

Vitreous IgM Autoantibodies Target Neurofilament Medium in a Spontaneous Model of Autoimmune Uveitis

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PURPOSE. Although the presence of IgG autoantibodies in the vitreous of spontaneous cases of equine recurrent uveitis (ERU) has been demonstrated, the potential role of IgM reactivities during ERU pathogenesis remains unexplored. The purpose of this study was to examine the presence of IgM autoantibodies in vitreous specimens of ERU-affected horses and to test their binding specificity to intraocularly expressed proteins.

METHODS. To test IgM autoantibody responses to retinal tissue, vitreous samples of eye-healthy controls and ERU patients were analyzed via two-dimensional Western blot analysis with equine retinal tissue as an antigen source. A candidate protein, the peptide neurofilament medium (NF-M), was identified via mass spectrometry and validated via enzyme-linked immunosorbent assay. Immunohistochemistry for NF-M expression was performed on healthy and ERU-affected retinal sections.

RESULTS. Whereas autoreactivity was never detected in the healthy vitreous samples, NF-M was specifically targeted by vitreous IgM autoantibodies in 44% of the ERU cases. Vitreous anti-NF-M IgG was detected in only 8% of the ERU samples, pointing to a persistent IgM response. In healthy horse retina, NF-M was located in the retinal ganglion cells and their processes, with additional staining in the outer plexiform layer. NF-M expression in ERU-affected retinas decreased considerably, and the remaining expression was limited to the nerve fiber layer.

CONCLUSIONS. Intraocular anti NF-M IgM autoantibodies occur with high prevalence in vitreous of spontaneous autoimmune uveitis cases. The IgM dominated response may indicate a thymus-independent response to NF-M and merits further investigation in ERU, as well as in its human counterpart, autoimmune uveitis. (*Invest Ophthalmol Vis Sci.* 2012;53:294–300) DOI:10.1167/iovs.11-8734

Equine recurrent uveitis (ERU) is a highly prevalent autoimmune disease in horses. Characterized by remitting–relapsing episodes of intraocular inflammation and closely resembling human autoimmune uveitis (AU), ERU is well established as a spontaneous model of human uveitis.¹ We already know that during the onset of disease, the eye's former immune privilege² is abolished, and the blood–retina barrier breaks down, allowing autoaggressive T-cells to invade the retinal tissue.^{3,4} Retinal proteins such as S-antigen (S-Ag), interphotoreceptor retinoid-binding protein (IRBP), and cellular retinaldehyde-binding protein (CRALBP), are targets in ERU that have been shown to induce ERU-like disease in horses.^{5–7} Because of its proximity to the protein pool of further potential targets among retinal proteins and its good availability, vitreous humor is a highly promising source specimen to further explore and elucidate the pathogenesis of ERU.⁸

One of the main factors contributing to ERU pathogenesis is the leaky blood–retinal barrier,⁹ which allows autoreactive T cells to invade the retina. T cells are known to be key players in ERU,¹⁰ but other cells, among them B cells, are also found in retinal tissue in ERU.⁹ The best known effector function of B cells is antibody production and in several autoimmune diseases, autoantibodies are known to directly contribute to pathogenesis.¹¹ In ERU, the presence of IgG antibodies against S-Ag and IRBP was previously observed in 72% of ERU-affected vitreous samples,¹⁰ whereas, in contrast, vitreous specimens from eye-healthy horses contained no IgG at all.¹⁰ The appearance of IgG antibodies is usually preceded by an initial IgM response.¹² This aspect is of special importance, since it is known that, in ERU, epitope-spreading takes place, leading to alterations in the antigen spectrum and subsequent new autoreactivity.¹³ Since IgM may point to newly emerging autoreactivity in the context of epitope-spreading and its upregulation in serum of ERU cases additionally caught our attention in a previous study,¹⁴ the purpose of this study was to determine whether intraocular IgM autoantibodies directed to retinal proteins are present in healthy and ERU-affected vitreous.

