

Suppressive role of PPAR γ in the IgE-dependent activation of mast cells

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Abstract

Mast cells (MCs) play a central role in IgE-dependent immune responses. PPAR γ is a nuclear receptor that is essential for adipocyte differentiation and insulin sensitivity. Although PPAR γ is expressed in activated MCs, the effect of PPAR γ suppression in IgE-mediated activation of MCs is largely unknown. In the current study, we evaluated the effect of PPAR γ knockdown on the function of IgE plus antigen (Ag)-stimulated MCs using siRNA-transfected bone marrow-derived MCs (BMMCs). We found that the mRNA expression level of cytokines in IgE/Ag-stimulated BMMCs was significantly increased in PPAR γ knockdown BMMCs, and IgE/Ag-mediated degranulation and the protein production level of TNF- α was moderately increased by PPAR γ knockdown, whereas the cell surface expression level of Fc ϵ RI was not affected by PPAR γ knockdown. Oral administration of pioglitazone (PPAR γ agonist) significantly suppressed body temperature change of mice in passive systemic anaphylaxis, supporting the inhibitory functions of PPAR γ in IgE/Ag-dependent activation of MCs *in vivo*. IgE-mediated up-regulation of mRNA levels of *Ptgs2* (encoding COX-2) was drastically enhanced in PPAR γ knockdown BMMCs. Although several prostaglandin (PG) derivatives are known to be ligands for PPAR γ , treatment with a COX inhibitor, acetyl salicylic acid, up-regulated the IgE-mediated increase of *Il13*, *Tnf* and *Ptgs2* mRNA levels in a synergistic manner with PPAR γ siRNA. Knockdown of COX-1 and/or COX-2 by siRNA showed that suppression of IgE/Ag-mediated activation was mainly dependent on COX-1. Taken together, these results indicate that PPAR γ suppresses IgE/Ag-induced transactivation of cytokine genes and the *Ptgs2* gene in MCs in a manner distinguishable from that of PGs.

Keywords: cyclooxygenase, Fc ϵ RI, IgE, mast cells, PPAR γ

Introduction

Mast cells (MCs), which express Fc ϵ RI on the cell surface, play a critical role in IgE-mediated immune responses, including allergic diseases and host defense against parasite infection. PPAR γ , which is known as NR1C3 belonging to the nuclear receptor (NR) superfamily, is a ligand-dependent nuclear transcription factor. PPAR γ is a well-known therapeutic target of type 2 diabetes because of its important roles in maintaining glucose homeostasis by regulating insulin sensitivity and adipocyte differentiation. Recently, studies regarding the expression and function of PPAR γ in immune-related cells have been reported (1–7). The PPAR γ expression is also observed in human (8) and mouse MCs (9). Although the roles of PPAR γ in the development and activation of MCs have been investigated mainly using PPAR γ agonists (8, 9), the involvement of PPAR γ in the function and gene expression of MCs is largely unknown. Furthermore, in some cases,

the agonistic compound exhibited suppressive effects on MCs in a PPAR γ -independent manner (10, 11). These observations prompted us to analyze the role of PPAR γ in MCs by knockdown experiments using siRNA.

A previous study using PPAR γ -deficient (+/–) bone marrow-derived MCs (BMMCs) and PPAR γ agonists showed the involvement of PPAR γ in the development and proliferation of BMMCs (12). Therefore, in the current study, we transfected siRNA into developed BMMCs to exclude the effect of PPAR γ in the development process.

Methods

Mice and cells

Bone marrow cells from C57BL/6 mice (Japan SLC, Hamamatsu, Japan) were maintained in RPMI-1640 medium

containing 5 ng ml⁻¹ IL-3 (BioLegend), 10% FBS (Biowest), 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 100 μ M 2-mercaptoethanol (Wako), 10 μ M Minimum Essential Medium (MEM) non-essential amino acid solution (Wako) and 100 μ g ml⁻¹ sodium pyruvate (Sigma-Aldrich) to generate BMMCs. All animal experiments were performed in accordance with the guidelines of the Institutional Review Board of Tokyo University of Science. The current study was specifically approved by the Animal Care and Use Committees of Tokyo University of Science.

siRNA transfection

A Neon transfection system (Invitrogen, Carlsbad, CA, USA) was used to introduce siRNAs into BMMCs as previously described (13). The following siRNAs were purchased from Invitrogen: siRNAs against *Pparg* (MSS207863 termed *Pparg* siRNA #1, and MSS207864 termed *Pparg* siRNA #2), *Ptgs1* (MSS208180), *Ptgs2* (MSS208183) and control siRNA (Stealth RNAi Negative Universal Control, #12935).

