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Procoagulant platelets promote immune evasion in triple negative breast cancer

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Abstract:
Triple-negative breast cancer (TNBC) is an aggressive tumor entity, in which immune checkpoint (IC) molecules are primarily synthesized in the tumor environment. Here, we report that procoagulant platelets bear large amounts of such immunomodulatory factors and that the presence of these cellular blood components in TNBC relates to pro-tumorigenic immune cell activity and impaired survival. Mechanistically, tumor-released nucleic acids attract platelets into the aberrant tumor microvasculature where they undergo procoagulant activation, thus delivering specific stimulatory and inhibitory IC molecules. This concomitantly promotes pro-tumorigenic myeloid leukocyte responses and compromises anti-tumorigenic lymphocyte activity, ultimately supporting tumor growth. Interference with platelet-leukocyte interactions prevented immune cell misguidance and suppressed tumor progression, nearly as effective as systemic IC inhibition. Hence, our data uncover a self-sustaining mechanism of TNBC in utilizing platelets to misdirect immune cell responses. Targeting this irregular multicellular interplay might represent a novel immunotherapeutic strategy in TNBC without side effects of systemic IC inhibition.

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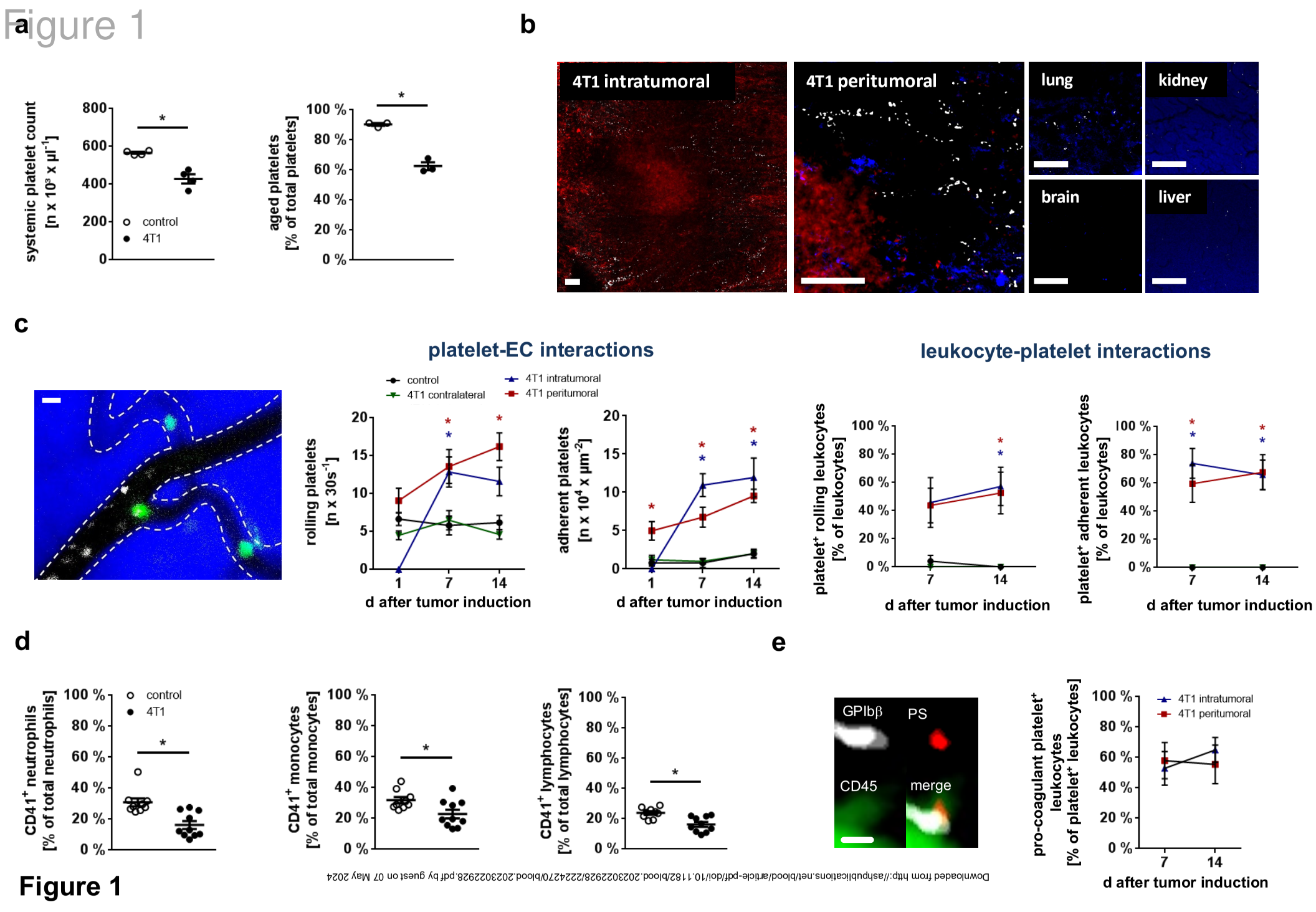
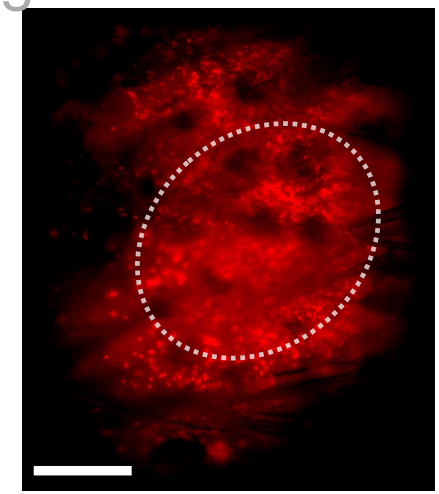
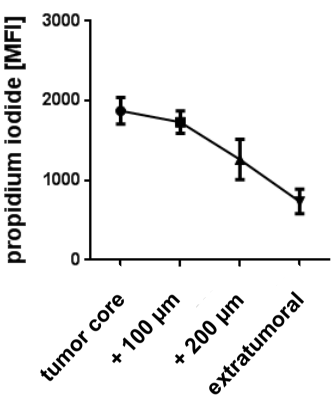
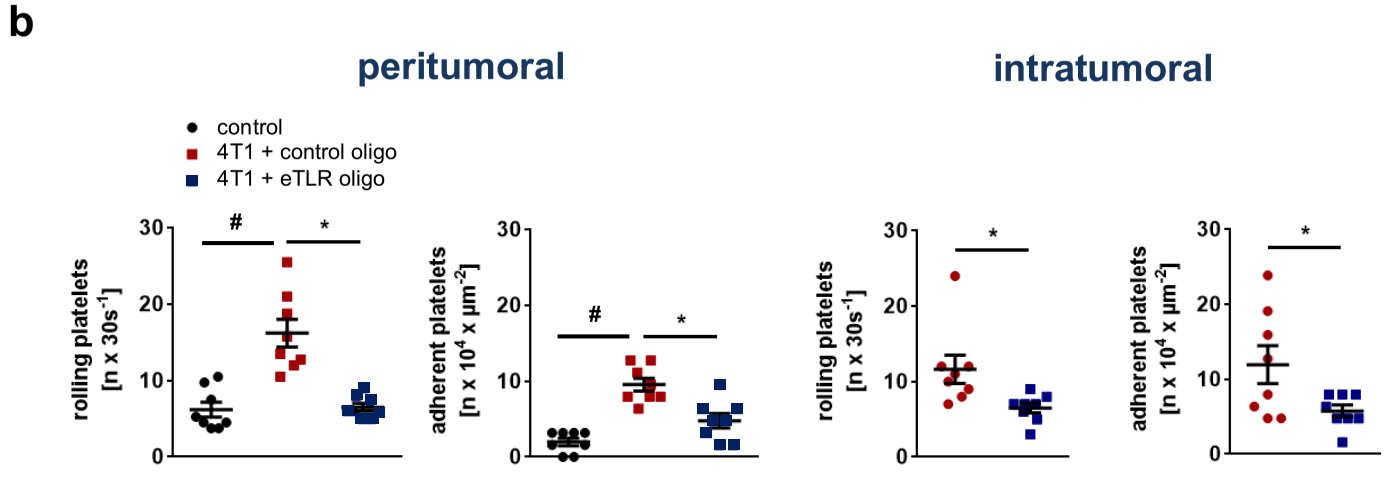
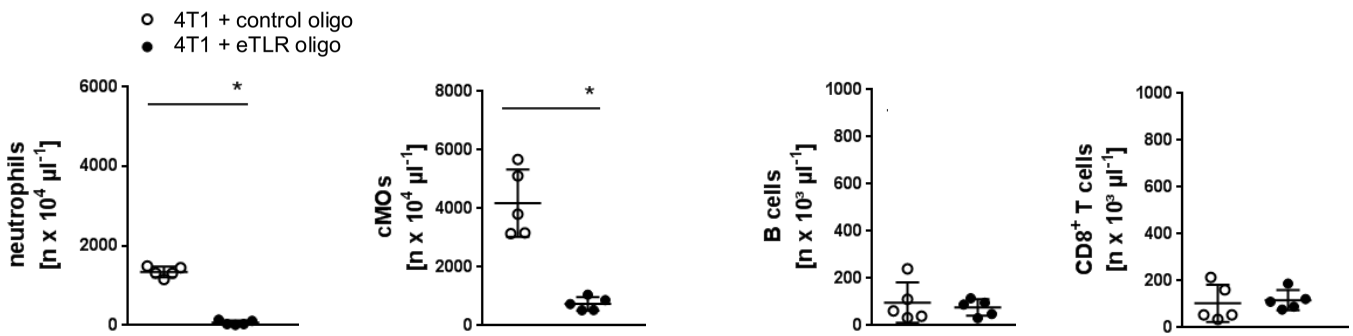


