A cross talk between class A scavenger receptor and receptor for advanced glycation end-products contributes to diabetic retinopathy

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Ma K, Xu Y, Wang C, Li N, Li K, Zhang Y, Li X, Yang Q, Zhang H, Zhu X, Bai H, Ben J, Ding Q, Li K, Jiang Q, Xu Y, Chen Q. A cross talk between class A scavenger receptor and receptor for advanced glycation end-products contributes to diabetic retinopathy. Am J Physiol Endocrinol Metab 307: E1153–E1165, 2014. First published October 28, 2014; doi:10.1152/ajpendo.00378.2014.—In response to hyperglycemia in patients with diabetes, many signaling pathways contribute to the pathogenesis of diabetic complications, including diabetic retinopathy (DR). Excessive production of inflammatory mediators plays an important role in this process. Amadori-glycated albumin, one of the major forms of advanced glycated end-products, has been implicated in DR by inducing inflammatory responses in microglia/macrophages. Our goal was to delineate the potential cross talk between class A scavenger receptor (SR-A) and the receptor for advanced glycated end-product (RAGE) in the context of DR. We show here that SR-A ablation caused an exacerbated form of DR in streptozotocin-injected C57BL/6J mice as evidenced by fundus imaging and electoretinography. Immunohistochemical staining and RT-PCR assay indicated that there was augmented activation of proinflammatory macrophages with upregulated synthesis of proinflammatory mediators in the retina in Sr−/− mice. Overexpression of SR-A suppressed RAGE-induced mitogen-activated protein kinase (MAPK) signaling, whereas RAGE activation in macrophages favored a proinflammatory (M1) phenotype in the absence of SR-A. Mechanistic analysis on bone marrow–derived macrophages and HEK293 cell line revealed that SR-A interacted with and inhibited the phosphorylation of mitogen-activated protein kinase kinase 7, the major kinase in the RAGE-MAPK-NF-κB signaling, thereby leading to diminished secretion of proinflammatory cytokines. Our findings suggest that the antagonism between SR-A and RAGE contributes to the pathogenesis of DR by nurturing a disease-prone macrophage phenotype. Therefore, specific agonist that boosts SR-A signaling could potentially provide benefits in the prevention and/or intervention of DR.

microglia/macrophages; inflammation; class A scavenger receptor, diabetic retinopathy

Diabetic Retinopathy (DR) is the main cause of vision loss and blindness in patients with diabetes (18). The more effective treatments for DR are urgently needed. Therefore, a clear understanding of the pathogenesis of DR would aid the development of novel and more efficient preventative/interventional strategies against DR.

Inflammation plays an important role in the development of diabetic complications (22, 37). Diabetes can induce an activation of resident microglia and infiltrated macrophages in the retina (1, 14, 38). Successful intervention of DR in vivo has been reported to accompany a decrease in inflammation (26, 28). Amadori-glycated albumin (AGA), one of the major forms of advanced glycated end-product (AGE) generated in the environment of hyperglycemia, is considered as a key inducer of proinflammatory response (3, 17, 22). The detrimental effects of AGE are largely mediated by their cognate receptor (2, 39). Receptor for AGE (RAGE) is a member of pattern recognition receptors (PRRs) superfamily surveilling intrinsic and extrinsic stress signals and directly corresponding host response (4). Once activated by AGEs, RAGE signals through different mitogen-activated protein kinases (MAPKs), which then relay the cues to nuclear factors, such as NF-κB and activator protein-1 (AP-1), to initiate transcriptional programs (8, 13, 21, 32). RAGE activation is associated with a proinflammatory and proangiogenic response. Its activity can be regulated at multiple levels (7, 15). Therefore, a definitive delineation of the underlying regulatory mechanism of RAGE signaling in the context of DR would offer invaluable insights into the development of more effective treatment options.

Class A scavenger receptor (SR-A) is another well-defined PPR primarily expressed in microglia/macrophages and is considered as an important regulator of cell migration, cell cycle, apoptosis, and gene transcription (5, 33). Mounting evidence has established a connection between SR-A expression/activity and chronic exposure to high levels of glucose both in vitro and in vivo (9, 16, 29). In addition, Usui et al. have demonstrated that SR-A is involved in the development of diabetic nephropathy (40). More recently, our laboratory has reported that deletion of SR-A deteriorates type 2 diabetes in mice as a result of desensitized insulin response (51). These data collectively argue for a pivotal role for SR-A in the maintenance (or disruption) of homeostasis during diabetes.

