Effects of *Strongyloides stercoralis* antigens on immune responses in dendritic cells

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วิธวินท์ สุทวีทรัพย์ : ผลของแอนติเจนจากพยาธิเส้นด้ายต่อการตอบสนองทางระบบภูมิคุ้มกันในเซลล์เดนได รติก. (Effects of *Strongyloides stercoralis* antigens on immune responses in dendritic cells) อ.ที่ปรึกษาหลัก : ผศ. ดร.วิวรพรรณ สรรประเสริฐ, อ.ที่ปรึกษาร่วม : ศ. พญ.สุรางค์ นุชประยูร,ดร.พิมพเยาว์ สดใส

โรคพยาธิเส้นด้ายเป็นโรคที่เกิดจากการติดเชื้อพยาธิเส้นด้าย *Strongyloides stercoralis* การติดเชื้อจะส่งผลร้ายถึงแก่ชีวิต หาก เกิดการติดเชื้อในผู้ป่วยที่มีความบกพร่องของระบบภูมิคุ้มกัน เช่น ผู้ป่วยผ่าตัดเปลี่ยนอวัยวะ ผู้ป่วยโรคแพ้ภูมิตัวเอง ผู้ป่วยที่ติดเชื้อไวรัส HIV หรือ HPV เป็นต้น ผู้ป่วยโรคพยาธิเส้นด้ายจะมีระดับของไซโตไคน์ที่เกี่ยวข้องกับการตอบสนองทางระบบภูมิคุ้มกันแบบ Th2 และ Treg สูง ไซ โตไคน์ดังกล่าวได้แก่ IL-4 IL-5 IL-9 IL-10 IL-13 และ TGF-β ภายหลังการรักษาโรคพยาธิเส้นด้ายด้วยยาพบว่าไซโตไคน์ดังกล่าวมีระดับที่ลดลง เท่ากับคนปกติ การที่ปรสิตสามารถดำรงชีวิตอยู่ในโฮสต์โดยไม่ถูกระบบภูมิคุ้มกันกำจัดออกได้นั้นเพราะปรสิตมีกลไกในการกด หลบหนี จาก การทำงานของระบบภูมิคุ้มกันของโฮสต์ รูปแบบการกดการทำงานของระบบภูมิคุ้มกันโดยปรสิตนั้น มีหลากหลายรูปแบบเช่น ลดการแสดงออก ของ co-stimulatory molecules ของ antigen presenting cells (APCs) กระตุ้นการตายของ APCs หรือ กระตุ้นการทำงานของระบบ ภูมิคุ้มกันแบบ Th2 หรือ Treg เป็นต้น Dendritic cells (DCs) คือเซลล์สำคัญที่ทำหน้าที่เชื่อมต่อการทำงานของระบบภูมิคุ้มกันโดยกำเนิด และระบบภูมิคุ้มกันแบบจำเพาะ DCs มีรูปแบบการทำงานโดยเขมือบสิ่งแปลกปลอม และนำ antigen เสนอให้กับ T cell โดยหลั่ง cytokines ที่เหมาะสมเพื่อให้เกิดการตอบสนองทางระบบภูมิคุ้มกันต่อไป อย่างไรก็ตามกลไกในการกดการทำงานของระบบภูมิคุ้มกันโดยพยาธิเส้นด้ายยัง ไม่ได้รับการศึกษามากนัก ผู้วิจัยจึงมุ่งเน้นในการศึกษากลไกของการกดการทำงานของระบบภูมิคุ้มกัน ในขั้นตอนการนำเสนอ antigen จากพยาธิ เส้นด้ายของ DCs ผู้วิจัยได้ทำการทดสอบ DCs ด้วย crude antigen (CA) จากพยาธิเส้นด้าย จากนั้นทำการวัดระดับการแสดงออกของยีน MHC-II CD40 CD80 TLR-2 TLR-4 IL-6 IL-10 TNF-α และ TGF-β ผลการศึกษาพบว่าระดับการแสดงออกของ MHC-II, CD40, CD80, TLR-2, TLR-4 ภายหลังการทำการทดสอบด้วย CA แล้วพบว่า การแสดงออกของยีนดังกล่าวไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติกับ negative control ในขณะที่รพดับการแสดงออกของ IL-6 ไม่เปลี่ยนแปลง ระดับการแสดงออกของ TNF-α เพิ่มขึ้นอย่างมีนัยสำคัญหลังการ ทดสอบ 1 ชั่วโมงด้วย CA นอกจากนี้ ยังพบระดับการแสดงออกของ IL-10 และ TGF-β ที่เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติอีกด้วย ผลการวัด ระดับการผลิต cytokines ด้วยเทคนิค Luminex multiplex assays พบว่า ระดับของ IL-10 และ TGF-β ในของเหลวเหนือ DC2.4 ที่ทดสอบ กับ CA และ แอนติเจนที่เป็นสารคัดหลั่งจากพยาธิเส้นด้าย มีระดับที่เพิ่มสูงขึ้นอย่างมีนัยสำคัญทางสถิติ น่าเสียดายที่ cofactor independent phosphoglycerate mutase (iPGM) ไม่สามารถเหนี่ยวนำการผลิต IL-10 และ TGF-β จาก DC2.4 ได้เช่นเดียวกับ CA และ แอนติเจนที่เป็น สารคัดหลั่งจากพยาธิเส้นด้าย นอกจากนี้ iPGM ไม่สามารถเหนี่ยวนำการผลิต TNF-α และ IL-6 จาก DC2.4 ได้เช่นกัน จากผลการศึกษาแสดง ให้เห็นว่า พยาธิเส้นด้ายสามารถเปลี่ยนแปลงการตอบสนองทางระบบภูมิคุ้มกัน โดยการเหนี่ยวนำให้ DC2.4 เกิดการผลิต cytokine ที่เกี่ยวข้อง กับการตอบสนองแบบ Treg ได้แก่ IL-10 และ TGF-β และ แอนติเจนที่มีบทบาทสำคัญในกลไกดังกล่าว เป็นแอนติเจนที่เป็นสารคัดหลั่งจาก พยาธิเส้นด้าย iPGM สามารถยับยั้งตอบสนองการอักเสบ แต่ไม่ได้กระตุ้นการกดการทำงานของระบบภูมิคุ้มกัน การกดการตอบสนองของระบบ ภูมิคุ้มกันในโฮสต์มีความซับซ้อนและมีความสัมพันธ์กับแอนติเจนจากปรสิต อย่างไรก็ตามพยาธิเส้นด้ายอาจมีกลไกในการกดการทำงานทาง ระบบภูมิคุ้มกันมากกว่านี้ การ co-culturing ระหว่างเซลล์เดนไดรติกและทีเซลล์ จะให้ข้อมูลเกี่ยวกับเปลี่ยนแปลงการตอบสนองทางระบบ ภูมิคุ้มกัน และนำไปสู่การพัฒนาการรักษาโรคพยาธิเส้นด้ายต่อไป

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Vittawin Sutaveesup : Effects of *Strongyloides stercoralis* antigens on immune responses in dendritic cells. Advisor: Asst. Prof. VIVORNPUN SANPRASERT, Ph.D. Co-advisor: Prof. SURANG NUCHPRAYOON, M.D.,MPH, Ph.D, PIMPAYAO SODSAI, Ph.D.

Strongyloidiasis is a parasitic disease caused by *Strongyloides stercoralis*. Infection in the immunocompromised hosts such as organ transplants, SLE patients, or HIV and HIPV infected patients can cause severe strongyloidiasis. Infected patients have high levels of Th2, and Treg related cytokines, including IL-4, IL-5, IL-9, IL-10, IL-13, and TGF-β. These cytokines are then decreased to normal levels after the treatments. Parasites have several mechanisms to suppress or evade the host's immune response, such as suppressing the expression of co-stimulatory molecules on antigen-presenting cells (APCs), stimulating apoptosis of APCs, or stimulating Th2 and Treg responses. Dendritic cells (DCs) play a crucial role in linking the innate and adaptive immune systems against pathogens by engulfing antigens and presenting to naïve T cells with appropriate cytokines. To understand the mechanisms of *S. stercoralis* to evade the host's immune responses, we studied the alteration of antigen presentation process and cytokines productions by DCs stimulated with *S. stercoralis* antigens. We treated DC2.4 cells with *S. stercoralis* L3s crude antigen (CA) and measured the expressions of genes related with antigen presentation and cytokine productions by quantitative real-time PCR; including MHC-II, CD40, CD80, TLR-2, TLR-4, IL-6, IL-10, TNF-α, and TGF-β. The results showed that the expression levels of MHC-II, CD40, CD80, TLR-2, TLR-4 after the stimulation by CA were not significantly different from negative controls. While the expression levels of IL-6 gene were not changed, TNF-α expression levels were significantly increased at 1 hr after CA treatment. Interestingly, the significant up-regulation of IL-10 and TGF-β expression of were detected in DC2.4 treated with CA. Luminex multiplex assays for detection of cytokine productions also showed significantly increased levels of IL-10 and TGF-β in the supernatant collected from DC2.4 treated with CA and excretory-secretory antigens (ES). Unfortunately, cofactor independent phosphoglycerate mutase (iPGM) antigen could not induced the IL-10 and TGF-β production from DC2.4. Like CA and ES antigens, iPGM could not induced the TNF-α and IL-6 production from DC2.4. Our results suggested that *S. stercoralis* modulates the host's immune responses through induction of Treg response-related cytokines from DCs, including IL-10 and TGF-β. The antigens that play the crucial roles in regulation mechanism is excretory secretory products of *S. stercoralis.* The iPGM antigen might involve in the suppression of inflammatory responses, but not involved in the regulatory responses. The immunomodulation of the immune responses in the hosts should be complex and related to several antigens from the parasites. Nevertheless, *S. stercoralis* may have more than one evasion mechanism. Co-culturing between DCs and T cells will give more information about the immunomodulation of *S. stercoralis* and lead to the development of novel medical treatment for strongyloidiasis.

Field of Study:	Medical Sciences	Student's Signature ....
Academic Year:	2019	Advisor's Signature ....
		Co-advisor's Signature ....
		Co-advisor's Signature ....

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CHAPTER I

INTRODUCTION

1. Background and Rationale

Strongyloidiasis is the parasitic disease caused by *Strongyloides stercoralis*. The number of patients is about 30-110 million of the population around the world, especially in a tropical and temperate country (1). Skin rash, skin inflammation, vomiting, and diarrhea are common symptoms of this disease. Some immunocompromised patients, such as organ transplants, SLE patients, or HIV HIPV infected patients, can cause severe strongyloidiasis. Dissemination, the larvae penetrate all over the patient's body, can cause wound in the GI tract, infection in the bloodstream, and several organ failures. About 90% of patients with severe strongyloidiasis are dead (2, 3).

Parasitism relationship can drive parasite-host coevolution. Hosts have immune system to eliminate pathogens and parasites. However, parasites develop their physiology to evade and regulate host's immune system in many ways, such as stimulate anti-inflammation response, inhibit APC function-associated gene. Thus, host immune system cannot expel the parasite of the body (3).

Excretory-secretory products (ESP) of some parasites can regulate host's immune system. *Brugia malayi* can evade the binding with APCs by inhibiting the expression of toll-like receptor (TLR) through produced ESPs. Moreover, *B. malayi* can stimulate programmed cell death (apoptosis) of dendritic cells (DCs) (4). *Ascaris suum* can to inhibit the adaptive immune response by inhibiting the production of T-lymphocyte, reduce production and secretion of inflammatory cytokines (5). Understanding of the host's immune regulation by parasites will be useful to improve the efficiency of parasitic treatment.

S. stercoralis can produce many ESPs. Strongylastacin, the metalloproteinase enzyme, has high expression levels in iL3 of S. stercoralis. This enzyme is used for tissue penetration of iL3 (6). Cofactor independent phosphoglycerate mutase (iPGM) used in glycolysis pathway (7). Thus, many leucocytes including dendritic cells (DCs) will react with these enzymes and present antigens to naïve T cells, and then stimulate the appropriate immune responses.

In this study, we treated in vitro murine DCs with crude antigens and recombinant strongylastacin and/or recombinant iPGM, and then measured expression levels of APC function-associated genes, and naïve T cell stimulation cytokines including IL-2, IL-4, IL-6, IL-10, tumor-necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), interferon-γ (IFN-γ), co-stimulatory molecules including major histocompatibility complex class II (MHC-II), cluster of differentiation 40 (CD40), CD80, as well as pattern recognition receptors including toll-like receptor-2 (TLR-2), and TLR-4 by qPCR method. Moreover, the levels of cytokines were measured by the Luminex multiplex assay to investigate mechanisms involved in the immune responses against S. stercoralis by dendritic cells.

2. Research Questions

1) Which mechanisms are involved in the immune responses against S. stercoralis by dendritic cells?

2) Which genes in dendritic cells are expressed during immune responses against S. stercoralis antigens?

3) Which cytokines are produced from dendritic cells in response to S. stercoralis antigens?

3. Hypothesis

The expression levels of anti-inflammatory cytokine genes, including IL-10, and TGF-β are increased, while the expression levels of pro-inflammatory cytokine genes, (including IL-6, TNF-α), and genes related with antigen presentation (including MHC-II, CD40, CD80, TLR-2, and TLR-4) are not affected when stimulating DCs with *S. stercoralis* antigens.

4. Objectives

1) To investigate the molecular effects of immune response against *S. stercoralis* by dendritic cells
2) To investigate the expression levels of cytokine genes and genes related with antigen presentation in dendritic cells during immune responses against *S. stercoralis* antigens
3) To investigate the cytokine production from dendritic cells in response to *S. stercoralis* antigens

5. Keyword

Strongyloides stercoralis
Strongylastacin
Independent phosphoglycerate mutase
Crude antigen
Dendritic cell
Quantitative real-time PCR

6. Benefits of the study

1) This study will be the first information on cytokines profile of DCs against S. *stercoralis* antigens.
2) Understanding of immunomodulation mechanisms by *S. stercoralis* antigens will be useful in development on strongyloidiasis treatment

CHAPTER II

LITERATURE REVIEW

1. **Strongyloidiasis**

Strongyloidiasis is one of the Neglected Tropical Diseases (NTDs) caused by soil-transmitted helminths named *"Strongyloides stercoralis"* Some immunocompetent patients with strongyloidiasis have symptoms in gastrointestinal and respiratory tract such as coughing, abdominal pain, vomiting, diarrhea or have not any sign. However, immunocompromised patients with strongyloidiasis can be severe and cause of death.(3)

About 30-100 million of the human population are infected, especially in a tropical country such as Africa, South America, and Southeast Asia. However, in a temperate and cold country such as England and Canada. They have a report on strongyloidiasis patients, which cause by traveling too (8). (Figure 1)
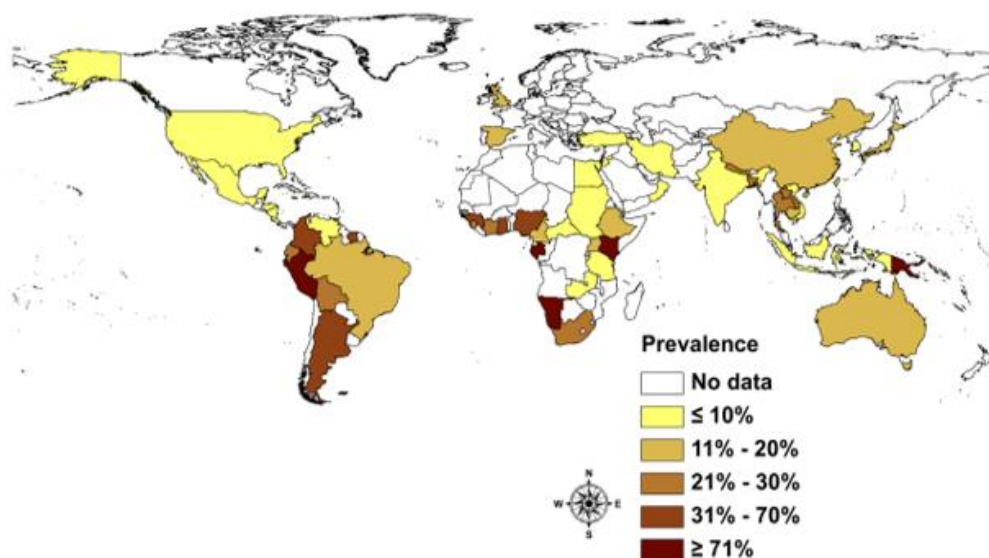
Figure 1 Geographic distribution of *S. stercoralis* infection

Source: NCBI, 2009

2. *S. stercoralis* life cycle

Human is the definitive host of *S. stercoralis*. The life cycle of *S. stercoralis* is complex and separated into the direct life cycle and the indirect life cycle.

