



Beneficial effect of dimethyl fumarate on experimental autoimmune uveitis is dependent of pro-inflammatory markers immunomodulation

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Abstract

Autoimmune uveitis is an inflammatory disease of the eye and is one of the major causes of blindness worldwide. Experimental autoimmune uveoretinitis (EAU) constitutes an animal disease model of human endogenous uveitis. In our study, we investigated the immunomodulatory effect of dimethyl fumarate (DMF) using bovine retinal extract-induced uveitis in a Female *Wistar* rats. To evaluate the *in vivo* efficacy, Female *Wistar* rats were divided into seven experimental groups: control group ($n=5$), consisting of non-immunized animals; Uveoretinitis ($n=5$), and DMF/Uveoretinitis groups ($n=15$), which received a subcutaneous injection of bovine retinal extract emulsified in complete Freund's adjuvant; MC group ($n=5$), treated by daily intragastric administration of methylcellulose 0.08% in tap water; DMF group, consisting of control positive group, rats received daily oral gavage administration of 500 μ L of dimethyl fumarate at 100 mg/Kg dissolved in 0.08% methylcellulose in tap water ($n=5$). On day 14 post immunization, the rats were then euthanized and associated indications were investigated to evaluate the therapeutic efficacy. Nitric oxide (NO) and TNF- α were assessed in plasma. Meanwhile, eyes were collected for histological and immunohistochemical studies. The retinal expression of iNOS, CD68, CD20, CD25, CD4, and CD8 was examined. Interestingly, DMF enhanced a significant reduction of NO and TNF- α production in the treated group. This effect was strongly related to the histological structure of eyes improvement. In the same context, a significant decrease of iNOS, CD68, and CD20 expression and CD25 increase expression were reported in retinal tissue of DMF/Uveoretinitis group in comparison to the immunized group. Collectively, our results indicate that DMF treatment has a beneficial effect in experimental autoimmune uveoretinitis and could constitute a good candidate for monitoring an ocular inflammatory diseases.

Keywords Experimental autoimmune uveoretinitis · Dimethyl fumarate · Nitric oxide · Proinflammatory markers

Introduction

Uveitis is a general term used for the inflammation of the uveal tissue (iris, ciliary body, and choroid). Anatomically it has been classified as anterior, intermediate and posterior or as panuveitis. Noninfectious uveitis is believed to be autoimmune or immune-mediated (Gery et al. 2002). Patients with autoimmune uveitis frequently showed the presence of immune responses against retinal soluble antigen (AgS), inter-photoreceptor retinoid-binding protein (IRBP), and recoverin (Bansal et al. 2015).

The delicate nature of ocular tissues makes it difficult to obtain tissue specimens in the study of the pathogenicity of uveitis diseases. However, over the past several decades, animal models of autoimmune uveitis have been developed (Bansal et al. 2015; Agarwal and Caspi 2004).

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Experimental autoimmune uveoretinitis (EAU) is T cell-mediated autoimmune disease that targets the neural retina and related tissues (Lee et al. 2014). It shares many common features in clinical and histological aspects with human uveitis (Agarwal et al. 2012). EAU is a Th1-dominant response. It was shown that the up-regulation of the Th2 cytokines production or down-regulation of the Th1 responses prevents inflammatory responses and protects against the EAU development (Caspi 2002; Nikoopour et al. 2019). In addition, many authors have reported the involvement of nitric oxide (NO) in mechanisms of pathogenesis of experimental autoimmune uveitis induced by IRBP, AgS and tropomyosin (Djeraba et al. 2010; Arroul-Lammali et al. 2012; Touri et al. 2019).

Currently, the treatment of uveitis is based on the use of corticosteroids and immunosuppressive agents. However, long-term application of these drugs frequently may have several intraocular and systemic side effects (Jonas et al. 2003; Moshfeghi et al. 2003). Furthermore, there are still a number of patients who do not respond to immunosuppressive treatment (Tian et al. 2011).

Oral dimethyl fumarate (DMF) is approved since 2014 in Europe for the treatment of multiple sclerosis based on a wide spectrum of immunomodulatory and neuroprotective effects with a favourable safety profile (Gold et al. 2012, b; Fox et al. 2012). DMF is rapidly hydrolyzed by esterases to its metabolite, monomethyl fumarate (MMF), after absorption in the small intestine (Litjens et al. 2004).

DMF exerts an immunomodulatory and a neuroprotective effects in the experimental animal models of acute and chronic autoimmune, demyelinating diseases of the peripheral nervous system, and in the autoimmune neuritis (Kiefer et al. 2001; Pitarokoili et al. 2019). Studies in experimental autoimmune encephalomyelitis, the mouse model for multiple sclerosis, and the experimental autoimmune neuritis, revealed a neuroprotective mechanism of action through the activation of the transcription factor Nrf2 (nuclear factor (erythroid derived 2)-related factor 2) via MMF (Stoof et al. 2001).

