Supplemental Data

Detailed Material and Methods

Blood cell count evolution

Blood was collected in EDTA from the retro-orbital venous plexus under 3% isoflurane anesthesia and analyzed using an automated counter (ABC Vet, Scil).

Preparation of platelet poor plasma and tumor extracts

Platelet-poor plasma (PPP) was prepared by centrifuging heparin-anticoagulated blood twice (1000 g for 5 min, 2000 g for 5 min with PGI2 (2 µg/mL) and apyrase (0,15 U/mL). Tumors were excised, weighed, homogenized in PBS supplemented with protease inhibitor cocktail (P8340, Sigma-Aldrich) at a volume-to-weight ratio of 5 µL/mg tumor, subjected to 3 freeze/thaw cycles and sonication, centrifuged at 5000 g for 5 minutes, and the resulting tumor extracts were collected and stored at -80°C.

Immunohistology of tumors

Tumors were harvested after intracardiac perfusion with zinc fixative [1], fixed in the same buffer for 48 hours, embedded in paraffin, and sectioned at 5 µm. Immunostainings were performed as described previously 19 using primary antibodies to CD45 (clone 30-F11; Biolegend), collagen IV (PA1-26148, ThermoFischer Scientific), PECAM-1 (clone MEC 13.3; BD Pharmingen), VE-Cadherin (AF1002, R&D System), ZO-1 (61-7300; Thermoscientific), F4/80 (clone BM8; Biolegend), CD68 (clone FA-11; Biolegend), CD163 (clone TNKUPJ; Invitrogen), CD8a (clone 53-6.7; Biolegend), PF4 (500-P05, Peprotech), anti-GPIX (M051-0, Emfret), VWF (A0082, DAKO), CD42b (R300, Emfret), and Ki67 (ab6326, Abcam). Apoptotic cells were detected by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL, Roche Applied Science). Tissue sections were counterstained with Hoechst 33342 (Life Technologies) and mounted in aqueous Fluorescent Mounting Medium (Ibidi). Images were acquired using a fluorescence microscope (Leica DMi8) and LasX software, and analyzed with Fiji.

Quantitative analysis of immunohistological stainings

Blood vessel density was quantified by counting PECAM-1-collagen IV-positive vessels across whole tumor sections from 7-12 different tumors per group. For quantification of leukocyte infiltration, CD45-positive cells were counted on 5 random fields (10x objective) per tumor.

The proliferative and apoptotic indexes were determined as the percentages of KI67- and TUNEL-positive nuclei relative to all DAPI-stained nuclei calculated from 10 random fields per tumor (acquired with a 40x objective for proliferation, 10X objective for apoptosis). Endothelial integrity was evaluated by scoring the continuity of PECAM-1, VE-cadherin, ZO-1 staining on a scale from 0 (highly discontinuous or absence of staining) to 3 (continuous staining). Scoring was performed by 3 independent observers blind to the treatment and mouse genotype on 3 images taken from random fields (63x objective) from 4-5 tumors per group.

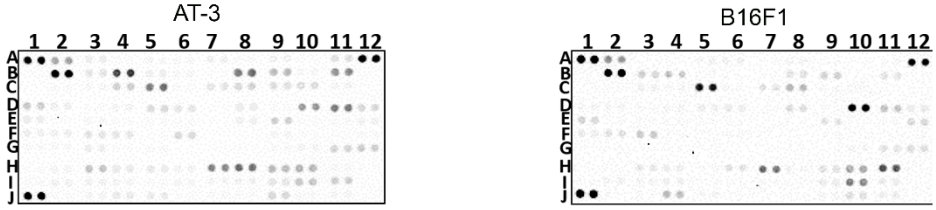
Supplemental References

1. Ho-Tin-Noé B, Carbo C, Demers M, Cifuni SM, Goerge T, Wagner DD. Innate Immune Cells Induce Hemorrhage in Tumors during Thrombocytopenia. Am J Pathol. 2009;175:1699–708.

