Ciglitazone, a Peroxisome Proliferator-Activated Receptor Gamma Ligand, Inhibits Proliferation and Differentiation of Th17 Cells

Dong Hyeok Kim[1,3,ψ], Hyun-ju Ihn[1,ψ], Chaerin Moon[1], Sang-Seok Oh[1,2], Soojong Park[1,2], Suk Kim[4], Keun Woo Lee[1,2,3] and Kwang Dong Kim[1,3,]*

[1]*Division of Applied Life Science,* [2]*BK21 Plus,* [3]*PMBBRC, Gyeongsang National University, Jinju 660-701, Republic of Korea;*
[4]*College of Veterinary Medicine, Gyeongsang National University, Jinju 660-701, Republic of Korea*

Abstract

Peroxisome proliferator-activated receptor gamma (PPARγ) was identified as a cell-intrinsic regulator of Th17 cell differentiation. Th17 cells have been associated with several autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease (IBD), and collagen-induced arthritis. In this study, we confirmed PPARγ-mediated inhibition of Th17 cell differentiation and cytokine production at an early stage. Treatment with ciglitazone, a PPARγ ligand, reduced both IL-1β-mediated enhancement of Th17 differentiation and activation of Th17 cells after polarization. For Th17 cell differentiation, we found that ciglitazone-treated cells had a relatively low proliferative activity and produced a lower amount of cytokines, regardless of the presence of IL-1β. The inhibitory activity of ciglitazone might be due to decrease of *CCNB1* expression, which regulates the cell cycle in T cells. Hence, we postulate that a pharmaceutical PPARγ activator might be a potent candidate for treatment of Th17-mediated autoimmune disease patients.

Key Words: Th17 cell, IL-17, PPARγ, CCNB1, Cell proliferation

INTRODUCTION

Peroxisome proliferator-activated receptor gamma (PPARγ) belongs to the nuclear receptor superfamily, which includes receptors for various lipid-soluble, small molecules that are most commonly generated as hormones or in the intermediary metabolic pathways (Evans *et al.*, 2004). PPARγ is most highly expressed in adipose tissues (white- and brown-adipose tissue), is a master regulator of adipogenesis, and is a potent modulator of whole-body lipid metabolism, and insulin sensitivity (Evans *et al.*, 2004; Tontonoz and Spiegelman, 2008). Recently, there have been a few reports of PPAR-γ mediated immune modulation. PPAR-γ activation in immune cells predominantly results in transrepression of proinflammatory gene expression (Schmidt *et al.*, 2010) and plays a role as a negative regulator

of dendritic cell maturation and function, contributing to CD4⁺ T cell anergy (Klotz *et al.*, 2007; Szatmari *et al.*, 2007).

Activation of PPARγ plays an anti-inflammatory role in various tissues, as demonstrated in liver inflammation (Moran-Salvador *et al.*, 2011; Gazit *et al.*, 2012), inflammatory bowel disease (IBD) (Hontecillas and Bassaganya-Riera, 2007; Guri *et al.*, 2010; Mwinyi *et al.*, 2012), and experimental autoimmune encephalomyelitis (EAE) (Niino *et al.*, 2001; Diab *et al.*, 2002). Recently, PPARγ was identified to be a cell-intrinsic regulator of Th17 cell differentiation. Th17 cells are known to be associated with EAE, IBD, allergic airway inflammation, experimental arthritis, and carrageenan-induced pleurisy (Becher and Segal, 2011; Newcomb and Peebles, 2013; Rossi and Bot, 2013; Ahmad *et al.*, 2014). The agonists of PPARγ include endogenous ligands such as the linoleic acid deriva-

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tive 13(S)-hydroxyoctadecadienoic acid (HODE) as well as several synthetic agonists such as pioglitazone, rosiglitazone, and troglitazone (Straus and Glass, 2007; Huang *et al.*, 1999). Therefore, these ligands need to be investigated for their regulatory effects in Th17-mediated inflammatory diseases.

In this study, we confirmed a PPARγ-mediated inhibition against differentiation and cytokine production in both the absence and presence of IL1β. We propose that a PPARγ ligand negatively regulates *CCNB1* expression, potentially regulating Th17 cell proliferation.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (age, 6-7 weeks) were purchased from Orient Bio Co. (Sungnam, Kyung Ki Do, Republic of Korea). All mice were kept at 23 ± 1°C with a 12 h light/dark cycle. They had free access to water and food and were acclimatized for at least 2 weeks before starting the experiments. All procedures using mice were reviewed and approved by the Animal Ethical Committee of Gyeongsang National University.

