Expression and Role of Nucleotide-Binding Oligomerization Domain 2 (NOD2) in the Ocular Surface of Murine Dry Eye

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Submitted: March 22, 2019
Accepted: May 18, 2019

PURPOSE. To investigate expression and role of nucleotide-binding oligomerization domain 2 (NOD2) in the ocular surface of experimental dry eye (EDE), which is a nod-like receptor member and is involved in innate immune response.

METHODS. C57/Bl6 female mice were divided into the groups: untreated (UT), EDE, and NOD2 knockout (KO) mice exposed to desiccating stress for 14 days. Clinical parameters and levels of inflammatory cytokine were measured at 3, 5, 7, and 14 days. Immunofluorescent staining for NOD2 and Western blot for RIP2 and NF-κB were performed at 14 days. Flow cytometry, PAS staining and TUNEL staining were performed.

RESULTS. After EDE induction, NOD2 was expressed in the corneal epithelium of the EDE group. The EDE group showed a significantly increased RIP2 expression compared to the UT and NOD2-KO groups. A significantly lower expression of NF-κB and lower levels of IL-1β, IL-6, IFN-γ, and TNF-α were noted in the NOD2-KO group than in the EDE group. The NOD2-KO group had lower CD11b+ and CD45+CCR5+ T cells, TUNEL-positive cells and corneal staining score and higher density of conjunctival goblet cell density, tear volume, and tear film break-up time than the EDE group. The UT group showed significant differences in inflammatory and clinical parameters compared to the EDE and NOD2-KO groups.

CONCLUSIONS. The NOD2 receptor pathway induced inflammation and apoptosis by activation of RIP2 and NF-κB on the ocular surface of EDE, thereby reducing tear secretion. Therefore, NOD2 pathway may be involved in the pathogenesis of dry eye.

Keywords: nucleotide-binding oligomerization domain 2 (NOD2), dry eye, RIP2, NF-κB

Dry eye (DE) is one of the most common reasons that cause the ocular discomfort. It is known as an immunoinflammatory disorder of the ocular surface, which accompanied by tear film instability and hyperosmolarity. Furthermore, studies have demonstrated that increased osmolarity induced the production of proinflammatory cytokines, chemokines, and matrix metalloproteinases, therefore promoting the epithelial cell apoptosis. Phosphorylation of the stress-activated mitogen-activated protein kinase signaling pathways, c-Jun N-terminal kinase, extracellular-regulated kinases, and p38, followed by activation of transcription factors, such as activator protein 1 and nuclear factor kappa B (NF-κB), results in increased levels of proinflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-8, and IL-6.1–5

Pattern recognition receptors such as toll-like receptors (TLRs) and nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) which are the innate immune response receptors, detect imminent dangers including microbial invasion, and environmental or endogenous noxious substances.6,7 Recent studies have been demonstrated that the corneal expression of TLRs resulted in corneal epithelial loss and thinning, and took part in the inflammatory response of DE.8,9

Whereas TLRs are transmembrane proteins with an extracellular or luminal binding domain, NLRs are intracellular cytosolic proteins which have recently been identified as key mediators of inflammatory and subsequent immune responses.10 A few studies have been suggested that expression of NLRP3 in the ocular surface increased in experimental and clinical DE and was involved in the pathway of inflammatory DE.11,12

NOD2, one of the NLRs, is sensitive to detect characteristic microbial products and danger signals. It has been demonstrated that NOD2 mediate proinflammatory responses by activating NF-κB.10 A previous study demonstrated that the elevated mRNA levels of NOD2 in the cornea was associated with infectious and herpetic stromal keratitis.13 Meanwhile, NOD2 can be involved in the chronic inflammatory disorders. A few studies have suggested that the expression of NOD2 was strongly associated with the Crohn’s disease.14–16

Although the role of TLRs and NLRP3 in the ocular surface or other inflammatory diseases have been widely investigated, the expression pattern of NOD2 in DE has been rarely studied.
Thus, the aim of this study was to investigate the expression and role of NOD2 in DE using an environmental desiccating stress with scopolamine injection model in C57BL/6 and NOD2 knockout (KO) mice.

METHODS

Mouse Model Design and Experimental Procedures

This research protocol was approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female C57BL/6 mice aged 6 to 8 weeks were used in the following experiments. During these experiments, animal behavior, food, and water intake were not restricted.

