Biochemical Pharmacology 79 (2010) 888–896

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Anti-allergic function and regulatory mechanisms of KR62980 in allergen-induced airway inflammation

Hee Yeon Won [a], Hyun Jung Min [a], Jin Hee Ahn [b], Sung-Eun Yoo [b], Myung Ae Bae [b], Jeong-Ho Hong [c], Eun Sook Hwang [a,*]

[a] College of Pharmacy and Division of Life and Pharmaceutical Sciences and Center for Cell Signaling & Drug Discovery Research, Ewha Womans University, 11-1 Daehyun-Dong, Sudaemun-Ku, Seoul 120-750, Republic of Korea
[b] Korea Research Institute of Chemical Technology, Daejeon 305-600, Republic of Korea
[c] School of Life Science and Biotechnology, Korea University, Seoul 136-701, Republic of Korea

ARTICLE INFO

Article history:
Received 20 August 2009
Accepted 27 October 2009

Keywords:
KR62980
PPARγ
Rosiglitazone
Th2 cell
Allergic airway inflammation

ABSTRACT

The ligand-activated transcription factor, peroxisome proliferator-activated receptor (PPAR)γ, and its ligands inhibit pro-inflammatory cytokine production by immune cells, thus exerting anti-inflammatory activity. As a non-thiazolidinedione PPARγ ligand, KR62980 has anti-diabetic and anti-adipogenic activities, but its anti-inflammatory function has yet to be characterized. In this study, we investigated the functions and mechanisms of KR62980 in the activation and differentiation of CD4+ T helper (Th) cells by comparing its effects with those of a thiazolidinedione PPARγ ligand, rosiglitazone. KR62980 dose-dependently and significantly suppressed TCR-triggered Th cell proliferation by suppressing IL-2/IL-2Rα-mediated signaling. Both KR62980 and rosiglitazone suppressed IFNγ production in a dose-dependent manner, whereas IL-4 gene expression was specifically suppressed by only KR62980. In addition, sustained KR62980 treatment diminished Th2 cytokine production by inhibiting c-Maf expression. *In vivo* administration of KR62980 in a model of allergic asthma significantly attenuated eotaxin-induced eosinophil infiltration, allergic cytokine production and collagen deposition in the lung. KR62980 also decreased goblet cell hyperplasia in the airway and mucous cell metaplasia in nasal epithelium, concurrent with decreases of allergic Th2 cytokines and IL-17 in the draining lymph node. In conclusion, a novel PPARγ ligand, KR62980, suppresses *in vitro* Th2 cell differentiation and attenuates *in vivo* OVA-induced airway inflammation, suggesting a beneficial role for KR62980 in the treatment of allergic asthma and allergic rhinitis.

1. Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily that regulates gene transcription upon ligand binding [1]. Ligand-activated PPARγ binds to the promoter region of target genes by interacting with other nuclear hormone receptors [2] and then activates target gene transcription. Although originally characterized as a key regulator for adipocyte differentiation to activate adipogenic gene transcription [3], PPARγ is known to induce insulin sensitization in diabetic and obese mice [4]. Considerable attention has been paid to thiazolidinedione (TZD) PPARγ ligands, such as rosiglitazone and pioglitazone, due to their potent anti-diabetic activity, but increasing numbers of reports have revealed their undesirable effects, including increased adipocyte differentiation, cardiac hypertrophy, edema, and hepatotoxicity [5–8]. Accordingly, several non-TZD partial PPARγ ligands such as GW0072 [9], N-(9-fluorenyl) methoxycarbonyl-L-leucine [10] and KR62980 [1-(transmethylimino-N-oxy)-6-(2-morpholinoethyoxy)-3-phenyl-1H-indene-2-carboxylic acid ethyl ester] [11] have been developed to circumvent the limitations of TZD PPARγ ligands and have been characterized as having potent anti-diabetic effects and less adipogenic activities [9,11].

PPARγ exerts anti-inflammatory activity that may be mediated by suppression of pro-inflammatory cytokine production in macrophages and dendritic cells [12–14] and by induction of apoptosis in monocytes [15]. PPARγ is also known to inhibit TCR-triggered T cell proliferation [16]. PPARγ ligands have proven therapeutic effects in animal models of inflammatory diseases such as arthritis, inflammatory bowl disease and asthma [17–21].

* Corresponding author. Tel.: +82 2 3277 4369; fax: +82 2 3277 3760.
E-mail address: eshwang@ewha.ac.kr (E.S. Hwang).

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.10.023

TZD PPARγ ligands suppress airway eosinophil adhesion [22] and allergen-induced airway inflammation [13,23,24] and also play protective roles in airway remodeling [21]. However, the regulatory mechanisms of the anti-inflammatory activities of TZD or non-TZD PPARγ ligands have yet to be clearly demonstrated.

CD4+ T helper (Th) cells are activated by TCR stimulation and subsequently differentiate into effector Th cells, Th1, Th2 and Th17 cells, which produce the signature cytokines IFNγ, IL-4 and IL-17, respectively [25,26]. Upon TCR stimulation, CD4+ Th cells increasingly produce IL-2 and induce T cell proliferation and expansion [27,28]. Increased IL-2 binds to IL-2 receptor (IL-2R)α and activates signal transducers and activator of transcription (STAT) 5-mediated signals within the cells [29,30]. Commitment of CD4+ Th cells into effector Th1, Th2 and Th17 cells is controlled by specific transcription factors: T-bet for Th1; GATA-3 and c-Maf for Th2; and RORγt for Th17 cells. Generation of effector Th cells induces protection against extracellular or intracellular pathogens, whereas overproduction of Th1, Th2 and Th17 cytokines is thought to cause chronic allergic and inflammatory immune responses [31,32]. Thus, suppression of cytokine production in activated CD4+ Th cells may be useful for the treatment of inflammatory immune disorders. Since ligand-induced PPARγ activation suppresses IL-2 [33], IFNγ [34] and IL-4 production in T cells [35], PPARγ may modulate CD4+ Th cell-mediated inflammatory immune responses.

In this study, we examined the effects of the non-TZD PPARγ ligand, KR62980, on activation and differentiation of CD4+ Th cells *in vitro* and on allergic airway inflammation *in vivo*. While both rosiglitazone and KR62980 suppressed the pro-inflammatory cytokine IFNγ in a dose-dependent manner, KR62980 more potently suppressed IL-2-induced cell proliferation via inhibition of IL-2Rα expression. In addition, KR62980, not rosiglitazone, selectively inhibited IL-4 and c-Maf-mediated Th2 cell differentiation and attenuated Th2-driven allergic inflammation *in vivo*. We suggest that KR62980 may play a beneficial role in modulating Th2-driven allergic airway inflammation through the significant suppression of Th2 cell development.

2. Materials and methods

2.1. Reagents

All cytokines and anti-cytokine antibodies were purchased from BD Biosciences (San Diego, CA). KR62980 was synthesized in the Korea Research Institute of Chemical Technology (Daejeon, Korea) and was provided endotoxin-free with 99% purity.

2.2. Mice

C57BL6 and Balb/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions at Ewha Womans University. Animal handling and experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines.

2.3. Isolation and activation of CD4+ Th cells

Single cell suspensions were prepared from lymph nodes and spleens of wild-type mice, and incubated with mouse anti-CD4 micro-beads according to the manufacturer's instructions (Miltenyi Biotech., Auburn, CA). Isolated CD4+ Th cells (>95% purity) were stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in the presence of recombinant human IL-2 (2 U/ml). The indicated amounts of rosiglitazone or KR62980 were added to the culture media concurrent with TCR stimulation.