In our current study, we investigated the ability of oral treatment with DMF to suppress EAU induced in rat by immunization with bovine retinal extract. The experiment was conducted to evaluate the immunomodulatory properties of DMF. In this sense, we analysed the systemic levels of NO and TNF- α secretion during the experimental autoimmune uveoretinitis. Histological changes and the expression of iNOS, CD68, CD20, CD25, CD4 and CD8 levels in retinal tissue from all experimental groups were assessed by semi-quantitative analysis using FIJI image processing software.

Materials and methods

Animals

A total of 35 female *Wistar* rats (4–6 weeks old) were purchased from the Pasteur Institute (Algiers, Algeria). These rats were acclimated for 1 week before the start of experiments and kept under normal conditions with a 12-h dark/light cycle with ad libitum access to food and water.

Disease induction and in vivo treatment with dimethyl fumarate

Animals ranging in weight from 160 to 180 g were divided into seven groups ($n = 5$ rat per group) as per Fig. 1. The control group (Ctrl) received no treatment. The Uveoretinitis and DMF/Uveoretinitis groups received a subcutaneous injection of bovine retinal extract emulsified in complete Freund's adjuvant. The DMF (25 mg/Kg)/Uveoretinitis, DMF (45 mg/Kg)/Uveoretinitis, and DMF (100 mg/Kg)/Uveoretinitis groups treated by daily oral gavage administration of 500 μ L of dimethyl fumarate (Biogen Idec, Cambridge, USA) at 25, 45 and 100 mg/Kg, respectively, dissolved in 0.08% methylcellulose in tap water starting after 2 days of immunization to day 12 p.i. The MC group received 500 μ L of methylcellulose 0.08% orally twice daily. The DMF group treated by daily oral gavage administration of 500 μ L of dimethyl fumarate at 100 mg/Kg dissolved in 0.08% methylcellulose in tap water. All our experiments were approved by Ethics Committee of the Thematic Research Agency in Health Sciences (N°58/DFPR/ATRSS).

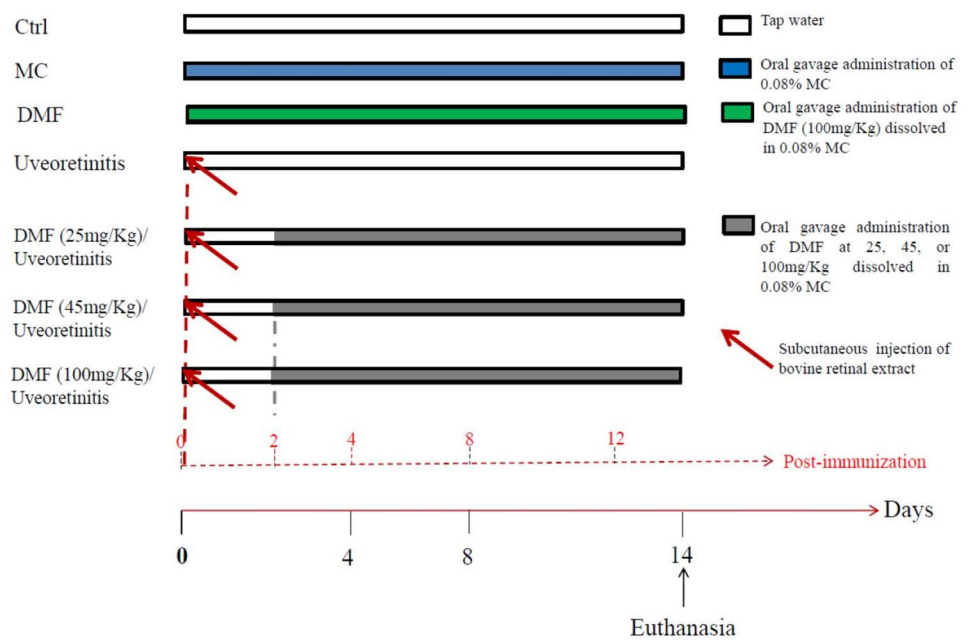
All animals were euthanized 14 days p.i. using inhalation of anesthesia gas.

Plasma collection

Wistar rats were anesthetized, and blood was collected via cardiac puncture. Blood was centrifuged to isolate plasma, which was kept at -20°C until use.

Nitrite measurement

The Griess reaction was used to determine nitrite levels in plasma as an indicator of NO production, as described previously (Touil-Boukoffa et al. 1998). Briefly, 100 mL of each sample was mixed with 50 mL of Griess reagent (5% sulfanilamide, 0.5% naphthylethylenediamine dihydrochloride, 20% HCl). Samples were incubated at room temperature for 20 min and the absorbance read at 543 nm by spectrophotometer. The nitrite concentration was determined

Fig. 1 Experimental plan

using a standard curve constructed with sodium nitrite [NaNO₂; (0–200) mmol/L].

