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2015 년 2 월

서울대학교 대학원

의학과 미생물학 전공

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February, 2015

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A thesis submitted to the Department of Medicine
in partial fulfillment of the requirements for the
Degree of Master of Science in Medicine
(Microbiology and Immunology)
at Seoul National University College of Medicine

January, 2015

Approved by Thesis Committee:

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이 논문을 의학석사 학위논문으로 제출함

2014 년 10 월

서울대학교 대학원

의학과 미생물학 전공

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정다운의 의학석사 학위논문을 인준함

2015 년 1 월

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ABSTRACT

Macrophages have important functions in tissue homeostasis, but the exact mechanisms regarding wide spectrum of macrophage phenotype remain unresolved. In this study, we report that naïve macrophages produce prostaglandin E_2 (PGE_2) endogenously, resulting in anti-inflammatory gene expression upon differentiation induced by macrophage colony stimulating factor (M-CSF). Cyclooxygenase (COX) inhibition by indomethacin reduced endogenous PGE_2 production of macrophages and subsequently reduced arg1, IL10 and Mrc1 gene expressions. Of note, PGE_2 phosphorylates CREB via EP2 and EP4 receptor ligation, thereby transcriptionally increasing C/EBPβ expression in Balb/c bone marrow derived macrophages (BMDM). Activated CREB directly binds to the CREB-responsive element of the C/EBPβ promoter, such that PGE_2 ultimately reinforces arg1, IL10 and Mrc1 gene expression. Cyclic AMP activator forskolin also phosphorylated CREB and induced the C/EBPβ cascade, but this was completely blocked by the PKA inhibitor, H89. Consequently, M-CSF grown macrophages inhibited T cell proliferation but the inhibition ability was reduced when the COX is inhibited by indomethacin or macrophage C/EBPβ expression was decreased by siRNA transduction. Our results collectively describe the molecular basis for homeostatic macrophage differentiation by endogenous PGE_2.

Keywords: Prostaglandin E_2, macrophage, CREB, C/EBPβ

Student number: 2013－21741

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LIST OF ABBREVIATIONS

PGE2: Prostaglandin E_2

CREB: cAMP response element-binding protein

C/EBPβ: CCAAT-enhancer-binding proteins beta

INTRODUCTION

Macrophages play a pivotal role in the innate immune system by sensing and killing microbes. Moreover, macrophages have important roles in almost every aspect of an organism's biology including development, tissue repair, and homeostasis (1). Tissue macrophages are highly heterogenous in terms of their functions and phenotypes as a consequence of adapting to different tissue environments, where they monitor the local environment and maintain homeostasis (2).

Historically, all tissue macrophages were considered to be derived from circulating monocytes that originate from hematopoietic stem cells (3). Recent studies revealed that a substantial portion of tissue macrophages arise from the yolk sac during embryogenesis, and are maintained by local proliferation (4). Regardless of their origin, macrophages acquire unique characteristics depending on their tissue specific microenvironment (5). Macrophage colony-stimulating factor (M-CSF) generally differentiates macrophages with homeostatic function in vivo (6). Likewise, tissue specific factors such as GM-CSF in lung alveoli and fat cell derived adipokines in adipose tissue also affect macrophage phenotypes. Infections greatly change local tissue microenvironments, particularly when blood derived monocytes infiltrate infected areas and differentiate into macrophages. Parasite induced IL-4 or IL-13 drive macrophages into alternatively activated M2 phenotypes, but most other microbes induce Th1 immune responses and drive macrophages into classically activated phenotypes, which contribute to many pathological processes (7). Accordingly, there is much interest in studying the exact factors that dictate macrophage phenotype changes for therapeutic applications.

Prostaglandin E_2 (PGE_2) is a lipid mediator derived from the metabolism of arachidonic acid by cyclooxygenase (COX). It is the most abundant prostanoid in most tissues, and it is a well-known regulator of numerous physiologic and pathophysiologic processes, including blood flow (8), parturition (9), fever and inflammation (10). While generally recognized as a mediator of active inflammation, promoting local vasodilation and local

attraction and activation of neutrophils, mast cells, and macrophages early during inflammation (11), it also promotes the induction of suppressive cytokine IL-10 and directly suppresses the production of multiple proinflammatory cytokines. This works to limit nonspecific inflammation as part of immune suppression associated with chronic inflammation (12). Importantly, its effects on macrophage function have been amply documented to be mainly suppressive (11), as evidence by the increase in the levels of the intracellular second messenger cAMP generated upon ligation of EP2 and EP4 receptors (13). Notably, M-CSF induces functional COX-1 in developing macrophages (14), but whether endogenous PGE_2 is generated during macrophage differentiation and how PGE_2 affects the signals that regulate specialized macrophage characteristics remain poorly defined.

Specifically, PGE_2 signals via four known receptors (EP1-EP4), which primarily use the cAMP/PKA/CREB signaling pathway for the suppressive and regulatory functions of PGE_2 (11). Once serine 133 of CREB is phosphorylated, CREB interacts with its coactivator protein, CREB-binding protein (CBP), or p300, to initiate transcription of CREB-responsive genes (15). Meanwhile, a recent study showed that the transcription factor C/EBPβ expression is induced by LPS mediated CREB activation and specifically regulates M2 macrophage-associated genes including arg1, IL10 and Mrc1 (16). Using a muscle injury model, CREB-mediated C/EBPβ expression was determined to be required for upregulating M2-specific genes in infiltrating macrophages and muscle regeneration. Though PGE_2 more potently activates CREB than LPS, the PGE_2/CREB-C/EBPβ axis and its effects on macrophage phenotype have not yet been examined.

We report here that M-CSF grown murine macrophages endogenously produce PGE_2 during differentiation. COX inhibition by indomethacin reduced endogenous PGE_2 production and subsequently reduced arg1, IL10 and Mrc1 gene expressions. PGE_2 phosphorylates CREB via EP2 and EP4 receptor ligation leading to cAMP/PKA activation. Phosphorylated CREB transcriptionally induces C/EBPβ and consequent arg1, IL10 and Mrc1 gene expressions. Lastly, M-CSF grown macrophages inhibit T cell proliferation

and this was reduced when COX activity was inhibited or when endogenous C/EBPβ was downregulated in differentiating macrophages. These results establish PGE_2 as the molecular basis of homeostatic macrophage differentiation with anti-inflammatory phenotype.

MATERIALS AND METHODS

Reagents

AH-23848, AH-6809, butaprost, CAY10598, forskolin, H89, PGE_2, SC-51089 and sulprostone were all purchased from Cayman Chemicals (Ann Arbor, MI). L-798,106 was obtained from Santa Cruz biotechnology (Dallas, Texas). Indomethacin was obtained from Sigma Aldrich (St. Louis, MO). All chemicals were dissolved in DMSO or ethanol and treated at the indicated concentration in Figure legends.

Cell line and cell culture

J774A.1 and RAW264.7 macrophage cell lines were generously provided by Dr. Cho NH (Seoul National University, Seoul, Korea). All cell lines were cultured at 37°C in a humidified incubator containing 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, NY) and 1% penicillin–streptomycin (Gibco).

