

Peroxisome proliferator-activated receptor γ -mediated suppression of dendritic cell function prevents the onset of atopic dermatitis in NC/Tnd mice

Kyungsook Jung, DVM,^a Akane Tanaka, DVM, PhD,^a Hiroshi Fujita, DVM,^a Akira Matsuda, DVM,^a Kumiko Oida, DVM,^a Kaoru Karasawa, DVM,^a Noriko Okamoto, MS,^a Keitaro Ohmori, DVM, PhD,^a Youngheun Jee, DVM, PhD,^b Taekyun Shin, DVM, PhD,^b and Hiroshi Matsuda, DVM, PhD^a *Tokyo, Japan, and Jeju, South Korea*

Background: Dendritic cells (DCs) are one of the key regulators for the initiation of allergic responses in patients with atopic dermatitis (AD), being strongly triggered by epithelial cell-derived thymic stromal lymphopoietin (TSLP). Because peroxisome proliferator-activated receptor (PPAR) γ acts as a negative regulator in immune cells, suppressive properties of PPAR γ in allergic responses have been proposed.

Objective: Because pieces of evidence must be organized to identify the exact role of PPAR γ in immune regulation, we explored the suppressive effects of a PPAR γ agonist on various functions of DCs and the onset of AD in a murine model.

Methods: Effects of rosiglitazone (RSG) on DCs that were derived from NC/Tnd mice, a model for human AD, were analyzed. RSG was administered to NC/Tnd mice to evaluate its preventive and therapeutic effects on the development of AD.

Results: RSG inhibited TSLP-induced DC maturation through downregulation of costimulatory molecules. TSLP-promoted expressions of chemokines in DCs were also suppressed by RSG treatment. Moreover, we showed the necessity of matrix metalloproteinase 9 in TSLP-promoted DC migration by using DCs derived from matrix metalloproteinase 9-deficient NC/Tnd mice, as well as the suppressive effect of PPAR γ in the process. Daily oral administration of RSG to NC/Tnd mice before the onset of AD revealed a significant reduction in severity of skin lesions and scratching behavior. In mice treated with RSG, both expression of TSLP in the skin and maturation and migration of DCs were markedly suppressed.

Conclusion: PPAR γ can be provided as an inhibitory regulator of TSLP-stimulated DCs in the onset of allergic reactions. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Mouse, atopic dermatitis, dendritic cells, peroxisome proliferator-activated receptor γ , thymic stromal lymphopoietin, matrix metalloproteinase 9

Atopic dermatitis (AD) is a chronic and relapsing skin disorder with allergic inflammation. Dendritic cells (DCs) in skin are one of the key regulators for the initiation of allergic responses as antigen-presenting cells (APCs), and a majority of patients with AD manifest strong polarization to T_H2-type responses, thereby resulting in the hyperproduction of IgE.¹⁻³ Furthermore, the hypersensitivity and barrier dysfunction of atopic skin play a pivotal role in the exacerbation of clinical conditions.⁴

One of the most critical factors for the local activation of skin DCs during allergic inflammation is thymic stromal lymphopoietin (TSLP),⁵⁻⁸ which is expressed in epithelial cells at barrier surfaces, activated bronchial smooth muscle cells, and activated mast cells.⁹ TSLP is highly expressed in proliferating keratinocytes in skin lesions of allergic skin diseases and in bronchial epithelial cells of asthmatic patients.¹⁰⁻¹² TSLP activates CD11c⁺ DCs and induces them to produce T_H2 cell-attracting CC chemokines, including thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC).^{8,13} Moreover, TSLP upregulated expression of OX40 ligand (OX40L), which engaged with OX40 on activated naive T cells on DCs and indirectly induced the expression of IL-4 and IL-13 and suppressed that of IFN- γ from T cells.^{6,7,14} In the process development into T_H2-like effectors is progressed.^{5,6} Matrix metalloproteinase (MMP) 9 produced from activated DCs is involved in the migration of Langerhans cells.¹⁵⁻¹⁷ Promotion of DC migration is crucial for the early development of allergic inflammation; however, the role of TSLP in DC migration remains unclear.

Peroxisome proliferator-activated receptors (PPARs) are the nuclear hormone receptor superfamily of ligand-activated transcription factors.¹⁸ PPAR γ is highly expressed not only in adipose tissue but also in most immune cells,¹⁹⁻²⁴ and its activation regulates various cell functions.²⁵ PPAR γ ligands downregulate functions of immune cells, including DC maturation, by repressing the expression of CD1a, CD40, CD83, CCR7, and the costimulatory molecule.²⁶⁻²⁸ These findings suggest that treatment with synthetic PPAR γ ligands might reduce allergic inflammation through suppression of DC functions to stimulate lymphocyte

From ^athe Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Graduate School, Institute of Agriculture, Tokyo University of Agriculture and Technology, and ^bthe College of Veterinary Medicine and Veterinary Medical Research Institute, Jeju National University.

Supported by a Grant-in-Aid for Scientific Research on Priority Areas A and a Grant-in-Aid for Scientific Research on Priority Areas B provided by the Japan Society for the Promotion of Science, Japan.

Disclosure of potential conflict of interest: A. Tanaka and H. Matsuda have received research support from the Japan Society for the Promotion of Science. The rest of the authors have declared that they have no conflict of interest.

Received for publication June 24, 2010; revised October 6, 2010; accepted for publication October 26, 2010.

Reprint requests: Akane Tanaka, DVM, PhD, and Hiroshi Matsuda, DVM, PhD, Laboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Graduate School, Institute of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwaicho, Fuchu, Tokyo, Japan. E-mail: akane@cc.tuat.ac.jp, hiro@cc.tuat.ac.jp.

0091-6749/\$36.00

© 2010 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2010.10.043

Abbreviations used

AD:	Atopic dermatitis
APC:	Antigen-presenting cell
BMDC:	Bone marrow–derived dendritic cell
DC:	Dendritic cell
DCCM:	Dendritic cell–conditioned medium
DLN:	Draining lymph node
FITC:	Fluorescein isothiocyanate
MDC:	Macrophage-derived chemokine
MMP:	Matrix metalloproteinase
NF:	Nuclear factor
OX40L:	OX40 ligand
PCI:	Picryl chloride
PGN:	Peptidoglycan
PPAR:	Peroxisome proliferator–activated receptor
RSG:	Rosiglitazone
SPF:	Specific pathogen free
TARC:	Thymus and activation-regulated chemokine
TSLP:	Thymic stromal lymphopoietin

proliferation and to prime antigen-specific T-cell responses; however, the exact role of PPAR γ in *in vitro* and *in vivo* models with susceptibility for AD has been still obscure.^{26–28}

In the current study we clearly demonstrated that treatment with the PPAR γ ligand rosiglitazone (RSG) suppressed the expression of various proinflammatory genes, including costimulatory molecules and attracting chemokines, in TSLP-stimulated DCs that were generated from NC/Tnd mice, a model for human AD.²⁹ We also demonstrated the crucial role of MMP-9 in TSLP-stimulated DC migration by using MMP-9–deficient NC/Tnd mice and showed that PPAR γ activation suppressed MMP-9–mediated DC migration. Daily oral administration of RSG effectively suppressed the development of clinical symptoms, indicating the clinical efficacy of RSG on AD in NC/Tnd mice. These observations indicate that PPAR γ is one of the favorable suppressors of early AD events, including TSLP production from keratinocytes, maturation and differentiation of DCs, and MMP-9–mediated migration of DCs to draining lymph nodes (DLNs), where they act as APCs.

METHODS

Mice

Conventional and specific pathogen–free (SPF) NC/Tnd mice (a registration name of NC/NgaTnd mice was changed to "NC/Tnd" in December 2010) with or without AD were maintained as previously described.²⁹ MMP-9^{−/−} 129/Sv mice³⁰ were generously provided by Professor Zena Werb (Department of Anatomy, University of California, San Francisco, Calif). Congenic MMP-9^{−/−} NC/Tnd mice were generated by backcrossing NC/Tnd mice onto MMP-9^{−/−} 129/Sv mice for more than 13 generations. MMP-9 deficiency in those mice was confirmed by means of genotyping and gelatin zymography.³⁰ All experiments with animals complied with the standards specified in the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

Preparation of bone marrow–derived DCs

Bone marrow–derived dendritic cells (BMDCs) were generated from bone marrow cells of SPF NC/Tnd mice, as described previously,^{31,32} with minor modifications. Briefly, isolated cells were cultured in dendritic cell–conditioned medium (DCCM; RPMI 1640 with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 2 mmol/L sodium pyruvate, antibiotics, 50 μ mol/L 2-mercaptoethanol, and 20 ng/mL GM-CSF). On day 3, another 10 mL of DCCM was

added. At day 6, 10 mL of medium was replaced. At day 8, nonadherent and loosely adherent cells were collected by means of gentle pipetting and centrifugation at 400g for 5 minutes at 20°C. In some experiments BMDCs were incubated with or without 10 μ mol/L RSG (Alexis Biochemicals, San Diego, Calif) for 8 days. These cells were suspended in fresh DCCM and were incubated in the presence of 20 ng/mL recombinant murine TSLP (R&D Systems, Minneapolis, Minn) or 1 μ g/mL anti-TSLP antibody (R&D Systems) for neutralization of TSLP activity.

