Tumorigenesis and Neoplastic Progression

Innate Immune Cells Induce Hemorrhage in Tumors during Thrombocytopenia

Benoit Ho-Tin-Noé,^{*†‡} Carla Carbo,^{*†§}
Mélanie Demers,^{*†‡} Stephen M. Cifuni,^{*†}
Tobias Goerge,^{*†‡} and Denisa D. Wagner^{*†‡}

From the Immune Disease Institute,^{*} Boston, Massachusetts; the Program in Cellular and Molecular Medicine,[†] Children's Hospital Boston, Boston, Massachusetts; the Department of Pathology,[‡] Harvard Medical School, Boston, Massachusetts; and the Servei d'Hemoteràpia-Hemostasia,[§] Hospital Clinic, Universitat de Barcelona, Barcelona, Spain

Platelets are crucial regulators of tumor vascular homeostasis and continuously prevent tumor hemorrhage through secretion of their granules. However, the reason for tumor bleeding in the absence of platelets remains unknown. Tumors are associated with inflammation, a cause of hemorrhage in thrombocytopenia. Here, we investigated the role of the inflamed tumor microenvironment in the induction of tumor vessel injury in thrombocytopenic mice. Using s.c. injections of vascular endothelial growth factor or tumor necrosis factor- α combined with depletion of neutrophils, we demonstrate that enhancing the opening of endothelial cell junctions was not sufficient to cause bleeding in the absence of platelets; instead, induction of tissue hemorrhage in thrombocytopenia required recruitment of leukocytes. Immunohistology revealed that thrombocytopenia-induced tumor hemorrhage occurs at sites of macrophage and neutrophil accumulation. Mice deficient in β_2 or β_3 integrins, which have decreased neutrophil and/or macrophage infiltration in their tumor stroma, were protected from thrombocytopenia-induced tumor hemorrhage, indicating that, in the absence of platelets, stroma-infiltrating leukocytes induced tumor vessel injury. This injury was independent of reactive oxygen species generation and of complement activation, as suggested by the persistence of tumor hemorrhage in C3- and nicotinamide adenine dinucleotide phosphate oxidase-deficient thrombocytopenic mice. Our results show that platelets counteract tumor-associated inflammation and that the absence of this platelet function elicits vascular injuries by tu-

mor-infiltrating innate immune cells. (Am J Pathol 2009, 175:1699–1708; DOI: 10.2353/ajpath.2009.090460)

Besides their fundamental role in primary hemostasis, platelets have been shown to support vascular barrier function and integrity during *in vitro* organ perfusion,¹ experimental angiogenesis,² cytotoxic T lymphocyte-dependent clearance of lymphocytic choriomeningitis virus,³ and inflammation.⁴ Recently, we reported that platelets are crucial regulators of tumor vascular homeostasis that continuously protect tumors from hemorrhaging and subsequent cell death.⁵ Prevention of tumor hemorrhage by platelets shows some similarities to that of hemorrhages at early stages of inflammatory reactions, suggesting that the inflamed microenvironment of tumors may play a role in the induction of tumor vessel damage in thrombocytopenic mice. In fact, in contrast to the mechanisms by which platelets promote primary hemostasis, neither firm platelet adhesion nor a platelet's ability to form thrombi is required for maintaining vascular integrity in both inflamed skin and tumors.^{4,5} We showed that transfusion of resting platelets prevented tumor bleeding in thrombocytopenic mice whereas transfusion of degranulated platelets did not.⁵ This pinpoints the importance of platelet granule secretion in protecting blood vessels. The soluble factors released by platelets may inhibit excessive endothelial permeability and/or prevent vascular injury induced by tumor-associated inflamma-

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Current address of B.H.-T.-N.: INSERMU698, Bichat Hospital, Paris, France; current address of T.G.: Department of Dermatology, University Hospital of Münster, Germany.

Address reprint requests to Denisa D. Wagner, Ph.D., Immune Disease Institute, 3 Blackfan Circle, Boston, MA 02115. E-mail: wagner@idi.harvard.edu.

tion. Besides vascular endothelial growth factor (VEGF), a permeability factor, platelet granules indeed also contain a variety of substances such as angiopoietin-1,^{5,6} transforming growth factor- β ,⁷ platelet basic protein,⁸ serpins,^{9,10} serotonin,¹¹ or sphingosin-1-P¹² that support vascular barrier function and/or have immunomodulatory properties.