Cytokines and antibodies

Recombinant murine IL-1β and IL-6 were purchased from PeproTech (Rocky Hill, NJ, USA). Recombinant human TGF-β1 was purchased from R&D systems (Minneapolis, MN, USA). PPARγ ligands (ciglitazone, rosiglitazone, and troglitazone) and trypan blue solution were obtained from Sigma-Aldrich (St. Louis, MO, USA). FITC-conjugated anti-mouse CD4 antibody, PE-conjugated anti-mouse IL-17 antibody, anti-mouse CD3 (145-2C11) antibody, and anti-mouse CD28 (37.51) antibody were purchased from BD Bioscience (Mississauga, ON, CA).

In vitro differentiation of Th17 T cells

Naïve CD4$^+$ T cells (CD4$^+$CD62L$^+$) were isolated from the lymph nodes and spleen of C57BL/6 mouse using MACS (Miltenyi Biotec, Auburn, CA, USA). Naïve CD4$^+$ T cells were cultured in IMDM (Sigma) supplemented with 10% FBS, 4 mM L-glutamine, 1 mM HEPES, 50 μM 2-mercaptoethanol, 100 units/mL Penicillin, 100 μg/ml Streptomycin, and 0.025 μg/mL amphotericin B at 37°C (CO$_2$ concentration at 5%). The cells were cultured with plate-bound anti-CD3 (145-2C11, 1 μg/mL) and anti-CD28 (37.51, 0.5 μg/mL) antibodies in flat-bottomed 96-well plates. Th17 cells were induced to undergo differentiation with rhTGF-β1 (2 ng/mL), rmIL-6 (40 ng/mL), 10% 11B11 culture supernatant (anti-IL-4 antibody), and 10% XMG1.2 culture supernatant (anti-IFN-γ antibody). In some instances, IL-1β (10 ng/ml) was added during and after Th17 differentiation.

Measurement of cytokine production

IL-17 and IL-22 production in the culture medium was determined using ELISA kit (eBiosciences, San Diego, CA, USA).

Flow cytometry analysis

For intracellular staining, cells were stimulated with 50 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich), 1 μg/mL ionomycin (Calbiochem, Nottingham, UK), and Golgi-Stop (BD Biosciences) for 5 h. After stimulation, the cells were washed and treated with 200 μl of 2.4G2 culture supernatant (anti Fc

receptor antibody) for 20 min at 4°C. Without washing, FITC-conjugated anti-mouse CD4 antibody was added to the cells and incubated for 30 min, followed by washing. For fixation and permeabilization, Cytofix/Cytoperm (BD bioscience) was used. After 20 min, cells were washed with permeabilization/wash buffer and stained with PE-conjugated anti mouse IL-17 antibody for intracellular cytokine staining.

Proliferation assay

Proliferation studies were performed using CellTiter 96® Aqueous One Solution cell proliferation assay (Promega, USA). Naïve T cells were seeded in 96-wells cell culture plates (1 × 10^5 cells/well) within the respective Th17 differentiation media (Group A: TGF-β + IL-6 + DMSO; Group B: TGF-β + IL-6 + ciglitazone). On day 4 and day 7, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent at 37°C for 4 hours, and absorbance was measured using a spectrophotometer at wavelength 490 nm. Sometimes polarized Th17 cells were restimulated on anti-CD3Ab coated plates for 2 days, and then the proliferation of cells was analyzed. To confirm the proliferation of Naïve T cells under Th17 differentiation condition, the cells were stained with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, USA) for the cell proliferation assay. The stained cells were analyzed using a FACS Calibur (BD Biosciences) and analyzed with Win MDI 2.9 software (TSRI Flow Cytometry Core Facility, La Jolla, CA, USA).

Quantitative real-time RT-PCR analysis (real-time RT-PCR)

Total RNA was isolated using the RNeasy kit (Qiagen). RNA was quantitated using a NanoDrop spectrophotometer (NanoDrop Technologies). RNA concentrations were equalized before making cDNA. cDNA was synthesized with an oligo dT primer and reverse transcriptase (Fermentas, Newington, NH, USA). Quantitative PCR was performed with CFX 384 real-time RT-PCR detection system (Bio-rad) using an Evagreen qRT-PCR master mixture (Bio-rad Laboratories, CA, USA). The results were normalized to β-actin levels. The primer sequences were as follows: *β-actin*, 5′-GGTCATCAC-TATTGGCAACG-3′ and 5′-ACGGATGTCAACGTCACACT-3′; *CCNB1*, 5′-ATGGTGCATTTTGCTCCTTC-3′ and 5′-CTTTGT-GAGGCCACAGTTCA-3′; *CCNE2*, 5′-CAGACTCTCCGCAA-GAAACC-3′ and 5′-GCCAAACCTCCTGTGAACAT-3′. Data were acquired on a CFX 384 and analyzed with the CFX manager software (Bio-rad).

