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Brief Report

Expansion of Myeloid Derived Suppressor Cells Contributes to Platelet Activation by L-Arginine Deprivation during SARS-CoV-2 Infection

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Abstract: Massive platelet activation and thrombotic events characterize severe COVID-19, highlighting their critical role in SARS-CoV-2-induced immunopathology. Since there is a well-described expansion of myeloid-derived suppressor cells (MDSC) in severe COVID-19, we evaluated their possible role in platelet activation during SARS-CoV-2 infection. During COVID-19, a lower plasmatic L-arginine level was observed compared to healthy donors, which correlated with MDSC frequency. Additionally, activated GPIIb/IIIa complex (PAC-1) expression was higher on platelets from severe COVID-19 patients compared to healthy controls and inversely correlated with L-arginine plasmatic concentration. Notably, MDSC were able to induce PAC-1 expression in vitro by reducing L-arginine concentration, indicating a direct role of PMN-MDSC in platelet activation. Accordingly, we found a positive correlation between ex vivo platelet PAC-1 expression and PMN-MDSC frequency. Overall, our data demonstrate the involvement of PMN-MDSC in triggering platelet activation during COVID-19, highlighting a novel role of MDSC in driving COVID-19 pathogenesis.

Keywords: COVID-19; MDSC; platelet; L-Arginine

1. Introduction

The ongoing COVID-19 pandemic due to the coronavirus SARS-CoV-2 remains a global health emergency. The clinical features of COVID-19 range from asymptomatic to severe pneumonia and fulminant disease [1], but the mechanisms responsible for this wide clinical presentation are not completely clear. In severe COVID-19, coagulation abnormalities appear, inducing a hypercoagulable state and an increased rate of thrombotic and thromboembolic events [2].

The high inflammatory response may contribute to the thrombotic complications by impairing procoagulant–anticoagulant balance, thus facilitating the development of microthrombosis and disseminated intravascular coagulation [3]. Further, the expression of SARS-CoV-2 receptor (angiotensin converting enzyme 2, ACE-2) on platelet membranes suggests a possible direct role of SARS-CoV-2 in platelet activation [4].

It has been shown that arginase I (Arg I) and nitric oxide synthase (iNOS) detract the microenvironment from arginine, inducing platelet activation, and impairing nitric oxide

synthesis [5]. The myeloid derived suppressor cells (MDSCs) population is one of the main producers of ArgI and iNOS [6]; they strongly expand early after SARS-CoV-2 infection and can predict the fatal outcome of the disease [7,8]. MDSCs, defined in humans as HLADR low/- CD11b+ CD14- CD33+ CD15+ (polymorphonuclear, PMN-MDSCs) or HLA-DR low/- CD11b+ CD14+ CD33+ (monocytic, M-MDSCs) are known to have the remarkable ability to reduce inflammation by suppressing innate and adaptive immune function through several mechanisms, including iNOS, Arg-1, nicotinamide adenine dinucleotide phosphate oxidase (NOX2), and transforming growth factor beta (TGF-β) [9].

In this study, we assessed the capability of PMN-MDSC to activate platelets during SARS-CoV-2 infection. Our results showed a novel role of PMN-MDSC from COVID-19 patients, being able to increase platelet activation by reducing L-arginine concentration, thus contributing to the platelet hyperactivity observed in severe COVID-19.

2. Materials and Methods

2.1. Study Population

SARS-CoV-2 infected patients (n = 62) were treated at the National Institute for Infectious Diseases "Lazzaro Spallanzani" (Rome, Italy). We enrolled SARS-CoV-2 positive patients without other infections such as HIV, HCV, HBV, MTB, and others. Pregnant women were also excluded. All patients were symptomatic, ranging from moderate (PO2/FIO2 > 200, n = 31, no ICU) to severe (n = 31, requiring intensive care unit admission, ICU). Median age was 65 years (range 22–95), and 60% were males. ICU and no ICU patients (63.3% and 70%, respectively) presented one or more co-morbidities. These included hypertension (ICU = 53.3%, no ICU = 36.7%), cardiovascular diseases (ICU = 26.7%, no ICU = 10%), obesity (ICU = 26.7%, no ICU = 23.3%), diabetes (ICU = 16.7%, no ICU = 10%), and cancer (ICU = 10%, no ICU = 16.7). Healthy individuals (HD, n = 9) were included as controls.