Measurement of systemic TNF- α level

The systemic level of TNF- α was determined in the plasma of rats using commercial enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions (Invitrogen, Camarillo, USA).

Histopathological analysis

Eyes were enucleated on day 14 following bovine retinal extract immunization and rinsed with phosphate-buffered saline (PBS), then fixed in 10% phosphate-buffered formaldehyde. Tissues were embedded in paraffin. Serial 5 μ m sections were cut through the papillary-optic nerve axis and stained by haematoxylin and eosin. At least four sections of each eye cut at different levels were prepared and evaluated histologically. The intensity of EAU was graded in a masked fashion on a scale of 0–4, as previously described (Caspi et al. 1988). In brief, focal nongranulomatous, monocytic infiltrations in the choroids, ciliary body, and retina were scored as 0.5. Retinal perivascular infiltration and monocytic infiltration in the vitreous were scored as 1. Granuloma formation in the uvea and retina, the presence of occluded retinal vasculitis, along with photoreceptor folds, serous detachment, and loss of photoreceptor were graded as 2. In addition, the formation of granuloma at the level of the retinal pigmented epithelium and the development of sub-retinal neovascularization were scored as 3 and 4, according to the number and the size of the lesions. Histopathological

diagnoses were performed in double blinded fashion by anatomopathologists.

Images were captured from each slide with a digital camera (Casio) on a light microscope (Motic) with 40 \times objectives.

Immunohistochemical procedures

The technical procedures for immunohistochemical study of iNOS, CD68, CD20, CD25, CD4 and CD8 expression included, in succession, the steps previously described (Labsi et al. 2016). The adopted protocol for immunohistochemical exam was adapted to local laboratory conditions (Labsi et al. 2016; 2018; 2019). Slides were submitted to immunohistochemistry for incubation with rabbit monoclonal antibodies against mouse CD68, CD20, CD25, CD4, CD8, and iNOS (Santa Cruz Biotechnology, Dallas, TX, USA; diluted 1:500). Indirect immunoperoxidase staining was performed according to the manufacturer's instructions (DAKO, Denmark A/S). The sections were counterstained with hematoxylin. Slides were covered with Faramount Mounting Medium (S3025, DAKO, Denmark A/S) and observed using a standard microscope (Motic). Pictures were taken using a digital camera (Casio) at 40 \times magnification. The percentage of cells positively stained for iNOS, CD68, CD20, CD25, CD4 and CD8 was scored semiquantitatively. The quantification of positive cells was performed using FIJI image processing software after calibration with a graduated slide. The quantification was carried by two observers. iNOS expression was evaluated on the basis of a four-point scale: '–': negative staining; '+': low expression, less than 10% positive cells; '++': moderate expression, 10–30%

