S100B immunization triggers NFκB and complement activation in an autoimmune glaucoma model

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In glaucoma, latest studies revealed an involvement of the complement system with and without an elevated intraocular pressure. In the experimental autoimmune glaucoma model, immunization with antigens, such as S100B, lead to retinal ganglion cell (RGC) loss and optic nerve degeneration after 28 days. Here, we investigated the timeline of progression of the complement system, toll-like-receptor 4 (TLR4), and the transcription factor nucleus factor-kappa B (NFκB). Therefore, rats were immunized with S100B protein (S100) and analyzed at 3, 7, and 14 days. RGC numbers were comparable at all points in time, whereas a destruction of S100 optic nerves was noted at 14 days. A significant increase of mannose binding lectin (MBL) was observed in S100 retinas at 3 days. Subsequently, significantly more MBL+ cells were seen in S100 optic nerves at 7 and 14 days. Accordingly, C3 was upregulated in S100 retinas at 14 days. An increase of interleukin-1 beta was noted in S100 aqueous humor samples at 7 days. In this study, activation of complement system via the lectin pathway was obvious. However, no TLR4 alterations were noted in S100 retinas and optic nerves. Interestingly, a significant NFκB increase was observed in S100 retinas at 7 and 14 days. We assume that NFκB activation might be triggered via MBL leading to glaucomatous damage.

Glaucoma is defined as a progressive optic neuropathy with changes at the optic nerve head, gradual retinal ganglion cell (RGC) death, and visual field loss. An elevated intraocular pressure (IOP) is still considered the main risk factor, but there are increasing evidences that other pathological factors are involved. Some glaucoma patients, who do not have an elevated IOP, also show glaucomatous damage. Glaucoma is a multifactorial disease and besides other factors recent studies revealed a contribution of immunological processes. To analyze these mechanisms, the experimental autoimmune glaucoma model (EAG) was developed. Here, immunization with ocular antigens leads to RGC loss and optic nerve degeneration without IOP elevation. In this model, autoreactive antibodies were detected in the retinas as well as in optic nerves. Furthermore, an increase in microglia cell numbers, the macrophages of the central nervous system (CNS), was noted in these retinas and optic nerves. This raises the question whether the microglia are an epiphenomenon or part of the degeneration process. For instance, retinal microglia can produce complement system proteins. The complement system, as part of the innate immune system, is activated via three distinct routes. The classical pathway can be initiated through the protein C1q, while the mannose-binding-lectin (MBL) induces the lectin pathway. The alternative pathway is spontaneously activated through cleavage of C3b. All three ways are assembled in the terminal pathway, which starts with the protein C3. Finally, the membrane attack complex (MAC) forms a pore in the target cell and forces its lysis. In the last years, studies showed a contribution of the complement system in glaucoma disease, e.g. deposits of complement components were observed in the human glaucomatous retina. Those depositions were also noted in ocular hypertension (OHT) animal models. Our group could also show an IOP-independent activation of the complement system in retinas and optic nerves.

One regulator of the innate immune system is the transcription factor nucleus factor-kappa-light-chain-enhancer of activated B cells (NFκB). It controls several cellular mechanisms such as proliferation, differentiation, survival, and apoptosis. In unstimulated cells, NFκB accumulates in the cytoplasm. After stimulation, the inhibitory protein IκBα dissociates from the NFκB complex and the transcription factor translocates into the nucleus.
Additionally, the levels of interleukin-1 beta (IL-1β) were evaluated via immunohistology and quantitative real-time PCR (qRT-PCR) on 3, 7, and 14 days after immunization.

We propose an activation of the complement system as well as an enhanced TLR4/NFκB pathway signaling in retinas and optic nerves of the EAG model before cell loss. To investigate this hypothesis, several cell types were used in our study.

### Results

#### No apoptotic retinal ganglion cells, but optic nerve degeneration.

At 14 days, Brn-3a stained retinal whole mounts revealed no statistically significant alterations in the S100 group (p > 0.05, supplement Fig. 1A,B). However, in total as well as in the three separate regions of the whole mounts (central, middle, peripheral), a slight decline of about 10% of RGCs was observable. The number of RGCs on retinal cross-sections was comparable in S100 and Co animals at 3, 7, and 14 days (p > 0.05, supplement Fig. 1C,D). Also, no changes were observed in cleaved caspase-3+ RGCs in the S100 group after all points in time (p > 0.05, supplement Fig. 1C,E).

