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Increased Complement Receptor-3 levels in monocytes and granulocytes distinguish COVID-19 patients with pneumonia from those with mild symptoms

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ABSTRACT

Background: The reasons why some patients with COVID-19 develop pneumonia and others do not are unclear. To better understand this, we used multiparameter flow cytometry to profile circulating leukocytes from non-immunocompromised adult patients with PCR-proven COVID-19 and specifically compared those with mild symptoms with those who had developed pneumonia.
Methods: Using clinically validated antibody panels we studied leukocytes from 29 patients with PCR-proven COVID-19. Ten were hypoxic requiring ventilatory support, eleven were febrile but otherwise well, and eight were convalescing having previously required ventilatory support. Additionally, we analysed patients who did not have COVID-19 but received ventilatory support for other reasons. We examined routine Full Blood Count (FBC) specimens that were surplus to routine diagnostic requirements; normal ranges were established in a historic group of healthy volunteers.
Findings: We observed striking and unexpected differences in cells of the innate immune system. Levels of CD11b and CD18, which together comprise Complement Receptor 3 (CR3), were increased in granulocytes and monocytes from hypoxic COVID-19 patients, but not in those with COVID-19 who remained well, or in those without COVID-19 but ventilated for other reasons. Granulocyte and monocyte numbers were unchanged, however Natural Killer (NK) cell numbers were two-fold higher than normal in COVID-19 patients who remained well.
Interpretation: CR3 is central to leukocyte activation and subsequent cytokine release in response to infection. It is also a fibrinogen receptor, and its over-expression in granulocytes and monocytes of patients with respiratory failure tables it as a candidate effector of both the thrombotic and inflammatory features of COVID-19 pneumonia, and both a biomarker of impending respiratory failure and potential therapeutic target. NK cells are innate immune cells that retain immunological memory. Rapid expansion of memory NK cells targeting common antigens shared with other Coronaviruses may explain why most patients with COVID-19 do not develop respiratory complications. Understanding the innate immune response to SARS-CoV-may uncover why most infected individuals experience mild symptoms, and inform a preventive approach to COVID-19 pneumonia in the future.

Introduction

The pneumonia and respiratory failure of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection follow an unusual clinical pattern. Respiratory deterioration occurs seven to eight days after initial symptoms, but even in high risk groups, it is not seen in all patients. In those eventually requiring ventilation, very high doses of oxygen are required to maintain adequate oxygenation despite relatively preserved lung compliance (Preckel et al., 2020; Berlin et al., 2020).

A detailed pathophysiological picture of COVID-19 patients with established respiratory failure has now emerged. In addition to a 'cytokine storm', deranged coagulation parameters and elevated inflammatory markers are common laboratory findings,

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and both pulmonary and cutaneous thrombotic microangiopathy with evidence of complement activation are reported *post mortem* (Berlin et al., 2020; Richardson et al., 2020; Varga et al., 2020; Magro et al., 2020; McGonagle et al., 2020; Yin et al., 2020; Li et al., 2020). Striking lymphopenia and reduced expression of HLA-DR in monocytes have also been described, although similar haematological abnormalities are also reported in patients with respiratory failure due to other infections (Giamarellos-Bourboulis et al., 2020). The cytokine storm and coagulopathy in particular have encouraged trials of novel therapies targeting these processes, although none has yet emerged as definitive treatment (Berlin et al., 2020).

In the absence of an effective vaccine, an ideal intervention to protect healthcare resources from being overwhelmed by COVID-19 in the future is one that prevents newly infected individuals from deteriorating *a priori*. Designing such an intervention requires detailed knowledge of the chain of events between initial infection with SARS-CoV-2 and the onset of pneumonia. The time course of respiratory symptoms and the high levels of several circulating cytokines suggest that COVID-19 pneumonia is triggered by the innate immune response to the virus, and to fully understand this, ideally one would serially profile peripheral blood leukocytes from newly infected patients comparing those who deteriorate with those who do not. However, undertaking such a study on the background of a global pandemic, where the majority of asymptomatic patients with PCR-proven SARS-CoV-2 infection are under home quarantine, is challenging.

We took an alternative approach to understanding the immune cellular basis for the alternative clinical outcomes in COVID-19 by taking a single "snapshot" profile of leukocytes from patients with PCR-proven infection, and comparing those who had either developed pneumonia or remained asymptomatic and stayed out of hospital.