Preparation of bone marrow-derived macrophages

Murine bone-marrow-derived macrophages (BMDM) were derived from 6~9-week-old female BALB/c mice. Isolated bone marrow cells were cultured for 3 days at a density of 10^6/ml in RPMI medium (Thermo, Waltham, Massachusetts) supplemented with 10% FBS (Gibco, Carlsbad, California), 10 units/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine (Gibco) and 20% L-cell conditioned medium (hereafter termed complete medium) at 37°C in a humidified atmosphere with 5% CO_2. All animal procedures were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institution of Animal Care and Use Committee of Seoul National University, Korea. The protocol was approved by the Seoul National University Institute Animal Care and Use Committee (Approval Number SNU-120904-8-1).

Western blotting

Cells were lysed in RIPA buffer with protease inhibitor and phosphatase inhibitor (GenDepot, Houston, TX). Protein samples were separated on a 12% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membrane (Millipore, Bedford, MA) and then washed with PBS containing 0.05% Tween 20 (PBST) and then blocked with PBS containing 5% nonfat dry milk for 1 h at room temperature. Membranes were then probed with primary antibodies including phospho-Ser133 CREB (#9191, Cell Signaling Technology, Danvers, MA), total CREB (#9197, Cell Signaling Technology), CEBPβ (#3087, Cell Signaling Technology) and actin (sc-47778, Santa Cruz biotechnology) at 1/2000 overnight. After washing three times, blots were incubated with HRP-conjugated appropriate secondary antibodies (Santa Cruz Biotechnology) at 1/5000 for 1 h at room temperature. Immunoreactivity was detected using the PICO Lucent detection kit (G Biosciences, St. Louis, MO).

RNA isolation and Quantitative real-time PCR

RNA was solubilized in TRIzol reagent (Invitrogen) and extracted according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA and the levels of mRNA were determined by quantitative real-time PCR using the Taqman Universal Master Mix II (Applied Biosystems) or SYBR Green PCR Master Mix (Applied Biosystems) on a Prism 7900HT. The relative amount of each mRNA was normalized by GAPDH expression. The primer and probe sequences are listed in Table 1. Murine GAPDH primers and probe sets (VIC) were obtained from Applied Biosystems.

ELISA

PGE_2 levels in BM-derived macrophage culture supernatants were measured by ELISA kit according to the manufacturer's instruction (Cayman Chemical).

Small interfering RNA transfection

Small interfering RNA (siRNA) for C/EBPβ and scramble control were obtained from Santa Cruz Biotechnology and transfected using Lipofectamine

RNAiMAX (Invitrogen) following the manufacturer's instructions. After 24h of J774 cell seeding, cells were treated with PGE2 (10μM) for the times indicated in figure legends. RNAs were harvested for RT-PCR to analyze downregulation of C/EBPβ expression.

Transient transfection

Raw 264.7 macrophage cells were plated in 12-well plates at a density of 2 x10^5/well. When the cells reached 60–70% confluence, cells were transiently transfected with wild-type CREB (pCMV-CREB Vector, Clontech), mutant CREB (pCMV-CREB133, Clontech), or a dominant-negative CREB (pCMV-KCREB, Clontech) vector using TransIT-LT1 Transfection reagent (Mirus Bio LLC, Madison) according to the manufacturer's instructions. After 24 h of transfection, cells were treated with PGE_2 (10μM) for another 4h.

Chromatin Immunoprecipitation(chIP)

Raw 264.7 cells were cross-linked with 1% formaldehyde in culture media for 10 min. Cross-linking was quenched by adding 0.125 M glycin to the medium and 10 min incubation. Cells were then washed, scraped in cold PBS, pelleted, and resuspended in ChIP lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) in a volume of 200 μl/million cells. Cell lysates were incubated on ice for 10 min. Chromatin was sheared by sonication for 10 s using water-sonication. After sonication, cellular debris was eliminated, and 200 μl lysate was diluted 10 times in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, and 0.01% SDS) and cleared by incubation with 30 μl protein A agarose beads (Santa Cruz Biotechnology) for 1 h at 4 °C with rotation. Four micrograms of anti-CREB antibody (Cell Signaling) or non-immune rabbit IgG (Santa Cruz Biotechnology) were added and lysates were incubated 16 h at 4 °C on rotation. Protein A bead solution was added and the lysates were incubated 4 h at 4 °C on a rotating wheel. Beads were collected by centrifugation and washed in ChIP dilution buffer, ChIP dilution buffer with 500 mM NaCl, and twice in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Finally, the beads were

resuspended in 100 µl TE and decrosslinked at 65 °C in the presence of 1 ng RNase for 4 h. Fifty nanograms of Proteinase K (Roche) and SDS to 0.5% were added and the samples were incubated 1 h at 42 °C. DNA was collected using QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Precipitated DNA was analyzed by PCR using the following conditions: 94 °C for 2 min, 30 times of (94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min), 72 °C for 5 min. The reactions were carried out with Promega Taq Polymerase as described by the manufacturer, using 2 µl DNA template and adding 5% DMSO. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

Flow Cytometry

To detect EP receptors, cells were fixed and permeabilized before incubation with rabbit polyclonal anti-EP receptor Abs (EP1: 101740, EP2: 101750, EP3: 101760, EP4: 101775, Cayman Chemicals). Primary Abs were detected with Alexa Fluor 488-conjugated anti-rabbit IgG (A21206, Molecular Probe). PE-conjugated anti-F4/80 (clone BM8, e-bioscience) and APC-Cy7 conjugated anti-CD11b (clone M1-70, e-bioscience) were also labeled for macrophage gating. Labeled cells were analyzed on a LSRII flow cytometer (BD Biosciences).

Co-culture Experiments

BM-derived primary macrophages were seeded in 100 mm dishes ($1x10^7$ cells/10ml). After 3 days of differentiation, macrophages were transiently transfected with scrambled control siRNA or C/EBPβ siRNA. After 24hr of transfection, transfected or non-transfected BM-derived macrophages were plated in 0.4 µm pore sized 24-transwell plates upper well at a density of 2 $x10^5$ cells /well. For co-culture experiment, CD4+ T cells were isolated from splenocytes of Balb/c mice using a T cell enrichment kit (Miltenyi Biotec) and labeled with CFSE (Invitrogen). T cells (2 $x10^6$ cells/well) were added into 24-well plate bottom wells in the presence of transfected macrophages or non-transfected macrophages. After indomethacin (20 µM or 100 µM) treatment to

the non-transfected group, T cells and macrophages were co-cultured for 4 days in the presence of anti-mouse CD3/CD28 antibodies (e-bioscience). T cell proliferation was examined using FACS analysis

Statistical analysis

Student's t-test or One-way ANOVA was used to determine statistical significance between groups using the GraphPad Prism Software (GraphPad, La Jolla, California, USA). P-value of less than 0.05 was considered to indicate statistical significance

RESULTS

Endogenous PGE_2 production is required for Arg1, IL10 and Mrc1 gene expression of macrophage.

