Original Article

Mycophenolate Mofetil and Cyclophosphamide Treatments Suppress Inflammation Intensity in an Experimental Model of Autoimmune Uveitis

(experimental autoimmune uveitis / autoimmunity / retinal antigen / uveitis / uveoretinitis / mouse / mycophenolate mofetil / cyclophosphamide / golimumab)

A. KLÍMOVÁ[1], P. SEIDLER ŠTANGOVÁ[1], J. HEISSIGEROVÁ[1], P. SVOZÍLKOVÁ[1], T. KUČERA[2]

[1]Department of Ophthalmology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic
[2]Institute of Histology and Embryology, First Faculty of Medicine, Charles University in Prague, Czech Republic

Abstract. In human, autoimmune uveitis is a leading cause of visual disability and ranks with diabetic retinopathy as a major source of blind registrations in developed countries. Since most cases of non-infectious uveitis are considered to be autoimmune or at least immune-mediated, the management of such patients has rested on appropriate immunosuppression. Some patients, however, despite maximal immunotherapy, fail to respond or are seriously intolerant of the drug therapies. Since its establishment 20 years ago, the model of experimental autoimmune uveoretinitis has served as a useful template for novel therapeutic approaches. The aim of our study was to compare the efficacy of mycophenolate mofetil and cyclophosphamide and golimumab treatment in the mouse model of experimental autoimmune uveitis. The intensity of intraocular inflammation was evaluated histologically in the treatment and control groups. Experimental autoimmune uveitis has been induced in mouse strain C57BL/6 by subcutaneous application of interphotoreceptor retinoid binding protein in complete Freund's adjuvant and pertussis toxin. The treatment was commenced on the day of uveitis induction. Cyclophosphamide was applied intraperitoneally in a single dose (100 mg/kg), mycophenolate mofetil intraperitoneally daily (30 mg/kg or 50 mg/kg), golimumab subcutaneously weekly (70 mg/kg). Sham intraperitoneal injection of a placebo (aqua pro injectione) and untreated mice with experimental autoimmune uveitis served as controls. The results show statistically significant suppression of experimental uveitis both with mycophenolate mofetil and with cyclophosphamide, and thus support its use in human medicine.

Introduction

Uveitis is an intraocular inflammation, which is one of the major causes of blindness in developed countries in patients of working age. It has been estimated that uveitis causes 10 % of the cases of blindness in these countries (Suttorp-Schulten and Rothova, 1996; Durrani et al., 2004). Since most cases of non-infectious uveitis are considered to be autoimmune or at least immune-mediated, the management of such patients has rested on appropriate immunosuppression. Some patients, however, despite maximal immunotherapy, fail to respond or are seriously intolerant of the drug therapies. Therefore, further research is needed to find optimal treatment for these patients.

The heterogeneity of the disease with a wide spectrum of clinical presentations is the major impediment to extensive human studies. Because of that, animal models of autoimmune uveitis have been developed to allow more detailed studies of this disease and help to

seek new immunological therapeutic strategies (Caspi et al., 1990a; Forrester et al., 1990, 1992). At present, the most widely used is the model of experimental autoimmune uveitis (EAU) in mice, where the uveitis is induced by subcutaneous application of interphotoreceptor retinoid binding protein (IRBP) in complete Freund's adjuvant (CFA) (Caspi et al., 1988). IRBP works as a retinal autoantigen (Avichezer et al., 2000; Broderick et al., 2002). The mouse strain C57BL/6, in which application of IRBP 1-20 causes a chronic mild inflammation, mainly involving the posterior segment of the eye (Xu et al., 2008), imitates posterior uveitis in humans probably most closely.

In the treatment of uveitis, cyclophosphamide (CPA) is probably the most powerful immunosuppressant used (Mochizuki et al., 1985; Suzuki et al., 1989). Its effect has been proved in rodent models of EAU (Caspi et al., 1990b) and in human medicine (Suelves et al., 2013).

The role of mycophenolate mofetil (MMF) remains unconvincing. MMF has been reported to be effective (Siepmann et al., 2006; Teoh et al., 2008; Doycheva et al., 2011) in the treatment of human autoimmune uveitis when compared to other immunosuppressants. Its effects in the treatment of severe uveitis are, however, rather disappointing when compared to its successful use in treatment of other ocular immunological diseases, such as ocular cicatrical pemphigoid and corneal transplantation (Zierhut et al., 2005). In an experimental model of EAU in Lewis rats (Chanaud et al., 1995; Dick et al., 1998), MMF was proved successful. As our model is probably the best to be compared to human autoimmune uveitis and as this drug has not been tested in mice with EAU, in this study, the efficacy of MMF has been compared with well-established treatment with CPA and with humanized monoclonal antibody golimumab. We have shown significant suppression of ocular inflammation with MMF and with CPA.

Material and Methods

Animals

The experimental use of animals was approved by The Commission for Animal Welfare of the First Faculty of Medicine of Charles University in Prague, Czech Republic, and the Ministry of Education, Youth and Sports according to animal protection laws. All the procedures were approved by the animal experimentation review committee.

Inbred female mice of the C57BL/6 strain 5 to 8 weeks old were obtained from the animal facility of the Centre of Experimental Biomodels (First Faculty of Medicine, Charles University in Prague, Czech Republic).

Induction of experimental autoimmune uveitis

The application of IRBP was conducted according to a standard protocol (Avichezer et al., 2000; Broderick et al., 2002). In brief: subcutaneous injection of 500 µg of IRBP 1-20 (interphotoreceptor retinoid binding protein,

also called retinol-binding protein 3 precursor fragment [Homo sapiens] H2N-GPTHLFQPSLVLDMAKVLLD-OH, New England Peptide, Gardner, MA) was applied. To dissolve the IRBP peptide, DMSO (dimethyl sulphoxide) (Sigma-Aldrich, St. Louis, MO) was used. IRBP was emulsified in ratio 1 : 1 with CFA (Difco Laboratories, Detroit, MI). The reactivity of the immune system was enhanced by intraperitoneal application of 1.2 µg pertussis toxin (List Biologicals, Campbell, CA) dissolved in PBS.

Study groups

In this study, four treatment groups and three control groups were used. In the treatment groups, two groups of mice were treated with MMF (Cellcept 1 g/5 ml, Roche, Welwyn Garden City, Great Britain). MMF was injected intraperitoneally daily in a dose of 30 mg/kg (32 eyes) and in a dose of 50 mg/kg (13 eyes). The following group of mice was treated with CPA (Endoxan 1 g, Baxter, Halle, Germany). CPA was injected intraperitoneally in a single dose of 100 mg/kg (29 eyes). The last treatment group was treated with golimumab (Simponi 50 mg, Janssen Biologics, Leiden, Netherlands). Golimumab was injected subcutaneously weekly in a dose of 70 mg/kg (16 eyes). We had three control groups: group without EAU induction (8 eyes), group with EAU without treatment (88 eyes) and group with EAU with sham treatment (aqua pro injection applied intraperitoneally; 14 eyes). The odd count of eyes in some groups was caused by a congenital anomaly, micropthalmos, which occurs relatively often in inbred animals.

Specimen preparation and histological processing

The mice were sacrificed following the ethical rules given by the law (§17) in the Czech Republic by cervical spine manipulation. The eyes were enucleated promptly post mortem on day 35 after the induction of EAU. Enucleation in mice was performed by dissecting the globe carefully from periocular tissue. The eyeballs were placed in gel medium (Tissue-Tek® O.C.T. Compound™, Sakura Finetek USA, Inc., Torrance, CA) and frozen in 2-methylbutane (Sigma-Aldrich) in nitrogen atmosphere. The samples, frozen to -70 °C, were then cut by a microtome Leica CM 1850 (Leica Microsystems Nussloch GmbH, Nussloch, Germany) to 7 µm thick slices. The sections were always cut from both eye peripheries and also through the optic nerve. The samples were stained with haematoxylin and eosin according to a standard protocol. The samples were taken from both eyes, since the intensity of inflammation may be asymmetric.

Clinical examination of uveitis

The clinical examination in living animals (bio-microscopy) was performed using a special endoscopic imaging system. The mice were examined in intraperitoneal general anaesthesia with ketamine 80 mg/kg

(Narkamon 50 mg/ml, Bioveta, Nitra, Slovakia) and xylazine 5 mg/kg (Rometar 20 mg/ml, Bioveta, Slovakia). The pupils were dilated by tropicamide (Unitropic 1% oph. gtt., Unimed Pharma, Bratislava, Slovakia) and phenylephrine hydrochloride (Neosynephrin-Pos 10 %, Ursahpharm, Říčany, Czech Republic) and anaesthetized with oxybuprocaine hydrochloride (Benoxi 0.4% Unimed Pharma, Unimed Bohemia, Czech Republic). An otoscope with external light source was connected to a camera (Paques et al., 2007). The otoscope was placed on the cornea covered with eye gel.

Statistical analysis

Data were analysed using GraphPad Prism Version 6.04 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). Kruskal-Wallis and Mann-Whitney non-parametric tests were used to assess differences between the data groups. The P value of < 0.05 was considered significant. Variation among column medians was significantly greater than expected by chance.

Results

The evaluation of the inflammation intensity, and hence the effect of EAU treatment, was evaluated by a histological scoring system. The histological specimens of eye sections stained with haematoxylin and eosin were evaluated microscopically by two experienced eye specialists. To assess the intensity of retinal inflammation, we used the histopathological grading score from 0 to 4, see Table 1 (Caspi et al., 1988; Dick et al., 1994; Thurau et al., 1997).

Evident histological signs of posterior uveitis are retinal folds, granulomas in the retina, often located in the corpus ciliare, then vasculitis, vitritis, retinal neovascularization and photoreceptor loss (Figs. 1–6). The retina

Fig. 1-7. Representative haematoxylin and eosin histological images of mouse retinal sections showing EAU features on day 35 after immunization. Scale bars are shown in the images.
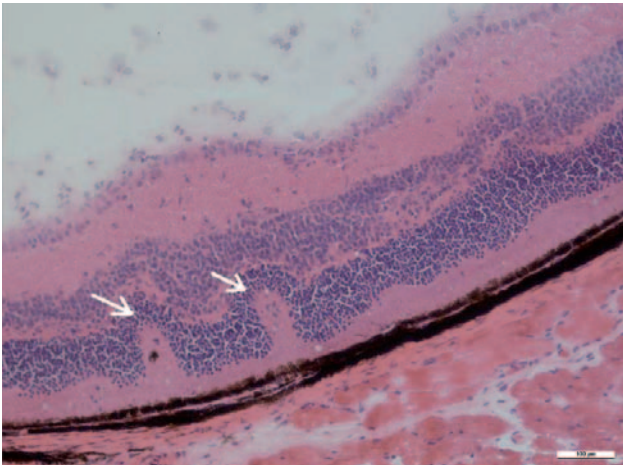

Fig. 1. Typical histological features of EAU are retinal folds (arrows), retinal layers are irregular in thickness and cellularity, inflammatory cells are present in the corpus vitreum.
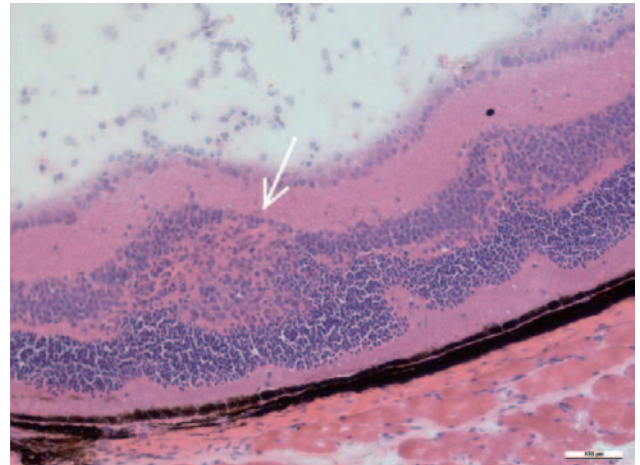
Fig. 2. Large retinal granuloma (arrow) is formed between the cellular layers in the retina, formation of retinal folds is apparent in the same location, inflammatory cells are present in the corpus vitreum.
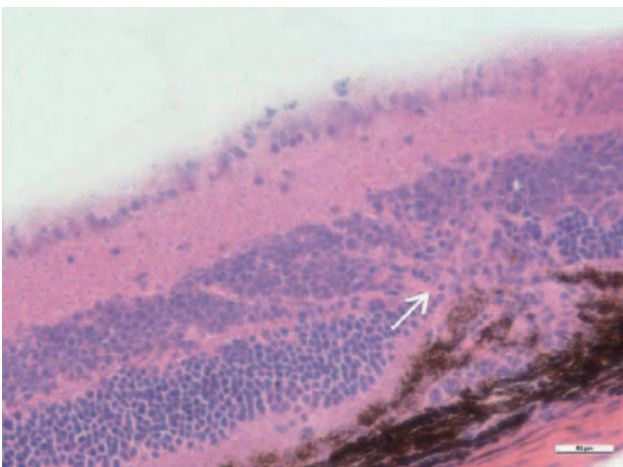
Fig. 5. Chorioretinitis in EAU is accompanied by new vessel formation; subretinal neovascularization (arrow) bursts from choroidal vessels through the retinal pigment epithelium and the Bruch's membrane into the retina.
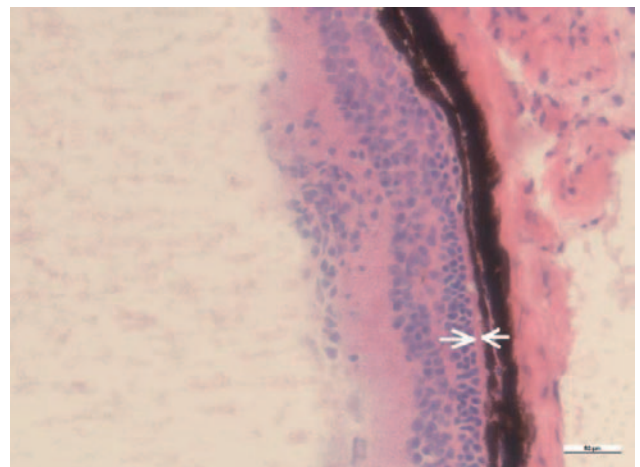
Fig. 6. The outer retinal layer with rods and cones is reduced by the inflammation, the significant photoreceptor loss (arrows) is a histopathological sign of advanced EAU.

Table 1. EAU scoring system

Grade	Description
0	No signs of inflammation
0.5	Small cellular infiltrate in ciliary body or sub-retinal space, non-granulomatous infiltrates in retina/choroid
1	Occasionally small retinal folds, single granulomatous infiltrate per section, mild perivasculitis, mild vitritis
2	Mild/moderate photoreceptor loss, 2 moderate-sized retinal folds per section, 1-2 granulomatous infiltrates per section, vasculitis < 10 % vessels, mild to moderate vitritis, cells in anterior chamber
3	Severe photoreceptor loss, 3 or more moderate or large retinal folds, more than 3 granulomatous infiltrates, vasculitis 10–50 % vessels, marked vitritis
4	Severe photoreceptor loss, extensive retinal folding or detachment, subretinal exudate, more than 3 large granulomas, vasculitis > 50 % vessels, severe vitritis

in a healthy mouse has clearly defined layers without any irregularities (Fig. 7).

