Online Data Supplement

Histidine-Rich Glycoprotein Prevents Septic Lethality through Neutrophil Regulation

Hidenori Wake, Shuji Mori, Keyue Liu, Kiyoshi Teshigawara, Masakiyo Sakaguchi, Kosuke Kuroda, Hideo Takahashi, Aiji Ohtsuka, Tadashi Yoshino, Hiroshi Morimatsu, and Masahiro Nishibori

Supplemental Methods

Reagents

Wortmannin, LY294002, SB239063, isoluminol, and horseradish peroxidase type IV were obtained from Sigma (St. Louis, MO). DAPI, Hoechst33342, Calcein-AM, Fluo-4, CM-H_2DCFDA, Phalloidin-Alexa584, DNaseI-Alexa488, BAPTA-AM, and Invivofectamine were obtained from Life Technologies. FR180204 and SP600125 were obtained from Merck (Darmstadt, Germany). fMLP was obtained from Peptide Institute, Inc. (Minoh, Japan). Toxin B from *C. difficile* was obtained from List Biological Laboratories (Campbell, CA). Anti-mouse Gr-1-FITC was obtained from eBioscience (San Diego, CA). Anti-human HRG monoclonal antibody (Rat) (#75-14) and anti-human HRG polyclonal antibody (Rabbit) were made in our laboratory.

Cell preparation

In accordance with the ethics approval and guidelines of Okayama University, written informed consent was obtained from healthy volunteers (n=5), and blood was drawn from the cubital vein. The blood was layered onto Polymorphprep (Axis-Shield, Oslo, Norway) prior to density-gradient centrifugation. The neutrophils purified were suspended in HBSS buffer.

The EA.hy926 cells (ATCC CRL-2922), a hybridoma of HUVEC and human epithelial cell line A549, were cultured in DMEM (ATCC) containing 10% FBS (Invitrogen). These cells were grown in a humidified atmosphere and passaged every 3-4 days.

Purification of HRG from human plasma

HRG was purified from human plasma (supplied by the Japanese Red Cross Society) as previously described (E1). In brief, human plasma was incubated with nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany) for 2 h at 4 °C with gentle shaking. The gel was packed into a column and washed successively with 10 mM Tris-buffered saline (TBS) (pH 8.0) containing 10 mM imidazole and then 10 mM Tris-buffer (TB) (pH 8.0) containing 1 M NaCl. Human HRG was eluted by 0.5 M imidazole in 10 mM TBS (pH 8.0). The protein extract was further purified by a Mono Q column (GE Healthcare, Little Chalfont, UK) with NaCl gradient. Purified human HRG was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with a human HRG-specific antibody.

Western blot analysis of HRG levels in serum

Mouse serum was electrophoresed on polyacrylamide gel (12.5%) and transferred onto a polyvinylidene diflouride (PVDF) membrane (Bio-Rad, Hercules, CA). After the membrane was stained with SYPRO Ruby (Life Technologies), it was blocked with 10% skim milk for 1 h and incubated overnight at 4 °C with rabbit anti-hHRG polyclonal Ab followed by anti-rabbit IgG goat polyclonal IgG-HRP (MBL, Nagoya, Japan) for 2 h at room temperature. The signals were finally visualized using an enhanced chemiluminescence system (Pierce Biotechnology, Rockford, IL).

In vivo RNAi experiments

Invivofectamine 2.0 reagent and HRG siRNA or negative control siRNA (Life Technologies, Carlsbad, CA) were mixed and incubated for 30 min at 50 °C. The mixtures were dialyzed by PBS and injected via tail veins of male C57BL/6N mice (7 weeks old). One week after injection, mouse blood was obtained by cutting the tail, and the plasma levels of HRG were determined using Western blotting. Then, a mild CLP sepsis (with one puncture) was induced in the RNAi-treated mice.

