Glaucoma-like damage induced by S100B injection is accompanied by microglial response

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Glaucoma is currently the second leading cause of blindness worldwide. Due to aging societies the number of patients suffering from this disease will further increase in the next years. Unfortunately, glaucoma can remain asymptomatic until it is rather far progressed, hence about 10–50% of patients are unaware they suffer from this disease. Glaucoma is a progressive optic neuropathy associated with changes at the optic nerve head, gradual retinal ganglion cell (RGC) death, and visual field loss. High intraocular pressure (IOP) is the main risk factor, but the exact pathomechanism of glaucoma remains unexplained to date. In addition to mechanical damage due to increased IOP, which can injure axons and disrupt the ocular blood flow, numerous other factors have been recorded that contribute to the development of glaucoma. In recent years, the role of the immune system has come into focus as a possible contributor to glaucoma pathology. The involvement of immunological changes in glaucoma disease is based on the detection of altered antibody titer in serum samples of primary open-angle or normal-tension glaucoma patients and tear film samples of primary open-angle glaucoma patients. Affected patients showed antibodies against proteins such as heat shock protein (HSP)60, HSP27, or S100B (Grus et al., 2010; Bell et al., 2013).

S100B is a calcium- and zinc-binding protein of the S100 protein family, which is primarily expressed by astrocytes, but also by microglia or oligodendrocytes in retina and optic nerve. S100B plays a role in cell processes like signal transduction, cell differentiation, regulation of cell motility, transcription, protein phosphorylation, calcium homeostasis, and apoptosis. This takes place via the concentration modulation of the secondary messenger substance calcium and the binding of S100 receptors, such as the receptor for advanced glycation end products (RAGE). In low concentrations, S100B has neuroprotective effects and can promote neurite growth. In high concentrations, on the other hand, S100B reveals neurotoxic characteristics. This is induced via microglia activation (Sorci et al., 2010).

Glaucoma-like damage after systemic S100B immunization: A so-called autoimmune glaucoma model was established based on the previously mentioned altered antibody patterns in glaucoma patients. In this model, systemic immunization with different ocular antigens, including an antigen homogenate from bovine optic nerve or HSP27 leads to the formation of antibodies against them and ultimately to a glaucoma-like damage of retinal structures. The IOP was not elevated in this autoimmune glaucoma model. Antibody deposits, loss of RGCs, and increased glial cell activity were noted in the retina. In addition, a degeneration of the optic nerve was observable (Bell et al., 2013).

Since glaucoma patients displayed increased antibody levels against the S100B protein, the effect of S100B immunization was investigated in the autoimmune glaucoma model. Glaucoma-like changes could also be recorded after systemic immunization with the S100B protein. Also, a significantly higher number of microglia was seen in the retinae of these animals as well as a higher microglia activation rate. This could be a hint for a destructive impact of retinal microglia in this model. Optic nerve neurofilament dissolution was also evident after S100B immunization (Noristani et al., 2016).

Effect of intravitreal S100B application on retina and optic nerve: In the next step, S100B was injected intravitreally to examine not only the influence of systemic immunization but also the local effects of ocular antigens. For this purpose, 14 and 21 days after intravitreal S100B injection, evaluations were carried out (**Figure 1A**). We noted that the RGC number as well as the β-III tubulin protein level was reduced in the S100B animals. Moreover, active apoptotic mechanisms could be detected 14 days after immunization. The optic nerve neurofilament structure was damaged starting 3 days after immunization (**Figure 1B**). Based on these findings we assume that S100B directly damages the optic nerve axons and consequently, RGC cell bodies degenerate (Kuehn et al., 2018).

