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Analysis of immunological effects of a non-specific immunostimulator, germanium biotite

비특이 면역증강제인 게르마늄 흑운모의 면역증진효과 분석

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By

Myunghwan Jung

Supervisor : Professor Han Sang Yoo, D.V.M., Ph.D.

A dissertation submitted to the faculty of the Graduate School of Seoul National University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Microbiology

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Department of Veterinary Medicine

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지도교수 유 한 상

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Abstract

Analysis of immunological effects of a non-specific immunostimulator, germanium biotite

Myunghwan Jung

(Supervisor: Han Sang Yoo, D.V.M., Ph.D.)

Department of Veterinary Medicine

The Graduate School

Seoul National University

Germanium biotite, a natural mineral within the mica group, is comprised of mainly silicate. It has been reported that use of this mineral as feed supplement could stimulate a non-specific immune system. The aims of the present study were to demonstrate the mechanism of the immune enhancing effects *in vitro* and to evaluate the immune stimulating effects of germanium biotite in cattle and pigs as an initial step towards the development of alternative feed supplement for prevention of diseases in livestock industry.

To demonstrate the mechanism of immune enhancing of germanium biotite, murine macrophage cell line, RAW 264.7, were treated with 100 μg/mL mica (germanium biotite) and changes in global gene expression upon mica treatment for 12 and 48 h were determined using microarrays. To evaluate the immune enhancing effects *in vivo*, the prophylactic effects of germanium biotite against respiratory diseases in cattle were investigated using challenge experiment with bovine herpesvirus type 1 (BHV- 1) and *Mannheimia haemolytica* serotype A1. The effects of the non-specific immune stimulator germanium biotite on foot-and-mouth disease (FMD) vaccination and immune system in cattle were also analyzed. In addition, the investigation of immune responses to FMD virus (FMDV) challenge in FMD vaccinated mini-pigs after oral administration of the germanium biotite was carried out using Andong strain.

Following the mica treatment, RAW 264.7 cells showed a change in an expression level of 1,128 genes after 48 h treatment. Specifically, genes associated with the cell cycle, DNA replication, and pyrimidine and purine metabolisms, were down-regulated in cells treated with mica. Mica treatment also up-regulated genes associated with lysosome and phagosome function, which are both required for macrophage activities. These results indicate that mica, major component of the germanium biotite, could activate macrophages, in part, through up-regulation of these pathways.

In challenge experiment of bovine respiratory diseases (BRDs), germanium biotite-fed group showed a lower cumulative clinical score (CCS) than the control group. In accordance with this clinical result, enhanced clearance of BHV-1, a low level of bacteria shedding, tempered superficial lesions, and moderated histopathological signs were observed in the germanium biotite-fed group, compared to the control group. These results indicate that germanium biotite has prophylactic effects against BRDs and could be a candidate for a new alternative feed supplement in cattle, through its effects as a non-specific immune stimulator.

In experiment of FMD vaccination and immune responses in cattle, it was found that high levels of IgG and IgA titers in serum and saliva were longstanding in the germanium biotite-fed group, compared to the control. Generally, higher virus-neutralizing antibody titers were observed in the germanium biotite-fed group. After feeding germanium biotite, subpopulation of the $CD4^+$ and major histocompatibility complex (MHC) class II^+ of lymphocytes and levels of interferon (IFN)-γ, interleukin (IL)-1α, IL-1β, and IL-4 gene expression were significantly increased in the feeding group. Feeding with germanium biotite increased lymphocyte proliferation at 23 weeks and lysozyme activity at 8 weeks after feeding. These results suggest that germanium biotite feeding could increase the protection against FMD virus infection *via* the induction of higher humoral and cellular immune responses in cattle.

Following the FMDV challenge, the germanium biotite-fed pigs showed high levels of IL-8 in serum, and increased cellular immune responses to stimulation with the Andong strain antigen compared to pigs of the control group. In addition, higher FMDV neutralizing antibody titers were detected in the germanium biotite-fed group than in the control group before the challenge. The findings of this study indicate that germanium biotite supplement might enhance immune responses to the FMD vaccine and FMDV challenge in pigs.

Taken together, germanium biotite oral administration showed enhancing the immune responses to challenge of BHV-1 and *Mannheimia haemolytica* serotype A1, FMD vaccination, and FMDV challenge. These immune enhancing effects may be related to its ability to activate non-specific immunity. It is also presumed that this enhanced non-specific immunity could be associated with, in part, macrophage activation by germanium biotite such as up-regulation in lysosome and phagosome pathways. Hence, germanium biotite could be a candidate for new alternative feed supplement to reinforce immunity, thereby reducing the risk of diseases outbreak in livestock industry.

Key words: Germanium biotite, non-specific immune system, immune stimulating effects, macrophage activation.

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APC	Antigen-presenting cells
AUC	Area under curve
BAP	Blood agar plate
BHV-1	Bovine herpesvirus type 1
BRD	Bovine respiratory disease
CCL	C-chemokine ligand
CCS	Cumulative clinical score
Cdc	Cell division cycle kinase
CDK	Cyclin-dependent kinase
ConA	Concanavalin A
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FE-SEM	Field emission scanning electron microscope
FITC	Fluorescein isothiocyanate
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus

GALT	Gut-associated lymphoid tissues
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
HRP	Horseradish peroxidase
IACUC	Institution Animal Care and Use Committee
IFN	Interferon
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
L^{pro}	Viral leader protein of foot-and-mouth virus
LPS	Lipopolysaccharide
MeV	MultiExperiment Viewer
MFP	Mica fine particles
MHC	Major histocompatibility complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NK cell	Natural killer cell
OIE	Office International des Epizooties
PANTHER	Protein Analysis Through Evolutionary Relationships
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD_{50}	Protective dose 50%

PE	Phycoerythrin
PI	Inhibition percentage
QIA	Animal and Plant Quarantine Agency
qRT-PCR	Quantitative real time reverse transcription PCR
QTC	Quality threshold clustering
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SAT	South African territories
SD	Standard deviation
SP	Structural protein
SPSS	Statistical Package for the Social Sciences
Th1	T helper type 1
Th2	T helper type 2
TNF	Tumor necrosis factor
TSB	Tryptic soy broth
VN	Virus neutralizing antibody

General introduction

The immune system has traditionally been divided into 2 branches: innate (or non-specific) and adaptive (or specific acquired) immunity [1]. Albeit appreciation of innate immunity was discovered at least the 1908 by Ilya Mechnikov, the innate immunity has been under appreciated and considered as only rapid and incomplete host defense until more definitive acquired immune response develops [1, 2]. However, as questions about how the innate immune system senses infection and empowers a protective immune response are being answered at molecular level, the central roles of innate immunity in the host defense against diseases have been palpable [1-5]. Therefore, researches on novel therapies to harness the power of the innate immune system have been actively in progress [6-10].

In accordance with the prohibition of antibiotic usage as supplementation in feed, studies for innate immunity have been also actively carried out in veterinary science, especially to seek for substances which can activate the non-specific immune abilities [11, 12]. Because antibiotics had been used as feed supplements to prevent infectious diseases in livestock industry [13], the ban of antibiotic feed supplement has aroused interest in activation of the non-specific immune, thereby

reducing the risk of infectious diseases such as respiratory diseases and foot-and-mouth disease (FMD).

Germanium biotite is a common phyllosilicate mineral within the mica group containing calcium, magnesium, ion, aluminum, germanium, and silicate (Table I) [14, 15]. Silicate, major component of germanium biotite, has shown enhancing effects on immune system associated with the innate immunity [16, 17]. Fibrogenic silicate (SiO_2) activated proinflammatory macrophages [18], and aluminosilicate (Al_2SiO_5) improved immune-cell differentiation [16]. Based on the previous studies, it could be presumed that germanium biotite has potential as a new alternative feed supplement for innate immune stimulation.

Bovine respiratory diseases (BRDs) are major economic problems in the cattle industry around the world due to high morbidity, mortality, low feed efficiency, prevention costs, and treatment [19, 20]. The diseases are caused by not only multi-factorial pathogens but also environmental conditions like immune depression. Bovine herpesvirus type 1 (BHV-1) and *Mannheimia haemolytica* serotype A1 have been described as major etiologic agents [19]. The suppression of the immune function renders the host susceptible to secondary infection like pneumonic mannheimiosis, thereby causing BRDs more easily.

FMD is a highly contagious and economically important disease that affects cloven-hoofed animals, and it is characterized by appetite loss, an increase in

body temperature, and vesicles in the mouth, tongue, hooves, and nipples [21, 22]. The disease is caused by the FMD virus (FMDV), which is a small, icosahedral, non-enveloped RNA virus classified within the genus *Aphthovirus* as a member of the family *Picornaviridae*. Since the devastating FMD outbreak in Korea at the end of 2010, cattle, pigs, and some small ruminants have been vaccinated with a trivalent FMD vaccine (O1 Manisa, A Malaysia 97, and Asia1 Shamir) at least 6 protective dose 50% (PD_{50}) [23]. Although the trivalent vaccine has induced high antibody titer in cattle, the vaccine showed a low rate of antibody formation in pigs in Korea from July 2011 to June 2012, according to type O ELISA assay [24]. Therefore, there is a growing need for strategies to enhance the immune responses to vaccination [25, 26].

The aims of the present study were to demonstrate the mechanism of the immune enhancing effects *in vitro* and to evaluate the immune stimulating effects of germanium biotite in cattle and pigs as an initial step towards the development of alternative feed supplement for prevention of disease in livestock industry. This is comprised of four chapters.

To demonstrate the mechanism of immune enhancing effects, murine macrophage cell line, RAW 264.7, were treated with 100 μg/mL mica and changes in global gene expression upon mica treatment for 12 and 48 h were determined using microarrays in chapter I. As immunostimulator, the prophylactic

effects of germanium biotite against respiratory diseases in cattle were investigated in chapter II. The effects of the non-specific immune stimulator germanium biotite on FMD vaccination and immune system in cattle were analyzed in chapter III. In chapter IV, the investigation of immunostimulating effects of the germanium biotite on immune responses to FMDV challenge in FMD vaccinated mini-pigs was carried out.

Literature review

I. Immune system

The immune system is defined as the host's defense against destructive foreign substances such as bacteria, viruses, parasites, and chemicals. Immune responses are commonly categorized as innate and adaptive responses [5]. The components and cells that comprise immune system could be classified as Figure I [1]. Innate immune system, as the first line of defense barrier against pathogen invasion, is characterized as non-specific and rapid responses, recognition of a diverse array of pathogens, and lack of any form of memory [1, 5, 27]. The adaptive immune system, which generally occurs after innate immune responses, develops over an individual's lifetime [5]. This antigen-specific immune system can not only recognize foreign but also retain the memory of the encounter. While innate immune system uses pre-existing receptors that can bind to molecules and molecular patterns commonly found on many different microbes, the adaptive immune system produces unique receptors and recognition molecules, thereby expressing a vast array of diverse receptors [28].

II. Immune stimulators and analysis of their effects

In terms of prevention and treatment of diseases, many kinds of methods including environmental control, pharmacotherapy, vaccination, and immunotherapy have been introduced in several studies [9-11, 29, 30]. Recently, as kind of immunotherapy, immune modulation has been highlighted as potential method of controlling diseases [8, 9, 17, 18]. Immune stimulator, a part of immune modulation, is drawing attention because it could enhance the overall resistance against many kinds of pathogens [11, 12]. Moreover, prophylaxis or treatment with antibiotics has become limited as antibiotic residues and emerging of antibiotic resistance bacteria [31-33]. Therefore, studies seeking for material which can stimulate immune system are actively in progress. This inquiry on the immunostimulators has been facilitated by not only growing the needs for immunostimulators but also development in tools for analyzing the immune responses. In particular, researches seeking for immunostimulators have been further accelerated according to that studies on the innate immune responses are actively in progress through not only immunological and functional approaches but also advanced biomolecular approaches such as transcriptomics, proteomics, and metabolomics.

II. 1. Immunostimulators

II. 1.1. Oligodeoxynucleotides

Oligodeoxynucleotides containing CpG motifs showed effects on T helper type 1 (Th1) activation [9, 34]. Tokunaga and his colleagues reported that DNA extracted from *Mycobacterium bovis* activated natural killer (NK) cells [34]. It was revealed that the ability to stimulate NK cells was limited to DNAs from invertebrates but not vertebrate and that particular sequences with CpG motifs were required for the activity [35]. CpG were also found to activate monocytes and macrophages, which could activate adaptive immune responses and stimulate NK cells [36, 37]. In addition, CpG conjugation with antigen increased phagocytosis activities of dendritic cells (DCs) and enhanced the interleukin (IL)-12 expression in DCs [9]. Therefore, it was presumed that activation of Th1 cells by CpG results from the increase of phagocytosis activity in antigen presenting cells such as macrophages. (Table II).

II. 1.2. Polysaccharides

High molecular weight polysaccharides extracted from plant components frequently were reported to enhance monocyte/macrophage activation *in vitro* [38]. Therefore, these molecules have been considered to contribute to immune enhancing effects of many botanicals [38]. β-glucans, kind of various polysaccharides, are natural polysaccharides with glucose as structural component, linked by β-glycosidic bonds. The cell wall of many plants (wheat, rye, barley, and oat), baker's and brewer's yeast (*Saccharomyces* genus), and Echinaceae members contains β-glucan structures [39]. It was reported that these structures could induce leukocyte activation, phagosome stimulation, cytotoxic activities, antimicrobial responses, and production of reactive oxygen species (ROS) [40]. Due to the abilities to effect on macrophages and other white blood cells, it is presumed that β-glucan could induce immune system enhancement and provide optimum resistance to pathogens [41, 42]. These immune enhancing effects are thought to be triggered by pattern recognition receptors of macrophage. The suggesting mechanism is that multiple glucan binding sites on macrophage interact with β-glucan, thereby enhancing the immune system (Table II) [40, 43].

II. 1.3. Probiotics

There are a number of studies showing enhanced effects of oral probiotics, especially acid bacteria, on systemic immune function [44-47]. Among the acid bacteria, bifidobacteria has been investigated as immunostimulator of gut-associated lymphoid tissues (GALT) [44, 45, 48]. It was reported that co-culture with bifidobacteria significantly increased the production of tumor necrosis factor (TNF)-α and IL-6 by macrophages and the production of IL-2 and IL-5 by stimulated $CD4^+$ T-cells [45]. These results were supported by the research showing increase of production nitric oxide, H_2O_2, IL-6 and TNF-α in macrophage cell line by bifidobacteria [48]. Similarly, culturing murine Peyer's patch cells with bifidobacteria resulted in increased proliferation and antibody production by B-cells and activated macrophage [44]. Schley and Field described that probiotics affect host through direct contact with GALT or activation of GALT by microbial substances of probiotics (e.g. cytoplasmic antigens, cell wall components) [5]. The suggesting mechanisms were demonstrated as stimulation of antibody secreting cell responses [49], enhancement of phagocytosis of pathogens [50, 51], and modification/enhancement of cytokine production/ natural complement activity [52, 53] by several studies (Table II).

II. 1.4. Fibers

The addition of fermentable fibers to the diet showed abilities to alter the function and structure of the gut and to modify the production of gut-derived hormones [30, 54]. It was reported that oral administration of the fermentable fiber modulated the type and function of cells from different regions of the GALT in dogs [55]. In addition, the fibers, contents of the diet, significantly altered the proportion of T-cells in GALT and their *in vitro* responses to mitogens [55]. Responding to mitogen stimulation, the higher and lower proliferation was observed in T- and B-cells, respectively [55]. A higher proportion of $CD8^+$ T-cell was measured after consuming the high fermentable fiber [55]. A number of the mechanisms for the immunostimulating effects of fermentable fibers have been proposed as follows: Direct contact of lactic acid bacteria or bacterial products with immune cells in the intestine; production of short-chain acid from fiber fermentation; modulation of mucin production (Table II) [30, 44, 46, 54, 55].

II. 1.5. Minerals

There are several minerals showing immune stimulating effects such as zeolite, germanium biotite, and anionic alkali mineral complex [12, 16, 56]. Zeolite has

been extensively used in various kinds of area, based on their capacities as catalysts, ion exchangers, and absorbents [16, 57-59]. Zeolite showed beneficial biological activities such as enhancement of growth rate, improved animals' health, and increase of feed efficiency in a previous study [57]. Moreover, immunostimulating effects associated with macrophage activation were reported in previous studies [57, 59-61]. Germanium biotite, a common phyllosilicate mineral within the mica group is comprised of mainly aluminosilicate, similarly to zeolite. It also showed immune stimulating effects in mice and pigs [16]. These immunostimulating effects were thought to be induced by macrophage activation. Hence, macrophage activation by zeolite and germanium biotite was postulated to be induced by silicate, the major constituent of zeolite and germanium biotite. Several studies reported that silicate may act as non-specific immunostimulators in manner similar to that of the superantigens such as potent T-cell mitogens and affinity to major histocompatibility complex (MHC) class II molecules [60, 61]. In addition, an anionic alkali mineral complex, Barodon® (BARODON-S.F. CORP, Korea), is also identified as a potential immune stimulant in terrestrial animals [12, 56, 62]. Barodon® is comprised with several minerals including silica, sodium, silver, and potassium in an alkali solution (pH 13.5) [12]. It was reported that Barodon® demonstrated immune modulatory effects on proliferation and activation of porcine immune cells [56]. The exact mode of action of Barodon® is

not clear, but it was postulated that its activities were related to the membrane-associated lymphoid tissue (Table II) [62].

II. 1.6. Herbs

Herbs were already used thousands of years ago for medical practice [63]. Several studies reported biological benefit effects of herbs such as antimicrobial [64], anti-inflammatory [65, 66], antioxidative [67, 68] activities. The herbs containing flavonoids, vitamin C, and carotenoids showed immunostimulating effects [69-71]. The most representative herbs as immunostimulators are echinacea, liquorice, garlic, and cat's claw. It was reported that these plants could improve the activities of lymphocytes, macrophages, and NK cells such as increase of phagocytosis or stimulation of interferon synthesis [69]. Essential oils extracted from these herbs showed the ability to improve the immune responses and induce the changes of the duodenal mucosa with beneficial effects for the animals [71]. However, the mechanism of these immune stimulating effects is not well established (Table II) [70, 71].

II. 2. Analysis of effects of immunostimulators

II. 2.1. Immunological analysis

II. 2.1.1. Analysis of antibody production

Antibodies circulating around the body in the blood and fluid play an important role in immune responses associated with protection. Antibodies bind antigens such as viruses, bacteria, and toxins, thereby reducing or neutralizing the harmless of antigens. In addition, opsonization by antibodies promotes phagocytosis, thus immune responses could be enhanced [28]. Antibodies are produced by activated B-cells by antigen exposures. These antigen exposures stimulated B-cells and multiple exposures induce secondary antibody responses by concurrent activation of both B- and T-cells [72]. Moreover, secondary antibody responses induce higher and more sustained antibody titer under the control of helper T-cells [28]. Therefore, measurements of antibody product abilities could be indicator reflecting the general situation of immune system following the administration of immunostimulators [73]. Antibody levels can be quantified using biochemical technique, ELISA, which is used commonly in immunology analysis to detect the present of an antibody or an antigen in samples.

II. 2.1.2. Cytokine analysis

The immune system is orchestrated interaction of a large number of cell *via* various receptors [74]. Immune responses result from these interactions which could be achieved through small proteins called cytokines. As cytokines play a prominent part in cell signals, analyses on changes in cytokine level could be indicators showing immune responses [75]. Therefore, a large number of studies on analysis of immune responses to immunostimulators have investigated the cytokines level changes following administration of immunostimulator *in vitro* and *vivo*. For example, several researches in effects of glucans on immune system using macrophages revealed that cytokine products of IL-1 and TNF-α were increased by glucan treatments, and thus could determine the mechanisms, in part, associated with enhanced phagocytosis of macrophages [76-88]. Analysis of cytokine levels can be carried out using mainly two methods, ELISA and real-time PCR. ELISA methods are commonly used for estimating cytokine product levels in sera or supernatants of cell culture media. On the other hand, real-time PCR is used for measurement of RNA expression levels in cells.

II. 2.2. Cell functional analysis

II. 2.2.1. Analysis of lysozyme activity

Macrophages play a central role in the immune system, and form a key barrier to host infection in both the innate and adaptive immune responses. [89] Due to intense phagocytic activity, phagocytosis of immunostimulator can be occurred by macrophages following the administration. Moreover, macrophages are considered one of the main entry routes of particles into the body [90, 91]. In addition, they have been known to induce the adaptive immune responses by antigen presentation to $CD4^+$ T-cells *via* MHC class II molecules [28]. Therefore, analyses of macrophage activity are considered as a large proportion in estimating the immune responses to immunostimulator. Macrophage activity can be measured by lysozyme activity assay. Lysozyme showing high hydrolytic reaction against Gram-positive bacteria is secreted into the blood by some phagocytes such as macrophages when phagocytosis is up-regulated [92]. Consequently, high level of lysozyme activity in the blood can be indicator of activation of phagocytosis by macrophages.

II. 2.2.2. Analysis of cell proliferative responses

Cell proliferative responses have been commonly investigated in immunological researches as indicators of activities of lymphocytes or peripheral blood mononuclear cells (PBMCs) [93]. Lymphocytes including B-, T-, and NK cells play an important role in the immune system. PBMCs, comprised of lymphocytes and monocytes, are also critical components in the immune system. Theses immune cells show proliferative responses when they are encountered specific antigens or stimulated by immune reactions [28, 93]. Therefore, proliferation of lymphocytes or PBMCs is commonly investigated in researches to immunostimulator effects on immune system. Proliferations of cells can be determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay used NAD(P)H-dependent cellular oxidoreductase enzymes which reduce tetrazolium dye MTT to its soluble formazan. These enzymes could reflect the number of viable cells under defined conditions. Therefore, production of formazan from a fixed quantity of MTT by the oxidoreductase enzymes can be considered as viable cell numbers [94].

II. 2.2.3. Analysis of cell subpopulation

Immune cells express specific molecules such as receptors and antigen presenting proteins on the surface in accordance with their types, thereby, conducting a series of own roles in immune system [95-101]. For this reason, subpopulation of immune cells according to specific molecules expressed on surface could reflect the function of immune system. Therefore, the subpopulation of cell could be the indicator for situation of immune system in research to effects of immunostimulators on immune system [96, 101-103]. Fluorescence-activated cell sorting (FACS) is well known as kind of flow cytometry, which could be applied to determine cell subpopulation. FACS, as a kind of flow cytometry, provides a method for detecting and sorting a cell population, based on the specific light scattering and fluorescent characteristics of cell.

II. 2.3. Biomolecular analysis

II. 2.3.1. Omics analysis

Biological processes are comprised of numerous converging signals that concertedly create a coherent effect [104]. For this reason, albeit individual

signaling could also elicit a physiological response, it is difficult to be assured of changes in biological responses of cell. Indeed, analysis of systemic biology has been focused on how molecular biology works as an integrated process [104, 105]. Recently, the systemic biology has advanced exponentially in accordance with progress in omics including transcriptomics, proteomics, and metabolomics [104]. Transcriptomic approaches, genome-wide gene expression analysis, can detect the up- and down-regulation of biosynthetic and signaling genes [106]. Proteomics analyses can identify enzymes and posttranslational protein changes, therefore, it has to date been mainly perceived as a drug development platform [107]. Metabolomic approaches, the comprehensive analysis of metabolites, can identify bioactive signaling molecules [104].

II. 2.3.2. Transcriptomic analyses

As RNA expressions could be indicators of activated and inactivated functions within cells, the changes in immune responses of immunocytes could be measured through the RNA expression levels [108, 109]. Therefore, identification of differently expressed genes and the characterization of their functions provide a promising window on the understanding of immune responses to stimulations such as infections, chemicals, and immunostimulators [110]. With the genome

project, global gene expression profiling has become applied to a wide range of experimental systems for understanding the genetic regulation of cellular processes and disease pathogenesis [111]. As global gene expression profiling approaches, transcriptomics, could provide a comprehensive view on all gene expression [105]. Using high through-put methods (such as microarrays), it is possible to measure the expression level of thousands of transcripts.

DNA microarrays are a promising technique for elucidating and interpreting the mechanistic roles of genes within cells [110]. This technique consists of a pre-defined arrangement of a large number of probe sequences, which serve as hybridization templates for RNA or DNA samples [105]. RNA expression levels can be measured by DNA microarray based approaches following the series of process such as reverse-transcription, amplification, biotin-NTP labeling, and hybridization. These approaches allow for parallel monitoring of gene expression levels, and have been extended to the study of gene regulation and interaction at whole-genome level [105]. Therefore, DNA microarrays have gained rapid acceptance in a variety of field for studying the roles of genes [105, 110]. Thousands of gene expression changes may be extracted from microarray analysis. Because changes of biological function and process in cells are derived from interaction among the large number of genes [112, 113], effective tools for analysis of raw data from microarray, such as clustering and pathway mapping,

are needed to obtain the significant information about biological function and process in cells. Clustering of differently expressed genes provides the specific patterns of gene responses, which present evidence of the scope of the cell responses to stimulation [110]. Pathways in cell process, formed by the interaction of genes, can be modulated by stimulating environment situation. Thus, identification of changes of these pathways can be attributed to present systemic reaction in cells [112, 113]. Indeed, the transcriptomics using microarray tool could present the regulatory network and the complex interaction in gene expression levels during the immune responses, thereby, providing the understanding of immune responses [105, 110].

III. Bovine infectious diseases in respiratory system

BRDs are major economic problems in the cattle industry around the world due to high morbidity, mortality, low feed efficiency, prevention costs, and treatment [19, 20]. These diseases are also called “shipping fever” and observed in 91% of transported calves [114]. After arrival, the typical symptoms of the BRDs, such as high fever (about 40-41.5℃), depression, decreased appetite, nasal and ocular discharge, and coughing, are presented highly in 1 to 3 weeks, and last for 12

weeks [115]. The etiology of BRDs is almost always polymicrobial associated with predisposing environmental or host risk factors.