Ezyme-linked immunosorbant assay (ELISA)

In accordance with the ethics approval and guidelines of Okayama University, written

informed consent was obtained from 10 healthy subjects and 5 septic patients. Human plasma HRG levels were assessed by ELISA using solid-phase-immobilized anti-HRG monoclonal antibodies (#75-14) and Ni-NTA labeled with HRP (Qiagen, Hilden, Germany)

In vivo neutrophil imaging

Neutrophils were stained with FITC-labeled anti-Gr-1 antibody (20 μg/mouse) via i.v. injection before the *in vivo* imaging experiments. Sham-operated or CLP-treated mice were anesthetized with isoflurane, the abdomen was opened, and the exposed mesentery vessels were observed by a high-speed scan spinning-disk confocal microscopy system. Heating pads were used to keep the body temperature at 37 °C.

High-speed scan spinning-disk confocal microscopy system

Images were acquired with an inverted microscope IX 73 (Olympus, Tokyo, Japan) using a x20/0.75 objective lens. The microscope was equipped with a CSU-X1 confocal scanner (Yokokawa, Tokyo, Japan) and an iXON3 EMCCD camera (Andor Technology, Belfast, Northern Ireland). Image acquisition software iQ2 (Andor Technology) was used to drive the confocal microscope.

Micro Channel Array Flow Analyzer (MC-FAN)

The blood or neutrophil samples were prepared as follows. Mice were sacrificed 24 h after CLP. Blood was obtained from the heart and mixed with ACD solution for the microcapillary passage test. Human whole blood was withdrawn from the cubital vein and mixed with ACD solution. The blood was incubated with rat anti-human HRG monoclonal antibody (clone # 75- 14) or rat IgG for 30 min at 37°C. The human neutrophils isolated were treated with one of the reagents (BSA, HSA, HRG, fMLP) for 1 h at 37°C. The whole blood or neutrophil samples were forced to flow through artificial micro-channels made of silicon (width 7 μm, depth 4.5 μm, length 30 μm) under a constant suction of -20 cm H_2O (Micro Channel Array Flow Analyzer; MC-FAN, MC Lab, Tokyo, Japan). The passage time of the 100 μl samples through microcapillaries was determined.

Immunohistochemistry

Immunohistochemical staining of Gr-1 was performed on 5 μm lung sections with FITC-labeled rat anti-mouse Gr-1 mAb. The lung edema on HE-stained sections was quantified using NIH image J. Gr-1-positive neutrophils were counted in four fields of

a lung section at x 200 magnification under a fluorescent microscope. The results were expressed as the number of Gr-1-positive neutrophils per square millimeter.

Real-time quantitative PCR

Total RNA was isolated from the mouse lung using an RNeasy mini kit (Qiagen, Hilden). Complementary DNA was synthesized with a Takara RNA PCR kit Ver. 3.0 (Takara Bio, Nagahama, Japan) according to the manufacturer's instructions. Real-time PCR was performed with a Light Cycler (Roche, Basel, Switzerland) according to the manufacturer's instructions. Reaction mixtures contained cDNA template, SYBR premix Ex Taq (Takara Bio), and sequence-specific primers (Supplemental Methods). GAPDH expression was used to normalize cDNA levels. The PCR products were analyzed by a melting curve to ascertain the specificity of amplification.

Primer sequence for real-time PCR

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_008084) F-5' TGA CGT GCC GCC TGG AGA AA 3', R-5' AGT GTA GCC CAA GAT GCC CTT CAG 3'; TNF-α (NM_013693) F-5' GAC CCT CAC ACT CAG ATC ATC CTT CT 3', R-5'

GCG CTG GCT CAG CCA CTC 3'; iNOS (NM_010927) F-5' GAT TTT GCA TGA CAC TCT TCA 3', R-5' GGA GCC ATA ATA CTG GTT GAT 3'; IL-6 (NM_031168) F-5' GAC CTG TCT ATA CCA CTT CAC A 3', R-5' CTC TGG AAG TTT CAG ATT GTT 3'; PAI-1 (NM_008871) F-5' CTA TGG CGT GTC CTC GGT GCT 3', R-5' CAT TCT TGT TCC ACG GCC CCA T 3'; Neutrophil elastase (NM_015779) F-5' CTA CTG GCA TTG TTC CTG GGT G 3', R-5' GCT GAC ATG ACG AAG TTC CTG G 3'; RAGE (NM_007425) F-5'CTA GAG CCT GGG TGC TGG TTC 3'; R-5' GTT TCC ATT CTA GCT GCT GGG GC 3'

