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Engagement of αIIbβ3 (GPIIb/IIIa) with ανβ3 Integrin Mediates Interaction of Melanoma Cells with Platelets

A CONNECTION TO HEMATOGENOUS METASTASIS[*][S]

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A mutual relationship exists between metastasizing tumor cells and components of the coagulation cascade. The exact mechanisms as to how platelets influence blood-borne metastasis, however, remain poorly understood. Here, we used murine B16 melanoma cells to observe functional aspects of how platelets contribute to the process of hematogenous metastasis. We found that platelets interfere with a distinct step of the metastasis cascade, as they promote adhesion of melanoma cells to the endothelium *in vitro* under shear conditions. Constitutively active platelet receptor GPIIb/IIIa (integrin αIIbβ3) expressed on Chinese hamster ovary cells promoted melanoma cell adhesion in the presence of fibrinogen, whereas blocking antibodies to aνβ3 integrin on melanoma cells or to GPIIb/IIIa significantly reduced melanoma cell adhesion to platelets. Furthermore, using intravital microscopy, we observed functional platelet-melanoma cell interactions, as platelet depletion resulted in significantly reduced melanoma cell adhesion to the injured vascular wall *in vivo*. Using a mouse model of hematogenous metastasis to the lung, we observed decreased metastasis of B16 melanoma cells to the lung by treatment with a mAb blocking the aν subunit of aνβ3 integrin. This effect was significantly reduced when platelets were depleted *in vivo*. Thus, the engagement of GPIIb/IIIa with aνβ3 integrin interaction mediates tumor cell-platelet interactions and highlights how this interaction is involved in hematogenous tumor metastasis.

Tumor metastasis occurs through a multistep process (1). The interaction of circulating tumor cells that have detached from the primary tumor with structures of the tissue microvasculature is a crucial step preceding the invasion of the target organ. The specific events determining tumor cell interactions with endothelial cells during hematogenous metastasis are well defined (2), whereas the contribution of other cell types such as platelets in this process is less well understood.

Besides their classical role in hemostasis and thrombosis, platelets have been implicated in various pathophysiological processes, including regeneration and inflammation or recently tumor metastasis (3–9). Both clinical and experimental evidence point to a role of platelets in the spread of cancer, as thrombocytopenia or anti-platelet treatments ameliorate experimental metastasis, and tumors provide a thrombogenic proinflammatory environment that promotes coagulation and endothelial cell activation (10, 11). Interestingly, patients with metastatic disease reveal increased platelet counts and significantly elevated numbers of activated platelets (12). Depending on the type of tumor, various aspects of cancer progression may be affected by platelets, including tumor cell proliferation (13), tumor angiogenesis (14), vessel stability within tumors (15), or immune evasion (16, 17). Moreover, platelets contribute to a specific gene expression profile of microvascular endothelial cells in the presence of tumor cells and consecutively to a permissive metastatic microenvironment (18). Integrins, a widely expressed family of transmembrane adhesion receptors, represent a central determinant for physiological platelet function. In particular, GPIIb/IIIa is involved in both cell-cell adhesion and thrombus formation at the vascular wall, establishing it as a therapeutic target in vascular diseases (19). For heterotypic cell-cell interactions between platelets and other cells such as leukocytes involving GPIIb/IIIa and fibrinogen, the amino acids

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Arg-Gly-Asp (RGD) seem to be of particular importance, as treatment with synthetic RGD peptides was shown to block integrin binding (20, 21). Interestingly, pharmacological inhibition of GPIIb/IIIa has been demonstrated to reduce tumor cell metastasis, although the underlying mechanisms remain elusive (22). Similar to GPIIb/IIIa (23), the integrin a$\nu\beta$3 has been implicated as a potential target in tumor metastasis, as blockade of a$\nu\beta$3 integrin on murine melanoma cells inhibits lung metastasis *in vivo*, in a time- and dose-dependent manner, which is mainly attributed to inhibition of angiogenesis (24). Currently, the direct relevance of GPIIb/IIIa interacting with a$\nu\beta$3 for melanoma metastasis has not been investigated. Collectively, these diverse observations reported in the literature have prompted us to investigate how platelets may participate in melanoma cell metastasis. We observed that platelets are relevant for melanoma cell adhesion and identified an interaction between the aforementioned integrins GPIIb/IIIa and $\alpha\nu\beta$3 as a relevant mechanism involved in adhesion events between platelets and melanoma cells.

EXPERIMENTAL PROCEDURES

Reagents

RGD and RAD peptides were from Calbiochem; anti-mouse aν integrin (CD51) mAb was from Biolegend; anti-mouse CD61 mAb and rat IgG1k isotype control were from ebioscience; anti-mouse CD31 mAb from was Biolegend; rat anti-mouse GPIIb/IIIa mAb and rat anti-GPIb mAb were from Emfret Analytics, and corresponding rat anti-mouse IgG was from Rockland Immunochemicals. Rabbit anti-mouse thrombocyte hyperimmune serum and control serum were purchased from Accurate Chemical & Scientific Corp.; 5-carboxyfluorescein diacetate succinimidyl ester (DCF)[5] was from Molecular Probes; fibrinogen was from Sigma. For fixation of tissues, OCT mounting medium (Tissue Tek) was used. Abciximab/c7E3 was from Lilly Pharmaceuticals. Cy2-goat anti-hamster IgG, Cy3-goat anti-rat IgG, and DAPI were from Dianova; CMFDA was from Invitrogen.

Mice

6–12-Week-old C57BL/6J female mice were originally purchased from Charles River Laboratories (Sulzfeld, Germany) and housed at the central animal facilities of the University of Heidelberg or the University of Tuebingen, Germany. Animal care and experimental procedures were approved by the institutional review boards and performed in accordance with the institutional guidelines for animal welfare.

Isolation of Murine and Human Platelets

Human platelets were isolated as described previously (25–27). Briefly, venous blood was drawn from the antecubital vein of healthy volunteers and collected in acid/citrate/dextrose buffer. After centrifugation at 430 × *g* for 20 min, platelet-rich plasma was removed, added to Tyrodes/HEPES buffer (2.5 mmol/liter HEPES, 150 mmol/liter NaCl, 1 mmol/liter KCl, 2.5 mmol/liter $NaHCO_3$, 0.36 mmol/liter NaH_2PO_4, 5.5 mmol/liter glucose, and 1 mg/ml BSA, pH 6.5), and centrifuged at 900 × *g* for 10 min. After removal of the supernatant, the resulting platelet pellet was resuspended in Tyrodes/HEPES buffer (pH 7.4 supplemented with 1 mmol/liter $CaCl_2$ and 1 mmol/liter $MgCl_2$). Murine platelets were isolated from pathogen-free C57BL/6J mice (Charles River Laboratories) as described previously (28).