III. 1. Agents and predisposing factors

BRDs are caused by not only multi-factorial pathogens but also environmental conditions [115]. BHV-1, infectious bovine rhinotracheitis virus (IBR), bovine virus diarrhea virus (BVDV), parainfluenza 3 (PI-3), and bovine respiratory syncytial virus (BRSV) are involved in etiologic agents of BRDs. The pathogenesis of BRDs are that the viruses are connected with establishing an environment that is favorable to colonization and replication by pathogenic bacteria resulting in pneumonia [115]. It was reported that etiologic virus of the BRDs may cause alteration in mucosal surface and modification of the immune system [116, 117], thereby elevating the risk of bacterial infection such as *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* [19, 118, 119]. Among the these etiologic viral and bacterial agents, BHV-1 and *Mannheimia haemolytica* serotype A1 have been described as major pathogens [19].

Predisposing factors as well as etiologic pathogens also play an important role in the BRDs [115, 120]. It was reported that infection of the pathogens is induced

by the predisposing factors [19, 20, 119]. One of the most important predisposing factors for BRDs is transportation. The transportation could induce the stress and the stress could lead to immunosuppression, thereby elevating the infection risk of pathogens of BRDs [19, 20, 115, 120, 121].

III. 2. BRDs control

Since the primary cause of BRDs is infection of etiological pathogens due to immune depression by transport stress, it could be prevented by reduction of stress, reinforcement of immunity, enhanced resistance to the pathogens. These efforts to reduce the incidence of BRDs can be achieved by preventive health program commonly referred to as “preconditioning” [115]. The preconditioning is a planned management program before shipment to the farm, containing weaning for a predetermined amount of time (usually 30 to 45 days), vaccination for various infectious agents, treatment with anthelmintics, acclimating to feed bunks and water troughs prior to transport [122]. Vaccination is a useful tool to reduce the risk of BRDs occurring. Ideally, vaccination is carried out 2 weeks prior to shipment [115, 122]. Supplyment of nutrition with good quality diet for calves is also considered as methods to control the BRDs [115]. Providing fodder containing high protein concentration, high level of energy, and various minerals

could reinforce the immunity and enhance resistance to etiologic pathogens. In addition, administration of antibiotics (metaphylaxis) on arrival is appropriate for high risk calves. The aim of metaphylaxsis is to reduce the incidence of acute-onset RBDs before generation of effective immune responses. The long acting antibiotics are usually used for metaphylaxis program and specific antibiotics administration should be introduced under the direction of a veterinarian.

III. 3. Role of innate immune system in BRDs

The immune responses to the etiologic pathogens of BRDs consist of innate and adaptive arms [117]. Among these immune responses, the innate immune responses showed characteristics such as rapid inducement, non-specific reaction, and no immunologic memory. However, the innate immune responses play prominent roles in protection against BRDs in view of recognition of antigens, ingestion and destruction many pathogens without the help of the adaptive immune responses, and activation of adaptive immune responses by antigen presentation [19, 117]. Phagocytosis is a critical innate defense mechanism and macrophages are the first phagocytes to encounter the pathogens [123]. Indeed, phagocytosis results in uptake and intracellular killing of the pathogens, as well as the secretion of pro-inflammatory cytokines including IL-1β, IL-8, and TNF-α.

Among these cytokines, IL-8 is a chemoattractant for neutrophils. Macrophages and neutrophils play a key role in the innate immunity considering the internalization and killing of pathogens which are essential pre-requisites for microbial antigen processing and presentation for the induction of a specific adaptive immune response against the pathogens [117, 124]. Macrophages and neutrophils also produce several toxic product such as superoxide anions, hydrogen peroxide, and nitric oxide that aid in the killing of the phagocytosed bacteria [123]. In addition, interferons (IFNs), well known as anti-viral effects, up-regulate immune function by binding to IFN receptor I or II on the cells, thereby suppressing viral replication and dissemination [125]. All things considered, as BRDs are caused by multi-factorial pathogens associating with environmental conditions, it could be suggested that activation of the innate immunity, non-specific immune responses, is also an effective control method for BRDs.

IV. Foot-and-mouth disease

FMD is an economically devastating disease of cloven-hoofed animals, including cattle, swine, sheep, and goats. The disease is characterized by oral and

pedal vesicles, appetite loss, and increase in body temperature [21, 22, 126]. Because FMD outbreak could lead to the limitation of international exchanges and cause mass-economic loss, it is on the list of notifiable infectious disease of animals of the Office International des Epizooties (OIE).

IV. 1. Agent and pathogenesis

FMDV is the etiologic agent of FMD, which is a single-strained positive-sense RNA virus in the genus *Aphthovirus* of the family *Picornaviridae* [126, 127]. It contains 4 structural proteins (SPs) and 10 non-structural proteins (NSPs). The capsid of the viron is comprised of 60 copies each of 4 SPs, which have been determined for a number of serotypes [126-128]. Among the these 4 SPs, VP1 to -3 SPs form the majority of the capsid structure and VP4 SP is buried within the capsid [128, 129]. Three of the NSPs (leader [L], 3C, and 2A), an 18 amino acid peptides, have proteinase activity and are involved in processing the viral polyprotein. Among these 3 NSPs, the viral leader proteinase (L^{pro}) limits the host innate response by inhibition of IFN-β induction. The replication of the FMDV is rapid after entry of virus and the disease can be transmitted *via* direct or indirect contact between FMDV-infected animals and susceptible animals [22]. The infected animals may become infectious at preclinical period [130] and the

clinical signs can be observed within 2 to 3 days after exposure and can last for 7 to 10 days [128]. Vesicles, typical clinical signs of FMD, develop at multiple sites, generally on the feet and tongue, and are usually preceded by fever. [128]. The virus is excreted during viremia; thereafter as serum antibody induces viremia decreases, and the animal ceases to be infectious as the lesions heal [130].

IV. 2. Antigenic variation and control of disease

Due to its high mutation rate, this virus exists as seven distinct serotypes (O, A, C, Asia 1, South African territories [SAT]1, SAT2, and SAT3) as well as numerous and constantly evolving subtypes, which shows a spectrum of antigenic diversity [22, 131]. Antigenic diversity and geographical distribution of the different foo-and-mouth disease virus serotypes were list in Table III [127] and seven major regional pools of FMDV were shown in Figure II. Infection or vaccination with one FMDV serotype does not protect against other serotypes, and it may even fail to protect fully against other subtypes within the same serotype [24, 132, 133]. These mean that the vaccines against FMD must match the circulating wild-type strains in order to provide sufficient protection. Therefore, vaccines are generally multivalent [127].

The outbreaks of FMD lead to devastating effects on livestock industry; therefore, most countries have applied "stamping-out with non-vaccination" policy to control the disease. However, non-vaccination can lead to high susceptibility to FMD in the domestic livestock population when the disease is introduced unexpectedly [131]. Consequently, this high level of susceptibility to FMD could have an adverse effect on the prevention of FMD spread. Therefore, FMDV vaccination can be considered as the resort of the control policy for FMD outbreaks. Live vaccines are good for preventing most viral diseases, but all FMDV vaccines used worldwide are inactivated [24]. Among the commercial FMDV vaccines currently in use, the O1 Manisa strain vaccine, which is free from NSPs of FMDV, is an oil adjuvanted vaccine containing SP antigens [134]. As the vaccines are killed, they typically must be given once or twice yearly in order to maintain effective herd immunity [24, 133, 135, 136]. Since outbreaks that occurred in 2010-2011, vaccination policy has been applied to control the FMD in Korea. The Korean national vaccination program is comprised of the fist-, booster-, and re-booster vaccination. The booster vaccination must be given 4 weeks after the first vaccination and the re-booster vaccination have to be carried out every 6 month after the booster vaccination, according to the Korean national vaccination programs [23, 24, 137].

IV. 3. Role of innate immune system in FMDV infection

FMDV infection elicits rapid humoral responses accompanied by clearance of virus-antibody complex [138]; this is considered the most important factor in conferring protection against FMD [132]. The induction of high levels of virus-neutralizing antibody (VN) in serum, which could be detectable as early as 2-4 days following infection, has been considered most important in protection [132], although protection can be witnessed in certain cases when the levels of such antibodies are low or undetectable [136, 138, 139]. This suggests that other immune factors may play an important role in FMDV protection. According to the previous literature, it is possible that the innate immune system has a critical role in defending against viral infection [138-140].

Among the immune cells associated with the innate immune system, macrophages play an important role in this immune system especially by presentation of antigen to $CD4^+$ T-cells. There is a suggestion that macrophages showed the central role in clearing the virus by phagocytosis of opsonized antigens [141, 142]. In addition, it was reported that macrophages could uptake FMDV without aid from antibodies although the opsonization by antibodies enhances the this activity [128, 138]. Previous study showed the release of small quantities of infectious virus by macrophages only during the first 10 h post-

infection [143]. Albeit it cannot be confirmed whether this virus was exocytosed "uptake" virus or would represent progeny virus, these small quantities of infectious virus could be considered as a kind of antigen presentation by infected macrophages, because the reduction of cellular protein synthesis induced by viral L^{pro} and NSPs were observed in these infected macrophages [138]. T-cell activities mediated by $CD4^+$ cells are required for protective immunity against FMDV as they participate in the production of antiviral antibodies [144]. Subsets of the $CD4^+$ MHC class II-restricted T-cells respond to activation by APC and antigens by producing a Th1 (IFN-γ) and T helper type 2 (Th2; IL-4, IL-5, and IL-13) responses [145]. Several studies have also demonstrated the presence of FMD-specific MHC class II-restricted responses in cattle and pigs [144-146].

In addition to macrophages and $CD4^+$ T-cells, the antiviral responses of $CD8^+$ T-cells were also detected following FMDV infection in previous studies [145, 147, 148]. These antiviral responses were through direct cytotoxicity or release of cytokines such as IFN-γ following vaccination, and that the antiviral responses were almost 100 times higher following re-stimulation [145, 147]. MHC class I-restricted $CD8^+$ T-cells also showed specific immune response to FMDV as memory cells; however, the correlation between FMDV-specific $CD8^+$ T-cell recognition and protection remains to be defined [147, 148].

Taken together, the results of these researches described the important of the innate immune system in protection against FMDV infection and control of the disease, particularly early stage. Therefore, it could suggest that targeting innate immune responses could represent a promising strategy to improve vaccine efficacy.

Table I. Composition of germanium biotite

Ingredients	%
Silicon dioxide (SiO_2)	61.90
Aluminum dioxide (Al_2O_3)	23.19
Iron oxide (Fe_2O_3)	3.97
Sodium oxide (Na_2O)	3.36
Calcium oxide (CaO)	< 2
Magnesium oxide (MgO)	< 2
Titanium oxide (TiO_2)	< 2
Other trace ingredients	< 2
Germanium	36 ppm

Table II. Effects of various immunostimulators and their suggesting mechanisms

Immunostimulators		Action and mechanisms	References
Oligodeoxynucleotides	Virus	1. Oligodeoxynucleotides containing CpG motifs showed effects on Th1 activation (monocytes/macrophages).	[35]
	Bacteria	2. CpG conjugation with antigen increased DC activities such as phagocytosis and IL-12 expression.	[34-37]
		3. Only DNAs from invertebrates could activate NK cells.	
	Silkworm	4. Immunestimulating effects are thought to be induced by NK celll activation through enhanced phagocytosis of macrophages.	[35]
Polysaccharides (glucans)	Algae	1. β-glucans could induce leukocyte activation.	[76]
	Mushroom and lichen	2. β-glucans could activate macrophages such as phagocytic, cytotoxic, antimicrobial activities and production of ROS.	[77-86]
	Plants	3. Enhanced macrphages abilities by glucans is thought to be induced by pattern recognition receptor of macrophages.	[87, 88]

Probiotics	Bifidobacterium	1. Inducement of production of TNF-α, IL-6, nitric oxide, and H_2O_2 in macrphages 2. Inducement of production of IL-2 and IL-5 in $CD4^+$ cells 3. Inducement of proliferation and antibody production of B-cells 4. Increase of permeation of antigens through Peyer's patches, thereby, stimulating the numberous IgA-committed B-cell population	[5, 44, 45, 48]
	Lactobacillius	4. Immunostimulating effects are thought to be associated with GALT.	[5, 46, 49, 50]
Others	Fibers	1. Ferrmentable fiber to the diet showed biological functions. 2. Modification of production of insuline, gut-derived hormones. 3. Alteration of T-cell responses in GALT responding th mitogens such as high T-cell proliferation and low B-cell proliferation. 4. Inducement of increase of proportion of $CD8^+$ T-cell	[30, 44, 46, 54, 55]

	5. Biological functions by fibers are thought to be elicited by direct interaction with lactic acid bacteria, short-chain acid production by fiber formentation, or enhanced mucin production.	
Minerals	1. Enhancement of growth rate, improvement of animals' health, and increasement of feed efficiency 2. Reinforcement of antibody production against antigens of PRRS and *Pasteurella multocida* type A 3. Increase of expression of TNF-α, IFN-γ, and IL-4 in macrophages 4. Increase of B-cell proportion in spleen 5. Immunostimulating effects are thought to be indcued by macrophage activation by enhanced phagocytosis.	[12, 16, 56-62]
Herbs	1. Inducement of the chagnes in duodenal mucosa with beneficial	[63-71]

effects such as angiogenesis prcess, lymphoid infiltration, and enlongation of villi.

2. Increase of lysozyme activities

3. Activation of lymphocytes, macrophages, and NK cells such as increase of phagocytosis or stimulation of interferon synthesis.

4. Biological benefits effects such as ntimicrobial, anti-inflammatory, and antioxidative activities

5. Biological benefits effects are thought to be induced by synergy effects of some ingredients of herbs such as flavonoids, vitamin C, and carotenoids (but not well established).

Table III. Antigenic diversity and geographical distribution of the different foot-and-mouth diease virus serotype

FMD type	Extent of diversity
O	The most prevalent serotype, with two main lineages: South America and 'Old Europe'; Middle East and Asia. There are several recognized vaccine strans (Manisa, BFS and Campos)
A	Secondly most prevalent serotype. High antigenic diversity. New antigenic variants emerge frequently.
C	Limited antigenic diversity and very restricted geographical distribution.
SAT 1-3	Highly genetically diverse. Limited endemic range with periodic excursions
Asia 1	Middle East and Asia. Limited antigenic diversity

[Domenech J, 2010]

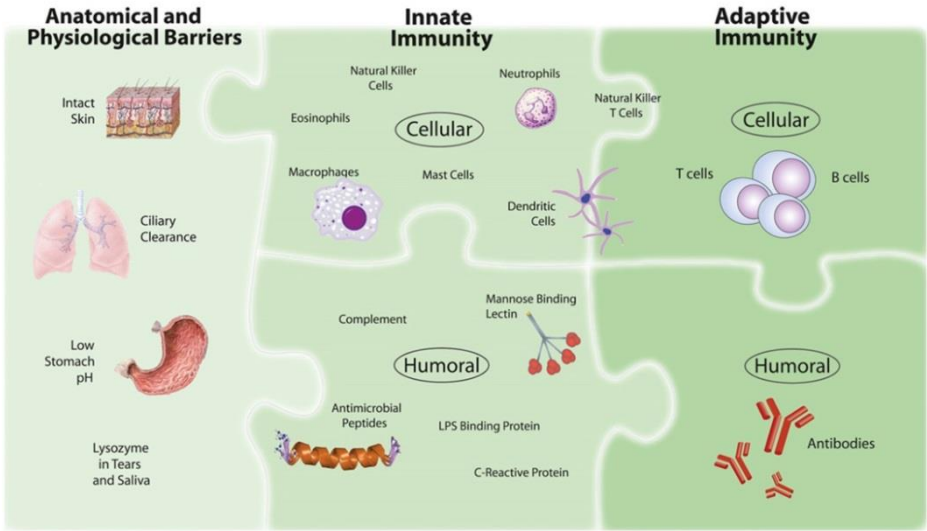

Figure I. Diagram of immune system. Immune defence system can be simplistically viewed as consisting of 3 levels: (1) anatomic and physiologic barriers; (2) innate immunity; and (3) adaptive immunity. Some elements are difficult to categorize becauseinnate immune component is enmeshed with adaptive immune component (NK cells and DCs) (Turvey and Broide, 2010).

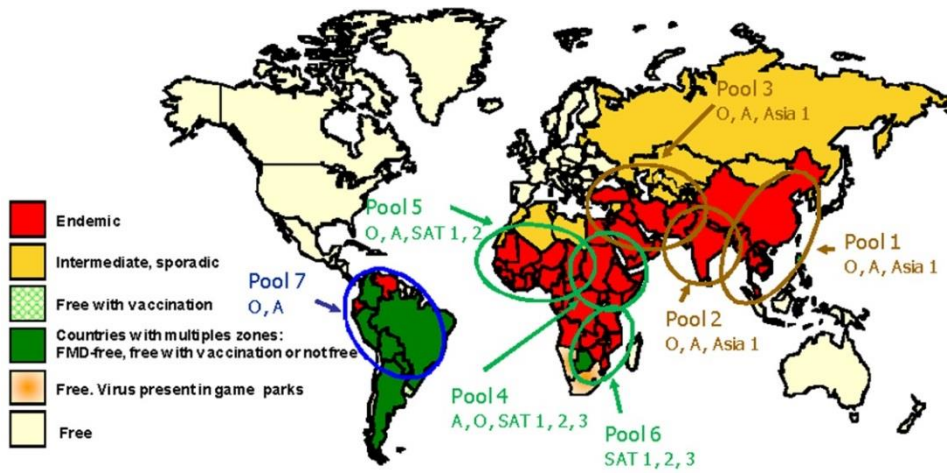

Figure II. Seven major regional pools of FMDVs have identified where circulation and evolution occure (OIE, 2012).

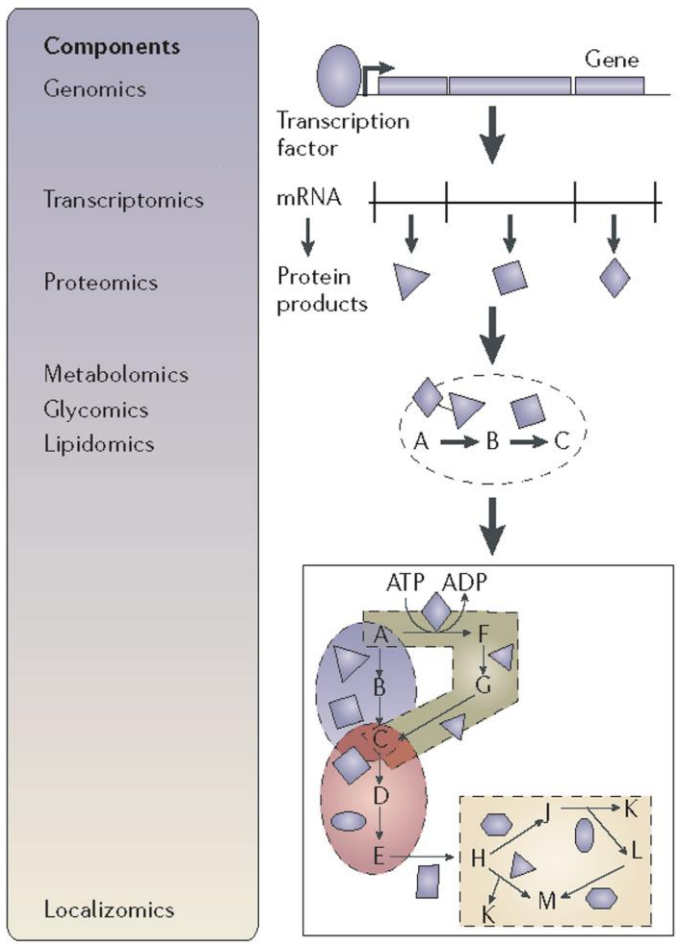

Figure III. Component in the cell described by 'Omics' data From the top, DNA (genomics) is first transcribed to mRNA (transcriptomics) and translated into protein (proteomics), which can catalyse reactions that act on and give rise to metabolites (metabolomics), glycoproteins and oligosaccharids (glycomics), and various lipids (lipidomics). Many of these components can be tagged and loclized within the cell (localizomics) (Joyce and Palsson, 2006).

Chapter I

Modulation of macrophage activities in proliferation, lysosome, and phagosome by the non-specific immunostimulator, mica

Abstract

It was reported that the aluminosilicate material mica activated macrophages and showed its immunostimulating effects. However, the mechanisms by which it exerts these effects are unclear. To address this, the effects of mica fine particles (MFP, 804.1 ± 0.02 nm) on the murine macrophage cell line, RAW 264.7 cells were evaluated. Specifically, RAW 264.7 cells were treated with 100 and 500 μg/mL MFP and their proliferative response was determined using the MTT assay. Changes in global gene expression upon MFP treatment for 12 and 48 h were also determined using microarrays. Following the MFP treatment, RAW 264.7 cells showed a low level of proliferation compared to nontreated cells ($p < 0.01$). There was a change in an expression level of 1,128 genes after 48 h treatment. Specifically, genes associated with the cell cycle, DNA replication, and pyrimidine

and purine metabolisms, were down-regulated in cells treated with MFP, which are resulted in reduction of cell proliferation. MFP treatment also up-regulated genes associated with lysosome and phagosome function, which are both required for macrophage activities. It could be speculated that activation of macrophages by mica is, in part, derived from up-regulation of these pathways.

Key words: lysosome pathway, macrophage, mica fine particle, microarray, and phagosome pathway

Introduction

Mica, which is well known for its immunostimulating effects, is a common aluminosilicate mineral containing calcium, magnesium, iron, aluminum, and silicates. The immunostimulating effects of mica have been proven through many previous studies [16, 17, 149, 150]. Mica has been used as feed supplement to improve immune activities [16, 17] and increase an absorption rate of high-protein nutrients [149, 150]. As described in the above study results, most studies using mica have been concentrated on the proof of the immune enhancement effects but few studies have been done on the mechanism of how the immunity enhancement effect is induced. It was reported that mica activated macrophages and the immunity enhancement effect was induced by this activation of the macrophages [18]. In addition, a number of studies have disclosed that mica acted as a superantigen, leads to T-cell activation and promotes phagocytosis of macrophages through high affinity binding to MHC class II molecules. This in turn leads to immune system activation via the induction of proinflammatory cytokines such as IL-1α, IL-6, and TNF-α [60, 61, 151]. Nonetheless, the precise mechanism by which mica modulates macrophage function and induces their activation remains to be determined.

Macrophages are critical effectors of the immune response, and carry out the removal of ‘non-self’ material via phagocytosis [89]. They are considered one of the main entry routes of particles into the body, and thereby play an important role in determining the biopersistence of foreign particles and in the associated inflammatory responses triggered by their phagocytic activities [90, 91]. They have been also known to participate in innate immunity and in the first line of immune defense [152]. Previous studies suggested that macrophages could interact with T-cells using cytokines secretion and receptors thereby modulating the immune responses [89, 153].

In this study, to investigate the mechanisms associated with mica-dependent activation of macrophages, MFP were manufactured and used to treat the murine macrophage cell line, RAW 264.7. Following the MFP treatment, global cell responses were investigated using a microarray approach. Through this genome-wide approach, cellular signaling pathways engaged following MFP treatment were demonstrated.

Materials and Methods

MFP preparation

The MFP were produced by Seobong Biobestech (Seoul, Korea), containing silicon dioxide (61.90 %), aluminum dioxide (23.19 %), iron oxide (3.97 %), sodium oxide (3.36 %), calcium oxide (< 2 %), magnesium oxide (< 2 %), titanium oxide (< 2 %), and 36 ppm germanium.

Morphology and size of MFP

The morphology of the MFP was observed using a field emission scanning electron microscope (FE-SEM; JSM-6700F, JEOL, Tokyo, Japan) following platinum-coating using Cressington 108 (Cressington, Watford, UK). The particle size of MFP was measured using the DLS-7000 spectrophotometer (Otsuka Electronics Ltd., Osaka, Japan).

Cell preparation and evaluation of effect of MFP on cell proliferation

The murine leukemic monocyte macrophage line, RAW 264.7, was obtained from Korea Cell Line Bank (KCLB No. 40071; Seoul, Korea) and grown at 37 ℃ in a 5 % CO_2 atmosphere in Roswell Park Memorial Institute medium (RPMI; Gibco, Carlsbad, CA, USA) 1640 containing 10 % fetal bovine serum (FBS, Gibco). The cells were then seeded at a concentration of 8.0×10^4 cells/cm^2 in 12-well plates with 1 mL of media containing 2 % FBS. Following 8 h culture, cells were incubated with 100 or 500 μg/mL of MFP for 0, 12, 24, or 48 h. After treatment with MFP, the cells were then incubated in FBS-free media with 0.5 mg/mL of MTT (Life Technologies, Carlsbad, CA, USA) for 4 h. Formed crystals were dissolved in 1 mL of dimethyl sulfoxide (DMSO; Sigma, USA) and 100-μL aliquots were transferred to 96-well plates. The plates were read on an Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA, USA), using a test wavelength of 590 nm and a reference wavelength of 620 nm. Cell numbers were calculated based on the standard curve generated from serially diluted cells. The effect of MFP on cell proliferation was expressed relative to the cell number at 0 h.

RNA preparation

RAW 264.7 cells cultured in RPMI 1640 containing 10 % FBS were seed at a concentration of 8.0×10^4 cells/cm^2 in T75 flasks with 20 mL of media containing 2 % FBS. Following an 8-h culture, cells were incubated with 100 μg/mL of MFP and collected at 0, 12, and 48 h post-stimulation. RNA was extracted using the RNeasy mini kit (Qiagen, Venlo, Limburg, Netherlands) as described by the manufacturer. All RNA samples were quantified, aliquoted, and stored at -80 ℃ until use. Purity and integrity of RNA samples was determined using denaturing gel electrophoresis, OD 260/280 ratio, and analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Microarray hybridization

RNA amplification, labeling, array hybridization, and scanning were carried out by Macrogen Inc. (Seoul, Korea). Amplification and purification of total RNA (550 ng) was performed using the Ambion Illumina RNA amplification kit (Ambion Inc., USA) per the manufacturer's recommendations in order to obtain biotinylated cRNA. Following quantification of the cRNA using the ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), 750 ng of labeled

cRNA samples were hybridized to each Illumina expression beadchip (Mouse WG-6 v2.0; Illumina Inc., San Diego, CA, USA) for 16–18 h at 58 ℃, which covers more than 45,200 transcripts, according to the manufacturer's instructions. After the bead array manual, detection of array signals was performed using Amersham Fluorolink Streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK). Arrays were scanned with an Illumina bead array Reader confocal scanner (Illumina Inc.) as described by the manufacturer.

