

REVIEW ARTICLE

COVID-19: Using high-throughput flow cytometry to dissect clinical heterogeneity

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Abstract

Here we consider how high-content flow cytometric methodology at appropriate scale and throughput rapidly provided meaningful biological data in our recent studies of COVID-19, which we discuss in the context of other similar investigations. In our work, high-throughput flow cytometry was instrumental to identify a consensus immune signature in COVID-19 patients, and to investigate the impact of SARS-CoV-2 exposure on patients with either solid or hematological cancers. We provide here some examples of our 'holistic' approach, in which flow cytometry data generated by lymphocyte and myelomonocyte panels were integrated with other analytical metrics, including SARS-CoV-2-specific serum antibody titers, plasma cytokine/chemokine levels, and in-depth clinical annotation. We report how selective differences between T cell subsets were revealed by a newly described flow cytometric T_{DS} assay to distinguish actively cycling T cells in the peripheral blood. By such approaches, our and others' high-content flow cytometry studies collectively identified overt abnormalities and subtle but critical changes that discriminate the immuno-signature of COVID-19 patients from those of healthy donors and patients with non-COVID respiratory infections. Thereby, these studies offered several meaningful biomarkers of COVID-19 severity that have the potential to improve the management of patients and of hospital resources. In sum, flow cytometry provides an important means for rapidly obtaining data that can guide clinical decision-making without requiring highly expensive, sophisticated equipment, and/or “-omics” capabilities. We consider how this approach might be further developed.

KEYWORDS

High-throughput flow cytometry, COVID-19, cancer, cell cycle, Ki-67, DNA dye

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1 | INTRODUCTION

At the start of 2020, it became increasingly clear that the COVID-19 pandemic, driven by the spread of the coronavirus SARS-CoV-2, would impact life as we knew it for the foreseeable future. Now, after almost 2 years, about 240 million infected persons, and 5 million deaths have been confirmed worldwide (<https://www.who.int/emergencies/diseases/novel-coronavirus-2019>). Even though most SARS-CoV-2-infected individuals are pauci- or asymptomatic, some present with severe manifestations that require hospitalization, often in relation with deterioration of lung function. Death can occur for complications including acute respiratory distress syndrome (ARDS), marked coagulopathy, hyperinflammation and multi-organ failure. It should be noted that acute COVID-19 patients can present with a variety of symptoms (respiratory, intestinal, vascular, etc.), and that severe disease and death are more frequent among males, elderly people, and patients with comorbidities (e.g., obesity, cardiopathy, etc.). Understanding the bases of such heterogeneity in both clinical presentation and symptom severity is challenging.

When the COVID-19 pandemic firstly hit Europe and our hospitals were filled to capacity, we recognized the importance of pivoting our scientific expertise toward making meaningful contributions to understanding how the virus affects us: thus, we focused on how SARS-CoV-2 might impact the immune system. We wondered whether the higher susceptibility of those with co-morbidities such as high body mass index (BMI) or those who were older might reflect immune variation, or whether there would be a COVID-19 immune signature that would be evident irrespective of patient heterogeneity. And we wondered whether variation in such a signature might correlate with or portend higher severity, offering insights that could improve our understanding and management of the disease. To this end, we promptly established a high-content, high-throughput flow cytometry study termed COVID-IP [1].

We recognize that an immense knowledge has been gained through complex analytical approaches, including transcriptomics, methylomics, proteomics, metabolomics, single cell RNA-seq, and mass cytometry, etc. [2–9]. For example, in one of the transcriptomics projects, RNAseq data obtained from 62 COVID-19 patients were compared with publicly available non-COVID data curated from 23 independent studies, resulting in the rapid identification of a set of genes shared with six other infections (influenza, respiratory syncytial virus (RSV), human rhinovirus (HRV), SARS-CoV-1, Ebola, dengue), as well as a set of not-shared genes [7]. Additionally, a large-scale proteomic study identified 27 potential biomarkers of COVID-19 severity among complement proteins, coagulation factors, inflammatory mediators, etc. highlighting the potential of proteome profiles to function as clinical classifiers [6].

In this context of rapidly evolving “-omics” technologies, we argue that flow cytometry remains an important means for rapidly obtaining informative biological data without requiring costly, highly sophisticated equipment. It is also a perfect tool to deconvolute cellular heterogeneity against the backdrop of a well-established flow cytometry-based classification of lymphoid and myeloid subsets/ phenotypes/ activation states. This is highly meaningful for COVID-19 patient management, considering the emerging association between

immunophenotype variations and clinical states. Furthermore, flow cytometry can be instrumental to a better understanding of COVID-19 pathogenesis, ultimately contributing to design new approaches for controlling SARS-CoV-2 replication and inhibiting immunopathology. Conversely, a current limitation of flow cytometry is its time-consuming data analysis process, often based on manual gating. Furthermore, flow cytometry data are often presented only in terms of cell percentages, even though the methodology allows for very accurate absolute cell counts/ μl , that is a particularly informative metrics in conditions of broad immune perturbations such as those of severe COVID-19 patients. In this article we will briefly discuss some key findings of the COVID-IP study, place them in the context of contemporaneous studies, and touch upon some possibilities for overcoming current limitation of flow cytometry and for further developing this methodology.

2 | HIGH-THROUGHPUT FLOW CYTOMETRY

To interrogate whether a consensus COVID-19 immune signature existed, a high-throughput flow cytometry platform (COVID-IP) was established in March 2020 to measure peripheral blood immunophenotypes in hospital-treated COVID-19 patients and controls [1] (Figure 1A). The COVID-IP platform was grounded in a high-throughput murine immunophenotyping platform that was developed to operate at a scale sufficient to facilitate a high-throughput immunogenetics screen conducted at the Wellcome Trust Sanger Institute [10]. That flow-cytometry-based screen permitted over 500 newly made single-gene knock-out mouse strains to be phenotyped over a 6-year period, as a result of which 140 immunoregulatory genes were identified, 80 of them never previously implicated in immunology [10]. There is no precedent for other immunophenotyping methods being able to operate at such high throughput.