Determination of PPAR γ activation by RT-PCR

BMDCs were incubated with RSG (0, 10, 20, and 50 μ mol/L) for 6 hours and incubated with or without RSG (20 μ mol/L) for 6 hours, followed by incubation with or without GW9662 (20 μ mol/L; Alexis Biochemicals, San Diego, Calif). Total RNA isolated from BMDCs was reverse transcribed into cDNA with oligo(dT)_{12–18} primers by using a PrimeScript RTase (Takara Bio, Shiga, Japan), according to the manufacturer's instruction. For *CD36* and *aP2*, the following primer sets were used: *CD36*, 5'-GAGCCATCCTTGAGCCTTCA-3' and 5'-TCAGATCCGAACACAGCGTA-3'; *aP2* (FABP4), 5'-GATGCCTTGTGGGAACCTG-3' and 5'-GAATTCCACGCCCAGTTTGA-3'. The relative intensities of each expression were calculated with the intensity of β -actin as an endogenous control.

Real-time PCR array

Immature BMDCs were incubated with or without TSLP (20 ng/mL) for 6 hours. RSG (20 μ mol/L) or vehicle was added 1 hour before incubation with TSLP. cDNA was generated by using an RT² First Strand Kit (SABiosciences, Frederick, Md). Relative expression levels of 84 genes relating to antigen presentation were analyzed with RT² Profiler PCR Array System (Dendritic & Antigen Presenting Cell PCR Array, #PAHS-406, SABiosciences) and 2 $\times$ SABiosciences RT² qPCR Master Mix (SABiosciences), according to the manufacturer's instruction. Fluorescence intensity was measured by using the ABI Prism 7000 Sequence Detector (Applied Biosystems, Tokyo, Japan). The relative expression level of the target gene was determined by using 2^{− $\Delta\Delta$ CT} and was normalized to an endogenous reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).¹ Comparisons of parameters among groups were made by means of 1-factor ANOVA, followed by a Tukey test.

Real-time RT-PCR

Total RNA (1–2 μ g) obtained from the skin and BMDCs was reverse transcribed into cDNA, as previously described. Reaction mixtures were amplified with a SYBR Premix Ex Taq II (Takara Bio) in the presence of 0.2 μ mol/L of each of the sense and antisense primers for *TSLP*, *MMP-9*, *TARC*, *MDC*, and *eotaxin 2* or with primers for β -actin (Takara Bio). The sequences of primers are as follows: (1) *TSLP*, forward 5'-CGAGCAAATCGAGGACTGTGAG-3' and reverse 5'-GCAGTCGTCATTGAGCGCTTC-3'; (2) *MMP-9*, forward 5'-GCCCTGGAAGTACACGACA-3' and reverse 5'-TTGGAACTCA CAGCCAGAAG-3'; (3) *TARC*, forward 5'-CCGAGAGTGCTCCCTG GATTA-3' and reverse 5'-AGCTTGCCCTGGACAGTCAGA-3'; (4) *MDC*, forward 5'-GGCACCTATCCAGTGCCACA-3' and reverse 5'-TGGTGGAC CAGCCTGAACTC-3'; (5) *eotaxin 2*, forward 5'-AATGCGGGGCTCTG CAAC-3' and reverse 5'-CCTGCCTTGGGACAGATGCT-3'; and (6) β -actin, forward 5'-CATCCGTAAGACCTCTATGCCAAC-3' and reverse 5'-ATG GAGCCACCGATCCACA-3'.

Flow cytometric analysis

Single cells from DLNs were blocked with anti-CD16/32 mAb (BD PharMingen, San Diego, Calif) and stained with fluorescein isothiocyanate (FITC)–conjugated anti-I-A/I-E (BD PharMingen), FITC–conjugated anti-CD80 (BD PharMingen) or phycoerythrin–conjugated anti-CD86 (BD PharMingen), phycoerythrin–conjugated anti-OX40L (BioLegend, San Diego, Calif), and FITC–conjugated anti-CD11c (BioLegend) antibodies. Positive reactions were analyzed by using the EPICS XL flow cytometer (Beckman Coulter, Hialeah, Fla).

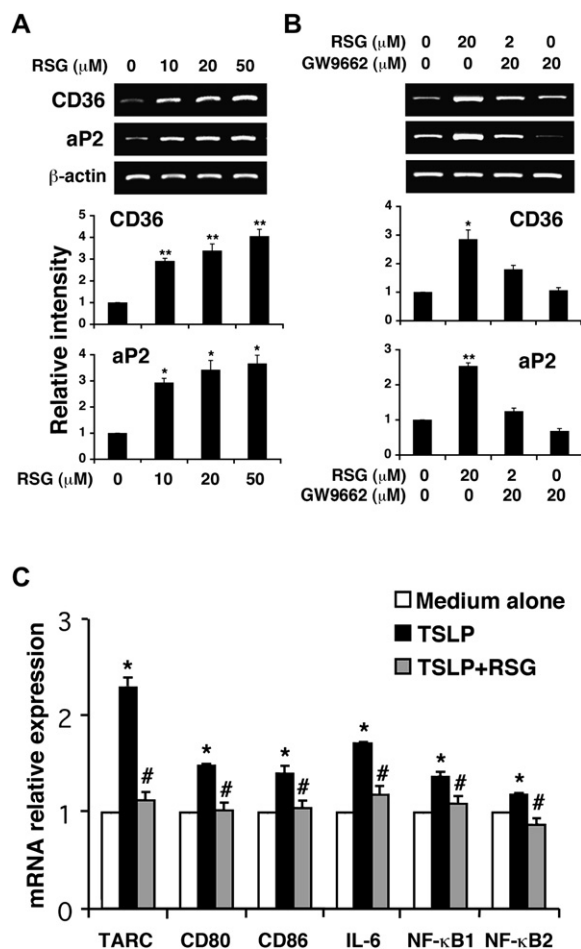


FIG 1. Suppression of expression of costimulatory molecules on DCs by PPAR γ activation. **A** and **B**, Effect of RSG on *CD36* and *aP2* gene expression with or without GW9662 was detected by means of RT-PCR. **C**, Genes up-regulated by TSLP were presented in the graph. Each bar represent means $\pm$ SEs of 3 individual experiments. * $P < .05$ compared with control mice. # $P < .05$ compared with TSLP-stimulated BMDs.

Gelatin zymography

MMP-9 levels were determined by means of gelatin zymography, as described previously,³³⁻³⁵ with proteins from 1×10^6 bone marrow cells. Images were analyzed with LAS-4000 IR multicolor and Multi Gauge Ver3.0 (Fujifilm, Tokyo, Japan).

Matrigel transmigration assay

Matrigel transmigration assays for mature DCs were performed by using Transwell cell-culture chambers (polycarbonate filters, 5-μm pore size; Corning Costar, Corning, NY), as previously described.^{35,36} BMDs harvested on day 8 were incubated with TSLP (20 ng/mL) for 48 hours to induce migration. RSG (20 μmol/L) was added to the cells 1 hour before TSLP treatment, and GW9662 (20 μmol/L) was added 1 hour before RSG addition. Complete medium was placed in the lower chamber of the Transwell. Mature DCs (1×10^5 cells) were suspended in serum-free RPMI 1640 medium in the upper chamber of the Transwell. After 22 hours, migrated cells were collected from the lower chamber and counted by using a light microscope (Nikon ECLIPS E600, Tokyo, Japan; 100 $\times$).

Skin tissue culture

Skin samples of 6-mm punch biopsy specimens were obtained from SPF NC/Tnd mice and cultured in RPMI 1640 containing 10% FCS and antibiotics.³⁷ Skin samples were incubated with 100 μg/mL peptidoglycan (PGN)

