Biochemical Pharmacology 115 (2016) 85–93

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Rosiglitazone-mediated dendritic cells ameliorate collagen-induced arthritis in mice

Sei-Hee Byun [a,1], Jun-Ho Lee [a,b,1], Nam-Chul Jung [b], Hyun-Ji Choi [a], Jie-Young Song [c], Han Geuk Seo [d], Jinjung Choi [e], Sang Youn Jung [e], Sangjin Kang [a], Yong-Soo Choi [a], Ji Hyung Chung [a], Dae-Seog Lim [a,*]

[a] Department of Biotechnology, CHA University, 335 Pangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do 463-400, Republic of Korea
[b] Pharos Vaccine Inc., 545 Dunchon-daero, Jungwon-gu, Seongnam, Gyeonggi-do 462-807, Republic of Korea
[c] Department of Radiation Cancer Sciences, Korea Institute of Radiological and Medical Sciences, 215-4 Gongneung-dong, Nowon-gu, Seoul 139-706, Republic of Korea
[d] Department of Animal Biotechnology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea
[e] Division of Rheumatology, Bundang CHA Medical Center, Yatap-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-712, Republic of Korea

ARTICLE INFO

Article history:
Received 23 March 2016
Accepted 17 May 2016
Available online 18 May 2016

Keywords:
Autoimmune diseases
Dendritic cells
Treg cells
Mouse models RA

ABSTRACT

Rosiglitazone is a selective ligand for peroxisome proliferator-activated receptor-gamma (PPAR-γ), which serves diverse biological functions. A number of autoimmune disease models have been used to examine the anti-inflammatory and immunosuppressive effects of tolerogenic dendritic cells (tDCs). The aim of the present study was to investigate whether rosiglitazone-mediated DC (Rosi-DC) therapy suppressed arthritis in a collagen-induced arthritis (CIA) mouse model.

Rosi-DCs were generated by treating immature DCs with TNF-α, type II collagen, and rosiglitazone. CIA mice then received subcutaneously (s.c.) two injections of Rosi-DCs. The severity of arthritis was then assessed histopathologically. The phenotypes of the DC and regulatory T (Treg) cell populations in CIA mice were determined by flow cytometry and the effect of Rosi-DCs on the secretion of autoimmunity-inducing cytokines was examined by ELISA.

Rosi-DCs expressed lower levels of DC-related surface markers than mature DCs. Histopathological examination revealed that the degree of inflammation in the paws of Rosi-DC-treated mice was much lower than that in the paws of PBS-treated CIA mice.

Taken together, these results clearly show that rosiglitazone-mediated DCs ameliorate CIA, most likely via the induction of antigen-specific Treg cells.

1. Introduction

Rheumatoid arthritis (RA) is a systemic, chronic autoimmune disease that primarily targets the synovial membranes [1]. RA is currently treated with immunosuppressive drugs and/or biologic agents such as methotrexate and infliximab. However, these therapeutic agents cause blanket immunosuppression, which increases the risk of infectious disease and cancer [2,3]. Therefore, new therapeutic approaches should aim to suppress inflammation.

The mouse model of type II collagen-induced arthritis (CIA) has proven to be a useful model of RA, since the characteristic cellular immune response is similar to that in human RA [4]. The main pathological features of CIA include proliferative synovitis accompanied by polymorphonuclear and mononuclear cell infiltration, pannus formation, cartilage degradation, bone erosion, and fibrosis.

Dendritic cells (DCs) are potent stimulators of adaptive immunity; however, mounting evidence suggests that DCs also establish and maintain immunological tolerance [5]. Tolerogenic DCs (tDCs) play an important role in inducing peripheral tolerance via specific mechanisms, including activation of regulatory T (Treg) cells, suppression of effector T cells, and negative modulation of Th1/Th2 immune responses [6–8]. Indeed, a promising new immunotherapeutic strategy aimed at attenuating pathogenic T cell responses is based on autologous tDCs [9]. The use of tDCs for immunotherapy is an attractive approach to treating autoimmune diseases in an antigen (Ag)-specific manner; this may avoid the need for steroids, which are associated with systemic immunosuppression and adverse effects.

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) belongs to the nuclear hormone receptor superfamily. PPAR-γ is highly expressed in adipose tissue, where it plays a role in regulating adipocyte differentiation, fatty acid storage, and glucose

* Corresponding author at: Department of Biotechnology, CHA University, 335 Pangyo-ro, Bundang-gu, Seongnam, Gyeonggi-do 463-400, Republic of Korea.
E-mail address: dslim@cha.ac.kr (D.-S. Lim).
[1] These authors contributed equally to this study.

http://dx.doi.org/10.1016/j.bcp.2016.05.009
0006-2952/

metabolism; it is also a target for anti-diabetic drugs [10]. A recent study showed that PPAR-γ protein was expressed by antigen presenting cells, monocytes, and macrophages, and plays a fundamental role in immune responses [11]. Indeed, synthetic PPAR-γ agonists suppress the production of inflammatory cytokines by these cells [12,13].

The majority of studies show that the maturation stage of DCs (which depends on culture conditions/cytokine environment, the presence of pharmacologic drugs, or the Ag concentration) is likely to determine their immunogenic and tolerogenic fates [9,14,15]. Our long-term aim is to develop tDC therapy strategies for the treatment of RA via the pharmacologic modification of DCs. The aim of the present study was to use rosiglitazone to generate tDCs (Rosi-DCs) and to examine their ability to regulate CIA in mice.

2. Materials and methods

2.1. Approvals of animal experiment

The protocols for the use of animals in these studies were approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University (Project No. IACUC140043) and all experiments were carried out in accordance with the approved protocols.

2.2. Mice

The study used female DBA/1J mice (6–8 weeks-of-age, each weighing 14–16 g). Mice were purchased from Orient Bio Inc. (Gyeonggi, Republic of Korea). All mice were housed under a 12 h/12 h light/dark cycle in a temperature- and humidity-controlled room.

2.3. Generation of bone marrow-derived DCs using rosiglitazone

DCs were generated from bone marrow progenitors obtained from DBA/1J mice as previously described, with some modifications [16]. Briefly, bone marrow cells were cultured in RPMI 1640 supplemented with HEPES (Lonza, USA), 10% fetal bovine serum (FBS, certified US origin) (Gibco, Life Technologies, Grand Island, NY, USA), antibiotics-antimycotics (Gibco), 55 nM 2-mercaptoethanol (Gibco), 20 ng/mL recombinant mouse (rm) GM-CSF (JW CreaGene, Gyeonggi, Korea), and 2 ng/mL rmIL-4 (JW CreaGene). Cells were maintained at 37 °C under an atmosphere containing 5% CO_2. On Days 3 and 6, half of the culture medium was replaced with fresh medium containing the same concentrations of GM-CSF and IL-4. To generate Rosi-DCs, bone marrow cells were cultured in RPMI 1640 supplemented with the components above and after 3 days of culture, treated with 10 μM rosiglitazone (Sigma–Aldrich, St. Louis, MO, USA). After 8 days of culture, Rosi-DCs were generated by additional incubation with 10 ng/mL rmTNF-α (BD Biosciences, Mountain View, CA, USA), 50 μg/mL type II collagen (or 50 μg/mL myosin) (Sigma–Aldrich), and 10 μM rosiglitazone (Sigma–Aldrich) for 4 h. After 8 days of culture in the absence of rosiglitazone, mature (m)DCs were generated by additional incubation with 1 μg/mL lipopolysaccharide (Sigma–Aldrich) and 50 μg/mL type II collagen (Sigma–Aldrich) for 24 h. Immature (im)DCs were harvested for this study after 8 days of culture in the absence of rosiglitazone. Rosi-DCs and mDCs were harvested at the same time and examined in further studies. On the other hand, to investigate whether rosiglitazone can block DC maturation, Rosi-DCs were further incubated for 18 h and harvested for this experiment.

