이학박사 학위논문

Role of platelet-activating factor and prostaglandin E2 signals in immunological characteristics of dendritic cells

수지상세포의 면역학적 특성에서 혈소판 활성화인자와 프로스타글란딘 E2 신호의 역할

2015 년 2 월

서울대학교 대학원

치의학과 면역 및 분자 미생물 전공

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Role of platelet-activating factor and prostaglandin E2 signals in immunological characteristics of dendritic cells

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A Thesis Submitted in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

February 2015

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ABSTRACT

Role of platelet-activating factor and prostaglandin E2 signals in immunological characteristics of dendritic cells

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Objectives

Dendritic cells (DCs) are professional antigen-presenting cells bridging innate and adaptive immunities. DCs are heterogeneous cell populations which conduct diverse functions in the regulation of immunological activation and tolerance. The immunological characteristics of DCs are distinctively determined depending on internal and external factors such as their origin, anatomical location, and local microenvironment. Among such factors, oxidized phospholipid products are known to regulate the differentiation and activation of DCs. Platelet-activating factor (PAF) and prostaglandin (PG) E2 are the representative inflammatory phospholipid products that are generated during early differentiation phase of DCs. However, little is known about the action mechanism of these lipid molecules in the differentiation, activation, and function of DCs that were investigated in the present study using human monocyte-derived DCs.

Methods

To investigate the role of PAF and PGE2 signals in immunological characteristics of DCs, $CD14^+$ monocytes isolated from peripheral blood mononuclear cells (PBMCs) were differentiated into immature DCs in the presence of antagonists or agonists for PAF receptor (PAFR), E-prostanoid receptor 2 (EP2), or EP4. The immature DCs were stimulated with heat-killed *Streptococcus pneumoniae* (HKSP) to examine their phenotypic maturation and cytokine production. Expression of co-stimulatory molecules, MHC class II, programmed death-ligand (PD-L) on the DCs was analyzed by flow cytometry. Amount of IL-12p70, TNF-α, and IL-10 in the culture media of DCs was measured by enzyme-linked immunosorbent assay (ELISA). To examine T lymphocyte-activating capacity of the DCs, unstimulated or HKSP-stimulated DCs were co-cultured with autologous $CD3^+$ T lymphocytes, and then proliferation, activation, and differentiation of T lymphocytes were analyzed by flow cytometry.

To ascertain the physiological relevance of PGE2 to the immunological characteristics of DCs, immature DCs were differentiated in the presence of umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) which constitutively produce PGE2. Separately, immature DCs were differentiated from UCB in which PGE2 is sustained in high levels. The immature DCs were stimulated with either heat-killed *Escherichia coli* (HKEC) or lipopolysaccharide (LPS) from *E. coli* to examine their maturation, cytokine production, and T lymphocyte-activating capacities. Expression of co-stimulatory molecules, MHC proteins, and PD-L on the DCs was analyzed by flow cytometry. Production of IL-12p70, TNF-α, and IL-10 by the DCs was analyzed by ELISA. To examine DC-mediated activation of T lymphocytes, unstimulated or HKEC-stimulated DCs were co-cultured with autologous T lymphocytes, and then the proliferation, activation marker expression,

and cytokine production of the T cells were analyzed by flow cytometry.

Results

DCs differentiated in the presence of PAFR antagonist, CV6209 (CV6209-DCs), showed decreased expression of CD1a, CD80, and PD-L1, but increased expression of CD86 and CD14 in comparison with those of DCs differentiated without CV6209 (control DCs). Phagocytic capacity of CV6209-DCs against HKSP were higher than that of control-DCs. In response to the stimulation with HKSP, CV6209-DCs weakly induced co-stimulatory molecules, MHC class II, and PD-Ls. In addition, CV6209-DC negligibly produced IL-12p70, TNF-α, and IL-10. When CV6209-DCs were co-cultured with autologous $CD3^+$ T lymphocytes, they weakly induced proliferation, activation marker expression, and cytokine production of the T lymphocytes compared to control DCs. Furthermore, CV6209-DCs preferentially induced differentiation of IL-10^+TGF-β^+ regulatory T lymphocytes. These results suggest that PAFR signaling is essential for the differentiation of immunogenic DCs which have typical stimulatory phenotypes and functions.

DCs differentiated in the presence of EP4 antagonist exhibited markedly attenuated expression of co-stimulatory molecules and IL-12p70 in responses to HKSP. When the DCs were co-cultured with autologous T lymphocytes, they weakly induced proliferation and activation of the T lymphocytes. However, significant differences in phenotypes and functions were not observed in DCs differentiated in the presence of EP2 antagonist in comparison with those of control DCs. Low doses of PGE2 preferentially activating EP4 potentiated expression of co-stimulatory molecules, production of IL-12, and T lymphocyte-activating capacity of the DCs. In contrast, high doses of PGE2 activating both EP2 and EP4 enhanced tolerogenic properties of DCs, weak induction of maturation markers, negligible production of IL-12, and

low T lymphocyte-activating capacity. Using the similar mechanism, UCB-MSCs

differently regulated the immunological characteristics of DCs by distinctively activating EP2 and EP4 signaling. Small number of UCB-MSCs activating EP4 enhanced stimulatory functions of DCs, whereas large number of UCB-MSC potentiated tolerogenic properties of DCs by activating EP2 signaling. These results indicate that PGE2 differently regulates immunological characteristics of DCs by distinctively activating its two different receptors.

UCB-DCs contained less $CD1a^+$ DCs than APB-DCs. UCB-DCs exhibited lower expression of CD80, MHC class I and II, and DC-SIGN, but higher endocytic activity, than APB-DCs. UCB-DCs stimulated with LPS weakly augmented the expression of maturation markers and production of IL-12 and TNF-α but potently expressed IL-10. When UCB-DCs were co-cultured with $CD14^+$ cell-depleted allogeneic PBMCs, they weakly induced the proliferation, surface expression of activation markers, and IFN-γ production of T lymphocytes compared with APB-DCs. UCB contained higher levels of PGE2 than APB, which might be responsible for tolerogenic phenotypes and functions of UCB-DCs. Indeed, APB-DCs prepared in the presence of PGE2 exhibited $CD1a^-CD14^+$ phenotypes with tolerogenic properties including weak maturation, impaired IL-12 production, and negligible T lymphocyte activation as UCB-DCs did. Taken together, UCB-DCs are suggested to have tolerogenic properties, which might be due to PGE2 highly sustained in UCB.

Conclusions

Activation of PAFR signal potentiates immunogenic properties of human monocytes-derived DCs. PGE2 differently regulates immunogenic and tolerogenic characteristics of DCs by distinctively activating its two different receptors, EP4 and EP2. PGE2 mediates immunomodulatory functions of UCB-MSCs that alters differentiation and activation of DCs. In addition, PGE2 sustained at high concentration in UCB preferentially induces tolerogenic characteristics of UCB-DCs. Conclusively, PAF and PGE2 efficiently mediate immunogenic and tolerogenic properties, respectively, of DCs, contributing to immune homeostasis.

Keyword: Human dendritic cells, Platelet-activating factor, Prostaglandin E2, Maturation of dendritic cells, T lymphocyte activation

Student Number: 2006-23508

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Abbreviations

Ant.EP2-DC	Dendritic cell differentiated in the presence of EP2 antagonist
Ant.EP4-DC	Dendritic cell differentiated in the presence of EP4 antagonist
APB	Adult peripheral blood
APB-DC	Dendritic cell derived from adult peripheral blood
APC	Allophycocyanin
cAMP	Cyclic adenosine monophosphate
CBMC	Cord blood mononuclear cell
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
COX	Cyclooxygenase
CTL	Cytotoxic T lymphocyte
CV6209/NS398-DC	Dendritic cell differentiated in the presence of CV6209 and NS398
CV6209-DC	Dendritic cell differentiated in the presence of CV6209
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular molecule-3-grabbing non-integrin
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
EP	E-prostanoid
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
GM-CSF	Granulocyte macrophage-colony stimulating factor
HKEC	Heat-killed *Escherichia coli*

HKSP	Heat-killed *Streptococcus pneumoniae*
HLA	Human leukocyte antigen
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
JAK2	Janus kinase 2
LB	Luria bertani
M-CSF	Macrophage-colony stimulating factor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
MSC	Mesenchymal stem cell
MSC-DC	Dendritic cell differentiated in the presence of mesenchymal stem cell-derived from umbilical cord blood
NK cell	Natural killer cell
PAF	Platelet-activating factor
PAF-DC	Dendritic cell differentiated in the presence of platelet-activating factor C-16
PAFR	Platelet-activating factor receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-L	Programmed death-ligand
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-Cyanin 5
PGE2	Prostaglandin E2
PGE2/PAF-DC	Dendritic cell differentiated in the presence of prostaglandin E2 and platelet-activating factor
PGE2-DC	Dendritic cell differentiated in the presence of prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A

PKB	Protein kinase B
PKC	Protein kinase C
PLA2	Phospholipase A2
PMA	Phorbol 12-myristate 13-acetate
SD	Standard deviation
SEM	Standard error of the mean
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor-β
TH1	Type 1 helper T lymphocyte
TH2	Type 2 helper T lymphocyte
TH17	Type 17 helper T lymphocyte
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T lymphocyte
UCB	Umbilical cord blood
UCB-DC	Dendritic cell derived from umbilical cord blood
UCB-MSC	Mesenchymal stem cell derived from umbilical cord blood

Chapter I. Introduction

1. Dendritic cells (DCs), regulators of immunological activation and tolerance

1.1. General characteristics of DCs

Dendritic cells (DCs) are one of the best antigen-presenting cells to bridge innate and adaptive immunities. DCs are heterogeneous cell populations that are present in most tissues and play a pivotal role in the clearance of invading pathogens and malignant cells before the initiation of antigen-specific adaptive immune responses. Although DCs differ in their origins, anatomical localization, phenotypes, and functions [1], most of the DC subtypes take up antigens and present them to naïve T lymphocytes. Maturation of DCs is an important process in the mediation of adaptive immune responses. In normal condition, DCs exist in an immature state. Immature DCs are characterized by high endocytic capacity, but low expression levels of co-stimulatory molecules, MHC proteins, and cytokines [2]. Recognizing microbial components or apoptotic cells through pattern-recognition receptors, DCs change phenotypes and functions to optimize themselves for stimulating naïve T lymphocytes. DCs up-regulate the expression of co-stimulatory molecules, MHC proteins, and cytokines [3]. Mature DCs migrate to T lymphocyte regions in draining lymph nodes and efficiently activate antigen-specific T effector cells by (i) presenting MHC-associated antigens to naïve T lymphocytes, (ii) providing co-stimulatory signals via CD80, CD86 and CD40, and (iii) creating a specific local environmental milieu by producing various cytokines such as interleukin (IL)-12, IL-6, and IL-10 [4]. Through these cellular processes, DCs mediate antigen-specific immunity or tolerance.

1.2. Immunological properties of immunogenic DCs and tolerogenic DCs

DCs can be functionally divided into two major subtypes. One is an immunogenic DC and the other is a tolerogenic DC. Immunogenic DCs are fully matured DCs that efficiently stimulate innate and adaptive immune responses. Expressing high levels of co-stimulatory molecules, MHC proteins, and inflammatory cytokines, the immunogenic DCs can preferentially induce differentiation of effector T lymphocytes [2]. In contrast, tolerogenic DCs display favorable expression of co-inhibitory molecules such as programmed death-ligand 1 (PD-L1) and PD-L2 [5], and production of anti-inflammatory cytokines including IL-10 and transforming growth factor (TGF)-β, which create immunosuppressive microenvironment prone to elicit regulatory T lymphocytes (Treg) [6, 7]. Tolerogenic DCs control excessive immune responses by attenuating differentiation and activation of immune cells including inflammatory macrophages and T lymphocytes [8]. Indeed, deficiency and/or malfunction of tolerogenic DCs have been implicated in the progression of uncontrolled inflammatory disorders such as autoimmune diseases and allergic diseases [9, 10].

2. Immunomodulating factors for differentiation and activation of DCs

2.1. Soluble factors

DCs developed at different conditions exhibit distinctive immunological features in their differentiation, activation, and function. Soluble mediators including cytokines, chemokines, and lipid metabolites importantly mediate these responses of DCs. Expression of the soluble mediators affecting DC properties are distinctively

regulated depending on the anatomical location and several extrinsic factors such as microbial infection [11]. Of the soluble mediators, granulocyte macrophage-colony stimulating factor (GM-CSF) is an essential cytokine for differentiation of inflammatory DCs which are predominantly induced at infections. Invading pathogens and the microbial components stimulate epithelial cells in the infected tissues to produce inflammatory cytokines such as GM-CSF and tumor necrosis factor (TNF)-α [12], which promote differentiation of the recruited DC progenitors into DCs. Indeed, combined stimulation with GM-CSF and IL-4 markedly differentiates IL-12-producing DCs and mediates induction of type 1 helper T lymphocytes (TH1) [13]. Furthermore, co-treatment with GM-CSF and IL-15 stimulates DCs to produce type 17 helper T lymphocytes (TH17)-eliciting cytokines such as IL-6, IL-1β, and IL-23 [13]. In contrast, IL-10 and TGF-β preferentially induce tolerogenic DCs that promote differentiation of Treg [14, 15]. Prostaglandin (PG) E2 and indoleamine 2,3-dioxygenase have also been reported to suppress differentiation of monocytes into immunostimulatory DCs [16, 17].

2.2. Cellular factors

Immunological characteristics of DCs can be determined by cellular interactions. Tissue-supporting stromal cells and epithelial cells provide progenitors of DCs with information about their anatomical locations through cell-to-cell contact manner or soluble factors and induce them to differentiate into tissue-specific DCs [18]. Additionally, some of cancer cells exploit DCs to evade immune surveillance [19] by modulating immunological characteristics of the cells. Multipotent mesenchymal stem cells (MSCs) are also characterized by their immunomodulatory functions. MSCs exhibit immune-evasive phenotypes, lacking co-stimulatory molecules and MHC class II [20, 21]. Furthermore, MSCs constitutively produce immuno-

suppressive mediators such as PGE2, IL-6, and indoleamine 2,3-dioxygenase, inhibiting normal development and immunostimulatory functions of DCs [22]. Indeed, previous studies have demonstrated that hematopoietic stem cells and monocytes exposed to MSCs or their culture media failed to acquire conventional phenotypes and functions of stimulatory DCs, which were mediated by MSC-derived IL-6, macrophage-colony stimulating factor (M-CSF), and/or PGE2 [23, 24]. MSCs also activated Notch signaling in DC progenitors and interfered with their normal differentiation into immunogenic DCs [25].

3. Immunological characteristics of DCs and monocytes from umbilical cord blood (UCB)

3.1. UCB

Umbilical cord is a specialized tissue connecting fetus to its mother. UCB is an important source for investigating immune responses of early life since it contains diverse types of immune cells and progenitors at the developmental stages [26]. Previous reports have shown that the cells derived from UCB exhibited compromised immune responses against allografts or microbes [27], which might be ascribed to the tissue-specific immunosuppressive microenvironment. Indeed, plasma of UCB contains less TH1-promoting cytokines such as IL-12, TNF-α, and IFN-γ, but more IL-6, IL-10, and PGE2 than that of adult peripheral blood (APB) [28]. Probably, the different composition of the plasma components between UCB and APB might be associated with distinct immune responses of neonates and adults. Furthermore, the tolerogenic milieu of UCB contributes to the protection of fetus from pathologic inflammation and maternal adaptive immunity. Nowadays, UCB is considered a promising allogeneic source of stem cells for transplantation [26] due to (i) high frequencies of hematopoietic progenitor cells [26], (ii) a low incidence of

graft versus host disease, and (iii) convenience of accessibility and human leukocyte antigen (HLA) matching. Indeed, UCB transplantation for some of hematopoietic disorders and metabolic diseases in children shows high rates of success [26].

3.2. DCs derived from UCB

It is important to understand immunological characteristics of DCs in early life for enhancing the efficacies of immunization conducted after birth and for clinical use of the fetal sources. DCs derived from UCB (UCB-DCs) exhibit distinctive immunological features from those of APB. UCB-DCs display a low level of co-stimulatory molecules, low responsiveness to microbial components, and low lymphocyte-activating capacity [29, 30]. In addition, UCB-DCs preferentially induce differentiation of Treg rather than effector T lymphocytes [31]. Many researchers have assumed that UCB-DCs with tolerogenic characteristics dampen efficacies of vaccination and increase susceptibilities to infections. In contrast, UCB-DCs with immunosuppressive characteristics alleviate immune rejection following transplantation of UCB [27]. However, the regulatory mechanisms determining immunological properties of UCB-DCs have not yet been understood well.

3.3. Monocytes in UCB

Blood monocytes are commonly used in the preparation of DCs for cell therapy since they can differentiate into diverse subtypes of DCs. Monocytes consist of two populations, $CD14^{+}CD16^{-}$ classical monocytes and $CD14^{+}CD16^{+}$ non-classical monocytes [32]. Non-classical $CD14^{+}CD16^{+}$ monocytes have been known as inflammatory monocytes of which the proportion is significantly increased in the blood of patients with systemic inflammatory diseases such as bacterial sepsis [33].

Interestingly, UCB and APB also exhibit difference in the ratio of $CD14^+CD16^+$ monocytes to $CD14^+CD16^-$ monocytes. UCB displays significantly reduced frequencies in $CD14^+CD16^+$ monocytes [34]. However, it is yet to be elucidated whether the lower proportions of $CD14^+CD16^+$ monocytes in UCB have a correlation with the tolerogenic immune responses of UCB-DCs and what factors are associated with the distinctive immunological features of UCB-DCs.

4. Platelet-activating factor (PAF)

4.1. Physiological functions of PAF

PAF is a representative phospholipid metabolite associated with platelet aggregation, inflammation, and allergic responses. Under physiological conditions, PAF mediates cell-to-cell adhesions, interactions, and signal transduction by autocrine and/or paracrine manner [35]. It is also involved in differentiation and activation of immune cells such as neutrophils, monocytes, and T lymphocytes [36-38]. Additionally, PAF regulates proliferation and apoptosis of tissue-supporting fibroblasts, epithelial cells, and stromal cells [39-41]. At infections, synthesis of PAF is markedly increased by microbial stimulation [42] or inflammatory cytokines such as GM-CSF and IL-1 [43, 44]. PAF facilitates leukocyte recruitment to the inflamed tissues by dilating blood vessels [45] and potentiates stimulatory functions of neutrophils, macrophages, and DCs infiltrated to the infective sites [46], which exacerbate inflammation. As PAF mediates hypotension, thrombosis, and multiple organ failure [45, 47], it has been considered as an etiologic agent of diverse inflammatory disorders including septic shock, necrotizing enterocolitis, autoimmune diseases, and cancer.

4.2. Synthesis and degradation of PAF

PAF is synthesized via two different enzymatic pathways. One is *de novo* pathway and the other is remodeling pathway. In the steady state, PAF is mainly synthesized by *de novo* pathway [48]. PAF-phosphocholinetransferase constitutively expressed in a variety of cell types transfers phosphocholine base group to 1-*O*-alkyl-2-acetyl-*sn*-glycerol and converts the phospholipid into PAF. Basal levels of PAF sustained in the human blood is about 18 ± 5 pg/ml [49], which is required for conducting its physiological functions. Under inflammatory conditions, most of PAF molecules are synthesized by remodeling pathway [48]. Microbial stimulation or inflammatory cytokines up-regulate the expression of cytosolic phospholipase A2 (cPLA2) [50]. This enzyme generates lyso-PAF by cleaving arachidonic acid from *sn*-2 position of phosphatidylcholine, a dominant phospholipid consisting plasma membrane of mammalian cells (40-50%), and then acetyltransferase converts the lyso-PAF into PAF by acetylating the free hydroxyl group in the *sn*-2 position [35]. PAF is an unstable molecule of which the half-life in the human blood is limited to a few minutes [51]. All the PAF molecules are rapidly converted into lyso-PAF by PAF-acetylhydrolase regardless their synthetic pathways.

4.3. PAF receptor and its signaling

PAF exerts physiological functions by binding to its single specific receptor, PAF receptor (PAFR), one of G protein-coupled receptor, which is expressed on diverse cell types including platelet, endothelial cells, granulocytes, monocytes, and lymphocytes [52]. PAFR recognizes an *sn*-1 ether bond, a short *sn*-2 acetyl group, and a phosphocholine head group of PAF or PAF-like lipids [35]. Activation of PAFR induces phosphatidylinositol turnover, raises intracellular calcium level, and activates protein kinase C (PKC) [35]. The rise of cytosolic calcium results in an increase of cPLA2, an enzyme needed for release of arachidonates, suggesting that

PAFR activation entails synthesis of eicosanoids including prostaglandins (PGs), thromboxane, and leukotrienes [53, 54]. In addition, PAFR signaling activates janus kinase 2 (JAK2)-mediated signaling transducers and activators of transcription (STAT) [55, 56] and mitogen-activated protein kinase (MAPK) members [57]. Upon the agonist binding, PAFR is rapidly desensitized by phosphorylation of the intracellular C-terminal region [58], PKC-dependent inactivation of phospholipase C-β3 pathway, a key downstream signal of PAFR [59], or arrestin-dependent internalization [60].

4.4. Roles of PAF in DC-mediated immune responses

PAF modulates immune responses of DCs and their progenitors. A previous study demonstrated that stimulation with PAF promoted human monocyte-derived Langerhans cells to express IL-6, IL-1β, and IL-23, leading to induction of TH17 cells [36]. In addition, PAF was reported to play an important role in the migration of DCs to draining lymph nodes. Epidermal Langerhans cells from PAFR-deficient mice displayed impairment in their migratory ability to lymph nodes [61]. In contrast, PAF seemed to regulate excessive immune activation of DCs by inducing expression of immunosuppressive mediators. Murine bone marrow-derived DCs up-regulated expression of PGE2 and IL-10 by PAFR-dependent manner when they were stimulated with LPS, which negatively controlled expression of co-stimulatory molecules, production of IL-12, and T lymphocyte activating ability of the DCs [62]. However, in an early phase of DC differentiation, the functions of PAF and PAFR signaling have not yet been determined on the differentiation, activation, and immunological functions of human monocyte-derived DCs.

5. PGE2

5.1. Physiological functions of PGE2

PGE2 is the most widely studied lipid mediator among PG isomers. It regulates various physiological processes such as inflammation, fever, pain, mucosal integrity, uterine contraction during parturition, and tumor growth [63-65]. Under inflammatory conditions, PGE2 plays an important role as a vasodilator that facilitates leukocyte influx to the infection sites [65] and exacerbates inflammation by activating the infiltrated granulocytes and macrophages. Recent studies have demonstrated crucial functions of PGE2 on the normal development of immune system and maintenance of immune homeostasis [65]. PGE2 supports survival, homing, and homeostatic proliferation of hematopoietic stem cells [66]. In addition, it contributes to resolution of inflammation by attenuating effector functions of inflammatory immune cells such as neutrophils, mast cells, and macrophages [65]. Furthermore, PGE2 promotes differentiation of immunosuppressive cells including myeloid-derived suppressor cells and Treg, creating immunosuppressive circumstance that tends to develop cancers [67].

5.2. Synthesis and degradation of PGE2

In the human blood, basal levels of PGE2 is around 54 $\pm$ 1 pg/ml [68], which is important for conducting aforementioned physiological functions. However, under inflammatory conditions, an approximate 5-fold increase in the plasma PGE2 levels are observed [68]. PGE2 is synthesized from arachidonic acids liberated from phospholipids in the plasma membrane under both normal and inflammatory

conditions. The free arachidonates are converted into PG precursor, PGH2, by cyclooxygenases (COX), and then PGE2 synthesis is completed by PGE synthase [69]. COX1 is constitutively expressed in most cell types and contributes to immediate synthesis of PGE2 in the steady states, whereas inducible COX2 is involved in the delayed production of PGE2 under inflammatory circumstances [70]. PGE2 is unstable lipid molecule that has rapid turnover rate *in vitro* and *in vivo* conditions because it is quickly degraded into 15-keto PGE2 by 15-hydroxyl PG dehydrogenase [69, 71]. The half-life of PGE2 *in vitro* condition is about 20 minutes, but that of *in vivo* condition is less than 10 minutes [72].

5.3. Receptors for PGE2 and the signaling

PGE2 has complex and multiple effects on the physiological responses of many cell types, which is ascribed to the presence of its four different receptors, E-prostanoid (EP) receptor 1, 2, 3, and 4 [73]. Each of the EP receptors has different affinity, downstream signals, and duration of activation [65]. Among the four types of EP receptors, EP2 and EP4 can bind to $G_s\alpha$ subunit of which the coupling results in the activation of cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling. However, EP4 also activates other intracellular signals including phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) or β-catenin pathways [74] because its long cytoplasmic tail can specifically bind to $G_i\alpha$, which are associated with EP4-specific functions distinct from those of EP2 activation. In addition, EP4 has higher affinity to its ligand (K_i = 7.3 ± 0.4 nM) than EP2 (K_i = 53 ± 1.8 nM) [75]. Upon agonist-induced activation, EP4 is rapidly desensitized by G protein-coupled receptor kinase and/or β–arrestin 1-dependent manner, while the expression of EP2 is sustained for longer period of time [75].

5.4. Roles of PGE2 in DC-mediated immune responses

With respect to the immune responses of DCs, PGE2 shows heterogeneous immunomodulatory effects depending on the differentiation and activation stages of the cells. In the early phases, PGE2 suppresses DC-mediated induction of TH1 and cytotoxic T lymphocytes (CTL) [67]. In line with this, DCs differentiated in the presence of PGE2 exhibits attenuation in the production of IL-12p70 but enhancement in the expression of IL-10, promoting differentiation of TH17 rather than TH1 [17]. In contrast, PGE2 has been used as an activating factor for already developed DCs [76, 77]. Combined treatment with PGE2 and other inflammatory cytokines such as IL-1β and TNF-α efficiently induces phenotypic maturation of DCs [78]. In addition, PGE2 potentiates migratory abilities of DCs toward draining lymph nodes by increasing expression of CCR7 on the cell surface [79]. However, underlying mechanisms by which PGE2 separately regulates DC-mediated immune responses have not yet been understood. Therefore, further studies are needed to clarify the exact roles of PGE2 in the differentiation, activation, and immunological function of DCs.

6. Aim of the present study

The aim of the present study is to investigate role of PAF and PGE2 signals in the immunological characteristics of DCs. Under the research aim, (i) role of PAFR signaling and (ii) role of different PGE2 signals via EP2 and EP4 in the differentiation, activation and function of human monocyte-derived DCs were investigated. Additionally, to examine physiological relevance of PGE2 to the immunological properties of DCs, (iii) immunomodulatory effects of UCB-MSCs constitutively producing PGE2 on the phenotypic and functional characteristics of DCs and (iv) immune responses of DCs derived from UCB in which PGE2 is

sustained in high levels were analyzed.

