Supplemental material

Supplemental materials and methods

Anti-HMGB-1 mAB binding assay

To confirm the binding specificity of the anti-high mobility group box 1 (HMGB-1) monoclonal antibody (mAB), the antibody was incubated in the dark for 2 hours on ice with EZ-Link Sulfo–NHS-LC-Biotin (ThermoFisher, Waltham, Massachusetts, USA) in a ratio of 2:1 respectively, while gently shaking. After incubation, unbound biotin was removed by 48-hour dialysis against phosphate buffered saline (PBS), using a 12-14 kDa semi-permeable membrane. A 96-well plate was then coated overnight at 4°C with recombinant HMGB-1 in concentrations ranging from 0 μg ml^{-1} to 20 μg ml^{-1} (Abcam, Cambridge, UK). After blocking all remaining binding spots for two hours at room temperature with 1% BSA/PBS and washing with 0.05% tween/PBS, the biotinylated anti-HMGB-1 mAB was added in concentrations ranging from 0 μg ml^{-1} to 10 μg ml^{-1}. Subsequently, the plate was incubated at room temperature for 30 minutes. After washing the reaction was initiated by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB), sodium acetate buffer, and hydrogen peroxide 3%. The reaction was stopped after 5 minutes by adding sulfuric acid 2M and optical density was measured.

Dose-response

Two separate dose-response experiments for anti-HMGB-1 mAB) and recombinant thrombomodulin (rTM) were performed using the same model of trauma and shock, as described in the manuscript. Mice were randomized between four doses of anti-HMGB-1 mAB and five doses of rTM or vehicle (N=6 per group). For anti-HMGB-1 mAB the following doses were tested: 0 μg g^{-1}, 0.4 μg g^{-1}, 1 μg g^{-1} and 2.5 μg g^{-1}. Doses were based on prior work using the same anti-HMGB-1 mAB.[1] For rTM the doses were 0 μg g^{-1}, 0.0017 μg g^{-1}, 0.01 μg g^{-1}, 0.06 μg g^{-1} and 0.36 μg g^{-1}. Adequate dose for both drugs was determined based primarily on rotational thromboelastometry (ROTEM). In addition, safety was assessed by measuring organ wet/dry ratios and mortality.

Organ wet/dry ratios

The left lung, part of the liver, and the left kidney were collected and wet weight was determined after the experiment (Pioneer PX series; Avantor, Radnor Township, Pennsylvania, USA). After drying the organs at 37 °C for seven days they were weighted again to determine wet/dry ratios.

Organ histopathology

After the experiment the right lobe of the lung was fixed in 4% formaldehyde and embedded in paraffin, after which 4-µm thick sections were cut and stained with haematoxylin and eosin. Sections were scored by a pathologist for lung oedema, interstitial inflammatory cell infiltration, endothelialitis, haemorrhage and the presence of thrombi or coagulation. The scale of each category consisted of a score of 0 (absent) to 3 (severe).

Plasma markers of inflammation

Soluble P-selectin (sCD62P), soluble intercellular adhesion molecule-1 (sICAM-1), interleukin 1β (IL-1β) and IL-6 were measured with Luminex according to the manufacturer's instructions (Bio-Techne, Minneapolis, Minnesota, USA).