2.4. Cytokine measurement

The levels of interleukin-1β (IL-1β), IL-6, IL-10 (Biolegend, CA, USA), IL-12p70, IL-4, interferon (IFN)-γ (BD Bioscience), IL-17A, and transforming growth factor (TGF)-β1 (eBioscience, CA, USA) were measured in the supernatant of cultures containing either lymphocytes or DCs alone using commercially available ELISA kits, according to the manufacturer's instructions.

2.5. Flow cytometry analysis

Fluorescently-conjugated monoclonal antibodies (mAbs) were used to examine the phenotype of the DCs and lymphocytes. Briefly, cells (1×10^5) were incubated in FACS buffer (0.2% BSA, 0.02% sodium azide in PBS) at 4 °C for 20 min along with the following mAbs: PE-conjugated CD11c (HL3), CD40 (3/23), CD80 (16-10A1), I-A^d/I-E^d (2G9), CD274 (MIH5), CD275 (HK5.3), FITC-conjugated CD4 (GK1.5), CD14 (rmC5-3), CD54 (3E2), H-$2D^b$ (28-14-8), CD86 (GL1), and APC-conjugated CD25 (PC61.5) (all from BD Bioscience). For intracellular staining, cells were fixed/permeabilized using an intracellular staining kit (BD Bioscience) or the Foxp3 staining buffer set (eBioscience) and then stained with PE-conjugated IFN-γ (XMG1.2), -IL-17A (TC11-18H10), and -Foxp3 (FJK-16 s) antibodies (all from BD Bioscience). To examine DC phagocytosis, cells were pulsed with FITC-dextran (Sigma–Aldrich) at 37 °C for 1, 2, or 4 h. Cell viability was examined by propidium iodide (BD Bioscience) staining. After staining, cells were washed with FACS staining buffer and examined in a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Data were analyzed using FlowJo software (TreeStar, Inc., San Carlos, CA, USA).

2.6. Western blot

Rosi-DCs and non-Rosi-DCs were lysed with radioimmunoprecipitation assay (RIPA) buffer (Tris base 50 mM, NaCl 150 mM, NP40 1%, sodium deoxycholate 0.25%, and EDTA 1 mM) containing a protease inhibitor cocktail (Amresco Inc., Cleveland, OH, USA) and phosphatase inhibitor cocktail set II (Merk Millipore, Billerica, MA, USA). The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated by SDS–PAGE and transferred to PVDF membranes (Thermo Scientific, Hudson, NH, USA). The membranes were blocked with 5% (w/v) skim milk in TBST and then incubated with primary antibodies (anti-p-Erk1/2, Erk1/2, p-JNK, JNK2, pp38, p38, NF-κBp65, PPAR-γ, or GAPDH; all diluted 1:1000; Cell Signalling Technologies, Danvers, MA, USA) overnight at 4 °C. The membranes were then washed with TBST and incubated with an HRP-conjugated secondary antibody; diluted1:2000; Cell Signalling Technologies) for 2 h at room temperature. The membrane was exposed to ECL reagents (Thermo Scientific) and signals were detected using a Luminescent image analyzer, LAS-4000 (FujiFilm, Tokyo, Japan).

2.7. Real-time PCR

RNA was isolated from Rosi-DCs and non-Rosi-DCs using TriZol reagent (Life Technologies, Mount Waverley, Australia), respectively. Total RNA was reverse transcribed using SensiFAST™ cDNA Synthesis Kit (Bioline, London, UK). cDNA samples were subjected to real-time PCR analyses with specific primers and a SensiFAST™ SYBR® Hi-ROX kit (Bioline) for PPAR-γ using real-time quantitative RT-PCR (Applied Biosystem, Foster City, CA, USA). The primers used for real-time PCR were as follow: PPAR-γ: forward – CTG GCC TCC CTG ATG AAT AAA G, reverse: AGG CTC CAT AAA GTC ACC AAA G; GAPDH: forward – AAC TTT GGC ATT GTG GAA GGG CTC, reverse:

TGG AAG AGT GGG AGT TGC TGT TGA. The normalized value for PPAR-γ mRNA expression was calculated as the relative quantity of PPAR-γ divided by the relative quantity of GAPDH. All samples were run in triplicate.

2.8. Co-culture of T lymphocytes and DCs

Splenocytes were isolated from DBA/1J mice and disaggregated in RPMI 1640 medium. $CD3^+$ T cells were isolated by passing the splenocytes through a nylon wool (Polysciences Inc., Warrington, PA, USA) column. Cells were co-cultured at a DC:lymphocyte ratio of 1:10. Purified $CD3^+$ T cells (1×10^6 cells/mL) were used as responders and Rosi-DCs or mDCs (1×10^5 cells/mL) were used as stimulators. Cells were co-cultured at 37 °C for 72 h in 2 mL of RPMI 1640 supplemented with 10% FBS.

2.9. CIA mice

Type II collagen (Sigma–Aldrich) was dissolved in 0.05 M acetic acid overnight at 4 °C and then emulsified in an equal volume of complete Freund's adjuvant (CFA; Sigma–Aldrich). To induce CIA, DBA/1J mice were immunized (subcutaneously (s.c.) into the base of the tail) with 100 μL of emulsion containing 200 μg of type II collagen. The mice were boosted with 200 μg type II collagen emulsified in incomplete Freund's adjuvant (IFA; Sigma–Aldrich) on Day 21 post-primary immunization. The CIA mice were then injected subcutaneously (s.c.) with 2×10^5 type II collagen-pulsed-Rosi-DCs, myosin-pulsed-Rosi-DCs, antigen-unpulsed Rosi-DCs, or conventional-tDCs (type II collagen-pulsed non-Rosi-DCs) on Days 21 and 29 (10 mice per group). Arthritis severity and foot thickness (all four paws) were observed three times (at intervals of 1 or more days) per week until Day 50 post-primary immunization using a triple-blind test. The severity of arthritis was expressed as the mean arthritis index, graded on a scale of 0–4 (0, no-arthritis; 1, light edema at the point of one finger; 2, edema at several points on a finger or in the joints of the wrist or ankle; 3, pervading edema involving the entire paw; 4, maximal pervading edema involving the entire paw and deformation of the joints (ankylosis) with impaired function). The maximum total arthritis score that each mouse could receive was 16. Five mice were randomly selected from each group and sacrificed on Day 35. The spleen, inguinal lymph nodes, blood, and paws were isolated and the immune status was examined.