F-actin distribution and cell surface structure in neutrophils

Neutrophils (5 x 10^6 cells/ml) were seeded onto poly L-lysine-coated cover glass (Matsunami, Tokyo, Japan) and incubated with one of the reagents (BSA, HSA, HRG, or fMLP: each at a final concentration of 1 μM) at 37 °C for 1 h. For actin staining, the neutrophils were fixed with 4% PFA and treated with 0.1% TritonX-100 followed by staining with Phalloidin-Alexa568 (F-actin), DNaseI-Alexa488 (G-actin), and DAPI (Nuclei). The samples were observed using a confocal microscope (LSM 510, Carl Zeiss, Oberkochen, Germany). For electron microscopy, the samples were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde for 24 h at 4 °C, then post-fixed using 1%

osmium tetroxide for 1 h at 4 °C. OsO_4 coating with an osmium coater HPC-1S (Vacuum Device, Mito, Japan) was used. Each sample was examined using an s-4800 scanning laser microscope (Hitachi, Tokyo, Japan).

Determination of HRG uptake into neutrophils

HRG and HSA were labeled by a fluorescein using a fluorescein-labeling kit-NH_2 (Dojindo, Kumamoto, Japan). The neutrophils or EA.hy926 cells were aliquoted to a 96-well plate and incubated with fluorescein-labeled HRG or HSA (each at a final concentration of 1 μM) at 37 °C for 1 h. The samples were assayed using In Cell Analyzer 2000, and the data on granule count were analyzed by In Cell Analyzer Workstation software.

Determination of ROS production

The neutrophil suspension with isoluminol (final concentration, 50 mM) and horse radish peroxidase type IV (final concentration 4U/ml) were aliquoted to a 96-well plate (BD, Franklin Lakes, NJ) in a volume of 100 μl (5×10^4 cells) with one of the reagents (BSA, HSA, HRG, each at a final concentration of 1 μM). Neutrophil extracellular ROS production was evaluated at 15 min after the start of incubation at 37 °C by the

measurement of chemiluminescence intensity using Flexstation3. Intracellular ROS was determined by pre-loading of CM-H_2DCFDA into neutrophils for 20 min.

Chemotaxis assay

The transfer of neutrophils to the vertical direction was examined using a 24-well Chemotaxicell chamber (Kurabo, Kurashiki, Japan). Neutrophils (5 x 10^5 cells in 100 μl) were added to the upper wells of the chamber and were separated by a polycarbonate membrane 5 μm in diameter pores from lower wells containing one of the reagents (BSA, HSA, HRG, or fMLP). Following 1 h incubation, the cells that had migrated to lower wells were counted under a light microscope. For the horizontal chemotaxis assay, neutrophils (5 x 10^5 cells/ 10 μl) were added to one well in agarose gel (5 mm thick) and were allowed to migrate toward the other well (2 mm from the former) containing a possible chemoattractant (1 μM). Two hours after the start of migration, the neutrophils were fixed and stained with Wright's stain, and the distance from the origin to the leading edge was measured.

Supplemental Reference

E1. Mori S, Takahashi HK, Yamaoka K, Okamoto M, Nishibori M. High affinity binding of serum histidine-rich glycoprotein to nickel-nitrilotriacetic acid: the application to microquantification. *Life Sci* 2003;73:93-102.

Supplemental Figure Legends

Figure E1

(A) Time-dependence of the spherical shape-inducing effects of HRG on neutrophils. The neutrophils were incubated with 1 μM of HRG for 5, 10, 15, 30, and 60 min after the addition of HRG, respectively. (B) The reversibility of a neutrophil's shape from flattened to spherical by the addition of HRG. The neutrophils were incubated in HBSS for 60 min and then HRG (1 μM) was added to the medium. Morphological changes were observed at 60 and 120 min after the addition of HRG. Scale bars, 20μm (A and B).