Methods

Subjects

Peripheral blood leukocyte immunophenotyping was performed in COVID-19 patients whose disease had followed one of three trajectories. "Hypoxic" patients required ICU Level 3 ventilatory support within 15 days of a PCR diagnosis of SARS-CoV2 infection; "Convalescing" patients had recovered following ICU care for COVID with minimal or absent oxygen requirements, but were at least 30 days from initial diagnosis; "Well" patients had mild symptoms prompting a positive PCR test within the previous 12 days, but were neither ever breathless nor hospitalised. No "Hypoxic" COVID-19 patient received either GCSF or drugs known to be toxic to either granulocytes or monocytes. As a control for the non-specific effects of hypoxia, we also studied 5 patients who did not have COVID-19 but required ventilatory support for other reasons; of these, 3 required mechanical ventilation after surgery, 1 had a pulmonary embolus and 1 had a chest infection following chemotherapy for a non-haematopoietic tumour for which he had received GCSF. The age distribution in each group is indicated in Table 2. No COVID-19 patient in this study had a prior history of malignancy or immunosuppression, and all also had LDH and D-Dimer measurements at the time blood was drawn.

Flow cytometry

The surplus material from routine Full Blood Count (FBC) specimens was prepared for analysis using the TQ-Prep whole blood lysis system (Beckman Coulter). Antibody staining was with the following Duraclone lyophilised antibody panels (Beckman Coulter): (i) IM T subset panel (cat. no. B53328). This is commercially available and in routine clinical use in our Institution; validated normal ranges for peripheral blood were previously established in a historical cohort of 33 healthy individuals with normal FBC. Details of the T cell subpopulations identified in this panel are provided in the supplementary material file. (ii) Myeloid orientation panel (cat. no. B38683). This antibody panel is in routine clinical use in our Institution for the diagnosis and monitoring of acute myeloid leukaemia. It is bespoke and not commercially available; details of the antibodies are shown in Table 1. We studied the following populations: monocytes $CD45^{+}CD13^{+}CD33^{++}CD15^{+/-}SSc^{int}$, granulocytes $CD45^{+}CD13^{+}CD33^{+}CD15^{++}SSc^{hi}$, NK cells $CD45^{++}CD2^{+}CD56^{+}SSc^{lo}$.

A minimum of 50,000 events were collected. Antigen expression levels in monocytes or granulocytes were calculated as corrected Mean Fluorescence Intensity (MFI): [raw value arithmetic mean fluorescence in monocytes or granulocytes - raw value arithmetic mean fluorescence in $CD45^{++}CD2^{+}CD56\text{-}SSc^{lo}$ T cells]. Normal ranges for MFI were established in the historic group of healthy volunteers whose FBC was used for the initial calibration of antibody panel.

Flow cytometry analysis was done with Kaluza software (Beckman Coulter). A Gaussian distribution was assumed for all continuous data including fluorescence intensities in the flow cytometry. Statistical analysis was by one-way ANOVA or *t*-test as appropriate (https://www.socscistatistics.com/tests/anova/default2.aspx, https://www.graphpad.com/quickcalcs/ttest1.cfm).

Results

Automated laboratory parameters in the four groups studied are shown in Table 2. We observed a trend towards higher circulating D-dimer in "hypoxic" COVID-19 patients compared to those who were not, or who were convalescing; however this did not meet the threshold for statistical significance in ANOVA or by pairwise *t*-test. Both COVID "hypoxic" and non-COVID "hypoxic" patients had significantly lower lymphocyte counts and significantly higher serum LDH than the COVID "well" group. The lymphopenia we observed in "hypoxic" COVID-19 patients is consistent with several other reports (Giamarellos-Bourboulis et al., 2020; Laing et al., 2020). However, lymphopenia was also a feature in the non-COVID hypoxic patients, and we suggest it to be a non-specific response to the physiological stresses of respiratory failure requiring ventilatory support (Cheadle et al., 1993).

Nonetheless, detailed comparison of lymphocyte compartments in the different groups of COVID-19 patients demonstrated that NK cell numbers were two-fold higher than normal in the "well" COVID group (Figure 1). We observed a trend towards increased NK cells in the "convalescing" group, but this was not statistically significant, and in the "hypoxic" COVID group they were in the normal range. All T cell compartments were either

Table 1
Duraclone Myeloid Orientation Panel.

