SUPPLEMENTARY MATERIALS

High-Mobility Group Box-1 Mediates Toll-like Receptor 4-Dependent Angiogenesis

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Expanded Methods

Animals

TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were originally generated by Dr. S. Akira (Osaka University, Osaka, Japan). These mice were backcrossed ten or more generations onto the C57BL/6 background, and were then intercrossed to obtain the knockout genotypes and WT mice (as control). Littermates of both sexes between 8 and 12 wk old were used in all experiments. Animals were kept in a specific pathogen-free (SPF) facility and given water and standard laboratory chow ad libitum. Animal care and use were in compliance with Institutional guidelines and with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents and Abs

Purified LPS from *E. Coli* 011:B4 strain and purified LPS from *Rhodobacter sphaeroides* (LPS-RS) were purchased from Invivogen (San Diego, CA, USA). Goat anti-mouse VEGF polyclonal

antibodies were from R&D Systems (Minneapolis, MN, USA). FITC-conjugated anti-mouse F4/80, PE-conjugated anti-mouse F4/80, Alexa Fluor® 488-conjugated anti-mouse TLR4 were from eBioscience, Inc. (San Diego, CA, USA). Goat anti-mouse PECAM-1 (CD31), FITC-conjugated goat anti-rabbit IgG polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cy3-conjugated donkey anti-goat IgG polyclonal antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). TRIzol reagent and Hoechst 33342 were from Invitrogen (Carlsbad, CA, USA). ExScriptTM RT reagent kit was from TaKaRa (TaKaRa Biotechnology Co. Ltd. DaLian, China). Brilliant SYBR® Green QPCR Master Mix was from Stratagene (La Jolla, CA USA). Goat anti-rabbit IgG-peroxidase antibody and rabbit anti-goat IgG-peroxidase antibody were purchased from Sigma Inc. (St. Louis, MO, USA). DAB substrate-chromogen was from Dako Cytomation, Inc. (Carpinteria, CA, USA). Most of other reagents such as salt and buffer components were analytical grade and obtained from Sigma.

Recombinant full-length HMGB1, Box A and anti-HMGB1 rabbit serum

The cloning, expression and purification of recombinant full-length HMGB1 and Box A and preparation of anti-HMGB1 rabbit serum were carried out as previously described.[1] The recombinant proteins were highly purified and functional. LPS content in both purified HMGB1 and Box A were less than 0.16 EU/mg (below the detection limit). High titer antiserum (1:2,000,000) against HMGB1 was obtained by immunizing rabbits with purified recombinant HMGB1.

Alkali-induced corneal injury model

Mice were anesthetized with i.p. administration of kessodrate. A 2-mm disc of filter paper saturated

with 1 N NaOH was placed onto the right cornea of each mouse for 40 s, followed by rinsing extensively with 25 ml of PBS. The corneal epithelia were removed using a corneal knife in a rotary motion parallel to the limbus by gently scraping over the corneal surface without injuring the underlying corneal stroma. Erythromycin ophthalmic ointment was instilled immediately following epithelial denudation. At the indicated time intervals (days 0, 2, 4, and 7), mice were killed and the corneas were removed from both eyes. These corneas were placed immediately into RNALate (Qiagen) and kept at -80°C until total RNA extraction. In another series of experiments, mice were killed at the indicated time points (days 0, 2, 4, 7 and 14) after alkali treatment and both eyes were entirely removed from each animal. These eyes were fixed in 10% neutral formalin buffer for histological analysis. The left eye of each mouse was used as an untreated control. In some experiments, recombinant HMGB1 (80 μg/ml), Box A (100 μg/ml), LPS (5 μg/ml) or LPS-RS (50 μg/ml) were in sterile LPS-free saline solution. Five microliters of these preparation or vehicle was applied topically to the alkali-treated eye twice a day for 7 days. In some experiments in WT mice, Box A (100 μg/ml) or vehicle (PBS) was administered to the alkali-treated eyes 30 min before LPS (5 μg/ml) administration, whereas the mice in parallel groups were administered with LPS-RS (50 μg/ml) prior to HMGB1 (80 μg/ml) exposure with the same procedures. The wounded eyes received PBS alone served as controls. Each experiment was repeated at least three times.

