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Dehydrodiconiferyl alcohol (DHCA) modulates the differentiation of Th17 and Th1 cells and suppresses experimental autoimmune encephalomyelitis

Junghun Lee, Jinyong Choi, Wonwoo Lee, Kyeongryang Ko, Sunyoung Kim*

Department of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea

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ABSTRACT

Dehydrodiconiferyl alcohol (DHCA), originally isolated from the stems of *Cucurbita moschata*, has previously been shown to exhibit anti-adipogenic and anti-lipogenic effects in 3T3-L1 cells and primary mouse embryonic fibroblasts (MEFs) (Lee et al., 2012). Here, we investigated whether synthetic DHCA could suppress the CD4 T helper 17 (Th17)-mediated production of the interleukin (IL)-17 protein. The results from RT-qPCR suggest that DHCA-mediated down-regulation of IL-17 occurred at the transcriptional level by suppressing the expression of RAR-related orphan receptor (ROR)γt, the master transcription factor involved in the differentiation of Th17 cells. Furthermore, such inhibition was mediated by the suppression of NF-κB activity. DHCA also inhibited the Th1-mediated production of interferon (IFN) γ by controlling the expression of a key transcription factor known to regulate the production of this cytokine, T-bet. In the mouse experimental autoimmune encephalomyelitis (EAE) model, DHCA showed significant therapeutic effects by inhibiting the infiltration of immune cells into the spinal cords, decreasing the differentiation of pathogenic Th17 and Th1 cells, suppressing the expression of various pro-inflammatory cytokines, and eventually ameliorating the clinical symptoms of EAE mice. Taken together, our data indicate that DHCA may be a potential candidate as an agent for the control of Th17 and Th1-mediated inflammatory diseases.

1. Introduction

Multiple sclerosis (MS) is one of the most severe autoimmune diseases. MS occurs primarily by the demyelination of axons in the brain and spinal cord, which could be mediated by various cell types, subsequently resulting in a variety of neurological symptoms such as cognitive disabilities, unstable mood or depression (Noseworthy et al., 2000).

Approximately, 2.5 million people suffer from MS worldwide, and the number of patients has been gradually increasing (Pugliatti et al., 2002). Current therapeutics have limited efficacies, as these drugs are generally used for the alleviation of pain symptoms rather than the complete recovery in the absence of severe side effects (Virley, 2005). For example, methotrexate and mitoxantrone ameliorate the relapse status of MS by up to 30%, but these drugs also show a high level of toxicity such as organ damage, stomatitis, hair loss and immunosuppression. Thus, efficient drugs with minimal side effects are highly needed.

Excessive inflammatory responses play a significant role in the development and progression of MS. A variety of immune cells are critically involved in the destruction of myelinated axons and subsequently to the central nervous system dysfunction. Among these various cell types, macrophages/microglia and CD4 T helper lymphocytes have been shown to directly damage neurons and other components of nervous system. Macrophages and microglial cells can be activated by a broad range of stimulations such as pathogens, reactive oxygen species (ROS) and autoantigens, resulting in not only the production of various pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, but also the activation of pathogenic T helper cells such as Th17 and Th1 cells. These subtypes of helper T cells have been reported to play a significant role in the pathogenesis of MS and such activation triggers the demyelination of axons and apoptosis of neuronal cells. (Sospedra and Martin, 2005; Gilgun-Sherki et al., 2004; Fletcher et al., 2010).

CD4 Th17 cells are recently characterized subtype of CD4 helper T cells, differentiated from naïve CD4 T cells by the stimulation

* Corresponding author at: Department of Biological Sciences, Building 504, Seoul National University, Gwanak-Gu, Seoul 151–742, Republic of Korea. Fax: +82 2 875 0907.
E-mail address: sunyoung@snu.ac.kr (S. Kim).

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of TCR activation in the presence of TGF-β and IL-6, subsequently leading to the expression of RORγt and RORα transcription factors (Yang et al., 2008). These proteins then induce the production of IL-17 cytokine (Korn et al., 2009; Bettelli et al., 2008). Several studies reported that the expression of RORγt is controlled by the transcription factor NF-κB and NF-κB-RORγt transcriptional axis plays a critical role in the induction of Th17 cells (Ruan et al., 2011; Chen et al., 2011).

It has been reported that the Th17-mediated production of IL-17 plays an important role(s) in the pathogenesis of MS, as mice carrying the deletion in this gene are resistant to rodent experimental models of MS (Korn et al., 2009). Upregulated levels of IL-17 have been shown in MS patients as well (McFarland and Martin, 2007). Other studies suggested that IL-17 amplifies inflammatory responses to induce the apoptosis of various cells including neurons (Gold and Luhder, 2008). Overall, IL-17 produced by Th17 cells might govern the inflammatory responses in the pathogenesis of MS.

After the discovery of pathogenic roles of Th17-mediated production of IL-17, various attempts are currently underway to discover novel therapeutics targeting IL-17 and RORγt (Hu et al., 2011). Recently, humanized antibodies against IL-17 or small molecules specifically inhibiting the activity of RORγt have been shown to improve the severity of EAE (Huh et al., 2011; Xu et al., 2011; Solt et al., 2011; Fan et al., 2011).

Animal models for the investigation of MS have been well established (Stromnes and Goverman, 2006; Miller et al., 2010). In particular, EAE mimics human demyelinating diseases. Autoreactive T cells specifically recognizing myelin-related proteins could be generated by the injection of the protein segments of myelin proteins, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein, in the presence of immune-activating adjuvants such as pertussis toxin. Antigen presenting cells phagocytose and present these peptides as an antigen, followed by the activation of various adaptive immune cells including Th17 cells. These cells subsequently promote excessive immune responses through the induction of various pro-inflammatory mediators, resulting in the destruction of myelin protein and dysfunction of neurons.

Dehydrodiconiferyl alcohol (DHCA), originally isolated from the stems of *Cucurbita moschata*, has been shown to contain anti-adipogenic and anti-lipogenic activities (Lee et al., 2012). In this study, it was investigated whether synthetic DHCA could suppress Th17 and Th1 cell differentiation, using primary CD4 T cells. Our data suggest that DHCA down-regulated the production of IL-17 and IFNγ by controlling the expression of RORγt and T-bet, respectively. The data from Western blot analysis showed that the inhibition of RORγt expression was mediated by inhibiting NF-κB activity. Consistent with these *in vitro* data, DHCA ameliorated the severity of the clinical symptoms of EAE by inhibiting the infiltration of immune cells, down-regulating the generation of MOG-specific Th17 and Th1 cells and suppressing the expression of various pro-inflammatory mediators in the spinal cord. Taken together, these data suggested that DHCA might be a useful starting point for developing efficient therapeutics for MS.

2. Materials and methods

2.1. Cell culture and reagents

Synthetic DHCA was produced by previously described methods (Hu and Jeong, 2006) and obtained from Biochemnet (Seoul, Korea). All antibodies and recombinant proteins used for the CD4 T cell isolation and Th17/Th1 differentiation were purchased from eBioscience (San Diego, CA, USA), RPMI and FBS were purchased from Cellgro (Manassas, VA, USA), streptomycin and penicillin were obtained from Gibco (Grand Island, NY, USA), myelin oligodendrocyte glycoprotein (MOG) and Pertussis toxin (PTX) from Sigma–Aldrich (St. Louis, MO, USA) and Freud's incomplete adjuvant and heat-killed *Mycobacterium tuberculosis* H37Ra were purchased from DIFCO (Detroit, MT, USA).