SR-A has been shown to have a cross talk with other PRRs in modulating physiological and pathological responses. For instance, virus sensing by Toll-like receptor (TLR) is crippled in macrophages without SR-A (47). In SR-A-deficient macrophages, lipopolysaccharide (LPS)-induced production of proinflammatory mediators, as a result of TLR4 activation, is attenuated (48). Currently, little is known whether and, if so, how SR-A communicates with RAGE in the pathogenesis of DR. Our results as summarized in this manuscript indicate that SR-A deletion exacerbates DR in mice with a shift of M1 microglia/macrophages differentiation. More importantly, we demonstrate that SR-A may negatively regulate the RAGE-NF-κB signaling by directly inhibiting mitogen-activated protein kinase kinase (MKK) 7 phosphorylation and macrophage polarization. Therefore, targeting SR-A may provide viable strategies in combating diabetic complications.
Glossary

**CoCl2** Cobalt chloride
**DR** Diabetic retinopathy
**MCP-1** Monocyte chemotactic protein 1
**MKK6** Mitogen-activated protein kinase kinase 6
**MKK7** Mitogen-activated protein kinase kinase 7
**PMB** Polymyxin-B
**SR-A** Class A scavenger receptor

**Methods**

*Animals.* Ten-week-old male C57Bl/6j mice and C57/6 background Sr-a<sup>−/−</sup> mice (Jackson Laboratories, Bar Harbor, ME) without carrying the rd8 mutation were used in this study. Mice were bred and maintained under specific pathogen free level conditions with a 12:12-h light-dark cycle and were fed regular chow diet and water. Diabetic mice were generated by a single intraperitoneal injection of streptozotocin (150 mg/kg in citrate buffer, 100 mmol/l, pH 4.5; Sigma) as previously described (50). Nondiabetic control mice were injected with an equal volume of vehicle. To determine the diabetes status of the animals, blood glucose was measured at day 7 after the streptozotocin injection. The fasting blood glucose level higher than 13 mmol/l was considered diabetic. All animal procedures were performed in accordance with the Principles of Laboratory Animal Care (National Institutes of Health publication 85–23, revised 1985).

*Cell culture.* Bone marrow-derived macrophages (BMDMs) were prepared as previously described (43). BMDMs were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 20% L929 cell supernatant, 0.5% RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum prepared as previously described (43). BMDMs were maintained in 13 mmol/l was considered diabetic. All animal procedures were performed in accordance with the Principles of Laboratory Animal Care (National Institutes of Health publication 85–23, revised 1985).

*Cell viability assay.* Cell viability was determined by cell counting kit-8 (CCK8) assay. Briefly, cells were treated with AGA or signaling molecule inhibitors (U-0126, SP-610025, or SB-203580; Sigma-Aldrich). Absorbance was measured using a microplate reader (SYNERGYmx; Biotek).

*DNA extraction.* Total DNA from retina tissues of mice or cells was extracted using RNaseo plus (TaKaRa, Shiga, Japan). RT reactions were performed using standard methods, and real-time PCR analysis was performed using SYBR Green (ABI Prism 7500 Sequence Detection System; Applied Biosystems). Quantities of all targets in test samples were normalized to 18S by standard curve calculation.

*Immunoprecipitation.* Antibody against SR-A (E20) (Santa Cruz Biotechnology), MKK7 (ABCAM), and normal goat IgG (Santa Cruz Biotechnology) were used. Cell extracts (500–1,200 µg) were incubated with 4 µg of antibody overnight at 4°C, followed by incubation with 40 µl of Protein A/G Plus-Sepharose beads (Santa Cruz Biotechnology) for 4 h at 4°C. The beads were washed with ice-cold PBS. Immunocomplexes were eluted by boiling in 2X SDS loading buffer for 5 min before Western blot analysis. Cobalt chloride (CoCl<sub>2</sub>), fucoidan, and LPS used in this study were purchased from Sigma-Aldrich.

*Luciferase assay.* HEK293 cells were transfected with His-tagged RAGE and SR-A plasmids. Cells were cultured in 24-well plates and on the second day transiently transfected with pGL3-NF-κB-Luc or an AP-1 promoter luciferase reporter plasmid. The plasmid pcDNA3.1 was used as control. In all experiments, the total amount of DNA was kept constant by adding various concentrations of the appropriate control plasmids. Luciferase activities were assayed 24–48 h after transfection using a luciferase reporter assay system (Promega). Experiments were routinely performed in triplicate wells and repeated three times.

