Platelet–monocyte interaction amplifies thromboinflammation through tissue factor signaling in COVID-19

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Platelet-monocyte interaction amplifies thromboinflammation through tissue factor signaling in COVID-19

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Abstract:
Accumulating evidence into the pathogenesis of COVID-19 highlight a hypercoagulability state with high risk of life-threatening thromboembolic complications. However, the mechanisms of hypercoagulability and their link to hyperinflammation remain poorly understood. Here we investigate functions and mechanisms of platelet activation and platelet-monocyte interactions in inflammatory amplification during SARS-CoV2 infection. We used a combination of immunophenotyping, single cell analysis, functional assays and pharmacological approaches to gain insights on mechanisms. Critically ill COVID-19 patients exhibited increased platelet-monocyte aggregates formation. We identified a subset of inflammatory monocytes presenting high CD16 and low HLA-DR expression as the subset mainly interacting with platelets during severe COVID-19. Single cell RNAseq analysis indicated enhanced fibrinogen receptor Mac-1 in monocytes from severe COVID-19 patients. Monocytes from severe COVID-19 patients displayed increased platelet binding and hyperresponsiveness to P-selectin and fibrinogen with respect to TFN-α and IL-1β secretion. Platelets were able to orchestrate monocyte responses driving TF expression, inflammatory activation and inflammatory cytokines secretion in SARS-CoV-2 infection. Platelet-monocyte interactions ex-vivo and in SARS-CoV-2 infection model in vitro reciprocally activated monocytes and platelets, inducing the heightened secretion of a wide panel of inflammatory mediators. We identified platelet adhesion as a primary signaling mechanism inducing mediator secretion and TF expression, while TF signaling played major roles in amplifying inflammation by inducing proinflammatory cytokines, especially TNF-α and IL-1β. Our data identify platelet-induced TF expression and activity at the crossroad of coagulation and inflammation in severe COVID-19.

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Platelet-monocyte interaction amplifies thromboinflammation through tissue factor signaling in COVID-19

Running Title: TF signaling amplifies inflammation in COVID-19

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Key points

Platelet-monocyte interaction engages a reciprocal activation loop that feeds thromboinflammation in COVID-19.

Platelet adhesion is a primary signaling mechanism for monocyte activation that is amplified by tissue factor–dependent signaling.

Abstract

Accumulating evidence into the pathogenesis of COVID-19 highlight a hypercoagulability state with high risk of life-threatening thromboembolic complications. However, the mechanisms of hypercoagulability and their link to hyperinflammation remain poorly understood. Here we investigate functions and mechanisms of platelet activation and platelet-monocyte interactions in inflammatory amplification during SARS-CoV2 infection. We used a combination of immunophenotyping, single cell analysis, functional assays and pharmacological approaches to gain insights on mechanisms. Critically ill COVID-19 patients exhibited increased platelet-monocyte aggregates formation. We identified a subset of inflammatory monocytes presenting high CD16 and low HLA-DR expression as the subset mainly interacting with platelets during severe COVID-19. Single cell RNAseq analysis indicated enhanced fibrinogen receptor Mac-1 in monocytes from severe COVID-19 patients. Monocytes from severe COVID-19 patients displayed increased platelet binding and hyperresponsiveness to P-selectin and fibrinogen with respect to TFN-α and IL-1β secretion. Platelets were able to orchestrate monocyte responses driving TF expression, inflammatory activation and inflammatory cytokines secretion in SARS-CoV-2 infection. Platelet-monocyte interactions ex-vivo and in SARS-CoV-2 infection model in vitro reciprocally activated monocytes and platelets, inducing the heightened secretion of a wide panel of inflammatory mediators. We identified platelet adhesion as a primary signaling mechanism inducing mediator secretion and TF expression, while TF signaling played major roles in amplifying inflammation by inducing proinflammatory cytokines, especially TNF-α and IL-1β. Our data identify platelet-induced TF expression and activity at the crossroad of coagulation and inflammation in severe COVID-19.

Keywords: Thromboinflammation, COVID-19, platelet-monocyte interaction, tissue factor signaling, inflammatory amplification.

Introduction

Hypercoagulability is central in pathophysiology and also a significant determinant of mortality in COVID-19 patients[1–4]. Pulmonary and extrapulmonary microvascular thrombosis is associated with multiorgan failure[5–7] occurring more frequently in COVID-19 than in influenza pneumonia[7,8]. While heparin treatment may be beneficial[9], persistent hypercoagulability and thromboinflammatory tissue damage have been reported despite prophylactic anticoagulation[5,10,11]. Markers of coagulation and inflammation, including D-dimers, TNF-α and IL-6 are early predictors of respiratory distress and mortality during COVID-19[12–16]. Overwhelming inflammatory activation ("cytokine storm") may both sustain and be amplified by hypercoagulability[17,18]. Nevertheless, the mechanisms of hypercoagulability in COVID-19 patients and how it is linked to hyperinflammation are still to be determined.

Platelets are blood cells classically known by their roles in thrombosis and hemostasis[19]. Beyond their hemostatic activities, platelets orchestrate inflammatory response, secreting inflammatory mediators and forming heterologous aggregates with leukocytes[19–22]. Activated platelets adhere to leukocytes reprogramming cellular functions through juxtracrine signals from P-selectin and fibrinogen-bearing integrins[23–25]. Severe COVID-19 evolves with platelet hyperactivity and increased platelet-monocyte, lymphocyte and neutrophil aggregates formation[26–29]. COVID-19 post mortem pathological findings show extensive areas of microvascular tissue thrombosis containing platelet-neutrophil complexes and NETosis[5,6]. Intravascular and airways NETosis is associated with case severity and mortality[6], and activated platelets in COVID-19 are a determinant to NET extrusion[5,30]. We have recently shown that increased platelet activation and platelet-monocyte interaction in severe COVID-19 induce pathologic expression of tissue factor (TF)[26], the main trigger of coagulation activation and thrombosis[31]. Interestingly, TF-expressing monocytes represent a subset of inflammatory monocytes highly expressing proinflammatory cytokines in people

116 living with HIV[18], but the participation of platelets in this monocyte subset
117 reprogramming remains unknown. Our central hypothesis is that platelet-induced
118 procoagulant and proinflammatory signaling in monocytes are linked, amplifying
119 inflammation and hypercoagulability in COVID-19.

120 Here we identified platelet and monocyte activation mechanisms involved in
121 reciprocal loops of cellular communication that feed the thromboinflammatory process
122 in COVID-19. We report new mechanisms of platelet-monocyte signaling involving
123 adhesion-mediated TF expression and activity, which drives activation and
124 proinflammatory cytokine secretion in monocytes. We stablish signaling pathways
125 linking coagulation and inflammation in severe COVID-19 by identifying novel
126 mechanisms of thromboinflammation associated with severity and mortality in critically
127 ill patients.

128

Material and methods

Human subjects

We prospectively enrolled a cohort of 68 RT-PCR confirmed mild (n = 22) to severe (n = 46) COVID-19 patients and 25 SARS-CoV-2-negative control subjects. Blood was obtained from the 46 patients with severe COVID-19 within 72 hours from ICU admission in three reference centers (Instituto Estadual do Cérebro Paulo Niemeyer, Hospital Copa Star and Leblon Campaign Hospital, all in Rio de Janeiro, Brazil). Severe COVID-19 was defined as those critically ill patients, presenting viral pneumonia on computed tomography scan and requiring oxygen supplementation through either a nonrebreather mask or mechanical ventilation. Twenty-two outpatients presenting mild self-limiting COVID-19 syndrome were also included. All patients had SARS-CoV-2 confirmed diagnostic through RT-PCR of nasal swab or tracheal aspirates. Peripheral blood samples were collected from 25 SARS-CoV-2-negative control volunteers. The characteristics of mild, severe and control participants are presented in **Table 1**. Mild and severe COVID-19 patients presented differences regarding the age and the frequency of comorbidities (**Table 1**), which is consistent with previous reports[32–34]. Subjects of older age and chronic noncommunicable diseases were also recruited in the SARS-Cov-2-negative control group to matched with mild and severe COVID-19 patients, except for hypertension and diabetes (**Table 1**).

All ICU-admitted patients received usual supportive care for severe COVID-19, including either noninvasive oxygen supplementation (n= 16) or mechanical ventilation (n= 30) (**Table S1**). Clinical information from all severe COVID-19 patients was collected using a standardized form - ISARIC/WHO Clinical Characterization Protocol for Severe Emerging Infections (CCP-BR)[35]. Clinical and laboratory data were

prospectively recorded and the primary outcome analyzed was 28-day mortality (n = 28 survivors and 18 nonsurvivors, **Table S2**). Sex, age and the frequency of comorbidities were not different between severe patients requiring mechanical ventilation or noninvasive oxygen supplementation neither between survivors and nonsurvivors (**Table S1** and **S2**). All clinical investigations were conducted according to the principles of the Declaration of Helsinki. The study protocol was approved by the National Review Board (Comissão Nacional de Ética em Pesquisa – CONEP 30650420.4.1001.0008), and informed consent was obtained from all participants or patients' representatives.

Monocyte adhesion on immobilized P-selectin or Fibrinogen

Monocyte adhesion assays were performed as previously described[36]. Briefly, 8-wells Lab-Tek plates were incubated overnight at 4 °C with PBS containing recombinant human albumin, P-selectin (10 µg/ml) or fibrinogen (100 µg/ml) and then blocked with albumin (10 mg/ml) for 4 hours at room temperature. The plates were washed twice with PBS containing 0.05 % Tween-20 and three times with PBS. Monocytes (1×10^5) from severe COVID-19 patients or control subjects were resuspended in 100 µL of M199 containing 10 mg/ml polymyxin B, plated on the coated surfaces and incubated overnight at 37 °C in a 5 % CO_2 atmosphere. After 12 hours post-plating, the supernatants were harvested, centrifuged to remove loose cells (500 x g for 15 min) and stored for further quantification of inflammatory mediators. Adherent cells were fixed with 4 % paraformaldehyde and the nuclei were stained with DAPI (1 µg/mL) and analyzed by fluorescence microscopy.

Platelet-monocyte ex vivo interaction

To examine whether platelets from COVID-19 patients modulate thromboinflammatory responses in monocytes from healthy volunteers, purified platelets and monocytes were incubated ex vivo at 37 °C in a 5% CO_2 atmosphere. Each experimental point contained 2 x 10^5 monocytes from a COVID-19 patient with 2 x 10^7 platelets from a healthy volunteer, or 2 x 10^5 monocytes from a healthy volunteer with 2 x 10^7 platelets from a COVID-19 patient. Control monocytes plus platelets from a different healthy volunteer were used as control. In selected experiments, platelet-monocyte interactions were performed in the presence of neutralizing antibodies against P-selectin (BBA30, R&D Systems) (20 µg/mL), TF (clone 10H10 or 5G9) (50 µg/mL), the anti-integrin $\alpha_{IIb}\beta_3$ monoclonal antibody abciximab (50 µg/mL), or isotype-matched IgG (50 µg/mL). Platelet-monocyte interactions were also performed in the presence of aspirin (100 µM, A5376, Sigma), clopidogrel (300 µM, PHR1431, Sigma), Ixolaris or DMSO (vehicle). After 0.5, 2 or 18 hours of interaction, cells were centrifuged, the supernatants were harvested and cells were fixed with 4 % paraformaldehyde for flow cytometry analysis as described above. The experiment was repeated using monocytes from 2-3 independent healthy volunteers with similar results, and a representative data from one of the donors is shown. Monoclonal anti-TF antibodies were kindly provided by Dr. Wolfram Ruf (Johannes Gutenberg University Medical Center, Mainz, Germany; and Department of Immunology and Microbiology, The Scripps Research Institute, La Jolla, CA). Ixolaris was expressed and purified as described[37].

Platelet-monocyte infection in vitro

SARS-CoV-2 was originally isolated from nasopharyngeal swabs of a confirmed case from Rio de Janeiro/Brazil (GenBank accession no. MT710714). The virus was amplified for 2 to 4 days in Vero E6 cell cultures in high glucose Dulbecco′s Modified Eagle′s Medium supplemented with 2% fetal bovine serum at 37˚C in 5% CO_2 atmosphere. Virus titers were determined by the tissue culture infectious dose at 50%

(TCID50/mL) and the virus stocks kept in -80˚C freezers until use. All procedures involving SARS-CoV-2 culture were performed in a biosafety level 3 (BSL3) facility. Platelets (2 x 10^7) and monocytes (2 x 10^5) were infected with SARS-CoV-2 separately or in combination (multiplicity of infection = 0.01 virus per monocyte). In selected experiments, platelet-monocyte co-cultures were infected in the presence of abciximab, anti-TF antibodies (clone 10H10 or 5G9), or isotype-matched IgG (50 µg/mL), or the PAR-1 inhibitor SCH79797 (5 µM, Tocris 1592), PAR-2 inhibitor AZ3451 (10 µM, Sigma SML2050) or DMSO (vehicle). After 12 h of infection, supernatants were harvested and stored for future analysis, and cells were fixed with 4 % paraformaldehyde for flow cytometry analysis as described in supplemental material.