2.2. Experimental animals

All animal protocols were performed in compliance with the guidelines set by the Institutional Animal Care and Use Committee of Seoul National University. Seven-week-old male C57BL/6 mice were purchased from Orientbio Inc. (Seongnam, Korea), and housed in an air-conditioned facility at Seoul National University with a fixed 12 h light/dark cycle. To prepare mouse splenocytes, mice were euthanized with CO_2 and spleens were isolated for further procedures.

2.3. Preparation and differentiation of primary naïve CD4 T cells

Naïve CD4 T cells ($CD4^+$ $CD44^{low}$ $CD25^{low}$) were prepared and differentiated into Th17 and Th1 cells by employing a modified version of the previously described method, respectively (Xiao et al., 2008; Jager et al., 2009). Briefly, to isolate naïve CD4 T cells, mouse splenocytes were obtained from the spleens of C57BL/J mice by physically mincing the tissue through a 40 μm nylon cell strainer (BD bioscience, Franklin Lakes, NJ, USA), and were subsequently cultured in RPMI-1640 in the presence of 200 U/ml penicillin G, 150 U/ml streptomycin and 10% FBS. The splenocytes were subsequently stained by the incubation with anti-CD4, anti-CD25 and anti-CD44 antibodies for 1 h on ice. The cells were then washed twice with cold PBS and subjected to a sorting procedure using a FACS Aria II (BD bioscience). $CD4^+$ $CD25^{low}$ $CD44^{low}$ cell populations were selected and purified to obtain naïve CD4 T helper cells.

To induce the differentiation of Th17 cells, sorted naïve CD4 T cells (5×10^5 cells) were cultured and stimulated with Th17 conditioning media which contained anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), TGF-β (1 ng/ml), IL-6 (30 ng/ml), anti-IFNγ (5 μg/ml) and anti-IL-4 (5 μg/ml). For the differentiation of Th1 cells, naïve CD4 T cells were treated with media containing anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), IL-2 (10 units/ml), IL-12 (10 ng/ml) and anti-IL-4 (5 μg/ml).

To measure the protein level of IL-17 or IFNγ, naïve CD4 T cells were stimulated with Th17 or Th1 conditioning media in the presence or absence of various concentrations of DHCA for 3 days. Supernatants were collected followed by IL-17 or IFNγ specific ELISA, respectively.

To determine the RNA level of IL-17A, IL-17F, RORγt, RORα, IFNγ and T-bet, total RNAs were obtained using TRIzol reagents (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Total cDNA was subsequently synthesized, using AMV reverse transcriptase (Takara, Tokyo, Japan), and subjected to real-time quantitative PCR (Thermal Cycler Device Real Time System, Takara) using SYBR Premix Ex Taq™ reagent (Takara). The cycle threshold (Ct) values of each gene were obtained and normalized to that of GAPDH. The following specific primers were used to detect the RNA levels of the respective genes:

IL-17A
forward 5′-TTT AAC TCC CTT GGC GCA AAA-3′
reverse 5′-CTT TCC CTC CGC ATT GAC AC-3′
IL-17F
forward 5′-GGG AGG TAG CAG CTC GGA AGA-3′
reverse 5′-GGT GGA CAA TGG GCT TGA CA-3′
RORγt
forward 5′-GAC CCA CAC CTC ACA AAT TGA-3′
reverse 5′-AGT AGG CCA CAT TAC ACT GCT-3′

RORα
forward 5′-GTG GAG ACA AAT CGT CAG GA-3′
reverse 5′-GAC ATC CGA CCA AAC TTG AC-3′
IFNγ
forward 5′-GAA CTG GCA AAA GGA TGG TGA-3′
reverse 5′-TGT GGG TTG TTG ACC TCA AAC-3′
T-bet
forward 5′-TGT GGA TGT GGT CTT GGT GG-3′
reverse 5′-ATA AGC GGT TCC CTG GCA T-3′
GAPDH
forward 5′-AGC CTC GTC CCG TAG ACA A-3′
reverse 5′-AAT CTC CAC TTT GCC ACT GC-3′.

2.4. Western blot analysis

Western blot analysis was performed as previously described (Lee et al., 2012). Briefly, naïve CD4 T cells were plated 5×10^5 cells/well in 48-well plates and stimulated with Th17 differentiation media in the presence of DHCA. Three hours later, cells were washed with cold PBS. Cell pellets were then collected by centrifugation at $800 \times g$ for 5 min and lysed using Phosphosafe Extraction buffer (Novagen, Madison, WI, USA). Cell lysates were obtained by centrifugation at $12{,}000 \times g$ for 10 min and total protein contents in the supernatant were determined by the Bradford assay. Aliquots of 20 μg proteins each were subjected to SDS-PAGE and the separated proteins were subsequently electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with Tris-buffered salin/ 0.05% Tween 20 (TBST) containing antibodies against I-κB (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin (1:100,000, Sigma) at 4 °C overnight. Membranes were then incubated with horsera dish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:100,000, Sigma) and visualized on films using ECL solution (Milipore, Billerica, MA, USA).

2.5. Mouse EAE model

EAE was induced in mice by immunization with MOG peptide$_{35-55}$ as previously described (Stromnes and Goverman, 2006). Antigen/adjuvant mixture solution was prepared by emulsifying 200 μg of MOG$_{35-55}$ peptide into complete Freund's adjuvant (CFA) containing 500 μg of heat-killed *Mycobacterium tuberculosis* H37Ra. C57BL/6 mice were anesthetized and 50 μl of the emulsified mixture was injected subcutaneously into two different sites on each hind flank of each mouse (designated as day 0). Pertussis toxin (PTX) powder was dissolved in PBS and intraperitoneally injected at day 0 and day 2. Clinical scores of each mouse were measured once a day. EAE symptoms were assessed and graded according to a previous report (Stromnes and Goverman, 2006): 0, normal; 0.5, partially limp tail; 1, fully paralyzed tail; 2, loss of normal movement; 2.5, one hind limb paralyzed; 3, both hind limbs paralyzed; 4, paralyzed forelimb; 5, moribund or death.

2.6. Hematoxylin & eosin staining

EAE-induced mice were sacrificed at day 21 post-immunization. For hematoxylin and eosin (H&E) staining, spinal cord tissues were isolated and fixed in 10% neutral buffered formalin solution. Fixed tissues were decalcified using Calci Clear Rapid (Natural Diagnostics, Somerville, NJ, USA), embedded in paraffin, sectioned into 4-μm thick slices and stained with H&E. Immune cell infiltration into the spinal cord was measured by counting the lesions of each spinal cord sample.

2.7. MOG re-stimulation

At day 21 post-immunization, splenocytes from the spleens of the sacrificed mice were obtained and cultured in RPMI media containing 10% FBS. The cells were plated at 5×10^6 in 24-well plates, followed by treatment with 10 μg/ml MOG$_{35-55}$ peptide for 3 days. The protein level of IL-17 or IFNγ was analyzed in the culture supernatants using IL-17 or IFNγ-specific ELISA, respectively.

