Characterization of eNOS-based Platelets Subpopulations in COVID-19 and the Impact of Platelet α-Granule Contents on Cancer Cell PD-L1

by

Amir Asgari

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University of Alberta

Abstract

Thrombosis significantly complicates numerous diseases that have inflammatory components. This condition, characterized by the formation of blood clots within blood vessels, can exacerbate a range of health issues, from cardiovascular diseases to systemic infections like COVID-19, and even cancer. Inflammation plays a critical role in thrombosis by enhancing platelet activation and aggregation, promoting endothelial dysfunction, and altering blood flow, which together increase the risk of clot formation. Earlier research in the Jurasz lab led to identification of two distinct platelet subpopulations distinguished by the presence or absence of endothelial nitric oxide synthase (eNOS). This led to the classification of platelets into eNOS-positive and eNOS-negative groups. It was further demonstrated that while eNOS-negative platelets initiate thrombotic reactions, while eNOS-positive platelets contribute to and ultimately limit thrombus growth. However, little is known about potential changes in the ratios of these platelet subpopulations in diseases.

In the context of severe COVID-19, which is characterized by platelet-rich microvascular thrombi, I investigated whether COVID-19-associated immune and inflammatory responses alter the balance of these platelet subpopulations. Platelets were isolated from age- and sex-matched hospitalized COVID-19 patients and COVID-19-negative controls. Platelet eNOS was measured by flow cytometry and plasma inflammatory cytokines (IFN-γ, TNF- α, IL-6, and IL-1β) by multiplex ELISA. COVID-19 patients demonstrated significantly elevated ratios of eNOS-negative to -positive platelets than controls and their ratios strongly correlated with disease severity (81.2 ± 2.8%: 19.2 ± 2.8% ICU vs. 66.0 ± 3.1%: 34.7 ± 3.5% non-ICU vs. 6.1 ± 1.3%: 93.5 ± 1.3% controls). Higher eNOS-negative to –positive platelet ratios were associated with enhanced platelet reactivity as measured by surface CD62P. Accordingly, COVID-19 patients demonstrated higher

TNF- α, IL-6, and IL-1β plasma concentrations than controls. Using the Meg-01 cell line, which demonstrates eNOS-negative and –positive subpopulations of cells, as a megakaryocyte model, demonstrated that inflammatory cytokines associated with COVID-19 promoted eNOS-negative Meg-01 formation and enhanced subsequent eNOS-negative platelet-like particle formation.

Further characterization of eNOS-based platelet subpopulations led to identification of α–Granule-enriched platelet subpopulations, marked by CD62P, with higher content of VEGF and PDGF in healthy donors. Further research revealed that these platelet subsets (α-granule-enriched platelets with higher content of VEGF and PDGF) were all characterized as eNOS-positive. Strikingly, COVID-19 patients' platelets demonstrated a higher overall mean in VEGF content and a greater percentage of VEGF-enriched platelet subpopulations compared to COVID-19-negative controls. COVID-19 patients demonstrated higher TNF-α plasma concentrations than COVID-19-negative controls. Incubation of Meg-01 cells with TNF-α led to the formation of a distinct Meg-01 subpopulation with higher intracellular VEGF levels (14.4 ± 3.1%).

Building on findings in platelet diversity, particularly the identification of eNOS-positive and eNOS-negative subpopulations with distinct VEGF and PDGF content, I investigated the impact of platelets on cancer cell evasion of the adaptive immune response.

Current understanding of platelets in cancer suggests they facilitate hematogenous metastasis by enabling cancer cells to evade the immune system, although the current knowledge of platelet function in modulating the adaptive immune system in cancer is limited. A major negative regulator of the adaptive response is the immune checkpoint protein Programmed Death Ligand 1 (PD-L1). As platelets secrete factors that may increase PD-L1 expression, we investigated whether they up-regulate cancer cell PD-L1, thus promoting immunoevasion, and whether common anti-platelet drugs inhibit this process. Platelets increased PD-L1 expression by

cancer cells, an effect counteracted by the simultaneous neutralization of platelet-derived VEGF and PDGF or the administration of eptifibatide, an anti-platelet agent. Furthermore, A549 lung carcinoma cells incubated with platelets showed diminished capacity to activate T-cells, an immunosuppressive effect reversed by eptifibatide.

In summary, altered ratios of eNOS-negative to eNOS-positive platelets may contribute to the thrombosis observed in COVID-19 and potentially other diseases with inflammatory components. Additionally, platelets may contribute to cancer cell immunoevasion, suggesting that anti-platelet drugs could be beneficial when used in combination with immune checkpoint inhibitor therapy targeting PD-L1. More research is needed to assess the impact of platelet heterogeneity on cancer cell immunoevasion.

Preface

This thesis is an original work by Amir Asgari. The studies were approved by UAlberta Health Research Ethics Board (Pro00100563 and Pro00089845)

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Chapter 1

General Introduction

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1.1 Introduction

1.1.1 Platelet Discovery

The discovery and understanding of platelets, also known as thrombocytes, trace back to the late 19th century, a period rich in medical discoveries, particularly in hematology. During this time, several physicians observed and described phenomena related to blood and its cellular components, then termed blood-corpuscles[1-3]. However, Dr. Giulio Bizzozero, an Italian pathologist, discovered platelets in 1882. He identified them as distinct elements in the blood, which he referred to as "third corpuscles" and noted their role in blood clotting, observing their behavior in capillary vessels under the microscope[3-5].

Bizzozero's observations highlighted platelets undergoing morphological changes after exposure to foreign surfaces, transforming from a crisp discoid shape to becoming spherical and extending filopodia as they activate. These changes facilitate the formation of aggregates and subsequent "white thrombi," a crucial process in the vascular repair mechanism. Human platelets, originating from bone marrow megakaryocytes, are anucleate cell fragments about 2-4μm in size. With a lifespan of 7-10 days in humans, and slightly less in mice, they are a transient yet essential component of the circulatory system[6]. At any given time, about two-thirds of the platelet population circulates in the blood, maintaining a physiological concentration of 150,000 to 450,000 per μl, while the rest are sequestered in the spleen as a reserve[7, 8].

1.1.2 Platelets Plasma Membrane, Internal Membrane and Cytoskeleton

The structure and functionality of platelets are intrinsically linked to their plasma membrane, internal membrane systems, and a highly specialized cytoskeleton. Each component

plays a vital role in ensuring platelets perform their critical functions in various physiological and pathophysiological process.

The exterior surface of circulating and resting platelets is covered by a plasma membrane that features a variety of receptors, including glycoproteins and glycolipids collectively referred to as the glycocalyx. This structure plays a crucial role in interactions with clotting factors and other cells, facilitating platelet adhesion, activation, and aggregation, which are essential processes for blood clot formation. The net negative charge on the exterior surface of platelets plays a pivotal role in preventing spontaneous aggregation by creating electrostatic repulsion between platelets and ECs or other blood components in circulation. This charge also facilitates the selective binding of positively charged clotting factors, which is essential for initiating and regulating the coagulation cascade during wound healing[9-11].

Beneath the glycocalyx, the platelet plasma membrane's bilayer composition is selectively permeable, with a distribution of phospholipids that supports crucial functions during platelet activation. Neutral phospholipids like phosphatidylcholine and sphingomyelin reside predominantly in the outer layer, while negatively charged phospholipids such as phosphatidylinositol and phosphatidylserine are sequestered in the inner layer, maintained by ATP-dependent flipases. Upon platelet activation, the asymmetry of the phospholipid bilayer is disrupted, leading to the externalization of phosphatidylserine (PS) on the platelet surface. The exposure of phosphatidylserine (PS) by a subset of platelets known as procoagulant platelets, in conjunction with calcium ions, is crucial for the assembly of vital coagulation complexes, particularly the tenase and prothrombinase complexes[12]. These complexes are central to the clotting process, accelerating the conversion of clotting factors into their active forms. The tenase complex, comprising factors VIIIa and IXa on the surface of pro-coagulant platelets, specifically

catalyzes the conversion of factor X to Xa[12]. Subsequently, the prothrombinase complex, formed by factors Va and Xa along with PS and calcium, facilitates the rapid generation of thrombin from prothrombin. The presence of PS thus not only supports these complexes but also enhances the pro-coagulant activity of platelets, making it a critical component in the regulation and propagation of the coagulation cascade[13].

The open canalicular system (OCS) and the dense tubular system (DTS) are two integral structural components found within platelets, the small blood cells essential for clotting and wound healing. Positioned internal to the plasma membrane, these complex membrane systems play crucial roles in the functionality of platelets during hemostasis and thrombosis[14, 15]. the OCS comprises an extensive network of membrane-bound channels that originate from invaginations of the plasma membrane. This system extends throughout the cytoplasm of the platelet, facilitating the transport of ions, molecules, and granules between the platelet interior and the external environment. It is especially critical for the rapid release of granular contents during platelet activation, which is vital for initiating and propagating the clotting cascade[16, 17]. Similar to the smooth endoplasmic reticulum in other cell types, the DTS in platelets is primarily responsible for the storage and release of calcium ions. This release is crucial for platelet activation, leading to changes in platelet shape, secretion of granules, and aggregation—fundamental components of platelet plug formation at sites of vascular injury. Additionally, the DTS is involved in the synthesis of molecules like thromboxane A2, which are essential for platelet function[18, 19]. Both the OCS and DTS are vital for the swift and effective response of platelets to vascular damage. The OCS enables the quick deployment of essential clotting factors and other molecules stored in platelet granules, while the DTS manages the calcium signaling that orchestrates these release mechanisms[16-19].

Supporting these membrane structures is the platelet cytoskeleton, a highly dynamic and complex network of structural proteins that plays a pivotal role in the various functions of platelets, which are crucial components of the blood coagulation system. This framework not only provides structural support to maintain the shape and integrity of platelets but also enables their ability to change shape rapidly, migrate, adhere to the vascular endothelium, and form aggregates necessary for blood clot formation. Central to the cytoskeletal architecture are actin and tubulin, along with a host of other associated proteins such as myosin, α-actinin, and filamin. Actin filaments, which are the most abundant proteins in platelets, form a dense web just beneath the plasma membrane and are instrumental in maintaining the platelet's discoid shape. During platelet activation, this actin network is dynamically reorganized to facilitate shape change, which is essential for platelet spreading and the formation of filopodia and lamellipodia, structures that enable platelets to make contact and interact with each other and the damaged vessel wall[20]. Additionally, spectrin is a key cytoskeletal protein in platelets, essential for maintaining their shape and mechanical stability. It forms a meshwork that supports membrane integrity during platelet activation and aggregation. Furthermore, spectrin aids in organizing membrane proteins crucial for effective signaling and function during clot formation[21, 22].

Moreover, the cytoskeleton is critically involved in the secretion of granular contents that promote coagulation and tissue repair. It also plays a key role in the intracellular signaling pathways that regulate platelet activation and the subsequent responses. Thus, these components collectively ensure that platelets are well-equipped to respond to endothelial damage, contributing to hemostasis through the formation of a platelet plug and the subsequent activation of the coagulation cascade.

1.1.3 Platelet Granules

Platelet secretory granules represent the heart of platelet function, serving as a dynamic and versatile repository of bioactive molecules. These granules are responsible for coordinating the cellular and molecular responses of platelets, allowing them to engage in complex processes at sites of vascular injury. Platelet granules, encompassing dense granules, lysosomes, T-granules, and notably, α-granules, serve as repositories for these essential factors[23-27]. These granules sequester substances ranging from adhesion molecules, growth factors, and enzymes to pro-inflammatory and immunomodulatory cytokines, which can be rapidly deployed upon platelet activation[26].

Dense granules, also known as δ-granules, are characterized by their high content of non-proteinaceous, small molecules. These include adenosine diphosphate (ADP), adenosine triphosphate (ATP), calcium ions, serotonin, and pyrophosphate. The release of these contents upon platelet activation plays a pivotal role in amplifying the aggregation response, modulating vascular tone, and influencing the inflammatory milieu. ADP and ATP, for example, act through purinergic receptors to enhance platelet aggregation and recruit additional platelets to the site of injury. Serotonin, on the other hand, acts as a vasoconstrictor and can modulate vascular tone and platelet function[28, 29].

Among these granules, the α-granules stand out for their structural complexity, functional diversity, and key role in platelet biology. α-granules are small membrane-bound compartments that harbor an extensive and diverse array of bioactive molecules. These granules contain a reservoir of growth factors, cytokines, adhesion molecules, clotting factors, and proteins involved in inflammation and tissue repair, representing a versatile arsenal for platelets[30-32].

α-granules are characterized by their heterogeneous content[33]. Their biogenesis is a complex process involving a multitude of regulatory mechanisms. α-granules originate in the megakaryocytes, the bone marrow progenitors of platelets, and undergo several stages of maturation and packing before being released into the bloodstream within newly formed platelets[25]. The dynamic and context-dependent nature of α-granule biogenesis plays a significant role in the diversity of molecules they contain[34].

The vast repertoire of bioactive molecules stored within α-granules allows platelets to exert a wide range of functions in hemostasis, inflammation, wound healing, tumor metastasis and angiogenesis[26]. Growth factors, such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), contribute to tissue repair and neovascularization[35, 36]. Cytokines, including transforming growth factor-beta (TGF-β) and interleukin-1β (IL-1β), regulate inflammatory responses[37]. Clotting factors, such as von Willebrand factor (vWF) and fibrinogen, are crucial for hemostasis[38]. The adhesive molecules P-selectin (CD62p) and CD40L (CD154) facilitate platelet-endothelial and platelet-leukocyte interactions[39, 40]. Thus, α-granules are involved in orchestrating various cellular and molecular processes.

α-granule secretion in platelets is a regulated process typically initiated by elevated intracellular calcium levels induced by physiological agonists (collagen, thrombin, and ADP) or permeabilization[41]. The precise mechanism by which calcium triggers α-granule release in platelets remains not fully defined[42]. This exocytosis process involves granule docking, SNARE protein engagement, membrane fusion, and granule content release[43]. Recent data suggests that primary fusion with the plasma membrane precedes compound fusion[43, 44]. SNARE proteins, including VAMPs (vesicle-associated membrane proteins), play pivotal roles in fusion pore formation[45-47]. The process is tightly controlled by chaperone proteins, docking factors, the

actin cytoskeleton, and posttranslational modifications of SNARE proteins to prevent inappropriate release[47-52].

1.1.4 Platelet Activation and Aggregation

Platelets are pivotal in hemostasis, working to minimize blood loss after vascular injury by forming a platelet plug. This process unfolds through three major phases: adhesion, activation, and aggregation. Each phase is characterized by distinct events: during adhesion phase platelets adhere to the injury site, activation triggers shape change and granule release, and aggregation involves the clumping of platelets to form a stable plug. These steps are supported by complex intracellular signaling and the activation of specific platelet receptors critical for each phase.

During the initiation phase, under normal conditions, platelets circulate alongside other cellular blood components, coated by a protective glycoprotein-rich layer that prevents their spontaneous activation. However, when damage occurs to a blood vessel, exposing the underlying extracellular matrix (ECM), components such as von Willebrand factor (vWF), collagen, and fibronectin become critical in mediating the adhesion of platelets. The platelets initially contact and adhere to the ECM through the interaction of vWF with the GPIb/IX/V complex. This interaction facilitates the rolling of platelets along the subendothelium, allowing them to either firmly adhere or return to circulation[53]. Although this interaction is rapid and reversible, it significantly reduces platelet velocity, enabling the engagement of collagen receptors like GPVI and integrin α2β1, which are essential for strong adhesion and trigger further platelet activation[54].

Following adhesion, the activation (extension) phase begins where initial weak signals from the GPIb-IX-V complex are soon bolstered by more potent signals from collagen receptors,

primarily through GPVI signaling. This activation pathway leads to the engagement of phospholipase C (PLC), generating second messengers such as inositol triphosphate (IP3) and diacylglycerol (DAG). These messengers are crucial for mobilizing intracellular calcium and activating protein kinase C (PKC), respectively, which are essential for platelet spreading, granule secretion at the site of vascular injury, and secondary activation of platelet integrins[55].

The aggregation (stabilization) phase is characterized by the release of various soluble agonists such as ATP, ADP, thromboxane A2 (TXA2), and fibrinogen from the degranulating platelets. These agonists bind to G-protein coupled receptors (GPCRs) on the platelet surface, inducing "inside-out" signaling that fully activates the platelets and recruits additional platelets to the growing aggregate. A key event in this phase is the activation of integrin αIIbβ3, which changes its conformation to bind fibrinogen more effectively, linking adjacent platelets and stabilizing the aggregate. This activation also exposes phosphatidylserine on the platelet surface, providing a procoagulant platform essential for the assembly of coagulation complexes and thrombin generation, leading to the conversion of fibrinogen into a fibrin mesh that reinforces the clot[55].

Through these phases, platelets orchestrate a rapid and robust response to vascular injury, effectively preventing excessive blood loss and initiating repair processes that restore the integrity of the blood vessel.

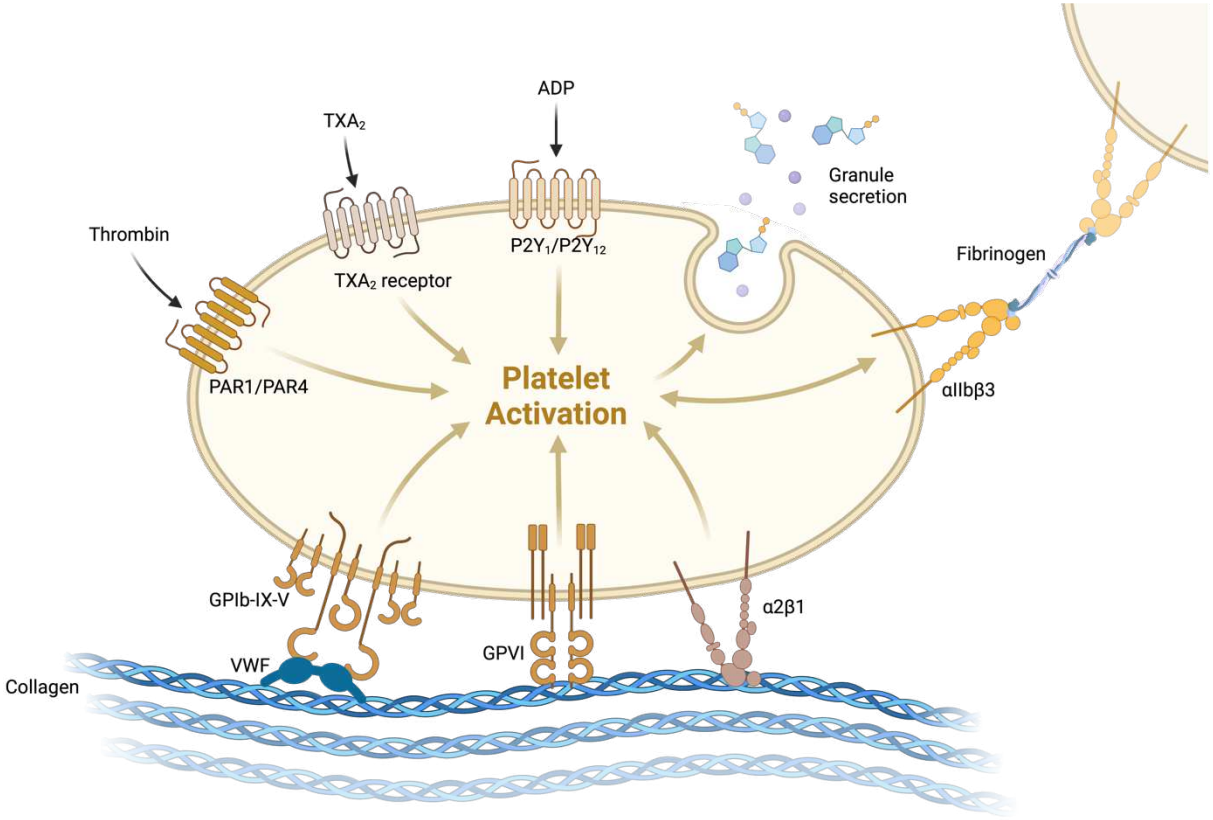


Figure 1.1.1 Platelet activation, Adhesion, granule secretion, and aggregation upon receiving a stimuli.

This figure illustrates the process of platelet activation, which is crucial for hemostasis and thrombosis. Upon vascular injury, platelets adhere to exposed subendothelial matrix proteins, such as collagen and von Willebrand factor, via surface receptors GPVI, integrin α2β1, and GPIb-IX-Vand. This adhesion triggers intracellular signaling pathways, leading to platelet shape change, granule release, and activation of the GPIIb/IIIa receptor. Activated GPIIb/IIIa binds fibrinogen, facilitating platelet aggregation and the formation of a stable platelet plug. Created with BioRender.com

1.1.5 Platelets and their Role in Hemostasis

Platelets are now recognized as key players in hemostasis, responsible for the physiological formation of a platelet plug at the site of vascular injury. Hemostasis is a critical process that prevents excessive haemorrhage upon damage to a vessel wall. This process involves the complex interplay of vessel wall components, circulating coagulation proteins, and platelets, which together form a plug of cells cross-linked with fibrin to effectively seal the injured site. Platelets, small, disk-shaped cell fragments derived from the fragmentation of megakaryocytes in the bone marrow, are the main cellular component in arterial thrombus, playing a pivotal role in this response. Conversely, a venous thrombus primarily comprises fibrin-trapped red blood cells.

Hemostasis is typically divided into two stages: primary hemostasis and secondary, or the hemostasis coagulation cascade. Primary hemostasis is initiated when platelets encounter the subendothelial matrix at the site of vascular injury, leading to rapid adhesion mediated by the interaction of platelet receptor GPIb-IX-V with immobilized von Willebrand factor (VWF). This adhesion is crucial under high shear stress conditions and is facilitated by the unique binding properties of VWF. Following adhesion, platelet activation occurs through receptors like GPVI, which binds collagen exposed in the subendothelium. Activation triggers a cascade of intracellular signaling, leading to the conformational activation of integrins, particularly αIIbβ3. This integrin binds various ligands including fibrinogen and VWF, promoting platelet aggregation. The aggregation process is further amplified by autocrine and paracrine signals from ADP and thromboxane A2, released by activated platelets, which recruit additional platelets to the injury site. Additionally, thrombin plays a dual role by activating platelets via protease-activated

receptors (PARs) and contributing to the formation of a fibrin meshwork that stabilizes the growing platelet plug. This coordinated response ensures the rapid formation of a platelet plug, effectively sealing the damaged vessel and preventing excessive blood loss while maintaining localized clot formation[56].

Following primary hemostasis, where a platelet plug is formed to temporarily seal vascular injuries, secondary hemostasis encompasses the coagulation cascade. During this process, tissue factor (TF) exposed upon vascular injury binds to factor VIIa, initiating a sequence that activates factors IX and X, ultimately leading to the formation of a stable fibrin clot. This cascade culminates in the cleavage of soluble fibrinogen by thrombin, leading to the formation of an insoluble fibrin mesh that stabilizes the initial platelet plug. Thrombin, a central serine protease, not only generates fibrin but also activates platelets and enhances the coagulation process through the positive feedback activation of factors XI, VIII, and V. Concurrently, it engages the anticoagulant pathway by binding to thrombomodulin on endothelial cells, thereby activating protein C, which, with cofactor protein S and factor V, inactivates procoagulant factors Va and VIIIa, ensuring that coagulation is localized and regulated. Additionally, the entire cascade is modulated by serpins like antithrombin and tissue factor pathway inhibitor, which inhibit thrombin and factor Xa, balancing the coagulation process and preventing excessive clot formation.[57, 58].

1.1.6 Role of Platelets in Coagulation Cascade

Platelets are fundamental to the coagulation cascade, also known as secondary hemostasis, which is crucial for stopping blood loss following vascular injury. This cascade begins shortly after the initial formation of the platelet plug, as detailed in Furie's intravital microscopy studies[59]. Coagulation involves a series of highly controlled enzymatic reactions governed by

clotting factors, primarily produced by the liver, that promote clot formation. These factors, typically serine proteases requiring calcium ions for activity, bind to phosphatidylserine on the activated platelet surface, facilitating their conversion from inactive precursors to active enzymes[60].

The coagulation cascade can be subdivided into three main phases: the generation of the prothrombinase complex, the conversion of prothrombin to thrombin, and the conversion of fibrinogen to fibrin, which stabilizes the primary platelet plug formed during primary hemostasis. The cascade is initiated through two pathways: the extrinsic pathway, triggered by exposure of tissue factor (TF) on damaged endothelial cells which reacts with factor VII (FVII) to form FVIIa, and the intrinsic pathway, which involves the activation of factors in a complex series with high-molecular-weight kininogen and prekallikrein on collagen[61, 62].

Thrombin, a key product of the coagulation cascade, not only converts fibrinogen into fibrin but also stimulates further platelet activation, creating a feedback loop that enhances the clotting process. This "thrombin burst" is crucial for rapidly producing the necessary fibrin to stabilize the clot, while thrombin also activates factor XIII, which cross-links fibrin strands, forming a robust and insoluble mesh[12, 62, 63].

Simultaneously, the fibrinolytic system is activated to counterbalance coagulation, preventing excessive clotting and facilitating the breakdown of clots as the vessel heals. Key components of this system include plasminogen, which is converted to plasmin to degrade fibrin clots into fibrin degradation products like D-dimers. The activity of the fibrinolytic system is tightly regulated by inhibitors such as alpha-2-antiplasmin and thrombin-activatable fibrinolysis inhibitor (TAFI), ensuring that fibrinolysis is appropriately timed. Overall, the coordinated actions

of platelets, coagulation factors, and fibrinolysis ensure that hemostasis is a finely tuned process[64].

1.1.7 Role of Platelets in Thrombosis

Thrombosis, the formation of an obstructive blood clot within a blood vessel, is a critical factor in tissue ischemia and occurs prominently in both arterial and venous circulations, each governed by distinct mechanisms and requiring different treatment approaches. Platelets play a central role in this process, particularly in arterial thrombosis, which is pivotal in cardiovascular disease (CVD) events such as myocardial infarction (MI) and stroke[65].

Arterial thrombosis occurs when blood clots form in arteries, the blood vessels that carry oxygen-rich blood from the heart to other parts of the body. This type of thrombosis is particularly dangerous because it can lead to acute events like myocardial infarction (heart attack) and ischemic stroke, which result from the sudden interruption of blood flow to critical tissues. Arterial thrombi are primarily composed of platelets, due to the high shear forces present in arteries, which promote platelet activation and aggregation more than fibrin formation. Risk factors for arterial thrombosis include atherosclerosis, hypertension, diabetes, smoking, and hyperlipidemia, which induce endothelial damage and dysfunction, enhancing thrombogenic conditions[66, 67].

Venous thrombosis occurs when a blood clot forms within a vein, obstructing blood flow and leading to conditions such as deep vein thrombosis (DVT) and pulmonary embolism. Unlike arterial thrombosis, which primarily involves platelets due to high shear forces in arteries, venous thrombosis typically occurs in areas of slower blood flow and is dominated by fibrin and red blood cells, forming what is often referred to as "red clots." Common risk factors for venous thrombosis include prolonged immobility, surgery, pregnancy, obesity, smoking, certain genetic factors that

affect blood clotting, and cancer. Venous thrombosis often develops in the deep veins of the legs or pelvis but can also occur in other areas, such as the central nervous system (CNS). CNS venous thrombosis can lead to significant morbidity due to potential complications like stroke or cerebral hemorrhage. Additionally, Venous thrombosis can result in pulmonary embolism, where the clot travels to and blocks a pulmonary artery, causing severe respiratory and cardiovascular complications. This process is influenced by Virchow's triad, which describes the three primary factors contributing to venous thrombosis: endothelial injury, hypercoagulability, and venous stasis. Endothelial injury, resulting from trauma, surgery, or inflammation, exposes subendothelial tissue and promotes platelet adhesion and activation. Hypercoagulability, caused by genetic disorders, certain medications, or medical conditions, increases the tendency for blood to clot. Venous stasis, due to prolonged immobility, obesity, or venous insufficiency, allows clotting factors to accumulate and interact, further increasing the risk of clot formation [67, 68].

Given the distinct pathophysiological mechanisms underpinning arterial and venous thrombosis, the treatments for these conditions also differ. Arterial thrombosis is typically managed with antiplatelet drugs to target the critical role of platelets in thrombus formation, while venous thrombosis is treated with anticoagulants that focus on the coagulation cascade[67, 69].

1.1.8 Thrombosis and Inflammation

Thrombosis, the formation of blood clots inside blood vessels, and inflammation, the body's response to injury or infection, are traditionally viewed through separate clinical lenses. However, emerging evidence suggests that the two are mutually reinforcing, with inflammation acting as a critical facilitator of thrombosis and vice versa[70]. Inflammation can promote thrombosis by inducing endothelial dysfunction, a condition in which the endothelium loses its

normal anti-thrombotic and anti-inflammatory capacity. Inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukins (IL-1β, IL-6, IL-8, and IL-17), can alter the anticoagulant properties of the endothelium and upregulate the expression of tissue factor (TF), a key initiator of the extrinsic coagulation cascade[70, 71]. Additionally, the presence of inflammatory cytokines in inflammation, such as IL-1β and TNF-α, promotes an adhesive phenotype in endothelial cells by inducing the expression of ICAM-1, VWF, P-selectin, and integrin αVβ3. This facilitates the recruitment of leukocytes and platelets to the endothelium, further promoting clot formation by activating and releasing tissue factor from monocytes[71].

Conversely, components of the coagulation system can enhance inflammatory responses. Thrombin, a serine protease generated during coagulation, possesses potent inflammatory properties. It can act directly on platelets and endothelial cells to stimulate the release of inflammatory mediators such as IL-6, IL-8, and MCP-1, and upregulate adhesion molecules like ICAM-1, VCAM-1, E-selectin, and P-selectin[72-76]. Moreover, the deposition of fibrin, a product of the coagulation cascade, not only functions as a scaffold for thrombus formation but also supports the migration of immune cells, such as neutrophils, macrophages, and T cells, into tissues[77-83]. This enhances the inflammatory response by providing a matrix for cell adhesion and movement[78, 83]. Additionally, fibrin degradation products (FDPs) generated during fibrinolysis can further promote inflammation by activating immune cells and stimulating the release of cytokines and chemokines, such as IL-1β, IL-12, and RANTES[84, 85].

This bidirectional relationship is particularly evident in conditions such as atherosclerosis, where the chronic inflammatory state of the vessel wall contributes to the development and rupture of atherosclerotic plaques, leading to thrombus formation. Similarly, in conditions like deep vein thrombosis and pulmonary embolism, inflammation within the vein wall can trigger thrombosis,

and the ensuing clot can induce further inflammatory responses from the vascular tissue[86]. This interplay is also seen in diseases such as cancer and COVID-19, where systemic inflammation and hypercoagulability enhance both thrombotic risks and inflammatory complications, complicating the clinical picture and management of these patients[87].

1.1.9 Pharmacological Inhibition of Platelet Function

Platelets play a central role in hemostasis and thrombosis, making them a key target for pharmacological intervention in conditions where platelet activation contributes to adverse clinical outcomes such as myocardial infarction, stroke, and other thrombotic events. The inhibition of platelet function is a cornerstone in the management of cardiovascular diseases, particularly in preventing the formation of arterial thrombi. Clinically used antiplatelet drugs work by various mechanisms to reduce platelet aggregation and subsequent clot formation, thus mitigating the risk of acute thrombotic events.

Among the most used antiplatelet drugs are aspirin, thienopyridines (such as clopidogrel and prasugrel), and glycoprotein IIb/IIIa inhibitors. Aspirin acts by irreversibly inhibiting cyclooxygenase (COX), with a preferential effect on COX-1 at low doses. This inhibition reduces the synthesis of thromboxane A2, a potent promoter of platelet aggregation, effectively impairing the platelet activation cascade, including arachidonic acid (AA)-driven aggregation, thus providing antithrombotic protection. At higher doses, aspirin also inhibits COX-2, which is involved in inflammation and pain. However, aspirin is not highly specific and affects both COX-1 and COX-2, leading to a range of therapeutic effects and side effects[88].

Ticagrelor, a cyclo-pentyltriazolo-pyrimidine, inhibits the P2Y12 receptor on platelets, thereby preventing platelet activation and aggregation. Thienopyridines work by irreversibly

blocking the P2Y12 component of ADP receptors on the platelet surface, which is important for the amplification of the aggregation signal. Ticagrelor, although similar in function, differs from clopidogrel and prasugrel as it is a reversible inhibitor of the P2Y12 receptor and does not belong to thienopyridine drug class. These drugs are particularly useful in patients undergoing percutaneous coronary interventions, as they help prevent platelet aggregation induced by arterial injury[89, 90].

Glycoprotein IIb/IIIa inhibitors, such as abciximab, eptifibatide, and tirofiban, represent a class of potent antiplatelet agents. They work by blocking the glycoprotein IIb/IIIa receptor, the final common pathway for platelet aggregation, where fibrinogen and other adhesive molecules bind to link platelets together. By inhibiting this receptor, these drugs prevent platelet aggregation regardless of the stimulus. This action also disrupts outside-in signaling, a process by which extracellular binding events (such as the binding of fibrinogen to the glycoprotein IIb/IIIa receptor) trigger intracellular signaling pathways that further activate platelets and stabilize the formation of the platelet plug. By blocking outside-in signaling, these inhibitors not only prevent the initial platelet aggregation but also impede the reinforcement of platelet activation and aggregation, providing a robust antithrombotic effect[91, 92].

In recent years, newer agents such as cangrelor—an intravenous, reversible P2Y12 inhibitor—and vorapaxar—a protease-activated receptor-1 (PAR-1) antagonist that blocks thrombin-induced platelet activation—have been introduced. These drugs offer alternative mechanisms and benefits, particularly in acute care settings or for patients who are unresponsive to other antiplatelet medications[90, 93].

1.1.10 Endogenous Negative Feedback Pathways

Platelets are crucial for hemostasis, but their excessive activation can lead to thrombosis, emphasizing the importance of regulatory mechanisms that maintain a balance in platelet activity. Among the various mechanisms that regulate platelet function, several key players include tissue inhibitor of metalloproteinases-4 (TIMP-4), matrix metalloproteinase-9 (MMP-9), prostacyclin (PGI2), and nitric oxide (NO) each contributing to a complex system of negative feedback that prevents excessive platelet aggregation and thrombus formation.

TIMP-4 plays a significant role in modulating platelet activity by inhibiting the activation of MMPs, which are involved in remodeling the extracellular matrix and can influence platelet function. Specifically, TIMP-4 regulates the activity of MMP-2 by forming a complex that prevents its activation on the platelet surface. This inhibition is crucial because active MMP-2 (and MMP-1) can enhance platelet activation and aggregation by cleaving and activating PAR1 at a non-canonical site, thus amplifying the thrombotic response[94]. By controlling MMP-2 activity, TIMP-4 helps to modulate the platelet response to vascular injury, maintaining a check on platelet activation and preventing excessive clot formation[95-97].

Matrix metalloproteinase-9 (MMP-9) plays a significant role in the regulation of platelet function and aggregation. It contributes to platelet function by providing a counterbalance to pro-aggregatory forces within the platelet itself. Upon partial activation, platelets release MMP-9, which then acts to inhibit further platelet aggregation. MMP-9 increases NO/cyclic GMP formation, leading to the inhibition of phosphoinositide breakdown and protein kinase C activity[98]. This biochemical cascade ultimately results in the inhibition of both the phosphorylation of P47 and intracellular Ca2+ mobilization[99]. By modulating intracellular signaling pathways and reducing calcium mobilization, MMP-9 effectively decreases platelet

activation, thereby regulating platelet function and aggregation[98]. The regulation of calcium levels by MMP-9 affects various downstream processes critical for platelet aggregation, highlighting its role in maintaining vascular stability by preventing excessive platelet clumping[98, 100].

Prostacyclin (PGI_2), a potent inhibitor of platelet aggregation, exerts its effects through the activation of the cAMP-PKA signaling pathway, which leads to a broad inhibition of platelet activation mechanisms. PGI2 increases the levels of cAMP, which in turn activates PKA, phosphorylating numerous targets that collectively reduce calcium levels within platelets, inhibit cytoskeletal changes, and decrease granule secretion. This broad inhibition of activation pathways not only prevents new platelet aggregation but also can disaggregate existing clumps, providing a powerful mechanism for controlling thrombus growth and stability[101-103].

1.1.11 Nitric Oxide and Platelet Function

An important negative-feedback pathway that limits platelet adhesion, aggregation, and thrombus formation is mediated by nitric oxide (NO), which may be generated by both endothelial cells and aggregating platelets themselves[104-106]. Nitric oxide (NO) is a highly diffusible free radical gas with a short half-life that plays an important role in many physiological and pathophysiological processes , including regulating vascular tone and signal transmission by neurons[107-111]. Importantly, it also plays a major role in immune function as well as within the hematopoietic system[112-116].

NO is produced enzymatically from the oxidation of L-arginine by an NADPH-dependent family oxidation-reduction enzymes called nitric oxide synthases or NOS[117-119]. These enzymes utilize flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-

)5,6,7,8-tetrahydro- L-biopterin (BH4) as cofactors to generate NO from the substrate L-arginine and co-substrates oxygen and NADPH. Three isoforms of nitric oxide synthase exist, including NOS I (nNOS, neuronal nitric oxide synthase), NOS II (iNOS, inducible nitric oxide synthase), and NOS III (eNOS, endothelial nitric oxide synthase) (Fig. 1.1.1)[120, 121]. Although all three enzymes bind to calmodulin (CaM), nNOS and eNOS bind CaM upon a rise intracellular Ca2+ concentration and become activated [122-124]. Further important for eNOS regulation is its localization to cell membrane caveole wherein the caveolae coat protein caveolin-1 is a tonic inhibitor of eNOS activity and recruitment of CaM and heat shock protein 90 displaces caveolin-1 leading to eNOS activation[125, 126]. eNOS activity is also widely regulated both positively and negatively via phosphorylation, with Ser1177 and Thr495 being the most widely studied of such sites. eNOS activating phosphorylation occurs in response to circulating mediators such as vascular endothelial growth factor, insulin, bradykinin and estrogen, as well as in response to sheer stress[127]. Similar to constitutive NOS enzymes, iNOS also binds to calmodulin; however, it does so even at basal levels of intracellular Ca2+ due to its high affinity for CaM[128]. nNOS and eNOS are constitutively expressed in different cells and tissues; while, under physiological conditions iNOS expression is limited[129-131], but can be induced in almost any cell type by proinflammatory proteins such as IL-1, TNF-α, IFN-γ, IL-2, IL-12, IL-18, CD40 ligand and Fas-ligand and pathogen-associated molecular patterns such as lipopolysaccharide[131].

Nitric Oxide Synthase isoforms

Figure 1.1.2 The isoforms of the nitric oxide synthase (NOS) enzyme.

Neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III) are equipped to produce nitric oxide (NO) in brief, pulsatile bursts, generating NO concentrations that vary from picomolar (pM) to nanomolar (nM) for nNOS and nanomolar (nM) to micromolar (μM) for eNOS. On the other hand, inducible NOS (iNOS or NOS II) is distinguished by its ability to produce NO at substantially higher levels, typically in the micromolar (μM) range, and does so continuously once the enzyme is expressed. (BH4: (6R-)5,6,7,8-tetrahydro-L-biopterin, a co-factor essential for NOS activity.)

In the 1980s, Radomski and Moncada demonstrated that NO potently inhibits platelet adhesion and aggregation[132-135], Subsequently, NOS and an NO signaling pathway were identified within platelets[136-139], and NO produced during aggregation was shown to inhibit further platelet recruitment[140, 141]. NO mediates most of its platelet inhibitory effects via cGMP generated by sGC (Fig 2)[142-144]. cGMP acts on PKG, which phosphorylates vasodilator-stimulated phosphoprotein (VASP) enabling VASP binding to the platelet cytoskeleton[145, 146]. Next, VASP inhibits integrin αIIbβ3 activation, preventing adhesion and aggregation[147, 148]. PKG signaling is also reported to suppress intracellular Ca2+ and integrin αIIbβ3 activation via inositol-1,4,5-triphosphate receptor-associated cGMP kinase substrate signaling[149, 150] and to suppress thromboxane receptor activation[151]. Platelet NOS activity has been attributed to eNOS[137, 152-154], although a few studies report iNOS in low amounts in platelets[152, 153] (there are no reports of platelet nNOS).

In the past 20 years, however, controversies arose over platelet NO signaling. The most relevant questioned platelet NO production and eNOS presence[155] and whether NO also has a stimulatory role in platelet activation[156]. To address these controversies, our lab previously investigated the hypothesis that some of these discrepancies may be explained by differences in platelet levels with and without eNOS signaling. This led to the identification of eNOSneg/low and eNOSpos/high platelet subpopulations in blood[157]. We demonstrated that eNOSneg/low platelets do not produce NO or produce it in low amounts. This platelet subpopulation also has a down-regulated sGC-PKG-VASP signaling pathway, initiates adhesion to collagen, and more readily activate integrin αIIbβ3, than eNOSpos/high platelets. eNOSpos/high platelets contain higher protein levels of sGC, PKG, and VASP and are more abundant (~80% of total platelets).

eNOSpos/high platelets also form the bulk of an aggregate via enhanced COX-1 signaling; however, they also ultimately limit aggregate size via NO generation.

Importantly, $ONOO^-$ also impacts platelet function[158], and its impacts may help explain some of the discrepant findings surrounding platelet NO function. At low concentrations, peroxynitrite was shown to mediate NO-dependent platelet inhibition; however, at higher concentrations it caused an increase in P-selectin exposure and platelet activation [159]. Consistently reducing peroxynitrite formation by suppressing NADPH oxidase, a major source of platelet superoxide generation, was shown to increase NO bioavailability and subsequent platelet inhibition [160]. Additionally, the structure of platelet aggregates, with a densely packed core and a more exposed dome, influences peroxynitrite levels and its effects, with higher concentrations and platelet activation more likely in the core[161].

Insufficient platelet NO production and a decrease in its bioavailability may also have important pathological consequences, particularly in the setting of acute coronary syndrome (ACS). Platelets from ACS patients have impaired NO production[162], and platelet NO production inversely correlates with increasing number of coronary artery disease risk factors [163]. Similarly, platelet refractoriness to the NO donor sodium nitroprusside predicts increased morbidity and mortality in patients with high-risk ACS [164]. Consistent with these findings, megakaryocytes from patients with normal coronary arteries have been reported to generate more NO in a Ca2+-dependent manner than megakaryocytes from patients with atherosclerosis, although megakaryocytes from atherosclerotic patients generate more NO in an iNOS-dependent manner [165-167]. Considering that platelets have a more limited transcriptome and capacity for new protein synthesis, and that iNOS protein has an extremely short half-life (< 2 hr) [168, 169], suggests that reduced platelet NO bioavailability within coronary artery disease may reflect a

reduction in megakaryocyte eNOS expression. Furthermore, NO formed from different NOS isoforms may play differing roles in megakaryocyte vs. platelet function. Hence, due to recent advances in understanding of platelet NO biology and its significance to pathology, a closer examination of NO-signaling in megakaryocytes is also needed.