COVID-IP featured 8 distinct flow cytometry panels collectively covering detailed analyses of lymphocytes: specifically, $\alpha\beta$ T cells, B cells, and $\gamma\delta$ T cells, including defined subsets of each, such as Th1, Th2, and Th17-effector cells; immunoglobulin-producing plasmablasts; memory lymphocyte subsets, and phosphoantigen-reactive V δ 2 T cells. The panels offered opportunities to track the cells' frequencies, activation, exhaustion, and cycling. Additionally, they provided data on discrete innate immune cell types (Figure 1B,C). The functionally and phenotypically tailored panels were internally tethered via specific common markers and reference samples. To further integrate a holistic view of the immune system, a broad ‘cell count’ panel was run on whole blood, allowing for the interpolation of accurate blood counts for the majority of cell subsets measured [1]. This provided the necessary data to corroborate the claim that many COVID-19 patients were severely lymphopenic [11, 12] but additionally showed that this applied very selectively to different lymphocyte subsets (Table 1). Additionally, there were very unexpected severity-related depletions of plasmacytoid dendritic cells (PDC) and basophils [1] (Table 1). Of note, the mechanistic basis of each of these is unknown and clearly merits follow up if we are to better understand human infectious

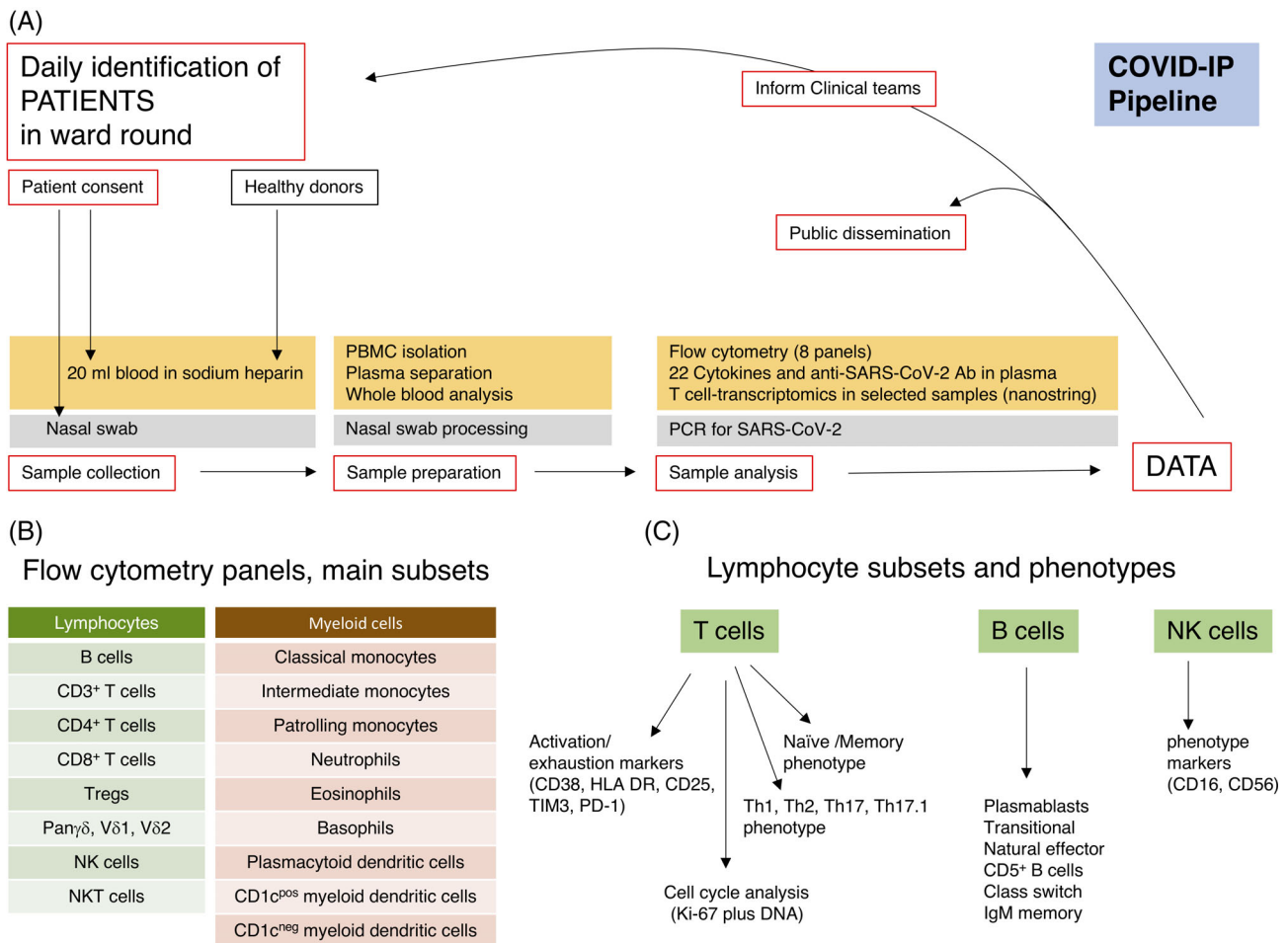


FIGURE 1 Scheme of the high-throughput flow cytometry platform in the COVID-IP study. (A) The COVID-IP study pipeline. (B) List of the main lymphocytes and myeloid cell populations analyzed. (C) Examples of further analysis of T, B, and NK cell subsets: markers, cell cycle, phenotype. For more details, see original Reference [1] [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.com)]

diseases and possibly other settings of hyperimmune activation. Indeed, the case could be made for developing animal models to investigate the impacts of such selective immunocyte depletions, even in the absence of virus infection.

