Endotoxemia shifts neutrophils with TIMP-free gelatinase B/MMP-9 from bone marrow to the periphery and induces systematic upregulation of TIMP-1

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Supplementary table 1: The effect of endotoxin shock on MMPs and TIMPs.

MMP/ Other	Model	Method/ trigger	Result	Detection method	Ref.
BLOOD					
MMP-2	Mice	CLP	protein levels =	WB	(1)
	Rats	LPS	protein levels ↓ (6-12h post LPS)	WB, zymography	(2)
	Pigs	live *E. Coli* LE392	protein levels =	zymography	(3)
	Human	patient samples	protein levels =	ELISA, zymography	(4, 5)
MMP-8	Human	patient samples	protein levels ↑	ELISA	(6)
MMP-9	Human cell culture (neutrophils)	LPS *E. Coli B6:026*/ LPS *E. Coli* O111:B4 / PepG *St aureus*	protein levels ↑	ELISA, WB, zymography	(7, 8)
	Mice	CLP	protein levels ↑	ELISA, WB	(1, 9)
	Rats	LPS *S. Typhosa* 0901	protein levels ↑ (from 1h post LPS, in ventricular tissue)	WB, zymography	(2)
	Rats	LPS *E.Coli* O55:B5 / CLP / LPS and/or PepG *S. aureus*	protein levels ↑	ELISA, zymography	(10, 11)

	Rats	CLP	gelatinolytic activity ↑	gelatin degradation assay with serine protease inhibitors	(10)
	Pig	live *E. Coli* LE392	protein levels ↑	zymography	(3)
	Baboons	LPS *E. Coli*	protein levels ↑	zymography	(12)
	Human	patient samples / LPS *E. Coli* / LPS *E. Coli* O113 / PepG S. aureus	protein levels ↑	ELISA, WB, zymography	(1, 4-8, 13)
TIMP-1	Human	patient samples / 28-day follow up patients	protein levels ↑	ELISA	(5, 6, 13)
TIMP-2	Human	patient samples	protein levels ↑	ELISA	(5)
	HEART				
MMP-2	Human cardiac fibroblasts	LPS	mRNA =	qRT-PCR	(14)
	Mice	CLP	mRNA ↑	qRT-PCR	(14)
	Rats	LPS *S. Typhosa* 0901	protein levels ↓ (in heart perfusate)	zymography	(15)
			protein levels ↓ (6-12h post LPS)	WB, zymography	(2)
MMP-9	Human cardiac fibroblasts	LPS	mRNA ↑, protein levels ↑	qRT-PCR, WB, zymography	(14)
	Mice	CLP	mRNA ↑	qRT-PCR	(14)

	Rats	LPS *S. Typhosa* 0901	protein levels ↑ (in heart perfusate)	zymography	(15)
BRAIN					
MMP-2	Cell culture (rat microglia)	LPS *Vibrio vulnificus* MO6-24/O / LPS *E. Coli* O26:B6	protein levels =	zymography	(16)
MMP-9	Cell culture (rat microglia)	LPS *Vibrio vulnificus* MO6-24/O / LPS *E. Coli* O26:B6	protein levels ↑	zymography	(16)
	Mice	LPS *E. Coli* 026:B6	mRNA =, protein levels ↑	RPA, zymography	(17)
TIMP-1	Mice	LPS *E. Coli* 026:B6	mRNA ↑	RPA *in situ* hybridization	(17)
LUNG					
MMP-2	Rats	CLP / LPS and/or PepG *S. aureus*	protein levels ↑	IHC	(18)
	Guinea Pigs	LPS	Protein levels ↑	zymography	(19)
	Pigs	LPS *E. Coli* 111:B4	protein levels ↑	zymography	(20)
MMP-9	MMP-9 $^{-}/^{-}$ mice	CLP	lung injury score ↓*	WB	(1)
	Mice	LPS *E. Coli* 0127:B8	activity of MMP-9 promotor =	Transgenic mouse model *luciferase*	(21)

