의학석사 학위논문

The generation of semi-mature dendritic cells by inhibiting acetyl-CoA carboxylases

Acetyl-coA carboxylase 억제를 통한 준성숙 수지상세포의 생성에 관한 연구

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A thesis of the Master's degree

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The generation of semi-mature dendritic cells by inhibiting acetyl-CoA carboxylases

by

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Approved by Thesis Committee:

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ABSTRACT

Introduction: The adoptive transfer of tolerogenic dendritic cells has been employed as a promising tool for transplantation as well as treatment of autoimmune diseases. Some different tolerogenic dendritic cells share the feature of semi-mature state. Also current studies have demonstrated that fatty-acid synthesis is important for dendritic cell activation while fatty-acid oxidation is necessary for maintaining immature state. Therefore, in this study, I focused on blocking acetyl-coA carboxylases which can inhibit fatty-acid synthesis and promote fatty-acid oxidation to generate the semi-mature dendritic cells which may have potential for immunotherapy.

Methods: Bone marrow cells (BMs) were isolated from 8-week-old C57BL/6 female mice then have been cultured for 8 days (in RPMI complete media with 10 ng/ml GM-CSF and 1.5 ng/ml IL-4) to differentiate to dendritic cells (DCs). At day 8, bone marrow-derived dendritic cells (BMDCs) were pre-incubated for 30 minutes with CP-640186 (CP), an acetyl-CoA carboxylase (ACC) inhibitor, following by LPS treatment up to 24hours for maturation. BMDCs and BMDCs with LPS treatment only were used as control groups. The effect of CP on BMDC maturation was investigated through DCs phenotypic and functional studies.

Results: The effect of CP to block ACCs in DCs was confirmed. Next, the expression of MHC class II and co-stimulatory molecules (CD80, CD86 and CD40) on LPS-treated BMDCs were down-regulated by CP with concentration dependent manner. Consistently, the secretion of pro-inflammatory cytokines (IL-6, IFN-γ, IL-12p70) also decreased. Interestingly, the chemokine receptor, CCR5 was up-regulated while the homing receptor, CCR7 was down-regulated in CP treatment groups.

Moreover, these groups also increased antigen uptake capacity. Finally, in the presence of CP, LPS-treated BMDCs failed to activate antigen-specific $CD4^{+}$T cells proliferation.

Conclusions: In summary, our data elucidates that DCs with semi-mature phenotype and characteristic of immature-like cells were generated by ACCs inhibition. This study has implication for the development of metabolism based-approaches in DC-mediated immunotherapies.

Key words: Semi-mature DCs, CP-640186, Acetyl-CoA carboxylase, Lipid metabolism.

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LIST OF ABBREVIATIONS

7AAD	7-Aminoactinomycin D
ACC	Acetyl-CoA carboxylase
APC	Allophycocyanin
BM	Bone marrow
BMDCs	Bone marrow-derived dendritic cells
CBA	Cytometric Bead Array
CCR	C-C chemokine receptor
CD	Cluster of Designation
CFSE	CarboxyfluoresceinSuccinimidyl Ester
CP	CP-640186
DCs	Dendritic cells
FACS	Fluorescence Activated Cell sorting
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FBS	Fetal bovine serum
FITC	Fluorescein Isothiocyanate
Foxp3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony-stimulating factor
IFN	Interferon
IL	Interleukin
imDCs	immature dendritic cells
LCs	Langerhans cells
LDs	Lipid droplets
LPS	Lipopolysaccharide
MACs	Magnetic-activated cell sorting
mDCs	mature dendritic cells

MHC-II	Major histocompatibility complex class II
OVA	Ovalbumin
PBS	Phosphate buffered Saline
PE	Phycoerythrin
PerCP	Peridinin-Chlorophy II protein complex
RPIM 1640	Rosewell Park Memorial Institute 1640
smDCs	semi-mature dendritic cells
TNF	Tumor necrosis factors

INTRODUCTION

1. Dendritic cells

Dendritic cells were known as “Langerhans cells” (LCs) when they were first described, in the late nineteenth century, by Paul Langerhans, a German pathologist, physiologist and biologist. In 1973, Ralph M. Steinman and Zanvil A. Cohn discovered the role of LCs in the adaptive immune response and termed them as “Dendritic cells” (DCs) (1).

DCs can be generated from myeloid and lymphoid precursor cells (2, 3). In its steady state, immature DCs (imDCs) circulate in peripheral blood and express some specific chemokine receptors such as CCR1, CCR2, CCR5 and CXCR1 to help them migrate to inflammatory sites (4). Once the DCs capture the invading pathogens or other foreign bodies, the antigen is processed and presented on their surface by the major histocompatibility complex (MHC). Subsequently, these inflammatory chemokine receptors are replaced by a homing receptor CCR7 for guiding DCs to the lymphoid tissues where they present antigens to T cells (4). Furthermore, they fully mature with a high number of MHC-peptide complexes on their surface (5). Other co-stimulatory molecules such as CD80, CD86 and CD40 are also up-regulated (6, 7). Following activation, mature DCs (mDCs) rapidly produce pro-inflammatory cytokines (TNF-α, IL-12, IL-6 etc.) (8). Afterwards, mDCs turn on both the innate and adaptive immunity responses (9).

However, there has been growing interest in DCs for studies and clinical applications since they were established as central players in not only immunity but also tolerance induction (10, 11). This special capacity strongly depends on their maturation stages. In other words, fully mature DCs can activate immune response. In contrast, imDCs can induce

immune tolerance. Recently, semi-mature DCs (smDCs) were found with tolerogenic potential higher than those of imDCs (12-14). The DCs which are locked in semi-maturation state express intermediate levels of MHC-II and co-stimulatory molecules, but a lack of pro-inflammatory cytokine secretion (15, 16). Due to the lack of the third signal, they fail to activate an immune response (17, 18). Beside this, smDCs may become more advantageous than imDCs in tolerance induction since the tolerogenic function of imDCs is unstable when injected to non-immunosuppressed recipients (19). Moreover, they have found that different tolerogenic DCs also display the semi-mature phenotype (20). Therefore, smDCs can be a prospective target for cell therapy in transplantation as well as treatment of autoimmune diseases.

2. Lipid metabolism in dendritic cells

In Greek, “metabolism” means “change”. It includes all kinds of chemical transformations within cells for sustainment of life such as growth, proliferation and responses to their environments. Also, the developmental stages of DCs show different roles in immune response and immune tolerance. Therefore, they may also rely on metabolic pathways to respond to stimuli.

Recently, research has shown that upon receiving distinct signals, the change in function of DCs also shows relative changes in metabolic pathways (21-24). The evidence is seen in fatty acid synthesis (FAS) which is important for DC precursor cells survival as their generation markedly decreases when FAS is blocked during the differentiation period (22). In its steady state, imDCs rely on fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) to induce ATP (25). In response to stimuli, together with changes in function, mDCs also make changes in cellular metabolism necessary for their adaptation. This includes dramatic increase of the glycolysis pathway which in turn leads to an increase in ATP as well as substrates for TCA cycle. However, this process is different from its immature state which uses OXPHOS to generate ATP. Instead, TCA cycle supplies citrate for de novo fatty acid synthesis and this was observed to be for ER and Golgi expansion (23). Thus, both glycolysis and FAS pathways are important for DCs activation in situ during inflammation. Additionally, the inhibition of FAS during DCs differentiation increases the secretion of pro-inflammatory cytokines, enhancing their capacity to activate not only allogeneic but also antigen-restricted T cells response without up-regulation of co-stimulatory molecules and MHC-II (22). Furthermore, the inhibition of FAS during

DCs maturation reduces the secretion of pro-inflammatory cytokines (23). Thus, lipid metabolism is important for DCs development and function. However, the role of lipid metabolism in DC semi-mature stages is still unknown.