2.4. Cell apoptosis assay

Cells were harvested, washed twice in phosphate-buffered saline (PBS) and incubated with phycoerythrin (PE)-conjugated annexin-V and 7-aminoactinomycin D for 20 min at room temperature (BD Biosciences). Cells were then analyzed by flow cytometry, and apoptotic cell populations were calculated using FACS Calibur and CellQuest software (BD Biosciences).

2.5. CFSE staining and cell proliferation assay

Single cell suspensions of CD4+ Th cells were incubated with 10 μM carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) at 37 °C for 10 min and then washed with PBS. CFSE-labeled CD4+ Th cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in the presence of either rosiglitazone or KR62980 for 48 h and the number of cell divisions was analyzed by dye dilution using flow cytometry.

2.6. Cell cycle analyses

Cells were fixed in ice-cold 70% ethanol, treated with RNase and incubated with propidium iodide (PI, Sigma–Aldrich Inc., St. Louis, MO) for 30 min. After washing the cells with PBS, cells were acquired by flow cytometry in the FACS Calibur and analyzed using the CellQuest and ModFit programs (BD Biosciences).

2.7. ELISA

Cell supernatants were collected and incubated on capture Ab-coated ELISA plates according to the manufacturer's instructions (BD Biosciences). Plates were subsequently incubated with biotinylated anti-cytokine Ab and alkaline phosphatase-conjugated streptavidin. Color changes of phosphatase substrates (Sigma–Aldrich Inc.) were measured on an ELISA plate reader (Molecular Devices, Palo Alto, CA). ELISA kits (R&D Systems Inc., Minneapolis, MN) were used to measure IL-5 (sensitivity <3.3 pg/ml) and IL-13 (sensitivity <1.5 pg/ml) in bronchoalveolar lavage fluid (BALF).

2.8. Real-time PCR

Total RNA was prepared using TRIzol reagent and reverse-transcribed into cDNA using a SuperScript II RT kit (Invitrogen, Carlsbad, CA). Transcribed cDNA template was diluted and mixed with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and specific primers as follows: β-actin-FWD 5′-aagcaggagtatgacgagtccg-3′, β-actin-REV 5′-cggaactaagtcatagtccgcc-3′, IL-4-FWD 5′-tcttgataaacttaattgtctctcgtcac-3′, IL-4-REV 5′-gcaggatgacaactagctggg-3′, IL-5-FWD 5′-agcacagtggtgaaagagacctt-3′, IL-5-REV 5′-tccaatgcatagctggtgattt-3′, IL-13-FWD 5′-agaccagactcccctgtgca-3′, IL-13-REV 5′-tgggtcctgtagatggcattg-3′, IFNγ-FWD 5′-agcaacagcaaggcgaaaa-3′, IFNγ-REV 5′-ctggacctgtgggttgttga-3′, eotaxin-FWD 5′-cagatgcaccctgaaagccata-3′, eotaxin-REV 5′-tgctttgtggcatcctggac-3′. Quantitative real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems).

2.9. Differentiation of CD4+ Th cells into Th1, Th2 and Th17 cells

Isolated CD4+ Th precursor cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in the presence of recombinant human IL-2 (2 U/ml). On day 1, cells were additionally treated with combinations of cytokine and anti-cytokines; IL-12 (2 ng/ml) and anti-IL-4 (5 μg/ml) for Th1;

IL-4 (10 ng/ml) and anti-IFNγ (5 μg/ml) for Th2; and anti-IFNγ (5 μg/ml), anti-IL-4 (5 μg/ml), TGFβ (5 ng/ml) and IL-6 (10 ng/ml) for Th17. Cells were cultured for an additional 4–5 days and subsequently re-stimulated with plate-bound anti-CD3 (1 μg/ml). Cells were treated with KR62980 upon TCR stimulation and media were refreshed on days 2 and 4. Cell supernatants were used for ELISA and cell pellets were harvested for real-time PCR analysis.

2.10. Immunoblot analysis

CD4+ Th cells were induced to differentiate into Th2 cells in the presence of 10 μM KR62980 for 48 h. Nuclear extracts were prepared and resolved by SDS-PAGE. Protein blots were incubated with specific antibodies against c-Maf, GATA-3, NFATp, and NF-kB p65 (Santa Cruz Biotech, Santa Cruz, CA).

2.11. OVA-induced allergic airway inflammation and BALF analysis

Balb/c mice (6–8 weeks of age) were intraperitoneally injected with 50 μg/ml OVA (0.1 ml at 500 μg/ml) complexed with alum on days 0 and 14. Mice were additionally treated with KR62980 (0, 10, 100 mg/kg) by intraperitoneal injection before receiving 50 μg of OVA intranasally on days 28, 30, 32, 35, and 37. Control animals received saline with alum without OVA. Total BALF (1 ml of PBS three times) was collected from the lungs and centrifuged. IL-5 and IL-13 levels were analyzed using high sensitivity ELISA kits (R&D Systems Inc.). Eosinophils were detected by May-Grunwald Giemsa staining and subsequently analyzed with a ZEISS microscope at 400×.

2.12. Lung histology analyses

Lung tissues were fixed in 10% formalin, dehydrated, mounted in paraffin, and sectioned at 10-μm thickness using a microtome (Leica Microsystems, Nussloch GmbH, Germany). Tissue sections were stained with hematoxylin & eosin, periodic acid-Schiff (PAS) and Masson's trichrome solutions (Sigma–Aldrich Inc.). The number of PAS+ cells per mm of base membrane was measured for quantitative analysis. Masson's trichrome-stained slides were photographed with an Olympus U-TV0.63XC digital camera (Olympus Corp, Melville, NY). Images were analyzed by MetaMorph 4.6 software (Universal Imaging Corp, Downingtown, PA) as previously described [36].

2.13. Statistical analysis

Statistical significance was determined by one-way ANOVA and, if necessary, group means were compared by post hoc analysis using the Tukey multiple comparison of means test. Data are given as means ± SEM of at least three independent experiments. Significance was set at a p value of less than 0.05.

3. Results

3.1. KR62980 decreases the number of TCR-triggered CD4+ Th cells but does not induce apoptotic cell death

KR62980 is a novel non-TZD PPARγ ligand containing the morpholino and phenyl indene moieties (Fig. 1A). In order to examine whether KR62980 influences the activation of CD4+ Th