The power of high-throughput flow cytometry was evident to many scientists in the field who used this technology to interrogate the peripheral blood of COVID-19 patients. A side-by-side comparison of the experimental schemes used in our and others' studies is shown in Table 2. The common theme was a high-throughput platform for ex vivo assessment of cell frequencies, and phenotypes, often (but not always) coupled to flow-cytometry-based quantification of cell numbers, and with functional assays requiring short-term activation for measurement of intracellular cytokines. In some cases, cell sorting by flow cytometry was performed for subsequent transcriptomics analysis.

Importantly from the perspective of scientific practice, these studies conducted independently and very rapidly in different countries discovered consistent immunological traits of COVID-19. Besides selective cases of COVID-19-associated cytopenia (above), they collectively reported highly activated T cell phenotype, changes in the B cell compartment, specifically transiently increased circulating plasma blasts, and strikingly abnormal monocyte traits (for detailed results,

see Table 1 and reference in Table 2). Beyond this consensus, some studies also reported information on specific cell subsets: for example, we showed severe depletion of V γ 9V δ 2 cells, and a shift toward V δ 1⁺ cells, which have been previously implicated in responses to virus infection (Table 1) [1].

Likewise, Rébillard and colleagues documented a high proportion of circulating promyelocytes, possibly reflecting a bone marrow response that could culminate in emergency hematopoiesis [13]. A dramatic increase of IL-6-producing CD14⁺ monocytes was observed by Giamarellos-Bourboulis and colleagues using a flow-cytometry-based intracellular cytokine assay [14], which was also deployed by several other studies, collectively showing increased IL-17-producing and IFN- γ -producing T cells [15, 16]. Two other groups and ourselves [1, 12, 15] also investigated whether circulating lymphocytes were in cell cycle or in a quiescent state, by use of different methods, as described later in this article.

A crucial aspect of the COVID-IP study was the integration of the flow cytometry platform with other analytical approaches, including the measurements of SARS-CoV-2-specific serum antibody titers, plasma cytokine/chemokine levels, and in-depth clinical annotation (Figure 1A). By integrating these data-sets, it proved possible to

TABLE 1 Main flow cytometry results of the COVID-IP study

(A) Lymphocytes		
	Counts	Phenotype/subpopulations
B cell compartment	Total B cells (variable) Plasmablasts (+ +)	Reduced CD19 expression by B cells Reduced frequency of natural effector and CD5 ⁺ B cells
CD3 ⁺ T cells	(- -)	see T cell subsets below
CD4 ⁺ T cells	(- -)	Reduced counts of Th17.1, Th1, Th17 cells Reduced counts of T _{EM} , T _{CM} , T _N cells Increased frequency of CD38 ⁺ HLA-DR ⁺ and CD25 ⁺ cells Increased frequency of cells in G ₁ and in S-G ₂ /M, particularly among T _{EM} cells
CD8 ⁺ T cells	(- - -)	Reduced counts of T _{EM} , T _{CM} , T _N , T _{EMRA} cells Increased frequency of CD38 ⁺ HLA-DR ⁺ and CD25 ⁺ cells Increased frequency of PD-1 ⁺ TIM-3 ⁺ cells Increased frequency of cells in G ₁ and in S-G ₂ /M, particularly among T _{EM} cells
Tregs	(-)	ND
γδ T cell compartment	Panγδ (- - -) Vδ1 (=) Vδ2 (- - -)	Increased frequency of Vδ1 and reduced frequency of Vδ2 T cells Increased frequency of CD45RA ⁺ CD27 ⁻ Vδ1 T cells Increased frequency of PD-1 ⁺ γδ T cells Increased frequency of γδ T cells in G ₁
NK cells	(- -)	Trend of slightly reduced frequency of CD56 ^{bright} CD16 ^{+/-} and increased frequency of CD56 ^{int} CD16 ⁺ cells
NKT cells	(- - -)	ND
(B) Myeloid cells		
	Cell counts	Phenotype /subpopulations
Monocytes	Total monocytes (-) Classical monocytes (-) Intermediate monocytes (+ +) Patrolling monocytes (variable)	Increased frequency of intermediate monocytes and reduced frequency of classical monocytes Reduced CD86 and HLA-DR expression, particularly by intermediate monocytes
Neutrophils	(+)	ND
Eosinophils	(=)	ND
Basophils	(- - -)	ND
Plasmacytoid dendritic cells (PDCs)	(- - -)	ND
Myeloid dendritic cells (mDCs)	CD1c ^{pos} mDCs (- - -) CD1c ^{neg} mDCs (+ + +)	Reduced frequency of CD1c ^{pos} mDCs and increased frequency of CD1c ^{neg} mDCs Increased frequency of CD1c ^{neg} mDCs in G ₁ and in S-G ₂ /M

Note: The table summarizes the most relevant findings on blood lymphocytes (A) and myeloid cells (B) obtained by the COVID-IP study. For more details, see original reference (1).