3. Controlling DCs fate and function by targeting both FAS and FAO pathways

In this context, current knowledge on DCs' metabolism shows that FAO and FAS are important for imDCs and mDCs respectively. Thus, it is possible that both FAS and FAO may together play a role in DCs semi-mature state. Briefly, lipid metabolism comprises of two main distinct pathways, fatty-acid synthesis in cytosol and long chain fatty-acid oxidation in mitochondria (Figure 1A). FAS is an anabolic pathway, which synthesizes fatty acid from acetyl-coA and malonyl-coA. FAO is a catabolic pathway, which breaks down fatty acid to acetyl-coA needed in the TCA cycle. FAS and FAO are tightly regulated by the key enzyme acetyl-coA carboxylase (ACC), which contains two isoforms ACC1 and ACC2 (Figure 1A). ACC1 is located in cytosol and ACC2 in the outer membrane of mitochondria (26, 27). The difference in location results in two different functions with ACC1 involved in fatty acid elongation and ACC2 in fatty acid oxidation inhibition (28, 29). Therefore, targeting ACCs may be a potential method to control DCs function and lock the cells in its semi-mature state.

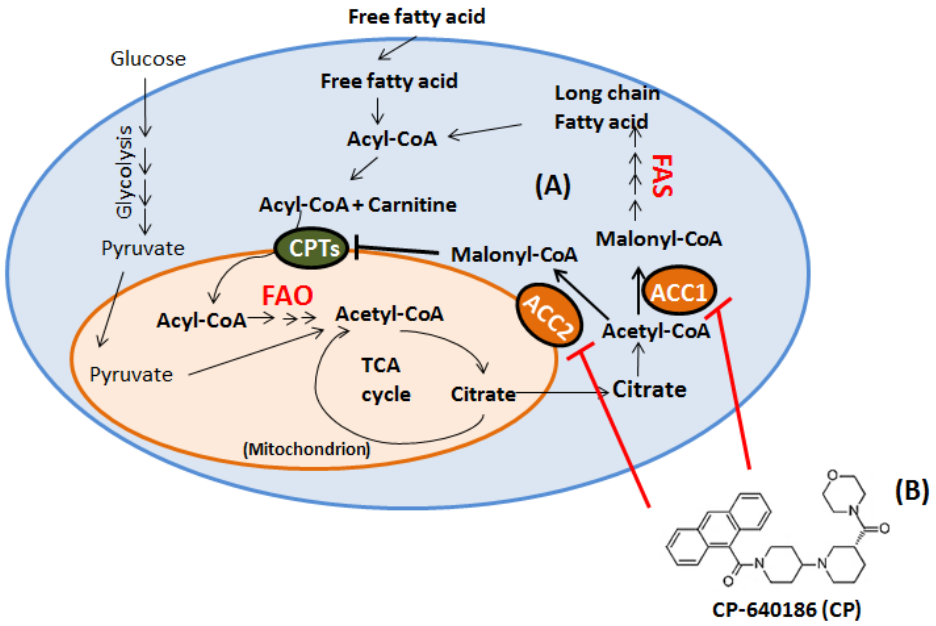


Figure 1. Schematic representation of metabolic pathways in dendritic cells and ACC inhibitor (CP-640186)

(A) Lipid metabolism comprises of two main distinct pathways, fatty acid synthesis (**FAS**) and fatty acid oxidation (**FAO**). These two pathways are tightly regulated by key enzyme acetyl-coA carboxylase (**ACC**), which contains two isoforms **ACC1** and **ACC2; (B) CP-640186** (CP) is an inhibitor of both ACC1 and ACC2 developed by Pfizer.

In this study, CP-640186 (CP), an inhibitor of both ACC1 and ACC2, was employed enabling us to look into the effect of FAS inhibition and FAO promotion during DCs maturation simultaneously (Figure 1B) (27). DCs for this experiment were differentiated from murine bone marrow (also called bone marrow-derived dendritic cells or BMDCs). CP was added before induction of DCs maturation by LPS. The effect of ACC-blockade was then investigated through DCs' phenotype and function. BMDCs and LPS-treated BMDCs were used as control groups. For DC phenotypic study, surface markers including MHC-II together with CD80, CD86 and CD40 were examined. DC migration chemokine receptors normally expressed on imDC and mDC such as CCR5 and CCR7 respectively were also tested. Next, to observe the functional maturation of BMDCs in the presence of CP, the secretion of pro-inflammatory cytokines upon stimulation, antigen uptake and T cell activation capacity were investigated. Furthermore, to confirm the effect of CP in controlling lipid metabolism, total neutral lipids of BMDCs was measured.

All these results revealed that DCs with semi-mature phenotype and low ability to active T cell response were generated successfully by inhibiting both ACC1 and ACC2. This study illustrates a new approach to induction of metabolic-induced semi-matured DCs which may have potential in DC-based immunotherapy application.

MATERIALS AND METHODS

Mice

8-week-old female C57BL/6 mice and female C57BL/6 mice with transgenic expression of OVA-specific $CD4^{+}$T cells (OT-II) were purchased from The Jackson Laboratory.

Mice were used for all experiments at 8 weeks old and conducted under protocols approved by Seoul National University Institutional Animal Care and Use Committee (SNU-150115-1)

Murine bone marrow cells isolation

BMDCs were isolated from 8-week-old B6 mice. Firstly, the femurs and tibias were separated from mice with the epiphysis intact. All muscle was removed and the bones washed with RPMI-1640 media. Both ends of femurs and tibias were trimmed carefully and all bone marrow cells flushed out using a 5ml syringe with a $30G_{1/2}$ needle. Finally, all cells were collected into a sterile 50ml Falcon tube using a 70μm cell strainer filter. BMDCs were washed once by RPMI-1640 complete media before seeding into a 6 well-plate for DC differentiation.

Bone marrow-derived dendritic cells differentiation

Bone marrow cells were adjusted to 10^6 cells/ml and cultured in a 6 well-plate (2 ml per well) with DC complete medium (RPMI 1640 with 10% FBS, L-glutamate, 2-ME, HEPES, penicillin/streptomycin, 10 ng/ml GM-CSF (Cat# 315-03) and 1,5 ng/ml IL-4 (Cat# 214-14) from Peprotech, USA at 37^0C in 5% CO_2 in order to differentiate to dendritic cells. On day 3, an additional 2 ml of fresh RPMI-1640 complete media with the same concentration of GM-CSF and IL-4 were added into each well. Suspension cells were harvested at day 6, centrifuged and replaced with new DC

complete medium at 10^6 cells/ml and 2 ml per well in a new 6 well-plate. Bone marrow-derived dendritic cells were harvested on day 8 for following experiments.

DC maturation

On day 8 of culture, BMDCs were transferred to a 96 wells-flat bottom plate at a density of $2x10^5$ cells per well in 200 µL of DC culture medium. 100 ng/ml LPS (Santa Cruz) was then added and cells were cultured at 37^0C in 5% CO_2 for 24 hours to induce DC maturation.

For examination of ACC blockade on DCs maturation and function, different doses of CP-640186 (MedChem Express) were pre-incubated with DCs for 30 minutes before stimulation for 24 hours with 100 ng/ml LPS.

Cytokine analysis

Supernatant from the DCs culture was collected after 24 hours incubation then centrifuged at 13,000 rpm, 4°C. Cytokines were detected by multiplexed bead-based immunoassays (CBA kits – BD Biosciences).

Cell sorting

In T cell proliferation experiment, $CD4^+$T cells were isolated from spleen of female C57BL/6 OT-II transgenic mice. Red blood cells were depleted by using red blood cell lysis buffer (Sigma-Aldrich, Buchs, Switzerland) then washed with PBS 1X buffer. Subsequently, cells were stained with biotin-conjugated antibody cocktails and anti-biotin microbeads following the negative selection protocol of the $CD4^+$T cells isolation kit (Miltenyi Biotec). Finally, conjugated cells were sorted by AutoMACS Pro Separator (Miltenyi Biotec).

CFSE staining

OT-II specific $CD4^+$T cells collected from AutoMACS Pro Separator were stained with 5μM CFSE (Bioscience) for 5 minutes in 37^0C then 5 minutes on ice. Afterwards, cold complete medium of up to 10ml was added for washing. Cells were centrifuged and adjusted to a density of $5x10^5$ cells/ml.

T cell proliferation

On day 8, BMDCs were treated with 10 μg/ml ovalbumin protein (MyBioSource) together with or without 100ng/ml LPS then incubated for 24 hours. In indicated wells, BMDCs were pre-incubated with CP-640186 for 30 minutes before stimulation by ovalbumin protein and LPS.