Raw data preparation and statistical analysis

The quality of hybridization and overall chip performance were monitored by visual inspection of an internal quality control check and the raw scanned data. Raw data were extracted using the Illumina GenomeStudio v2009.2 (Gene Expression Modulev1.5.4, Illumina Inc.) and selected based on a p-value < 0.05 with no fail-count (sample count of detection p-value ≥ 0.05). These selected gene signal values were transformed by logarithm and normalized by the quantile method. The comparative analysis between the test and control samples was performed using fold change. Genes showing more than 2-fold increase or decrease were considered to be significantly altered. All data and visualization of differentially expressed genes were carried out using R2.4.1 (www.r-project. org).

Microarray data analysis

Gene set enrichment analysis for genes showing significant altered expression was performed using Protein Analysis Through Evolutionary Relationships (PANTHER) (http://www.pantherdb.org). Differentially expressed genes were categorized by biological process and molecular function using the PANTHER classification database by means of Fisher's exact test to detect coordinated changes in pre-specified sets of related genes. Clustering analyses of differently expressed genes were investigated using MultiExperiment Viewer version 4.9.0 software (MeV; Institute for Genomic Research, Boston, MA, USA). Based on these altered genes, unsupervised hierarchical clustering was conducted to determine the relation according to MFP treatment period. In addition, the patterns of changes in gene expression were also analyzed through quality threshold clustering (QTC) method.

The changes in cell process are derived from pathways formed by the interaction of genes [112, 113]. Therefore, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping of genes involved each cluster was also carried out in order to analysis systemic information representing functional aspects of each of patterns (http://www.genome.jp/kegg/tool/map_pathway2.html). For a pathway mapping term to be considered significantly, pathway represented by fewer than 10

genes were filtered out, thereby identifying the most robustly affected pathways [90]. Dataset of microarray results has been deposited in Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63827) and accessible through GEO series accession number GSE63827.

Verification of microarray results

To verify the microarray results, 3 genes that were identified as being up-regulated and associated with lysosome and/or phagosome pathways and 4 genes that showed down-regulation and involved in cell cycle, pyrimidine metabolism, and/or purine metabolism (Table 1.1) were randomly selected and subjected to quantitative real time RT-PCR (qRT-PCR). Total RNA (1 µg), the remainder of microarray analysis, was submitted for synthesis of cDNA using QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. The qRT-PCR reaction was carried out with 1 µL of cDNA using the Rotor-Gene SYBR Green PCR kit (Qiagen) and Rotor-Gene Q real time PCR cycler (Qiagen). The cDNA was amplified under following conditions: 45 cycles at 95 ℃ for 15 s followed by 45 s at 60 ℃. The fluorescence was detected during the extension phase and the expression level was compared to a non-stimulated RAW 264.7 cell

control by the $2^{-\Delta\Delta C}{}_{T}$ method using the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a reference [154].

Statistics

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed by using the Student's *t*-test or repeated measures of ANOVA using Statistical Package for the Social Sciences version 19.0 software (SPSS, USA). The statistical significance of differences was set at value of $p < 0.05$.

Results

Morphology and size of MFP

SEM micrographs revealed that MFP were irregular polygons with a rough surface. The average MFP particle size was 804.1 ± 0.02 nm, which places these substances in the 'fine particles' class as described by the United States Environmental Protection Agency (Figure 1.1).

Effect of MFP on cell proliferation

MFP-treated cells showed proliferative responses until 24 h (Figure 1.2). All MFP-treated cells showed significant increases in cell numbers except treatment with 500 μg/mL for 48 h which showed significant decrease ($p < 0.01$). Nontreated cells demonstrated same proliferation pattern as MFP 100 μg/mL treatment, showing high level of proliferative responses compared to the MFP treatment ($p < 0.01$). An appropriate concentration was defined as 100 μg/mL at which a significant decrease of cell population was not observed for 48 h. In addition, we defined early and late treatment time points as 12 h and 48 h, respectively.

Microarray analysis of differentially expressed genes after stimulation with MFP

Following MFP treatment for 12 h and 48 h, expression of 1,165 of 30,854 genes examined showed greater than 2-fold changes. Among the 1,165 genes, 116 and 569 genes were up-regulated, and 20 and 559 genes were down-regulated at 12 h and 48 h, respectively (Figure 1.3). Figure 1.4 shows the median of normalized hybridization signals of the genes with altered transcription between the MFP 100 μg/mL treated cells for 12 h or 48 h and for 0 h. Red dots indicate an expression

level change of ≥ 2 or ≤ 2-fold for both up- and down-regulated genes with $p < 0.05$. As shown in the graph, a larger number of red dots, indicating more than 2-fold changes in expression, were observed in the MFP-treated cells at 48 h compared to treatment for 12 h.

Gene set enrichment analysis

Each of the up- and down-regulated genes were categorized by using gene set enrichment analysis using the PANTHER classification database to detect coordinated changes in pre-specified sets of related genes. This revealed that 10 molecular function categories (Figure 1.5) and 13 biological process categories (Figure 1.6) were associated with transcriptional changes following treatment with MFP 100 μg/mL. Most of the differentially up- and down-regulated genes were involved in two molecular function categories (binding and catalytic activity) and two biological process categories (metabolic and cellular processes).

Clustering analyses

The 1,165 genes that were differentially expressed were subjected to unsupervised hierarchical clustering. As shown Figure 1.7A, experimental groups

could be divided into two groups by 0 and 12 h versus 48 h based on gene expression. QTC analysis revealed that gene expression patterns according to MFP treatment were classified under six clusters (Figure 1.7B). Cluster 1, 2, 3, 4, 5, and 6 contained 524, 504, 57, 36, 34, and 10 genes, respectively. Cluster 1 and 2 presented the genes showing continuous up- and down-regulation, respectively. The genes involved cluster 3 and 5 showed no noticeable expression changes at 12 h, however, down- and up-regulation were observed at 48 h in two clusters, respectively. The genes showing up-regulation at 12 h and then down-regulation at 48 h were classified as cluster 4. Cluster 6 demonstrated the genes that showed no noticeable expression change since down-regulation at 12 h.

Analyses of affected pathways

In order to elucidate whole chains of events caused by differently expressed genes involved each cluster of QTC, KEGG pathway mapping was conducted with significant regulated genes in each cluster. Among pathways constructed using the differentially expressed genes classified as cluster 3, 4, 5, and 6, there was no pathway represented by more than 10 genes. In case of differentially regulated genes involved cluster 1 and 2, total 114 genes were listed on 9 pathways. Pathways of cell cycle, lysosome, phagosome, pyrimidine metabolism, purine

metabolism, cell adhesion molecules, DNA replication, endocytosis, and antigen processing and presentation were represented by 26, 24, 21, 19, 16, 14, 16, 11, and 10 genes, respectively. In addition, lysosome, phagosome, cell adhesion molecules, endocytosis, and antigen processing and presentation pathways were mainly associated with cluster 1 whereas cell cycle, purine metabolism, pyrimidine metabolism, and DNA replication pathways were represented by mainly genes of cluster 2. Figure 1.8 and 1.9 conducted using KEGG mapper show the pathways of cell cycle and phagosome, respectively. The genes of *Gadd45*, *Cip1* (*Cdkn1a*), and *Mdm2* involved in cell cycle pathway showed gene expression pattern of cluster 1 (Figure 1.8). In addition, down-regulation in gene expression associated with microtubule activity was observed in Figure 1.9.

Validation of microarray data

To verify the microarray results, qRT-PCR was also conducted using the same experimental RNA samples with 7 selected genes that showed altered expression and involved lysosome, phagosome, cell cycle, pyrimidine metabolism, and/or purine metabolism functions. As shown Figure 1.10, all genes tested by qRT-PCR showed same direction in expression levels as the microarray results.

Discussion

The immune enhancement effects of mica are clear based on the results of several previous studies [16-18, 60, 61]. Aluminosilicate, one of the major ingredients of mica, is known to play an important role in stimulating an immune response via inducing the activation of macrophages [16, 18, 60, 61]. Nonetheless, few studies have addressed the mechanism of macrophage activation by mica. Therefore, investigation to macrophage signaling networks that are modulated by mica treatment was conducted in this study.

As shown in Figure. 2, the number of genes that showed changes in gene expression level at 48 h increased more than those of 12 h. In addition, unsupervised hierarchical clustering results showed that gene expression pattern of 0- and 12-h treated cells formed high level of similarity compared to 48 h. On the other hand, gene expression at 48 h was different from those of 0- and 12-h treated cells. Together these data reveal that a 12-h MFP treatment has no noticeable effect on gene expression, whereas a 48-h treatment elicits significant changes. The QTC analysis showed that differently expressed genes by MFP treatment could be classified into 6 clusters. As the results, most of up- and down-regulated genes were involved in cluster 1 and 2; therefore, it could be thought that MFP-treated cells were mainly affected by the genes of these clusters. The KEGG mapping

analysis also supported this suggestion. All of the identified pathways, which are represented by more than 10 genes, were mostly conducted with the genes involved cluster 1 and 2. Based on the results, it was determined that the pathways of cell cycle, lysosome, phagosome, pyrimidine metabolism, purine metabolism, cell adhesion molecules, DNA replication, endocytosis, and antigen processing and presentation were significantly affected by MFP treatment.

Among the identified pathways, the cell cycle pathway, which contained the largest number of genes showing different expression, is involved in cell division and proliferation (Figure 1.8). The key regulatory enzymes in the cell cycle pathway are cell division cycle kinases (*Cdc*s) and cyclin-dependent kinases (*CDK*s) [155-157]. Genes related to the cell cycle pathway, such as *Cdkn2c*, were generally down-regulated after MFP treatment, and exhibited an expression pattern that placed them in cluster 2. On the other hand, *Cdkn1a*, *Gadd45*, and *Mdm2*, which are cell cycle-related genes that were all up-regulated, were classified into cluster 1. This result was thought that up-regulation of *Cdkn1a*, *Gadd45*, and *Mdm2* was associated with the p53 pathway. It was known that the tumor suppressor p53 plays an important role in cellular stress response pathway [158-160]. p53 activity is kept low under non-stressed conditions by its predominant negative regulator, *Mdm2* [158]. However, under certain circumstances such as cell stress, drug treatment, or hypoxia, p53 protein is accumulated and is activated in

order to prevent uncontrolled growth. *Cdkn1a* and *Gadd45*, as target genes of p53 pathway, were up-regulated according to activation of the p53 pathway, thereby down-regulating the cell cycle pathway (Figure 1.8). *Mdm2* were also up-regulated according to the p53 protein accumulation as negative feedback although *Mdm2* is negative regulator of the p53 pathway [158]. KEGG mapping also showed that reduced expression of *Cdk1* (*Cd2a*) leads to down-regulation of *Plk1*, which has critical function during mitosis [161]. In addition, it could be found that *Espl1* having a role in chromosome segregation was inhibited by down-regulation of *Anapc5* and *Cdc20* in the cell cycle pathway (Figure 1.8) [162]. *Mcm* genes, that were down-regulated after MFP treatment in this study, have been postulated to couple DNA replication to cell cycle progression [163, 164]. *Pola2*, *Pold1*, *Pold2*, *Pole*, *Pole3*, and *Prim2*, which are associated with DNA polymerases and participate in pathways of DNA replication, pyrimidine metabolism, and purine metabolism [165], were also down-regulated in this study. In addition, other genes related to pyrimidine and purine metabolisms were also down-regulated. Since pyrimidine and purine nucleotides are essential precursors for RNA and DNA synthesis [166], it could be postulated that DNA replication pathway were also attenuated by down-regulation of pyrimidine and purine metabolism. Subsequently, all above results showed that down-regulated genes by MFP treatment were mainly associated with pathway of cell cycle, DNA replication, and pyrimidine and purine

metabolisms that are known to affect cell proliferation [158, 160, 163, 165, 166]. Based on the KEGG mapping, some of genes associated with cell cycle pathway including *Anapc5*, *Cdc20*, *Cdc25a*, *Cdc45l*, *Cdc6*, *Cdc7*, *Cdkn2c*, *Dbf4*, *E2f2*, *Espl1*, *Orc6l*, and *Plk1* were involved in cell growth as well as death. It could be considered that changes of expression level in these genes are more related to cell growth and/or inhibition of cell proliferation than apoptosis and/or necrosis, because RAW 264.7 cells treated with 100 μg/mL of MFP for 12 and 48 h showed high cell numbers in MTT assay compared with 0 h. Moreover, there was no significant difference among the cell numbers at 12, 24, and 48 h. In addition, the genes associated with cell cycle showing altered gene expression were mostly classified as cluster 2 showing pattern of continuous down-regulation. This result was supported by the MTT assay results that showed continuous low proliferation rate in MFP-treated RAW 264.7 cells compared to the nontreated cells (Figure 1.2). To sum up, it could suggest that RAW 264.7 cells showed decrease of cell proliferation, responding to MFP treatment.

KEGG mapping analysis revealed that up-regulated genes by MFP treatment were predominantly associated with the lysosome and phagosome pathways. A large number of genes involved the lysosome pathway were coupled with the phagosome pathway. Most of up-regulated genes in pathways of endocytosis and antigen processing and presentation were also involved in the phagosome pathway.

Lysosomal acid hydrolases and lysosomal membrane proteins are essential for lysosome function [167, 168]. In this study, genes encoding lysosomal acid hydrolases and lysosomal membrane were up-regulated by MFP treatment. Lysosomal activities play an important role in the processing of peptides and degradation of biomacromolecules that bind MHC class II molecules, and thereby enhance the activities of antigen receptors on T-cells [168, 169]. This activities of antigen processing and presentation are crucial for immunity to pathogens [169]. Beside, lysosomes are crucial for the maturation of phagosome to phagolysomes in phagocytosis, which is important for cellular defense against pathogens [167]. As innate immunity, macrophage-driven phagocytosis initiates signaling cascades that connect the innate and adaptive immunity pathways to elicit a sustained immune response. Moreover, antigen presentation by macrophages is dependent on phagosome activity [170] and enhanced phagocytosis is an indicator of macrophage activation [89]. Corresponding with these results, KEGG mapping also showed that the phagosome pathway was up-regulated by MFP treatment (Figure 1.9). MFP treatment induced up-regulation of *Atp6v0a1*, *Atp6v1a*, *Atp6v1d*, and *Atp6v1g1*, which were known to play a critical role in receptor-mediated endocytosis by providing the acidic endosomal environment in phagosome (Figure 1.9) [171]. It could be suggested that the up-regulation of lysosome- and phagosome-associated genes observed in this study may indicate enhanced

macrophage activities, which in turn would enhance the responses of antigen presentation [169, 172, 173]. This is also supported by data showing the up-regulation of genes associated with cell adhesion molecules (*Pvrl2*, *Pvrl3*, *Cd40*, *Cd80*, *Cd86*, *Cd274*, *Itgb7*, and *Sdc3*) that are expressed on the cell surface and play a critical role in immune responses and inflammation [174]. Moreover, genes associated with antigen presentation such as *Cd74*, *Ctsl*, *Ctss*, *H2-D1*, *H2-K1*, *H2-Q6*, *H2-Q7*, *H2-Q8*, *H2-T23*, and *Hspa2* were also up-regulated by MFP treatment (Figure 1.11). Previous studies showed that enhanced antigen presentation in macrophages can reinforce the activities of T- and B-cells, thereby strengthening the immune response [89, 175, 176]. In addition, the genes associated with the lysosome and phagosome pathways were mostly involved in cluster 1. However, genes associated with microtubule activity of the phagosome pathway showed different expression pattern classes. These latter classes included *Actb*, *Tfrc*, *Tuba1a*, *Tuba1b*, *Tubb2c*, *Tubb5*, and *Tubb6*, which were all down-regulated (Figure 1.9). Microtubules are known to play roles in late phagosome maturation, such as fusion with endocytic organelles and phagolysosome formation [177]. Additionally, fusion of the phagosome with the lysosome and endosome in activated macrophages is delayed [170]. Therefore, down-regulation of microtubule expression may be indicative of early phagosome status in activated macrophages. In summary, the immune-enhancing effects of mica are likely

induced, at least in part, by the activation of macrophages through up-regulation of the pathways mentioned above (Figure 1.11).

These results could be believed as the specific responses of macrophages to MFP simulation. There were several studies on cell-particle interactions using a microarray tool [90, 178-180]. Water et al. (2009) demonstrated that RAW 264.7 cells showed altered regulation in genes expression associated with chemokines and inflammation, responding to stimulation of 500 nm of silica particles [90]. Murine cells isolated from branchoalveolar lavage containing macrophages, lymphocytes, and neutrophils showed different gene expression involved in immune, inflammatory, and cytoskeleton organization, responding to 580 nm of urban particulate matter [180]. In case of human macrophages, it was reported that altered gene expression related to cytokines and signal transduction was observed in U937 cells following the stimulation of polyethylene particles (1,710-2,580 nm) [179]. In addition, stimulation with fine ambient particles (2,500 nm) showed effect of gene modulation involved in metal binding and oxidative stress in human alveolar macrophages [178]. However, changes of cell systemic function including up-regulation of lysosome and phagosome pathways were not reported in previous that showed responses of cell to the fine particles stimulation. Therefore, it could be believed that the up-regulation of lysosome and phagosome pathway identified

in this study result from the specific responses of macrophage to MFP stimulation, not common reaction against fine particles.

In this study, the effects of MFP on gene expression profile were analyzed using RAW 264.7 murine macrophages, as this cell type plays a critical role in the immune system. Genes associate with pathways related to the cell cycle, DNA replication, and pyrimidine and purine metabolism, were down-regulated, consistent with a mica-dependent reduction of proliferation. Mica also up-regulated genes associated with lysosome and phagosome function in RAW 264.7 cells (Figure 1.11). It could be speculated that macrophage activation is due to up-regulation of some of these pathways, and by extension that the positive effects of mica in the immune system are mediated through increased lysosomal and phagosomal activity in macrophages.

Table 1.1. Primer used for qRT-PCR.

Genes		Primers (5'-3')	Pathway	Accession no.
Pla2g1	F	CAGTTTCCCGATGGTGTGGA	Lysosome	NM_133792.2
	R	CCGTTTTCATTTGGGGCTCG		
Lamp2	F	GTTCCTAGGAGCCGTTCAGTC	Lysosome / Phagosome	NM_001017959.1
	R	TCATCCCCACAACTGCTTCC		
Arsb	F	GTGCGCCGATTGAGTCTTTG	Lysosome	NM_009712.3
	R	AACAGTGGTTTCTCCGGTGG		
Nme1	F	GACCGCCCCTTCTTTACTGG	Pyrimidine and purine metabolisms	NM_008704.2
	R	CCTCCCAGACCATAGCAACC		
Rrm1	F	ACGAAGCACCCTGACTATGC	Pyrimidine and purine metabolisms	NM_009103.2
	R	TGGCAGAATTCAGGCGATCC		
Ccne1	F	TTCGGGTCTGAGTTCCAAGC	Cell cycle	NM_007633.2
	R	TGCAAAAACACGGCCACATT		
Espl1	F	CAAGCCGCGACTTTTGCC	Cell cycle	NM_001014976.1
	R	GCAAGCCCTCAGGATGGTAT		

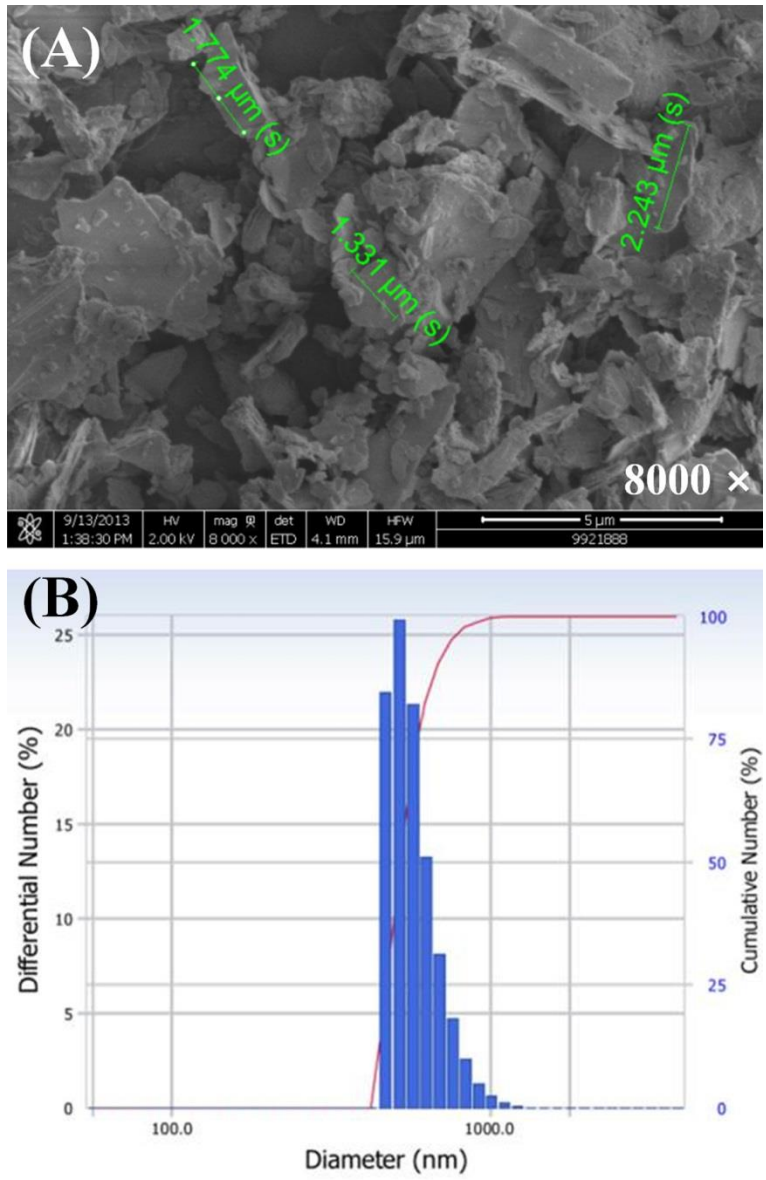

Figure 1.1. SEM microphotographs (A) and the diameter distribution of mica fine particles (B).

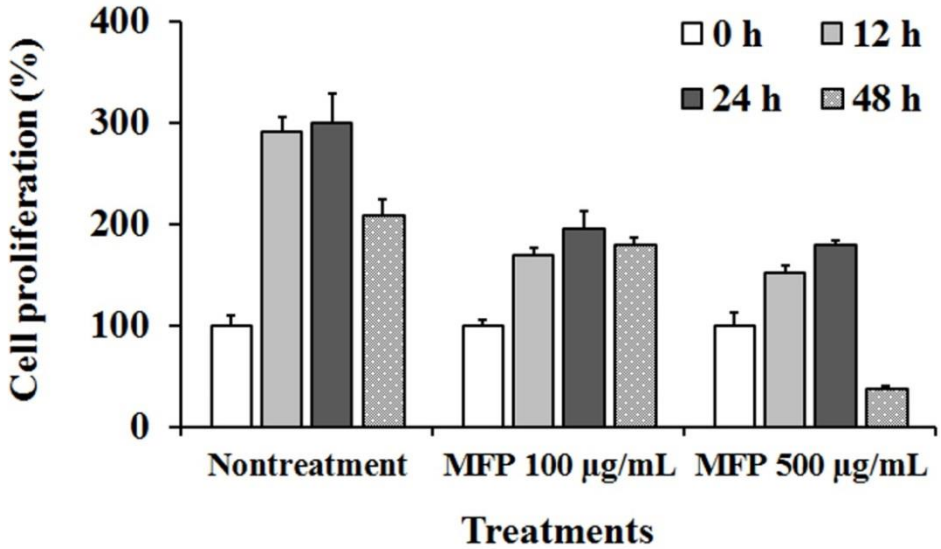

Figure 1.2. Proliferation of RAW 264.7 cells after treatment with 100 and 500 µg/mL of MFP. Cell proliferation was measured using the MTT assay.

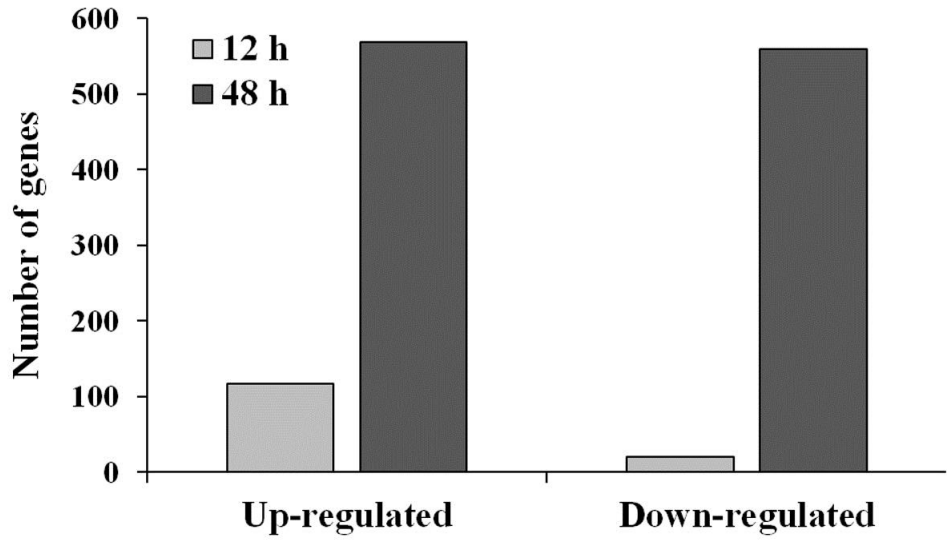

Figure 1.3. Different gene expression after treatment with 100 μg/mL of MFP. Count of up-and down-regulated genes in RAW 264.7 cells treated with MFP 100 μg/mL compared to nontreated. (0 h; $p < 0.05$, |fold change | ≥ 2).