For direct life cycle, infective third-stage larvae (iL3) on soil penetrate through human skin and get into the blood circulation system, lung, and finally habitat in gastrointestinal tract. When iL3 gets into human small bowel, the larvae molt and fully develop into the adult stage called "Parasitic female". The adult parasite can produce a large number of dispensable fertilization offspring (parthenogenesis). Their offspring will be hatching from eggs before going outside from adult parasite (ovoviviparity) and contaminate in the host's feces. This stage of larva calls rhabditiform larva (first stage larva; L1). L1 in feces develops into iL3, which can infect another human host. However, in some situations, such as lacking the human host, *S. stercoralis* can grow in the indirect lifecycle, L1 develops into a free-living adult which live in the soil (3).

Moreover, L1 can develop into iL3 without getting off the host body. L1 penetrates from the intestine's wall to the lung, gastrointestinal, and fully develops into the adult stage (autoinfection). Autoinfection with *S. stercoralis* cause hyperinfection followed by chronic infection. Therefore, hyperinfection patients have a high risk of death from the penetration of *S. stercoralis* larvae through a vital organ such as liver, thyroid, lung, urinary tract, or CNS (disseminated strongyloidiasis) (Figure 2). Many studies suggested that autoinfection is correlated with an immune deficiency in the host, especially in immunocompromised or the patient who take immune suppression drugs, HTLV-1, and HIV infection (1).
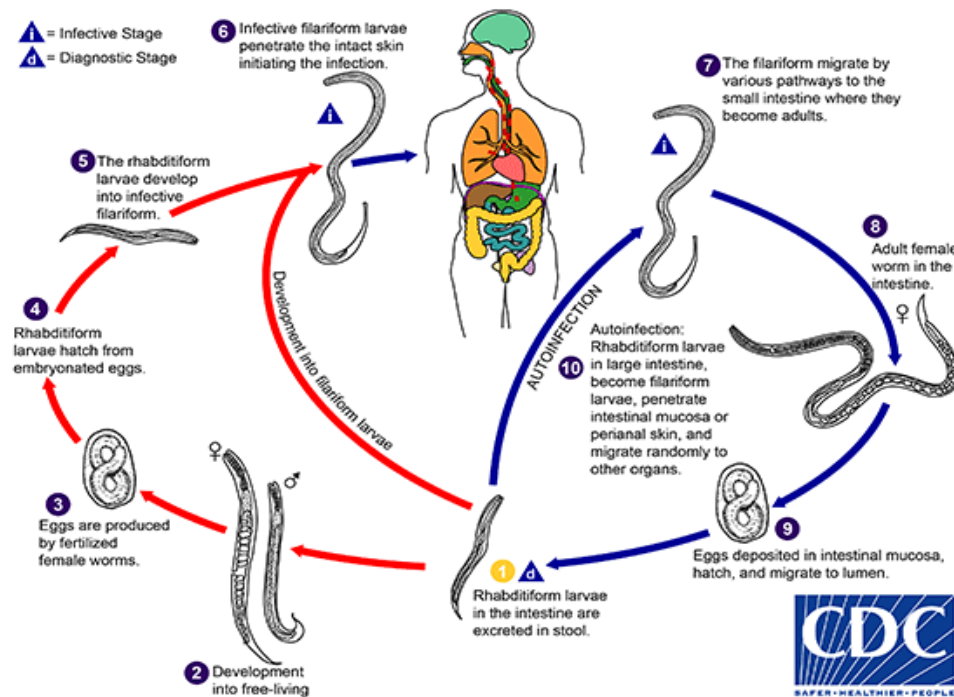
Figure 2 Life cycle of *S. stercoralis.*
Source: CDC, 2009

3. Immune responses in patients with strongyloidiasis

Infection with *S. stercoralis* stimulates the host's immune responses to kill the larvae and expel the adult parasite by activating both innate and adaptive immune response (9).

In the innate immune responses, neutrophils can kill the larvae by secreting myeloperoxidase through activation of interleukin-17 (IL-17) and by parasite's antigens. Then, activated neutrophils secrete macrophage inflammatory protein 2 (MIP-2) to kill the larvae (10). Eosinophils also have a potential role in killing many parasitic helminths through granulation. Major basic protein (MBP) and eosinophil cationic protein (ECP) can affect only penetrated larvae (L3$^+$) but not in iL3. The mechanism of eosinophil to kill L3$^+$ is the activation of interleukin-5 (IL-5) from Th2 cell then, eosinophils move toward and kill the larvae by inducing of C3 protein on larvae's skin.

Moreover, antigen-presenting cells (APCs) play a crucial role in the adaptive immune response by presenting antigen to naïve T-cells. Naïve T-cells differentiate into many subtypes depend on cytokines secreted by APCs. Finally, the mature helper T cells stimulate B cells to secrete antibodies resulting in expelling and killing the parasites.

Eosinophils can act as antigen presenting cell to stimulate parasite-specific Th2 response. Patients have high levels of IL-4, IL-5, specific immunoglobulin G (IgG), and immunoglobulin M (IgM). These cytokines and immunoglobulins have a role in killing parasitic worms, especially in soil-transmitted helminths. Therefore, patients with parasitic helminths usually have eosinophilia and high levels of these cytokines.

Although the host's immune system has the potential to kill the parasites, parasites can survive in the human host for a long time. *Strongyloides* spp. has many mechanisms to modulate the host's immune system. *S. ratti* can stimulate mouse hosts to produce Foxp3+ regulatory T cells (T-reg). As a result, they decrease inflammatory cytokines such as IL-9, which play a critical role in killing the larvae by stimulating mast cell in the mucosa. Moreover, a high level of IL-9 can inhibit the biding of the BTLA-HVEM regulatory receptor on $CD4^+$ T lymphocyte. Thus, *S. ratti* larvae can evade from mast cells and inhibit T lymphocyte (11, 12).

S. venezuesis decreases the severity of Th1 response through stimulating Th2 polarization as a result of increasing in anti-inflammatory cytokines such as IL-4 and IL-5 in the hosts. After the inhibition of Th2 response in mouse, the larvae are quickly expelled. These results showed that stimulation of Th2 responses is the important mechanism for *Strongyloides* spp. to survive in the hosts (4).

Previous studies suggested that cytokine levels in patients infected with *S. stercoralis* are different between pre-treatment and post-treatment. In the pre-treatment group, the levels of proinflammatory cytokines, including IFN-γ, TNF-α, and IL-1, are significantly decreased. In contrast, the levels of anti-inflammatory cytokines, including IL-4, IL-5, IL-9, IL-10, IL-13, and TNF-α are significantly increased. In the post-treatment group, the levels of proinflammatory cytokines, including IFN-γ, TNF-α, IL-

1, IL-2, and IL-17, are significantly increased while anti-inflammatory cytokines including IL-4, IL-5, IL-9, IL-10, IL-13, and TGF-β are significantly decreased (13).

Dendritic cells (DCs) are the specialized APCs that play a crucial role in the stimulation of naïve T cells. DCs are ordinary found on human skin. Thus, DCs directly contact with the larvae of *S. stercoralis*, which infect human host by skin penetration, and then produce appropriate cytokines together with co-stimulatory molecules to stimulate naïve T cells. However, the study of the relationship between *S. stercoralis.* and DCs is limited (14).

4. Dendritic cell

DCs are first described in the 1970s by Ralph Steinman. DCs distribute around many organs such as lymph nodes, skin, respiratory tract, and gastrointestinal tract. Hematopoietic progenitor cells in the bone marrow generate immature DCs. In this stage, DCs able to endocytosis but cannot stimulate naïve T cell (14).

The antigen-binding molecules on DCs surface named toll-like receptor (TLRs) can bind with a wide range of antigen types. When TLRs bind with the antigens, DCs engulf the antigens into the cytoplasm and digest. Then, immature DCs develop to mature DCs, which stimulate naïve T cells by combining antigens and presenting molecules such as major histocompatibility complex (MHC) (Figure 3).
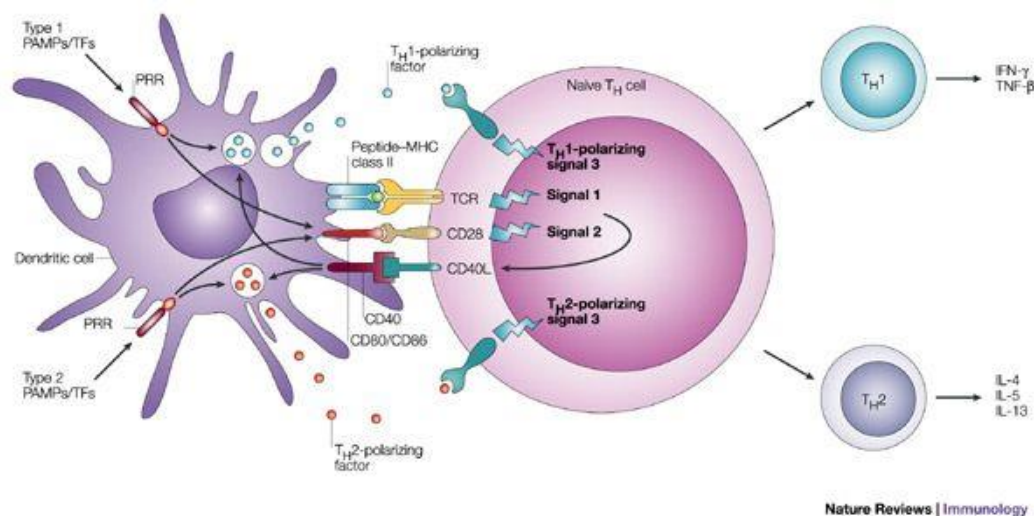
Figure 3 Dendritic cell stimulate naïve t cell
Source: Martien L. Kapsenberg, 2003

Mature DCs present combined antigen, some co-stimulatory molecules such as CD40, CD80, CD86. They secrete cytokines to communicate with naïve T cells. Then, naïve T cells differentiate into many subtypes of helper T cells. Helper T cells move toward and stimulate B cell resulting in a proliferation of B cells. Finally, B cells differentiate to plasma cells and secrete antibodies into the bloodstream (15).

Recently, another subtype of DCs called semi-mature DCs is discovered. Semi-mature DCs have immune tolerance characteristics. The role of semi-mature DCs is regulation of the excessive immune response through stimulating regulatory T-cell (Treg) differentiation. The appropriate response of DCs is the key to prevent the human from infections, autoimmune diseases, and allergic responses.

Although DCs play a crucial role against many diseases, including parasitic infections, some parasites can modulate DCs in many ways for their survival.

5. **DCs modulation by protozoan parasites**

5.1 *Plasmodium spp.*

Plasmodium spp. can produce many proteins to modulate DCs; such as p38 mitogen-activated protein (MAP) kinase, hemozoin. These proteins can inhibit generation and proliferation of DCs, as well as can decrease MHC-II, CD40, CD54, CD80, and CD84 expression on DCs. Moreover, *Plasmodium* spp. can stimulate secretion of anti-inflammatory cytokines, including IL-4, IL-10, and IL-12, resulting in stimulating Th2 responses. Therefore, the parasites successfully survive in the hosts and cause the diseases (16).

5.2 *Leishmania spp.*

Leishmania donovani inhibits maturation of DCs through regulating the expression of maturation gene, decreasing proinflammatory cytokines (IL-6 and IL-12), decreasing co-stimulatory molecules CD40, CD83. In contrast, it stimulates expression of an anti-inflammatory cytokines, IL-10, through a high level of phosphoglycan protein produced from *L. donovani* (17).

The effects of parasitic protozoa on DCs were summarized in Table 1.

Table 1 The effects of parasitic protozoa on DCs
(Edited from C´esar A et al., 2010 (17)

Parasites	Products	Effects on DCs
P. berghei	Infection	Regulate T cell priming
P. yoelii	Infection	Regulate T cell priming Shorter DC/T cell interaction
	Soluble factor	Stimulate Treg Inhibit IL-12

P. vivax	Infection	Stimulate Treg
P. chabaudi	Infection	Stimulate Th2 An increasing number of CD8-DC
P. falciparum	Merozoite	Inhibit IL12, IFN-γ Stimulate ERK, IL-10
	Hemozoin	Inhibit Immature differentiation Inhibit Immature migration
	Soluble extract	Inhibit IL-12
L. major	Promastigote	Inhibit IL-10
	LPGs, PGs	Inhibit IL-12, IFN-γ Inhibit motility migration Stimulate IL-4, IL-10
	ES	Inhibit IL-10, IL-12
L. amazonensis	Promastigote	Stimulate ERK Inhibit IL-12
L. mexicana	Promastigote	Inhibit IL-12
L. donovani	Promastigote	Inhibit CD11, CD51, CD86, IL-12
	ES	Inhibit IL-10, IL-12
Trypanosoma cruzi	Infection and soluble factor	Inhibit T cell priming Inhibit IL-12, TNF-α Decreasing of DC number

	Trypomastigote	Stimulate TGF-β, IL-10
	GIPLs	Inhibit IL-10, IL-12, TNF-α
Toxoplasma gondii	Tachyzoites	Stimulate motility migration Inhibit T cell priming
	Soluble factor	Stimulate DC attraction
	LXA4	Stimulate SOC2 Inhibit IL-12
Giardia lamblia	Parasite extract	Stimulate CD40, CD80, CD86 Stimulate IL-12, TNF-α Inhibit IL-10

6. DCs modulation by parasitic helminths

6.1 Trematode

Excretory-secretory products (ESPs) from a blood fluke, *Schistosoma mansoni,* stimulate Th2 and Treg responses in mice. ESP of *S. mansoni* assembles from many substances such as soluble egg antigen (SEA), which can stimulate Jagged 2, and phosphatidylserine lipid. These substances can bind with TLR2 and stimulate Th2 and Treg response. Inhibition of TLR2 in murine DCs, resulting in inhibition of Treg response, while Th2 response still occurs (18).

6.2 Cestode

Echinococcus granulosus stimulates Th2 response and downregulates the expression of MHC-II to decrease the antigen presentation rate. Similarly, *Taenia crassiceps* can stimulate Th2 response by parasite's glycoprotein (19).

6.3 Nematode

Antigens of a filarial worm, *Brugia malayi*, inhibit expression of IL-12, TLR3, TLR4 and stimulate apoptosis of human DCs but not effect in macrophages. ESP of *Nippostrongylus brasiliensis* stimulate Th2 response in mice through stimulation of CD86 and OX40L. *Ascaris suum* secretes many substances to regulate the expression of inflammatory cytokines, CD40, and CD86 (17, 20).

The effects of parasitic nematodes on DCs show are summarized in Table 2

Table 2 The effects of parasitic helminths on DCs
(edited from C´esar A et al., 2010) (17)

Parasites	Products	Effects on DCs
B. malayi	Alive microfilariae	Inhibit TLR-4, TLR-3 Inhibit IL-8 Stimulate TNF-**α**, IL-1 Stimulate SOCS1, SOCS3 Stimulate DC apoptosis
	Microfilarial Ag	Stimulate CD40, CD80, MHC-I
N. braziliensis	NES	Stimulate OX40L Stimulate CD86 Stimulate Th2
A. suum	PI	Inhibit IL-12
	PCF	Inhibit CD40, CD86
	PC	Inhibit TNF-**α**
A. vitae	ES-62	Simulate TLR4, CD80 Stimulate Th2
Heligmosomoides polygyrus	ES	Stimulate IL-4, IL-10 Stimulate Treg

In conclusion, both protozoan and helminthic parasites regulate DCs functions in many ways, such as inhibition of Th1 response, stimulation of Th2/Treg response, inhibition of antigen-receptor binding, and decreasing of DCs population. Understanding of immune evasion of *S. stercoralis* will be useful to develop the treatment of strongyloidiasis.