Here, we investigated the role of tumor-associated inflammation in tumor bleeding in thrombocytopenic mice. Our results show that stroma-infiltrating neutrophils and macrophages induce tumor vessel damage in thrombocytopenia. We further demonstrate that maintenance of vessel integrity by platelets exceeds their impact on endothelial junctions and involves inhibition of vascular damage induced by infiltrating leukocytes.

Materials and Methods

Reagents

Fetal bovine serum was from the American Type Culture Collection (Manassas, VA). Penicillin/streptomycin and high-glucose Dulbecco's modified Eagle's medium were from Invitrogen (Carlsbad, CA). Medium titanium skinfold chambers were from APJ Trading (Ventura, CA). Polyclonal anti-GPIb α rat IgG R300 and polyclonal nonimmune rat IgG C301 were from emfret Analytics (Eibelstadt, Germany). Evans blue, hematoxylin, eosin, prostacyclin (PGI₂), myeloperoxidase (MPO), tetramethylbenzidine, recombinant mouse tumor necrosis factor- α (TNF- α), hexadecyltrimethylammonium bromide, trypsin-EDTA, and Drabkin's reagent were from Sigma-Aldrich (St. Louis, MO). 4',6-Diamino-2-phenylindole (DAPI) was from Molecular Probes/Invitrogen (Eugene, OR). Recombinant mouse VEGF-164 was from R&D Systems (Minneapolis, MN).

Animals

All animal procedures described in this study were performed using 6- to 8-week-old C57BL/6J and BALB/c female mice (The Jackson Laboratory, Bar Harbor, ME), except in experiments using dorsal skinfold chamber for which 12-week-old BALB/c male mice were used. Mice deficient in β_3 integrins on BALB/c background¹³ and in CD18 on C57BL/6J background^{14,15} were bred and housed in our animal facility. Mice deficient in complement component 3 (C3^{-/-}) on C57BL/6J background¹⁶ were a gift from Dr. Michael C. Carroll (Immune Disease Institute, Boston, MA). NADPH oxidase-deficient mice on a C57BL/6J background (gp91phox^{-/-}, strain name, B6.129S6-Cybbtm1Din/J)¹⁷ were obtained from The Jackson Laboratory. All experimental procedures involving the use of mice were approved by the Animal Care and Use Committee of the Immune Disease Institute.

Cell Culture

Lewis lung carcinoma (LLC) cells (CRL-1642) and 4T1 mammary gland tumor cells (CRL-2539) were purchased from the American Type Culture Collection. Cells were cul-

tured at 37°C in a humidified atmosphere of 5% CO₂, high-glucose (4.5 g/L) Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% glutamine.

Tumor Cell Implantation

One hundred microliters of LLC at 1.0×10^7 /ml in Dulbecco's modified Eagle's medium were injected s.c. into the backs of 6- to 8-week-old syngenic C57BL/6J female mice. For 4T1 cells, 100 μ l of tumor cells at 1.5×10^5 /ml in Dulbecco's modified Eagle's medium were injected into the mammary glands of 6- to 8-week-old syngenic BALB/c female mice.

Induction of Thrombocytopenia

Thrombocytopenia was induced by an intravenous injection of 2.5 μ g/g mouse of the platelet-depleting antibody¹⁸ (polyclonal anti-mouse GPIb α rat IgG; emfret Analytics). Control mice were injected with a nonimmune rat polyclonal IgG (emfret Analytics). Thrombocytopenia was evaluated by flow cytometry. The i.v. injection of the depleting antibody resulted in $\geq 97\%$ reduction in circulating platelets at 1 hour postinjection in all mice, and platelet counts remained $\leq 3\%$ for over 24 hours.^{4,18}

Depletion of Neutrophils

Neutrophils depletion was induced by intravenous injection of 5 μ g/g mouse of rat anti-Ly6G/C (RB6-8C5; eBioscience, San Diego, CA). Control mice were injected with PBS. Neutropenia was evaluated 24 hours postinjection by flow cytometry using a FITC-conjugated anti-mouse neutrophils mAb (Cedarlane Laboratories, Hornby, Ontario, Canada). The i.v. injection of the neutrophil depleting antibody resulted in $\geq 94\%$ reduction in circulating neutrophils in all mice. For long-term (11 days) depletion of neutrophils in tumor-bearing animals, the neutrophil depleting antibody was injected every 3 days starting 2 days before tumor cell implantation.