Quantification of mRNA

Reverse transcription and quantitative PCR were performed as previously described (13, 14). The following TaqMan primers (Applied Biosystems) were used: *Pparg* (SYBR), *Il4* (Mm00445259_m1), *Il6* (Mm00446190_m1), *Il13* (Mm00434204_m1), *Tnf* (#Mm00443258_m1), *Ptgs1* (SYBR), *Ptgs2* (SYBR), *Actb* (#4351315) and *Gapdh* (#4352339E). The nucleotide sequences of SYBR primers are as follows: *Pparg* (forward; 5'-gcccttgggtgactttatgga-3', and reverse; 5'-gcagcaggtgtgtcttgatg-3'), *Ptgs1* (forward; 5'-tgcatgtggctgtggatgtcatca-3', and reverse; 5'-cactaagacagaccgcgtcatctcca-3') and *Ptgs2* (forward; 5'-cagacaacataaactgcgccttt-3', and reverse; 5'-gggatacacctctctccaccaatg-3').

Flow cytometric analysis

Cell surface expression levels of Fc ϵ RI and c-kit were determined by a MACS Quant (Miltenyi Biotech) using PE-labeled anti-mouse Fc ϵ RI α Ab (MAR-1, eBioscience) and FITC-labeled anti-c-kit antibody (Ab) (2B2, BioLegend). To detect CD4⁺/Foxp3⁺

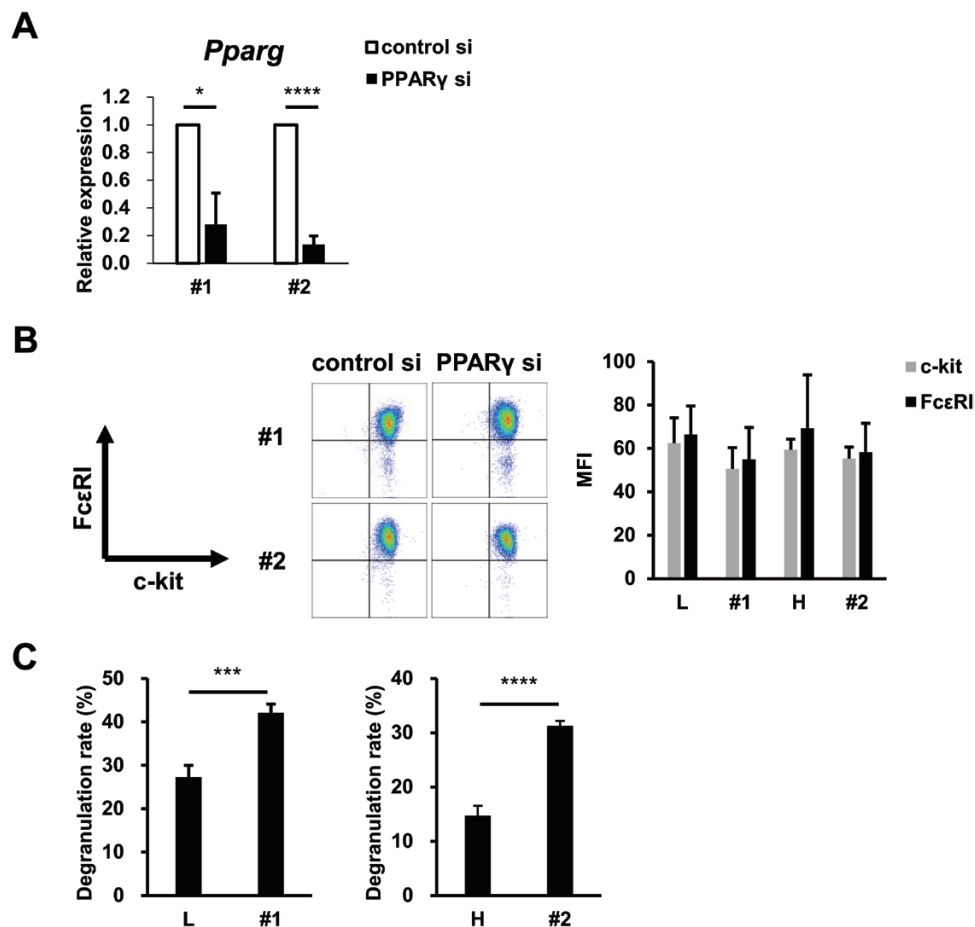


Fig. 1. Effects of PPAR γ knockdown on cell surface expression levels of Fc ϵ RI and Fc ϵ RI-mediated degranulation of BMMCs. (A) mRNA levels of *Pparg* in siRNA-transfected BMMCs. #1; BMMCs transfected with PPAR γ siRNA #1 (closed bar) or its control siRNA (open bar), #2; BMMCs transfected with PPAR γ siRNA #2 (closed bar) or its control siRNA (open bar). (B) Cell surface expression levels of Fc ϵ RI and c-kit. MFI, mean fluorescence intensity. Typical dot plots (left) and the mean $\pm$ SD of MFIs obtained in five independent experiments (right). L; GC-low control siRNA for PPAR γ #1, #1; PPAR γ siRNA #1, H; GC-high control siRNA for PPAR γ #2, #2; PPAR γ siRNA #2. (C) Degranulation degree of IgE-stimulated BMMCs. L; GC-low control siRNA, #1; PPAR γ siRNA #1, H; GC-high control siRNA, #2; PPAR γ siRNA #2. Cells were analyzed at 48 h (A, B and C #1) or 72 h (C #2) after siRNA transfection. The data in (A) and (C) represent the mean $\pm$ SD of triplicate samples. Similar results were obtained in four independent experiments (A and C). * P < 0.05, *** P < 0.001, **** P < 0.0001.

cells, FITC-labeled anti-mouse CD4 Ab (GK1.5, BioLegend), allophycocyanin-labeled anti-mouse Foxp3 Ab (3G3, TOMBO Bioscience) and a Foxp3/Transcription factor staining buffer kit (#TNB-0607-KIT, TOMBO Bioscience) were used.

