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นางสาวนวพร วรศิลป์ชัย

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IMMUNE RESPONSE IN PYTHIOSIS PATIENTS TREATED WITH *PYTHIUM INSIDIOSUM ANTIGEN* (PIA)

Miss Navaporn Worasilchai

A Dissertation Submitted in Partial Fulfillment of the Requirements
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การรักษาแบบ immunotherapy โดยใช้ *Pythium insidiosum* antigen (PIA) เป็นหนึ่งในวิธีการรักษาแบบผสมผสานสำหรับผู้ป่วยติดเชื้อโรค *P. insidiosum* ควบคู่ไปกับการผ่าตัดและการใช้ยาปฏิชีวนะ การรักษาด้วยวิธีดังกล่าวจะให้ PIA ทั้งหมด 7 ครั้ง โดยฉีดครั้งแรก ณ วันที่ 0 หลังจากผู้ป่วยได้รับการวินิจฉัยแล้วว่าเป็นโรค pythiosis และจากนั้นจะฉีดอีก 6 ครั้ง ณ เดือนที่ 0.5, 1, 1.5, 3, 6, 12 หลังจากการฉีดครั้งแรก ภายใต้สมมุติฐานว่าวัคซีนจะกระตุ้นให้เกิดการเปลี่ยนแปลงของการตอบสนองทางภูมิคุ้มกันของร่างกายจากการตอบสนองผ่านทาง T-helper2 (Th2) ไปเป็น Th1 ควบคู่ไปกับอาการทางคลินิกที่ดีขึ้นดังที่ได้มีการศึกษาก่อนหน้าในม้าและกระต่าย สำหรับการศึกษาการตอบสนองทางภูมิคุ้มกันหลังผู้ป่วยได้รับวัคซีนในคนนั้นยังมีอยู่น้อยมาก การศึกษานี้จึงมีวัตถุประสงค์ที่จะติดตามการตอบสนองทางภูมิคุ้มกันของผู้ป่วยผ่านรูปแบบการหลั่งไซโตไคน์ (การตอบสนองผ่านระบบ CMI), รูปแบบการหลั่ง *P. insidiosum* specific antibody; *Pi*-Ab (การตอบสนองผ่านระบบ HMI) และติดตามระดับของสาร (1,3)-β-D-glucan (สารประกอบโพลีแซคคาไรน์ พบในผนังเซลล์ของเชื้อ *P. insidiosum* และ PIA) ที่เปลี่ยนแปลงในตัวอย่างน้ำเหลืองผู้ป่วยทั้งก่อนและหลังได้รับวัคซีน ณ ระยะเวลาต่างๆ การศึกษานี้ผู้วิจัยได้รวบรวมผู้ป่วย pythiosis ที่มารับการรักษาในช่วงเดือนมกราคม พ.ศ. 2554 ถึงเดือนมิถุนายน พ.ศ. 2559 ในผู้ป่วย vascular pythiosis ทั้งหมด 50 ราย เราพบความสัมพันธ์ระหว่างระดับของสารชีวภาพ และลักษณะอาการแสดงทางคลินิกของผู้ป่วย ในผู้ป่วยที่ไม่มีอาการทางคลินิกที่บ่งบอกถึงการกลับเป็นซ้ำของโรคมีการเปลี่ยนแปลงของระดับไซโตไคน์จากกลุ่ม Th2 (IL-4, IL-5) ไปเป็นกลุ่ม Th1 (IFN-γ) พร้อมกับการเพิ่มขึ้นของ IL-10, IL-17 หลังรับการรักษาด้วย PIA ในด้านของการตอบสนองผ่านระบบ HMI ผู้ป่วยกลุ่มนี้ทั้งหมดมีระดับของแอนติบดีที่จำเพาะต่อเชื้อ *P. insidiosum* สูงตลอดระยะเวลา 1 ที่ได้รับ PIA (ELISA value; EV>6) ควบคู่ไปกับการลดลงของสาร (1,3)-β-D-glucan สำหรับผู้ป่วยอีกกลุ่มหนึ่งที่มีการติดเชื้อแบบเฉพาะที่ (local infection) จำนวน 30 รายนั้น ไม่พบการตอบสนองทางภูมิคุ้มหลังการติดเชื้อ แต่อย่างไรก็ตามการตอบสนองผ่านทางทั้ง CMI และ HMI เกิดขึ้นหลังจากผู้ป่วยได้รับ PIA แม้ว่าการตอบสนองทางภูมิคุ้มกันเหล่านั้นจะไม่พบความสัมพันธ์กับผลการรักษาของผู้ป่วยก็ตาม