2.8. RT-qPCR

To isolate total RNA from the spinal cords, frozen spinal cord tissues were homogenized, vortexed vigorously and incubated in TRIzol reagent according to the manufacturer's instructions. Total cDNA was synthesized using AMV reverse transcriptase (Takara) followed by RT-qPCR (Thermal Cycler Device Real Time System, Takara) using SYBR Premix Ex Taq^{TM} reagent (Takara). The cycle threshold (Ct) values of each gene were obtained and normalized to that of GAPDH. Primers used to detect the RNA levels of IL-17A, IFNγ, RORγt and GAPDH were the same as those used in *in vitro* study. The specific primer sequence to analyze the level of granulocyte-macrophage colony-stimulating factor (GM-CSF) is as follows:

GM-CSF
forward 5′-TCG TCT CTA ACG AGT TCT CCT-3′
reverse 5′-CCT GCT CGA ATA T CT TCA GGC-3′

2.9. Statistical analysis

All individual experiments were repeated at least 3 times. All values from the *in vitro* experiments are presented as the mean ± SD of triplicate samples and the differences between two groups were statistically analyzed using one-way ANOVA. *p*-Values were calculated and a value less than 0.05 was considered statistically significant. The differences between the *in vivo* experimental groups were analyzed using two-way ANOVA followed by the Bonferroni *post-hoc* test.

3. Results

3.1. Effects of DHCA on the differentiation of CD4 Th17 cells

To test whether DHCA could control the differentiation of Th17 cells, naïve CD4 T cells (CD4$^+$ CD44^{low} CD25^{low}) were isolated from splenocytes using FACS Aria II followed by the differentiation into Th17 cells in the presence of various concentrations of DHCA for 3 days. The culture supernatants were obtained and the protein level of IL-17 was analyzed using IL-17 specific ELISA. Naïve CD4 T cells exhibited an undetectable level of the IL-17 protein, while it was highly produced in that of differentiated Th17 cells. DHCA, however, significantly decreased the production of IL-17 in a dose-dependent manner, with an estimated IC$_{50}$ of 1.67 μM (Fig. 1A).

To examine the time kinetics of the DHCA-mediated suppression of IL-17, naïve CD4 T cells were stimulated with TGF-β and IL-6 in the presence or absence of DHCA for 1, 2 and 3 days. When cells were induced to differentiate into Th17 cells, the production level of IL-17 was up-regulated starting from day 1 and was markedly increased by day 3, when compared with that of undifferentiated control cells (Fig. 1B). The pattern of the IL-17 protein production in DHCA-treated cells was similar but the fold of increase was significantly lower than that in untreated cells on day 2 and 3 (Fig. 1B).

To test if DHCA might exhibit such inhibitory effects by directly interacting with cytokines or antibodies present in the stimulation media, naïve CD4 T cells were stimulated with TGF-β, IL-6 to induce Th17 differentiation. Three days later, cells were washed with fresh culture media followed by treatment with various concentrations

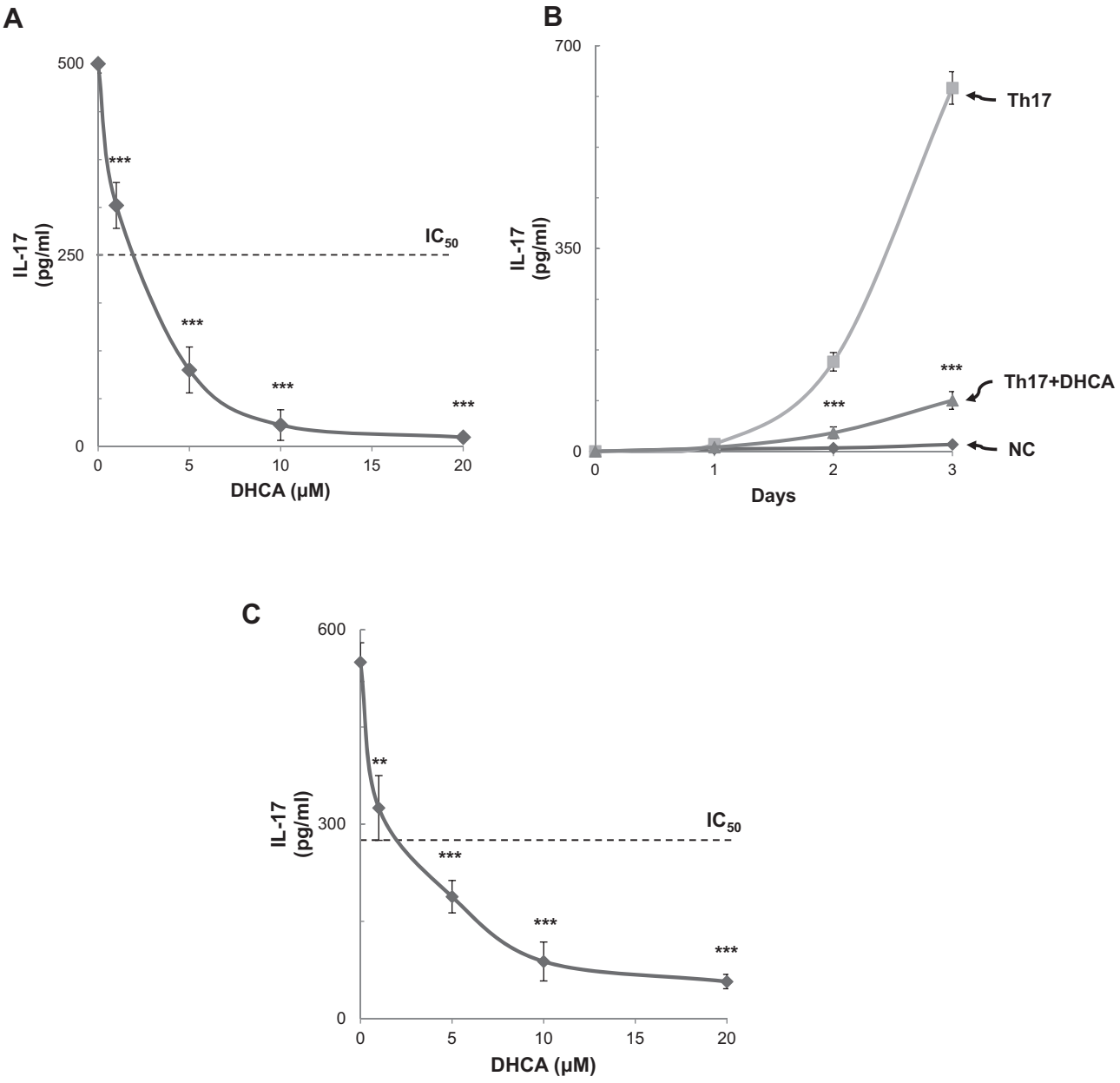

Fig. 1. Effects of DHCA on the production of IL-17 A and B. Naïve CD4 T cells isolated from mouse splenocytes were stimulated with TGF-β and IL-6 in the presence of various concentrations of DHCA for three days, or in the presence of 20 μM DHCA for selected time points . The culture supernatants were collected and analyzed with IL-17 specific ELISA. C. Naïve CD4 T cells were differentiated into Th17 cells for 3 days, and then treated with various concentrations of DHCA. The culture supernatants were subjected to IL-17 ELISA.

of DHCA for 24 h. The culture supernatants were collected followed by ELISA for IL-17. As shown in Fig. 1C, the protein level of IL-17 was markedly increased in differentiated Th17 cells, but DHCA treatment down-regulated the production of IL-17 in a dose-dependent manner. Taken together, these data indicated that DHCA controlled the Th17-mediated production of IL-17 in a dose- and time-dependent manner without interacting with molecules contained in the Th17 stimulation media.