Stimulation of MCs

BMMCs ($\times 10^6/1$ ml) were incubated in the presence of $0.2 \mu\text{g ml}^{-1}$ anti-TNP mouse IgE (clone IgE-3, BD Bioscience, San Jose, CA, USA) for 2 h. After washing with culture medium, cells were re-suspended in 1 ml culture medium containing 30 ng TNP-BSA as an antigen (Ag) (LSL, Tokyo, Japan). Cells and culture supernatant were harvested at the indicated time points and subjected to RT-qPCR to determine mRNA levels and ELISA, respectively.

For the degranulation assay, Tyrode's buffer was used instead of culture medium. Thirty minutes after TNP-BSA

stimulation, the culture supernatant was collected, and β -hexosaminidase activity in the supernatant was determined as previously described (15).

Enzyme-linked immunosorbent assay

The concentrations of TNF- α and IL-13 were determined by ELISA kits (#430904 for TNF- α , BioLegend, #DY413 for IL-13, R&D, respectively). The PGD $_2$ and PGE $_2$ concentrations were also measured by ELISA (#512031 and #514010, Cayman Chemical).

Passive systemic anaphylaxis

C57BL/6 mice were orally administrated 25, 50 and 100 mg kg^{-1} day $^{-1}$ PPAR γ agonist pioglitazone (#P1901, Tokyo Chemical Industry Co. Ltd, Tokyo) solubilized in 0.5% methyl cellulose 400 solution (#131-17811, Wako) for 5 days. Mice

