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Immunologic characterization of COVID-19 patients with hematological cancer

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The rapid spread of the coronavirus disease 2019 (COVID-19) pandemic is having a profound impact in oncologic care, with recent analyses suggesting that cancer patients may have an increased risk of severe complications, including hospitalization, respiratory failure and death. Severe events from initial onset of COVID-19 appear to be more frequent in individuals with blood malignancies vs other cancer types. Furthermore, an increased risk of death in patients with hematological cancer infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been identified in some studies, though unconfirmed in others. Here, we analyzed outcomes and immune response to SARS-CoV-2 in a large series of 513 patients with COVID-19 that included ten cases with blood cancer. This study confirms that the later have greater risk of fatal COVID-19 and shows for the first time that hematological patients display specific immune alterations that could compromise a response to the infection.

The immune system reacts to viral infection with cellular and humoral responses. Thus, myelo- and lympho-suppression caused by cancer itself as well as cytotoxic treatment may pose a challenge to COVID-19 patients with hematological tumors. Indeed, lymphopenia has been associated with worse prognosis in the general population and preliminary data showed lower neutrophil and lymphocyte counts in COVID-19 patients bearing hematological cancer. However, there are conflicting results supporting that both worsening of lymphopenia during COVID-19 and prior to infection had a beneficial impact on survival. Accordingly, the role of some antineoplastic drugs (e.g., inhibitors of PD-1, BTK, JAK1/2, XPO-1 and tyrosine kinases, as well as thalidomide) in mitigating the harmful immune response associated with severe COVID-19 is being investigated. At the same time, it was found that checkpoint inhibitor immunotherapy is a risk factor for severe outcomes in COVID-19 patients with cancer. Thus, greater knowledge on the immune status of hematological patients may be useful to optimize prevention, risk stratification and treatment strategies.

From March to May 2020, 513 consecutive patients with a SARS-CoV-2 positive result had peripheral blood (PB) samples taken at presentation for immune profiling using multidimensional flow cytometry (MFC). Additional PB samples were collected during follow-up in 167 of the 513 cases. Data was analyzed with a semi-automated pipeline that performs batch-analyses of MFC data to avoid variability intrinsic to manual analysis, and unveils full cellular diversity based on unbiased clustering. In PB samples from 14 COVID-19 patients, higher-resolution MFC was performed to characterize antigen-dependent differentiation of B and T cells. Furthermore, various myeloid subsets and antigen-presenting cells were isolated by
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FACS for transcriptome analysis using RNAseq (all methods described in Supplemental Material). The Clínica Universidad de Navarra Ethics Committee approved the protocol and informed consent forms, required prior to patient enrollment. The study was conducted per the ethical principles of the Declaration of Helsinki.

Of the 513 COVID-19 patients included in this study, 10 had a hematological tumor (Supplemental Table 1). These patients showed similar frequency of hospitalization than those without an active tumor (80% vs 77%). By contrast, the frequency of hematological cases requiring intensive care (50%) and dying from COVID-19 (30%) was significantly higher to that observed in patients without hematological cancer (5% and 4%, respectively) (Table 1). These outcomes are consistent with previous results observed in the general population and some series of hematological patients infected by SARS-CoV-2.2,4,5

Studying immunological biomarkers has become notoriously important in COVID-19, since immunopathology was suggested as a primary driver of morbidity and mortality in these patients.11 However, there are no studies analyzing the immune response to SARS-CoV-2 at presentation and during follow-up in hematological cases. Here, we performed a holistic and unbiased analysis of MFC data that enabled the systematic quantification of 17 cell types (including 5 myeloid and 12 lymphoid subsets), in all 680 PB samples corresponding to 513 COVID-19 patients taken at presentation and during follow-up (Figure 1A). Those with hematological tumor showed significantly decreased percentages of classical monocytes, immunoregulatory NK cells, double-positive T cells, and B cells, when compared to COVID-19 patients without hematological cancer (Figure 1B). Similarly, hematological patients tended to have also reduced absolute cell counts of various cell types, reaching statistical significance in double-positive T cells (Supplemental Figure 1). While the aspects of cancer and its treatment conferring risk of severe COVID-19 have remained largely unknown 3, this study exposes for the first time that hematological patients show significant alterations in the relative distribution of specific innate and adaptive cell types, which could compromise an initial response to the infection.