7. Roles of secreted cytokines, and co-stimulatory molecules

7.1 Tumor necrosis factor (TNF-α) and interferon γ (IFN-γ)

Patients with inflammatory diseases such as Crohn's disease, rheumatoid arthritis, and uveitis have a high level of TNF-α. Inhibition of TNF-α can enhance IFN production, which plays a crucial role in viral infection and autoimmune disease. However, a recent study showed the anti-inflammatory effects of TNF-α. The stimulation of APC with TGF-β shows an increasing level of TNF-α, while the production of IL-12 is decreased, resulting in interruption of Th1 response. Thus, the duration of inflammatory responses depends on TNF-α. Besides pro-inflammation activity, TNF-α has some regulatory activities (21, 22).

Another pro-inflammatory cytokine IFN-γ, induces the expansion of CD8^{+} T cells, which cause direct tissue damage and promoted the production of IgG in mice. Moreover, stimulation of DCs with viruses and SLE immune complexes containing nucleic acids that bind TLRs results in secretion of both IL-6 and IFN. Thus, a combination of IFN and IL-6 can cause a destructive immune environment to eliminate viral infected cells or cells in SLE patients (23).

7.2 Interleukin-6 (IL-6) and transforming growth factor-beta (TGF-β)

Varies types of immune cells, such as both T and B lymphocyte, fibroblast, monocyte, tumor cell, and DCs, can secrete IL-6. This cytokine stimulates the proliferation and differentiation of both B cell and T cell. A combination of TGF-β and IL-6 stimulates Th17 differentiation, which has a pro-inflammatory function. However, IL-6 and TGF-β have another different role. TGF-β stimulates the expression of Foxp3, resulting in Treg formation, while IL-6 inhibits expression of Foxp3. Patients with autoimmune and inflammatory disease also show a high level of IL-6 in serum.

As a regulatory cytokine, TGF-β plays a crucial role in many functions of the immune cell, especially in regulatory T cell differentiation and induction of immunological tolerance subtype of DCs. Infection with some helminthic parasites can cause a high level of TGF-β and IL-10. Moreover, mice in which T cells express negative TGF-β are lost helminthic infection properties. Interestingly, some helminthic parasites produce TGF-β homolog or mimicry molecules, resulting in immune evasion of the parasite (21, 22, 24, 25).

7.3 Interleukin-10 (IL-10)

IL-10 is the important regulatory cytokine that tolerogenic semi-mature DCs secrete to terminate the excessive T cell response to prevent tissue damage and chronic inflammation. Initially, IL-10 identified as an inhibitor of IFN-γ. In the skin, Il-10 prevents the hypertensive of an immune response to antigen. The regulation mechanism of IL-10 is inhibition of DCs maturing and reduce the production of pro-inflammatory cytokines, including IL-2, IL-12, IFN-γ, TNF, IL-1, and IL-6, while immune suppression gene is stimulated. Thus, IL-10 is the essential cytokines that play a critical role in inflammatory suppression and promote regulatory T cell production to maintain immune homeostasis.

Moreover, many studies suggest that infection with some parasites can stimulate the production of IL-10 and TGF-β. Thus, immune suppression is one of the invasion mechanisms of the parasite to survive from an aggressive host's immune response (21, 22, 26).

7.4 Cluster of differentiation 80 and 40 (CD80, CD40)

The differentiation of Th1 and Th2 requires appropriate cytokines and co-stimulatory molecules CD80/CD86. Thus, the absence of CD80/CD86 naïve T cells can affect only differentiation but not affect cell proliferation.

Among many types of costimulatory molecules, CD40 enhances the function of DCs make DCs more effective in the role in APC, such as up-regulate MHC-II and CD80/CD86. However, CD40 also has a regulatory function on both B cell and T cell. CD40-CD40L interaction between DCs and T cells provides regulation on both DCs and T cells. The expression of CD40 is essential for regulatory function. Anti-CD40 or CD40L can stimulate Th1 polarizing cytokine IL-12 in both human and murine DCs. However, DCs do not show a high expression level of DCs when an encounter with some parasitic pathogen, including *Plasmodium falciparum* and *Typanosoma cruzi in vitro* (27).

8. Strongylastacin

iL3 of *S. stercoralis* penetrate host skin with 10 cm/hr velocity. Skin penetration of *S. stercoralis* requires histolytic proteinase enzymes from iL3. The previous proteomic analysis about secreted ESP component shows many types of protein (Table 3)(28), including zinc metalloproteinase named strongylastacin (accession no. 17865392). Interestingly, this enzyme plays a crucial role in the digestion of the extracellular compartment. Inhibition of expression of strongylastacin can inhibit the migration rate of *S. stercoralis* up to 98% (29, 30).

The bioinformatic study gives detail about the structure of strongylastacin, 45 kDa, 1,134 base pairs. Moreover, strongylastacin has four conservative domains, including signal domain, peptidase domain, EGF domain-containing Ca^{2+} binding site, and CUB domain. These domains refer to the properties of strongylastacin, including secreted protein, proteinase, Ca^{2+} enzyme cofactor, and ligand of the cell's receptor, respectively (Figure 4). Moreover, strongylastacin can stimulate the secretion of IgE and histamine. Thus, an allergic response occurs in some patients (31).

Strongylastacin is studied in many views but not in immunomodulation roles. So, this study aims to study the immunomodulation roles of both strongylastacin compared to *S. stercoralis* crude antigen.
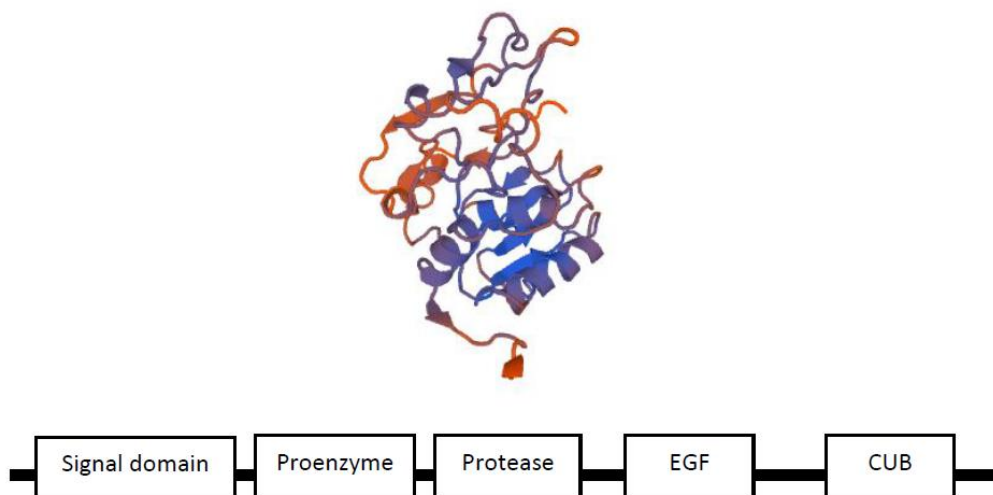

Figure 4 3D structure of strongylastacin *(ExPASy)*

Table 3 Proteomic analysis of *S. stercoralis* iL3
(modified from Macilla, et al. 2010)

Type of protein	NCBI ID number
Myosin heavy chain	157748701
Myosin N	157759896
Actin	14278147
Paramyosin	547976
Tropomyosin	1208409
Elongation factor EF-1 alpha	119152
Histone 2a	194039792
Histone 4	170588045
Adenine Nucleotide Translocator	17542128
Phosphoenolpyruvate carboxykinase (PEPCK)	130763
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	6016070
Succinate dehydrogenase	2282574
Cytrate synthase	17555174
Arginine kinase	31247902
ATP synthase	170591793
ATP: guanido phosphotransferase	32566409
Enolase	47211348
Propionyl Coenzyme A carboxylase alpha (pcca-1)	17567343
Vacuolar H ATPase family member (vha-13)	17565854
Mitochondrial carrier protein	133901794
Guanine nucleotide-binding protein	232136
Galectin	1935060
14-3-3 like protein 1	72179591
Hsp70	9438176
Elongation factor EF-1-TPA	199600272
IgG & IgE immunoreactive antigen	2290388
Metalloproteinase precursor (strongylastacin)	17865392

9. **Cofactor independent phosphoglycerate mutase (iPGM)**

Cofactor independent phosphoglycerate mutase (iPGM) is a catalytic enzyme in the glycolysis pathway. IPGM express in various plants and invertebrates especially in nematodes such as *Caenorhabditis elegans*, *Leishmania Mexicana*, *Trypanosoma brucei*, *Brugia malayi*, *Wuchereria bancrofti*, *Dirofilaria immitis*, *Onchocerca volvulus*, *Loa loa,* and *S. stercoralis.* Inhibition of IPGM gene in *C. elegans* results in larvae lethality and abnormality morphology.

IPGM of *S. stercoralis* is now described as an enzyme composed of 500 amino acids. The molecular size of IPGM is about 60kDa. IPGM of *S. stercoralis* similar to IPGM of *C. elegans*. Immunization of Bm-IPGM induces mixed Th1/Th2 response in BALB/c *Mastomys coucha*. Moreover, anti-Bm-IPGM can kill the infective larvae and microfilarial of *B. malayi* through the ADCC mechanism (37-39). Therefore, understanding the immunomodulation of host's immune response by IPGM might be useful for developing the treatment of strongyloidiasis.

CHAPTER III

MATERIAL AND METHOD

1. Isolation of infective larva stage 3 (iL3) of *S. stercoralis.*

iL3 larvae of *S. stercoralis* were obtained by a modified agar plate culture technique. The stool samples of infected gerbils were placed on the center of agar plates. The agar plates were incubated at 25 °C for 5-10 days. iL3 larvae on agar plates were observed under the microscope to confirm the stage of growth. iL3 larvae on plates were separated by washing with buffered saline solution (BU saline) (22mM of KH_2PO_4, 50mM of Na_2HPO_4, 70mM of NaCl). Then, iL3 were decontaminated by low melting agarose technique. Briefly, one volume of 3% low melting agarose in BU saline was mixed with 2 volumes of iL3. The parasite-agarose solution was poured on clean petri dish plates and placed at room temperature until they were completely solidified. Then, BU saline was added to the plates on solidified agar. The active-iL3 larvae migrated from the agar to the solution. The active parasites in solution were collected and transferred to a 15-ml conical centrifuge tube. Then, iL3 in solution was centrifuged at 2,000 RPM for 10 minutes and washed with BU saline. The pellet of the larvae was stored at -80 °C until use and some of pellet were preserved in TRIzol[®] reagent to preserve RNA. (32)

2. Preparation of *S. stercoralis* crude antigen.

The larvae pellet was washed with phosphate buffer saline (PBS) containing protease inhibitor cocktails (Sigma-Aldrich, MO, USA). The larvae were homogenized by sonication technique at 10-second pulse, 10-second pause for 10 minutes. The homogenate was centrifuged at 12,000 RPM for 10 minutes. The supernatant was collected and filtered through a 0.22 **μ**m non-binding protein filter and use as the

crude antigen. Finally, the protein concentration was measured by bicinchoninic acid (BCA) protein assay (Thermo Scientific, MA, USA) and kept at -80 °C until use.

3. Preparation of recombinant strongylastacin

3.1 Cloning of strongylastacin from *S. stercoralis*.

RNA of *S. stercoralis* was extracted using TRIzol® reagent method. Reverse transcription was performed using ImProm-II™ Reverse Transcription System (Promega, U.S) to get cDNA of *S. stercoralis*. The coding sequence of strongylastacin gene was cloned from cDNA of *S. stercoralis* into pET100/D-TOPO expression vector (in vitro gen). The recombinant plasmid was transformed into DH5-alpha E. coli strain for maintenance and Origami *E. Coli* strain for expression. Origami greatly enhance disulfide bond formation in protein synthesis. Features of pET100/D-TOPO are including T7lac promoter for high-level, IPTG-inducible expression of the gene of interest, directional TOPO cloning site, N-terminus fusion tags for detection and purification of recombinant fusion protein, protease recognition site for cleavage of the fusion tag from the recombinant protein of interest, *lacI* gene encoding the lac depressor to reduce basal transcription from the T7 lac promoter, and from lacUV5 promoter in the *E. Coli* chromosome, ampicillin and kanamycin resistance marker for colony selection, and pBR322 origin for low-copy replication and maintenance in *E. Coli*. (figure 5)
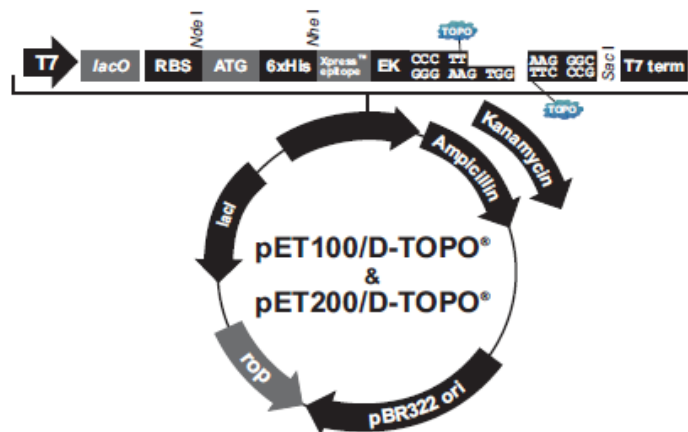
	pET100/D-TOPO®
T7 promoter	209-225
T7 promoter/priming site	209-228
lac operator (lacO)	228-252
Ribosome binding site (RBS)	282-288
Initiation ATG	297-299
Polyhistidine (6xHis) region	309-326
Xpress™ epitope	366-389
EK recognition site	375-389
TOPO® Cloning site (directional)	396-409
T7 reverse priming site	466-485
T7 transcription termination region	427-555
bla promoter	856-954
Ampicillin (*bla*) resistance gene	955-1815
Kanamycin resistance gene	---
pBR322 origin	2022-2757
ROP ORF (complementary strand)	3001-3192
lacI ORF (complementary strand)	4507-5595

Figure **5** Map and diagram features of the pET100/D- TOPO® expression vector

Source: Thermofisher, 2010

3.2 Expression of strongylastacin.

A single colony of *E. Coli* was inoculated into LB broth containing 100 ug/ml ampicillin and inoculated at 28 $^{\circ}$C with 150 rpm shaking for 16-24 hours. The overnight culture was diluted 1:100 into LB broth containing 100 ug/ml ampicillin and 1% glucose. The culture was inoculated at 28 $^{\circ}$C until an OD600 of 0.5-0.7. Then 5 ml of cells was collected to be negative control. Recombinant strongylastacin was induced to express by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration 0.8 mM into culture and incubated at 28 $^{\circ}$C with 150 rpm shaking for 24 hours. The pellet was washed with PBS buffer and proceeds to sonication step.

3.3 Extraction of strongylastacin from *E. coli* by sonication

The pellet was resuspended with 30 ml PBS buffer containing protease inhibitor cocktails for His-tagged protein purification (Sigma-Aldrich, MO, USA). Then, 3 mg of lysozyme was added and incubate on ice for 10 minutes. The cells were sonicated on ice with 10 seconds pulse, 10 seconds pause for 5 minutes sonication time (Vibra-Cell[tm] 750W). The cell lysate was centrifuged at 14,000 rpm for 30 minutes at 4 $^{\circ}$C. Supernatant was collected and immediately performed purification step.

3.4 Purification of recombinant strongylastacin by Ni-NTA agarose

The supernatant was purified by affinity chromatography Ni-NTA purification system (Qiagen) under native control. Supernatant was filtrated through a 0.45-μm membrane. The column was assembly according to the manufacturer's instruction. Then, 1 ml of 50% Ni-NTA Superflow resin slurry was pipetted into the empty column by avoiding air bubbles. After that, the column was equilibrated with 5 column volumes of binding buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 10 mM imidazole). Then, the lysate was applied into the column. The bottom outlet of the column was adjusted to collect the lysate as flow-through. Six column volumes of washing buffer1 (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 30 mM imidazole) was poured into the column, followed by 5 column volumes of washing buffer2 (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 60 mM imidazole). The recombinant protein was eluted with 6 volumes of elution buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 200 mM imidazole). Purity of recombinant strongylastacin was determined by SDS-PAGE and Western blot analysis with anti-His antibodies respectively.

