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Development of Immune Modulatory Adjuvants for Antigen Presenting Cells and Immunological Role of CD30 in Adaptive Immunity

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항원제시세포의 면역조절 보조제 발굴과

적응면역반응에서 CD30의 면역학적 역할 규명 연구

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이 논문을 이학박사학위신청 논문으로 제출함

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CONTENTS

II. MATERIALS AND METHODS.....15

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

APCs	Antigen presenting cells
β-GA	β-Glycyrrhetinic acid
COX-2	Cyclooxygenase-2
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
ESH	Ethanolic extract of *S. horneri (Turner)* C. Agardh
FACS	Fluorescence activated cell sorter
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
iDCs	Immature DCs
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinases
KO	Knockout
LPS	Lipopolysaccharide
mDCs	Mature DCs

MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Histocompatibility complex
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NF-κB	Nuclear factor-κB
NK	Natural killer
NO	Nitric oxide
SDS-PAGE	SDS-polyacrylamide gel eletrophoresis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PI	Propidium iodide
PE	Phycoerythrin
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PRRs	Pattern-recognition receptors
PVDF	Polyvinylidene difluoride
rm	Recombinant murine
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
TCR	T cell receptor
Th1	T helper cell type 1

Th2	T helper cell type 2
TLR	Toll-like receptor
TRAF	Tumor necrosis factor receptor-associated factor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TNFRSF	TNFR superfamily
WT	Wild-type

ABSTRACT

Development of Immune Modulatory Adjuvants for Antigen Presenting Cells and Immunological Role of CD30 in Adaptive Immunity

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The immune response is a host defense mechanism against foreign pathogens and tumors. Immunity is composed of two major responses, the "innate immune response" and "adaptive immune response". The innate immune response is non-specific. For example, inflammation is an innate immunological response induced by macrophages, which is characterized by fever, vasodilatation, and inflammatory cytokine production. In addition, the innate immune response plays a role in presenting antigenic information to the adaptive arm of the immune response, which induces the antigen-specific immune response. Dendritic cells (DCs) are powerful antigen presenting cells (APCs) that present antigen-specific peptides to T cells. In contrast to the innate immune response, the

adaptive immune response is antigen-specific, and is divided into cell-mediated and humoral immunity. Many studies have shown that these immune responses are tightly associated with health; however, aberrant immune responses are associated with many types of disease including diabetes, sepsis, cardiovascular disease, allergy, and cancer. It has been suggested that these immune-related diseases might be cured or prevented through modulation of the immune response. Therefore, many researchers have attempted to understand the regulation of immunological functions in various aspects. The two major ways to regulate the immunological functions are via chemical modulation and genetic modulation. The present study identified new immunomodulatory chemicals and genetic regulators for innate immunity and adaptive immunity.

Sargassum horneri (Turner) C. Agardh is a brown algae and galangin is a member of the flavonols found in *Alpinia officinarum*. In the present study, ethanolic extracts of *S. horneri* (Turner) C. Agardh (ESH) and galangin exhibited anti-inflammatory effects on RAW 264.7 macrophages *in vitro*. Treatment with ESH and galangin resulted in reduced nitric oxide (NO) production as well as reduced expression of pro-inflammatory genes such as interleukins, inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-stimulated macrophages. These anti-inflammatory activities were mediated by the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways.

Baicalin is a flavonoid found in *Scutellaria baicalensis,* and 18β-glycyrrhetic acid (β-GA) is a triterpenoid compound derived from Licorice root. The present

study investigated whether baicalin and β-GA could influence surface molecule expression, antigen uptake capacity, and cytokine production on DCs, and induce subsequent T cell differentiation. Baicalin and β-GA significantly suppressed the expression of surface molecules, CD80, CD86, major histocompatibility complex (MHC) class I, and MHC class II and reduced the levels of IL-12 production in LPS-stimulated DCs. Moreover, baicalin or β-GA-treated DCs showed an impaired induction of the T helper type 1 (Th1) immune response. These findings provided clues to important immunopharmacological functions of baicalin and β-GA, which have ramifications for the development of therapeutic adjuvants for the treatment of DC-related acute and chronic diseases.

CD30, also called tumor necrosis factor receptor superfamily (TNFRSF) 8, is a co-stimulatory molecule of immune cells. In a previous study, CD30 was shown to be expressed on lymphoid cells such as lymphoma cells, and activated T cells and B cells. Although CD30 is a marker for several diseases including lymphoma and allergy, it is also related to helper type 2 (Th2) responses in adaptive immunity. The present study investigated the immunological role of CD30 in $CD4^+$ T cells and tumor immunity using $CD30^{-/-}$ mice. CD30 deficiency did not affect immune cell populations or DC maturation. However, the lack of CD30 caused $CD4^+$ T cell proliferation and activation, and decreased survival of the MC-38 murine colon cancer tumor metastasis model. These data indicate that CD30 is involved in$CD4^+$ T cell function and tumor immunity.

Taken together, it was concluded that various natural compounds could regulate the immunological functions of APCs such as macrophages and DCs

through modulation of immune response-related gene expression, expression of co-stimulatory molecules, and pro-inflammatory cytokine production. Furthermore, the regulation of DCs resulted in T cell polarization, which would influence the fate of the adaptive immune response. In addition, CD30 deficiency was shown to suppress T cell activation and anti-tumor immunity. Our findings suggest that ESH, galangin, baicalin, and β-GA have potential as immunological therapeutic adjuvants, while CD30 might be a genetic modulator of adaptive immunity.

국문초록

항원제시세포의 면역조절 보조제 발굴과 적응면역반응에서 CD30의 면역학적 역할 규명 연구

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면역반응은 외부로부터 침입한 항원 또는 종양으로부터 신체를 보호하기 위한 숙주의 방어기작으로, 선천면역반응과 적응면역반응 두 가지로 구분된다. 선천면역반응은 비특이적 면역반응으로, 대식세포에 의해 유발되는 염증반응은 대표적인 면역학적 징후이며 열, 혈관확장, 염증성 사이토카인의 생성을 동반한다. 또한, 선천면역반응에서는 항원 특이적인 면역반응을 위하여 적응면역반응에 항원의 정보를 제공한다. 수지상세포는 강력한 항원제시세포이며 T 세포에 항원특이성에 대한 정보를 제공한다. 선천면역반응과 달리 적응면역반응은 항원 특이적 면역반응으로 세포매개성 면역반응과 체액성 면역반응으로 나뉜다. 현재까지, 수많은 연구를 통해서 이러한 면역반응들이 인체의 건강과 당뇨, 패혈증, 심혈관질환, 알러지, 종양 등 다양한 질병과 밀접한 연관이 있다고 알려져 왔으며, 이러한 질병은

면역반응을 조절함으로써 완화될 수 있다고 보고 있다. 따라서 많은 사람들은 다양한 측면에서 면역반응을 조절할 수 있는 방법을 찾고자 하였으며, 크게 화학적 조절방법과 유전적 조절방법으로 볼 수 있다. 본 연구에서는 새로운 선천면역반응과 적응면역반응에 대한 새로운 화학적 조절인자와 유전적 조절인자를 찾고자 하였다.

괭생이모자반은 갈조류에 속하는 해조류이며 galangin은 양강에서 유래된 flavonol의 일종이다. 본 연구에서는 쥐의 대식세포인 RAW 264.7 세포주를 이용하여 괭생이모자반의 에탄올 추출물(ESH)와 galangin의 항염증 효과를 확인하였다. ESH와 galangin은 각각 200 μg/ml 과 50 μM까지 대식세포에 독성을 나타내지 않았으며 지질다당체 (LPS)가 처리된 대식세포에서 염증의 지표인 일산화질소뿐만 아니라 인터루킨, inducible nitric oxygenase (iNOS), cyclooxygenase (COX-2)의 생성을 저해하였다. 이러한 항염증성 활성은 mitogen-activated protein kinase (MAPK) 신호전달 경로 억제를 통해서 나타남을 확인하였다.

Baicalin은 황금에서 유래되는 flavonoid의 일종이며 18-Glycyrrhetic acid는 감초에서 유래되는 triterpenoid의 일종이다. 본 연구에서는 bacalin과 β-GA가 수지상세포의 세표표면분자 발현, 항원포식능, 사이토카인 생성능과 T 세포 분화 유도능에 영향을 미칠 수 있는지 확인하였다. Baicalin과 β-GA는 LPS에 의해 자극된 수지상세포에서 세포표면 분자인 CD80, CD86, MHC class I, MHC class II의 발현뿐만 아니라 IL-12의 생성능을 억제하였다. 또한 baicalin과 β-GA는 수지상세포의 Th1 면역반응을 유도할 수 있는 능력을 저해하였다. 이러한 연구 결과는 baicalin과 β-GA가 수지상세포에 의해 매개되는 과민성 면역질환에 대하여 치료용 보조약물로 개발될 수 있는 가능성을 제시하였다.

CD30은 TNRFSF8이라고도 알려져 있으며 면역세포 표면에 발현되는 공동자극인자로 알려져 있다. 기존에 보고된 바에 의하면, CD30은 주로 림프종,

활성화된 T 세포, B 세포 등 림프계 세포에 주로 발현되는 것으로 알려져 있다. CD30이 림프종과 다양한 질환의 지표로 알려져 있음에도 불구하고 Th2 면역반응에도 밀접한 연관이 있다고 알려져 있다. 본 연구에서는 CD30결핍 마우스를 통해 $CD4^+$ T 세포와 종양면역에 대한 CD30의 면역반응 기능에 대하여 확인하였다. CD30의 결핍은 비장에 존재하는 면역세포들의 수와 LPS를 통한 수지상세포의 활성에는 영향을 미치지 않았다. 그러나 CD30의 결핍은 $CD4^+$ T 세포의 증식과 활성을 저해하는 효과를 나타내었고 쥐의 대장암세포인 MC-38을 주입한 마우스에서 정상 쥐에 비하여 생존율이 낮아지는 효과를 나타내었다. 이러한 결과는 CD30이 $CD4^+$ T 세포의 활성과 종양면역에 관련이 있으며 CD30이 결핍될 경우, $CD4^+$ T cell의 활성이 억제됨을 통해서 종양세포에 더 취약해짐을 알 수 있다.

결론적으로, 다양한 천연물은 면역관련 유전자, 공동자극인자, 사이토카인 생성 등의 조절을 통해서 대식세포와 수지상세포와 같은 항원제시세포의 면역학적 기능을 조절할 수 있다. 더욱이 수지상세포 조절은 T 세포의 극성을 결정하는 능력을 가지며, 이는 적응면역반응의 방향을 결정하는데 중요한 요소이다. 또한, 공동자극인자인 CD30의 결핍은 T 세포의 활성과 항종양 면역반응을 저해하는 효과를 가짐을 관찰할 수 있다. 이러한 연구 결과들은 ESH, galangin, baicalin과 β-GA와 같은 천연물이 면역학적 치료보조제로써의 가능성을 가지며 CD30 분자가 적응면역반응에서 유전적 조절인자가 될 수 있음을 제시한다.

I. INTRODUCTION

1. Immunity

Immunity is the biological defense mechanisms against infection, disease, or other biological attack. The mammalian immune response consists of two arms:antigen-specific adaptive immunity and non-specific innate immunity (Kindt, Goldsby et al. 2007). These two arms of the immune response involve various soluble mediators and diverse cell types. The innate immune response includes soluble components such as cytokines, chemokines, and complement, and has cellular components including macrophages, dendritic cells (DCs), granulocytes (basophils, neutrophils and eosinophils), mast cells, and natural killer (NK) cells. By comparison, the adaptive immune response consists of antibodies as soluble factors, and $CD4^+$ T cells and $CD8^+$ T cells as cellular components (Fig. 1) (Biron, Byron et al. 1989; Dranoff 2004; Daha 2011).

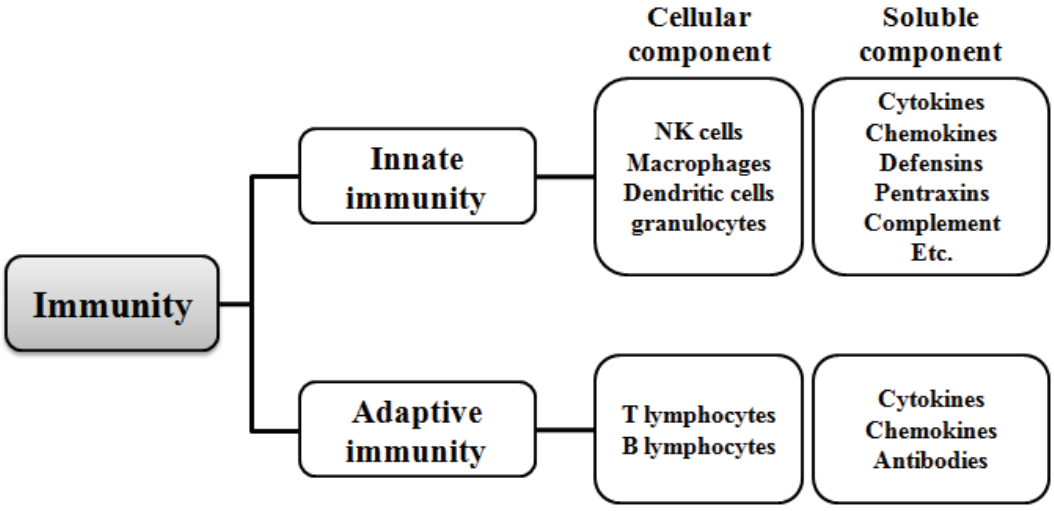

Fig. 1. Two types of immune responses and the major components.

2. Innate immunity (Inflammation)

Innate immunity isthe first line of immune defense and operates independently of antigen specificity against infections and microbial invasion, and proceeds through physical, chemical, and cellular processes. First, pathogens encounter the skin as a physical barrier, where pathogens gain entry through a breach in the barrier. Upon entry, a chemical defense is triggered, and chemical signals (cytokines and chemokines) are secreted that recruit immune cells such as phagocytes to the site of entry. The innate immune response consists of myeloid cells (monocytes, macrophages, neutrophils, eosinophils, basophils, DCs and platelets)and specialized functions such as phagocytosis and secretion of cytokines (Yutin, Wolf et al. 2009). Phagocytes such as macrophages and DCs, also called Antigen presenting cells (APCs), engulf and eliminate the pathogens, or present the molecular patterns of the antigen on the cellular surface. APCs also release inflammatory cytokines such as interleukins (IL) and interferons (IFN) that induce inflammation to attack the viruses and bacteria. Through these processes, the innate immune response limits the expansion and spread of pathogens within the host. In addition, APCs present antigen peptides from the pathogen in the context of MHC molecules on the cell surface, and particularly DCs, which uniquely induce T cell priming and initiation of adaptive immune responses (Fig. 2) (Goldsby, Kindt et al. 2000).

In the innate immune response, inflammation is a major response to infection, which is characterized by fever, vasodilatation, and swelling of the infected or

injured regions. Inflammation is triggered by complement and pro-inflammatory cytokines such as ILs released from activated phagocytes and macrophages, which are the primary immune cell types in inflammation (Parker and Picut 2005). However, inflammation is a two-edged sword because of the necessity for balance between "acute inflammation" and "chronic inflammation". Inflammation is meant to be a beneficial process, the main purpose of which is to eliminate the pathogen and restore the tissue after injury. After clearance of the pathogen, inflammation is resolved by homeostatic reactions (Medzhitov 2008; Norling and Serhan 2010). However, if inflammation fails to resolve and becomes "chronic inflammation", diseases such as parenchyma necrosis, cancer, autoimmune disease, and cardiovascular diseases may result (Fig. 3) (Yan, Anderson et al. 2006; Munoz, Janko et al. 2010; Mason and Libby 2014).

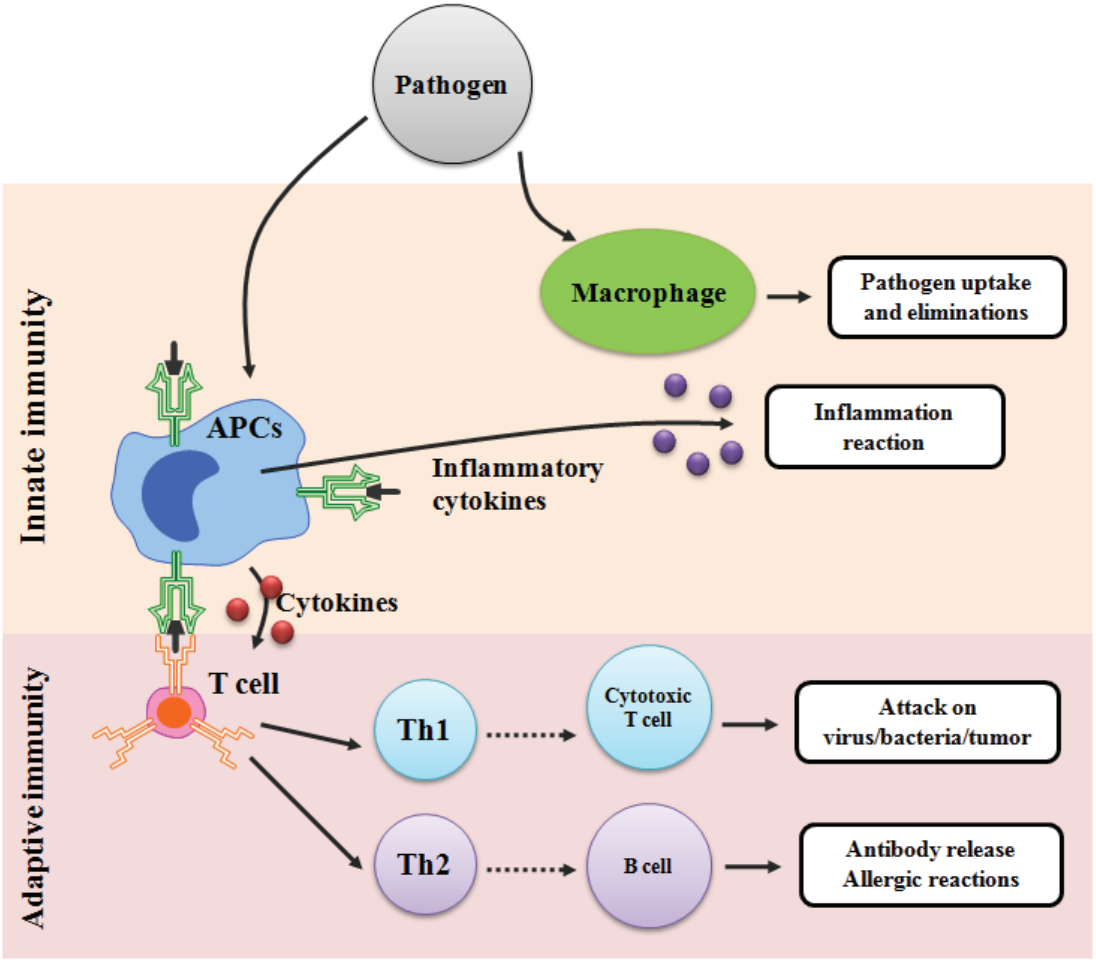

Fig. 2. The mechanism of innate immunity and adaptive immunity.

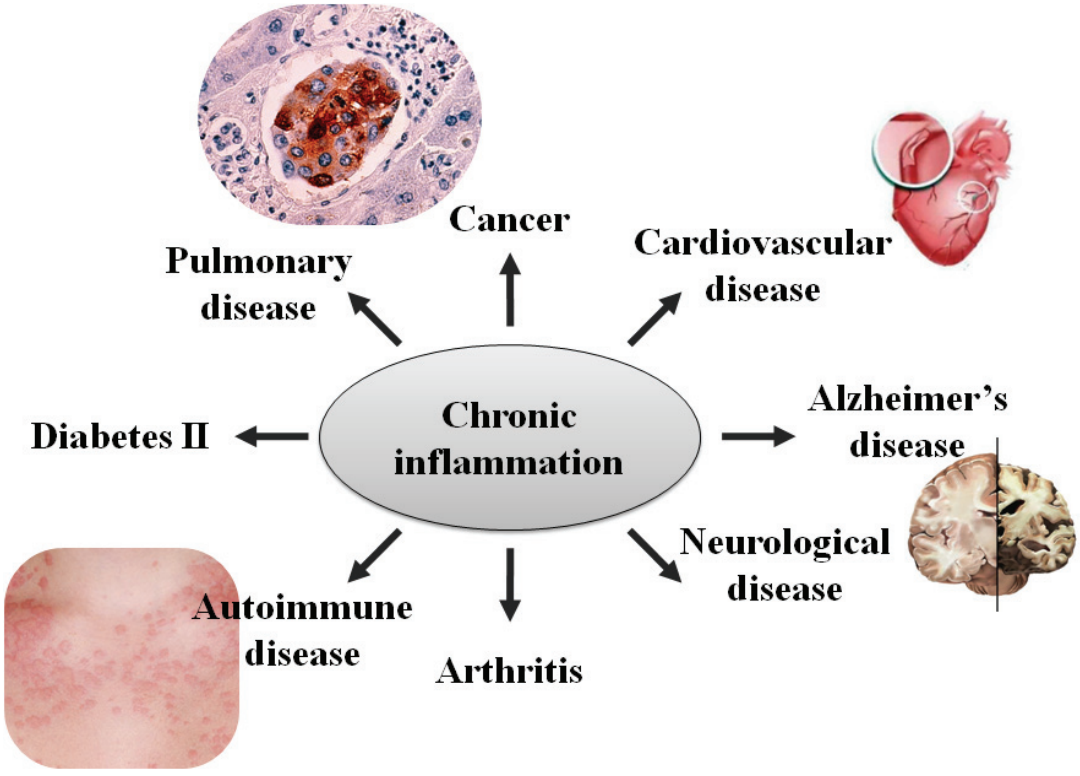

Fig. 3. Acute inflammation and chronic inflammation.

3. Antigen Presenting Cells

(1) Macrophage

Macrophages are a type of white blood cell that originates from monocytes, which are derived from myeloid progenitor cells (Fig. 4). These cells migrate by ameboid movement from blood to tissue and are found in all tissues where they act as sentinels. Macrophages engulf and digest pathogens, infected cells that have died, and malignant cells by a process called phagocytosis. They play a critical role in innate immunity especially in infections and inflammation, and also help initiate adaptive immunity by recruiting other immune cells such as lymphocytes (Gordon 1998; Sheikh and Plevy 2010; Locati, Mantovani et al. 2013). Through pattern-recognition receptors (PRRs),macrophages recognize and eliminate pathogens through pathogen-associated molecular patterns (PAMPs) expressed on infected or stressed cells. During these processes, macrophages produce inducible nitric oxide synthase (iNOS), a key marker of inflammation, and inflammatory cytokines such as IL-1β, IL-6 and TNF-α. These inflammatory mediators induce vasodilatation, recruitment of other immune cells, and subsequently restore the inflamed tissues (Honda, Takaoka et al. 2006). Beyond the pivotal role they play in innate immune responses, macrophages also exhibit anti-inflammatory activity through cytokines. Recently, macrophages were classified into M1 macrophages and M2 macrophages, based on function and cytokine/chemokine profiles. M1 macrophages are involved in inflammatory responses and induced by foreign antigens or IFN-γ, and M2

macrophages are anti-inflammatory and are induced by IL-4 and IL-10 (Akira, Uematsu et al. 2006; Moreira and Hogaboam 2011).

(2) Dendritic cell

DCs are APCs that also are derived from myeloid progenitor cells, have immune sentinel characteristics, and play a critical role in adaptive immune response (Fig. 4). DCs are exclusively professional APCs, because they act as mediators between innate and adaptive immunity. DCs are the most potent APCs, which is why DCs are specialized to uptake and present antigens, and have the ability to initiate T cell responses against both microbial pathogens and tumors (Banchereau and Steinman 1998; Lo and Clare-Salzler 2006). Immature DCs (iDCs) capture and process foreign antigens in the peripheral tissues, and subsequently become mature DCs (mDCs) that can present antigens to T cells. The mDCs migrate into lymphoid organs, where they stimulate naïve T cells through antigen-presenting major MHC molecules and co-stimulatory molecules; the activated T cells then migrate into target tissues (Austyn 1998; Spits and Di Santo 2011).

The migration of DCs is related with decreased antigen uptake, increased half-life of surface MHC-peptide complexes, up-regulation of co-stimulatory molecules, altered expression of chemokine receptors, and production of cytokines that are crucial effectors of T cell differentiation (Aarvak and Natvig 2001). Antigens captured by DCs are transported to and concentrated in the peripheral lymphoid organs for presentation to antigen-specific T cells.

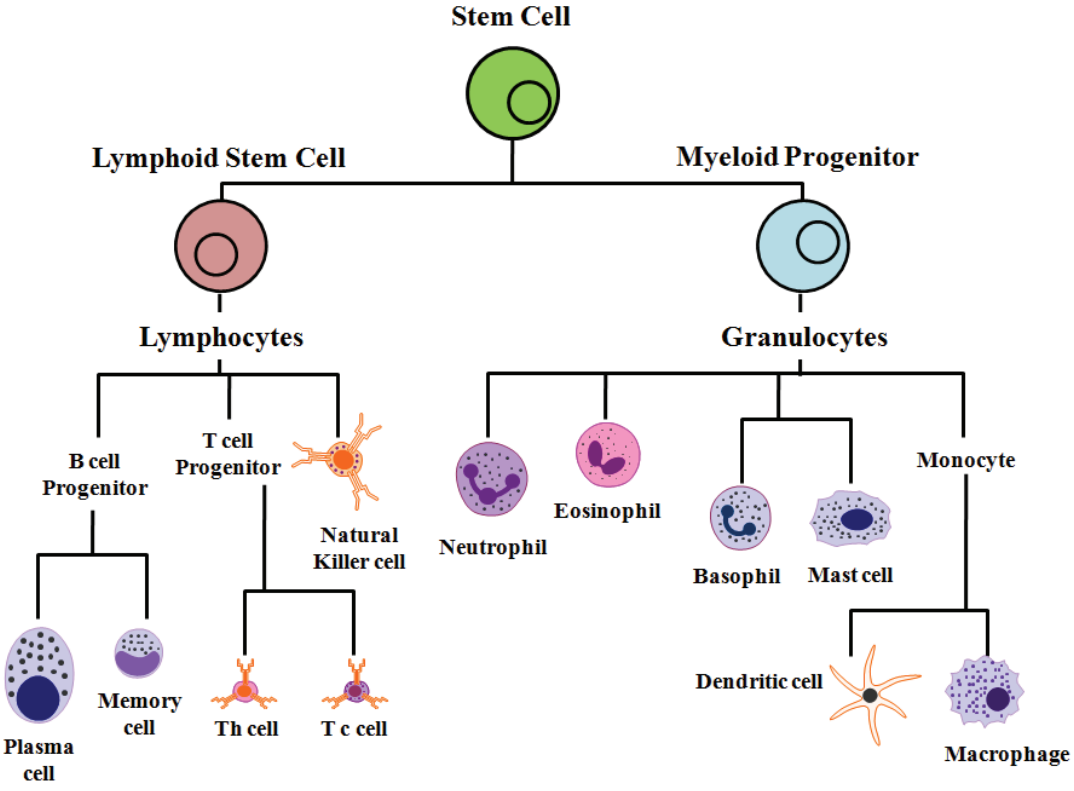

Fig. 4. Development of immune cells.

