Angiogenesis Mediated by Toll-Like Receptor 4 in Ischemic Neural Tissue

Chang He, Yuying Sun, Xiangrong Ren, Qing Lin, Xiao Hu, Xi Huang, Shao-Bo Su, Yizhi Liu, Xialin Liu

Objective—Activation of the immune system via toll-like receptors (TLRs) is implicated in atherosclerosis, microvascular complications, and angiogenesis. However, the involvement of TLRs in inflammation-associated angiogenesis in ischemic neural tissue has not been investigated. The goal of this study is to determine the role of TLR4 signaling in oxygen-induced neovascularization in retina, a neural tissue.

Methods and Results—In oxygen-induced retinopathy model, we found that retinal neovascularization was significantly attenuated in TLR4−/− mice. The further study revealed that the absence of TLR4 led to downregulation of proinflammatory factors in association with the attenuated activation of glia in the ischemic retina, which was also associated with reduced expression of high-mobility group box-1, an endogenous ligand for TLR4. The application of high-mobility group box-1 to the ischemic retina promoted the production of proinflammatory factors in wild-type but not TLR4−/− mice. High-mobility group box-1 treatment in vitro also significantly promoted the production of proinflammatory factors in retinal glial cells from wild-type mice, but much less from TLR4−/− mice.

Conclusion—Our results suggest that the release of high-mobility group box-1 in ischemic neural tissue initiates TLR4-dependent responses that contribute to neovascularization. These findings represented a previously unrecognized effect of TLR4 on angiogenesis in association with the activation of glia in ischemic neural tissue. (Arterioscler Thromb Vasc Biol. 2013;33:330-338.)

Key Words: angiogenesis ■ glial cells ■ HMGB1 ■ Toll-like receptor 4

Increasing evidence has demonstrated that inflammation is an important pathogenetic factor in the development of vascular injury and angiogenesis. The inflammatory responses often present in angiogenesis can be called inflammation-associated neovascularization.1,2 Activation of the immune system via toll-like receptors (TLRs) is implicated in several vascular-related diseases, including atherosclerosis,3,5 diabetic nephropathy,4 and tumor angiogenesis.7 The mechanism of TLR mediation of inflammation-associated angiogenesis, however, has not been investigated in ischemic neural tissue.

TLRs, a family of evolutionarily preserved pattern recognition receptors, have emerged as a key component of the innate immune and adaptive immune system.5,9 Pathological angiogenesis is linked to inflammatory responses through the production of proinflammatory or proangiogenic mediators by the involved cells, which might express TLRs.14,10 Among the 13 TLRs, TLR2 and TLR4 have been reported to play a critical role in inflammation-associated angiogenesis.4,11

It has been shown that, in the absence of pathogens, TLR4 signaling can be activated by molecules released by injured tissue. These molecules, called damage-associated molecular patterns, include high-mobility group box-1 (HMGB1), a nearly ubiquitous chromatin component that is passively released by necrotic cells, retained by cells undergoing programmed death (apoptosis), and actively secreted by cells in profound distress.12-14 HMGB1 has been shown to enhance vessel density in skin wounds of diabetic mice and to increase the release of angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).15,16 Beijnum reported the role of HMGB1 in tumor angiogenesis by showing that its overexpression is associated with an increased angiogenic potential of endothelial cells.7 In a previous study, we also found that corneal injury can initiate HMGB1 release, which is involved in corneal angiogenesis by activating TLR4 signaling.17

TLR4 has been reported to have broad expression in the central nervous system.18-21 In addition to being expressed on the classic immune cells, TLRs are also expressed on the endothelial, neuronal, and glial cells, exerting their inflammatory effects in various vascular and neural pathological conditions, including atherosclerosis,22 ischemia-reperfusion injury,23 Alzheimer disease,24 and the model of acute and chronic seizures in the central nervous system.21 The retina is a neural tissue comprising large numbers of neurons, glia,
and microvascular network. It is highly sensitive in response to oxygen deficiency. Because the HMGB1-TLR4 axis has been implicated in the inflammatory process of many disease models, and the proinflammatory role of TLR4 in several vasculature-related disorders has been reported, we sought to investigate whether TLR4 is an important mediator in retinal angiogenesis.

The presence of TLR4 on retinal pigment epithelium cells, photoreceptors, and glia and the resultant TLR signaling in retinal cells also suggest that these molecules may play an important role in innate and adaptive immune responses within the retina. Moreover, it has been reported that proinflammatory cytokines, such as transforming growth factor (TGF)-β, interleukin (IL)-1β, and IL-6, were rapidly upregulated under ischemic conditions in astrocytes or Müller cells, microglia, and endothelial cells in the retina in rat and mouse models. TLR4 signaling activation might be connected with the upregulation of those proinflammatory or angiogenic cytokines.