Supplemental tables

Assay	Buffer		Platelet identifying marker		Platelet activation marker		Agonist		WB	Total volume	Incubation Time	Blocking buffer	
Platelet activation 1	TB + $CaCl_2$ (2 mM final)	13 µl	CD41-APC	1 µl	JON/A-PE	4 µl	Control	1µl	1 µl	20 µl	15 min	TB	200 µl
Platelet activation 2	TB + $CaCl_2$ (2 mM final)	13 µl	CD41-APC	1 µl	JON/A-PE	4 µl	ADP (20 µM final)	1 µl	1 µl	20 µl	15 min	TB	200 µl
Platelet activation 3	TB + $CaCl_2$ (2 mM final)	13 µl	CD41-APC	1 µl	JON/A-PE	4 µl	PAR4-AP (100 µM final)	1 µl	1 µl	20 µl	15 min	TB	200 µl
Platelet activation 4	TB	16 µl	CD41-APC	1 µl	CD62P-FITC	1 µl	Control	1 µl	1 µl	20 µl	15 min	TB	200 µl
Platelet activation 5	TB	16 µl	CD41-APC	1 µl	CD62P-FITC	1 µl	ADP (20 µM final)	1 µl	1 µl	20 µl	15 min	TB	200 µl
Platelet activation 6	TB	16 µl	CD41-APC	1 µl	CD62P-FITC	1 µl	PAR4-AP (100 µM final)	1 µl	1 µl	20 µl	15 min	TB	200 µl
Assay	**Buffer**		**Platelet identifying marker**		**Leukocyte identifying marker**		**Neutrophil identifying marker**		**WB**	**Total volume**	**Incubation time**	**Blocking buffer**	
Platelet-leukocyte aggregates Single 1	TB	1 µl	CD41-APC	1 µl	CD45	1 µl			1 µl	8 µl	15 min	BD FACS lysis	200 µl
Platelet-leukocyte aggregates Single 2	TB	1 µl	CD41-APC	1 µl			Ly6G	1 µl	5 µl	8 µl	15 min	BD FACS lysis	200 µl
Platelet-leukocyte aggregates combined			CD41-APC	1 µl	CD45	1 µl	Ly6G	1 µl	5 µl	8 µl	15 min	BD FACS lysis	200 µl

Table S1: Incubation conditions for platelet activation and platelet-leukocyte aggregates. *All antibodies were used at undiluted concentration. TB, Tyrode's buffer buffer (NaCl 137 mM, HEPES 10 mM, KCl 2.8 mM, $MgCl_2$ 1 mM, $NaHCO_3^-$ 12 mM, Na_2HPO_4 0.4 mM, glucose 5 mM and bovine serum albumin 0.35%, WB, whole blood; ADP, adenosine diphosphate; PAR4-AP, protease activated receptor 4 - activating peptide.*

	VEH (mAB)	0.4 µg g^{-1}	1.0 µg g^{-1}	2.5 µg g^{-1}
General				
Weight (g)	27.8 (26.7 – 28.9)	28.1 (25.7 – 30.6)	29.2 (27.7 – 29.6)	28.4 (26.4 – 29.4)
Blood withdrawn (µl)	650 (500 – 730)	680 (550 – 710)	600 (590 – 790)	630 (590 – 710)
Arterial blood gas				
pH	7.28 (7.20 – 7.36)	7.31 (7.27 – 7.34)	7.32 (7.22 – 7.36)	7.28 (7.10 – 7.36)
pCO_2 (kPa)	3.8 (2.6 – 4.0)	3.8 (3.6 – 4.4)	3.9 (2.9 – 4.4)	3.4 (2.8 – 4.1)
pO_2 (kPa)	21.9 (18.7 – 28.0)	24.5 (21.3 – 25.9)	26.7 (21.7 – 27.5)	24.1 (21.3 – 28.7)
HCO_3^- (mM)	13.2 (7.5 – 16.5)	14.9 (13.1 – 16.2)	12.5 (11.3 – 15.4)	11.2 (8.2 – 14.2)
BD (mM)	12.4 (7.8 – 18.5)	10.4 (8.6 – 12.0)	10.8 (9.4 – 15.0)	14.2 (10.0 – 19.5)
Hb (mM)	6.9 (6.4 – 6.9)	6.8 (6.2 – 7.6)	7.0 (6.2 – 7.1)	6.7 (6.0 – 6.9)
K^+ (mM)	6.0 (5.8 – 7.5)	5.6 (4.9 – 6.0)	6.2 (5.7 – 6.8)	6.3 (5.6 – 7.4)
Ca^{2+} (mM)	0.98 (0.85 – 1.05)	0.97 (0.91 – 0.99)	1.00 (0.91 – 1.15)	1.05 (1.03 – 1.06)
Glucose (mM)	11.3 (10.2 – 12.3)	9.5 (7.9 – 11.6)	9.0 (8.8 – 11.7)	11.4 (9.8 – 12.8)
Rotational thromboelastometry				
CT (s)	36 (31 – 44)	40 (34 – 42)	39 (30 – 45)	36 (34 – 41)
CFT (s)	113 (104 – 125)	112 (87 – 130)	98 (78 – 110)	125 (115 – 160)
Alpha (°)	73 (72 – 78)	74 (68 – 79)	76 (73 – 82)	75 (71 – 76)
A5 (mm)	33 (31 – 34)	35 (32 – 38)	36 (34 – 39)	32 (27 – 34)
MCF (mm)	46 (44 – 48)	53 (47 – 56)	55 (48 – 57)	44 (40 – 48)
LI30 (%)	100 (100 – 100)	100 (99 – 100)	100 (98 – 100)	100 (98 – 100)
LI60 (%)	98 (95 – 100)	100 (93 – 100)	99 (91 – 100)	94 (92 – 96)
Organ wet/dry				
Lung	3.7 (3.5 – 3.8)	3.8 (3.6 – 3.9)	3.8 (3.4 – 4.2)	4.0 (3.4 – 4.7)
Kidney	3.8 (3.7 – 4.1)	3.6 (3.5 – 3.7)	3.6 (3.6 – 3.7)	3.6 (3.4 – 4.0)
Liver	3.2 (3.2 – 3.2)	3.1 (3.1 – 3.2)	3.2 (3.1 – 3.4)	3.3 (3.2 – 3.7)