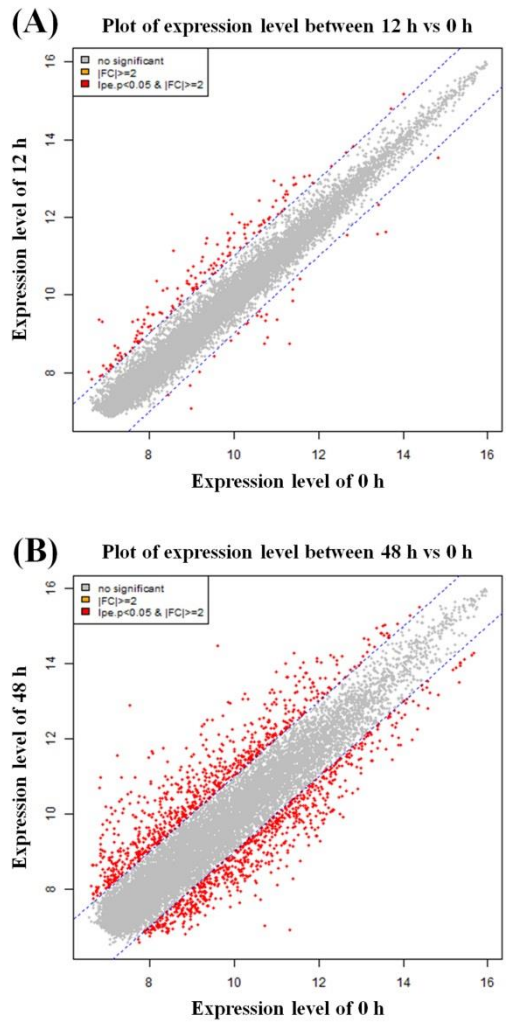

Figure 1.4. (A) and (B) showed plots of the expression level between nontreated cells versus those treated with 100 μg/mL MFP for 12 and 48 h, respectively. Red dots present an expression level change of ≥ 2 or ≤ -2-fold for both up- and down-regulated genes. Expression levels were calculated by base 2 logarithm of normalized hybridization signals from each sample.

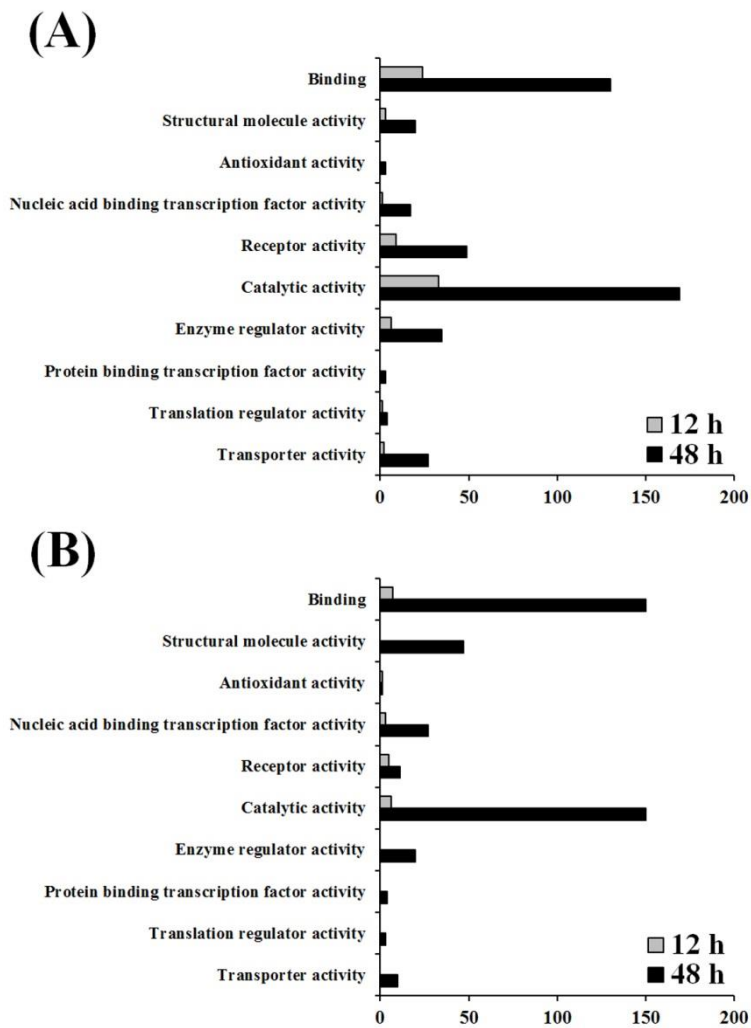

Figure 1.5. Categorization by molecular function of genes showing significant regulation. Up-regulated transcripts (A) and down-regulated transcripts (B) in RAW 264.7 macrophage at 12 h and 48 h post treatment with 100 µg/mL MFP. (p < 0.05, Fisher's exact test)

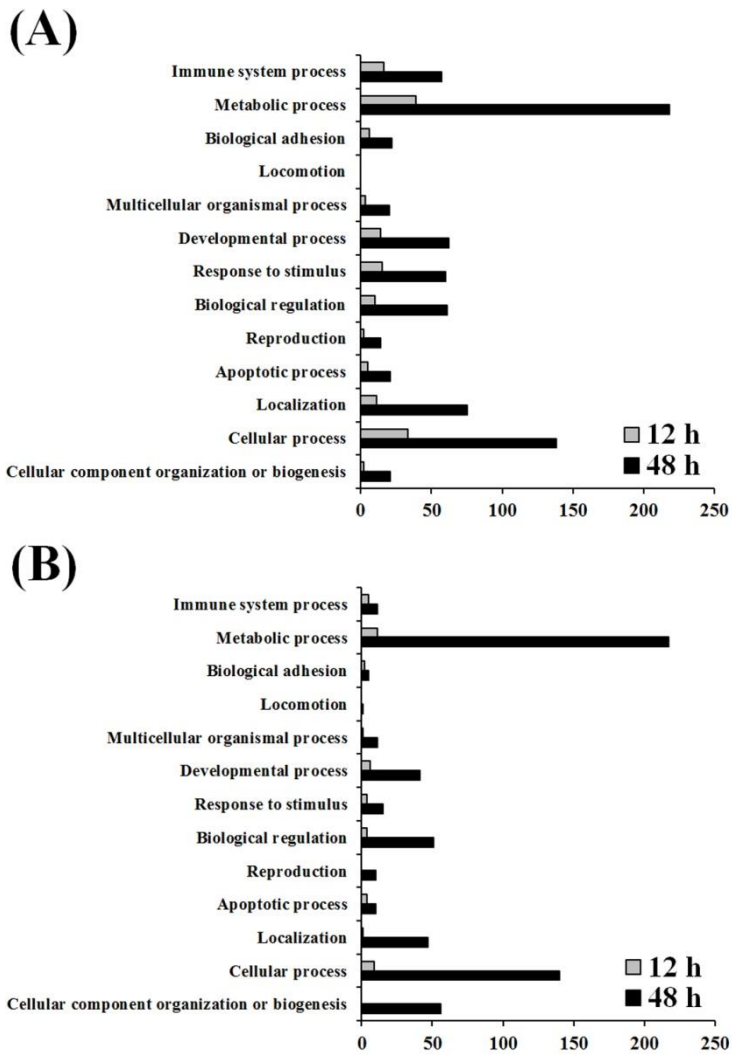

Figure 1.6. Categorization by biological process of genes showing significant regulation. Up-regulated transcripts (A) and down-regulated transcripts (B) in RAW 264.7 macrophage at 12 h and 48 h post treatment with 100 μg/mL MFP. ($p < 0.05$, Fisher's exact test)

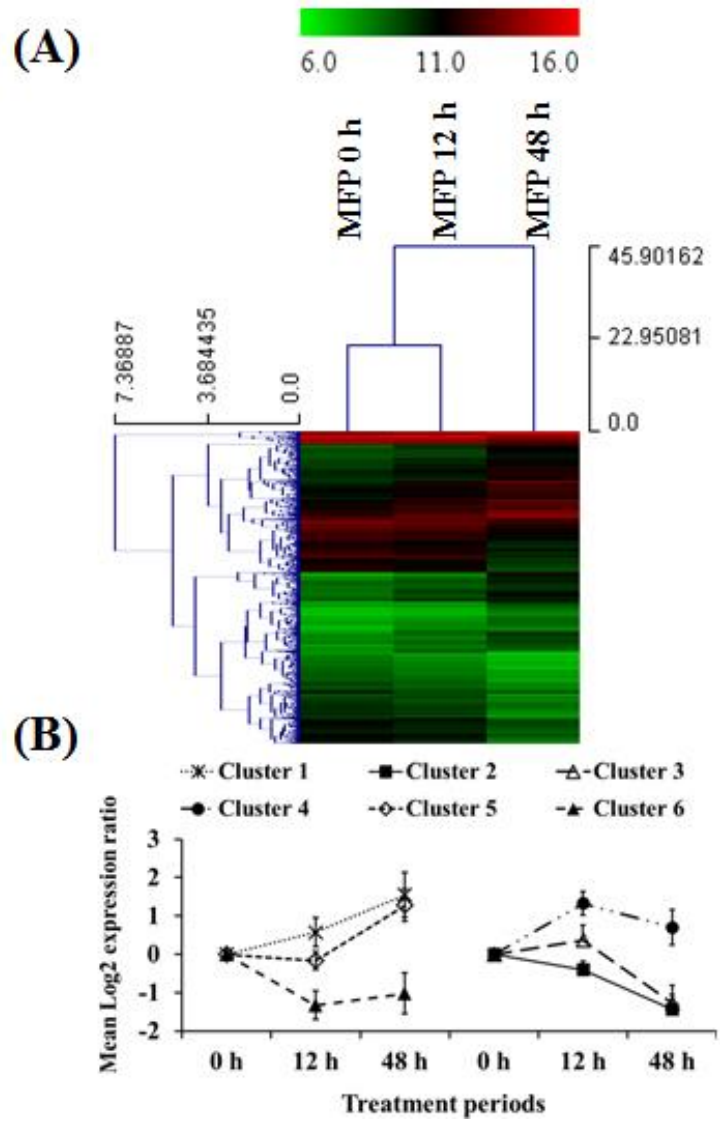

Figure 1.7. Unsupervised hierarchical clustering (A) and expression pattern profiles according to quality threshold clustering (B). The clustering results were determined using differentially expressed genes after treatment with 100 μg/mL of MFP. ($p < 0.05$, |fold change | ≥ 2)

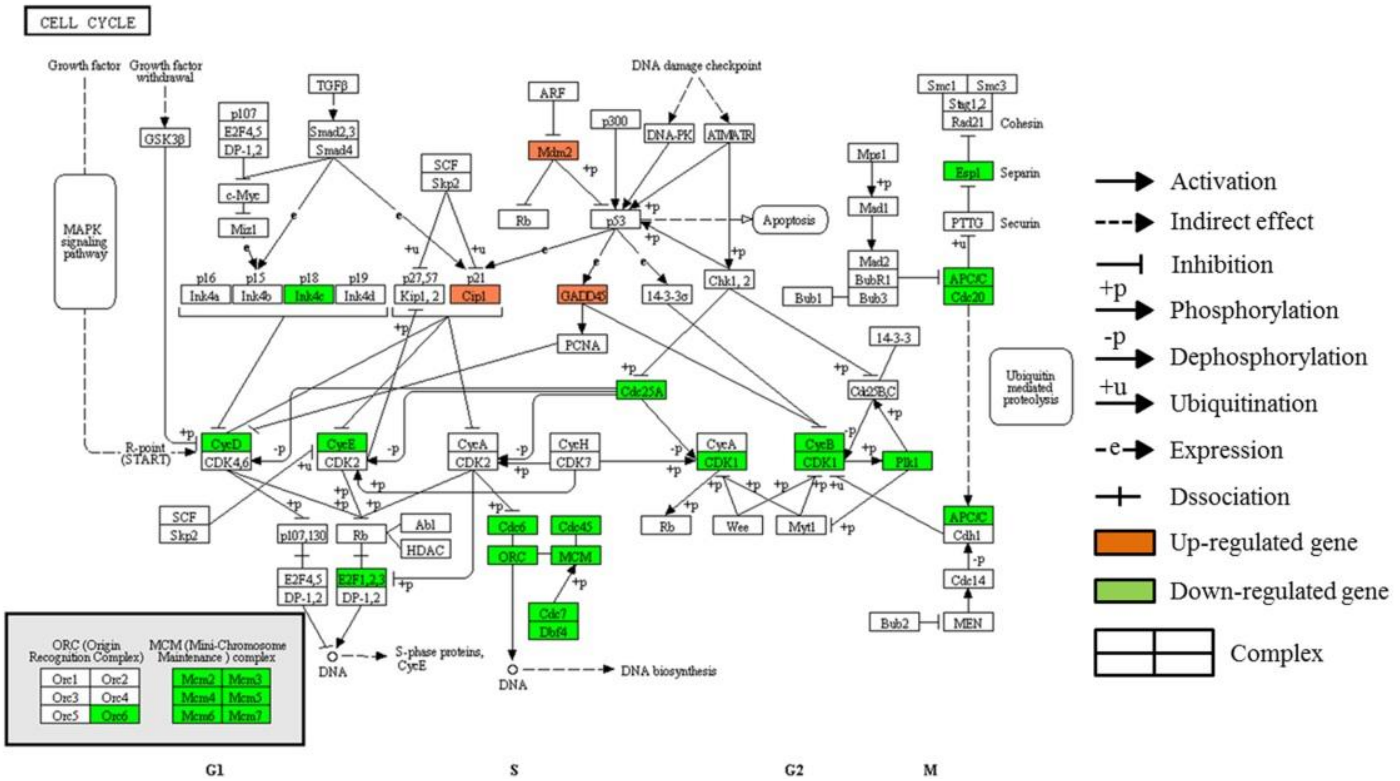

Figure 1.8. Genes showing altered expression in the cell cycle pathway after treatment of MFP 100 µg/mL. Activation of p53 enhances expression of Gadd45 and Cip1 (*Cdkn1a*). Up-regulation of Gadd45 and Cip1 inhibit CycD (*Ccnd3*).

CycE (*Ccne1* and *2*) and CycB (*Ccnd3*) are also down-regulated by Cip1 and Gadd45, respectively. Reduced expression of CDK1 leads to down-regulation of Plk1, which has critical function during mitosis. In addition, down-regulation of Cdc6, ORC (*Orc6l*), Cdc45 (*Cdc45l*), MCM, and EfF2 result in reduction of DNA synthesis. Reduced expression of APC/C (*Anapc5*) and Cdc20 down-regulates expression of Espl (*Espl1*), thereby affecting on chromosome segregation. This pathway map was conducted using KEGG mapper.

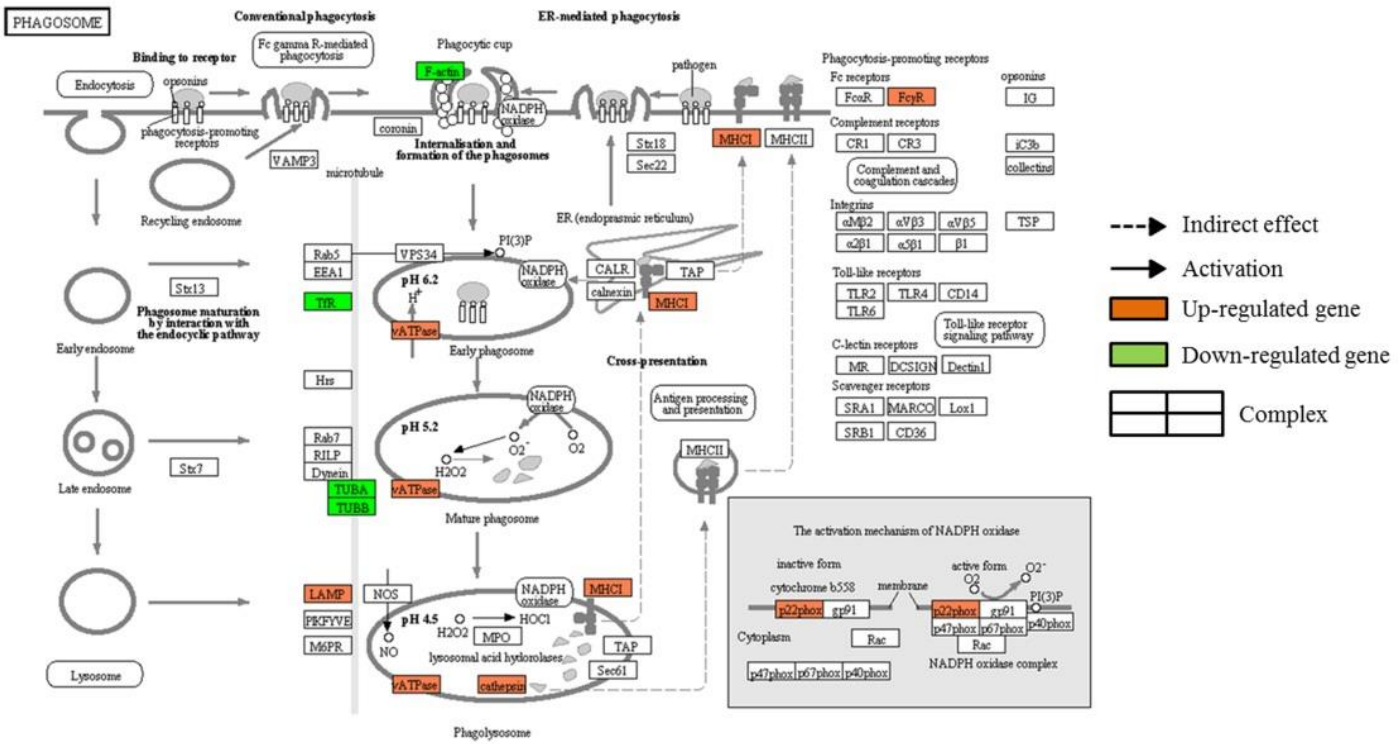

Figure 1.9. The genes showing altered expression in the phagosome pathway after treatment of MFP 100 μg/mL. The MFP treatment down-regulated genes associated with microtubule activity such as F-actin (*Actb*), TfR (*Tfrc*), TUBA

(*Tuba1a* and *1b*), and TUBB (*Tubb2c*, *5*, and *6*). Activation of NADPH oxidase by up-regulation of p22phox (*Cyba*) enhances catabolic activities in phagosome. vATPase (*Atp6v0a1*, *Atp6v1a*, *Atp6v1d*, and *Atp6v1g1*), which was up-regulated by MFP treatment, plays an critical role in receptor-mediated endocytosis by providing the acidic endosomal environment in phagosome. As proteases, cathepsin (*Ctsl* and *Ctss*) were also up-regulated by MFP treatment. MHC class I (*H2-D1*, *-K1*, *-Q6*, *-Q7*, *-Q8*, and *-T23*) and FcγR (*Fcgr1*, *2b*, *3*, and *4*), which play role in phagosome activation as antigen presenting molecules and phagocytosis-promoting receptors, were also up-regulated by MFP treatment. This pathway map was conducted using KEGG mapper.

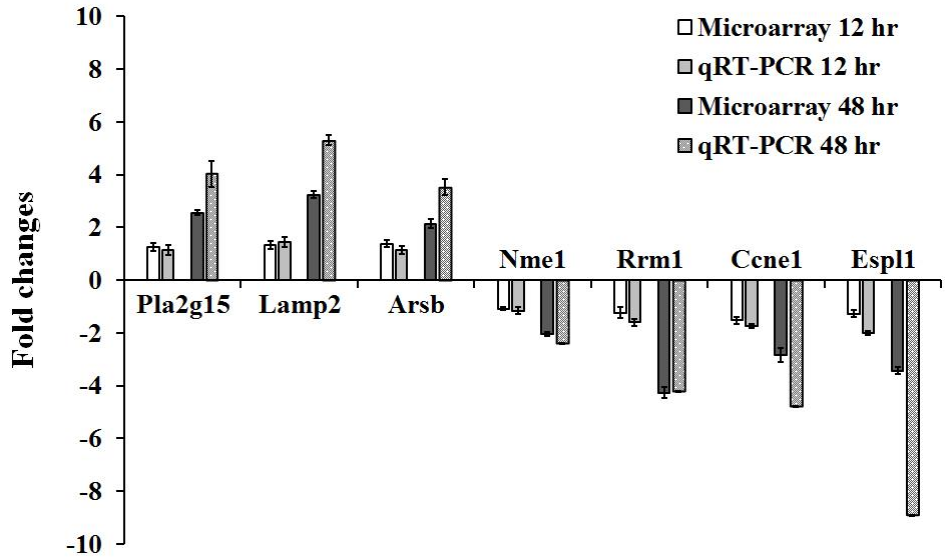

Figure 1.10. Validation of microarray data *via* quantitative RT-PCR. The relative expression level was normalized to the level of GAPDH expression using the $2^{-\Delta\Delta CT}$ method.

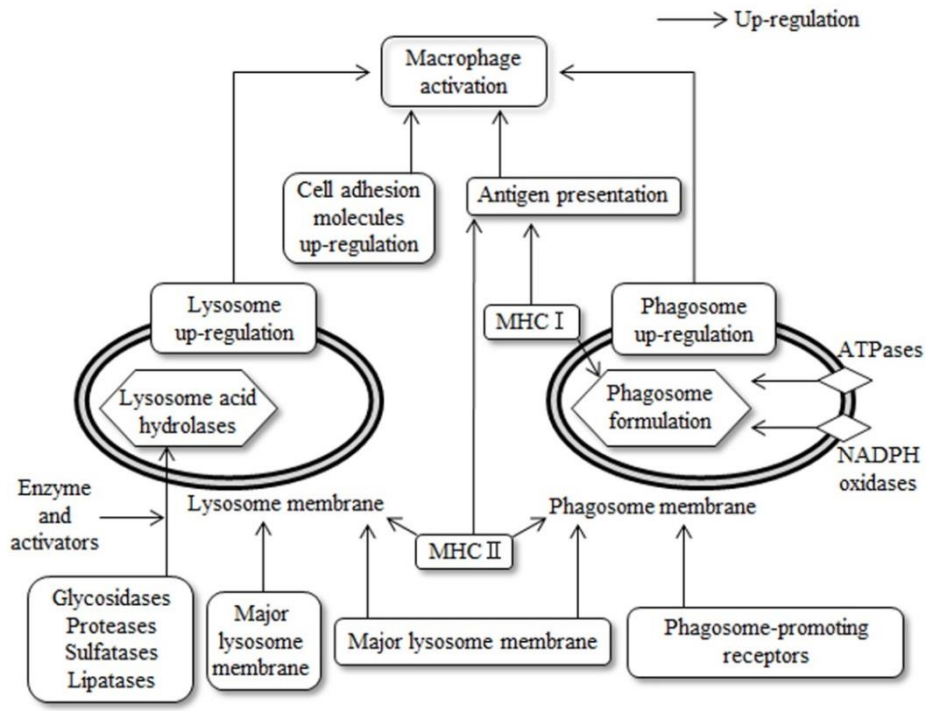

Figure 1.11. Suggesting diagram of activation pathways. This diagram was determined through the analysis of genes whose expression level was altered in RAW 264.7 cells that were treated with MFP 100 μg/mL. MFP treatment up-regulated the pathways of lysosome and phagosome. The pathway mapping was conducted using KEGG database.

Chapter II

The effects of germanium biotite supplement as a prophylactic agent against respiratory infection in cattle

Abstract

Germanium biotite, a natural mineral, is comprised of mainly silicate. This mineral showed activities of increase in feed efficiency and non-specific immunostimulating in previous studies. The aims of the present study were to evaluate the prophylactic effects of germanium biotite against respiratory diseases in cattle as a feed supplement and investigate the possibilities of the substitution of antibiotics with germanium biotite as feed additives. To achieve this purpose, bovine herpesvirus-1 (BHV-1) and *Mannheimia haemolytica* serotype A1 were experimentally inoculated into cattle. After challenge, germanium biotite showed a lower cumulative clinical score than the control group. In accordance with this clinical result, enhanced clearance of BHV-1, a low infection rate of *Mannheimia haemolytica* serotype A1, tempered superficial lesions, and moderated

histopathological signs were observed in the germanium biotite group, compared to the control group. These results of the present study indicate that germanium biotite has prophylactic effects against bovine respiratory disease and could be a candidate for a new alternative feed supplement in cattle, through its effects as a non-specific immune stimulator.

Keywords: Bovine herpesvirus-1, cattle, germanium biotite, immune stimulator, *Mannheimia haemolytica* serotype A1

Introduction

The consumption of livestock products has been increasing along with increased per capita income. This growth in consumption has driven an increase in the important of the safety of different food commodities. Antibiotics have been used as feed supplements to improve the rate of gain and feed efficiency and to prevent infectious diseases [13, 181]. However, due to concern about antibiotic residue and emerging antibiotic resistance [31, 32], use of antibiotics as feed additives has been banned in the European Union [33] and Korea. For this reason, studies seeking for substances which can activate the non-specific immune ability in place of antibiotics are actively in progress.

Germanium biotite is a common phyllosilicate mineral containing calcium, magnesium, ion, aluminum, germanium, and silicate [14, 15]. It has been reported that immune cells stimulated by silicate were associated with the non-specific immunostimulating ability of biotite. Fibrogenic silicate (SiO_2) activated proinflammatory macrophages [18], and aluminosilicate (Al_2SiO_5) improved immune-cell differentiation [16]. These previous studies suggest that germanium biotite has potential as a new alternative feed supplement for non-specific immunostimulators, prophylactic agents, and remedial agents.

BRDs are major economic problems in the cattle industry around the world due to morbidity, mortality, low feed efficiency, prevention costs, and treatment [19, 20]. The disease is caused by not only multi-factorial pathogens but also environmental condition, and BHV-1 and *Mannheimia haemolytica* serotype A1 have been described as major etiologic agents [19]. *Mannheimia haemolytica* serotype A1 causing BRDs in young calves has been detected in the respiratory tract of healthy cattle. Although the microorganism naturally exists as commensal of the respiratory tract, it is also considered the major bacterial agent of BRDs in calves [119]. As host immunity becomes weaker as a result of stress or infection with other pathogens, the microorganism may become infective and play an important role in BRDs, also known as shipping fever in calves. BHV-1 is also a major etiological agent of BRDs along with *Mannheimia haemolytica* serotype A1 [118]. BHV-1 is an α-herpesvirinae subfamily member that causes significant economic problems in the cattle industry [19, 20]. Following infection with BHV-1, the virus could be latent in sensory ganglia and reactivated both by stressful environments and administration of glucocorticoids [182]. In addition, BHV-1 depresses cell-mediated immunity in the host by repressing expression of MHC class I that acts in association with $CD8^+$ T-cell recognition of infected cells and transporter associated with antigen presentation [183, 184]. The suppression of the

immune function renders the host susceptible to secondary infection like pneumonic mannheimiosis, thereby causing BRDs more easily.

