ANIMAL CELLS AND SYSTEMS
2024, VOL. 28, NO. 1, 37–44
https://doi.org/10.1080/19768354.2024.2304557

Eugenol ameliorates uveitis in mice with experimental autoimmune encephalomyelitis through the suppression of key inflammatory genes

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ABSTRACT

Visual impairment associated with uveitis is among the potential complications in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). Bioinformatics analyses have shown that some hub genes are closely associated with the molecular mechanisms underlying uveitis in EAE. This study evaluated whether 4-allyl-2-methoxyphenol (eugenol) can mitigate the pathogenesis of uveitis in EAE through the interruption of key uveitogenic gene expression. Myelin oligodendrocyte glycoprotein$_{35–55}$ (MOG) peptide-immunized C57BL/6 mice were injected intraperitoneally with eugenol. The eyeballs and spinal cords of EAE mice with or without eugenol treatment were collected simultaneously and immunohistochemical and molecular biological analyses were conducted. Eugenol treatment significantly ameliorated hindlimb paralysis. Ionized calcium-binding adapter molecule 1 (Iba-1) immunohistochemistry showed that the inflammatory response was significantly reduced in the uvea of eugenol-treated EAE mice compared with vehicle-treated controls. Eugenol also significantly reduced the expression of key uveitogenic genes including *C1qb* and *Tyrobp*. The suppressive effect of eugenol on inflammation was also observed in the spinal cord, as determined by the suppression of Iba-1-positive microglial cells. Together, these results suggest that the ameliorative effect of eugenol against EAE uveitis is associated with the suppression of key proinflammatory genes, which may represent targets for the treatment of uveitis.

ARTICLE HISTORY
Received 9 November 2023
Revised 31 December 2023
Accepted 8 January 2024

KEYWORDS
Anti-inflammation; eugenol; experimental autoimmune encephalomyelitis; neuroinflammation; uveitis

Introduction

Experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), is characterized by the infiltration of autoimmune T cells into the tissues of the central nervous system (CNS) (Shin et al. 2012), which secrete proinflammatory cytokines with a concurrent burst of reactive oxygen species (ROS) (Jung et al. 2020; Kim et al. 2020). In addition to CNS inflammation, the uvea is occasionally involved during the course of EAE. with the infiltration of immune cells into the iris and retina (Adamus et al. 1997; Adamus et al. 2001; Hong et al. 2023). Recently, transcriptome profiling of the eyeball in a mouse EAE model identified key genes involved in the pathogenesis of uveitis in EAE, including *C1qb*, *Ctss*, and *Tyrobp*, which may be therapeutic targets for EAE-uveitis (Hong et al. 2023). Although the key uveitogenic genes in EAE have been identified, their functional roles in the pathogenesis of EAE-uveitis remain to be evaluated. A number of anti-inflammatory, antioxidant, and immunomodulatory compounds have been tested for their capacity to ameliorate EAE paralysis in animal models (Kim et al. 2010; Jung et al. 2020; Kim et al. 2020) as therapeutic candidates for the amelioration of autoimmune diseases.

Eugenol (4-allyl-2-methoxyphenol), a major phenolic constituent of clove essential oil, has been shown to have a variety of therapeutic properties, among which its anti-inflammatory, antioxidant, and antibacterial effects have been examined (Aburel et al. 2021; Nisar et al. 2021). Eugenol has been reported to ameliorate ischemia-induced brain injury in CNS inflammation

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Supplemental data for this article can be accessed https://doi.org/10.1080/19768354.2024.2304557.

models (Won et al. 1998; Sun et al. 2020), as demonstrated by antioxidant activity in cultured neurons (Wie et al. 1997) and anti-inflammatory activity via suppression of the nuclear factor kappa B (NF-κB) pathway (Zhai et al. 2022).