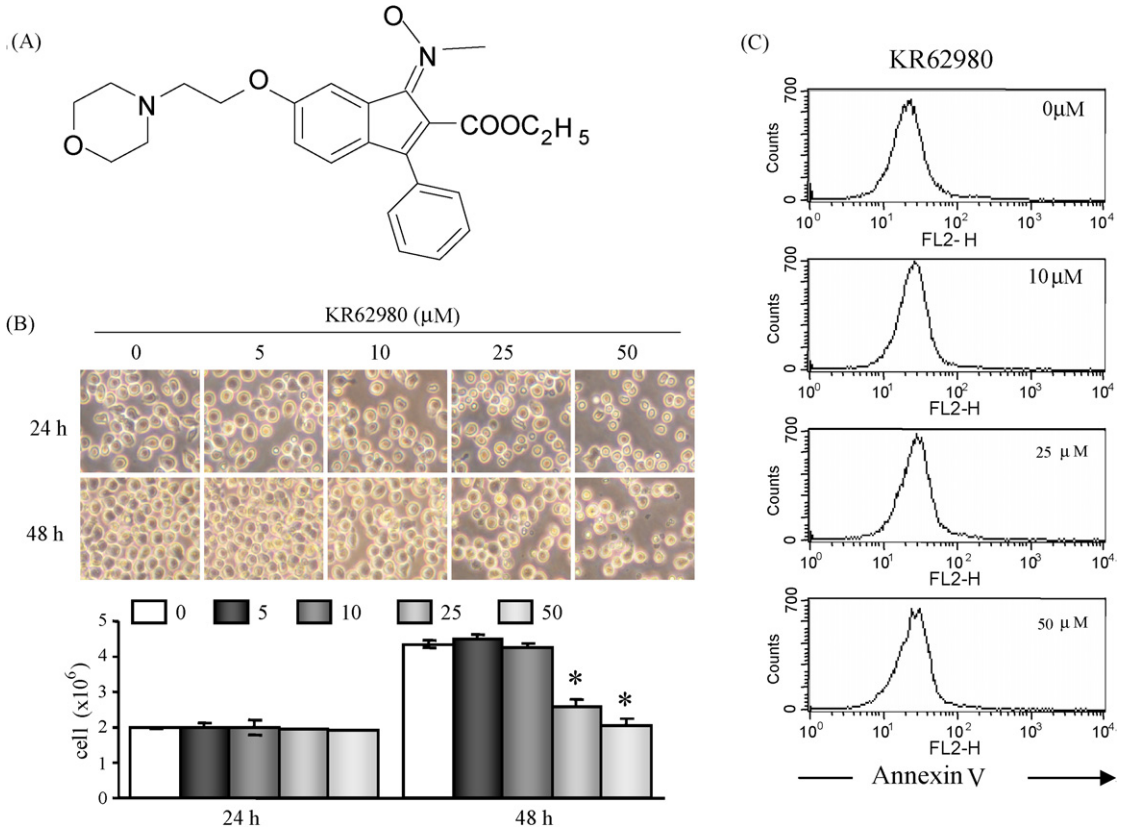

Fig. 1. Effects of KR62980 on the activation of CD4+ Th cells. (A) Chemical structure of KR62980. CD4+ Th cells were isolated from lymph node of C57BL6 mice and stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in the presence of various concentrations of KR62980. (B) Activated CD4+ Th cells were observed at 24 and 48 h after TCR stimulation with 200× magnification using standard optical microscopy. Viable cells were counted in the Trypan blue exclusion assay (n = 3). (C) Cells were harvested at 48 h and stained with annexin-V and 7-AAD using a cell apoptosis assay kit. Apoptotic cells were analyzed by flow cytometry.

cells, primary CD4+ Th cells were treated with various amounts of KR62980 concurrent with TCR stimulation. CD4+ Th cells were well activated upon TCR stimulation and highly proliferative at 48 h. At concentrations below 10 μM, KR62980 had no effect on the activation and proliferation of CD4+ Th cells, whereas diminished numbers of CD4+ Th cells were observed at 25 and 50 μM KR62980 (Fig. 1B). We performed cell apoptosis assays with annexin-V binding and found no induction of apoptosis by KR62980. These results suggest that the decrease in the numbers of KR62980-treated CD4+ Th cells is not mediated by cell apoptosis.

3.2. Anti-proliferative activity of KR62980 via suppression of IL-2Rα expression

By labeling CD4+ Th cells with CFSE, we next tested whether KR62980 suppresses cell proliferation. While CFSE-labeled CD4+ Th cells proliferated rapidly, resulting in four divisions at 48 h post-TCR stimulation, KR62980, like rosiglitazone, suppressed CD4+ Th cell division and dose-dependently reduced cell division (Fig. 2A). Further cell cycle analysis verified that KR62980 diminished the G1- to S-phase transition of the cell cycle (Fig. 2B). Since TCR-triggered T cell proliferation is critically associated with IL-2 production and IL-2R-mediated signaling [37], we investigated IL-2 levels and IL-2Rα expression in the presence of rosiglitazone or KR62980. Rosiglitazone attenuated IL-2 production and IL-2Rα expression, but only very moderately at 50 μM. However, KR62980 potently diminished IL-2 production and suppressed IL-2Rα expression (Fig. 2C and D), suggesting that KR62980 suppresses IL-2-dependent T cell proliferation by suppressing IL-2Rα expression.

3.3. KR62980, but not rosiglitazone, decreases IL-4 production

As PPARγ ligands are known to suppress pro-inflammatory cytokine production, we examined the IFNγ and IL-4 levels produced by TCR-triggered CD4+ Th cells of C57BL6 (Th1-prone) or BALB/c (Th2-prone) mice. The Th1-prone pro-inflammatory cytokine IFNγ was decreased by both rosiglitazone (IC_{50}, 40.2 μM) and KR62980 (IC_{50}, 10.9 μM) at the levels of protein and gene transcription (Fig. 3A and B). In addition, KR62980 dose-dependently suppressed allergic cytokine IL-4 expression via inhibition of gene transcription (IC_{50}, 17.7 μM), whereas rosiglitazone did not (Fig. 3C and D). These results imply that unlike rosiglitazone, KR62980 may have potent anti-allergic activity through its suppression of IL-4 gene expression.