				gene under *mmp9* promotor control	
	Rats	LPS *E. Coli* O55:B5 / CLP /LPS	protein levels ↑	IHC	(11, 18)
	Guinea Pigs	LPS	Protein levels ↑	zymography	(19)
	Pigs	LPS *E. Coli* / LPS *E. Coli* 111:B4	protein levels ↑	zymography	(20)
LIVER					
MMP-2	Mice	LPS *E. Coli* 026:B6	protein levels =	zymography	(17)
	Rats	CLP	protein levels ↑	WB	(10)
MMP-9	Mice	LPS *E. Coli* 0127:B8 / LPS *E. Coli* 026:B6	MMP-9 promotor activation ↑; mRNA ↑; protein levels ↑	RPA, zymography, Transgenic mouse model *luciferase* gene under *mmp9* promotor control	(17, 21)
	Rats	LPS *E. Coli* O55:B5 / CLP / LPS	protein levels ↑	IHC, WB, zymography	(10, 11)
MMP-13	Mice	LPS E. Coli O26.B6	mRNA ↑	RPA	(17)
MMP-14	Mice	LPS E. Coli O26:B6	mRNA ↑	RPA	(17)
TIMP-1	Mice	LPS *E. Coli* 026:B6	mRNA ↑	RPA	(17)
TIMP-3	Mice	LPS *E. Coli* 026:B6	mRNA ↑	RPA	(17)
KIDNEY					

MMP-2	Mice	LPS E. Coli O26:B6	protein levels =	zymography	(17)
MMP-3	Mice	LPS E. Coli O26:B6	mRNA ↑	RPA	(17)
MMP-9	Mice	LPS *E. Coli* 0127:B8 / LPS *E. Coli* 026:B6	MMP-9 promotor activation =; mRNA ↑; protein levels ↑	RPA, zymography	(17, 21)
	Rats	LPS *E. Coli* O55:B5	protein levels ↑	IHC	(11)
MMP-10	Mice	LPS *E. Coli* O55:B5	mRNA ↑	RPA	(17)
MMP-13	Mice	LPS E. Coli O26.B6	mRNA ↑	RPA, *in situ* hybridization	(17)
MMP-14	Mice	LPS E. Coli O26:B6	mRNA ↑	RPA	(17)
TIMP-1	Mice	LPS *E. Coli* 026:B6	mRNA ↑	RPA	(17)
SPLEEN					
MMP-2	Mice	LPS *E. Coli* 026:B6	Protein levels =	zymography	(17)
MMP-9	Mice	LPS *E. Coli* 0127:B8 / LPS *E. Coli* 026:B6	MMP-9 promotor activity =; mRNA ↑; Protein levels =	RPA, *in situ* hybridization, zymography, Transgenic mouse model *luciferase* gene under *mmp9* promotor control	(17, 21)

MMP-13	Mice	LPS E. Coli O26.B6	mRNA ↑	RPA, *in situ* hybridization	(17)
MMP-14	Mice	LPS E. Coli O26:B6	mRNA ↑	RPA	(17)
TIMP-1	Mice	LPS *E. Coli* 026:B6	mRNA ↑	RPA, *in situ* hybridization	(17)

CLP; cecal ligation/perforation, **IHC**; immunohistochemistry, **LPS**; Lipopolysaccharides, **PepG**; peptidoglycan, **RPA**; RNase protection assay, **WB**; Western blot, ↑; increased, =; unchanged, ↓; decreased.

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Supplementary Methods

Protein extraction and quantification

Tissues were homogenized and proteins extracted with a precellys lysing kit (Bertin Technologies). Tissue samples were placed in hard tissue homogenizing CK28 tubes (Bertin Technologies). 1 ml of assay buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0,01% tween-20 and pH 7.4) was added to all tubes and homogenization was done using the Precellys®24 (2x 5s at 6000g, Bertin Technologies). To precipitate all tissue debris, the tubes were centrifuged at 20800 g and 4°C for 15 minutes. The supernatant, containing soluble proteins, was collected. Prior to zymography analysis, the total protein content of all samples was determined by using a standard Bradford assay (Bio-Rad).

RNA extraction, preparation of cDNA and qPCR

RNA was extracted from tissue with the Qiagen RNeasy mini kit (Qiagen). The quantity and quality of the extracted RNA was examined with a NanoDrop ND-1000 spectrophotometer (Isogen). Next, equal amounts of RNA were converted to cDNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems) and a GeneAmp PCR system 9700 (Applied Biosystems). Finally, qPCR was performed using TaqMan® fast universal PCR master mix (Applied Biosystems), PrimeTime® predesigned qPCR assays (IDT) and a 7500 Fast Real-Time PCR System (Applied Biosystems). qPCR was performed using the following PrimeTime® predesigned qPCR assays (IDT): *Mmp2* (Mm.PT.58.7728513), *Mmp9* (Mm.PT.58.45834688.g), *Tbp* (Mm.PT.58.17504874), *Timp1* (Mm.PT.58.43416496), *Timp2* (Mm.PT.58.8927491), *Timp3* (Mm.PT.58.5548479); *Timp4* (Mm.PT.58.8559650), *Mpo* (Mm.PT.58.5251395) and *Elane* (Mm.PT.58.6682392.gs).