Effective viral clearance requires CD8 effector T cell-mediated killing of virally infected cells as well as CD4 T cell-dependent enhancement of CD8 and B-cell responses. Interestingly, deep phenotypic characterization of T and B cell compartments in PB of COVID-19 patients with (N = 4) or without (N = 10) hematological cancer showed that the relative distribution of antigen-dependent maturation stages within the T cell compartment was generally similar between both groups (Supplemental Figure 2A). By contrast, hematological cases displayed alterations in several B cell subsets that reached statistical significance in memory B
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cells expressing IgG and IgA subclasses (Supplemental Figure 2B). These findings are important because the humoral immune response is critical for the clearance of SARS-CoV-2 and could be a major part of the memory response that prevents reinfection.\textsuperscript{11}

We next compared immune kinetics from presentation to last follow-up in COVID-19 patients with and without hematological tumor (N = 6 and 161, respectively), depicting those with favorable or fatal outcome (Figure 1C). There was a profound variation from the first to the latest PB sample in the relative distribution of all immune cell types in COVID-19 patients bearing hematological tumor when compared to other cases. Although immune kinetics were quite variable, cancer patients dying from COVID-19 tended to have increasing numbers of neutrophils counterbalanced by reduced percentages of other immune cell types vs those who survived (Figure 1C). Additional studies in larger series are warranted to confirm these findings; if so, individualized management of COVID-19 patients with hematological tumor according to their immune status at presentation and during follow-up should be investigated to improve outcomes in this population.

In an attempt to confirm these findings in a larger series, we were able to analyze PB samples from five additional COVID-19 cases with blood cancer from other Hospitals who also performed immune profiling by MFC. A detailed analysis of major immune cell types in the 15 hematological cases vs the 503 COVID-19 patients without blood cancer, confirmed recurrent altered distribution of basophils, eosinophils, neutrophils, monocytes, NK, T and B lymphocytes as well as circulating PCs (Figure 2). Furthermore, such alterations appeared to be more profound in deceased cases (two with acute myeloid leukemia and two with lymphoma) and reached statistical significance regarding B cell distribution. Humoral immunity is critical for the clearance of SARS-CoV-2, as evidenced by the rapid and near-universal detection of virus-specific neutralizing antibodies. Thus, very low B cell numbers in patients with blood cancer because of tumor expansion and/or specific drugs (e.g. immuno[chemo]therapies targeting B cell and PC antigens) could emerge as another biomarker to predict disease severity after SARS-CoV-2 infection, together with advanced age and comorbidities that commonly affect hematological cases. Noteworthy, the median age of COVID-19 patients with or without blood cancer was 73 and 60 years, respectively (P = .049), which could also have contributed to poorer outcomes in the former.

SARS-CoV-2 infection of respiratory epithelial cells has been shown to activate monocytes, macrophages and dendritic cells \textsuperscript{11}, with an increasing number of studies suggesting that heightened inflammation is a defining feature of severe COVID-19. Thus, we specifically aimed at comparing the transcriptional profile of myeloid and
antigen-presenting cells in COVID-19 patients with (N = 3) or without (N = 10) hematological tumor. Unsupervised hierarchical analysis of RNAseq data from basophils, myeloid and plasmacytoid dendritic cells, classical and non-classical monocytes and neutrophils showed considerable clustering of samples from hematological cases (Supplemental Figure 3A). Furthermore, a variable number of differentially expressed genes was found in all six cell types between COVID-19 patients with or without blood cancer (Supplemental Figure 3B). Genes related to NF-κB and STAT transcription factors as well as genes encoding Toll-like receptors and proinflammatory interleukin receptors, all of which described to be implicated in the response and evasion of innate sensing by coronaviruses 11, were differentially expressed in many of these cell types. Although preliminary, these data suggest that myeloid and antigen-presenting cells could be phenotypically altered in COVID-19 patients with hematological tumor.