On day 9, BMDCs were harvested into 15ml falcon tubes, washed by 10 ml PBS 1X then fixed with 2% paraformaldehyde (diluted 1:1 in PBS 1X) for 15 minutes in room temperature. 4% paraformaldehyde was purchased from Santa-cruz. Fixed BMDCs were then washed 3 times by complete medium, tubes were changed for the last wash and the concentration adjusted to 10^5 cells/ml.

OT-II specific $CD4^+$T cells and BMDCs were co-cultured at a ratio of 1:2 in 96 wells-U bottom for 5 days at 37^0C in 5% CO_2 and blocked from light. On day 5, cells were harvested and checked by FACS analysis.

(In indicated experiments, $CD4^+$T cell and DCs co-culture was conducted without DCs fixation)

Foxp3 staining

After 5 days of T cells-DCs co-culture, cells were harvested and a routine stain with anti-CD4 performed. Cells were then fixed with fixation buffer (eBioscience) and stained with anti-Foxp3 for FACS analysis.

Antigen uptake

BMDCs after stimulation by LPS with or without CP-640186 for 24 hours were collected into e-tubes and put on ice for 10 minutes. Subsequently, 10 μg/ml Alexa fluor 488 conjugated ovalbumin (Molecular Probe) was added into each test tube and incubated at 37^0C (water pool), the control sample was left on ice. After the antigen uptake process, cells were washed by cold FACS buffer and stained with BMDCs' surface maker as part of the routine protocol for flow cytometry. All procedures were performed at 4^0C and protected from light. Cells were re-suspended by 2% paraformaldehyde in PBS 1X for FACS analysis.

Bodipy staining

BMDCs after stimulation by LPS with or without CP-640186 for 24 hours were harvested, washed by FACs buffer and blocked with 2.4G2 for 15 minutes in room temperature. Cells were then stained with surface marker together with 10ug/ml bodipy (Molecular Probe) for 15 minutes at 37^0C. Samples were protected from light. Cells were washed and re-suspended with 2% paraformaldehyde in PBS 1X for FACS analysis.

Flow cytometry for surface marker

BMDCs after differentiation and maturation as well as CP-640186 treatment were harvested in separate test tubes, Fc non-specific binding blocked by 2.4G2 in room temperature for 15 minutes and washed again by FACS buffer (0.5% BSA, 0.1% sodium azide in PBS 1X). Cells were

stained with different cocktails of antibodies depending on the purpose of each experiment for 30 minutes on ice and protected from light. After that, cells were washed twice by FACS buffer and re-suspended for FACS analysis directly or stained with 5ul of 7-AAD (Biolegend) for 5 minutes in the dark before immediate analysis by FACS.

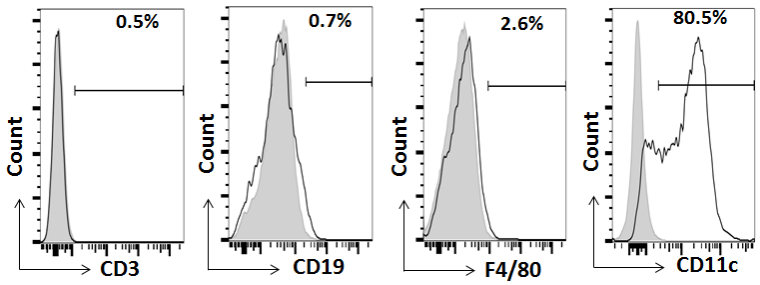
Antibodies used for flow cytometry analysis were as follows: anti-CD45 (30-F11), anti-CD3e (145-2c11), anti-CD80 (16-10A1), anti-CD4(RM4-5), was purchased by BD Pharmingen; anti-CD19 (eBio1D3), anti-F4/80 (BM8), anti-CD11c (N418), anti-MHC class II (I-A,I-E, clone M5/114.15.2), anti-CD86 (GL1), CCR5, CCR7, anti-FoxP3 (FJK-16a) and all isotype controls were purchased by eBioscience; anti-CD40 (clone 3/23) and 7-AAD were from BioLegend.

RESULTS

1. Validation of bone marrow-derived dendritic cells differentiation and maturation

First of all, the quantity and quality of BMDCs were checked to validate whether these cells can be used for subsequent experiments. After 8 days of culture, all suspension cells were gently harvested and stained with anti-CD45, anti-CD11c, anti-CD3, anti-CD19 and anti-F4/80 as markers of leukocytes, DCs, T cells, B cells and macrophages respectively. Isotype control antibodies for each marker were used as negative controls. All CD11c, CD3, CD19 and F4/80 populations were shown in $CD45^+$ gated population. Only the expression of DC's marker (CD11c) was detected in levels up to 80% compared to the other markers of T cell, B cell or macrophage (Figure 2A). Subsequently, BMDCs was incubated with 100 ng/ml LPS for 24 hours to induce full maturation. The expression of MHC class II as well as co-stimulatory molecules including CD80, CD86 and CD40 in the LPS treated group showed marked increment compared to the BMDCs only group (Figure 2B). The morphology of BMDCs with dendrites was confirmed by light microscopy (Figure 2C).

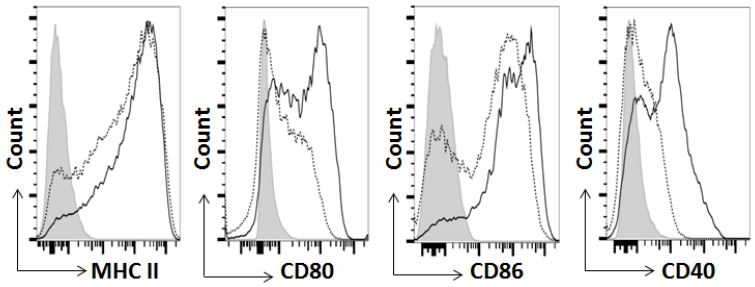
A



Isotype control antibody

Antibody against surface marker

B



Negative control

BMDC only

BMDC + LPS

C

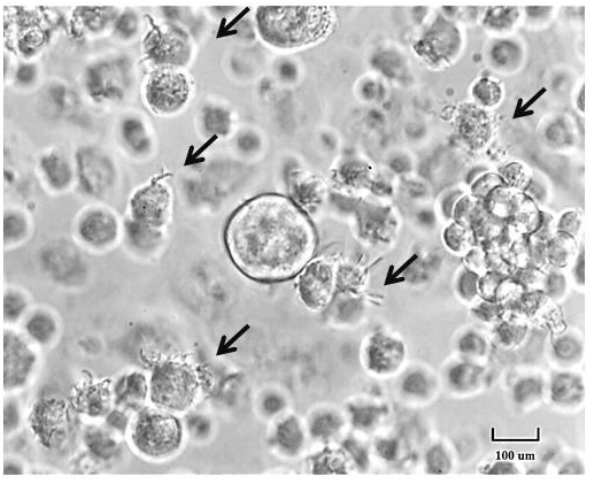
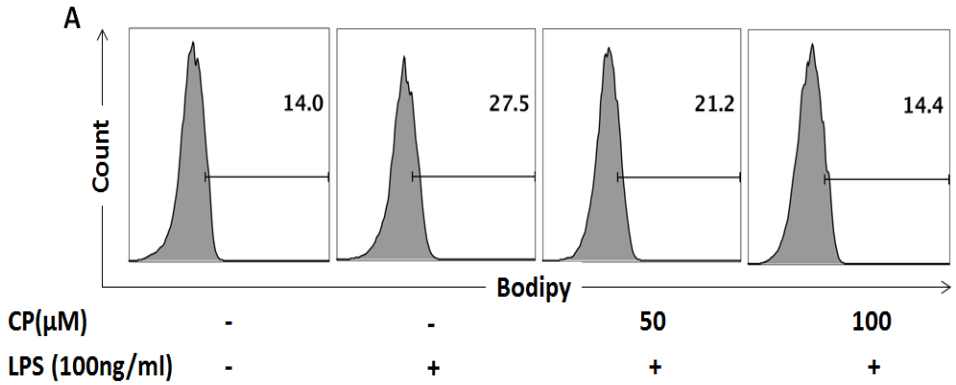


Figure 2. **BMDCs were generated with high purity and immature phenotype**

(A) The expression of DCs, T cells, B cells and macrophage surface markers after 8 days of differentiation from murine bone marrow. (B) The expression of MHC-II and co-stimulatory molecules in immature (dot line) and mature (black line) DCs. (C) Morphology of BMDCs on day 8 with dendrites was observed by light microscopy.