In the experimental dry eye (EDE) model, mice received subcutaneous injection of 0.5 mg/0.2 mL scopolamine hydrobromide (Sigma-Aldrich Corp., St. Louis, MO, USA) four times a day (8 AM, 11 AM, 2 PM, and 5 PM) for 14 days with exposure to an air draft and 30% ambient humidity, as previously described.17–21 The mice were divided into three groups as follows: untreated (UT) control mice that were not exposed to desiccating stress; wild EDE mice exposed to desiccating stress; and NOD2-KO mice exposed to desiccating stress. Each group consisted of six animals, and the experiments were performed on three independent sets of mice.

Immunofluorescent Staining

Immunofluorescent staining was performed in cryosections of the eye and adnexa. Sections were fixed in acetone at −20°C, and then incubated at 4°C overnight with mouse monoclonal anti-NOD2 antibody (1:50, Santa Cruz Biotechnology, Dallas, TX, USA). The next day, samples were incubated with AlexaFluor 488-conjugated chicken anti-mouse (1:200, catalog no. A21200; Invitrogen, Eugene, OR, USA) for 1 hour and incubated for 2 hours at room temperature with secondary antibodies, the immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL Blotting Analysis System; Amersham, Arlington Heights, IL, USA). Anti-β-actin was used as an internal control.

Multiplex Immunobead Assay

A multiplex immunobead assay (Luminex 200; Luminex Corp., Austin, TX, USA) was used to measure concentrations of TNF-α, IL-1β, IFN-γ, and IL-6 in the conjunctiva (n = 3), as previously described.20 Conjunctival tissues were collected and pooled in lysis buffer containing protease inhibitors for 30 minutes. The cell extracts were centrifuged at 14,000g for 15 minutes at 4°C, and the supernatants were stored at −70°C before use. The supernatants were added to wells containing the appropriate cytokine bead mixture that included mouse monoclonal antibodies specific for the cytokines and chemokines for 60 minutes. After three washes, the biotinylated secondary antibody mixture was applied for 30 minutes in the dark at room temperature. The reactions were detected after addition of streptavidin-phycocerythrin using an analysis system (xPONENT; Luminex Corp.). The concentrations of these factors in the tissues were calculated using standard curves of known concentrations of recombinant mouse cytokines.

Flow Cytometry

Flow cytometry was performed to determine the number of CD11b+, CD4+CCR5+ T-cells and DCF-DA from the cornea, conjunctiva, and lacrimal gland (n = 3) using a previously described method.22 Tissues from each group were teased and shaken at 37°C for 60 minutes with 0.5 mg/mL collagenase type D (Roche Applied Science, Indianapolis, IN, USA). After grinding with a syringe plunger and passage through a cell strainer, cells were obtained, centrifuged, and resuspended in PBS with 1% bovine serum albumin. After washing, the samples were incubated with fluorescein-conjugated anti-CD4 antibody (BD Biosciences, San Jose, CA, USA), phycoerythrin-conjugated anti-CCR5 antibody (BD Biosciences), and isotype control antibody at 37°C for 30 minutes. The number of CD11b+ and CD4+CCR5+ T-cells were counted using a cytometer (FACSCalibur with Cell Quest software; BD Biosciences).

Histologic Analysis

For periodic acid-Schiff (PAS) staining, conjunctival tissue was fixed in 4% paraformaldehyde overnight and embedded in paraffin. Serial sections 6-μm thick were cut from each sample. The sections were deparaffinized and stained with the 0.5% PAS for identification of goblet cells. Sections from each group were photographed with a microscope equipped with a digital camera. The number of positively stained goblet cells in the superior and inferior conjunctiva was counted in three sections from each eye by using image analysis software (Media Cybernetics, Silver Spring, MD, USA). The data are presented as the average number of goblet cells per millimeter.