Statistical analysis

Data were analyzed using unpaired Student's *t*-test or ANOVA. Results were considered statistically significant if *p* <0.05.

RESULTS

PPARγ ligands inhibit Th17 cell differentiation in a concentration-dependent manner

To confirm whether various PPARγ ligands could inhibit Th17 cell differentiation, we treated cells with PPARγ ligands (25 μM; Ciglitazone, Rosiglitazone, and Troglitazone) under conditions of Th17 cell differentiation for 7 days. The cells were subsequently stained with α-CD4 and α-IL-17 antibod-
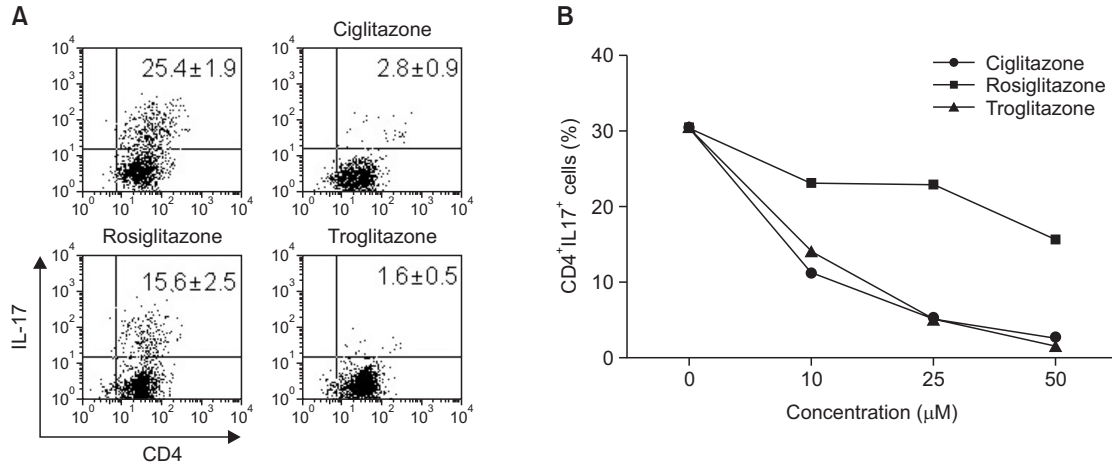
Fig. 1. PPARγ ligands inhibit Th17 cells differentiation. (A) Naïve CD4$^+$ T cells were purified and differentiated into Th17 cells with or without 25 μM of ciglitazone, rosiglitazone, and troglitazone. After 7 days, IL-17 producing CD4$^+$ cells were analyzed using FACS. (B) Data from IL-17-producing cells with various concentrations of PPARγ ligand are indicated. One-way ANOVA was used for statistical analysis ($p<0.01$).
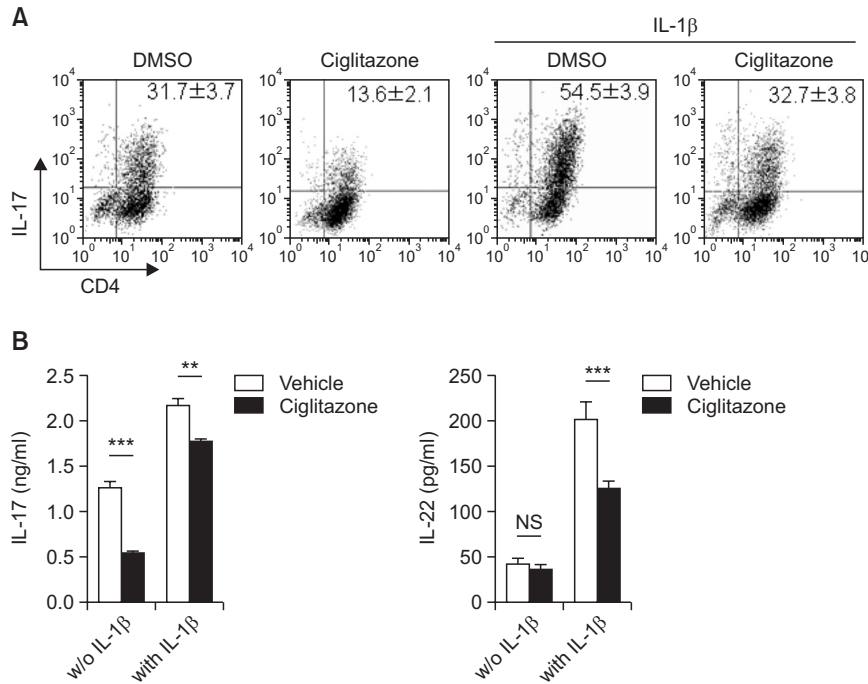