Abbreviation: ND, not done.

identify components of the COVID-19 immune signature that correlated with disease severity. Against some a priori assumptions, strong anti-SARS-CoV-2 IgM and IgG antibody responses were not included among such components since they were commonly made by patients irrespective of disease status [1, 17–19]. Likewise, other studies reported that patients showed strong SARS-CoV-2 reactive T cell responses across a range of disease severity [20]. Given the scale of T cell dysregulation, such data argue that any failings in making immuno-protective anti-viral responses are more likely due to defective immune regulation rather than impaired immune recognition. Indeed, there have been claims that patients with severe COVID-19 were delayed in their production of antibodies [21], and/or that their T cell responses may be unhelpfully dominated by low avidity CD4⁺ T

cell with cross-reactivities to previously encountered common cold coronaviruses (CCCoV) [22]. However, in seeming contrast to this study, it was also reported that functional CCCoV-cross reactive T cells might be protective against SARS-CoV-2, and that older persons, in whom SARS-CoV-2 infection presents a greater risk of severe COVID-19, have a lower frequency or complete lack of such T cells [23].

By contrast to the uncertain and unresolved associations of disease-severity with virus-specific B and T cell traits, there were unequivocal and significant correlations of severity with several innate and innate-like immunological traits including the above-mentioned basophil, PDC and Vγ9Vδ2 cell depletions. Furthermore, the status of some traits on the day of hospital admission appeared to anticipate

TABLE 2 High-throughput flow cytometry in COVID-19 studies

Hospitalized patient cohort analyzed by flow cytometry (N)	Lymphoid subsets	Myeloid subsets	Measurements	Analysis of flow cytometry data	References
COVID-19 (54); bacterial Community Acquired Pneumonia (CAP) with sepsis (104); historical H1N1 influenza (21).	CD4 ⁺ T; CD8 ⁺ T; B; NK.	Monocytes; Neutrophils.	Cell numbers; Cell percentages; Intracellular cytokines.	Manual gating (not shown); Correlation with clinical status, serum analytes, Ab and cytokines.	[14]
COVID-19 (35); Recovered from SARS-CoV-2 positive test; no hospitalization (7); Healthy controls (HC, 12).	CD4 ⁺ T; CD8 ⁺ T; Tregs; B; Plasmablasts; NK; ILC; MAIT.	Monocytes; Neutrophils; Eosinophils; Immature granulocytes; Dendritic cells.	Cell percentages; Cell types within main subsets; Cell phenotype; Non-quiescent state (Ki-67); Intracellular granzyme/perforin.	Manual gating (strategy in supplementary material); Correlation with clinical status. (Note: Ig heavy chain sequencing in selected samples).	[12]
COVID-19 (125); Recovered from SARS-CoV-2 positive test, no hospitalization (60); HC (36). Repeated sampling for longitudinal analysis when possible.	CD4 ⁺ T; CD8 ⁺ T; B; Plasmablasts; non B/non T.	Monocytes; Neutrophils; Basophils; Eosinophils (all clinically measured).	Cell percentages; Cell types within main subsets; Cell phenotype; Non-quiescent state (Ki-67); Intracellular cytokines.	Manual gating (gating strategy partially shown in main figure); Unsupervised analysis (FlowSOM); Correlation with clinical status, clinically measured blood cell counts, serum analytes, Ab, cytokines and chemokines.	[15]
COVID-19 (113); HC (108). Repeated sampling for longitudinal analysis when possible.	CD4 ⁺ T; CD8 ⁺ T; B; NK; NKT.	Monocytes; Neutrophils; Eosinophils; Dendritic cells.	Cell numbers; Cell percentages; Cell types within main subsets; Cell phenotype; Intracellular cytokines.	Manual gating (strategy in supplementary material); Unsupervised analysis (FlowCT); Correlation with clinical status, viral load in nasal swab, serum analytes, Ab, cytokines and chemokines.	[16]
COVID-19 (63); SARS-CoV-2 Negative Low Respiratory Tract Infections (LRTI, 10); HC, positive for anti-SARS-CoV-2 Ab (23); HC, negative for anti-SARS-CoV-2 Ab (32). Repeated sampling for longitudinal analysis when possible.	CD4 ⁺ T; CD8 ⁺ T; $\gamma\delta$ T; Tregs; B; Plasmablasts; NK; NKT.	Monocytes; Neutrophils; Basophils; Eosinophils; Dendritic cells.	Cell numbers; Cell percentages; Cell types within main subsets; Cell cycle (DNA/Ki-67). Cell sorting for transcriptomics by nanosttring (T cell subsets from selected samples).	Manual gating (strategy in supplementary material); Correlation with clinical status, viral load in nasal swab, serum analytes, Ab, cytokines and chemokines.	[1]
COVID-19 (50); SARS-CoV-2 Negative with respiratory symptoms (22); HC (49). Repeated sampling for longitudinal analysis when possible.	CD4 ⁺ T; CD8 ⁺ T; $\gamma\delta$ T; B; NK; NKT.	Monocytes; Neutrophils; Dendritic cells; Promyelocytes.	Cell percentages; Cell types within main subsets; Cell phenotype.	Manual gating versus data-driven analysis (strategy in supplementary material) Unsupervised analysis; Correlation with clinical status.	[13]
COVID-19 (868, admitted to hospital); SARS-CoV-2 Negative with respiratory infections (24); HC (36). Repeated sampling for longitudinal analysis when possible.	CD4 ⁺ T; CD8 ⁺ T; B; Plasmacells; NK.	Monocytes; Neutrophils; Basophils; Eosinophils; Dendritic cells.	Cell percentages; Cell types within main subsets; Cell phenotype. Cell sorting for transcriptomics by RNAseq (basophils, myeloid and plasmacytoid DC, classical and non-classical monocytes, and neutrophils from selected samples).	Semi-automated workflow for analysis (strategy in supplementary material); Unsupervised analysis; Correlation with clinical status, and clinically measured blood cell counts.	[24]

Note: A simplified scheme of the experimental approaches used by some compelling studies to interrogate peripheral blood from COVID-19 patients by high-throughput flow cytometry. For each study, we listed here the main lymphoid and myeloid subsets analyzed, the measurements performed by flow cytometry, and the quantitative methods used for flow cytometry data analysis. See text for a summary of the main findings of the cited studies, and original references for detailed results. We apologize for leaving out many studies of the vast literature.