*RNA interference.* Small-interfering RNA (siRNA) kits for RAGE and MKK7 were purchased from Santa Cruz Biotechnology. For each siRNA experiment, we used more than two sequences to knock down the target genes and selected the most efficacious one or two sequences for the further experiments. A scrambled siRNA was used as a negative control. BMDMs were seeded 1 day before transfection of Opti-MEM (Invitrogen) without serum supplemented with Lipofectamine 2000 (Invitrogen). Various concentrations of siRNA were added for 48 h before collection for experiments.

**Enzyme-linked immune-sorbent assay.** Culture supernatant of BMDMs and tissue lysates were determined using mouse tumor necrosis factor (TNF)-α and vascular endothelial growth factor (VEGF) enzyme-linked immune-sorbent assay (ELISA) kits (eBioscience) according to the manufacturers’ protocols.

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*Dual-labeled immunofluorescence BMDMs.** BMDMs were plated in the glass bottom cell culture disc (NEST, Wuxi, China). Primary antibody [MKK7 1:100 (Abcam), SR-A 1:100 (BDcam)] and secondary antibody were added before being incubated with DAPI (Sigma-Aldrich) according to the manufacturer’s protocols. Images were taken using a confocal BMDMs excited at 488 and 543 nm. All of the images were acquired with a ×63 oil immersion objective on a laser-scanning confocal microscope (ZEISS LSM 790).

*Immunohistochemistry stain.** To examine the microglia/macrophages in retina, eyes were removed from dead mice and evaluated immunohistochemically. The rabbit polyclonal antibodies against F4/80 (Abcam) and against CD68 (Cell Signaling) were used as primary antibodies. Sections were exposed to the appropriate secondary antibodies according to the manufacturers’ protocols.

*Hematoxylin and eosin stain.** Eyes were immediately placed in Davidson’s fixative (11% glacial acetic acid, 2% neutral buffered formalin, and 32% ethanol in H<sub>2</sub>O) for 24 h, transferred to 70% ethanol for 24 h, and stored in PBS at 4°C. Eyes were embedded in paraffin, sectioned to 4 µm, and stained with hematoxylin and eosin (H&E) as described in detail previously (50).

*Flow cytometry.** BMDMs were incubated with FITC-labeled antibody against F4/80 (AbD; Serotec, Kidlington, UK), Alexa Fluor 647-labeled antibody against CD11c (eBioscience), and Alexa Fluor 488-labeled antibody against CD301 (eBioscience). After PBS washing, labeled BMDMs were detected by flow cytometry (BD FACSCalibur). Each panel depicts data gathered from 10,000 individual cells. Gating strategy on M1 or M2 BMDM was consistent with the previous work (20).

*Fundus fluorescein angiography.** Fundus fluorescein angiography (FFA) was performed using a fundus camera (Heidelberg V2) after topical administration of one drop each of 1% tropicamide and 2.5% phenylephrine ophthalmic solutions for pupil dilation. Mice were anesthetized with a combination of ketamine and xylazine and then
injected intraperitoneally with 10% sodium fluorescein dye after pupil dilation. The passage of fluorescein through the retinal vascular system was serially recorded using a fundus camera equipped with exciter and barrier filters suitable for FFA. Tortuosity analyses and statics were assessed up to third-generation vessel branches (44).

**Electroretinography.** Electroretinography (ERG) was recorded to evaluate the retina function as described previously (49). Wild-type (WT) control (n = 15), Sr-a−/− control (n = 16), WT diabetic (n = 16), and Sr-a−/− diabetic (n = 15) mice were measured under anesthetized conditions (100 mg/kg ketamine and 9 mg/kg xylazine ip and proparacaine hydrochloride 0.5% eye drops) after being kept in the dark for 24 h. Eyes were dilated with atropine sulfate (1%). The signal for record was amplified 2,000 times through a grass P511 amplifier with a band pass frequency of 0.1–1,000 Hz and digitized for 250 ms at a sampling rate of 15,000 samples/s. ERG waves were recorded by at least five stimuli, separated by 30 s. The intensity of a Ganzfeld flash stimulation was 9 cd/m² attenuated by a 0.3-log unit neutral density filter. The A wave amplitude of the ERG signal was measured from the baseline to the first negative trough, whereas the B wave was calculated from the A wave trough to the maximum positivity that followed it, on a signal that had previously gone through the low-pass filter to reduce the contamination of the overlying oscillatory potentials.