In a preliminary study, we found that endogenous cyclooxygenase 1 and 2 are elevated throughout M-CSF grown macrophage differentiation. We therefore hypothesized that differentiating macrophages produce PGE_2 endogenously. To confirm macrophage mediated PGE_2 production during naïve differentiation, we serially collected culture supernatants after bone marrow cell seeding at differentiation days 1, 3, 5 and 7. As expected, macrophages produced PGE_2 continuously during differentiation (Fig. 1A). To test if PGE_2 production is mediated by endogenous cyclooxygenases, we treated the cells with indomethacin throughout the culture period. PGE_2 production rates increased following maturation and indomethacin significantly reduced PGE_2 synthesis in macrophages. We then examined C/EBPβ as well as several anti-inflammatory C/EBPβ target gene expressions after indomethacin treatment in macrophages to see the effects of reduced PGE_2 synthesis. Unless indicated, we used bone marrow cells at differentiation day 3 under M-CSF to reveal the effects of PGE_2 during macrophage differentiation more clearly. At this stage, F4/80 positive myeloid cell population is approximately half of that at more mature stage (Supplementary Figure S1). Surprisingly, cyclooxygenase inhibition by indomethacin rapidly decreased C/EBPβ gene expression from 30 min, reaching 10% of control after 8 hr of indomethacin treatment (Fig. 1B). This suggests that endogenous C/EBPβ production is mostly derived from the effect of microenvironmental PGE_2 on differentiating macrophages. C/EBPβ target genes showed similar results. Arg1 gene expression decreased 12 hr after cyclooxygenase inhibition (Fig. 1C). IL10 and Mrc1 gene expression levels were almost abolished 8 hr after indomethacin treatment (Fig. 1D, E), indicating that these genes are directly induced by cyclooxygenase produced during macrophage differentiation. Taken together, cyclooxygenase mediates endogenous PGE_2 production, and therefore C/EBPβ, Arg I, IL10 and Mrc1

gene expressions in macrophages.

PGE2 phosphorylates CREB and increases C/EBPβ.

To confirm the exact mechanism of PGE_2 mediated anti-inflammatory gene expressions, we examined time dependent CREB phosphorylation after PGE_2 treatment in macrophages. As expected, 10 μM concentration of PGE_2 resulted in phosphorylated CREB starting at 10 min after treatment, which peaked at 30 min followed by a gradual decrease (Fig. 2A). To confirm the relationship of PGE_2 and C/EBPβ in macrophages, we next examined whether PGE_2 could increase C/EBPβ expression level by Western blotting. PGE_2 clearly increased C/EBPβ protein expressions from 4 hr to 12 hr of treatment (Fig. 2B). This result matched with mRNA expression, which showed increased C/EBPβ mRNA levels starting from 2 hr of PGE_2 treatment (Fig. 2C). J774A.1 cells showed similar results but C/EBPβ mRNA synthesis was detected much earlier after PGE_2 treatment (Fig. 2D). These results show that PGE_2 increases C/EBPβ through CREB phosphorylation in macrophages.

PGE_2 activates CREB through EP4 and EP2 receptors.

Before investigating the exact signaling pathway of PGE_2 in macrophages, we examined EP 1~4 receptor expressions in BMDMs from Balb/c mice. The two G_s-coupled receptors, EP2 and EP4, signal downstream to the adenylate cyclase-triggered cAMP/PKA/CREB pathway, which mediates the anti-inflammatory and suppressive activities of PGE_2 (17,18). In contrast, low-affinity EP1 and high-affinity EP3 are not coupled to G_s and lack cAMP-activating functions (19). Unexpectedly, FACS analysis showed that EP3 is the most abundant EP receptor, with EP4 second (Fig. 3A). EP1 and EP2 expressions were similar and detectable. Macrophages from C57BL/6 mice also showed the same results (Supplementary Figure S2). To examine which EP receptor is related with C/EBPβ upregulation following PGE_2, EP agonists and antagonists were used (Fig. 3B). EP1/3 agonist Sulprostone did not phosphorylate CREB. On the other hand, EP2 agonist Butaprost and EP4 agonist CAY10598 strongly phosphorylated CREB in macrophages. EP2 and

EP4 agonists showed dose dependent CREB phosphorylation (unpublished observations) and the EP4 agonist was more potent. Accordingly, C/EBPβ was upregulated only in EP2 and EP4 agonist treated macrophages and the EP4 agonist was also the most potent C/EBPβ inducer. Next we pre-treated macrophages with EP antagonists 30 min before PGE_2 treatment and analyzed CREB phosphorylation levels. Paralleling agonist results, EP2 antagonist AH-6809 and EP4 antagonist AH-23848 inhibited PGE_2 mediated CREB phosphorylation as well as C/EBPβ upregulation. EP1 antagonist SC-51089 and EP3 antagonist L-798,106 could not abrogate PGE_2 mediated CREB phosphorylation or C/EBPβ upregulation. These results indicate that PGE_2 activates CREB and increases C/EBPβ mainly through EP4 and, to a lesser degree, EP2 receptors, in macrophages.

PGE_2 activates CREB through the cAMP/PKA signaling pathway.

To confirm whether PGE_2 increases C/EBPβ through the cAMP/PKA signaling pathway in macrophages, we treated adenylyl cyclase activator forskolin (10 μM) and checked CREB phosphorylation and C/EBPβ upregulation (Fig. 4A). PKA inhibitor H-89 abrogated both the PGE_2 and forskolin effects on CREB phosphorylation as well as C/EBPβ upregulation. These results confirmed that PGE_2 uses the typical cAMP/PKA pathway to increase C/EBPβ in macrophages. Next, we examined direct CREB involvement in transcriptionally upregulated C/EBPβ (Fig. 4B, C). We overexpressed CREB, a dominant negative mutant KCREB that can be phosphorylated at serine 133 but cannot enter the nucleus, and the CREB133 vector which cannot be phosphorylated at serine 133 in RAW264.7 cells. After 24 hr post-transfection, PGE_2 was added and phospho-CREB was detected by Western blot. Wild-type CREB overexpressed cells showed increased total CREB proteins as well as phosphorylated CREB (Fig. 4B). However, increased CREB expression did not further upregulate C/EBPβ induced by PGE_2 compared with empty vector control, possibly because endogenous CREB levels already saturate C/EBPβ CRE binding sites. In contrast, KCREB and CREB133 vector increased total CREB, but KCREB

did not increase phosphorylation while CREB133 abolished phosphorylation. These mutant CREB overexpressions diminished C/EBPβ upregulation by PGE_2. CHIP PCR of C/EBPβ CRE after CREB pull down showed enhanced C/EBPβ promoter binding of CREB with PGE_2 treatment but wildtype CREB overexpression could not further increase CRE binding as in Fig. 4B (Fig. 4C). However, mutant CREB overexpressed groups showed decreased CRE binding indicating that CREB directly induces C/EBPβ transcription after PGE_2 treatment. Together, these results indicate that PGE_2 upregulates C/EBPβ in macrophages through cAMP/PKA activation and CREB phosphorylation is followed by C/EBPβ promoter CRE binding by activated CREB.

PGE_2 induces Arg1, IL10, MRC1 gene expression through C/EBPβ.

Because C/EBPβ is known to induce several M2 macrophage markers (20), we examined whether PGE_2 increased Arg1, IL10 and Mrc1 gene expressions via C/EBPβ induction. Macrophages were transfected with scrambled control siRNA or C/EBPβ siRNA for 24 hr. First, we confirmed that PGE_2 upregulates C/EBPβ in scramble siRNA transfected macrophages but not in C/EBPβ knockdown macrophages (Fig. 5A). C/EBPβ siRNA efficiency was confirmed by Western blot (Fig. 5B). Next we investigated ArgI (Fig. 5C), IL10 (Fig. 5D) and Mrc1 (Fig. 5E) gene expressions in both groups after PGE_2 treatment. Arginase 1 mRNA level was increased up to 13 times compared to control macrophages 8 hr after PGE_2 treatment, but was increased only 3 times in the C/EBPβ knockdown macrophages (Fig. 5C). IL-10 mRNA level was increased by more than 60 times 12 hr after PGE_2 treatment in control cells and only 3 times in the C/EBPβ knockdown macrophages (Fig. 5D). Macrophage mannose receptor Mrc1 gene expression was increased more than 110 times at 12 hr after PGE_2 in control macrophages, but increased only 10 times in the C/EBPβ knockdown cells (Fig. 5E). These results support our hypothesis that PGE_2 potentiates anti-inflammatory phenotype of macrophage through C/EBPβ upregulation and Arg1, IL10, Mrc1 gene expressions.