Table S2: Dose-response of anti-HMGB1 mAB. *VEH, vehicle for anti-high mobility group box-1 (HMGB-1) monoclonal antibody (mAB); BD, base deficit; Hb, haemoglobin; CT, clotting time; CFT, clot formation time; A5, amplitude at 5 min after CT; MCF, maximum clot firmness; LI30, lysis index at 30 min after CT; LI60, lysis index at 60 min after CT. Data are presented as median (interquartile range).*

	VEH (rTM)	0.0017 µg g^{-1}	0.01 µg g^{-1}	0.06 µg g^{-1}	0.36 µg g^{-1}
General					
Weight (g)	27.0 (26.2 – 27.5)	27.0 (26.1 – 28.0)	28.1 (25.7 – 28.9)	26.9 (25.9 – 27.8)	27.6 (26.2 – 28.8)
Blood withdrawn (µl)	580 (500 – 710)	580 (500 – 660)	580 (550 – 700)	630 (540 – 700)	580 (480 – 630)
Arterial blood gas					
pH	7.21 (7.09 – 7.22)	7.25 (7.16 – 7.33)	7.19 (7.19 – 7.29)	7.24 (7.02 – 7.32)	7.25 (7.02 – 7.33)
pCO_2 (kPa)	5.1(4.4 – 6.4)	5.1 (4.4 – 6.1)	4.4 (3.3 – 6.4)	5.2 (4.8 – 5.7)	5.3 (4.0 – 6.1)
pO_2 (kPa)	30.0 (28.4 – 31.6)	27.3 (25.2 – 28.8)	26.7 (24.3 – 29.2)	27.6 (25.1 – 30.0)	28.1 (21.9 – 30.9)
HCO_3^- (mM)	13.3 (11.5 – 18.8)	16.1 (13.3 – 19.6)	14.9 (10.3 – 18.2)	16.5 (14.6 – 17.8)	19.1 (9.0 – 20.1)
BD (mM)	13.3 (8.9 – 17.4)	9.3 (7.4 – 13.8)	11.4 (8.8 – 17.0)	10.7 (8.2 – 12.5)	7.8 (5.4 – 20.7)
Hb (mM)	6.4 (6.3 – 7.5)	6.3 (5.8 – 6.7)	6.6 (6.0 – 7.1)	6.9 (6.4 – 7.1)	6.7 (6.0 – 7.2)
K^+ (mM)	6.1 (5.9 – 7.1)	6.1 (5.7 – 6.6)	6.6 (5.9 – 7.4)	6.2 (5.4 – 6.5)	5.4 (5.2 – 6.1)
Ca^{2+} (mM)	1.06 (1.00 – 1.14)	1.04 (0.92 – 1.12)	1.04 (0.86 – 1.07)	0.99 (0.95 – 1.14)	1.08 (1.01 – 1.19)
Glucose (mM)	11.1 (10.8 – 11.1)	11.3 (8.6 – 12.3)	7.8 (5.9 – 11.4)	12.9 (9.2 – 13.1)	11.1 (8.4 – 14.3)
Rotational thromboelastometry					
CT (s)	35 (31 – 36)	35 (34 – 38)	39 (37 – 50)	41 (36 – 53)	38 (33 – 43)
CFT (s)	89 (67 – 140)	89 (75 – 134)	113 (92 – 134)	97 (88 – 154)	94 (78 – 122)