Eugenol has been shown to downregulate Th1 cytokines (tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12)) in nicotine-treated macrophages with concurrent activation of Th2 responses (IL-10, transforming growth factor-beta (TGF-β)) (Kar Mahapatra et al. 2011), which are also important factors in the progression of autoimmune diseases, including EAE (Xie et al. 2018) as a model of human MS (Rodríguez-Sáinz Mdel et al. 2002). Eugenol has also been shown to attenuate collagen-induced arthritis by decreasing levels of free radicals and proinflammatory cytokines (Mateen et al. 2019).

This study examined whether key uveitogenic gene expression was altered in the eyeball after treatment with eugenol in the course of EAE.

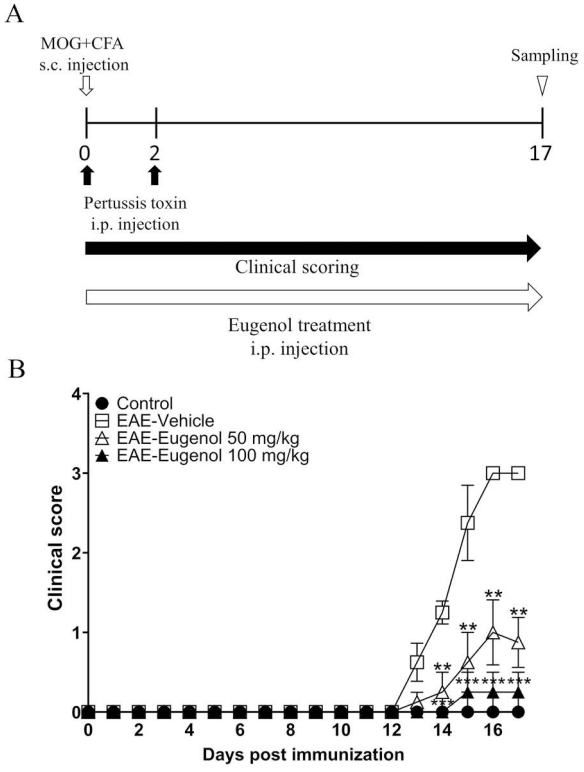

Figure 1. Experimental schedule and clinical signs in experimental autoimmune encephalomyelitis (EAE) mice with or without eugenol treatment. (A) Eugenol treatment schedule. (B) Clinical scores after EAE induction in mice treated with 50 and 100 mg/kg eugenol. Eugenol treatment at both doses significantly reduced paralysis severity from day 14–17 post-immunization. Data are presented as means ± standard errors of the mean (SEM) ($n = 4$ per group). ** $p < 0.01$, *** $p < 0.001$ vs. EAE + vehicle group. CFA, complete Freund's adjuvant; i.p., intraperitoneal; MOG, myelin oligodendrocyte glycoprotein$_{35\text{-}55}$; s.c., subcutaneous.

Materials and methods

Animals

Male C57BL/6 mice (8–10 weeks old; Samtako, Gyeonggi-do, Korea) were maintained in our facility under normal conditions (12-h light/dark cycle, temperature, 23°C ± 2°C). All animal protocols conformed to international laws and National Institutes of Health policies, including the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, 1985, revised 1996). All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Jeju National University (permission no.: 2022-0062).

Induction of EAE

Experimental animals were subcutaneously injected with 200 µL of myelin oligodendrocyte glycoprotein$_{35\text{-}55}$ (MOG) peptide at 1 mg/mL (Koma Biotech, Seoul, Korea) emulsified in complete Freund's adjuvant (CFA; Sigma-Aldrich, St. Louis, MO, USA) supplemented with *Mycobacterium tuberculosis* H37Ra (5 mg/mL; Difco Laboratories, Franklin Lakes, NJ, USA). All mice were injected intraperitoneally with 500 ng of pertussis toxin (List Biological Laboratories, Campbell, CA, USA) on the day of induction and again 2 days later. After immunization, all mice were weighed and observed daily for clinical signs of EAE, divided into seven clinical stages as follows: 0, no signs; 1, floppy tail; 2, mild paraparesis; 3, severe paraparesis; 4, tetraparesis; 5, moribund condition or death due to EAE.