The clinical evaluation of inflammation features of EAU by ophthalmoscopic examination of the retina *in vivo* is not a main focus of this article. The clinical signs of intraocular inflammation are seen clearly on the photographs of the retina, taken by the endoscopic fundus imaging system. The clinical grading system of EAU was described thoroughly by Xu et. al. (2008). The signs seen on our fundus image are focal infiltrations along the reti-

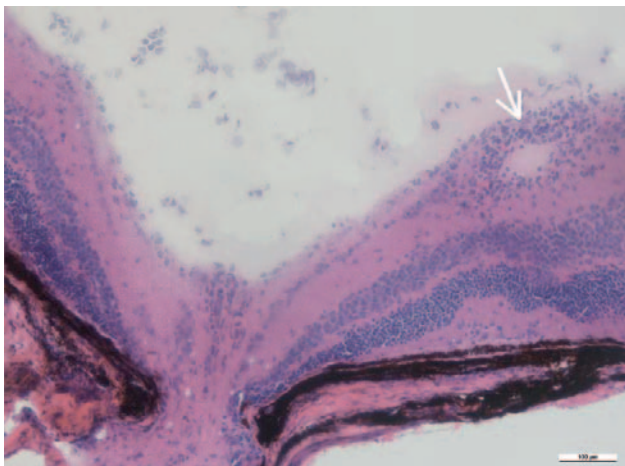*Fig. 3*. Vasculitis with thick infiltration of the retinal vessel wall (arrow) is located close to the optic nerve.
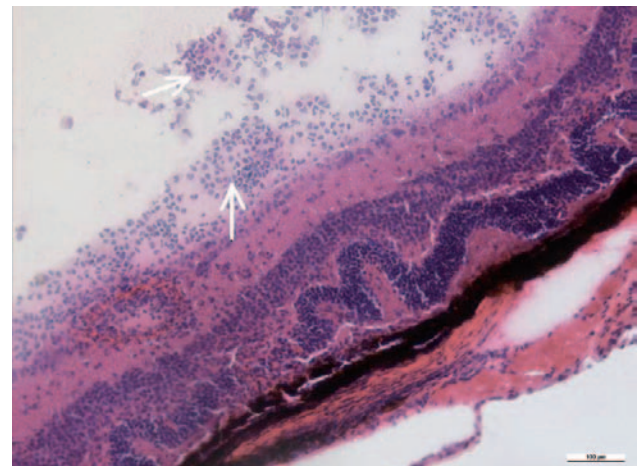

Fig. 4. Histological image showing grade 4 EAU: severe vitritis is represented by a collection of inflammatory cells in the corpus vitreum (arrows), severe vasculitis with wall infiltration and several retinal folds.
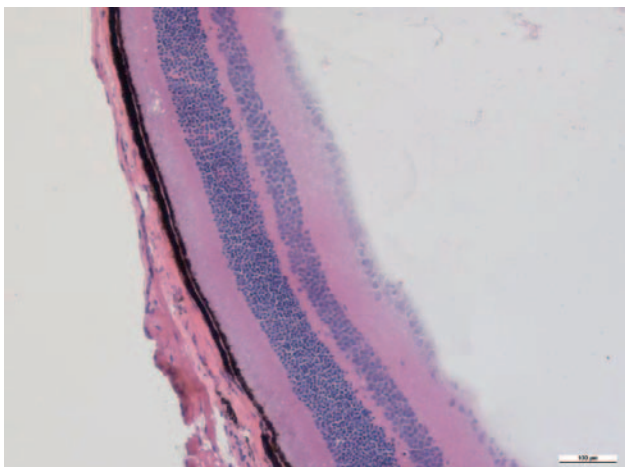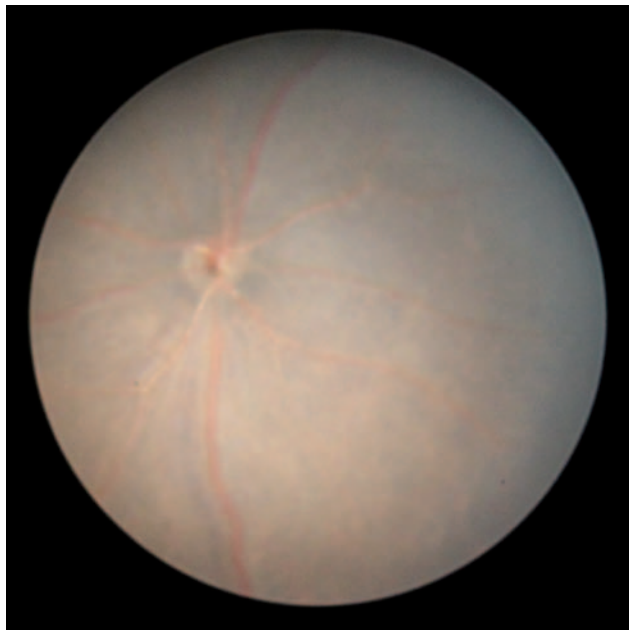

Fig. 7. Histological section of healthy retina with regular retinal layers.

Fig. 8-9. Mouse retinal images showing the posterior pole of the fundus were taken using the endoscopic fundus imaging system.

Fig. 8. Normal mouse fundus image taken from an 8-week-old healthy control mouse. The image was taken during general anaesthesia after pupil dilatation using mydriatic drops.
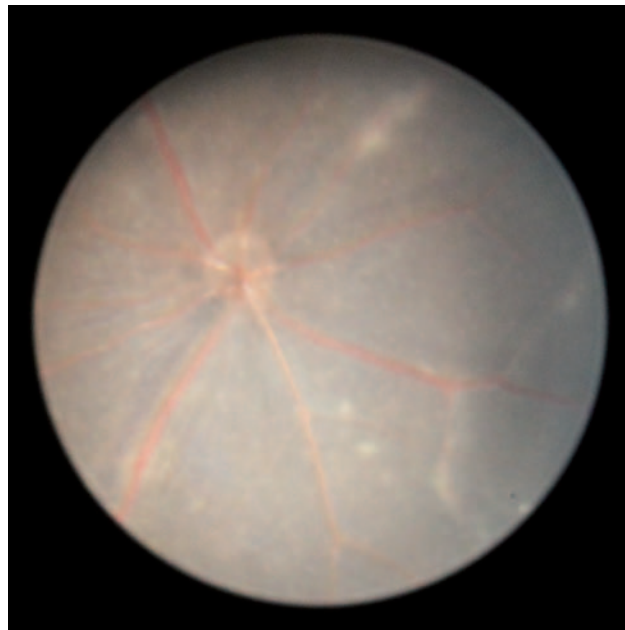
Fig. 9. Retinal changes during EAU in a C57BL/6 mouse are shown in a fundus image taken from day 28 post immunization. Several linear lesions and a small lesion show mild inflammation.

nal vessels (vasculitis), round lesions of various sizes (choroiditis) and oedema of the optic nerve (Figs. 8 and 9).

The aim of our study was to assess the efficacy of MMF compared to well-established treatment with CPA and humanized monoclonal antibody golimumab. The results of our study are outlined in a graph (Fig. 10). The treatment with CPA represented a positive control and it reflects the fact that all eyes in this group (29 eyes) were completely treated. This result was very highly significant ($P < 0.0001$) when compared to all control groups, including the sham treated group.

Treatment with antimetabolite drug MMF was applied in two dosage regimens. The lower dose of MMF (30 mg/kg; 32 eyes) resulted in statistically significant suppression of inflammation ($P < 0.05$) when compared to all control groups, including the sham treated group. The higher dose of MMF, 50 mg/kg (13 eyes), suppressed the EAU intensity; however, the results did not reach statistical significance. We presume that the higher dose did not prove statistical significance due to the high number of eyes in the control group. MMF in both doses was well tolerated by mice; as a side effect, we observed only a local fur loss in most of the animals. Our study thus supports the use of MMF in autoimmune posterior uveitis in humans.

Golimumab treatment has not shown any effect in rodent models due to its human origin and we used golimumab only as a negative control. From histological evaluation it is obvious that golimumab induced rather more severe inflammation; the results, however, were not statistically significant.

Discussion

Uveitis is a sight-threatening condition which in humans is difficult to treat. The immunosuppressants and biological therapy applied according to the present knowledge do not always prove effective in patients with autoimmune uveitis. As a vision-saving regimen, there are several treatment options.

CPA is a cytotoxic alkylating agent, which inhibits DNA replication. CPA is a strong cytostatic and immunosuppressive drug, it eliminates proliferating lymphoid cells, but also some quiescent cells. In humans, the suppressive effect of CPA has been known for a long time. CPA is used in patients in the case of failure of other immunomodulatory regimens or as a first line therapy in fulminant or life-threatening diseases (Khan et al., 2013; Suelves et al., 2013). Suzuki et al. (1989) observed in rats that application of CPA on the day before immunization markedly suppressed EAU development. Our study on EAU in mice confirmed an absolute anti-inflammatory effect of CPA, which was applied intraperitoneally in a dose of 100 mg/kg.

The main aim of our study was to evaluate the effect of MMF on uveitis in the mouse model of EAU. MMF is an antimetabolite which reversibly inhibits purine biosynthesis necessary for B- and T-cell growth. The treatment of non-infectious uveitis with MMF was proved successful in several reported case series in humans: Siepmann (2006), Teoh et al. (2008), Daniel et al. (2010) or Doycheva et al. (2011). Its effect in uveitis, however, is not as marked as in corneal diseases (Zierhut et al.,
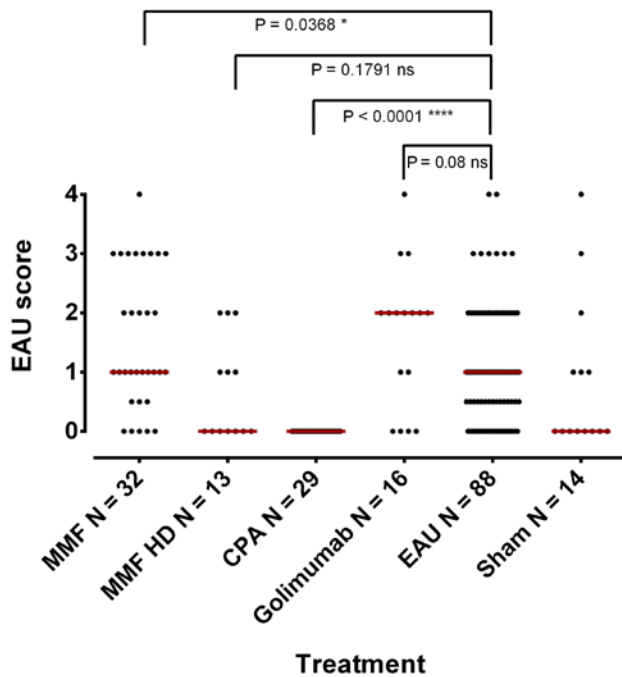
Fig. 10. The effectivity of EAU treatments. Comparison of the inflammation score (grade 0 to 4) acquired by histological evaluation on day 35 post immunization in the treated groups and eyes with EAU without treatment. The medians are displayed as red lines. The comparison between the groups was assessed using Mann-Whitney statistical test and the results are represented by ns, stands for non-significant, and significant outcomes that are displayed as * (P < 0.05) and **** (P < 0.0001). The median of inflammation score in EAU untreated mice is 1.0.
Abbreviations: MMF – mycophenolate mofetil, HD – high dose, N – number of eyes, CPA – cyclophosphamide, EAU – experimental autoimmune uveitis.

2005). In the literature even rather low success rate of uveitis therapy with MMF is reported as successful. In the everyday practice, MMF has got its place among the variety of immunosuppressants, and for some types of uveitis, it is recommendable. The main role of MMF is, however, rather supportive, and its effect in monotherapy in sight-threatening uveitis is more than questionable.

We have therefore tested MMF effects in the experimental model of uveitis. In the past, MMF was used in the EAU model in Lewis rats, and EAU induction was performed by bovine S-antigen (Chanaud et al., 1995; Dick et al., 1998). The rats were gavage fed with 30 mg/kg/day MMF on days 0–13 after immunization, which markedly suppressed the inflammation. In the control group 10 of 11 rats developed severe inflammatory changes; in the treated group 2 of 11 rats developed disease of lower intensity and later onset. Dick et al. (1998) also immunized the Lewis rats with bovine antigen and MMF was applied using gastric lavage in a dose of 30 mg/kg/day on days 7 to 20. The rats displayed a delay in disease onset with reduced clinical severity scores (1.38) compared to the control group (3.4) and animals developed a second peak of clinical disease around the day

28–32 post immunization. Histologic examination showed protection of photoreceptor loss in outer segments with MMF treatment. However, to date MMF has not been applied in the mouse EAU model. In our study, two dose regimens of MMF were used, one which better reflects the human dosage of this drug (30 mg/kg) and one with a higher dose (50 mg/kg). The lower dose of MMF suppressed experimental uveitis to a degree of statistical significance (P < 0.05) when compared to the sham treated group of EAU mice and of EAU untreated mice. The higher dose did not reach statistical significance, presumably because of a comparison with a higher number of control eyes examined (13 eyes with 50 mg/kg MMF versus 88 eyes with EAU).

Golimumab is a humanized anti-TNF-α monoclonal antibody approved for treatment of inflammatory bowel disease since 2013. The advantage of golimumab compared to other anti-TNF-α is the application which is performed subcutaneously once a month. Promising results in humans were described in a case series with severe autoimmune uveitis (Miserocchi et al., 2013). There are no reports published concerning golimumab in the EAU rodent model, as this drug should have no effect on rodents due to its humanized structure. Our observation shows rather enhancement of uveitis in the treatment group, which however did not reach statistical significance. Our hypothesis about the enhanced pro-inflammatory effect of golimumab is that the fully humanized antibody anti-TNF-α applied to mice causes a strong immunological reaction.

The establishment of a reproducible model of experimental autoimmune uveitis opens up many research possibilities. Uveitis induced in the C57BL/6 mouse strain is mild and chronic and closely resembles autoimmune uveitis in humans. In this study, we have confirmed the efficacy of CPA therapy for posterior uveitis. Our results show statistically significant suppression of experimental uveitis with MMF, which supports its use in human medicine. The model of EAU in mice is a very useful template for novel therapeutic approaches in the future.

Acknowledgements

References

Avichezer, D., Silver, P. B., Chan, C. C., Wiggert, B., Caspi, R. R. (2000) Identification of a new epitope of human IRBP that induces autoimmune uveoretinitis in mice of the H-2b haplotype. *Invest. Ophthalmol. Vis. Sci.* **41**, 127-131.

Broderick, C., Hoek, R. M., Forrester, J. V., Liversidge, J., Sedgwick, J. D., Dick, A. D. (2002) Constitutive retinal

CD200 expression regulates resident microglia and activation state of inflammatory cells during experimental autoimmune uveoretinitis. *Am. J. Pathol.* **161**, 1669-1677.

Caspi, R. R., Roberge, F. G., Chan, C. C., Wiggert, B., Chader, G. J., Rozenszajn, L. A., Lando, Z., Nussenblatt, R. B. (1988) A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens. *J. Immunol.* **140**, 1490-1495.

Caspi, R. R., Chan, C. C., Wiggert, B., Chader, G. J. (1990a) The mouse as a model of experimental autoimmune uveoretinitis (EAU). *Curr. Eye Res.* **9 (Suppl)**, 169-174.

Caspi, R. R., Chan, C. C., Leake, W. C., Higuchi, M., Wiggert, B., Chader, G. J. (1990b) Experimental autoimmune uveoretinitis in mice. Induction by a single eliciting event and dependence on quantitative parameters of immunization. *J. Autoimmun.* **3**, 237-246.

Chanaud, N. P., Vistica, B. P., Eugui, E., Nussenblatt, R. B., Allison, A. C., Gery, I. (1995) Inhibition of experimental autoimmune uveoretinitis by mycophenolate mofetil, an inhibitor of purine metabolism. *Exp. Eye Res.* **61**, 429-434.

Daniel, E., Thorne, J. E., Newcomb, C. W., Pujari, S. S., Kaçmaz, R. O., Levy-Clarke, G. A., Nussenblatt, R. B., Rosenbaum, J. T., Suhler, E. B., Foster, C. S., Jabs, D. A., Kempen, J. H. (2010) Mycophenolate mofetil for ocular inflammation. *Am. J. Ophthalmol.* **149**, 423-432.e1-2.

Dick, A. D., Cheng, Y. F., Liversidge, J., Forrester, J. V. (1994) Immunomodulation of experimental autoimmune uveoretinitis: a model of tolerance induction with retinal antigens. *Eye (Lond)*, **8 (Pt 1)**, 52-59.

Dick, A. D., Kreutzer, B., Laliotou, B, Forrester, J. V. (1998) Effects of mycophenolate mofetil on nasal mucosal tolerance induction. *Invest. Ophthalmol. Vis. Sci.* **39**, 835-840.

Doycheva, D., Zierhut, M., Blumenstock, G., Stuebiger, N, Deuter, C. (2011) Long-term results of therapy with mycophenolate mofetil in chronic non-infectious uveitis. *Graefes Arch. Clin. Exp. Ophthalmol.* **249**, 1235-1243.

Durrani, O. M., Meads, C. A., Murray, P. I. (2004) Uveitis: a potentially blinding disease. *Ophthalmologica* **218**, 223-236.

Forrester, J. V., Liversidge, J., Dua, H. S., Towler, H., McMenamin, P. G. (1990) Comparison of clinical and experimental uveitis. *Curr. Eye Res.* **9 (Suppl.)**, 75-84.

Forrester, J. V., Liversidge, J., Dua, H. S., Dick, A., Harper, F., McMenamin, P. G. (1992) Experimental autoimmune uveoretinitis: a model system for immunointervention: a review. *Curr. Eye Res.* **11 (Suppl.)**, 33-40.

Khan, I. J., Barry, R. J., Amissah-Arthur, K. N., Carruthers, D., Elamanchi, S. R., Situnayake, D., Murray, P. I., Denniston, A. K., Rauz, S. (2013) Ten-year experience of

pulsed intravenous cyclophosphamide and methylprednisolone protocol (PICM protocol) in severe ocular inflammatory disease. *Br. J. Ophthalmol.* **97**, 1118-1122.

Miserocchi, E., Modorati, G., Pontikaki, I., Meroni, P. L., Gerloni, V. (2013) Long-term treatment with golimumab for severe uveitis. *Ocul. Immunol. Inflamm.* **22**, 90-95.

Mochizuki, M., Nussenblatt, R. B., Kuwabara, T., Gery, I. (1985) Effects of cyclosporine and other immunosuppressive drugs on experimental autoimmune uveoretinitis in rats. *Invest. Ophthalmol. Vis. Sci.* **26**, 226-232.

Paques, M., Guyomard, J. L., Simonutti, M., Roux, J. M., Picaud, S., Le-Gargasson, J. F., Sahel, J. A. (2007) Panretinal, high resolution color photography of the mouse fundus. *Invest. Ophthalmol. Vis. Sci.* **48**, 2769-2774.