Antigen	CD2	CD11b	CD13	CD15	CD18	CD33	CD34	CD45	CD56	CD117
Clone	39C1.5	Bear1	Immu103.44	80H5	7E4	7E4 D3HL60.251	581	J33	N901	104D2D1
Fluoro-chrome	APC-a750	FITC	PC5.5	PB	PE	PC7	ECD	KrO	APC-A700	APC

Antigen specificities with respective clone identities and fluorochromes in the Duraclone B38683 antibody panel.

Table 2
Baseline laboratory parameters.

	COVID "well" n=11 (age 35–60)	COVID "hypoxic" n=10 (age 29–82)	COVID "convalescing" n=8 (age 52–72)	Non-COVID "hypoxic" n=5 (age 22–84)
Total WCC (3.0–10.0 × 10^9/L)	7.01 (3.73–10.35 × 10^9/L)	7.00 (2.01–13.1 × 10^9/L)	8.54 (3.01–14.31 × 10^9/L)	12.12 (3.48–34.50 × 10^9/L)
Monocytes (0.1–1.0 × 10^9/L)	0.54 (0.32–0.84 × 10^9/L)	0.35 (0.13–0.63 × 10^9/L)	0.54 (0.17–1.20 × 10^9/L)	0.56 (0.25–1.00 × 10^9/L)
Granulocytes (2.0–7.5 × 10^9/L)	4.50 (2.31–6.88 × 10^9/L)	6.04 (1.83–11.99 × 10^9/L)	6.55 (1.72–11.44 × 10^9/L)	10.93 (3.00–32.15 × 10^9/L)
Lymphocytes (1.2–3.65 × 10^9/L)	1.83 (1.08–2.77 × 10^9/L)	0.68 (0.24–1.15 × 10^9/L)	1.19 (0.32–2.21 × 10^9/L)	0.51 (0.2–1.17 × 10^9/L)
LDH (135–225 IU/L)	205 (148–254 IU/L)	400 (256–561 IU/L)	380 (255–563 IU/L)	312 (199–578 IU/L)
D-Dimer (0–550 μg/L)	887 (190–6260 μg/L)	4128 (610–17070 μg/L)	2524 (540–6330 μg/L)	not available

The mean values and ranges for each parameter in each group are shown. Normal ranges are in the left-hand column. Lymphocyte counts were significantly lower (f-ratio 8.86, p = 0.0002) and serum LDH significantly higher (f-ratio 8.00, p = 0.006) in both hypoxic groups compared to COVID "well" patients. Differences in D-Dimer (f-ratio 2.35, p = 0.12), automated total white cell count (WCC, f-ratio 1.17, p = 0.34), granulocyte count (f-ratio 1.85, p = 0.16) and monocyte count (f-ratio 1.64, p = 0.20) were not significant (f-ratio 2.35, p = 0.12).

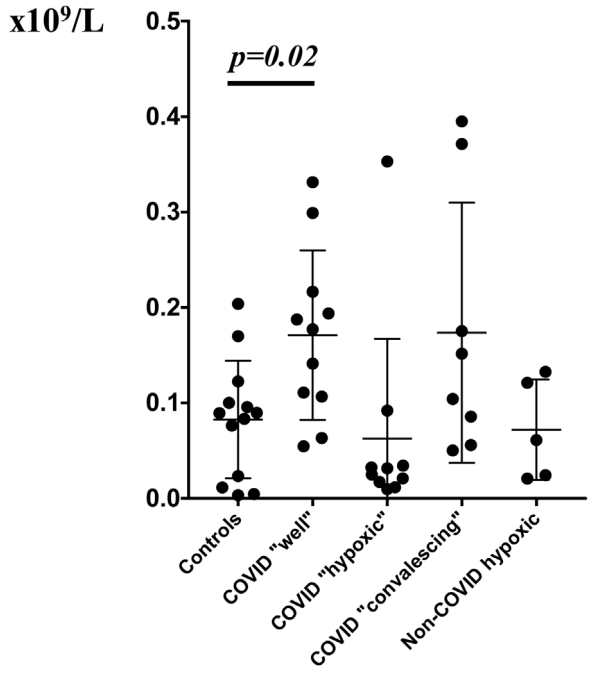

Figure 1. NK cell numbers.
Absolute numbers of NK cells in each group. NK cells are significantly increased in the COVID "well" group, compared to healthy controls. Mean 0·17 × 10^9/L in COVID "well" (range 0·05–0·30 × 10^9/L) *vs* 0·08 × 10^9/L in normal controls (range 0·005–0.21 × 10^9/L). Error bars indicate mean +/− SD. (significance levels <0·05; f-ratio 3.23, p = 0.02).

reduced or at the lower limit of normal in both groups requiring ventilatory support, and were preserved in the "well" COVID group with the exception of CD4+ central memory T cells, which were reduced (see Supplementary Material). As with NK cells, we saw an upward trend in all T cell compartments in "convalescing" patients.