Experimental groups and procedures

To determine whether the drug would have therapeutic effects in EAE-induced mice, eugenol (50 or 100 mg/kg) or vehicle (2% Tween 80) in saline was administered intraperitoneally from day 0 post-immunization (D0PI) until D17PI (Figure 1(A)). The mice were divided into four groups to investigate the effects of eugenol (50 and 100 mg/kg, E51791; Sigma-Aldrich) on the uvea and spinal cord: sham control ($n = 4$ mice per group), EAE + vehicle ($n = 4$ mice per group), EAE + 50 mg/kg eugenol ($n = 4$ mice per group) and EAE + 100 mg/kg eugenol ($n = 4$ mice per group).

Tissue preparation

The mice were euthanized with 95% CO_2 gas from D17PI. The spinal cord and eyes ($n = 4$ mice per group) were removed and fixed in 4% paraformaldehyde (spinal cord) or Tokuda-Baron fixative (eyes) composed of 100 mL of glacial acetic acid, 150 mL of 99% ethyl alcohol, 100 mL

of 40% neutral buffered formalin, and 650 mL distilled water (Tokuda et al. 2018), and stored at 4°C overnight for histopathological examination. The samples were stored at −80°C until analysis by reverse-transcription polymerase chain reaction (RT–PCR) ($n = 4$ mice per group).

Histological examination

The fixed tissues of spinal cords and eyes were embedded in paraffin wax and cut into 5-μm-thick sections with a rotary microtome (RM 2135; Leica, Nussloch, Germany). After deparaffinization with xylene and 100%–70% ethanol, the sections were stained with hematoxylin and eosin.

Immunohistochemical and immunofluorescence staining

Immunohistochemistry was performed as described in our previous study (Kim et al. 2022; Kim et al. 2023). Briefly, the deparaffinized spinal cord sections were incubated with rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1) primary antibody (1:1000, 019-19741; Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan) overnight at 4°C. The slides were incubated with biotinylated goat anti-rabbit IgG secondary antibody based on the primary antibody for 45 min. A commercial avidin–biotin complex kit (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) was applied for 45 min. A 3-3′-diaminobenzidine substrate kit (DAB kit, SK-401; Vector Laboratories) was used to visualize the peroxidase reaction and the sections were counterstained with hematoxylin for 30 s.

Immunofluorescence staining was performed. Briefly, eyeball sections were incubated overnight with rabbit anti-Iba-1 antibody (1:1000, 019-19741; Fujifilm Wako Pure Chemical Industries), as a specific marker for macrophages and microglia. After incubation with primary antibody, sections were incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (1:50; Sigma-Aldrich). The slides were observed under a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Semi-quantitative analysis of immunohistochemistry and immunofluorescence results

Iba-1 immunoreactivity in the spinal cord and uvea was quantified using ImageJ v1.53t (National Institutes of Health, Bethesda, MD, USA) (Schneider et al. 2012), as described in our previous study (Ahn et al. 2016). Three different regions of the spinal cord and uvea (iris and ciliary body) of each of four animals per group were photographed at 20× magnification. The immunopositive area per total area [(Positive area/total area) × 100 (%)] was measured. The results are reported as means ± standard error of the mean (SEM).

RT–PCR analysis

Total RNA was extracted from the eyeballs of each group (n = 4 per group) at D17PI using Qiazol Lysis Reagent (Qiagen, Hilden, Germany). cDNA was transcribed from purified RNA using the 5× First Strand cDNA Synthesis Master Mix (CellSafe, Kuala Lumpur, Malaysia). Subsequent analyses were performed using a quantitative PCR machine (Bio Molecular Systems, Upper Coomera, Australia) and 2× Quantity SYBR Green PCR Master Mix (PhileKorea Co., Ltd., Seoul, Korea), involving 40 cycles of denaturation at 95°C, annealing at 60°C, and extension at 72°C for 10 s each. Primer sequences are presented in Supplementary Table 1.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as means ± SEM. Means were compared using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Significance was evaluated at a level of $p < 0.05$.