Cell Lines

Murine B16/F1 melanoma cells (B16) and B16 cells sequentially transduced with cDNA encoding either CXCR4 (B16-CXCR4) in the pLNCX2 retroviral vector (Clontech) or pLNCX2 empty vector alone (B16-pLNCX2) or with cDNA encoding firefly (*Photinus puralis*) luciferase (B16-luc), as described previously (29), were the kind gifts from Dr. Sam Hwang (Medical College of Wisconsin). CHO cells expressing wild-type or mutated and thereby activated GPIIb/IIIa (αIIbβ3) integrin were a kind gift from Dr. Karl-Heinz Peter (Baker Heart Research Institute, Melbourne, Australia) (30, 31). Tumor cell lines, the murine endothelial cell line b.End.3, and the human melanoma cell line (MV3) were cultured as described previously (32, 33) or grown as described by the supplier.

Transmigration Assay

Transmigration of B16 cells was performed as described previously (29, 34). Briefly, transmigration assays were performed using 6.5-mm transwells with 8-μm pore size (Costar, Bodenheim, Germany). b.End.3 cells were seeded on transwell filters 2 days prior to the assay and grown to confluence in the upper compartment in a humidified atmosphere (37 °C, 5% CO_2). 600 μl of medium containing SDF-1 (200 ng/ml) or isolated platelets (1×10^7) was added to the lower compartment of the transwell system. 2×10^5 B16-CXCR4 or B16-pLNCX2 control cells, respectively, were added to the upper compartment on top of the endothelial monolayer in a total volume of 100 μl. After incubation for 3 h at 37 °C, cells from the upper chamber were removed with a cotton swab, and the filters were removed and stained with crystal violet. After washing the filters in distilled water, they were mounted on glass coverslips, and the number of transmigrated B16 cells was quantified on the lower side of the filter by cell counting using an inverted microscope (Zeiss Axiovert 200 M).

Adhesion Assays

Static Adhesion Assay—Static adhesion assays and dynamic adhesion assays were performed as described previously (25, 35, 36). For static adhesion, 96-well polystyrene plates (Falcon) were coated with murine or human platelets (1×10^8/ml) for 2 h, washed with Tyrode's buffer to remove nonadherent platelets, and blocked for 30 min with 1% BSA in Tyrode's buffer. During the adhesion process, platelets become activated, which was verified using flow cytometry (supplemental Fig. 5). To this end, adherent platelets were incubated with a phycoerythrin-conjugated rat anti-mouse P-Selectin antibody (10 μg/ml). The platelets were then removed from the well using trypsin, washed, and analyzed using a FACSCalibur (BD Biosciences). In the case of human platelets, wells had been pre-coated with

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[5] The abbreviations used are: DCF, 2′,7′-dichlorofluorescein; CMFDA, 5-chloromethylfluorescein diacetate.

collagen (10 μg/ml). Subsequently, murine B16 melanoma cells (5×10^4/well) or human MV3 melanoma cells (5×10^4/well) were added and allowed to adhere for 40 min under cell culture conditions. After careful washing with PBS, adherent melanoma cells were counted and quantified per visual field. In some experiments, melanoma cells were preincubated for 30 min with blocking mAbs to murine CD51 (αν integrin), or platelets were preincubated with blocking mAbs to murine CD61 (β3 integrin) or to GPIIb/IIIa (JON/A (37)) or control IgG, RGD peptide, or control RAD peptide as indicated in the figure legends. To exclude a difference in the number of adherent platelets by the treatment with JON/A or RGD, and as a consequence a reduction in melanoma cell adhesion, the number of adherent platelets after final washing was assessed using an automated whole blood analyzer (Sysmex Se 9000, Kobe, Japan; supplemental Fig. 6). Cells were collected from the well for analysis using trypsin.

Flow Chamber Assay—Interaction of B16 cells to platelets on murine endothelial cells (b.End.3 cells) was measured in a parallel plate flow chamber (Chromaphor, Ascheberg, Germany) at shear rates of 500 s^{-1}, principally as described before (38). Briefly, CMFDA-labeled B16 cells (2×10^5 cells/ml) were perfused over confluent b.End.3 monolayers and preincubated with freshly isolated murine platelets (1×10^8/ml) or Tyrode's buffer (control), respectively, for 4 h under cell culture conditions. In some experiments, platelets or melanoma cells were preincubated for 30 min with blocking mAbs to murine CD51 (aν integrin), murine CD61 (β3 integrin) or control IgG, RGD peptide or control RAD peptide as indicated in the figure legends. The number of adherent cells per visible field was visualized by video microscopy. Images were recorded and evaluated off-line for quantification at the indicated time points; adherent cells appear in yellow after merge of frames 50 and 55 in the off-line analysis at the respective time points after shear flow was started.

Static Adhesion to CHO Cells Expressing Resting or Activated GPIIb/IIIa—CHO cells (2×10^5/well) expressing wild-type or mutated and thereby activated GPIIb/IIIa integrin, as described previously (30, 31), were grown to confluency in 6-well plates (Nunc). In these cells, a constitutive high affinity state of GPIIb/IIIa was achieved by deleting the GFFKR region in the integrin α (GPIIIa) subunit (39). This region is highly conserved in the integrin protein family, and deleting the same region in other integrins (*e.g.* Mac-1) has resulted in a constitutive high affinity state (40). Wells were washed with warm media to remove nonadherent CHO cells. B16 cells (5×10^4/well) were stained with CellTracker Orange (1:1000; Invitrogen) for 10 min to distinguish melanoma cells from CHO cells, washed, and added to the adherent CHO cells. In selected experiments CHO cells were preincubated with Abciximab/C7E3 inhibiting GPIIb/IIIa (10 μg/ml, Lilly) (30) or control IgG for 30 min. In some experiments, fibrinogen (300 μg/ml) was added to the assay system prior to studying adhesion of melanoma cells to CHO cells as indicated in the figure legends. After 45 min, nonadherent melanoma cells were removed by repeated washings, and adherent melanoma cells were quantified by direct counting using a Zeiss Axiovert fluorescence microscope. In all experiments, nonspecific binding to CHO cells was assessed and was subtracted to calculate specific binding.

In Vitro [^{3}H]Thymidine Proliferation Assay—B16 cells, harvested in the exponential growth phase, were incubated at indicated numbers with freshly isolated and washed murine platelets (1×10^7/well) or cell culture media (control) in 96-well round-bottom plates (Falcon) under cell culture conditions. After 2 days, B16 proliferation was assayed by [^{3}H]thymidine (5 μCi/ml) incorporation followed by scintillation counting (MicrobetaTriLux, PerkinElmer Life Sciences).

