Article

Spleen Tyrosine Kinase Mediates Microglial Activation in Mice With Diabetic Retinopathy

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Purpose: Diabetic retinopathy (DR) is a leading cause of blindness in developed countries, in which microglial activation is involved. However, the mechanism of microglial activation in DR remains largely unknown.

Methods: We used Cx3cr1^{CreERT2}; Syk$^{fl/fl}$ mice to knockout microglial spleen tyrosine kinase (Syk) in the retina of mice (cKO mice) after streptozotocin injection to induce diabetes. We also isolated primary retinal microglia from wild-type and cKO mice, respectively, to explore the role of microglial Syk in DR.

Results: The deletion of microglial Syk in the retina of mice or in the primary retinal microglia inhibited microglial activation and inflammatory response, eventually leading to the improvement of DR by regulating the expressions of interferon regulatory factor 8 (Irf8) and Pu.1 both in vivo and in vitro.

Conclusions: The deletion of microglial Syk in the retina effectively ameliorated microglial activation-induced DR, suggesting the potential of microglial Syk as a therapeutic target for DR.

Translational Relevance: Microglial spleen tyrosine kinase might serve as a potential therapeutic target for diabetic retinopathy.

Introduction

Diabetic retinopathy (DR), also known as diabetic eye disease, is a common diabetic complication in which damage occurs in the retina, owing to high blood glucose.[1] The retina is the light-sensitive tissue of the blood vessels at the back of the eye, and DR caused by retinal damage is a leading cause of blindness in developed countries. At the beginning, DR might cause no symptoms or only mild vision problems, but eventually progresses to blindness.[1,2] Approximately 80% of patients who suffered diabetes for more than 20 years eventually developed DR. Moreover, the incidence of DR was positively correlated with the duration of diabetes. Therefore, proper treatment is required to delay its progression.[3,4]

The development of DR is accompanied with the activation of retinal microglia, which was mediated by Amadori-glycated albumin (AGA).[5] Spleen tyrosine kinase (Syk) as a member of tyrosine kinase family is an enzyme that could transmit signals from varieties of cell surface receptors to the cells. Emerging evidence has demonstrated that Syk plays an important role in regulating multiply immune response-related signaling pathways. Moreover, the SyK signaling pathway was discovered to mediate epithelial mesenchymal migration induced by cigarette smoke in human retinal pigment epithelial cells.[6] Previous study revealed that Syk along with Dendritic cell-associated C-type lectin-1 contributed to inflammatory activation after ischemic stroke in mice.[7] Furthermore, Syk inhibitor R406 ameliorated DR by reducing retinal inflammation via inhibiting the activation of nuclear factor kappa-light-chain-enhancer of activated B cells in streptozotocin (STZ)-induced diabetic rats.[8] However, the cellular and molecular mechanisms of microglial Syk in DR as well as its role in microglial activation remained largely unknown. Therefore, in this study, we used Cx3cr1^{CreERT2}; Syk$^{fl/fl}$ mice to knockout microglial Syk

in the retina followed by the STZ injection to induce DR in mice, and combined with in vitro studies, explored the role of microglial Syk in microglial activation, as well as the development of DR.

Methods

DR Mouse Model

Cx3cr1[CreERT2-EYFP/CreERT2-EYFP] (#021160) and Syk^{fl/fl} (#029863), purchased from the Jackson Laboratory (Bar Harbor, ME), were used to acquire Cx3cr1^{+/+}; Syk^{fl/fl} (wild type [WT]) and Cx3cr1[CreERT2-EYFP/+]; Syk^{fl/fl} (cKO) mice respectively. Seven-week-old male WT and cKO mice were orally administrated with 20 mg/kg tamoxifen (#T5648, Sigma Aldrich, St Louis, MO) dissolved in dimethyl sulfoxide/Kolliphor-EL/5% sucrose solution (1:3:6) daily for 5 consecutive days. On day 14 from the first dose of tamoxifen, mice were intraperitoneally injected with 50 mg/kg STZ (V900890, Sigma Aldrich) or phosphate-buffered saline (PBS) after 4-hour fasting daily for another 5 consecutive days. Blood samples were collected from tail vein and fasting blood glucose levels were measured using glucometer (OneTouch UltraEasy, Johnson & Johnson, New Brunswick, NJ). STZ-injected mice with fasting blood glucose of greater than 250 mg/dL were selected as diabetic mice for the following studies.[9] At week 10, which was 8 weeks after diabetes induction, after collecting urine for 24 hours, blood, and bilateral retinas of mice were collected and left retina was cut into halves for immunofluorescent staining and enzyme-linked immunosorbent assay (ELISA) respectively, and the right retina was also cut into halves for Western blot and reverse transcriptase PCR (RT-PCR). All the animal procedures were under the guideline of Gucheng County Hospital.

Flow Cytometry

Mouse peripheral blood was collected by cardiac puncture, and cells were centrifuged and lysed in red blood cell lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA), then frozen in liquid nitrogen using Cryostor media CS5 (Biolife, Sarasota, FL). Before flow cytometry, peripheral blood cells were thawed in a 37°C water bath and followed by the staining of fluorophore-conjugated anti-Gr-1 and anti-F4/80 (BioLegend, San Diego, CA) and analyzed by flow cytometry (LSR Fortessa, BD Biosciences, Franklin Lakes, NJ). Viable cells were determined by 7-AAD^- staining and analyzed further. Peripheral blood polymorphonuclear leukocyte population was represented as Gr-1^{++} F4/80^{−} cells in the peripheral blood.

ELISA

Retinal tissue collected from mice were lysed in 100 μL of lysis buffer with protease inhibitor cocktail as previously described[10] and homogenized by a Mini Bead Beater (BiosSpec Products, Bartlesville, OK). After centrifuging at $12,000 \times g$ for 10 minutes, the supernatant was used to measure the levels of tumor necrosis factor-α (TNF-α), IL-1β, and chemokine (C-C motif) ligand 2 (CCL2) in the retinal tissue. The conditional medium was also used to determine these cytokines' secretions using mouse TNF-α ELISA kit (R&D, Minneapolis, MN), mouse IL-1β ELISA kit (R&D), mouse IL-6 ELISA kit (R&D), and mouse CCL2 ELISA kit (R&D), respectively, following the manufacturer's instructions. Serum creatine and blood urea nitrogen were measured using SCR ELISA kit (cat# YS04392B, YaJi Biological, China) and blood urea nitrogen ELISA kit (cat# C013-2, YaJi Biological, China) respectively.

