Immature neutrophils and high anti-SARS-CoV-2 IgG levels are hallmarks of severe and critical COVID-19 infection

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Title: myelopoiesis in severe COVID-19

Word count: 2521

Abstract: 194

References: 45
Table: 3
Figure: 3
ABSTRACT

Introduction
High neutrophil-to-lymphocyte ratio was reported as a biomarker for severe COVID-19 infection. Here, we correlate SARS-CoV-2 nasopharyngeal viral load, blood count parameters, lymphocyte typing and serology of infected patients according to the severity of the disease.

Material and methods
Patients with qRT-PCR-confirmed SARS-CoV-2 infection and available blood cell counts, serology (immunofluorescence and ELISA) and lymphocyte typing (total lymphocyte count, B, T, CD4, CD8 and NK cells counts) were included. Three groups of patients were defined and compared, those with mild, moderate and severe or critical disease.

Results
Seventy-seven patients were included, 18 had mild, 28 moderate and 31 severe/critical COVID-19 infection. Eight patients died. SARS-CoV-2 nasopharyngeal viral load was similar in the three groups. Multivariate analysis showed that initial eosinopenia (<0.04 G/L) was significantly associated severe/critical disease (OR: 4.6; 95 IC [1.1-19] p<0.05) as well as neutrophilia (OR: 6.9; 95 IC [1.6-29] p<0.05), myelemia (OR: 13; 95 IC [4.2-40.5] p<0.001) and high anti-SARS-CoV-2 antibody titers (OR: 4.8; 95 IC [1.4-16.7]; p<0.05).

Conclusion
The egress of immature granulocytes, so called "myelemia", together with neutrophilia and high anti-SARS-CoV-2 IgG antibodies, preceded by profound eosinopenia and lymphopenia (T, B, CD4) are hallmarks of severe and critical COVID-19 infection.
**Key words:** COVID-19, SARS-COV-2, immature neutrophils, humoral response, myelemia
Introduction

The unprecedented outbreak of COVID-19 has been declared a public health emergency of international concern, resulting in 42,990,580 reported cases and 1,155,437 deaths as of October 25th 2020 (Coronavirus disease (COVID-2019) situation reports). It has recently been estimated that nearly 80% of patients with SARS-CoV-2 infection have mild disease, i.e. no or few symptoms, while about 20% have evolved toward severe COVID-19 infection (Huang et al., 2020). Two major phenomena contribute to the severity of the disease. The first is coagulopathy, driven by endothelial cell injury, thrombo-inflammation phenomena and renine angiotensine aldosterone system deregulation. The second is the exacerbated immune response often associated with a cytokine storm (Melenotte et al., 2020; Qin et al., 2020). These phenomena are respectively reported in the second and third phase of the disease, promoting both thromboembolic events, acute respiratory distress syndrome and multi-organ failure (phase IV), whereas the SARS-CoV-2 viral load is declining (Melenotte et al., 2020). Irreversible organ lesions may finally occur, sometimes resulting in death (Xu et al., 2020).

Several biological markers of severity have been proposed, including high levels of D-dimer, fibrinogen, troponin, interleukin-6, eosinopenia, high neutrophil/lymphocyte ratio and high anti-SARS-CoV-2 antibodies (Melenotte et al., 2020; Okba et al., 2020; Tanni et al., 2020; Zhang et al., 2020). Recently, using high-tech tools, immature granulocytes have been identified as a marker of the severity of COVID-19 infection, while the T cell immune response plays a crucial role in the healing of the disease (Schulte-Schrepping et al., 2020a; Silvin et al., 2020). Elevation of CD8 and CD4 T cells is involved in the effectiveness of the immune response, and CD4 and CD8 T-cell lymphopenia are significantly associated with poor clinical outcome (Melenotte et al., 2020). Here, by analyzing SARS-CoV-2 nasopharyngeal viral load, blood count data, lymphocyte typing and serology of COVID-19 patients, we correlated immune biological parameters to the clinical outcome. In patients presenting severe/critical COVID-19
infection, we observed deep initial eosinopenia and peripheral lymphopenia, followed by neutrophilia, myelemia and high anti-SARS-CoV-2 IgG titers, indicating emergency activation of the bone marrow.
Material and method