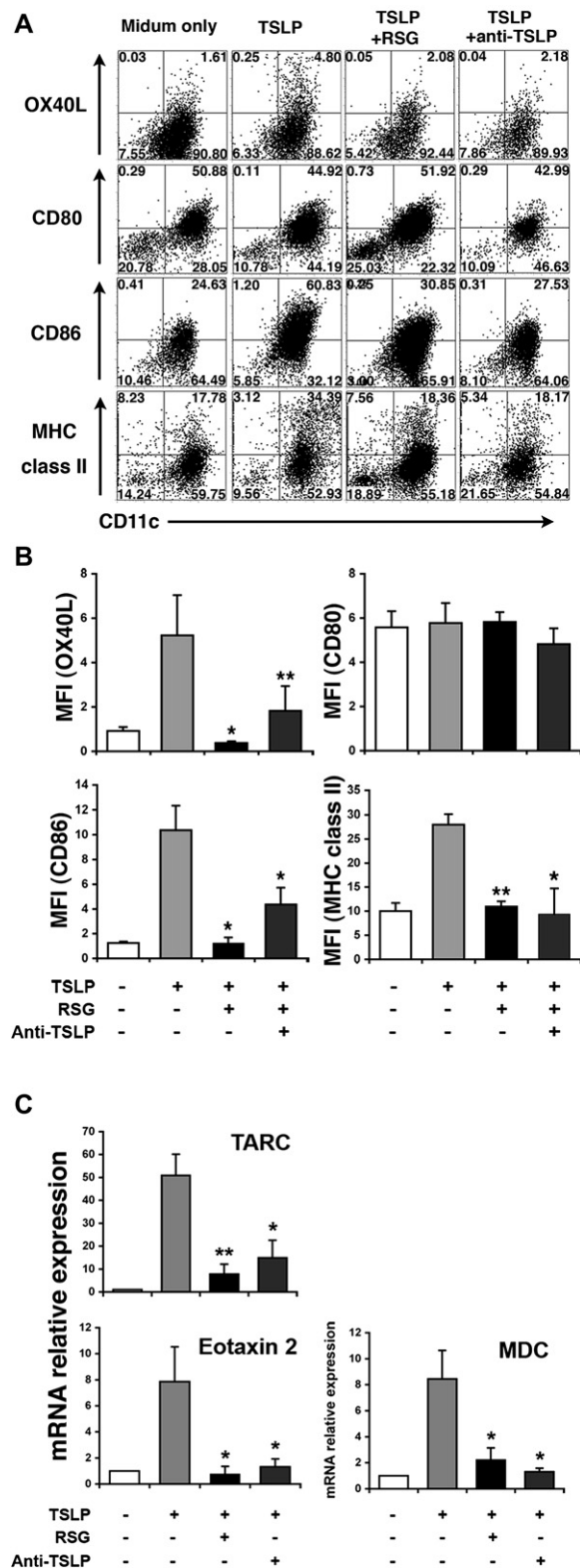


FIG 2. Suppression of TSLP-induced expression of costimulatory molecules by PPAR γ activation. **A**, Flow cytometric analysis of maturation markers. **B**, Each bar represent means $\pm$ SEs of 3 individual experiments. * $P < .05$ and ** $P < .01$ compared with TSLP alone. MFI, Mean fluorescence intensity. **C**, Real-time RT-PCR for RSG's effect on TSLP-induced expression of chemokines. Data represent means $\pm$ SEs of 3 different experiments. * $P < .05$ and ** $P < .01$ compared with TSLP-stimulated cells.

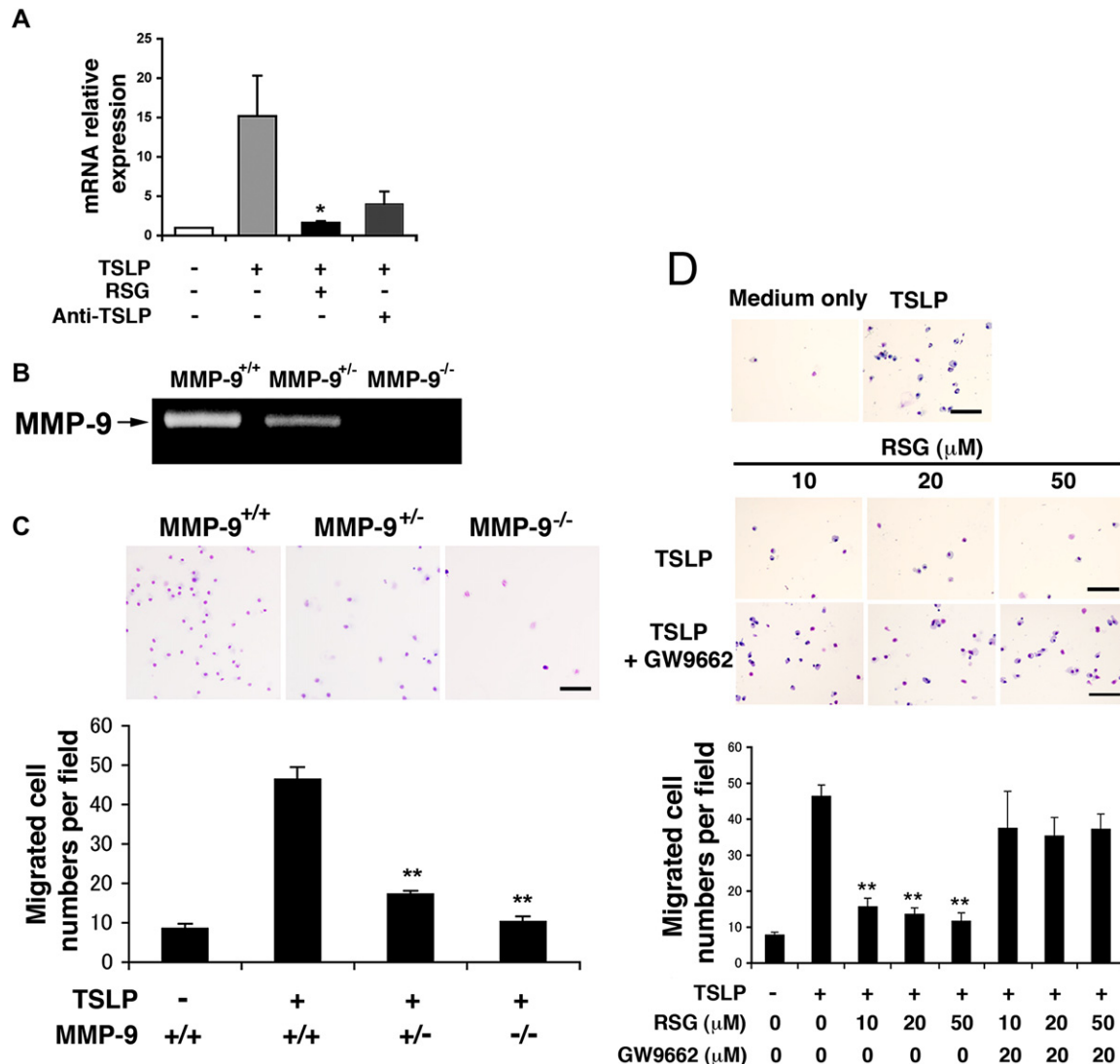


FIG 3. Suppression of MMP-9-mediated DC migration by PPAR γ activation. **A**, Suppression of *MMP-9* in TSLP-stimulated DCs. Each value was calculated with medium alone as 1. **B**, Detection of MMP-9 by means of gelatin zymography. **C**, Reduced transmigration of BMDCs generated from MMP-9^{-/-} NC/Tnd mice. **D**, Prevention of TSLP-stimulated migration of BMDCs by RSG. The average number of cells in 5 fields was calculated. Bars = 50 μ m. Data represent means $\pm$ SEs of 3 different experiments. * $P < .05$ and ** $P < .01$ compared with control mice.

from *Staphylococcus aureus* (Sigma, St Louis, Mo) for 6 hours to induce TSLP expression. RSG (20 mol/L) was applied 1 hour before adding PGN to the culture to examine the inhibitory effect of RSG on TSLP expression. Skin samples were pretreated with GW9662 (20 μ mol/L) for 1 hour before adding RSG to block PPAR γ . After incubation, skin samples were immediately frozen in liquid nitrogen and crushed into small pieces by using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Total RNA was isolated with ISOGEN (Nippongene, Tokyo, Japan), according to the manufacturer's instructions.

Treatment of NC/Tnd mice with RSG

RSG was dissolved in 0.25% carboxymethyl cellulose (Sigma-Aldrich) and orally applied to conventional NC/Tnd mice from 5 weeks of age once a day at a dose of 10 mg/kg body weight every day for 7 weeks. Vehicle alone (0.25% carboxymethyl cellulose) was applied as a control. The clinical symptoms of mice were recorded every week, according to the previously described criteria.²⁹ Scratching behavior of mice was quantified by using a SCLABA-Real system (Noveltec, Inc, Kobe, Japan) at the indicated weeks of age according to the manufacturer's instructions.³⁸ Blood was collected

from the caudal vena cava at the end of the experiments, and plasma was stored at -80°C until use. DLNs were collected every 2 weeks for flow cytometric analysis. For histologic analysis, the remaining DLNs were preserved at -80°C with OCT compound (Sakura Finetek, Tokyo, Japan) until use.

Histologic and immunohistochemical analysis

Hematoxylin and eosin, toluidine blue, and Congo red stainings were performed as previously described.¹ Frozen sections were incubated with affinity-purified anti-CD4 antibody (BD PharMingen), anti-Mac-1 antibody (BD PharMingen), and rat anti-langerin antibody (clone 929F3; Dendritics, Dardilly, France) to detect CD4⁺ T cells, macrophages, and DCs. Each preparation was then incubated with peroxidase-conjugated streptavidin and stained in a substrate solution containing hydrogen peroxide and 3-amino-9-ethylcarbazole (Dako, Glostrup, Denmark). Numbers of each cell were counted by using a microscope at a magnification of $\times 400$ and were expressed as the total number of cells in 5 fields. Cryosections of DLNs were incubated with rat anti-langerin antibody, according to the manufacturer's recommendation. Positive reactions were identified with a peroxidase-conjugated

streptavidin and DAB enhancer (Dako). For phenotypic analysis of DCs in DLNs, cryosections were double-stained with rat anti-langerin/CD207 antibody (clone 929F3; Dendritics, Dardilly, France) and hamster anti-CD103 antibody (clone 2E7; Serotec, Oxford, United Kingdom). For detection of TSLP, affinity-purified rat anti-mouse TSLP antibody (R&D Systems) was used.

Measurement of total plasma IgE levels

Total plasma IgE levels were measured by using a sandwich ELISA, according to the previously described method.^{1,39}

Statistical analysis

Comparisons of 2 parameters were analyzed by using the Student *t* test. Comparisons of parameters among groups were made by means of 1-way ANOVA, followed by the Tukey test. *P* values of less than .05 were considered significant.