**Retinal permeability.** Retinal permeability was assessed according to previous studies (25). In brief, mice were anesthetized, and 30 mg/kg Evans Blue (Sigma) solution was administered via the femoral vein. After 2 h, mice were killed and perfused with citrate-buffered saline. Evans Blue dye in retinal sections was eluted in 0.4 ml formamide (Sigma) for 18 h at 70°C. The extract was ultracentrifuged at 70,000 rpm through a 5-kDa filter for 1 h. Absorbance was measured at 620 nm. Background absorbance was measured at 740 nm. Data are presented as fold change mean ± SE of at least 6 mice/group.

**Cell death assay.** Cell death in retina was measured by Cell Death Detection plus ELISA (Roche Diagnostics, Mannheim, Germany), which detect apoptotic but not necrotic cell death. Retina samples were prepared and assayed according to the manufacturer’s instructions.

**Statistical analysis.** Data are expressed as means ± SE. Statistical significance between two groups was determined using two-tailed Student’s test. ANOVA was used to determine the significance among groups. P < 0.05 was considered statistically significant.

**RESULTS**

**SR-A deficiency aggravates DR in mice.** To investigate the role of SR-A in the pathogenesis of DR, C57Bl/6j mice were induced to develop diabetes by intraperitoneal injection of streptozotocin. In keeping with a previous study (40), there was no significant difference in levels of fasting blood glucose and GHbA1c between WT and Sr-a−/− mice from the onset of diabetes (data not shown). However, Sr-a−/− mice appeared to have developed a more serious form of DR by 13 wk after the onset of diabetes. H&E staining analysis showed that, compared with WT littermates, the number of cells in the retinal ganglion cell layer (GCL) was more severely decreased in Sr-a−/− mice under diabetic conditions. In addition, the reduction of outer and inner plexiform layer thickness in Sr-a−/− mice was more preeminent than the WT mice, indicative of a more robust structural change (Fig. 1, A–D). In vivo imaging also showed that retinal vessels became more disordered and tortuous (Fig. 1E), accompanied by pathological phenomena of more vascular exudates in the Sr-a−/− diabetic groups (Fig. 1F). Increased DNA fragmentation was also found in the diabetic knockout group (Fig. 1H). These data all pointed to a deteriorated retinal function in the absence of SR-A. Indeed, diabetes resulted in extended explicit times, a phenotype that was worsened in Sr-a−/− mice as opposed to WT mice, revealing decreased visual function (Fig. 1I). Thus, SR-A deficiency aggravates DR, leading to deteriorated retinal function in mice.

**AGA induces a skewed M1 microglia/macrophages polarization in Sr-a−/− mice.** SR-A is primarily expressed in microglia/macrophages, which play an important role in the inflammatory responses in retinopathy (35). To evaluate the effect of SR-A on microglia/macrophage activities in the context of DR, we performed immunohistochemical staining of F4/80 and CD68, markers of microglia/macrophages, in mouse retina sections. Microglia/macrophages activation was more vigorous in SR-A ablation diabetic retina (Fig. 2A). Macrophage polarization, of which SR-A is a primary modulator, influences cellular inflammatory response (20). Therefore, we examined the expression levels of M1 and M2 markers in the retina. As shown in Fig. 2B, M1 markers (e.g., II-1β, II-6, and Mcp-1) were universally upregulated in the retina of diabetic Sr-a−/− mice compared with diabetic WT mice. Meanwhile, expression levels of M2 markers were not significantly altered (Fig. 3A).

