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IL-27 affects helper T cell responses via regulation of PGE_2 production by macrophages

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ABSTRACT

IL-27 is a heterodimeric cytokine that regulates both innate and adaptive immunity. The immunosuppressive effect of IL-27 largely depends on induction of IL-10-producing Tr1 cells. To date, however, effects of IL-27 on regulation of immune responses via mediators other than cytokines remain poorly understood. To address this issue, we examined immunoregulatory effects of conditional medium of bone marrow-derived macrophages (BMDMs) from WSX-1 (IL-27Rα)-deficient mice and found enhanced IFN-γ and IL-17A secretion by $CD4^+$ T cells as compared with that of control BMDMs. We then found that PGE_2 production and COX-2 expression by BMDMs from WSX-1-deficient mice was increased compared to control macrophages in response to LPS. The enhanced production of IFN-γ and IL-17A was abolished by EP2 and EP4 antagonists, demonstrating PGE_2 was responsible for enhanced cytokine production. Murine WSX-1-expressing Raw264.7 cells (mWSX-1-Raw264.7) showed phosphorylation of both STAT1 and STAT3 in response to IL-27 and produced less amounts of PGE_2 and COX-2 compared to parental RAW264.7 cells. STAT1 knockdown in parental RAW264.7 cells and STAT1-deficiency in BMDMs showed higher COX-2 expression than their respective control cells. Collectively, our result indicated that IL-27/WSX-1 regulated PGE_2 secretion via STAT1–COX-2 pathway in macrophages and affected helper T cell response in a PGE_2-mediated fashion.

1. Introduction

IL-27 is a novel heterodimeric cytokine consisting of EBI-3 and p28 which are structurally related to the IL-12/IL-23 subunits p40 and p35/p19, respectively [1]. IL-27 is produced by activated antigen presenting cells (APCs) including dendritic cells (DCs) and macrophages [2–4]. IL-27 signals through its heterodimeric receptor consisting of WSX-1 and gp130 [2–4]. Initial studies demonstrated that IL-27 plays a role in Th1 induction and enhances proliferation of naïve $CD4^+$ T cells [1–4]. Recent studies, however, have demonstrated the anti-inflammatory function of IL-27 by generating Tr1 cells [5]. In addition to its regulation of T cell function, IL-27 also regulates APCs in an autocrine manner [4].

Prostaglandins (PGs) are a group of biologically active lipid mediators that are derived from arachidonic acid, and mediate a variety of functions. While cyclooxygenase (COX)-1 constitutively exists and mediates PG production in various tissues/organs, COX-2 is induced in immune cells, such as macrophages, by stimulation including growth factors, and mediates PG production in inflammatory venues [6]. Among various PGs produced, PGE_2 has a dominant role in inflammation/immune response [7,8]. Interestingly, recent reports have demonstrated that PGE_2 promotes Th1 differentiation and Th17 expansion *in vitro* via its receptors, EP2 and EP4 [9,10]. Regulation of PGs, especially PGE_2, may thus be a potent target of anti-inflammation strategies.

To date, functions of IL-27 on lipid mediators are poorly understood. In this study, we firstly demonstrated that lack of IL-27 signaling resulted in enhanced production of PGE_2 by bone marrow-derived macrophages (BMDMs), which led to increased production

Abbreviations: DC, dendritic cell; PG, prostaglandin; COX, cyclooxygenase; BMDM, bone marrow-derived macrophage; BM, bone marrow; CM, conditioned medium; mPGES-1, microsomal prostaglandin E_2 synthase-1; cPGES-1, cytosolic prostaglandin E_2 synthase; H-PGDS, hematopoietic prostaglandin D synthase; APCs, antigen presenting cells.

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of IL-17A and IFN-γ by $CD4^+$ T cells. We also demonstrated that IL-27 negatively regulated COX-2 expression in a STAT1-dependent way. These findings clearly showed that IL-27 regulates PGE_2 production by macrophages and "indirectly" regulates Th1/17 differentiation. IL-27 modulation of PGE_2 production is a novel mechanism by which IL-27 shows its anti-inflammatory/immunosuppressive function.

2. Materials and methods

2.1. Mice

Female *WSX-1*$^{-/-}$ mice were generated as described previously [11]. Female *STAT1*$^{-/-}$ mice were provided by Dr. Yoshimura (Keio University, Japan). C57BL/6J mice as a control were purchased from Japan CLEA, Inc. Mice were maintained under specific pathogen-free conditions and used between 8 and 14 weeks of age. All experiments were approved by the Animal Care and Use Committee at Nippon Boehringer Ingelheim Co., Ltd. or Saga University.

2.2. Reagents

Recombinant murine (rm) M-CSF was purchased from eBioscience. rmIL-27 was purchased from R&D systems. LPS (Re595) was purchased from Sigma–Aldrich. Indomethacin, NS-398, GW627368X, AH6809, anti-COX-1, anti-mPGES-1 were purchased from Cayman Chemicals. SC-58125, anti-β-actin, anti-pSTAT1 were purchased from Santa Cruz Biotechnology. Anti-COX-2, anti-pSTAT3, anti-STAT1 and anti-STAT3 were purchased from Cell Signaling Technology. Anti-CD3ε (145-2C11) and anti-CD28 (37.51) were purchased from BD Biosciences.