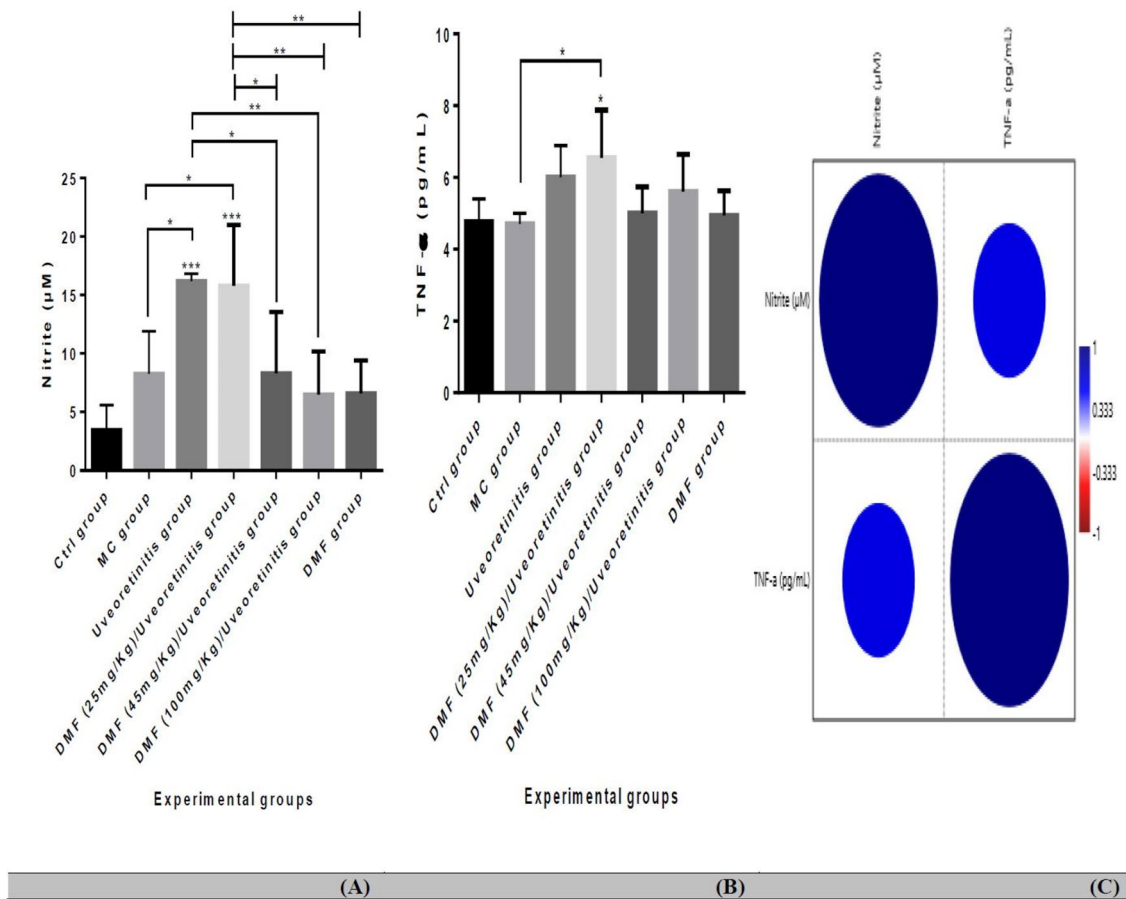


Fig. 2 Oral administration of dimethyl fumarate reduced the systemic production of nitrite (A) and TNF- α (B), with $r=0.61$, $P<0.005$ (C). Data are presented as mean \pm SD. P values from One-way ANOVA test are indicated (* $P<0.05$, ** $P<0.005$, *** $P<0.001$)

positive cells; ‘+++’: intense expression, more than 30% positive cells.

Statistical analysis

An ANOVA or Kruskal–Wallis one-way analysis of variance tests were used to compare the treatment effects between all groups. Probability values of $P\leq 0.05$ were considered to be statistically significant. We have used the statistical software Past version 4.03 and GraphPad Prism 6.

Results

Dimethyl fumarate treatment reduced systemic levels of nitric oxide and TNF- α

We investigated the effect of DMF on NO production in EAU. Nitrite measurement in plasma of rats showed a significant increase in the Uveoretinitis group compared to the Ctrl group (16.2 ± 0.3 vs. 3.4 ± 1.0 μ M, *** $P<0.001$;

Fig. 2A). Oral administration of DMF at doses of 45 and 100 mg/Kg to immunized rats with bovine retinal extract caused a significant decrease in systemic NO level in comparison with the untreated-Uveoretinitis group (8.3 ± 5.2 vs. 16.2 ± 0.6 μ M, * $P<0.05$ and 6.5 ± 3.7 vs. 16.2 ± 0.6 μ M, ** $P<0.005$, respectively; Fig. 2A).

To analyze the effect of DMF on proinflammatory cytokine production in vivo, we measured the level of TNF- α in plasma (Fig. 2B). The systemic TNF- α level was increased in the Uveoretinitis group in comparison with the Ctrl, MC and DMF groups (Fig. 2B). DMF treatment at a dose of 45 mg/Kg attenuates the systemic TNF- α level (5.0 ± 0.3 vs. 6.0 ± 0.4 pg/mL; Fig. 2B). There is an important correlation between the Nitrite and TNF- α systemic production during EAU development ($r=0.61$, $P<0.005$; Fig. 2C).

