The effect of a histone deacetylase 6 inhibitor on experimentally induced uveitis in vivo mouse model

Min Kim

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Koh, Hyoung Jun

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Min Kim

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This certifies that the Doctoral Dissertation of Min Kim is approved.

[Signature]

Thesis Supervisor : Koh, Hyoung Jun

[Signature]

Thesis Committee Member#1 : Lee, Hyung Keun

[Signature]

Thesis Committee Member#2 : Lee, Jun Haeng

[Signature]

Thesis Committee Member#3: Yoon, Ho Geun

[Signature]

Thesis Committee Member#4: Jung, Ho Sung

The Graduate School
Yonsei University

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ABSTRACT

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The Graduate School, Yonsei University

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Autoimmune uveitis is a clinically heterogeneous group of intraocular inflammation that often leads to visual deterioration, retinal destruction and blindness and remains a therapeutic challenge. Active CD4+ T cells, particularly Th1 and Th17 cells infiltrate the eye and play a crucial role in the pathogenesis of autoimmune uveitis. Current treatments for non-infectious autoimmune uveitis include glucocorticoids or immunosuppressive agents. However, the long-term use of these agents could lead to severe ocular or systemic side effects and there is a need for more effective, targeted and less harmful medications to treat autoimmune uveitis.

Histone deacetylase (HDAC)6 has been shown to be involved in regulation of inflammatory and immune responses, and the genetic or pharmacologic disruption of HDAC6 resulted in downregulation of IL-6 and TNF. However, the role of HDAC6 inhibitors in uveitis and the underlying immunomodulatory mechanisms remain undetermined yet. Therefore, I investigated the

anti-inflammatory efficacy of CKD4, a histone deacetylase 6 inhibitor, in experimental autoimmune uveitis mice model by analyzing immune cell infiltration, histological changes of retina, as well as changes in profile of inflammatory cytokines. Interphotoreceptor binding protein(IRBP) induced EAU model was successfully created and demonstrated clinical features of autoimmune uveitis. CKD4 alleviated the clinical severity of experimental uveitis and limited the development of chorioretinitis and inflammatory cell infiltrations in the retina. HDAC6 and CD4+ markers were co-localized within the same cells, indicating that HDAC6 was highly expressed in CD4+ T cells in EAU. The treatment with CKD4 significantly reduced detection of HDAC6 and CD4+ T cells within the inner and outer retina, and also increased the detection of acetylated alpha-tubulin in EAU model, as shown by immunohistochemistry. The mRNA expression of HDAC6, IFN-γ, IL-17 and TNF-α, were significantly increased in the EAU mouse retina and the treatment with CKD4 led to their significant decrease. Proinflammatory cytokines including IFN-γ, and IL-17A were significantly elevated in EAU mouse model and treatment with CKD4 led to a significant reduction of IFN-γ and IL-17A. Flow cytometry analysis showed that the treatment with CKD4 eyedrops suppressed the percentage of CD4+IFN-γ +Th1 cells and CD4+IL-17+ Th17 cells in the retina of EAU mouse. Western blot showed a prominent downregulation of HDAC6 protein level in EAU mouse retina and increased acetylation of α-tubulin in EAU mouse retina. In conclusion, I found that HDAC6 inhibitor, CKD4, ameliorated the clinical severity of experimental

uveitis and also attenuated the infiltration of T lymphocytes in the EAU. It reduced the expression of HDAC6 and various inflammatory cytokines including IFN-γ, IL-17, and TNF-α. Thus, this study suggested the potential therapeutic role of CKD4 in the treatment of autoimmune uveitis.

Key words: Histone deacetylase 6 (HDAC), HDAC6 inhibitor, CKD4, uveitis, experimental autoimmune uveitis, inflammation, autoimmunity

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I. INTRODUCTION

Uveitis is a clinically heterogeneous group of intraocular inflammation that often leads to visual deterioration, retinal destruction and blindness. It is one of the important causes that contributes to the visual impairment affecting approximately 200 per 100,000 in the population in developed countries. Uveitis and its complications account for up to 10-35% of severe visual impairment.[1, 2] It is caused by either autoimmune mechanisms or infectious agents. In human autoimmune uveitis, eye may be the only organ involved, or uveitis may be a part of syndrome involving multiple tissues or a group of diseases that may differ in clinical presentation.[3]

In autoimmune uveitis, although its exact pathogenesis is not known, the involvement of auto-antigens or autoantibodies had been speculated for years. The eye is known to possess ocular immune privilege and this is believed to have evolved to protect vision from damage caused by inflammatory insults.[4] However, despite such immune privilege, humans still develop autoimmune uveitis, which remains a paradox to be solved. During the formation of central immunologic tolerance, the process of thymic education eliminates the T cells that happen to express antigen receptors with high affinity to self, and spares those with low affinity to self for the purpose of host antimicrobial defense.

Self-reactive cells that escape the central tolerance are dealt with peripheral tolerance, but retinal antigens residing within the healthy eye are largely sequestered behind the blood–retinal barrier as part of ocular immune privilege, hindering the normal functioning of peripheral tolerance. This results in persistence of a population of nontolerant T cells that can be activated by a chance exposure to antigen, either as a result of ocular trauma or in the form of a microbial mimic whose molecular structures happen to resemble self. The breakdown of self-tolerance to immunologically privileged antigens found in the retina of the eye is speculated to lead to subsequent pathologic processes that ultimately manifest as autoimmune uveitis.[5] Self activated T-lymphocytes from peripheral blood of uveitis patients were shown to respond to antigenic proteins expressed in the retina, and these responses seem to play a crucial role in the pathogenesis of autoimmune uveitis.[5] They migrate into the eye and cause tissue damage when the intraocular immune balance is broken by environmental stimuli.[6]

Inflammation and the immune response are regulated by various host suppressor mechanisms, including the production of anti-inflammatory cytokines by cells of the innate immune system and the generation of regulatory T cells.[7, 8] Investigations in animal models have shown that active CD4+ T cells, particularly Th1 and Th17 cells infiltrate the eye and play a crucial role in the pathogenesis of autoimmune uveitis.[9] Also, in humans, activated CD4+ T cells contribute to the development of various uveitides when environmental stimuli break the intraocular immune balance.[10]

Because of unclear etiology of autoimmune uveitis and the limited availability of human tissue samples due to the ethical considerations, the use of animal model to study uveitis is necessary. Experimental autoimmune uveitis(EAU) induced by interphotoreceptor retinoid-binding protein(IRBP) in mice provides an important method for investigating the pathogenesis and treatment strategy for human uveitis in both clinical and histopathological

aspects.[11-13] In this model, during the immune activation phase, lymphocytes get activated in the peripheral lymphoid organs and during the immune effector phase, the activated T cells migrate and infiltrate the eye, eventually leading to destruction of tissues within the eye.[14, 15]

Current treatments for non-infectious autoimmune uveitis include glucocorticoids or immunosuppressive agents. However, the long-term use of these agents could lead to severe ocular or systemic side effects and there is a need for more effective, targeted and less harmful medications to treat autoimmune uveitis.[16] Moreover, a significant portion of patients do not respond to these treatments. Therefore, more effective treatments with less side effects are needed to prevent irreversible visual impairment for uveitis patients with suboptimal response to conventional therapy.

Histone deacetylases (HDAC) and histone acetyltransferase play important roles in the regulation of gene transcription.[17] The positively charged lysine in the N-terminal of histones gets neutralized by acetylation of histone by histone acetyltransferase. Histones do not bind to the negatively charged phosphate groups in DNA and as a result, the binding affinity between the histones and DNA backbone is reduced, leading to enhanced gene transcription by promoting the binding of transcription factors to DNA.[18, 19] Conversely, HDAC are a class of enzymes that remove acetyl groups from an N-acetyl lysine amino acid on a histone, allowing the histones to wrap the DNA more tightly and thereby repressing gene transcription. HDAC proteins are classified into four classes based on DNA sequence similarity and function. Class I,II, and IV HDACs are considered “classical” HDACs that have a zinc-dependent active site and whose activities are inhibited by trichostatin A. Class III enzymes are a family of nicotinamide adenine dinucleotide (NAD+)-dependent proteins known as sirtuins and are not affected by TSA.[20] Class I HDACs (HDACs 1-3, and 8) are found primarily in the nucleus, with HDAC8 being found in both nucleus and the cytoplasm. Class II HDACs (HDAC4-7,9,10) can shuttle

between the nucleus and the cytoplasm, thus found in both the nucleus and the cytoplasm.[21, 22] Class III HDACs (SirT 1–7) are considered a separate type of enzymes due to their distinct structures and different mechanisms of action. Their enzymatic activity depends on the cofactor NAD+. Class IV HDAC (HDAC11) is structurally similar to class I and II HDACs.[23] HDAC6 belongs to class II HDACs and is a cytoplasmic, microtubule-associated enzyme. It contains two homologous catalytic domains and an ubiquitin binding domain at the C-terminal end.[24] It deacetylates tubulin, heat shock protein(Hsp)90, and cortactin. It regulates acetylation of various proteins by forming complexes with other partner proteins, and is, therefore, involved in a variety of biological processes, including cell migration and immune response.[25] HDAC6 plays an important role in cell motility by catalyzing a-tubulin deacetylation, which leads to retraction of the cilium of the cell, a necessary step prior to mitosis of the cell.[26] These various functions of HDAC6 make it an attractive potential therapeutic targets in various diseases including cancer, inflammation and autoimmune diseases.