Statistical analysis

Statistics were performed using GraphPad Prism software version 7. All the numerical variables were tested regarding their distribution using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used to compare differences among 3 or more groups following a normal (parametric) distribution, and Tukey's post-hoc test was used to locate the differences between the groups. Comparisons between 2 groups were performed using the Student t-test for parametric distributions or the Mann-Whitney *U* test for nonparametric distributions.

Results

Platelet-monocyte interaction associates with monocyte activation and immune dysfunction in COVID-19

We have recently described novel mechanisms of platelet activation and platelet-induced monocyte TF expression that were associated with hypercoagulability and mortality in severe COVID-19 patients[26]. We then investigated the relationship of platelet-monocyte aggregate formation and monocyte inflammatory phenotypes during severe COVID-19. Interaction with platelets was assessed by the expression of the platelet marker CD41 on the classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{high}CD16^{+}$) and nonclassical ($CD14^{low}CD16^{+}$) monocyte subsets. As shown in **Figure 1A**, COVID-19 patients presented increased levels of platelet-monocyte aggregates specifically in CD16-positive intermediate and nonclassical monocytes. In addition, platelet-monocyte aggregates formed preferentially with HLA-DR-negative monocytes (**Figure 2B** and **Supplemental Figure S1**). These data highlight a strong association of platelet-monocyte aggregate formation with monocyte inflammatory activation and immune dysfunction in severe COVID-19.

Monocytes from COVID-19 patients secrete proinflammatory cytokines in response to P-selectin and fibrinogen

Considering the relationship between monocyte immunoinflammatory phenotype and interaction with platelets in severe COVID-19 (**Figure 1 A-B**), we investigated the expression of monocyte adhesion molecules that mediate platelet-leukocyte aggregate formation. Single cell RNA analysis has shown that the fibrinogen receptor Mac-1 subunits integrin α_M (ITGAM) and integrin β_2 (ITGB2) transcripts are increased in

monocytes from severe COVID-19 patients (**Figure 1C** and **Supplemental Figure S2**). We confirmed through flow cytometry that Integrin α_M (CD11b) expression is increased on monocytes from severe COVID-19 patients compared to patients with mild COVID-19 or control subjects (**Figure 1D**), indicating increased Mac-1 expression. Importantly, Mac-1 expression was higher in mechanically ventilated patients compared to patients under noninvasive oxygen supplementation (**Figure 1E**), and in patients that evolved with mortality compared to hospital discharge (**Figure 1F**).

To gain insights on how monocytes from severe COVID-19 patients respond to the molecules that mediate platelet-monocyte aggregate formation, we performed monocyte adhesion assays on P-selectin or fibrinogen coated surfaces. As expected, monocytes from healthy volunteers showed increased adhesion to recombinant P-selectin and fibrinogen when compared to recombinant human albumin (**Figure 2A**). Monocytes from severe COVID-19 patients, on the other hand, were more adhesive and secreted higher levels of IL-6, IL-10 and MCP-1/CCL2 regardless of the surface on which they were adhered (**Figure 2A-B** and **Supplemental Figure S3A-B**). Importantly, monocytes from severe COVID-19 patients were more responsive to P-selectin and fibrinogen coated surfaces regarding the secretion of TNF-α, IL-1β, IL-8, MIP-1α and MIP-1β, as compared to control monocytes (**Figure 2C-D** and **Figure S3D-F**). These data indicate that monocytes from severe COVID-19 patients present higher responsiveness to P-selectin and fibrinogen regarding inflammatory cytokine secretion, especially TNF-α, IL-1β and IL-8.

Platelet adhesion and induction of TF expression precede monocyte inflammatory activation

We have recently shown that activated platelets from severe COVID-19 patients induce monocyte TF expression[26]. We hypothesized that besides procoagulant pathways,

platelet-monocyte interaction also orchestrates inflammation in COVID-19. To confirm this hypothesis, we incubated monocytes from healthy volunteers with platelets from severe COVID-19 patients ex vivo (**Figure 3A**), and evaluated the kinetics of monocyte TF and CD16 expression. Platelets from severe COVID-19 patients rapidly formed aggregates with control monocytes and induced TF expression up to 2 hours post-interaction, as compared to platelets from heterologous healthy volunteers (gray and red lines in **Figure 3B-C**). The interaction with platelets from severe COVID-19 patients also increased CD16 expression on control monocytes, even though at a later time-point (**Figure 3D**).

As previously reported, monocytes from severe COVID-19 patients present increased aggregation with platelets and higher TF expression at baseline (white symbols in **Figure 3B-C**) [26]. Interestingly, when monocytes from severe COVID-19 patients were exposed to platelets from healthy volunteers, platelet-monocyte aggregates formation and TF expression were further enhanced (black lines in **Figure 3B-C**), indicating that platelet-monocyte aggregates from severe COVID-19 patients recruit resting platelets to amplify TF expression. Even though the addition of control platelets potentiated aggregate formation and TF expression by COVID-19 monocytes, these were transient responses, while the interaction of control monocytes with COVID-19 platelets was sustained (**Figure 3B-C**). Collectively, these data suggest that platelet-mediated monocyte procoagulant and proinflammatory activation follow different kinetics and involve a complex set of signals influenced by infection-driven phenotypes of both platelets and monocytes.

Platelet-monocyte interaction drives inflammatory mediator secretion in COVID-19

Previous studies from our group and others have demonstrated the ability of activated platelets to regulate monocyte transcription and secretion of inflammatory mediators[36,38–40]. To characterize the pattern of inflammatory mediator secretion by platelet-monocyte aggregates in COVID-19, monocytes from healthy volunteers were exposed to platelets from severe COVID-19 patients or platelets from a different healthy volunteer. Monocytes from severe COVID-19 patients were also incubated with platelets from control participants (**Figure 4A**). The levels of cytokines and eicosanoids were quantified at 18-hours post-interaction. As shown in **Figure 4A-C** and **Supplemental Figure S4A**, increased secretion of the proinflammatory cytokines TNF-α, IL-1β and IL-8/CXCL8 was observed in monocytes from healthy volunteers that interacted with platelets from severe COVID-19 patients, but not with control platelets. Furthermore, monocytes from healthy volunteers exposed to platelets from COVID-19 patients, or monocytes from COVID-19 patients exposed to platelets from healthy volunteers secreted heightened levels of IL-10 and PGE_2, which was not observed when control monocytes were exposed to control platelets (**Figure 4D-E**). Platelet-monocyte interactions also increased the secretion of the cytokines IL-1RA and IL-6, the chemokine CCL2/MCP-1 and the platelet-derived factors PF4/CXCL4 and PDGF regardless the source of the cells (from COVID-19 or from healthy donors) (**Figure 4F-I** and **Supplemental Figure S8C-D**). These data highlight an inflammatory cytokine pattern that is characteristic of platelet-monocyte interactions involving platelets or monocytes from COVID-19 patients (**Figure 4A**).

Platelets respond to SARS-CoV-2 and orchestrate monocyte activation in vitro

We next investigated the platelet and monocyte responses to SARS-CoV-2 separately and in combination. Platelets, monocytes or platelet-monocyte co-cultures (100 platelets per monocyte) were incubated with SARS-CoV-2 in vitro (MOI of 0.01 virus per monocyte and 0.0001 virus per platelet) (**Figure 5A**). Platelet exposure to SARS-

CoV-2 significantly increased platelet activation and secretion of granule-stored factors (**Figure 5B-C**). Importantly, the conjunct cytokines secreted by monocytes incubated in the presence of platelets showed increased diversity compared to monocytes infected alone (**Figure 5D**). While monocytes exposed to SARS-CoV-2 alone enhanced the secretion of TNF-α, MIP-1α and MIP-1β, monocytes incubated in the presence of platelets showed higher secretion of the inflammatory cytokines IL-1β, IL-18 and IL-1RA, and the chemokines IL-8/CXCL8, MIG/CXCL9, IP10/CXCL10, MCP-1/CCL2 and MCP-3/CCL7 (**Figure 5D and Supplemental Figure S9C-I**). Monocytes infected in the presence of platelets also displayed increased CD16 and TF expression as compared to monocytes alone (**Figure 5D**). HLA-DR downregulation was a monocyte response to SARS-CoV-2 independent on the presence of platelets (**Figure 5D**). These data highlight platelet recognition and response to SARS-CoV-2 and platelet ability to reprogram monocyte responses to virus.

Platelet-monocyte interaction reciprocally activates platelets

An important step of our investigation was to examine whether platelet-monocyte interaction could also impact on the secretion of platelet-derived mediators. Interestingly, the secretion of PDGF, PF4 and TXB_2, mediators produced exclusively by platelets, was increased when platelets from healthy volunteers interacted with monocytes from severe COVID-19 patients or from different control subjects (**Figure 4H-I** and **Supplemental Figure S4D**). Platelets from COVID-19 patients were also responsive to the interaction with monocytes from healthy volunteers by releasing PF4/CXCL4 and PDGF, as compared to platelets alone (**Figure 4H-I**). Similarly, platelets exposed to SARS-CoV-2 in vitro in the presence of monocytes secreted higher levels of PF4/CXCL4, sCD62P, PDGF and RANTES/CCL5 than platelets exposed to SARS-CoV-2 only (**Figure 5 E-F**). Comparable results were observed with platelets from healthy volunteers stimulated with thrombin in vitro (**Supplemental**

Figure 5K-M), indicating that platelet activation by interaction with monocytes is not a COVID-19 exclusive feature. These data show that platelet-monocyte adhesion induces two-way signals that impact not only the monocytes but also the platelets, increasing the secretion of stored and newly-synthesized platelet factors.

Platelets from COVID-19 patients activate monocytes through TF-dependent and independent signaling

We have recently shown that P-selectin and integrin α_{IIb}/β_3 play major roles in platelet-induced TF expression in monocytes in severe COVID-19[26]. Besides its roles in coagulation, monocyte TF expression and activity have been implicated in inflammatory cytokine production and immune activation[18]. Considering the earlier kinetics of platelet-induced TF compared to CD16 expression on monocytes (**Figure 3**), we hypothesized that platelet-induced TF expression might contribute to monocyte inflammatory responses during platelet-monocyte aggregate formation. To investigate whether TF is involved on platelet-monocyte signaling, we performed ex vivo platelet-monocyte coculture in the presence of a neutralizing anti-P-selectin antibody, the anti-α_{IIb}/β_3 abciximab, and a pair of isotype-matched antibodies against distinct epitopes of TF that impair TF direct signaling (clone 10H10) or coagulation activation (clone 5G9)[41]. In addition, we performed ex vivo platelet-monocyte interaction in the presence of the anti-platelet drugs aspirin and clopidogrel. As shown in **Figure 6A**, we identified patterns of platelet-induced monocyte activation depending not only on P-selectin- and integrin α_{IIb}/β_3-mediated adhesion, but also on TF activity, leading to increased CD16 expression and TNF-α and IL-1β secretion (**Figure 6A**). We have also identified platelet-mediated monocyte responses depending only on P-selectin- and integrin α_{IIb}/β_3, regardless of TF activity, leading to the secretion of IL-10, IL-8/CXCL8, MIP-1α/CCL3 and MCP-1/CCL2 (**Figure 6A** and **Supplemental Figure 6**). Even though aspirin treatment effectively inhibited platelet TXA_2 synthesis and PF4 secretion, aspirin

or clopidogrel were both unable to impair platelet-induced monocyte activation and secretion (**Figure 6B-C** and **Supplemental Figure S7**). Importantly, the secretion of the platelet-derived mediators PDGF, basic FGF and HGF were inhibited by anti-P-selectin and/or abciximab (**Figure 6A**), reassuring the notion that platelet-monocyte adhesion reciprocally signals to platelets, activating platelet secretion.

We then investigated whether Ixolaris, a small molecule from the saliva of the tick *Ixodes scapularis* that blocks TF coagulant and signaling activities[18,42], also inhibit monocyte activation during platelet-monocyte aggregate formation. Exposure of control monocytes to platelets from severe COVID-19 patients in the presence of Ixolaris significantly impaired platelet-induced CD16 expression (**Figure 6C**). In addition, treatment with Ixolaris completely blunted P-selectin- or fibrinogen-induced TNF-α secretion in monocytes from severe COVID-19 patients (**Figure 6D**), while the secretion of MIP-1α, MIP-1β and G-CSF was enhanced (**Supplemental Figure S8**).