To clarify the role of SR-A in the phenotypic modulation of microglia/macrophages in diabetic retina, we used AGA to treat BMDMs (22, 23). The AGA used was endotoxin free, and treatment with AGA did not influence cell viability (Fig. 1G). Instead, AGA treatment resulted in a significant increase in mRNA levels of M1 markers in Sr-a−/− BMDMs with a concomitant decrease of M2 markers, including Il-10, Cd16/32, and Cd206 (Fig. 2, C and B). Similar changes were confirmed by measurements of protein levels of these cytokines in the culture medium and diabetic retinal tissue (Fig. 3, F and G). Furthermore, FACS analysis showed that SR-A ablation caused a significant increase in F4/80+ and ly6c+ (M1) BMDMs and a simultaneous decrease in F4/80− and Cd301+ (M2) BMDMs following AGA treatment (Fig. 3, D and E). When M1 phenotype was evaluated with a different criterion (F4/80+/CD11c+), we reached the same conclusion that SR-A deletion promotes M1 macrophage polarization in response to AGA treatment (Fig. 3C). Collectively, these data indicated that SR-A deletion might help shape a proinflammatory microenvironment in the retina by influencing microglia/macrophages polarization.

**SR-A selectively suppresses AGA-induced JNK signaling in macrophages.** To understand the mechanism behind the modulation of macrophage polarization by SR-A in response to hyperglycemia, we examined MAPK signaling in the mouse retina. As shown in Fig. 4A, JNK activation was unregulated, whereas ERK activation was suppressed in Sr-a−/− diabetic mice compared with WT diabetic mice. In the meantime, p38 phosphorylation remained unaltered with or without SR-A. These changes were accompanied by increased phosphorylation of IkBa and augmented activities of NF-κB and AP-1 in diabetic Sr-a−/− mice as evidenced by gel shift assay (Fig. 4, B–D).

These data were further validated in vitro in AGA-treated BMDMs. In response to AGA treatment, Sr-a−/− BMDMs exhibited a stronger phosphorylation of JNK in SR-A-deficient BMDMs compared with WT BMDMs. There was not a statistical difference in p38 phosphorylation between these two AGA-treated groups. Treatment with AGA did not alter the
phosphorylation status of ERK1/2, c-raf, and AKT in BMDMs (Fig. 4F). However, AGA induced a stronger IkBα phosphorylation and higher binding activities of NF-κB and AP-1 in Sr-α/−/− BMDMs compared with WT cells (Fig. 4, E, G, and H). Thus, these data indicated that SR-A deficiency may lead to an activation of JNK-NF-κB signaling in microglia/macrophages under the condition of diabetes.

**SR-A suppresses the RAGE-mediated signaling pathway by inhibiting MKK7.** RAGE is another receptor for AGA that is also linked to JNK-NF-κB signaling in microglia/macrophages and contributes to retinal inflammation in diabetes (36). We investigated the potential cross talk between SR-A and RAGE in macrophages. When SR-A was overexpressed in HEK293 cells along with RAGE, it dose-dependently suppressed phosphorylation of JNK and p38 in the presence of AGA without altering either RAGE expression or ERK phosphorylation (Fig. 5A). However, there was no impact of SR-A on activities of JNK and MKK7 in SR-A-expressing HEK293 cells (Fig. 5B). To evaluate a broader biological significance of AGA-induced changes, we transfected NF-κB and AP-1 reporter constructs for MAPKKs. Based on this finding, we postulated that SR-A inhibits RAGE signaling by suppressing MKK7 phosphorylation.

**RAGE is required for AGA-induced inflammatory response in macrophages.** To verify the causal role of RAGE in AGA-induced MAPK activation and inflammatory factor release, endogenous RAGE was knocked down by RNA interference. We found that silencing of RAGE was sufficient to suppress AGA-induced phosphorylation of JNK and p38 even in Sr-α/−/− BMDMs (Fig. 7A). In accordance, knock down of RAGE resulted in a decrease in proinflammatory factors, including IL-1β, Tnf-α, and Vegf (Fig. 7, B–D). These results strongly support that RAGE is required for AGA-induced activation of JNK and p38 kinases and consequently induction of proinflammatory mediators.

**MKK7 is essential for AGA-induced inflammatory response in macrophages.** Finally, we tackled the question whether MKK7 is dispensable for AGA-induced synthesis of proinflammatory mediators. Transfection of MKK7-targeting siRNA decreased MKK7 expression in BMDMs (Fig. 7E). Meanwhile, AGA-induced production of IL-1β, Tnf-α, and Vegf was blunted in both WT and Sr-α/−/− BMDMs (Fig. 7, F–H). In light of these results, we found that SR-A may interact with the MKK7-JNK-NF-κB pathway to influence the hyperglycemia-associated macrophage phenotype.