Results

Eugenol reduced hindlimb paralysis associated with EAE

The sham control mice did not exhibit any clinical signs during the experiment. However, compared to the EAE + vehicle group, the EAE + eugenol group exhibited significant improvement in clinical signs between D14PI and D17PI (Figure 1(B)). This trend continued throughout the experimental period, suggesting that eugenol may have therapeutic effects in EAE mice.

Histopathological examination of the spinal cord and uvea

Histopathological observations revealed well-preserved histological structure and the absence of inflammatory responses in the spinal cord (Figure 2(A)) and uvea (Figure 2(D)) of sham control mice. However, a number of inflammatory cells were found in the perivascular cuffing region and parenchyma of the spinal cord in the EAE + vehicle group (Figure 2(B)) compared to the EAE + eugenol groups (Figure 2(C)). Infiltration of inflammatory cells in the uvea was observed in the EAE + vehicle group compared to the EAE + eugenol group (Figure 2(E, F)).

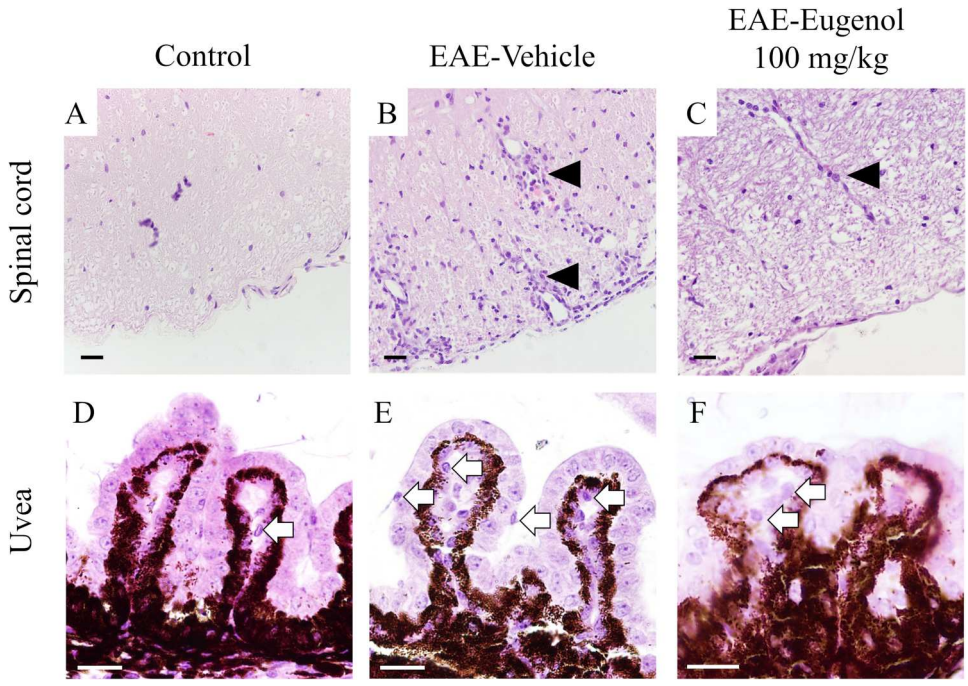

Figure 2. Histopathological examination of the spinal cord and uvea in sham control and EAE mice with or without eugenol treatment. (A) No evidence of inflammatory cells was observed in the spinal cord of sham control. (B) Inflammatory cells (B, arrowheads) were observed in the perivascular region and parenchyma of the spinal cord in the EAE + vehicle group. (C) Fewer inflammatory cells (C, arrowhead) were detected in EAE + eugenol groups than in the EAE + vehicle group. (D) No signs of inflammatory cells were observed in the uvea of sham control, except for occasional resident cells (D, arrow). (E) Infiltration of inflammatory cells (E, arrows) was observed in the uvea in the EAE + vehicle group. (F) However, fewer inflammatory cells (F, arrows) were found in the uvea in the EAE + eugenol groups. Scale bar = 20 μm.