In this study, we established the retinal angiogenesis model through oxygen-induced retinopathy (OIR) in TLR4−/− and wild-type (WT [C57BL/6J]) mice to determine the significance of HMGB1-TLR4 in the angiogenesis process of ischemia-induced retinal neovascularization (RNV). We found that TLR4 signaling was involved in the process of RNV. TLR4-deficiency retarded the HMGB1-TLR4 signaling responses and resulted in decreased RNV. HMGB1 from injured tissues triggered TLR4-dependent responses, also involving the activation of local astrocytes or Müller cells, and the generation of a proinflammatory or proangiogenic factors. Our results revealed a role for HMGB1-TLR4 signaling in promoting neovascularization in neural tissue.

Methods

An expanded Methods section is available in the online-only Data Supplement.

Animals

TLR4−/− mice were originally produced by Dr S. Akira (Osaka University, Osaka, Japan). Newborn animals of both TLR4−/− and WT (C57BL/6J) (littermates of both sexes between 7 and 24 days old) from mature mice were used in all experiments. Animals were kept in a specific pathogen-free facility in Animal Laboratories of Zhongshan Ophthalmic Center, Guangzhou, China. All experiments were approved by the Institutional Animal Care and Use Committee.

Recombinant Full-Length HMGB1

The cloning, expression, and purification of recombinant full-length HMGB1 were carried out as previously described. The recombinant proteins were highly purified and functional. In addition, to exclude lipopolysaccharide (LPS) contamination, we measured LPS content in purified HMGB1 by the chromogenic Limulus amebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD). The LPS content was <0.16 EU/mg (below the detection limit).

Established Retinal Angiogenesis Model in Mouse

According to a previously described method, we used the following procedures to produce the OIR model. On postnatal day 7 (P7), the mouse pups (both TLR4−/− and WT) and their nursing mother were placed in an airtight incubator (own production) ventilated by a mixture of oxygen and air to a final oxygen fraction of 75±5%. OIR mice were returned to normal room air at P12.

Quantification of RNV in the Flat-Mounted Retinas

Twenty TLR4−/− and 20 WT-OIR mice were used for imaging quantification of RNV. On P12, P17, and P21, for fluorescein isothiocyanate (FITC)-dextran angiography, animals were anesthetized, and 1 mL high molecular weight FITC–dextran (diluted to concentration 50 mg/mL in PBS) was injected into the left ventricle and allowed to circulate. The neurosensory retinas were carefully removed and the radial cuts were made in the peripheral retina to allow whole flat-mounting on glass slides. For the Griffonia Simplicifolia (GS)-isolecitin staining, the retinas were incubated for 2 hours at room temperature with biotinylated GS isolecitin B4 diluted in PBS (1:100). Retinal whole mounts were examined by fluorescence microscopy (BH2-RFC, Olympus, Hamburg, Germany). According to a previously described method, quantitative analysis of the vasculature and vascular obliteration were performed on the flat-mounted FITC-dextran–perfused or GS isolecitin staining retinas from each group by using a computerized system with Image-Pro Plus 5.1 software (Media Cybernetics).

Retinal Cross-Sections and Quantitation of Pre-ILM Neovascular Cell Nuclei

According to a previously described method, on P12, P17, and P21, the animals (10 in each group) were euthanized and both eyes were enucleated and fixed, serially sectioned for preinternal limiting membrane (ILM) nuclei counting. Over 50 serial 6-μm paraffin-embedded axial sections were obtained, and the standard hematoxylin and eosin staining was performed. All retinal vascular cell nuclei anterior to the ILM were counted in each section, using a fully masked protocol. The mean of the 10 counted sections yielded average neovascular cell nuclei per 6-μm section per eye.

Immunofluorescence Staining

The enucleated animal eyes were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Fine Technical) overnight for cryosectioning, and then 5-μm serial sections were cut. The slides were incubated with primary antibodies TLR4, HMGB1, glial fibrillary acidic protein (GFAP), and CD31 in a humidified chamber overnight at 4°C in a double immunofluorescence staining manner (1:100 mouse anti-TLR4, 1:100 goat polyclonal anti-HMGB1; 1:500 rabbit anti-GFAP, and 1:100 rat anti-CD31 antibody). Then the slides were rinsed 3× with 0.01% phosphate-buffered saline and tween-20 and incubated with fluorescently labeled secondary antibodies.

Real-Time Quantitative RT-PCR for Detecting Gene Expression

Retinal specimens from 2 mice (4 eyes) were pooled together. Total RNAs were extracted from the mice retinal or cultured Müller cells, and the cDNA was prepared. Real-time polymerase chain reaction (PCR) was performed on an Applied Biosystems StepOne Real-Time PCR System using the comparative threshold cycle quantification method. Each reaction contained 12.5 μL of 2×SYBR green Master Mix, 300 nmol/L oligonucleotide primers for HMGB1, nuclear factor-kB, VEGF, b-FGF, TGF-β1, IL-1β, IL-4, TLR4, receptor for advanced glycation end products (RAGE) (Table I in the online-only Data Supplement) 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.