2.3. Cells preparation

BMDMs were prepared from bone marrow (BM) suspension of femurs and tibias of mice. Briefly, BM cells were cultured in RPMI1640 supplemented with 10% FBS and 10 ng/ml murine M-CSF for 6 days and used as macrophages. Naïve $CD4^+$ T cells were isolated from splenocytes of C57BL/6J mice by MACS beads, $CD4^+$-$CD62L^+$ T Cell Isolation Kit II, mouse (Miltenyi Biotech). The murine macrophage cell line RAW264.7 cells were obtained from the RIKEN Cell Bank (Japan).

2.4. LPS stimulation of macrophages

BMDMs (2×10^5 cells or 4×10^4 cells/well) were seeded at 24- or 96-well plate, respectively. Cells were pretreated with or without indomethacin or COX-2 inhibitors (NS-398, SC-58125) for 10 min, and then stimulated with LPS for additional 20 h. Culture supernatants and cells were collected and used for qPCR, Western blotting, cytokine and PGE_2 assay.

2.5. $CD4^+$ T cells culture with conditional medium of BMDMs

Naïve $CD4^+$ T cells (5×10^4 cells/well) were cultured with conditioned medium (CM) of BMDM in the presence of anti-CD3ε (precoated, 1 μg/ml) and anti-CD28 (0.5 μg/ml). Cells were pretreated with or without EP2 antagonist (AH6809) or EP4 antagonist (GW627368X) for 10 min. After 4 days culture, culture supernatants were collected and used for cytokine ELISA.

2.6. Establishment of mWSX-1-expressing RAW264.7 cells

RAW264.7 cells were transfected with murine WSX-1-FLAG plasmid by Lipofectamine 2000 (Life technologies). After 2 days, transfected cells were selected by resistance to neomycin (G418, Sigma–Aldrich) for more than 2 weeks.

2.7. siRNA transfection to RAW264.7 cells

RAW264.7 cells (2×10^4 well/well) were transfected with 30 nM siRNA (Qiagen) for mouse STAT1 (pools of SI02668862, SI02688763, SI02710729, SI02735054) and STAT3 (pools of SI01435294, SI01435301, SI01435287, SI01435308) by Lipofectamine RNAiMAX (Life technologies). AllStars Negative Control siRNA was used as a negative control.

2.8. PGE_2 and cytokine assays

PGE_2 was measured by homogeneous time resolved fluorescence (HTRF) assay (Cisbio Bioassay). Cytokines were determined by ELISA. ELISA kits for IL-17A, IFN-γ, and IL-4 were from eBioscience and for TNF-α, IL-6 and IL-12p40 were from R&D systems.

2.9. Quantification of eicosanoids

BMDMs (2×10^5 cells/well) were stimulated with LPS for 20 h. Supernatants were collected and stored at −80 °C. The amounts of eicosanoids were measured as described previously [12]. In brief, the samples were diluted with 2 ml of methanol and 7 ml of water containing 0.1% formic acid containing a mixture of deuterium-labeled eicosanoids as internal standards, and then loaded on Oasis HLB cartridges (Waters). The column was washed with 1 ml of water, 1 ml of 15% methanol, 1 ml of petroleum ether, and then eluted with 0.2 ml of methanol containing 0.1% formic acid. Eicosanoids levels were quantified by reversed phase HPLC–electrospray ionization-tandem mass spectrometry method.

2.10. Western blotting analysis

Cells (2×10^5 cells) were lysed and samples were boiled and electrophoresed with 10–20% polyacrylamide SDS–PAGE, transferred to PVDF membrane (Bio-Rad), and blotted with primary antibodies followed by HRP-conjugated secondary antibodies. Immunoblots were visualized with Western Lightning Plus ECL (Perkin Elmer) and densitometric analysis was performed using an image analyzer LAS-3000 (Fujifilm).

2.11. RNA isolation and PCR

Total RNA was extracted with RNeasy mini kits (Qiagen) and was reverse transcribed using PrimeScript RT reagent (Takara Bio) and analyzed for gene expression by RT-PCR or quantitative real-time PCR using an ABI PRISM 7000 (Applied Biosystems). The level of gene expression was normalized to GAPDH expression.

2.12. Plasmid construct

For the STAT1-FLAG and WSX-1-FLAG, murine STAT1 and murine WSX-1 cDNAs were amplified and ligated into pCMV14-3xFLAG (Sigma).

2.13. Statistical analysis

Data were presented as the mean ± SEM. Student's *t* test was used to determine the significance of differences.