3.5 Protein profile analysis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Ten percentages of resolving gel and four percentages of stacking gel were freshly prepared as follow:

10% of Resolving gel (for 2 gels)

30% Acrylamide	2,667	µl
Distilled water	3,739	µl
1.5 M Tris-HCl, pH 8.8	2,000	µl
10% SDS	80	µl
10% ammonium sulfate (APS)	40	µl
TEMED	8	µl

4% of Stacking gel (for 2 gels)

30% Acrylamide	462	µl
Distilled water	2,100	µl
0.5 M Tris-HCl, pH 6.8	882	µl
10% SDS	35	µl
10% ammonium sulfate (APS)	17.5	µl
TEMED	5	µl

When the gels had already set, the protein samples were mixed with ¼ volume of 4X reducing sample buffer (62.5mM Tris-HCl, pH 6.8; 8% w/v SDS; 40% v/v glycerol; 0.005% bromophenol blue; 10% 2-mercaptoethanol), denatured at 95°C for 5 minutes. Each protein fraction was loaded into prepared gels to separate protein. Polyacrylamide gel electrophoresis was carried out in 1X running buffer (0.25 M Tris-HCl, 1.92 M glycine, 1% w/v SDS) at 80 volts for 120 minutes.

Coomassie Brilliant Blue staining

The resolving gel area was soaked in Coomassie Brilliant Blue solution (0.25% w/v Coomassie Brilliant Blue R-250, 45% methanol, 10% glacial acetic acid) for 45 minutes with gentle shaking. After that, the staining solution was removed. The gel was destained with destaining solution (10% glacial acetic, 30% methanol) for 2 hours with gentle shaking for 2 hours. The destaining solution was changed 2-3 times until fully destained. Finally, the gel was captured with camera.

Western blot analysis

The separated protein in resolving gel was transferred from polyacrylamide gel to nitrocellulose membrane (Millipore, Darmstadt, Germany). The membrane, the thick filter paper, and the gel were presoaked in transfer buffer (20 mM Tris, 150 mM Glycine, 20% methanol) for 10 minutes. Both of wet membrane and equilibrated gel were sandwiched between sheet of thick filter papers, and then were placed on Trans-Blot SD cell (Bio-Rad, CA, USA). The proteins were transferred at 15 volts for 2 hours. The nitrocellulose membrane was blocked with 5% non-fat dry milk (Carnation) in TBST (0.2M Tris base, 1.5M NaCl, Tween 0.05% v/v) for 1 hour at room temperature. The blot was then incubated with mouse anti-His antibody (Thermo Scientific, MA, USA) diluted in blocking buffer (1:1,000) at room temperature for 2 hours with 3D gentle shaking. After washing in 3 changes of TBST for 5 minutes, the blot was incubated in secondary antibody, goat anti-mouse antibody conjugated with HRP (Thermo Scientific, MA, USA), diluted in the blocking buffer (1:5,000) for 2 hours at room temperature. Then, the blot was washes with 3 changes of TBST for 5 minutes. Finally, the blot was soaked in 1-Step™ Ultra TMB-Blotting Solution (Thermo Scientific, MA, USA) in dark at room temperature for 3 minutes. The solution was then removed, washed in distilled water, and dried overnight at room temperature.

3.6 Removal of unwanted small molecules from recombinant strongylastacin by dialysis

Dialysis is a method to remove unwanted small molecules including reducing agents and salts. The purified strongylastacin was dialyzed by using the SnakeSkin™ Dialysis Tubing (Thermo Scientific, MA, USA). The dialysis Tubing was presoaked in PBS pH 7.4. Then, it was closed one of the bottoms ends with SnakeSkin™ Dialysis Tubing clip. After that, the sample was poured in the tube and closed the opened end of another side. The dialysis tubing with the sample was soaked in 500 volumes of the dialysis buffer (PBS pH 7.4) overnight at 4 °C. The sample was collected and stored at -20 °C until use.

4. Recombinant independent phosphoglycerate mutase (iPGM) preparation.

The *E. coli* BL21 containing *ipgm* gene was kindly provided by Ms. Phinyarat Suksomphak. The recombinant iPGM was prepared together with recombinant strongylastacin.

4.1 Expression of iPGM.

A single colony of *E. Coli* was inoculated into LB broth containing 100 ug/ml ampicillin and inoculated at 28 °C with 150 rpm shaking for 16-24 hours. The overnight culture was diluted 1:100 into LB broth containing 100 ug/ml ampicillin and 1% glucose. The culture was inoculated at 28 °C until an OD600 of 0.5-0.7. Then 5 ml of cells was collected to be negative control. Recombinant strongylastacin was induced to express by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration 0.8 mM into culture and incubated at 28 °C with 150 rpm shaking for 24 hours. The pellet was washed with PBS buffer and proceeds to sonication step.

4.2 Extraction of iPGM from *E. coli* by sonication

The pellet was resuspended with 30 ml PBS buffer containing protease inhibitor cocktails for His-tagged protein purification (Sigma-Aldrich, MO, USA). Then, 3 mg of lysozyme was added and incubate on ice for 10 minutes. The cells were sonicated on ice with 10 seconds pulse, 10 seconds pause for 5 minutes sonication time (Vibra-Cell[tm] 750W). The cell lysate was centrifuged at 14,000 rpm for 30 minutes at 4 °C. Supernatant was collected and immediately performed purification step.

4.3 Purification of recombinant iPGM by Ni-NTA agarose

The supernatant was purified by affinity chromatography Ni-NTA purification system (Qiagen) under native control. Supernatant was filtrated through a 0.45-μm membrane. The column was assembly according to the manufacturer's instruction. Then, 1 ml of 50% Ni-NTA Superflow resin slurry was pipetted into the empty column by avoiding air bubbles. After that, the column was equilibrated with 5 column volumes of binding buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 10 mM imidazole). Then, the lysate was applied into the column. The bottom outlet of the column was adjusted to collect the lysate as flow-through. Six column volumes of washing buffer1 (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 60 mM imidazole) was poured into the column, followed by 5 column volumes of washing buffer2 (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 100 mM imidazole). The recombinant protein was eluted with 6 volumes of elution buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 200 mM imidazole). Purity of recombinant strongylastacin was determined by SDS-PAGE and Western blot analysis with anti-His antibodies respectively.

4.4 Protein profile analysis and dialysis

Recombinant iPGM was analyzed and dialyzed with the same condition as recombinant strongylastacin (3.5-3.6).

5. DC 2.4 cell culture and antigen stimuli

DC2.4 cells (Merk, MO, USA) were cultured in RPMI1640 with 10% fetal bovine serum, 0.0025 mM beta-mercaptoethanol, 1x essential amino acid and 1x antimycotic-antibiotic medium (Thermo Scientific, MA, USA). DC2.4 cells were thawed from freezing medium in 37°C water bath until completely thaw and then transferred to a 15-ml-conical tube. The cell suspension was centrifuged at 2,500 rpm for 3 minutes to remove the freezing medium. The cell pellet was resuspended in phosphate buffer saline (PBS) then centrifuged at 2,500 rpm for 3 minutes to remove PBS. The cell pellet was mixed with 7 ml complete medium, then transferred into a T75 cell culture flask and added 3 ml of complete medium. The cultured flask was put into 37 $^{\circ}$C 5% CO_2 incubators for cell expansion. DC2.4 cells were observed by an inverted-microscope and changed medium every 2-3 days.

When DC2.4 attached to the wall of cultured flask reaches to 80-90%, DC2.4 cells were seeded by trypsinization and transferred to 6-well culture plate for 4e5 cell/well incubate in 37 $^{\circ}$C 5% CO_2 incubator overnight. The next day, DC2.4 cells will be increased to 1e^6 cell/well.

DC2.4 were treated with 20 ug/ml of *S. stercoralis* crude antigen (CA), 5 ug/ml of LPS, and PBS for 1, 3, 6, 12, 16, 24 hours. LPS was used as positive control to confirm the typically responses of DC2.4. Cells were collected and preserved in TRIzol® reagent at -80 $^{\circ}$C for RNA extraction.

6. RNA extraction and cDNA synthesis

Total RNA of treated DC2.4 cells was obtained by TRIzol® reagent RNA extraction method. First added 0.2 ml of chloroform left for 3 minutes and centrifuged at 12,000 g for 15 minutes and pipetted the aqueous phase for RNA extraction.

Transferred aqueous was adding 0.5 ml of isopropanol incubated for 10 minutes and centrifuged at 12,000 g for 10 minutes. Then, removed the supernatant and washed RNA pellet with 1 ml of 75% ethanol and centrifuged at 7,500 g for 5

minutes, removed ethanol. RNA pellet was dried in laminar flow for 10 minutes, finally, resuspended RNA pellet in 20 μl ultrapure water. The quality and quantity of extracted RNA were determined by RNA agarose gel electrophoresis method and nanodrop spectrophotometer.

To synthesize complementary DNA (cDNA), The extracted RNA was converted by SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Scientific, MA, USA). The chemicals were mixed and placed in thermal cycler start with 25 $^{\circ}$C for 10 minutes, 42 $^{\circ}$C for 60 minutes, and 85 C$^{\circ}$ for 5 minutes, respectively. Synthesized cDNA was determined by polymerase chain reaction using the control primer of housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

7. Quantitative real-time polymerase chain reaction (qPCR) analysis

qPCR reactions were performed in 96-well fast qPCR plate (Applied biosystems, America) each well contain 5 μl of SYBR master mix (Applied biosystems, America), 0.5 μl of cDNA, 2 μl of forward and reverse primer (table 4) and 2.5 μl of ultrapure water. Then put the plate in plate centrifuge to remove air bubbles. Finally, put the plate into Quanstudio™ Flex real-time PCR system and start with 50 $^{\circ}$C 2 minutes, 95 $^{\circ}$C 2 minutes, and the 40 cycles of 95 $^{\circ}$C 15 seconds, 60 $^{\circ}$C 15 seconds, 72 $^{\circ}$C 1 minute respectively. Following with melt curve analysis, 95 $^{\circ}$C 15 seconds, 60 $^{\circ}$C 1 minute, and 95 $^{\circ}$C 15 minutes, respectively. Gene expression can be calculated by $2^{-\Delta\Delta CT}$ method.

Table 4 List of genes and their sequences (33-35)

Gene	Forward	Reverse	Product size (bp)	Reference
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	141	Primer-BLAST
IL-10	AGCCGGGAAGACAATAACTG	CATTTCCGATAAGGCTTGG	189	Jin X, et al. (2019)
TNF-α	GGCAGGTCTACTTTGGAGTCATTG	ACATTCGAGGCTCCAGTGAATTCGG	300	Kim T, Kang B, Cho D, Kim S. (2003)
TGF-β	AACTATTGCTTCAGCTCCACAG	AGTTGGCATGGTAGCCCTTG	115	Jin X, et al. (2019)
IFN-γ	GGAACTGGCAAAAGGATGGTGAC	GCTGGACCTGTGGGTTGTTGAC	219	Jin X, et al. (2019)
MHC-II	AGTGAAAGGGGTTGAGCGTC	CCGCTCACACCAGATTAAGGT	223	Primer-BLAST
CD40	GCTATGGGGCTGCTTGTTGA	ATGGGTGGCATTGGGTCTTC	232	Morgado P, *et al.* (2014)
CD80	GGCAAGGCAGCAATACCTTA	CTCTTTGTGCTGCTGATTCG	94	Merav E, *et al.* (2010)
TLR2	TTGTTCCCTGTGTTGCTGGT	GGATAGGAGTTCGCAGGAGC	141	Primer-BLAST
TLR4	TGCCAACATCATCCAGGAAGG	CAGAAGATGTGCCTCCCCAG	267	Primer-BLAST
GAPDH	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA	171	Jin X, et al. (2019)

qPCR reactions were performed to determine relative quantitative expression of CD40, CD80, IL-6, IL-10, TNF-α, TGF-β, TLR-2, TLR-4, MHC-II genes in 96-well fast qPCR plate (Applied biosystems, America). Each well containing 5 μl of SYBR master mix (Applied biosystems, America), 0.5 μl of cDNA, 2 μl of forward and reverse primer, and 2.5 μl of ultrapure water. The reactions was amplified for 40 cycles in Quanstudio^{TM} Flex real-time PCR system with the standard PCR parameters (Applied biosystems profile: 50 ^{o}C for 2 minutes one cycle, 95 ^{o}C for 2 minutes one cycle, 95 ^{o}C for 15 seconds one cycle, 60 ^{o}C for 15 seconds 40 cycles, and 72 ^{o}C for 1 minute one cycle). Dissociation curves were analyzed to ensure products specificity and amplicon based on T_{m} (melting temperature of a PCR product) values. The data analyzed by plotting the ΔRn fluorescence signal versus the cycle number. The threshold cycle (Ct) values calculated from midpoint of log ΔRn were used to determine relative quantification of gene expression by $2^{-\Delta\Delta CT}$ method (fold change). GAPDH was used as a reference gene. The H$_2$O negative control was used as a reference sample.

$$\Delta CT = CT_{avg}(\text{a target gene}) - CT_{avg}(\text{a reference gene}).$$

$$\Delta\Delta CT = \Delta CT(\text{a target sample}) - \Delta CT(\text{a reference sample})$$

7. Luminex multiplex assays

Luminex multiplex assays were performed using multiplex assay kits for the Luminex$^{®}$ platform. (Thermo Scientific, MA, USA). 25 μl of 1x antibody beads were pipetted into 96-well plate. Then, incubate the plate on the magnet for 60 seconds. The plate was washed twice with 200 μl of wash solution. The supernatant was diluted with RPMI 1640. Then, 50 μl of supernatant, 50 μl of assay diluent, and 50 μl of incubation buffer were pipetted into the plate. Then, shake in the dark for 2 hr. The plate was washed twice with 200 μl of wash solution.

Then, 100 μl of 1x detector antibody was pipetted into the plate, and shake in the dark for 1 hr. The plate was washed twice with 200 μl wash solution.

Then, 100 μl of 1x SA-PE was pipetted into the plate and shake in the dark for 30 minutes. The plate was washed 3 times with 200 μl wash solution.

Finally, add 150 μl of wash solution into the plate, shake the plate for 3 minutes, and put the plate into Bio-plex 200 multiplex suspension array system (Bio-Rad, CA, USA). The concentration of cytokines was calculated by comparing absorbance with the standard curve.

8. Statistical analysis

The two-way ANOVA was performed in GraphPad Prism 8 to compare differences in ΔCT for genes expression measurement and $p < 0.05$ were considered significant.

The one-way ANOVA was performed in GraphPad Prism 8 to compare differences in cytokines concentration cytokines production measurement and $p < 0.05$ were considered significant.