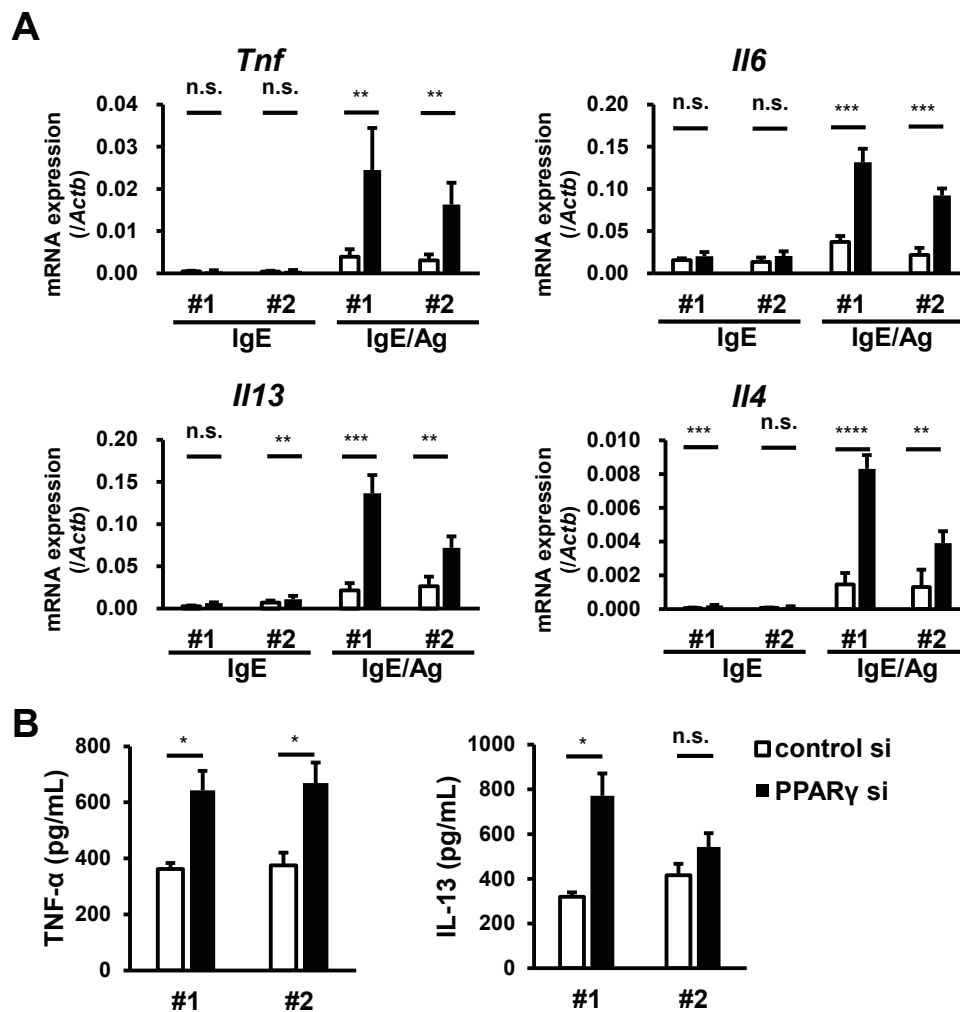


Fig. 2. PPAR γ knockdown enhances IgE-induced expression of cytokines in BMMCs. (A) mRNA levels of cytokines in siRNA-transfected BMMCs. Cells were subjected to IgE/Ag stimulation at 48 h after siRNA transfection and were harvested at 1 h after the addition of Ag to determine mRNA levels of *Tnf*, *Il6*, *Il13* and *Il4*. IgE; not stimulated (without Ag), IgE/Ag; stimulated with IgE plus Ag. Open bars in #1; GC-low control siRNA, closed bars in #1; PPAR γ siRNA #1, open bars in #2; GC-high control siRNA, closed bars in #2; PPAR γ siRNA #2. (B) The amount of cytokines released from Ag-stimulated BMMCs. Cells were subjected to IgE/Ag stimulation at 48 h after siRNA transfection and culture media were collected at 3 h after the addition of Ag to determine the concentration of TNF- α and IL-13. #1; BMMCs transfected with PPAR γ siRNA #1 (closed bars) or its control siRNA (open bars), #2; BMMCs transfected with PPAR γ siRNA #2 (closed bars) or its control siRNA (open bars). The data represent the mean $\pm$ SD of triplicate samples (A and B). Similar results were obtained in five (A) and two (B) independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, n.s. not significant.

were intravenous injection (i. v.) injected with 3 μ g anti-TNP mouse IgE on day 5. The body temperature of each mouse was measured every 10 min just after challenge by 20 μ g TNP-BSA, which was i.v. injected at 24 h after IgE injection.

Statistical analysis

We used a two-tailed Student's *t*-test to compare two samples (Figs 1, 2, 4A, 5A, 5D left and 5E) and Turkey's multiple comparison test to compare multiple samples (Figs 4B, 5B, 5C and 5D right). We performed Dunnett's multiple comparison test for the evaluation of body temperature in passive systemic anaphylaxis (PSA) (Fig. 3). *P* values < 0.05 were considered significant.

Results and discussion

Effects of PPAR γ knockdown on Fc ϵ RI expression and Fc ϵ RI-mediated degranulation in BMMCs

First, we evaluated the effect of PPAR γ siRNA on PPAR γ mRNA levels using BMMCs at 4 h after stimulation with anti-TNP IgE and TNP-BSA because quantitative PCR showed that PPAR γ mRNA was non-detectable in steady-state BMMCs and Fc ϵ RI-mediated stimulation up-regulated PPAR γ mRNA levels in BMMCs (data not shown), which was consistent with a previous study (9). As shown in Fig. 1(A), both siRNAs, #1 and #2, carrying different nucleotide sequences, significantly reduced mRNA levels of PPAR γ . Next, we confirmed that cell surface expression levels of Fc ϵ RI and c-kit were not affected by PPAR γ siRNA transfection (Fig. 1B). Under these experimental conditions, Fc ϵ RI-mediated degranulation of BMMCs was significantly increased by PPAR γ knockdown (Fig. 1C). Although PPAR γ mRNA was not detected in steady-state BMMCs, a small amount of PPAR γ may be enough for suppression of the very early responses including degranulation.

Effects of PPAR γ knockdown on Fc ϵ RI-mediated production of cytokines in BMMCs

In addition to degranulation, IgE-dependent stimulation induces the transactivation of cytokines in MCs. To evaluate the effect of PPAR γ knockdown on cytokine production levels in IgE-stimulated MCs, we determined mRNA levels of *Il4*, *Il6*, *Il13* and *Tnf* in siRNA-transfected BMMCs at 1 h after Fc ϵ RI cross-linking. As shown in Fig. 2(A), stimulation with IgE plus Ag up-regulated the mRNA levels of cytokines in control siRNA-transfected BMMCs, which were drastically enhanced in PPAR γ siRNA transfectants.

To further investigate whether increased cytokine mRNA levels are reflected in the amount of secreted cytokine proteins, we determined the concentration of TNF- α and IL-13 in culture media by ELISA (Fig. 2B). As expected, the levels of secreted TNF- α (#1 and #2) and IL-13 proteins (#1) were increased by PPAR γ knockdown.

These results indicate that PPAR γ suppresses the transcription and release of cytokines in MCs.