RESULTS

Suppression of costimulatory molecules on DCs through PPAR γ activation

First, the time-dependent effect of RSG on *CD36* and *aP2* expression was examined,⁴⁰⁻⁴² and the most potent effect was obtained after 6 hours of incubation (see Fig E1 in this article's Online Repository at www.jacionline.org). When BMDCs were treated with various concentrations of RSG for 6 hours, the expression of *aP2* and *CD36* was significantly increased (Fig 1, A). However, when cells were pretreated with a PPAR γ antagonist, GW9662, for 1 hour before RSG treatment, the expression of both genes was completely suppressed (Fig 1, B). When splenic DCs were preincubated with RSG for 24 hours before LPS stimulation, expression levels of MHC class II, CD80, and CD86 were reduced (see Fig E2 in this article's Online Repository at www.jacionline.org). No differences in the viability of DCs in each group were mentioned (data not shown).⁴³ Among 84 genes, TSLP promoted mRNA expression of *IL6*, nuclear factor (*NF*) κ B1, and *NF*- κ B2, as well as *TARC*, *CD80*, and *CD86* (Fig 1, C). RSG treatment significantly suppressed TSLP-induced transcription of these genes (Fig 1, C), although RSG alone did not affect their expression (data not shown).

Inhibition of TSLP-stimulated activation of BMDCs through PPAR γ activation

When BMDCs originating from SPF NC/Tnd mice were cultured in the presence of TSLP for 48 hours, the expression levels of MHC class II, CD86, and OX40L were upregulated (Fig 2, A and B). Unlike LPS stimulation, CD80 protein expression was not induced by TSLP (Fig 2, A and B). The expression of these maturation markers was suppressed by the addition of neutralizing antibodies against TSLP to the culture medium (Fig 2, A and B). The efficacy of TSLP in BMDC maturation was markedly inhibited by RSG treatment (Fig 2, A and B).

TSLP-stimulated BMDCs expressed high levels of mRNA for the T_H2-cell attracting chemokines, including *TARC*, *MDC*, and *eotaxin 2*; however, addition of anti-TSLP antibody to the culture eliminated this effect (Fig 2, C). Like anti-TSLP antibody, RSG treatment completely suppressed TSLP-induced expression of these chemokines (Fig 2, C).

Inhibitory effect of RSG on migration of TSLP-stimulated BMDCs

Because MMPs play an important role in migration of epidermal Langerhans cells,^{36,44} we examined the effects of

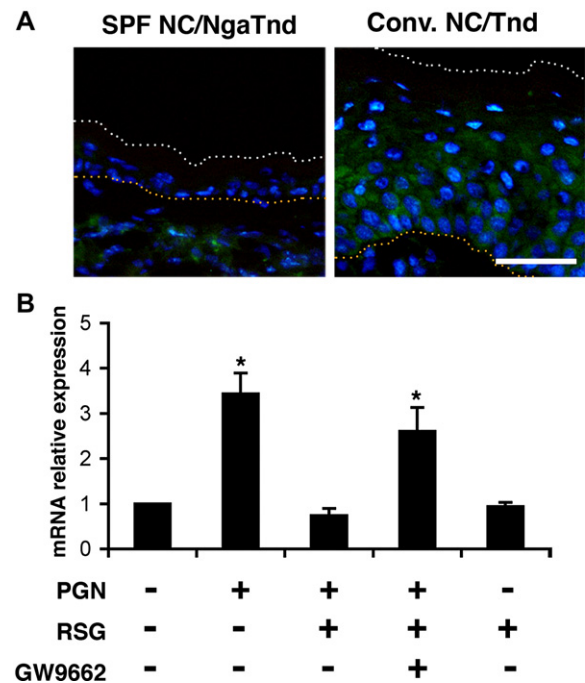


FIG 4. TSLP expression in the skin of NC/Tnd mice. **A**, TSLP expression in the skin of NC/Tnd mice with severe AD. Yellow dotted lines, Dermal/epidermal junction. Original magnification = $\times 100$. Bars = 50 μ m. **B**, TSLP expression in skin specimens cultured with PGN. The fold change was calculated with medium alone as 1. Data represent means $\pm$ SEs of 3 different experiments. **P* < .05 compared with medium alone.

TSLP on MMP-9 expression in DCs. *MMP9* mRNA expression was increased in TSLP-activated BMDCs; however, its increase was completely suppressed by RSG treatment (Fig 3, A). Next, we attempted to clarify the essential roles of MMP-9 in DC migration by using *MMP-9*^{-/-} NC/Tnd mice. Unlike *MMP-9*^{+/+} NC/Tnd mice, *MMP-9* activity was not present in bone marrow cells collected from *MMP-9*^{-/-} NC/Tnd mice (Fig 3, B). Migration of BMDCs generated from *MMP-9*^{+/+} NC/Tnd mice was upregulated by TSLP stimulation; on the other hand, the numbers of migrated DCs were significantly reduced in BMDCs from *MMP-9*^{-/-} NC/Tnd mice, even under TSLP stimulation (Fig 3, C). Pretreatment with RSG significantly suppressed TSLP-induced migration of BMDCs from *MMP-9*^{+/+} NC/Tnd mice through Matrigel in a dose-dependent manner (Fig 3, D). The suppressive effect of PPAR γ was contradicted by the addition of GW9662 to the culture (Fig 3, D).

Suppression of TSLP expression in the skin through PPAR γ activation

TSLP was undetectable in the skin of NC/Tnd mice without AD; however, a high expression of TSLP was found in proliferating keratinocytes of NC/Tnd mice with AD (Fig 4, A). *TSLP* mRNA was highly expressed in conventional NC/Tnd mice with AD as compared with those of SPF NC/Tnd mice and BALB/c mice without skin lesions (see Fig E3 in this article's Online Repository at www.jacionline.org). Moreover, the expression levels of *TSLP* mRNA in NC/Tnd mice with AD were significantly higher compared with those in BALB/c mice with picryl chloride (PCI)-induced contact dermatitis (see Fig E3). When

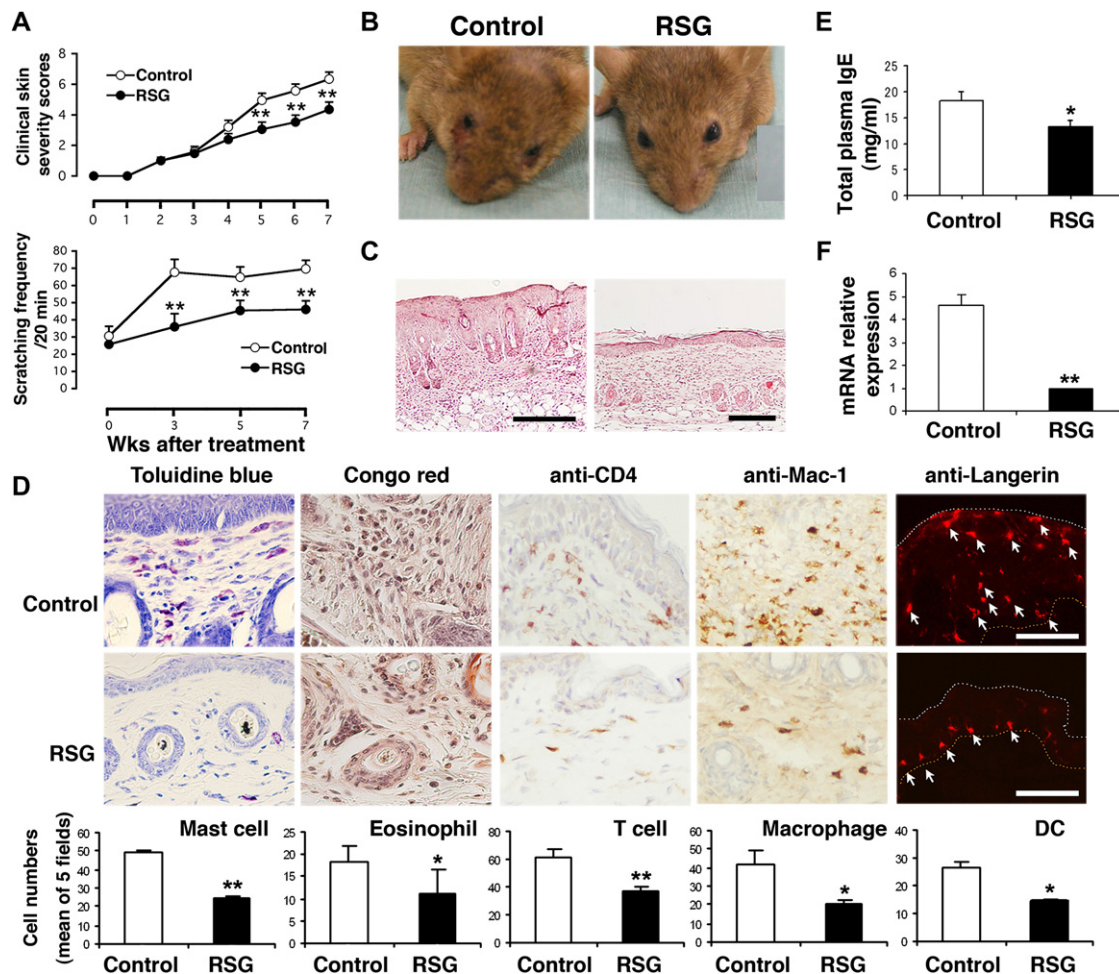


FIG 5. Reduction of early development of AD in NC/Tnd mice by means of oral administration of RSG. **A**, Clinical skin severity scores (top) and scratching frequencies (bottom). Each point represents means $\pm$ SEs (n = 18). ** P < .01 compared with control mice. **B**, Typical features of mice treated with (right) or without (left) RSG. **C**, Microscopic features of skin with hematoxylin and eosin staining. Bar = 100 μ m. **D**, Histologic analysis of affected skin. White arrows indicate langerin-positive cells in the epidermis. Original magnification = $\times 100$. Bars = 50 μ m. Each column represents means $\pm$ SEs of 3 different experiments with 5 mice. * P < .05 and ** P < .01 compared with control mice. **E**, Plasma total IgE levels. Each value represents means $\pm$ SEs of 3 different experiments with 5 to 7 mice. * P < .05 compared with control mice. **F**, Relative mRNA expression levels of *TSLP* in the skin. Data represent means $\pm$ SEs of 5 mice. * P < .05 and ** P < .01 compared with control mice.