2.10. Histopathological examination

Paws from each group were fixed in 10% neutral formalin, decalcified in 15% EDTA solution, and embedded in paraffin. Serial sections (5 μm) were prepared and stained with hematoxylin and eosin (H&E).

2.11. Statistical analysis

Statistical analysis was performed using GraphPad software (GraphPad Prism v5.0; GraphPad Software, San Diego, CA, USA). Data were analyzed by Paired *t*-test or a one-way ANOVA followed by the Newman–Keuls test. Results were expressed as mean ± SEM. A *p*-value of <0.05 was considered significant.

3. Results

3.1. The characterization of Rosi-DCs

Rosi-DCs expressed significantly lower levels of CD40, CD54, CD80, and CD86 than mDCs and rosiglitazone treatment did not induce cell death (as determined by PI staining) (Fig. 1A). Fully mature DCs (mDCs) were not exposed to rosiglitazone. Cytokine production is an important mechanism by which DCs regulate immune responses; therefore, we examined cytokine profile of DCs. Rosi-DCs produced lower levels of pro-inflammatory cytokines (IL-1β, IL-6, and IL-12p70) than mDCs (Fig. 1B). DCs did not produce the anti-inflammatory cytokine, IL-10 (data not shown). Phagocytic analysis revealed that both Rosi-DCs and mDCs showed markedly reduced phagocytic activity compared with imDCs (Fig. 1C).

3.2. Rosi-DCs induce T cell tolerance upon co-culture

We next performed a series of functional co-culture experiments to investigate the effect of Rosi-DCs on Treg cell expansion and T cell polarization. Compared with mDCs, Rosi-DCs markedly increased the $FoxP3^+CD4^+CD25^+$ Treg cell population and reduced the Th1/Th17 cell population (Fig. 2A–C). Additionally, compared with mDCs, Rosi-DCs inhibited the production of IFN-γ and IL-17A. By contrast, Rosi-DCs produced higher levels of TGF-β1. However, no Th2 cytokines (IL-4 and IL-10) were detected (Fig. 2D). These results suggest that Rosi-DCs induce immune tolerance upon co-culture with T cells.

3.3. Rosiglitazone induces tolerance by blocking DC maturation

As an alternative means of assessing the maturation-blocking effect of rosiglitazone on DCs, we stimulated Rosi-DCs with TNF-α for 24 h. Rosi-DCs expressed lower levels of CD80 (B7-1) and CD86 (B7-2) than Rosi-untreated DCs (non-Rosi-DCs). However, there was no difference in MHC II expression between Rosi-DCs and non-Rosi-DCs (Fig. 3A). Additionally, the rosiglitazone treatment markedly decreased phosphorylation of Erk1/2, p38 MAPK, and NF-κBp65 in DCs. However, no significant difference of p-JNK level was observed between non-Rosi-DCs and Rosi-DCs (Fig. 3B). Quantitative real-time PCR (qRT-PCR) and Western blot data revealed that PPAR-γ mRNA/protein levels were markedly increased in Rosi-DC, compared with non-Rosi-DC (Fig. 3B and C). Moreover, Rosi-DCs markedly inhibited production of IL-12 (Fig. 3D) and significantly increased the FoxP3-positive Treg cell population, compared with the cases of non-Rosi-DCs (Fig. 3E). These results clearly demonstrate that rosiglitazone inhibits DC maturation and increases tolerogenicity.

3.4. Therapeutic effects of type II collagen-pulsed Rosi-DCs in CIA mice

CIA mice are a well-established model for evaluating therapeutic interventions against autoimmune arthritis. To examine the therapeutic effects of Rosi-DCs, mice received two injections of type II collagen-pulsed Rosi-DCs (a primary injection followed by a booster). Other groups of mice received antigen-mismatched (myosin-pulsed) or Ag-unpulsed Rosi-DCs according to the same schedule to confirm the antigen specificity of Rosi-DCs. The experiments revealed that treatment of CIA mice with a type II collagen-pulsed Rosi-DCs abrogated the severity of arthritis (Fig. 4A), whereas disease severity in CIA mice injected with antigen-mismatched or Ag-unpulsed Rosi-DCs was similar to that observed in PBS-treated CIA mice. Additionally, the treatment of CIA mice with a type II collagen-pulsed Rosi-DCs showed a more significant anti-rheumatic effect compared to the treatment with conventional tDCs (type II collagen-pulsed non-Rosi-DCs). Paw thickness followed a trend similar to that observed for the clinical score (Fig. 4B). Mice were sacrificed at Day 35 post-primary immunization and the organs and paws examined histologically. The paws of non-arthritic mice were normal, whereas those of arthritic mice showed evidence of severe disease, including cartilage erosion and bone resorption. Sections from type II collagen-pulsed Rosi-DC-treated mice showed a clear joint space, with a normal