Study design

COVID-19 infection was confirmed by SARS-CoV-2 specific qRT-PCR assay on nasopharyngeal swab as previously described (Lagier et al., 2020, 37). A treatment with hydroxychloroquine in addition to azithromycin was proposed to all qRT-PCR positive patients in the absence of contraindication (drug interactions and heart rhythm disturbances) (Lagier et al., 2020, 37). Symptomatic patients were followed up on an outpatient basis at 6 and 10 days after diagnosis or were hospitalized depending on the severity of the disease. Clinical data, age, sex, hospitalization and outcome were collected retrospectively from medical record. We included all patients for whom blood cell count, serology and lymphocyte typing (total lymphocyte, B, T, CD4, CD8 and NK cells counts) were available.

Patients

Among the included patients, three groups were defined according to the severity of the disease (https://www.who.int/publications/i/item/clinical-management-of-covid-19). The first group had mild disease and favorable clinical outcome (https://www.who.int/publications/i/item/clinical-management-of-covid-19). The second group consisted of patients with moderate COVID-19 infection requiring hospitalization in a conventional medical unit (CMU) but not in an intensive care unit (ICU) (https://www.who.int/publications/i/item/clinical-management-of-covid-19). The third group was comprised of patients diagnosed with severe or critical COVID-19 infection hospitalized in ICU or in CMU (https://www.who.int/publications/i/item/clinical-management-of-covid-19). Groups of patients were adjusted according to the age. This study was approved by the internal review board (Ethical Committee of the IHU Méditerranée Infection, reference 2020-13). According to European General Data Protection Regulation No 2016/679, patients were
informed of the potential use of their medical data and they could refuse. The analysis of collected data followed the reference methodology MR-004 registered on N° MR 5010010520 in the AP-HM register. It was performed according to the good clinical practices recommended by the Declaration of Helsinki and its amendments.

**Laboratory investigations**

The initial blood cell count (Sysmex, Roissy, France) was performed at the time of diagnosis. Myelemia, defined as the detection of immature granulocytes in peripheral blood, *i.e.* the egress of myelocytes, metamyelocytes and/or promyelocytes from the bone marrow to the peripheral blood, was systematically reported on the blood count result when present. An in house indirect immunofluorescent assay was performed for the detection of anti-SARS-CoV-2 IgG antibodies, as previously reported, without specification of their neutralizing capabilities (Edouard et al., 2020). An anti-SARS-CoV-2 ELISA assay for detection of IgG antibodies (Euroimmun, Bussy-Saint-Martin, France) was performed retrospectively on the available serum using Elispeed automate (Euroimmun, Bussy-Saint-Martin, France) (Beavis et al., 2020). ELISA ratio was defined as the optical density of the serum sample/the cut-off optical density (Beavis et al., 2020). Lymphocyte typing was performed on EDTA *in extenso* anticoagulated blood samples using the Aquios flow cytometer (Beckman Coulter, Villepinte, France).

**Statistical analysis**

The kinetics of the biological parameters were carried out including all available data over time, for each group, considering the onset of symptoms as day 1. We used multivariate analysis, Chi-squared, Fisher exact test, ROC analysis and principal component analysis (Prism 7.0, IBM SPSS 20, ExcelStat 2). All tests were two- sided and p<0.05 was considered as
significant. The logistic regression model was used, and multivariate models were adjusted for sex and age at baseline.
Results

Characteristics of patients

Seventy-seven patients were included, 36 were men and the mean age was 65±13 years (Table 1). Eighteen had a mild COVID-19 infection, 28 a moderate and 31 a severe/critical (18 severe and 13 critical). All outpatients had a mild disease whereas patients who had a moderate or a severe/critical disease were all hospitalized. Obesity was present in 15 patients (19.4%). Patients with a moderate or a severe/critical disease had a lower initial eosinopenia (<0.1 G/L) than those with a mild disease (Table 1). Neutrophil counts were significantly higher in patients with a severe/critical disease than in those with a moderate and a mild disease (Table 1 & Figure 1). Total lymphocyte, T, and CD4 T cell counts were significantly lower in severe/critical group than in others (Table 1). Myeloma was observed in 31 patients, 40% were hospitalized, 8 presented a moderate (34%) and 23 a severe/critical disease (74%) (Chi²<0.001). Myeloma was significantly higher in the severe/critical group than in the moderate one (0.24±0.06 vs 0.04±0.01, t test <0.01) and was detected at 20±9 and 13±11 days after the onset of symptoms, respectively. SARS-CoV-2 nasopharyngeal viral load at diagnosis was similar in the three groups (Table 1).