Platelet-monocyte interaction activates monocytes and platelets through TF-PAR1/2 signaling

Finally, we investigated TF-mediated platelet-monocyte signaling in response to SARS-CoV-2 infection in vitro. Similar to monocytes exposed to platelets from COVID-19 patients, TF expression in response to SARS-CoV-2 was dependent on integrin-mediated platelet adhesion (**Figure 7A**). Enhanced expression of CD16, TNF-α and IL-1β in platelet-monocyte cocultures were dependent on both integrin α_{IIb}/β_3 and TF-dependent signaling, while MCP-1/CCL2 secretion depended only on integrin signaling but not TF activity (**Figure 7B**). The secretion of the platelet-derived mediators PDGF and sCD62P was also inhibited by blocking the integrin α_{IIb}/β_3 and TF coagulation activity with anti-TF 5G9 clone (**Figure 7B**). To gain insights on the mechanisms of TF-mediated signaling in platelet-monocyte interaction, we exposed platelet-monocyte co-

cultures to SARS-CoV-2 in the presence of the PAR1 and PAR2 selective inhibitors SCH79797 and AZ3451, respectively. As shown in **Figure 7C**, monocyte activation and proinflammatory cytokine secretion depended majorly on PAR1, while CD16 expression and IL-1β secretion also dependent on PAR2 activation. Importantly, PAR-1 inhibition also reduced platelet-monocyte aggregate formation (CD41+ monocytes), PDGF and sCD62P secretion, indicating a role in platelet activation (**Figure 7C**). Collectively, these data dissect novel pathways of platelet-delivered proinflammatory signaling to monocytes through P-selectin- and integrin α_{IIb}/β_3, that amplifies platelet and monocyte activation by driving TF expression and signaling through PAR1 and 2 (**Figure 7D**).

Discussion

A state of hypercoagulability with high frequency of thromboembolic complications has emerged as a key pathological feature of COVID-19[2,3]. Even though coagulation disturbances are common features of critically ill patients, their frequencies are particularly higher in severe COVID-19[1,7,12,43–46]. Histopathological analysis of COVID-19 deaths or nonhuman primate infection models have revealed lung thromboinflammatory features including neutrophil and macrophage infiltration, NET-containing pulmonary microvascular thrombosis, and endothelial inflammation with platelet-fibrin deposition[5–7,47–49]. These thromboinflammatory vascular occlusions are almost ten times increased in lungs from COVID-19 fatalities compared to those from influenza pneumonia[7,8,49]. Importantly, interaction with platelets is key for monocyte and neutrophil thromboinflammatory activities in COVID-19, including in driving TF expression contributing to hypercoagulability state[5,26,30]. Here, we provide novel evidence of a platelet-induced proinflammatory amplification program in monocytes through adhesion molecules and TF-dependent signaling. Moreover, activated monocytes from COVID-19 patients recruit and activate platelets, consistent with a dysregulated amplification loop that is associated with severity and mortality in COVID-19 patients.

Immune profiling of severe COVID-19 patients has revealed an expansion of intermediate and nonclassical monocytes that fail to engage the adaptive immunity due to lower HLA-DR expression[12,50,51]. This monocyte inflammatory program was also associated with poor outcomes in the COVID-19 patients in our cohort (data not shown). Our findings support the idea that these monocyte subsets are the ones preferentially interacting with platelets during severe COVID-19. Consistently, combined single-cell transcriptome and surface proteome approaches have shown the expansion of $CD16^+$ nonclassical monocytes highly expressing complement and type I IFN transcripts and forming aggregates with platelets[52]. By single-cell RNA-seq and

447 flow cytometric analysis we identified monocytes highly expressing the leukocyte
448 integrin Mac-1 heterodimer, a fibrinogen receptor that participates in platelet-monocyte
449 aggregate formation[16]. These monocyte phenotypic changes may explain the
450 increased affinity to platelets and higher responsiveness to P-selectin and fibrinogen
451 during severe COVID-19, leading to TF expression and proinflammatory cytokines
452 secretion.

453 While monocytes from severe COVID-19 patients are highly responsive to
454 platelets, the platelet activation status in COVID-19 also contributes to interaction with
455 monocytes leading to TF expression and inflammatory activation. We and others have
456 previously reported the ability of activated platelets to modulate monocyte
457 secretion[36,38–40]. We have demonstrated beforehand that platelet-monocyte aggregates
458 formation reprogram monocyte cytokine production in dengue[36,40]. We now report
459 similar results in monocytes interacting with platelets in SARS-CoV-2 infection, except
460 for a more proinflammatory profile marked by higher levels of TNF-α and IL-1β.
461 Interestingly, differential signaling was required for the secretion of distinct cytokines
462 and chemokines in this model. Platelet adhesion through P-selectin and integrin α_{IIb}/β_3
463 is a primary signal for the secretion of a wide range of mediators, including pro- and
464 anti-inflammatory cytokines and chemokines and monocyte TF expression. TF, in its
465 turn, signals to foster monocyte CD16 expression and pro-inflammatory cytokine
466 production through PAR1 and PAR2 activation. TF-dependent PAR1 signaling was
467 also involved in platelet activation during platelet-monocyte aggregate formation
468 (**Figure 7D**). In addition to platelet-monocyte interaction, proinflammatory factors may
469 also contribute to hyperinflammation and hypercoagulability, including in driving TF
470 expression in monocytes[17,18]. Our data describe complex mechanisms of platelet-
471 monocyte interaction that depend on contact-mediated signaling and are amplified by
472 TF-driven inflammatory signaling.

The mechanisms underlying platelet activation in severe COVID-19 are not yet completely understood. Our data demonstrate that platelets are responsive to SARS-CoV-2 in vitro, even though to a lower extent compared to platelets from COVID-19 patients. Of note, SARS-CoV-2 RNA, proteins and virions have been detected in platelets from infected patients, indicating the feasibility of SARS-CoV-2-induced platelet activation in natural infections[53–55]. Previous studies have described that exposure to SARS-CoV-2 activates platelets in vitro[56,57], which may involve canonical interaction through ACE-2[56,58], but also alternative receptors as CD42 and CD147[58–60]. Similar to previously reported observations[61,62], monocytes were also responsive to SARS-CoV-2 in vitro in the present work. Importantly, our experiments revealed that monocytes infected together with platelets display amplified inflammatory activation and secrete higher levels of inflammatory cytokines. Reports from our group and others have indicated that the inflammatory mediators in COVID-19 patients' plasma also activate platelets[26,28,63]. In whole blood from healthy volunteers reconstituted with COVID-19 plasma, IL-6 receptor blocking by tocilizumab inhibits platelet activation, platelet-leukocyte aggregates formation and TF expression[28]. Therefore, viral and inflammatory factors clearly contribute to platelet activation, which in turns amplifies inflammation in COVID-19 by reprogramming monocyte responses. We show that platelet-monocyte interaction activates both platelets and monocytes through mechanisms requiring TF-mediated PAR1 and 2 signaling, feeding hyperinflammation and hypercoagulability in a reciprocal amplification loop.

In summary, we describe a monocyte proinflammatory program depending on platelet-induced TF-mediated signaling during COVID-19. These platelet-monocyte responses were associated with severity and mortality in a cohort of ICU-admitted patients. However new studies are still necessary to unravel the clinical relevance of these mechanisms. Based on the potential involvement of these cellular and molecular events in pathophysiological mechanisms of hyperinflammation and hypercoagulability,

500 TF-mediated initiation of coagulation and inflammatory signaling may represent a target
501 for therapeutic intervention in future clinical research in COVID-19.

502

Authors Contributions:

E.D.H. performed experimental design and execution, data analyses and manuscript writing; R.M.G., L.P., I.G.A.Q., M.M.C., C.Q.S., J.R,T, V.C.S., S.S.G.D. and L.T. performed part of the experiments and data analysis; H.T.I.N. and I.C. performed data analysis; P.K. and C.R. performed patient inclusion, clinical management, clinical and laboratorial data compilation and patient classification; B.B.A., H.T.I.N., R.Q.M., T.M.L.S. and F.A.B performed experimental design and manuscript reviewing; P.T.B. performed experimental design, manuscript reviewing and directed the study. P.T.B. and E.D.H. conceptualized the study. All authors reviewed and critically edited the manuscript.

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Data Sharing Statement: All data are available within the manuscript and supplemental material. For original data please contact the corresponding authors.

Figure legends:

Figure 1: Platelet monocyte interaction associate with monocyte inflammatory activation in COVID-19. (**A**) The percentage of platelet-monocyte complexes among classical, intermediate and nonclassical monocyte subsets from SARS-CoV-2-negative control participants and patients with mild to severe COVID-19 syndrome. (**B**) The percentage of platelet-monocyte complexes in HLA-DR-positive or negative monocytes from severe COVID-19 patients. (**C**) The Log2 fold change of the transcripts for P-selectin and fibrinogen receptors P-selectin glycoprotein ligand 1 (SELPG), integrin β_1 (ITGB1), integrin β_2 (ITGB2), integrin α_X (ITGAX) and Integrin α_M (ITGAM) in monocytes from severe COVID-19 patients. * Means $p < 2.5x10^{-13}$. (**D-F**) The percentage of CD11b-positive monocytes in blood from (**D**) SARS-CoV-2-negative control participants and patients with mild to severe COVID-19 syndrome; or from severe COVID-19 patients stratified according to (**E**) the requirement of invasive mechanical ventilation or noninvasive O_2 supplementation or (**F**) the 28-day mortality outcome as survivors or nonsurvivors. The horizontal lines in the box plots represent the median, the box edges represent the interquartile ranges and the whiskers indicate the minimal and maximal value in each group. * indicates $p < 0.05$ compared to control in the same monocyte subset; # indicates $p < 0.05$ between selected groups.

Figure 2: Monocytes from severe COVID-19 patients are hyperresponsive to P-selectin and fibrinogen regarding inflammatory cytokine secretion. Monocytes (1×10^5) from severe COVID-19 patients or control participants were plated on recombinant human albumin, P-selectin or fibrinogen coated surfaces. (**A**) The number of monocytes (DAPI, nuclei) adhered on each condition is shown. Scale bar represents 100 µm. (**B-D**) The concentration of (**B**) MCP-1/CCL2, (**C**) TNF-α and (**D**) IL-1β in each condition. Bars represent mean ± standard error of the mean of monocytes from 5 independent control participants and 6 independent severe COVID-19 patients. #

indicates $p < 0.05$ compared to monocytes from control participants in the same condition; * indicates $p < 0.05$ compared albumin.

Figure 3: Platelet-monocyte aggregates formation, TF expression and CD16 expression follows differential kinetics in COVID-19. (**A**) Monocytes from healthy volunteers (control monocyte) were incubated in the absence of platelets (open circles) or with platelets from severe COVID-19 patients (COVID-19 platelets, red circles) or from a different healthy volunteer (control platelets, gray circles) for the indicated time-points. Monocytes from COVID-19 patients (COVID-19 monocyte) were also incubated in the absence of platelets (open squares) or with platelets from healthy volunteers (control platelets, black squares). The percentage of (**B**) platelet-monocyte aggregates formation, (**C**) TF-expressing monocytes and (**D**) CD16-positive monocytes are shown. Dots represent mean ± standard error of 4-6 platelet and monocyte combinations from COVID-19 patients or control participants. All experiments were repeated with cells from at least 2 independent control participants exposed to platelets or monocytes from the same COVID-19 patients with similar results, and a representative data from one of the donors is shown. # indicates $p < 0.05$ compared to baseline; * indicates $p < 0.05$ compared to control monocytes exposed to control platelets.

Figure 4: Platelet-monocyte interactions increase the secretion of inflammatory mediators in COVID-19. (**A**) Monocytes from healthy volunteers (control monocyte) were incubated with platelets from severe COVID-19 patients (COVID-19 platelets) or from a different healthy volunteer (control platelets) for 18 hours and the indicated inflammatory mediators were quantified in the supernatants. Monocytes from COVID-19 patients (COVID-19 monocyte) were also incubated with platelets from healthy volunteers (control platelets). The concentration of (**B**) TNF-α (**C**) IL-1β, (**D**) IL-10, (**E**)

586 PGE_2, (**F**) IL-6, (**G**) MCP-1/CCL2, (**H**) PDGF and (**I**) PF4/CXCL4 are shown. Bars
587 represent mean ± standard error of the mean of 6-12 platelet and monocyte
588 combinations from COVID-19 patients or control participants. All experiments were
589 repeated with cells from 2 independent control participants exposed to platelets or
590 monocytes from the same COVID-19 patients with similar results, and a representative
591 data from one of the donors is shown. * indicates $p < 0.05$ between selected groups.

592

593 **Figure 5: Platelets respond to SARS-CoV-2 and modulate monocytes activation**
594 **in vitro.** (**A**) Platelets, monocytes and platelet-monocyte cocultures were kept
595 uninfected or exposed to SARS-CoV-2 overnight. (**B**) The percentage of P-selectin in
596 uninfected and SARS-CoV-2-infected platelets. (**C**) The fold change in platelet
597 activation markers and mediator secretion after SARS-CoV-2 infection as compared to
598 uninfected platelets. (**D**) The fold change in platelet and monocyte activation markers
599 and mediator secretion after SARS-CoV-2 infection as compared between infected and
600 uninfected monocytes (left panel), infected and uninfected platelet-monocyte cocultures
601 (middle panel) or in infected co-cultures compared to monocytes infected alone (right
602 panel). (**E**) The fold change in platelet activation markers and mediator secretion in
603 SARS-CoV-2 infected co-cultures compared to platelets infected alone. (**F**) Soluble P-
604 selectin (sCD62P) concentration in platelets, monocytes or platelet-monocyte
605 cocultures after SARS-CoV-2 infection. Bars represent mean ± standard error of the
606 mean of platelets and/or monocytes from 4 independent donors. * indicates $p < 0.05$
607 compared to uninfected platelets or between selected groups.