METHODS

Samples

Vitreous samples of ERU cases were obtained during therapeutic pars plana vitrectomy from horses diagnosed with ERU¹⁰ and treated at the Equine Clinic of LMU Munich. ERU-affected eyes providing ERU samples for retina sections used in immunohistochemistry were also from horses treated for ERU at the clinic. Control vitreous samples and healthy retinal specimens for proteome preparation and immunohistochemistry were derived from eyes obtained at a local abattoir. Eyes were considered normal based on the diagnosis of the veterinarian present at the abattoir, medical histories of the horses as provided by their owners, and preliminary histologic analysis. Vitreous samples

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were stabilized with protease inhibitor (Roche, Mannheim, Germany) and stored at -20°C until used. Eyes used for healthy and ERU-affected retinal sections were prepared according to a standardized protocol.¹⁵ Retina specimens were prepared as previously described.⁴ All horses were treated according to the ethical principals and guidelines for scientific experiments on animals according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. No experimental animals were used in the study.

A total of 37 unique control vitreous samples and 95 unique ERU-affected vitreous samples were analyzed. Western blot analysis was performed using 25 different control samples and 50 different ERU-affected samples. For the ELISA assay, 20 different control vitreous samples and 72 different ERU-affected samples were used. Immunohistochemical analysis was performed using 12 healthy retinal sections derived from 10 eyes and 9 retinal sections derived from 9 eyes affected by ERU.

SDS-Polyacrylamide Gel Electrophoresis

Retinas were solubilized in 2DE lysis buffer (9 M urea, 2 M thiourea, 1% DTE, 4% CHAPS, and 2.5 mM each of EGTA and EDTA), and protein content was quantified with the Bradford assay (Sigma-Aldrich, Deisenhofen, Germany). For one-dimensional gel electrophoresis, retinal protein (40 μg per gel) was resolved by 10% SDS-PAGE. For two-dimensional (2D) gel electrophoresis, dry strips (Immobiline, pH 3–11 NL, 11 cm; GE Healthcare, Freiburg, Germany) were rehydrated overnight in lysis buffer containing 100 μg retina, additional 1% ampholyte carriers (Pharmalyte, pH 3–11; GE Healthcare), and 0.5% bromophenol blue. Isoelectric focusing (IEF) was performed (Multiphor; GE Healthcare) at 14 kV/h at 20°C , followed by separation on SDS gels (10%) at a constant 45 V per gel at 4°C . The gels were used for Western blot analysis or as master gels for candidate protein detection.

Western Blot Analysis

For Western blot analysis, gels resulting from SDS-PAGE were blotted semidry on PVDF membranes (GE Healthcare). After nonspecific binding was blocked with 1% polyvinylpyrrolidone in PBS-T (PBS containing 0.05% Tween 20) for 1 hour at room temperature, blots were incubated with vitreous samples as a primary antibody source (controls or ERU-affected, dilution 1:100) overnight at 4°C . The blots were washed subsequently and incubated with secondary antibody for 1 hour at room temperature (goat anti-horse-IgM POD, dilution 1:10,000; AbD Serotec, Düsseldorf, Germany). Signals were detected via enhanced chemoluminescence on x-ray film (Christiansen, Planegg, Germany).

Mass Spectrometry

Selected spots from 2D analysis were excised, destained, and processed by proteolysis with trypsin, as described before,⁴ and analyzed by MALDI-TOF peptide mass fingerprinting and MS/MS on a MALDI-TOF/TOF tandem mass spectrometer (4700 Proteomics Analyzer; Applied Biosystems, Inc. [ABI], Darmstadt, Germany). For positive-ion reflector mode spectra, 2500 laser shots were averaged and processed with external calibration. PMF spectra were not smoothed, and background was not subtracted. Monoisotopic peak masses were automatically determined within the mass range 800 to 4000 kDa with a signal-to-noise ratio minimum set to 5 and the local noise window width at m/z 200. Up to seven of the most intense ion signals with signal-to-noise ratio above 30 were selected as precursors for MS/MS acquisition excluding common trypsin autolysis peaks and matrix ion signals. In MS/MS positive-ion mode, 4000 spectra were averaged with 1 kV collision energy, collision gas air at a pressure of 1.6×10^6 torr and default calibration. Monoisotopic peak masses were automatically determined with a signal-to-noise ratio minimum set to 10 and the local noise window width at m/z 200. Combined PMF and MS/MS queries were performed using the MASCOT database search engine (ver. 1.9; Matrix Science Ltd., London, UK) embedded in proteomics application software (GPS-Explorer Software; ABI) on the Ensemble horse database