3. Results

3.1. WSX-1-deficient BMDM conditioned medium promoted IFN-γ and IL-17A secretion by $CD4^+$ T cells

LPS stimulation of BMDMs induced expression of IL-27 subunits, EBI-3 (peak at around 12 h after stimulation) and p28 (peak

as early as 3–6 h after stimulation) (Supplemental Fig. 1A). LPS stimulation also induced expression of WSX-1 (data not shown). LPS stimulation of macrophages not only induced IL-27R expression but also primed the cells responsive to IL-27 stimulation by STAT1 activation. IL-27 stimulation of LPS-pre-treated BMDMs, but not untreated BMDMs, induced phosphorylation of both STAT1 and STAT3 (Supplemental Fig. 1B). These results demonstrated that IL-27 production is induced by LPS stimulation in BMDMs and that IL-27 acts on macrophages in an autocrine manner.

To investigate whether IL-27/WSX-1 signaling regulates T cell functions "indirectly" via macrophages, we examined the effects of CM derived from LPS-stimulated WSX-1-deficient BMDMs on the cytokine production by $CD4^+$ T cells. As shown in Fig. 1, CM derived from WSX-1-deficient BMDMs enhanced production of both IFN-γ and IL-17A by $CD4^+$ T cells as compared to that from control BMDMs. These data suggested that a substance(s) in the CM affected IFN-γ and IL-17 production by T cells.

3.2. WSX-1-deficient BMDMs produced higher level of PGE_2

For the enhanced production of IFN-γ and IL-17A by $CD4^+$ T cells in the presence of WSX-1-deficient macrophage-derived CM, augmented production of cytokines including IL-23 and IL-12 by WSX-deficient cells may be responsible. However IL-23 production was not detected by ELISA system and IL-12p70 production from WSX-1-deficient BMDMs and control BMDMs was comparable (data not shown). As IL-27-mediated lipid mediator production was implicated [13,14], we focused on PGs and other lipid mediators differentially produced by wild-type and WSX-1-deficient macrophages. We first examined PGE_2 production by HTRF assay to find higher production of PGE_2 in CM of WSX-1-deficient BMDMs (Fig. 2A). We then performed a multiple analysis of lipid mediators in the CM of BMDMs by mass spectrometry-based quantitation technique to investigate the factor(s) responsible for the enhanced production of IFN-γ and IL-17A. Among various lipid mediators analyzed (Supplemental Fig. 2), secretion of PGE_2, PGD_2 and 11-HETE were augmented in the CM of WSX-1-deficient BMDMs stimulated with LPS stimulation and WSX-1 deficient BMDMs as compared with CM of wild-type BMDMs (Fig. 2B). COX-2 and microsomal PGE synthase-1 (mPGES-1) but not COX-1, cytosolic prostaglandin E_2 synthase (cPGES)-1 or mPGES-2, were important for PGE_2 production [6] and hematopoietic prostaglandin D synthase (H-PGDS) is critical for LPS-induced PGD_2 production in BMDMs [15]. Expression of both COX-2 and mPGES-1 was induced in BMDMs by LPS stimulation, while expression of H-PGDS was remarkably suppressed after LPS stimulation (Fig. 2C). When compared between in wild-type and WSX-1-deficient macrophages, expression of COX-2 was further enhanced in WSX-1-deficient BMDMs (Fig. 2C, top), while mPGES-1 expression level was rather reduced in WSX-1-deficient cells (middle). H-PGDS expression was not affected by WSX-1-deficiency (bottom). Western blot analysis also showed higher COX-2 production, but not COX-1 and mPGES-1, in WSX-1-deficient BMDMs compared to wild-type BMDMs (Supplemental Fig. 3). Taken together, it is highly likely that the enhanced PGE_2 and PGD_2 secretion by WSX-1-deficient BMDMs is dependent on COX-2 up-regulation in the absence of IL-27 signaling, but not mPGES-1 or H-PGDS expression.

3.3. PGE_2 in the CM is responsible for the enhanced production of IFN-γ and IL-17A by $CD4^+$ T cells

We speculated that enhanced IFN-γ and IL-17A secretion by $CD4^+$ T cells cultured with CM of WSX-1-deficient BMDMs was caused by PGE_2. To prove this hypothesis, we inhibited PGE_2 synthesis in BMDMs with a COX-2 inhibitor, NS-398 (Fig. 3A). NS-398 inhibited production of PGE_2 by both wild-type and WSX-1-deficient BMDMs almost completely, but not cytokines such as IL-12p40, IL-6 (Fig. 3C). The CM of BMDMs treated with NS-398 dramatically reduced its activity to induce IFN-γ and IL-17A production by $CD4^+$ T cells, suggesting the role of PGE_2 in the augmented cytokine production (Fig. 3B. right-most columns). COX-2 inhibitors, however, can reduce not only PGE_2 but also PGD_2 secretion by BMDMs, as reported previously [15]. To further make clear the primary involvement of PGE_2, PGE_2 signaling in $CD4^+$ T cells was inhibited by EP2 and EP4 antagonists during culture of $CD4^+$ T cells in the presence of the CM from BMDMs. As shown in Fig. 3B, both EP2 and EP4 antagonists (AH6809 and GW627368, respectively) remarkably reduced IFN-γ and IL-17A secretion by $CD4^+$ T cells. Taken together, these results demonstrated that WSX-1-deficient BMDMs facilitated IFN-γ and IL-17A secretion by $CD4^+$ T cells through enhanced production of PGE_2 by BMDMs, in which COX-2 expression was augmented by WSX-1-deficiency.