QRT-PCR revealed no alterations in the expression level of Pou4f1 and Bax/Bcl-2 (p > 0.05, supplement Fig. 1F,G, Table 1). In regard to SM1-32 labeling in the optic nerve, no changes occurred in the S100 group after 3 and 7 days (p > 0.05, Fig. 1A,B). However, a significantly higher SM1-32 score was noted in S100 animals at 14 days (p = 0.007). The S100B optic nerve staining showed no alterations between both groups throughout the study (p > 0.05, Fig. 1C,D).

#### Slight complement activation via the lectin pathway in retinas after immunization.

For retinal C3, no changes were noted in the S100 group after 3 and 7 days (p > 0.05, Fig. 2A,B). Also, no alterations were seen in C3 mRNA expression levels at these points in time (p > 0.05, Fig. 2C). At 14 days, significantly more C3+ cells could be observed in the S100 animals (p = 0.04). Additionally, a significant upregulation of C3 mRNA was detected at this point in time (p < 0.001, Table 1). C3 cell numbers on optic nerves were comparable in both groups throughout the study (p > 0.05, Fig. 2D,E).

In regard to retinal MAC expression, the immunohistological data revealed no alterations in the S100 group at all points in time (p > 0.05, supplement Fig. 2A,B). MAC was often co-localized with Brn-3a+ cells (supplement Fig. 3). Also, the C5 mRNA expression remained unaltered at 3, 7, and 14 days (p > 0.05, supplement Fig. 2C).

### Table 1. Analyses of mRNA levels via quantitative real-time PCR. Significant values are marked in bold.

|  | 3 days | 7 days | 14 days |
|---|---|---|---|
| Bax/Bcl-2 | 0.99 (0.67–1.44) | 0.89 (0.76–1.17) | 0.84 (0.77–0.96) |
| P-value | 0.9 | 0.4 | 0.07 |
| C3 | 0.57 (0.20–1.16) | 0.73 (0.53–1.07) | 1.56 (1.33–1.87) |
| P-value | 0.1 | 0.2 | <0.001 |
| C5 | 0.84 (0.50–1.31) | 1.47 (1.06–1.98) | 0.79 (0.62–0.96) |
| P-value | 0.5 | 0.3 | 0.3 |
| Factor B | 2.22 (1.41–3.82) | 0.76 (0.54–1.26) | 0.54 (0.37–1.09) |
| P-value | 0.02 | 0.3 | 0.3 |
| Mbl | 1.66 (1.31–2.22) | 0.91 (0.73–1.09) | 0.48 (0.32–0.839) |
| P-value | 0.847 | 0.6 | 0.08 |
| NEMO | 0.76 (0.55–1.05) | 1.00 (0.87–1.21) | 0.93 (0.86–0.99) |
| P-value | 0.09 | 0.99 | 0.2 |
| Pou4f1 | 0.83 (0.57–1.31) | 1.33 (1.06–1.67) | 0.97 (0.86–1.07) |
| P-value | 0.4 | 0.2 | 0.8 |
| Myd88 | 1.48 (0.99–2.44) | 0.86 (0.72–1.13) | 0.92 (0.75–1.29) |
| P-value | 0.1 | 0.3 | 0.7 |
| Tlr4 | 0.80 (0.52–1.22) | 1.16 (0.90–1.42) | 0.94 (0.85–1.08) |
| P-value | 0.3 | 0.3 | 0.4 |

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In the optic nerves, MAC staining was comparable in both groups at all points in time (p > 0.05, supplement Fig. 2D,E).

Regarding retinal factor B, no alterations were observed in the S100 animals at 3, 7, and 14 days (p > 0.05, Fig. 3A,B). Interestingly, an upregulation of factor b mRNA was noted in the S100 retinas at 3 days (p = 0.02, Fig. 3C, Table 1). At 7 and 14 days, the expression went back to control level (p > 0.05).