Intravital Fluorescence Video Microscopy

Intravital microscopy and induction of platelet accumulation to study platelet- melanoma cell interaction *in vivo* were performed principally as described before (26). Wild-type C57BL/6J mice (Charles River Laboratories) were anesthetized by intraperitoneal injection of a solution of midazolam (5 mg/kg body weight; Ratiopharm), medetomidine (0.5 mg/kg body weight; Pfizer), and fentanyl (0.05 mg/kg body weight, CuraMed Pharma GmbH). Polyethylene catheters (Portex) were implanted into the left jugular vein to administer platelet-depleting serum or control, respectively, and DCF-labeled B16 cells (2×10^5 cells/250 μl). The serum was given 30 min before the cells were injected to achieve sufficient platelet depletion. Platelet accumulation at the vascular wall was induced by temporary ligation of the supplying vessels for 60 min. Before and after induction of ischemia-reperfusion, the cell vascular wall interactions were visualized by *in vivo* video microscopy. In a control experiment to exclude thrombotic occlusion of the intestinal vessels, animals were treated similarly. Instead of staining melanoma cells with DCF, rhodamine-6G was injected to visualize any potential thrombus formation and thus exclude thrombi in this setting, which might interfere with melanoma cell adhesion. As a positive control, we treated mice locally with $FeCl_3$ to induce extensive thrombus formation (supplemental Fig. 4). All images were recorded and evaluated off-line.

In Vivo Metastasis Assay

In vivo metastasis assays using B16-luc cells were performed principally as described previously (29, 32, 41). Briefly, B16-luc cells in the exponential growth phase were harvested by trypsinization and washed twice with PBS before injection. Cell viability was >95% as determined by trypan blue dye exclusion. Platelet-depleting serum or control serum was administered intraperitoneally into C57BL/6 mice and randomly distributed into experimental groups as specified, 24 h before and 48 h after tumor inoculation. For footpad injections, cells (4×10^5 in 20 μl of PBS) were injected into the left hind footpads of mice. In this setting, a second experiment, for which platelets were continuously depleted over a longer period of 12 days, was conducted. Successful platelet depletion was monitored in the peripheral blood (data not shown). Tumor growth was measured with a caliper at the indicated time points after tumor inoculation. For B16-luc metastasis to the lungs, B16-luc cells (2×10^5 cells/200 μl of PBS) were injected into the lateral tail veins of mice. Mice were euthanized at the indicated time points for gross inspection and quantification of tumor burden in the lungs or the popliteal lymph nodes by using an *in vitro* bioluminescence system (MicroBeta TriLux Luminescence Counter, PerkinElmer Life Sciences) principally as described previously (29, 32). Figures show luciferase activity measurements in relative light units reflecting tumor burden.

Immunofluorescence Microscopy

Mice were intravenously injected with murine B16 melanoma cells (2×10^5 cells in 200 μl of PBS), and melanoma cells were allowed to circulate for 60 min. Before sacrificing mice and extracting the lungs, mice were perfused with PBS to efficiently remove circulating blood, including circulating platelets, thus allowing for the assessment of only endothelium-associated platelets or platelets permanently adherent to the endothelium of the lungs. Lung tissue was embedded into OTC (Tissue Tek®, Sakura) mounting medium and flash frozen at −80 °C. 6-μm tissue sections were air-dried, fixed in acetone for 5 min at −80 °C, rehydrated with PBS for 5 min at room temperature, and blocked with 3% skim milk in 5% goat serum for 2 h in a humidified chamber at 4 °C. Sections were then stained with hamster anti-mouse CD31 mAb (Serotec, 1:50), a rat anti-mouse GPIIb/IIIa antibody (JON/A, Emfret, 1:50), or the respective isotype control IgGs in blocking buffer with 0.3% Triton X (Neolab) for 1 h in a humidified chamber at 4 °C. After thorough washing in PBS, sections were incubated with Cy2-labeled goat anti-hamster IgG (Dianova) and Cy3-labeled goat anti-rat IgG (Dianova) at 1:250 and DAPI nuclear stain (Dianova) at 1:5000 in PBS for 30 min. After thorough washing in PBS, sections were covered with mounting medium, dried at room temperature overnight, and analyzed by immunofluorescence microscopy using a Leica microscope.

Statistical Methods

p values were based on unpaired two-sided Student's t tests unless otherwise specified. Significance was assumed at $p < 0.05$.

RESULTS

Here, we examined the functional relevance of platelets in hematogenous metastasis using a murine B16 melanoma cell line. When melanoma cells were injected intravenously into mice, platelets could be visualized adherent to the lung endothelium (supplemental Fig. 1). It is known that platelets express and secrete the CXC chemokine SDF-1α (CXCL12), which has recently been implicated in attracting circulating cells to the vascular wall (27, 42, 43). Expression of the cognate receptor for SDF-1α, CX-chemokine receptor 4 (CXCR4) has been shown to crucially support organ-selective hematogenous metastasis of melanoma cells to the lung (32). Therefore, we considered that soluble signaling molecules secreted by platelets, such as SDF-1α, might contribute to the directional chemotaxis and transmigration properties of B16 melanoma cells. As shown in Fig. 1*A*, the presence of freshly isolated murine platelets did not enhance B16 melanoma transmigration. We observed only a minor increase in transmigrating B16 melanoma cells overexpressing CXCR4 (B16-CXCR4) in the presence of platelets, whereas the addition of high concentrations of recombinant SDF-1α to the lower chamber resulted in a significant increase in transmigrated tumor cells (Fig. 1*A*).

Next, we investigated the influence of platelets on melanoma cell adhesion under static conditions. Platelets were allowed to adhere to the polystyrene surface of 96-well flat-bottom plates for 30 min before adding melanoma cells, and after careful washing, the amount of adherent melanoma cells was quantified. The presence of adherent platelets significantly increased melanoma cell adhesion, as shown for mouse platelets and murine B16 melanoma cells (Fig. 1*B*). Similarly, collagen-immobilized human platelets mediated adhesion of human melanoma cells (MV3) under static conditions (supplemental Fig. 2).

After metastasizing cells have entered the target organ, they undergo subsequent proliferation. Although selection pressure from the host, in the form of growth factors, might play an important role in the formation of metastatic foci, the proliferation characteristics of [^{3}H]thymidine-pulsed B16 melanoma cells remained unaltered when co-cultured with isolated murine platelets (Fig. 1*C*). In addition, we did not observe a significant alteration in the growth of subcutaneously implanted luciferase-expressing murine B16 melanoma tumors in the footpad of syngeneic C57BL/6J mice after *in vivo* depletion of platelets (Fig. 1*D*). Efficient platelet depletion was achieved with an intraperitoneal injection of rabbit anti-mouse platelet serum (depletion of over 97% at 24 h post-injection) as described previously (supplemental Fig. 3*A*) (44) and sustained for at least 72 h. Importantly, the number of circulating total leukocytes was unaltered by platelet depletion (supplemental Fig. 3*B*). Furthermore, *ex vivo* bioluminescence analysis of the harvested draining popliteal lymph nodes on day 21 after tumor inoculation revealed that the metastatic spread of tumor cells via the lymphatics was unaffected by changes in platelet counts of the host (Fig. 1*E*). Thus, although we found no significant platelet-mediated effect on the transmigrational and proliferative characteristics of B16 melanoma cells *in vitro* or the subcutaneous growth of tumors and lymphatic metastasis *in vivo*, platelets significantly increased the adhesion capacity of B16 melanoma cells.