3.4. Augmentation of IL-27R signaling in macrophage resulted in inhibition of PGE_2 production

To directly show that IL-27 inhibits PGE_2 production by macrophages, we generated RAW264.7 macrophage cell lines overexpressing mWSX-1. Upon LPS stimulation, COX-2 induction was

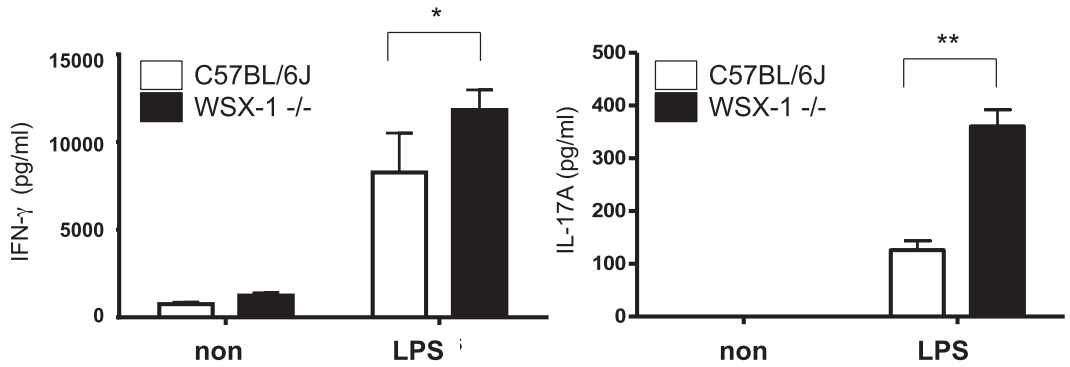

Fig. 1. Increases in IFN-γ and IL-17A production by $CD4^+$ T cells in the presence of conditioned medium from WSX-1-deficient macrophages. BMDMs (4×10^4 cells/well) of C57BL/6J and *WSX-1*$^{-/-}$ mice were stimulated with LPS (1 ng/ml) for 20 h. Culture supernatants were collected and put into cultures where naïve $CD4^+$ T cells (5×10^4 cells/well) of C57BL/6J mice were stimulated with anti-CD3 and anti-CD28 antibodies for 4 days. IFN-γ and IL-17A productions were examined. Results are representative of three independent experiments. Values are mean ± SEM, $n = 3$, $^*P < 0.05$ and $^{**}P < 0.01$.