Intraocular Injections With HMGB1

On P12, when TLR4−/− or WT-OIR mice were returned to room air, they received intravitreal injections of HMGB1 in the right eye (twenty in each group). Intravitreal injections were performed using a 5-μL Hamilton syringe with a 32-gauge needle, according to a previously described method. The eye was punctured at the upper
Retinal Glial Cell Culture and Treatment With HMGB1

Retinal glial cells were obtained from isolated retinas of postnatal day P7–12 WT and TLR4−/− mice, using a method modified from Hicks and Courtois.46 Briefly, retinas were dissected and cut into small pieces and incubated with culture medium. Müller cells were identified by immunofluorescence staining with GS (glutamine synthetase). After continuous incubation at 37°C in a humidified environment with 5% CO2 for 2 days, Müller cells were washed with PBS, and cells were suspended in new medium. Müller cells were cultured at hypoxic condition (treated with CoCl2). Then 1.0 μg/mL of recombinant HMGB1 or 0.1 μg/mL LPS (Sigma, St Louis, MO) and blank vehicle were added. For each treated well, cells were incubated for 24 hours, 36 hours, 48 hours, and 72 hours. After being washed with PBS, cells were split with 1 mL trizol (invitrogen) and exact RNA used for reverse transcriptase-PCR. In addition, Polymyxin B (10 μg/mL), a cyclic cationic polypeptide antibiotic known to specifically block the biological effect of LPS, was also included in Müller cells culture before HMGB1 treatment as a control group to exclude the LPS contamination.

Statistic Analysis

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 Ischemia-Induced Retinal Neovascularization in OIR Model

To evaluate the involvement of TLR4 in ischemia-induced retinal angiogenic response, we first examined neovascularization in TLR4−/− deficient mice in an OIR model. Figure 1 shows FITC-dextran–perfused retinas from 12, 17, and 21-day-old mice that had been exposed to hyperoxia from P7 to P12. In control WT mice, oxygen induction caused rapid RNV, which peaked at P17; however, in TLR4−/− deficient mice, a significant attenuated neovascularization was observed. In contrast, the normal vessels are more apparent in TLR4−/− mice. Figure 2 shows typical immunostaining with GS isoelectin, demonstrating the highest level of RNV at P17 (Figure 2A and 2B). The macroscopic RNV in WT mice reached a maximal level at P17 after oxygen induction, whereas a relatively small number of vessels appeared somewhat neovascular in TLR4-deficient OIR mice. Figure 2E and 2F are the representative photographs, illustrating neovascular cell nuclei anterior to the ILM. Consistent with the FITC-dextran–perfused retinas and GS-isolectin staining imaging analysis, the number of neovascular cell nuclei in the TLR4−/− group is significantly decreased compared with the counterpart control (Figure 2G). In addition, we performed CD31 staining to further characterize neovascular cells as shown in the Figure I in the online-only Data Supplement. These observations indicate that TLR4 is involved in pathological retinal angiogenesis in the OIR model.

The Expression of TLR4 and HMGB1 in Ischemic Retina

Immunofluorescence analysis showed markedly enhanced TLR4 expression in ischemic retina compared with the lower level expression of TLR4 in normal retina in non-OIR mice (Figure 3). Large numbers of TLR4-positive cells were displayed in the ischemic retina among the different layers, including ganglion cell layer, photoreceptors, and retinal pigment epithelium. To test whether HMGB1 is involved in ischemic retinopathy, we evaluated HMGB1 expression in the retina before and after ischemic injury in TLR4−/− mice and WT mice. Figure 4 shows representative immunofluorescence staining of the retinal cryosections labeled with HMGB1, demonstrating the HMGB1 expression in both OIR models. HMGB1 mRNA expression analysis revealed that ischemic injury significantly enhanced the HMGB1 mRNA expression in WT-OIR mice. Interestingly, however, the magnitude of HMGB1 mRNA expression was markedly lower in TLR4−/− mice concomitantly throughout a 5-day period of observation (Figure 5). These results indicated that inactivation of TLR4 might result in impaired injury-induced HMGB1 production.

In addition, we have examined RAGE (another major receptor for HMGB1) expression in retina. Figure II in the online-only Data Supplement shows that there was a very low RAGE expression in normal and ischemic retina of OIR model. However, there was a remarkably increased expression of TLR4 mRNA in OIR retina.