608

609 **Figure 6: Platelets from COVID-19 patients activate monocytes through surface**
610 **interaction and TF mediated signaling.** (**A**) Monocytes from healthy volunteers were
611 incubated with platelets from severe COVID-19 patients for 18 hours in the presence of

anti-P-selectin (anti-CD62P) neutralizing antibody, the anti-α_{IIb}/β_3 antibody abciximab, anti-TF clone 10H10, anti-TF clone 5G9 or isotype matched IgG. The percent inhibition on platelet-monocyte aggregate formation (CD41+ monocytes), monocyte CD16 expression and on cytokine release is shown for each condition. (**B-C**) Control monocytes were exposed to platelets from severe COVID-19 patients in the presence of the anti-platelet drugs aspirin, clopidogrel, the TF inhibitor Ixolaris or DMSO (vehicle). The percent inhibition on platelet-monocyte aggregate formation, monocyte CD16 expression and on cytokine release (**B**) and the percentage of monocytes expressing CD16 (**C**) are shown for each condition. (**D**) Monocytes from severe COVID-19 patients were adhered on recombinant human albumin, P-selectin or fibrinogen-coated surfaces in the presence of Ixolaris or vehicle. The concentration of TNF-α secreted at each condition is shown. Bars represent mean ± standard error of the mean of monocytes of monocytes exposed to platelets from 3-6 independent COVID-19 patients. * indicates $p < 0.05$ compared to isotype-matched IgG (**A**), vehicle (**B**) or albumin (**C**). # indicates $p < 0.05$ between selected groups.

Figure 7: Platelet-monocyte interaction induces monocyte and platelet activation through TF-dependent PAR signaling. Platelet-monocyte cocultures were exposed to SARS-CoV-2 overnight in the presence of the anti-α_{IIb}/β_3 antibody abciximab, anti-TF clone 10H10, anti-TF clone 5G9 or isotype matched IgG. (**A**) The percentage of monocytes expressing TF in platelet-monocyte cocultures exposed SARS-CoV-2 in the presence of abciximab or isotype control IgG. (**B**) The percent inhibition on platelet-monocyte aggregate formation (CD41+ monocytes), monocyte CD16 expression and cytokine release from platelets and monocytes is shown for each condition. (**C**) Platelet-monocyte cocultures were exposed to SARS-CoV-2 overnight in the presence the PAR1 inhibitor SCH79797, the PAR2 inhibitor AZ3451 or DMSO (vehicle). The percent inhibition on platelet-monocyte aggregate formation, monocyte CD16

639 expression and cytokine release from platelets and monocytes is shown for each
640 condition. (**D**) Schematic representation of platelet-monocyte signaling through P-
641 selectin and integrin α_{IIb}/β_3 surface interaction and TF-mediated inflammatory
642 amplification through PAR1 and PAR2 during severe COVID-19.

643 **Table 1:** Characteristics of COVID-19 patients and control donors.

Characteristics[1]	Control (n=25)	Mild (n=22)	Severe (n=46)
Age, years	48 (39 – 58)	41 (32 – 50)	58 (47 – 66)
Sex, male	10 (40 %)	9 (41 %)	23 (50 %)
Respiratory support			
Oxygen supplementation	0 (0 %)	0 (0 %)	16 (35 %)
Mechanical ventilation	0 (0 %)	0 (0 %)	30 (65 %)
SAPS II	–	–	60 (47 – 68)
PaO_2/FiO_2 ratio	–	–	152 (127 – 280)
Vasopressors[2]	0 (0 %)	0 (0 %)	16 (35 %)
Time from symptom onset to blood sample, days	8 (6 – 16)		11 (7 – 16)
28-day mortality	0 (0 %)	0 (0 %)	18 (39 %)
Comorbidities			
Obesity	2 (8 %)	1 (5 %)	10 (22 %)
Hypertension	2 (8 %)	4 (18 %)	25 (54 %)*
Diabetes	0 (0 %)	1 (5 %)	16 (35 %)*
Cancer	0 (0 %)	0 (0 %)	4 (9 %)
Heart disease[3]	0 (0 %)	0 (0 %)	3 (7 %)
Presenting symptoms			
Cough	0 (0 %)	8 (36 %)	26 (57 %)
Fever	0 (0 %)	8 (36 %)	29 (63 %)
Dyspnea	0 (0 %)	3 (14 %)	29 (63 %)
Headache	0 (0 %)	7 (32 %)	5 (11 %)
Anosmia	0 (0 %)	6 (27 %)	11 (24 %)
Laboratory findings at admission			
Leukocytes, x 1000/μL	–	7,6 (6,2 – 15,5)	13 (9,1 – 18,4)
Lymphocytes, cells/μL	–	2,156 (2,015 – 2933)	1,057 (567 – 1540)
Monocytes, cells/μL	–	447 (308 – 620)	672 (473 – 848)
Platelet count, x 1000/μL	–	188 (26 – 198)	194 (155 – 268)

644 [1]Numerical variables are represented as the median and the interquartile range, and
645 qualitative variables are represented as the number and the percentage.
646 [2]Dopamine, epinephrine/norepinephrine, vasopressin or phenylephrine.
647 [3]Coronary artery disease or congestive heart failure.

*p < 0.05 compared to control. The qualitative variables were compared using the two tailed Fisher exact test, and the numerical variables using the t test for parametric and the Mann Whitney U test for nonparametric distributions.

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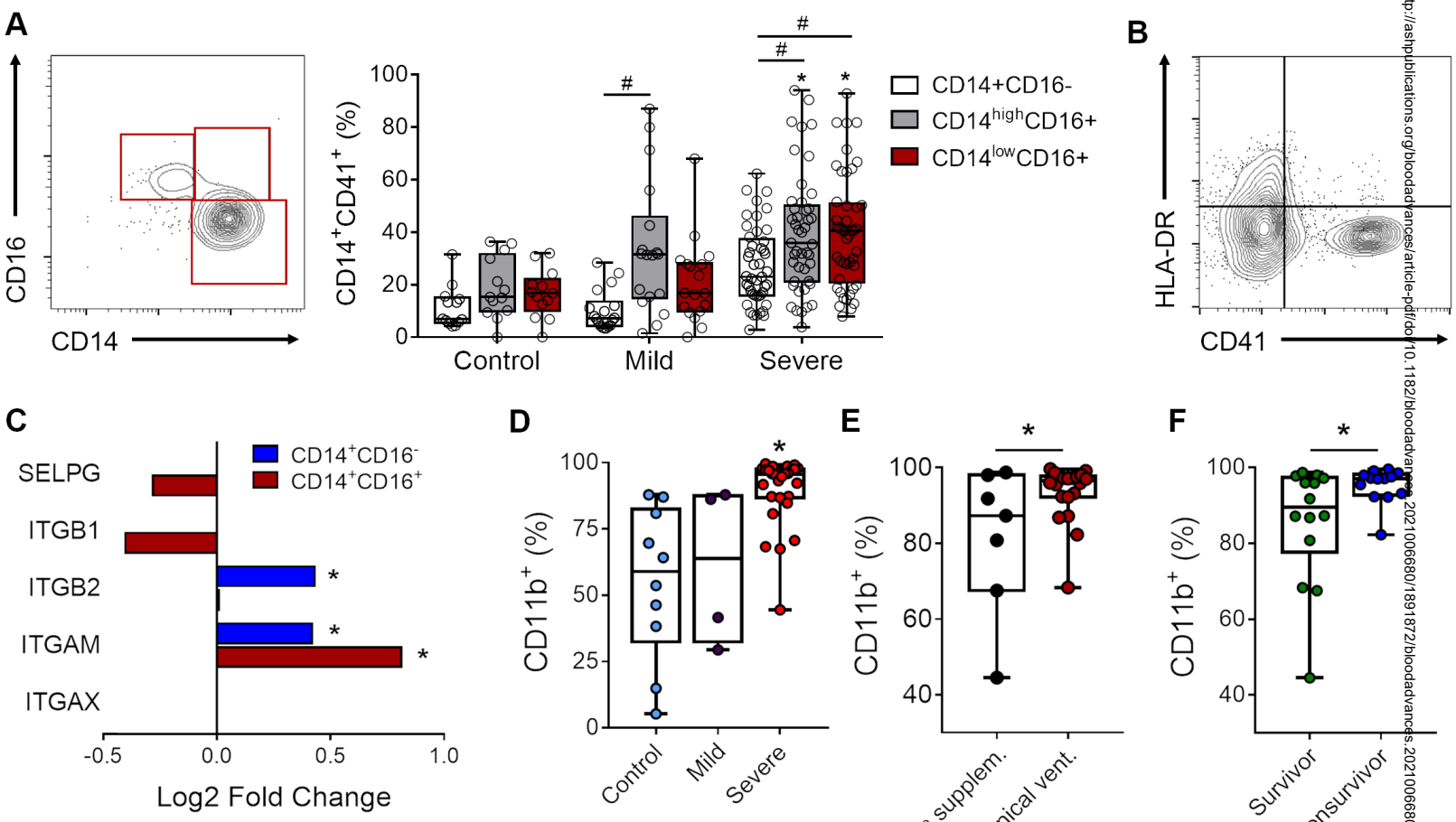
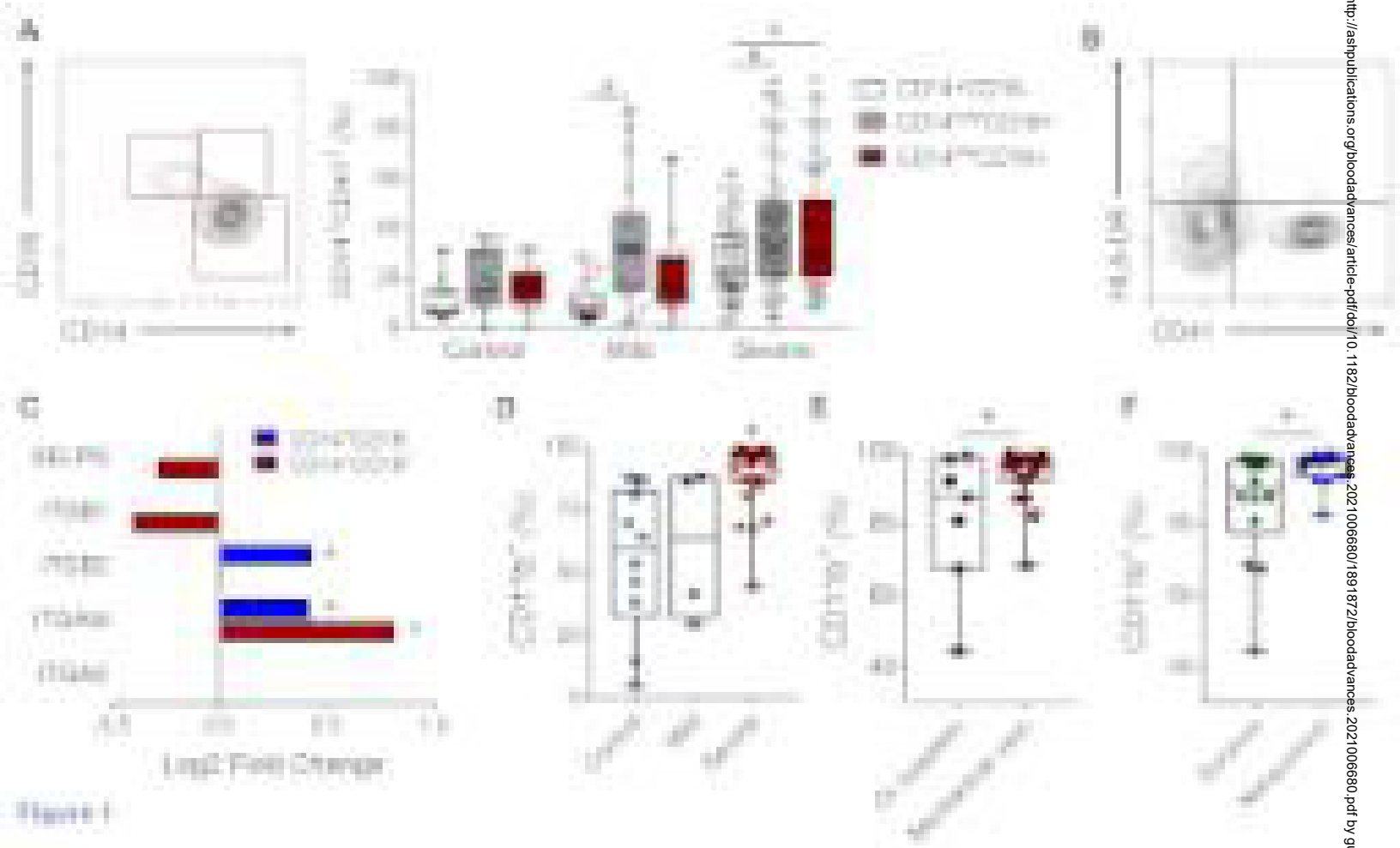