In Vivo Imaging of Skin Bleeding

Dorsal skinfold chambers and surgical preparation were performed as described previously.¹⁹ After 2 days of recovery, mice were injected i.v. with either the control or the platelet-depleting antibody. Two hundred nanograms of either recombinant VEGF or TNF- α were topically applied on the conjunctive tissue below the striated skin muscle layer of the remaining skin 30 minutes later. A subset of mice was also injected i.v. with 100 μ l of 5% Evans blue 10 minutes after the stimulation with VEGF or TNF- α , and Evans blue leakage was visually assessed 20 minutes later. Skin was photographed through dorsal skinfold chambers 12 hours after stimulation using an upright microscope (Axioplan; Zeiss, Oberkochen, Germany) with a $\times 2.5$ magnification objective and recorded by an attached digital camera (AxioCam HSc).

Subcutaneous Injections of VEGF and TNF- α

Two hundred nanograms of recombinant VEGF or TNF in PBS were injected s.c. into the backs of 6- to 8-week-old C57BL/6J female mice. Control or platelet-depleting IgG was injected i.v. 5 minutes later.

Determination of Intraskin and Intratumor Hemoglobin Content

At 12 hours postinjection of either the control or the platelet-depleting IgG, skin biopsies and tumors were excised from sacrificed animals, weighed, homogenized in Drabkin's reagent (Sigma-Aldrich), and centrifuged ($2000 \times g$; 10 minutes), and hemoglobin content of supernatants was measured by absorbance reading at 540 nm.

Determination of MPO Activity

At 12 hours postinjection of either the control or the platelet-depleting IgG, skin biopsies and tumors were excised from sacrificed animals, and MPO activity was analyzed as described previously.²⁰ Briefly, skin biopsies and tumors were weighed, blended, in 50 mmol/L potas-

sium phosphate buffer, centrifuged, resuspended, and sonicated in potassium phosphate buffer supplemented with 50 mmol/L hexadecyltrimethylammonium bromide. After centrifugation of the cell lysates, MPO activity was assessed in the supernatant by adding tetramethylbenzidine and absorbance reading at 450 nm after stopping the reaction with 0.3 N sulfuric acid.

Immunohistology of LLC Tumors

Subcutaneous tumors were harvested from sacrificed animals, fixed in zinc fixative (100 mmol/L Tris-HCl containing 37 mmol/L zinc chloride, 23 mmol/L zinc acetate, and 3.2 mmol/L calcium acetate), paraffin embedded, and sectioned. Tumor sections were stained with H&E, and leukocyte infiltration was studied by immunostaining of neutrophils, macrophages, and T lymphocytes using an anti-GR1 (clone RB6-8C5; eBioscience), an anti-MPO (Abcam, Cambridge, MA), an anti-F4/80 (clone C1: A3-1; Cedarlane Laboratories), an anti-CD4 (GK1.5; BD Pharmingen, San Diego, CA), and an anti-CD8a mAb (5H10-1; BD Pharmingen). Counterstaining with DAPI was performed to visualize all nuclei. After washing, the slides were mounted with Gel/Mount aqueous mounting