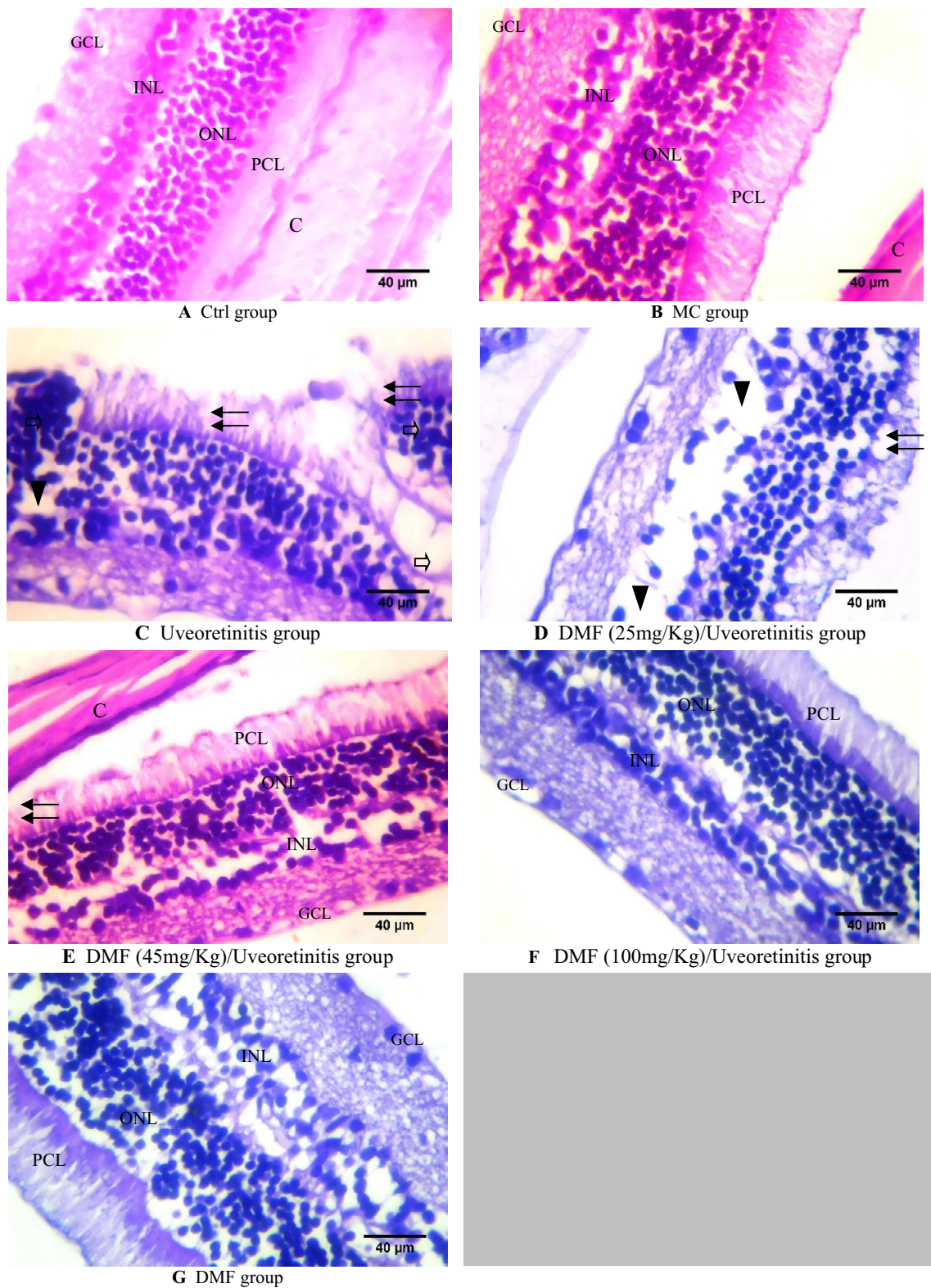


Fig. 3 Representative photomicrographs (×40) of H and E stained rat's retinal tissue. The bovine retina extract-induced morphological changes in the architecture of retina of Wistar rats. square fragmentation and alteration of the ONL; (◻): structural disorganization of the

INL; square destruction of photoreceptors cells layer. *GCL* ganglion cells layer, *INL* inner nuclear layer, *ONL* outer nuclear layer, *PCL* photoreceptors cells layer, *C* choroid

Retina architecture was improved in rats treated with dimethyl fumarate

In our study, histological changes were evaluated using Hematoxylin and eosin staining. Histological analysis of eye isolated from EAU, showed fragmentation and alteration of the outer nuclear layer (ONL), structural disorganization of the inner nuclear layer (INL), and destruction of photoreceptors cells layer (PCL), in retina (Fig. 3C).

Interestingly, the oral administration of DMF at doses of 45 and 100 mg/Kg to rats with autoimmune uveitis caused an improvement in the histological structure in retina sections relative to untreated immunized-rats (Fig. 3E, F). We showed a significant important histology damage score (HAI) in Uveoretinitis and DMF (25 mg/Kg)/Uveoretinitis groups in comparison with the Ctrl and MC groups (Fig. 4). Histopathological evaluation revealed that 25 mg/Kg of DMF is too low and have no effect on bovine retinal extract-induced uveitis (Fig. 3D). Indeed, A decrease in HAI was shown in the DMF (45 mg/Kg)/Uveoretinitis and DMF (100 mg/Kg)/Uveoretinitis groups compared with the Uveoretinitis group [0.5(35.9) vs. 3.0(57.5) and 0.25(33.7) vs. 3.0(57.5), respectively; Fig. 4].

Wistar rats from control, MC and DMF groups exhibited normal retinal tissue (Fig. 3A, B and G).

Anti-inflammatory and immune-modulatory effects of dimethyl fumarate therapy

To evaluate the effect of DMF on inflammatory reaction in our model of autoimmune uveitis, we examined the retinal expression of iNOS, CD68, CD20, CD25, CD4 and CD8.

