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

Qing Lin, Xiaop Ping Yang, Dan Fang, Xiangrong Ren, Hongyan Zhou, Jiazhu Fang, Xialin Liu, Shiyou Zhou, Feng Wen, Xiaohong Yao, Ji Ming Wang, Shao Bo Su

Objective—Inflammation is closely linked to angiogenesis, and Toll-like receptors (TLRs) are the key mediators of inflammatory responses. However, the impact of TLRs on angiogenesis is incompletely understood. In this study, we determined the involvement of TLRs in angiogenesis.

Methods and Results—In a mouse model of alkali-induced corneal neovascularization (CNV), we found that CNV was attenuated in TLR4−/− but not TLR2−/− mice. Further study revealed that the absence of TLR4 led to decreased production of proangiogenic factors in association with reduced accumulation of macrophages at the site of wounds, which was associated with reduced expression of high-mobility group box-1 (HMGB1) protein, an endogenous ligand for TLR4. Topical application of HMGB1 to the injured cornea promoted CNV with increased macrophage accumulation in wild-type mice but not in TLR4−/− mice. HMGB1 treatment in vitro also promoted the production of proangiogenic factors by mouse macrophages in a TLR4-dependent manner. Furthermore, antagonists of HMGB1 and TLR4 reduced CNV and macrophage recruitment in the injured cornea of wild-type mice.

Conclusion—Our results suggest that the release of HMGB1 in the wounds initiates TLR4-dependent responses that contribute to neovascularization. Thus, targeting HMGB1-TLR4 signaling cascade may constitute a novel therapeutic approach to angiogenesis-related diseases. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: angiogenesis ■ macrophages ■ HMGB1 ■ inflammation ■ Toll-like receptor

Angiogenesis is a normal process in growth, development, and wound healing. It also participates in a variety of pathogenesis, such as cancer, atherosclerosis, diabetic retinopathy, rheumatoid arthritis, and corneal neovascularization (CNV). Pathological angiogenesis is linked to inflammatory responses through the production of proangiogenic mediators by innate and adaptive immune cells that express Toll-like receptors (TLRs).

TLRs are a family of pattern recognition receptors that recognize distinct molecular patterns associated with microbial pathogens, including viral or bacterial nucleic acids, lipopolysaccharide (LPS) or lipoteichoic acids, and flagellin. Thus far, 11 TLRs in human and 13 TLRs in mouse have been identified, and some of their endogenous ligands, such as heat shock proteins, surfactant protein A18, extracellular matrix components, and high-mobility group box-1 (HMGB1), are known. Recent studies showed that TLRs might be involved in angiogenesis. It is reported that TLR4 expressed on liver endothelial cells regulates the invasive capacity of liver endothelial cells by promoting extracellular protease production. Activation of TLR4 by endotoxin could induce the proliferation and differentiation of endothelial progenitor cells. Previous study also showed that activation of TLR4 by LPS induced the production of proangiogenic factor vascular endothelial growth factor (VEGF) by macrophages. However, the role of endogenous TLR4 ligands released from damaged tissues in angiogenesis remains to investigate.

HMGB1 is 1 of the endogenous ligands for TLR4. It is either passively released by injured or necrotic cells or actively secreted by monocyte/macrophages. HMGB1 has not only been demonstrated as a late mediator of sepsis but also been implicated as a putative danger signal involved in the pathogenesis of a variety of noninfectious inflammatory conditions, including autoimmunity, cancer, trauma, hemorrhagic shock, and ischemia-reperfusion injury. HMGB1 has been reported to signal mainly through 3 putative receptors, including the receptor for advanced glycation end products (RAGE), TLR2, and TLR4. Recent studies have indicated that HMGB1 might be a proangiogenic factor in tissue injury by stimulating the sprouting, proliferation, and chemotaxis of endothelial cells. HMGB1 also attracts endothelial progenitor cells and hematopoietic stem
cells to the sites of tissue injury and tumors to promote neovascularization.\textsuperscript{19,20} HMGB1 enhanced vessel density in skin wound or promoted collateral blood flow in ischemic hindlimbs of diabetic mice and increased the release angiogenic factors, such as VEGF and basic fibroblast growth factor (b-FGF), by macrophages or cultured human cardiac fibroblasts.\textsuperscript{21–24} These proangiogenic functions of HMGB1 were reported to be mediated by the RAGE signaling pathway.\textsuperscript{19,25} Mice deficient in TLR4 or RAGE but not TLR2 had a drastically reduced production of inflammatory cytokines in response to HMGB1 administration. In contrast, TLR2-deficient mice displayed an increased production of inflammatory cytokines.\textsuperscript{26} These results suggest that TLR4 but not TLR2 is a receptor involved in the induction of inflammatory processes by HMGB1 in vivo.

