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REVIEW

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Platelet extracellular vesicles in COVID-19: Potential markers and makers

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Abstract

Platelets and platelet extracellular vesicles (pEV) are at the crossroads of coagulation and immunity. Extracellular vesicles are messengers that not only transmit signals between cells, but also provide information about the status of their cell of origin. Thus, pEVs have potential as both biomarkers of platelet activation and contributors to pathology. Coronavirus Disease-19 (COVID-19), caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a complex disease affecting multiple organs and is characterized by a high degree of inflammation and risk of thrombosis in some patients. In this review, we introduce pEVs as valuable biomarkers in disease with a special focus on their potential as predictors of and contributors to COVID-19.

KEYWORDS
calcium modulators, HDT, pCAMKII

1 | INTRODUCTION

Extracellular vesicles (EV) are small membrane-bound vesicles that contain molecules from their cell of origin. As EVs can be internalized by cellular recipients, they are suggested to mediate cellular signaling.[1,2] The two most described EV-subtypes are microvesicles and exosomes.[3] Microvesicles are produced by plasma membrane budding and shedding, have a diameter ranging from approximately 100 to 1000 nm[2,4–6] and generally expose phosphatidylserine (PS) although there are exceptions.[7–10] They bud from cells activated by numerous inflammatory triggers.[11] Exosomes are typically smaller than microvesicles[9] and are released by cells from multivesicular bodies in an exocytosis-dependent mechanism.[6,12,13]

Platelets are anucleated cell fragments with a diameter of 1 to 3 μm and are produced by megakaryocytes.[14–16] They prevent bleeding and interact with pathogens and immune cells thereby assisting immune responses.[15,16] Platelets and megakaryocytes are the major sources of circulating EVs.[17–19] Similar to platelets, platelet EVs (pEVs) were first recognized as procoagulant entities.[20–22] However, their roles now appear to be more diverse and pEV subtypes may fine tune both coagulation and inflammation.[1] pEVs have also been identified in bone marrow,[23] lymph,[24,25] and synovial fluid.[26,27] This suggests that pEVs also enable platelets to transmit signals into tissues that are normally inaccessible to platelets.

Coronavirus Disease-19 (COVID-19) is caused by respiratory tract infection with coronavirus SARS-CoV-2.[28] COVID-19 was declared a pandemic by the World Health Organization in March 2020[29] and is now recognized as a complex disease involving high levels of inflammation and thrombosis.[28,30–32] Platelet hyperactivation[33–35] and an increase in pEVs circulating[33–37] in blood of COVID-19 patients is now documented.[33–37]

Herein, we review the current knowledge concerning pEVs and related blood-borne EVs as biomarkers and contributors to pathologies. In particular, we will review (i) pEVs as a biomarker in COVID-19, (ii) how they may be induced in COVID-19, and (iii) how they may contribute to COVID-19 pathology. Figure 1 recapitulates the concepts presented in the review.

2 | HISTORY OF EXTRACELLULAR-VESICLE RESEARCH

Peter Wolf[22] provided the first description of EVs as small, procoagulant lipid particles that can be separated from platelets by

Abbreviations: ACE2, angiotensin-converting enzyme 2; CD147, cluster of Differentiation 147; CLEC—2, C-type lectin-like receptor 2; COVID-19, Coronavirus Disease-19; DAMPs, damage-associated molecular patterns; EVs, extracellular vesicles; HMGB1, high-mobility Group Protein 1; Ig, immunglobulin; MDA, malondialdehyde; OxLDL, oxidized low-density lipoprotein; pEVs, platelet extracellular vesicles; PS, phosphatidylserine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SLE, systemic lupus erythematosus; TF, tissue-factor; TLR, toll-like receptor.