HDAC inhibitors are potent epigenetic modifiers and has been demonstrated to be effective in the treatment of inflammatory and autoimmune diseases, as well as in certain malignancies.[27] They are believed to exert their anti-inflammatory and immunosuppressive effects by blocking the cell cycle, cell growth and inhibiting cell differentiation, resulting in cell apoptosis. The inhibition of HDAC6 was also found to increase the suppressive activity of regulatory T cells (Treg cells) in inflammation.[28] Both in vivo and in vitro experiments showed that HDAC inhibitors downregulate cytokines, such as IL-1b, IL-2, IL-6, IL-12, IL-17, IL-23, TNF-α, and IFN-γ.[29] They have been approved for the treatment of cutaneous T-cell lymphoma and shown to attenuate GVHD and anti-CD3 antibody triggered cytokine storm syndrome through the modulation of T-cell functions in murine models.[27, 30]

However, a broad spectrum HDAC inhibitors could cause severe adverse effects and inhibition of specific HDACs by member specific HDAC inhibitors may achieve more favorable outcome with less side effects. Recently, HDAC6 has been shown to be involved in regulation of inflammatory and immune responses, and that genetic or pharmacologic disruption of HDAC6 resulted in downregulation of IL-6 and TNF.[31] However, the therapeutic efficacy of HDAC6 inhibitor on autoimmune uveitis has not been investigated previously.

In this study, I aim to investigate the anti-inflammatory efficacy of a HDAC6 inhibitor in EAU by analyzing immune cell infiltration, histological changes of retina, as well as changes in profile of inflammatory cytokines. I hypothesize that HDAC6 inhibitor would demonstrate its therapeutic effect in EAU by resulting in improvement of uveitis and downregulation of inflammatory cytokines by T-lymphocytes via inhibition of deacetylation by HDAC6.

II. MATERIALS AND METHODS

1. Animals

Male C57BL/6 wildtype mice, approximately 6-8 weeks old, were obtained from from Orient Bio (Seoul, South Korea). They were maintained in pathogen-free air-conditioned room with a 12 h light-dark cycle with water and food available ad libitum until they were used for experiments. The research protocol was approved by the Yonsei University Health System Institutional Animal Care and Use Committee (Permit number: LML 11-18) and is in compliance with the Association for Research in Vision and Ophthalmology animal policy (ARVO statement for the use of animals in ophthalmic and vision research).

2. Induction of EAU in mice

Each mouse is subcutaneously injected in both footpads with 200 μg human IRBP $_{651-670}$(LAQGAYRTAVDLESLASQLT Peptron, South Korea) mixed with CFA (Complete Freund's Adjuvant, Sigma Aldrich, St Louis, MO, USA) (1:1) using 23-gauge needle at day 0. Human IRBP $_{651-670}$ were emulsified in 50μl of CFA containing 2.5mg of Heat-killed *Mycobacterium Tuberculosis* strain H37Ra (Difco, Detroit, MI, USA) mixed with 25μL of PBS. CFA is critical for disease induction. In addition, 0.2 μg/0.1 ml of pertussis toxin from Bordetella pertussis (Sigma-Aldrich, St. Louis, MO) were given intraperitoneally on day 0 and day 2 as an adjuvant for additional inflammatory stimulus to induce autoimmune uveitis.

3. Treatment of EAU by HDAC6 inhibitor, CKD4

CKD4 is a novel histone deacetylase 6 inhibitor. On day 11 after the development of experimental autoimmune uveitis with IRBP, CKD4 was administered twice a day as an eyedrop.

4. Clinical Assessment of EAU after treatment with CKD4

At day 21 after immunization with IRBP, funduscopic camera was used to take fundus pictures of the mice retina from each group and the degree of inflammation was classified into 4 different stages according to Caspi's criteria as previously described and the clinical score was assessed in a masked fashion. The mice were euthanized and the eyes are then enucleated and prepared for histopathological assessment.

5. Histopathologic examination

The mice were euthanized 21 days after immunization and the eyes were then enucleated and immersed in PBS with 6% glutaraldehyde solution for 24-48 hours at room temperature and prepared for histopathological assessment. The

fixed and dehydrated tissues were embedded in paraffin. Then, 5-μm thick sagittal sections were cut at different levels and were stained with hematoxylin and eosin (H&E) for histopathologic examination.

6. Immunohistochemical staining

5-μm thick sections were made from paraffin block and incubated with primary antibodies, anti-CD3 rabbit polyclonal antibody (1:100; Abcam, Cambridge, MA, USA) and anti-CD45R rat (B220) antibody (1:100; Abcam), anti-CD4 rat monoclonal antibody(1:100; Abcam), anti-HDAC6 rabbit monoclonal antibody(1:500; Cell Signaling Technology, MA, USA) and anti-acetylated α-tubulin mouse monoclonal antibody(1:1000; Sigma Aldrich, St. Louis, MO, USA) at 37 C for one hour. Anti-Rabbit FITC (1:100; Biolegend, San Diego, CA, USA) and anti-rat Alexa 594 (1:100; Biolegend) were used as the secondary antibody and incubated at 4℃ for overnight for quantitative and qualitative analysis of immune cell infiltration. Images were examined and captured with confocal laser scanning microscope (LSM780; Zeiss, Germany).

7. Quantitative Real-time Polymerase Chain Reaction (PCR)

The retina and choroids were carefully dissected from the enucleated mouse eyes. The total RNA was extracted from the retina after treating with Trizol reagent (Invitrogen, Carlsbad, CA, USA) for lysis. RNA samples were reverse transcribed to cDNA using PrimeSctipt RT Master (#RR036A; Takara, Dalian, China). Real-time PCR was performed using SYBR Premix Ex Taq (#RR420A; Takara) with gene specific primers(IL-1β, TNF-α, IFN-γ, IL-17, HDAC1, HDAC2, HDAC4, HDAC6) and StepOnePlusReal-Time PCR System (Applied Biosystems, Foster City, CA, USA). All the procedures were performed according to the manufacturer's guidelines and the nucleotide sequence of primers are shown in table 1.

Table 1. Nucleotide sequence of primers for quantitative RT-PCR

mHDAC6	F 5'-AAGTGGAAGAAGCCGTGCTA-3' R 5'-CTCCAGGTGACACATGATGC-3'
mHDAC1	F 5'-GGACCGGTTAGGTTGCTTCA-3' R 5'-TTCGTAAGTCCAGCAGCGAG-3'
mHDAC2	F 5'-CTATCCCGCTCTGTGCCCTA-3' R 5'-CCTCCTTGACTGTACGCCAT-3'
mHDAC4	F 5'-CCAGACACCCCTTGTCACAG-3' R 5'-GAATGGATGGGGACACCCTG-3'
mIFN-γ	F 5'-ACAAAGATGGCAGAGCACGA-3' R 5'-TCCACCAACATGTGCGGTTT-3'
mIL-1β	F 5'-AAGGGCTGCTTCCAAACCTTTGAC-3' R 5'-ATACTGCCTGCCTGAAGCTCTTGT-3'
mTNF-α	F 5'-AGCCGATGGGTTGTACCTTGTCTA-3' R 5'-TGAGATAGCAAATCGGCTGACGGT-3'
mβ-actin	F 5'-AGGGAAATCGTGCGTGACAT-3' R 5'-AACCGCTCGTTGCCAATAGT-3'

8. Enzyme Linked Immunosorbent Assay (ELISA)

The inflammatory cytokines were measured by ELISA. Spleen tissues secured from 3 mice of each group were minced and prepared for 520 μl of SHEM with 80 μl of protease inhibitor cocktail. The tissues were passed through a 100-μm cell strainer to get single cells suspension by crushing with forceps and collecting the cell suspension in 5 ml phosphate-buffered saline (PBS). Splenocyte mononuclear cells were isolated by density gradient centrifugation using Histopaque-1083 (Sigma Chemicals, St. Louis, MO, USA). Mononuclear cells were seeded in a concentration of 5 x 10^5 cells/200 $\mu\ell$ well in Roswell Park Memorial Institute (RPMI)-1640 without fetal bovine serum (FBS) medium at 37℃ in 5% CO^2 for 72 hrs. Then, the prepared cell supernatants were used for ELISA of IFN-γ (#430804, Biolegend), IL-17A (#432501, Biolegend), and TGF-β (#MB100B, R&D Systems, Minneapolis, MN, USA) measurement by ELISA followed by the manufacturer protocol.