The expression of iNOS, CD68, CD20, CD25 and CD4 was shown in retina (Fig. 5). We observed a little expression of CD8 in retinal tissue in some cases (Fig. 5). Importantly, the expression of pro-inflammatory markers (iNOS, CD68, CD20) was significantly increased in the Uveoretinitis group compared with the control groups ($P < 0.005$; Table 1). This increase in expression in retina was significantly attenuated by DMF treatment ($P < 0.05$; Table 1, Fig. 5D, I, N). In retinal tissue of the DMF (45 mg/Kg)/Uveoretinitis, Ctrl, MC and DMF groups, there was, strikingly, no significant difference in iNOS, CD68, CD20, CD4 and CD8 expression. In the same way, we observed with interest a significant expression of Treg marker (CD25) in retinal tissue of DMF(45 mg/Kg)/Uveoretinitis group compared with Ctrl and MC groups ($P < 0.005$; Table 1, Fig. 5P, Q, S).

Discussion

Uveitis is comprised of a variety of a heterogeneous group of disorders with uncertain etiology and pathogenesis. Because of the limit in obtaining samples at ocular level, various

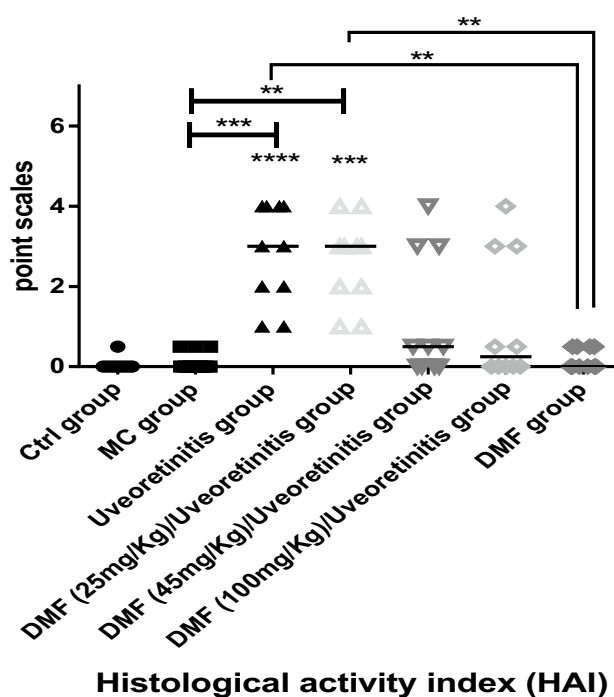


Fig. 4 Improvement of the histological structure by administration of dimethylfumarate in rats immunized with bovine retinal extract. The histological activity index (HAI) was calculated as described in Material and Methods. Data are presented as median (range). P values from Kruskal–Wallis test are indicated (** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$)

EAU animal models have been developed for studying and understanding this pathology. Numerous therapeutic agents have been discovered, which have been put into successful clinical use (Bansal et al. 2015).

Dimethyl fumarate is commonly prescribed as a first-line agent with favourable safety and efficacy profiles. The potential benefits of DMF against other diseases appears pathogenically different (Kourakis et al. 2020). In the study outlined here, we examined the potential anti-inflammatory and immunomodulatory effect of DMF on the experimental model of autoimmune uveitis with regards to systemic levels of NO and TNF- α production, retina histological changes, and local expression of iNOS, CD68, CD20, CD25, CD4 and CD8.

Dimethyl fumarate is a methyl ester of fumaric acid registered for the treatment of relapsing forms of Multiple Sclerosis (Kappos et al. 2008; Gold et al. 2012; Michell-Robinson et al. 2015) and psoriasis (Litjens et al. 2004; Reszke and Szepietowski 2020). DMF influences a variety of cell types and molecules pathways involved in inflammatory mechanisms. Indeed, Wilms et al. (2010) demonstrated that DMF exerts an inhibitory effect on the proinflammatory response characterised by increased microglial and astrocytic synthesis of NO, IL-1, IL-6 and TNF- α in an in vitro model