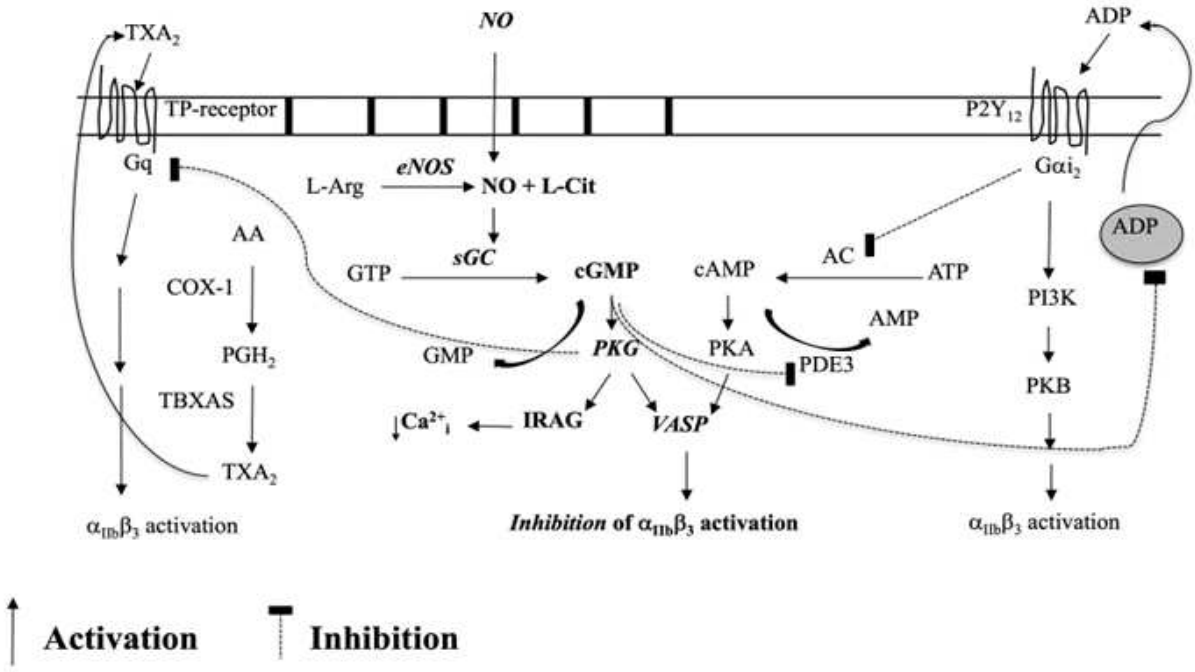


Figure 1.1.3 Platelet eNOS-NO-sGC-PKG-signaling.

The process through which nitric oxide (NO) prevents platelets from activating mainly uses the signaling pathway involving soluble guanylate cyclase (sGC) and protein kinase G (PKG). PKG acts by adding a phosphate group to the vasodilator-stimulated phosphoprotein (VASP), which then allows VASP to attach to the platelet's internal framework, thereby blocking the activation of the αIIbβ3 complex. Additionally, PKG can inhibit the activation of αIIbβ3 by two other methods: it interferes with the signaling pathway linked to the inositol-1, 4, 5-triphosphate receptor-associated cGMP kinase substrate, and it prevents the activation of thromboxane receptors.

1.1.12 Basic Megakaryocyte Biology

Megakaryocytes are large (50-100μM), multi-nucleated cells that are responsible for releasing platelets into the blood [170]. They are characterized by a multilobulated nucleus accumulating 2n, 4n, 8n, 16n, 32n, up to 128n DNA content and constitute ~0.01% of bone marrow cells [8]. Recent evidence sheds light on the different roles of megakaryocytes in various physiological and pathophysiological processes. Until recently, it has been thought that megakaryocytes solely serve as progenitors or precursor cells responsible for platelet production. This simple notion may no longer be valid as recent reports have uncovered various roles of megakaryocytes in the immune response and in modulating the proliferation and differentiation of different cell lineages, particularly osteoblasts and osteoclasts within the bone marrow [171-173]. Megakaryocytes are capable of antigen endocytosis and, ultimately, its presentation within the MHC I to CD8+ T cells [174]. Moreover, recent studies revealed that megakaryocytes release several immune-modulatory cytokines, including TGF-β and IL-1, and express co-stimulatory molecules such as CD40L and B7-2 (CD86) on their surface, suggesting they act as antigen presenting cells (APCs) within the bone marrow microenvironment [25, 173, 175-177]. Of interesting note, recent evidence suggests that megakaryocytes may act as the first line of defense against cancer metastasis to the bone [171, 172]. Therefore, like platelets, which are increasingly recognized for their diverse roles beyond hemostasis[178-181], megakaryocytes may have functions beyond platelet production. As such a greater understanding of how important chemical mediators influence megakaryocytes in platelet production and newly recognized functions is required.

According to conventional or classic hematopoiesis, hematopoietic stem cells (HSCs) give rise to megakaryocyte-biased progenitors after passing through several strict commitment points

or lineage-biased steps like a hierarchical-branched tree [182, 183]. However, more recent evidence demonstrates that although hematopoietic stem cells are capable of reconstituting all blood cell lineages, they may exhibit megakaryocyte or platelet-biased phenotypic and functional characteristics. Therefore, these multipotent progenitor cells, may bypass differentiation pathways and directly give rise to megakaryocyte or platelet-committed progenitors at a very early step in differentiation [184-190]. Consistently, bone marrow transplantation in humans demonstrates that platelet reconstitution takes place earlier than that of other blood cell lineages [191].

It has been reported that HSC differentiation toward megakaryocytes and their maturation, endomitosis, and invaginated membrane system (IMS) development takes place in the osteoblastic niche; whereas later generation of proplatelets, megakaryocytye-platelet intermediate pseudopodia-like structures, requires vascular niche localization [8]. Within these bone marrow niches HSC/megakaryocyte progenitor cell interactions with microenvironment extracellular matrix proteins help to regulate megakaryocyte differentiation and platelet production. HSC interaction with type I collagen within the osteoblastic niche via VLA-2 (Integrin α2β1) promotes commitment to megakaryocyte-biased progenitor formation and maturation, but suppresses megakaryocyte terminal development which results in proplatelet generation [192-194]. Similarly, megakaryocyte glycoprotein (GP) VI – collagen I interaction has been shown to inhibit proplatelet formation [195]. However, double knockout of collagen receptors (GPVI-/- integrin α2β1-/-) shows no difference in megakaryocyte distribution, size, or blood platelet levels compared to that of wild type mice, suggesting other regulatory mechanism may exist to suppress ectopic proplatelet generation within the osteoblastic niche [196]. In contrast, the vascular niche contains extracellular matrix proteins including collagen IV, fibronectin, fibrinogen, and von Willebrand factor which induce proplatelet generation [197-200]. Other factors, including megakaryocyte-active mitogens

such as fibroblast growth factor 4 (FGF-4) and the chemokine stromal cell-derived factor 1 (SDF-1) also promote survival, maturation, and platelet production from megakaryocytes by facilitating their chemotaxis toward and affinity for bone marrow sinusoid endothelial cells [201, 202]. Once in the vascular niche several hypotheses have been proposed to explain the mechanism behind the proplatelet extension from megakaryocytes into the lumen of bone marrow blood vessels [203-205]. A concentration gradient of sphingosine-1 phosphate (S1P) has been shown to exist at the contact site between the megakaryocytes and sinusoidal blood, which directs proplatelets into lumens in a sphingosine-1-phosphate receptor 1 (S1prP1)-dependent manner [206]. Ultimately, blood flow shear forces facilitate proplatelet release from the megakaryocyte and their fission to produce platelets.

In addition to extracellular matrix proteins, various soluble factors have been proposed to play important roles in regulating megakaryopoiesis and thrombopoiesis. Of these particularly important is the glycoprotein hormone thrombopoietin (TPO). Through its receptor c-mpl, which is expressed on the most primitive HSCs, TPO plays a key role in megakaryocyte differentiation from HSCs and their maturation toward platelet generation [182, 207]. TPO plays a central role in maintaining platelet/megakaryocyte-biased HSCs, as TPO knockout (TPO –/–) bone marrow cells give rise to lymphoid-biased bone marrow reconstitution in irradiated recipient mice. As such c-mpl and TPO knockout mice demonstrate 90% reductions in megakaryocyte and platelet numbers[208], while loss of function mutations to Mpl within humans cause congenital amegakaryocytic thrombocytopenia, resulting in a severe phenotype only rescued by bone marrow transplantation. In addition to TPO, several other factors have been identified which promote megakaryocyte proliferation and maturation, including interleukin 3(IL-3), interleukin 6(IL-6), and stem cell factor (SCF)[209-211].

TPO also induces megakaryocyte polyploidization, which results in the accumulation of lipids and proteins required for the constitution of a vast invaginated membrane network connected to the megakaryocyte surface membrane. This membrane network forms the surface membrane of proplatelets and the cytoskeletal ultrastructure that supports the elongation of proplatelet tubular structures [170, 212-214]. The process of proplatelet formation and the release of platelets into the sinusoidal blood vessels in the bone marrow is highly regulated [215], During this process, cytoskeletal proteins, including β1-tubulin, dynein, F-actin, and myosin II, facilitate proplatelet generation by providing assembly lines for elongation, organelle transportation, and ultimately platelet release [202, 214, 216, 217]. Of particular importance is the role of the transcription factor NF-E2 and its expression of β1-tubulin that plays a pivotal role in proplatelet biogenesis, structure, and function by polymerizing into microtubule bundles and coils that extend throughout these cytoplasmic extrusions. Consequently, NF-E2 and β1-tubulin knockout mice suffer from thrombocytopenia because of a significant reduction in proplatelet formation [216, 218-223]. TPO also induces reactive oxygen species (ROS) production, which play an important role driving HSC differentiation toward mature megakaryocytes and platelet production. This ROS generation likely involves nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases (NOXs) and increased oxygen tension resulting in enhanced tyrosine phosphorylation, proliferation, and polyploidization[224, 225]. Moreover, NF-E2 in addition to expressing platelet genes also maintains a moderate expression of cytoprotective genes allowing for ROS accumulation during megakaryocytic maturation[226]. The initiation of platelet formation from mature megakaryocytes has also been shown to be governed by a reciprocal interplay between mitochondrial dynamics and ROS, in which increased ROS levels stimulate mitochondrial fission, leading to the production of more mitochondrial ROS [227]. Most recently, a role for ROS has also been identified in the

pulling of megakaryocyte intravascular proplatelet extensions by so-called “plucking” neutrophils to enhance platelet formation[228].

However, it is worth noting that platelet generation via megakaryocyte proplatelet formation at steady state may differ mechanistically from platelet production in response to stress or injury. Stress thrombopoiesis, or the process of platelet production under inflammatory or acute thrombocytopenia conditions, can occur much faster than physiological platelet production [229, 230]. This may occur in part due to the presence of platelet- or megakaryocyte-primed hematopoietic stem cells (HSCs) in the bone marrow that can bypass the traditional route of multi-step lineage-biased progenitor differentiation and give rise to platelets more quickly [185, 186, 230-232]. However, equally important to stress thrombopoiesis is whether platelet generation proceeds through or bypasses the need for TPO and classic proplatelet formation. Although proinflammatory cytokines, such as IL-1β, can upregulate the expression of TPO and other megakaryocyte-related transcription factors to further promote platelet production[233], recent studies have shown that in response to IL-1α megakaryocytes undergo rupture to rapidly produce platelets in a TPO-independent manner after platelet loss or inflammatory stimulus[234, 235]. This rupture-dependent thrombopoiesis also displays caspase-3 dependence[234], and platelet generation differences at stress vs. steady-state may help to explain whether or not megakaryocyte apoptosis needs to be restrained for platelet generation and which apoptotic pathways may or may not be involved[236-239].

While much is known about the roles of extracellular matrix proteins, soluble protein mediators, and even gaseous chemical mediators such as ROS in megakaryopoiesis and thrombopoiesis, relatively little is known of the role of nitric oxide (NO) in these processes. This

is somewhat surprising considering NO's pleiotropic biological activity, and the important role it plays regulating hematopoiesis and platelet function [104, 240-243]

1.1.13 Nitric Oxide Synthases in Megakaryocytes

As described above constitutive (Ca2+-dependent) and inducible NOS isoforms have been identified in both human bone marrow megakaryocytes [165] and within the Meg-01 megakaryoblastic cell line [244]. Treatment of Meg-01 with proinflammatory cytokines IL-1β and TNF-α also revealed a reciprocal relation between constitutive and inducible NOS activity consistent with an increase in iNOS expression and a down-regulation of constitutive NOS expression. The Ca2+-dependent NOS in megakaryocytes/blasts likely corresponds to eNOS as its expression has been confirmed by RT-PCR and immunostaining within Meg-01[245].

Moreover, like in platelets, both eNOSneg/low and eNOSpos/high Meg-01 subpopulations have been identified [245, 246]. Research has shown that the pro-inflammatory cytokine IFN-γ can attenuate eNOS levels in megakaryocytes/blasts, leading to the formation of eNOS-negative cells. These cells subsequently give rise to more reactive eNOS-negative platelets, which are typically associated with increased thrombotic activity. Conversely, the anti-inflammatory cytokine IL-10 acts to counterbalance the effects of IFN-γ. It enhances eNOS expression in megakaryocytes/blasts, promoting the generation of eNOS-positive platelets[247].

1.1.14 Effect of NO on Differentiation and Proliferation of Megakaryocytes

Early research demonstrated that high concentrations (μM) of the NO donor DETA/NO induce apoptosis of bone marrow-derived CD34+ progenitor cells and that iNOS-generated NO may in part mediate hematopoietic suppression by proinflammatory cytokines IFN-γ and TNF-α

[240]. Treatment of human bone marrow-derived and TPO-cultured CD34+ cells, as well as mononuclear cells, with IFN-γ and TNF-α reduces the number of CD41+ cells after 12 days of culture, while high NO-donor concentrations inhibit the outgrowth of megakaryocytes derived from these cells by inducing their apoptosis [116]. Prostacyclin treatment and cAMP signaling protect megakaryocytes outgrown from CD34+ cells from NO-induced apoptosis [248], while TPO, 5-hydroxytryptamine, and IL-11 appear to protect megakaryocytic cell lines from apoptosis induced by high NO concentrations achieved by NO-donors or iNOS induction[249]. Altogether, these results suggest that in absence of protective factors and under inflammatory-like conditions up-regulation of iNOS expression and increased NO concentrations induce apoptosis of progenitor cells preventing their differentiation toward megakaryocytes. Whether the pro-apoptotic effects of NO are mediated via cGMP-dependent or non-cGMP-dependent mechanisms remains to be fully elucidated, as does the contribution of peroxynitrite and of the apoptotic pathways involved. Moreover, potential cross-talk between pathways that retard NO-induced apoptosis of megakaryocytes and their progenitors also needs further investigation [250].

1.1.15 Effect of NO on Platelet Production by Megakaryocytes

Similar to the limited number of studies investigating the role of NO in megakaryopoiesis, there is also a paucity of data with regards to NO's role in thrombopoiesis. Early work by Loscalzo and colleagues demonstrated that treatment of the Meg-01 cell line with high NO concentrations as achieved by utilizing the NO-donor S-nitrosoglutathione (GSNO) or by treating the Meg-01 cell line with proinflammatory cytokines (IFN-γ, TNF-α, and IL-1β) induces the generation of CD41+ platelet-sized particles in culture with a capacity to aggregate [115]. Moreover, platelet particle generation by Meg-01 is further enhanced if the Meg-01 are pretreated with TPO prior to

stimulation, although TPO treatment alone was not able to promote platelet particle generation consistent with its role in megakaryocyte maturation [115]. The mechanism by which high NO concentrations induce platelet-sized particle formation was reported to be cGMP-independent, and interestingly, was associated with the generation of distinct Meg-01 derived annexin-V and propidium iodide positive apoptotic bodies. This finding led the authors to hypothesize that NO-induced apoptosis is related to the process by which megakaryocytes produce platelets, although as also noted by the authors it is not clear whether the observed apoptosis is a result of removal of spent megakaryocytes or whether apoptosis and platelet production are simultaneous events [114, 115]. Lastly, of note, the authors identified that iNOS null mice demonstrate platelet counts nearly half of that of their wild-type or eNOS null counterparts further exemplifying the important role of NO in platelet production.

Consistent with the findings of Loscalzo and colleagues, intravenous infusion of L-nitroarginine or N(G)-nitro-L-arginine methyl ester, both NOS inhibitors, to rats results in thrombocytopenia or decreased platelet counts [251]. More recently, CD226 whole body or platelet/megakaryocyte specific knockout mice have been shown to have elevated platelet and megakaryocyte (bone marrow and spleen) counts compared to wild-type controls [252]. Notably, the platelets from CD226 -/- mice demonstrated greater aggregation response to thrombin compared to platelets from WT mice, attributed to their reduced eNOS levels and decreased ability to generate NO. Currently, it is unknown whether the potential alterations in megakaryocyte-platelet NO-signaling in these mice impact their megakaryo- or thrombopoiesis. However, considering that platelet function may be regulated by low NO concentrations attributable to eNOS activity while high NO concentrations associated with iNOS appear to have profound effects on megakaryocytes and their potential to produce platelets, it is tempting to speculate whether these

two NOS isoforms have differential function in megakaryocytes vs. platelets. Specifically, the role of iNOS and its increased expression may be of particular importance to platelet production in cases of inflammation/infection-induced secondary (reactive) thrombocytosis (Fig 1.1.3).

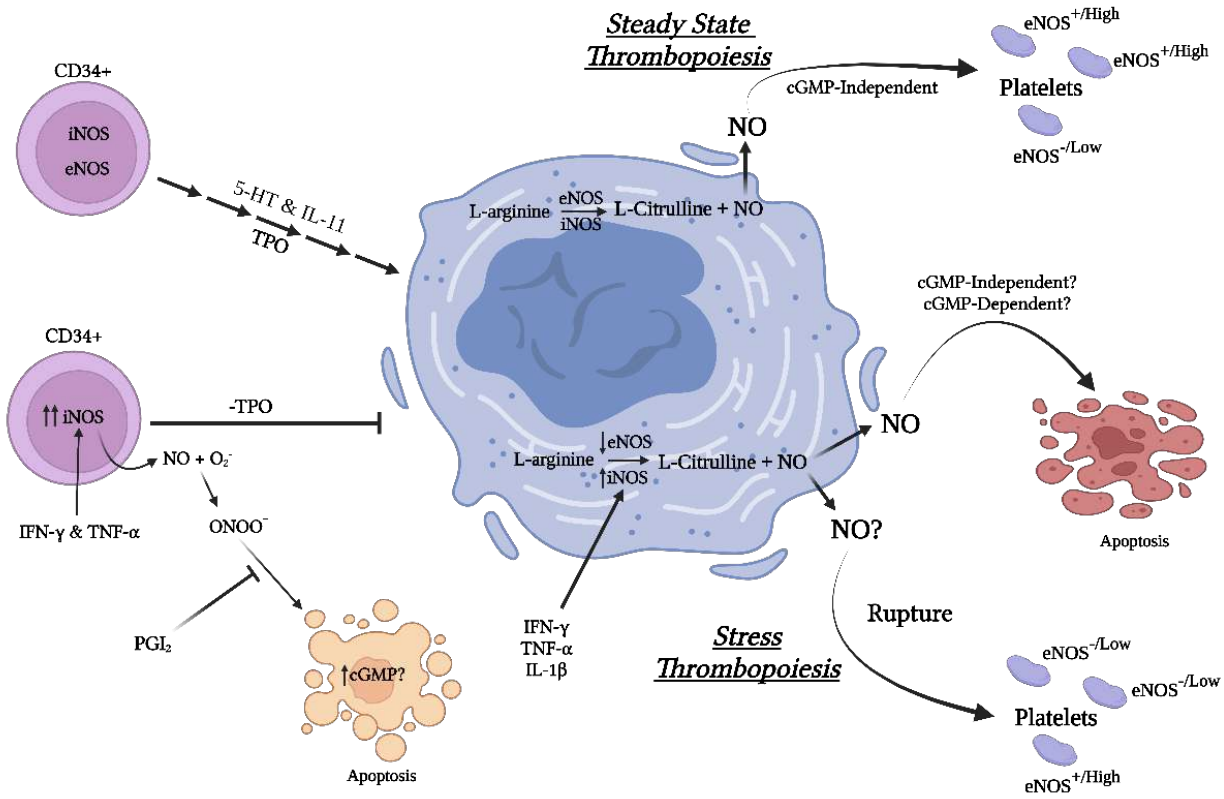


Figure 1.1.4 Steady-state thrombopoiesis vs. stress thrombopoiesis

Cartoon summarizing the impact of Nitric oxide derived from iNOS and eNOS on megakaryopoiesis and thrombopoiesis under stressed and non-stressed conditions. Created with BioRender.com

1.1.16 Introduction to COVID-19

Coronavirus disease 2019 (COVID-19), caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has previously been identified as a global health crisis of unprecedented scale (January 30, 2020 - May 5, 2023), although it is currently not considered a global emergency by the World Health Organization (WHO). As of the latest reports, it has infected over 774 million people across every continent, leading to more than 7 million deaths worldwide[253-256]. This pandemic has not only strained healthcare systems but also brought significant economic, social, and psychological challenges globally[257].

SARS-CoV-2 belongs to the Coronaviridae family, a group of enveloped, positive-sense, single-stranded RNA viruses known for their distinctive crown-like appearance under electron microscopy, attributed to the viral spike (S) proteins projecting from their surface[258]. The spike proteins are essential for the virus's capacity to invade host cells, as they interact with specific host cell proteins. Among these, the ACE2 (angiotensin-converting enzyme 2) receptor, TMPRSS2 (transmembrane protease, serine 2), and cathepsins stand out as key players. ACE2 serves as the primary docking site for the virus, binding the receptor-binding domain of the spike protein with high affinity. Once the virus is anchored to ACE2, the spike protein must be primed to facilitate viral and cellular membrane fusion, a process significantly mediated by TMPRSS2. This serine protease cleaves the spike protein, triggering a conformational change that promotes fusion. In parallel, in environments where TMPRSS2 is less abundant, cathepsins, which are lysosomal proteases, can alternatively cleave the spike protein within endosomes after the virus has been endocytosed. These dual pathways for spike protein activation—surface-based via TMPRSS2 and endosomal via cathepsins—illustrates a redundancy that enhances the efficiency of viral entry[259-262]. This interaction facilitates viral entry and replication within host cells,

primarily affecting the respiratory tract but also capable of systemic involvement, leading to widespread tissue and organ damage[263].

The genome of SARS-CoV-2 encodes several structural and non-structural proteins, each playing a unique role in the virus's life cycle, pathogenicity, and interaction with the host immune system. Among these, the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins are integral to the virus's structure and its ability to infect host cells[264]. Non-structural proteins, including the RNA-dependent RNA polymerase (RdRp), play crucial roles in both viral replication and the evasion of the host's immune response. The RdRp is instrumental in copying the viral RNA genome, a critical step in viral replication. Beyond replication, it also contributes to immune evasion by interfering with the host's interferon signaling pathways. For instance, it blocks the nuclear translocation of the transcription factor IRF3, inhibiting the activation of interferon-stimulated genes. Additionally, other non-structural proteins like NSP1 and NSP14 suppress host mRNA translation and reduce IFN receptor expression, respectively, while proteins like NSP8 and NSP9 disrupt normal protein trafficking. Furthermore, ORF6 impedes the nuclear import of STAT1 and STAT2, effectively crippling the host's ability to mount an effective antiviral response. These evasion strategies underscore the virus's capability to manipulate the host's cellular machinery to enhance its survival and proliferation [265-267].

The rapid dissemination of SARS-CoV-2 can be attributed to several factors, including its high reproductive number (R0), efficient human-to-human transmission, and the presence of asymptomatic carriers[268, 269]. Moreover, the virus has exhibited a notable capacity for genetic variation, with several variants of concern (VOCs) emerging and spreading globally. These variants, characterized by mutations in the spike protein and other genomic regions. These VOCs have demonstrated increased transmissibility, altered disease severity, and varying degrees of

resistance to neutralizing antibodies[270]. Among these, the Alpha (B.1.1.7) and Delta (B.1.617.2) variants have significantly impacted the trajectory of the pandemic, due to mutations in their spike proteins that confer advantages in transmissibility, pathogenicity, and, in some cases, evasion of immune responses[271-273].

The Alpha variant, first identified in the United Kingdom, and the Delta variant, first detected in India, have both demonstrated increased transmissibility, attributed to specific mutations in their spike proteins. The Alpha variant carries the N501Y mutation in the receptor-binding domain (RBD) of the spike protein, enhancing its binding affinity for ACE2 and potentially increasing the viral load in infected individuals. Additionally, the deletion of two amino acids (Δ69-70) in the spike protein has been linked to immune evasion and increased infectivity. The Delta variant, on the other hand, harbors the L452R and P681R mutations, among others, which not only improve ACE2 receptor binding but also appear to aid the virus in evading the host immune response, contributing to breakthrough infections even among vaccinated individuals[271-275].

COVID-19 is primarily recognized for its profound impact on the respiratory system, with complications ranging from mild symptoms reminiscent of the common cold to severe manifestations such as lung injury and acute respiratory distress syndrome (ARDS)[276-278]. These severe respiratory complications are the leading cause of morbidity and mortality in affected individuals[279-281]. The pathogenesis of these respiratory complications is complex, involving viral replication, hyperinflammation, and immune system dysregulation, which together contribute to the tissue damage and functional impairments observed in the lungs[280, 282, 283].

COVID-19 extends beyond respiratory symptoms, manifesting a wide range of extrapulmonary effects that underscore its systemic impact. These include complications within

the cardiovascular, renal, gastrointestinal, hepatic, neurological, and dermatological systems[284]. The cardiovascular system, in particular, is acutely affected, with patients exhibiting conditions such as myocarditis, arrhythmias, acute coronary syndromes, and thromboembolic events[285-287]. These cardiovascular issues are largely the result of the hyperinflammatory state and coagulopathy observed in severe cases of COVID-19, which precipitate enhanced clot formation and vascular inflammation[288, 289].

Among the notable pathological hallmarks of severe COVID-19 is microvascular thrombosis[290]. This condition involves the formation of clots in the small vessels of the circulatory system, leading to impaired microcirculation and contributing to the progression of organ dysfunction. The presence of both venous and arterial thrombotic events further exacerbates the clinical scenario, elevating the risk of organ failure and mortality[291, 292].

Platelets, central regulators of hemostasis and thrombosis, have been found to play a pivotal role in the development of microvascular thrombosis among COVID-19 patients[293, 294]. Their function, extending beyond mere blood clot formation to include substantial involvement in the body's immune response, places them at the heart of the thromboinflammatory processes observed in COVID-19. The activation of platelets and their interaction with endothelial cells and leukocytes under the inflammatory milieu of SARS-CoV-2 infection contributes to the formation of thrombi, highlighting the critical nexus at which platelets operate to mediate the balance between protective hemostasis and pathological thrombosis.

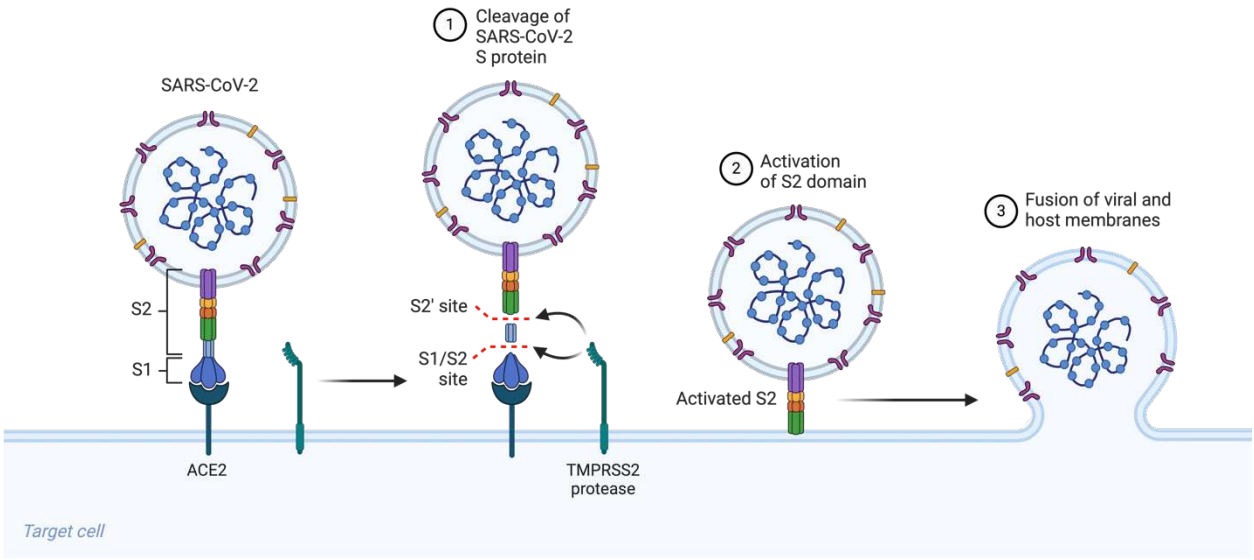


Figure 1.1.5 Mechanims of SARS-CoV-2 viral entry

This figure illustrates the steps involved in the entry of SARS-CoV-2 into a host cell. The process begins with the viral spike (S) protein binding to the ACE2 receptor on the surface of the target cell. The host cell protease, TMPRSS2, then cleaves the S protein at the S1/S2 site, facilitating further processing. Cleavage at the S2' site activates the S2 domain of the spike protein, which undergoes a conformational change necessary for membrane fusion. The activated S2 domain mediates the fusion of the viral envelope with the host cell membrane, allowing the viral RNA to enter the host cell and initiate infection. Created with BioRender.com

1.1.17 Role of Platelets in COVID-19

During the early stages of the COVID-19 pandemic, clinicians and researchers quickly recognized an alarming pattern of clotting abnormalities among patients, particularly those with severe forms of the disease[295]. This clotting, often associated with worse outcomes and increased mortality, highlighted an aspect of SARS-CoV-2 infection that extended beyond the direct viral attack on respiratory tissues. The role of platelets in this process became a focal point for understanding the broader impacts of the virus on the body's systems.

Platelets, traditionally known for their role in hemostasis (the process of stopping bleeding), were found to have a far more complex function during COVID-19. They are central to thromboinflammation, a critical and often detrimental feature of severe COVID-19 cases. Thromboinflammation refers to the intertwined dynamics of thrombosis (the formation of blood clots) and inflammation, which can both defend against infections and exacerbate the infection or the pathology. In the context of COVID-19, activated platelets contribute to this process not just by forming clots, but also by releasing inflammatory molecules that can enhance vascular and immune system disturbances. This dual role of platelets thus contributes to a vicious cycle where inflammation leads to thrombosis and vice versa, significantly impacting the pathology of COVID-19 and highlighting their importance beyond mere blood clotting[296, 297].

As platelet activation and aggregation are often observed in COVID-19 patients, various mechanisms have been reported to contribute to these phenomena[298]. In addition to the interactions mediated by complement components such as C3a and C5a with their corresponding receptors on platelets, C3aR and C5aR, thrombin and the binding of anti-SARS-CoV-2 immunoglobulins to the FcγRIIa receptor on platelets are significant factors in platelet activation[70, 299-301]. Thrombin, a potent coagulation factor, directly stimulates platelets,

enhancing their aggregation and secretion activities[302]. Concurrently, the immune response to SARS-CoV-2 involves the production of specific immunoglobulins that can bind to the FcγRIIa receptors on platelets[301, 303]. This binding promotes platelet activation, further amplifying their role in thrombosis and inflammation.

Upon activation, platelets interact extensively with endothelial cells, facilitated by the viral spike protein's interaction with the ACE2 receptor on these endothelial cells. Endothelial cells, which line the blood vessels, express the ACE2 receptor. When a virus, such as SARS-CoV-2, binds to ACE2 via its spike protein, it can induce endothelial cell activation and damage[304]. This engagement triggers a series of reactions including the expression of adhesion molecules such as P-selectin, which play a critical role in promoting platelet aggregation and recruitment of other immune cells[304]. Activated platelets express procoagulant phosphatidylserine and release various cytokines, including Platelet Factor 4 (PF4/CXCL4), RANTES/CCL5, IL-8, IL-1β, and TNF-α[305]. PF4 is particularly noteworthy as it not only exacerbates the disease's severity but also interacts with endothelial ultra-large von Willebrand Factor (ULVWF) multimers, increasing their resistance to cleavage by ADAMTS13[306]. This interaction enhances the stability of ULVWF multimers, facilitating the assembly of complement convertases and promoting further platelet adhesion and aggregation[307, 308].

IL-8, secreted by activated platelets, plays a pivotal role in the inflammatory cascade of COVID-19[309, 310]. It not only recruits and activates neutrophils but also promotes NETosis—a process where neutrophils expel their nuclear contents and proteolytic enzymes. This contributes significantly to thrombus formation, central to severe COVID-19 complications such as acute organ failure and myocardial infarction[311]. These NETs provide a a structural scaffold that, along with fibrin and von Willebrand factor (VWF), is crucial for the development of venous

thrombi[312, 313]. This structural support is foundational for clot formation within blood vessels. Also, the negatively charged extracellular nucleic acids within NETs facilitate the assembly of various coagulation factors, enhancing both the intrinsic and extrinsic coagulation pathways[314]. Key factors involved include FXIa, FIXa, and the thrombin/anti-thrombin complex, all of which contribute to the clotting process[314]. Furthermore, NETs promote the exposure of tissue factor (TF), a critical initiator of the coagulation cascade. In COVID-19, heightened levels of TF expression are observed in neutrophils and on NETs, reinforcing the thrombotic pathway. This process is partly influenced by the complement system, which can induce NETosis through activation of the C5a receptor[315-318].

Additionally, NETs play a pivotal role in platelet activation—a mechanism observed not only in COVID-19 but also in other diseases, such as viral influenza, deep vein thrombosis (DVT), and cancer-associated thrombosis[319-321]. In the specific context of COVID-19, this exaggerated platelet activation significantly contributes to the severe thrombotic complications often seen in patients[322]. NETs also interact with neutrophil-derived extracellular vehicles (EVs), creating a platform that increases thrombin generation through the intrinsic pathway of coagulation. This interaction further amplifies the likelihood of clot formation, underlining the multifaceted role of NETs in promoting thrombosis during severe COVID-19 infections[314].

Of important note, when neutrophils are attracted to an inflammation site by IL-8, they undergo degranulation near endothelial cells, releasing proteolytic enzymes like myeloperoxidase (MPO), proteinase 3 (PR3), neutrophil elastase (NE), and cathepsin G (CG)[323]. These enzymes can induce endothelial cell permeabilization and apoptosis, exacerbating as exposure prolongs. The resulting apoptosis compromises the endothelial barrier's integrity, exposing the underlying

subendothelium to platelets and leukocytes, thereby potentially escalating the inflammatory response[323].

1.1.18 Impact of COVID-19 on Endothelial Cells

The COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, has highlighted the critical role of endothelial dysfunction in the pathophysiology of the virus, extending beyond the respiratory symptoms to include significant vascular complications[324]. SARS-CoV-2 induces a complex array of molecular mechanisms that contribute to endothelial cell activation and the subsequent inflammatory responses[325]. Evidence of endothelial dysfunction in COVID-19 includes increased markers of endothelial inflammation and injury such as circulating endothelial cells, von Willebrand factor, P-selectin, E-selectin, angiopoietin-2 (Ang-2), and intercellular adhesion molecule 1 (ICAM-1), which remain elevated even in convalescent patients[324]. The endothelial glycocalyx (eGC), a protective meshwork that covers endothelial cells and is crucial for maintaining vascular homeostasis, is also disrupted in COVID-19 patients, further compromising vascular integrity[326].

One of the key pathways implicated in SARS-CoV-2-induced endothelial dysfunction involves the vascular endothelial growth factor (VEGF). It has been reported that the spike protein of SARS-CoV-2 induces VEGF production in enterocytes through the Ras-Raf-MEK-ERK signaling pathway, leading to vascular hyperpermeability and intestinal inflammation[327]. These effects were shown to be reversible with the inhibition of the ERK/VEGF pathway, highlighting the paracrine action of VEGF secreted by SARS-CoV-2-infected cells in triggering endothelial cell (EC) activation[327]. Another significant pathway is the activation of Toll-like receptor 4 (TLR4). In COVID-19 patients, both mild and severe, the TLR4/p38 MAPK14/RELA/IL-1β

pathway has been found activated in circulating endothelial cells[328, 329]. The SARS-CoV-2 spike protein further mediates endothelial activation, monocyte adhesion, nitric oxide production, and phosphorylation of p38 MAPK, NF-κB, and eNOS in ACE2-deficient endothelial cells through TLR4, a process that can be blocked by TLR4 antagonists[330, 331].

Moreover, the interaction of the spike protein with integrin α5β1 on ECs directly induces endothelial inflammation. This binding activates NF-κB and increases leukocyte adhesion and endothelial hyperpermeability, effects that are mitigatable with integrin α5β1 inhibitors[332]. The systemic impact of this interaction is highlighted by increased expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 across multiple organs following intravenous injection of the spike protein. Additionally, the cleavage of NF-κB essential modulator (NEMO) by the main protease of SARS-CoV-2 (M^{pro}) represents an alternative mechanism of brain endothelial cell activation. This process has been associated with endothelial cell death and disruption of the blood-brain barrier in COVID-19 patients and animal models[333]. This highlights the specificity of NEMO-dependent cell survival pathways in different subsets of endothelial cells, suggesting a targeted mechanism of SARS-CoV-2-induced endothelial damage particularly in the brain[333].

Finally, the exposure of endothelial cells to inflammatory cytokines following SARS-CoV-2 infection contributes significantly to endothelial injury. Cytokines such as IL-6, interferons, and TNF-α have been implicated as major drivers of reactive oxygen species (ROS) production in endothelial cells. These cytokines activate various pathways, including NOX2 and protein kinase C, to induce ROS production, thereby exacerbating endothelial dysfunction and contributing to the severe outcomes observed in COVID-19 patients[334].

1.1.19 Impact of COVID-19 on Vasculature Permeability

Vascular permeability, a crucial physiological mechanism, governs the flow of fluids, solutes, and cells across the endothelial barrier of blood vessels. This process is essential for maintaining homeostasis, enabling efficient nutrient delivery, waste removal, and immune system functioning[335-337]. However, when dysregulated, it can lead to pathological conditions such as pulmonary edema, a life-threatening complication characterized by the accumulation of excess fluid in the lungs' alveolar spaces[338, 339]. In the context of COVID-19, caused by the SARS-CoV-2 virus, vascular permeability takes on a pivotal role in the disease's progression and severity[340-342].

The relationship between COVID-19 and increased vascular permeability, particularly leading to pulmonary edema, involves a complex interplay of inflammatory responses and endothelial dysfunction[297, 343-346]. The pathophysiology of pulmonary edema in COVID-19 involves a complex interplay between viral-induced direct lung injury, systemic inflammation, and endothelial dysfunction [347-350]. SARS-CoV-2 enters the host cells via the ACE2 receptor, predominantly expressed in lung alveolar epithelial cells[351, 352]. The viral replication triggers an intense immune response, leading to cytokine release syndrome (CRS) or "cytokine storm," which results in widespread inflammation, increased vascular permeability, and vascular leakage[339, 353, 354]. Furthermore, COVID-19 has been associated with coagulopathy, characterized by microvascular thrombosis and endothelial injury, contributing to the pathogenesis of pulmonary edema[291, 339, 355].

1.1.20 Pulmonary Edema in COVID-19: Role of Platelets

The role of platelets in the pathogenesis of pulmonary edema in COVID-19 patients has garnered significant interest[356]. As described, platelets, beyond their well-known function in hemostasis and thrombosis, play a crucial role in immune responses and inflammation. In the context of COVID-19, platelets contribute to both coagulation and inflammation, processes that are critically involved in the development of pulmonary edema[357].

Platelets can interact with leukocytes, facilitating their recruitment and activation within the pulmonary vasculature[358]. This not only contributes to the inflammatory milieu but also to the formation of microthrombi, exacerbating endothelial injury and increasing vascular permeability, thus facilitating the extravasation of fluid into the alveoli[358, 359]. Moreover, the hypercoagulable state induced by COVID-19, partly mediated by platelet activation, plays a significant role in the pathogenesis of pulmonary edema[360-362]. The formation of microthrombi within the pulmonary circulation impairs blood flow, leading to hypoxia and further promoting fluid leakage into the lung interstitium and alveoli. Importantly, the interaction between platelets and the endothelium becomes particularly relevant in COVID-19. The virus-induced endothelial dysfunction leads to an increased expression of ICAM-1, VCAM-1, E-selectin, and P-selectin, promoting leukocyte adhesion and potentially enhancing platelet adhesion and aggregation[71, 363]. Activated platelets release an array of bioactive compounds from their granular stores, notably including pro-inflammatory cytokines and chemokines, further amplifying the immune response and contributing to the cytokine storm observed in severe cases[364].

1.1.21 Vascular Endothelial Growth Factor

VEGFs belong to a family of conserved glycoproteins that are connected by disulphide bonds and secreted in soluble form[365]. This term (VEGF) serves as a nomenclature encompassing a family of closely related VEGF polypeptides and refers to the initial and prototypical growth factor, previously recognized as vascular permeability factor (VPF)[366]. VEGFs act as primary orchestrators in the modulation of vascular development and regulating blood and lymphatic functionality in various physiological and pathophysiological processes[367-371].

The regulation of VEGF expression is a multifaceted process influenced by a variety of factors, including hypoxia, which significantly upregulates VEGF via the stabilization of hypoxia-inducible factors (HIFs) that bind to hypoxia-responsive elements in the VEGF gene promoter[372-374]. TNF-α and IL-6 are key cytokines that modulate VEGF expression, with TNF-α notably enhancing VEGF transcription through the NF-κB pathway. This involves the phosphorylation and degradation of IκB proteins, allowing NF-κB to enter the nucleus and bind to the VEGF gene promoter[374, 375]. Furthermore, TNF-α activates the MAPK pathway, contributing to VEGF expression by promoting AP-1 transcription factor activation[376]. Additionally, growth factors such as TGF-β, alongside genetic factors and signaling pathways, including PI3K/Akt, MAPK, and Notch, play critical roles in VEGF regulation[376, 377]. This complex regulatory network marks the sophisticated mechanisms controlling VEGF levels, which are pivotal in angiogenesis and vascular permeability across various physiological and pathological states.

