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RESEARCH PAPER

# Platelets modulate CD4$^+$ T-cell function in COVID-19 through a PD-L1 dependent mechanism

**Ana Paletta**[1] | **Facundo Di Diego García**[1] | **Augusto Varese**[1] | **Fernando Erra Diaz**[1] | **Julián García**[2] | **Juan Carlos Cisneros**[3] | **Guillermina Ludueña**[4] | **Ignacio Mazzitelli**[1] | **Andrea Pisarevsky**[4] | **Gonzalo Cabrerizo**[1] | **Álvaro López Malizia**[1] | **Alejandra G. Rodriguez**[3] | **Nicolás Lista**[3] | **Yesica Longueira**[1] | **Juan Sabatté**[1] | **Jorge Geffner**[1] | **Federico Remes Lenicov**[1] | **Ana Ceballos**[1]

[1]Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS), Universidad de Buenos Aires (UBA)-CONICET, Buenos Aires, Argentina

[2]División C, Hospital de Enfermedades Infecciosas Francisco Muñiz, Buenos Aires, Argentina

[3]Unidad de Terapia Intensiva, Hospital de Enfermedades Infecciosas Francisco Muñiz, Buenos Aires, Argentina

[4]Departamento de Medicina Interna, Hospital de Clínicas, Universidad de Buenos Aires, Buenos Aires, Argentina

**Correspondence**
Ana Ceballos, INBIRS, Facultad de Medicina, Universidad de Buenos Aires C1121ABG, Ciudad de Buenos Aires, Argentina.
Emails: anaceballos1@gmail.com; aceballo@fmed.uba.ar

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**Summary**

Severe COVID-19 is associated with a systemic inflammatory response and progressive CD4$^+$ T-cell lymphopenia and dysfunction. We evaluated whether platelets might contribute to CD4$^+$ T-cell dysfunction in COVID-19. We observed a high frequency of CD4$^+$ T cell–platelet aggregates in COVID-19 inpatients that inversely correlated with lymphocyte counts. Platelets from COVID-19 inpatients but not from healthy donors (HD) inhibited the upregulation of CD25 expression and tumour necrosis factor (TNF)-α production by CD4$^+$ T cells. In addition, interferon (IFN)-γ production was increased by platelets from HD but not from COVID-19 inpatients. A high expression of PD-L1 was found in platelets from COVID-19 patients to be inversely correlated with IFN-γ production by activated CD4$^+$ T cells cocultured with platelets. We also found that a PD-L1-blocking antibody significantly restored platelets' ability to stimulate IFN-γ production by CD4$^+$ T cells. Our study suggests that platelets might contribute to disease progression in COVID-19 not only by promoting thrombotic and inflammatory events, but also by affecting CD4$^+$ T cells functionality.

**KEYWORDS**
CD4$^+$ T cell–platelet aggregates, CD4$^+$ T cells, COVID-19, PD-L1, platelets

## INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an emerging pathogen responsible for the coronavirus disease 2019 (COVID-19). Severe COVID-19 is associated with an overactive inflammatory process where the host's immune response seems to play a critical role in disease progression and pathogenesis.[1–10] Severe COVID-19 cases exhibit increased levels of pro-inflamatory cytokines (IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, G-CSF, GM-CSF, IP10, MCP1, IFN-γ and TNF-α) in plasma.[2,4,5,7,10] Furthermore, a progressive reduction and functional exhaustion of T cells is observed.[1–8,10] The drivers of these pathological processes remain poorly understood.

Ana Paletta and Facundo Di Diego García contributed equally to this work.

Patients with COVID-19 pneumonia exhibit coagulation abnormalities.[11–13] A hypercoagulable state has been reported as the major pathologic event in COVID-19,[1,11–14] and thromboembolic complications were listed among the life-threatening complications of the disease.[13,14] Platelets show an activated phenotype in COVID-19 patients requiring intensive care.[11–13] They might contribute to COVID-19 pathogenesis not only by promoting a hypercoagulable state and thromboembolic complications,[1,11–13] but also by regulating the function of immune cells. In fact, a large body of evidence indicates that platelets are able to modulate the function of either innate or adaptive immune cells.[12,15–20] They can mediate the immune responses both indirectly through the release of cytokines and antimicrobial peptides and directly through their interaction with neutrophils, monocytes and lymphocytes.[19,21–23] Previous studies have shown that platelets modulate the phenotype and function of monocytes and neutrophils in patients with severe COVID-19.[11,12,24] Circulating aggregates of platelets with either monocytes or neutrophils were shown to be increased in COVID-19 patients.[11,12,24] Whether platelets were also able to modulate the function of T cells contributing to the T-cell dysfunction in severe COVID-19 remains to be elucidated.

Here, we analysed the immunoregulatory action exerted by platelets from COVID-19 patients on the function of $CD4^+$ T cells. We observed that platelets from COVID-19 patients but not from healthy donors (HD) inhibited the up-regulation of CD25 expression and tumour necrosis factor (TNF)-α production by $CD4^+$ T cells. Interferon (IFN)-γ production was increased by platelets from HD but not from COVID-19 patients. Interestingly, a high expression of PD-L1 was found in platelets from COVID-19 patients and an inverse correlation was observed between platelet PD-L1 expression and IFN-γ production by activated $CD4^+$ T cells cocultured with platelets. Furthermore, we found that a blocking antibody directed to PD-L1 restored the platelets' ability to stimulate IFN-γ production by $CD4^+$ T cells. Our present results suggest that platelets might contribute to severe COVID-19 through a PD-L1-dependent mechanism.

## MATERIALS AND METHODS

### Study participants

Age- and sex-matched HD ($n$ = 35) and COVID-19 patients ($n$ = 62) were included in our study. HD and patients were admitted at 'Hospital de Clínicas José de San Martín' and 'Hospital Muñiz', Buenos Aires, Argentina. None of the HD presented comorbidities or acute infections at the time of sampling. All patients had SARS-CoV-2 infection confirmed by polymerase chain reaction (PCR) of nasopharyngeal swabs. Patients were classified according to the WHO criteria.[25] COVID-19 patients were all hospitalized and comprised moderate (29.0%) and severe (71.0%%) infections ($n$ = 62, 61.3% men; age range 26–88 years). Moderate inpatients were treated with little or no requirement for supplemental oxygen, and severe patients required high-flow oxygen, mechanical ventilation, and/or multi-organ support. Over 50% of patients (56.5%) were admitted into the intensive-care unit (ICU). Patients were hospitalized and were enrolled within 72 h of hospital or ICU admission. Disease severity was recorded at the time of sampling. Patient characteristics are shown in Table 1. Patients with oncological comorbidities or other infectious diseases were excluded from this study. None of the study participants had been vaccinated when the samples were collected.

