2020 년 2 월
석 사 학 위 논 문

Functional Study on Microglia and Dendritic Cells in Neuroinflammation and Oxidative stress

조선대학교 대학원

생명과학과

조 준 휘

Functional Study on Microglia and Dendritic Cells in Neuroinflammation and Oxidative stress

신경 염증 및 산화 스트레스에서 미세아교세포와
수지상세포에 대한 기능 연구

2020 년 2 월 25 일

조선대학교 대학원

생명과학과

조 준 휘

Functional Study on Microglia and Dendritic Cells in Neuroinflammation and Oxidative stress

지도교수 이 준 식

이 논문을 이학석사학위 신청 논문으로 제출함

2019 년 10 월

조선대학교 대학원

생명과학과

조 준 휘

조준휘의 석사학위논문을 인준함

위원장 조선대학교 교 수 전 택 중 (인)

위 원 조선대학교 교 수 이 준 식 (인)

위 원 조선대학교 교 수 조 광 원 (인)

2019 년 11 월

조선대학교 대학원

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Ag	Antigen
APCs	Antigen presenting cells
BBB	Blood-brain barrier
CNS	Central nervous system
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 kinase
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
iDCs	Immature DCs
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IKK	IκB kinase
JNK	c-JUN N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase

MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium
mDCs	Mature DCs
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMP	Mitochondrial membrane potential
NAC	N-Acetyl-L-Cysteine
NF-κB	Nuclear factor-kappa-B
NO	Nitric oxide
PBS	Phosphate-buffered saline
PI	Propidium iodide
PE	Phycoerythrin
PVDF	Polyvinylidene difluoride
rm	Recombinant murine
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TCR	T cell receptor
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WT	Wild-type

ABSTRACT

Functional Study on Microglia and Dendritic Cells in Neuroinflammation and Oxidative stress

Cho, Jun Hwi

Advisor : Prof. Lee, Jun Sik, Ph.D.

Department of Life Science,

Graduate School of Chosun University

Antigen presenting cells (APCs) are responsible for recognizing and presenting information on external antigens and pathogens. These cells are known to play a role in linking innate and adaptive immune responses. APCs can transmit external antigens to T cells and can initiate or regulate immune responses. In addition, APCs corresponding to each tissue site has a different immunological role and function. Therefore, in present study, I studied the immunological function of microglia and dendritic cells (DCs) as immune regulators in neuroinflammation and oxidative stress.

In the part I, *Sargassum horneri* (Turner) C. Agardh is a brown algae species that exerts anti-inflammatory activity toward murine macrophages. However, the anti-neuroinflammatory effects and the mechanism of *S. horneri* (Turner) C. Agardh extract on microglia cells are still unknown. I investigated the anti-neuroinflammatory effects of

S. horneri extract on BV-2 microglia. *S. horneri* extract cytotoxicity and nitric oxide (NO) production were measured by Annexin V/PI staining and the Griess assay, respectively. mRNA expression level of pro-inflammatory factors were determined by reverse transcription-polymerase chain reaction (RT-PCR), and cytokine production was evaluated by enzyme-linked immunosorbent assays (ELISAs). Protein expression level of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) were detected by western blot analysis. *S. horneri* extract was not cytotoxic to BV-2, and it significantly decreased lipopolysaccharide (LPS)-induced NO production. Moreover, *S. horneri* extract treatment reduced mRNA level of pro-inflammatory cytokines including inducible NO synthase, cyclooxygenase-2, interleukins (ILs)-1β and -6, and tumor necrosis factor-α in LPS-stimulated microglia cells in a dose-dependent manner. *S. horneri* extract also diminished the protein expression of iNOS, COX-2, and cytokines including TNF-α and IL-6. *S. horneri* extract elicited anti-neuroinflammatory effects by inhibiting phosphorylation of extracellular signal-regulated kinase (ERK), p38, and NF-κB-p65. These results suggested that *S. horneri* extract exerted anti-neuroinflammatory effects on LPS-stimulated microglia cell activation by inhibiting MAPKs phosphorylation and NF-κB signaling.

In part II, I examined whether the function of DCs could be regulated by redox status regulation. Oxidative stress induced age-related disorders by cellular dysfunctions, DNA damage and expression of senescence-related factors. In addition, immune senescence also shows decreased immunological functions and imbalance of adaptive immune responses. Therefore, I studied the immunological function of DCs in response to oxidative stress. As shown in the results, the expression of co-stimulatory molecules in DCs was increased by low concentration of oxidative stress. However, high

concentration of oxidative stress was applied to DCs, the expression of co-stimulatory molecules decreased and cytotoxicity was also observed.

In addition, oxidative stress induced the production of ROS in DCs, but decreased antigen uptake capacity. IL-12 production of DCs is an important marker of DCs activation and involved in the T cell priming as well as the polarization of Th1/Th2. As a result of measuring the amount of IL-12 produced by DCs in the oxidative stress state, it was observed that IL-12 production was reduced by LPS stimulation. These results are attributed to cytotoxicity, as oxidative stress impairs mitochondrial membrane potential (MMP, Ψm) in DCs, increasing Bax expression and cleaved caspase-3/-9 expression. For reason of this, I determined whether oxidative stress regulation restored the function of DCs. As a result, NAC (N-Acetyl-L-Cysteine) treatment restored the oxidative stress-induced cytotoxicity, ROS production, and MMP. Also, NAC treatment rehabilitated the co-stimulatory molecules expression of DCs in oxidative stress.

In conclusion, the regulation of microglia is important for the regulation of neuroinflammation, and the damage of DCs function caused by oxidative stress was observed to be restored by antioxidant factors.

국문초록

신경 염증 및 산화스트레스에서 미세아교세포와 수지상세포의 기능 연구

조준휘

지도교수 : 이준식

생명과학과

조선대학교 대학원

항원제시세포 (APC)는 외부 항원 및 병원체에 대한 정보를 인식하고 제시하는 역할을 하는 세포이다. 이러한 APC는 선천성 면역 및 적응성 면역 반응을 연결해주는 역할을 하는 것으로 알려져 있다. APC는 외부 항원을 T 세포에게 전달할 수 있고 면역 반응을 개시 또는 조절할 수 있다. 또한, 각 조직 부위에 상응하는 APC는 상이한 면역학적 역할 및 기능을 갖는다. 따라서, 본 연구에서는 신경 염증 및 산화 스트레스에서 면역 조절자로서 미세아교세포 (Microglia) 및 수지상세포 (Dendritic cells, DC)의 면역 기능을 연구 하였다.

괭생이 모자반은 쥐에서 유래된 대식세포에 대하여 항 염증 활성을 나타내는 갈조류로 알려져 있으나, 미세아교세포에 대한 괭생이 모자반 추출물 (*S. horneri* extract)의 항 신경염증 효과 및 메커니즘은 아직 연구되지 않았다. 따라서 나는 BV-2 미세아교세포에 대한 *S. horneri* extract의 항 신경

염증 효과를 연구하였다. *S. horneri* extract의 세포 독성 및 일산화질소 (NO)의 생성은 각각 Annexin V/PI staining 및 Griess assay를 통해 측정하였다. 전 염증성 인자들의 mRNA 발현 수준을 역전사-중합 효소 연쇄반응 (RT-PCR)에 의해 결정하고, 사이토카인 생성을 ELISA 기법에 의해 평가하였다. MAPKs와 NF-κB의 단백질들의 발현 수준은 western blot 분석에 의해 확인하였다. *S. horneri* extract는 BV-2 미세아교세포에 대한 세포 독성이 없었으며, 지질 다당체 (LPS)에 의한 NO의 생성을 현저하게 감소시켰다. 또한, *S. horneri* extract의 처리는 LPS로 자극된 미세아교세포에서 IL-1β, IL-6, iNOS, COX-2 및 TNF-α 등의 전 염증성 인자들의 mRNA 수준을 농도 의존적으로 감소시켰다. 또한 *S. horneri* extract는 iNOS와 COX-2의 단백질 발현 수준을 감소시켰고, TNF-α 및 IL-6를 포함한 사이토카인의 발현 또한 감소시켰다. *S. horneri* extract는 ERK, p38 및 p65의 인산화를 억제함으로써 항 신경 염증 효과를 이끌어 냈다. 이러한 결과는 *S. horneri* extract가 MAPKs 인산화 및 NF-κB의 신호전달을 억제함으로써 LPS로 자극된 미세아교세포의 활성화에 대해 항 신경 염증 효과를 가진다는 것을 시사한다.

Part II에서는 DC의 기능이 산화/환원 상태 조절에 의해 조절 될 수 있는지 여부를 조사하였다. 산화스트레스는 세포 기능 장애, DNA 손상 및 노화 관련 인자의 발현에 의해 노화 관련 장애를 유발 한다. 또한, 면역 노화는 면역기능을 감소시키고, 적응성 면역 반응의 불균형을 초래한다. 따라서 본 연구에서는 산화 스트레스에 의한 DC의 면역 기능 변화을 연구하였다. 그 결과, 저농도의 산화 스트레스 DC에서 공동 자극 분자의 발현이 증가되었으나, 고농도의 산화 스트레스 DC에서 공동 자극 분자의 발현이 감소하였고 세포 독성도 관찰되었다.

또한, 산화스트레스는 DC에서 ROS의 생성을 유도하였으나, 항원포식능을 감소시켰다. DC의 IL-12의 생성은 DC의 활성화의 중요한 지표이며 T 세포 반응 개시 뿐만 아니라 Th1/Th2의 극성화에 관여한다.

산화스트레스 상태에서 DC에 의해 생성된 IL-12의 양을 측정한 결과, LPS 자극에 의해 IL-12의 생성이 감소 된 것으로 관찰되었다. 산화스트레스가 DC에서 미토콘드리아 막 전위 (MMP, Ψm)를 손상시켜 Bax 발현 및 cleaved caspase-3/-9의 발현을 증가시키기 때문에 이러한 결과는 산화스트레에 의한 DC의 세포 독성이 미토콘드리아의 세포사멸 신호전달 경로에 의해 나타난다는 것을 알 수 있다. 따라서, 항산화 물질에 의한 산화스트레스 조절이 DC의 기능을 회복시키는지 여부를 확인하고자 NAC (N-Acetyl-L-Cysteine)를 처리하여 실험을 진행하였다. NAC 처리는 산화스트레스에 의한 세포 독성, ROS 생성 및 손상된 MMP (Ψm)를 회복시켰을 뿐만 아니라, NAC 처리는 산화스트레스에서 DC의 공동 보조 자극 분자들의 발현을 회복시켰다.

결론적으로, 미세아교세포의 조절은 신경 염증의 조절에 중요한 역할을 하며, 산화스트레스에 의해 야기된 DC 의 기능의 손상은 항 산화 인자에 의해 회복되는 것으로 관찰되었다.

I. INTRODUCTION

1. Immune response

The immune system is traditionally divided into innate and adaptive components, each with different roles and functions (Medzhitov & Janeway Jr, 2000). In biology, immune response is the balanced state of various microcellular or multicellular organisms having suitable biological defenses to correspond disease, antigen, infection, or biological invasion (Sompayrac, 2019).

A) Innate immune response

The innate immunity, also known as innate immune system is the nonspecific immune response. It plays role of primary immune response and include of various immune cells which protect the host from invasion and infection by antigen or microbes (Grivennikov, Greten, & Karin, 2010). The various immune cells related to innate immunity traditionally recognize and respond to infectious agents. The role of recognition in innate immunity is basically the detection of constitutive and preserved products of metabolism (Medzhitov, 2001).

Phagocytosis is critical for innate immune system. Some innate immune cells such as macrophages and DCs perform two essential immune functions by ingesting pathogens or any antigens (Sbarra & Karnovsky, 1959). First, they present a death pathway by transferring partially ingested antigens and pathogens to lysosomes that are high in hydrolytic enzymes and also targeting phagocytic oxidase complexes to phagolysosomes. Second, DCs in phagocytic leukocytes allow antigens to migrate into both MHC I and II compartments through phagocytosis. Thus, phagocytosis acts

as an innate immune effector and plays a dual role in linking the innate and adaptive immune responses (Greenberg & Grinstein, 2002).

(1) Neuroinflammatory response and microglia

Neuroinflammation is an essential event to enable recovery from innate immune responses following events such as nervous tissue damage or microbial infection. It starts with signals such as brain damage from infection, trauma, and/or autoimmunity (Morganti-Kossmann, Satgunaseelan, Bye, & Kossmann, 2007). Neuroinflammation can also be chronic. In neuroinflammation, it plays an important role in cells or molecules, and many molecules such as microglia, pattern-recognition receptors and cytokines (Sorrenti, Giusti, & Zusso, 2018). These neuroinflammation mediators damage the blood-brain-barrier (BBB) of the central nervous system (CNS) (Broux, Gowing, & Prat, 2015).

Microglial cells are well known as representative cells of the CNS (Lehnardt, 2010). They are also the major effector cells that mediate neuroinflammation. Activated microglia usually secrete inflammatory cytokines from several neuroinflammatory diseases (Sasaki, 2016). Microglia secretes anti-inflammatory factors or mediators that affect BBB integrity (Hung et al., 2017). When microglial cells are continuously activated, immune cells become concentrated in the brain (Shastri, Bonifati, & Kishore, 2013). They are responsible for phagocytosis in the brain where they account for 10% to 15% of brain cells. Activated microglia secrete inflammatory cytokines in several neuroinflammatory diseases (Sasaki, 2016). They can actively change their morphology by reacting to infection conditions or signals. Microglia maintain small size in their resting inactive state, but morphology changes in response to damage or pathogens, and they migrate through chemotaxis to become

phagocytic cells (Skaper, Facci, Zusso, & Giusti, 2017). Activated microglia phagocytose apoptotic cells by releasing pro-inflammatory and anti-inflammatory cytokines or other molecules (Fu, Shen, Xu, Luo, & Tang, 2014). Neurons that have undergone apoptosis secrete various factors such as soluble factors and extracellular membrane proteins, which in turn induce microglial activity. This process is known as the self-propelling cycle (Fig. 1.). (Block, Zecca, & Hong, 2007). Murine microglial BV-2 cells involved in the inflammatory response stimulate Toll-like receptor-4 in response to lipopolysaccharide (LPS) and activate mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) (Chan et al., 2017). Once activated, these signaling pathways produce pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) and stimulate the expression of enzymes including inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) to produce a variety of inflammatory molecules such as nitric oxide (NO) (Geng et al., 2017). Although these inflammatory mediators are needed to remove viruses and tumors, their overproduction can cause serious damage to tissues and nerves and lead to chronic inflammatory diseases (Chan et al., 2017). Inhibiting inflammatory mediator production may be one way to combat excessive inflammation.

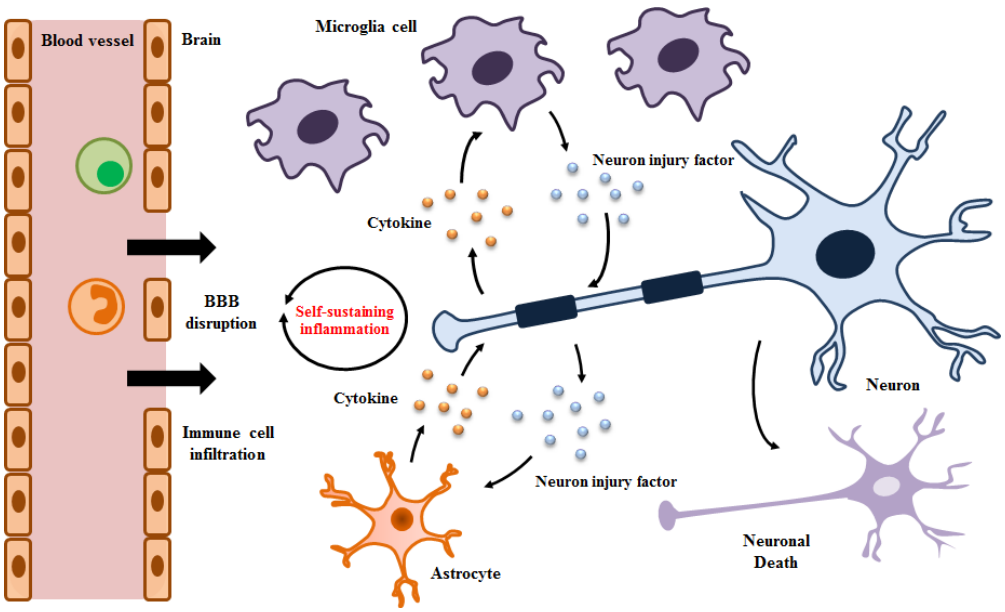


Figure 1. Mechanism of neuroinflammatory response

B. Adaptive immune response

Innate immune response systems provide an important mechanism for the rapid recognition and removal of pathogens (Steinman & Hemmi, 2006). Adaptive immune responses, on the other hand, have evolved to provide more specific recognition mechanisms for both self/non-self-antigens. Adaptive immunity is based on the interaction between antigen presenting cells such as dendritic cells and T and B lymphocytes. It is characterized by facilitating the regulation of host homeostasis, immunologic memory, and antigen specific immunologic mechanism pathways (e Sousa, 2004). The development and activation of lymphocytes occurs in the lymphatic system, which includes the lymphatic system. Rearrange and assemble gene segments during development to generate genes for encoding specific antigen receptors on T and B lymphocytes (Joffre, Nolte, Spörri, & Sousa, 2009). Another function of adaptive immunity, except specificity, is the generation of immunological memory. During the first reaction to antigens or pathogens, memory T and B cells are produced and established. In secondary reactions with the same antigens or pathogens, memory T and B cells are activated quickly to create a strong and fast defense mechanism (Fig. 2.). (Bonilla & Oettgen, 2010).

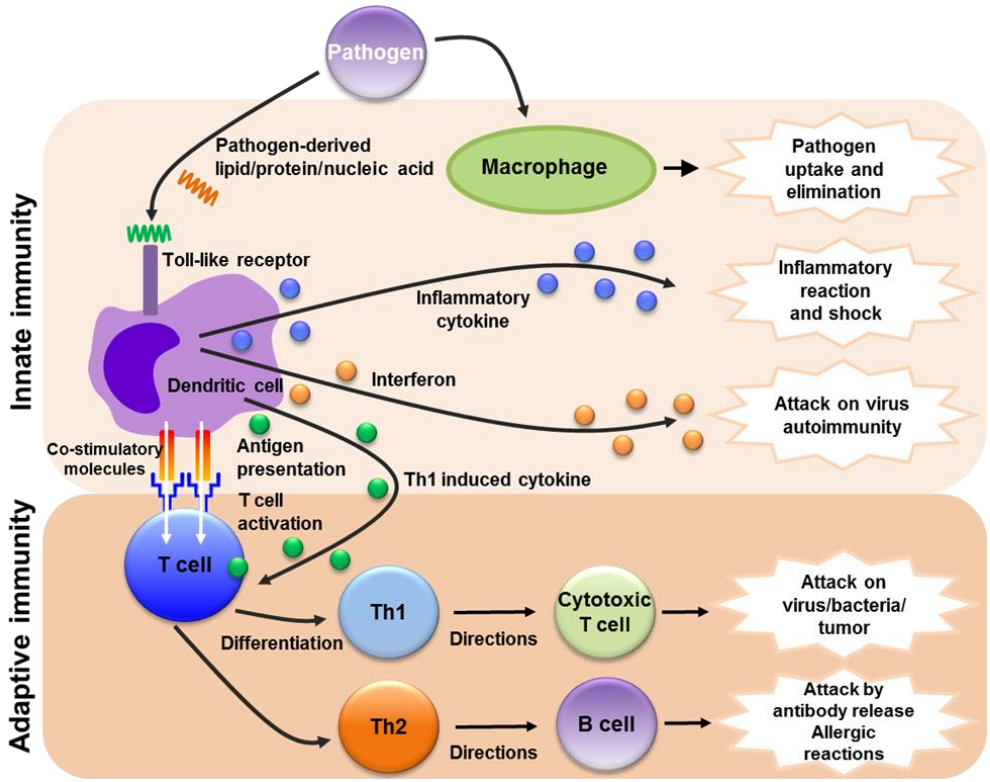


Figure 2. Innate and adaptive immunity

(1) Dendritic cells

Dendritic cells (DCs) are representative initiators and regulators of immune responses belonging to antigen presenting cells (Steinman, 1991). DCs are produced continuously in hematopoietic stem cells of bone marrow, and immature DCs are widely distributed in both lymphoid and non-lymphoid tissues (Fig. 3.). Innate recognition of pathogens, antigens and infections can activate dendritic cells and induce adaptive immune responses (Kushwah & Hu, 2011). DCs are activated either directly by antigens or pathogens, or indirectly by inflammation-related mediators produced by different cells that recognize these molecules. In addition, DCs may be activated by an incomplete environment in cells or by certain stress molecules (Y.-J. J. C. Liu, 2001). These cells are also specialized antigen presenting cells that have the ability to stimulate naïve T cells to produce primary immune responses (Y.-J. Liu, Kanzler, Soumelis, & Gilliet, 2001). Of the above processes, understanding which process causes DCs to be active for the immune response is thought that effector T cells will have an improved strategy for the immune responses.