2. Inhibitor effect of CP-640186 (CP) on DCs' lipid metabolism

Next, to confirm the effect of CP on ACCs to block FAS but improve FAO, lipid droplets (LDs) accumulation within DCs was measured following CP treatment. DCs were harvested on day 8 and pre-incubated with CP for 30 minutes, followed by LPS treatment of up to 24 hours. On day 9, cells were stained by Bodipy, together with DC surface marker CD11c, for FACS analysis. As expected, LDs were reduced following CP treatment in a concentration-dependent manner (Figure 3A). Moreover, as FAO and FAS pathways are each important for imDCs and mDCs respectively, LDs accumulation was observed to be lower in imDCs than in mDCs (Figure 3A, B). Notably, when CP was pre-incubated in LPS-treated BMDCs group, the amount of LDs in DCs significantly decreased at a 100 μM concentration of CP (Figure 3B). Thus, these data showed that CP blocked LDs accumulation in LPS-treated DCs.


A
Count
14.0
27.5
21.2
14.4
Bodipy
CP(μM) - - 50 100
LPS (100ng/ml) - + + +

B

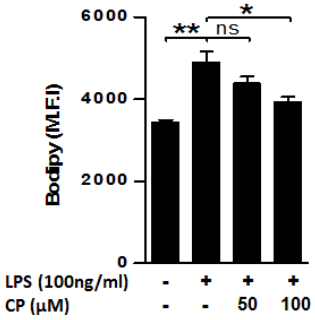

6000
4000
2000
0
Bodipy (MFI)
**
ns
*
LPS (100ng/ml) - + + +
CP (μM) - - 50 100

Figure 3. Lipid droplets (LDs) accumulation in LPS-treated DCs decreased following CP treatment

(A) LDs accumulated in BMDCs alone (black line), LPS-treated BMDCs alone (red line) and LPS-treated BMDCs with different concentration of CP (gray histogram). (B) Bodipy staining experiment was repeated three times independently and mean fluorescence intensity observed. Statistical data was calculated by using one-way ANOVA followed by Tukey's comparison test. Error bars denote the mean± S.D. *$p<0.05$, **$p<0.01$ when compared to the control.

3. Mapping experimental design for phenotypic and functional study on semi-mature DCs generated by CP treatment:

To investigate whether semi-mature DCs can be generated by the inhibition of ACCs after 8 days of differentiation from murine bone marrow, BMDCs were gently harvested (only non-suspension cells) for further studies (Figure 4A). In order to induce DCs resistance to stimuli, CP was pre-incubated with DCs for 30 minutes before LPS or LPS with OVA treatment. BMDCs only and LPS-treated or LPS+OVA-treated groups were used as controls (Figure 4B). All groups were then continuously incubated for up to 24 hours. On day 9, cells phenotype and function were examined (Figure 4C).

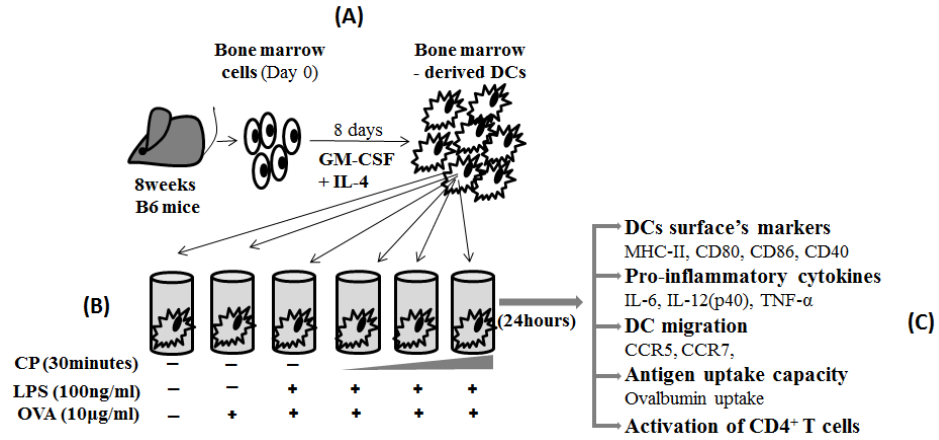


Figure 4. Schematic representation of experimental design

(A) Bone marrow cells were isolated from 8-week-old naive B6 mice then differentiated into BMDCs for 8 days. (B) On day 8, BMDCs were gently harvested and treated by CP, LPS and OVA under the indicated conditions. (C) DCs phenotype and function were subsequently tested.

4. Targeting ACC signaling pathway suppresses co-stimulatory molecules expression during BMDCs maturation

Firstly, the effect of ACC-blockade on DC's phenotype was examined by using different concentrations of CP (20-50-100-200 μM). The results showed that the expression of MHC-II and all other co-stimulatory molecules (CD80, CD86 and CD40) in LPS-treated DCs were down-regulated in the presence of CP in a concentration-dependent manner (Figure 5A). The expression of MHC-II showed a slight decrease from CP 20 to 50μM and was effectively suppressed at CP 100 μM (Figure 5B). Meanwhile, the inhibition effect on CD80 was minor up to 100μM (Figure 5C). Interestingly, levels of CD86 molecule were dramatically decreased at 20 μM and showed almost complete suppression with levels similar to imDCs at 50 and 100 μM (Figure 5D). Additionally, the expression of CD40 fluctuated between 20 and 50μM before a dramatic decrease at CP 100 μM (Figure 5E). However, all co-stimulatory molecules as well as MHC-II were inhibited at CP 200 μM (Figure 5B-E). Hence, these results showed that the inhibition of ACCs to suppress the maturation process of BMDCs is dose-dependent but differs between each type of surface marker.