Figure 1

Figure 1



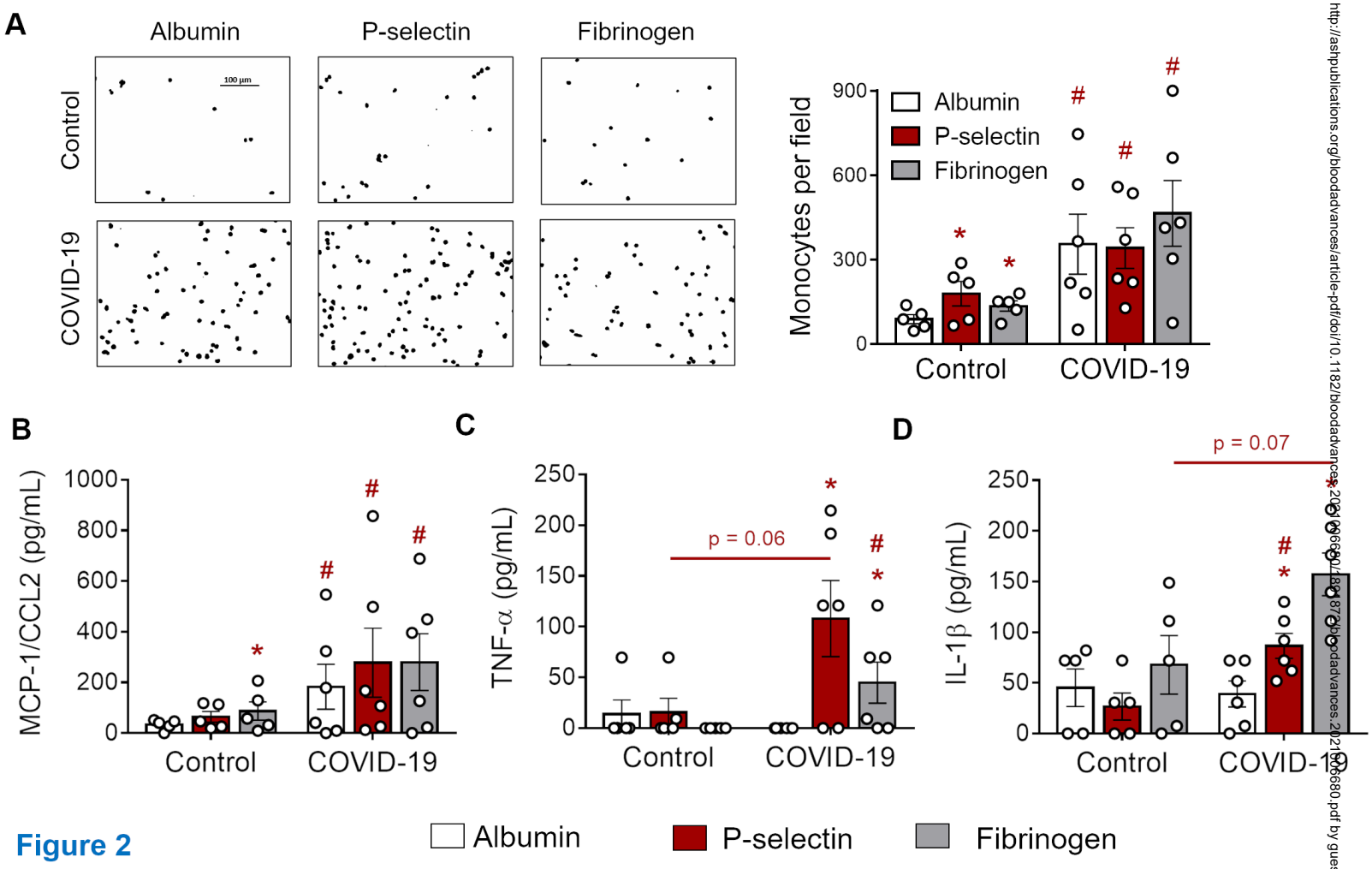


Figure 2

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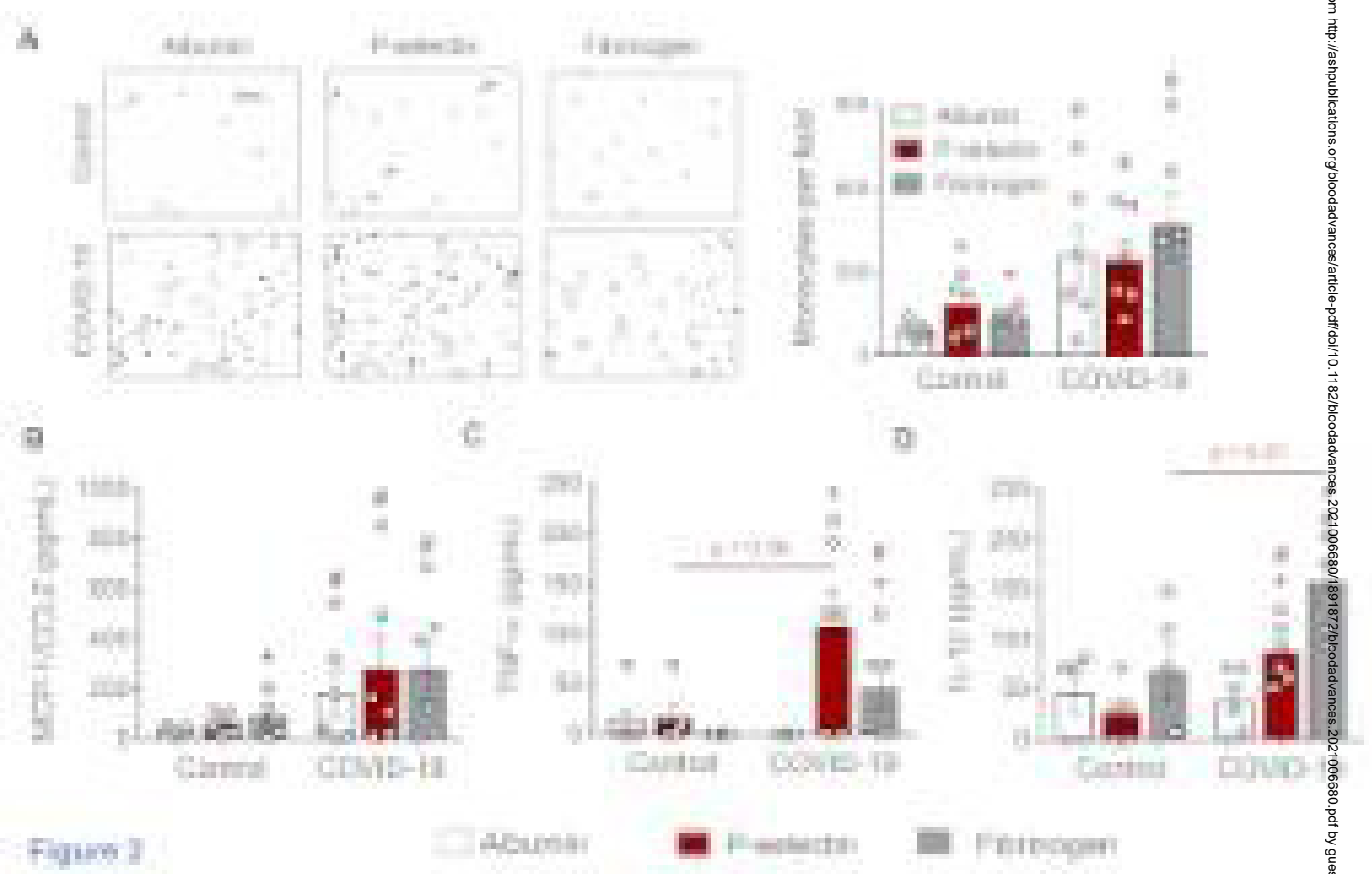

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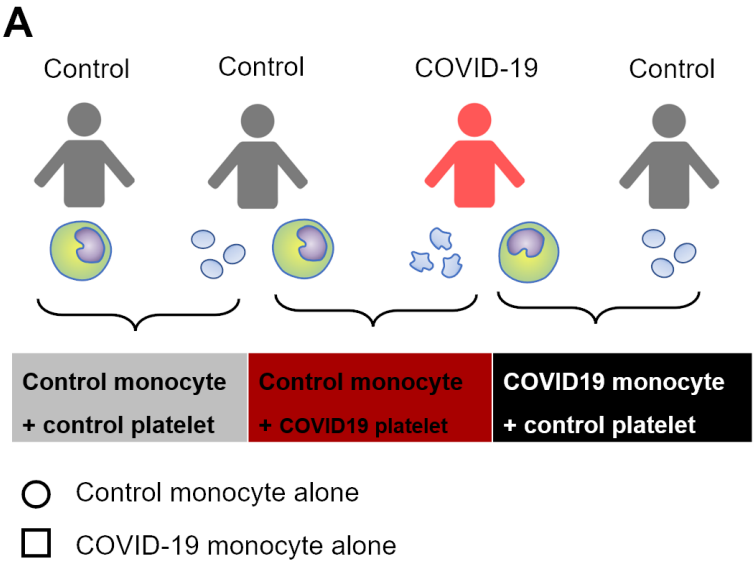

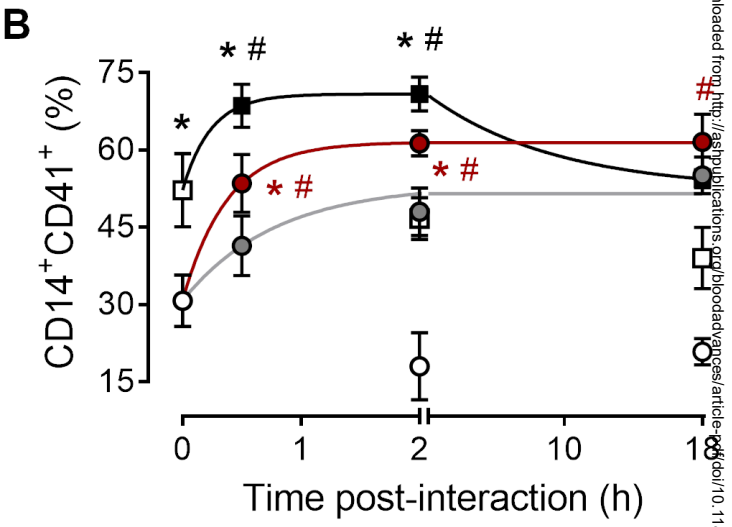

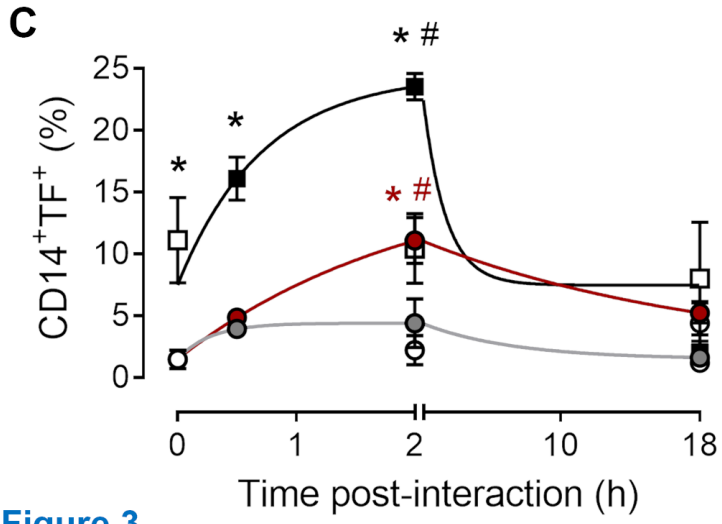

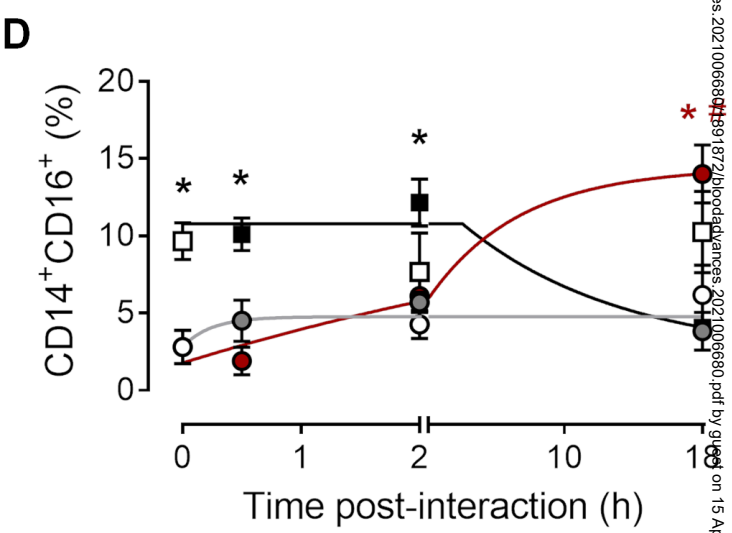

Figure 3

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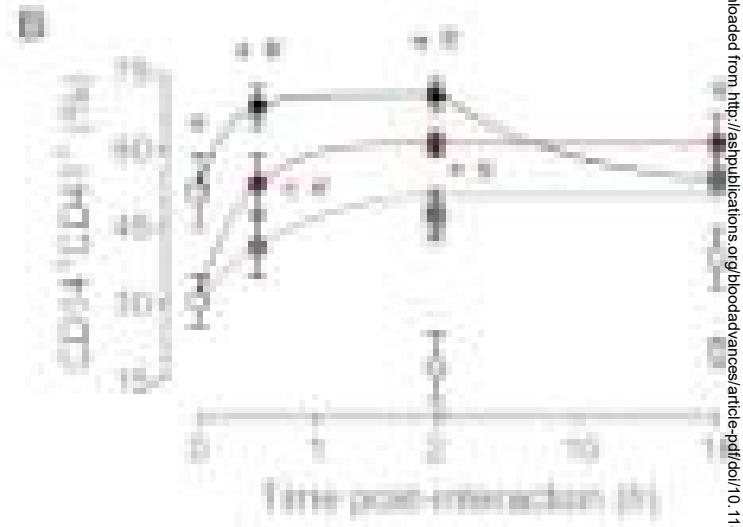