DCs induce the function of antigen presentation or T cells stimulation through a process that enhances the overall function of the cell itself. However, these processes do not include the expression of DCs specific gene products of B cells that express B cell receptors (BCR) or T cells that express T cell receptors (TCR) (Mellman, 2013). DCs are functionally distinct immature and mature form. In immature DCs, they are commonly found in peripheral tissues for the invasion of antigens or for the death of host cells, and accumulate antigens bound to cells because they perform a function of phagocytosis corresponding to endocytosis (Rescigno et al., 1997). In the case of immature DCs, it is not possible to present the information of the antigen accumulated in the T cells. Usually they synthesize both MHC class I and II, but are

inefficient in producing MHC complexes for immature DCs. In addition, MHC class II is converted to lysosomes through partial ubiquitination, and immature DCs do not express ligands to co-stimulatory molecules and secrete small amounts of immune-related cytokines (Trombetta, Ebersold, Garrett, Pypaert, & Mellman, 2003). When TLR ligands induced by antigens or pathogens develop, immature DCs undergo maturation and activate T cells from cells capable of presenting antigen information (López et al., 2004). After this transient up-regulation, the endocytosis is down-regulated to receive information from new antigens or pathogens. Therefore, ubiquitination of MHC class II or other molecules is stopped and the MHC complex remains on the cell surface (Chow, Toomre, Garrett, & Mellman, 2002). During DCs maturation, migration is partially induced by up-regulation of chemokine receptors and up-regulation of the production of co-stimulatory molecules and immune-related cytokines (C.-H. Chen et al., 2006). After that, they move to the T cell-rich site and stimulate naïve T cells response (Benvenuti et al., 2004). So, DCs migrate to lymphoid tissue during maturation and stimulate naïve T cells by signaling through major histocompatibility complex (MHC) and co-stimulatory molecules such as CD80 and CD86 (Mi Eun Kim et al., 2013).

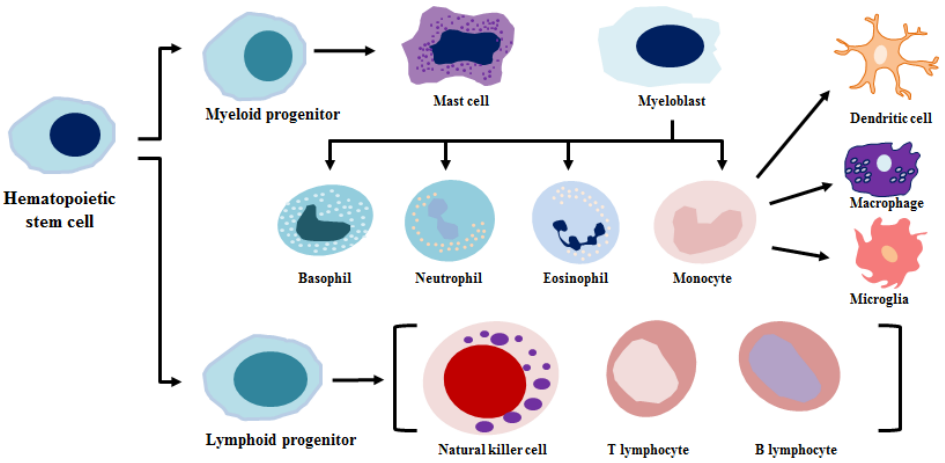


Figure 3. Immune cells

(2) Immune checkpoint

Immune checkpoints are regulators for the activation of immune responses. This plays an important role in the regulation and maintenance of the basic homeostasis of the immune response and in the prevention of autoimmunity (Li et al., 2016). Uncontrolled immune responses by pathogens, antigens, or mutations or overexpression factors can result in tissue damage or other immune-related diseases. To prevent these consequences, the magnitude of the immune responses is controlled by the balance between the stimulatory and suppression signals. These global signals are defined as “immune checkpoints”, which are necessary to control the tolerability of the immune response and to protect the host from tissue damage or external risk factors (Postow, Callahan, & Wolchok, 2015).

In the case of pathogens or external antigens, information is communicated to T cells through interactions between major histocompatibility complex (MHC) and T cell receptor (TCR) (Pardoll, 2012). It also represents an important key signal for activating T cells afterwards. First, the activation of T cells requires interaction between the B7 family of APCs and CD28, a co-stimulatory molecules present in T cells (Jenkins, Barbie, & Flaherty, 2018). It then acts as a negative regulator in the immune responses, which acts as several co-receptors at different molecular checkpoints. Specifically, CTLA-4, which is involved in the inactivation of T cells, is derived from T cells at the time of initial reaction against the antigens and binds to the B7 family with high affinity. The PD-1/PD-L1 signaling pathway, which is maintained by effector T cells and regulates inflammatory responses in tissues, is not initially involved in T cell activation (T. Chen, Razak, Bedard, Siu, & Hansen, 2015). Thus, regulatory mechanisms through these signaling pathways are used in tumor cells to protect normal tissues from several collateral disruptions and to avoid

immune responses. As described above, in response to TCR, signaling mechanisms through co-stimulatory molecules after recognition of specific antigens induce T cell activation/inactivation. As a result, the last signaling mechanism is induced by cytokines and chemokines and is involved in T cell polarization (Fig. 4.). (Rouwet, Lutgens, & Biology, 2018).

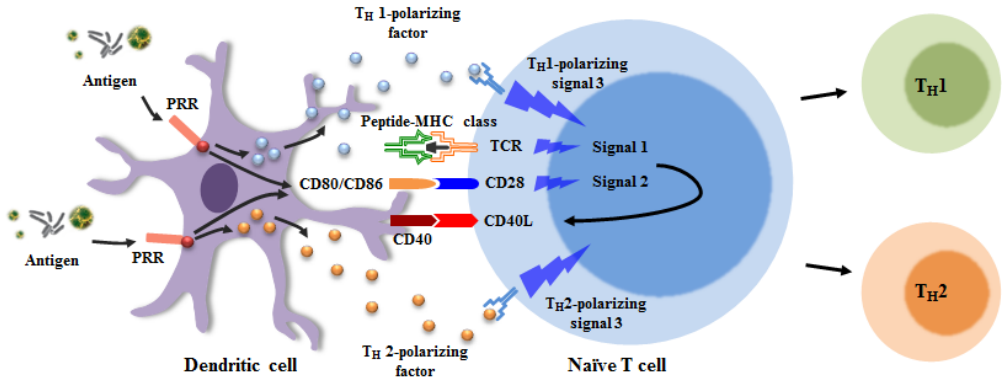


Figure 4. DCs control of T cell polarization

(3) T cells

T cells are lymphocytes developed by the thymus that play a central role in the immune responses (Alberts et al., 2002). T cells are distinguished from other lymphocytes by presence or absence of t cell receptor (TCR) on the cell surface, which develops into various types of t cells by moving from the bone marrow-derived precursor cells to the thymus (Kapsenberg, 2003). Specifically, different types of t cells perform a variety of immunological functions that form and control immune responses, where t cells differentiation proceeds beyond the thymus.

One of these immune-related functions is immune response mediated cell death that occurs in several ways. Representatively known as “killer T cells”, $CD8^+$ T cells can be described as cytotoxic and can directly kill not only cancer cells but also virus-infected cells. In addition, $CD8^+$ T cells produce an immune response using signaling cytokines to infiltrate other immune cells (Germain, 2002). $CD4^+$ T cells, which belong to another subset of t cells, function as “helper T cells”. $CD4^+$ helper T cells have a mechanism that indirectly kills cells identified from the outside, unlike $CD8^+$ T cells, which directly induce apoptosis (Parnes, 1989). These cells are responsible for determining how to respond to specific external risks in the immune responses. Like $CD8^+$ T cells, $CD4^+$ helper T cells directly affect B cells through signaling through cytokines and indirectly affect other immune cells (Bierer, Sleckman, Ratnofsky, & Burakoff, 1989). Regulatory T cells, which belong to another subset of T cells, are called Treg, and function to provide a mechanism by which immune cells recognize invading cells as “self/non-self”. That is, it functions to regulate immune cells from inappropriately responding to their immune response, and for this reason, Regulatory T cells function as “suppressor” (Abbas et al., 2013).

2. Inflammatory signaling pathway.

A. Mitogen-activated protein kinases (MAPKs) pathway

Mitogen-activated protein kinases (MAPKs) are a family of essential serine/threonine kinases that allow cell surface receptors to perform functions of changes in transcriptional programs (Muzio, Polentarutti, Bosisio, Kumar, & Mantovani, 2000). MAPKs are a three component kinase module consisting of MAPK, upstream MEK, and MEKK, which couples signals from cell surface receptors into downstream signal pathways. The classifications of three functions of MAPKs are characterized including ERK, JNK and p38MAPKs. As reported over the last decade, extensive research has established that their proteins play an important role in the functional regulation of cellular processes, including cell migration, growth, differentiation, proliferation and survival (Y. Sun et al., 2015).

MAPKs are activated by excessive cellular stimulation, which often suggests that there are certain and specific controls on the function of activating downstream signaling pathways (Seger & Krebs, 1995). MAPKs bind the upper signal MAP2K and lower signal pathways through cell surface interactions via docking motifs external to the catalytic region. Their binding sites or docking motif sites play an important role in determining functional substrate specificity of MAPKs (Zhang & Liu, 2002). The docking motif is usually located at the phosphorylation site of MAPKs, which consist of serine/threonine residues and proline, and the surrounding amino acids enhance the specificity of recognition. In addition, recognition and binding of the scaffold protein of the MAPKs signaling complex regulates the duration and location of MAPKs activation to determine the outcome of a particular signaling pathway. For example, the JIP1 protein functions as a scaffold that converts

proteins JNK1/2, MKK1, and MKK7 into MAPKs signal modules. Similar to the JIP1 protein, other scaffold proteins such as JIP2, JIP3, Axin form complexes of specific signals related to JNK activation and promote assembly (Rasheed, Akhtar, Haqqi, & therapy, 2010). Another scaffold protein, MP1, promotes activation of ERK1 through interaction with ERK1 and MEK1. This protein is involved in the activation of ERK1, but suggests how scaffold proteins such as MP1 but not ERK2 can confer signal specificity. Recently, KSR-1 has also been identified as a scaffold protein that regulates the ERK MAPKs signaling pathway (Qi & Elion, 2005). This protein is capable of interacting with Raf-1, MEK1/2 and ERK1/2, and part of MEK1/2 is involved in cell maintenance. When activated by RAS, KSR translocate to the plasma membrane along with MEK1/2, forming and activating the aRaf/MEK/ERK complex in close proximity to Raf-1 and the ERK1/2 sub-signal pathway. In the description so far, no scaffold has been mentioned for members of the p38MAPKs signaling pathway (Mainiero et al., 2000). However, some of the JNK-related scaffold proteins are known to be components of the p38MAPKs signaling pathway. For example, it has been shown that JIP2 can activate p38 MAPKs signaling pathway by binding to p38α and p38δ. In addition, MEKK3 protein, a specific scaffold for the p38MAPKs pathway, bind to MEK3 and p38 and responds to stress resulting in the activation of p38 (Fig. 5.).

B. Nuclear factor kappa-B (NF-κB) pathway

NF-κB plays an important role in not only the inflammatory response but also various immune response, tumor formation, and autoimmune diseases (Wuertz, Vo, Kletsas, & Boos, 2012). NF-κB is present in the cytoplasm in an inactive state bound to Iκ-Bα, but in response to external stimuli such as LPS, Iκ-Bα is phosphorylated,

and the separated NF-κB translocates to the nucleus where it is involved in the production of NO and pro-inflammatory cytokines (Gilmore, 2006). The NF-κB family in mammals usually consists of five related transcription factors, p50, p52, p65, c-Rel and RelB1, 2. These transcription factors share information through 300 amino acid domains called RHD homology domains (Lee, Dimtchev, Lavin, Dritschilo, & Jung, 1998). The RHD domain is a system in which each subunit forms a homodimer or heterodimer and binds to a promoter or enhancer region to regulate its expression. For RelA, c-RelB, C-terminal transcriptional activation domains (TADs) can be included to regulate expression of target genes. Thus, their homodimers inhibit transcription unless RelA, c-Rel or related transcriptional activators such as RelB or Bcl-3 are bound to the TAD protein (Wijayanti et al., 2004). Unlike other NF-κB subunits, p50 or p52 are derived from the respective precursor p105 and p100. NF- κB proteins are usually inhibited by IκB proteins present in the cytoplasm (Zandi, Rothwarf, Delhase, Hayakawa, & Karin, 1997). The two signaling pathways of NF-κB are divided into classical pathways and alternative pathways and induce activation. Dimers of NF-κB are generally activated by phosphorylation mediated by IKK of IκB, which results in the degradation of proteasome IκB. This allows the transcription factors of the activated NF-κB subunit to migrate into the nucleus and induce the expression of the target gene (Israël, 2010). This activation of NF-κB induces the expression of the IκBα gene and functions as negative feedback to sequester the NF-κB subunit, which terminates the signal if there is no persistent activation signal pathway (Fig. 5.).

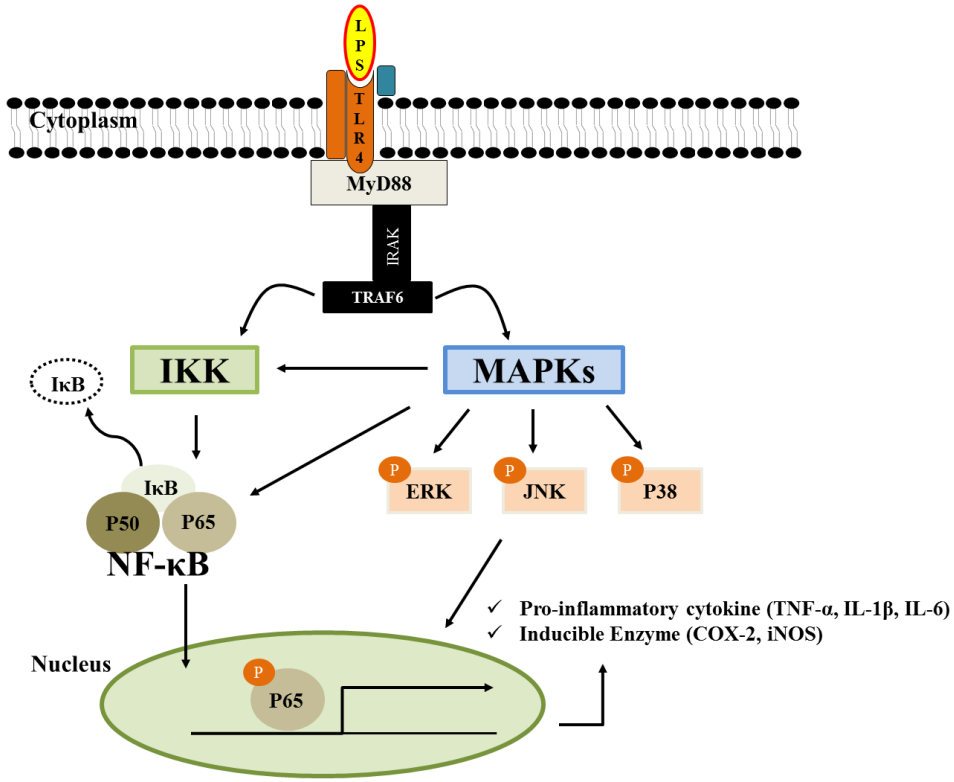


Figure 5. MAPKs and NF-κB signaling pathway

3. Oxidative stress

Free radicals generated in the human body have a positive effect when the proper position, time and amount are produced, and the defense mechanism of our body can also eliminate the toxicity caused by free radicals (H. J. E. P. T. Sies & Integration, 1997). Our body regulates the amount of free radicals in the body by itself, and excessive exercise of external stimulation causes a rapid increase in the generation of reactive oxygen species (ROS), or the ability to remove them, causing various diseases caused by harmful oxygen (H. Sies, 2000). Therefore, the side effects of the harmful oxygen are called oxidative stress. That is, harmful oxygen rapidly increases during the energy generation process of the human body, causing bad effects, or such a situation is called oxidative stress. Oxidative stress results in an imbalance in biological functions that detoxify or repairs the response of the entire reactive oxygen species (ROS) (H. J. A. C. I. E. i. E. Sies, 1986). In cells that maintain a normal redox state, the disorder causes toxicity by producing free radicals or peroxides through the action of damaging various components of the cell, including lipids, proteins, and DNA. In the case of oxidative stress caused by oxidative metabolism, not only DNA strand damage but also base damage is caused, and the basic damage is indirectly caused by ROS (Finkel & Holbrook, 2000). In addition, some of the ROS acts as an intercellular messenger in the redox signaling mechanism, and as a result, oxidative stress can cause disruption of the normal cell signaling mechanism (Fig. 6.). (Maritim, Sanders, Watkins Iii, & toxicology, 2003).

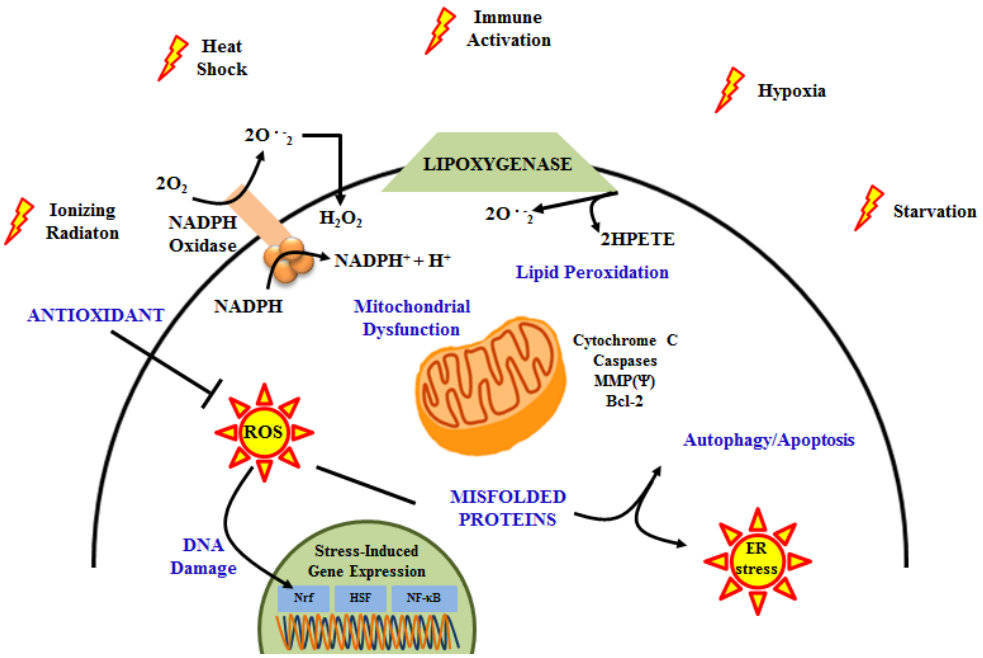


Figure 6. Origins and consequences of oxidative stress

A. Redox status in immune response

Many molecules that activate reactive oxygen and reactive nitrogen species (ROS/RNS) or redox play an important role in the immune responses. Redox reactions regulate and form immune responses, which additionally perform the function of initiating cell killing or cell repair processes. Imbalances in their production and elimination in the interaction between reactive oxygen and reactive nitrogen species (ROS/RNS) play an important role in pathophysiological reactions (Gostner, Becker, Fuchs, & Sucher, 2013). At present, the importance of ROS/RNS produced by respiratory destruction among antigen defense mechanisms has been reported a lot. However, to avoid further damage, the increased production of ROS/RNS must be controlled temporally and spatially (Matés et al., 2002). If this adjustment is not made, oxidation of biomolecules results in toxicity to self-cells, which in turn can lead to cell death. Redox imbalances usually lead to diseases characterized by immune activation such as autoimmune disorders, infections, neurodegenerative diseases and allergies (Buttke & Sandstrom, 1995). ROS/RNS and redox molecules, at lower levels, act as inducers of the oxidative stress pathway to activate immune responses or to regulate many physiological processes through detoxification. Therefore, the balance of redox status is intrinsic marker for cellular and body homeostasis (Calabrese et al., 2010).