4. Adaptive Immunity

Adaptive immunity is specific to foreign antigens or self-antigens, and provides the long term protective responses. Adaptive immunity is usually divided into two major arms, 'cell-mediated immune response' and 'humoral immune response', depending on associated immune cells and components such as T cells and B cells. T cells and B cells strongly regulate the adaptive immune responses *via* directional action of lymphocytes and non-directional actions such as secretion of cytokines and antibodies (Alberts 2002; Bonilla and Oettgen 2010). In adaptive immunity, DCs capture and process antigens, and present the antigens in the context of MHC class I and II. In the cell-mediated immune response, the antigen-loaded MHC class I and II bind to the T cell receptor (TCR) of the $CD8^+$ T cells and $CD4^+$ T cells, respectively, and transfer the antigenic information. Subsequently, the $CD4^+$ T cells become polarized into T helper cell type 1 (Th1) or T helper cell type2 (Th2). Th1 cells support the activation and development of $CD8^+$ T cells into cytotoxic T cells (CTLs) to kill virus-infected or tumor cells *via* cytokines. Th2 cells contribute the switching of B cells into plasma cells that produce antibodies against target antigens. In the humoral immune response, B cells activated by Th2 cytokines differentiate into antibody-secreting plasma cells, and the antibodies bind to antigens to promote antigen clearance (Kindt, Goldsby et al. 2007).

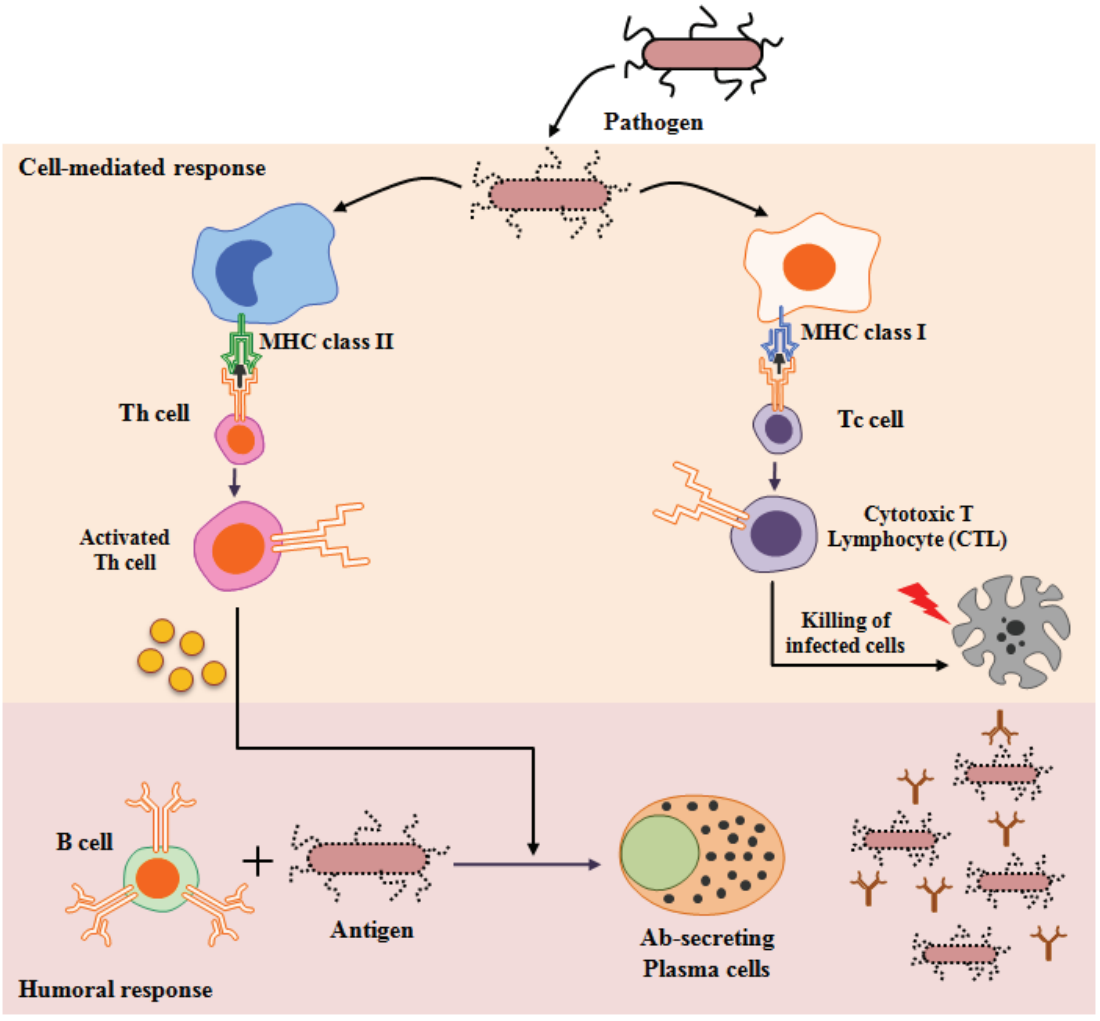

Fig. 5. Cell-mediated immunity and humoral immunity in adaptive immune response.

(1) T cells

T cells (T lymphocytes) are a type of lymphocyte characterized by the presence of TCRs on the cell surface (Cooper and Alder 2006). The cells originally come from the bone marrow or fetal liver, to mature in the thymus (hence the name "T cell"). T cells play a pivotal role in cell-mediated immunity. T cells originate from hematopoietic progenitor cells in the fetal liver or after birth, the bone marrow, which then migrate to the thymus where they divide into large populations of immature thymocytes. The early thymocytes express neither CD4 nor CD8 (double-negative cells). During the development/education process, thymocytes express both CD4 and CD8 (double-positive cells), then they eventually become single positive cells ($CD4^+CD8^-$ or $CD4^-CD8^+$ cells). The single positive T cells can migrate from the thymus to peripheral tissues. $CD4^+$ T and $CD8^+$ T cells are the most essential and include a wide range of T cell categories including $CD4^+$ T cells, $CD8^+$ T cells and regulatory T cells (Mathis and Benoist 2009).

(2) APCs – T cell interaction

iDCs respond to inflammatory cytokines that are released from the activated cells at the injured or infected site, and then undergo a morphological change to become mDCs. mDCs show decreased antigen uptake function (phagocytosis) and increased antigen processing and presenting functions. In addition, they have higher expression of MHC class I, II, and co-stimulatory molecules. mDCs leave the site of inflammation/injury to transfer to local lymph nodes and migrate into

lymphoid organs. The mDCs prime naïve T cells *via* a 'three-signal' mechanism. Signal 1 is where the T cell receives the pathogen information through the TCR-MHC complex. The TCR binds to an antigen-specific peptide presented in the context of MHC class I or II expressed on the DC surface. Signal 2 is generated by a co-stimulatory molecule such as a CD molecule on the surface of the DC or T cell, which contributes to stabilization of the 'immune synapse'. Signal 3 is mediated by immunological factors such as cytokines or other molecules released from DCs. This step induces the functional polarization of the T cell (Grakoui, Donermeyer et al. 1999; Amsen, Blander et al. 2004; Corthay 2006)

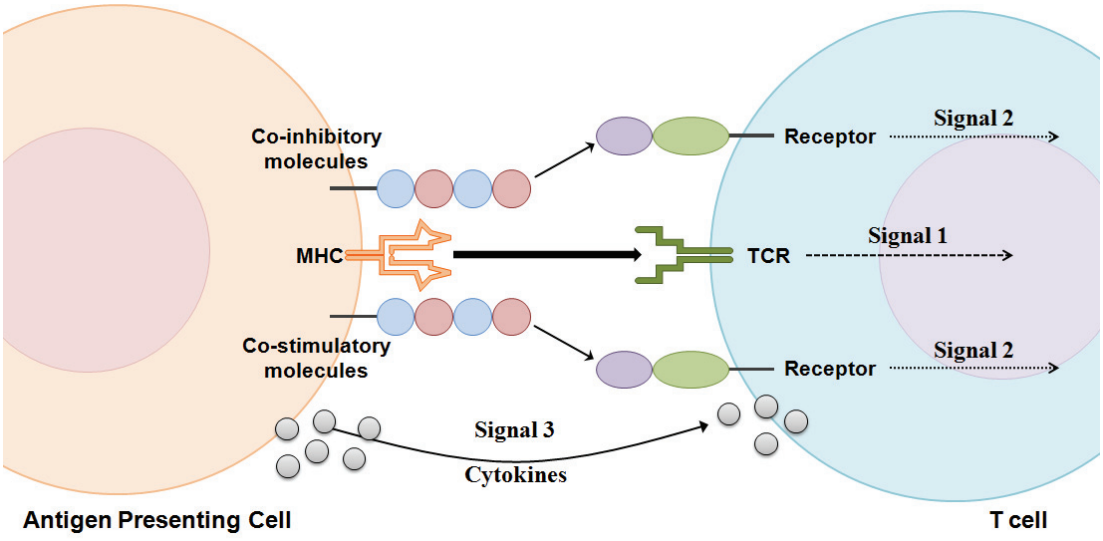

Fig. 6. The interaction between DCs and T cells.

II. MATERIALS AND METHODS

(1) Reagents

Galangin, baicalin, 18β-glycyrrhetinic acid, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Tri Reagent and Griess Reagent were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

(2) Extraction of *Sargassum horneri* (Turner) C. Agardh

The brown algae *Sargassum horneri* (Turner) C. Agardh was collected from the Sea of Wando, Korea, twice in February in 2012. The marine brown algae *S. horneri* (Turner) C. Agardh was washed with pure water four times to completely remove salt, then dried and homogenized by a blender. One hundred grams of dried *S. horneri* (Turner) C. Agardh powder was dissolved in 70% ethanol and incubated in a shaking incubator at 37℃ for 3 days; this procedure was performed twice. The ethanolic extract was filtered using qualitative filter paper, and concentrated to a minimum volume using a rotary evaporator. The remaining solvent was removed by lyophilization of the concentrated samples, and the lyophilized powder was dissolved in 100% ethanol

(3) Cell culture

The RAW 264.7 murine macrophage cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Briefly, cells were cultured at 37℃ in the presence of 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM)

supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml streptomycin, 4 mM L-glutamine and 1 mM sodium pyruvate

(4) ESH treatment

ESH was reconstituted in 100% ethanol and then diluted to the desired concentration in DMEM (final ethanol concentration 0.2% v/v). An equal final concentration of ethanol was used for the control (untreated) samples.

(5) Galangin treatment

Galangin was reconstituted in dimethyl sulfoxide (DMSO) and then diluted to the desired concentration in DMEM (final DMSO concentration 0.05% v/v). The same final concentration of DMSO was used for the control (untreated) samples.

(6) Cell viability assay

Cell viability was measured by colorimetric MTT assay. The cells (5×10^4 cells/well) were seeded in 96-well culture plates with DMEM. Cells were treated with the various concentrations of ESH (0 to 200 μg/ml) and incubated at 37℃ for 24 hr. After treatment, medium containing ESH was removed and MTT (0.5 mg/ml) solution was added to each well. After incubation at 37℃ for 4 hr, MTT solution was removed and the formazan product was dissolved in solubilization solution (1:1=DMSO:ethanol), which turned color. Absorbance was quantified by an Enzyme-linked immunosorbent assay (ELISA) microplate reader at 570 nm.

(7) NO assay

RAW 264.7 cells (5×10^4 cells/well) were seeded in a 96-well culture plate in DMEM. Cultured cells were pretreated with various concentrations of ESH (0 to 200 μg/ml) for 2 hr followed by incubation for 22 hr in the absence or presence of LPS(1 μg/ml). After incubation, the cultured medium was mixed with an equal volume of 1 X Griess Reagent and incubated for 15 min at room temperature. After incubation, absorbance was measured using an ELISA microplate reader at 540 nm.

(8) Reverse transcription (RT)-PCR

RAW 264.7 cells (1×10^6 cells/well) in DMEM were seeded in a 6-well culture plate. Cultured cells were pretreated with various concentrations of ESH (0 to 200 μg/ml) for 2 hr followed by incubation for 6hr in absence or presence of LPS(1 μg/ml). After incubation, the cells were collected by centrifugation and total RNA was isolated from the ESH-treated cells using TRI Reagent according to the manufacturer's protocol. To synthesize cDNA, 0.5 μg of total RNA was primed with oligo(dT) in a reaction mix containing M-MLV RTase, dNTP, and reaction buffer (Promega, WI, USA) according to the manufacturer's protocol. PCR for inflammatory cytokines including IL-1β, iNOS, and COX-2 was performed using the primers listed in Table 1.The primers were specifically designed for target genes (Bioneer, Daejeon, Korea). The cDNA was amplified using an e-Taq DNA Polymerase kit (Solgent, Daejeon, Korea) and the primers on a Gene Atlas G02 gradient thermal cycler (Astec, Tokyo, Japan). PCR

products were separated on 1% agarose gels and visualized by fluorescent dye on a UV transilluminator.

(9) Quantitative real-time PCR

iNOS, COX-2, IL-6, TNF-α, and GAPDH PCR primers used were as listed in Table 1. Quantitative amounts of each gene were normalized against GAPDH as a housekeeping gene. Real-time PCR with SYBR Green was performed using an Exicycler™ 96 (Bioneer Corporation, Daejeon, South Korea). Reactions were performed in a total volume of 20 μl containing10 μl 2 ×iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1 μl of each primer at 10 μM concentration and 1 μM of reverse-transcribed cDNA template. The protocols used were as follows: denaturation (95℃ for 3min), amplification repeated 40 times (95℃ for 15 sec, 55℃ for 30 sec, 72℃ for 30 sec and acquisition temperature for 15sec). For each sample, Ct (crossing point) values were calculated as the Ct of the target gene minus Ct of the GAPDH gene. Gene expression was derived according to the equation $2^{-\Delta\Delta CT}$, and changes in gene expression were expressed relative to basal expression.

Table. 1. Primers used in RT-PCR.

Target		Sequence
iNOS	F	5'-TTCATGCTAATCGAAGG-3'
	R	5'-GGUGUUAAGGCTAGCTGA-3'
COX-2	F	5'-TGGGTGTGAAGGGAAATAAGG-3'
	R	5'-CATCATATTTGAGCCTTGGGG-3'
IL-6	F	5'-CGCACTAGGTTTGCCGAGTA-3'
	R	5'-CCTTTCTACCCCAATTTCCA-3'
TNF-α	F	5'- GGCCT CTCACCTTGTTGCC-3'
	R	5'-ATGACCCGTAGGGCGATTAC-3'
GAPDH	F	5'-CAAAATGGTGAAGGTCGGTG-3'
	R	5'-CGTTGATGGCAACAATCTCC-3'

(10) Western blot analysis

Following pretreatment with 200 μg/ml ESH (or vehicle), the cells were exposed to LPS (1 μg/ml). Following 15, 30 or 45 min of incubation at 37℃, cells were washed twice with cold phosphate buffered saline (PBS) and lysed with modified RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50mMTris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 100 mM sodium fluoride) for 30 min at 4℃. Lysates were cleared by centrifuging at 14,000 × g for 15 min at 4℃. The protein content of the lysates was determined using the Micro BCA assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was placed into a blocking solution (5% nonfat dry milk) at room temperature for 1 hr. After blocking, primary antibodies (listed in Table 2) were added and blots were incubated for 90 min at room temperature. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the secondary antibodies. Visualization was performed using the Enhanced Chemiluminescence (ECL) detection system and exposed to radiographic film. Pre-stained blue markers were used for molecular weight determination.

Table. 2. Primary antibodies used in Western blotting.

1st antibody	2nd antibody	Dilution ratio	Company (Cat No.)
ERK1/2	Rabbit	1:2000	Cell signaling #9102
phopho-ERK1/2	Mouse	1:2000	Santa Cruz Sc-7378
p38	Rabbit	1:2000	Cell signaling #9212
phospho-p38	Mouse	1:2000	Santa Cruz Sc-7973
NF-κB-p65	Mouse	1:2000	Santa Cruz Sc-8008
phospho-NF-κB-p65	Rabbit	1:2000	Santa Cruz Sc-101749
β-actin	Mouse	1:5000	Santa Cruz Sc-47778

(11) Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells (5×10^4 cells/well) were seeded in a 96-well culture plate. Cells were pretreated with various concentrations of ESH for 2 hr followed by incubation in the absence or presence of LPS (1 μg/ml) for 22 hr. IL-1β released into the supernatants was quantified using the Mouse IL-1β ELISA MAX™ Deluxe Sets (BioLegend, San Diego, CA, USA), according to the manufacturer's protocol. Absorbance was measured by ELISA microplate reader at 405 nm wavelength.

(12) Animals and chemicals

Male 8-week-old CD30$^{-/-}$(B6.129P2-Tnfrsf8^{tm1Mak}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Male 8–12-week-old C57BL/6 (H-2K^b and I-A^b) and BALB/c (H-2K^d and I-A^d) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). All mice were housed in a specific pathogen-free environment within our animal facility for at least 1 week before use. All mouse work was approved by the IACUC and was performed in our IACUC-approved facility.

(13) Reagents and Abs

Recombinant mouse (rm)GM-CSF and rmIL-4 were purchased from R&D Systems (Minneapolis, MN USA). Dextran-FITC (molecular mass, 40,000), and LPS (from Escherichia coli 055:B5) were obtained from Sigma-Aldrich. Cytokine ELISA kits for murine IL-12p70, IFN-γ, IL-4, and IL-2 were

purchased from BD Pharmingen (Rockville, MD, USA). FITC- or PE-conjugated mAbs were used to detect the expression of CD11c (HL3), CD80 (16-10A1), CD86 (GL1), $IA^b\beta$-chain (AF-120.1), $H2K^b$ (AF6-88.5), intracellular IL-12p40/p70 (C15.6), and IL-10 (JESS-16E3) by flow cytometry. The Abs and isotype-matched control mAbs, were purchased from eBiosciences (San Diego, CA, USA).

(14) Generation and culture of DCs

DCs were generated from murine bone marrow cells. Briefly, bone marrow was flushed from the tibiae and femurs of male C57BL/6 and depleted of red cells with ammonium chloride. The cells were plated in 6-well culture plates (1×10^6 cells/ml; 3 ml/well) in OptiMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 10 mM HEPES (pH 7.4), 20 ng/ml rmGM-CSF and rmIL-4 at 37℃, 5% CO_2. On day 3 of culture, floating cells were gently removed, and fresh medium was added. On day 6 of culture, non-adherent cells and loosely adherent proliferating DCs aggregates were harvested for analysis or stimulation, or, in some experiments, re-plated in 60-mm dishes (1×10^6 cells/ml; 5 ml/dish). On day 6, 80% or more of the non-adherent cells expressed CD11c. In certain experiments, to obtain highly purified populations for subsequent analyses, the DCs were labeled with bead-conjugated anti-CD11c mAb (MiltenyiBiotec, Gladbach, Germany) followed by positive selection through paramagnetic columns (LS columns; MiltenyiBiotec)

according to the manufacturer' s instructions. The purity of the selected cell fraction was >95%

(15) Stimulation of DC by β-GA

β-GA and baicalin were dissolved in DMSO, or DMSO alone (0.01% v/v) and were added separately to cultures of isolated DCs in 6-well plates (1×0^6 cells/ml; 2ml/well). DMSO was deemed non-toxic for DCs (data not shown). For analysis of apoptosis, DCs were stimulated with LPS (1 μg/ml) or not stimulated. In addition, apoptosis was analyzed over time by staining of phosphatidylserine translocation with FITC-annexin V in combination with a propidium iodine kit according to the manufacturer's instructions.

(16) Stimulation of DC by baicalin

On day 6, BM-DC were harvested, washed with PBS, and resuspended in fluorescence activated cell sorter (FACS) washing buffer (1% fetal bovine serum in PBS). The cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4℃ then stained with the following antibodies; phycoerythrin (PE)-conjugated anti-H-$2K^b$(MHC class I), anti-I-A^b (MHC class II), anti-CD80, and anti-CD86 with FITC-conjugated anti-CD11c (BD PharMingen, San Diego, CA) for 30 min at 4℃. The stained cells were analyzed using a FC500 flow cytometer (Beckman Coulter, Brea, CA).

(17) Flow cytometric analysis

DCs were first blocked with 10% (v/v) normal goat serum for 15 min at 4℃ and then stained with FITC-conjugated $CD11c^+$ antibody for 30 min at 4℃. The cells stained with the appropriate isotype-matched Ig were used as negative controls. The cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Pharmingen) according to manufacturer' s instructions. Intracellular IL-12p40/p70 and IL-10 expression was stained with fluorescein R-PE-conjugated antibodies (PharMingen) in a permeabilization buffer. The cells were analyzed using a FACSCalibur flow cytometer with the CellQuest program.

Murine IL-12p70 or IL-4 from DC and IL-2, IFN-γ as well as IL-4 from co-cultured T cells were measured using an ELISA kit (BD Pharmingen) according to the manufacturer's instructions.

(18) Cytokines assay

Responder T cells, which participate in allogeneic T cell reactions, were isolated by passing mononuclear cells from the spleens of BALB/c mice through a MACS column (MiltenyiBiotec). Staining with FITC-conjugated anti-CD4 antibody (BD Pharmingen) revealed that they were highly enriched for$CD4^+$ cells (>95%). DC were treated with 50 μg/ml mitomycin C (Sigma) for 1hrand added in graded doses to 1×10^5 allogeneic T cells in U-bottomed 96-wellmicrotiter culture plates. During the last 18 hr of the 72h culture period, cell proliferation was quantified by addition of 0.5 μCi (methyl^{3}H) thymidine (NEN-DuPont, Boston, MA) per well. The cells were harvested onto glass fiber filters (InotechBiosystems, Zurich, Switzerland) and the radioactivity was measured in

a scintillation counter. Results are presented as mean c.p.m. of triplicate cultures.

(19) Mixed Lymphocyte Reaction

Responder T cells, which participate in allergenic T cell reactions, were isolated passing through mononuclear cells from spleen of BALB/c mice in a MACS column (Miltenyi Biotec). Staining with FITC-conjugated anti-CD4 antibody (BD Pharmingen) revealed that they consisted mainly of $CD4^+$ cells (>95%). DC were treated with 50 μg/ml mitomycin C (Sigma) for 1 hr and added in graded doses to 1×10^5 allergenic T cells in U-bottomed 96-well microtiter culture plates. During the last 18 of the 72 hr culturing, cell proliferation was quantified by (^{3}H)Thymidine (NEN-DuPont, Boston, MA) uptake of cells 0.5 μCi of (methyl^{3}H)Thymidine. The cells were harvested onto glass fiber filters (Inotech Biosystems, Zurich, Switzerland) and the radioactivity was measured in a scintillation counter. Results are presented as mean c.p.m. of triplicated cultures.

(20) Experimental lung metastasis model and survival study

The MC-38 cell line, a murine colon adenocarcinoma in a C57BL/6 mouse, cultured in DMEM containing 10% FBS, 200 IU/ml penicillin, 200 μg/ml streptomycin, 4 mM L-glutamine and 1 mM sodium pyruvate. A total of 1×10^6 MC-38 cells was injected into the mice tail vein for experimental metastasis experiment.

(21) T cell stimulation and intracellular cytokine staining

Whole splenocyte were isolated from WT and $CD30^{-/-}$ mouse spleen and cultured in RPMI-1647 containing 10% FBS, 200 IU/ml penicillin, 200 μg/ml streptomycin, 4 mM L-glutamine and 1 mM sodium pyruvate. Cells were stimulated with 1 μg/ml PMA for 5 days and treated with BD GolgiPlug™ (BD Biosciences). Cells were washed; Fc blocked; stained with CD4 and CD62L or IFN-γ in permeabilization buffer for 30 min. The stained cells were analyzed using a FC500 flow cytometer (Beckman coulter, Brea, CA).

(22) Statistical analysis

All results were expressed as the mean ± SD of the indicated number of experiments. Statistical significance was estimated using a Student's *t*-test for unpaired observations, and the differences among groups were compared by one-way ANOVA, followed by Bonferroni's *post hoc* test. The categorical data from the fertility test were subjected to Chi-square test. A P value of < 0.05 was considered significant.

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III. RESULTS

Part I. Immune modulatory adjuvants for antigen presenting cells

1. Anti-inflammatory effects of ethanolic extracts from *Sargassum horneri* (Turner) C. Agardh on lipopolysaccharide-stimulated macrophage activation *via* NF-κB pathway regulation

a. Introduction

Inflammation is the main manifestation of the innate immune response to pathogens. Macrophages have an essential role in inflammation and activated macrophages produce various pro-inflammatory factors, including cytokines and nitric oxide (NO)during an inflammatory response. NO is synthesized in mammalian cells by NO synthase (NOS) and has a critical role in many diseases that involve inflammation including diabetes and autoimmune diseases (Wiklund, Iversen et al. 1999; Manzi and Wasko 2000; Park, Jeon et al. 2012). Various stimuli iNOS including lipopolysaccharide (LPS) released during the inflammatory process, leading to production of NO by macrophages (Evans 1995; Robinson, Zhang et al. 1999; Jung, Kim et al. 2010; Park, Kim et al. 2010).