**DISCUSSION**

With a list of diverse ligands, SR-A positions itself as a versatile stressor capable of coordinating a wide range of pathophysiological processes. Investigations in recent years have confirmed that SR-A-dependent regulation of macrophage phenotypes contributes to the pathogenesis of hypertension, atherosclerosis, cardiac hypertrophy, carcinogenesis, and stroke (5, 20, 46). Now, we present evidence that SR-A, by tuning RAGE-mediated MKK7-JNK-NF-κB signaling in macrophages, participates in the development of DR.

Macrophage-related inflammation is involved in the pathogenesis of diabetes and its complications. However, the role of infiltrated macrophages in development of DR is still disputable. The present study suggests a positive role of macrophages in DR. Macrophages in the diabetic retina may be due to the breakdown of the blood-ocular barrier, in-
creasing vessel permeability or intraocular bleeding, allowing circulating macrophages to enter into the retina. In addition, increased expression of inflammatory cytokines or reactive oxygen species can also promote vascular leakage and/or occlusion (1, 11, 14). At this point, they are likely interdependent.

SR-A activity is invariably and intimately associated with macrophage phenotypic modulation. Recent investigations by our laboratory and others have made a strong case for SR-A-mediated macrophage polarization in the pathogenesis of myocardial infarction (20), stroke (46), lung carcinogenesis (5), insulin resistance (51), and diabetic nephropathy (50). Of note, SR-A deletion exerts

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**Fig. 2. Effects of SR-A on AGA-induced BMDM phenotypic changes.**

**A:** Immunohistochemical staining of F4/80 and CD68 in mouse retina sections. Representative images are 1 of at least 5 independent experiments. All data are means ± SE. *P < 0.05.**

**B**: Transcription level of Tnf-α, Il-1β, Il-12, Mcp-1, vascular endothelial growth factor (Vegf), and Il-6 in the normal or diabetic mouse retina. mRNA levels of cytokines were measured by RT-PCR. *P < 0.05.

**C**: mRNA levels of Tnf-α, Il-1β, Il-12, Mcp-1, Vegf, and Il-6 in BMDMs. Cells were treated with 200 μg/ml AGA or BSA for 6 h. *P < 0.05.
distinct effects on stress-induced organ injuries. For instance, we observed that SR-A deficiency leads to alleviation of ischemic cerebral injury but exacerbates ischemic myocardial injury (20, 46). Similarly, Usui and colleagues have shown that Sr-a/−/− mice are protected from diabetic nephropathy, in contrast to our assertion that SR-A loss-of-function contributes to a deteriorated phenotype of DR (40). These apparently inconsistent observations made in various animal models seem to reinforce the notion that regulation of macrophage function by SR-A is influenced by both the intracellular signaling network and extracellular cues. Indeed, ample evidence seems to support, at least in vitro, the model in which SR-A can either promote or dampen the MAPK signaling pathway depending on the specific receptor SR-A communicates with. Ohnishi et al. have shown that SR-A deficiency enhances the phosphorylation of p38, ERK, and JNK when TLR4 is activated (34). We demonstrate here that activation of RAGE by AGA evokes stronger phosphorylation of JNK, but not ERK or p38, in SR-A-deficient macrophages. Therefore, macrophage can be educated to exert either a proinflammatory or an anti-inflammatory role as a consequence of the different inputs received and different cross talks forged by SR-A.