9. Fluorescence-Activated Cell Sorter(FACS) of T lymphocytes (Immune cell identification and cytokine expression in the EAU retina)

- At day 21 of EAU induction, cells were isolated from the mouse retina or draining lymph nodes for identification of immune cells and cytokine expression. To determine intracellular expression of IFN-γ and IL-17, cells were stimulated with 1μg/ml Bredfeldin A(BD Biosciences, San Jose, CA, USA) for 6 hrs. The cells were then harvested, fixed, permeabilized and stained with anti-IFN-γ and anti-IL-17. Fluorochrome-conjugated mAbs were used to detect the expression of CD4, IL-17, and IFN-γ. After staining, all the samples were subjected to flow cytometry analysis(FACSCanto II, USA) and the results were analyzed with Cell Quest software(BD Biosciences).

10. Western Blotting

Mouse retinae were isolated and incubated with RIPA buffer(Biosesang, Seongnam, Korea) containing protease inhibitor mixture (100 ml/retina; Roche Bioscience, Palo Alto, CA, USA) at 4℃ for 20 min. Supernatant was collected after centrifuge and the quantification of protein expression level of HDAC6, acetylated α-tubulin, and total α-tubulin was performed using a protein assay according to the manufacturer's instructions. 30㎍ of protein was mixed with 5X sample buffer and SDS-PAGE was performed on 10% acrylamide gel (stacking gel 80v, separating gel 120v). It was transferred to PVDF membrane using Trans-blot electrophoretic transfer (Bio-Rad, Hercules, CA, USA). After blocking the membrane with 3% bovine serum albumin (BSA) for 1 hour at room temperature, blots were washed and incubated overnight at 4℃with specific primary antibodies: ACE α-tubulin (#T6793; Sigma Aldrich) 55kDa 1:2000, Total α-tubulin (#2144; Cell Signaling Technology) 52kDa 1:1000, HDAC6 (#7612; Cell Signaling Technology) 52kDa 1:1000, and β-actin (#bs-oo61R; Bios, Beijing, China) 42kDa 1:1000. Blots were incubated with secondary antibodies for 3 hours at room temperature Anti-Mouse

IgG-Peroxidase (#32430; Thermo Scientific, Rockford, IL, USA) 1:250 and Anti- Rat HRP (#P0450; Dako, Hamburg, Germany). Blots were visualized and filmed with AFGA CP1000. β-actin was used as an internal reference.

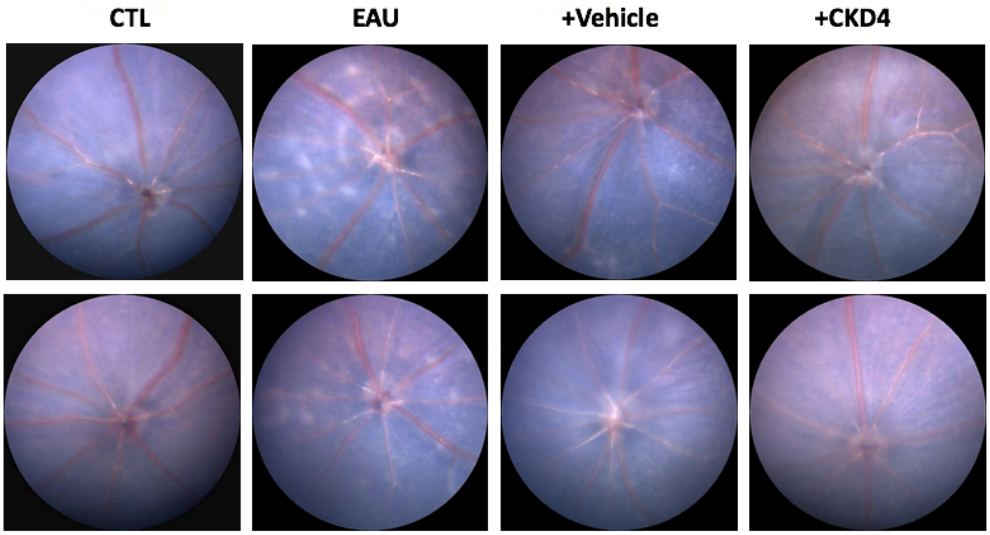
III. RESULTS

1. HDAC6 inhibitor, CKD-4, decreases the clinical severity of EAU

IRBP $_{651-670}$ immunization generated characteristic signs of uveitis, including chorioretinal infiltrations and vascular sheathing suggestive of retinal vasculitis.

To examine the therapeutic effects of CKD-4 on the mouse EAU model, CKD-4 was administered twice a day as an eyedrop from day 11 to day 21 after IRBP $_{651-670}$ immunization. Figure 1A demonstrates that treatment with CKD-4 attenuates the severity of inflammation in the EAU mice compared with the control group or the vehicle treated group. There was a significant improvement of clinical scores in the CKD-4 treated group ($p<0.05$; Figure 1B). Histopathologic analysis of EAU eyes showed severe inflammatory cell infiltration, disorganization of inner and outer retinal structure, retinal folding with detachments in the control group and vehicle group, whereas the treatment with CKD-4 attenuates the infiltration of inflammatory cells in the mouse experimental uveitis. (Figure 1C)

A



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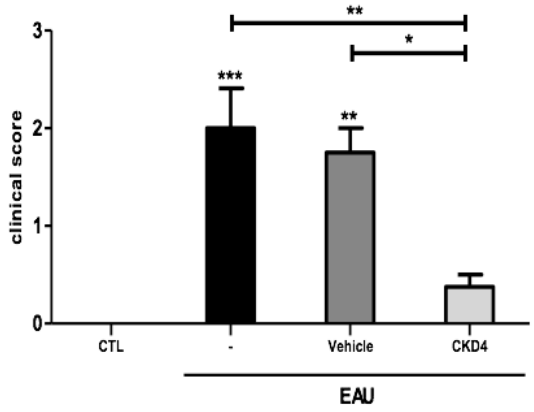