TUNEL Staining

A TUNEL assay was a method for detecting DNA fragmentation by labeling the 3’-hydroxyl termini in the double-stranded DNA breaks generated during the apoptotic cascade, widely used to identify and quantify apoptotic cells. After tissue preparation with paraffin embedded and paraformaldehyde fixed material, staining was performed using a commercially available kit (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI, USA) according to the manufacturer’s protocols with modifications.23–24 The nuclei were visualized with 4’,6-diamidino-2-phenylindole (DAPI) present in the mounting medium (ProLong Gold Antifade Mounting Medium; Invitrogen, Carlsbad, CA, USA). Digital images of representative areas were captured with a Leica laser scanning confocal microscope.
Evaluation of Tear Film and Ocular Surface Parameters

Tear volume was measured using phenol red–impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA, USA), as previously described. The threads were placed in the lateral canthus for 20 seconds. Tear volume, expressed in millimeters of thread dampened by tears and turned red, was measured using a microscope (SMZ 1500; Nikon, Melville, NY, USA). A standard curve was generated to convert distance into volume.

After instilling 1 μL of 1% sodium fluorescein into the inferior conjunctival sac, tear film break-up time (TBUT) was recorded in seconds using slit lamp biomicroscopy (BQ-900; Haag-Streit Bern, Switzerland) under cobalt blue light. Ninety seconds later, punctate staining of the corneal surface was evaluated in a masked fashion. Each cornea was divided into four quadrants that were scored individually. The corneal fluorescein staining score was calculated using a 4-point scale: 0, absent; 1, slightly punctate staining <30 spots; 2, punctate staining >30 spots, but not diffuse; 3, severe diffuse staining but no positive plaque; and 4, positive fluorescein plaque. The four area scores were added to generate a final score (possible maximum score of 16 points).

Statistical Analysis

The Statistical Package for Social Sciences (SPSS, version 17.0, Chicago, IL, USA) was used for statistical analyses. Results are presented as mean ± SD. The data were analyzed using 1-way ANOVA with a Tukey post-hoc analysis; P < 0.05 was considered statistically significant.

RESULTS

Immunofluorescent Staining for the NOD2 Expression

Magnified images of the representative corneal sections stained with NOD2 (green) and counterstained with DAPI (blue) are demonstrated in Figure 1A. The mean NOD2 expression cell counts in the corneal epithelium at day 14 were 5.0 ± 0.3 cells/100 μm, 13.0 ± 1.7 cells/100 μm, and 2.0 ± 0.2 cells/100 μm in the UT, EDE, and NOD2-KO groups, respectively (Fig. 1B). A significant increased corneal NOD2 expression was noted in the EDE group, compared to the UT (P < 0.01) and NOD2-KO groups (P < 0.01).

Expression of RIP2 and NF-κB in Corneal and Conjunctival Tissues

To investigate involvement of RIP2 and NF-κB activation, we identified the expression of phosphorylated RIP2 and phosphorylated NF-κB p65 in corneal and conjunctival tissues. (Figs. 2A, 2B). In the EDE group, we found that the relative protein expression of RIP2 and NF-κB in the cornea and conjunctiva were significantly increased after desiccating stress (all P < 0.05). In both tissues, the NOD2-KO group showed a lower expression of RIP2 and NF-κB than in the EDE group and a higher expression of NF-κB than in the UT group after desiccating stress (all P < 0.05).

Inflammatory Cytokine Levels in Conjunctival Tissue

The EDE and NOD2-KO groups had a significantly higher level of IL-1β, IL-6, IFN-γ, and TNF-α than the UT group at 7 and 14 days, and the EDE group had a significantly higher level of IL-1β, IL-6, and IFN-γ than the UT group at 5 days (all P < 0.05). In addition, IL-1β, IL-6, IFN-γ, and TNF-α levels in the NOD2-KO group were significantly lower than those in the EDE group at 7 and 14 days (all P < 0.05, Figs. 3A–D).
Flow Cytometric Analysis

Histograms of percentages of CD11b$^+$ and CD4$^+$CCR5$^+$ T cells in the conjunctiva from representative samples from the UT, EDE, and NOD2-KO groups are shown in Figures 4 and 5. The EDE and NOD2-KO groups had a higher number of CD11b$^+$ and CD4$^+$CCR5$^+$ T cells compared to the UT group (all $P < 0.01$). The percentages of CD11b$^+$ and CD4$^+$CCR5$^+$ T cells were significantly lower in the NOD2-KO groups than in the EDE group (all $P < 0.01$).