CHAPTER IV

RESULTS

1. Isolation of infective stage larvae of *S. stercoralis* (iL3s)

Stools of infected gerbil were culture by modified agar plate technique for 5-10 days to get a humorous amount of iL3 (Figure 6). Both crude antigen and RNA were extracted from obtained iL3. Extracted RNA was used to synthesize the recombinant strongylasticin.
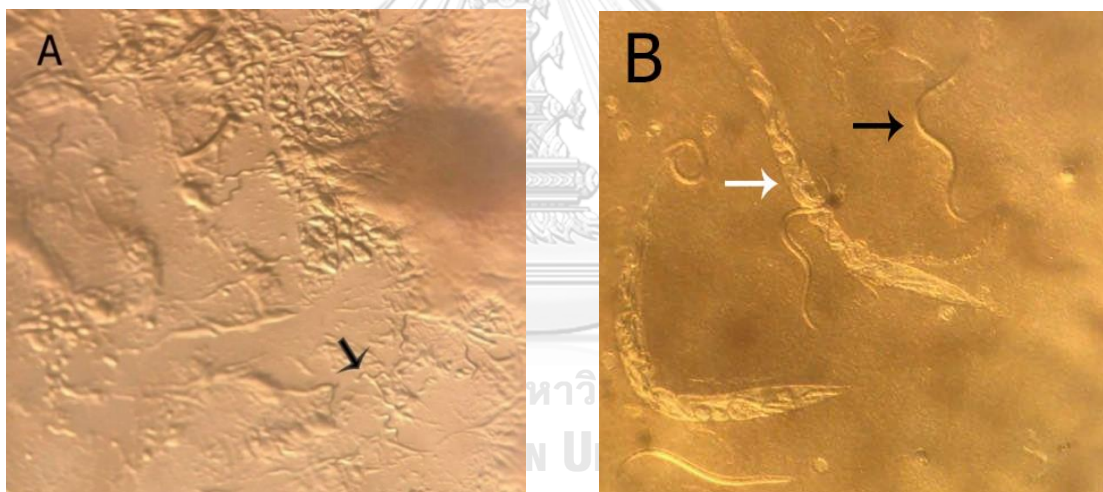

Figure 6 *S. stercoralis* from modified agar plate culture technique
(A) iL3 larvae (black arrow) under stereo microscopic observation, (B) iL3 larvae (black arrow), and free-living stage giving birth of L1 larvae (white arrow) under stereo microscopic observation

2. Morphological observation of DC2.4 cells

After DC2.4 treatment with LPS, and *S. stercoralis* crude antigen DC2.4 cells were observed under an inverted light microscope. DC showed a "spiky" morphology related to mature DCs phenotype (Figure 7).
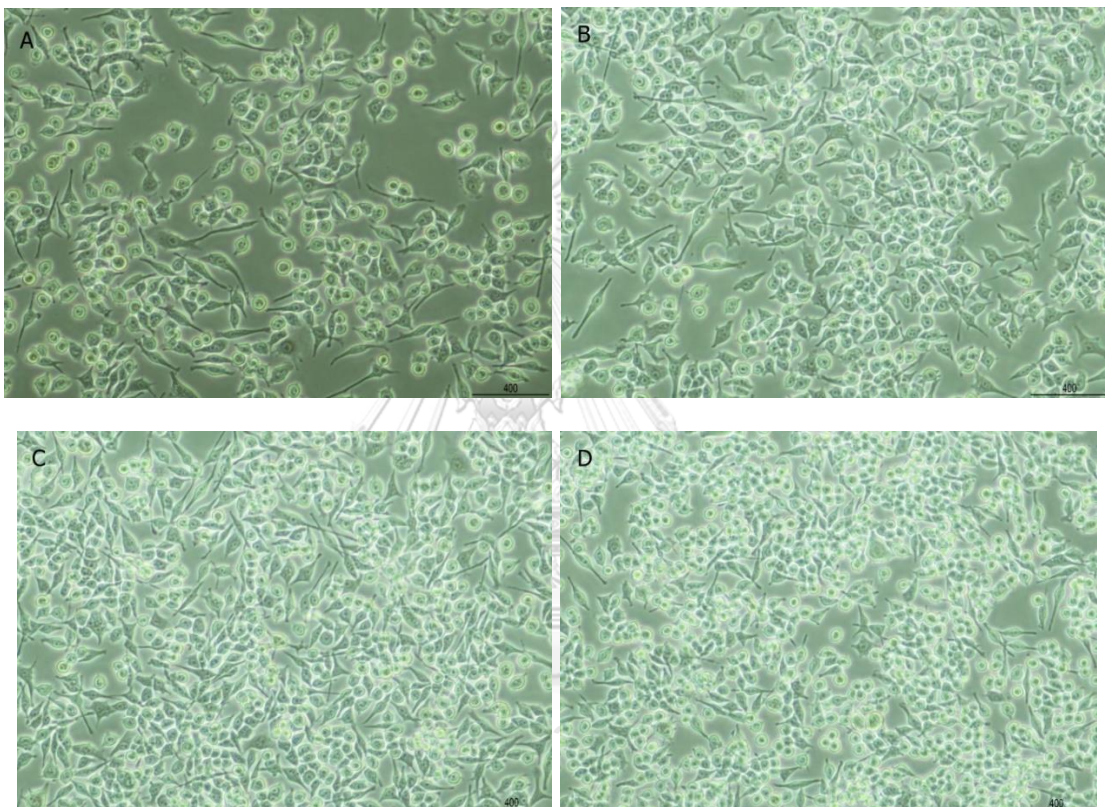

Figure 7 Morphological observation of DC2.4 cells in varies condition
(A) before treatment, (B) 16 hr after treatment with LPS (5 **μ**g/ml), (C) 16 hr after treatment with *S. stercoralis* crude antigen) (20 **μ**g/ml), (D) 16 hr of negative control

3. Expression of cytokines and costimulatory molecules after treatment of DC2.4 cells with the crude antigen of *S. stercoralis*

3.1 Primer quality check.

Conventional PCR reactions were prepared using cDNA from DC2.4, and primers including GAPDH, TNF-**α**, TGF-**β**, IFN-**γ**, IL-2, IL-4, IL-6, IL-10, IL-12, CD40, CD80, TLR2, TLR4, and MHC-II were performed to confirm the quality of primers. PCR products were loaded into 1% agarose gel with GeneRuler™ DNA ladder mix (Thermo Scientific, MA, USA), and GeneRuler™ 1kb DNA ladder (Thermo Scientific, MA, USA). The size of amplified products were showed on Figure 8.
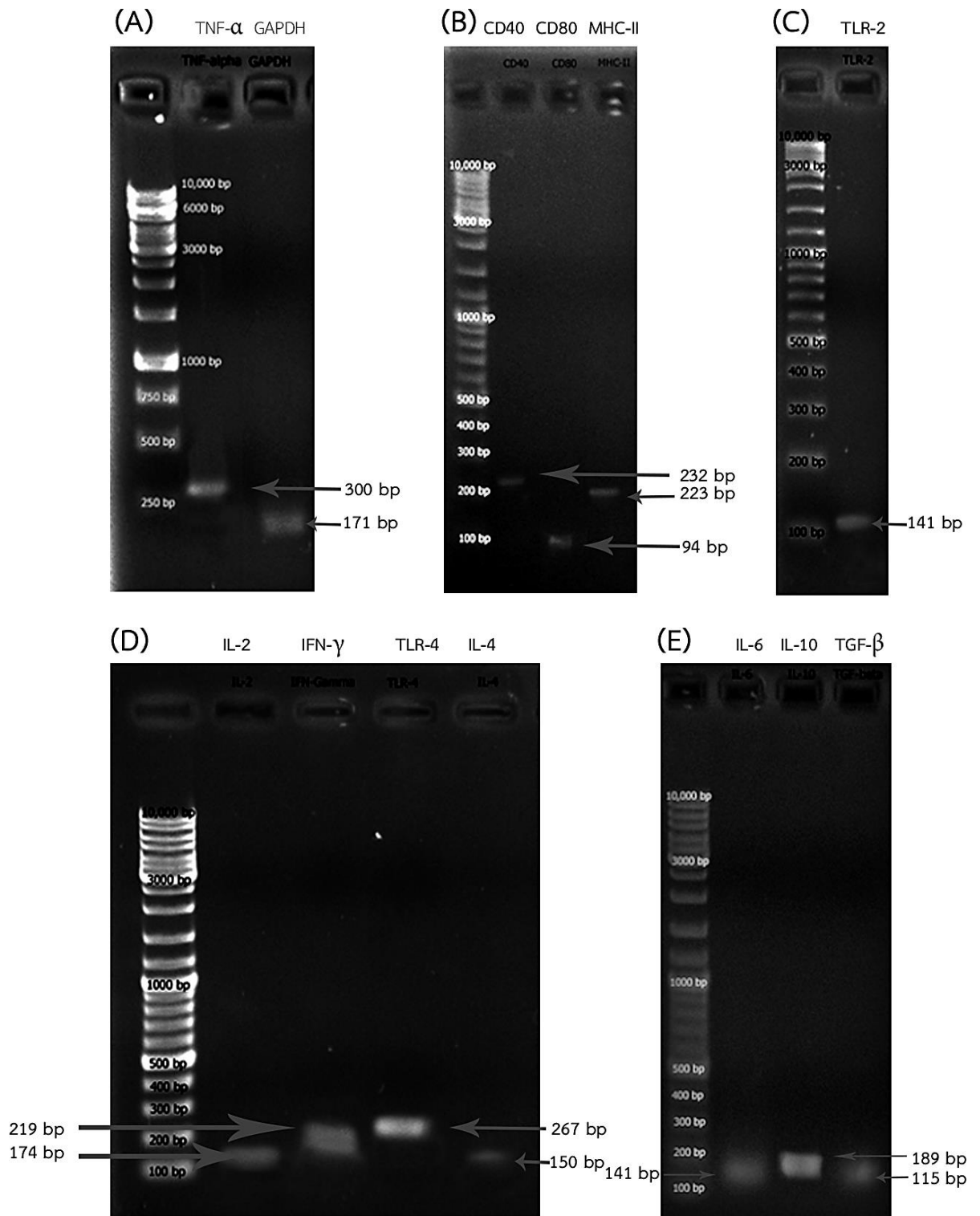
Figure 8 Agarose gel electrophoresis of the interested genes
including TNF-α (A), GAPDH (A), CD40 (B), CD80 (B), MHC-II (B), TLR-2 (C), IL-2 (D),
IFN- γ (D), TLR-4 (D), IL-4 (D), TLR-2 (D), IL-6 (E), IL-10 (E), TGF-β (E)

3.2 Amplification plot and Melt curve analysis

The amplification plot represented the accumulation of PCR products throughout the experiment and gives the mean of cycle threshold (CT) that use for calculating the expression of the gene. Melt curve analysis represents the peak of the plot relate to the purity of PCR products. Three repeats were performed. The amplification plot and melt curve were analyzed. The melt curves of each gene showed a single peak related to the purity of the amplicon (Figure 9).
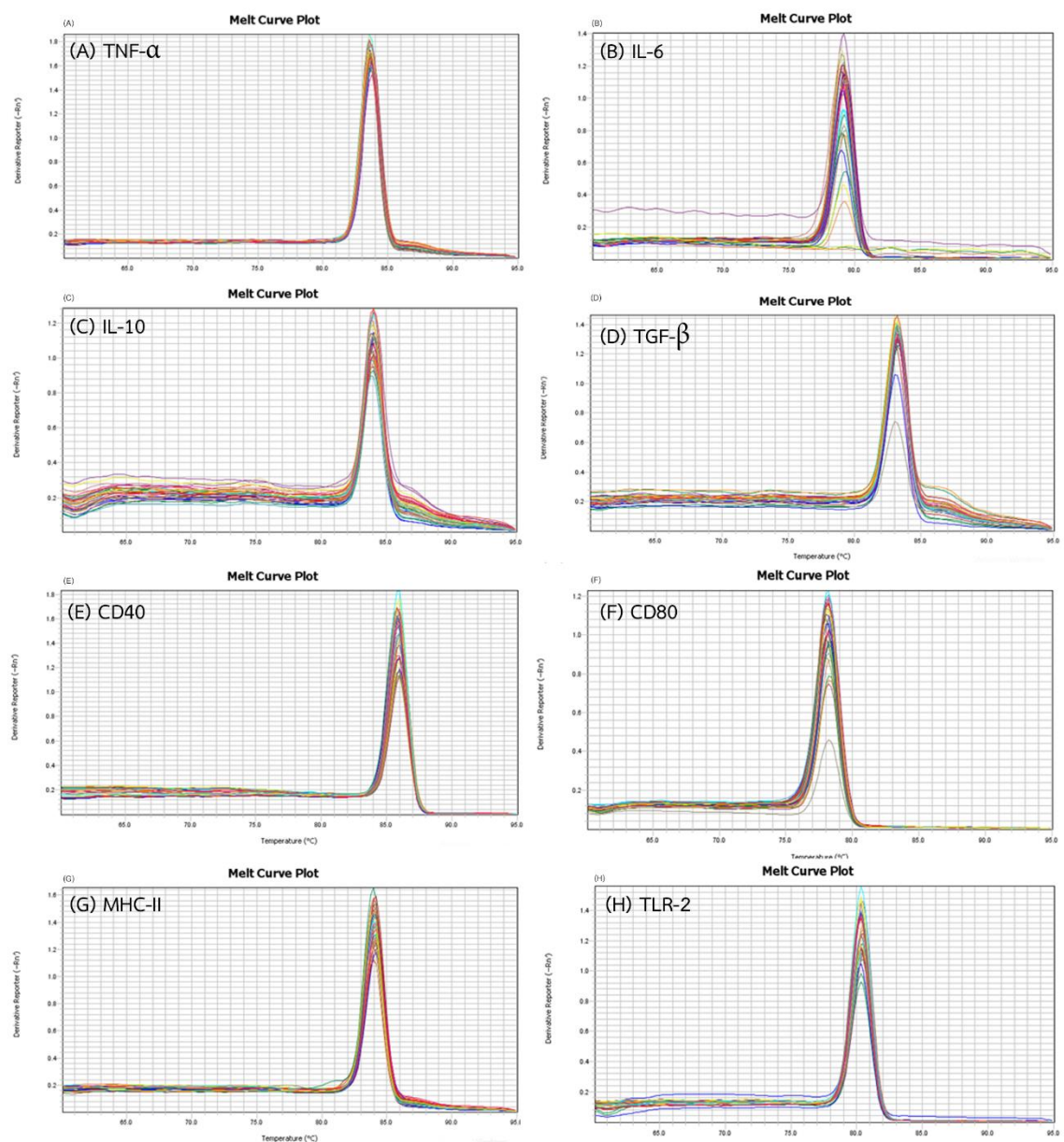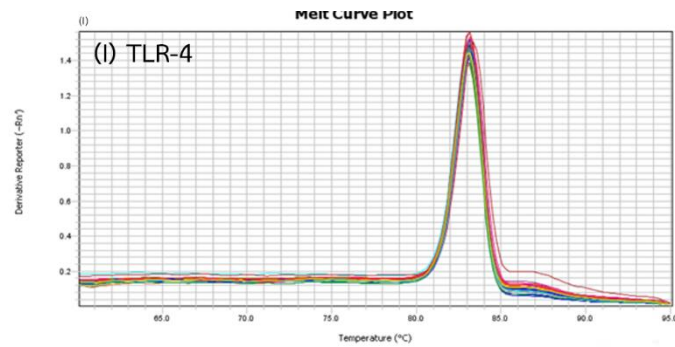
Figure 9 Melt curve analysis

of TNF-α (A), **IL-6 (B), IL-10 (C),** TGF-β (D), CD-40 (E), CD-80 (F), MHC-II (G), TLR-2 (H)

and TLR-4 **(I)**

3.3 Gene expression calculated by the $2^{-\Delta\Delta ct}$ method

The acquired CT mean of each repeat was calculated in the $2^{-\Delta\Delta ct}$ method and plot into a bar graph with fold change in Y-axis and each treatment and time point in X-axis.

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Expression levels of TNF-α gene in DC2.4 cells

Both DC2.4 cells treated with LPS and *S. stercoralis* crude antigen (CA) showed a significantly high level of expression of TNF-α gene in the first hour ($P<0.0001$) and tended to decrease after 1hr. At 3-hour treatment, both DC2.4 cells treated with LPS and CA still showed significantly increased expression levels of TNF-α compared to negative control. At 6-hour treatment, only DC 2.4 cells treated with LPS still showed significantly increased expression levels of TNF-α gene compared to the negative control ($P<0.01$), while expression of TNF-α in CA experiment was not significantly different from negative control. However, there was a significant difference between CA and LPS experiments ($P<0.01$) (Figure 10).

Figure 10 Expression levels of TNF-α gene in DC2.4 cells exposed to *S. stercoralis* crude antigen (CA) (20 μg/ml), LPS (5 μg/ml), or media alone for 1,3,6,12, and 16 hours.

* significantly different ($P<0.05$) compared to control by using two-way ANOVA.
** significantly different ($P<0.01$) compared to control by using two-way ANOVA.
*** significantly different ($P<0.001$) compared to control by using two-way ANOVA.
**** significantly different ($P<0.0001$) compared to control by using two-way ANOVA.
.. significantly different ($P<0.01$) compared to treatment group by using two-way ANOVA.

