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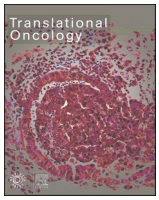

Revealing profile of cancer-educated platelets and their factors to foster immunotherapy development

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ABSTRACT

Among multiple hemostasis components, platelets hyperactivity plays major roles in cancer progression by providing surface and internal components for intercellular crosstalk as well as by behaving like immune cells. Since platelets participate and regulate immunity in homeostatic and disease states, we assumed that revealing platelets profile might help in conceiving novel anti-cancer immune-based strategies. The goal of this review is to compile and discuss the most recent reports on the nature of cancer-associated platelets and their interference with immunotherapy. An increasing number of studies have emphasized active communication between cancer cells and platelets, with platelets promoting cancer cell survival, growth, and metastasis. The anti-cancer potential of platelet-directed therapy has been intensively investigated, and anti-platelet agents may prevent cancer progression and improve the survival of cancer patients. Platelets can (i) reduce antitumor activity; (ii) support immunoregulatory cells and factors generation; (iii) underpin metastasis and, (iv) interfere with immunotherapy by expressing ligands of immune checkpoint receptors. Mediators produced by tumor cell-induced platelet activation support vein thrombosis, constrain anti-tumor T- and natural killer cell response, while contributing to extravasation of tumor cells, metastatic potential, and neovascularization within the tumor. Recent studies showed that attenuation of immunothrombosis, modulation of platelets and their factors have a good perspective in immunotherapy optimization. Particularly, blockade of intra-tumoral platelet-associated programmed death-ligand 1 might promote anti-tumor T cell-induced cytotoxicity. Collectively, these findings suggest that platelets might represent the source of relevant cancer staging biomarkers, as well as promising targets and carriers in immunotherapeutic approaches for combating cancer.

Abbreviations: 5HT, 5-hydroxytryptamine; AML, acute myeloid leukemia; Ang-1, angiopoietin 1; bFGF, basic fibroblast growth factor; BFU-MK, burst-forming unit; BM, bone marrow; CAF, cancer-associated fibroblast; CCL, C-C motif chemokine ligand; CEPs, cancer-educated platelets; CLEC2, C-type lectin-like receptor-2; CXCL, C-X-C motif chemokine ligand; ECs, endothelial cells; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; EPA, extravasated platelet aggregation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GAPR, glycoprotein A repetitions predominantly; G-CSF, granulocyte colony-stimulating factor; GITR, glucocorticoid-induced TNF receptor family-related protein ligand; GITRL, GITR ligand; GITRL, glucocorticoid-induced tumor necrosis factor receptor–related protein ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; GP, glycoproteins; HSC, hematopoietic stem cell; ICI, immune checkpoint inhibitor; IL, interleukin; MDSCs, myeloid derived suppressor cells; MEP, megakaryocyte–erythroid progenitors; MK, megakaryocytes; CFU-MK, colony-forming unit MK; MKP, precursor; ITP, immune thrombocytopenia; MSCs, mesenchymal stromal cells; NET, neutrophil extracellular trap; NK, natural killer; NKG2D, natural killer group 2D; NSCLC, non-small cell lung cancer; OSCC, oral squamous cell carcinoma; PAF, platelet-activating factor; PDGF, platelet-derived growth factor; PD-L1, programmed death-ligand 1; PF4, platelet factor 4; PSGL-1, P-selectin ligand 1; PMP, platelet microparticle; Spp1, secreted phosphoprotein 1; TAM, tumor-associated macrophages; TCIPA, tumor cell-induced platelet activation; TF, tissue factor; TGF-β, transforming growth factor-β; TLT-1, triggered receptor expressed on myeloid cell–like (TREM)-like transcript 1; TME, tumor microenvironment; TNF-α, tumor necrosis factor; TPO, thrombopoietin; TSP-1, thrombospondin 1; VEGF, vascular endothelial growth factor; VTE, venous thromboembolism; PAR, platelet-activating receptors; vWF, von Willebrand factor.

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Introduction

The platelet concept has shifted from unique hemostatic and procoagulant roles to a broader function including defense against pathogens, immune regulation, and finally, participation in malignant diseases. Platelets have been recognized as important participants in inflammation, innate immunity, growth and development, vascular integrity and angiogenesis, wound healing, and finally, cancer [1]. These anuclear blood cells function as reporters and transporters within the tumor microenvironment (TME) [2]. Cancer induces a prothrombotic activity of the hemostatic system, and vice versa the clotting system activation supports the cancer development and progression. Tumor tissue has many prothrombotic features involved in background of the cancer-induced hemostatic dysregulation. Namely, oncogenes control hemostatic protein expression by tumor cells and together with tumor-derived tissue factor (TF)-positive microparticles are an important players in cancer-associated thrombosis. Moreover, the changes in the TME in the presence of TF affect dormant tumor cells to shift to a malignant phenotype. However, the coagulopathy of cancer is complex, with both thrombotic and bleeding complications contributing to morbidity and mortality. In cancer patients, plasma thrombotic markers are dysregulated with activation of blood coagulation and fibrinolysis following the cancer development and progression. Moreover, TF within the stromal compartment of the TME pointed out the importance of the hemostasis and cancer interaction [1,3,4].