In a follow-up project our research group, an increased rate of NfκB, a transcription factor that regulates multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammatory responses, was observed in RGCs 14 days after S100B injection. Retinae in the S100B group displayed almost twice as many nuclear factor kappa-light-chain-enhancer of activated B^+ (NfκB) cells as both control groups (both: $P = 0.04$; **Figure 1B**). This indicates that NfκB activation might be associated with neuronal degeneration in this model. In the same study, an increased concentration of interleukin (IL)-1β was detected in serum of S100B animals after 14 days and also a tendency towards an IL-1β increase was detected in the aqueous humor. In addition, a highly increased number of retinal microglia expressing IL-1β could be noted in S100B retinae at 14 days. More precisely, significantly more IL-1β expressing microglia were observed in the S100B group in comparison to the PBS ($P = 0.01$) and the naïve group ($P = 0.02$). Furthermore, the number of microglia as well as the number of active microglia was increased in the retina and the optic nerve at 14 days. A strong microglia response was especially noted in optic nerves of S100B animals. The number of microglia, labelled with an anti-Iba1 antibody via immunohistology, was more than two-fold higher in the optic nerves of the S100B group than in the naïve and the PBS control groups (both: $P < 0.001$). Active microglia were visualized with a double staining of $ED1^+$ and $Iba1^+$ cells. Interestingly, the number of active microglia was much higher in the S100B group than in the controls (both: $P < 0.001$). Later on, after 21 days, microglia numbers as well as the rate of active microglia in both tissues were comparable within the groups (**Figure 1B**). Furthermore, the macroglia in retinae and optic nerves were analyzed after intravitreal S100B injection. Neither at 14 nor at 21 days, any differences regarding the occurrence of macroglia were observed between the retinae of the different groups. In contrast, in the optic nerve a slight astrogliosis, via glial fibrillary acidic protein (GFAP) staining, was detected in the S100B group in comparison to the naïve group after 14 days ($P = 0.03$), where differences in the GFAP area were not seen between the S100B optic nerves and the ones of the PBS group.