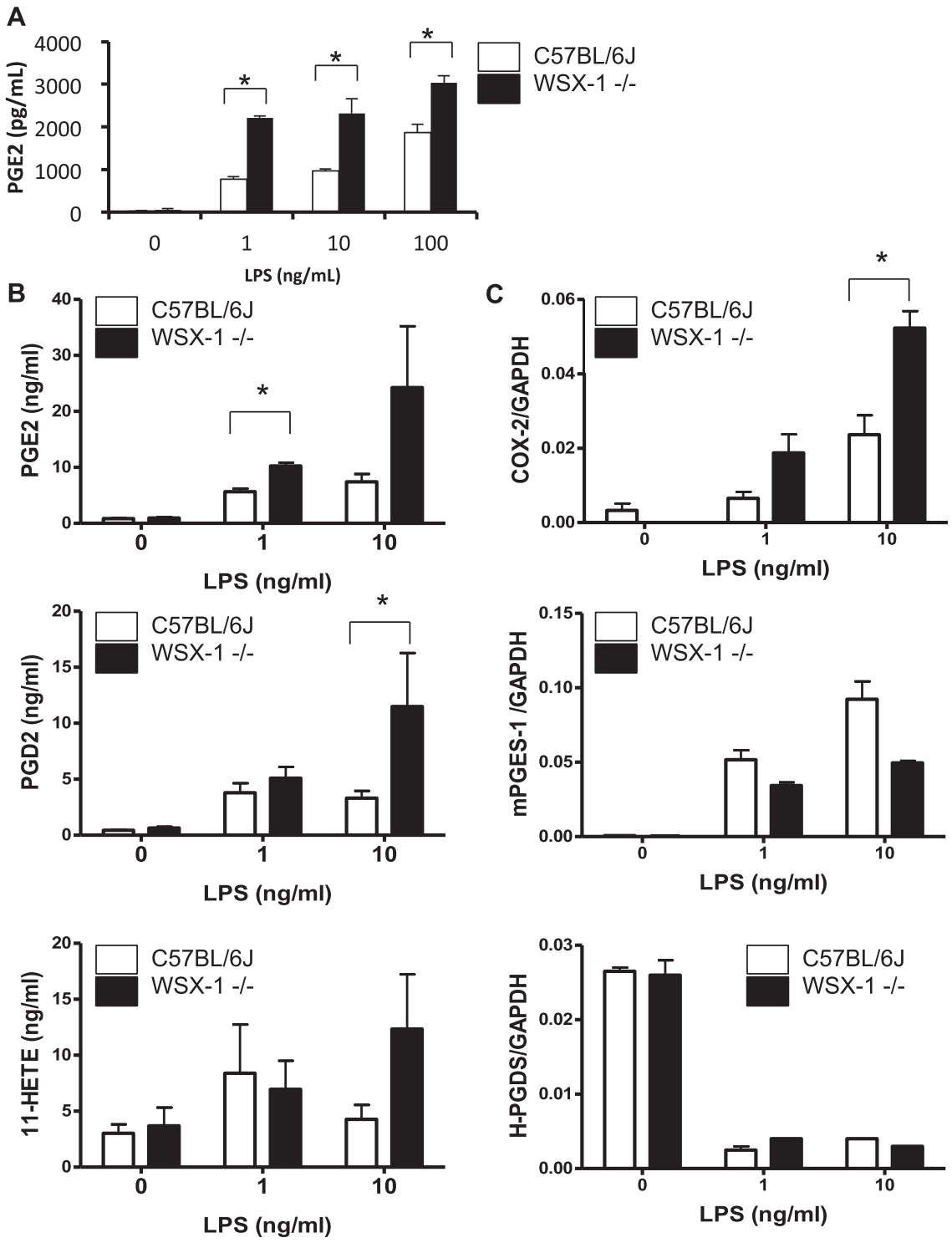

Fig. 2. Enhanced production of PGE_2 and COX-2 by WSX-1-deficient macrophages. (A) BMDMs (2×10^5 cells/ml) of C57BL/6J and *WSX-1*$^{-/-}$ mice were stimulated with LPS at indicated concentration for 20 h. Culture supernatants were collected and PGE_2 production determined. Results are representative of two independent experiments. Values are mean ± SEM, $n = 3$, $^*P < 0.05$. (B) BMDMs were stimulated as in (A). Culture supernatants were collected and examined for lipid mediator concentration by mass spectrometry analyses. Results are representative of two independent experiments. Values are mean ± SEM, $n = 3$, $^*P < 0.05$. (C) BMDMs stimulated as in (A) were collected and expression of *COX-2*, *mPGES-1*, and *H-PGDS* genes were analyzed. Results are representative of two independent experiments. Values are mean ± SEM, $n = 3$, $^*P < 0.05$.

less evident in mWSX-1-RAW264.7 cells than in parental RAW264.7 cells. Similarly and perhaps more apparently, LPS-induced production of PGE_2 was markedly reduced in mWSX-1-RAW264.7 cells as compared with parental RAW264.7 cells (Fig. 4A), demonstrating the inhibitory effects of IL-27 signaling on mWSX-1 expression on PGE_2 production.

3.5. COX-2 expression and PGE2 production are negatively regulated by Stat1 in RAW264.7

The anti-inflammatory and/or immunosuppressive function of IL-27 is largely dependent on STAT1 and in part on STAT3 [16,17]. To investigate which STAT, STAT1 or STAT3, regulates