Siepmann, K., Huber, M., Stübiger, N., Deuter, C., Zierhut, M. (2006) Mycophenolate mofetil is a highly effective and safe immunosuppressive agent for the treatment of uveitis: a retrospective analysis of 106 patients. *Graefes Arch. Clin. Exp. Ophthalmol.* **244**, 788-794.

Suelves, A. M., Arcinue, C. A., González-Martin, J. M., Kruh, J. N., Foster, C. S. (2013) Analysis of a novel protocol of pulsed intravenous cyclophosphamide for recalcitrant or severe ocular inflammatory disease. *Ophthalmology* **120**, 1201-1209.

Suttorp-Schulten, M. S., Rothova, A. (1996) The possible impact of uveitis in blindness: a literature survey. *Br. J. Ophthalmol.* **80**, 844-848.

Suzuki, I., Takahashi, S., Fujii, Y., Fuyama, S., Arai, S. (1989) Experimental autoimmune uveoretinitis (EAU) in rats: isolation of S-antigen, EAU susceptibility of rat strains, genetic control of EAU induction, and effects of cyclophosphamide and irritation on EAU. *Jpn. J. Ophthalmol.* **33**, 13-26.

Teoh, S. C., Hogan, A. C., Dick, A. D., Lee, R. W. (2008) Mycophenolate mofetil for the treatment of uveitis. *Am. J. Ophthalmol.* **146**, 752-60.

Thurau, S., R., Chan, C. C., Nussenblatt, R. B., Caspi, R. R. (1997) Oral tolerance in a murine model of relapsing experimental autoimmune uveoretinitis (EAU): induction of protective tolerance in primed animals. *Clin. Exp. Immunol.* **109**, 370-376.

Xu, H., Koch, P., Chen, M., Lau, A., Reid, D. M., Forrester, J. V. (2008) A clinical grading system for retinal inflammation in the chronic model of experimental autoimmune uveoretinitis using digital fundus images. *Exp. Eye Res.* **87**, 319-326.

Zierhut, M., Stübiger, N., Siepmann, K., Deuter, C. M. (2005) MMF and eye disease. *Lupus* **14 (Suppl 1)**, 50-54.

The critical points in induction of experimental autoimmune uveitis

Aneta Klimova[a], Petra Seidler Stangova[a], Petra Svozilkova[a], John V. Forrester[b,c] , Izabela Klaska[b], Jarmila Heissigerova[a]

Background. Autoimmune uveitis is a leading cause of visual impairment in developed countries in patients of working age. Animal models of experimental autoimmune uveitis (EAU) have been established to serve as a useful template for novel therapeutic approaches.

Methods. Experimental autoimmune uveitis is induced in C57BL/6 mice by subcutaneous application of interphotoreceptor retinoid binding protein in complete Freund's adjuvant and pertussis toxin. Clinical and histological grading is used to assess the inflammation intensity of EAU.

Results. The protocol of induction of EAU in mice hides several important aspects, which are crucial for developing the disease. These details have to be addressed to ensure reproducible disease induction. We describe our experience in establishing the model by pointing out the critical steps in EAU protocol which we found important.

Conclusion. The mouse model of EAU has practical value for preclinical studies, is robust and well established. However, the induction of inflammation of the eye can be quite challenging when important details of the protocol are not recognized and adhered to.

[a]*Department of Ophthalmology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic*
[b]*Section of Immunology and Infection, Institute of Medical Sciences, University of Aberdeen, United Kingdom*
[c]*Immunology and Virology Program, Centre for Ophthalmology and Visual Science, The University of Western Australia, Crawley, Western Australia, Australia and Centre for Experimental Immunology, Lions Eye Institute, Nedlands, Western Australia, Australia*
Corresponding author: Aneta Klimova, e-mail: aneta.klimova@volny.cz

INTRODUCTION

Uveitis is an intraocular inflammation affecting mostly people of working age. In spite of expanding therapeutic possibilities 10% of patients have severe visual handicap[1]. Clinical studies are difficult to perform for ethical reasons, therefore animal models were developed. Models of experimental autoimmune uveoretinitis are representative for various forms of human intraocular inflammation and serve as a useful template for novel immunotherapeutical approaches.

Mouse models of experimental autoimmune uveitis (EAU) have been established by Rachel Caspi more than 20 years ago[2,3]. After rat, rabbit, guinea pig and primate uveitis models the mouse model is most sophisticated and versatile. Thanks to the many genetically manipulated strains and variety of reagents, it is possible to perform research in the mouse model to extent, which is impossible to achieve in other species[4]. The development of experimental autoimmune uveitis helps to understand the mechanisms of autoimmune disease and to find more specific treatment with fewer side effect[5].

Although the model of EAU is quite robust, the protocol of induction has to be followed precisely. Successful induction of EAU is based on careful laboratory preparation of the emulsified antigen as well on proper subcutaneous application of the antigen emulsion.

MATERIALS AND METHODS

The uveitis in mice is induced by subcutaneous application of the retinal autoantigen interphotoreceptor retinoid binding protein (IRBP) or its uveitogenic peptide, available in several versions according to a mouse strain[6] in complete Freund's adjuvant (CFA). The reactivity of immune system is furthermore enhanced by intraperitoneal application of pertussis toxin (PT) depending on the mouse strain.

The susceptibility to IRBP induced EAU model varies with mouse strain, the most susceptible is B10.RIII, followed by B10.A. The C57BL/6 strain is only moderately susceptible[4], but provides mild posterior uveitis, which has a longer duration of the disease and the unaffected anterior eye segment allows to examine retina clearly. C57BL/6 is a common mouse strain available in knockout, transgenic and gnotobiotic form. Some strains (e.g. BALB/c) are resistant to EAU (ref.[7]).

We used inbred female mice strain C57BL/6 6 to 8 weeks old, since we wished to induce a chronic mild inflammation, mainly involving the posterior segment of the eye[8]. This imitates most closely posterior uveitis in humans and is therefore most appropriate to test new therapeutical strategies. Noticeably, for induction of experimental autoimmune uveitis is appropriate to use the mouse substrain C57BL/6J. The substrain C57BL/6N

carries the rd8 retinal degeneration mutation and mice congenitally lack photoreceptor cells[7].

The peptide inducing experimental autoimmune uveitis in C57BL/6 mouse strain, also called retinol-binding protein 3 precursor fragment ([Homo sapiens] H2N-GPTHLFQPSLVLDMAKVLLD-OH, New England Peptide, Gardner, MA, USA) is commercially prepared as a fine powder. We recommend preparing the peptide emulsion in the original plastic container with the appropriate dose of the protein in milligrams since freeze dried peptide adheres readily to surfaces due to electrostatic effects and so is difficult to weigh to the correct amount. Dimethyl sulphoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA) is used to dissolve the IRBP. This solution is further diluted by sterile distilled water, slowly in 3 steps. After each step the solution centrifuged for at least 3 min at 500 rpm. This produces a clear solution without particles or precipitates. The IRBP solution can be stored in freezer under -20 ˚C for several months or longer.

The IRBP solution is mixed 1:1 with complete Freund's adjuvant (CFA, Sigma Aldrich, St. Louis, MO, USA), which can be strengthened by additional Mycobacteria tuberculosis either by adding 25 milligrams of heat-dried H37RA Mycobacteria tuberculosis organisms in 10 mL of CFA, containing 1 mg of Mycobacterium tuberculosis in 1 mL. The other possibility, which we prefer, is to remove the top 7 mL of pure oil from the 10 mL vial of CFA after sedimentation of Mycobacteria at the bottom of the vial. With first method we reach the concentration of Mycobacterium tuberculosis in oil 3.5 mg/ mL, with the second 3.3 mg/ mL.

We have found that a consistently reproducible emulsion depends on effective mixing of the suspension. The final product should be uniform (no separation of phases), white, stiff and viscous[9] to stimulate a strong antigen response in mice. We use a plastic tube (Tub PTFE 2xHub, Hamilton Company, Reno, NV, USA) connected on both sides to glass, 1 mL volume, Hamilton syringes. It is recommended to use glass syringes, since equivalent 1 mL plastic (insulin) syringes are not PTFE (polytetrafluor ethylene)-manufactured and particles precipitate on the plastic surface, thus losing material. The syringe-hub-syringe must be air free to avoid a froth forming. Thorough manual mixing takes around 15 minutes (equivalent to 1000 passes through the syringes). Ultrasonic emulsification mixing[10] reduces the time at this stage but requires short pulse of ultrasound to avoid damaging the peptide. The quality of emulsification is tested by placing a drop of emulsion on a cold water surface (a well-prepared emulsion will not spread and remains as droplets).

Subcutaneous application of 50 μL of emulsion (500 μg of IRBP per mouse) in each hind leg is performed using a short intradermal needle (BD Microlance Hypodermic Needle 26G x 3/8"). A shorter needle provides more comfortable subcutaneous instillation. A small uniform swelling will appear under the skin. The needle should be held in position under the skin for 2-3 s before removal so as to avoid backflush of emulsion. The subcutaneous swelling should remain palpable at least 2 weeks.

The injury of adjacent muscle may be severe and must be avoided by ensuring that the injection is correctly placed subcutaneously and not intramuscularly.

Since the *in vivo* procedure has to be performed carefully and precisely and may be moderately painful we prefer to work with mice under short mild anesthesia and analgesia by ketamine intraperitoneally in dose 2 mg/kg (Narkamon 50 mg/ mL, Bioveta, Nitra, Slovakia).

Disease induction is optimized by intraperitoneal application of 0.5-2 μg PT per mouse (List Biologicals, Campbell, CA, USA) at the day of induction. Lyophilized PT from *Bordetella pertussis* (stored in 4 ˚C) is dissolved in sterile distilled water, and can be stored in 4 ˚C. Immediately before administration PT is further diluted in cold PBS (phosphate buffered saline), transported on ice, but warmed to room temperature for the intraperitoneal injection. In general, this attention to detail in preparing the PT is very important since its potency for experimental autoimmune uveitis may be diminished. Similar observations have been made in the induction of experimental autoimmune encephalomyelitis (EAE) (ref.[11]).

The experimental use of animals was approved by The Commission for Animal Welfare of the First Faculty of Medicine of Charles University in Prague, Czech Republic, and the Ministry of Education, Youth and Sports according to animal protection laws. All the procedures were approved by the animal experimentation review committee.

RESULTS AND DISCUSSION

By following the above protocol the EAU in C57BL/6 mice is induced, which is normally a mild chronic disease of low intensity of inflammation. The intensity of inflammation varies from lab to lab. On average grades severity varies between grades 1-2 [2.13 (ref.[12]), between 1 and 2 (ref.[8]), 1.2 (ref.[13]), 1.63 (ref.[14]).] In our experience, using the above protocol the mean histological score is 2.0.

Even when following the above procedure precisely the incidence of disease can be variable. Other factors which affect this are age and sex. We use 6 to 8 weeks old mice as in other reports, while Xu et al. (ref.[8]) used 8 to 12 week old mice. In general, female mice are preferred since autoimmune diseases are more prevalent in females. There are no publications comparing the intensity of uveitis between male and female mice.

The susceptibility of a specific mouse strain to induction of EAU may also vary with the housing conditions of mice. Chronically stressed mice (elevated corticosteroid levels suppress the inflammation) or animals with acute disease (elevated circulating interferons) may fail to develop EAU (ref.[7]).

A complication of oily adjuvant instillation is injection site ulceration[15]. These usually develop 2 to 3 weeks after injection and have been reported in induction of EAE and other models. Frequency of occurrence varies from 0.5% to 20% of animals. Most ulcers are small (up to 4 mm in size) but rarely large confluent ulcers may

occur. Ulcer development is reduced by systemic immunosuppressive therapy and there is no correlation between ulcers formation and intensity of inflammation (personal observation).

CONCLUSION

A reliable, standardized protocol and procedure for induction would be valuable for its further use in research on disease pathogenesis and treatment. The interpretation of the results should take into account the variation in protocol and procedure used in each experiment. It is therefore important to include the possible variation in the evaluation of the results.

ABBREVIATIONS

EAU, Experimental autoimmune uveitis; IRBP, Interphotoreceptor retinoid binding protein; CFA, Complete Freund's adjuvant; PT, Pertussis toxin; EAE, experimental autoimmune encephalomyelitis.

Author contribution: AK: manuscript writing and literature search, PSS: data collection, PS: study design and manuscript writing, JVF, IK: model development support, JH: study design and manuscript writing.
Conflict of interest statement: The authors declare there are no conflicts of interest regarding the publication of this article.

REFERENCES

1. Durrani OM, Meads CA, Murray PI. Uveitis: a potencially blinding disease. Ophthalmologica 2004;218:223-36.
2. Caspi RR, Roberge FG, Chan CC, Wiggert B, Chader GJ, Rozenszajn LA, Lando Z, Nussenblatt RB. A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens. J Immunol 1988;140:1490-5.
3. Caspi RR, Chan CC, Wiggert B, Chader GJ. The mouse as a model of experimental autoimmune uveoretinitis (EAU). Curr Eye Res 1990; 9(Suppl.):169-74.
4. Caspi RR. Animal models of autoimmune and immune-mediated uveitis. Drug Discov Today Dis Mod 2006;3:3-10.
5. Klimova A, Seidler Stangova P, Heissigerova J, Svozilkova P, Kucera T. Mycophenolate mofetil and cyclophosphamide treatments suppress inflammation intensity in an experimental model of autoimmune uveitis. Folia Biol (Praha) 2014;60:228-34.
6. Caspi RR, Silver PB, Luger D, Tang J, Cortes LM, Pennesi G, Mattapallil MJ, Chan CC. Mouse models of experimental autoimmune uveitis. Ophthalmic Res 2008;40:169-74.
7. Agarwal RK, Silver PB, Caspi RR. Rodent models of experimental autoimmune uveitis. Methods Mol Biol 2012;900:443-69.
8. Xu H, Koch P, Chen M, Lau A, Reid DM, Forrester JV. A clinical grading system for retinal inflammation in the chronic model of experimental autoimmune uveoretinitis using digital fundus images. Exp Eye Res 2008;87:319-26.
9. Stromnes IM, Goverman JM. Active induction of experimental allergic encephalomyelitis. Nat Protoc 2006;4:1810-9.
10. Maatta JA, Nygardas PT, Hinkkanen AE. Enhancement of experimental autoimmune encephalomyelitis severity by ultrasound emulsification of antigen/adjuvant in distinct strains of mice. Scand J Immunol 2000;51:87-90.
11. Bernard CC, Carnegie PR. Experimental autoimmune encephalomyelitis in mice: immunologic response to mouse spinal cord and myelin basic proteins. J Immunol 1975;114: 1537-40.
12. Oh JY, Kim TW, Jeong HJ, Lee HJ, Ryu JS, Wee WR, Heo JW, Kim MK. Intraperitoneal infusion of mesenchymal stem/stromal cells prevents experimental autoimmune uveitis in mice. 2014;2014:624640. doi: 10.1155/2014/624640.
13. Keino H, Kezuka T, Takeuchi M, Yamakawa N, Hattori T, Usui M. Prevention of experimental autoimmune uveoretinitis by vasoactive intestinal peptide. Arch Ophthalmol 2004;122:1179-84.
14. Kitamei H, Kitaichi N, Yoshida K, Nakai A, Fujimoto M, Kitamura M, Iwabuchi K, Miyazaki A, Namba K, Ohno S, Onoé K. Association of heat shock protein 70 induction and the amelioration of experimental autoimmune uveoretinitis in mice. Immunobiology 2006;212:11-8.
15. Whitehouse M. Oily adjuvants and autoimmunity: now time for reconsideration? Lupus 2012;21:217-22.

KLINICKÉ PROJEVY EXPERIMENTÁLNÍ AUTOIMUNITNÍ UVEITIDY

Klímová A.[1], Seidler Štangová P.[1], Svozílková P.[1], Kučera T.[2], Heissigerová J.[1]

[1]Oční klinika, 1. lékařská fakulta, Univerzita Karlova v Praze a Všeobecná fakultní nemocnice v Praze, přednosta doc. MUDr. Bohdana Kalvodová, CSc.
[2]Ústav histologie a embryologie, 1. lékařská fakulta, Univerzita Karlova v Praze, přednosta doc. MUDr. Tomáš Kučera, Ph.D.

Autoři práce prohlašují, že vznik i téma odborného sdělení a jeho zveřejnění není ve střetu zájmu a není podpořeno žádnou farmaceutickou firmou.