The aims of the present study were to investigate characteristics of germanium biotite, known to the non-specific immune stimulating mineral, about prophylactic effect in BRDs infected cattle. A BHV-1 and *Mannheimia haemolytica* serotype A1 challenge experiment in cattle was performed to examine the prophylactic effect of germanium biotite against BRDs. Finally, an aim was to explore the possibilities of the substitution of germanium biotite for antibiotics as a feed supplement.

Materials and Methods

Source of feed supplements

Germanium biotite provided by Seobong Biobestech (Seoul, Korea) was comprised of silicon dioxide (61.90%), aluminum dioxide (23.19%), iron oxide (3.97%), sodium oxide (3.36 %), calcium oxide (< 2%), magnesium oxide (< 2%), titanium oxide (< 2%), and 36 ppm germanium.

Cattle and challenge experiment design

Korean native cattle of three months of age were used for this study. The cattle were randomized into two groups: the control group (n=3), fed with a normal commercial feed without any antibiotics, and the germanium biotite group (n=3), fed with the commercial feed supplemented with 0.5% germanium biotite. The cattle of two groups were housed in individual rooms allowed easy access to water and feed. Before the challenge, cattle were confirmed as negative against respiratory pathogens to be challenged. After feeding with those fodders for two weeks, the cattle were challenged with BHV-1 (5.0×10^9 PFU) and *Mannheimia haemolytica* serotype A1 (1.0×10^{10} CFU) three times at 24 h intervals. To equalize natural infectious condition, all cattle were challenged through nasal spray.

Preparation of respiratory pathogens

The BHV-1 used in this study was isolated from a calf with naturally occurring shipping fever by Animal and Plant Quarantine Agency (QIA, Anyang, Korea). Before challenge, the virus was propagated and titrated in Bovine kidney cells (MDBK, ATCC CCL-22) [182, 185]. *Mannheimia haemolytica* serotype A1 was also provided by QIA. The identity of the bacterium was confirmed by using

multiplex PCR as previously described [186, 187]. The pathogen was grown on a blood agar plate (BAP) in a CO_2 incubator at 37℃ and subcultured in tryptic soy broth (TSB; Difco, USA) to obtain a sufficient quantity of challenge agent. Before challenge, the cultured bacteria were pelleted by centrifugation, washed three times in sterile phosphate buffered saline (PBS), and resuspended in PBS. The final concentration of approximately 1.0×10^{10} CFU was confirmed according to the standard curve of CFUs versus optical density [188]. After each challenge, plate counts were used to confirm bacterial concentration.

Clinical monitoring after respiratory pathogens challenge

The body conditions and clinical signs of cattle were recorded each 24 h throughout the experiment period. Rectal temperatures, degree of nasal discharge, cough frequency degree, and respiratory rate were scored using the criteria established by Hodgson *et al*. (Table 2.1) [189]. The measured scores of each cattle were accumulatively added and considered as cumulative clinical score (CCS).

Measurement of challenged pathogens clearance

After challenge, nasal swab samples were collected after every two days and each sample was resuspended in 1 mL PBS and diluted in 10-fold steps to 10^{-4}. Aliquots (50μl) of each dilution were used for experiment samples. Real-time PCR was used in measurement of virus clearance. DNA extraction from each sample was performed using the Accuprep genomic DNA extraction kit (Bioneer, Seoul, Korea) according to the manufacturer's instructions and used template for real-time PCR. Primers and probes for quantitative real-time TaqMan PCR, real-time PCR standard of BHV-1 genomes, and condition were developed and validated as described previously [185]. Multiplex PCR was used in confirmation of bacterial infection using specific primers (Table 2.2). DNA extraction from aliquots of sample was performed according to the a previous study [190]. DNA extracts were used for the PCR template as previously described [186]. The total isolated bacteria counts were measured by CFU counting. Aliquots (50μl) of each sample dilution were applied to BAP and incubated at 37℃ for 16 h. The viable counts were determined and expressed as CFU/swab. The area under curve (AUC) of bacteria counting graph was calculated for confirmation of total shedding bacteria throughout the experiment.

Necropsy

On day 12^{th} post challenge, all cattle were humanely euthanized and their lungs were collected. The percentages of superficial lesion area were calculated by comparison with total lung area. The lungs and tracheas were collected from cattle, and then representative samples were placed in 10% formal-saline for histopathological examination using standard techniques. Microscopic lesion scores were calculated using a described scoring system [191]. For viral and bacterial examination, tissue samples (approximately 1 g) were homogenized in 9 mL PBS and diluted in 10-fold steps up to 10^{-4}, and then aliquots (50μl) of each dilution were used for experiment samples for pathogen examination.

Statistical analysis

The data were expressed as mean ± SD, and statistical differences between the groups were analyzed with a student's *t*-test; whereas, for microscopic lesion score, a nonparametric test (Mann-Whitney *U* test) was performed using Statistical Package for the Social Sciences version 17.0 software (SPSS, Chicago, IL, USA). Differences were considered significant when probability values of $p < 0.05$ were obtained.

Results

Clinical signs after challenge in experiment cattle

The CCSs of all cattle were 0 before challenge. The CCSs, however, appeared to increase after challenge and lower CCSs than those of the control group were observed in the germanium biotite group throughout the experimental period (Figure 2.1).

Viral and bacterial clearance

On D+2 following the challenge, BHV-1 was detected in all cattle. After D+6, the BHV-1 genomic quantity of the germanium biotite group decreased, showing a lower level than the control group (Figure 2.2A). The viral genome load in tracheas of the germanium biotite group was lower than the control group at post-mortem, while higher level than in the control group was observed in lungs of the germanium biotite group (Figure 2.2C). *Mannheimia haemolytica* serotype A1 was detected on D+2 by multiplex PCR in all cattle except for two of the germanium biotite group (Figure 2.4). Infection of two cattle in the control group and one cattle in the germanium biotite group was confirmed until D+10. *Mannheimia*

haemolytica serotype A1 was identified in lungs from one cattle of the control group at post-mortem, but not identified in other cattle. The numbers of total bacteria isolated from nasal discharge in the germanium biotite and control groups showed no significant differences, but higher AUC than in the germanium biotite group was observed in the control group. The AUC of control and germanium biotite groups was 41.3 ± 0.5 and 38.3 ± 0.7, respectively (Figure 2.2B).

Gross pathology and histopathologic analysis

Lung lesions of cattle varied from brown to purple dark pink often associated with congestion and most lesions were presented in apical lobes. The germanium biotite group showed lower distribution of surface lesions and their severity as compared to the control group. Compared to the control group, low hepatization of lung was confirmed in the germanium biotite group by palpation. Cattle in the germanium biotite group showed a significant decrease in the percentages of superficial lesion area compared to the control group ($p < 0.01$). The percentages of lesion area in the control and germanium biotite groups were 34.8% ± 4.6 and 10.2% ± 3.0, respectively (Figure 2.3). In microscopic lung lesions, type 2 pneumocyte hypertrophy and hyperplasia, alveolar wall thickening, and neutrophils filtration were observed in all lungs of challenged cattle, showing moderate signs in the

germanium biotite group (Figure 2.5). The microscopic lesion score of the germanium biotite group (2.06 ± 0.23) was low compared to the control group (4.55 ± 0.22) (Table 2.3).

Discussion

BRDs, along with diarrhea a major cause of calf deaths, are caused by multi-factors mainly immune depression. BHV-1 and *Mannheimia haemolytica* serotype A1 are usually isolated from cattle suffering from BRDs [19, 20], even though *Mannheimia haemolytica* serotype A1 is also detected in healthy cattle [119]. In the cattle industry, antibiotics have been used as a feed supplement to prevent both BRDs and secondary infections. Virus infection, however, cannot be prevented by using antibiotics, and the use of antibiotics has been gradually limited because of antibiotic residue and emergence of antibiotic resistance. In addition, there are restrictions on vaccination against viruses especially, live vaccines, in view of vaccine infection, latent infection, pathogen carrier, and serological differentiation from wild infection [192-194]. For these reasons, activation of non-specific immunity has been considered for controlling BRDs in place of antibiotics.

Silicate, the major constituent of germanium biotite, has been studied for its non-specific immune enhancing effects [60, 61]. It was reported that macrophages could be stimulated and release large amounts of TNF-α by silicate *in vitro* [18]. It was also reported that relative mRNA expression levels of IFN-γ, IL-4 and TNF-α produced mainly by T-cell and macrophages could increase significantly in splenocytes of aluminosilicate orally primed mice. In addition, aluminosilicate orally primed mice showed high antibody production levels when they were exposed to formalin-killed *Pasteurella multocida* type A antigen. Moreover, oral ingestion of aluminosilicate showed enhancing effects on reinforcing clearance of porcine circovirus type 2 of experimentally infected pigs [16]. The results of these studies of immune activities of silicate make us postulate that germanium biotite could have prophylactic effects on BRDs occurred mainly with relevance to immunosuppression. However, a natural mineral like germanium biotite has not been studied for its prophylactic effect against BRDs. In this study, the first analysis of the prophylactic effect of germanium biotite against BRDs in experimentally infected cattle was conducted.

To investigate exact clinical signs, the normal temperature limit (39.0-40.0℃) confirmed in a previous study [189] was modified because situations in the present experiment, like rearing environments and breed of cattle, differed from those in the previous study. Based on this information, the normal temperature limit was

modified in the present experiment according to the mean of temperatures measured in preinoculated cattle.

Following the challenge, the germanium biotite group showed alleviated clinical signs compared to the control group. In accordance with these clinical results, the germanium biotite group showed enhanced clearance of BHV-1 and a low infection rate of *Mannheimia haemolytica* serotype A1 compared to the control group. The germanium biotite group also showed lower of clearance of total bacteria in nasal swab samples. At post-mortem, however, the germanium biotite group showed a higher quantity of BHV-1 in lungs than the control group and a lower quantity in tracheas than the control group. It appeared that there was a greater amount of virus shedding in the trachea in the control group, even though a high quantity of BHV-1 was detected in lungs of the germanium biotite group compared to the control group. Moreover, it was reported that severe clinical signs were usually accompanied by a high titer of virus shedding [195]. These clinical and clearance results correspond with superficial lesion area data and microscopic lesion analysis in the present study. Significantly higher percentages of normal area than the control group were observed in the germanium biotite group ($p < 0.01$). In microscopic lung lesions, the germanium biotite group showed moderate signs of neutrophils filtration and bronchiolar exudates compared to the control group. Neutrophils filtration in alveoli and bronchial exudates are typical signs of

bronchopneumonias, which are generally caused by bacteria. Therefore, it could be inferred that germanium biotite primed cattle are protected from secondary infections caused mainly by bacteria like *Mannheimia haemolytica* serotype A1.

These results suggest that ingestion of germanium biotite had prophylactic effects against BHV-1 and *Mannheimia haemolytica* serotype A1 in cattle. It can be presumed that the prophylactic effects of germanium biotite against challenged BRDs pathogens are associated with its stimulating activities on non-specific immune response. In conclusion, results of this study indicated that germanium biotite has the potential to activate innate immunity thereby could be a good alternative to antibiotics as a feed supplement for cattle.

Table 2.1. The criteria for the scoring of respiratory clinical sign.

Sign	**Description**	**Score**
Temperature	38.0-39.5℃	0
	39.5-40.0℃	1
	40.0-40.5℃	2
	40.51-41.0℃	3
	41.0℃ >	4
Degree of nasal discharge	Absent	0
	Mild	1
	Moderate	2
	Severe	3
Cough frequency degree	Absent	0
	Mild	1
	Moderate	2
Respiratory rate	< 50	0
	50-60	1
	60-70	2
	70-80	3
	80 >	4

This criteria was modified from Hodgson *et al*. (1995).

Table 2.2. Primers and probes of PCR for detection of the respiratory pathogens.

Pathogens	**Oligonucleotide**		**Sequences (5′-3′)**	**Size**
BHV-1	BHV-1 (F)[a]		TGTGGACCTAAACCTCACGGT	97 bp
	BHV-1 probe[b]		AGGACCGCGAGTTCTTGCCGC	
	BHV-1 (R)[c]		GTAGTCGAGCAGACCCGTGTC	
Mannheimia	Lkt	(F)	GCAGGAGGTGATTATTAAAGTGG	206 bp
haemolytica		(R)	CAGCAGTTATTGTCATACCTGAAC	
serotype A1	HP	(F)	CGAGCAAGCACAATTACATTATGG	90 bp
		(R)	CACCGTCAAATTCCTGTGGATAAC	
	16s	(F)	GCTAACTCCGTGCCAGCAG	~304 bp
		(R)	CGTGGACTACCAGGGTATCTAATC	

[a] Forward primer; [b] 6-Carboxyfluorescein-labeled; [c] Reverse primer

Table 2.3. Microscopic lesion scores in challenged cattle.

Groups	**Control**	**Germanium biotite**
Scores (± SD)	5.00 (± 0.26)	2.50 (± 0.23)
	4.30 (± 0.34)	1.75 (± 0.30)
	4.36 (± 0.28)	1.92 (± 0.38)
Means (± SD)	4.55 (± 0.22)	2.06 (± 0.23) **

The microscopic lesion scores were calculated using a previous scoring system (Opriessnig *et al*., 2004). The control group shows higher lesion scores than the germanium biotite group significantly ($^{**}P < 0.01$).

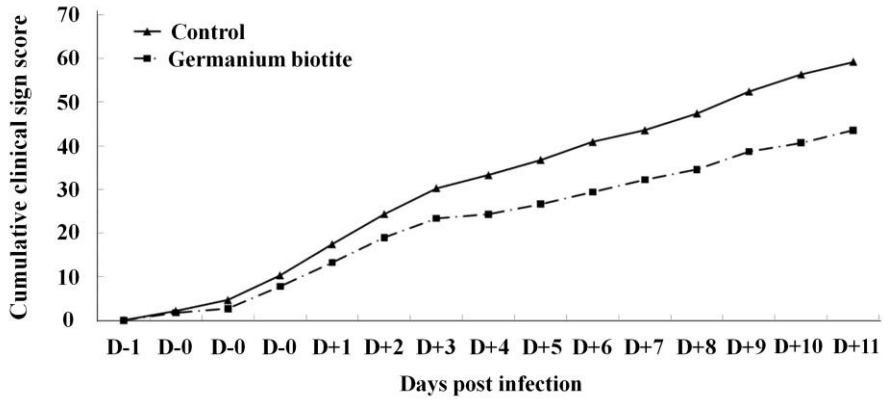

Figure 2.1. Cumulative clinical sign scores after challenge. Cumulative clinical sign score was calculated from each clinical sign. The control group showed higher level than the germanium biotite group.

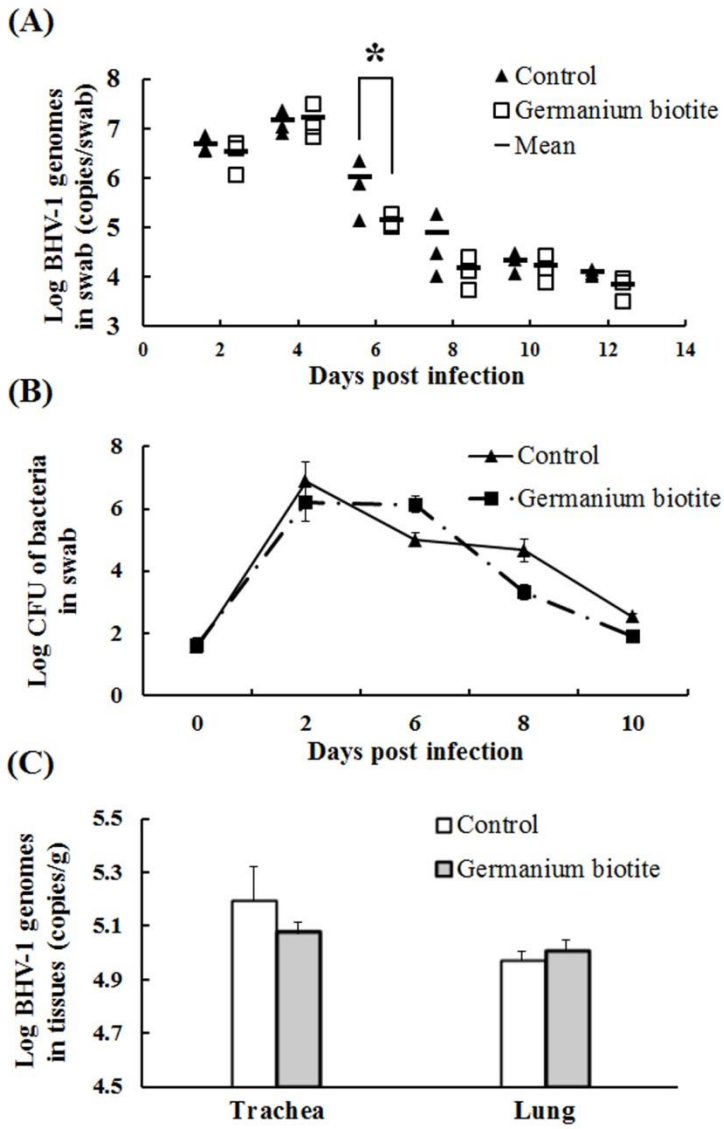

Figure 2.2. Clearance of challenge pathogens. **(A)** Clearance of BHV-1 in experimentally infected calves. **(B)** Clearance of total bacteria in experimentally infected calves. **(C)** Quantity of BHV-1 genomes in trachea and lung at post-mortem. (*$p < 0.05$)

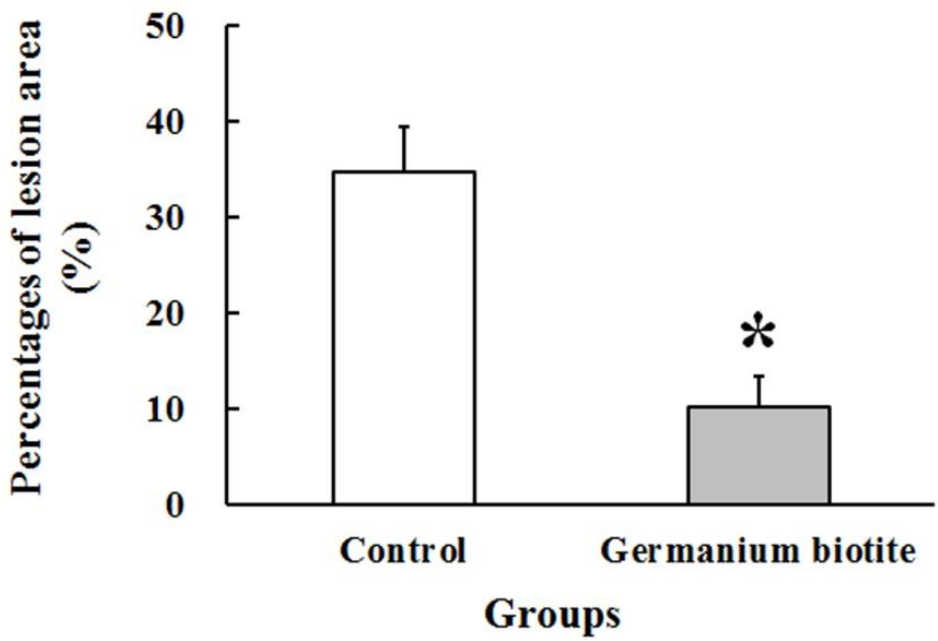

Figure 2.3. Percentages of superficial lesion area in experimentally infected cattle. The germanium biotite group showed significantly lower percentages of lesion area than the control group. (*$p < 0.05$)

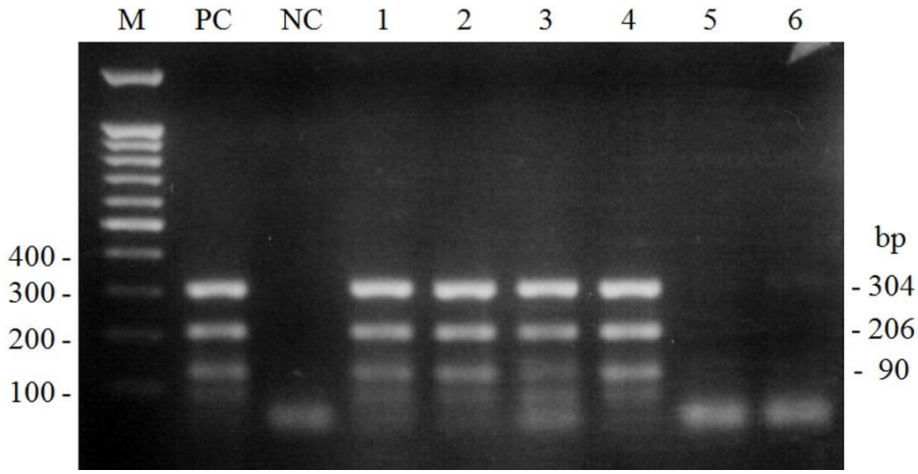

Figure 2.4. The confirmation of *Mannheimia haemolytica* serotype A1 infection of cattle on D+2 using multiplex PCR. Lines PC and NC, Positive control and negative control, respectively, lines 1-3; calves in control group and lines 4-6; calves in germanium biotite administrated group. PCR amplification regions of 304-bp, 206-bp, and 90-bp account for 16S rDNA gene, leukotoxin gene, and unknown hypothetical protein of *Mannheimia haemolytica* serotype A1. *Mannheimia haemolytica* serotype A1 is detected in all cattle of the control group (1-3) and one cattle of the germanium biotite group (4-6).

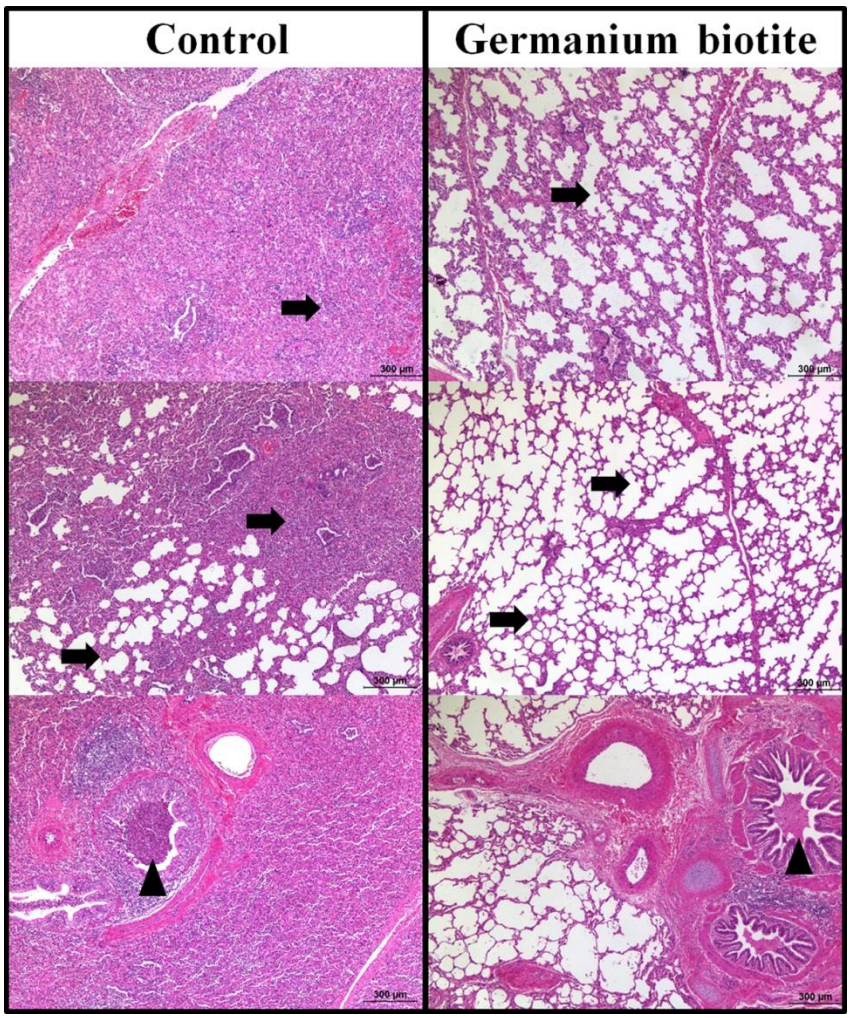

Figure 2.5. Histopathological features of lung tissue samples in challenged cattle. Severe neutrophils filtration and thickened alveolar septa (arrows) are observed in lungs of the control group compared with the germanium biotite group. Bronchiole of the control group also contains more numerous neutrophils (arrowhead) compared with the germanium biotite group (arrowhead).

Chapter III

Supplementation of dietary germanium biotite enhances induction of the immune responses by foot-and-mouth disease vaccine in cattle

Abstract

After the outbreak of FMD in Korea in 2010, a vaccination policy has been applied to control the disease. In addition, several non-specific immune stimulators have been used without any scientific evidence that they would enhance the immune response after FMD vaccination and/or protect against FMD. Based on the current situation, the aim of this study was to evaluate the effect of the non-specific immune stimulator germanium biotite on FMD vaccination and immune responses in cattle. To achieve a goal, immune responses to FMD vaccination, such as levels of IgG and IgA, antibody duration, and virus-neutralizing titers were investigated after germanium biotite feeding. The PBMC typing and proliferative response after stimulation with ConcanavalinA (ConA) and lipopolysaccharide (LPS), the cytokines expression level of PBMC, and the lysozyme activity in the serum were

measured to evaluate the immune enhancing effects of germanium biotite in cattle following its administration.

Following the first vaccination, high level of IgG (at 4 weeks) and IgA (at 2 and 31 weeks) titers in serum and saliva were observed in the germanium biotite-feeding group ($p < 0.05$). The germanium biotite group also showed high and longstanding inhibition percentage value in ELISA assay at 31 weeks after the first vaccination ($p < 0.05$). Generally, higher virus-neutralizing antibody titers were observed in the feeding group at 20 and 31 weeks after vaccination. Following the feeding germanium biotite, the germanium biotite group showed increased subpopulation of $CD4^+$ lymphocytes and major histocompatibility complex (MHC) class II^+ cells in PBMCs at 23 week, responding to stimulation of LPS and ConA. The levels of IFN-γ (at 3 and 8 weeks), IL-1α (at 3 11, and 23 weeks), IL-1β (at 3, 8, and 11 weeks), and IL-4 (at 8 and 11 weeks) gene expression were also significantly increased in the feeding group ($p < 0.01$ and $p < 0.05$). Feeding with germanium biotite increased the lymphocytes' proliferative response to the stimulation of ConA and LPS at 23 weeks and lysozyme activity at 9 weeks after feeding.