To determine the contribution of platelets to the adhesive capacity of tumor cells under flow conditions, we analyzed interactions of B16 cells with platelets on murine endothelial cells (b.End.3) *in vitro* by using a parallel plate flow chamber. The presence of platelets resulted in significantly more CMFDA-labeled B16 melanoma cells adhering to the endothelial monolayer under shear flow conditions compared with the absence of platelets (Fig. 2, *A* and *B*). Next, we monitored adhesion of circulating B16 melanoma cells to platelets under more physiological conditions *in vivo* using intravital microscopy. In the applied model, platelet accumulation at the vascular wall was provoked by ischemia-reperfusion injury induced by transient ligation of the mesenteric artery (26), and thus, platelet-melanoma cell interaction could be studied *in vivo*. Subsequently, fluorescently labeled B16 melanoma cells were injected into the jugular vein of syngeneic C57BL/6J mice after platelet depletion or control treatment. Adhesion of fluorescent tumor cells was directly assessed by real time intravital video microscopy of the mesenteric microvasculature. In line with the findings from our *in vitro* experiments, off-line video

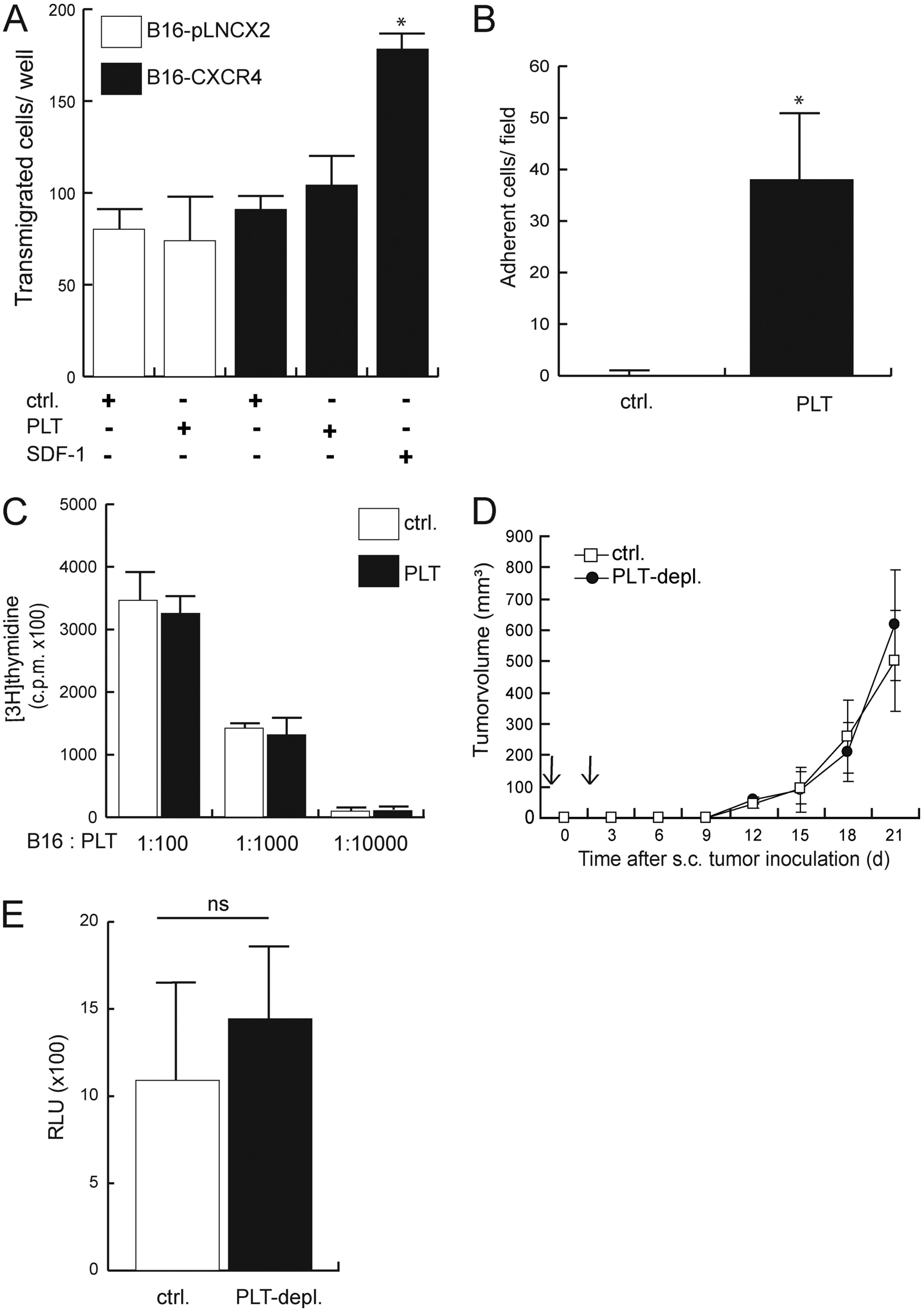

FIGURE 1. **Platelets mediate melanoma cell adhesion *in vitro* but have no effect on melanoma cell transmigration or proliferation.** *A,* murine endothelial (b.End.3) cells were grown to confluence in transwell chambers. Murine B16 melanoma cells were added to the upper well and allowed to migrate toward the lower well containing buffer, platelets, or SDF-1 (200 ng/ml). B16 melanoma cells overexpressing CXCR-4 or control vector (B16-pLNCX2) were applied. No significant difference was observed regarding B16 transmigration in the absence or presence of platelets. *, $p < 0.05$ in the presence of SDF-1. *B,* static adhesion of B16 cells in the presence (*PLT*) or absence (*ctrl.*) of immobilized murine platelets (1×10^8/well). Data are representative of four experiments, each with three analyzed wells/condition (means ± S.D.). *, $p < 0.05$. *C,* proliferation of B16 cells was measured by [^{3}H]thymidine incorporation after 48 h of co-culture with freshly isolated murine platelets (*PLT*) (2×10^7/well) at the indicated ratios or with Tyrode's buffer (*ctrl.*). Data from three experiments, each with three analyzed wells/condition (means ± S.D.), are shown. No significant difference was observed between groups. *D,* B16-luc tumor cell formation following subcutaneous inoculation of 4×10^5 B16-luc cells in 20 μl of PBS into the left footpad of C57BL/6 mice. *Arrows* indicate time points of intraperitoneal injection of 200 μl of anti-murine platelet-hyperimmune serum (1:10, *closed circles*) to deplete (*depl.