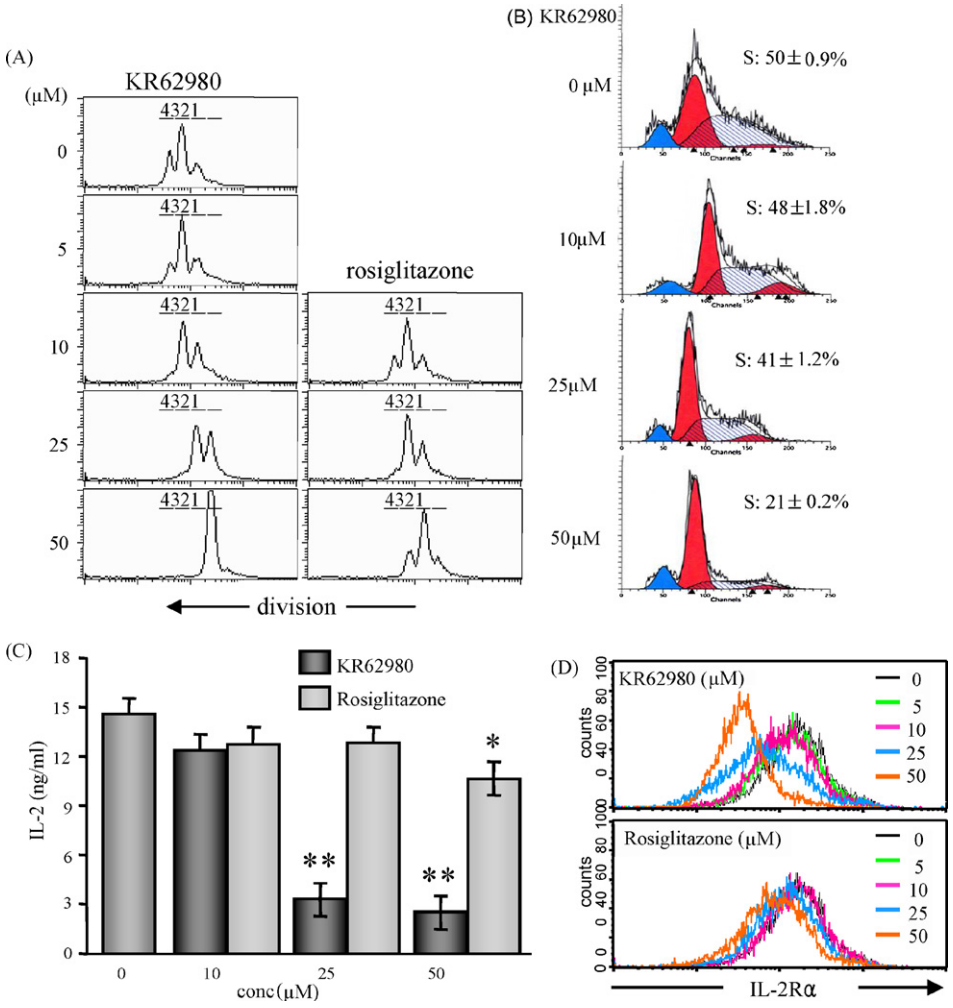

Fig. 2. Anti-proliferative function of KR62980 in CD4+ Th cells. (A) CD4+ Th cells (1×10^7) were labeled with CFSE dye and stimulated with TCR for 48 h in the presence of the indicated amounts of KR62980 or rosiglitazone. Proliferating cells were analyzed using the FACS Calibur system. Data shown are one representative of three independent experiments and numbers indicate the number of cell divisions. (B) Proliferating CD4+ Th cells upon TCR stimulation were fixed in ethanol and incubated with PI. Cell cycle progression was analyzed by CellQuest and flow cytometry. Cell populations in G1 to S transition phase were calculated using the ModFit program and reported as means ± SEM ($n = 3$). (C) Cell supernatant was collected from 48-h stimulated CD4+ Th cells in the presence of KR62980 or rosiglitazone and used for ELISA of IL-2 cytokine. Data are given as means ± SEM ($n = 3$). (D) Cells treated with different concentrations of KR62980 or rosiglitazone were harvested at 48 h after TCR stimulation, incubated with PE-conjugated IL-2Rα Ab, and then analyzed by flow cytometry.

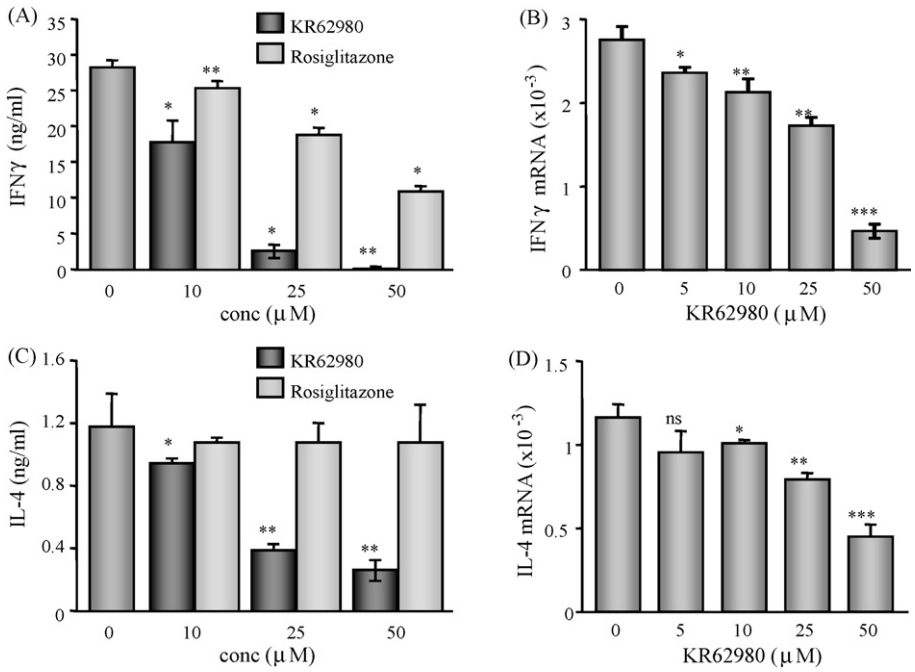

Fig. 3. Suppression of IL-4 production by KR62980. CD4+ Th cells were isolated from either C57BL6 (Th1-prone, A and B) or Balb/c (Th2-prone, C and D) mice and activated with anti-CD3 and anti-CD28 in the presence of KR62980 or rosiglitazone for 48 h. Cell supernatants were collected for measuring the cytokines IFNγ (A) and IL-4 (C) by ELISA. Cells were also harvested for total RNA preparation. Two μg of total RNA was used for reverse transcription and subsequent real-time PCR. Relative expression levels of IFNγ (B) and IL-4 (D) were calculated after normalization to the levels of β-actin. Data are reported as means ± SEM ($n = 4$). Statistical significance was analyzed by ANOVA. ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

3.4. KR62980 specifically ameliorates Th2 cell development

The significant decrease of IL-4 with KR62980 inspired us to investigate whether KR62980 affects Th cell differentiation into Th1, Th2 or Th17 cells. In order to avoid the potent anti-proliferative effect of KR62980 in TCR-triggered CD4+ Th cells, cells were treated and refreshed with 10 μM KR62980 during Th cell differentiation. Sustained treatment with 10 μM KR62980 had no significant effect on IL-2 production, but reduced IFNγ-producing Th1 cell differentiation (Fig. 4A); however, Th17 cell development was not affected by KR62980, as evidenced by the lack of changes in IL-2 and IL-17 levels (Fig. 4B). Interestingly,