Stimulation of toll-like receptors (TLRs) by LPS leads to activation of the mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB pathways. Activation of MAPK and NF-κB have important activities as transcription factors for iNOS and as mediators that induce cellular responses and pro-inflammatory cytokines, especially IL-1β, IL-6, and tumor necrosis factor (TNF)-α. Therefore, reduction of NO and pro-inflammatory cytokine production has been suggested as an effective strategy for the treatment of inflammatory diseases (Welters, Fimiani et al. 2000; Pearson, Robinson et al. 2001; Hoffmann and Baltimore 2006; Kawai and Akira 2006; Tsatsanis, Androulidaki et al. 2006; Cha, Moon et al. 2009).

Sargassum is a large genus with more than 150 species described. It is the most conspicuous brown algae in tropical and subtropical waters. In a few papers, it was reported that *Sargassum fulvellum* has anti-inflammatory activities and promotes the differentiation of PC12 cells (Ina, Hayashi et al. 2007; Kang, Khan et al. 2008). *S. horneri* (Turner) C. Agardh is found near the coast of the Pacific Ocean and in the adjoining seas of China, Korea, and Japan (Yoshida, Murase et al. 2004). It has been reported that *S. horneri* (Turner) C. Agardh shows anti-oxidant, anti-tumor, and anti-viral activities (Hoshino, Hayashi et al. 1998; Shao, Chen et al. 2014). However, neither an anti-inflammatory activity nor the mechanism by which *S. horneri* (Turner) C. Agardh acts on macrophages has been reported.

This study was performed to identify the anti-inflammatory effects of an ethanolic extract of *S. horneri* (Turner) C. Agardh (ESH) on the regulation of NO

and cytokine production in macrophages stimulated by LPS, and further examined the potential mechanisms by which ESH modulates NO production. Wealso investigated whether ESH can regulate COX-2 expression and pro-inflammatory cytokine production including IL-1β in LPS-activated macrophages. It was determined that ESH reduced the LPS-induced production of NO and pro-inflammatory cytokines in macrophages by inhibiting ERK and p38 phosphorylation, and NF-κB activation.

b. Results

(1) ESH inhibited NO production in LPS-stimulated macrophages

First the cytotoxicity of ESH in macrophages was examined using MTT assay. Cells were cultured in the presence of various concentrations of ESH (25, 50, 100, and 200 μg/ml). When cells were treated with 25 to 200 μg/ml of ESH for 24 hr, cytotoxicity was not observed at any concentration (Fig. 7). To investigate whether ESH could regulate NO production, NO levels in LPS-stimulated macrophages were measured after ESH treatment. Macrophages were pretreated with ESH for 2 hr and then stimulated by LPS (1 μg/ml). LPS treatment induced significant NO production compared with control (Fig. 8). However, pretreatment of macrophages with ESH significantly inhibited LPS-induced NO production in a dose-dependent manner. These results indicated that at the concentrations tested, ESH inhibited NO production in LPS-stimulated macrophages.

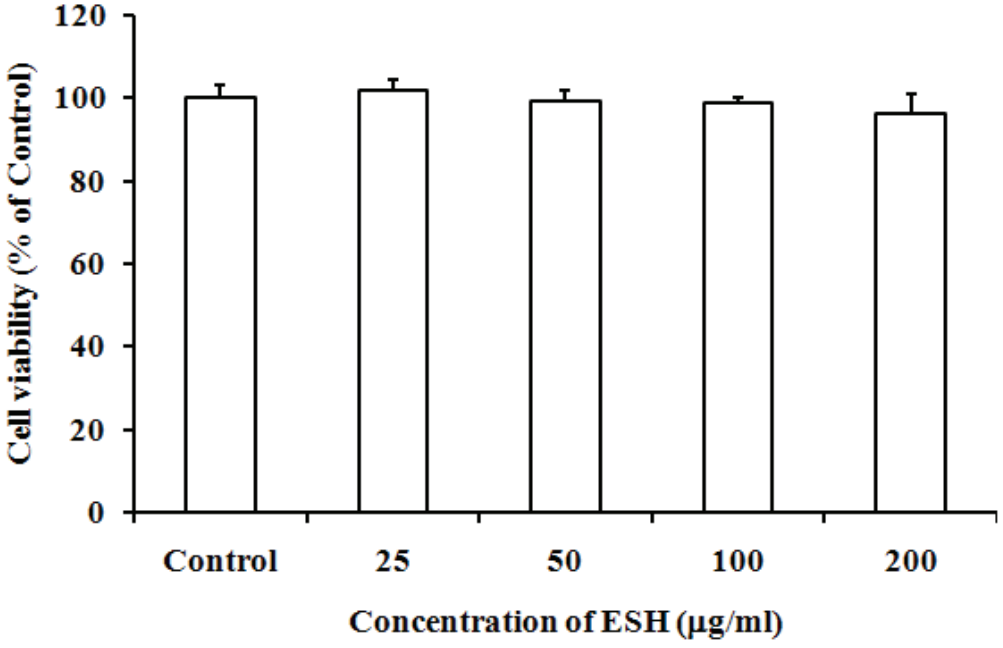


Fig. 7. ESH has no effect on cell viability in RAW 264.7 murine macrophages. RAW 264.7 cells were plated in 96-well cell culture plates. Various concentrations of ESH and control (0.2% ethanol) were added, and viable cell numbers were assessed by MTT assay after 24 hr incubation, as described in the *Materials and Methods*. Data are reported as viable cells numbers, expressed as a percentage of control cells that were exposed to 0.2% ethanol. The data represent the average (±SD) of four replicate wells and are representative of three separate experiments.

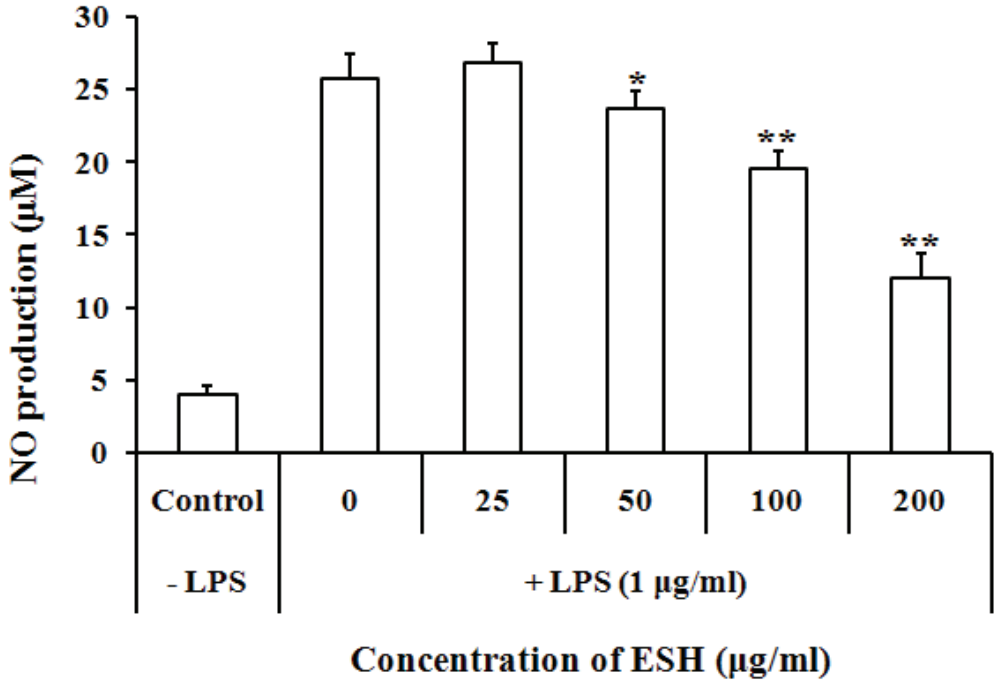


Fig. 8. ESH decreases LPS-induced NO production in RAW 264.7 murine macrophages. RAW 264.7 cells were plated in 96-well cell culture plates. Cells were pretreated with various concentrations of ESH (0 to 200 μg/ml) for 2 hr and then incubated for 22 hr in the presence or absence of LPS (1 μg/ml). Supernatants were mixed with Griess reagent, and absorbance was measured by ELISA microplate reader. The data represent the average (±SD) of four replicate wells and are representative of three separate experiments ($^{**}P < 0.01$ *vs.* LPS only groups).

(2) ESH suppressed pro-inflammatory cytokine expression in LPS-stimulated macrophages

IL-1β is a pro-inflammatory cytokine that is released from macrophages upon stimulation with LPS or other inflammatory mediators. To examine the effects of ESH on IL-1β mRNA expression and production, the RT-PCR and ELISA assays were performed using LPS-stimulated macrophages. For RT-PCR, macrophages were pretreated with or without ESH for 2hr and then stimulated with LPS for 6 hr. As shown in Fig. 9A, stimulation of macrophages with LPS increased the expression level of IL-1β, which was significantly reduced by ESH in a dose-dependent manner. In addition, to measure the effect of ESH on IL-1β production, macrophages were pretreated with or without ESH for 2hrand then stimulated with LPS for 24 hr. The release of IL-1β was reduced by ESH treatment of LPS-activated macrophages (Fig. 9B).

Next, to investigate whether the inhibitory effect of ESH on the production of iNOS and COX-2 occurred at the level of gene expression, the mRNA levels of iNOS and COX-2 in macrophages stimulated with LPS in the presence or absence of ESH were quantitated. After treatment with LPS, iNOS and COX-2 mRNA expression was significantly increased in macrophages. However, pretreatment with various concentrations of ESH prior to LPS suppressed the LPS-induced increase in iNOS and COX-2 mRNA expression in a dose-dependent manner (Fig. 9A). This result suggests that ESH inhibited the expression of inflammatory genes at the transcriptional level.

Fig. 9. ESH inhibits LPS-induced pro-inflammatory genes in RAW 264.7 murine macrophages. (A) RAW 264.7 cells were pretreated with 200 μg/ml ESH for 2 hr and stimulated with LPS for 6 h; the mRNA expression levels of inflammatory genes were determined by RT-PCR. ESH treatment reduced expression of IL-1β, COX-2, and GAPDH (used as the internal control). The data are representative of four separate experiments. (B) Supernatants were examined for cytokine release, and IL-1β was measured by ELISA. Data are expressed as the average (±SD) of triplicate cultures ($^{**}P < 0.01$ *vs.* LPS only groups).

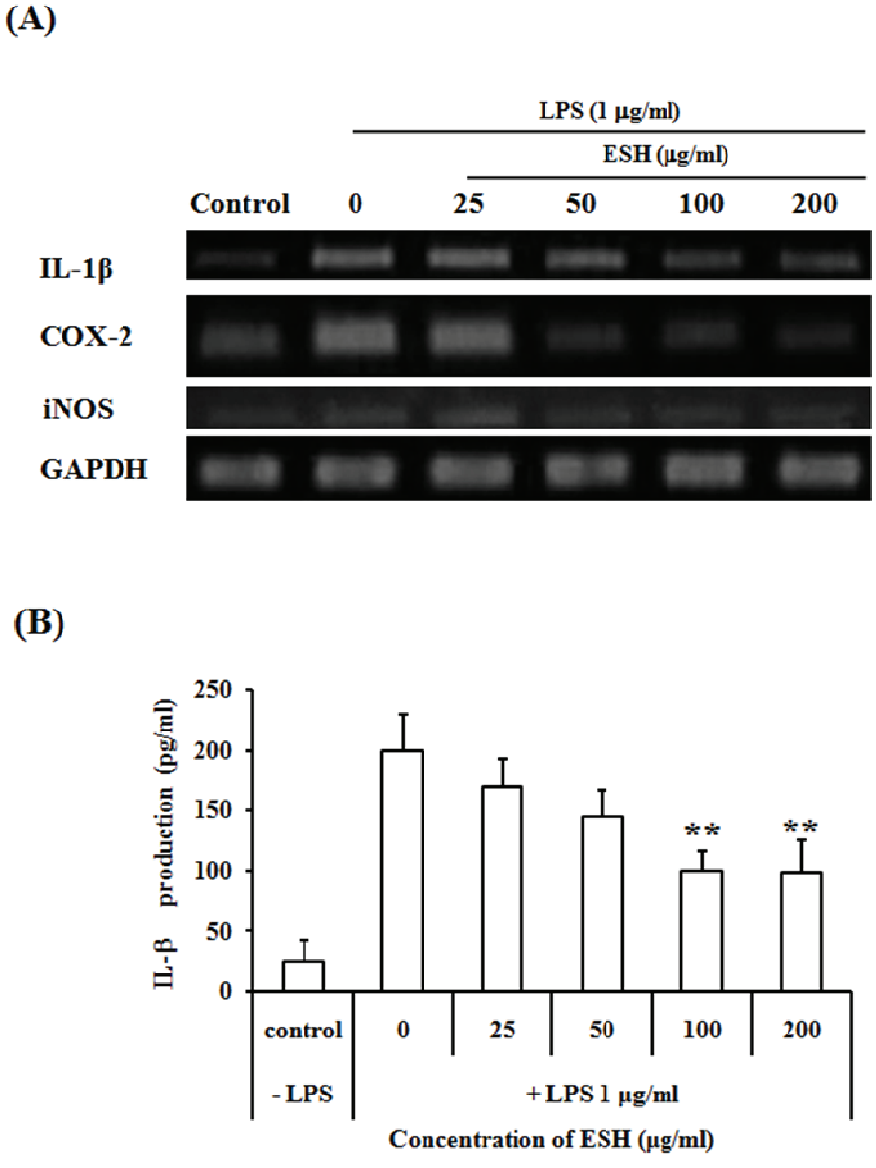


Fig. 9. (continued)

(3) ESH treatment inhibited LPS-induced activation of ERK

MAPK pathway constituents such as ERK1/2 and p38 are involved in the regulation of inflammatory mediators *via* the activation of transcription factors, especially NF-κB. To investigate the molecular mechanism of inhibition by ESH, the level of phosphorylated MAPK was determined in LPS-activated macrophages. As shown in Fig. 10A, phosphorylation of ERK1/2 and p-p38 was increased after 15, 30 and 45 min of LPS-induced macrophage activation. Treatment with ESH strongly inhibited the LPS-induced phosphorylation of ERK1/2 and slightly reduced p38 phosphorylation. This result suggests that ESH affected the ability of ERK and p38to induce pro-inflammatory genes production in LPS-stimulated macrophages.

(4) ESH attenuated LPS-stimulated NF-κB activation

NF-κB activation plays critical roles in the induction of inflammatory mediators and cytokines such as iNOS, COX-2, and IL-1βin LPS-treated macrophages. For this reason, the effects of ESH on activation of NF-κB subunit NF-κB-p65 were determined in LPS-stimulated macrophages. As shown in Fig. 10B, ESH inhibited the LPS-stimulated phosphorylation of the NF-κB subunit NF-κB-p65 in macrophages. This result indicates that the ERK pathway in conjunction with the NF-κB pathway may contribute to the inhibitory effects of ESH on the down-regulation of pro-inflammatory mediators such as iNOS, COX-2, and IL-1β.

Fig. 10. ESH inhibits LPS-induced ERK, p38, and NF-κB-p65 phosphorylation. RAW 264.7 cells were starved in serum free DMEM and pretreated with 200 μg/ml ESH for 2 hr. Cells were then stimulated with 1 μg/ml LPS for 15, 30, and 45 min. Cells were lysed and equal amount of whole cell proteins were separated by SDS-PAGE and transferred to PVDF membranes. (A) Membranes were probed with anti-ERK, anti-p-ERK, anti-p-38, anti-p-p38, and anti-β-actin antibodies. (B) To determine NF-κB translocation, whole cell lysates were probed by anti-NF-κB-p65, anti-NF-κB-p-p65, and anti-β-actin antibodies. β-actin was used as the internal control. The results are from one representative experiment, out of four that showed similar patterns ($^{\#\#}P < 0.01$ vs. unstimulated macrophages; $^{**}P < 0.01$ *vs.* LPS-stimulated macrophages).

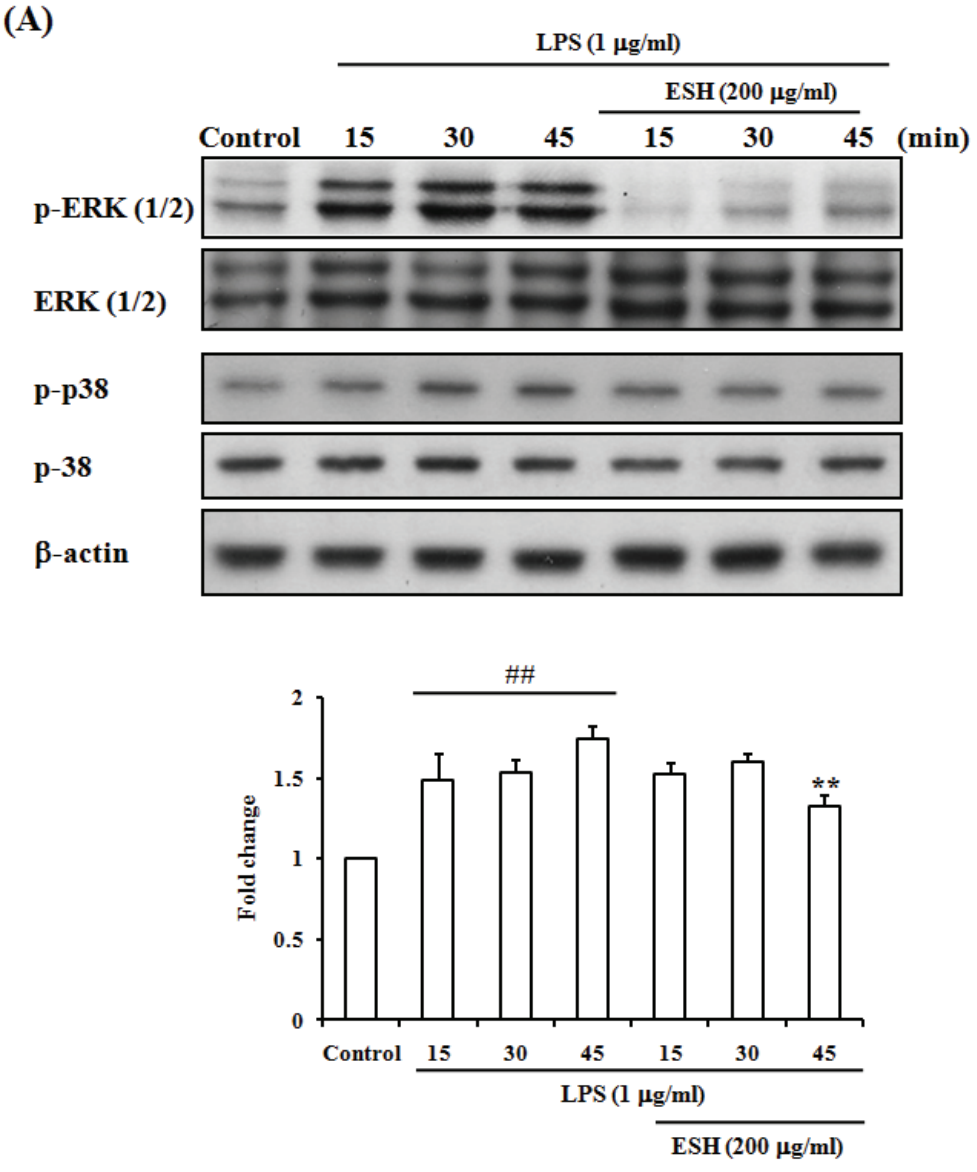


Fig. 10. (continued)

(B)

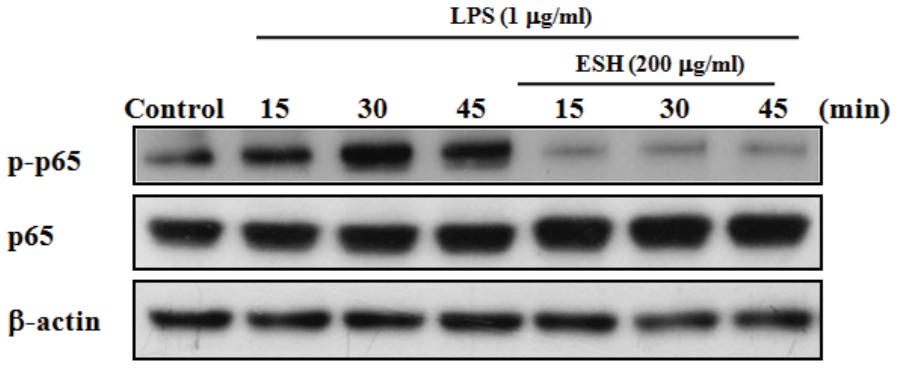


Fig. 10. (continued)

c. Discussion

Macrophages are one of the major cell types involved in the innate immune response that infiltrate into sites of chronic inflammatory disease, such as in rheumatoid arthritis, fibrosis, and atherosclerosis (Wiklund, Iversen et al. 1999; Manzi and Wasko 2000; Park, Jeon et al. 2012). In the present study, it was demonstrated that ESH from the brown algae *S. horneri* (Turner) C. Agardh has effective anti-inflammatory activities by inhibiting the production of pro-inflammatory mediators such as NO, iNOS, COX-2, and IL-1β in LPS-activated macrophages. Moreover, our results demonstrated that ESH suppressed the expression of iNOS, COX-2, and IL-1β mRNA as well as the production of IL-1β protein. ESH inhibited LPS-stimulated ERK and p38 phosphorylation, which may account for its anti-inflammatory activity by suppressing NF-κB-p65activation.

NO is a reactive molecule and molecular marker of inflammation. It is generated not only by products of different NO metabolites, but also *via* iNOS expression. NO is generated in high quantities by activated macrophages in inflammatory sites, which is important in the process of macrophage activation and is related with acute/chronic inflammation (Evans 1995; Robinson, Zhang et al. 1999; Jung, Kim et al. 2010; Park, Kim et al. 2010). For this reason, the inhibition of NO and PGE_2 production by decreasing iNOS and COX-2 protein expression is a promising therapeutic strategy and for the development of anti-inflammatory agents. In the present study, It was shown that ESH inhibited the production of NO and expression of iNOS as well as expression in LPS-activated

macrophages. The inhibitory effects of ESH were caused by decreased iNOS and COX-2 mRNA expression in a dose-dependent manner.

MAPKs, serine/threonine kinases, comprise a primary signaling pathway for iNOS and COX-2 up-regulation by various stimuli. It has been reported that MAPK phosphorylation is closely associated with LPS-induced iNOS and COX-2 expression (Welters, Fimiani et al. 2000; Pearson, Robinson et al. 2001; Hoffmann and Baltimore 2006; Kawai and Akira 2006; Tsatsanis, Androulidaki et al. 2006; Cha, Moon et al. 2009). Moreover, the activation of IκB/NF-κB is associated with the MAPK signaling pathway. It is well known that degradation of IκB-α following IκB-α phosphorylation and the rapid translocation of NF-κB-p65 and NF-κB-p50 subunits are essential processes for the activation of NF-κB as a transcription factor in response to various stimuli. LPS-activated NF-κB and MAPK could regulate the expression of pro-inflammatory factors including iNOS, TNF-α, COX-2, IL-1β, and IL-6 in macrophages (Welters, Fimiani et al. 2000; Pearson, Robinson et al. 2001; Hoffmann and Baltimore 2006; Kawai and Akira 2006; Tsatsanis, Androulidaki et al. 2006; Cha, Moon et al. 2009). Therefore, the effect of ESH on the phosphorylation of MAPK and NF-κB subunit NF-κB-p65 was examined in LPS-stimulated macrophages. As shown in Fig. 10, it was found that phosphorylation of ERK1/2and NF-κB-p65 were significantly decreased in LPS-stimulated macrophages by pretreatment with ESH. These results suggest that ESH inhibited the activation of NF-κB, ERK1/2 and p38.

In summary, our results demonstrated that ESH isolated from *S. horneri*

(Turner) C. Agardh suppressed NO synthesis through the down-regulation of iNOS gene expression in LPS-stimulated macrophages. Moreover, suppression of iNOS and COX-2 expression involved the inhibition of the ERK1/2, p38 and NF-κB signaling pathway in LPS-stimulated macrophages. Therefore, our findings suggest the potential for ESH as an important therapeutic agent for the treatment of aberrant inflammation.

2. Anti-inflammatory effects of galangin on lipopolysaccharide-activated macrophages *via* ERK and NF-κB pathway regulation

a. Introduction

Inflammation is a complex biological response to harmful stimuli that is associated with many pathophysiological conditions. Macrophages play an essential role in local host defense and inflammatory responses. In response to inflammatory stimuli, activated macrophages produce various pro-inflammatory factors, including cytokines and NO. NO is synthesized by mammalian cells and plays an important role in many diseases such as inflammation, hypertension, diabetes, rheumatoid arthritis, bowel disease, and atherosclerosis (Manzi and Wasko 2000; Park, Kim et al. 2010; Park, Jeon et al. 2012; Kolesov, Korkotashvili et al. 2013). NO is produced by iNOS. iNOS can elicit NO production during inflammation, and COX-2 is believed to be responsible for the synthesis of PGE_2 in various models of inflammation (Moncada 1999; Chun, Choi et al. 2012). Moreover, iNOS is responsible for the excessive production of NO by macrophages during the inflammatory process that is stimulated by LPS. LPS-activated TLR leads to the phosphorylation of MAPK. Activation of MAPK is an important signal for induction of iNOS and cellular responses to extracellular signals, as well as the regulation of pro-inflammatory cytokines during cellular responses, especially IL-1β, IL-6, and TNF-α. Therefore, treatment with anti-inflammatory agents to reduce NO and pro-inflammatory cytokine production has been suggested as an effective strategy for various

inflammatory diseases (Welters, Fimiani et al. 2000; Pearson, Robinson et al. 2001; Kawai and Akira 2006; Elicabe, Arias et al. 2011).

Flavonoids are comprised by more than 4000 polyphenol compounds that occur naturally in various foods including vegetables, herbs, grains, and fruits. These compounds have a common phenylbenzopyrone structure and are divided into flavonols, flavones, isoflavones, and flavanonols according to the saturation level and opening of the central pyran ring (Leonard, Yan et al. 2006; Wang, Lee et al. 2009). These polyphenol compounds exhibit extensive biological activities including anti-oxidant, anti-inflammatory, anti-carcinogenic (Seelinger, Merfort et al. 2008; Xie, Kang et al. 2012).