Expression levels of IL-6 gene in DC2.4 cells

The expression levels of IL-6 gene in DC2.4 cells were non-significantly increased only in DC2.4 cells treated with LPS, while expression levels of IL-6 gene in DC2.4 cell line treated with CA and PBS control was not changed (Figure 11).

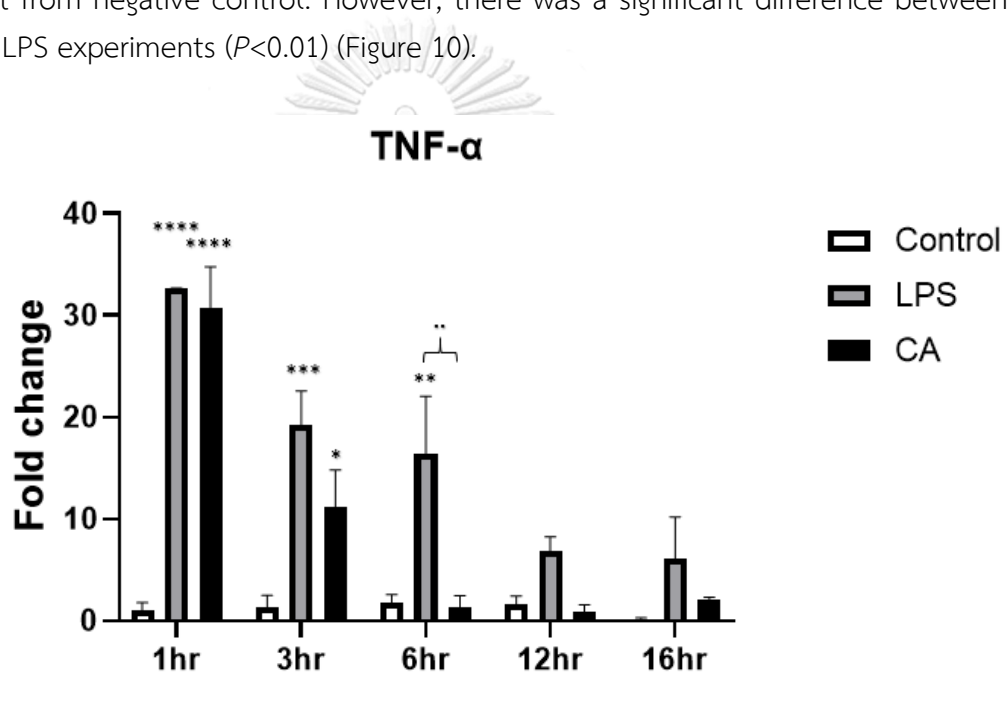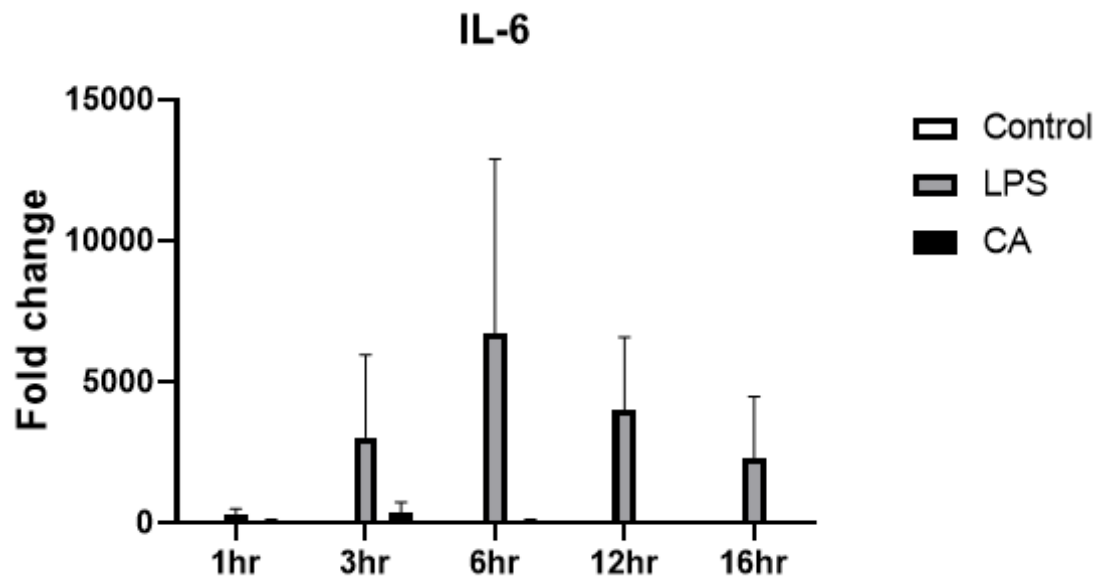Figure 11 Expression levels of IL-6 gene in DC2.4 cells exposed to S. stercoralis crude antigen (CA) (20 µg/ml), LPS (5 µg/ml), or media alone for 1,3,6,12, and 16 hours.

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Expression levels of IL-10 gene in DC2.4 cells

The expression levels of IL-10 gene in DC2.4 cells treated with CA were significantly increased in 1 hour after treatment ($P<0.001$) and showed a significant difference between CA and LPS experiments ($P<0.05$). However, these levels tended to decrease to normal levels at 3 hours after treatment. In contrast, expression levels of IL-10 gene in LPS experiment showed a significantly increased ($P<0.01$) and showed a significant difference between CA and LPS experiments ($P<0.01$) (Figure 12).

Figure 12 Expression levels of IL-10 gene in DC2.4 cells

exposed to *S. stercoralis* crude antigen (CA) (20 μg/ml), LPS (5 μg/ml), or media

alone for 1,3,6,12, and 16 hours.

** significantly different ($P<0.01$) compared to control by using two-way ANOVA.

*** significantly different ($P<0.001$) compared to control by using two-way ANOVA.

. significantly different ($P<0.05$) compared to treatment group by using two-way ANOVA.

Expression levels of TGF-β gene in DC2.4 cells

The expression levels of TGF-β gene in DC2.4 cells treated with CA were significantly increased in 1 hour after treatment ($P<0.001$) and showed a significant difference between CA and LPS experiments ($P<0.05$). However, the expression levels of TGF-β gene tended to decrease to normal levels after 3 hours after CA treatment. In contrast, expression of TGF-β gene in LPS experiment still showed a significantly increased at 3 hours and 6 hours. Moreover, there was a significant difference between CA and LPS experiments ($P<0.01$). (Figure 13).
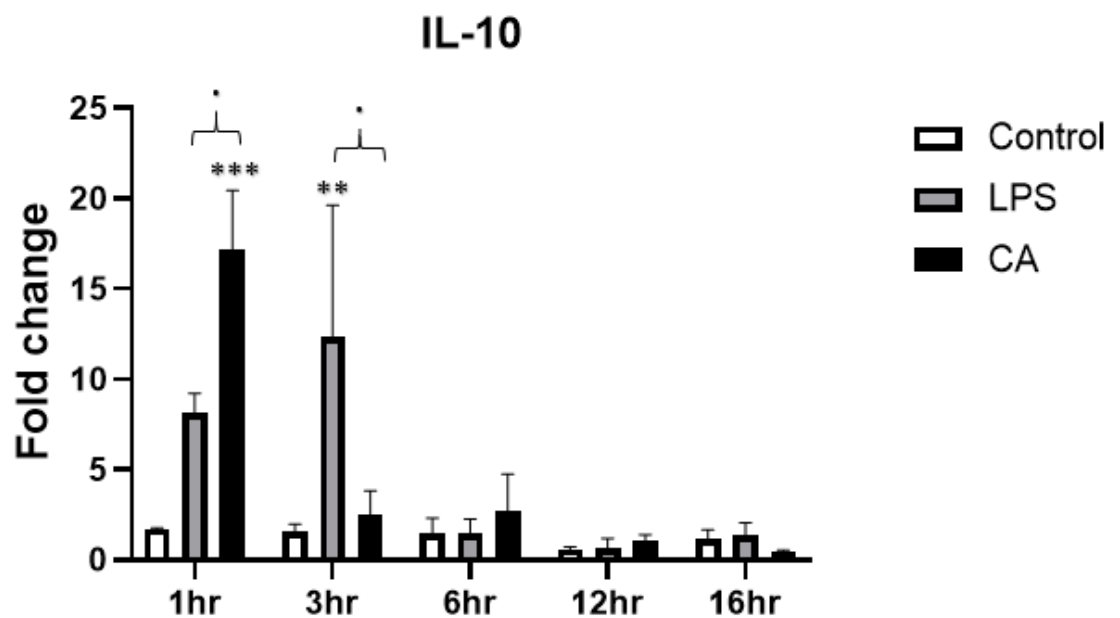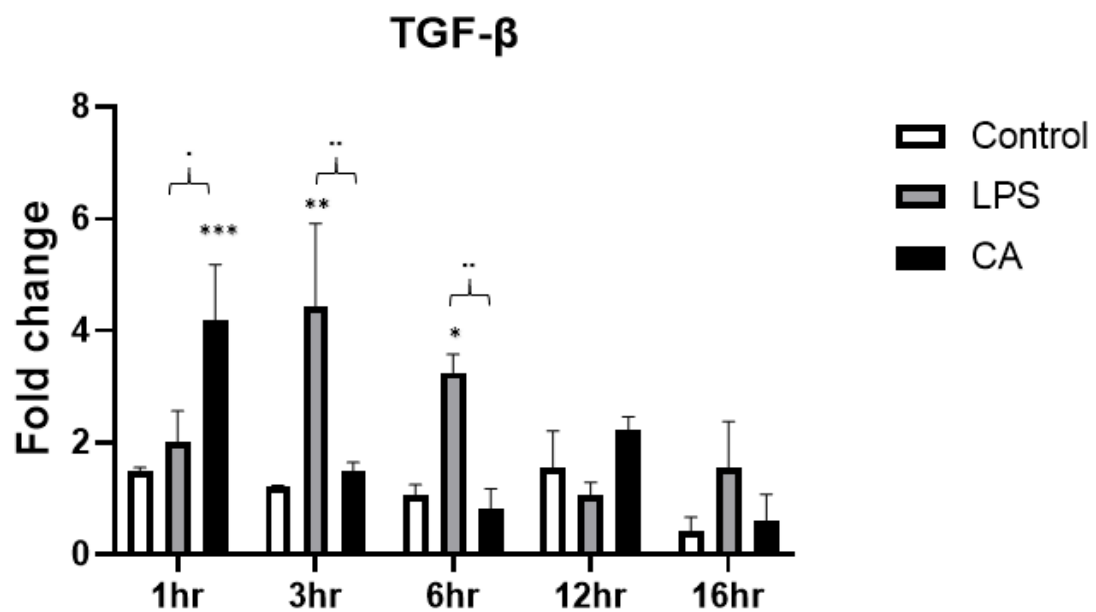

Figure 13 Expression levels of TGF-β gene in DC2.4 cells
exposed to *S. stercoralis* crude antigens (CA) (20 μg/ml), LPS (5 μg/ml), or media
alone for 1,3,6,12, and 16 hours.

* significantly different ($P<0.05$) compared to control by using two-way ANOVA.
** significantly different ($P<0.01$) compared to control by using two-way ANOVA.
*** significantly different ($P<0.001$) compared to control by using two-way ANOVA.
. significantly different ($P<0.05$) compared to treatment group by using two-way ANOVA.
.. significantly different ($P<0.01$) compared to treatment group by using two-way ANOVA.

Expression levels of MHC-II gene in DC2.4 cells

The expression levels of MHC-II gene in both DC2.4 cells treated with LPS and CA were not significantly different from the PBS control. Although, the expression levels of MHC-II gene in LPS experiment were increased at 16hr, but it was not significant (Figure 14).

Figure **14** Expression levels of MHC-II gene in DC2.4 cells

exposed to *S. stercoralis* crude antigens (CA) (20 **μ**g/ml), LPS (5 **μ**g/ml), or media alone for 1,3,6,12, and 16 hours.

Expression levels of TLR-2 gene in DC2.4 cells

The expression levels of TLR-2 gene in DC2.4 cells treated with LPS were significantly increased at 3 hours after treatment ($P<0.05$). However, the expression levels of TLR-2 gene in DC2.4 cells treated with CA were not significantly different from PBS control at any time point ($P>0.05$) (Figure 15).

Figure 15 Expression levels of TLR-2 gene in DC2.4 cells

exposed to *S. stercoralis* crude antigens (CA) (20 μg/ml), LPS (5 μg/ml), or media

alone for 1,3,6,12, and 16 hours.

* significantly different ($P<0.05$) compared to control by using two-way ANOVA.

Expression levels of TLR-4 gene in DC2.4 cells

The expression levels of TLR-4 on both LPS and CA experiments were not significantly different compared to the PBS control at any time point (Figure 16).
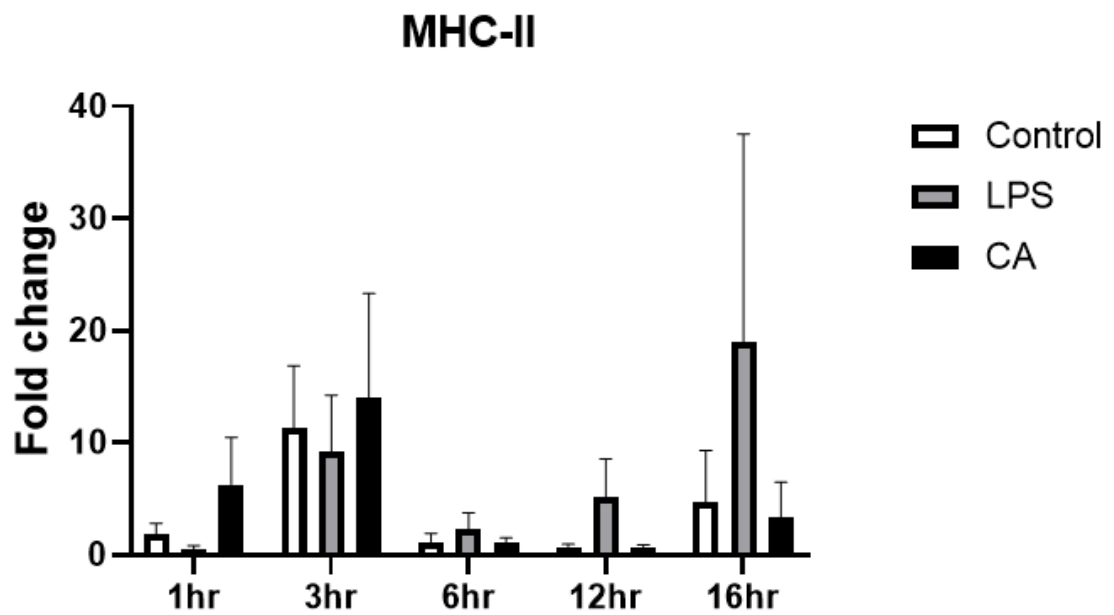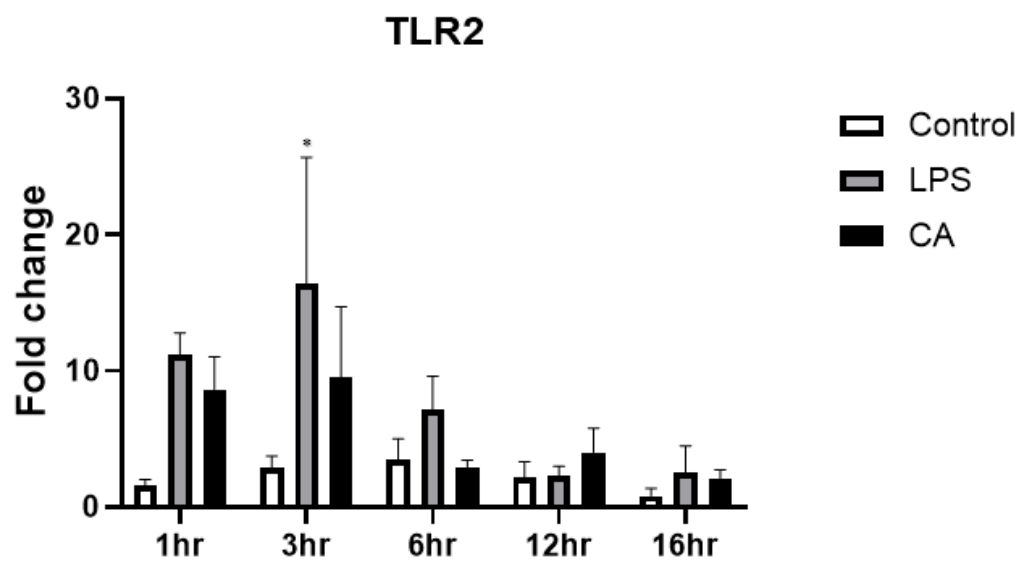

Figure 16 Expression levels of TLR-4 gene in DC2.4 cells exposed to *S. stercoralis* crude antigens (CA) (20 μg/ml), LPS (5 μg/ml), or media alone for 1,3,6,12, and 16 hours.