![Figure 1](http://atvb.ahajournals.org/)

Figure 1. Representative pictures of the retinal flat-mount with fluorescein isothiocyanate (FITC)-dextran perfusion. The typical appearance of ischemic retinopathy at P12, P17, and P21 of the wild-type oxygen-induced retinopathy (OIR) model is shown in the Top: The central avascular area is a typical feature of the OIR model at P12. The blood vessel tufts, presumed retinal neovascularization, as well as blood vessel tortuosity are shown obviously at P17. The neovascularization was withdrawn with time at P21. As compared with the wild-type (WT) mice, significantly attenuated neovascularization and avascular (vascular obliteration) area were observed in the FITC-dextran–perfused retinas from Toll-like receptor (TLR) 4−/− OIR mice. Moreover, in contrast, the normal vessels are more apparent in TLR4−/− mice.
TLR4 Is Associated With the Glia Activation in OIR

Given that TLR4 is expressed on glia to mediate inflammatory responses, and that activation of glial cells, such as astrocytes and Müller cells, are related to the new blood vessel growth, we examined the effects of TLR4 on Müller cell activation in ischemic retina in TLR4-deficiency mice. Double staining showed that these activated TLR4 positive astrocytes or Müller cells are strongly positive for TLR4. In contrast, less GFAP immunoreactivity in Müller cells processes was observed in TLR4−/− OIR model (Figure 3). Obviously, there is a strong GFAP expression in the WT OIR model at P17, but significant attenuated expression in TLR4−/− mice. In addition, there are some double positive cells with CD31 and TLR4, indicating CD31+ cells (endothelial cells) can also express TLR4 (Figure III in the online-only Data Supplement).

Similarly, Figure 4 revealed that HMGB1 was significantly increased and distributed in ILM, ganglion cell layer, and inner nuclear layer of the retina with more intensive GFAP positive astrocytes and Müller cells in WT OIR model, whereas less HMGB1 was released in TLR4−/− OIR retina with a less GFAP immunoreactivity of astrocytes or Müller cells. These observations indicate that TLR4 is critical for activation of astrocytes and Müller cells in ischemic retina.

TLR4 Is Required for the Ischemic-Induced Proinflammatory Factor Gene Expression In Vivo

Retinal glial cells are a rich source of growth factors. Müller cell activation often resulted in release of proinflammatory or proangiogenic factors. Markedly attenuated Müller cell activation implied that proinflammatory or proangiogenic factor expressions might be downregulated in ischemic TLR4−/− retina. To test this hypothesis, quantitative real-time PCR (n=10) was used to examine the changes in mRNA expression of these proinflammatory or proangiogenic factors, including nuclear factor-kB, VEGF, b-FGF, TGF-β1, IL-1β, and IL-6 in ischemic retina. Ischemic injury markedly increased retinal mRNA expression of these proinflammatory or proangiogenic factors, including nuclear factor-kB, VEGF, b-FGF, TGF-β1, IL-1β, and IL-6 in ischemic retina. Ischemic injury markedly increased retinal mRNA expression of these proinflammatory or proangiogenic factors, including nuclear factor-kB, VEGF, b-FGF, TGF-β1, IL-1β, and IL-6 in ischemic retina. Ischemic injury markedly increased retinal mRNA expression of these proinflammatory or proangiogenic factors, including nuclear factor-kB, VEGF, b-FGF, TGF-β1, IL-1β, and IL-6 in ischemic retina.

The number of GFAP+ cell nuclei anterior to ILM was significantly increased in TLR4−/− OIR model (Figure 3). Obviously, there is a strong GFAP expression in the WT OIR model at P17, but significant attenuated expression in TLR4−/− mice. In addition, there are some double positive cells with CD31 and TLR4, indicating CD31+ cells (endothelial cells) can also express TLR4 (Figure III in the online-only Data Supplement).

HMGBl Activated TLR4 and Promoted the Angiogenic Factor Expression

In vivo, we performed intraocular injection with HMGB1 in an OIR model. We found significantly increased mRNA expression for VEGF, b-FGF, and IL-6 in the WT-OIR model, which was consistent with our in vitro observations. These results suggest that the TLR4 signaling pathway plays a critical role in proangiogenic factor production by activating glia in the ischemia retina. The reliability of the quantitative reverse transcriptase-PCR data were further verified, as similar results were also obtained by normalizing quantitative reverse transcriptase-PCR results with expression of the β-actin, another selected housekeeping gene (Data shown in Figure IV in the online-only Data Supplement).
model compared with the TLR4−/− OIR mouse model (Figure 7A). We also examined the expression of TLR4 in cultured Müller cells at hypoxic condition and the changes of expression of some proinflammatory cytokines in HMGB1-treated Müller cells through quantitative real-time PCR. Figure V in the online-only Data Supplement shows the mRNA expression of TLR4 in Müller cells at hypoxic condition in vitro; TLR4 expression is enhanced in Müller cells treated with HMGB1 for 72 hours. Importantly, there is no significant decrease in TLR4 mRNA expression in the group treated with HMGB1+ polymyxin B as compared with HMGB1 treatment group, indicating that the enhanced expression of TLR4 induced by HMGB1 was not attributable to LPS contamination.