(EquCab2 assembly: 22,641 sequences; 12,722,273 residues; downloaded from the Ensembl public database, <http://www.ensembl.org/index.html>) with the following parameter settings: 65-ppm mass accuracy, 0.8-Da fragment mass tolerances, trypsin cleavage, with one missed cleavage allowed. Carbamidomethylation was set as a fixed modification, and oxidation of methionines and deamination of asparagine and glutamine were allowed as variable modification. A protein was regarded as identified if the probability-based MOWSE score was significant (>70 ; $P < 0.05$), if the matched peptide masses were abundant in the spectrum and if the theoretical masses of the significant hit fit the experimentally observed values.

Enzyme-Linked Immunosorbent Assay

Polystyrene 96-well flat-bottomed plates (Nunc Maxisorp; Fisher Scientific GmbH, Schwerte, Germany) were coated with native bovine neurofilament medium (NF-M) polypeptide (Hoelzel Biotech, Cologne, Germany) or porcine neurofilament light (NF-L, MW 68; Biomol, Hamburg, Germany). For coating, lyophilized protein was reconstituted according to the manufacturer's instructions and diluted at 1 $\mu\text{g}/\text{mL}$ in NaHCO_3 buffer (pH 9.6). Each well was incubated with 100 μL of this dilution overnight at 4°C . Plates were blocked with 200 μL per well 0.5% gelatin at 37°C for 1 hour to prevent nonspecific binding. Vitreous samples as primary antibody source were diluted 1:100 in PBS-T, and the wells were incubated at 37°C for 1 hour (100 $\mu\text{L}/\text{well}$) and washed with PBS-T (three times, 300 $\mu\text{L}/\text{well}$). For both the IgM and IgG assays, wells were incubated with the respective secondary antibody, 50 $\mu\text{L}/\text{well}$ for 1 hour at 37°C . We used either goat anti-horse-IgM POD antibody (dilution 1:100,000; AbD Serotec) or goat anti-horse IgG Fc POD antibody (dilution 1:100,000; Biozol, Eching, Germany). To enable later correction for blank values, each plate contained four wells in which primary antibody incubation was omitted. Using tetramethylbenzidine (Sigma-Aldrich) as a substrate, we measured optical density (OD) at 450 nm with a microplate reader after stopping the reaction with 1 M sulfuric acid (50 $\mu\text{L}/\text{well}$). The cutoff was set at a fivefold increased SD above the average OD of vitreous samples from healthy horses. The frequency of positive reactions in control samples and ERU samples were compared by using Fisher's exact two-tailed test; differences were significant at $P < 0.05$ (GraphPad Prism 5.04 software; Statcon, Witzenhausen, Germany).

Immunohistochemistry

Posterior eyecups were fixed in Bouin's solution (Sigma-Aldrich, Deisenhofen, Germany), as previously described.¹⁶ The tissue blocks¹⁵ obtained were sectioned (8 μm) and subsequently mounted on coated slides (Superfrost; Menzel, Braunschweig, Germany). Heat antigen retrieval was performed at 99°C for 15 minutes in 0.1 M EDTA-NaOH buffer (pH 8.0). To prevent nonspecific antibody binding, sections were blocked with 1% BSA in TBS-T containing 5% goat serum for 40 minutes at RT before incubation with monoclonal mouse anti-160-kDa neurofilament medium antibody (dilution 1:100; Biozol), followed by goat anti-mouse-IgG-Fc secondary antibody labeled with horseradish peroxidase (dilution 1:200; Jackson ImmunoResearch, West Grove, PA). Antibody binding was visualized using a peroxidase substrate kit (Vector-VIP SK-4600; Linaris, Wertheim, Germany), resulting in a purple stain. Cell nuclei were counterstained with Mayer's hematoxylin (Sigma-Aldrich). The sections were then fixed via successive immersion steps in increasing alcohol concentrations and covered with glass coverslips (Fluka Eukitt mounting medium; Sigma-Aldrich). For comparison, healthy ($n = 12$) and ERU-affected retinas ($n = 9$) were examined via tissue microarray. This technique provided the advantage of high comparability between the staining of retinal sections, as all tissue specimens examined were mounted and stained on the same slide (Alphamatrix; Beecher, Rödermark, Germany).¹⁷ Images were recorded with a scanner (Mirax Scan; Carl Zeiss Meditec, Göttingen, Germany) and analyzed (Panoramic Viewer 1.15.00.53 software; Intas, Göttingen, Germany).