3.2. Effects of DHCA on the key transcription factors involved in the Th17 cell differentiation

IL-17A and IL-17F are well known to play a key role in the pathogenesis of Th17-mediated inflammatory diseases and their expression has been shown to be regulated mainly at the transcription level by transcription factors, RORγt and RORα (Yang et al., 2008; Korn et al., 2009; Ivanov et al., 2006). To investigate whether DHCA-mediated inhibition of IL-17 production occurred at the transcription level, naïve CD4 T cells were stimulated with TGF-β, IL-6 and various concentrations of DHCA for 3 days. Total RNA was prepared and RT-qPCR was performed using specific primers for IL-17A and IL-17F, respectively. The basal level of both IL-17A and IL-17F was very low, while their expression level was significantly increased in differentiated Th17 cells. DHCA treatment, however, down-regulated the RNA level of both genes in a dose-dependent manner and consistent with the protein data, treatment with 20 μM DHCA decreased the expression level of IL-17A and IL-17F to an almost undetectable level (Fig. 2A).

To understand the mechanism underlying this phenomenon, the RNA level of RORγt and RORα was analyzed as these master transcription factors have been shown to be regulated at the RNA level to control the expression of IL-17 (Yang et al., 2008). RNA used in

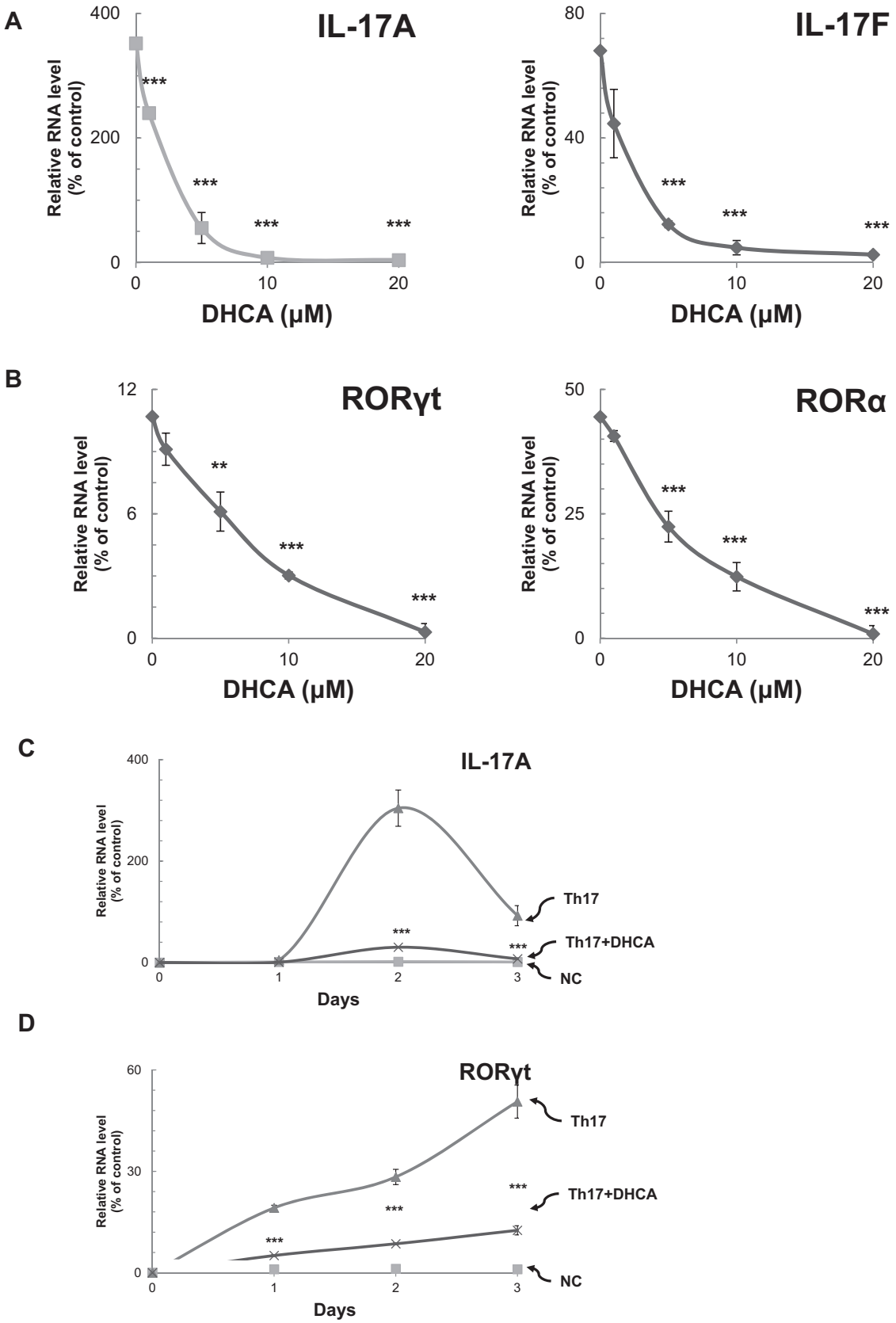

Fig. 2. Effects of DHCA on the expression of IL-17A and IL-17F A. Effects of DHCA on the expression of IL-17A and IL-17F. Naïve CD4 T cells were purified followed by stimulation with TGF-β and IL-6 in the presence of various concentrations of DHCA. Three days later, total RNA was prepared and analyzed with RT-qPCR, using a specific primer against IL-17A and IL-17F, respectively. The expression level of each gene was normalized to that of GAPDH. B. Effects of DHCA on the expression of RORγt and RORα. The same total RNA used for the analysis of IL-17A/F as shown was further analyzed using specific primers to RORγt and RORα, respectively. C and D. Time kinetics of IL-17A and RORγt expression. Naïve CD4 T cells were stimulated with Th17 conditioning media in the presence of 20 μM DHCA at appropriate times. Total RNAs were prepared, and the expression level of IL-17A and RORγt was analyzed using primers specific to IL-17A and RORγt, respectively.

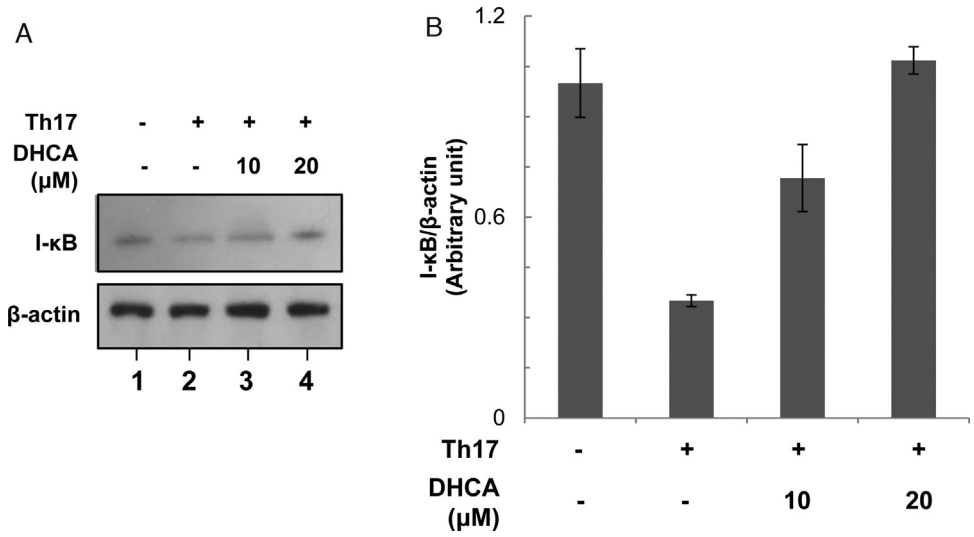

Fig. 3. Effect of DHCA on the activity of NF-κB transcription factor Naïve CD4 T cells were prepared from mouse splenocytes and stimulated with TGF-β and IL-6 in the presence of various concentrations of DHCA for 3 h. Total cell lysates were obtained and subjected to Western blot hybridization using an antibody against I-κB. The protein level of β-actin was used as a control.