Abbreviations: CAP, community acquired pneumonia; HC, healthy controls.

disease deterioration: these included T-cytopenia and a triad of elevated IL-6, IL-10, and IP-10 [1]. Similarly, other studies provided a global view of the immune alterations in COVID-19 by combining flow cytometry with other approaches (Table 2), and offered candidate biomarkers for risk of death, for example a reduced B-cell percentage [24]. It is also to be noted that applying flow cytometry to COVID-19 offered a rare chance to observe the human immune system in action, in the heat of battle with an infectious pathogen. We shall illustrate this by now considering some selected findings.

3 | CELL CYCLE ANALYSIS OF T CELLS: THE T_{DS} ASSAY

Despite the lack of evidence for SARS-CoV-2 directly infecting T cells, T cell pathology was a common feature of COVID-19, with severe depletion of defined T cell subsets, for example, CD8⁺ and IFN- γ -producing CD4⁺ Th1 and Th17.1 cells and relative preservation of others, including CD4⁺ Th2 cells and CD4⁺ T-regulatory cells (Table 1) [1]. Notably, very similar phenotypes were observed in our study of cancer patients with COVID-19, considered below [25], and somewhat related observations have been made in other severe respiratory infection and in sepsis. The factors underpinning these changes are unknown, but given their selectivity, we questioned whether it might simply reflect differences in activation-induced cell death. We therefore investigated the cells' activation status and proliferation in some detail.

To measure T cell cycling at high throughput, we employed a newly refined flow cytometry method, based on dual staining for Ki-67 and DNA content [1, 26]. The dual staining method exploited DNA content to discriminate between cells in the G₁ phase of cell cycle (having 2n DNA), from cells in the S-G₂/M phases (having 2n < DNA ≤ 4n), all of which stain positively for Ki-67, in contrast to cells in the quiescent G₀ phase which are Ki-67^{negative} [1]. This in contrast with other studies that relied on the more common use of Ki-67 as a single proliferation marker for B and T cells (see References [12, 15] in Table 2). Ki-67 expression was also examined by other authors who focused their analysis on T cell subsets [27, 28].

The possibility that peripheral blood T cells might include a fraction of actively proliferating cells in the S-G₂/M phases of cell cycle, and that this fraction might reflect protective or pathologic immune responses had precedent in our studies of vaccinated mice [29], and of patients with Type 1 diabetes (T1D), or infectious mononucleosis (IM) [26]. In this context, T cells in the S-G₂/M phases of cell cycle were collectively termed T_{DS} (for T cells in S phase in Sanguine; "T Double S"), and the dual staining method for their assessment was named the T_{DS} assay [26].

The use of the T_{DS} assay in COVID-IP was instrumental in showing highly selective differences in the status of different T cell subtypes. For example, $\gamma\delta$ T cells and conventional $\alpha\beta$ T cells each showed ~5–10-fold increased percentages of cells in G₁, but only $\alpha\beta$ T cells showed clear transition to S-G₂/M, that is, T_{DS} cells, possibly reflecting the diverse activation response and/or recirculation

pathways of the two types of T cells [1]. Likewise, $\alpha\beta$ T_{DS} cells were relatively enriched within effector memory CD4⁺ and CD8⁺ T cells (CD4⁺ T_{EM} and CD8⁺ T_{EM}) compared to other subsets (Table 1). Notably, the stark increase of CD4⁺ T_{EM} cells in G₁ and in S-G₂/M were prominent traits of the immune signature of severe COVID-19 disease [1].

A refined gating strategy was used for analysis of the T_{DS} assay data (Reference [1], example in Figure 2A). In fact, according to the T_{DS} assay principles, and in contrast with commonly used flow cytometry strategies for analyzing peripheral blood lymphocytes, T cell analysis was not restricted to single cells having low FSC and SSC [26, 29, 30]. Rather, cell aggregates were excluded on a DNA-A/DNA-W plot (Figure 2A, left), and a "relaxed" gate used on the FSC-A/SSC-A plot (Figure 2A, 4th plot from left, red gate), instead of the commonly used "narrow" gate (black gate in the same plot, shown for comparison). Using this strategy, cells in G₁ and in S-G₂/M, that is, T_{DS} cells, were consistently detected in non-naïve CD4⁺ T and CD8⁺ T cells from COVID-19 patients (Reference [1], example of a patient with severe disease in Figure 2B). That most of the actively cycling T cells from COVID-19 patients had high FSC-A and especially high SSC-A is shown by the FSC-A/SSC-A plots of these cells (Figure 2C). Indeed, 60%–80% of proliferating cells in S-G₂/M were out of a "narrow" gate on the FSC-A/SSC-A plot, and even cells in G₁ were partially excluded by the "narrow" gate (Figure 2C). Thus, this analysis of human T cell status during ongoing COVID-19 confirmed the importance of a "relaxed" gate for analysis of T cell proliferation, as was reported for T1D patients and for vaccinated mice [26, 29]. In contrast to COVID-19 patients, healthy donors showed almost no proliferation in non-naïve CD4⁺ and CD8⁺ T cells (Reference [1], example in Figure 2D).