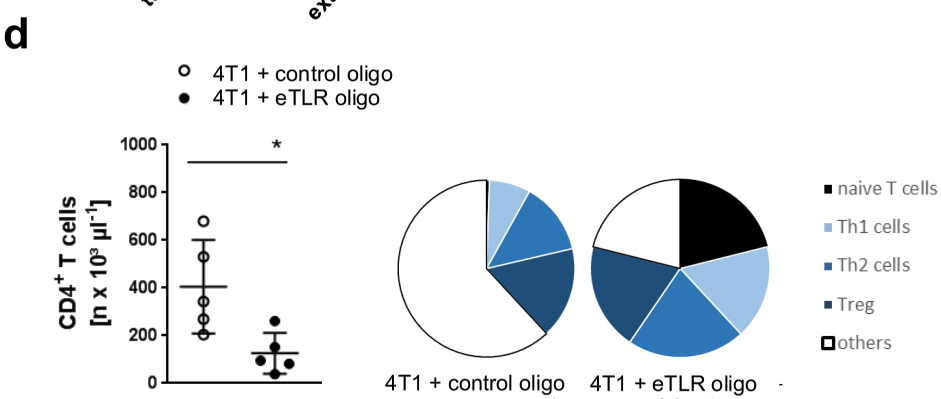
Figure 1

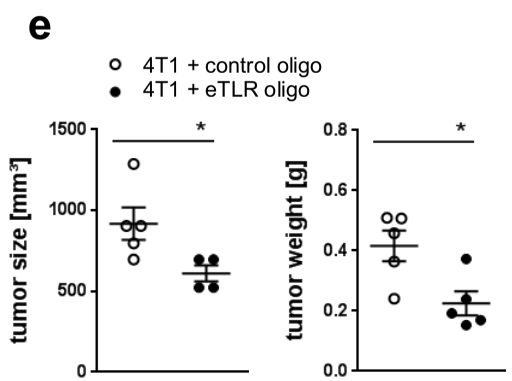


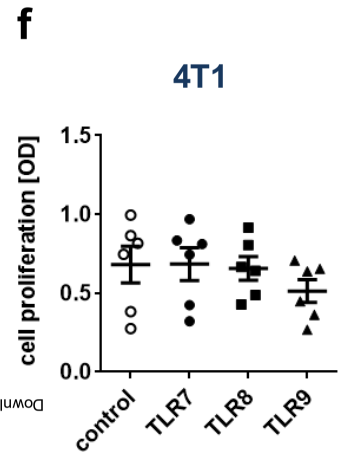


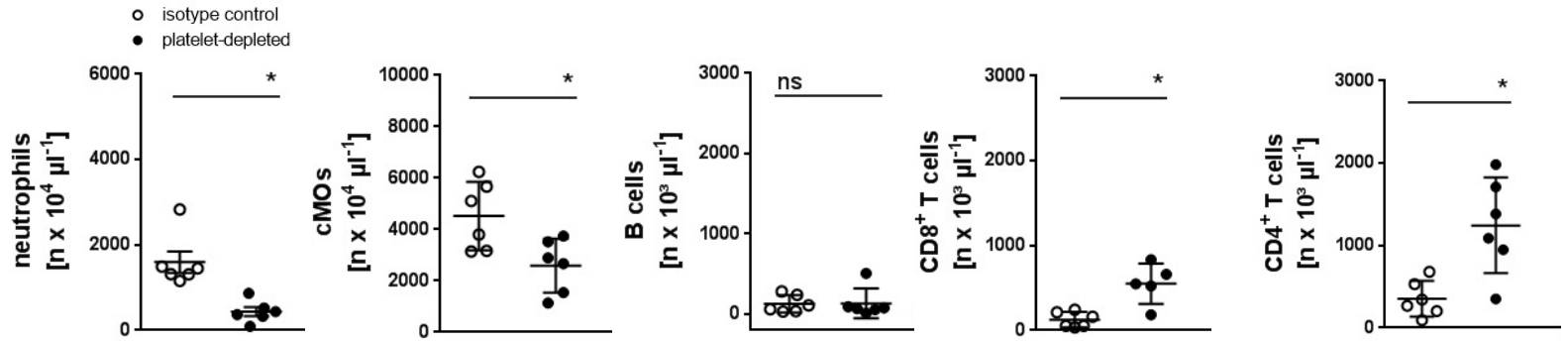


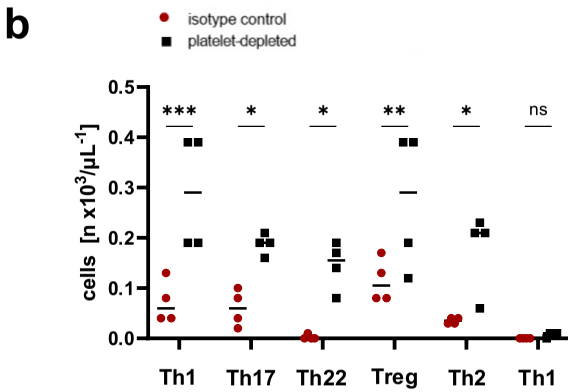


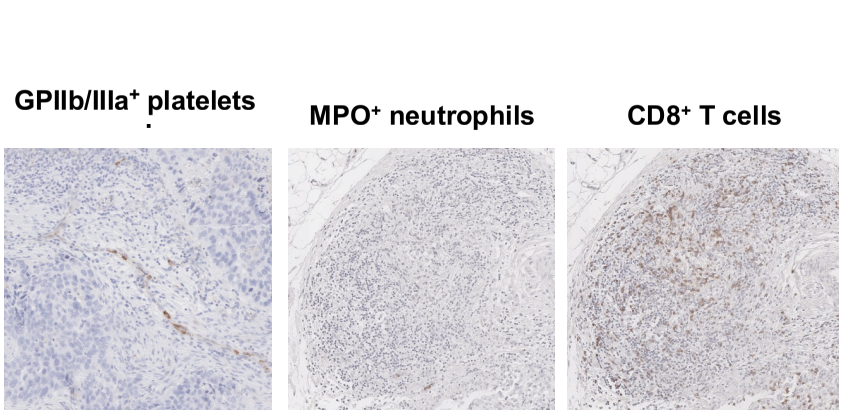


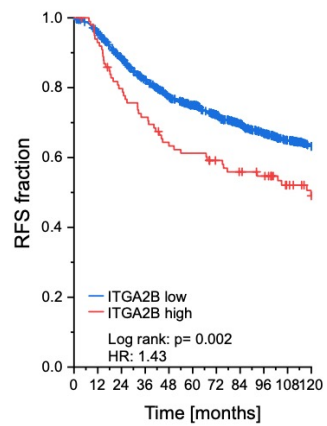


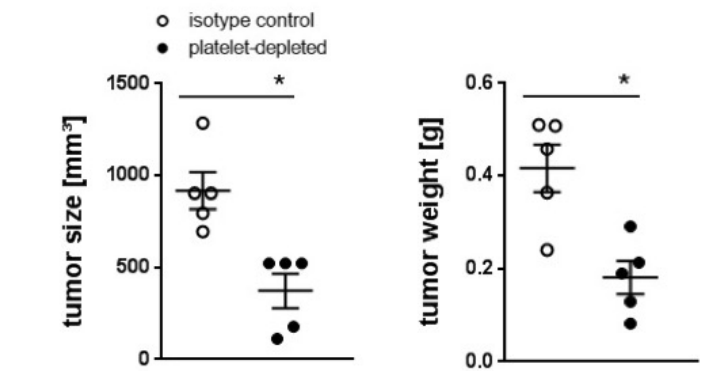












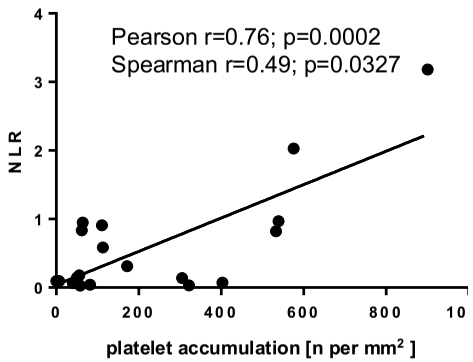