Supplemental figures

Cytokine Proteome Profiler array

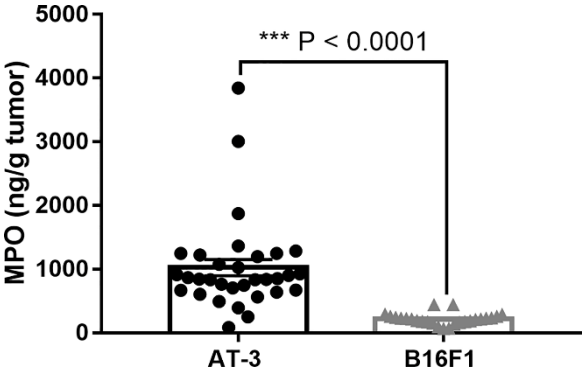
	1	2	3	4	5	6	7	8	9	10	11	12
A	Pos. ctrl	Adiponectin	Amphiregulin	Angiopoietin-1	**Angiopoietin-2**	**Angiopoietin-like 3**	BAFF	C1qR1	MCP-1	MIP-1α/β	**RANTES**	Pos. Ctrl
B		CCL6	Eotaxin	**MCP-5**	TARC	MIP-3β	MIP-3α	6Ckine	CCL22	**CD14**	**CD40**	
C		CD160	Chemerin	**Chitinase 3-like 1**	TF	C5a	Complement Factor D	**CRP**	**Fractalkine**	CXCL1	CXCL2	
D	**CXCL9**	**CXCL10**	CXCL11	CXCL13	CXCL16	**Cystatin C**	**DLK-1**	DPPIV	EGF	Endoglin	**Endostatin**	**Fetuin A**
E	**FGF acidic**	FGF-21	Flt-3 Ligand	Gas6	G-CSF	GDF-15	GM-CSF	HGF	**ICAM-1**	IFN-γ	IGFBP-1	IGFBP-2
F	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1β	**IL-1ra**	IL-2	IL-3	IL-4	IL-5	**IL-6**	IL-7
G	IL-10	IL-11	**IL-12 p40**	IL-13	IL-15	IL-17A	IL-22	IL-23	IL-27 p28	IL-28A/B	**IL-33**	LDLR
H	Leptin	LIF	**Lipocalin-2**	LIX	M-CSF	MMP-2	MMP-3	MMP-9	**Myeloperoxidase**	Osteopontin	**Osteoprotegerin**	PD-ECGF
I	PDGF-BB	Pentraxin 2	Pentraxin 3	Periostin	**Pref-1**	Proliferin	PCSK9	RAGE	RBP4	Reg3G	Resistin	
J	Pos. ctrl	E-Selectin	P-Selectin	Serpin E1	Serpin F1	**Thrombopoietin**	TIM-1	TNF-α	**VCAM-1**	VEGF	WISP-1	Neg. Ctrl