We then focused on other cells of the innate immune system, namely monocytes and granulocytes. Their absolute numbers as quantified in the FBC were not significantly different between patient groups, however in the flow cytometry study we found that expression levels of the integrins CD11b and CD18, which together comprise Complement Receptor 3 (CR3), were significantly higher in both cell types in COVID-19 patients who were hypoxic requiring respiratory support (Figure 2). No other immunophenotypic changes were observed. "Convalescing" patients had intermediate levels of monocyte CD11b, but monocyte CD18 and the levels of both proteins on granulocytes had returned to normal. Levels were not elevated in patients who had SARS-CoV2 infection but remained well, and in those requiring ventilatory support for reasons other than COVID-19. Taken together these observations suggest that increased expression of CD11b/CD18 on granulocytes and monocytes is related to COVID-19 rather than hypoxia *per se*, which may up-regulate CD11b (Class and Natoli, 2016; Jibiki et al., 2002). However, we found that when blood was drawn, there was no significant difference in oxygenation-recorded to the nearest 30 min interval-between either group of ventilated patients (Mean Sa O2 94·6% in the COVID-19 cohort *vs* 94·8% in the non-COVID cohort. p = 0.92). This further supports the notion that increased CR3 on monocytes and granulocytes is specific to patients with both COVID-19 infection and respiratory failure.

Discussion

The unique design of our study has afforded insights into a hitherto unappreciated element of immune cell activation in COVID-19, and more importantly a specific association with severe pneumonia and hypoxia, not seen in those with only mild symptoms. We suggest that this highlights the central importance of the early innate immune response in determining clinical course. Our key novel observations are (i) that monocytes and granulocytes in patients with COVID-19 *and* established respiratory failure show increased levels of CR3 independently of both peripheral oxygen saturation and absolute monocyte and granulocyte counts, and (ii) NK cells are increased in COVID-19 patients who remain out of hospital with mild symptoms only.

Monocytes and granulocytes mediate initial immune responses to pathogens, are central to inflammatory processes following tissue damage and regulate haemostasis at sites of injury (Class and Natoli, 2016), and CR3 is a key component of these processes (Carvelli et al., 2020). Our data table monocyte and granulocyte activation as a cellular biomarker for respiratory complications in COVID-19, possibly by driving thrombotic microangiopathy due to increased binding of fibrinogen. CR3 is also responsible for complement-mediated leukocyte recruitment, cytokine production and phagocytosis – all of which are increased in severe COVID-19, and which also contribute to respiratory failure. Consistent with this, Carvelli et al. report that in COVID-19, circulating C5a levels increase proportional to clinical severity, although levels of the C5 receptor in monocytes and granulocytes do not change