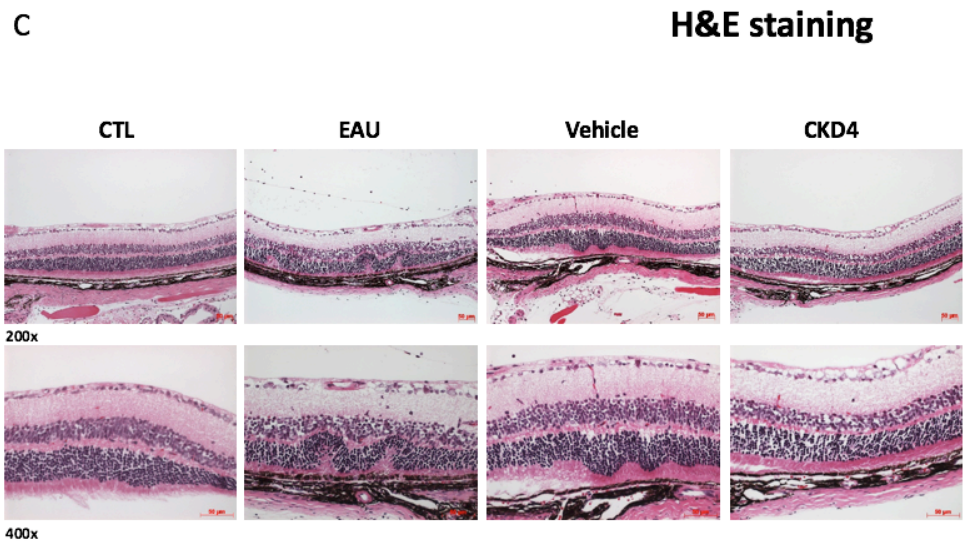
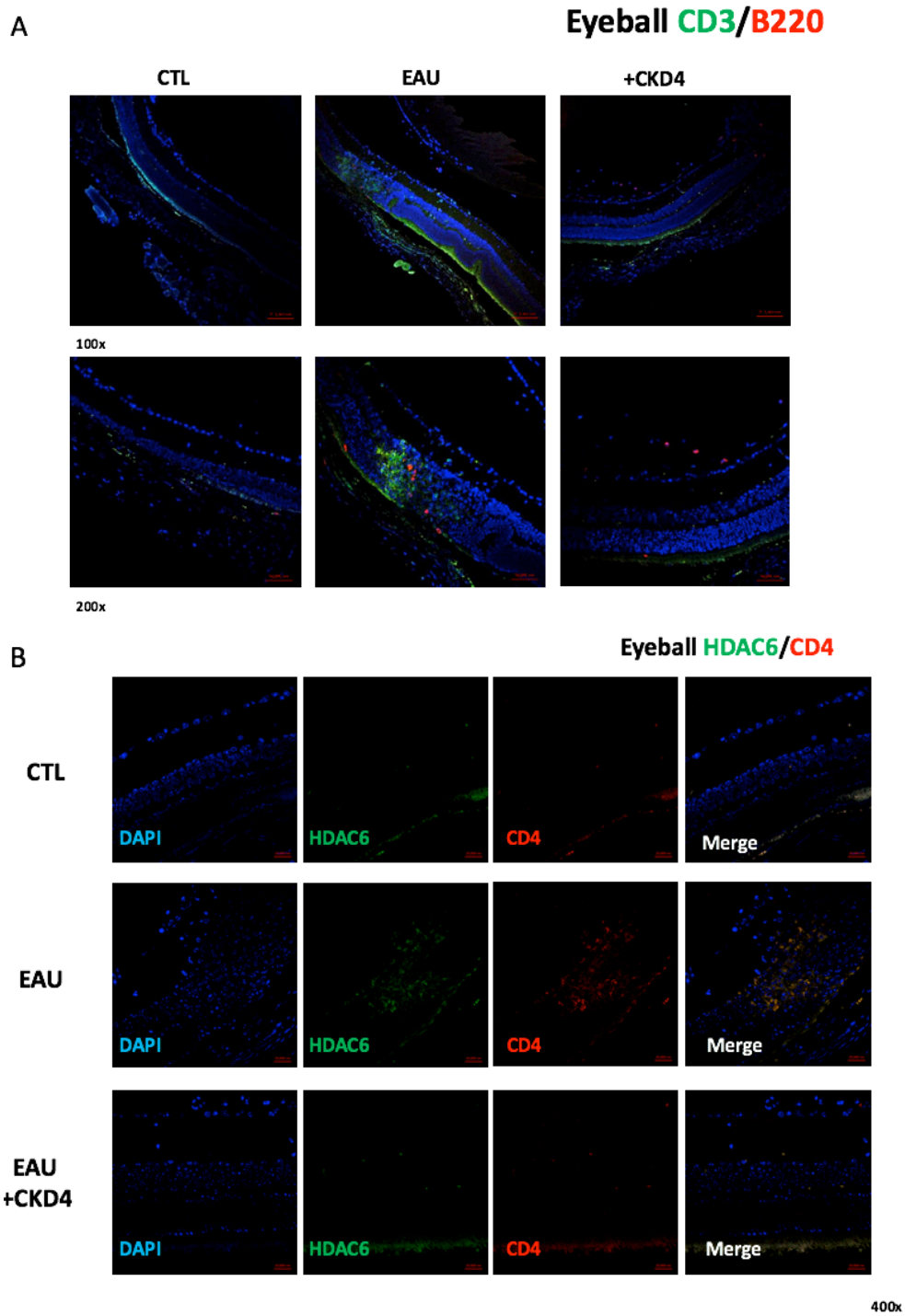
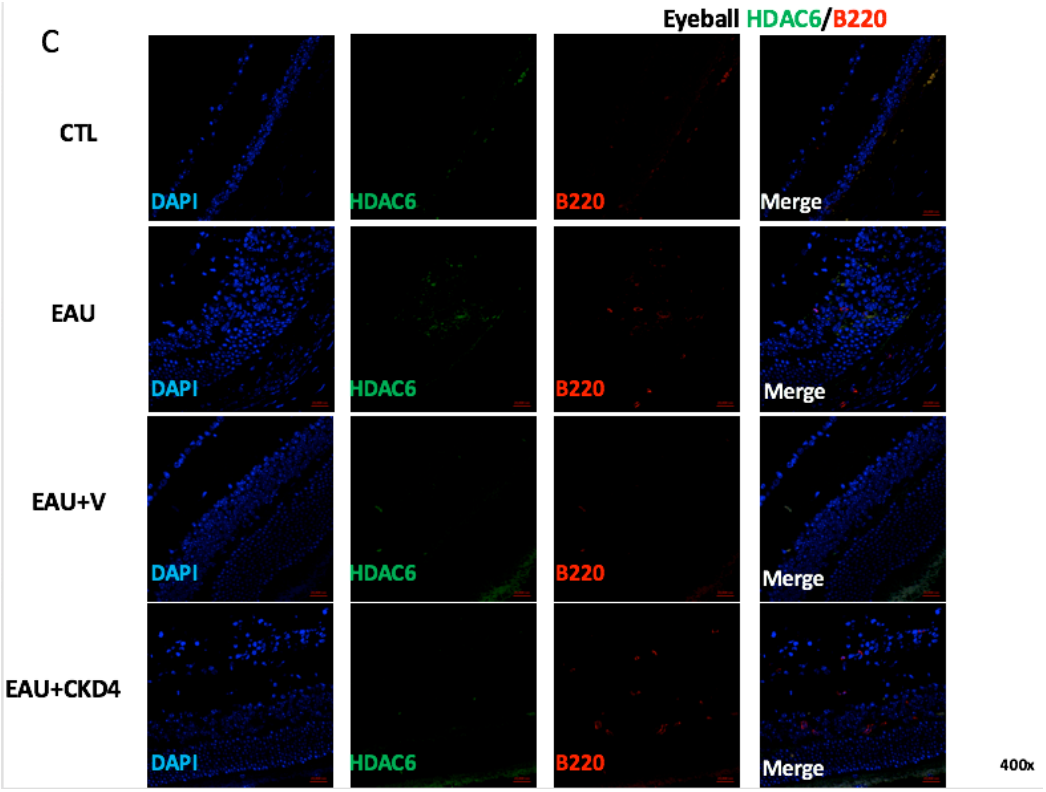


Figure 1. Effect of HDAC6 inhibitor, CKD-4, on the EAU mouse retina. (A) Representative images of funduscopic examinations show that the treatment with CKD-4 led to significant improvement of retinal vasculitis and chorioretinal infiltration when compared with the control group or vehicle treated group. (B) The treatment with CKD-4 led to significant reduction of clinical score in the EAU mice compared to the control group or the vehicle treated group. (C) The treatment with CKD-4 led to a significant reduction of inflammatory cellular infiltration. CTL, control group; EAU, experimental autoimmune uveitis; CKD-4, HDAC6 inhibitor; *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$

2. CKD-4 suppresses T lymphocyte infiltration in the mouse EAU.

Immunohistochemical staining analysis showed marked increase of CD3+ inflammatory cell infiltration in the mouse retina in EAU group, whereas treatment with CKD4 suppressed the number of CD3+ cells in the mouse retina. (figure 2A). However, the detection of B220 were only slightly increased in the EAU group and there was no significant difference between the EAU group and the CKD4 treated group. Also, EAU revealed prominent staining of HDAC6 and CD4+ markers within the mouse retina, with both markers being mainly co-localized within the same cells. The treatment with CKD4 significantly reduced detection of HDAC6 and CD4 within the inner and outer retina.(figure 2b). On the other hand, immunohistochemical staining pattern was quite different for HDAC6 and B220, both of which were detected in different cells within the retina in EAU model and the treatment with CKD4 seems to reduce detection of HDAC6, but does not seem to have any significant effect on B220. (Figure 2C) Treatment with CKD4 significantly increased the detection of acetylated alpha-tubulin in EAU model compared to EAU with no treatment. (Figure 2D)





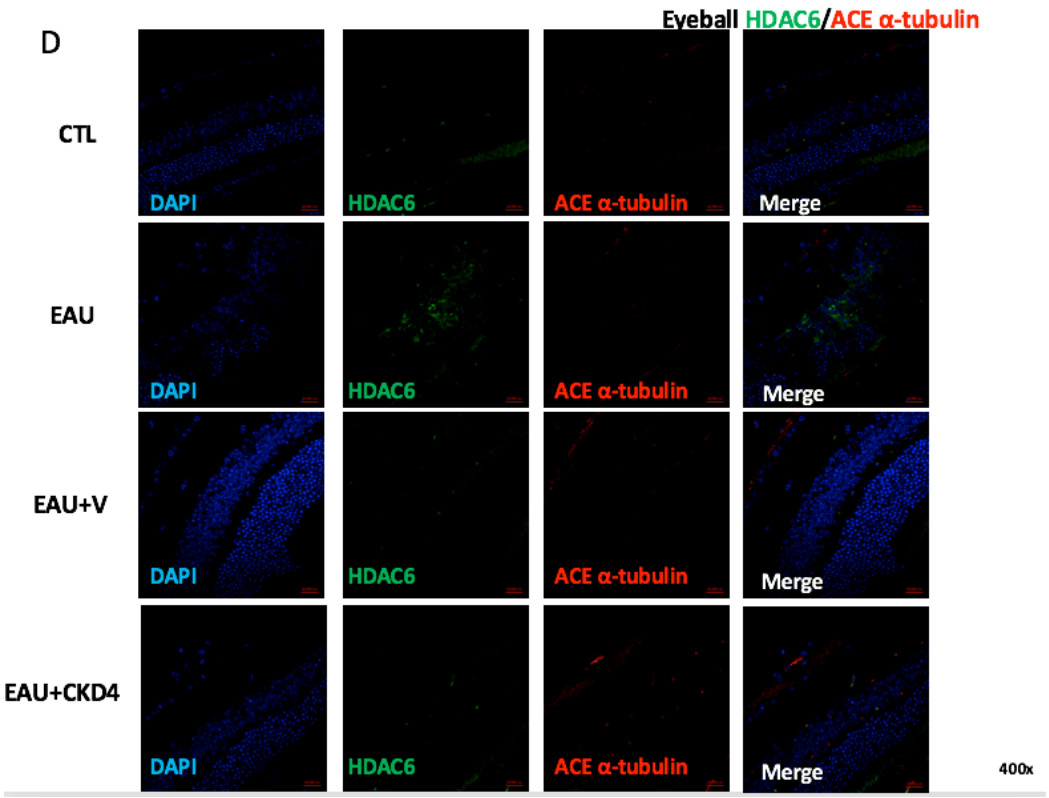


Figure 2. Effect of CKD-4 on the EAU mouse retina using immunohistochemical staining analysis. (A) CD3+ inflammatory cell infiltration is observed in the mouse retina in EAU group, whereas treatment with CKD4 suppressed the number of CD3+ cells in the mouse retina. (B) HDAC6 and CD4+ markers are co-localized within the same cells and their detection is decreased after treatment with CKD-4. (C) B220 detection was not significantly altered after CKD-4. (D) Acetylated alpha-tubulin was increased after treatment with CKD4. CTL, control group; DAPI, 4',6-diamidino-2-phenylindole; V, vehicle; HDAC6, histone deacetylase 6; ACE, acetylated; CKD-4, HDAC6 inhibitor;