### Ethics statement

Our study was approved by the Ethics Committee of the Hospital Francisco J. Muñiz and Hospital de Clínicas José de San Martín (Argentina), in line with the Declaration of Helsinki (Fortaleza 2013). All participants or their legal guardians provided written informed consent.

### Isolation of platelets, $CD4^+$ T cells and cocultures of $CD4^+$ T cells and platelets

Platelet–lymphocyte aggregates were evaluated by flow cytometry in whole-blood samples. Blood samples were collected into heparin vacutainer tubes (BD Pharmingen) and immediately processed to obtain platelets and $CD4^+$ T cells. Platelet-rich plasma (PRP) was obtained by blood sample centrifugation. To avoid leukocyte contamination, only the top 75% of the PRP was collected. Platelets were isolated from PRP supplemented with PGI2 (75 nM), washed and resuspended in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 0.1 mM non-essential amino acids (complete culture medium; all from Life Technologies). $CD4^+$ T cells were purified using the RosetteSep™ Human $CD4^+$ T Cell Enrichment Cocktail (Stem Cell Technologies). $CD4^+$ T cells ($1.5 \times 10^5$/200 µl) were stimulated with anti-CD3/anti-CD28/anti-CD2 monoclonal antibodies (mAb)-coated-beads (Miltenyi Biotec) and cultured with platelets from a single healthy donor or a COVID-19 patient using a 1:100 ratio in complete culture medium. All cocultures were performed with fresh platelets and $CD4^+$ T cells immediately after purification. Experiments were performed with $CD4^+$ T cells and platelets from single donors. $CD4^+$ T cells were obtained from single HD except otherwise indicated. All cocultures were allogeneic except for a set of experiments with autologous cocultures ($CD4^+$ T cells and platelets from the same donor). Supernatants were harvested at 48 or 96 h post stimulation and concentrations of IFN-γ, TNF-α, interleukin (IL)-17 and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA; BioLegend). When indicated, platelets were pre-incubated for 30 min with a PD-L1-blocking antibody (10 µg/ml) (clone 29E.2A3, BioLegend). Labelled antibodies directed to CD4, CD62p, CD25, PD-1 and PD-L1 were from BD Pharmingen.

**TABLE 1** Clinical characteristics of healthy donors and inpatients with COVID-19

| | HD | COVID-19 inpatients |
|---|---|---|
| Demographic data | | |
| *n* | 35 | 62 |
| Age, years (range) | 50 (26–65) | 50 (26–88) |
| Male (%) | 54.2% | 61.3% |
| Intensive care unit (%) | None | 56.5% |
| Disease severity | | |
| Mild | – | – |
| Moderate | – | 29.0% |
| Severe | – | 71.0% |
| Laboratory parameters (mean ± SEM) | | |
| Leukocytes ($mm^3$) | – | 6998 ± 185 |
| Neutrophills ($mm^3$) | – | 6431 ± 236 |
| Lymphocytes ($mm^3$) | – | 813 ± 45 |
| Neutrophil to lymphocyte ratio | – | 11.43 ± 0.82 |
| Platelets ($mm^3$) | – | 217543 ± 8627 |
| Ferritin (ng/ml) | – | 1650 ± 149 |
| C-reactive protein (mg/l) | – | 10.63 ± 1.12 |
| D-Dimer (ng/ml) | – | 1548 ± 299 |
| LDH (U/L) | – | 555 ± 152 |
| Comorbidities (%) | | |
| Hypertension | 0 | 27.4% |
| Chronic obstructive pulmonary disease | 0 | 8.6% |
| Chronic kidney disease | 0 | 6.5% |
| Cardiovascular disease | 0 | 21.0% |
| Obesity | 0 | 29.0% |
| Diabetes | 0 | 27.4% |
| Days of symptoms at admission | – | 6.12 ± 1.10 |
| Blood sampling (days after diagnosis by PCR) | 0 | 8.33 ± 1.04 |
| Days of hospitalization | None | 17.61 ± 3.86 |
| Treatments (%) | | |
| NSAIDs | – | 16.1% |
| Antibiotics | – | 74.1% |
| Enoxaparin | – | 46.8% |
| Hydroxychloroquine | – | 1.6% |
| Remdesivir | – | – |
| Corticosteroids[a] | – | 66.1% |

Abbreviations: HD, healthy donor; LDH, lactate dehydrogenase; NSAID, non-steroidal anti-inflammatory drug; PCR, polymerase chain reaction.

[a]Corticosteroids used include prednisone, betamethasone and dexamethasone.

## Flow cytometry

Phycoerythrin (PE)-, fluorescein isothiocyanate (fitc)-, allophycocyanin (apc)- or peridinin chlorophyll (PerCP)-conjugated mAbs directed to CD4, CD62p, CD25, PD-1 and PDL-1 (BD Pharmingen) were used. In all cases, isotype-matched control mAbs were used, and a gate was defined in the analysis to exclude non-viable cells and debris. Analyses were performed using a FACS Canto II flow cytometer (BD Bioscience) and FlowJo X v10.0.7r2 software. Results are expressed as the percentage of positive events. Labelled antibodies directed to CD4, CD62p, CD25, PD-1 and PD-L1 were from BD Pharmingen.

## Quantitation of cellular apoptosis and viability by flow cytometry

Apoptosis and viability of cells were evaluated by flow cytometry using an AnnexinV -FITC/propidium iodide kit, according to manufacturer's instructions (BD Bioscience).