Alpha (°)	79 (73 – 84)	77 (70 – 83)	74 (72 – 78)	73 (65 – 80)	77 (69 – 80)
A5 (mm)	37 (30 – 40)	36 (30 – 40)	32 (30 – 36)	30 (29 – 36)	37 (31 – 39)
MCF (mm)	52 (41 – 58)	51 (44 – 57)	52 (47 – 55)	48 (45 – 51)	55 (50 – 57)
LI30 (%)	100 (94 – 100)	100 (96 – 100)	100 (100 – 100)	100 (100 – 100)	100 (100 – 100)
LI60 (%)	90 (87 – 100)	99 (91 – 100)	100 (98 – 100)	98 (95 – 100)	100 (94 – 100)
Organ wet/dry					
Lung	4.1 (3.5 – 4.2)	3.8 (3.4 – 4.3)	3.6 (3.0 – 4.2)	3.7 (3.3 – 4.1)	3.4 (2.8 – 4.1)
Kidney	3.5 (3.3 – 3.6)	3.4 (3.4 – 3.5)	3.5 (3.3 – 3.6)	3.7 (3.5 – 3.9)	3.8 (3.5 – 3.9)
Liver	3.2 (3.1 – 3.3)	3.2 (3.1 – 3.2)	3.2 (3.0 – 3.3)	3.2 (3.1 – 3.3)	3.2 (3.1 – 3.4)

Table S3: Dose-response of recombinant thrombomodulin. *VEH, vehicle for recombinant thrombomodulin (rTM); BD, base deficit; Hb, haemoglobin; CT, clotting time; CFT, clot formation time; A5, amplitude at 5 min after CT; MCF, maximum clot firmness; LI30, lysis index at 30 min after CT; LI60, lysis index at 60 min after CT. Data are presented as median (interquartile range).*

	VENT	TS	TS+AB	TS+TM	TS+COMBI
IL-1β (pg ml^{-1})	< 91.6	< 91.6	< 91.6	< 91.6	< 91.6
IL-6 (pg ml^{-1})	517 (322 – 898)	357 (251 – 696)	390 (161 – 1452)	380 (181 – 801)	147 (89 – 255)*
sICAM-1 (ng ml^{-1})	28.6 (21.8 – 33.6)*	16.0 (13.0 – 18.4)	18.3 (15.0 – 19.0)	16.6 (14.5 – 21.2)	16.2 (9.3 – 17.9)
sCD62P (ng ml^{-1})	27.7 (24.1 – 30.9)*	18.8 (16.7 – 20.4)	19.3 (16.8 – 23.0)	19.3 (17.3 – 25.3)	16.9 (12.6 – 21.4)
Lung wet/dry	4.2 (4.1 – 4.3)	4.1 (4.0 – 4.2)	4.1 (3.9 – 4.5)	4.0 (3.8 – 4.1)	4.0 (3.8 – 4.2)
Kidney wet/dry	3.7 (3.6 – 3.8)	3.8 (3.7 – 3.2)	3.8 (3.7 – 3.9)	3.7 (3.7 – 3.8)	3.8 (3.7 – 3.9)
Liver wet/dry	3.2 (3.1 – 3.3)	3.3 (3.2 – 3.3)	3.3 (3.3 – 3.3)	3.3 (3.2 – 3.3)	3.3 (3.3 – 3.5)