Cyclooxygenases and C/EBPβ in macrophages suppress T-cell proliferation.

Finally we investigated the functional implication of macrophage endogenous PGE_2 production on T cell proliferation. Macrophage Arginase 1 suppresses T cell proliferation by depleting local arginine which is needed for T cell proliferation (21). IL-10 can also suppress T cell activation (22). Because we observed PGE_2 mediated Arg1 and IL10 gene transcription, we next examined the functional implication of these effects on T cell suppression. Syngeneic splenic $CD4^+$ T cells were isolated and labeled with CFSE dye before co-culture with macrophages. Anti-CD3 and anti-CD28 antibodies were applied to T cells to measure proliferation. Macrophages were attached to the upper well of transwell plates and co-cultured with T cells for 4 days. Highly proliferating T cells ($CFSE^{low}$ cells) were gated for our control experiment (Fig. 6A). We first attempted direct PGE_2 treatment of the co-culture environment, but this experimental group was omitted due to the massive inhibition of PGE_2 on T cell proliferation. When C/EBPβ knockdown macrophages were co-cultured with T cells, the $CFSE^{low}$ T cell population increased in a dose dependent manner (Fig. 6B). In accordance with this, when indomethacin was applied for 4 days, the $CFSE^{low}$ T cell population also increased dose dependently. Indomethacin treatment of T cells without macrophage co-culture did not affect $CFSE^{low}$ percentages (data not shown). In summary, endogenous C/EBPβ and cyclooxygenase derived PGE_2 in macrophages have roles on inhibition of T cell proliferation.

Table 1. Primer sequences used in this study.

Target gene		sequence
CEBPβ	Forward	5'-CGT TTC GGG ACT TGA TGC AAT C-3’
	Reverse	5'-CAA CAA CCC CGC AGG AAC AT-3'
IL-10	Forward	5'-CCA GGG AGA TCC TTT GAT GA-3'
	Reverse	5'-CAT TCC CAG AGG AAT TGC AT-3'
Mrc-1	Forward	5'-AAT GAA GAT CAC AAG CGC TGC-3'
	Reverse	5'-TGA CAC CCA GCG GAA TTT CT-3'
Arginase I	Forward	5’-GTC TGG CAG TTG GAA GCA TCT-3’
	Reverse	5’-GCA TCC ACC CAA ATG ACA CA-3’
	Probe (FAM)	5’-TGG CCA CGC CAG GGT CCA C-3’

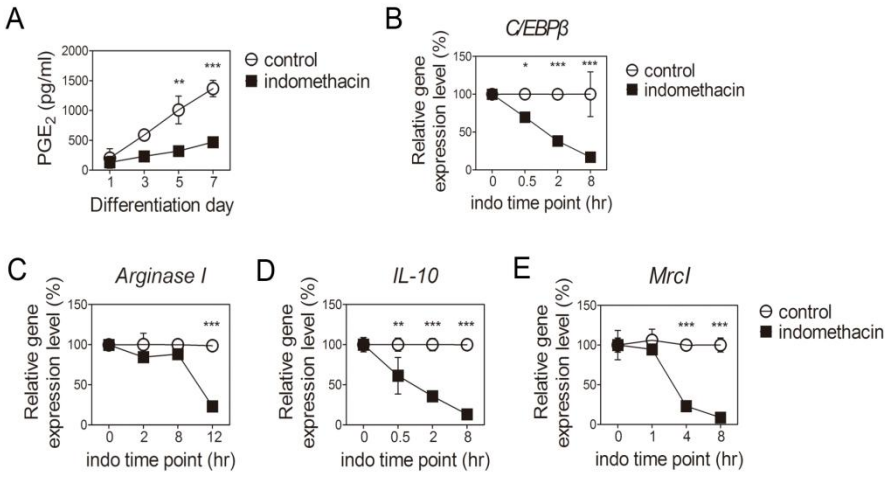

FIGURE 1. Endogenous PGE_2 production is required for Arg1, IL10 and Mrc1 macrophage gene expression. (A) PGE_2 production of bone marrow cell derived macrophages at the indicated differentiation day with M-CSF supplementation in the presence of indomethacin (100 μM) or not. Supernatants were harvested and PGE_2 levels were determined by EIA. (B-E) Macrophages at differentiation day 3 were treated with indomethacin (100 μM) for the indicated time points and RNAs were isolated. Relative mRNA levels of C/EBPβ (B), Arg1 (C), IL10 (D) and mrc1 (E) were measured by quantitative real-time PCR and normalized by GAPDH. Error bars, SD, *, $p<0.05$, **, $p<0.005$, ***, $p<0.001$ analyzed by one-way ANOVA with Bonferroni correction, n=3. Representative of three independent experiments.

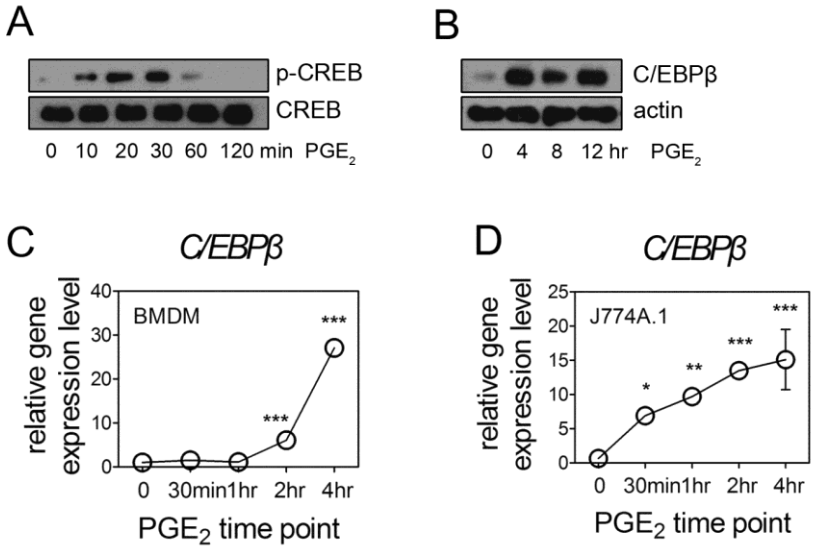

FIGURE 2. PGE_2 phosphorylates CREB and increases C/EBPβ expression. (A and B) Bone marrow derived macrophages (BMDMs) were treated with 10 μM of PGE_2 and examined at the indicated time points. Phospho-CREB (p-CREB), C/EBPβ and actin expressions were detected by Western blotting. (C and D) Time course of C/EBPβ mRNA expression in BMDMs (C) and J774A.1 macrophage cell lines (D) after PGE_2 (10 μM) treatment. Data represents relative fold changes of PGE_2-treated cells compared with control, after normalization with endogenous GAPDH control. Error bars, SD, **, $p<0.005$, ***, $p<0.001$ analyzed by one-way ANOVA with Bonferroni correction, n=3. Representative of three independent experiments.

