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Short communication

Patients with moderate to severe COVID-19 have an impaired cytokine response with an exhausted and senescent immune phenotype

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Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), is asymptomatic or mild in most but leads to moderate or severe disease requiring oxygen therapy in approximately 20% with symptomatic infection (World Health Organization. COVID-19 Clinical Management. Living Guidance. World Health Organization;, 2021). Mortality is associated with age and comorbidity including diabetes and hypertension (Zhou et al., 2020), hypothesised to be driven by cellular exhaustion and senescence (Mathew et al., 2020; Nehme et al., 2020). Identifying biomarkers of immune dysfunction that can predict an individual's response to the virus remains an important goal.

We aimed to determine whether biomarkers of immune function and immune cell exhaustion and senescence could predict clinical outcomes of unvaccinated patients admitted to hospital with moderate to severe COVID-19.

QuantiFERON Monitor (QFM; QIAGEN, Dusseldorf, Germany) is a commercially available cytokine release assay designed to measure global immune function using innate and adaptive immune stimulation followed by measurement of interferon (IFN)-γ release by ELISA. QFM was performed according to manufacturer's instructions with an additional heat inactivation step before IFN-γ ELISA. In brief, 1 mL heparinised whole blood was added to the supplied sterile QFM tube. QFM lyosphere (containing anti-CD3 and TLR 7/8 ligand) was added, mixed well and incubated for 18–24 h at 37 °C. Serum was incubated at 56 °C for 20 min with the addition of protease inhibitor (Merck Life Science UK Limited, Dorset, UK). Heat inactivation was mandated by our institution's Health and Safety Committee to ensure viral inactivation of viable SARS-CoV2 within the serum (Xiling et al., 2021). Samples from healthy controls were treated in exactly the same way as patient samples including heat inactivation.

500 μL of heparinised whole blood was stained with combinations of fluorochrome-conjugated monoclonal antibodies to CD3 (PerCP-eFluor710; ThermoFisher Scientific, Paisley UK), PD-1 (PE; BD Biosciences, Wokingham, UK), CD4 (PE-Cy7), CD8 (APC-Cy7), KLRG-1 (Brilliant Violet 421) and CD57 (Brilliant Violet 510; all Biolegend, London, UK). Samples were lysed and fixed (ThermoFisher Scientific), washed and then analysed on a BD Lyric (BD Biosciences) flow cytometer. Fluorescence minus one controls were used for gating. Cells were gated on $CD3^+$ and then percentage positive PD-1, KLRG-1 and CD57 cells was determined on $CD4^+$ and $CD8^+$ cells (supplementary Fig. 1).

Consecutive patients admitted to University Hospitals Plymouth NHS Trust with a primary diagnosis of COVID-19 were recruited from April to May 2020 and in February 2021. Patients received standard clinical care including randomisation into interventional trials. Clinical and routine laboratory parameters were collected at baseline and participants were followed up to determine admission to the Intensive Care Unit (ICU) and in-hospital mortality. Healthy controls were recruited from healthcare and laboratory workers.

Forty-one patients were recruited to the study with mean age 69, 46% female (supplementary Table 1). Eleven patients had pre-existing lung disease, 9 had diabetes mellitus and 13 hypertension. Eleven patients required oxygen therapy at admission, 1 patient required ICU admission and 7 (17%) patients died during the index hospital admission. Twelve healthy volunteers were recruited to the study with mean age 58, 50% female (supplementary Table 1).

Thirty-one patients had a valid QFM performed at baseline with a median IFN-γ of 1.2 IU/ml compared to 10 age-matched healthy controls with a median IFN-γ of 25 IU/ml ($p < 0.0001$; Fig. 1). There was no difference between patients who survived to discharge and those who died during hospital admission (2.2 v 2.2 IU/ml; $p = 0.98$). IFN-γ levels

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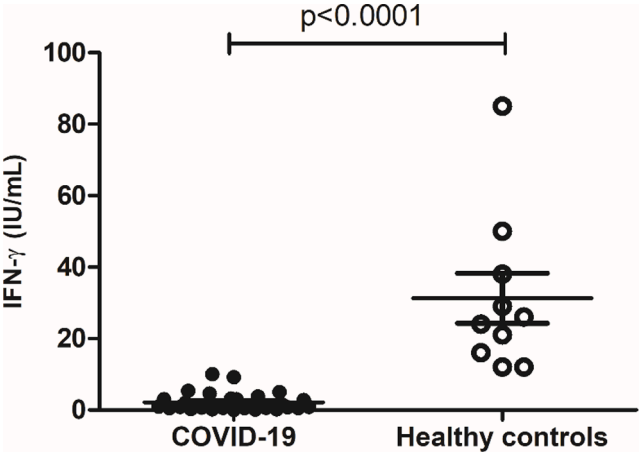

Fig. 1. IFN-γ release measured by QuantiFERON Monitor in 31 patients with moderate to severe COVID-19 and 10 healthy controls.

were negatively correlated with C-reactive protein (CRP; spearman's rho = -0.35; p = 0.05) but were not associated with any other parameter.