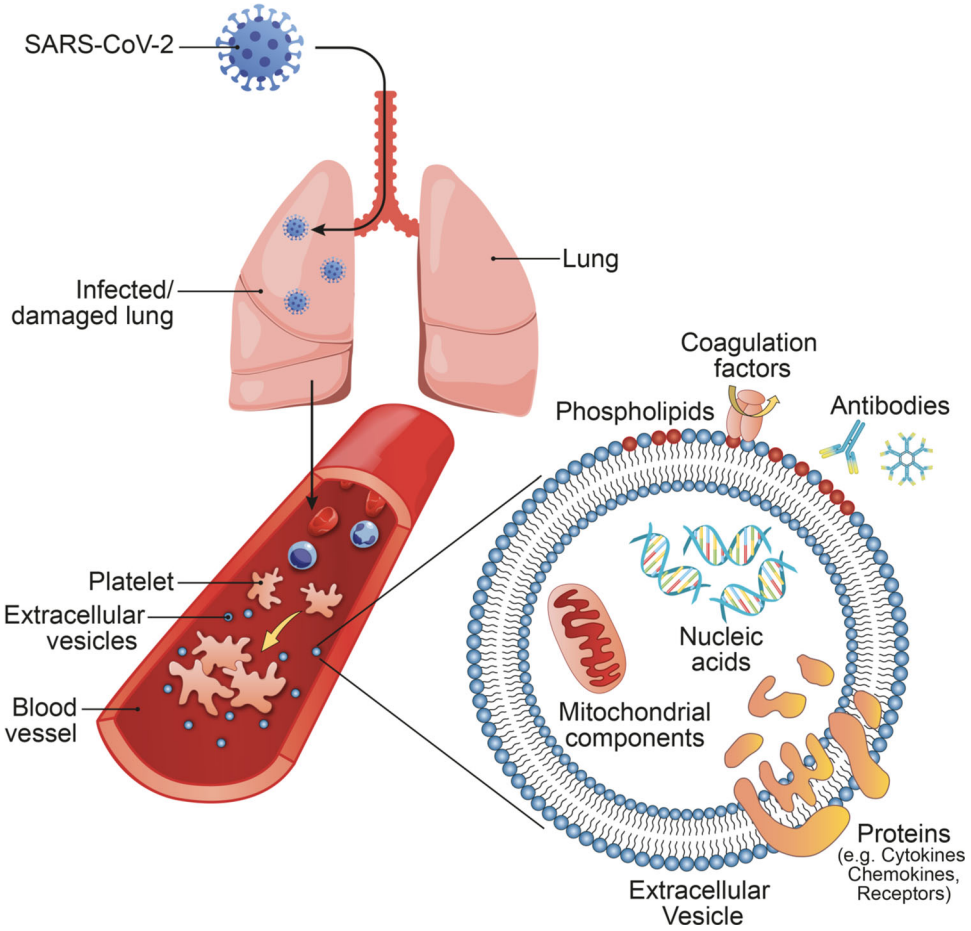

FIGURE 1 SARS-CoV-2 infection of lungs and subsequent damage may activate cells in the blood and induce platelet activation, aggregation, and extracellular vesicle release. Extracellular vesicles carry a diverse array of signaling molecules that can influence immune responses and coagulation

differential centrifugation. Subsequent studies and electron microscopic analysis identified two main classes of vesicles released from cells: exosomes and microvesicles. [13,38–40]During the last 20 years, technological progress has transformed the study of EVs. Advances in flow cytometry—the most commonly used method to detect and quantify EVs—have enabled the analysis of EVs at a higher resolution than ever before, further revealing EV complexity. EVs have historically been categorized into major subclasses such as "microvesicle/microparticle" and "exosomes." However, these narrow definitions have become problematic. Indeed, the vocabulary and methodology describing EVs have expanded at a rapid pace and may lead to confusion upon retrospective examination of EV studies.[3,41,42] Therefore, the International Society of Extracellular Vesicles (ISEV) recommends the use of the umbrella term "extracellular-vesicle (EV)" unless specific investigations permit to determine whether EVs were liberated from the plasma membrane by budding or implicated exocytosis, and to include a detailed description of the isolation and detection methods used in the study of EVs.[3,41,42]

Notably, interpretations of historical studies may have changed with advancements in the technologies and methods used to detect EVs. A common discrepancy is the concentration of EVs per microliter in healthy plasma, with a reported concentration of 200 up to 10^9 EVs/μL, which likely depends on the isolation and detection techniques used.[19,43] The pEV concentration in healthy plasma has been conservatively estimated at around 11,500/μL by cryo-electron microscopy.[19] The most commonly used methods of EV isolation and detection are differential centrifugation and flow cytometry, respectively. However, common pitfalls associated with these techniques are the risk of co-isolation and detection of EVs and lipoproteins, the potential of overlooking particularly small EVs due to insufficient resolution, and the risk of damaging EVs during isolation at high centrifugal forces.[43,44]