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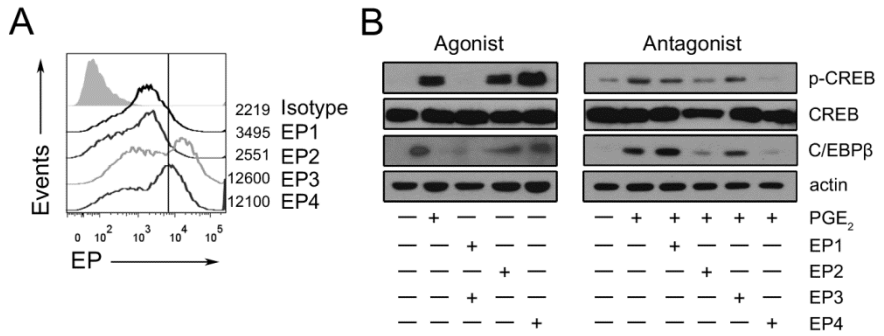


FIGURE 3. PGE_2 activates CREB through EP4 and EP2 receptors. (A) EP receptor expression in BMDMs. Macrophages were fixed and st ained with EP1~4 antibodies followed by rabbit Ax488 secondary antibo dy. Histograms were obtained by Flow cytometry. Mean fluorescence in tensities of each EPs are indicated on right of histograms. (B) BMDMs were treated with PGE_2 (10 μM) for 30 min or the EP receptor agoni sts sulprostone (EP1/3 agonist, 10 μM), butaprost (EP2 agonist, 50 μM) and CAY10598 (EP4 agonist, 10 μM) for 1 hr. Cells were pre-treated with EP receptor antagonist SC-51089 (EP1 antagonist, 10 μM), AH-6 809 (EP2 antagonist, 10 μM), L-798,106 (EP3 antagonist, 10μM) and A H-23848 (EP4 antagonist, 10 μM) for 30 min before PGE_2 (10μM) tre atment, and then phospho-CREB and C/EBPβ were detected by Western Blot

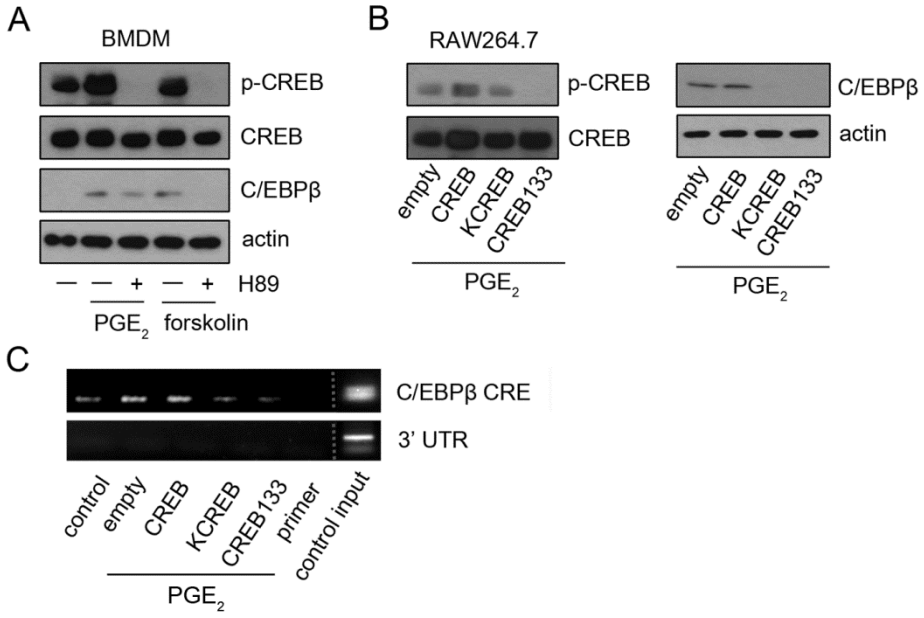


FIGURE 4. PGE_2 activates CREB through the cAMP/PKA signaling pathway. (A) BMDMs were treated with PGE_2 (10 μM) or cAMP acti vator forskolin (10 μM) for 30min (CREB) or 2 hr (C/EBPβ) after pret reatment with PKA inhibitor H-89 (40 μM). Expression of phospho-CR EB, CREB, C/EBPβ and actin were detected by Western blot. (B and C) Raw264.7 cells were transfected with wild-type CREB (CREB Vecto r), dominant-negative CREB (KCREB) or mutant CREB (CREB133) vec tors. After 24 hr, cells were treated with PGE_2 (10 μM) for 30min (p-CREB) or 4 hr (C/EBPβ). (B) Expression of phospho-CREB and C/EB Pβ were detected by Western Blot. (C) ChIP of CREB on the C/EBPβ promoter in Raw 264.7 macrophages. PCR specific for a 140-bp DNA fragment that spans the CRE elements in the C/EBPβ promoter was per formed. Amplification of a 200-bp fragment in the 3'UTR was used as a control.

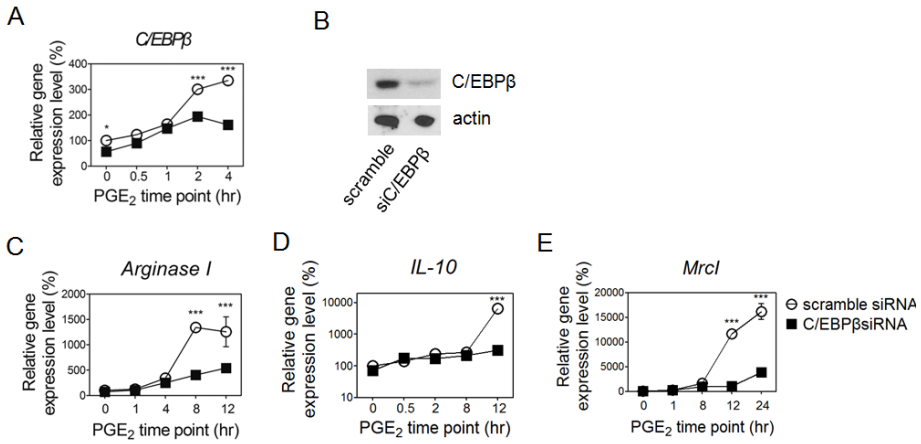


FIGURE 5. PGE_2 induces Arg1, IL10 and Mrc1 macrophage gene expression through C/EBPβ. J774A.1 cells were transfected with scrambled control siRNA or C/EBPβ siRNA. After 24 hr, cells were treated with PGE_2 (10 μM) and RNAs were isolated at the indicated time points. Relative mRNA levels of C/EBPβ (A), Arginase I (C), IL-10 (D) and mrc1 (E) were measured by quantitative real-time PCR. Macrophages were quantified using the relative CT method after endogenous GAPDH normalization. (B) C/EBPβ protein reduction by siRNA transduction was analyzed by Western blot. Error bars, SD, *, $p<0.05$, ***, $p<0.001$ analyzed by one-way ANOVA with Bonferroni correction, n=3. Representative of three independent experiments.