3. Effects of CKD4 on the gene expression level of HDAC6 and inflammatory cytokines.

To further evaluate the beneficial effect of CKD4 on the EAU retina, real-time PCR was performed to measure the mRNA levels of HDAC6, IL-1β, IFN-γ, IL-17, and TNF-α. Total RNA was extracted from harvested cells and cDNA was synthesized by RT-PCR and then amplified. mRNA expression of HDAC6, IL-1β, IFN-γ, IL-17 and TNF-α were significantly increased in the EAU mouse retina and the treatment with CKD4 led to their significant decrease. (Figure 3)

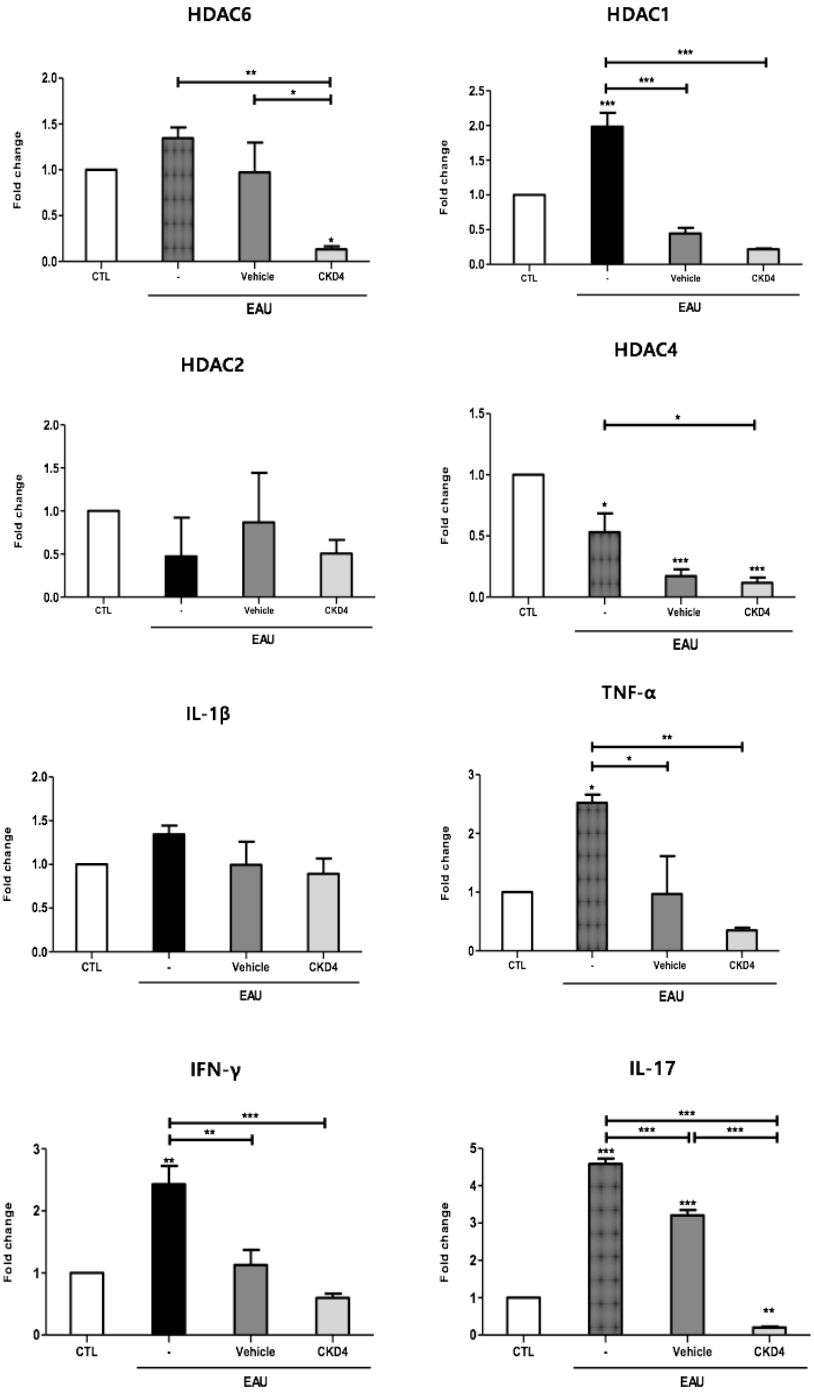


Figure 3. Effect of CKD-4 on the gene expression level of HDAC6 and various inflammatory cytokines by qPCR. mRNA expression of HDAC6, IFN-γ, IL-17, and TNF-α, were significantly increased in the EAU mouse retina and the treatment with CKD4 led to their significant decrease. CTL, control group; EAU, experimental autoimmune uveitis; HDAC, histone deacetylase; CKD-4, HDAC6 inhibitor; PCR, polymerase chain reaction. *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$

4. Effect of CKD4 on regulation of inflammatory cytokines

The level of inflammatory cytokines was upregulated in EAU mouse model and significantly downregulated after treatment with CKD4, as shown by ELISA.

IFN-γ, IL-17A, and TGF-β were significantly elevated in EAU mouse model. Treatment with CKD4 led to a significant reduction of IFN-γ and IL-17A, but not TGF-β (Fig.4).