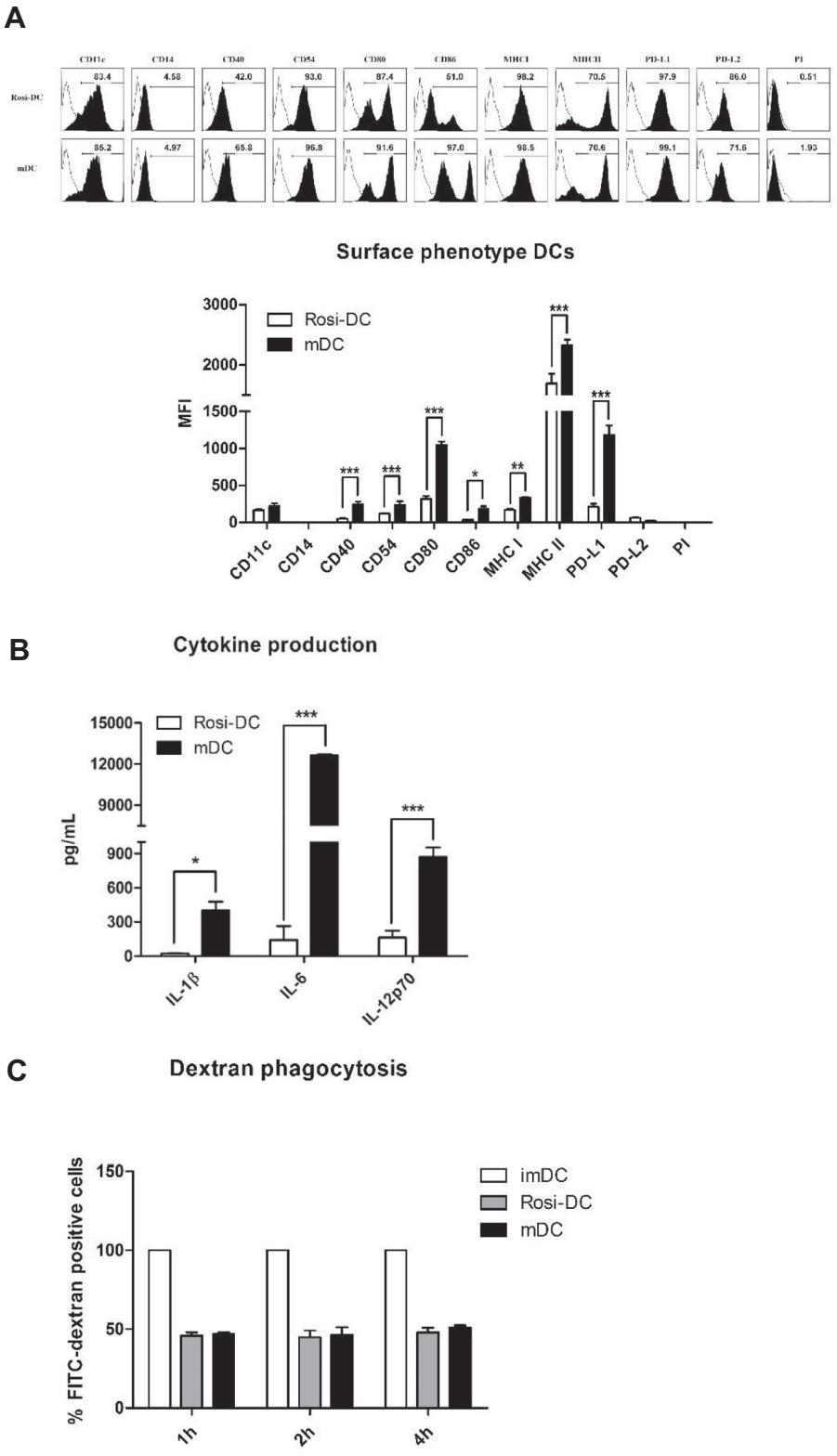

Fig. 1. Characterization of rosiglitazone-mediated DCs. (A) DC subsets (Rosi-DCs and mDCs) were stained with the indicated fluorescently-conjugated antibodies and analyzed by flow cytometry. Data are presented as histograms (data are representative of ten independent DC preparations). The bar graphs show mean fluorescence intensity, expressed as mean ± SEM (n = 10 independent DC preparations). (B) Pro-inflammatory cytokines in the culture supernatants of DCs were analyzed by ELISA. Data are expressed as mean ± SEM (n = 5 independent DC preparations) of triplicate experiments. (C) Each DC subset was incubated with FITC-dextran for the indicated times and the percentage of FITC-dextran-positive cells determined by flow cytometry. The bar graphs show the mean fluorescence intensity, expressed as mean ± SEM (n = 3 independent DC preparations). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

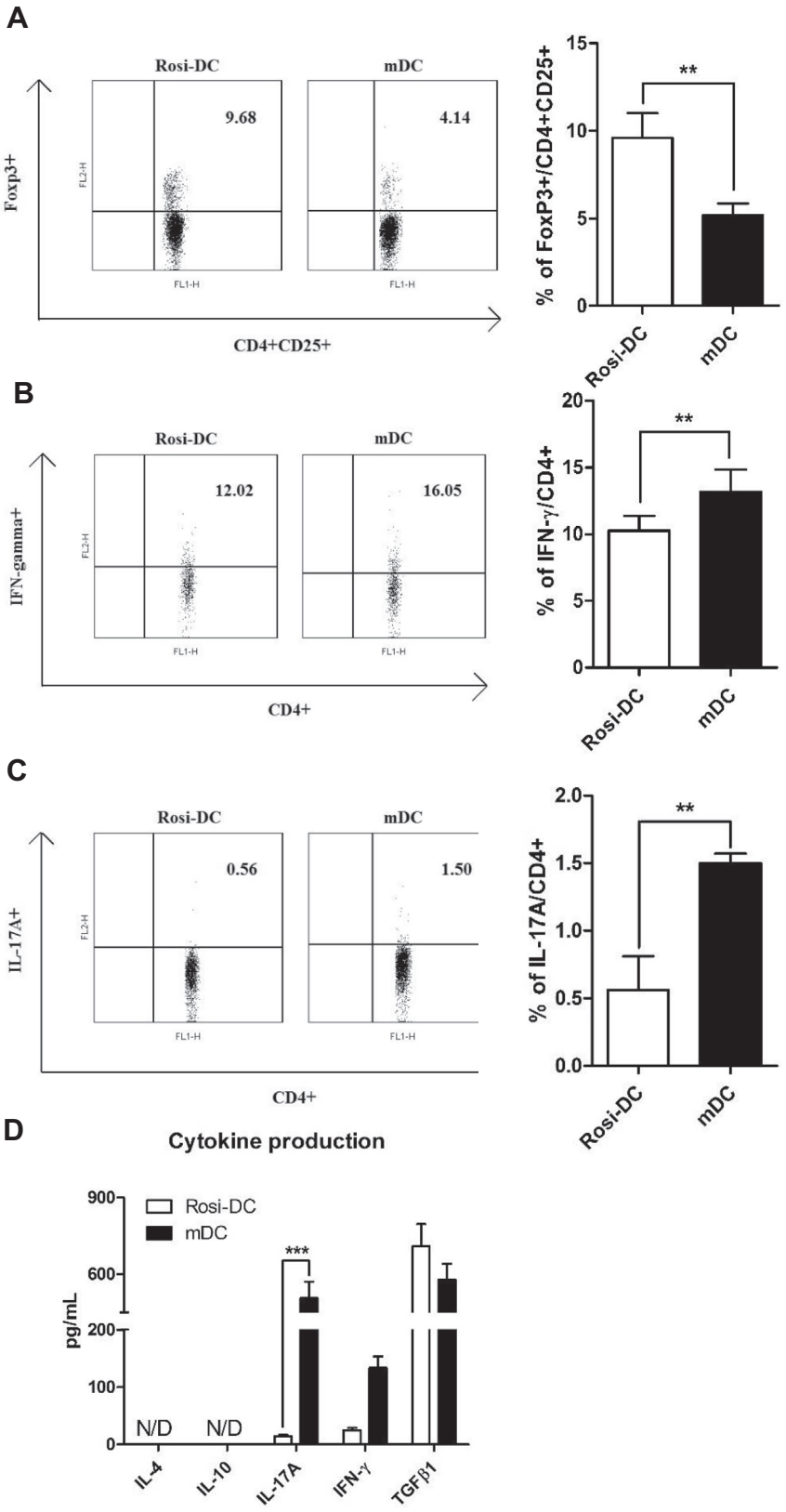

Fig. 2. Immunosuppressive characteristics of Rosi-DCs. (A) Each DC subset was co-cultured with $CD4^+$ T cells (isolated from splenocytes obtained from naïve DBA/1J mice) for 72 h. The stimulator:responder ratio was 1:10. To identify Treg analysis, cells were stained with anti-CD4 and anti-CD25 antibodies in Foxp3 staining buffer and then analyzed by flow cytometry (data are representative of ten independent DC preparations). The bar graph shows the percentage of $CD4^+CD25^+Foxp3^+$ cells (mean ± SEM of ten independent co-culture preparations). Cells were intracellularly stained with anti-CD4 and anti-IFN-γ or anti-IL-17A antibodies and analyzed by flow cytometry to detect Th1 (B) and Th17 (C) cells (data are representative of five independent co-culture preparations). (D) Cytokine levels in the supernatants after 72 h of co-culture were measured by ELISA. Data are expressed as mean ± SEM (n = 5 independent co-culture preparations performed in triplicate). $^{**}P < 0.01$; $^{***}P < 0.001$.