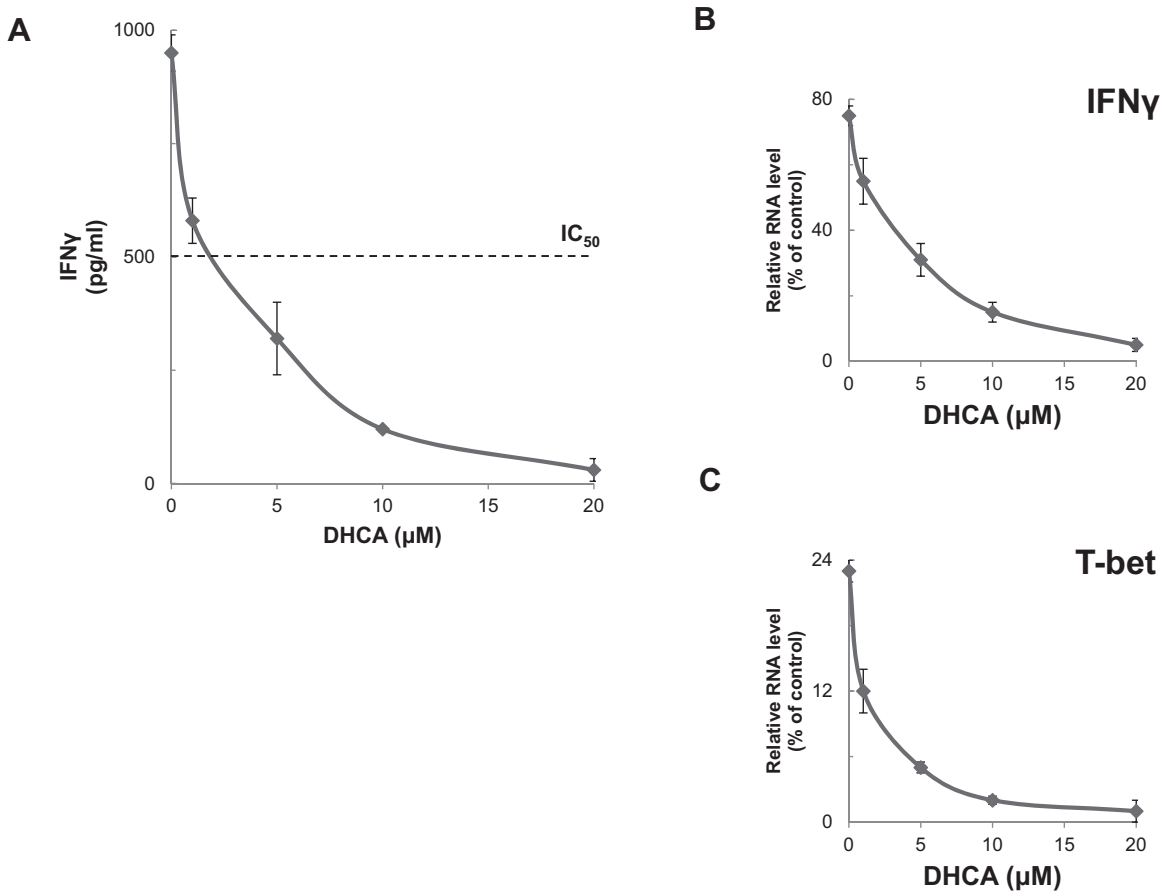

Fig. 4. Effects of DHCA on the differentiation of Th1 cells. Effect of DHCA on the production of IFNγ. Naïve CD4 T cells were stimulated with Th1 conditioning media in the presence of various concentrations of DHCA for 3 days. The culture supernatants were subjected to IFNγ specific ELISA, and total RNAs were prepared for analyzing the level of IFNγ and T-bet using specific primers.

Fig. 2A was further analyzed by RT-qPCR, using specific primers for RORγt and RORα, respectively. As shown in Fig. 2B, the expression level of RORγt and RORα was up-regulated when cells were differentiated into Th17 cells, but DHCA treatment decreased their expression in a dose-dependent manner (Fig. 2B).

To examine the time kinetics of DHCA-mediated transcriptional control of IL-17A and RORγt, naïve CD4 T cells were differentiated into Th17 cells in the presence or absence of DHCA for 1, 2 and 3 days. Total RNAs were prepared and subjected to RT-qPCR using specific primers for IL-17A and RORγt, respectively. When cells

Table 1
DHCA-mediated suppression of MOG-induced EAE diease.

Group		N	Incidence(%)	Day on onset	Mean clinical score	Mean high score
Naïve		5	0	N.D.	0	0
Vehicle		6	100	10.66 ± 0.69	1.31 ± 0.40	2.33 ± 0.25
DHCA	50	6	83.33	13.17 ± 0.43*	0.92 ± 0.37	1.5 ± 0.42
(mg/kg)	100	7	71.42	16.67 ± 0.47***	0.57 ± 0.24*	1.5 ± 0.27
	300	6	0***	N.D.***	0***	0***
FTY720	1 mg/kg	7	0***	N.D.***	0***	0***

Statistic analysis was performed using two-way ANOVA with Bonferoni *post-hoc*. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

were induced to differentiate into Th17 cells, the RNA level of IL-17A was relatively low at day 1, but dramatically increased at day 2 and then decreased at day 3. DHCA treatment, however, suppressed the expression of IL-17A at all time points (Fig. 2C). In contrast to the expression kinetic of IL-17A, the RNA level of RORγt was gradually up-regulated during the whole period. Similar to the case of IL-17A, the RNA level of RORγt in DHCA-treated group was down-regulated at all time points (Fig. 2D). These data suggested that DHCA might control the transcription level of RORγt, consequently leading to the inhibition of IL-17A expression.

It has been reported that the activity of NF-κB plays a key role in the expression of RORγt (Ruan et al., 2011; Chen et al., 2011). Under normal conditions, I-κB binds to NF-κB, preventing its translocation to nucleus. When stimulated, however, upstream signaling kinases such as IKKα/β becomes activated and phosphorylates I-κB, resulting in the degradation of I-κB, translocation of NF-κB, and activation of the NF-κB signaling pathway (Hacker and Karin, 2006). Because DHCA suppressed the expression of RORγt, it was further tested whether this lignan compound could affect the activity of NF-κB. Naïve CD4 T cells were sorted from mouse splenocyte and treated with Th17 stimulation (TGF-β and IL-6) and various concentrations of DHCA for 3 h. Total cell lysates were obtained followed by Western blot analysis, using an I-κB-specific antibody. The protein level of I-κB decreased in cells stimulated with TGF-β and IL-6. DHCA treatment, however, increased the level of I-κB in a dose-dependent manner, up to a level comparable with that of un-stimulated control cells at a concentration of 20 μM (Fig. 3A). Taken together, these data suggested that DHCA decreased the production of IL-17 by controlling the expression and the activity of key transcription factors involved in Th17 differentiation.

