HHS Public Access

Author manuscript

Exp Eye Res. Author manuscript; available in PMC 2022 October 04.

Published in final edited form as:

Exp Eye Res. 2022 July ; 220: 109108. doi:10.1016/j.exer.2022.109108.

TNFAIP3 may be key to TLR4-activation of the inflammasome in the retinal vasculature

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Abstract

The goal of these studies were to determine whether tumor necrosis factor, alpha-induced protein 3 (TNFAIP3) regulated toll-like receptor 4 (TLR4) actions on the NOD-like receptor protein 3 (NLRP3) inflammasome. Western blotting was done on retinal lysates from TLR4 floxed and endothelial cell specific TLR4 knockout mice for TNFAIP3, TLR4, and NLRP3 pathway proteins. Retinal endothelial cells (REC) were grown in normal (5 mM) and high glucose (25 mM) and treated with TNFAIP3 siRNA, followed by Western blotting for TLR4 and NLRP3 pathway proteins. Loss of TLR4 in endothelial cells increased TNFAIP3 levels, while decreasing NLRP3 pathway proteins. High glucose culturing conditions increased TLR4 and NLRP3 proteins, which were also increased by TNFAIP3 siRNA. Data demonstrate that TLR4 regulates NLRP3 pathway proteins. TNFAIP3 can regulate TLR4 and the NLRP3 pathway. TNFAIP3 may offer a new target for therapeutic development against retinal inflammation.

Keywords

Retinal endothelial cells; Toll-like receptor 4; Tumor necrosis factor induced protein 3; Inflammation; Diabetes

1. Introduction

The importance of inflammation in the pathogenesis of diabetic retinopathy has been appreciated for the past 2 decades. Both innate and specific chemokines have been shown to be highly involved in the diabetic retinal damage (Joussen et al., 2004; Steinle, 2020; Tang and Kern, 2011). We and others have previously shown that tumor necrosis factor alpha (TNFα) is highly involved in the diabetic retina (Joussen et al., 2009; Zhang et al., 2013). Others have shown that TNFAIP3 regulates TNFα (Xie et al., 2022). One interesting finding from the previous work showed that TNFAIP3 regulated TRAF6 in retinal endothelial cells

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Contributions

Liu performed studies, edited text, proofread final draft; Jiang performed studies and edited text; Steinle conceptualized the work, got the funding, wrote the text.

(REC), which matches the literature (Momtazi et al., 2019). Others have shown that TLR4 and TRAF6 are involved in diabetic retinopathy in retinal ganglion cells (Hu et al., 2017).

TRAF6 is part of the toll-like receptor 4 (TLR4) signaling cascade (Momtazi et al., 2019). We have recently showed that loss of TLR4 is key to retinal damage using diabetic cdh5Cre-TLR4 mice where TLR4 is eliminated in the endothelial cells (Seidel et al., 2021). Additionally, our recent studies have shown that Epac1 can regulate TNFAIP3, TLR4 (Liu et al., 2021b), and the NLRP3 inflammasome *in vitro* (Jiang et al., 2017). Our findings agree with existing literature showing an established link between TLR4 and the NLRP3 inflammasome in the retina (Liu et al., 2021a). Thus, TNFAIP3 likely plays a role in inflammatory pathway in the diabetic retina.

In addition to focusing on the NLRP3 inflammasome, we investigated whether TLR4 regulated NIMA related kinase 7 (Nek7) and puringeric 2X7 receptor (P2X7R). We chose to focus on Nek7, as others Nek7 was shown to activate NLRP3 following potassium efflux in NLRP3 and Nek7 knockout mice and hematopoietic cells (He et al., 2016). A genome-wide CRISPR screen in macrophages showed that Nek7 was a key player in NLRP3 inflammasome activation (Schmid-Burgk et al., 2016). We recently reported that exchange protein activated by cAMP 1 (Epac1) and protein kinase A (PKA) regulate Nek7 (Liu et al., 2021b, 2022b), which followed our previous finding that Epac1 regulated TLR4. Similarly for P2X7R, we have shown that both Epac1 and PKA can regulate P2X7R levels in retinal endothelial cells (Liu et al., 2022a). We explored P2X7R as others showed that P2X7 plays a strong role in activation of the NLRP3 inflammation and renal inflammation in type 2 diabetic subjects and renal cells (Solini et al., 2013). Studies in geographic atrophy models showed that P2X7 signaling mediated NLRP3 inflammasome activation (Fowler et al., 2014; Kerur et al., 2013).