Chapter II. Materials and Methods

1. Reagents and chemicals

Ficoll–Paque PLUS and HetaSep solution were purchased from GE Healthcare (Uppsala, Sweden) and Stem Cell Technology (Vancouver, Canada), respectively. Keratinocyte-serum free media (K-SFM) and D-media (formula 78-5470EF) were obtained from GIBCO BRL (Grand Island, NY, U.S.A.). Endothelial growth medium (EGM)-2 SingleQuot was purchased from Lonza (Walkersville, MD, U.S.A.). RPMI 1640 and penicillin-streptomycin solution were obtained from HyClone (Logan, UT, U.S.A.). Anti-human CD14 magnetic bead and anti-human CD3 magnetic bead were purchased from BD Biosciences (San Diego, CA, U.S.A.). Trypsin-EDTA and EDTA solution were purchased from GIBCO. Fetal bovine serum (FBS) was obtained from GIBCO or HyClone. Recombinant human GM-CSF was purchased from Peprotech (Rocky Hill, NJ, U.S.A.) or R&D Systems (Minneapolis, MN, U.S.A.). Recombinant human IL-4 was from R&D Systems. 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and FITC-conjugated dextran (MW 40,000) were obtained from Molecular Probes (Eugene, OR, U.S.A.). Dimethyl sulfoxide (DMSO), red blood cell (RBC) lysing buffer, hydrocortisone, N-acetyl-L-cystein, calcium chloride, insulin, ascorbic acid, NS398, phorbol myristate acetate (PMA), ionomycin, brefeldin A, paraformaldehyde, and PGE2 were obtained from Sigma Aldrich Inc. (St. Louis, MO, U.S.A.). PF04418948 and L-161,982 were obtained from Tocris Bioscience (Bristol, UK). CV6209 and PAF C-16 were obtained from Wako (Osaka, Japan) and Enzo Life Science

(Farmingdale, NY, U.S.A.), respectively. Saponin was obtained from Fluka Chemie GmbH (Steinheim, Germany). Ultra-pure LPS from *E. coli* serotype K12 was purchased from Invivogen (San Diego, CA, U.S.A).Difco™ Luria Bertani (LB) broth, BBL™ blood agar base, and Bacto™ Todd Hewitt broth (THB) were purchased from BD Biosciences (Sparks, MD, U.S.A.). Enzyme-linked immunosorbent assay (ELISA) kits for human IL-12p40 and IFN-γ were obtained from R&D Systems. Human IL-12p70, TNF-α, and IL-10 ELISA kits were purchased from BioLegend (San Diego, CA, U.S.A.). PGE2 competitive ELISA kit was from Arbor assays (Ann Arbor, MI). PE-labeled anti-human CD1a antibody, APC-labeled anti-human CD14 antibody, FITC-labeled anti-human CD80 antibody, PE-labeled anti-human CD83 antibody, APC-labeled anti-human CD86 antibody, APC-labeled anti-human PD-L1 antibody, PE-labeled anti-human PD-L2 antibody, PE-labeled DC-SIGN antibody, APC anti-human CD25 antibody, APC-labeled anti-human CD3 antibody, Alexa Fluor® 647-labeled anti-human IFN-γ, Alexa Fluor® 647-labeled anti-human IL-17A, APC-labeled anti-human IL-4 antibody, PE-labeled anti-human TGF-β antibody, APC-labeled anti-human IL-10 antibody, and Alexa Fluor® 647-labeled anti-human FOXP3 antibody were purchased from BioLegend. Alexa Fluor® 647 labeled anti-human CD205 antibody, FITC-labeled anti-human CD206 antibody, FITC-labeled anti-human HLA-DR, DP, DQ antibody for MHC class II and PE-Cy5 anti-human HLA-A, B, C antibody for MHC class I were obtained from BD Biosciences. All isotype-matched antibodies were purchased from BD Biosciences and BioLegend. COX2 (C-20) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A). PE-labeled anti-goat IgG antibody was obtained from Jackson ImmunoResearch (West Grove, PA, U.S.A.).

2. Preparation of heat-killed bacteria

Escherichia. coli BL21 (DE3) was obtained from Stratagene (La Jolla, CA). Single colony of *E. coli* was taken by streaking on LB agar and inoculated in LB broth. Bacteria were cultured in LB broth media at 37°C until mid-log phase and harvested by centrifugation. The bacterial cells were suspended in phosphate-buffered saline (PBS) and killed by heating at 60°C for 1 h. To confirm the complete killing, heat-treated bacteria were plated onto LB agar plates and cultured overnight at 37°C. No bacterial colonies were observed. *Streptococcus pneumoniae* (ATCC6330) was obtained from Americal Type Culture Collection (Manassas, VA, USA). *S. pnemoniae* was streaked on a blood agar plate and single colony of the bacteria was taken and inoculated in THB. The bacteria were cultured at 37°C until mid-log phase and harvested by centrifugation. Bacterial cells were suspended in PBS and killed by heating at 75°C for 1 h. To confirm the complete killing, heat-treated bacteria were plated onto blood agar plates and cultured overnight at 37°C. No bacterial colonies were observed.

3. Carboxyfluorescein succinimidyl ester (CFSE) labeling of bacteria

Heat-killed *E. coli* (HKEC, 1×10^9 CFU/ml) and heat-killed *S. pneumoniae* (HKSP, 1×10^9 CFU/ml) were suspended in 1 ml PBS containing 10 μM CFDA-SE and incubated for 15 min at 37°C to for fluorescent labeling of the bacterial cells. The bacterial suspensions were centrifuged at 2,991 × *g* for 5 min and supernatant was discarded. The bacteria were washed with PBS once, and then re-suspended in PBS to adjust to 1×10^9 CFU/ml.

4. Isolation of UCB-derived mesenchymal stem cells (UCB-MSCs)

RBCs in UCB were removed using HetaSep solution, and then mononuclear cells were isolated by density-gradient centrifugation using Ficoll–Paque PLUS. Mononuclear cells were suspended in D-media containing EGM-2 and 10% FBS at a density 2×10^5 cells/ml and seeded in 6-well cell culture plates for 3 days. Non-adherent cells were removed, and spindle-shaped adherent colony-forming cells were cultured until the cells were 40-50% confluent. To check whether the isolated cells were MSCs, expression of MSC markers on the cells was analyzed by flow cytometry (FACSCalibur, BD Biosciences). To further expand MSCs, the cells were cultured in K-SFM supplemented with bovine pituitary extract, recombinant human epidermal growth factor, hydrocortisone, N-acetyl-L-cysteine, calcium chloride, insulin, and ascorbic acid to further expand the cells.

5. Generation of monocyte-derived DCs

5.1. Isolation of monocytes

APB and UCB were provided from the "Red Cross" and from "ALL CORD, the public cord blood bank of Seoul Metropolitan Boramae Medical Center (Seoul, Korea)", respectively. All experiments using human blood were conducted under the approval of the Institutional Review Board of the Seoul National University. To obtain peripheral blood mononuclear cells (PBMC) or cord blood mononuclear cells (CBMC), APB and UCB were two-fold diluted with PBS and overlaid on Ficoll–Paque PLUS. PBMCs and CBMCs were obtained by collecting buffy-coat after density-gradient centrifugation with brake off at $479 \times g$, for 20 min at room temperature. The mononuclear cells were washed with PBS three times to remove platelets, and then treated with RBC lysing buffer for 5 min to eliminate remained RBCs.

5.2. Magnetic separation of $CD14^+$ monocytes

Mononuclear cells were washed with PBS containing 2 mM EDTA, and then incubated with anti-human CD14 magnetic beads for 30 min at room temperature. The cells were suspended in PBS containing 2 mM EDTA and 1.5% FBS, and then CD14-expressing cells were isolated by magnetic separation. Residual magnetic beads in the isolated cells were removed and the $CD14^+$ monocytes were suspended in RPMI 1640 media containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at a density of 2×10^6 cells/ml. To differentiate the monocytes into immature DCs, human recombinant GM-CSF and IL-4 were added to the monocytes. $CD14^-$ fractions were collected and frozen at -80°C to further use for mixed leukocyte reaction (MLR) analysis.

5.3. Differentiation of DCs in the presence of PAF or its receptor antagonist

To examine functions of PAFR signaling on differentiation and activation of DCs, the $CD14^+$ monocytes (2×10^6 cells/ml) were treated with either CV6209 (2 μM) or PAF C-16 (2 μM) in the presence of human recombinant GM-CSF (5 ng/ml, R&D system) and IL-4 (18 ng/ml). After 24 h, the monocytes were washed with PBS to remove residual CV6209 or PAF C-16 and cultured in RPMI 1640 media containing GM-CSF and IL-4 for another 4 days to differentiate them into immature DCs. To induce maturation of the immature DCs, DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24 h in the presence of GM-CSF (2.5 ng/ml) and IL-4 (9 ng/ml).

5.4. Differentiation of DCs in the presence of PGE2 or its receptor antagonists

To examine role of endogenous PGE2 signaling in differentiation and activation of DCs, $CD14^+$ monocytes (2×10^6 cells/ml) were treated with either PF04418948 (5 and 10 μM) or L-161,982 (5 and 10 μM) and differentiated into immature DCs for 5 days in the presence of human recombinant GM-CSF (5 ng/ml, R&D system) and IL-4 (18 ng/ml). To examine effects of exogenous PGE2 on phenotypes and functions of DCs, the monocytes (2×10^6 cells/ml) were stimulated with PGE2 (0.01, 0.1, 1, 10, and 100 nM) in the presence of GM-CSF (5 ng/ml, R&D system) and IL-4 (18 ng/ml) for 24 h, and then the cells were washed with PBS to remove the residual PGE2. The unstimulated and PGE2-stimulated monocytes were differentiated into immature DCs for another 4 days. The immature DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24 h in the presence of GM-CSF (2.5 ng/ml) and IL-4 (9 ng/ml) to induce maturation of the cells.

5.5. Differentiation of DCs in the presence of UCB-MSCs

To explore immunomodulatory effects of UCB-MSCs, $CD14^+$ monocytes (2×10^6 cells/ml) were co-cultured with UCB-MSCs at various MSC to monocyte ratios ($1:10^6$, $1:10^5$, $1:10^4$, $1:10^3$, $1:10^2$, and 1:30), and then the monocytes were differentiated into immature DCs in the presence of human recombinant GM-CSF (50 ng/ml, Peprotech) and IL-4 (18 ng/ml) for 5 days. The DCs (2.5×10^5 cells/ml) were stimulated with either *E. coli* LPS (100 ng/ml) or HKEC (1×10^6 CFU/ml) for 24 h in the presence of GM-CSF (25 ng/ml) and IL-4 (9 ng/ml) to induce maturation of the cells.

5.6. Differentiation of APB-DCs and UCB-DCs

$CD14^+$ monocytes isolated from PBMCs or CBMCs were suspended in RPMI at a 2×10^6 cells/ml. To differentiate the monocytes into immature DCs, the cells were

stimulated with human recombinant GM-CSF (50 ng/ml, Peprotech) and IL-4 (18 ng/ml) for 5 to 6 days. The immature DCs (5×10^5 cells/ml) were stimulated with either *E. coli* LPS (100 ng/ml) or HKEC (1×10^6 CFU/ml) for 24 h to 48 h in the presence of GM-CSF (25 ng/ml) and IL-4 (9 ng/ml) to induce maturation of the cells.

6. Morphologic analysis of DCs

Monocytes were differentiated into immature DCs for 5 to 6 days. Morphologies of DCs were analyzed by digital inverted fluorescence microscope (Nikcon) and SPOT microscope camera software (SPOT Imaging Solutions). Sizes and granularity of DCs were analyzed by flow cytometry and FlowJo software (TreeStar). More than seven thousands cells were acquired for each sample and dead cells were gated out.

7. Phenotypic analysis of DCs

DCs (5×10^4 cells) were harvested and treated with PBS containing 1% human plasma to block Fc receptors expressed on the DCs. DCs were stained with fluorochrome-conjugated monoclonal antibodies specific for CD1a, CD14, CD80, CD86, HLA-DR, DP, DQ, PD-L1, PD-L2, DC-SIGN, CD206, and CD205 for 30 min at 4°C, and then washed with PBS once. To examine phenotypic changes of DCs upon LPS or bacterial stimulation, unstimulated or stimulated DCs were stained with antibodies specific for their maturation markers including CD80, CD83, CD86, HLA-DR, DP, DQ, and PD-L1 for 30 min at 4°C. Geometric mean fluorescence intensity (MFI) of the cells was analyzed by flow cytometry (FACSCalibur, BD Bioscience). More than seven thousands cells were acquired for each sample and dead cells were gated out. All the flow cytometic data were

analyzed by FlowJo software.

8. Phagocytosis assay

To analyze phagocytic abilities of DCs, the DCs (5×10^4 cells) were cultured with CFSE-labeled heat-killed bacteria (5×10^6 CFU) in 20 μl PBS for 1 h at 4°C and 37°C, respectively, and then the cells were washed with PBS once. MFI of the cells was analyzed by flow cytometry as described above. The actual phagocytosis (net MFI) of the DCs was calculated by subtracting the MFI at 4°C for bacterial binding from the MFI at 37°C for bacterial internalization. All the flow cytometic data were analyzed by FlowJo software.

9. Dextran-FITC uptake assay

Endocytic capacity of monocytes, unstimulated DCs, and LPS-stimulated DCs were measured using a dextran-FITC uptake assay. Briefly, the cells were washed with PBS and suspended in complete RPMI 1640 containing dextran-FITC (1 mg/ml) and incubated for 1 h at 4°C or 37°C to measure non-specific or specific uptake, respectively. Then, the cells were washed three times with ice-cold PBS containing 2% FBS, and the MFI of the cells was analyzed by flow cytometry as described above. The actual uptake (net MFI) was calculated by subtracting the MFI at 4°C from the MFI at 37°C. All the flow cytometic data were analyzed by FlowJo software.

10. Cytokine quantification

DCs (2.5×10^5 cells/ml) were stimulated with either LPS (100 ng/ml) or heat-killed bacteria (2.5×10^6 CFU/ml) for 24 h and the supernatant was collected. Amount of

IL-12p70, TNF-α, and IL-10 in the culture supernatant was measured by ELISA according to manufacturer's instructions. In some experiments for characterizing UCB-DCs, APB or UCB-DCs (1×10^5 cells/ml) were stimulated with either LPS (100 ng/ml) or HKEC (1×10^6 CFU/ml) for 24 h or 48 h, and then amount of IL-12p40, IL-12p70, TNF-α, and IL-10 in the culture supernatant was measured by ELISA according to manufacturer's instructions. To quantify cytokine production by PBMCs cultured with DCs, unstimulated, LPS-stimulated, or HKEC-stimulated DCs (2×10^4 cells) were co-cultured with $CD14^+$ cell-depleted autologous or allogeneic PBMCs (2×10^5 cells) for 3 days, and then the culture supernatant was subjected to ELISA to measure amount of IFN-γ and IL-10.

11. Autologous MLR assay

To isolate $CD3^+$ T lymphocytes from PBMCs, $CD14^+$ cell-depleted PBMCs were incubated with anti-human CD3 magnetic bead for 30 min at room temperature. The cells were suspended in PBS containing 2 mM EDTA and 1.5% FBS and separated on magnetic field. $CD3^+$ cells were enriched by positive selection and labeled with CFDA-SE (10 μM) for 15 min at 37°C. DCs (2.5×10^5 cells/ml) were stimulated with heat-killed bacteria (2.5×10^6 CFU/ml) for 12 h, and the supernatant was removed. The unstimulated or heat-killed bacteria-stimulated DCs (5×10^4 cells) were co-cultured with the CFSE-labeled autologous T lymphocytes (5×10^4 cells) for 4 days. To measure proliferation and activation of T lymphocytes by the stimulation with DCs, the T lymphocytes were stained with anti-human CD25 antibodies for 30 min at 4°C and subjected to flow cytometic analysis.

12. Allogeneic MLR assay

PBMCs were incubated in PBS containing CFDA-SE (10 μM) for 15 min at 37 °C. DCs (2.5×10^5 cells/ml) were treated with HKEC (1×10^6 CFU/ml) for 24 h. The unstimulated and HKEC-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled allogeneic PBMCs (5×10^5 cells) for 3 to 5 days. On day 3, the culture supernatant was collected and subjected to ELISA for measuring the amount of IFN-γ and IL-10 (R&D Systems). On day 5, CFSE-labeled PBMCs were stained with anti-human CD3 antibodies to detect T lymphocytes among the mixed leukocytes, and proliferation of the $CD3^+$ cells and $CD3^-$ cells was analyzed by flow cytometry. To examine the expression of activation markers on T lymphocytes, PBMCs were stained with anti-human CD3 antibodies, anti-human CD25 antibodies, and anti-human HLA-DR, DP, DQ antibodies, and the cells were analyzed by flow cytometry.

13. Analysis of intracellular cytokine expression of T lymphocytes

DCs (2.5×10^5 cells/ml) were stimulated with heat-killed bacteria (2.5×10^5 cells/ml) for 12 h, and then the unstimulated or heat-killed bacteria-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous T lymphocytes (5×10^4 cells) for 4 days. The T lymphocytes were re-stimulated with PMA (0.5 μM) and ionomycin (1 μg/ml) for 4 h in the presence of brefeldin A (1 μg/ml) before harvest. To analyze intracellular cytokine expression in the T lymphocytes, the cells were fixed with PBS containing 4% paraformaldehyde for 15 min at 4°C and washed with PBS once. The T lymphocytes were permeabilized by treatment with PBS containing 0.1% saponin for 15 min at 4°C, and then the cells were stained with Alexa Fluor® 647-labeled anti-human IFN-γ antibodies, Alexa Fluor® 647-labeled anti-human IL-17A, or APC-labeled anti-human IL-4 antibodies for 30 min at 4°C. Expression of intracellular cytokines in the T lymphocytes was analyzed by flow

cytometry and FlowJo software.

14. Analysis of intracellular COX2 in monocytes

14.1. COX2 in monocytes treated with PAFR antagonist or agonist

$CD14^+$ monocytes (5×10^5 cells/ml) were stimulated with either CV6209 (2 μM) or PAF C-16 (2 μM) in the presence of GM-CSF (5 ng/ml, R&D system) and IL-4 (18 ng/ml) for 4 h, 16 h, or 24 h. The monocytes were washed with PBS once, and then the cells were suspended in PBS containing 4% paraformaldehyde and incubated for 15 min at 4°C. The monocytes were permeabilized with PBS containing 0.1% saponin for 15 min at 4°C, and then the cells were stained with primary anti-human COX2 antibodies for 30 min followed by stain with PE-labeled anti-goat IgG antibodies for another 30 min at 4°C. Expression of COX2 in the monocytes was analyzed by flow cytometry and FlowJo software.

14.2. COX2 in monocytes co-cultured with UCB-MSCs

$CD14^+$ monocytes (5×10^5 cells/ml) were co-cultured with UCB-MSCs at various MSC to monocyte ratios ($1:10^5$, $1:10^4$, $1:10^3$, $1:10^2$, and 1:10) in the presence of GM-CSF (50 ng/ml, Peptrotech) and IL-4 (18 ng/ml) for 24 h. The monocytes were stained with APC-labeled anti-human CD14 antibodies to distinguishably detect monocytes from UCB-MSCs. Then, the monocytes and UCB-MSCs were fixed with PBS containing 4% paraformaldehyde for 15 min at 4°C and permeabilized by treatment with PBS containing 0.1% saponin for 15 min at 4°C. The cells were stained with primary anti-human COX2 antibodies for 30 min followed by stain with PE-labeled anti-mouse IgG antibodies for another 30 min at 4°C. Expression of COX2 in the monocytes or UCB-MSCs was analyzed by flow cytometry and

FlowJo software.

15. PGE2 quantification

To measure concentration of PGE2 in human plasma, heparinized UCB and APB were centrifuged at 1,320 × *g* for 10 min at 4°C to obtain the plasma. The plasma was used to determine PGE2 levels using a PGE2 ELISA kit according to the manufacturer's instruction. To measure amount of PGE2 produced by UCB-MSCs and/or monocytes, $CD14^+$ monocytes (5×10^5 cells/ml) were co-cultured with UCB-MSCs at various MSC to monocyte ratios ($1:10^5$, $1:10^4$, $1:10^3$, $1:10^2$, and 1:10) in the presence of GM-CSF (50 ng/ml, Peptrotech) and IL-4 (18 ng/ml) for 24 h. Amount of PGE2 in the culture supernatant was measured by PGE2 ELISA kit according to the manufacturer's instruction.

16. Statistical analysis

Experimental data were compared using Student's *t*-test or ANOVA test (Tukey's multiple comparison test). *P* values under 0.05 were considered statistically significant. Results are indicated as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM)

Chapter III. Results

1. Role of PAF signal in immunological characteristics of DCs

1.1. Activation of PAFR is critical for DCs to acquire typical phenotypes and phagocytic abilities.

Monocytes highly produce PAF in response to GM-CSF during their differentiation into DCs [80, 81]. However, functions of PAF and its signaling on the immunological characteristics of DCs have not yet been elucidated. To examine the role of PAFR signaling in determining phenotypes and phagocytic capacities of DCs, $CD14^+$ monocytes were differentiated into DCs in the absence or presence of PAFR antagonist, CV6209, and then morphologies, phenotypes, and phagocytic abilities of the DCs were analyzed. DCs differentiated in the presence of CV6209 (CV6209-DCs) showed smaller sizes and lower granularity than those of control DCs differentiated without CV6209 (control DCs) (Fig. 1A and B). CV6209-DCs displayed unique phenotypes distinct from those of control DCs. CV6209-DCs

contained less $CD1a^{+}CD14^{-}$ DCs than control DCs (Fig. 2A). In addition, CV6209-DCs exhibited significantly lower expression of CD80 and PD-L1 but higher expression of CD86 than those of control DCs, while the expression of MHC class II and PD-L2 was comparable to those of control DCs (Fig. 2B). With respect to the phagocytic abilities of the DCs, CV6209-DCs showed markedly enhanced uptake abilities to HKSP at 37˚ (Fig. 3A). However, significant differences in the expression of phagocytosis-related receptors including DC-SIGN, CD205, and CD206 by the DCs was not observed (Fig. 3B). These results indicate that the activation of PAFR is crucial for the generation of DCs with typical morphologic, phenotypic, and phagocytic characteristics.

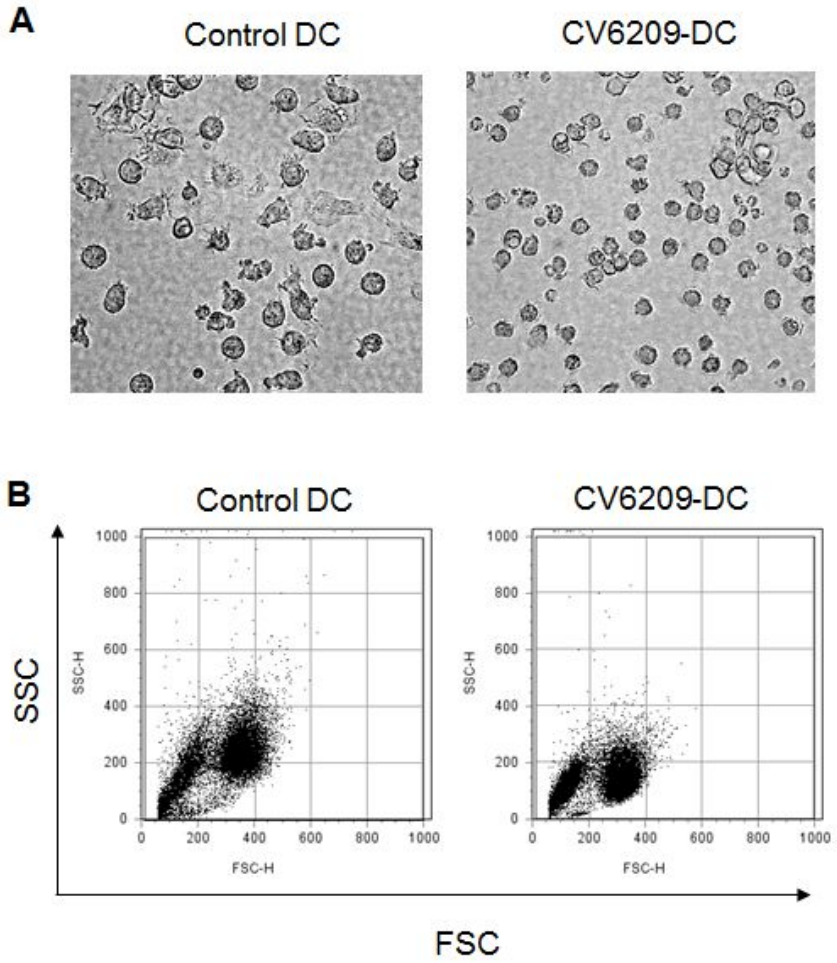

Figure 1. Morphologies of CV6209-DCs are different from those of control DCs. Monocytes (2×10^6 cells/ml) were treated without or with CV6209 (2 μM) for 24 h in the presence of GM-CSF and IL-4. After removal of residual CV6209, the monocytes were further differentiated into DCs in the presence of GM-CSF and IL-4 for another 4 days. (A) Morphologies of the DCs were analyzed using optical microscopy. (B) Sizes and granularity of the DCs were analyzed by flow cytometry. These results are representative of three independent experiments.

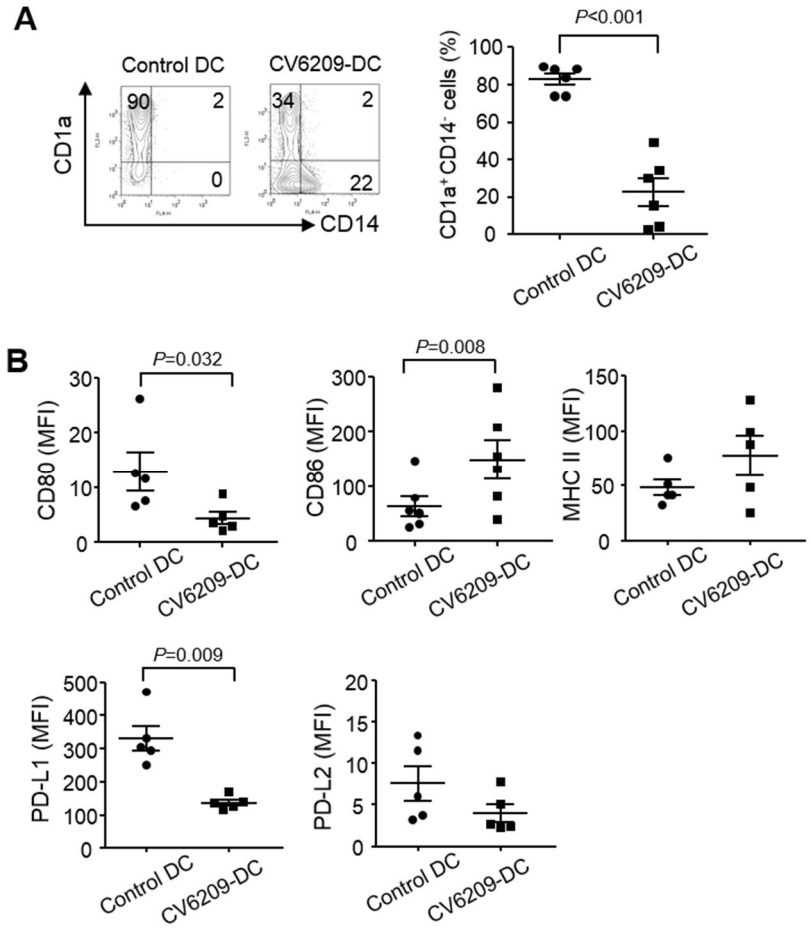

Figure 2. Inactivation of PAFR signaling inhibits differentiation of monocytes into DCs with typical phenotypes. Monocytes (2×10^6 cells/ml) were treated without or with CV6209 (2 μM) in the presence of GM-CSF and IL-4. After removal of residual CV6209, the monocytes were further differentiated into DCs for another 4 days. DCs (5×10^4 cells) were stained with monoclonal antibodies specific for DC markers and subjected to flow cytometric analysis. (A) Expression of CD1a and CD14 on the DCs was analyzed by flow cytometry. Numbers on histograms indicate the percentage of cells in each quadrant. Scatter plot on the right of the histograms indicates average of $CD1a^+CD14^-$ cells of each treatment group (n=6). (B) Expression of CD80 CD86, MHC class II, PD-L1, and PD-L2 was analyzed by flow cytometry. MFI of these markers was indicated in the scatter plots (n=5). Statistical differences in the MFI of control DCs and CV6209-DCs were analyzed by

Student's paired *t*-test.