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NAVAPORN WORASILCHAI: IMMUNE RESPONSE IN PYTHIOSIS PATIENTS TREATED WITH *PYTHIUM INSIDIOSUM ANTIGEN* (PIA). ADVISOR: ASSOC. PROF. ARIYA CHINDAMPORN, Ph.D., CO-ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., DR. RANGSIMA REANTRAGOON, M.D., Ph.D., 128 pp.

Immunotherapy using *Pythium insidiosum* antigen (PIA) is one of the combination treatment for *P. insidiosum* infected patients along with amputation and certain antifungal agents. The protocol of first prime (day0) after diagnosis and six booster doses (at 0.5, 1, 1.5, 3, 6, 12 months; mo.) along a year period were performed based on previous study. The switching of T-helper2 (Th2) to Th1 after immunotherapy along with the clinical improvement in equine and rabbit models were revealed whereas studies in human are very limited. To investigate the immune response in PIA treated pythiosis patients, cytokine profile (CMI response), *P. insidiosum* specific antibody; *Pi*-Ab (HMI response) and (1->3)-β-D-glucan (polysaccharide found in *P. insidiosum*'s cell wall component and PIA) in serum were examined before prime dose and each booster. Here, vascular and ocular pythiosis treated with immunotherapy during January 2011- July 2016 was recruited in this study. Among fifty vascular cases recruited in this study, the correlation between the tested parameters and the clinical outcome was found. The switching of Th2 (IL-4, IL-5) to Th1 (IFN-γ) cytokines along with the increasing of IL-10, IL-17 after PIA immunotherapy were found in patients with no sign of disease recurrent. Regarding HMI aspect, all of them demonstrated high constant level (ELISA value; EV>6) of *Pi*-Ab together with the decreasing trend of (1,3)-β-D-glucan level. Regarding, thirty ocular pythiosis, local infection, were enrolled in this study. Very less immune response was showed after infection. However, it clearly showed that both CMI and HMI were activated after PIA treatment even no correlation was found between the tested parameters and the clinical outcome.

Field of Study:	Medical Microbiology	Student's Signature	....
Academic Year:	2017	Advisor's Signature	....
		Co-Advisor's Signature	....
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Chapter I

Introduction

Human pythiosis is a life-threatening disease caused by "*Pythium insidiosum*" which can be found in stagnant water related to agricultural area or rice field in (sub) tropical or temperate regions (1, 2). In the infective stage, *P. insidiosum* is in form of zoospores. These zoospores can invade the host skin via injured lesion or soft tissue such as cornea leading to the "*P. insidiosum* infection" (3, 4). Presently, based on the clinical manifestation, two major forms of human pythiosis have been described: vascular form and localized form (3, 5, 6). In vascular pythiosis, after the invasion of *P. insidiosum* through the skin, it will germinate and spread through the blood vessel with ascending direction toward the main artery. With the rapid growth of this pathogen, arterial obstruction or aneurysm resulting chronic ischemia or gangrene ulcer of the extremities organ is the outstanding pathogenesis (4). As it was reported so far, most of the vascular pythiosis patients had underlying hemoglobinopathy complicated by iron overload or hemochromatosis such as thalassemia or aplastic anemia/paroxysmal nocturnal hemoglobinuria (PNH). Regarding the localized form, ocular infection, is the major pathogenesis, have been reported. Patients usually visit the doctors by the symptoms of pain and redness eye. By the confocal microscopy, positive branching hyphae are presented. Among patients from both groups, some characteristics are shared. There are 1) the history of water exposure, 2) rapid progression lesion with 3) ineffective treatment by using antifungal agents (6).