Cancer-educated platelets (CEPs) directly interact with tumor cells, contributing to their dissemination and angiogenesis, and regulating the state of TME, being promising biomarker carriers and basis for anticancer therapy. It has been found that circulating platelets are activated ("primed") in advanced stages of breast cancer development, along with increased formation of platelet-tumor cell aggregates, extramedullary hematopoiesis, and metastasis during aging. Importantly, blocking antibodies were able to target platelet-tumor cell aggregation induced by the increased thrombin in metastatic breast cancer [5]. An interaction between activated platelets and tumor cells adversely affects the prognosis of cancer patients, leading to an increased cancer recurrence rate and decreased overall survival rate [3,6]. As numerous cancers are caused by pathological (chronic) inflammation, therapies targeting the crosstalk between hemostasis and inflammation could be used to prevent cancer development. Thus, elucidation of the molecular basis of cancer-platelet crosstalk would further have important implications in the development of anti-cancer immunotherapy.

Thrombopoiesis and platelet structure

To maintain a platelet population, the adult human bone marrow (BM) must produce an estimated 100 billion platelets daily. The life span of platelets in circulation is short (average of 7 days) [7]. Platelets are generated in the process of thrombopoiesis by the large, polyploid, and hematopoietic stem cell (HSC)-derived megakaryocytes (MKs) in BM. This process is initiated with HSC's commitment towards megakaryocyte–erythroid progenitors (MEPs) via common myeloid progenitors, and further differentiation into MKs, requiring interleukin (IL)−3 along with thrombopoietin (TPO) (Fig. 1). Because of the difference in their proliferative potential and by analogy to erythroid progenitors, burst-forming units MK (BFU-MK) and colony-forming units MK (CFU-MK) are thought to represent the primitive and mature progenitors restricted to the megakaryocyte lineage, respectively [8]. During the fragmentation of mature MK membrane, pseudopodia projections (proplatelets) are arised, where the entire cytoplasmic component participates (including membranes, organelles, granules, and soluble macromolecules). Finally, platelets are formed as anuclear mature blood cells and exocytosis of platelet granules is their main tool of platelet activities. Platelet granule exocytosis is a well-regulated secretion, by which upon agonist stimulation, cargo stored in platelet granules is released [8]. Platelet activation depends on the presence of collagen and a blood protein - von Willebrand factor (vWF). The biogenesis of platelet granules begins in MKs, but maturation continues in blood platelets. At least three major types of granules are found in platelets—α-granules, δ(dense)-granules, and lysosomes, as well as peroxisomes and recently described T granules [9]. α-Granules are unique to platelets and are mostly saturated with proteins of membrane-associated receptors (for example, αIIbβ3 and P-selectin) and soluble cargo (platelet factor 4 (PF4) and fibrinogen). Exocytosis of δ(dense)- granule (containing calcium, adenyl nucleotides, and serotonin) is fastest and most sensitive to agonists, whereas exocytosis of lysosomes containing proteolytic enzymes appears to be slow [9].

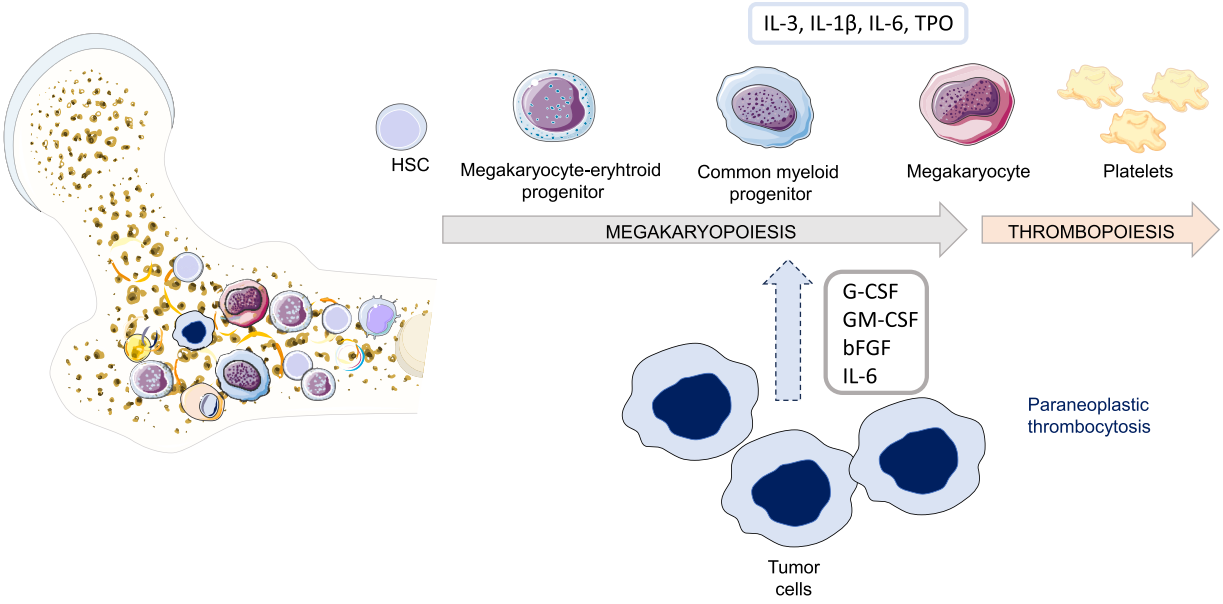

Fig. 1. Normal thrombopoiesis and platelet generation in cancer. Hematopoietic stem cell (HSC) niche MKs in bone marrow and platelet generating MKs in blood. Platelets are generated in the thrombopoiesis by the large, polyploid, and HSC-derived MKs. Paraneoplastic thrombocytosis occurs in many solid tumors.