Immune impairment in patients with hematological malignancies has been well-documented.12 Moreover, there is growing body of evidence supporting that immune defects emerge in pre-malignant stages such as monoclonal B-cell lymphocytosis (MBL) and monoclonal gammopathy of undetermined significance (MGUS) 13,14, which are present in 3% or more of individuals older than 50-years. Thus, our results unveiling altered immune profiles in COVID-19 patients with benign and malignant hematological cancer could be relevant to a considerable number of elderly adults worldwide. However, while MBL and MGUS cases displayed immune alterations similar to patients with malignant tumor (Figure 2), their outcome was favorable as compared to patients with malignant disease, in line with recent observations 15. Accordingly, this study should foster further investigation to clarify if all hematological cases or only those with hematological malignancies are at risk of severe COVID-19.
ACKNOWLEDGEMENTS

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DISCLOSURE OF CONFLICTS OF INTEREST

All authors declare no competing financial interests.
REFERENCES


Leukemia. 2018;32(12):2701-2705.

**TABLES**

**Table 1.** Outcome of patients diagnosed with COVID-19 (N = 513) regarding hospitalization, need of intensive care and survival.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>No tumor (N = 503)</th>
<th>Hematological tumor (N = 10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospitalization, N (%)</td>
<td>385 (77%)</td>
<td>8 (80%)</td>
<td>.324</td>
</tr>
<tr>
<td>Intensive care, N (%)</td>
<td>27 (5%)</td>
<td>5 (50%)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Death, N (%)</td>
<td>20 (4%)</td>
<td>3 (30%)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Immune response in patients with COVID-19 and hematological cancer. 
(A) Schematic representation of semi-automated analysis of flow cytometric data and clustering of 17 immune cell types systematically identified in a total of 513 patients diagnosed with coronavirus disease 2019 (COVID-19). (B) Relative distribution of immune cell types in COVID-19 patients with \( N = 10 \), dots) and without \( N = 503 \), boxes) blood cancer. (C) Ratio between the percentage of each immune cell type at last follow-up and presentation in COVID-19 patients with \( N = 6 \), dots) and without \( N = 161 \), line) hematological tumor. Blue dots indicate alive patients and red dots represent deceased patients. Dashed line represents no variation over time (ratio = 1). PCs: plasma cells
*, \( P < .05 \); **, \( P < .01 \); ***, \( P < .001 \)

Figure 2. Distribution of major immune cell types in patients with COVID-19 according to type of hematological cancer and outcome. Patients were classified according to the pre-malignant vs malignant stage of the disease, tumor type and outcome. Grey boxes represent the distribution of COVID-19 patients without blood cancer, and dots correspond to hematological patients. Black asterisks indicate significant differences between patients without and with hematological tumor, and red asterisks represent significant differences between alive and deceased patients with hematological cancer. No significant differences were observed between pre-malignant and malignant stages.
*, \( P < .05 \); **, \( P < .01 \)
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SUPPLEMENTAL METHODS

Multidimensional flow cytometry (MFC). EDTA anti-coagulated peripheral blood (PB) samples were stained with the 8-color combination of the monoclonal antibodies (mAb) CD3-V450, CD45-OC515, CD20-FITC, CD16-PE, CD4-PerCPCy5.5, CD19-PECy7, CD56-APC, CD8-APCH7, lysed for 30 min and measured directly without centrifugation and washing steps to minimize risk of infection in a FACSCanto II flow cytometer (Beckton Dickinson –BD– Biosciences, San Jose, CA, USA) using the FACSDiva 6.1 software (BD, San Jose, CA, USA). Data was analyzed using automated clustering (described below). In a subset of COVID-19 patients (N = 14), PB B and T cells were characterized using EuroFlow panels for primary immunodeficiencies 1, and samples were processed with the EuroFlow lyse-wash-and-stain standard sample preparation protocol (SOP) adjusted to 10^5 nucleated cells. Data was acquired in a FACSLyric flow cytometer (BD Biosciences, San Jose, CA) using the FACSSuite v1.3.0.6137 software (BD), and analyzed with the Infinicyt software (Cytognos SL, Salamanca, Spain).