Galangin (3,5,7-trihydroxyflavone) is a member of the flavonols and is found in India root, *Alpinis officinarum*, which is an herbal medicine in Asia, and propolis, which is naturally produced by honeybees (Gwak, Oh et al. 2011). Many studies have suggested that galangin has beneficial biological activities such as anti-cancer, anti-oxidant, anti-fibrotic activity (Tolomeo, Grimaudo et al. 2008; Zhang, Luo et al. 2010; Kim, Jeon et al. 2012; Wen, Wu et al. 2012; Ha, Kim et al. 2013; Wang, Gong et al. 2013; Zhang, Tang et al. 2013). However, anti-inflammatory activity and the mechanisms of action of galangin on macrophages have not been investigated.

This study was performed to verify the effect of galangin in the regulation of NO and inflammatory cytokine production in LPS-stimulated macrophages and further to examine the potential mechanisms by which galangin modulated NO production. Whether galangin regulates iNOS expression and pro-inflammatory

cytokine production including IL-1β, IL-6, and TNF-α in LPS-stimulated macrophages was also investigated. Indeed, galangin reduced the LPS-induced production of NO and pro-inflammatory cytokines in macrophages through the inhibition of ERK phosphorylation.

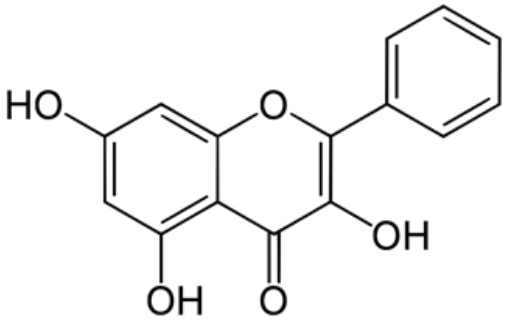
b. Results

(1) Galangin inhibited NO production in LPS-stimulated macrophages

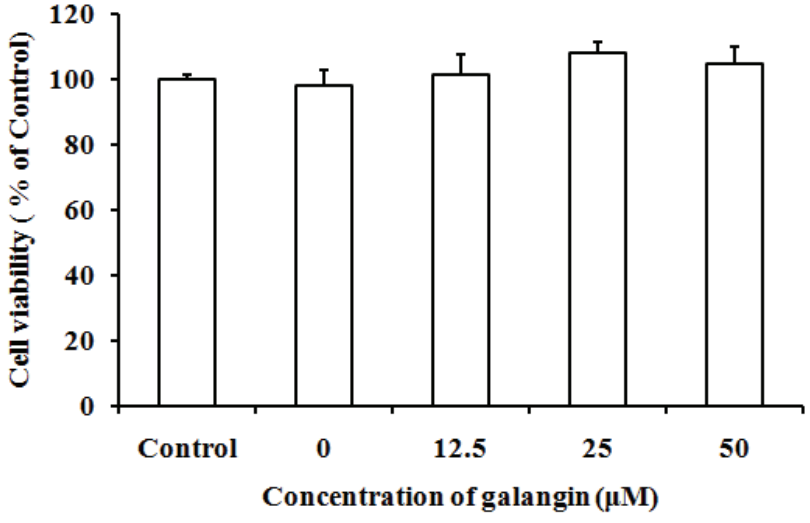
First, the cytotoxicity of galangin in macrophage was examined using an MTT assay. The cells were incubated in the presence of various concentration of galangin (0, 12.5, 25, and 50μM) for 24 hr. Galangin did not show cytotoxic effect at any concentration up to 50 μM (Fig. 11). To investigate whether galangin regulated NO production, NO production was measured in LPS-stimulated macrophages with or without galangin treatment. Pretreatment of macrophages with galangin for 2 hr was followed by stimulation with LPS (200ng/ml). LPS treatment significantly induced NO production compared with control (Fig. 12). However, the pretreatment of macrophages with galangin significantly inhibited LPS-induced NO production in a dose-dependent manner. These results suggest that at the concentrations of galangin tested, NO production in LPS-stimulated macrophages was inhibited.

Fig. 11. Galangin has no effect on RAW 264.7 murine macrophage viability. (A) Structure of galangin. (B) RAW 264.7 cells were plated in 96-well cell culture plates. Various concentrations of galangin and control (0.05% DMSO) were added, and viable cell numbers were assessed by MTT assay after 24 h, as described in the *Materials and Methods*. Data are reported as viable cell numbers, expressed as a percentage of control cells that were exposed to 0.05% DMSO. The data represent the average (±SD) of four replicate wells and are representative of three separate experiments.

(A)



(B)



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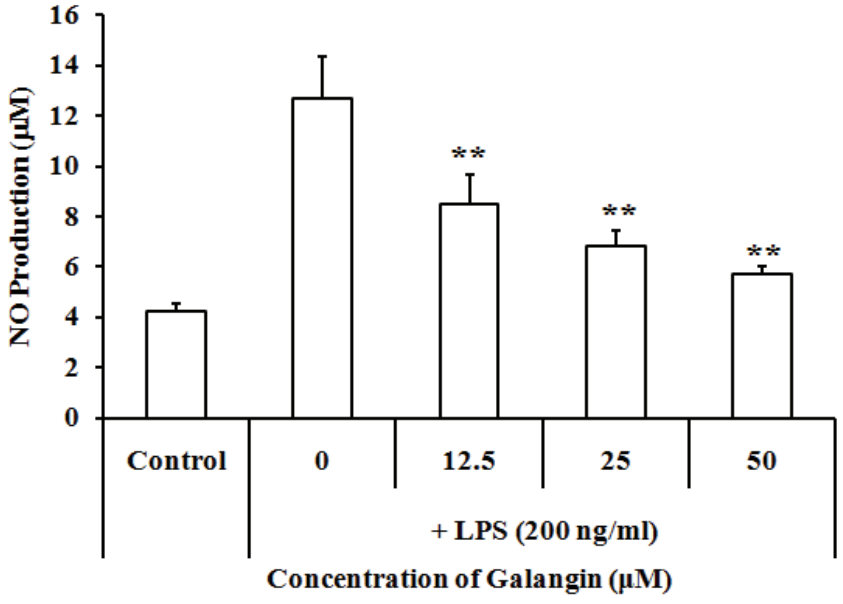


Fig. 12. Galangin decreases LPS-induced NO production in RAW 264.7 murine macrophages. RAW 264.7 cells were plated in 96-well cell culture plates. Cells were pretreated with various concentrations of galangin (0 to 50 μM) for 2 hr and then incubated for 22 hr in the presence or absence of LPS (200 ng/ml). Supernatants were mixed with Griess reagent, and absorbance was measured using an ELISA microplate reader. The data represent the average (±SD) of four replicate wells and are representative of three separate experiments ($^{**}P < 0.01$ *vs.* LPS only groups).

(2) Galangin suppressed the expression of pro-inflammatory factors

IL-1β is a pro-inflammatory cytokine that is released from macrophages under inflammatory conditions including LPS stimulation. To identify the effects of galangin in IL-6, TNF-α, iNOS, and COX-2 expression, RT-PCR and ELISA assays were used to measure the levels of mRNA expression in macrophages pretreated with or without galangin for 2 hr followed by stimulation with LPS for 6 hr. As shown in Fig. 13, stimulation of LPS stimulation of macrophages increased the expression levels of IL-6 and TNF-α, which was significantly reduced by galanginin a dose-dependent manner. Next, to investigate whether the inhibitory effect of galangin on the production of iNOS and COX-2 occurred at the level of pro-inflammatory gene expression, the mRNA and protein expression levels of iNOS and COX-2 were measured in macrophages stimulated with LPS in the presence or absence of galangin. Upon treatment with LPS, the mRNA and protein expression of iNOS and COX-2 were significantly increased in macrophages. However, co-treatment of cells with LPS and various concentrations of galangin suppressed only the LPS-induced mRNA and protein levels of iNOS in a dose-dependent manner (Fig. 14A). This result suggests that galangin inhibited production of the iNOS at the mRNA and protein levels, but had no effect on COX-2 at either the mRNA or protein level.

(3) Galangin inhibited LPS-induced ERK and NF-κB-p65phosphorylation

MAPK such as ERK1/2 and p38 are involved in the signaling pathways leading to the regulation of inflammatory mediators *via* activation of transcription factors,

especially NF-κB. Therefore, to investigate the molecular mechanism behind the inhibitory effects of galangin on inflammatory mediators, the phosphorylation of MAPK was determined in LPS-stimulated macrophages. As shown in Fig. 8B, phosphorylation of ERK1/2 and p38 MAPK was increased at 15, 30 and 45 min in LPS-induced macrophages. Treatment with galangin strongly inhibited LPS-induced phosphorylation of ERK1/2. However, p38 MAPK was not altered by treatment with galangin. This result indicates that the ERK pathway may contribute to the inhibitory effects of galangin on the induction of pro-inflammatory genes in LPS-stimulated macrophages.

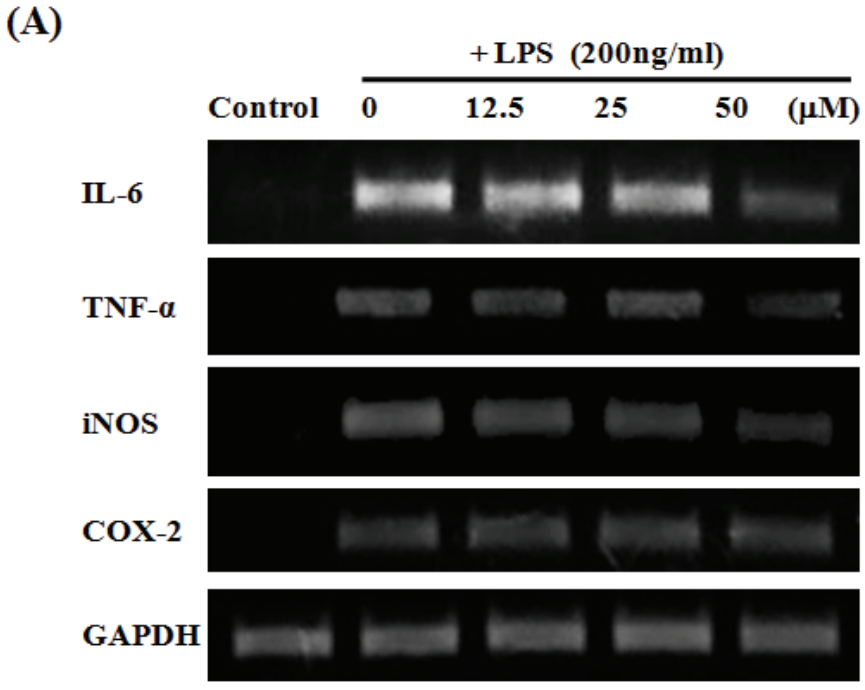


Fig. 13. Galangin inhibits LPS-induced pro-inflammatory genes in RAW 264.7 murine macrophages. RAW 264.7 cells were pretreated with 50 μM galangin for 2 hr and stimulated with LPS for 6 hr; mRNA expression levels of inflammatory genes were determined by (A) RT-PCR and (B) RT-qPCR. GAPDH was used as an internal control. The data represent the average (±SD) of four replicate wells and are representative of three separate experiments ($^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* LPS only groups).

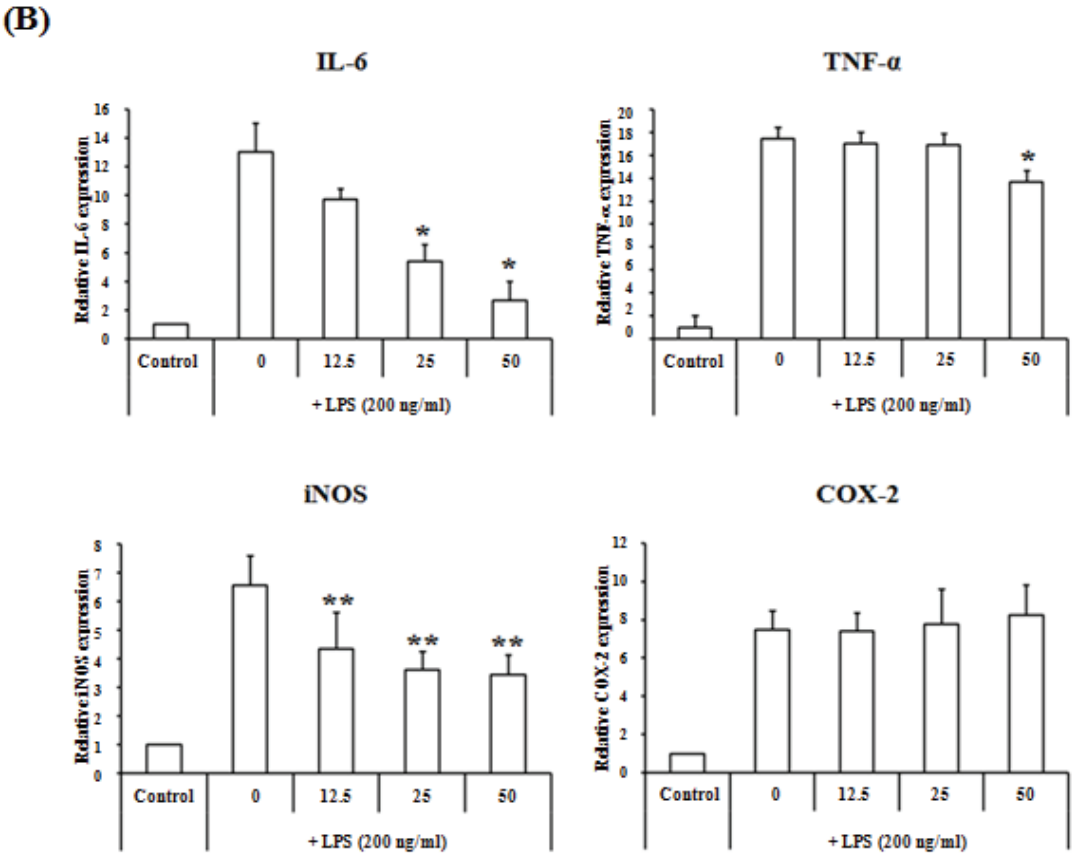


Fig. 13. (continued)

Fig. 14. Galangin inhibits iNOS expression and ERK phosphorylation in LPS-stimulated macrophages. (A) RAW 264.7 cells were pretreated with various concentrations (0 to 50 μM) of galangin and stimulated with 200 ng/ml LPS for 24 hr. (B) RAW 264.7 cells were starved in serum free DMEM; 50 μM galangin was added after 2 hr, and cells were stimulated with 200 ng/ml LPS for 15, 30, and 45 min. Cells were lysed, and equal protein amounts were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were probed with primary antibodies (p-ERK1/2, p-p38, p-JNK1/2, p-NF-κB-p65), and β-actin was used as an internal control. The results are representative of four experiments. (C) RAW 264.7 cells were pretreated with ERK inhibitor (20 μM) and galangin (50 μM) and stimulated with 200 ng/ml LPS for 24 hr.

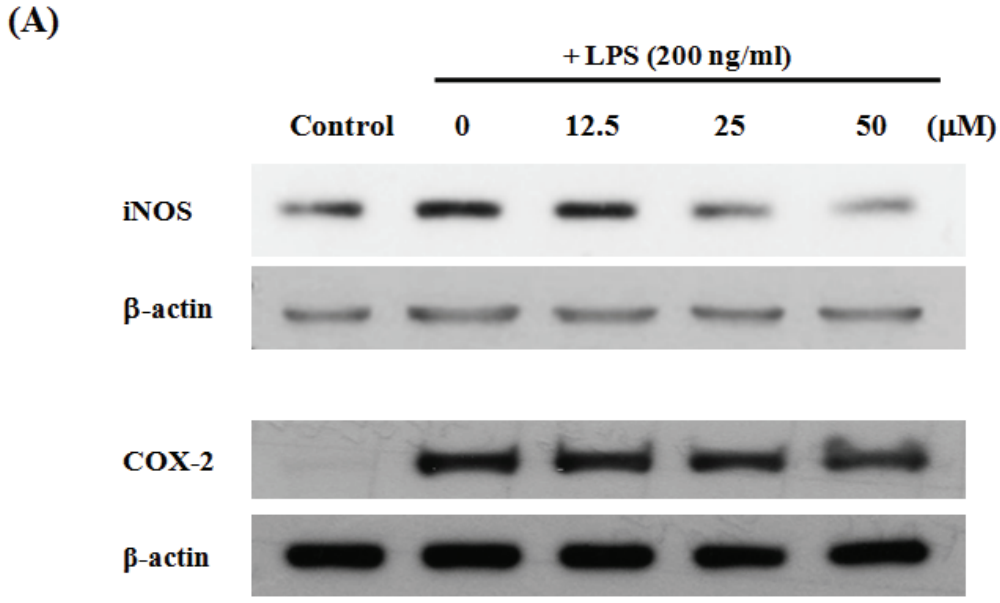
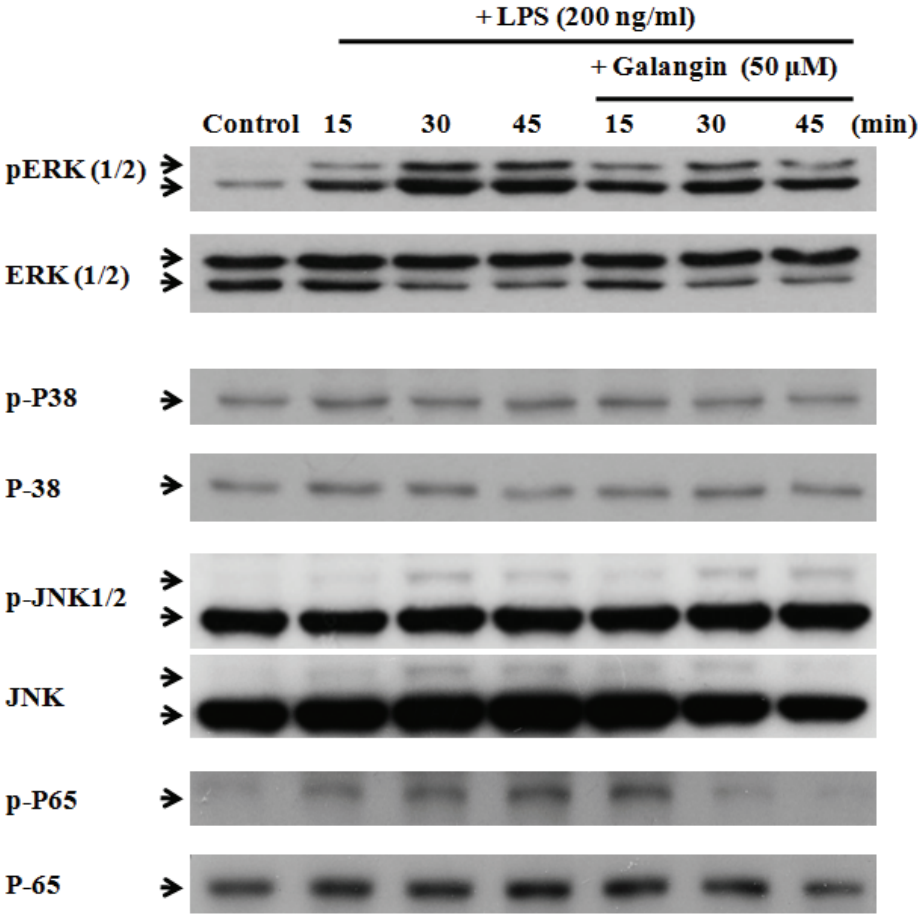


Fig. 14. (continued)

(B)



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(C)

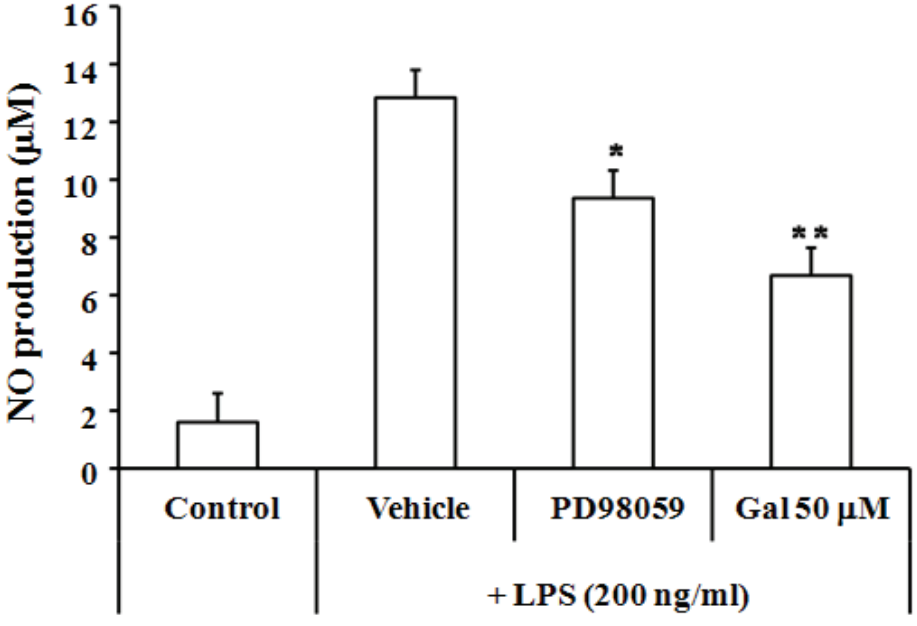
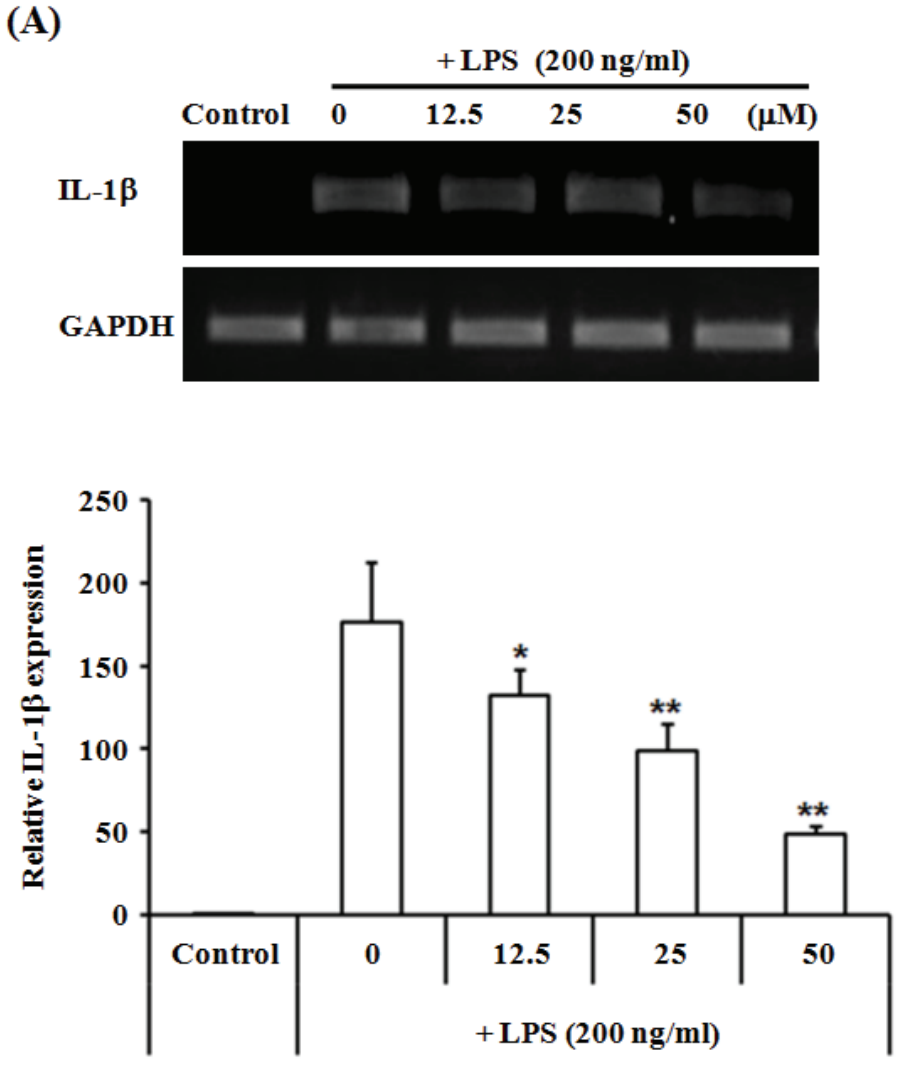


Fig. 14. (continued)

(4) Galangin attenuated IL-1β mRNA expression levels

IL-1β is a pro-inflammatory cytokine that is released from macrophage cells upon stimulation with LPS or other inflammatory mediators. To examine the effects of galangin in IL-1β expression and production, RT-PCR and ELISA assays were used to measure IL-1β levels in cell lysate or culture media of macrophages pretreated with or without galangin for 2 hr followed by stimulation with LPS for 6 or 24 hr, respectively. As shown in Fig. 9A, stimulation of macrophages with LPS increased the expression level of IL-1β, which was significantly reduced by galangin in a dose-dependent manner. Moreover, the secreted level of IL-1β was reduced by galangin treatment of LPS-activated macrophages (Fi.g 9B). These results indicate that galangin inhibited production and release of IL-1β at the transcriptional level.

Fig. 15. Galangin attenuates mRNA expression and secretion of IL-1β from RAW 264.7 murine macrophages. RAW 264.7 cells were pretreated with various concentrations (0 to 50 μM) of galangin, and stimulated with LPS for (A) 6 hr or (B) 24 hr. The mRNA expression level of IL-1β was determined by RT-PCR, and supernatants were examined for IL-1β release by ELISA. Data are expressed as pg/ml ± SD of triplicate cultures ($^{**}P < 0.01$ *vs.* LPS only groups).