Many reactive molecules disrupt the immune response signaling cascade by modifying the activity of enzymes, transcription factors, and redox-related sensing molecules. Representative reactive species include hydrogen peroxide (H_2O_2), ROS as hydrogen peroxide anion ($O_2^{\bullet -}$) and RNS such as nitrogen dioxide ($NO_2^{\bullet}$), nitrogen peroxide and nitrogen trioxide (Rose et al., 2012). Recently, other toxic gases, CO and H2S, have been reported as immunomodulators. To protect cells from these toxic

gases or reactive species, antioxidant molecules serve as redox buffers. Such redox buffers are typically glutathione/glutathione disulfide (GSH/GSSG), cysteine/cysteine (Cys/CySS) and thioredoxins (Trx) (Ortona et al., 2014).

As a result, reactive molecules such as ROS/RNS play a role in protecting against antigens and pathogens, but in small amounts they act as signaling molecules. Many redox molecules are immunomodulators that regulate inflammatory signaling pathways and cell metabolism and are involved in immune suppression or recovery processes (A. Sun, Nie, & Xing, 2012). Usually the effect or process involved depends on the intracellular situation or the time and location of the exposure. Redox signaling pathways involved in the immune response regulate the capacity of immune cells by participating in the regulation of metabolic functions (De la Fuente, Cruces, Hernandez, & Ortega, 2011). This redox imbalance is involved in the progression and development of several pathological states, almost through anti-oxidation situations.

4. Dysfunction of immune response

Dysfunction of the immune response can typically be defined as an immune-related disease. These immune-related diseases are most common in allergic diseases such as allergic rhinitis, eczema, and asthma (Good, 1967). But they can actually be explained by hypersensitivity to external antigens and allergens. Frequently, substances such as pollen, mold, or animal hair are misunderstood as antigens in the body and attacked. Other dysfunctions of the immune response also include autoimmune diseases such as rheumatoid arthritis (Matarese, De Placido, Nikas, & Alviggi, 2003). These autoimmune diseases are the result of an overactive immune system that recognizes and attacks normal tissues as if they were external antigens. Representative autoimmune diseases include rheumatoid arthritis, type 1 diabetes, Hashimoto’s thyroiditis, and

systemic lupus. This dysfunction can also cause inflammatory diseases or cancer (Carbone & Ohm, 2002). Reportedly, immune deficiency can occur in a state where the immune system is less functional than normal, resulting in recurrent and threatening infections (Etzioni, 2003). Usually these immune deficiencies are the result of acquired immunodeficiency, such as HIV/AIDS, severe combined immunodeficiency, or side effects of immunosuppressant (Nixon, Landay, & AIDS, 2010).

When describing the dysfunction of the immune response for the purpose of diagnosis and treatment, this is basically an indication that the immune system is not functioning normally (Rosenblat & McIntyre, 2015). In general, immunodeficiency diseases are diagnosed with a blood test that can measure the functional activity or level of immune components. For allergic diseases, skin tests or blood tests can be used to identify the antigen causing the symptoms. In hypersensitivity or autoimmune reactions, drugs such as immunosuppressant or corticosteroids that reduce the immune response are commonly used (Campbell et al., 2003). Treatment in some immunodeficiency states is typical of the injection of monoclonal antibodies against infection. Monoclonal antibodies, used to modulate the overall immune response that triggers inflammation, are proteins that can be incorporated into the body. It is also used in cancer treatment by transporting toxins, drugs or radioactive substances directly to cancer cells (Sica & Bronte, 2007).

5. *Sargassum horneri* (Turner) C. Agardh

Sargassum horneri (Turner) C. Agardh is a brown algae found in coastal areas of Asia such as Korea, China, Japan that has various effects in the body (M. E. Kim et al., 2015; Sanjeewa et al., 2017). Studies suggest that *S. horneri* (Turner) C. Agardh can help prevent osteoporosis and control cholesterol, blood pressure, and hyperlipidemia

(M. Yamaguchi, 2006; Yang et al., 2014). Cosmetics and functional foods containing *S. horneri* (Turner) C. Agardh are known to have anti-aging, anti-allergic, anti-acne and whitening effects (Fujiwara et al., 2016; J. A. Kim, Ahn, Kong, & Kim, 2012). However, no study has assessed its effects on inflammation mediated by murine microglia. Here I investigated the anti-inflammatory activity of *S. horneri* extract in the setting of LPS-induced inflammation.

II. MATERIALS AND METHODS

1. Reagents

The compound TRI Reagent and Griess reagent, 33-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), were purchased from Sigma Chemical (St. Louis, MO, USA). The antibody Anti-β-actin, anti-caspase 3, anti-Bax, anti-Bcl-2, anti-AIF, anti-PARP-1, anti-p-p65, anti-p65, anti-i-NOS, anti-COX-2, anti-p-p38, anti-p-ERK, anti-p-JNK, and anti-JNK were purchased Santa Cruz (Santa, CA, USA), and anti-caspase 8, anti-caspase 9, anti-ERK, and anti-p38 were purchased Cell signaling (Cell signaling, MA, USA). Recombinant mouse (rm) granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Invitrogen Corporation (Carlsbad, CA, USA). Lipopolysaccharide (LPS) (from Escherichia coli 055:B5) and Dextran-FITC (molecular mass, 40,000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). FITC-or PE- conjugated mAbs used to detect the expression of anti-CD11c, anti-CD80, anti-CD86, anti-MHC I, and anti-MHC II and purchased from Biolegend (San Diego, CA, USA) and eBioscience (San Diego, CA, USA).

2. Cell culture

Murine microglial cells, BV-2, were cultured. And 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.

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

Sargassum horneri (Turner) C. Agardh belonging to the brown algae was collected from the Sea of Wando, Korea. *S.horneri* (Turner) C. Agardh was washed three to four times in running water to remove salt completely. After that, it was completely dried and ground to a blender. The prepared 100 g of dried *S. horneri* (Turner) C. Agardh was dissolved in 70% ethanol and shaking for 3 days. After repeating the dissolving process twice, the ethanol extract was filtered using a filter paper, and concentrated to a minimum volume using an evaporator. The remaining solvent was lyophilized and dissolved in 100% ethanol for cells to make samples. *S. horneri* (Turner) C. Agardh was reconstituted in ethanol and then diluted to the desired concentration in DMEM (final ethanol concentration 0.3%). Control groups without ethanolic extract of *S. horneri* (Turner) C. Agardh (*S. horneri* extract) used 100% ethanol solvent.

4. Isolation and generation of DCs

Immature DCs were obtained from the bone marrow of C57BL/6. DCs were cultured in RPMI 1640 medium (welgene, Gyeongsangbuk-do, South Korea), containing 1% penicillin/sterptomycin, 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium bicarbonate and 4 mM L-glutamine and rmGM-CSF (20 ng/ml) at 37℃ in presence of 5% CO_2. The DCs (2×10^6 cells/well) were seeded in 6-well culture plates with RPMI 1640 containing rmGM-CSF (20 ng/ml). rmGM-CSF is replaced with newly fresh media every 2 days for 6 days. On day 6, immature DCs were stimulated by treatment with H_2O_2 and after 2 h with LPS (100 ng/ml).

5. Cell viability analysis

Cell viability was measured by Annexin V/PI staining. The DCs (2 x 10^6 cells/well) were seeded in 6-well culture plates with RPMI 1640 containing rmGM-CSF (20 ng/ml). Cells were treated with the various concentrations hydrogen peroxide (H_2O_2) (100, 250, and 500 μM) and incubated in 37℃ for 24 h. The BV-2 cells (2 x 10^6 cells/well) were seeded in 6-well culture plates with DMEM. Cells were treated with the various concentrations *S. horneri* extract (0~300 μg/ml) and incubated in 37℃ for 24 h. In the case of BV-2, cells were treated with the various concentrations *S. horneri* extract (0~300 μg/ml) and incubated in 37℃ for 24 h. After 24 h, the cells were detached and centrifuged at 20℃ and 2000 rpm for 3 min. In DCs, treat group was incubated for 24 h with H2O2 treatment and NAC, antioxidant, was pretreated for 1 h and then cells were incubated with H2O2 24 h. After 24 h, the cells were detached and centrifuged at room temperature and 2000 rpm for 3 min. Wash 2~3 times with warm PBS, staining using Annexin V/PI Kit, and measure using FACS (Fluorescence activated cell sorter).

6. NO assay

BV-2 cells (3 x 10^4 cells/well) were seeded in 96-well culture plate in DMEM. Cultured cells were pretreated with various concentration of *S. horneri* extract (0~300 μg/ml) for 2 h and then cells were incubated for 22 h in absence or presence of LPS (200 ng/ml). After incubation, the cultured medium was mixed with an equivalent volume of Griess Reagent and incubated for 15 min at room temperature. After 15 min incubation, absorbance was measured using an ELISA microplate reader at 540 nm of absorbance.

7. Enzyme-Linked Immunosorbent Assay (ELISA)

BV-2 cells (3×10^4 cells/well) were seeded in 96-well culture plate. Cells were pretreated with various concentration of *S. horneri* extract (0~300 μg/ml) for 2 h, and then cells were incubated for 22 h in absence or presence of LPS (200 ng/ml). After incubation, supernatant was used for samples. The quantification of TNF-α and IL-6 release was measured by Mouse IL-6, TNF-α ELISA MAX Deluxe Sets (BioLegend), according to manufacturer's protocol. Briefly, standards and samples were incubated for 1 h and Avidin-HRP bind to detection antibody. To visualization, substrate solution was added to each well, and then the reaction was stopped by stop solution (2 N H_2SO_4). Absorbance was measured by ELISA microplate reader at 405 nm wavelength.

8. Immunohistochemistry

The mice were intra-cardially perfused with 10 mM phosphate-buffered saline (PBS) and subsequently 4% paraformaldehyde. The brain was extracted and post-fixed in 4% paraformaldehyde for 24 h at 4℃. Fixed brain was transferred to 30% sucrose solution. These brain samples were embedded in OCT for frozen sections, and then coronally sectioned at 40 μm using a freezing microtome (MICROM, Walldorf, Germany). Brain sections were washed and blocked with blocking solution for 30 min at room temperature. The sections were incubated with anti-ionized calcium-binding adapter molecule 1 (Iba-1, microglia maker) antibody and glial fibrillary acidic protein (GFAP, astrocytes maker) (Wako Chemical USA, Inc.; Richmond, VA, USA) in TBS-TS at 4% overnight. Brain sections were washed with TBS and incubated with anti-mouse IgG labeled with Alexa Fluor 488 and 568 for 3 h at room temperature,

respectively. Confocal fluorescence images were acquired FV10i fluoview confocal microscope (Olympus, Tokyo, Japan).

9. Surface marker analysis

On day 6, BM-DCs were harvested and washed with phosphate-buffered saline (PBS) and rewashed with flow cytometry washing buffer (1% fetal bovine serum in PBS). The DCs were stained with Fluorescein isothiocyanate (FITC)-conjugated anti-CD11c with phycoerythrin (PE)-conjugated anti-CD80, anti-CD86, anti-H-$2K^b$ (MHC class I), and anti-I-A^b (MHC class II) (eBioscience, CA, USA) (Biolegend) for 30 min at 4℃. Stained DCs were measured by FC500 and analyzed by Flowjo Cytometry Analysis Software V10.0 (BD biosciences, CA, USA).

10. Intracellular ROS measurement

On day 6, the cells treated with the H_2O_2 were harvested and washed twice with 1X PBS. After washing with 1X PBS treated with 25 μM dichlorofluorescein diacetate (DCFDA) and stained for 30 min. As a final step, the stained cells were fixed at 1% paraformaldehyde (PFA) and measured with FC500 Flow cytometry (Beckman Coulter, Brea, CA, USA). The production of ROS was expressed as mean fluorescence intensity (MFI), which was analyzed by the Flowjo Cytometry Analysis Software V10.0 (BD biosciences, CA, USA).

11. Mitochondrial membrane potential (MMP, Ψm) assay

MMP (Ψm) was measured by tetramethylrhodamine, methyl ester (TMRM) staining. DCs (2×10^6 cells/well) were seeded in 6-well culture plate in RPMI

1640 containing rmGM-CSF (20 ng/ml) and treated with H_2O_2. Cells were harvested at 0, 15, 30, 60, 120 and 180 min after H_2O_2 treatment. After washing twice with 1X PBS, the fluorescent dye TMRM ester was stained for 30 min. After 30 min, the stained cells were washing twice with 1X PBS and MMP (Ψm) was expressed as MFI values, which was measured by FC500 Flow cytometry (Beckman Coulter, Brea, CA, USA). The measured results were analyzed by the Flowjo Cytometry Analysis Software V10.0 (BD biosciences).

12. Antigen uptake assay

Antigen uptake assay was measured by dextran-FITC uptake ability assay. The DCs were seeded in 6-well culture plate (2×10^6 cells/well) in RPMI 1640 media containing rmGM-CSF (20 ng/ml). The cultured cells were treated with the various concentrations H_2O_2 (0, 100, 250, and 500 μM) and incubated in 37℃ for 12 h in the presence of 5% CO_2. Treatment group was incubated for 12 h with LPS (100 ng/ml) treatment 2 h after treatment with H_2O_2. After 12 h, BM-DCs were harvested and washed with 1X PBS, then stained with dextran-FITC into 4℃ and 37℃. In the case of the 4℃ stained with dextran-FITC, it was used as a negative control for endocytic activity. After 30 min of staining, cells were washed at 1X PBS and fixed at 1% PFA for 5 min. Then cells were stained with a PE-conjugated anti-CD11c antibody for 30 min. Stained cells were measured by FC500 flow cytometry and analyzed by Flowjo Cytometry Analysis Software V10.0 (BD biosciences).

13. Mixed lymphocyte reaction (MLR) assay

Basic co-culture DCs and T cells begins with splenocyte isolation from BALB/c mice. After collecting the spleen and grinding the slide glass in a dish containing cold media to collect the cells, centrifuge for 3 min at 1500 rpm. Incubate the collected cells with 3 ml/spleen of RBC lysing buffer for 3 min at room temperature. The obtained splenocytes were washed with MACs buffer (10 mM PBS pH 7.2 containing 2 mM EDTA, 0.5% BSA) and incubated with 20 µl CD4 bead antibody for 15 min at 4℃. After the incubation, wash once with MACS buffer and filter the CD4 positive T cell which was reacted through the magnetic column. Finally, the column was separated, MACS buffered was added and the cells were separated and centrifuged at 1500 rpm for 3 min. After washing the isolated T cells with media, incubation with 1.25 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 8 min at 37℃ was stained.

Oxidative stress was induced by treating H_2O_2 (0, 100, and 250 µM) with DCs differentiated for 6 days. After 12 h, oxidative stress induced DCs harvested and co-cultured at 96-well u-bottom plate with T cells at a ratio of 1:1 and 1:10. [DC: T cell = 2×10^4 cells: 2×10^4 cells (1:1), DC: T cell = 2×10^3 cells: 2×10^4 cells (10:1)]. After 3 days of co-cultured, the mixed cells were harvested and washed with 1X PBS. After washing, the mixed cells were stained with a Cy7-conjugated anti CD3 antibody for 30 min. Stained mixed cells were measured by FC500 flow cytometry and analyzed by Flowjo Cytometry Analysis Software V10.0 (BD biosciences).

14. Reverse transcription (RT)-PCR

BV-2 cells (5×10^5 cells/well) were seeded in 12-well culture plate in DMEM. Cultured cells were pretreated with various concentrations of *S. horneri* extract (0~300

μg/ml) for 2 h and then cells were incubated for 6 h in absence or presence of LPS. After incubation, the cells were collected by centrifugation and total RNA was isolated form H_2O_2-treated cells and *S. horneri* extract-treated cells using TRIZOL reagent according to manufacturer's protocol. To synthesize cDNA, 0.1 μg of total RNA was primed with oligo dT and reacted with mixture of dNTP, M-MLV RTase and reaction buffer (Promega, WI, USA). To measure the mRNA level of inflammatory chemokines including CCR1, CCR3, CCR5, and CCR7, I designed the primers for target genes (Bioneer, Daejeon, Korea). And cDNA was amplified using Gene Atlas G02 gradient thermal cycler system (Astec Fukuoka, Japan) e-Taq DNA Polymerase kit (Solgent, Daejeon, Korea) and the primers. And then, PCR products were visualized by fluorescent dye and UV transilluminator.

15. Western blot analysis

DCs (5×10^6 cells/dish) were seeded in 100 mm culture dish in RPMI 1640 containing rmGM-CSF (20 ng/ml). Cultured cells stimulated 100, 250, and 500 μM in rmGM-CSF media RPMI 1640 by H_2O_2 treatment. BV-2 cells (2×10^6 cells/dish) were seeded in 60 mm culture dish in DMEM. Cultured cells starvated 4 h in serum free media DMEM and exposed to LPS (200 ng/ml) in the absence or presence of 300 μg/ml of *S. horneri* extract pretreatment. Following 15, 30, and 45 min (*S. horneri* extract) of incubation at 37℃, cells were washed twice with cold PBS and lysed with modified RIPA buffer containing (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 g/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 14000 xg for 15 min at 4℃. The protein content of cell lysates was determined using the Micro BCA assay kit (Pierce, Rockford, IL, USA).

Equivalent amounts of proteins were separated by 8~10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) transfer membrane. The membrane was placed into a blocking solution (5% skim milk or 2% BSA) at room temperature for 1 h. After blocking, anti-caspase 3, anti-caspase9, anti-bax, and anti-β-actin antibodies overnight (Santa Cruze Biotechnology, CA, USA) (Cell signaling, MA, USA) were used as the primary antibodies. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Santa Cruze Biotechnology) were used as the secondary antibodies. Band detection was performed using the enhanced chemiluminescence (ECL) detection system and exposed to radiographic film. Pre-stained blue markers were used for molecular weight determination.

16. Statistical analysis

The results are presented as the mean ± standard deviation. The data were analysis of variance (ANOVA) followed by Scheffe's post-hoc test using SPSS. The statistically significant differences were considered at $p<0.05$.

Table. 1. Primers used in RT-PCR

Target gene	primer	Sequence
COX-2	Forward	5'-TGGGTGTGAAGGGAAATAAGG-3'
	Reverse	5'-CATCATATTTGAGCCTTGGGG-3'
i-NOS	Forward	5'-CTTGCCCCTGGAAGTTTCTC-3'
	Reverse	5'-GCAAGTGAAATCCGATGTGG-3'
IL-1β	Forward	5'-GTGTCTTTCCCGTGGACCTT-3'
	Reverse	5'-TCGTTGCTTGGTTCTCCTTG-3'
TNF-α	Forward	5'-GGCCTCTCTACCTTGTGCC-3'
	Reverse	5'-TAGGCGATTACAGTCACGGC-3'
IL-6	Forward	5'-CCTTCCTACCCCAATTTCCA-3'
	Reverse	5'-CGCACTAGGTTTGCCCACTA-3'
GAPDH	Forward	5'-TGCACCACCAACTGCTTAG-3'
	Reverse	5'-GGATGCAGGGATGATGTTC-3'

Table. 2. Primary antibodies used in western blotting

1'st antibody	2'nd antibody	Titer	Company (Cat NO.)
phospho-ERK1/2	Mouse	1 : 2000	Santa Cruz Sc-7378
ERK1/2	Rabbit	1 : 2000	Cell signaling #9102
phospho-p38	Mouse	1 : 2000	Santa Cruz Sc-166182
p38	Rabbit	1 : 2000	Cell signaling #9212
phospho-NF-κB-p65	Rabbit	1 : 1000	Santa Cruz Sc-33020
NF-κB-p65	Mouse	1 : 2000	Santa Cruz Sc-8008
NOS2	Rabbit	1 : 2000	Santa Cruz Sc-8310
COX-2	Mouse	1 : 2000	Santa Cruz Sc-166475
Bax	Mouse	1 : 2000	Santa Cruz Sc-526
Caspase 3	Rabbit	1 : 2000	Santa Cruz Sc-7148
Caspase 9	Mouse	1 : 2000	Cell signaling #9508
β-actin	Mouse	1 : 5000	Santa Cruz Sc-47778

III. RESULTS

Part I. *S. horneri* extract attenuates neuroinflammatory response through inhibition of MAPKs and NF-κB pathway.