Mouse cornea and cultured human corneal epithelial cells express TLR2, TLR3, TLR4, TLR5, and TLR9.\textsuperscript{27} Animal model studies indicate that development of inflammation and injurious responses in cornea depends on TLR4 signaling.\textsuperscript{28,29} Because inflammation is a key process linked to angiogenesis,\textsuperscript{2} in this study, we investigated the role of HMGB1-TLR4 signaling in angiogenesis using a model of CNV in mice. We found that TLR4 but not TLR2 was involved in the development of CNV. HMGB1 from injured tissues triggered TLR4-dependent responses, including the recruitment of macrophages and the generation of proangiogenic factors. Our results addressed an essential role for HMGB1-TLR4 signaling in promoting neovascularization.

**Methods**
A detailed description of the methods is given in the Supplemental Materials, available online at http://atvb.ahajournals.org.

**Animals**
TLR2\textsuperscript{−/−} and TLR4\textsuperscript{−/−} mice were backcrossed onto the C57BL/6 background and then intercrossed to obtain the knockout genotypes and wild-type (WT) mice (control littermates). Littermates of both sexes between 8 and 12 weeks old were used in all experiments. Animals were kept in a specific pathogen-free facility. Animal care and use were in compliance with institutional guidelines.

**Recombinant Full-Length HMGB1, 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.\textsuperscript{30}

**Alkali-Induced Corneal Injury Model**
Mice were anesthetized. A 2-mm disc of filter paper saturated with 1 N NaOH was placed onto the right cornea of each mouse for 40 seconds, followed by rinsing extensively with 25 mL of PBS. In some experiments, recombinant HMGB1, Box A, LPS, or *Rhodobacter sphaeroides* LPS (LPS-RS) was applied topically to the alkali-treated eye twice a day for 7 days.

**Histology**
Immunohistochemical analyses were performed using anti-HMGB1 or anti-CD31 antibodies (Abs). As the immunofluorescence analysis...
for macrophage infiltration, the cryostat sections of isolated eyes were immunostained with fluorescein isothiocyanate–conjugated anti-F4/80 Ab. For 2-color analyses, sections were incubated with the combinations of anti-F4/80 and anti-TLR4, anti-F4/80 and anti-HMGB1, anti-CD31 and anti-TLR4, or anti-CD31 and anti-HMGB1 Abs. For an immunocytochemical analysis of VEGF expression, murine peritoneal macrophages were harvested and stimulated with recombinant HMGB1 for 24 hours and then subjected to immunocytochemical study.

Biomicroscopic Examination
Eyes were examined with a slit lamp (Zeiss, Germany) 7 and 14 days after alkali injury, and microscopic assessment was done by observers without prior knowledge of the procedures.

Enumeration of CNV
The sections were stained using anti-CD31 polyclonal Abs, and the numbers and sizes of the CNV were determined.

Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction
Total RNAs were extracted from the corneas or cultured peritoneal macrophages, and real-time polymerase chain reaction (PCR) was performed. The tested mRNA expression was finally determined after correction with GAPDH expression. Each measurement of a sample was conducted in duplicate.

Reproducibility and Statistical Analysis
Experiments were repeated at least 3 times. Results were highly reproducible. Representative results are shown in the figures. The means and SEM were calculated on all parameters determined in the study. Data were analyzed statistically using 1-way ANOVA or 2-tailed Student t test. A value of P<0.05 was accepted as statistically significant.

Results

TLR4 Is Involved in Mouse Alkali Injury–Induced CNV
To test the involvement of TLR4 in angiogenic response, we first examined neovascularization in TLR4-deficient mice with alkali-induced corneal injury. In control mice, alkali burn caused a rapid neovascularization, with limbal vessels sprouted into corneas 7 days after burn (Figure 1A). However, in TLR4-deficient mice, few new blood vessels grew into the cornea on day 7 (Figure 1A). The macroscopic CNV in WT mice reached a maximal level at 14 days after injury, whereas only a small number of vessels appeared near the limbal area in TLR4-deficient mice (Figure 1A). Immunohistochemical analysis using anti-CD31 Ab showed that vascular areas were increased to a greater extent in WT mice than in TLR4−/− mice (Figure 1B to 1D). These observations indicate that TLR4 is involved in the development of new vessels in alkali-induced injured cornea.