Immunohistochemical and immunofluorescence staining for Iba-1 in the spinal cord and uvea

Immunohistochemical staining was performed using an antibody against Iba-1, which is a marker for macrophages and microglia, in the spinal cord (Figure 3) and uvea (Figure 4). It was difficult to detect Iba-1-positive cells in the spinal cord (Figure 3(A)) and uvea (Figure 4(A)) of sham control mice. Reduced Iba-1 immunoreactivity was detected in the spinal cord of mice in the EAE + eugenol groups (Figure 3(C)) compared to the EAE + vehicle group (Figure 3(B)). Fewer Iba-1-positive cells were detected in the uvea of the EAE + eugenol groups (Figure 4(C)) than in the EAE + vehicle group (Figure 4(B)).

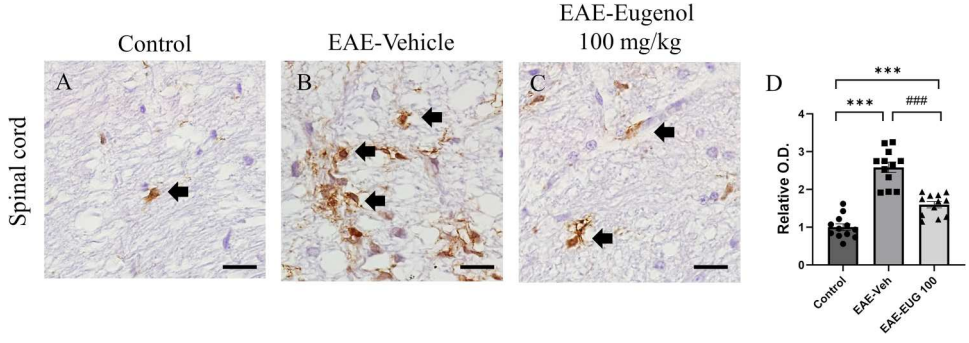

Figure 3. Immunohistochemical examination of the spinal cord of sham control and EAE mice with or without eugenol treatment. (A) Iba-1-positive resident microglia (A, arrow) were detected in the normal spinal cord. (B) Increased numbers of Iba-1-positive cells (B, arrows) were detected in the spinal cord in the EAE + vehicle group. (C) The EAE + eugenol groups showed decreased Iba-1 immunoreactivity in the spinal cord (C, arrows) compared to the EAE + vehicle group. (D) Relative numbers of Iba-1-positive cells in the spinal cord in sham control and EAE mice with or without eugenol treatment. Scale bar = 20 μm. Data are means ± SEM ($n = 4$ per group). *** $p < 0.001$ vs. sham control group; ### $p < 0.001$ vs. EAE + vehicle group.