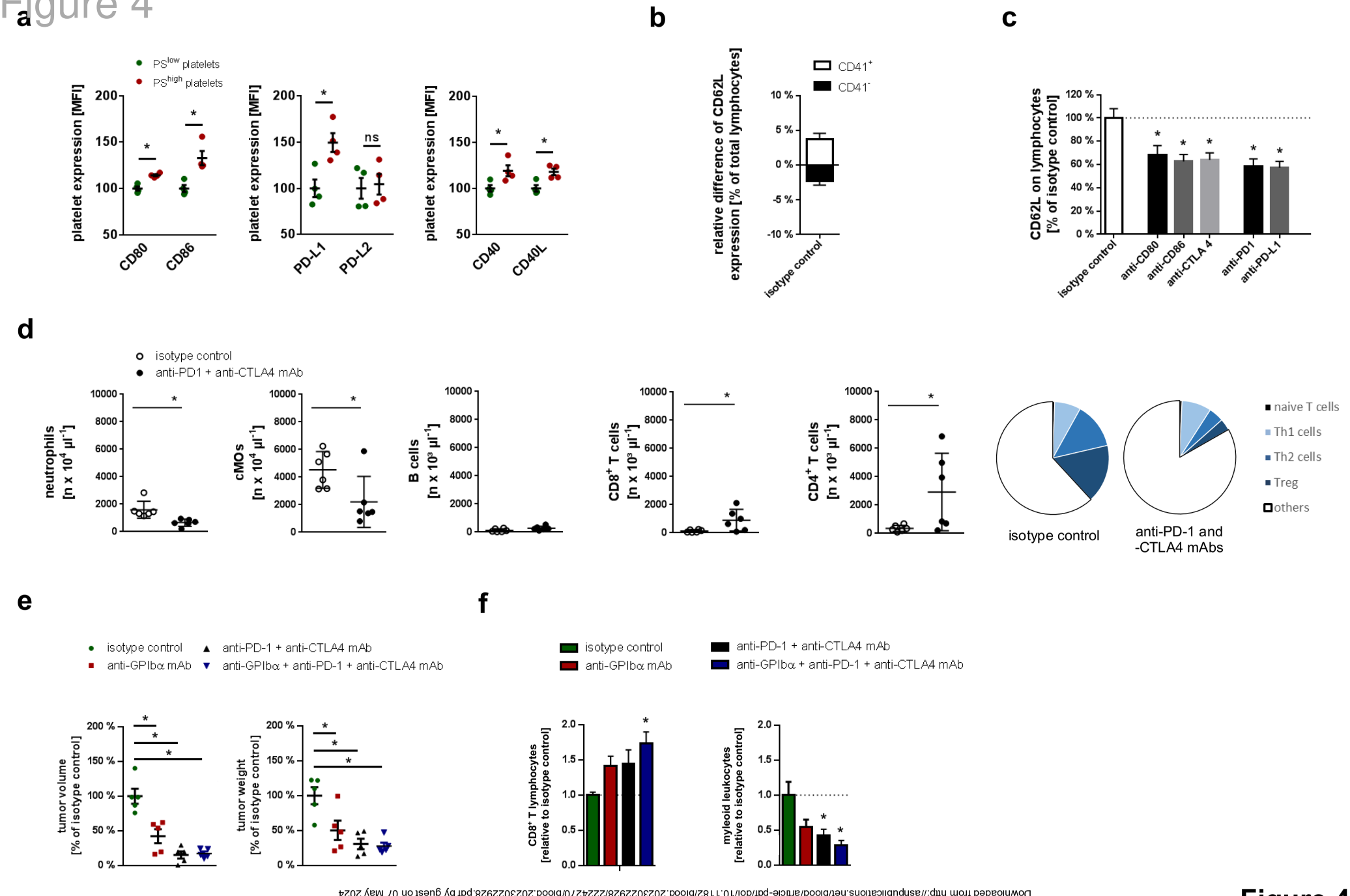


Figure 3



Procoagulant platelets promote immune evasion in triple negative breast cancer

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Key points

- Procoagulant platelets deliver large amounts of immune checkpoint molecules to malignant tumors.
- Procoagulant platelets employ these immune checkpoint molecules to misguide immune cell responses, thus promoting tumor progression.