P. insidiosum is an aquatic fungus-like organism which belongs to the class Oomycetes, kingdom Stramenopila. Even their morphological structures and physiological properties are similar to true fungi as they develop mycelia, they show

closely relate to diatom and algae more than true-fungi by the phylogenetic analysis base on internal transcribed spacer (ITS) region (5, 7-9). This is the reason why pythiosis treatment using antifungal drugs alone is not effective. So far during no standard treatment has been established for pythiosis treatment, most of the physicians usually use the combination of 1) antifungal agents, mainly by systemic itraconazole (Sporal®), systemic terbinafine (Lamisil®), 2) amputation and 3) immunotherapy by *P. insidiosum* antigen (PIA) (6).

PIA is a crude antigen composes of both endo- and exo- antigen. Originally, PIA has been developed for animal pythiosis as a therapeutic vaccine. By using PIA, it can promote a switching of T-helper 2 (Th2) to Th1 cytokine production resulting in a favorable clinical response. In equine model, the switching of Th2 to Th1 response after immunotherapy is presented along with the decreasing number of *P. insidiosum* hyphae and the switching of eosinophil to be mononuclear cells, macrophage and lymphocyte at the infected skin called “kunker” are presented. (6, 10-13). Until the year 1998, PIA was used for the first time as an immunotherapy to a surgically unresponsive deep tissue infection invading the carotid artery patient who failed by other treatments. Fortunately, this patient responded well and recovered from the disease. Thus this is the starting point that PIA has been used in human so far (13). In 2004, Wanachiwanawin *et al*. published the retrospective study of 8 human PIA-treated pythiosis cases. Only one survived case was studied the immune response after PIA immunotherapy and he showed the “immunomodulation” of Th2 (IL-4, IL-5) to Th1 (IFN-γ) response. This phenomenon is demonstrated along with the changing of eosinophilic reaction to be mononuclear cells, macrophage and lymphocytes and the decreased level of immunoglobulin E (IgE) in the blood circulation (13).

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Besides the cell-mediated immune (CMI) respond, humoral immune (HMI) response is another system supported the pathogen clearance system of the host. However, very little information of HMI respond in PIA treated pythiosis patients has been studied. We knew only PIA treatment resulting in the increased level of *P. insidiosum* specific IgG antibody (*Pi*-Ab) in blood circulation (14).

Even PIA immunotherapy has been used in human since the year 1998, the problem of this treatment is its efficacy which is vary depended on the host. Around 97%-100% treatment efficacy was proposed in cattle and equine whereas in human cases, around 50%-60% of treatment efficacy has been reported (15-17). So far there are 2 publications reported the factors associated with PIA treatment. “Free margin amputation” is the determining factor of survival among vascular cases whereas “age of patients” is the determining factor of the saved globe among ocular cases (6, 18). However, so far the study of immune response in pythiosis patients who treated with PIA is very limited. Therefore, in this study we focused on the immune response in PIA treated pythiosis patients which is one of the essential data for the development of pythiosis treatment.

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To develop the effective pythiosis treatment, standard disease evaluation is another factor need to be established. Currently, *computed tomography* (CT) angiogram is the method used for pythiosis monitoring by observing the arterial obstruction. However, the 2 major limitations of CT angiogram are 1) the expensive test which cost around 50,000 baht per test and 2) the unavailable service in the local hospitals where most of the patients live. In this study, we are interested in “(1,3)-β-D-glucan” which we hypothesized that (1,3)-β-D-glucan level in pythiosis patients with significant disease burden or patients who retained fungal elements within their blood

circulation system would remain high based on the previous studies in animal models. The studies in rabbit models revealed serum BG level was correlated with severity of invasive pulmonary aspergillosis and BG level in cerebrospinal fluid might be useful in monitoring of therapeutic response in homogenous *Candida* meningoencephalitis.

In summary, here, we plan to investigate the immune response in PIA immunotherapy-treated pythiosis patients with both vascular and ocular form by following their CMI (serum cytokines profile) and HMI (*Pi*-Ab profile) response parallel with the biomarker suggesting the disease status along with a year course of immunotherapy.