Fig. 2. Ciglitazone inhibited the enhancement of Th17 differentiation induced by IL-1β. Naïve CD4$^+$ T cells were differentiated to Th17 in the presence or absence of IL-1β for 4 days. To test whether a PPARγ ligand inhibits IL-1β-mediated Th17 differentiation, 25 μM ciglitazone was added to the differentiation medium. On day 4, cells were harvested for CD4 and IL-17 staining (A), and cell supernatant was used for determination of IL-17 and IL-22 using ELISA (B). Statistically significant differences are indicated by asterisks (**$p<0.01$; ***$p<0.001$).

ies, and were analyzed for determination of Th17 cell differentiation. As shown in Fig. 1A., the PPARγ ligands inhibited Th17 cell differentiation. The inhibitory effect was dependent on the concentration of ligands, and ciglitazone and troglitazone were found to be more effective than rosiglitazone (Fig. 1B).

PPARγ ligand inhibits IL-1β-mediated enhancement of Th17 cell differentiation

IL-1β plays an important role in regulation of early Th17 cell differentiation, and in inducing autoimmune disease and EAE

(mediated by Th17 cells) (Chung *et al.*, 2009). In this study, we tested whether a PPARγ ligand could inhibits IL-1β-mediated enhancement of Th17 cell differentiation. At the beginning of Th17 cell differentiation, naïve T cells were incubated in the presence or absence of IL-1β supplemented with a PPARγ ligand (ciglitazone). Ciglitazone effectively attenuated Th17 cell differentiation and offset the enhancement of Th17 differentiation induced by IL-1β (Fig. 2A). IL-22 and IL-17 levels, which are enhanced by IL-1β, were attenuated by ciglitazone (Fig. 2B).
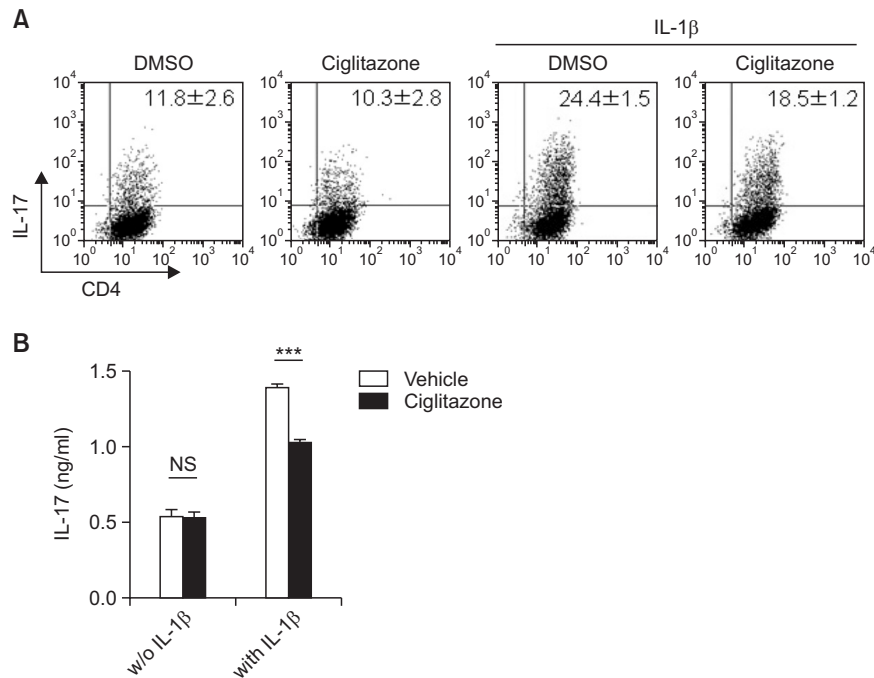
Fig. 3. IL-1β-mediated activation of Th17 cells after polarization is attenuated by ciglitazone. Naïve CD4⁺ T cells were differentiated using the Th17 cell differentiation medium. (A) On day 4, the cells were harvested and then further restimulated with plate bound anti-CD3 over-night in the presence or absence of IL-1β or/and ciglitazone (25 μM). Following 2 days restimulation, IL-17 producing cells were analyzed using FACS. (B) Restimulated cell supernatant was collected and IL-17 production was measured. Statistically significant differences are indicated by asterisks (***p<0.001).