Western Blot

Retinal tissues from WT and cKO mice or treated cells were lysed in RIPA buffer supplemented with proteinase inhibitor cocktail (Sigma Aldrich). After centrifuging at $12,000 \times g$ for 15 minutes, the protein was obtained from the supernatant, and then Western Blot was performed as previously described.[11] All the antibodies used were Irf8 (#5628), Pu.1 (#2758), Syk (#13198), phosphor-Syk (#2710) (Cell Signaling Technology, Danvers, MA), cleaved vaspase 3 (#AF7022, Affinity Biotech, Cincinnati, OH) and β-actin (#20536-1-AP, Proteintech, Rosemont, IL).

RT-PCR

Total messenger RNA (mRNA) was extracted from lysed retina of mice or treated microglial cells using SV Total RNA Isolation kit (Promega, Madison, WI), then transcribed into cDNA using iScript reagents (Bio-Rad, Hercules, CA), then RT-PCR was performed as described previously.[12] The expressions of all genes were normalized to *GAPDH*. The sequences of primers were:

Aif1, Forward: GACGTTCAGCTACTCTGACTTT;
Reverse: GTTGGCCTCTTGTGTTCTTTG;
Irf8, Forward: CGGGGCTGATCTGGGAAAAT;
Reverse: CACAGCGTAACCTCGTCTTC;
Spi1, Forward: GGAGAAGCTGATGGCTTGG;

Reverse: CAGGCGAATCTTTTTCTTGC;
GAPDH, Forward: AGGTCGGTGTGAACG-
GATTTG;
Reverse: TGTAGACCATGTAGTTGAGGTCA;
Tnfa, Forward: CCAAGGCGCCACATC TCCCT;
Reverse: GCTTTCTGTGCTCATGGTGT;
IL1b, Forward: GCAACTGTTCCTGAACTCAACT;
Reverse: ATCTTTTGGGGTCCGTCAACT;
Ccl2, Forward: TTAAAAACCTGGATCGGAAC-
CAA;
Reverse: GCATTAGCTTCAGATTTACGGGT;
Ly86, Forward: ATTCTGAACTACTCCTATCCC-
CTTT;
Reverse: GGCCGGCATAGTATATCTGTTCT;
Lyn, Forward: TCAAATCTGACGTGTGGTCCT;
Reverse: CATCACATCTGCGTTGGTTC;
Ncf4, Forward: GCCCCTGTTCAAAGACCTG;
Reverse: ACAGCAGCCTAACCAAGTCC;

Cell Culture

Male and female WT and cKO mice aged 4 weeks were gavaged with 20 mg/kg tamoxifen for 5 consecutive days. On day 14 from the first dose, eyeballs were collected from mice. After removing the lens and the cornea, the retina was dissected from the eyeclip under microscope. Eight retinas together were mechanically dissociated by harsh aspiration in precold medium, then digested by 5 µg/mL collagenase type A in DMEM/F12 + Glutamax medium with 10% fetal calf serum and penicillin/streptomycin for 1 hour at 37°C. After washing by serum-free DMEM/F12 medium, the mixed single-cell suspension was filtered through a 100-µm cell strainer (BD Falcon, London, UK), then seeded into 24-well plate in DMEM/F12 + Glutamax medium with 20% L929 and 20% fetal calf serum. After 10 days' culture, nonadhesive cells were removed after replacing the medium and adhesives, which were microglial cells, kept growing with monitoring their morphology.[13]

Cells were treated with 500 µg/mL AGA or PBS for 12 hours, or treated with 25 mM glucose or 25 mM mannitol and Syk inhibitor R406 (10 µM) (#S2194, Selleck, Houston, TX) or vehicle for 24 hours, then treated cells were harvested for Western blot, PCR analysis, or taken images by phase contrast microscope, and conditional medium was collected for measuring cytokines.

Immunofluorescent Staining

After fixing in 4% paraformaldehyde, frozen retinal sections were blocked with 10% goat serum after the incubation of the primary antibodies overnight.

Then, after washing in PBS three times, sections were incubated with secondary antibody followed the DAPI staining for 10 minutes. Slides were examined under confocal microscope (LSM 510, Carl Zeiss AG, Jena, Germany). Images were obtained from at least four mice per group. The cell number was analyzed by Image Pro Plus 6.0 software.[14]

Preparation, Imaging, and Quantification of Microglial Morphology

Frozen retinal sections were stained with Iba-1 overnight followed by the incubation with Alexa Fluor 488-conjugated anti-rabbit antibody (1:1000) at room temperature for 2 hours, then were imaged using a fluorescence microscope. To quantify individual microglia, the images were captured at 400× magnification with a confocal microscope. Four images from the center and four from the peripheral retina were captured, and the length and the number of microglia processes were quantified by ImageJ software.

Statistical Analysis

Data were analyzed by the Student t test, one- or two-way analysis of variance and an appropriate post hoc test using GraphPad Prism 7 software and represented as means $\pm$ standard error of the mean. A P value of less than 0.05 was considered as statistically significant.

Results

Deletion of Microglial Syk Decreased Its Upregulation in the Retina of STZ-induced Diabetic Mice