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Chapter II
Rational

Since the year 1998 that PIA has been used in the first human case (19), the study of immune response in PIA-treated pythiosis patients is very limited (5). To improve the efficacy of PIA treatment, the basic information of immune response is essential leading to the research question of this study that "How do the CMI and HMI immune system in PIA treated pythiosis patients respond?".

Based on the previous publication, *P. insidiosum* is one of the pathogens which can activate the immune response of host after infection (20). In equine models and a human case study, *P. insidiosum* infection can activate the CMI respond via Th2 cytokine production (13, 17). However, after PIA immunotherapy, the switching of Th2 to Th1 response is demonstrated along with the clinical favorable effect which presently, standard disease evaluation is not available. Several methods for disease evaluation have been practiced. There are 1) directly monitoring of the existent of *P. insidiosum* hyphae at the infected site (17), 2) observing for the eosinophilic condition (13, 17) and 3) monitoring of the serum IgE level (13). In this study we were interested in "(1,3)-β-D-glucan", an immunogenic polysaccharide (21). We believed that the level of (1,3)-β-D-glucan can indicate the severity of pythiosis based on previous publications. In fungemia cases, during the process of fungal replication, (1,3)-β-D-glucan was released in blood circulation leading to the positive serum (1,3)-β-D-glucan (22). In term of HMI response, the capable of *P. insidiosum* infection to induce *Pi*-Ab was proved. However, there was only one case study proposed the increased level of

Pi-Ab after PIA treatment (14). By those limited data, it led us to design the experiment based on 3 objectives as below

- Characterize serology profile in pythiosis patients treated with PIA.
- Characterize cytokine production profile in pythiosis patients treated with PIA.
- Identify the pythiosis marker which can be applied as patients' evaluation.

By those 3 objectives, we hypothesized that

In vascular pythiosis cases, prior PIA immunotherapy, the level of Th2 cytokines, *Pi*-Ab and (1,3)-β-D-glucan were higher than the healthy control. After PIA immunotherapy, we expected to see the immunomodulation of Th2 to Th1 cytokines, the high constant level of *Pi*-Ab along with the decreased level of (1,3)-β-D-glucan suggesting the pathogen clearance of host.

In ocular pythiosis cases, since the ocular pythiosis was the local infection at immunoprivileged site, non-significant difference of cytokines profile, *Pi*-Ab and (1,3)-β-D-glucan level between ocular pythiosis cases and the healthy control were expected prior PIA immunotherapy. However, after PIA immunotherapy started, all the cases were treated with combination therapy including PIA which contained 2 pg/ml of (1,3)-β-D-glucan per a dose of PIA (2 mg/ml of PIA). Thus we expected the increased level of (1,3)-β-D-glucan along with the increased level of Th1 cytokines and *Pi*-Ab.

Chapter III
Review literatures

1. Background and history of *Pythium* spp.

Pythium spp. was classified in the class oomycetes, means “egg fungi” which refers to the large round oogonia. Class oomycetes is a large group of terrestrial and aquatic eukaryotic organism. For decades, it has been classified as true-fungi based on their morphology: filamentous vegetative growth, production of mycelia and spore formation in both sexual and asexual stages (23-27). Until the year 1969, phylogenetic analysis of oomycetes by internal transcribed spacer (ITS) nucleic acid sequence was performed (23-27). The obtained results indicated that oomycetes showed closely related to photosynthetic organism, such as diatom and brown algae more than true-fungi (9). Subsequently, oomycetes also demonstrate some different cell components compared to the true-fungi such as cell walls, cytoplasmic membrane etc. By those findings, class oomycetes is re-classified and grouped as fungus-like or parafungus organism which belong to the kingdom Stramenopila by Kreisel *et al*.

Oomycetes evolves with both saprophytic and pathogenic lifestyles including some of the most notorious pathogens of plants as either facultative or obligatory parasitic (4). It can be found worldwide both in fresh and saltwater habitats. This is the reason why oomycetes also has been known as a water mould (4). Presently, oomycetes is the only one class which is grouped in the phylum oomycota. This class can be arranged into seven orders as follow: Leptomitales, Rhipidiales, Saprolegniales, Peronosporales, Sclerosporales, Salilagenidiales and Pythiales (5). Regarding the member in order Pythiales, it contains both genus *Pythium* and genus *Phytophthora*.