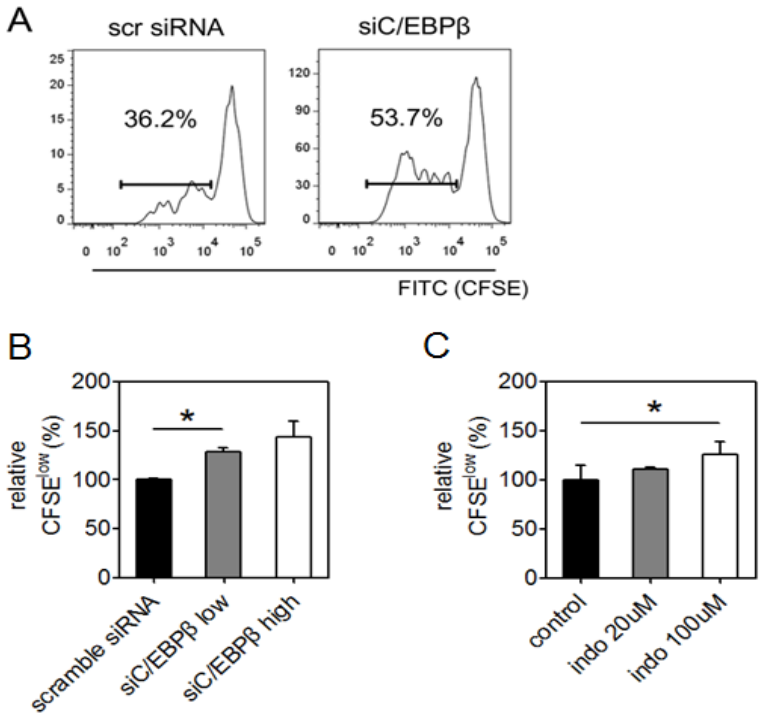


FIGURE 6. Endogenous cyclooxygenase and C/EBPβ in macrophages suppress T-cell proliferation. BMDMs were plated in the upper wells of 24-transwell plates at a density of 2 x10^5 cells /well. CD4+ T cells were isolated from Balb/c splenocytes and labeled with CFSE before seeding in the bottom well at a density of 2 x10^6 cells /well. T cells and macrophages were co-cultured for 4 days in the presence of anti-mouse CD3/CD28 antibodies. (A) T cell proliferation was analyzed by Flow cytometry. Only 7-AAD negative T cells were included and $CFSE^{low}$ percentages were analyzed. (B) Macrophages were transfected with scrambled control siRNA or C/EBPβ siRNA for 24 hr before co-culture. (C) Macrophages were treated with indomethacin (20 μM or 100 μM) during the co-culture period. Error bars, SD, *, $p<0.05$ analyzed by student t-test, n=3. Representative of three independent experiments.

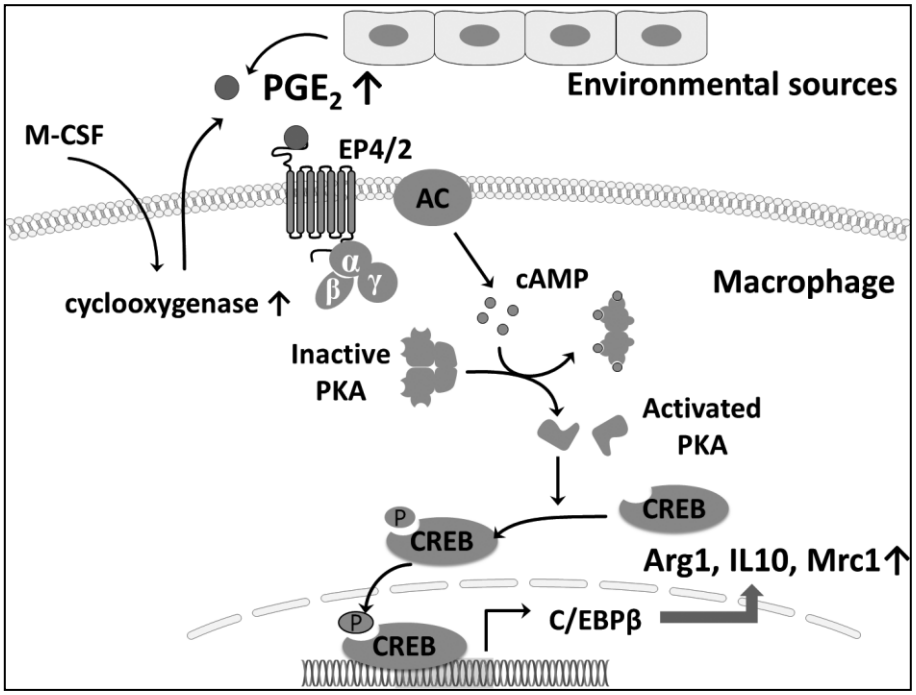
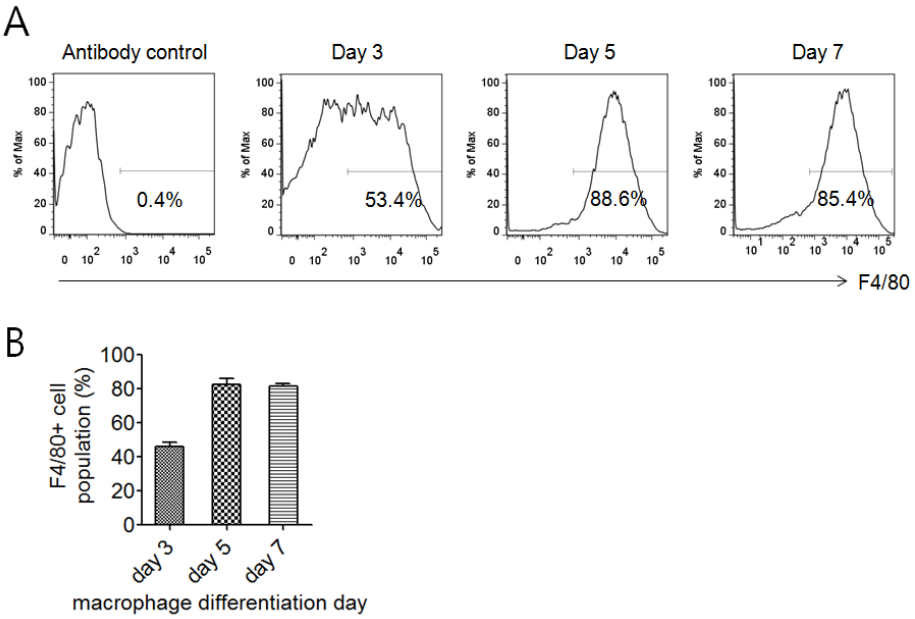


FIGURE 7. Schematic representation of proposed mechanism by which endogenous PGE_2 promotes anti-inflammatory macrophage differentiation through the CREB/CEBPβ cascade. Schematic figure shows the effect of endogenous PGE_2 on macrophage differentiation. M-CSF derived cyclooxygenase induces PGE_2 production in macrophages. Other cell types may also produce PGE_2 in vivo. Signaling by EP2 and EP4 is triggered by PGE_2 binding, and then adenylate cyclase is activated, leading to an increase in intracellular cAMP levels, which in turn activates PKA. Activated PKA then causes phosphorylation of CREB. This leads to transcription of C/EBPβ which promotes Arg1, IL10 and Mrc1 gene expression. Thus, endogenous PGE_2 promotes anti-inflammatory macrophage differentiation through the CREB/CEBPβ cascade.