Figure 1. Induction of tissue hemorrhage in the skin of thrombocytopenic mice requires recruitment of leukocytes. **A:** Mice carrying skinfold chambers were injected with either control or platelet-depleting IgG, and VEGF or TNF- α was topically applied at time 0. Photographs of the skin were taken 12 hours after stimulation. Only TNF- α -treated skin of thrombocytopenic mice bled. Bar, 500 μ m. **B:** Comparison of neutrophil infiltration in biopsies from PBS-, VEGF-, and TNF- α -injected skin as assessed by their MPO content 12 hours after the injections ($n = 6$). **C:** Treatment with an anti-GR1 mAb prevented neutrophil infiltration in TNF- α -injected skin ($n = 9$ –12). **Inset:** Depletion of circulating neutrophils before TNF- α injections was assessed by FACS using a fluorescein isothiocyanate-conjugated IgG to neutrophils 24 hours after injecting the anti-GR1 mAb. **Arrow** indicates the absence of circulating neutrophils in anti-GR1-treated mice as compared with control mice. **D:** Comparison of hemoglobin content in biopsies from PBS and TNF- α -injected skin from untreated and anti-GR1-treated control and thrombocytopenic mice ($n = 5$ –9); representative photographs of TNF- α -treated skin are shown.

medium (Biomed, Foster City, CA) and observed under an epifluorescence microscope. For H&E staining, slides were mounted with DPX Mountant (Fluka BioChemika, Buchs, Switzerland) and observed in light microscopy.

Tumor-infiltrating macrophages were quantified by counting F4/80-positive cells on tumor sections counterstained with DAPI (three to five random sections per tumor from at least four different mice per group at $\times 100$ magnification).

Statistical Analysis

Data are presented as mean $\pm$ SEM and were analyzed by analysis of variance followed by Fisher's protected least-significant difference test. When two samples were compared, unpaired two-tailed Student's *t*-test was applied. Values of $P < 0.05$ were regarded as statistically significant.

Results

Neutrophil Infiltration but Not Induction of Vascular Permeability in the Skin Causes Hemorrhage in Thrombocytopenia

VEGF with vascular permeability activity²¹ and TNF- α with proinflammatory properties²² are two factors abundantly expressed in solid tumors that may affect tumor vessel integrity. We compared their ability to induce hemorrhage when applied topically onto the conjunctive tissue below the skin of control and thrombocytopenic mice. Application of either VEGF or TNF- α led to an increase in vascular permeability as indicated by Evans

blue leakage in control and thrombocytopenic mice (data not shown). However, although bleeding was observed in the skin of thrombocytopenic animals treated with the proinflammatory cytokine TNF- α , there was no hemorrhage upon application of VEGF (Figure 1A). Measurement of MPO activity in the treated area showed that only TNF- α induced recruitment of neutrophils (Figure 1B). To investigate the role of neutrophils in TNF- α -induced skin bleeding in thrombocytopenic mice, we depleted neutrophils before s.c. injection of TNF- α . Twenty-four hours after the i.v. injection of an anti-GR1 antibody that recognizes neutrophils and the GR1-positive inflammatory monocyte subset,^{23,24} a $\geq 94\%$ reduction in circulating neutrophils was observed by flow cytometry (Figure 1C, inset). This abolished neutrophil infiltration to the TNF- α -injected skin as its MPO activity was similar to that of PBS-injected skin (Figure 1C). As a result, TNF- α -induced skin hemorrhage in thrombocytopenic mice was prevented in neutrophil-depleted mice (Figure 1D). Altogether, these results show that opening of endothelial cell junctions with VEGF was not sufficient to induce tissue hemorrhage in thrombocytopenia and that infiltration of GR1-positive leukocytes in the tissue was required.

Thrombocytopenia-Induced Tumor Hemorrhage Colocalizes with Peritumoral Neutrophils and Macrophages and Develops in Close Proximity to Areas Containing Large Numbers of Dead Leukocytes

Leukocyte infiltration in the tumor stroma is one of the features of tumor-associated inflammation.²⁵ We investi-

Figure 2. Thrombocytopenia-induced tumor hemorrhage colocalizes with accumulation of innate immune cells in the tumor stroma. **A:** Photographs of 9-day-old s.c. LLC tumors 18 hours after the injection of either the control (left panel) or platelet-depleting IgG (right panel). Bar, 5 mm. **B:** H&E staining of LLC tumors from control (left panel) and thrombocytopenic mice (right panel). Arrows indicate leukocytes at the periphery of tumors. Star indicates area of hemorrhage in the platelet-depleted sample. Bar, 50 μ m. **C:** Merge picture of phase-contrast image, DAPI staining (blue), and anti-Ly6G immunostaining for neutrophils (green) in the hemorrhagic area of an LLC tumor from a thrombocytopenic mouse. Arrows show accumulation of neutrophils at the interface between tumor cells and hemorrhage. Scale bar, 100 μ m.