1. *S. horneri* extract inhibits NO production in LPS-stimulated BV-2 microglial cells.

I investigated effect of *S. horneri* extract on cell viability and NO regulation. Annexin V/PI staining showed that *S. horneri* extract was not cytotoxic at doses up to 300 μg/ml (Fig. 7A.). To determine the effect of *S. horneri* extract on NO production, BV-2 microglial cells were pretreated with *S. horneri* extract for 2 h and then stimulated with LPS (200 ng/ml). NO production was increased in LPS compared to control, but NO level decreased in a dose-dependent manner with *S. horneri* extract treatment (0~300 μg/ml) (Fig. 7B.). These results indicate that *S. horneri* extract effectively inhibits NO production in LPS-stimulated BV-2 microglial cells.

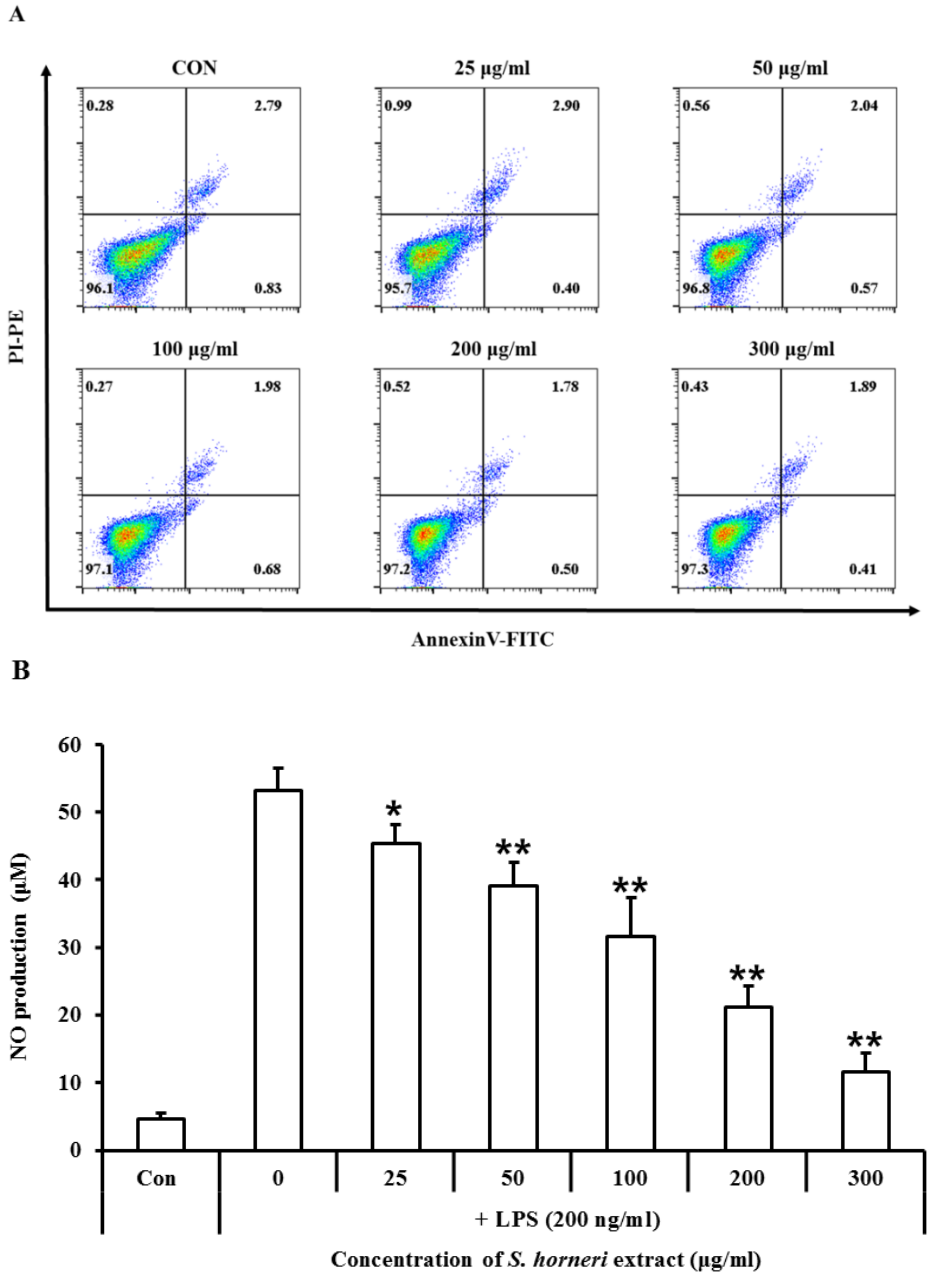


Figure 7. (Continued)

Figure 7. Effect of *S. horneri* extract on NO production in LPS-stimulated BV-2 microglial cells. The BV-2 cells were cultured in the same way as the *Materials & Methods*. (A) The BV-2 cells (2×10^6 cells/well) were seeded in 6-well culture plates with DMEM. Cells were treated with the various concentrations *S. horneri* extract (0~300 μg/ml) and incubated in 37℃ for 24 h and stained with Annexin V–FITC and PI–PE. (B) After *S. horneri* extract treatment with microglial cells stimulated with LPS (200 ng/ml), NO production level was confirmed by Griess assay. Fluorescence intensity was measured by FC500 flow cytometry and presented as a percentage of each quadrant and NO production was measured by microplate reader at 540 nm wavelength.

2. *S. horneri* extract reduces LPS-induced iNOS and COX-2 mRNA/protein expression.

Since *S. horneri* extract inhibits NO production, I assessed its impact on mRNA expression of iNOS, which is a pro-inflammatory enzyme producing NO, and cyclooxygenase (COX)-2. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot were performed to confirm that *S. horneri* extract inhibits the expression of pro-inflammatory enzymes. BV-2 microglial cells were pretreated for 2 h with *S. horneri* extract and then stimulated with LPS (200 ng/ml) for 6 h. In protein expression, BV-2 microglial cells were pretreated with *S. horneri* extract (0~300 μg/ml) for 2 h and then stimulated with LPS (200 ng/ml). As shown in Fig. 8A, iNOS and COX-2 gene expression were increased in LPS-stimulated BV-2 microglial cells, but decreased in *S. horneri* extract treatment (Fig. 8A.). Protein levels of iNOS and COX-2 were also increased in LPS-stimulated BV-2 microglial cells and then decreased with *S. horneri* extract (0~300 μg/ml) treatment (Fig. 8B.). These results indicate that *S. horneri* extract inhibits mRNA and protein expression of iNOS and COX-2 in LPS-stimulated BV-2 microglial cells.

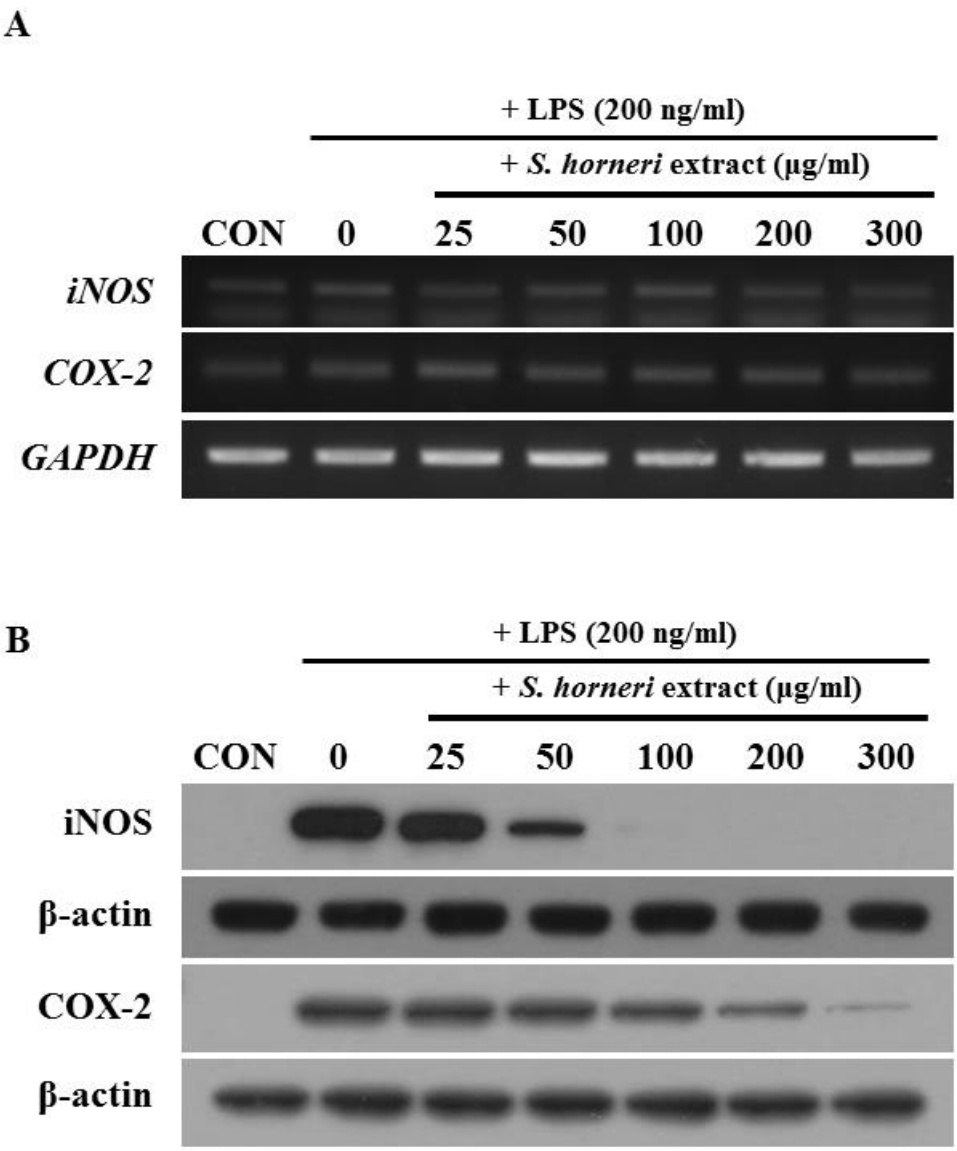


Figure 8. (Continued)

Figure 8. Effects of *S. horneri* extract on gene and protein expression levels of iNOS and COX-2 in LPS-stimulated microglial cells. BV-2 cells (5×10^5 cells/well) were seeded in 12-well culture plate in DMEM. Cultured BV-2 microglial cells were pretreated with various concentration of *S. horneri* extract (0, 25, 50, 100, 200, and 300 μg/ml) for 2 h and then cells were incubated for 6 h in absence or presence of LPS. RNA was isolated from *S. horneri* extract-treated cells using TRIZOL reagent and analyzed by RT-PCR. iNOS and COX-2 gene expression was determined, respectively (A). *S. horneri* extract was pretreated with microglial cells for 2 h, then incubated with LPS (200 ng/ml) for 22 h. iNOS and COX-2 protein level expression was measured by western blot analysis (B).

3. *S. horneri* extract reduces LPS-induced pro-inflammatory genes.

I next used RT-PCR to measure mRNA level of the pro-inflammatory substances IL-6, TNF-α and IL-1β and determined whether their expressions were inhibited by *S. horneri* extract treatment. BV-2 microglial cells were pretreated for 2 h with *S. horneri* extract and then stimulated with LPS (200 ng/ml) for 6 h. As shown in Fig. 9, expression of pro-inflammatory genes such as IL-6, TNF-α, and IL-1β were increased in LPS-stimulated BV-2 microglial cells, but dose-dependently decreased in *S. horneri* extract treatment (Fig. 9). These results indicate *S. horneri* extract inhibits mRNA expression of pro-inflammatory genes in LPS-stimulated BV-2 microglial cells.

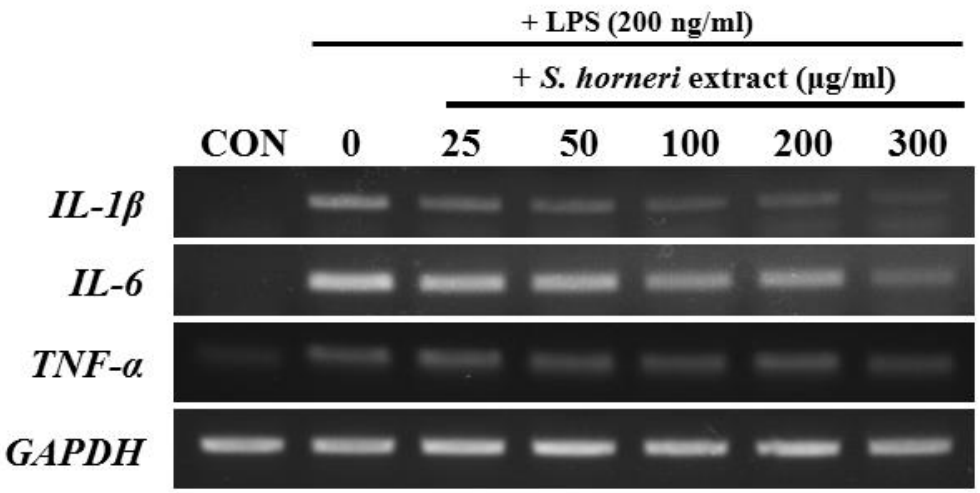


Figure 9. The effect of *S. horneri* extract on pro-inflammatory factors in LPS-stimulated BV-2 microglial cells. BV-2 microglial cells were seeded in 12-well culture plate (5 x 10^5 cells/well) in DMEM media. Cultured BV-2 cells were pretreated with *S. horneri* extract (0, 25, 50, 100, 200, and 300 μg/ml) for 2 h, and incubated with LPS (200 ng/ml) for 6 h. Total RNA was isolated form *S. horneri* extract-treated cells using TRIZOL reagent according to manufacturer's protocol. IL-1β, IL-6, and TNF- α gene expression levels were confirmed, respectively.

4. *S. horneri* extract reduces LPS-induced pro-inflammatory cytokine levels.

Pro-inflammatory cytokines production was measured to determine whether the production of IL-6, TNF-α and IL-1β was decreased by *S. horneri* extract treatment. IL-6 and TNF-α were pro-inflammatory cytokines expressed by BV-2 microglial cells following exposure to LPS and other inflammatory stimuli. The results confirmed that IL-6 and TNF-α produced from LPS-stimulated BV-2 microglial cells were significantly decreased by pretreatment with *S. horneri* extract (Fig. 10.). Therefore I suggest that IL-6 and TNF-α production can be inhibited by *S. horneri* extract treatment, suggesting that the compound has anti-inflammatory effects.

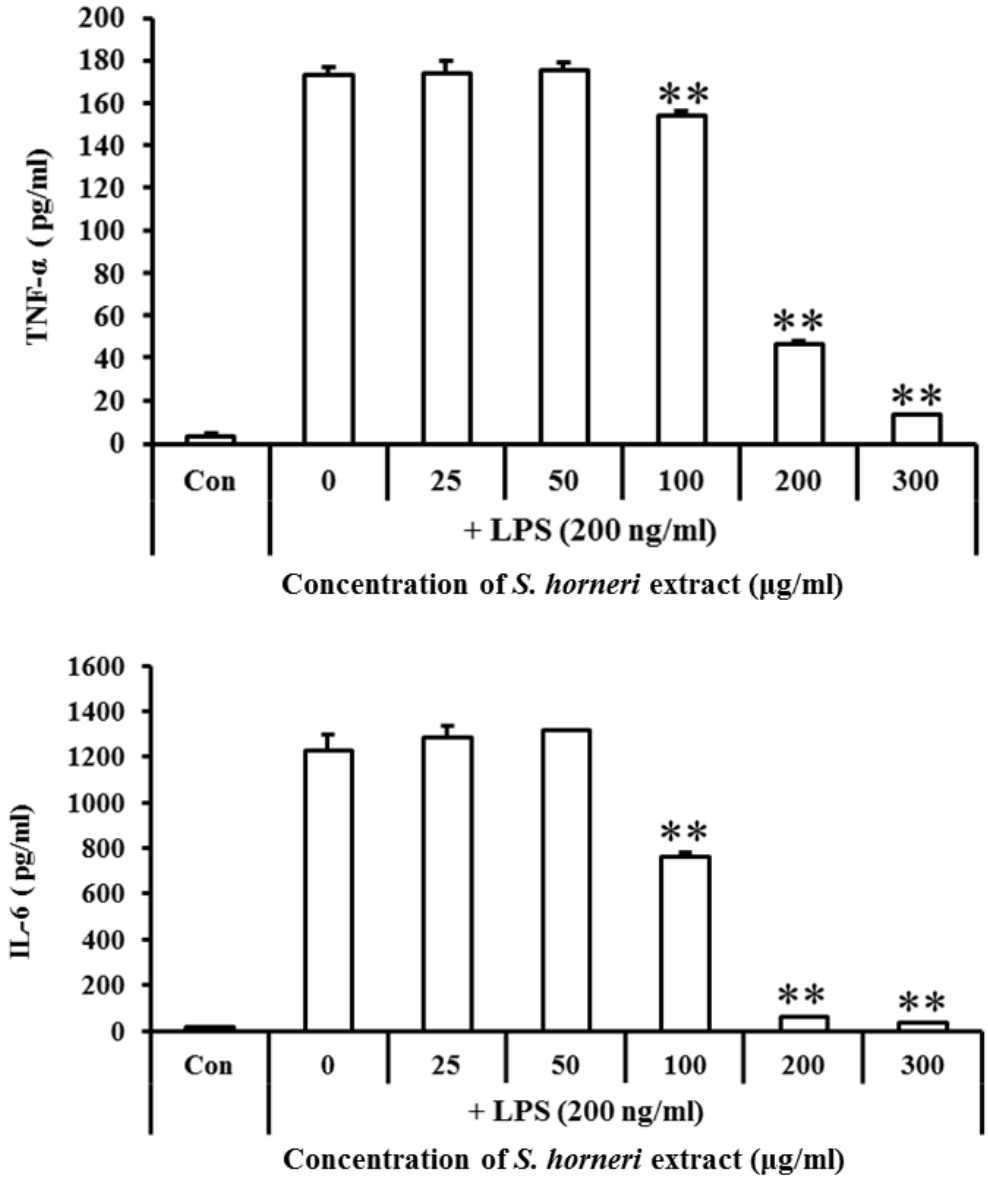


Figure 10. (Continued)

Figure 10. The effect of *S. horneri* extract on IL-6 and TNF-α production in LPS-stimulated BV-2 microglial cells. BV-2 microglial cells were seeded in 96-well culture plate (3×10^4 cells/well). Cells were pretreated with various concentration of *S. horneri* extract (0~300 μg/ml) for 2 h, and cells were incubated for 22 h in LPS (200 ng/ml); the cytokine (IL-6, TNF-α) production levels in the supernatants were measured by ELISA.

5. *S. horneri* extract treatment inhibits phosphorylation of p38, ERK, MAPKs, and NF-κB.