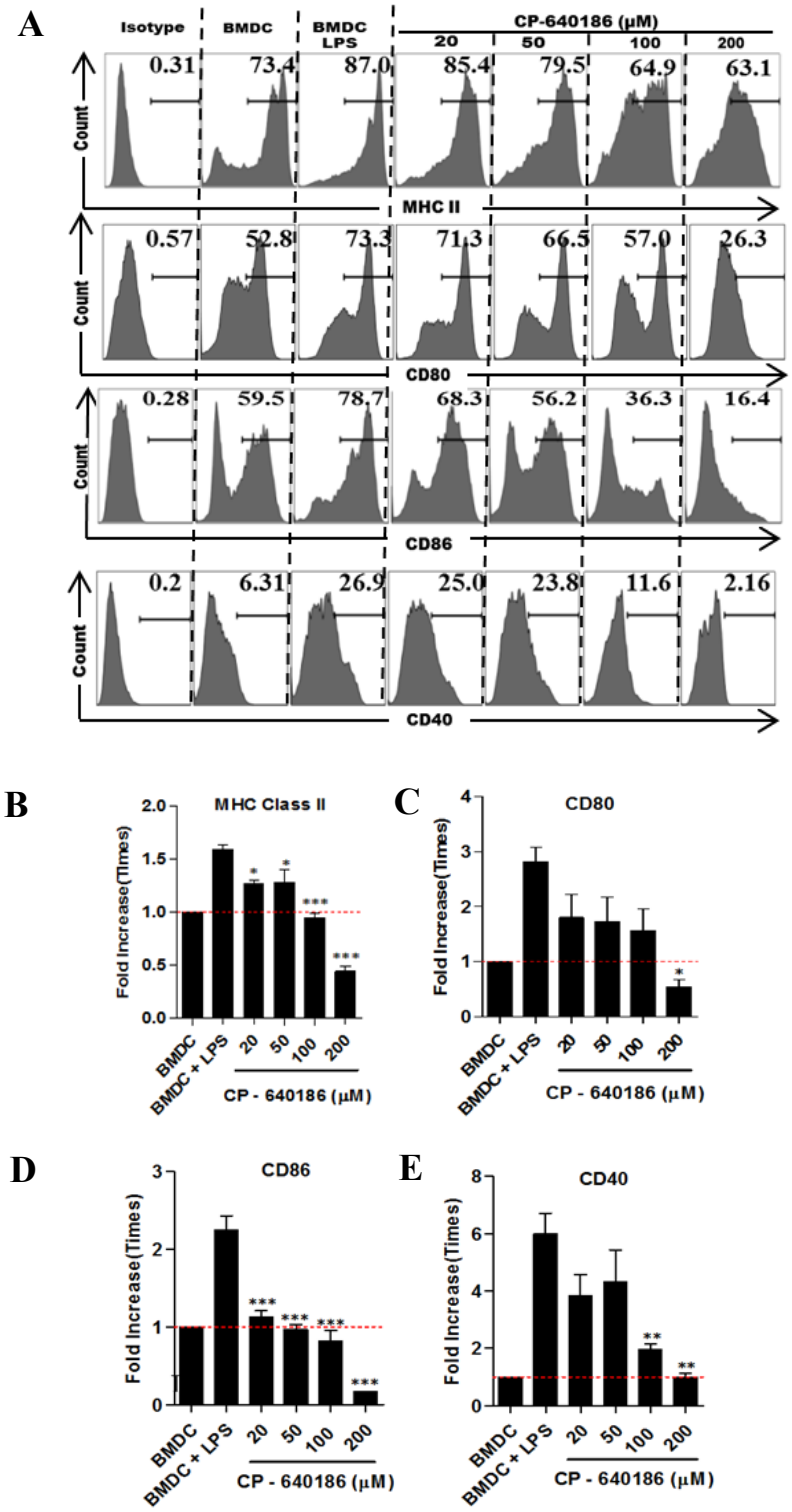

A
Isotype
BMDC
BMDC LPS
CP-640186 (μM)
20
50
100
200
Count
MHC II
0.31
73.4
87.0
85.4
79.5
64.9
63.1
CD80
0.57
52.8
73.3
71.3
66.5
57.0
26.3
CD86
0.28
59.5
78.7
68.3
56.2
36.3
16.4
CD40
0.2
6.31
26.9
25.0
23.8
11.6
2.16
B
MHC Class II
Fold Increase(Times)
BMDC
BMDC + LPS
CP - 640186 (μM)
C
CD80
D
CD86
E
CD40

Figure 5. MHC-II and co-stimulatory molecules expression during BMDCs maturation were suppressed by ACCs inhibitor

(A) Representative histogram of MHC-II and co-stimulatory molecules expression at different CP-concentration. (B-E) The expression of MHC-II, CD80, CD86 and CD40 respectively by fold increase of mean fluorescence intensity compared to the level of BMDCs. (the results indicate three independent experiments). Statistical data was calculated by using one-way ANOVA followed by Tukey's comparison test. Error bars denote the mean± S.D. *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to the control.

5. The down-regulation of DC surface markers is not affected by cell death

For further functional studies, CP concentration was optimized to be able to suppress DC maturation but not induce cell death. On day 8, BMDCs were harvested and divided into two groups. Different doses of CP was pre-incubated for 30 minutes into both groups (dosage as indicated) but only the second group was treated with 100 ng/ml LPS. Both groups were then incubated again for another 24 hours. FACs analysis was carried out for the two groups with BMDCs only or LPS-treated BMDCs used as the controls. Late apoptosis induction was tested with 7-AAD. Notably, the dead cell population was negligible up to CP concentration of 100 μM (Figure 6). The population increased to 40% only in a very high dose of CP at 200 μM in both groups (Figure 6). Therefore, CP 50 and 100 μM were selected for further studies. These data also suggested that FAS might be essential for the survival of not only mature but also immature DCs since cell death of about 40% of the population was induced at high inhibition dose (200 μM).

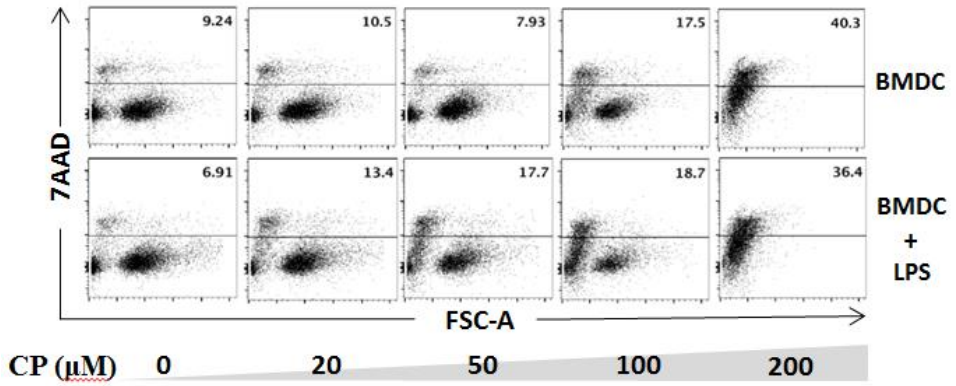


Figure 6. ACCs inhibition should be strictly optimized

BMDCs (upper panel) and LPS-BMDCs (lower panel) in the presence of CP were tested for late apoptosis induction by FACS analysis. All these results were shown from the $CD11c^+$ gated population.

6. ACC-blockade decreased the pro-inflammatory cytokine secretion in dendritic cells

One of the most important functions of DCs to control both innate and adaptive immune response is their ability to secrete a large amount of cytokines. In this study, IL-12, TNF-α and IL-6 were tested. To measure cytokine secretion, DCs were prepared and stimulated by LPS as described in Figure 4B, the supernatant was collected and analyzed using a CBA kit. Interestingly, the secretion of all tested cytokines was significantly decreased from CP 50 μM (Figure 7). Especially, IL-6 (Figure 7B) and IL-12 (Figure 7C) were suppressed more effectively than TNF-α (Figure 7A) from CP 50 μM. Thus, these results showed that ACC-blockade also suppress cytokine secretion in LPS-treated DCs.

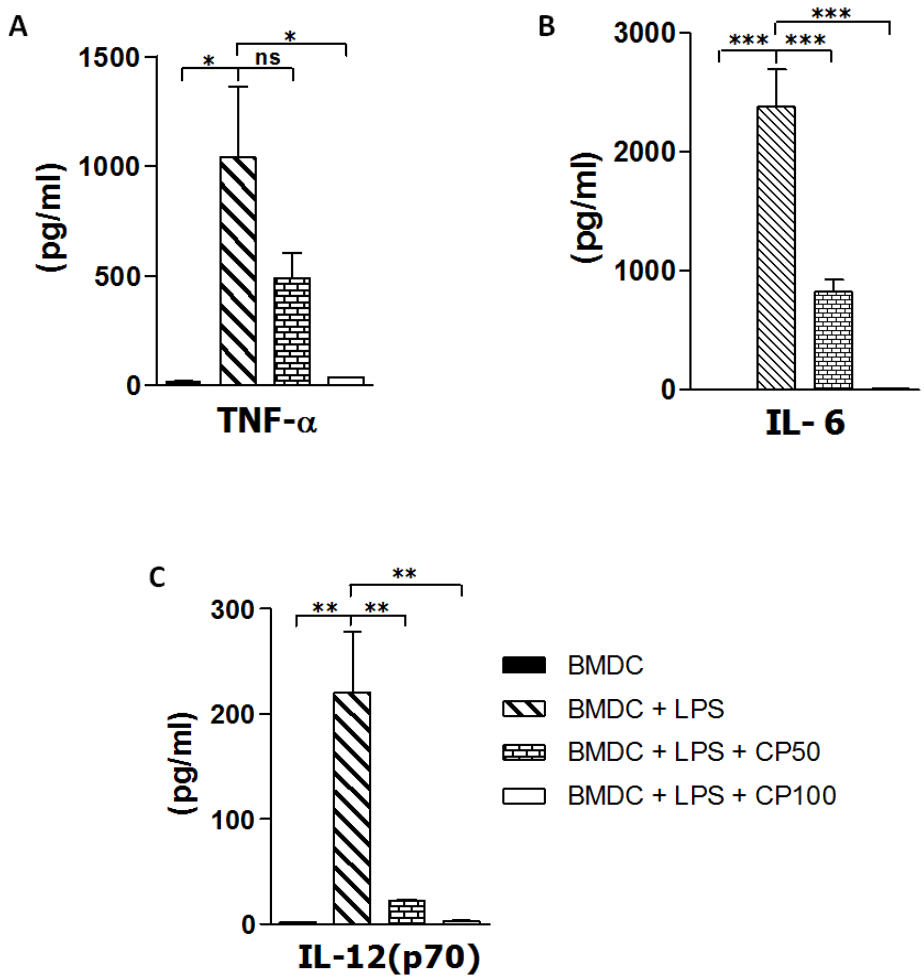


Figure 7. ACC-blockade (CP-640186) decreased the pro-inflammatory cytokine secretion in DCs.