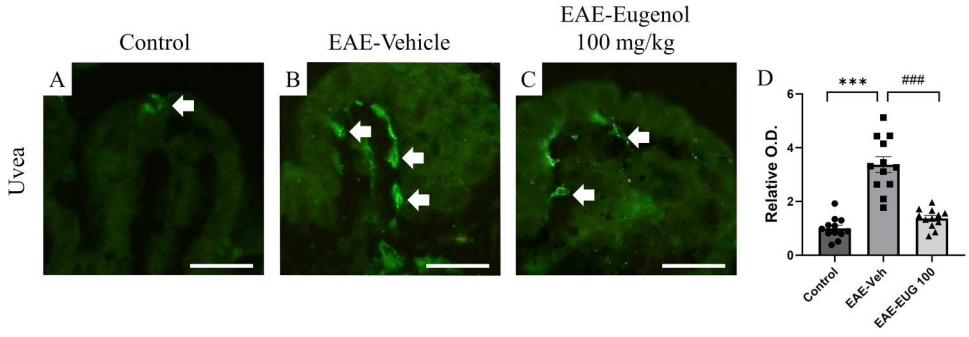

Figure 4. Immunofluorescence staining of the uvea in sham control and EAE mice with or without eugenol treatment. (A) Iba-1-positive cells (A, arrow) were detected in sham control uvea. (B) Increased numbers of Iba-1-positive cells (B, arrows) were found in the EAE + vehicle group. (C) However, fewer Iba-1-positive cells (C, arrows) were observed in the EAE + eugenol groups compared to the EAE + vehicle group. (D) Relative proportions of Iba-1-positive cells in the uvea in sham control and EAE mice with or without eugenol treatment. Scale bar = 20 μm. Data are means ± SEM ($n = 4$ per group). *** $p < 0.001$ vs. sham control group; ### $p < 0.001$ vs. EAE + vehicle group.

Eugenol affects the expression of key EAE-uveitis genes

To assess whether eugenol suppresses the expression of key genes related to EAE-uveitis and enhances the expression of downregulated genes, quantitative RT–PCR was performed at D17PI to measure the levels of several key genes (Hong et al. 2023) (Figure 5). Compared to the EAE + vehicle group, the EAE + eugenol 100 mg/kg group exhibited significantly reduced expression levels of key EAE-uveitis genes, including complement c1q beta chain (*C1qb*) (Figure 5(A)), cathepsin S (*Ctss*) (Figure 5(B)), integrin subunit alpha M (*Itgam*) (Figure 5(C)), integrin beta 2 (*Itgb2*) (Figure 5 (D)), and TYRO protein tyrosine kinase-binding protein (*Tyrobp*) (Figure 5(F)). Notably, the expression of downregulated EAE-uveitis genes, including *Hapln1* and *Ndst4*, exhibited no significant changes compared to the EAE + vehicle group (Figure 5(G, H)). Additionally, the expression levels of *C1qb* and *Ctss* significantly differed between the control and EAE-eugenol 50 mg/kg groups, indicating that eugenol 50 mg/kg eugenol treatment was insufficient to suppress these genes to control levels (Figure 5(A, B)). Similarly, the expression of *Tyrobp*, *Hapln1*, and *Ndst4* significantly differed between the control and EAE-eugenol 50 and 100 mg/kg groups, indicating that neither eugenol dosage adequately restored their expression to control levels (Figure 5(F-H)). These findings suggest that eugenol modulates the expression of key genes associated with EAE-uveitis.

Discussion

This is the first study to demonstrate that eugenol can ameliorate the progression of MOG-induced EAE. Uveitis is occasionally found in EAE (Adamus et al. 1997; Adamus et al. 2001). The pathogenetic mechanisms of most disorders in uveitis are related to oxidative stress. Although the molecular mechanism of action of eugenol in EAE has not been elucidated, the anti-inflammatory and antioxidant effects of eugenol have been proposed to be involved in its effects on EAE. Eugenol has been shown to have neuroprotective effects in brain ischemic injury, possibly mediated via its antioxidant activity (Won et al. 1998). An immunomodulatory effect of eugenol was also reported in rheumatoid arthritis models (Mateen et al. 2019). Based on these previous studies, we investigated whether eugenol would show neuroprotective and immunomodulatory effects in a mouse EAE model of MS.

Behavioral amelioration of hindlimb paralysis in eugenol-treated EAE mice largely corresponds to treatment with a variety of essential oils (Alberti et al. 2014; Mateen et al. 2019; Rezapour-Firouzi et al. 2020; Lee et al. 2023) in EAE models (Alberti et al. 2014; Rezapour-Firouzi et al. 2020; Lee et al. 2023). The mechanism underlying behavioral amelioration in the present study is likely related to the effects of eugenol, including its anti-inflammatory (Jung et al. 2023; Liu et al. 2023; Wang et al. 2023; Zhu et al. 2023) and antioxidant activity (Wang et al. 2023). The ameliorative effect of eugenol in EAE was further demonstrated by histopathological and immunohistochemical analyses of Iba-1, which is a marker of microglia, in EAE lesions. The results indicated that eugenol ameliorated paralysis in EAE partly through the suppression of macrophage/microglial activity in the spinal cord.