Expression levels of CD-40 gene in DC2.4 cells

The expression levels of CD-40 gene in DC2.4 cells treated with LPS were significantly increased at 6hr after the treatment (*P*<0.01) and showed a significant difference between LPS and CA experiment (P<0.01). However, the expression level of CD-40 gene in DC2.4 cells treated with CA were not significantly different at any timepoint compared to the negative control (Figure 17).
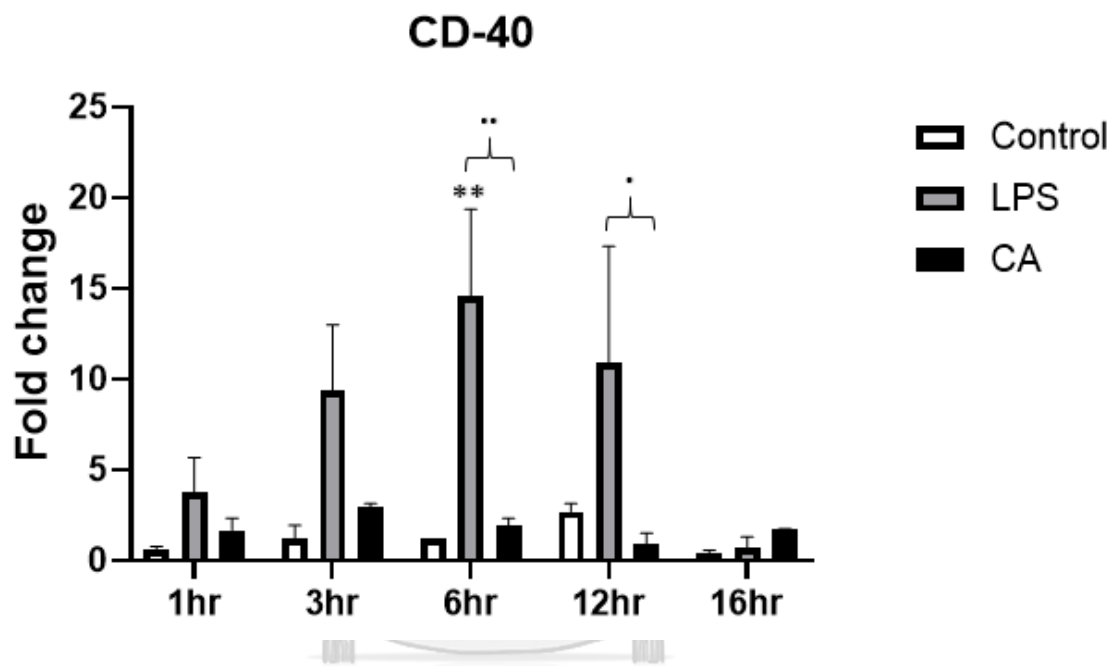
Figure 17 Expression levels of CD-40 gene in DC2.4 cells
exposed to *S. stercoralis* crude antigens (CA) (20 μg/ml), LPS (5 μg/ml), or media alone for 1,3,6,12, and 16 hours.

* significantly different (*P*<0.05) compared to control by using two-way ANOVA.
** significantly different (*P*<0.01) compared to control by using two-way ANOVA.
*** significantly different (*P*<0.001) compared to control by using two-way ANOVA.
. significantly different (*P*<0.05) compared to treatment group by using two-way ANOVA.
.. significantly different (*P*<0.01) compared to treatment group by using two-way ANOVA.

Expression levels of CD-80 gene in DC2.4 cells

The expression levels of CD-80 gene in DC2.4 cells treated with LPS were significantly increased at 12 hours after treatment ($P<0.0001$) and showed a significant difference between LPS and CA experiment ($P<0.0001$). The expression of CD-80 gene in DC2.4 cells treated with CA were significantly increased at 16 hours after treatment ($P<0.05$) and showed a significant difference between LPS and CA experiment ($P<0.01$). (Figure 18).
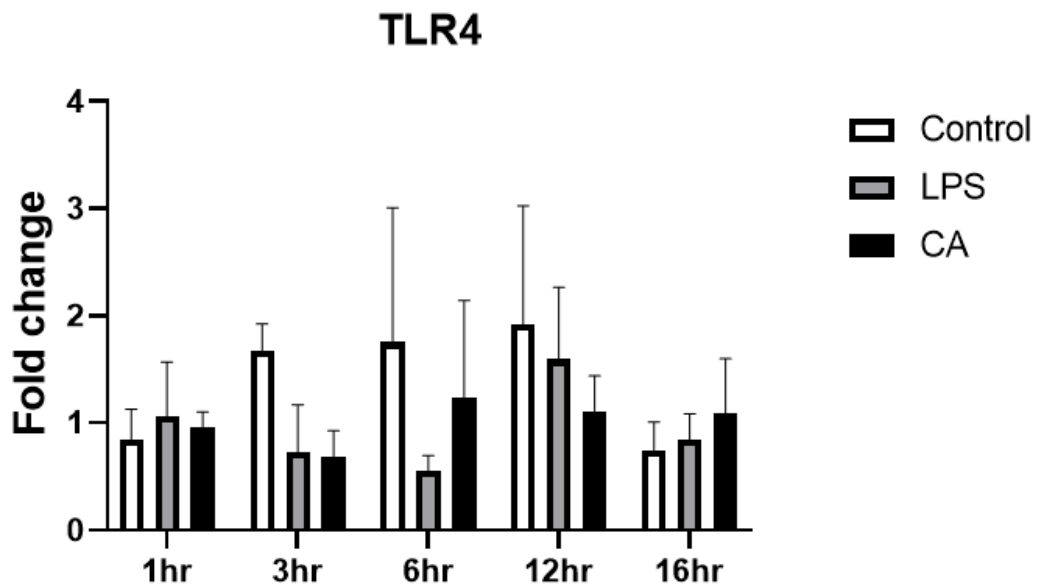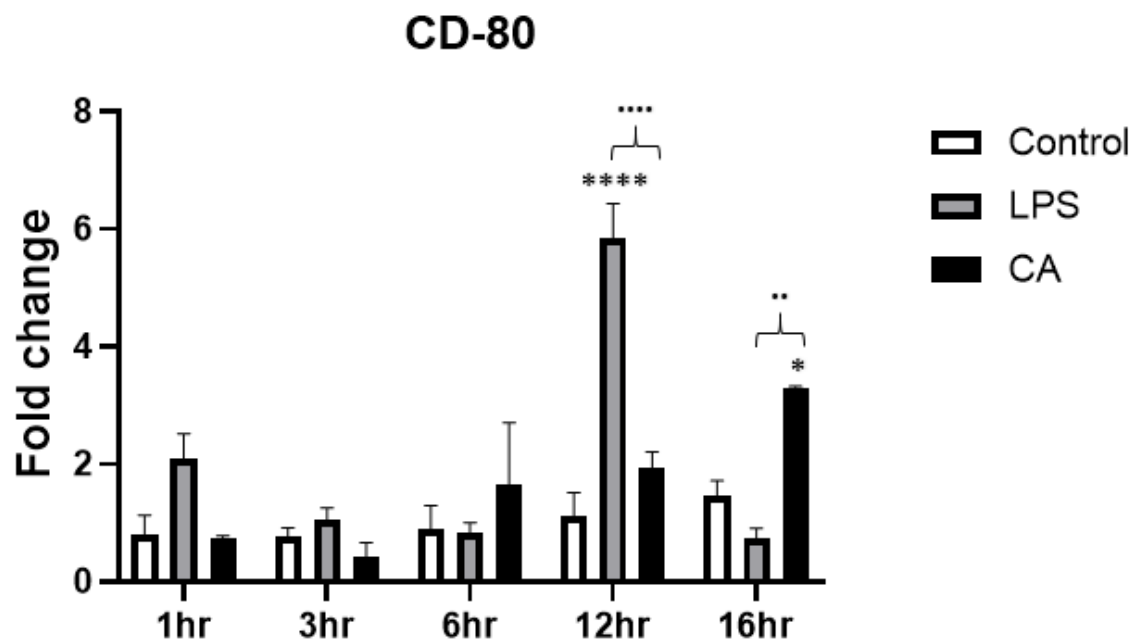

Figure **18** Expression levels of CD-80 gene in DC2.4 cells
exposed to *S. stercoralis* crude antigens (CA) (20 μg/ml), LPS (5 μg/ml), or media
alone for 1,3,6,12, and 16 hours.

* significantly different ($P<0.05$) compared to control by using two-way ANOVA.
**** significantly different ($P<0.0001$) compared to control by using two-way ANOVA.
.. significantly different ($P<0.01$) compared to treatment group by using two-way ANOVA.
.... significantly different ($P<0.0001$) compared to treatment group by using two-way ANOVA.

For the alteration of antigen presentation process, we found that LPS stimulation significantly increased the expression levels of TLR2, CD-40, and CD-80 genes at 3, 6, and 12 hours after treatments, respectively (Figure 15, 17, and 18). In contrast, stimulation with *S. stercoralis* crude antigen (CA) could not affect the expression levels of TLR2, and CD-40 genes compared to the negative control (Figure 15, and 17). Interestingly, we found lately significant upregulation of CD-80 gene at 16hr after CA treatment (Figure 18). Unfortunately, we did not detect any significant changes of expression levels of MHC-II and TLR-4 genes after stimulating LPS and CA. These results suggested that *S. stercoralis* crude antigen from *S. stercoralis* could not stimulate both pattern recognition receptors including TLR-2 and TLR-4 and co-stimulatory molecules including MHC-II, CD40, and CD80 in DC2.4 cells.

For cytokine gene expression, we studied the expression levels of naïve T cell stimulating cytokine genes, including TNF-α, IL-6, TGF-β, and IL-10. We found that the expression levels of IL-6 genes were not significantly changed at any timepoint (Figure 11), while expression levels of TNF-α gene were significantly increased at 1 hour and 3 hours after CA treatment (Figure 10). Interestingly, the expression levels of IL-10 gene were also significantly increased at 1 hour after CA treatment and tended to decrease to normal levels (Figure 12). Interestingly, expression levels of TGF-β gene were significantly increased at 1 hour after CA treatment (Figure 13). Therefore, our result suggested that crude antigen from *S. stercoralis* could induce Treg response by inducing the IL-10 and TGF-β production from DCs. These changes are related to immunomodulation roles of *S. stercoralis* in the regulation of immune response through stimulating expression of regulation cytokine IL-10 and TGF-β which required for Treg differentiation. To confirm the increasing of IL-10, TGF-β and other high expression levels of cytokine genes, the cytokine levels in supernatant were measured by Luminex multiplex assays. Additionally, we studied some candidate antigens which share the same immunomodulation roles as *S. stercoralis* crude antigen. In this study, we focused on crude antigens, excretory-secretory antigens (ES antigens), strongylastacin, and iPGM.

4. **Expression of recombinant strongylastacin in Origami E. coli**

Colony PCR screening showed the correct-size band of strongylastacin amplicon (1134 bp) (Figure 19). The extracted plasmid was sequenced and compared to the FASTA nucleotide with BlastN (NCBI, USA). The sequence showed 99% of identities to homolog base pair with metalloproteinase precursor (Figure 20). However, more than ten times repeating in expression and purification step. We failed to get the confidently recombinant strongylastacin. The Coomassie blue staining showed the correct-size band of strongylastacin (45 kDa), but we did not find any specific band by the western blot analysis (Figure 21).

Because we failed to prepare recombinant strongylastacin, we studied another antigen candidate antigen, iPGM, which highly expresses in all stages of *S. stercoralis*. The *E. coli* BL21 containing *iPGM* gene was kindly provided by Ms. Phinyarat Suksomphak. Recombinant iPGM antigen was prepared and showed a big correct-size band (61 kDa) in both Coomassie blue technique and nitrocellulose from the western blot (Figure 22).
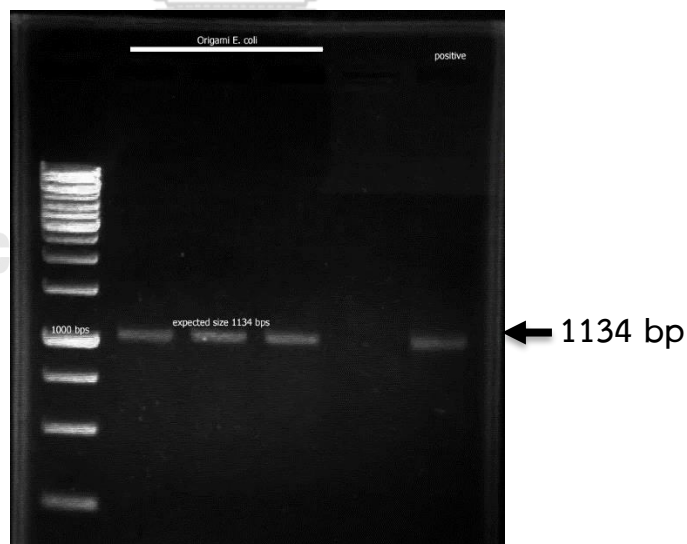

Figure 19 Agarose gel electrophoresis of colony PCR screening
for recombinant strongyastacin ligation and transformation

Strongyloides stercoralis metalloproteinase precursor, mRNA, partial cds

Sequence ID: AF118022.2 Length: 1134 Number of Matches: 1

Range 1: 59 to 1134 GenBank Graphics   ▼ Next Match ▲ P

Score	Expect	Identities	Gaps	Strand
1916 bits(1037)	0.0	1066/1078(99%)	9/1078(0%)	Plus/Minus

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Query  57    TTATTTAAAACTTTTGAACTTAAGTGAAAGACTGTCAGAAAAGGATTTTCCAACATATCT   116
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Sbjct  1134  TTATTTAAAACTTTTGAACTTAATTGAAAGACTGTCAGAAAAGGATTTTCCAACATATCT   1075

Query  117   TATCACAATATAATTATTTTCAGATACAAATTCTTTTGCACTTATTTTTCCACATAACAT   176
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Query  177   TGCTCCAGAAACAGCTTTATCAGCAAGGTATTTTATTTCTAATCCTGAACCAGGTTGGCA   236
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Query  897   AATAAAGGATACACATCTATTTCCTGGTCTATAAACTATTCCACCTTTAGTAAATCTATT   956
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294   AATAAAGGATACACATCTATTTCCTGGTCTATAAACTATTCCACCTTTAGTAAATCTATT   235

Query  957   AGTTTTTCTAAATCTTATACATGTATCTCTTTCTAATGTTT-CAATCCCTTTTGTAATTG   1015
             ||||||||||||||||||||||||||||||||||||||||| |||| || ||||||||||
Sbjct  234   AGTTTTTCTAAATCTTATACATGTATCTCTTTCTAATGTTTCAAT-CCTTTTGTAATTG    176

Query  1016  TATCTTCATTTATTCCATCCTCGACATAATAATCTAT--GAGATGACCATTTAAGTG-AA   1072
             ||||||||||||||||||||||||||||||||||||  |||||||||||||||||| ||
Sbjct  175   TATCTTCATTTATTCCATCCTCGACATAATAATCTATTGGAAATGACCATTTAAGTGGAA   116