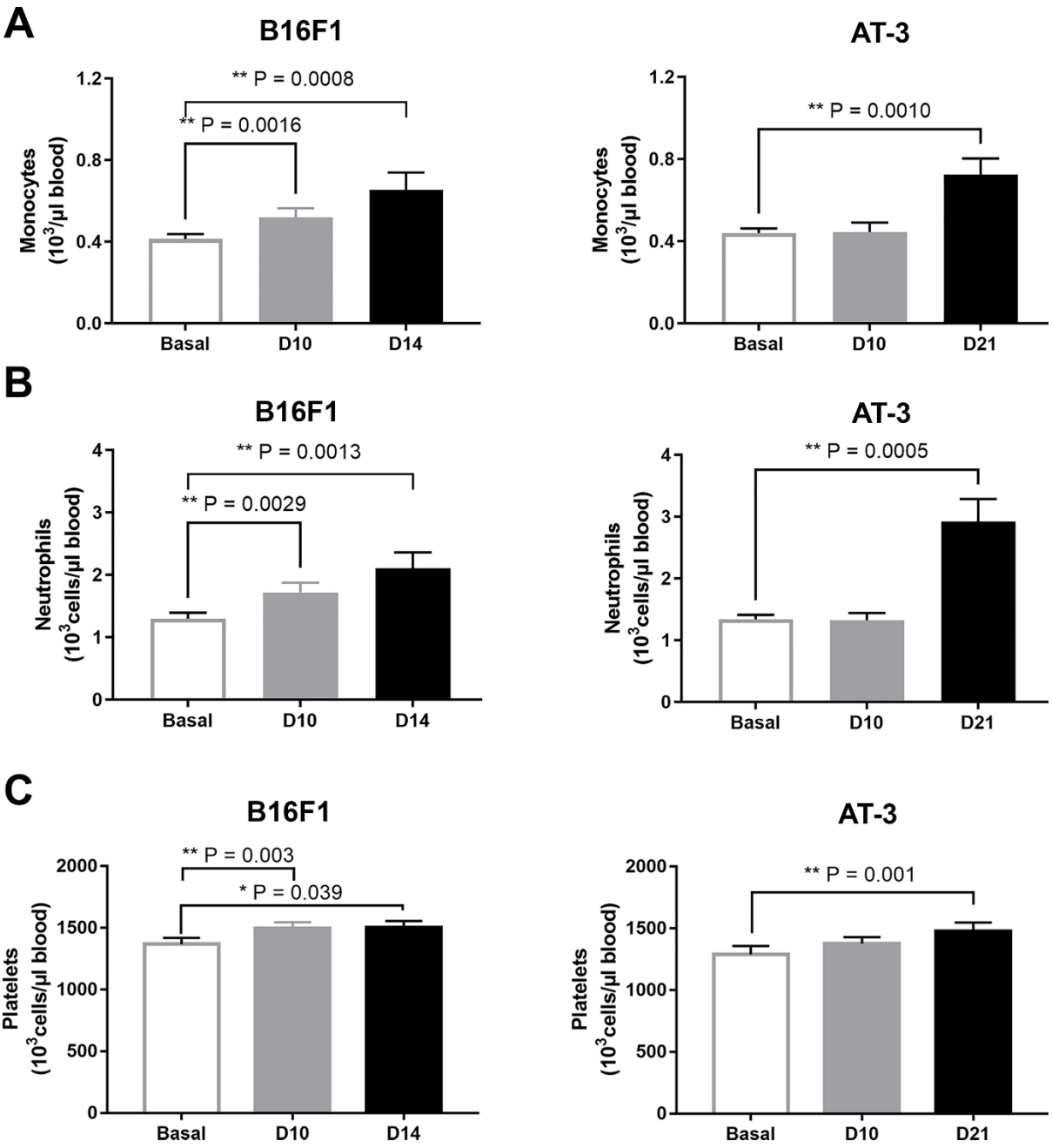
Angiogenesis Proteome Profiler array

	1	2	3	4	5	6	7	8	9	10	11
A	Pos. ctrl		**ADAMTS1**	**Amphiregulin**	Angiogenin	Angiopoietin-1	Angiopoietin-3	TF	**CXCL16**		Pos. ctrl
B		Cyr6	DLL4	DPPIV	**EGF**	**Endoglin**	Endostatin	Endothelin-1	FGF acidic	FGF basic	
C		KGF	CX3CL1	GM-CSF	HB-EGF	**HGF**	IGFBP-1	**IGFBP-2**	**IGFBP-3**	IL-1α	IL-1β
D		**IL-10**	CXCL-10	KC	Leptin	**MCP-1**	MIP-1α	MMP-3	MMP-8	MMP-9	NOV
E		**Osteopontin**	**PD-ECGF**	PDGF-AA	PDGF-AB	Pentraxin-3	PF4	PIGF-2	Prolactin	**Proliferin**	
F	Pos. ctrl	SDF-1	**Serpin E1**	Serpin F1	Thrombospondin-2	**TIMP-1**	TIMP-4	**VEGF**	VEGF-B	Neg. ctrl	