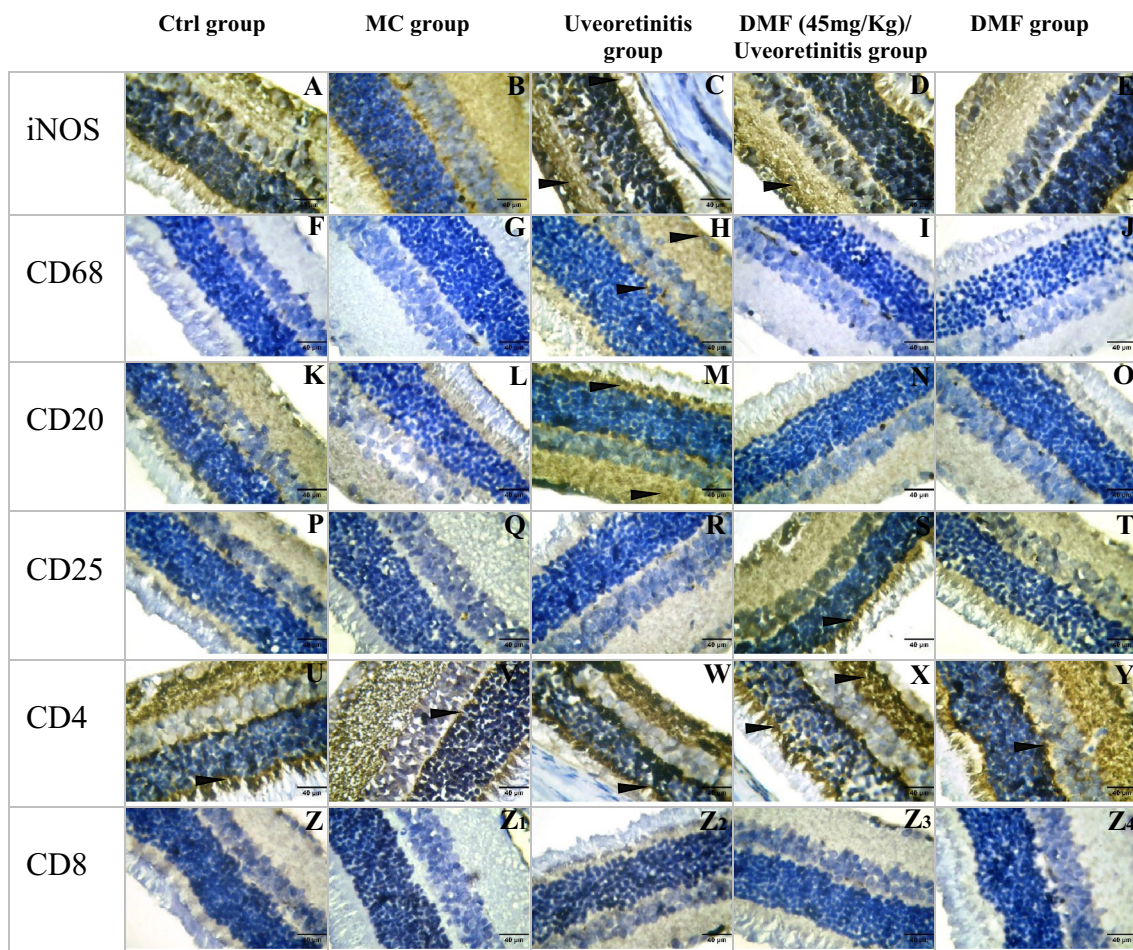


Fig. 5 Representative images ($\times 40$) of immunohistochemical analysis for iNOS, CD68, CD20, CD25, CD4, CD8 expression in retina. **A, B, E** retina sections of Ctrl, MC and DMF groups with low expression of iNOS, scored as “+”. **C** retina section of Uveoretinitis group showed diffuse expression of iNOS, scored as “++”. **D** retina section of DMF (45 mg/Kg)/Uveoretinitis group showed low expression of iNOS, graded as “+”. **F, G, J, K, L, O** retina sections of Ctrl, MC and DMF groups with low expression of CD68 and CD20. **H, M** an intense expression of CD68 and CD20 was observed in Uveoretinitis group. **I, N** Treatment with DMF reduced the intensity of CD68 and

CD20 expression observed in DMF (45 mg/Kg)/Uveoretinitis group. **P, Q, R, T** The expression of CD25 was lower in retina sections of Ctrl, MC, Uveoretinitis and DMF groups. **(S)** Treatment with DMF increased the intensity of CD25 expression observed in DMF (45 mg/Kg)/Uveoretinitis group. **U, V, W, X, Y** an important expression of CD4 was shown in all experimental groups. **Z, Z1, Z4** no expression of CD8 in retina sections of Ctrl, MC and DMF groups. **Z2, Z3** a little expression of CD8 in retina sections of Uveoretinitis and DMF (45 mg/Kg)/Uveoretinitis groups. (\blacktriangleright): Arrows indicate positive staining

of brain inflammation. In our conditions, we reported with interest a significant decrease of systemic NO and TNF- α level after DMF treatment. In the same line, we showed a positive correlation between the NO and the TNF- α systemic production (Fig. 2C).

As previously reported by our team, during EAU activity, a significant proportion of damages in ocular structures was observed and attributed mainly to nitric oxide (Djeraba et al. 2010; Arroul-Lammali et al. 2012; Touri et al. 2019). In addition, TNF- α , has been shown to be an important cytokine involved in alteration of ocular tissue (Dick et al. 2004). The histological structure of retina showed an alteration and disorganization of different layers (Fig. 3C).

Interestingly, the damage observed in retina architecture with significant expression of iNOS, CD68 and CD20, was related to establishment of inflammatory reaction ($P < 0.005$, Table 1, Fig. 5C, H and M). Heiligenhaus et al. (2011) have used an anti-CD20 monoclonal antibody (rituximab) for the treatment of severe uveitis associated with juvenile idiopathic arthritis.