Thus, the goal of these studies was to explore the role of TNFAIP3 in endothelial cell specific TLR4 knockout mice and in REC to determine whether TNFAIP3/TLR4 regulate the NLRP3 inflammasome. We also investigated whether TLR4 regulates Nek7 and P2X7R.

2. Methods

2.1. TLR4 floxed and cdh5Cre-TLR4 mice

All animal procedures were followed the Association for Research in Vision and Ophthalmology requirements and were approved by the Institutional Animal Care and Use Committee of Wayne State University, and all studies conform to NIH guidelines. TLR4 floxed mice (B6(Cg)-Tlr4$^{tm1.1Karp}$/J mice) and B6 FVB-Tg (cdh5-cre)7Mlia/J Cre mice were purchased from Jackson Laboratories. After 2 generations, the TLR4 floxed mice were bred with the Cdh5-Cre mice to generate conditional knockout mice where TLR4 is knocked out in vascular endothelial cells (Liu et al., 2017b; Seidel et al., 2021).

2.2. Retinal endothelial cells (REC)

Primary human retinal endothelial cells (REC) were purchased from Cell Systems Corporation (CSC, Kirkland, Washington) and grown as we have described previously (Jiang et al., 2018).

Cells in each culture conditions were transfected with TNFAIP3 siRNA or scrambled siRNA using methods we have used previously (Liu et al., 2021c).

2.3. Western blotting

Whole retinal lysates from mice or cell culture lysates were collected into lysis buffer as we have done previously (Liu et al., 2017a). Primary antibodies used were TNFAIP3, TLR4, NLRP3, ASC, IL-1β, cleaved caspase 1, Nek7, P2X7R (Abcam, Cambridge, MA) or beta actin (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were processed on an Azure C500 (Azure Biosystems, Dublin, CA). Western blot data were assessed using Image Studio Lite software.

2.4. ELISA analyses for IL-1β

The IL-1β ELISA (R&D Systems, Menomomie, WI) was done according to manufacturer's instructions, with the exception that ELISA were done overnight at 4C.

2.5. Statistics

One-way ANOVA with Tukey's post-hoc test or unpaired T-tests were used for statistical analyses produced by Prism software (Graph-Pad, San Diego, CA). Data are presented as mean ± SEM with representative blots shown. $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Loss of TLR4 in endothelial cells regulated NLRP3 pathway proteins

Since TLR4 can activate the NLRP3 inflammasome pathway, we measured TNFAIP3, TLR4, and NLRP3 pathway proteins in mice without TLR4 in the endothelial cells. Fig. 1 shows that loss of TLR4 increased TNFAIP3 levels (A), while decreasing TLR4 levels (B) in whole retinal lysates. Loss of TLR4 also decreased NLRP3 (C), ASC (D), cleaved caspase 1 (E) and IL-1β (F). These data suggest that TNFAIP3 regulation of TLR4 may offer a new way to reduce retinal inflammation.

3.2. Loss of TLR4 in endothelial cells decreased Nek7 and P2X7R levels

We recently showed that Nek7 and P2X7R are increased in REC grown in high glucose (Liu et al., 2022a, b). In this study, we measured Nek7 and P2X7R in mice lacking TLR4 in endothelial cells. Fig. 2 shows that loss of TLR4 in endothelial cells significantly decreased both Nek7 (A) and P2X7R (B) levels in whole retinal lysates.

3.3. TNFAIP3 regulates TLR4 levels in REC

To support our findings from the TLR4 mice, we also grew REC in normal and high glucose and treated some with TNFAIP3 siRNA. Fig. 3A is a control showing successful knockdown of TNFAIP3 with siRNA. Fig. 3B shows that TLR4 levels are increased in REC grown in high glucose only or treated with TNFAIP3 siRNA.