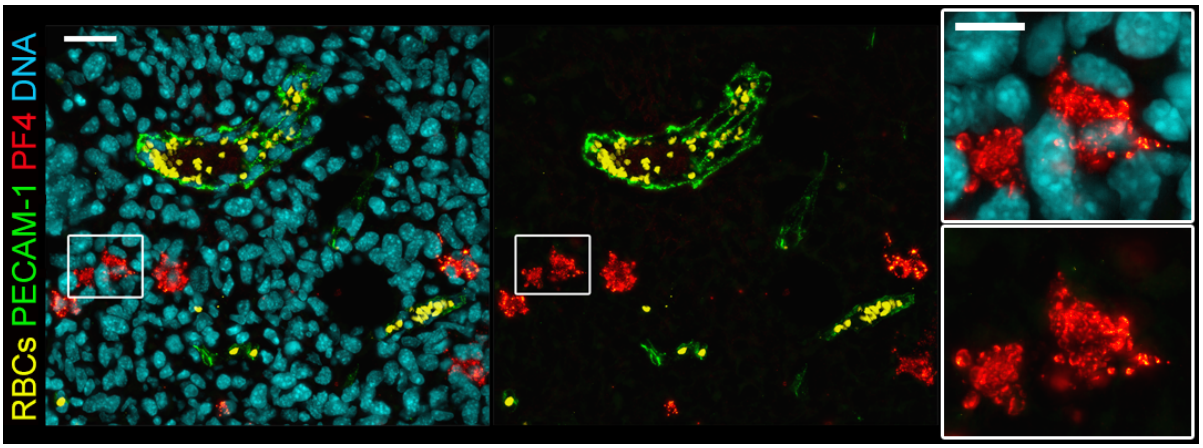
Supplemental Figure S1. Cytokine and angiogenic factor expression using Proteome Profiler arrays (R&S Systems). List and location of proteins analyzed on cytokine and angiogenic array membranes, and representative pictures of membranes probed for AT-3 and B16F1 tumor extracts. Proteins showing statistically significant differences are highlighted in bold text.



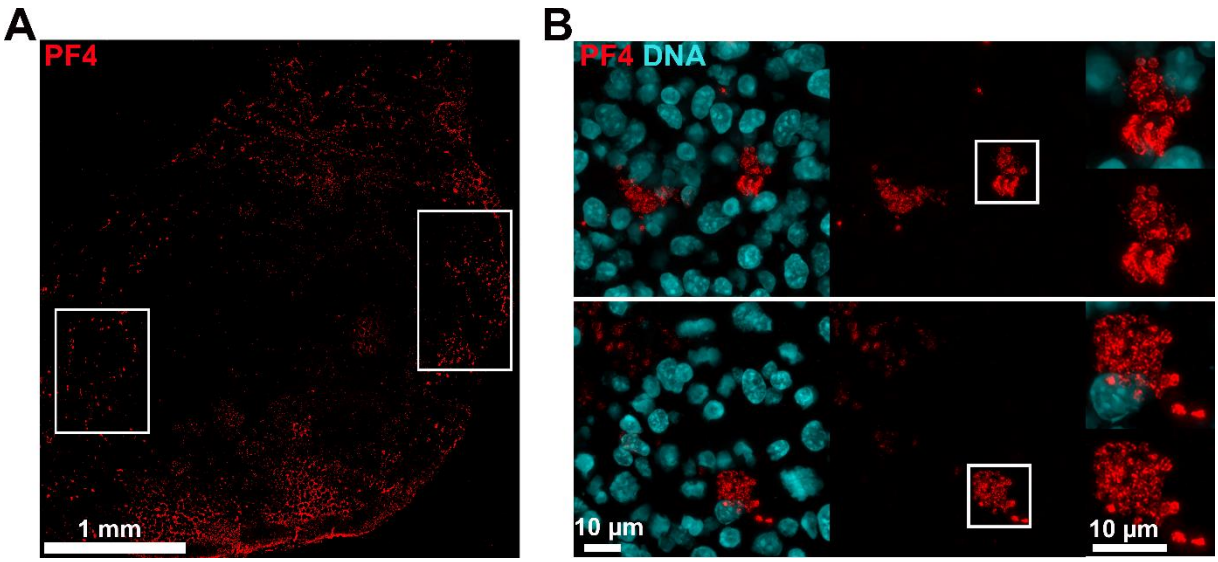
Supplemental Figure S2. Comparison of intratumoral myeloperoxidase content between AT-3 and B16F1 tumors. Myeloperoxydase (MPO) levels in AT-3 (n=32) and B16F1 tumor extracts (n=25), as evaluated by ELISA.