Abstract

Triple-negative breast cancer (TNBC) is an aggressive tumor entity, in which immune checkpoint (IC) molecules are primarily synthesized in the tumor environment. Here, we report that procoagulant platelets bear large amounts of such immunomodulatory factors and that the presence of these cellular blood components in TNBC relates to pro-tumorigenic immune cell activity and impaired survival. Mechanistically, tumor-released nucleic acids attract platelets into the aberrant tumor microvasculature where they undergo procoagulant activation, thus delivering specific stimulatory and inhibitory IC molecules. This concomitantly promotes pro-tumorigenic myeloid leukocyte responses and compromises anti-tumorigenic lymphocyte activity, ultimately supporting tumor growth. Interference with platelet-leukocyte interactions prevented immune cell misguidance and suppressed tumor progression, nearly as effective as systemic IC inhibition. Hence, our data uncover a self-sustaining

mechanism of TNBC in utilizing platelets to misdirect immune cell responses. Targeting this irregular multicellular interplay might represent a novel immunotherapeutic strategy in TNBC without side effects of systemic IC inhibition.

Introduction

Breast cancer is the most prevalent oncological disorder in women worldwide and one of the most common solid cancers overall. Thereof, triple negative breast cancer (TNBC) accounts for up to 20% of the cases. These tumors do not express the therapeutically targetable receptors for estrogen (ER), progesterone, or human epidermal growth factor receptor 2 (HER2) and belong to the most aggressive breast cancer entities.[1]

The immune system protects the organism against the development of malignant tumors. In particular, cytotoxic T lymphocytes (CTLs) recognize neoplastic cells and eliminate them through the release of perforins and granzymes. Furthermore, Th1-polarized $CD4^+$ T lymphocytes collaborate with CTLs in cytotoxic killing, increase antigen presentation in neoplastic cells, and induce anti-tumor activity in peritumoral macrophages. Strategies to enhance responses of these anti-tumorigenic immune cells (*e.g.,* 'immune checkpoint inhibitors' (ICI)) have already been proven effective in clinical trials for different cancer entities.[2] Here, primarily interference with interactions of tumor-released *programmed death-ligand 1* (PD-L1) with *programmed death receptor 1* (PD-1) expressed on the surface of CTLs and/or of tumor-released

CD80 and CD86 with their receptor *cytotoxic T-lymphocyte-associated protein-4* (CTLA-4) increased the anti-tumor activity of CTLs, ultimately compromising malignant growth.[2] In TNBC, however, PD-L1 expression is largely restricted to the tumor environment while tumor cells produce only low amounts of this IC molecule.[3] Importantly, more than 10% of anti-PD-L1/PD-1 ICI-treated patients experience severe or life-threatening autoimmune-related side effects due to compromised homeostatic PD-L1 activity in healthy tissues causing excessive local CTL activity. The prevalence of these side effects is further enhanced upon addition of other ICI (*e.g.*, anti-CTLA-4 antibodies) to single ICI treatment.[4] Consequently, the benefit of ICI in TNBC is subject of controversial discussions.[5] Moreover, distinct leukocyte subsets are able to interfere with CTL activity or even increase B cell-mediated pro-tumorigenic humoral responses (*e.g.*, Th2 T cells; regulatory T cells, T_{reg}). Specifically, myeloid leukocytes (*e.g.*, neutrophils, monocytes) exhibit potent pro-tumorigenic properties by producing pro-proliferative, pro-angiogenic, and immunosuppressive factors, notwithstanding that these immune cells are also able to exhibit anti-tumorigenic properties.[6,7] Although tremendous insights have been gained into the individual anti- and pro-tumorigenic functions of different immune cell populations, it remains poorly understood how specific leukocyte subsets reach malignant lesions.

Besides their essential role in hemostasis, platelets are increasingly recognized to participate in tumorigenesis.[8-11] To this end, platelets release growth factors and angiogenic mediators[12,13,14], reduce tumor cell apoptosis and anoikis,[15] induce gene expression[16] and epithelial-mesenchymal transition (EMT) in malignant cells,[17-19] as well as protect tumor cells from immune responses,[20-23] thus promoting tumor progression and metastasis.[24] Accordingly, cyclooxygenase inhibitors (*e.g.,* acetylsalicyclic acid)[25-27] or adenosine diphosphate (ADP) receptor antagonists (*e.g.,*

clopidogrel, ticagrelor, or prasugrel) have been reported to exhibit beneficial effects in the prevention of different malignancies including breast cancer.[28] Importantly, activated platelets acquire distinct phenotypes in thrombosis: Whereas 'aggregatory' platelets tighten the platelet plug, 'procoagulant' platelets expose negatively charged phospholipids (particularly phosphatidylserine (PS)), on their surface upon contact to subendothelial collagen to promote plasmatic coagulation.[29-31] The role of these activated platelet phenotypes in cancer, however, remained unclear.

Reciprocal interactions with platelets are well-known to support the extravasation of immune cells to the perivascular tissue under inflammatory conditions.[32-38] The functional relevance of platelets for the regulation of immune cell responses in malignant tumors, however, is still elusive. Interestingly, platelets have recently been reported to express IC molecules including PD-L1.[39-41] With respect to the already known pro-tumorigenic effects of platelets and their established role for immune cell trafficking in inflammation, we hypothesize that these anucleate cell particles employ their specific immunomodulatory properties to promote tumor progression in TNBC.

Methods

Systemic trafficking dynamics of platelets including their interplay with immune cells in the intra-/peritumoral microvasculature were analyzed in female BALB/c mice using orthotopic (mammary fat pad: flow cytometry, confocal microscopy) and heterotopic (auricle: *in vivo* microscopy) syngeneic TNBC models (4T1 tumor cells) at early tumor stages. To characterize the underlying mechanisms, flow cytometry in a mouse peritoneal assay, *in vivo* microscopy in a mouse cremaster muscle assay, and different *in vitro* assays were employed. Towards translational perspectives, immunohistochemical analyses in human breast cancer samples, flow cytometry in primary human platelets, and bioinformatic analyses of published transcriptomic data were performed.

A detailed Methods section is provided in Supplemental data.

All animal experiments were performed according to German law for animal protection and approved by the local government authorities ('Regierung von Oberbayern').

Results

Procoagulant platelets accumulate in the tumor microvasculature

In the present study, we hypothesize that platelets employ their immunomodulatory properties to regulate tumor growth in TNBC. To prove this, we first sought to follow the tracks of platelets in an orthotopic syngeneic mouse model of TNBC at early stages. Flow cytometry revealed that the total number of circulating platelets initially declined in tumor-bearing animals as compared to healthy controls, before increasing (**Tab. S1a**). A pulse-labeling approach further documented that the proportion of aged platelets of total circulating platelets was significantly diminished in diseased animals (**Fig. 1a**), pointing to a reduced circulatory time of these anucleate cell particles in the peripheral blood. In this context, confocal microscopy of tissue sections demonstrated that platelets primarily accumulate in tumors, but barely in the major peripheral organs of the diseased animals (**Fig. 1b; Tab. S1b**). These events were associated with a (compensatory) increase in bone marrow megakaryocyte and platelet content (**Tab. S1b, c**) as well as slight splenic organ enlargement (**Tab. S1d**) and enhanced splenic accumulation of platelets (**Tab. S1e**), collectively unveiling an accelerated platelet turnover in diseased animals. In particular, *in vivo* microscopy in a heterotopic 4T1 breast cancer model (mouse auricle) documented that platelets (together with neutrophils, lymphocytes, and – to a lesser degree – with classical monocytes (cMOs), but not with non-classical monocytes; **Fig. S1a**) increasingly roll and adhere on the endothelial surface of peritumoral and, later on, of the developing intra-tumoral aberrant microvessels, with the progression of the tumors (**Fig. 1c**). Importantly, the accumulating immune cells were preferentially bound to adherent platelets (**Fig. 1c; video S1**). As a result of these events, less aggregates of platelets and immune cells were formed in the peripheral blood of diseased animals than in

healthy controls (**Fig. 1d**). Interestingly, immunostaining and flow cytometry of tumor tissue homogenates revealed that there is an almost 100-fold higher content of platelets exhibiting high surface levels of PS (**Tab. S1f**) than in the peripheral blood, which is indicative for the interactive 'procoagulant' phenotype of these anucleate cell particles (exhibiting a larger cell size, more nucleic acid content, as well as higher surface levels of P-selectin, GPIIb/IIIa, and coagulation factor Xa as opposed to non-procoagulant platelets including platelets with 'aggregatory' or resting phenotypes; **Fig. S1b**).[29-31] Hence, circulating platelets 'settle down' in malignant lesions, where they interact with immune cells upon procoagulant activation (**Fig. 1e**).