To explore the role of microglial Syk in DR, we first knockout microglial Syk in the retina of mice by gavage 20 mg/kg of tamoxifen into Cx3cr1$^{CreERT2-EYFP/+}$; Syk$^{fl/fl}$ (cKO) mice for 5 consecutive days, and on day 14 from the first dose of tamoxifen the mice were intraperitoneally injected with 50 mg/kg STZ to induce diabetes. A diagram of the experimental procedure was showed in Figure 1A. Then, we measured the mRNA levels of Syk from week 0 to week 10. Compared with WT mice, cKO mice displayed a stable decrease in Syk expression from week 2 to week 10 after the injection of tamoxifen (Fig. 1B), which indicated that Syk in the microglia was effectively deleted in cKO mice. Next, we explored the role of deleting microglial Syk in the development of DR. STZ injection significantly
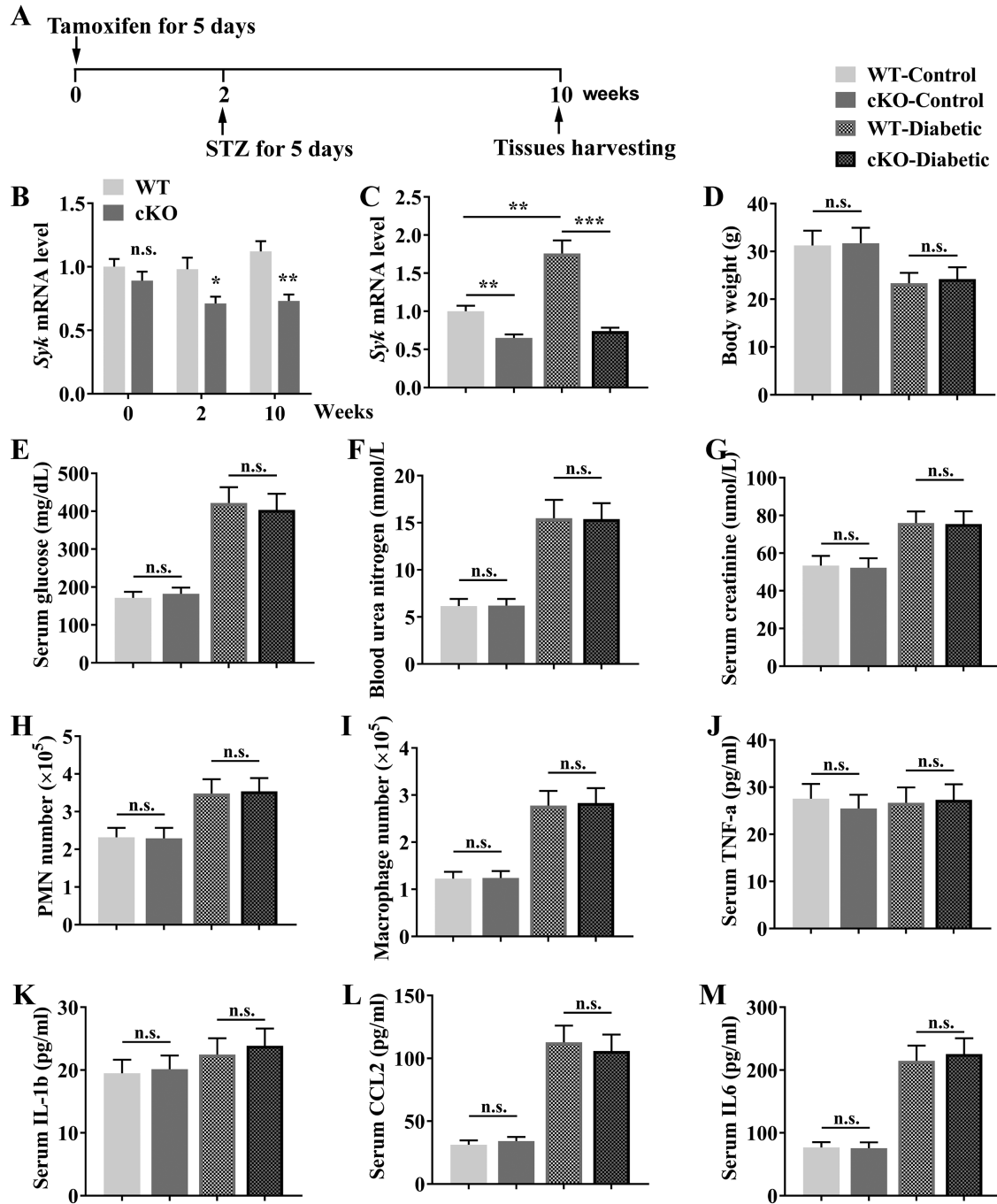
Figure 1. Deletion of microglial Syk repressed its upregulation in retina by STZ-induced mice. (**A**) The diagram of experiment procedure. (**B**) The mRNA level of Syk in the retina at indicated time points post the administration of tamoxifen. Four mice per group for the 0-week and 2-week time points, and five mice per group for 10-week points. (**C** through **E**) The DR was induced in Cx3cr1^{+/+}; Syk^{fl/fl} (WT) and Cx3cr1[CreERT2-EYFP/+]; Syk^{fl/fl} (cKO) mice. The mRNA level of Syk in the retina (**C**), body weight (**D**), blood glucose (**E**), blood urea nitrogen (**F**), serum creatinine (**G**), peripheral blood polymorphonuclear leukocytes (PMN; Gr1^{hi}) number (**H**), peripheral blood macrophage number (F4/80[+]) (**I**), serum TNF-α (**J**), IL-1β (**K**), and CCL2 (**L**) and IL6 (**M**) protein level were determined 8 weeks post diabetic induction. There were five mice for the control groups and six mice for the diabetic groups. n.s., not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

increased the Syk expressions in STZ-injected WT mice in comparison with control-treated WT mice at week 10, which could be restored by the deletion of microglial Syk in the retina of cKO mice (Fig. 1C).

In the meantime, diabetic mice showed no changes in body weight (Fig. 1D) or serum glucose (Fig. 1E) after the deletion of microglial Syk in the retina, suggesting that the deletion of microglial Syk in the retina

did not ameliorate diabetes in STZ-induced diabetic mice. In addition, similar findings could be observed in blood urea nitrogen (Fig. 1F), serum creatinine (Fig. 1G), peripheral blood polymorphonuclear leukocytes number (Fig. 1H), peripheral blood macrophage number (Fig. 1I), serum TNF-α levels (Fig. 1J), IL-1β (Fig. 1K), and CCL2 (Fig. 1L) and IL-6 (Fig. 1M) protein levels, which indicated that the specific deletion of Syk in Cx3Cr1-positive cells (mainly microglia) did not alter peripherally the systemic response to STZ induction, including diabetes and peripheral inflammation. These data revealed that the deletion of microglial Syk inhibited its upregulation in the retina of STZ-induced diabetic mice.

Deletion of Microglial Inhibited Microglial Activation in the Retina of Diabetic Mice

Previous studies demonstrated that the microglia were activated in DR and accelerated the progression of DR. Therefore, we assessed the effect of microglial Syk on the microglial activation during DR. RT-PCR data and immunofluorescent staining demonstrated that there was no difference on *Aif1* expressions (the coding gene of Iba-1, microglial marker) in the retina between WT and cKO mice, which indicated that WT and cKO mice had the similar microglial numbers in the retina (Figs. 2A through 2C). Notably, STZ injection remarkably increased *Aif1* expressions in the retina of WT mice, which was consistent with the previous study wherein DR activated microglia in the retina; however, this microglial activation was significantly inhibited in the retina of STZ-injected cKO mice, which suggested that the deletion of microglial Syk blocked the microglial activation in the retina of STZ-induced diabetic mice.

Moreover, we analyzed the morphology of microglia to further assess the role of Syk in the activation of microglia using stacked images of cross-cut retinas. The deletion of Syk in microglia reversed the changes of microglial morphology inhibited by diabetes, including the process numbers (Fig. 2D) and length (Fig. 2E), suggesting the function of Syk in microglial activation. Furthermore, Syk deletion in retinal microglia partially inhibited the expansion of macroglia (GFAP+) induced by diabetes (Figs. 2F and 2G), which might contribute to microglial function in the microenvironment and cellular interaction in the diabetic retina.