3.4. TNFAIP3 regulates NLRP3 pathway proteins

Since others have reported that TLR4 can regulate the NLRP3 pathway and TNFAIP3 can reduce TLR4, we wanted to measure NLRP3 proteins after TNFAIP3 siRNA transfection. Fig. 4 shows western blotting for NLRP3 (A), ASC (B), cleaved caspase 1 (C) and IL-1β (D). To support the findings of activation of the NLRP3 inflammasome, we also measured IL-1β by ELISA. The ELISA data matched the Western blot findings showing increased IL-1β data in HG and after TNFAIP3. High glucose increased the pathway proteins, which were further increased by TNFAIP3 siRNA.

3.5. TNFAIP3 regulated Nek7 and P2X7R in REC in culture

Because we found that TLR4 regulated Nek7 and P2X7R in mouse retina, we also wanted to investigate whether TNFAIP3 siRNA altered Nek7 and P2X7R in REC grown in normal and high glucose. Fig. 5 shows that high glucose increased Nek7 and P2X7R levels in the REC. Transfection with TNFAIP3 siRNA further increased Nek7 (A) and P2X7R (B), suggesting that these proteins are involved in this pathway.

4. Discussion

We recently reported that loss of TLR4 in endothelial cells protected the retina against diabetes-induced damage, focusing on permeability, neuronal, and vascular changes (Seidel et al., 2021). These studies were designed to investigate whether TLR4 was key to NLRP3 inflammasome actions in the retina, as well as whether TNFAIP3 can regulate TLR4.

Data from the present study demonstrated that TNFAIP3 levels were increased in whole retinal lysates from mice with TLR4 eliminated in endothelial cells. TLR4 levels are reduced in whole retinal lysates from these mice. The loss of TLR4 in the endothelial cells also reduced NLRP3 signaling proteins. While we focused these studies on REC, literature suggests that TNFAIP3 plays a role in other ocular cells. A role for TNFAIP3 has been shown in RPE cells (Hu et al., 2020) and microglial cells (Gao et al., 2021). Future work can explore TNFAIP3/TLR4 in other cell types after diabetes.

In addition, loss of TLR4 also reduced Nek7 and P2X7 receptor levels in the retinal lysates. We recently published that Nek7 lies upstream of the NLRP3 inflammasome in REC (Liu et al., 2022b). These data suggest that TLR4 regulates the NLRP3 pathway in the retinal vasculature. To expand those findings, we also treated REC in normal and high glucose with TNFAIP3 siRNA. The data demonstrate that TNFAIP3 regulated TLR4 and NLRP3 signaling pathways.

Our findings on TNFAIP3 and the NLRP3 inflammasome match well with the existing literature. Work in pristine-induced lupus in mice showed that A20 (TNFAIP3) overexpression reduced NFkB and NLRP3 in macrophages (Li et al., 2015). Result were similar in a arthritis model (Vande Walle et al., 2014). Similarly, A20 deficiency exacerbated NLRP3-induced damage in microglia in a mouse multiple sclerosis model (Voet et al., 2018). Focusing on endothelial cells, work shows that beclin-1 induced TNFAIP3 to reduce NLRP3 inflammasome actions in a model of microvascular injury (Sun et al., 2021). Others have suggested that TLR4 can regulate the NLRP3 inflammasome in the diabetic kidney

(Liu et al., 2021d). The role of TLR4 in the inflammasome has recently been reported in diabetic mouse retina (Eissa et al., 2021). Our findings add to this literature to link TNFAIP3 to TLR4 and the NLRP3 inflammasome in the retina.

Future work will expand the studies on TNFAIP3 *in vivo*. We would prefer to have run IL-1β ELISA in the mouse samples, but it takes much more protein to do this. We only ran a Western blot on these samples, but we did blot for cleaved caspase 1 in the mice, suggesting activation of the NLRP3 inflammasome. Additionally, since TNFAIP3 has actions in microglia, we can explore other cell types in culture in future experiments. Additionally, the work in retinal endothelial cells occur only *in vitro*, which cannot completely mimic *in vivo* conditions.