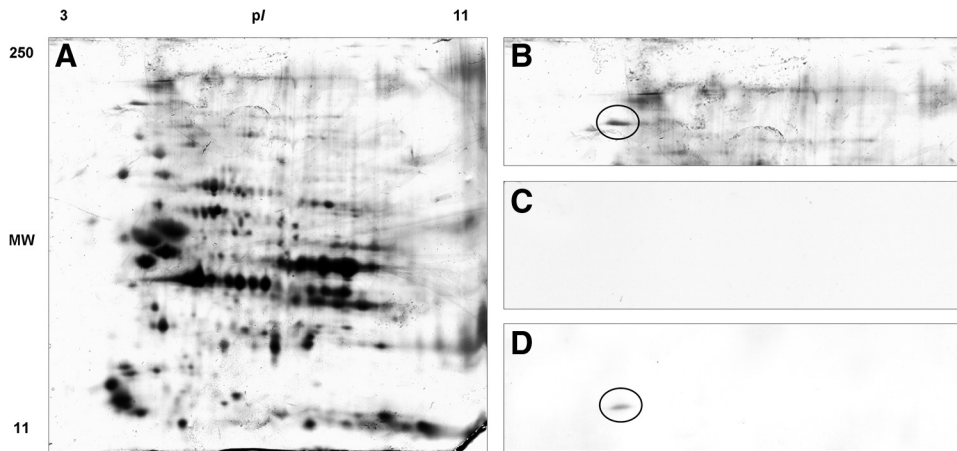


FIGURE 1. Autoreactivity of vitreal IgM antibodies to the retinal proteome. (A) 2D gel displaying the map of the equine retinal proteome, silver stained. (B) Zoom-in view of (A), showing the area of the 2D gel containing proteins with MW of ~80 to 250 and a pI of ~3 to 9. Circle: spot identified as NF-M by tandem mass spectrometry. (C) Western blot of a 2D-gel incubated with control vitreous sample showing no IgM reaction. (D) Exemplary Western blot of a 2D-gel incubated with vitreous sample from ERU-affected horse. Circle: reaction to NF-M appearing repeatedly among ERU samples.

RESULTS

IgM Autoantibodies in ERU-Affected Vitreous Samples Target NF-M

To determine the presence and possible binding targets of IgM autoantibodies in vitreous samples, we used an antigen source consisting of equine retinal proteins (Figs. 1A, 1B) subjected to 2D PAGE and subsequent Western blot analysis. Whereas control vitreous samples were consistently negative for IgM antibodies (Fig. 1C), ERU-affected specimens showed a variable binding pattern, ranging from no signal at all to a multitude of proteins in a single sample. One protein spot was particularly notable, as reactions to it were repeatedly detected in vitreous samples of ERU cases (Fig. 1D). It was located at a rather high molecular mass (~100) and a rather acidic pI of 4 to 5 on 2D blots. Alignment to a silver-stained 2D master gel of retinal proteome (Fig. 1A) and subsequent unambiguous identification with tandem mass spectrometry revealed NF-M polypeptide (MW 95, pI 4.84, protein score 264; Ensembl database accession number: ENSECAP00000019857) as the targeted protein (Fig. 1B).