DAMPs promote platelet trafficking into the tumor microvasculature

Cell death occurs frequently in malignant tumors, leading to the release of damage-associated molecular patterns (DAMPs; *e.g.,* nucleic acids, ADP) into the extracellular space.[42,43] Accordingly, *in vivo* propidium iodide staining in our heterotopic 4T1 breast cancer model demonstrated a broad deposition of extracellular nucleic acids in the tumor environment as assessed by *in vivo* microscopy (**Fig. 2a**). With respect to these observations, we hypothesized that the nucleic acid-recognizing endosomal toll-like receptors (eTLR)-7, -8, and -9 initiate the trafficking of platelets and immune cells into malignant lesions. In line with this assumption, inhibition of these eTLRs by blocking oligonucleotides significantly decreased the recruitment of platelets (**Fig. 2b**), neutrophils, cMOs, and $CD4^+$ T cells (but not of CTLs or B cells) into the tumor microvasculature as compared to control oligonucleotide-treated animals (**Fig. 2c, d**). These immunomodulatory effects of the blocking oligonucleotides were associated with significantly reduced tumor size and weight **(Fig. 2e)**. Of note, exposure of TLR7, -8, or -9 agonists to 4T1 cancer cells did

not alter 4T1 tumor cell proliferation *in vitro* (**Fig. 2f**), collectively implying that tumor-released nucleic acids recruit platelets and immune cells into malignant lesions through eTLRs and subsequently support tumor progression. Importantly, ADP receptor blockade did not significantly alter procoagulant platelet (**Fig. S2a**) or immune cell (**Fig. S2b**) accumulation in the orthotopic TNBC model, but decreased non-procoagulant platelet accumulation (**Fig. S2a**) and, partially, tumor growth (**Fig. S2c**). Further, surface levels of sialic acid on platelets (which decrease with chronological platelet ageing and modulate systemic immune responses)[44] were not significantly altered upon ADP receptor blockade (**Fig. S2d**), collectively pointing to non-immunomodulatory pro-tumorigenic effects of non-procoagulant platelets.

Towards a more comprehensive mechanistic understanding of these eTLR-dependent processes, we employed flow cytometry analyses in a mouse peritoneal assay and *in vivo* microscopy in the mouse cremaster muscle. Here, agonists of TLR7, -8, or -9 (but not of TLR3) induced the trafficking of neutrophils, cMOs, and lymphocytes (**Fig. S3a**) as well as of platelets (**Fig. S3b**) to the site of application as compared to controls. *In vitro* analyses further revealed that agonism of these eTLRs directly activates neutrophils, cMOs (indicated by increased surface expression of Mac-1; **Fig. S4a**), and lymphocytes (indicated by L-selectin shedding; **Fig. S5**), but does not directly activate platelets (indicated by unchanged surface expression of activated GPIIb/IIIa) or microvascular endothelial cells (indicated by unaltered ICAM-1, VCAM-1, E-selectin, and P-selectin surface expression). Instead, agonism of TLR7, -8, or -9 profoundly induced the production of the cytokine tumor necrosis factor (TNF) in macrophages (**Fig. S4b**) that, in turn, activated endothelial cells to induce the expression of adhesion molecules on their surface (**Fig. S4c, d**). Accordingly, endothelial expression of these molecules in the microvasculature was more pronounced in the vicinity of perivascular macrophages (**Fig. S4e**), collectively

initiating endothelial interactions with platelets and immune cells. Thus, nucleic acids mediate platelet and immune cell responses *via* direct and indirect eTLR-dependent effects.

Platelets differentially regulate responses of distinct immune cell subsets

In our TNBC model, antibody-mediated depletion of neutrophils or cMOs attenuated tumor growth, whereas depletion of CTLs supported tumor progression (**Fig. S6**). To characterize the functional relevance of platelets for responses of these immune cells in TNBC, we performed experiments in thrombocytopenic animals (**Tab. S2a**). Here, antibody-mediated depletion of platelets almost completely abolished the migration of pro-tumorigenic neutrophils and cMOs into orthotopically grown 4T1 tumors, whereas the infiltration of anti-tumorigenic CTLs was significantly enhanced as compared to isotype control antibody-treated tumor-bearing mice (**Fig. 3a**). To a lesser extent, tumor infiltration by Th17 and Th22 cells, of anti-tumorigenic Th9 cells, as well as of pro-tumorigenic T_{reg} and Th2 cells, but not of B cells or anti-tumorigenic Th1 cells (**Fig. 3a, b**), were intensified in thrombocytopenic animals. The resulting overall immunomodulatory effect of platelet depletion was associated with a significant reduction in tumor size and weight as compared to isotype control antibody-treated controls (**Fig. 3c**). Our findings were confirmed in the more reductionist peritoneal assay as eTLR-dependent extravasation of neutrophils and cMOs to the peritoneal cavity was significantly attenuated upon platelet depletion, whereas responses of lymphocytes were significantly elevated (**Fig. S7a**). Importantly, antibody-mediated depletion of neutrophils (**Tab. S2b**) significantly diminished the recruitment of cMOs into the peritoneal cavity, but did not alter lymphocyte responses (**Fig. S7b**). Thus, platelets differentially regulate innate and adaptive immune cell responses in the

tumor microenvironment and (subsequent) tumor progression. Most interestingly, immunohistochemical analyses of human TNBC samples documented that intravascular accumulation of platelets positively correlates with high neutrophil-CTL ratios in the perivascular tumor immune cell infiltrate (**Fig. 3d**), confirming our experimental data and translating them into human disease. Accordingly, gene expression data from the METABRIC breast cancer cohort (**Fig. S8a**) indicate that recurrence free (**Fig. 3e**) and overall (**Fig. S8b**) survival of breast cancer patients with high RNA expression of the platelet surrogate marker integrin α_{IIb} (ITGA2B/CD41) in their tumors is significantly impaired as compared to patients with low ITGA2B RNA levels. Interestingly, $ITGA2B^{high}$ expressing METABRIC cases were enriched with the $ER^{+}/HER2^{high}$-proliferating subtype and histological grade 3 (**Fig. S8a, c**). On the molecular level, ITGA2B expression positively correlated with expression of gene sets involved in proliferation, DNA synthesis, and repair and negatively correlated with gene sets associated with immune mechanisms and inflammation (**Fig. S8d**). Noteworthy, tumoral ITGA2B RNA expression did not positively correlate with expression of the endothelial cell marker PECAM-1, whereas tumor RNA expression of PECAM-1 positively correlated with expression of PD-L1, CD80, CD86, CD40, and CD40L (**Fig. S8e**) – collectively suggesting that higher expression levels of ITGA2B do not simply reflect higher tumor vascularization.