Thrombopoiesis and platelet disorders in cancer

Intratumoral MKs were detected in various solid tumors and a subset of platelets may originate from the tumor tissue itself [10]. Tumors can affect thrombopoiesis and megakaryopoiesis directly by secretion of a variety of growth factors, such as TPO, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and basic fibroblast growth factor (bFGF) [11]. Additionally, tumor-derived IL-6 can influence increased TPO production in the liver, thereby indirectly supporting thrombopoiesis [12] (Fig. 1). Thrombocytopenia (low platelet production) or hematological malignancies, can lead to the accumulation of MK precursors (MKPs) and/or early MKs in the BM; indicating that the developmental arrest could result in MKP hyperplasia and affect functional platelet biogenesis [9,13]. For instance, thrombocytopenia is linked to poor prognosis in multiple myeloma (MM) patients. Namely, serine released from myeloma cells into the BM microenvironment, is a key metabolic factor that suppresses megakaryopoiesis and thrombopoiesis in myeloma patients as showed in vitro and in vivo [14]. Chemo- and radiotherapies, and autoimmune disorders often lead to secondary thrombocytopenia in cancer patients, and thus, platelet transfusion is necessary to stop or prevent bleeding. However, before being transfused, platelets are stored in blood banks, which leads to their activation. As activated platelets promote metastasis [15], and the proliferation of cancer cells (our unpublished results as well), the effect of platelet transfusion remains controversial and there is a lack of agreement on transfusion strategies. Additionally, thrombocytopenia is also caused by chemo- or radiotherapy. Immune thrombocytopenia (ITP) is characterized by the excessive destruction of platelets and MKs dysmaturity mediated by humoral and cellular immunity. On the other side, thrombocytosis and increased platelet activity are seen in cancer patients and were first noticed by *Reiss et al.* in 1872 [4]. Cancer-associated thrombosis is a life-threatening state and major cause of mortality in cancer patients, and the most common type is venous thromboembolism (VTE). Thrombocytosis-associated massive platelet production can appear in response to various tumor-derived and systemic factors. It is of paramount interest to identify platelet-activating receptors (PARs) as potential targets for anti-cancer drugs designed to prevent the unwanted formation of platelet aggregates [16].

Direct contact between platelets and tumor cell-induced platelet activation (TCIPA)

Up to now, several molecules involved in direct contact of platelets and tumor cells have been identified. Direct interactions between platelets and tumor cells are mainly mediated by platelet membrane glycoproteins (GPVI, GPIb- IX -V), integrins (α2β1, α6β1), and lectins (P-selectin, C-type lectin-like receptor-2 (CLEC-2)). αIIbβ3 (GPIIb/IIIa) is responsible for tumor cell–platelet interactions through fibronectin and vWF on cancer cells, and ADAM9 triggers platelet activation by binding to integrin α6β1 on platelets [11,17]. TCIPA can be triggered by TF expressed on tumor cells or tumor microparticles, as well as by tumor-secreted thrombin, thromboxane A2, ADP, cathepsin B, matrix metalloproteinase-2, and other secreted factors [7,11]. TCIPA is partly responsible for the observed pro-thrombotic state in some cancer patients and correlates with their metastatic potential [11]. TCIPA triggers shape change and degranulation, releasing a plethora of thrombogenic, angiogenic, and inflammatory factors that can further enforce platelet activation in an autocrine and paracrine manner. The effect of TCIPA is mediated by multiple agents: soluble P-selectin, thrombospondin 1 (TSP-1), β-thromboglobulin, CD40 ligand, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), angiopoietin (Ang)−1, matrix proteins, C—C motif chemokine ligand 17 (CCL17), C-X-C motif chemokine ligand