Table S4. Inflammation and organ oedema. *VENT, ventilation + vehicle; TS, trauma and shock + vehicle; TS+AB, trauma and shock + anti-high mobility group box-1 (HMGB-1) monoclonal antibody (mAB); TS+TM, trauma and shock + recombinant thrombomodulin (rTM); TS+COMBI, trauma and shock + anti-HMGB-1 mAb + rTM; IL-6, interleukin-6; IL-1β, interleukin-1 bet; sICAM-1, soluble intercellular adhesion molecule-1; sCD62P, soluble P-selectin. Data are presented as median (interquartile range). *P<0.05 compared to TS.*

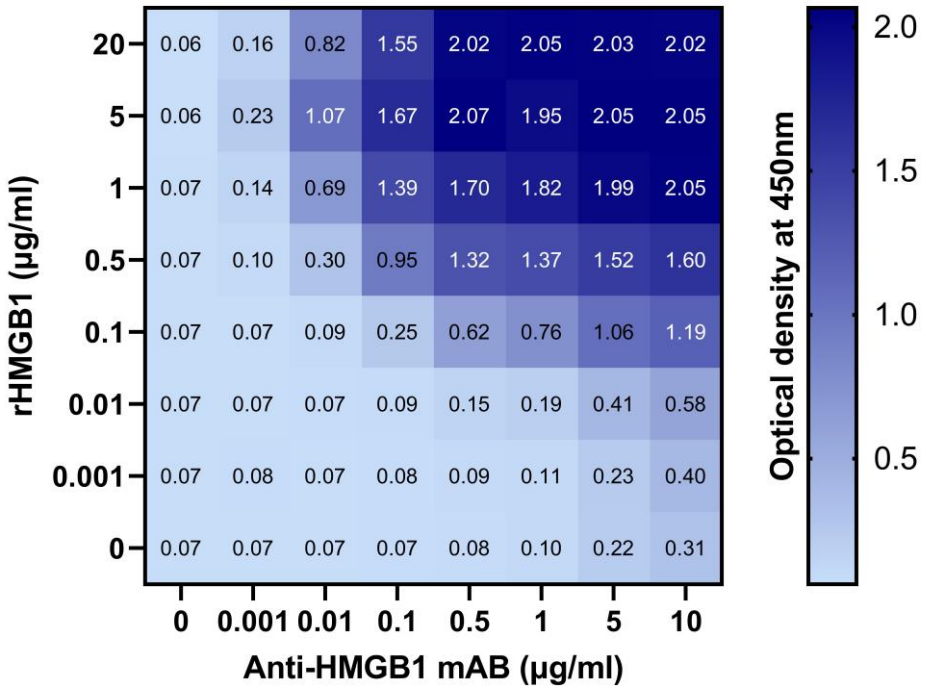

Figure S1. Binding specificity of anti-HMGB-1 mAB. *Anti-high mobility group box-1 (HMGB-1) monoclonal antibody (mAb) used in these animal experiments binds recombinant mouse HMGB-1 in a dose-dependent manner.*