## RNA-seq analysis

FASTQ files were downloaded from BioProject (accession #PRJNA634489).[11] Reads were pseudo-aligned to the transcriptome using Kallisto.[26] The rest of the analysis was performed as described previously.[27] Briefly, transcript expression values were summarized using tximport (http://bioinformatics.sdstate.edu/idep92/). Differential expression analysis was performed in IDEP92 using the Bioconductor package DESeq2 with a fold change (FC) cut-off of 2 and false discovery rate (FDR) < 0.05. The top 500 highly variable genes were used for Hierarchical Clustering. Heatmaps were plotted using Morpheus (https://software.broadinstitute.org/morpheus).

## Statistical analysis

The Mann–Whitney test was used to determine the significance of differences between the two groups. For paired analyses, the Wilcoxon test was used. For multiple analyses, the Kruskal–Wallis test was used. Experimental data were plotted and analysed by GraphPad Prism v. 8.02 (GraphPad Software, San Diego, CA, USA). Results are shown as mean ± SEM.

# RESULTS

## Platelets from healthy donors and COVID-19 inpatients differentially modulate $CD4^+$ T-cell function

All patients were diagnosed with COVID-19 by PCR amplification of SARS-CoV-2 viral RNA obtained from nasopharyngeal or oropharyngeal swabs. Patients were all hospitalized (inpatients) as described in Table 1, and classified according to WHO criteria.[25] We first evaluated the frequency of $CD4^+$ T cell–platelet aggregates in whole blood samples from

COVID-19 patients and HD measuring the expression of CD4 and CD62p (P-selectin) by flow cytometry. Upon activation, platelets express a large amount of P-selectin which is rapidly mobilized from α-granules to the platelet surface.[28] Increased expression of P-selectin promotes the formation of platelets and leukocyte aggregates through the P-selectin glycoprotein ligand-1 (PSGL-1).[11] A higher frequency of $CD4^+$ T cell–platelet aggregates was observed in samples from COVID-19 patients measuring CD62p expression. Both moderate and severe patients showed increased $CD4^+$ T cell–platelet aggregates (Figure S1A). Besides, CD42b and CD41b expressions were measured in whole-blood samples from COVID-19 patients and HD. We also observed a higher frequency of $CD4^+$ T-cell aggregates in samples from COVID-19 patients (Figure S1A,B). Fluorescence minus one (FMO) controls for CD4, CD62p, CD42b and CD41 are shown in Figure S1D. Similar results were obtained when aggregation between platelets and monocytes was analysed (Figure S1E) and also with polymorphonuclear cells, as reported previously by our group.[24] $CD4^+$ T cell–platelet aggregates frequency showed negative correlation with lymphocyte and neutrophil counts, and positive correlation with neutrophil:lymphocyte ratio but not with leukocyte or platelet counts (Figure 1C–G). Interestingly, the lack of correlation between $CD4^+$ T cell–platelet aggregates and platelet counts reinforces the idea that platelet–leukocyte aggregation is a consequence of altered platelet functionality and not of changes in quantity of cells. The comparison between CD62p and CD41 expression in aggregates showed that lymphocytes aggregate mainly with activated platelets, although there is a proportion of $CD62p^-CD41^+$ platelets (30%) aggregated to lymphocytes (data not shown).

Although elderly people present an increased risk to develop a severe disease,[1,2] we did not observe any correlations between $CD4^+$ T cell–platelet aggregation and the age of the study participants or the treatment they received (data not shown).

We then evaluated whether platelets were able to regulate $CD4^+$ T-cell function. Isolated $CD4^+$ T cells from HD were stimulated with anti-CD3/anti-CD28/anti-CD2-coated beads in the absence or presence of platelets from HD or COVID-19 inpatients (allogeneic cocultures) (Figure 2A). Then, CD25 expression and TNF-α production were evaluated. Platelets from COVID-19 inpatients, but not from HD, significantly inhibited the upregulation of CD25 in $CD4^+$ T cells and TNF-α production (Figure 2B,C). We demonstrated that the presence of the platelet aggregates did not mask the detection of CD25 (Figure S2A,B). It has been reported that platelets enhance the IFN-γ production by $CD4^+$ T cells.[18,20] As expected, in our assays, analysis of IFN-γ production revealed that it was increased by platelets from HD, but interestingly no increment of IFN-γ production was observed using platelets from COVID-19 patients (Figure 2D). In contrast with these results, we found that platelets from HD and COVID-19 patients modulated IL-17 and IL-10 production in a similar way: both enhanced IL-17 and inhibited IL-10 production (Figure 2E,F). Moreover, we observed that platelets from HD and COVID-19 patients did not modulate the proportion of $CD4^+CD25^+FoxP3^+$ T cells (data not shown). Culture with platelets did not induce any change in $CD4^+$ T-cell viability (Figure S2C). As controls, platelets from HD or COVID-19 patients were cultured alone and were not able to produce TNF-α, IFN-γ, IL-17 or IL-10 (Figure 2C–F). Interestingly, in the whole set of results, platelets from COVID-19 patients differ from HD only in those characteristics associated with the TH1 phenotype. This suggests that COVID-19 platelets could be compromising TH1 functionality.

To determine if platelet–lymphocyte interactions within a single donor might reproduce the results observed in allogeneic cocultures, we evaluated the IFN-γ production in autologous cocultures. Similar results to allogeneic cocultures were observed when $CD4^+$ T cells and platelets were isolated from the same donor: IFN-γ production was increased by platelets from HD but not by platelets from COVID-19 patients (Figure 2G).