3 | pEV AS MARKERS AND MAKERS

pEVs, as a component of liquid biopsies, show potential as biomarkers in autoimmune diseases, cancer, cardiovascular diseases, and infectious diseases.[25,45–55] The presence of pEVs is documented in synovial fluid in rheumatoid arthritis,[26] and increased levels of circulating pEVs correlate with disease activity.[56] Moreover, an increase in pEV concentrations was found in lymph in murine models of atherosclerosis and autoimmune arthritis.[24-27] The number of pEVs is increased in blood in

systemic lupus erythematosus (SLE), and higher levels are suggested to associate with declining kidney function.[45] In addition to pEV concentration, pEV content can be used as a biomarker. The protein composition of EVs in disease can be distinguished from that of EVs in healthy controls. For instance, as activated platelets can translate and produce interleukin-1 (IL-1), this cytokine can be packaged into pEVs, which can augment inflammation.[26,57] Lipid mediators of inflammation, such as prostaglandins and leukotrienes, can also be transported or generated by pEVs given the latter's content of enzymatic machinery and fatty acids.[58] EVs can be released from activated and dying cells, and may therefore, carry self-antigens and damage-associated molecular patterns (DAMPs). Such EVs could have a role as potential biomarkers and may contribute to disease.

Indeed, pEVs are associated with autoantibodies in SLE, which suggests that they bear autoantigens and may facilitate cellular activation through activation of Fc receptors.[59–61] Moreover, platelets and their pEVs can contain DAMPs such as high-mobility group protein 1 (HMGB1),[62] S100A8/9,[63,64] and mitochondrial DAMPs.[59] Another prominent DAMP that can be found on EVs are oxidation-specific epitopes.[65] The latter result from oxidation of polyunsaturated fatty acids and are commonly found on oxidized low-density lipids (OxLDL).[65,66] For instance, malondialdehyde (MDA)-modifications of EVs may be the product of phosphatidylcholine peroxidation during EV-biogenesis. These MDA epitopes are inflammatory/immunogenic and recognized by a subset of germline-encoded (natural) IgM antibodies.[65,66] Low levels of natural IgM antibodies and high levels of MDA + EV and OxLDL are associated with an increased risk of cardiovascular disease.[65] EVs and MDA + EVs[65] are elevated in acute myocardial infarction (STEMI)[46] and in acute coronary syndrome,[48] and as such are likely indicative of tissue damage.

pEVs may be useful biomarkers in cancers associated with thrombotic risks and may enhance metastasis.[52–54] Of particular interest are prostate cancer cells, which are reported to release tissue-factor (TF)-associated EVs (TF + EV).[67] TF is the main initiator of coagulation,[68] and cancer cell TF + EVs may thereby induce platelet activation,[68] leading to the release of pEVs. In the case of coagulation initiated by cancer, TF + EVs are more likely the causative agents in this pathology as opposed to pEVs. However, detection of TF-protein expressed on EVs (TF + EV) is notoriously difficult and generally only achieved through indirect determination of TF-activity.[69,70] Considering these challenges, pEV-quantification is a potential surrogate marker of TF-activity and platelet activation in cancer and other diseases.

Table 1 provides an overview of the different techniques used to identify platelet EV and other blood-borne EV in the literature cited in this section. As indicated in Table 1, pEVs are most commonly isolated from platelet-poor or platelet-free plasma obtained by differential centrifugation, subsequently labeled for platelet-specific markers (primarily CD41) and detected by flow cytometry. Ideally, it is recommended to perform a two-step centrifugation protocol on whole-blood to obtain platelet-free plasma.[44] Plasma prepared this way can be frozen and stored for long-term.[44]

4 | pEVS AS BIOMARKERS IN COVID-19

While pEVs have been most extensively studied as biomarkers in noninfectious diseases, pEVs are also found in association with viral infections.[49–51,71,72] Influenza virus H1N1 activates platelets and induces the release of pEVs by a mechanism that implicates thrombin and the activation of FcgRIIa by antibodies directed against this virus.[49] pEVs may contribute to the propagation of HIV, as they can transport C-X-C chemokine receptor type 4 (CXCR4), a coreceptor for HIV, to other cells.[50] In dengue virus infection, pEVs may be released in a C-type lectin-like receptor 2 (CLEC−2)-dependent manner by platelets[51] and thereby show potential as biomarkers of disease severity[71] by distinguishing between patients who may or may not require platelet transfusion.[72] pEVs in infections and sepsis are discussed in more detail elsewhere.[73]