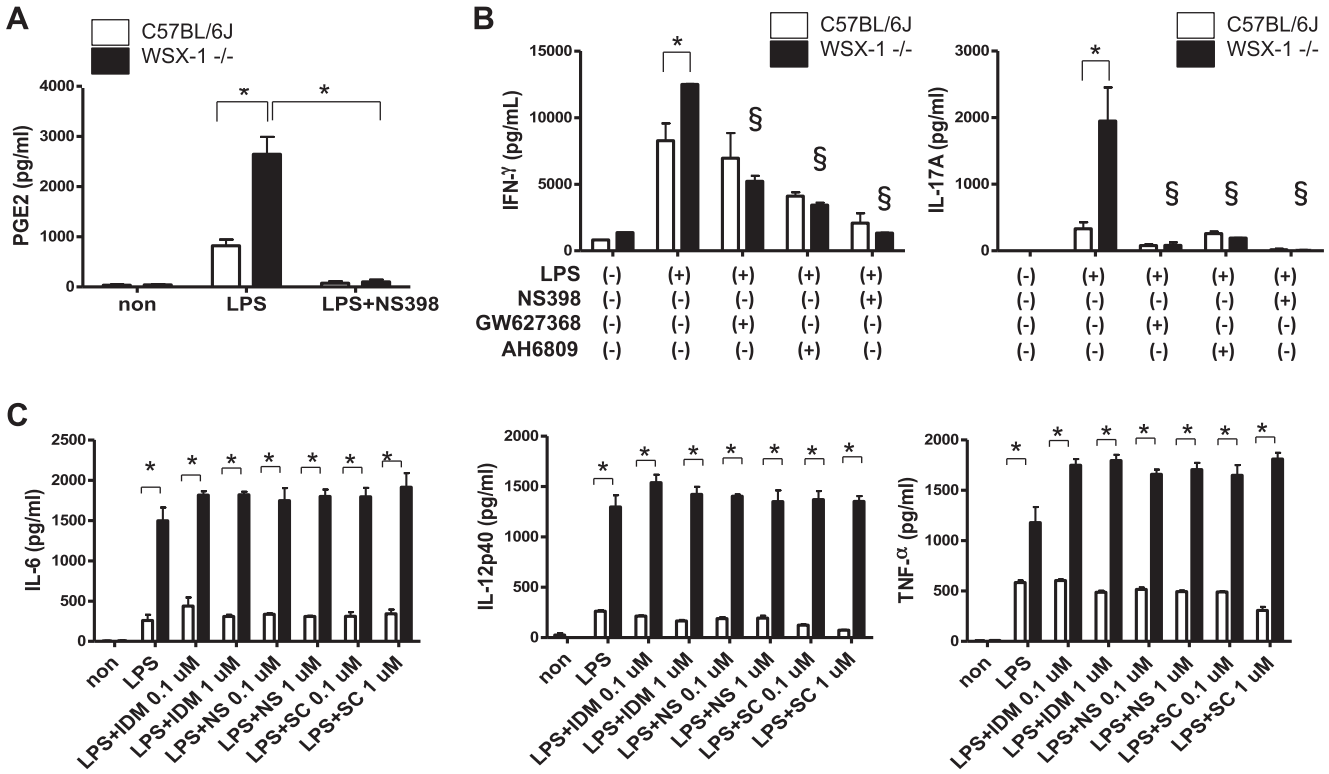

Fig. 3. Reduction in the augmented production of IFN-γ and IL-17A by PGE_2 inhibition. (A) BMDMs (4×10^4 cells/well) of C57BL/6J and *WSX-1*$^{-/-}$ mice were stimulated with LPS (1 ng/ml) with or without COX-2 inhibitor, NS-398 (100 nM) for 20 h. PGE_2 production in the supernatants was determined. Results are representative of three independent experiments. Values are mean ± SEM, $n = 3$, $^*P < 0.05$. (B) Naïve $CD4^+$ T cells (5×10^4 cells/well) were stimulated in the presence of supernatants from (A) with anti-CD3 and anti-CD28 antibodies. Cells were pre-treated with or without COX-2 inhibitor, NS-398, EP2 antagonist (AH6809), or EP4 antagonist (GW627368X) for 10 min. After 4 days of culture, culture supernatants were collected and IFN-γ and IL-17A production determined. Results are representative of two independent experiments. Values are mean ± SEM, $n = 3$, $^*P < 0.05$, $^{\S}P < 0.05$ as compared between with and without inhibitors. (C) BMDMs stimulated with LPS in the presence or absence of indomethacin (IDM) or COX-2 inhibitors (NS-398 and SC-58125) for 20 h. Cytokine production in the supernatants was determined. Open columns; C57BL/6J and closed columns; *WSX-1*$^{-/-}$. Results are representative of two independent experiments. Values are mean ± SEM, $n = 3$, $^*P < 0.05$.

COX-2 expression and PGE_2 secretion downstream of the IL-27R in macrophages, knockdown of STAT1 and STAT3 by siRNA were performed in RAW264.7 cells. As shown in Fig. 4B, siSTAT1 markedly increased PGE_2 secretion by RAW264.7 cell while no such increase was observed in STAT3-knockdown cells. COX-2 expression was also enhanced by siSTAT1 but not by siSTAT3 (Fig. 4C. Densitometric intensities; 66.52, 87.13, and 58.81 for siNega, siSTAT1, and siSTAT3, respectively). To confirm that STAT1 is involved in the regulation of COX-2 expression in macrophages, STAT1-deficient BMDMs were examined for LPS-induced COX-2 expression and PGE_2 production. Just like STAT1 knockdown cells, STAT1-deficient BMDMs stimulated with LPS produced higher amounts of PGE_2 and COX-2 protein over control BMDMs, similar to the results obtained in WSX-1-deficient BMDMs (Fig. 4D and E. Densitmetric intensities for COX-2 in E; 48.24, 56,07, and 73.57 for C57BL/6, WSX-1$^{-/-}$, and STAT1$^{-/-}$, respectively). Finally, we confirmed the effects of IL-27 on PGE_2 production. In RAW264.7 cells treated with irrelevant siRNA (siNega), IL-27 treatment reduced LPS-induced PGE_2 secretion (Fig. 4F, left columns). Taken together, these data indicated that IL-27-mediated activation of STAT1 negatively regulates COX-2 expression and PGE_2 production by BMDMs and RAW264.7 cells.

4. Discussion

Here we firstly reported that IL-27 negatively regulated PGE_2 production from murine macrophages and it resulted in the modulation of IFN-γ and IL-17A production from $CD4^+$ T cells *in vitro*.