PCI was applied repeatedly onto the skin, *TSLP* expression in affected sites was more accelerated in SPF NC/Tnd mice than in BALB/c mice. When skin specimens isolated from SPF NC/Tnd mice were incubated with PGN from *S aureus*, *TSLP* expression was markedly increased (Fig 4, B). However, pretreatment of skin pieces with RSG reduced *TSLP* expression (Fig 4, B). GW9662 canceled the suppressive effect of RSG (Fig 4, B).

Prevention of early development of AD in NC/Tnd mice by RSG treatment

Established AD in conventional NC/Tnd mice was not improved by means of oral administration of RSG (see Fig E4 in this article's Online Repository at www.jacionline.org). On the other hand, when RSG was applied from 5 weeks of age (before the onset of AD), AD development was delayed (Fig 5, A, top panel). In addition to the clinical symptoms, the increase in frequency of

scratching behavior was suppressed (Fig 5, A, lower panel). Clinical skin severity scores in RSG-treated mice were 3.9 ± 0.4 and those of control mice were 6.9 ± 0.5 after 7 weeks of treatment. Severe clinical symptoms were observed in control mice but not in RSG-treated mice (Fig 5, B). No toxicity was recognized in mice treated with RSG for 7 weeks (see Table E1 in this article's Online Repository at www.jacionline.org). RSG treatment reduced epidermal hyperplasia, parakeratosis, dermal edema, and infiltration of inflammatory cells in the skin (Fig 5, C). At the affected skin lesions, the numbers of mast cells, eosinophils, CD4⁺ cells, and Mac-1⁺ cells were also significantly decreased in RSG-treated mice compared with those in control mice (Fig 5, D). Langerin-positive DCs accumulated at inflammatory sites on the skin of NC/Tnd mice with AD (Fig 5, D), as reported in human patients.^{45,46} However, the numbers of langerin-positive DCs were reduced in RSG-treated mice (Fig 5, D). Plasma total IgE levels were increased in control mice at the end of the

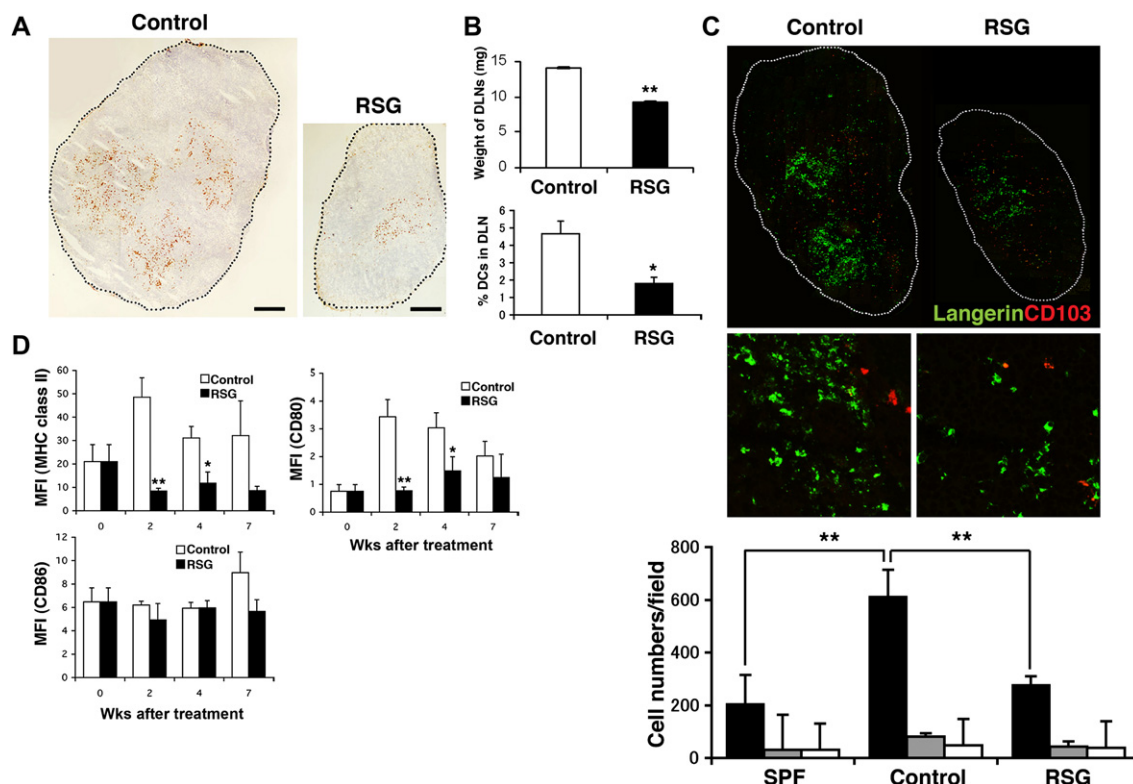


FIG 6. Suppression of DC migration and maturation in RSG-treated NC/Tnd mice. **A**, Shapes and langerin-positive cells (brown) in DLNs. Original magnification = $\times 100$. Bar = 200 μ m. **B**, Weight of DLNs and percentages of CD11c⁺ cells in DLNs. Data represent means $\pm$ SEs of 3 different experiments with 5 mice. **C**, Photos of langerin-positive (green) cells, CD103-positive (red) cells, or both in the DLNs. Each bar in the graph represents means $\pm$ SEs of 3 mice in each group. Black column, Langerin-positive CD103⁻ cells; gray column, langerin-positive CD103⁺ cells; white column, langerin-negative CD103⁺ cells. **D**, Expression levels of costimulatory molecules in DLNs during RSG administration. Each point represents means $\pm$ SEs of 6 mice in each group. * $P < .05$ and ** $P < .01$ compared with control mice. MFI, Mean fluorescence intensity.

experiment,²⁹ whereas the increase in plasma total IgE levels in RSG-treated mice was suppressed (Fig 5, E). Moreover, TSLP expression levels were reduced in the skin of RSG-treated mice (Fig 5, F).

Decreased accumulation of DCs in DLNs of RSG-treated mice

The weight and size of the axillary lymph nodes were significantly lower in RSG-treated mice compared with those of control mice (Fig 6, A and B). The numbers of accumulated CD11c⁺ DCs in DLNs were markedly decreased in RSG-treated mice (Fig 6, A and B). Phenotypic analysis of migrated DCs revealed that langerin-positive CD103⁻ DCs were significantly increased in DLNs of control mice (Fig 6, C).^{47,48} On the other hand, numbers of langerin-positive CD103⁺ DCs and langerin-negative CD103⁺ DCs were unchanged. Accumulation of langerin-positive CD103⁻ DCs was markedly suppressed in DLNs of RSG-treated mice (Fig 6, C). CD11c⁺ DCs in DLNs were isolated from RSG-treated and control mice every 2 weeks to examine the efficacy of PPAR γ activation *in vivo*. In RSG-treated mice MHC class II and CD80 expression was decreased compared with that seen in control mice (Fig 6, D). On the other hand, CD86 expression was not altered by RSG treatment (Fig 6, D).

DISCUSSION

PPAR γ has been shown to exhibit anti-inflammatory activities in different models of inflammatory diseases, such as atherosclerosis, inflammatory bowel disease, or multiple sclerosis.^{49,50} Recently, it has been reported that systemic application a PPAR γ ligand ameliorated experimental skin allergy in mice⁵¹ and clinical symptoms in patients with AD⁵²; however, the exact mechanism remains unclear. In this study we first demonstrated that PPAR γ acted as an effective suppressor to regulate DC functions during the initiation stage of AD by using atopic NC/Tnd mice.

First, we showed that RSG inhibited DC expression of costimulatory molecules, such as MHC class II, CD80, and CD86, as well as OX40L, indicating that the T-cell priming abilities of DCs were abrogated. TSLP-induced expression of OX40L, MHC class II, and CD86, as well as TARC, eotaxin 2, and MDC, in DCs was also reduced by RSG treatment. TSLP also promoted gene expression of proinflammatory molecules in DCs, including IL-6, NF- κ B1, and NF- κ B2, which were significantly suppressed by means of incubation with RSG. These results suggest the role of PPAR γ as a downregulator in the initiation of allergic inflammation.