Procoagulant platelets deliver immune checkpoint molecules into tumors

Recently, IC molecules have been detected on the surface of platelets.[39-41] Here, we show that particularly procoagulant platelets bear large amounts of the inhibitory IC molecules PD-L1, CD80, and CD86 (but not of PD-L2) as well as of the stimulatory IC molecules CD40 and CD40L on their surface as compared to non-procoagulant

platelets or immune cells isolated from the peripheral blood of tumor-bearing mice (**Fig. 4a; Fig. S9**). Similar results were obtained in primary human platelets (**Fig. S10**). Confocal microscopy further documented that platelets in the aberrant microvasculature of orthotopically raised 4T1 tumors co-localize with exposed collagen (**Fig. S11a**). Interestingly, collagen-stimulated procoagulant activation of platelets associated *in vitro* particularly with high surface expression of PD-L1, which was mediated by the platelet receptors GPVI and GPIIb/IIIa as well as through cyclophilin D (cypD)- and scramblase transmembrane protein (TMEM) 16F-dependent pathways (**Fig. S11b, c**). Accordingly, blockade of GPVI and – to a lesser extent – of GPIIb/IIIa significantly attenuated the accumulation of procoagulant platelets in 4T1 tumors (**Fig. S11d**). In addition, blockade of GPVI or GPIIb/IIIa diminished intratumoral numbers of non-procoagulant platelets and myeloid leukocytes while increasing intratumoral lymphocyte responses (**Fig. S11d, e**). As a consequence, tumor growth was strongly impaired (**Fig. S11f**).

Furthermore, procoagulant platelets exhibited higher expression levels of the immunomodulatory cytokines CCL5 and TGF-□ than non-procoagulant platelets (**Fig. S12**). Binding of platelets to T lymphocytes significantly decreased the activation status of these immune cells (indicated by elevated surface expression of the activation marker L-selectin (**Fig. 4b**) as well as by reduced surface expression of the activation markers CD25 and CD69, the cytokine interferon (IFN)-□, the pro-migratory integrin CD49d, and the proliferation marker Ki-67 (**Fig. S13**). Antibody blockade of the inhibitory IC molecule receptors PD-1 and CTLA-4 consequently attenuated these inhibitory effects of platelets (**Fig. 4c; Fig. S13**), suggesting that platelet-dependent, PD-1/CTLA-4 ligand-mediated inhibition of T lymphocyte activation compromises diverse T lymphocyte functions. Concomitant interference with the PD-L1 receptor PD-1 and the CD80/CD86 receptor CTLA-4 accordingly

increased eTLR-dependent trafficking of CTLs to the level of platelet-depleted animals in the peritoneal assay, whereas blockade of the CD40L receptor CD40 expectedly attenuated responses of myeloid leukocytes and lymphocytes (**Fig. S14**). Moreover, concomitant inhibition of PD-1 and CTLA-4 significantly enhanced tumor infiltration by anti-tumorigenic CTLs and Th1 cells and reduced (pro-tumorigenic) myeloid leukocyte responses as well as tumor growth in the orthotopic 4T1 breast cancer model as compared to isotype control antibody-treated tumor-bearing animals (**Fig. 4d, e**). In line with the low IC molecule expression of the poorly immunogenic 4T1 tumor cells, combined PD-1 and CTLA-4 inhibition achieved comparable immunomodulatory and tumor-suppressing effects as antibody-mediated platelet depletion (**Fig. 4e, f; Fig. 3a, b**). Importantly, upon platelet depletion, remaining viable tumor cells exhibited a slightly enhanced proliferation status (indicated by average Ki-67 expression; **Fig. S15a**) as compared to control animals (which might be due to the survival of the 'fittest', most proliferative cells), but unaltered EMT status (measured by EpCAM expression; **Fig. S15a**) or – in line with the lack of IC molecule expression in human TNBC cells – tumor cell expression of IC molecules **Fig. S15b**) as well as vascularization of tumors (**Fig. S15c**) as identified in the orthotopic and heterotopic 4T1 tumor models. Thus, our experimental data suggest that procoagulant platelets primarily modulate immune cell responses by delivering IC molecules into the tumor microvasculature. As a consequence, numbers of viable tumor cells were significantly decreased in platelet-depleted animals (**Fig. 4e; S15d**).

GPIb blockade and PD-1/CTLA-4 inhibition exhibit similar effects in experimental TNBC

Interestingly, as a consequence of intratumoral procoagulant platelet activation (**Fig. S11**), the proportion of procoagulant platelets of total platelets bound to lymphocytes was significantly higher in 4T1 tumors than in the peripheral blood (**Fig. S16a**). Towards a translational perspective, we therefore tested different platelet molecules for their ability to interfere with the formation of platelet-lymphocyte complexes in the systemic circulation prior to procoagulant platelet activation in the tumor. Using blocking antibodies and inhibitors, we show that the platelet receptor GPIb□, but not GPVI, GPIX, GPIIbIIIa, ICAM-2, P-selectin, PSGL-1, and P2Y12 or the enzyme cyclooxygenase, facilitates the binding of procoagulant platelets to circulating lymphocytes (**Fig. S16b**). Confocal microscopy analyses further indicate that platelet GPIb□ interacts with von Willebrand factor (vWF)-decorated $CD3^+$ T cells (**Fig. S16c, d**). Accordingly, *a priori* blockade of GPIb□ showed similar immunomodulatory and tumor-suppressing effects as combined PD-1- and CTLA-4 inhibition in the orthotopic TNBC model (**Fig. 4e, f**). Importantly, blocking or platelet-depleting antibodies efficiently bound to platelets, but not to 4T1 tumor cells (**Fig. S16e**). Most interestingly, triple blockade of GPIb□, PD-1, and CTLA-4 did not significantly enhance tumor infiltration by anti-tumorigenic CTLs or further attenuate (pro-tumorigenic) myeloid leukocyte responses and suppression of tumor growth as compared to isolated ICI treatment. This lack of synergistic effects of IC inhibitors and GPIb□ blockade in the 4T1 tumor model, in which ligands of PD-1 and CTLA-4 are predominantly expressed by platelets, suggests that platelets may promote tumor progression in this experimental model mainly *via* their immunomodulatory properties. As a therapeutic perspective, treatment of existing tumors with anti-GPIb□□or anti-PD-1/CTLA-4 antibodies significantly reduced tumor progression (**Fig. S17a**) and enhanced tumoral CTL accumulation, but without affecting the presence of myeloid leukocytes in the tumors (**Fig. S17b**), which might be due to the

dominance of intravascular lymphocyte responses in established tumors (**Fig. S1a**). Similar results were obtained in an orthotopic mouse model of poorly immunogenic head and neck squamous cell carcinoma (**Fig. S18**), suggesting platelet-mediated immune checkpoint molecule delivery to malignant tumors as a more general pathological mechanism. Moreover, despite low numbers of platelets in peripheral organs of orthotopically 4T1 tumor-bearing mice (**Fig. 1b; Tab. S1b**), procoagulant platelet activation was also detected at sites of tumor metastases in bone marrow (7.9±0.2% of total platelets), liver (11.8±0.8% of total platelets), and lungs (12.5±0.6% of total platelets). Platelet depletion critically modulated neutrophil (but not cMO, $CD4^+$ T cell, CTL, and B cell) accumulation (**Fig. S19a**) and (subsequently) compromised metastatic seeding of breast cancer cells (**Fig. S19b**), further indicating that platelets exhibit common and distinct immunoregulatory effects in primary tumors and at sites of tumor metastasis. This might be explained by the contribution of tissue-specific properties to the control of immune cell responses (*e.g.*, the presence of DAMPs and inflammatory cytokines in tumors as opposed to homeostatic cytokines in peripheral tissues during initial metastatic stages).