Significantly more MBL⁺ cells were noted in the S100 retinas after 3 days (p < 0.0001, Fig. 3D,E). MBL was often co-localized with active microglia (supplement Fig. 3). Additionally, the qRT-PCR analysis revealed an upregulation of Mbl mRNA at this point in time (p = 0.047, Fig. 3F). At 7 days, the number of MBL⁺ cells in both groups was similar (p > 0.05). This was still the case at 14 days (p > 0.05). No changes were observed regarding Mbl expression at 7 and 14 days (p > 0.05, Table 1). In the optic nerves, the number of MBL⁺ cells remained unaltered after 3 days (p > 0.05, Fig. 3G,H). At 7 days, significantly more MBL⁺ cells were observed in the S100 optic nerves (p = 0.03). An increased number of MBL⁺ cells was still noted after 14 days (p = 0.02).

Increase of retinal NFκB. At 3 days, no changes were noted in regard to NFκB labeling in the S100 group (p > 0.05). More NFκB⁺ cells were observed after 7 days (p = 0.03). Also, a higher number of NFκB⁺ cells was revealed in the S100 retinas at 14 days (p = 0.03, Fig. 4A,B). The qRT-PCR data showed no alterations in Nfκb expression after 3 and 14 days (p > 0.05, Table 1), while a slight trend pointed towards an upregulation was noted at 7 days (p = 0.18, Fig. 4C). Furthermore, analyses of Myd88 expression levels revealed a slight upregulation at 3 days (p = 0.1, Table 1). At 7 and 14 days, the expression of Myd88 remained unaltered (p > 0.05, Fig. 4D). The mRNA expression levels of NEMO showed no changes at all points in time (p > 0.05, Fig. 4E, Table 1).
No TLR4 alterations in retinas and optic nerves. The analysis of TLR4+ cells in the retinas showed no changes in the S100 group after 3, 7, and 14 days (p > 0.05, Fig. 5A,B). Also, the expression of Tlr4 mRNA remained unaltered in the S100 retinas at all points in time (p > 0.05, Fig. 5C, Table 1). The quantification of TLR4+ cells in the optic nerves also revealed no differences between both groups at all points in time (p > 0.05, Fig. 5D,E).

Enhanced levels of IL-1β in aqueous humor. ELISA analysis of IL-1β in sera of S100B animals revealed comparable levels as in control animals at all points in time (p > 0.05, Fig. 6A). Also, the concentration levels of IL-1β in the aqueous humor were comparable in both groups at 3 and 14 days (p > 0.05, Fig. 6B). Nevertheless, 7 days after immunization, a significantly higher concentration of IL-1β was measured in the aqueous humor of S100B animals (p = 0.03).