Effects of administration of a PPAR γ agonist on allergic responses *in vivo*

In order to clarify whether the above-mentioned inhibitory function of PPAR γ is observed in IgE-dependent allergic

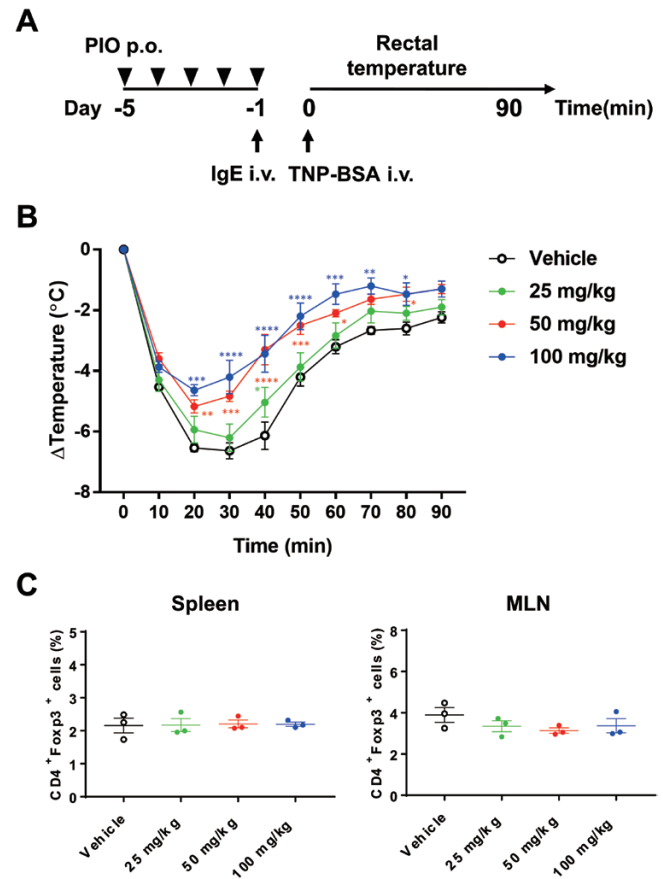


Fig. 3. PPAR γ agonist alleviated IgE-dependent anaphylaxis of mice. (A) Schematics of the schedule of pioglitazone (PIO) administration and PSA induction. p.o., per os. (B) Change in body temperature after Ag challenge. Vehicle ($n = 3$); p.o. administration of 200 μ l methyl cellulose. 25 ($n = 3$), 50 ($n = 3$) and 100 ($n = 3$) mg kg $^{-1}$ day $^{-1}$ of pioglitazone was p.o. administered. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 versus vehicle. (C) Frequency of CD4 $^{+}$ /Foxp3 $^{+}$ T cells per total cells in MLN and spleen.

responses *in vivo*, we examined the effect of administration of pioglitazone (an agonist for PPAR γ) on PSA. As shown in Fig. 3(B), body temperature of control mice, which were pre-injected with anti-TNP IgE, was promptly decreased under -6° C and peaked at 30 min after Ag injection. Under this experimental condition, oral administration of 50–100 mg kg $^{-1}$ day $^{-1}$ pioglitazone significantly suppressed the decrease of body temperature (Fig. 3B). Previously, it was reported that PPAR γ was crucial for transactivation of *Aldh1a2* in dendritic cells, resulting in the induction of Treg cells (16). To confirm a possibility that pioglitazone may accelerate Treg development, we determined the CD4 $^{+}$ /Foxp3 $^{+}$ frequency and found that the percentage of Treg cells in mesenteric lymph node (MLN) and spleen was not affected by pioglitazone administration in the current study (Fig. 3C).

These results demonstrate that PPAR γ exhibits inhibitory functions in IgE-dependent activation of MCs *in vivo*.

Involvement of PPAR γ in COX expression in BMMCs

MCs express two cyclooxygenases, COX-1 and COX-2, which contribute to the production of prostaglandins (PGs) and their derivatives. In addition to the major role of

PG-related eicosanoids as modulators of cell function via G protein-coupled receptors on the cell surface, several derivatives, such as 15-deoxy- $\Delta^{12,14}$ -PGJ₂, also function as ligands for PPAR molecules (17). Then, we determined the mRNA levels of *Ptgs1* and *Ptgs2* in PPAR γ siRNA transfectants. As shown in Fig. 4(A), the level of constitutively expressed *Ptgs1* mRNA was not affected by PPAR γ knockdown. In contrast, *Ptgs2* mRNA levels that were increased upon stimulation were strikingly up-regulated in PPAR γ knockdown MCs. These results suggest that PPAR γ plays a suppressive role in COX-2 expression.

To clarify the involvement of COXs in PPAR γ -mediated gene suppression, PPAR γ siRNA-transfected BMMCs were stimulated by IgE plus Ag in the presence or absence of a non-steroid anti-inflammatory drug, acetylsalicylic acid (ASA). IgE-dependent transactivation of *Tnf*, *Il13* and *Ptgs2* in control BMMCs was increased by ASA pre-treatment (Fig. 4B). These results indicate that COX-mediated products

are involved in the suppression of IgE-induced transactivation in MCs. When ASA was used in PPAR γ knockdown BMMCs, the knockdown-dependent enhancement of gene expression was further accelerated (Fig. 4B), suggesting that COX-dependent suppression was still present in PPAR γ knockdown conditions.

From these results, we demonstrate that PPAR γ and COX are involved in the reduction of the activation of MCs and that the effect of COX on IgE-dependent gene expression is not fully dependent on PPAR γ because the inhibition of COX increases IgE-dependent transactivation even in PPAR γ knockdown MCs.