The study was approved by the institutional review board (approval number: 9/2020) and signed written informed consent was obtained from patients.

2.2. Plasma Samples Preparation

Heparin anti-coagulated whole blood samples were centrifuged at 100× g for 15 min and platelet rich plasma (PRP) was collected for further use.

2.3. PBMC and PMN-MDSC Isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparin-treated whole blood by density gradient centrifugation (Lympholyte-H, Cederlane, Burlington, ON, USA) PBMC were suspended in RPMI 1640 (Corning Incorporated, NewYork, NJ, USA) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (EuroClone, Milan, Italy), penicillin/streptomycin solutions, and 2 mmol/L L-glutamine (Corning Incorporated, New York, NJ, USA).

PMN-MDSCs were isolated by using CD15 microbeads (MiltenyiBiotec, Bergisch Gladbach, Germany) according to the manufacturer's procedure. Purity was >90% as verified by flow-cytometry (data not shown).

2.4. Platelets-PMN-MDSC Culture

Purified PMN-MDSCs (2×10^5) were seeded in 96-well plate (Corning-Incorporated, New York, NJ, USA) in the above described medium without FBS. Twenty microliters of PRP from HD were added and cultured at 37 °C. After 4 h, platelet activation was evaluated by flowcytometry, and supernatants were collected for L-Arginine quantification.

2.5. Flow Cytometry

Platelet activation was analyzed by using anti-human REAfinity CD41 APC and anti-human REAfinity activated GPIIb/IIIa complex (PAC-1 PE mAb, Bergisch Gladbach,

Miltenyi Biotec) on ice in the dark. After 15 min, 1% paraformaldehyde was added and samples were acquired by Cytoflex Flow Cytometer (Beckman-Coulter, Brea, CA, USA).

MDSC frequency was evaluated by staining PBMC with customized Duraclon tubes, (FITC-CD11b, ECD-HLA-DR, PC5.5-CD14, PC7-CD33, KrO-CD45, APC-CD80, DRAQ7, APC-alexa750-CD56, APC-alexa750-CD19, APC-alexa750-CD3, Pacific-Blue-CD15, and Beckman-Coulter) following manufacturer's procedures. Data were acquired by CytoFlex flow-cytometer and analyzed by CytExpert (Beckman-Coulter, Brea, CA, USA).

2.6. L-arginine Quantification

Plasma samples and co-culture supernatants were centrifuged at 2000 rpm for 10 min to eliminate platelets and debris. L-arginine level was evaluated by UPLC-MS/MS by using Kairos Amino Acid Kit (Waters, Milford, MA, USA) according to the manufacturer's instruction. Chromatographic separation was performed using an ACQUITY-UPLC system, followed by detection on a Xevo-TQD (Waters, Milford, MA, USA).

2.7. Statistical Analysis

GraphPad Prism version 8.00 (GraphPad Software) was used to perform statistical analyses. The non-parametric Kruskal-Wallis with Dunn's post hoc test or the Wilcoxon matched-pairs signed rank test were used. Correlations were evaluated with the non-parametric Spearman test. The $p < 0.05$ was considered significant.