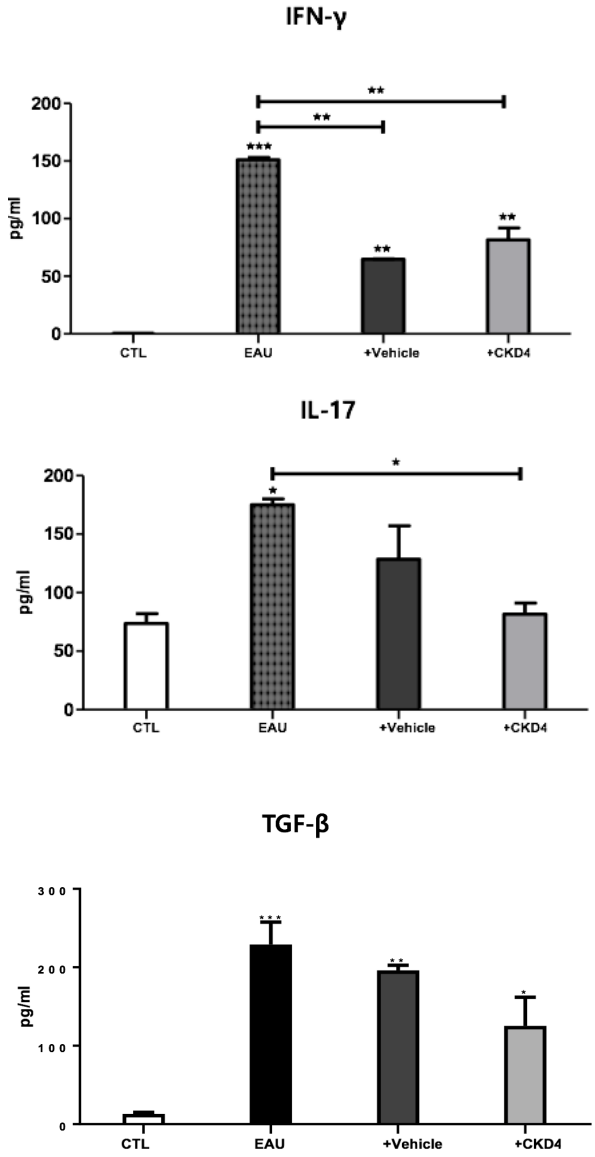
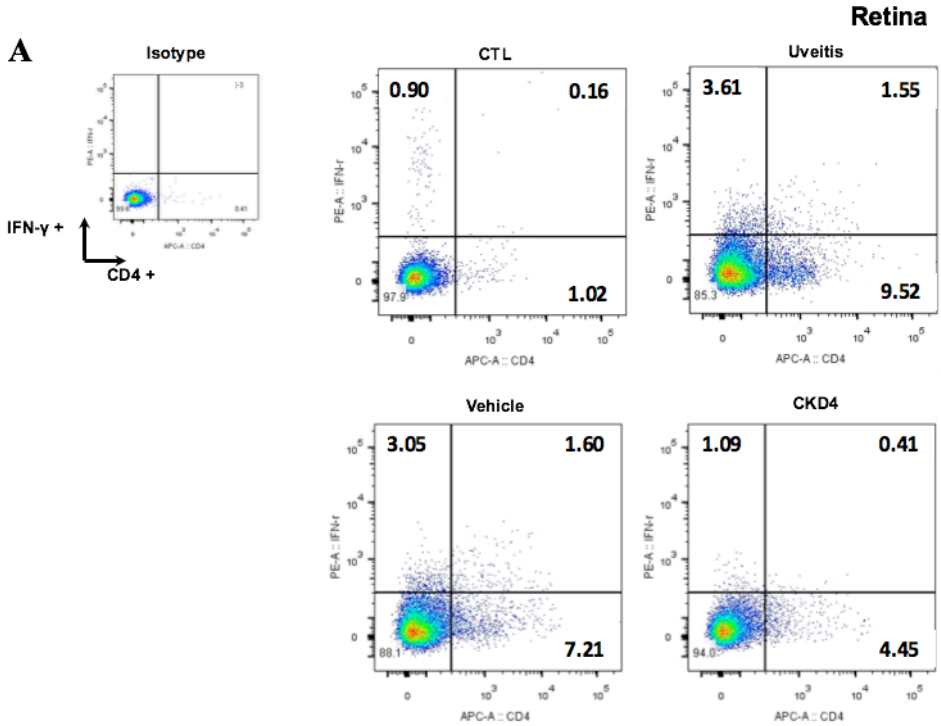
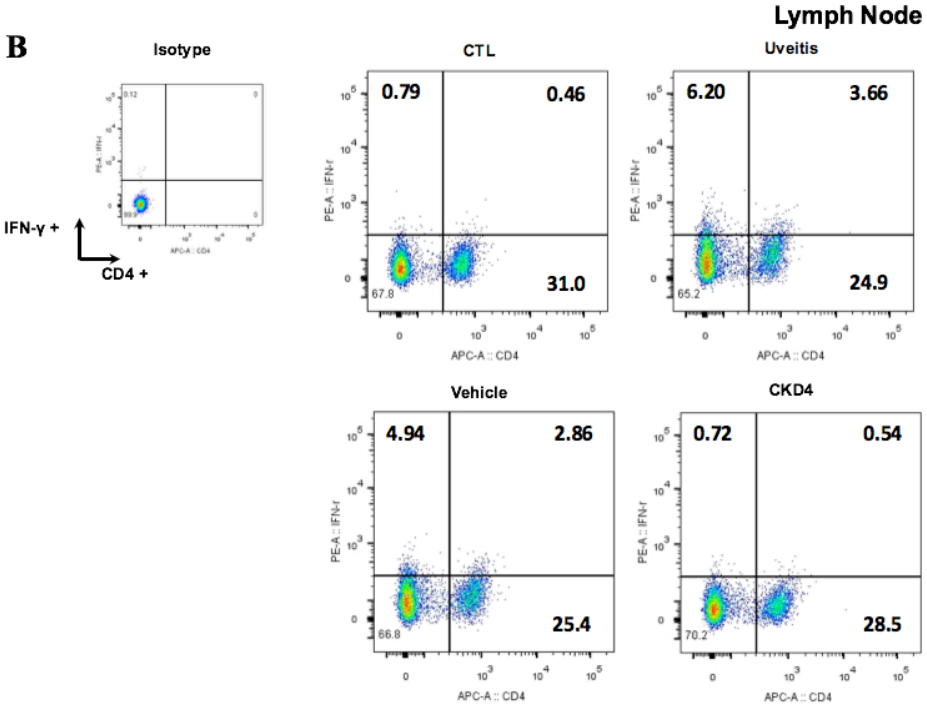


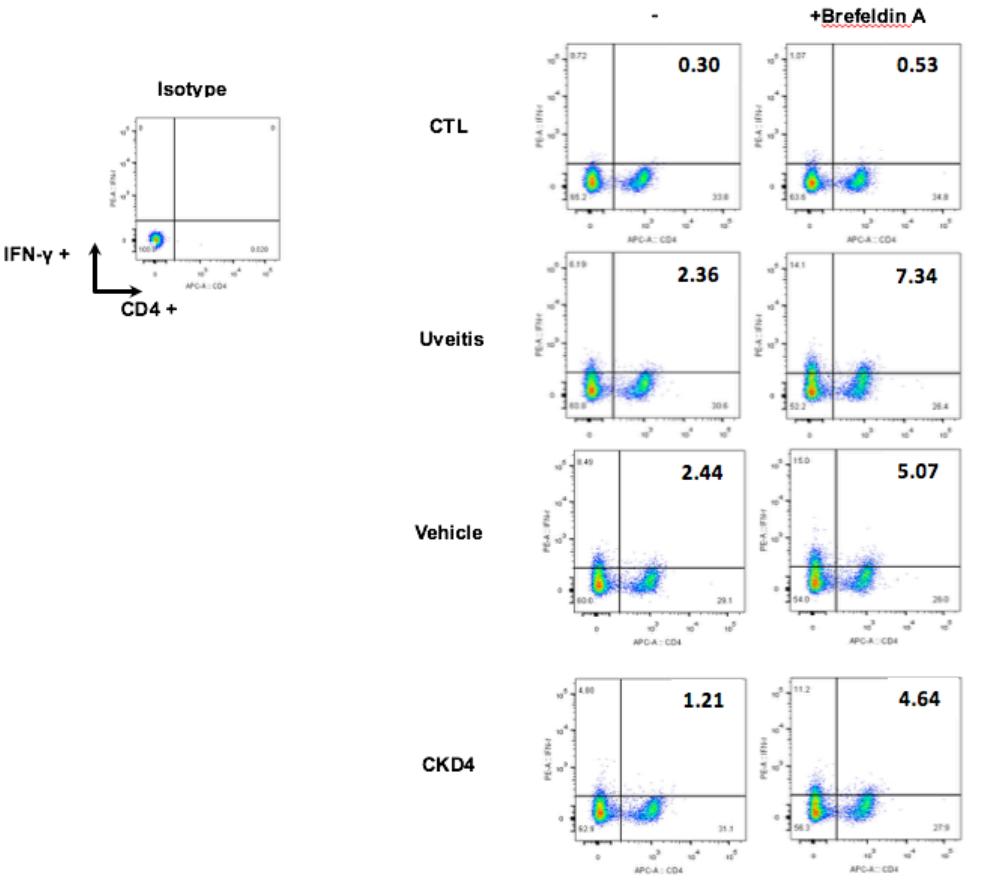
Figure 4. Effect of CKD-4 on the level of various inflammatory cytokines in EAU mouse retina shown by ELISA. Treatment with CKD4 led to a significant reduction of IFN-γ and IL-17A, but not TGF-β in EAU mouse model. *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$

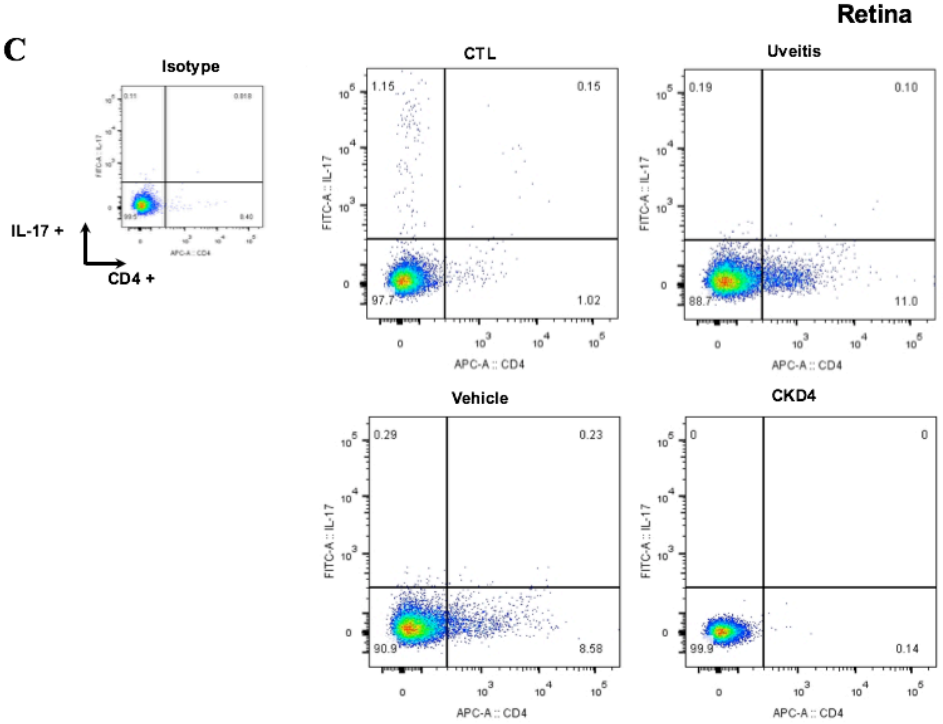
5. Immune cell identification and cytokine expression in the EAU mouse retina.