Gelatin zymography

Prior to gelatin zymography, the protein extracts were first spiked with a low molecular weight (±50 kDa) form of MMP-9 (MMP-9ΔHemOG) as a loading control and subsequently pre-purified using a previously described gelatin affinity chromatography method (1). MMP-9ΔHemOG lacks both the C-terminal hemopexin domain (Hem) and O-glycosylated linker (OG) and was recombinantly produced in Sf9 cells as previously described (2). Briefly, gelatin-Sepharose beads (GE Healthcare) were washed three times with equilibration buffer (50 mM Tris, 0.5 M NaCl, 10 mM $CaCl_2$, 0.01% tween 20 and pH 7.5) and diluted 1/2 in equilibration buffer. Next, 20 µl of this solution was added to each well of a 96-well high-throughput

chromatography plate. Samples and spiking (480 pg MMP-9 ΔHemOG, an MMP-9 deletion mutant, to calculate the recovery of the prepurified samples) were added to the columns and this suspension was incubated at room temperature for 30 minutes. After incubation the columns were rinsed three times with washing buffer 1 (50 mM Tris, 0.5 M NaCl, 10 mM $CaCl_2$, 0.05% tween 20 and pH 7.5) and twice with washing buffer 2 (50 mM Tris, 10 mM NaCl, 0.05% Tween 20 and pH 7.5). Finally, gelatinases were eluted in 20 µl non-reducing loading buffer and stored at -20°C. Gelatin zymography was performed as previously described (1,3). Briefly, zymography gels were prepared consisting of an 8% acrylamide separating gel with incorporation of 1 mg/ml gelatin (Sigma Aldrich G1890) and a 5% stacking gel. Next, the gels were placed in an electrophoresis system (running buffer: 25 mM Tris, 192 mM glycine and 0.1% SDS) and the samples, diluted in non-reducing loading dye, were added to the wells and electrophoretically separated. The gels were removed from the gel system and washed twice for 20 minutes in re-activation buffer containing 2.5% Triton-X-100. During this step, SDS is washed away which allows the proteins to partially refold. Next, the refolded gelatinases will degrade the gelatin in the gel during an overnight incubation (± 16h) in 50 mM Tris-HCl, 10 mM $CaCl_2$ and pH 7.5 at 37°C. Finally, the gels were stained with 0.1% Coomassie Brilliant Blue R-350 (GE Healthcare). Zymograms were analysed densitometrically using the ImageQuant TL software (GE Healthcare).

Two-dimensional zymography

Bone marrow and lung lysates (containing 8 ng of proMMP-9 monomer, based on semi-quantitative zymography) were incubated with gelatin-Sepharose (GE Healthcare Life Sciences) for 30 minutes, and by shaking at room temperature. The gelatin-bound fraction was washed with the use of 50 mM Tris pH 7.4, 0.05% Tween-20 and eluted in 8 M ureum and 2% N-cyclohexyl-3-aminopropanesulfonic acid (CAPS). Immobilized 3-10 non-linear pH gradient strips (ThermoFisher) were incubated with the obtained gelatin-Sepharose-purified fraction, after the addition of broad range ampholytes (Bio Rad) for 1 hour at room temperature to rehydrate in a ZOOM IPGRunner Cassette (ThermoFisher). Afterwards, isoelectric focusing was performed in the ZOOM IPGRunner system (ThermoFisher) for 20 min at 200 V, 15 min at 450 V, 15 min at 750 V and 75 min at 2000 V. The strips were loaded onto 10 % SDS-PAGE gels co-polymerised with 1 mg/ml gelatin. Gelatin zymography was performed as described previously (1,3).

Gelatin degradation assay

Bone marrow extracts were treated with 10 µM elastase inhibitor (Elastase Inhibitor IV, Calbiochem) to inhibit gelatinolytic activity derived from elastase or with 10 µM SB-3CT (sc-205847, Santa Cruz Biotechnology) to inhibit MMP-2 and MMP-9 gelatinolytic activity. Subsequently these mixtures were incubated with 2.5 µg/ml fluorogenic gelatin (DQ™ Gelatin from pig skin, ThermoFisher Scientific) and fluorescence was measured at (ex. 485 nm/em. 530 nm) as previously described (4).