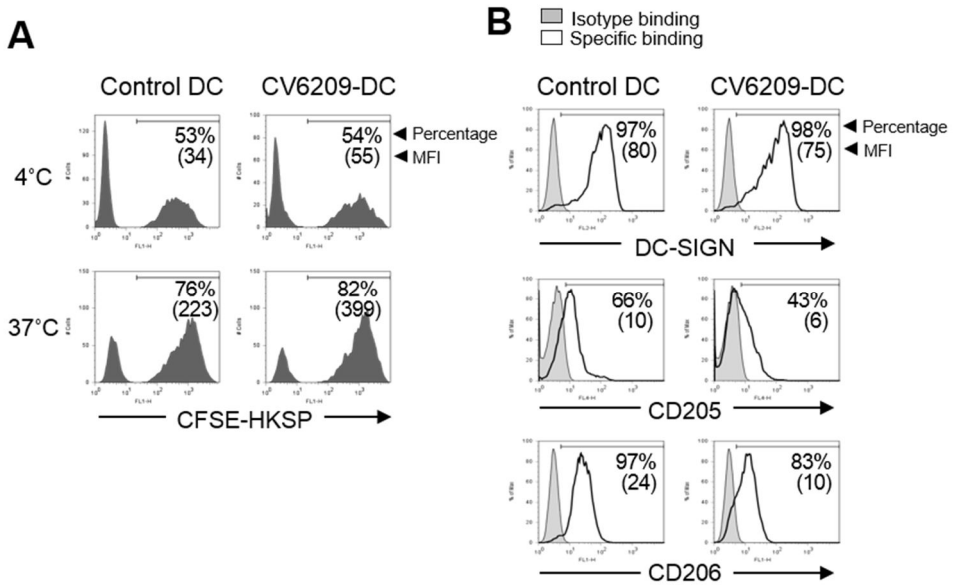

Figure 3. Inactivation of PAFR signaling enhances phagocytic ability of DCs. Monocytes (2×10^6 cells/ml) were treated without or with CV6209 (2 μM) in the presence of GM-CSF and IL-4. After removal of residual CV6209, the monocytes were further differentiated into DCs for another 4 days. (A) DCs (5×10^4 cells) were incubated with CFSE-labeled HKSP (5×10^6 CFU) for 1 h at 4˚C and at 37˚C, respectively, followed by flow cytometric analysis. Numbers on the histograms indicate the percentage of CFSE-positive DCs (*upper*) and MFI (*lower*, in parenthesis) of the DCs. Actual phagocytosis can be calculated by subtracting MFI at 4˚C from MFI at 37˚C. (B) Expression of DC-SIGN, CD205, and CD206 on the DCs was analyzed by flow cytometry. Gray-filled area and open area indicate isotype binding and specific binding, respectively. Numbers in the histogram indicates the percentage of positive cells (*upper*) and MFI (*lower*, in parenthesis) of the cells, respectively. These results are representative of three independent experiments.

1.2. PAFR signaling is crucial for enhancing immunogenic properties of DCs.

Maturation of DCs is an important process for the mediation of antigen-specific adaptive immune responses, which is accompanied with increase of MHC proteins, co-stimulatory molecules, and cytokines [2]. To ascertain the function of PAFR signaling on maturation and activation of DCs, phenotypic changes and cytokine production of CV6209-DCs was compared to those of control DCs. When the DCs were stimulated with HKSP, CV6209-DCs did not efficiently increase expression of co-stimulatory molecules including CD80, CD83, and CD86, and MHC class II (Fig. 4A). Additionally, HKSP-stimulated CV6209-DCs displayed lower expression of co-inhibitory molecules such as PD-L1 and PD-L2 than control DCs (Fig. 4A). Furthermore, IL-12p70, TNF-α, and IL-10 were not detected in response to HKSP, control DCs highly expressed these cytokines (Fig. 5). These results suggest that PAFR activation at early phase of DC differentiation is crucial for DCs to efficiently induce phenotypic maturation and cytokine production.

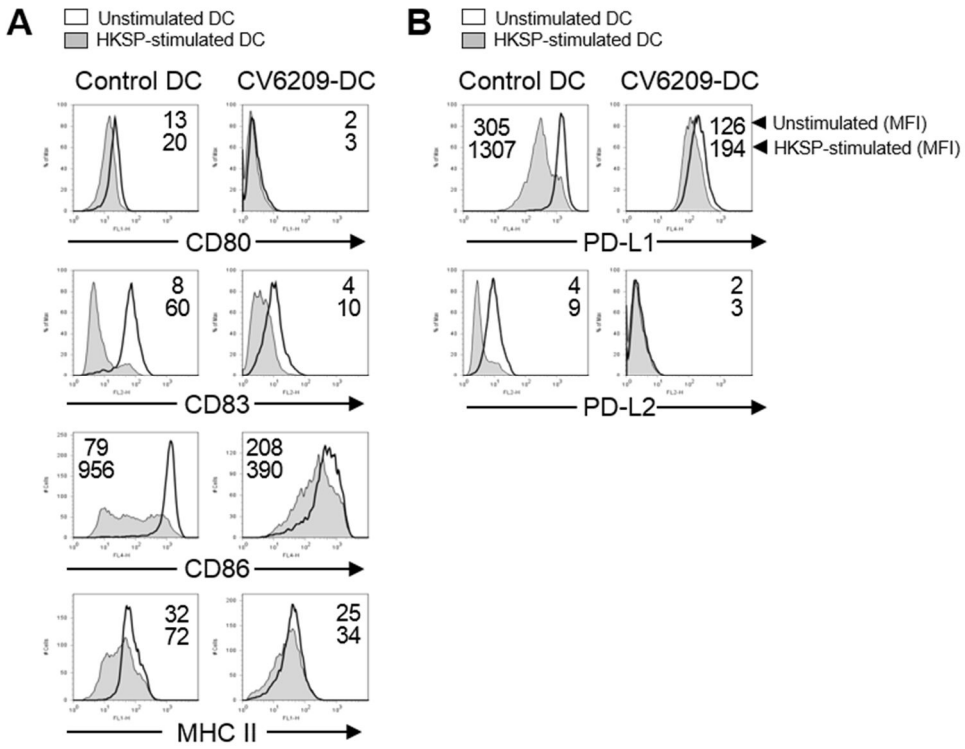

Figure 4. CV6209-DCs exhibit impairment in their maturation. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24 h and stained with monoclonal antibodies specific for maturation markers of the cells. Expression of (A) co-stimulatory receptors including CD80, CD83, CD86, and MHC class II, and (B) co-inhibitory receptors including PD-L1 and PD-L2 was analyzed by flow cytometry. Gray-filled area and open area indicate unstimulated and HKSP-stimulated DCs, respectively. Numbers on the histograms indicate MFI of the unstimulated DCs (*upper*) and MFI of the HKSP-stimulated DCs (*lower*). These results are representative of five similar experiments.

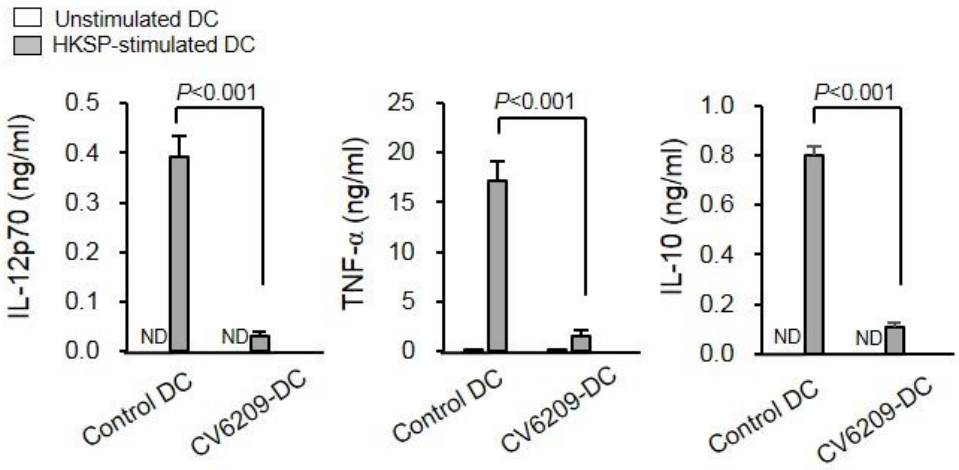

Figure 5. Inactivation of PAFR signaling abolishes cytokine production of DCs. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24 h and the amount of IL-12p70, TNF-α, and IL-10 in the culture supernatant was measured by ELISA. Levels of the cytokines were indicated as mean ± SD and statistical differences between the compared experimental groups were analyzed Student's unpaired *t*-test. Representative data are shown among three similar experiments. ND denotes 'not detected'.

Functionally mature DCs efficiently induces proliferation and activation of T lymphocytes [2]. Activated T lymphocytes highly express activation markers including CD69 and CD25 on the surface, and inflammatory cytokines such as IFN-γ and IL-17 [82]. To ascertain role of PAFR signal in the DC-mediated activation of T lymphocytes, unstimulated or HKSP-stimulated CV6209-DCs were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes, and then proliferation, CD25 expression, and cytokine production of the T lymphocytes were analyzed. HKSP-stimulated CV6209-DCs weakly induced proliferation and CD25 expression of the $CD3^+$ T lymphocytes (Fig. 6A). In addition, $CD4^+$ Th and $CD8^+$ CTL were less activated by CV6209-DCs (Fig. 6B and C). Furthermore, T lymphocytes co-cultured with CV6209-DCs did not produce IFN-γ, IL-17A, or IL-4 (Fig. 7A to C). These results indicate that blockade of PAFR signaling hampers T lymphocyte-activating capacities of DCs. Taken together, activation of PAFR is critical for the induction of stimulatory properties in DCs with regard to their phenotypic maturation, cytokine production, and T lymphocyte-activating capacities.

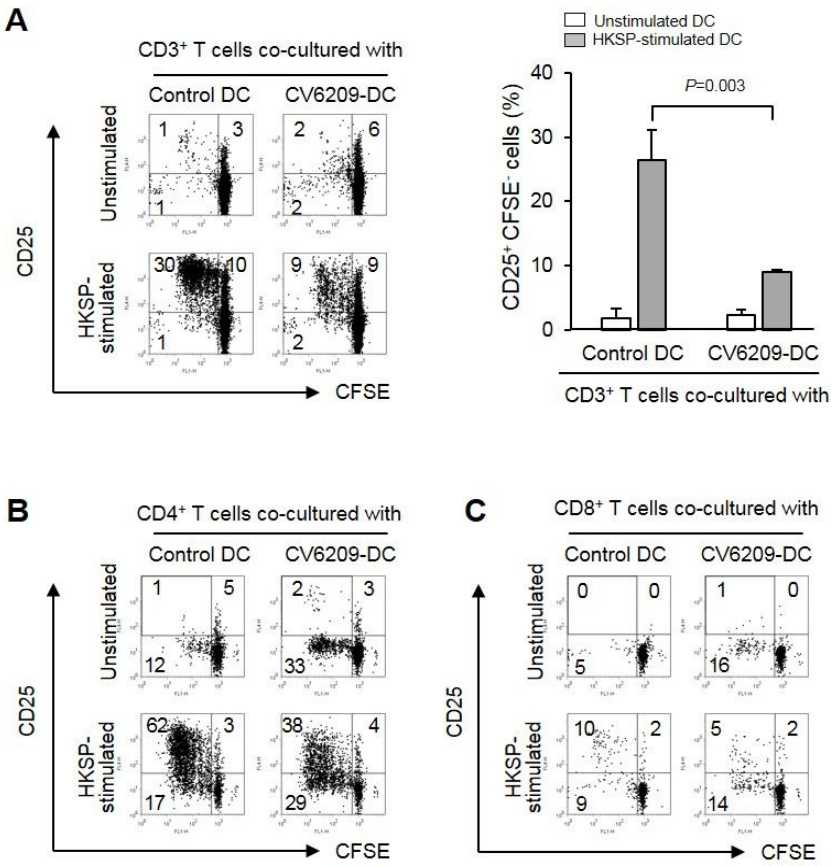

Figure 6. Inactivation of PAFR signaling hampers DC-mediated proliferation and activation of autologous T lymphocytes. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h, and then the unstimulated or HKSP-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (5×10^4 cells) for 4 days. (A) Proliferation and CD25 expression of the $CD3^+$ T lymphocytes were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant. Bar graph on the right of the histograms represents the mean value of triplicate measurements. Frequencies of the activated T lymphocytes were indicated as mean ± SD and statistical differences between the compared experimental groups were analyzed by Student's unpaired *t*-test. Result shown is representative of five independent experiments. N.S. denotes 'not significant'. (B and C) Proliferation and CD25 expression of $CD4^+$ Th and $CD8^+$ CTL were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant.

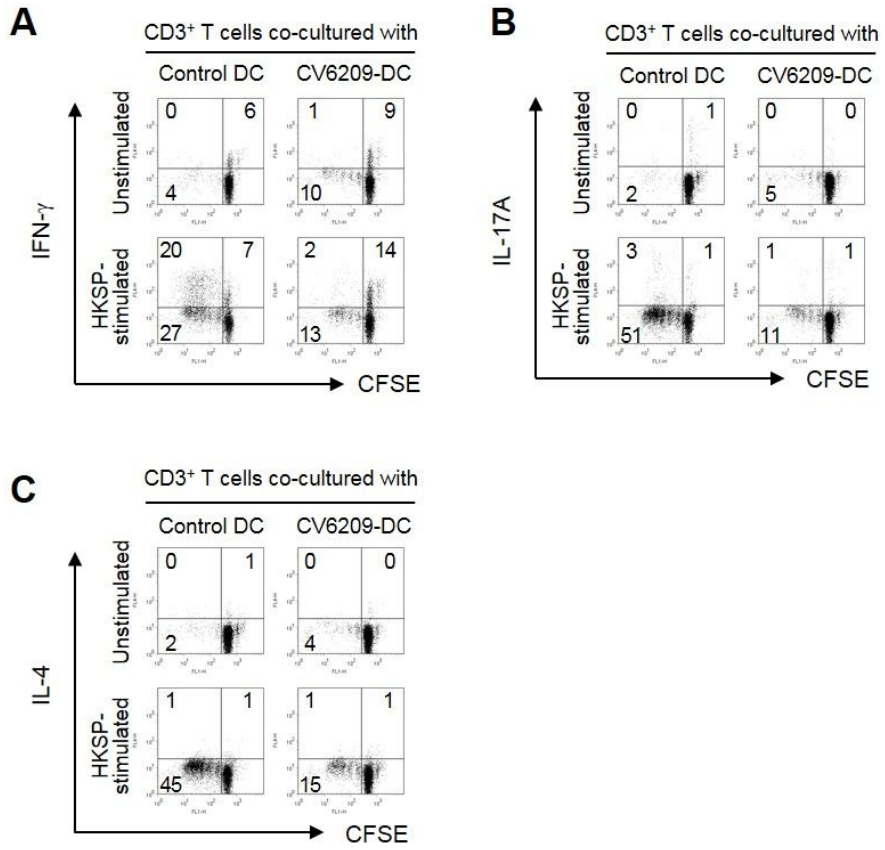

Figure 7. Inactivation of PAFR signaling impairs DC-mediated induction of IFN-γ-producing T lymphocytes. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h, and then the unstimulated or HKSP-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (5×10^4 cells) for 4 days. The T lymphocytes were re-stimulated with PMA (0.5 μM) and ionomycin (1 μg/ml) for 4 h before harvest in the presence of brefeldin A (1 μg/ml), and then the frequencies of (A) IFN-γ- producing T lymphocytes, (B) IL-17A- producing T lymphocytes, or (C) IL-4-producing T lymphocytes in the mixed cells were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of T lymphocytes in each quadrant. Representative data are shown among three similar experiment.

1.3. Blockade of PAFR renders DCs to mediate tolerogenic immune responses.

Tolerogenic DCs have been reported to induce Treg which usually express forkhead box P3 (FOXP3) or anti-inflammatory cytokines such as TGF-β and IL-10 [83, 84] and efficiently control excessive activation of T lymphocytes. To further examine whether CV6209-DCs have tolerogenic properties, suppressive functions of CV6209-DCs on the activation of T lymphocytes were examined. CV6209-DCs were stimulated with HKSP, and then the cells were mixed with HKSP-stimulated control DCs at various ratios indicated in Fig. 8A. The mixed DCs were subsequently co-cultured with autologous $CD3^+$ T lymphocytes, and then the proliferation and CD25 expression of the T lymphocytes were analyzed. CV6209-DCs potently suppressed the proliferation and activation of the T lymphocytes induced by control DCs (Fig. 8A). To verify whether the suppressive functions on T lymphocyte activation was specific to CV6209-DCs, HKSP-stimulated monocytes, instead of CV6209-DCs, were mixed with HKSP-stimulated control DCs at 1:2 and 1:1. The mixed cells were co-cultured with autologous T lymphocytes and the proliferation and CD25 expression of the T lymphocytes were analyzed. Contrary to CV6209-DCs, monocytes could not suppress T lymphocyte proliferation and activation induced by control DCs (Fig. 8B). To unravel regulatory mechanisms by which CV6209-DCs inhibit T lymphocyte proliferation and activation, differentiation of Treg was analyzed. CV6209-DCs remarkably increased frequencies of $IL\text{-}10^+TGF\text{-}\beta^+$ T lymphocytes among the total T lymphocytes (Fig. 9A) but could not efficiently induce $FOXP3^+$ T lymphocytes (Fig. 9B). Considering that CV6209-DCs potently exerted suppressive functions on T lymphocyte activation and induced $IL\text{-}10^+TGF\text{-}\beta^+$ Treg, inactivation of PAFR signaling appears to potentiate tolerogenic characteristics of DCs.

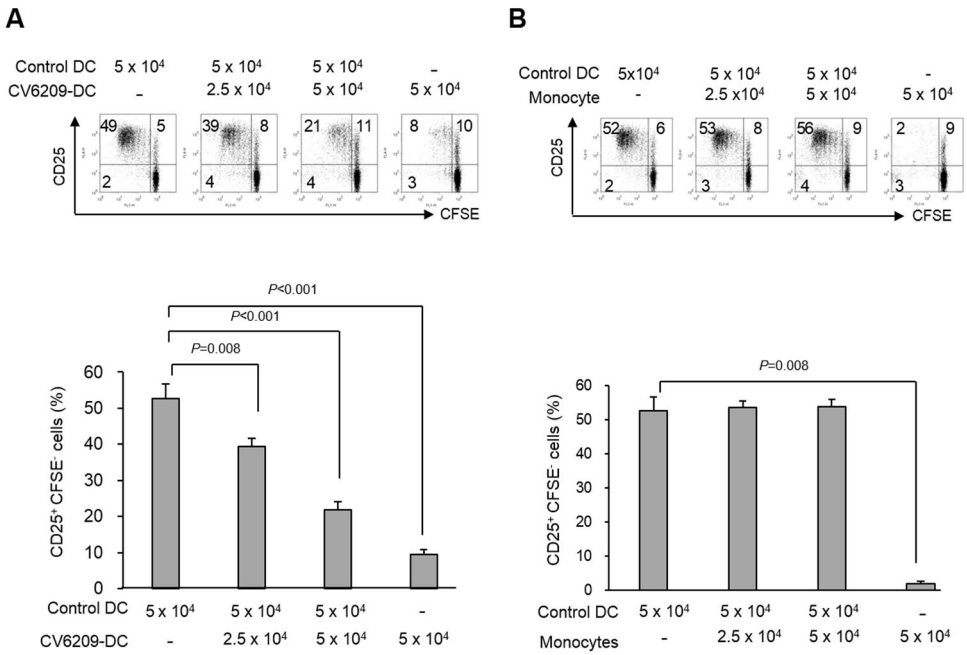

Figure 8. CV6209-DCs exert immunosuppressive functions on proliferation and activation of autologous T lymphocytes. DCs (5×10^4 cells) or undifferentiated monocytes (5×10^4 cells) derived from same donor were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h. (A) HKSP-stimulated control DCs (5×10^4 cells) and HKSP-stimulated CV6209-DCs (5×10^4 cells) were cultured separately or simultaneously at various culture ratios indicated in the figure. The DCs were co-cultured with CFSE-labeled autologous T lymphocytes (5×10^4 cells) for 4 days. Proliferation and CD25 expression of the T lymphocytes were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant. (B) HKSP-stimulated control DCs (5×10^4 cells) and HKSP-stimulated monocytes (2.5×10^5 cells/ml) were cultured separately or simultaneously at various ratios as indicated in figure. The mixed cells were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (2.5×10^5 cells/ml) for 4 days. Proliferation and CD25 expression of the T lymphocytes were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant. Data shown are representative of three similar experiments.

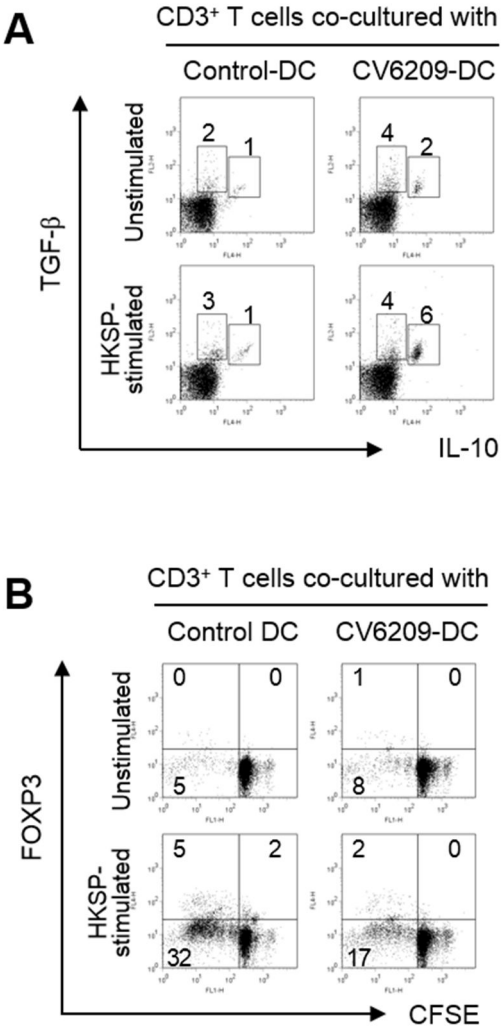

Figure 9. CV6209-DCs induce IL-10$^+$ TGF-β$^+$ regulatory T lymphocytes. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h, and the unstimulated or HKSP-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous CD3$^+$ T lymphocytes (5×10^4 cells) for 4 days. (A) The T lymphocytes cultured with the DCs were treated brefeldin A (1 μg/ml) for 4 h before harvest, and then the frequencies of IL-10 and/or TGF-β-producing T lymphocytes were analyzed by flow cytometry. (B) Intracellular expression of FOXP3 in the T lymphocytes was analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant.

1.4. Prostaglandins partially contribute to DC-mediated tolerogenic immune responses upon PAFR inactivation.

GM-CSF potently stimulates monocytes to produce PAF and PGs [80, 81]. Accumulating evidence has suggested that PGs including PGE2 and PGJ2 potentiate regulatory/tolerogenic functions of DCs [67, 85]. To determine whether inactivation of PAFR signaling in monocytes change the expression levels of PGE2-synthetic enzyme induced by stimulation with GM-CSF, the monocytes were treated either with CV6209 or PAF C-16 in the presence of GM-CSF and IL-4, and then the expression of PGE2 synthetic enzyme, COX2, was analyzed. In response to GM-CSF and IL-4, the monocytes markedly up-regulated COX2 expression by time-dependent manner. However, treatment with either CV6209 or PAF C-16 did not alter the expression levels of the COX2 in the monocytes (Fig. 10). To examine whether endogenously generated PGE2 contribute to potentiating tolerogenic characteristics of CV6209-DCs, PGE2 synthesis was blocked by treatment with selective COX2 inhibitor, NS398, during differentiation of the CV6209-DCs (CV6209/NS398-DCs). CV6209/NS398-DCs contained more $CD1a^{+}$ cells than CV6209-DCs (Fig. 11A). When the cells were stimulated with HKSP, CV6209/NS398-DCs showed higher expression levels of CD80, CD83, CD86, and PD-L1 than CV6209-DCs (Fig. 11B). Moreover, they exhibited slight but significant augmentation in the production of IL-12p70 and IL-10 in comparison with those of CV6209-DCs (Fig. 11C). With respect to the induction of T lymphocyte activation, CV6209/NS398-DCs showed higher stimulating potencies in proliferation and CD25 expression of the T lymphocytes than CV6209-DCs (Fig. 12). These results suggest that the endogenously generated PGE2 partially contribute to enhancing tolerogenic characteristics of CV6209-DCs.

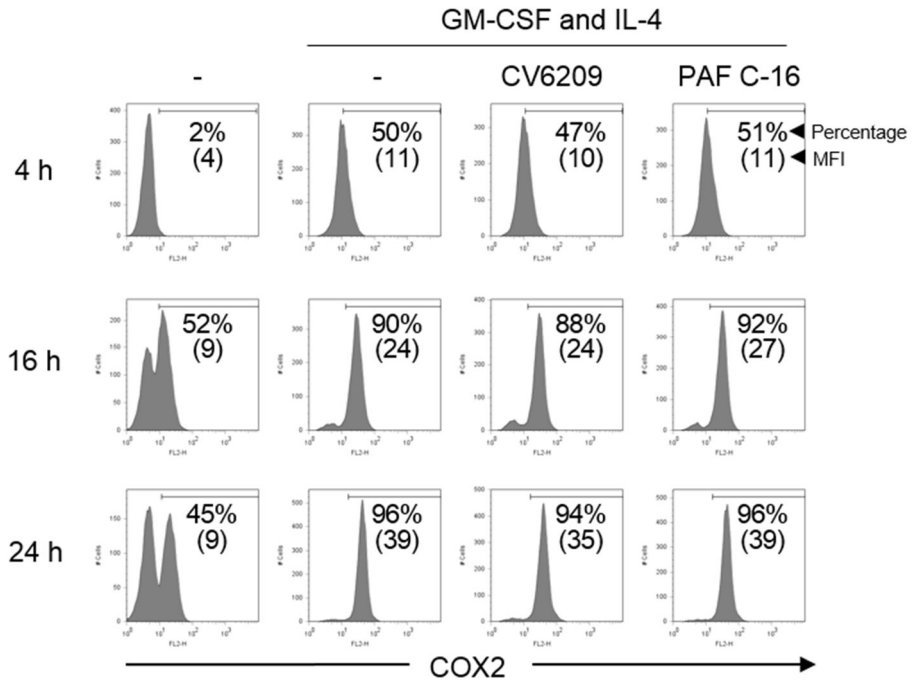

Figure 10. Treatment with GM-CSF and IL-4 increases COX2 in monocytes. Monocytes (5×10^5 cells/ml) were pretreated with either CV6209 (2 μM) or PAF C-16 (2 μM) for 1 h, and then the cells were stimulated with GM-CSF (5 ng/ml) and IL-4 (18 ng/ml) for 24 h. Expression of COX2 in the monocytes were analyzed by flow cytometry. Numbers in the histogram indicates the percentage of COX2-positive cells (*upper*) and MFI (*lower*, in parenthesis) of the cells, respectively.

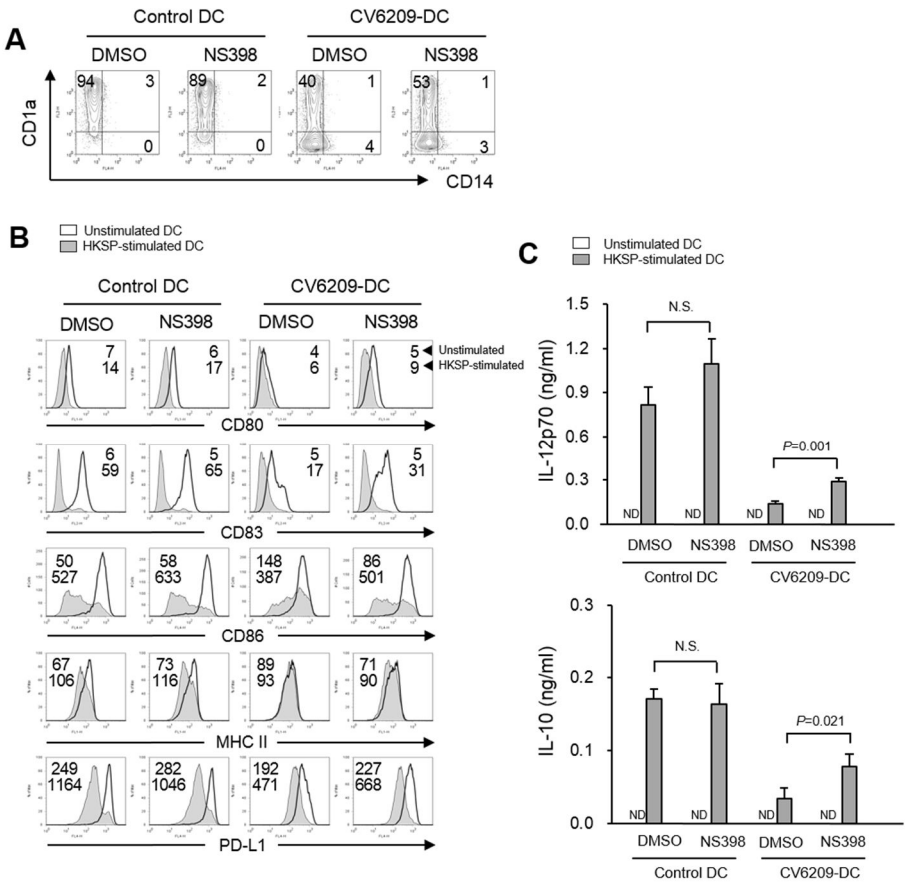

Figure 11. Blockade of PGE2 synthesis partially restores stimulatory phenotypes and cytokine-producing capacities of CV6209-DCs. Monocytes (2×10^6 cells/ml) were pretreated with NS398 (5 μM) for 1 h, and then treated with CV6209 (1 μM) for 24 h in the presence of GM-CSF and IL-4. After removal of the NS398 and CV6209, the monocytes were further differentiated into DCs for another 4 days. (A) Expression of CD1a and CD14 on the DCs was analyzed by flow cytometry. Numbers on histograms indicate the percentage of the cells in each quadrant. (B) DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24 h, and then the expression of CD80, CD83, CD86, MHC class II, and PD-L1 was analyzed by flow cytometry. Gray-filled area and open area indicate unstimulated and HKSP-stimulated DCs, respectively. Numbers on the histograms indicate MFI of unstimulated- (*upper*) and HKSP-stimulated cells (*lower*). (C) Amount of IL-12p70 and IL-10 in culture supernatant was quantified by ELISA. ND and N.S. denote 'not detected' and 'not significant', respectively.

