Summary of the Final Examination Results

(Form 14)

Report No.	Diploma No. 629		Applicant	Ela Novianti
Examination Committee Members	Chief Examiner	MIYATA Atsuro	Degree	Doctor of Philosophy in Medical Science
	Examiner	KUWAKI Tomoyuki	Examiner	HASHIGUCHI Teruto
	Examiner	NISHIO Yoshihiko	Examiner	HARA Hiromitsu

The chief and four examiners interviewed applicant Ela Novianti on February 3rd, 2022. The applicant was asked to explain the thesis and answer related questions. We asked the following questions and received satisfactory answers.

Question 1. Why did you choose to use three types of cells? Are there any specific reasons?
Answer: The aim of this study is to examined whether atractylenolide III (AIII) can modulate inflammatory responses induced by LPS. Since microglial cells exist only in the brain and are responsible for brain inflammation, we chose both mouse microglia cell line (MG6 cells) and primary cultured microglia cells (PMC) to determine the effects of AIII on brain inflammation models. As a reference, Mouse macrophage cell line RAW 264.7 cells were used to examine the effecs of AIII in the peripheral system.

Question 2. Are there any different effects of AIII on those three types of cells?
Answer: In our present study, the results showed no difference in the effects of AIII in all types of cells being used. AIII inhibited the expressions of cytokines (TNF-α, IL-1β, and IL-6) and enzymes (iNOS and COX-2) induced by LPS in MG6 cells, PMC, and RAW 267.4 cells.

Question 3. Are there any previous publications regarding the effects of AIII in in vivo study?
Answer: Yes, there are *in vivo* studies that examined the effects of AIII or herbal medicine containing AIII. For instance, AIII exert a gastroprotective effects on ethanol-induced gastric ulcer *in vitro* and *in vivo* (Wang et al., Journal of Pharmacy and Pharmacology, 2010, 62: 381-388) and oral administration of AIII in LPS-injected mice suppressed the serum levels of TNF-α and IL-6 (Kwak et al., Evidence-Based Complementary and Alternative Medicine 2018, 2018: 4041873). However, many *in vivo* studies of AIII were conducted in peripheral organs. Still *in vivo* researches are necessary to be attempted to determine the impact of AIII on brain inflammation models.

Question 4. Did you examine the effects of AIII on other TLRs?
Answer: No, I didn't examine the effects of AIII on TLRs other than TLR4. However, some herbal medicines containing the rhizome of Atractylodes macrocephala also have an effect on the expression of TLR2 (Song et al., Evidence-Based Complementary and Alternative Medicine 2020, 2020: 6538156) and TLR7 (Cheng et al., Molecular Medicine Reports 2016, 14: 3704-3710).

Question 5. Are there any other publications showing the anti-inflammatory effect of AIII with inducers other than LPS?
Answer: Yes, there are. For example, AIII exerts an anti-inflammatory effect in phorbol 12-myristate 13-acetate- and calcium ionophore A23187-stimulated human mast cells (HMC)-1 (Kang et al., Journal of Natural Products 2011, 74(2): 223-227) and in thymic stromal lymphopoietin (TSLP)-induced HMC-1 cells (Yoou et al., Food and Chemical Toxicology 2017, 106: 78-85). AIII also downregulated cytokines productions in bleomycin-induced pulmonary fibrosis (Huai and Ding, Immunopharmacology and Immunotoxicology 2020, 42(5): 436-444).

Question 6. How much concentration/ conversion dose of AIII will be used if you ~~will~~ conduct an in vivo study?
Answer: Based on the previous study, the recommended dose of Atractylodes macrocephala for a 60 kg adult human is 8-24 g of raw plant per day or 3-9 g of extract per day. The dose for mouse can be determined as follows: a human equivalent dose of 50-150 mg/kg × 12.3 (the conversion coefficient) = a mouse dose of 615-1,845 mg/kg. The content of AIII in Atractylodes macrocephala was 0.565 mg/g extract (Kwak et al., Evidence-Based Complementary and Alternative Medicine 2018, 2018: 4041873). Based on this dose range, the optimum dose for in vivo study is 0.282-1.41 mg/kg.