The VEGF family consists of five structurally related factors: VEGFA (the prototypical member, also referred to as VEGFA165), VEGFB, VEGFC, VEGFD, and placenta growth factor

(PlGF). Of note, VEGF family members primarily exist as homodimeric polypeptides, although instances of naturally occurring heterodimers involving VEGFA and PlGF have been reported. VEGFs exert their function by binding to three structurally related VEGF receptor tyrosine kinases, each consisting of an extracellular ligand-binding region with an Ig-like domain, a transmembrane domain, and a tyrosine kinase (TK) domain in the cytoplasmic region. These receptors are known as VEGFR1 (FLT1), VEGFR2 (KDR, FLK1), and VEGFR3 (FLT4). The interaction between VEGFs and VEGFRs leads to the phosphorylation of specific tyrosine residues within distinct intracellular domains. These receptors exhibit both overlapping and distinct expression patterns[378-380].

1.1.22 Platelet-Derived Growth Factor

Platelet-Derived Growth Factors (PDGFs) represent a group of four cystine-knot-type growth factors, namely PDGF-A, -B, -C, and -D. These four PDGF chains have the ability to form disulphide-bonded dimers through either homo- or heterodimerization, resulting in the description of five different dimeric isoforms, namely PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD[381]. These factors are responsible for regulating the proliferation of connective tissue cells, including fibroblasts and smooth muscle cells[382-384]. Additionally, PDGF regulates vascular permeability indirectly through its role in vascular stability and angiogenesis. PDGF promotes the proliferation and migration of pericytes and smooth muscle cells to endothelial cell layer, reinforcing vessel wall integrity[385-387]. By strengthening the vascular wall and supporting endothelial cell function, PDGF can modulate the permeability of blood vessels, maintaining the balance between permeability for physiological processes and the prevention of excessive leakage that could lead to edema.

These actions of PDGFs are mediated through their interaction with the tyrosine kinase receptors PDGF receptor-α and PDGF receptor-β. These receptors are categorized within the class III receptor tyrosine kinases (RTKs) and exhibit varying expression patterns and physiological functions. Similar to all Receptor Tyrosine Kinases (RTKs), the PDGFR receptor family exhibits a modular architecture. This architecture involves the utilization of the extracellular domain for ligand recognition, a single transmembrane helix to transmit structural and informational cues from outside the cell, and an effector tyrosine kinase domain that responds to extracellular signals. This domain undergoes phosphorylation to initiate downstream recruitment and signaling events. PDGFRα signaling primarily governs processes such as gastrulation and the development of multiple organs, including the lung, intestine, skin, testis, kidney, bones, and neuroprotective tissues[388]. In contrast, PDGFRβ signaling is predominantly recognized for its pivotal role in early hematopoiesis and the formation of blood vessels[389-391].Platelets and the growth factors they release during thrombosis impact not only COVID-19 but also other inflammatory diseases, particularly cancer.

1.1.23 Historical Perspective of Platelets in Cancer

Beyond hemostasis and infection, the connection between the connection between platelets and cancer, a subject of considerable intrigue and study in modern oncology, has its roots in observations made over a century ago. Armand Trousseau, a French physician, was among the first to note a peculiar link in 1865. He observed that patients with occult malignancies often presented with excessive blood clotting, a phenomenon that would later bear his name as Trousseau's syndrome[392]. This observation was crucial in establishing a relationship between coagulation abnormalities and cancer. Building upon Trousseau's initial findings, Leopold Rises further

established the connection between thrombocytosis and solid tumors, emphasizing the role of platelets in cancer beyond their traditional function in hemostasis[393]. This path was further explored by Levin and Conley in their large-scale study, which analyzed patients with high platelet counts in the absence of other conditions. Remarkably, almost 40% of these patients were found to have undiagnosed malignancies, particularly in organs such as the stomach, colon, lungs, breasts, and ovaries[394]. This study not only reinforced the association between thrombocytosis and cancer but also suggested thrombocytosis as a potential marker for undiagnosed malignancies.

Building on the foundational observations of Trousseau, Rises, and Levin and Conley, the study of platelets in cancer progressed significantly. Recent research, including Bailey *et al*. identified increased platelet counts as predictors of undiagnosed malignancies and indicators of poorer prognosis in various cancers such as ovarian, lung, and breast[395]. Studies like Stone *et al*. (2012) revealed mechanisms like tumor-derived IL-6 stimulating thrombopoietin production, leading to thrombocytosis in ovarian cancer[396]. Moreover, the risk of thromboembolism in cancer patients, highlighted by Khorana *et al*. emphasized the need for better risk assessment and management strategies[397]. These studies highlight how platelets contribute to cancer progression and metastasis, with mechanisms like tumor-derived IL-6 leading to thrombocytosis and increased thromboembolism risks, as shown by Stone *et al*. and Khorana *et al*. Together, they emphasizegas the need for deeper molecular studies into platelets' roles in oncology, shifting research focus to more detailed functional analyses.

1.1.24 Interactions Between Platelets and Tumor Cells and Their Influence on Cancer Spread

The exploration of platelet interactions with cancer cells holds essential significance in oncology research, primarily because it reveals crucial insights into the mechanisms of cancer metastasis and proliferation. Platelets, often recognized for their role in hemostasis/thrombosis, have emerged as key players in tumor biology. Their interaction with cancer cells contributes to the formation of metastases, which is a leading cause of cancer-related mortality[398]. By understanding how platelets adhere to, activate, and protect circulating tumor cells, researchers can unravel the complex cellular dialogues that facilitate cancer spread.

Historically, the study of platelet-tumor cell interactions has evolved from recognizing basic adhesion and aggregation processes to understanding sophisticated molecular pathways. Earlier research primarily focused on the observation that tumor cells can induce platelet aggregation, a phenomenon first identified in the 1960s by Gasic and Stewart, known as Tumor-Cell Induced Platelet Aggregation (TCIPA), and this ability correlates with the metastatic potential of cancer cells [399]. In this context, various studies have shown that the type and characteristics of cancer cells influence how circulating tumor cells (CTCs) activate platelets, indicating that the mechanisms of platelet activation vary significantly across different cancers.

Direct interactions between cancer cells and platelets form one key aspect of this process. Cancer cells can directly activate platelets through several methods. The secretion of thrombin, a potent platelet agonist, is a primary example[400-402]. Thrombin activates platelets by cleaving their PAR1 and PAR4 receptors, setting off a series of events that lead to platelet activation[403-406]. Another important pathway is the secretion of adenosine diphosphate (ADP), which specifically targets P2Y1 and P2Y12 receptors on platelets, playing a significant role in platelet

activation and aggregation[407-412]. Furthermore, platelet-derived TXA2 upon activation by cancer cells was shown to mediate endothelial activation via the P2Y2 receptor leading to the recruitment of prometastatic monocytes/macrophages that contribute to the formation of pulmonary premetastatic niche[413] (Fig 3.1.1).

The complexity of this process is further increased by the involvement of various platelet-derived factors, such as MMP-2, and the function of adhesion molecules in platelets like integrins and P-selectin[414-421]. These factors and molecules facilitate platelet adhesion and aggregation, thereby contributing to the protective cloaking of cancer cells[422-425]. Additionally, a crucial direct interaction in platelet activation is highlighted by the engagement of C-type lectin-like receptor-2 (CLEC-2) on platelets by Podoplanin present on cancer cells[426-428].

Expanding on these mechanisms, tumor cells can also induce platelet activation and aggregation indirectly. Indirect activation involves the release or induction of classic platelet agonists involved in hemostasis or coagulation pathways. This includes the conversion of prothrombin into thrombin, facilitated by cancer procoagulant (CP), a factor X activator, and tissue factors (TF) expressed by tumor cells, which initiate the extrinsic coagulation pathway[429, 430].

Furthermore, tumor cells can influence platelet activation through interactions with innate immune cells. For example, carcinoma mucins can bind to selectins on both platelets and neutrophils, facilitating their interaction and leading to further platelet activation and aggregation through the release of agents such as cathepsin G from neutrophils[431]. Additionally, neutrophil extracellular traps (NETs) have been implicated in platelet activation, suggesting a complex interplay of interactions in the tumor environment[432-435].

The activation of platelets by cancer cells, through mechanisms like thrombin and ADP secretion or surface protein interactions, is a pivotal event in the complex process of cancer

progression. This activation is not an end in itself but a crucial trigger for a cascade of interactions that amplify the metastatic potential of cancer cells. Once activated, platelets assume a multifaceted role in the oncological milieu. Beyond their well-established function in inducing the epithelial-mesenchymal transition (EMT), which endows cancer cells with migratory and invasive capabilities, platelets contribute to several other critical aspects of cancer metastasis[436]. They offer a protective shield to tumor cells circulating in the bloodstream, guarding them against shear stress and immune surveillance. This protective role is crucial for the survival of circulating tumor cells (CTCs) in the harsh environment of the vascular system[437]. Furthermore, activated platelets enhance the adhesion of these tumor cells to the vascular wall at potential metastatic sites, a key step in the establishment of new tumor colonies. They also facilitate the extravasation and invasion of tumor cells at these metastatic niches, aiding in the colonization process[438]. Beyond the immediate vicinity of tumor cells, platelets influence the broader tumor microenvironment by regulating angiogenesis, thus providing the necessary blood supply for the growth of new metastases[439]. Therefore, the activation of platelets by cancer cells sets in motion a series of interactions that not only contribute to the spread of cancer but also influence the tumor microenvironment, affecting tumor growth and metastasis.

1.1.25 Platelet Role in Regulating EMT in Cancer Cells

EMT is characterized by cancer cells in the primary tumor gaining invasive capability, leading to their detachment, migration into the nearby stroma, and eventual intravasation into the blood or lymphatic system. This process involves significant changes in cellular architecture, including cytoskeletal rearrangement, loss of epithelial polarity, and reduction in tight cell–cell adhesion, which collectively enhance the cells' motility and invasion capability. Central to EMT is

the alteration of cellular adhesion molecules, exemplified by the reduction of E-cadherin and the increase of N-cadherin levels on the cell surface. These changes facilitate the detachment of cancer cells from the primary epithelium and their transition towards a more motile and invasive mesenchymal phenotype[440, 441].

Platelets have been identified as key players in inducing EMT in tumor cells, leading to a more invasive and resistant phenotype. Platelets can interact with cancer cells through various mechanisms, thereby influencing the EMT process. One significant pathway involves the secretion of TGFβ by platelets, which upon release, can induce EMT and subsequent metastasis in various types of cancer cells, including breast, colon carcinoma, and ovarian cancer cells[442]. The TGFβ/Smad pathway in tumor cells, activated by TGFβ from platelets, synergizes with the NF-κB pathway activated through direct platelet-tumor cell interaction, culminating in a pro-metastatic invasive mesenchymal phenotype[443, 444] (Fig 3.1.1). In another study by Zuo *et al*., platelets and MCF-7 breast cancer cell line direct interaction via integrin α2β1 promote invasion and induce EMT by stimulating the Wnt-β-catenin signaling axis. They suggested that the activated Wnt-β-catenin pathway can directly and indirectly, through stimulating TGF-β1expression and subsequent autocrine signaling, up-regulate the expression of EMT associated transcription factors, including Snail and Slug[442].

Platelets have been shown to significantly enhance the invasive capabilities of MCF7 breast cancer cells, as evidenced by a marked increase in MMP-9 production driven by activated PKC. This study revealed that the increase in MMP-9 was primarily due to *de novo* synthesis within the cancer cells, leading to a threefold increase in their invasion capabilities. Building on these findings, subsequent research focused on prostate cancer cell lines demonstrated that platelets also increase invasion and migration through the up-regulation of mesenchymal markers

such as pro-MMP2 and pro-MMP9. These collective insights highlight the crucial role of platelets in promoting tumor cell aggressiveness across various cancer types[406]. Consistently, platelet-derived PDGF was shown to increase invasion/migration of Cholangiocarcinoma cell line via up-regulation of MMP-2 and MMP-9 expression[445]. Also, direct platelet contact with gastric cancer cell line was demonstrated to induce invasion via up-regulating EMT-related gene expression such as matrix metalloproteinase 9 (MMP9) [446]. Moreover, platelets were shown to induce EMT on HT-29 via diminishing the expression of E-cadherin, up-regulating snail1 expression, and increasing the mRNA level of MMP-2 and MMP-10. Importantly, TRAP-activated platelet /releasate increases invasion potency of SK-OV-3 ovarian cancer cell line by inducing uPA and VEGF secretion by these cells[447].

In a study by Spillane *et al*., they investigated the effect of the platelets on 15 epithelial cell lines from 7 different types of cancer, including breast, cervix, lung, melanoma, ovary, prostate, and thyroid, by measuring the expression of various EMT and stemness-related genes. They reported that at least two EMT-related genes were up-regulated in 13 out of 15 cell lines, suggesting that platelets increase the mesenchymal-like phenotype in these cell lines. Also, by screening a panel of EMT-associated genes, they indicated that platelets induce/enhance mesenchymal phenotype through modulating the expression of 5 essential genes, including TNC, CD73, PAI1, CCL2, and PLEK2 in human and mouse ovarian cancer cells[442].

Additionally, platelets induce EMT through their interaction with cancer cell podoplanin via the C-type lectin-like receptor-2 (CLEC-2)[437, 448]. Moreover, platelets secrete microparticles containing microRNA like miR-939, which upon internalization by ovarian cancer cells, can alter the expression of EMT-related genes, causing a reduction in E-cadherin levels and an increase in mesenchymal markers such as vimentin and fibronectin[449].

In summary, the role of platelets in facilitating epithelial-mesenchymal transition (EMT) underscores a critical aspect of cancer metastasis. Through EMT, platelets empower tumor cells with enhanced motility and invasiveness, enabling them to breach primary tumor boundaries and survive in new environments. This transition is a testament to the intricate ways in which cancer cells manipulate their surrounding elements, like platelets, to promote their own survival and spread. However, the influence of platelets in the journey of a cancer cell extends beyond just initiating EMT. As tumor cells enter the bloodstream, they face challenges, including immune surveillance and the physical stresses of circulation. This protective action of platelets is pivotal for the survival of CTCs in the vascular system, further illustrating the multifaceted role of platelets in the metastatic cascade and the complex interplay between cancer cells and their microenvironment.

1.1.26 Platelets in Tumor Metastasis: A Crucial Role in Immune Modulation and CTC Survival

Tumor metastasis, a key determinant of cancer progression, is inherently a highly inefficient process[437, 450-453]. The majority of CTCs die shortly after entering the bloodstream, confronted by numerous challenges such as anoikis, which is programmed cell death induced upon detachment from the extracellular matrix, blood shear forces, and attacks from the immune system, particularly from cells of the innate immune response [437, 452-455]. Natural Killer (NK) cells, key players in innate immunity, are remarkably effective in eliminating circulating tumor cells (CTCs), as demonstrated in studies where depletion of NK cells led to increased metastatic formations. Platelets play a significant role in this context by forming a protective shield around CTCs, which is crucial for helping these cells evade detection and elimination by NK cells. This

mechanism provides one of the ways tumor cells circumvent the immune surveillance system.[456, 457].

A study by Nieswandt *et al*. showed that platelet depletion reduces metastasis *in vivo*, which was counteracted by NK-cells depletion *in vivo*[456]. One strategy that cancer cells employ to escape CD8+ T-cell cytotoxicity is downregulation of MHC I to avoid tumor-associated antigen presentation to these immune cells[458]. However, MHC-I downregulation sensitizes these cancer cells to NK-cell mediated cytotoxicity. Placke *et al*. observed that platelet confer MHC-I to tumor cells, thereby providing these malignant cells with a 'pseudonormal' phenotype that can evade antitumor activity of NK cells[459]. Platelets store a member of the TNF family glucocorticoid-induced TNF-related ligand (GITRL) in their α-granule. By receiving stimuli, platelet translocate this ligand to their membrane, where it can interact with its receptor, GITR, on NK cells. According to Placke *et al*., accumulation of activated platelet on cancer cells confers these cells a pseudoexpression of GITRL that suppresses NK-cell mediated cytotoxicity and inhibits IFN-γ secretion[460].

Moreover, platelets secrete TGF-β, which diminishes the levels of NKG2D on NK cells, further inhibiting their ability to recognize and attack tumor cells[461]. Active platelet releasates containing sheddases like ADAM10 and ADAM17 induce the shedding of NKG2D ligands on tumor cells, thereby reducing NK cell-mediated recognition. In addition, platelets downregulate critical NK cell receptors, such as CD226 and CD96, thereby impairing efficient NK cell cytotoxicity against tumor cells[462]. In this context, Cluxton *et al*. reported that platelet-derived TGF-β downregulate the expression of CD226/CD155, a signaling axis that mediates prolonged stable contact between NK-cell and cancer cells, therefore impairing efficient NK-cell cytotoxicity against malignant cells[463].

Beyond NK cells, platelets also interact with other immune cells like neutrophils, which can promote both the proliferation and dissemination of tumor cells. Neutrophils, capable of infiltrating primary tumors, can form clusters with some CTCs, leading to an inflammatory interaction that enhances tumor cell proliferation[464-466]. Furthermore, platelets contribute to the trapping of CTCs by neutrophil extracellular traps (NETs), promoting adhesion to the vasculature and subsequent metastasis formation[465, 467]. Labelle *et al*. demonstrated a key sequence of events where the formation of platelet-cancer cell heteroaggregates leads to the initial arrest of these aggregates in the endothelium. Following this arrest, platelets secrete CCL5 and CCL7, which in turn recruit granulocytes in a CXCR-2 dependent manner. This sequence highlights a critical pathway in the interaction between platelets, cancer cells, and the immune system. Therefore, platelet-recruited granulocytes provide a permissive microenvironment for cancer cell extravasation and metastasis called "early metastatic niche" which occurs before monocyte/macrophage recruitment[468].

Platelets play an important role in metastasis by impacting the integrity of the endothelium, thereby aiding the survival and trans endothelial migration (TEM) of circulating tumor cells (CTCs). By carrying and releasing factors that alter the endothelial barrier, platelets facilitate the disruption needed for CTCs to exit the bloodstream. This disruption may include loosening of endothelial junctions, cytoskeletal rearrangements, and induction of endothelial cell apoptosis or necroptosis. These changes increase vascular permeability, allowing CTCs to migrate through the endothelium and invade distant tissues. Schumacher *et al*. reported that ATP secretion from the dense granule of activated platelet by tumor cells promotes CTCs trans-endothelial migration and subsequent metastasis. They further revealed that this effect was mediated through P2Y2 receptor activation on endothelial cells via platelet-derived ATP [469]. Ward *et al*. observed that cancer-

related CD97, a G-protein coupled receptor, causes platelet activation and subsequent ATP secretion, resulting in endothelial barrier disruption and increased vascular permeability. Also, they showed that platelet-derived LPA promotes invasion potency and motility of cancer cells in an LPAR/CD97 dependent manner [470]. In agreement with this finding, secretion of autotaxin (ATX), the enzyme that produces LPA, from α-granule of activated platelet by cancer cells and its interaction with integrin αVβ3 on malignant cells have been shown to promote TEMs and subsequent breast cancer bone metastasis [471, 472]. Furthermore, platelets were shown to enhance 12-HETE biosynthesis by tumor cells, potentiating endothelial cell retraction through PKC-dependent cytoskeletal rearrangement [473-476] (Fig 3.1.1).

Given platelets' established role in shielding circulating tumor cells (CTCs) from natural killer (NK) cells and influencing innate immune responses, it is plausible to consider their potential effects on the adaptive immune system. This speculation is supported by platelets' ability to secrete various immune-modulating factors, which could potentially interact with and influence cells of the adaptive immune response. One key mediator is CD40 Ligand (CD40L), which platelets release in its soluble form to enhance T cell activation and differentiation by interacting with CD40 on T cells and antigen-presenting cells. Another important mediator, TGF-β, suppresses T cell proliferation and activation while enhancing regulatory T cell functions, contributing to immune tolerance.

Additionally, platelets are known to directly interact with lymphocytes, suggesting they could play a role in modulating adaptive immune functions. These interactions and the secretion of specific mediators like CD40L and TGF-β not only facilitate communication with lymphocytes but also potentially modulate their behavior in the context of immune surveillance. Although our current understanding of platelet interaction with adaptive immunity, especially in the context of

cancer, is limited, the evidence of platelet involvement in innate immunity leads to a plausible hypothesis that platelets could similarly protect CTCs from adaptive immune responses. This gap in knowledge presents an important area for future research, as understanding platelet function in the adaptive immune response could reveal new dimensions of their role in cancer progression and potential therapeutic targets.

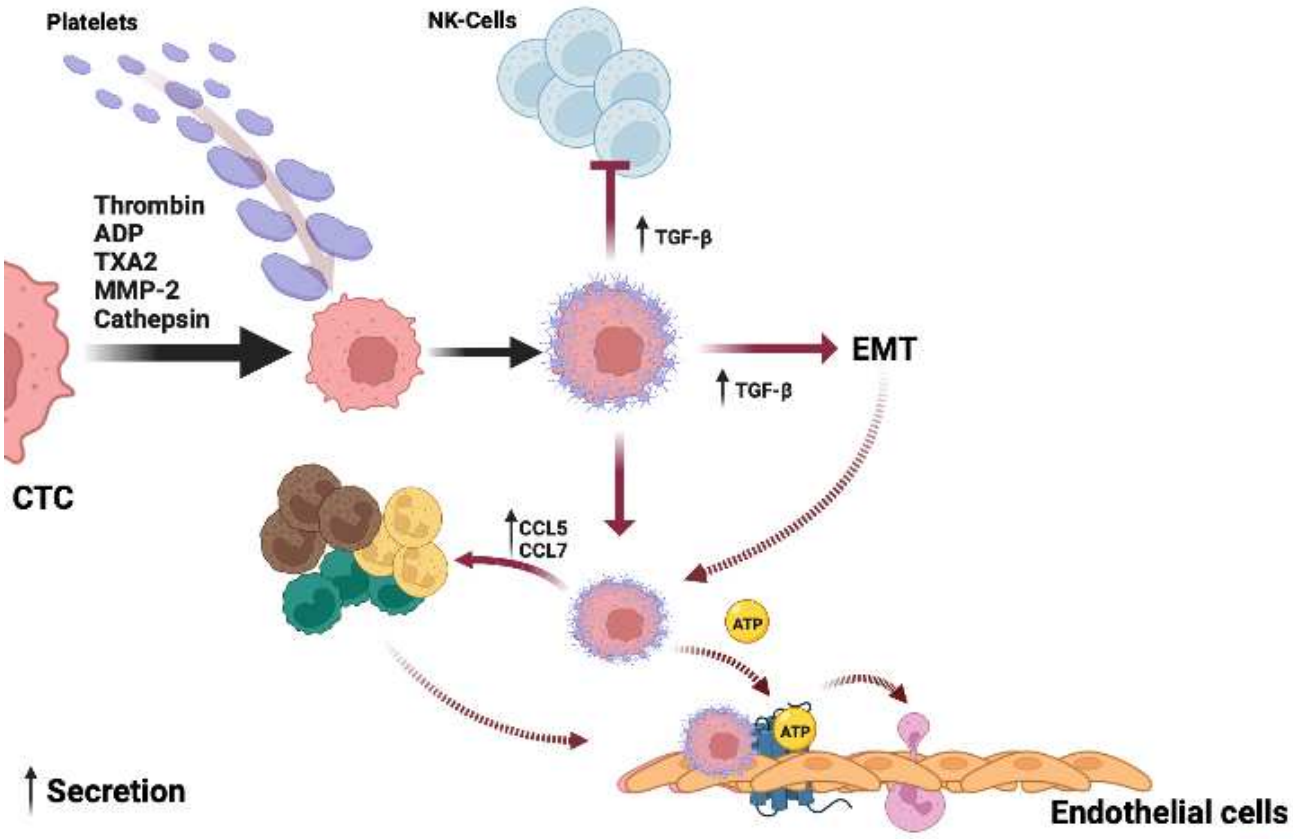


Figure 1.1.6 Interaction between platelet and tumor cells in blood circulation.

Platelets protect CTC against shear stress and immune system response via various mechanisms, including physical shielding, transferring MHC-I, and secretion of Immuno-suppressive cytokines. Also, platelets induce EMT via secreting TGF- β and facilitate cancer cells -endothelial interaction via recruiting granulocyte. Moreover, platelets promote Transendothelial migration of arrested CTC via opening endothelial barrier via releasing ATP. Created with BioRender.com

1.1.27 Programmed Death-Ligand 1(PD-L1)

Immune checkpoints are critical regulators in the immune system, playing a pivotal role in maintaining immune homeostasis and preventing autoimmunity. These checkpoints are essentially molecular brakes that, when engaged, can inhibit or dampen immune responses. They are found on various immune cells, such as T cells and APCs and are crucial for the modulation of adaptive immunity[477].

Immune checkpoints can be broadly classified into two categories: stimulatory and inhibitory. Stimulatory checkpoints boost immune responses, while inhibitory checkpoints, like PD-1 (Programmed cell death protein 1), are crucial in preventing overactive immune responses, thereby maintaining immune tolerance and preventing tissue damage.[478, 479].

PD-L1, a ligand for PD-1, is a member of the B7 co-stimulatory molecule family and is found on the surface of various cell types. Notably, it is expressed on hematopoietic cells like T cells, B cells, and macrophages, as well as on non-hematopoietic cells, including vascular endothelial cells and hepatocytes. The expression of PD-L1 can be significantly upregulated in response to pro-inflammatory cytokines. This upregulation is particularly notable in many types of cancer cells, such as those found in lung, ovarian, colon, and melanoma cancers, indicating a key role in tumor immunology[480-482]. PD-1, a prominent immune checkpoint, plays a significant role in downregulating immune responses and promoting self-tolerance by suppressing T cell inflammatory activity. This mechanism is vital under normal physiological conditions but can be detrimental in the context of cancer and infectious diseases. Tumor cells, for instance, can exploit these checkpoints to evade immune surveillance. They do this by expressing PD-L1, the ligand for PD-1, thereby inhibiting T cell activity and allowing the tumor to grow and spread unchecked[483-489].

In the environment of a tumor, cancer cells often express high levels of PD-L1. This overexpression of PD-L1 is critical in the interaction with the PD-1 receptors located on the surface of tumor-infiltrating lymphocytes (TILs). When PD-L1 binds to PD-1 on these TILs, it transmits immunosuppressive signals that essentially "put the brakes" on the immune response of these cells[490]. This is especially detrimental in the case of tumor-associated antigen-specificCD8+ T lymphocytes, which are pivotal in the immune system's ability to identify and destroy tumor cells. CD8+ T cells are known for their role in directly killing infected or malignant cells, and their inhibition via the PD-1/PD-L1 interaction is a sophisticated means by which tumors evade immune-mediated destruction[491, 492].

PD-L1 expression in cancer cells, a key regulator of immune responses, is upregulated through various intricate mechanisms involving inflammatory cytokines such as IL-6 and Tumor Necrosis Factor-alpha (TNF- α), oncogenic signaling pathways(PI3K/Akt and MAPK pathways), and interactions within the tumor microenvironment (TME)[493-496]. This process significantly contributes to the ability of cancer cells and tumor stromal cells to evade the immune system, a characteristic observed in many types of cancer[478].

Toll-like receptor (TLR) signaling is involved in the upregulation of PD-L1, influencing immune response mechanisms in the cellular environment. This signaling, activated by pathogen-associated molecular patterns, along with the accumulation of substances like NAD+, cAMP, and ROS in the tumor milieu, contributes significantly to the increased expression of PD-L1 [497-499]. Moreover, several oncogenic signaling pathways, including EGFR, MET, ALK, RAS–MEK–ERK (MAPK), and PI3K–AKT–mTOR, are also known to induce PD-L1 expression in various cancer types, highlighting the intersection of oncogenesis and immune regulation[478, 500-503].

In addition to these factors, cytokines such as TGF-β and IL-6 play a pivotal role. TGF-β, produced by different cells within the TME, enhances PD-L1 expression[494, 495, 504, 505]. Similarly, IL-6 contributes to the upregulation of PD-L1 via JAK activation in multiple cancer types and immune cells, underlining the diverse cytokine-mediated pathways influencing PD-L1 expression[506-510].

The IFN-γ pathway stands out among these mechanisms due to its dual role in antitumor immunity and in facilitating immune evasion by tumors. IFN-γ, mainly produced by T cells and natural killer cells, is a key inducer of PD-L1 expression through the JAK–STAT–IRF1 signaling axis. The activation of this axis begins with IFN-γ binding to its receptor, leading to the activation of Janus kinases (JAKs). This activation triggers the phosphorylation and dimerization of Signal Transducer and Activator of Transcription (STAT) proteins, which then translocate to the nucleus. Inside the nucleus, STAT proteins, particularly STAT1, induce the transcription of Interferon Regulatory Factor 1 (IRF1)[511-520].

The STAT pathway, pivotal in this process, is also stimulated by growth factors like VEGF and PDGF, which platelets secrete upon activation. VEGF is a potent angiogenic factor that primarily acts on endothelial cells to promote their proliferation, migration, and survival, all of which are essential steps in angiogenesis[521, 522]. VEGF exerts its effects by binding to its receptors (VEGFRs) on the surface of endothelial cells. This binding triggers a cascade of downstream signaling pathways, including the activation of the STAT proteins. STAT proteins are critical transcription factors that regulate the expression of genes involved in cell survival, proliferation, and angiogenesis. The activation of STAT signaling by VEGF plays a key role in mediating its angiogenic effects[523-525].

Similarly, PDGF is known for its role in regulating cell growth and division, impacting a range of cell types like fibroblasts, smooth muscle cells, and endothelial cells. Upon binding to its receptors (PDGFRs), PDGF activates several signaling pathways, with the STAT pathway being one of the critical routes[521, 526, 527]. Through STAT activation, PDGF influences cell proliferation and migration, essential for wound healing and tissue repair. This pathway is also implicated in various diseases, including fibrosis and atherosclerosis[445, 523-525, 528].

1.1.28 Rationale

The COVID-19 pandemic has underscored the critical need to comprehend complex disease mechanisms, especially those involving the vascular system. One such mechanism is microvascular thrombosis, which plays a significant role in the morbidity and mortality associated with the disease [529]. Identifying and characterizing the molecular pathways that lead to microvascular thrombosis are crucial steps in developing effective treatments. For instance, the role of endothelial dysfunction, platelet activation, and inflammatory responses are key areas of focus. By studying these mechanisms, researchers can identify specific molecules or signaling pathways that can be targeted by therapeutic agents. Platelets play a pivotal role in these processes due to their involvement in thrombus formation and modulation of inflammation[293, 294].

An important negative-feedback mechanism that regulates platelet adhesion, aggregation, and thrombus formation is mediated by nitric oxide (NO), which can be produced by both endothelial cells and aggregating platelets[104-106]. Research has identified that platelets vary in their ability to produce NO based on the presence or absence of endothelial nitric oxide synthase (eNOS), leading to the classification of eNOS-positive and eNOS-negative platelet subpopulations. It has been observed that eNOS-negative platelets are more reactive and more likely to initiate thrombotic reactions, and thrombosis is a critical factor in the pathogenesis of the vascular complications in COVID-19 [245]. However, the mechanisms behind the formation of these eNOS-based platelet subpopulations are not fully understood, even though both eNOS-positive and eNOS-negative subpopulations were initially identified in the megakaryoblastic Meg-01 cell line.

Studies have shown that incubation of Meg-01 cells with cytokines IL-1β and TNF-α reveals a reciprocal relationship between the activities of constitutive and inducible nitric oxide

synthase (NOS) isoforms. This interaction indicates the presence of both isoforms in human bone marrow megakaryocytes and Meg-01 cells [530, 531].Also, this relationship suggests that an increase in inducible NOS (iNOS) expression, coupled with a decrease in constitutive NOS, likely affects eNOS levels due to cellular signaling interference. Specifically, when iNOS expression is upregulated under conditions of inflammation or immune response—triggered by cytokines such as IL-1β and TNF-α—it can lead to several pathways that negatively impact eNOS expression/activity. Similar regulatory patterns have been observed in endothelial cells, where cytokines such as IFNγ and IL-10 counter-regulate eNOS expression[532, 533]. Moreover, some studies suggest that endothelial cells and megakaryocytes share a common ancestral cell during development, potentially making them sister cells with shared signaling pathways[534].

Our current understanding acknowledges the existence of eNOS-positive and eNOS-negative platelet subpopulations. This part of the study aims to determine if and how their ratio changes in COVID-19 patients compared to COVID-19 negative controls. Additionally, our study aims to uncover the underlying mechanisms responsible for these alterations. Specifically, we will investigate whether COVID-19-associated inflammatory cytokines are contributing to these shifts in platelet subpopulation dynamics.

Building on the current understanding of eNOS-based platelet subpopulations, little is known about differences in the granule number or contents of these platelet subpopulations. This is important because platelets serve as an arsenal, storing various bioactive agents that, upon release, play a crucial role in various processes. Specifically, α-granules within platelets store critical growth factors such as Vascular Endothelial Growth Factor (VEGF) [366]. The central role of VEGF in promoting vascular permeability is crucial in the pathogenesis of conditions like endothelial cell barrier disruption and subsequent edema, which are prevalent in severe COVID-

19 cases, highlighting the potential impact of platelets on disease progression[534, 535]. Given that platelets inherit most of their protein content from their progenitor megakaryocytes and considering that studies have shown NF-kB activation in megakaryocytes can increase VEGF production[536], it is plausible that the upregulation of cytokines such as TNF-α, IL-1β, and IL-6 in COVID-19 could lead to enhanced VEGF content within platelets. These cytokines are known to activate NF-kB, suggesting that inflammatory conditions in COVID-19 could indirectly influence VEGF content within platelets through cytokine-mediated NF-kB activation in megakaryocytes[537-542]. Of important note, hypoxemia and tissue hypoxia often associated with severe COVID-19 can further hypoxia-inducible factors (HIFs) and increase VEGF expression within megakaryocytes and subsequently platelets[372-374]. Preliminary data suggested that eNOS-positive platelets are more granular; however, it is unknown whether platelets constitute a novel subpopulation enriched with α-granules/growth factors. This study aims to characterize these platelets and determine if they undergo changes under inflammatory conditions, using COVID-19 as a model. Additionally, this section of the thesis explores the mechanisms that regulate the vascular endothelial growth factor (VEGF) content in platelets.

As platelets have been identified as crucial players in inflammatory and immune response associated with COVID-19, their involvement extends beyond viral infections to include other realms of inflammatory pathology: cancer. Platelets are known to play a pivotal role in hematogenous metastasis, primarily through mechanisms such as tumor cell-induced platelet aggregation (TCIPA) [399]. This interaction not only promotes metastasis by secreting factors that create a favorable microenvironment for cancer cells but also protects them from the innate immune system, particularly from natural killer (NK) cell-mediated cytotoxicity[459, 460, 462, 543]. This protection is facilitated through several pathways, including physical shielding by

platelet coats, modulation of immune receptors through membrane-bound and soluble factors, and the suppression of NK cell activity via specific ligand-receptor interactions[459, 460, 462, 543].

Despite extensive research into how platelets affect innate immunity, their role in modulating the adaptive immune system, particularly in cancer, is less understood. A significant regulator of the adaptive immune response is Programmed Death Ligand 1 (PD-L1), a transmembrane protein that inhibits T-cell activation and proliferation by interacting with its receptor, PD-1, on T-cells. This interaction results in reduced T-cell efficacy and anergy, a mechanism exploited by several cancers to evade immune surveillance[481, 482]. Platelets, which are rich in growth factors like vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) that can stimulate signal transducer and activator of transcription (STAT) signaling pathways, factors that are known to increase PD-L1 expression in response to factors like IFN-γ[519, 520] [481, 519, 520].

Given this background, this section of the thesis aims to investigate whether platelets, known to secrete factors that can increase PD-L1 expression, up-regulate PD-L1 in cancer cells thereby promoting immunoevasion from adaptive immunity. Additionally, this part of thesis investigates whether common anti-platelet drugs can counteract or neutralize the platelet-mediated up-regulation of PD-L1 on cancer cells, thereby restoring the adaptive immune response against these cells.

1.1.29 Hypotheses

1. Increased platelet reactivity in COVID-19 patients is associated with elevated eNOS-negative to eNOS-positive platelet ratios, and to determine if these elevated ratios result from the action of inflammatory cytokines on megakaryocytes/blasts. This elevated ratio occurs as a result of the action of inflammatory cytokines on megakaryocytes/blasts.
2. A novel growth factor/α-granule-enriched platelet subpopulation exists and is up-regulated in COVID-19 due to the strong inflammatory conditions associated with the infection
3. Upon platelet activation, Secreted Vascular endothelial growth factor (VEGF) and Platelet-derived growth factor (PDGF) increase PD-L1 expression on cancer cells, therefore promoting cancer cell immunoevasion potency

1.1.30 Objectives

1. To determine and compare the ratio of eNOS-negative to eNOS-positive platelets in COVID-19 patients (ICU and non-ICU) and COVID-19-negative healthy control.
2. To determine plasma concentration of inflammatory and anti-inflammatory cytokine including IFN-γ, TNF-α, IL-6, IL-1β, and IL-10.
3. To determine plasma concentration of total nitrite/nitrate ($NO2^-$ and $NO3^-$) COVID-19 COVID-19 patients (ICU and non-ICU) and COVID-19-negative healthy control.
4. To determine whether DAF-FM-negative and -positive Meg-01 cells correspond to eNOS-negative and –positive Meg-01, respectively.
5. To determine whether eNOS-negative Meg-01 cells exist at the mRNA level.
6. To determine whether inflammatory cytokines known to down-regulate eNOS expression (TNF-α, IL-6, and IL-1β) decrease eNOS expression in megakaryocyte/blast and promote the formation of eNOS-negative megakaryocytes/blasts.
7. To determine if there exists a novel growth factor/α-granule-enriched subpopulation within platelets.
8. To characterize VEGF and PDGF expression in eNOS-positive and eNOS-negative platelets.
9. To characterize VEGF expression in isolated platelets from COVID-19 patients and COVID-19 negative controls.
10. To measure plasma concentration of TNF-α in COVID-19 patients and COVID-19 negative controls.
11. To characterize the effect of TNF-α on VEGF expression in Meg-01 cell line.

12. To determine whether platelets increase PD-L1 expression on cancer cells (A549 cell line and 786-O cell line).
13. To determine whether the increased platelet-induced PD-L1 surface expression on cancer cells occurs due to transcriptional expression or whether this surface increase simply results from cancer cell-bound platelets.
14. To identify platelet-derived growth factors that promote cancer cell PD-L1 expression.
15. Evaluating the effect of anti-platelet drugs on platelet-induced PD-L1 expression by cancer cells.
16. To determine the effect of platelet-mediated PD-L1 expression by cancer cells on primary T-cell and Jurkat cells activation.

Chapter 2

COVID-19 Promotes Generation of Pro-thrombotic eNOS-Negative Platelets: Potential Role of Inflammatory Response

Part of this chapter is submitted for Publication in:

Amir Asgari, Aleksandra Franczak, Alex Herchen, Glen Jickling, and Paul Jurasz. COVID-19 Promotes Generation of Pro-thrombotic eNOS-Negative Platelets: Potential Role of Inflammatory Response. Under revision by Thrombosis Research journal.

Abstract

Background. Platelet-rich microvascular thrombi are common in severe COVID-19. Endogenous nitric oxide (NO)-signaling limits thrombus formation and previously we identified platelet subpopulations with a differential ability to produce NO based on the presence or absence of endothelial nitric oxide synthase (eNOS). eNOS expression is counter-regulated by cytokines, and recent reports demonstrate that COVID-19-associated immune/inflammatory responses affect the transcriptome profile of megakaryocytes and their platelet progeny.

Objectives: We investigated whether the ratio of eNOS-negative to –positive platelets increases in COVID-19 patients and whether this change may be due to the actions of pro-inflammatory cytokines on megakaryocytes.

Methods: Platelets were isolated from hospitalized COVID-19 patients and COVID-19-negative controls. Platelet eNOS was measured by flow cytometry and plasma inflammatory cytokines by electrochemiluminescence immunoassay (ECLIA). Megakaryocytes from eNOS-GFP transgenic mice and the Meg-01 cell line were characterized to identify an appropriate model to study eNOS-based platelet subpopulation formation in response to inflammatory cytokines.

Results: COVID-19 patients demonstrated significantly elevated ratios of eNOS-negative to -positive platelets compared to controls and their ratios correlated with disease severity. Higher eNOS-negative to –positive platelet ratios were associated with enhanced platelet reactivity as measured by surface CD62P. Accordingly, COVID-19 patients demonstrated higher TNF-α, IL-6, and IL-1β plasma concentrations than controls. Inflammatory cytokines associated with COVID-19 promoted eNOS-negative Meg-01 formation and enhanced subsequent eNOS-negative platelet-like particle formation.

Conclusions: COVID-19 patients have a high eNOS-negative to –positive platelet ratio, likely as a result of inflammatory response reducing megakaryocyte/blast eNOS expression, which predisposes them to thrombosis.

Key words

COVID-19, platelets, thrombosis, nitric oxide synthase, inflammatory cytokines

2.1 Introduction

Coronavirus disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has infected over 774 million people worldwide with more than 7 million global deaths [544-546]. Although COVID-19 is mainly characterized as a disease with respiratory system complications such as lung injury and acute respiratory distress syndrome, microvascular thrombosis is also a key pathological feature of severe COVID-19 [547]. Additionally, venous and arterial thrombotic events are also common which may contribute to organ failure and mortality [291, 292]. Platelets are major components of microvascular thrombi associated with COVID-19 and in addition to forming occlusive thrombi their role in inflammation is well documented [293, 294].

An important negative-feedback pathway that limits platelet adhesion, aggregation, and thrombus formation is mediated by nitric oxide (NO), which may be generated by both endothelial cells and aggregating platelets themselves [104-106]. Previously, we reported on platelet subpopulations with differential abilities to produce NO based on the presence/absence of endothelial nitric oxide synthase (eNOS-positive and eNOS-negative platelets) revealing that eNOS-negative platelets are more reactive than eNOS-positive platelets and that they initiate thrombotic reactions [245]. However, It is not known how these different eNOS-based platelet subpopulations arise, although initially both eNOS-positive and –negative subpopulations have been identified in the megakaryoblastic Meg-01 cell line. Constitutive and inducible isoforms of NOS have also been found in human bone marrow megakaryocytes and in Meg-01 cells [530, 531], and treatment of Meg-01 with IL-1β and TNF-α revealed a reciprocal relation between constitutive and inducible NOS activity, consistent with an increase in iNOS expression and a down-regulation of constitutive NOS expression likely corresponding to eNOS. These findings are

also consistent with those observed in endothelial cells wherein pro-inflammatory and anti-inflammatory cytokines such as IFNγ and IL-10 counter-regulate eNOS expression [532, 533].