Furthermore, the effects of exogenous HMGB1 on expression of those proinflammatory or proangiogenic factors by Müller cells obtained from TLR4−/− and WT mice were evaluated. Figure 7B shows that HMGB1 markedly increased the expression of the genes for VEGF, b-FGF, and TGF-β1 in Müller cells. In contrast, retinal glia cells from TLR4−/− mice showed markedly reduced expression of genes for VEGF, b-FGF; in particular, the magnitudes in VEGF expression were ≈3-1 fold decreased in TLR4−/− Müller cells compared with WT Müller cells after treatment with HMGB1 for 72 hours. These results are consistent with observations in vivo, indicating that HMGB1 can induce the production of angiogenic factors by Müller cells via TLR4-dependent mechanisms.
Ischemia-induced RNV has been reported to be associated with inflammation. This study demonstrated that activation of TLR4 signaling-associated inflammatory response contributes to neovascularization in the retina of the ischemia-induced angiogenesis model. 

Without exogenous pathogen-associated ligands, TLR4 could be activated by HMGB1, an endogenous agonist produced by damaged tissue or infiltrating immune cells in the injured sites. In this study, the findings revealed that oxygen-induced retinal injury significantly enhanced the HMGB1 mRNA expression in wild-type (WT) oxygen-induced retinopathy (OIR) mice compared with a relative lower expression in toll-like receptor (TLR) 4−/− mice at both P12 (2.52±0.54 vs 1.55±0.39, *P<0.05, n=10) and P17 (1.85±0.37 vs 0.98±0.32 *P<0.05, n=10).

**Discussion**

Ischemia-induced RNV has been reported to be associated with inflammation. This study demonstrated that activation of TLR4 signaling-associated inflammatory response contributes to neovascularization in the retina of the ischemia-induced angiogenesis model.

Without exogenous pathogen-associated ligands, TLR4 could be activated by HMGB1, an endogenous agonist produced by damaged tissue or infiltrating immune cells in the injured sites. In this study, the findings revealed that oxygen-induced retinal injury promotes the expression of HMGB1 and TLR4, indicating that ischemia initiates HMGB1-TLR4 signaling pathway in the retina. Furthermore, HMGB1 release is associated with TLR4 expression, and TLR4 ablation suppresses the release of HMGB1, indicating that their interaction may regulate RNV through an autocrine or paracrine mechanism. Exogenous HMGB1 intraocular injection enhanced the production of proinflammatory factors in the WT-OIR model, but not in TLR4−/− OIR mice, further suggesting that HMGB1-TLR4 signaling plays an important role in immune-inflammation-mediated retinal angiogenesis.

To date, 3 putative HMGB1 receptors have been reported: RAGE, TLR4, and TLR2. Our data showed that there was a very low RAGE expression in normal and ischemic retina of OIR model, however, a remarkably increased expression of TLR4 mRNA in OIR retina (Figure II in the online-only Data Supplement). Furthermore, we found HMGB1-induced angiogenesis was suppressed in TLR4−/− mice, but not in TLR2−/− mice (data not shown). These data suggest that TLR4 may play a predominant role as a major receptor of HMGB1 in RNV.

HMGB1-TLR4 axis has been implicated in the inflammatory process of several vasculature-related disease models. This study demonstrated that activation of TLR4 signaling-associated inflammatory response contributes to neovascularization in the retina of the ischemia-induced angiogenesis model.

Without exogenous pathogen-associated ligands, TLR4 could be activated by HMGB1, an endogenous agonist produced by damaged tissue or infiltrating immune cells in the injured sites. In this study, the findings revealed that oxygen-induced retinal injury promotes the expression of HMGB1 and TLR4, indicating that ischemia initiates HMGB1-TLR4 signaling pathway in the retina. Furthermore, HMGB1 release is associated with TLR4 expression, and TLR4 ablation suppresses the release of HMGB1, indicating that their interaction may regulate RNV through an autocrine or paracrine mechanism. Exogenous HMGB1 intraocular injection enhanced the production of proinflammatory factors in the WT-OIR model, but not in TLR4−/− OIR mice, further suggesting that HMGB1-TLR4 signaling plays an important role in immune-inflammation-mediated retinal angiogenesis.
The downstream pathway of the TLR4 signaling regarding VEGF production and its effect on angiogenesis has been demonstrated in angiogenic response in damaged tissue.56,57 The study of ischemic neural tissue, indicating that there will be a new paradigm in exploring the role and mechanism of immune-inflammatory response mediated by glia in angiogenesis, particularly in ischemic neural tissue.

In summary, using an acute oxygen-induced ischemic retinopathy model in TLR4−/− and WT mice, we discovered an unrecognized pathway involving HMGB1 and its interaction with TLR4 in retinal angiogenesis. Our results suggest that TLR4-deficiency could retard the HMGB1-TLR4–mediated response and downregulate the expression of nuclear factor-κB and VEGF, resulting in decreased neovascularization. In addition, retinal glia activation is involved in the TLR4-associated inflammatory-angiogenesis in the retina. These results represent a previously unrecognized effect of TLRs on RNV and reveal a new potential mechanism of angiogenesis associated with glia activation in ischemic neural tissue. It means that the extent of RNV with ischemia depends on the immuneinflammation-associated TLR4 gene background to some extent. Thus, targeting the HMGB1-TLR4 signaling cascade may constitute a novel therapeutic approach to angiogenesis-related diseases.