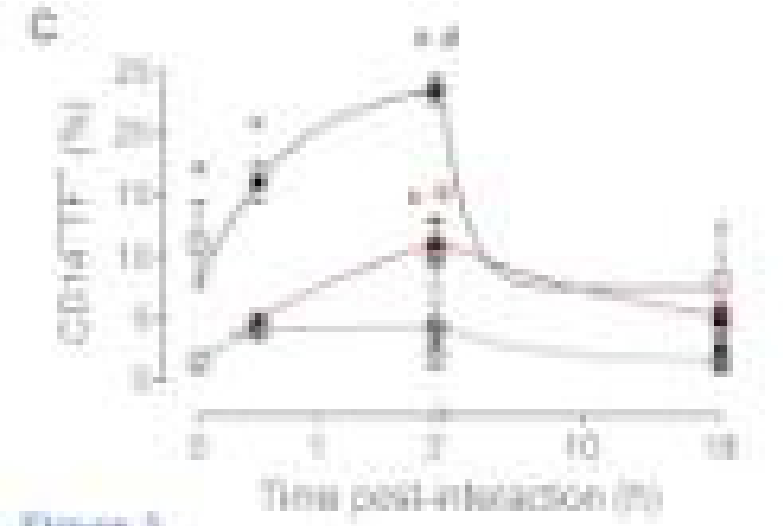

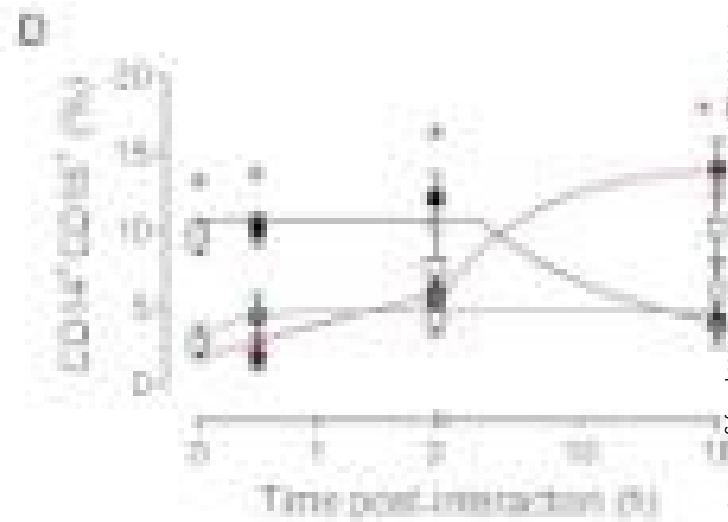

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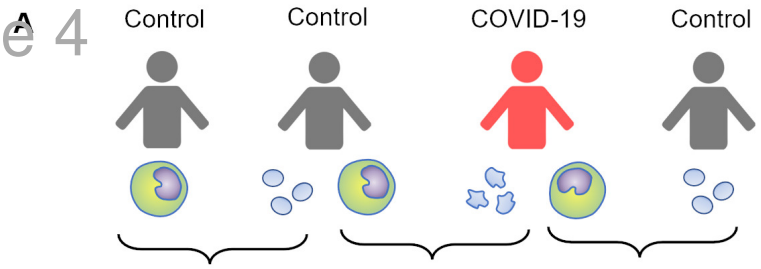
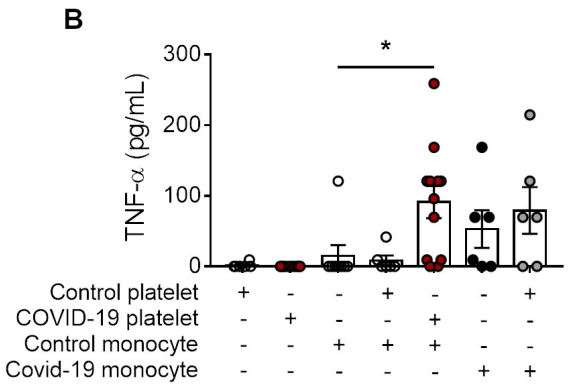
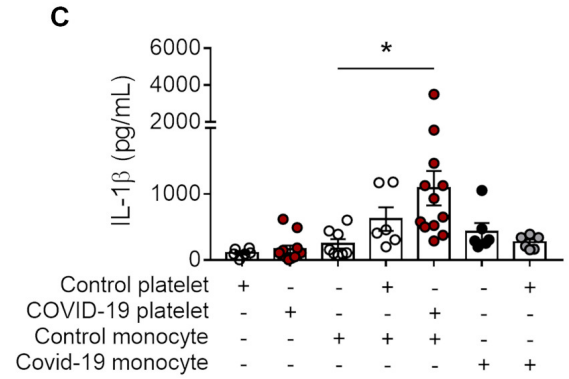
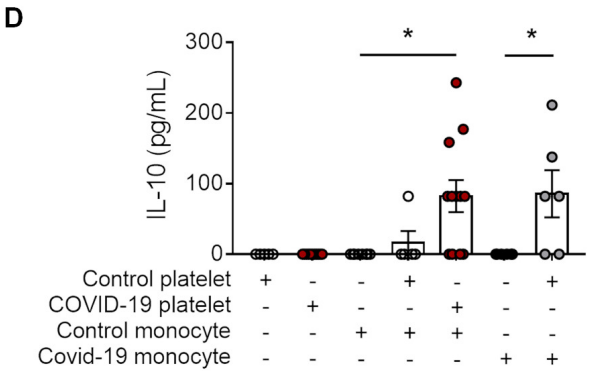
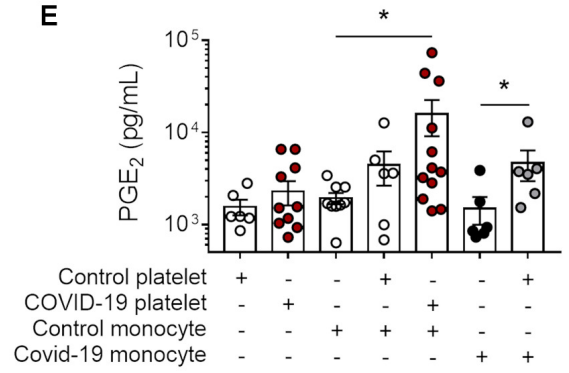


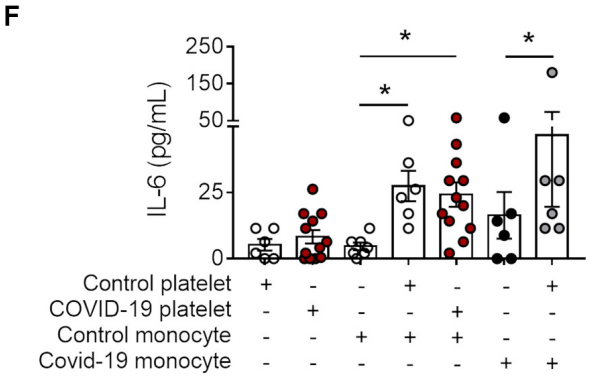
	Control platelet + control monocyte	COVID19 platelet + control monocyte	Control platelet + COVID19 monocyte
TNF-α	–	↑	–
IL-1β	–	↑	–
IL-8	–	↑	–
VEGF	–	↑	–
IL-10	–	↑	↑
PGE_2	–	↑	↑
IL-6	↑	↑	↑
MCP-1	↑	↑	–
IL-1RA	↑	↑	–
PDGF	↑	↑	↑
PF4	↑	↑	↑
TXB_2	↑	↑	–

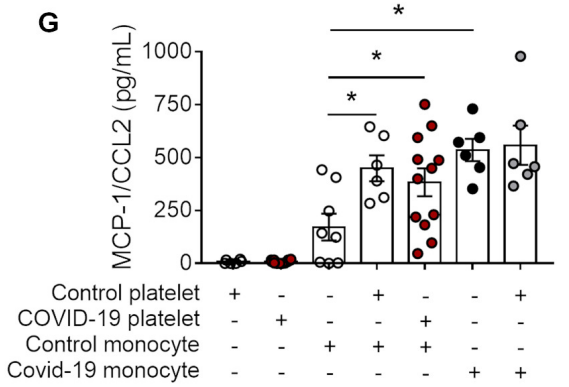


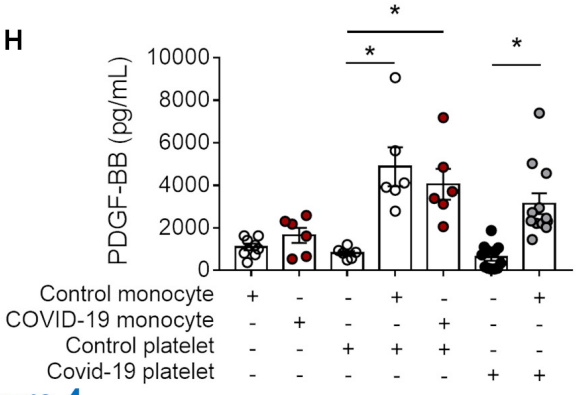












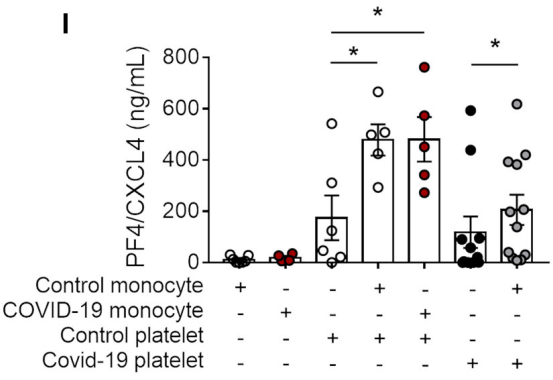


Figure 4

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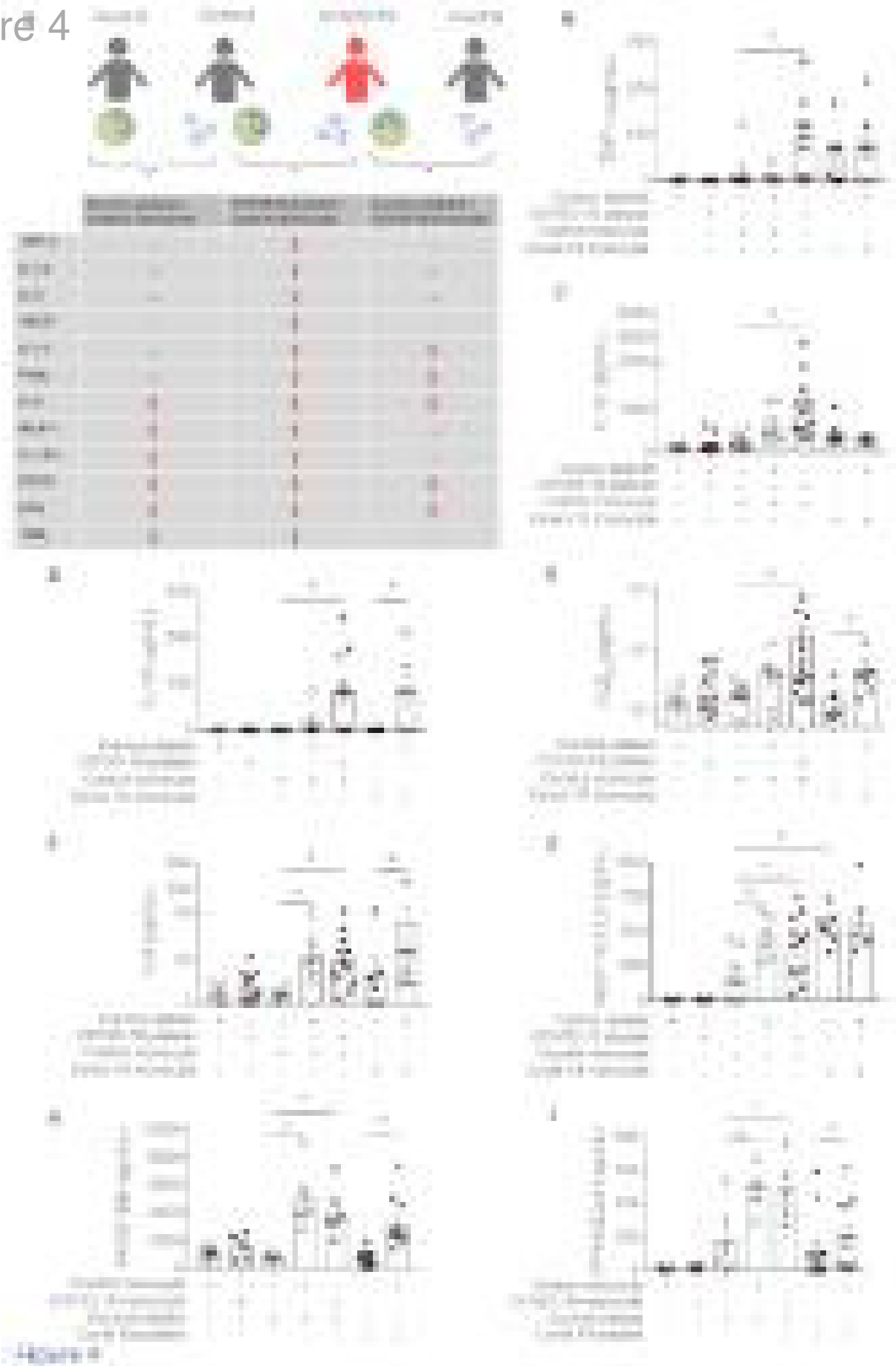