The high FSC-SSC profile of activated/proliferating T cells is an often-neglected issue that can potentially lead to an underestimation of Ki-67⁺ cells, or even of T cells with an activated phenotype, in human peripheral blood. As an example, Ki-67⁺ T cells were quantified after applying a "narrow" gate on the FSC-A/SSC-A plot in some recently published studies on COVID-19 [27, 28] and on T cell response to SARS-CoV-2 vaccination [31]. For the data in Figure 2, we estimated that replacing the "relaxed" with a "narrow" FSC-A/SSC-A gate in the gating strategy (Panel A, 4th from the left, black gate instead of red gate), would result in a 1.34- and 1.03-fold reduction in the percentage of Ki-67⁺ cells in CD4⁺ and CD8⁺ T_{EM}, respectively (from 16.22% to 12.09% in CD4⁺ T_{EM}; from 60.33% to 58.62% in CD8⁺ T_{EM}). The diverse extent of reduction depends on the different proportions of Ki-67⁺ cells in G₁ and in S-G₂/M in the two cell populations (Figure 2B), highlighting that the "narrow" gate can bias a correct comparison between different cell subsets from the same donor, and potentially between a single cell subset from different donors.

In sum, COVID-19 investigation affirmed the utility of the T_{DS} flow cytometry assay of peripheral blood that is easily and broadly implemented [1, 25, 32]. The prospect that peripheral blood T cells might circulate while in the process of duplicating their DNA is not currently described in text book descriptions which typically consider the cells to be quiescent *passengers en route* between lymphoid and