In addition, numerous studies reported that the apoptosis of retinal neural cells were observed, leading to the development of DR. Our study also indicated that the expressions of cleaved caspase 3 (a marker of cell apoptosis), which were significantly provoked in the retinal microglia of diabetic mice were remarkably diminished in the retinal microglia of cKO mice (Figs. 2H and 2I). All these data revealed that Syk-induced microglial activation promoted the pathogenesis of DR, which could be inhibited by s deleting Syk in retinal microglia.

Deletion of Microglial Syk Decreased Inflammation in the Retina of STZ-Induced Diabetic Mice

Owing to the importance of inflammation in DR, we further explored the effect of deleting microglial Syk on inflammation in STZ-induced diabetic mice, and we determined the release of cytokines, including TNF-α, IL-1β, and CCL2. The mRNA levels and secretions of these cytokines in the retinal tissue were all significantly increased in STZ-treated WT mice, which were all remarkably decreased in STZ-injected cKO mice, with no alternation between WT and cKO mice before STZ injection (Figs. 3A through 3F). These data revealed that deletion of microglial Syk effectively decreased the inflammation in the retina of STZ-induced diabetic mice.

Syk Was Activated by AGA in Primary Retinal Microglia

To further evaluate the role of microglial Syk in the retina, we isolated primary microglial cells from the retina of WT or cKO mice. The mRNA and protein levels of Syk were lower in primary microglia isolated from cKO mice than the ones from WT mice (Figs. 4A and 4B), and Syk expressions as well as phosphor-Syk expression were significantly induced 12 hours after AGA stimulation in WT cells (Figs. 4B and 4D). At the meanwhile, cKO cells still had low expressions of Syk and p-Syk after AGA treatment (Figs. 4A through 4D), which demonstrated that Syk was activated by AGA in primary retinal microglia isolated from WT cells, and this activation was inhibited in cKO mice-isolated microglia because of the deletion of microglial Syk.