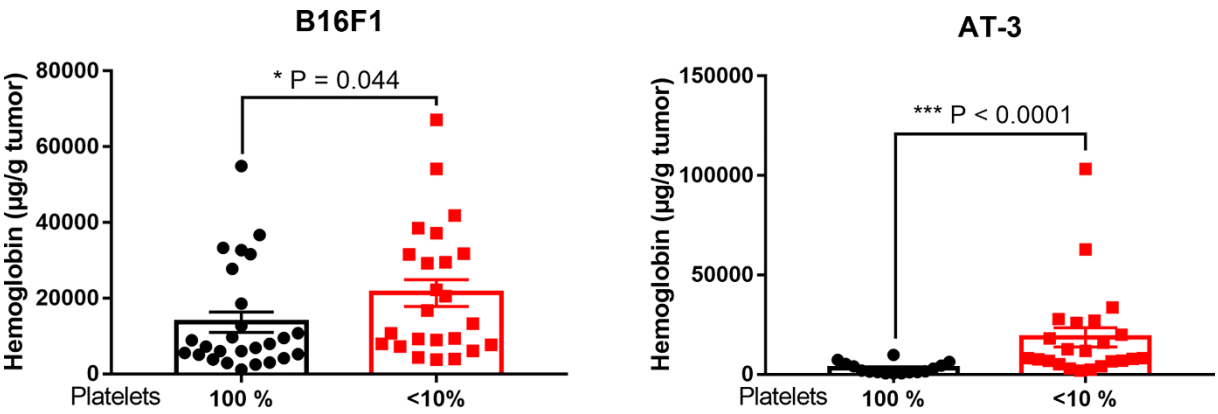
Supplemental Figure S3. Evolution of monocyte, neutrophil and platelet counts in B16F1 and AT-3 tumor-bearing mice. A-C. Evolution of blood monocytes (**A**), neutrophils (**B**) and platelets (**C**) following implantation of B16F1 and AT-3 cells. n=29 mice with B16F1 tumors; n =12 mice with AT-3 tumors.



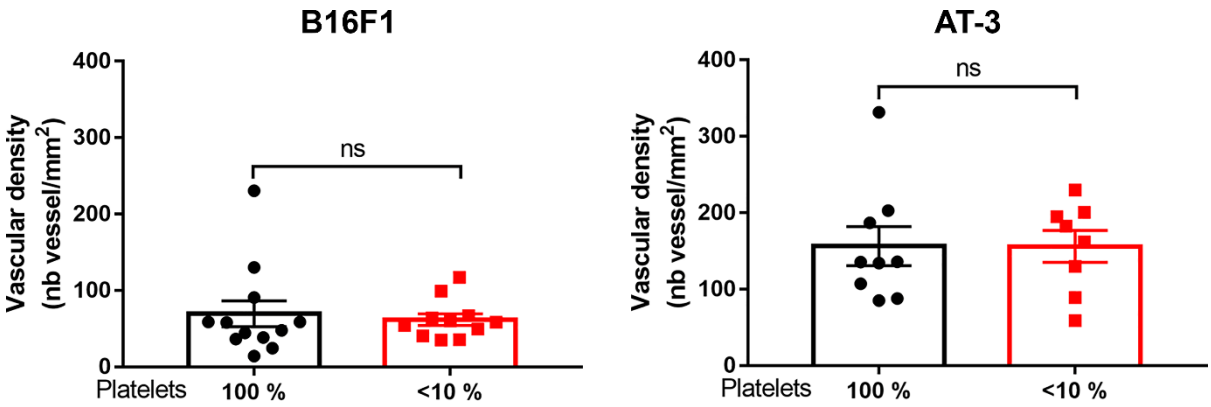
Supplemental Figure S4. Extravascular PF4-positive platelet clusters in the microenvironment of AT-3 mammary tumors. Representative images of PF4 and PECAM-1 staining in AT-3 tumors showing the presence of extravascular platelet clusters. White boxes show higher magnification of PF4-positive platelets.