The secretion of cytokines TNF-α (A), IL-6 (B) and IL-12 (C) was examined in three independent experiments. Statistical data was calculated using one-way ANOVA followed by Tukey's comparison test. Error bars denote the mean± S.D. *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to the control.

7. ACC-blockade modified CCR-expression pattern in dendritic cells

Next, immature DCs express several kinds of CCR patterns including CCR5 to maintain at the site of infection. However, this chemokine receptor is replaced by CCR7 in mature DCs, hence CCR5 and CCR7 are used to distinguish the maturity of DCs. The effect of ACC-blockade on expression levels of CCR5 and CCR7 were examined to determine the migration capacity of DCs. CP treated group showed a significant increase of CCR5 expression to levels of 70% and 80% at doses of CP 50 and 100μM respectively (Figure 8, left panel). Interestingly, CCR7 was down-regulated but still remained at an intermediate level between BMDCs and LPS-treated groups (Figure 8, right panel). These results indicated the migration capacity of mature DCs treated with CP was similar to immature-like DCs.

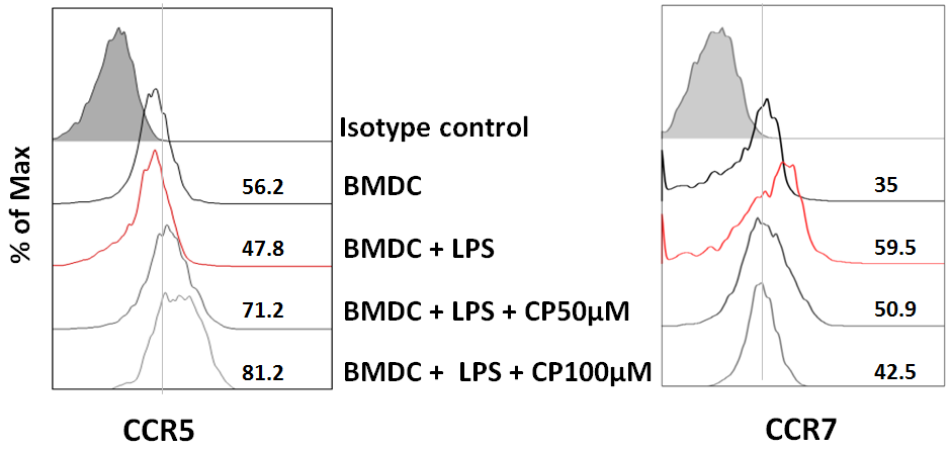


Figure 8. The migration capacity of mature DCs was reversed by ACC inhibitor

The modification of CCR5 (left panel) and CCR7 (right panel) expression on DCs in different conditions was observed by FACS analysis. 7AAD was used to exclude apoptotic cells. This experiment was conducted twice and similar results were obtained.

8. Antigen uptake capacity is recovered in LPS-treated DC by blocking ACC

Following CP treatment, DCs were shown to have markers indicating low maturation, low pro-inflammatory cytokines secretion and the ability to maintain in the site of inflammation. Therefore, there is a possibility that CP-treated DCs may be able to capture antigens like imDCs. To further investigate, DCs were prepared (Figure 4A, B) and ovalbumin protein conjugated with fluorescence Alexa-fluor 488 was added on day 9. ImDCs incubated with conjugated OVA-Alexa-fluor 488 was put on ice as a control. Surprisingly, in the presence of CP, antigen uptake capacity was dramatically recovered and even increased to levels higher than those seen in imDCs (Figure 9). These results strongly demonstrated that by blocking ACC, LPS-treated DCs showed enhanced antigen uptake capacity, a characteristic of imDCs.

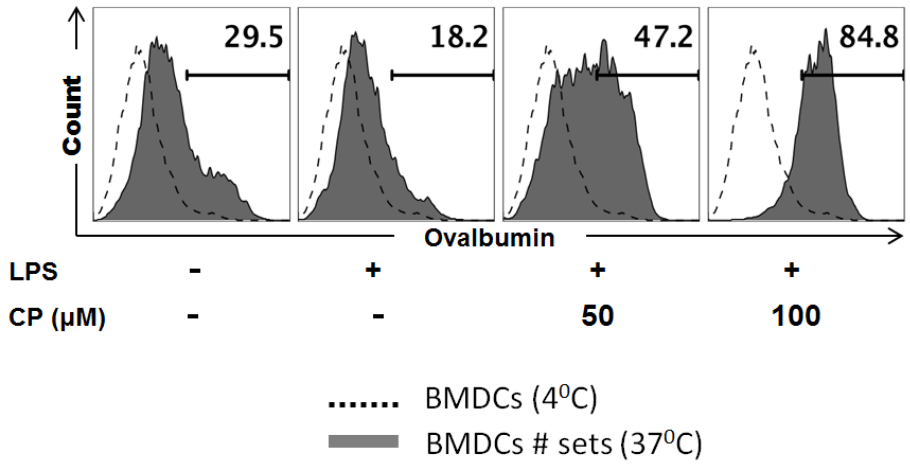


Figure 9. LPS-treated DC enhanced antigen uptake capacity by blocking ACC

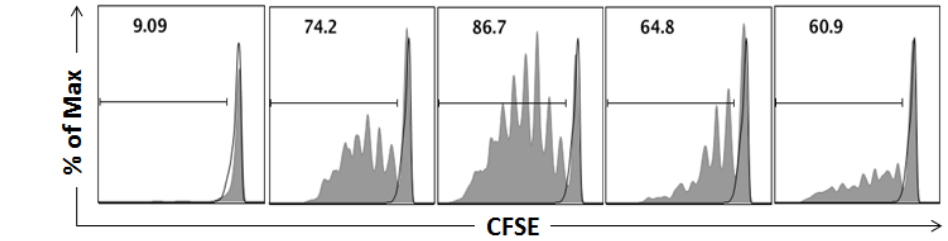
Antigen uptake capacity of DCs was tested by using conjugated OVA-Alexa fluor 488. Immature DCs were incubated with antigen at 4^0C as a control (dash line). All other samples (gray histogram) were incubated with antigens at 37^0C for 1 hour and stained with DC surface marker (CD11c) for FACS analysis. This experiment was conducted twice and similar results were obtained.

9. Dendritic cell failed to activate antigen-restricted $CD4^+$T cell proliferation in the presence of ACCs inhibitor

The function of DCs as the professional antigen presenting cells for activation of T cells to induce adaptive immune response was examined. DCs ability to activate T cell proliferation at a time point on day 9 (24 hours after exposed to stimuli including LPS and OVA as described in figure 4B) was compared between different conditions of DCs. On day 9, before co-culture, DCs were fixed with 2% paraformaldehyde. OT-II specific $CD4^+$T cells were isolated by MACS negative selection then labeled with CFSE. Subsequently, $CD4^+$T cells and DCs-loaded antigen were co-cultured for 5 days. T cell proliferation was detected on day 5 by FACS analysis. These results showed that, in the presence of CP 50 and 100μM, T cell proliferation was less than that in LPS+OVA treated group (Figure 10A).

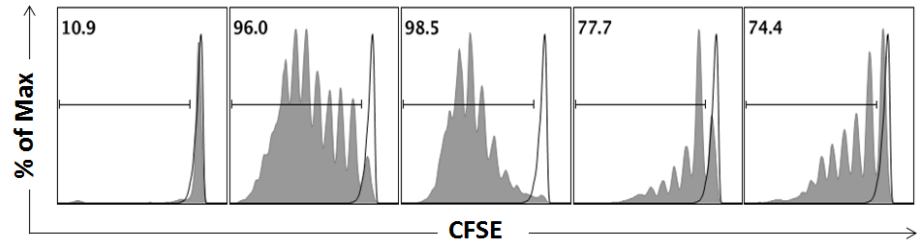
Finally, DCs were further investigated in order to test whether they can be used for in vivo study. DCs-T cell co-culture experiment was repeated without DCs fixation step. And Foxp3 expression in $CD4^+$T cells after 5 days co-culture was tested as well. Surprisingly, T cell proliferation was lowest in CP 50μM and not 100 μM (Figure 10B). Foxp3 population was also slightly increased in CP 50 μM (Figure 10C). Additionally, the increase of Foxp3 expression was only observed when DCs was not fixed (Figure 10D). These data greatly demonstrated that DCs failed to activate T cell proliferation by ACCs inhibitor.