Discussion

Complement activation in glaucoma pathogenesis. Several studies indicate an involvement of the immune system in the pathogenesis of glaucoma. Besides findings of autoantibody patterns and depositions, also an activation of microglia was noted in both human glaucoma and in experimental animal studies. Therefore, a contribution of the complement system in glaucoma disease seems likely. In the study presented here, we noted an activation of the complement system via the lectin pathway, before any cell loss or optic nerve degeneration occurred. This is consistent with previous findings from our group, where the complement system was
Figure 3. Activation through the lectin pathway. (A) Retinas were labeled with an antibody against factor B (red) 3, 7, and 14 days after immunization. Cell nuclei were stained with DAPI (blue). (B) No alterations in factor B+ cell numbers were noted in S100 animals compared to controls at all points in time (p > 0.05). (C) The qRT-PCR analyses revealed an upregulation of factor b mRNA at 3 days (p = 0.02). No changes were observed at 7 and 14 days (p > 0.05). (D) Retinal cross-sections were stained with anti-MBL (green) and DAPI (blue) at 3, 7, and 14 days. (E) At 3 days, significantly more MBL+ cells were observed in S100 retinas (p < 0.0001). No changes were noted later on (p > 0.05). (F) Expression levels of Mbl measured with qRT-PCR at 3, 7, and 14 days. At 3 days, a significant increase of Mbl mRNA expression was detected in S100 retinas (p = 0.048). Mbl went back to control level later on (p > 0.05). (G) Optic nerve sections were labeled with an anti-MBL antibody (green) and cell nuclei with DAPI (blue) 3, 7, and 14 days after immunization. (H) The number of MBL+ cells remained unaltered in S100 optic nerves at 3 days (p > 0.05). After 7 days, significantly more MBL+ cells were noted in the S100 group (p = 0.03) and were still present at 14 days (p = 0.02). Abbreviation: GCL = ganglion cell layer. Values are mean ± SEM for immunohistology and median ± quartile + maximum/minimum for qRT-PCR. Scale bars: 20µm.
also initiated through the lectin route following immunization with an optic nerve homogenate antigen (ONA) \(^\text{19}\). MBL, an initiator of the lectin pathway, is a C-type lectin belonging to the collectins\(^\text{30}\). An increase of MBL\(^+\) cells in the retina was observed at 3 days and an enhancement of NF-\(\kappa\)B at 7 and 14 days. Since collectins are able to trigger NF-\(\kappa\)B activation, it is possible that in this model NF-\(\kappa\)B is triggered via MBL, which subsequently might lead to cell death. Interestingly, in human glaucoma as well as in OHT models, the classical pathway was upregulated\(^\text{43,44}\). However, proteomic analysis of human glaucoma donor retinas also revealed an upregulation of proteins linked to the lectin pathway\(^\text{15}\). Additionally, we found a slight increase of the alternative pathway via qRT-PCR analyses. An inappropriate alternative pathway response is known to occur in eye diseases like age-related macular degeneration (AMD). Here, factor B related genes are upregulated, while factor H genes are downregulated\(^\text{17,31,32}\). However, proteomic analysis of human glaucoma donor retinas also revealed an upregulation of proteins linked to the lectin pathway\(^\text{15}\). Furthermore, enhanced levels of the pro-inflammatory cytokine IL-1\(\beta\) were observed in S100 animals \((p = 0.04)\). At all points in time, the expression of Nf\(\kappa\)B mRNA remained unchanged \((p > 0.05)\). Abbreviation: GCL = ganglion cell layer. Values are mean \(\pm\) SEM for immunohistology and median \(\pm\) quartile + maximum/minimum for qRT-PCR. Scale bars: 20\(\mu\)m.

**Figure 4.** Increased NF-\(\kappa\)B staining. (A) Retinas were labeled with an anti-NF-\(\kappa\)B antibody (green) and DAPI (blue). (B) At 3 days, no changes were observed in regard of the number of NF-\(\kappa\)B\(^+\) cells in the S100 group \((p > 0.05)\). After 7 days, significantly more NF-\(\kappa\)B\(^+\) cells were noted in S100 animals \((p = 0.03)\). A significant increase of NF-\(\kappa\)B\(^+\) cells was still revealed in this group after 14 days \((p = 0.03)\). (C) QRT-PCR analysis revealed no differences in Nf\(\kappa\)B mRNA expression at all points in time \((p > 0.05)\). However, a slight trend towards an upregulation could be observed at 7 days \((p = 0.18)\). (D) The expression levels of Myd88 showed a trend towards an upregulation at 3 days \((p = 0.1)\). At 7 and 14 days, no alterations were noted \((p > 0.05)\). (E) At all points in time, the expression of NEMO mRNA remained unchanged \((p > 0.05)\). Abbreviation: GCL = ganglion cell layer. Values are mean \(\pm\) SEM for immunohistology and median \(\pm\) quartile + maximum/minimum for qRT-PCR. Scale bars: 20\(\mu\)m.

**Enhanced NF-\(\kappa\)B signaling without TLR4 contribution.** The transcription factor NF-\(\kappa\)B is constitutively expressed in the CNS and is activated in various neurological diseases, such as Morbus Alzheimer, Morbus Parkinson, or Huntington’s disease\(^\text{37-40}\). It is known that components of NF-\(\kappa\)B are TLR induced genes that undergo positive feedback regulation by NF-\(\kappa\)B\(^\text{41}\). In human glaucomatous retinas, an upregulation of TLRs in microglia and astrocytes was reported\(^\text{45}\). It could also be observed that TLR4 signaling and NF-\(\kappa\)B activation accompanied by complement upregulation is a key mechanism of neuroinflammation\(^\text{42}\). An increase of NF-\(\kappa\)B was observed in the trabecular meshwork of glaucoma patients\(^\text{43,44}\). In microglia, NF-\(\kappa\)B controls migration to the site of injury due to expression of \(\beta\)-integrin CD11a. In our study, we noted an increase of NF-\(\kappa\)B in the retinas 7 and 14 days after immunization. This is followed by an activation of microglia at 14 days, which was previously described\(^\text{6}\). Furthermore, enhanced levels of the pro-inflammatory cytokine IL-1\(\beta\) were observed in S100 aqueous humor at 7 days. Yoneda et al. noted that IL-1\(\beta\) plays an important role in mediating ischemic and excitotoxic damage in glaucomatous retina\(^\text{45}\). Several studies indicate that IL-1\(\beta\) is secreted by microglia in photo-oxidative damage\(^\text{46-48}\), neovascular AMD\(^\text{19}\), retinitis pigmentosa\(^\text{49}\), and retinal detachment\(^\text{50}\). But not only microglia/macrophages, also NF-\(\kappa\)B was found to play multiple roles in the induction of IL-1\(\beta\) transcription\(^\text{52}\).