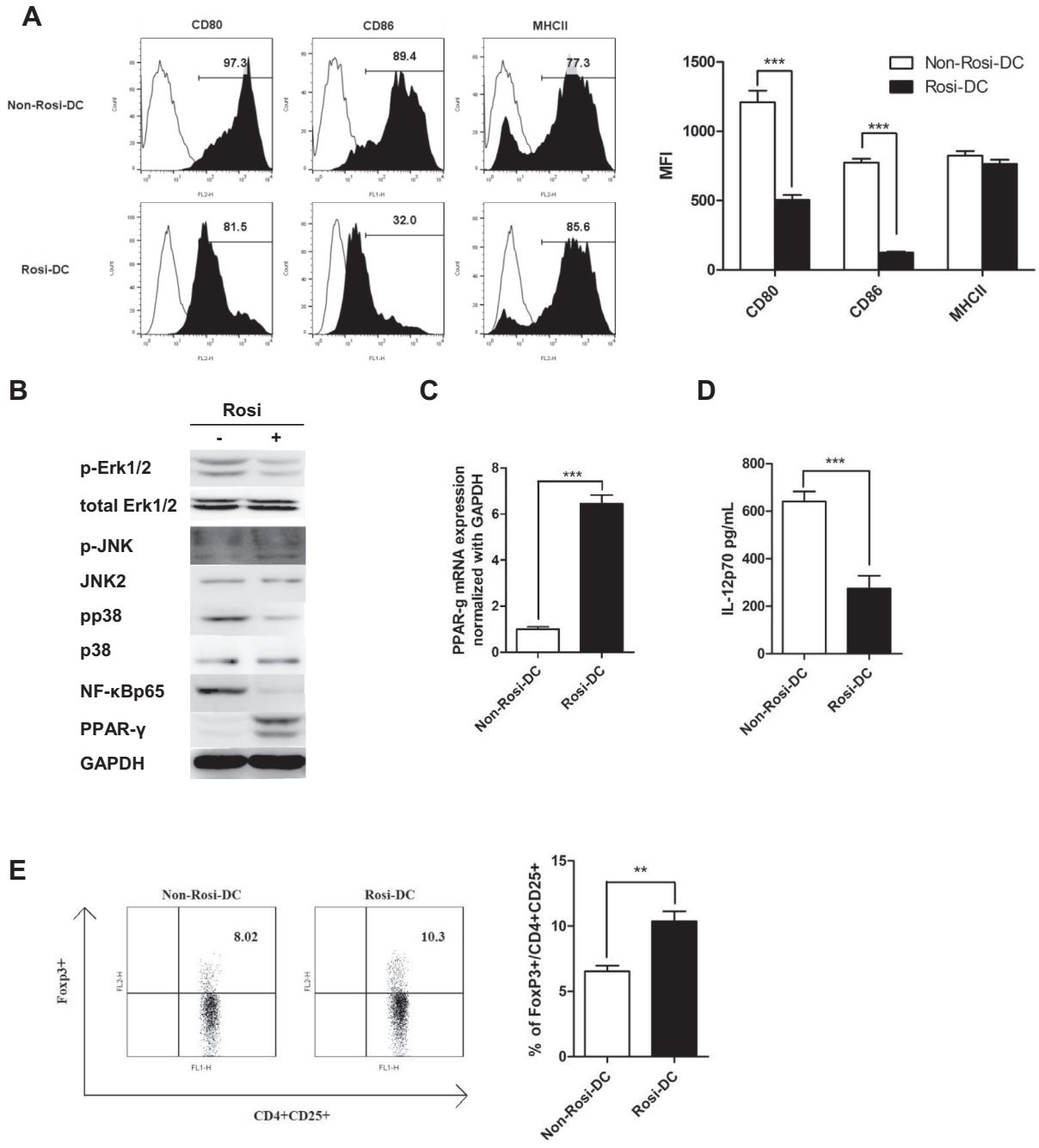

Fig. 3. Rosiglitazone blocks dendritic cell maturation. (A) Each DC subset was generated by treating imDCs with 10 ng/mL rmTNFα and 50 μg/mL type II collagen for 24 h in the presence or absence of rosiglitazone. The expression of co-stimulatory molecules on Rosi-DCs and non-Rosi-DCs was detected by flow cytometry (data are representative of three independent DC preparations). The bar graphs show mean fluorescence intensity, expressed as mean ± SEM (n = 3 independent DC preparations). (B) Western blot analysis of MAPKs and NF-κBp65. Data shown are representative of at least three independent experiments. (C) PPAR-γ mRNA levels in Rosi-DCs and non-Rosi-DCs determined by qRT-PCR. (D) IL-12 in the culture supernatants of DCs were analyzed by ELISA. Data are expressed as mean ± SEM (n = 5 independent DC preparations) of triplicate experiments. (E) DCs were co-cultured with $CD3^+$ T cells and the $CD4^+CD25^+Foxp3^+$ Treg cell population was detected by flow cytometry (data are representative of three independent co-culture preparations). The bar graph shows the mean percentage of $CD4^+CD25^+Foxp3^+$ cells (n = 3 co-culture preparations). $^{**}P < 0.01$; $^{***}P < 0.001$.

cartilage interface; also, the connective tissue surrounding the joints showed only a minor mixed inflammatory cell infiltrate (Fig. 4C). These results suggest that antigen-pulsed Rosi-DCs are potent inhibitors of CIA progression.