In conclusion, these studies had 3 major findings. TLR4 regulates the NLRP3 inflammasome in the retinal vasculature of mice. Secondly, TNFAIP3 regulated TLR4 and NLRP3 inflammasome pathway proteins in retinal endothelial cells. Thirdly, TNFAIP3 regulates Nek7 and P2X7 levels retinal endothelial cells (Fig. 6). These studies establish that additional work on TNFAIP3 is needed to investigate further mechanisms on the diabetic retina *in vivo*.

Acknowledgements

These studies were funded by R01EY028442 (JJS), R01EY030284 (JJS), and P30EY04068 (LDH) and an unrestricted grant from Research to Prevent Blindness.

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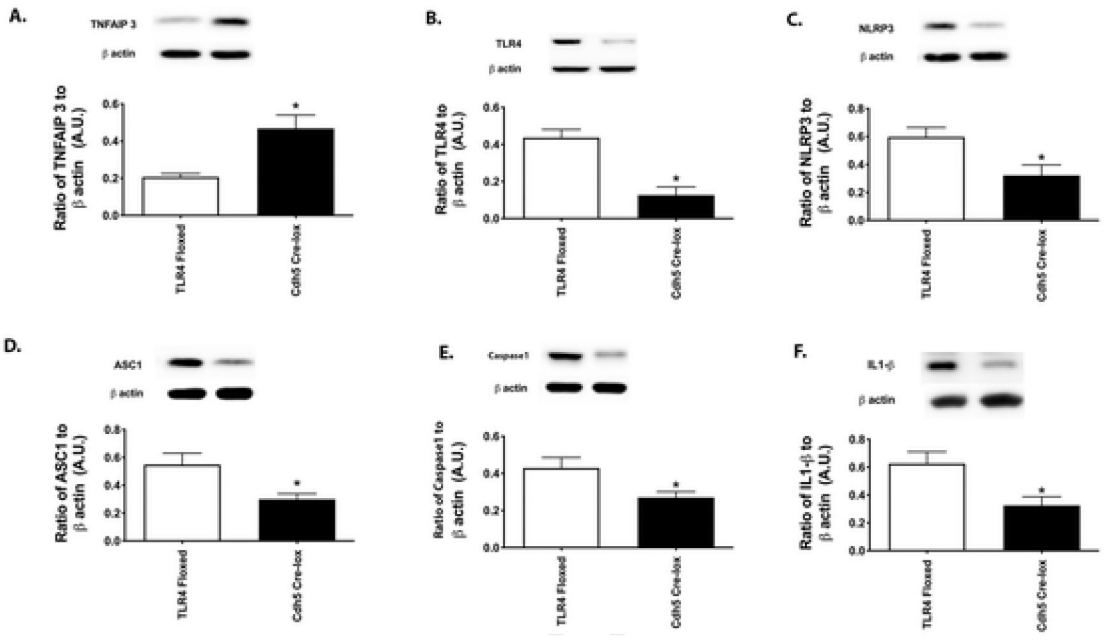

Fig. 1.

Loss of toll-like receptor 4 (TLR4) regulates NOD-like family protein 3 (NLRP3) pathway proteins. Western blot results for TNF-alpha induced protein 3 (TNFAIP3, A), TLR4 (B), NLRP3 (C), apoptosis-associated speck protein 1 (ASC1, D), caspase 1 (E) and interleukin-1-beta (IL-1β, F) in whole retinal lysates from TLR4 floxed and cdh5-Cre TLR4 mice (TLR4 CreLox). *P < 0.05 vs. TLR4 floxed. N = 5; Data are mean ± SEM.