Vitreous IgM Response to NF-M Outweighs the IgG Response

To verify our findings from the experimental set of 2D Western blots, we used an indirect ELISA, enabling us not only to confirm that NF-M was the actual targeted protein, but also to test whether the observed high prevalence of reactions would hold true in a large-scale test setup. First, we examined the frequency of vitreal IgM reactions toward NF-M in controls and ERU samples. All controls tested were negative, whereas in 44% of ERU diseased samples an IgM reaction toward NF-M was detected (Fig. 2). Since 44% of ERU cases had intraocular NF-M-specific IgM autoantibodies, we were next interested to determine whether these IgM responses were followed by intraocular IgG responses. Therefore, we tested the IgG reactivity toward NF-M in the same vitreous samples as used for the IgM assay. All control samples were negative again, whereas 8% of uveitic samples were anti-NF-M/IgG positive (Fig. 2). Of note, compared with the large proportion of intraocular IgM-positive ERU cases (44%), the number of IgG reactions (8%) was strikingly low (Fig. 2). Half of these IgG-positive samples were IgM/IgG double positive, and the other half had exclusively IgG antibodies. ELISA results showed that IgM in ERU-affected vitreous samples specifically target NF-M with high frequency. Since NF-M shares structural similarities with NF-L that could result in antibody cross-reactivity,^{18,19} we controlled the specificity of autoantibodies to NF-M specificity by testing

the same vitreous specimen in ELISAs coated with the NF-L subunit. Again, none of the control samples showed any reactivity as well as none of the tested ERU samples, thus confirming that NF-M autoantibodies did not bind to NF-L.

Reduced Expression of NF-M in ERU-Affected Retina

As ELISAs showed NF-M to be such a frequent target for vitreal IgM autoantibodies in ERU-affected samples, we were very interested in exploring its expression pattern in the target tissue retina. Alterations in expression strength or location may give us valuable clues concerning the role of NF-M in ERU pathophysiology and also indicate which cells may be most affected by it. Our immunohistochemical analysis of NF-M expression in equine retina showed the retinal ganglion cell (RGC) layer and nerve fiber layer of healthy retinal tissue to be the sites with the strongest and most extensive signal, whereas moderate expressions in the inner plexiform layer (IPL) and outer plexiform layer (OPL) were also observed (Fig. 3A). Notably, not all RGC somata stained for NF-M. Most of the unstained cells appeared to be small RGCs.¹⁵ The scattered expression foci in the IPL showed signals of varying intensity, with an overall signal intensity that appeared to be slightly below that in the nerve fiber layer. We can report that NF-M distribution in healthy equine retina resembles that in porcine retina, especially with expression in the RGCs, and that, in

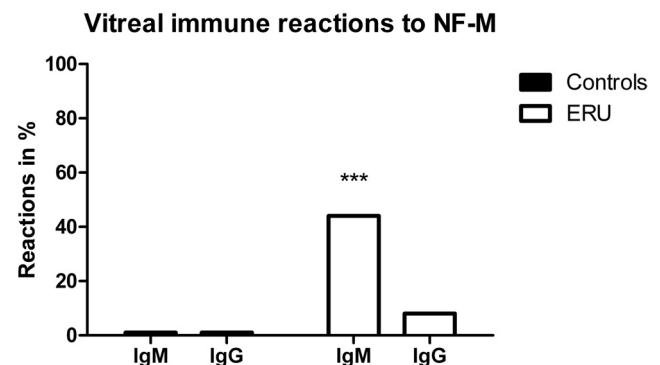


FIGURE 2. Reactivity toward NF-M in indirect ELISA, comparing control and ERU-affected vitreous samples. The percentage of IgM and IgG reactivities in all tested vitreous samples is presented. No reactions were observed in the control samples. Reactions were observed in the ERU-affected samples. Of all the ERU samples, 44% tested IgM positive and 8% tested IgG positive. In the ERU samples, IgM reactions were observed much more frequently than IgG reactions.