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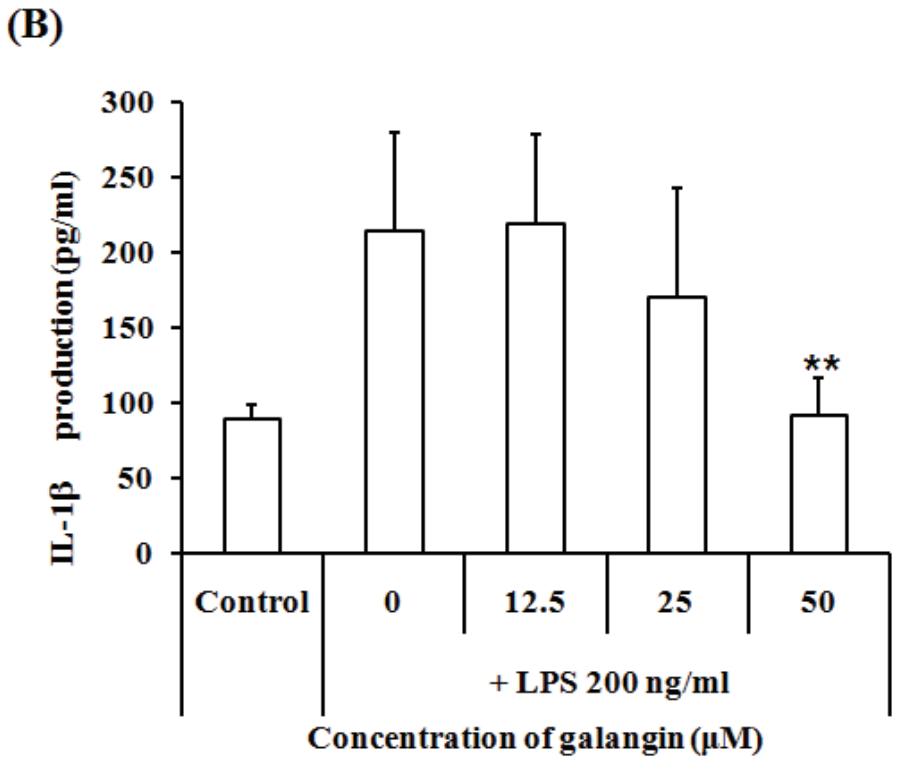


Fig. 15. (continued)

c. Discussion

Macrophages are one of the major immune cell types that reside in sites of inflammation and can induce chronic inflammatory diseases such as fibrosis, atherosclerosis, rheumatoid arthritis, and diabetes (Wiklund, Iversen et al. 1999; Steinberg and Schertzer 2014; Wynick, Petes et al. 2014). In the present study, It was demonstrated that galangin inhibited the production of pro-inflammatory mediators such as NO, iNOS, TNF-α, IL-6, and IL-1β in an LPS-stimulated macrophage cell line *in vitro*. Moreover, our results demonstrated that galangin reduced expression of iNOS and IL-1β mRNA as well as the production of IL-1β, NO, and iNOS. Galangin inhibited LPS-stimulated phosphorylation of ERK.

NO is a potent reactive factor in biological systems and inflammatory responses. NO can be regulated by iNOS expression as well as by other NO metabolites. In addition, NO is generated by stimulated macrophages and in inflammatory sites. NO and PGE_2 play critical roles in macrophage activation and are associated with both acute and chronic inflammation (Evans 1995; Robinson, Zhang et al. 1999; Welters, Fimiani et al. 2000). For this reason, the suppression of NO and PGE_2 production by the inhibition of iNOS and COX-2 protein expression can be a very important therapeutic approach in the development of anti-inflammatory agents. In the present study, it was shown that galangin inhibited the production of NO and expression of iNOS, but not COX-2 expression, in LPS-activated macrophages (Fig. 12 and Fig. 13). The mechanism of inhibition of galangin was *via* decreased iNOS mRNA expression in a dose-dependent manner.

MAPKs represent a highly conserved family of protein serine/threonine kinases that have important roles in iNOS regulation in various biological systems. Several studies have shown that the phosphorylation of MAPK is involved in LPS-induced iNOS expression (Tsatsanis, Androulidaki et al. 2006; Zhou, Shin et al. 2008). MAPK activated by LPS is known to control the expression of cell survival genes as well as pro-inflammatory enzymes and cytokines such as iNOS, TNF-α, COX-2, IL-1β, and IL-6. In LPS-stimulated macrophages, expression of iNOS and COX-2 was dependent on MAPK activation (Kim, Ko et al. 2013; Li, Su et al. 2013; Shi, Cao et al. 2014). Therefore, the effect of galangin on the phosphorylation of MAPK was examined in LPS-activated macrophages. As shown in Fig. 14, it was found that the phosphorylation of ERK1/2 was significantly inhibited in LPS-stimulated macrophages that had been pretreated with galangin. These results suggested that galangin inhibited the activation of ERK1/2.

In summary, our results indicate that galangin suppressed the down-regulation of iNOS, IL-1β, IL-16, and TNF-α gene expression in LPS-stimulated macrophages. Moreover, the inhibition of iNOS and IL-1β expression was related to the inhibition of the ERK1/2 pathway in an LPS-stimulated macrophage cell line. Although further investigation is required to clarify the detailed mechanism, this study provides important information regarding the use of galangin as a candidate therapeutic agent against inflammation.

3. 18β-Glycyrrhetinic acid from licorice root impairs dendritic cell maturation and Th1 immune responses

a. Introduction

DCs are professional APCs, playing key roles as immune sentinels and as initiators of T cell responses against microbial pathogens and tumors (Lee, Jeong et al. 2007; Carrion, Scisci et al. 2012; Sawant, Hensel et al. 2012). An extensive body of research is currently focused on the biological activities of DCs, specifically regarding their possible clinical use as cellular adjuvants for the treatment of chronic infectious disease and tumors, as well as for the restoration of immune homeostasis (Mattsson, Yrlid et al. 2011; Joshi, Duhan et al. 2012).

As potent APCs, DCs possess immune sentinel properties, which enable the induction of primary immune responses and the activation of T cell responses against pathogens and tumors. This is important because the generation of an optimal immune response often requires the presence of $CD4^+$ and $CD8^+$ T cells as well as the expression of T cell-specific antigens on APCs (Reichardt, Dornbach et al. 2010; Rosenits, Keppler et al. 2010; Li, Kim et al. 2012). DCs are highly responsive to both bacterial products and inflammatory cytokines, such LPS and TNF-α (Lee, Kim et al. 2007). While in the immature state, DCs have been shown to effectively capture and process exogenous antigens within peripheral tissues, where they then begin to mature. iDCs do not induce primary immune responses, as they have low expression of co-stimulatory molecules. However, upon stimulation, DC maturation is associated with high expression of

major histocompatibility complex (MHC) class I and II, decreased or absent antigen uptake, the up-regulation of co-stimulatory molecules, and IL-12 production. Intriguingly, recent reports have suggested that functional modulators of DCs could act as therapeutic adjuvants for DC-associated immune diseases (Rutella and Locatelli 2011; Stenger, Turnquist et al. 2012; Zheng, Cao et al. 2012).

It was previously reported that a variety of phytochemicals exhibit profound immunoregulatory activities both *in vitro* and *in vivo*, particularly for DCs (Kim, Jeong et al. 2007; Lee, Jung et al. 2007; Lee, Kim et al. 2007). One of these, 18β-glycyrrhetinic acid (β-GA), is a natural triterpenoid compound derived from *Licorice root* extract and the hydrolysis product of glycyrrhizin. Many of the properties of glycyrrhizin can be attributed to β-GA, and it has been shown to possess several beneficial pharmacological activities, such as anti-tumor and anti-viral activities, and immunomodulatory ability (Asl and Hosseinzadeh 2008; Kuang, Zhao et al. 2012). Until now, the immune cellular targets of β-GA have remained unknown, thereby creating the opportunity for research aimed at characterizing the global function of β-GA, particularly as it relates to DC maturation and immune-regulation.

In the present study, it was sought to characterize the effects of a non-cytotoxic dose of β-GA on the functional and maturational properties of murine bone marrow-derived DCs. It was found that β-GA suppressed the expression of co-stimulatory molecules and MHC class I and II in these cells during LPS-induced maturation. Moreover, treatment of DCs with β-GA, impaired Th1

responses through the regulation of Th1-inducing cytokines on $CD4^+$ T cells. Thus, our findings demonstrate that β-GA treatment inhibits the phenotypic and functional maturation of DCs.

b. Results

(1) β-GA inhibits LPS-stimulated DC maturation

In an initial series of experiments, it was investigated whether β-GA could influence the maturation of DCs. Bone marrow-derived DCs were cultured for 6 days in media supplemented with granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4. Different concentrations of β-GA were added to the culture on day 6 with or without 200 ng/ml of LPS. β-GA was determined to be cytotoxic to DCs at concentrations in excess of 20 μM (Fig. 16A); however, there were no marked differences in the percentage of dead cells when the concentrations were all set to ≤ 40 μM. Next, the effects of different physiological concentrations of β-GA on DC maturation was assessed by measuring the protein expression levels of co-stimulatory molecules B7-1 (CD80), B7-2 (CD86), and MHC class I and II in presence or absence of LPS. As shown in Fig. 16B, treatment with various concentrations of β-GA (1 μM, 10 μM, and 20 μM) slightly attenuated the expression of CD80, CD86, MHC class I, and MHC class II on DCs, all of which are normally up-regulated during DCs maturation. These same molecules were also up-regulated within 24 hr of LPS exposure (Fig. 16C). Notably, a significant down-regulation of CD80, CD86, and MHC class I was observed in DCs treated with 20 μM of β-GA in the presence of LPS.

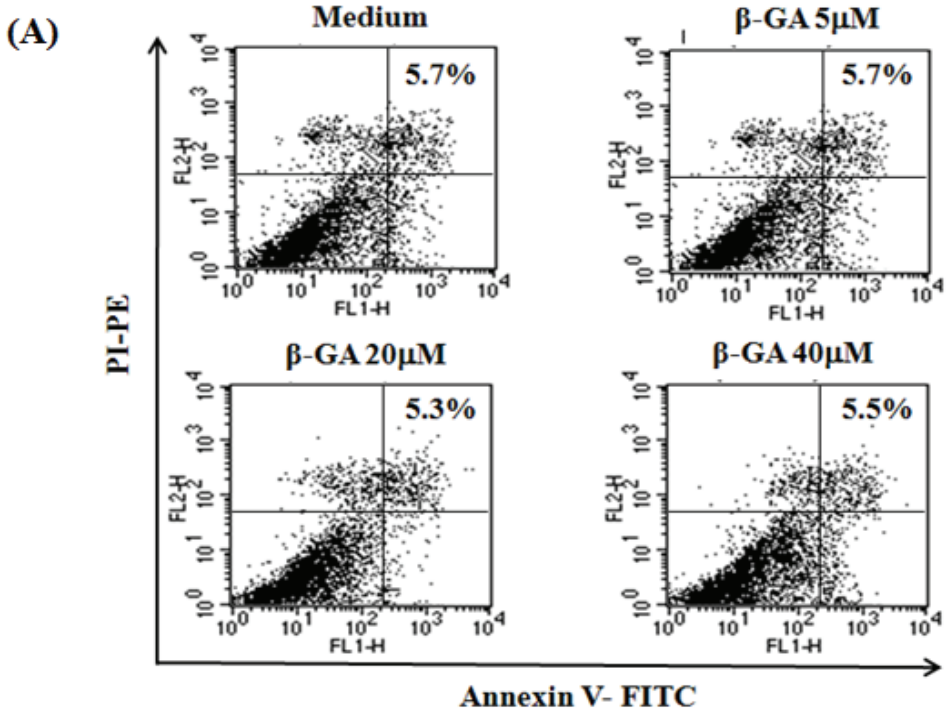
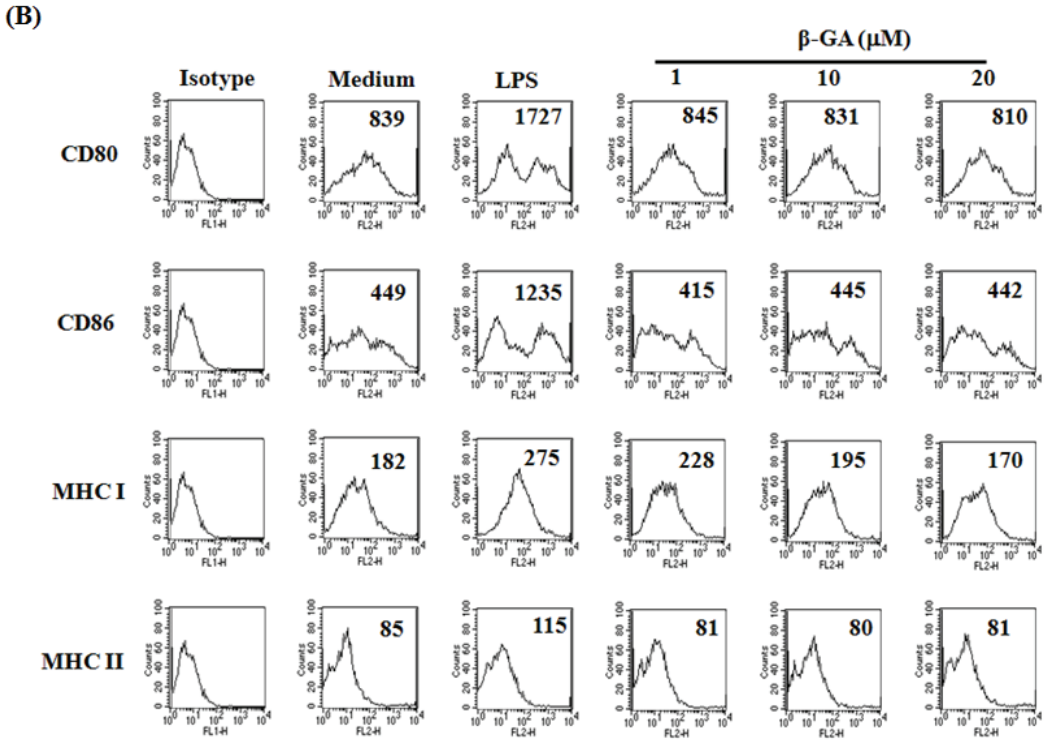


Fig. 16. β-GA impairs the expression of co-stimulatory molecules MHC class I and II during DCs maturation. DCs were generated as described in the *Materials and Methods*. On day 6, β-GA was added at concentrations of 1, 10, 20 or 40 μM for 24 hr. DCs were stained for CD11c, annexin-V, and PI. The percentage within each positive cell represents the incidence of annexin-V^+PI^+. (A) β-GA exerts no influence on cytotoxicity in $CD11c^+$ DCs. (B) The expression of surface molecules was analyzed by two-color flow cytometry. (C) Histograms indicate cells exposed to media alone (gray bar) or cells stimulated for 24 hr with 200 ng/ml LPS (black bar) in the presence of 20 μM β-GA (white and black bar) on day 6. The results are representative of three separate experiments ($^{**}P < 0.01$ *vs.* LPS-treated DCs).



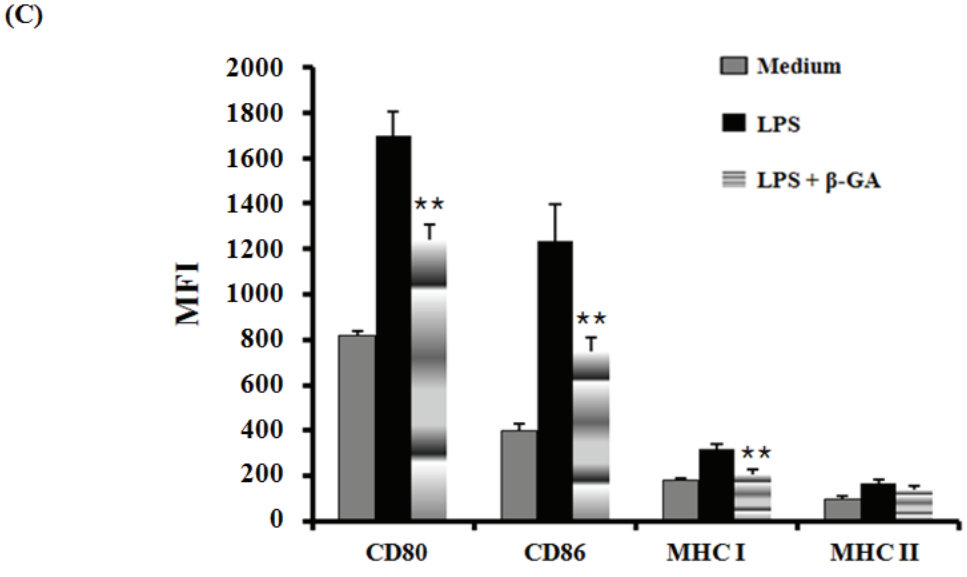


Fig. 16. (continued)

(2) β-GA impairs the secretion of IL-12 secretion during LPS-induced DC maturation

IL-12 is an important marker of DC activation, and can be used to select Th1-dominant adjuvants (Lee, Kim et al. 2007). Thus, the effect of β-GA on the production of pro-inflammatory cytokines, such as IL-12, was examined in LPS-stimulated DCs; specifically intracellular levels of IL-12p40/70 and IL-10 were measured in β-GA-treated DCs (Fig. 17A). Intracellular staining of $CD11c^+$ DCs with PE-labeled anti-IL-12p40/p70 or FITC-anti-IL-10 monoclonal antibodies (mAbs) revealed that DCs stimulated with LPS and 20 μM β-GA expressed lower levels of IL-12p40/p70 than those stimulated with LPS alone, whereas IL-10 was barely detectable under any condition. When the supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA), it was found that β-GA exerted a significant inhibitory effect on the secretion of bioactive IL-12p70 in LPS-stimulated DCs (from 72 ± 10.1 pg/ml to 32 ± 4.5 pg/ml) (Fig. 17B), indicating that β-GA impairs the ability of DCs generate bioactive IL-12p70 in response to LPS stimulation.

Fig. 17. β-GA impairs IL-12 production and secretion during LPS-stimulated DCs maturation. DCs were stimulated with β-GA (10 and 20 μM) for 24 hr in the absence or presence of LPS. (A) Cells were analyzed by intracellular cytokine staining and gated for $CD11c^+$ positive DCs. (B) DCs (1×10^5 cells/ml) were cultured for 24 hr, and the production of bioactive IL-12p70 and IL-10 were analyzed by ELISA from the culture supernatants. The results are representative of three separate experiments ($^{**}P < 0.01$ *vs.* LPS-stimulated DCs).

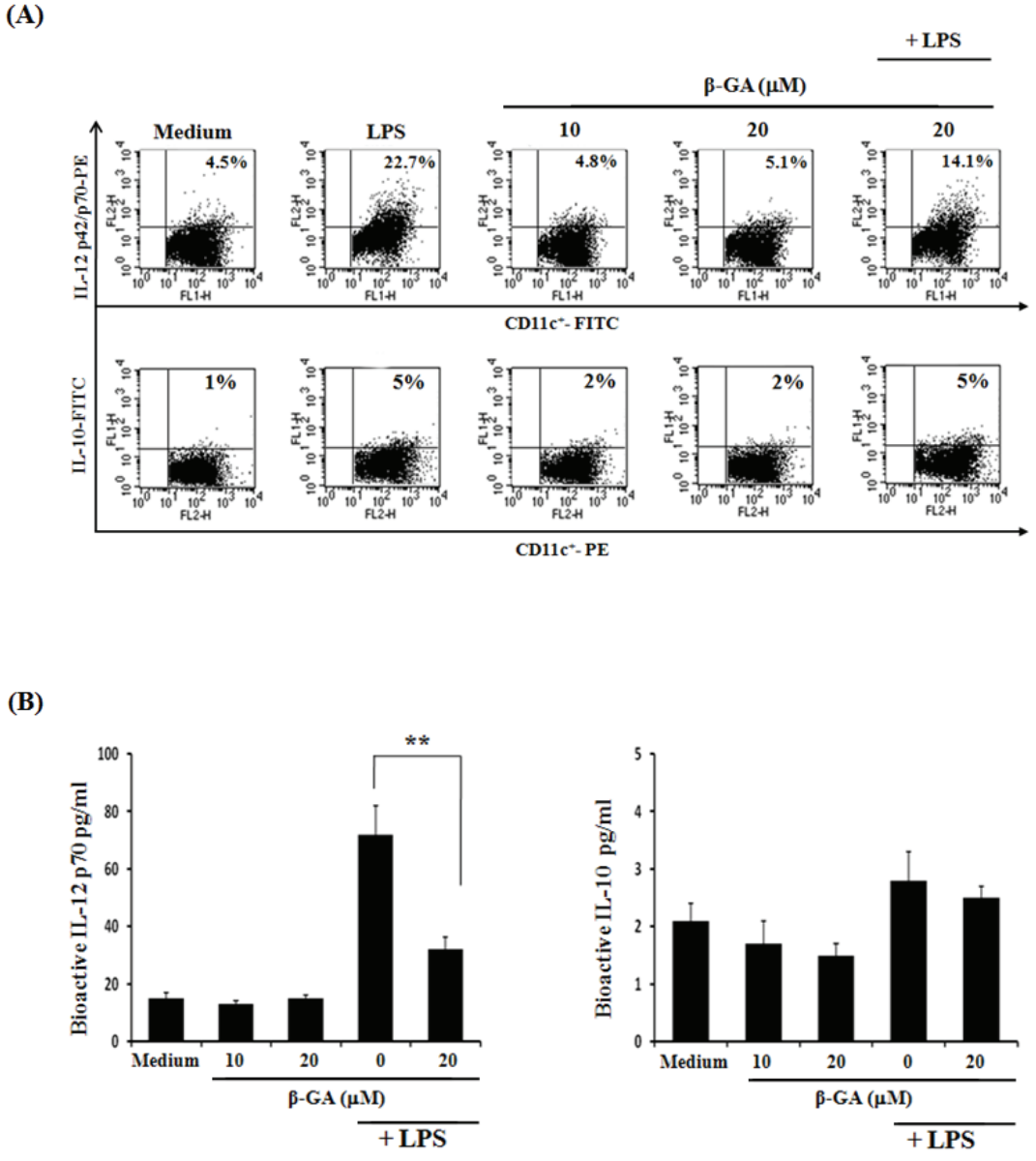
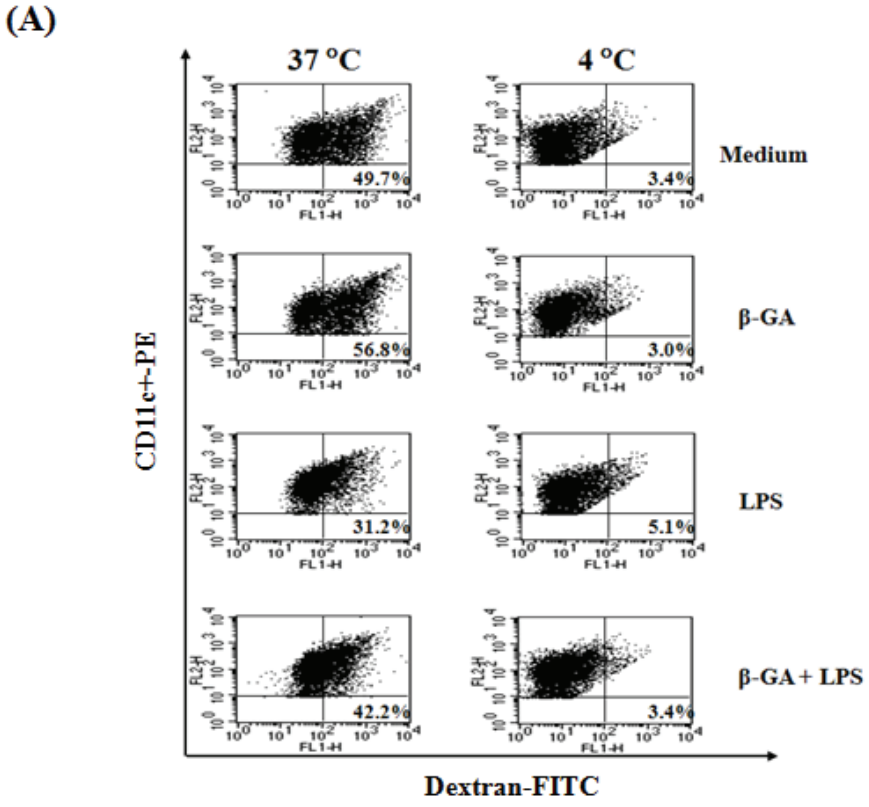


Fig. 17. (continued)

(3) β-GA enhances the immature state of DCs with high endocytic capacity

The altered expression of DC surface molecules and the observed changes in IL-12 production in response to β-GA indicate that treatment with this compound leads to a profound inhibition of DC phenotypic and functional maturation. However, these results do not exclude the possibility that β-GA might induce a general inhibition of the physiological functions of DCs. Thus, it was investigated whether stimulation with β-GA altered the ability of DCs to capture antigens. DCs were exposed to β-GA in the absence or presence of LPS in culture media containing dextran-FITC to measure their endocytic activity. It was observed that the percentage of double positive cells ($CD11c^{+}$-PE with dextran-FITC) for β-GA-treated and non-treated DCs (media only) was identical. Furthermore, the percentage of LPS-stimulated DCs was lower than the percentage of untreated DCs, and the β-GA-treated DCs exhibited a high endocytic capacity for dextran-FITC, as compared to LPS-stimulated DCs (Fig. 18A and B). These results indicate that the β-GA-treated DCs are both phenotypically and functionally immature. A set of experiments identical to the above-mentioned experiments were also performed at 4℃, indicating that the uptake of dextran-FTIC by the DCs is inhibited at low temperatures. Collectively, these results suggest that β-GA enhances DC immaturity.

Fig. 18. β-GA-treated DCs exhibit enhanced endocytic capacity. DCs were treated with 20 μM β-GA with or without LPS (200 ng/ml) for 24 hr. (A) Endocytic activity was assessed by FITC-dextran treatment followed by flow cytometry. DCs were washed and stained with a PE-conjugated anti-$CD11c^+$ antibody. The endocytic activity of the controls was determined after exposure to FITC-dextran at 4℃. The number represents the percentages of FITC-dextran/$CD11c^+$-PE double positive cells. (B) The profile of endocytic capacity displayed as a histogram using the percentages of FITC-dextran/$CD11c^+$-PE double positive cells. The results shown are representative of three separate experiments ($^{\#\#}P < 0.01$ vs. unstimulated DCs [medium only]; $^{**}P < 0.01$ *vs.* LPS-stimulated DCs).