Syk Was Induced by High Glucose in the Microglia

To further explore the role of Syk in the retinal microglia, we treated WT microglia cells with 25 mM high glucose (HG) for 24 hours. Compared with mannitol-treated ones, HG-treated cells showed a significant increase on the protein levels of both Syk
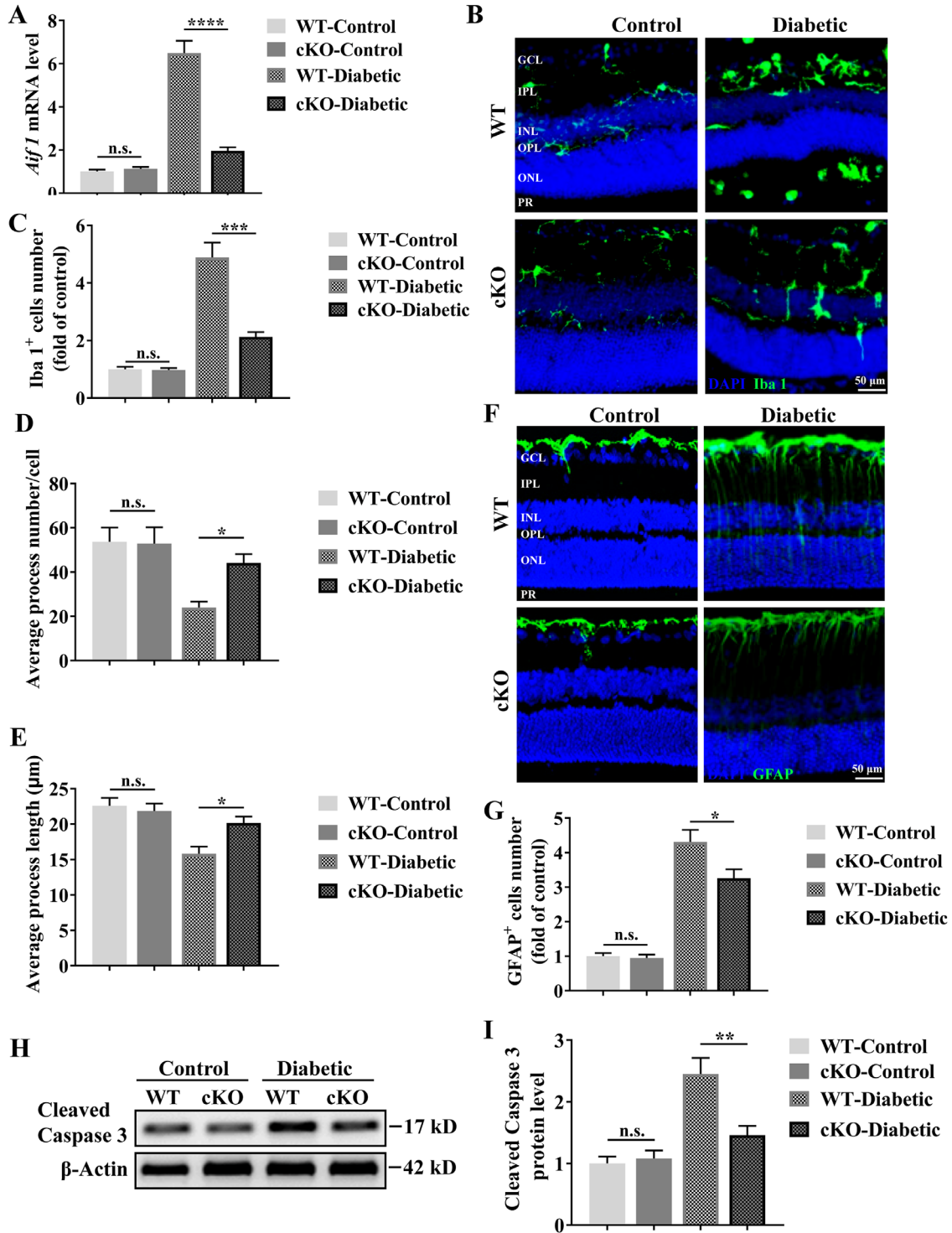
Figure 2. Deletion of microglial Syk ameliorated microglial activation in the retina of diabetic mice. DR was induced in Cx3cr1^{+/+}; Syk^{fl/fl} (WT) and Cx3cr1[CreERT2-EYFP/+]; Syk^{fl/fl} (cKO) mice. The mRNA level of *Aif1* (the coding gene of Iba 1) (**A**), microglia number (Iba 1[+]) (**B, C**) and macroglia number (GFAP[+]) (**F** and **G**) in retina were determined by RT-PCR and immunofluorescent staining, respectively. (Green, Iba 1 or GFAP; Blue, DAPI). Scale bar = 50 μM. And the average process number (**D**) and length (**E**) of microglia were quantified. The protein level of cleaved Caspase 3 was determined by western blot (**H** and **I**). There were five mice for the control groups and six mice for the diabetic groups. n.s., not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.
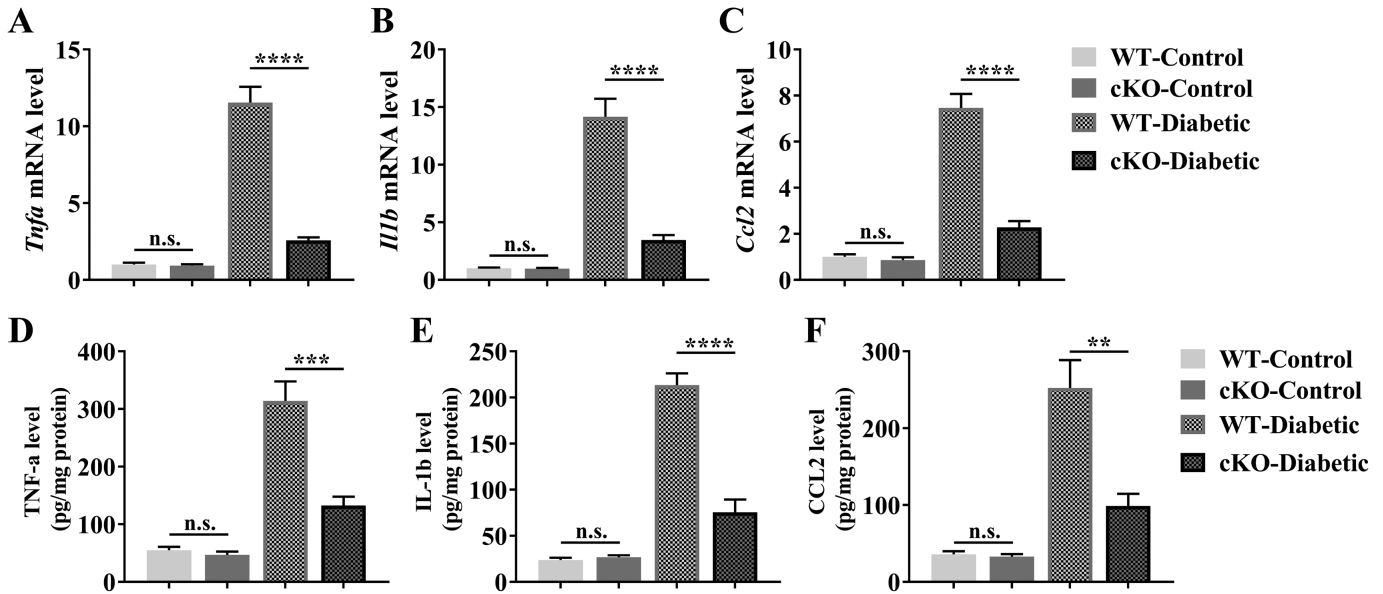
Figure 3. Deletion of microglial Syk reduced the inflammation in the retina of STZ-induced diabetic mice. DR was induced in WT and cKO mice for 8 weeks. Retinas were harvested for detecting the mRNA levels of Tnfa (**A**), Il1b (**B**) and Ccl2 (**C**) by RT-PCR, and the protein levels of TNF-α (**D**), IL-1β (**E**) and CCL2 (**F**) by ELISA. There were five mice for the control groups and six mice for the diabetic groups. n.s., not significant. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.
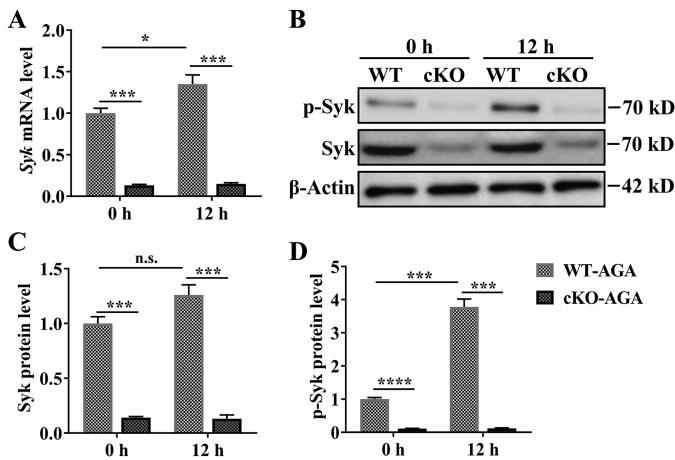

Figure 4. Syk was activated by AGA in primary retinal microglia. Primary retinal microglia from WT and cKO mice were treated with 500 μg/mL AGA for 12 hours. The control (0 hour group) and treated (12 hour group) microglia were harvested for detecting the mRNA level of Syk (**A**) by RT-PCR and the protein level of Syk (**B, C**) and p-Syk (**B, D**) by Western blot. (*n* = 3.) n.s., not significant. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

suggested that AGA or HG-induced Syk could activate retinal microglia.

Deficiency of Syk Decreased Inflammation Induced by AGA in Primary Retinal Microglia

We subsequently assessed the effect of microglial Syk deficiency in the inflammation in vitro. Consistent with our previous data, there was no difference on cytokines secretions between WT and cKO cells (Fig. 6), and these secretions in the conditional medium were dramatically induced by AGA in WI microglia, which were restored in cKO microglia (Figs. 6A through 6C). Therefore, deficiency of microglial Syk also decreased inflammation in the primary retinal microglia.

Deficiency of Microglial Syk Inhibited Microglial Activation in the Retina by Decreasing the Expressions of Irf8 and Pu.1