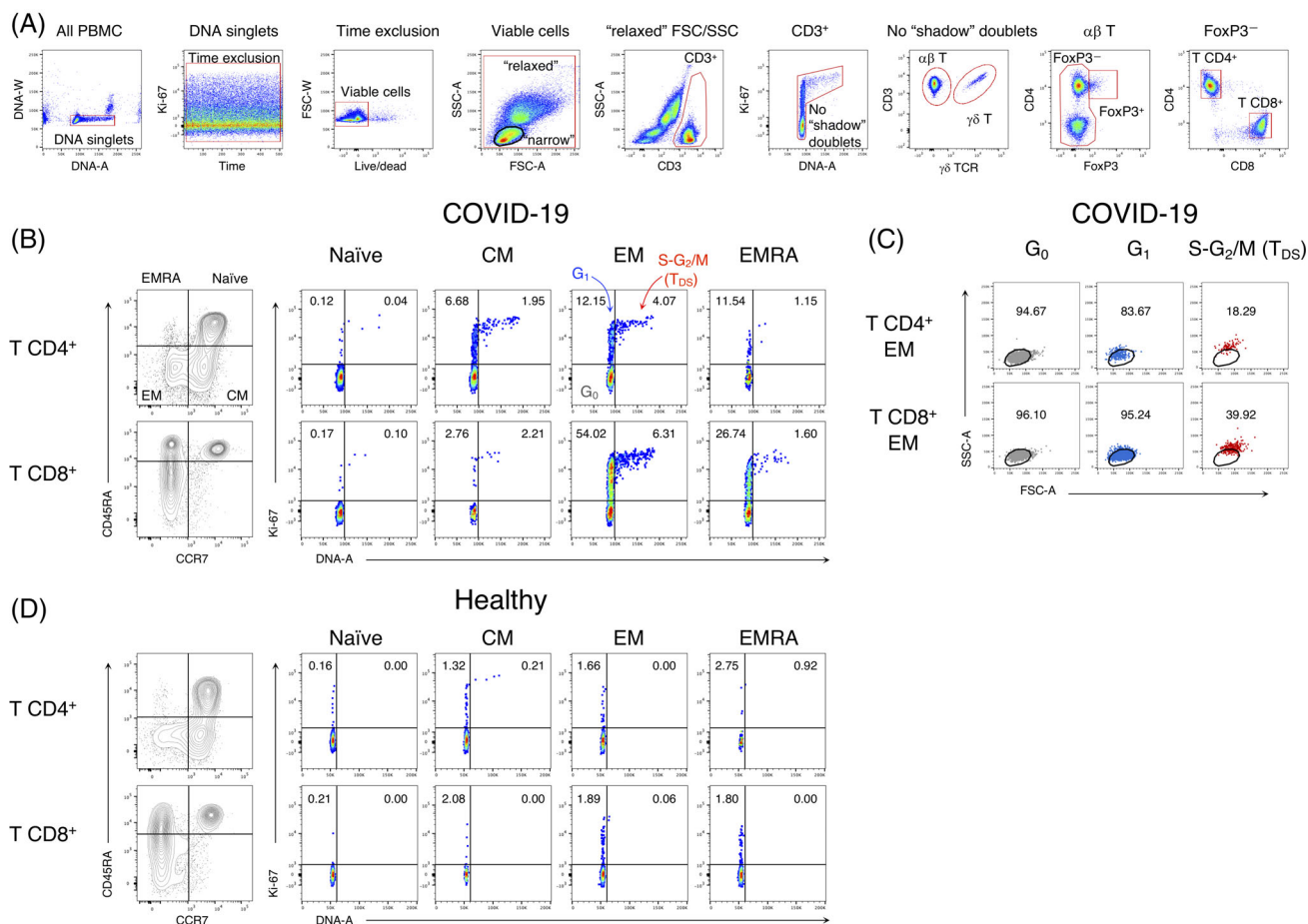


FIGURE 2 Example of CD4⁺ T_{EM} and CD8⁺ T_{EM} cell cycle analysis by the T_{DS} assay, as in the COVID-IP study. PBMCs from COVID-19 patients and healthy controls were stained with the live/dead dye eFluor 780, the DNA dye Hoechst-33,342, and a cocktail of fluorochrome conjugated mAbs, as described Reference [1]. An example of flow cytometry analysis of a patient with severe COVID-19 (A–C) is shown, in comparison with a healthy donor (D). (A) Gating strategy: (1) DNA-A/-W singlets. Single cells having $2n \leq$ DNA content $\leq 4n$ were selected on the DNA-area (A) versus (vs) DNA-width (W) plot; (2) Time exclusion. Stable acquisition over time (seconds) was monitored on the time vs Ki-67 plot and any events collected in case of pressure fluctuations were excluded; (3) Viable cells. Dead cells were excluded on the FSC-W vs live/dead plot; (4) FSC-A/SSC-A “relaxed” gate. A “relaxed” gate (in red) was used on the FSC-A vs SSC-A plot, to include highly activated and cycling lymphocytes [29]; a “narrow” gate (in black) is shown for comparison; (5) CD3⁺ T cells. CD3⁺ T cells were gated on the CD3 versus SSC-A plot; (6) Refined singlets. A few remaining doublets composed by one cell sitting on top of another (so-called shadow doublets) were excluded as Ki-6^{int/+} events having $>2n$ DNA content [26]; (7) $\alpha\beta$ T cells. $\alpha\beta$ and $\gamma\delta$ T cells were gated on the $\gamma\delta$ TCR versus CD3 plot; (8) FoxP3⁻ cells. FoxP3⁻ and FoxP3⁺ cells were gated on the FoxP3 versus CD4 plot; (9) T CD4⁺ and T CD8⁺ cells. T CD4⁺ and T CD8⁺ cells were gated on the CD4 versus CD8 plot. This gating strategy was used as a base for the subsequent cell cycle analysis of T subsets [1]. (B and D) Cell cycle of naïve/memory subsets. The following naïve/memory subsets were identified among T CD4⁺ (top left) and T CD8⁺ (bottom left) cells: CD45RA⁺ CCR7⁺ Naïve, CD45RA⁻ CCR7⁺ central memory (CM), CD45RA⁻ CCR7⁻ effector memory (EM), and CD45RA⁺ CCR7⁻ (EMRA). Cell cycle phases of each T CD4⁺ (top) and T CD8⁺ (bottom) naïve/memory subset were defined on DNA-A vs Ki67-A plot as follows: cells in G₀ were identified as DNA 2n/Ki67⁻ (bottom left quadrant); cells in G₁ as DNA 2n/ Ki67⁺ (upper left quadrant); cells in S-G₂/M (or T_{DS} cells) as DNA $> 2n$ /Ki67⁺ (top right quadrant). (C) FSC-A/SSC-A “narrow” gate. The panels represent FSC-A vs SSC-A plots of T CD4⁺ and T CD8⁺ EM cells in G₀, in G₁ and in S-G₂/M (or T_{DS} cells), as indicated. A “narrow” gate was used to select lymphocytes with low FSC-A/SSC-A (see panel A). Numbers indicate percentage of cells in the gated region. Unpublished data in relation to [1] [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

extralymphoid organs [30]. Moreover, the assay provided valuable insights beyond T cells. Specifically, many studies have considered the conspicuously dysregulated state of the myelomonocytic compartment in COVID-19 [14, 16, 33, 34], and have suggested this to be an underpinning component of COVID-19 severity. In that regard, applying the T_{DS} assay methodology to myelomonocytic cells identified a dramatic increase of actively proliferating CD11c⁺ CD1c^{neg} dendritic cells for which there is rare if any precedent, but which may constitute a pathognomonic marker (Table 1).

4 | IMMUNOPHENOTYPING IN CANCER PATIENTS WITH COVID-19

The application of high throughput, high-content flow cytometry was particularly helpful in investigating the impact of SARS-CoV-2 exposure on patients with cancer, as it provided a state-of-the-art platform for accurately tracking immune cell population changes during acute infection and recovery. Given the common perception of immune compromise caused by cancer and/or its treatment, there was

understandable concern about the capacity of cancer patients to respond to infection. Flow cytometry offered a means to displace conjecture with data. In studying SARS-CoV-2-exposed persons with hematological versus solid malignancies [25], it was found that those in the latter cohort displayed the signature mix of immunoprotective and immunopathological traits of SARS-CoV-2-exposed subjects without cancer, including the concurrent proliferation, activation, exhaustion and depletion of selective T cell subsets considered above. This again illustrates how the profile of an immune response to a virus infection can be dominant to other concurrent co-morbidities, including cancer. This notwithstanding, the balance of activation versus exhaustion seemed much more skewed toward the latter in patients with hematological cancers, associated with which they had generally worse outcomes, in some agreement with other studies [35–39]. Patients with hematological cancers also showed distinguishing increases in CD56^{hi} NK cells [25].

Overt B cell cytopenia was observed in solid and hematological cancer patients, and in only a small number of cases could this be only partially attributable to either anti-CD20 therapy (in patients with B cell cancers) or underlying malignancy [25, 39]. Flow cytometry offered the opportunity to effectively track cell populations within the B cell compartment. Immunophenotyping showed that cytopenia was not limited to reduced memory B cells, but that naïve, effector and transitional B cell numbers were also compromised in solid cancer patients, while naïve B cells were mostly affected in hematological cancer patients [25]. The susceptibility of naïve B cells again questions activation-induced cell death as a sole basis for lymphopenia.