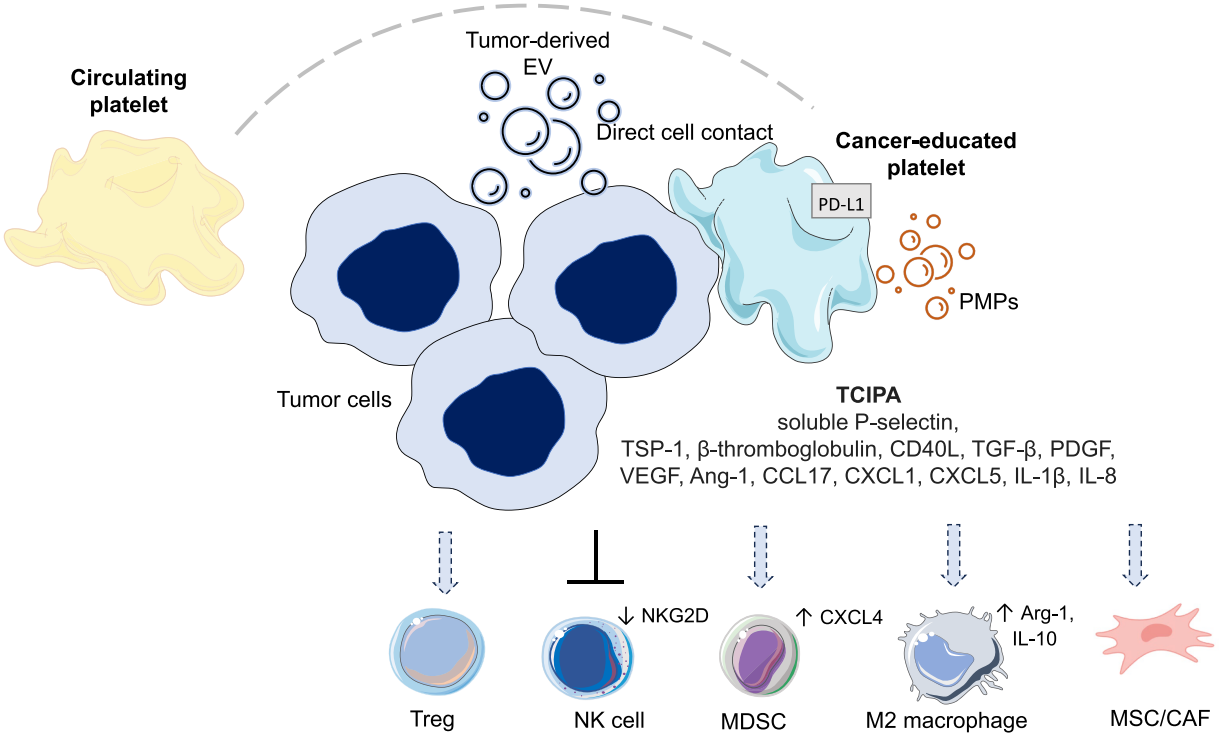

Fig. 2. Immune context of TCIPA. In an early step of TCIPA, cancer cells trigger platelet granule and extracellular vesicle release to facilitate cancer cell survival in circulation. In turns, platelet profile is reprogrammed toward cancer-educated platelet. Platelet microparticles (PMPs) are produced upon activation in the blood of cancer patients. TCIPA is followed by the attenuated anti-tumor immunity via inhibition of T lymphocytes and NK cells, and macrophage polarization towards M2 tumor-associated macrophages (TAMs). TGF-β derived by platelets reduces natural killer group 2D (NKG2D) receptor expression and inhibits their antitumor reactivity. CXCL4 produced by CEPs induces myeloid derived suppressor cells (MDSCs) generation. Platelet-induced MSC trans-differentiation into CAF-like cells through TGF-β production.

(CXCL) 1, and CXCL5 (Fig. 2) (Table 1). These factors have been shown to be significantly elevated in the blood of some cancer patients and to be associated with an increased risk of deep vein thrombosis [11]. Additional proteins have been identified on platelet exosomes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which can function as a membrane fusion protein and serves as a plasminogen receptor. By releasing fibrin, plasmin can suppress natural killer (NK) cell activity, and thus GAPDH represents a potential source of immune suppression [4,18]. P-selectin, a C-type lectin expressed on the surface of platelets, interacts with P-selectin ligand 1 (PSGL-1) and other mucins on cancer cells, while another mucinous glycoprotein, podoplanin (PDPN), which is upregulated in various types of cancer, can bind to the CLEC-2 receptor on platelets and induce TCIPA [11]. Furthermore, direct contact via platelet membrane molecules CLEC-2 or integrin β3 also promotes tumor growth in vitro and in vivo [19,20]. By providing pro-coagulant surface, releasing bioactive molecules, and interacting with leukocytes, CEPs contribute to TME and cancer-associated immunothrombosis, which further supports cancer progression [21]. Furthermore, adhesion molecules on platelets help intravascular arrest of platelet-tumor cell aggregates and extravasation of tumor cells. In addition, platelet-derived factors that increase vascular permeability, such as VEGF, PDGF, ATP/ADP, and lysophosphatidic acid, support the transmigration of tumor cells [6,22] (Table 1). In contact with tumor cells, numerous proteins, lipids, growth factors, cytokines, and proteases released from platelet α and δ granules can directly or indirectly affect neoangiogenesis process [7,23]. Some of these platelet-derived factors promote vascular sprouting and neovascularization of tumors, and platelet-rich fibrin matrix provides structural support for migrating endothelial cells. Other factors, like serotonin and Ang-1, induce vessel maturation and stabilization, and maintain tumor vascular integrity [24]. In response to activation signals, platelet microparticles (PMPs) can also be generated, accounting for 70 to 90 % of all extracellular vesicles in the blood of cancer patients. Cancer-induced PMPs are involved in angiogenesis, metastasis and multidrug resistance [25].

Table 1
Specific engagement of CEP-produced factors in cancer progression.

Platelet-derived factors in TME	Functions
VEGF	Support transmigration of tumor cells [22,26]; Macrophages recruitment to TME [27,28]; Neovacularization [29]
CXCL5	Granulocyte recruitment [30];Thromboinflammation [31, 32]
CXCL7	Granulocyte recruitment [30];Thromboinflammation [31, 32]
TGFβ	Inhibits specific anti-tumor cytotoxic T cells [6]; Thromboinflammation [31,32]; Decreases NKG2D receptor NKs [33]; Pre-metastatic niche formation in bone [34]; Increases Tregs production [32]; Epithelial-mesenchymal transition (EMT) [35]; Induction of CAF formation [36]; Immunotherapy target [37,38]
EGF	Upregulates tumor cell PD-L1; Immunotherapy target [39, 40]
PDGFBB	Support transmigration of tumor cells [6,22]; Neovacularization [29]
CXCL4	Thromboinflammation [31,32]; Regulates CD4+ *T* cell activation [41]; Induces MDSC production [42]; Supports profibrotic macrophage differentiation [43]
CCL5	Regulates CD4+ *T* cell activation [41]
IL-1β	Supports cancer-activated platelets [39]; Thromboinflammation [31,32]; Attracts monocytes in metastatic niche [44]
P-selectin	TCIPA induction [11]; T-cell response inhibition [45,46]; NET formation and establishment of pro-coagulant environment [47,48]
IL-8	Thromboinflammation [31,32]; NET formation and establishment of pro-coagulant environment [47,48]; Incresed during immunotherapy of cancer patients with VTE [49]; Increases breast cancer cell invasiveness [50]
CXCL12	Thromboinflammation [31,32]

Collectively, numerous important mediators are produced by TCIPA, and most of them aggravate anti-tumor immune response, contributing to tumor growth and progression.