These results suggest that germanium biotite feeding could increase the protection against FMDV infection via the induction of higher humoral and cellular immune responses in vaccinated cattle.

Key words: FMD, germanium biotite, immune responses, cattle

Introduction

FMD is a highly contagious and economically important disease that affects cloven-hoofed animals, and it is characterized by appetite loss, an increase in body temperature, and vesicles in the mouth, tongue, hooves, and nipples [21, 22]. The disease is caused by the FMDV, which is a small, icosahedral, non-enveloped RNA virus classified within the *Aphthovirus* genus as a member of *Picornaviridae*. The clinical severity of the disease varies with the strain of FMDV, infection dose, species, and individual susceptibility of the host [131]. The disease can be transmitted via direct or indirect contact between FMDV-infected animals and susceptible animals [22].

Due to its high mutation rate, this virus exists as seven distinct serotypes (O, A, C, Asia 1, SAT1, SAT2, and SAT3) as well as numerous and constantly evolving subtypes, which shows a spectrum of antigenic diversity [22, 131]. Therefore, one major problem in controlling FMD is antigenic variation, as infection or vaccination with one FMDV serotype does not protect against other serotypes, and it may even fail to protect fully against other subtypes within the same serotype [24, 132, 133]. This problem has been raised by the experimental and field data of previous researchers on vaccination, including both single and multivalent vaccines [131, 196, 197].

Despite the powerful effects of vaccines, which have mitigated enormous FMD outbreaks, the current vaccines have many problems to overcome such as the long time required to induce antibodies and short duration of immunity when applying them to emergency and routine vaccination programs [24, 196-198]. Therefore, strategies to improve the immune response to vaccination have included using higher vaccine doses or increasing the number of doses, using different routes of administration, accelerating the dosing schedule, and using adjuvants such as antigen delivery systems and various immunostimulators [25].

The immune response against FMDV is related to circulating humoral antibody titers; this is considered the most important factor in conferring protection against FMD [132]. The importance of cell-mediated immunity also has been recognized in the induction of humoral immunity and the clearance of FMDV. T-cell responses mediated by $CD4^+$ T-cells are required for protective immunity against FMDV as they participate in the production of antiviral antibodies [144]. Subsets of the $CD4^+$ MHC class II-restricted T-cells respond to activation by antigen-presenting cells and antigens by producing a Th1 (IFN-γ) and Th2 (IL-4, IL-5, and IL-13) responses [145]. Several studies have also demonstrated the presence of FMD-specific MHC class II-restricted responses in cattle and pigs [144-146, 148]. The antiviral responses of $CD8^+$ T-cells were also detected following FMDV infection in previous studies [145, 147, 148]. These antiviral responses were through direct

cytotoxicity or release of cytokines such as IFN-γ following vaccination, and that the antiviral responses were almost 100 times higher following re-stimulation [147]. MHC class I-restricted $CD8^+$ T-cells also showed specific immune response to FMDV as memory cells; however, the correlation between FMDV-specific $CD8^+$ T-cell recognition and protection remains to be defined [145, 147]. In addition, the innate immune systems, as well as adaptive immune system, play an important role in immune responses to FMDV infection [126, 138]. The innate immune system, characterized by non-specific responses, is associated with early protection against FMD and is involved in the formation of adaptive immune response to FMDV infection [126, 138].

Germanium biotite, as a well-known feed supplement, is a common phyllosilicate mineral that contains calcium, magnesium, iron, aluminum, and silicate. It has been reported that the effects of non-specific immune stimulating of biotite are associated with the immune cells being stimulated by silicates [16, 17]. Fibrogenic silicates (SiO_2) activated proinflammatory macrophages, and aluminosilicate improved immune-cell differentiation [18]. Aluminosilicates act as a non-specific immunostimulator that is similar to a superantigen such as potent T-cell mitogens and affinity to MHC class II molecules [61, 151]. Indeed, proinflammatory macrophages, which belong to MHC class II antigen–presenting cells, are activated by fibrogenic silicate particulates [18]. Thus, previous studies

have suggested that germanium biotite has a potential as a new supplement for immunostimulators, prophylactic agents, and remedial agents [16, 17].

Since the devastating FMD outbreak in Korea at the end of 2010, cattle, pigs, and some small ruminants have been vaccinated with a trivalent FMD vaccine (O1 Manisa, A Malaysia 97, and Asia1 Shamir) at least 6 PD_{50} [23]. To improve the vaccine efficacy, several non-specific immune stimulators have been used in Korea without any scientific understanding of their effects. Therefore, as a first step to understanding the immunological mechanism and improve the vaccine's efficacy, the effects of a non-specific immune stimulator, germanium biotite, in relation to the FMD vaccination in cattle were examined.

Materials and Methods

Experimental animals and vaccination

This experiment was carried out with 89 cattle from 6–8 months in age (Korean Native Cattle) at three different farms, which were located in different provinces in Korea. Forty-five cattle were raised with feed supplemented with 0.5% germanium biotite (SoltoB, Seobong Biobestech Co, Seoul, Korea; germanium biotite group)

from 1 week before vaccination to the end of the experiment, while the rest were raised without supplementation (control group). Control and germanium biotite groups were arranged at each farm (Table 3.1). All the cattle were intramuscularly vaccinated with 2 mL of Decivac FMD DOE trivalent vaccine (InterVet, Germany). A booster vaccination was given 4 weeks after the first vaccination, according to the national vaccination policy of Korea. Samples of blood, saliva, and feces were collected before vaccination and up to 6 months after the second vaccination. Feces and saliva were collected by swabbing with a cotton swab. Bleeding was carried out from the jugular vein. Sera were collected and stored at -20℃ until use after inactivation at 56℃ for 30 min. The cotton swabs were immersed in 1 mL of PBS for 1 h at 4℃, and the supernatants were collected after centrifugation at 1,500 × g for 15 min at 4℃; the supernatants were stored at -20℃ and used as samples for the ELISA analysis of FMDV-specific IgA.

The germanium biotite consisted of silicon dioxide (61.90%), aluminum dioxide (23.19%), iron oxide (3.97%), sodium oxide (3.36%), calcium oxide (< 2%), magnesium oxide (< 2%), titanium oxide (< 2%), and 36 ppm germanium. All procedures in the animal experiment in this study were approved by the Institution Animal Care and Use Committee (IACUC) of Seoul National University (SNU-111210-1).

Measurement of antibody against FMDV

The antibody titers against FMDV were measured using the PrioCHECK FMDV type O ELISA kit (Prionics, Switzerland) following the manufacturer's protocols. The serotype O antibody titer using this ELISA kit is the criterion for assessing seropositivity in the national policy of Korea. The antibody titer against FMDV was indicated by the inhibition percentage (PI) value. The high PI value presents a high level of antibody titer, and the sera that showed more than 50% of the PI value were considered to be seropositive in the ELISA assays. FMDV-specific IgG in the serum were measured using the PrioCHECK FMDV type O ELISA kit (Prionics) with 1:40 diluted serum and horseradish peroxidase (HRP)-conjugated sheep anti-bovine IgG (Bethyl, USA). In addition, FMDV-specific IgA in the saliva and feces was measured using the PrioCHECK FMDV type O ELISA kit (Prionics) with 1 x samples extracted by PBS from cotton swabs and HRP-conjugated rabbit anti-bovine IgA (Bethyl). All procedures for detecting FMDV-specific IgG and IgA followed the manufacturer's protocols with a 1:3,000 dilution of each immunoglobulin-specific conjugate.

Analysis of virus-neutralizing antibody titer

The VN titer was measured in eight randomly selected sera from the germanium biotite and seven sera from the control groups of Farm B at 20 and 31 weeks after vaccination (Tables 1 and 3). Fifty microliters of twofold serially diluted bovine sera were reacted with 50 μL of the FMDV Andong strain ($100TCID_{50}$), a serotype O FMDV Korean isolate from the 2010 outbreak, at 37℃ for 1 h. After incubation, 50 μL of complete Dulbecco's Modified Eagle Medium (Gibco, USA) with 5% fetal bovine serum containing 1×10^4 LFBK cells/ml was added to the reaction mixture. After incubation of the reaction mixtures at 37℃ for 48 h, the VN titer was determined as the final dilution of serum showing no cytopathic effect.

Analysis of peripheral blood mononuclear cells subsets

Among the 89 cattle, analysis of the peripheral lymphocyte profiles was conducted with ten Korean native cattle (IACUC permission no. SNU-111210-1). Five of them were fed with a commercial feed supplemented with 0.5% germanium biotite (germanium biotite group), while the rest were fed without the supplement (control group). PBMCs were cultured from the heparinized whole blood of the experiment cattle using the Histopaque® -1077 Hybri-Max™ (Sigma, USA) system,

as described by the manufacturer. The PMBCs were then stimulated with DPBS (Gibco), ConA (5 g/ml, Sigma) for CD3, CD4, CD8, and MHC class I and II and LPS (5 g/ml, Sigma) for CD79a staining for 96 h to characterize the phenotype of PBMC responding to stimulation with mitogens. The procedures for cell fixation and antibody reaction to detect the cell markers were performed as previously reported [96] using the DPBS with 0.2% Tween® 20 (Sigma) as the permeabilisation solution. To prevent a non-specific reaction of the Fc receptor with the detection antibodies, the fixed cells were reacted with normal Ig from the antibody-producing host for 10 min and reacted with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated cell surface marker detection antibodies. After the reaction, the number of cells reacting with the FITC or PE conjugate was analyzed using Flow cytometric analysis (BD, USA). The monoclonal antibodies used for the staining of the cells surface markers are listed in Table 3.2.

Analysis of cytokine gene expression in PBMC

Total RNAs were extracted from PBMCs with the RNeasy mini kit (Qiagen, USA), and cDNAs were synthesized with the reverse transcription kit (Qiagen). The relative expression levels of IFN-γ, IL-1α, IL-1β, IL-4, IL-6, and IL-10 were quantitated using the Rotor-Gene SYBR Green PCR kit (Qiagen) and Rotor-Gene

Q (Qiagen), as previously reported [199]. The expression levels were normalized by β-actin expression using the $2^{-\Delta\Delta C}{}_{T}$ method [154].

Analysis of PBMC proliferation

A lymphocyte proliferation assay was conducted with the PBMCs collected from the experiment cattle. Ninety microliters of PBMCs (5×10^6 cells/ml) in a 96-well plate were incubated with ConA (5 μg/ml) and LPS (5 μg/ml) at 37℃ for 96 h in a 5% CO_2 atmosphere. Absorbance at 560 nm was measured 2 h after the addition of 10 μL of 10× Prestoblue (Invitrogen, USA). Proliferation of the PBMCs was compared based on this absorbance.

Analysis of lysozyme activities

Lysozyme activities in the serum were measured based on the dissolution ability against *Micrococcus lysodeikticus* (Sigma) [92]. The activity was calculated based on the standard curve generated from the serially diluted lysozyme solution.

Statistical analysis

The data were expressed as mean ± SD. A student's *t*-test and repeated measures of ANOVA were performed for statistical analysis of the data. All statistical analyses of data were carried out using Statistical Package for the Social Sciences version 19.0 software (SPSS, USA). A probability value of $p < 0.05$ was considered significant.

Results

Antibody responses to FDMV

In the analysis of the duration of antibody levels and the secretion of IgA after FMD vaccination, the PI values started to increase with the first vaccination, and the values were significantly increased by a booster vaccination at 4 weeks, with the highest PI values at 10 weeks, and this continued steadily up to 28 weeks, regardless of whether the germanium biotite was administered. However, the values decreased at 31 weeks in the control group when the re-booster vaccination

was required, while the values continued at 31 weeks in the germanium biotite group (Figure 3.1).

FMDV-specific IgA in the saliva of the germanium biotite group was generally higher than in the control group, although there were no significant differences between the groups at some points during this experiment (Figure 3.2). However, IgA could not be detected in the feces of the two groups with the kit used (data not shown).

In the analysis of the early response of IgG, both groups showed a steady increase in the IgG antibody level up to 3 weeks after vaccination; after this point, the level decreased (Figure 3.3). The IgG level in the germanium biotite group was higher than in the control group, although the difference was not significant. However, there was a significantly higher IgG antibody level at 4 weeks after vaccination when the booster vaccination was required (Figure 3.3, $p < 0.05$).

Virus neutralization antibody against the heterologous FMDV strain

As shown in Table 3.3, the sera of the control group had a titer of less than 16 at both points. On the other hand, while the germanium biotite group had a VN titer of 16 or less at 20 weeks after vaccination, the VN titer in the group was found to have increased at 31 weeks after vaccination.

Analysis of peripheral blood mononuclear cell subpopulation

Changes in the subpopulations of five different lymphocytes were analyzed based on the administration of the germanium biotite. At 23 weeks, $CD4^+$ lymphocytes and MHC class II^+ cells in the germanium biotite group were significantly higher than in the control group ($p < 0.01$), while $CD79a^+$, $CD3^+$, and $CD8^+$ lymphocytes exhibited no differences between the two groups (Table 3.4).

Analysis of cytokine gene expression

The gene expression of IFN-γ, IL-1α, IL-1β, and IL-4 was significantly higher in the germanium biotite group during this experiment (Figure 3.4). The significant high levels of IFN-γ were determined in the germanium biotite group at 3 and 8 weeks, compared to the control ($p < 0.01$). In case of IL-1α and IL-1β, significant differences were observed at 3, 11, and 23 weeks and at 3, 8, and 11 weeks, respectively ($p < 0.05$ and $p < 0.01$). The germanium biotite group also showed significant high levels of IL-4 at 8 and 11 weeks, compared to the control ($p < 0.01$). However, there were no noticeable differences in the expression levels of IL-6 and IL-10 between the two groups although a significant difference in IL-6 expression was observed at 3 week. The germanium biotite feeding induced a

significant increase in IFN-γ gene expression at a relatively early stage and in IL-4 at a late stage, while increases in IL-1α and IL-1β gene expression were observed throughout the experimental period.

Lymphocyte proliferation

Higher lymphocyte proliferation in the germanium biotite group was observed by LPS and ConA stimulation at 23 weeks compared to the control (Figure 3.5, $p < 0.05$).

Lysozyme activity

Of the three points at which the lysozyme activity was measured in the serum, significantly higher lysozyme activity was observed in the germanium biotite group at 9 weeks after starting the feeding (Figure 3.6, $p < 0.05$). The increase was observed at a relatively early stage after germanium biotite feeding.

Discussion

The current FMD vaccines that are used in cattle need to be administered every six months, in accordance with the duration of the antibody, to protect against the disease [24, 133, 135, 136]. The diversity of FMD serotypes has also raised issues related to the efficacy of the current FMD vaccines. Furthermore, there is considerable argument about whether the trivalent vaccines show protective abilities against the Andong strain through the cross-reactivity of antibodies induced by trivalent vaccination [23]. Based on the current knowledge of FMD vaccines, the need for additional materials and/or methods has been raised with a view to overcoming the problem. Therefore, non-specific immune stimulators for reinforcement of immune responses to vaccination have received attention as a possible solution to this issue. In the disastrous FMD outbreak in Korea in 2010 [23], many non-specific immune stimulators were used without any scientific evidence that they would enhance protection against FMDV infection. Although non-specific immune stimulators have been used, their efficiency is still controversial. Therefore study on the effects of a non-specific immune stimulator, germanium biotite on FMD vaccination in cattle was carried out.

Previous studies reported that a re-booster vaccination was needed 6 months after the booster vaccination based on the decrease of antibody titer against FMDV

[24, 133, 135, 136]. Coinciding with these previous studies, this study observed a decreased PI value in the control group at 31 weeks. However, the germanium biotite group showed a continued high level of PI value for 31 weeks. In order to elevate the antibody level, a booster vaccination was needed 4 weeks after the first vaccination. At that time, the germanium biotite group showed a high level of IgG antibody compared to the control. This experimental result closely matches with previous studies' findings on the improvement of antibody production by germanium biotite [16, 17]. Furthermore, germanium biotite feeding showed a high level of IgA in the saliva, which could provide a beneficial effect when it comes to protecting against FMDV infection in cattle [200]. In addition, the IgG response suggested that the point at which to administer the booster injection in the FMD vaccine program for cattle is the same as indicated in previous studies [24, 136]. However, the increase in antibody duration suggests that the current FMD vaccine program should be reconsidered in animals fed germanium biotite. In this study, sampling of sera after 31 weeks from only first and boosting vaccinated cattle was limited, due to national FMD control policy by which re-booster vaccination carried out every 6 month in cattle. The decline point of PI 6 month after booster vaccination could not be identified. Therefore, the investigation of PI value changes for a longer period should be conducted in the future study to determine the re-booster vaccination timing.

Along with these antibody responses, a VN test was performed using the Andong strain, which is an isolate from the outbreak in Korea in 2010. Following the vaccination, 20 weeks was the mid-point of the period showing a high PI value and 31 weeks was the time point that showed a difference in PI values between the two experiment groups. To ensure the reliability of VN test and reduce the variation, cattle in Farm B were selected and sera of half the number of scale of each group in Farm B were taken from the same cattle in each collection date. As shown in Table 3.3, all experimental groups showed a low VN titer. These low VN titers might be due to using the heterologous FMDV. The immunological relationship between the O1 Manisa vaccine and the Andong strains was relatively low or moderate (*r*-value of approximately 0.3) [137]. A previous study considered an *r*-value in the range of 0.3–1.0 as indicative of reasonable levels of cross-reactivity [201]. Therefore, the low VN titers observed in this study could be explained by the low level of the *r*-value between the two strains. However, in comparison with the control group, the increase of VN antibodies in the germanium biotite group suggests that protectivity against FMDV infection might be increased through the supplementation of germanium biotite [24, 131, 198].

Although the importance of antibodies in protection against FMDV infection is well known [24, 131, 198], a specific antibody does not guarantee sterile immunity or clinical protection against FMDV infection [135, 198]. Moreover, protection

against FMDV infection has been observed in the absence of a detectable specific humoral response [202]. Therefore, it is reasonable to consider a possible protective role for innate immune responses in FMD infection and for control of the disease [136, 145, 146, 203]. In this study, high lysozyme activity—indicating macrophage activity—was observed in the germanium biotite group compared to the control group. This result corresponds with the findings of previous studies, which that the macrophage activation effects of silicate (SiO_2) and aluminosilicate, which are major components of germanium biotite [16-18]. Along with macrophage activation, a high proliferative response of lymphocyte to the mitogen stimulation was observed in the germanium biotite group compared to the control group, thereby indicating that the marker level of lymphocyte activity in the germanium biotite group was higher than in the control group. Through the analysis of PBMC subset stimulation with ConA and LPS, it can be presumed that the proliferative responses of the T-cells were predominately associated with $CD4^+$ and MHC class II^+ cells, as the $CD4^+$ and MHC class II^+ cells were significantly increased at 23 weeks after stimulation with ConA. The important role of $CD4^+$ T-cells in the induction of the antibody response in ruminants following infection or vaccination with a virus or a viral peptide has been demonstrated [144, 145]. Furthermore, MHC class II–restricted T-cells ($CD4^+$) play an important role in the immune responses to the FMDV antigen and the activation of macrophages [203].

These results show the increase of activation markers for innate immune responses associated with cell-mediated immunity in the germanium biotite group. In addition, previous studies reported that the cross-reactivities against a heterologous virus were mainly induced by T-cell responses to FMDV vaccination [146]. Therefore, it could be postulated that the increase of VN titer against Andong strain in the germanium biotite group compared to the control might result from enhanced T-cell responses due to germanium biotite feeding.

The increase of the expression of the IFN-γ, IL-1α, IL-1β, and IL-4 genes in the germanium biotite group was in agreement with the results that demonstrated the activation of immune cells associated with cell-mediated immune responses. The increase of the IFN-γ expression level and the $CD4^+$ T-cell subpopulation in results of this study is closely correlated with a previous report that indicated that $CD4^+$ T-cells were the major proliferating phenotype and IFN-γ producing cells [145]. The induction of IFN-γ expression has been observed in antigen-specific T-cell activation [204], MHC class II–restricted T-cells ($CD4^+$) [133], and activation of macrophages [203]. This immune cell activation induced by IFN-γ expression could also be observed in this study through the increases in the $CD4^+$ and MHC class II^+ cell subpopulations, lysozyme activity, and lymphocyte proliferation level. In addition, the antiviral activity of IFN-γ against FMDV contributed to the control of FMDV replication and the spread of the virus within the host via activation of

NK cells and macrophages [205]. IL-1α and IL-1β are mainly produced in activated macrophages and promote Th2 immune cells, which represent the major source of IL-4 [206]. IL-4 also promotes Th2 cell growth and enhances MHC class II expression in B-cells [206]. These results suggest that germanium biotite induces Th1 and Th2 responses through the activation of macrophages and $CD4^+$ MHC class II–restricted T-cells [21, 206]. Considering these results, it can be presumed that supplementation of germanium biotite may activate cell-mediated immunity, thereby enhancing the induction of the immune responses by FMD vaccination and the protectivity against FMDV infection. However, the effects of germanium biotite on T-cell activation through FMDV specific T-cell responses could not be investigated. Therefore, an inquiry on the effects of germanium biotite on immune responses to FMDV vaccination through a comparative study of T-cell responses using FMDV antigen were needed to evaluate the specific effects in a further research.

In addition, a large difference in the appearance of the MHC class II subpopulation was observed in the experiment cattle between two time points, before and after the administration of the germanium biotite. Previous studies showed that the MHC class II subpopulation in the PBMCs of Holstein at 5–7 and 15–16 months could be estimated at about 32.2% and 10.7%, respectively [99, 207].

Based on these reports, it can be presumed that the significant difference in this study could be caused by the age change in the experiment calves.

The results of this study suggest an enhancement of the immune responses to FMD vaccination through the supplementation of dietary germanium biotite such as IgG, IgA, and VN test. It can be presumed that these enhancements are induced by the immunostimulating effects of the germanium biotite, including the activation of macrophages and $CD4^+$ MHC class II–restricted cells, as well as the induction of cytokines, which can activate Th1 and Th2 immune cells. In conclusion, the findings of this study indicate that dietary germanium biotite could be used to improve the efficacy of the FMD vaccine.

Table 3.1. Experiment design for grouping, sampling, and analysis

Farms	Number of cattle		Samples	Analysis of
	Control	Germanium biotite		
A	26	22	Serum, saliva, feces	Inhibition percentage, ELISA for antibody levels (IgG and IgA), lysozyme activity
B	13	18	Serum, saliva, feces	Inhibition percentage, ELISA for antibody levels (IgG and IgA), lysozyme activity, virus neutralization test
C	5	5	Serum, saliva, feces, PBMC	Inhibition percentage, ELISA for antibody levels (IgG and IgA), lysozyme activity, PBMC (phenotype, cytokines, proliferation,

Among 89 cattle at 6–8 months of age, 45 cattle were raised with feeds supplemented with 0.5% germanium biotite from 1 week before vaccination to the end of experiment (germanium biotite group) and the rest of them were raised without supplementation (control group).

Table 3.2. Antibodies used to detect the cell surface markers of PBMCs.

Target	Antibody	Company
CD79a	PE-conjugated mouse anti-bovine CD79a	Abcam, UK
CD3	FITC-conjugated hamster anti-bovine CD3	Abcam
CD4	PE-conjugated mouse anti-bovine CD4	US Biological, USA
CD8	PE-conjugated mouse anti-bovine CD8	LifeSpan BioSciences, USA
MHC class I	FITC-conjugated mouse anti-bovine MHC class I	Novus Biological, USA
MHC class II	Mouse anti-bovine MHC class II [a]	Abcam
Fc receptor blocking	Polyclonal mouse IgG fraction	Abcam
	Monoclonal Armenian hamster IgG fraction	Abcam

[a] Mouse anti-bovine MHC class II antibody was conjugated with PE using an EasyLink B-Phycoerythrin conjugation kit (Abcam)

Table 3.3. Virus neutralization antibody titers in sera after vaccination.

Group	Samples	20 weeks	31 weeks
Control	1	< 16	< 16
	2	< 16	< 16
	3	< 16	< 16
	4	< 16	< 16
	5	< 16	< 16
	6	< 16	< 16
	7	< 16	< 16
Germanium biotite	1	< 16	16
	2	16	< 16
	3	16	32
	4	16	26
	5	16	< 16
	6	16	< 16
	7	< 16	26
	8	< 16	32

The selected sera were taken from the same cattle in each collection date. All cattle were 6-month-old when the first vaccination was conducted. The sera selected from the germanium biotite group show a higher virus-neutralizing antibody titer than the control group.

Table 3.4. Subsets of peripheral blood mononuclear cells (PBMCs) after mitogen stimulation.

	Before administration		**23 weeks after administration**		Stimulators
	Control	**Germanium biotite**	**Control**	**Germanium biotite**	
$CD79a^+$	8.40% (± 0.17)	7.90% (± 0.14)	6.70% (± 0.53)	4.70% (± 1.01)	LPS
$CD3^+$	59.50% (± 0.49)	57.80% (± 0.53)	93.50% (± 0.04)	93.10% (± 0.28)	ConA
$CD4^+$	14.10% (± 0.72)	13.00% (± 0.22)	15.40% (± 0.58)	23.80% (± 0.52)**	ConA
$CD8^+$	12.50% (± 0.74)	10.40% (± 1.30)	14.30% (± 1.40)	10.20% (±1.21)	ConA
MHC I^+, II^+	43.00% (± 0.45)	37.50% (± 0.12)	8.80% (± 0.17)	10.20% (± 0.15)**	ConA

The data were expressed as mean ± standard deviation (SD).