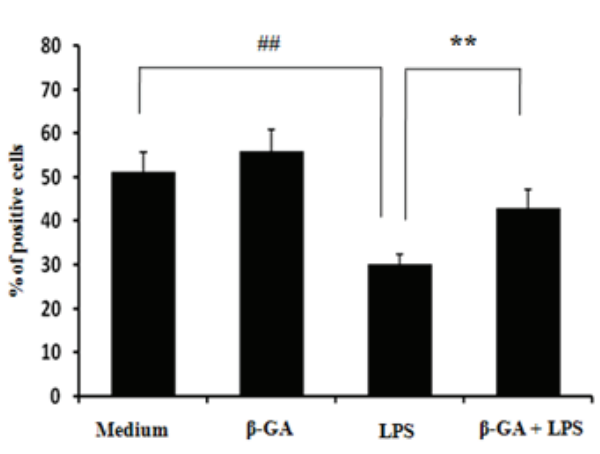


Fig. 18. (continued)

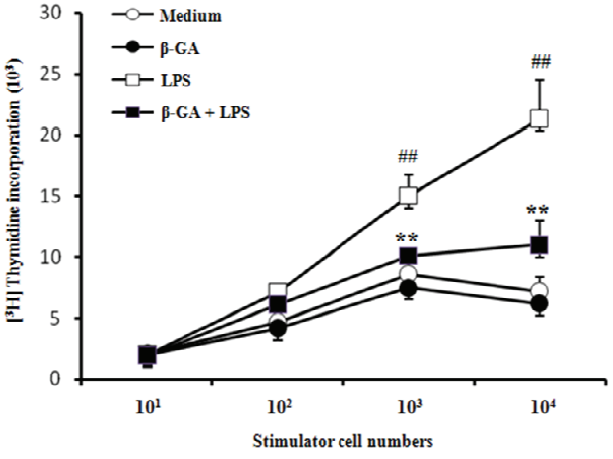
(4) β-GA inhibits DC-mediated induction of Th1 polarization

In order to determine whether β-GA can exert any detectable effects on allogeneic T cell stimulation, DCs were treated with β-GA for 24 hr and subsequently incubated with allogeneic $CD4^+$ T cells. As shown in Fig. 19, LPS-treated DCs elicited more profound proliferative responses in allogeneic $CD4^+$ T cells than untreated DCs, whereas β-GA appeared to impair these proliferative responses induced by LPS-activated DCs. Therefore, while LPS-induced maturation profoundly promotes the allostimulatory capacity of DCs, β-GA treatment significantly impairs this activity.

Additionally, because β-GA was observed to inhibit the production of IL-12, a Th1-inducing cytokine, it was next attempted to characterize the primary T cell response induced by DCs that were matured in the presence of β-GA. Naïve allogeneic $CD4^+$ T cells primed by mDCs differentiated into Th1 lymphocytes, which produced high levels of IFN-γ and low levels of IL-4. In contrast, naïve $CD4^+$ T cells primed with DCs that had matured in the presence of β-GA displayed lower levels of IFN-γ production (Fig. 19B). These results suggest that the effect of β-GA on the T cell differentiating properties of DCs is primarily due to an inhibition of IL-12 production.

Fig. 19. β-GA impairs the Th1 response and IFN-γ production. DCs were incubated for 24 hr in media alone or media containing β-GA (20 μM), LPS (200 ng/ml), or LPS with β-GA. These cells were washed and co-cultured with allogenic $CD4^+$ T cells, and the mixed leukocyte reaction was incubated for 4 days. (A) Background levels of [^{3}H]Thymidine uptake were determined by measuring reactions without the stimulator. (B) The cells were examined for cytokine production *via* ELISA after 48 hr. The results are representative of three separate experiments ($^{**}P < 0.01$ *vs.* LPS-stimulated DCs).

(A)



(B)

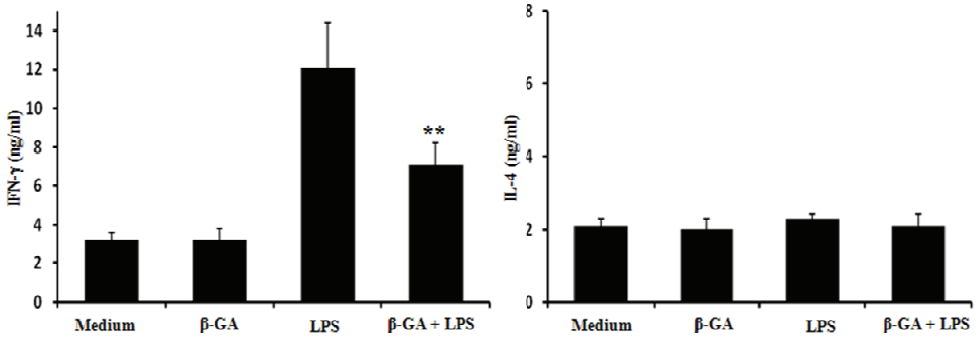


Fig. 19. (continued)

c. Discussion

To the best of our knowledge, this is the first report investigating the effects of β-GA on the phenotypic and functional maturation of DCs. Specifically, the present study investigated how β-GA influences the phenotypic and functional maturation of bone marrow-derived DCs. β-GA is a natural triterpenoid compound derived from *Licorice root* extract, and researchers have shown that this molecule exerts biological effects, including anti-viral, anti-tumor, and immunomodulatory properties (Asl and Hosseinzadeh 2008; Kuang, Zhao et al. 2012). However, it is unknown whether β-GA can modulate DC maturation pathways. DCs are potent APCs and interact with T cells to promote cell-mediated immune responses. Therefore, a variety of functional assays were utilized to characterize the function and phenotype of β-GA-treated DCs, including the expression of MHC and co-stimulatory molecules, IL-12 production, and endocytic capacity, and to elucidate the effect of β-GA treatment on DC-mediated immune responses, as it relates to Th1 or Th2 polarization and T cell proliferation.

It was observed that β-GA inhibited LPS-induced DC maturation, and this suppressive activity can likely be attributed to a non-specific inhibitory effect. Therefore, it was assessed that β-GA-treated DCs affect on uptake FITC-dextran *via* mannose receptor-mediated endocytosis and found that the endocytic capacity of β-GA-treated DCs was profoundly elevated, suggesting that β-GA suppressed both the phenotypic and functional maturation of DCs. The structure of β-GA is similar to that of cortisone, and therefore, the ability of β-

GA to prevent DC maturation might be the basis for the anti-inflammatory effects of licorice.

Polarization of the helper T cell-mediated immune response toward Th1 or Th2 differentiation depends on specific cytokines secreted by activated DCs. Specifically, IL-12 exerts multiple immune-regulatory functions, including activation of the Th1 subset, which plays a pivotal role in the induction of inflammation. High levels of IL-12 will induce differentiation of naïve $CD4^+$ T cells into Th1 cells and simultaneously block the development of the Th2 lineage (Lipscomb, Chen et al. 2009; Jeong, Hong et al. 2011). The secretion of bioactive IL-12p70 requires the coordinated expression of two of its subunits, namely p35 and p40, which are encoded by two separate genes and are regulated independently. It was found that LPS-treated DCs displayed enhanced IL-12 production and secreted the bioactive form IL-12p70, whereas β-GA exerted inhibitory effects on the production of both intracellular IL-12p40/p70 and secretion of bioactive IL-12p70. In contrast, β-GA and LPS had no effect on IL-10 production in DCs. Therefore, our data indicate that β-GA can inhibit IL-12 production from DCs in the presence of LPS, further confirming the inhibitory effects of β-GA on the DCs maturation and suggesting it can directly inhibit Th 1 responses.

In accordance with this, our study also demonstrated that while LPS-stimulated DCs skewed naïve $CD4^+$ T cells toward developing into IFN-γ-producing Th1 cells, naïve $CD4^+$ T cells stimulated by β-GA-treated DCs generated lower level of IFN-γ, although IL-4 production was unaffected. It was

also observed that β-GA significantly impaired the capacity of these $CD4^+$ T cells to proliferate and initiate the Th1 response. This suggests that β-GA is a potent immune-modulator of the Th1 response, and the inhibition of DC-mediated Th1 polarization might constitute a β-GA-associated immunosuppressive mechanism. Consequently, β-GA-mediated inhibition of IL-12 generation in LPS-stimulated DCs may also contribute to the induction of an immunosuppressive state.

In this study, a variety of β-GA-mediated effects were observed on DCs. Namely, β-GA can inhibit the production of the pro-inflammatory cytokine, IL-12, resulting in the inhibition of T cell activation. These findings illustrate the immunopharmacological functions of β-GA and suggest that exposure to this readily available drug may constitute a nontoxic and highly effective means for the modulation of DC immunoregulatory capacity.

4. Baicalin from *Scutellaria baicalensis* impairs Th1 polarization through inhibition of dendritic cell maturation

a. Introduction

DCs are powerful APCs that possess immune sentinel properties and initiate T cell responses against pathogens and tumors (Lee, Jung et al. 2007; Markey, Banovic et al. 2009). They have also been reported to have possible clinical use as cellular adjuvants for the treatment of chronic infectious disease and tumors, as well in therapies to restore immune homeostasis (Machida, Kitamoto et al. 2010; Mattsson, Yrlid et al. 2011; Zeng, Xia et al. 2011; Joshi, Duhan et al. 2012; van de Laar, Coffer et al. 2012). As potent APCs, DCs initiate immune responses and the activation of T cell responses against specific antigens, and the presence of $CD4^+$ and $CD8^+$ T cells, as well as the expression of T cell-specific antigens on APCs, are required for optimal generation of DC-dependent immune responses (Vinuesa, Linterman et al. 2010). DCs located in peripheral tissues generally tend to be both functionally and phenotypically immature, and iDCs do not induce primary immune responses, as they have low expression of co-stimulatory molecules. While in the immature state, DCs have been shown to effectively capture and process exogenous antigens within infected peripheral tissues, and during maturation, DCs migrate to the lymphoid organs where they stimulate naïve T cells by signaling through the MHC and co-stimulatory molecules. DCs can also be matured by bacterial products and exposure to inflammatory cytokines, such as LPS and TNF-α. During stimulation, there are

numerous phenotypic changes that act as markers of DC maturation, including elevated expressions of MHC class I and II, decreased or absent antigen uptake, up-regulation of co-stimulatory molecules, and IL-12 production. Notably, recent studies have suggested that molecules that act as functional modulators of DCs might be effective therapeutic adjuvants for DC-associated immune diseases.

Baicalin is a major flavonoid constituent of the traditional oriental medicinal herb Baikal skull cap (*Scutellaria baicalensis* Georgi), which is known as "HwangKeum" in Korea and "Ogon in Japan. Baicalin has been widely used for the treatment of various diseases, such as hepatitis, pneumonia, allergies, diabetes, and cancer (Waisundara, Hsu et al. 2008; Jung, Kim et al. 2012). Previous studies have demonstrated that baicalin possesses a wide range of biological and pharmacological activities, including anti-inflammatory, antioxidant, and anti-cancer properties (Shen, Chiou et al. 2003; Franek, Zhou et al. 2005; Zhou, Wang et al. 2009; Lin, Wu et al. 2010; Zhu, Wang et al. 2012). Evidence suggests that baicalin can act by suppressing the functions of various inflammatory cells, such as lymphocytes, and inhibiting the production of pro-inflammatory mediators, such as IL-6 and TNF-α (Lin, Wu et al. 2010; Zhu, Wang et al. 2012). However, until recently, the immune cellular targets of baicalin have remained enigmatic, creating the opportunity to investigate the function of baicalin, as it relates to DCs maturation and immune-regulatory activities.

In the present study, it was sought to characterize the effects of a non-

cytotoxic dose of baicalin on the functional and maturational properties of murine bone marrow-derived DCs. Our findings demonstrate that baicalin treatment inhibits the phenotypic and functional maturation of DCs. Thus, this available drug may constitute a simple, inexpensive, and highly effective means to exploit the immunostimulatory capacity of DCs.

b. Results

(1) Baicalin inhibits LPS-induced DCs maturation

First, it was investigated whether baicalin could influence DC maturation. Bone marrow-derived monocytes were differentiated into DCs in media supplemented with GM-CSF and IL-4 for 6 days. Bone marrow-derived DCs have certain characteristics similar to myeloid DCs and, unlike plasmacytoid DCs, are a major stimulator of T cells. On day 6, cultures were treated with various concentrations of baicalin with or without 200 ng/ml of LPS. Baicalin (Fig. 20A) was determined to be cytotoxic to DCs at concentrations in excess of 30 μM (data not shown), and there were no marked differences in the percentage of dead cells when the concentrations were all set to ≤ 30 μM. To determine whether baicalin has a regulatory effect on DC maturation, the protein expression levels of co-stimulatory molecules B7-1 (CD80), B7-2 (CD86), and MHC class I and II were determined in LPS-stimulated DCs treated with baicalin. As shown in Fig. 20B, treatment with various baicalin concentrations attenuated the expression of CD80, CD86, MHC class I, and MHC class II on $CD11c^+$ DCs, all of which are normally up-regulated during DCs maturation. These molecules were also up-regulated within 24 hr of LPS exposure (Fig. 20.C, thick lines). Additionally, treatment with 20 μM baicalin in the presence of LPS impaired the expression of the MHC class I and II co-stimulatory molecules, and interestingly, a significant down-regulation of CD80, CD86, and MHC class I was also observed under these conditions (Fig. 20C, thin lines).

Fig. 20. Baicalin inhibits the expression of co-stimulatory molecules MHC class I and II during DCs maturation. (A) The chemical structure of baicalin. (B) DCs were generated as described in the *Materials and Methods*. On day 6, baicalin was added at concentrations of 5, 10, or 20 μM for 24 hr, and the expression of surface molecules was analyzed by two-color flow cytometry. (C) Histograms indicate media (control) or cells stimulated for 24 hr with 200 ng/ml LPS in the presence or absence of 20 μM baicalin (gray line, isotype control; thick line, LPS treatment; thin line, baicalin plus LPS treatment) . The results are representative of three separate experiments.

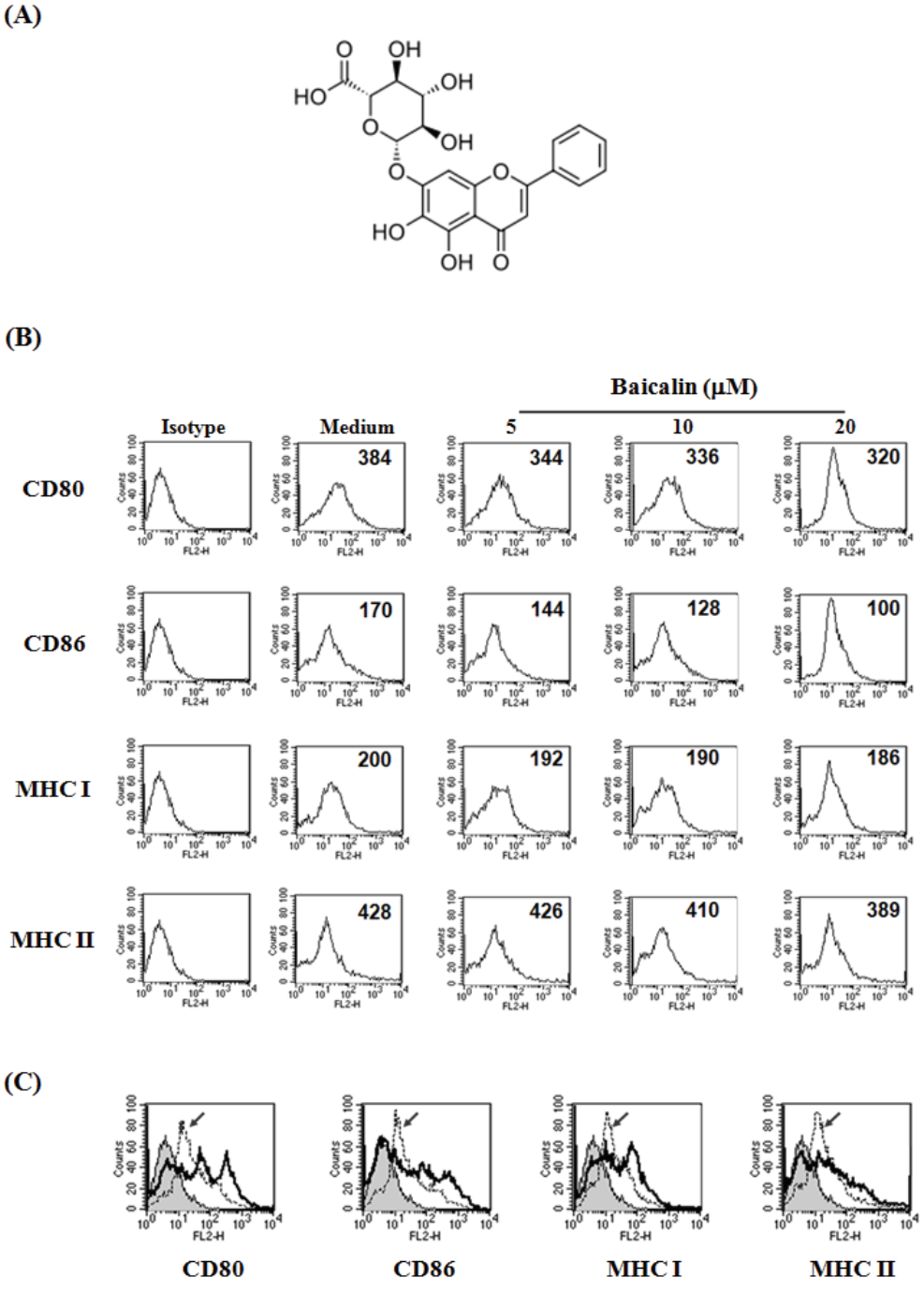


Fig. 20. (continued)

(2) Baicalin impairs the secretion of IL-12 during LPS-induced DC maturation

It has previously been shown that DCs, macrophages, and monocytes function as sources of pro-inflammatory molecules such as IL-12 (Lee, Jung et al. 2007). Thus, the ability of baicalin to modulate pro-inflammatory cytokine expression was examined in LPS-stimulated DCs. In particular, IL-12 expression has been identified as a specific marker of DC activity and the Th1 immune response. It is also considered to be an important marker for DC maturation and can be used to select Th1 dominant adjuvants. Therefore, the production of both intracellular IL-12p40/70 and bioactive IL-12p70 was measured in baicalin-treated DCs. As shown in Fig. 21A, intracellular staining of $CD11c^+$ DCs with PE-labeled anti-IL-12p40/p70 or FITC-anti-IL-10 mAbs revealed that DCs stimulated with 20 μM baicalin expressed IL-12p40/p70 less abundantly than the LPS-stimulated DCs, whereas IL-10 was barely detectable. The inhibitory effects of baicalin on the bioactive form of IL-12 (IL-12p70) were also confirmed by ELISA. As shown in Fig. 21B, high levels of IL-12p70 were secreted upon LPS stimulation of DCs (63.3 ± 1.4 pg/ml) for 24 h, whereas baicalin (34 ± 1.5 pg/ml) attenuated these effects. Conversely, IL-10 secretion was barely detectable 24 hr after stimulation with 200 ng/ml of LPS. These results indicate that baicalin treatment impairs the ability of DCs to produce IL-12p70 and suppresses the functional maturation of LPS-stimulated DCs.

Fig. 21. Baicalin impairs IL-12 production and secretion during LPS-induced DCs maturation. (A) DCs were stimulated by baicalin (20 μM) for 24 hr in the absence or presence of LPS. $CD11c^+$ DCs were subsequently analyzed by intracellular cytokine staining. The cells were gated for $CD11c^+$ positive DCs. (B) DCs (1×10^5 cells/ml) were cultured for 24 hr, and the production of bioactive IL-12p70 and IL-10 were analyzed by ELISA from the culture supernatants. The results are representative of three separate experiments ($^{\#\#}P < 0.01$ *vs.* unstimulated DCs [medium only]; $^{**}P < 0.01$ vs. LPS-stimulated DCs).

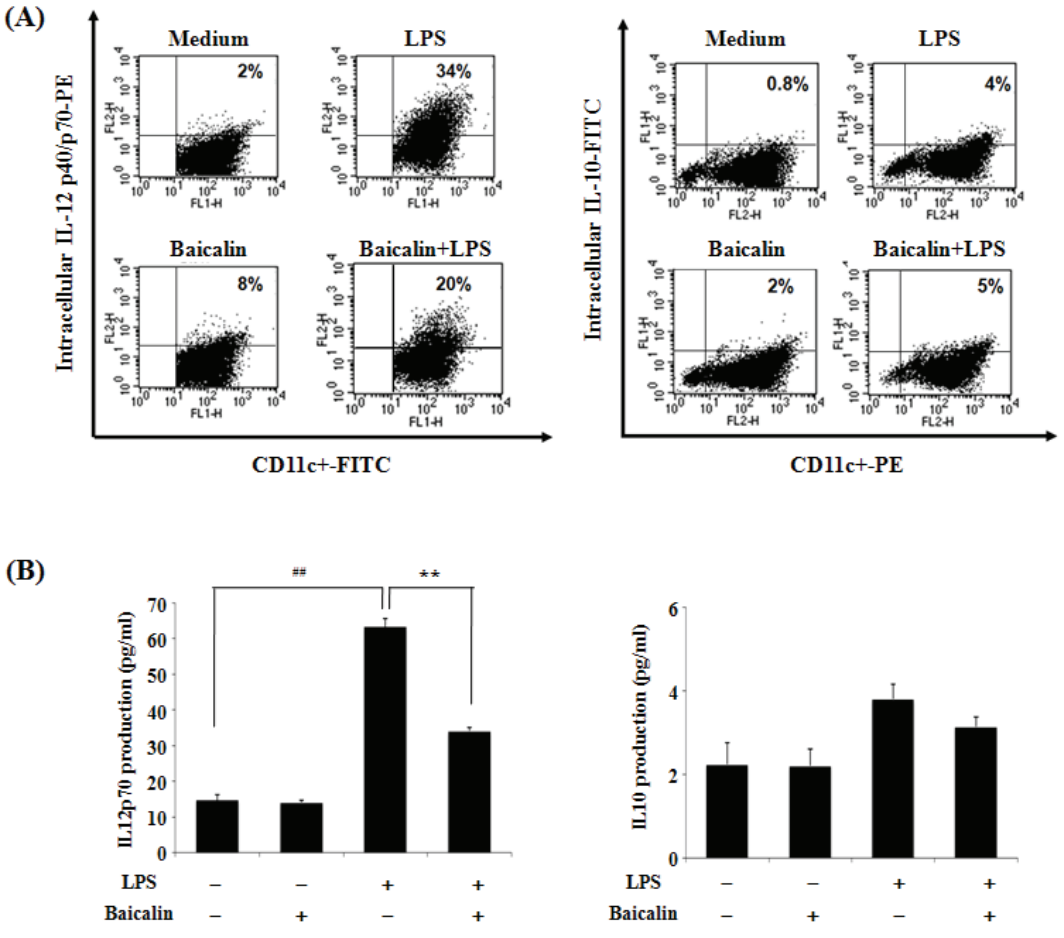


Fig. 21. (continued)

(3) Baicalin enhances the immature state of DCs with high endocytic capacity

The baicalin-mediated down-regulation of co-stimulatory surface molecule expression and inhibition of IL-12 production indicates that treatment with this compound leads to a profound inhibition of DC phenotypic and functional maturation. However, these results do not exclude the possibility that baicalin induces a general inhibition of the physiological functions of DCs. Thus, it was next investigated whether treatment with baicalin alters the ability of DCs to capture antigens. DCs were treated with baicalin in the absence or presence of LPS in culture media containing dextran-FITC to measure their endocytic activity. Based on the uptake of FITC-conjugated dextran, the percentage of double positive cells ($CD11c^{+}$-PE with dextran-FITC) did not differ between baicalin-treated and untreated DCs (medium only). Furthermore, the percentage of LPS-stimulated DCs was lower than the percentage of untreated DCs, and baicalin-treated DCs exhibited a high endocytic capacity for dextran-FITC, as compared to LPS-stimulated DCs (Fig. 22A and B). These results indicate that baicalin-treated DCs are both phenotypically and functionally immature A set of identical experiments were also performed at 4℃, and the results of these indicated that the uptake of dextran-FITC by DCs was inhibited at lower temperatures. Collectively, these experiments suggest that baicalin enhances DC immaturity.

Fig. 22. Baicalin-treated DCs exhibit enhanced endocytic capacity. DCs were treated with 20 μM baicalin with or without LPS (200 ng/ml) for 24 hr. (A) Endocytic activity was assessed by FITC-dextran treatment followed by flow cytometry. DCs were washed and stained with a PE-conjugated anti-$CD11c^+$ antibody. The endocytic activity of the controls was determined after exposure to FITC-dextran at 4℃. The number represents the percentages of FITC-dextran/$CD11c^+$-PE double positive cells. (B) The profile of endocytic capacity displayed as a histogram using the percentages of FITC-dextran/$CD11c^+$-PE double positive cells. The results are representative of three separate experiments ($^{\#\#}P < 0.01$ vs. unstimulated DCs [medium only]; $^{**}P < 0.01$ *vs.* LPS-stimulated DCs).