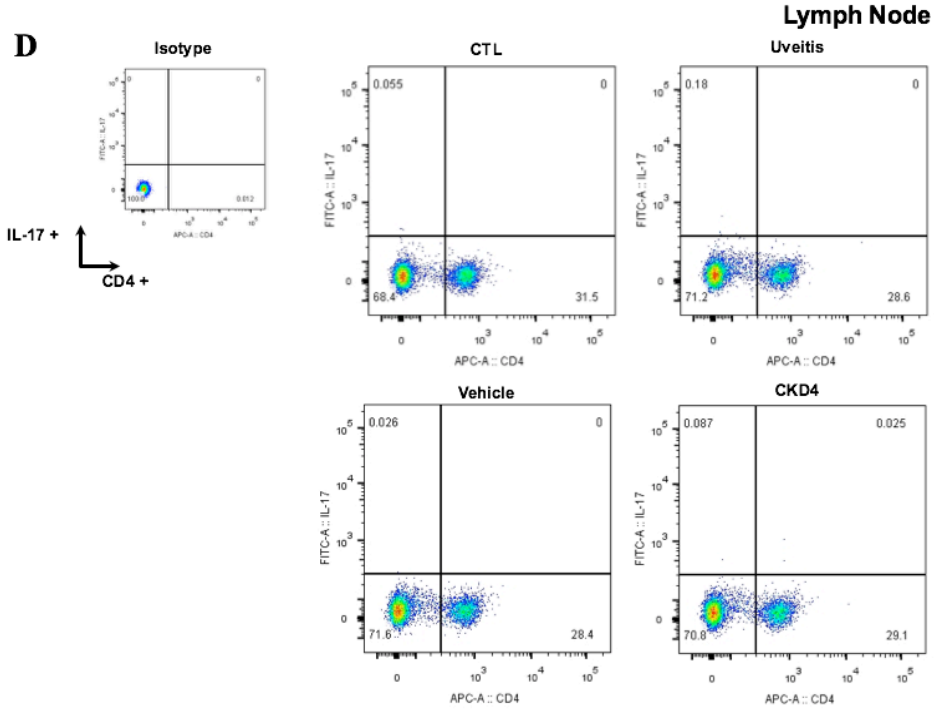
Treatment with CKD4 down regulated proinflammatory cytokines. I examined the T cell subsets in the retina and lymph nodes of EAU and CKD4 treated mice using flow cytometry and found that the number of CD4+IFN-γ+ cells were significantly increased in the EAU retina and the treatment with CKD4 eyedrops suppressed the percentage of CD4+IFN-γ+Th1 cells (Figure 5A). Similarly, CD4+IFN-γ+Th1 cells were increased in the lymph node of EAU mouse, and the treatment with CKD4 eyedrops resulted in its significant decrease(Figure 5B). CD4+IL-17+Th17 cells were also significantly increased and CKD4 significantly decreased the CD4+IL-17+Th17 cell population in the EAU mouse retina(Figure 5C) and lymph node(Figure 5D).











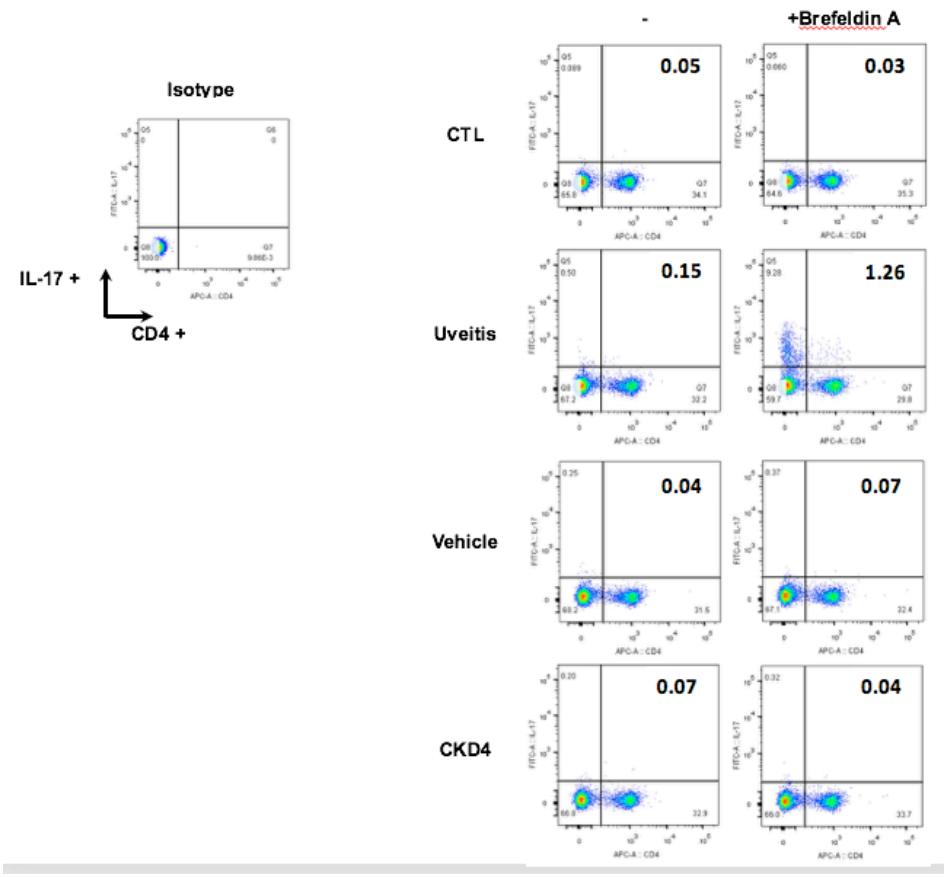


Figure 5. Immune cell identification and effect of CKD-4 on regulation of inflammatory cytokines by flow cytometry. (A) the number of CD4+ IFN-γ+cells were significantly increased, but the treatment with CKD4 eyedrops suppressed CD4+IFN-γ+Th1 cells in the EAU mouse retina(A) and lymph nodes(B). CD4+IL-17+Th17 cells were also significantly increased and CKD4 decreased the CD4+IL-17+Th17 cell population in the EAU mouse retina(C) and lymph node(D).

6. Downregulation of HDAC6 protein level in EAU mouse retina

HDAC6 protein level was assessed by western blot in control and EAU mouse retina. The treatment with CKD4 led to a prominent downregulation of HDAC6 protein level in EAU mouse retina (Figure 6). Acetylation of α-tubulin was noted immediately after 10 minutes in EAU after treatment with CKD4. (Figure 6)

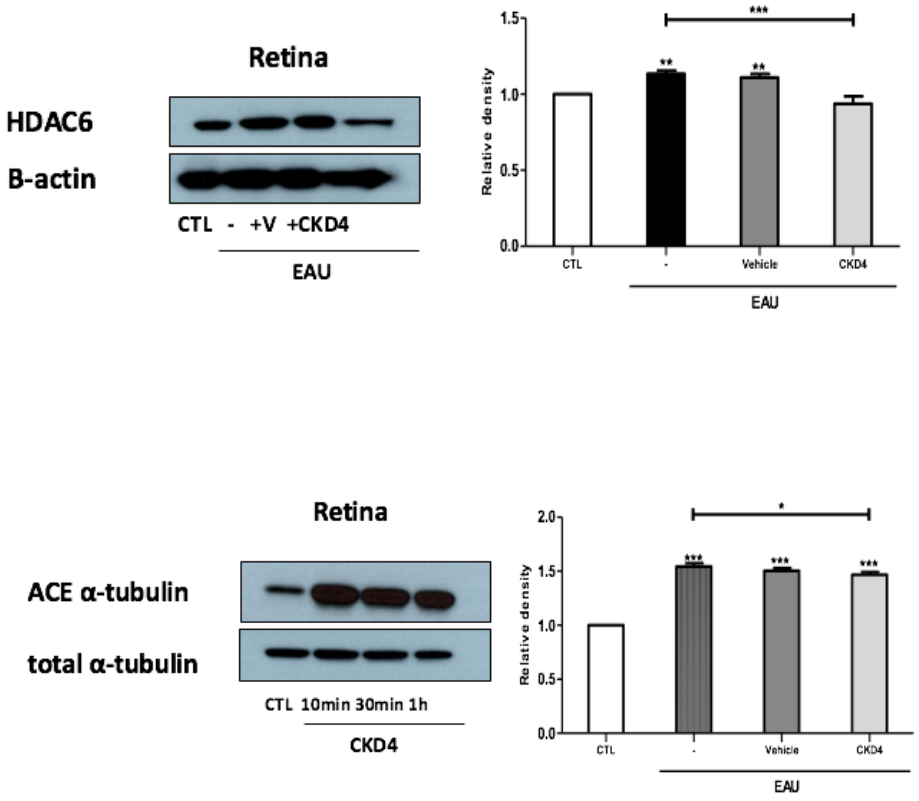


Figure 6. Effect of CKD-4 on the protein expression level of HDAC6 and acetylation of α-tubulin in EAU mouse retina by western blot.