3. Results

3.1. Plasmatic L-Arginine in COVID-19 Patients was Correlated to PMN-MDSC Frequency

We evaluated the plasmatic concentration of L-arginine in patients with moderate (no ICU) and severe (ICU) COVID-19 and HD. A lower plasmatic L-arginine level was observed in both patient groups compared to HD (Figure 1a). Moreover, L-arginine was lower in ICU compared to no ICU patients, suggesting possible association with disease severity. PMN-MDSC percentage was evaluated by flow-cytometry (Figure 1b). A strong negative correlation was found between L-arginine level and PMN-MDSC frequency (Figure 1c), indicating that PMN-MDSC may be involved in the plasmatic L-arginine deprivation during COVID-19. To assess the role of arginine level in driving platelet activation, the expression of PAC-1 on CD41+ platelets was analyzed in a subgroup of SARS-CoV-2 infected patients and HD by flow-cytometry. The representative plots in Fig 1D show PAC-1 expression on platelets from ICU, non ICU patients, and HD. Cumulative analysis shows that the expression of PAC-1 on platelets was higher in ICU patients compared to HD (Figure 1e). In no ICU patients, an intermediate PAC-1 level was observed (Figure 1e), suggesting a higher platelet activation state in more severe patients. The expression of PAC-1 on platelets directly correlated with plasmatic L-arginine (Figure 1f), indicating that arginine shortage may be involved in platelet activation observed in severe COVID-19.