Supplementary Figure 1. F4/80$^+$ cell populations of differentiating bone marrow derived macrophages. Bone marrow cells from Balb/c mice were differentiated under M-CSF supplementation. At the indicated time points, attaching cells were scrapped and stained with F4/80 antibody followed by analysis using flow cytometry. (A) F4/80$^+$ cell populations are shown in histograms. (B) Graph indicates each F4/80$^+$ population percentages of differentiating macrophages

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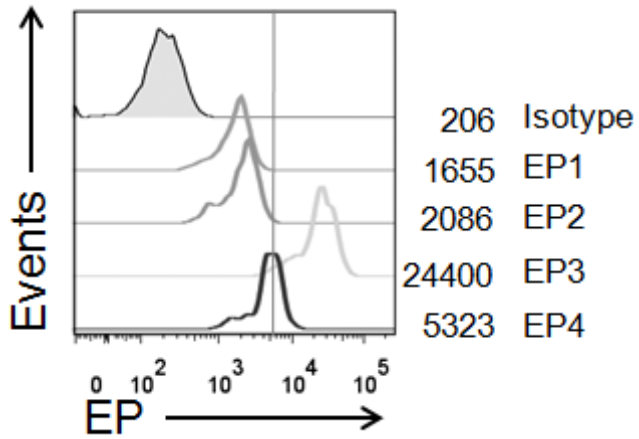
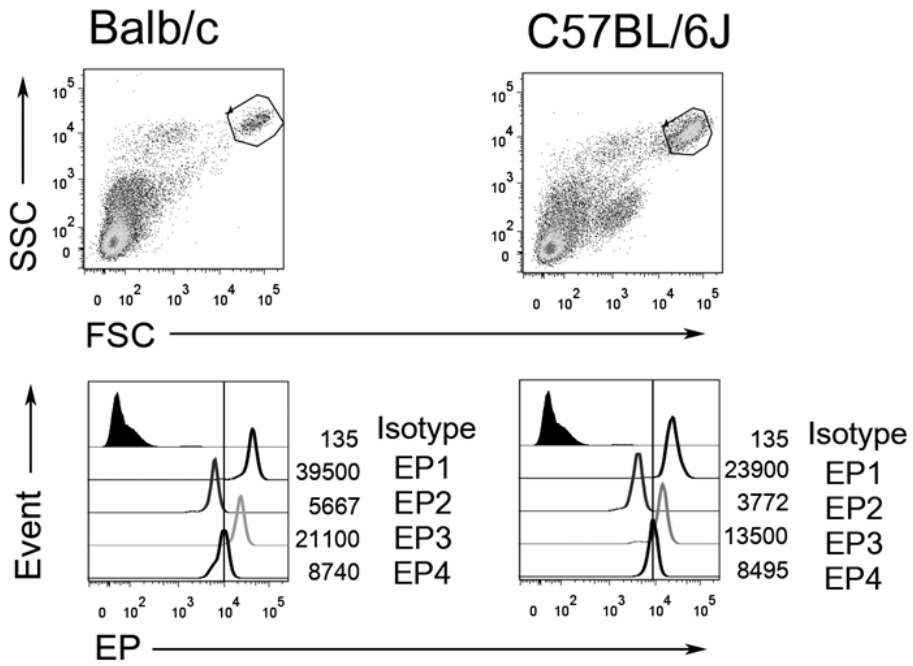


Supplementary Figure 2. EP receptor expression on bone marrow derived macrophages from C57BL/6J. Macrophages at differentiation day 3 were fixed and stained with EP1~4 antibodies followed by rabbit Ax488 secondary antibody. Histograms were obtained by Flow cytometry. Mean fluorescence intensities of each EPs are indicated on right of histograms.



Supplementary Figure 3. EP receptor expression on peritoneal macrophages of Balb/c and C57BL/6J mice. Peritoneal macrophages from Balb/c and C57BL/6J mice were fixed and stained with EP1~4 antibodies followed by rabbit Ax488 secondary antibody as well as F4/80 and CD11b antibodies. Macrophages were gated for F4/80 and CD11b double positive population and analyzed for EP receptor expression. Histograms were obtained by Flow cytometry. Numbers next to the histograms indicate mean fluorescence intensity

DISCUSSION

Macrophage phenotype is an emerging hot issue, because they serve central roles in the pathologies of various diseases including atherosclerosis, diabetes mellitus and cancer. For example, fat deposits progressively polarize macrophage into inflammatory phenotype by means of adipokines, leading to a systemic glucose insensitive state (23). Cancer cell derived M-CSF, CCL2 or PDGF-C lead to the generation and survival of immune-suppressive tumor associated macrophages, which function in angiogenesis and anti-tumor immunity (24, 25). Numerous efforts have been directed at finding key molecules governing macrophage polarization (Akt1/2, CARKLE, NF-kB, IRF5, IRF4, etc) in order to manipulate macrophage phenotypes to overcome related diseases. Here, we demonstrate for the first time that endogenous PGE_2, an essential homeostatic factor, is a key mediator of immune suppressive macrophage phenotype and delineate the specific signals that regulate Arg1, IL10, Mrc1 gene expression. Specifically, M-CSF grown macrophages have an anti-inflammatory phenotype via the endogenously provided PGE_2/CREB-C/EBPβ signaling cascade as illustrated in our schematic representation (Fig. 7).

C/EBPβ expression is abundant in such macrophages as primary bone marrow derived macrophages, J774A.1, and RAW264.7 macrophage-like cell lines. The exact role of C/EBPβ in macrophage differentiation has been examined in a few studies. While macrophage populations of C/EBPβ knockout mice were normal in the blood, spleen, lymph nodes, skin and liver, the peritoneal and alveolar subpopulations were reduced, suggesting that the role of C/EBPβ is tissue specific (26). Another study showed that C/EBPβ deficiency does not affect the number nor the migration of macrophages in muscle (16). It did however affect the activity of macrophages in injury repair, demonstrating that C/EBPβ is also involved in macrophage repair functions, not just macrophage survival or differentiation. Consistent with this, we also observed in this study that C/EBPβ knockdown using siRNA in macrophages undergoing differentiation did not affect survival rates (data not shown).

C/EBPβ was instead specifically involved in macrophage anti-inflammatory phenotype. It regulated Arg1, IL10 and Mrc1 gene expression (Fig. 5). Functionally, macrophage inhibition of T cell proliferation was impaired with lower expression of C/EBPβ (Fig. 6). These results are supported by several reports that demonstrate that E3 ubiquitin ligase neuregulin receptor degradation protein 1 (Nrdp1) promotes M2 macrophage polarization by ubiquitinating C/EBPβ (27). Arginase 1 induction, which thwarts effective immunity against intracellular pathogens (28), was well known target of C/EBPβ (29). These studies and our current study collectively suggest that C/EBPβ is critical in naïve homeostatic macrophage function.