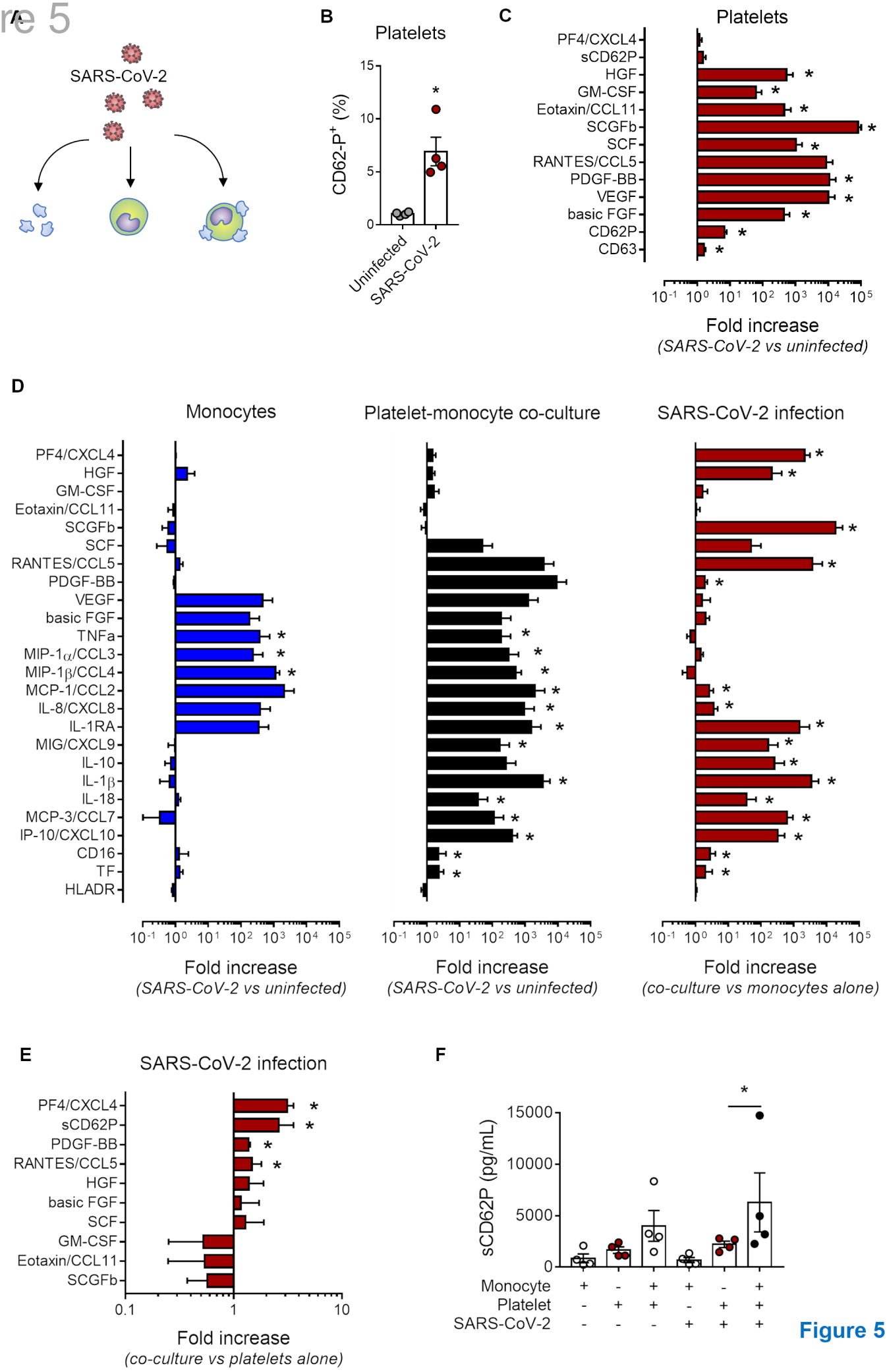

Figure 5

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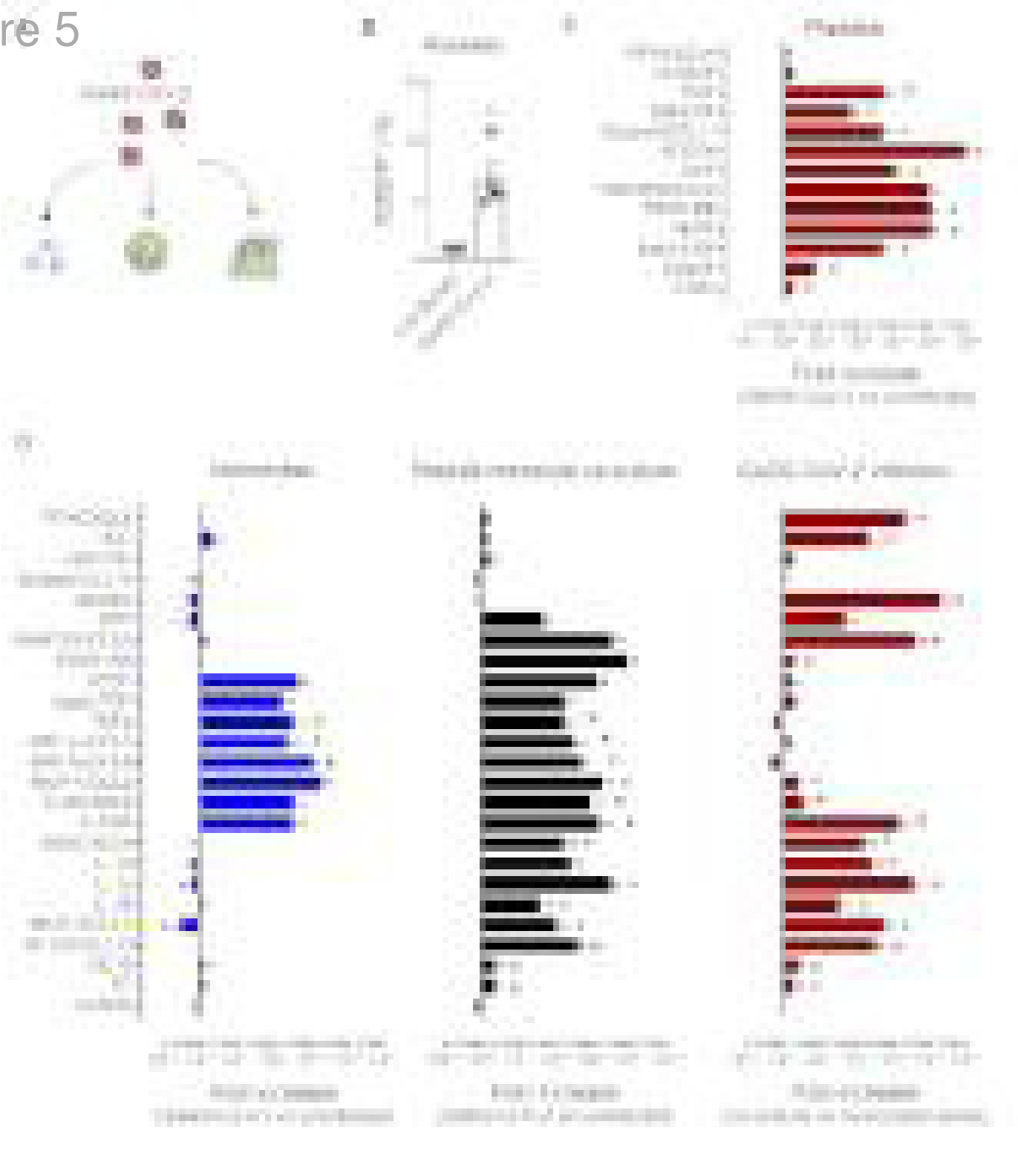

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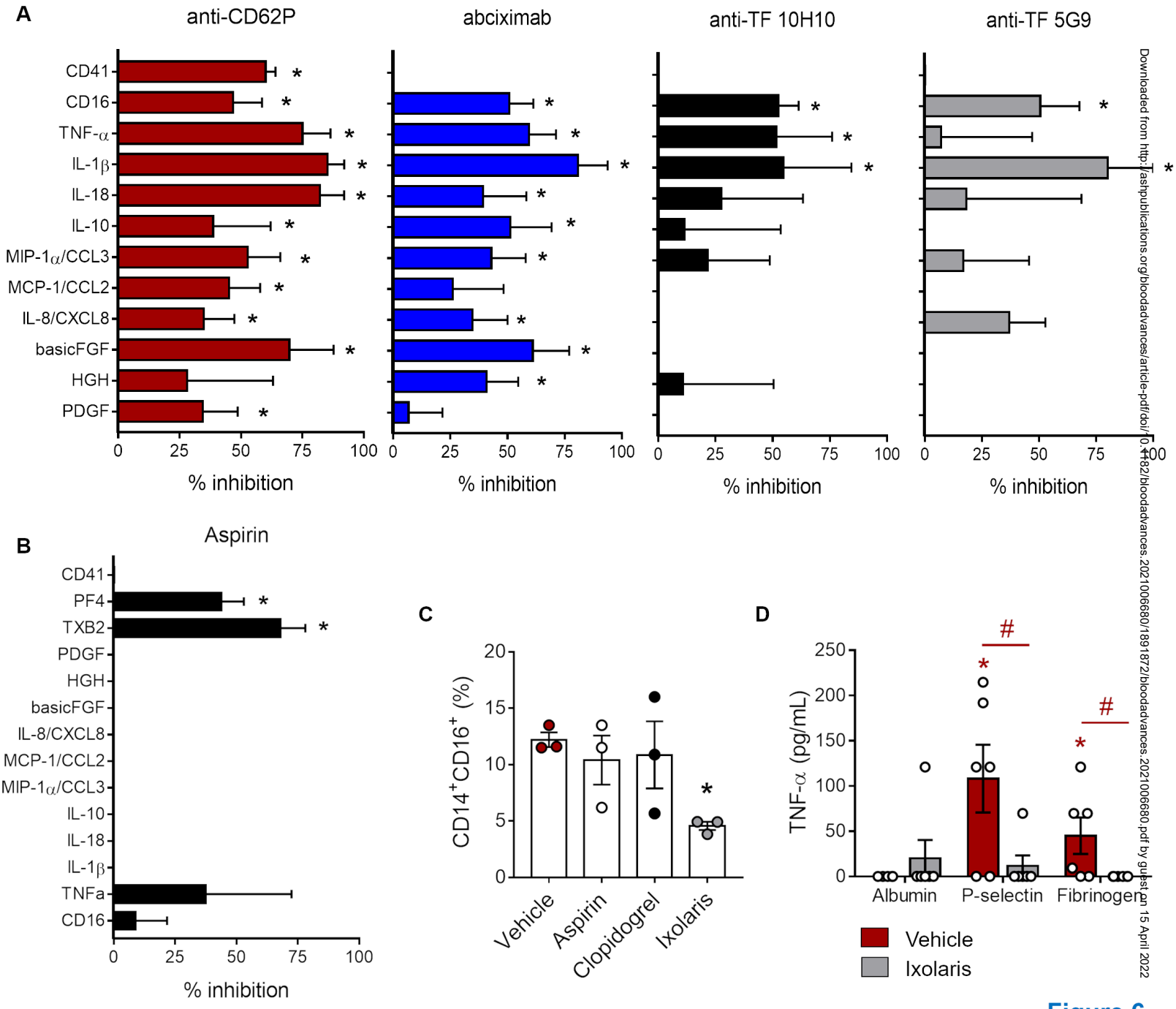