Query  1073  GA-TTTTTGTAACAGATTTTTTT-ACTCT-GTTTCAATAAGATTCTAAATTATTTCTAT   1127
             || |||||||||||||||||||| ||||| ||| |||| |||||||||||||||||||
Sbjct  115   GATTTTTTGTAACAGATTTTTTTTACTCTTGTTTCAAT-AGATTCTAAATTATTTCTAT    59
```

Figure 20 Sequencing alignment of recombinant plasmid
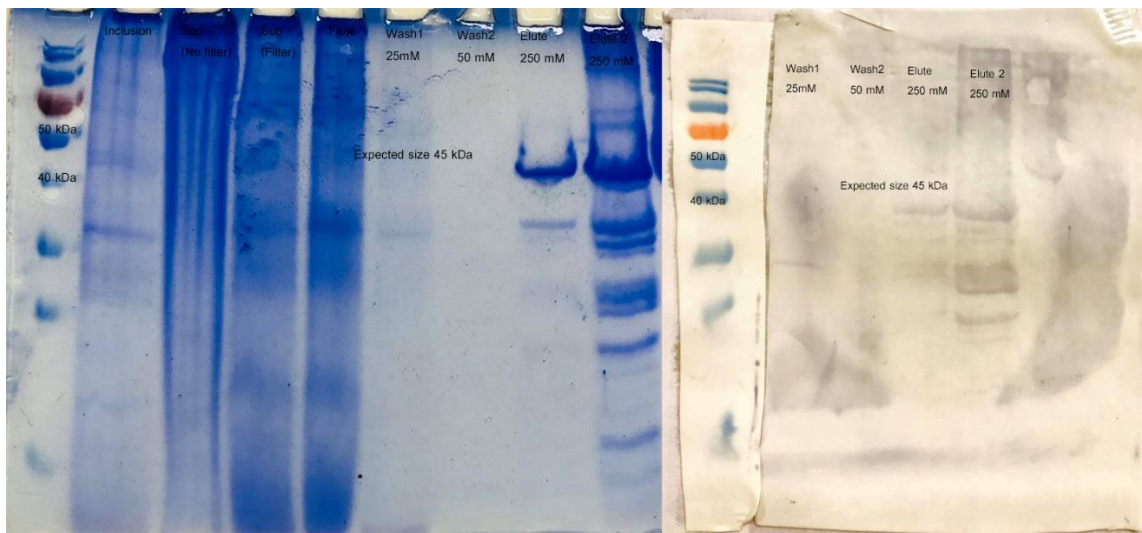extracted from Origami *E. coli*

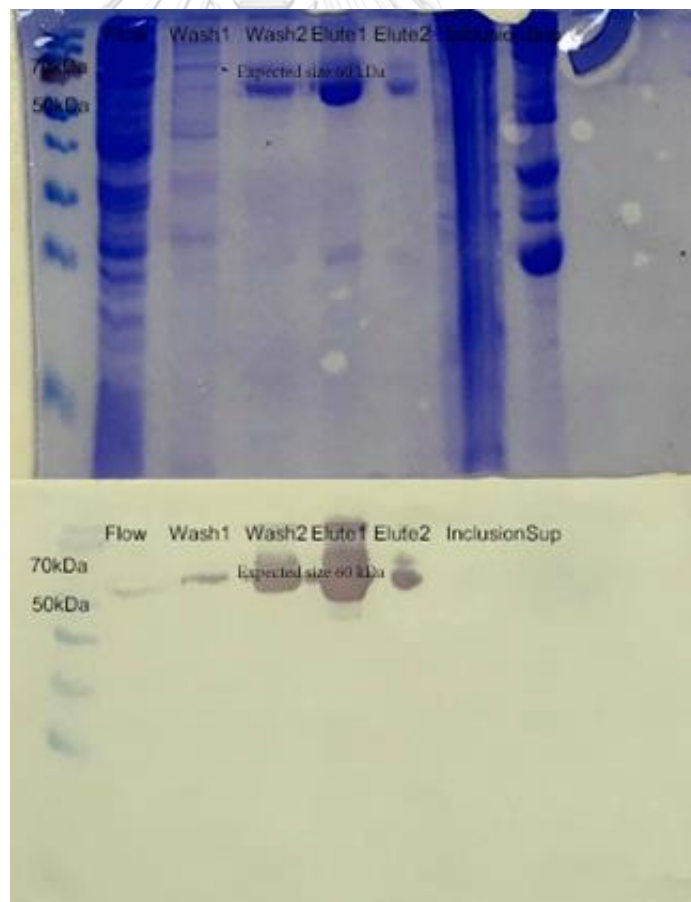Figure 21 Coomassie blue staining and antibody staining of recombinant strongylastacin.

Figure 22 Coomassie blue staining and antibody staining of recombinant iPGM.

5. Luminex multiplex assays

To study the role of iPGM antigen in the immunomodulation of cytokine production from DCs, we treated DC2.4 cells with crude antigen, iPGM and lived iL3 of *S. stercoralis* in trans well to represented secreted antigen for 24 hours. Supernatants were collected to measure cytokines levels by Luminex multiplex assays compared to LPS and negative control.

Pro-inflammatory cytokine production from DC2.4 cells

Only DC2.4 cells stimulated with LPS produced significantly levels of TNF-α, and IL-6 (P<0.05) (Figure 23). There was no antigen from *S. stercoralis*, including crude antigen (CA), secretory antigens from live iL3s, and iPGM antigen, could stimulate DC2.4 cells to produce TNF-α and IL-6 production. Statistical test shows a differentiation between treatment groups.

Figure **23** TNF-α (A) and IL-6 (B) production in supernatants collected from DC2.4 cells treated with *S. stercoralis* crude antigen (20ug/ml), LPS (5ug/ml), iPGM (20ug/ml), iL3 *of S. stercoralis* (3,000 larvae/ml) for 24 hours detected by Luminex multiplex assay.

Y-axis represents the concentration in pg/ml unit.

*** significantly different (P<0.001) compared to control by using one-way ANOVA**.**

. significantly different (P<0.05) compared to treatment group by using one-way ANOVA**.**

Regulatory cytokine production from DC2.4 cells

The supernatants collected from LPS, CA, and iL3 experiments showed a significantly increased levels of IL-10 production, while the levels of IL-10 production in iPGM experiment were not changed (Figure 24A). Similarly, the supernatants from CA and iL3 experiments showed a significantly increased the concentration of TGF-β, while the levels of TGF-β in iPGM were not different (Figure 24B).
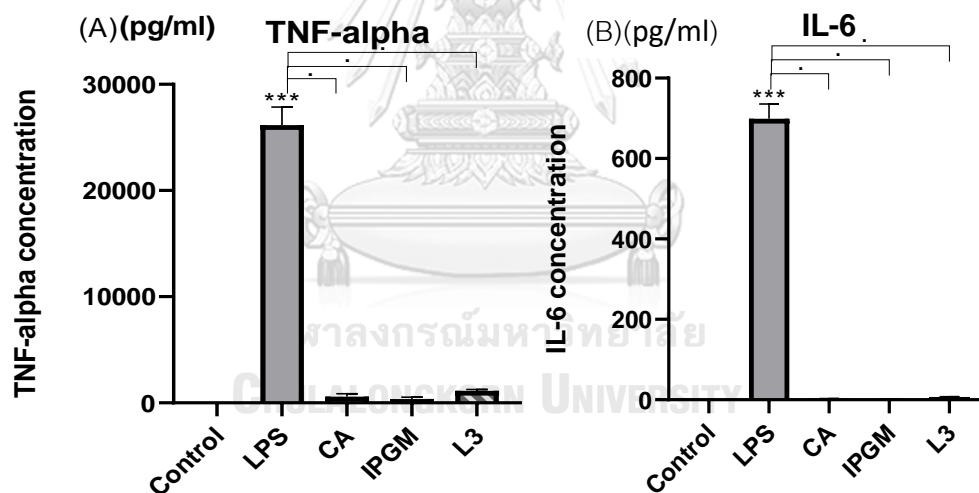

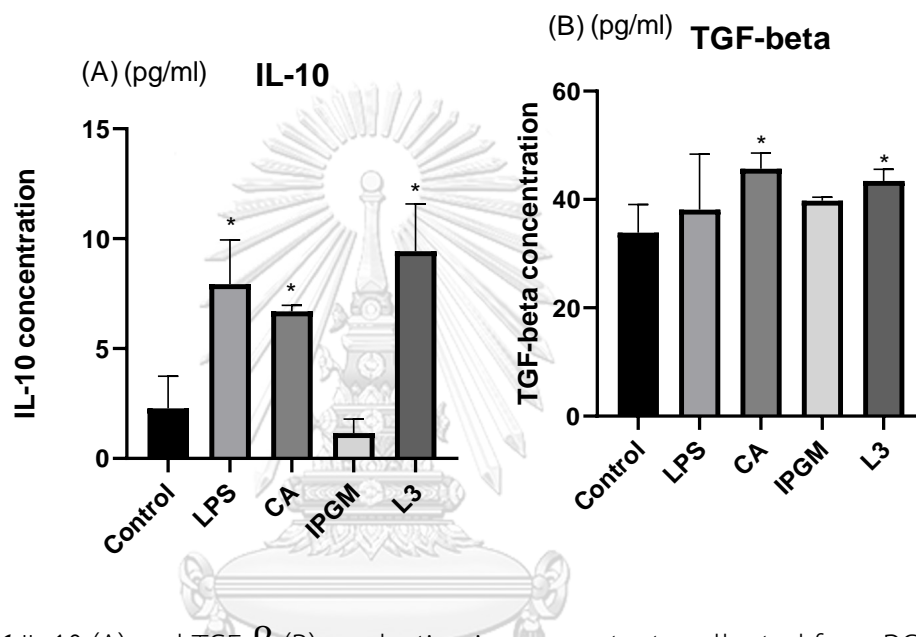Figure *24* IL-10 (A) and TGF-β (B) production in supernatants collected from DC2.4 cells treated with *S. stercoralis* crude antigen (20ug/ml), LPS (5ug/ml), iPGM (20ug/ml), iL3 *of S. stercoralis* (3,000 larvae/ml) for 24 hours detected by Luminex multiplex assay.

Y-axis represents the concentration in pg/ml unit.
* significantly different (*P*<0.05) compared to control by using one-way ANOVA.

CHAPTER V

DISCUSSION

Our results suggested that crude antigens and secretory antigens from *S. stercoralis* can induce anti-inflammatory cytokines including TGF-β and IL-10 production from DCs. These two cytokines play a crucial role in regulating the human immune system by promoting the production of regulatory T cells for Treg response.

TGF-β is multifunctional cytokines and has both pro-inflammatory and anti-inflammatory roles depended on other secreted cytokines. For pro-inflammatory functions, the secretion of the low levels of TGF-β synergies with IL-6 from DCs can induce the production of Th17 cells. For anti-inflammatory roles, secretion of a high level of TGF-β synergies with IL-10 from DCs can induce the production of regulatory T cells. According to our results, DCs treated with LPS showed high levels of both gene expression and cytokine production of TGF-β and IL-6, while DCs treated with *S. stercoralis* crude antigen and excretory-secretory antigens from iL3 induced high levels of both TGF-β and IL-10 production. These results emphasized the regulatory responses against the *S. stercoralis* crude antigen and excretory-secretory antigens from iL3.

IL-10 is also the important regulatory cytokine which tolerogenic semi-mature DCs secrete to terminate the excessive T cell response to prevent tissue damage and chronic inflammation. In the skin, IL-10 prevents the hypertensive of an immune response to antigen. Thus, IL-10 is the essential cytokines that play a critical role in inflammatory suppression and promote regulatory T cell production to maintain immune homeostasis. Treg cells can produce a high amount of IL-10 for self-maintenance and reduce the aggressive inflammatory response of the immune system. Immune regulation is an important pathway to save the host's cell from a destructive immune response and make the host survive from autoimmune disease.

In the same way, many parasites can survive from the host's immune system by modulating the host immune system in many ways. Stimulation of regulatory cytokines is one of the immunomodulation mechanisms of some helminthic parasites, such as *Clonorchris sinensis* (36).

Interestingly, our results also showed the high expression levels of TNF-α in both DC2.4 cells treated with LPS and *S. stercoralis* crude antigen (Figure 10). Unfortunately, we did not find significant levels of TNF-α production in supernatant collected from DC2.4 cells treated with *S. stercoralis* crude antigen. TNF-α plays a pivotal role in the survival of DCs. The TNF-α can both trigger the apoptosis-related gene and inhibit the apoptotic pathway depended on TNF-α concentration and TLR-stimuli. TNF-α can also induce the production of TGF-β. The synergy between TNF-α and TGF-β can maintain DCs survival. After *S. stercoralis* crude antigen stimulation, the expression levels of TNF-α was significantly high at 1 hour and 3 hours, then tended to decrease conformed to the immunomodulation mechanism of *S. stercoralis*. In contrast, DCs stimulated with LPS showed the higher expression levels of TNF-α than in DCs stimulated with CA. Our result suggested that increasing TNF-α in the crude antigen experiment was not for a chronic inflammatory response but related to DCs survival (23).

In this study, we would like to investigate which antigens of *S. stercoralis* are involved in the immunomodulation of DCs. We selected 4 candidate antigens including crude antigen, excretory-secretory antigens, strongylastacin and iPGM. However, we failed to produce recombinant strongylastacin and it lasted for two years of trying to express strongylastacin with many adjustments. One of the hypotheses is that strongylastacin may require a post-transcriptional modification for correct protein folding. Protein expression in the eukaryote expression system, such as in yeast, might solve the problem. Therefore, we investigated the molecular effects of immune response against *S. stercoralis* crude antigen, excretory-secretory antigens, strongylastacin and iPGM by dendritic cells. Our result showed that, same as crude antigen and excretory-secretory antigens, iPGM did not induce inflammatory cytokine production from DC2.4 cells. Unfortunately, iPGM did not induce the production of IL-

10 and TGF-β from DC2.4 cells like the *S. stercoralis* CA and excretory-secretory antigens from iL3s. These results suggested that the antigens that play the crucial roles in regulation mechanism is excretory secretory products of *S. stercoralis.* The iPGM antigen might involve in the suppression of inflammatory responses in the hosts, but not involved in the regulatory responses. The immunomodulation of the immune responses in the hosts should be complex and related to several antigens from the parasites.

The other targets of host immune responses manipulation by helminth parasites are pattern recognition receptors and co-stimulatory molecules. In this study, we found gene stimulation only in LPS experiment but not in antigens stimulation. The expression of TLR-2 and TLR-4 was not showing a significant difference between LPS and CA experiments. In contrast, the expression level of CD40 and CD80 showed a significant difference between LPS and CA experiments. The expression results suggested that crude antigen from *S. stercoralis* cannot trigger co-stimulatory molecules CD40 and CD80.

However, we found that crude antigen from iL3 larvae could trigger the high expression levels of both TNF-α and IL-10 genes. The fold changes of TNF-α and IL-10 were about 30 and 15 times, respectively at 1 hour after treatment. Interestingly, the concentration of TNF-α after CA treatment was undetectable. This might be due to the incomplete translation of mRNA into the active protein. Therefore, antigens from *S. stercoralis* might modulate post- transcriptional modification of some cytokines in the hosts.

In conclusion, our results suggested that *S. stercoralis* modulate the host's immune responses by introducing Treg response-related cytokines, including IL-10 and TGF-β. We also attempt to find the specific antigen that plays a crucial role in this modulation pathway. Excretory-secretory antigens from lived iL3 showed the same cytokine production pattern with crude antigens. The result suggested that antigens that play a crucial role in this modulation pathway are excretory-secretory products from iL3 of *S. stercoralis*. However, iPGM might not play the crucial role in this mechanism. Nevertheless, *S. stercoralis* may have more than one evasion mechanism.

More information about IL-2, IL-4, and IFN-**γ**, which require interaction from naïve T cell's co-stimulatory molecules and cytokines is still needed to be investigated. Co-culturing between DCs and T cells will also give more information about the immunomodulation of *S. stercoralis* and lead to the development of novel medical treatment for strongyloidiasis.

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