Inflammatory signature of platelet-tumor cell interactions

Platelets are fundamental components of the inflammatory system that, upon activation express numerous mediators, cytokines, and growth factors to modulate both innate and adaptive immune systems [39,51]. The commonly used platelet products in clinical practice are apheresis and/or concentrated platelets, both having similar effects on platelet increase, hemostatic effect, and side effects after transfusion. Multiple studies reported pro-inflammatory changes in platelets as well as in MKs in both pre-malignant states and cancer conditions. The inflammatory switch of platelets appears to be one of the most important cancer-instructed "education" of platelets. Morphological changes coupled with upregulation of inflammatory response and hypoxia genes in platelets can be observed in early-stage acute myeloid leukemia (AML) mouse model, associated with impaired mitochondrial oxidation in platelets [26]. Importantly, recent study showed that breast cancer cells can induce generation of pro-inflammatory platelets that occur via alterations to BM MKs. Thus, analyses in spontaneous murine model of breast cancer (PyMT model) by using single-cell RNA sequencing of sorted $CD41^{+}DAPI^{low}$ BM cells, demonstrated the pro-inflammatory phenotype of MKs from tumor-bearing mice. Particularly, inflammation-related *Ctsg*, *Lcn2*, *S100a8*, and *S100a9* transcripts are increased in MKs from tumor-bearing mice [52]. Also, soluble factors derived from breast cancer cell lines (MCF-7, MDA-MB-468 and SK-BR-3) stimulated immune checkpoint molecule glucocorticoid-induced tumor necrosis factor receptor–related protein ligand (GITRL) expression in megakaryoblastic/megakaryocytic MEG-01 cells, suggesting that tumor cells can induce immune related changes in MKs as well [53]. Moreover, platelet-derived TGF-β may specifically be involved in tumor metastasis and in the regulation of inflammatory cell functions. Particularly, TGF-β produced by platelets, declines anti-tumor T- and NK-cell response. Thus, platelets demonstrated capacity for regulation of anti-tumor immune response through the TGF-β/glycoprotein A repetitions predominantly (GARP) axis. Expressed in activated regulatory T cells and platelets, the TGF-β receptor inhibits specific anti-tumor cytotoxic T cells, which results in immune suppression [6]. TGF-β derived from platelets decreases natural killer group 2D (NKG2D) receptor expression on the surface of NKs thus inhibiting their antitumor reactivity [4]. In addition, tumor-associated immune cells significantly modulate platelet profiles.Thus, extracellular chromatin released through neutrophil extracellular traps (NETs) triggers cancer-associated thrombosis in breast cancer patients [54]. NETs provide a scaffold and stimulus for thrombus formation, while P-selectin, cellular or soluble, promotes NET genesis through binding to PSGL-1 [47]. From these data, we can conclude that by responding to TME cells, platelets play active roles in (anti-) tumor immunity, influencing regulatory immune cells, and expressing ligands of immune checkpoint receptors.

Role of platelet-tumor cell interaction in metastatic niche formation

Platelets can extravasate from tumor vasculature to TME [15]. Platelet extravasation is an important phenomenon that supports tumor metastasis after cessation of therapy in ovarian [15], lung and esophageal carcinoma [55]. Specifically, increased production of thrombopoietic cytokines in TME leads to paraneoplastic thrombocytosis, which fuels tumor growth and metastasis. IL-6 secreted by ovarian cancer cells stimulates hepatic TPO production. This triggers thrombopoiesis in the bone marrow, giving rise to thrombocytosis. Administration of anti-platelet antibody can lead to reduction in tumor spread [12]. One mechanism by which the platelet-fibrin(ogen) axis contributes to

metastatic potential is by impeding elimination of tumor cells by NK cell [30]. Additionally, platelet-derived factors are critically important for the formation of pre-metastatic niche. Thromboxane A2 produced by cyclooxygenase 1 in tumor-activated platelets contributes to formation of pulmonary premetastatic niche by endothelial activation, tumor cell adhesion to the endothelium, and recruitment of metastasis-promoting monocytes/macrophages [56]. In prostate cancer, TSP-1-dependent activation of TGF-β1 was shown to regulate pre-metastatic niche formation in bone and tumor-induced bone turnover [34]. Moreover, platelet secretion of CXCL5 and CXCL7 chemokines and subsequent granulocyte recruitment is essential for the formation of early metastatic niche in lung. Thus, specific inhibition of platelet-granulocyte interactions and mediators might represent a valuable anti-cancer therapy [57]. Namely, the interplay of CEPs and immune cells collectively creates an inflammatory milieu at both primary tumor and metastasis site promoting tumor cell proliferation and invasion [58]. On the other hand, platelets might cooperate with residing endothelial and mesenchymal stromal cells affecting tumor cells migration, metastasis, and blood vessel formation. Together, we can conclude that platelets cooperate with TME-residing immune and stromal cells to organize pre- and metastatic niche important for cancer persistence and progression.

Interplay of platelets and cancer-associated cells

Within the perplexed TME different cell types communicate and bidirectionally shape their phenotype and functional properties. As expected, residing and/or recruited cells to the tumor site, such as different stromal and immune cells, interact with platelets contributing to tumor development and progression [58]. CEPs release various proinflammatory cytokines, including CXCL4, CXCL5, CXCL7, CXCL12, IL-8, IL-1β and TGF-β, that potently attract and activate immune cells [31,32] (Fig. 2) (Table 1). When present in TME, immune cells engage in complex interactions with activated platelets leading to phenotype conversion and modulation of leukocyte effector functions.

