Tissue-Infiltrating Neutrophils Constitute the Major *In Vivo* Source of Angiogenesis-Inducing MMP-9 in the Tumor Microenvironment

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Supplementary Material

Supplementary Methods

Cytological and immunofluorescent analyses of isolated cells

Isolated neutrophils, TANs, macrophages and TAMs were placed on glass cover slips or slides pre-coated with poly-L-lysine, allowed to adhere, washed, and fixed in cold methanol for 10 min. The adherent cells were stained with the Protocol HEMA-3 kit (Fisher Diagnostics, Middletown, VA). After magnetic beads isolations, the freshly isolated cells were immunostained with avidin-FITC to detect biotin-conjugated mAbs on the surface (Sigma). Freshly isolated cells were also immunostained with fluorophore-conjugated mAbs against myeloid cell markers to assess their purity in the flow cytometer (Bekton Dickinson) or immunofluorescent microscope (Carl Zeiss AxioImager M1m). The isolated cells with high purity (90-95%) were used immediately in the *in vivo* angiogenesis assays and/or used for accumulation of their respective secretates.

Flow cytometry analysis of TAMs for cell surface markers

Freshly isolated TAMs and TAMs from *in vitro* cultures were resuspended in FACScan buffer (PBS/0.5% BSA/0.02% sodium azide) and pretreated with 10-20 μg/mL of Fc-Block, to prevent binding of primary antibodies to Fc receptor abundant on monocytic cells. After 10-min incubation on ice, fluorochrome-conjugated primary mAbs against Ly6G, F4/80, MMR (CD206), CD11b or control IgG (**Suppl. Table 4**), were added to the cells at 10 μg/mL without removal of Fc-Block for 45-60 min. If non-conjugated mAbs were used, the cells were washed of Fc-Block and primary mAbs and incubated with the corresponding species-specific fluorochrome-conjugates antibodies (**Suppl. Table 5).** Following 45-60 min incubation on ice, stained cells were washed and resuspended in FACScan buffer

supplemented with 0.3 μg/mL propidium iodide (Sigma) to exclude dead cells from analysis. At least 10,000 viable cells were analyzed per run. The levels of MFI were determined against IgG control in a FACScan flow cytometer (Becton Dickinson) equipped with CellQuest software (Becton Dickinson).

Tumor histology and immunohistochemistry

Overall histological examination of tumors was performed on deparaffinized 5-8 μm-thick tissue sections stained with H&E.

For immunohistochemical (IHC) examination, deparaffinized sections were first incubated for 30 min with 1% H_2O_2 to quench endogenous peroxidase and then endogenous biotin was blocked using a Biotin Blocking System (Dako, Glostrup, Denmark). Following blocking in 1% BSA, tumor sections were stained for mouse neutrophil Ly6G, mouse macrophage F4/80, or mouse endothelial CD31 with corresponding non-conjugated rat mAb (**Suppl. Table 4**), applied at 2-5 μg/mL PBS/1% BSA for overnight incubation at 4^{o}C. After washes in PBS/0.05% Tween 20, secondary HRP-conjugated antibodies (**Suppl. Table 5**) were applied in PBS/1% BSA for 2 hr at RT. Following washes in PBS/0.05% Tween 20, HRP-containing complexes were detected with a DAB substrate kit (Vector Laboratories, Burlingame, CA).

Histological and immunohistochemical examinations were performed by using an Olympus CKX-41 microscope equipped with Olympus U-LS30-3 video camera (Olympus America, Center Valley, PA) and Infinity capture software (Lumenera, Ottawa, Canada) or an Olympus BX60 microscope (Olympus America, Melville, NY) equipped with a digital DVC video camera and high performance ImageJ plugin acquisition software (DVC Company, Austin, TX). Images were processed using Adobe Photoshop software.