TLR4 Is Required for the Recruitment of Macrophages in CNV
Macrophages have been known to participate in the development of neovascularization in various tissues, including cornea.31–35 Given the fact that TLR4 is expressed on macrophages to mediate inflammatory responses,28 we examined
the effects of TLR4 deficiency on macrophage accumulation in injured corneas. There was no obvious accumulation of macrophages in the uninjured corneas of WT and TLR4−/− mice (day 0) (Figure 2A). F4/80+ macrophages started to accumulate on day 1 and reached a peak on day 4 after injury (Figure 2A and 2B). Although the kinetics of macrophage infiltration in the wounds were similar in control and TLR4−/− animals (Figure 2B), the infiltration of macrophages in TLR4−/− wounded corneas was significantly attenuated (Figure 2B). Decreased infiltration of macrophages may imply that chemokine expression is downregulated in TLR4−/− wounds. To test this hypothesis, quantitative PCRs were used to examine changes in mRNA expression of chemokines MIP-2/CXCL2, MIP-1α/CCL3, and MCP-1/CCL2, which have been shown to play a critical role in macrophage recruitment.36 All 3 chemokine genes tested were upregulated in the wounded corneas. However, these expressions were significant reduced in TLR4−/− mice (Supplemental Figure I). These observations indicate that in damaged corneas, TLR4 is critical for macrophage accumulation, which may be attributed to the TLR4-induced production of chemokines.

**TLR4 Is Required for Alkali Injury–Induced Proangiogenic Factor Gene Expression**

Macrophages are a rich source of growth factors.31 Markedly decreased infiltration of macrophages also implied that proangiogenic factor expression might be downregulated in wounded TLR4−/− corneas. To test this hypothesis, quantitative PCRs were used to examine the changes in mRNA expression of 3 angiogenic factors, VEGF, transforming growth factor (TGF) β1, and b-FGF, in injured corneas. Alkali injury markedly increased intracorneal mRNA expression of these 3 proangiogenic factors in WT and TLR4−/− mice (Figure 2C to 2E). The level of enhancement was significantly lower in TLR4−/− corneas, with the expression of VEGF increased up to 49-fold on day 2 in WT mice but only 10-fold in the TLR4−/− mice (Figure 2C). These results suggest that TLR4 plays a critical role in proangiogenic factor production by infiltrating macrophages in the wounded corneas.

**The Expression of HMGB1 and TLR4 in Injured Cornea**

We examined TLR4 expression in corneas after alkali-induced injury and found that the mRNA of TLR4 was significantly upregulated in wounded corneas (Figure 3A). Large numbers of TLR4-positive cells were found in the limbus, epithelium, and stroma of the wounded corneas before and after injury (Figure 3A). The expression of HMGB1 in corneas after alkali injury. A, Quantitative reverse transcription–PCR to assess TLR4 gene expression in corneas of WT mice. Data represent means±SEM (n=6). *P<0.05 vs normal corneas. B, Immunofluorescence analysis of TLR4 protein expression in intact or wounded cornea from WT mice. Magnification ×400. C, Real-time PCR analysis of HMGB1 expression in wounded corneas at the indicated time points. Results were expressed as mean±SEM (n=6) of fold increase over control. *P<0.05 vs WT mice. D, HMGB1 protein expression in injured corneas. Whole eyes were obtained and processed for immunohistochemical analysis using an anti-HMGB1 Ab. Magnification ×400. E, Real-time PCR analysis of RAGE expression in wounded corneas. All values represent mean±SEM (n=6). *P<0.05 vs intact corneas.
CNV. Wounding induced a transient and moderate upregulation in the RAGE mRNA expression in the corneas on day 2 (Figure 3E). These data indicate that TLR4 plays a major role in angiogenesis after the corneal injury.