Effects of COX knockdown on Fc ϵ RI-mediated activation of BMMCs

ASA treatment enhanced Fc ϵ RI-mediated transactivation in MCs (Fig. 4B). To further clarify the involvement of COX-1 and

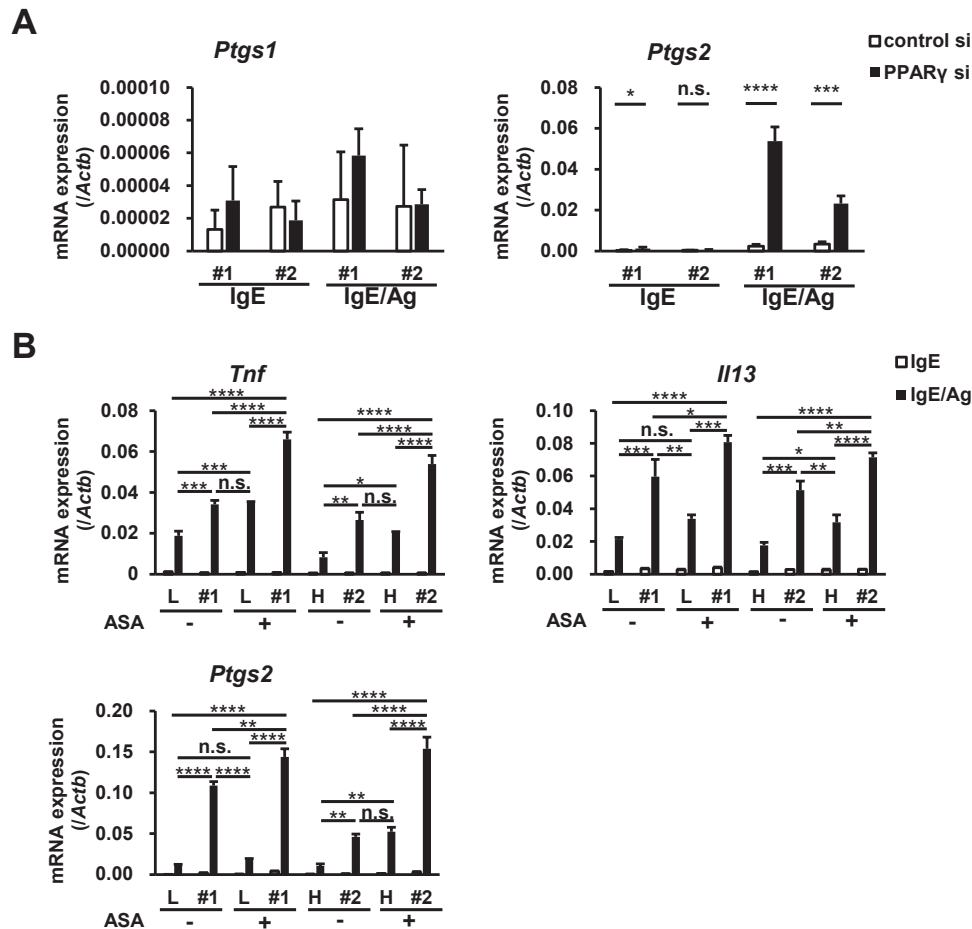


Fig. 4. COX inhibition and PPAR γ knockdown synergistically up-regulated IgE-mediated transactivation in BMMCs. (A) mRNA levels of *Ptgs1* and *Ptgs2* in PPAR γ siRNA-transfected BMMCs. Transfection, stimulation and harvest of cells were performed in the same schedule as that in Fig. 2(A). IgE; no stimulation (without Ag), IgE/Ag; stimulated with IgE plus Ag. #1; BMMCs transfected with PPAR γ siRNA #1 (closed bar) or its control siRNA (open bar), #2; BMMCs transfected with PPAR γ siRNA #2 (closed bar) or its control siRNA (open bar). * P < 0.05, *** P < 0.001, **** P < 0.0001, n.s. not significant. (B) Effects of PPAR γ siRNA transfection and ASA treatment on IgE-induced up-regulation of *Tnf*, *Il13* and *Ptgs2* mRNAs. BMMCs transfected with siRNA were maintained in the presence (+) or absence (–) of ASA throughout this experiment. Open bars (IgE); not stimulated (without Ag), closed bars (IgE/Ag); stimulated with IgE plus Ag. L; GC-low control siRNA, #1; PPAR γ siRNA #1, H; GC-high control siRNA, #2; PPAR γ siRNA #2. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, n.s. not significant. The data represent the mean $\pm$ SD of triplicate samples (A and B). Similar results were obtained in three (A) and two (B) independent experiments.