3.5. Effect of Rosi-DC therapy on antigen-specific Treg cell induction and Th1/Th17 cell inhibition

Effective suppression of immune responses by Treg cells requires that these cells migrate to the appropriate site, respond to antigen, and down-regulate the immune responses responsible for increased disease severity. To examine the *in vivo* effects of type II collagen-pulsed Rosi-DCs on the Treg and Th1/Th17 cell populations, we harvested splenocytes and inguinal lymph nodes from cells from Rosi-DCs-immunized mice at Day 35 post-primary immunization. The splenocytes and lymph node cells were then cultured with type II collagen (50 μg/mL) for 72 h and the $FoxP3^+CD4^+CD25^+$ Treg cell population evaluated by flow cytometry. The percentage of Treg cells in the spleens and inguinal lymph nodes of mice vaccinated with type II collagen-pulsed Rosi-DCs

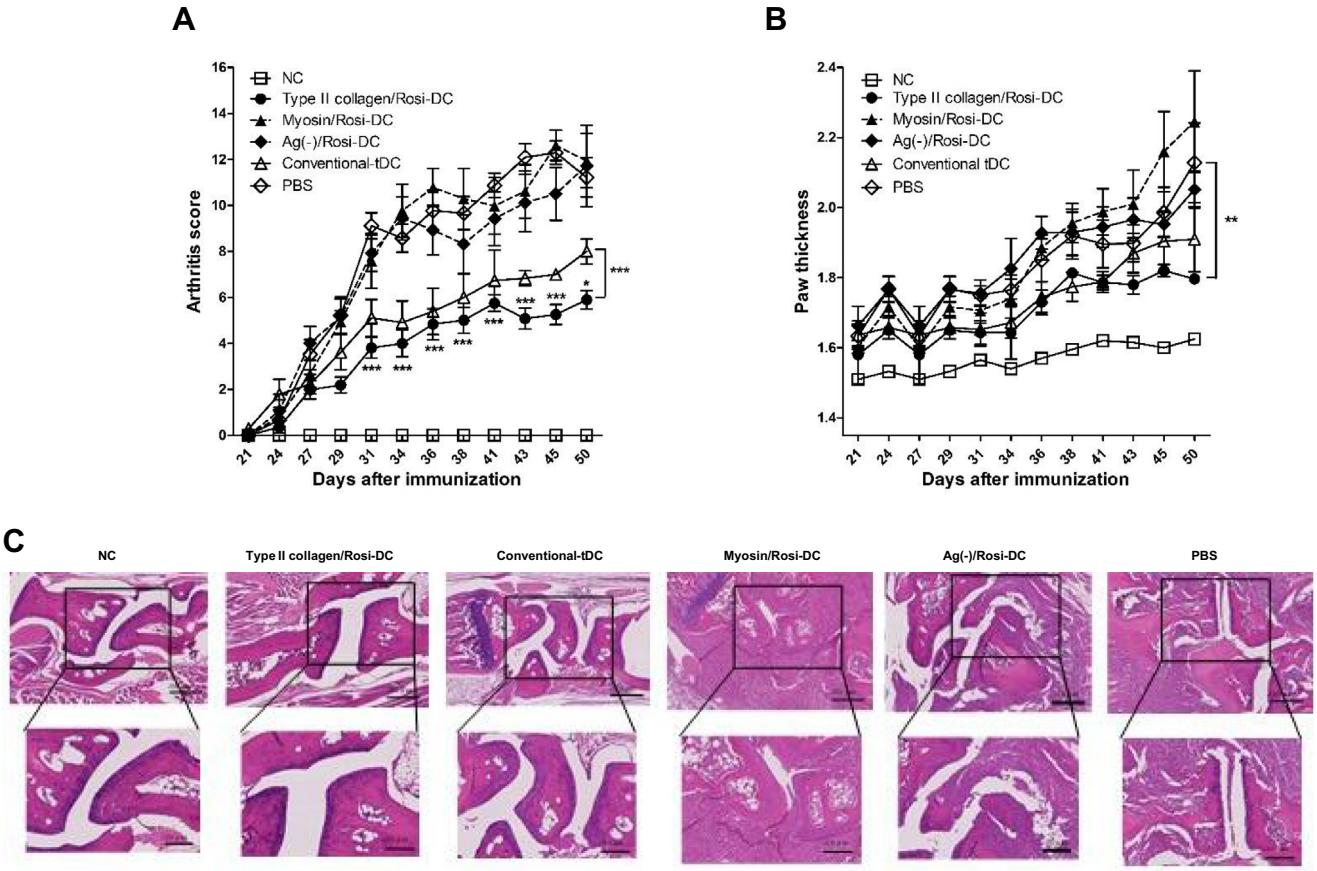

Fig. 4. Injecting CIA mice with type II collagen-pulsed Rosi-DCs suppresses CIA development. Twenty-one days and 29 days after the primary injection with type II collagen, mice received a subcutaneously (s.c.) injection of 2×10^5 type II collagen-pulsed Rosi-DC, myosin-pulsed Rosi-DC, Ag-unpulsed Rosi-DC, conventional-tDC (type II collagen-pulsed non-Rosi-DC), or PBS. Arthritis incidence was assessed by clinical scoring and by measuring paw thickness from Days 21 to 50. (A) Disease severity was graded on a scale of 0–4 (see Section 2 for details). (B) Footpad thickness was measured three times per week. Data are expressed as mean ± SEM (n = 10 mice per group). (C) Paws were removed from each group of mice and fixed for 2 days in 4% formalin, decalcified for 18 days in 14% EDTA, dehydrated, and embedded in paraffin blocks. Serial sections (5 μm) were cut and stained with hematoxylin and eosin (H&E). Long scale bars: 200 μm; short scale bars: 100 μm. Data shown are representative of at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

was markedly higher than that in mice injected with myosin-pulsed or Ag-unpulsed Rosi-DCs and PBS-treated CIA mice (Fig. 5A and B). Treatment with type II collagen-pulsed Rosi-DCs resulted in a reduction in the percentage of Th1 and Th17 cells within the splenocyte population (Fig. 5C and D), a finding that supports the *in vitro* data. Also, IFN-γ levels decreased and IL-10 levels increased significantly in mice injected with in type II collagen-pulsed Rosi-DCs (Fig. 5E). These results imply that type II collagen-pulsed Rosi-DCs induce an increase in the number of type II collagen-specific Treg cells and reduce the number of pathogenic Th1 and Th17 cells *in vivo*.

4. Discussion

PPARs are ligand activated transcription factors belonging to the nuclear hormone receptor superfamily, which includes the classic steroid, thyroid, and retinoid hormone receptors as well as many orphan receptors [17]. The three members of the PPAR subfamily are PPAR-α, PPAR-γ, and PPAR-β/δ. PPAR-α is expressed mainly in the liver, whereas PPAR-γ is expressed in adipose tissue, macrophages, and DCs; PPAR-β/δ is ubiquitously expressed [18]. The phenotype and functional heterogeneity of DCs primarily stems from the diverse tissue microenvironments in which they reside. Changes in the local tissue environment alter the extracellular lipid milieu, which in turn modifies intracellular lipid metabolism. Nuclear receptors receive extracellular and intracellular lipid signals, resulting in gene expression [19]. Thus, similar to the case for macrophages, microenvironmental stimuli define a broad range of DC subsets that differ in terms of function, location, migratory properties, maturity, and activation status [20].