Intragastric administration of DMF in *Wistar* rats caused an amelioration of tissue architecture and an important decrease of inflammatory cells infiltration (CD68, CD20). These observations correlate with down-regulation of iNOS expression and up regulation of the Treg markers (CD4 and

Table 1 Percentages of iNOS, CD68, CD20, CD25, CD4, CD8 expression in retina sections of Ctrl, MC, Uveoretinitis, DMF (45 mg/Kg)/Uveoretinitis, and DMF groups

	Ctrl group	MC group	Uveoretinitis group	DMF (45 mg/Kg)/Uveoretinitis group	DMF group
iNOS	5.9 (34.4)	3.2 (16.0) *	9.6 (40.2) ##	4.6 (21.2) ‡	3.1 (15.6) ‡
CD68	0.01 (19.2)	0.0 (16.2)	3.3 (45.5) *** ####	0.1 (29.5) ns	0.0 (17.1) ‡
CD20	0.0 (17.4)	0.01 (18.3) *	22.1 (45.5) *** ####	0.2 (30.2) ‡	0.0 (16.0) ‡
CD25	0.1 (17.7)	0.1 (14.4)	0.7 (25.1)	2.7 (43.2) ** ### ns	0.37 (26.9)
CD4	14.6 (27.3)	13.4 (16.9)	17.9 (31.4)	16.4 (31.7) ns	13.6 (20.1)
CD8	0.0 (17.8)	0.0 (17.8)	0.2 (38.8) ** ##	0.1 (33.3) ns	0.0 (19.7) *

Data are presented as median (range). *p* values Kruskal–Wallis test are indicated (**P* < 0.05 vs. Ctrl group, ***P* < 0.005 vs. Ctrl group, ****P* < 0.001 vs. Ctrl group, *****P* < 0.0001 vs. Ctrl group, ##*P* < 0.005 vs. MC group, ###*P* < 0.001 vs. MC group, ####*P* < 0.0001 vs. MC group, ns not significant vs. Uveoretinitis group, †*P* < 0.05 vs. Uveoretinitis group, **P* < 0.05 vs. Uveoretinitis group, †*P* < 0.005 vs. Uveoretinitis group, ‡*P* < 0.0001 vs. Uveoretinitis group)

CD25) in DMF/Uveoretinitis group. Pitarokoili et al. (2019) demonstrated that DMF exhibited an immunomodulatory effect by an increase of regulatory T cells (CD4⁺ CD25⁺), exerting a protective role in experimental neuritis.

We noted a little expression of CD8 in Uveoretinitis and DMF/Uveoretinitis groups. Calder et al. (1993) suggest that depleting CD8⁺ cells had no effect on the course of disease and that CD8⁺ cells do not play a crucial role in the immunoregulation of EAU.

Fumarates are potent therapeutic compounds that exert pleiotropic immunomodulatory effects. Multiple pathways have been implicated in mediating these effects, the cytoprotective and anti-oxidant responses through the strong potentiation of the Nrf2 pathway, and the anti-inflammatory effect by inhibition of NF-κβ pathway (Gillard et al. 2015). DMF attenuate colon injury, pro-inflammatory cytokine and adhesion molecule production, and NF-κβ signalling, in experimental rodent models of colitis (Casili et al. 2015). In mouse models of Parkinson's disease, DMF reduced damage and degeneration to preserve neuronal populations by upregulating Nrf2-dependent antioxidant genes and suppressing NF-κβ-mediated inflammation (Lastres-Becker et al. 2016; Campolo et al. 2017). In the same context of inflammatory conditions, it was reported that COX-2 plays an important role in inflammation during experimental autoimmune uveitis (Shiratori et al. 2004). Lal et al (2021) have reported that

DMF treatment reduced the COX-2 levels. The anti-arthritis activity of DMF may be mediated by the activation of the Nrf2/HO-1 pathway, which reduced oxidative damage and inflammation.

NF-κβ is recognized as a central mediator in the pathophysiology of many chronic inflammatory diseases, which lead to an increased risk of ocular inflammation. Moreover, the expression of TNF-α and iNOS are transcriptionally regulated by the transcription factor NF-κβ. DMF showed inhibition of NF-κβ activity in an Nrf2-independent manner (Gillard et al. 2015).

In conclusion, our analysis of local retinal inflammatory responses using an experimental autoimmune uveitis could be in part related to the increased expression of inflammatory markers such as iNOS, CD68, CD20 and CD4. Dimethyl fumarate represents a promising approach for the treatment of EAU. Our results suggest that DMF appears to be more effective in reducing inflammatory effects in the retina and could constitute a good candidate for monitoring an acute ocular inflammatory diseases.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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