It was again the case that against the backdrop of immune dysregulation, strong anti-SARS-CoV-2 IgM and IgG antibodies could be made, particularly by most solid cancer patients. By contrast, many hematological cancer patients failed to mount a protective antibody response, and even in those who did, there was often failure to clear the virus for weeks or months [25, 39, 40]. Interestingly, it was reported that those with a greater number of CD8⁺ T cells had improved survival, including those treated with anti-CD20 therapy [39]. This has profound implications with regard to vaccination, as CD8⁺ T cell responses against the virus may give some protection even in the absence of humoral responses [31, 39]. Moreover, there was a legacy of long-standing immune dysregulation that distinguished the patients from non-virus exposed persons with the same types of hematological cancers and treatment modalities. Included among these legacies was an evident increase of CD8⁺ T_{EM} cells in G₁, detected by the T_{D5} flow cytometry assay [32], that was not observed in solid cancers [25].

Although the number of flow cytometry studies looking at cancer patients with COVID-19 remains relatively small, there are some clear and provocative implications. First, that routine cancer care can probably be sustained for those with solid cancers with limited added risk, whereas treatment centres for patients with blood cancers should guard against a high risk of prolonged virus exposure between patients and their carers. Second, that immune response to SARS-CoV-2 vaccination might be partially impaired in some

patients with solid cancers, but more profoundly impaired in those with hematological cancers. This prospect was confirmed in our recent study on the safety and immunogenicity of the COVID-19 mRNA vaccine BNT162b2 (Pfizer-BioNTech) in oncological settings for which interim results were reported [41]. That study showed that two doses of BNT162b2 vaccine were required for seroconversion in most patients with solid cancer, whereas the immunogenicity of either one or two doses of the vaccine was often very poor in patients with hematological malignancies [41]. In contrast, the vast majority of healthy individuals developed strong anti-SARS-CoV-2 IgG and SARS-CoV-2-reactive T cell responses at 3–5 weeks after a single vaccine dose [41]. A reduced seroconversion rate of patients with hematological cancers following SARS-CoV-2 vaccination was likewise reported by another study [42]. The extent of protection provided by vaccine-induced T cells in seronegative patients remains to be determined.

5 | PREDICTION OF COVID-19 DISEASE TRAJECTORY

One of the major aspects of healthcare provision in a pandemic is resource allocation and planning; for example, predicting future burden on Intensive Care Unit (ICU) beds and ventilators, and anticipating duration of hospitalization. Any capacity to accurately predict disease progression can allow for more appropriate prioritization of care. Currently our understanding of a patient's risk of developing severe COVID-19 is based on very broad demographic considerations; age, BMI, sex, ethnicity etc. coupled with relatively variably measured clinical parameters; history of hypertension, blood oxygen saturation, C Reactive Protein (CRP), lymphocyte counts etc. [43–46]. Several laboratory parameters including CRP and lymphocyte counts are integrated into the ISARIC 4C mortality score [47], the only scoring system that thus far show promise within a living systematic review of prognostic models.

Thus, much attention has been given to the potential utility of additional prognostic markers or signatures that may more accurately reflect the direct interaction of the host with the virus. Those include virus and host genetics [48], and serum cytokine levels, for example, IFN α , IL-6, and IP-10 [1, 49, 50]. To these may be added changes in specific blood cell populations that can be accurately quantitated by high-throughput flow cytometry, contingent on appropriate panel design. Indeed, across highly heterogeneous patient groups, consensus severity-related parameters emerged, including flow cytometry determined T cell quantification [1].

There is much opportunity for this approach to be developed further. More specifically, one can envision the development of databases that record typical values of discrete immune parameters for individuals of defined ages, gender, BMI etc., whereupon one can track the degree of deviation from this “norm” that any one person displays early in their response to SAR-CoV-2 exposure. One might then envision that different degrees of deviation may constitute useful prognostic indicators of disease progression or response to

treatment in myriad yet defined settings. In this regard, automated analysis can provide an extremely useful tool to overcome some limitations of the manual gating approach, including biased cell subset identification, and long processing time [51]. An advanced workflow for high-throughput flow cytometry should also include data preprocessing and quality checking before cell population identification [10, 52].

These principles have been applied by a recent COVID-19 study on >900 individuals among patients and controls, enabling the identification of reduced B-cell and non-classical monocyte percentages as independent prognostic factors (Table 2, [24]). Notably, the widespread implementation of automatic pipelines for high-throughput flow cytometry in different studies would facilitate both data sharing between researchers working at various institutions, and metadata comparison across diverse projects, ultimately greatly advancing the speed of discovery in keeping with the demands imposed by rapidly emerging pandemic infections.

6 | CONCLUDING REMARKS

In response to the COVID-19 pandemic, researchers across the globe have re-focused research efforts into understanding the immune response to SARS-CoV-2. The emerging picture shows that a delayed or impaired IFN-I response is a strong determinant of viral evasion, while antibody response follows the expected pattern, and T cell response correlates with B cell response; the role of pre-existing adaptive immunity to common-cold coronaviruses remains to be determined [53]. The scale of the data generated by high-throughput flow cytometry, in individual studies and in meta-analyses, when coupled with ever-more incisive clinical annotation, offers the opportunity to better understand a patient's clinical condition, and the potential to identify prognostic cellular immune signatures. Collectively, these can lead to more personalized patient care, reduce the burden on health care systems, and suggest novel therapeutic modalities rather than the re-purposing of broadly acting drugs such as dexamethasone or anti-IL-6. Indeed, high-content flow cytometric methodology when applied at appropriate scale and throughput, as was the case in COVID-IP, may provide a prototype for the utilization of cellular immunology to understand and predict disease progression across multiple disease states.

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

A.C.H. is a board member and equity holder in ImmunoQure, AG., and Gamma Delta Therapeutics, and is an equity holder in Adaptate Biotherapeutics. I.d.M.d.B. is employed by GSK. T.S.H. and A.G.L. are employed by Melio Health Ltd.

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