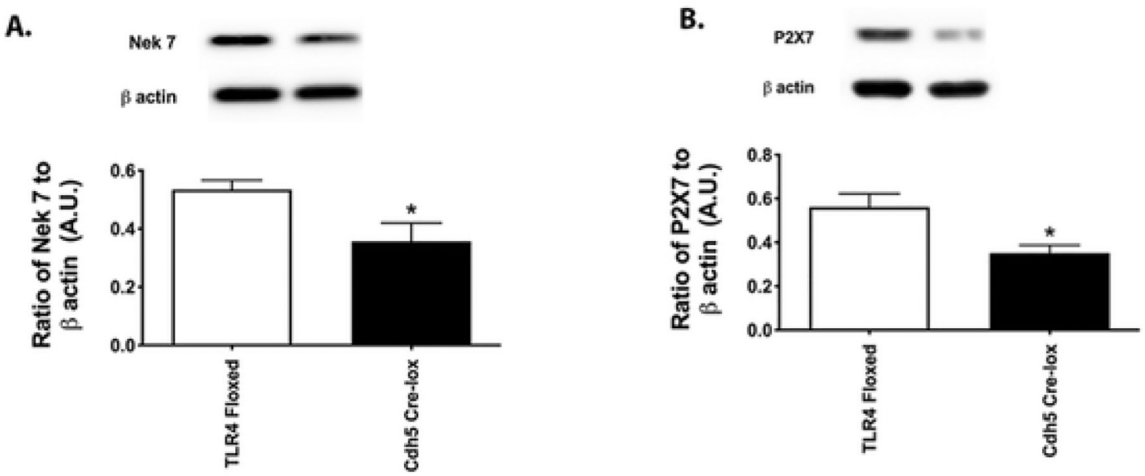

Fig. 2.

Loss of toll-like receptor 4 (TLR4) in endothelial cells reduced NIMA related kinase 7 (Nek7) and purinergic 2X7 receptor (P2X7R). Western blot results for Nek7 (A), and P2X7R (B) in whole retinal lysates from TLR4 floxed and cdh5-Cre TLR4 mice (TLR4 CreLox). *P < 0.05 vs. TLR4 floxed. N = 5; Data are mean ± SEM.

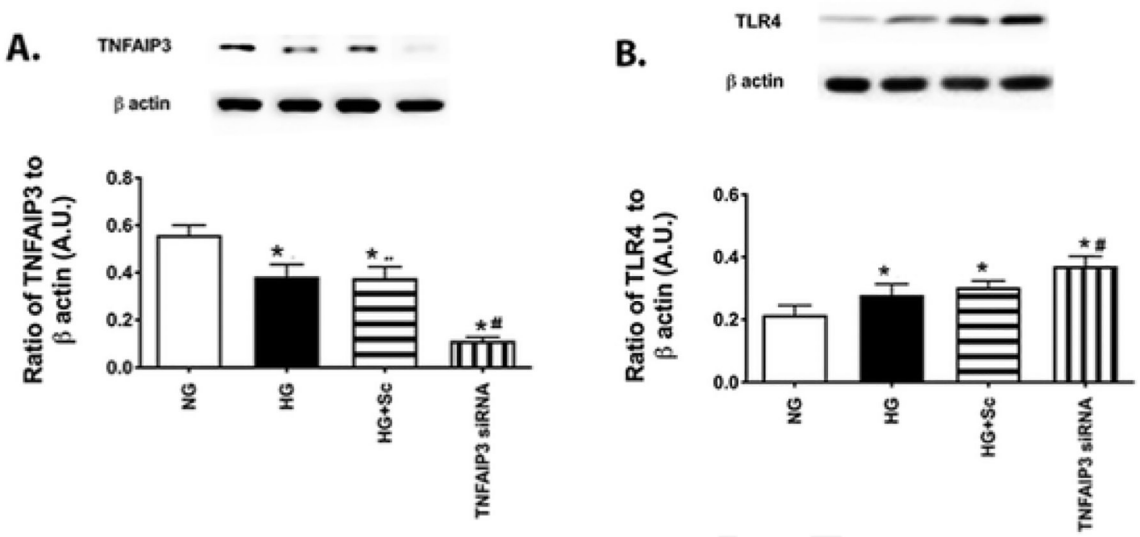

Fig. 3.

TNF-alpha induced protein 3 (TNFAIP3) siRNA regulates of toll-like receptor 4 (TLR4). Western blot results for TNFAIP3 (A) and TLR4 (B) in retinal endothelial cells (REC) grown in normal glucose (NG), high glucose (HG), high glucose with scrambled siRNA (HG + Sc) or high glucose with TNFAIP3 siRNA. *P < 0.05 vs. NG; #P < 0.05 vs. HG. N = 6. Data are mean ± SEM.