gated whether tumor-infiltrating leukocytes could also play a role in thrombocytopenia-induced tumor hemorrhage. Induction of platelet depletion in mice bearing 8-day-old LLC tumors led to extensive hemorrhage, which occurred mostly at the tumor-stroma interface (Figure 2, A and B). Interestingly, massive accumulation of leukocytes was found in the same areas, as revealed by H&E and anti-GR1 staining (Figure 2, B and C). We further analyzed the recruitment of leukocytes within and surrounding tumors by staining tumor sections with antibodies to F4/80, GR1, CD4, or CD8 for detection of macrophages, neutrophils, and T4 and T8 lymphocytes, respectively. Stroma-infiltrating leukocytes accumulating at the interface between the tumor and the hemorrhage were neutrophils and macrophages (Figures 2C and 3, A and C), whereas no CD4 and CD8 T lymphocytes were detected in this area (data not shown). The presence of neutrophils near the edge of tumor hemorrhage was confirmed using anti-lactoferrin and anti-MPO antibodies that gave staining patterns similar to those obtained with the anti-GR1 antibody (data not shown).

No difference in intratumor neutrophil infiltration was found between LLC tumors from control and platelet-depleted mice as assessed by measurement of intratumor MPO content (Figure 3, A and B). Similarly, immunofluorescence analysis of F4/80-positive cells on tumor sections from 8-day-old LLC tumors showed no differ-

ence in intratumor macrophage infiltration between control and mice rendered thrombocytopenic 18 hours before (Figure 3, C and D). This indicates that tumor neutrophil and macrophage contents are not affected by acute thrombocytopenia and suggests that platelets may prevent vascular damages originating from infiltrated innate immune cells at tumor sites.

Interestingly, large areas of dead neutrophils and macrophages were observed next to the hemorrhagic regions of thrombocytopenic tumors (Figure 4A, lower panel). The absence of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells (data not shown) suggested that leukocytes most likely died by a necrotic pathway in these regions. These areas were filled with ghost-like neutrophils and macrophages characterized by either no nuclei or irregularly shaped nuclei stained most faintly with DAPI (Figure 4, B and C). The presence of extracellular MPO in leukocyte necrotic areas indicated that granular content had been released from the neutrophils in these regions (Figure 4D). Because of the absence of defined nuclei in leukocyte necrotic areas and their uneven distribution within tumors, quantification was difficult. However, large leukocyte necrotic areas were present in both tumors from control and thrombocytopenic mice (Figure 4A). The proximity between the hemorrhagic areas and the leukocyte necrotic sites suggests that platelets may be needed to inhibit potential vessel-injuring products released from the dying leukocytes in these regions.

Figure 3. Leukocyte infiltration in tumors is not affected by acute thrombocytopenia. At day 8 after subcutaneous implantation of LLC tumor cells, mice were injected with either the control or the platelet-depleting IgG. Tumors were excised 18 hours later for histological staining and measurement of MPO content. **A:** Merge picture of DAPI staining (blue) and anti-GR1 immunostaining for neutrophils (green). **Arrows** point to accumulation of neutrophils. Scale bar = 100 μ m. **B:** Quantification of neutrophil infiltration in LLC tumors from control and thrombocytopenic mice by measurement of tumor MPO content. ($n = 13$). **C:** Merge picture of DAPI staining (blue) and anti-F4/80 immunostaining for resident macrophages (orange). Scale bar = 100 μ m. **Arrows** indicate accumulation of macrophages. **D:** Immunofluorescence quantification of F4/80-positive cells in tumor sections from LLC tumors from control and thrombocytopenic mice. ($n = 5$ tumors per group).