MAPKs such as p38, JNK, ERK plays important roles in regulating cell growth, division, stress, and cytokine-mediated cellular responses; they are involved in signal pathways that regulate inflammatory mediators through transcription factor activation. To determine the mechanism of inhibitory effect of *S. horneri* extract on inflammatory mediators, I confirmed that the BV-2 microglial cells inhibited the activation of the LPS-induced MAPKs. p38 and ERK phosphorylation were increased in BV-2 microglial cells stimulated by LPS for 15, 30, and 45 min, but this was inhibited by *S. horneri* extract (Fig. 11A.). NF-κB is a transcription factor that regulates the intracellular synthesis of various molecules including the expression of inflammatory cytokines and iNOS. So, I asked whether the anti-inflammatory activity of *S. horneri* extract was due to inhibited NF-κB activity. Phosphorylation of p65 was increased in the cytosol of LPS-stimulated BV-2 cells after 15, 30, and 45 min, but p65 phosphorylation was decreased in the nucleus. This suggests that *S. horneri* extract inhibited NF-κB migration to the nucleus upon activation (Fig. 11B.). Collectively, these results indicate that the inhibitory effect of the *S. horneri* extract on pro-inflammatory cytokines production in LPS-induced BV-2 cells is mediated by p38, ERK, and NF-κB signaling.

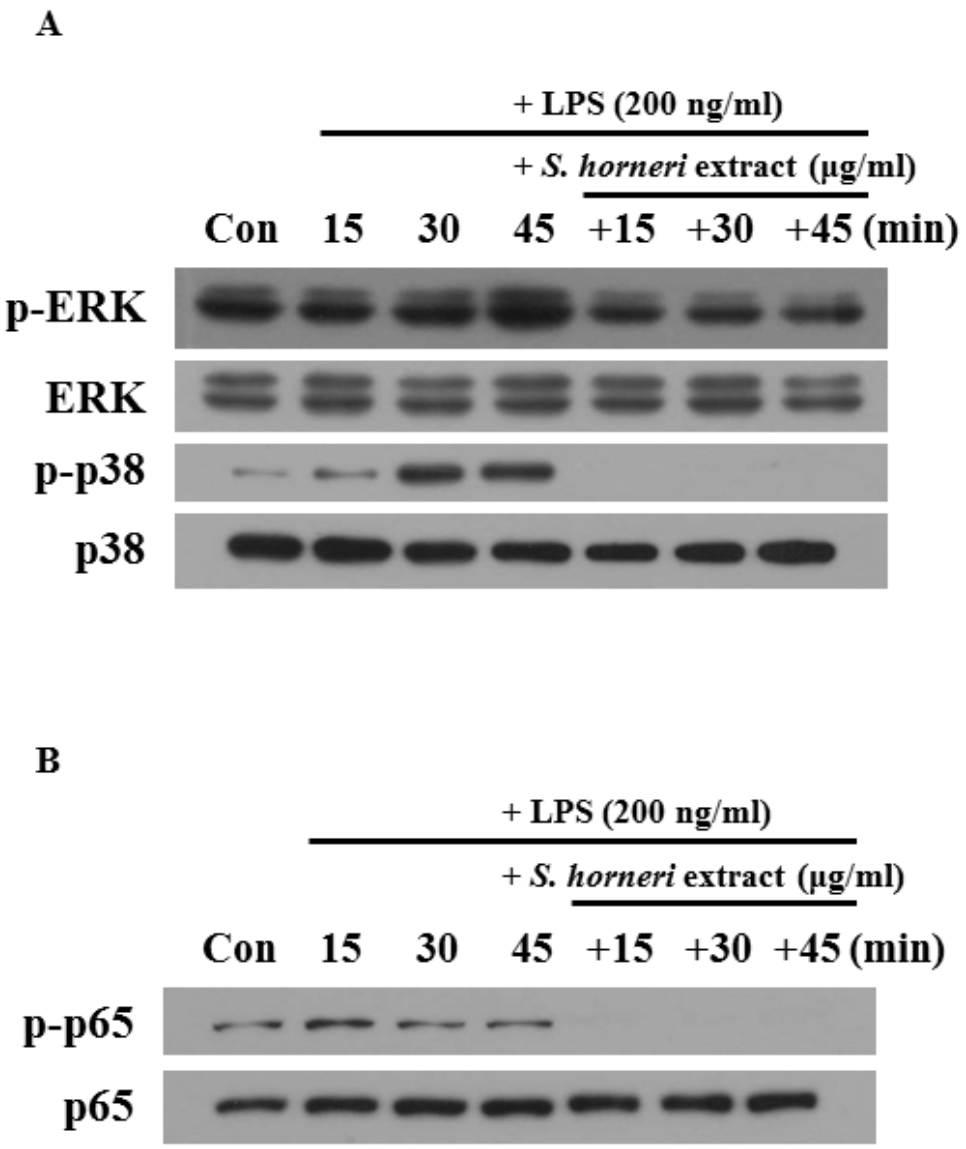


Figure 11. (Continued)

Figure 11. The effect of *S. horneri* extract on phosphorylation of ERK, p38, and (NF-κB, p65) in LPS-stimulated BV-2 microglial cells. BV-2 microglial cells (2 x 10^6 cells/dish) were seeded in 60 mm culture dish in DMEM. Cultured cells were pre-treated with various concentrations of *S. horneri* extract (0, 25, 50, 100, 200, and 300 μg/ml) for 2 h, and exposed to LPS (200 ng/ml). The expression of phosphorylated proteins was determined by western blot analysis. Total extract were measured with related antibodies specific for p-ERK1/2, p38 MAPKs, and p-p65 (NF-κB). β-actin was used as loading control for total fraction.

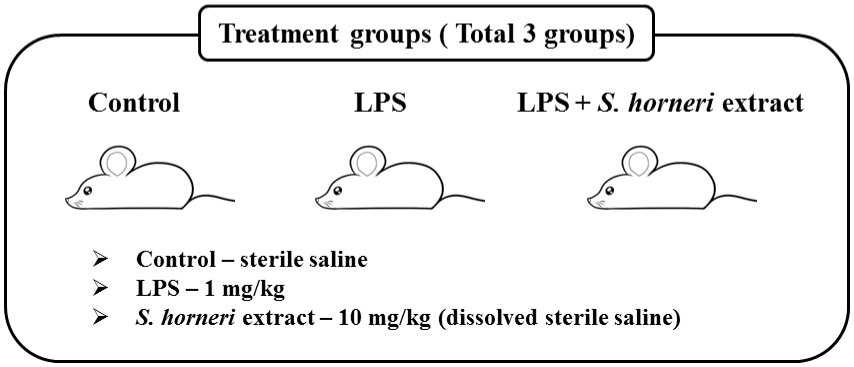
6. *S. horneri* extract attenuates astrocyte and microglia activation in LPS-administrated mouse brain.

In order to investigate the anti-neuroinflammatory effect of *S. horneri* extract, experiments were conducted in mice brains through animal experiments. Astrocytes and microglial cells, which reside in the brain stimulated by LPS, were identified by immunostaining in the mice brain. GFAP, used as a marker protein of astrocytes and Iba-1, which is a marker protein of microglia, were expressed higher in LPS-injected groups in hippocampus than in the control group. The activation of LPS-induced astrocytes and microglia in hippocampus was significantly reduced in the *S. horneri* extract-administered group (Fig. 12.). These results suggest that *S. horneri* extract has anti-neuroinflammatory effect by inhibiting the activation of astrocytes and microglia.

A

Day 0 ~ 2 (3 days)	Day 3	Day 4
S. horneri extract treatment by I.P.	LPS sensitization	Sacrifice

- **Day 0 – starting experiment**
- **Day 3 – LPS injection ; intraperitoneal injection (I.P.)**
- **Day 4 – sacrifice**



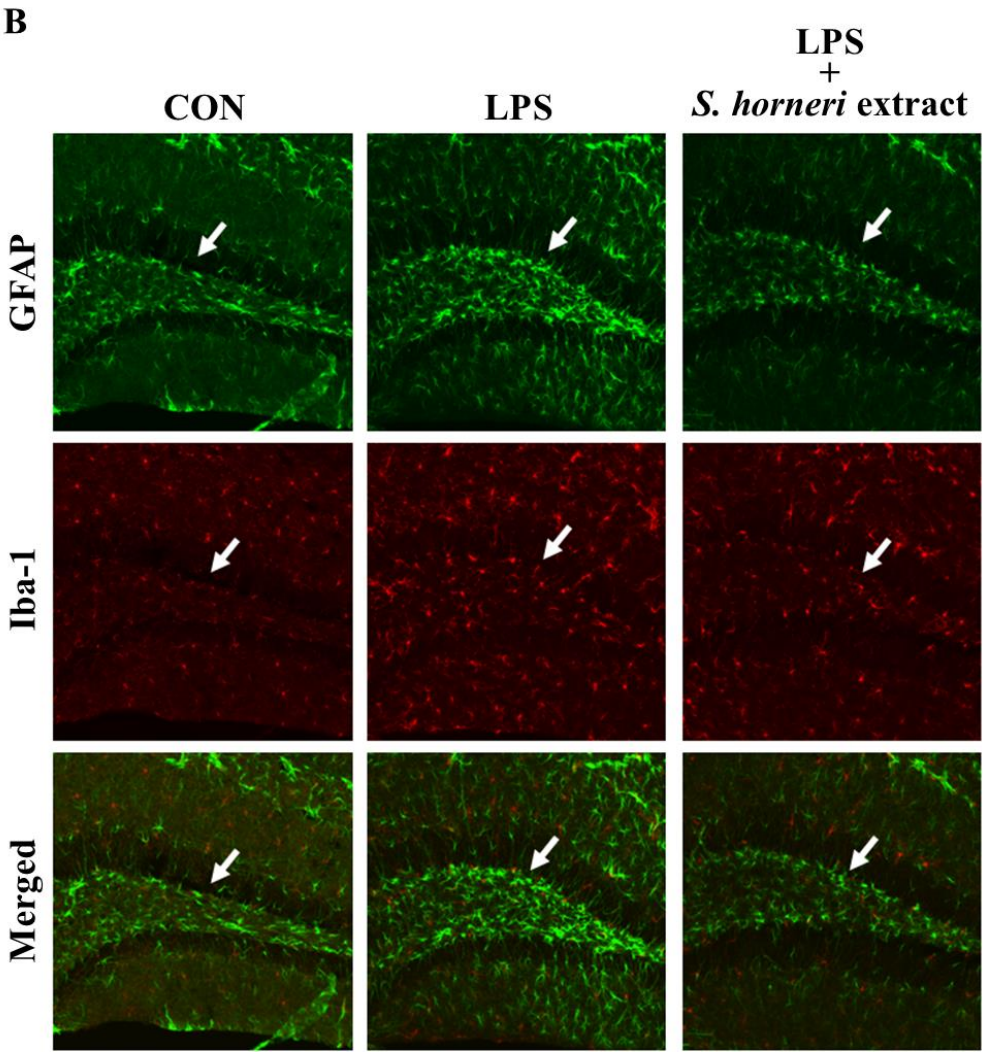


Figure 12. The effect of *S. horneri* extract on astrocyte and microglia activation in LPS-challenged mice brain. C57BL/6 wild type mice were randomly divided into three groups. The control group injected the same amount of solvent used to dissolve the *S. horneri* extract. In the drug-treated groups, in vivo studies were designed by administering LPS (1 mg/kg) and *S. horneri* extract (10 mg/kg).

Part II. Redox status regulation restores oxidative stress-induced dendritic cell dysfunction.

1. Oxidative stress induces DCs death

First, I investigated the cytotoxicity of oxidative stress on DCs. DCs were treated with various concentrations of H_2O_2 (0, 100, 250, and 500 μM) for 24 h and analyzed to an Annexin V/PI staining. Annexin V binds to the cell membrane where apoptosis proceeds. In apoptosis progresses, the cells expose the internal PS to outside and Annexin V binds to PS. PI is a marker of necrosis and binds to the nucleus. As shown in Figure 13, low levels of oxidative stress was not cytotoxic but showed high oxidative stress levels cytotoxicity. These results suggest that the concentration of oxidative stress is an important factor for cytotoxicity.

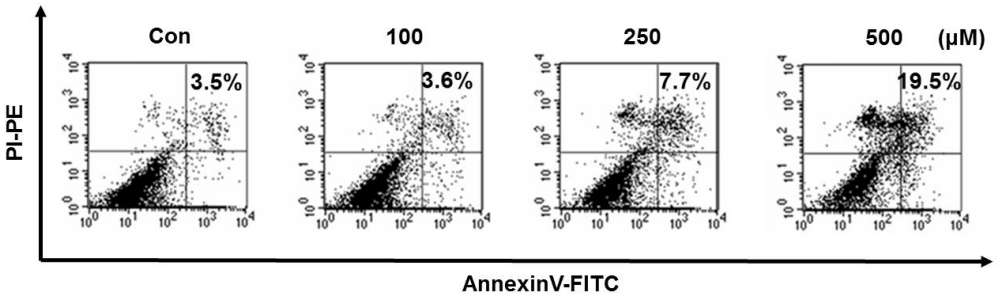


Figure 13. Oxidative stress reduces the viability of DCs in a dose-dependent manner. DCs were differentiated in the same way as the *Materials and Methods*. The DCs (2 x 10^6 cells/well) were seeded in 6-well culture plates with RPMI 1640 containing rmGM-CSF (20 ng/ml). On day 6, DCs were treated with H_2O_2 for 24 h and stained with Annexin V–FITC and PI–PE. Fluorescence intensity was measured by FC500 flow cytometry and presented as a percentage of each quadrant.

2. Oxidative stress increases ROS production in DCs.

Since ROS is known as the key to oxidative stress, I measured the amount of intracellular ROS production in DC by oxidative stress. I determined intracellular ROS production by DCFDA measurement. DCFDA is diffused in to cells, oxidized by oxidative radicals, and de-acetylated, resulting in the conversion of DCF to fluorescent materials. Through this, the level of ROS generated in cells could be measured. DCs were differentiated in media containing GM-CSF. On day 6, Various concentration of H_2O_2 (100, 250, and 500 μM) were treated to DCs to induce oxidative stress. As shown in Fig. 14, as the concentration of oxidative stress increased, the amount of intracellular ROS production was increased. Therefore, it was confirmed that cytotoxicity was shown by the generation of ROS.

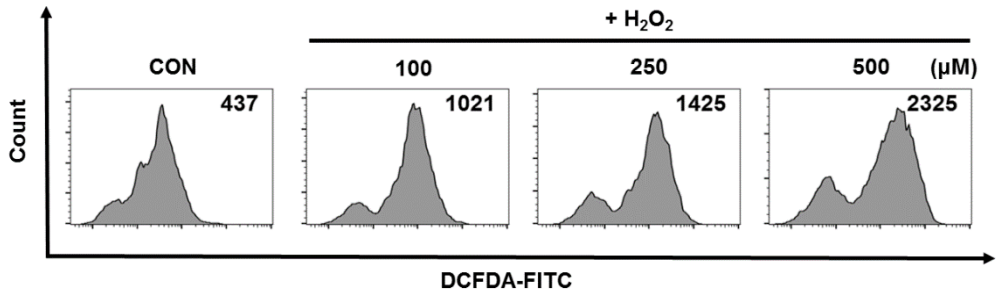


Figure 14. Oxidative stress increases the intracellular ROS production of DCs in a dose-dependent manner. DCs were differentiated in the same way as the *Materials and Methods*. DCs were treated with H_2O_2 (100, 250, and 500 μM) for 1 h to induce oxidative stress, and DCF-DA was treated after 30 min of H_2O_2 treatment and reacted for 30 min. ROS production level was measured by FC500 flow cytometry analysis using DCF-DA. The result is representative of repeated three independent experiments.

3. High level of oxidative stress reduces DCs phenotypical function.

In previous experiments, the association between cytotoxicity and ROS production was confirmed. Next, I investigated whether oxidative stress regulates the expression of co-stimulatory molecules of DCs. BMDCs from monocytes were differentiated into media containing GM-CSF for 6 days. On day 6, H_2O_2 was treated with 100, 250, and 500 μM of DCs to give oxidative stress. Then, the protein expression levels of co-stimulatory molecules such as CD80 (B7-1), CD86 (B7-2), MHC class I, and MHC class II were determined in oxidative stress-stimulated DCs. As shown in Fig. 15, low levels of oxidative stress increased the expression of costimulatory molecules, while high levels of oxidative stress decreased the expression of costimulatory molecules. These results suggest that oxidative stress affects the expression of costimulatory molecules and cytotoxicity in DCs.

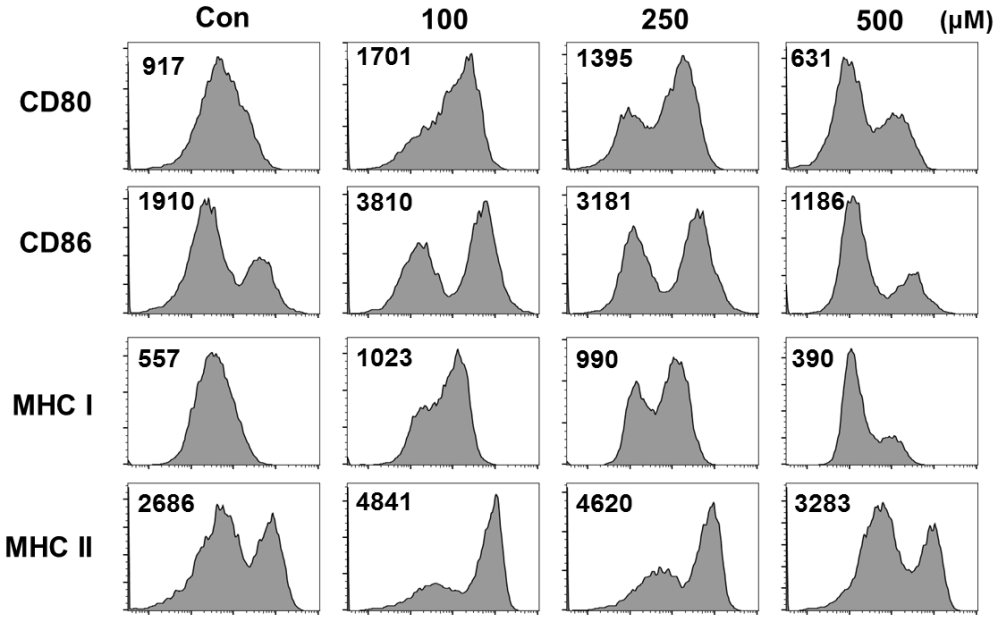


Figure 15. High level of oxidative stress reduces the expression of DCs surface markers. DCs were differentiated in the same way as the *Materials and Methods*. DCs (2×10^6 cells/well) were seeded in 6-well culture plate in RPMI 1640 containing rmGM-CSF (20 ng/ml). On day 6, DCs were stimulated H_2O_2 (100, 250, and 500 μM) for 12 h to induce oxidative stress. Co-stimulatory molecules in DCs were analyzed by FC500 flow cytometry in two-colors (CD11c-FITC, CD80and86, MHC I and II-PE).

4. Oxidative stress reduces the IL-12 production on LPS-stimulated DCs.

IL-12 is known to be an important marker of DCs activation and may be the basis for polarization of Th1/Th2 in determining phenotype. Therefore, I tried to measure the amount of IL-12 produced by oxidative stress in LPS-stimulated DCs (Fig. 16.). DCs (2×10^6 cells/well) were seeded in 6-well culture plate in RPMI 1640 containing rmGM-CSF (20 ng/ml). Cultured DCs were treated with H_2O_2 (100, 250, and 500 μM) for 24 h in the presence of LPS (100 ng/ml). Harvested cells were fixed and permeabilized through Cytofix/Cytoperm kit according to manufacturer's instructions. As shown in Fig. 16, intracellular staining of $CD11c^+$ DCs with PE-conjugated anti-IL-12p40/p70 mouse antibodies showed that LPS (100 ng/ml) and H_2O_2-induced oxidative stress (100, 250, and 500 μM) co-treated group expressed lower level of IL-12p40/p70 production compared with LPS alone group. These results indicate that oxidative stress impairs IL-12 production levels in LPS-stimulated DCs.