Automated clustering. We used FlowSOM (version 1.14.1) 2 for automated clustering. Briefly, it is based on a four-step approach: 1) reading data; 2) building a self-organizing map (SOM) for clustering and dimensionality reduction; 3) building a minimum spanning tree to connect nodes according to their similarity; and 4) computing an automated meta-clustering by grouping similar nodes. The meta-clustering step is critical for the definition of cell populations. In this phase, groups of similar nodes are “fused” to obtain more consistent populations following specific algorithms. We used the ConsensusClusterPlus 3 (version 1.46.0) package separated from FlowSOM to obtain better control of each function. Clonal B cells clustering according to their bright CD19 expression and dim reactivity for CD20 were excluded from the whole B-cell cluster in patients with B-cell lymphoproliferative disorders. However, it should be noted that the combination of monoclonal antibodies described above and the number of cells measured per sample, are not empowered to detect small B-cell clones in subjects with unknown history of monoclonal B-cell lymphocytosis.

Fluorescence-activated cell sorting (FACS). Various myeloid subsets and antigen-presenting cells were stained using the mAb combination – HLADR-PacB, CD45-OC515, CD15-FITC, CD203c-PE, CD33-PerCPCy5.5, CD16-PECy7, CD123-APC, CD14-APCH7 – and isolated in a MoFlo Astrios EQ sorter (Beckman Coulter, CA,
USA). Based on its six-way sorting, basophils, myeloid and plasmacytoid dendritic cells, classical and non-classical monocytes and neutrophils were simultaneously isolated from PB samples of 13 COVID-19 patients. All cell types were successfully isolated in all cases except for plasmacytoid dendritic cells and basophils in 2 patients. Cells were stored in Lysis/Binding Buffer (Invitrogen™, CA, USA).

**RNA sequencing (RNAseq) and data analysis.** RNAseq was performed using a protocol adapted from massively parallel single-cell RNA-sequencing [4], which enabled preparing libraries with as few cells as starting material. Briefly, we barcoded RNA from each sample in a retrotranscription (RT) reaction with AffinityScript Multiple Temperature Reverse Transcriptase (Agilent, Santa Clara, CA, USA) and different RT primers. After qPCR, cDNA with similar Ct values were pooled together. cDNA was purified with SPRlselect 1.2X (Beckman Coulter –BC–, Brea, CA, USA) and amplified using the T7 polymerase (New England Biolabs – NEB–, Ipswich, MA, USA) and the T7 promoter as template, previously introduced in the RT reaction. Samples were incubated for 16 hours at 37°C. RNA molecules were fragmented with 2 µL of 10X Zn²⁺ fragmentation buffer (Ambion™, ThermoFisher, Waltham, MA, USA) for 1 min at 70°C and purified with SPRlselect 2X. Afterwards, a ssRNA adaptor (Illumina, San Diego, CA, USA) was ligated to the 3'-end of the RNA fragments in presence of DMSO, 100 mM ATP, 50% PEG and T4 RNA ligase I (NEB, Ipswich, MA, USA) for 2 hours at 22°C. A second RT reaction was performed with AffinityScript Multiple Temperature Reverse Transcriptase and resulting cDNA was purified with SPRlselect 1.5X. Finally, cDNA was amplified with 12.5 µL Kappa Hifi ready mix + 1µL of primer mix at 25 µM per sample and purified with SPRlselect 0.7X. Qubit, TapeStation and qPCR analyses were done as quality controls and the final library products at 4 nM were sequenced in a NextSeq (Illumina, San Diego, CA, USA).

Differential gene expression across all pairwise comparisons (hematological vs no cancer) of sorted immune populations was analyzed with Deseq2 R package (version 1.28.1). [5] Data is available in the GEO database with the accession number GSE153610.

**Statistical analysis.** The Kruskal-Wallis and Mann Whitney tests were used to estimate the statistical significance observed between groups. Statistical analyses were performed using the GraphPad Prism software (version 7, San Diego, CA, USA), SPSS (version 25.0.0, IBM, Chicago, IL, USA) and R (versions 3.5.1 and 4.0.0 for MFC and RNAseq studies, respectively). P values < .05 were considered as statistically significant.
REFERENCES
**Supplemental Table 1.** Demographics and clinical course of patients with COVID-19 and hematological cancer (N = 10).