Tumor Vessel Damage in Thrombocytopenic Mice Is Independent of Leukocyte-Derived Reactive Oxygen Species and of Complement Activation

Production of reactive oxygen species (ROS) by phagocytes and activation of the complement system represent two important mechanisms by which the innate immune system kills microbial pathogens but also damages surrounding tissues.^{26,27} To investigate whether thrombocytopenia-induced tumor vessel damages were mediated by phagocyte-derived ROS and/or complement activation, we used NADPH oxidase-deficient mice (gp91^{phox}^{-/-}) that are characterized by a defective phagocyte respiratory burst¹⁷ and C3^{-/-} mice because component C3 plays a central role in all three pathways of complement activation. Neither NADPH oxidase- nor C3-deficient mice bearing 8-day-old LLC tumors were protected against thrombocytopenia-induced tumor hemorrhage. Wild-type, NADPH oxidase-, and C3^{-/-} mice rendered thrombocytopenic had a similar increase in their tumor hemoglobin content (4.6 ± 0.8 , 4.5 ± 0.2 , and 3.8 ± 0.6 -fold increase, respectively, relative to control tumors from nonthrombocytopenic wild-type mice, $n = 5$; Supplemental Figure 1, see <http://ajp.amjpathol.org>). This suggests that thrombocytopenia-induced tumor vessel injuries are independent of NADPH oxidase-derived ROS and of complement activation.

Figure 4. Thrombocytopenia-induced tumor hemorrhage develops in close proximity to areas containing dead leukocytes. **A:** Phase contrast, DAPI, and anti-GR1 staining of 9-day-old LLC tumors from control and thrombocytopenic mice. Hemorrhage and leukocyte necrotic areas containing GR1-positive neutrophils with faint DAPI staining are indicated. Scale bar = 100 μ m. **B–D,** DAPI, anti-GR1 (**B**), anti-F4/80 (**C**), anti-MPO (**D**), and corresponding merged pictures of viable and necrotic areas of LLC tumors from thrombocytopenic mice. Scale bar = 20 μ m. **Arrows,** extracellular DNA of neutrophils (**B**), faint DAPI staining of macrophage nuclei (**C**), and extracellular MPO (**D**) in necrotic areas in proximity to the hemorrhage. Scale bars = 20 μ m.

Mice with Decreased Numbers of Tumor-Infiltrating Neutrophils and Macrophages Are Protected from Thrombocytopenia-Induced Hemorrhage: Importance of Leukocyte Integrins

To further investigate the role of innate immune cells in the induction of tumor hemorrhage in the absence of platelets, we implanted tumors and induced acute thrombocytopenia in mice with genetic defects affecting leukocyte migration into inflamed tissues. Because tumor leukocyte content may affect tumor growth¹³ and to prevent differences due to variability in tumor size between

knockout and wild-type mice, only age-matched tumors of similar size were compared.

β_2 Integrin-deficient ($CD18^{-/-}$) mice are a well established model for leukocyte adhesion deficiency type I.¹⁵ $CD18^{-/-}$ mice lack adherence and transmigration of neutrophils in tumors^{28,29} and in several models of cutaneous inflammation.^{30,31} In agreement with these reports, we found that tumor neutrophil infiltration measured by intratumor MPO content was reduced significantly in size-matched 10-day-old LLC tumors from $CD18^{-/-}$ mice as compared with wild-type C57BL/6J mice (Figure 5A). However, the tumor hemoglobin content (as an index of

Figure 5. $CD18^{-/-}$ mice have reduced levels of tumor-infiltrating neutrophils and are protected against thrombocytopenia-induced tumor bleeding. **A:** Comparison of neutrophil infiltration in 10-day-old LLC tumor from wild-type and $CD18^{-/-}$ mice determined by MPO content. ($n = 9$). **B:** Comparison of intratumor hemoglobin content among control and platelet-depleted wild-type and $CD18^{-/-}$ mice 12 hours after the injection of the control or platelet depleting IgG on day 10 ($n = 6$ –11). Representative photographs of tumors are shown below.