Previous studies proved that transcriptional factors interferon regulatory factor 8 (Irf8) and Pu.1 were two key mediators that regulated microglial activation in central nervous system[15]; therefore, here we addressed whether Irf8 and PU.1 were involved in Syk-mediated microglial activation in the retina. There was no difference between WT and cKO cells (Figs. 7A through 7D). Nevertheless, stimulation with AGA significantly

and p-Syk in the WT microglia (Figs. 5A through 5C). In addition, specific Syk inhibitor R406 alone had no effect on the activation of microglia, but had a significant inhibition on microglial activation caused by either AGA or HG (Figs. 5D through 5F), which
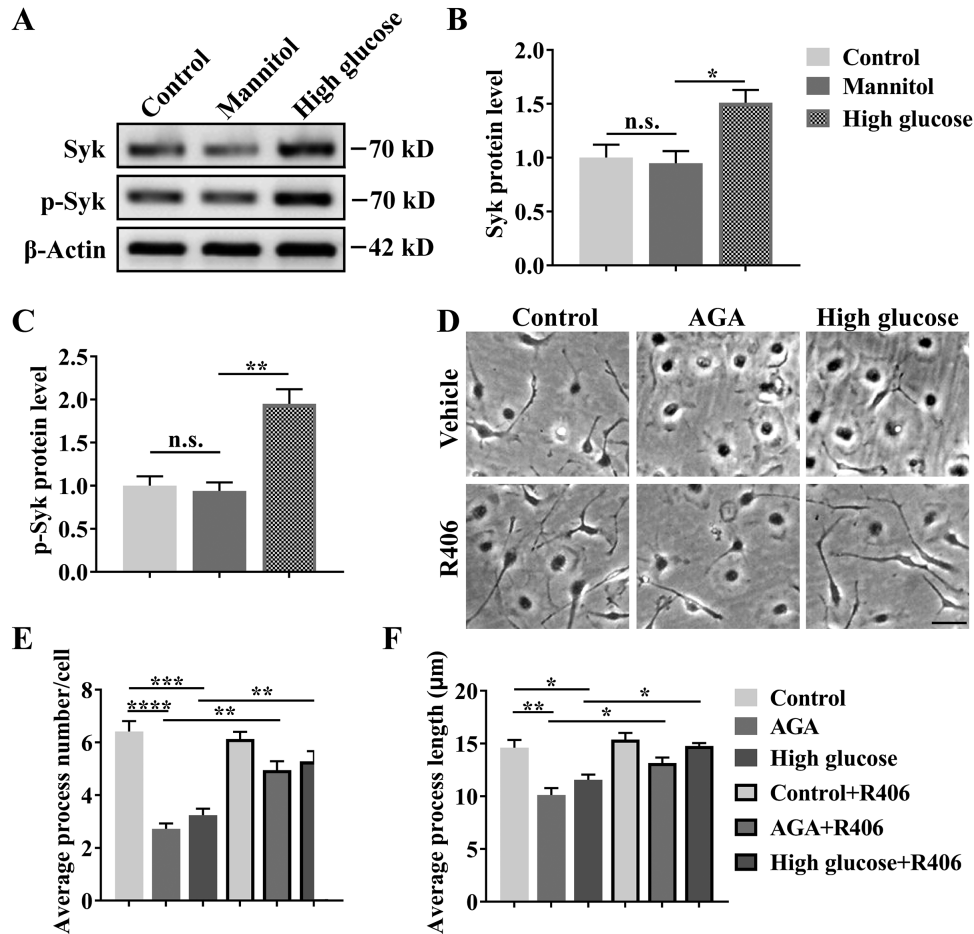
Figure 5. Inhibition of Syk ameliorated microglial activation in vitro. (**A** through **C**) Primary retinal microglia from WT mice were treated with 25 mM mannitol or 25 mM glucose for 24 hours, then harvested for detecting the protein level of Syk (**A, B**) and p-Syk (**A, C**) by Western blot. ($n = 3$.) (**D** through **F**) Primary retinal microglia from WT mice were treated with 500 µg/mL AGA or 25 mM glucose and Syk inhibitor R406 (10 µM) or vehicle for 24 hours, then were taken images by phase contrast microscope (**D**). Scale bar, 10 µm. And the average process number (**E**) and length (**F**) of microglia were quantified. ($n = 3$.) n.s., not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

induced both the mRNA and protein levels of Irf8 and *Spi1* (the coding gene of Pu.1)/Pu.1 in WT cells, which indicated that the microglia was activated in WT cells. These increased expressions of Irf8 and *Spi1* were restored in cKO cells, suggesting that microglial activation was inhibited (Fig. 7E). We further explored the expressions of Irf-8 and Pu.1-targeted genes, which were also microglial activation-related genes, including common marker for immune process (*Ly86*), cell morphogenesis (*Lyn*), and phagocytosis (*Ncf4*). Lyn was recognized as the marker of morphogenesis and Ncf4 was the marker of phagocytosis. The increase on the mRNA levels of these genes in AGA-treated WT cells, were inhibited in AGA-treated cKO cells (Figs. 7F through 7H). These data suggested that a deficiency of microglial Syk might inhibit microglial activation in the retina by decreasing the expressions of Irf8 and Pu.1.

Deletion of Microglial Syk Inhibited the Expressions of Irf8 and Pu.1 in the Retina of STZ-induced Diabetic Mice

Next, we measured the mRNA and protein levels of Irf8 and Pu.1 in vivo. WT and cKO mice displayed similar expressions on Irf8 and *Spi1*/Pu.1 (Figs. 8A through 8E). The expressions of these two factors were greatly increased in the retina of STZ-induced diabetic mice and inhibited in the retina of STZ-injected cKO mice. Additionally, Irf8 and Pu.1-targeted genes also had higher expressions in the retina of STZ-induced diabetic WT mice than that of control-treated WT or cKO mice, and this increase was diminished after the deletion of microglial Syk in the retina of STZ-induced mice (Figs. 8F through 8H), which further confirmed that the deletion of microglial Syk inhibited microglial
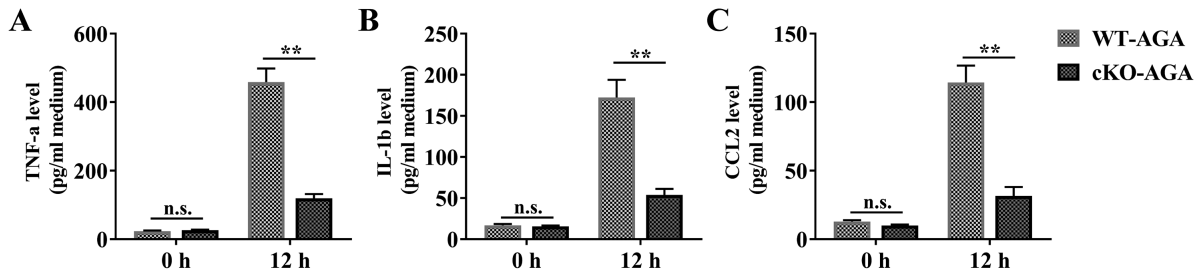
Figure 6. Deficiency of Syk decreased the inflammation induced by AGA in primary retinal microglia. Primary cultured retina microglia from WT and cKO mice were treated with 500 µg/mL AGA for 12 hours. The conditional medium of control (0 hour group) and treated (12 hour group) microglia was harvested for determining the protein levels of TNF-α (**A**), IL-1β (**B**), and CCL2 (**C**) by ELISA. ($n = 3$.) n.s., not significant. **$P < 0.01$.