## PD-L1 expression enables platelets from COVID-19 inpatients to modulate IFN-γ production by $CD4^+$ T cells

We then re-analysed available RNA-seq datasets of isolated platelets from COVID-19 patients previously published (PRJNA634489).[11] Unsupervised clustering of the samples showed that platelets from COVID-19 patients shared a similar transcriptional profile that differs from that of HD platelets, although it did not separate ICU from non-ICU patients (Figure 3A). As expected for a viral infection, platelets from COVID-19 patients were mainly enriched in transcripts associated with type I interferon response (IFITM3, IFITM1, IFITM2 and IFI27; Figure 3B). Looking for a candidate that could explain our previous results, we evaluated the expression of ligands that interact with $CD4^+$ T cells' inhibitory receptors (PD-L1, HVEM, Galectin 9, GITRL, CD155)[29] in platelets from HD and COVID-19 patients (Figure 3C). We observed that PD-L1 was upregulated in COVID-19 patients (Figure 3C). Moreover, among these inhibitory transcripts, PD-L1 showed the highest fold change, 7.67 ($\log_2$ fold change = 2.94; $p = 0.0319$; Figure 3B). PD-L1/PD-1 signalling is a well-characterized inhibitory pathway that can also be activated by PD-1 interaction with PD-L2. When binding to its ligands, PD-1 can activate intracellular signalling pathways and inhibit the activation of immune cells, to even exhaust the immune cell and thus maintain immune system homeostasis. The PD-L1/PD-1 signalling pathway is an important component of tumour immunosuppression, which can inhibit the activation of T lymphocytes and enhance the immune tolerance of tumour cells, thereby achieving tumour immune escape.[30] To validate these results, PD-L1 and CD62p expressions were measured by flow cytometry in purified platelets from HD and COVID-19 patients. Consistent with the RNAseq datasets, we found that platelets from COVID-19 patients showed

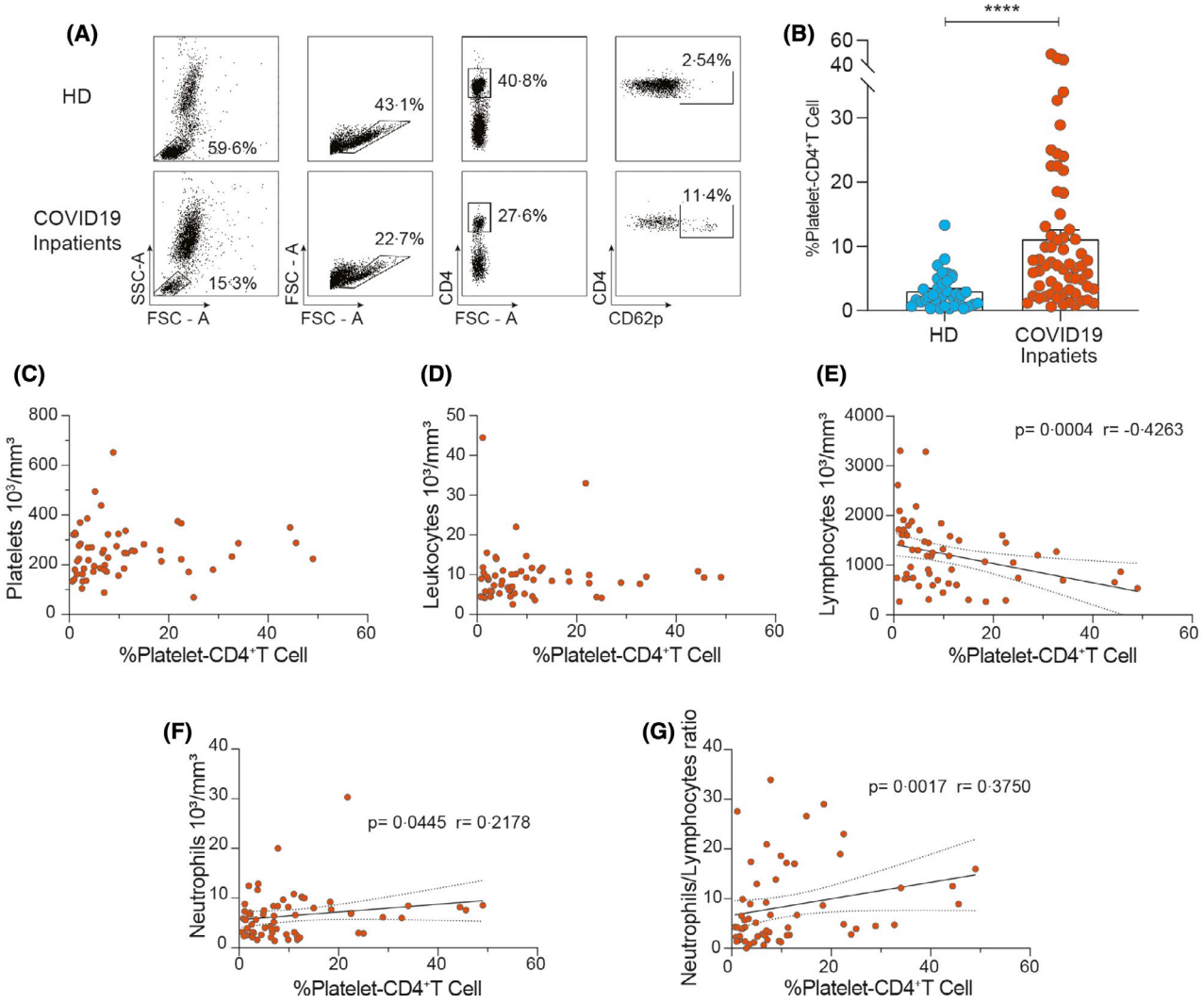


**FIGURE 1** COVID-19 increases platelet–$CD4^+$ T-cell aggregates. (A–F) Whole blood samples from COVID-19 patients and healthy donors (HD) were collected and the expression of CD62p (P-selectin) and CD4 was analysed by flow cytometry. Platelet–$CD4^+$ T-cell aggregates were identified as $CD4^+$ $CD62p^+$ leukocytes. (A) Representative dot plots and (B) bar graph showing the percentage of platelet–$CD4^+$ T-cell aggregates in HD ($n = 35$) and COVID-19 inpatients ($n = 62$). (C) Platelet count, (D) leukocyte count, (E) lymphocyte count, (F) neutrophil count and (G) neutrophil:lymphocyte ratio plotted against percentage of platelet–$CD4^+$ T-cell aggregates ($n = 62$). Bars represent the mean ± SEM. Mann–Whitney test was used in (B). Linear regression and Spearman correlation were calculated according to the distribution of the dots in (C–G). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ [Colour figure can be viewed at wileyonlinelibrary.com]

a higher expression of PD-L1 compared to HD platelets (Figure 3D). We also observed an elevated expression of P-selectin in platelets from COVID-19 patients (Figure 3D), and the percentage of PD-L1 and P-selectin double-positive platelets was also higher in COVID-19 patients (Figure 3D). It was demonstrated previously that the treatment with low doses of thrombin, which activates platelets, increases P-selectin expression and triggers the expression of PD-L1 in platelets.[31] In line with this observation, we found a positive correlation between P-selectin and PD-L1 expression in platelets (Figure 3E). We also observed that the expression of PD-L1 by platelets was similar in moderate and severe COVID-19 patients (Figure S3A). The expression of PD-L2 and CD4 was also measured and platelets from HD and COVID-19 patients did not express PDL-2 or CD4 (Figure S3B,C).