MMPs are essential for DC migration through the basement membrane and extracellular matrix^{53,54} and are suggested to play a crucial role in DC migration during allergic responses.⁵⁵

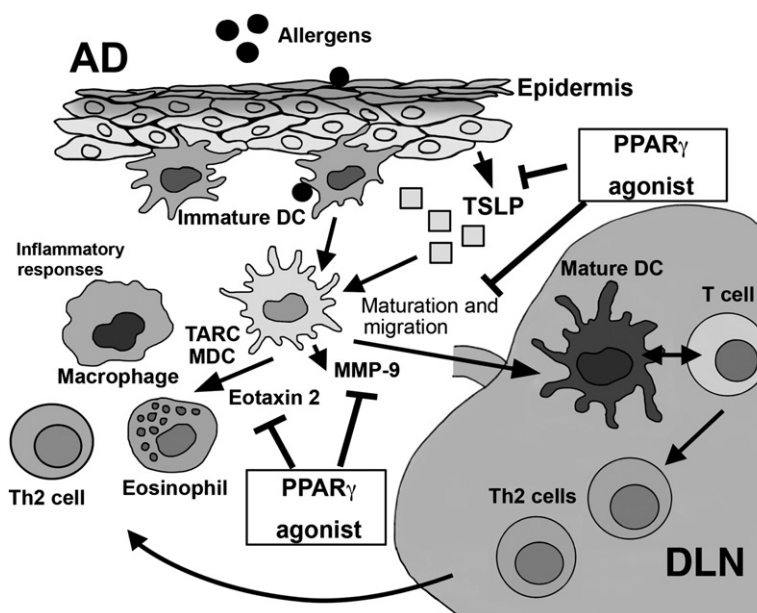


FIG 7. Hypothesis: invasion of allergens triggers the epithelial cells to produce TSLP. TSLP activates immature DCs to produce chemokines, which cause inflammatory responses, and MMP-9, which is responsible for migration of DCs to DLNs. PPAR γ activation might modulate the early atopic inflammation through inhibition of both function and production of TSLP, as well as downregulation of MMP-9 production.

Moreover, MMP-9 levels have been reported to increase in the skin and plasma obtained from patients with AD.⁵⁶ In this study we first generated BMDCs from MMP-9-deficient NC/Tnd mice and provided evidence that MMP-9 is essential for TSLP-stimulated DC migration. Our results showed that the PPAR γ agonist reduced MMP-9 production and TSLP-induced migration of DCs.

By using tissue cultures, we found that TSLP expression was induced in the skin of NC/Tnd mice by the addition of PGN, which was inhibited by RSG. Because the components of *S aureus* aggravate clinical symptoms of AD as superantigens,² an increase in the TSLP level might induce DC activation and AD development. Therefore PPAR γ might be an important negative regulator in the early events seen in patients with AD.

Finally, we clearly demonstrated that systemic application of RSG prevented the development of AD in NC/Tnd mice, reducing scratching behavior in the animals. After 7 weeks of treatment, keratinocyte hyperplasia and infiltration of inflammatory cells were dramatically reduced in RSG-treated mice, indicating the alleviation of dermatitis clinically and histologically. In particular, migration and maturation were effectively suppressed. According to results from immunohistochemical examinations, we speculated that DCs predominantly affected by TSLP might be langerin-positive epidermal DCs, which play pivotal roles in the development of AD. On the other hand, application of RSG to NC/Tnd mice with established AD had no beneficial effects. In the murine model of asthma, PPAR γ was expressed in airway epithelium on antigen sensitization and nebulization with its agonist-decreased airway hyperresponsiveness.⁵⁷ Recently, PPAR γ was reported to be involved in alleviation of skin inflammation and allergic dermatitis.⁵⁸ These observations indicate that PPARs might be critical downregulators of the initiation of allergic responses.

Our hypothesis is shown in Fig 7. In the skin of patients with AD, keratinocytes might be susceptible to release of TSLP on stimulation of antigens and irritants. TSLP derived from keratinocytes induces maturation and differentiation of DCs in the skin, and activated DCs might produce MMP-9 for their migration to DLNs, where DCs act as APCs. Activated DCs secrete chemokines and induce recruitment of leukocytes to the site of inflammation. In early events in AD development, PPAR γ plays a role as a downregulator against TSLP production and TSLP-initiated immune responses through suppression of DC functions. Thus PPAR γ might be an important therapeutic target in the early development of AD.

We thank Professor Zena Werb (Department of Anatomy, University of California, San Francisco, Calif) for providing MMP-9-deficient mice. We are also grateful for her enthusiastic reviewing of our manuscript. We deeply appreciate Dr Tomoaki Ikeda (National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan) for valuable suggestions and discussions.

Clinical implications: Agonists for PPAR γ prevent the onset and exacerbation of AD in NC/Tnd mice by controlling DC functions that are activated by TSLP during the initial stage of AD.

REFERENCES

1. Tanaka A, Muto S, Jung K, Itai A, Matsuda H. Topical application with new NF-kappaB inhibitor improves atopic dermatitis in NC/NgaTnd mice. *J Invest Dermatol* 2007;127:855-63.
2. Boguniewicz M, Leung DY. Recent insights into atopic dermatitis and implication for management of infectious complications. *J Allergy Clin Immunol* 2010;125:4-13.
3. Beltrani VS. Suggestions regarding a more appropriate understanding of atopic dermatitis. *Curr Opin Allergy Clin Immunol* 2005;5:413-8.
4. Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. *J Clin Invest* 2004;113:651-7.