Ciglitazone attenuated the maintenance of Th17 pheno-type by IL-1β after polarization

To investigate whether ciglitazone could inhibit the maintenance of Th17 phenotype after polarization, we treated Th17 cells differentiated and restimulated with anti-CD3 antibody with ciglitazone, in the presence or absence of IL-1β. Ciglitazone inhibited the amount of IL-17-producing Th17 cells enhanced by IL-1β, while it had no effect against polarized Th17 cells in the absence of IL-1β (Fig. 3). These data suggested that ciglitazone might have no effect against already polarized Th17 cells, and might inhibit only IL-1β-mediated stimulatory signaling after polarization.

Ciglitazone attenuated Th17 cell function through regulation of cell cycle

Under Th17 cell differentiation conditions, ciglitazone showed an inhibitory activity in terms of IL-17-producing cell numbers and the amounts of cytokines in the presence or absence of IL-1β. At first we did MTS assay to determine proliferative activity of the cells under Th17 differentiation (Fig. 4A) and Th17 activation condition (Fig. 4B). Ciglitazone-mediated inhibitory effect was shown at both of them and these results proposed that ciglitazone might inhibit the proliferation of differentiating cells into Th17 and of polarized Th17. To confirm whether ciglitazone can decrease cell proliferation during Th17 differentiation, we labeled cells with CFSE and then induced differentiation under indicated conditions. As shown in Figure 4C, ciglitazone-treated cells (bold line) had a relatively low proliferative activity in the presence or absence of IL-1β. The inhibitory activity of ciglitazone might be due to a de-crease in CCNB1, which regulated the G2/M phase of the cell cycle (Fig. 4D). These observations indicate that ciglitazone might attenuate IL-17-producing Th17 cell number, and the enhancement of Th17 cells by IL-1β through regulation of the cell cycle in T cells under these conditions.

DISCUSSION

Th17 cells have been known as a key mediator in the pathology of several autoimmune diseases, including EAE, IBD, and collagen-induced arthritis in humans (Lock *et al*., 2002; Tzartos *et al*., 2008) as well as in mouse models (Cua *et al*., 2003; Murphy *et al*., 2003; Yen *et al*., 2006). Therefore, regulation or inhibition of Th17-differentiation and activation might be an important strategy to treat autoimmune diseases. In this study, we confirmed that a PPARγ ligand could inhibit Th17 cell differentiation in both the presence and absence of IL1β, and could attenuate Th17 activation induced by IL-1β after polarization.