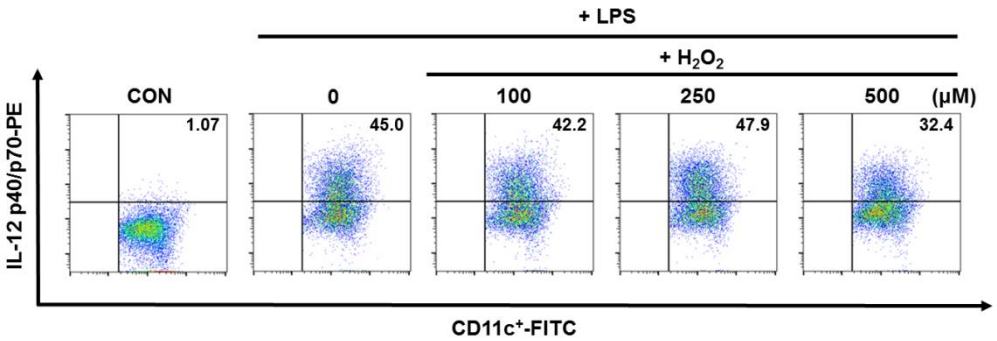


Figure 16. Oxidative stress impairs IL-12 production during LPS-stimulated DCs maturation. Cultured DCs were differentiated for 6 days using the previous *Materials & Methods*. DCs (2×10^6 cells/well) were seeded in 6-well culture plate and treated with H_2O_2 (100, 250, and 500 μM) for 24 h in the presence or absence of LPS (100 ng/ml). Cultured cells were measured by intracellular cytokine staining and gated for $CD11c^+$ DCs.

5. Oxidative stress changes antigen uptake capacity of DCs.

The altered expression of surface molecules of DCs and the alteration of IL-12 production in response to oxidative stress have been shown to affect DCs phenotype and functional maturation. However, these results don't exclude the possibility that oxidative stress induces the inhibition of the physiological function of DCs. Therefore, I confirmed the change of DCs antigen capture ability by induced oxidative stress. DCs (2×10^6 cells/well) were seeded in 6-well culture plate in RPMI 1640 containing rmGM-CSF (20 ng/ml). Cultured cells were treated with the various concentrations H_2O_2 (0, 100, 250, and 500 μM) in presence of LPS (100 ng/ml). H_2O_2 was pretreated for 2 h and LPS was treated. After 24 h, BM-DCs were harvested and stained with dextran-FITC into 4℃ and 37℃. As shown in Fig. 17, as the level of oxidative stress increased, it was confirmed that the antigen uptake capacity was increased in DCs. In addition, the percentage at DCs-stimulated with LPS was lower than the control, and DCs given oxidative stress, showed higher endocytic capacity than DCs-stimulated with LPS. These results show that the change in antigen uptake capacity by oxidative stress is due to cell membrane or functional damage rather than activation of DCs.

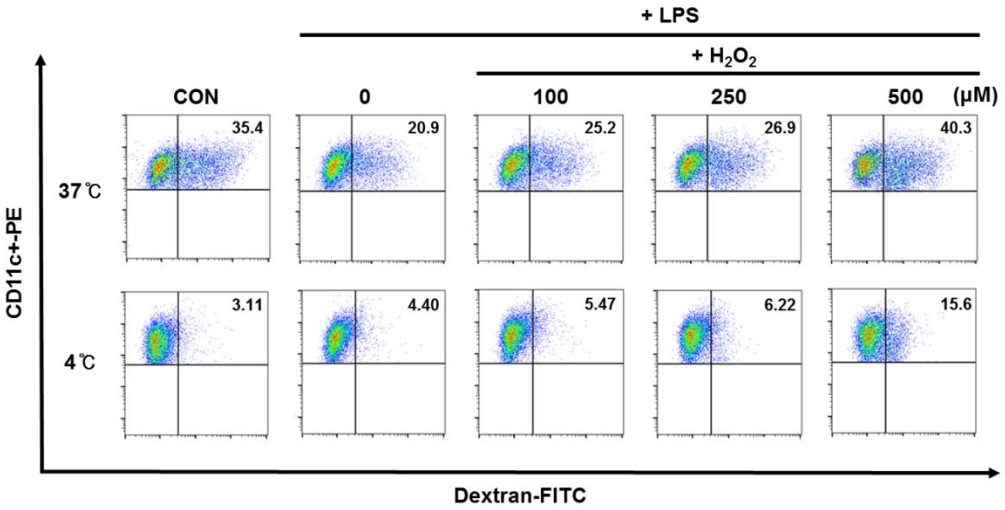


Figure 17. Oxidative stress induces high endocytic capacity in DCs. Cultured DCs were differentiated for 6 days using the previous *Materials & Methods*. DCs (2×10^6 cells/well) were seeded in 6-well culture plate in RPMI 1640 containing rmGM-CSF (20 ng/ml). Cells were treated with or without 100 ng/ml LPS for 24 h following pre-treatment H_2O_2 (100, 250, and 500 μM). The capacity of uptake of FITC-conjugated dextran and PE-conjugated $CD11c^+$ DCs was measured by FC500 flow cytometry. Stained FITC-dextran at 4℃ was used as a negative control (non-specific binding). The ability of antigen uptake capacity was represented as the number of FITC-dextran and PE-$CD11c^+$ double positive cells.

6. Low level of oxidative stress enhances DCs-mediated $CD4^+$ T cells proliferation.

To determine how oxidative stress regulates the maturation of DCs to stimulate allogeneic $CD4^+$ T cells, DCs were incubated with oxidative stress for 24 h and incubated with allogeneic $CD4^+$ T cells. As a result, $CD4^+$ T cells exposed to LPS-stimulated DCs showed a larger proliferative response than those cultured with control DCs (Fig. 18.). However, $CD4^+$ T cells by DC stimulated with low levels of oxidative stress increased proliferation, but it is necessary to confirm whether they induce an immune response by antigen specificity.

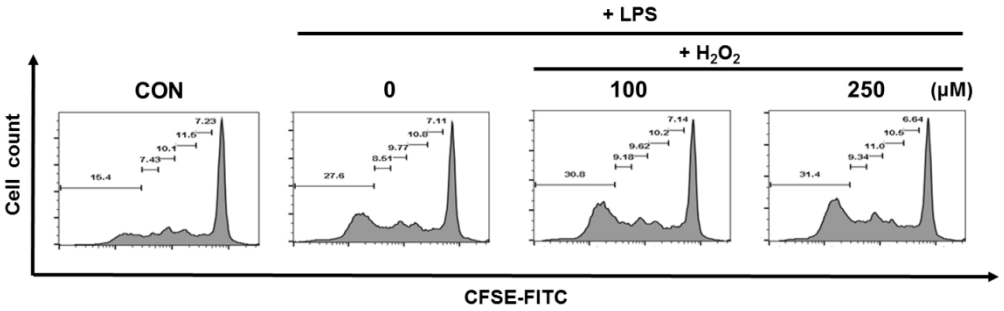


Figure 18. Oxidative stress changed DCs-mediated $CD4^+$ T cells proliferation. Differentiated DCs were treated with H_2O_2 (100, 250, and 500 μM) induced oxidative stress for 24 h in the presence or absence of LPS (100 ng/ml). Isolated T cells were stained with 1.25 μM CFSE. Cells were washed with washing buffer and co-cultured with allogeneic $CD4^+$ T cells, and the mixed leukocyte reaction was incubated for 3 days. After 3 days of co-cultured, the mixed cells were stained with a Cy7-CD3 antibody and measured by FC500 flow cytometry.

7. Oxidative stress impairs mitochondrial membrane potential (MMP, Ψm) of DCs.

Intracellular production of ROS, an important component of oxidative stress, was confirmed, and it was determined that high level of oxidative stress impairs the expression of DCs surface molecules. In addition, the production of IL-12, which may be the basis for the phenotype and polarization of DCs, was impaired, resulting in a reduction in antigen uptake due to DCs physiological functions. Mitochondria is a major factor of oxidative stress as a major producer of ROS, it was confirmed whether mitochondrial-mediated dysfunction appeared in oxidative stress induced DCs. Next, the change of MMP by oxidative stress was attempted. DCs were differentiated by incubating for 6 days in the media containing GM-CSF. On day 6, DCs induced oxidative stress at a concentration of 500 μM of H_2O_2 and treated with timely. After oxidative stress induced at various times (15, 30, 60, 120, and 180 min), TMRM fluorescence was stained for 30 min. As shown in Fig. 19, result showed that H_2O_2 fixed at the highest concentration and treated in time course, MMP was decreased. These results indicate that oxidative stress induced DCs cytotoxicity was confirmed by the change of MMP.

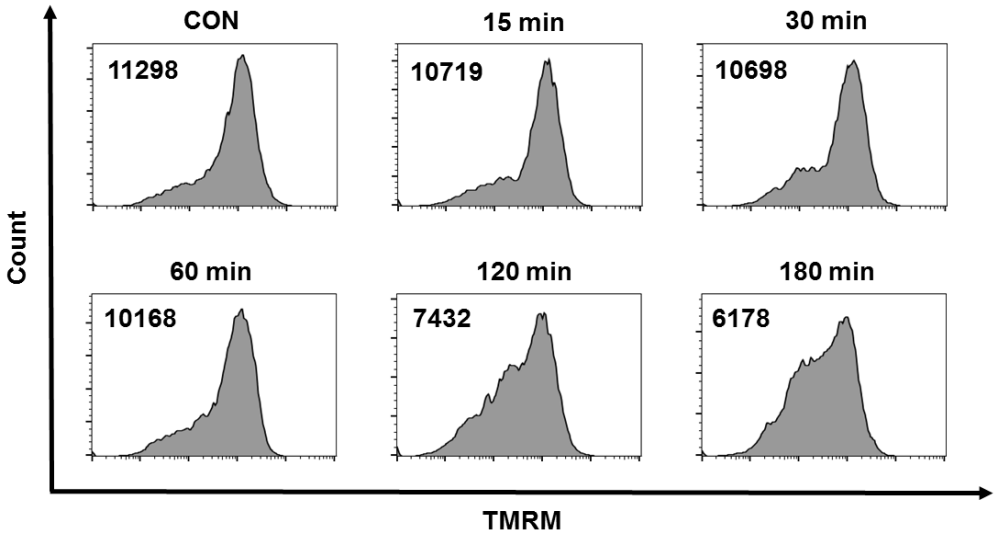


Figure 19. Oxidative stress impairs MMP (Ψm). DCs were differentiated for 6 days using the previous *Materials and Methods*. DCs (2×10^6 cells/well) were seeded in 6-well culture plate in RPMI 1640 containing rmGM-CSF (20 ng/ml). DCs were treated timely (15, 30, 60, 120, and 180 min) with H_2O_2 fixed at a maximum concentration of 500 μM. After oxidative stress induced, TMRM fluorescence was stained at 25 nM for 30 min and analyzed by FC500 flow cytometry.

8. Oxidative stress induces DCs death via mitochondrial-mediated apoptotic pathway.

Since oxidative stress caused a change in MMP and showed cytotoxicity of DCs, I tried to identify apoptosis-related signaling proteins cause by oxidative stress. DCs (5×10^6 cells/dish) were seeded in 100 mm culture dish in RPMI 1640 containing rmGM-CSF (20 ng/ml). Cultured cells stimulated H_2O_2 (100, 250, and 500 μM) treatment for 24 h in RPMI 1640 (contained rmGM-CSF). Oxidative stress triggered DCs apoptosis via induced the expression of mitochondria-mediated apoptotic proteins, including caspase, Bax, and Bcl-2. As shown in Fig. 20, oxidative stress not only increased Bax expression but also activated caspase 3 and caspase 9.

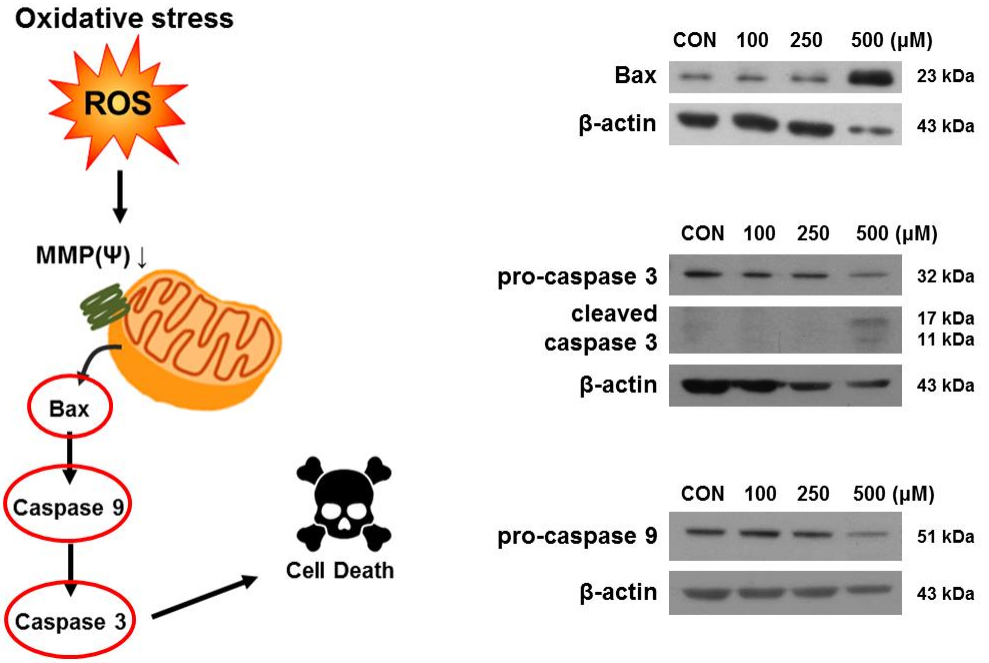


Figure 20. Oxidative stress triggers DCs death via mitochondrial-mediated apoptotic proteins, including bax, caspase 3, and caspase 9. DCs (5×10^6 cells/dish) were seeded in 100 mm culture dish in RPMI 1640 containing rmGM-CSF (20 ng/ml). DCs that differentiated for 6 days, cultured cells were treated with various concentration of H_2O_2 (100, 250, and 500 μM) for 24 h. The expression of mitochondria-mediated proteins was determined western blot analysis. The extract were confirmed with related antibodies specific for Bax, caspase3 and caspase9, β-actin was used as loading control for total fraction.

9. Treatment of NAC inhibits oxidative stress-induced DCs death.

Usually, antioxidants work after cysteine is transported by the Alanine-Serine-Cysteine (ASC) system and absorbed into cells. However, N-Acetyl-L-Cysteine (NAC) does not require any active transport to deliver cysteine to the cell. The antioxidant NAC, known as a cell-permeable compound, is absorbed into the cell and quickly hydrolyzed, regenerating glutathione (GSH) to regulate the redox state and destroy H_2O_2. Therefore, NAC, which has an antioxidant effect through the direct removal of free radicals and the production of GSH and cysteine, was a good candidate for antioxidant. DCs were differentiated by incubating for 6 days in the media containing GM-CSF. On day 6, DCs induced oxidative stress at a concentration of 500 μM of H_2O_2 and treated with antioxidant NAC. The antioxidant NAC was pretreated with 1, 2, and 5 mM for 1 h. As shown in Fig. 21, when I treated with antioxidant NAC, DCs death rate was restored (Fig. 21.). These results suggest that NAC treatment restores oxidative stress-induced cell death.

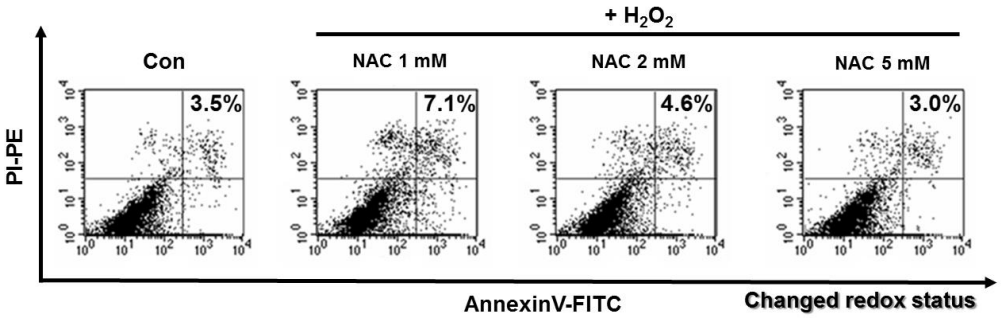


Figure 21. Treatment of NAC restores the cell death in oxidative stress-induced DCs. DCs were differentiated in the same way as the *Materials and Methods*. On day 6, DCs were treated with high concentration of H_2O_2 (500 μM) for 24 h and stained with Annexin V – FITC and PI – PE. The antioxidant NAC was pretreated with 1, 2, and 5 mM for 1 h. Fluorescence intensity was measured by flow cytometry FC500 and presented as a percentage of each quadrant.

10. NAC treatment decreases ROS production in oxidative stress-induced DCs.

Next, I determined whether antioxidant NAC could reduce the production of increased DCs intracellular ROS in oxidative stress induced DCs. DCs were differentiated in media containing GM-CSF. On day 6, the DCs were treated with different concentration of H_2O_2 (100, 250, and 500 μM) to induce oxidative stress. NAC, an antioxidant, was pretreated to a concentration of 2 mM 1 h before H_2O_2 treatment. As shown in Fig. 22, changed redox status by NAC treatment, ROS level was decreased and similar to basal level. Therefore, this results show that the production of DCs intracellular ROS was decreased in a dose dependent manner by NAC treatment.

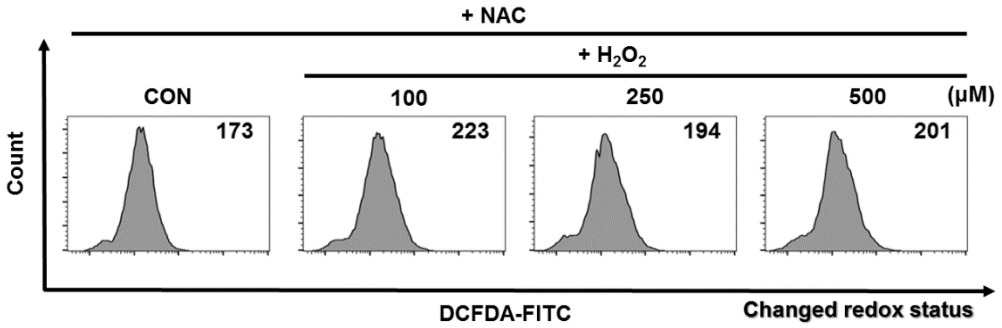


Figure 22. NAC treatment reduces oxidative stress-induced increased intracellular ROS production. DCs were differentiated for 6 days using the previous *Materials and Methods*. DCs were treated with H_2O_2 (100, 250, and 500 μM) for 1 h to induce oxidative stress, and DCF-DA was treated after 30 min of H_2O_2 treatment and reacted for 30 min. NAC, an antioxidant, was pretreated to a concentration of 2 mM 1 h before H_2O_2 treatment. ROS production level was measured by FC500 flow cytometry analysis using DCF-DA. The result is representative of repeated three independent experiments.

11. NAC inhibits MMP (Ψm) damage in oxidative stress-induced DCs.

I have previously identified mitochondrial-mediated dysfunction in oxidative stress-induced DCs. Therefore, it was confirmed that mitochondrial-mediated dysfunction induced by oxidative stress was recovered by treatment with the antioxidant NAC. DCs were differentiated by incubating for 6 days in the media containing GM-CSF. On day 6, DCs induced oxidative stress at a concentration of 500 μM of H_2O_2 and treated with timely. Antioxidant NAC was pretreated for 1 h before oxidative stress induced. As shown in Fig. 23, MMP (Ψm) impaired by mitochondrial-mediated dysfunction due to oxidative stress was found to be recovered by treatment with the antioxidant NAC. These results confirmed that oxidative stress induced MMP (Ψm) and cell death through the induction of mitochondrial dysfunction but was restored by NAC treatment (Fig. 23.).