Figure E2

(A) HRG's effects on vertical transfer of neutrophils through micropores. Purified neutrophils (5 x 10^5 cells) were added to the upper part of a Boyden chamber. The transfer of neutrophils into a lower chamber containing HRG (0.03-1 μM), BSA (1 μM), HSA (1 μM), and fMLP (1 μM) was measured at 37 °C 60 min later. The results shown are the means ± SEM of three experiments. * $P<0.05$ and ** $P<0.01$ *vs.* HBSS. (B) HRG's effects on the horizontal migration of neutrophils in agarose gel. The purified neutrophils (5 x 10^5 cells) were added to one well in agarose gel (5 mm thick)

and were allowed to migrate toward the other well (2 mm apart) containing a possible chemoattractant (1 μM). The results shown are the means ± SEM of three experiments. ** P<0.01 *vs.* HBSS.

Figure E3

(A) Binding of fluorescein-labelled HRG on EA.hy926. The Hoechst33342-stained EA.hy926 cells were incubated with fluorescein-HRG or fluorescein-HSA for 60 min, and the cells were photographed under fluorescent microscopy after washing. Differential interference contrast microscopic observation was performed. (B) The even distribution of fluorescein-HRG binding was observed on EA.hy926. Scale bars, 20 μm (A and B).

Figure E4

Effects of increasing concentrations of IL-8 and C5a on the shapes of HRG-induced neutrophils. The neutrophils were incubated with increasing concentrations of IL-8 or C5a (1- 100 ng/ml) in the presence of HRG (1 μM). Scale bar, 20 μm.

Figure E5

Scheme of the effect of HRG on septic condition. A plasma protein HRG decreased markedly in septic mice with high lethality. Supplementary treatment with HRG improved the survival of septic mice. HRG kept circulating neutrophils quiescent morphologically and functionally.

Supplemental Video Legends

Video E1

Behavioral changes in sepsis mice treated with HRG. Sepsis was induced by CLP and the mice were treated with PBS, HSA (20 mg/kg, i.v.), or HRG (20 mg/kg, i.v.). The locomotor activity of the mice in each group was monitored by a video camera 24 h after the induction of sepsis.

Video E2

In vivo imaging of circulating neutrophils in the venules in CLP sepsis mice. FITC-labelled anti-Gr-1 antibody (1 mg/kg) was injected i.v. to CLP mice treated with PBS or HRG (20 mg/kg, i.v.). The immunostained neutrophils in the mesenteric venules were observed by a high-speed scan spinning-disk confocal microscopy system.