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Disclosures
None.
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Supplement Material

Expanded Materials and Methods

Animals

TLR4−/− mice were originally produced by Dr. S. Akira (Osaka University, Osaka, Japan). These mice were backcrossed ten or more generations onto the C57BL/6J background, and were then intercrossed to obtain the knockout genotypes and WT mice (as control). Newborn animals (littermates of both sexes between 7 and 24 days old) from mature mice were used in all experiments. Animals were kept in a specific pathogen-free (SPF) facility and given water and standard laboratory chow ad libitum in Animal Laboratories of Zhongshan Ophthalmic Center, Guangzhou, China. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center.

Reagents and Abs

High molecular weight Fluorescein isothiocyanate–dextran (FITC-dextran) was purchased from Sigma-Aldrich, St. Louis, MO. Biotinylated Griffonia Simplicifolia isolectin B4 (GS isolectin) was purchased from Vector Laboratories, Burlingame, CA. mouse anti-TLR4 was purchased from Abcam, Cambridge, MA. rabbit anti-GFAP polyclonal antibody was purchased from Dako, Denmark. Goat polyclonal anti-HMGB1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rat
anti-CD31 antibody was purchased from Becton Dickinson. Alexa Fluor® 594 Donkey Anti-Rabbit IgG (H+L), Alexa Fluor® 594 Donkey Anti-Rat IgG (H+L), Alexa Fluor® 488 Donkey Anti-mouse IgG (H+L), Alexa Fluor® 488 Donkey Anti-goat IgG (H+L), TRIzol reagent and DAPI were purchased from Invitrogen (Carlsbad, CA, USA). ExScriptTM RT reagent kit was purchased from TaKaRa (TaKaRa Biotechnology Co. Ltd. DaLian, China). Brilliant SYBR® Green QPCR Master Mix was purchased from Stratagene (La Jolla, CA USA). Most of the other reagents such as salt and buffer components were analytical grade and obtained from Sigma. Purified LPS from E. Coli 011:B4 strain was purchased from Sigma Inc. (St. Louis, MO, USA).

Recombinant full-length HMGB1 preparation

The cloning, expression and purification of recombinant full-length HMGB1 were carried out as previously described. Genes coding for human HMGB1 were cloned by PCR and inserted into pET28a vectors for transformation of Escherichia coli BL21. The E. coli expressed proteins were then highly purified with a Ni2+-NTA column and the endotoxin content was removed. In addition, to exclude LPS contamination, we measured LPS content in our purified HMGB1 by the chromogenic Limulus amebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD). The LPS content was less than 0.16 EU/mg (below the detection limit).

Established retinal angiogenesis model in Mouse
The OIR model has been widely studied as an animal model of ischemia-induced retinal neovascular disease. According to a previously described method of Smith et al.,38 we used the following procedures to produce the OIR model. On postnatal day 7 (P7), the mouse pups (both TLR4<sup>−/−</sup> and WT) and their nursing mother were placed in an airtight incubator (own production) ventilated by a mixture of oxygen and air to a final oxygen fraction of 75 ± 5%. Oxygen levels were checked at least three times a day. OIR mice were returned to normal room air at P12. TLR4<sup>−/−</sup> mice and WT mice underwent the OIR procedure, and untreated WT mice were used as normal controls to evaluate the HMGB1 and TLR4 expression in retina. The mouse retinas were isolated for retinal neovascularization evaluation, histology analysis, immunohistochemical staining, and RNA extraction from isolated retinas for further study.

**Quantification of retinal neovascularization in the flat-mounted retinas**

The TLR4<sup>−/−</sup> and WT-OIR mice were used for imaging quantification of retinal neovascularization. On P12, P17 and P21, for FITC-dextran angiography, animals were anesthetized, and approximately 1 ml high molecular weight Fluorescein isothiocyanate–dextran (FITC-dextran, diluted to concentration 50 mg/mL in PBS) was injected into the left ventricle and allowed to circulate for approximately 2 minutes, after which the animals were euthanatized. The eyeballs were then enucleated and put into 4% paraformaldehyde to fix for 45 min at room temperature. The anterior segment was cut off and the neurosensory retina carefully removed.
Radial cuts were made in the peripheral retina to allow whole flat-mounting on glass slides. Then the retina in the liquid which can avoid fluorescent quenching was covered with a coverslip. In addition, for the GS-isolectin staining, the retinas were washed with phosphate buffer solution (1×PBS) and incubated for 2 h at room temperature with biotinylated Griffonia Simplicifolia isoelectin B4 diluted in PBS (1:100). Retinas were washed with PBS three times at 10-min intervals.