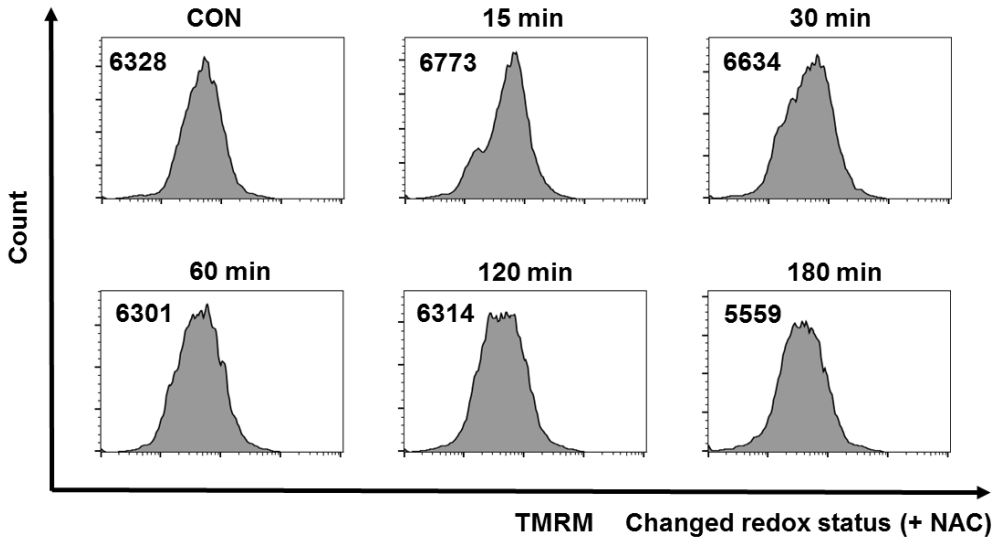


Figure 23. Treatment of NAC restores oxidative stress-induced impaired MMP (Ψm). DCs were differentiated in the same way as the *Materials and Methods.* DCs (2 x 10^6 cells/well) were seeded in 6-well culture plate and treated with high concentration of H_2O_2 (500 μM). Antioxidant NAC was pretreated for 1 h before oxidative stress induced. After oxidative stress induced, TMRM fluorescence was stained at 25 nM for 30 min and analyzed by FC500 flow cytometry.

12. NAC treatment restores the co-stimulatory molecules expression of DCs.

I attempted to determine whether the changes of DC costimulatory molecules cause by oxidative stress were restored by NAC treatment. BMDCs from monocytes were differentiated into media containing GM-CSF for 6 days. On day 6, H_2O_2 was treated with 100, 250, and 500 μM of DCs to give oxidative stress. Then, the expression levels of co-stimulatory molecules such as CD80 (B7-1), CD86 (B7-2), MHC class I, and MHC class II were determined in oxidative stress-stimulated DCs. As shown in Fig. 24, it was determined that the decrease of costimulatory molecules expression of DC by high concentration of oxidative stress was recovered by NAC treatment.

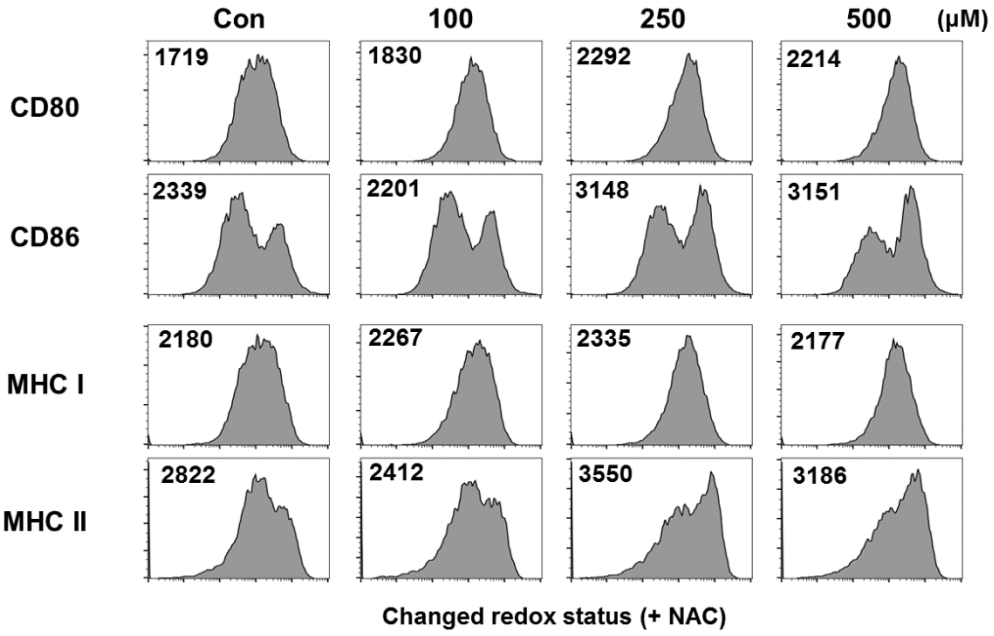


Figure 24. Treatment of NAC restores reduced expression of surface molecules in oxidative stress-induced DCs. DCs were differentiated in the same way as the *Materials and Methods.* DCs (2 x 10^6 cells/well) were seeded in 6-well culture plate in RPMI 1640 containing rmGM-CSF (20 ng/ml). On day 6, DCs were stimulated H_2O_2 (100, 250, and 500 μM) for 12 h to induce oxidative stress. Antioxidant NAC was pretreated with 2 mM for 1 h. Co-stimulatory molecules in DCs were analyzed by FC500 flow cytometry in two-colors (CD11c-FITC, CD80 and 86, MHC I and II-PE).

IV. DISCUSSION

Antigen-presenting cells (APCs) play a role in initiating the immune system by presenting externally invading antigens or pathogens (Knight & Stagg, 1993). In addition, it is known the play a role of messenger connecting the innate and adaptive immune response through antigen presentation (Kapsenberg, Teunissen, Stiekema, & Keizer, 1986). However, not all APCs regulate the immune response in the same way. Therefore, in this study, I identified two functions of morphologically and functionally different antigen-presenting cells as immune regulators.

In part I, in the first study, I studied to find neuroinflammatory inhibitors through the regulation of microglia. *Sargassum horneri* (Turner) C. Agardh is seaweed that exerts various effects in the body. Previous studies reported that *S. horneri* (Turner) C. Agardh can prevent osteoporosis and help control cholesterol, blood pressure, and hyperlipidemia (M. J. I. J. F. S. N. D. Yamaguchi, 2013) (Son et al., 2019). Cosmetics and functional foods containing *S. horneri* (Turner) C. Agardh are known to have anti-aging, anti-allergic, anti-acne, and whitening effects (Jesumani et al., 2019). However, no mechanism has been described for how it could regulate murine microglia activity. I investigated the effect of *S. horneri* extract isolated from *S. horneri* (Turner) C. Agardh on inflammatory mediator production. Stimulation of the murine microglial cell line BV-2 enhanced iNOS and NO expression. NO is a highly reactive factor involved in vasorelaxation, neurotransmission, and cell-mediated immune responses. NO production mediates the inflammatory response and exerts cytotoxic activity against external pathogens. LPS-induced BV-2 cells act as inflammatory mediators downstream of NO to induce pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α; they also enhance inflammation by secreting COX-2. However, NO

overexpression aggravates various inflammatory diseases. Therefore, substances that inhibit inflammatory mediators that produce NO and pro-inflammatory cytokines may be useful for treating various immune diseases. MAPKs include JNK, ERK, and p38 and play important roles in various cellular processes such as cell differentiation, proliferation, and inflammation (Zhang & Liu, 2002). Inactive MAPK remains in the cytosol and it activated by phosphorylation in response to LPS or other stimuli, at which point it translocates to the nucleus and stimulates cytokine production (Plotnikov, Zehorai, Procaccia, & Seger, 2011). In this study, *S. horneri* extract pretreatment reduced ERK and P38 activities.

NF-κB plays important roles in inflammatory reactions and in various immune responses, tumorigenesis, and autoimmune diseases (O'Neill & Kaltschmidt, 1997). NF-κB is present in the cytoplasm in an inactive state bound to Iκ-Bα, but in response to external stimuli such as LPS, Iκ-Bα is phosphorylated, and the separated NF-κB translocate to the nucleus where it is involved in the production of NO and pro-inflammatory cytokines (Magnani, Crinelli, Bianchi, & Antonelli, 2000). In this study, I observed that NF-κB migration into the nucleus was decreased following pretreatment with *S. horneri* extract.

Overall, our results show that *S. horneri* extract isolated from *S. horneri* (Turner) C. Agardh inhibited the activation of MAPK and NF-κB pathways including ERK and p38 in LPS-activated BV-2 cells, thereby inhibiting NO and the pro-inflammatory cytokines IL-6 and TNF-α. These findings indicate that *S. horneri* extract may be an effective treatment for inflammatory diseases.

In part II, in the second study, I examined whether DCs dysfunctions could be modulated by regulating the redox status. Free radicals produced in the human body have a positive effect when the right time, position and amount are produced, and our

body's immune mechanism can eliminate the toxicity caused by free radicals (Slater, 1988). Excessive exercise in our bodies also leads to the ability to rapidly increase or eliminate the production of ROS, leading to various diseases caused by harmful oxygen (Cadenas, Davies, & Medicine, 2000). Therefore, the side effect of such harmful oxygen is called oxidative stress. Oxidative stress caused by these free radicals has been reported to induce aging-related diseases (H. Sies, 2000)

Therefore, oxidative stress caused by free radicals was selected as the most representative aging model for immune senescence (Feng, He, Ochi, & development, 2001). The functional decline in immune responses that can occur through aging-related diseases has the same effect as dysfunction of adaptive immune response. For reason of this, I focused on DCs, which functions as a control tower by fundamentally regulating and activating T cells involved in adaptive immune response. Oxidative stress induced DCs death in a dose-dependent manner. In addition, the expression of intracellular ROS of DCs was increased by oxidative stress. As a result of confirming the maturation of DCs through expression of co-stimulatory molecules, it was determined that functional dysfunction occurred due to the decrease of the expression of surface markers at high level but increased at low level of oxidative stress. As the physiological function of DCs, when the antigen uptake ability was confirmed, the antigen uptake ability changed by oxidative stress. This could lead to damage to the dextran-mannose receptor or impair to the cell membrane. IL-12 production of DCs is important marker of DCs activation and involved in the T cell priming and the polarization of Th1/Th2 (Merrick et al., 2005). as a result of measuring the amount of IL-12 produced, it was determined that the amount of IL-12 production was reduced by oxidative stress. In addition, I investigated the signaling pathways for oxidative stress-induced DCs dysfunction and death. Oxidative stress impairs DCs viability through the

induction of mitochondrial mediated apoptotic pathways. NAC, a cysteine precursor, has been reported to promote the production of GSH and directly eliminate free radicals (Lavoie et al., 2008). In addition, it has been reported that inhibition of free radical oxidative stress-related activation is regulated through changes in the redox status. Therefore, I confirmed that NAC treatment could modulate the functional change of DCs by oxidative stress. As a result, NAC treatment restored the oxidative stress-induced cytotoxicity, ROS production, and MMP. Moreover, NAC treatment regulated the co-stimulatory molecules expression of DCs in oxidative stress.

In conclusion, microglia and DCs are APCs that play an important role in regulating immune responses in neuroinflammation and oxidative stress, and suggest that they can be important factors in the prevention and treatment of neurodegenerative and immunesenescence-related diseases.

V. REFERENCES

Abbas, A. K., Benoist, C., Bluestone, J. A., Campbell, D. J., Ghosh, S., Hori, S., . . . Roncarolo, M. G. (2013). Regulatory T cells: recommendations to simplify the nomenclature. *Nature immunology, 14*(4), 307.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Lymphocytes and the cellular basis of adaptive immunity. In *Molecular Biology of the Cell. 4th edition*: Garland Science.

Benvenuti, F., Lagaudrière-Gesbert, C., Grandjean, I., Jancic, C., Hivroz, C., Trautmann, A., . . . Amigorena, S. J. T. J. o. I. (2004). Dendritic cell maturation controls adhesion, synapse formation, and the duration of the interactions with naive T lymphocytes. *172*(1), 292-301.

Bierer, B. E., Sleckman, B., Ratnofsky, S., & Burakoff, S. (1989). The biologic roles of CD2, CD4, and CD8 in T-cell activation. *Annual review of immunology, 7*(1), 579-599.

Block, M. L., Zecca, L., & Hong, J. S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci, 8*(1), 57-69. doi:10.1038/nrn2038

Bonilla, F. A., & Oettgen, H. C. (2010). Adaptive immunity. *Journal of Allergy and Clinical Immunology, 125*(2), S33-S40.

Broux, B., Gowing, E., & Prat, A. (2015). Glial regulation of the blood-brain barrier in health and disease. *Semin Immunopathol, 37*(6), 577-590. doi:10.1007/s00281-015-0516-2

Buttke, T. M., & Sandstrom, P. A. J. F. r. r. (1995). Redox regulation of programmed cell death in lymphocytes. *22*(5), 389-397.

Cadenas, E., Davies, K. J. J. F. R. B., & Medicine. (2000). Mitochondrial free radical generation, oxidative stress, and aging. *29*(3-4), 222-230.

Calabrese, V., Cornelius, C., Maiolino, L., Luca, M., Chiaramonte, R., Toscano, M., & Serra, A. J. N. r. (2010). Oxidative stress, redox homeostasis and cellular stress response in Meniere's disease: role of vitagenes. *35*(12), 2208-2217.

Campbell, J., Strohbehn, R., Weaver, E., Borg, B., Russell, L., Pozo, F. P., . . . Quigley, J. (2003). Methods and compositions for treatment of immune dysfunction disorders. In: Google Patents.

Carbone, J., & Ohm, D. J. O. (2002). Immune dysfunction in cancer patients. *16*(1 suppl 1), 11-18.

Chan, C. K., Tan, L. T., Andy, S. N., Kamarudin, M. N. A., Goh, B. H., & Kadir, H. A. (2017). Anti-neuroinflammatory Activity of Elephantopus scaber L. via Activation of Nrf2/HO-1 Signaling and Inhibition of p38 MAPK Pathway in LPS-Induced Microglia BV-2 Cells. *Front Pharmacol, 8*, 397. doi:10.3389/fphar.2017.00397

Chen, C.-H., Floyd, H., Olson, N. E., Magaletti, D., Li, C., Draves, K., & Clark, E. A. J. B. (2006). Dendritic-cell-associated C-type lectin 2 (DCAL-2) alters dendritic-cell maturation and cytokine production. *107*(4), 1459-1467.

Chen, T., Razak, A., Bedard, P., Siu, L., & Hansen, A. J. A. o. O. (2015). A systematic review of immune-related adverse event reporting in clinical trials of immune checkpoint inhibitors. *26*(9), 1824-1829.

Chow, A., Toomre, D., Garrett, W., & Mellman, I. J. N. (2002). Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. *418*(6901), 988.

De la Fuente, M., Cruces, J., Hernandez, O., & Ortega, E. J. C. p. d. (2011). Strategies to improve the functions and redox state of the immune system in aged subjects. *17*(36), 3966-3993.

e Sousa, C. R. (2004). Activation of dendritic cells: translating innate into adaptive immunity. *Current opinion in immunology, 16*(1), 21-25.

Etzioni, A. J. A. r. (2003). Immune deficiency and autoimmunity. *2*(6), 364-369.

Feng, R., He, W., Ochi, H. J. M. o. a., & development. (2001). A new murine oxidative stress model associated with senescence. *122*(6), 547-559.

Finkel, T., & Holbrook, N. J. J. N. (2000). Oxidants, oxidative stress and the biology of ageing. *408*(6809), 239.

Fu, R., Shen, Q., Xu, P., Luo, J. J., & Tang, Y. (2014). Phagocytosis of microglia in the central nervous system diseases. *Mol Neurobiol, 49*(3), 1422-1434. doi:10.1007/s12035-013-8620-6

Fujiwara, T., Nishida, N., Nota, J., Kitani, T., Aoishi, K., Takahashi, H., . . . Hato, N. (2016). Efficacy of chlorophyll c2 for seasonal allergic rhinitis: single-center double-blind randomized control trial. *Eur Arch Otorhinolaryngol, 273*(12), 4289-4294. doi:10.1007/s00405-016-4133-z

Geng, Y., Zhu, S., Cheng, P., Lu, Z. M., Xu, H. Y., Shi, J. S., & Xu, Z. H. (2017). Bioassay-guided fractionation of ethyl acetate extract from Armillaria mellea attenuates inflammatory response in lipopolysaccharide (LPS) stimulated BV-2 microglia. *Phytomedicine, 26*, 55-61. doi:10.1016/j.phymed.2017.01.005

Germain, R. N. (2002). T-cell development and the CD4–CD8 lineage decision. *Nature Reviews Immunology, 2*(5), 309.

Gilmore, T. D. J. O. (2006). Introduction to NF-κB: players, pathways, perspectives. *25*(51), 6680.

Good, R. A. J. H. P. (1967). Disorders of the immune system. *2*(1), 38-53.

Gostner, J. M., Becker, K., Fuchs, D., & Sucher, R. J. R. R. (2013). Redox regulation of the immune response. *18*(3), 88-94.

Greenberg, S., & Grinstein, S. (2002). Phagocytosis and innate immunity. *Current opinion in immunology, 14*(1), 136-145.

Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell*, *140*(6), 883-899.

Hung, T. H., Shyue, S. K., Wu, C. H., Chen, C. C., Lin, C. C., Chang, C. F., & Chen, S. F. (2017). Deletion or inhibition of soluble epoxide hydrolase protects against brain damage and reduces microglia-mediated neuroinflammation in traumatic brain injury. *Oncotarget*, *8*(61), 103236-103260. doi:10.18632/oncotarget.21139

Israël, A. J. C. S. H. p. i. b. (2010). The IKK complex, a central regulator of NF-κB activation. *2*(3), a000158.

Jenkins, R. W., Barbie, D. A., & Flaherty, K. T. J. B. j. o. c. (2018). Mechanisms of resistance to immune checkpoint inhibitors. *118*(1), 9.

Jesumani, V., Du, H., Pei, P., Zheng, C., Cheong, K.-L., & Huang, N. J. I. j. o. b. m. (2019). Unravelling property of polysaccharides from Sargassum sp. as an anti-wrinkle and skin whitening property. *140*, 216-224.

Joffre, O., Nolte, M. A., Spörri, R., & Sousa, C. R. e. (2009). Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. *Immunological reviews*, *227*(1), 234-247.

Kapsenberg, M. L. (2003). Dendritic-cell control of pathogen-driven T-cell polarization. *Nature Reviews Immunology*, *3*(12), 984.

Kapsenberg, M. L., Teunissen, M. B., Stiekema, F. E., & Keizer, H. G. J. E. j. o. i. (1986). Antigen-presenting cell function of dendritic cells and macrophages in proliferative T cell responses to soluble and particulate antigens. *16*(4), 345-350.

Kim, J. A., Ahn, B. N., Kong, C. S., & Kim, S. K. (2012). Protective effect of chromene isolated from Sargassum horneri against UV-A-induced damage in skin dermal fibroblasts. *Exp Dermatol*, *21*(8), 630-631. doi:10.1111/j.1600-0625.2012.01535.x

Kim, M. E., Jung, Y. C., Jung, I., Lee, H. W., Youn, H. Y., & Lee, J. S. (2015). Anti-inflammatory effects of ethanolic extract from Sargassum horneri (Turner) C.

Agardh on lipopolysaccharide-stimulated macrophage activation via NF-kappaB pathway regulation. *Immunol Invest, 44*(2), 137-146. doi:10.3109/08820139.2014.942459

Kim, M. E., Kim, H. K., Park, H.-Y., Kim, D. H., Chung, H. Y., & Lee, J. S. (2013). Baicalin from Scutellaria baicalensis impairs Th1 polarization through inhibition of dendritic cell maturation. *Journal of pharmacological sciences, 121*(2), 148-156.

Knight, S. C., & Stagg, A. J. J. C. o. i. i. (1993). Antigen-presenting cell types. *5*(3), 374-382.

Kushwah, R., & Hu, J. J. I. (2011). Complexity of dendritic cell subsets and their function in the host immune system. *133*(4), 409-419.

Lavoie, S., Murray, M. M., Deppen, P., Knyazeva, M. G., Berk, M., Boulat, O., . . . Copolov, D. J. N. (2008). Glutathione precursor, N-acetyl-cysteine, improves mismatch negativity in schizophrenia patients. *33*(9), 2187.

Lee, S.-J., Dimtchev, A., Lavin, M. F., Dritschilo, A., & Jung, M. J. O. (1998). A novel ionizing radiation-induced signaling pathway that activates the transcription factor NF-κB. *17*(14), 1821.