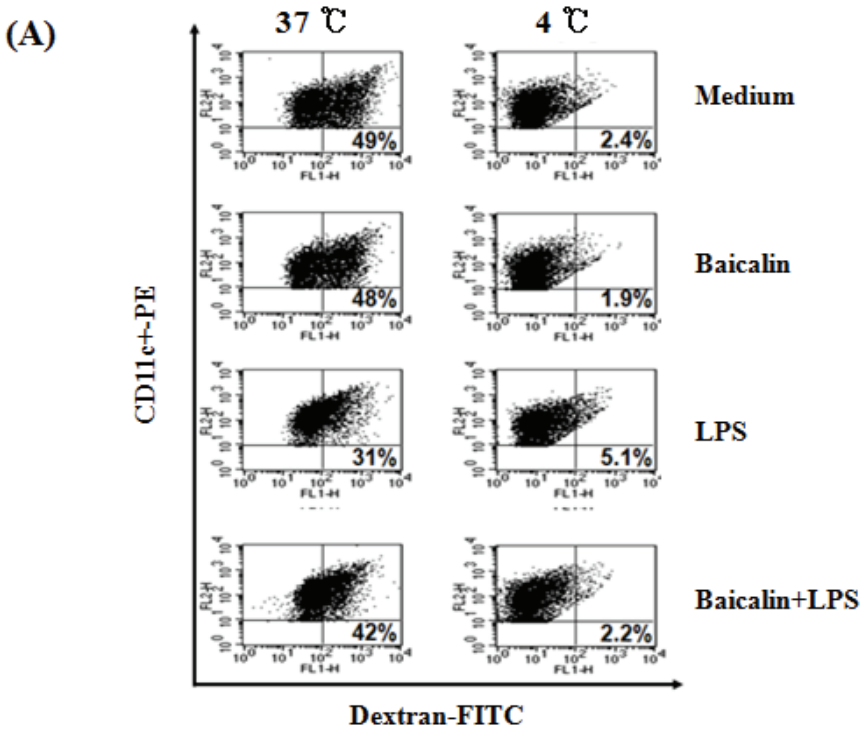


Fig. 22. (continued)

(4) Baicalin inhibits DC-mediated induction of Th1 polarization

To determine whether baicalin inhibits the maturation of DCs into fully functional APCs for allogeneic $CD4^+$ T cell stimulation, DCs were treated with baicalin for 24 hr and subsequently incubated with allogeneic $CD4^+$ T cells. As shown in Fig. 23, $CD4^+$ T cells exposed to LPS-treated DCs exhibited greater proliferative responses than those incubated with untreated DCs, whereas baicalin appeared to inhibit these proliferative responses. Therefore, while LPS-induced maturation profoundly promoted the allostimulatory capacity of DCs, baicalin treatment significantly impaired this response.

In addition, because baicalin was observed to inhibit the production of IL-12, a Th1-inducing cytokine, it was next attempted to characterize the primary T cell response induced by DCs that were matured in the presence of baicalin. Naïve allogeneic $CD4^+$ T cells primed by mDCs differentiated into Th1 lymphocytes, which generated high levels of IFN-γ and low levels of IL-4. In contrast, $CD4^+$ T cells primed with DCs that had matured in the presence of baicalin displayed inhibited IFN-γ and IL-2 production (Fig. 23B). These results suggest that the effect of baicalin on the T cell differentiating abilities of DCs is primarily due to an inhibition. of IL-12 production.

(A)

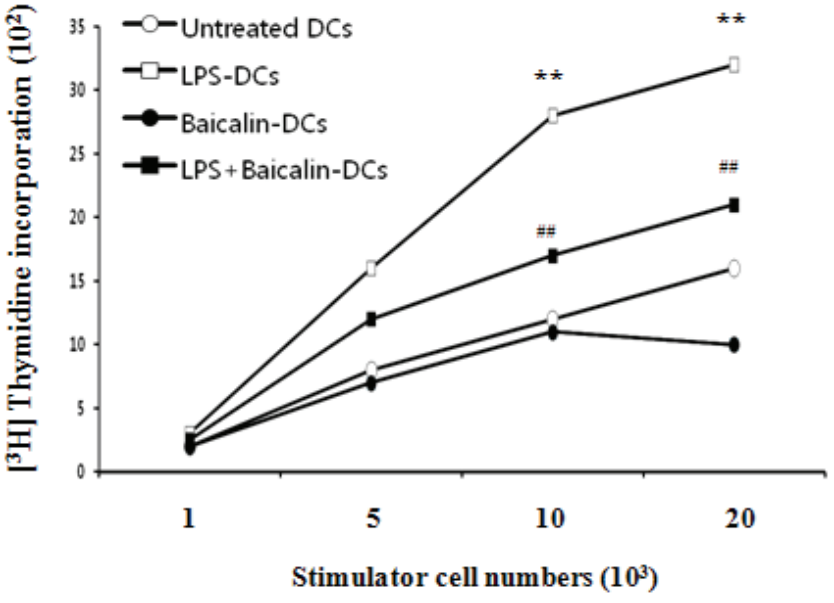


Fig. 23. Baicalin impairs the Th1 response and cytokine production. DCs were incubated for 24 hr in media alone or media containing baicalin (20 μM), LPS (200 ng/ml), or LPS with baicalin. These cells were washed and co-cultured with allogenic $CD4^+$ T cells, and the mixed leukocyte reaction was incubated for 4 days. (A) Background levels of [^{3}H]Thymidine uptake were determined by measuring reactions without stimulus. (B) The cells were examined for cytokine production *via* ELISA after 48 hr. The results shown are from one representative experiment out of three ($^{\#\#}P < 0.01$ *vs.* unstimulated DCs [medium only]; $^{**}P < 0.01$ vs. LPS-stimulated DCs).

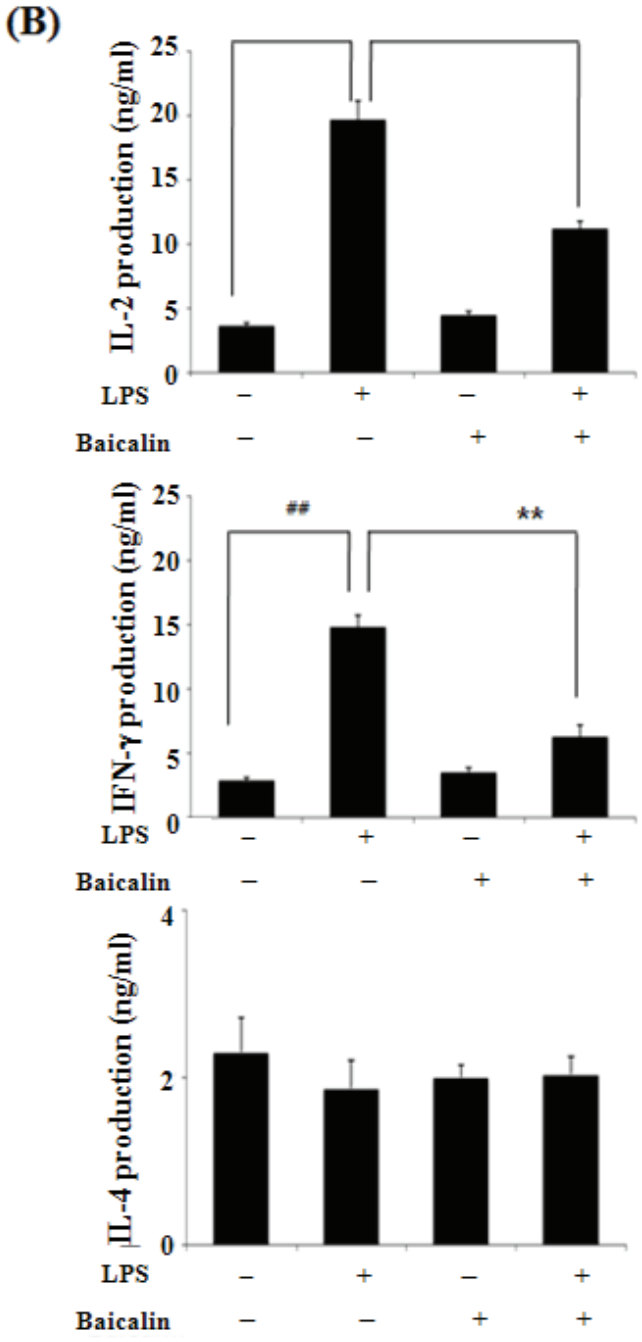


Fig. 23. (continued)

c. Discussion

This study investigated the effects of baicalin on the phenotypic and functional maturation of bone marrow-derived DCs. DCs are known to play important roles in immunotherapy and the establishment of hypersensitivity and transplantation tolerance (Bianco, Kim et al. 2009; Aspord, Leccia et al. 2012; Land 2012; Palucka and Banchereau 2012). Baicalin is a major flavonoid constituent of *S. baicalensis* Georgi, a traditional oriental medicinal herb known as Baikal skull cap, and researchers have shown that it exerts a variety of biological effects, including anti-inflammatory and anti-cancer activities (Ikezoe, Chen et al. 2001; Franek, Zhou et al. 2005; Lin, Wu et al. 2010; Zhu, Wang et al. 2012). Nevertheless, there are few published reports investigating the mechanism underlying the immunoregulatory effect of baicalin. Zhang H *et al.* have reported that baicalin induces DC apoptosis *in vitro* at concentrations of 50 μM (Zhang, Jiao et al. 2011). However, in our study, However, there were no marked differences in the percentage of dead cells when the concentrations were all set to ≤ 30 μM. These discrepancies may also be due to different sources of baicalin and/or different cell culture systems. Some studies have shown that baicalin acts through toll-like receptor 4 (TLR4) to modulate T and B cells and promote regulatory T cell differentiation (Gong, Sun et al. 2011; Yang, Yang et al. 2012). However, the exact pathways that are modulated in response to baicalin to alter the function of DCs, which are potent APCs that interact with T cells for cell-mediated immune responses, are still unclear. Moreover, the effects of baicalin on the maturation and immunological response of DCs remain largely unknown. Therefore, a variety of functional assays were used to ascertain the function and

phenotype of baicalin-treated DCs and to determine how baicalin influences the DC-mediated immune response in terms of Th1 or Th2 polarization. To be sure the observed effects of baicalin could be attributed to DCs and not to contaminating cells in the bone marrow-derived cell cultures, the DCs were purified at greater than 95%. Our results indicate that baicalin is a potent inhibitor of LPS-induced DC maturation that impairs the Th1 immune response and provide new insights into the immunopharmacological potential of baicalin.

During maturation, DCs up-regulate co-stimulatory molecules, such as CD80, CD86, and MHC class I and II, allowing for effective antigen presentation to naïve T cells. Moreover, mDCs produce and secrete specific cytokines to activate innate effector cells and direct the development and differentiation of specific T helper cell subsets. Although it was previously reported by Zhang *et al.* that baicalin treatment had no effect on expression levels of CD80 and CD86 in LPS (500 ng/ml)-stimulated DCs (Zhang, Jiao et al. 2011). It was found that treatment of DCs with 20 μM baicalin in the presence of LPS (200 ng/ml) impaired the expression of the CD80 and CD86, as well as MHC class I and II molecules, indicating that baicalin has the ability to inhibit LPS-induced DC maturation. This suppressive effect of baicalin on DC maturation can likely be attributed to a non-specific inhibitory effect, as it was observed that the endocytic capacity of baicalin-treated DCs, as measured by uptake of FITC-dextran *via* mannose receptor-mediated endocytosis, was profoundly elevated. This demonstrates that baicalin can suppress both the phenotypic and functional maturation of DCs.

Polarization of the helper T cell-mediated immune response toward Th1 or Th2 differentiation depends on specific cytokines secreted by activated DCs. High levels of IL-12 will induce differentiation of naïve $CD4^+$ T cells into Th1 cells and simultaneously block the development of the Th2 lineage (Sanecka, Ansems et al. 2011). The secretion of bioactive IL-12p70 requires the coordinated expression of two of its subunits, namely p35 and p40, which are encoded by two separate genes and are regulated independently. Here, it was observed that DCs stimulated with LPS displayed enhanced IL-12 production and secreted the bioactive form IL-12p70, while baicalin exerted inhibitory effects on the production of intracellular IL-12p40/p70 and bioactive IL-12p70. In contrast, baicalin and LPS had no effect on IL-10 production in DCs. Further, although LPS-stimulated DCs skewed naïve $CD4^+$ T cells toward developing into IFN-γ-producing T cells for Th1 polarization, naïve $CD4^+$ T cells stimulated by DCs treated with both LPS and baicalin generated lower levels of IFN-γ; IL-4 production was unaffected. Consequently, baicalin significantly impaired the capacity of these cells to proliferate and initiate the Th1 response. This suggests that baicalin is a potent immune-modulator of the Th1 response, and the inhibition of DC-mediated Th1 polarization might constitute a baicalin-associated immunosuppressive mechanism. Our results further suggest that the baicalin-mediated inhibition of IL-12 production in LPS-stimulated DCs may also contribute to the induction of an immunosuppressive state.

In conclusion, this study demonstrated a variety of baicalin-mediated effects on DCs. Specifically, baicalin inhibited DC maturation and pro-inflammatory

cytokine production, resulting in the inhibition of T cell activation. Our current data also show that baicalin affected the capability of DCs to determine Th1/Th2 polarization through the regulation of IL-12 and IFN-γ production. These findings help to elucidate the immunopharmacological functions of baicalin and suggest this may act as a highly effective means for modulating the immunoregulatory capacity of DCs. Taken together, our results suggest new implications for manipulating DC function for potential immunotherapeutic applications.

Part II. Genetic regulation of adaptive immunity

1. CD30 deficiency impairs anti-tumor activity through $CD4^+$ T cell responses

a. Introduction

CD30 is a 120 kDa glycosylated type I transmembrane protein originally identified on Reed-Sternberg cells in Hodgkin's lymphoma. Expression of this protein has also been reported on non-Hodgkin's lymphomas, neoplastic cells, and normal cells, and it is most commonly expressed on lymphoid cells (Schwab, Stein et al. 1982; Stein, Mason et al. 1985). CD30 is a co-stimulatory molecule for T cells and is a member of the tumor necrosis factor (TNF) receptor (TNFR) superfamily (TNFRSF). It binds to CD30L, which is also called CD153 or TNFSF8. TNFRSF members are involved in many signal transduction pathways that are are meditated primarily through interaction with TNF receptor-associated (TRAF) proteins, and can induce the activation of NF-κB (Baker and Reddy 1996; Bazzoni and Beutler 1996). CD30 can interact with TRAF1, 2, 3, and 5, and these proteins are subdivided into two groups dependent on their binding domain. TRAF1, 2 and 3 bind to the C-terminal 42 amino acids of CD30, and TRAF2, which is major factor for CD30-mediated NF-κB [A62], also binds to D2 domain. TRAF5 is another major signal transduction factor involved in CD30-dependent NF-κB activation, and it binds to the D3 domain.

Although CD30 is a major marker of for lymphomas, it has also been reported to act in pro-Th2 signaling in non-lymphoma cells, including allergy and virus infected cells (Leonard, Tormey et al. 1997; Bengtsson 2001). In the case of tumor immunity, the goal is to induce effector T cells directed against the cancer cells, which will suppress expansion of the malignant cell and eliminate the tumor. DCs are powerful APCs that have a critical role in the initiation and regulation of tumor-specific immunity (Waldmann 2003). These cells capture self or non-self antigens and present the processed antigen peptide on MHC class I-peptide complexes. Effector T cells, such as cytotoxic T lymphocytes (CTLs), recognize peptide-MHC class I complexes on cancer cells with the T cell receptor (TCR), and destroy them with granzyme B and perforin (Steinman and Swanson 1995; Dieli, Gebbia et al. 2003; Finn 2008). It is not known if CD30 can suppress $CD4^+$ T cells responses and/or inhibit $CD4^+$ T cell-mediated tumor immunity on non-lymphoid tumor metastases. Therefore, the present study investigated the role of CD30 on $CD4^+$ T cells using a tumor metastasis model in $CD30^{-/-}$ mice.

b. Results

(1) Mice with CD30 deficiency have normal immune cells populations

To investigate the immunological role of CD30 in normal T cell activity, $CD30^{-/-}$ mice on a C57BL/6 (Black six, B6) background, which is previously described by Amakawa *et al.*(Amakawa, Hakem et al. 1996), and normal B6 mice were used as mutant and wild-type (WT). The mice were utilized in a tumor metastasis model. Initially, the expression of the *Cd30* gene in mutant and WT mice was determined by PCR genotyping of tail tissue, and it was confirmed that *Cd30* was completely deleted in $CD30^{-/-}$ mice (Fig. 24). And then, the population and development of naïve immune cells in spleen was ascertained by monoclonal antibodies against CD4, CD8 and B220, to identify T and B lymphocytes, and flow cytometry analysis (Fig. 25A). Additionally, mAbs directed against CD11c, F4/80, and Ly6G were used for the detection of myeloid cells, including DCs, macrophages, and neutrophils (Fig. 25B). CD62L was used as an activation marker for immune cells, and $CD62L^{low}$ cells were considered to be in the activated state. Generally, it was observed that lymphoid cells were predominant in the spleen, as compared with myeloid cells, and $CD30^{-/-}$ mice did not show differences in either the total number of each cell type or the expression of CD62L, as compared to WT. This result indicates that the lack of CD30 does not change the development of immune cell populations and their activation.

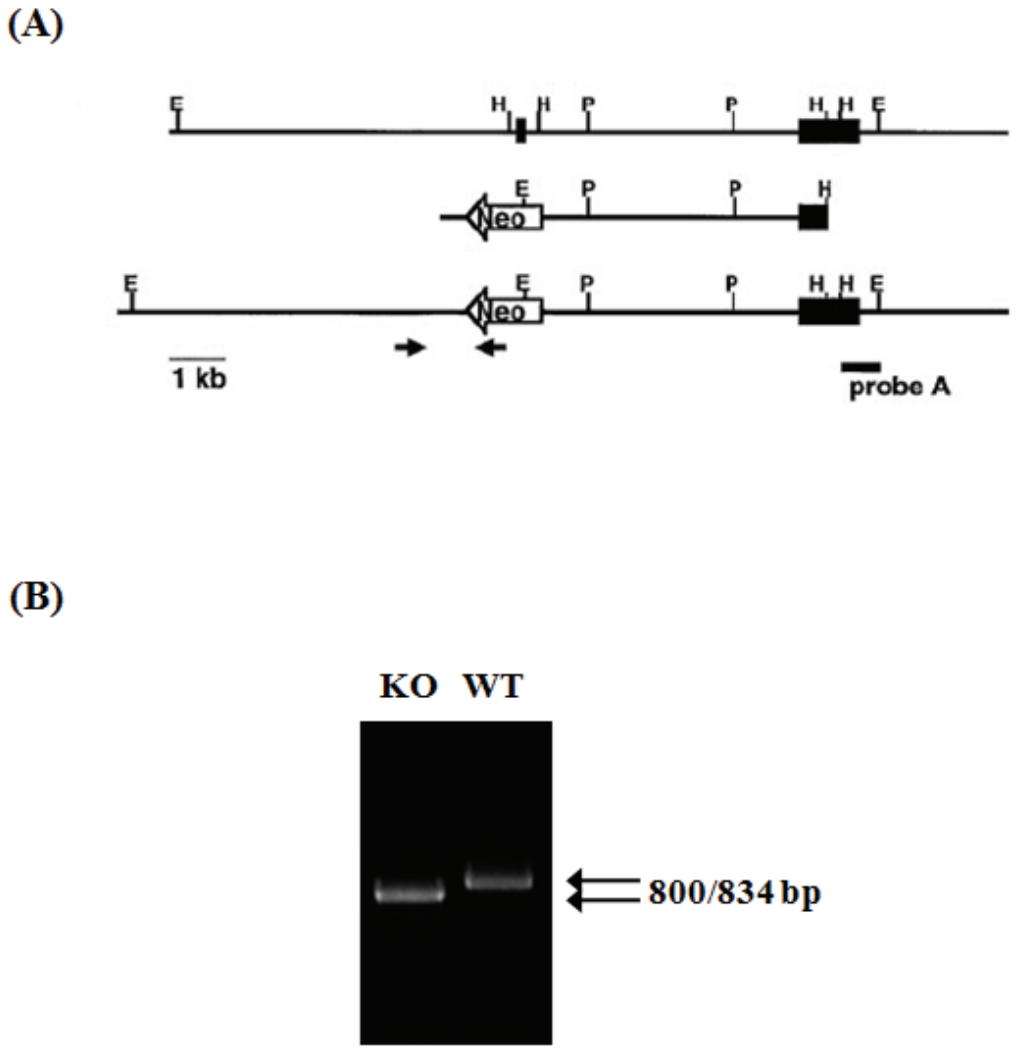


Fig. 24. Genotype analysis of CD30−deficient mice by PCR. (A) Genetic mapping of the partial CD30 locus, the targeting construct, and the targeted locus (Amakawa, Hakem et al. 1996). (B) Genotype analysis of WT and $CD30^{-/-}$ mice. PCR was used to classify the CD30 genotypes; KO designates the CD30 knockout PCR product and WT indicates the WT CD30 allele.

Fig. 25. Lack of CD30 has no effect on development and numbers of immune cells in the spleen. The population and development of immune cells were stained with lineage markers and CD62L as activation marker. Cells were sorted into six types of immune cells: (A) lymphoid cells, including $CD4^+$ T cells, $CD8^+$ T cells, and B cells (B) and myeloid cells, including macrophages, DCs, and neutrophils, using lineage-specific markers. Cells were then classified as activated ($CD62L^{low}$) or inactivated ($CD62L^{hi}$). The quadrants shown were set based on isotype control staining.

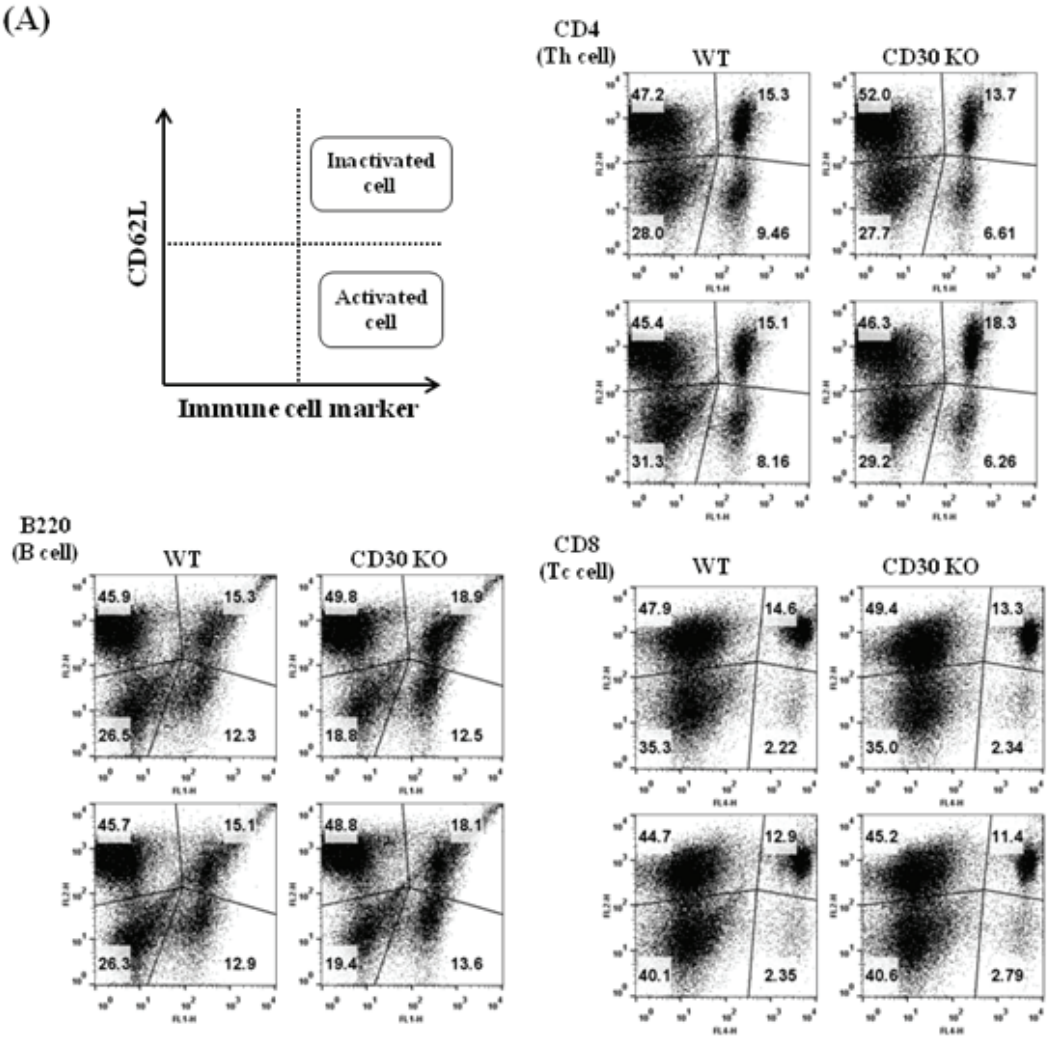


Fig. 25. (continued)

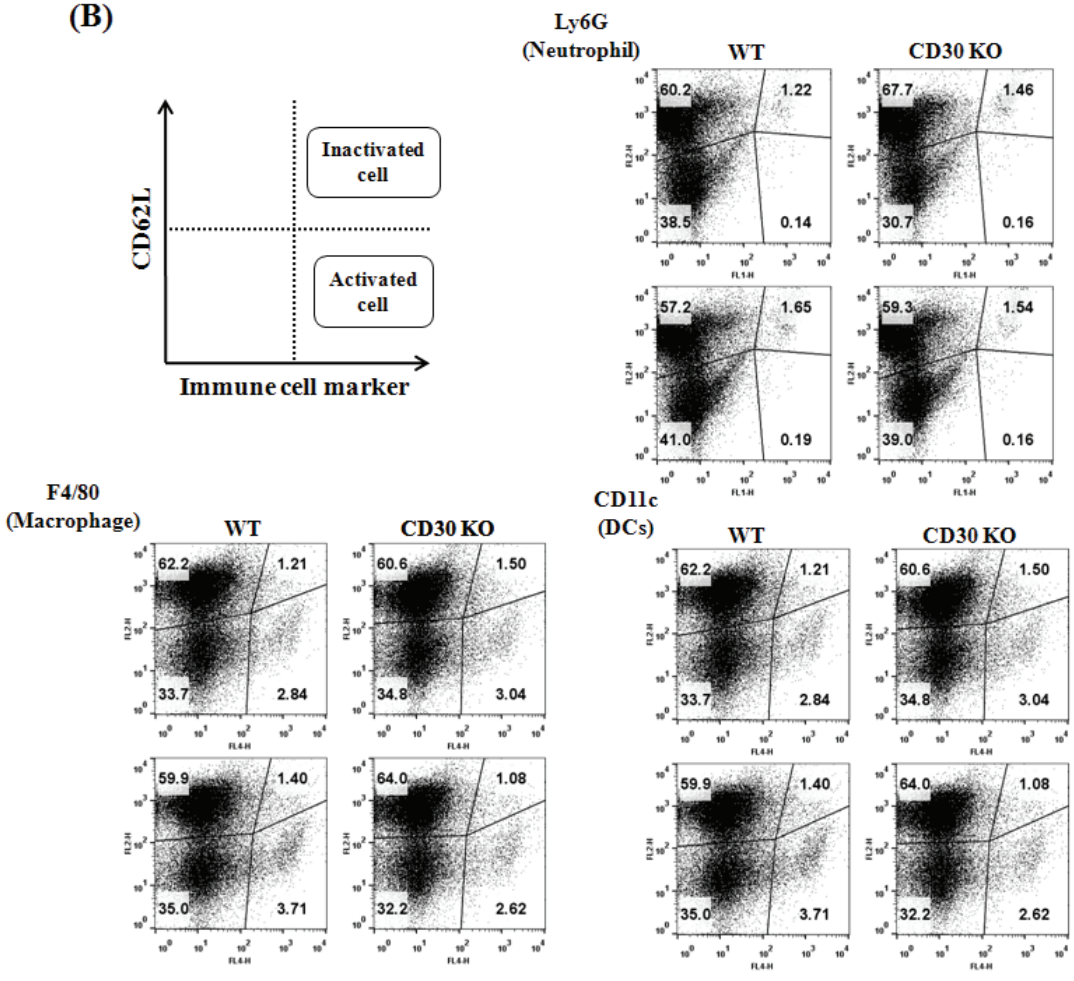


Fig. 25. (continued)

(2) CD30 deficiency results in spontaneous up-regulation of CD80 and MHC class II

It has previously been reported that although CD30 is not expressed on monocytes and DCs, CD30L (CD153) is expressed on activated monocyte/macrophages (Horie and Watanabe 1998). However, the role of CD30 in DC activation and/or maturation remains unknown. In order to determine whether CD30 plays a role in DC maturation by LPS stimuli, monocytes were isolated from murine bone marrow and differentiated into iDCs for 6 days. Under normal conditions, LPS-stimulated DCs show increased co-stimulatory molecule expression, and in accordance with this, it was observe that DCs matured in the presence of LPS for 24 hr, show elevated expression of MHC classes I and II, CD80, and CD86, as measured by flow cytometry (Fig. 26). CD30 deficient DCs were also activated by LPS treatment and similarly showed increased expression of these proteins (Fig. 26). However, cells with a CD30 deficiency displayed slight spontaneous increases in CD80 and MHC class II expression. Although these data indicate that lack of CD30 does not influence on DC maturation, it does not absolutely exclude the possibility that the spontaneous increase in CD80 and MHC class II expression does not influence DC function.