Discussion

Platelets substantially contribute to the pathogenesis of malignant tumors. Their systemic trafficking dynamics in cancer, however, remain obscure. In the present study, we show that the circulatory time of platelets in the peripheral blood decreases already in early stages of experimental TNBC – despite (reactively) enhanced bone marrow megakaryocytopoiesis and stable splenic platelet clearance – as these cellular blood components massively accumulate in the aberrant tumor microvasculature. Here, platelets heavily interacted with neutrophils, cMOs, lymphocytes, and endothelial cells, resembling platelet-immune cell interactions in inflamed tissue.[32-34] Most interestingly, the majority of these interactive platelets in the tumor environment exhibited a highly reactive 'procoagulant' phenotype, which shares distinct phenotypic properties with the hyper-reactive young, reticulated subset of platelets[45] and is triggered by exposure to subendothelial collagen in defective vessels.[29-31] Hence, platelets instantly traffick to neoplastic lesions in early TNBC, where they undergo procoagulant activation. At later stages of disease, reactive thrombocytopoiesis might further intensify tumoral platelet responses and even overcompensate the initial decline of circulating platelets, ultimately leading to the clinically reported thrombocytosis and thrombotic events in advanced stages of breast cancer.[46-48]

DAMPs are host biomolecules that are released in a variety of pathologies [49] including cancer as a consequence of inflammation, cell death, and tissue destruction.[42,43] Upon recognition of these specific molecular signals by eTLRs, distinct inflammatory programs are initiated, mediating the activation of cells.[35] In our experiments, we expectedly detected an enormous accumulation of extracellular nucleic acids in the tumor environment. These tumor-released DAMPs (but not ADP,

which is also known to be liberated by necrotic cells) attracted platelets and immune cells into the tumor environment by instructing perivascular macrophages through eTLRs to induce the expression of distinct adhesion molecules on microvascular endothelial cells capable of recruiting and activating circulating platelets and immune cells.[50] Noteworthy, previous experimental studies suggested that activation of endosomal TLR7 and/or TLR8 primarily stimulates anti-tumorigenic immune responses.[51] In clinical trials, however, eTLR agonists showed beneficial effects only in precancerous skin lesions, but did not improve survival of (head and neck) cancer patients.[51] This lacking anti-tumorigenic effect of eTLR agonism might be due to the concomitant induction of eTLR-dependent pro-tumorigenic platelet responses in invasive tumors.

Under inflammatory conditions, platelets are well-known to promote the migration of immune cells to their target destination *via* diverse molecular interactions.[32-38] The role of these anucleate cell particles for the recruitment of immune cells to malignant tumors is still unclear. Here, we unveil a previously unrecognized immunomodulatory function of platelets that concurrently promotes the trafficking of innate immune cells into neoplastic lesions while inhibiting responses of adaptive immune cells, independently of effects on tumor cell proliferation, EMT, and angiogenesis. Most importantly, analyses in human TNBC samples clearly confirmed our experimental results as the presence of platelets in tumors was associated with a pro-tumorigenic perivascular immune cell milieu. Accordingly, transcriptomic data from the METABRIC cohort document particularly impaired survival of individuals with high tumoral expression levels of the platelet surrogate marker ITGA2B, presumably originating form a more aggressive subtype as indicated by enrichment of histological grade 3, the $ER^{+}/HER2^{high}$-proliferating subtype, and gene sets associated with proliferation, DNA synthesis and repair. Furthermore, enrichment of ITGA2B across

all tumor stages and different breast cancer subtypes point to a more general role of platelets in this tumor entity.

IC molecules control immune cell responses under homeostatic and pathological conditions.[52] Whereas stimulatory ICs enhance immune reactions,[34] inhibitory ICs dampen immune cell activity. In cancer, particularly interactions of inhibitory PD-1 and PD-L1 as well as of CTLA-4 and its ligands CD80 and CD86 drive adaptive immune cell evasion.[52] Extending previous observations,[39-41] we here demonstrate that primarily procoagulant platelets expose large amounts of these IC molecules on their surface as compared to non-procoagulant platelets or other blood cells, which is mediated *via* GPVI, GPIIb/IIIa, cypD, and TMEM16F. Accordingly, platelets attenuated the activity of anti-tumorigenic T cells and promoted neutrophil responses (which are pro-tumorigenic in experimental TNBC)[53,54] in malignant lesions by employing these immunomodulatory factors, ultimately fueling tumor growth. Opposite lymphocyte supporting effects of platelets observed in viral infection[36-38] might be explained by context-specific differences in the models employed.

Most interestingly, we here demonstrate that platelet depletion achieved similar immunomodulatory and tumor-suppressing effects as compared to combined PD-1 and CTLA-4 inhibition in our model of TNBC. Consequently, targeting interactions of platelets and immune cells might represent a promising treatment strategy in TNBC without side effects of systemic ICI. In line with this assumption, we show that blockade of the platelet receptor GPIb nearly reached the immunomodulatory effects as platelet-depletion or combined PD-1 and CTLA-4 inhibition in experimental TNBC. This approach might be particularly beneficial in individual tumors lacking tumor cell expression of IC molecules,[40] in which primarily platelets deliver these molecular factors to malignant lesions, and in paraneoplastic thrombocytosis[55] as

GPIba additionally supports thrombopoietin-dependent platelet production.[56] Of note, anti-platelet strategies might enhance the risk of hemorrhage, albeit they do not exhibit the severe or life-threatening autoimmune-related adverse effects observed in ICI-treated patients.[4] Although hereditary GPIba or vWF defects associate with severe bleeding episodes,[57,58] pharmacological GPIb blockade might be safe as it did not increase the risk of intracranial hemorrhage in experimental cerebral ischemia[52,59] – in contrast to conventional anti-platelet drugs and besides targeting procoagulant platelet activation *via* GPVI, cypD, or TMEM16F.

In conclusion, our findings uncover a previously unrecognized immunomodulatory activity of procoagulant platelets in the tumor microvasculature that concurrently promotes pro-tumorigenic myeloid immune cell responses and impedes anti-tumorigenic T cell activity, thus supporting TNBC progression. Targeting this self-sustaining mechanism of such malignant neoplasms effectively interferes with their expansion, hence providing a novel strategy to counteract immune evasion in TNBC without side effects of systemic ICI.