Interestingly, a negative correlation was found between platelet PD-L1 expression and IFN-γ production by $CD4^+$ T cells cultured with platelets (Figure 4A). Besides, the correlation between platelet PD-L1 expression and the production of IFN-γ by $CD4^+$ T cells (without platelets) from the same HD or COVID-19 patient was evaluated. We also observed a negative correlation between platelet PD-L1 expression and IFN-γ production by $CD4^+$ T cells (Figure 4B). Although these results suggested that PD-L1 could be a contributing factor to our observations, it is not sufficient to confirm that the decreased IFN-γ production is its consequence. To evaluate the role of PD-L1 expressed by platelets in the modulation of $CD4^+$ T-cell function, we first analysed the expression of PD-1 in $CD4^+$ T cells. As expected, activation of $CD4^+$ T cells resulted in the upregulation of PD-1 expression regardless of the

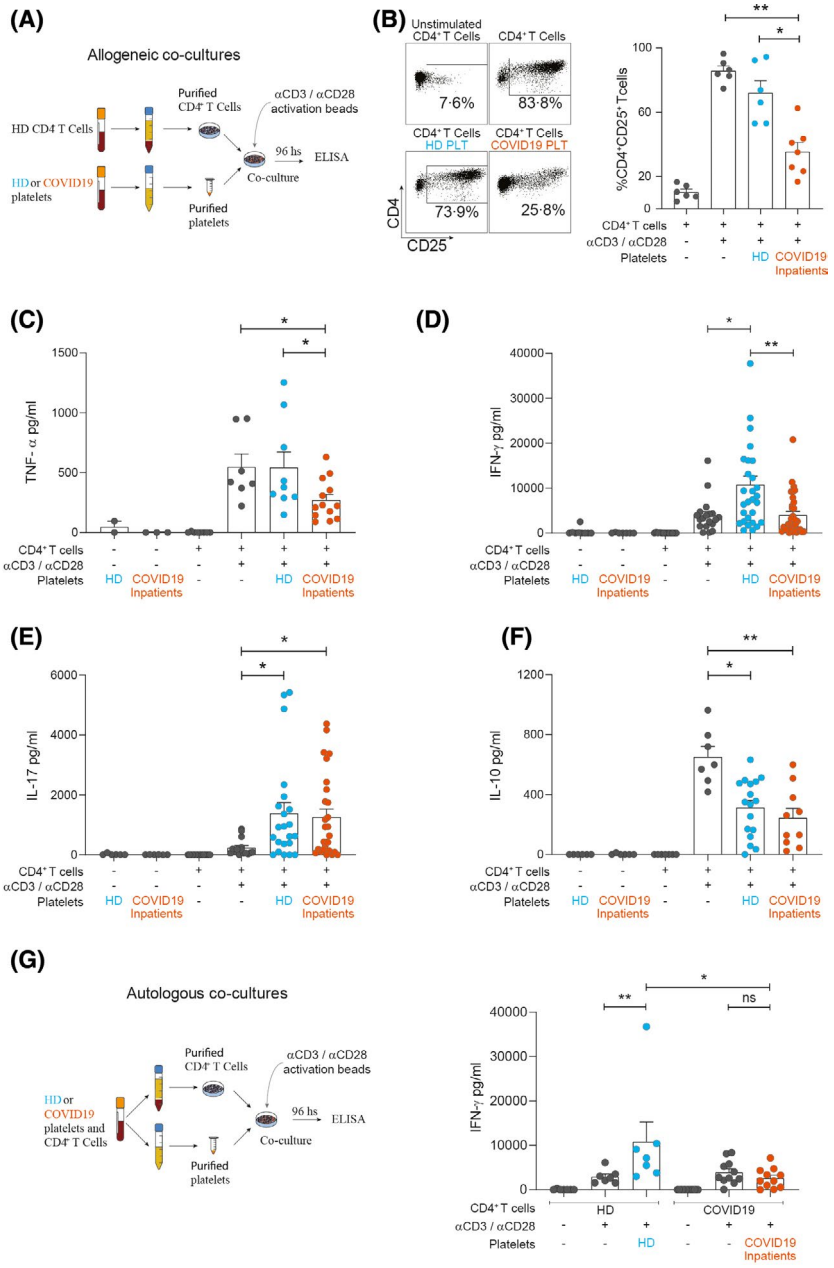


**FIGURE 2** Platelets from healthy donors and COVID-19 inpatients differentially modulate $CD4^+$ T-cell function. (A–G) Peripheral blood samples were collected from healthy donors (HD) or COVID-19 inpatients. $CD4^+$ T cells ($1.5 \times 10^5$) were cocultured with heterologous platelets from HD ($n$ = 7–21) or hospitalized COVID-19 patients ($n$ = 7–26) (1:100 $CD4^+$ T cell:platelet ratio), and stimulated with anti-CD3/anti-CD28-coated beads ($1.5 \times 10^5$ beads/$1 \times 10^6$ $CD4^+$ T cells). $CD4^+$ T cells were obtained from HD except indicated otherwise. (A) Schematic diagram of the experimental design. (B) CD25 expression was measured by flow cytometry 96 h after stimulation ($n$ = 6–7). Dot plot from a representative experiment and a bar graph showing percentages are shown. (C) TNF-α production was measured by enzyme-linked immunosorbent assay (ELISA) 48 h after stimulation ($n$ = 2–13). (D) IFN-γ ($n$ = 7–33), (E) IL-17 ($n$ = 6–27) and (F) IL-10 ($n$ = 6–17) production was measured by ELISA 96 h after stimulation. (G) IFN-γ was measured in autologous cocultures ($CD4^+$ T cells and platelets from the same donor) from HD ($n$ = 7) or COVID-19 patients ($n$ = 11). Bars represent the mean ± SEM. Kluskal-Wallis test was used in (B–G). $^*p < 0.05$ and $^{**}p < 0.01$ [Colour figure can be viewed at wileyonlinelibrary.com]