Immunohistochemical analysis

The OCT-embedded tissues were cut into 8-μm- thick slices and mounted on poly-L-lysine-coated slides, and subjected to immunohistochemical staining. Immunohistochemical analyses were performed using anti-HMGB1, or -CD31 Abs. The sections were incubated with Abs at a

concentration of 0.5–5 μg/ml at 4°C overnight. After incubation with peroxidase conjugated secondary Ab, the chromogen diaminobenzidine tetrahydrochloride (DAB) was added, until color had developed sufficiently, sections were counterstained with Mayer's hematoxylin.

Immunofluorescence analysis

For one color staining for macrophage infiltration, the frozen cryostat sections of isolated eyes were immunostained with FITC-conjugated anti- F4/80 Ab and counterstained with Hoechst 33342 and mounted. The numbers of positive cells were counted on five randomly chosen fields of corneal sections in each animal at 200-fold magnification, by an examiner without any prior knowledge of the experimental procedures. The numbers of positive cells per mm^2 were calculated. For two color analyses, the frozen cryostat sections were incubated with the combinations of PE-conjugated anti-F4/80 and Alexa Fluor® 488-conjugated anti-TLR4, PE-conjugated anti-F4/80 and rabbit anti-HMGB1, goat anti-CD31 and Alexa Fluor® 488-conjugated anti-TLR4, or goat anti-CD31 and rabbit anti-HMGB1 Abs overnight at 4°C. After being rinsed with PBS, the sections were further incubated with the combination of FITC-conjugated goat anti-rabbit IgG and Cy3-conjugated donkey anti-goat IgG (1/100) for 40 min at room temperature. After counterstain with Hoechst 33342, the sections were visualized in a dualchannel mode on an Olympus fluorescence microscope.

Biomicroscopic examination

Eyes were examined with a slit-lamp (Zeiss, Germany) 7, 14 days after alkali injury. In brief, under anesthesia, photographs of the corneas were obtained using a digital camera (ORCA ER; Hamamatsu, Japan) that was linked to an operating microscope. Microscopic assessment was done by two

independent observers without prior knowledge of the experimental procedures.

Enumeration of CNV

The fixed cryosections (8-μm thick) were stained using anti-CD31 polyclonal Abs and the numbers and sizes of the CNV were determined by an examiner with no knowledge of the experimental procedures. Briefly, images were captured with a digital camera and imported into Adobe Photoshop (version 7.0). Then, the number of neovascular tubes per mm^2 and the proportions of CNV in the hot spots were determined using NIH Image analysis software version 1.62 (National Institutes of Health, Bethesda, MD). The sections were from the central region of the cornea. The numbers and areas of CNV were evaluated on at least two sections from each eye.

Real-time quantitative RT-PCR

Total RNAs were extracted from the corneas or cultured peritoneal macrophages with a TRIzol reagent kit and the cDNA was prepared by reverse transcriptase. Real-time PCR was performed on an Applied Biosystems StepOne Real-Time PCR System using the comparative threshold cycle (CT) quantification method. Each reaction contained 12.5 μl of 2×SYBR green Master Mix, 300 nM oligonucleotide primers (Supplementary Table I) synthesized by Invitrogen Biotechnology Co. Ltd, (Shanghai, China), 10 μl of 1 in 10 dilution of the cDNA and water, to a total of 25 μl. The thermal cycling conditions included an initial denaturation step at 95 ^{o}C for 10 min, 40 cycles at 95 ^{o}C for 30 s, 60 ^{o}C for 1min, and 72 ^{o}C for 1min. The tested mRNA expression in each sample was finally determined after correction with GAPDH expression. Each measurement of a sample was conducted in duplicate.

Murine peritoneal macrophage isolation and culture

Specific pathogen-free male WT or TLR4-deficient mice were injected i.p. with 2 ml of sterile 3% thioglycolate medium (Sigma-Aldrich). The peritoneal macrophages were harvested 3 days later. The resultant cell preparation consisted of >95% macrophages as verified by immunocytochemical analysis on the cell preparation immunostained with FITC-conjugated anti-F4/80 antibody (Supplementary Figure VI). The cells were suspended in antibiotic free RPMI 1640 medium containing 10% FBS and cultured in 24-well plates in a humidified incubator at 37°C in 5% CO_2. Two hours later, nonadherent cells were removed and the medium was replaced. The cells were then stimulated with the 1.0 μg/ml of recombinant HMGB1 for 12 h. Total RNAs were extracted from the cultured cells and subjected to quantitative RT-PCR as described above. For an immunocytochemical analysis of VEGF expression, murine macrophages were seeded onto the wells of a Lab-Tec chamber slide with eight wells (Nalge Nunc) at 5×10^4 cells/well. After adhesion, the cells were stimulated with 1.0 μg/ml of recombinant HMGB1 for 24 h in a 37°C incubator with 5% CO_2 and then subjected to immunocytochemical study.