Therefore, we hypothesized that increased platelet reactivity in COVID-19 patients may be associated with elevated eNOS-negative to eNOS-positive platelet ratios, and that these elevated ratios occur as a result of the action of inflammatory cytokines on megakaryocytes/blasts.

2.2 Material and Methods

2.2.1 Reagents

Prostacyclin, propidium iodide (PI), 5-azacytidine, human recombinant interleukin-10 and human recombinant interferon-γ, human recombinant thrombopoietin (TPO), L-Arginine, N-nitro-L-arginine methyl ester hydrochloride (L-NAME), Tyrode's buffer, goat serum, human plasma fibrinogen and Eptifibatide acetate were obtained from Millipore-Sigma (Ontario, Canada). Human recombinant interleukin-6, TNF-α, and interleukin-1β were purchased from Thermo Fisher Scientific (California, USA). Alexa Fluor™ 488 conjugated goat anti-mouse IgG (cat # A11001), PE-conjugated goat anti-rat IgG (cat # A10545), iNOS monoclonal antibody (clone CXNFT, cat #14-5920-82), 4-Amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM)-diacetate, and CellTracker™ Green CMFDA Dye, RNase A and Lipofectamine 2000 were purchased from Thermo Fisher Scientific (IL, USA). V-PLEX human proinflammatory panel I (cat # K15052D-1) was purchased from Meso Scale Diagnostics (Maryland, USA). Anti-human CD42b-PE (clone HIP1, cat # 555473), mouse IgG1-PE (clone MOPC-21, cat # 556650), BV421 Mouse Anti-Human CD62P (Clone AK-4, cat # 564037) were purchased from BD Biosciences (Ontario, Canada). Sensiscript RT Kit, RNeasy mini kit was ordered from Qiagen (Ontario, Canada). Antibodies recognizing human eNOS at the C- (clone M221, cat # ab76198) and N- (clone 6H2, cat # ab91205) terminals, mouse IgG1 isotype control (clone ICIGG1, cat # ab91353) were obtained from Abcam (MA, USA). EBM-2 Basal Medium, EGMTM-2 MV Microvascular Endothelial Cell Growth Medium SingleQuots supplements and hydrocortisone were purchased from Lonza (Walkersville, MD, USA). Rat anti-mouse CD41-PerCP/Cy5.5 conjugated antibody (MWReg30 clone) and rat PerCP/Cy5.5 IgG1 isotype control were obtained from Biolegend (San Diego, CA, USA).

Measuring plasma concentration of NOx. To measure the concentration of total nitrite/nitrate (plasma NO2- and NO3-) in plasma of COVID-19 positive and COVID-19 negative controls, the Griess assay was performed according to the manufacturer's protocol (Cayman Chemicals, USA).

2.2.2 Participant Recruitment and Platelet Isolation

The study was approved by the UAlberta Health Research Ethics Board (Pro100563). Fixed platelet samples and frozen archived EDTA plasma samples from de-identified hospitalized qPCR-confirmed COVID-19 patients (n = 25) were obtained from the Canadian BioSample Repository (CBSR, UAlberta) as part of the COVID-19 Surveillance Collaboration study (Pro00100207). Platelets and plasma were obtained from non-ICU (n=12) hospitalized COVID-19 patients throughout January 2021 and ICU (n=13) hospitalized COVID-19 patients throughout September 2021. Following informed consent venous blood samples were collected from control volunteers (n = 11) between November 3, 2021 and December 17, 2021. Venous blood was collected into tubes with EDTA (Monoject Covidien, cat. no. 8881311446) and then prostacyclin (0.075 μg ml-1) was added to whole blood in an EDTA tube, followed by centrifugation at 250g for 5 minutes to isolate the platelet-rich plasma (PRP). Next, prostacyclin (0.3 μg ml-1) was added to PRP, and platelets were pelleted at 900g for 5 minutes. Finally, platelet pellets were resuspended in the fixation buffer (Tyrode's buffer containing 4% paraformaldehyde) for further analysis.

Additionally, following informed consent nasal-mid-turbinate specimens (to confirm COVID-19-negative status) were collected from control participants at time of blood draw. Nasal mid-turbinate specimens were collected using flocked nylon swabs and stored in a viral transport medium (HBSS, 2% FBS, 100μg/ml gentamicin, 0.5μg/ml amphotericin B) at -80°C until analysis, according to the Interim Guidelines for Collecting and Handling of Clinical Specimens for

COVID-19 Testing (Centers for Disease Control and Prevention, CDC, United States). Total RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen), and cDNA was synthesized using Sensiscript® Reverse Transcriptase Kit (Qiagen). Then, PCR was performed using a Bio-Rad S1000 Thermal Cycler with 2019-nCoV_N1 primer pair (Forward - GAC CCC AAA ATC AGC GAA AT and Reverse - TCT GGT TAC TGC CAG TTG AAT CTG), and RNAse P primer pair (Forward - AGA TTT GGA CCT GCG AGC G and Reverse - GAG CGG CTG TCT CCA CAA GT) as endogenous nucleic acid extraction control. Primers were synthesized by Thermo Fisher Scientific, United States. For positive control, 2019-nCoV_N Positive Control plasmid (Integrated DNA Technologies) was used. Finally, PCR products were separated on a 1% agarose gel and visualized following SYBR™ Safe staining (Thermo Fisher) using a Bio-Rad VersaDoc MP5000 molecular imager.

2.2.3 Detection of Cellular eNOS by Flow Cytometry

Cells (platelets, Meg-01 cells) were fixed in 4% paraformaldehyde in Tyrode's buffer for 20 minutes at room temperature and centrifuged at 900g for 10 minutes to pellet cells. The paraformaldehyde solution was removed and discarded, and cells were washed (3x) and resuspended in the wash buffer (0.3% BSA in a PBS buffer with 0.05% Tween 20). Samples were then permeabilized using 0.1% Triton X-100 in PBS buffer for 10 minutes at room temperature on a sample rotator and then centrifuged at 900g for 10 minutes to pellet cells. The triton X-100 solution was discarded, and cells were washed and centrifuged (3x 900g for 10 minutes) and resuspended in wash buffer. Next, the samples were blocked with blocking buffer (5.0% goat serum in PBS buffer with 0.05% Tween 20) for 2 hours at room temperature on a sample rotator. Subsequently, samples were centrifuged at 900g for 10 minutes, the blocking buffer was discarded,

and cells were resuspended in the wash buffer. Next, blocked platelets or Meg-01 were incubated with primary antibodies: mouse anti-eNOS clones (M221 at 1.25 μg/ml or 6H2 at 5 μg/ml) or concentration-matched isotype controls. at room temperature. In some experiments platelets or Meg-01 were incubated with anti-iNOS antibody (clone CXNFT) or concentration-matched isotype control for 1 hour (10 μg/ml). The samples were washed with wash buffer (3x) and incubated with secondary antibodies Alexa Fluor 488-conjugated goat F(ab')2 fragments anti-mouse IgG (15 μg/ml) and PE-conjugated goat anti-rat IgG (10 μg/ml) for 1 hour in the dark at room temperature. Meg-01 were then washed (3x) and resuspended in a PBS buffer and flow cytometry was performed using Quanta SC (Beckman Coulter) or a LSRFortessa X-20 (Becton Dickinson) flow cytometers. For analyzing platelets, anti-CD42b-PE antibody (1:100) was added to the sample and incubated for 15 minutes, then diluted to a final volume of 1 ml with PBS buffer before flow cytometry analysis using the LSRFortessa X-20 (Becton Dickinson) flow cytometer.

2.2.4 Meg-01 Cell Culture and Platelet-Like Particle Generation

The human megakaryoblastic cell line, Meg-01, was purchased from ATCC and cultured at 37 °C in a humidified atmosphere with 5% CO2 in 90% RPMI-1640 with gentamicin (0.05 mg mL-1), penicillin (0.06 mg mL-1), streptomycin (0.01 mg mL-1), and 10% fetal bovine serum (FBS). The cells were supplied with fresh medium and subcultured three times each week. To generate platelet-like particles (PLPs) the Meg-01 were grown in T25 flasks using 5 ml of full medium for a duration of 8 days. To induce PLP generation, Meg-01 were treated every 48 hours with 100 ng/ml of human thrombopoietin (TPO). Simultaneously, every 48 hours, the cells also received treatments with the cytokines IFN-γ and IL-10 or TNF- α, IL-6, and IL-1β. Then, the Meg-01 and generated PLPs were centrifuged at 200 g for 10 minutes to pellet Meg-01. Next, the

PLP containing upper layer was centrifuged at 1800 g for 20 minutes. The supernatant was discarded, and the PLP pellet was washed once with Wash Buffer (0.3% BSA in PBS containing 0.05% Tween 20) and centrifuged at 10,000g for 5 minutes after the wash. The generated PLPs were then stained for eNOS expression and analyzed as by flow cytometry as described above [245].

2.2.5 eNOS Knock-Down in Meg-01

Human NOS3 (eNOS) or scrambled siRNA (1μM) each (Horizon Discovery, Canada) were transfected into Meg-01 cells using Lipofectamine 2000 reagent (Thermo Fisher Scientific, USA) and incubated for 3 days. After 3 days, eNOS expression and NO production were assessed using flow cytometry as described above.

2.2.6 Fibrinogen Adhesion Assay

Microscope coverslips were pre-coated with human plasma fibrinogen (at a concentration of 10 μg/ml) and left to incubate overnight at 4°C. Meg-01 generated PLPs were stained with CMFDA at 5 μM concentration (5-Chloromethylfluorescein diacetate, Thermo Fisher Scientific) for 30-minute at 37°C. Subsequently, an equal number of CMFDA-stained PLPs (5 × 105/100μl) were added to fibrinogen-coated coverslips and incubated for 1 hour at 37°C. The adhered PLPs were imaged using a spinning disk confocal microscope (WaveFX, Olympus IX-81), and the number of PLPs were quantified using Image J.

2.2.7 Measuring Plasma Concentration of NOx

To measure the concentration of total nitrite/nitrate (plasma NO2- and NO3-) in plasma of COVID-19 positive and COVID-19 negative controls, the Griess assay was performed according to the manufacturer's protocol (Cayman Chemicals, USA).

2.2.8 Assessing Plasma Concentration of Inflammatory and Anti-Inflammatory Cytokines

For measuring plasma concentration of IFNγ, TNF-α, IL-6, and IL-1β in the plasma of COVID-19 positive and COVID-19 negative controls, multiplex ECLIA (V-PLEX Human Proinflammatory Panel I (4-Plex), cat # K15052D) was performed based on the manufacturer's protocol (Meso Scale Diagnostics, USA). Additionally, IL-10 plasma concentration was evaluated using Human IL-10 Quantikine ELISA kit (R&D Systems, USA).

2.2.9 Staining Meg-01 Cells with DAF-FM

Meg-01 cells, totalling 5 × 105, were counted and subsequently centrifuged at 130g for 7 minutes at room temperature. Following centrifugation, the medium was discarded, and the cells were resuspended in PBS. This resuspension and centrifugation process at 130g for 7 minutes was repeated twice to ensure thorough washing. The cells were then resuspended in 500 µl of PBS and DAF-FM dye was added to the cells, achieving a final concentration of 10 µM. The cells treated with DAF-FM were incubated for 30 minutes at 37°C in the dark. After the incubation period, the stained cells were washed twice with PBS buffer to remove excess dye. Subsequently, the cells were resuspended in 500 µl of PBS. The final preparation of stained cells was then analyzed using the LSRFortessa™ X-20 flow cytometer (Becton Dickinson).

2.2.10 Flow RNA for Assessing eNOS and iNOS mRNA Expression

For assessing eNOS and iNOS mRNA expression in Meg-01 cells PrimeFlow RNA assay protocol from Thermo Fisher Scientific was followed. In brief, initially, 1 × 106 cells are counted and suspended in PBS, followed by centrifugation at 130g for 7 minutes at room temperature. This step precedes the fixation process, where Fixation Buffer 1 is prepared by blending equal volumes of PrimeFlow RNA Fixation Buffer 1A and 1B, resulting in 1 mL of mixture per sample. This mixture is then added to each cell sample, which is subsequently incubated at 4°C for 30 minutes. A centrifugation at 800g for 5 minutes at 4°C follows, after which the supernatant is discarded, leaving the cells in a minimal volume.

The permeabilization phase involves preparing a 1X PrimeFlow RNA Permeabilization Buffer with RNase Inhibitors. This is achieved by diluting the 10X PrimeFlow RNA Permeabilization Buffer to a 1X concentration with RNase-free water and adding PrimeFlow RNase Inhibitors at a 1/100 dilution. The cells are treated with 1 mL of this permeabilization buffer and incubated for 20 minutes at 4°C. After incubation, the cells are centrifuged at 800 x g for 5 minutes at 4°C, and the supernatant is discarded. This permeabilization step is repeated to ensure optimal cell membrane permeability, with a total incubation time of 45 minutes.

Following permeabilization, a secondary fixation is conducted using PrimeFlow RNA Fixation Buffer 2. This involves mixing 125 µL of PrimeFlow RNA Fixation Buffer 2 (8X) with 875 µL of PrimeFlow Wash Buffer for each sample, followed by an incubation for 60 minutes in the dark at room temperature. The cells are then centrifuged at 800 x g for 5 minutes at room temperature, and the supernatant is aspirated, leaving approximately 100 µL of buffer.

For the hybridization of target probes, specific thawed probes (20X) for eNOS(5µl) and iNOS (5 µl) are added to the cell suspension along with Target Probe Diluent to reach a final volume of 100 µL. This mixture is incubated for 2 hours at 40°C, with a mid-point inversion to ensure even mixing. Following incubation, 1 mL of PrimeFlow RNA Wash Buffer is added, and the cells are centrifuged at 800 x g for 5 minutes at room temperature. The wash step is designed to remove unbound probes, and the supernatant is aspirated, leaving a small volume for resuspension. Next, cells were resuspended in 1 mL of PrimeFlow RNA Wash Buffer containing RNase Inhibitors (100X) and then centrifuged at 800 x g for 5 minutes at room temperature, and the supernatant is aspirated, leaving approximately 100 µL of buffer.

The amplification process involves two main steps: pre-amplification and amplification, each followed by specific washing protocols to further purify the sample. For pre-amplification, 100 µL of PrimeFlow RNA PreAmp Mix is added to the cell suspension, which is incubated for 1.5 hours at 40°C. After this incubation, the cells are washed three times with PrimeFlow RNA Wash Buffer to eliminate any unbound PreAmp Mix. Each wash involves adding 1 mL of Wash Buffer, centrifuging at 800 x g for 5 minutes at room temperature, and aspirating the supernatant down to approximately 100 µL.

Following pre-amplification, the amplification phase employs 100 µL of PrimeFlow RNA Amp Mix for each sample, with a subsequent 1.5-hour incubation at 40°C. Post-incubation, the cells are subjected to two washes under the same conditions used after pre-amplification to remove excess Amp Mix and ensure that only specifically bound amplification products remain.

For the labeling phase, after diluting PrimeFlow RNA Label Probes 1/100 in Label Probe Diluent, 100 µL of this solution is added to each cell suspension, and the samples are incubated for 1 hour at 40°C. Following the incubation, two final washes are conducted with PrimeFlow

RNA Wash Buffer to remove any unbound label probes. Each wash consists of adding 1 mL of Wash Buffer, centrifuging at 800 x g for 5 minutes at room temperature, and carefully aspirating the supernatant to leave about 100 µL. The cells are then washed with 1ml of PBS and resuspended in 500 µL of this buffer. Finally, samples are ready for analysis by LSRFortessa™ X-20 flow cytometry.

To demonstrate the specificity of the target probes for eNOS and iNOS, an additional step was incorporated after the final fixation process. RNase A, at a concentration of 50 µg/ml, was introduced to digest the total RNA within Meg-01 cells. This digestion was carried out at a temperature range of 4°C overnight. Subsequently, the standard protocol, as previously detailed, was resumed the following day to detect eNOS and iNOS.

2.2.11 Fluorescence Activated Cell Sorting (FACS)

For separating viable NO-producing from non-NO-producing Meg-01 cells, fluorescence-activated cell sorting (FACS) was performed. Meg-01 cells were washed (2x) in PBS buffer. Next, DAF-FM diacetate (10 µM) and Proprium Iodide (PI) were added to the cell suspension in PBS and incubated for 30 minutes at 37°C in the dark. Subsequently, stained cells were washed (2x) with PBS buffer. Cells were resuspended in phenol red-free RPMI and viable NO-producing from non-NO-producing Meg-01 cells were sorted by FACS Aria III (Becton Dickinson).

2.2.12 Statistics

Statistics were performed using GraphPad Prism 8 software, and all means are reported with SEM. Two-tailed Student's T-tests and Ordinary and Repeated Measures One-way ANOVA with either Dunnett's multiple comparisons test or Tukey's multiple comparison test were

performed where appropriate. A *P*-value less than 0.05 was considered as significant. All reported Ns are independent experiments consisting of platelets obtained from different blood donors and different cell line passage numbers.

2.3 Results

2.3.1 COVID-19 Increases the Ratio of eNOS-Negative to eNOS-Positive Platelets

COVID-19 patients were sampled upon hospital admission and their characteristics and those of COVID-19 negative controls are given in Table 2.3.1. Overall, study participant groups were well matched except for a small but significantly increased incidence of prior anxiety/depression within the COVID-19 non-ICU patient group. Importantly, there was no significant difference in use of anti-platelet drugs such as acetylsalicylic acid between COVID-19 non-ICU and ICU patient groups (Table 2.3.2). The percentage of eNOS-positive platelets within the blood of both ICU and non-ICU COVID-19 patients was significantly lower than that of COVID-19 negative controls (COVID-19 ICU 19.2 ± 2.8% vs. COVID-19 non-ICU 34.7 ± 3.5% vs. COVID-19 negative controls 93.5 ± 1.3%, P-value < 0.0001; Fig. 2.3.1A & B). Accordingly, the percentage of eNOS-negative platelets of COVID-19 patients (non-ICU and ICU) was also significantly higher than that of the controls and these levels correlated with disease severity as based on ICU admission (COVID-19 ICU 81.2 ± 2.8% vs. COVID-19 non-ICU 66.0 ± 3.1% vs. COVID-19 negative controls 6.1 ± 1.3% controls, P-value < 0.0001; Fig. 2.3.1A & C). Interestingly, the increase in percentage of eNOS-negative platelets in COVID-19 was also associated with reduced eNOS content within the eNOS-positive subpopulation as evident by the leftward shift of the eNOS-positive peak (Fig. 2.3.1A & D). Subsequently, plasma nitrate and nitrite (NOx) levels were measured as markers of circulating NO levels. COVID-19 ICU patients were found to have significantly decreased NOx levels relative to COVID-19 negative controls (COVID-19 ICU 0.4 ± 0.03 μM vs. COVID-19 negative controls 0.55 ± 0.05 μM) (Fig. 2.3.1E),

and a trend toward lower NOx levels in non-ICU patients compared to COVID-19 negative controls was also observed (COVID-19 Non-ICU 0.49 ± 0.03 μM vs. COVID-19 negative controls 0.55 ± 0.05 μM). Importantly, consistent with an elevated ratio of eNOS-negative to –positive platelets and reduced plasma NO metabolites, ICU COVID-19 patients demonstrated increased platelet reactivity as measured by a significantly higher percentage of surface CD62P-positive platelets (Fig. 2.3.1F), although non-ICU platelets were unavailable for CD62P analysis. Importantly, potent in vitro activation of control platelets from healthy individuals did not increase the percentage of eNOS-negative platelets demonstrating that changes in eNOS levels of COVID-19 patients were likely not due to its loss upon activation (Fig. 2.3.2A and B).

Having previously demonstrated that eNOS-negative platelets are not platelets undergoing cell death [245], we investigated whether the elevated ratio of eNOS-negative to eNOS-positive platelets in COVID-19 could be due to the counter-regulation of megakaryocyte/blast eNOS-expression by pro- and anti-inflammatory cytokines. Therefore, the plasma concentrations of inflammatory and anti-inflammatory cytokines known to regulate the transcriptional expression of eNOS were measured including IFNγ, TNF-α, IL-6, IL-1β, and IL-10 in COVID-19 patients (ICU and non-ICU) and COVID-19 negative controls. Consistent with a COVID-19 associated inflammatory response plasma concentrations of TNF-α, IL-6, and IL-1β, were significantly higher in COVID-19 patients compared to COVID-19 negative controls, as was the immunomodulatory cytokine IL-10, but not IFNγ (Fig. 2.3.3A-E).

Table 2.3.1 Clinical demographics of study participants

Characteristic	COVID-19 negative controls (N =11)	COVID-19 Non-ICU Patients (N = 12)	COVID-19 ICU Patients (N = 13)	*P*-value
Age (years)	59.6+14.1	67.6±16.6	53.3±15.2	0.0815
Male	7/11 (63.6%)	9/12 (75.0%)	8/13 (61.5%)	0.7504
Immunology/Allergy	0/11 (0.0%)	3/12 (25.0%)	1/13 (7.7%)	0.1442
Asthma	0/11 (0.0%)	0/12 (0.0%)	1/13 (7.7%)	0.4026
Chronic Obstructive Pulmonary Disease (COPD)	0/11 (0.0%)	1/12 (8.3%)	1/13 (7.7%)	0.6260
Obstructive sleep apnea	1/11 (9.1%)	1/12 (8.3%)	1/13 (7.7%)	0.9924
Diabetes	0/11 (0.0%)	4/12 (33.3%)	5/13 (38.5%)	0.0683
Stroke (TIA/CVA)	0/11 (0.0%)	0/12 (0.0%)	1/13 (7.7%)	0.4026
Myocardial Infarction (MI)	0/11 (0.0%)	1/12 (8.3%)	1/13 (7.7%)	0.6260
Coronary Artery Disease (CAD)	1/11 (9.1%)	2/12 (16.7%)	1/13 (7.7%)	0.7504
History of Coronary Artery Bypass Graft (CABG)	1/11 (9.1%)	1/12 (8.3%)	0/13 (0.0%)	0.5479
History of Percutaneous Coronary Intervention (PCI)	0/11 (0.0%)	0/12 (0.0%)	0/13 (0.0%)	>0.9999
History of Cardiac Surgery	0/11 (0.0%)	1/12 (8.3%)	0/13 (0.0%)	0.3575
Heart Failure	0/11 (0.0%)	1/12 (8.3%)	0/13 (0.0%)	0.3575
Valve Disease	0/11 (0.0%)	1/12 (8.3%)	0/13 (0.0%)	0.3575
Hypertension	3/11 (27.3%)	4/12 (33.3%)	4/14 (30.8%)	0.9513
Atrial Fibrillation	0/11 (0.0%)	0/12 (0.0%)	1/13 (7.7%)	0.4026
Dyslipidemia	1/11 (9.1%)	2/12 (16.7%)	3/13 (23.1%)	0.6573
Chronic Kidney Disease	0/11 (0.0%)	1/12 (8.3%)	1/13 (7.7%)	0.6260
Liver Disease	0/11 (0.0%)	1/12 (8.3%)	0/13 (0.0%)	0.3575
Cancer	0/11 (0.0%)	3/12 (25.0%)	1/13 (7.7%)	0.1442
Anxiety/Depression	0/11 (0.0%)	3/12 (25.0%)	0/13 (0.0%)	**0.0379**
History of Solid Organ Transplant	0/11 (0.0%)	0/12 (0.0%)	2/13 (15.4%)	0.1536
HIV Positive	0/11 (0.0%)	0/12 (0.0%)	0/13 (0.0%)	>0.9999
Immunocompromised	0/11 (0.0%)	1/12 (8.3%)	1/13 (7.7%)	0.6260
Tonsillectomy	1/11 (9.1%)	2/12 (16.7%)	1/13 (7.7%)	0.7504

Table 2.3.1. Data are presented as mean ± SEM or frequencies (%), unless stated otherwise. Comparisons were performed by using the ANOVA test or Chi-squared test/Fisher's exact test. Samples missing due to incomplete questionnaire data collection are not included in the analyses.

Table 2.3.2. Frequently administered & relevant medications of study participants

Medication	COVID-19 Non-ICU Patients (N = 12)	COVID-19 ICU Patients (N = 13)	*P*-value
Acetaminophen	0/12 (0.0%)	5/13 (38.5%)	**0.0391**
Acetylsalicylic acid	0/12 (0.0%)	2/13 (15.4%)	0.4800
Azithromycin	7/12 (58.3%)	9/13 (69.2%)	0.6882
Dexamethasone	5/12 (41.7%)	8/13 (61.5%)	0.4338
Electrolyte solution	1/12 (8.3%)	6/13 (46.2%)	0.0730
Fentanyl	1/12 (8.3%)	3/13 (23.1%)	0.5930
Furosemide	0/12 (0.0%)	6/13 (46.2%)	**0.0149**
Hydromorphone	1/12 (8.3%)	6/13 (46.2%)	0.0730
Insulin	2/12 (16.7%)	1/13 (7.7%)	0.5930
Ipratropium	0/12 (0.0%)	4/13 (30.8%)	0.0957
Lidocaine	2/12 (16.7%)	2/13 (15.4%)	>0.9999
Magnesium Sulfate	2/12 (16.7%)	1/13 (7.7%)	0.5930
Midazolam	1/12 (8.3%)	6/13 (46.2%)	0.0730
Norepinephrine	2/12 (16.7%)	5/13 (38.5%)	0.3783
Pantoprazole	2/12 (16.7%)	7/13 (53.8%)	0.0968
Polyethylene glycol	0/12 (0.0%)	4/13 (30.8%)	0.0957
Potassium chloride	3/12 (25.0%)	5/13 (38.5%)	0.6728
Prednisone	0/12 (0.0%)	0/13 (0.0%)	>0.9999
Propofol	2/12 (16.7%)	8/13 (61.5%)	**0.0414**
Rocuronium	2/12 (16.7%)	7/13 (53.8%)	0.0968
Salbutamol	0/12 (0.0%)	5/13 (38.5%)	**0.0391**
Sodium chloride	2/12 (16.7%)	4/13 (30.8%)	0.6447
Tinzaparin	3/12 (25.0%)	1/13 (7.7%)	0.3217

Table 2.3.2. Data are presented as mean ± SEM or frequencies (%), unless stated otherwise. Comparisons were performed by using the independent-samples t test or Chi-squared test/Fisher's exact test. Samples missing due to incomplete questionnaire data collection are not included in the analyses.

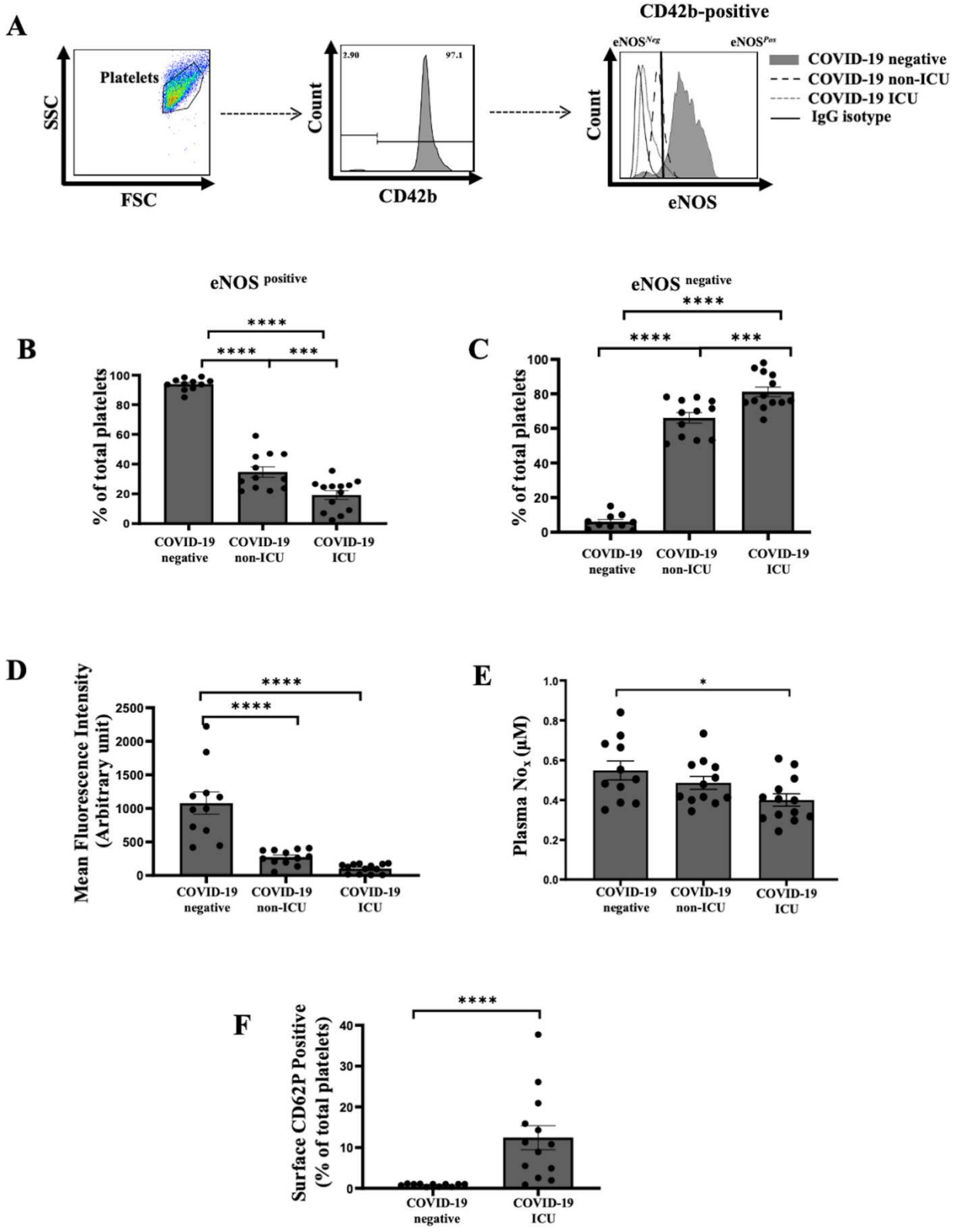

A
Platelets
SSC
FSC
Count
CD42b
2.90
97.1
CD42b-positive
eNOS^Neg
eNOS^Pos
eNOS
COVID-19 negative
COVID-19 non-ICU
COVID-19 ICU
IgG isotype
B
eNOS positive
% of total platelets
C
eNOS negative
D
Mean Fluorescence Intensity (Arbitrary unit)
E
Plasma Nox (µM)
F
Surface CD62P Positive (% of total platelets)

Figure 2.3.1 Elevated ratios of eNOS-negative to –positive platelets in COVID-19 Patients. **(A)** Representative platelet flow cytometry gating strategy and histograms demonstrating changes in eNOS-based platelet subpopulations in COVID-19 patients along with summary data for eNOS-positive **(B)** and eNOS-negative **(C)** platelet subpopulations. Statistics: one-way ANOVA with Tukey's multiple comparison test. *** *P*-value < 0.001, **** *P*-value < 0.0001 **(D)** Summary data comparing MFI for eNOS demonstration reduced eNOS protein in platelets isolated from COVID-19 patients stratified by ICU admission and COVID-19 negative controls. Statistics: one-way ANOVA with Tukey's multiple comparison test. **** *P*-value < 0.0001 **(E)**. Summary data demonstrating reduced plasma concentrations of nitrite and nitrate (NOx) in COVID-19-positive ICU patients. Statistics: One-way ANOVA with Dunnett's multiple comparison test, **P*-value < 0.05. **(F)** Summary data demonstrating significant increase in surface CD62P on platelets from COVID-19 ICU patients compared to COVID-19 negative controls. Statistics: unpaired, two-tailed Student's t-tests. **** *P*-value < 0.0001.

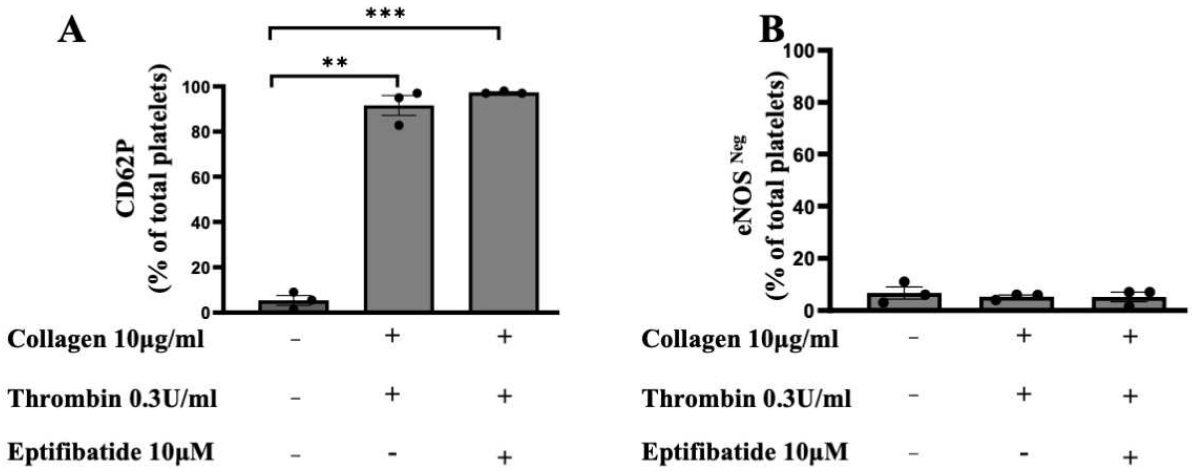


Figure 2.3.2. Assessment of percent eNOS-negative platelets post-activation by collagen and thrombin.

(A) Summary data demonstrating percent surface CD62P positive platelets upon and **(B)** percent eNOS-negative platelets following activation by collagen (10µg/ml) and thrombin (0.3U/ml) in the absence and presence of Eptifibatide (10µM). Statistics: one-way ANOVA with Dunnett's multiple comparisons test $^{**}P$-value < 0.01, $^{***}P$-value < 0.001.

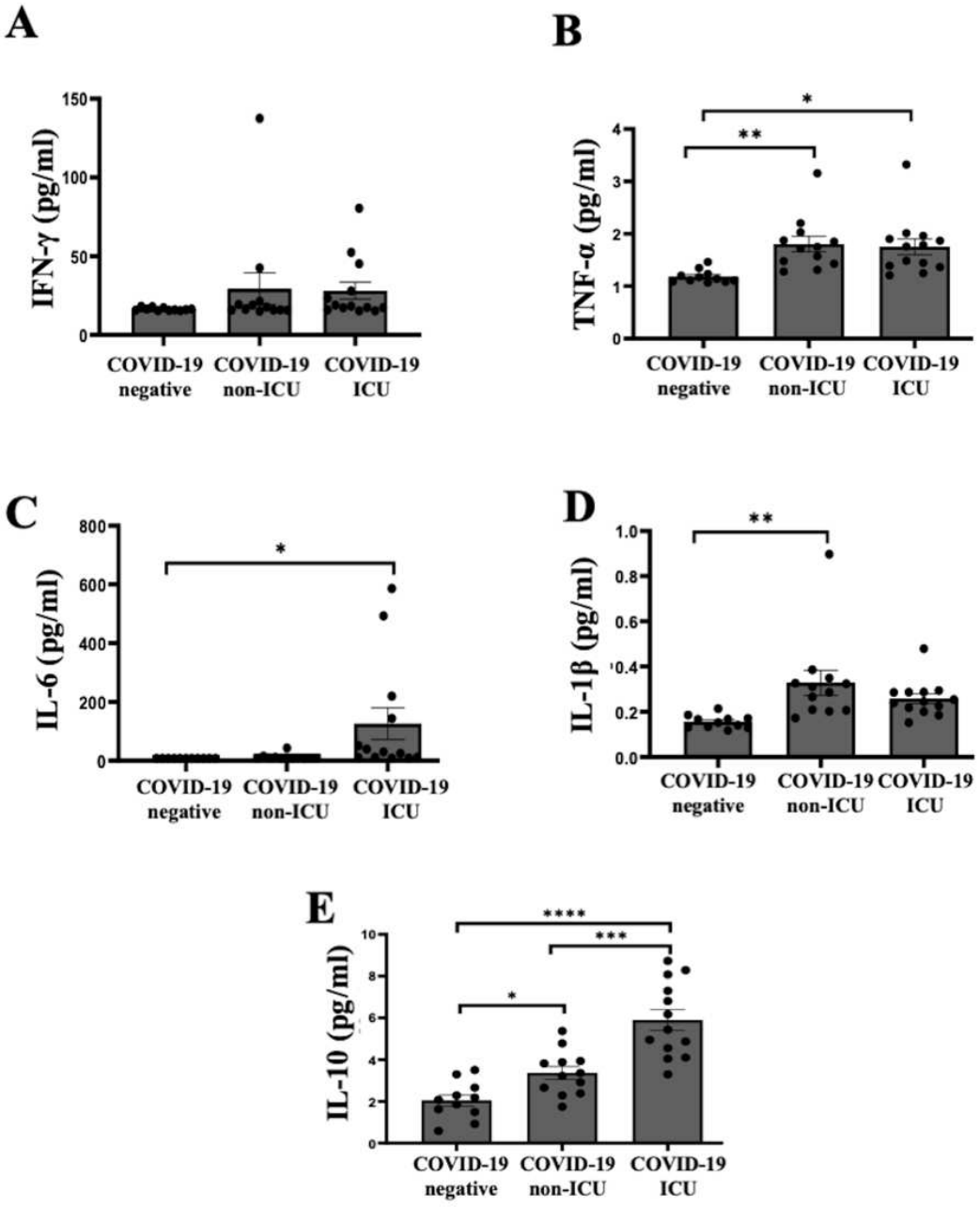

A
IFN-γ (pg/ml)
150
100
50
0
COVID-19 negative
COVID-19 non-ICU
COVID-19 ICU
B
TNF-α (pg/ml)
4
3
2
1
0
*
**
COVID-19 negative
COVID-19 non-ICU
COVID-19 ICU
C
IL-6 (pg/ml)
800
600
400
200
0
*
COVID-19 negative
COVID-19 non-ICU
COVID-19 ICU
D
IL-1β (pg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
**
COVID-19 negative
COVID-19 non-ICU
COVID-19 ICU
E
IL-10 (pg/ml)
10
8
6
4
2
0
****
***
*
COVID-19 negative
COVID-19 non-ICU
COVID-19 ICU

Figure 2.3.3 Assessment of pro- and anti-inflammatory cytokine concentrations in the plasma of COVID-19 patients and COVID-19-negative controls

(A) No significant difference in the concentration of plasma IFNγ between COVID-19 negative controls and COVID-19 positive patients. **(B-D)** Summary data demonstrating plasma concentrations of TNF-α, IL-6, and IL-1β were significantly higher in COVID-19 patients than in COVID-19-negative controls. Statistics: one-way ANOVA with Dunnett's multiple comparisons test **P*-value < 0.05, ***P*-value < 0.01. **(E)** Summary data demonstrating plasma concentration of IL-10 in COVID-19-positive and COVID-19-negative controls. One-way ANOVA with Tukey's multiple comparison test, **P*-value < 0.05, ****P*-value < 0.001, **** *P*-value < 0.0001.

2.3.2 Characterizing Live Nitric Oxide-Producing and Non-Producing Meg-01 Cells Based on eNOS Expression

Previous studies have demonstrated and characterized[245] the presence of distinct subpopulations of megakaryocytes (MEG-01 cells) based on the presence or absence of endothelial nitric oxide synthase (eNOS), dividing them into eNOS-positive and eNOS-negative groups. This distribution closely matches the ratio of eNOS-positive to eNOS-negative observed in platelets from healthy donors. To confirm these findings, controls experiments demonstrated that FACS-sorting of DAF-FM-stained cells into positive and negative subpopulations followed by staining and flow cytometry for eNOS showed DAF-FM-negative and -positive cells to correspond to eNOS-negative and –positive Meg-01, respectively (Fig. 2.3.4A & B). Lastly, siRNA-mediated knock down of eNOS resulted in reduced eNOS-protein levels in eNOS-positive Meg-01 (Fig. 2.3.4C), again confirming the specificity of the antibodies and presence of eNOS-positive and negative Meg-01 subpopulations.