Immunohistochemistry

First the slides were deparaffinised in the Leica autostainer XL (Leica Biosystems). Next, the slides were subjected to heat retrieval for 10 min at 97°C and pH 6 in the PT Link (DAKO), followed by 10 min incubation with peroxidase-blocking reagent (DAKO) and were washed twice for 5 minutes in wash buffer (DAKO). Next the primary antibody, goat anti-mouse MMP-9 (R&D Systems), was diluted 100 times in antibody diluent (DAKO) to have a final concentration of 1 µg/ml and 200 µl was added to each slide and incubated for 30 minutes. After incubation, the slides were washed twice for 5 minutes in wash buffer (DAKO). Thereafter the secondary antibody, polyclonal rabbit anti-goat (DAKO), was diluted 20 times in antibody diluent and 10% N-hydroxysulfosuccinimide was added. 200 µl of the secondary antibody was added to the slides, incubated for 30 minutes, followed by a double wash step for 5 min with wash buffer. Finally, 3,3' diaminobenzidine was added to the slides and incubated for 6 minutes. Counterstaining and mounting of the slides was performed in the Leica autostainer XL and Leica CV5030 respectively. Finally, the tissue was imaged using a Leica Dmlb microscope.

Flow cytometry

Red blood cells of the spleen, bone marrow and blood cell suspensions were lysed by two incubations (5 and 3 min at 37°C) with 0.83% NH_4Cl solution. Remaining cells were washed twice with ice-cold PBS containing 2% FCS. Next, $0.5x10^6$ cells were incubated for 15 min with Fc-receptor-blocking antibodies anti-CD16/anti-CD32 (BD Biosciences Pharmingen, San Diego, CA, USA) and Zombie aqua BV510 (death cells staining) (BioLegend, San Diego, CA, USA). After washing with PBS supplemented with 2% FCS, cells were stained for 30 min with the indicated conjugated antibodies. FITC-conjugated anti-Gr1, PE-conjugated anti-F4/80, APC-conjugated anti-CD11b, PE-Cy7-conjugated anti-CD3 and PerCp Cy5.5-conjugated anti-CD19 were purchased from eBioscience (San Diego, CA, USA). Cells were washed twice and fixed with

0.37% formaldehyde in PBS and analysed with a FACS Fortessa flow cytometer. Data were processed with the FlowJo software (Becton Dickinson Labware, Franklin Lakes, NJ, USA).

References to 'supplementary Methods'