Immunofluorescent examination of tumors was performed on frozen sections kept at -80^{o}C till staining procedures. Tissue sections were air-dried and fixed for 10 min in cold methanol (kept at -20^{o}C). After dehydration in PBS and blocking in PBS/2% BSA, primary fluorochrome-conjugated antibodies against murine MMP-9, F4/80, Ly6G, CD31, NG2 or desmin **(Suppl. Table 4**), were applied individually or in combination. After overnight incubation, non-bound antibodies were washed and cell nuclei were stained with Hoechst 33342 (NucBlue, Molecular Probes, Eugene, OR) for 5-10 min. After brief wash in PBS, stained sections were embedded in ProLong Gold antifade reagent (Life Technologies, Eugene, OR). Immunofluorescence analyses were performed in either a Carl Zeiss

AxioImager M1m microscope equipped with Axiovision Re.4.6 software (Carl Zeiss Microscopy, Thornwood, NY) or an Olympus IX51 microscope (Olympus, Center Valley, PA) equipped with a Macrofire mono camera and Picture Frame capture software (Optronics; Goleta, CA).

For quantification purposes, from 45 to 55 images from 4 to 6 individual tumors from at least 2 independent experiments were acquired to meet criteria for statistical significance.

Gelatin zymography

Zymographic analyses were preformed on TAN releasates, SF CM from TAMs or cell lysates. For quantification purposes, the material was analyzed as produced by the known number of cells (namely, cells per lane) and compared within in the same gel to the known amount of recombinant murine proMMP-9 (ng per lane). The loaded proteins were separated by SDS-PAGE on 10% gelatin-impregnated Novex gels (Invitrogen) under non-reducing conditions. After electrophoresis, the gels were washed 2x in 2.5% Triton X-100 for 30 min and then incubated overnight at 37^0C in 50 mM Tris buffer, pH 7.5, supplemented with 100 mM NaCl, 5 mM $CaCl_2$, and 0.2% Brij 35. The gels were stained with 0.25% Coomassie Blue G-250 in 10% acetic acid and destained in distilled water to visualize bands of gelatinolytic activity.

Western blotting

Western blot analyses were performed under reducing conditions (100 mM DTT, 10 min boiling) by separating proteins released or produced by the known number of cells. For quantification purposes, the mixture of recombinant murine proMMP-9 (#72069; Anaspec, Fremont, CA) and recombinant murine TIMP-1 (gift from Dr. Christopher M. Overall) were loaded at different amounts into the lanes of the same 4-20% SDS-PAGE gels. After separation, the proteins were transferred to the PVDF membranes and blocked with 5% milk in PBS/0.05% Tween 20. Using stained molecular wt. markers as reference, the membranes were cut at the middle section (around 50-kDa), to separate the upper portion that would contain 105-kDa proMMP-9 monomers and lower portion that would contain 28-kDa TIMP-1. These portions were incubated with 1 μg/mL of anti-mouse MMP-9 or anti-murine TIMP-1 antibodies, respectively (**Suppl. Table 4**). The expression of 135-kDa iNOS and 38-kDa α-arginase-1 was analyzed in a similar manner with the corresponding primary antibodies applied respectively to the upper and lower portions of the same blot. After incubating with

the corresponding species-specific secondary HRP-conjugated antibodies, the membranes were subjected to chemiluminiscence analysis with Thermo Pico 34080 Kit according to the manufacturer's instructions. Where indicated, equal loading for cell lysates was verified by re-staining of the stripped membrane with the antibody against murine β-actin.