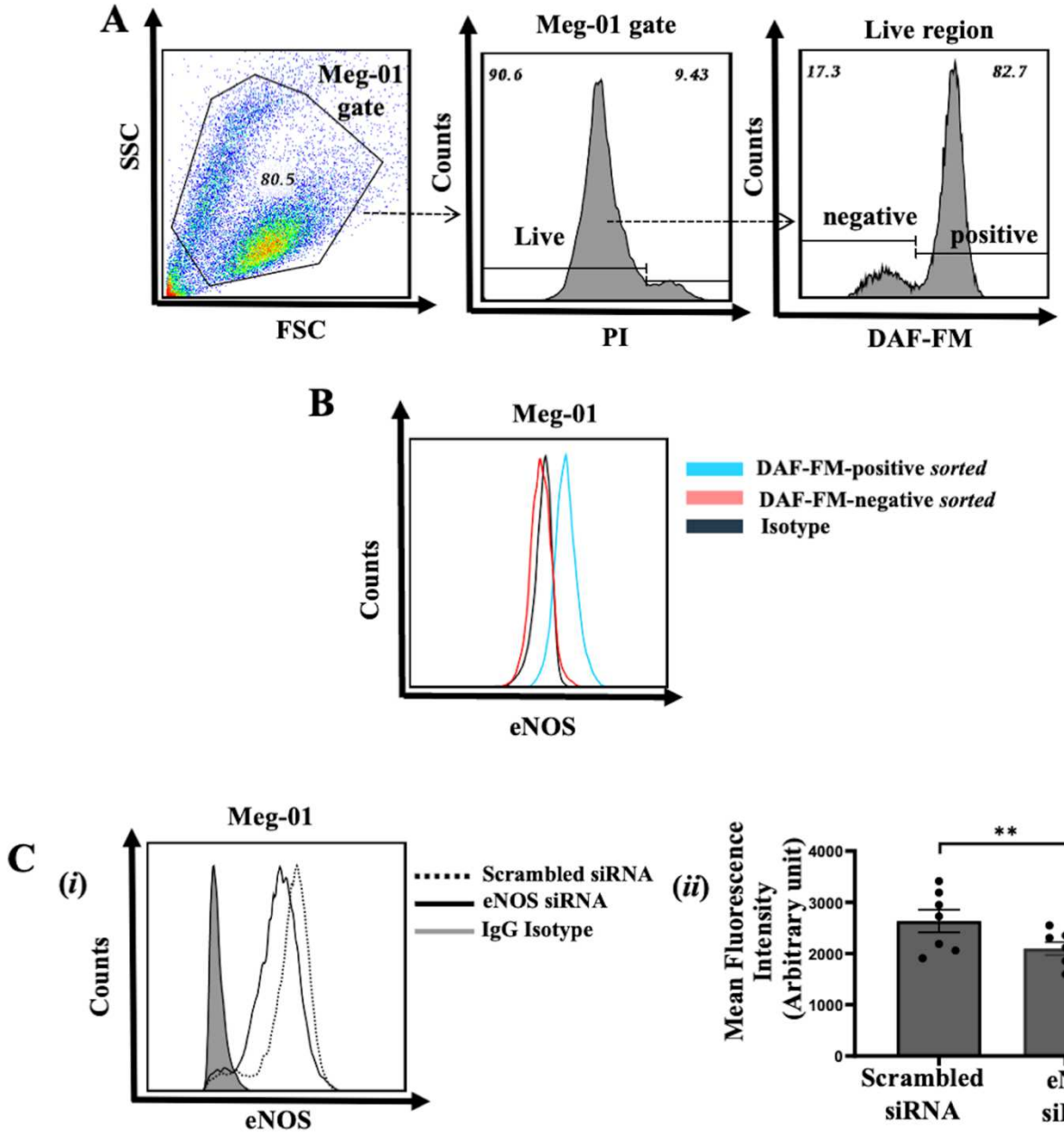

A
Meg-01 gate
SSC
FSC
80.5
Counts
Meg-01 gate
90.6
9.43
Live
PI
Live region
17.3
82.7
negative
positive
DAF-FM
B
Meg-01
Counts
eNOS
DAF-FM-positive sorted
DAF-FM-negative sorted
Isotype
C
(i)
Meg-01
Counts
eNOS
Scrambled siRNA
eNOS siRNA
IgG Isotype
(ii)
Mean Fluorescence Intensity (Arbitrary unit)
4000
3000
2000
1000
0
**
Scrambled siRNA
eNOS siRNA

Figure 2.3.4. Evaluating the presence of eNOS in live Nitric Oxide-producing and non-producing Meg-01 cells

(E) Representative dot plot and histograms depicting the gating strategy for sorting viable Meg-01 cells based on PI and DAF-FM. **(F)** Representative overlay histograms of IgG isotype control and eNOS protein detected in DAF-FM-negative and DAF-FM-positive sorted Meg-01. **(G)** ***(i)*** Representative histograms and ***(ii)*** summary data demonstrating siRNA-mediated knockdown of eNOS protein. Statistics: paired, two-tailed Student's t-tests N=7, ***P* value < 0.01.

2.3.3 At COVID-19-Relevant Concentrations Inflammatory Cytokines Enhance eNOS-Negative Platelet-Like Particle Release from Meg-01

Next, we investigated whether inflammatory cytokines up-regulated during COVID-19 can induce changes in eNOS-positive to -negative ratios of Meg-01 and released platelet-like particles similar to IFNγ At concentrations measured in our cohorts of COVID-19 patients, a combination of IL-6, IL-1β, and TNF-α reduced the percentage of eNOS-positive and increased the percentage of eNOS-negative Meg-01 (Fig. 2.3.5A and B) similar to high concentrations of IFNγ alone (Fig. 2.3.5B). Analogously, the combination of these three pro-inflammatory cytokines reduced the percentage of eNOS-positive and increased the percentage of eNOS-negative platelet-like particles released from Meg-01 similar to high concentrations of IFNγ alone (Fig. 2.3.5C-E). Functionally, platelet-like particles released from Meg-01 stimulated by the three pro-inflammatory cytokines demonstrated greater adhesion to fibrinogen than those obtained from control Meg-01, similar to control platelet-like particles incubated with the NOS inhibitor L-NAME (Fig. 2.3.5F and G).

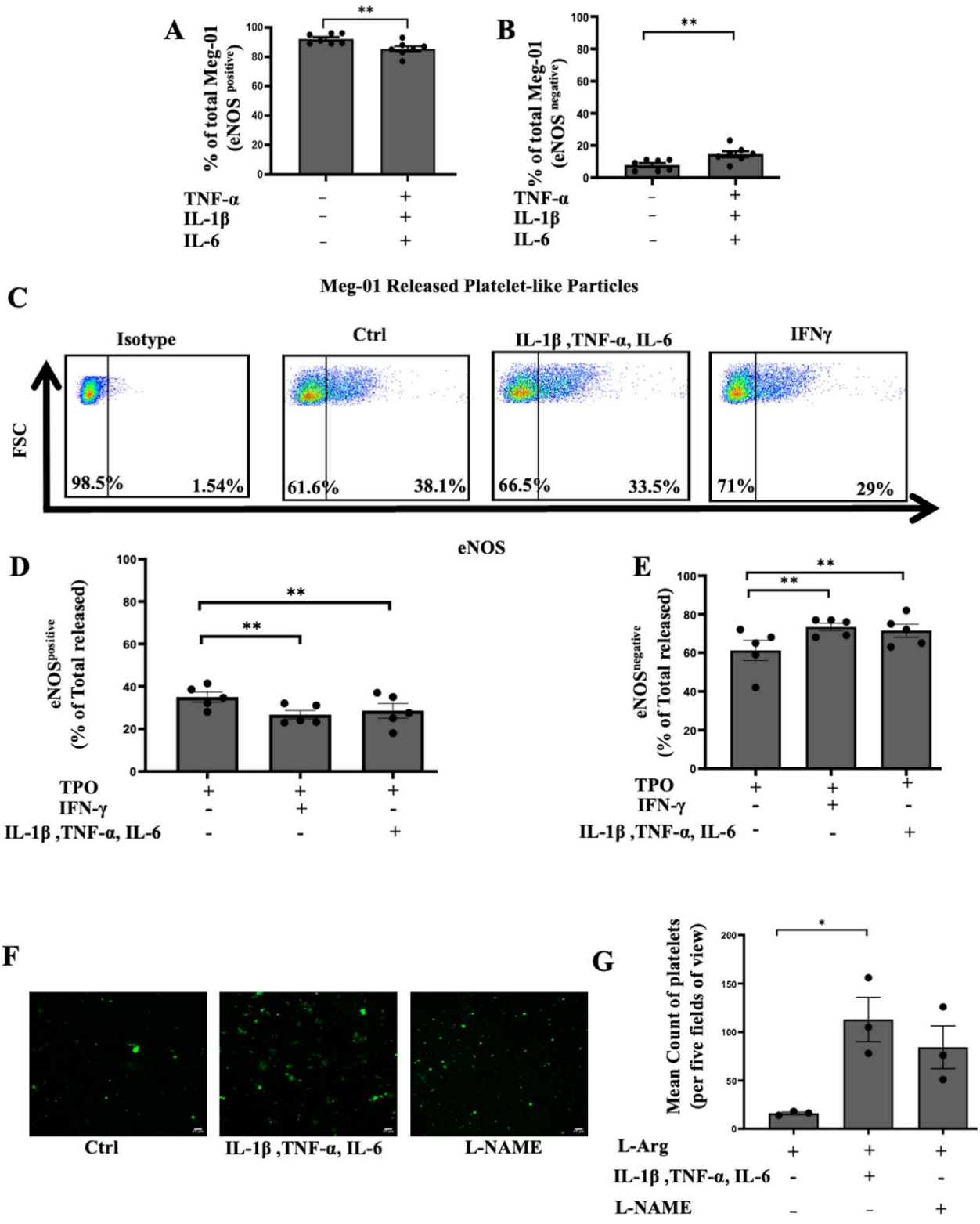

A
% of total Meg-01 (eNOS positive)
**
TNF-α
IL-1β
IL-6
B
% of total Meg-01 (eNOS negative)
**
TNF-α
IL-1β
IL-6
C
Meg-01 Released Platelet-like Particles
Isotype
Ctrl
IL-1β ,TNF-α, IL-6
IFNγ
FSC
98.5%
1.54%
61.6%
38.1%
66.5%
33.5%
71%
29%
eNOS
D
eNOSpositive (% of Total released)
**
**
TPO
IFN-γ
IL-1β ,TNF-α, IL-6
E
eNOSnegative (% of Total released)
**
**
TPO
IFN-γ
IL-1β ,TNF-α, IL-6
F
Ctrl
IL-1β ,TNF-α, IL-6
L-NAME
G
Mean Count of platelets (per five fields of view)
*
L-Arg
IL-1β ,TNF-α, IL-6
L-NAME

Figure 2.3.5. COVID-19-associated inflammatory cytokines increase the ratios of eNOS-negative to -positive Meg-01 and their released platelet-like particles.

Flow cytometry summary data demonstrating a decrease in **(A)** eNOS-positive and **(B)** an increase in eNOS-negative Meg-01 cells after incubation with TNF-α (0.1ng/ml), IL-6 (1ng/ml), and IL-1β (0.1ng/ml). Statistics: paired, two-tailed Student's t-tests N=5, ***P* value < 0.01. **(C)** Representative flow cytometry dot plots and summary data demonstrating eNOS-positive **(D)** and eNOS-negative **(E)** platelets-like particles released from Meg-01 incubated with inflammatory cytokines (IFNγ (10ng/ml), TNF-α (0.1ng/ml), IL-6 (1ng/ml), and IL-1β (0.1ng/ml)). Statistics: one-way ANOVA with Tukey's multiple comparisons test. N = 5. ***P*-value < 0.01. **(F)** Representative confocal microscopy images and **(G)** summary data demonstrating increased adhesion to fibrinogen by platelet-like particles released from Meg-01 incubated with inflammatory cytokines (TNF-α, IL-6, and IL-1β) compared to controls. Platelet like particle that generated from Meg-01 cells were treated with L-NAME as control. Statistics: one-way ANOVA with Dunnett's multiple comparisons test. N = 3. **P*-value < 0.05.

2.3.4 COVID-19 Does Not Increase the Percentage of iNOS-Positive Platelets

Lastly, as megakaryocytes are also known to express the inducible NOS isoform and severe COVID-19 is associated with a strong pro-inflammatory response [165, 548, 549], we investigated whether iNOS protein is up-regulated in COVID-19 platelets using an iNOS selective antibody (Fig. 2.3.6). Although only platelets from ICU COVID-19 patients were available for analysis, there was no significant difference in the percent iNOS-positive platelets between COVID-19-negative controls and ICU patients (controls 11±1.4% vs. COVID-19 ICU 13±1.6%; Fig. 2.3.7A). In line with these null results, incubation of Meg-01 with a combination of IL-6, IL-1β, and TNF-α did not significantly change the percent iNOS-positive Meg-01 (Fig. 2.3.7B). Interestingly, iNOS-positive Meg-01 were all eNOS-positive suggesting NOS-positive and NOS-negative subpopulations exist, and that low levels of iNOS are likely present as the iNOS-positive population largely overlapped with isotype control antibody (Fig. 2.3.7B). Consistent with these findings, flow RNA analysis of Meg-01 cells revealed that 80-90% of cells were positive for both eNOS and iNOS mRNA and that approximately 10-15% of Meg-01 were eNOS mRNA-negative and iNOS mRNA-positive (Fig. 2.3.7C and D).

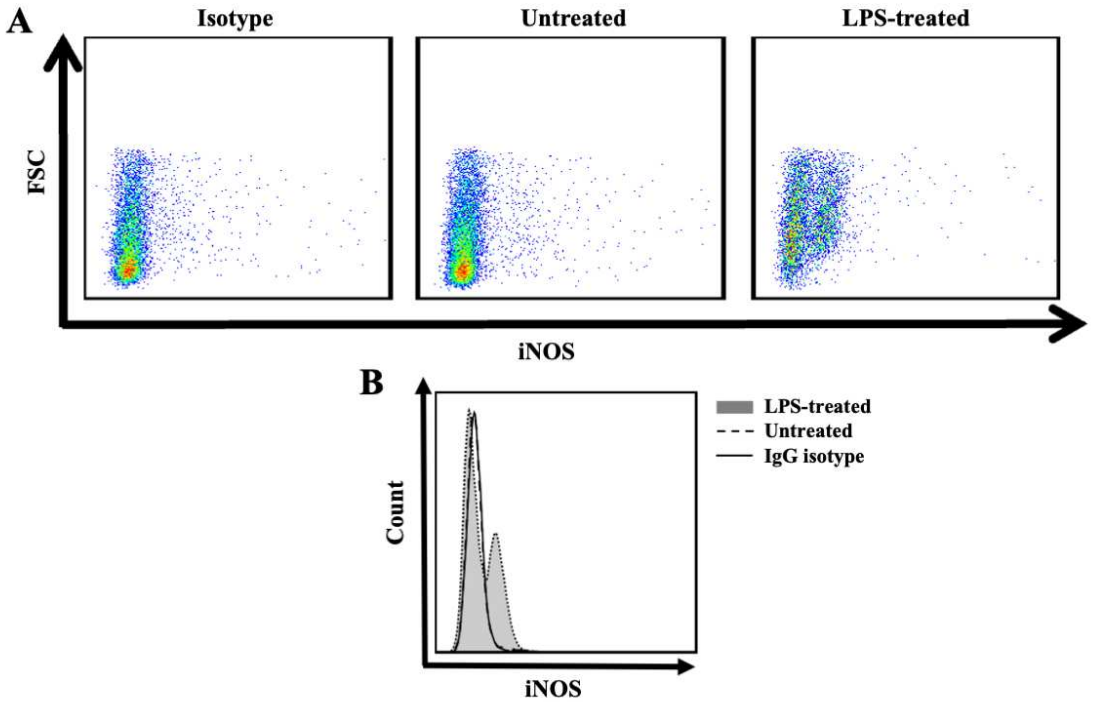
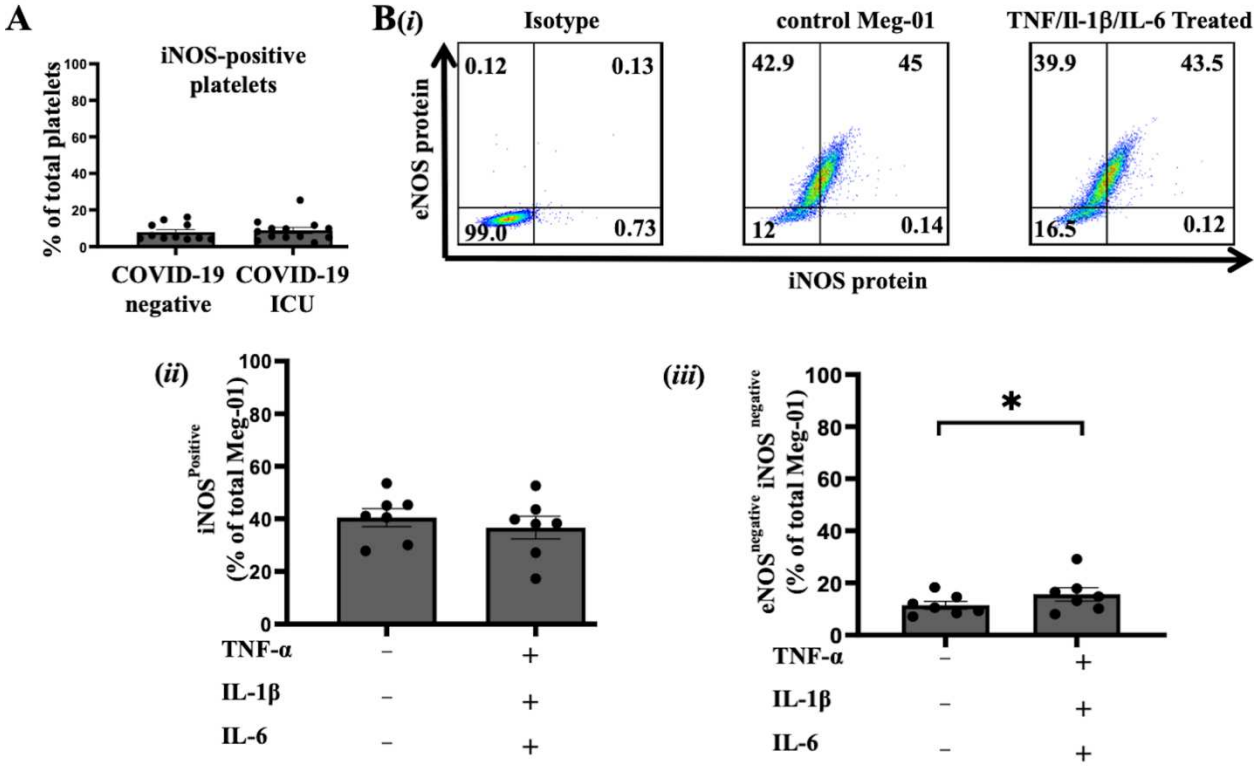
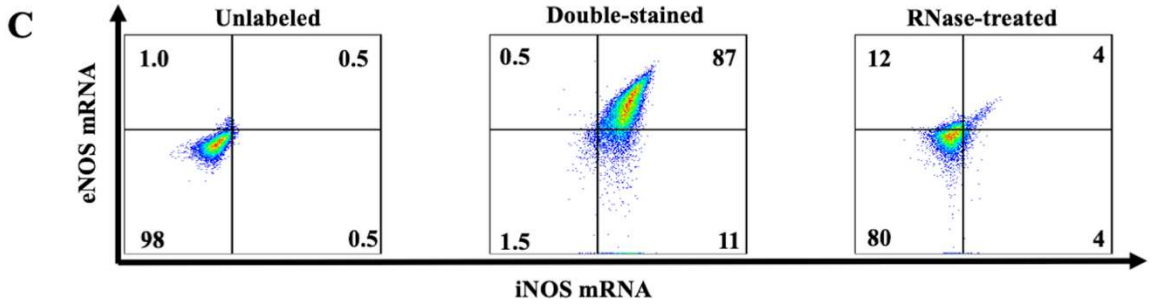


Figure 2.3.6. Evaluating anti-iNOS antibody in LPS-induced PBMCs.

Isolated PBMCs were treated with LPS at a concentration of 1 µg/ml for 3 days. Then, cells were stained using PE-conjugated monoclonal anti-iNOS antibody. **(A)** Dot plot and **(B)** histogram demonstrating the specificity of the anti-iNOS monoclonal antibody in detecting the expression of this enzyme in LPS-induced leukocytes.


A
iNOS-positive platelets
% of total platelets
COVID-19 negative
COVID-19 ICU
B(i)
Isotype
control Meg-01
TNF/Il-1β/IL-6 Treated
eNOS protein
iNOS protein
0.12
0.13
99.0
0.73
42.9
45
12
0.14
39.9
43.5
16.5
0.12
(ii)
iNOS Positive (% of total Meg-01)
TNF-α
IL-1β
IL-6
(iii)
eNOS negative iNOS negative (% of total Meg-01)
*


C
Unlabeled
Double-stained
RNase-treated
eNOS mRNA
iNOS mRNA
1.0
0.5
98
0.5
0.5
87
1.5
11
12
4
80
4

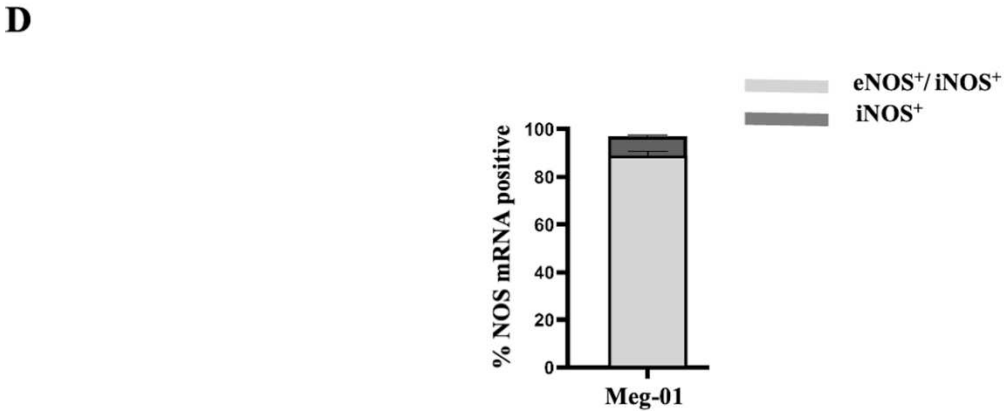

D
eNOS+/ iNOS+
iNOS+
% NOS mRNA positive
Meg-01

Figure 2.3.7. Effects of up-regulated inflammatory cytokines during COVID-19 (TNF- α, IL-6, and IL-1β) on eNOS and iNOS levels in Meg-01 and platelets.

(A) Summary data demonstrating the percentage of iNOS expression in platelets isolated from ICU admitted COVID-19 patients and COVID-19 negative healthy controls. Statistics: unpaired, two-tailed Student's t-tests. Ns, *P*-value > 0.05. **(B)** ***(i)*** Representative flow cytometry dot plots demonstrating changes in eNOS and iNOS protein expression in Meg-01 cells upon treatment with up-regulated inflammatory cytokines during COVID-19 (TNF-α (0.1ng/ml), IL-6 (1ng/ml), and IL-1β (0.1ng/ml)). Flow cytometry summary data demonstrating changes in Meg-01 iNOS-positive subpopulation ***(ii)***, and eNOS-negative iNOS-negative ***(iii)*** subpopulation upon treatment with up-regulated inflammatory cytokines during COVID-19 (TNF-α, IL-6, and IL-1β). Statistics: paired, two-tailed Student's t-tests. N = 7, **P*-value < 0.05 and ***P*-value < 0.01. **(C)** Representative flow cytometry dot plots demonstrating changes in eNOS and iNOS mRNA expression upon Rnase treatment. **(D)** Summary data demonstrating significant detection of NOS mRNA (eNOS and iNOS) in Meg-01 cells. N = 3.

2.4 Discussion

COVID-19 is commonly associated with the development of thrombotic complications including not only microvascular thrombosis, but also venous and arterial thrombosis [550] that can lead to life-threatening cardiovascular complications including venous and arterial thromboembolism, pulmonary embolism, ischemic stroke, and myocardial infarction. This higher rate of thrombotic events in COVID-19 patients is associated with an increased presence of platelets with a hyperactivated phenotype [551-555]. A number of studies have investigated the mechanism behind this platelet hyperactivity. One of these studies showed that COVID-19 alters the transcriptome profile of platelets and reprograms them toward a more prothrombotic phenotype characteristic of large and immature platelets, an affect attributed to direct infection of megakaryocytes by SARS-Cov-2 [555]. While another transcriptomic study of COVID-19 patient platelets demonstrated an enrichment of pathways associated with protein ubiquitination, antigen presentation, and mitochondrial dysfunction and attributed the hyperactive platelet phenotype to be partly due to increased MAPK pathway activation and subsequent thromboxane A2 production, while also noting an basal increase in reactive oxygen species [556]. Still another demonstrated increased circulating megakaryocytes with an increase in IFITM3 [557], whose expression has been shown to increase fibrinogen endocytosis and platelet reactivity [558]. However, none of these studies have investigated whether dysfunction of endogenous platelet negative feedback pathways, such as that mediated by NO, contribute to COVID-19-associated platelet hyper-reactivity, and whether the ratio of platelet subpopulations with differential abilities to produce NO are altered in COVID-19. Considering the well-documented hyper-inflammatory response associated with severe COVID-19 [559], and the counter-regulation of eNOS and iNOS expression by inflammatory cytokines [560, 561], we investigated whether platelet ratios of eNOS-positive

to eNOS-negative platelets are altered in moderate to severe SARS-Cov-2 infection and whether this contributes, in part, to increased platelet reactivity in COVID-19.

As previously described in a young adult healthy population [245], eNOS-positive platelets were the predominant platelet subpopulation in COVID-19-negative controls, while eNOS-negative platelets formed the predominant subpopulation in COVID-19 patients, with those in ICU exhibiting nearly a complete reversal in eNOS-positive to eNOS-negative ratios. Moreover, eNOS-positive platelets within COVID-19 patients demonstrated lower levels of eNOS compared to control platelets, suggesting reduced eNOS expression by megakaryocytes during SARS-Cov-2 infection and subsequent inheritance by platelets. Consistent with these findings COVID-19 ICU patients demonstrated a significantly lower concentration of plasma NO metabolites and increased platelet reactivity compared to controls. Further in line with our hypothesis COVID-19 patients demonstrated increased plasma concentrations of inflammatory and immunomodulatory cytokines compared to COVID-19-negative controls despite dexamethasone treatment in half the COVID-19 patients.

To identify the mechanism by which COVID-19 decreases eNOS-positive platelet levels, we previously sought to establish an appropriate model of eNOS-based platelet subpopulation generation by investigating the presence of eNOS-negative and eNOS-positive megakaryocytes within the bone marrow of eNOS-GFP mice as well as within the human megakaryoblastic cell line Meg-01[247]. Although a previous study by Freedman *et al*. demonstrated the presence of eNOS in mouse bone marrow and platelets, it did not identify the bone marrow cell subtype or the proportion of mouse platelets containing eNOS [562]. Unfortunately, but in agreement with our previous findings which demonstrated that approximately 2% of platelets derived from eNOS-GFP transgenic mice are eNOS-positive, less than five percent of eNOS-GFP transgenic mouse

megakaryocytes contained eNOS ruling out their utility to study eNOS-based platelet subpopulation generation from megakaryocytes[247]. As DNA methylation of the eNOS promoter can determine the extent of eNOS expression between different cell types [563, 564], we previously also investigated whether differences in DNA methylation could account for the species difference in eNOS protein detection in human megakaryoblasts vs. mouse megakarocyte/blasts[247]. Indeed, incubation with a DNA methyltransferase inhibitor enhanced the number of eNOS-GFP positive mouse megakaryoctyes in culture. In contrast, eNOS was readily detectable in human Meg-01 megakaryoblasts in a proportion of cells similar to that found for platelets from COVID-19-negative controls, consistent with reports of constitutive NOS activity by both human bone marrow megakaryocytes and Meg-01 cells [530, 531]. Hence, we previously also characterized the Meg-01 cell line for subpopulations of eNOS-protein expressing cells, NO production, viability, size, ploidy and decrease in eNOS content in response to IFNγ. IFNγ has been shown to be generated by coronary artery infiltrating T-cells and to be a central mediator of vascular eNOS dysregulation [565-567], as well as a factor capable of megakaryocytic differentiation [568].

Next, we utilized TPO-stimulated Meg-01 cells as an experimental model of platelet subpopulation generation in response to pro-inflammatory cytokines. Although Meg-01 are predominantly eNOS-positive, surprisingly in absence of pro-inflammatory cytokines Meg-01 generated a greater ratio of eNOS-negative to –positive platelet-like particles. It is not clear whether this is due to a limitation in platelet production under culture conditions, incorrect mix of cytokines, or the differentiation status of the Meg-01, which are a megakaryoblastic cancerous cell line. However, similar to IFNγ increasing the number of eNOS-negative Meg-01, Incubating these cells with IL-6, IL-1β, and TNF-α increased the percent of eNOS-negative platelet-like particles

generated from Meg-01, an effect that was reversed by IL-10 in a concentration-dependent manner. IL-10 has previously been shown to increase eNOS transcripts, protein, and NO production by endothelial cells and to overcome impairments in eNOS expression and acetylcholine-induced vasorelaxation caused by both endothelin-1 and TNF-α [569-571]. Similar to IFNγ a combination of IL-6, IL-1β, and TNF-α at concentrations measured in our COVID-19 patient population resulted in a decrease in eNOS-positive and an increase in eNOS-negative Meg-01 accompanied by analogous changes in eNOS-positive to eNOS-negative platelet-like particles generated from Meg-01. These results indicate that COVID-19-associated pro-inflammatory cytokines may be, in part, responsible for the changes in ratio of eNOS-positive to eNOS-negative platelets observed in COVID-19 patients. Moreover, the increased propensity of platelet-like particles generated from Meg-01 stimulated by the three pro-inflammatory cytokines to adhere to fibrinogen, similar to control PLPs incubated with L-NAME, further suggests that the increased ratio of eNOS-negative to eNOS-positive platelets observed in COVID-19 patients may contribute to the platelet hyperactivity associated with SARS-Cov-2 infection.

Interestingly, although the plasma concentrations of IL-6, IL-1β, and TNF-α in COVID-19 patient blood samples were elevated compared to non-COVID-19 controls, the most significantly elevated cytokine measured was IL-10, which we demonstrate increased the percent of eNOS-positive PLPs generated in response to IFNγ[247]. Consistent with our finding, elevated IL-10 levels are a hallmark of the cytokine storm associated with SARS-Cov-2 infection and are associated with disease severity [344, 572-575]. Although IL-10 has been “canonically classified as an anti-inflammatory cytokine” [576], at high concentrations it has been shown to also have proinflammatory and immunostimulatory effects [577-580]. Apart from it’s immunomodulatory roles, IL-10 has also been shown to promote the growth of megakaryocyte and megakaryocyte-

mixed colonies in hematopoietic colony forming assays [581], although clinically recombinant IL-10 has been demonstrated to induce thrombocytopenia [582].

Lastly, we focused on platelet eNOS as it is the predominant NOS isoform found in platelets and responsible for regulating platelet function. Indeed, a comparison of iNOS positive platelets between ICU COVID-19 patients and COVID-19-negative controls demonstrated only a small portion of platelets to be iNOS-positive and no significant differences between the two study groups. Fascinatingly, eNOS mRNA expressing Meg-01 were all also iNOS mRNA positive, although at the protein level eNOS-positive Meg-01 appear to be only weakly iNOS positive with the iNOS-specific signal overlapping with isotype control antibody. Hence, unlike eNOS, little of the iNOS protein present in megakaryocytes maybe passed down to platelets. This would be consistent with the short half-life of iNOS mRNA (6 hr) [583], and even shorter half-life of iNOS-protein (< 2 hr) [169, 584]. Conversely, under basal conditions eNOS mRNA has been reported to have a half-life of 48 hours [585], although it may be shortened by TNF-α Further, as eNOS protein co-localizes with caveolin-1 in platelet membranes it is likely stable in its inactivated state until intracellular Ca^{2+} concentrations rise upon platelet activation and eNOS becomes activated [245, 586]. Although not currently investigated but considering that iNOS maybe constitutively expressed at low levels in some organ/cell types [118, 587, 588], that it and eNOS are found in megakaryocytes[531], and that iNOS knock out mice have half the platelet count of their wild-type counterparts and eNOS knockouts [589], suggests that iNOS and eNOS may play distinct roles within the hemostatic system. Specifically, iNOS with its short half-life and potential to produce high NO concentrations may contribute to platelet production by megakaryocytes. While eNOS with its more stable protein and capacity to generate NO at low concentrations contributes

to the negative-regulation of platelet function, a function lost under inflammatory conditions such as COVID-19 resulting in altered ratios of eNOS-negative to –positive platelet ratios.

Our study has a number of limitations. First, our non-ICU COVID-19 patient platelets and plasma were obtained just prior to the start of the alpha SARS-Cov-2 variant driven wave of COVID-19 while ICU COVID-19 platelets and samples were obtained from patients during the delta driven wave. Hence, it is not clear whether the different variants differentially impacted eNOS-based platelet subpopulations generation in COVD-19 patients. Second, we did not investigate whether a cause of platelet eNOS loss is platelet death as SARS-Cov-2 infection has been demonstrated to initiate programmed cell death pathways including apoptosis in platelets [590]. However, we have previously demonstrated that eNOS-negative platelets are not platelets undergoing apoptosis [245], and mouse platelets lacking eNOS are not apoptotic. Also, it should be noted that platelets are not the only source of NO in plasma and down-regulation of eNOS in endothelial cells due to COVID-19 might also contribute to the observed lower concentration of NO_x in plasma and therefore the platelet hyperactivity. Additionally, it is not clear whether different ratios of eNOS-positive to –negative platelet-like particles would have been obtained with cultured primary human megakaryocytes exposed to COVID-19 relevant cytokines. Lastly, due to the relatively low numbers of platelet-like particles produced by Meg-01 in culture we were unable to perform platelet function testing such as light-transmittance aggregometry or a flow-chamber assay.

However, overall, our data suggest that the up-regulation of inflammatory cytokines (TNF-α, IL-6 and IL-1β) in COVID-19 attenuates megakaryocyte/blast eNOS levels and promotes the formation of megakaryocytes/blasts lacking eNOS that give rise to more reactive eNOS$^-$negative platelets. Therefore, the increased eNOS-negative to -positive platelet ratio may predispose

COVID-19 patients to developing thrombotic events. Finally, future research will need to validate if the ratio of eNOS-negative/eNOS-positive platelet subpopulations are altered and accompany the inflammation leading up adverse cardiovascular events such as myocardial infarction and stroke. As well as, whether only eNOS-negative megakaryocytes/blasts generate eNOS-negative platelets or whether both eNOS-based platelet subpopulations arise from eNOS-positive megakaryocytes/blasts.

Conflicts of Interest

The authors have no conflicts of interest to disclose with respect to the content of this manuscript.

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Authorship Contributions

The following authors contributed to the study design: A.A. and P.J. The following authors performed experiments: A.A., A.F.., A.H., A. R-B.., P.J. The following authors contributed to the analysis and interpretation of data: A.A., A.H., and P.J. The following authors contributed to the critical writing or revising the intellectual content: A.A., and P.J. The final draft of the manuscript was approved by P.J.

Chapter 3

Identification of a Novel Growth Factor-Enriched Platelet Subpopulation: Potential Implications for COVID-19

Abstract

Background: While previous research into platelet subpopulations has focused primarily on platelet reactivity, little is known about platelet subpopulations in the context of granule and growth factor content. Recent studies revealed that platelets contain various numbers of α-granules. Therefore, each platelet contains different levels of stored bioactive substances that mediate platelet function in various physiological and pathophysiological processes. Some of the main complications of COVID-19 are microthrombosis and pulmonary edema due to increased vascular permeability. Vascular endothelial growth factor (VEGF), largely stored in platelet α-granules, plays a central role in promoting vascular permeability.

Aims: To characterize a novel growth factor/α-granule-enriched platelet subpopulation and investigate whether changes in this subpopulation occur under inflammatory conditions with COVID-19 as an example. To identify a potential mechanism by which this growth factor-enriched platelet subpopulation may arise.

Methods: Platelets were isolated from healthy individuals and age- and sex-matched COVID-19-ICU patients and COVID-19-negative controls. Platelets were intracellularly stained for CD62P, VEGF and PDGF. Plasma TNF-α were evaluated using ELISA. Lastly, the VEGF expression in TNF-α-treated Meg-01 was assessed using flow cytometry.

Results: α−Granule-enriched platelet subpopulations (2.5 – 5.1%), marked by CD62P, exist with high levels of VEGF and PDGF in healthy donors. Additionally, COVID-19 patients' platelets demonstrated a higher overall mean in VEGF content and a greater percentage of VEGF-enriched platelet subpopulations compared to COVID-19-negative controls. COVID-19 patients demonstrated higher TNF-α plasma concentrations than COVID-19-negative controls. Treatment

of Meg-01 cells with TNF-α led to the formation of a distinct subpopulation with higher intracellular VEGF levels (19.8±1.7%).

Conclusion: Preliminary data suggests the existence of a novel α-granule-enriched platelet subpopulation with higher VEGF and PDGF content. Significant alteration of VEGF-enriched platelets subpopulation was observed in COVID-19. This change may be due to the upregulation of TNF-α and subsequent induction of VEGF expression in megakaryocytes; predisposing COVID-19 patients to higher vascular permeability and pulmonary edema upon thrombosis.

3.1 Introduction

Studies about platelet subpopulations have focused primarily on platelet reactivity, little is known about platelet subpopulations in the context of platelet granules and their growth factor content[591]. Upon activation, platelets release a vast array of bioactive substances from their secretory granules, including α-granules, δ-granules (dense granules), and lysosomes. Among these, α-granules are particularly significant due to their content and diverse functionality. They store a wide range of proteins such as growth factors, cytokines, and clotting factors(fibrinogen, FII, FV and FXI) , which are pivotal in mediating platelet functions related to various physiological and pathophysiological processes[24].

The α-granules in platelets are rich sources of specific growth factors, notably platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) [366]. VEGF, initially identified as vascular permeability factor, is crucial for vascular development and the regulation of blood and lymphatic vessel functions[367-371]. It not only enhances vascular permeability but also promotes angiogenesis, essential for tissue repair and regeneration[592, 593]. VEGF induces angiogenesis by activating the VEGFR2 pathway on endothelial cells, triggering downstream signaling through the PI3K/Akt and MAPK pathways that facilitate endothelial cell survival, proliferation, and migration[35, 594, 595]. Additionally, VEGF modulates endothelial barrier permeability through the PLCγ pathway, increasing intracellular calcium levels and disrupting cell-cell junctions, particularly affecting VE-cadherin[535]. Normally, vascular permeability is essential for maintaining homeostasis, facilitating the transfer of fluids, solutes, and cells across the endothelial barrier. This physiological process is pivotal for nutrient delivery, waste removal, and immune response efficiency[596, 597]. However, dysregulation of this process in the lung can lead to pulmonary edema, a severe condition characterized by the accumulation of

fluid in the alveoli of the lungs leading to hypoxemia, which has been frequently observed in severe COVID-19 cases[342, 598, 599]. The COVID-19 pandemic (COVID-19), caused by the SARS-CoV-2 virus, has highlighted critical aspects of vascular biology, particularly the regulation of vascular permeability and its implications for severe respiratory conditions like pulmonary edema[600, 601].

Endothelial barrier integrity is crucially modulated by a variety of factors through complex signaling pathways. Inflammatory cytokines such as TNF-α, IL-1, and IL-6 disrupt the barrier by activating NF-κB and JAK/STAT pathways, leading to changes in adhesion molecule expression, such as vascular endothelial (VE)-cadherin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), and junction protein phosphorylation[539-542, 602, 603]. Thrombin affects permeability through PAR-1 activation, triggering phospholipase C and protein kinase C signaling, which alters cytoskeletal dynamics and junctional organization[604, 605]. Histamine contributes to increased permeability via H1 and H2 receptor activation, promoting calcium influx and nitric oxide production, which temporarily opens endothelial junctions[606, 607]. Additionally, hypoxic conditions stabilize hypoxia-inducible factors (HIFs), enhancing vascular endothelial growth factor (VEGF) expression, which further disrupts intercellular junctions and increases permeability [608-611].

VEGF, previously recognized as vascular permeability factor (VPF), act as primary orchestrators in the modulation of vascular development and regulating blood and lymphatic functionality in various physiological and pathophysiological processes[367-371, 610]. VEGF, predominantly expressed in the alveolar epithelium, shows the highest mRNA levels in the lungs of both animals and humans, with alveolar protein levels being 500 times higher than those in plasma[612-614]. Other cells like smooth muscle cells, macrophages, and endothelial cells also

contribute to VEGF production[612]. Notably, VEGF is stored in platelets within α-granules, highlighting platelets as crucial for mobilizing VEGF to regulate vascular functions[615, 616].

The regulation of vascular endothelial growth factor (VEGF) expression is governed by various environmental and molecular factors, with each component finely tuning VEGF's role in vascular dynamics. Hypoxia significantly upregulates VEGF through the stabilization of hypoxia-inducible factors (HIFs), which bind to hypoxia-responsive elements in the VEGF gene promoter, enhancing its expression[372-374].. This hypoxic regulation is complemented by the effects of key cytokines such as TNF-α and IL-6. TNF-α enhances VEGF transcription via the NF-κB pathway, involving the phosphorylation and degradation of IκB proteins, which permits NF-κB to enter the nucleus and stimulate VEGF gene activity[374, 375]. This cytokine also activates the MAPK pathway, promoting VEGF expression by activating the AP-1 transcription factor[376]. Similarly, IL-6 upregulates VEGF through the JAK/STAT3 pathway, with STAT3 directly enhancing VEGF transcription[617, 618].Additionally, Platelet-Derived Growth Factor (PDGF) has been reported to enhance VEGF expression, a process that plays a significant role in angiogenesis and vascular development. This interaction is particularly evident in the context of tumor angiogenesis, where PDGF not only stimulates the recruitment and stabilization of perivascular cells but also up-regulates VEGF production[619-621].

Given that platelets inherit most of their protein content from their progenitor megakaryocytes and considering that studies have shown NF-kB activation in megakaryocytes can increase VEGF production[536], we hypothesized that the upregulation of cytokines such as TNF-α, commonly observed in COVID-19, could lead to enhanced VEGF content within platelets. This increase might result in the formation of a platelet subpopulation that is particularly enriched in VEGF, potentially influencing vascular permeability following activation during the infection.

3.2 Materials and Methods:

3.2.1 Reagents

Prostacyclin was obtained from Millipore-Sigma (Ontario, Canada). Human recombinant TNF-α, anti-PDGF A polyclonal antibody, and anti-VEGF polyclonal antibody were purchased from Thermo Fisher Scientific (California, USA). V-PLEX human proinflammatory panel I (cat # K15052D-1) was purchased from Meso Scale Diagnostics (Maryland, USA). BV421 Mouse Anti-Human CD62P (Clone AK-4, cat # 564037) was purchased from BD Biosciences (Ontario, Canada). The Sensiscript RT Kit and RNeasy mini kit were ordered from Qiagen (Ontario, Canada). Antibodies recognizing human eNOS at the N-terminal (clone 6H2, cat # ab91205), mouse IgG1 isotype control (clone ICIGG1, cat # ab91353), Goat Anti-Rabbit IgG H&L (PE) preabsorbed (cat # ab72465) were obtained from Abcam (MA, USA).

3.2.2 Participant Recruitment and Platelet Isolation

Participant recruitment, along with the isolation of platelets and plasma, was conducted as detailed in section 2.2.2.

3.2.3 Flow Cytometry

The fixation, permeabilization, and blocking of cells, including platelets and Meg-01 cells, were performed as described in section 2.2.3. Then, blocked platelets and Meg-01 cells were incubated with primary antibodies: Rabbit anti-VEGF Polyclonal or Rabbit anti-PDGF Polyclonal Antibody was used at a concentration of 10 μg/ml (1:100) for 3 hours and 1 hour at room temperature on a sample rotator, respectively. The samples were washed with wash buffer (3x)

and incubated with secondary antibody Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, PE at a concentration of 10 μg/ml for 1 hour in the dark at room temperature. Meg-01 cells were washed (3x) and resuspended in a PBS buffer and analyzed using flow cytometry (LSRFortessa X-20, BD). For analyzing platelet, Anti-CD42b-PE antibody (1:100) was added to the sample and incubated for 15 minutes, then diluted to a final volume of 1 ml with PBS buffer before flow cytometry analysis.

Additionally, for staining intracellular CD62p (P-selectin) as a marker of α-granules, fixed and permeabilized platelets were incubated with BV421 Mouse Anti-Human CD62P at room temperature for 15 minutes and then diluted to a final volume of 1 ml with PBS buffer before flow cytometry analysis.