SOUHRN

Úvod: Autoimunitní uveitida je závažné zrak ohrožující onemocnění, které u části pacientů zůstává rezistentní ke konvenční imunosupresivní anebo biologické léčbě. Výzkum z posledních let zlepšil terapeutické možnosti tohoto onemocnění a významný podíl na tomto vývoji má základní výzkum na experimentálních modelech. Cílem této práce je prezentovat klinické a histologické projevy zánětu na modelu experimentální autoimunitní uveitidy (EAU) u myši.
Metodika: EAU byla indukována u myší kmene C57BL/6 subkutánní aplikací IRBP (interphotoreceptor retinoid binding protein) v kompletním Freundově adjuvans a pertusovým toxinem aplikovaným intraperitoneálně. Intenzita zánětu byla hodnocena *in vivo* pomocí speciálního zobrazovacího systému s využitím otoskopu. Bulby byly enukleovány *post mortem* 35. den po indukci EAU a intenzita zánětu hodnocena histologicky barvením kryořezů metodou hematoxylin-eozin.
Výsledky: Fotodokumentace zobrazuje klinické projevy uveitidy *in vivo* i histologické známky zánětu získané *post mortem*.
Závěr: Zavedení stabilního a reprodukovatelného modelu EAU umožňuje podrobně studovat imunopatogenetické mechanismy zánětu a jejich cílenou regulaci. Podobnost zánětlivých změn s nálezy pacientů se zadní uveitidou autoimunitní etiologie umožňuje výsledky výzkumu na tomto experimentálním modelu aplikovat v humánní medicíně.
Klíčová slova: experimentální autoimunitní uveitida, autoimunita, myši

SUMMARY

THE CLINICAL SIGNS OF EXPERIMENTAL AUTOIMMUNE UVEITIS

Introduction: Autoimmune uveitis is a sight threatening disease which in many cases fails to respond to conventional immunosuppressive or biological therapy. The research in experimental models of autoimmune uveitis helps to find new therapeutical strategies. The aim of this study is to present the clinical and histological signs of experimental autoimmune uveitis (EAU) in mice.
Methods: EAU was induced in C57BL/6 mice by subcutaneous application of IRBP (interphotoreceptor retinoid binding protein) in complete Freund's adjuvant and intraperitoneal application of pertussis toxin. Clinical evaluation of uveitis was performed in vivo using special imaging system with otoscope. Histological evaluation of uveitis was performed at day 35 post induction of EAU on hematoxylin and eosin stained frozen sections. Clinical and histological grading was used to assess the inflammation intensity of EAU.
Results: The intensity of inflammation is depicted on representative fundus images and histological images of retina at day 35 post induction.
Conclusion: The model of EAU is robust and reproducible and allows us to study the immunopathological mechanisms of inflammation and its regulation. The inflammatory signs in our model are similar to findings of posterior uveitis of autoimmune etiology in humans, thus we may apply our experimental results in human medicine.
Key words: experimental autoimmune uveitis, autoimmunity, mice

MUDr. Aneta Klímová
Oční klinika, 1. lékařská fakulta,
Univerzita Karlova v Praze a VFN
U Nemocnice 2
128 08 Praha 2
Telefon: 737 800 058
e-mail: aneta.klimova@volny.cz

ÚVOD

Uveitida je spolu s diabetickou retinopatií hlavní příčinou slepoty v rozvinutých zemích u lidí v produktivním věku. Uveitida představuje desetiprocentní podíl na slepotě v těchto zemích (20, 9), a to i přes stále se rozšiřující terapeutické možnosti.

Etiologii autoimunitní uveitidy u člověka je velice obtížné zjistit. Přibližně u 25 % pacientů se autoimunitní uveitida pojí se systémovým onemocněním typu sarkoidózy, ankylozující spondylitidy, roztroušené sklerózy, systémového lupus erythematodes, Wegenerovy granulomatózy apod. Téměř u poloviny případů však etiologii nezjistíme.

Základní léčebnou modalitou autoimunitní uveitidy je imunosuprese. Výzkum v posledních letech významně zlepšil terapeutické možnosti a nemalý podíl na tomto vývoji má základní výzkum uveitid na experimentálních modelech.

První model uveitidy u potkanů (18) využíval aplikaci Freundova adjuvans obsahujícího mykobakteria a později i endotoxinu (10). Od té doby byl vyvinut model autoimunitní uveitidy u potkanů, u morčat, u koní a u primátů imunizací jedním ze sítnicových antigenů – arrestinem neboli

S-antigenem v kompletním Freundově adjuvans (22, 12). Jeho aplikace experimentálnímu zvířeti vyvolala onemocnění s klinickým obrazem velmi podobným uveitidě u lidí. U běžně používaných kmenů myší však S-antigen zánětlivé oční onemocnění nevyvolává. Až po objevu intraretinálního vazebného peptidu (IRBP) byl vyvinut experimentální model autoimunitní uveitidy u myší (4). Od té doby vznikly četné varianty prvního základního myšího modelu, který spočívá v intraperitoneální či subkutánní aplikaci antigenu (IRBP).

V našich experimentech byla indukována EAU pomocí IRBP humánního původu. Protein IRBP se nachází v interfotoreceptorové matrix, která slouží k transportu derivátů vitaminu A mezi fotoreceptory a retinálním pigmentovým epitelem. Strukturu proteinu IRBP tvoří čtyři evolučně staré domény, o kterých se předpokládá, že vznikly genovou duplikací (1).

Další možností indukce EAU je adoptivní přenos T lymfocytů z imunizovaných donorů, dále „humanizovaná" forma EAU u transgenních kmenů myší, EAU indukovaná injekcí dendritických buněk, které in vitro zrály v přítomnosti antigenu, a nakonec model spontánní uveitidy u myší, kterým chybí gen pro autoimunitní regulátor (AIRE), a u athymických „nude" myší s implantovaným krysím embryonálním thymem (6).

Námi používaný model experimentální uveitidy je velmi podobný obrazu zadní uveitidy či panuveitidy u lidí – s vitritidou, choroiditidou, retinitidou a vaskulitidou. Posouzení stupně uveitidy je založeno na hodnocení in vivo biomikroskopicky, tak post mortem histologicky (7).

Cílem této práce je prezentovat klinické a histologické projevy experimentální uveitidy u myši. Podle jejich stupně se provádí standardizované hodnocení intenzity zánětu, které slouží například k posouzení účinnosti nové léčby.

METODIKA

Myši C57BL/6

Samice inbredního kmene myší C57BL/6 ve věku 5 až 8 týdnů byly dodány z Centra pro experimentální biomodely (1. lékařská fakulta, Univerzita Karlova, Praha). Použití laboratorních zvířat pro tento projekt bylo schváleno odbornou komisí pro práci s pokusnými zvířaty 1. lékařské fakulty Univerzity Karlovy v Praze a se zvířaty bylo nakládáno v souladu se zákonem č. 246/1992 Sb., na ochranu zvířat proti týrání, na základě osvědčení Ministerstva zemědělství.

Indukce EAU

Aplikace IRBP, který působí jako autoantigen, byla prováděna podle standardního protokolu (2, 3). Subkutánně se aplikuje 500 µg IRBP 1-20 (interphotoreceptor retinoid binding protein, New England Peptide, Gardner, USA). Pro rozpuštění peptidu byl použit DMSO (dimethyl sulfoxid) (Sigma Aldrich, St. Louis, USA). IRBP byl emulzifikován v poměru 1:1 s kompletním Freundovým adjuvans (Difco, USA) obsahujícím mykobakterie. Reaktivita imunitního systému byla podpořena intraperitoneální aplikací 1,2 µg pertusového toxinu (List Biologicals, Campbell, USA), rozpuštěného v PBS (phosphate buffered saline).

Klinické vyšetření uveitidy

Biomikroskopické vyšetření zvířat in vivo bylo prováděno pomocí otoskopu (obr. 1). Myši byly vyšetřovány v celkové intraperitoneální kombinované anestezii ketaminem 80 mg/kg (Narkamon 50 mg/ml, Bioveta, Slovensko) a xylazinem 5 mg/kg (Rometar 20 mg/ml, Bioveta, Slovensko). Pro dilataci zornice byl lokálně aplikován tropikamid (Unitropic 1% oph. gtt., Unimed Pharma, Slovensko) a phenylephrin (Neosynephrin-Pos 10 %, Ursapharm, Česká republika). Na rohovku pokrytou vrstvou viskomateriálu byl přikládán otoskop připojený k zevnímu zdroji světla a k fotoaparátu s předsazenou čočkou +4,0 dioptrie (19).

Obr. 1 Biomikroskopické vyšetření sítnice u myši

Histologické zpracování materiálu

Zvířata byla usmrcena podle etických pravidel daných zákonem České republiky cervikální dislokací. Oči byly enukleovány vystříháním ze spojivkového vaku bezprostředně post mortem 35. den po indukci EAU. Oči byly vloženy do gelového media (Tissue-Tek® O.C.T. Compound™, USA) a zamrazeny ve 2-methylbutanu (Sigma Aldrich, St. Louis, USA) v atmosféře tekutého dusíku. Vzorky zmrazené na -70 °C byly krájeny na mikrotomu (Leica CM 1850) na 7 µm silné řezy. Řezy byly krájeny vždy z periferie a z oblasti optického nervu obou očí a barveny metodou hematoxylin-eozin podle standardního protokolu. Vzorky byly odebrány z obou očí, vzhledem k tomu, že zánět může být asymetrický.

VÝSLEDKY

Subkutánní aplikace IRBP v kompletním Freundově adjuvans potencovaná intraperitoneálním pertusovým toxinem vyvolala u citlivých myších kmenů projevy zadní uveitidy, mimořádně i panuveitidy.
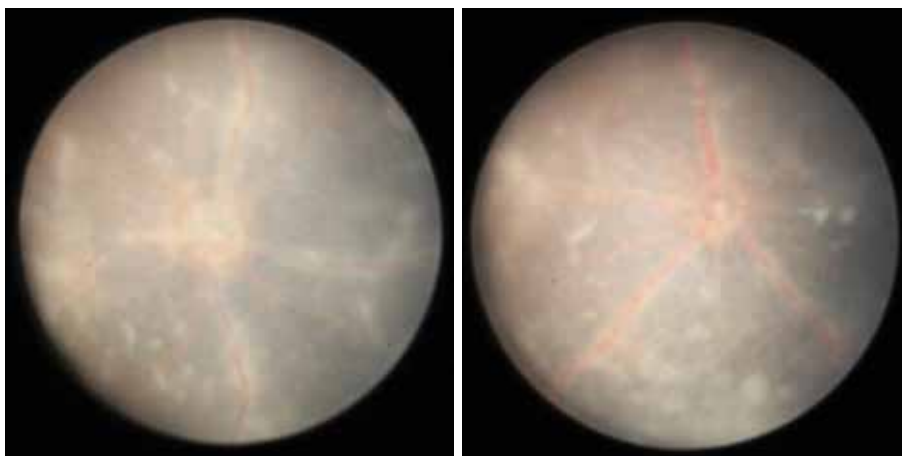
Hodnocení intenzity zánětu bylo provedeno klinicky in vivo nebo histologicky post mortem.

Klinické projevy EAU

Klinické známky zadní uveitidy jsou zřetelné na fotografiích sítnice vytvořených pomocí zobrazovacího systému oto-

Obr. 2 a 3 Fyziologický fundus u myši

Obr. 4 a 5 Fotografie sítnice 20. den po indukci EAU, jsou přítomny infiltráty v sítnici, opouzdřené cévy a edém terče zrakového nervu

Obr. 6 Zadní synechie na fotografii předního segmentu přítomné 25. den po indukci EAU

**Obr. 7** Totální serózní amoce 25. den po indukci EAU

Obr. 8 Fotografie fundu 25. den po indukci EAU, na sítnici jsou patrné granulární infiltráty, opouzdřené cévy a edém terče zrakového nervu

skopu. Známky zánětu na našich snímcích zachycují vaskulitidu, choroiditidu a edém optického nervu (obr. 2–12).

Klinický systém hodnocení intenzity uveitidy popisuje Xu et. al. (23). Hodnotí ve škále 1 (minimální zánět) až 4 (silný zánět) 4 parametry: velikost a tvar infiltrátů sítnice, zánětlivé změny zrakového nervu, stupeň opouzdření cév a strukturální změny sítnice (atrofie/jizvení).

Biomikroskopicky byla u našich myší nejvyšší intenzita uveitidy pozorována 25. až 28. den po indukci, a to průměrně stupeň 3. Na začátku pozorování (14. den) byl u někte-
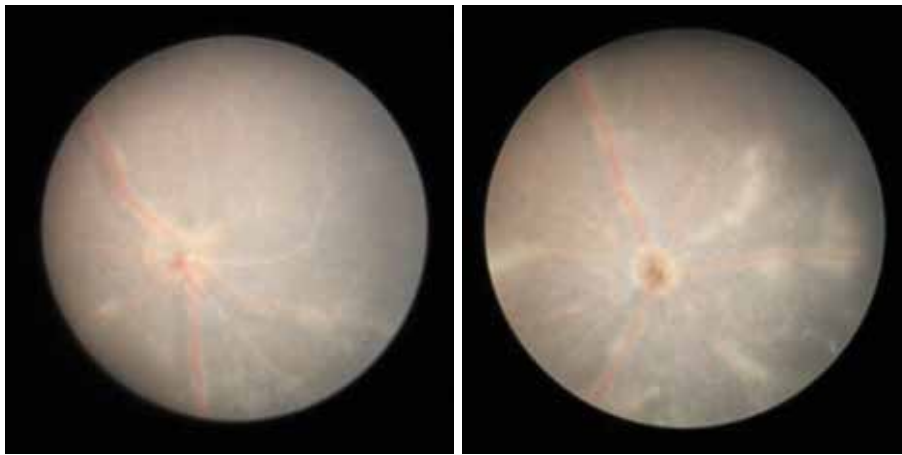
Obr. 9 a 10 Fotografie fundu 35. den po indukci EAU, sítnice s převažující atrofií, granulární i lineární infiltráty ubývají, edém terče zrakového nervu přetrvává
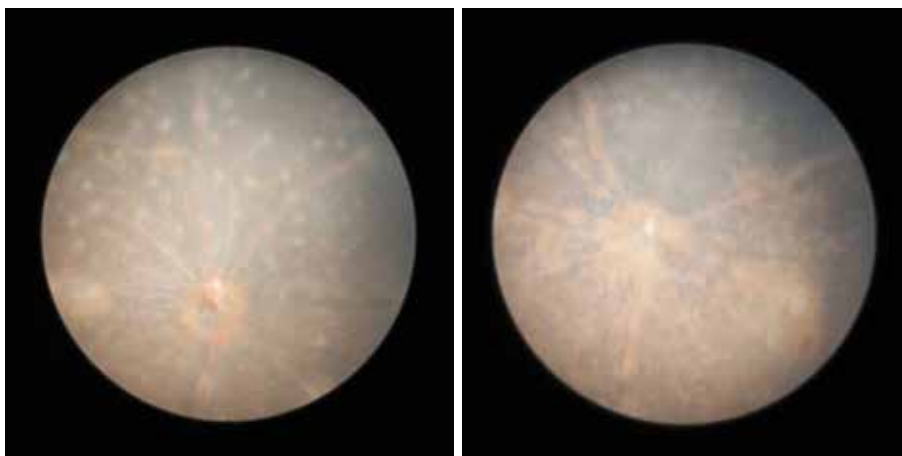
**Obr. 11 a 12** Fotografie fundu 60. den po indukci EAU, dominuje atrofie sítnice, infiltráty jsou více organizované, papila zrakového nervu je ohraničená
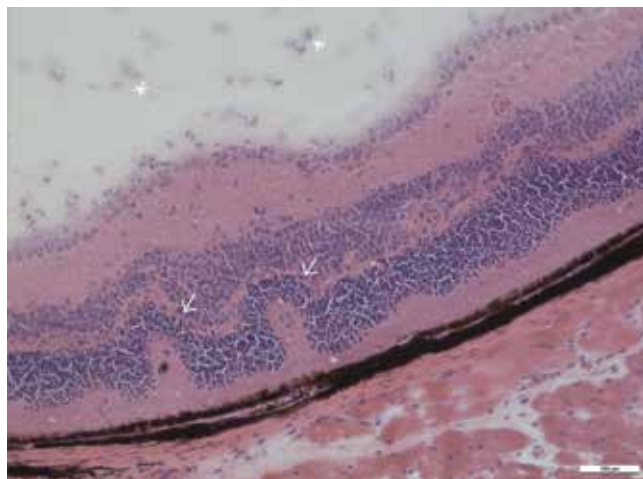
Obr. 2– 12 Fotografie sítnice u zdravých myší a u myší v různých intervalech po indukci EAU zobrazují dynamiku zánětlivých projevů
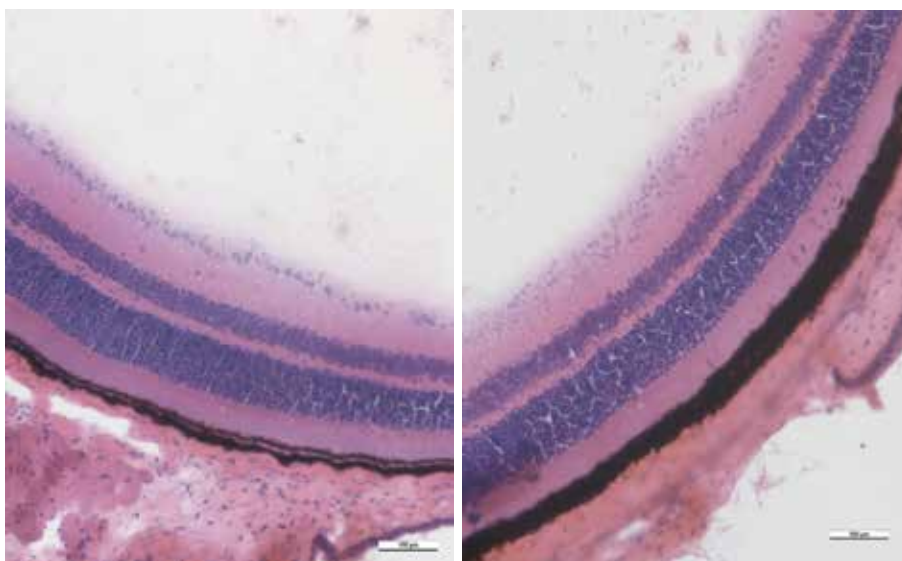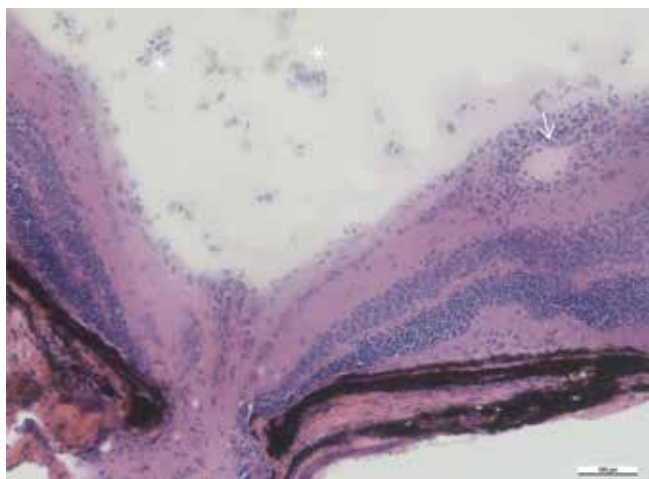