Immunofluorescent serological test was first performed on the primary serum, whereas globally, ELISA serological test was performed later, more than four weeks after the onset of symptoms for all patients and on available sera only (n=60) (Table 1). IgG anti-SARS-CoV-2 antibodies (ELISA) were significantly higher in the severe/critical group than in the mild one (Table 1).

Eight patients died (4 men, mean age 75±17 years, 2 obese). All presented initial eosinopenia. Myeloma was detected in 5 of these patients (mean ± SD : 0.49±0.62 G/L). Viral load at diagnosis was 27±2 as expressed in cycle threshold (Ct) values. Deceased patients had CD4 and CD8 T-cell lymphopenia, mean ± SD was 314 ± 162/mm³ and 136±111/mm³ respectively. Delay between the onset of symptoms and first positive PCR, lymphocyte typing
and serology (ELISA) were 6±32, 6±4 and 16±12 days respectively. Only one deceased patient had a positive immunofluorescence serology, IgG: 200, performed 3 weeks after the onset of symptoms. Other immunofluorescent tests were negative and were performed on the early serum (<14 days after the onset of symptoms). By contrast, three of the deceased patients had positive serology (ELISA) performed at 7, 29 and 15 days with a high correspondent antibody level (13, 10 and 2.36 respectively) for only two of them. Three had negative ELISA test performed <10 days following the onset of symptoms.

**Kinetics of biological parameters since the onset of symptoms**

Viral load kinetics on nasopharyngeal samples was similar in all three groups (Figure 1). Two days after symptom onset, blood count analysis was available for 3/18, 9/28 and 8/31 of the mild, moderate and severe/critical groups, respectively. All patients had initial eosinopenia (Figure 1). Elevation of eosinophils was detected in the severe/critical group 15 days after the onset of symptoms. Lymphopenia persisted until day 12 in severe/critical affected patients (Figure 1). Severe/critical patients presented higher neutrophil levels than others throughout the follow up and neutrophilia was observed 14 days after symptoms onset.

**Receiver Operating Characteristic analysis**

Neutrophilia, myelemia, CD4-T cells, CD8-T cells and total T-cell lymphopenia were significantly correlated with death (p<0.05, Table 2). Myelemia, neutrophilia, high levels of IgG (ELISA), T cells and CD4 T-cells lymphopenia were significantly associated with severe/critical disease (Table 2).

**Principal component analysis and multivariate analysis**
The principal component analysis showed that severe/critical affected patients were those
with myelemia, high levels of neutrophils, high IgG titer (ELISA) and lymphopenia (B, T, CD4,
CD8) (Figure 2).

Using a multivariable analysis, myelemia was found to be significantly associated with a
13-fold increased risk of severe/critical diseases and with CD4 T- and B-cell lymphopenia
(Table 3). High levels of IgG anti-SARS-CoV-2 (ELISA) antibodies were found to be
significantly associated with severe/critical diseases regardless of the sex and age (Table 3). B-
cell lymphopenia was found to be significantly associated with high levels of IgG detected by
immunofluorescence and ELISA (Table 3).
**Discussion**