presence of platelets (Figure 4C). When allogeneic cultures of $CD4^+$ T cells and platelets were performed in the presence of a blocking antibody directed to PD-L1, we found a significant recovery of COVID-19 platelets' ability to stimulate IFN-γ production by $CD4^+$ T cells (Figure 4D). Taking into account the kinetics of PD-1 expression in activated $CD4^+$ T cells that reaches a maximum of five days upon stimulation (Figure 4E), we performed a new set of experiments. First, we activated $CD4^+$ T cells for five days and then added platelets at this time point, when we also re-stimulated the lymphocytes. The results showed that platelets from HD did not increase IFN-γ production as observed in Figure 2C, probably because with the re-stimulation lymphocytes it had already reached high

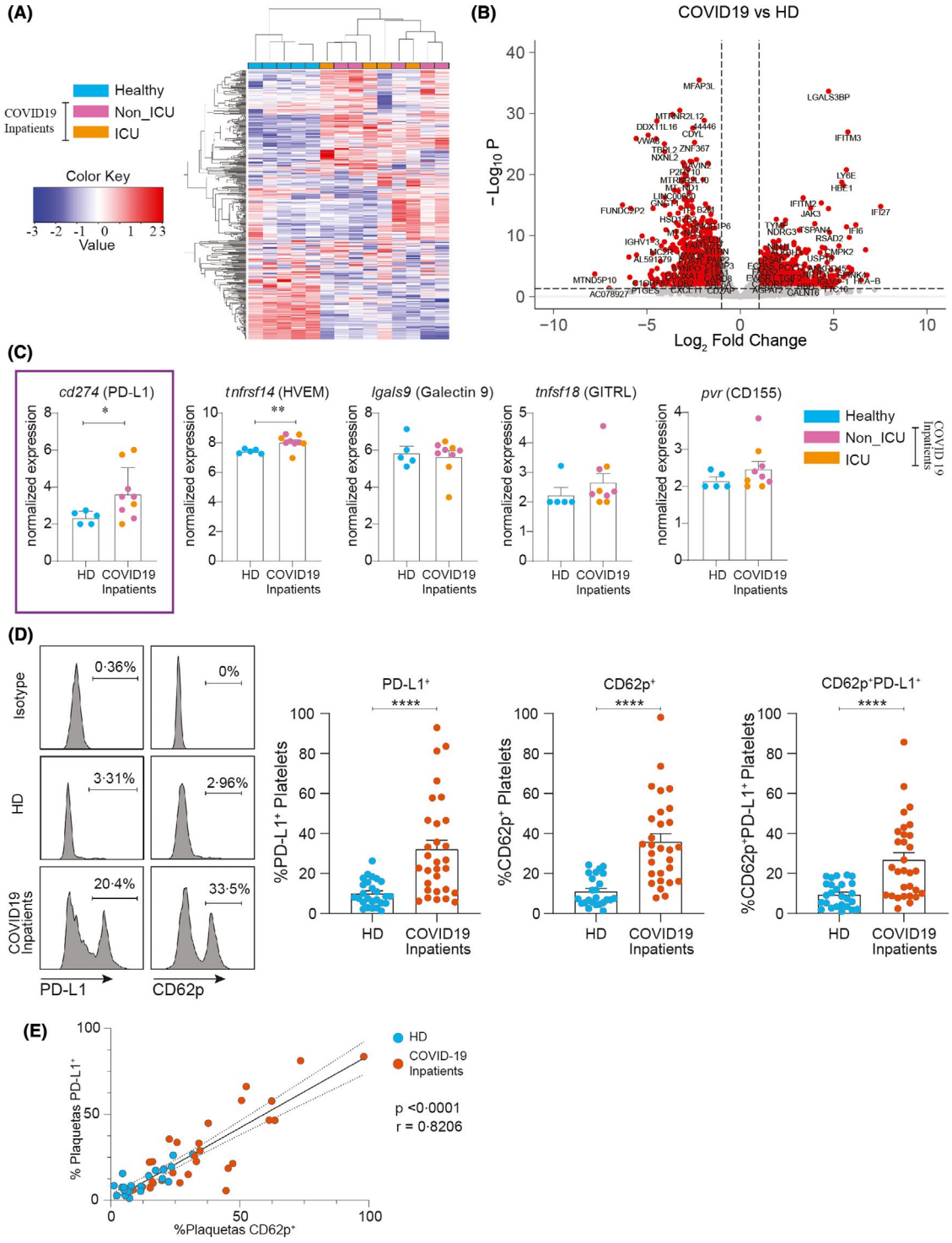


**FIGURE 3** Platelets from COVID-19 inpatients show an increased PD-L1 expression (A–C); an available RNAseq data set of purified platelets from healthy donors (HD) ($n = 5$), non-ICU ($n = 5$) and ICU ($n = 4$) COVID-19 patients was analysed.[11] (A) Heat map of the top 500 highly variable genes is shown. Red indicates increased relative expression and blue indicates decreased relative expression. (B) Volcano plot showing significantly increased and decreased transcripts of COVID-19 vs HD. (C) Bar graph showing normalized expression [$\log_2$(TPM + 1)] of PD-L1, HVEM, galectin 9, GILTR and CD155 in HD ($n = 5$) and COVID-19 patients ($n = 9$). (D) Peripheral blood samples were collected from HD or COVID-19 inpatients. PD-L1 and P-selectin (CD62p) expression was evaluated by flow cytometry. Representative histograms and bar graphs showing percentages of PD-L1 and CD62p and double-positive $CD62p^+PD\text{-}L1^+$ platelets expressing platelets in HD ($n = 26$) and COVID-19 patients ($n = 30$) are shown. (E) Proportion P-selectin-positive platelets plotted against percentage of PD-L1-positive platelets ($n = 56$). Bars represent the mean ± SEM. The Mann–Whitney test was applied in (C) and (D). Linear regression and Spearman correlation were calculated according to the distribution of the dots in (E). *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$ [Colour figure can be viewed at wileyonlinelibrary.com]