Retinal whole mounts were examined by fluorescence microscopy (BH2-RFC, Olympus, Hamburg, Germany). Total images of flat-mounted retina were produced from 5-8 pieces of images acquired using a fluorescence microscope. Images were obtained using a high-resolution charge-coupled device (CCD) camera (DP30BP; OLYMPUS, Japan) via a computerized system. According to a previously described method,\textsuperscript{39-41} quantitative analysis of the vasculature and vascular obliteration were performed on the flat-mounted FITC-dextran-perfused or GS isolectin staining retinas from each group by using a computerized system with Image-Pro Plus 5.1 software (Media Cybernetics, USA). In brief, the area of vascular obliteration was measured by careful delineation of the avascular zones in the central retina of the fluorescein-dextran-perfused retinas by two individuals using a fully masked protocol. Similarly, the area of neovascularization (tufts) was carefully delineated by using confocal images focused on the preretinal plane and selecting tufts based on pixel intensities. Selected regions were then summed to generate the total area of neovascularization and the total area was calculated using Image-Pro Plus.1. Student’s t test was used to statistically compare the different experimental groups.
Retinal Cross-Sections and Quantitation of Pre-ILM neovascular cell Nuclei

According to a previously described method\textsuperscript{38,42,43} on P12, P17 and P21, the animals were sacrificed and both eyes were enucleated and fixed in the 4% buffered formaldehyde for 4-6 hours, serially sectioned in their entirety for pre-ILM nuclei counting were as described in Smith et al. Standard hematoxylin and eosin (H&E) staining was performed. Over 50 serial 6-μm paraffin-embedded axial sections were obtained starting at the optic nerve head. After staining with hematoxylin and eosin, 10 intact sections of equal length, each 30 μm apart, were evaluated for a span of 300 μm. All retinal vascular cell nuclei anterior to the internal limiting membrane were counted in each section, using a fully masked protocol. The mean of the 10 counted sections yielded average neovascular cell nuclei per 6-μm section per eye. No vascular cell nuclei anterior to the internal limiting membrane were observed in normal, unmanipulated animals.

Immunofluorescence Staining

The animal eyes were enucleated and embedded in Tissue-Tek OCT compound (Sakura Fine Technical) overnight for cryosectioning and then 5-μm serial sections were cut. Frozen sections of mouse eyes were dried at room temperature and fixed in cold acetone for 10 min. Then the fixed sections were incubated in 0.5% Triton X-100 for 5 min. Sections were washed with phosphate-buffered saline (PBS) and blocked with 5% BSA in PBS for 1 hour at room temperature in a moist chamber. The slides
were then incubated with appropriate primary antibodies overnight at 4 °C. Then the slides were rinsed three times with PBS/0.01% Tween-20 (PBST) and incubated with fluorescently labeled secondary antibodies at room temperature for 1 h. Slides were thoroughly washed with PBS/Tween-20 three times at 5-min intervals and cell nuclei were stained with DAPI at a dilution of 1:2000 in PBS for 5 min. For double staining between TLR4 or HMGB1 and GFAP, the sections were incubated with both primary antibodies (TLR4 at 1:100 or HMGB1 at 1:100 and GFAP at 1:500) overnight and then Alexa Fluor® 594 Donkey Anti-Rabbit IgG (H+L) at 1:400 and Alexa Fluor® 488 Donkey Anti-mouse IgG (H+L) at 1:400 or Alexa Fluor® 488 Donkey Anti-goat IgG (H+L) at 1:400 for 1 hour. For double staining between TLR4 and CD31, 1:100 mouse anti-TLR4 and 1:100 rat anti-CD31 antibodies were applied overnight, and then Alexa Fluor® 594 Donkey Anti-Rat IgG (H+L) and Alexa Fluor® 488 Donkey Anti-mouse IgG (H+L) at 1:400 were applied for 1 hour. Finally, the slides were mounted and analyzed by a Zeiss LSM 510 META Confocal microscope (Carl Zeiss, Oberkochen, Germany). Fluorescence pictures were taken with identical exposure settings. For negative control, slides stained with BSA in place of primary antibodies showed no signals.

**Real-time quantitative RT-PCR for detecting gene expression**

Retinal specimens from 2 mice (4 eyes) were pooled together. Total RNAs were extracted from the mice retinal and cultured Müller cells with a TRIzol, and the cDNA was prepared by reverse transcriptase reagent kit (TaKaRa Biotechnology Co.
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 for HMGB1, NF-kB, VEGF, b-FGF, TGF-β1, IL-1β, IL-6, TLR4, RAGE (Supplementary Table 1) 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 50° for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15s, 60 °C for 30s. The tested mRNA expression in each sample was finally determined after correction with GAPDH expression. Each measurement of a sample was conducted in duplicate. The reliability of the qRT-PCR data was further verified by normalizing qRT-PCR results with expression of the β-actin, another selected housekeeping genes.