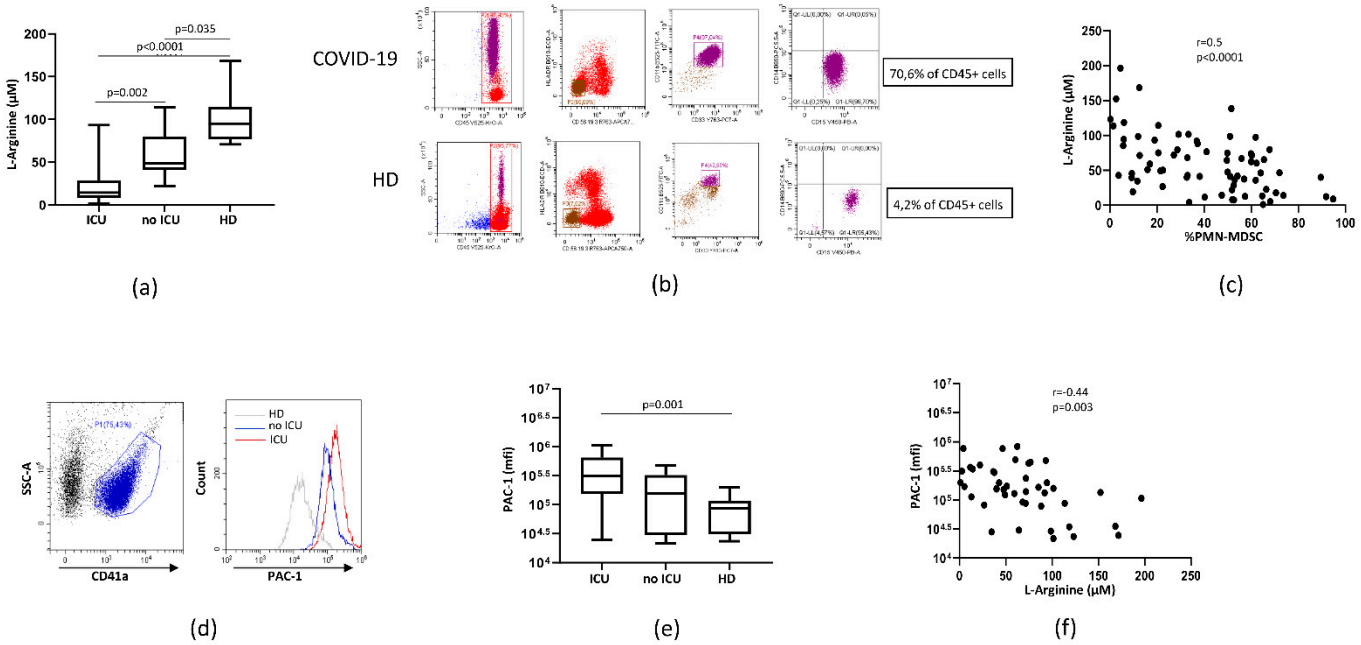

Figure 1. Plasmatic L-arginine level in SARS-CoV-2 patients correlated with PMN-MDSC frequency. (**a**) Plasmatic Arginine level of SARSCoV-2 ICU (ICU, n = 31) and no ICU (no ICU, n = 31) patients and healthy donors (HD, n = 9). Results are shown as box and whiskers plot. Kruskal-Walliswith Dunn's post hoc test was applied. (**b**) Flow-cytometry gating strategy used to identify MDSC among PBMC. Dead cells were excluded by selecting DRAQ7neg cells. One representative COVID-19 patient and one HD are shown. (**c**) Correlation between plasmatic L-arginine level and PMN-MDSC percentage from SARSCoV-2 infected patients and HD. Non-parametric Spearman correlation was applied. (**d**) Representative plots of the adopted gating strategy to evaluate platelet activation. Platelets were selected as CD41a+ (SSC/CD41a plot), and PAC-1 expression (mean fluorescence intensity, mfi) was evaluated. (**e**) PAC-1 expression (mfi) on platelets from ICU (n = 21), no ICU (n = 15) patients, and HD (n = 9). Results are shown as box and whiskers plot. Kruskal-Walliswith Dunn's post hoc test was applied. (**f**) Correlation between PAC-1 platelet expression (mfi) and L-arginine plasmatic level.

3.2. PMN-MDSC Induced Platelet Activation by Reducing L-Arginine

Next, we evaluated whether PMN-MDSCs were directly involved in platelet activation. We found a positive correlation between PAC-1 platelet expression and PMN-MDSC frequency (Figure 2a), suggesting that PMN-MDSC may be involved in arginine deprivation. To confirm that PMN-MDSC may induce platelet activation, we cultured platelet rich plasma (PRP) from HD with PMN-MDSCs isolated from COVID-19 patients and, after 4 h, the expression of PAC-1 on platelets was evaluated by flow cytometry. We found that PMN-MDSCs from COVID-19 were able to activate resting healthy platelets by increasing the expression of PAC-1 (Figure 2b,c). As a control, PBMC depleted from MDSCs were used and no effect was observed. Accordingly, a reduction of L-arginine in the culture supernatants (Figure 2d) was found, indicating that PMN-MDSCs may activate platelets by depriving L-arginine from the microenvironment.