Figure 6

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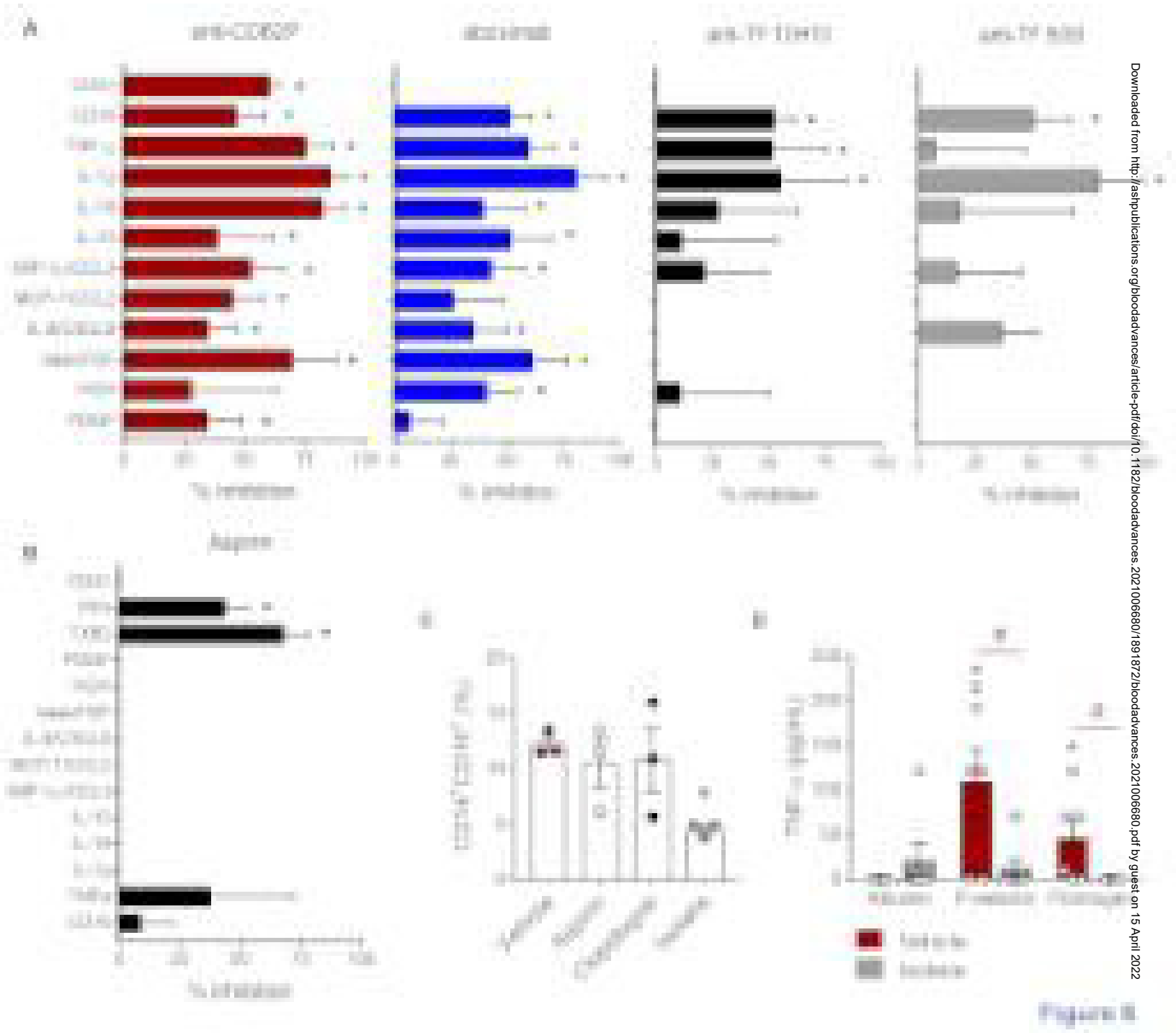
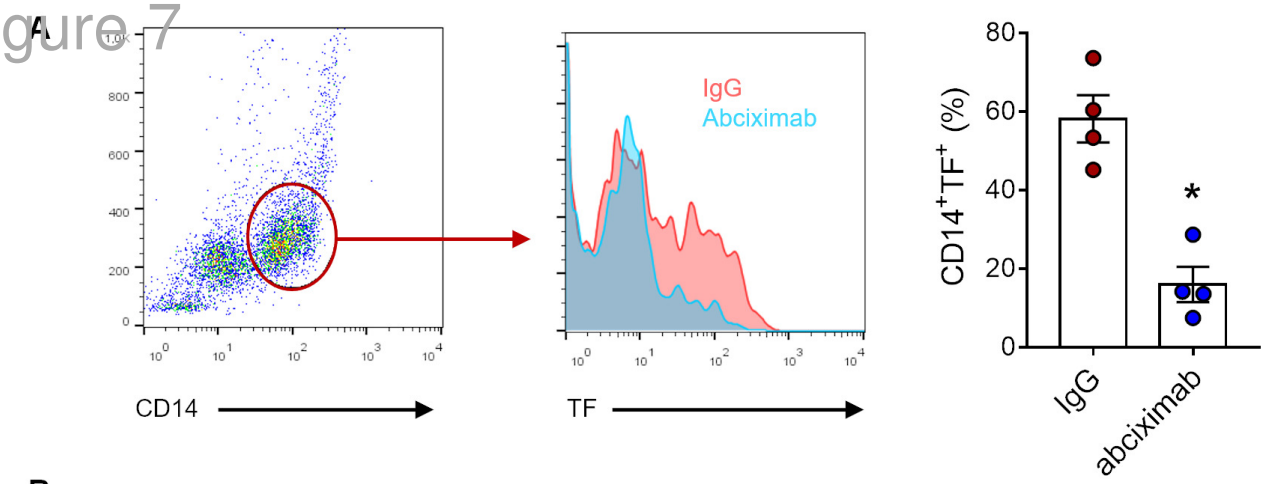
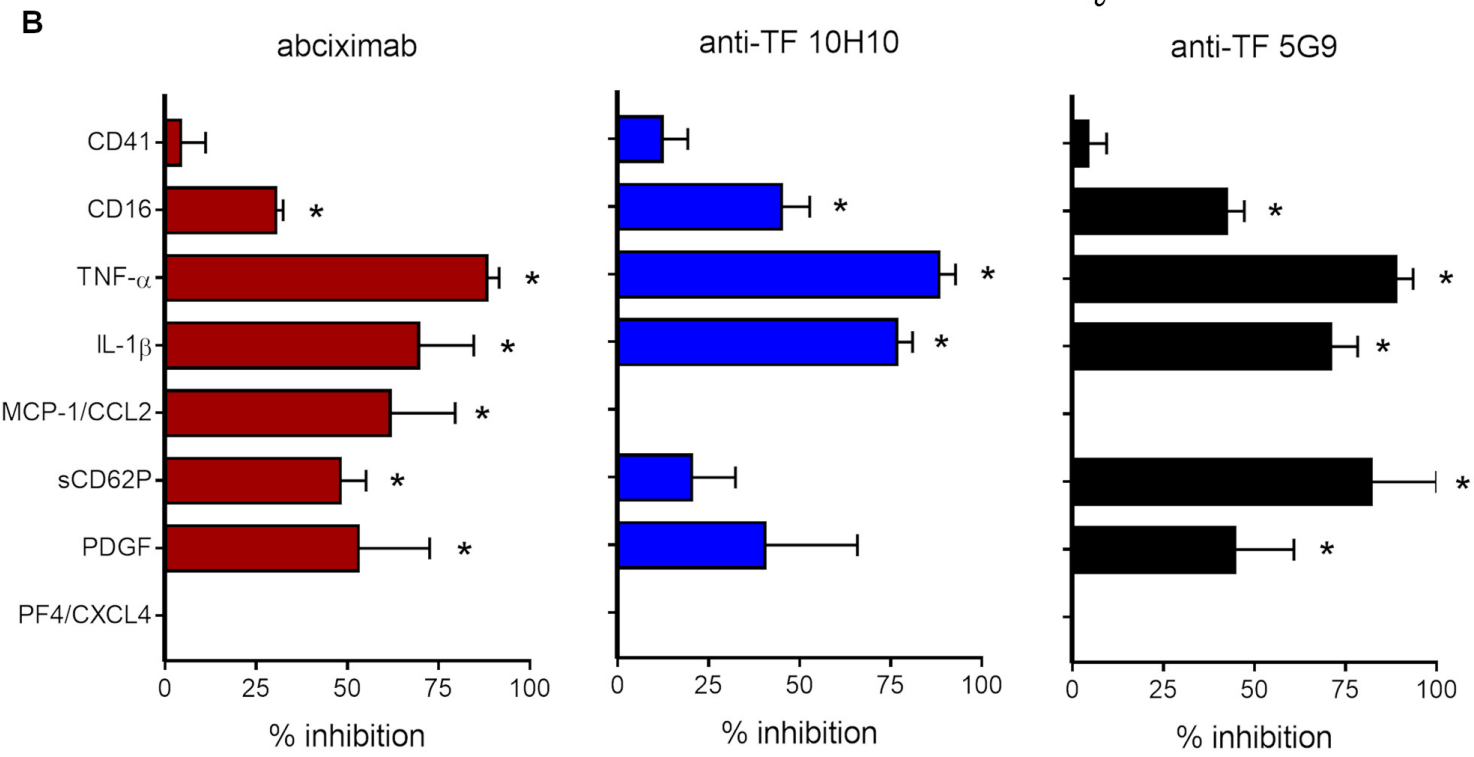
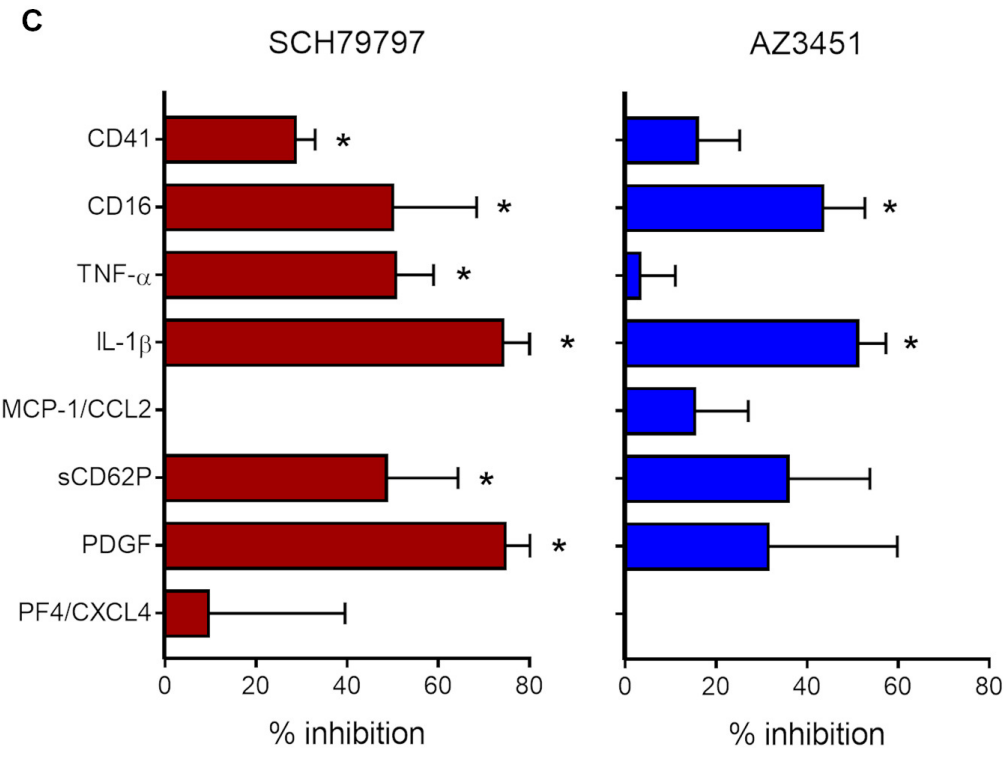


Figure 7







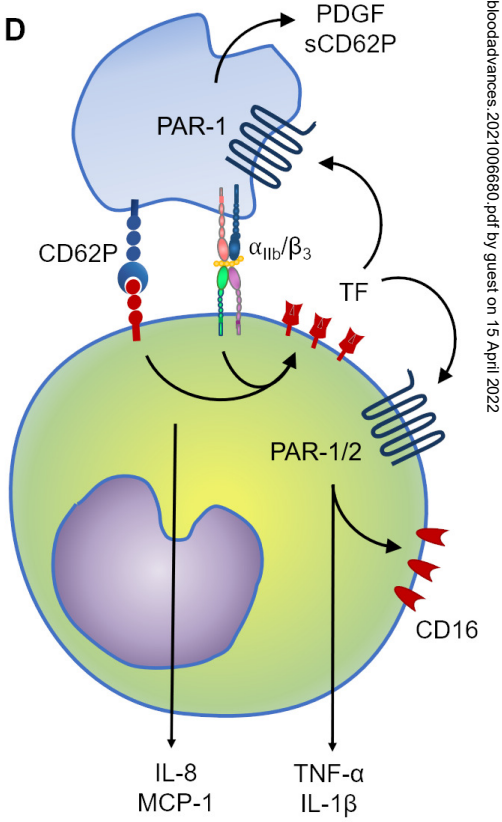


Figure 7

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