Figure 6. $\beta_3^{-/-}$ mice have reduced tumor-infiltrating macrophages and are protected against thrombocytopenia-induced tumor bleeding. **A:** Merge pictures of DAPI staining (blue) and anti-F4/80 immunostaining (orange) for resident macrophages in two 8-day-old 4T1 breast carcinomas from wild-type and $\beta_3^{-/-}$ mice. Scale bar = 100 μ m. **Arrows** show accumulation of macrophages in tumors of wild-type mice. **B:** Quantification of F4/80-positive cells on tumor sections from 8-day-old 4T1 breast carcinoma from wild-type and $\beta_3^{-/-}$ mice ($n = 4$ tumors per genotype). **C:** Comparison of neutrophil infiltration in 8-day-old 4T1 tumor from wild-type and $\beta_3^{-/-}$ mice by measurement of their MPO content ($n = 6$). **D:** Comparison of intratumor hemoglobin content among control and platelet-depleted wild-type and $\beta_3^{-/-}$ mice ($n = 8-14$). Representative photographs of tumors are shown below.

vascularization) was similar in nonthrombocytopenic wild-type and CD18^{-/-} mice, indicating that there was no difference in the extent of vascularization of LLC tumors between these mice (Figure 5B). Interestingly, when rendered thrombocytopenic on day 10 after tumor implantation, CD18^{-/-} mice appeared protected from tumor bleeding, as revealed by both macroscopic observation and reduced intratumor hemoglobin content when compared with wild-type thrombocytopenic mice (Figure 5B). These results show that tumors with reduced neutrophil infiltration are partially protected against thrombocytopenia-induced hemorrhage. This indicates that CD18-containing leukocytes, likely neutrophils, damage the tumor vessels and such damage can be prevented by platelets. Supporting this hypothesis, induction of acute thrombocytopenia in wild-type mice carrying 9-day-old LLC tumors and pretreated with the neutrophil depleting antibody starting 2 days before tumor cell implantation and then at 3-day intervals caused significantly less bleeding than was seen in tumors from wild-type mice pretreated with PBS (2.77 ± 0.47 - versus 5.23 ± 0.54 -fold increase in tumor hemoglobin content relative to control tumors from nonthrombocytopenic wild-type mice, $P < 0.02$, $n = 8$). Thus, experimental neutrophil depletion also provided partial protection from thrombocytopenia-induced tumor bleeding.

β_3 Integrins are required for arrest and extravasation of monocytes/macrophages at sites of inflammation³² including tumors.¹³ Of note, despite the hemostatic defect of $\beta_3^{-/-}$ mice, no spontaneous bleeding was observed in

4T1 breast tumors from nonthrombocytopenic $\beta_3^{-/-}$ mice (Figure 6D). This result further confirms our previous observation indicating that prevention of tumor hemorrhage by platelets is independent of thrombus formation.⁵ Consistent with a previous report,¹³ we found that 4T1 tumors implanted in $\beta_3^{-/-}$ mice showed several-fold fewer infiltrating macrophages than 4T1 tumors from wild-type BALB/c mice ($13.1 \pm 2.1\%$ versus $2.5 \pm 0.8\%$ F4/80-positive cells per tumor section, $P < 0.0004$; Figure 6, A and B). In contrast, tumor neutrophil infiltration of $\beta_3^{-/-}$ mice was similar to that of wild-type BALB/c mice (Figure 6C). Interestingly, $\beta_3^{-/-}$ mice were partially protected from thrombocytopenia-induced tumor hemorrhage as shown by decreased tumor hemoglobin content and macroscopic observation (Figure 6D). These results indicate that macrophages also promote tumor bleeding observed in thrombocytopenia.

Discussion

Recently, we demonstrated the continuous requirement of platelets for the prevention of bleeding in solid tumors.⁵ This effect was independent of thrombus formation and relied on the secretion of their granule content.⁵ In the present study, we tried to identify which cell type platelets counteract to prevent tumor hemorrhages. Platelets have long been known to enhance the vascular barrier function through the release of vasoactive mediators, such as angiopoietin-1 or sphingosin-1-P, that tighten endothelial