Gene expression analysis by quantitative real time RT-PCR analysis

Relative expression levels of genes for murine arginase-1, *Arg1*, and iNOS, *NOS2*, were determined by a quantitative RT-PCR (qRT-PCR). Total RNA was extracted from the cells with TRIzol (Invitrogen) and 2 μg of isolated RNA was reverse-transcribed using the RNA to cDNA EcoDry Premix (#639549; Clontech). The resulting cDNA was analyzed by qRT-PCR in an iCycler iQ (Bio-Rad). Each reaction contained 60 ng of cDNA as template, LightCycler 480 SYBR Green Master Mix (#04707516001; Roche), and each of forward and reverse primers used at 0.4 μM. PCR conditions included heating for 5 min at 95^{o}C, followed by 40 cycles of 30 s at 95^{o}C, 30 s at 60^{o}C, and 60 s at 72^{o}C. The sequences of forward and reverse primers are presented in **Suppl. Table 6**. A melt curve analysis was performed to ensure specific amplification. For each target gene, relative levels of expression were normalized against housekeeping gene signal (murine β-actin), generating ΔCt value (ΔCt=Ct target gene – Ct reference gene). Differences in gene expression levels between cell types were calculated according to the formula $2^{-\Delta Ct}$ as described [1]. The relative gene expression was analyzed in comparison to control (parental tumor cells) according to the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt=ΔCt experimental setting - ΔCt control setting [2].

References

1. Muller PY, Janovjak H, Miserez AR, Dobbie Z: Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques* 32, 1372-1374, 1376, 1378-1379 (2002).

2. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408 (2001).

Supplementary Tables

Supplementary Table 1. ***Isolation of F4/80-positive TAMs from L929 and B16 tumors***

Tumor Type	**Host *Mmp9* Genotype**	**Total cells per mg of dissociated tumor, x 10^{-3}**	**Purity of isolated TAMs, % F4/80^{+} cells**	**F4/80^{+} TAMs per 1x10^{8} dissociated tumor cells, x 10^{-6}**
L929	**WT**	**197.0 ± 28.1** (n=3)[a]	**93.8 ± 2.0** (n=7)	**29.03 ± 5.40** (n=3)
B16	**WT**	**33.8 ± 8.2** (n=5)	**90.2 ± 1.0** (n=5)	**3.54 ± 2.00** (n=4)
	KO	**22.7 ± 0.5** (n=2)	**93.7 ± 1.2** (n=2)	**1.87 ± 0.29** (n=2)

[a] **n**, number of independent experiments

Supplementary Table 2. ***Confirmatory analysis of isolated TAMs after in vitro cultivation: mean fluorescence intensity and percentage of positive cells***

Tumor type	**Days in culture**	**Mean Fluorescence Intensity (% of gated cells)**		
		F4/80	**CD206 (MMR)**	**CD11b**
L929	**2 to 3**	**195-350 (92.6 ± 0.2%)** (n=3)[a]	**20 (90.0%)** (n=1)	**40-360 (89.2 ± 2.8%)** (n=2)
LLC	**2 to 5**	**60-139 (97.5 ± 1.2%)** (n=3)	**n.d**[b]	**72-84 (97.0%)** (n=2)

[a] **n,** number of independent experiments

[b] **n.d**., not determined

Supplementary Table 3. ***Isolation of TAMs and TANs from LLC tumors grown in WT and Mmp9-KO mice***

Host *Mmp9* Genotype	**Total cells per mg of dissociated tumor, x 10^{-3}**	**Tumor-Associated Leukocyte Type**	**Purity of isolated leukocytes, %**	**Isolated leukocytes per 1x10^{8} dissociated tumor cells, x 10^{-6}**
WT	**225.9 ± 30.3** (n=7)[a]	**TAMs** ($F4/80^{+}$)	**95.1 ± 1.6** (n=6)	**11.65 ± 0.97** (n=6)
		TANs ($Ly6G/1A8^{+}$)	**94.5 ± 2.3** (n=4)	**2.78 ± 0.59** (n=8)
***Mmp9*-KO**	**189.1 ± 20.8** (n=2)	**TAMs** ($F4/80^{+}$)	**92.6 ± 4.6** (n=3)	**11.92** (n=3)
		TANs ($Ly6G/1A8^{+}$)	**91.5 ± 3.8** (n=2)	**2.12 ± 0.53** (n=2)