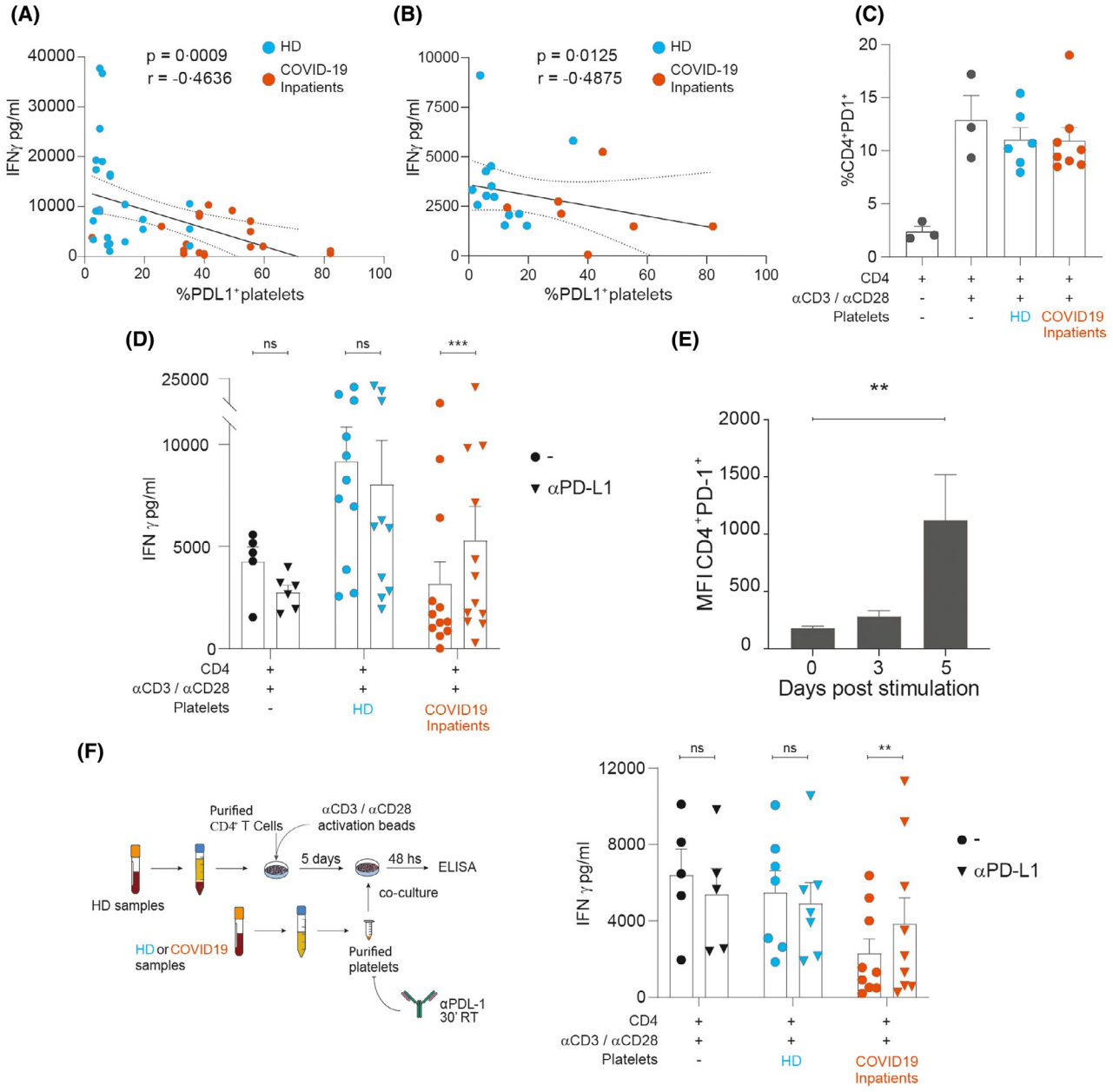


**FIGURE 4** PD-L1 expression enables platelets to modulate IFN-γ production by $CD4^+$ T cells. (A–D) $CD4^+$ T cells ($1.5 \times 10^5$) were cocultured with heterologous platelets from healthy donors (HD) ($n = 5–13$) or from hospitalized COVID-19 patients ($n = 7–12$) (1:100 $CD4^+$ T cell:platelet ratio), and stimulated with anti-CD3/anti-CD28-coated beads ($1.5 \times 10^5$ beads/$1 \times 10^6$ $CD4^+$ T cells). (A) IFN-γ production in cocultures of $CD4^+$ T cells and platelets from HD (blue) or COVID-19 (red) patients plotted against the percentage of PD-L1-expressing platelets are shown ($n = 42$). (B) IFN-γ production in cultures of $CD4^+$ T cells (without platelets) from HD (blue) or COVID-19 (red) patients plotted against the percentage of PD-L1 expressed in platelets from the same donor are shown ($n = 21$). (C) PD-1 expression was measured in $CD4^+$ T cells by flow cytometry in cocultures after 96 h post stimulation ($n = 3–8$). (D) Platelets were pre-incubated for 30 min at room temperature with a blocking antibody directed to PD-L1 (10 μg/ml) and then added to the culture. IFN-γ production was measured by enzyme-linked immunosorbent assay (ELISA) 96 h post stimulation ($n = 5–12$). (E) Purified $CD4^+$ T cells were stimulated with anti-CD3/anti-CD28-coated beads ($1.5 \times 10^5$ beads/$1 \times 10^6$ $CD4^+$ T cells). PD-1 expression was measured by flow cytometry at 0, 3 or 5 days after stimulation ($n = 4–5$). (F) Platelets from HD ($n = 9$) or COVID-19 patients ($n = 10$) were pre-incubated for 30 min at room temperature with αPD-L1 -blocking antibody (10 μg/ml), and then added to five-day-stimulated $CD4^+$ T cells ($1.5 \times 10^5$ $CD4^+$ T cells at 1:100 $CD4^+$ T cell:platelet ratio). The cocultures were restimulated ($1.5 \times 10^5$ beads/$1 \times 10^6$ $CD4^+$ T cells) and 48 h post stimulation IFN-γ production was measured by ELISA ($n = 5–9$). Bars represent the mean ± SEM. Linear regression and Spearman correlation were calculated according to distribution of the dots in (A) and (B). The Kruskal–Wallis test was used in C–F. The Wilcoxon test was used to compare the platelet + lymphocyte cocultures with or without αPDL1-blocking antibody in (D) and (F). *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$ [Colour figure can be viewed at wileyonlinelibrary.com]