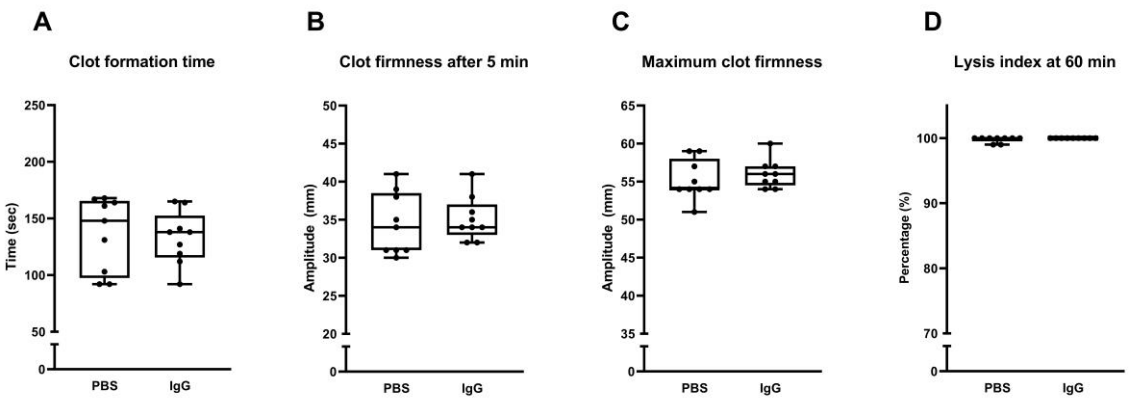

Figure S2. Effect of immunoglobulin G (IgG) on rotational thromboelastometry (ROTEM) ex-tem. *Data presented as median (interquartile range). Mouse whole blood, collected in trisodium citrate 109 mM (1/9 v/v ratio) was incubated with either Purified Mouse IgG2b, κ Isotype Ctrl Antibody 12.5 µg ml*$^{-1}$ *(Biolegend, USA) or phosphate-buffered saline (PBS). A) ROTEM ex-tem clot formation time, B) ROTEM ex-tem clot firmness after 5 minutes, C) ROTEM ex-tem maximum clot firmness, D) ROTEM ex-tem lysis index at 60 minutes. Data presented as median (interquartile range).*

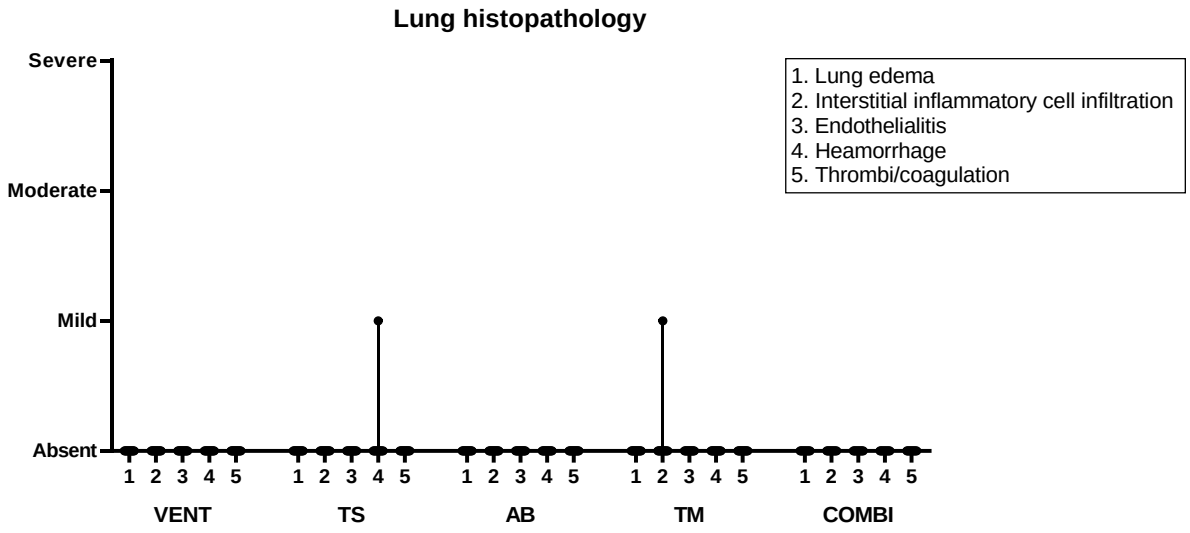

Figure S3. Lung histopathology scores. *Data are presented as median with range (all points are shown).*

Reference

1. Zhou H, Wang Y, Wang W, Jia J, Li Y, Wang Q, et al. Generation of monoclonal antibodies against highly conserved antigens. PloS one. 2009;4(6):e6087-e.