Several studies point to EVs and pEVs as potential biomarkers in COVID-19.[33,36,37] Increased levels of circulating pEVs have been observed in patients with SARS-CoV-2 infection.[33,37] One particular study reported higher numbers of circulating pEVs in patients with nonsevere and severe COVID-19 in comparison with healthy individuals after determining pEV levels in platelet-free plasma.[33] It is intriguing that the increase in pEVs was less pronounced in patients with severe disease relative to those with nonsevere disease.[33] Normalization of pEV numbers to platelet counts still revealed significantly increased levels of pEVs in severe COVID-19, pointing to increased EV-biogenesis during COVID-19.[33] Cappellano et al.[37] evaluated pEVs as a biomarker for SARS-CoV-2 infection in hospitalized patients and found significantly elevated levels of pEVs in COVID-19 patients compared with healthy controls. Moreover, they reported that increased levels of circulating pEVs could distinguish SARS-CoV-2-infected patients from patients with suspected COVID-19 who tested negative for SARS-CoV-2 infection at the time of hospitalization. Despite a significant difference in pEV concentration, detection of viral infection by pEV-quantification is unlikely to replace PCR as the gold standard to detect SARS-CoV-2 infection. For these analyses, the investigators quantified pEVs in whole blood in the absence of additional purification steps to reduce sample manipulation. The reduction of preanalytical procedures may minimize the risk of damaging EVs, although the use of whole blood may lead to the generation of EVs after collection. Thus, although the approach used by Cappellano et al.[37] may be useful for laboratory analyses performed within hours of blood sampling, as intended by the authors, platelet-free plasma by two-step centrifugation protocols[44] is necessary for long-term storage and sample biobanking.

It is interesting to note that we and others observed that absolute numbers of pEVs[33,36] and PS-exposing (PS+) pEVs[33] were significantly lower in severe COVID-19 when compared with nonsevere COVID-19.[33,36] PS + EVs are considered procoagulatory since they provide a negatively charged surface for initiation and maintenance of coagulation.[74] Notably, a decrease in PS+ EVs has also been reported in multiple organ dysfunction syndrome and sepsis, which might point to common mechanisms in these diseases.[75] COVID-19 is indeed a complex disease affecting many organs distant from the lungs and is

TABLE 1 Detection of platelet EV in different diseases

Context	Refs.	Source	Isolation method	(p)EV-identification	Detection
Arthritis	[25]	Lymph (Mouse)	2 × 2500 × *g*, RT → EV in SUP	CD41 (CellTracker: not-platelet-specific)	Flow-Cyt., Cryo-EM
Arthritis	[26]	Synovial Fluid	2 × 600 × *g*, 30 min, RT → EV in SUP	CD41	Flow-Cyt.
Arthritis	[27]	Blood, Synovial Fluid	Blood: 2 × 2500 × *g* Synovial Fluid: 650 x *g*, 20 min→EV in SUP	CD41	Flow-Cyt., EM, NTA, MassSpec
Arthritis	[47]	Blood, Platelets, Megakaryocytes	Blood → 2 × 2500 × *g*, 15 min, RT → EV in SUP	CD41, CLEC2, GPVI	Flow-Cyt., Western-Blot
Arthritis	[56]	Blood	1550 × *g*, 20 min, RT → SUP 2 × 17,570 × *g*, 30 min, 20°C → EV in pellet	CD61	Flow-Cyt.
Atherosclerosis	[24]	Lymph (Mouse)	1200 × *g*, 10 min, 4°C → analysis of EV in SUP	CD41 (AnV: not-platelet-specific)	Flow-Cyt., Cryo-EM
Cancer (Gastric)	[52]	Blood	1550 × *g*, 15 min, RT → EV in SUP	CD41	Flow-Cyt.
Cancer (Prostate)	[53]	Blood	2 × 28,00 × *g*, 15 min, RT → EV in SUP	CD41	Flow-Cyt.
Cancer (Breast)	[54]	Platelet Concentrates	2000 × *g*, 15 min, 4°C → SUP 2400 × *g*, 15 min, RT → EV in pellet	CD41, CD62p, CXCR4	Flow-Cyt.
Myocardial Infarction	[46]	Blood	Plasma → 2500 × *g*, 10 min, RT → SUP 20,000 × *g*, 30 min, RT → EV in pellet	CD61, CD31, CD42b, (CD14, CD142, AnV: not-platelet-specific)	Flow-Cyt.
Myocardial Infarction	[65]	Blood	Blood: 2000 × *g*, 30 min → 21,000 × *g*, 30 min → EV in pellet Blood: 2000 ×*g*, 30 min → 13,000 × *g*, 2 min → 18,000 × *g*, 30 min → EV in pellet	CD41 (CD235, CD14, CD31: not-platelet-specific)	Flow-Cyt.