Obr. 13 a 14 Histologický řez sítnicí zdravé myši s pravidelně uspořádanými vrstvami, zvětšení 20x
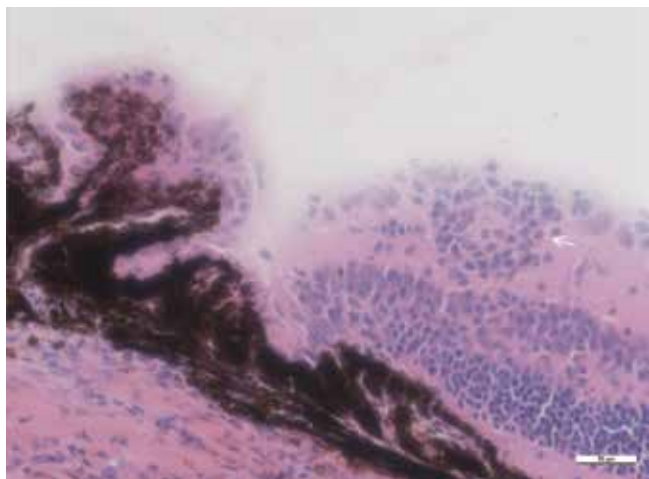
Obr. 15 Zánětlivé ložisko ve vnitřní vrstvě sítnice v blízkosti ciliárního tělesa (šipka), zvětšení 40x
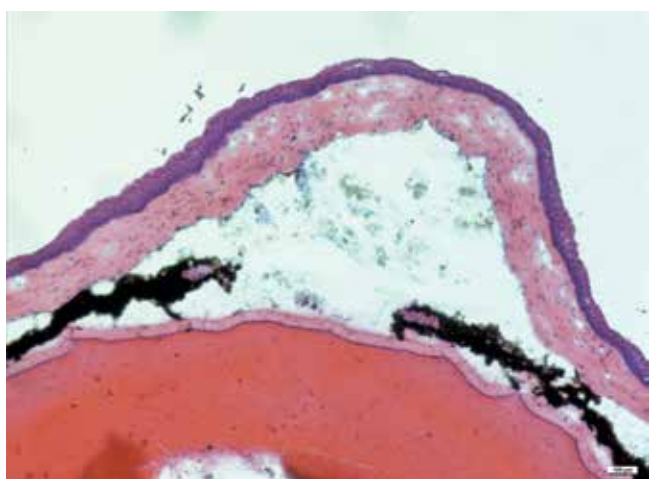

Obr. 16 Retinální záhyby v zevních vrstvách sítnice (šipky), ve sklivci jsou přítomny zánětlivé buňky (hvězdičky), zvětšení 20x

Obr. 17 Infiltrace v okolí sítnicové cévy v blízkosti terče zrakového nervu (šipka), ve sklivci jsou zánětlivé buňky (hvězdičky), zvětšení 20x
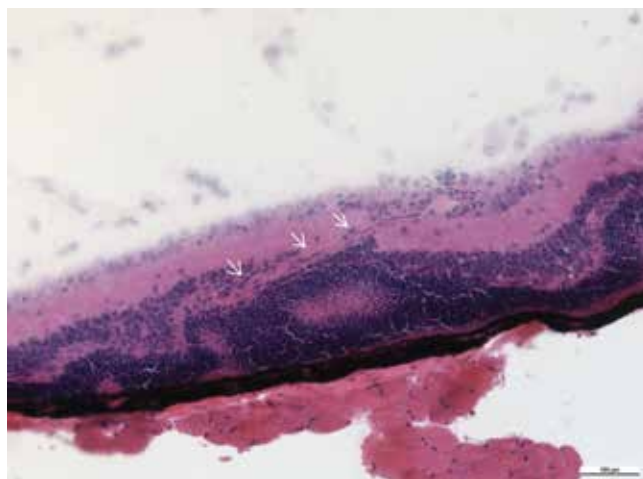
Obr. 18 Novotvořená céva prochází vrstvami sítnice (šipky), zvětšení 20x

Obr. 19 Histologický řez přední částí oka, komorová tekutina je zkalená, duhovka je překrvená, jsou přítomny přední i zadní synechie. Za duhovkou je čočka, která fyziologicky vyplňuje velkou část oka. Zvětšení 10x

Obr. 13–19 Histologické řezy očí u zdravých myší a u myší 35. po indukci EAU barvené hematoxylinem a eozinem. Měřítko je uvedené na snímcích, linka odpovídá velikosti 100 μm.

rých očí ojediněle zachycen infiltrát nebo byl přítomný mírný edém terče, v celkovém hodnocení je klasifikován průměrně stupeň 0. V dalším vývoji 20. až 21. den je u některých očí patrný výrazný edém terče zrakového nervu, opouzdření cév a bělavé sítnicové lineární či granulární zánětlivé léze (obr. 4 a 5), většina očí zůstává bez zánětlivých změn, průměrně je stupeň zánětu 1. Na vrcholu zánětu 25. až 28. den jsou u většiny očí výrazné projevy zánětu (obr. 6, 7, 8). Od 35. dne jsou na sítnici přítomny atrofické změny (obr. 9 a 10), které se v čase stupňují (obr. 11 a 12).

Histologické projevy zánětu

Mezi histologické známky zadní uveitidy patří retinální záhyby, zánětlivá ložiska v sítnici, často lokalizovaná v blízkosti ciliárního tělíska, vaskulitida, vitritida, neovaskularizace sít-

nice či ztráta fotoreceptorů (obr. 15–18). Sítnice u zdravých myší má zřetelně oddělené vrstvy sítnice bez nepravidelností (obr. 13 a 14).

Mikroskopické hodnocení intenzity uveitidy na řezech barvených metodou hematoxylin-eozin se provádí podle zavedeného systému hodnocení od stupně 0 (žádný zánět) do stupně 4 (silný zánět) podle Caspi (4, 21, 8).

Histologicky jsou v našem pozorování maximální zánětlivé změny přítomny 35. den po indukci. Intenzita zánětu posuzovaná histologicky u našich myší je 1,0 až 2,0.

DISKUSE

Autoimunitní uveitida zahrnuje celou řadu jednotek, které se liší klinickým obrazem a průběhem onemocnění. Vzhledem k tomu, že se navíc jedná o vzácné onemocnění, je v humánní medicíně velice obtížné autoimunitní uveitidu studovat. Bylo vyvinuto několik experimentálních modelů, které umožňují detailnější studium uveitid (4, 23, 6). Tyto modely pomohly za posledních 40 let objasnit vliv genové výbavy na průběh nitroočního zánětu, zkoumat základní mechanismy patogeneze uveitid a vyzkoušet nové strategie imunologické léčby (11, 17).

Experimentální autoimunitní uveitida u myší představuje reprodukovatelný model, který otevírá další možnosti v oblasti výzkumu zadní uveitidy autoimunitní etiologie. Uveitida indukovaná u myší kmene C57BL/6 je mírná a chronická a blízce připomíná autoimunitní uveitidu u lidí (5). Tím se liší od staršího modelu akutní uveitidy u myší kmene B10.RIII, kde vzniká prudká krátkodobá panuveitida. Na tomto modelu je vrchol zánětu 12. až 15. den a není možné vyšetřit fundus biomikroskopicky pro zkalená optická média (13).

Citlivost běžně používaného kmene myší C57BL/6 k sítnicovému peptidu (IRBP) je poměrně variabilní. Úspěch indukce ovlivňuje více faktorů, například pohlaví a stáří myší nebo podmínky chovu. V našem souboru máme myši staré 5 až 8 týdnů, zatímco někteří autoři preferují myši ve stáří 8 až 12 týdnů (23). Obvykle se pracuje se samicemi, a to vzhledem k vyšší prevalenci autoimunitních onemocnění u žen. Dosud nebyla publikována práce srovnávající u myší intenzitu zánětu EAU mezi pohlavím. Neúspěšná indukce uveitidy může být důsledek chronického stresu (vyšší hladiny kortikosteroidů potlačují zánět) nebo akutního onemocnění (zvýšené cirkulující interferony) (1).

Intenzita vyvolaného zánětu hodnocená histologicky u kmene myší C57BL/6 kolísá mezi jednotlivými laboratořemi v závislosti na protokolu indukce uveitidy. Průměrně autoři uvádějí stupeň zánětu 1 až 2: Kim et al. (15) 2,13, Xu et al. (15) 1 až 2, Keino et al. (14) 1,2, Kitamei et al. (16) 1,63. V našich experimentech byl při histologickém hodnocení zaznamenán průměrný stupeň zánětu 1 až 2.

Pozoruhodné je, že intenzita zánětu ve škále 1 až 4 zjištěná při klinickém vyšetření otoskopem je vyšší než při hodnocení histologickém (23), což potvrzuje také naše sledování. Tato diskrepance může být vysvětlena například rozdílným rozsahem hodnocené sítnice. Při klinickém vyšetření je přehlédnuta celá sítnice. Při histologickém hodnocení je posuzováno 8 až 10 řezů sítnice jednoho oka silné 7 μm, nemusí být proto všechny léze zachyceny.

Výhodou modelu chronické EAU u myší C57BL/6 je relativně dlouhá doba aktivity zánětu trvající přibližně 3 až 4 týdny. Biomikroskopicky byla u našich myší nejvyšší intenzita zánětu 25. až 28. den po indukci. Histologicky byly prokázány maximální zánětlivé změny 35. den po indukci. Naše pozorování korelují s výsledky jiných autorů, Xe et al. (23) zaznamenává vrchol zánětu kolem 25. dne po indukci, Caspi (4) popisuje aktivní zánět od 3. do 7. týdne po indukci.

Postupný vývoj klinických a histologických projevů zánětu v našem pozorování odpovídá popisu v práci kolektivu autorů Xu et al. (23). V počátečních stadiích (14. den po indukci) je klinicky přítomný mírný edém terče zrakového nervu, na histologickém řezu je zrakový terč bez viditelných strukturních změn. Na vrcholu zánětu (25. den po indukci) je klinicky patrný výrazný edém terče zrakového nervu, opouzdření cév a bělavé sítnicové lineární léze. Na histologických řezech tyto změny odpovídají infiltraci zrakového nervu zánětlivými buňkami, vaskulitidě a retinálním záhybům. V pozdním stádiu (80. den po indukci) dominují v klinickém obraze sítnicové jizvy, histologicky byla prokázána glióza a ztráta zevních segmentů fotoreceptorů.

Minimální či žádné postižení předního segmentu u našeho modelu umožňuje lepší přehlednost a snadnější biomikroskopické vyšetření sítnice i při vysoké aktivitě zánětu.

ZÁVĚR

Klinické a histologické hodnocení intenzity zánětu na modelu EAU je zásadní pro využití tohoto modelu v základním výzkumu. Zavedení stabilního a reprodukovatelného modelu EAU umožňuje podrobně studovat imunopatogenetické mechanismy zánětu a jejich cílenou regulaci, což může přispět k efektivní léčbě nitroočních zánětů v humánní medicíně.

LITERATURA

1. **Agarwal R.K., Silver P.B., Caspi R.R.:** Rodent Models of Experimental Autoimmune Uveitis. Autoimmunity: Methods and Protocols, Methods in Molecular Biology, vol. 900, Springer Science+Business Media New York; 2012, Chapter 22, 443–469.
2. **Avichezer D., Silver P. B., Chan C. C., Wiggert B. et al.:** Identification of a new epitope of human IRBP that induces autoimmune uveoretinitis in mice of the H-2b haplotype. Invest Ophthalmol Vis Sci, 41; 2000: 127–131.
3. **Broderick C., Hoek R. M., Forrester J. V. et al.:** Constitutive retinal CD200 ex-

pression regulates resident microglia and activation state of inflammatory cells during experimental autoimmune uveoretinitis. Am J Pathol, 161(5); 2002: 1669–77.

4. **Caspi R. R., Roberge F. G., Chan C. C.:** A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens. J Immunol, 140(5); 1988: 1490–1495.

5. **Caspi R. R.:** Animal models of autoimmune and immune-mediated uveitis. Drug Discovery Today: Disease Models, Volume 3, Issue 1, 2006, 3–9.

6. **Caspi R. R., Silver P. B., Luger D. et al.:** Mouse models of experimental autoimmune uveitis. Ophthalmic Res, 40; 2008: 169–74.

7. **Chan C. C., Caspi R. R., Ni M. et al.:** Pathology of experimental autoimmune uveoretinitis in mice. J Autoimmun, 3; 1990: 247–55.

8. **Dick A. D., Cheng Y. F., Liversidge J. et al.:** Immunomodulation of experimental autoimmune uveoretinitis: a model of tolerance induction with retinal antigens. Eye (Lond), 8 (Pt 1), 1994: 52–59.

9. **Durrani O. M., Meads C. A., Murray P. I.:** Uveitis: a potencially blinding disease. Ophthalmologica, 218; 2004: 223–36.

10. **Forrester J. V., Worgul B. V., Merriam G. R., Jr.:** Endotoxin-induced uveitis in the rat. Albrecht Von Graefes Arch Klin Exp Ophthalmol, 213; 1980: 221–33.

11. **Forrester J. V., Liversidge J., Dua H. S. et al.** (1992): Experimental autoimmune uveoretinitis: a model system for immunointervention: a review. Curr Eye Res, 11 (Suppl.): 33–40.

12. **Hirose S., Singh V. K., Donoso L. A. et al.:** An 18-mer peptide derived from the retinal S antigen induces uveitis and pinealitis in primates. Clin Exp Immunol, 77; 1989: 106–11.

13. **Jiang H.R., Lumsden L., Forrester J.V.:** Macrophages and dendritic cells in IRBP-induced experimental autoimmune uveoretinitis in B10RIII mice. Invest Ophthalmol Vis Sci, 40 (13); 1999: 3177–85.

14. **Keino H., Kezuka T., Takeuchi M., et al.:** Prevention of experimental autoimmune uveoretinitis by vasoactive intestinal peptide. Arch Ophthalmol. Aug, 2004; 122(8): 1179–84.

15. **Kim T.W., Jeong H.J., Lee H.J. et al.:** Intraperitoneal Infusion of Mesenchymal Stem/Stromal Cells Prevents Experimental Autoimmune Uveitis in Mice. Mediators Inflamm, 2014: 624-640.

16. **Kitamei H., Kitaichi N., Yoshida K.** et al.: Association of heat shock protein 70 induction and the amelioration of experimental autoimmune uveoretinitis in mice. Immunobiology, 2006; 212(1):11–8.

17. **Klímová A., Seidler Štangová, J. Heissigerová et al.:** Mycophenolate Mofetil and Cyclophosphamide Treatments Suppress Inflammation Intensity in an Experimental Model of Autoimmune Uveitis. Folia Biologica (Praha), 2014; 60: 228–234.

18. **Lalive dE-Z.:** Experimental uveitis in the rat after subcutaneous injection of Freund's adjuvant. Histological changes in the uvea. Ophthalmologica, 155; 1968: 271–89.

19. **Paques M., Guyomard J. L., Simonutti M. et al.:** Panretinal, High Resolution Color Photography of the Mouse Fundus. Invest. Ophthalmol Vis Sci, 48; 2007: 2769–2774.

20. **Suttorp-Schulten M. S., Rothova A.:** The possible impact of uveitis in blindness: a literature survey. Br J Ophthalmol, 80; 1996: 844–848.