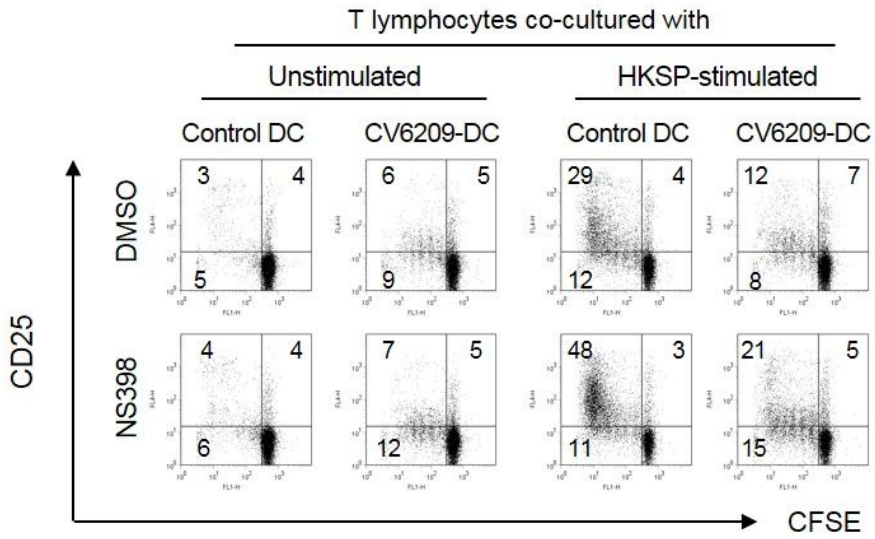

Figure 12. Blockade of PGE2 synthesis partially restores T lymphocyte-activating capacity of CV6209-DCs. Monocytes (2×10^6 cells/ml) were pretreated with NS398 (5 μM) for 1 h, and then treated with CV6209 (1 μM) for 24 h in the presence of GM-CSF and IL-4). After removal of the NS398 and CV6209, the monocytes were further differentiated into DCs for another 4 days. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h, and then the unstimulated or HKSP-stimulated DCs were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (2.5×10^5 cells/ml) for 4 days. Proliferation and CD25 expression of the T lymphocytes were analyzed by flow cytometry. Numbers on the histogram indicate the percentage of T lymphocytes in each quadrant.

1.5. PAF and PGE2 exhibit antagonistic effects on differentiation and activation of DCs.

To ascertain regulatory relations between PAF and PGE2 signaling in differentiation and activation of DCs, immature DCs were differentiated in the presence of PGE2 and/or PAF C-16, and then the phenotypes and functions of the cells were analyzed. DCs differentiated with PGE2 (PGE2-DCs) showed $CD1a^{-}CD14^{+}$ phenotypes rather than $CD1a^{+}CD14^{-}$ phenotypes. However, DCs differentiated in the presence of both PGE2 and PAF C-16 (PGE2/PAF-DCs) exhibited higher frequencies in $CD1a^{+}CD14^{-}$ cell population than PGE2-DCs (Fig. 13A). When the DCs were stimulated with HKSP, PGE2-DCs could not efficiently augment the expression of CD83, CD86, and PD-L1, but PGE2/PAF-DCs exhibited higher levels in the expression of these markers than PGE2-DCs (Fig. 13B). In addition, HKSP-stimulated PGE2-DCs could not produce IL-12p70, but co-treatment with PAF C-16 partially recovered the IL-12p70-producing abilities of PGE2-DCs (Fig. 14A). In contrast, PGE2-DCs highly produced IL-10 in response to HKSP-stimulation, but PGE2/PAF-DCs less produced IL-10 than PGE2-DCs (Fig. 14B). To examine T lymphocyte-activating capacities of the DCs, PGE2-DCs and PGE2/PAF-DCs were co-cultured with autologous $CD3^{+}$ T lymphocytes, and then proliferation and activation of the T lymphocytes were analyzed. Expressing low levels of co-stimulatory molecules and cytokines necessary for T lymphocyte activation, PGE2-DCs could not efficiently induce proliferation, CD25 expression, and IFN-γ production of the autologous T lymphocytes. However, PGE2/PAF-DCs exhibited higher potencies in eliciting proliferation and activation of the T lymphocytes than PGE2-DCs (Fig. 15A). Moreover, frequency of the IFN-γ-producing T lymphocytes induced by PGE2/PAF-DCs were higher than that induced by PGE2-DCs (Fig. 15B). These results suggest that PAF and PGE2 signaling antagonistically regulate

immunostimulatory functions of DCs.

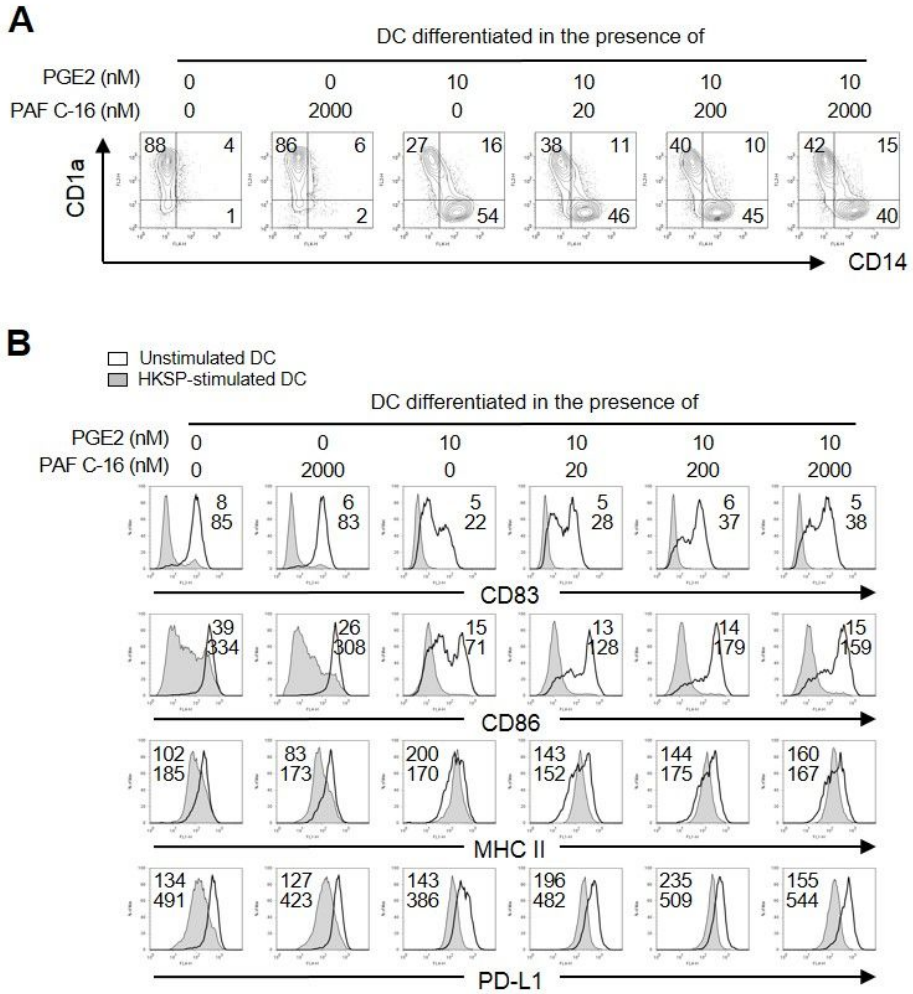

Figure 13. PAF and PGE2 antagonistically regulate phenotypes of DCs. Monocytes (2×10^6 cells/ml) were stimulated with PGE2 (10 nM) in the absence or presence of PAF C-16 (0.02, 0.2, and 2 μM) for 24 in the presence of GM-CSF and IL-4 for 24 h. After removal residual PGE2 and PAF C-16, the monocytes were differentiated into DCs for another 4 days. (A) Expression of CD1a and CD14 on DCs was analyzed by flow cytometry. Numbers on histograms indicate the percentage of the cells in each quadrant. (B) The DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24, and then the expression of CD80, CD83, CD86, MHC class II, and PD-L1 was analyzed by flow cytometry. Gray-filled area and open area indicate unstimulated and HKSP-stimulated DCs, respectively. Numbers

on the histograms indicate MFI of unstimulated- (*upper*) and HKSP-stimulated cells (*lower*).

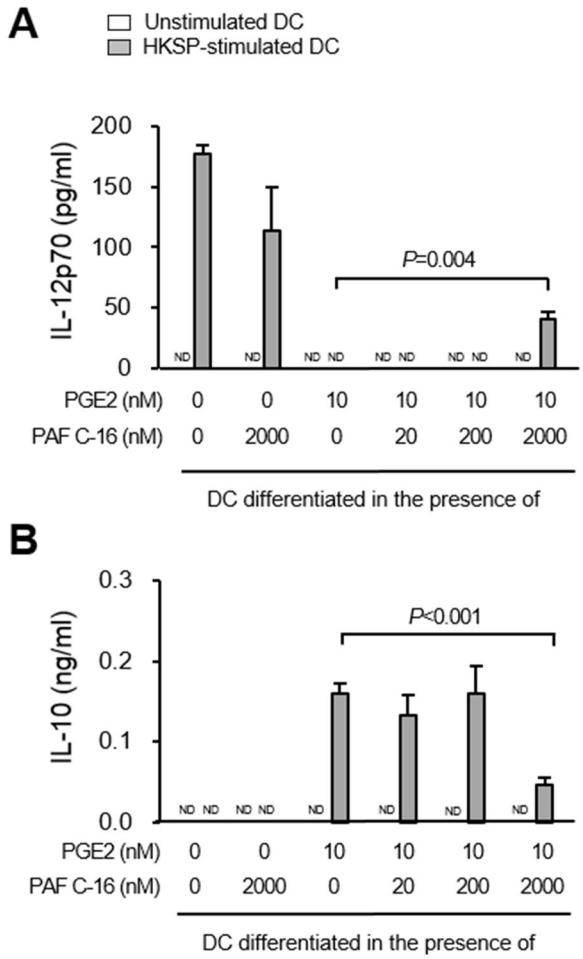

Figure 14. PAFR and PGE2 signals antagonistically regulate maturation of DCs. Monocytes (2×10^6 cells/ml) were stimulated with PGE2 (10 nM) in the absence or presence of PAF C-16 (0.02, 0.2, and 2 μM) for 24 in the presence of GM-CSF and IL-4. After removal of residual PGE2 and PAF C-16, the monocytes were further differentiated into DCs for another 4 days. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24, and then the amount of (A) IL-12p70 and (B) IL-10 in the culture supernatant was measured by ELISA. Levels of the cytokines were indicated as mean ± SD and statistical differences between the compared experimental groups were analyzed Student's unpaired *t*-test. ND denotes 'not detected'.

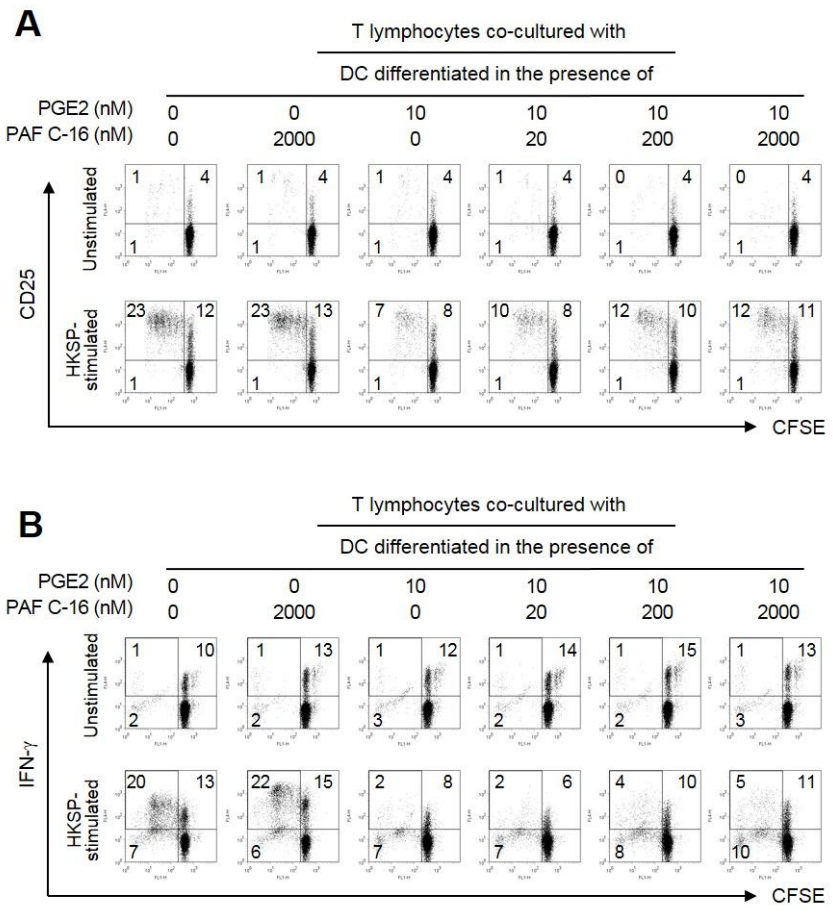

Figure 15. PAFR and PGE2 signals antagonistically regulate T lymphocyte-activating capacity of DCs. Monocytes (2×10^6 cells/ml) were stimulated with PGE2 (10 nM) in the absence or presence of PAF C-16 (0.02, 0.2, and 2 μM) for 24 in the presence of GM-CSF and IL-4. After removal of the residual PGE2 and PAF C-16, the monocytes were further differentiated into DCs for another 4 days. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 12, and then the unstimulated and HKSP-stimulated DCs were co-cultured with autologous $CD3^+$ T lymphocytes (2.5×10^5 cells/ml) for 4 days. (A) Proliferation and CD25 expression of the T lymphocytes were analyzed by flow cytometry. (B) The T lymphocytes were re-stimulated with PMA (0.5 μM) and ionomycin (1 μg/ml) for 4 h before harvest in the presence of brefeldin A (1 μg/ml), and IFN-γ-producing T lymphocytes were analyzed by flow cytometry. Numbers on the histogram indicate the percentage of T lymphocytes in each quadrant.

2. Role of PGE2 signals in immunological characteristics of DCs

2.1. Activation of EP4 signaling is essential for immunostimulatory phenotypes and functions of monocyte-derived DCs.

Monocytes produce PGE2 mediated by GM-CSF stimulation during their differentiation into DCs [80, 81]. PGE2 binds to EP2 and EP4 predominantly expressed on the monocytes. However, it is not yet clearly known about the functions of the different PGE2 receptor signals on differentiation and activation of human monocyte-derived DCs. To investigate roles of EP2 and/or EP4 signals in the regulation of DC differentiation and activation, monocytes were differentiated into DCs in the presence of EP2 antagonist (ant.EP2-DCs), EP4 antagonist (ant.EP4-DCs), or DMSO as a vehicle control (control DCs). Both ant.EP2-DCs and ant.EP4-DCs displayed similar morphologies and sizes to those of control DCs (Fig. 16A and B). Expression levels of CD1a and CD14 on ant.EP2-DCs and ant.EP4-DCs were also comparable to those of control DCs (Fig. 17A). In addition, the DCs exhibited similar expression levels of other phenotypic markers including CD86, MHC class II, CD11c, PD-L1, and PD-L2 (Fig. 18B). To examine functions of the endogenous PGE2 signaling in phagocytic ability of DCs, ant.EP2-DCs and ant.EP4-DCs were co-cultured with CFSE-labeled HKSP at 4˚C and 37˚C, respectively. Phagocytic activity of ant.EP2-DCs against HKSP was comparable to that of control DCs, but that of ant.EP4-DCs was slightly decreased (Fig. 18A). Additionally, no significant differences in the expression of phagocytosis-related receptors including CD206, DC-SIGN, and CD205 were observed in ant.EP2-DCs and ant.EP4-DCs compared to those of control DCs (Fig. 18B). These results indicate that inactivation of endogenous PGE2 signaling through EP2 or EP4 does not alter morphologic, phenotypic and phagocytic characteristics of DCs.

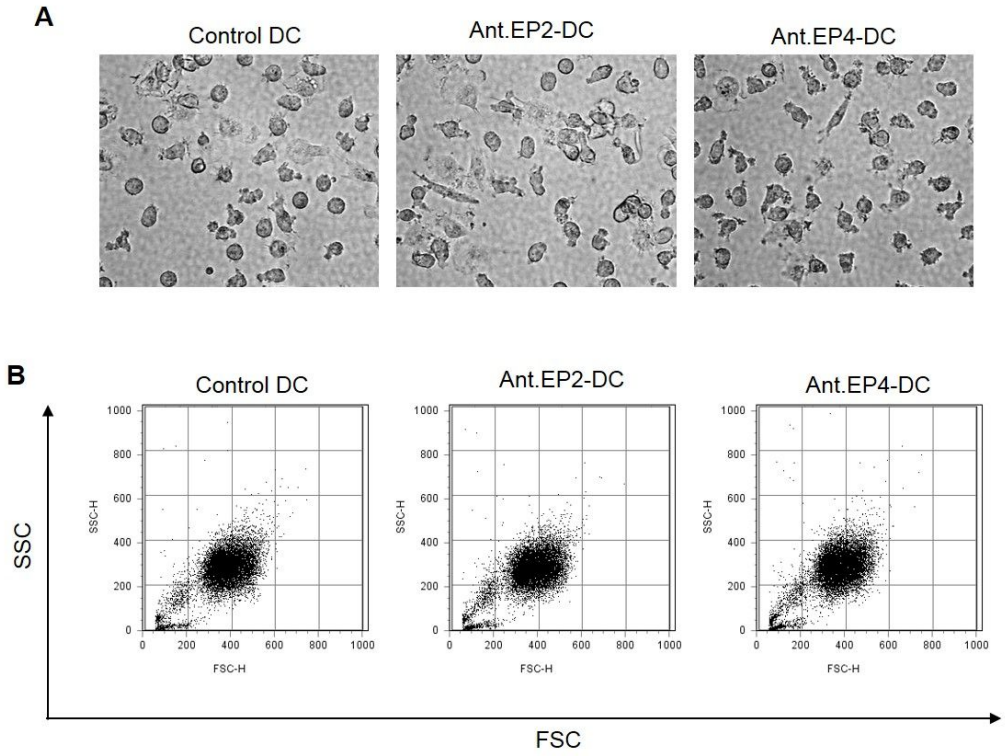

Figure 16. Morphologies of DCs differentiated in the presence of PGE2 receptor antagonists are comparable with those of control DCs. Human monocytes (2×10^6 cells/ml) were differentiated into immature DCs in the presence of EP2 antagonist (PF04418948, 10 μM) or EP4 antagonist (L-161,982, 10 μM) for 5 days. As a vehicle control, the monocytes were treated with 0.05% DMSO, and then the cells were differentiated into DCs. (A) Morphologies of DCs were analyzed by optical microscopy. (B) Sizes and granularity of the DCs were analyzed by flow cytometry.

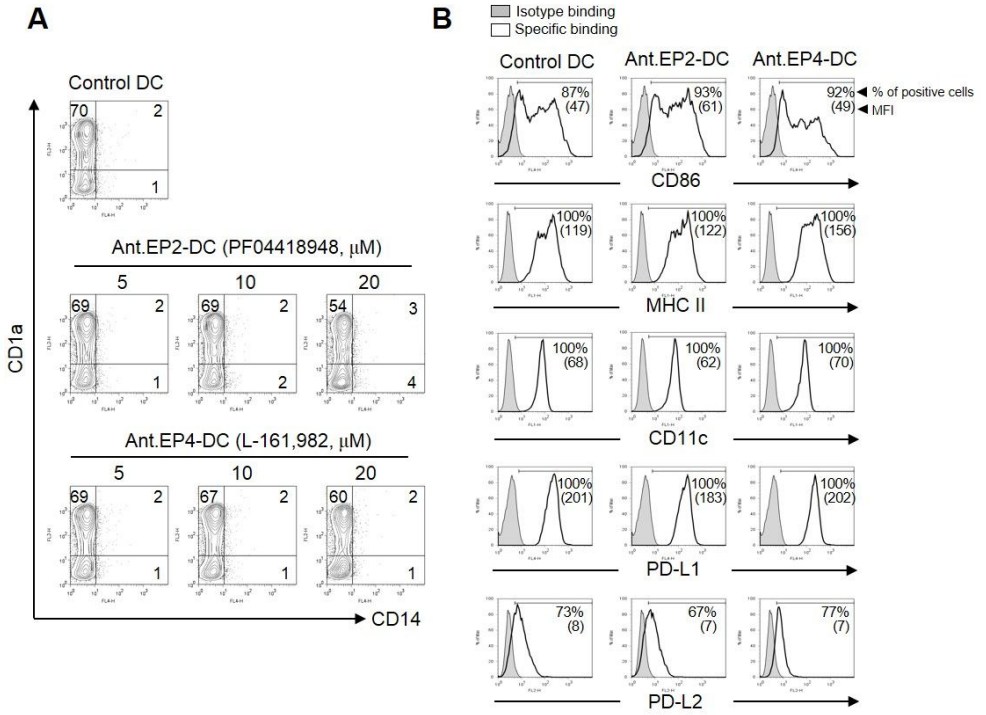

Figure 17. Phenotypes of DCs differentiated in the presence of PGE2 receptor antagonists are similar to those of control DCs. Human monocytes (2×10^6 cells/ml) were differentiated into DCs in the presence of either PF04418948 (5, 10, and 20 μM) or L-161,982 (5, 10, and 20 μM) for 5 days. As a vehicle control, the monocytes were treated with 0.05% DMSO, and then differentiated into DCs. (A) Expression of CD1a and CD14 on the DCs was analyzed by flow cytometry. Numbers on the histograms indicate percentage of the cells in each quadrant. (B) Expression of CD86, MHC class II, CD11c, PD-L1, and PD-L2 on ant.EP2-DCs (PF04418948, 10 μM), ant.EP4-DCs (L-161,982, 10 μM), and control DCs was analyzed by flow cytometry. Gray-filled area and open area indicate isotype binding and specific specific binding, respectively. Numbers in the histogram indicates the percentage of positive cells (*upper*) and MFI (*lower*, in parenthesis) of the cells.

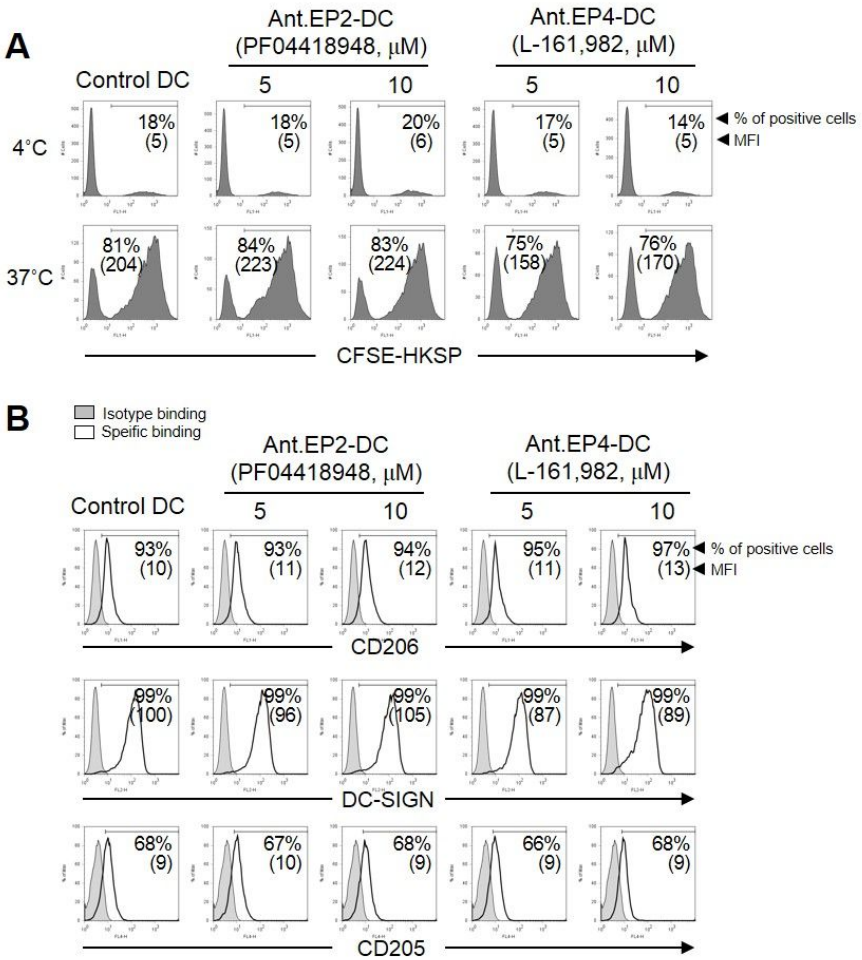

Figure 18. Inactivation of EP4 signaling attenuates phagocytic abilities of DCs. Human monocytes (2×10^6 cells/ml) were differentiated into DCs in the presence of PF04418948 (5 and 10 μM) or L-161,982 (5 and 10 μM) for 5 days. As a vehicle control, the monocytes were treated with 0.05% DMSO, and then differentiated into DCs. (A) DCs (5×10^4 cells) were incubated with CFSE-labeled HKSP (5×10^6 CFU) for 1 h at 4˚C (bacterial binding) and at 37˚C (bacterial uptake), respectively, followed by flow cytometric analysis. Actual phagocytosis can be calculated by subtracting MFI at 4˚C from MFI at 37˚C. (B) Expression of CD206, DC-SIGN, and CD205 was analyzed by flow cytometry. Gray-filled area and open area indicate isotype binding and specific specific binding, respectively. Numbers in the histograms indicate the percentage of positive cells (*upper*) and MFI (*lower*, in parenthesis) of the cells, respectively.

To ascertain immunological functions of ant.EP2-DCs and ant.EP4-DCs, the DCs were stimulated with HKSP, and the expression of maturation markers including co-stimulatory molecules, MHC class II, and programmed death-ligands on the DCs was analyzed. Ant.EP4-DCs stimulated with HKSP exhibited remarkably attenuated induction of CD83 and CD86 in comparison with those of control DCs (Fig. 19A). With respect to the expression of co-inhibitory molecules, PD-L1 and PD-L2 were slightly reduced in ant.EP4-DCs (Fig. 19B). On the other hand, HKSP-stimulated ant.EP2-DCs did not show significant differences in the expression levels of these markers compared to those of HKSP-stimulated control DCs (Fig. 19A and B). Next, production of pro-inflammatory and anti-inflammatory cytokines by the DCs were measured. Ant.EP4-DCs exhibited markedly reduced production of IL-12p70 but enhanced expression of IL-10 by the stimulation with HKSP. Additionally, ant.EP2-DCs showed increase in the production of IL-10 but not IL-12p70 (Fig. 20A and C). However, no significant differences in the production of TNF-α were observed among the DCs (Fig. 20B). Taken together, endogenous PGE2 signaling through EP4 is essential factor for inducing phenotypic maturation and IL-12p70 production of the DCs.