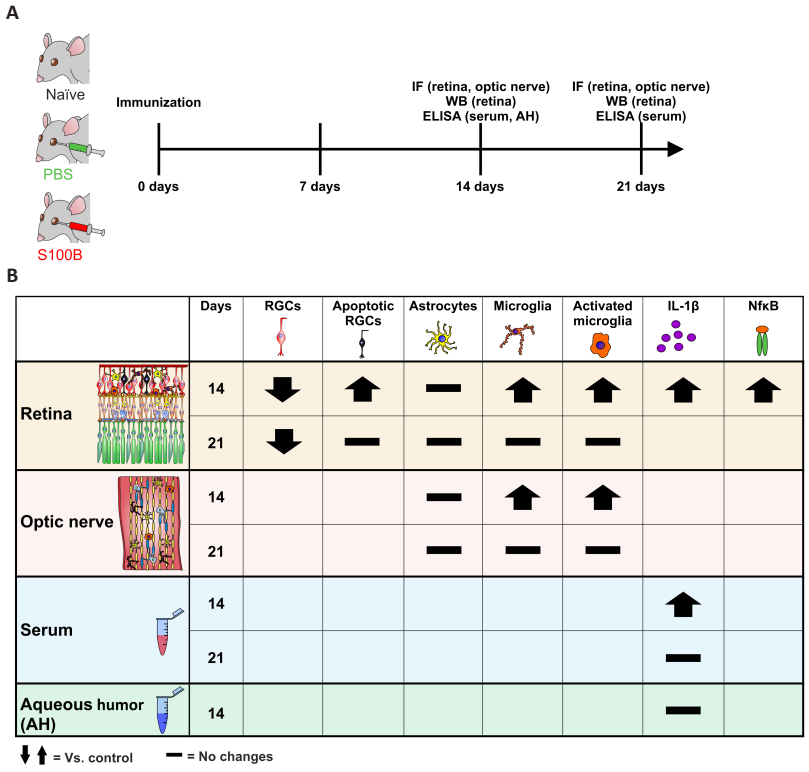

Figure 1 | **Effects of intravitreal S100B injection on retina and optic nerve.**
(A) At day 0, S100B or phosphate buffer saline (PBS) (control) was intravitreally injected in rats. Untreated eyes served as a naïve control group. Retina, optic nerve, and serum samples were explored at two points in time, 14 and 21 days after the injection using immunofluorescence (IF), and western blot (WB) for retina and optic nerve tissue, or enzyme-linked immunosorbent assay (ELISA) for serum. The aqueous humor (AH) was analyzed via ELISA at 14 day. (B) Pathological changes after intravitreal S100B injection are displayed. These changes are presented in relation to the PBS and the naive group at the respective time, as there were no significant differences between these two control groups. 14 days after S100B application, retinal ganglion cell (RGC) loss, a larger number of apoptotic RGCs, and an increase in microglia numbers as well as activated microglia was noted. We also observed an increased production of interleukin (IL)-1β and NfκB in the retina. After 21 days, only a loss of RGCs was detectable in the retina. In the optic nerve, 14 days after S100B injection, an increased number of microglia and activated microglia was observed, which was no longer detectable after 21 days. Astrocytes were not affected in the retina or in the optic nerve at any of the examination points. The IL-1β level in S100B serum was upregulated after 14 days, but not at 21 days. In the IL-1β analysis of AH samples revealed comparable levels in all groups.

In sum, intravitreal injection of S100B induces a strong inflammatory response after 14 days. This includes activation of the NfκB signaling pathway, which probably leads to microglia activation and IL-1β upregulation. Also, in glaucoma patients, IL-1β is considered an essential pro-inflammatory cytokine which produced by activated microglia, and therefore seems to promote the progression of glaucoma (Williams et al., 2017). It can be assumed that S100B activates NfκB in a RAGE-dependent manner. The sirtuin pathway, which is also upregulated in the S100B animals (Grotegut et al., 2020b), could act as a link between the S100B/RAGE complex and NfκB, since NfκB is also a target of sirtuins (Preyat and Leo, 2013). Then, the pro-inflammatory mechanisms likely triggered the optic nerve degeneration and apoptosis of RGCs (Grotegut et al., 2020a). Moreover, it is known that the toxic effect of S100B apparently relies on caspase activation or mitochondrial dysfunction, which is due to cytochrome C release. Label-free quantitative proteomics analysis after intravitreal injection of S100B also showed increased caspase activation and mitochondrial dysfunctions in the examined animals. Thus, these points of attack seem to play an important role after intravitreal injection of S100B.

However, it is still not known if the noted inflammation, more precisely microglia activation, is the main degenerative mechanism triggered by the intravitreal S100B application. In order to get to the bottom of this, microglia were inhibited in S100B animals in another study and the effects were analyzed.

Microglia inhibition through minocycline protects RGCs: Microglial activation is one of the first events in glaucomatous neurodegeneration (Zeng and Shi, 2018) and thus represent an interesting starting point for therapeutic intervention. *In vivo* and *in vitro* studies have demonstrated that minocycline can reduce the activity of microglia, T-cells, and macrophages. Minocycline was already used to inhibit the inflammatory response of microglia and thereby increased the neuronal survival in different glaucoma and retinal degeneration models, like the DBA/2J mouse, ischemia/reperfusion model, or the translimbal photocoagulation laser model (Karlstetter et al., 2015; Wei et al., 2019). Based on our previous findings, we hypothesized that a microglia inhibition will lead to a survival of neuronal cells in retina and optic nerve. Therefore, minocycline was used in a follow-up project of our group to inhibit microglia in the S100B model. Consequently, immunofluorescence evaluations were performed in the retina and optic nerve to investigate both the microglia response and the possible neuronal survival. These analyzes showed that minocycline treatment led to a reduced activation of microglia and a lower number of microglia in retina and optic nerve. This in turn decreased the number of apoptotic signals in both tissues, saving some RGCs and protecting optic nerve neurofilament.