In the present study, CM from WSX-1-deficient BMDMs after LPS stimulation induced higher amount of IFN-γ and IL-17A from $CD4^+$ T cells (Fig. 1). WSX-1-deficient BMDMs produced higher level of PGE_2 and cytokines such as IL-6 and IL-12p40, than control BMDMs, (Figs. 2A and 3). Inhibition of PGE_2 production by COX-2 inhibitors from BMDMs or abolishment of PGE_2 signaling in $CD4^+$ T cells by EP2 and EP4 antagonists dramatically reduced IFN-γ and IL-17A secretion without reduction of IL-6 and IL-12p40 (Fig. 3). It was thus suggested that higher level of PGE_2 from WSX-1-deficient BMDMs dominantly promoted Th1 and Th17 differentiation, indicating an important role for IL-27 in PG-mediated regulation of Th differentiation. Previous reports demonstrated pivotal involvement of PGE_2 in Th1 and Th17 differentiation. In human systems, PGE_2 was required for IL-23- and IL-1β-induced IL-17 production in a monocytes/naïve $CD4^+$ T cells co-culture systems [10]. *In vivo*, Yao et al. reported that EP4 antagonism suppressed inflammation in disease models, where both of Th1 and Th17 cells were involved in pathogenesis [9]. These results indicated that PGE_2 signal is important for the pathogenesis of autoimmune or inflammatory diseases through induction of Th1 and Th17 differentiation. Together with data shown in the current study, it was thus demonstrated that IL-27/WSX-1 regulates Th1 and Th17 differentiation by inhibition of PGE_2 secretion by macrophages. DCs are also very potent APCs. To determine whether BM-derived DCs could also supply PGE_2 during T cell activation/differentiation, we compared the level of PGE_2 from BMDMs and BMDCs, and found that BMDCs produced PGE_2 ten times less than BMDMs (data not shown). It was thus highly likely that macro-