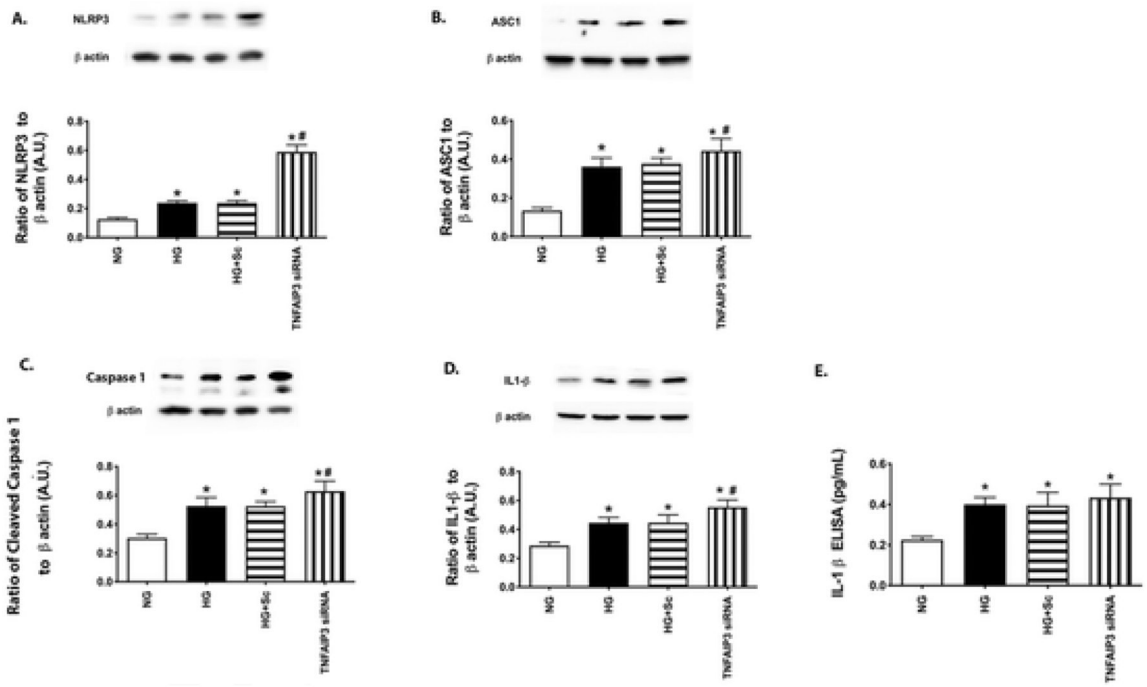

Fig. 4.

TNF-alpha induced protein 3 (TNFAIP3) siRNA regulated NOD-like family protein 3 (NLRP3) pathway proteins. Western blot results for NLRP3 (A), apoptosis-associated speck protein 1 (ASC1, B), caspase 1 (C) and interleukin-1-beta (IL-1β, D) in retinal endothelial cells (REC) grown in normal glucose (NG), high glucose (HG), high glucose with scrambled siRNA (HG + Sc) or high glucose with TNFAIP3 siRNA. Panel E shows ELISA results from the same cell groups. *P < 0.05 vs. NG; #P < 0.05 vs. HG. N = 6. Data are mean ± SEM.