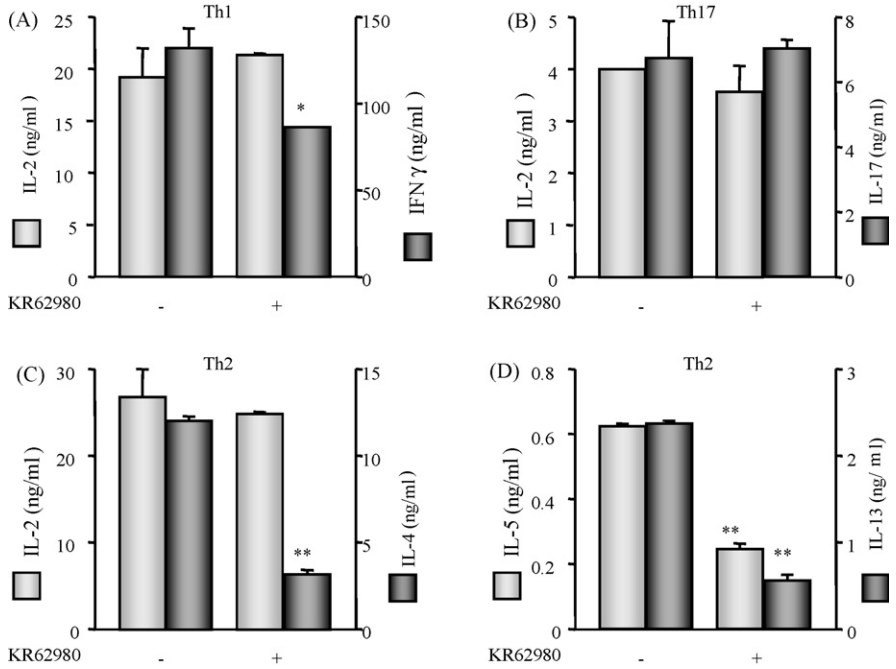

Fig. 4. KR62980-mediated inhibition of Th2 cell differentiation. CD4+ Th cells isolated from lymph node were activated and induced to differentiate into effector Th cells as described in Section 2. Differentiating Th cells were incubated with 10 μM KR62980 and refreshed every 2 days during Th cell differentiation. Differentiated Th1, Th2 or Th17 cells were re-stimulated with anti-CD3 for 24 h and cell supernatants were harvested for cytokine measurements by ELISA. Cell supernatants were used for measuring cytokine levels: IL-2 and IFNγ from Th1 cells (A), and IL-2 and IL-17 from Th17 cells (B). (C and D) Th2 cell supernatants were used for determining IL-2, IL-4, IL-5 and IL-13 (C and D) levels by ELISA. Data are reported as means ± SEM ($n = 3$). Statistical significance was analyzed by Student's t-test. ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.005$.

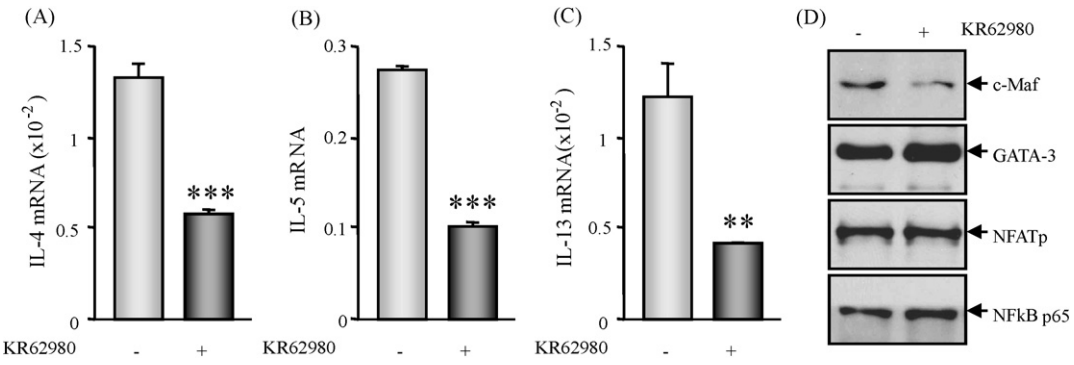

Fig. 5. Attenuation of c-Maf expression by KR62980. CD4+ Th cells isolated from Balb/c mice were incubated with 10 μM KR62980 during Th2 cell development. Differentiated Th2 cells were re-stimulated with anti-CD3 and harvested for RNA preparation and subsequent real-time PCR assays. Gene transcription levels of IL-4 (A), IL-5 (B) and IL-13 (C) were determined from the activated Th2 cells. Data are reported as means ± SDs ($n = 4$). ns, not significant; $^{**}p < 0.005$; $^{***}p < 0.0005$. (D) CD4+ Th cells were differentiated into Th2 cells in the absence or presence of 10 μM KR62980 for 72 h. The nuclear proteins were isolated from activated Th2 cells and resolved by SDS-PAGE. Protein blots were subsequently incubated with specific Abs against c-Maf, GATA-3, NFATp and NF-kB p65.

constant treatment with KR62980 drastically reduced IL-4 production without affecting IL-2 levels in the differentiation of Th2 cells (Fig. 4C). In addition, the other major Th2 cytokines, IL-5 and IL-13, were also decreased by 60 and 80%, respectively, with KR62980 (Fig. 4D), supporting the notion that KR62980 ameliorates Th2 cell development.

3.5. Attenuation of c-Maf-mediated Th2 cell differentiation by KR62980

Corresponding to the decreased protein levels of IL-4, IL-5 and IL-13 cytokines with KR62980 treatment, the gene transcription levels of IL-4, IL-5 and IL-13 were substantially inhibited by KR6290 (Fig. 5A–C). To explain the inhibitory mechanisms of KR62980 in Th2 cytokine gene transcription, the expression levels of active transcription factors in the nucleus were determined. The expression levels of the T cell-specific transcription factors, NFATp and NF-kB p65, and of GATA-3, a master regulator of Th2 cell development, were not influenced by KR62980, but the level of c-Maf, another Th2-specific transcription factor [38,39], was comparatively decreased by treatment with KR62980 (Fig. 3D). These results suggest that the suppression of Th2 cell differentiation by KR62980 may be mediated by a reduction in c-Maf expression.

3.6. Attenuation of eosinophil infiltration by KR62980 in OVA-induced airway inflammation model

Since KR62980 substantially suppressed c-Maf-induced Th2 cell development *in vitro*, we assessed whether KR62980 modulated Th2 cell-related immune responses in an allergen-induced airway inflammation model. BALB/c mice were immunized with OVA and injected with PBS or KR62980 before intranasal administration of OVA. As shown in Fig. 6A, OVA challenge in sensitized animals markedly increased the infiltration of immune cells such as neutrophils, macrophages, lymphocytes and eosinophils in the BALF. While most infiltrating immune cells were not influenced by KR62980, eosinophils were markedly decreased by treatment with KR62980 (Fig. 6B). The dominant eosinophil-chemoattractant, eotaxin, was also attenuated in the lungs of KR62980-treated mice (Fig. 6C).

3.7. KR62980-mediated reduction of Th2-driven allergic cytokines in the lung

Next, the allergic cytokines in the lung were assessed by ELISA and Multiplex assay system. The protein levels of the allergic cytokines, IL-5 and IL-13, in the BALF were significantly attenuated by KR62980 injection (Fig. 7A and B). Moreover, *ex vivo* analysis of cytokine production confirmed that KR62980 diminished IL-4 and IL-5 cytokines in draining lymph node (Fig. 7C and D). IL-17, which is a pivotal pro-inflammatory cytokine in allergic airway inflammation, was also decreased in the KR62980-treated group (Fig. 7E). These results support the *in vivo* suppression of Th2 cytokine production by KR62980.

3.8. Amelioration of inflammation in the lung and nasal cavity by KR62980

Histological analyses of lung sections using PAS and trichrome staining revealed profound goblet cell hyperplasia and dense collagen deposition in airways that were sensitized and challenged with OVA (Fig. 8A). However, KR62980 treatment decreased both goblet cell-mucus secretion and collagen deposition in the lung interstitium around the airways (Fig. 8A). Quantitation of PAS+ cells and Trichrome+ area revealed statistical differences between the vehicle- and KR62980-treated groups (Fig. 8B). In addition, inflammatory responses induced by OVA in nasal mucous membranes were significantly alleviated by KR62980 (Fig. 8C), demonstrating that KR62980 potently attenuates allergen-induced airway inflammation *in vivo*.