A



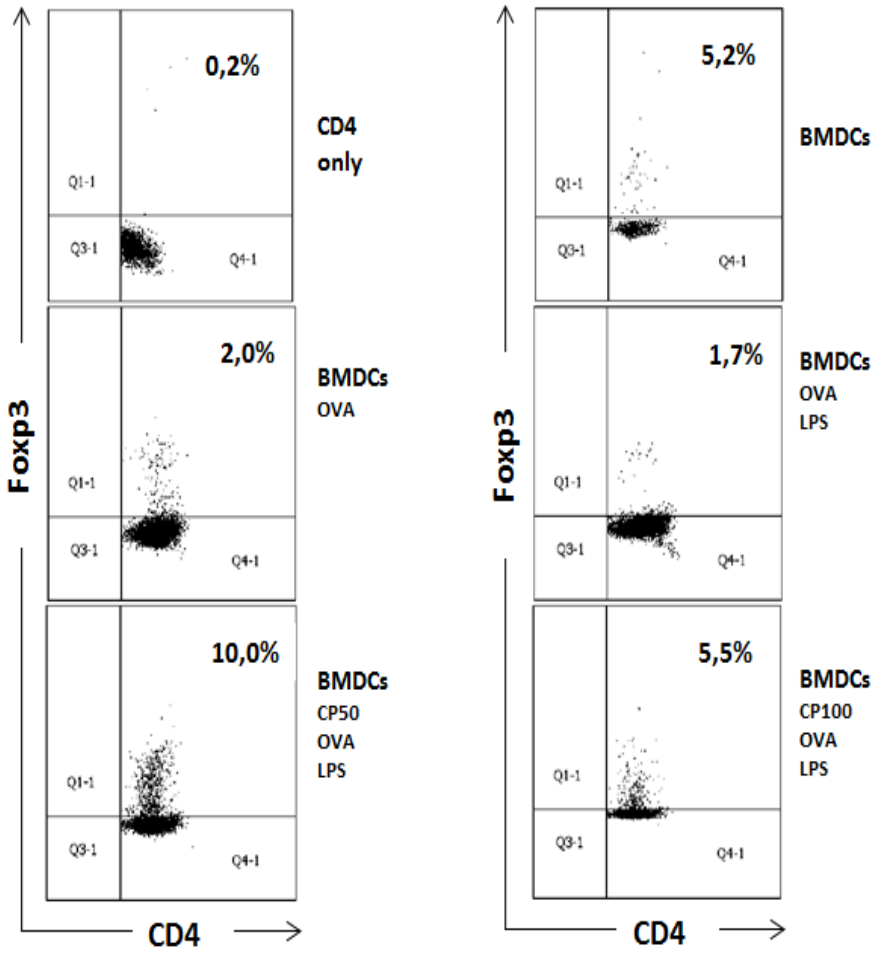
CP(μM)	-	-	-	50	100
OVA(10μg/ml)	-	+	+	+	+
LPS(100ng/ml)	-	-	+	+	+

B

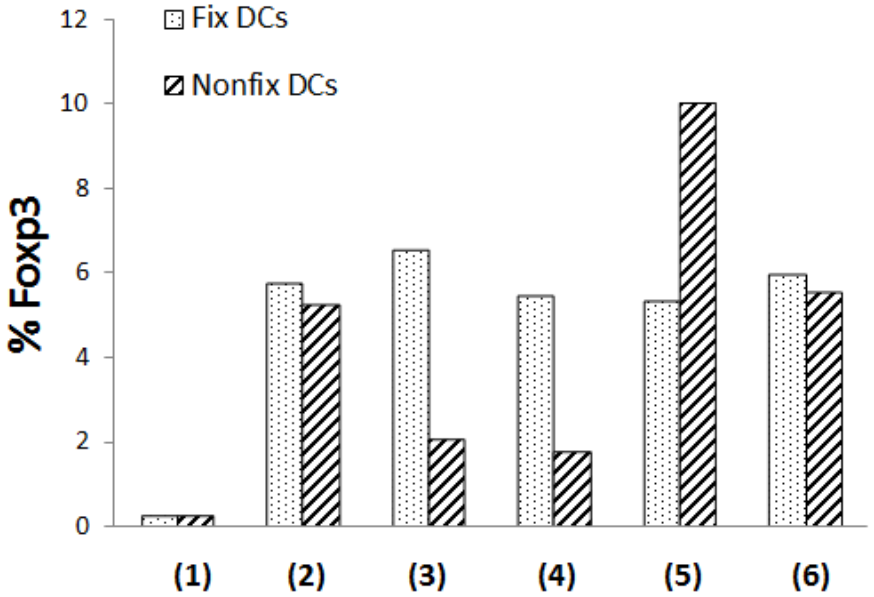


CP(μM)	-	-	-	50	100
OVA(10μg/ml)	-	+	+	+	+
LPS(100ng/ml)	-	-	+	+	+

C


0,2%
CD4
only
2,0%
BMDCs
OVA
10,0%
BMDCs
CP50
OVA
LPS
5,2%
BMDCs
1,7%
BMDCs
OVA
LPS
5,5%
BMDCs
CP100
OVA
LPS
Foxp3
CD4
Q1-1
Q3-1
Q4-1

D



CD4	$5x10^4$	$5x10^4$	$5x10^4$	$5x10^4$	$5x10^4$	$5x10^4$
DCs	0	10^4	10^4 **OVA**	10^4 **LPS+OVA**	10^4 **CP50 LPS+OVA**	10^4 **CP100 LPS+OVA**

Figure 10. DCs lost their ability to activate T cell proliferation by ACCs inhibitor

For T cell proliferation assay, $CD4^+$T cells co-cultured with DCs which were harvested on day 9 then fixed with 2% paraformaldehyde (A) or non-fixed (B). $CD4^+$T cell proliferation was examined by CFSE staining method. $CD4^+$T cells expressing only $CFSE^{high}$ was used as a control (black line). The proliferating cells were gated in the $CFSE^{low}$ population (gray histogram). Different conditions of DCs co-culture with T cells are labeled under each histogram respectively. (C) Foxp3 expression in $CD4^+$T cells after 5 days of co-culture with non-fixed DCs. (D) Bar chart comparing Foxp3 expression in $CD4^+$T cells after 5 days of co-culture between fixed DCs (black-dot bar) and non-fixed DCs (black-slant bar). The table below bar chart indicates the different conditions of each group. These experiments were conducted twice and similar results were obtained.

DISCUSSION

This study demonstrates that semi-mature DCs can be generated by optimizing ACC inhibition dose. The evidence is that semi-mature DCs (smDCs) were shown as MHC-II^{intermediate}CD86^{low}CD80^{intermediate}CD40^{high} at CP 50 μM and MHC-II^{low}CD86^{low}CD80^{intermediate}CD40^{low} at CP 100μM (Figure 5B-E). These phenotypic modifications are not due to cell death since the 7AAD positive population is minor up to 100 μM of CP treatment (Figure 6). However, as FAS is essential for both survival of imDCs and mDCs, experiments to optimize the use of ACC inhibitor in DCs should be strictly controlled (Figure 6, right-top and bottom panel). In addition, blocking both ACC1 and ACC2 can modify DC phenotype as well as its functions. Firstly, ACCs-blockage induced a high expression of chemokine receptor CCR5 but showed a slight decrease of CCR7 on LPS-treated DC (Figure 8) different from that of its natural maturation process (4, 30). Comparing to a previous study by *Braun et al.*, the interaction of CD47 and SIRP-α on LPS-treated DCs after 24 and 48 hours was investigated. Results showed that MHC-II and other co-stimulatory molecules exhibited slight decrease at 24 hours and was completely down-regulated to the level seen in imDCs at 48 hours Observations by *Braun et al.* on the expression of CCR5 and CCR7 at 24 hours and 48 hours were similar to results obtained in this study (16). However, in this study, expression of CCR5 was observed to be enhanced to levels higher than those in imDCs but no similar observation was found in *Braun et al.* (Figure 8, left panel). Moreover, the observed antigen uptake capacity of smDCs (16) was not induced as effectively as those in our study (Figure 9). A possible explanation could be due to a difference in signaling pathways leading to a different effect on the DCs.