Myelemia and neutrophilia are markers of severity of COVID-19 infection as they are associated with a 13 and 7-fold higher risk of severe/critical disease, respectively. SARS-CoV-2 was reported to induce profound alterations and deregulation in the myeloid cell compartment (Beavis et al., 2020; Schulte-Schrepping et al., 2020a). Indeed, single cell RNA sequencing and cell proteomics enabled the identification of an emergency myelopoiesis generating immature and dysfunctional neutrophils and monocytes (Mann et al., 2020; Schulte-Schrepping et al., 2020b, 2020a). An immune key signature was identified with shifts in neutrophil to T cell ratio, elevated serum IL-6, MCP-1 and IP-10, modulation of CD14+ monocyte phenotype and function (Mann et al., 2020; Schulte-Schrepping et al., 2020b, 2020a). In addition, in severe COVID-19 patients, immature CD10^low^CD101^−^CXCR4^+/-^ neutrophils with immunosuppressive profile were reported (Silvin et al., 2020). Here, myelemia correlates with T and B cells lymphopenia in severely/critically-affected patients. Myelemia was described in infectious and haematological diseases, such as acute bacterial infection, sepsis, myeloid leukaemia, myeloproliferative syndrome, bone marrow metastatic cancer and myelofibrosis (Venet et al., 2020). It was also identified as a severity marker of sepsis (Venet et al., 2020). Immature granulocytes were yet reported to have immunosuppressive activity (T cell killing functions, low phagocytosis ability, high release neutrophil extracellular traps, sepsis deterioration, immune suppression in Epstein Barr Virus infection) (Guérin et al., 2014; Ayres et al., 2019; Katahira et al., 2019; Venet et al., 2020). We report here the identification of myelemia, *i.e.* the circulation of immature granulocytes, possibly with immunosuppressive properties, as a biological marker of COVID-19 severity.

Neutrophilia as a predictive biological marker of mortality can be linked to bacterial co-infection. In a recent report on 92 SARS-CoV-2 infected patients hospitalized in ICU, 28% had a bacterial co-infection (Contou et al., 2020). *Staphylococcus aureus, Haemophilus influenzae,*
*Streptococcus pneumoniae* and *Enterobacteriaceae* were documented (Contou et al., 2020).

Moreover, neutrophilia is involved in lymphocyte and monocyte paralysis as in the immunothrombosis phenomena by secreting and releasing neutrophils extracellular traps (Middleton et al., 2020; Vitte et al., 2020). In severe cases, in contrast to the high levels of neutrophils and immature granulocytes observed, SARS-CoV-2 induced lymphopenia and eosinopenia.

Initial eosinopenia was significantly lower in the severe/critical patient group than in the others. In the literature, eosinopenia was observed in 29.5% of children with mild COVID-19 symptoms and in 53% to 73% of confirmed COVID-19 adult patients (Lagier et al., 2020, 37; Tanni et al., 2020). Eosinopenia, as persistent eosinopenia, was found to be significantly associated with severe COVID-19 infection and poor clinical outcome (Du et al., 2020; Lagier et al., 2020). Outside COVID-19, eosinopenia was reported as a predictive marker of ongoing bacterial infections and in patients hospitalized for chronic obstructive pulmonary exacerbation, eosinopenia was predictive of the length of stay and mortality (Shankar-Hari et al., 2017; Sun et al., 2020). It was also identified in 16% of patients infected with influenza (Tanni et al., 2020).

The antiviral properties of eosinophils have been evidenced. By acting against the single-stranded RNA viruses and by inducing proliferation and activation of CD8 T cells in response to the virus exposition, eosinophils may contribute to viral clearance (Flores-Torres et al., 2019; Jesenak et al., 2020). Here, we observed an increase in the number of eosinophils on day 15 in the severe/critical patient group along with neutrophilia and myelemia, which corroborates the observation of Lucas et al. regarding eosinophils and neutrophils and supports bone marrow activation of this granulocyte lineage (Yale IMPACT Team et al., 2020).

Patients with a severe/critical disease presented lymphopenia as previously reported (Tan et al., 2020). Interestingly, we identified B-cells and CD4 T cell lymphopenia as being significantly associated with severe/critical COVID-19 infection and myelemia, whereas B-cell lymphopenia was additionally associated with high levels of anti-SARS-CoV-2 antibodies.
Increased mortality has been reported in patients with CD3+ T-cells <800/µL, CD4+ T-cells <300/µL, and CD8+ T-cells <400/µL (Diao et al., 2020). Several hypotheses were proposed to explain the decrease in the lymphocyte pool observed in severe cases in response to the infection. Spleen and lymph nodes follicle destruction, with necrotic and apoptotic B and T-cells widely distributed in the decomposed follicles, has been described in the post mortem examination of COVID-19 patients, but not in controls; a fact that has already been observed in sepsis patients (Hotchkiss et al., 2001; Chen et al., 2020). In addition, the sequestration of activated lymphocytes in injured tissues and the immune exhaustion of lymphocytes in the bone marrow may result in peripheral lymphopenia (Chen et al., 2020; Melenotte et al., 2020). Lymphocyte depletion induced by SARS-CoV-2 infection is thus multifactorial and may occur in primary and secondary lymphoid organs as in injured tissues. Nevertheless, as the peripheral B-cell lymphocyte pool is depleted, in response to cytokine storm syndrome, plasmablast proliferation can generate autologous and heterologous antibodies from the bone marrow (Chau et al., 2020).