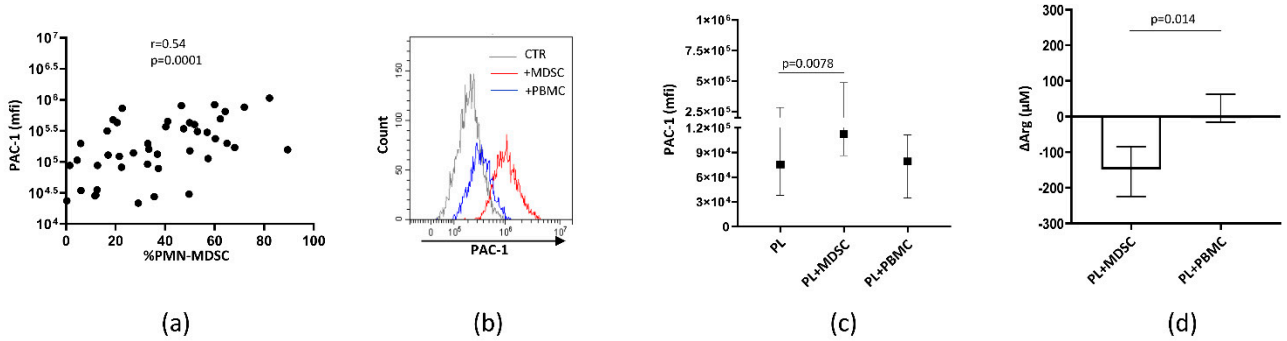

Figure 2. PMN-MDSC induced platelet activation by reducing L-arginine. (**a**) Correlation between PAC-1 platelet expression (mfi) and PMN-MDSC percentage from SARSCoV-2 infected patients (ICU n = 21, no ICU n = 15) and HD (n = 9). Non-parametric Spearman correlation was applied. (**b**) Representative histogram plot showing PAC-1 expression on platelets after culture with or without PMN-MDSC or PBMC depleted from MDSCs (PBMC). (**c**) PAC-1 expression on platelets from HD (n = 6) after culture with PMN-MDSC or PBMC from SARSCoV-2 infected patients (n = 6). Results are shown as median and IQR. Wilcoxon matched-pairs signed rank test was applied. (**d**) L-arginine reduction (ΔArg) calculated as the difference between L-arginine concentration in the presence of MDSCs or PBMC and platelets alone. Results are shown as median and IQR. A Wilcoxon matched-pairs signed rank test was applied.

4. Discussion

Patients with severe COVID-19 commonly present thrombotic disorders, and these conditions have been associated with a higher mortality rate [10]. Moreover, severe COVID-19 is characterized by a strong neutrophilia that persists overtime. Among neutrophils, a strong inflammatory-driven expansion of PMN-MDSCs was observed in severe patients, which significantly reduces the adaptive immune response to SARS-CoV-2 and predicts a fatal clinical outcome [7,8].

In this paper, we analyzed an MDSC function never explored before, to the best of our knowledge, and showed that PMN-MDSCs from COVID-19 patients may be involved in platelet activation by reducing L-arginine availability, highlighting a new interplay between immune regulatory cells and platelet function.

According to previous published data [11], a decrease of L-arginine in the plasma from COVID-19 patients was found. In the present work, we also observed that L-arginine level inversely correlated with PMN-MDSC frequency and with platelet activation. Moreover, PMN-MDSC frequency directly correlated with platelet activation, suggesting a role of PMN-MDSCs in platelet activation during COVID-19. This hypothesis is corroborated by the high level of the enzymes involved in L-arginine catabolism, Arg I, and iNOS, expressed by PMN-MDSCs in COVID-19 patients [7]. Our in vitro experiments further provide a formal proof of the direct role of PMN-MDSC in inducing platelet activation through L-arginine consumption.

Platelet activation has been described during SARS-CoV-2 infection, and it is known to contribute to thromboembolic complications. Besides hyper-inflammation, factors such as a direct SARSCoV-2 infection and antibody-mediated mechanisms have been proposed to contribute to platelet hyperactivity [4]. In the present study, we demonstrated the PMN-MDSC as a new player in platelet homeostasis, highlighting an unprecedented function of the PMN-MDSC during COVID-19. Correlations between PMN-MDSC frequency with L-arginine concentration and with platelet activation have been shown during other infections such as severe fever caused by a bunyavirus [12]. Herein, we demonstrated that PMN-MDSC, by decreasing L-arginine, might directly contribute to platelet activation, shedding light on a novel role of PMN-MDSCs besides immune suppression.

5. Conclusions

Our findings demonstrate the direct involvement of PMN-MDSCs in platelet activation during COVID-19, confirming the MDSC expansion as one of the main events driving

COVID-19 pathogenesis. These results also reveal new therapeutic perspectives targeting MDSC number and function, a promising strategy already under evaluation in cancer patients [13,14].

Author Contributions: A.S. and C.A. contributed to conceptualization; A.S. and C.A. contributed to experimental design; G.G. (Germana Grassi), S.G., V.B., E.C. and R.C. contributed to flow cytometry experiments and analysis; A.S., G.G. (Germana Grassi), S.N., E.T. and D.M. contributed to functional experiments; A.S. performed statistical analysis; L.M., E.N., G.G. (Gabriele Garotto) and A.B. contributed to patient management; A.S., G.G. (Germana Grassi), and C.A. wrote the paper; M.B. and G.I. contributed to revising the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of National Institute for Infectious Diseases "Lazzaro Spallanzani" (approval number: 9/2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Publicly available datasets were analyzed in this study. This data can be found here: [https://rawdata.inmi.it/].

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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