In this study, smDCs expressed CCR5 with the ability to capture antigens but did not secrete pro-inflammatory cytokines (Figure 7). Therefore, usage in the treatment for autoimmune diseases can be considered since they can migrate to the tissues and capture antigens but may not activate T cell proliferation (Figure 10).

Our results are in agreement with another study on the blocking of enzymes in the FAS pathway by C75 or inhibition of ACC1 by TOFA during DCs maturation resulting in decreased secretion of inflammatory cytokines (23). However, in that study, they did not further investigate on the other functions of DCs. Interestingly, another study has also reported that inhibition of FAS, via ACC1 only, during DCs generation showed the down-regulation of MHC-II and B7-1, B7-2 molecules, enhancement of antigen uptake capacity but increased inflammatory cytokines. Therefore, immune response was induced by BMDCs without need for further stimuli (22). These different results reveal that lipid metabolism pathway plays an important but distinct role in different periods of DC's life cycle. However, other functions or surface markers of DCs when FAS was inhibited during DC maturation were not shown in those studies. Furthermore, these studies are limited as FAS and FAO pathways in lipid metabolism are closely linked and studies on FAS cannot disregard the influence of FAO. Particularly, the optimization of CP concentration to balance FAS inhibition/FAO promotion showed that DCs phenotype and functions were modified in a dose dependent manner. The evidence showed that difference in strength of inhibition and promotion of the respective pathways led to a significant difference in the surface markers expressed in DCs. The data also strongly suggested a dose-dependent difference in function of DCs which is proven in CP 50 μM and CP 100 μM. Thus, not

only FAS but also FAO should be considered and strictly controlled to generate semi-mature DCs, since it affects DCs function and even survival (Figure 6-10).

Finally, it was interest to note that there were differences observed between fixed and non-fixed DCs. Slight differences such as the increase in percentage of Foxp3 was seen only in non-fixed DCs (Figure 10D). Notably, smDCs showed a loss in their capacity to activate T cell proliferation (Figure 10A, B). In the case of fixed DCs, DCs can activate T cell proliferation at CP 50 μM more than that in CP 100 μM (Figure 10A). This observation may be explained by their lower expression of MHC-II and co-stimulatory molecules as well as the loss in their capacity to secrete cytokines in CP 100 μM than in CP 50 μM (Figure 5, 7). Interestingly, in the case of non-fixed DCs, the opposite was observed where a greater inability to activate T cells was found in DCs pre-treated with CP 50 μM rather than 100 μM (Figure 10B). This difference observed between fixed and non-fixed DCs could be due to the cells' ability to secrete cytokines as inhibition effect on cytokine secretion in CP 50 μM is reduced by half relative to CP100 μM. Therefore, in the case of non-fixed DCs, during the co-culture period, they may be able to actively secrete cytokines or molecules which can further suppress their ability to activate T cell response (Figure 10B). However, for fixed DCs, they rely on the levels of MHC-II loaded antigen and co-stimulatory molecules to activate T cell proliferation and is therefore not as susceptible to inhibition of cytokine secretion (Figure 10A). Additionally, this explanation can be supported by current knowledge on active secretion of molecules for immune tolerance by tolerogenic DCs which share the same semi-mature phenotype (20) as

cells in this study. However, further studies must be conducted to prove this reasoning and for the determination of the exact molecules involved.

In summary, all observations support the important role of both FAS and FAO in DCs maturation and function. By partially blocking the action of ACC1 and ACC2, important isoenzymes in lipid metabolism, DCs can be held at its semi-mature state. This allows for the control of DC function in (1) the expression of MHC-II and co-stimulatory molecules, (2) the secretion of cytokine as well as (3) activation of T cell proliferation. By targeting both ACC1 and ACC2 with only a single inhibitor, CP, this study introduces a promising smDCs phenotype and provides a more efficient and precise method to enhance the role of DCs in immunotherapy. Further studies in vivo can improve use of this phenotype in clinical application and in further development of dendritic cell therapy metabolism-based approaches in areas such as transplantation and treatment of autoimmune diseases.

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초 록

연구배경: 준성숙 (semi-mature) 또는 미성숙 (immature) 단계의 수지상 세포는 항원-특이적 T 세포 면역관용을 유도할 수 있다고 알려져 있다. 면역관용 유도능이 있는 이러한 수지상 세포 (Dendritic cell)을 이용하여 이식거부반응의 억제나 자가면역질환 등의 치료에 응용하려는 연구들이 많이 시도되고 있다. 본 연구는 지방 대사과정 중 지방산 (fatty acid)의 합성 (synthesis)과 산화 (oxidation)에 중요한 역할을 하는 acetyl-CoA carboxylase (ACC)가 수지상세포의 성숙과정에 미치는 영향에 대해 알아보았다. 또한 acetyl-CoA carboxylase 가 억제된 상황에서 생성된 준성숙 수지상세포가 시험관내에서 (*in vitro*) 항원특이적 T 세포의 증식과 활성에 미치는 영향에 대하여 알아 보았다.

재료 및 방법: 8 주령의 C57BL/6 마우스 골수에서 분리한 골수세포를 GM-CSF (10 ng/ml)와 IL-4 (1.5 ng/ml)가 포함된 RPMI 배지에서 8 일간 배양 및 분화시켜 골수유래 미성숙 수지상세포를 획득하였다. 8 일간 배양된 골수유래 미성숙 수지상세포는 24 시간동안 LPS (Lipopolysaccharide) 자극을 줌으로서 성숙 수지상세포로의 분화를 유도하였다. Acetyl-CoA Carboxylase 의

활성을 저해하기 위해 CP-640186 이라는 Acetyl-CoA Carboxylase 활성 저해제를 사용하였다. 그 후 미성숙수지상세포, 성숙 수지상세포 그리고 acetyl-CoA carboxylase 의 억제 화합물인 CP-640186 를 처리한 수지상세포의 세포표면인자 발현량, 사이토카인 분비능, 항원섭취능력 (antigen-uptake capacity), 항원특이적 T 세포 증식능력등을 비교하였다.

결 과: 미성숙수지상세포에서는 LPS 의 자극에 의해 성숙수지상세포로 변화되는 과정에서 지방산의 합성이 증가되었다. 이에 반해 지방산 합성과 산화에 관여하는 acetyl-CoA carboxylase 가 저해된 수지상세포에서는 성숙수지상세포에 비해 지방산 합성이 현저히 감소되었다. Acetyl-CoA carboxylase 저해제를 사용한 수지상 세포는 성숙 수지상세포에 비해 MHC-class II 와 보조자극분자 (co-stimulatory molecule)인 CD80, CD86, CD40 의 발현량이 감소되었다. 동시에 염증성 사이토카인인 IL-6, IFN-γ, IL12p70 의 발현 또한 CP-640186 의 사용량에 비례하여 억제되었다. 흥미롭게도 케모카인 수용체 (chemokine receptors) 중, lymph node 귀소 수용체 (homing receptor)인 CCR7 의 세포표면 발현은 acetyl-CoA carboxylase 사용에 의해

감소되었지만 CCR5 의 발현은 오히려 증가되었다. Acetyl-CoA carboxylase 의 억제에 의해 수지상세포의 항원섭취능은 증가되었다. 따라서 acetyl-CoA carboxylase 의 억제에 의해 미성숙 수지상 세포는 성숙과정에서 준성숙 수지상세포로 변화되었음이 세포표면인자, 항원 섭취능력 및 염증성 사이토카인의 발현량등을 통해 확인되었다. 이렇게 만들어진 준성숙 수지상세포는 성숙 수지상세포에 비해 ovalbumin-specific $CD4^{+}T$ 세포의 증식을 항원특이적으로 저해 하였다.

결론: 지방산의 합성과 산화에 중요한 acetyl-CoA carboxylase 를 저해함으로써 준성숙 수지상세포를 만들수 있음을 이 실험을 통해 밝혔다. 이러한 방법을 통하여 얻어진 준성숙 수지상세포는 수지상세포를 응용한 면역치료에 응용될 수 있을 것이다.

주요어: 준성숙 수지상 세포, CP-640186, Acetyl-CoA carboxylase, 지방대사

학생번호: 2013 – 22591