Next, we examined the eyeballs of mice with EAE with or without eugenol treatment. The results of histological and immunohistochemical analyses of Iba-1 in the uvea revealed that the infiltration of Iba-1-positive cells was significantly reduced, suggesting a reduction of

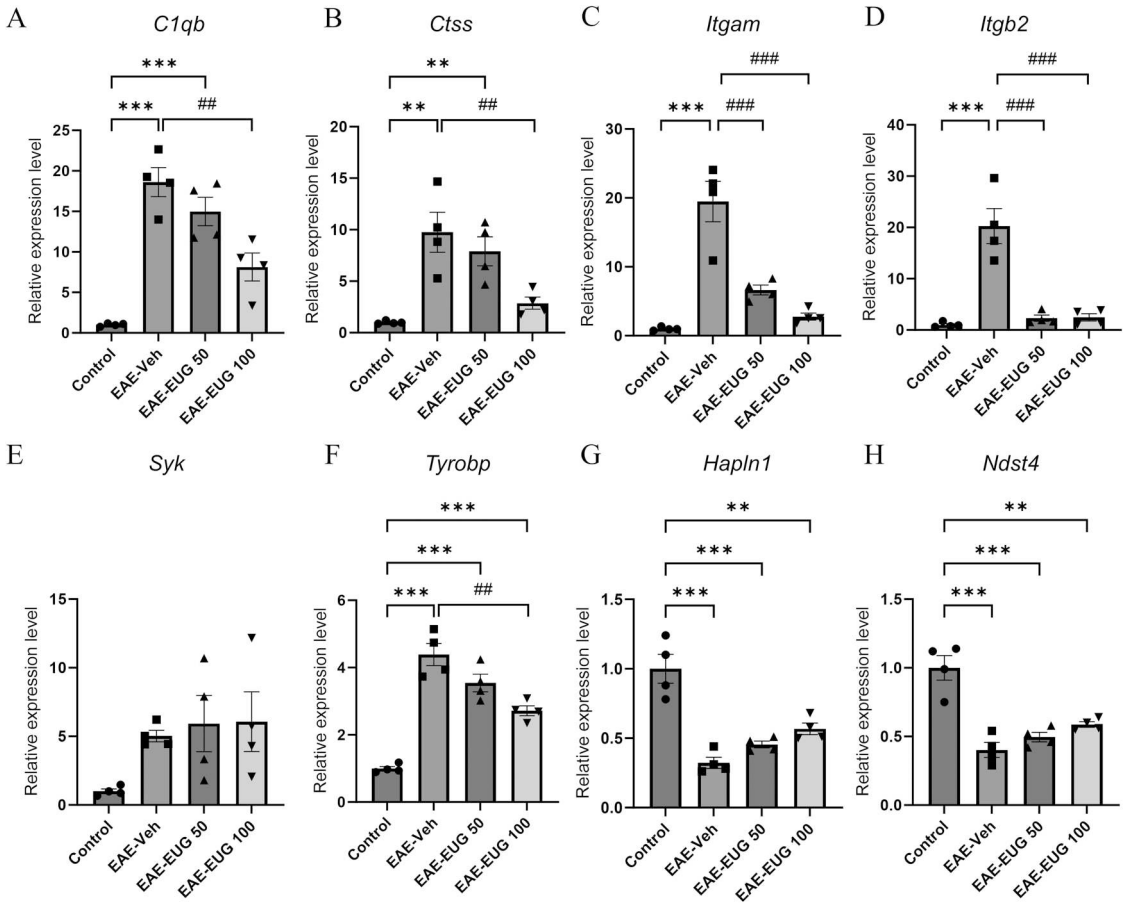

Figure 5. Expression changes in genes related to EAE-uveitis in the uvea of sham control and EAE mice with or without eugenol treatment. (A-H) Bar graphs show relative expression levels of six key genes including (A) *C1qb*, (B) *Ctss*, (C) *Itgam*, (D) *Itgb2*, (E) *Syk*, and (F) *Tyrobp* and two downregulated EAE-uveitis-related genes including (G) *Hapln1* and (H) *Ndst4*. Data are means ± SEM ($n = 4$ per group). ** $p < 0.01$, *** $p < 0.001$ vs. sham control group; ## $p < 0.01$ and ### $p < 0.001$ vs. EAE + vehicle group.

inflammation in the uvea of EAE mice treated with eugenol, as observed in the spinal cord in the same mice. These findings suggested that eugenol systemically reduces the inflammatory response in EAE mice.

Consistent with the amelioration of inflammation in the uvea, the present study focused on changes in the expression of previously identified key uveitogenic genes in EAE (Hong et al. 2023). As expected, most genes upregulated in EAE were suppressed in the eugenol-treated mice, suggesting that these genes are uveitogenic in EAE.

Transcriptomics analyses have been performed in experimental autoimmune uveitis (EAU) in Lewis rats; genes including *Casp1*, *Casp4*, and *Casp12* were shown to be closely associated with inflammation in the iris (Li et al. 2022). In the present study, we found that key genes in EAE-uveitis in mice were distinct from those in the rat EAU model. This discrepancy may be attributable to differences in the models and/or immunogenic antigens (MOG-EAE in C57Bl/6 mice vs. interphotoreceptor retinoid-binding protein (IRBP)-induced EAU in Lewis rats). These results indicate that the key genes in uveitis are case-dependent. Single-cell transcriptomics analyses identified genes including *Ctss* and *Tyrobp* as key genes in IRBP-induced EAU in mice (Zhu et al. 2023). In mice, uveitogenic genes in EAE appear to be mainly organ-specific; therefore, *Ctss* and *Tyrobp* appear to be common key genes in uveitis regardless of their autoimmune origin. The effects of eugenol on EAE-uveitis were mediated by the suppression of *Ctss* and *Tyrobp*, suggesting that these genes are potential therapeutic targets for EAE-uveitis, as similarly reported in EAU (Zhu et al. 2023). The mechanisms by which eugenol ameliorates uveitis in EAE and EAU are not fully understood (Zhu et al. 2023). However, eugenol treatment suppressed the expression of *Ctss* and *Tyrobp* in uveitis in both EAE-uveitis and EAU mouse models (Zhu et al. 2023).

The main focus of this study was to confirm the influence of eugenol treatment on key molecules in EAE. Other upregulated genes, including *C1qb*, *Itgam, and Itgb2* were found to be suppressed in eugenol-

treated EAE-uveitis. However, the expression of the downregulated genes in EAE-uveitis (*Hapln1* and *Ndst4*) remained unchanged after eugenol treatment, indicating its limited role in the restoration of *Hapln1* and *Ndst4* gene expression. Furthermore, the expression levels of genes related to axon projection and guidance (*Halpn1* and *Ndst4*) in EAE-uveitis remained unchanged by the end of the experimental period. Therefore, further studies are required to determine the time course of changes in the expression of these genes, as neuronal changes have been reported, including decreases in ganglion cells in the retina in MS (Denis et al. 2022; Miscioscia et al. 2022) and EAE (Rayatpour et al. 2022). The molecular mechanism underlying the effects of eugenol on the key genes examined in this study remains to be determined. Together, our results indicate that eugenol ameliorated uveitis in EAE through the suppression of key uveitogenic molecules.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by National Research Foundation of Korea: [grant no NRF-2019R1A2C1087753].

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