(Continues)

TABLE 1 (Continued)

Context	Refs.	Source	Isolation method	(p)EV-identification	Detection
Multiple Sclerosis	[55]	Blood	160 × *g*, 10 min, RT → SUP 2000 × *g*, 6 min → EV in SUP	CD41, CD62p	Flow-Cyt.
SLE	[45]	Blood	Platelet-poor plasma → 2000 ×*g*, 20 min, RT → SUP 13,000 × *g*, 2 min, RT → in SUP	CD41 (CD40L, VCAM, HMGB1, CD142, C4d, AnV: not-platelet-specific)	Flow-Cyt.
SLE	[59]	Blood	282 × *g*, 10 min → 2500 × *g*, 20 min → 3 × 3200 × *g*, 5 min, RT → EV in SUP	CD41 (mitotracker: not-platelet-specific)	Flow-Cyt.
SLE	[60]	Blood	1800 × *g*, 10 min, 21°C → 3000 × *g*, 10 min, 21°C → 18,890 × *g*, 30 min, RT → EV in pellet	Proteomics-analysis	MassSpec
SLE	[61]	Blood	1800 × *g*, 10 min, 21°C → 3000 × *g*, 10 min, 21°C → 19,000 × *g*, 30 min, 22°C → EV in pellet	CD41 (IgG1, IgM, C1q, AnV: not-platelet-specific)	Flow-Cyt., MassSpec
Viral infection (H1N1)	[49]	Washed Platelets	In supernatant with platelets	CD41	Flow-Cyt.
Viral infection (HIV)	[50]	Washed Platelets	Platelet Suspension → 2 × 2000 × *g*, 15 min, 4°C → SUP 28,000 ×*g*, 1h, 4°C → EV in pellet	CD41, CXCR4	Flow-Cyt.
Viral infection (Dengue)	[51]	Washed Platelets	1500 × *g*, 15 min, RT → 20,000 × *g*, 40 min, 4°C → EV in pellet; SUP 100,000 × *g*, 40 min, 4°C → EV in pellet	CD41, CD62p, CD63	Flow-Cyt., NTA

Abbreviations. AnV, Annexin-V; EM , electron microscopy; Flow-Cyt., flow cytometry; MassSpec, mass spectrometry; NTA, nanoparticle tracking analysis; RT, room temperature; SUP, supernatant.

characterized by a high degree of thrombosis. Thus, the observed decrease in PS+ pEVs may suggest increased consumption of such EVs in patients with more severe disease as PS is also an "eat-me" signal for cellular removal.[33,36,76] In summary, PS+ pEVs may be a biomarker in COVID-19 that distinguishes different stages of disease activity, especially with regard to coagulation and organ damage.[30–32]

Table 2 provides an overview of the isolation and detection techniques used for the analysis of platelet EV in COVID-19 in the literature cited in this section.

5 | WHAT TRIGGERS THE RELEASE OF pEVS IN COVID-19?

5.1 | SARS-CoV-2-mediated platelet activation via receptors of innate and adaptive immunity

Considering the high abundance of platelets in blood and their ability to detect pathogens, platelets have also become recognized as an important component of the immune response to microbial invasion. Platelets express various pattern-recognition receptors, including functional expression of toll-like receptors (TLR1, TLR2, TLR3, TLR4, TLR6, TLR7, TLR9)[77,78] and mRNA for all 10 TLRs.[79] TLR2 and TLR4 are not primarily known to recognize viruses, but a role for TLR4 and TLR2 signaling on platelets has been shown for dengue virus and cytomegalovirus infection.[80,81] The TLR4 ligand lipopolysaccharide induces EV-release by monocytic cells,[10] but it is not known if TLR2 or TLR4 activation induces EV release by platelets. In fact, TLR2 and TLR4 engagement is not sufficient for platelet activation, but instead primes or sensitizes platelets to respond to other stimuli, or induces protein translation by platelets.[57,77] Since SARS-CoV-2 is a single-stranded RNA (ssRNA) virus[82] and double-stranded RNA (dsRNA) is considered to be an intermediate product of viral replication,[82] TLR3 (dsRNA) and TLR7 (ssRNA) are candidate receptors for SARS-CoV-2 recognition by platelets. Engagement of ssRNA by platelet TLR7[83–85] and ssRNA by platelet TLR3[86] is also known. TLR3 activation has been associated with EV release by different cell types,[87–89] but has not been described for platelets. Moreover, activation of TLR3 and TLR7 does not induce typical platelet activation, such as granule content release and exposure of activated GPIIbGPIIIa, as seen in response to thrombin stimulation. These interactions may be more similar to the priming or sensitization effect of TLR2 and TLR4.[77,90] Of note is that SARS-CoV-2 RNA has been found in association with platelets in some COVID-19 patients.[33,34] Thus, TLR3 and TLR7 may be attractive targets for SARS-CoV-2 platelet interactions and priming of platelets in COVID-19. In a recent study,[91] transmission-electron-microscopy revealed that SARS-CoV-2 may be taken up by platelets and locate in phagosome-like structures. TLR3 and TLR7 are endosomal receptors and could thereby come in contact with SARS-CoV-2 RNA, but an actual interaction has not been shown in platelets. Damaged capillaries in the alveolar wall could be contact sites for SARS-CoV-2 and the blood. DAMPs, such as DNA liberated by activated or NETosing cells,[92] may also contribute to platelet activation and pEV release in COVID-19. Indeed, while