4. Discussion

Many investigators have demonstrated the anti-inflammatory functions and therapeutic potential of PPARγ agonists, and TZD PPARγ ligands have been successfully used to treat inflammatory diseases such as colitis, psoriasis and chronic obstructive pulmonary disease [13,40–43]. The regulatory mechanisms of the anti-inflammatory effects of PPARγ agonists have also been extensively studied. A natural PPARγ agonist, 15-deoxy-delta-prostaglandin J2, suppresses TNFα and G-CSF production in monocytes and smooth muscle cells [12] and ameliorates *de novo* synthesis of protein involved in cell cycle progression and NF-kB-mediated inflammatory gene expression [12,44]. In addition, the TZD PPARγ agonist, rosiglitazone can directly inhibit IFNγ expression by interfering with c-Jun activation in the IFNγ gene promoter [34]. In this study, rosiglitazone also diminished IFNγ production in activated Th cells. Moreover, KR62980, a non-TZD PPARγ ligand, potently suppressed IFNγ expression, suggesting that the regulatory mechanism of KR62980 in IFNγ suppression is similar to that of rosiglitazone.

In addition to IFNγ suppression, KR62980 substantially inhibited Th cell proliferation and Th2-derived IL-4 production,

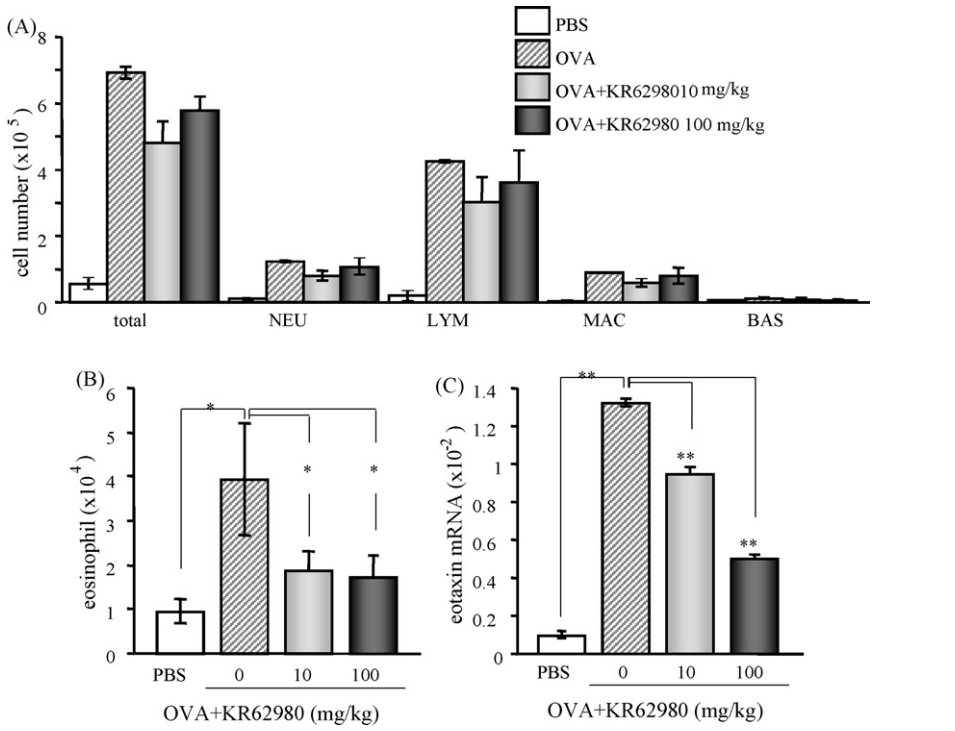

Fig. 6. Reduction of eotaxin-induced eosinophil infiltration by KR62980. Balb/c mice ($n = 6$ per group) were immunized with OVA and intraperitoneally injected with KR62980 (0, 10, 100 mg/kg) before intranasal administration of OVA. BALF was collected from the lung and centrifuged. (A) Cell numbers of neutrophils (NEU), lymphocytes (LYM), macrophages (MAC), and basophils (BAS) in BALF were determined using a hemocytometer and reported as means ± SEM for six mice. (B) Inflamed eosinophils in BALF were counted and reported as means ± SEM ($n = 6$). (C) Total RNA was prepared from the lung and used for reverse transcription. Relative expression levels of eotaxin were determined by real-time PCR and statistical significance was analyzed by ANOVA ($n = 6$). $^{*}p < 0.05$; $^{**}p < 0.005$.

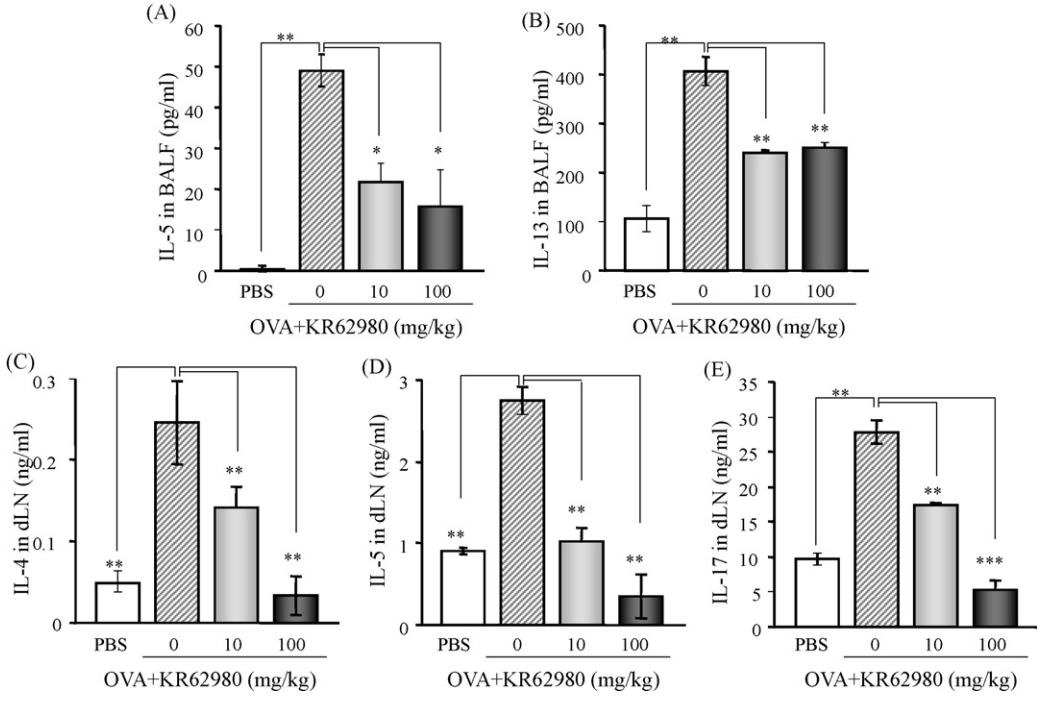

Fig. 7. Diminished allergic inflammatory cytokine production by KR62980. Balb/c mice ($n = 6$) were sensitized and challenged with OVA and injected with or without KR62980. (A) BALF from each group was collected and used to measure the cytokines IL-5 (A) and IL-13 (B) with ELISA kits ($n = 6$). (B) Single cell suspensions (5×10^6 cells) were isolated from the draining lymph nodes of mice ($n = 4$) and stimulated with anti-CD3 for 24 h. Cytokines IL-4 (C), IL-5 (D) and IL-17 (E) were measured in the cell supernatants. Data are expressed as means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

whereas rosiglitazone did not affect IL-2 and IL-4 production. As IL-2/IL-2Rα-mediated signaling is critical for inducing Th cell differentiation, significant reduction of IL-2Rα expression by treatment with KR62980 may be subject to the regulatory mechanism acting in the inhibition of IL-2-dependent Th cell proliferation. Separately, the decreased IL-4 expression and Th2 cell differentiation with KR62980 may be mediated by suppression of c-Maf, a Th2-specific transcription factor. These findings