<table>
<thead>
<tr>
<th>Patient - Disease</th>
<th>Sex</th>
<th>Age</th>
<th>Clinical course until immune monitoring</th>
<th>Time between anticancer treatment and COVID-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - IgM MGUS</td>
<td>M</td>
<td>74</td>
<td>Asymptomatic</td>
<td>NA</td>
</tr>
<tr>
<td>2 - CLL</td>
<td>M</td>
<td>77</td>
<td>Early stage, untreated</td>
<td>NA</td>
</tr>
<tr>
<td>3 - IgG MGUS</td>
<td>M</td>
<td>88</td>
<td>Asymptomatic</td>
<td>NA</td>
</tr>
<tr>
<td>4 - CLL</td>
<td>M</td>
<td>62</td>
<td>Early stage, untreated</td>
<td>NA</td>
</tr>
<tr>
<td>5 - CLL</td>
<td>M</td>
<td>57</td>
<td>Early stage, untreated</td>
<td>NA</td>
</tr>
<tr>
<td>6 - AML</td>
<td>F</td>
<td>64</td>
<td>Secondary to MDS that was secondary to NHL. Studied in CR after allogenic stem cell transplantation</td>
<td>1 month</td>
</tr>
<tr>
<td>7 - DLBCL</td>
<td>F</td>
<td>31</td>
<td>Studied in CR after 6 cycles with R-CVP</td>
<td>12 months</td>
</tr>
<tr>
<td>8 - Follicular lymphoma</td>
<td>F</td>
<td>71</td>
<td>Studied after 1 cycle of R-CVP</td>
<td>On treatment</td>
</tr>
<tr>
<td>9 - MBL</td>
<td>M</td>
<td>89</td>
<td>Asymptomatic</td>
<td>NA</td>
</tr>
<tr>
<td>10 - MDS</td>
<td>M</td>
<td>86</td>
<td>Newly-diagnosed</td>
<td>NA</td>
</tr>
</tbody>
</table>

MGUS, monoclonal gammopathy of undetermined significance; CLL, chronic lymphocytic leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; NA: not applicable; NHL: non-Hodgkin lymphoma; CR, complete remission; DLBCL, diffuse large B-cell lymphoma; R-CVP, rituximab, cyclophosphamide, vincristine and prednisone; MBL, monoclonal B-cell lymphocytosis
Supplemental Figure 1. Absolute number of immune cell types in COVID-19 patients with (N = 10) and without (N = 503) blood cancer.

PCs: plasma cells

*, P < .05
Supplemental Figure 2. Antigen-specific differentiation of adaptive immune cells after SARS-CoV-2 infection. Relative distribution of various (A) T and (B) B cell subsets in COVID-19 patients without cancer (N = 10) and those with hematological malignancies (N = 4).

TEMRA: T cell effector memory CD45RA+; GC: germinal center; PCs: plasma cells

*, P < .05
Supplemental Figure 3. Transcriptional status of myeloid and antigen-presenting cells in patients with COVID-19 and hematological cancer. (A) Unsupervised correlation (Pearson’s method) heatmap based on RNAseq data from basophils, myeloid and plasmacytoid dendritic cells, classical and non-classical monocytes as well as neutrophils from COVID-19 patients with (N = 3) or without (N = 10) hematological cancer. (B) Volcano plots based on gene expression of basophils, myeloid and plasmacytoid dendritic cells, classical and non-classical monocytes as well as neutrophils from COVID-19 patients with (N = 3) or without (N = 10) hematological cancer. Each dot corresponds to an individual gene. Differentially expressed genes (minimum log<sub>2</sub> fold-change and adjusted \( P \) value < .05) were given a unique color for each cell type: basophils (dark yellow, n=112 infra/19 overexpressed), myeloid (green, n=9/1) and plasmacytoid (dark blue, n=495/43) dendritic cells, classical (cyan, n=188/56) and non-classical (grey, n=26/13) monocytes as well as neutrophils (red, n=436/81). Differentially expressed genes encoding selected transcription factors, Toll-like and interleukin receptors are indicated.
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A

B

Over-expressed in haem. tumors  ↔  Over-expressed in non-haem. tumors