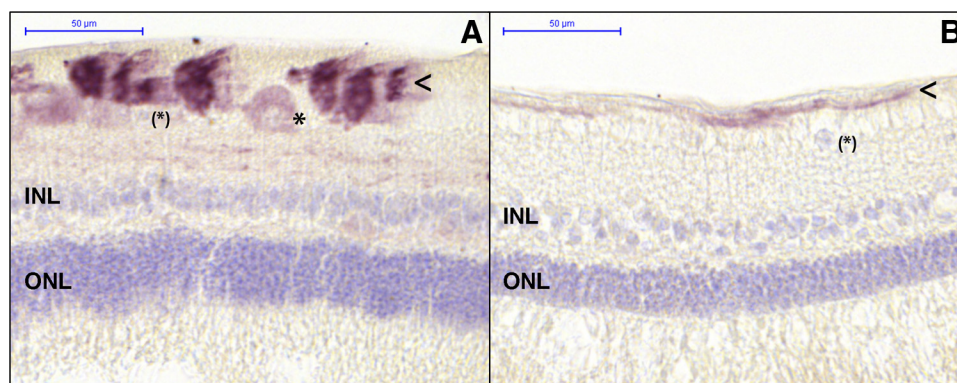


FIGURE 3. Changes in NF-M expression (purple) in ERU-affected retina. Both sections represent the central retina. (A) NF-M expression in a healthy retina with the predominant signal in the nerve fiber layer (open arrowhead) and most RGCs (*). Note the unstained RGC (* in brackets). Additional staining is visible in the IPL fiber structures and OPL cells and fibers. Cell nuclei (blue) were stained with hematoxylin. (B) Representative image of an ERU-affected retina with absent NF-M signal in the OPL and IPL and the negative RGC (* in brackets). Residual NF-M expression occurred only in the nerve fiber layer (open arrowhead).

addition, NF-M is present in the equine OPL, as it is in that of the pig.²⁰ However, in retinal tissue affected by ERU (Fig. 3B), NF-M expression was strongly decreased in all samples, the extreme being a complete loss of NF-M staining. The only exception was one case without detectable changes in NF-M expression. A consistent feature in ERU specimens was the absence of NF-M staining in IPL and OPL. Also, NF-M positive RGC somata were detected less frequently than in healthy retinas. NF-M expression in the nerve fiber layer was still detectable in ERU-affected sections, except for the aforementioned NF-M-negative specimen. Intensity and distribution of the NF-M signal in ERU-affected nerve fiber layers were variable, however. In one sample, only overall staining intensity in the nerve fiber layer was reduced, with no changes in distribution pattern, whereas in several others, we observed a reduction in both the distribution pattern to isolated residual foci and a reduction of signal intensity to moderate to low.

DISCUSSION

ERU is currently the only spontaneous animal model for human AU,⁸ a disease characterized by remitting-relapsing inflammation of the inner eye, ultimately leading to blindness, in which pathogenesis and origin are not entirely clarified to date.^{21,22} Besides the great similarities in clinical appearance and the spontaneous occurrence of both diseases, the most impressive proof of the high transferability and extremely valuable contribution of ERU to AU research was the demonstration that CRALBP, an autoantigen detected in the ERU model,⁷ is a highly prevalent autoantibody target in AU, as well.³ Research in the field of autoimmunity and studies investigating autoreactivities are mostly focusing on serum antibody profiling, an all-purpose source material available in relatively large amounts that can be obtained in an ethical way, even repeatedly, from most animals, human patients, and control subjects alike. However, antibody profiling that is focused on source material close to the target tissue has been shown to yield highly interesting results in other autoimmune diseases such as rheumatoid arthritis, in which synovial autoantibodies were investigated.²³ In the field of ophthalmology, vitreous meets the criteria for source material very well, as it is in direct contact with the retina. The vitreous body is a structure that greatly merits further investigation in AU and ERU, as in addition to its immediate proximity to the target tissue, it shows unique immunologic properties.⁸ A great advantage of the horse