*) platelets or control serum (*open squares*). Tumor volumes (mm^3) were measured using a caliper at the indicated time points. Data are from two experiments (means ± S.D.) with 4 to 5 mice per group. No significant difference was observed between groups. *E,* B16-luc tumor metastasis to draining popliteal lymph nodes (*LN*). On day 21 following subcutaneous inoculation of B16-luc (4×10^5 cells/20 μl of PBS) into the left footpad of C57BL/6 mice, draining popliteal lymph nodes were processed to determine the metastatic tumor burden by bioluminescence measurements (in relative light units, *RLU*). Data are means ± S.D. from two experiments with 4 to 5 mice per group. No significant (*n.s.*) difference was observed between animals after platelet depletion (*PLT-depl.*) and control mice (*ctrl.*).

analysis revealed that the capacity of circulating tumor cells to adhere to the injured vascular wall was strongly diminished in the absence of platelets, indicating that circulating melanoma cells interact with platelets *in vivo* (Fig. 2, *C* and *D*). Thrombus formation, which might have influenced melanoma cell adhesion in this setting, was excluded (supplemental Fig. 4).

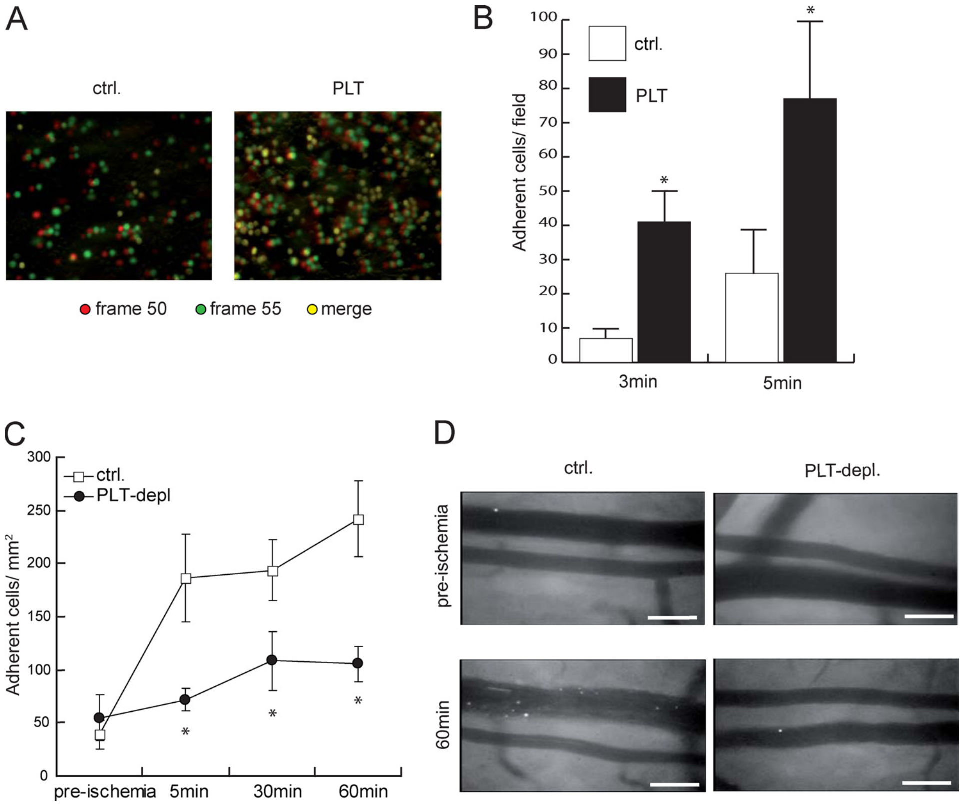

FIGURE 2. **Melanoma cells interact with platelets under flow conditions and *in vivo*.** *A* and *B,* firm adhesion of CMFDA-labeled B16 cells (2×10^5/ml) was assessed after 3 and 5 min of flow at a shear rate of 500 s^{-1} on a monolayer of b.END.3 cells preincubated with freshly isolated murine platelets (*PLT,* 1×10^8/ml) or Tyrode's buffer (*ctrl.*). *A,* adherent cells appear in *yellow* after merge of frames 50 and 55 in the off-line analysis at the respective time points after the perfusion was started. *B,* quantification of B16 cell adhesion experiments to a monolayer of b.END.3 cells. Data are representative of four individual experiments with similar results. *, $p < 0.05$, means ± S.D. is depicted. *C* and *D,* intravital video microscopy demonstrating the *in vivo* kinetics of firm adhesion of i.v. injected DCF-labeled B16 cells (2×10^5 cells in 250 μl of PBS/mouse i.v.) to the endothelium of mesenteric vessels of C57BL/6J mice. Intravital video microscopy was performed before ligation of a jejunal branch of the superior mesenteric artery and at the indicated time points after reperfusion. Mice were depleted of platelets 30 min before tumor inoculation. Data are pooled from two experiments with 6–8 mice per group (means ± S.D.). *, $p < 0.05$ compared with control group. *D,* representative pictures of intravital video microscopy analysis before perfusion and at 60 min after reperfusion. *Scale bar,* 100 μm.