Platelets and lymphoid cells

CEPs actively influence and interact with cells of lymphoid lineages, T lymphocytes and NK cells. Different direct and indirect mechanisms are involved in $CD4^+$ *T* cell activation upon interplay with platelets, including CD40L-CD40 interactions, secretion of 5-hydroxytryptamine, CXCL4, CCL5 and platelet-activating factor (PAF) [41]. Formation of platelet aggregates with $CD4^+$ *T* cells established through integrins (αIIbβ3), P-selectin, and lectins (e.g., CLEC-2) can disturb their interactions with antigen presenting cells [45,46]. However, additional studies are needed to confirm implication of these pathways in an interplay of tumor-infiltrating platelets and T cells. Thus far, platelet-secreted TGF-β is recognized as a central immunosuppressive player of tumor microenvironment [59]. Higher numbers of platelets are found to inhibit cytotoxic T cells and increase Tregs through TGF-β/GARP axis and lactate production as evidenced both in vitro and in vivo in mouse melanoma and colorectal cancer models [32]. In addition, Mulet and colleagues showed that platelet-derived PAF4 reduced $CD4^+$and $CD8^+$ *T* lymphocyte cytotoxic activity in pleural metastasis favoring tumor progression in lung adenocarcinoma [60]. Recent study also demonstrated that lower numbers of $CD8^+$ *T* cells along with increased platelets stimulate breast cancer metastasis through platelet-secreted CXCL4 which induces myeloid derived suppressor cells (MDSCs) generation [42]. Other literature data linking platelets to patient's tumor immune cell content correlated elevated levels of programmed death-ligand 1 (PD-L1) on platelets with lower numbers of tumor-infiltrating T cells in PD-L1-positive lung cancer patients thus indicating platelet-associated PD-L1 contribution to tumor immune evasion [61] (Fig. 2).

Platelets also reduce the anti-tumor effect of NK cells in different manner. Importantly, when tumor cells enter the circulation, platelets may provide the mechanism to escape NK cell surveillance and increase the metastatic potential [30] by binding to tumor cells and subsequently translocating MHC class I to their surface. Moreover, platelets secrete TGF-β which downregulates the expression of NKG2D leading to reduction of NK cell cytotoxicity and IFN-γ production [33,62] (Fig. 2). Collectively, CEPs and their produced factors impair anti-tumor immunity mediated by lymphoid cells.

Platelets and myeloid cells

Additional evidences indicate that platelet achieve their suppressing roles in anti-tumor immunity by direct communication with myeloid cells. Here, we summarized existing findings on impact of platelets on monocyte, macrophage, neutrophils and MDSCs. Platelets secrete high amounts of CXCL4 in vivo, and support profibrotic secreted phosphoprotein 1 ($Spp1^+$) macrophage differentiation. Applying single nuclear RNA sequencing it is showed that macrophages orchestrate fibroblast activation [43]. In the context of TME, interactions of activated platelets and monocytes have also been reported. Namely, platelet-tumor cell aggregates recruited monocytes and macrophages to early lung metastatic niche in murine melanoma models [63]. Also, in murine model of colon cancer, complex interactions of tumor cells with neutrophils and platelets have been demonstrated to activate endothelium facilitating establishment of metastatic niche through increase of CCL5, which attracts monocytes [44]. VEGF released by activated platelets was also shown to promote the recruitment of circulating macrophages to tumor sites in cervical, mammary, and skin cancers [27,28]. It has also been shown that intratumoral platelets promoted growth and progression of colorectal cancer leading to JNK/STAT1 signaling activation, which further promoted C5 transcription and activated the C5a/C5aR1 axis in tumor-associated macrophages (TAMs). Shifting TAMs toward M2 polarization and impairing the phagocytosis of TAMs was followed by the increased mRNA levels of Arg1 and IL-10 in sorted TAMs [64,65] (Fig. 2). By applying colon cancer and breast cancer mouse models, it has been shown that the controlled TAM depletion might support delivery of anti-PD-1 protein (aPD-1) antibody-conjugated platelets to attenuate tumor recurrence after treatment. Namely, inflammatory environment after surgery can trigger platelet activation to support the release of PMPs conjugated with aPD-1 antibodies and their binding to PD-1 receptors for re-activation of T cells. Thus, it can be speculated that immunotherapeutic efficacy against tumor recurrence of aPD-1 antibody-conjugated platelets could be supported by depletion of TAMs [66]. The presence of platelet-bound neutrophils in circulation of non-small cell lung cancer (NSCLC) patients have been found to correlate with poor patient prognosis [67]. Previous studies showed that neutrophils exert complex functions upon interactions with platelets inhibiting NK cells and cytotoxic T-cells at later tumor stages [68]. Importantly, interactions between platelet P-selectin and neutrophil PSGL-1 induce neutrophil activation, IL-8 secretion, and formation of NETs which contribute to establishment of pro-coagulant environment [47,48]. During metastasis NETs also protect cancer cells from shear stress and immune system in the circulation [69]. Also, surgical stress can favor interactions between NETs and platelet-coated-circulating tumor cells via TLR4-ERK5 axis facilitating tumor cell dissemination [69]. However, the mechanisms of platelet-monocyte/macrophage and platelet/neutrophil interactions in tumorigenesis and cancer progression remains to be further explored. Additionally, extravasated platelet aggregation (EPA) plays a key role in the TME and tumor progression. EPA induces epithelial-mesenchymal transition (EMT) via TGF-β (44, 62), and recruits immunosuppressive cells, including Tregs cells and MDSCs. Accordingly, analyses of tissue from gastric cancer patients showed that EPA affects immunosuppression by recruiting MDSCs in the tumor microenvironment via the secretion of soluble factor TGF-β [70,35] (Fig. 2). Connection between VTE, immunotherapy and increased MDSCs and IL-8 levels, has also been observed in cancer patients with developed VTE during immunotherapy. MDSCs possess procoagulant

properties and the release of IL-8 by tumor and other cells in the TME is linked to MDSC accumulation within tumors of patients receiving immune checkpoint inhibitor (ICI). Moreover, it has been suggested that MDSCs with activation of coagulation pathways can impair anti-tumor immunity [71]. From these findings, it can be concluded that platelets support myeloid cells recruitment to TME, and their further conversion towards immunosuppressive profile and finally, metastatic site establishing.