junctions.^{1,6,33–35} Absence of this antipermeability function was proposed to cause extravasation of red blood cells from postcapillary venules in thrombocytopenic patients.³⁶ However, extravasation of red blood cells does not occur only through the endothelium but also through the basement membrane, which implies degradation or rupture of the basal lamina surrounding vasculature. Our results show that acute exposure to the proinflammatory cytokine TNF- α abundantly expressed by tumors causes bleeding in thrombocytopenic mice in contrast to the proper permeability factor VEGF (Figure 1). This indicates that bleeding during thrombocytopenia is not likely induced by enhanced vascular permeability but that it requires additional factors such as leukocyte recruitment that can be elicited, for example, by TNF- α (Figure 1). In fact, we show that depletion of neutrophils abolished the ability of TNF- α to cause bleeding in thrombocytopenic mice (Figure 1). We consistently found that thrombocytopenia-induced tumor bleeding arose from regions where neutrophils and macrophages accumulated (Figures 2 and 3). Furthermore, tumors from CD18^{-/-}, $\beta 3$ ^{-/-}, or neutrophil-depleted mice had reduced neutrophil and/or macrophage content and were protected from the thrombocytopenia-induced bleeding (Figures 5 and 6). Altogether, our results indicate that, in thrombocytopenic mice, tumor vessel damage is induced by stroma-infiltrating neutrophils and macrophages.

Diapedesis of leukocytes has been shown to cause vessel damages.³⁰ On the other hand, platelets have been reported to promote leukocyte infiltration into chronically inflamed tissues.^{37,38} In our model, neutrophil and macrophage tumor contents were unaffected by the induction of acute thrombocytopenia (Figure 3). This indicates that prevention by platelets of tumor vessel damage does not result from any decrease of leukocyte recruitment into tumor stroma.

Neutrophils and macrophages contain a variety of products such as matrix metalloproteinases, serine proteases, and ROS-generating enzymes capable of vascular injury.³⁹ Platelets can modulate the activation of phagocytes and prevent the release of their harmful content.⁴⁰ Platelets have also been shown to inhibit the activity of leukocyte-derived proteases and ROS through the secretion of serpins, tissue inhibitor of metalloproteinases and ROS scavengers from their granules.^{10,40–45} Recently, it was shown that angiogenic factors and immunomodulatory cytokines can be differentially secreted by platelets.^{46–48} Tumor cells may stimulate platelets to specifically release factors affecting activities of tumor resident neutrophils and macrophages. Our results show that C3- and NADPH oxidase-deficient mice are not protected from thrombocytopenia-induced tumor bleeding. This suggests that vessel damages inflicted by tumor leukocytes are independent of ROS generation and complement activation and perhaps points to a role for leukocyte-derived proteases in this phenomenon. We noted that hemorrhage primarily occurs in leukocyte rich regions and near large necrotic areas filled with ghost-like neutrophils and macrophages (Figure 4). Platelets may inhibit the cytotoxic content released from live and

dead leukocytes and thus prevent tumor vessel injuries.

Accumulating evidence indicates that tumor-infiltrating leukocytes may favor tumor development by promoting angiogenesis and suppressing adaptive immunity.^{49–51} However, it has been also proposed that when appropriately stimulated macrophages and neutrophils would exert tumoricidal activities.^{29,49,51–53} It is possible that because of the negative impact of leukocytes on tumor vessels, mice deficient in adhesion receptors important for leukocyte recruitment grow larger primary tumors.¹³ Our study indicates that platelets are essential to limit the negative effects of the tumor-associated inflammation and that absence of platelets elicits vessel injuries by the tumor infiltrating innate immune cells. Inhibition of this platelet function may help in redirecting tumor infiltrating macrophages and neutrophils toward tumor destruction. In fact, we showed previously that thrombocytopenia-induced tumor hemorrhage results in reduced tumor cell proliferation and increased apoptosis in the hemorrhagic areas,⁵ and it has been shown that vascular destabilizing agents and TNF- α have synergistic antitumor effects.^{54,55} Thus, when the tumor is localized outside major organs, induction of intratumor bleeding may represent a potential antitumor strategy.

On the other hand, hemorrhage during chemotherapy-induced thrombocytopenia is a serious clinical problem, especially when it occurs in the central nervous system or in major organs.^{56,57} For this reason, prophylactic platelet transfusions are routinely administered to thrombocytopenic cancer patients.⁵⁸ Our results suggest that anti-inflammatory agents may prevent thrombocytopenia-induced hemorrhage in solid tumors, where leukocyte infiltration occurs. However, bleeding outside the tumor zone may not be affected. Clinical data indicating whether bleeding in thrombocytopenic cancer patients is intratumoral or not are lacking and could help determine whether anti-inflammatory agents could be beneficial.

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