In hemostasis, thrombosis, and inflammation, integrins are indispensable for platelet functions such as adhesion to the extracellular matrix and heterotypic cell-cell interaction with other cell types (4). Both integrin expression and activation on tumor cells have been linked to tumor progression (45–47). Platelets express several integrins, but the fibrinogen receptor GPIIb/IIIa is the most abundant. Once activated, it forms a bridge to proteins such as fibrinogen and mediates cell-cell and cell-matrix interactions (21). The amino acid motif Arg-Gly-Asp (RGD) is essential for integrin-mediated cell-cell adhesion events, as it mediates fibrinogen binding and heterotypic integrin bridging (20). Indeed, we found that preincubation with RGD, but not control peptide, significantly reduced B16 melanoma cell adhesion to immobilized murine platelets (Fig. 3*A*). Control experiments showed that after adhesion platelets are activated as assessed by increased staining for the platelet activation marker P-Selectin (supplemental Fig. 5). Moreover, a strong reduction in platelet-mediated adhesion of B16 melanoma cells was achieved after preincubation of murine platelets with a blocking anti-CD61 (β3 integrin) mAb or an anti-GPIIb/IIIa Ab (JON/A) (Fig. 3, *B* and *C*) (37). To exclude that reduced melanoma cell adhesion was caused by a reduction in the number of present platelets after incubation with RGD or anti-GPIIb/IIIa Ab, the number of platelets in the well was analyzed and showed no difference between control and inhibitory protein (supplemental Fig. 6). Similarly, inhibition of the $\alpha\nu$-integrin (CD51) subunit, highly expressed on the surface of B16 melanoma cells (data not shown) by a monoclonal antibody, reduced the increase in platelet-mediated adhesion of B16 melanoma cells (Fig. 3*D*). However, when we preincubated platelets with an anti-$\alpha\nu$-integrin (CD51) antibody, we observed no difference in melanoma cell adhesion to platelets (Fig. 3*E*). To further investigate the relevance of the platelet fibrinogen receptor in platelet-B16 cell interactions, CHO cells expressing wild-type or mutated and thereby activated GPIIb/IIIa integrins were used to study B16 cell adhesion (30, 31). Our results indicate that both fibrinogen receptor activation and fibrinogen are necessary for adhesion of melanoma cells (Fig. 3*F*). When fibrinogen was added to CHO cells expressing the activated GPIIb/IIIa receptor, we observed a significant increase in melanoma cell adhesion. Equal amounts of added fibrinogen did not result in increased melanoma cell adhesion in the resting state of the GPIIb/IIIa receptor. The increase in CHO-melanoma cell adhesion could be reversed by inhibition of the GPIIb/IIIa receptor with abciximab (C7e3) (30), whereas control IgG did not reduce melanoma cell adhesion to the activated GPIIb/IIIa receptor (Fig. 3*F*). Along with these findings, exposure of B16 melanoma cells to RGD peptide substantially

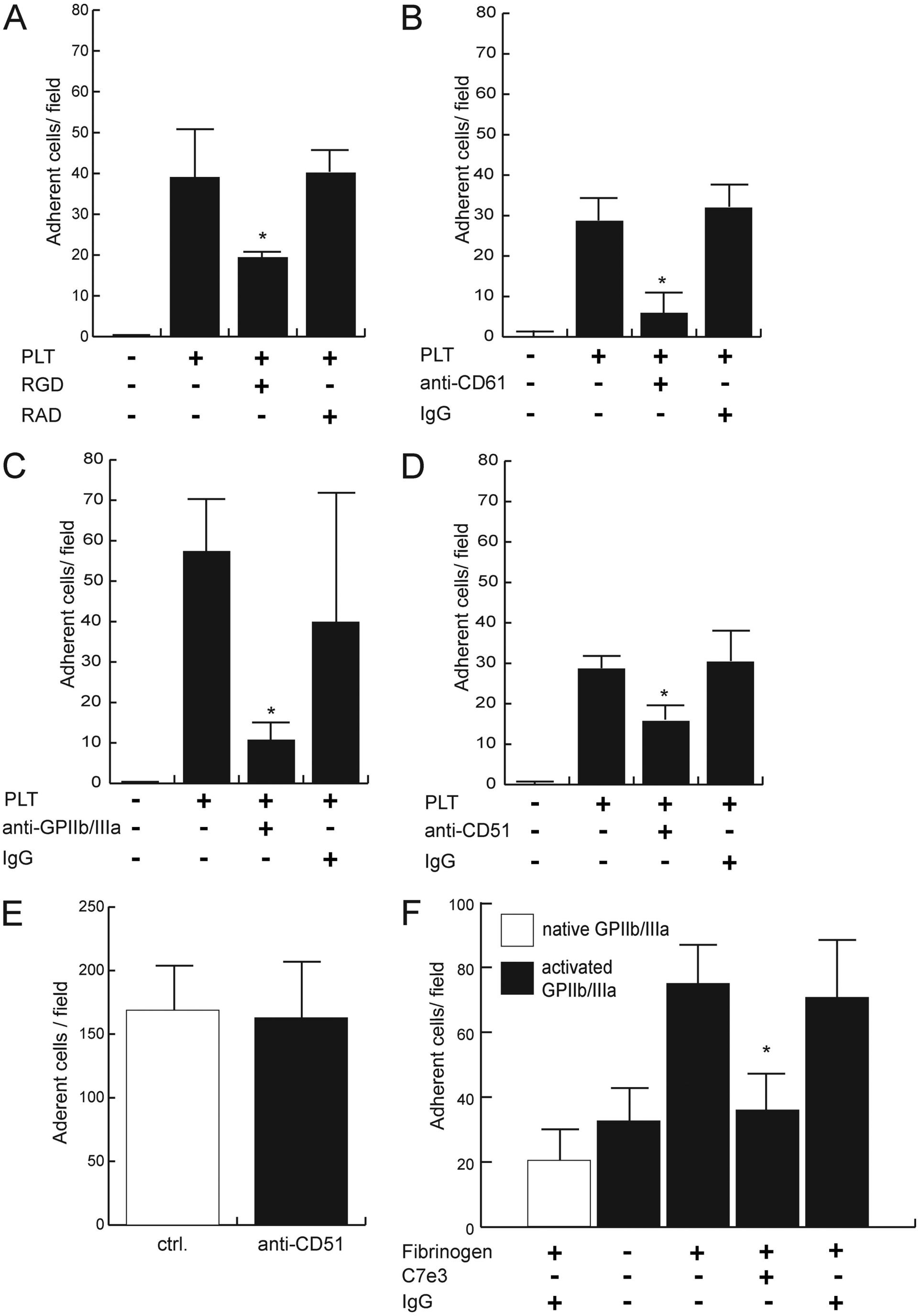

FIGURE 3. **Inhibition of GPIIb/IIIa on platelets and $\alpha\nu\beta3$ on melanoma cells inhibits platelet-melanoma cell interaction.** *A–E*, static adhesion of B16 cells in the presence or absence of immobilized murine platelets (*PLT*) (1×10^8/well). *A*, B16 cells were exposed to RGD peptide or RAD control peptide (10 μg/ml) prior to the assay. Data are representative of three experiments (means ± S.D. of three analyzed wells/condition). *, $p < 0.05$. *B–D*, similarly, adhesion of B16 cells to immobilized platelets was assessed after addition of blocking anti-CD61 mAb (10 μg/ml; or hamster IgG isotype control) (*B*), anti-GPIIb/IIIa mAb (JON/A, 10 μg/ml; or rat IgG isotype control) (*C*), and preincubation of B16 cells with anti-CD51 mAb (10 μg/ml; or rat IgG1 isotype control) (*D*). Data are representative of three experiments, each with three analyzed wells/condition (means ± S.D.). *, $p < 0.05$. *E*, furthermore, adhesion of B16 cells to immobilized platelets was assessed after preincubation of the immobilized platelets with blocking anti-CD51 mAb (10 μg/ml; or IgG1 isotype control (*ctrl.*)). Data are representative of three experiments, each with three analyzed wells/condition (means ± S.D.), and no significant difference was observed between groups. *F*, CHO cells bearing native or activated GPIIb/IIIa receptor (30, 31) were grown to confluency. B16 cells were labeled with a fluorescent dye, and adhesion to the CHO cells was assessed in the absence or presence of fibrinogen and a blocking antibody to the platelet fibrinogen receptor GPIIb/IIIa (c7E3) or IgG control. The number of adherent melanoma cells to surface-adherent CHO cells is presented. Nonspecific binding to CHO cells was assessed and was subtracted to calculate specific adhesion. The means ± S.D. ($n = 6$) is shown. *, $p < 0.05$.