TABLE 2 Detection of platelet EVs in COVID-19

Context	Ref	Source	Isolation Method	EV-identification	Detection
EV-quantification	[33]	Blood	200 × *g*, 15 min → 1000 × *g*, 10 min → EV in SUP	CD41 (AnV: not-platelet specific)	Flow-Cyt.
EV-quantification and EV-TF-activity	[36]	Blood	FACS: 2 × 2500 × *g*, 15 min, RT → EV in SUP TF-activity assay: 2 × 2500 × *g*, 15 min, RT → 3 × 70,000 × *g*, 90 min, 4°C → EV in pellet	CD41 (CD31, CD146, CD15, IgG1: not-platelet specific)	Flow-Cyt., Other: TF-activity assay
EV-quantification	[37]	Blood	EV were analyzed directly in whole-blood	CD41 (CD31, Phalloidin: not-platelet specific)	Flow-Cyt.

Abbreviations. AnV, Annexin-V; Flow-Cyt., flow cytometry; RT, room temperature; SUP, supernatant; TF, tissue factor.

stimulation of TLR9 (recognizes unmethylated CpG DNA) induced oxidative stress in platelets, it also enhanced pEV release when platelets were activated by immune complexes recognized by FcγRIIa.[59] Moreover, antibody-mediated recognition of SARS-CoV-2 via FcγRIIa may play a role in platelet activation and pEV-release, as has been shown for H1N1.[49]

Protein in the envelope of viruses are often heavily glycosylated, which may aid in the evasion of adaptive immune responses.[93] Indeed, the SARS-CoV-2 spike (S) protein has multiple glycosylation sites[94] that may be relevant to its function and interactions with target cells. In multiple variants of SARS-CoV-2, mutations to glycosylation sites affect infectiveness.[95] Of note, is that platelets express CLEC-2 (C-type lectin-like type II) and DC-SIGN,[96] which are relevant in HIV-1 and dengue virus infections[51,96] and platelets may release EVs in a CLEC-2-dependent manner.[51]

5.2 | Direct SARS-CoV-2 platelet interaction via known and putative SARS-CoV-2 receptors

The primary receptor for SARS-CoV-2 in humans is ACE2 (Angiotensin-converting enzyme 2),[97,98] which is ubiquitously expressed by type II epithelial cells in the upper and lower respiratory tract.[99] The typical cellular entry route for SARS-CoV-2 is engagement with ACE2 via the Spike protein, subsequent cleavage of the latter by the serine protease TMPRSS2 to enable fusion of the viral and cellular membrane resulting in infection of the target cell.[100] While expression of ACE2 has been shown for vascular endothelial cells and lung macrophages,[99] physiological expression by platelets or megakaryocytes is controversial.[101] Moreover, Koupenova et al.[91] recently reported that SARS-CoV-2 may be taken up by platelets through both ACE2-dependent and independent mechanisms. Cluster of Differentiation 147 (CD147) has been proposed as an alternative receptor for SARS-CoV-2,[102] is commonly expressed in circulating cells, and is associated with risk factors of severe COVID-19 such as obesity, asthma, and chronic obstructive pulmonary disease (COPD).[102,103,] CD147 is indeed functionally expressed on platelets,[104,105] but its relevance to SARS-CoV-2 infection has been called into question.[106] Other intriguing targets for direct SARS-CoV-2 platelet interaction are the integrins. Platelet integrin GPIIbGPIIIa binds the three amino acid motif Arg-Gly-Asp (RGD) present in physiological ligands (e.g., fibrinogen, von Willebrand Factor), which is crucial for platelet aggregation.[107,108] Platelet integrin GPIIbGPIIIa is important in platelet responses and is implicated in pEV-release triggered by FcγRIIA or GPVI receptor activation.[25,26,] Furthermore, the Spike protein sequence of SARS-CoV-2 contains an RGD-motif (403-405: Arg-Gly-Asp) within the receptor-binding domain.[109,110] Thus, platelet integrin GPIIbGPIIIa presents another alternative target receptor for SARS-CoV-2.