stimulation levels. However, platelets from COVID-19 patients were able to decrease IFN-γ. Under these experimental conditions, blockade of PD-L1 also significantly prevented the inhibitory effect exerted by platelets from COVID-19 patients on the production of IFN-γ by $CD4^+$ T cells (Figure 4F).

## DISCUSSION

Severe COVID-19 is characterized by massive cytokine release mediated mainly by innate immune response and T-cell loss.[1–6] The exaggerated host inflammatory response is incapable of viral clearance, and instead aggravates respiratory distress and damage to other organs.[1,2] Platelet hyperactivity may contribute to COVID-19 pathophysiology through increased platelet–platelet and platelet–leukocyte interactions.[11,12] Consistent with these reports, compared with HD, we observed a significant increase of $CD4^+$ T cell–platelet aggregates in COVID-19 inpatients, with moderate and severe disease.[11,12,24] A previous report also showed that monocyte–platelet aggregation had a major impact on monocyte function during COVID-19, thus increasing the tissue factor expression.[12] Similar results have been observed in patients with bacterial sepsis,[32] a syndrome with many parallels with severe COVID-19,[33] and HIV.[34] However, understanding the role of the platelets in the modulation of lymphocyte functionality in COVID-19 remains elusive.

A large body of evidence indicates that platelets regulate T-cell function through different mechanisms mediated by either platelet surface molecules such as P-selectin and CD40L or the release of a number of mediators such as enzymes, cytokines (platelet factor 4, RANTES, and TGF-β) or extracellular vesicles.[15,17–20] In fact, coculture of platelets with $CD4^+$ T cells has shown to promote the differentiation of TH1 and TH17 cells and the production of IFN-γ and IL-17.[17,18,20] Platelets have also been reported to enhance the function of regulatory T cells.[15,17,18,20]

In the present study, we found that platelets from hospitalized COVID-19 inpatients acquire an immunomodulatory profile different from that shown by HD platelets. Platelets from hospitalized COVID-19 patients inhibited the upregulation of CD25 expression and the production of TNF-α by activated $CD4^+$ T cells. Moreover, also contrasting with the observation made with HD platelets, they did not increase IFN-γ production by $CD4^+$ T cells. Surprisingly, the proportion of $CD25^+Foxp3^+$ cells and IL-10 and IL-17 production was not altered when platelets were from a COVID-19 patient instead of a HD. These observations suggest that platelets from COVID-19 patients could specifically hinder TH1 responses, which plays an important role in antiviral immunity. Interestingly, we found that platelets from COVID-19 patients show a higher expression of PD-L1. The inability of COVID-19 platelets to increase IFN-γ production by $CD4^+$ T cells was almost completely restored by pretreating platelets with a blocking antibody directed to PD-L1, revealing its role in the modulation of $CD4^+$ T-cell function. PD-L1 expression in platelets was first shown in leukaemia patients.[35] To our knowledge, no previous reports have analysed the role of PD-L1 expressed in platelets in COVID-19. It was previously shown that platelets are able to suppress T-cell-mediated cytotoxicity against tumour cells and that this effect could be partially rescued with the addition of PD-L1 neutralizing antibody.[31]

A lack of understanding of the underlying mechanisms driving both a dysregulated production of inflammatory cytokines by innate immune cells and a progressive dysfunction and loss of T cells has hindered the rational design of new and more effective therapeutic strategies in COVID-19 patients. Beyond cancer treatment, immune checkpoint inhibitors have been recently tested to restore immunocompetence in sepsis. In fact, the anti-PD-1 mAb nivolumab has shown to partially restore lymphocyte count and function in septic patients without increasing the cytokine storm.[36] It has been recently reported that progression to COVID-19 is associated with an increased expression of PD-L1 in different cell types such as epithelial cells, monocytes, neutrophils, and $CD4^+$ T cells.[37] Moreover, severe COVID-19 has shown to be associated to an increase in PD-L1 in serum levels.[37] Our present results suggest that platelets might contribute to disease progression in patients with COVID-19 through a PD-L1-dependent mechanism. Together, these observations suggest that restoring exhausted T cells by blockade of PD-1/PD-L1 interactions might be considered as an adjuvant therapeutic strategy in severe COVID-19.

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### CONFLICT OF INTERESTS

All authors report no potential conflicts.

### AUTHOR CONTRIBUTIONS

Ana Ceballos, Federico Remes Lenicov, Ana Paletta, Facundo Di Diego García: conceptualization; Ana Paletta, Facundo Di Diego García, Fernando Erra Diaz, Ignacio Mazzitelli, Álvaro López Malizia: methodology; Ana Paletta, Facundo Di Diego García, Ana Ceballos, Augusto Varese, Julián García, Juan Carlos Cisneros, Guillermina Ludueña, Alejandra G. Rodriguez, Nicolás Lista, Andrea Pisarevsky: investigation; Ana Paletta, Facundo Di Diego García, Fernando Erra Diaz, Ana Ceballos: formal analysis; Ana Ceballos, Ana Paletta, Facundo Di Diego García: writing; Ana Ceballos, Federico Remes Lenicov, Augusto Varese, Juan Sabatté, Jorge Geffner: review and editing; Ana Ceballos: funding acquisition; Juan Carlos Cisneros, Guillermina Ludueña, Gonzalo Cabrerizo: resources; Ana Ceballos, Federico Remes Lenicov, Jorge Geffner: supervision.

### ORCID

*Ana Ceballos* https://orcid.org/0000-0003-4564-7361

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## SUPPORTING INFORMATION

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