TLR4-Positive Macrophages and Endothelial Cells Express HMGB1

In line with the previous observation that TLR4 is expressed on intracorneally infiltrating macrophages during fungal keratitis,28 we observed that a substantial proportion of F4/80-positive macrophages expressed TLR4 in injured cornea on day 2 postinjury (Supplemental Figure IIA). HMGB1 protein was detected in macrophages at the same time (Supplemental Figure IIA). The expression of HMGB1 by TLR4-positive macrophages in cornea suggests that triggering HMGB1-TLR4 signaling pathway may create a positive feedback loop to regulate macrophage activation. Moreover, TLR4 protein was also detected on most CD31-positive endothelial cells at the wound sites 7 days after injury, and HMGB1 was coexpressed on a minority of endothelial cells (Supplemental Figure IIB). The HMGB1-TLR4 intensity in macrophages was stronger than that in endothelial cells. These observations suggest that the recruitment of macrophages may be a key step in CNV formation.

HMGB1 Promotes CNV in a TLR4-Dependent Manner

We next used pharmacological and genetic approaches to examine the effects of the TLR4 endogenous agonist HMGB1 on angiogenesis in vivo. Recombinant HMGB1 was topically administered to wounded corneas after alkali injury. Because CNV reaches a peak on day 14 after injury (Figure 1A), we chose this time point to compare the effect of HMGB1. HMGB1 showed a notable effect on the enhancement of CNV in WT mice at 2 weeks after the injury (Figure 4A and Supplemental Figure IIIA). Similar results were observed in the corneal cryosections with anti-CD31 staining (Figure 4A, 4C, and 4D). Concomitantly, topical administration of HMGB1 augmented the number of intracorneal macrophages (Figure 4E and 4F) at the early stage of alkali-induced wounds. However, HMGB1 treatment failed to enhance CNV and macrophage accumulation in the wounds of TLR4−/− animals (Figure 4B to 4F and Supplemental Figure IIIA). Similar to HMGB1, treatment with LPS, the exogenous ligand for TLR4, also enhanced CNV and macrophage accumulation in WT mice but not in TLR4−/− mice (Figure 4A to 4F and Supplemental Figure IIIA). These data indicate that activation of TLR4 by HMGB1 enhanced the recruitment macrophages, which in turn may produce angiogenic factors and promote CNV.

HMGB1-Induced Angiogenic Factor Expression by Murine Peritoneal Macrophages Is TLR4 Dependent

Because TLR4-mediated signaling can enhance the functions of macrophages,28 we next examined the effects of exogenous HMGB1 on angiogenic factor expression by primary peritoneal murine macrophage. HMGB1 markedly increased the
HMGB1 with antagonist activity. Box A topical instillation reduced alkali-induced CNV at 2 weeks after the injury (Figure 6A to 6C and Supplemental Figure III B). We then tested the effects of the TLR4 antagonist LPS-RS on CNV. LPS-RS also reduced the degree of CNV in WT mice (Figure 6A to 6C and Supplemental Figure III B). Box A and LPS-RS were also highly effective in inhibiting alkali injury-induced cornea macrophage infiltration in WT mice (Figure 6D and 6E). These results confirm macrophages as the central effector cell type in HMGB1/TLR4-mediated angiogenesis in CNV. Furthermore, LPS-RS pretreatment significantly suppressed HMGB1-enhanced CNV, whereas Box A pretreatment reduced LPS-aggravated CNV in WT mice (Supplemental Figure V), suggesting that the effect of HMGB1 on promoting CNV is TLR4 dependent.

**Discussion**

To our knowledge, this study was the first demonstration of an essential role of the HMGB1-TLR4 signal pathway in angiogenesis in vivo.

In the vasculature, TLR4 is expressed in different cell types, such as endothelial cells, smooth muscle cells, adventitial fibroblasts, dendritic cells, macrophages, and endothelial progenitor cells. TLR4 signaling has been shown to play an important role in the pathogenesis of atherosclerosis and the models of hepatic, pulmonary, brain myocardial, and renal ischemia/reperfusion injury. In ocular surface tissues, activation of TLR4 by LPS mediates inflammatory responses in keratits. TLR4 is also involved in corneal injury, including corneal incision, corneal epithelium scraping, and corneal suture. In vitro, LPS-induced TLR4 signaling in primary cultures of the epithelial cells and fibroblasts from cornea conjunctiva also induces the production of inflammatory cytokines, chemokines, growth factors, and adhesion molecules. Our findings showed that topical treatment with LPS promotes alkali-induced CNV in WT mice but not in TLR4−/− mice. In specific pathogen-free conditions without any exogenous agonist treatment, mice deficient in TLR4 showed reduced CNV, suggesting that under sterile conditions without PAMPs, TLR4 could be activated by endogenous agonists produced by damaged tissues or infiltrating immune cells in the injured cornea, such as HMGB1.