CTL, control group; EAU, experimental autoimmune uveitis; HDAC, histone deacetylase; V, vehicle; CKD-4, HDAC6 inhibitor;

IV. DISCUSSION

Histone deacetylases (HDAC) plays important roles in the regulation of gene transcription.[17] Epigenetic regulation using HDAC inhibitors has been suggested in the treatment of autoimmune diseases.[27] However, the use of pan-HDAC inhibitor harbors potential for unwanted adverse effect. Recently, among many different classes of HDAC, HDAC6 was shown to play an important role in the regulation of autoimmune diseases.[31] Moreover, there has been no previous studies performed on the use of HDAC inhibitor for the treatment of autoimmune uveitis and in particular, HDAC6 inhibitor. Therefore, I hypothesized that a novel HDAC6 inhibitor, CKD4 could exert beneficial effect on the treatment of EAU. Our study showed that CKD4 significantly improved the clinical score of EAU and histopathological findings by ameliorating the progression of inflammation mostly driven by Th1 and Th17 cells in EAU mouse model.

IRBP induced EAU model was first reported in 1986 by Gery et al and has been used as a classic EAU model since then. IRBP acts as a strong uveitogenic antigen and in our study, IRBP $_{651\text{-}670}$ immunization successfully created EAU model as demonstrated by clinical features of autoimmune uveitis including chorioretinal infiltrations and vascular sheathing suggestive of retinal vasculitis. Histopathological finding also clearly confirmed the successful induction of autoimmune uveitis as revealed by severe inflammatory cell infiltration, disorganization of inner and outer retinal structure, retinal folding with detachments from the mouse retina. In EAU, CKD4 alleviated the clinical severity of experimental uveitis and limited the development of chorioretinitis and inflammatory cell infiltrations in the retina.

In autoimmune uveitis, T-lymphocytes, especially CD4+ T cells are known to play an important role.[9] These findings are in agreement with our results from qualitative analysis of immunohistochemistry showing that predominant population of inflammatory cells in the area of inflammation are T-cell origin. Furthermore, HDAC6 and CD4+ markers were mainly co-localized within the

same cells, indicating that HDAC6 were highly expressed in CD4+ T cells in inflammatory conditions. The treatment with CKD4 significantly reduced detection of HDAC6 and CD4 within the inner and outer retina. Treatment with CKD4 also increased the detection of acetylated alpha-tubulin in EAU model.

Based on the cytokine secretion profile, CD4+ T cells are further divided into Th1 and Th17 cells, both of which were shown to infiltrate the eye and play a crucial role in the pathogenesis of autoimmune uveitis.[9 32, 33] Th1 cells produce the proinflammatory cytokines IL-6, IFN-γ, TNF-α and promote macrophage activation and are involved in cell mediated immunity. Th17 cells produce Il-17 and IL-22, promoting inflammatory response in the retina during the course of EAU. Downregulation of these pro-inflammatory cytokines by HDAC inhibitors have been demonstrated in previous studies.[34, 35] To further explore this, the mRNA levels of proteins and cytokines by qRT-PCR were also measured and found elevated expression of HDAC1, HDAC6, IFN-γ, TNF-α and IL-17 in EAU. The treatment with CKD4 in EAU significantly reduced expression of HDAC6, IFN-γ, TNF-α and IL-17 in EAU. To identify which vital cytokines are involved in the anti-inflammatory effect of CKD4 for the treatment of EAU, the protein levels of pro-inflammatory mediators and anti-inflammatory mediators were measured. IFN-γ, IL-17A, and TGF-β were significantly elevated in EAU mouse model. Treatment with CKD4 led to a significant reduction of IFN-γ and IL-17A, but not TGF-β.

The T lymphocyte phenotypes in EAU mouse retina and lymph node were identified by flow cytometry, and the percentage of CD4+IFN-γ+ cells were significantly increased in the EAU retina and the treatment with CKD4 suppressed the percentage of CD4+IFN-γ+Th1 cells. Similarly, CD4+IL-17+ Th17 cells were also significantly increased in the retina of EAU mouse, and CKD4 significantly decreased the CD4+IL-17+Th17 cell population in the EAU mouse retina. Consequently, our data suggests that CKD-4 alleviates the experimental uveitis by decreasing the infiltration of Th1 and Th17 T cell population into the retina. In other words, pathogenic T-cell clonal expansion was suppressed by treatment with CKD-4. This finding is also supported by a previous study using pan-HDAC inhibitor.[36]

Findings from our study collectively suggest that CKD-4 alleviates EAU in mouse by repressing the function of two specific T cell population, Th1 and Th17 cells.

The potential mechanism by which HDAC6 inhibitor exerts its anti-inflammatory effects remains unclear at this point. Blockage of signaling pathways responsible for the effector T cell subset differentiation was suggested to be one possible mechanism.[37] In another study, proliferation of T effector cells was shown to be inhibited by restoring immune balance through T reg cells, as CTLA-4 expression and function of Treg cells were increased by HDAC6 inhibitor in rheumatoid arthritis mouse model.[38] HDAC6 plays an important role in cell motility by catalyzing α-tubulin deacetylation, which leads to retraction of the cilium of the cell and encourages cell motility, a necessary step prior to mitosis of the cell.[26] HDAC6 inhibitor could inhibit deacetylation of α-tubulin in inflammatory cells, thereby interfering with cell migration and immune response and exerting its anti-inflammatory action.[25] Based on these findings and our study result, I speculate that the downregulation of T-cell mediated inflammation in the EAU mouse retina following treatment with CKD-4 may be through the mechanism of inhibiting CD4 T-lymphocytes clonal expansion and migration into the inflamed retina through the action of CKD-4 on microtubule rearrangement.

There are several limitations in our study. HDAC6 inhibitor is also known to affect the function of regulatory T cells, but the role of regulatory T cells, and other cells such as macrophage and natural killer cells in EAU were not investigated in our study. Although possible hypothesis was suggested, the exact mechanism by which HDAC6 remains to be seen in the future studies. Potential adverse effects associated with the use of HDAC6 inhibitor could not be evaluated in our study. Further studies are needed to clarify the immunosuppressive mechanisms of CKD-4 in EAU and establish its role as a potential therapeutic agent for autoimmune uveitis in the future.

V. CONCLUSION

In this study, HDAC6 inhibitor, CKD4, ameliorated the clinical severity of experimental uveitis in EAU. It attenuated the infiltration of CD4+ T lymphocytes in the retina and reduced the expression of HDAC6 and various inflammatory cytokines including IFN-γ, IL-17, and TNF-α. Thus, our study suggested the potential therapeutic role of CKD4 in the treatment of autoimmune uveitis.

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ABSTRACT(IN KOREAN)

실험적으로 유도된 마우스 포도막염 모델에서 히스톤 탈아세틸화 6 억제제의 치료효과

<지도교수 고 형 준>

연세대학교 대학원 의학과

김 민

자가면역성 포도막염은 안구내 조직에서 반복적이고 만성적으로 염증을 유발하고 진행 시에 시력저하 및 실명을 유발할 수 있는 심각한 질환이다. 병리기전으로는 활동성의 면역세포들이 안구를 침범하게 되는데, 특히 Th1, Th17 세포들이 자가면역 포도막염의 병태생리에서 중요한 역할을 한다. 현재 포도막염의 치료로는 스테로이드와 면역억제제등이 사용되고 있지만, 장기간 사용에 따른 안구 및 전신적 부작용이 따르고, 이러한 치료에도 반응하지 않는 불응성 포도막염의 치료를 위해서 더 효과적인 치료방법의 개발이 요구된다. 최근 히스톤 탈아세틸화 6가 염증성 질환 및 자가면역질환의 병태생리에 관여하는 것으로 밝혀졌고 이를 억제하였을때 염증세포에서 인터루킨6 와 TNF-α 의 감소를 유도하는 것으로 밝혀졌다. 따라서 본 연구에서는 실험적으로 유도된 마우스포도막염 모델에서 히스톤 탈아세틸화 6 억제제인 CKD4 가 포도막염에서 치료효과를

보일 수 있는지 알아보고자 하였다. 실험적으로 유도된 포도막염 모델에서는 임상적으로 관찰되는 포도막염의 전형적인 소견들이 관찰됨을 확인할 수 있었고, 특히 CD4+ T 세포에서 히스톤 탈아세틸화6가 증가하였다. CKD4 를 점안하였을때 포도막염의 임상적 소견들이 호전되었고 조직검사상에서도 염증성 세포의 침윤과 CD4+ 세포에서 히스톤 탈아세틸화6 가 감소하는 것을 확인하였다. CKD4 의 치료후 염증성 사이토카인인 인터페론 감마와 인터루킨 17이 의미있게 감소하였고, 유세포분석상 인터페론 감마와 인터루킨 17을 분비하는 Th1 세포와 Th17 세포가 감소하는 것을 확인하였다. 따라서 본 연구결과는 자가면역 포도막염에서 히스톤 탈아세틸화 6 억제제인 CKD4 의 치료제로서의 가능성을 시사하는 것이라 하겠다.

핵심되는 말 : 포도막염, 자가면역, 히스톤 탈아세틸화 6, 히스톤 탈아세틸화 6 억제제, 염증