3.3. Effects of DHCA on the differentiation of CD4 Th1 cells

Based on the data that DHCA might control the differentiation of Th17 cells, the effect of this lignan molecule on the differentiation of Th1 cells was investigated as they also play a crucial role in the pathogenesis of T-cell mediated inflammatory diseases (Moss et al., 2004). Naïve CD4 T cells ($CD4^{+}$ $CD44^{low}$ $CD25^{low}$) were isolated and differentiated into Th1 cells in the presence of various concentrations of DHCA for 3 days. The culture supernatants were collected, and the protein level of IFNγ was analyzed by ELISA. The level of IFNγ was very low in naïve CD4 T cells but was highly increased in differentiated Th1 cells. When cells were treated with DHCA, however, its level was significantly decreased in a dose-dependent manner with the IC_{50} value being approximately 2.84 μM (Fig. 4A).

Because the expression of IFNγ was reported to be regulated mainly at the transcriptional level (Schoenborn and Wilson, 2007), the RNA level of this molecule was further analyzed. Naïve CD4 T cells were stimulated with Th1 conditioning media and treated with various concentrations of DHCA for 3 days. Total RNAs were isolated followed by RT-qPCR analysis using specific primers against IFNγ. As shown in Fig. 4B, the basal level of IFNγ in naïve T cells was undetectable, but Th1 stimulation markedly increased the RNA level of IFNγ. DHCA treatment, however, decreased the expression level of IFNγ in a dose-dependent manner.

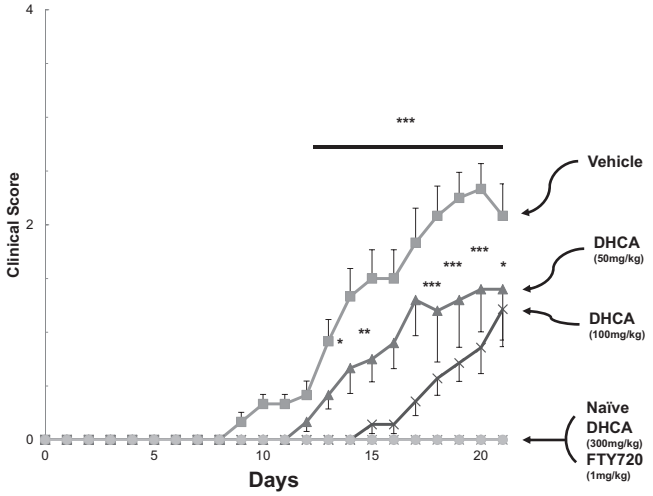

Fig. 5. Effect of DHCA on the mouse EAE model. C57BL/J mice were immunized with MOG_{35-55} in the presence of CFA (day 0). PTX was injected at day 0 and 2. Various concentrations of DHCA were dissolved in vehicle (62.5% PBS and 37.5% DMSO) and administered to the immunized mice via intraperitoneal injection. FTY720 was orally introduced as a positive control. The clinical scores of the mice were measured once a day. The values are shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* EAE/Vehicle mice as determined by two-way ANOVA.

To investigate the underlying mechanisms, the RNA level of transcription factor T-bet was analyzed as it plays a key role in the differentiation of Th1 cell (Szabo et al., 2000). RNA used in Fig. 4B was further analyzed using a specific primer to T-bet. Naïve CD4 T cells expressed little, but its level was increased by Th1 stimulation. DHCA treatment decreased the RNA level of T-bet in a dose-dependent manner (Fig. 4C). Taken together, these data suggested that DHCA might control the differentiation of Th1 as well as Th17 cells.

3.4. Effects of DHCA on the mouse EAE model

Mouse EAE model has been reported to mimic human demyelinating diseases such as MS, and various pro-inflammatory molecules produced by immune cells are involved in the pathogenesis of this disorder (Constantinescu et al., 2011). It has recently been shown that Th17 and Th1 cells produce IL-17 and IFNγ, respectively, and these cytokines play a key role in the development of MS and mouse EAE (Gold and Luhder, 2008; Jager et al., 2009; Komiyama et al., 2006). Based on our *in vitro* data showing that DHCA suppressed the production of IL-17 and IFNγ, it was further investigated whether DHCA could produce therapeutic effects in the mouse EAE model. Seven-weeks-old C57BL/6 mice were immunized with MOG_{35-55} in the presence of CFA and PTX. One day after immunization, various concentrations of DHCA were administered daily *via* intraperitoneal injection. FTY720, a positive control, was administered orally. The clinical scores were measured once a day. As shown in Fig. 5, mice in the vehicle-treated group showed clinical symptoms starting at day 9, which gradually increased and

reached a peak at day 20 post-immunization. Treatment with 50 and 100 mg/kg body weight (b.w.) DHCA delayed the onset of disease for 3 and 6 days, respectively. The overall pattern of the clinical scores of each experimental group was similar to that of the vehicle-treated group, but their mean values were significantly lower over the entire duration of the EAE experiment (Fig. 5 and Table 1). Interestingly, mice injected with 300 mg/kg (b.w.) DHCA did not show any clinical symptom during the entire duration of the EAE experiment (Fig. 5). These data suggest that DHCA might effectively suppress the development of MOG-induced EAE model.

It has been reported that resident or infiltrated immune cells, including microglia and CD4 T cells in the spinal cord produce a