Question 7. Why did you check only the phosphorylation of MAPK and NF-kB in microglia, but not in RAW 264.7 cells?
Answer: The aim of this study is to examine whether AIII can modulate inflammatory responses induced by LPS, especially in brain inflammation, and elucidated the mechanism. To achieve this goal, we used only microglia cells (MG6 cells and PMC) to check the phosphorylation of MAPK and NF-kB. As we know that RAW 264.7 cells is a model for peripheral inflammation, therefore we didn't check the effects of AIII on the phosphorylation of MAPK and NF-kB in RAW 264.7 cells. We also didn't do the comparison of its effect on between microglia and macrophage. In stead, previous sudy showed that AIII inhibits NF-kB and MAPKs pathways in LPS-induced RAW 264.7 cells (Ji et al., Immunopharmacology and Immunotoxicology 2016, 38(2): 98-102).

Question 8. In your result, AIII significantly decreases the increases of p38 MAPK and JNK phosphorylation induced by LPS in MG6 cells and does not affect the NF-kB phosphorylation. Are there any other publications showing similar results?
Answer: Fan and his colleague demonstrated that Eucommia ulmoides Oliv. exerts an anti-inflammatory effect in a Parkinson's mouse model through the inhibition of p38/JNK-Fosl2 gene expression (Fan et.al., Journal of Ethnopharmacology 2020, 260: 113016). However, in that study, they didn't examine the effecs of Eucommia ulmoides Oliv. on the NF-kB gene expression.

Question 9. In your result, LPS 5 ng/mL itself only increases the low levels of the phosphorylation of NF-kB. Is it a phenomenon that we usually encounter?
Answer: Previous study revealed that in MG6, the change in NF-kB phosphorylation by LPS seems to be weak, although they used a 200 times higher concentration of LPS than ours. (Sato et al., International Immunology 2007, 19(8): 901-911). On the other hand, Hong demonstrated that in BV2, another cell line of the same mouse microglia as MG6, LPS 1 μg/mL (20 times higher than our LPS concentration) increases NF-kB phosphorylation (Hong et.al., International Immunopharmacology 2022, 105: 108566). Immortalization seems to make a difference between two cell lines, although both cell lines are used for general analysis.

Question 10. In your result, why did LPS+AIII treatment increase the phosphorylation of NF-kB?
Answer: Altough it is now accepted that sesquiterpene lacton compounds harbours anti inflammatory effects by inhibiting the phosphorylation of NF-kB in most cases, some of them might paradoxically increase NF-κB phosphorylation. In this study, LPS+AIII treatment increases the phosphorylation of NF-kB. The dosage of LPS, and AIII, time course and type of cell being used might be responsible for this discrepancy. I would like to conduct further investigation to figure it out.

Question 11. The production of IL-1β by LPS requires activation of the inflammasome. Can you explain the mechanism of inflammasome acivation?
Answer: LPS has been found to trigger the activation of a distinct type of inflammasome, known as the "non-canonical" inflammasome. When LPS is present in abundance or is contained within vacuoles, guanylate-binding proteins (GBPs) promote vacuolar lysis, causing entry of LPS into the cytosol of the cell. Caspase-4 (also known as caspase-11 in mice) and caspase-5 detect cytocolic LPS. They become activated and promote pyroptosis through cleavage of the pore-forming protein gasdermin D (GSDMD), and trigger a secondary activation of the canonical NLRP3 inflammasome for cytokine release (Downs et al., Molecular Aspects of Medicine 2020, 76: 100924).

Question 12. Which phosphorylation of NF-kB did you examine in this study?
Answer: In this study, we examined the phosphorylation of NF-kB using the phospho- NF-kB p65 (Ser536) antibody. This antibody is widely used to detect the activation of NF-kB signaling in various types of cells, including microglia, by western blotting analysis. RelA/p65 protein, a main subunit in the NF-kB family, is mostly phosphorylated at the transcriptional activation domain (TAD) of the carboxy-terminus (such as Ser536) during inflammation (Sasaki et al., J. Biol. Chem 2005, 280: 34538-34547; Christian et al., Cells 2016, 5: 12; Pan et al., Oncotarget 2017, 8: 46249-46261). Moreover, previous studies reported that LPS induced the phosphorylation of the p65 trans-activation domain on Ser536 in monocytes/macrophages (Yang et al., J. Immunol. 2003; 170: 5630-5635) and the NF-kB p65 (Ser536) activity is associated with TLR4 signaling (Wang et al., Cancer Res. 2011, 71: 1325-1333). Therefore, based on those studies, we examined the effects of AIII on LPS-induced inflammation on TLR4 and cytokines expression in MG6 cells using phospho- NF-kB p65 (Ser536) antibody.