**Intraocular injections with HMGB1**

On P12, when TLR4−/− or WT-OIR mice were returned to room air, they received intravitreal injections of HMGB1 in the right eye. Phosphate-buffered saline with 0.025% bovine serum albumin (PBS-BSA) was the control vehicle for injection. Mice were anesthetized with subcutaneous 50 mg kg−1 ketamine and 5 mg kg−1 xylazine and placed under a microsurgical microscope. Intravitreal injections were performed using a 5-μl Hamilton syringe with a 32-gauge needle, according to a previously described method. The eye was punctured at the upper nasal limbus, and a volume of 0.5 μl of HMGB1 solution (1.0 μg/ml of recombinant HMGB1) or control
vehicle, respectively, was injected in one eye of each mouse.

**Retinal Glial cell culture and treatment with HMGB1**

Retinal glial cells were obtained from isolated retinas of postnatal day P7-12 WT and TLR4⁻/⁻ mice, using a method modified from Hicks and Courtois.46 Briefly, retinas were dissected and cut into small pieces and incubated in Dulbecco’s modified Eagle’s medium (DMEM,Gibco) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 g/ml), and L-glutamate(0.3g/ml) at 37°C in humidified 5% CO2. The new culture medium (DMEM medium with 10% FBS) was added to cells after seeding 4-6d, and was then changed every third day. When isolated glial cells were grown to confluence in a 35-mm dish (Corning, Corning, NY), the cells were passaged 3-5 times before all experiments were performed. Müller cells were identified by immunofluorescence staining with GS (glutamine synthetase). After continuous incubation at 37°C in a humidified environment with 5% CO2 for two days, Müller cells were washed with PBS, and cells were suspended in new medium in a 6-well plate. Müller cells were cultured at hypoxic condition by treatment with CoCl2. We next tested a range of cobalt concentrations between 50 and 200 μM and established that 150 μM CoCl2 was optimal for TLR4 induction. In the new media, 1.0 μg/ml of recombinant HMGB1 or 0.1μg/ml lipopolysaccharide (LPS) (Sigma, St Louis, MO) and blank vehicle were added. For each treated well, cells were incubated for 24 h, 36 h, 48 h,72 h. After being washed with PBS, cells were split with 1ml trizol (invitrogen) and exact RNA used for RT-PCR. In addition, Polymyxin B
(10μg/ml), a cyclic cationic polypeptide antibiotic known to specifically block the biological effect of LPS, was also included in Müller cells culture before HMGB1 treatment as a control group to exclude LPS contamination.
<table>
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Figure I  Immunofluorescence staining of ischemic retina with anti-CD31

Immunofluorescence staining of retinal sections showed capillary profiles immunoreactivity for CD31 in the OIR model and normal control. There is remarkably capillary tufts (neovascular cells anterior to ILM) in the ischemic retina in OIR model, which were indentified with CD31 positive staining (indicated with arrows). ILM: internal limiting membrane.
Real-time PCR analysis of TLR4 and RAGE expression in ischemic retinas at P17 in OIR model revealed that there was a very low RAGE expression in normal and ischemic retina. However, there was a remarkably increased expression of TLR4 mRNA in OIR retina as compared to normal retina. Results were expressed as mean ± SEM (n=10) of fold increase over control. * p < 0.05
Figure III  

Double color-Immunofluorescence staining with TLR4 and CD31

CD31 labeled cells were detected either in normal or in ischemic retina. There are significantly increased CD31 positive cells in the ischemic retina as compared to the normal retina. Double positive cells with CD31 and TLR4 indicated CD31 + cells (vascular endothelial cells) can also express TLR4.
Figure IV

The changes in mRNA expression of proinflammatory factors in OIR model by quantitative RT-PCR.

Histograms represent quantification of qRT-PCR analysis of mRNA expression. The average value for each sample was normalized to the amount of β-actin. Ischemic injury markedly increased retinal mRNA expression of proinflammatory factors, including NF-κB, VEGF, b-FGF, TGF-β1, IL-1β, IL-6 in ischemic retina in WT and TLR4-/− mice at P12 and P17, but to different extents. Results were expressed as mean ± SEM (n=10) of fold increase over control. * indicates P<0.05)
Figure V  The expression of TLR4 in cultured Müller cells

Quantitative real time PCR analysis revealed the changes of mRNA expression of TLR4 in cultured Müller cells at hypoxic conditions. The expression of TLR4 is increased after HMGB1 treatment 72 hours; and there is no significant decrease in mRNA expression of TLR4 in the group treated with HMGB1+ polymyxin B as compared with HMGB1 treatment group, indicating that the enhanced expression of TLR4-induced by HMGB1 was not due to contaminating LPS (Figure S5). Results were expressed as mean ± SEM (n=10) of fold increase over control. * indicates P<0.05. PB: Polymyxin B)