Regarding TLR4, we could not note any alterations in retinas and optic nerves in the EAG model throughout the study. Possibly, an upregulation of TLR4 could be observed at subsequent points in time, since a loss of RGCs is not measurable before day 28 in this model\(^\text{6,19}\). Nonetheless, these results strengthen the hypothesis that NF-\(\kappa\)B plays a crucial role in glaucoma pathogenesis.
Conclusion
We conclude that the early activation via the lectin complement pathway triggers NFκB signaling with subsequent microglia response in this EAG model. The combination of these mechanisms seems to initiate the IOP-independent RGC death and optic nerve degeneration. Therefore, an inhibition of complement components might lead to protection of RGCs and optic nerve fibers. However, further studies are required to gain more knowledge regarding the interaction of NFκB/complement signaling components and their contribution to glaucoma like RGC death.

Methods
Animals. All procedures concerning animals adhered to the ARVO statement for the use of animals in ophthalmic and vision research. All experiments involving animals were approved by the animal care committee of North Rhine-Westphalia, Germany, and were performed in accordance with relevant guidelines and regulations.

Male Lewis rats (Charles River, Sulzfeld, Germany), 6 weeks of age, were used for the experiments and kept under environmentally controlled conditions with free access to chow and water. Detailed observations and health checks, including eye exams, were performed regularly, as described previously53.

Immunizations. Rats received 1 mg/ml S100B (Sigma Aldrich, Munich, Germany)7,8. The antigen was mixed with incomplete Freund’s adjuvant (200 µl) plus 3 µg pertussis toxin (both Sigma Aldrich). The animals of the control group (Co) were injected with 0.9% NaCl in Freund’s adjuvant and pertussis toxin.

For tissue dissection, animals were sacrificed with an overdose CO₂ at 3, 7, and 14 days after immunization.

Retinal ganglion cell counts via whole mounts. 14 days after immunization, eyes were fixed in 4% paraformaldehyde (PFA) for one hour and then prepared as whole mounts (n = 6/group)19. The following steps...

Figure 5. No TLR4 alterations in retina and optic nerve. (A) Retinal cross-sections were labeled with an anti-TLR4 antibody (red) and DAPI (blue) at 3, 7, and 14 days. (B) No alterations in the number of TLR4+ cells were observed in the S100 retinas at all points in time (p > 0.05). (C) Also, no altered Trl4 mRNA expression could be noted at all points in time via qRT-PCR (p > 0.05). (D) To determine TLR4 expression in optic nerves, sections were stained with anti-TLR4 (red) and DAPI (cell nuclei, blue). (E) After 3 days, no changes were seen regarding the TLR4 expression in the S100 animals (p > 0.05). Also, no alterations were detected later on (p > 0.05). Abbreviation: GCL = ganglion cell layer. Values are mean ± SEM for immunohistology and median ± quartile + maximum/minimum for qRT-PCR. Scale bars: 20 µm.
were performed at 20 °C on a thermo shaker (70 rpm). First, the whole mounts were blocked with 10% donkey serum and 0.5% Triton-X in PBS for 90 min. Then, they were incubated with the RGC marker Brn-3a 54 (1:300; Santa Cruz, CA, USA) overnight, followed by a 2 hour incubation of donkey anti-goat Alexa Flour 488 (1:1000; Dianova, Hamburg, Germany). From each of the four whole mount arms, three photos were captured (central, middle, and peripheral) with an Axiocam HRc CCD camera on an Axio Imager M1 fluorescence microscope (Zeiss, Jena, Germany). Cells were counted using ImageJ software (V 1.43 u; NIH, Bethesda, MD, USA).