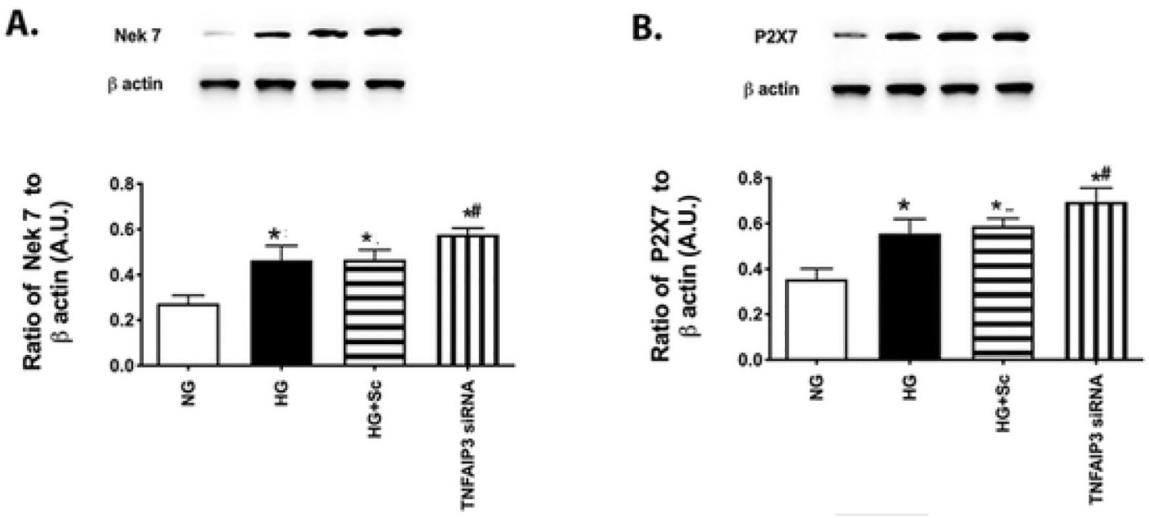

Fig. 5.

TNF-alpha induced protein 3 (TNFAIP3) siRNA regulated NIMA related kinase 7 (Nek7) and purinergic 2X7 receptor (P2X7R) in retinal endothelial cells. Western blot results for Nek7 (A), and P2X7R (B) in retinal endothelial cells (REC) grown in normal glucose (NG), high glucose (HG), high glucose with scrambled siRNA (HG + Sc) or high glucose with TNFAIP3 siRNA. *P < 0.05 vs. NG; #P < 0.05 vs. HG. N = 6. Data are mean ± SEM.

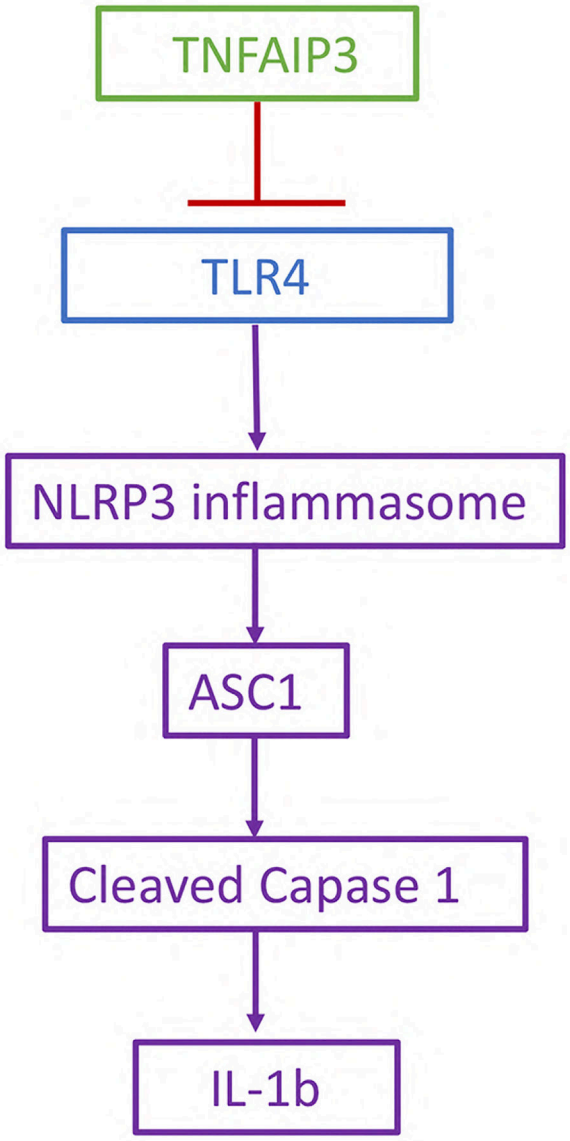

Fig. 6.
Schematic of the pathway of TNFAIP3, TLR4, and NLRP3 in the retina.