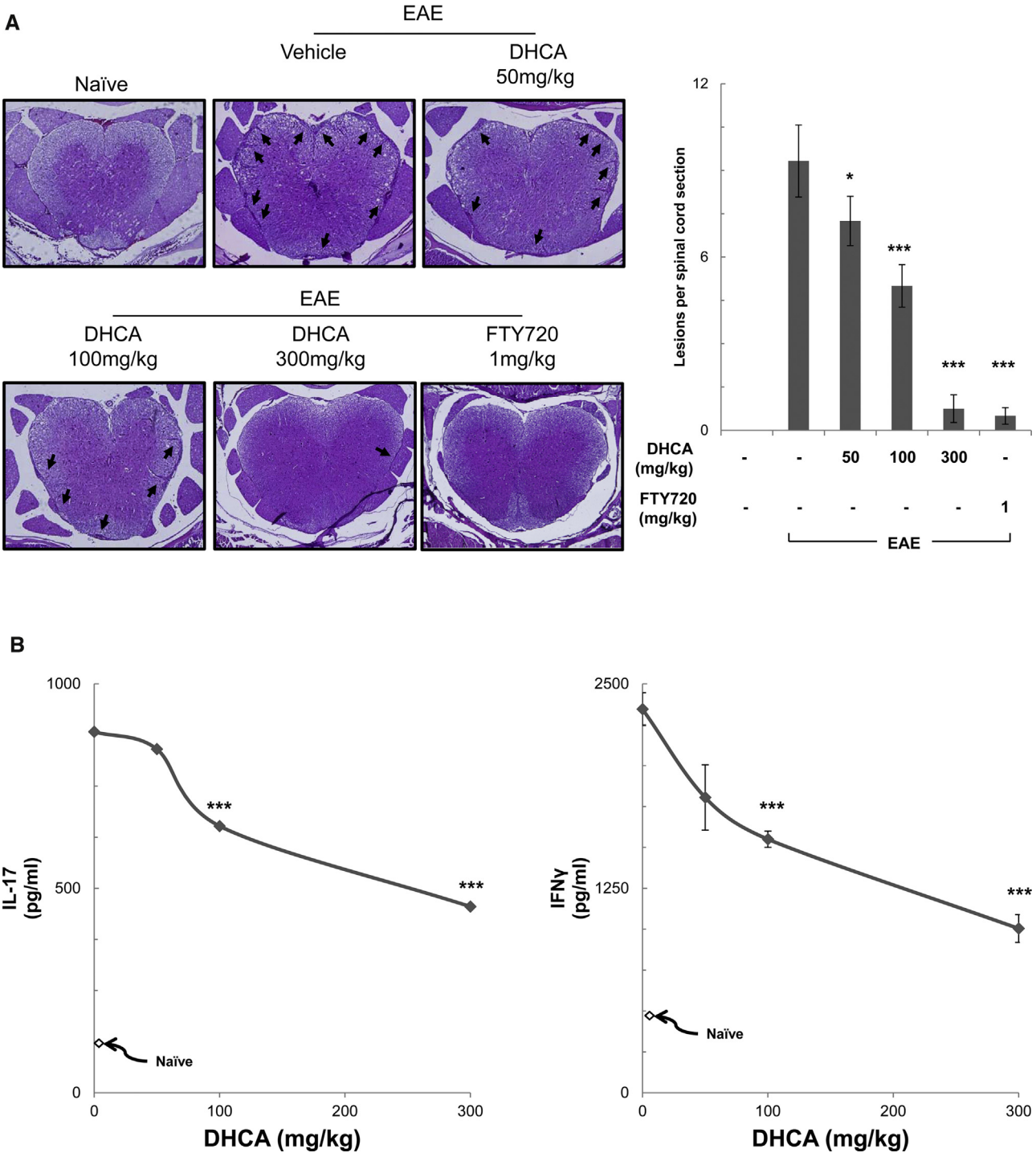

Fig. 6. Histological and molecular characterization of DHCA-mediated suppression of EAE A. Effect of DHCA on the infiltration of immune cells. To analyze the effect of DHCA on the infiltration of immune cells, spinal cords were dissected at day 21 post-immunization and the lumbar regions of the spinal cords were fixed in paraformaldehyde and embedded in paraffin, followed by H&E staining. The lesions containing infiltrated cells were counted and quantified. B. Effect of DHCA on the re-stimulated Th17/Th1 cells. EAE-induced mice were euthanized on day 21 and the splenocytes were isolated from spleens. The splenocytes were subsequently re-stimulated with 10 μg/ml $MOG_{35–55}$. Three days later, the supernatants were collected to measure the protein levels of IL-17A and IFNγ, respectively. C–E. Effect of DHCA on the expression of pro-inflammatory genes in the spinal cord. EAE-induced mice were euthanized on day 21 and the spinal cords prepared. Total RNA was prepared from the thoracic segment of spinal cords followed by the RT-qPCR analysis using specific primers. The RNA level of each gene was normalized to that of GAPDH.

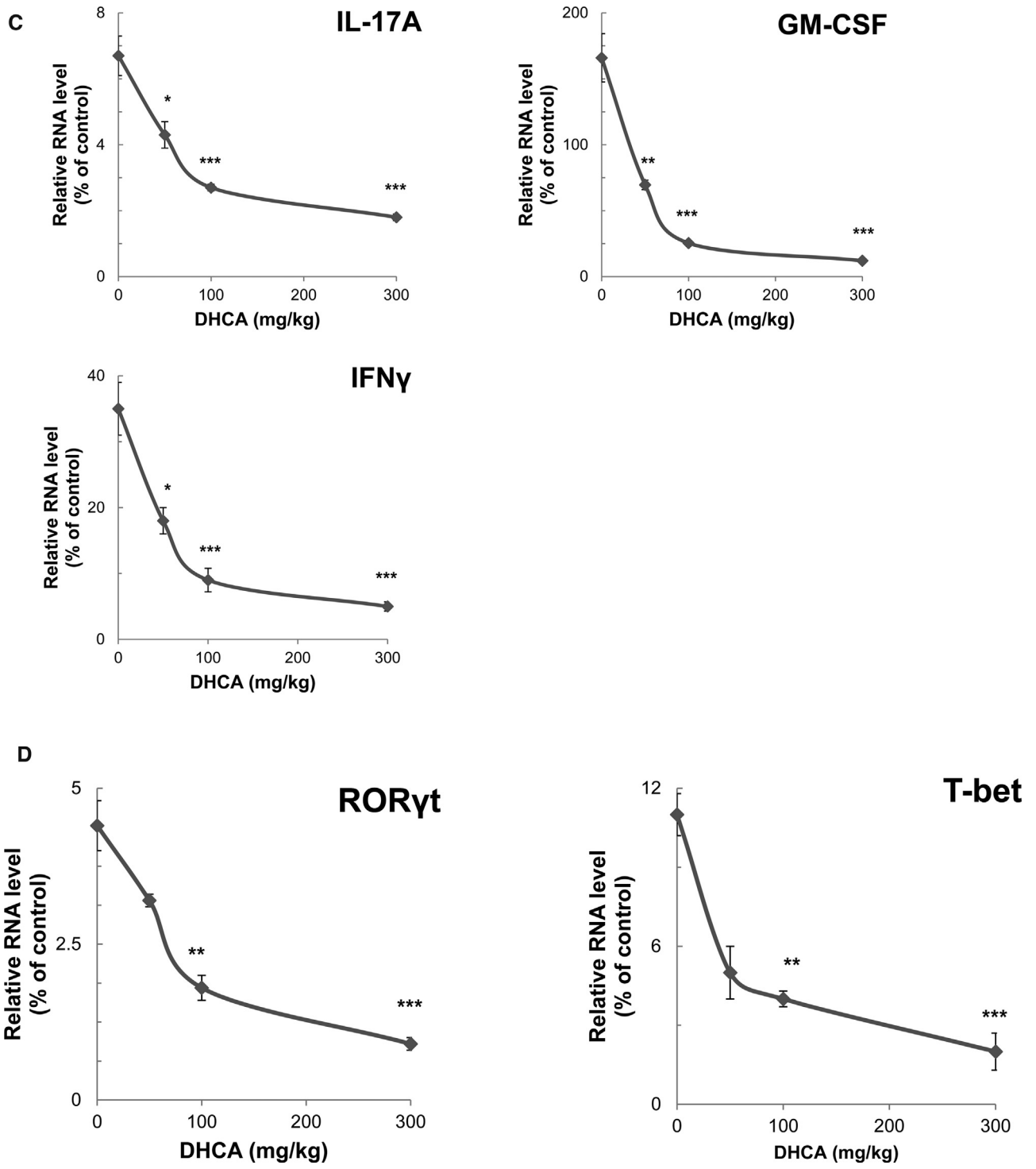

Fig. 6. (*Continued*).

large amount of inflammatory mediators, resulting in the destruction of the myelin sheath in mouse EAE (Hickey, 1991; Hickey et al., 1991). To analyze whether DHCA affected the infiltration of immune cells, mice were euthanized at day 21 post-immunization. Spinal cords were dissected and the lumbar regions were fixed in paraformaldehyde, embedded in paraffin and stained with H&E. As shown in Fig. 6A, fixed segments of spinal cords from vehicle-treated mice showed an increased number of lesions with infiltrated immune cells when compared to that of naïve control mice, which was down-regulated by DHCA treatment in a dose-dependent manner.