[a] **n,** number of independent experiments

Supplementary Table 4. ***Primary polyclonal antibodies (Abs) and monoclonal Abs (mAbs) used in this study***

Host species	Antibody	Murine antigen	Format	Source
Rat	mAb 1A8	Neutrophil-specific antigen **Ly6G**	Non-conjugated	BD Pharmingen, San Diego, CA
			FITC-conjugated	Biolegend, San Diego, CA
			Biotin-conjugated	
Rat	mAb BM8	Macrophage-specific antigen **F4/80**	Non-conjugated	Invitrogen, Frederick, MD
			AlexaFluor 488-conjugated	Biolegend, San Diego, CA
			Biotin-conjugated	Biolegend, San Diego, CA
Rat	mAb M1/70	Myeloid cell marker **CD11b** (**Mac-1**)	Non-conjugated	eBioscience, San Diego, CA
			FITC-conjugated	BD Pharmingen San Diego, CA
Rat	mAb C068C2	**CD206** (**MMR**, Macrophage mannose receptor)	AlexaFluor 488-conjugated	Biolegend, San Diego, CA
Rat	mAb MEC 13.3	Endothelial marker **CD31**	Non-conjugated	BD Pharmingen, San Diego, CA
Rat	mAb 2.4G2	**CD16/CD32** (Fc receptor)	Non-conjugated	BD Pharmingen, San Diego, CA
	mAb 93			Biolegend, San Diego, CA
Rat	mAb AB5320	**NG2** (pericyte marker)	Non-conjugated	Millipore, Billerica, MA
Rat	Whole IgG	**Control IgG**	Non-conjugated	Jackson ImmunoResearch, West Grove, PA
Rabbit	mAb D8281	**Desmin** (pericyte marker)	Non-conjugated	Sigma, St. Louis, MO
Goat	Ab AF980	**TIMP-1**	Non-conjugated	R&D Systems, Minneapolis, MN
Rabbit	Ab38898	**MMP-9**	Non-conjugated	Abcam, Eugene, OR
Rabbit	Ab AV45673	**Arginase-1**	Non-conjugated	Sigma, St. Louis, MO
Rabbit	Ab Poly6221	**β-actin**	Non-conjugated	Biolegend, San Diego, CA
Rabbit	Whole IgG	**Control IgG**	Non-conjugated	Jackson ImmunoResearch, West Grove, PA
Goat	Ab sc-18351	**iNOS**	Non-conjugated	Santa Cruz, Santa Cruz, CA
Goat	Whole IgG	**Control IgG**	Non-conjugated	Jackson ImmunoResearch, West Grove, PA

Supplementary Table 5. ***Secondary species-specific antibodies (Abs) used in this study***

Host species	Antibody	Target IgG	Conjugate	Source
Donkey	Poly4064	**Rabbit** IgG	DayLight 488	Biolegend, San Diego, CA
Goat	Poly4054	**Rat** IgG	DayLight 488	Biolegend, San Diego, CA
Goat	115-035-146	**Mouse** IgG	HRP	Jackson ImmunoResearch Laboratories, West Grove, PA
Goat	111-035-144	**Rabbit** IgG	HRP	
Donkey	HAF109	**Goat** IgG	HRP	R&D Systems, Minneapolis, MN

Supplementary Table 6. ***Primer sequences for murine genes***

Gene	Encoded Protein	Forward Primer	Reverse Primer
ActB	Beta-actin (β-actin)	CTC TGG CTC CTA GCA CCA TGA AGA	GTA AAA CGC AGC TCA GTA ACA GTC CG
Arg1	α-Arginase-1 (arginase-1)	CAG AAG AAT GGA AGA GTC AG	CAG ATA TGC AGG GAG TCA CC
Nos2	Nitric oxide synthase, inducible (iNOS)	CCC TTC AAT GGT TGG TAC ATG G	ACA TTG ATC TCC GTG ACA GCC