model ERU in this approach is that, via vitrectomy, intraocular autoantibodies are available for research in ample amounts and almost immediately after sampling. We were able to gain information about differentially expressed proteins in healthy and ERU-affected vitreous in a previous study, discovering changes in the matrix metalloproteinase pathway^{8,24} in ERU that were accompanied by a rise in vitreal IgM content. Another study examining intraocular samples in ERU showed that vitreal IgG content is increased in ERU samples²⁴ and that autoreactive immune cells and IgG autoantibodies were present in ERU-affected vitreous specimens, but not in vitreous samples from healthy eyes.¹⁰ Yet, IgG antibodies are probably not the only type involved in autoimmune processes occurring in ERU. Serum IgM is upregulated in ERU,¹⁴ which probably points to an important role for IgM autoantibodies in ERU, as well. Thus, the purpose of this study was to examine whether intraocular IgM autoreactivity is detectable, using vitreous as autoantibody source material. Western blot analysis on a preliminary test sample set showed that IgM reactions were very frequent in ERU-affected vitreous samples, showing a great diversity in their binding patterns. Among this variety of reactions, one protein spot was bound repeatedly and was subsequently identified as NF-M (Fig. 1B). Moreover, the identification of NF-M as intraocular IgM autoantibody target for nearly every second ERU-affected case, supports the possible role of IgM autoantibodies in ERU. In a large set of samples, ELISAs verified that NF-M was indeed the protein targeted by intraocular IgM with a high prevalence (44%) in ERU. In addition, we observed that compared to the high prevalence of IgM reactions, the prevalence of vitreal IgG response toward NF-M is almost negligible. ERU-affected vitreous samples used in these assays represented horses in a variety of different disease stages, and the number of positive IgM reactions among them is so high that the discrepancy between the scarce IgG-positive and the frequent IgM-positive reactions is not likely to be a transient occurrence in the course of disease. An IgM response is usually seen as the first line of defense, indicating an acute response to an antigen.¹² One of the most famous events in immunology is the switch of antibody isotype that is often observed if antigen exposure continues or reoccurs—that is, the initial IgM response is switched to an IgG response with enhanced antibody affinity.^{12,25} However, the intraocular ERU IgM antibodies analyzed in this study were obtained during vitrectomy, which is a therapeutic procedure undertaken in clinical quiescent state, making our observation of an IgM-dominated response even

more remarkable. Evidence thus strongly points to an IgM response toward NF-M that persists over an uncommonly long period.

IgM is a very potent activator of the complement system,^{12,26} which is known to contribute to tissue destruction and promote inflammation in AU^{27,28} and was also shown to be involved in ERU.^{14,29} Via complement activation, the presence of an IgM response toward NF-M may aggravate the process of intraocular inflammation as a whole and thus cause damage that impacts ocular structures and cell populations beyond those that express NF-M.

Immunohistochemistry revealed that in healthy equine retina, the strongest signals were elicited in the nerve fiber layer followed by the NF-M signals in the RGC layer, which showed a homogeneous distribution in stained cell bodies (Fig. 3A). Signals in the IPL were the least intense by comparison. As the equine nerve fiber layer contains mostly RGC axons,¹⁵ we can conclude that in equine retina, RGC axons most probably show a somewhat stronger expression of NF-M than the soma and RGC dendrites. Interestingly, we found several RGC bodies that did not stain for NF-M, most of them were small RGCs.¹⁵ Equine RGCs are known to possess processes that extend into the IPL,¹⁵ which clearly stain for NF-M (Fig. 3A) and are probably the NF-M-positive structures observed here in the IPL. However, since processes of bipolar cells or amacrine cells are also known to spread into the equine IPL¹⁵ and these cells have been shown to be able to express NF proteins in other mammalian species,^{30–32} we cannot exclude them as a source of the observed NF-M staining in the IPL. The NF-M positive fiber structures and cell bodies we detected in the OPL are most likely horizontal cells. Our findings in equine retina place NF-M distribution in the horse very close to that in the pig, which is an established model organism in human glaucoma research.^{20,33–35} In the porcine retina, NF-M is also predominantly expressed in RGC axons, dendrites, and cell bodies, with smaller RGC somata staining less frequently. The NF-M signal in the OPL that was also detected in the pig has been attributed to horizontal cells.²⁰