Flow cytometry in 13 patients (mean age 64; 46% female; 2 with underlying chronic lung disease; 1 death) and 12 healthy controls was consistent with previous reports that there was greater expression of PD-1 on $CD8^+$ T cells from patients with COVID-19 compared to healthy controls (median expression 39% vs 21%; p = 0.04) (Diao et al., 2020). There were no differences in expression of PD-1, CD57 or KLRG-1 on $CD4^+$ T cells or KLRG-1 on $CD8^+$ T cells. CD57 expression was significantly higher on $CD8^+$ T cells compared to healthy controls (42% vs 25%; p = 0.007; supplementary Fig. 1).

Patients with moderate to severe COVID-19 admitted to hospital universally had impaired cytokine release using the QuantiFERON Monitor test. Furthermore, a marker of T cell senescence was increased in $CD8^+$ T cells in patients with COVID-19 compared to healthy controls.

QFM is a validated cytokine release assay measuring global immune response to both innate and adaptive stimuli that has been used to monitor immune function after solid organ transplantation to guide immunosuppression (Sood and Testro, 2014). A pilot study of QFM as predictor of ICU usage in patients with severe COVID-19 pneumonia also found reduced IFN-γ production in COVID-19 patients compared to controls, but no association with clinical outcomes (Blot et al., 2020).

It is likely that the heat inactivation step resulted in protein loss. Median IFN-γ production from age-matched controls in this study was 25 IU/ml compared to 423 IU/ml in 212 unselected healthy controls without heat treated samples (Sood et al., 2014), suggesting over 90% protein loss with heat inactivation. Therefore, our QFM results are not directly comparable to other studies. However, as all samples from both patients and controls were treated equally, comparisons between groups are still valid.

Previous studies have demonstrated that protein and gene expression of T cell exhaustion markers are associated with increasing patient age and severity of COVID-19 (Mathew et al., 2020; Diao et al., 2020). $CD8^+$ T cells play an important role in viral clearance but chronic activation of these cells can result in ongoing cytokine production but lack of proliferation. CD57 is expressed on $CD8^+$ T cells that are chronically activated and increases in frequency with age and correlates with senescence (Champagne et al., 2001). The role of immune senescence is supported by experimental evidence and is hypothesised to explain the increased severity and mortality rate in older people (Nehme et al., 2020). In the current study, we show that expression of a cell surface marker of cellular senescence is increased on $CD8^+$ T cells in patients with COVID-19 compared to matched controls. These findings are supported by a report investigating immune senescence in 22 hospitalised patients with severe COVID-19, which demonstrated an increase in the proportion of $CD57^+$ $CD4^+$ and $CD8^+$ T cells in patients compared to controls (Arcanjo et al., 2021). Interestingly, these $CD57^+$ T cells co-expressed the exhaustion marker PD-1 and the $CD8^+$ T cells had SARS-CoV-2-specific T cell responses when stimulated with viral antigens, suggesting that the virus may drive a senescent and exhausted cellular phenotype. Our study, in a similar population of COVID-19 patients, concurs with the findings of increased $CD57^+$ expression on $CD8^+$ T cells but this did not meet significance in $CD4^+$ T cells (9.2% vs 4.4% in patients vs controls; p = 0.10; supplementary Fig. 1C), likely due to the small sample size.

We acknowledge that this was a single centre pilot study. However, participants were recruited and followed-up prospectively and are representative of hospitalised patients with moderate to severe COVID-19.

Taken together, these data indicate that significant immune paresis (impaired cytokine response) is present in all cases of moderate to severe COVID-19 and is associated with an exhausted and senescent cellular phenotype. These findings require validation in a larger independent sample and in patients with milder disease. Further study to understand the mechanisms of how SARS-CoV2 induces exhaustion and senescence may yield novel targets for therapy.

Author contributions

Study conception: ADD, DS, MEC. Participant recruitment: PM, HT. Experimental work: ADD, DF, PB, LdPS, KL. Drafting of manuscript: ADD. Finalising manuscript: all authors

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CRediT authorship contribution statement

Ashwin D. Dhanda: Conceptualization, Methodology, Investigation, Resources, Formal analysis, Project administration, Writing – original draft, Writing – review & editing. **Dan Felmlee:** Investigation, Resources, Writing – review & editing. **Paula Boeira:** Investigation, Resources, Writing – review & editing. **Prebashan Moodley:** Investigation, Resources, Writing – review & editing. **Huey Tan:** Investigation, Resources, Writing – review & editing. **Leticia De Paula Scalioni:** Investigation, Resources, Writing – review & editing. **Kristen Lilly:** Investigation, Resources, Writing – review & editing. **David A. Sheridan:** Conceptualization, Methodology, Writing – review & editing. **Matthew E. Cramp:** Conceptualization, Methodology, Writing – review & editing.

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.

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