3.2.4 Assessing Plasma Concentration of TNF-α Using Multiplex ELSIA

For the quantification of TNF-α in the plasma of COVID-19-positive patients and healthy controls, procedures were followed as described in section 2.2.8.

3.2.5 Cell Culture

The Meg-01 cell culture was conducted as detailed in section 2.2.4. Meg-01 cells were cultured in RPMI media supplemented with 2% FBS for evaluating VEGF expression upon TNF-α treatment. To do that, Cells were seeded at a density of 250,000 cells/ml in T-25 culture flasks, 24 hours prior to the treatment. Then, Cells were treated with recombinant human TNF-α at 1ng/ml concentration. After 48 hours of incubation, cells were then harvested and prepared for intracellular staining, targeting their VEGF content.

3.2.6 Statistics

Statistical analyses were also performed according to the methods outlined in section 2.2.15.

3.3 Results

3.3.1 Assessment of α-Granule Enrichment Platelets by CD62P Expression and Growth Factor Content

As illustrated in Fig 3.3.1A-D, a small platelet subset has been identified that is distinctly enriched in VEGF (Fig 3.3.1D) and PDGF (Fig 3.3.1E). Additionally, dual staining for interacellular CD62P(P-selectin), an established α-granule marker, along with VEGF or PDGF, demonstrated that these VEGF- and PDGF-enriched platelet subsets exhibit higher mean fluorescence intensity (MFI) compared to the dim subset (CD62P MFI in $VEGF^{Bright}$ 31136 ± 3800 vs CD62P MFI in $VEGF^{Dim}$ 7198 ± 1088, Fig 3.3.1F). Notably, size comparison between $VEGF^{Bright}$ and $VEGF^{Dim}$ platelets showed no significant differences, indicating that the enhancement in growth factor content within α-granules does not simply reflect larger platelets. (Fig 3.3.1G).

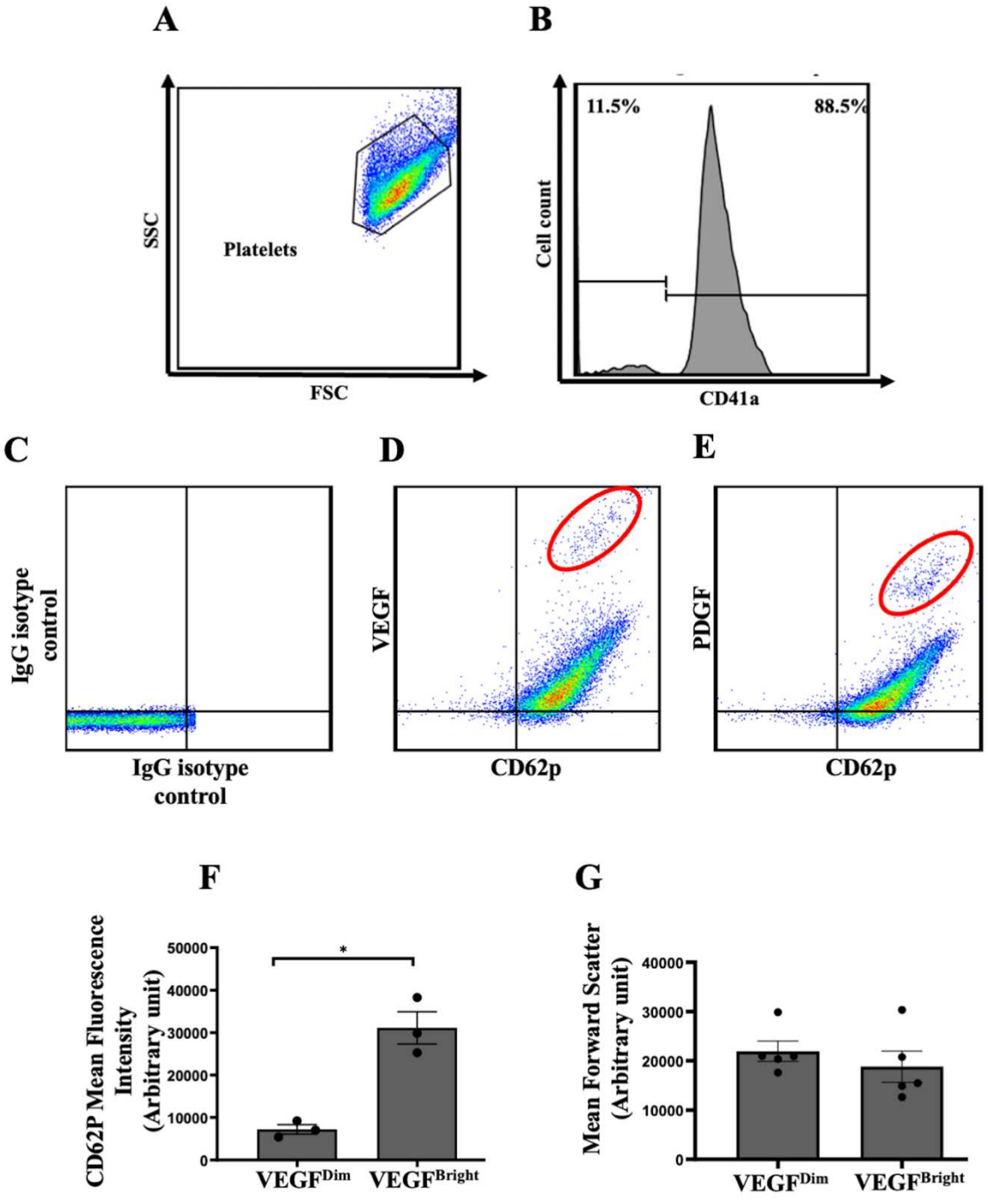
A
B
SSC
Platelets
FSC
11.5%
88.5%
Cell count
CD41a
C
D
E
IgG isotype control
IgG isotype control
VEGF
CD62p
PDGF
CD62p
F
G
CD62P Mean Fluorescence Intensity (Arbitrary unit)
50000
40000
30000
20000
10000
0
*
VEGFDim
VEGFBright
Mean Forward Scatter (Arbitrary unit)
40000
30000
20000
10000
0
VEGFDim
VEGFBright

Figure 3.3.1. Flow cytometric analysis of α-granule-enriched platelet subpopulations in healthy donors.

Platelets were gated on forward and side scatter (FSC/SSC) to identify the population **(A)**, confirmed by CD41a expression **(B)**. Subsequent dot plots demonstrate isotype control **(C)**VEGF **(D)** and PDGF **(E)** distribution within CD62P+ platelets, with red ellipses highlighting the subpopulations with high content of these growth factors. **(F)** Summary data demonstrating significantly higher expression of CD62p in $VEGF^{Bright}$ (VEGF-enriched) compared to $VEGF^{Dim}$. Statistics: paired, two-tailed t-tests. N = 3. **P*-value <0.05. **(G)** Comparative analysis of FSC, indicates no significant size difference between $VEGF^{dim}$ and $VEGF^{bright}$ platelets, suggesting that α-granule enrichment, as indicated by VEGF and PDGF content, does not affect the physical size of the platelets. Statistics: paired, two-tailed t-tests. N = 5. NS. *P*-value >0.05.

3.3.2 Differential Expression of VEGF and PDGF in eNOS-Positive and eNOS-Negative Platelet Subpopulations

Using flow cytometry, as illustrated in Fig 3.3.1, platelets were gated for size and granularity (Fig. 3.3.2 A) and their identity was confirmed through CD41a staining (Fig. 3.3.2B). Next, the levels of VEGF and PDGF were characterized in platelet subpopulations distinguished by the presence or absence of endothelial nitric oxide synthase (eNOS). The results indicated that eNOS-positive platelets contain significantly higher levels of VEGF (eNOS-negative: 64.5 ± 14.3 vs. eNOS-positive: 1462 ± 289.2; Fig. 3.3.2C and E) and PDGF (eNOS-negative: 27.7 ± 5.3 vs. eNOS-positive: 364 ± 54; Fig. 3.3.2D and F) compared to the eNOS-negative subpopulation. Notably, the subset of platelets that was enriched with VEGF (red gate: 2.3 ± 1.1%, Fig 3.3.2 C) and PDGF (red gate: 5.1 ± 1.4%, Fig. 3.3.2 D) was further identified within eNOS-positive platelets.

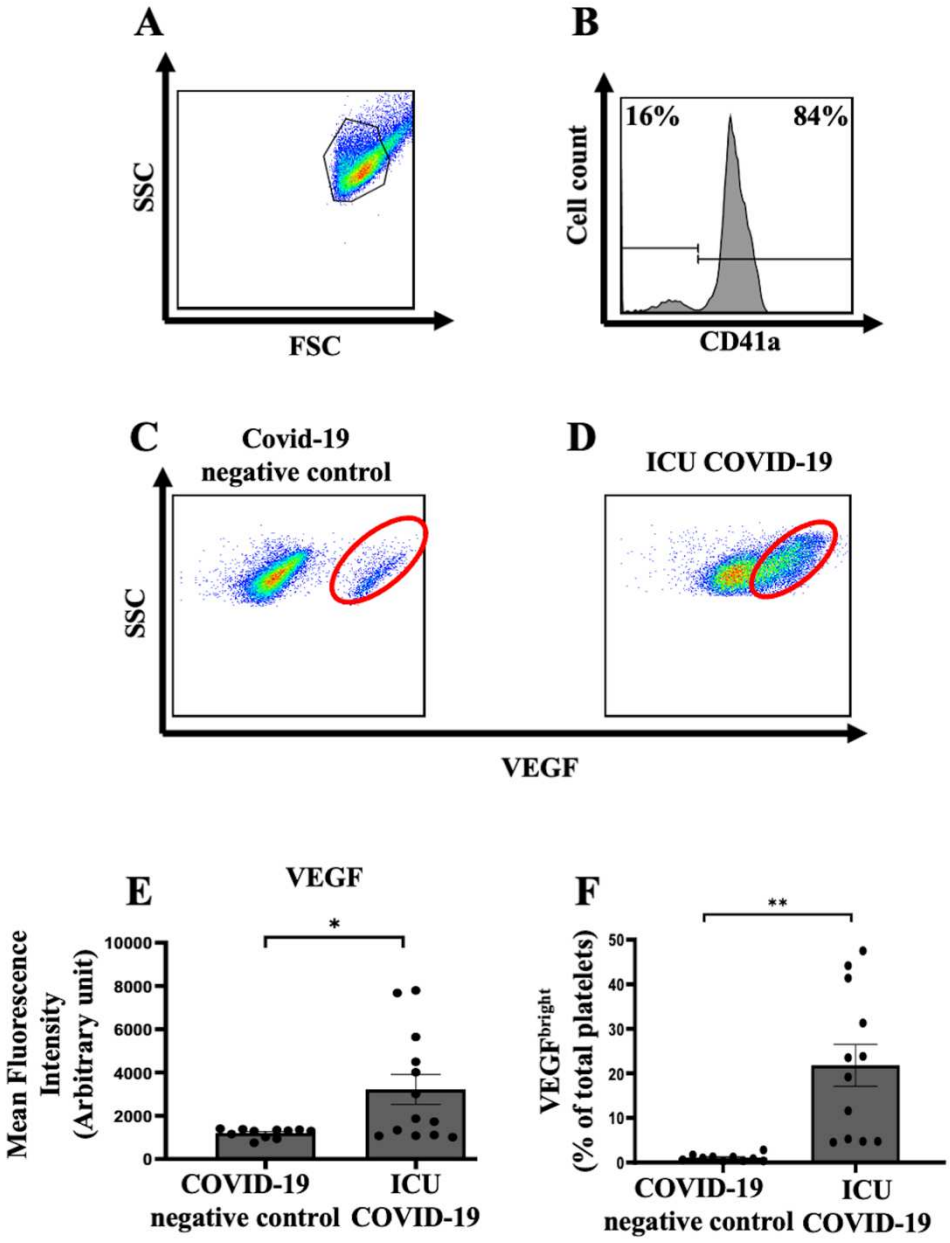
A
SSC
FSC
B
16%
84%
Cell count
CD41a
C
Covid-19
negative control
SSC
D
ICU COVID-19
VEGF
E
VEGF
*
Mean Fluorescence Intensity (Arbitrary unit)
10000
8000
6000
4000
2000
0
COVID-19 negative control
ICU COVID-19
F
**
VEGFbright (% of total platelets)
50
40
30
20
10
0
COVID-19 negative control
ICU COVID-19

Figure 3.3.2 Flow cytometric analysis of eNOS-positive and -negative platelet subpopulations by VEGF and PDGF content.

(A) Representative dot plot of forward scatter (FSC) vs. side scatter (SSC) and **(B)** CD41a expression histogram representing the platelet flow cytometry gating strategy. Representative dot plots showing the distribution of VEGF **(C)** and PDGF **(D)** within the eNOS-positive and eNOS-negative platelet subpopulation. Summary data demonstrating the eNOS-positive platelet subpopulation containing higher amount of VEGF **(E)** and PDGF **(F)** in comparison with eNOS-negative platelet subpopulation. Statistics: paired, two-tailed t-tests. N = 5 (VEGF) and N=3 (PDGF). **P*-value <0.05, ***P*-value <0.01.

3.3.3 Characterizing VEGF Protein Content in Isolated Platelets from COVID-19 Patients and COVID-19-negative Controls

As TNFα and hypoxia can increase VEGF gene expression, and increased plasma TNFα concentrations and hypoxia/hypoxemia are characteristics of severe COVID-19, we sought to compare platelet VEGF content in a previously characterized population of COVID-19 ICU patients and COVID-19-negative controls (Table 3.3.1 and 3.3.2)[622]. Flow cytometry analysis demonstrated that platelets from COVID-19 patients exhibit higher mean fluorescence intensity, indicating elevated VEGF content compared to those from COVID-19-negative controls (COVID-19 ICU patients: 3221 ± 693 vs. COVID-19-negative controls: 1204 ± 64.2, arbitrary units, Fig 3.3.3A-D). This was further supported by the increased percentage of $VEGF^{bright}$ platelet subpopulations among patients (COVID-19 ICI patients 23.8 ± 4.1% vs. COVID-19 negative controls 1.8 ± 0.3%, Fig 3.3.3A-C and E).

Table 3.3.1 Clinical demographics of study participants

Characteristic	COVID-19 negative controls (N =11)	COVID-19 ICU Patients (N = 13)	*P*-value
Age (years)	59.6+14.1	53.3±15.2	0.304
Male	7/11 (63.6%)	8/13 (61.5%)	>0.999
Immunology/Allergy	0/11 (0.0%)	1/13 (7.7%)	>0.999
Asthma	0/11 (0.0%)	1/13 (7.7%)	>0.999
Chronic Obstructive Pulmonary Disease (COPD)	0/11 (0.0%)	1/13 (7.7%)	>0.999
Obstructive sleep apnea	1/11 (9.1%)	1/13 (7.7%)	>0.999
Diabetes	0/11 (0.0%)	5/13 (38.5%)	**0.041**
Stroke (TIA/CVA)	0/11 (0.0%)	1/13 (7.7%)	>0.999
Myocardial Infarction (MI)	0/11 (0.0%)	1/13 (7.7%)	>0.999
Coronary Artery Disease (CAD)	1/11 (9.1%)	1/13 (7.7%)	>0.999
History of Coronary Artery Bypass Graft (CABG)	1/11 (9.1%)	0/13 (0.0%)	>0.999
History of Percutaneous Coronary Intervention (PCI)	0/11 (0.0%)	0/13 (0.0%)	>0.9999
History of Cardiac Surgery	0/11 (0.0%)	0/13 (0.0%)	>0.999
Heart Failure	0/11 (0.0%)	0/13 (0.0%)	>0.999
Valve Disease	0/11 (0.0%)	0/13 (0.0%)	>0.999
Hypertension	3/11 (27.3%)	4/14 (30.8%)	0.983
Atrial Fibrillation	0/11 (0.0%)	1/13 (7.7%)	>0.999
Dyslipidemia	1/11 (9.1%)	3/13 (23.1%)	0.596
Chronic Kidney Disease	0/11 (0.0%)	1/13 (7.7%)	>0.999
Liver Disease	0/11 (0.0%)	0/13 (0.0%)	>0.999
Cancer	0/11 (0.0%)	1/13 (7.7%)	>0.999
Anxiety/Depression	0/11 (0.0%)	0/13 (0.0%)	>0.999
History of Solid Organ Transplant	0/11 (0.0%)	2/13 (15.4%)	0.482
HIV Positive	0/11 (0.0%)	0/13 (0.0%)	>0.999
Immunocompromised	0/11 (0.0%)	1/13 (7.7%)	>0.999
Tonsillectomy	1/11 (9.1%)	1/13 (7.7%)	>0.999

Table 3.3.1. Data are presented as mean ± SEM or frequencies (%), unless stated otherwise. Comparisons were performed by using the independent-samples t test or Chi-squared test/Fisher's exact test. Samples missing due to incomplete questionnaire data collection are not included in the analyses.

Table 3.3.2. Frequently administered & relevant medications of study participants

Medication	COVID-19 ICU Patients (N = 13)
Acetaminophen	5/13 (38.5%)
Acetylsalicylic acid	2/13 (15.4%)
Azithromycin	9/13 (69.2%)
Dexamethasone	8/13 (61.5%)
Electrolyte solution	6/13 (46.2%)
Fentanyl	3/13 (23.1%)
Furosemide	6/13 (46.2%)
Hydromorphone	6/13 (46.2%)
Insulin	1/13 (7.7%)
Ipratropium	4/13 (30.8%)
Lidocaine	2/13 (15.4%)
Magnesium Sulfate	1/13 (7.7%)
Midazolam	6/13 (46.2%)
Norepinephrine	5/13 (38.5%)
Pantoprazole	7/13 (53.8%)
Polyethylene glycol	4/13 (30.8%)
Potassium chloride	5/13 (38.5%)
Prednisone	0/13 (0.0%)
Propofol	8/13 (61.5%)
Rocuronium	7/13 (53.8%)
Salbutamol	5/13 (38.5%)
Sodium chloride	4/13 (30.8%)
Tinzaparin	1/13 (7.7%)

Table 3.3.2. Data are presented as frequencies (%). Samples missing due to incomplete questionnaire data collection are not included in the analyses.

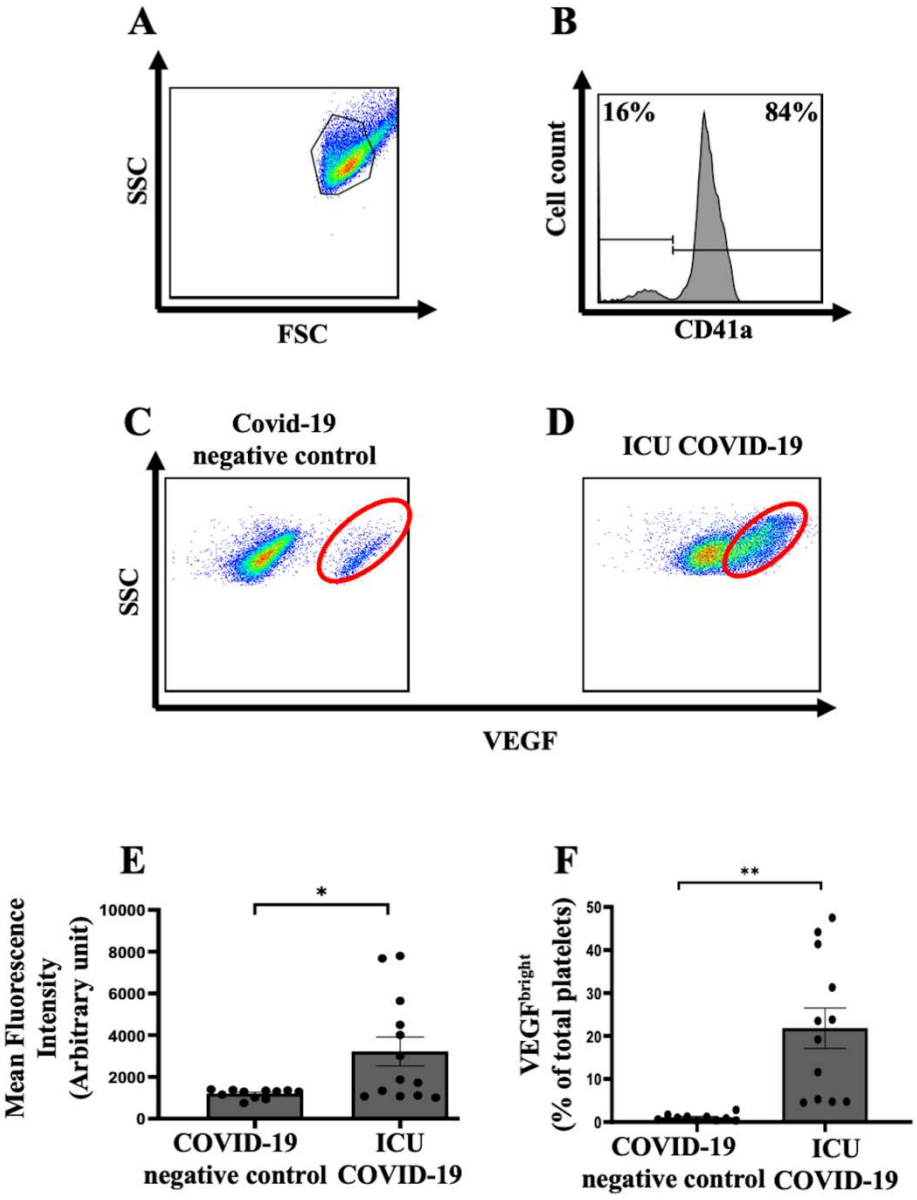
A
SSC
FSC
B
16%
84%
Cell count
CD41a
C
Covid-19
negative control
SSC
D
ICU COVID-19
VEGF
E
Mean Fluorescence Intensity (Arbitrary unit)
10000
8000
6000
4000
2000
0
*
COVID-19 negative control
ICU COVID-19
F
VEGFbright (% of total platelets)
50
40
30
20
10
0
**
COVID-19 negative control
ICU COVID-19

Figure 3.3.3. COVID-19 increases VEGF^{bright} platelet subpopulation.

Platelets were gated on forward and side scatter (FSC/SSC) to identify the population (**A**), confirmed by CD41a expression (**B**). Representative dot plot showing VEGF content of platelets in COVID-19 negative controls **(C)** and COVID-19 patients **(D)**. Summary data demonstrating COVID-19 patients' total platelets population contain higher VEGF content compared to COVID-19 negative controls **(E)**. Statistics: unpaired, two-tailed t-tests. **P*-value <0.05. Summary data demonstrating a significantly higher percentage of VEGF-enriched platelet subpopulations in COVID-19 patients compared to COVID-19 negative controls **(F)**. Statistics: unpaired, two-tailed t-tests. ***P*-value <0.01.

3.3.4 Induction of VEGF Expression by TNF-α Treatment in Meg-01 Cells

Previously, plasma TNF-α levels were found to be significantly higher in our COVID-19 ICU patient population than in the COVID-19 negative controls, correlating with the heightened inflammatory response often seen in these patients. Therefore, to explore the potential mechanisms behind the increased formation of VEGF-enriched platelet subpopulations in COVID-19 patients Meg-01 were incubated with TNFα. Incubation of Meg-01 cells with TNF-α led to the formation of a distinct subpopulation with higher intracellular VEGF levels (TNFα 14.4 ± 3.1% vs. control 3.6 ± 1.3%, Fig 3.3.4A-C).

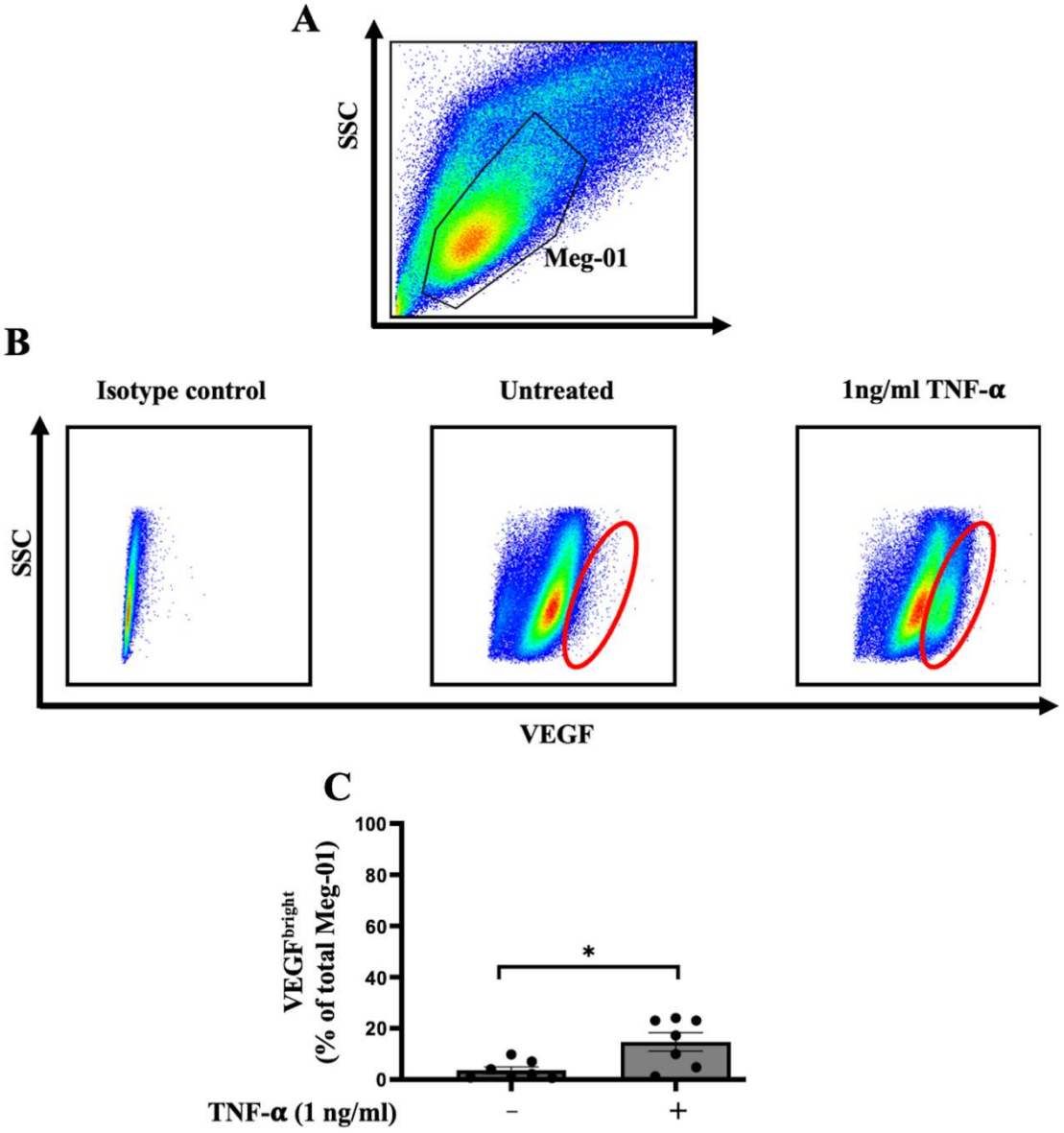
A
SSC
Meg-01
B
Isotype control
Untreated
1ng/ml TNF-α
SSC
VEGF
C
VEGF^bright (% of total Meg-01)
100
80
60
40
20
0
*
TNF-α (1 ng/ml)
–
+

Figure 3.3.4. TNF-α increases VEGF expression in Meg-01 cells.

(A) representative dot plot demonstrating Meg-01 cells gating strategy. **(B)** representative dot plot demonstrating an increase in VEGF content (red gate) of TNF-α-treated Meg-01. **(C)** Summary data demonstrating a significant increase in VEGF content of TNF-α-treated Meg-01 cells. Statistic: unpaired, two-tailed t-tests. N = 7, **P*-value <0.05.

3.4 Discussion

Our data suggest the existence of an α-granule-enriched platelet subpopulation, as indicated by intracellular CD62p expression, with elevated VEGF and PDGF content, and this platelet subset specifically exists within eNOS-positive platelets. This discovery aligns with a growing body of literature that recognizes the heterogeneity of platelet populations and their diverse roles in various physiological and pathological processes[623]. Recent studies have increasingly focused on the functional specialization of platelets, particularly noting the variation in granule content and its impact on platelet function[245, 624-629]. For instance, research has highlighted the role of platelets in angiogenesis, wound healing, tumour metastasis, and inflammation, largely mediated by the secretion of growth factors like VEGF and PDGF[630].

Alpha-granule content is derived from both *de novo* protein synthesis in megakaryocytes and endocytosis of plasma proteins[631, 632]. The collection of bioactive components with a-granules spans a broad range, including factors with contrasting functional properties[33, 629, 633, 634]. For instance, α-granules carry pro-angiogenic factors like Angiopoietin, EGF, FGF, IGF, PDGF, and VEGF, which facilitate angiogenesis and tissue regeneration. Conversely, they contain anti-angiogenic agents such as angiostatin, endostatin, LAMP-2 (Lysosome-associated membrane protein 2), and TIMPs (Tissue inhibitor of metalloproteinase), which suppress angiogenesis. This heterogeneity raises questions regarding whether α-granules are a homogeneous population or consist of subpopulations with distinct functional roles that are selectively released in a manner aligned with the physiological context.

Current debates in the field revolves around whether α-granules constitute a single homogeneous population or whether they are organized into subpopulations with distinct functions. Studies have reported differential localization of various proteins (vWF, Fibirnogen, pro

and anti-angiogenic factors) within α-granules, suggesting the possibility of thematic release, where specific cargo can be differentially released in a contextually appropriate manner[626, 629, 635, 636]. However, other research using high-resolution imaging techniques like electron microscopy suggests a single homogenous distribution of cargo within α-granules, indicating differentially zoned within the same α-granule not cargo proteins that are present in different α-granules [637-641].

The concept of heterogeneity in α-granule content across different platelets, as opposed to within individual platelets, gains critical importance in the context of pathological conditions like severe COVID-19, which are characterized by blood vessel inflammation, clotting disorders, and endothelial dysfunction[642]. The preliminary data indicating a subpopulation of platelets with an increased concentration of VEGF and PDGF in their α-granules suggest a notable variance in the granule composition from one platelet to another. This variation points to a regulatory mechanism in megakaryocytes, where certain platelets, perhaps as a response to specific physiological or pathological stimuli, are primed with a distinct set of bioactive molecules.

Additionally, an important aspect for further investigation is whether platelets enriched with growth factors are indeed reticulated platelets. Reticulated platelets are typically identified by their residual RNA content, indicating recent production and suggesting a young age within the circulating platelet pool[643, 644]. Conclusively determining whether growth factor-enriched platelets are reticulated or not remains an open question, necessitating further exploratory studies.

In the context of COVID-19, a disease characterized by systemic inflammation and hypoxic conditions, the observed increase in VEGF-enriched platelets is particularly noteworthy. VEGF plays a vital role in increasing vascular permeability, processes that are often dysregulated in COVID-19 and contribute to its severe manifestations, such as pulmonary edema and

microvascular thrombosis[645-647]. The significant alteration in the VEGF-enriched platelet subpopulation suggests that these platelets could be responding to the unique pathological environment created by the COVID-19 infection. This environment, potentially marked by factors such as hypoxia and elevated levels of inflammatory cytokines like TNF-α, is known to induce VEGF expression[372-374, 648]. Therefore, the increase in VEGF-rich platelets could be viewed as an adaptive response to the up-regulation of pro-inflammatory cytokines like TNF-α in infectious and other inflammatory diseases. However, this adaptation may exacerbate vascular complications such as microthrombosis and endothelial dysfunction.

Our results demonstrate that COVID-19 patients had higher plasma concentrations of TNF-α compared to COVID-19-negative controls, indicating a significant inflammatory response. This elevated TNF-α level aligns with other findings that associate COVID-19 with a heightened inflammatory response[548, 649-651]. Evidence indicates that activating NF-κB in megakaryocytes significantly increases VEGF production, so elevated TNF-α levels in COVID-19, which activate NF-κB, may stimulate megakaryocytes to produce more VEGF and raise VEGF content in platelets.

Further supporting this hypothesis is our observation that the incubation of Meg-01 cells with TNF-α resulted in the formation of a distinct subpopulation of Meg-01 characterized by higher intracellular VEGF levels. This finding indicates that TNF-α, a cytokine elevated in COVID-19[652, 653], can induce an increase in VEGF within the megakaryocytes. This induction likely contributes to the formation of a VEGF-enriched platelet subpopulation in the circulation, particularly under inflammatory conditions such as those seen in COVID-19. Additionally, TNF-α induces an adhesive phenotype in endothelial cells by upregulating adhesion molecules such as P-selectin, VWF, and integrin αVβ3, leading to the recruitment of leukocytes and platelets[654-

656]. Recruited neutrophils can generate neutrophil extracellular traps (NETs), with histones, particularly H3 and H4, causing platelet activation[657, 658]. The activation and release of tissue factor (TF) from monocytes will activate the extrinsic coagulation pathway, generating thrombin, which activates platelets and promotes microthrombosis[659]. As a result, activated platelets may potentially release elevated levels of VEGF, which could disrupt the endothelial barrier and allow plasma proteins and neutrophils to pass through, causing edema. It should be noted, however, that while we observed significantly increased VEGF content in platelets from COVID-19 patients, the direct impact on endothelial barrier disruption requires further investigation.

To maintain vascular integrity, a balance between pro-permeability and anti-permeability factors is essential. Upon activation, platelets release both enhance and decrease vascular permeability such as angiopoietin-1 (Ang-1)[660] and Sphingosine-1-phosphate (S1P)[661-663], which work to stabilize and protect the endothelium. However, studies have indicated that plasma from COVID-19 patients induces greater endothelial hyperpermeability, irrespective of disease severity[664, 665]. This suggests that in SARS-CoV-2 infection, the balance of platelet factors may be tilted toward those that promote permeability, as part of the plasma bioactive molecules originate from platelets, which upon activation, secrete them into the plasma. This shift, suggesting that the SARS-CoV-2 virus may alter the plasma fingerprint to favor pro-permeability agents, significantly contributes to chronic endothelial dysfunction characteristic of long COVID. This dysfunction leads to increased vascular permeability, permitting more fluids and harmful substances to enter tissues and facilitating the infiltration of white blood cells (WBCs) into vascular walls. These processes exacerbate issues with vascular and barrier integrity, leading to a pro-thrombotic state. The recruited WBCs further drive inflammation, potentially resulting in the formation of microthrombi that can obstruct the microvasculature. This cycle of endothelial

activation, persistent inflammation, thrombosis, and tissue damage complicates the resolution of long COVID symptoms and adds to its persistence and management complexity. The interplay between altered vascular permeability, thromboinflammation, and immune cell recruitment is likely a critical factor in the prolonged recovery and varied symptoms observed in long COVID patients, reflecting a profound impact on systemic health post-infection[666].

Based on studies, plasma PDGF (Platelet-Derived Growth Factor) levels are consistently upregulated across all COVID-19 patients, although there is a notable negative correlation with disease severity; more severe cases exhibit lower PDGF levels compared to milder ones[667]. Furthermore, there are strong positive correlations between plasma PDGF levels and key markers of endothelial dysfunction, such as P-selectin and sCD40L[668]. Particularly in severe COVID-19, both plasma and bronchoalveolar lavage (BAL) fluid show significantly elevated levels of PDGF-AB/BB[669]. Further investigation is required to determine if the consistent elevation of plasma PDGF levels in COVID-19 is partly due to platelets being enriched with PDGF, which, upon activation, is secreted into the plasma. Additionally, the significance of the increase in PDGF plasma concentration needs to be explored, particularly regarding its role in disrupting the endothelial barrier and contributing to subsequent pulmonary edema in Covid-19.

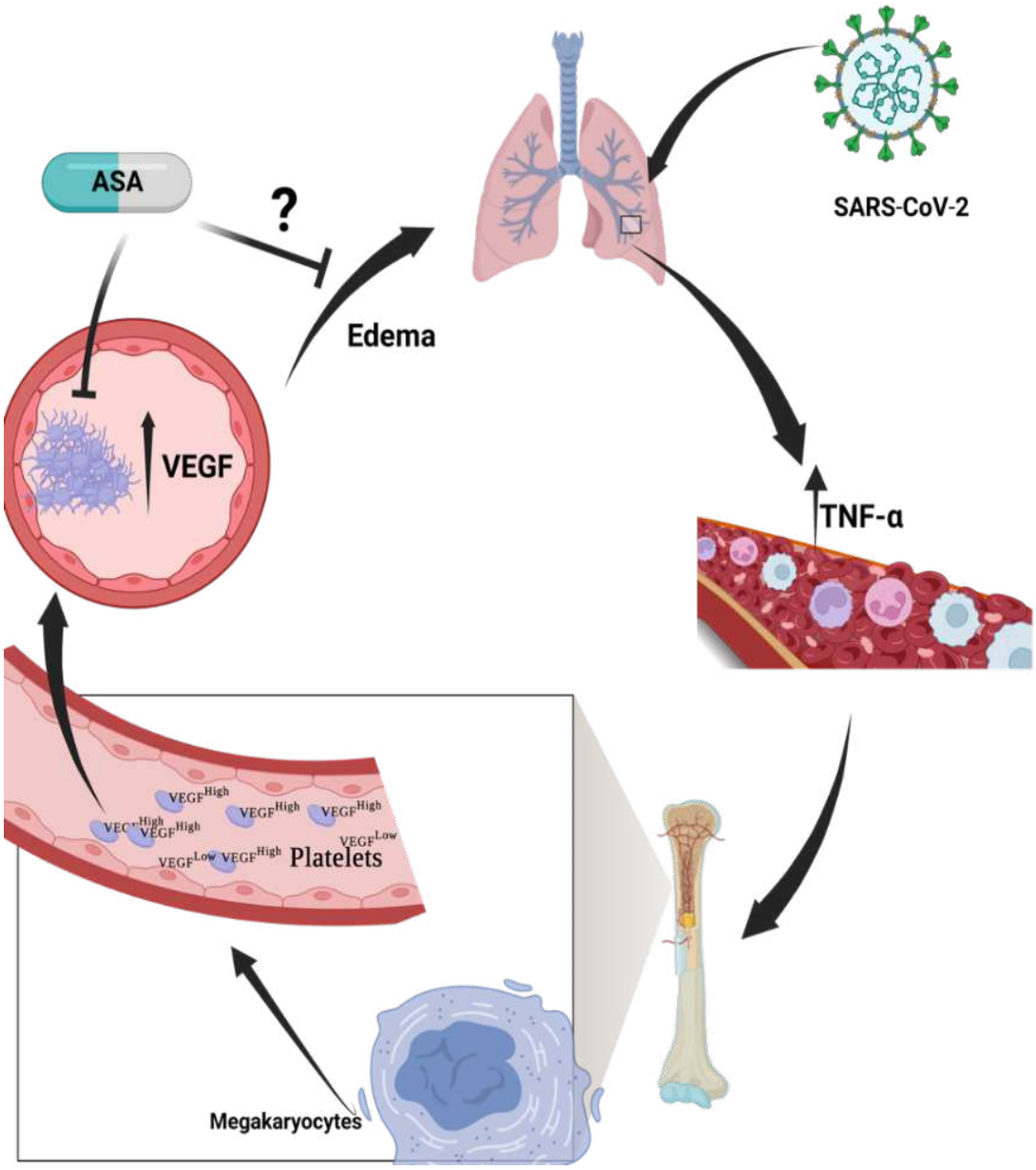

Figure 3.4.1 Cartoon summarizing the proposed model explaining the generation of VEGF^{bright} (VEGF^{high}) platelets from megakaryocytes.

Created with BioRender.com

Chapter 4

Platelets Stimulate Programmed Death-Ligand 1 Expression by Cancer Cells: Inhibition by Anti-Platelet Drugs

Platelets Stimulate Programmed Death-Ligand 1 Expression by Cancer Cells: Inhibition by Anti-Platelet Drugs

A. Asgari[1], G. Lesyk[1], E. Poitras[2], N. Govindasamy[3], K. Terry[2], Rachel To[1], V. Back[1], J.K. Rudzinski[3], J.D. Lewis[3], and P. Jurasz[1,2,4,5]

[1]Faculty of Pharmacy and Pharmaceutical Sciences, [2]Department of Pharmacology, [3]Department of Oncology, [4]Cardiovascular Research Centre, [5]Mazankowski Alberta Heart Institute, University of Alberta, Edmonton, Alberta, T6G-2E1, Canada.

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Corresponding author: Paul Jurasz, Faculty of Pharmacy and Pharmaceutical Sciences, 3-142E Katz Group-Rexall Centre, 11361–87 Avenue, Edmonton, AB, Canada, T6G-2E1. Tel: 780-492-2120; Email: jurasz@ualberta.ca

Abstract

Platelets help facilitate hematogenous metastasis in part by promoting cancer cell immunoevasion, although our understanding of platelet function in modulating the adaptive immune system in cancer is limited. A major negative regulator of the adaptive response is the immune checkpoint protein Programmed Death Ligand 1 (PD-L1). As platelets secrete factors that may increase PD-L1 expression, we investigated whether they up-regulate cancer cell PD-L1, thus promoting immunoevasion, and whether common anti-platelet drugs inhibit this process. Platelets were isolated from human volunteers. A549 lung, PD-L1 null A549, and 786-O renal cancer cells were incubated with and without platelets, and cancer cell PD-L1 expression was measured by qPCR and flow cytometry. Additionally, platelet-cancer cell incubations were performed in the presence of common anti-platelet drugs, and with growth factor neutralizing antibodies. Following incubation with platelets, A549 cells were co-cultured with Jurkat cells and interleukin-2 (IL-2) levels were measured by flow cytometry as a marker of T-cell activation. Platelets increased PD-L1 mRNA and surface protein expression by A549 and 786-0 cells. Combined neutralization of VEGF and PDGF prevented the platelet-induced up-regulation of PD-L1 by A549 cells, as did the anti-platelet drug eptifibatide. A549 incubated with platelets demonstrated a reduced ability to activate human T-cells and this effect was reversed by eptifibatide. As platelets promote immunoevasion of the adaptive immune response by increasing cancer cell PD-L1 expression and as anti-platelet drugs prevent this immunoevasive response, the investigation of anti-platelet drugs as adjuvant therapy to immune checkpoint inhibitors may be warranted in the treatment of cancer.