Lehnardt, S. (2010). Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. *Glia, 58*(3), 253-263. doi:10.1002/glia.20928

Li, Y., Li, F., Jiang, F., Lv, X., Zhang, R., Lu, A., & Zhang, G. (2016). A mini-review for cancer immunotherapy: molecular understanding of PD-1/PD-L1 pathway & translational blockade of immune checkpoints. *International journal of molecular sciences, 17*(7), 1151.

Liu, Y.-J., Kanzler, H., Soumelis, V., & Gilliet, M. (2001). Dendritic cell lineage, plasticity and cross-regulation. *Nature immunology, 2*(7), 585.

Liu, Y.-J. J. C. (2001). Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *106*(3), 259-262.

López, C. B., Moltedo, B., Alexopoulou, L., Bonifaz, L., Flavell, R. A., & Moran, T. M. J. T. J. o. I. (2004). TLR-independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses. *173*(11), 6882-6889.

Magnani, M., Crinelli, R., Bianchi, M., & Antonelli, A. J. C. d. t. (2000). The ubiquitin-dependent proteolytic system and other potential targets for the modulation of nuclear factor-kB (NF-kB). *1*(4), 387-399.

Mainiero, F., Soriani, A., Strippoli, R., Jacobelli, J., Gismondi, A., Piccoli, M., . . . Santoni, A. J. I. (2000). RAC1/P38 MAPK Signaling Pathway Controls β1 Integrin–Induced Interleukin-8 Production in Human Natural Killer Cells. *12*(1), 7-16.

Maritim, A., Sanders, a., Watkins Iii, J. J. J. o. b., & toxicology, m. (2003). Diabetes, oxidative stress, and antioxidants: a review. *17*(1), 24-38.

Matarese, G., De Placido, G., Nikas, Y., & Alviggi, C. J. T. i. m. m. (2003). Pathogenesis of endometriosis: natural immunity dysfunction or autoimmune disease? , *9*(5), 223-228.

Matés, J. M., Pérez-Gómez, C., de Castro, I. N., Asenjo, M., Márquez, J. J. T. i. j. o. b., & biology, c. (2002). Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. *34*(5), 439-458.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nature Reviews Immunology, 1*(2), 135.

Medzhitov, R., & Janeway Jr, C. (2000). Innate immunity. *New England Journal of Medicine, 343*(5), 338-344.

Mellman, I. J. C. i. r. (2013). Dendritic cells: master regulators of the immune response. *1*(3), 145-149.

Merrick, A., Errington, F., Milward, K., O'Donnell, D., Harrington, K., Bateman, A., . . . Selby, P. J. B. j. o. c. (2005). Immunosuppressive effects of radiation on human

dendritic cells: reduced IL-12 production on activation and impairment of naive T-cell priming. *92*(8), 1450.

Morganti-Kossmann, M. C., Satgunaseelan, L., Bye, N., & Kossmann, T. (2007). Modulation of immune response by head injury. *Injury, 38*(12), 1392-1400. doi:10.1016/j.injury.2007.10.005

Muzio, M., Polentarutti, N., Bosisio, D., Kumar, P. M., & Mantovani, A. (2000). Toll-like receptor family and signalling pathway. In: Portland Press Ltd.

Nixon, D. E., Landay, A. L. J. C. O. i. H., & AIDS. (2010). Biomarkers of immune dysfunction in HIV. *5*(6), 498.

O'Neill, L. A., & Kaltschmidt, C. J. T. i. n. (1997). NF-kB: a crucial transcription factor for glial and neuronal cell function. *20*(6), 252-258.

Ortona, E., Maselli, A., Delunardo, F., Colasanti, T., Giovannetti, A., Pierdominici, M. J. A., & signaling, r. (2014). Relationship between redox status and cell fate in immunity and autoimmunity. *21*(1), 103-122.

Pardoll, D. M. J. N. R. C. (2012). The blockade of immune checkpoints in cancer immunotherapy. *12*(4), 252.

Parnes, J. R. (1989). Molecular biology and function of CD4 and CD8. In *Advances in immunology* (Vol. 44, pp. 265-311): Elsevier.

Plotnikov, A., Zehorai, E., Procaccia, S., & Seger, R. J. B. e. B. A.-M. C. R. (2011). The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *1813*(9), 1619-1633.

Postow, M. A., Callahan, M. K., & Wolchok, J. D. J. J. o. c. o. (2015). Immune checkpoint blockade in cancer therapy. *33*(17), 1974.

Qi, M., & Elion, E. A. J. J. C. S. (2005). MAP kinase pathways. *118*(16), 3569-3572.

Rasheed, Z., Akhtar, N., Haqqi, T. M. J. A. r., & therapy. (2010). Pomegranate extract inhibits the interleukin-1β-induced activation of MKK-3, p38α-MAPK and transcription factor RUNX-2 in human osteoarthritis chondrocytes. *12*(5), R195.

Rescigno, M., Winzler, C., Delia, D., Mutini, C., Lutz, M., & Ricciardi-Castagnoli, P. J. J. o. l. b. (1997). Dendritic cell maturation is required for initiation of the immune response. *61*(4), 415-421.

Rose, S., Melnyk, S., Trusty, T. A., Pavliv, O., Seidel, L., Li, J., . . . treatment. (2012). Intracellular and extracellular redox status and free radical generation in primary immune cells from children with autism. *2012*.

Rosenblat, J., & McIntyre, R. J. A. P. S. (2015). Are medical comorbid conditions of bipolar disorder due to immune dysfunction? , *132*(3), 180-191.

Rouwet, E. E., Lutgens, E. E. J. A., Thrombosis,, & Biology, V. (2018). Immune Checkpoints in Atherosclerosis: Toward Immunotherapy for Atheroprotection. *38*(8), 1678-1688.

Sanjeewa, K. K., Fernando, I. P., Kim, E. A., Ahn, G., Jee, Y., & Jeon, Y. J. (2017). Anti-inflammatory activity of a sulfated polysaccharide isolated from an enzymatic digest of brown seaweed Sargassum horneri in RAW 264.7 cells. *Nutr Res Pract, 11*(1), 3-10. doi:10.4162/nrp.2017.11.1.3

Sasaki, A. (2016). Microglia and brain macrophages: An update. *Neuropathology*. doi:10.1111/neup.12354

Sbarra, A. J., & Karnovsky, M. (1959). The biochemical basis of phagocytosis. *J biol chem, 234*, 1355-1362.

Seger, R., & Krebs, E. G. J. T. F. j. (1995). The MAPK signaling cascade. *9*(9), 726-735.

Shastri, A., Bonifati, D. M., & Kishore, U. (2013). Innate immunity and neuroinflammation. *Mediators Inflamm, 2013*, 342931. doi:10.1155/2013/342931

Sica, A., & Bronte, V. J. T. J. o. c. i. (2007). Altered macrophage differentiation and immune dysfunction in tumor development. *117*(5), 1155-1166.

Sies, H. (2000). What is oxidative stress? In *Oxidative stress and vascular disease* (pp. 1-8): Springer.

Sies, H. J. A. C. I. E. i. E. (1986). Biochemistry of oxidative stress. *25*(12), 1058-1071.

Sies, H. J. E. P. T., & Integration. (1997). Oxidative stress: oxidants and antioxidants. *82*(2), 291-295.

Skaper, S. D., Facci, L., Zusso, M., & Giusti, P. (2017). Neuroinflammation, Mast Cells, and Glia: Dangerous Liaisons. *Neuroscientist, 23*(5), 478-498. doi:10.1177/1073858416687249

Slater, T. F. (1988). Free radical mechanisms in tissue injury. In *Cell Function and Disease* (pp. 209-218): Springer.

Sompayrac, L. M. (2019). *How the immune system works*: Wiley-Blackwell.

Son, M., Oh, S., Lee, H. S., Ryu, B., Jiang, Y., Jang, J. T., . . . Byun, K. J. M. d. (2019). Pyrogallol-Phloroglucinol-6, 6'-Bieckol from Ecklonia cava Improved Blood Circulation in Diet-Induced Obese and Diet-Induced Hypertension Mouse Models. *17*(5), 272.

Sorrenti, V., Giusti, P., & Zusso, M. (2018). A Model of Systemic Inflammation to Study Neuroinflammation. *Methods Mol Biol, 1727*, 361-372. doi:10.1007/978-1-4939-7571-6_27

Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. *Annual review of immunology, 9*(1), 271-296.

Steinman, R. M., & Hemmi, H. (2006). Dendritic cells: translating innate to adaptive immunity. In *From Innate Immunity to Immunological Memory* (pp. 17-58): Springer.

Sun, A., Nie, S., & Xing, D. J. P. P. (2012). Nitric oxide-mediated maintenance of redox homeostasis contributes to NPR1-dependent plant innate immunity triggered by lipopolysaccharides. *160*(2), 1081-1096.

Sun, Y., Liu, W.-Z., Liu, T., Feng, X., Yang, N., Zhou, H.-F. J. J. o. R., & Transduction, S. (2015). Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *35*(6), 600-604.

Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M., & Mellman, I. J. S. (2003). Activation of lysosomal function during dendritic cell maturation. *299*(5611), 1400-1403.

Wijayanti, N., Huber, S., Samoylenko, A., Kietzmann, T., Immenschuh, S. J. A., & signaling, r. (2004). Role of NF-kB and p38 MAP kinase signaling pathways in the lipopolysaccharide-dependent activation of heme oxygenase-1 gene expression. *6*(5), 802-810.

Wuertz, K., Vo, N., Kletsas, D., & Boos, N. J. E. C. M. (2012). Inflammatory and catabolic signalling in intervertebral discs: the roles of NF-KB and MAP kinases. *23*, 103-119.

Yamaguchi, M. (2006). Regulatory mechanism of food factors in bone metabolism and prevention of osteoporosis. *Yakugaku Zasshi, 126*(11), 1117-1137.

Yamaguchi, M. J. I. J. F. S. N. D. (2013). Marine alga Sargassum horneri component and bone homeostasis: role in osteoporosis prevention. *2*(1), 9-14.

Yang, Q., Yang, R., Li, M., Zhou, Q., Liang, X., & Elmada, Z. C. (2014). Effects of dietary fucoidan on the blood constituents, anti-oxidation and innate immunity of juvenile yellow catfish (Pelteobagrus fulvidraco). *Fish Shellfish Immunol, 41*(2), 264-270. doi:10.1016/j.fsi.2014.09.003

Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., & Karin, M. J. C. (1997). The IkB kinase complex (IKK) contains two kinase subunits, IKKa and IKKb, necessary for IkB phosphorylation and NF-kB activation. *91*(2), 243-252.

Zhang, W., & Liu, H. T. J. C. r. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *12*(1), 9.

감사의 글

2015 년 12 월 면역학이라는 분야에 대한 궁금함에 연구실에 처음 들어와 학부 2 학년 말부터 3 학년, 4 학년, 석사 2 년이 순식간에 지나갔습니다. 졸업을 앞두고 돌아보니, 지난 4 년이라는 시간은 저에게 하루도 빠짐없이 가치 있고 보람 있는 시간이었습니다. 그래서 제가 학위과정 동안 공부하면서 저에게 많은 도움을 주신 감사한 분들에게 이렇게 짧게나마 글로 마음을 전하고자 합니다.

먼저, 면역학이라는 분야에 관심을 가지고, 연구실 생활을 하면서 연구활동을 끊임없이 지지해 주시며 부족한 저를 이 자리까지 이끌어 주시고 지도해 주신 이준식 교수님 정말 너무 감사 드립니다. 스스로 한계를 정하지 마라는 말씀과 여러 충고들이 학위 기간 동안 의지를 다질 수 있는 큰 원동력이 되었습니다. 또한 저의 논문을 심사해주시고 충고와 격려를 아끼지 않으신 전택중 교수님, 조광원 교수님 감사 드립니다. 수업시간과 복도에서 마주치는 시간 동안 많은 조언과 따뜻한 말씀들이 큰 도움이 되었습니다. 늘 진취적이시고 여러 분야에 관심을 갖게 도와주신 윤성명 교수님, 매사에 열정적이시며 학위 동안 많은 관심을 가져주시고 조언을 아끼지 않으신 박현용 교수님 감사 드립니다. 항상 끊임없는 관심과 따뜻한 격려로 다독여주시며 지도학생처럼 잘 챙겨주신 송상기 교수님 감사 드립니다. 교수님의 관심과 따뜻한 격려가 큰 힘이 되었습니다. 언제나 밝은 미소로 응원해주시는 조태오 교수님, 이현화 교수님께도 감사 드립니다. 교수님들의

훌륭한 지도와 관심 속에 부족한 제가 무사히 학위과정을 마무리 할 수 있게 되었습니다. 정말 감사 드립니다.

항상 옆에서 연구에 대한 열정과, 조언, 격려를 아끼지 않고 다독여주시고 이끌어주신 저희 연구실에 꽃, 김미은 박사님 정말 감사 드립니다. 연구실 생활에 있어 많은 부분을 희생하고 인내하시면서도 부족한 저를 위해, 후배들을 위해 생활적인 부분들까지도 하나하나 신경 써주셔서 감사 드립니다.

학위 하는 동안 함께 열심히 공부하고 같이 고생한 연구실 식구들에게도 감사의 말을 전하고 싶습니다. 항상 선배가 아닌, 정말 식구처럼 챙겨주고 응원해주는 푸름이누나, 친형처럼 큰 일, 작은 일 구분 없이 자주 연락하고 챙겨주는 우리 주용이형 너무 고맙습니다. 두 분 앞 길에 행복한 일만 있기를 바랍니다. 학부 동기이자 학위과정 선배인 인애야, 공부하는 동안 그리고 졸업하고 나갔음에도 자주 연락해서 응원해주고 챙겨줘서 너무 고맙다. 여러 일들에 같이 슬퍼해주고 화도 내주고 큰 힘이 되었어 고마워. 가장 긴 시간 연구실 생활을 같이한 순효형, 우여곡절도 많고 이런 일 저런 일 많이 있었지만 얼굴 보면 응원 많이 해주고 격려해줘서 고마워. 지금은 각자의 길을 걷고 있지만, 같이 연구실 생활을 했던 친구, 후배들, 동생들도 너무 고맙습니다. 항상 응원해주고 칭찬도 많이 해주는 내 친구 재헌아 벌써 우리가 7 년이 넘어간다. 연구실 생활 1 년 동안 같이 의지도하고 고민도 많이 하고 밖에서는 둘도 없는 친구로 격려해주고 힘이 되어줘서 고맙다. 같이 연구하면서 오빠 고생한다고 이래저래 많이 챙겨주고 응원해준 승희야 소연아 너희들한테도 너무 고마워. 승희는 외국생활 잘 마무리하고 건강하게

귀국해서 하고 싶은 일 꼭 했으면 좋겠고, 소연이는 앞으로 하게 될 학위과정 탈 없이 무사히 잘 마무리 했으면 좋겠다. 막둥이 경민아, 해주고 싶은 말도 많고 알려주고 싶은 것도 많은데 시간이 얼마 없네. 훌륭하신 교수님과 박사님에게 많이 배우고 익혀서 열심히 하길 바란다. 그리고 늘 가까이에서 날 배려하고, 이해해주고, 힘든 일이 있을 때 같이 힘들어 해주고 고민해주며 묵묵히 응원해준 지선아, 너무 너무 고마워. 지선이도 하고 싶은 일, 이루고자 하는 목표 이룰 수 있도록 오빠가 많이 응원할게.

다른 연구실이지만 정말 둘도 없는 내 친형 같은 우리 영빈이형, 요한이형, 수민이형, 학부과정부터 학위 과정 동안 기쁜 일, 슬픈 일, 모든 순간들을 같이 웃고 울어줘서 너무 큰 힘이 되었어. 이제는 얼굴만 봐도 무슨 생각을 하는지 아는 사이라서 더 소중하고 많이 고마워. 영빈이형, 요한이형 박사과정 잘 마무리하고 수민이형도 석사과정 무사히 마무리 하길 바랄게. 한참 먼저 박사과정 마무리하고 졸업하신 신구형, 석사과정 마무리하고 졸업한 현웅이형, 방헌이형, 지금도 박사과정 열심히 공부하고 있는 주원이형, 항상 응원해주고 격려해줘서 너무 고마워요. 대학원 동기인 평화, 한 학기 먼저 마무리한 건혁이, 학위 과정 마무리 하느라 너무 고생 많았고 고맙다. 모든 일에 열심히 하는 친구 동주, 덤벙거리지만 열심히 하는 경민이, 둘 다 별 탈 없이 학위 과정 잘 마무리 할거라 믿어. 힘내자. 늘 성실하고 응원 많이 해준 안지, 묵묵히 열심히 공부하고 있는 원범이, 하준이, 지성이, 지현이, 상철이, 준한이, 광철이, 잠시 쉬고 있지만 다시 열심히 공부할 성은이도 너무 고마워.

학부과정부터 석사과정까지 쉬지 않고 달려가는 내가 대단하다며 격려해주고, 위로해주며 지금까지도 응원해주는 둘도 없는 친구 같은 윤호형, 우리 벌써 8 년이 넘어가네. 너무 고맙고 앞으로 서로 뭐든 열심히 해보자. 연구실 생활과, 학위 과정을 응원해주고 격려해준 학과 동생들, 후배들 채영이, 다솜이, 정옥이, 지희, 시원이, 민서, 규원이, 석주, 준영이, 성훈이, 다른 동생들도 다들 너무 고맙다. 너희들의 앞날에 좋은 일만 있기를 바랄게.

힘들다고 지친다고 징징거리고 전화해서 귀찮게 해도 다 받아주고 격려해준 친구 유준아 너무 고맙다. 이제 조금은 한가해 질 테니 자주 보자. 한창 의대생활 하면서 바쁜 생활에 치여있는 명재야, 친구 자랑스러워 해줘서, 열심히 해서 마무리 잘하라고 응원해줘서 너무 고맙다. 바빠도 연락 자주하고 살자.

마지막으로, 공부하는 동안 단 한번도 싫은 내색 안 하시고 의심 없이, 조건 없이 저를 믿어주시고 지지해주셨던 부모님께 너무 감사 드립니다. 수동적이 아닌, 제 스스로 능동적인 선택을 할 수 있게 도와주시고 제 의견이나 고민을 누구보다 잘 들어주시고 존중해주시고 응원해 주셨기에 제가 지금 이 자리까지 올 수 있었습니다. 우리 엄마, 아빠 항상 존경하고 감사하고 사랑합니다. 사랑하는 내 동생 주연이, 공부하느라 많이 힘들 텐데 지친 기색 없이 내색 안하고 열심히 하는 모습이 보기 좋다. 오빠가 학위 하는 동안 연구하는 오빠 자랑스럽고 멋있다며 응원해주는 모습에 감동했고 고마웠어. 앞으로도 열심히 해서 서로 자랑스러운 오빠, 동생이 되어보자. 엄마처럼, 아빠처럼, 저를 항상 아들대하듯 챙겨주시는 우리 이모, 이모부에게도 너무 감사합니다. 잘하고 있다고 늘 격려해주시고

응원해주셔서 큰 힘이 되었습니다. 나윤이 누나, 준원이도 너무 고마워. 멀리서도 응원해주고 날 자랑스러워 해주는 모습이 공부하는 동안 큰 도움이 되었어. 떨어져있지만 늘 조언과 위로를 아끼지 않고 여러모로 많이 챙겨주시는 큰 삼촌, 작은 삼촌, 큰 외숙모, 작은 외숙모, 너무 감사 드려요. 명절이나 가족행사 때마다 응원해주시고 다독여주셔서 큰 힘을 얻을 수 있었습니다. 군복무 중인 우리 재욱이, 열심히 공부하고 있는 성욱이, 준호 항상 응원해줘서 고맙다. 자랑스러운 형이 될게. 사랑하는 우리 할머니, 늘 제 끼니 걱정하시고 공부하느라 애쓴다고 격려해주시고 응원해주셔서 제가 항상 든든했어요. 너무 너무 감사하고 건강하세요 할머니!

다시 한번, 그 동안 저를 응원해주시고 힘이 되어주신 모든 분들께, 그리고 미처 감사한 마음을 전하지 못한 분들에게도 감사의 말씀을 드리며 이 논문을 바칩니다. 감사합니다.