To analyze the effects of DHCA on EAE at the molecular level, splenocytes were isolated and stimulated with 10 μg/ml MOG peptide to activate MOG-specific Th17 and Th1 cells. Three days later, supernatants were collected and subjected to an IL-17 and IFNγ-specific ELISA. The basal level of these proteins was undetectable in splenocytes isolated from the naïve group, but its level was significantly increased in those isolated from vehicle-treated mice (Fig. 6B). The protein level of IL-17 and IFNγ, however, was reduced

in the DHCA-treated groups in a dose-dependent manner, and 300 mg/kg of DHCA decreased the production level of IL-17 and IFNγ up to 60% and 45%, respectively (Fig. 6B). These data suggested that DHCA might suppress the generation of MOG-specific Th17 and Th1 cells, resulting in the prevention or alleviation of EAE development.

To further examine the anti-inflammatory effects of DHCA, the gene expression level of IL-17A, GM-CSF and IFNγ was determined as these proteins are produced in the spinal cord by infiltrated Th17 and Th1 cells at the transcriptional level, contributing to the progression of the EAE (Jager et al., 2009; Komiyama et al., 2006; El-Behi et al., 2011). Total RNA was prepared from the thoracic segment of the spinal cords and the RNA level of IL-17A, GM-CSF and IFNγ was measured by RT-qPCR, using gene-specific primers. As shown in Fig. 6C, the level of IL-17A, GM-CSF and IFNγ was significantly increased by 6, 166 and 35-fold, respectively, in vehicle-treated EAE mice, but their level was highly decreased in mice treated with DHCA in a dose-dependent manner. The RNA level of GM-CSF in mice treated with 300 mg/kg (b.w.) DHCA was reduced by almost 90%.

Because RORγt and T-bet plays an important role in the differentiation of Th17 and Th1 cells, respectively, the RNA expression level of these transcription factors was also examined. The expression level of RORγt and T-bet was increased in EAE-induced mice by 4.4 and 11-fold, but decreased by the treatment with DHCA in a dose-dependent manner (Fig. 6D). Taken together, these data suggested that DHCA might have therapeutic effects in mouse EAE by inhibiting the infiltration of immune cells, controlling the generation of MOG-specific Th17 cells and suppressing the expression of pro-inflammatory genes in the spinal cords.

4. Discussion

Our data indicate that DHCA could down-regulate the *in vitro* differentiation of Th17 and Th1 cells by suppressing the expression of the transcription factor RORγt and T-bet, respectively. In addition, DHCA alleviated the severity of clinical symptoms, probably by inhibiting the infiltration of immune cells into spinal cords, controlling the generation of MOG-specific pathogenic CD4 Th17 and Th1 cells and suppressing the expression of various pro-inflammatory genes in the spinal cord.

The activation of various types of T helper cells has been reported to be involved in the development of both MS and mouse EAE (Fletcher et al., 2010). Th17 and Th1 cells are highly pathogenic, as these cells play a crucial role in intensifying the disease severity by inducing demyelination in the central nervous system in a distinctive, but intertwined, manner (Domingues et al., 2010). Th17 cells produce a large amount of IL-17 and GM-CSF, while Th1 cells express a high level of IFNγ and IL-12. These cytokines might have different downstream mechanisms, but their common consequences are the induction of excessive inflammatory responses and the activation of various immune cells in the CNS (Komiyama et al., 2006; El-Behi et al., 2011; Ferber et al., 1996).

In our mouse EAE model, the fold-induction of IL-17 RNA in the spinal cord was relatively low (6.7-fold) compared with that of GM-CSF (165.9-fold). The magnitude of the decrease in the RNA level of GM-CSF was also significantly higher than that of IL-17. For example, treatment with 300 mg/kg (b.w.) DHCA reduced the level of GM-CSF 14-fold, while decreasing the level of IL-17 3.7-fold. Therefore, the effect of DHCA on IL-17 might not be a major cause of DHCA-mediated suppression of EAE development. In the context of MS/EAE pathogenesis, the expression of IL-17 seems to be quite different from that of other genes such as GM-CSF (Hesske et al., 2010). The gene expression kinetics of the latter cytokine is consistent with the increasing clinical score. However, IL-17 expression was significantly increased during early time points when the mice did not show any clinical symptoms, but was markedly reduced, to nearly basal levels, at the time point when the clinical score of the mice reached a peak (Hofstetter and Forsthuber, 2010; Momcilovic et al., 2008).

In our experiments, the effects of DHCA on the RNA level of inflammatory genes in the CNS were investigated when the mice exhibited a high level of disease severity. The expression of IL-17 might have been reduced at the time of sample collection. Further experiments would be necessary to determine the level of IL-17 at early and late time points.

Pathogenic Th17 cells produce unusually high levels of not only IL-17 but also GM-CSF, which underlie the severity of the EAE model (Spolski and Leonard, 2009; McGeachy, 2011). GM-CSF, originally known as a growth factor for granulocytes and myeloid cells, has recently been reported to play a critical role in the progression of EAE by the induction of the pathological properties of Th17 cells. This cytokine also induces the recruitment and infiltration of a large number of immune cells to local inflammation sites, subsequently resulting in undesirable immune responses and eventually leading to CNS impairment (McGeachy, 2011).

Our data from RT-qPCR assays indicate that the RNA levels of GM-CSF were increased by 160-fold in EAE-induced mice, consistent with previous reports, and treatment with 300 mg/kg (b.w.) DHCA reduced these levels by 14-fold. Because DHCA also decreased the infiltration of immune cells into the spinal cord and down-regulated the expression of various inflammatory genes, it is possible that DHCA might regulate the Th17-mediated expression of GM-CSF to control pro-inflammatory responses and consequently the clinical severity of EAE.

It has been reported that Th17 cells produce a large amount of GM-CSF protein, which is regulated mainly at the transcription level (Codarri et al., 2011). The key transcription factors involved in the regulation of GM-CSF gene expression include NF-κB, AP-1, NFAT and STAT5 (Kimura et al., 2009; Gilmour et al., 2007; Thomas et al., 1997). In Th17 cells, the production of GM-CSF has been reported to be dependent on the expression of RORγt (Codarri et al., 2011). Because DHCA inhibits the differentiation of Th17 cells by controlling the expression of RORγt *in vitro*, DHCA might also reduce the Th17-mediated production of GM-CSF in a RORγt-dependent manner.

Our data suggest that a dose of 300 mg/kg (b.w.) DHCA could effectively prevent the development and progression of EAE by controlling a wide range of inflammatory responses mediated by Th17 cells. Thus, DHCA might have potential as an effective therapeutic targeting MS. Because a variety of chronic diseases results from the induction of excessive inflammatory responses, it would be worthwhile to test whether DHCA might also have therapeutic benefits in other models of inflammatory disease models such as inflammatory bowel disease, psoriasis or systemic lupus erythematosus.

Conflict of interest

None.

Authorship contributions

Participated in research design: J. Lee and S. Kim.

Conducted experiments: J. Lee, J. Choi, W. Lee and K. Ko.

Contributed new reagents or analytic tools: J. Lee, J. Choi and S. Kim.

Performed data analysis: J. Lee, J. Choi, W. Lee and S. Kim.

Wrote or contributed to the writing of the manuscript: J. Lee and S. Kim.

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