5. Wang YH, Ito T, Wang YH, Homey B, Watanabe N, Martin R, et al. Maintenance and polarization of human Th2 central memory T cells by thymic stromal lymphopoietin-activated dendritic cells. *Immunity* 2006;24:827-38.
6. Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* 2005;202:1213-23.
7. Jenkins SJ, Perona-Wright G, Worsley AGF, Ishii N, MacDonald AS. Dendritic cell expression of OX40 ligand acts as a co-stimulatory, not polarizing, signal for optimal Th2 priming and memory induction in vivo. *J Immunol* 2007;179:3515-23.
8. Soumelis V, Reche RA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell-mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;7:673-80.
9. Miyata M, Hatsushika K, Ando T, Shimokawa N, Ohnuma Y, Katoh R, et al. Mast cell regulation of epithelial TSLP expression plays an important role in the development of allergic rhinitis. *Eur J Immunol* 2008;38:1487-92.
10. Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol* 2005;6:1047-53.
11. Al-Shami A, Solski R, Kelly J, Keane-Myers A, Leonard WJ. A role for TSLP in the development of inflammation in an asthma model. *J Exp Med* 2005;202:829-39.
12. Yoo J, Omori M, Gyarmati D, Zhou B, Aye T, Brewer A, et al. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J Exp Med* 2005;202:541-9.
13. Elentner A, Finke D, Schmuth S, Chappaz S, Ebner S, Malissen B, et al. Langerhans cells are critical in the development of atopic dermatitis-like inflammation in symptoms in mice. *J Cell Mol Med* 2009;13:2658-72.
14. Omori M, Ziegler S. Induction of IL-4 expression in CD4⁺ T cells by thymic stromal lymphopoietin. *J Immunol* 2007;178:1396-404.
15. Darmanin S, Chen J, Zhao S, Cui H, Shirkoohi R, Kubo N, et al. All-trans retinoic acid enhances murine dendritic cell migration to draining lymph nodes via the balance of matrix metalloproteinases and their inhibitors. *J Immunol* 2007;179:4616-25.
16. Yen JH, Khayrullina T, Ganea D. PGE2-induced metalloproteinase-9 is essential for dendritic cell migration. *Blood* 2008;111:260-70.
17. Ratzinger G, Stoitzner P, Ebner S, Lutz MB, Layton GT, Rainer C, et al. Matrix metalloproteinases 9 and 2 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin. *J Immunol* 2002;168:4361-71.
18. Zapata-Gonzalez F, Rueda F, Etriz J, Domingo P, Villarroja F, Madariaga AD, et al. 9-cis-retinoic acid (9cRA), a retinoid X receptor (RXR) ligand, exerts immunosuppressive effects on dendritic cells by RXR-dependent activation: inhibition of peroxisome proliferators-activated receptor γ blocks some of the 9cRA activities, and precludes them to mature phenotype development. *J Immunol* 2007;178:6130-9.
19. Braissant O, Foulle F, Scotto C, Dauca M, Wahli W. Differential expression of peroxisome proliferators-activated receptors (PPARs): tissue distribution of PPAR- α , - β , and - γ in the adult rat. *Endocrinology* 1996;137:354-66.
20. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor- γ is a negative regulator of macrophage activation. *Nature* 1998;391:79-82.
21. Sugiyama H, Nonaka T, Kishimoto T, Komoriya K, Tsuji K, Nakahata T. Peroxisome proliferator-activated receptors are expressed in human cultured mast cells: a possible role of these receptors in negative regulation of mast cell activation. *Eur J Immunol* 2000;30:3363-70.
22. Schlezinger JJ, Jensen BA, Mann KK, Ryu HY, Sherr DH. Peroxisome proliferator-activated receptor gamma-mediated NF-kappa B activation and apoptosis in pre-B cells. *J Immunol* 2002;169:6831-41.
23. 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.
24. Woerly G, Honda K, Loyens M, Papin JP, Auwerx J, Staels B, et al. Peroxisome proliferator-activated receptors alpha and gamma down-regulate allergic inflammation and eosinophil activation. *J Exp Med* 2003;198:411-21.
25. Lehrke M, Lazar MA. The many face of PPAR γ . *Cell* 2005;123:993-9.
26. Klotz L, Dani I, Edenhofer F, Nolden L, Evert B, Paul B, et al. Peroxisome proliferators-activated receptor γ control of dendritic cell function contributes to development of CD4⁺ T cell anergy. *J Immunol* 2007;178:2122-31.
27. Nencioni A, Grunebach F, Zobywalski A, Denzlinger C, Brugger W, Brossart P. Dendritic cell immunogenicity is regulated by peroxisome proliferator-activated receptor gamma. *J Immunol* 2002;169:1228-35.
28. Jakobsen MA, Petersen RK, Kristiansen K, Lange M, Lillevang ST. Peroxisome proliferators-activated receptor α , δ , γ 1 and γ 2 expressions are present in human monocyte-derived dendritic cells and modulate dendritic cell maturation by addition of subtype-specific ligands. *Scand J Immunol* 2006;63:330-7.
29. Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, et al. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* 1997;9:461-6.
30. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, et al. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 1998;93:411-22.
31. Lutz MB, Kulkutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999;223:77-92.
32. Bleier JI, Pillarisetty VG, Shah AB, DeMatteo RP. Increased and long-term generation of dendritic cells with reduced function from IL-6-deficient bone marrow. *J Immunol* 2004;172:7408-16.
33. Sallusto F, Lanzavecchia A. Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. *J Exp Med* 1999;189:611-4.
34. Tanaka A, Arai K, Kitamura Y, Matsuda H. Matrix metalloproteinase-9 production, a newly identified function of mast cell progenitors, is down-regulated by *c-kit* receptor activation. *Blood* 1999;94:2390-5.
35. Tang J, Liu J, Zhou C, Alexander JS, Nanda A, Granger DN, et al. MMP-9 deficiency enhances collagenase-induced intracerebral hemorrhage and brain injury in mutant mice. *J Cereb Blood Flow Metab* 2004;24:1133-45.
36. Darmanin S, Chen J, Zhao S, Cui H, Shirkoohi R, Kubo N, et al. All-trans retinoic acid enhances murine dendritic cell migration to draining lymph nodes via the balance of matrix metalloproteinases and their inhibitors. *J Immunol* 2007;179:4616-25.
37. Bogiatzi SI, Fernandez I, Bichet JC, Marloie-Provost MA, Volpe E, Sastre X, et al. Proinflammatory and Th2 cytokines synergize to induce thymic stromal lymphopoietin production by human skin keratinocytes. *J Immunol* 2007;178:3373-7.
38. Ishii I, Kurozumi S, Orito K, Matsuda H. Automatic scratching pattern detection for laboratory mice using high-speed video images. *IEEE Trans Autom Sci Eng* 2008;5:176-82.
39. Matsumoto M, Ra C, Kawamoto K, Sato H, Itakura A, Sawada J, et al. IgE hyperproduction through enhanced tyrosine phosphorylation of Janus kinase 3 in NC/Nga mice, a model for human atopic dermatitis. *J Immunol* 1999;162:1056-63.
40. Han J, David PH, Xiaoye Z, Gotto AM, Nicholson AC. Regulation of peroxisome proliferators-activated receptor- γ -mediated gene expression. *J Biol Chem* 2002;277:23582-6.
41. Pelton PD, Zhou L, Demarest KT, Burris TP. PPARgamma activation induces the expression of the adipocyte fatty acid binding protein gene in human monocytes. *Biochem Biophys Res Commun* 1999;262:456-8.
42. Fu Y, Luo N, Lopes-Virella MF. Oxidized LDL induces the expression of ALBP/ap2 mRNA and protein in human THP-1 macrophages. *J Lipid Res* 2000;41:2017-23.
43. Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, et al. Activation of proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages. *J Biol Chem* 1998;273:25573-80.
44. Larsen CP, Steinman RM, Witner-Pack M, Hankins DF, Morris PJ, Austyn JM. Migration and maturation of Langerhans cells in skin transplants and explants. *J Exp Med* 1990;172:1483-93.
45. Dubrac S, Schmuth M, Ebner S. Atopic dermatitis: the role of Langerhans cells in disease pathogenesis. *Immunol Cell Biol* 2010;88:400-9.
46. Novak N, Bieber T. The role of dendritic cell subtypes in the pathophysiology of atopic dermatitis. *J Am Acad Dermatol* 2005;53(suppl):S171-6.
47. Henri S, Poulin LF, Tanoutounour S, Ardouin L, Williams M, de Bovis B, et al. CD207⁺CD103⁺ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J Exp Med* 2010;207:189-206.
48. Merad M, Ginhoux F, Collin M. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol* 2008;12:935-47.
49. Natarajan C, Muthian G, Barak Y, Evans RM, Bright JJ. Peroxisome proliferators-activated receptor- γ -deficient heterozygous mice develop and exacerbated neural antigen-induced Th1 response and experimental allergic encephalomyelitis. *J Immunol* 2003;171:5743-50.
50. Peyrin-Biroulet L, Beisner J, Wang G, Nuding S, Oommen ST, Kelly D, et al. Peroxisome proliferators-activated receptor gamma activation is required for maintenance of innate antimicrobial immunity in the colon. *Proc Natl Acad Sci U S A* 2010;107:8772-7.

51. Dahten A, Koch C, Ernst D, Schnoller C, Hartmann S, Worm M. Systemic PPAR γ ligation inhibits allergic immune response in the skin. *J Invest Dermatol* 2008;128:2211-8.
52. Behshad R, Cooper KD, Korman NJ. A retrospective case series review of the peroxisome proliferators-activated receptor ligand rosiglitazone in the treatment of atopic dermatitis. *Arch Dermatol* 2008;144:84-8.
53. Ratzinger G, Stroitzner P, Ebner S, Lutz MB, Layton GT, Rainer C, et al. Matrix metalloproteinase 9 and 2 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin. *J Immunol* 2002;168:4361-71.
54. Kouwenhove M, Ozenci V, Tjernlund A, Pashenkov M, Homman M, Press R, et al. Monocyte-derived dendritic cells express and secrete matrix-degrading metalloproteinases and their inhibitors and are imbalanced in multiple sclerosis. *J Neuroimmunol* 2002;126:161-71.
55. Vermaelen KY, Cataldo D, Tournoy K, Dhulst A, Louis R, Foidart JM, et al. Matrix metalloproteinase-9-mediated dendritic cell recruitment into the airways is a critical step in mouse model of asthma. *J Immunol* 2003;171:1016-22.
56. Devillers ACA, Toorenenbergen AW, Heerenbrink GJK, Mulder PGH, Oranje AP. Elevated levels of plasma matrix metalloproteinase-9 in patients with atopic dermatitis: a pilot study. *Clin Exp Dermatol* 2007;32:311-3.
57. Honda K, Marquillies P, Capron M, Dombrowicz D. Peroxisome proliferator-activated receptor gamma is expressed in airways and inhibits features of airway remodeling in a mouse asthma model. *J Allergy Clin Immunol* 2004;113:882-8.
58. Staumont-Sallé D, Abboud G, Brénuchon C, Kanda A, Roumier T, Lavogiez C, et al. Peroxisome proliferator-activated receptor alpha regulates skin inflammation and humoral response in atopic dermatitis. *J Allergy Clin Immunol* 2008;121:962-8.

METHODS

Determination of PPAR γ activation by means of RT-PCR

Generated BMDCs were incubated with 20 μ mol/L RSG for 0, 3, 6, 12, and 24 hours. One microgram of total RNA isolated from BMDCs was reverse transcribed into cDNA with oligo(dT)₁₂₋₁₈ primers by using a PrimeScript RTase (Takara Bio), according to the instructions of the manufacturer. For CD36 and aP2, the primer sets described were used. The relative intensities of each expression were calculated as described.

Real-time RT-PCR

Total RNA (1–2 μ g) obtained from the was reverse transcribed into cDNA, as previously described. Reaction mixtures were amplified with a SYBR Premix Ex Taq II (Takara Bio) in the presence of 0.2 μ mol/L of each of the sense and antisense primers for TSLP or with primers for β -actin (Takara Bio). The relative expression level of the target gene was given by $2^{-\Delta\Delta CT}$ and was normalized to an endogenous reference.^{E1}

Preparation of splenic DCs

Single cells were obtained from the spleens of NC/Tnd mice, and splenic DCs were enriched by means of magnetic cell sorting with murine anti-CD11c microbeads (clone N418; Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions (purity >93%). Isolated cells were cultured with 100 ng/mL recombinant murine GM-CSF (Wako Pure Chemicals, Osaka, Japan) for 24 hours. DCs were preincubated with or without 10 μ mol/L RSG for 24 hours, and DC maturation was induced by means of incubation with 1 μ g/mL LPS (L6761, Sigma-Aldrich) for a further 24 hours. Expression of surface molecules was assessed by means of flow cytometry.

Treatment of NC/Tnd mice with RSG

Mice at 8 weeks of age (after development of skin lesions) were used to evaluate the therapeutic effect of RSG on established AD. RSG was dissolved in 0.25% carboxymethyl cellulose (Sigma-Aldrich) and orally applied to conventional NC/Tnd mice from 8 weeks of age once a day at a dose of 10 mg/kg body weight every day for 6 weeks. Vehicle alone (0.25% carboxymethyl

cellulose) was applied as a control. The clinical symptoms of mice were recorded every week according to the previously described criteria.^{E2} Scratching behavior of mice was quantified by using a SCLABA-Real system (Noveltec, Inc) at the indicated weeks of age, according to the manufacturer's instructions.^{E3}

Induction of contact dermatitis in BALB/c mice

To induce hyperkeratosis by contact dermatitis, 1% PCI (Tokyo Chemical Industry, Tokyo, Japan) in an ethanol–acetone mixture (3:1) was applied to shaved dorsal skin, as described previously, with modifications.^{E4} After 5 days, dorsal skin samples were removed and immediately frozen in liquid nitrogen and crushed into small pieces by using a Multi-Beads Shocker (Yasui Kikai). Total RNA was isolated with ISOGEN (Nippongene), according to the manufacturer's instructions. Real-time PCR analysis was performed as described above.