Interplay of platelets and stromal cells

Besides immune cells, platelets communicate with different cell populations within TME stroma, such as endothelial cells (ECs) and mesenchymal stromal cells (MSCs). In this section, we summarized current knowledge on these interactions. Namely, ECs establish important interactions with platelets. Activated platelets have been shown to release PDGF-BB and VEGF which stimulate vascular ECs proliferation and differentiation thus promoting neovascularization and tumor growth [49]. Indirectly, platelets stimulate neutrophil migration by activating ECs to secrete Weibel-Palade vesicles and express P-selectin, contributing to tumor development [29]. Also, platelet dense granules release ATP which binds to P2Y2 receptors on ECs stimulating vascular permeability and tumor cells extravasation to metastasis site [72]. As for MSCs, common constituents of healthy tissue and TME [73], studies implied importance of their interactions with platelets. Platelet-derived factors were shown to stimulate immunosuppressive functions of MSCs, inducing, in parallel, their metabolic adaptations, senescence and susceptibility to apoptosis [74]. However, the contribution of these mechanisms to mutual interactions between MSCs and platelets in tumor niche remains to be determined. So far, platelet impact on MSCs' capacity to support gastric cancer proliferation, migration, and metastasis in vitro and in vivo was shown [75], evidencing cancer cells and MSCs interplay in platelet activation, along with platelet-induced MSC transdifferentiation into cancer-associated fibroblast (CAF)-like cells through TGF-β production. In vitro and in vivo studies in mice melanoma model showed that tumor-educated platelets induced migration of MSCs to secondary metastatic site where they contribute to vessel-like structure formation (vascular mimicry), ultimately favoring metastasis process [76]. Also, the role of TSP-1/TGF-β1 axis in platelets interactions with bone marrow cells was found to contribute to pre-metastatic niche formation and tumor-induced bone turnover in murine model of prostate cancer [34]. Platelets may influence other important constituents of TME, such as CAFs, as shown in pancreatic cancer and metastasis of gastric cancer [70], which impact on tumor progression is currently unknown. Taken together, platelets can contribute to neovascularization in TME and formation of CAFs which directly support metastatic process and cancer progression. However, it is clear that additional studies have to be performed and molecular background of these events has to been further investigated and clarified.

Platelets in cancer patient immunotherapy

Targeting platelets and their products appears to be a promising anticancer strategy, while platelet homeostasis is an important indicator for immunotherapy performance. Genetic targeting of platelets supports adoptive T cell therapy of cancer. It is reported that TGF-β and lactate act as major platelet-derived soluble factors to exhaust $CD4^+$ and $CD8^+$ *T* cell functions. Thus, deletion of TGFβ-docking receptor glycoprotein A repetitions predominant (GARP) gene Lrrc32 at the tumor site can protect immunity against cancer implying combined cancer immunotherapy to target both TGFβ and GARP in platelets [32].

Moreover, in the following section, we will discuss the interference of platelets and immunotherapies in cancer patients. Due to promising pre-clinical and clinical trial results, immune checkpoint inhibitors (ICIs) are part of Food and Drug Administration approved immunooncology therapies. Yet not all patients benefit from ICI therapy [36]. ICI therapies that target the PD-1, or its ligand (PD-L1) have shown unprecedented rates of durable clinical responses in patients with various cancer types. Moreover, it has been suggested that platelet counts might be a predictive biomarker for immunotherapy. Thus, patients with thrombocytosis respond better to anti-PD-1/PDL-1 therapy [40]. It appears that PD-L1 expression increases in both tumor cells and platelets in cancer patients and further blocks anti-tumor T cell response [77,78]. Expression of PD-L1 in metastatic breast cancer patients on both tumor cells and platelets, could present a promising predictive marker to determine which patients should receive ICI, and as a pharmacodynamics biomarker during the treatment ([77]. However, treatment of lung cancer patients with the PD-L1 inhibitor fully humanized antibody (atezolizumab) caused a decrease in PD-L1 expression on platelets and peripheral blood mononuclear cells, without affecting whole platelet counts or leukocyte counts in human lung and head and neck squamous cell cancer [78]. Coagulation process activated by TF and complement component factors can alter T cell responses leading to immunotherapy resistance and side effects [79]. Retrospective study showed high incidence of thromboembolism in melanoma patients receiving ICI and particularly with combination therapy [80]. Yet, another retrospective study revealed a higher rate of VTE in NSCLC patients treated with platinum-based chemotherapy when compared to those receiving ICI [81]. On the other side, development of grade 1 thrombocytopenia is associated with improved overall survival in metastatic patients treated with ICI, when compared to patients without thrombocytopenia [82]. Promising effects of novel inhibitors of immunothrombosis have been observed. Thus, M7824, a fusion protein against PD-L1 and TGF-β, is first-in-class bifunctional fusion protein composed of a monoclonal antibody against PD-L1 fused to a TGF-β. Basically, this protein saturates peripheral PD-L1 and sequesters any released plasma TGF-β1, -β2, and -β3, and this activity has been shown in cervical and pancreatic cancer [83]. Another type of immunotherapy, applying monoclonal antibody, has been identified to be initiator of thrombosis in NSCLC patients. Namely, empowerment of T lymphocytes by humanized monoclonal immunoglobulin (Ig) G4 directed against human cell surface receptor PD-1, was found to induce acute (inflammatory) thrombosis development [37].