Fig. 3. Effects of SR-A on AGA-induced BMDM phenotypic changes. A: transcription level of Arg-1, Cd16/32, Il-10, and Cd206 in the mouse retina. *P < 0.05. B: transcription level of Arg-1, Cd16/32, Il-10, and Cd206 in BMDMs. *P < 0.05. Cells were treated with 200 μg/ml AGA or BSA. C: flow cytometry analysis for differentiation of AGA-induced BMDMs. Cells were treated with 200 μg/ml AGA for 24 h. Relative percentage of F4/80- and CD11c-positive BMDMs is presented. *P < 0.05. D: flow cytometry analysis for differentiation of AGA-induced BMDMs. Cells were treated with 200 μg/ml AGA or BSA for 24 h. Relative percentages of F4/80- and ly6c-positive (M1 type) BMDMs are presented. *P < 0.05. E: flow cytometry analysis for F4/80- and CD301-positive (M2 type) BMDMs. Relative percentage are presented. *P < 0.05. F: levels of TNF-α and VEGF produced by BMDMs. Cells were treated with 200 μg/ml AGA for 24 h. Levels of cytokines in the culture medium were measured by ELISA. *P < 0.05. G: ELISA measurements of TNF-α levels in retina by total protein in each group determined by ELISA assay. *P < 0.05.
Fig. 4. Changes in signal molecule activities in the mouse retina. A: Western blot of mitogen-activated protein kinase (MAPK) signaling in the mouse retina. Representative blots are presented. Results from three independent experiments are quantitatively analyzed. Results are expressed as means ± SE of triplicate samples. *P < 0.05. B: Western blot of phosphorylated (p)-IkBα and IkBα in the mouse retina. Results are expressed as means ± SE of triplicate samples. *P < 0.05. C: NF-κB DNA-binding activities in nucleoprotein from the mouse retina lysates determined by EMSA. Results are expressed as means ± SE of triplicate samples. *P < 0.05. D: activator protein-1 (AP-1) DNA-binding activities in nucleoprotein from the mouse retina lysates determined by EMSA. Results are expressed as means ± SE of triplicate samples. *P < 0.05. E: Western blot of p-IκBα and IκBα in BMDMs Cells were incubated with 200 μg/ml AGA for 30 min. *P < 0.05. F: Western blot of signal molecules in BMDMs. Cells were incubated with 200 μg/ml AGA at 37°C for 0, 15, 30, and 60 min for Western blot of signal molecules in cell lysates. Representative blot is 1 of 3 independent experiments. G: NF-κB DNA-binding activities in nucleoprotein from BMDM lysates determined by EMSA. Results are expressed as means ± SE of triplicate samples. *P < 0.05. H: AP-1 DNA-binding activities in nucleoprotein from BMDM lysates determined by EMSA. Results are expressed as means ± SE of triplicate samples. *P < 0.05.
Several recent publications seem to echo our conclusion. Davis and colleagues demonstrate that macrophage-specific deletion of JNK attenuates insulin resistance in mice that can be attributed to diminished M1 polarization (19). In addition, RAGE-null mice display improved nerve regeneration in a model of diabetic neuropathy because of enhanced M2 trans-differentiation (27). On the contrary, Marchetti et al. have shown that selective M2 polarization promotes tissue repair in ischemic retinopathy (30). Therefore, a strong case can be made for the SR-A-RAGE interplay in determining macrophage function and the progression of DR.

MKK7 specifically signals to activate the JNK pathway (12). MKK7 is responsive to a range of stimuli, including IL-1β, TNF-α and cellular stresses (31). Of intrigue, MKK7 can be activated by signals of nutrition surplus, such as saturated fatty acids, amino acid, fructose, and glucose, indicating that MKK7 might contribute to the pathogenesis of metabolic diseases (10, 24, 42). Consistently, our data shown here suggest that SR-A can inhibit MKK7 activation in macrophages. This likely explains the exacerbated phenotype observed in Sr-a-deficient mice, since MKK7 activation can lead to unchecked angiogenesis and inflammation (41, 45). In addition, our data also suggest that MKK7 can directly influence macrophage phenotype after the engagement of RAGE, since MKK7 knock down by siRNA suppressed production of proinflammatory cytokines (Fig. 6, F and G). To our knowledge, this is the first clear demonstration that MKK7, downstream of RAGE activation, can contribute to M1 macrophage polarization. In contrast, Bhattacharjee et al. have reported that MKK3/MKK6 can signal to p38 to promote M2 macrophage differentiation by IL-13 (6). It is interesting that knock down of RAGE inhibited activation of JNK, p38, and IκBα even in the absence of its ligand (Fig. 6A), suggesting a close linkage between RAGE and the inflammatory signaling. It might be that inhibition of
JNK, p38, and IkBα signaling causes a dampening of inflammatory response to the receptor-mediated signal. In light of our new finding, it is reasonable to speculate that extracellular signals may instruct different MKKs to form separate conduits to influence macrophage phenotype. Additional studies are warranted to dissect these.

In summary, our report suggests that SR-A serve as an important checking mechanism in microglia/macrophages.
posed to AGEs to rein in RAGE-dependent inflammation. Because SR-A relies on MKK7 to oppose macrophage polarization following RAGE activation, the development of small-molecule agonist that can enhance the purported SR-A-MKK7 interaction may provide potential therapeutic solutions to the treatment of DR.

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DISCLOSURES

All the authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS

REFERENCES


