Immature granulocytes can help the diagnosis of pulmonary bacterial infections in patients with severe COVID-19 pneumonia

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Abstract
During COVID-19, immature granulocyte (IG) concentration is heterogeneous with higher concentrations than those found in bacterial sepsis. We investigated the relationship between IG levels at ICU admission and on days 7 (± 2) and 15 (± 2) and associated pulmonary bacterial infections in intensive care unit (ICU) patients hospitalized for an acute respiratory distress syndrome (ARDS) related to SARS-CoV-2. Patients with associated pulmonary bacterial infection had a peak of IGs. IG thresholds of 18% or 2 G/L allowed discriminating patients with ventilator associated pneumonia with 100% sensitivity and specificity. Our study supports that IGs could help identifying pulmonary bacterial infections in this population.

Keywords: Biomarker, COVID-19, Immature granulocytes, Secondary infections, Ventilator-associated pneumonia

Introduction
Patients hospitalized in the intensive care unit (ICU) with an acute respiratory distress syndrome (ARDS) related to SARS-CoV-2 frequently develop associated pulmonary bacterial infections, including ventilator-associated pneumonia (VAP) which diagnosis is challenging in this clinical setting [1, 2]. In bacterial sepsis and severe COVID-19, the myeloid cell compartment is dysregulated and circulating levels of immature granulocytes (IG) may increase [3, 4]. The range of IG increase appears highly variable in COVID-19 [4, 5]. We previously showed that septic patients exhibit higher IG levels than patients with severe SARS-CoV-2 infection [3, 5]. We hypothesized that IG levels heterogeneity could be related to the development of pulmonary bacterial infections in patients mechanically ventilated for a SARS-CoV2-induced ARDS.

Methods
Between December 2020 and March 2021, consecutive patients without known immunosuppression who required invasive mechanical ventilation for severe COVID-19 pneumonia were prospectively enrolled. The evolution of peripheral blood leukocyte populations were studied, from ICU admission to day 7 (± 2) and day 15 (± 2). Using flow cytometry, leukocyte populations were discriminated with CD3 for the T cells, CD19 for the B cells, CD14 for the monocytes, and CD16 for the granulocytes. CD45 was used to identify the hematopoietic cells and CD64 was used as a marker of neutrophils activation. Immature granulocytes or “band cells” were characterized as CD45+CD3−CD19−CD14−CD16dim/neg (Fig. 1). Monocyte expression of HLA-DR, CD4+ and CD8+ T lymphocytes counts were also analyzed.

An independent committee blindly adjudicated the diagnosis of pulmonary bacterial infections during the
Fig. 1  Example of flow cytometry biparametric histograms showing the gating strategy used to identify immature granulocytes (IG) in peripheral blood of COVID-19 patients. The examples shown here are merged data of one same patient on day 0 (in blue dot) and day 7 (in red dot) to illustrate gating strategy. A Hematopoietic cells were selected on specific morphological parameter (Side Scatter channel, reflecting the granularity of the cytoplasm) and expression of CD45 (a pan-leukocyte marker). B Hematopoietic cells positive for CD14 (monocyte maker) were considered as monocytes, and the ones positive for CD3 (T cell marker) as T lymphocytes. The red square corresponds to cells that are negative for these two markers (Not T not mono). C Side scatter (cytoplasm granularity) and CD19 (B cell marker) were used to separate the neutrophils (red gate) and the B lymphocytes, respectively. D Neutrophils were divided into two subtypes (i) mature granulocytes strongly positive for CD16 (CD16+) and (ii) Immature granulocyte (IG) low or negative for CD16. CD64 was used as an activation marker.

Table 1  Study population

| Demographics | Study population (n = 19) | Bacterial pulmonary infection (n = 12) | No bacterial infection (n = 7) |
|--------------|--------------------------|--------------------------------------|-------------------------------|
| Age          | 72 [63;74.5]             | 72.5 [59.5;73.8]                     | 70 [65;74.5]                 |
| Gender       |                          |                                      |                              |
| Male, n (%)  | 13 (68)                  | 10 (83)                              | 3 (43)                       |
| BMI, kg/m²   | 26.8 [24.5;31.5]         | 26.4 [23.4;31.2]                     | 27 [26.29.8]                 |
| BMI > 30, n (%) | 6 (32)              | 4 (33)                               | 2 (29)                       |
| Comorbidities, n (%) |                  |                                      |                              |
| Hypertension | 6 (32)                   | 3 (25)                               | 3 (43)                       |
| Diabetes     | 6 (32)                   | 4 (33)                               | 2 (29)                       |
| COPD         | 2 (11)                   | 2 (17)                               | 0                            |
| Chronic heart failure | 1 (5)                  | 1 (8)                                | 0                            |
| Chronic renal failure | 2 (11)             | 1 (8)                                | 1 (14)                       |
| Immunosuppression | 1 (5)                 | 0                                    | 1 (14)                       |
| ICU admission |                          |                                      |                              |
| SAPS II      | 29 [26;35.5]             | 29 [26.5;33.5]                       | 33 [26.39]                   |
| SOFA score   | 2 [2;4]                  | 2 [2;4]                              | 3 [2;3]                      |
| Days from onset of disease to ICU admission | 8 [6;11]        | 7.5 [4.75;9]                         | 10 [9;15.5]                  |
| Steroids before ICU admission, n (%) | 3 (16)            | 2 (17)                               | 1 (14)                       |
| Mechanical ventilation at ICU admission, n (%) | 5 (26)            | 4 (22)                               | 1 (14)                       |
| Severe ARDS at ICU admission, n (%) | 7 (37)            | 3 (25)                               | 4 (57)                       |
| Steroids, n (%) | 3 (16)                 | 2 (17)                               | 1 (14)                       |
| Dead at ICU discharge, n (%) | 6 (32)            | 6 (50)                               | 0                            |