Discussion

Our study used Cx3cr1^{CreERT2}; Syk$^{fl/fl}$ mice to knockout microglial Syk in the retina after diabetes induction, combined with in vitro studies to evaluate the role of microglial Syk in microglial activation, as well as the progression of DR. Our data demonstrated that the deletion of microglial Syk inhibited microglial activation both in vivo and in vitro, and also suppressed the secretions of inflammatory cytokines, eventually leading to the improvement of DR.

Our group for the first time established new mice, which had a deletion of Syk in the retinal microglia. Our data clearly showed that the knockout efficacy of Syk in the retinal microglia was stable within 2 to 10 weeks after tamoxifen induction. The expression of Syk was decreased by approximately 30% to 40% in cKO mice, because besides microglia, varieties of cells in the retina also expressed Syk, which resulted in the incomplete decrease of Syk in the retina after deleting Syk in the retinal microglia. Although Syk might be mostly expressed in other cells of the retina, instead of the microglia, our study strongly suggested the importunateness of microglia Syk in the pathogenesis of DR. Moreover, besides retinal microglia, Syk in the brain microglia peripheral monocytes and dendritic cells were deleted in our cKO mice, but the cKO mice still had high blood glucose levels and low body weight induced by STZ, which indicated that the deletion of microglial Syk did not improve diabetes, and Syk in the peripheral system might had less of an impact on DR. These data, combined with in vitro studies, indicated that the novel knockout mice effectively facilitated in

evaluating the role of microglial Syk in the microglial activation, as well as DR. In addition, a more in-depth characterization of the mice could clearly assess a potential impact on those cells and systemic inflammation or vascular perturbation, especially in the context of diabetes. For example, Cx3cr1CreERT2;Ai14 mice will help to assess dynamic changes of Cre-induced gene expression in the periphery brain and retina, which is our limitation in this project, and we might explore that in the future.

Increasing evidence shows that microglial activation in the retina played a critical role in the progression of DR.[16,17] More and more attention has been paid to this new field.[18] In recent years, modulating microglial activation in the retina became a new therapeutic strategy to treat DR.[19,20] In this study, we also aimed to explore the new mechanism of microglial activation in DR, and tried to find out a new promising therapeutic target to treat patients with DR.

Syk was first known to relay adaptive immune receptor signaling. Nevertheless, emerging evidence proved that Syk also regulated other diverse biological functions, such as innate immune recognition, cellular adhesion, platelet activation, osteoclast maturation, and vascular development.[21] Owing to its importance in these physiological processes, Syk knockout mice died during embryonic development around the second trimester. Syk and its downstream signaling pathways in the microglia were reported to mediate neuroinflammatory injury in ischemic stroke[22] and microglial cell dysfunction in Alzheimer's disease.[23] Although a previous publication indicated that the Syk inhibitor R406 could ameliorate DR in diabetic rats,[8] the relationship between microglial Syk and DR was still largely unknown. Therefore, our current study focused mainly on the role of microglial Syk in the progression of DR, and proved that deletion of microglial Syk could effectively delay the development of DR.
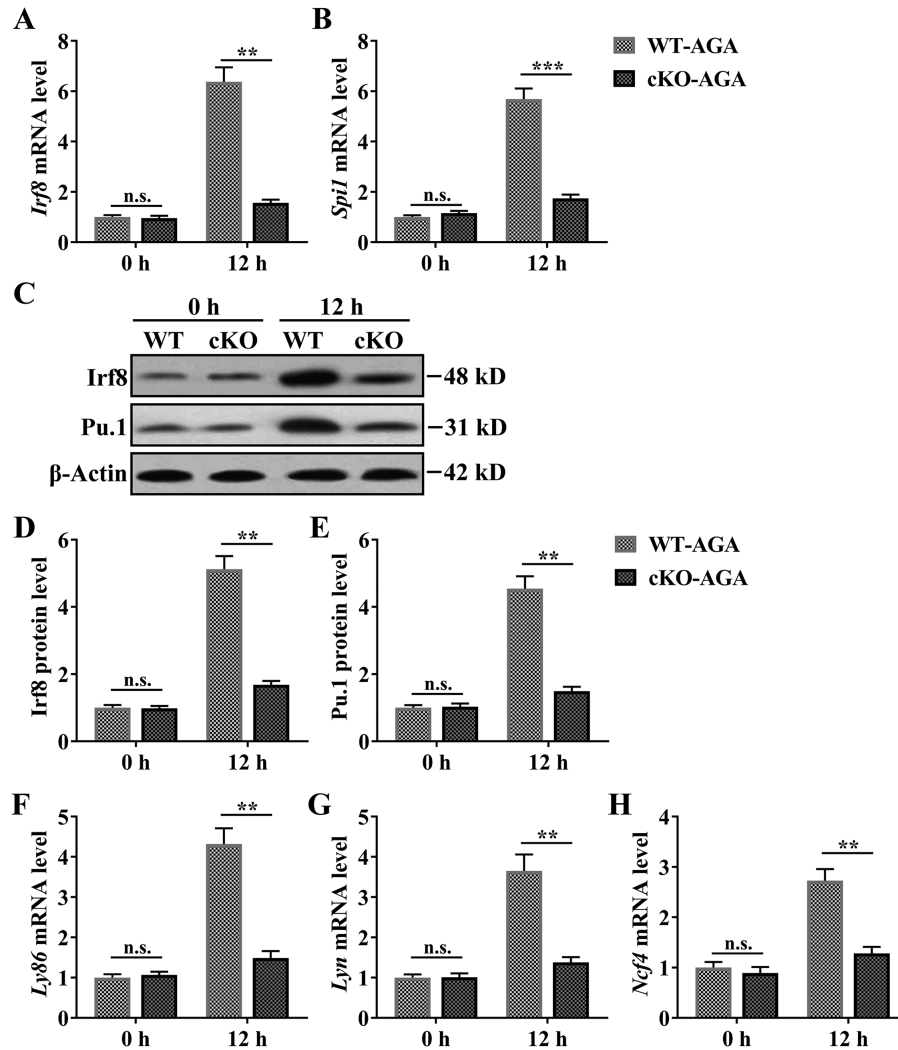
activation by suppressing Irf8 and Pu.1 expression in the retina.