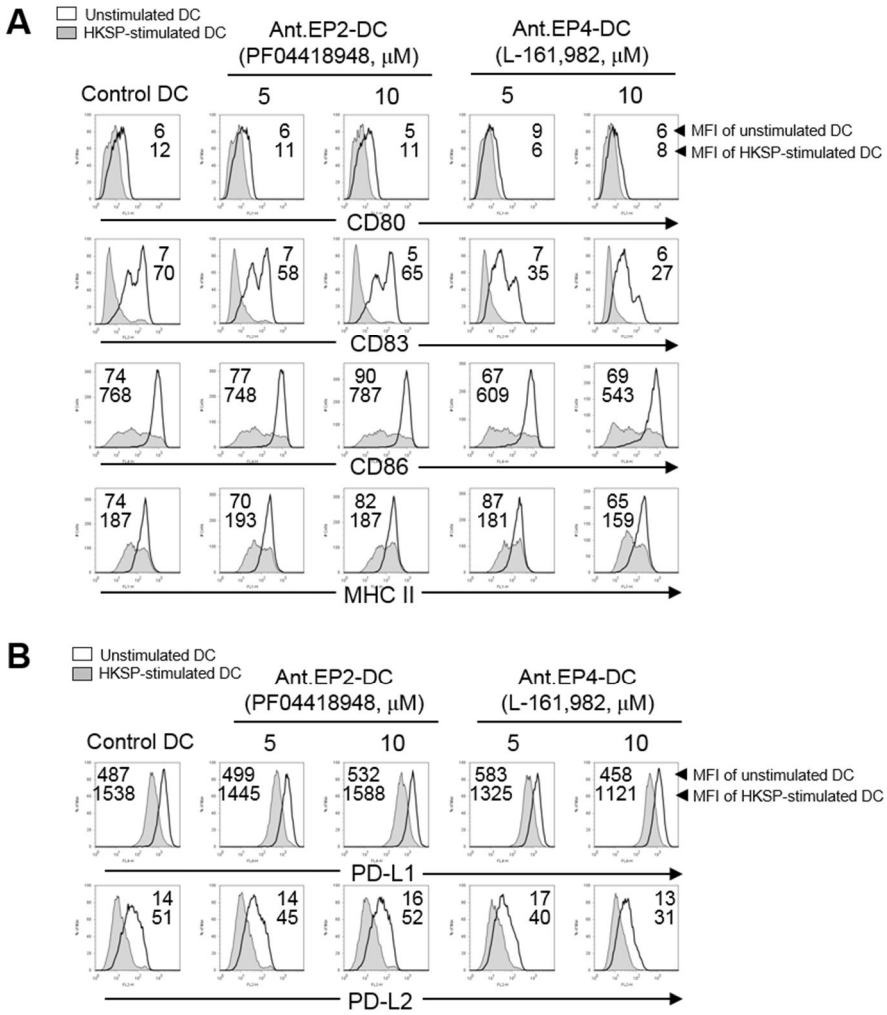

Figure 19. Inactivation of EP4 signaling attenuates maturation of DCs upon stimulation with HKSP. Monocytes (2×10^6 cells/ml) were treated with PF04418948 (5 and 10 μM), L-161,982 (5 and 10 μM), or DMSO (0.05%), and then the cells were differentiated into DCs in the presence of GM-CSF and IL-4 for 5 days. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24 h. (A) Expression of CD80, CD83, CD86, MHC class II, (B) PD-L1, and PD-L2 was analyzed by flow cytometry. Gray-filled area and open area indicate unstimulated and HKSP-stimulated DCs, respectively. Numbers on each histogram indicate MFI of the DCs (*upper*: MFI of unstimulated DCs, *lower*: MFI of HKSP-stimulated DCs).

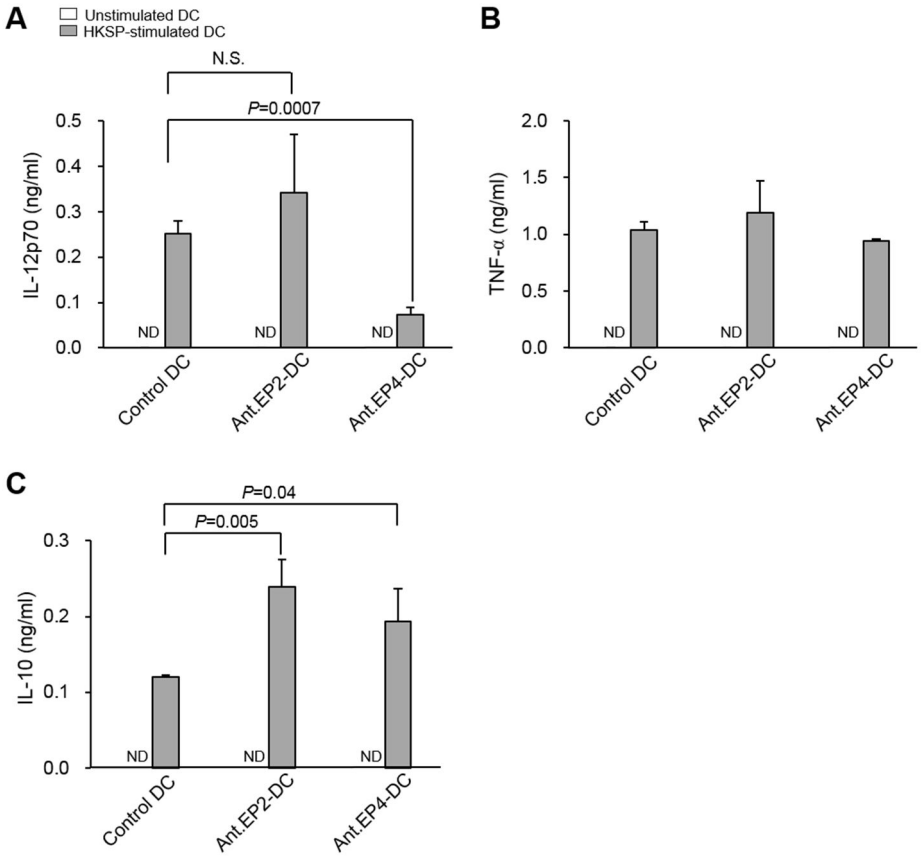

Figure 20. Inactivation of EP4 signaling reduces HKSP-induced IL-12p70 production of DCs. Monocytes (2×10^6 cells/ml) were treated with PF04418948 (10 μM), L-161,982 (10 μM), or DMSO (0.05%), and then the cells were differentiated into DCs in the presence of GM-CSF and IL-4 for 5 days. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24 h. The amount of (A) IL-12p70, (B) TNF-α, or (C) IL-10 in the culture supernatant was quantified by ELISA. Levels of the cytokines were indicated as mean ± SD and statistical differences between the compared experimental groups were analyzed Student's unpaired *t*-test. Representative data are shown among three similar experiments. ND and N.S. denote 'not detected' and 'not significant', respectively.

2.2. EP4 signaling is important for DC-mediated T lymphocyte activation.

To examine T lymphocyte-activating capacities of ant.EP2-DCs and ant.EP4-DCs, the DCs were stimulated with HKSP and co-cultured with autologous $CD3^+$ T lymphocytes, and then proliferation, activation marker expression, and cytokine production of the T lymphocytes were analyzed. Ant.EP2-DCs did not show significant differences in the induction of proliferation and activation of $CD3^+$ T lymphocytes compared to control DCs (Fig. 21A). However, ant.EP4-DCs exhibited attenuated T lymphocytes activating capacities (Fig. 21B). In addition, subsets of T lymphocytes including $CD4^+$ Th and $CD8^+$ CTL exhibited attenuated proliferation and expression of CD25 when they were co-cultured with ant.EP4-DCs (Fig. 22A and B). Furthermore, ant.EP4-DCs negligibly induced differentiation of IFN-γ-producing T lymphocytes (Fig. 23). These results indicate that endogenous PGE2 signaling via EP4 is important for inducing immunogenic DCs stimulating T lymphocyte proliferation and activation.

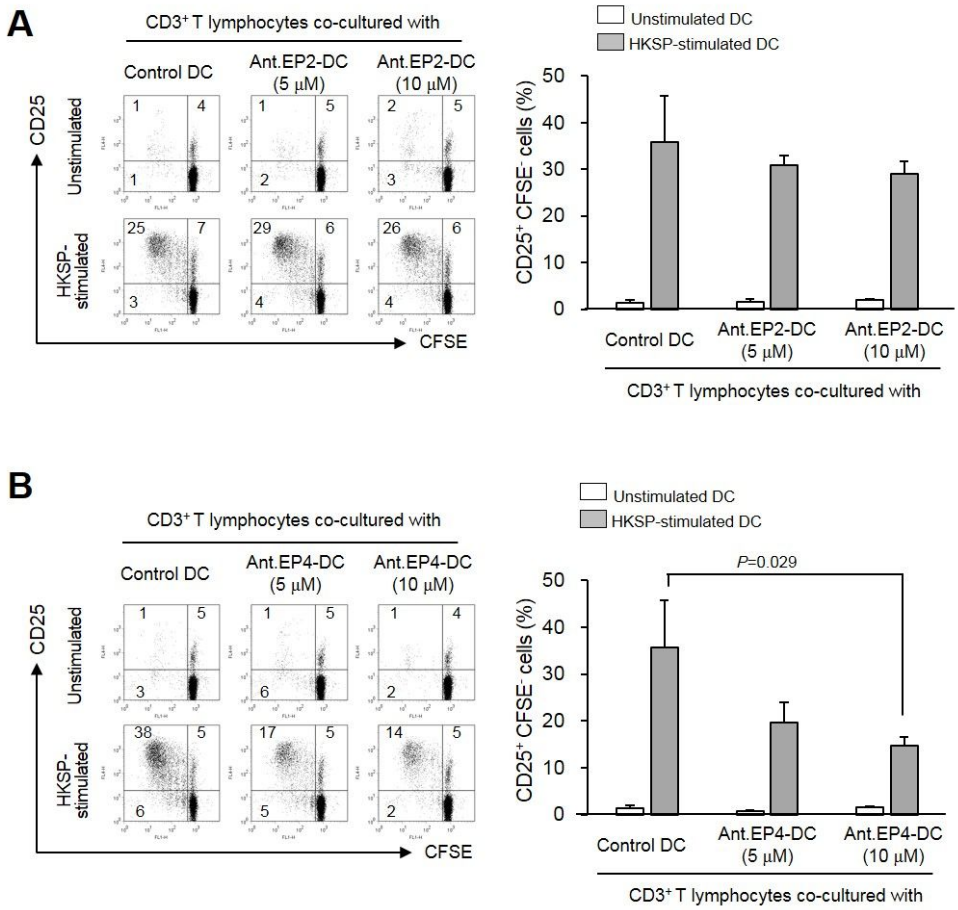

Figure 21. Inactivation of EP4 signaling impairs DC-mediated proliferation and activation of autologous T lymphocytes. Monocytes (2×10^6 cells/ml) were treated with either PF04418948 (5 and 10 μM) or L-161,982 (5 and 10 μM) and differentiated into DCs for 5 day. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h, and then the unstimulated or HKSP-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (5×10^4 cells) for 4 days. Proliferation and CD25 expression of the T lymphocytes induced by (A) ant.EP2-DCs or by (B) ant.EP4-DCs were analyzed by flow cytometry. Numbers on the histograms indicate percentage of cells in each quadrant. Bar graphs on the right panel represent the mean value of triplicate measurements. Frequencies of the activated T lymphocytes were indicated as mean ± SD and statistical differences between the compared experimental groups were analyzed Student's unpaired *t*-test. Representative data are shown among three similar experiments.

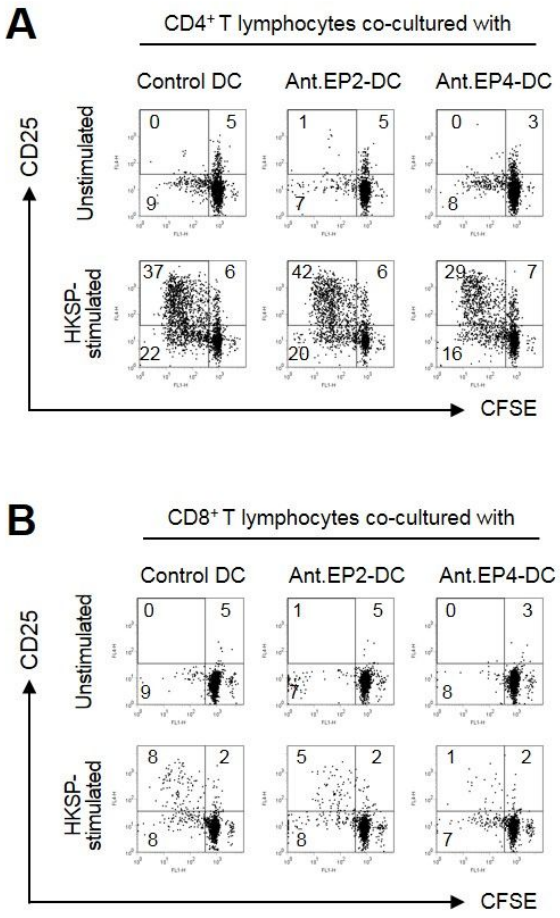

Figure 22. Blockade of EP4 signaling attenuates DC-mediated proliferation and activation of T lymphocyte subsets. Monocytes (2×10^6 cells/ml) were treated with either PF04418948 (10 μM) or L-161,982 (10 μM), and then differentiated into DCs for 5 day. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h, and then the unstimulated or HKSP-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (5×10^4 cells) for 4 days. Proliferation and CD25 expression of (A) $CD4^+$ T lymphocytes and (B) $CD8^+$ T lymphocytes were analyzed by flow cytometry. Numbers on the histograms indicate percentage of cells in each quadrant.

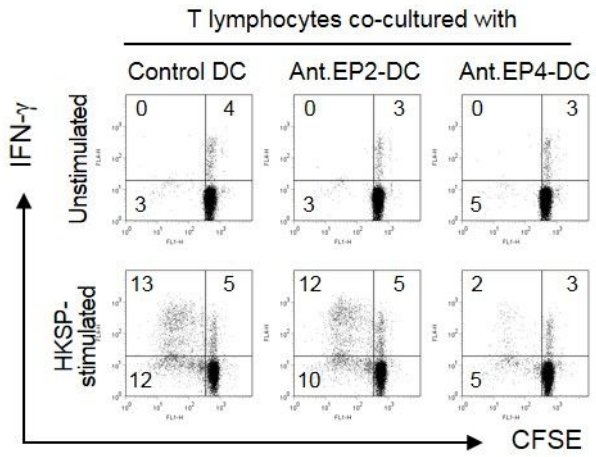

Figure 23. Inactivation of EP4 signaling hampers DC-mediated induction of IFN-γ-producing T lymphocytes. Monocytes (2×10^6 cells/ml) were treated with PF04418948 (10 μM), L-161,982 (10 μM), or DMSO (0.05%), and then the cells were differentiated into DCs in the presence of GM-CSF and IL-4 for 5 days. DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h, and then the unstimulated or HKSP-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (5×10^4 cells) for 4 days. The T lymphocytes were re-stimulated with PMA (0.5 μM) and ionomycin (1 μg/ml) for 4 h before harvest in the presence of brefeldin A (1 μg/ml), and then the frequencies of IFN-γ-producing T lymphocytes were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of T lymphocytes in each quadrant.

2.3. Blockade of EP4 signaling potentiates tolerogenic properties of DCs.

To ascertain whether inactivation of EP4 signaling renders the DCs to be tolerogenic, suppressive functions of ant.EP4-DCs were examined in the activation of T lymphocytes. Ant.EP4-DCs and control DCs were separately stimulated with HKSP, and then the cells were mixed together at control DC to ant.EP4-DC ratios 10:1, 2:1, and 1:1. The mixed DCs were subsequently co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes, and then the proliferation and activation marker expression of the T lymphocytes were analyzed. Ant.EP4-DCs potently suppressed proliferation and CD25 expression of T lymphocytes induced by control DCs as well as they merely exhibited weak T lymphocyte-activating capacities (Fig. 24A). To verify whether the suppressive effects on T lymphocytes activation were specific to ant.EP4-DC, HKSP-stimulated monocytes, instead of ant.EP4-DCs, were mixed with control DCs as indicated culture ratios (10:1, 2:1, and 1:1) and co-cultured with T lymphocytes. Unlike ant.EP4-DCs, monocytes could not suppress the activation of T lymphocytes induced by control DCs (Fig. 24B). These results suggest that inactivation of EP4 signaling triggers tolerogenic DCs with potent suppressive effects on activation of T lymphocytes.

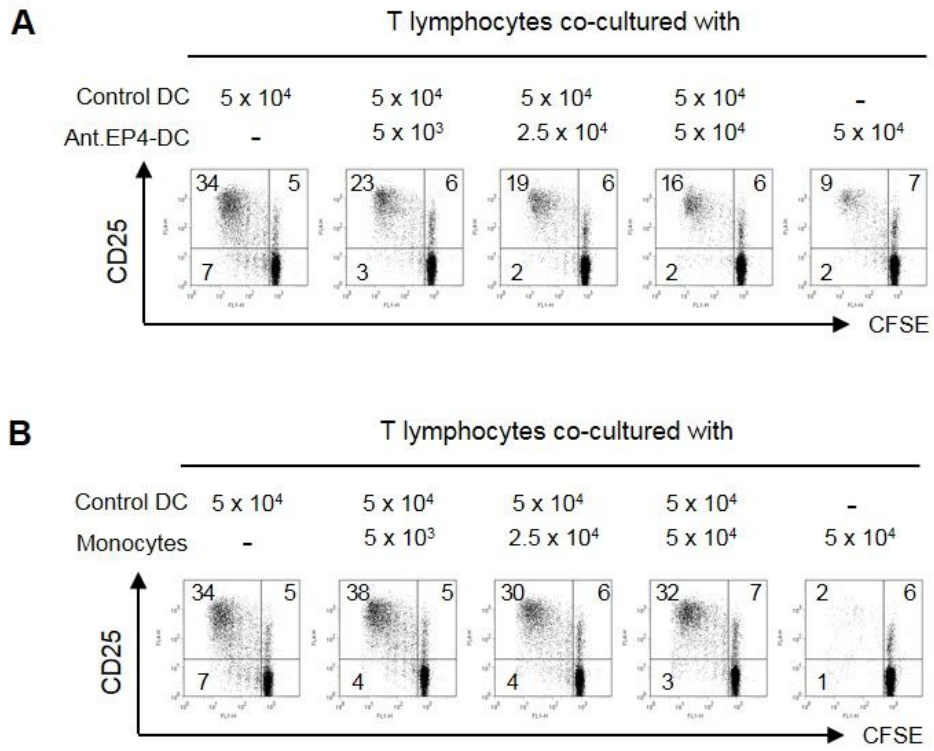

Figure 24. DCs differentiated in the presence of EP4 antagonist exert suppressive functions on the activation of autologous T lymphocytes. DCs (5×10^4 cells) or undifferentiated monocytes (5×10^4 cells) derived from same donor were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h. (A) HKSP-stimulated control DCs (5×10^4 cells) and HKSP-stimulated ant.EP4-DCs (5×10^4 cells) were cultured separately or simultaneously at various culture ratios indicated in the figure. The DCs were co-cultured with CFSE-labeled autologous T lymphocytes (5×10^4 cells) for 4 days. (B) HKSP-stimulated control DCs (5×10^4 cells) and HKSP-stimulated monocytes (2.5×10^5 cells/ml) were cultured separately or simultaneously at various ratios as indicated in figure. The mixed cells were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (2.5×10^5 cells/ml) for 4 days. Proliferation and CD25 expression of the T lymphocytes were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant.

2.4. PGE2 distinctively regulates immunological characteristics of DCs by selectively activating of EP2 and/or EP4 signals.

Previous findings have shown that DCs differentiated from PGE2-stimulated monocytes produced IL-10 rather than IL-12p70, and weakly activated T cell responses [17]. However, some of the former studies demonstrated positive effects of PGE2 in DC-mediated type I T cell responses [86]. To clarify the specific functions of PGE2 on determination of the immunological characteristics of DCs, monocytes were stimulated with PGE2 at various concentrations and differentiated into DCs. Similar expression levels of CD1a and CD14 were observed in DCs differentiated from monocytes stimulated with low doses of PGE2 (~1 nM, $PGE2^{low}$-DCs) to those of control DCs differentiated without PGE2. However, $CD1a^{-}CD14^{+}$ cells were increased in DCs differentiated with high doses of PGE2 (10 to 100 nM, $PGE2^{high}$-DCs) (Fig. 25A). $PGE2^{low}$-DCs efficiently up-regulated expression of CD80, CD83, and CD86 in response to the stimulation with HKSP, but $PGE2^{high}$-DCs exhibited weak induction of these markers even with HKSP stimulation (Fig. 25B). Significantly enhanced production of IL-12p70 was observed in $PGE2^{low}$-DCs stimulated with HKSP, but the IL-10-producing abilities of the cells were markedly reduced. In contrast, $PGE2^{high}$-DCs highly produced IL-10 in response to HKSP rather than IL-12p70 (Fig. 26A and B). Concomitantly, $PGE2^{low}$-DCs potentiated the proliferation, CD25 expression, and IFN-γ production of autologous T lymphocytes, but $PGE2^{high}$-DCs scarcely induced these responses in the T lymphocytes (Fig. 26C and D). These results suggest that PGE2 differently regulates immunological characteristics of DCs depending on its concentrations.

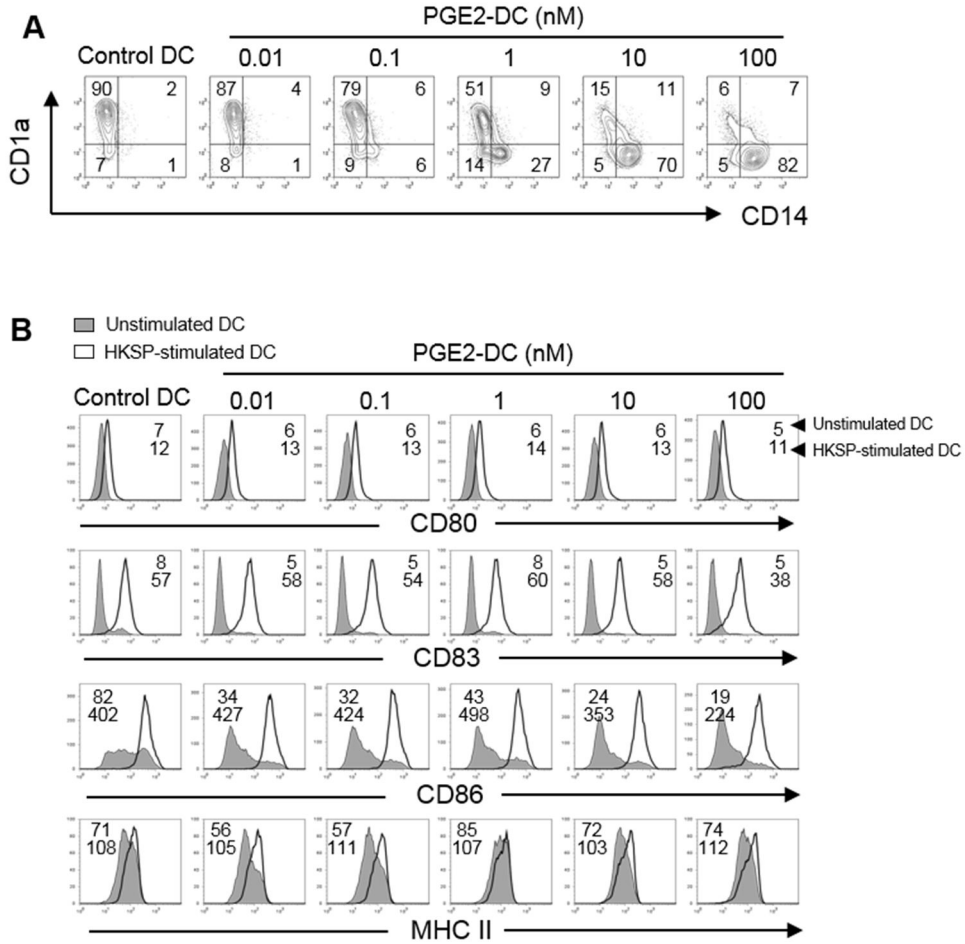

Figure 25. PGE2 alters expression of phenotypic markers of DCs. Monocytes (2×10^6 cells/ml) were stimulated with various concentrations of PGE2 (0.01, 0.1, 1, 10, and 100 nM) for 24 h in the presence of GM-CSF and IL-4. After removal of residual PGE2, the monocytes were further differentiated into DCs for another 4 days. (A) Expression of CD1a and CD14 on the DCs was analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant. (B) DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24 h and expression of CD80, CD83, CD86, and MHC class II was analyzed by flow cytometry. Gray-filled area and open area indicate unstimulated DCs and HKSP-stimulated DCs, respectively. Numbers on the histograms indicate MFI of the unstimulated DCs (*upper*) and MFI of HKSP-stimulated DCs (*lower*).

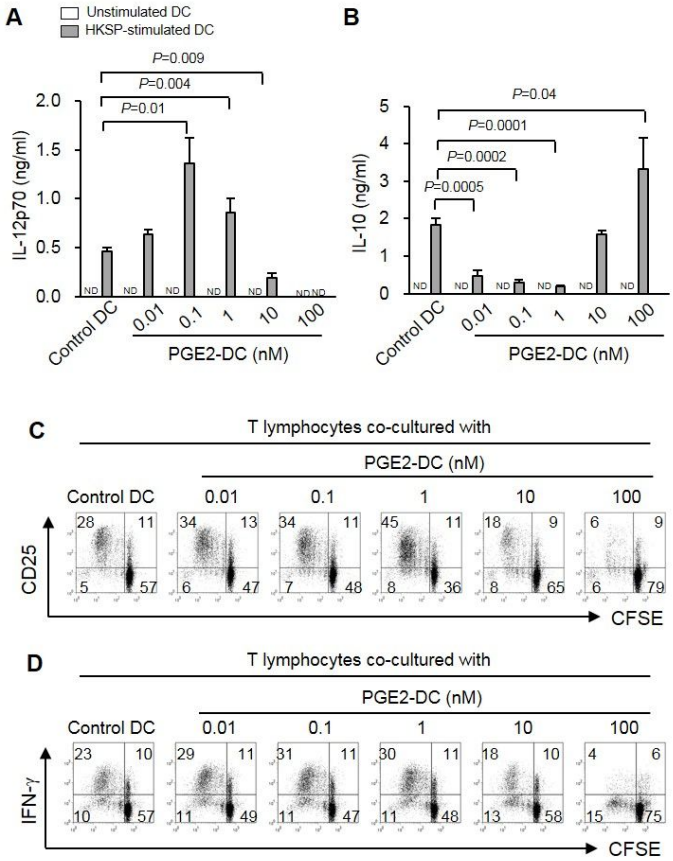

Figure 26. P PGE2 differently regulates DC-mediated immune responses depending on its concentrations. Monocytes (2×10^6 cells/ml) were stimulated with various concentrations of PGE2 (0.01, 0.1, 1, 10, and 100 nM) for 24 h in the presence of GM-CSF and IL-4. After removal of residual PGE2, the monocytes were further differentiated into DCs for another 4 days. (A and B) DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 12 h or 24 h, and then the amount of IL-12p70 and IL-10 in the culture supernatant was measured by ELISA. (C and D) The HKSP-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (5×10^4 cells) for 4 days. (C) Proliferation and CD25 expression on the T lymphocytes were analyzed by flow cytometry. (D) The T lymphocytes were re-stimulated with PMA (0.5 μM) and ionomycin (1 μg/ml) for 4 h in the presence of brefeldin A (1 μg/ml) before harvest, and then frequencies of IFN-γ-producing T lymphocytes were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of T lymphocytes in each quadrant.