Previously, we demonstrated that TNF-α-treated DCs prevent experimental autoimmune myocarditis and arthritis in mice via the enrichment of FoxP3$^+$ regulatory T cells [16,21]. FoxP3$^+$ regulatory T cells have a profound ability to regulate responses and are capable of inhibiting pathogenic T cell responses. The results presented herein show that Rosi-DCs have a clear therapeutic effect in an established CIA model. Injecting mice with type II collagen-pulsed Rosi-DCs after the onset of disease led to a significant reduction in both the severity and progression of arthritis, whereas treatment with myosin-pulsed on non-Ag-pulsed Rosi-DCs led to disease exacerbation. Rosi-DCs modulated the immune response in a type II collagen-specific manner. In addition, Rosi-DCs showed lower expression of co-stimulatory molecules (CD80, CD86, and CD40) than mDCs. The results of the present study suggest that rosiglitazone induces tolerance by blocking DC maturation. The PDL-1-2/PD1 binding interaction strongly inhibits T cells and induces Treg cell differentiation [22], and is essential for maintaining peripheral tolerance. However, although PD-L1 expression by Rosi-DCs is higher than that by mDCs, the levels of PD-L2 expression were not measurable. Indeed, our results strongly demonstrated that the rosiglitazone treatment suppressed the production of inflammatory factors *via* marked downregulation of Erk1/2, pp38, and NF-κB in DCs, thus supporting the tolerogenic environment of DCs.

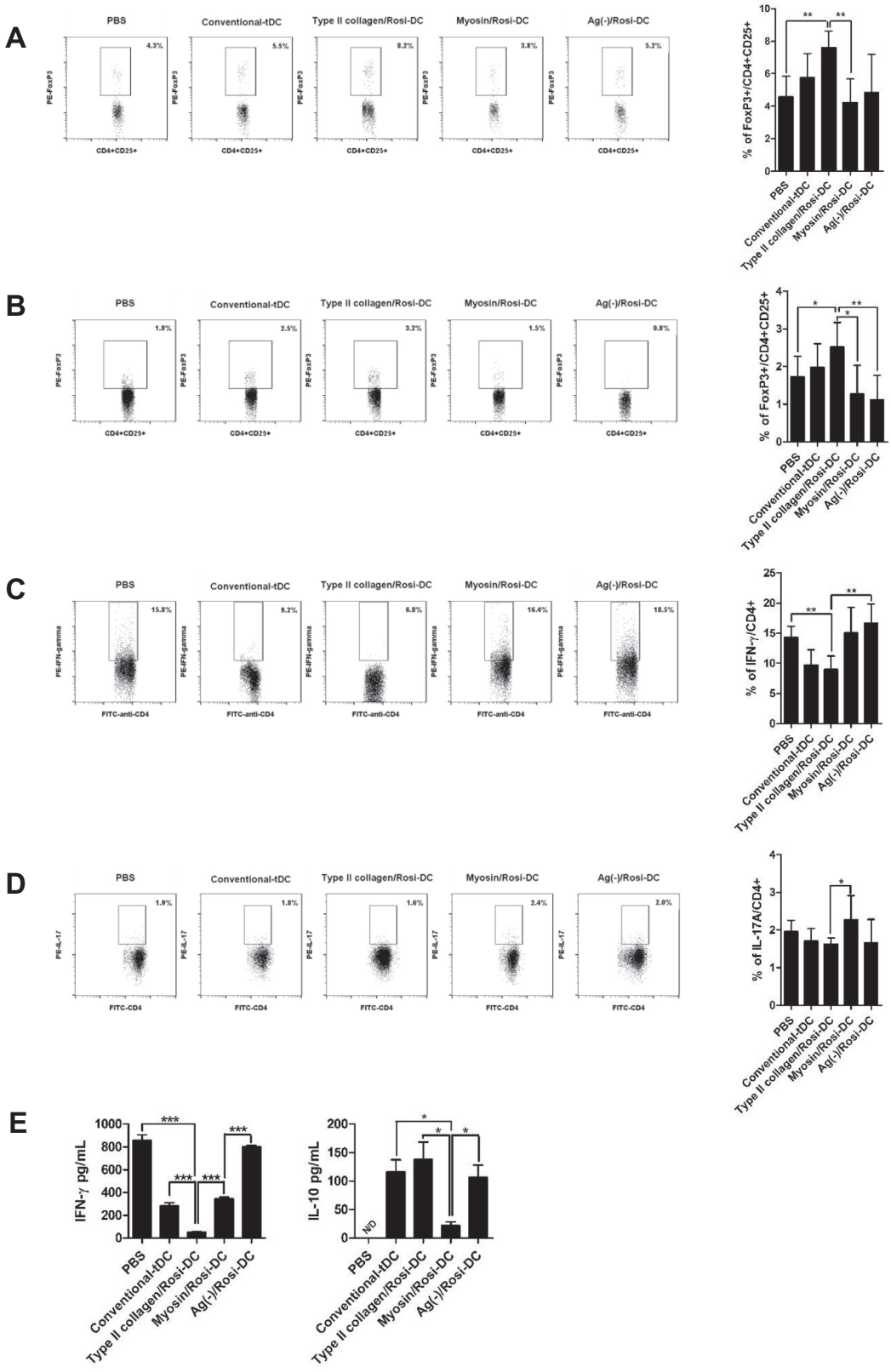

Fig. 5. *Ex-vivo* immune status of type II collagen-pulsed Rosi-DC-vaccinated CIA mice. Cells were isolated from the spleens and inguinal lymph nodes of each group of CIA mice and cultured with 50 μg/mL type II collagen for 72 h. (A) Ag-specific $CD4^+CD25^+Foxp3^+$ Treg cells within the splenocyte population from CIA mice. (B) The Ag-specific $CD4^+CD25^+Foxp3^+$ Treg cell population within the inguinal lymph node population from CIA mice. The percentage of induced Th1 cells (IFN-γ-producing $CD4^+$ T cells) (C) and Th17 cells (IL-17A-producing $CD4^+$ T cells) (D) within the splenocyte population from CIA mice was examined by flow cytometry (data are representative of ten mice per group) and data expressed as mean ± SEM. (E) The levels of IFN-γ or IL-10 in the splenocyte culture supernatants were measured by ELISA. Data are expressed as mean ± SEM of triplicate samples. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Recent evidence suggests that Th1 and Th17 cells are key players in the pathogenesis in CIA [23]. Mice deficient in the Th17 cell-associated molecules, IL-17A, IL-17R, or IL-23p19, suffer less severe arthritis than their wild-type counterparts [24]. Furthermore, treatment with type II collagen-pulsed tDCs decreases the proportion of Th17 cells in CIA mice and simultaneously reduces the disease severity and progression [9]. The *in vivo* studies performed herein showed that treatment with type II collagen-pulsed Rosi-DCs resulted in a decrease in the percentages of IFN-γ-producing $CD4^+$ T cells and IL-17-producing $CD4^+$ T cells.

In parallel with this therapeutic effect, injection of Rosi-DCs led to a significant increase in the $FoxP3^+CD4^+CD25^+$ regulatory T cell population both *in vivo* and *in vitro*. Treg frequency and function can be measured in the peripheral blood of RA patients as well as at the site of inflammation. Studies investigating circulating Tregs in RA report variable results, particularly with regard to Treg inhibitory function [25]. When naïve $CD4^+$ T cells recognize Ag on interdigitating DC they remain within the lymph node and proliferate in the paracortex [26]. However, increased Treg levels are consistently reported at the local site of inflammation. Rosi-DCs therapy also increased the proportion of $FoxP3^+$ regulatory T cells, suggesting that a shift from a pathogenic to a suppressive T cell phenotype may contribute to the suppression of arthritis [27–29]. For the above reasons, DC therapies for autoimmune diseases aim to either diminish the inflammatory potential of T cells or to enhance their tolerogenic characteristics.