Figure 7. Deficiency of Syk inhibited microglial activation in the retina by downregulating the expression of Irf8 and Pu.1. Primary retina microglia from WT and cKO mice were treated with 500 μg/mL AGA for 12 hours. The control (0 hour group) and treated (12 hour group) microglia were harvested for detecting the mRNA (**A, B**) and protein level (**C** through **E**) of Irf8 (**A, C**, and **D**) and *Spi1* (the coding gene of Pu.1)/Pu.1 (**B, C**, and **E**) by RT-PCR and Western blot, respectively. The mRNA level of *Ly86* (**F**), *Lyn* (**G**), and *Ncf4* (**H**) was determined by RT-qPCR. ($n = 3$.) $**P < 0.01$, $***P < 0.001$.

Here, we demonstrated that the deletion of microglial Syk had no effect on fasting blood glucose levels or body weight of mice with STZ-induced diabetes, which indicated that the deletion of Syk in the retinal microglia could not ameliorate diabetes in mice with STZ-induced diabetes. This conclusion was not consistent with previously published articles. Numerous reports demonstrated that Syk and its signaling pathways were involved in diabetes,[24] and microRNA-136 improved renal fibrosis in diabetic rats by mediating Syk.[25] Moreover, Syk was suggested as therapeutic target for autoimmune diabetes.[26] However, our data suggested that the deletion of microglial Syk had no effect on diabetes, because Syk was deleted only in the retinal microglia, and until

now microglial activation in the retina was only proved participate in DR instead of diabetes.[27] Our mice had a deficiency on microglial Syk in the retina, not the whole body, and that might be the reason that cKO mice cannot ameliorate diabetes.

Additionally, the inflammation mediated by the microglial activation could lead to vascular defects in DR.[28,29] Therefore, it is an important and interesting point whether Syk in the retinal microglial contributes to the vascular defects in DR, which remains unknown as yet. However, our study was only focused on the role of Syk in microglial activation, and this finding may influence indirectly the vascular defects in the retina by regulating microglial function and inflammation, which might need to be explored further.
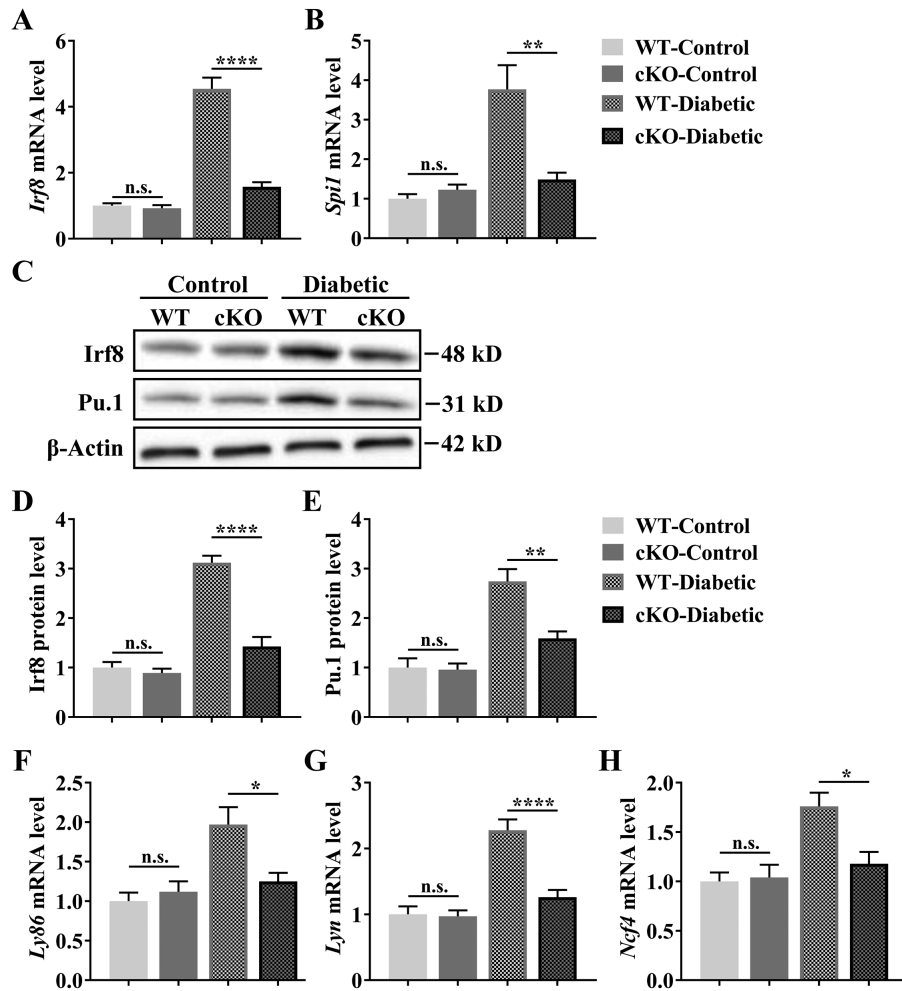
Figure 8. Deletion of microglial Syk inhibited the expressions of Irf8 and Pu.1 in the retina of STZ-induced diabetic mice. DR was induced in Cx3cr1^{+/+}; Syk^{fl/fl} (WT) and Cx3cr1[CreERT2-EYFP/+]; Syk^{fl/fl} (cKO) mice for 8 weeks. The mRNA (**A, B**) and protein level (**C** through **E**) of Irf8 (**A, C,** and **D**) and *Spi1* (the coding gene of Pu.1)/Pu.1 (**B, C,** and **E**) in the retina were determined by RT-PCR and Western blot, respectively. The mRNA level of *Ly86* (**F**), *Lyn* (**G**), and *Ncf4* (**H**) was determined by RT-qPCR. There were five mice for the control groups and six mice for the diabetic groups. n.s., not significant. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

Moreover, the current study also revealed that Syk mediated microglial activation by regulating two transcriptional factors, namely, Irf8 and Pu.1. Although Irf8 and Pu.1 were two key factors that mediated microglial activation in the central nervous system,[15] there was still no publication about the relationship between Syk and Irf8 or Pu.1 in DR. Our study has provided the new molecular mechanism of Syk in the microglial activation, as well as in DR. However, how Irf8 or Pu.1 mediated by the Syk-regulated microglial activation and DR remain poorly understood. There was evidence revealed that Irf8 and Pu.1 could directly target each other's gene transcription and enhance the expression of microglial activation-related genes in neurodegenerative condi-

tion.[30] However, in the retinal microglia of DR, the mechanism might be completely different and this needs further exploration for the more detailed mechanism.

Conclusions

We established new mice with deletion of microglial Syk in the retina to explore the role of microglial Syk in microglial activation and DR. Our study revealed that deletion of microglial Syk could ameliorate DR by inhibiting microglial activation through Irf8 and Pu.1 both in vivo and in vitro, therefore, microglial

Syk might serve as a potential therapeutic target for DR.

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