To ascertain the mechanism by which different doses of PGE2 oppositely regulate the immunological properties of the DCs, it was examined whether the DC precursors selectively activate EP2 and EP4 signals in response to different concentrations of PGE2. Monocytes were stimulated with either low dose (1 pM, $PGE2^{low}$-DCs) or high dose (50 nM, $PGE2^{high}$-DCs) of PGE2 in the presence of its receptor antagonists, and then the cells were differentiated into DCs. As previous data shown, $PGE2^{low}$-DCs and $PGE2^{high}$-DCs exhibited different potencies in the induction of IL-12p70 and T lymphocyte activation (Fig. 27A to C). $PGE2^{low}$-DCs potentiated production of IL-12p70, T lymphocyte proliferation, and activation. However, the enhanced stimulatory functions were abrogated in $PGE2^{low}$-DCs differentiated with EP4 antagonist (Fig. 27B and C). In contrast, $PGE2^{high}$-DCs containing less $CD1a^{+}CD14^{-}$ cells showed attenuated IL-12p70 production and T lymphocyte-activating capacities. Treatment with EP2 antagonist increased frequency of the $CD1a^{+}CD14^{-}$ cells in $PGE2^{high}$-DCs (Fig. 27A). In addition, treatment with either EP2 antagonist or EP4 antagonist partially restored IL-12p70-production and T lymphocyte-activating capacities of $PGE2^{high}$-DCs. These results indicate that low doses of PGE2 potentiate immunogenic properties of the DCs by selectively activating EP4 signaling, but high doses of PGE2 diminish the stimulatory characteristics of the cells by co-activating EP2 and EP4 signals. Taken together, PGE2 differentially regulates immunogenic and tolerogenic characteristics of DCs by distinctively activating EP4 and/or EP2 signals.

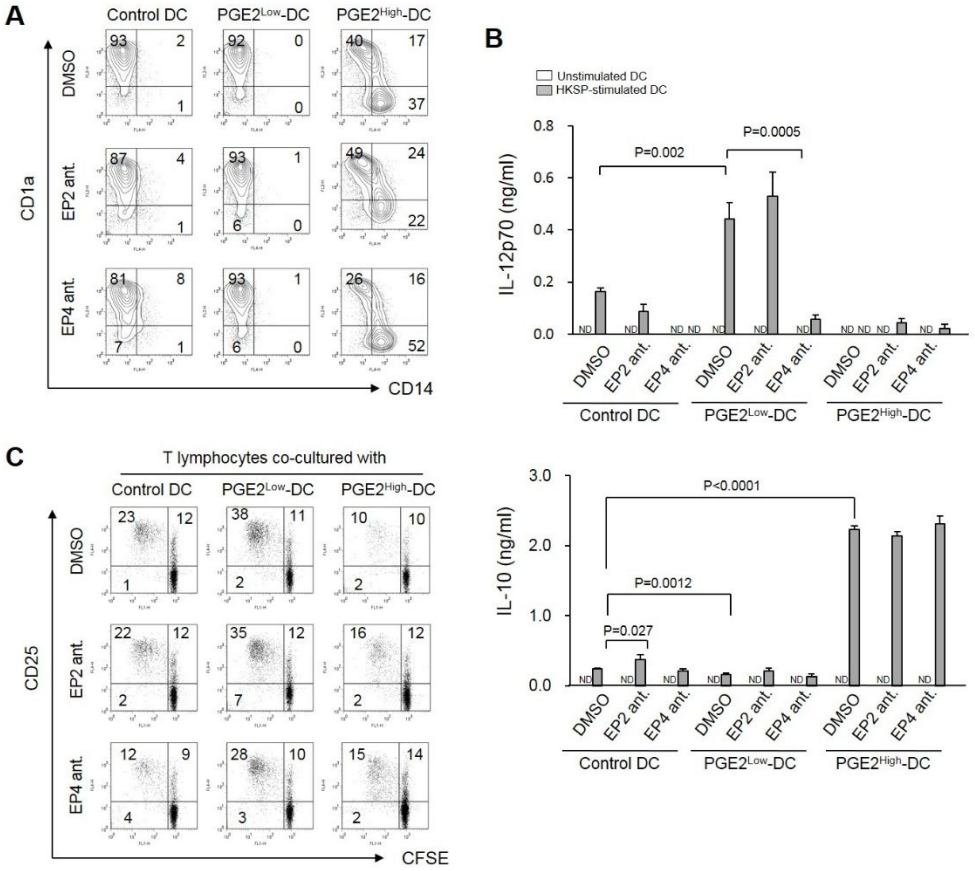

Figure 27. PGE2 distinctively modulates DC-mediated immune responses by selective activation of EP2 and/or EP4 signals. Monocytes (2×10^6 cells/ml) were pretreated with either EP2 antagonist (5 μM) or EP4 antagonist (5 μM) for 1 h, and then the cells were stimulated with PGE2 (1 pM or 50 nM) for 24 h in the presence of GM-CSF and IL-4. After removal of residual PGE2, the monocytes were further differentiated into DCs for another 4 days. (A) Expression of CD1a and CD14 on the DCs was analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant. (B) DCs (2.5×10^5 cells/ml) were stimulated with HKSP (2.5×10^6 CFU/ml) for 24 h, and then the amount of IL-12p70 and IL-10 in the culture supernatant was measured by ELISA. (C) The HKSP-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (5×10^4 cells) for 4 days. Proliferation and CD25 expression on the T lymphocytes were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of T lymphocytes in each quadrant.

3. Immunomodulatory effects of human UCB-MSCs on immunological characteristics of DCs

3.1. UCB-MSCs modulate phenotypic characteristics of DCs.

Human MSCs have been known to suppress activation of various immune cells including T lymphocytes and natural killer (NK) cells [22, 87]. In the present study, immunomodulatory effects of MSCs derived from UCB (UCB-MSCs) on differentiation and activation of DCs were examined. Monocytes were co-cultured with UCB-MSCs at various MSC to monocyte ratios and differentiated into DCs (MSC-DCs). Similar expression levels of CD1a and CD14 was observed in MSC-DCs differentiated at low MSC to monocyte ratios ($1:10^6$ to $1:10^4$, MSC^{low}-DCs) compared to those of control DCs differentiated without UCB-MSCs. However, MSC-DCs differentiated at high MSC to monocyte ratios ($1:10^3$ to 1:30, MSC^{high}-DCs) displayed $CD1a^{low/-}$and $CD14^+$ phenotypes (Fig. 28A). MSC^{high}-DCs expressed higher expression levels of CD80, MHC class II, DC-SIGN, PD-L1 and PD-L2, but lower levels of CD86 than control DCs. On the other hand, MSC^{low}-DCs showed similar expression levels of these molecules compared to those of control DCs (Fig. 28B). These results indicate that MSC-DCs differentiated at different MSC to monocyte ratios exhibit different expression levels of their phenotypic markers, suggesting that UCB-MSCs alter phenotypic characteristics of DCs.

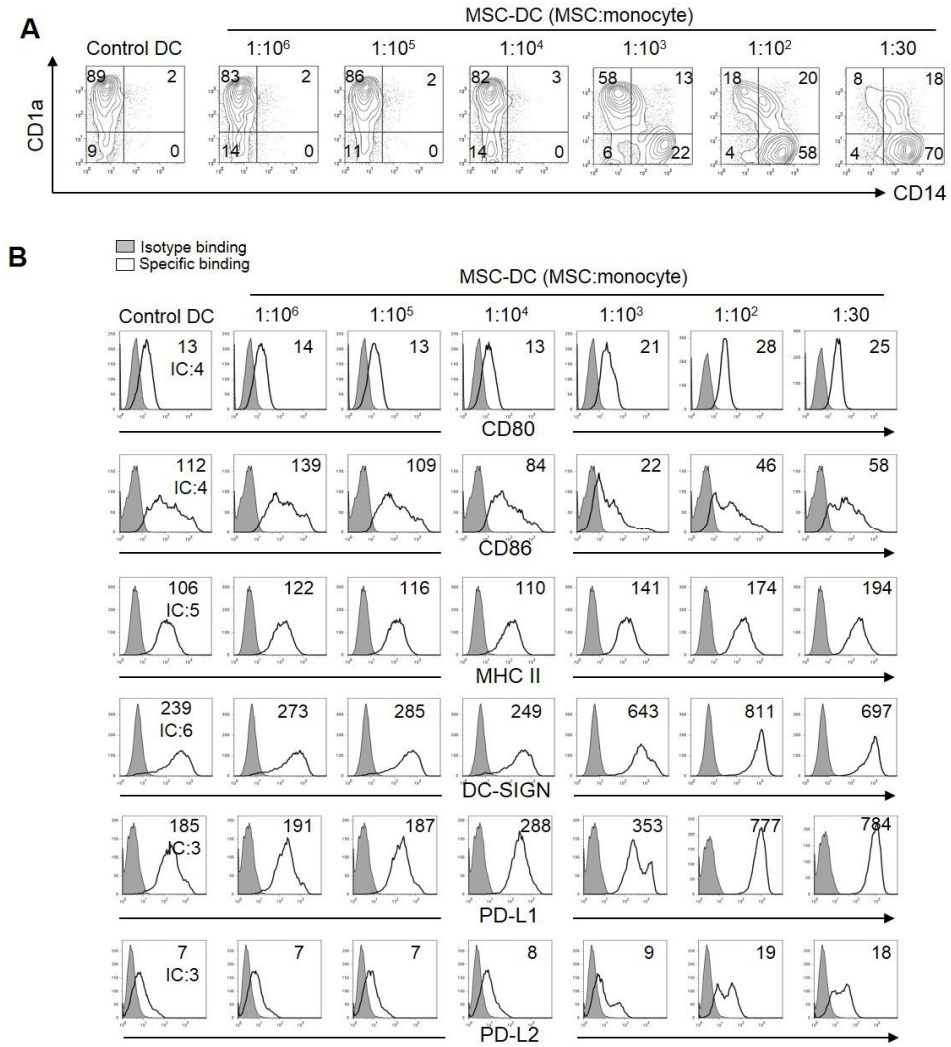

Figure 28. UCB-MSCs alter phenotypes of DCs. Monocytes (2×10^6 cells/ml) were co-cultured with UCB-MSC at various MSC to monocyte ratios ($1:10^6$, $1:10^5$, $1:10^4$, $1:10^3$, $1:10^2$, and 1:30), and the monocytes were differentiated into DCs in the presence of GM-CSF and IL-4 for 5 days. (A) Expression of CD1a and CD14 on the DCs was analyzed by flow cytometry. Numbers on the histograms indicate the percentage of the cells in each quadrant. (B) Expression of phenotypic markers of DCs including co-stimulatory molecules, MHC class II, DC-SIGN, and programmed death-ligands was analyzed by flow cytometry. Gray-filled area and open area indicate isotype binding and specific binding, respectively. Numbers on the histograms indicate MFI of DCs.

3.2. DCs differentiated at different MSC to monocyte ratios exhibit distinctive immunological characteristics.

To examine immunological characteristics of MSC-DCs, phenotypic changes, cytokine production, and T lymphocyte-activating capacities of the DCs were analyzed. MSC^{low}-DCs stimulated with LPS efficiently up-regulated expression of CD83, CD86, and PD-L1 on the surface, whereas MSC^{high}-DCs showed attenuated induction of CD83 and CD86, but enhanced expression of PD-L1 (Fig. 29). In response to LPS, MSC^{low}-DCs and MSC^{high}-DCs exhibited difference in the production of IL-12p70. MSC^{low}-DCs showed higher expression level of IL-12p70 than that of control DCs, but MSC^{high}-DCs could not produce IL-12p70 (Fig. 30A). However, both MSC^{low}-DCs and MSC^{high}-DCs attenuated production of TNF-α and IL-10 compared to those of control DCs (Fig. 30B and C). When MSC-DCs were co-cultured with either autologous PBMCs or T lymphocytes, MSC^{low}-DCs and MSC^{high}-DCs oppositely regulated activation of T lymphocytes. PBMCs co-cultured with LPS-stimulated MSC^{low}-DCs showed enhanced production of IFN-γ with slight increase of IL-10 expression. In contrast, PBMCs co-cultured with LPS-stimulated MSC^{high}-DCs preferentially expressed IL-10 rather than IFN-γ (Fig. 31A and B). In addition, MSC^{low}-DCs efficiently induced proliferation and expression CD25 of the T lymphocytes more than control DCs did. However, MSC^{high}-DCs failed to elicit proliferation and activation of the T lymphocytes (Fig. 31C). All these results suggest that MSC-DCs differentiated at different MSC to monocyte ratios have different stimulatory potencies in phenotypic maturation, cytokine production, and T lymphocyte activation.

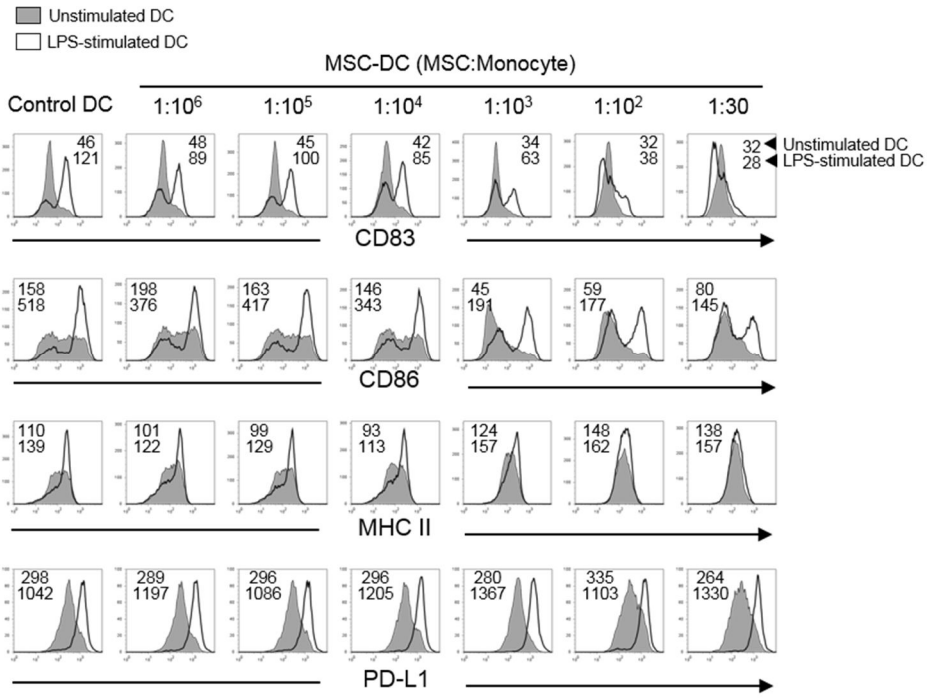

Figure 29. UCB-MSCs attenuate maturation of DCs. Monocytes (2×10^6 cells/ml) were co-cultured with UCB-MSC at various MSC to monocyte ratios ($1:10^6$, $1:10^5$, $1:10^4$, $1:10^3$, $1:10^2$, and 1:30), and the monocytes were differentiated into DCs in the presence of GM-CSF and IL-4 for 5 days. DCs (2.5×10^5 cells/ml) were stimulated with LPS (100 ng/ml) for 24 h, and then the expression of CD80, CD83, MHC class II, and PD-L1 on the DCs were analyzed by flow cytometry. Gray-filled area and open area indicate unstimulated cells and LPS-stimulated cells, respectively. Numbers on the histograms indicate MFI of the unstimulated (*upper*) and LPS-stimulated (*lower*) DCs.

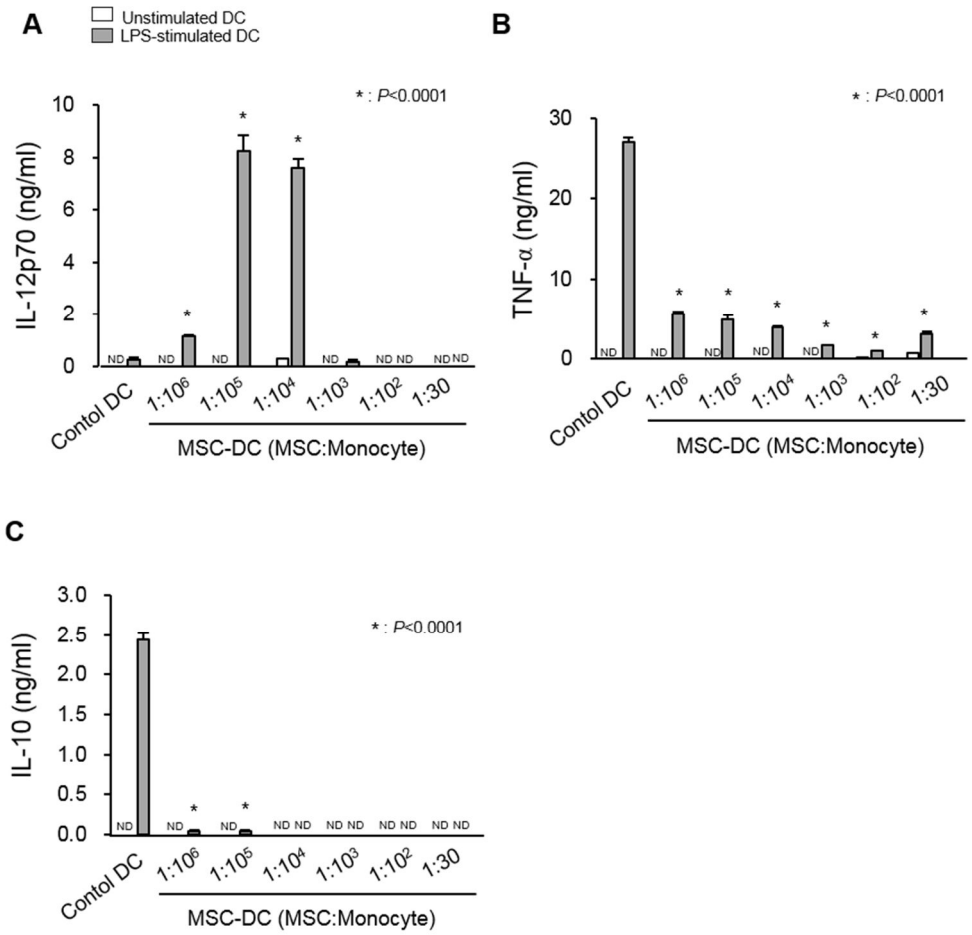

Figure 30. UCB-MSCs attenuate cytokine-producing capacity of DCs except for IL-12p70. Monocytes (2×10^6 cells/ml) were co-cultured with UCB-MSC at various MSC to monocyte ratios ($1:10^6$, $1:10^5$, $1:10^4$, $1:10^3$, $1:10^2$, and 1:30), and the monocytes were differentiated into DCs in the presence of GM-CSF and IL-4 for 5 days. DCs (2.5×10^5 cells/ml) were stimulated with LPS (100 ng/ml) for 24 h. Amount of (A) IL-12p70, (B) TNF-α, and (C) IL-10 in the culture supernatant was measured by ELISA. Levels of the cytokines were indicated as mean ± SD and statistical differences between the compared experimental groups were analyzed Student's unpaired *t*-test.

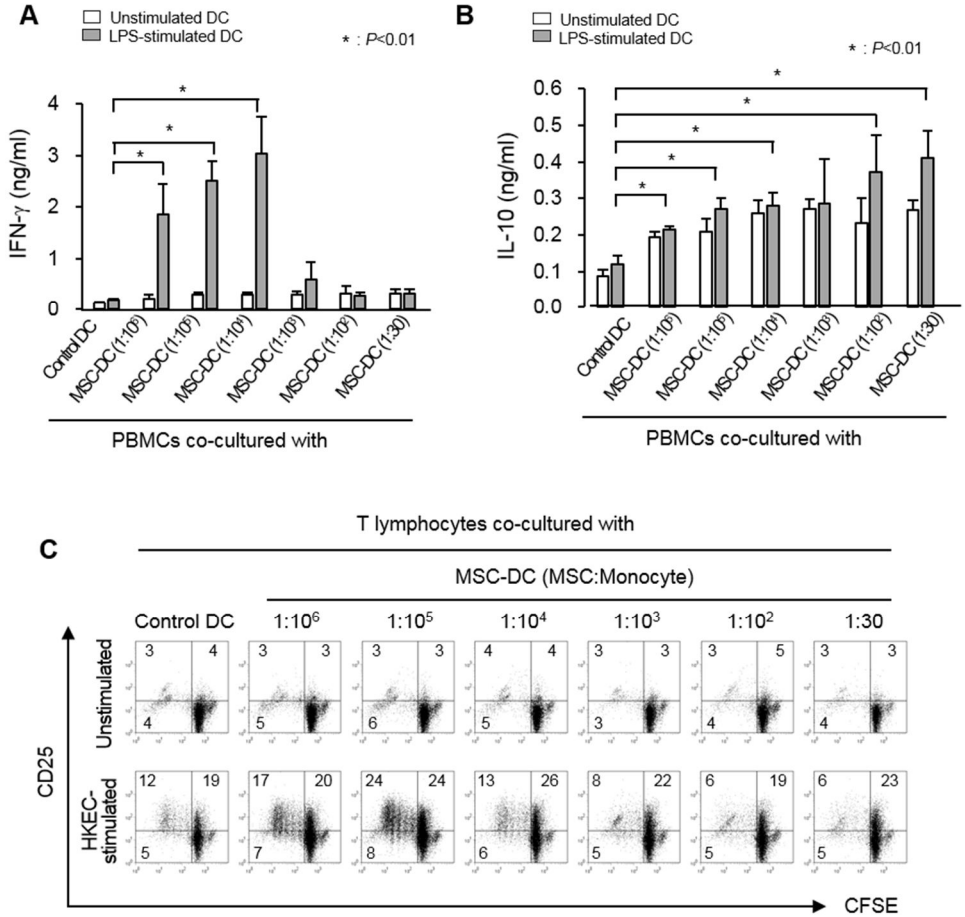

Figure 31. DCs differentiated at different MSC to monocyte ratios exhibit distinctive stimulating potencies in T lymphocyte activation. Monocytes (2×10^6 cells/ml) were co-cultured with UCB-MSC at various MSC to monocyte ratios ($1:10^6$ to 1:30) and differentiated into DCs in the presence of GM-CSF and IL-4 for 5 days. (A and B) The DCs (2.5×10^5 cells/ml) were stimulated with LPS (100 ng/ml) for 24 h, and then the DCs were co-cultured with monocyte-depleted autologous PBMCs for 3 days. Amount of IFN-γ and IL-10 in the culture supernatant was measured by ELISA. (C) DCs (2.5×10^5 cells/ml) were stimulated with HKEC (2.5×10^6 CFU/ml) for 12 h, and then the unstimulated and HKSP-stimulated DCs were co-cultured with CFSE-labeled autologous $CD3^+$ T lymphocytes (2.5×10^5 cells/ml) for 4 days. Proliferation and expression of CD25 of T lymphocytes were analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant.

3.3. PGE2 mediates phenotypic and functional alteration of DC induced by UCB-MSCs.

Previously, MSCs were reported to exert their immunomodulatory functions by PGE2-dependent manner [22, 24]. In the present study, involvement of PGE2 in the regulatory mechanism by which UCB-MSCs modulate immunological characteristics of DCs was investigated. Expression of COX2 was examined in monocytes and UCB-MSCs when they were cultured separately or simultaneously to determine the major producer of PGE2. Monocytes increasingly expressed COX2 at co-culture with UCB-MSCs (Fig. 32A). Also, co-culture with monocytes enhanced the expression of COX2 in UCB-MSCs (Fig. 32B). Concomitantly, higher level of PGE2 was detected in co-culture of UCB-MSCs and monocytes than in the separate culture (Fig. 32B). To ascertain the specific roles of PGE2 in determining immunological characteristics of MSC-DCs, MSC^{low}-DCs and MSC^{high}-DCs were differentiated in the presence of COX2 inhibitor, NS398, and then the phenotypic and functional changes of the DCs were analyzed. Treatment with NS398 partially restored expression level of CD1a (Fig. 33A) and increased LPS-induced expression of CD83 and CD86 on MSC^{high}-DCs (Fig. 33B). In addition, MSC^{high}-DCs differentiated with the COX2 inhibitor efficiently augmented proliferation and CD25 expression of the autologous T lymphocytes (Fig. 33D). On the other hand, inhibition of COX2 in MSC^{low}-DCs showed different effects from those in MSC^{high}-DCs. Treatment with NS398 abolished an enhancement of IL-12p70 production and T lymphocyte-activating abilities of MSC^{low}-DCs (Fig. 33C and D). These results indicate that PGE2 derived from UCB-MSCs and/or monocytes differently regulate immunological characteristics of MSC^{low}-DCs and MSC^{high}-DCs.

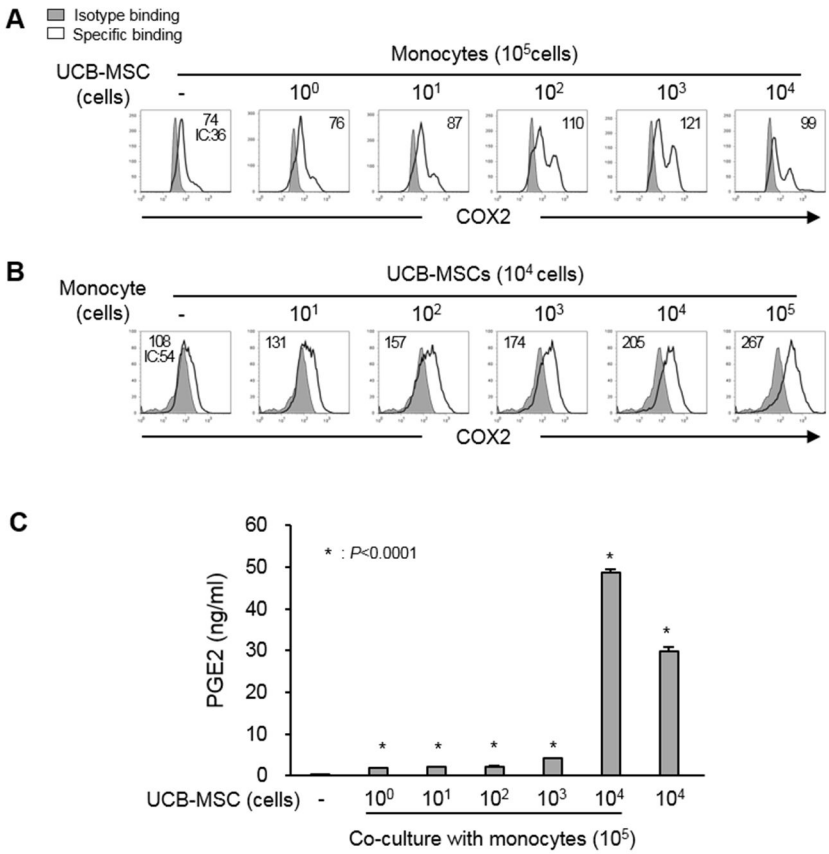

Figure 32. UCB-MSCs augment expression of COX2 and PGE2 by monocytes. (A) Monocytes (1×10^5 cells) were cultured without or with various numbers of UCB-MSCs (from 10^0 to 10^4 cells) for 18 h. (B) UCB-MSC (1×10^4 cells) were cultured without or with various numbers of monocytes (from 10^1 to 10^5 cells) for 18 h. The expression of COX2 in the $CD14^+$ monocytes and $CD14^-$ UCB-MSCs was analyzed by flow cytometry. Gray-filled area and open aria indicate isotype binding and specific binding, respectively. Numbers on the histograms indicate MFI of the cells. (C) The monocytes (1×10^5 cells) were cultured without or with indicated number of UCB-MSCs for 18 h, and then the amount of PGE2 in the culture supernatant was analyzed by ELISA. Levels of PGE2 were indicated as mean ± SD and statistical differences between the compared experimental groups were analyzed Student's unpaired *t*-test.