Modulation of platelet and their factors has good perspective in immunotherapy optimization. Intra-tumoral platelet-derived PD-L1 regulates the growth of PD-L1-negative tumor cells and blockade of platelet binding to PD-L1-negative tumor cells promotes T cell-induced cytotoxicity [84]. Also, TGF-β is not only involved in tumor-mediated changes in platelet functions. Although T cell-based immunotherapy based on T cell-recruiting bispecific antibody (bsAb) made important progress in immunooncology therapies, many patients do not respond to treatment. Thus, a clinical study NCT04104607 [85] has shown that prostate-specific membrane antigen (PSMA) × CD3 bsAb decreased total platelet amount, while platelet activation was present. Specifically, platelet activation impaired bsAb-mediated $CD4^+$ and $CD8^+$ T-cell reactivity, leading to inhibition of tumor cell lysis, while blockade of TGF-β could restored T-cell reactivity [85]. Interestingly, platelets increase PD-L1 on ovarian tumor cells both directly (contact-dependent via NF-κB signaling) and indirectly (via TGF-β released from platelets through TGF-βR1/Smad signaling) [40]. Importantly, there is a huge need for the development of platforms that can overcome the limitations of current strategies for ICI delivery. Recently, a platform based on the possibility of PMPs delivering aPD-1 antibodies into the TME has been established. This thrombosis-mediating navigation system combined platelet membrane, paclitaxel, and nanoparticles in a patient-derived xenograft (PDX) orthotopic breast cancer model. It was shown that recruited anti-PD-1 antibody-conjugated platelets led to elevation of cytokines with anti-tumor activities, IFN-γ and TNF-α [86]. As for the most frequent cancers, within the following part, we will provide an overview on the capacity of CEPs to modify immunotherapies.

Lung cancer. Circulating platelets interact with lung cancer cells where PD-L1 protein is transferred from tumor cells to platelets in a

fibronectin 1, integrin α5β1 and GPIbα-dependent manner. Thus, PD-L1 on platelets has an important role in tumor immune evasion [61]. Although suggested, immunoregulatory role of platelets in cancer is not fully understood. In NSCLC patients, it has been found that platelets possess triggered receptor expressed on myeloid cell–like (TREM)-like transcript 1 (TLT-1). Additionally, in syngeneic tumors in immunocompetent mice, binding of TLT-1 to T lymphocytes led to exhaustion of $CD8^+$ *T* lymphocyte fraction, thus supporting lung cancer growth. Importantly, anti–TLT-1 antibody prevented exhaustion of patients' T cells from platelet-induced immunosuppression ex vivo and reduced tumor growth in mice in vivo. The activation-promoting genes (GZMB, IL2, IL12B, IFNG, and TNF) involved in antitumor responses and the CXCR family genes (CXCR1, CXCR2, and CXCR3) associated with improving antitumor responses by effector T lymphocytes were also downregulated in treated $CD8^+$ *T* lymphocytes. Thus, NSCLC platelet TLT-1 suppresses $CD8^+$ *T* lymphocytes, whereas extracellular TLT-1 can be targeted using an antibody-based therapeutic approach to prevent platelet-mediated suppression of patient $CD8^+$ *T* lymphocytes [86].

Breast cancer. Luminal breast carcinoma) can load circulating platelets with proinflammatory and proangiogenic factors rendering them pro-tumorigenic. Such educated platelets can then systemically promote tumor growth and vascularization, arising malignant phenotype of otherwise indolent tumor [87]. Platelets induce high expression of pro-inflammatory interleukin 8 (IL-8, CXCL8), thus promoting invasiveness via AKT signaling in breast cancer cells. Aspirin treatment of platelets lowers IL-8 production by breast cancer cells in patients [88]. High blood platelet-to-lymphocyte ratio has been associated with failed ICI treatment in breast cancer cells. Moreover, platelets induce increased protein and gene expression of PD-L1 by breast cancer cells, which can be abolished by anti-platelet agents, such as aspirin and ticagrelor. EGF-neutralizing antibody and cetuximab (EGF receptor monoclonal antibody) inhibited platelet-induced increases in PD-L1 in breast cancer cells [36]. Another platelet-associated immune checkpoint is glucocorticoid-induced TNF receptor family-related protein ligand (GITR). GITR is expressed by platelets and acts as known context-dependent modulator of T cells and NK cells. Ligand of GITR (GITRL) is found to be increased on blood platelets in breast cancer patients when compared to healthy donors, and its expression varied during different cancer stages [53].

Conclusion remarks and further readings

It can be concluded that platelet and mediators released in TME represent promising targets and vectors for novel anti-cancer strategies development. Moreover, profiling of transcriptome of cancer platelets can improve cancer disease stratification [50]. Some novel platforms have been recently described to advance the discovery of novel anti-platelet therapeutics against tumor metastasis and chemoresistance [2]. Another important finding revealed that platelets uptake prothrombotic small extracellular vesicles from aggressive cancer cells. These platelet-associated cancer biomarkers appear to have perspective diagnostic and prognostic potential [89]. Therefore, further research of platelets in cancer will bring novel knowledge on disease development as well as novel drug design.

CRediT authorship contribution statement

Drenka Trivanović: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Slavko Mojsilović:** Investigation, Visualization, Writing – original draft, Writing – review & editing. **Nikola Bogosavljević:** Investigation, Visualization, Writing – original draft, Writing – review & editing. **Vladimir Jurišić:** Writing – review & editing. **Aleksandra Jauković:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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