21. **Thurau S. R., Chan C. C., Nussenblatt R. B. et al.:** Oral tolerance in a murine model of relapsing experimental autoimmune uveoretinitis (EAU): induction of protective tolerance in primed animals. Clin Exp Immunol, 109; 1997: 370–376.

22. **Wacker W. B., Kalsow C. M.:** Autoimmune uveo-retinitis in the rat sensitized with retina photoreceptor cell antigen. Int Arch Allergy Appl Immunol, 45; 1973: 582–92.

23. **Xu H., Koch P., Chen M. et al.:** A clinical grading system for retinal inflammation in the chronic model of experimental autoimmune uveoretinitis using digital fundus images. Exp Eye Res, 87; 2008: 319–326.

The Microbiota Determines Susceptibility to Experimental Autoimmune Uveoretinitis

Authors:

Jarmila Heissigerova, Assoc. Prof., MD, PhD, MBA[1*], Petra Seidler Stangova, MD[1*], Aneta Klimova, MD[1], Petra Svozilkova, Assoc. Prof., MD, PhD[1], Tomas Hrncir, MD, PhD[2], Renata Stepankova, MA, PhD, DSc[2], Miloslav Kverka, MD, PhD[2,3], Helena Tlaskalova-Hogenova, Prof., MD, PhD, DSc[2] and John V. Forrester, Prof., MD, FRCSE, FRCSG, FRCOphth[4,5]

[1]Department of Ophthalmology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic

[2]Institute of Microbiology of the Czech Academy of Sciences, v.v.i., Prague and Novy Hradek, Czech Republic

[3]Institute of Experimental Medicine of the Czech Academy of Sciences, v.v.i., Prague, Czech Republic

[4]Section of Immunology and Infection, Institute of Medical Sciences, University of Aberdeen, United Kingdom

[5]Immunology and Virology Program, Centre for Ophthalmology and Visual Science, The University of Western Australia, Crawley, Western Australia, Australia and Centre for Experimental Immunology, Lions Eye Institute, Nedlands, Western Australia, Australia

*both authors contributed equally to the research

Corresponding Author: Jarmila Heissigerova, Dept. of Ophthalmology, Charles University, U Nemocnice 2, Praha 2, 12808, Czech Republic. E-mail: j.heissigerova@yahoo.co.uk

Abstract:

The microbiota is a crucial modulator of the immune system. Here, we evaluated how its absence or reduction modifies the inflammatory response in the murine model of experimental autoimmune uveoretinitis (EAU). We induced EAU in germ-free (GF) or conventionally housed (CV) mice, and in CV mice treated with a combination of broad-spectrum antibiotics either from the day of EAU induction or from one week prior to induction of disease. The severity of the inflammation was assessed by fundus biomicroscopy or by histology, including immunohistology. The immunophenotyping of T cells in local and distant lymph nodes was performed by flow cytometry. We found that GF mice and mice where the microbiota was reduced one week before EAU induction were protected from severe autoimmune inflammation. GF mice had lower numbers of infiltrating macrophages and significantly less T cell infiltration in the retina than CV mice with EAU. GF mice also had reduced numbers of IFN-γ and IL-17-producing T cells and increased numbers of regulatory T cells in the eye-draining lymph nodes. These data suggest that the presence of microbiota during auto-antigen recognition regulates the inflammatory response by influencing the adaptive immune response.

1. Introduction:

Considerable effort has been made to understand mechanisms leading to the initiation of autoimmune diseases, and yet the reason for the loss of self-tolerance is still not fully clarified. One hypothesis is that infection triggers autoimmune disease in genetically predisposed individuals by crossreactivity between self and foreign antigens due to similarity between foreign and self-antigenic epitopes [1]. A second proposal is that infections may also trigger autoimmune disease through „bystander" activation of autoreactive T cells, in which self-antigen released during tissue damage is presented by activated innate immune cells or B cells, for instance during mycobacterial infection [2, 3].

Uveitis or intraocular inflammation is a sight-threatening condition, which affects mostly people of working age. Despite improved therapeutic possibilities 10% of patients become blind [4, 5]. Many cases of intraocular inflammation are directly caused by infections such as toxoplasmosis, viral infections and other pathogens. However, in around 50% of cases the etiology remains unknown. Some cases may be associated with systemic diseases such as sarcoidosis, multiple sclerosis, and ankylosing spondylitis in which infections as triggers of autoimmunity are also implicated. Specific agents such as Chlamydophila, Human Herpes Virus 6, and Epstein-Barr virus, are suggested in either the development or progression of multiple sclerosis [6]. However, both for „idiopathic" uveitis and uveitis associated with systemic disease, the search for infectious causes is frequently fruitless.

Accordingly, animal models of autoimmune diseases have been developed most of which utilize immunization with an evolutionarily conserved „autoantigen" together with one or more adjuvants which usually comprise a component of an infectious agent such as heat-killed mycobacteria and pertussis toxin [7]. Adjuvants are required in order to activate innate immune cells via pathogen recognition receptors which include at least four classes of receptors, particularly toll-like receptors and C-type lectins. The need for adjuvants in generating experimental models implicates infectious agents in the induction of autoimmune disease and in this context, there has been considerable interest in the role of the microbiome in modulating susceptibility to autoimmune diseases.

The explosion of research into the nature of the microbiome using molecular genetic techniques to type and classify microbiota has revealed that commensal organisms exist in specific sites or niches in the body and are specific for each site. Initial studies on the gut microbiome in the context of inflammatory bowel disease revealed the role of the gut microbiome in maintaining immunological homeostasis in situ, thereby reducing the risk of

inflammatory bowel disease [8]. These studies have been extended to other autoimmune disease-models and for this purpose the use of germ-free (GF) mice has been extremely valuable [9-13]. Interestingly, the susceptibility to experimental autoimmune disease varied with the type of the disease: for instance experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein was found to be less severe in GF mice [14], while diabetes mellitus in non-obese diabetic mice was more severe [15-17]. Caspi et al. [18] and Nakamura et al. [19] have previously reported in a surrogate model of GF mice that alteration of the gut microbiota by using a combination of orally-administered broad-spectrum antibiotics modulates the severity of EAU. More recently work from the same laboratory has used an interphotoreceptor retinoid binding protein (IRBP)-transgenic mouse model of spontaneous uveitis in antibiotic treated and GF mice to demonstrate suppression of EAU [20]. Although this work describes a spontaneous model of EAU, which is considered by some to be more clinically relevant than the complete Freund´s adjuvant (CFA)-induced model, the mice contain an artificially high number of IRBP-specific CD4 T cells (>20% in the IRBP-161H clone). However, in CV mice and in humans, the precursor frequency of any particular antigen specific T cell is known to be very low, if not rare [21]. It is therefore possible that in the IRBP-transgenic spontaneous model of uveitis, the sheer weight of numbers of antigen specific T cells predicates a greatly increased risk of microbial antigen-induced T cell receptor cross reactivity, which would then permit entry of activated T cells into tissue sites and further activation via cognate antigen.

We have therefore investigated whether reduction in the gut microbiome, either by antibiotic use as previously reported or as found in GF mice, modifies EAU induction in CV mice in which T cell activation is induced with a mycobacterial adjuvant (CFA) and is dependent on innate immune cell activation and signaling via the C-type lectin, dectin-1 [22]. We report that the severity of EAU is markedly reduced in GF mice and in mice which have been pretreated with antibiotics to reduce microbial burden but not when the microbial burden is reduced after activation of T cells has been induced.

2. Materials and methods:

2.1. Animals

We used inbred male and female mice of the C57BL/6J strain (5 to 8 weeks old). Mice were housed either at the conventional animal facility of Department of Pharmacology, First

Faculty of Medicine, Charles University in Prague, where untreated CV mice were compared with CV mice treated with broad-spectrum antibiotics; or in the Laboratory of Gnotobiology at the Institute of Microbiology Academy of Sciences, Czech Republic, Novy Hradek, where experiments comparing GF and CV mice were performed.

The mice were rederived into GF conditions using Caesarean section and bred in sterile Trexler-type plastic isolators for many generations. The bedding, food pellets, and water were sterilized by gamma irradiation (25 kGy) or autoclaving. The long-term colonies of GF mice were supplied with sterile water and food pellets, HD2 extruded diet (Fitmin, Czech Republic), ad libitum. The GF status of colonies was evaluated weekly as fecal samples and cotton swabs from the isolator interior were tested for the presence of aerobic and anaerobic bacteria, mold, and yeast. The CV mice, which served as CV controls at this facility, were fed with the same diet and regularly tested for the absence of potential mouse pathogens, including strains of *Helicobacter muridarum* and *H. hepaticus*, according to internationally recognized standards (FELASA).

The use of animals for these experiments was approved by The Commission for Animal Welfare of the First Faculty of Medicine of Charles University in Prague, Czech Republic, and the Ministry of Education, Youth and Sports and by The Animal Care And Use committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic, according to animal protection laws.

2.2 EAU induction

EAU was induced by subcutaneous inoculation of IRBP peptide 500 μg per mouse in complete Freund´s adjuvant in conjunction with intraperitoneal application of pertussis toxin (PT) 0.6 μg according to a standard protocol [23, 24]. In brief, IRBP peptide 1-20 (interphotoreceptor retinoid-binding protein, also called retinol-binding protein 3-precursor fragment [Homo sapiens] H2N-GPTHLFQPSLVLDMAKVLLD-OH, New England Peptide, Gardner, USA) dissolved in DMSO (dimethyl sulfoxide, Sigma Aldrich, St. Louis, USA) was emulsified in ratio 1:1 with CFA (Difco, USA) and the solution was applied subcutaneously.

2.3 Antibiotic treatment

To reduce the microbial load, the mice were treated with broad-spectrum antibiotics (mixture of 500 mg/l of metronidazole (B. Braun, Czech Republic) and ciprofloxacin 100 mg/l (Ciprinol, Krka, Czech Republic)) in the drinking water as previously described [25].

Metronidazole (nitroimidazole) has a limited spectrum of activity that encompasses various protozoans and most Gram-negative and Gram-positive anaerobic bacteria. Ciprofloxacin is a second-generation fluoroquinolone, with a spectrum of activity, which includes Gram-negative and Gram-positive bacterial pathogens. To establish the importance of the microbiota with respect to disease induction, we initiated treatment either one week prior to EAU induction or on the day of EAU induction. In both experimental schedules, the antibiotic treatment continued until the end of the experiment.

2.4 Clinical evaluation

In vivo clinical examination (fundus biomicroscopy) was performed using the TEFI imaging system [26-28]. An additional +4.0 diopter lens between the camera and the otoscope was used. During the procedure, the mice were under general anesthesia (ketamine 80 mg/kg and xylazine 5 mg/kg (both Bioveta, Slovakia) intraperitoneally). The fundi were imaged through a dilated pupil (tropicamide, Unitropic 1% oph. gtt., Unimed Pharma, Slovakia) and phenylephrine (Neosynephrin-POS 10% oph. gtt., Ursapharm, Czech Republic). The otoscope was applied to the cornea using eye gel carbomerum (Vidisic gel, Bausch and Lomb, Czech Republic). A single image of the posterior central retinal part from each eye was taken, transferred to a computer for analysis.

The inflammation was graded as described previously – see Table 1 [28]. Retinal inflammatory changes were evaluated separately for the optic disc, retinal vessels and retinal tissue changes from the central part of the retina (Table 1). The mean overall clinical inflammation grade was then averaged. All samples were evaluated by two experienced ophthalmologists (PSS, AK), and the discussed consensus of the two evaluations used.

2.5 Histological evaluation

The mice were sacrificed on day 35 and the eyes were enucleated, and immediately immersed in Tissue-Tek® O.C.T. Compound^Tm (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen in 2-methylbutane (Sigma Aldrich, St. Louis, USA) in liquid nitrogen. The samples were stored at −70 °C until sectioning to 7 μm thick slices (at -19 to -21°C). Sections were taken from both eye peripheries and centrally through the optic nerve. The samples were cut with a cryostat (Leica CM 1850) and stained with hematoxylin and eosin. These samples were then evaluated by two experienced ophthalmologists and graded using a standardized scoring system as previously published [7, 29, 30] and modified by the authors (Table 2).

Eyes with congenital defects, such as microphthalmia or cataract, have been excluded from evaluation, which led to odd numbers in some graphs.

2.6 Immunohistochemistry

The immunohistochemistry was performed on six randomly selected mice from each group. T-lymphocytes were detected using a three-step immunoperoxidase method with polyclonal rabbit anti-human CD3 (Dako Denmark A/S, Glostrup, Denmark) diluted 1:200 in PBS containing 1.5% normal goat serum. This antibody is cross-reactive with mouse antigens [31]. Visualization of primary antibody binding was performed using secondary biotinylated anti-rabbit antibody (Dako) and the Vectastain Elite ABC kit standard (Vector Laboratories, USA).

Macrophages were detected using a three-step immunoperoxidase method with monoclonal rat anti-mouse F4/80 antibody (clone BM8, Abcam, Cambridge, Great Britain) diluted 1:100 in PBS containing 1.5% normal goat serum. Visualization of primary antibody binding was performed using secondary biotinylated anti-rat antibody (Abcam) and the Vectastain Elite ABC kit standard (Vector Laboratories, USA). Positive cells were counted in two sections per eye, one from periphery and one from the center, to obtain quantitative data.

2.7 Immunophenotyping by flow cytometry

Mouse mesenteric and cervical lymph nodes were separately harvested, mashed into cell suspension, washed in complete RPMI medium, filtered through a 70 μm cell strainer. For detection of regulatory T cells, the cell suspensions were washed, labeled with Fixable Viability Dye (eBioscience), blocked with anti-CD16/CD32 antibody, stained for surface CD4 and CD25, fixed and permeabilized overnight with Fixation/Permeabilization buffer (eBioscience) and stained for intracellular FoxP3. To analyze intracellular cytokine production, cells (2×10^6 cells/ml in complete RPMI) were incubated for 5 hours with 50 ng/ml PMA, 500 ng/ml ionomycin (both from Sigma-Aldrich), and 2 μM Monensin (eBioscience). After the incubation, the cells were washed, labeled with a viability dye, blocked, stained for surface CD4, fixed and permeabilized as described above. Next, the cells were stained for intracellular cytokines with antibodies against IFN-γ, IL-17, and TNF-α. The data were acquired on a FACSCalibur flow cytometer and analyzed with FlowJo software. The cytokines were analyzed while gating on viable CD4$^+$ cells. All monoclonal antibodies were purchased from eBioscience (San Diego, USA).

2.8 Data analysis

Data were analyzed using GraphPad Prism Version 6.04 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Kruskal-Wallis and Mann-Whitney non-parametric tests were used to evaluate differences between the groups and p<0.05 was considered significant.

3. Results

3.1 The severity of EAU is reduced in GF mice

EAU was induced with sterile reagents in either GF or CV mice and the level of inflammation at day 35 by fundoscopy and histology was compared. EAU was significantly reduced in GF mice compared to CV controls. By clinical fundoscopy, no inflammation was observed in the GF mice at day 35 post induction, whereas in control CV mice, severe inflammation was observed as extensive signs of chorioretinal lesions, vascular sheathing (vasculitis) and vitreous haze (Table 1 and Fig. 1a; p<0.001). On histological evaluation, minimal to no signs of uveitis were observed in GF mice compared to severe uveitis in CV mice (Table 2 and Fig. 1b; p<0.001).

3.2. EAU in mice treated with antibiotics

Since GF mice appeared to develop less severe EAU disease than CV housed mice, we speculated whether reduction in microbial load using antibiotic therapy would have the same effect as GF state. We performed two experiments by administering antibiotics in CV mice either from the day of EAU induction or from one week prior. Mice treated with metronidazole and ciprofloxacin (see Methods) commencing one week prior to EAU induction and continued for the course of the experiment (a treatment which significantly reduces microbial burden [25]) had significantly lower levels of EAU compared to controls both clinically (Fig. 2a; maximal difference observed at day 35; p<0.05) and histologically (Fig. 2b; p<0.05). In contrast, mice treated with the same antibiotic regime but commencing on the day of immunization showed little difference in the level of EAU compared to controls (Fig. 3a,b).

3.3 Immunohistology of the eyes

Immunohistological studies of the eyes performed on GF mice and littermate controls at day 35 (see Methods) showed that qualitatively there was no difference in the nature of the cell infiltrate in the retina and choroid, which was composed of T cells and macrophages, distributed as individual cells or small cell aggregates (granulomas). However, quantitatively there was a significant reduction in CD3+T cells (Fig. 4a; $p<0.05$) and a similar but non-significant reduction in F4/80+ macrophages (Fig. 4b; $p=0.093$) in the GF mice compared to the controls. Immunohistology was also performed on eyes of mice treated with metronidazole and ciprofloxacin from one week before or on the day of EAU induction (see Methods; Fig. 4c,d,e,f). Our data show that there was no significant qualitative or quantitative difference in the numbers and distribution of CD3+ T cells (Fig. 4c,e) or F4/80+ macrophages (Figure 4d,f), when compared to controls.