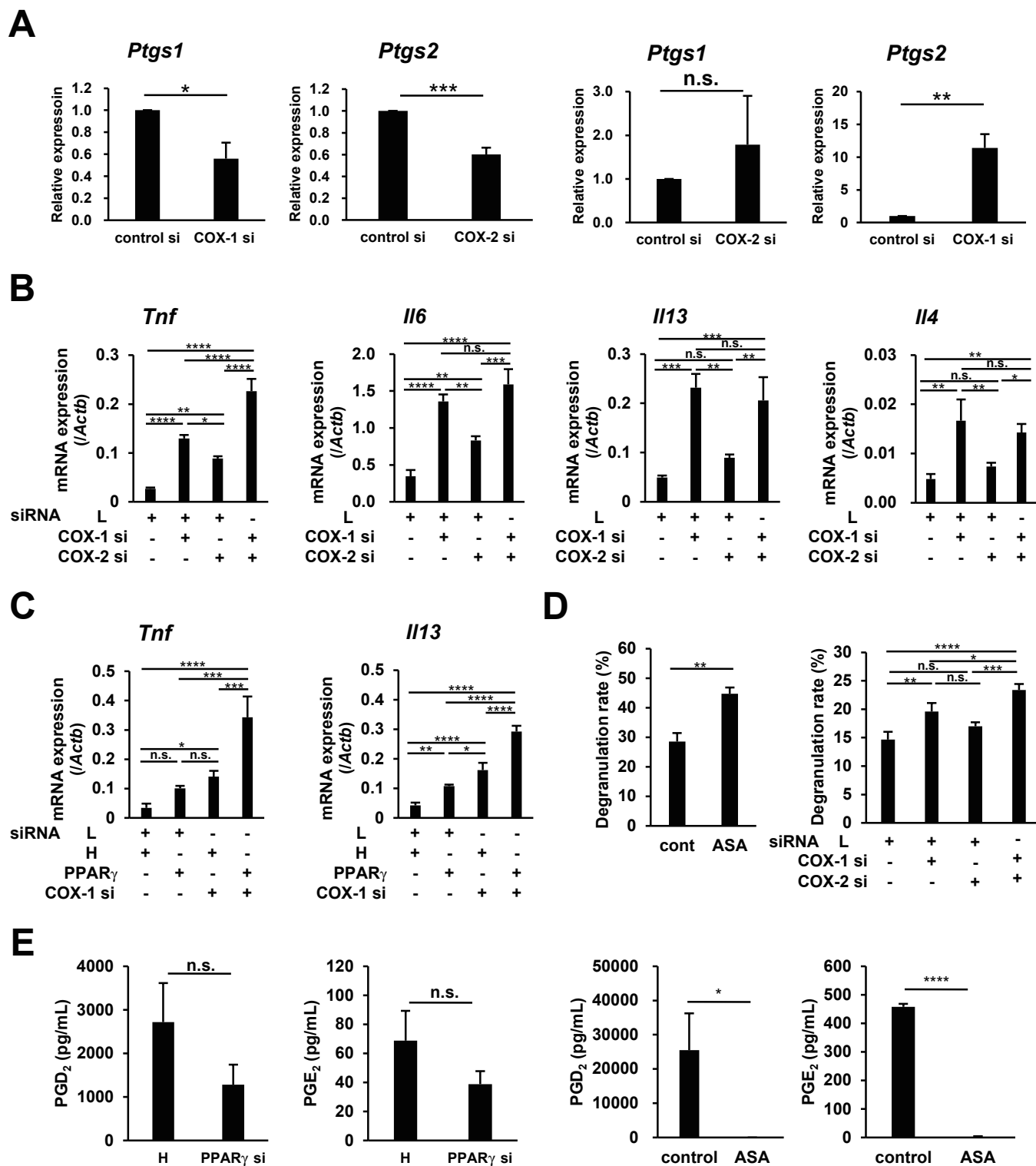


Fig. 5. Effects of COX knockdown on Fc ϵ R1-mediated activation of BMMCs. (A) mRNA levels of *Ptgs1* and *Ptgs2* in BMMCs which were transfected with *Ptgs1* siRNA or *Ptgs2* siRNA. *Ptgs1* mRNA in steady state (without Ag) and *Ptgs2* mRNA at 1 h after stimulation (with Ag) were shown. (B) Effects of COX-1 and/or COX-2 knockdown on Fc ϵ R1-mediated transactivation of cytokine genes in BMMCs. Control siRNA was used in single knockdown to adjust total amount of siRNA (200 pmol). (C) Effects of PPAR γ and/or COX-1 knockdown on Fc ϵ R1-mediated transactivation in BMMCs. +; 200 pmol siRNA was transfected. Control siRNA was used in single knockdown to adjust total amount of siRNA. (D) Effects of ASA treatment and COX knockdown on Fc ϵ R1-mediated degranulation of BMMCs. (E) The amount of PGD $_2$ and PGE $_2$ released from PPAR γ knockdown BMMCs and from ASA-treated BMMCs. After IgE/Ag stimulation of cells ($5.0 \times 10^5/100 \mu\text{l}$ Tyrode's buffer) for 1 h, culture supernatants were harvested. Cells were used at 48 h after siRNA transfection (A, B, C, D right and E left). ASA was added in culture media at 24 h (D left) or 2 h (E right) before the addition of IgE. After 2 h incubation in the presence of IgE, cells were stimulated with Ag for 1 h (B and E). The data represent the mean $\pm$ SD of triplicate samples, and similar results were obtained in two independent experiments (A, B, C, D and E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, n.s. not significant.