BMI body mass index, COPD chronic obstructive pulmonary disease, ICU intensive care unit, SPAS Simplified Acute Physiology Score, SOFA sequential organ failure assessment, ARDS acute respiratory distress syndrome
ICU stay based on clinical findings (fever, new onset of purulent endotracheal sputum or modification of sputum characteristics, auscultation abnormalities, increasing need of oxygen therapy), biological abnormalities (hyperleukocytosis, decreased PaO2/FIO2), radiological data (new onset or worsening of pulmonary infiltrate), and microbiological documentation. Pulmonary bacterial infections diagnosed within the first 2 days of ICU stay were considered as co-infections, while those diagnosed later were reported as VAP.

Results

Nineteen patients ventilated for severe COVID-19 were studied (72 [63.0–74.5] y.o; SAPS II: 29 [26.0–35.5]; mortality rate: 32%). Severity scores, comorbidities and steroids use were similar, irrespective of the presence of a pulmonary bacterial infection (Table 1). Two patients were admitted to ICU with a pulmonary bacterial co-infection, whereas 10 patients developed VAP (median diagnosis: 6.5 [4.3–7.8] days). On ICU admission, patients without pulmonary co-infection (n = 17) exhibited markedly lower circulating IG levels in absolute count (0.40 ± 0.75 G/L) and in percentage (3.22 ± 3.78%) than those with bacterial co-infection (2.30 G/L and 9.37 G/L in absolute count, or 75% and 84% in percentage) (Fig. 2A). In the two patients with bacterial co-infection at admission, IG absolute numbers and frequencies decreased with time (Additional file 1: Figure S1). On day 7, patients who developed VAP on day 7, and were close to those observed on ICU admission (8.7 ± 5.6% and 1.75 ± 1.13 G/L; Fig. 2C). IG levels were moderately correlated to the SOFA score (Sequential Organ Failure Assessment) (Fig. 3; Spearman test r = 0.62). Total neutrophil count tended to be increased in patients with VAP but without reaching statistical significance. No significant difference was noticed on day 7 in the neutrophil to lymphocyte ratio (NLR) (Fig. 2D). CD4 and CD8 T lymphocyte counts as well as HLA-DR monocyte expression were similar between patients (Fig. 2E, F).
Discussion

Our results, even if obtained on a small cohort, suggest a strong association between level of IGs and pulmonary bacterial infection. Provided consolidation, they indicate that the peak of IG observed in case of bacterial associated infection could be a characteristic reaction of COVID-19.

In contrast to previous studies [4, 6, 7], IG levels were not associated with clinical severity in our patients as reflected by the absence of correlation between IG level and SOFA score. Nevertheless, the occurrence of secondary bacterial infection was not documented in these studies [4, 6, 7]. Associated pulmonary bacterial infections affect approximatively 50% of ventilated patients with severe COVID-19 [8]. This incidence appears superior to that observed in influenza or non-viral pneumonia [9]. In the clinical setting of acute viral ARDS, bacterial infection is associated with an increased risk of death [10]. In addition, radiological and clinical criteria are often inconclusive and, the diagnosis as well as the start of antibiotics mainly rely on microbiological documentation [2, 11]. Due to the severity of SARS-CoV-2 pneumonia in ICU patients, the occurrence of an associated bacterial pulmonary infection requires early antibiotic therapy. Nevertheless, inappropriate antibiotic prescription could favor the emergence of multidrug resistant bacteria which could also jeopardize outcome. Therefore, a reliable biomarker would be highly beneficial in the context of the challenging diagnosis of associated bacterial infection in severe COVID-19.

Even if our results need to be validated by further larger scale studies, our proof of concept study supports that IGs could be an interesting biomarker of bacterial over-infections in ICU patients with severe COVID-19, especially since more and more hospitals have access to flow cytometry.
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