Video E3

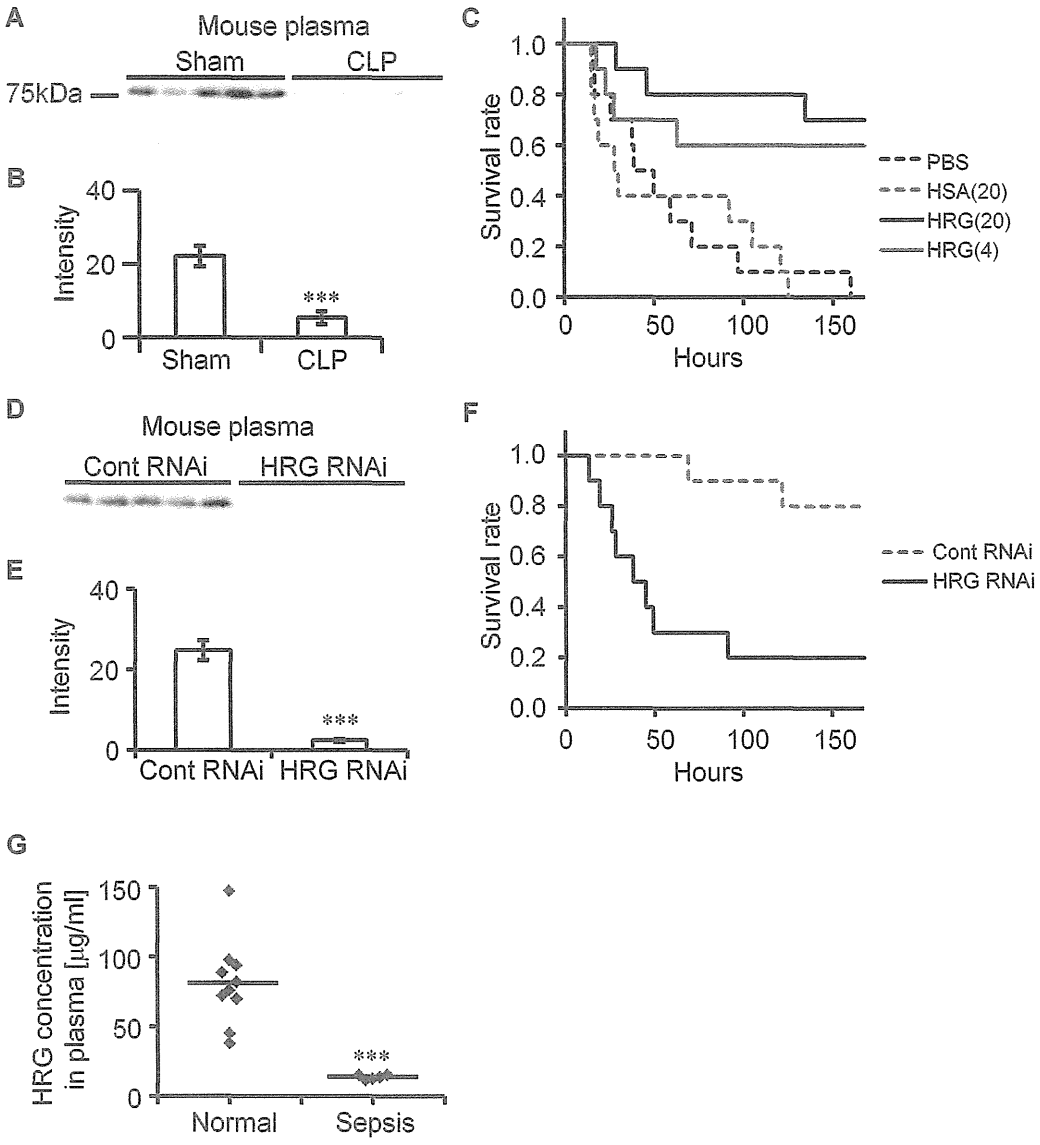
HRG's effects on the passage of blood through microchannels. Whole blood of CLP mice treated with PBS, HSA (20 mg/kg, i.v.), or HRG (20 mg/kg, i.v.) was withdrawn from the abdominal aorta 24 h after CLP and applied to a MC-FAN. The passage of blood cells was monitored by a CCD camera

Video E4

HRG's effects on the passage of neutrophils in microcapillaries. The purified human neutrophils were incubated with BSA, HSA, HRG, or fMLP at 1 μM for 60 min at 37 °C and applied to a MC-FAN. The passage of neutrophils through microcapillaries was monitored by microscope-CCD camera system in the apparatus.