COX-2 in Fc ϵ RI-mediated activation, we examined the effect of COX-1 and/or COX-2 knockdown on IgE/Ag-stimulated BMMCs. Transfection of siRNA against *Ptgs1* and *Ptgs2* significantly reduced mRNA level of *Ptgs1* and *Ptgs2*, respectively (Fig. 5A). Although *Ptgs2* siRNA did not exhibit an apparent effect on *Ptgs1* mRNA levels, the amount of *Ptgs2* mRNA in *Ptgs1* siRNA-transfected BMMCs was over 10-fold of that in control BMMCs (Fig. 5A). As shown in Fig. 5(B), COX-1 knockdown markedly up-regulated IgE-mediated transactivation of the *Tnf*, *Il4*, *Il6* and *Il13* genes. In contrast, COX-2 knockdown moderately increased mRNA levels of *Tnf* and *Il6*, which caused further increase of *Tnf* and *Il6* mRNAs in a synergistic manner with COX-1 knockdown, and did not affect mRNA levels of *Il13* and *Il4*. From these results, we demonstrate that COX-1 rather than COX-2 is involved in the suppression of Fc ϵ RI-mediated transactivation in MCs. To clarify whether COX-1 siRNA induces the similar effect to that observed in the case of ASA treatment (Fig. 4B), we examined the mRNA levels of *Tnf* and *Il13* in BMMCs, which were transfected with COX-1 and/or PPAR γ siRNAs, and found that the double knockdown of PPAR γ and COX-1 significantly enhanced the mRNA transactivation compared with the single knockdown of PPAR γ or COX-1 (Fig. 5C). These results indicate that PPAR γ suppresses IgE/Ag-induced transactivation of cytokine genes in MCs in a manner distinguishable from that of eicosanoids, which were mainly produced by COX-1. ASA treatment enhanced Fc ϵ RI-mediated degranulation, suggesting that COX-derived eicosanoid(s) suppresses not only the transactivation of cytokine genes but also the degranulation reaction (Fig. 5D left). Knockdown experiments using *Ptgs1* and/or *Ptgs2* siRNA indicate that COX-1 is mainly required for suppression of degranulation (Fig. 5D right). To evaluate the effect of PPAR γ knockdown on levels of PGD₂ and PGE₂ produced from MCs, we determined concentrations of these PGs in the culture supernatant of IgE/Ag-activated MCs. As shown in Fig. 5(E) left, a significant difference was not observed between the amount of PGs released from PPAR γ knockdown MCs and that of control MCs. In contrast, ASA treatment completely blocked the release of PGD₂ and PGE₂ (Fig. 5E right). These results suggest that the effect of PPAR γ knockdown on the activation of MCs is not associated with production levels of PGD₂ and PGE₂. In a recent study by Karasuyama's group, the early-phase production of PGD₂, PGE₂ and 11-HETE by IgE/Ag-stimulated BMMCs is completely abrogated by SC-560, a COX-1 inhibitor, but not affected by celecoxib, a COX-2 inhibitor (18). There are several reports showing anti-allergic effects of PGs such as a study regarding food allergy, in which PGD₂ derived from MCs exhibits anti-allergic effects (19). It is also reported that cysteinyl leukotrienes are overproduced in PGE₂-deficient mice, resulting in aspirin-exacerbated respiratory disease-like pathology (20). Alternatively, other eicosanoids such as Resolvins and Protectins, which are produced from ω 3 fatty acids, may be involved in suppression of MC activation. Further detailed analyses of COX-1-mediated eicosanoids, which may suppress MC activation in an autocrine manner, would be useful for the prevention and/or treatment of IgE-dependent allergic diseases.

In regard to the mechanism by which PPAR γ functions as a negative regulator of cell activation, it is known that PPAR γ

antagonizes the activities of transcription factors including AP-1, STAT and NF- κ B in macrophages (1). The function of inducible transcription factors involved in IgE-mediated gene expression may be competitively suppressed by PPAR γ in MCs. Detailed analyses using the ChIP assays will clarify the effect of PPAR γ on the recruitment of related transcription factors to target genes. Alternatively, considering the function of PPAR γ as a transactivator, it is possible that PPAR γ up-regulates the expression of a negative regulator of IgE-mediated activation. The expression of the phosphatase SHIP was not affected by PPAR γ knockdown in our preliminary experiment (data not shown). The expression of Srcin1, a Src-inhibitory protein that was recently identified as a target of PPAR γ in MCs (21), was not detected in our experimental condition. We will conduct further analyses to clarify the mechanism by which PPAR γ suppresses gene expression and activation of MCs.

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Authorship contribution

K.N. performed most of the experiments and analyzed the data. K.K. designed the research and performed the experiments. R.M. and T.Y. performed the experiments. C.N. designed the research and wrote the paper.

Conflicts of interest statement: the authors declared no conflicts of interest.

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