Key words: Platelets, Hemostasis, Immune system, Neoplasm metastasis, Pharmacology

4.1 Introduction

Platelets play an important role promoting hematogenous metastasis [670, 671]. This occurs as a result of tumor cell-induced platelet aggregation (TCIPA), which facilitates platelet secretion of factors that contribute to generating a favourable microenvironment for metastasize cancer cells both within blood vessels and newly forming metastatic niches [672-674]. One mechanism by which TCIPA creates a cancer cell favourable microenvironment is via the platelet ability to protect malignant cells from the innate immune system and prevent natural killer (NK) cell-mediated cell death [459, 460, 462, 543]. Although much is known of how platelets affect the NK-mediated innate immune response, our understanding of platelet function in modulating the adaptive immune system in cancer is limited. A major negative regulator of the adaptive immune response is Programmed Death Ligand 1 (PD-L1; B7-H1), an immune checkpoint B7 family transmembrane protein that contributes to T-cell inhibition. PD-L1 interacts with its receptor PD on T-cells resulting in reduced T-cell proliferation, cytokine secretion, and T-cell anergy [481, 482]. Certain cancers including lung and kidney exploit PD-L1 as an immune evasive mechanism [675, 676]. PD-L1 expression by cells is increased in response to interferons including IFN-γ[481] and by activation of intracellular pathways that stimulate signal transducer and activator of transcription proteins (STAT) signaling [519, 520]. Notably, platelets have an abundant store of growth factors that stimulate STAT-signaling, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), which they secrete upon aggregation [523-525]. Therefore, we hypothesized that cancer cell-activated platelets increase the transcriptional expression of cancer cell PD-L1 and that this may enhance cancer cell immune evasion of the adaptive response. Further, we proposed that common anti-platelet agents such as acetylsalicylic acid, Prasugrel active metabolite, and Eptifibatideinhibit this platelet-induced up-regulation of

cancer cell PD-L1 and as consequence promote the adaptive immune cell response against them.

4.2 Materials and Methods:

4.2.1 Reagents

Prostacyclin, acetylsalicylic acid (ASA), Eptifibatideacetate, propidium iodide (PI), human recombinant interferon-γ, and PMA (Phorbol 12-myristate 13-acetate) were obtained from Millipore-Sigma (Oakville, Ontario, Canada). Phytohemagglutinin-L (PHA-L) Solution (500X), FITC-conjugated Anti-CD3 (clone UCHT1; cat# 11-0038-42), PE- conjugated anti-IL-2 (clone MQ1-17H12; 12-7029-42) and Brefeldin A Solution (1000X) were purchased from Thermo Fisher Scientific (Carlsbad, California, USA). Opti-MEM™ I Reduced Serum Medium, Cas9 plus reagent, Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent were ordered from Thermo Fisher Scientific (Whitby, Ontario, Canada). Prasugrel active metabolite (PAM) was obtained from Sirius Fine Chemicals SiChem GmbH (Bremen, Germany). Human PD-L1 (B7-H1) phycoerythrin (PE)-conjugated monoclonal antibody (clone 130021; cat# FAB1561P), goat anti-human PD-L1 polyclonal antibody, goat anti-human VEGF polyclonal antibody (cat# AF-293-NA) , goat anti-human PDGF(AA) polyclonal antibody (cat# AF-221-NA), and goat anti-human PDGF (BB) polyclonal antibody (cat# AF-220-NA), human recombinant VEGF165, and human recombinant PDGF-AB were purchased from R&D Systems (Minneapolis, MN, USA), and phycoerythrin-conjugated mouse IgG1 isotype control (MOPC-21, cat# IC002P) was obtained from BD Biosciences (Mississauga, Ontario, Canada). Sensiscript reverse transcriptase was ordered form Qiagen (Toronto, Ontario, CA). Common Alt-R® CRISPR-Cas9 tracrRNA conjugated with ATTO™ 550, Alt-R® crRNA, and Nuclease Free Duplex buffer were obtained from Integrated DNA Technologies or IDT (Coralville, Iowa, USA). S. pyogenes Cas9 Nuclease was purchased from NEB (Whitby, Ontario, Canada). Lympholyte-H Cell Separation Medium was obtained from Cedarlane (Burlington, Ontario, Canada).

4.2.2 Platelet and Peripheral Blood Mononuclear Cell Isolation

Approval for the study was obtained from the University of Alberta Human Research Ethics Board. Following informed consent, blood was collected from healthy volunteers who had not taken any drugs affecting platelet function for 14 days prior to the study. Platelets from both male and female donors were utilized and donor ages ranged from 22 – 45 years of age. Prostacyclin-washed platelets were prepared in sterile DMEM or RPMI-1640 as described previously [671]. Briefly, prostacyclin (0.075 μg ml-1) was added to citrated whole blood followed by centrifugation at 250g for 20 minutes to isolate platelet rich plasma (PRP). Next, prostacyclin (0.3 μg ml-1) was added to PRP, and platelets were pelleted at 900g for 10 minutes. The platelet pellet was subsequently washed 3 times with medium and re-suspended at 2.5 x 108 ml-1. The platelet suspensions were allowed to rest for 1 hour at room temperature for the platelet inhibitory effects of prostacyclin to dissipate.

As ASA and PAM are irreversible platelet inhibitors, for some experiments PRP was divided into four centrifuge tubes and incubated with vehicle, ASA (100 μM), or PAM (10 μM) for 20 minutes at room temperature prior to proceeding with platelet isolation.

For other experiments involving assessment of T-cell activation, anticoagulated blood was slowly added to Lympholyte-H Cell Separation Medium and centrifuged at 600g for 20 minutes. The isolated ring of peripheral blood mononuclear cells (PBMCs) was then resuspended in RPMI 1640 and centrifuged once again at 200g for 10 minutes. Finally the cells were suspended in RPMI and counted .

4.2.3 Cell Culture

Human A549 lung adenocarcinoma and 786-O renal cell adenocarcinoma cells were purchased from ATCC and cultured as described previously at 37 °C in a humidified atmosphere with 5% CO2 in 90% DMEM (A549) or RPMI-1640 (786-O) with gentamicin (0.05 mg mL-1), penicillin (0.06 mg mL-1), streptomycin (0.01 mg mL-1), and 10% fetal bovine serum (FBS) [671]. The cells were supplied with fresh medium, and subcultured three times each week. 786-O and A549 cells between passages 8 – 16 and 17-27, respectively, were utilized for experiments.

4.2.4 Cancer Cell-Platelet Co-Incubation Experiments

Platelet suspensions in serum-free medium supplemented with gentamicin (0.05 mg mL-1), penicillin (0.06 mg mL-1), and streptomycin (0.01 mg mL-1) were added to T25-cell culture flasks containing subconfluent A549 or 786-O cells. Platelets were incubated with cancer cells at 37 °C in a humidified atmosphere with 5% CO2 for 24 hours. For experiments utilizing pharmacological platelet inhibitors, Eptifibatideacetate (10 μM) was added directly to platelet-A549 containing flasks at the start of the incubation, as Eptifibatideis a reversible inhibitor. After 24 hours, platelets were removed by gentle washing, and cancer cells were detached from flasks using EDTA (7 mM) in DMEM with 10% FBS and gentle shaking. Subsequently, the cells were pelleted at 300 g for 5 minutes and washed three times with a flow cytometry binding buffer consisting of PBS with 5% BSA and 5 mM EDTA.

4.2.5 Flow Cytometry

Measurement of PD-L1 protein on the surface of A549 and 786-0 cells was performed with a Quanta SC flow cytometer (Beckman Coulter, Mississauga, Ontario, CA) as well as with a

LSRFortessa X-20 (Becton Dickinson, US). Briefly, A549 or 786-O (5 x 105/sample) cells were incubated with PE-conjugated PD-L1 antibody or isotype control (2.5 μg ml-1) in the dark for 15 minutes. Subsequently, samples were washed once with and re-suspended in 1 ml of binding buffer. Fluorescence was induced with a 488 nm argon laser and detected on FL2 (575 nm BP filter on Quanta) or (Y586 BP filter on LSRFortessa X-20). Residual non-specific IgG binding was subtracted from PD-L1-specific events. In some experiment, cancer cells were additionally stained with PI (5 μg ml-1), and PI-negative cells were gated and percent PD-L1 positive cells were determined. Compensation was performed using Cell Lab Quanta analysis software to account for fluorophore spectral overlap.

4.2.6 RT-PCR and qPCR

Total RNA was isolated from the A549 and human platelets using Qiagen RNeasy MiniKit according to manufacturer's protocol (Qiagen, CA). The RNA was reverse transcribed using Sensiscript reverse transcriptase. Thereafter, PCR was performed using a Bio-Rad (Bio-Rad, CA) S1000 thermal cycler with human PD-L1 primer pairs (Forward - CCT GGC TGC ACT AAT TGT CT and Reverse - CAC ATC CAT CAT TCT CCC TTT TC) or GAPDH primer pairs (Forward - GAG AAG GCT GGG GCT CAT TT and Reverse - AGT GAT GGC ATG GAC TGT GG) as endogenous control. Reaction products were separated on a 2% agarose gel and visualized following ethidium bromide staining using a Bio-Rad VersaDoc MP5000 molecular imager.

Quantitative PCR was performed as described previously using SYBR Green PCR Master Mix and the ABI PRISM 7900HT (Applied Biosystems Inc., CA).[677] The relative fold change in PD-L1 expression compared to the endogenous control GAPDH was calculated using the $2^{-\Delta\Delta Ct}$ method.

4.2.7 PD-L1 Knockout Using CRISPR/Cas9 in the A549 Cell Line

Target site sequences for Crispr/cas9 cleavage were identified using the online synthego CRISPR Design Tool (https://www.synthego.com). Accordingly, potential gRNA's (5′ACCTACTGGCATTTGCTGAA3′, 5′ TAGGGCATTCCAGAAAGATG 3′) with "NGG" as The Protospacer Adjacent Motif or PAM sequences for PD-L1 were selected and synthesized as Target-specific Alt-R® crRNA by IDT. The gene knock out procedure was performed according to the Alt-R CRISPR-Cas System Protocol from IDT. Briefly, for gRNA construction crRNA's and Alt-R® CRISPR-Cas9 tracrRNA, which is conjugated with ATTO™ 550 fluorescent dye, were mixed at the 1:1 ratio in Nuclease Free Duplex buffer at a final concentration of 5µM and stored at -20°C. CRISPR/Cas 9 ribonucleoprotein compartment (RNP) was generated by adding S. pyogenes Cas9 Nuclease into Opti-MEM media containing gRNA's and Cas9 plus reagent and incubated at room temperature for 10 minutes. Then, the RNP was transfected into A549 cells using Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent. After 24h, ATTO™ 550 positive transfected cells were single cell sorted using a FACS Area III (Becton Dickinson, USA) into a 96-well plate for clonal expansion.

After 2-3 weeks, expanded clones were harvested and Screened for PD-L-1 expression using PE-conjugated anti-PDL-1 antibody. For further confirmation of PD-L1 knock out., genomic DNA of the selected clone was extracted and exon 2 of PD-L1 was amplified and sequenced using specific primers (Forward -5′GGGAAAAAGCATTGACAGGTTG 3′ and Reverse -5′ GTAGAAAGAAGACTTTGCCATTG 3′) in order to confirm CRISPR/Cas9 mediated gene disruption.

4.2.8 Antibody-Mediated Neutralization of VEGF And PDGF

For neutralizing VEGF, goat anti-human VEGF antibody (1 μg/ml) was added to platelet and A549 co-cultures. Similarly, goat anti-human PDGF-AA (0.7μg/ml) and goat anti-human PDGF-BB (0.3μg/ml) antibodies were combined and used to neutralize all platelet-stored PDGF dimeric isoforms. Platelet-A549 co-cultures were incubated with antibodies for 24h, after which the surface expression of PD-L1 on A549 cells was analyzed using flow cytometry.

4.2.9 Jurkat and T-cell Activation Assays

A549 cells were co-cultured without and with platelets, as well as with Eptifibatide(10 μM)- or ASA (100 μM)-inhibited platelets. After 24h platelets were washed away from the culture and the A549 cells were incubated for 6 hours with pre-activated Jurkat cells (PMA 25 ng/ml and PHA-L 1μg/ml) at a 2:1 effector to target ratio or PBMCs cells (PMA 25 ng/ml and PHA-L 1μg/ml) at a 4:1 effector to target ratio, in the presence of Brefeldin A (10 μg/ml). Then the Jurkat cells or PBMCs were harvested and washed with PBS containing 2% FBS (Wash buffer). Subsequently, the Jurkat or PBMCs were fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes. After removing PFA, 0.1% triton X-100 in PBS was used to permeabilize the cells. Subsequently, the Jurkat or PBMCs were stained with FITC-conjugated Anti-CD3 (10 μg/ml) and PE-conjugated anti-IL-2 (1.25 μg/ml) antibodies and incubated at 4°C for 45 minutes. Finally, the Jurkat or PBMCs were washed with wash buffer and re-suspended in PBS and analyzed by flow cytometry using a LSRFortessa X-20 instrument. Data was analyzed with FlowJo 10.5.3 software (FlowJo, LLC, CA, USA).

4.2.10 Immunoblot

Immunoblot of platelet and A549 lysates to detect PD-L1 was performed under reducing/denaturing conditions as described previously[678]. Briefly, lysed platelet and A549 samples were subjected to 10% SDS-PAGE. After electrophoresis and transfer of gel onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA), the membranes were blocked overnight in blotting buffer with 5% non-fat milk. Subsequently, membranes were incubated with polyclonal anti-PD-L1 antibody (2 μg/ml) for 2 hours at room temperature. Subsequently, anti-goat horseradish peroxidase–labeled antibodies were used as the secondary antibody (Sigma-Aldrich). The immunoreactive bands were visualized with an ECL Plus kit (Amersham Biosciences, San Francisco, CA). The membrane was further probed with a β-actin-HRP antibody (1:35,000) (Sigma-Aldrich), which was used as a loading control. Chemiluminescence was detected using a VersaDoc MP5000 molecular imager with Quantity One software (Bio-Rad).

4.2.11 statistics

Statistics were performed using GraphPad Prism 8 software, and all means are reported with SEM. Two-tailed Student's T-tests and Ordinary and Repeated Measures One-way ANOVA with either Dunnett's multiple comparisons test or Tukey's multiple comparison test were performed where appropriate. A *P*-value less than 0.05 was considered as significant. All reported N's are independent experiments consisting of platelets obtained from different blood donors and different cell line passage numbers.

4.3 Results

4.3.1 Platelets Induce PD-L1 Surface Expression on Cancer Cells

To assess the effects of platelets on cancer cell PD-L1 expression, first we performed our co-incubation experiments in serum free medium, as FBS is rich in platelet-derived growth factors that are secreted during the clotting of fetal bovine blood. Under these conditions although less than 5% of A549 express PD-L1 (Fig 4.3.1A and B) their co-incubation with platelets resulted in a significant increase in the number of A549 expressing PD-L1 on their surface (2.7 ± 1.4% vs. 11.3 ± 2.5% of A549). To further demonstrate that platelets cause an up-regulation of PD-L1 expression by cancer cells, and not simply prevent loss of PD-L1 due to serum-starved conditions, we compared the effects of platelets to that of full medium on cancer cell PD-L1 surface expression. Compared to medium containing 10% FBS, platelets caused an increase in the percentage of A549 positive for PD-L1 on their surface (Fig. 4.3.2). As cancer cell PD-L1 positivity within non-small cell lung cancer can range widely with reports of 1 to >50% of cancer cells expressing PD-L1on their surface [679-682], likely due to tumor-associated inflammation, we next investigated whether platelets could further enhance IFNγ-stimulated PD-L1 expression by A549. Importantly, platelets potently increased IFNγ-stimulated PD-L1 surface expression by A549 (Fig 4.3.1D). To confirm that the platelet promotion of PD-L1 expression by cancer cells is not an epiphenomenon limited to platelet-lung cancer cell interactions, we co-incubated 786-O renal cell adenocarcinoma cells with and without platelets. Similar to results obtained with A549, platelets caused an increase in the percentage of 786-Oexpressing PD-L1 on their surface (11.2 ± 3.1% vs. 21.8 ± 4.8% of 786-O) (Fig 4.3.1E).

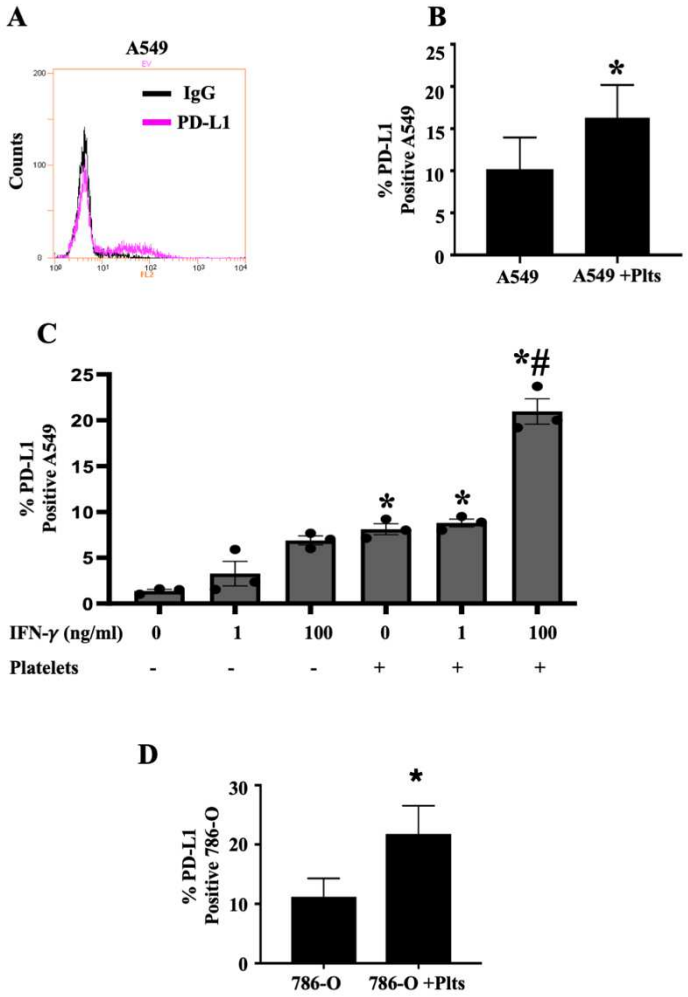


Figure 4.3.1. Platelets induce surface expression of PD-L1 on cancer cells.

(A) Representative flow cytometry histogram demonstrating detection of PD-L1 on the A549 surface membrane. **(B)** Summary data demonstrating platelets increase the percentage of A549 expressing PD-L1. N = 6. **(C)** Summary data demonstrating platelets further enhance IFNγ-stimulated PD-L1 expression by A549. N = 3. *, $P < 0.05$ vs. control. #, $P < 0.05$ vs. IFNγ 100 ng/ml. **(D)** Summary data demonstrating platelets increase the percentage of PD-L1 expressing 786-O. N = 3. *, P < 0.05. Plts – platelets. (Data in **A**, **B** and **D** were obtained with the assistance of E. Poitras, JK Rudzinski, and N. Govindasamy)

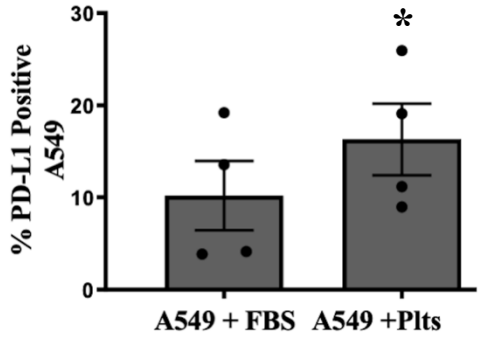


Figure 4.3.2. Comparing PD-L1 expression on A549 cells treated with FBS and Platelets.

Summary data demonstrating that elevated level of PDL-1 is not due to Serum starvation. N = 4.

**P*-Value < 0.05. Plts – platelets. Data were obtained with the assistance of V. Back.

4.3.2 Platelets Increase Cancer Cell PD-L1 at mRNA Levels

We next investigated whether the increased platelet-induced PD-L1 surface expression on cancer cells occurs due to transcriptional expression, or whether this surface increase simply results from cancer cell-bound platelets as a small percentage of platelets have also been demonstrated to express PD-L1 on their surface [683, 684]. Accordingly, A549 co-incubated with platelets showed significantly higher copy number of PD-L1 gene transcripts in comparison with control A549 cells (Fig 4.3.3A), while a control RT-PCR experiment demonstrated that only A549 expressed PD-L1 mRNA and not platelets (Fig 4.3.3B). Unlike after a 24h incubation, a 1h incubation of A549 with platelets did not induce a significant increase in A549 PD-L1 surface expression (Fig. 4.3.4), consistent with a need for gene transcription, translation, and *de novo* protein synthesis. Importantly, CRISPR/Cas9 -mediated PD-L1 knock out A549 when incubated with platelets did not cause a significant increase in PD-L1 on the A549 surface indicating that platelets increase the transcriptional expression of PD-L1 by A549 (Fig 4.3.3C). Further consistent with this data, only 2% of platelets isolated from healthy humans demonstrated to be PD-L1 surface positive , and immunoblot confirmed known glycosylated (~45 KDa) forms of PD-L1 in A549 and platelet lysates (Fig. 4.3.5A-C) [685] as well as potentially known dimers (~100 KDa).

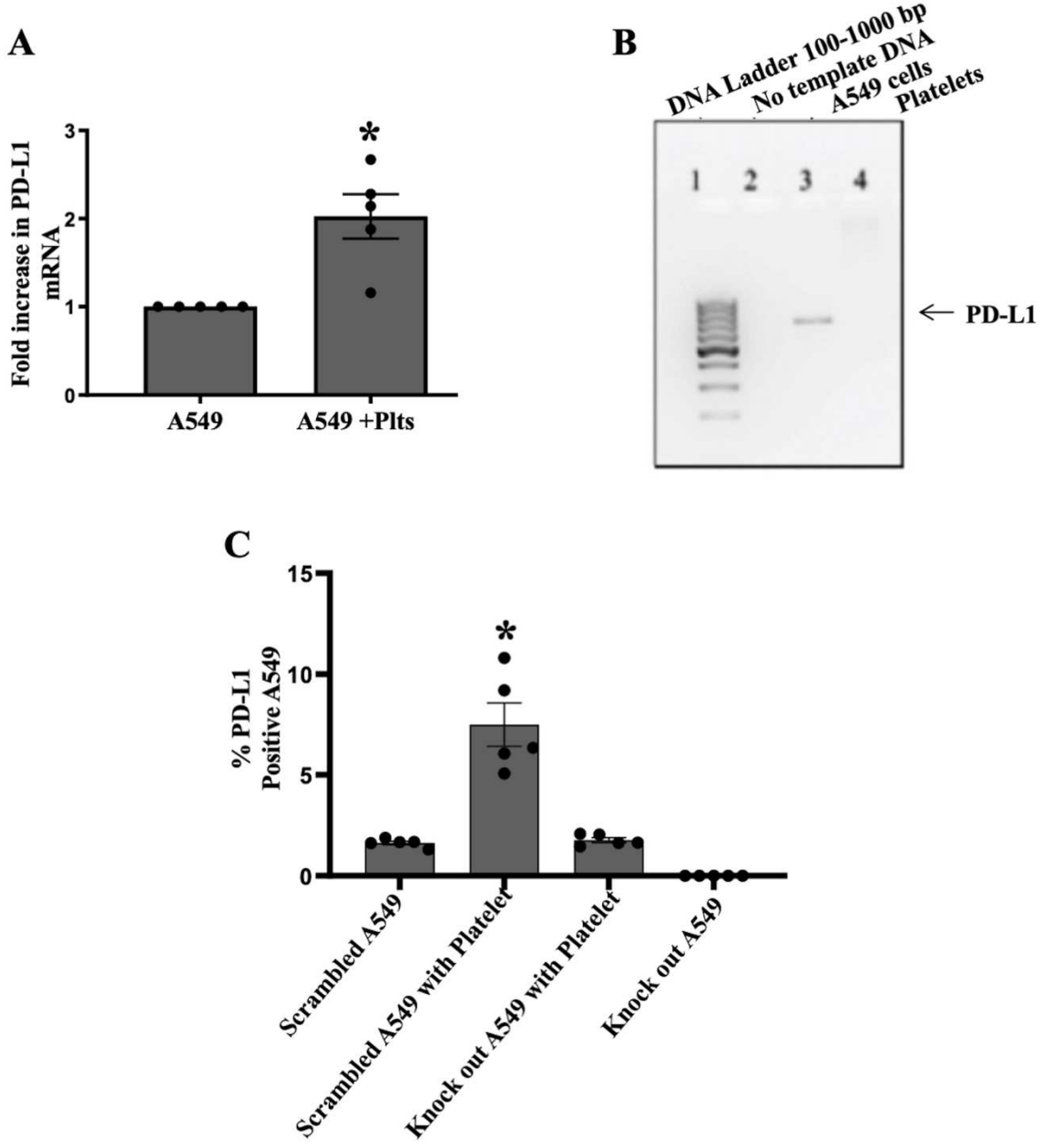

A
Fold increase in PD-L1 mRNA
3
2
1
0
*
A549
A549 +Plts
B
DNA Ladder 100-1000 bp
No template DNA
A549 cells
Platelets
1
2
3
4
PD-L1
C
% PD-L1 Positive A549
15
10
5
0
*
Scrambled A549
Scrambled A549 with Platelet
Knock out A549 with Platelet
Knock out A549

Figure 4.3.3 Platelets stimulate an increase in cancer cell PD-L1 mRNA.

(A) Summary data demonstrating platelets increase A549 PD-L1 mRNA. **(B)** A control experiment demonstrating that platelets do not express PD-L1 mRNA. Plts – platelets. **(C)** Comparison of PLD-1 expression by control scrambled and PD-L1 knock out A549 cells co-cultured in the presence or absence of platelets. N = 5. *, $P < 0.05$ vs. all other groups. (Data in **A** and **B** were obtained with the assistance of G. Lesyk)

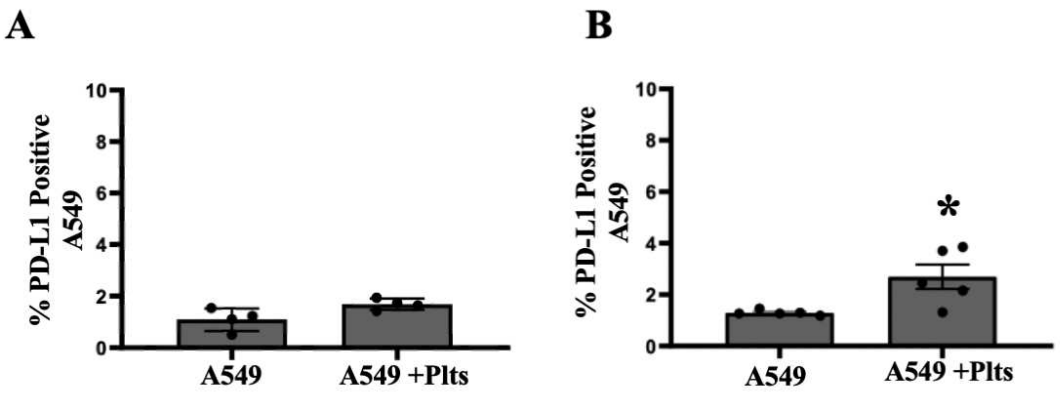


Figure 4.3.4 Surface expression of PD-L1 on A549 after 1 and 48 hour incubations with platelets.

(A) Summary data demonstrating A549 surface PD-L1 levels are not significantly elevated after a 1-hour incubation with platelets. N = 4. $P > 0.05$. **(B)** Summary data demonstrating A549 surface PD-L1 levels remain significantly elevated after a 48 hour incubation with platelets. N = 5. *, $P < 0.05$. Plts – platelets.

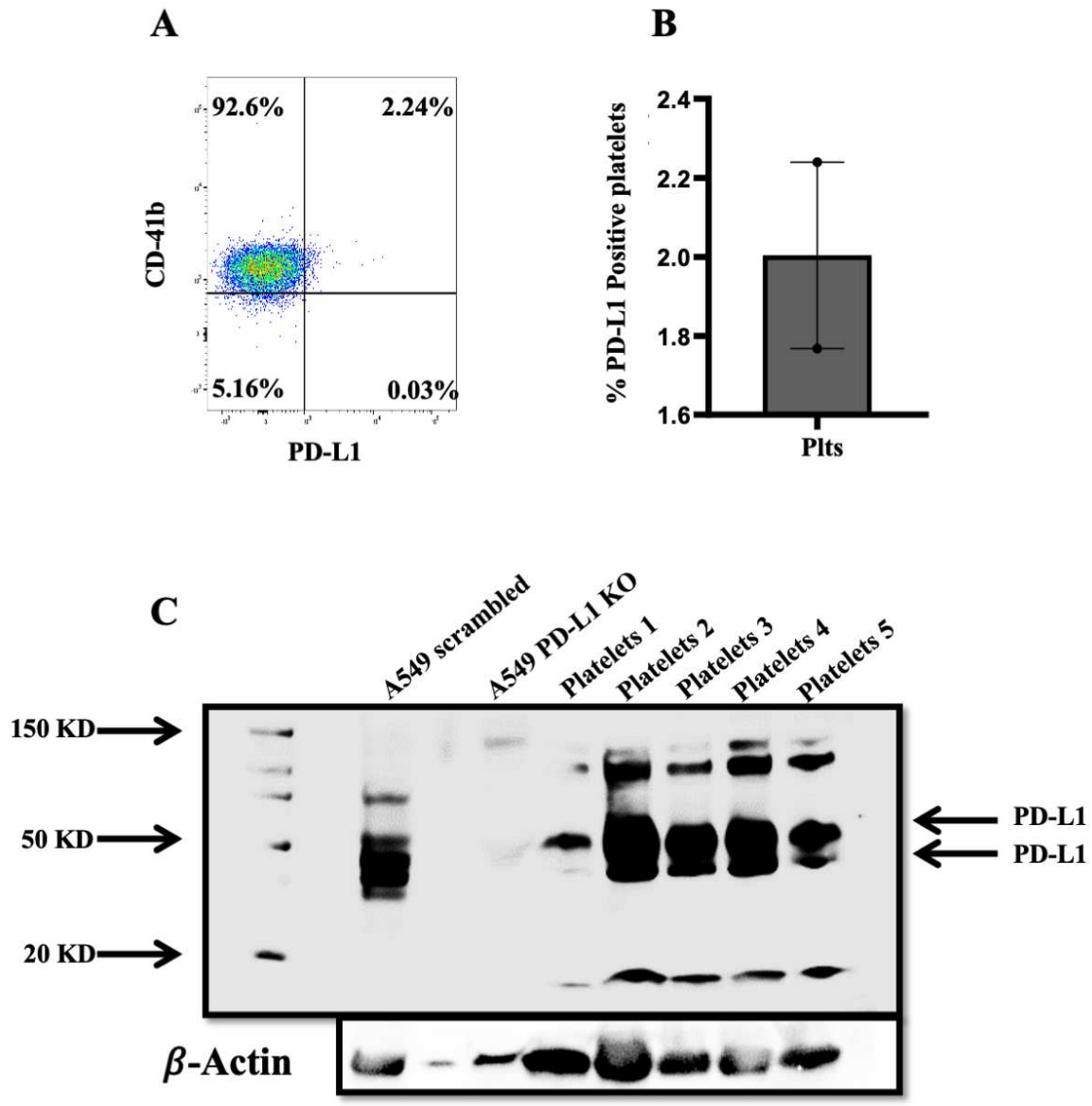


Figure 4.3.5 Evaluation of PD-L1 expression on platelets.

A representative dot plot and **(B)** summary data demonstrating PD-L1 expression on CD41b-positive platelets. N = 3. **(C)** Detection of PD-L1 by immunoblot in A549 and platelet lysates from 5 donors. A549 CRISPR scrambled and PD-L1 knockout lysates serve as positive and negative controls, respectively.

4.3.3 Identification of Platelet-Derived Growth Factors that Promote Cancer Cell PD-L1 Expression.

To assess whether VEGF and PDGF released from platelets enhances PD-L1 expression by cancer cells, neutralizing VEGF and PDGF antibodies were added to co-cultured A549 cells and platelets. In comparison to A549 incubated with platelets, incubation with a combination of both anti-VEGF with anti-PDGF antibodies resulted in a significant reduction in PD-L1 expression on A549 cells (6.7 ± 1.3% vs. 3.9 ± 0.8% PD-L1 positive; $P < 0.05$). No significant difference in PD-L1 surface expression was observed when either VEGF- or PDGF-neutralizing antibodies alone were utilized during the co-incubation of platelet and A549 cells (Fig. 4.3.6A). Consistent with these results both recombinant VEGF165 and PDGF-AB increased PD-L1 expression on A549 (Fig. 4.3.6B).

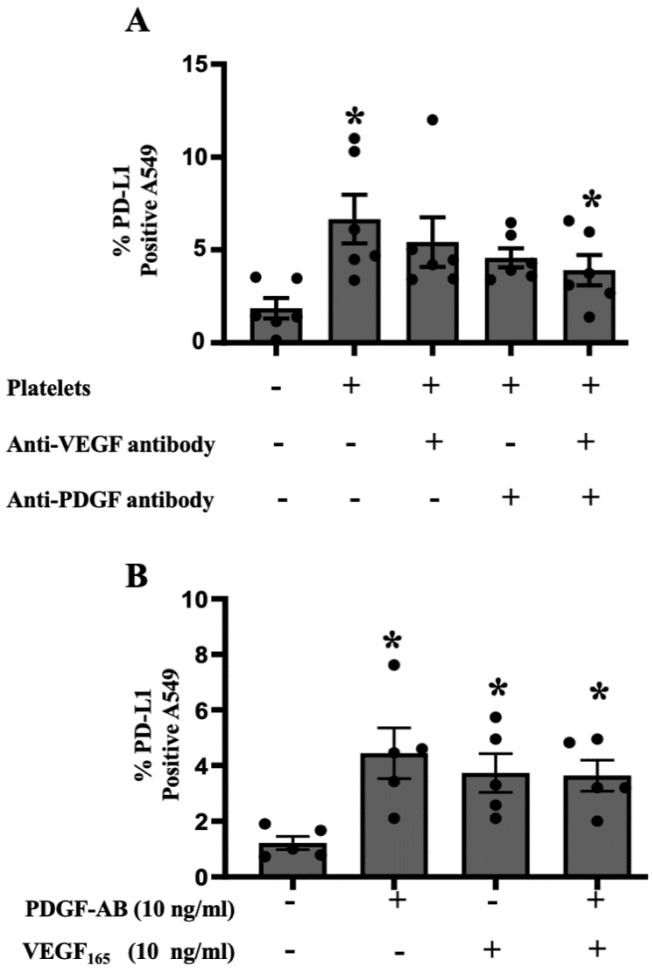

A
% PD-L1 Positive A549
15
10
5
0
*
*
Platelets - + + + +
Anti-VEGF antibody - - + - +
Anti-PDGF antibody - - - + +
B
% PD-L1 Positive A549
10
8
6
4
2
0
*
*
*
PDGF-AB (10 ng/ml) - + - +
VEGF165 (10 ng/ml) - - + +

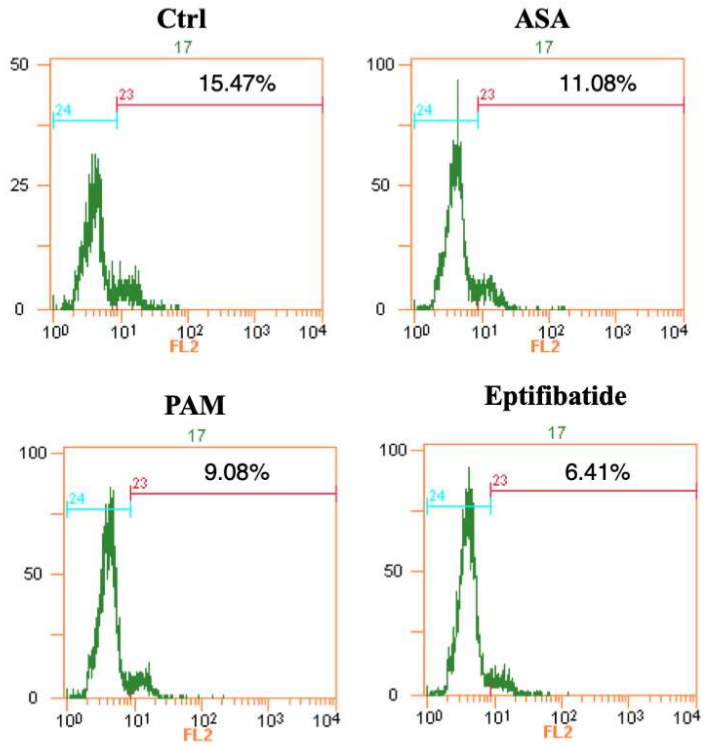
Figure 4.3.6 VEGF and PDGF secreted from platelets induces PD-L1 surface expression on A549 cells.

(A) Summary data demonstrating the effects of VEGF and PDGF neutralization on platelet-induced PD-L1 expression by A549 cells. Anti-VEGF antibody was used at (1 μg/ml) and anti-PDGF antibody represents the combined use of anti-human PDGF-AA (0.7μg/ml) and goat anti-human PDGF-BB (0.3μg/ml) antibodies in the co-culture of platelets with A549 cells. N = 6. *, $P < 0.05$ vs. indicated groups. **(B)** Summary data demonstrating recombinant $VEGF_{165}$ and PDGF-AB increase PD-L1 expression on A549. N = 5. *, $P < 0.05$ vs. control.

4.3.4 Evaluating the Effect of Anti-Platelet Drugs on Platelet-Induced PD-L1 Expression by Cancer Cells

We utilized clinically relevant anti-platelet drugs to investigate whether they would reduce the platelet-induced PD-L1 expression by cancer cells. We used ASA and Prasugrel active metabolite (PAM) to inhibit the platelet cyclooxygenase-1 and P2Y12 pathways, respectively, as well as eptifibatide to block integrin α_{IIb}/β_3, which mediates the final step of aggregation. eptifibatide was most effective at inhibiting platelet-induced PD-L1 expression by cancer cells (Control 13.3 ± 1.1% vs. ASA 9.4 ± 0.9% vs. PAM 10.0 ± 1.6% vs. Eptifibatide7.2 ± 0.6%) (Fig. 4.3.7A and B). In absence of platelet stimulation of A549, Eptifibatide did not significantly reduce PD-L1 expression (Fig. 4.3.8), although basal PD-L1 surface levels expression was low.

A


Ctrl
17
15.47%
24
23
50
25
0
10^0 10^1 10^2 10^3 10^4
FL2
ASA
17
11.08%
24
23
100
50
0
10^0 10^1 10^2 10^3 10^4
FL2
PAM
17
9.08%
24
23
100
50
0
10^0 10^1 10^2 10^3 10^4
FL2
Eptifibatide
17
6.41%
24
23
100
50
0
10^0 10^1 10^2 10^3 10^4
FL2

B

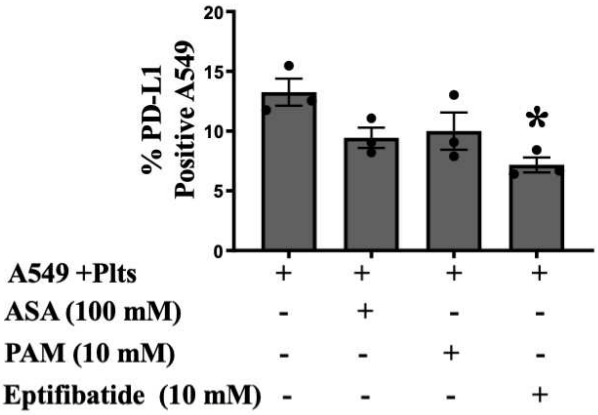

% PD-L1 Positive A549
20
15
10
5
0
*
A549 +Plts + + + +
ASA (100 mM) - + - -
PAM (10 mM) - - + -
Eptifibatide (10 mM) - - - +

Figure 4.3.7 Anti-platelet drugs prevent platelet-induced PD-L1 expression by A549 cells. **(A)** Representative flow cytometry histograms and **(B)** summary data demonstrating the effects of common anti-platelets drugs on the percentage of A549 expressing PD-L1 surface protein. N = 3. *, $P \leq 0.05$ vs. A549 + Plts control. Plts – platelets. (Data was obtained with the assistance of E. Poitras)

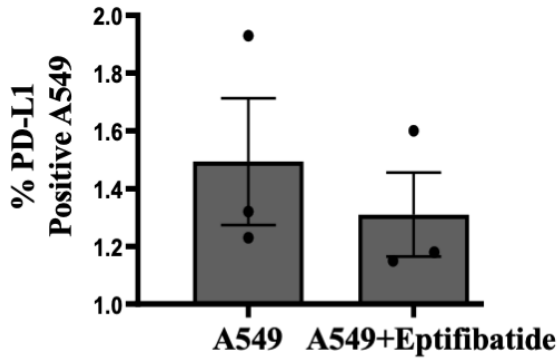


Figure 4.3.8 Surface expression of PD-L1 on A549 in the presence of Eptifibatide.

Summary data demonstrating A549 surface PD-L1 levels are not significantly decreased upon incubation with Eptifibatide in absence of a platelet stimulus. N = 3. P > 0.05. Plts – platelets.