Video E5

Effects of anti-HRG Ab on the passage of human whole blood in microcapillaries. The human whole blood was treated with anti-HRG Ab (40 μg/ml) or control IgG for 30 min at 37 °C and applied to MC-FAN. The passage of blood cells through the microcapillaries was monitored as described above.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Sham
CLP
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0.6
0.4
0.2
0.0
0
50
100
150
Hours
PBS
HSA(20)
HRG(20)
HRG(4)
D
Mouse plasma
Cont RNAi
HRG RNAi
E
Intensity
40
20
0
***
Cont RNAi
HRG RNAi
F
Survival rate
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
Hours
Cont RNAi
HRG RNAi
G
HRG concentration in plasma [µg/ml]
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100
50
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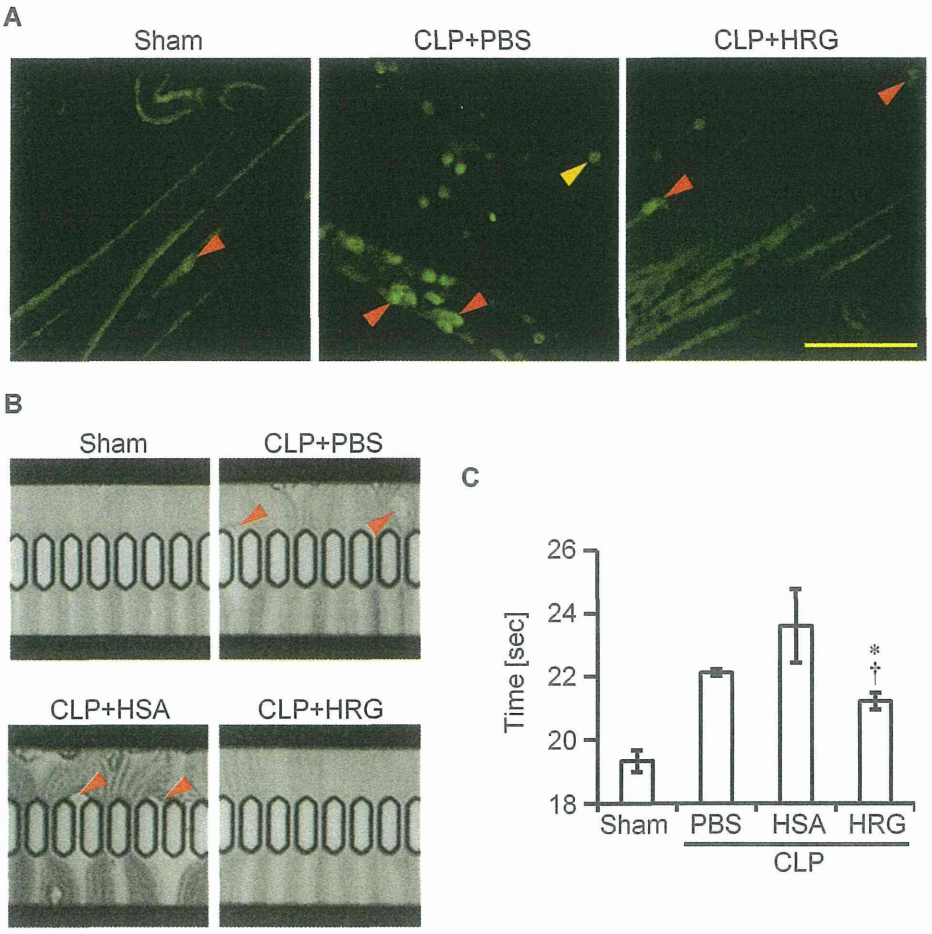