Figure 2. Western blot analysis of RIP2 and NF-kB expression in the UT, EDE, and NOD2-KO groups at day 14. Representative immunoblots of cell lysates showing detection of phosphorylated RIP2 and NF-kB with β-actin loading control in the cornea and conjunctiva (A). Relative protein expression of RIP2 and NF-kB in the (B) cornea and conjunctiva ($*P < 0.05$).

Figure 3. Levels of (A) IL-1β, (B) IL-6, (C) IFN-γ, and (D) TNF-α in the conjunctiva of the UT, EDE, and NOD2-KO groups ($*P < 0.05$).
Conjunctival Goblet Cell Density

Representative histologic findings showing the conjunctival goblet cells in each group are presented in Figure 6A. The mean goblet cell counts at day 14 were $27.3 \pm 3.7$ cells/100 μm, $8.3 \pm 2.1$ cells/100 μm, and $16.0 \pm 2.8$ cells/100 μm in the UT, EDE, and NOD2-KO groups, respectively (Fig. 6B). The EDE and NOD2-KO groups showed a significantly lower density of conjunctival goblet cells compared to the UT group (all $P < 0.05$).

Figure 4. Flow cytometry showing (A) representative histograms of CD11b$^+$ cells, and (B) frequency of CD11b$^+$ cells in the cornea, conjunctiva, and lacrimal gland of the UT, EDE, and NOD2-KO groups at 14 days (*$P < 0.05$).

Figure 5. Flow cytometry showing CD4$^+$CCR5$^+$T cells in the cornea, conjunctiva, and lacrimal gland of the UT, EDE, and NOD2-KO groups at day 14 (*$P < 0.05$).
0.01). Goblet cell density in the NOD2-KO group was higher than that in the EDE group ($P < 0.01$).

Counts of TUNEL Positive Cells in Corneal and Conjunctival Tissues

Magnified images of the representative corneal sections stained with TUNEL (green) and counterstained with DAPI (blue) are demonstrated in Figure 7A. The mean apoptotic cell counts in the cornea were $6.0 \pm 1.5$ cells/100 $\mu$m, $14.8 \pm 2.8$ cells/100 $\mu$m, and $10.2 \pm 1.3$ cells/100 $\mu$m, and the counts of conjunctiva were $2.3 \pm 0.8$ cells/100 $\mu$m, $20.3 \pm 3.8$ cells/100 $\mu$m, and $9.7 \pm 1.5$ cells/100 $\mu$m in the UT, EDE, and NOD2-KO groups, at day 14 (Fig. 7B). Apoptotic cell counts in the cornea and conjunctiva were significantly higher in the EDE and NOD2-KO groups compared to the UT group (all $P < 0.01$). In addition, the NOD2-KO group had lower apoptotic cell counts in the cornea ($P = 0.03$) and conjunctiva ($P < 0.01$) than the EDE group.

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**FIGURE 6.** PAS staining of (A) representative specimens and (B) mean conjunctival goblet cell densities in the UT, EDE, and NOD2-KO groups after 14 days of induction. (*$P < 0.05$).

**FIGURE 7.** TUNEL staining of (A) representative photographs and (B) mean numbers of TUNEL-positive cells in the cornea and conjunctiva of UT, EDE, and NOD2-KO groups at 14 days (*$P < 0.05$).
Clinical Parameters in the Ocular Surface

Three days after EDE induction, the mean tear volume was 0.043 ± 0.005 μL in the UT group, 0.039 ± 0.006 μL in the EDE group, and 0.040 ± 0.005 μL in the NOD2-KO group. Five days after induction, the mean tear volume was 0.042 ± 0.002 μL, 0.036 ± 0.001 μL, and 0.037 ± 0.002 μL in the UT, EDE, and NOD2-KO groups, respectively. Seven days after EDE induction, the mean tear volume was 0.042 ± 0.006 μL in the UT group, 0.036 ± 0.001 μL, and 0.037 ± 0.002 μL in the EDE and NOD2-KO groups, respectively. At 14 days after induction, the mean tear volume was 0.041 ± 0.003 μL in the UT group, 0.019 ± 0.002 μL in the EDE group, and 0.015 ± 0.003 μL in the NOD2-KO group. The NOD2-KO groups showed a higher tear volume than the EDE group at 7 and 14 days, and the EDE and NOD2-KO groups showed a lower tear volume than the UT group (both P < 0.01; Fig. 8A).