To gain novel insights into altered protein expressions, liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis of retina tissue was carried out to characterize changes induced by S100B as well as minocycline treatment. Since the effect of minocycline appears to be dose-dependent, we used two different concentrations in the intravitreal S100B model. The low dose consisted of 13.5 mg/kg body weight minocycline and the high dose was 25 mg/kg body weight. In this study, we confirmed a dose-dependent effect of minocycline in the intravitreal S100B model and noted, as already mentioned, that the inhibition of microglia achieved a mild neuronal protection. Furthermore, a total of 733 proteins were identified in all the groups using proteomic analysis and more than 142 were found to be significantly differently abundant between some of the groups. The S100B group showed a lot of alterations when compared to the PBS control group. Canonical protein pathway analysis of the S100B group versus the PBS group demonstrated the association of, for example, the sirtuin signaling pathway, mitochondrial dysfunction, glycolysis, or oxidative phosphorylation. Hence, degenerative properties of S100B were

identified, which caused an imbalance in glycolysis and mitochondrial function. Comparing the two minocycline groups with the S100B retinae, altered proteins were associated with for example glycogen degradation or the sirtuin signaling pathway. One of the most significantly altered proteins in the proteomic analysis was pyruvate kinase, a distinct reduction was noted in the S100B group. We then analyzed the pyruvate kinase area via immunofluorescence staining and noted a significantly smaller pyruvate kinase$^+$ area in the S100B group than in controls. In contrast, the minocycline groups showed a similar pyruvate kinase$^+$ staining area as the control groups. Thus, S100B seems to affect pyruvate kinase and thus the energy balance regulation of retinal cells (Grotegut et al., 2020b).

Microglial reactivity is a common and early hallmark in several degenerative retinal diseases (Karlstetter et al., 2015). Our analyzes confirm the importance of S100B and the resulting activation of microglia for this study and glaucoma.

It is known that S100B can bind to the RAGE receptor leading to NfκB activation and a pro-inflammatory microglia response. S100B can increase NfκB and IL-1β levels, which indicates that microglia were active in a pro-inflammatory manner (Grotegut et al., 2020a). This mechanism is also conceivable for the degeneration of RGCs and the optic nerve via the S100B-RAGE-NFκB pathway. Since the inhibition of the microglia cannot completely inhibit RGC death and optic nerve degeneration, this suggests that further mechanisms are triggered by S100B. It is possible that S100B directly leads to caspase activation or mitochondrial dysfunction, like lower pyruvate kinase activity, via the RAGE or Toll-like receptor receptor activation.

Conclusion and future perspective: Glaucoma is characterized by the progressive and irreversible loss of RGCs and their axons, but the underlying damage pathways are not completely explored yet. Immunological processes and inflammation are involved in glaucoma pathogenesis, but their exact meaning has not been fully clarified.

In recent studies, we observed that intravitreal application of S100B leads to RGCs loss. After 14 days, we also noted increased numbers of NfκB$^+$ cells, more IL-1β expression, and higher microglia counts in S100B animals. After 21 days, these changes were no longer detectable. S100B seems to activate the NfκB pathway possibly by binding the RAGE, which in turn triggers a higher IL-1β production (**Figure 1B**). This inflammatory environment leads to microglia activation and further production of proinflammatory cytokines. Consequently, optic nerve axons and RGCs degenerate. To further analyze the degenerative potential of microglia, treatment with minocycline was applied to this animal model. This led to a reduced microglia response. In addition, the loss of RGCs decreased and optic nerve neurofilament was protected. Retinal proteomic analysis indicated that in addition to inflammatory processes S100B dysregulates proteins that are responsible for mitochondrial dysfunction.

In summary, microglia can be a degenerative factor and their inhibition protects neuronal cells from glaucoma-like damage. At the same time, it was noted that in the intravitreal S100B model, further degenerative factors triggering mitochondrial and metabolic dysfunctions were altered.

Since the low concentration of minocycline protected RGCs and optic nerve neurofilament, but could not completely stop the degeneration, it could be an interesting additive therapy for neurodegenerative eye diseases like glaucoma. It might be possible to combine a minocycline treatment with already established IOP lowering therapies. This could, on the one hand, inhibit the harmful influence of an exceeding microglial response and, on the other hand, support the survival of RGCs. Before that, however, further analyzes are needed to determine the molecular pathways to define the phenotypes of inflammation and dissolution of the retinal cells in order to specifically modulate these cellular processes. Such studies will help to implement therapeutic strategies.

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