserved PBMCs of pre-COVID-19 donors and post-recovery state patients who have severely suffered from COVID-19-associated CS are tested for CR against SARS-CoV-2 antigens.

5. Conclusions

We introduce an adaptable *in vitro* human cytokine release assay, a protoype for assessment of cytokine storm risk, with COVID-19 as a model. The assay is interchangeable between cell lines and PBMCs and reveals that it can differentiate cytokine release patterns and cell surface markers induced by specific proteins or peptides of an agent and determine the most potent inducing region. The *in vitro* cytokine data are comparable with clinical cytokine data, in this case in a COVID-19 model. Further studies may prove this assay to be useful as a safe *in vitro* pre-screening assay for susceptibility to cytokine storm induced by virulent contagious microbial agents.

CRediT authorship contribution statement

Masih Alam: Methodology, Investigation, Formal analysis, Visualization, Project administration, Writing – original draft. **Rawshan Choudhury:** Methodology, Investigation, Validation, Writing – review & editing. **Robert-Jan Lamers:** Conceptualization, Funding acquisition, Formal analysis, Writing – original draft, Supervision.

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.

Data availability

Data will be made available on request.

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