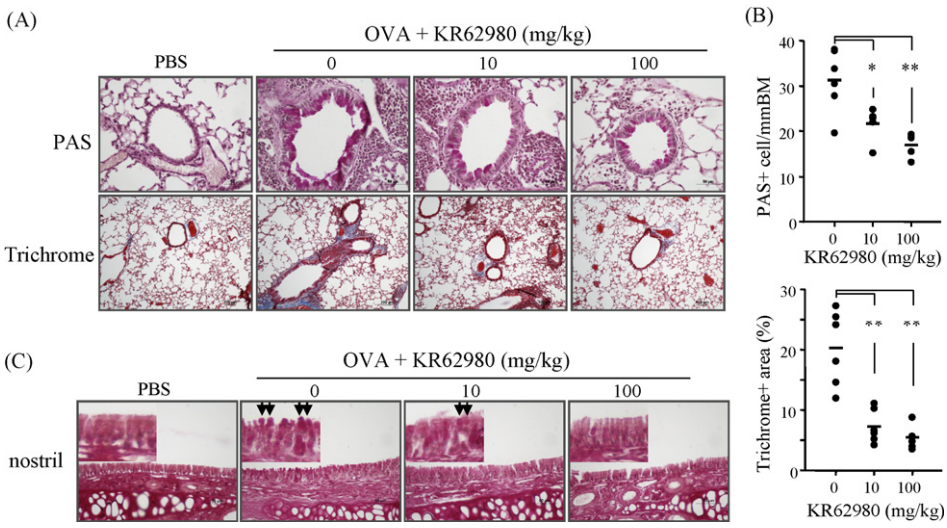

Fig. 8. Attenuation of inflammation in the lung and nasal cavity by KR62980. Lung tissues were harvested from mice treated with or without KR62980 as in Fig. 6. Lung tissues fixed in 10% formalin were sectioned and stained with PAS and Masson's trichrome solution (A) ($n = 6$). Scale bars show 50 and 100 μm in upper and lower panels, respectively. (B) PAS+ cells and Trichrome+ area were calculated as described in Section 2 ($n = 6$). $^{*}p < 0.05$; $^{**}p < 0.005$. (C) The nasal cavities were decalcified, fixed, sectioned, and stained with PAS. Mucous membrane lining the nostril is shown with the 50-μm scale bar.

indicate that KR62980, unlike rosiglitazone, may have distinct roles in the regulation of Th cell proliferation and Th2 cell differentiation via different mechanisms.

Our data strongly indicate that KR62980 plays a pivotal role in controlling allergic airway inflammation *in vivo* by reducing eotaxin-induced eosinophil infiltration and by attenuating mucus-secreting goblet cell hyperplasia and collagen deposition in the airways. Prolonged and continuous treatment with TZD PPARγ agonists in inflammatory diseases provoke undesirable side effects including weight gain, edema, myocardial infarction and exacerbation of cardiac dysfunction [45,46]; thus, safer PPARγ agonists are in development [11,47–49]. KR62980 may provide an alternative to the TZD PPARγ ligands in treating inflammatory diseases such as allergic asthma, rhinitis or chronic obstructive pulmonary disease. Sustained treatment with KR62980 exerts no adipogenic effects, which are prominently observed with TZD PPARγ ligands, but rather antagonizes the adipogenic activity induced by rosiglitazone [50]. Moreover, the anti-diabetic function of KR62980 is as efficient as that of TZD PPARγ ligands, and no toxicity, including mutagenic potential or hepatotoxicity, has been observed [11].

Collectively, we propose that KR62980 may be distinguished from rosiglitazone in terms of its functional anti-allergic mechanisms and may have powerful beneficial effects in the treatment of Th2-driven allergic inflammatory diseases such as allergic asthma and allergic rhinitis.

Conflicts of interest

The authors state no conflicts of interest.

Acknowledgements

This work was supported by the Center for Biological Modulators of the 21st Century Frontier R&D program, Basic Science Research program (KRF-2008-314-E00283) and partially by the NCRC program (R15-2006-020) of KOSEF funded by MEST.

References

[1] Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988;240:889–95.
[2] Dubuquoy L, Dharancy S, Nutten S, Pettersson S, Auwerx J, Desreumaux P. Role of peroxisome proliferator-activated receptor gamma and retinoid X receptor heterodimer in hepatogastroenterological diseases. Lancet 2002;360:1410–8.
[3] Spiegelman BM, Flier JS. Adipogenesis and obesity: rounding out the big picture. Cell 1996;87:377–89.
[4] Mukherjee R, Davies PJ, Crombie DL, Bischoff ED, Cesario RM, Jow L, et al. Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. Nature 1997;386:407–10.
[5] Scheen AJ. Combined thiazolidinedione-insulin therapy: should we be concerned about safety? Drug Saf 2004;27:841–56.
[6] Martens FM, Visseren FL, Lemay J, de Koning EJ, Rabelink TJ. Metabolic and additional vascular effects of thiazolidinediones. Drugs 2002;62:1463–80.
[7] Kobayashi M, Iwata M. Evaluation of thiazolidinedione derivative drugs for safety. Nippon Rinsho 2001;59:2228–32.
[8] Chui PC, Guan HP, Lehrke M, Lazar MA. PPARgamma regulates adipocyte cholesterol metabolism via oxidized LDL receptor 1. J Clin Invest 2005;115:2244–56.
[9] Oberfield JL, Collins JL, Holmes CP, Goreham DM, Cooper JP, Cobb JE, et al. A peroxisome proliferator-activated receptor gamma ligand inhibits adipocyte differentiation. Proc Natl Acad Sci USA 1999;96:6102–6.
[10] Rocchi S, Picard F, Vamecq J, Gelman L, Potier N, Zeyer D, et al. A unique PPARgamma ligand with potent insulin-sensitizing yet weak adipogenic activity. Mol Cell 2001;8:737–47.
[11] Kim KR, Lee JH, Kim SJ, Rhee SD, Jung WH, Yang SD, et al. KR-62980: a novel peroxisome proliferator-activated receptor gamma agonist with weak adipogenic effects. Biochem Pharmacol 2006;72:446–54.
[12] Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 1998;391:82–6.
[13] Belvisi MG, Hele DJ, Birrell MA. Peroxisome proliferator-activated receptor gamma agonists as therapy for chronic airway inflammation. Eur J Pharmacol 2006;533:101–9.
[14] Faveeuw C, Fougeray S, Angeli V, Fontaine J, Chinetti G, Gosset P, et al. Peroxisome proliferator-activated receptor gamma activators inhibit interleukin-12 production in murine dendritic cells. FEBS Lett 2000;486:261–6.
[15] Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, et al. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. J Biol Chem 1998;273:25573–80.
[16] Schmidt S, Moric E, Schmidt M, Sastre M, Feinstein DL, Heneka MT. Anti-inflammatory and antiproliferative actions of PPAR-gamma agonists on T lymphocytes derived from MS patients. J Leukoc Biol 2004;75:478–85.
[17] Ueki S, Matsuwaki Y, Kayaba H, Oyamada H, Kanda A, Usami A, et al. Peroxisome proliferator-activated receptor gamma regulates eosinophil functions: a new therapeutic target for allergic airway inflammation. Int Arch Allergy Immunol 2004;134(Suppl. 1):30–6.
[18] Serhan CN, Devchand PR. Novel antiinflammatory targets for asthma. A role for PPARgamma? Am J Respir Cell Mol Biol 2001;24:658–61.
[19] Scher JU, Pillinger MH. 15d-PGJ2: the anti-inflammatory prostaglandin? Clin Immunol 2005;114:100–9.
[20] Park SJ, Lee YC. Peroxisome proliferator-activated receptor gamma as a novel therapeutic target in asthma. J Asthma 2008;45:1–8.
[21] Lee KS, Park SJ, Kim SR, Min KH, Jin SM, Lee HK, et al. Modulation of airway remodeling and airway inflammation by peroxisome proliferator-activated receptor gamma in a murine model of toluene diisocyanate-induced asthma. J Immunol 2006;177:5248–57.