Serum biochemistry

Serum biochemistry was performed by using VetScan VS2 (Abaxis, Union City, Calif), according to the manufacturer's instructions. Blood samples were collected from control or RSG-treated mice (n = 14 in each group) at the end of oral administration. Serum samples were isolated and stored at -20°C until use.

REFERENCES

- E1. Tanaka A, Muto S, Jung K, Itai A, Matsuda H. Topical application with new NF-kappaB inhibitor improves atopic dermatitis in NC/NgaTnd mice. *J Invest Dermatol* 2007;127:855-63.
- E2. Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, et al. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* 1997;9:461-6.
- E3. Ishii I, Kurozumi S, Orito K, Matsuda H. Automatic scratching pattern detection for laboratory mice using high-speed video images. *IEEE Trans Automa Sci Eng* 2008;5:176-82.
- E4. Kawamoto K, Paliwal V, Ramabhadran R, Szezepanik M, Tsuji RF, Matsuda H. IL-2 is produced by antigen-presenting cells stimulated with soluble $\alpha\beta$ TCR and restores impaired Th1 responses. *Int Immunol* 2000;12:103-12.

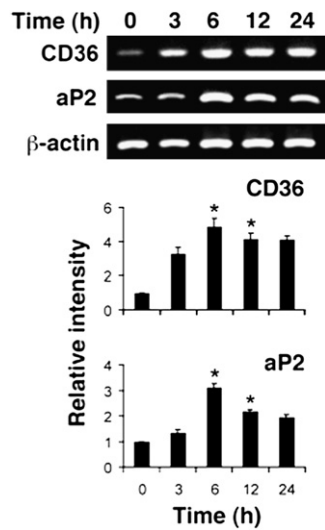


FIG E1. Time-dependent expression of PPAR γ -associated genes. Time-dependent effect of RSG (20 μ mol/L) on expression of *CD36* and *aP2* in BMDCs were analyzed by using the PCR procedure. Each *bar* in the diagrams represents means $\pm$ SEs of 3 individual experiments. * P < .05 compared with control mice.

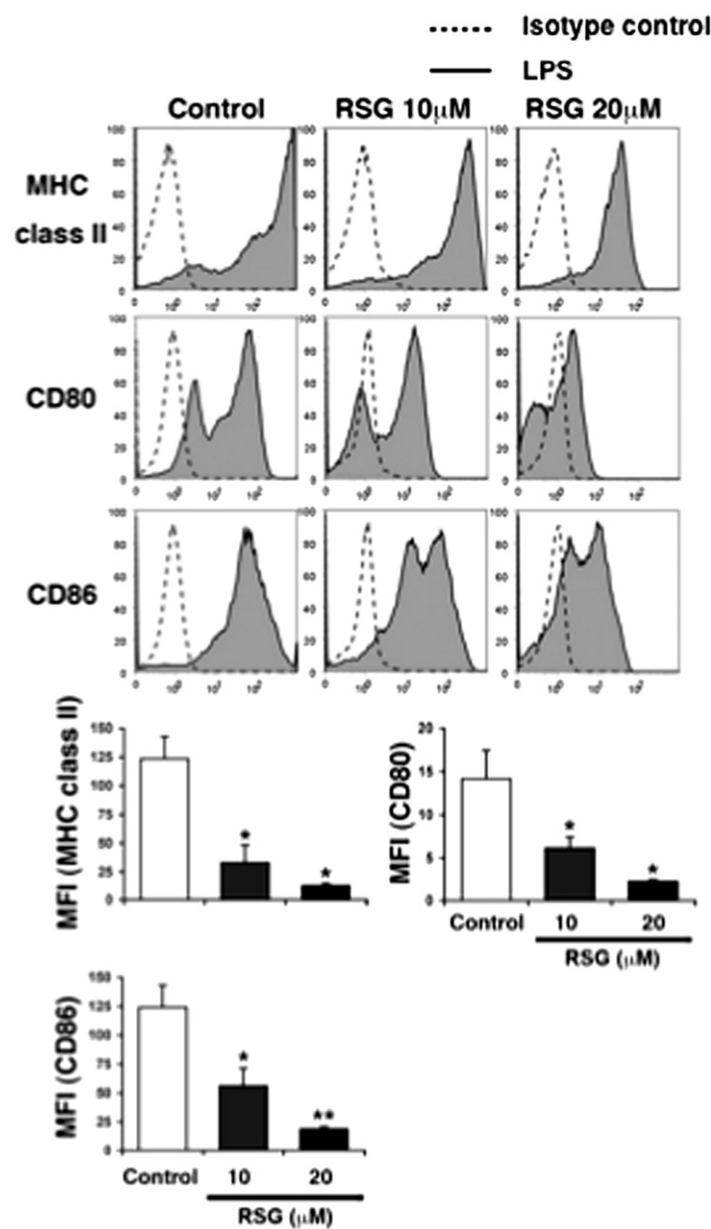


FIG E2. Preparation of splenic DCs. LPS-induced expressions of costimulatory molecules on splenic CD11c⁺ DCs were suppressed by RSG treatment. Each bar in the diagrams represents means $\pm$ SEs of the mean fluorescence intensity (MFI) from 3 individual experiments. * P < .05 and ** P < .01 compared with control mice.

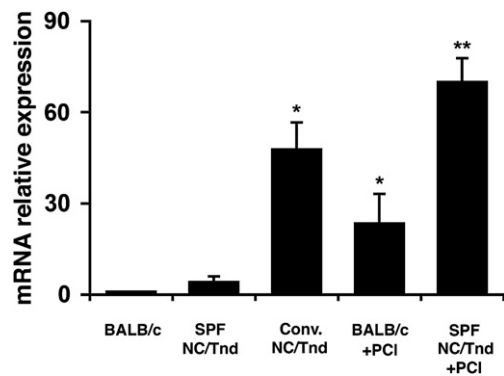


FIG E3. Increased expression of *TSLP* in skin of NC/Tnd mice with AD. *TSLP* mRNA was quantified by means of real-time RT-PCR in skin of BALB/c, SPF NC/Tnd, and conventional NC/Tnd mice with or without PCI application. The fold change in expression is standardized to expression levels of diluent-treated BALB/c mice as 1. Data represent means $\pm$ SEs of 3 individual experiments. * $P < .05$ and ** $P < .01$ compared with data from diluent-treated BALB/c mice.

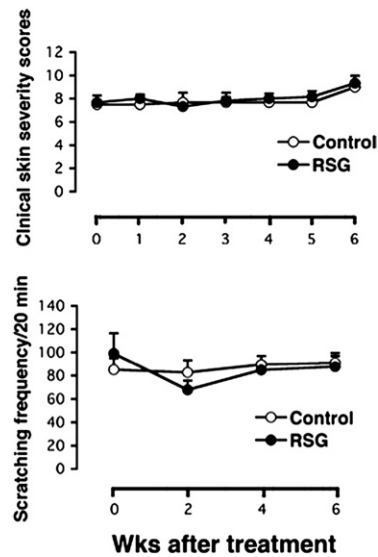


FIG E4. No effect of PPAR γ activation on NC/Tnd mice with severe AD. Clinical skin severity scores (*upper panel*) and scratching frequencies (*bottom panel*) were recorded and quantified during RSG application for 6 weeks. Each point represents means $\pm$ SEs of clinical scores or scratching frequencies of 18 mice with AD in each group.

TABLE E1. Serum biochemistry

	Control mice	RSG-treated mice
ALB (g/dL)	2.6 ± 0.13	2.8 ± 0.05
ALP (U/L)	58.6 ± 5.2	60.1 ± 3.5
ALT (U/L)	14.4 ± 0.7	12.6 ± 1.0
AMY (U/L)	769.0 ± 35.4	824.4 ± 41.6
TBIL (mg/dL)	0.3 ± 0.1	0.2 ± 0.0
BUN (mg/dL)	32.9 ± 2.5	32.2 ± 1.3
CA (mg/dL)	9.7 ± 0.2	9.8 ± 0.2
PHOS (mg/dL)	9.9 ± 0.6	10.1 ± 0.6
CRE (mg/dL)	0.2 ± 0.0	0.2 ± 0.0
GLU (mg/dL)	142.9 ± 8.2	139.4 ± 10.5
TP (g/dL)	4.9 ± 0.2	5.0 ± 0.1
GLOB (g/dL)	2.3 ± 0.2	2.2 ± 0.1

No statistical difference was identified in every result from each group, indicating less toxicity in long-term administration of RSG.

ALB, Albumin; *ALP*, alkaline phosphatase; *ALT*, alanine aminotransferase; *AMY*, amylase; *BUN*, blood urea nitrogen; *CA*, calcium; *CRE*, creatinine; *GLOB*, globulin; *GLU*, glucose; *PHOS*, phosphorus; *TBIL*, total bilirubin; *TP*, total protein.