Retina and optic nerve histology. Retinas and optic nerves were fixed in 4% PFA for 1 (retina) or 2 hours (optic nerves), dehydrated in sucrose, and embedded in Tissue Tek (Thermo Fisher, Waltham, CA, USA). Cross-sections of the retina (10 µm) and longitudinal optic nerve sections (4 µm) were cut with a Cryostat (Thermo Fisher) and mounted on Superfrost slides (Thermo Fisher).

Immunohistology of retinal and optic nerve sections. To identify the different cell types in the retina and the optic nerve, specific immunofluorescence antibodies were applied (retina: n = 5–6/group, optic nerve: n = 6–8/group, 6 sections/staining; Table 2) 19. Briefly, retina and optic nerve sections were blocked with a solution containing 10–20% donkey and/or goat serum and 0.1% Triton-X in PBS. Primary antibodies were incubated at room temperature overnight. Incubation using corresponding secondary antibodies was performed for 1 h on the next day. Nuclear staining with 4′, 6 diamidino-2-phenylindole (DAPI) was included to facilitate the orientation on the slides. Negative controls were performed by using secondary antibodies only.

Histological examination of retinas and optic nerves. All photographs were taken with a fluorescence microscope (Axio Imager M1). In the retina, two photos of the peripheral and two of the central part of each section were captured for each point in time. In the optic nerve, three photos were captured (proximal, middle, and distal). The images were transferred to Corel Paint Shop Pro (V13, Corel Corporation, CA, USA) and equal excerpts were cut out. RGCs, cleaved caspase 3+ RGCs, complement factors, NFκB+, and TLR4+ cells were counted using ImageJ software. The neurofilament marker SMI-32 was evaluated via a scoring system ranging from 0 (intact structure) to 2 (loss of structural integrity, fissures, many retraction bulbs) 8,55,56. Measurement and analysis of S100B area was performed using ImageJ software as described previously 53,57,58. Briefly, images were transformed into grayscale. To minimize interference with background labeling, a rolling ball radius of 50 pixels was subtracted. Then, for each picture, a suitable lower threshold was set. The ideal threshold was obtained when
Table 2. Primary and secondary antibodies used for immunohistochemistry.

| Antibody       | Company       | Tissue  | Dilution | Antibody Company  | Tissue  | Antibody Company  | Tissue  |
|----------------|---------------|---------|----------|-------------------|---------|-------------------|---------|
| Anti-Brn-3a    | SySy          | Retina  | 1:1000   | Donkey anti-rabbit Alexa Fluor 888 | Retina  | Invitrogen       | Retina  |
| Anti-Brn-3a    | Santa-Cruz    | Retina  | 1:100    | Goat anti-mouse Alexa Fluor 888 | Retina  | Invitrogen       | Retina  |
| Anti-C3        | Cedarlane     | Retina  | 1/500    | Goat anti-rabbit IgG Cy3 | Retina  | Linaris          | Retina  |
| Anti-C5b-9 (MAC) | Biozol    | Retina  | 1/100    | Donkey anti-mouse DyLight 488 | Retina  | Invitrogen       | Optic nerve |
| Anti-cleaved caspase 3 | Sigma-Aldrich | Retina  | 1/300    | Donkey anti-rabbit Alexa Fluor 555 | Retina  | Invitrogen       | Retina  |
| Anti-ED1       | Millipore     | Retina  | 1/250    | Donkey anti-mouse Alexa Fluor 488 | Retina  | Invitrogen       | Retina  |
| Anti-factor B  | TECOmedical   | Retina  | 1/1000   | Donkey anti-goat Cy3 | Retina  | Abcam            | Retina  |
| Anti-MBL       | Biozol        | Retina  | 1/100    | Donkey anti-rabbit Alexa Fluor 555 | Retina  | Invitrogen       | Retina  |
| Anti-NeuN      | Millipore     | Retina  | 1/500    | Donkey anti-chicken Alexa Fluor 488 | Jackson ImmunoResearch | Retina  |
| Anti-NFkB p50  | Santa-Cruz    | Retina  | 1/500    | Goat anti-mouse Alexa Fluor 888 | Retina  | Invitrogen       | Optic nerve |
| Anti-S100B     | Novus Biological | Optic nerve | 1/100    | Donkey anti-rabbit Alexa Fluor 555 | Retina  | Invitrogen       | Optic nerve |
| Anti-SMI-32    | Biolegend     | Optic nerve | 1/2000   | Goat anti-mouse Alexa Fluor 488 | Retina  | Invitrogen       | Optic nerve |
| Anti-TLR4      | Abcam         | Retina  | 1/400    | Donkey anti-rabbit Alexa Fluor 555 | Retina  | Invitrogen       | Retina  |