Supplemental Figure S5. Extravascular platelet clusters in AT-3 tumors from severe and chronic thrombocytopenic mice. A. Representative image of immunostaining for platelet factor 4 (PF4) in AT-3 tumor section from mice with chronic severe thrombocytopenia. White boxes stress the abundance of platelet clusters at the periphery of AT-3 tumors. **B.** Immunostaining for platelets (PF4) showing the presence of extravascular platelet clusters in AT-3 tumors from mice with chronic severe thrombocytopenia. White boxes show higher magnification of PF4-positive platelets.



Supplemental Figure S6. Severe and chronic thrombocytopenia increases tumor bleeding in both B16F1 and AT-3 tumors. Comparison of hemoglobin content in B16F1 and AT-3 tumors from control and thrombocytopenic mice. n=26 (100%) and 24 (<10%) B16F1 tumors; n=16 (100%) and 23 (<10%) AT-3 tumors.



Supplemental Figure S7. Vessel density B16F1 and AT-3 tumors is not affected by severe and chronic thrombocytopenia. Quantification of vascular density in B16F1 and AT-3 tumors from control and thrombocytopenic mice. n=12 (100%) and 11 (<10%) B16F1 tumors; n=9 (100%) and 8 (<10%) AT-3 tumors.

Supplemental Movie legends 1-10

Supplemental Movie 1-2-3

Wild-type mice implanted with AT-3 tumors were injected intravenously with fluorochrome conjugated antibody to GPIX and FITC-dextran (2000 kDa) and were observed through a dorsal skinfold chamber at different magnifications.

Supplemental Movie 4

Wild-type mice implanted with AT-3 tumors were injected intravenously with fluorochrome conjugated antibody to GPIX, Gr-1 and PECAM-1. Platelet-tumor vessel interactions were observed in angiogenic sprouts.

Supplemental Movie 5-6

Wild-type mice implanted with B16F1 tumors were injected intravenously with fluorochrome conjugated antibody to GPIX and FITC-dextran (2000 kDa) and were observed through a dorsal skinfold chamber at different magnifications.

Supplemental Movie 7

Wild-type mice implanted with B16F1 tumors were injected intravenously with fluorochrome conjugated antibody to GPIX and Gr-1.

Supplemental Movie 8

Non-tumor bearing mice were injected intravenously with fluorochrome conjugated antibody to GPIX and FITC-dextran (2000 kDa) and were observed through a dorsal skinfold chamber.

Supplemental Movie 9

Wild-type mice implanted with AT-3 tumors were injected intravenously with fluorochrome conjugated antibody to GPIX and fibrin. Platelets associated with fibrin deposit were observed in the microcirculation.

Supplemental Movie 10

Wild-type mice implanted with B16F1 tumors were injected intravenously with fluorochrome conjugated antibody to GPIX and Gr-1. Platelets in interaction with migrating neutrophils were observed (white arrow). Occlusive microthrombi were observed in the tumor vasculature.