Reproducibility and statistical analysis

Experiments were repeated at least three times. Results were highly reproducible. Representative results were shown in figures. The means and SEM were calculated on all parameters determined in the study. Data were analyzed statistically using one-way ANOVA or two tailed Student's t test. A value of $p < 0.05$ was accepted as statistically significant.

Supplementary Table I Sequences *of primers for real time PCR*

Primer	Forward primer	Reverse primer	bp
TLR4	5′-CCTCCAACTGTCTACCAGTTCC-3'	5′-GCCTGGCTAAGTTATTGTGC-3'	230
HMGB1	5′- CTTCGGCCTTCTTCTTGTTCT-3'	5′- GGCAGCTTTCTTCTCATAGGG -3'	152
RAGE	5′- GGACCCTTAGCTGGCACTTAGA -3'	5′- GAGTCCCGTCTCAGGGTGTCT -3'	99
VEGF	5′- TGCTCG TGGCTGCCTTCT-3'	5′-CAGGAAGTGGGAGGGTCAGA-3'	67
b-FGF	5′- CGCCCAGACAGAAGTCATAG-3'	5′-TCCTCCTTTCCAGGTCAGTTA-3'	132
TGFβ1	5′-TGCACCCAAACCGAAGTCAT-3'	5′-TTGTCAGAAGCCAGCGTTCAC-3'	177
GAPDH	5′-TGAGCAAGAGAGGCCCTATC-3'	5′-AGGCCCCTCCTGTTATTATG-3'	93

Supplementary Figure Legends

Supplementary Figure I *Chemokine expression of TLR4-deficient mice during wound healing.* Real-time PCR analysis was used to examine changes in mRNA expression of chemokine MIP-2/CXCL2 (A), MCP-1/CCL2 (B) and MIP-1α/CCL3(C) in wounds on day 1, 3, 5 post injury in WT and $TLR4^{-/-}$ mice. The relative changes were normalized against the housekeeping gene (GAPDH) and calculated using the $2^{-\triangle\triangle}$CT method. Results were from three separate experiments performed in duplicate and expressed as mean ± SEM of fold increase over control.* $p < 0.05$; statistically significant difference in values compared with WT mice groups. Representative results from three independent experiments with four animals in each group are shown.

Supplementary Figure II *Double-color immunofluorescence analysis of injured corneas.* (A) Corneas were obtained from WT mice 2 days after the injury. The samples were immunostained with a combination of anti-F4/80 and -TLR4 (upper panels) or anti-F4/80 and -HMGB1 (lower panels) antibodies. (B) Corneas were obtained from WT mice 7 days after the injury. The samples were immunostained with anti-CD31 and -TLR4 (upper panels) or anti-CD31 and -HMGB1 (lower panels) antibodies. The nuclei of cells in the samples were visualized by Hoechst 33342 staining as shown in left panels. Signals were digitally merged in right panels. Arrows indicate the double positively stained cells. Original magnification, ×400.

Supplementary Figure III *The effect of LPS, HMGB1, LPS-RS and Box A on alkali injury-induced CNV.* 14 days after alkali burn, the images (lateral view) were taken with slit lamp to show CNV in

(A): PBS-, LPS-, HMGB1-treated eyes of WT (left panels) and $TLR4^{-/-}$ (right panels) mice or in (B): PBS-, LPS-RS-, or Box A-treated eyes of WT mice. Representative results from six individual mice in each group are shown. Original magnification of images, ×32.

Supplementary Figure IV *Alkali injury-induced CNV in WT and TLR2-deficient mice.*

(A) Macroscopic appearance of WT (upper panels) and TLR2-deficient mouse eyes (lower panels) 2 wk after alkali injury. Images were taken with slit lamp to show the lateral (left panels) and frontal (middle panels) view of each eye. Corresponding cryosections from corneal tissues were immunostained with anti-CD31 Ab (right panels). Representative results from six individual mice in each group are shown. Original magnification of images, ×400. (B)-(C) Quantitative analysis of data presented in (A). The CNV numbers per mm^2 in hot spots (B) and % CNV areas in hot spots (C) were determined. Each value represents the mean ± SEM (n=6 animals). * $p < 0.05$; statistically significant difference in values compared with WT mice groups.