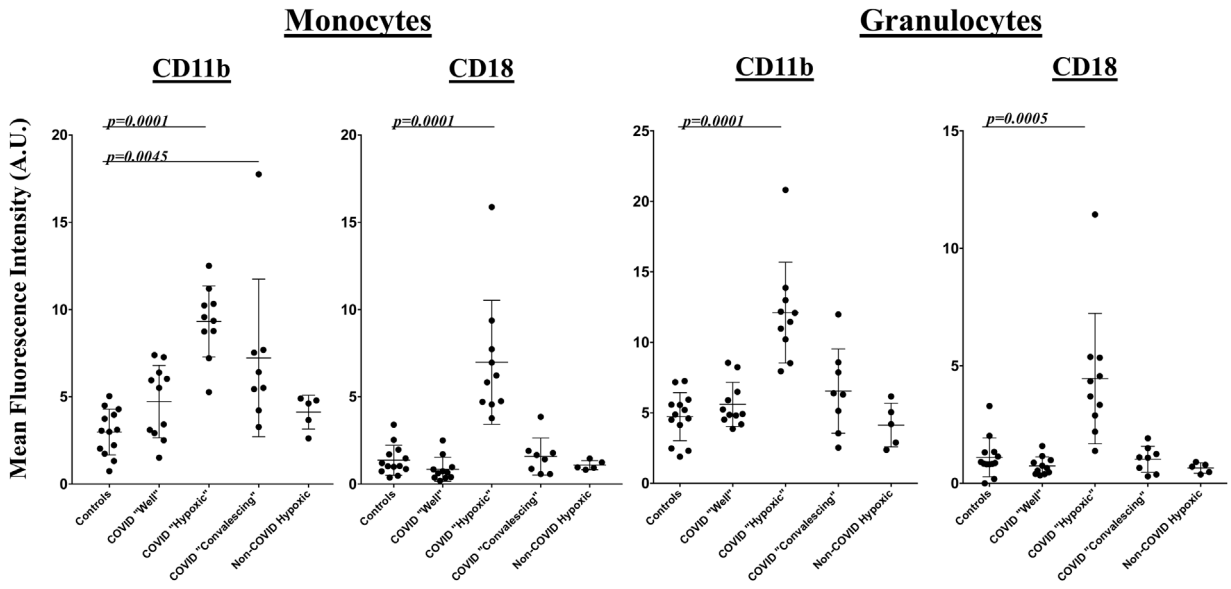

Figure 2. Surface antigen density of CD11b and CD18 in Monocytes and Granulocytes.
Mean Fluorescence intensity of CD11b and CD18 expression (Arbitrary Units) in peripheral blood monocytes. Controls are the historical cohort of healthy volunteers with normal blood counts used for validation of the assay. Both monocytes and granulocytes were otherwise immunophenotypically normal. Error bars indicate mean +/− SD. Bold horizontal bars indicate groups with significantly different expression levels and relevant p values (significance levels <0·05; CD11b f-ratio 12·61. CD18 f-ratio 20·48).

(Carvelli et al., 2020). Specific antagonists for C5a and C5 are at an early stage of development, however our results suggest that targeting of CR3 could be either a preventive or a therapeutic strategy in COVID-19 pneumonia. Targeting could be achieved non-specifically by drugs such as Colchicine that temporarily inhibit granulocyte function (Angelidis et al., 2018), or directly by readily available drugs such as Simvastatin - a known CR3 antagonist (Vorup-Jensen and Jensen, 2018).

NK cells are components of the innate immune system that mediate the rapid cytolytic response to virally infected cells. Unlike other lymphocytes they lack antigen specific receptors, however they have some adaptive features, such as self-renewal and rapid proliferation in response to re-stimulation, and are thus responsible for non-specific immunological memory to viral antigens (Adams et al., 2016). SARS-CoV-2 shares some viral epitopes with other Coronaviruses, and our results raise the possibility that some infected individuals who remain well do so because they have generated NK memory cells following earlier Coronavirus infections and are able to mount a more effective early innate immune response to this virus.

Although adaptive immune responses to SARS-CoV2 may determine subsequent long-term immunity (Takuya et al., 2020), it is the innate immune response that dictates the early clinical course, and our findings encourage further study of the potentially central role of mononuclear phagocytes in the generation and execution of both afferent and efferent immune responses in COVID-19 infection. Finally, it suggests the serial tracking of both CR3 expression and NK cells in individual patients with COVID-19 as potential biomarkers for disease severity. Data gleaned from such approaches may help reduce the incidence of pneumonia, informing a mechanistic, preventative approach to the management and effective therapy of COVID-19 and likely similar viruses in the future.

Conflict of interest

Dr Gant reported receiving personal fees from Gilead and Bio Mérieux. The other authors reported no Conflicts of Interest.

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Ethical approval

All specimens used in this study were taken as part of routine clinical care, and were either reported as such or further analysed as they became surplus to requirements and destined for destruction.

Author contributions

RG and VAG had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Concept and design: RG, VAG, TE.

Acquisition, analysis, or interpretation of data: All authors.

Drafting of the manuscript: RG, VAG, TE.

Critical revision of the manuscript for important intellectual content: All authors.

Administrative, technical, or material support: All authors.

Supervision: All authors.

CRediT authorship contribution statement

Rajeev Gupta: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Vanya Alasdair Gant:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Bryan Williams:** Data curation, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing. **Tariq Enver:** Conceptualization, Formal analysis,

Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2020.08.004.

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