Question 13. What are the upstream kinases of the phospho- NF-kB p65?
Answer: A number of kinases have been identified that phosphorylate NF-kB p65 on Ser536 which includes IKKα, Ribosomal Subunit S6 Kinase 1 (RSK1), IKKβ, IKKε and NF-κB activating kinase (NAK)/TANK-binding kinase 1 (TBK1). Phosphorylation at Ser536 in the transactivation domain (TAD) of p65 leads to enhanced transactivation, through increased CBP/p300 binding and acetylation at K310 of p65. This phosphorylation induces a conformational change, which impacts p65 ubiquitination and stability, as well as protein interactions (Chistian et al., Cells 2016, 5: 12).

Question 14. Which transcription factors of p38 and JNK are activated in this study?
Answer: p38 is supposed to act synergistically with JNK. Thus, similar as JNKs, p38 might contribute to AP-1 activity through induction of c-jun and c-fos via phosphorylation of ATF-2 and Elk-1, respectively (Mielke and Herdegen, Progress in Neurobiology 2000, 61(1): 45-60). However, in this study, we didn't examine the effects of AIII on c-jun/c-fos/AP-1 activation.

Question 15. Can you explain the mechanism of CCK-8?
Answer: Cell Counting Kit-8 (CCK-8) is a sensitive colorimetric assays for the determination of cell viability in cell proliferation and cytotoxicity assays by using a Highly water-soluble tetrazolium salt, WST-8. CCK-8 measures the metabolic activity of living cells. WST-8, is reduced by dehydrogenase activities in living cells to give a yellow-color formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye, generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells.

Question 16. Is there any evidence that showed the TLR4-induced microglia activation is involved in the CNS inflammation? If yes, in what condition?
Answer: Overactivated microglia causes oxidative stress and damage to CNS cells. As microglial activation is widely controlled by pathogen-recognition receptors, TLR4 is implicated in the microglia-mediated CNS inflammation that occurs in many brain pathologies. In addition, TLR4 recognizes some endogenous molecules released by injured tissues and necrotic cells, called Damage-associated molecular patterns (DAMPs), which are known to cause various sterile inflammations. For example, in Alzheimer's disease (AD), amyloid-β (Aβ) oligomers act as endogenous ligand and directly induce microglial activation through several receptors, including TLR4 (Stewart et al., Nature Immunology 2010, 11: 155–161). Moreover, the high mobility group box 1, a typical DAMP protein which is initiator and activator of neuroinflammatory responses, has been involved in the pathogenesis of neurodegenerative diseases through binding to the TLR4 (Paudel et al., Cells 2020, 9: 383).

Question 17. How will you elucidate the mechanism by which AIII regulates TLR4 expression?
Answer: Expression of TLR4 gene is controlled by two distinct molecular mechanisms. First, TLR4 is regulated by transcriptional activation. Second, regulation of TLR4 gene can also result from posttranscriptional stabilization of mRNA. We can examine either endogenous or exogenous micro RNAs (miRNAs) and short interfering RNAs (siRNAs) which affect the downregulation of TLR4 mRNA expressions. First, we examine whether AIII treatment on microglia followed by LPS activation increases expression of candidate miRNA which directly targets TLR4, then we try to inhibit the selected miRNA by siRNA or miRNA inhibitor and verify the recovery of the TLR4 expression as we can expect inhibition of this miRNA will block the efficacy of AIII. We also should analyze the transcriptional regulation of TLR4 by checking the effects of AIII on activity of transcription factors for TLR4 expression. PU.1, AP-1, and GATA-1 transcription factors were known to regulate the transcription of TLR4 gene. PU.1 and AP-1 were found to regulate positively TLR4 gene expression, while GATA-1 was reported to inhibit TLR4 gene transcription (Roger et al., Biochem J. 2005, 387: 355–365; Lichtinger et al., Journal of Biological Chemistry 2007, 37: 26874-26883).

Question 18. Did you examine materials (drug or medicine) other than AIII that downregulates TLR4 expression?
Answer: No, I didn't. However, previous studies showed that some drugs, such as statin and resolvin D2, downregulates TLR4 expression and signaling via inhibition of protein prenylation and induction of microRNA-146a, respectively (Methe et al., Arterioscler Thromb Vasc Biol. 2005, 25:1439-1445; Croasdell et al., FASEB Journal 2016, 30(9): 3181-3193).

From the above results, the five reviewers confirm that the applicant possesses the academic skills and knowledge required to complete the doctoral course and is qualified to receive the degree of Doctor of Philosophy (Medical Science).