[22] Hirasawa H, Chiba T, Ueki S, Kamada Y, Ito W, Takeda M, et al. The synthetic PPARgamma agonist troglitazone inhibits eotaxin-enhanced eosinophil adhesion to ICAM-1-coated plates. Int Arch Allergy Immunol 2008;146(Suppl. 1):11–5.
[23] Lee SY, Kang EJ, Hur GY, Jung KH, Jung HC, Kim JH, et al. Peroxisome proliferator-activated receptor-gamma inhibits cigarette smoke solution-induced mucin production in human airway epithelial (NCI-H292) cells. Am J Physiol Lung Cell Mol Physiol 2006;291:L84–90.
[24] Narala VR, Ranga R, Smith MR, Berlin AA, Standiford TJ, Lukacs NW, et al. Pioglitazone is as effective as dexamethasone in a cockroach allergen-induced murine model of asthma. Respir Res 2007;8:90.
[25] Rengarajan J, Szabo SJ, Glimcher LH. Transcriptional regulation of Th1/Th2 polarization. Immunol Today 2000;21:479–83.
[26] Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol 2007;25:821–52.
[27] Oravecz T, Monostori E, Kurucz E, Takacs L, Ando I. CD3-induced T-cell proliferation and interleukin-2 secretion is modulated by the CD45 antigen. Scand J Immunol 1991;34:531–7.
[28] Sereti I, Anthony KB, Martinez-Wilson H, Lempicki R, Adelsberger J, Metcalf JA, et al. IL-2-induced CD4+ T-cell expansion in HIV-infected patients is associated with long-term decreases in T-cell proliferation. Blood 2004; 104:775–80.
[29] Van Parijs L, Refaeli Y, Lord JD, Nelson BH, Abbas AK, Baltimore D. Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity 1999;11:281–8.
[30] Lin JX, Leonard WJ. The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene 2000;19:2566–76.
[31] Boniface K, Blom B, Liu YJ, de Waal Malefyt R. From interleukin-23 to T-helper 17 cells: human T-helper cell differentiation revisited. Immunol Rev 2008;226:132–46.
[32] Wang YH, Liu YJ. The IL-17 cytokine family and their role in allergic inflammation. Curr Opin Immunol 2008;20:697–702.
[33] Clark RB, Bishop-Bailey D, Estrada-Hernandez T, Hla T, Puddington L, Padula SJ. The nuclear receptor PPAR gamma and immunoregulation: PPAR gamma mediates inhibition of helper T cell responses. J Immunol 2000;164:1364–71.
[34] Cunard R, Eto Y, Muljadi JT, Glass CK, Kelly CJ, Ricote M. Repression of IFN-gamma expression by peroxisome proliferator-activated receptor gamma. J Immunol 2004;172:7530–6.
[35] Chung SW, Kang BY, Kim TS. Inhibition of interleukin-4 production in CD4+ T cells by peroxisome proliferator-activated receptor-gamma (PPAR-gamma) ligands: involvement of physical association between PPAR-gamma and the nuclear factor of activated T cells transcription factor. Mol Pharmacol 2003;64:1169–79.
[36] Park JW, Min HJ, Sohn JH, Kim JY, Hong JH, Sigrist KS, et al. Restoration of T-box-containing protein expressed in T cells protects against allergen-induced asthma. J Allergy Clin Immunol 2009;123:479–85.
[37] Kuhn DJ, Dou QP. Direct inhibition of interleukin-2 receptor alpha-mediated signaling pathway induces G1 arrest and apoptosis in human head-and-neck cancer cells. J Cell Biochem 2005;95:379–90.
[38] Ho IC, Lo D, Glimcher LH. c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4-dependent and -independent mechanisms. J Exp Med 1998;188:1859–66.
[39] Ho IC, Hodge MR, Rooney JW, Glimcher LH. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. Cell 1996;85:973–83.
[40] Patel HJ, Belvisi MG, Bishop-Bailey D, Yacoub MH, Mitchell JA. Activation of peroxisome proliferator-activated receptors in human airway smooth muscle cells has a superior anti-inflammatory profile to corticosteroids: relevance for chronic obstructive pulmonary disease therapy. J Immunol 2003;170:2663–9.
[41] Su CG, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, et al. A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response. J Clin Invest 1999;104:383–9.
[42] Pershadsingh HA. Peroxisome proliferator-activated receptor-gamma: therapeutic target for diseases beyond diabetes: quo vadis? Expert Opin Investig Drugs 2004;13:215–28.
[43] Saubermann LJ, Nakajima A, Wada K, Zhao S, Terauchi Y, Kadowaki T, et al. Peroxisome proliferator-activated receptor gamma agonist ligands stimulate a Th2 cytokine response and prevent acute colitis. Inflamm Bowel Dis 2002; 8:330–9.
[44] Na HK, Surh YJ. Peroxisome proliferator-activated receptor gamma (PPAR-gamma) ligands as bifunctional regulators of cell proliferation. Biochem Pharmacol 2003;66:1381–91.
[45] Home PD, Pocock SJ, Beck-Nielsen H, Gomis R, Hanefeld M, Jones NP, et al. Rosiglitazone evaluated for cardiovascular outcomes–an interim analysis. N Engl J Med 2007;357:28–38.
[46] Nissen SE, Wolski K, Topol EJ. Effect of muraglitazar on death and major adverse cardiovascular events in patients with type 2 diabetes mellitus. J Am Med Assoc 2005;294:2581–6.
[47] Burgermeister E, Schnoebelen A, Flament A, Benz J, Stihle M, Gsell B, et al. A novel partial agonist of peroxisome proliferator-activated receptor-gamma (PPARgamma) recruits PPARgamma-coactivator-1alpha, prevents triglyceride accumulation, and potentiates insulin signaling in vitro. Mol Endocrinol 2006;20:809–30.
[48] Higgins LS, Mantzoros CS. The development of INT131 as a selective PPAR-gamma modulator: approach to a safer insulin sensitizer. PPAR Res 2008; 2008:936906.
[49] Chandalia A, Clarke HJ, Clemens LE, Pandey B, Vicena V, Lee P, et al. MBX-102/JNJ39659100, a novel non-TZD selective partial PPAR-gamma agonist lowers triglyceride independently of PPAR-alpha activation. PPAR Res 2009;2009: 706852.
[50] Jung H, Lee MS, Jang EJ, Ahn JH, Kang NS, Yoo SE, et al. Augmentation of PPARgamma-TAZ interaction contributes to the anti-adipogenic activity of KR62980. Biochem Pharmacol 2009;78(10):1323–9.