Compared to our findings in healthy retinal tissue, NF-M expression in ERU-affected retinal tissues decreased in 89% of examined cases. A common observation was the vanishing of NF-M signal in the OPL and IPL. Although the exact significance of RGCs and their neurofilaments in AU remains to be clarified, an involvement of these two structures in glaucoma pathogenesis has been demonstrated in the past in the pig model.^{33,36} There, the NF-M signal in immunohistochemical analysis decreased in optic nerve head sections of eyes subjected to elevated intraocular pressure and in retina sections in a focal retinal ischemia experiment, as shown in the pig model.^{33,34} Interestingly, the decrease in neurofilament content in RGCs can be detected early, before alterations in axonal transport occur,^{34,35} which can lead to cell death by apoptosis.³⁷ Thus, the decrease in NF content in RGCs has been proposed as an early marker or warning sign of impending axonal damage.^{33,34} With respect to the decrease in NF-M content that we observed in equine retina tissue affected by ERU, from our point of view, there are two mechanisms that could be responsible for this finding. The first possibility features RGCs as the central factor. Damaged RGCs may downregulate NF-M expression, as observed in porcine glaucoma models,^{33–35} but we can also hypothesize that in ERU, damaged RGCs or their axons release NF-M fragments into the adjacent fluid compartment, the vitreous body, similar to the way the heavy neurofilament subunit NF-H is released into the vitreous after retinal degeneration in humans.³⁸ These released fragments may in turn expose additional antigen material to invading immune cells, keeping the autoimmune attack going. The second possibility is that NF-M is directly destroyed in the wake of the autoimmune attack

directed toward it. Cells expressing NF-M may be damaged or destroyed, as well, preventing a reconstitution of former expression levels and sites. These two mechanisms do not exclude each other and can occur concurrently to some degree. This open question could be solved, for example, by further investigation of the role of RGCs in AU or by determining NF content in ERU-affected vitreous in future experiments. However, as we do know that NF-M is a target for vitreal IgM autoantibodies, downregulation of expression is not likely to be the only factor that contributes to its decrease in the retinal tissue in ERU.

There is a surprising discrepancy in the IgM and IgG NF-M responses (Fig. 2), strongly arguing that there was no IgM/IgG switch. Persistent IgM responses can occur by B-cell activation through so-called thymus-independent (TI) antigens.³⁹ Antigens belonging to the TI type 2 (TI-2) category can directly stimulate mature B cells, by cross-linking several of their IgM receptors.⁴⁰ TI-2 antigens are a heterogeneous group, but share some common characteristics, including high molecular mass and repetitive epitope structures, the prototype being polysaccharide antigens or bacterial flagella.^{39,40} Considering its structural properties, NF-M as an antigen may meet the criteria for TI-2 activation. NF-M belongs to the group of intermediate filaments and is a subunit of the NF triplet, an essential part of the neuronal cytoskeleton that is composed of a heavy (NF-H), a light (NF-L), and a middle (NF-M) weight subunit,⁴¹ with α -internexin as a putative fourth element.⁴² Possessing post-translational modifications, including glycosylations,^{43,44} featuring a repetitive motif in its amino acid sequence,⁴⁴ and assembling to form polymeric repetitive filament structures,⁴¹ NF-M may be fit to elicit a TI-2 response. Although it is the NF-L subunit, that has been described as a target for serum autoantibodies in glaucoma patients^{45–47} and the neurofilament triplet has been shown to occasionally induce antibodies that cross-react among the subunits,^{48,49} we confirmed by ELISA that vitreal IgM autoantibodies specifically target NF-M and do not react with NF-L. A TI-2-like activation of B cells by NF-M may thus explain the dominance, as well as the prolonged persistence, of IgM in the autoimmune response toward NF-M. The unique immunoregulatory microenvironment in the eye⁵⁰ may in some way influence B cells or plasma cells, as well, and further influence the response toward IgM domination, instead of switching to an IgG response. In addition, as changes in NF-M expression probably occur in ERU-affected retinal specimens (Fig. 3B), existing posttranslational modifications may also be altered and trigger or perpetuate the autoimmune attack.⁵¹

CONCLUSIONS

This study demonstrated that in ERU, intraocular IgM autoantibodies occur and, moreover, that they can specifically target retinal self proteins. The identification of NF-M as an autoantibody target and its subsequent demise in ERU-affected retina point to a possible involvement of RGCs in AU, as this cell population is the main source of NF-M in the healthy equine retina. The prevalence of NF-M reactive vitreal IgM autoantibodies is high, and therefore in our opinion the roles of both NF-M and vitreal IgM in the pathology of ERU and AU absolutely merit further investigation.

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