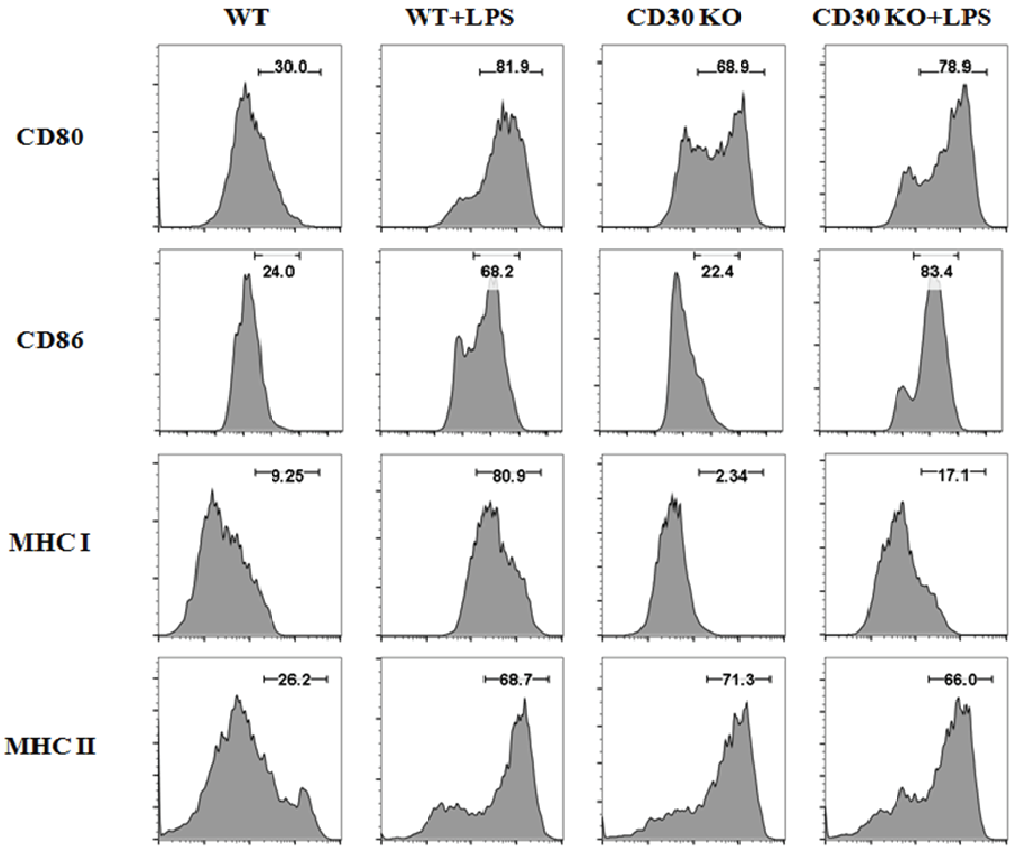


Fig. 26. The effect of CD30 deficiency on expression of co-stimulatory molecules in DC maturation. DCs were harvested and analyzed using two-color flow cytometry. The cells were gated on $CD11c^{+}$. DCs were stimulated 200 ng/ml LPS for 24 hr, and surface molecule expression was analyzed. The histogram shows the expression of surface molecules, and the numbers indicate MFI.

(3) CD30 deficiency impairs $CD4^+$ T cell proliferation and activation

In previous studies, CD30 has been found to be primarily expressed in resting lymphoid cells, including $CD4^+$ and $CD8^+$ T cells, which increase in number when activated (Manetti, Annunziato et al. 1994). Therefore, it was investigated whether CD30 deficiency impairs $CD4^+$ T cell activation and cytokine production. Splenocytes were isolated from $CD30^{-/-}$ and WT mice and stimulated with 1 μg/ml phorbol 12−myristate 13−acetate (PMA) for 5 days. These were stained with CD4 and CD62L antibodies in order to distinguish activated $CD4^+$ T cells ($CD4^+CD62L^-$) from inactivated $CD4^+$ T cells ($CD4^+CD62L^+$). It was found that both $CD4^+CD62L^-$ and $CD4^+CD62L^+$T cells from WT mice expand upon PMA treatment (Fig. 27), demonstrating that T cells of WT mice are normally activated by stimuli. However, neither activated nor inactivated $CD4^+$ T cells from CD30 deficient mice were increased by PMA stimulation. Interestingly, unlike WT $CD4^+$ T cells, CD30 deficient $CD4^+$ T cells did not secrete elevated levels of IFN−γ in response to PMA stimulation. These data indicate that the lack of CD30 results in reduced IFN−γ production from activated $CD4^+$T cells, as compared to WT $CD4^+$ T cells, and suggest that CD30 likely affects the Th1 or CTL immune response.

Fig. 27. Analysis of $CD4^+$ T cell activation in cells from CD30 deficient mice. PMA-stimulated T cells from the spleen of WT and $CD30^{-/-}$ mice were analyzed for activation and cytokine production. Percentages of CD62L or IFN-γ^+ cells are shown in representative dot plots from flow cytometry analysis.

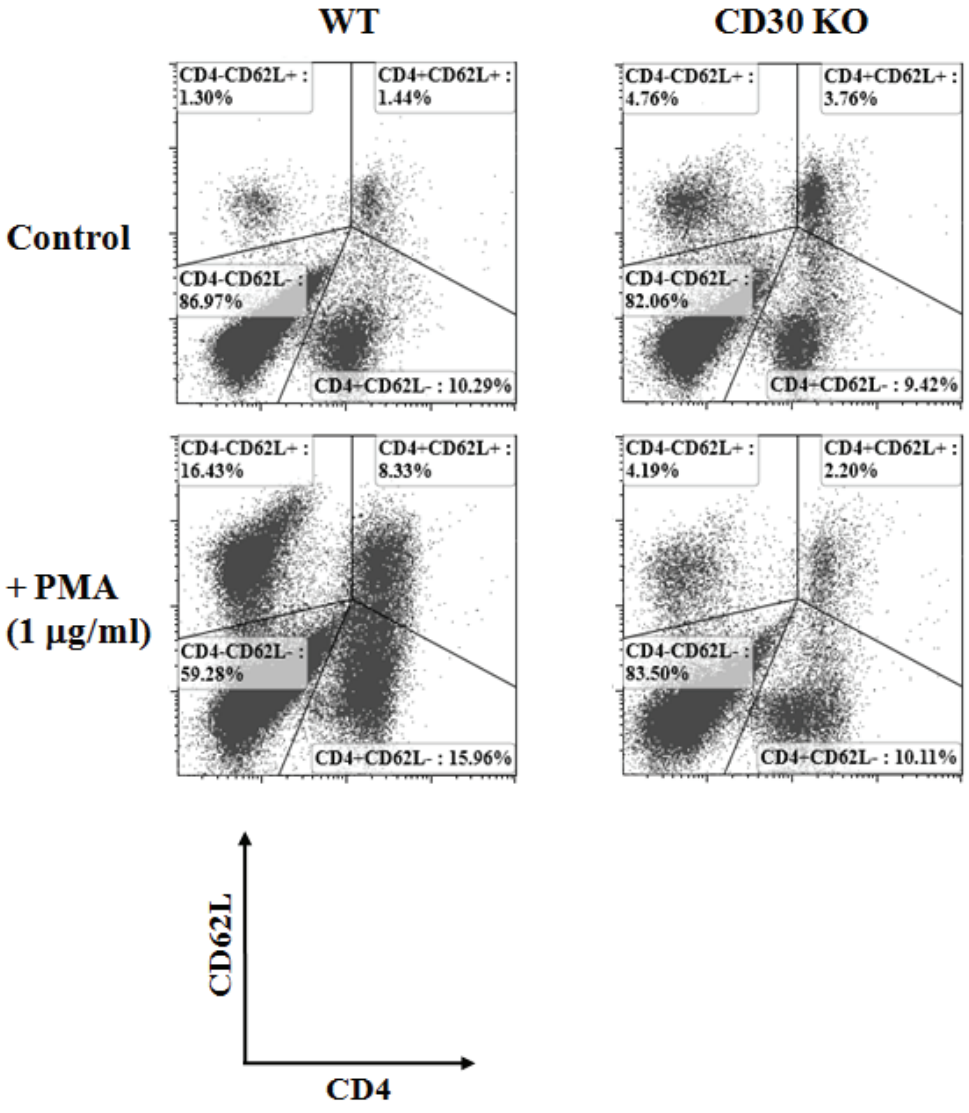


Fig. 27. (continued)

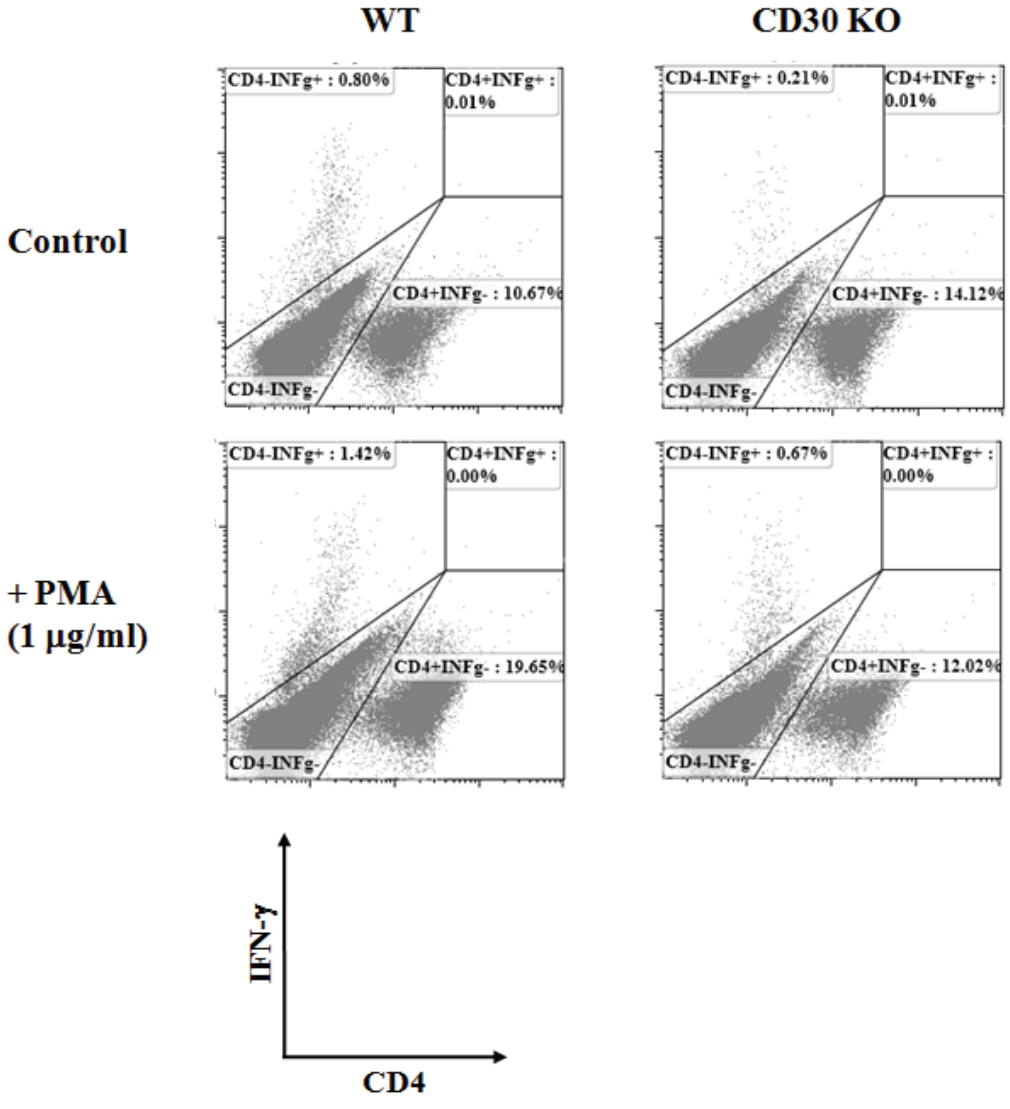


Fig. 27. (continued)

(4) CD30 deficiency is vulnerable to cancer metastasis

$CD4^+$ T cells, also called helper T cells, are essential for the adaptive immune response. These can differentiate into different subsets, including Th1 and Th2 cells, which induce CTL and B cell activation, respectively. Therefore, to investigate the impairment of T cell immunological function in $CD30^{-/-}$ mice *in vivo,* survival study was performed using the MC−38 (murine colon cancer cells) tail vein tumor metastasis model. It was found that CD30 deficient mice had a significantly higher death rate than WT mice, consistent with the T cell activation results (Fig. 28). These data suggest that lack of CD30 increases susceptibility to tumor challenge *via* impairment of T cell activation in mice.

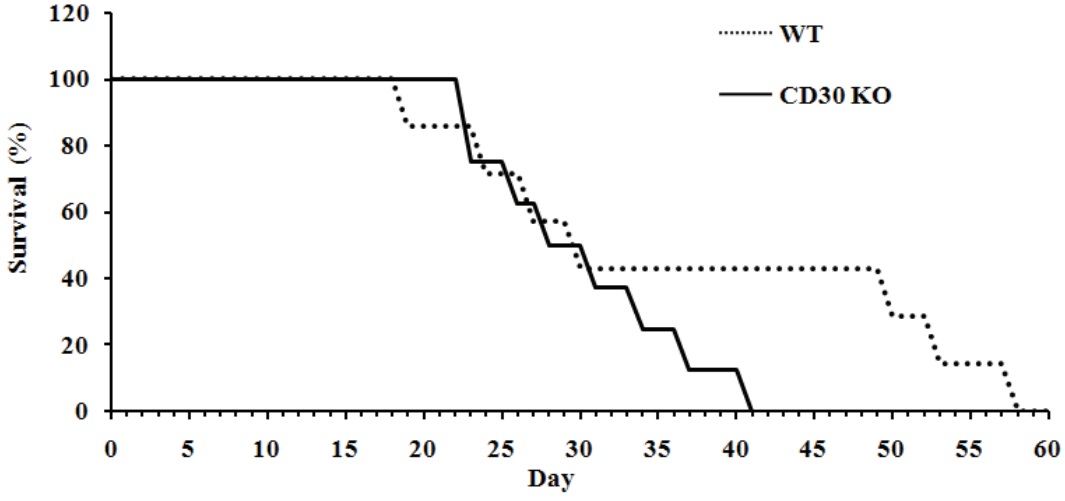


Fig. 28. CD30 knockout (KO) mice display decreased survival rate after MC-38 challenge. Survival of mice injected with MC-38 cells (2×10^6/mice) in the tail vein (n = 10) is shown. Data are representative of two separate experiments.

c. Discussion

The present study revealed that CD30 deficiency promotes metastatic tumor growth in an *in vivo* cancer model, likely *via* inhibition of $CD4^+$ T cells. Although a number of previous studies have shown that CD30 is the major marker of Reed-Sternberg cells of Hodgkin's disease, as well as some other lymphomas, it has also been considered as a potential Th2-specific marker (Manetti, Annunziato et al. 1994; Leonard, Tormey et al. 1997). However, the precise immune-regulatory functional role of CD30 in regards to activation of $CD4^+$ T cells was not fully understood. Here, it was shown that CD30 deficiency significantly suppressed PMA-stimulated $CD4^+$ T cell activation *in vitro* , and in mice, lack of CD30 resulted in a significant impairment of tumor immunity to lung metastasis in the MC-38 tumor model.

T cells play a critical role in immunity by initiating and amplifying the adaptive immune response. In addition, these cells play a critical role in DC-based cancer immunotherapy by directly, or indirectly, killing and destroying cancer cells (Steinman and Swanson 1995; Dieli, Gebbia et al. 2003). The anti-tumor activity of T cells requires co-stimulatory molecules, which have crucial roles both in their activation and polarization, and determine their stimulatory/inhibitory fate (Barach, Lee et al. 2011; Abadi, Jeon et al. 2013). CD30 is one such co-stimulatory molecule that is mainly expressed on lymphoid cells, such as activated T cells, lymphomas, and infected B cells, and is thought to be involved in T cell activation *via* Th2 factors (Schwab, Stein et al. 1982; Stein, Mason et al. 1985; Leonard, Tormey et al. 1997). Therefore, it was sought to determine the

immune-regulatory functional role of CD30 as it relates to the adaptive immune response and anti-tumor responses using CD30 deficient mice.

CD30 is not expressed on monocytes, macrophages, and DCs, and here it is shown that CD30 deficiency did not affect the population of CD4, CD8, B220, Ly6G, CD11c, and F4/80 positive cells on isolated splenocytes from $CD30^{-/-}$ mice. Moreover, expression of CD80, CD86, and MHC class I and II on DCs were also not affected by CD30 deficiency. These results are in agreement with previous reports suggesting that CD30 has no effect on the population of lymphoid and myeloid lineage cells and is not involved in LPS-stimulated DC maturation. In contrast, CD30 is mainly expressed on lymphoid cells, such as T cells, and it was found that PMA-activated $CD4^{+}$ T cells from CD30 deficient mice show dysfunction in both proliferation and activation, and in addition, produce less IFN-γ than identically treated cells from WT mice. This inhibition of T cell activation, proliferation, and cytokine production suggests that lack of CD30 might result in weaker induction of Th1 responses in adaptive immunity. Both Th1 and Th2 responses are essential for the DC-based tumor immunity, and MC-38 challenged tumor metastasis model shows that CD30 deficient mice are vulnerable to tumor metastasis and display lower survival rate, as compared with WT mice. Taken together, these data suggest that CD30 deficiency leads to tumor growth susceptibility *via* suppression of the Th1 response, and thus, this protein is important in the regulation of anti-tumor immunity.

V. CONCLUSION

The goal of this present study was to investigate potential immune adjuvants and genetic modulators of immune responses. Immunity is divided into two primary types, innate and adaptive immunity. The present study focused on the interaction between APCs and T cells, which is a critical step in the regulation adaptive immunity. In innate immunity, macrophages engulf the foreign pathogen by phagocytosis. These activated macrophages increase NO production, a marker of inflammation, and secrete various inflammatory factors, including IL-1β, IL-6, and TNF-α. The inflammatory response contributes to recovery of the injured/infected regions in peripheral tissues and could be resolved by homeostasis. However, if inflammatory responses are not resolved, chronic inflammation may develop, and this is thought to be an underlying cause for several diseases, such as cancer, cardiovascular disease, diabetes, and various autoimmune diseases. During adaptive immunity, DCs uptake self-antigen (tumor or normal tissue) or non self-antigen (foreign pathogen) and present the antigen peptide on MHC class I or II molecules on the cell surface. This provides information of the relevant antigens and initiates antigen-specific adaptive immunity *via* interaction with T cells. Under normal conditions, T cells receive antigen information from DCs and become polarized into Th1 or Th2 cells. These act as effector T cells, subsequently attacking the infected peripheral tissues or cancer cells, depending on antigen-specific information. Cancer cells, however,

continuously try to escape the anti-tumor immunity through immune suppressive factors, including TGF-β, VEGF, and myeloid derived suppressor cells (MDSCs). In contrast, T cells obtaining normal self antigens from DCs could attack normal peripheral tissues and induce several autoimmune diseases, such as allergic disease, rheumatoid arthritis, and diabetes. Therefore, it is critical to maintain the balance of adaptive immune responses, depending on pathophysiological conditions.

In part I of this study, it was investigated whether various phytochemicals, including ESH, galangin, baicalin, and β-GA can have an effect on macrophages and/or DCs. It was found that treatment of LPS-stimulated murine macrophages with ESH and galangin reduced inflammatory responses *via* decreases in NO and inflammatory cytokines. Baicalin and β-GA suppressed DC maturation by affecting expression of co-stimulatory molecules, antigen uptake, and cytokine production, resulting in an impairment of Th1 immune responses in LPS-stimulated DCs.

In the part II, the present study identified a genetic modulator of adaptive immune responses. Loss of CD30, a co-stimulatory molecule, did not affect immune cell populations in the spleen of CD30 deficient mice, as compared with WT mice. However, CD30 deficiency spontaneously induced CD80 and MHC class II expression and impaired activation of $CD4^+$ T cells. As a likely result of this, CD30 deficiency resulted in an impairment of anti-tumor immunity and decreased survival in a murine tumor metastasis model.

In conclusion, it was found that ESH, galangin, baicalin, β-GA, and the CD30 molecule could regulate immune responses *via* modulation of specific immune cells, such as macrophages, DCs, and T cells. These results suggest that these chemical and genetic modulators might function as effective adjuvants in immunological therapeutics.

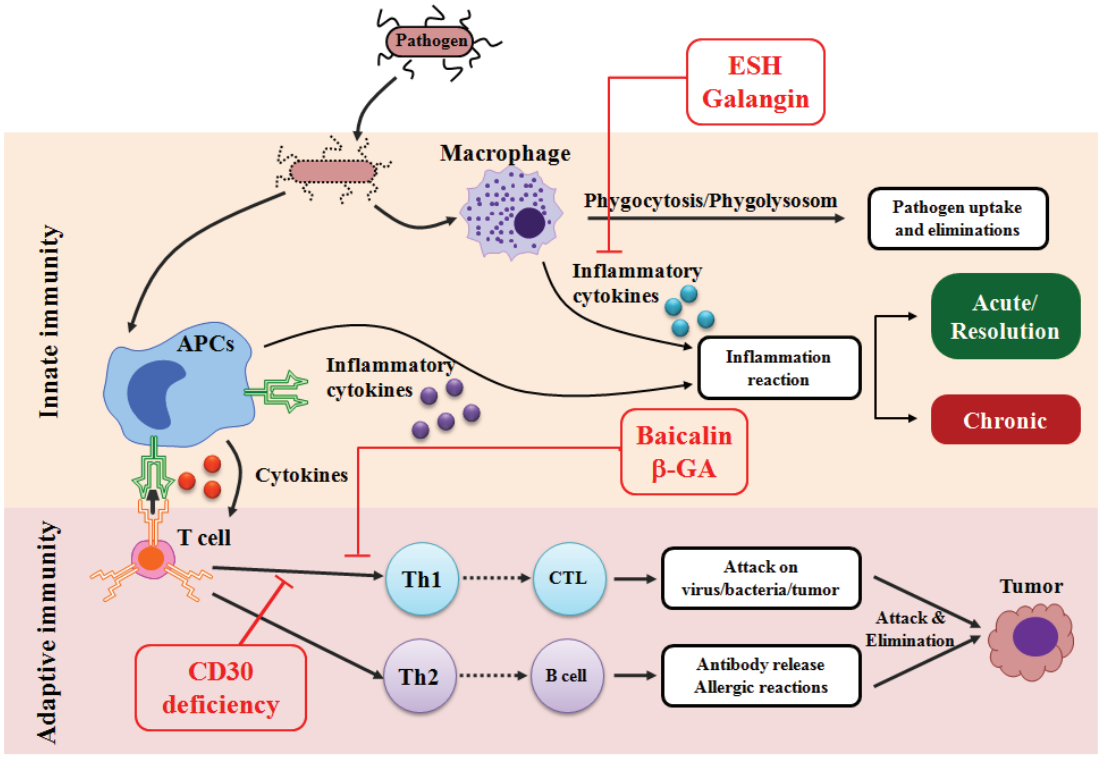


Fig. 29. A diagram for summary of the present study.

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