HMGB1 is a nuclear protein that acts as a cytokine when released into the extracellular milieu by necrotic and inflammatory cells. A large body of evidence indicates that HMGB1 is required for the development or progression of inflammation in the absence of infection, as occurs during experimental autoimmune arthritis, cerebral ischemia, hemorrhagic shock, pancreatitis, sterile hepatic necrosis, and other conditions that lead to inflammation and tissue injury. Recently, HMGB1 has been recognized as a putative proangiogenic factor mediated by RAGE based on the ability of HMGB1 to induce endothelial cell sprouting, proliferation, and chemotaxis. HMGB1 may also attract endothelial progenitor cells and hematopoietic stem cells to the sites of injured tissues and tumors. Blockade of HMGB1 binding to RAGE with soluble RAGE or anti-RAGE Ab inhibited angiogenesis. HMGB1 has been implicated in several

**Alkali-Induced CNV Is Not Dependent on TLR2**

Because TLR2 has also been reported to recognize HMGB1, we analyzed alkali-induced corneal angiogenesis in TLR2−/− mice. We found that CNV developed in TLR2 mutant mice was not different from that in WT animals (Supplemental Figure IV). These data suggest that CNV induction is primarily dependent on TLR4 but not on TLR2.

**Reduction of CNV and Macrophage Recruitment by Antagonists of HMGB1 and TLR4**

We then attempted to block the activity of endogenous HMGB1 by topical administration of Box A, a fragment of TLR4 and TGFβ1 markedly reduced expression of the genes for VEGF, b-FGF, and TGFβ1 in peritoneal macrophages, with an increase in the expression of the VEGF gene of 40.5-fold as compared with untreated cells (Figure 5A). Immunofluorescence analysis showed that HMGB1-stimulated macrophages exhibited increased VEGF protein expression as compared with untreated cells (Figure 5B). In contrast, macrophage from TLR4−/− mice showed markedly reduced expression of the genes for VEGF, b-FGF, and TGFβ1; in particular, the magnitudes in VEGF expression were approximately 80% lower in the TLR4−/− macrophage compared with the WT macrophage (Figure 5A). This is associated with a decrease of VEGF protein expression in TLR4−/− macrophages (Figure 5B). These results confirmed the observations in vivo, indicating that HMGB1 induces the production of angiogenic factors by macrophage via TLR4-dependent mechanisms.

Figure 5. The effects of HMGB1 on angiogenic factor expression by murine peritoneal macrophages. A, Peritoneal macrophages from WT and TLR4−/− mice were incubated with 1.0 μg/mL recombinant HMGB1 for 12 hours. The levels of VEGF, b-FGF, and TGFβ1 mRNA were determined by quantitative reverse transcription–PCR, and data represent means ± SEM. **P<0.05 vs WT mice. B, Peritoneal macrophages from WT mice were stimulated in the absence (upper panels) or presence of HMGB1 (lower panels) for 24 hours. The cells were stained with anti-VEGF Ab and then Cy3-conjugated secondary Ab and Hoechst 33342. Original magnification, ×400.
vasculature-related diseases, including tumor, and diabetes, arthritis, and atherosclerosis. In our study, we found that injury induced the expression of HMGB1 in mouse cornea. We revealed that exogenous HMGB1 enhanced CNV and macrophage recruitment. Treatment with Box A inhibited CNV with reduced macrophage accumulation in WT mice but not in TLR4-deficient mice, indicating that HMGB1-enhanced CNV was TLR4 dependent. Furthermore, LPS-RS inhibited HMGB1-enhanced CNV and Box A inhibited LPS-promoted CNV in WT mice (Supplemental Figure V). Because Box A functions by competitive binding to the receptors for HMGB1, including TLR4, LPS-RS is known as a specific antagonist for TLR4, these findings confirmed the involvement of HMGB1 and TLR4 in CNV formation.