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Authorship Contributions

JBC, FH, BS, ZW, KS, SB, SM, JL, VS, JH, MC, LAM, CB, and GZ performed experiments and contributed to data analysis and interpretation. RK, LN, WW, KL, BU, and CAR wrote the manuscript. CAR conceived and supervised the study. All authors read and approved the manuscript.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

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Figure legends

Figure 1. Platelet trafficking in experimental breast cancer. (**a**) Systemic platelet count as well as proportion of aged platelets of total platelets in the peripheral blood of tumor-free control or orthotopic 4T1 tumor bearing mice as assessed by flow cytometry, quantitative data are shown (mean±SEM for n=3-4 mice per group; *p<0.05 vs. control). (**b**) Representative confocal microscopy images of GPIb□$^+$ platelets (white) in the intra- and peritumoral microvasculature of 4T1 tumors (tdTomato-transduced fluorescent tumor cells; red) or tumor-free lungs, brain, kidneys, and liver (parenchymal structure in blue; scale bars: 100□m). (**c**) Interactions of platelets (white), endothelial cells (EC; broken lines), and leukocytes (green) in the peri- and intra-tumoral microvasculature of 4T1 tumors (blue) implanted into the left auricle, in the right tumor-free ear, or in the left ear of tumor-free mice as assessed by *in vivo* microscopy, a representative image (scale bar: 10μm) and quantitative data are shown (mean±SEM for n=6 mice per group; *p<0.05 vs. control). (**d**) Proportion of neutrophils, monocytes, and lymphocytes bound to platelets of total neutrophils/monocytes/lymphocytes in the peripheral blood of mice 10d after 4T1 tumor induction, quantitative data are shown (mean±SEM for n=10 mice per group; *p<0.05 vs. control). (**e**) Proportion of leukocytes bound to procoagulant platelets (PS^+ GPIb□$^+$ cells) of leukocytes ($CD45^+$ cells) bound to platelets (GPIb□$^+$ cells) accumulating in the intra- and peritumoral microvasculature, quantitative data and a representative *in vivo* microscopy image is shown (mean±SEM for n=3 mice per group).

Figure 2. Effects of tumor-released extracellular nucleic acids on platelet and immune cell trafficking in experimental breast cancer. (**a**) Extracellular nucleic

acids (red) released into the environment of 4T1 tumors as assessed by propidium iodide staining and *in vivo* microscopy on day 10 after tumor induction, a representative image (scale bar: 100µm) and quantitative data for the fluorescence intensity relative to the distance from the tumor core (dotted line) are shown (mean±SEM). (**b**) Endothelial cell interactions of platelets in the peri- and intratumoral microvasculature of 4T1 tumors implanted into the left auricle in mice treated with blocking eTLR oligonucleotides or control oligonucleotides and of the left auricle of tumor-free mice, quantitative data are shown (mean±SEM for n=7 mice per group; #p<0.05 vs. control *p<0.05 vs. control oligo). Infiltration by (**c**) neutrophils, classical monocytes (cMOs), B cells, $CD8^+$ T cells, and (**d**) $CD4^+$ T cell subsets as assessed by flow cytometry as well as (**e**) size and weight of orthotopic 4T1 tumors, quantitative data are shown (mean±SEM for n=5 mice per group; *p<0.05 vs. control oligo). (**f**) Proliferation of 4T1 tumor cells exposed to agonists of TLR-7, -8, or -9 as assessed by a MTT assay, quantitative data are shown (mean±SEM for n=6 mice per group).

Figure 3. Effects of platelets on immune cell trafficking in breast cancer. (**a**) Infiltration by neutrophils, classical monocytes (cMOs), B cells, $CD8^+$ T cells, $CD4^+$ T cells, and (**b**) $CD4^+$ T cell subsets as assessed by flow cytometry as well as (**c**) size and weight of orthotopic 4T1 tumors in mice receiving platelet depleting or isotype control antibodies, quantitative data are shown (mean±SEM for n=4-6 mice per group; *p<0.05/**p<0.01/p<0.001 vs. isotype control; ns=not significant). (**d**) Correlation of the ratio of extravascular MPO^+ neutrophils and $CD8^+$ T cells (NLR=neutrophil/lymphocyte ratio) and intravascularly accumulated integrin □3^+ platelets as assessed by immunohistochemical analyses of human TNBC samples,

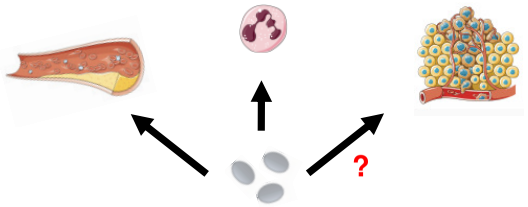
representative images and quantitative data are shown. (**e**) Recurrence free survival (RFS) of breast cancer patients from the METABRIC breast cancer cohort exhibiting ITGA2B^{low} and ITGA2B^{high} expression levels (z-value ≥ 1.5 served as cut-off).

Figure 4. Synergistic effects of anti-platelet therapy and immune checkpoint inhibition in experimental breast cancer. (**a**) Surface expression of CD80, CD86, PD-L1, PD-L2, CD40, and CD40L on PS^{low} or PS^{high} platelets from the peripheral blood of mice, quantitative data are shown (mean±SEM for n=3 mice per group; *p<0.05 vs. PS^{low}). (**b**) Activation status of platelet-bound (CD41^{+}) or -unbound (CD41^{-}) lymphocytes isolated from orthotopically raised 4T1 tumors as assessed by L-selectin/CD62L surface expression in flow cytometry, quantitative data are shown (mean±SEM for n=3 mice per group). (**c**) Activation status of platelet-bound (CD41^{+}) lymphocytes as assessed by L-selectin/CD62L surface expression in flow cytometry upon antibody blockade of CD80, CD86, CTLA4, PD-L1, or PD-1, quantitative data are shown (mean±SEM for n=3 mice per group). (**d**) Infiltration by neutrophils, classical monocytes (cMOs), B cells, CD8^{+} T cells, and CD4^{+} T cell subsets of orthotopic 4T1 tumors in mice receiving anti-PD-1 and -CTLA4 mAbs or isotype control antibodies as assessed by flow cytometry, quantitative data are shown (mean±SEM for n=5 mice per group; *p<0.05 vs. isotype control). (**e**) Relative volume and weight of tumors as well as (**f**) relative infiltration by lymphocytes and myeloid leukocytes of orthotopically grown 4T1 tumor in mice receiving anti-GPIb□ mAbs, and/or anti-PD-1 and -CTLA4 mAbs, or isotype control antibodies, quantitative data are shown (mean±SEM for n=5 mice per group; *p<0.05 vs. isotype control).

Procoagulant Platelets in Malignant Tumors

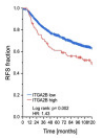
Background

Beyond their essential hemostatic properties, platelets control immune responses in inflammation. Their immunomodulatory role in cancer remains obscure.

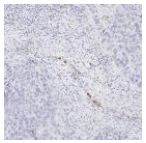


Methods

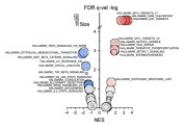
Patient cohorts →



Clinical data

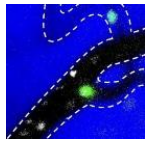


Immuno-histochemistry

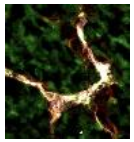


RNA sequencing

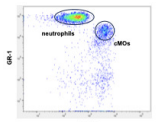
Mouse models →



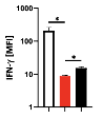
***In vivo* microscopy**



Confocal microscopy



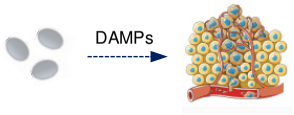
Flow cytometry →



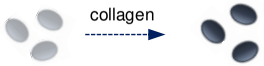
***In vitro* assays**

Findings

Recruitment to tumors

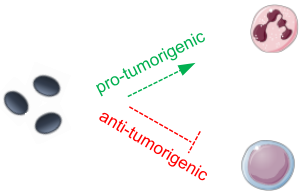


Procoagulant activation

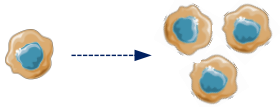


Release of immune checkpoint molecules

Misguidance of immune cells



Tumor progression



Conclusions: 1) Procoagulant platelets deliver large amounts of immune checkpoint molecules to malignant tumors. 2) Procoagulant platelets employ these immune checkpoint molecules to misguide immune cell responses, thus promoting tumor progression.

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Blood Visual Abstract