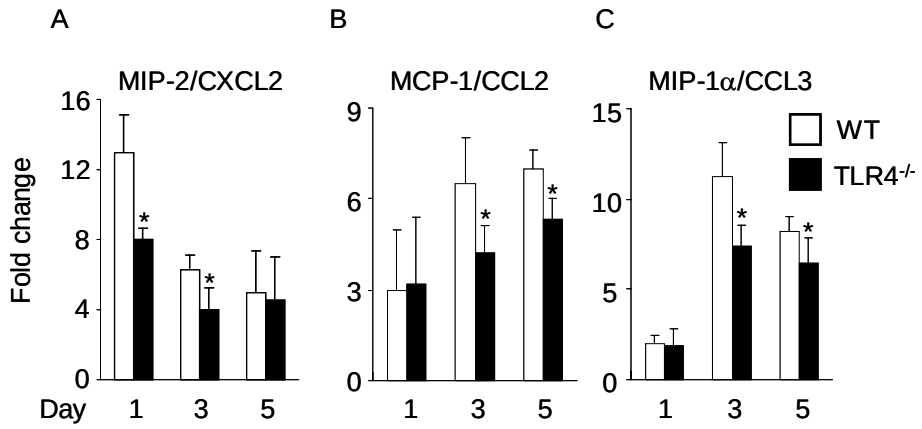
Supplementary Figure V *The effects of LPS-RS on HMGB1-enhanced CNV and Box A on LPS-enhanced CNV in WT mice.* (A)-(C) Macroscopic images of CNV in WT mice two weeks after alkali injury are shown. (A) Wounded eyes received PBS treatment as a control. (B) Wounded eyes received HMGB1 application followed the treatment with LPS-RS (lower panels) or vehicle (PBS) (upper panels) for 30 min. (C) Wounded eyes received LPS application followed the treatment with Box A (lower panels) or PBS (upper panels) for 30 min. Images were taken with slit lamp to show the lateral (left panels) and frontal (middle panels) view of each eye. Corresponding cryosections of the corneal tissues were immunostained with anti-CD31 Ab (right panels). Original magnification, ×400.

(D)-(E) Quantitative analysis of neovascularization in (A)-(C). The CNV numbers per mm^2 in hot spots (D) and % CNV areas in hot spots (E) were determined. Each value represents the mean ± SEM (n=6). * $p < 0.05$, PBS + HMGB1 vs LPS-RS + HMGB1, or PBS + LPS vs Box A + LPS.

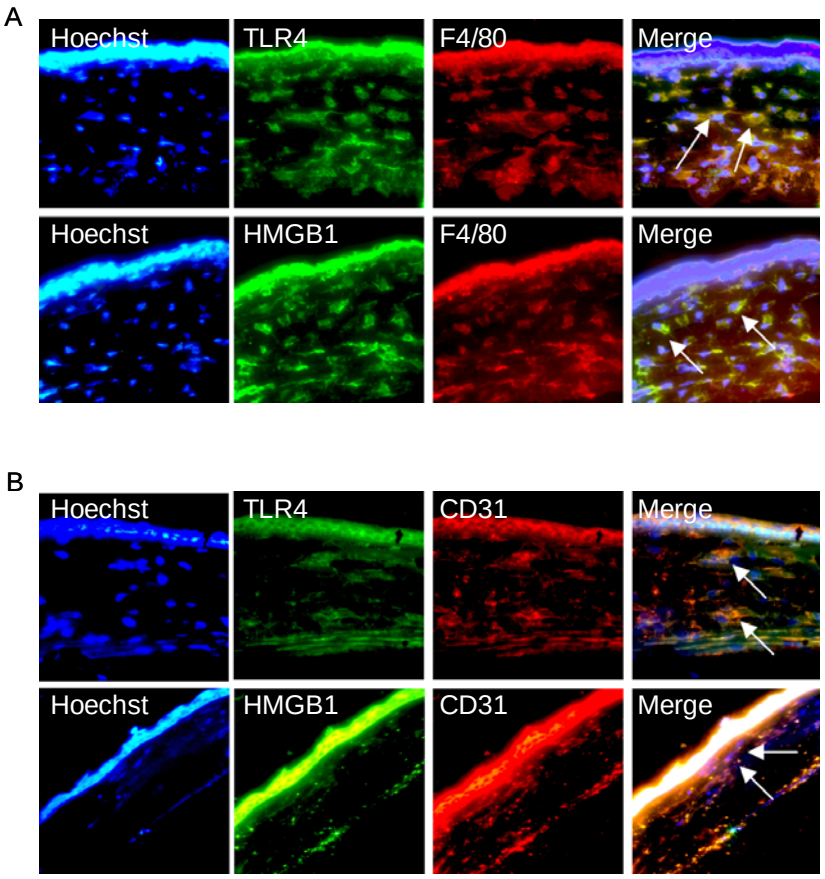
Supplementary Figure VI *Murine peritoneal macrophage determination.* Mouse peritoneal macrophages were harvested and seeded on uncoated coverslips in Petri dishes. The nuclei of the cells were visualized by Hoechst 33342 staining as shown in upper panels. The resultant cell preparation consisted of greater than 95% macrophages, as verified by immunocytochemical analysis with FITC-conjugated anti-F4/80 antibody shown in lower panels. Original magnification of images, ×400.