3.4 Flow cytometry of the cervical and mesenteric lymph nodes

Since in CFA immunized mice, T cell activation occurs extra-ocularly with clonal expansion in the skin draining lymph nodes beginning as early as 6 days post-immunization [32] and subsequently in the eye-draining nodes as disease develops, we evaluated the phenotypes of lymph node cells by flow cytometry. In eye-draining cervical lymph nodes of CV mice, we observed an expansion of IFN-γ-producing ($p<0.01$) and IL-17-producing CD4$^+$ T cells ($p<0.01$) and a reduced percentage of Foxp3$^+$ Tregs ($p<0.01$) in CV mice at day 35 post-immunization with CFA and IRBP. However, the observed T cell expansion was considerably reduced in GF mice, which were similarly immunized. Interestingly, the percentage of CD4$^+$TNF-α$^+$ T cells was similar in both CV and GF mice. Cell populations in non-eye draining mesenteric lymph nodes showed a small increase in the percentage of IFN-γ-producing CD4$^+$ T cells in CV mice ($p<0.05$) which was significantly greater than IFN-γ+ T cells in GF mice but there was no difference in IL-17 producing T cells in the mesenteric lymph nodes (Fig. 5).

4. Discussion

The gut microbiota plays a significant role in the development of many inflammatory diseases, both in the gut [33, 34] and in distant organs [10, 35-38]. Continuous host-microbiota interactions determine the type and robustness of mucosal immune responses [39].

This is particularly important during the early stages of development, when the presence of microbiota is crucial for the maturation of the immune system in adult life [40]. As mentioned in the Introduction, the presence of microbiota usually enhances inflammation in most animal models of colitis, multiple sclerosis, arthritis or ankylosing spondylitis [13, 14, 41, 42], but it decreases the inflammation in models of type 1 diabetes [17].

Here, we show that the severity of the ocular inflammation in a murine model of autoimmune uveoretinitis is significantly lowered if the bacterial load is reduced either by rearing the mice in GF conditions (Fig. 1), or by prophylactic treatment with broad-spectrum antibiotics (metronidazole and ciprofloxacin; Fig. 2). Similar results were recently reported in the IRBP-transgenic mouse model of spontaneous uveitis [20]. However, the mode of uveitis pathogenesis in this model is dependent on a high peripheral precursor frequency of antigen specific T cells (around 20% antigen specific T cells are required in the periphery for expression of disease) which is far in excess of antigen specific T cell precursor frequency in normal mice and humans [21]. Our data now show that in the standard model of EAU induced by IRBP in CFA, which more closely resembles human uveitis [43], GF mice have markedly reduced but not completely suppressed disease. We also show as has been reported previously [18, 19] that reducing the bacterial load by administration of broad-spectrum antibiotics, EAU is significantly reduced in severity. Importantly, in our study, when antibiotics were administered from the time of EAU induction, there was no significant effect on EAU severity. These experiments suggest that the pre-existing microenvironment, particularly of the gut, has a significant role in determining the level of susceptibility to EAU and in addition that broad spectrum antibiotic treatment may at least partially modify this environment, although not as effectively as in gem free mice. This might be relevant for human medicine as treatment of uveitis patients with antibiotics in the past has not been limited only to infectious forms of uveitis [44]. The timing of the antibiotic treatment in patients, however, was always initiated after the development of immune response. In addition, some antibiotics, such as metronidazole, are known to suppress certain aspects of cell-mediated immunity, even when administered orally [45]. These experiments do not exclude the role of microbiota in the establishment of the immune response, since commensal microbes can produce molecules that regulate the immune system and these microbes may also be influenced by oral antibiotics [45-47]. In our experiments, when average water consumption and average mouse weight is calculated, the exposure to metronidazole was approx. 65 mg/kg for either gender of mice. Although, this amount may have some minor suppressive effect on cellular immunity, the resistance of GF mice to EAU and the sensitivity of CV mice treated from the day of EAU

induction suggest that it is more the effect on the microbiota than the immunosuppressive effect of metronidazole.

The surface of the eye, as for all mucosal surfaces, is colonized by microbes [48], and it is possible that the conjunctival microbiome may influence the development of ocular inflammation. However, such an event is unlikely unless there is a breach of the ocular surface barrier and any effect would likely be mediated through breakdown of the blood ocular barrier, in a similar manner that must apply to the gut microbiome. In this context, Horai et al. [20] have suggested that IL-17 producing antigen-specific T cells in the gut lamina propria are activated through their T cell receptor via non-cognate microbial antigen derived from commensal bacteria. These activated cells then have the possibility to cross the blood retinal barrier and induce disease. In our experiments, T cells are a prominent infiltrating subset in the retina/choroid and both IFN-γ-producing (Th1) and IL-17-producing T cells are found in the eye-draining lymph node. However only a small induction of Th1 and no induction of Th17 cells was observed in the mesenteric lymph nodes draining the gut (Fig. 5). In contrast, we have previously shown that T cell activation by CFA during EAU induction is mediated via dectin-1 [22], a known activator of IL-17 producing T cells and that both Th1 and Th17 cells are involved. In addition, other innate receptors activated by mycobacterial proteins such as Mincle have been identified all of which appear to act via the CARD9 signaling complex [49]. We therefore propose, in the CFA model of EAU described here, that antigen specific Th1 and Th17 cells are activated locally in the skin-draining lymph node from the immunization site, which then traffic through many tissues and sites including the gut, where the gut microbiome amplifies their activation status in a bystander fashion, rather than by inducing antigen-specific T cells via commensal microbial antigen. Inflammation is a strictly compartmentalized process, although there is often some systemic reflection of this event. Therefore, we compared T cells in the eye, the local (cervical) and the distant (mesenteric) lymph nodes between GF and CV mice 35 days after the EAU induction. Using immunohistochemistry, we found that GF mice have significantly less CD3+ T cells and a similar but not significant reduction in F4/80+ macrophages in their eyes when compared with CV mice (Fig. 4). In both groups, the localization of cells was similar, with T cells either clustered in granulomas or scattered in inner and outer retinal layers, and macrophages located in the inner retinal layers. These data suggest that the lower eye infiltration with T cells in GF mice is the consequence, and not the cause of the reduced level of EAU. The higher number or regulatory T cells in the cervical lymph nodes of GF mice, but

not in mesenteric lymph nodes, also suggests that these cells are attracted to the local site of inflammation, and may regulate the local immune response by bystander suppression.

5. Conclusions

In the current study, we show that the absence of microbiota or decrease of bacterial load before induction of inflammation significantly decreases the susceptibility of mice to EAU induced by IRBP in CFA. We show that reduction in the microbial burden induces changes in the strength of the T cell response in GF mice, with reduced T cell infiltration in the retina and also reduced Th1 and Th17-type T cell numbers in the eye-draining lymph node. This effect was not reiterated in antibiotic treated mice suggesting that the reduction in the microbiota in antibiotic-treated mice was incomplete.

We propose that the presence of the microbiota promotes organ specific autoimmunity by amplifying the activation of antigen specific T cells when these cells are induced in the secondary lymphoid organs as would occur in human disease. These results support the notion that the microbiota is important in pathogenesis of auto-antigen induced uveitis and that treatment with antibiotics may constitute an adjunct therapy for sight-threatening uveitis.

Acknowledgements

References

1. M. Blank, O. Barzilai, Y. Shoenfeld, "Molecular mimicry and auto-immunity," *Clinical Reviews in Allergy & Immunology*, vol. 32, no. 1, pp. 111-118, 2007.
2. H. Saiga, Y. Shimada, K. Takeda, "Innate immune effectors in mycobacterial infection," *Clinical and Developmental Immunology*, vol. 2011, pp. 347594, 2011.

3.   W. Van Eden, G. Wick, S. Albani, et al., "Stress, heat shock proteins, and autoimmunity: how immune responses to heat shock proteins are to be used for the control of chronic inflammatory diseases," *Annals of the New York Academy of Sciences*, vol. 1113, pp. 217-237, 2007.

4.   A. Rothova, "Inflammatory cystoid macular edema," *Current Opinion in Ophthalmology*, vol. 18, no. 6, pp. 487-492, 2007.

5.   M. S. Suttorp-Schulten, M. J. Jager, A. Kijlstra, "Recent developments in the treatment of posterior uveitis," *Ocular Immunology and Inflammation*, vol. 4, no. 4, pp. 207-217, 1996.

6.   S. Pawate, S. Sriram, "The role of infections in the pathogenesis and course of multiple sclerosis," *Annals of Indian Academy of Neurology*, vol. 13, no. 2, pp. 80-86, 2010.

7.   R. R. Caspi, F. G. Roberge, C. C. Chan, et al., "A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens," *Journal of Immunology*, vol. 140, no. 5, pp. 1490-1495, 1988.

8.   A. Biswas, J. Wilmanski, H. Forsman, et al., "Negative regulation of Toll-like receptor signaling plays an essential role in homeostasis of the intestine.," *European Journal of Immunology*, vol. 41, no. 1, pp. 182-194, 2011.

9.   T. Hrncir, R. Stepankova, H. Kozakova, et al., "Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice," *BMC Immunology*, vol. 9, pp. 65, 2008.

10.   H. Tlaskalová-Hogenová, R. Stěpánková, H. Kozáková, et al., "The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases," *Cellular & Molecular Immunology*, vol. 8, no. 2, pp. 110-120, 2011.

11.   K. Berer, M. Mues, M. Koutrolos, et al., "Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination," *Nature*, vol. 479, no. 7374, pp. 538-541, 2011.

12.   W. S. Garrett, C. A. Gallini, T. Yatsunenko, et al., "Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis," *Cell Host & Microbe*, vol. 8, no. 3, pp. 292-300, 2010.

13. H. J. Wu, I. I. Ivanov, J. Darce, et al., "Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells," *Immunity*, vol. 32, no. 6, pp. 815-827, 2010.

14. Y. K. Lee, J. S. Menezes, Y. Umesaki, et al., "Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis," *Proceedings of the National Academy of Sciences of the USA*, vol. 108 Suppl 1, pp. 4615-4622, 2011.

15. E. Denou, K. Lolmède, L. Garidou, et al., "Defective NOD2 peptidoglycan sensing promotes diet-induced inflammation, dysbiosis, and insulin resistance," *EMBO Molecular Medicine*, vol. 7, no. 3, pp. 259-274, 2015.

16. J. G. Markle, D. N. Frank, S. Mortin-Toth, et al., "Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity," *Science*, vol. 339, no. 6123, pp. 1084-1088, 2013.

17. L. Wen, R. E. Ley, P. Y. Volchkov, et al., "Innate immunity and intestinal microbiota in the development of Type 1 diabetes," *Nature*, vol. 455, no. 7216, pp. 1109-1113, 2008.

18. R. R. Caspi, R. Horai, C. Zárate-Bladés, et al., "Activation of Autoreactive T Cells by Endogenous Commensal Microflora Provokes Spontaneous Autoimmunity in the Immunologically Privileged Eye," *Investigative Ophthalmology & Visual Science*, vol. 55, no. 13, pp. 4600-4600, 2014.

19. Y. Nakamura, C. Metea, H. Gruner, et al., "Altering the gut microbiota ameliorates experimental autoimmune uveitis," *Investigative Ophthalmology & Visual Science*, vol. 55, no. 13, pp. 2497-2497, 2014.

20. R. Horai, C. R. Zárate-Bladés, P. Dillenburg-Pilla, et al., "Microbiota-Dependent Activation of an Autoreactive T Cell Receptor Provokes Autoimmunity in an Immunologically Privileged Site," *Immunity*, vol. 43, no. 2, pp. 343-353, 2015.

21. N. J. Tubo, A. J. Pagan, J. J. Taylor, et al., "Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection," *Cell*, vol. 153, no. 4, pp. 785-796, 2013.

22. S. Stoppelkamp, D. M. Reid, J. Yeoh, et al., "Murine pattern recognition receptor dectin-1 is essential in the development of experimental autoimmune uveoretinitis," *Molecular Immunology*, vol. 67, no. 2 Pt B, pp. 398-406, 2015.

23. D. Avichezer, P. B. Silver, C. C. Chan, et al., "Identification of a new epitope of human IRBP that induces autoimmune uveoretinitis in mice of the H-2b haplotype," *Investigative Ophthalmology & Visual Science*, vol. 41, no. 1, pp. 127-131, 2000.

24.   C. Broderick, R. M. Hoek, J. V. Forrester, et al., "Constitutive retinal CD200 expression regulates resident microglia and activation state of inflammatory cells during experimental autoimmune uveoretinitis," *American Journal of Pathology*, vol. 161, no. 5, pp. 1669-1677, 2002.

25.   K. Klimesova, M. Kverka, Z. Zakostelska, et al., "Altered gut microbiota promotes colitis-associated cancer in IL-1 receptor-associated kinase M-deficient mice," *Inflammatory Bowel Diseases*, vol. 19, no. 6, pp. 1266-1277, 2013.

26.   M. Paques, J. L. Guyomard, M. Simonutti, et al., "Panretinal, high-resolution color photography of the mouse fundus," *Investigative Ophthalmology & Visual Science*, vol. 48, no. 6, pp. 2769-2774, 2007.

27.   D. A. Copland, M. S. Wertheim, W. J. Armitage, et al., "The clinical time-course of experimental autoimmune uveoretinitis using topical endoscopic fundal imaging with histologic and cellular infiltrate correlation," *Investigative Ophthalmology & Visual Science*, vol. 49, no. 12, pp. 5458-5465, 2008.

28.   H. Xu, P. Koch, M. Chen, et al., "A clinical grading system for retinal inflammation in the chronic model of experimental autoimmune uveoretinitis using digital fundus images," *Experimental Eye Research*, vol. 87, no. 4, pp. 319-326, 2008.

29.   S. R. Thurau, C. C. Chan, R. B. Nussenblatt, et al., "Oral tolerance in a murine model of relapsing experimental autoimmune uveoretinitis (EAU): induction of protective tolerance in primed animals," *Clinical & Experimental Immunology*, vol. 109, no. 2, pp. 370-376, 1997.

30.   A. D. Dick, Y. F. Cheng, J. Liversidge, et al., "Immunomodulation of experimental autoimmune uveoretinitis: a model of tolerance induction with retinal antigens," *Eye (Lond)*, vol. 8 (Pt 1), pp. 52-59, 1994.

31.   M. Jones, J. L. Cordell, A. D. Beyers, et al., "Detection of T and B cells in many animal species using cross-reactive anti-peptide antibodies," *Journal of Immunology*, vol. 150, no. 12, pp. 5429-5435, 1993.

32.   H. R. Jiang, L. Hwenda, K. Makinen, et al., "Sialoadhesin promotes the inflammatory response in experimental autoimmune uveoretinitis," *Journal of Immunology*, vol. 177, no. 4, pp. 2258-2264, 2006.

33.   D. Pagliari, C. A. Piccirillo, A. Larbi, et al., "The Interactions between Innate Immunity and Microbiota in Gastrointestinal Diseases," *Journal of Immunology Research*, vol. 2015, pp. 898297, 2015.

34. D. Pagliari, R. Urgesi, S. Frosali, et al., "The Interaction among Microbiota, Immunity, and Genetic and Dietary Factors Is the Condicio Sine Qua Non Celiac Disease Can Develop," *Journal of Immunology Research*, vol. 2015, pp. 123653, 2015.

35. M. Kverka, H. Tlaskalova-Hogenova, "Two faces of microbiota in inflammatory and autoimmune diseases: triggers and drugs," *APMIS*, vol. 121, no. 5, pp. 403-421, 2013.

36. R. Bonamichi-Santos, M. V. Aun, R. C. Agondi, et al., "Microbiome and Asthma: What Have Experimental Models Already Taught Us?," *Journal of Immunology Research*, vol. 2015, pp. 614758, 2015.

37. M. H. Nelson, M. A. Diven, L. W. Huff, et al., "Harnessing the Microbiome to Enhance Cancer Immunotherapy," *Journal of Immunology Research*, vol. 2015, pp. 368736, 2015.

38. G. Giorgetti, G. Brandimarte, F. Fabiocchi, et al., "Interactions between Innate Immunity, Microbiota, and Probiotics," *Journal of Immunology Research*, vol. 2015, pp. 501361, 2015.

39. K. Honda, D. R. Littman, "The Microbiome in Infectious Disease and Inflammation," *Annual Review of Immunology*, vol. 30, no. 1, pp. 759-795, 2012.

40. C. H. Hansen, D. S. Nielsen, M. Kverka, et al., "Patterns of early gut colonization shape future immune responses of the host," *PLoS One*, vol. 7, no. 3, pp. e34043, 2012.

41. T. Hudcovic, R. Stěpánková, J. Cebra, et al., "The role of microflora in the development of intestinal inflammation: acute and chronic colitis induced by dextran sulfate in germ-free and conventionally reared immunocompetent and immunodeficient mice," *Folia Microbiologica (Praha)*, vol. 46, no. 6, pp. 565-572, 2001.

42. Z. Reháková, J. Capková, R. Stěpánková, et al., "Germ-free mice do not develop ankylosing enthesopathy, a spontaneous joint disease," *Human Immunology*, vol. 61, no. 6, pp. 555-558, 2000.