reduced the platelet-mediated adhesion of CMFDA-labeled melanoma cells to a endothelial monolayer (b.END.3 cells) under flow conditions, compared with control peptide or untreated B16 melanoma cells (Fig. 4*A*). Moreover, we observed that inhibition of CD61 blocked the increase of melanoma cell adhesion to platelets, when perfused over murine endothelial cells (Fig. 4*B*). Similarly, preincubation of melanoma cells with a blocking antibody to aν integrin (CD51)

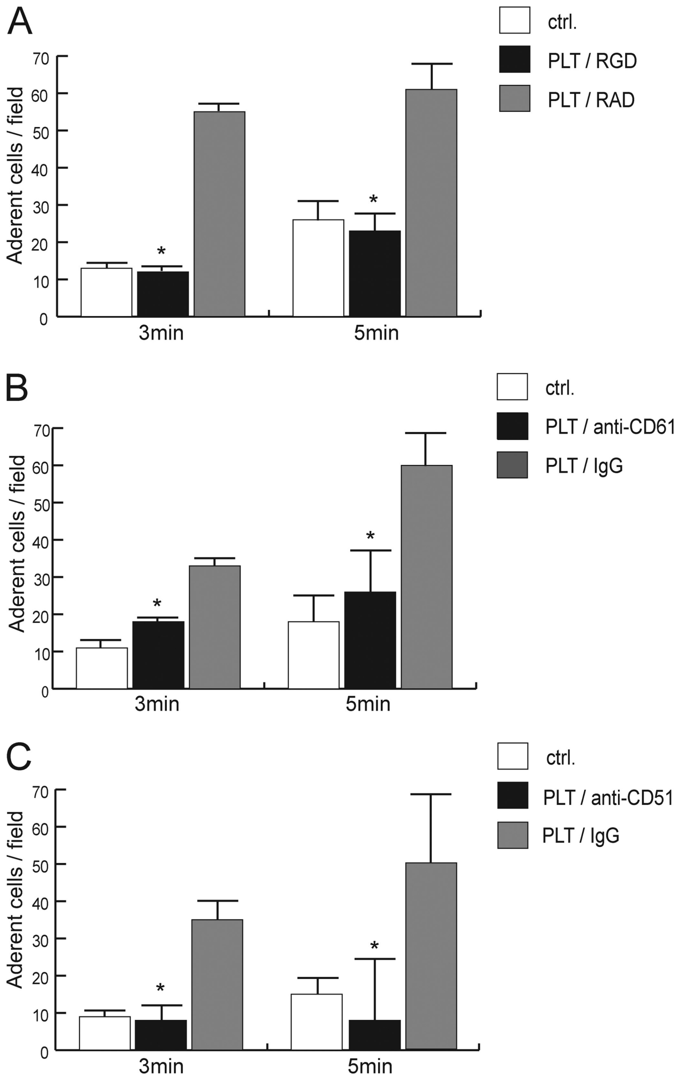

FIGURE 4. **Inhibition of GPIIb/IIIa on platelets and $\alpha\nu\beta3$ on melanoma cells inhibits platelet-melanoma cell interaction under flow conditions.** *A–C*, firm adhesion of CMFDA-labeled B16 cells (2×10^5/ml) on a monolayer of b.END.3 cells preincubated with freshly isolated murine platelets (*PLT*, 1×10^8/ml) or Tyrode's buffer (*ctrl.*) was assessed at shear rates of 500 s^{-1} at the indicated time points. *A*, B16 cells were exposed to RGD peptide or RAD control peptide (10 μg/ml) prior to the assay. Data are representative of four experiments with similar results (means ± S.D.). *, $p < 0.05$. *B* and *C*, similarly, adhesion of B16 cells to bEND.3 cells was assessed in the presence (*PLT*) or absence (*ctrl.*) of freshly isolated murine platelets after preincubation of platelets with blocking anti-CD61 ($\beta3$ integrin) mAb (10 μg/ml; or hamster IgG isotype control) (*B*), as well as preincubation of B16 cells with anti-CD51 ($\alpha\nu$ integrin) mAb (10 μg/ml; or rat IgG1 isotype control) (*C*). Data are representative of four experiments with similar results (means ± S.D.). *, $p < 0.05$.

reduced the platelet-mediated increase of melanoma cell adhesion compared with control IgG (Fig. 4*C*).

To assess the potential relevance of our findings for metastasis *in vivo*, we injected luciferase-transduced murine B16 melanoma cells intravenously and evaluated metastasis to the lung after 3 weeks by luminescence analysis of lung lysates *ex vivo*. Previous studies have shown that targeting $\alpha\nu\beta3$ integrin can reduce metastasis *in vivo* (24, 48). Similarly, we observed decreased metastasis of B16 melanoma cells to the lung by treatment with a blocking anti-$\alpha\nu$ integrin (CD51) mAb (Fig. 5). However, this effect was significantly reduced in the absence of platelets *in vivo* (*i.e.* we observed no difference in tumor metastasis between mice treated with anti-CD51 mAb compared with control IgG, when mice were treated with anti-platelet serum). Overall, our results highlight the importance of the GPIIb/IIIa receptor on platelets for the interaction with melanoma cells through $\alpha\nu\beta3$ integrin.