At 3 days, values for TBUT were 4.28 ± 0.32 seconds in the UT group, 3.89 ± 0.45 seconds in the EDE group, and 4.14 ± 0.76 seconds in the NOD2-KO group. At 5 days, values for TBUT were 4.00 ± 0.31, 3.63 ± 0.42, and 3.67 ± 0.55 seconds in the UT, EDE, and NOD2-KO group in the respectively. At 7 days, values for TBUT were 3.96 ± 1.28, 1.64 ± 0.79, and 2.25 ± 0.68 seconds in the respective groups. At 14 days, the respective values were 3.93 ± 0.33, 1.12 ± 0.24, and 1.65 ± 0.50 seconds, in respective groups. The EDE and NOD2-KO groups showed a significant decrease in TBUT compared to the UT group at 7 and 14 days (both P < 0.01). TBUT was higher in the NOD2-KO group than in the EDE group at 7 and 14 days (both P < 0.01). The EDE and NOD2-KO groups showed a significant decrease in TBUT compared to the UT group at 7 and 14 days (both P < 0.01). TBUT was higher in the NOD2-KO group than in the EDE group at 7 and 14 days (both P < 0.01).

The EDE and NOD2-KO groups showed a higher tear volume than the EDE group at 7 and 14 days, and the EDE and NOD2-KO groups showed a lower tear volume than the UT group (both P < 0.01).

At 3 days, the corneal fluorescein staining scores were 2.69 ± 0.79 in the UT group, 2.94 ± 0.85 in the EDE group, and 2.75 ± 1.13 in the NOD2-KO group. At 5 days, the corneal fluorescein staining scores were 3.51 ± 1.15, 5.31 ± 2.87, and 4.50 ± 2.03 in the respective groups. At 7 days, the corneal fluorescein staining scores were 3.81 ± 1.28, 11.88 ± 2.83, and 8.88 ± 1.99 in the UT, EDE, and NOD2-KO groups, respectively. At day 14, the staining scores were 4.25 ± 1.24, 15.06 ± 0.85, 11.63 ± 2.40 in the respective groups (Figs. 8C, 8D). The EDE and NOD2-KO groups showed a higher corneal staining scores than the UT group at 7 and 14 days (both P < 0.01). The staining score was lower in the NOD2-KO group than in the EDE group at 7 and 14 days (both P < 0.01).

DISCUSSION

Ocular surface inflammation is now considered to be the main pathophysiology in DE development. Many studies have demonstrated that DE increases the ocular surface expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α.26,27 These inflammatory mediators appear to initiate an inflammatory cascade on the ocular surface, evidenced by increased expression of immune activation and adhesion molecules (HLA-DR and intracellular adhesion molecule-1) by the conjunctival epithelia.2,3 In the meantime, increased expression of inflammatory cytokines promotes the infiltration of additional CD11b+, CD11c+, CD45+ T cells in the ocular surface.28,29

Recently, the importance of innate immunity and its balance with adaptive immunity in DE pathogenesis have been highlighted. Pattern recognition receptors including TLRs and NLRs have been demonstrated to play an important role in triggering innate immune system, which results in the production of pro-inflammatory cytokines.30 Among NLRs, it has been reported that NLRP3 which was activated by increased reactive oxygen species (ROS) upregulated corneal and conjunctival IL-1β gene expression in environment-
induced murine DE.\textsuperscript{31} Furthermore, NLRP3 inflammasome-mediated innate immune responses triggered by increased ROS generation induced IL-1β-associated inflammation in hyperosmotic stressed human corneal epithelial cells and tear fluid samples of environment-induced DE patients.\textsuperscript{32} ROS-NLRP3-IL-1β signaling pathway might play a role in environment-induced DE development.