Table 3. Oligonucleotide sequences. The listed oligonucleotide pairs were used in quantitative real-time PCR experiments, while β-actin and cyclophilin served as housekeeping genes. The predicted amplicon sizes are given. Abbreviations: F = forward, R = reverse, acc. no. = accession number, bp = base pair.
the grayscale picture and the original one corresponded. Afterwards, the mean value of the lower threshold was calculated, and this number was used for final analysis. The percentage of the labeled area was measured between these defined thresholds: lower threshold: 11.61; upper threshold: 247.44.

**RNA preparation and CDNA synthesis.** For RNA preparation, retinas from every point in time were isolated, transferred into lysis buffer containing 2-mercaptoethanol (Sigma-Aldrich), and snap frozen in liquid nitrogen (n = 3–6/group). Total RNA was extracted with the Gene Elute Mammalian Total RNA Miniprep Kit according to the manufacturer’s instructions (Sigma-Aldrich) and digested with RNase-free DNase I (Sigma-Aldrich). Quality and quantity of RNA were assessed by measuring the ratio of absorbance values at 260 and 280 nm (BioSpectrometer®8, Eppendorf, Hamburg, Germany). Total RNA (1 µg) was used for reverse transcription with a cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA).

**Quantitative real-time PCR.** The designed oligonucleotides are shown in Table 3. Real-time-PCR (Roche Applied Science, Mannheim, Germany) analyses were performed using SYBR Green I and the Light Cycler®9 96 (Roche Applied Science)19,59,60. Oligonucleotide concentration was optimized to a final concentration of 200 nM and combined with 200 ng retinal cDNA per well. We set up two reactions per RNA sample (duplicates) with a final volume of 20 µl per single reaction61–63. Each qRT-PCR was performed in duplicates from each retina and for each point in time and repeated twice (n = 3–6/group). The average threshold cycle (Ct) values of the two independent experiments were used to calculate the ratios for the target genes64. In order to obtain amplification efficiencies of different primer sets, we generated standard curves by a twofold dilution series with template amounts ranging from 5 to 125 ng cDNA per well. The Ct values of two reference genes (β-actin and cyclophilin) were taken into account.

**Measurement of IL-1β in serum and aqueous humor.** Levels of the pro-inflammatory cytokine IL-1β in serum (n = 7/group) and aqueous humor (n = 4–6/group) were measured using a commercially available enzyme immunoassay kit 3, 7, and 14 days after immunization (ELISA; R&D systems, MN, USA). All samples were used undiluted. Each assay was performed according to the manufacturer’s instruction. All measurements were performed on a microplate reader (AESKU Reader with Gen5 ELISA software, AESKU.DIAGNOSTICS, Wendelstein, Germany)65.

**Statistics.** Immunohistological and ELISA data are presented as mean ± SEM. The S100 group was compared to the Co group via two-tailed Student’s t-test using Statistica Software (Version 13, Dell, Tulsa, OK, USA). Regarding qRT-PCR, data are presented as median ± quartile + minimum/maximum and were assessed using REST® software (Qiagen, Hilden, Germany)64. P-values below 0.05 were considered statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001.

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**Author Contributions**
S.R. performed experiments, analyzed data and wrote the manuscript, J.R. analyzed data and revised the manuscript, M.G. and I.G. performed experiments and analyzed data, G.S. performed experiments, A.F. and H.B.D. revised the manuscript, S.C.J. designed the study and revised the manuscript. All authors have approved the final article.

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