Supplementary Figures

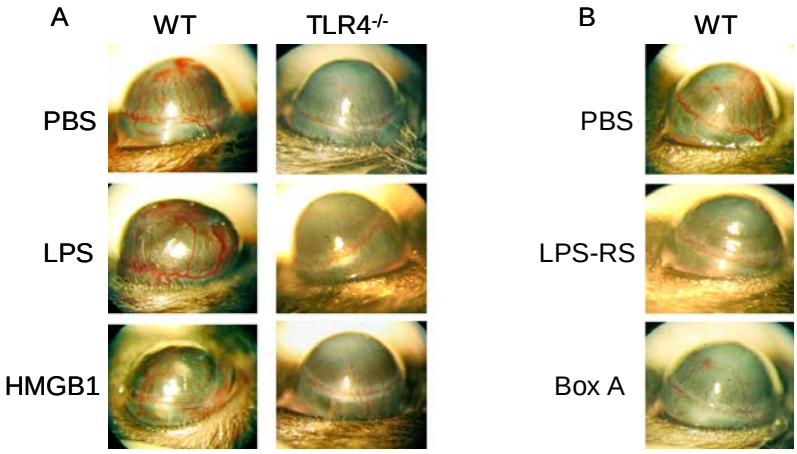
Supplementary Figure I



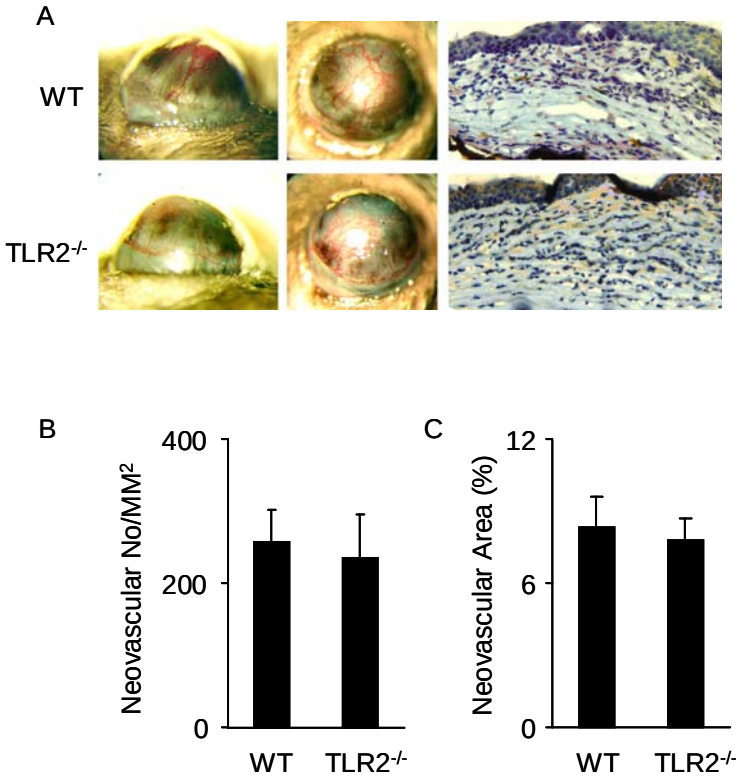
Supplementary Figure II



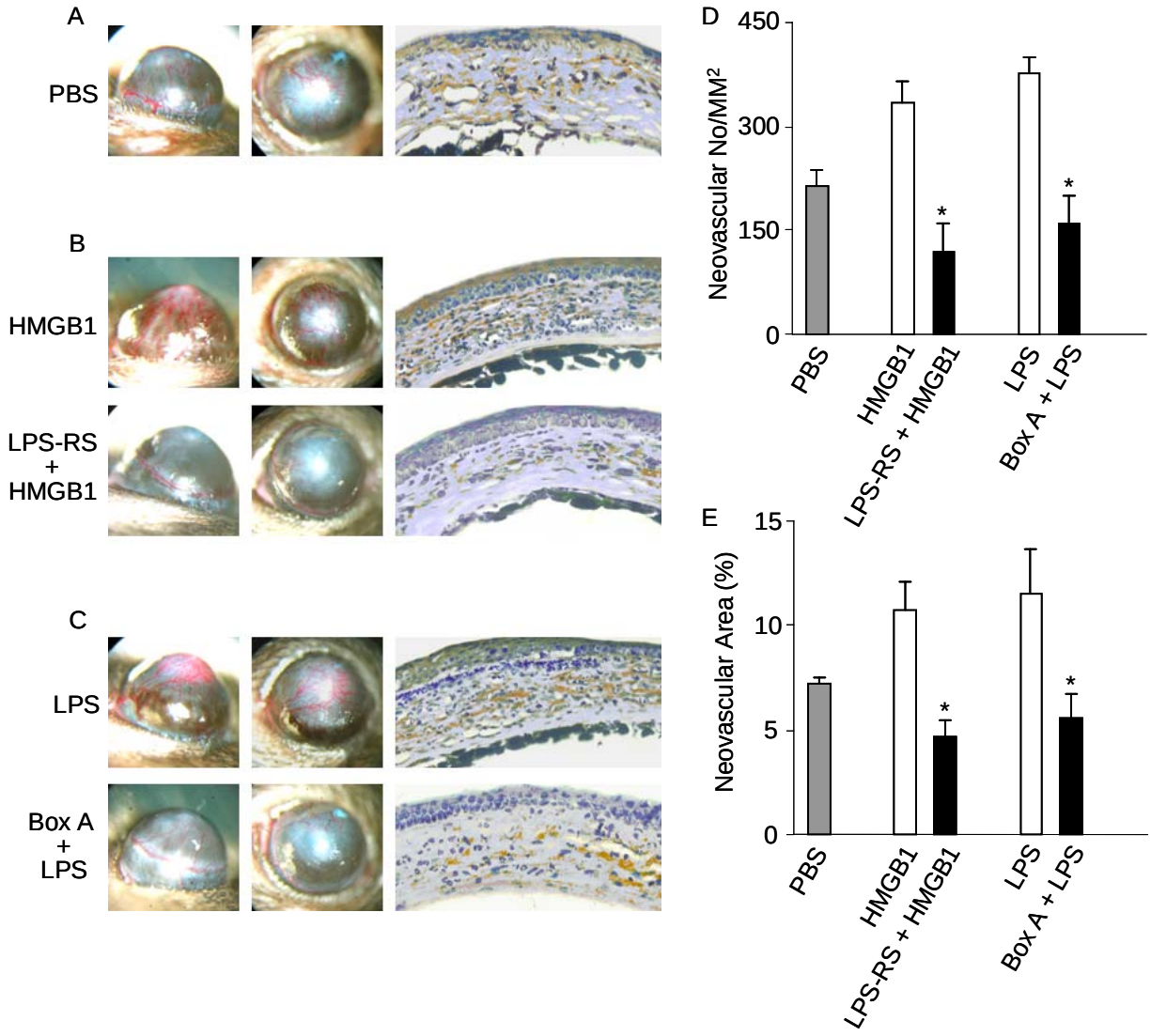
Supplementary Figure III



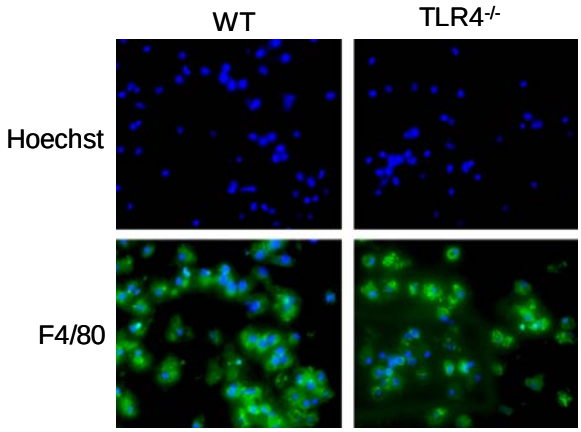
Supplementary Figure IV



Supplementary Figure V



Supplementary Figure VI



Reference

1. Lin Q, Fang J, Fang D, Li B, Zhou H, Su SB. Production of recombinant human HMGB1 and anti-HMGB1 rabbit serum. *Int Immunopharmacol*; In press.