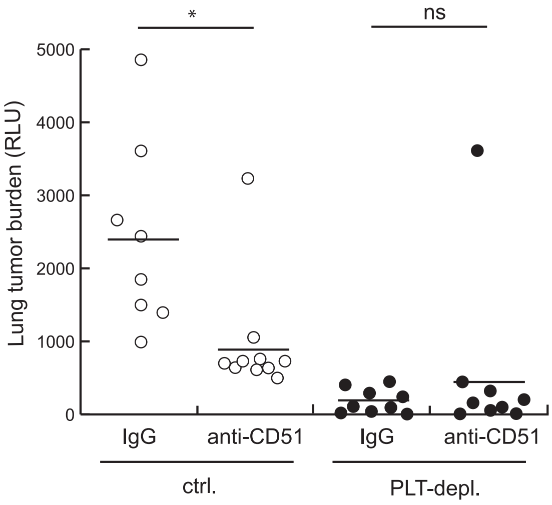

FIGURE 5. **Effect of $\alpha\nu\beta3$ inhibition on tumor metastasis is reduced in absence of platelets *in vivo*.** B16-luc cells (2×10^5 cells in 200 μl of PBS/animal) were injected i.v. into C57BL/6 mice, and lung tumor burden was assessed on day 21 by detection of luciferase activity *ex vivo* in lysates of the lung tissue by bioluminescence measurements (in relative light units, *RLU*). Animals had been injected intraperitoneally with anti-murine platelet-hyperimmune serum (*open circles*) for platelet (*PLT*) depletion or with control (*ctrl.*) serum (*filled circles*) 24 h before and 48 h after tumor cell inoculation. Where indicated, B16-luc cells were exposed to monoclonal anti-CD51 (10 μg/ml) or rIgG1k isotype control (10 μg/ml) for 30 min and washed with DMEM prior to i.v. injection. Data are pooled from two experiments with a total of 8–10 animals/group. *, $p < 0.05$, *ns* = nonsignificant compared with IgG control in the presence or absence of platelets.

DISCUSSION

Various molecular details responsible for the reciprocal relationship between metastasizing tumor cells and soluble components of the coagulation cascade have already been described (49). However, the involvement of platelets, the cellular component of thrombi, in the pathogenesis of cancer metastasis is still poorly understood. The metastatic process is thought to occur as a cascade of distinct sequential steps, including tumor cell chemotaxis, adhesion, extravasation, and finally proliferation of the metastasizing cells. Although the factors involved in each step may vary, the mechanisms mediating the firm arrest of tumor cells to the vascular wall may be among the most important determinants. Here, we provide evidence that platelets interact with melanoma cells both *in vitro* and *in vivo* and that the interaction of integrin GPIIb/IIIa expressed on platelets and $\alpha\nu\beta3$ on melanoma cells contributes to the molecular interface between these two cells types, which may be of relevance for melanoma cell metastasis.

Platelets in patients with newly diagnosed metastatic disease display an activated state measured by P-Selectin expression (12), indicating that these cells are potentially an important factor in tumor metastasis. A study by Wang and Zhang (13) using various tumor cell lines suggested that platelets directly influence tumor cell proliferation in an MHC-independent manner. Furthermore, platelets are crucial regulators of tumor vessel stability and prevent intra-tumor hemorrhage thereby affecting tumor cell viability (15). In turn, it has been reported that human melanoma cells can cause platelet aggregation (50). In our experiments, local tumor progression was not affected by thrombocytopenia at least when platelets were depleted in the early phase after subcutaneous tumor cell implantation. Surprisingly, although platelets have been reported to provide chemotactic signals to various cell types, including leukocytes or stem cells (4, 28, 36, 51), we observed no effect of platelets on B16 melanoma transmigration. In contrast, we observed a robust increase in adhesivity of murine and human melanoma cells in the presence of platelets in various settings *in vitro*. Adherent platelets present potent adhesion receptors such as JAM-C, P-Selectin, GPIbα, or GPIIb/IIIa to other circulating cells providing a platform for a potent cellular bridging mechanism to the vascular wall (20, 25, 52–56). Recently, glycoprotein Ibα (GPIbα), the second most abundant receptor expressed on platelets, has been reported to be significantly involved in melanoma metastasis, as the functional absence of GPIbα correlated with a clear reduction in the number of lung metastatic foci *in vivo* (57). In contrast, another report showed that GPIbα inhibition led to a significant increase in pulmonary metastasis, improved survival, and pulmonary arrest of tumor cells (58), presumably reflecting the complexity of tumor cell-platelet-endothelial interactions and manifesting the need for continued experimental studies. Here, we confirm by intravital microscopy that platelet depletion results in significantly decreased melanoma cell adhesion to the vascular wall *in vivo*, demonstrating an interaction of melanoma cells with platelets *in vivo*. Once adherent to the vessel wall, such as after endothelial injury, the platelet fibrinogen receptor GPIIb/IIIa becomes activated and contributes to thrombus formation and wound healing (3, 59). Interestingly, we observed that inhibition of GPIIb/IIIa resulted in both the reduced adhesion of melanoma cells to immobilized platelets and the reduced adhesion to endothelial cells under shear flow conditions in the presence of platelets. Similarly to GPIIb/IIIa, integrin $\alpha\nu\beta3$ can bind RGD proteins and mediate cell aggregation or cell adhesion in a homotypic or heterotypic fashion (21, 60). Stable arrest and adhesion strengthening of circulating neutrophils to surface-adherent platelets in flow requires interactions of integrins with fibrinogen bound to platelet GPIIb/IIIa (20).

It is clear that $\alpha\nu\beta3$ expressed on tumor cells regulates a broad range of cellular functions such as survival, apoptosis, migration, or angiogenesis contributing to tumorigenicity (61, 62). Regarding cell adhesion, expression of integrin $\alpha\nu\beta3$ promotes a metastatic phenotype in human melanoma by supporting specific adhesive properties of the tumor cells (63). Using an RGD peptide and CHO cells expressing resting or constitutively active GPIIb/IIIa (30, 31), we demonstrated that melanoma cells adhere to platelets in an integrin-dependent fashion. These experiments furthermore indicate that both integrins interact with each other in the presence of fibrinogen.

Furthermore, experiments using blocking antibodies to GPIIb/IIIa or $\alpha\nu\beta3$, respectively, revealed a significant involvement of these surface receptors in melanoma cell/platelet adhesion. These findings indicate an important potential mechanism, whereby interactions between GPIIb/IIIa on platelets and $\alpha\nu\beta3$ on tumor cells support metastatic spread.

Besides contributing to cell-cell interactions, platelets may influence melanoma cell metastasis in a paracrine fashion. As platelets contain a very broad range of paracrine mediators (3, 4, 8, 9), future studies will have to address the distinct contribution of soluble platelet-derived factors for hematogenous melanoma metastasis. Furthermore, it will be interesting to investigate, how circulating melanoma cells influence activation of platelets. In extension to our studies, it will have to be evaluated whether stability of platelet adhesion and platelet receptor binding capacity is an important factor for melanoma cell adhesion augmented by platelets.

Our results demonstrate the relevance of the interaction between melanoma cells and platelets for hematogenous tumor cell metastasis and may indicate potential novel molecular targets for patients at risk for metastatic tumor spread. Although further detailed studies are needed to address this concept, in particular, the identification of novel adhesion receptors involved in platelet-melanoma cell cross-talk, such as the interaction of GPIIb/IIIa with $\alpha\nu\beta3$, may reveal promising new pharmacological approaches to block tumor metastasis.

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