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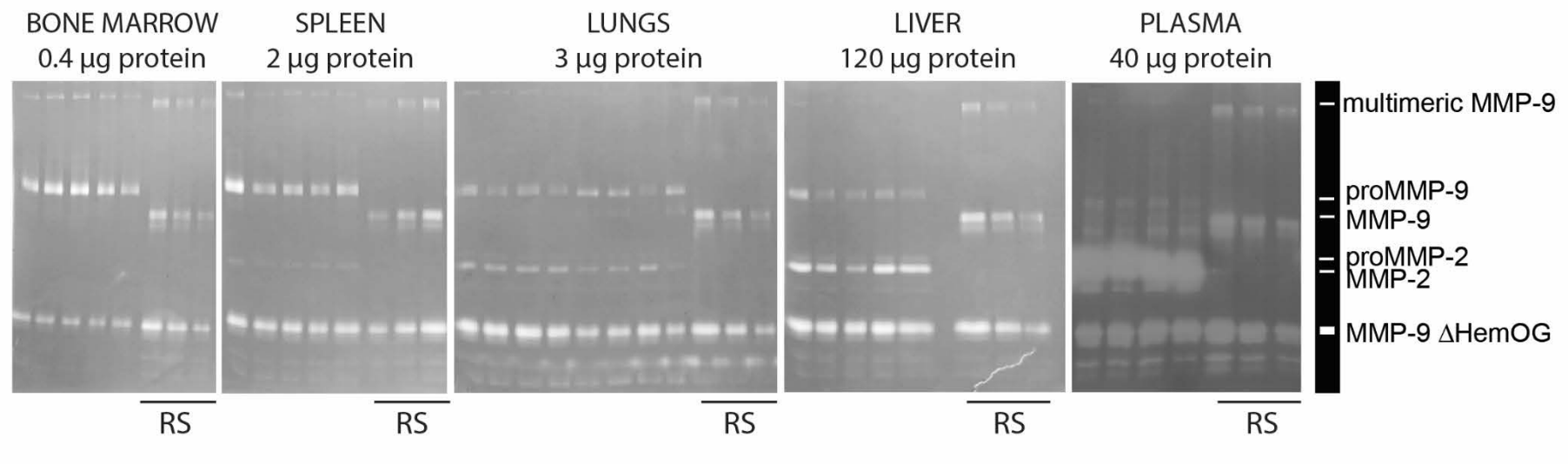
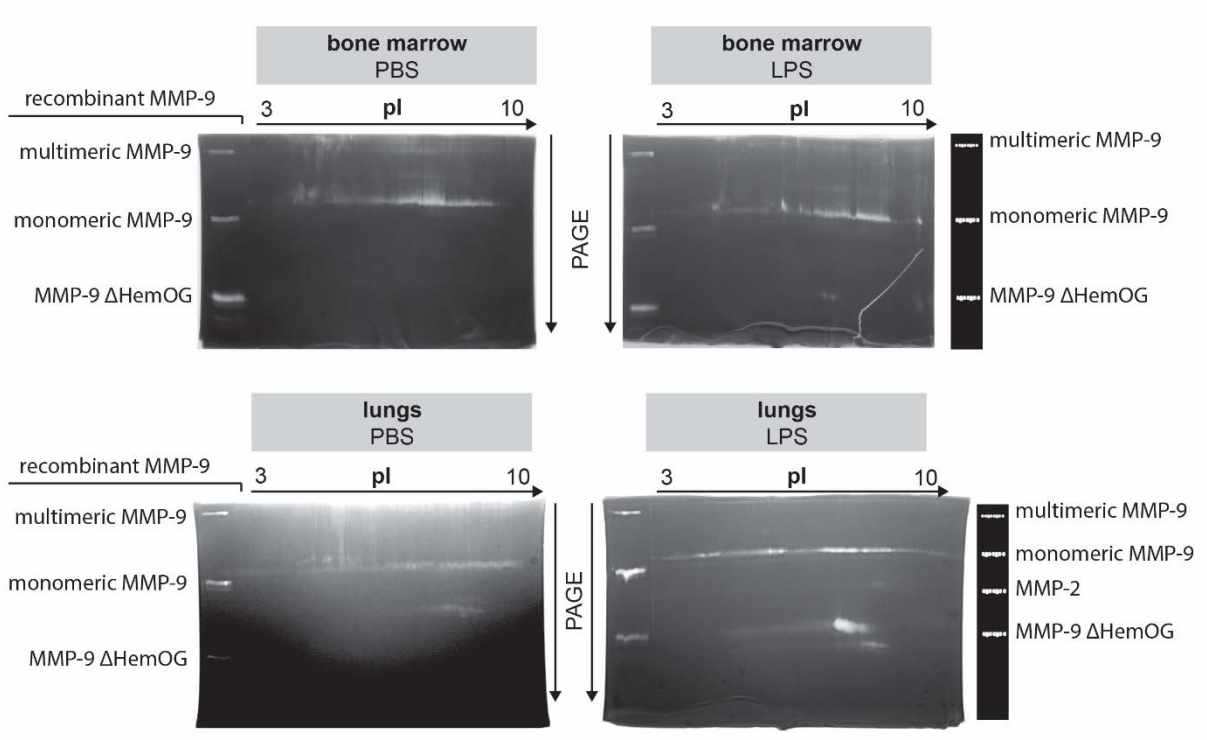
Supplementary Figure 1: Representative zymograms of bone marrow, spleen, lungs, liver and plasma of control mice.



Representative gelatin zymography gels of bone marrow, spleen, lung, liver and plasma of control mice. Each lane represents the analysis of the sample from a single mouse. Each sample was spiked with an internal processing and loading control (MMP-9ΔHemOG) and each gel has three lanes of recombinant MMP-9 standard protein (RS), including multimeric, monomeric and MMP-9 ΔHemOG proteins, to serve as a molecular weight marker and standard (10, 5 and 3 pg). The loading quantity of the samples corresponded with, respectively, 0.4 µg (bone marrow), 2 µg (spleen), 3 µg (lung), 120 µg (liver) and 40 µg (plasma) of total protein.



Supplementary Figure 2: Bidimensional zymography analysis of bone marrow and lung samples of mice *i.p*. injected with LPS or control mice (PBS injection). Samples were first separated based on charge by isoelectrofocussing under a pI (isoelectric point) gradient of 3 to 10. Next, samples were separated according to size by standard gelatin zymography techniques. The lung LPS sample was spiked with an internal control (MMP-9ΔHemOG = MMP-9 form lacking the C-terminal hemopexin and O-glycosylated domains) and each gel has one left lane of recombinant MMP-9 standard protein, including multimeric, monomeric and MMP-9 ΔHemOG proteins, to serve as molecular weight markers. Because the tissular levels of MMPs change after LPS stimulation and because different tissues contain different amounts of MMPs, we titrated MMP-9 in preliminary experiments, as to allow equivalent visualization of all isoforms of the enzyme. For this reason, different quantities had to be loaded for each 2D-gelatin zymography gel. The loading quantities of the samples corresponded with, 10 µg and 140 µg (bone marrow) and 288 µg and 130 µg (lung) of total protein for PBS and LPS treatments, respectively.