High levels of IgG antibodies, whether tested precociously with immunofluorescence or secondary with ELISA were significantly associated with severe/critical COVID-19 infection, which is consistent with the literature data. Elevation of anti-SARS-CoV-2 antibodies has been identified in severe patients and IgG seroconversion was reported to occur concomitantly with acute respiratory distress syndrome (Liu et al., 2019; Okba et al., 2020; Zhang et al., 2020). Some authors have questioned the deleterious effects of these antibodies in severe cases, as high levels of anti-spike IgG have been shown to cause acute lung damage in macaques monkeys (Liu et al., 2019). In addition, antiphospholipid autoantibody secretion were reported in patients with severe COVID-19 infection having clinical manifestations known to be associated with antiphospholipid syndrome: cerebral microhaemorrhage, purpuric rash, thrombosis and catastrophic antiphospholipid antibody syndrome (Bertin et al., 2020; Cárdenas Suri and Jimomila Bening, 2020; Shoskes et al., 2020). Even more recently, Bastard et al. identified the
presence of neutralizing IgG auto-Abs against IFN-ω, the 13 types of IFN-α, or both, at the onset of critical COVID-19 pneumonia (Bastard et al., 2020).

Initial viral load on nasopharyngeal sample as viral load kinetics on nasopharyngeal sample were similar in the 3 groups. The association between high nasopharyngeal viral load and COVID-19 severity has been debated in the literature, where some reports have identified high viral load as being associated with intubation and mortality (Magleby et al., 2020). Our results are in line with those previously published by To et al. and Lee et al., in which viral load was not associated with disease severity (Lee et al., 2020; To et al., 2020).

Finally, all these data converge to sustain the hypothesis of a bone marrow emergency activation as follows (Figure 3). SARS-CoV-2 infection induce initial eosinopenia and lymphopenia, which is particularly marked in severe cases, testifying the peripheral depletion and the central exhaustion of both lineages. Then, in response to the cytokine storm syndrome (8-12 days), emergency myelopoiesis stimulates the production of granulocytes, while antibody producing cells generate high levels of anti-SARS-CoV-2 antibodies (Channappanavar and Perlman, 2017). Neutrophils were identified as immature and immunosuppressive and IgG antibody neutralizing capacity needs to be further clarified.

**Conclusion**

Initial deep eosinopenia, lymphopenia (B, CD4 T cell) followed by neutrophilia, myelemia and high anti-SARS-CoV-2 IgG antibodies are prognostic markers for COVID-19 severe and critical COVID-19 disease. Emergency bone marrow activation generates immature neutrophils and high levels of anti-SARS-CoV-2 specific antibodies to the detriment of the lymphocyte lineage. Myelemia is a simple biological marker of severity attesting of this bone marrow activation.
Conflicts of interest
The authors have no conflicts of interest to declare.

Funding source:
URMITE, IHU Méditerranée Infection.
This work was supported by the French Government under the « Investissements d’avenir » (Investments for the Future) programme managed by the Agence Nationale de la Recherche (ANR, fr: National Agency for Research), (reference: Méditerranée Infection 10-IAHU-03). This work was also supported by Région Provence-Alpes-Côte d’Azur and European funding FEDER PRIMMI (Fonds Européen de Développement Régional - Plateformes de Recherche et d'Innovation Mutualisées Méditerranée Infection).
Cléa Melenotte was supported by the RHU Lumière for its post doctoral fellowship.

Contributions: CM wrote the manuscript and designed the work, JCL, MM and PP provided clinical management of inpatients and ambulatory patients, MG provided clinical management of intensive care unit patients, SE performed serological tests, PC supervised virological diagnostics, MD, LZ and DR supervised the work.
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