There are some reports where PPARγ activation could impair differentiation of Th17 cells. The inhibitory function of PPARγ on Th17 cells has focused on RORγt expression, which was necessary for Th17 cell differentiation (Yang *et al*., 2008). PPARγ activation is thought to prevent removal of SMRT (silencing mediator of retinoid and thyroid hormone receptor). SMRT is bound to the RORγt promoter and inhibits transcription of the gene. Thus, PPARγ activation can suppress RORγt expression, thereby inducing Th17 cell differentiation (Hwang, 2010; Klotz and Knolle, 2011), whereas the expression of the transcription factors determining Th1 (T-
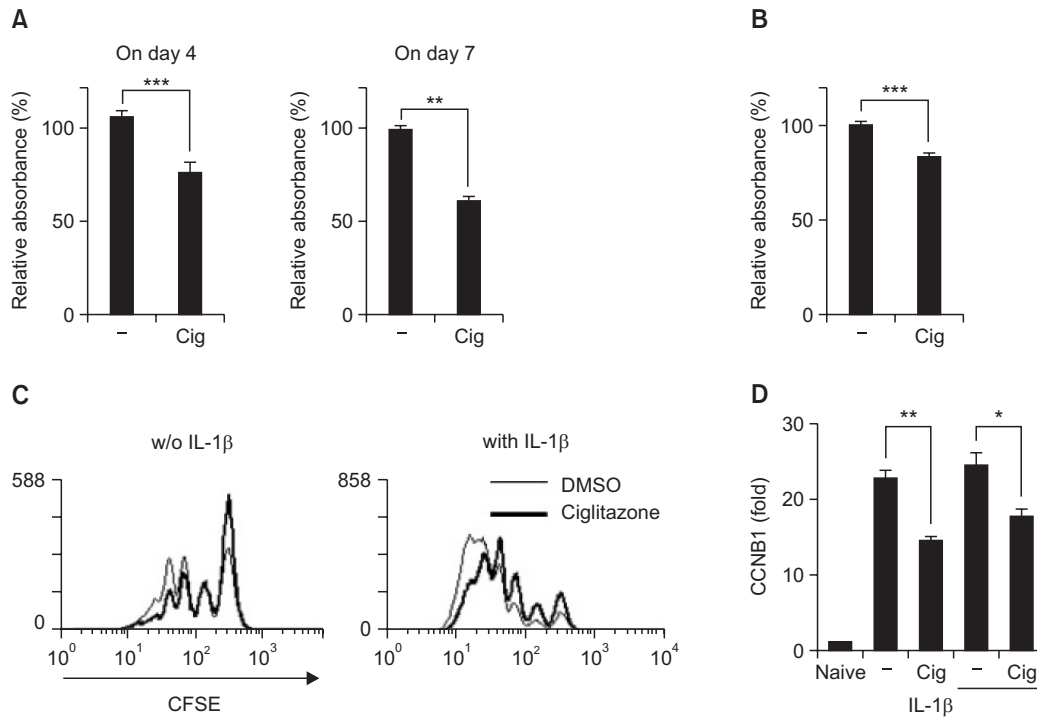
Fig. 4. Ciglitazone decreased cell proliferation during Th17 cell differentiation. Naïve T cells were seeded in 96-wells cell culture plates (1×10^5 cells/well) within the indicated Th17 differentiation condition. On day 4 and day 7, cells were incubated with MTS reagent for 4 hr (A). Sometimes the differentiated Th17 cells were restimulated with anti-CD3 Ab for 2 days and the proliferation was analyzed (B). To confirm whether ciglitazone can decrease cell proliferation during Th17 differentiation, CFSE-labeled Naïve CD4$^+$ T cells were cultured under Th17 differentiation in the presence or absence of IL-1β or/and ciglitazone (25 μM). (C) On day 4, the intensity of CFSE in IL-17 producing cells were analyzed using FACS. (D) After 2 days of Th17 cell differentiation, mRNA expression was assessed by real-time RT-PCR. Statistically significant differences are indicated by asterisks (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

bet), Th2 (GATA-3), and Treg (Foxp3), was not influenced by the PPAR activation (Klotz *et al*., 2009). We also confirmed that PPARγ ligand could induce/inhibit Th17 cell differentiation at an early stage (Fig. 1). Although modulation of Th17 cell differentiation is important to regulate several autoimmune diseases, IL-1 signaling was known to be important in pathology mediated by Th17 cells as well as in early Th17 cell differentiation (Chung *et al*., 2009). Ciglitazone, a PPARβ ligand, showed an inhibitory activity against IL-1β-mediated enhancement of Th17 cell differentiation (Fig. 2) and activation of Th17 after polarization (Fig. 3). Under conditions of Th17 cell differentiation, ciglitazone treated cells had less cell density than that of the control group. Upon examining cell proliferation of IL-17 producing cells, we found ciglitazone inhibited both the proliferation rate of IL-17 producing cells and the expression of CCNB1, which regulates the cell cycle. Although we could not identify the mechanisms by which the PPARγ ligand could attenuate CCNB1 expression in Th17 cells, there are earlier reports of PPARγ ligand-mediated inhibition of cyclin B expression resulting in attenuated cell proliferation in several cell lines (Strakova *et al*., 2004; Yeh *et al*., 2011).

Unfortunately, it is known that pharmaceutical PPARγ ligands induce severe side effects such as fluid retention, weight gain, bone loss, and congestive heart failure in clinical applications. Nevertheless, recent studies also provide opportunities to develop newer classes of molecules that reduce or eliminate the side effects (Ahmadian *et al*., 2013). Thus, we propose that pharmaceutical PPARγ activators might be potent candidates for treatment Th17-mediated autoimmune disease, meriting further investigation.

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