Sepsis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CLP+HRG
B
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C
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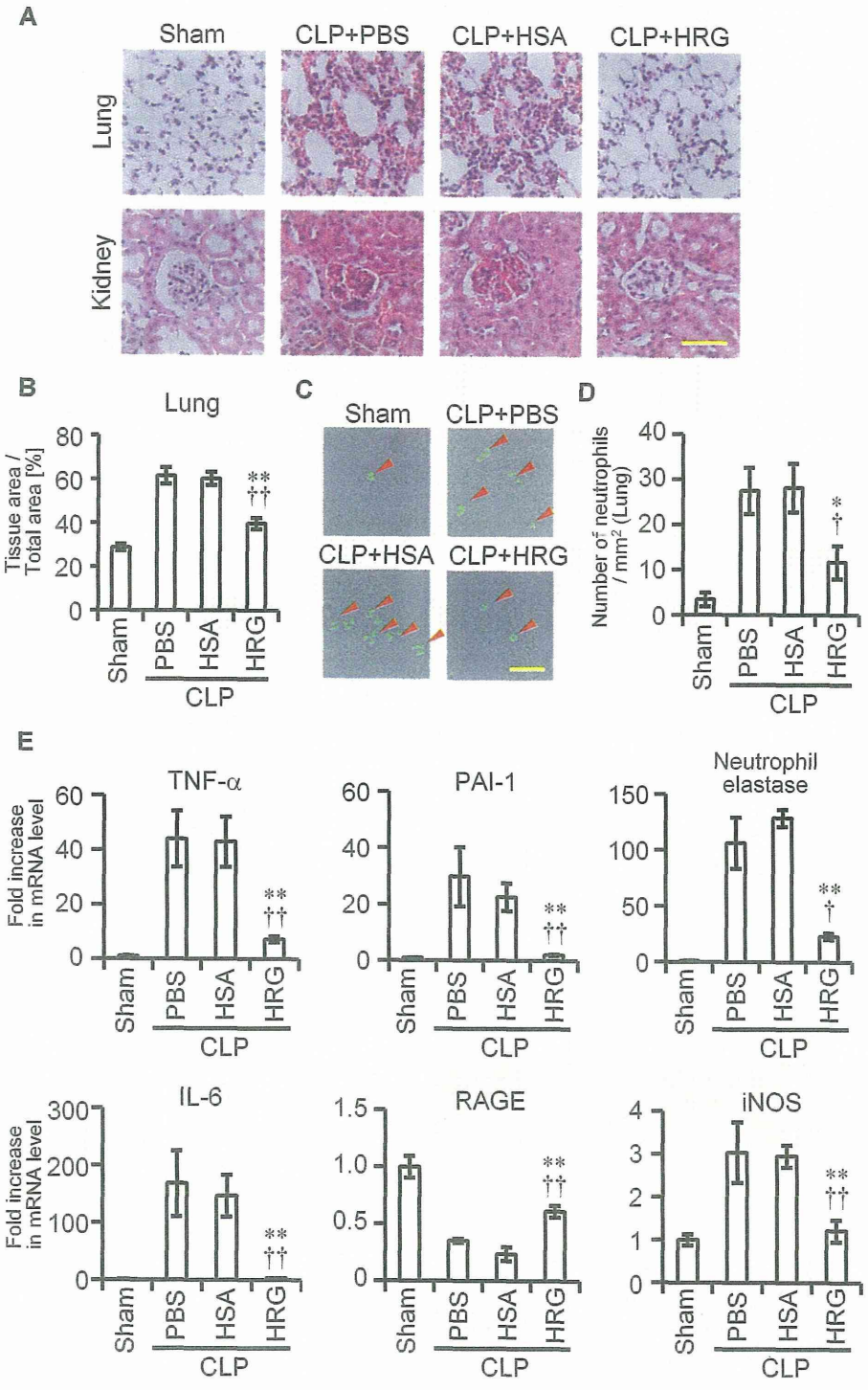
22
20
18
Sham
PBS
HSA
HRG
CLP
*
†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CLP+PBS
CLP+HSA
CLP+HRG
Lung
Kidney
B
Lung
Tissue area / Total area [%]
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60
40
20
0
**
††
Sham
PBS
HSA
HRG
CLP
C
Sham
CLP+PBS
CLP+HSA
CLP+HRG
D
Number of neutrophils / mm² (Lung)
40
30
20
10
0
†*
Sham
PBS
HSA
HRG
CLP
E
TNF-α
Fold increase in mRNA level
60
40
20
0
**
††
PAI-1
60
40
20
0
**
††
Neutrophil elastase
150
100
50
0
**
†
IL-6
300
200
100
0
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††
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iNOS
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3
2