Intriguingly, PGE_2 transcriptionally upregulates C/EBPβ in macrophages (Fig. 2). To our knowledge, this is the first report demonstrating macrophage C/EBPβ protein level regulation by PGE_2. More compellingly, macrophages produce PGE_2 during M-CSF induced differentiation. Endogenous PGE_2 clearly autologously affect C/EBPβ gene expression, and subsequent Arg1, IL10 and Mrc1 gene expression in differentiating macrophages (Fig. 5). PGE_2 potentially originates from local stromal cells as well as epithelial cells, which are also possible candidates for affecting macrophage phenotype in vivo (11). Most studies regarding PGE_2 effects on macrophage have focused on mature macrophage functions. For example, PGE_2 is known to inhibit macrophage phagocytosis through EP2/cAMP (30), exert anti-inflammatory effects by reducing pro-inflammatory cytokines and enhances IL-10 (31-34), decreases bacterial killing through NADPH inhibition (35), and enhances cell membrane stability (36). Kerisztian Nemeth et al. underscored the effect of PGE_2 on immune inhibition in a systemic sepsis model when they proved that bone marrow stromal cells attenuate sepsis via PGE_2-dependent reprogramming of host macrophages to increase their IL-10 production (37). Only one study thus far has investigated the effect of PGE_2 on macrophage maturation (38). They found that EP2 was the most abundant PGE_2 receptor expressed by bone marrow derived macrophages and that PGE_2 restrains macrophage maturation via EP2/PKA signaling as determined by decreased F4/80 expression in EP2 deficient mice. In this context, our study conflicts with some of these results.

First of all, we found that the most abundant EP receptors on murine macrophages are EP3 and EP4 (Fig. 3, Supplementary Fig. S2), not EP2. Zbigniew Zaslona *et al.* examined EP expression in macrophages from C57BL/7 by mRNA levels, but our method using antibody labeling and FACS analysis is arguably more reliable in determining actual EP receptor surface expression. Furthermore, peritoneal macrophages from both Balb/c and C57BL/6 mice express more EP4 than EP2 (Supplementary Fig. S3). EP4 is also more potent for PGE_2 positive signaling than EP2, thus EP4 can partially counter EP2 deficiency. Another point is that F4/80 is not a reliable marker for macrophage maturation. GM-CSF grown mature macrophages express lower levels of F4/80 than M-CSF grown macrophages, indicating that its expression varies depending on macrophage phenotype. Our data supports that endogenous PGE_2 directs macrophage to the anti-inflammatory phenotype via EP2 and EP4 (primarily E4).

These findings mechanistically agree with the association of PGE_2 with the CREB-C/EBPβ cascade given that EP2 and EP4 are coupled to a stimulatory G protein α subunit (Gαs) (13). Ligand binding results in the exchange of GDP for GTP on the Gαs protein and its subsequent dissociation from the βγ subunit complex (39). The free Gαs subunit stimulates the enzyme adenylyl cyclase (AC) to catalyze the cyclization of ATP to generate cAMP. cAMP dependent PKA activation phosphorylates CREB (40) and transcriptionally induces C/EBPβ. Our results clearly show that AC activator forskolin phosphorylated CREB and enhanced C/EBPβ. In addition, PKA inhibitor H89 inhibited CREB phosphorylation and decreased C/EBPβ, and serine 133 mutant CREB could not bind C/EBPβ CRE sufficiently (Fig. 4). Given that C/EBPβ knockdown efficiency was incomplete (Fig. 5), it is understandable that Arg1 and IL10 were only partially inhibited following PGE_2 activation. Indomethacin on the other hand decreased Arg1, IL10, Mrc1 genes more than C/EBPβ knockdown (Fig. 1). There are several possible reasons for these differences. PKA is known to activate p105 (34) and salt inducible kinase 2 (31). PGE_2 is thus likely able to alternatively induce IL10 gene expression aside from the CREB-C/EBPβ cascade. CREB alone can also induce IL-10

without CEBPβ activity (41), demonstrating the insufficiency of C/EBPβ knockdown alone to completely inhibit Arg1, IL10, Mrc1 gene expressions.

In a related study, we recently investigated long term COX inhibition mediated macrophage M1 polarization (under revision). This study adds another mechanistic level by showing that COX mediated PGE_2 production primarily differentiates macrophage to the anti-inflammatory phenotype. Besides macrophages, PGE_2 affects various other types of immune cells including NK cells (42, 43) and T cells (44). We also observed that long term COX-2 inhibitor treatment enhanced macrophage anti-tumor immunity and reduced regulatory T cell populations in breast tumor microenvironment (45). In concert with these studies, endogenous PGE_2 effects on differentiating macrophage shown in this study supports the general immune inhibitory function of PGE_2. This action is mainly mediated by the EP4/PKA/CREB-C/EBPβ cascade. Together, our study has important implications for future efforts examining macrophage phenotypes in various PGE_2 altered pathological states.

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국문 초록

큰포식세포는 대부분의 장기에 상주하며 조직의 항상성 유지에 관여한다. 이러한 역할을 수행할 수 있는 것은 큰포식세포가 주위 환경에 민감하게 반응하여 자신의 특성을 변화시킬 수 있기 때문이며, 장기에 존재하는 큰포식세포가 각기 고유한 특징을 갖는 것 또한 장기마다의 미세 환경이 다르기 때문이다. 시기 적절한 큰포식세포의 활동이 제대로 이루어지지 못할 경우 만성 감염이나 조직의 손상 등의 병리학적 결과로 이어질 수 있어, 큰포식세포의 성격을 결정하는 인자의 규명을 위한 연구가 활발하게 진행되고 있다. 그러나 큰포식세포의 미세환경에 대한 영향과 단핵구로부터의 분화기전에 대한 이해는 의외로 부족한 실정이다. 따라서 본 연구에서, 연구자는 체내 항상성 유지에 관여하는 중요한 지질매개인자인 prostaglandin E_2(PGE_2)가 큰포식세포를 항염증의 성격을 갖도록 분화시킨다는 가정 하에 연구를 진행하였다. 그 결과, 단핵구 유래 큰포식세포가 PGE_2를 생산하고 분비하며, PGE_2가 M-CSF에 의해 큰포식세포로 분화하는 과정에 항염증성 큰포식세포의 마커 발현을 촉진시키는 것을 발견하였다. 또한 PGE_2는 Balb/c 마우스 골수 유래 큰포식세포에서 PGE_2수용체인 EP2 와 EP4를 활성화시켜 하위 신호전달과정에서 전사인자인 CREB의 인산화를 야기하였으며 이는 전사적으로 항염증성 큰포식세포와 관련한 전사인자인 C/EBPβ의 발현을 증가시켰다. 더 나아가 PGE_2가 이러한 CREB-C/EBPβcascade를 통해 arg1, IL10 그리고 Mrc1을 포함한 항염증성 큰포식세포 유전자 발현을 증가시킴을 확인하였다. 그리고 cyclic AMP를 활성화시키는 forskolin을 처리하였을 때 CREB이 인산화되어 C/EBPβ cascade 가 유도되었으며 반대로 PKA 억제제인 H89를 처리하였을 때 완전하게 CREB 활성화가

저해되었다. 또한, NSAIDs의 일종인 indomethacin에 의해 COX activity를 저해하면 PGE_2 분비량이 감소함과 동시에 항염증성 큰포식세포의 유전자 발현이 감소하는 것을 확인하였다. 종합해보면, 본 연구자는 이번 연구를 통해 큰포식세포가 M-CSF에 의한 분화 과정 중 PGE_2를 합성 분비하고, 여기서 분비된 PGE_2는 CREB-C/EBPβ cascade 활성화로 이어지는 신호전달과정으로 항염증성 큰포식세포의 분화를 촉진할 수 있다는 것을 확인하였다.

주요어 : Prostaglandin E_2 (PGE_2), 항염증성 큰포식세포, CREB, C/EBPβ

학 번 : 2013-21741