Macrophages, the main source of HMGB1 under inflammatory conditions, are proposed to play central roles in tissue repair based on the observations that these cells produce various growth and angiogenic factors. It has also been reported that macrophages are essential in corneal and choroidal neovascularization because they produce angiogenic mediators. Previous reports have shown that systemic or local macrophage depletion inhibited pathological neovascularization in several ocular models. In our study, the majority of TLR4- and HMGB1-positive cells were F4/80 positive cells in the injured corneas. Moreover, in vitro, the main HMGB1-induced angiogenic factor from murine peritoneal macrophages of WT mice but not of TLR4-/- mice was VEGF. These observations support the hypothesis that HMGB1 activates macrophages in a positive feedback mechanism to sustain inflammation and angiogenesis cascade under pathological conditions. Recent studies showed that the receptor binding of HMGB1 is TLR4 dependent and the cysteine in position 106 within the HMGB1 B box is required for the binding. Consistently, our results, suggest that the corneal injury induced the extracellular release of HMGB1, which may act as an autocrine or paracrine mediator to stimulate the infiltrating macrophages to produce more chemokines, proangiogenic factors, and HMGB1 itself in a TLR4-dependent manner, which contributes to the neovascularization.

To date, 3 putative HMGB1 receptors, RAGE, TLR2, and TLR4, have been reported. Although TLR2 was expressed in normal human and mouse corneal epithelial cells, our data indicate that there is no effect of deleting TLR2 on induction of CNV. One possibility, as suggested, is that TLR2 acts as a receptor for nucleosome-bound HMGB1 but not for free HMGB1. Our data also showed that in murine cornea, RAGE mRNA is inducible after injury. Although the kinetics of alkali injury-induced RAGE expression were similar to those of TLR4, the magnitudes were markedly lower, and TLR4 is sufficient to promote CNV, suggesting that TLR4 may play a major role in this process. However, the possibility that HMGB1 may interact with the other purported cellular receptors in the process of CNV development requires further investigation.

Recently, HMGB1-TLR4 signaling has been implicated in the inflammatory process of a vast array of disease models. HMGB1-TLR4 interactions also regulate the processing and presentation of tumor antigens by dendritic cells, which is considered as important for the death-driven immunoadjuvant effects of chemotherapy. More recently, the proinflammatory role of TLR4-HMGB1 axis in the model of acute and chronic seizures or skin tumor development has also been established. A 2-step inflammation-induced skin tumorigenesis model showed that carcinogen-induced inflammation was dependent on endogenous HMGB1 but not on skin bacteria–associated LPS. In addition, we found that
TLR4 was involved in the aberrant angiogenesis in an oxygen-induced retinopathy model (data not shown). Because TLR4 and HMGB1 are detected in the retina, our data suggest that the HMGB1–TLR4 signaling cascade may also contribute to the retinal neovascularization induced by ischemic injury.

In conclusion, targeting the HMGB1–TLR4 signaling pathway may be a novel therapeutic approach for a variety of vasculature-related disorders, such as atherosclerosis, diabetic retinopathy, rheumatoid arthritis, psoriasis, cancer, and as CNV.

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Disclosures
None.

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SUPPLEMENTARY MATERIALS

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

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Shiyou Zhou, Feng Wen, Xiaohong Yao, Ji Ming Wang and Shao Bo Su

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). ExScript™ 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. 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 mm² 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 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 1min, and 72 °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% CO₂.

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 × 10⁴ 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% CO₂ 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

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<thead>
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<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
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</table>
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⁻ⁿCOR{} 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 mm² 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

A

MIP-2/CXCL2

Fold change

Day 1 3 5

B

MCP-1/CCL2

Fold change

Day 1 3 5

C

MIP-1α/CCL3

Fold change

Day 1 3 5

WT

TLR4−/−

*
Supplementary Figure II

A

B
Supplementary Figure III
Supplementary Figure IV

A

WT

TLR2−/

B

C

WT

TLR2−/

Neovascular No/MM²

Neovascular Area (%)

0

6

12
Supplementary Figure V

A

PBS

B

HMGB1

LPS-RS + HMGB1

LPS

Box A + LPS

D

Neovascular No/MM²

E

Neovascular Area (%)

PBS  HMGB1  LPS  Box A + LPS

Box A  LPS

HMGB1  LPS-RS + HMGB1  LPS  Box A + LPS

*
Supplementary Figure VI
Reference