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0
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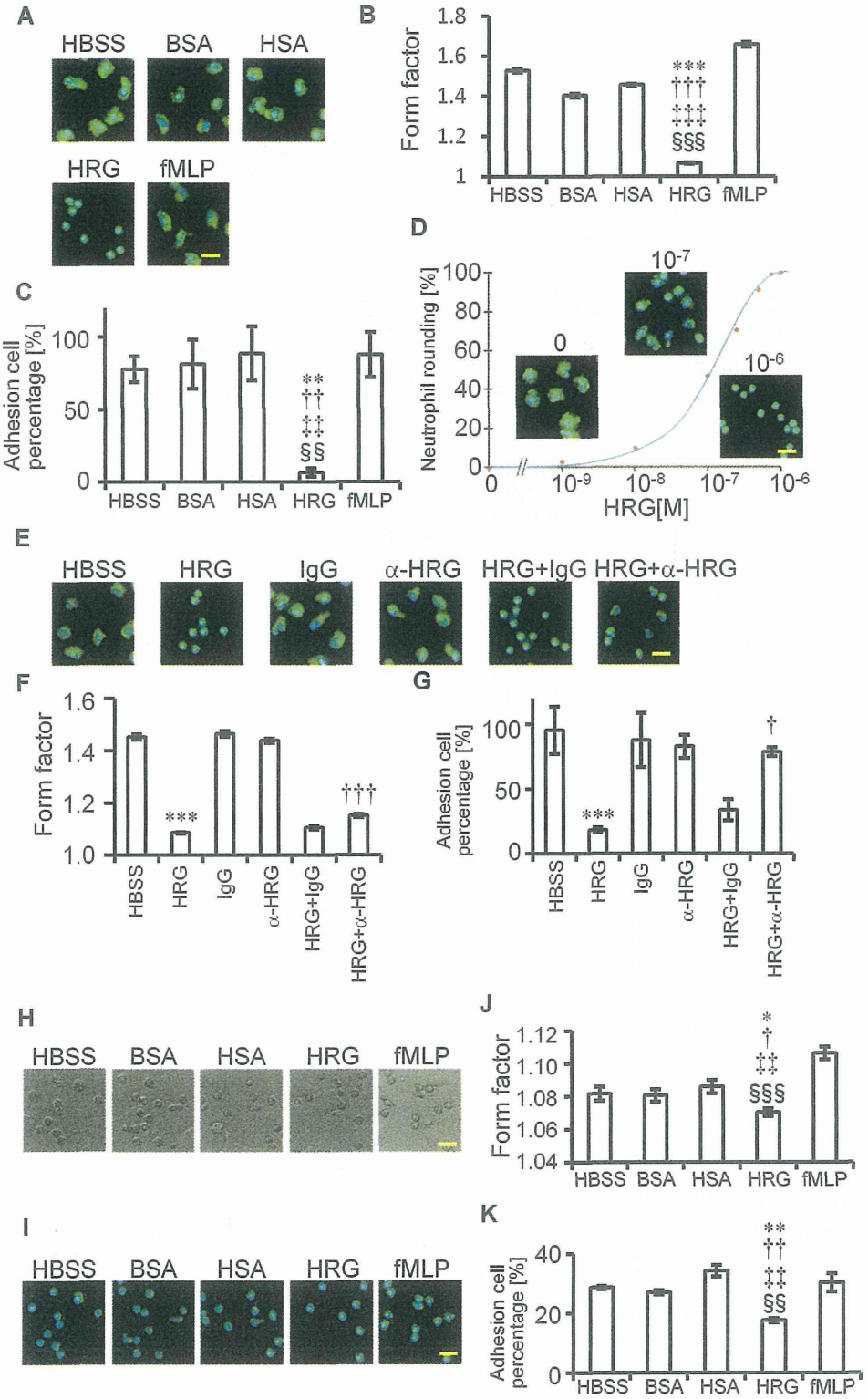
††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‡‡‡
§§§
HBSS BSA HSA HRG fMLP
C
Adhesion cell percentage [%]
100
50
0
**
††
‡‡‡
§§
HBSS BSA HSA HRG fMLP
D
Neutrophil rounding [%]
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80
60
40
20
0
0
10-7
10-6
10-9
10-8
10-7
10-6
HRG[M]
E
HBSS
HRG
IgG
α-HRG
HRG+IgG
HRG+α-HRG
F
Form factor
1.6
1.4
1.2
1.0
***
†††
HBSS HRG IgG α-HRG HRG+IgG HRG+α-HRG
G
Adhesion cell percentage [%]
100
50
0
***
†
HBSS HRG IgG α-HRG HRG+IgG HRG+α-HRG
H
HBSS
BSA
HSA
HRG
fMLP
J
Form factor
1.12
1.10
1.08
1.06
1.04
*
†
‡‡
§§§
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HBSS
BSA
HSA
HRG
fMLP
K
Adhesion cell percentage [%]
40
20
0
**
††
‡‡
§§
HBSS BSA HSA HRG fMLP