NOD1/2 which are another NLRs member can also mediate innate immune responses such as induction of inflammation, autophagy, or cell death.\textsuperscript{34} In ophthalmology, NOD2 was known to promote inflammation the Blau syndrome-associated uveitis.\textsuperscript{35} NOD2 activates NF-κB pathway through RIP2 kinase.\textsuperscript{36} Similar to other mucosal barriers, the epithelial NF-κB pathway acts as a pivotal regulator of downstream immune reaction in several corneal and conjunctival diseases including DE.\textsuperscript{37,38} We previously reported that a significantly increased expression of NOD1, inhibitor kappa B kinase-alpha, and NF-κB was detected in the conjunctiva of patients with DE including Sjögren syndrome.\textsuperscript{39} In addition, the level of NOD1 expression in the conjunctiva was significantly correlated with symptom, Schirmer test, and corneal fluorescent scores, showing its association with the disease severity. Therefore, the NOD1/2 signaling innate immune pathway may also be involved in induction of inflammation in DE.

In the present study, we found that NOD2 was expressed in the ocular surface epithelium of EDE mice. A significantly decreased expression of RIP2 and NF-κB was observed in the NOD2-KO group compared to the EDE group. Conjunctival IL-1β, IFN-γ, IL-6, and TNF-α levels and CD11b+ cell counts were significantly lower in the NOD2-KO group than in the EDE group. A few previous studies have also been reported that expression of NOD2 resulted in enhanced production of IL-1β, IFN-γ and IL-6.\textsuperscript{40,41} Our results suggest that NOD2 is expressed in the corneal epithelium of EDE, and the expression can be upregulated by environmental desiccating stress and pharmacological stimulation. In addition, increased NOD2 participates in the inflammatory response to ocular surface desiccating stress.

The activation of innate immune system results in T cell activation, and the release of pro-inflammatory cytokines, consequently activating adaptive immune system.\textsuperscript{32} Strikingly, there is increasing evidence that DE is an immune-mediated inflammatory disease that is mediated primarily by CD4+ T cells.\textsuperscript{42,43} Also, it is generally recognized that ocular surface apoptosis as well as inflammation plays an important role in the pathogenesis of DE.\textsuperscript{43} In our study, the percentage of CD4+CCR5+ T cells showed a significant decrease in the NOD2-KO group compared to the EDE group. In DE, CD4+ T cells infiltrate the ocular surface after inflammatory events and secrete proinflammatory cytokines. Specifically, IL-6 and IFN-γ are known to exacerbate the conjunctival goblet cell loss during desiccating stress.\textsuperscript{45} Our results revealed a significantly higher density of goblet cells and lower number of apoptosis cells in the NOD2-KO group compared to the EDE group. These results suggested that NOD2 pathway may involve in the T cell activation, thereby decreasing conjunctival goblet cells and promoting epithelial apoptosis.

Increased levels of inflammatory cytokines including IL-6, IL-1, and TNF-α have been found in the ocular surface of DE.\textsuperscript{46,47} Moreover, it was demonstrated that IL-6 played an important role in the pathogenesis of DE and highly correlated with ocular surface parameters including Schirmer I Test, TBUT, and cornea fluorescein staining in DE patients.\textsuperscript{48} Our results showed significantly higher tear volume and TBUT and lower corneal staining scores in NOD2-KO mice compared to EDE mice. We suggest that the expression of NOD2 promotes production of inflammatory cytokines such as IL-6 and TNF-α, thereby decreasing tear secretion and volume and enhancing further ocular surface damage. In this study, clinical evaluations between EDE and NOD2-KO groups were significantly different, but the difference was slight. Beside NOD2 receptor pathway, environmental desiccating stress with scopolamine may have activated pattern recognition receptors, such as TLRs and NLRP3, which were also involved in the pathogenesis of DE and promoted corneal epithelial defects.

In conclusion, the expression of NOD2 in the ocular surface could significantly increase the production of inflammatory cytokines and infiltration of CD11b+ and CD4+ T cells through activation of RIP2 and NF-κB. Also, it promoted ocular surface apoptosis and decreased tear secretion, inducing DE. Our study suggested that the NOD signaling pathway-associated innate immune response participates in the pathogenesis of DE.

Acknowledgments

Supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2017R1A2B4003367), and CNUH Biomedical Research Institute (BCRI19038). The authors alone are responsible for the content and writing of the paper.

Disclosure: Y. Li, None; R. Jin, None; L. Li, None; H.J. Yoon, None; J.-H. Choi, None; J.-H. Park, None; Z. Liu, None; W. Li, None; Z. Li, None; K.C. Yoon, None

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Investigative Ophthalmology & Visual Science