5.3 | pEV-release independent of direct SARS-CoV-2 platelet interaction

Platelets may not be stimulated to release pEVs solely upon direct interaction with SARS-CoV-2. Indeed, excessive inflammatory responses and tissue damage, particularly, in the lung and lung microvasculature, are observed in COVID-19 patients.[111,112] Of interest is that autopsies of 21 patients[113] revealed inflammatory damage and microthrombi in multiple organs (lungs, heart, liver, kindeys, brain), while SARS-CoV-2 infected cells were absent from most of the affected tissues. Notably, SARS-CoV-2 viral RNA copies in a range of 63 to 6,310 copies per milliliter of blood have been detected in a quarter of hospitalized COVID-19 patients.[114] Given that the concentration range of platelets in blood is 150 × 10ˆ8 to 450ˆ8 per mL of blood, platelets would outnumber virus by a factor of 23,771 to 7.14 × 10ˆ6. This would make direct interactions of SARS-CoV-2 with platelets rare events, although the amount of SARS-CoV-2 in the blood may be underestimated viral as RNA may have been degraded.[91] Dissemination of SARS-CoV-2 throughout the circulation is not excluded, but may not be the primary path taken by the virus to affect platelet function and pEV release. As discussed by Chen et al.,[115] infection of cells in the lungs (pneumocytes, epithelial cells) and nearby vasculature (endothelial cells) causes the production of inflammatory cytokines contributing to an immune response leading to tissue damage.[116,117] At the same time, damage to tissue may lead to the liberation of DAMPs, including, but not limited to, mitochondria and mitochondrial components and oxidized phospholipids, and TF associated with EVs, which would further amplify the inflammatory and coagulation cascade that stimulates platelet activation and subsequent pEV release. Moreover, the overwhelming inflammation may affect endothelial barrier integrity and thereby lead to increased expression of soluble thrombomodulin, soluble P-selectin, and von Willebrand factor.[117] Indeed, elevated levels of TF activity associated with EVs have been reported in COVID-19, which may directly contribute to excessive coagulation.[36,70,118] In addition, ACE2, the primary receptor for SARS-CoV-2 infection in humans, is important in the regulation of the renin–angiotensin–aldosterone system and a deficiency of ACE2 is linked to enhanced risk of inflammation and thrombosis.[98] As COVID-19 is increasingly viewed as a thromboinflammatory disease,[32] it is conceivable that platelet activation in COVID-19 may be a consequence of the inflammation, organ damage, and pathological activation of the coagulation cascade, rather than a consequence of direct virus–platelet interaction. Moreover, at later stages of the disease, secondary effects caused by the tissue damage and inflammatory response, as opposed to viral presence, might become decisive in determining disease outcome. Thus, changes to the presence and activity of pEVs may represent potential risk markers, as in other diseases.[25,45–51,119]

6 | THE CONTRIBUTION OF pEVS TO COVID-19

The first descriptions of EVs referred to their procoagulant abilities, which are primarily attributed to exposure of the negatively charged phospholipid, PS.[120–122] PS exposure on platelets and pEVs supports propagation of coagulation.[120–122] In COVID-19, studies report the presence of antiphospholipid antibodies, such as anticardiolipin antibodies, similar to those seen in antiphospholipid syndrome and SLE.[123–126] These antibodies may target PS-exposing pEVs, thereby,

forming immune complexes for cellular activation through Fc receptors. Negatively charged surfaces may also initiate the "intrinsic pathway" of coagulation.[121] However, the main cellular initiator of coagulation is TF.[121] While some procoagulant activity of circulating pEVs has been associated with TF in the past,[127] it has been suggested that platelets may acquire TF from other cells via TF-expressing EVs in a P-selectin glycoprotein ligand-1-dependent manner.[128] However, expression of TF by platelets and pEVs is controversial and direct detection of TF displayed by EVs is challenging.[69] The concentration of TF may be below the detection limit of flow cytometric approaches, but may be sufficiently high to initiate coagulation as concentrations as low as 20 fM suffice to initiate coagulation.[129] Furthermore, TF may be present in an inactive ("encrypted") or active ("decrypted") state.[121] Therefore, it is necessary to determine TF biological activity to confirm its role in a biological process.