4.3.5 Platelets Suppress Immune Cell Activation by Upregulating Cancer Cell PD-L1 Surface Expression

The effect of platelet-mediated PD-L1 expression by cancer cells on immune cell activation was determined by measuring expression of IL-2 by Jurkat cells incubated with A549. Higher expression of PD-L1 on A549 as a result of platelet co-incubation lead to a significant reduction in IL-2 expression by Jurkat cells in comparison to control A549 cancer cells (Fig. 4.3.9A). However, this immunoevasive effect was reversed by A549 co-cultured with Eptifibitide-inhibited platelets (Control 12.4 ± 1.4% vs. with platelets 8.9 ± 1.5% vs. with Eptifibitide-inhibited platelets 10.8 ± 1.4% IL-2-positive Jurkat; $P < 0.05$) (Fig. 4.3.9B), consistent with the ability of Eptifibatideto reduce platelet-mediated PD-L1 expression by A549 cells. Additionally, unlike Eptifibitide, treatment of platelets with ASA did not counteract the ability of platelets to reduce IL-2 expression by Jurkat cells, consistent with ASA's weaker ability to inhibit platelet-mediated PD-L1 surface expression by A549. (Fig. 4.3.10). Similarly, A549 co-incubated with platelets caused a significant reduction in IL-2 expression by human primary T-cells (CD3+) in comparison to control A549 cancer cells, an effect that was once again reversed by A549 co-cultured with Eptifibitide-inhibited platelets (Control 18.3 ± 1.5% vs. with platelets 14.9 ± 1.3% vs. with Eptifibitide-inhibited platelets 16.9 ± 1.2% IL-2-positive CD3+ cells; $P < 0.05$) (Fig. 4.3.5C and Fig. 4.3.11)

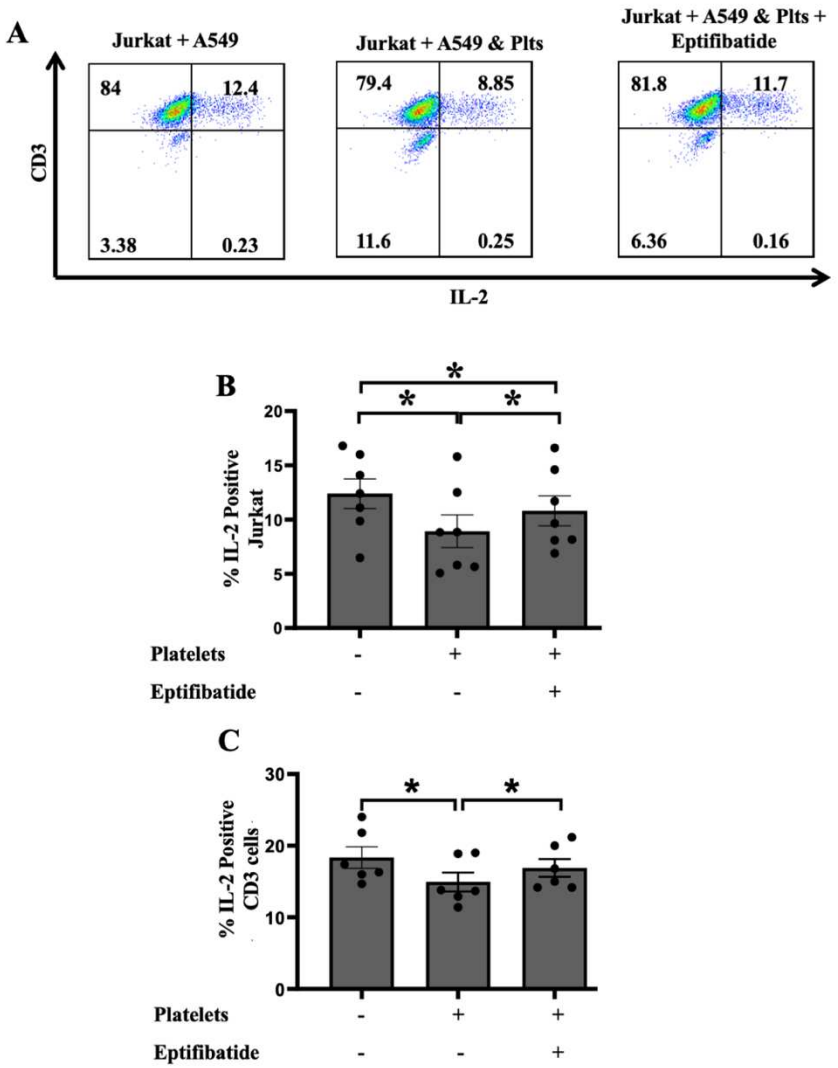


Figure 4.3.9 Platelets suppress T-cell activation by A549 cell an effect reversed by eptifibatide.

(A) Representative flow cytometry dot plots and **(B)** summary data demonstrating IL-2 expression in Jurkat cells after 6 hour of co-culture with A549 cells that were pre-treated with/without platelets and eptifibatide (10 μM). N = 7. **P*-value ≤ 0.05 vs. indicated groups. **(C)** Summary data demonstrating IL-2 expression in $CD3^+$ T-cells isolated from PBMCs after 6 hour of co-culture with A549 cells that were pre-treated with/without platelets and eptifibatide (10 μM). N = 6. **P*-value ≤ 0.05 vs. indicated groups.

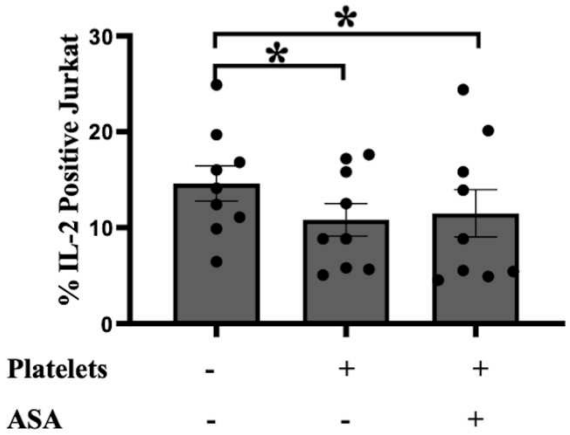


Figure 4.3.10 Analyzing IL-2 expression in Jurkat cells co-cultured with A549 after co-incubation with ASA-Inhibited Platelets.

Summary data demonstrating IL-2 expression in Jurkat cells after 6 hour of co-culture with A549 cells that were pre-treated with/without platelets and ASA (100 mM). N = 9. *, P <0.05 vs. indicated groups.

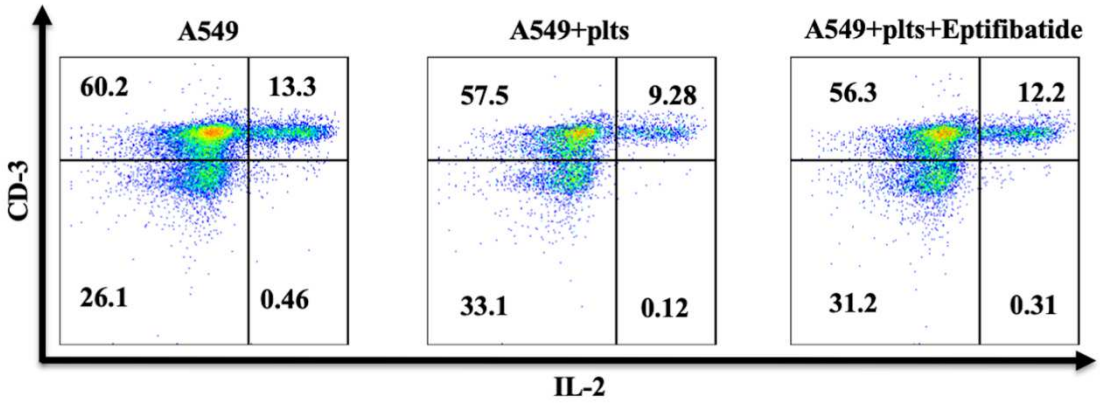


Figure 4.3.11 Platelets suppress T-cell activation by A549 cell an effect reversed by eptifibatide.

Representative flow cytometry dot plots demonstrating IL-2 expression in $CD3^+$ T-cells after 6 hours of co-culture with A549 cells that were pre-treated with/without platelets and eptifibatide (10 μM).

4.4 Discussion

In the current study we investigated the effect of platelets on cancer cells with regards to their PD-L1 expression and potential evasion of the adaptive immune response. Our results revealed that PD-L1 expression is up-regulated on the surface of cancer cells upon their interaction with platelets and this up-regulation reduces T-cell activation. Although only a small proportion of cancer cells express PD-L1 on their surface, a 2 – 4-fold increase in the number of cancer cells expressing it, caused by platelets, likely reflects a highly significant effect size considering that metastasis is an extremely inefficient and selective process in which less than 0.01% of circulating cancer cells succeed [450, 451]. In support of such a view is a recent study demonstrating that among urothelial carcinoma patients PD-L1 positivity was associated with shorter metastasis-free and overall survival in those with high platelet counts [686], while another study of patients with metastatic malignancies treated with immune checkpoint inhibitors showed that a mild thrombocytopenia (grade 1; nadir platelet count 75 – 150 x 10^3/μl blood) was associated with increased overall survival compared with non-thrombocytopenic patients [687]. Using platelets derived from healthy donors we showed that platelets increase cancer cell PD-L1 mRNA in addition to surface protein. This suggests that transcriptional activation of PD-L1 expression occurs in cancer cells following platelet interactions. Consistent with our current findings that few platelets contain PD-L1 on their surfaces, other recent studies have also demonstrated that only a small percentage of platelets (< 10%) from healthy human donors express PD-L1 on their surface, although this percentage can increase in patients with lung cancer, head and neck squamous cell carcinoma, and myeloproliferative neoplasms [683, 684]. Nonetheless, our data utilizing CRISPR-generated PD-L1 null A549 cells revealed that the primary mechanism by which platelets increase

cancer cell PD-L1 is via an up-regulation of its mRNA, and not due to platelet binding that may occur during TCIPA.

Upon activation and aggregation platelets secrete numerous growth factors that may stimulate transcriptional expression of PD-L1 by cancer cells including VEGF and PDGF [523]. Indeed, simultaneous blockade of secreted VEGF and PDGF during platelet-cancer cell co-culture experiments reduced cancer cell PD-L1 surface expression by approximately half. Hence, combined these α-granule stored growth factors play a crucial role in the induction of PD-L1 expression by cancer cells, likely via STAT-mediated signaling. It is not clear as to which platelet-derived factors are responsible for the remaining PD-L1 inducing effect as platelets from healthy donors secrete negligible amounts of IFNγ[688]. Other candidates include platelet-derived TGFβ which is known to down-regulate the innate immune response vs. cancer cells and has recently also been demonstrated to potentially regulate PD-L1 expression [689]. Although canonical TGFβ signalling is associated with activation of Smad, physical interaction of STAT1 with TGFβ-receptors has been reported to mediate crosstalk between the two pathways at least in ovarian cancer [690]. Alternatively, platelet-cancer cell integrin-mediated interactions may, in part, also be responsible for up-regulating cancer cell PD-L1 expression as recently cancer cell β3-integrin signalling has been shown to enhance PD-L1 expression [691]. Therefore, it is likely that multiple platelet factors may contribute to increasing cancer cell PD-L1 expression.

As platelet aggregation and secretion of growth factors can be inhibited to varying degrees by anti-platelet agents [523, 692], we utilized clinically relevant anti-platelet drugs to investigate whether they would reduce the platelet-induced PD-L1 expression by cancer cells and consequently restore T-cell activation in response to the cancer cells. In comparison to ASA and prasugrel active metabolite, eptifibatide was most effective at inhibiting platelet-induced PD-L1

expression by cancer cells suggesting that multiple platelet signalling pathways are involved in mediating this effect as it blocks the final step of aggregation. Therefore, dual anti-platelet therapy or α_{IIb}/β_3 blockade may be required to effectively inhibit VEGF and PDGF secretion and/or the platelet-cancer cell interactions that enhance PD-L1 expression.

Interestingly, anti-PD-L1 antibodies have recently been conjugated to platelets and anti-PD-L1 released platelet-derived microparticles shown to reduce cancer regrowth and metastatic spread in experimental models [693]. Whether such a therapy would benefit from adjuvant anti-platelet drugs or hindered by them was not investigated, however, our data demonstrate that integrin αIIb/β3 blockade and subsequent down-regulation of PD-L1 expression by cancer cells may lead to reduced immunoevasive capability and a more vigorous immune response against malignant cells. Finally, there is growing rationale for the use of adjuvant anti-platelet therapy in cancer, although the effects of not all anti-platelet drugs are equal [523, 672, 674]. Therefore, the possible addition of anti-platelet agents to novel immune checkpoint inhibitors may warrant careful investigation, particularly in patients that do not respond well to these new drugs and/or those already at risk of cancer-associated thrombosis. In this respect, an exploratory study involving NSCLC patients demonstrated an initial association between anti-platelet and immune checkpoint inhibitor treatment and longer progression-free survival and a trend toward better overall survival [694].

A limitation of our study is that we investigated the effect of platelets on a single B7 family member, PD-L1, although at present there are ten members of the B7 family that have been identified and other family members are also utilized by cancer cells for immunoevasion [695]. Nonetheless, in PD-L1, we focused on the most extensively investigated inhibitory ligand as a starting point for future research. Another limitation of our study is that we demonstrated increased

platelet-dependent PD-L1 expression within two cancer cell lines. Future studies will need to confirm this platelet-dependent mechanism beyond lung and renal cancer. Lastly, future studies should also focus on the potential metastasis promoting effects of platelet-induced B7 family protein expression by cancer cells, and conversely the potential immune-stimulatory and anti-metastatic effects of anti-platelet drugs.

Author Contributions

A. Asgari, G. Lesyk, E. Poitras, K. Terry, V. Back, J.K. Rudzinski, J.D. Lewis, and P. Jurasz contributed to concept and design. A. Asgari, G. Lesyk, E. Poitras, N. Govindasamy, Rachel To, V. Back, J.K. Rudzinski, and P. Jurasz contributed to the analysis and interpretation of data. A. Asgari, G. Lesyk, J.K. Rudzinski, and P. Jurasz contributed to the critical writing or revising the intellectual content. P. Jurasz gave final approval of the version to be published.

Conflicts of Interest

The authors have no conflicts of interest to disclose with respect to the content of this manuscript.

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Chapter 5

General discussion

5.1 Discussion

Platelets are small, anucleate cell fragments crucial to numerous physiological and pathophysiological processes, including hemostasis, wound healing, inflammation (notably in conditions like COVID-19 and sepsis), and the progression and metastasis of cancer. They contribute to these processes through the release of a variety of bioactive molecules, either stored within their granules or embedded in their membranes, playing vital roles in both normal bodily functions and disease states. The growing recognition of platelets' biochemical and functional complexity—despite their simple cellular structure—suggests that distinct subpopulations of platelets may have specialized roles, varying in size, density, and biochemical properties, each potentially influencing clinical outcomes and therapeutic strategies. For instance, variations in platelet response to anti-platelet drugs among different subpopulations can affect treatment efficacy and patient safety, emphasizing the importance of personalized medicine. Specifically, identifying platelet subpopulations that are more prothrombotic can be critical. By targeting these prothrombotic subpopulations with specific anti-platelet drugs that predominantly inhibit their activity, it is possible to reduce the incidence of microthrombosis—a common and severe symptom of COVID-19. Moreover, the ability to identify and characterize these platelet subpopulations opens the door to developing new diagnostic markers and therapeutic targets. Specific platelet subpopulations might be more prominent or functionally active in particular diseases, such as cardiovascular disorders or cancer, serving as potential indicators for disease presence, progression, or even prognostic purposes, thereby advancing both diagnostic and therapeutic aspects of medical care.

An important negative-feedback pathway that limits platelet adhesion, aggregation, and thrombus formation is mediated by nitric oxide (NO), which may be generated by both endothelial

cells and aggregating platelets themselves [104-106]. Previously, we reported on platelet subpopulations with differential abilities to produce NO based on the presence/absence of endothelial nitric oxide synthase (eNOS-positive and eNOS-negative platelets) revealing that eNOS-negative platelets are more reactive than eNOS-positive platelets and that they initiate thrombotic reactions [245]. Considering the well-documented hyper-inflammatory response associated with severe COVID-19[559], and the counter-regulation of eNOS and iNOS expression by inflammatory cytokines[560, 561], in the second chapter of this thesis, I explored whether platelet ratios of eNOS-positive to eNOS-negative platelets are altered in moderate to severe SARS-CoV-2 infection and whether this contributes, in part, to increased platelet reactivity in COVID-19.

In the effort to study how COVID-19 influences eNOS-positive platelet levels, a pivotal transition was made from using mouse models to employing the human megakaryoblastic cell line Meg-01. This shift was necessitated by prior investigations which established that mouse models, including eNOS-GFP transgenic mice, were not suitable for studying eNOS-based platelet subpopulation dynamics due to the negligible presence of eNOS in mouse platelets and megakaryocytes, rendering them ineffective for research objectives[245]. In contrast, the human Meg-01 cell line emerged as a suitable model, exhibiting detectable levels of eNOS in a significant proportion of cells—similar to findings in platelets from COVID-19-negative individuals. This model's compatibility with research goals was further supported by reports confirming NOS activity in human megakaryocytes and Meg-01 cells[530, 531].

In the second chapter of this thesis, it was established that COVID-19 patients show significantly elevated ratios of eNOS-negative to eNOS-positive platelets compared to controls, indicating that the predominant platelet subpopulation in COVID-19 patients is eNOS-negative,

whereas in non-COVID-19 controls, it is predominantly eNOS-positive. These altered ratios correlate strongly with disease severity and are associated with increased platelet reactivity, as indicated by elevated surface expression of CD62P.

A shift in the ratio of these subpopulations could have significant implications for microvascular thrombosis and platelet reactivity, particularly in COVID-19 where inflammation and endothelial dysfunction are prominent. An increased proportion of eNOS-negative platelets, possibly more prone to activation, could exacerbate thrombotic events in these patients. This ratio also offers potential as a biomarker for assessing thrombosis risk, aiding in the prediction and management of thrombotic complications. Furthermore, it opens avenues for tailored anti-thrombotic therapy, where treatment can be specifically directed towards inhibiting the more reactive subpopulation of platelets. However, while this approach holds promise, it requires cautious application, considering the complex dynamics of platelet behavior in inflammatory states and the critical balance necessary to avoid excessive bleeding while preventing thrombosis. Thus, further research is essential to validate these subpopulations as therapeutic targets and biomarkers in the clinical management of COVID-19.

Building on the insights into the dynamics of platelet subpopulations in COVID-19, repurposing oral phosphodiesterase-5 (PDE-5) inhibitors, such as sildenafil and tadalafil, presents a strategic therapeutic approach. Initially approved for pulmonary hypertension[696] and later for erectile dysfunction, these agents are now recognized for their broad pharmacological effects, including anti-apoptotic, anti-inflammatory, antioxidant, and immunomodulatory actions[697, 698]. These properties make them particularly appealing for COVID-19 treatment due to their ability to modulate the nitric oxide (NO)/soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway, which can reduce pulmonary vasoconstriction and potentially

mitigate severe complications associated with COVID-19[699]. The established safety and tolerance profile of these drugs is crucial for rapid repurposing. They are effective in various COVID-19 severity levels and against different variants due to their mechanism of action, and by modulating platelet activation and aggregation, they could lessen thrombotic events, aligning with the observed changes in platelet behavior in COVID-19[700, 701]. However, despite promising preclinical data, there is a scarcity of robust clinical trials substantiating their effectiveness specifically for COVID-19, and the repurposing process can be impeded by high costs, regulatory hurdles, and the need for extensive real-world evidence to confirm efficacy and safety in new indications. Typical dosing for sildenafil is 20 mg three times a day for pulmonary hypertension; doses might need adjustment based on its use for COVID-19[702]. Tadalafil, known for a longer half-life, might offer a dosing advantage, typically administered as 5 mg once daily[702]. Given their potential benefits, further rigorous clinical trials are essential to determine optimal dosing and to validate the efficacy of PDE-5 inhibitors in COVID-19, ensuring their place as an therapeutic option against this and potentially other viral outbreaks.

Furthermore, it was measured that COVID-19 patients exhibit higher plasma levels of TNF-α, IL-6, and IL-1β compared to controls. These elevated levels of inflammatory cytokines are linked to the promotion of eNOS-negative Meg-01 cell formation and subsequently, the increased production of eNOS-negative platelet-like particles. Given the elevated cytokine levels in COVID-19 patients, particularly TNF-α, IL-1β, and IL-6, it becomes imperative to explore their broader role beyond altering eNOS expression in platelets. These cytokines likely influence other key factors within these cellular fragments, which could be crucial in modulating common COVID-19 complications such as microthrombosis and vascular leakage.

Among the factors stored in platelets that can modulate vascular permeability are PDGF and VEGF. Initially characterized as Vascular Permeability Factor (VPF) [366, 385-387], VEGF plays a pivotal role in weakening the endothelial cell barrier and enhancing vascular permeability. Before analyzing the effects of COVID-19-associated inflammatory cytokines on the VEGF content in platelets, it is crucial to understand the baseline presence and status of these factors in healthy individuals. The current research has identified α-granule-enriched subpopulations of platelets marked by CD62P, which exhibit high levels of VEGF and PDGF. Further characterization of these platelets has shown them to be eNOS-positive, delineating a distinct profile for VEGF-high/PDGF-high platelets.

In patients with COVID-19, platelets demonstrated an increased overall mean in VEGF content and a higher proportion of VEGF-enriched platelet subpopulations compared to controls who were COVID-19-negative. Notably, in healthy donors, eNOS-positive platelets were more VEGF enriched that eNOS-negative platelets. However, the predominant subpopulation of platelets in COVID-19 ICU patients is the eNOS-negative subpopulation, constituting approximately 80%. This observation indicates a significant alteration in the character of platelet subpopulations—a dysregulation that leads to the eNOS-negative subpopulation being enriched in VEGF to a greater extent compared to the eNOS-positive subpopulation. These platelets have a strong potential to disrupt the endothelial barrier in the capillary-alveolar membrane in the lungs of COVID-19 patients, as opposed to the less reactive eNOS-positive platelets, which contain almost all the VEGF in healthy donors.

VEGF expression has been shown to be induced by TNF-α[376] and hypoxia[372-374], conditions prevalent in COVID-19. Activation of NF-kB, a transcription factor that can be activated by TNF-α, has been shown to induce VEGF-A expression in megakaryocytes[536],

which subsequently is inherited by their progeny, the platelets. Significant increases in plasma TNF-α concentrations were observed in COVID-19 patients, and incubation of Meg-01 cells with TNF-α resulted in the formation of a distinct Meg-01 subpopulation with elevated intracellular VEGF levels. These data suggest that the increased VEGF content observed in COVID-19 ICU patient platelets may be due to the upregulation of TNF-α. This upregulation induces VEGF expression in megakaryocytes, which is then inherited by their progeny, the platelets. This may predispose COVID-19 patients to increased vascular permeability and potential pulmonary edema upon thrombosis. Moreover, based on the mechanisms described in the context of COVID-19, it is plausible to speculate that similar changes in platelet subpopulations might occur in other infectious diseases, such as influenza, that involve similar inflammatory conditions. TNF-α and hypoxia, conditions often present in severe respiratory infections including influenza, suggest a potential for similar mechanisms to influence platelets' subpopulation across these diseases. If influenza or other respiratory infections significantly elevate TNF-α levels, similar to what is observed in COVID-19, they could potentially also drive the formation of distinct platelet subpopulations with elevated VEGF content.

In the final chapter of this thesis, the role of platelets in immune reactions relevant to cancer was investigated. In the realm of oncology, platelets play a key role in promoting hematogenous metastasis, primarily through mechanisms such as tumor cell-induced platelet aggregation (TCIPA) [399]. One mechanism by which TCIPA creates a cancer cell-favorable microenvironment is via the platelets' ability to protect malignant cells from the innate immune system and prevent natural killer (NK) cell-mediated cell death. Although much is known about how platelets affect the NK-mediated innate immune response [459, 460, 462, 543], our understanding of platelet function in modulating the adaptive immune system in cancer is limited.

A major negative regulator of the adaptive immune response is Programmed Death Ligand 1 (PD-L1; B7-H1), an immune checkpoint B7 family transmembrane protein that contributes to T-cell inhibition[481, 482].

In the first part of this chapter, the influence of platelets on PD-L1 expression by cancer cells was evaluated. It was demonstrated that platelets can induce a 2–4-fold increase in PD-L1 expression on cancer cells. This finding is particularly significant given the selective nature and overall inefficiency of the metastatic process. Although only a minority of cancer cells express PD-L1, the observed upregulation is noteworthy. By nature, metastasis is an exceedingly selective and inefficient process, with less than 0.01% of circulating cancer cells successfully establishing secondary tumors[450, 451].

Using platelets derived from healthy donors, we have demonstrated that platelets increase cancer cell PD-L1 mRNA and surface protein levels. This observation suggests that transcriptional activation of PD-L1 expression in cancer cells is initiated following interactions with platelets. However, this process raises a significant debate over the exact mechanism by which platelets mediate the up-regulation of PD-L1 in cancer cells. The central question is whether this upregulation is facilitated by the direct transfer of PD-L1 from platelets to cancer cells due to attachment or via a mechanism in which platelets release specific factors or integrin interactions that induce or activate PD-L1 gene expression in cancer cells.

Consistent with our current findings, which show limited PD-L1 presence on platelets, recent studies corroborate that a minimal proportion of platelets from healthy individuals (less than 10%) express PD-L1. Interestingly, this proportion is notably elevated in various cancer conditions, such as lung cancer, breast cancer, head and neck squamous cell carcinoma, myeloproliferative neoplasms, and ovarian cancer[683, 684]. Hinterleitner *et al.*'s analysis of

platelets from 64 healthy donors and 128 NSCLC patients revealed a significant difference in PD-L1 expression: a median of 0.29% in healthy donors versus 0.89% in NSCLC patients[703]. Colarusso *et al*. examined 9 healthy donors, 23 Stage I-III NSCLC patients, and 64 Stage IV NSCLC patients, finding elevated platelets PD-L1 levels in Stage IV patients[704]. This trend was also observed by Park *et al*. in their study, which included six healthy donors and patients with non-small cell lung cancer (NSCLC). They reported only marginal PD-L1 expression in platelets from healthy donors, but a significant, more than tenfold increase in expression in cancer patients[705]. Additionally, Darga *et al*. did not detect PD-L1 on platelets from 12 healthy donors but found PD-L1 expressio by platelets in 28% of from 124 metastatic breast cancer patients[706]. Similarly, Rolfes *et al*. observed PD-L1 upregulation in 37 HNSCC patients compared to 6 healthy donors, with lung cancer patients also showing increased PD-L1 expression on platelets[683].

Nonetheless, our investigations, particularly with CRISPR-generated PD-L1 null A549 cells, shed light on the complex interactions between platelets and cancer cells in the tumor microenvironment. We observed that platelets are capable of increasing PD-L1 expression in cancer cells, primarily through mRNA up-regulation. Additionally, the work of Zaslavsky *et al*. contributes to this understanding by demonstrating that the co-incubation of various PD-L1 negative cancer cell lines (such as UMUC-5, MCF-7, PANC-1, VCaP, 22RV1) with platelets results in an up-regulation of PD-L1 on the cancer cells. This up-regulation was attributed to the membrane fusion between PD-L1 positive platelets and cancer cells, potentially occurring during tumor-cell induced platelet aggregation (TCIPA) [707]. Complementing this, Hinterleitner's research sheds light on the reverse scenario, wherein blood platelets in contact with lung cancer cells, both in vitro and *in vivo*, take up PD-L1 from the cancer cells. This process is mediated

through fibronectin, integrin α5β1, and GPIbα, and interestingly, it transpires without substantial platelet activation or degranulation[703].

Linking this understanding to the therapeutic potential of anti-platelet drugs, it becomes clear that targeting integrin αIIb/β3 could reduce PD-L1 expression on cancer cells, thereby diminishing their ability to evade the immune system. This could potentially enhance the effectiveness of immune checkpoint inhibitors (ICIs), leading to a more robust immune response against tumors. This strategy shows promise particularly for patients who do not respond adequately to standard ICI therapy or are at an elevated risk of thrombosis. Preliminary clinical outcomes in NSCLC patients support this approach, indicating that combining anti-platelet drugs with ICIs could improve both progression-free and overall survival[694].

Blockade of VEGF and PDGF during platelet-cancer cell co-culture experiments significantly reduced PD-L1 expression on cancer cells by about half, highlighting the importance of these α-granule-stored growth factors, likely mediated through STAT signaling. In another study, Cho *et al*. conducted a study on specific ovarian cancer cell lines like SKOV3, OVCAR8, HeyA8, A2780, OVCAR432, and OVCAR4[708]. Their research uncovered additional layers in the platelet-cancer cell interaction. They found that direct contact between platelets and these cancer cells led to an increase in PD-L1 expression, primarily through the activation of NF-κB and Smad2/3 signaling pathways, a distinct mechanism from the VEGF and PDGF pathways identified in our study. This was further evidenced by the increased phosphorylation of P65 and Smad2/3 in the cancer cells post-exposure to platelets[708]. Notably, the absence of significant STAT1/3 phosphorylation suggested a different mode of action compared to the one we observed. Additionally, Cho *et al*. observed that indirect exposure to platelets, akin to the tumor microenvironment, enhanced PD-L1 expression mainly through the TGFβR1/Smad signaling

pathway, again differing from our findings and adding to the complexity of the platelet-cancer cell dynamics.

Also, Guo *et al*. explored the mechanisms behind platelet-induced upregulation of PD-L1 expression in cancer cells, focusing specifically on the role of the EGF/EGFR pathway. They conducted experiments using various cancer cell lines, including MCF7, MDA-MB-468, and A549 lung cancer cells, to explore this intricate interaction. Their research revealed that the response of cancer cells to platelets is closely tied to their EGFR expression levels. In cell lines with lower EGFR expression, such as MCF7, there was no notable increase in PD-L1 expression following exposure to platelets. This finding contrasted sharply with the response in higher EGFR-expressing cells like MDA-MB-468, which showed a significant upregulation of PD-L1 when co-cultured with platelets. In the case of A549 lung cancer cells, while there was an increase in PD-L1 expression upon exposure to platelets, the response was less pronounced compared to the MDA-MB-468 cell line, indicating a variable response among different cell types based on their EGFR status. This upregulation was diminished when EGFR was knocked out in MDA-MB-468 cells, using CRISPR technology, underscoring a role for the EGF/EGFR pathway in the platelet-mediated upregulation of PD-L1 by some cancer cells [709].

In light of these findings, our understanding of the intricate interplay between platelets and cancer cells in the modulation of PD-L1 expression has significantly deepened. The diverse pathways identified – from VEGF and PDGF involvement to NF-κB, Smad2/3, and EGF/EGFR signaling dynamics – underscore the complexity of this interaction. Recent insights into platelet-cancer cell integrin-mediated interactions, such as the enhancement of PD-L1 expression via β3-integrin signaling, reveal a multifaceted mechanism of PD-L1 regulation[691]. This body of evidence suggests that multiple platelet-derived factors, each following its own unique signaling

pathway or engaging in crosstalk among pathways, collaboratively contribute to the upregulation of PD-L1 on tumor cells.

Next, a comparison of the effectiveness of various anti-platelet drugs, including aspirin (ASA), prasugrel active metabolite (PAM), and eptifibatide, our study revealed that eptifibatide, fibrinogen receptor (GPIIb/IIIa) inhibitor, was the most effective in inhibiting platelet-induced PD-L1 expression in cancer cells. Further studies by other groups have demonstrated that anti-platelet agents, particularly aspirin and ticagrelor, can significantly reduce the interaction between platelets and cancer cells, thereby diminishing PD-L1 expression [707-709]. Further supporting this notion, research has highlighted the role of specific platelet adhesion molecules in the process of PD-L1 transfer. Inhibiting these molecules, notably GPIbα and integrin α5ß1, has a significant impact on PD-L1 transfer, emphasizing the nuanced mechanisms at play [691, 703]. However, not all inhibitors show the same level of effectiveness; for example, targeting integrin αIIb/β3 (GPIIbIIIa) demonstrated only a marginal effect on PD-L1 transfer, underscoring the variability in response to different anti-platelet strategies[703]. Additionally, the findings from ovarian cancer studies, where aspirin and ticagrelor led to a substantial decrease in PD-L1(+) cells in tumors[708], further corroborate the potential of anti-platelet drugs in modulating PD-L1 expression and improving the efficacy of cancer immunotherapies. This suggests that anti-platelet drugs could be an effective adjunct in treatments involving immune checkpoint inhibitors (ICIs), potentially reducing the risk of thrombosis associated with cancer and its treatment.

Of particular importance in our study is the evaluation of whether up-regulation of PD-L1 on the A549 cell line enhances their capacity for immune evasion against Jurkat cells and influences IL-2 expression within these T cells. It is crucial to acknowledge the presence of a major histocompatibility complex (MHC) mismatch between the A549 cells and the effector cells used,

including Jurkat cells and primary T cells isolated from peripheral blood mononuclear cells (PBMCs). This mismatch arises because MHC molecules are highly polymorphic and vary widely among individuals, playing a pivotal role in the immune system's ability to distinguish self from non-self[710]. The presence of this mismatch is essential for simulating the realistic immune interactions that could occur during transplant rejection or in autoimmune settings, thus providing a critical framework for our observations on PD-L1 mediated immunomodulation.

The collective evidence from various studies, including our own, underscores the effectiveness of anti-platelet drugs such as ASA (aspirin), prasugrel, and eptifibatide in reducing PD-L1 expression on cancer cells. However, the varying extent of their efficacy points to a complex interplay among multiple signaling pathways in platelet-cancer cell interactions. Consequently, a tailored approach, possibly involving a combination of different anti-platelet agents, might be crucial to maximize their therapeutic potential in enhancing immune responses against cancer. Careful consideration of dosage is essential to balance efficacy with the risk of bleeding, particularly in cancer patients who may be more susceptible to such complications.

In conclusion, the findings from this thesis elucidate the complex interactions between platelets and cancer and highlight the functional changes in platelets in COVID-19 as an example of an inflammatory disease. In COVID-19 patients, a significant shift towards a higher eNOS-negative to eNOS-positive platelet ratio was observed, likely due to an inflammatory-mediated reduction in megakaryocyte eNOS expression, which may predispose these patients to thrombosis. Additionally, there is evidence of a novel α-granule-enriched platelet subpopulation with elevated VEGF and PDGF levels in healthy donors. A notable change in the subpopulation of VEGF-enriched platelets was observed in COVID-19 patients, potentially driven by TNF-α upregulation, leading to increased VEGF expression in megakaryocytes. This mechanism may contribute to

heightened vascular permeability and the risk of pulmonary edema following thrombosis. Furthermore, the thesis highlights that platelets can enhance PD-L1 mRNA and surface protein expression in cancer cells, such as A549 and 786-0. Neutralization of VEGF and PDGF, as well as the use of the anti-platelet drug eptifibatide, effectively prevented this platelet-induced up-regulation of PD-L1, thereby inhibiting the suppression of human T-cell activation by cancer cells.

5.2 Limitation of Study

Our study has a number of limitations. First, our non-ICU COVID-19 patient platelets and plasma were obtained just prior to the start of the alpha SARS-Cov-2 variant driven wave of COVID-19 while ICU COVID-19 platelets and samples were obtained from patients during the delta driven wave. Hence, it is not clear whether the different variants differentially impacted eNOS-based platelet subpopulations generation in COVD-19 patients. Second, we did not investigate whether a cause of platelet eNOS loss is platelet death as SARS-Cov-2 infection has been demonstrated to initiate programmed cell death pathways including apoptosis in platelets [590]. However, we have previously demonstrated that eNOS-negative platelets are not platelets undergoing apoptosis [245], and mouse platelets lacking eNOS are not apoptotic. Also, it should be noted that platelets are not the only source of NO in plasma and down-regulation of eNOS in endothelial cells due to COVID-19 might also contribute to the observed lower concentration of NO_x in plasma and therefore the platelet hyperactivity. Additionally, it is not clear whether different ratios of eNOS-positive to –negative platelet-like particles would have been obtained with cultured primary human megakaryocytes exposed to COVID-19 relevant cytokines. Lastly, due to the relatively low numbers of platelet-like particles produced by Meg-01 in culture we were unable to perform platelet function testing such as light-transmittance aggregometry or a flow-chamber assay.

In Chapters two and three also utilized Meg-01 cells, a megakaryocyte cell line, as a model system. While this cell line provides a cell model that facilitates the study of specific biochemical pathways, it must be noted that cancer cells can be more complex and variable than megakaryocytes from bone marrow. Although it is technically possible to obtain and assess bone marrow-derived megakaryocytes, the highly invasive nature of bone marrow biopsies makes it

difficult to secure such samples routinely. This limitation affects the ability to directly incubate primary megakaryocytes with TNF-α, thus restricting the generalizability of the findings to the physiological conditions present in COVID-19 patients. Another limitation is the absence of functional analyses with live platelets isolated from COVID-19 patients, particularly in terms of their potential to increase endothelial cell permeability compared to platelets from healthy controls. This gap arises from the risks associated with viral transmission, which prevent the handling of blood drawn from COVID-19 patients and the isolation of platelets in standard laboratory settings.

In chapter four, the study concentrated on PD-L1 due to its prominence and extensively documented role in mediating immune evasion by tumors. PD-L1, a member of the B7 family, plays a crucial role in inhibiting T-cell activation[481, 482], thereby facilitating tumor survival and progression. However, it's important to note that the B7 family encompasses ten members, each with distinct roles in the immune landscape[695]. This diversity in function highlights a limitation in our study: by focusing solely on PD-L1, we have explored just one facet of a complex system. Other B7 family members, utilized by cancer cells for immunoevasion, remain unexamined in the context of platelet-cancer interactions.

Additionally, our investigation demonstrated increased platelet-dependent PD-L1 expression within two specific cancer cell lines, representing lung and renal cancers. While these findings provide valuable insights into the potential role of platelets in cancer progression, the applicability of this mechanism across the diverse spectrum of cancer types remains to be determined. The heterogeneity inherent in cancer suggests that the interactions between platelets and cancer cells, and the consequent effects on PD-L1 expression, may vary significantly across different tumor types.

5.3 Future Direction

The findings from second chapter lay the groundwork for numerous potential research directions. One particularly compelling avenue involves assessing the efficacy of current anti-platelet agents on eNOS-negative and eNOS-positive platelets. This investigation could illuminate whether these therapies impact both subpopulations equally, offering a foundation for the development of novel inhibitors targeting eNOS-negative platelets specifically. Such research could also shed light on the role of eNOS subpopulation ratios in the varied responses observed with anti-platelet therapy, contributing valuable insights into personalized medicine approaches for thrombotic disorders.

While the Meg-01 cell line has proven instrumental in our studies, the pursuit of validation using primary human megakaryocytes exposed to COVID-19-related cytokines represents a critical next step. This approach would not only mirror *in vivo* conditions more closely but also enhance the relevance of our model for eNOS-based platelet subpopulation research. Expanding this validation to include bone marrow-derived megakaryocytes from both healthy donors and patients with inflammatory diseases, and related cardiovascular complications, could further refine our understanding of megakaryocyte and platelet biology under disease conditions. Furthermore, identifying and incorporating animal models that closely resemble the human platelet phenotype could provide deeper insights into the systemic effects of platelet subpopulations *in vivo*, thereby enhancing the translational potential of this research for clinical applications. Although initial attempts with mouse models were not successful due to species differences, exploring other animal models that more accurately replicate the human platelet phenotype is crucial for advancing this research and boosting its clinical relevance.

Finally, future research should focus on validating whether alterations in the ratio of eNOS-negative to eNOS-positive platelet subpopulations are linked to inflammation and consequently to adverse cardiovascular events such as myocardial infarction and stroke. This ratio holds promise as a biomarker for both diagnosing and prognosticating various inflammatory conditions, including cardiovascular diseases and other viral infections. Investigating the significance of this ratio across a spectrum of diseases could provide valuable insights into underlying disease mechanisms, thus offering dual benefits: enhancing early diagnosis and enriching prognostic evaluations.

As we advance our understanding of the changes in VEGF content within platelets in the context of COVID-19, particularly noting the increase in VEGF-enriched platelet subpopulations, future research directions present several intriguing possibilities. One key area of exploration is the mechanistic basis of platelet alteration in response to inflammatory stimuli, specifically the role of TNF-α, IL-1β, and IL-6 in modulating platelet function and content. Further studies are needed to delineate how these inflammatory mediators influence the biogenesis and differentiation of megakaryocytes, and consequently, how they impact the composition and functionality of the resulting platelets. This line of investigation will be crucial in understanding the pathophysiological mechanisms underlying the vascular complications seen in COVID-19 and potentially other inflammatory and infectious diseases. Moreover, a deeper exploration of the signaling pathways and molecular mechanisms driving the upregulation of VEGF within platelets could provide new insights into targeted strategies. Specifically, these strategies could aim at inhibiting or suppressing VEGF-enriched platelets to mitigate adverse outcomes such as the disruption of the endothelial barrier or vascular permeability following platelet activation, while minimally impacting their crucial role in hemostasis.

Building upon the significant finding of increased VEGF-enriched platelet subpopulations in COVID-19 patients, a crucial area for future clinical research involves examining the potential therapeutic benefits of commonly used antiplatelet drugs. Agents like aspirin, P2Y12 antagonists (such as clopidogrel), and GPIIb/IIIa antagonists (like eptifibatide) have established roles in inhibiting platelet aggregation and function. A hypothesis worth exploring is the potential for antiplatelet drugs to mitigate COVID-19-related complications by specifically targeting the reduction of VEGF release from platelets, particularly from those subpopulations where VEGF is highly enriched. Aspirin and other antiplatelet drugs, by inhibiting platelet activation, could potentially lower the overall release of VEGF and other pro-inflammatory factors from platelets, thereby alleviating some of the vascular complications associated with COVID-19, such as thrombosis and inflammation.

The identification of a novel platelet subpopulation enriched in VEGF and PDGF holds significant potential for advancing wound healing therapies, particularly in the treatment of chronic and complex wounds such as diabetic ulcers, venous ulcers, pressure sores, and non-healing surgical wounds. These specialized platelets, with their enhanced VEGF and PDGF content, may offer superior efficacy in promoting tissue repair and regeneration compared to traditional treatments. Chronic or complex wounds, characterized by their failure to progress through the normal stages of healing and prolonged inflammatory phase, often challenge conventional healing methods. Platelet-rich plasma (PRP) therapy, while beneficial, faces limitations such as variability in efficacy and lack of standardization. In contrast, using this specific subpopulation of enriched platelets could provide a more controlled and effective therapeutic option by directly applying platelets that are naturally higher in essential growth factors. This approach could enhance natural

healing processes by promoting angiogenesis, modulating inflammation, and stimulating cell proliferation more effectively than standard PRP.

Building on the insights and limitations highlighted in the final chapter, future research directions emerge with a clear path toward expanding our understanding of platelet-cancer cell interactions. First, the potential roles of platelet-induced expression of B7 family proteins such as CD80 and CD86, which enhance the immunoevasion capabilities of cancer cells by suppressing T-cells through their CTLA-4 (Cytotoxic T-Lymphocyte-Associated Protein 4) receptors and subsequently promoting metastasis, present an intriguing avenue for further exploration. Concurrently, the investigation should also encompass the immune-stimulatory and anti-metastatic effects of anti-platelet drugs. These dual aspects offer a promising therapeutic window that could be leveraged to inhibit cancer progression while bolstering the body's immune response against tumors.

A critical enhancement to future studies would be the incorporation of *in vivo* models to validate and expand upon the in vitro findings. Specifically, employing mouse models of cancer metastasis could offer invaluable insights into the therapeutic potential of combining anti-platelet drugs with immune checkpoint inhibitors (ICIs). By introducing cancer cells into these models and subsequently administering a regimen of anti-platelet drugs and ICIs, the impact of adding an anti-platelet drug to standard ICI therapy on tumor growth, size, and the number of metastatic foci can be explored. Such models are invaluable for observing the real-time effects of platelet interactions on tumor growth, metastasis, and response to therapies, providing a comprehensive understanding that bridges the gap between in vitro and *in vivo* observations and clinical applicability.

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