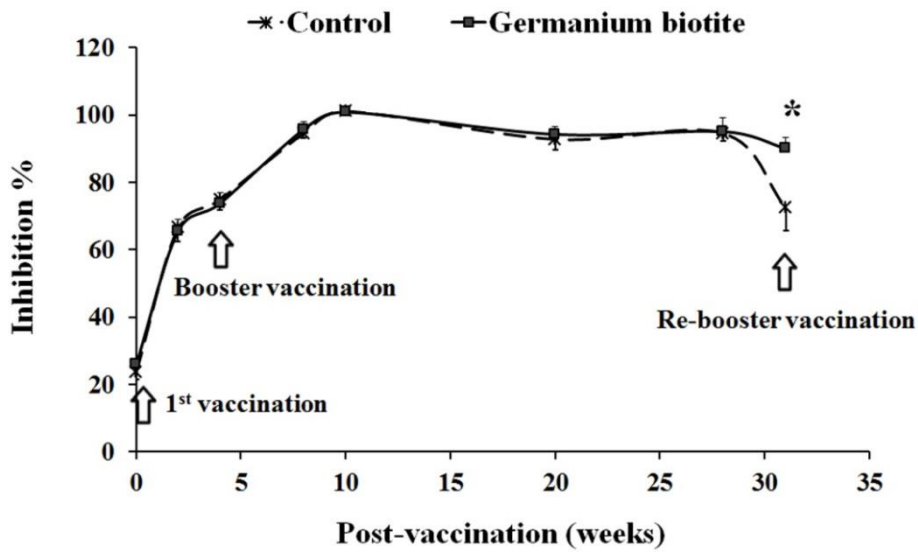

Figure 3.1. Inhibition percentage (PI) of the antibody against FMDV serotype O in both the germanium biotite-feeding group and the non-feeding group after vaccination. The high PI value indicates a high level of antibody titer, and the sera that showed more than 50% of the PI value were considered to be seropositive in the ELISA assays. (*$p < 0.05$)

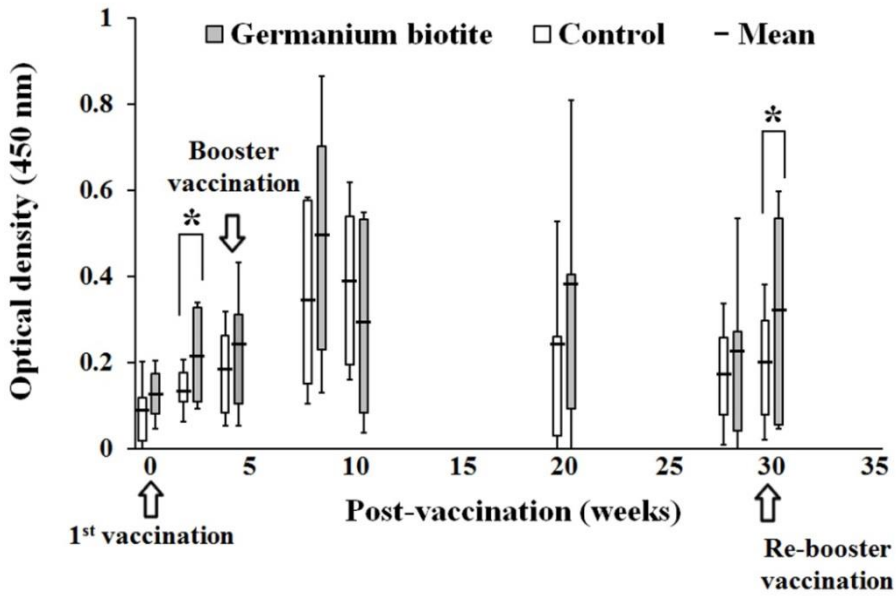

Figure 3.2. The FMDV serotype O-specific IgA response in the saliva of cattle of the germanium biotite and the control groups after vaccination with Decivac FMD DOE trivalent vaccine. (*$p < 0.05$)

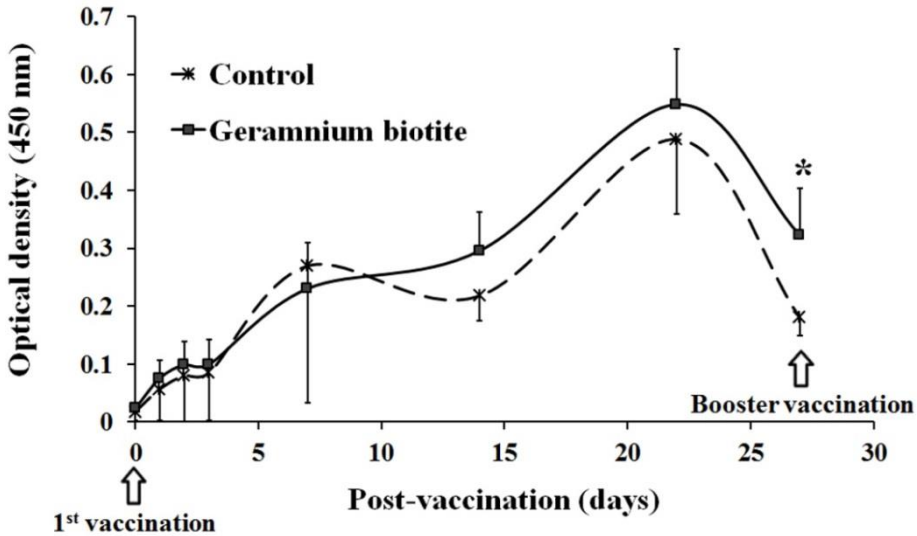

Figure 3.3. Induction of FMDV serotype O-specific IgG in the cattle of the germanium biotite and control groups after vaccination with Decivac FMD DOE trivalent vaccine. (*$p < 0.05$)

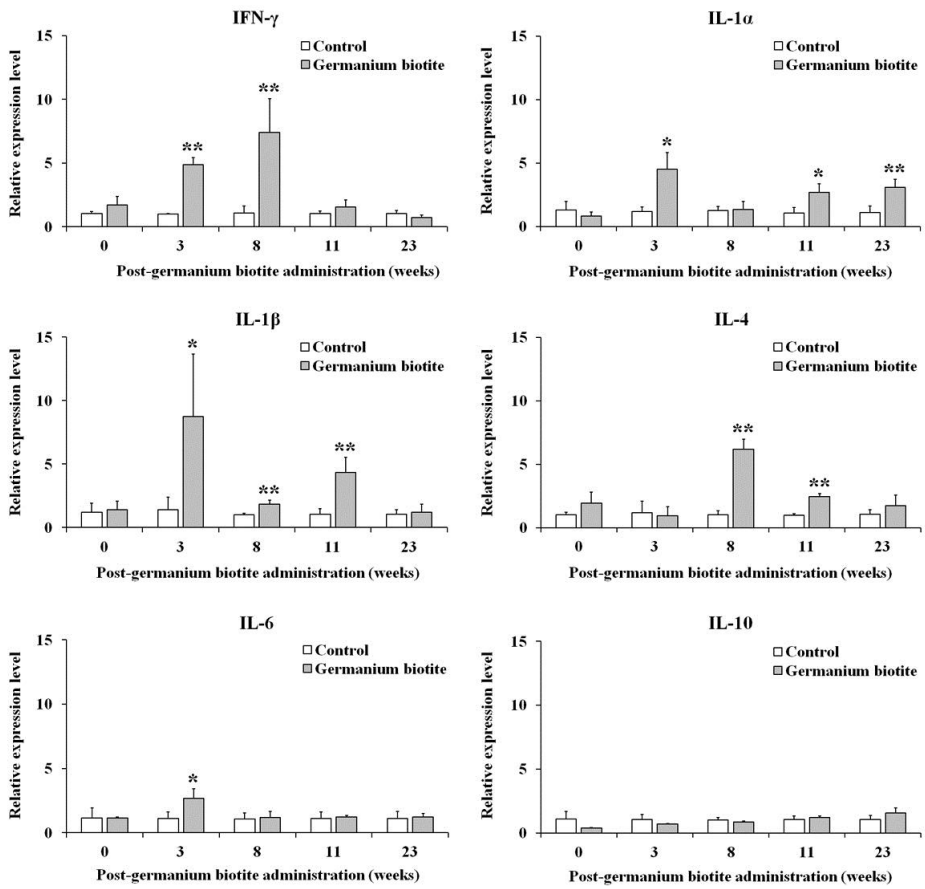

Figure 3.4. Analysis of gene expression of cytokines IFN-γ, IL-1α, IL-1β, IL-4, IL-6, and IL-10. Total RNA were purified from PBMCs purified from cattle vaccinated and fed with the germanium biotite supplement or without the supplement. (*$p < 0.05$ and **$p < 0.01$)

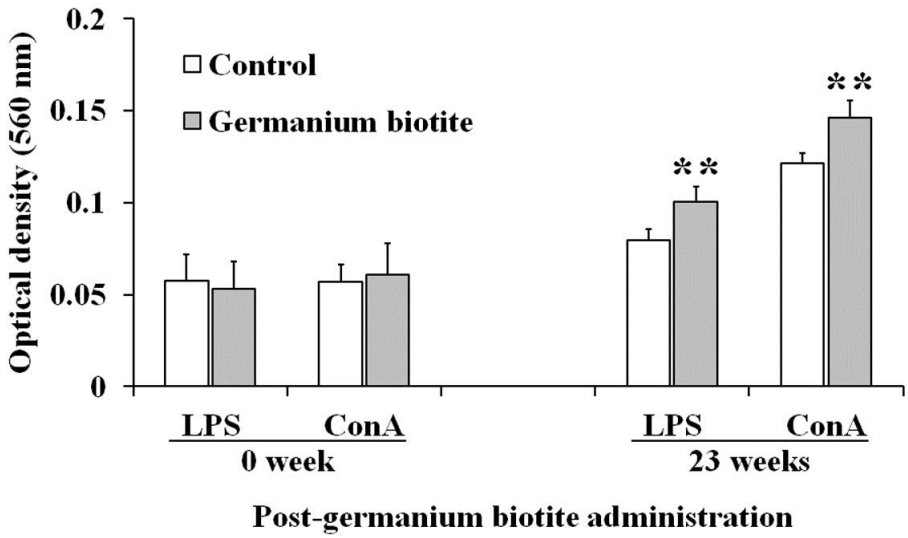

Figure 3.5. Changes of proliferative responses to stimulation of ConA and LPS in PBMCs. PBMCs were separated from the vaccinated cattle that were fed with the germanium biotite supplement or without the supplement at 0 and 23 weeks. The proliferation of the PBMCs was estimated after stimulation with LPS or ConA. (**$p < 0.01$)

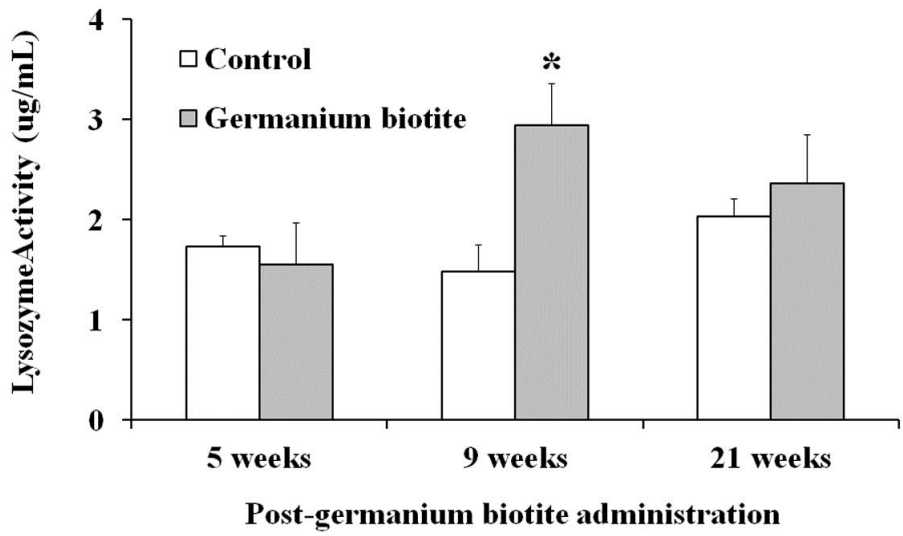

Figure 3.6. Changes of lysozyme activities in serum after oral administration of germanium biotite. The lysozyme activities of the sera from the vaccinated cattle that were fed with the germanium biotite supplement or without the supplement were measured based on the dissolution ability against *Micrococcus lysodeikticus*. (*$p < 0.05$)

Chapter IV

Effects of germanium biotite supplement on immune responses of vaccinated mini-pigs to foot-and-mouth disease virus challenge

Abstract

Since the outbreaks of FMD in South Korea in 2010–2011, a trivalent vaccine has been used as a routine vaccination. Despite the high efficacy of the trivalent vaccine, low antibody formation was reported in the pig industry and there is considerable concern about the ability of the vaccine to protect against the Andong strain responsible for outbreaks in Korea in 2010. To overcome these problems, immunostimulators have been widely used to improve vaccine efficacy in Korea, although without any scientific evidence. Based on the current situation, the aim of this study was to investigate the effects of germanium biotite, a feed supplement used to enhance the immune system, on the immune responses to FMD vaccination through the Andong strain challenge experiment in trivalent vaccinated pigs. Following the challenge, the germanium biotite-fed pigs showed high levels of IL-

8 in serum, and increased cellular immune responses to stimulation with the Andong strain antigen compared to nonsupplemented pigs. In addition, higher FMDV neutralizing antibody titers were detected in the germanium biotite-fed group than in the nonsupplemented group before the challenge. The findings of this study indicate that germanium biotite supplement might enhance immune responses to the FMD vaccine in pigs.

Key words: Foot-and-mouth disease, germanium biotite, vaccine, FMDV challenge, pigs

Introduction

Outbreaks of FMD have devastating effects on the livestock industry and limit international exchanges. Therefore, most countries have applied a "stamping-out with nonvaccination" policy to control the disease [131]. However, nonvaccination can lead to high susceptibility of domestic livestock populations to FMD if the disease is introduced unexpectedly [131], and make it more difficult to prevent the spread of FMD. Therefore, a prophylactic FMD vaccination can be considered as the resort of the control policy for FMD outbreaks.

Among commercial FMD vaccines currently in use, a trivalent vaccine, which is composed of O1 Manisa, A Malaysia 97, and Asia1 Shamir, has been used as a routine vaccination in South Korea since the outbreaks that occurred in 2010–2011 [23, 131]. Several studies have reported that multivalent vaccines could provide clinical protection against FMD and mitigate large FMD outbreaks [23, 24, 131, 196]. Although the trivalent vaccine has induced high antibody titer in cattle, the vaccine showed a low rate of antibody formation in pigs in Korea from July 2011 to June 2012, according to type O ELISA assay [24]. Furthermore, there is considerable concern about the abilities of trivalent vaccines to protect against the Andong strain, which is responsible for outbreaks in Korea in 2010 [23]. Therefore,

there is a growing need for strategies to enhance the immune responses to vaccination [25, 26].

In accordance with efforts to improve immune responses to vaccination, various immunostimulators have been used as feed supplement in Korea. Aluminosilicate, the major constituent of germanium biotite, has been studied for its beneficial biological activities [60, 61, 208]. It was reported that aluminosilicate showed effects of non-specific immunostimulating similar to superantigens [60, 61]. As mimic superantigens, aluminosilicate induced increase of intracellular Ca^{2+} level in the immune cells, thereby activating the cells as potent T-cell mitogens. These potent T-cell mitogens showed high affinity to regions of MHC class II molecules [151], thereby activating proinflammatory macrophages, which belong to MHC class II antigen-presenting cells [18].

Macrophages, cells of the innate immune system, are one of the primary sensors of danger in the host and activate, orient, and regulate adaptive immune responses [89]. It was also reported that macrophages change their physiology in response to environmental cues and play an important role in the host immune responses [89, 209]. Based on the reported effects of aluminosilicate, the germanium biotite has been considered as non-specific immunostimulator and used for feed supplement to reinforce the immune activities. As feed supplement, germanium biotite showed

prophylactic effects against respiratory infection in calves and antiviral effects against porcine reproductive and respiratory syndrome virus in pigs [17].

It was also demonstrated that dietary aluminosilicate supplement could enhance humoral immune response in mice and reinforce antiviral activity against porcine circovirus type 2 in experimentally infected pigs [16]. These findings of enhanced immune activities through germanium biotite supplementation could be associated with stimulating effects on the immune cells of aluminosilicate. Thus, previous studies have suggested that the germanium biotite could be used as feed supplement to improve the efficacy of the FMD vaccine through its potential for immunostimulator. However, there is no scientific understanding of how germanium biotite exerts its effects on immune responses to FDM vaccination.

Clinical signs of protection and specific antibody responses are important parameters when assessing the efficacy of FMD vaccines in challenge experiments [136, 139, 210, 211]. Previous studies reported a correlation between protection and specific antibody levels [198, 212]. In addition, it was reported that emergency FMD vaccination showed protection against FMDV, although FMDV-specific antibody was not detectable [136, 139]. This suggests that other immune factors may play an important role in FMDV protection. According to the previous literature, it is possible that the innate immune system has a critical role in defending against viral infection [138-140].

The current study investigated the effects of germanium biotite on immune responses to FMD vaccination in pigs through an FMDV challenge experiment. The aim of the study was to explore the ability of germanium biotite to enhance immune responses to the FMD vaccine when it was used as a feed supplement.

Materials and Methods

Feed supplement

Germanium biotite (Seobong Biobestech, Seoul, Korea) contains silicon dioxide (61.90%), aluminum dioxide (23.19%), iron oxide (3.97%), sodium oxide (3.36%), calcium oxide (< 2%), magnesium oxide (< 2%), titanium oxide (< 2%), and 36 ppm germanium.

Experimental animals and vaccination

Five-week-old white/black Yucatan mini-pigs (< 10 kg; Optipharm Medipig, Korea) were randomized into three groups as follows: a nonvaccinated negative control group (Nv group, n=5), which received no feed supplementation or FMD

vaccination; an only-vaccinated group (Ov group, *n*=5), which received the FMD vaccination but no feed supplementation; and a germanium biotite-supplemented vaccinated group (Gv group, *n*=5), which received the FMD vaccination and 0.5% of germanium biotite. The Gv group was provided with germanium biotite for the entire period of the experiment. Any difference in clinical signs and body conditions between the Gv and Ov groups, which might be due to side effects of germanium biotite, was not observed for two months period of experiment in this study. The FMD vaccination was carried out with 2 mL of Aftopor® (Merial, UK) when the animals were 6 weeks old, according to the manufacturer's instructions. The vaccine was double-oil-emulsion (water-in-oil-in-water [W/O/W]) adjuvanted trivalent vaccine (O1 Manisa, A Malaysia 97, and Asia1 Shamir) containing at least $6PD_{50}$ per dose.

FMDV challenge experiment

The FMDV challenge was performed with the Andong strain (serotype O), which was responsible for the 2010–2011 outbreaks in Korea [23], 5 weeks after the vaccination (35 days postvaccination [dpv]) as previously reported [210]. A seeder pig was randomly selected from the Nv group and administered an intradermal injection of 0.8×10^5 $TCID_{50}$ FMDV into the heel bulb 48 h before

housing with the other experimental pigs. After confirmation of vesicular lesions in the seeder pig, all the experimental pigs (< 20 kg) were exposed to the seeder pig for 16 hr, with direct contact. The seeder pig was then removed from the experiment and euthanized for ethical reasons. Clinical signs were monitored. Sera, saliva, and nasal discharge were collected every day before the challenge and until 8 days after the challenge (8 days postinfection, [dpi]). Saliva and nasal discharge were collected by swabbing with BD™ Universal Viral Transport (BD, USA) as described by the manufacturer. The pigs found to be infectious (presence of vesicular lesions) were moved to an isolator cage as soon as the lesions were observed to prevent an overwhelming challenge by the infected pigs, and all cages were disinfected twice a day with Virkon-S® (Bayer Korea, Korea) to prevent reinfection by shedding virus, according to the manufacturer's instructions. This challenge experiment was conducted in the Containment Research Laboratory (Biosafety level 3) of the QIA (Anyang, Korea). All the animal experimental procedures were approved by the Seoul National University Institutional Biosafety Committee (SNUIBC-P120220-1) and the Institution Animal Care and Use Committee of Seoul National University (SNU-120223-8).

Clinical monitoring after FDMV challenge

The clinical signs of the challenged pigs were recorded every 24 h throughout the experiment period. Rectal temperature, depression, and vesicular lesions were monitored, and each criterion of clinical signs was given one point [213]. The measured scores of each challenged pig were cumulatively added and considered as the CCS.

Measurement of viremia and virus shedding

Total RNA was extracted from the sera, saliva, and nasal discharge using the MagNA Pure 96 system® (Roche, Germany). The RNA of the FMDV in isolated total RNA of each sample was detected using the AccuPower® FMDV real-time RT-PCR kit (BioNeer, USA) and CFX96 Touch™ real-time PCR detection system (Rio-Rad, USA). All the procedures were performed according to the manufacturer's instructions, and a Cq value of < 40 cycles for real-time RT-PCR was considered positive for FMDV. The presence of live FMDV in sera, saliva, and nasal discharge at 4 dpi were investigated by a plaque assay using a continuous bovine kidney cell line (LFBK cell line) as previously reported [214, 215].

Measurement of antibody against FMDV

The presence of antibody titers against SPs of FMDV in inactivated sera was assayed using the PrioCHECK FMDV type O ELISA kit (Prionics, Switzerland). Antibody titers against FMDV in sera were indicated by the PI, and all procedures were performed following the manufacture's protocols. Sera with a PI value greater than 50% were considered seropositive in the ELISA assays.

Virus-neutralizing antibodies

Sera collected at -17, -10 (18 and 25 dpv), 4, and 8 dpi were inactivated at 56° C for 30 min. Two-fold serially diluted sera (50 µl) collected at -17 dpi were reacted with 50 µL of FMDV O1 Manisa strain ($100TCID_{50}$) at 37° C for 1 hr. The sera at -10, 4, and 8 dpi were also diluted and incubated with 50 µL of FMDV Andong strain ($100TCID_{50}$) at 37° C for 1 hr. After incubation, 1.0×10^4 of LFBK cells/ml in Dulbecco's Modified Eagle Medium (Gibco, USA) with 5% FBS (Gibco) were added to the reaction mixture. Following the incubation, VN titers were determined as the final dilution of serum showing no cytopathic effects.

Cytokine product responses in sera and peripheral blood mononuclear cells

IFN-γ (R&D system, USA), IL-8 (R&D system), and chemokine ligand 2 (CCL2, MyBioSource, USA) ELISA kits were used to investigate the cytokine product levels in the sera collected at 0, 1, 3–5, and 7 dpi, as described by the manufacture. PBMCs were isolated from the pigs at 8 dpi using the Histopaque®-1077 HybriMax™ (Sigma, USA) system and cultured in 5.0×10^6 cells/ml in Roswell Park Memorial Institute medium (Sigma) 1640 with 5% FBS. Following the stimulation with the FMDV antigen of the Andong strain (provided by QIA, 2.5 μg/ml) for 96 hr, supernatants of the cultured media were used for analysis of the cytokine product levels in the PBMCs. IFN-γ (Kingfisher Biotech Inc., USA), IL-8 (Kingfisher Biotech Inc.), and CCL2 (Kingfisher Biotech Inc.) ELISA kits were used for investigation of the cytokine product levels in the PBMCs, as described by the manufacture.

PBMC proliferation responses to stimulation with the FMDV antigen

Ninety microliters of PBMCs (5.0×10^6 cells/ml) isolated from the pigs at 8 dpi were cultured in a 96-well plate and stimulated with the FMDV antigen (Andong strain, 2.5 μg/ml) for 96 hr. Absorbance at 560 nm was measured 2 h after the

addition of 10 μL of 10× Prestoblue® (Invitrogen, USA). The proliferation of the PBMCs was compared based on this absorbance.

Statistical analysis

The data were presented as mean ± SD. Statistical differences between the groups were analyzed with the Student's *t*-test and repeated measures of ANOVA using Statistical Package for the Social Sciences version 19.0 software (SPSS, USA). Differences were considered significant when a probability value of $p < 0.05$ was obtained.

Results

Scores for clinical signs

Two days after the challenge, the rectal temperature of the Nv group started to increase, and all the experimental pigs showed signs of depression. Vesicular lesions were observed in the Nv group from 2 to 8 dpi. The signs of depression disappeared from 5 dpi in the Ov and Gv groups. The CCS of the Nv group was

significantly higher than that of the Ov and Gv groups from 5 dpi. There was no significant difference in the CCSs of the Ov and Gv groups (Figure 4.1).

Viremia and virus shedding

Viremia was confirmed in the sera of all the pigs in the Nv group at 2–8 dpi and in one pig in the Gv group at 3–7 dpi by real-time RT-PCR. FMDV was detected in the saliva and nasal discharge of all the pigs in the Nv group at 2–8 dpi and 3–8 dpi by real-time RT-PCR, respectively. However, FMDV was not present in the saliva or nasal discharge of the Ov and Gv groups. Live FMDV was isolated from sera, saliva, and nasal discharge samples only in the Nv group at 4 dpi.

Antibody titers against FMDV

Before the challenge, the Ov and Gv groups showed more than 80% of PI values against SPs of FMDV, and there was no change in the PI values after the challenge (Figure 4.2A). The VN antibody titers against the O1 Manisa and the Andong strain showed a tendency to increase at -17 and -10 dpi, respectively, in the Gv group compared to the Ov group. However, the VN antibody titers exhibited a tendency

to decrease in the Gv group compared to the Ov group at 4 and 8 dpi (Figure 4.2B, $p = 0.17$).

Analysis of cytokine product levels

Levels of serum IL-8 product were higher in the Gv group than in the Ov group at 3, 4, 5, and 7 dpi, and low levels of CCL2 were observed in the Gv group compared to the Ov group (Figure 4.3B and C, $p = 0.1$). Serum IFN-γ was close to or below detectable levels of the ELISA system (Figure 4.3A). PBMCs cultured from the Gv group showed the tendency of high levels of IL-8 product and low levels of CCL2 product compared to the Ov group ($p < 0.05$) following the stimulation with the FMDV antigen (Andong strain) (Figure 4.4). There were no significant differences in the IFN-γ product levels between the Ov and Gv groups (Figure 4.4).

Analysis of PBMC proliferation responses

Following stimulation for 96 hr, the PBMCs cultured from the Gv group showed high levels of proliferative responses to FMDV antigen compared to the Ov group (Figure 4.5, $p < 0.05$).