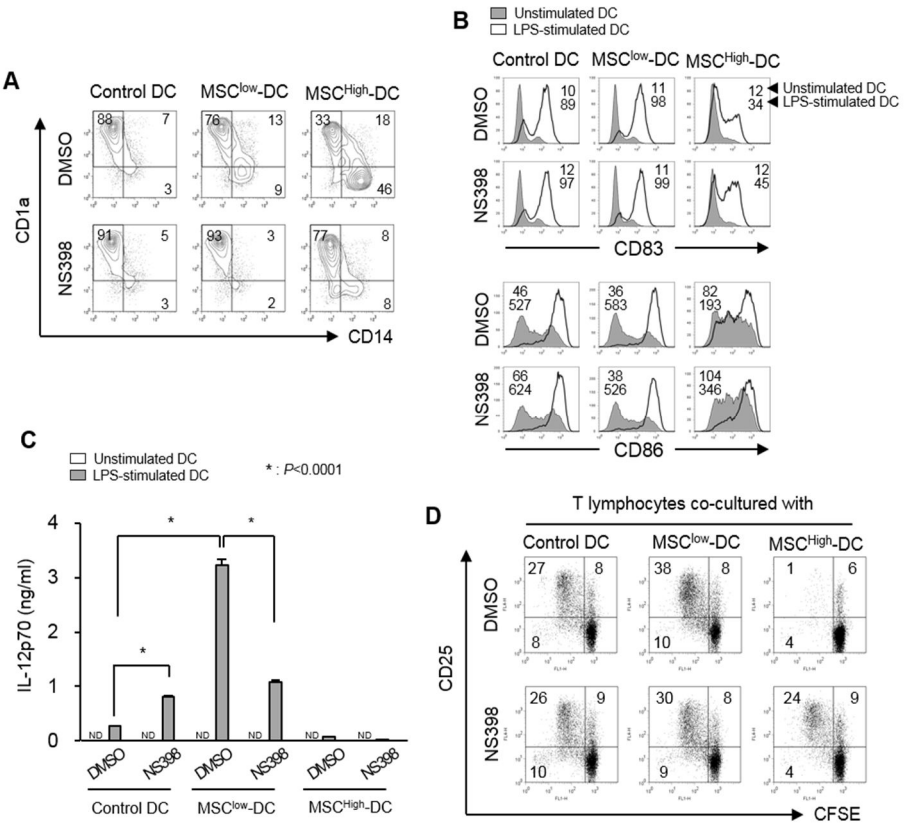

Figure 33. Blockade of PGE2 synthesis abolishes immunomodulatory effects of UCB-MSCs on DC-mediated immune responses. Monocytes (2×10^6 cells/ml) were pretreated with NS398 (5 μM) for 1 h and co-cultured with UCB-MSCs (MSC to monocyte ratios, $1:10^5$ or 1:30), and then the monocytes were differentiated into DCs for 5 days. (A) The expression of CD1a and CD14 on the DCs was analyzed by flow cytometry. Numbers on the histograms indicate the percentage of cells in each quadrant. (B and C) DCs (2.5×10^5 cells/ml) were stimulated with LPS (100 ng/ml) for 24 h, and (B) expression of CD83 and CD86 was analyzed by flow cytometry. Numbers on the histograms indicate MFI of unstimulated DC (*upper*) and LPS-stimulated DC (*lower*). (C) Amount of IL-12p70 was measured by ELISA. (D) DCs (2.5×10^5 cells/ml) were stimulated with HKEC (2.5×10^6 CFU/ml) for 12 h and co-cultured with CFSE-labeled autologous T lymphocyte (2.5×10^5 cells/ml) for 4 days, and then the expression of CD25 and proliferation of the T lymphocytes was analyzed by flow cytometry. Numbers in the histograms indicate the percentage of cells in each quadrant. These data are representative of three similar experiments.

3.4. UCB-MSCs distinctively modulate immunological properties of DC by selectively activating EP2 and/or EP4.

PGE2 conduct various cellular functions in monocytes by stimulating EP2 and/or EP4 receptor signaling [73]. Here, it was examined whether the PGE2 produced by UCB-MSCs and/or monocytes selectively activates its receptor signals through EP2 and/or EP4 and distinctively regulates immunological properties of MSC-DCs. MSC^{low}-DCs and MSC^{high}-DCs were differentiated in the presence of either EP2 antagonist or EP4 antagonist, and then the expression of maturation markers and T lymphocyte-activating capacities of the DCs were analyzed. Inactivation of PGE2 signals through EP2 and EP4 receptors restored HKEC-induced CD83 and CD86 expression on MSC^{high}-DCs (Fig. 34A). In addition, the DC-mediated proliferation and CD25 expression of the T lymphocytes was markedly increased upon inactivation of EP2 and EP4 signaling in MSC^{high}-DCs (Fig. 34C). On the other hand, the enhanced T lymphocyte-activating functions of MSC^{low}-DCs were abrogated only by treatment with EP4 antagonist (Fig. 34C). These data suggest that large number of UCB-MSCs induce DCs with low immunostimulatory functions by activating both EP2 and EP4 signaling, whereas relatively small number of UCB-MSCs potentiate stimulatory properties of the DCs by solely activating EP4 signaling. Taken together, UCB-MSCs differently regulate immunogenic characteristics of DCs by selectively activating PGE2 signals through EP2 and/or EP4.

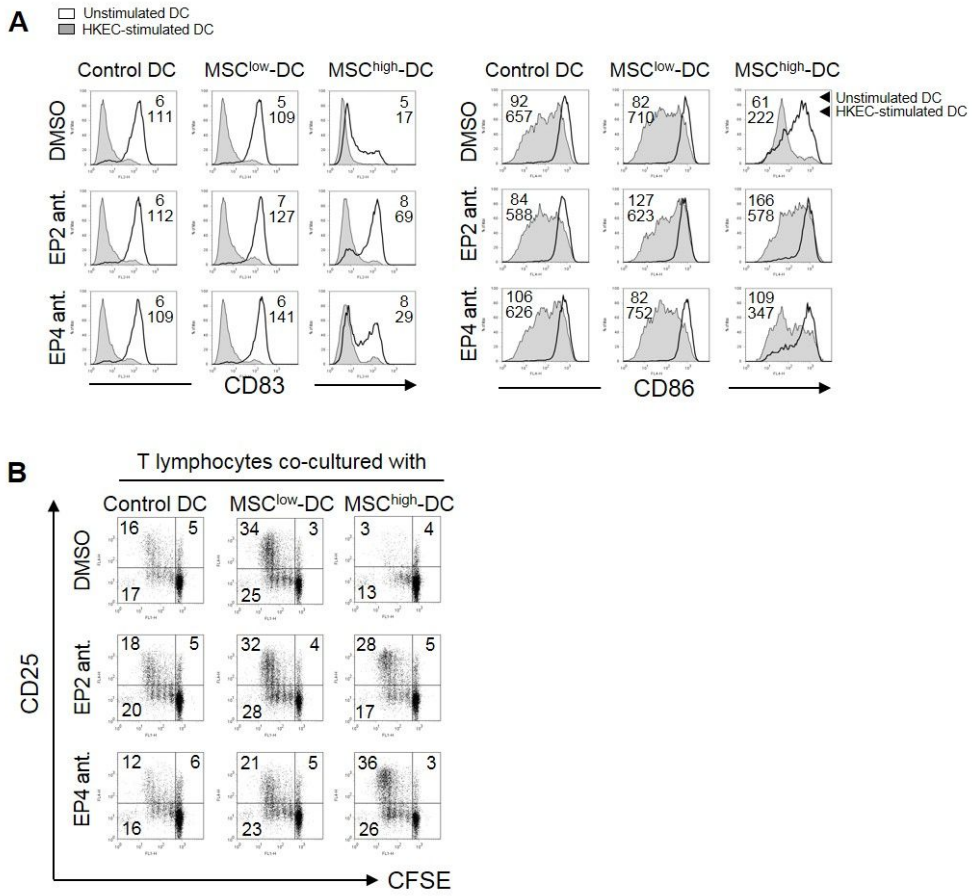

Figure 34. UCB-MSCs separately regulate DC-mediated immune responses by selective activation of EP2 and/or EP4. Monocytes (2×10^6 cells/ml) were pretreated either with EP2 antagonist (5 μM) or EP4 antagonist (5 uM) for 1 h, and then the cells were differentiated into DCs in the presence of UCB-MSCs (MSC to monocyte ratios, $1{:}10^5$ and 1:30) for 5 days. (A) DCs (2.5×10^5 cells/ml) were stimulated with HKEC (2.5×10^6 CFU/ml) for 24 h, and then the expression of CD83 and CD86 on the cells was analyzed by flow cytometry. Gray-filled area and open area indicate unstimulated and HKEC-stimulated DCs, respectively. Numbers on the histograms indicate MFI of unstimulated (*upper*) and HKEC-stimulated (*lower*) cells. (B) The HKEC-stimulated DCs (5×10^4 cells) were co-cultured with CFSE-labeled autologous T lymphocytes (5×10^4 cells) for 4 days and proliferation of T lymphocytes was analyzed by flow cytometry. Numbers on the histograms indicate the percentage of the proliferated cells.

4. Immunological characteristics of human UCB-derived DCs

4.1. UCB-DCs display distinctive features from APB-DCs in phenotypes and endocytic capacity.

The morphology, phenotypes, and endocytic activity of UCB-DCs and APB-DCs were comparatively investigated. Microscopic analyses showed that monocytes and DCs from both UCB and APB displayed similar morphologies (Fig. 35A). UCB-derived monocytes exhibited lower expression of CD14 than APB-derived monocytes. When the monocytes were differentiated into DCs, UCB-DCs displayed less $CD1a^+$ cells but more $CD14^+$ cells than APB-DCs (Fig. 35A and B). UCB-DCs showed significantly lower levels of CD80, MHC class I, MHC class II, and DC-SIGN than APB-DCs, while the expression of CD86, CD205, and CD206 was not significantly different from those of APB-DCs (Fig. 35C to I). UCB-DCs displayed better endocytic activity than APB-DCs (Fig. 35J). These results indicate that UCB-DCs exhibited lower expression of CD1a, CD80, MHC class I and II, and DC-SIGN but higher CD14 expression and endocytic capacity than APB-DCs.

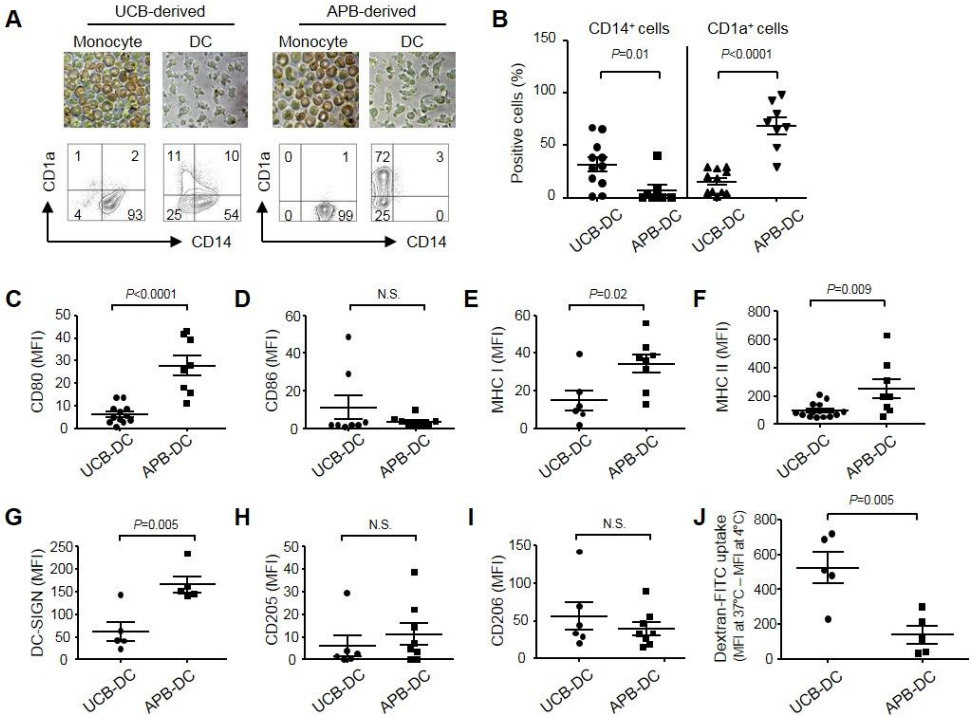

Figure 35. UCB-DCs display distinctive features from APB-DCs in phenotypes and endocytic capacity. $CD14^+$ monocytes (2×10^6 cells/ml) isolated from UCB and APB were differentiated into DCs in the presence of human GM-CSF (800 U/ml) and IL-4 (500 U/ml) for 6 days. (A) Morphology of monocytes and DCs from UCB and APB was observed by microscopic analyses (*upper panel*). The expression of CD1a and CD14 on the cell surface was analyzed before and after differentiation (*lower panel*). (B) Percentages of $CD14^+$ cells or $CD1a^+$ cells in UCB-DCs and in APB-DCs were shown in a scatter plot. Expression of (C) CD80, (D) CD86, (E) MHC class I, (F) MHC class II, (G) DC-SIGN, (H) CD205, and (I) CD206 on the DCs were analyzed by flow cytometry and net MFI of the cells was shown in scatter plots. Net MFI was obtained by subtracting MFI of isotype from that of each protein expression. (J) The cells (1×10^5 cells) were suspended in 50 µl of PBS containing dextran-FITC (1 mg/ml) for 1 h at 4°C (non-specific uptake) and 37°C (specific uptake). Endocytic activity of the DCs was examined by flow cytometric analysis. The vertical axis indicates net MFI values of the DCs subtracting MFI at 4°C from MFI at 37°C. Statistical differences between the two experimental groups were analyzed by Student's *t*-test. The results are shown as mean ± SEM. N.S. denotes not significant.

4.2. LPS weakly induces the maturation of UCB-DCs and their expression of inflammatory cytokines in comparison with those of APB-DCs.

Since maturation of DCs is an important process in the mediation of immune responses, the phenotypic changes and cytokine expression of DCs were examined. LPS-stimulated UCB-DCs showed poor induction of co-stimulatory molecules and MHC proteins, while APB-DCs exhibited significant augmentation in the expression of these molecules (Fig. 36A and B). UCB-DCs still sustained high endocytic activity even after LPS stimulation, while LPS-matured APB-DCs showed remarkably decreased endocytic activity (Fig. 36C). The expression of phagocytosis-related receptors (CD205 and CD206) was minimally changed in LPS-treated UCB-DCs (Fig. 36D). UCB-DCs produced significantly lower IL-12p40 and TNF-α but higher IL-10 in response to LPS than APB-DCs did (Fig. 36E). HKEC-stimulated UCB-DCs weakly induced IL-12p70 in comparison with HKEC-stimulated APB-DCs (Fig. 36F). Taken together, these results indicate that UCB-DCs exhibit less mature phenotypes than APB-DCs in response to LPS or HKEC.

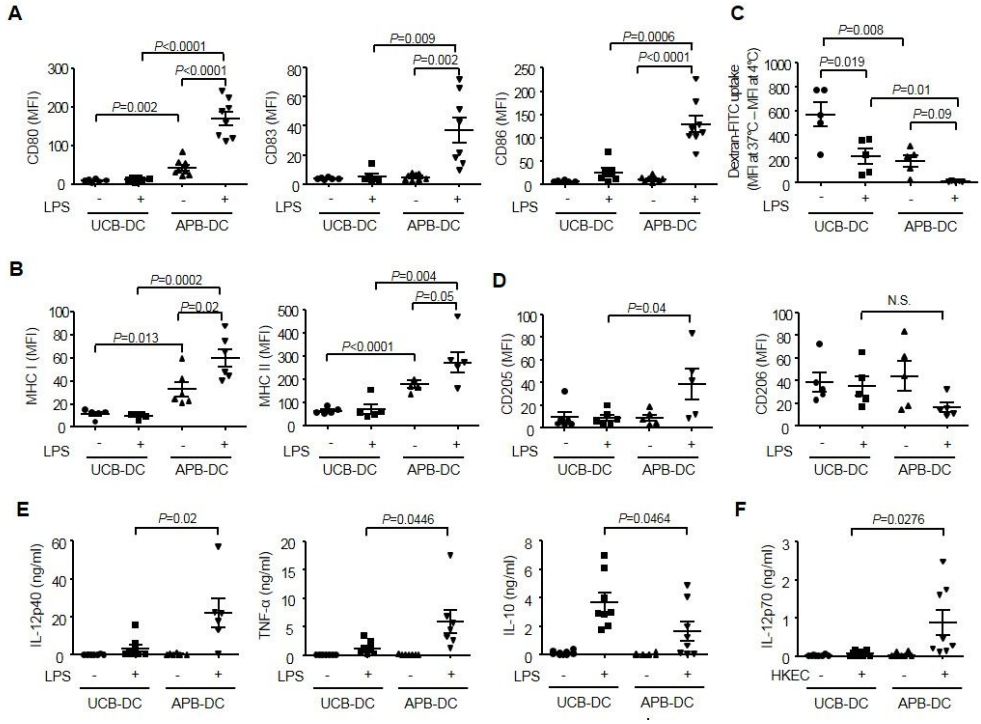

Figure 36. LPS weakly induces the maturation of UCB-DCs and their expression of inflammatory cytokines in comparison with those of APB-DCs. (A and B) DCs (5×10^5 cells/ml) were stimulated with LPS (100 ng/ml) for 48 h. Expression of DC maturation markers (CD86, CD80, CD83, and MHC class I and II) on the cell surface was analyzed by flow cytometry. (C) Unstimulated and LPS-stimulated DCs (1×10^5 cells) were suspended in PBS containing dextran-FITC (1 mg/ml, 50 µl) for 1 h at 4°C and 37°C and subjected to flow cytometric analysis. The vertical axis indicates the net MFI values of the DCs subtracting MFI at 4°C from MFI at 37°C. (D) The expression of phagocytosis-related receptors (CD205 and CD206) was analyzed by flow cytometry. (E) DCs (5×10^5 cells/ml) were stimulated with LPS (100 ng/ml) for 48 h, and the amount of IL-12p40, TNF-α, and IL-10 in the culture supernatant was measured by ELISA. (F) The DCs (2.5×10^5 cells/ml) were stimulated with HKEC (1×10^6 CFU/ml), and the amount of IL-12p70 in the culture supernatant was determined by ELISA. The statistical differences among the experimental groups were analyzed by ANOVA. *P* values under 0.05 were considered statistically significant. The results are shown as mean ± SEM. N.S. denotes not significant.

4.3. UCB-DCs weakly induce proliferation and activation of allogeneic T lymphocytes.

One of the major functions of DCs is to stimulate naïve T lymphocytes to differentiate into various effector T lymphocytes [88]. To examine T lymphocyte-activating capacities of DCs, unstimulated or HKEC-stimulated DCs were co-cultured with $CD14^+$ cell-depleted allogeneic PBMCs. HKEC-stimulated UCB-DCs induced less proliferation of $CD3^+$ T lymphocytes and $CD3^-$ cells in PBMCs than APB-DCs (Fig. 37A). In addition, HKEC-stimulated UCB-DCs weakly induced IFN-γ production, while that of HKEC-stimulated APB-DCs by PBMCs was potently induced. However, HKEC-stimulated UCB-DC and APB-DC exhibited no significant difference in IL-10 expression of PBMCs (Fig. 37B). Concomitantly, UCB-DCs poorly induced $CD25^+$MHC class II^+ T lymphocytes, while APB-DCs increased those cell populations (Fig. 37C). These results indicate that UCB-DCs weakly induced activation of allogeneic T lymphocytes in comparison with APB-DCs.

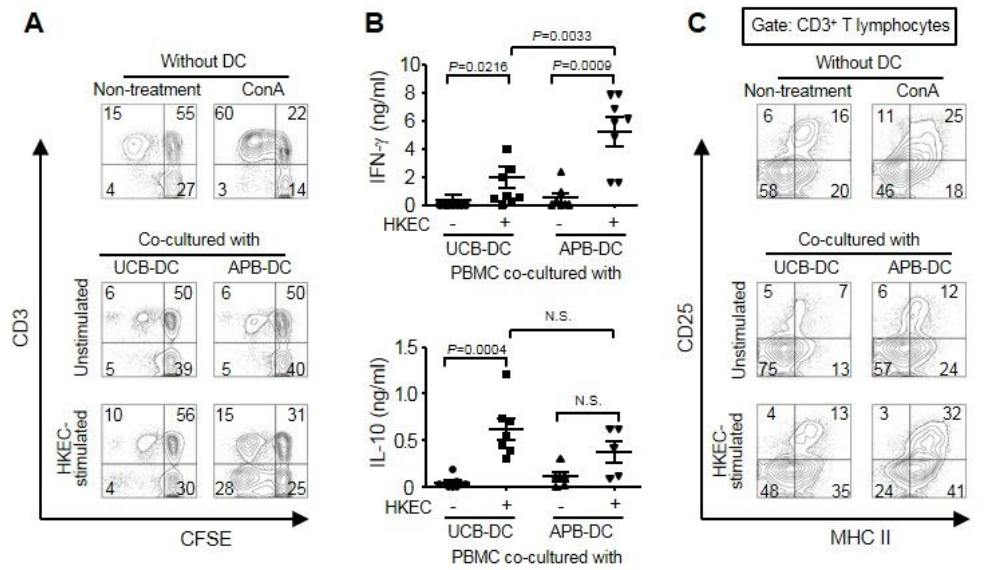

Figure 37. UCB-DCs weakly induce proliferation and activation of T cells. DCs (5×10^4 cells) were stimulated with HKEC (1×10^6 CFU/ml) for 24 h, and the allogeneic PBMCs (5×10^5 cells) were co-cultured with the stimulated DCs for 3 to 5 days. (A) Cells were stained with anti-human CD3 antibodies to detect T lymphocytes from the PBMCs. Proliferation of T lymphocytes among the PBMCs was analyzed by the CFSE-dilution assay followed by flow cytometric analysis. Numbers on the histograms indicate the percentage of cells on each quadrant. (B) The amount of IFN-γ (*upper panel*) and IL-10 (*lower panel*) in the supernatant of PBMCs co-cultured with UCB-DCs or APB-DCs was measured by ELISA. Statistical differences among the experimental groups were analyzed by ANOVA. *P* values under 0.05 were considered statistically significant. The results are shown as mean ± SEM. N.S. denotes not significant. (C) Expression of T lymphocyte activation markers (CD25 and MHC class II) on $CD3^+$ cells was analyzed by flow cytometry. The number in each quadrant indicates the percentage of cells. The results shown are representative of three similar experiments.

4.4. Reduced frequencies of $CD14^+CD16^+$ monocytes in UCB correlate with increased level of blood PGE2.

$CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes exhibit different immunological properties when they differentiate into DCs [89]. To unravel the mechanism responsible for the distinct phenotypes and functions of UCB-DCs, monocyte subtypes of UCB and APB were analyzed. Frequencies of $CD14^+CD16^+$ monocytes were significantly lower in UCB than in APB (Fig. 38A). PGE2 is a potent immuno-modulator that regulates differentiation and activation of various immune cells and is known to exist at a high level in UCB [90]. Thus, PGE2 concentration in the plasma of UCB and APB was measured and examined whether it was related to the frequencies of $CD14^+CD16^+$ monocytes. Higher amounts of PGE2 were detected in the plasma of UCB than in that of APB (Fig. 38B). The PGE2 level of UCB plasma was inversely correlated with the percentage of $CD14^+CD16^+$ monocytes ($R^2 = 0.834$) (Fig. 38C). To examine whether PGE2 is directly involved in the regulation of $CD14^+CD16^+$ monocyte differentiation, $CD14^+$ monocytes isolated from APB were cultured with or without PGE2. An increase of $CD14^+CD16^+$ cells was observed in the monocytes cultured in the absence of PGE2 (Fig. 38D), which is concordant with the previous report [91]. In contrast, $CD14^+CD16^+$ cells were decreased in the monocytes cultured in the absence of PGE2 (Fig. 38D). These results suggest that the reduced frequencies of $CD14^+CD16^+$ monocyte in the UCB have relevance to the levels of PGE2 in the plasma.

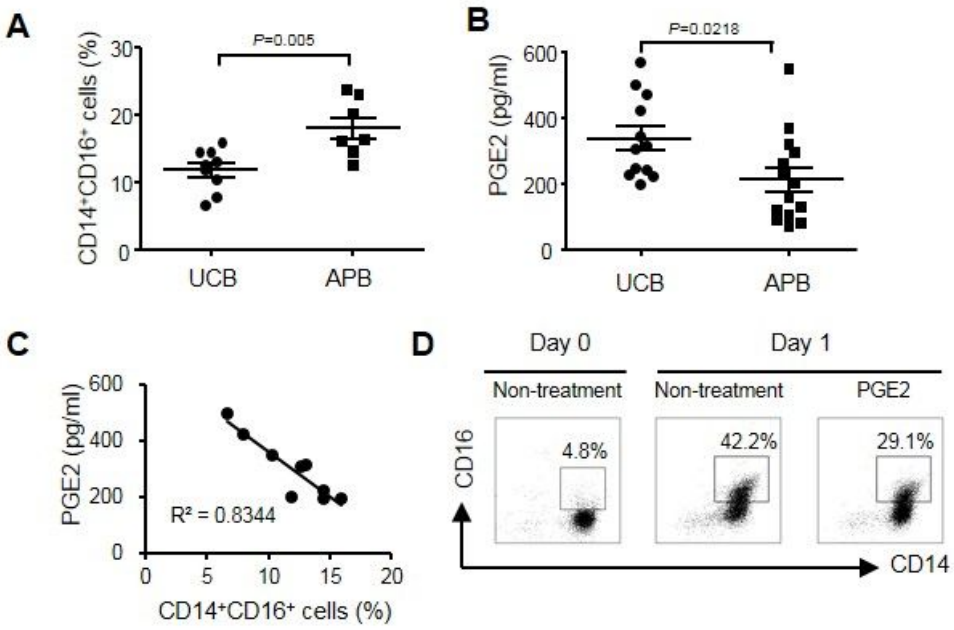

Figure 38. Reduced frequencies of $CD14^+CD16^+$ monocytes in UCB correlate with increased level of blood PGE2. (A) Mononuclear cells were isolated from UCB and APB, and the subtypes of monocytes were analyzed by flow cytometry. The scatter plot shows frequencies of $CD14^+CD16^+$ monocytes in the UCB and APB. (B) The amount of PGE2 in the plasma of UCB and APB was quantified by ELISA. Statistical differences were analyzed by Student's *t*-test. The results are presented as mean ± SEM. (C) The correlation between plasma PGE2 levels and frequencies of the $CD14^+CD16^+$ monocytes in UCB was analyzed. (D) Monocytes isolated from APB were cultured in RPMI 1640 containing 10% FBS with or without PGE2 (1 μM) for 24 h. Changes in the expression of CD16 on $CD14^+$ monocytes were analyzed by flow cytometric analysis. The results shown are representative of five independent experiments.

4.5. PGE2 induces differentiation of monocytes into UCB-DC-like cells.

To further investigate the role of PGE2 in phenotypic and functional characteristics of DCs, APB-monocytes were differentiated into DCs in the absence (control DCs) or presence of PGE2 (PGE2-DCs). PGE2-DCs preferentially expressed CD14 rather than CD1a, while control DCs expressed CD1a but not CD14 (Fig. 39A). PGE2-DCs displayed higher endocytic activity than control DCs (Fig. 39B). Unlike control DCs, PGE2-DCs weakly augmented the expression of co-stimulatory molecules in response to LPS (Fig. 39C). PGE2-DCs expressed low IL-12p70 but remarkably high IL-10 in response to HKEC. In contrast, the HKEC-stimulated control DCs efficiently up-regulated IL-12p70 but weakly induced IL-10 (Fig. 39D). HKEC-stimulated PGE2-DCs poorly induced allogeneic T lymphocyte proliferation when compared to that of APB-DCs (Fig. 39E). Furthermore, PGE2-DCs induced lower levels of IFN-γ but more IL-10 in the co-culture with allogeneic PBMCs than control DCs (Fig. 39F). These results show that monocytes exposed to PGE2 are differentiated into DCs similar to UCB-DCs.