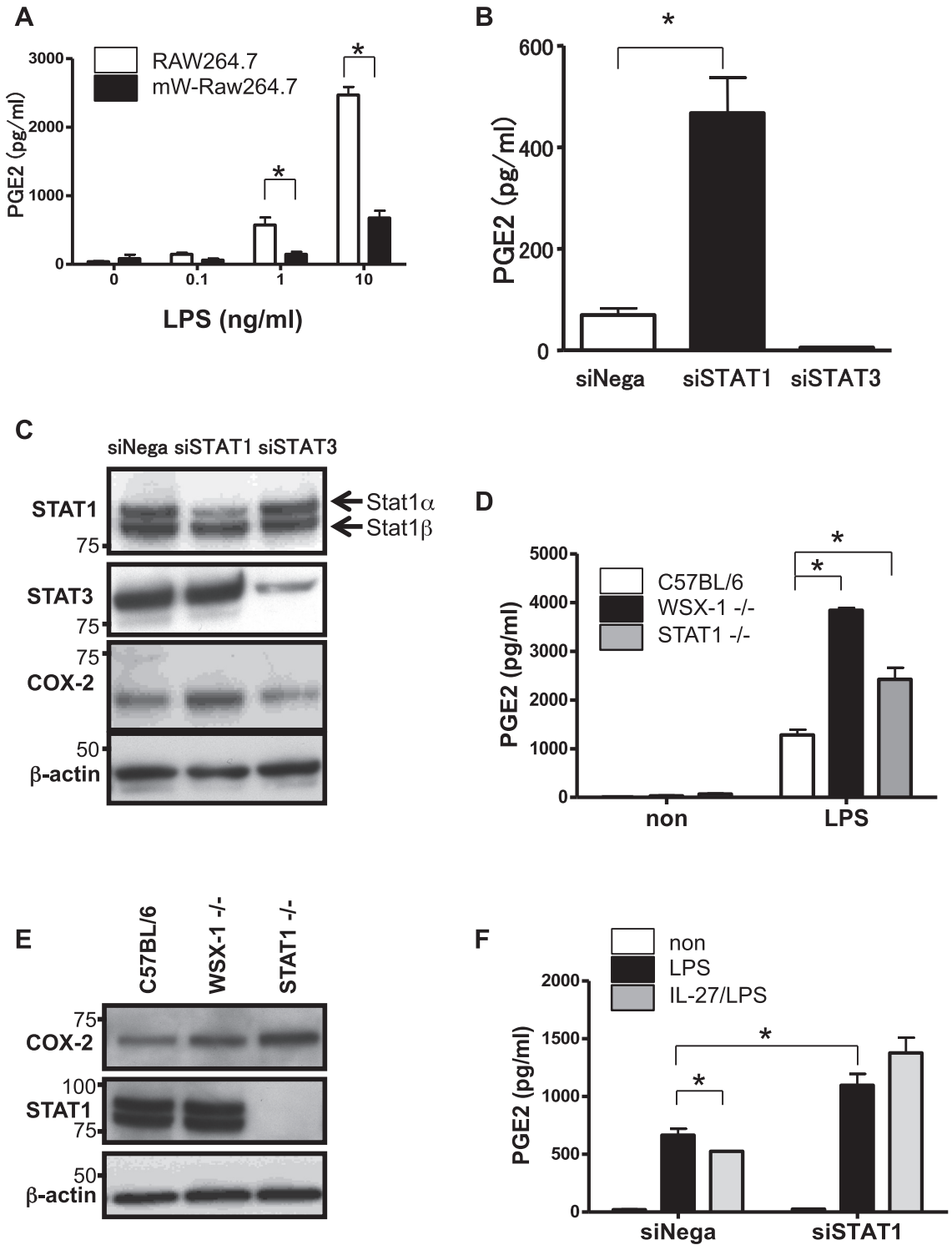

Fig. 4. STAT1-dependent regulation of PGE_2 and COX-2 production by over-expression of WSX-1. (A) RAW264.7 cells and mWSX-1-RAW264.7 cells were stimulated with LPS and PGE_2 secretion examined. Results are representative of three independent experiments. Values are mean ± SEM, $n = 3$, $^*P < 0.05$. (B) RAW264.7 cells were transfected with siRNAs (30 nM) for negative control, STAT1 or STAT3. PGE_2 secretion in response to LPS was determined. Results are representative of two independent experiments. Values are mean ± SEM, $n = 3$, $^*P < 0.05$. (C) Cells in (B) was examined for STAT1, STAT3, COX-2, β-actin expressions. Results are representative of two independent experiments. (D) BMDMs from C57BL/6J, *STAT1*$^{-/-}$, and *WSX-1*$^{-/-}$ mice were stimulated with LPS. Culture supernatants were collected and PGE_2 secretion determined. Values are mean ± SEM, $n = 3$, $^*P < 0.05$. (E) Cells in (D) were examined for COX-2, STAT1, and β-actin expressions. Results are representative of two independent experiments. (F) RAW264.7 cells were transfected with siRNAs for negative control or STAT1. After 48 h, cells were cultured with or without rmIL-27 (20 ng/ml) for 2 h and further cultured with or without LPS (1 μg/ml) for 20 h and PGE_2 secretion determined. Values are mean ± SEM, $n = 3$, $^*P < 0.05$.

phages are the major PGE_2-providing cells during T cell activation, more dominant than DCs. In addition, it was also reported that PGE_2 induced IL-23 production by DCs [18]. It is possible that augmented production of PGE_2 by WSX-1-deficient BMDMs enhanced IL-23 production by DCs to promote Th1 and Th17 differentiation synergistically with the direct effect of PGE_2 on $CD4^+$ T cells.

In WSX-1 deficient BMDMs, the expression of COX-2 but neither mPGES-1 nor H-PGDS was increased compared to control BMDMs (Fig. 2C). It was reasonable because WSX-1-deficient BMDMs produced not only PGE_2 but also PGD_2 and 11-HETE, since these are the products of COX-2 (Fig. 2B) [19]. To address if COX-2 is regulated by IL-27/WSX-1 signaling, we established mWSX-1-RAW264.7 cells, macrophage cell lines over-expressing murine WSX-1 gene, and revealed that COX-2 expression and PGE_2 secretion were decreased compared to parental RAW264.7 cells (Fig. 4A and Supplemental Fig. 4C), clearly demonstrating that IL-27R signaling negatively regulates COX-2 expression. In line with these results, IL-27 transduction resulted in reduction of COX-2 expression and PGE_2 secretion in lung carcinoma cell lines [14]. We also examined the level of other COX-2-dependent products such as $PGF_2\alpha$ and 6-keto-$PGF_1\alpha$ a of PGI_2). Their production levels, however, were very low in BMDMs (Supplemental Fig. 2).

In BMDMs and mWSX-1-RAW264.7 cells, both STAT1 and STAT3 were phosphorylated in response to IL-27 stimulation (Supplemental Figs. 1B and 4B). In RAW264.7 and BMDMs, siRNA and gene deletion study with knockout mice revealed that STAT1 is a negative regulator for COX-2 expression and PGE_2 secretion downstream IL-27R (Fig. 4B, D and E). Although we did not address whether STAT1 directly regulated COX-2 transcription, COX-2 promoter region contains STAT-binding element, namely IFN-γ-activated sequences (GAS) and site-directed mutation in GAS abolished COX-2 transcriptional activity mediated by STAT3/STAT5 in human colonic epithelial cells [20]. It is thus highly possible that STAT1 activated downstream IL-27R directly binds to the promoter region of COX-2 and regulate COX-2 transcription.

In summary, we demonstrated that IL-27/WSX-1 negatively regulated PGE_2 secretion by murine BMDMs, which was mediated by STAT1-induced inhibition of COX-2 expression. Reduction in PGE_2 then resulted in the reduced Th1 and Th17 differentiation *in vitro*. These results indicated that IL-27/WSX-1 regulates Th differentiation by direct effect on T cells but also by "indirect" effects on macrophages via regulation of lipid mediator production in an autocrine manner.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.096.

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