Discussion

Germanium biotite, which showed the effects of non-specific immunostimulating in previous studies [16-18, 60, 61, 208], is a well-known feed supplement in pigs industry in Korea. Since germanium biotite was applied to feed supplement in pigs, there was no reported side effect. The aim of this study was to determine the potential of germanium biotite as a feed supplement to enhance immune responses to the FMD vaccine. To shed light on this issue, trivalent (O1 Manisa, A Malaysia 97, and Asia1 Shamir) FMD-vaccinated pigs, with and without germanium biotite supplement, were challenged with the FMDV Andong strain. A previous study, reported that it was difficult to infect a vaccinated pig through intradermal inoculation in the bulb of the heel [213]. Therefore, the nonvaccinated seeder pig in this study had direct contact with the experimental animals [210].

Following the challenge, no significant difference was observed in the CCS of the Ov and Gv groups, as shown Figure 4.1. One pig in the Gv group showed vesicular lesions and viremia at 3–7 dpi, but all the other pigs were protected throughout the challenge. In this study, the trivalent vaccine had a 90% protection rate. It was postulated that the infected pig in the Gv group were related to the variation of challenge conditions, such as the method of challenge, skin injury, and

stress [214, 216], considering previous studies that reported high immunity following vaccination [23, 24, 131, 196].

Before the challenge, the VN antibody titers against the O1 Manisa and Andong strains showed a tendency to increase in the Gv group compared to the Ov group. However, following the challenge, they exhibited a tendency to increase in the Ov group compared to Gv group (Figure 4.2B, $p = 0.17$). The low VN antibody titers against the Andong strain in the Gv group may reflect reduced replication of the virus, as cross-reactivity of antibodies in response to O1 Manisa strain vaccination would have induced neutralization of the Andong strain before the challenge. However, after the challenge, VN antibody was produced mainly by virus infection. The high VN antibody titer following the challenge might represent a kind of sero-conversion. Previous studies reported that a low level of sero-conversion may be an indicator of reduced viral replication [131, 217].

Innate immune responses play an important role in FMDV infection, as well as adaptive immune responses. [136, 139]. Thus, cytokine responses are very important because both innate and adaptive immune responses are driven and regulated by cytokine production [136]. Among the cytokines, IFN-γ, IL-8, and CCL2 play a key role in viral protection and clearance [136, 139, 218-221]. The present study investigated the level of IL-8 product in the sera and found a high level in the Gv group compared to that of the Ov group (Figure 4.3B, $p = 0.1$).

However, IFN-γ activities were not detectable in this study (Figure 4.3A). This result corresponds with that of a previous study that demonstrated no IFN-γ activity in FMDV-vaccinated and challenged pigs [139].

On the other hand, levels of CCL2 product in sera were lower in the Gv group than in the Ov group (Figure 4.3C, $p = 0.07$). These results are presumed that CCL2 production is associated with the development of polarized Th2 responses [219, 222], whereas previous studies reported that the immunostimulating effects of germanium biotite were related to Th1 responses [16, 223].

Measurement of immune response levels using immune cells that originate from immunized animals has been found to be an important method of predicting FMD protection following vaccination and challenge [148]. In the present study, the PBMCs of the Gv group showed the tendency of high level of IL-8 production, which was previously found to be consistently elevated in FMDV-vaccinated pig [136], compared to the Nv and Ov groups after stimulation with FMDV antigen (Figure 4.4). In common with the level of CCL2 in sera, lower CCL2 productive response than the Ov group was found in Gv group following the FMDV stimulation. The increase in the PBMCs responses of the Gv group to FMDV antigen was also observed in the proliferation responses to FMDV antigen stimulation compared to the Ov group (Figure 4.5, $p < 0.05$). The high level of

proliferation responses of PBMCs in the Nv group compared to those in the Ov and Gv groups may be due to overdose stimulation by viremia of FMDV.

In this study, pigs administered germanium biotite orally in feed showed improved immune responses to FMD vaccination and challenge. The results may be attributed to the induction of enhanced immune responses to FMD vaccination by the immunostimulating effects of germanium biotite. In conclusion, the findings of this study indicate that germanium biotite can be used as feed supplement to enhance the efficacy of the FMD vaccine in pigs.

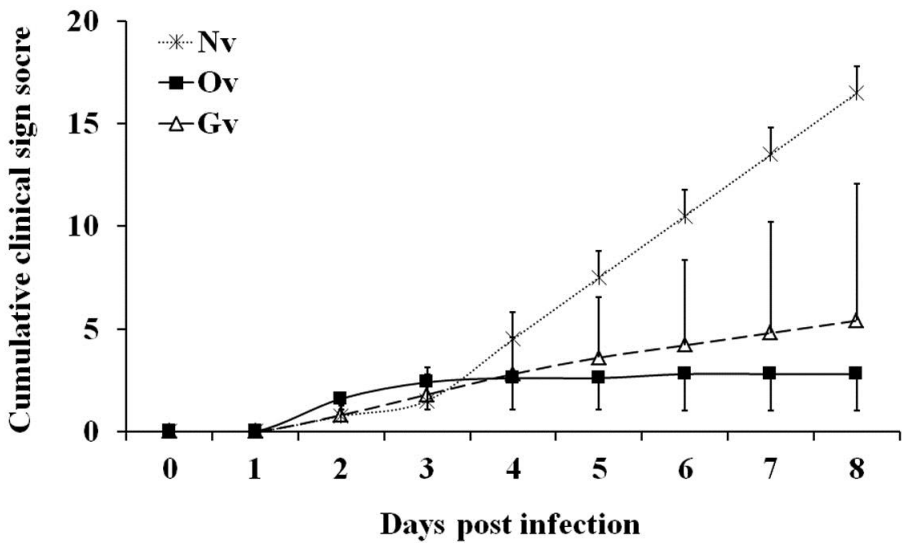

Figure 4.1. Cumulative total clinical sign scores were calculated for rectal temperature, depression, and vesicular lesions after challenge with the Andong strain. The clinical signs were recorded every 24 hr. Nv, nonvaccinated group; Ov, only-vaccinated group; Gv, germanium biotite-supplemented vaccinated group.

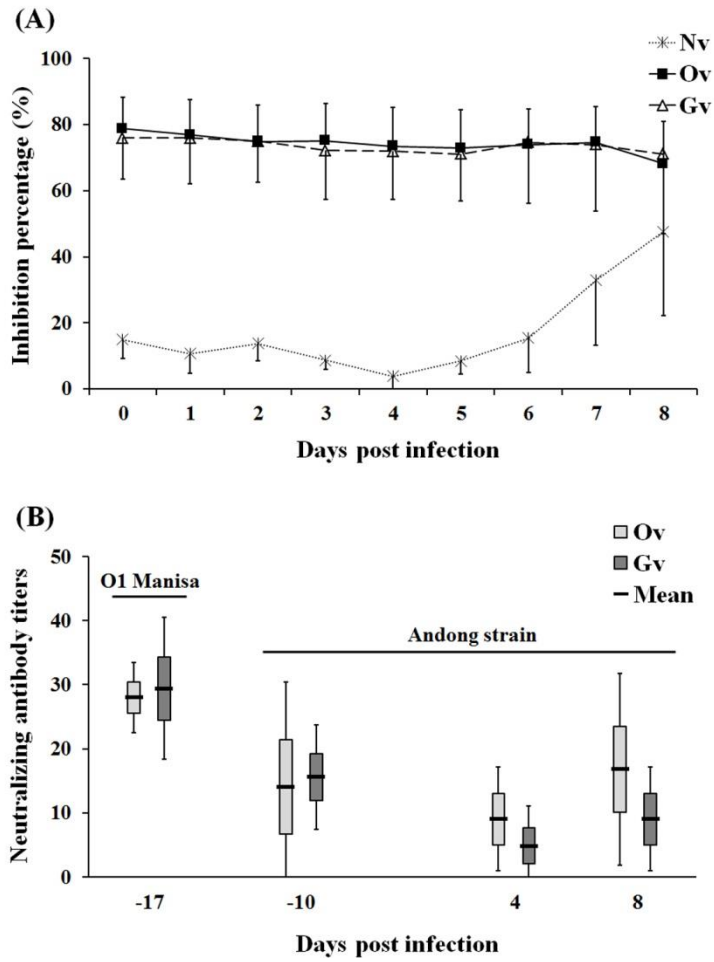

Figure 4.2. Changes of antibodies levels after challenge with the Andong strain. (A) Antibody levels against structural proteins of type O FMDV following challenge where a PI value of > 50% was considered positive. (B) Virus neutralizing antibody titers against the O1 Manisa at -17 dpi (18 dpv) and Andong strain at -10 (25 dpv), 4, and 8 dpi. Nv, nonvaccinated group; Ov, only-vaccinated group; Gv, germanium biotite-supplemented vaccinated group.

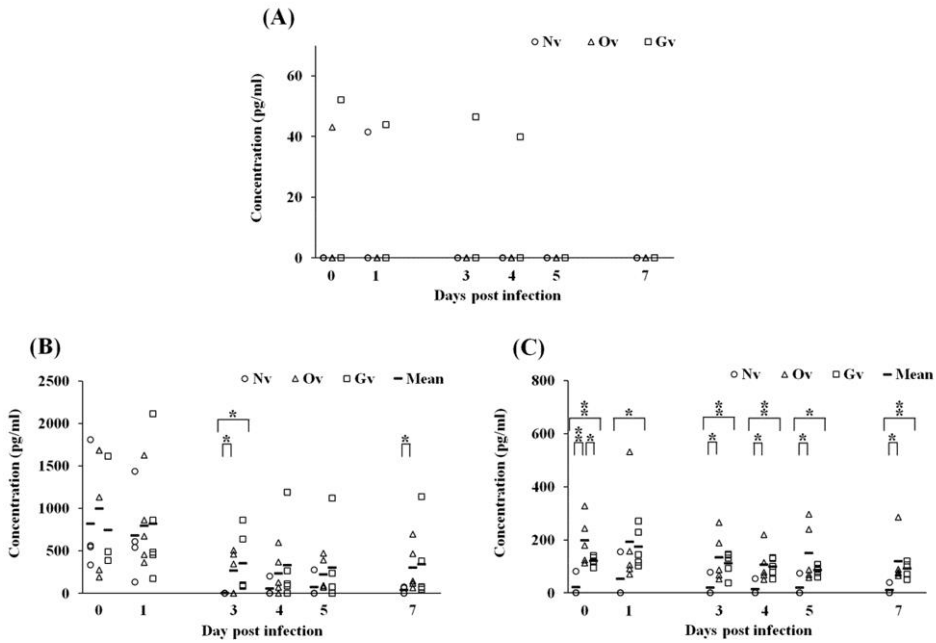

Figure 4.3. Changes of cytokines levels in serum after challenge with the Andong strain. Product levels of IFN-γ (A), IL-8 (B), and CCL2 (C) in sera were investigated using an EILISA kit after FMDV challenge. Serum IFN-γ was close to or below detectable levels of the ELISA system. Nv, nonvaccinated group; Ov, only-vaccinated group; Gv, germanium biotite-supplemented vaccinated group. (*$p < 0.05$ and **$p < 0.01$)

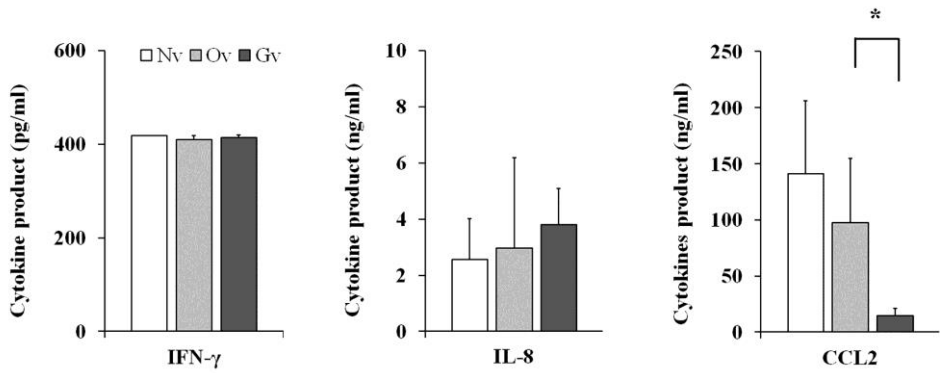

Figure 4.4. Cytokines product responses to stimulation with FMDV antigen in PBMCs. PBMCs were cultured from pigs at 8 dpi and stimulated with FMDV antigen (Andong strain) for 96 hr. Following the stimulation, the production levels of IFN-γ (A), IL-8 (B), and CCL2 (C) were measured. Nv, nonvaccinated group; Ov, only-vaccinated group; Gv, germanium biotite-supplemented vaccinated group. (*$p < 0.05$)

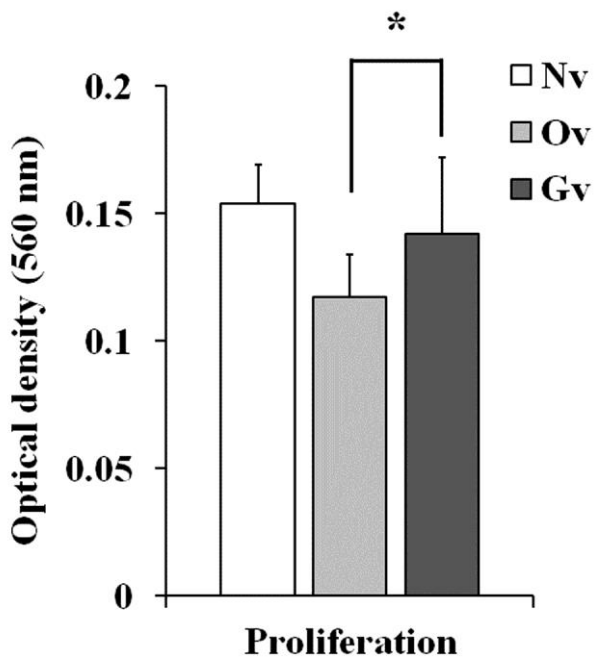

Figure 4.5. Proliferative responses to stimulation with FMDV antigen in PBMCs. PBMCs were cultured from pigs at 8 dpi and stimulated with FMDV antigen (Andong strain) for 96 hr. Nv, nonvaccinated group; Ov, only-vaccinated group; Gv, germanium biotite-supplemented vaccinated group. (*$p < 0.05$)

General conclusions

As researches on novel therapies to harness the power of the innate immune system to control diseases are actively in progress, there is a need to develop and evaluate the new approaches that can stimulate innate immune system, and thereby prevent outbreak of diseases. Silicate, major component of germanium biotite, has shown enhancing effects on immune system. Based on these beneficial effects, the aims of the present study were to demonstrate mechanism of the immune stimulating effects of germanium biotite and to evaluate the immune enhancing effects in cattle and pigs. To achieve the goals, global gene expression changes in RAW 264.7 cells after treatment of mica (germanium biotite) was investigated, along with effects of the germanium biotite on respiratory diseases of cattle, FMD vaccinated cattle, and immune responses to FMDV challenge in pigs.

Following the treatment with the mica, RAW 264.7 cells showed up-regulation of lysosome and phagosome function. As feed supplement, the germanium biotite showed prophylactic effects on challenge of BHV-1 and *Mannheimia haemolytica* serotype A1 in cattle such as moderated clinical sings, enhanced clearance of BHV-1, low level of bacteria shedding, tempered superficial lesions, and

moderated histophathological lesions. In FMD vaccinated cattle, the germanium biotite increased efficacy of FMD vaccine such as high levels antibodies and increased antibody duration. Moreover, germanium biotite-fed cattle showed increases of $CD4^+$ and MHC class II^+ expression in PBMCs, high levels of cytokines (IFN-γ, IL-1α, IL-1β, and IL-4) expression, enhanced lymphocyte proliferation, and reinforced lysozyme activity. In addition, germanium biotite-fed pigs showed high levels of IL-8, and low levels of CCL2 in serum, and increased cellular immune responses to stimulation with the Andong strain antigen after challenge of Andong strain. Before challenge, high levels of VN titers were detected in the germanium biotite-fed pigs.

This study suggests that germanium biotite enhanced the immune responses to challenge of BHV-1, *Mannheimia haemolytica* serotype A1, and FMDV and FMD vaccination. These results are thought to be derived, in part, from macrophage activation associated with the innate immunity such as increase of lysozyme activity and up-regulation of lysosome and phagosome functions. Therefore, the germanium biotite could be a candidate for new alternative feed supplement to reinforce immunity, thereby reducing the risk of diseases outbreak in livestock industry, even though the limit problems to use natural minerals due to environmental issues such as mineral residues in feces have yet to be solved. In addition, since many other cells as well as macrophages also play an important role

in immune responses, further researches based on other immunocytes such as DCs and NK cells are needed to demonstrate overall effects of the germanium biotite, as a non-specific immunostimulator, on immune system (Figure IV).

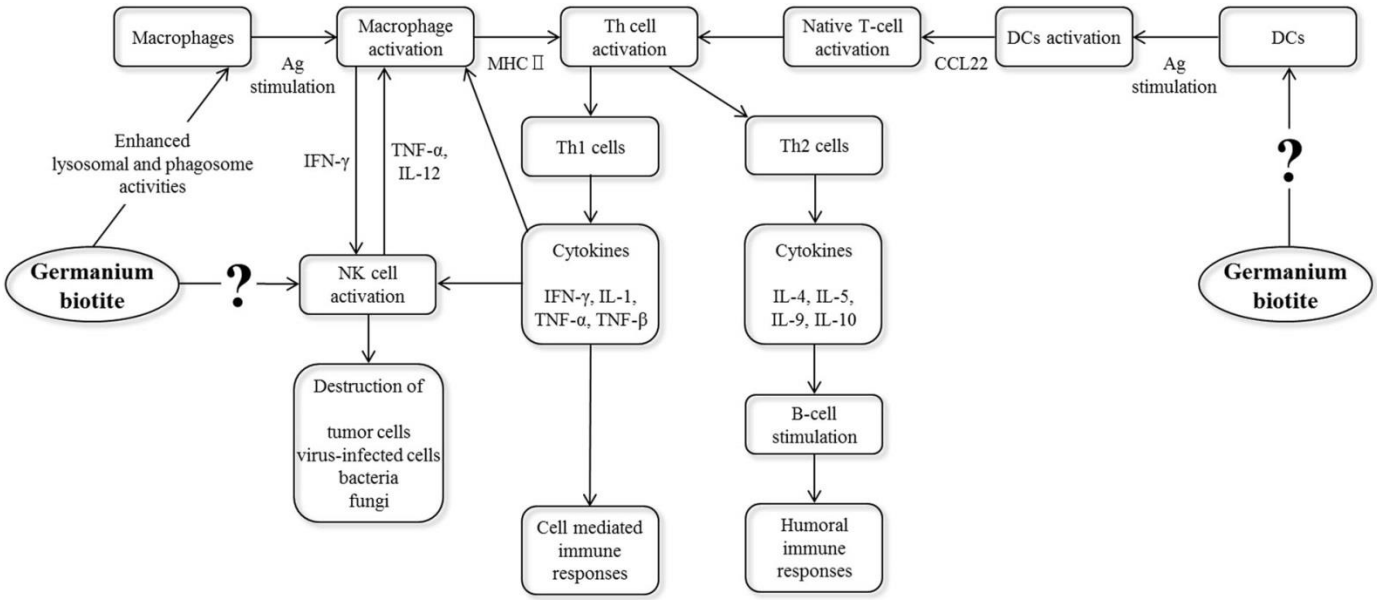

Figure IV. Diagram of immune responses affected by germanium biotite. This study showed that the germanium biotite could reinforce macrophage activities such as lysosomal and phagosomal activities. Therefore, it might be presumed that these reinforced macrophage activities, in part, induced the enhanced immune responses to challenge of BHV-1, *Mannheimia haemolytica* serotype A1, and FMDV and FMD vaccination. In addition, since many other cells as well as

macrophages also play an important role in immune responses, further researches based on other immunocytes such as DCs and NK cells are needed to demonstrate overall effects of the germanium biotite, as non-specific immunostimulator, on immune system.

국문초록

비특이 면역증강제인 게르마늄 흑운모의 면역증진효과 분석

정 명 환

(지도교수: 유한상, D.V.M., Ph.D.)

서울대학교 대학원

수의학과 수의미생물학 전공

게르마늄 흑운모는 실리케이트가 주성분인 마이카 종류의 천연 광물질로, 사료 첨가제 형태의 이용은 비특이 면역을 증가시킬 수 있음이 보고되어 왔다. 본 연구에서는 축산업에서 질병예방을 위한 사료 첨가제 개발의 첫 단계로서, 게르마늄 흑운모의 면역증강효과를 시험관내에서 확인하여 메커니즘을 밝히고, 소와 돼지에서 게르마늄

흑운모의 사료 첨가제 이용으로 유발되는 면역 증강 효과를 평가하는 것이다.

게르마늄 흑운모의 면역증강효과의 세포 수준의 메커니즘을 규명하기 위하여 마우스 대식세포주인 RAW 264.7 세포를 100 μg/mL 의 마이카(게르마늄 흑운모)로 자극하여 12 시간 및 48 시간 동안의 유전자 발현 변화를 microarray 의 실험방법으로 조사하였다. 또한 소와 돼지에서의 게르마늄 흑운모의 면역증강효과를 평가하기 위하여, bovine herpesvirus type 1 (BHV-1)과 *Mannheimia haemolytica* serotype A1 을 이용한 소 호흡기 질병 병원체의 공격접종에 대한 게르마늄 흑운모의 예방효과를 평가하였다. 더불어 소에서 구제역 백신에 대한 면역반응과 소의 면역계에 게르마늄 흑운모가 미치는 영향을 분석하였으며, 구제역 백신을 접종한 미니돼지의 구제역 안동주 바이러스 공격접종에 대한 면역반응에 게르마늄 흑운모가 미치는 영향을 분석하였다.

48 시간 동안 마이카로 자극된 RAW 264.7 세포에서는 대조군에 비해 1,128 개의 유전자 발현이 달라졌으며, 발현이 달라진 유전자로 인해 세포주기, DNA 복제, pyrimidine 및 purine 의 합성에 관여하는 경로의 활성이 감소된 것으로 분석되었다. 또한 대식세포의 활성화에 필요 조건인 라이소자임 및 대식 작용의 활성이 증가된 것으로 분석되었다. 마이카의 자극에 의한 유전자 발현 변화 분석을 통해, 게르마늄

흑운모가 대식세포를 활성화 시킬 수 있고, 이러한 대식세포의 활성화에는 라이소자임 및 대식 작용의 활성화 증가가 관여함을 확인할 수 있었다.

소 호흡기 질병 병원체 공격접종 실험에서 게르마늄 흑운모 투여군은 대조군에 비해 완화된 임상증상을 보였다. 또한 게르마늄 흑운모 투여군은 대조군에 비해 증가된 BHV-1 의 청소율과 낮은 수준의 세균 분비, 완화된 폐병변 및 병리조직학적 소견을 보여 임상증상의 결과와 부합되는 결과를 보였다. 이러한 결과를 통해 게르마늄 흑운모가 비특이 면역증강 효과를 통해 소 호흡기 질병에 대한 예방 효과를 보임을 확인할 수 있었으며, 이를 바탕으로 게르마늄 흑운모가 새로운 사료첨가제 후보물질로서의 가능성이 있음을 확인할 수 있었다.

소에서 구제역 백신에 대한 면역반응과 소의 면역계에 게르마늄 흑운모가 미치는 영향 분석 실험에서 게르마늄 흑운모 투여군은 대조군에 비해 높은 수준의 혈청내 IgG 와 타액내 IgA 를 보였으며, 항체가의 유지기간에서도 대조군에 비해 길어진 것이 확인되었고, 중화항체가의 경우도 게르마늄 흑운모의 군이 대조군에 비해 높게 관찰되었다. 게르마늄 흑운모의 투여 후, 말초혈액 단핵구의 $CD4^+$ 및 MHC class II^+ 의 발현이 증가하였고 IFN-γ, IL-1α, IL-1β 및 IL-4 의 유전자 발현도 증가하였다. 또한 게르마늄 흑운모의 투여군의 말초혈액

단핵구는 투여 후 23 주째에 대조군에 비해 높은 증식반응을 보였으며, 게르마늄 흑운모 투여군의 말초혈액 내 lysozyme 의 활성도 투여 후 8 주째에 대조군에 비해 증가된 것이 관찰되었다. 이러한 결과를 통해 게르마늄 흑운모의 투여가 체액성 면역과 세포성 면역을 활성화 시켜 구제역 바이러스 감염에 대한 방어율을 높일 수 있을 것으로 판단되었다.

구제역 바이러스 공격접종 실험에서 게르마늄 흑운모 투여군 돼지는 대조군 돼지에 비해 높은 수준의 혈청내 IL-8 과 안동주 바이러스에 대한 증가된 세포성 면역반응을 보였다. 또한 공격접종 전, 대조군에 비해 높은 안동주 바이러스에 대한 중화 항체가가 게르마늄 흑운모 투여군에서 관찰되었다. 이러한 결과를 통해 게르마늄 흑운모의 투여로 돼지에서 구제역 바이러스의 공격접종에 대한 향상된 면역 반응을 유도할 수 있음을 확인 할 수 있었다.

이상 결과를 종합하여 볼 때, 게르마늄 흑운모의 경구 투여 이용은 소 호흡기 질병 병원체 (BHV-1 및 *Mannheimia haemolytica* serotype A1), 구제역 백신접종, 구제역 바이러스 공격접종에 대한 면역반응의 향상을 유발할 수 있음을 확인할 수 있었다. 이러한 향상된 면역반응은 게르마늄 흑운모가 비특이 면역을 활성화 시키는 것과 관련 있는 것으로 생각되며, 이러한 비특이 면역증강효과에서 라이소자임 및 대식 반응의 증가와 같은 대식세포의 활성이 큰 역할을 하는 것으로 판단된다.

이러한 결과를 바탕으로 게르마늄 흑운모는 면역력을 향상시켜 축산업에서 질병 발생의 위험을 감소 시킬 수 있는 새로운 사료첨가제의 후보물질이 될 수 있을 것으로 판단되었다.

핵심어: 게르마늄 흑운모, 비특이 면역, 면역증강효과, 대식세포의 활성

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