In conclusion, the results presented herein show that type II collagen-pulsed Rosi-DCs ameliorate the inflammation associated with CIA *via* the induction of Treg cell population. These results suggest that rosiglitazone-mediated DCs hold promise as a novel therapeutic strategy for RA.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

SH Byun and NC Jung performed *in vivo* experiments and supervised DC characterization studies, analyzed data; JH Lee and HJ Choi performed *ex vivo* immune status experiments and analyzed data; JY Song and HG Seo supervised DC and T cell related laboratory assay and experiment design; JJ Choi, SY Jung, YS Choi, SJ Kang, and JH Jung supervised histopathology analysis and contributed to study design; SH Byun, NC Jung, and DS Lim contributed to the writing of the manuscript; DS Lim supervised the research design and laboratory activities.

Acknowledgements

This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (Grant No. HN14C0082).

References

[1] I.B. McInnes, J.R. O'Dell, State-of-the-art: rheumatoid arthritis, Ann. Rheum. Dis. 69 (2010) 1898–1906.
[2] F. Flores-Borja, C. Mauri, M.R. Ehrenstein, Restoring the balance: harnessing regulatory T cells for therapy in rheumatoid arthritis, Eur. J. Immunol. 38 (2008) 934–937.
[3] A.K. Imperato, C.O. Bingham 3rd, S.B. Abramson, Overview of benefit/risk of biological agents, Clin. Exp. Rheumatol. 22 (2004) S108–S114.
[4] J.M. Stuart, A.S. Townes, A.H. Kang, Collagen autoimmune arthritis, Annu. Rev. Immunol. 2 (1984) 199–218.
[5] R.M. Steinman, D. Hawiger, M.C. Nussenzweig, Tolerogenic dendritic cells, Annu. Rev. Immunol. 21 (2003) 685–711.
[6] K. Mahnke, T.S. Johnson, S. Ring, A.H. Enk, Tolerogenic dendritic cells and regulatory T cells: a two-way relationship, J. Dermatol. Sci. 46 (2007) 159–167.
[7] R.A. Maldonado, U.H. von Andrian, How tolerogenic dendritic cells induce regulatory T cells, Adv. Immunol. 108 (2010) 111–165.
[8] S. Rutella, S. Danese, G. Leone, Tolerogenic dendritic cells: cytokine modulation comes of age, Blood 108 (2006) 1435–1440.
[9] J.N. Stoop, R.A. Harry, A. von Delwig, J.D. Isaacs, J.H. Robinson, C.M. Hilkens, Therapeutic effect of tolerogenic dendritic cells in established collagen-induced arthritis is associated with a reduction in Th17 responses, Arthritis Rheum. 62 (2010) 3656–3665.
[10] R. Mukherjee, L. Jow, G.E. Croston, J.R. Paterniti Jr., Identification, characterization, and tissue distribution of human peroxisome proliferator-activated receptor (PPAR) isoforms PPARgamma2 versus PPARgamma1 and activation with retinoid X receptor agonists and antagonists, J. Biol. Chem. 272 (1997) 8071–8076.
[11] H. Martin, Role of PPAR-gamma in inflammation. Prospects for therapeutic intervention by food components, Mutat. Res. 690 (2010) 57–63.
[12] M. Ricote, A.C. Li, T.M. Willson, C.J. Kelly, C.K. Glass, The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation, Nature 391 (1998) 79–82.
[13] C. Jiang, A.T. Ting, B. Seed, PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines, Nature 391 (1998) 82–86.
[14] H. Jonuleit, E. Schmitt, K. Steinbrink, A.H. Enk, Dendritic cells as a tool to induce anergic and regulatory T cells, Trends Immunol. 22 (2001) 394–400.
[15] X. Zhang, T. Moyana, M. Quereshi, J. Xiang, Conversion of tolerogenic CD4-8-dendritic cells to immunogenic ones inducing efficient antitumor immunity, Cancer Biother. Radiopharm. 21 (2006) 74–80.
[16] D.S. Lim, M.S. Kang, J.A. Jeong, Y.S. Bae, Semi-mature DC are immunogenic and not tolerogenic when inoculated at a high dose in collagen-induced arthritis mice, Eur. J. Immunol. 39 (2009) 1334–1343.
[17] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, et al., The nuclear receptor superfamily: the second decade, Cell 83 (1995) 835–839.
[18] L. Michalik, J. Auwerx, J.P. Berger, V.K. Chatterjee, C.K. Glass, F.J. Gonzalez, et al., International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors, Pharmacol. Rev. 58 (2006) 726–741.
[19] M. Kiss, Z. Czimmerer, L. Nagy, The role of lipid-activated nuclear receptors in shaping macrophage and dendritic cell function: from physiology to pathology, J. Allergy Clin. Immunol. 132 (2013) 264–286.
[20] D. Hashimoto, J. Miller, M. Merad, Dendritic cell and macrophage heterogeneity in vivo, Immunity 35 (2011) 323–335.
[21] J.H. Lee, T.H. Kim, H.E. Park, E.G. Lee, N.C. Jung, J.Y. Song, et al., Myosin-primed tolerogenic dendritic cells ameliorate experimental autoimmune myocarditis, Cardiovasc. Res. 101 (2014) 203–210.
[22] L.M. Francisco, P.T. Sage, A.H. Sharpe, The PD-1 pathway in tolerance and autoimmunity, Immunol. Rev. 236 (2010) 219–242.
[23] E. Lubberts, IL-17/Th17 targeting: on the road to prevent chronic destructive arthritis? Cytokine 41 (2008) 84–91.
[24] S. Nakae, A. Nambu, K. Sudo, Y. Iwakura, Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice, J. Immunol. 171 (2003) 6173–6177.
[25] G. Mijnheer, B.J. Prakken, F. van Wijk, The effect of autoimmune arthritis treatment strategies on regulatory T-cell dynamics, Curr. Opin. Rheumatol. 25 (2013) 260–267.
[26] K.A. Pape, E.R. Kearney, A. Khoruts, A. Mondino, R. Merica, Z.M. Chen, et al., Use of adoptive transfer of T-cell-antigen-receptor-transgenic T cell for the study of T-cell activation in vivo, Immunol. Rev. 156 (1997) 67–78.
[27] C.A. Lawson, A.K. Brown, V. Bejarano, S.H. Douglas, C.H. Burgoyne, A.S. Greenstein, et al., Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood, Rheumatology 45 (2006) 1210–1217.
[28] J.M. van Amelsfort, K.M. Jacobs, J.W. Bijlsma, F.P. Lafeber, L.S. Taams, CD4(+) CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid, Arthritis Rheum. 50 (2004) 2775–2785.
[29] D. Cao, R. van Vollenhoven, L. Klareskog, C. Trollmo, V. Malmstrom, CD25brightCD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease, Arthritis Res. Ther. 6 (2004) R335–R346.