43. J. V. Forrester, H. Xu, L. Kuffová, et al., "Dendritic cell physiology and function in the eye," *Immunological Reviews*, vol. 234, no. 1, pp. 282-304, 2010.

44. J. Preisova, "[Treatment of sympathetic ophthalmia with penicillin]," *Ceska Oftalmologie*, vol. 8, no. 3, pp. 151-157, 1952.

45. D. I. Grove, A. A. Mahmound, K. S. Warren, "Suppression of cell-mediated immunity by metronidazole," *International Archives of Allergy and Applied Immunology*, vol. 54, no. 5, pp. 422-427, 1977.

46. Y. Furusawa, Y. Obata, S. Fukuda, et al., "Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells," *Nature*, vol. 504, no. 7480, pp. 446-450, 2013.

47. R. Martín, F. Chain, S. Miquel, et al., "The commensal bacterium Faecalibacterium prausnitzii is protective in DNBS-induced chronic moderate and severe colitis models," *Inflammatory Bowel Diseases*, vol. 20, no. 3, pp. 417-430, 2014.

48. Q. Dong, J. M. Brulc, A. Iovieno, et al., "Diversity of bacteria at healthy human conjunctiva," *Investigative Ophthalmology & Visual Science*, vol. 52, no. 8, pp. 5408-5413, 2011.

49. E. J. Lee, B. R Brown., E. E. Vance, et al., "Mincle Activation and the Syk/Card9 Signaling Axis Are Central to the Development of Autoimmune Disease of the Eye," *Journal of Immunology*, vol. 196, no. 7, pp. 3148-3158, 2016.

Figure 1: Severity of ocular inflammation in germ-free (GF) and conventional (CV) mice 35 days post EAU induction.

(a) Representative photographs of retinal fundus of GF (grade 0) and CV (grade 2) mouse. The figure show small and linear lesions (arrow), minimal optic disc inflammation and moderate vascular cuffing (arrowheads).

(b) Quantification of the clinical EAU score is displayed below. By clinical fundoscopy, no inflammation was observed in the GF mice (3 animals) at day 35 post induction, whereas in control CV mice (6 animals), severe inflammation was observed. The red line in the graphs represent mean. *** $p < 0.001$ (Mann-Whitney test).

(c) Representative microphotographs of hematoxylin and eosin-stained retina of GF and CV mouse. The figure shows a large infiltrate (star) located in inner retinal layer, mild vitritis (arrow) and small retinal folds (arrowhead).

(d) Quantification of histological EAU score is displayed below. On histological evaluation, minimal to no signs of uveitis were observed in GF mice (6 animals) compared to severe uveitis in CV mice (6 animals). The red line in the graphs represent mean. *** $p < 0.001$ (Mann-Whitney test).
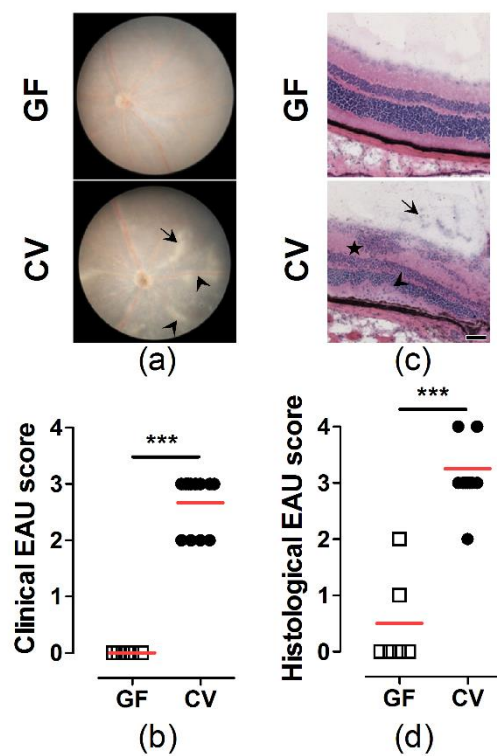
Figure 2: Reduced severity of EAU in mice treated with antibiotics (ATB) from one week before EAU induction (15 animals). Mice treated with metronidazole and ciprofloxacin commencing one week prior to EAU induction and continued for the course of the experiment had significantly lower levels of EAU compared to controls (15 animals) both clinically and histologically.

(a) Representative photographs of retinal fundus at day 14, 21, 28 and day 35 post EAU induction show the development of ocular pathology in ATB and control mice.

(b) Quantification of clinical EAU score is displayed below. The red lines in the graphs represent mean. * $p<0.05$ (Mann-Whitney test).

(c) Representative microphotographs of hematoxylin and eosin-stained retina of ATB-treated and control mice at day 35 post induction. Fewer signs of inflammation are present in ATB-treated compared to control mice, including cells in the vitreous (arrows) and small retinal folds (arrowheads), retinal neovascularization (black star) and vasculitis (white star).

(d) Quantification of histological EAU score is shown. The red lines in the graphs represent mean. * $p<0.05$ (Mann-Whitney test).
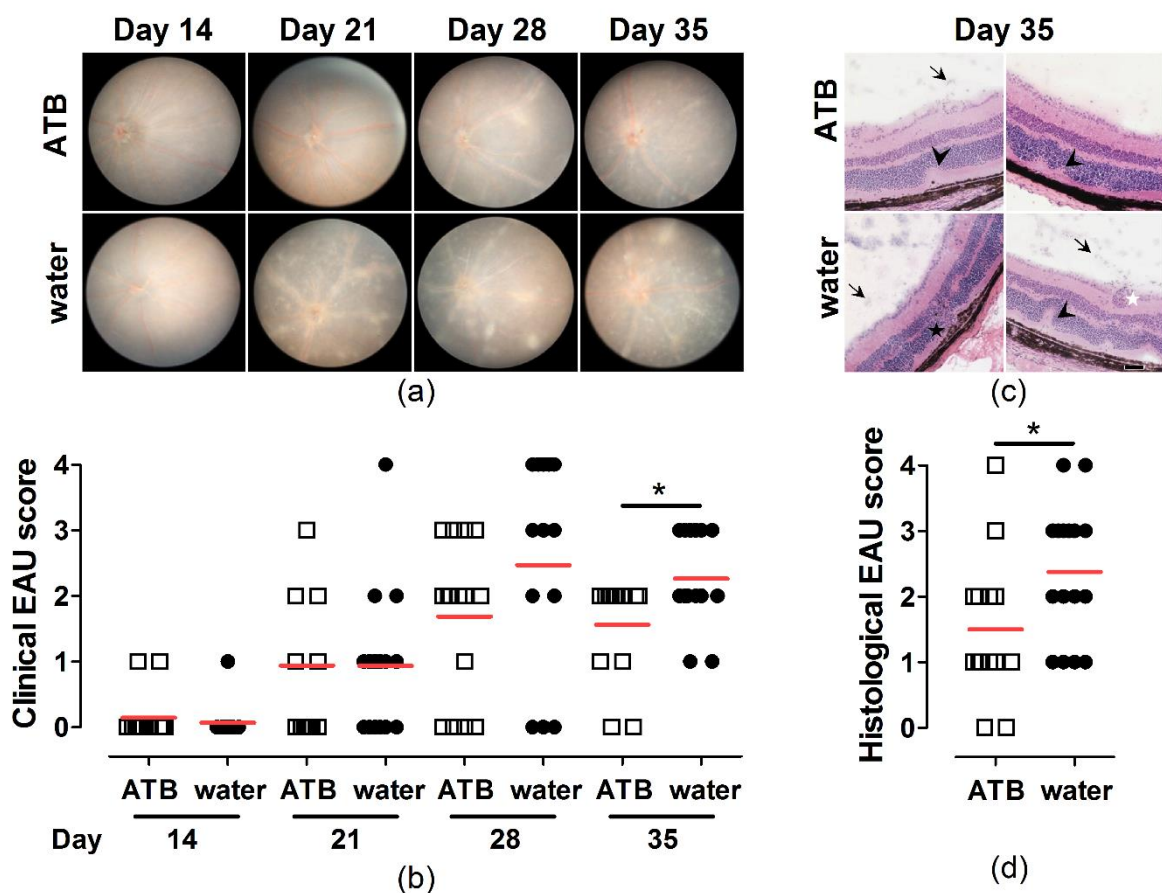
Figure 3: Antibiotics (ATB) administered from the day of EAU induction do not reduce the EAU severity.

Quantification of (a) clinical and (b) histological EAU score at the day 35 post-induction is shown. The clinical data are from one of several independent experiments (ATB 11 mice, water 15 mice). The red lines in the graphs represent mean.
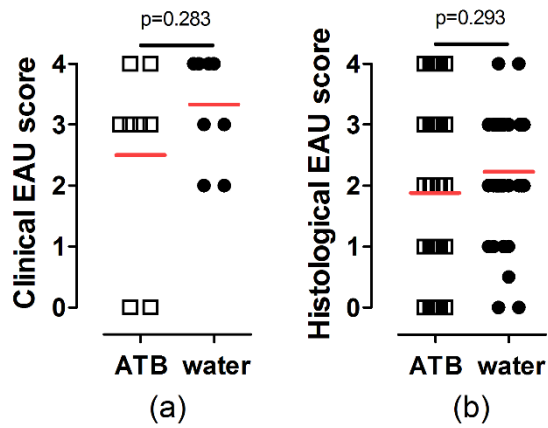
Figure 4: T cell and macrophage infiltration of the retina from germ-free (GF) mice (a,b), mice treated with antibiotics (ATB) administered from one week (c,d) or from the day of EAU induction (e,f) and conventional (CV, water) mice 35 days post EAU induction.

(a,c,e) CD3$^+$ cells (T lymphocytes) are shown both distributed as single cells in inner and outer retinal layers (arrows), in the vitreous (arrowheads) and concentrated as clumps in granulomas (stars).

(b,d,f) F4/80$^+$ cells (macrophages) are either present as single cells, in inner retinal layers (arrows), or accumulated in the periphery of granulomas (stars).

Each point in the graphs below shows the sum of all positive cells counted in two sections, one from periphery and one from the center, from one randomly selected eye. The red lines in the graphs represent mean. * $p<0.05$ (Mann-Whitney test).
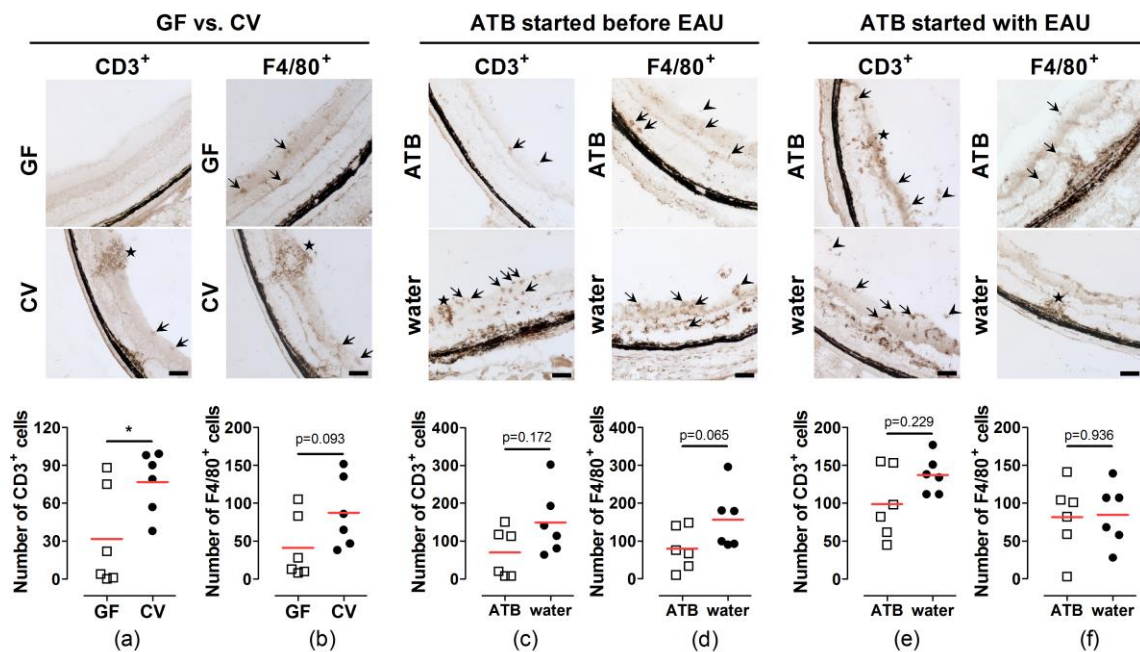
Figure 5: Flow cytometric analysis of lymphocyte populations in cervical and mesenteric lymph nodes.

In the cervical lymph nodes of conventional (CV) mice, (a) the percentage of IFN-γ and IL-17-producing CD4+ T cells increased and (c) the percentage of regulatory Foxp3-expressing CD4+ T cells decreased compared to germ free (GF) mice. In the mesenteric lymph nodes of CV mice, the environment was less pro-inflammatory showing only a small but significant increase of IFN-g-producing CD4+ T cells compared to GF mice. In both cervical and mesenteric lymph nodes, (b) the percentage of TNF-a-producing CD4+ T cells remained unchanged. The dot plots are representative of two independent experiments.

The column graphs summarize the frequency of lymphocyte subpopulations in (d) the cervical and (e) the mesenteric lymph nodes. Each graph represents data from two independent experiments. * p<0.05, ** p<0.01 (Mann-Whitney test).
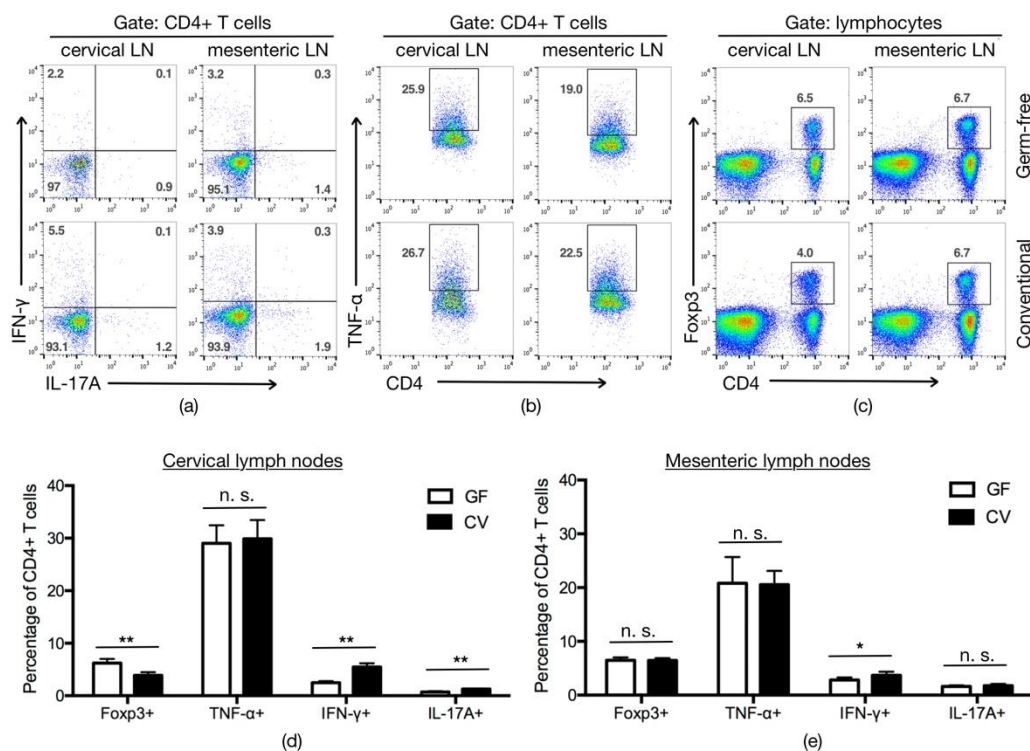
Table 1: Clinical evaluation of retinal changes during EAU

Features	Grade 1	Grade 2	Grade 3	Grade 4
Retinal Tissue Infiltrates	1-4 small lesions or 1 linear lesion	5-10 small lesions or 2-3 linear lesions	≥10 small lesions or ≥3 linear lesions	linear lesions confluent
Optic Disc	minimal inflammation	mild inflammation	moderate inflammation	severe inflammation
Retinal Vessels	engorged vessels with no perivascular cuffing	engorged vessels and 1-4 mild cuffing	≥4 mild cuffings or 1-3 moderate cuffings	≥3 moderate cuffings or ≥1 severe cuffing

Table 2: Histopathological scoring system

Features	Grade 0,5	Grade 1	Grade 2	Grade 3	Grade 4
Non-granulomatous Infiltrate	small in the ciliary body/retina/choroid	-	cells in AC	-	subretinal exudate
Vasculitis	-	occ./mild	≥2 vessels	≥10-50%	≥50%
Vitritis	-	mild	mild/moderate	marked	severe
Retinal Folds	-	occ.	2	≥3	extensive or detachment
Granulomas	-	-	1-2	≥3	≥3
Photoreceptor Loss	-	-	mild/moderate	severe (≥60%)	severe (≥60%)