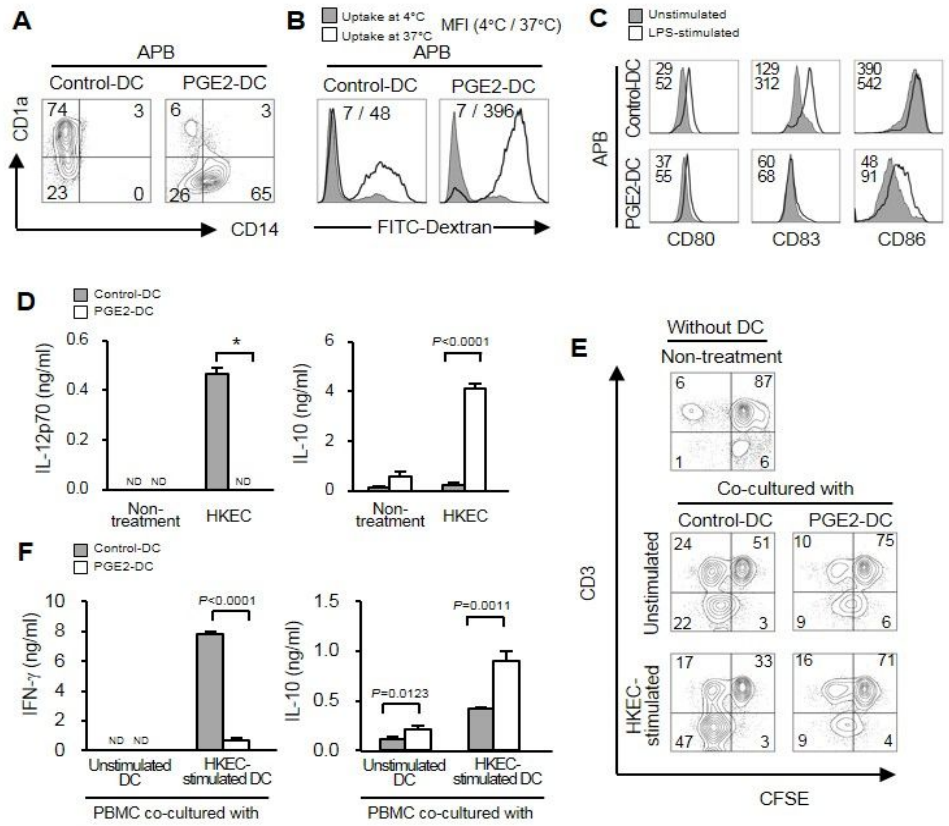

Figure 39. PGE2 induces differentiation of monocytes into UCB-DC-like cells.

Monocytes (2×10^6 cells) isolated from APB were differentiated into DCs in the presence or absence of exogenous PGE2 (10 nM) for 6 days, and the expression of CD14 and CD1a on the DCs was analyzed by flow cytometry. (B) Endocytic activity of the DCs was examined by dextran-FITC uptake assay followed by flow cytometric analysis. (C) Expression of maturation markers (CD80, CD83, and CD86) on DCs stimulated with LPS (100 ng/ml) for 24 h was analyzed by flow cytometry. (D) DCs (2.5×10^5 cells/ml) were stimulated with HKEC (1×10^6 CFU/ml) for 24 h, and the amount of IL-12p70 and IL-10 in the culture supernatant was measured by ELISA. DCs (5×10^4 cells) were stimulated with HKEC (1×10^6 CFU/ml) for 24 h, and the unstimulated or HKEC-stimulated DCs were co-cultured with allogeneic PBMC (5×10^5 cells) for 3 to 5 days. (E) Proliferation of T lymphocytes in the PBMCs was analyzed by flow cytometry. (F) The amount of IFN-γ and IL-10 in the culture supernatant was measured by ELISA. The results shown are representative of three independent experiments.

Chapter IV. Discussion

In the present study, it was investigated the role of PAF and PGE2 signals in the immunological characteristics of DCs with respect to their differentiation, activation, and function. Activation of PAFR was essential for eliciting immunostimulatory properties in DCs considering the results that the DCs differentiated in the presence of PAFR antagonist failed to acquire typical phenotypes and immunostimulatory functions of DCs. Most phenotypic and functional characteristics of the DCs differentiated in the presence of PAFR antagonist were considerably similar to those of previously identified tolerogenic DCs [6, 67, 92]. On the other hand, PGE2 signals through its two different receptors, EP2 and EP4, showed different effects on the regulation of DC functions. Activation of EP4 by low doses of PGE2 was crucial for immunogenic characteristics of DCs, while the activation of EP2 by high doses of PGE2 was important for triggering tolerogenic properties in the cells. In addition, UCB-MSCs distinctively modulated characteristics of DCs by selectively activating EP4 and EP2 signals. Furthermore, DCs derived from UCB in which the level of PGE2 was highly sustained exhibited immunosuppressive characteristics in their phenotypic maturation, cytokine production, and T lymphocyte-activating ability. These results suggest that PAF and PGE2 complementarily regulate immunological characteristics of human DCs and contribute to immune homeostasis.

Inactivation of PAFR signaling potentiated tolerogenic properties of DCs. Expression of CD1a, typical phenotypic marker of immunogenic DCs, was significantly decreased in CV6209-DCs. Additionally, expression of co-stimulatory molecules, production of cytokines such as IL-12 and TNF-α, and activation of T lymphocytes by CV6209-DCs were lower than those by control DCs. These characteristics of CV6209-DCs are in line with those of tolerogenic DCs generated

in vitro conditions [6, 92]. Previous studies have shown that DCs exhibiting immunosuppressive properties little expressed CD1a on the cell surface, whereas they showed high levels of other phenotypic markers for DCs, MHC class II, CD86, and DC-SIGN [93, 94]. The $CD1a^{low/-}$ DCs was reported to display low levels of maturation markers, production of pro-inflammatory cytokines, and weak T lymphocyte-activating capacities compared to those of immunostimulatory $CD1a^{+}$ DCs [127, 128]. Moreover, $CD1a^{low/-}$ DCs were also reported to induce diverse types of Treg [94, 95], indicating that the $CD1a^{low/-}$ DCs preferentially mediate tolerogenic immune responses. Considering that CV6209-DCs preferentially elicited IL-10^{+}TGF-β^{+} Treg, inactivation of PAFR signaling in the early phase of differentiation seems to impart tolerogenic characteristics to DCs.

Atypical differentiation of DCs upon blockade of PAFR signaling seems to be ascribed to impairment of intracellular signals required for normal development of DCs. GM-CSF has been known to primarily stimulate JAK2 in DC progenitor cells and subsequently activate both STAT and PI3K pathways to differentiate the progenitors into DCs [96]. PAF was also reported to activate JAK2-mediated STAT signaling pathway [55] and modulate GM-CSF-induced immune responses in many cell types [97, 98]. Previous studies have shown that treatment with PAFR antagonists such as WEB2086 and CV6209 efficiently blocked phosphorylation of JAK2 in human monocytic cells and murine macrophage-like cells, respectively [55, 56]. Taken together, the failure of acquiring immunostimulatory properties for DCs might be due to inhibitory effects of PAFR antagonist on the GM-CSF-induced activation of JAK2 and its downstream signals.

In this study, enhanced production of IL-12p70 and elicitation of TH1 were

observed in DCs differentiated in the presence of low doses of PGE2. However, treatment with EP4 antagonist, but not EP2 antagonist, abolished the stimulatory properties of the DCs, indicating that selective activation of EP4 signaling by low doses of PGE2 potentiates immunostimulatory functions of DCs. Although little has been known about stimulatory characteristics of PGE2 particularly on DC-mediated TH1 responses so far, a previous study showed that treatment with low dose of PGE2 polarized naïve T lymphocytes toward IFN-γ-producing TH1 cells, which were predominantly mediated by EP4 activation [86]. The authors of the study proposed that PGE2 might enhance the action of IL-12 since they could not observe the same responses of the PGE2-treated T lymphocytes without IL-12. Recently, PGE2 has been reported to augment the expression of receptors for IL-12 and IFN-γ by EP4-dependent manner [99]. These previous and the present findings suggest that the activation of EP4 signaling potentiates TH1 responses by enhancing production of IL-12 by DCs.

Contrary to EP4 signaling, activation of EP2 by high doses of PGE2 potentiated tolerogenic characteristics of DCs. DCs differentiated in the presence of high concentrations of PGE2 little produced IL-12 and could not augment T lymphocyte proliferation and activation. However, treatment with EP2 antagonist markedly abrogated the tolerogenic properties of the DCs exposed to PGE2. In addition, the blockade of EP4 signaling partially abolished the effects of the high-dose PGE2. Previous studies also have demonstrated that PGE2-mediated suppression of IL-12 production and TH1 elicitation by DCs involves the activation of EP2 and/or EP4 [100, 101]. Furthermore, EP2 signaling have been reported to attenuate activation of NK cells [102], cytotoxicity of T lymphocytes [103], and inflammation of activated macrophages [104]. Activation of EP2 augments cAMP in many cell types, which

usually suppresses inflammatory functions of immune cells [105, 106]. Indeed, cAMP elevation in DCs attenuated IL-12 production of the cells by potentiating IL-10 expression [105, 107]. Therefore, it is convincing that immunosuppressive functions of DCs are mainly mediated by EP2 activation.

The dual effects of PGE2 have previously been reported in many cell types. Expression of IL-23 was oppositely regulated in DCs depending on concentrations of PGE2 [108]. In addition, TNF-α production in murine macrophages [109], differentiation of rat chondrocytes [110], and water-chloride absorptive functions of colon epithelial cells were differently regulated by different concentrations of PGE2 [111]. The opposite functions of PGE2 by concentrations may be due to the presence of multiple EP receptors on those cell types that have different affinity, duration of activation, and downstream signaling. Since EP4 has higher affinity to PGE2 than EP2 [112], low doses of PGE2 selectively activate EP4 but high doses of PGE2 can stimulate both EP2 and EP4. EP4 is rapidly desensitized following PGE2 binding, whereas the EP2 signaling is prolonged relatively for a long time [75]. With respect to downstream signaling of those receptors, EP4 preferentially activates PI3K pathway, which is crucial for regulating expansion, survival, and stimulatory functions of DCs [96]. However, EP2 activates cAMP/protein kinase A (PKA) signaling pathway, mediating anti-inflammatory and immunosuppressive functions of PGE2 on DCs [65]. Taken together, the differences in the properties of EP2 and/or EP4 signaling may result in distinctive immunological characteristics of DCs differentiated in the presence of PGE2.

In the regulation of differentiation, activation, and function of DCs, PAF and PGE2 showed mutually antagonistic effects. Treatment with PAF partially

abrogated tolerogenic properties of DCs differentiated in the presence of PGE2. Additionally, DCs differentiated in the presence of PAFR antagonist restored their stimulatory phenotypes and functions by blocking the action of PGE2, indicating that the antagonistic interactions between PAF and PGE2 in the regulation of DC characteristics. Although these two lipid mediators have exhibited similar effects on eliciting inflammatory responses or developing cancers [113, 114], they showed antagonistic effects on certain immune responses. PGE2 potentiated migratory ability of DCs by up-regulating expression of metalloprotease-9 [115], but PAF retained DCs to stay in peripheral tissues. In allergic responses, PAF exacerbated allergen-induced inflammation by accelerating neutrophil infiltration [116], while PGE2 restrained allergic pulmonary inflammation [117]. PAF and PGE2 also showed opposite effects on platelet aggregation [118]. The antagonism between two lipid molecules may be an important part of regulatory strategies for maintaining immune homeostasis. Specific mechanisms by which these lipid mediators exert antagonistic functions on those physiological responses should be further investigated.

UCB-MSCs distinctively modulated immunological characteristics of DCs toward both immunity and tolerance. Previously, MSCs have been reported to predominantly suppress differentiation and activation of DCs and other immune cells such as T lymphocytes and NK cells [22, 23, 87]. Although MSCs produce diverse immuno- modulatory mediators including PGE2, IL-6, and M-CSF [23, 24, 119], they predominantly utilized PGE2 for suppressing immune responses of DCs [24]. In agreement with the previous findings, here, it was shown that maturation, cytokine production, and T lymphocyte-activating ability were markedly attenuated in DCs differentiated in the presence of large number of UCB-MSC, which were

mediated by EP2 signaling. Contrary to this, IL-12 production and TH1-inducing ability were markedly potentiated in DCs differentiated in the presence of small number of UCB-MSCs and these responses were resulted from the activation of EP4 signaling. However, in the mouse liver, activation of EP4 by MSC-derived PGE2 elicited tolerogenic characteristics in DCs, contributing to alleviation of hepatic inflammation induced by bacterial infection [120]. The discrepancy between the present and the previous findings may be ascribed to differences in the species, source of the DC progenitors and MSCs, and affinity of EP4 to PGE2.

In clinical fields, MSCs have widely been used as cell therapeutics for degenerative diseases [121] or immune-related disorders [122, 123]. As MSCs possess potent immunosuppressive functions, their application so far was restricted to some inflammatory disorders such as graft versus host disease and autoimmune diseases [122, 123]. The present study identified the novel functions of MSCs on enhancing stimulatory properties of DCs. Some of recent findings have shown that MSCs were capable of exerting both immunostimulatory and immunosuppressive functions depending on stages of development or activation states of the target immune cells. In early phases of DC differentiation, MSCs potently inhibited DC progenitors to differentiate into conventional DCs with stimulatory characteristics [23, 87]. However, MSCs rather induced mild maturation of DCs at the late stages of differentiation [124]. In addition, MSCs also distinctively regulated cytotoxic activity of NK cells at different activation states [22, 125]. Given that DCs and NK cells are important sentinels for detecting and eliminating malignant cells such as cancer cells and infected cells, MSCs could be applied to immunocompromised disorders such as cancers and infectious diseases.

In this study, UCB-DCs contained more $CD1a^-$ cells and weakly expressed the maturation markers and inflammatory cytokines in response to LPS or HKEC, which are crucial for the activation of subsequent adaptive immunity. These results are in keeping with the previous report that UCB-DCs showed lower expression level of co-stimulatory molecules, MHC class II, and IL-12p70 in response to LPS or dying cells including apoptotic cells and necrotic cells [126]. Therefore, UCB-DCs might be less potent than APB-DCs in the induction of adaptive immunity due to inadequate provision of co-stimulatory signals and immunostimulatory cytokines to T lymphocytes necessary for the generation of effector T lymphocytes. Indeed, it was observed that UCB-DCs weakly induced the proliferation and activation of T lymphocytes at the co-culture of UCB-DCs and allogeneic PBMCs. All these properties of UCB-DCs seem to be associated with those of $CD1a^-$ DCs, in light of the fact that $CD1a^-$ DCs even in response to LPS displayed a decrease in the expression of CD83, CD86, and IL-12p70 and activation of T lymphocytes [127, 128].

PGE2 levels are highly sustained in the human placenta and UCB to maintain pregnancy and help in uterine contractions during parturition [129]. Considering that PGE2 levels soar transiently in both maternal and fetal circulation during periparturition [63], exposure of immune cells and their precursors to high amounts of PGE2 seems to be inevitable. Here, it was found that the precursors of UCB-DCs were exposed to high concentrations of PGE2, which would enhance tolerogenic properties during differentiation. In addition, UCB contained a lower number of $CD14^+CD16^+$ monocytes, which are known as activated monocytes, than APB. The PGE2 level in the UCB plasma has inverse correlation with the frequencies of $CD14^+CD16^+$ cells and treatment with PGE2 indeed suppressed the differentiation of $CD14^+CD16^-$ monocytes into $CD14^+CD16^+$ monocytes. Concomitant with the

present findings, reduction of inflammatory $CD14^{+}CD16^{+}$ monocytes in UCB and its implications for weak immune responses of newborns to infections has been reported [34]. Taken together, these data suggest that sustaining high PGE2 levels in UCB might be a plausible strategy for protecting the immunocompromised neonates from excessive inflammation.

Immunogenic DCs protect host from infectious diseases and cancers by stimulating innate and adaptive immunities, whereas tolerogenic DCs efficiently control the excessive activation of inflammatory cells including effector T lymphocytes, and NK cells [8]. However, both types of DCs could place the host in danger if their stimulatory or suppressive functions are uncontrolled. Indeed, pathogenesis of some inflammatory disorders such as autoimmune diseases and allergic diseases are closely associated with hyperactivation of immunogenic DCs [130]. In contrast, functional deficiency of the immunogenic DCs creates tolerogenic condition prone to develop immunocompromised diseases such as cancers [131, 132]. Since DCs are key players that finely tune immunity and tolerance, mechanisms for DC differentiation, activation, and regulation of their immunological properties need to be extensively investigated.

In the present study, distinctive roles of PAF and PGE2 in the immunological characteristics of DCs were determined. In the steady state, PAF and EP4-mediated PGE2 signaling contribute to the enhancement of immunostimulatory properties of DCs. In contrast, PGE2 potentiates tolerogenic properties of DCs by activating both EP2/EP4 signaling under inflammatory conditions or cancer progression (Fig. 40). Although the present study proposes that DC-mediated immune homeostasis is potently associated with the PAF and PGE2, the physiological relevance should be verified using *in vivo* models. Therefore, PAF and PGE2 can be applied to the

development of DC-targeting therapeutic agents for the prevention and treatment of inflammatory diseases and cancers.

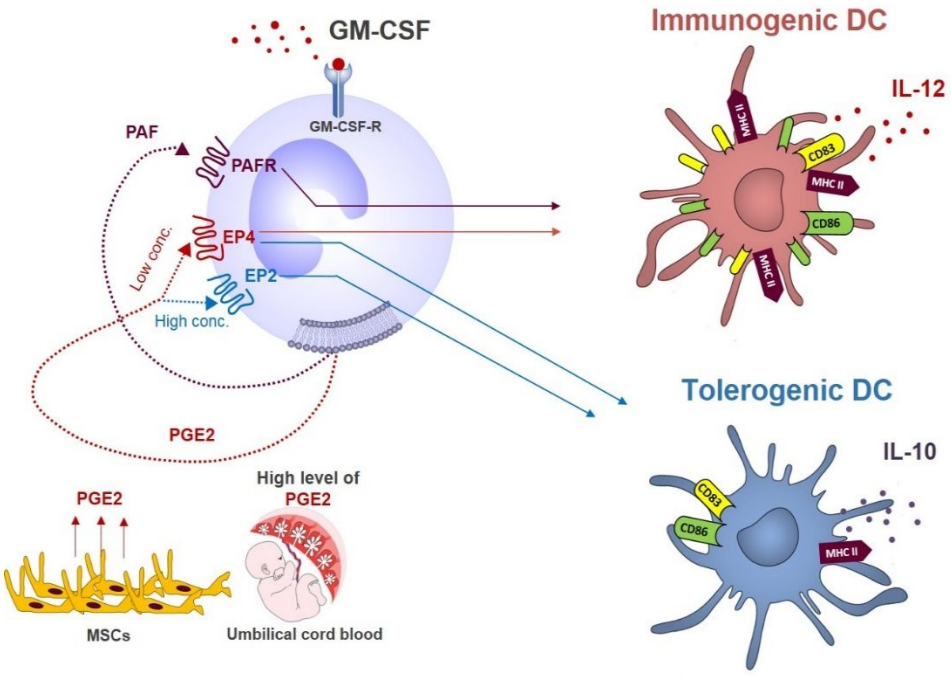

Figure 40. PAFR signal and PGE2 signals differently regulate immunological characteristics of DCs and contribute to immune homeostasis. Activation of PAFR signaling potentiates immunostimulatory functions of DCs. PGE2 differently regulates immunogenic and tolerogenic characteristics of DCs by distinctively activating its two different receptors, EP4 and EP2. Activation of EP4 by low concentrations of PGE2 enhances immunostimulatory characteristics of DCs, while the activation of EP2 by high concentrations of PGE2 suppresses stimulatory properties of the cells. PGE2 mediates immunomodulatory functions of UCB-MSCs that altered differentiation, activation, and functions of DCs. Finally, PGE2 sustained at high concentration in UCB preferentially induces tolerogenic properties of UCB-DCs.

Chapter V. References

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국문초록

수지상세포의 면역학적 특성에서 혈소판 활성화인자와 프로스타글란딘 E2 신호의 역할

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목 적

수지상세포는 선천성 면역과 획득면역을 연결해주는 전문적인 항원제시세포로서 면역학적 활성과 내성 조절에서 핵심적인 역할을 수행한다. 수지상세포의 면역학적 특성은 전구세포의 유래, 해부학적 위치, 국소 미세환경과 같은 인자에 따라서 다르게 조절된다. 이러한 요소들 중에서, 인지질 산화물들은 수지상세포의 분화, 활성화, 기능 조절 전반에서 중요하게 작용한다. 혈소판 활성화인자와 프로스타글란딘 E2는 대표적인 염증성 인지질 매개인자로서 수지상세포의 초기 분화단계에서 생성된다. 하지만 현재까지 수지상세포의 분화 및 활성화를 비롯한 면역학적 특성 조절에서 이들의 역할과 상호작용 기전은 잘 알려져 있지 않다. 본 연구에서는 인체 단핵구 유래 수지상세포의 면역학적 특성에서 혈소판 활성화인자와 프로스타글란딘 E2의 기능과 상호 조절기전을 조사하였다.

실험방법

혈소판 활성화인자와 프로스타글란딘 E2의 신호전달에 따른 수지상세포의 면역학적 특성 조절을 연구하기 위해서 말초혈액으로부터 분리한 $CD14^+$ 단핵구에 혈소판 활성화인자 수용체와 프로스타글란딘 E2 수용체의 길항제 또는 작용제를 각각 처리한 후, 미성숙 수지상세포로 분화시켰다. 병원성 미생물의 자극에 따른 세포의 성숙 및 싸이토카인 발현 양상을 알아보기 위해서 열을 가하여 사멸시킨 폐구균으로 미성숙 수지상세포를 자극한 후, 세포표면의 동시자극/억제 분자 및 MHC 단백질의 발현을 유세포 분석기를 이용하여 측정하였다. 또한, 효소면역측정법을 이용하여 IL-12p70, TNF-α, IL-10의 생성량을 분석하였다. 수지상세포에 의한 T 림프구의 활성화 양상을 조사하기 위해서 열-사멸 폐구균으로 자극한 수지상세포 또는 자극을 하지 않은 미성숙 수지상세포를 각각 자가 유래 T 림프구와 함께 배양하고, T 림프구의 증식, 활성화 마커 발현, 세포 내 싸이토카인 생성 양상을 유세포 분석기를 이용하여 측정하였다.

수지상세포의 면역학적 특성과 프로스타글란딘 E2의 생리학적 연관성을 규명하기 위해서 프로스타글란딘 E2를 지속적으로 생성하는 제대혈 유래 중간엽 줄기세포와 $CD14^+$ 단핵구를 동시 배양하고 이를 미성숙 수지상세포로 분화시켰다. 또한, 프로스타글란딘 E2의 농도가 높게 유지되고 있는 제대혈로부터 분리한 $CD14^+$ 단핵구를 미성숙 수지상세포로 분화시켰다. 병원성 미생물의 자극에 따른 수지상세포의 성숙 및 싸이토카인 발현 양상을 알아보기 위해서 열을 가하여 사멸시킨 대장균으로 각 수지상세포를 자극한 후, 세포표면의 동시자극/억제 분자 및 MHC 단백질의 발현을 유세포 분석기를 이용하여 측정하였다. 또한, 효소면역측정법을 이용하여 IL-12p70, TNF-α, IL-10의 생성량을 분석

하였다. 수지상세포에 의한 T 림프구의 활성화 양상을 조사하기 위해서 열-사멸 대장균으로 자극한 수지상세포 또는 자극을 하지 않은 미성숙 수지상세포를 각각 T 림프구와 함께 배양하고 T 림프구의 증식과 활성화 마커의 발현을 유세포 분석기를 이용하여 측정하였다.

결 과

혈소판 활성화인자 수용체의 길항제를 처리한 후 분화 유도한 수지상세포는 대조군 수지상세포에 비해서 CD1a, CD80, PD-L1을 적게 발현하였고 CD86과 CD14는 더 많이 발현하고 있었으며, 열-사멸 폐구균에 대한 높은 탐식 능력을 보였다. 혈소판 활성화인자 수용체 신호가 불활성화된 수지상세포는 열-사멸 폐구균으로 자극 시, 동시자극/억제 분자 및 MHC class II를 대조군 수지상세포에 비해서 약하게 발현하였고 IL-12p70, TNF-α, IL-10과 같은 싸이토카인을 거의 생성하지 않았다. 뿐만 아니라 자가 유래 T 림프구의 증식, 활성화 마커 발현, 싸이토카인 생성 역시 효과적으로 유도하지 않았다. 이들은 오히려 IL-10과 TGF-β를 생성하는 조절 T 세포의 분화를 촉진시켰다. 이 결과들은 혈소판 활성화인자 수용체를 매개한 세포 내 신호전달이 단핵구가 면역학적 활성을 가지는 수지상세포로 분화하는데 필수적이라는 것을 의미한다.

프로스타글란딘 E2의 수용체인 EP4의 길항제를 처리한 후 분화 유도한 수지상세포는 열-사멸 폐구균으로 자극 시, 대조군 수지상세포에 비해서 동시자극분자의 발현과 IL-12p70의 생성 능력이 낮았다. 또한, 이들을 자가 유래 T 림프구와 혼합하여 배양하여 T 림프구 활성화 양상을 분석해본 결과, 대조군 수지상세포에 비해서 약한 T 림프구 증식 및 활성화 능력을 나타내었다. 반면, EP2의 길항제를 처리한 후 분화 유도한 수지상세포는 대조군 수지상세포과 비교해 보았을 때 표현형과 기능에서

유의적인 차이를 보이지 않았다. EP4와 EP2의 활성화는 단핵구에 처리한 프로스타글란딘 E2의 농도에 따라서 다르게 조절되었는데, 낮은 농도의 프로스타글란딘 E2는 EP4를 활성화 시켜서 수지상세포의 동시자극분자 발현, 싸이토카인 생성, T 림프구 증식 등 면역학적 활성을 증가시켰다. 반대로, 높은 농도의 프로스타글란딘 E2는 EP2와 EP4의 활성화를 매개하여 상기 수지상세포의 면역반응을 감소시켰다. 프로스타글란딘 E2의 각기 다른 수용체 활성화 양상에 따른 상반된 효과는 제대혈 유래 중간엽 줄기세포의 수지상세포 분화 및 활성화 조절에서도 관찰되었는데, 중간엽 줄기세포와의 혼합배양에 따른 단핵구 내 EP4의 활성화는 수지상세포의 면역학적 활성을 증가시켰고, 반대로 EP2의 활성화는 수지상세포의 면역학적 내성을 강화시켰다. 이러한 결과들로부터 프로스타글란딘 E2가 각기 다른 수용체 활성화를 매개하여 수지상세포의 면역학적 특성이 다르게 조절한다는 것을 알 수 있다.

제대혈 유래 수지상세포는 성인의 말초혈액 유래 수지상세포에 비해서 CD1a, CD80, MHC class I, MHC class II, DC-SIGN의 발현 수준이 낮게 관찰되었지만 항원탐식작용은 말초혈액 유래 수지상세포 보다 높게 나타났다. LPS로 자극 시, 제대혈 유래 수지상세포는 성숙마커, IL-12p70, TNF-α의 발현은 낮게 생산하였지만 IL-10의 발현은 현저히 증가시켰다. 동종 타가 유래 혼합백혈구와 동시 배양한 제대혈 유래 수지상세포는 말초혈액 유래 수지상세포에 비해서 T 림프구의 증식, 활성화 마커 발현, IFN-γ의 생성을 약하게 유도하였다. 제대혈에서는 성인의 말초혈액에서보다 높은 수준의 프로스타글란딘 E2가 검출되었는데, 이는 제대혈 유래 수지상세포의 표현형 및 면역 내성 유도 능력과 높은 상관관계가 있는 것으로 나타났다. 실제로, 높은 농도의 프로스타글란딘 E2의 존재 하에 분화 유도한 수지상세포는 표현형과 싸이토카인 발현 양상 그리고 T 림프구

활성화 유도 반응에서 제대혈 유래 수지상세포와 유사한 특성을 나타내었다. 이 결과들은 제대혈 내의 프로스타글란딘 E2가 수지상세포의 면역학적 내성 조절에 관여한다는 것을 나타낸다.

결 론

이상의 연구결과들로부터 다음과 같은 결론을 도출할 수 있다. 혈소판 활성화인자 수용체의 신호 활성화는 인간 단핵구 유래 수지상세포의 면역학적 활성을 향상시킨다. 반면, 프로스타글란딘 E2는 수용체인 EP4와 EP2의 활성화 양상에 따라서 수지상세포의 면역학적 활성과 내성을 다르게 조절한다. 또한 프로스타글란딘 E2는 제대혈 유래 중간엽 줄기세포에 의한 수지상세포의 분화, 활성화, 및 기능 변화에서 핵심 조절인자로 작용할 뿐만 아니라, 면역학적 내성을 나타내는 제대혈 유래 수지상세포의 분화 및 특성 조절에서도 중요한 역할을 수행한다. 결론적으로 혈소판 활성화인자와 프로스타글란딘 E2 수용체 활성화에 따른 세포 내 신호전달은 수지상세포의 활성과 내성을 효과적으로 조절하여 면역학적 항상성 유지에 기여한다.

주요어: 인체 수지상세포, 혈소판 활성화인자, 프로스타글란딘 E2, 수지상세포의 성숙, T 세포 활성화

학 번: 2006-23508