Given the high prevalence of thrombosis in COVID-19,[30–32] TF activity associated with EVs and its involvement in COVID-19 pathology is of high interest.[36,70,118,130] Several studies[36,70,118,131] report a significant increase in TF activity associated with EVs from COVID-19 patients when compared with healthy controls. Moreover, EV-TF activity was significantly higher in COVID-19 than in sepsis.[36,118] Guervilly et al.[36] found a higher EV-TF activity in severe compared with nonsevere (moderate) COVID-19 and a TF activity of more than 78.3 fM associated with thromboembolic events. Moreover, they observed fibrinolytic activity of EVs in COVID-19, but found no significant change between nonsevere (moderate) and severe disease, indicating that the balance is shifted toward coagulation in severe disease.[36] Krishnamachary et al.[131] reported enrichment of TF-protein and TF-activity associated with EVs isolated from COVID-19 patients with severe disease. When stratifying COVID-19 patients with severe disease by mortality, no differences in EV-TF-activity was noted, as the level of EV-TF-activity was approximately 50 pM in both survivors and nonsurvivors.[131] These data appear to contrast with Guervilly et al.,[36] who reported far lower levels of EV-associated TF-activity in COVID-19. While the discrepancy might be explained by an overestimation of TF activity measured with commercial assays in absence of blocking antibodies to ensure of the specificity of the measurements,[132–134] these studies suggest that high EV-TF activity may be associated with increased severity and mortality in COVID-19.[36,70,131]

Guervilly et al.[36] reported no differences in overall EV levels after comparing moderate and severe COVID-19, with the exception of significantly lower levels of pEVs in severe COVID-19.[36] This is consistent with observations made by Zaid et al. who also reported significantly lower levels of total and PS+ pEVs in severe COVID-19 compared with nonsevere COVID-19.[33] However, the procoagulant activity of EVs was increased in COVID-19, and was thought to depend on the expression of active TF given that the fibrinolytic activity of EVs remained unchanged in these patients.[36] A recent interesting in vitro study by Wang et al.[130] found that infection of human monocyte-derived macrophages with SARS-CoV-2 spike protein pseudovirus increased TF activity at the cell surface and stimulated the release of EVs with associated TF activity.[130] This study suggests that TF activity depended on "decryption" (activation) of TF by hydrolysis of sphingomyelin via acid sphingomyelinase (ASMase), and not on increased TF protein expression or externalization of PS.[130] This lends further support to a role for SARS-CoV-2 in the "decryption"' of TF and the subsequent release of EVs with associated, active TF.[130] These observations are of clinical importance given the low femtomolar concentrations of EV-associated TF activity reported in COVID-19 patients[36] that are nevertheless sufficient to predict an increased thrombotic risk. The absolute quantification of TF + EVs, or identification of the cellular origin of TF-exposing EVs is challenging and requires careful study. Thus, the distinction of "decrypted" from "encrypted" TF in association with EVs, by the quantification of TF activity, may be a more relevant risk marker in COVID-19.

7 | CONCLUDING REMARKS

EVs have attracted interest as biomarkers and players in COVID-19 pathology. As COVID-19 is a complex disease affecting multiple organs and is characterized by a high degree of thrombosis, the study of platelet activation and the involvement of procoagulant EVs has drawn interest. A growing number of independent studies found that quantification and characterization of pEVs and other types of blood-borne EVs carry potential as biomarkers of COVID-19 disease severity and as predictors of outcome. While high levels of circulating EVs appear to be found during all stages of the disease, the presence of TF activity associated with EVs may predict disease severity and initiate the "extrinsic" or "TF" pathway of coagulation and, thereby, directly contribute to the high thrombotic risk in COVID-19. Conversely, the PS-exposing pEVs may stimulate inflammation if targeted by antiphospholipid antibodies, or support the propagation of coagulation due to the exposure of negatively charged surfaces. However, what remains unclear, is the pathway of pEV biogenesis in COVID-19 as the relevance of direct SARS-CoV-2-platelet interaction has not been established. Excessive production of active TF and damaged surfaces, culminating in coagulation initiation and propagation, may be the most probable trigger for high levels of platelet activation and pEV biogenesis in COVID-19. Thus, determination of plasma pEV levels provides an opportunity for a surrogate marker for excessive coagulation and inflammation. Important challenges to overcome are the clear identification of circulating EVs and their physical separation into distinct populations to assign specific functions to individual EV-populations and to define their role in COVID-19. In summary, these observations indicate directions for future studies of EV involvement in COVID-19.

AUTHOR CONTRIBUTIONS

FP wrote the manuscript. EB supervised and reviewed the manuscript. LF reviewed the manuscript. All authors read and approved the final version of the manuscript for submitting to the Journal of Leukocyte Biology

CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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