Presently, more than 200 species in the genus *Pythium* have been described worldwide (28, 29). Most of them are noted as causative agents in the root of crop plants whereas only one species in the genus *Pythium* named *"P. insidiosum"* is revealed as pathogenic agents in mammals and birds called pythiosis. Although there is one publication reported the successful isolation of *P. aphanidermatum* from the invasive wound in a 21-years old army who injured during combat operations in Afghanistan in 2011 (30), it was quite difficult to prove whether it was the real causative agent or just being a contamination one. And also no any evidence of pythiosis caused by non-*P. insidiosum* has been documented so far.

The first mammalian case with pythiosis caused by *P. insidiosum* is reported in 1884 by British veterinarian named Smith who was working with horses in Indonesia. However, at that time the isolate was unable to identify. After that, this pathogen was later isolated by Dutch scientists who work with horses in the same country named Hoogkamer in 1901. At that time this disease has been recognized by various names depend on the origin such as bursattee/bursatte (India), espundia (Latin America), equine phycomycosis (Australia, USA), granular dermatitidis (Japan), hyphomycosis destruens equi (Indonesia), leeches (USA), swamp cancer (Australia, USA) and summer sores (Australia, Latin America, USA). Until the year 1961, it was named *"Hyphomyces destruens"* (*H. destruens*) which was developed from hyphomycosis destruens by Bridges and Emmons. However, this name is not published because Bridges and Emmons though that this organism was zygomycete so the disease was called phycomycosis. In 1974, Austwick and Copland observed some zoospores in the cultures on sabouraud dextrose agar after induced by some supplement contained media so they thought that *H. destruens* should belong to the class oomycete and

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genus *Pythium*. This was the reason why Chandler *et al*. defined the name of its disease as “Pythiosis” in 1980. In the same year, *P. insidiosum* is isolated from a horse and found some similar morphology against *P. gracile* by Ichitani and Amemiya. In 1987, De Cock *et al*. described the oomycetes with sexual sporulation as *P. insidiosum*. Moreover, Shipton *et al*. showed that *P. destruens*, isolated from Australian horse with pythiosis in 1987, was considered as the same species as *P. insidiosum* in the present (5).

2. Biology of *Pythium insidiosum*

By naked eye observation, *P. insidiosum* presents as mycelium form which is very similar to mould, true-fungi (5). However, the study on their cytoskeleton components found that microtubules and actin filament including mitochondria and golgi-bodies are observed around the tips of active growing hyphae. Those characteristics are one of the specific oomycetes characteristic which differ from true-fungi.

Similar to other fungi, *P. insidiosum* can grow on several fungal and bacterial culture media without antifungal/antibacterial agents. On Sabouraud Dextrose agar (SDA) and Sheep Blood agar (BA), *P. insidiosum* grows rapidly as submerged with white to colorless colonies (Figure 1A, 1B). The short aerial filaments with the fine radiate pattern or hair-like colonies are demonstrated in Sabouraud Dextrose broth (SB) (Figure 1C). Regarding microscopic characteristic, broad non-septate hyphae ranging from 4 to 10 microns in diameter are observed by direct examination using 10% potassium hydroxide preparation (10% KOH preparation) (Figure 1D) or by Lactophenol Cotton Blue (LCB) wet mount (Figure 1E). Only in water culture, zoosporangia with size 45-700 microns x 3-4 microns are produced together with filamentous and undifferentiated from assimilative hyphae. A vesicle with zoosporogenesis extrasporangial was

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approximately 20-60 microns in diameter. Encysted zoospores were 8-12 microns in diameter. Their oogonia are freely formed, sub-globose and intercalary (30). Their optimum temperature is ranging from $28^{\circ}C$ to $35^{\circ}C$. Cultivation at too low ($\leq 8^{\circ}C$) or too high ($\geq 42^{\circ}C$) temperatures resulting in death or inhibit growth of the organism (31).

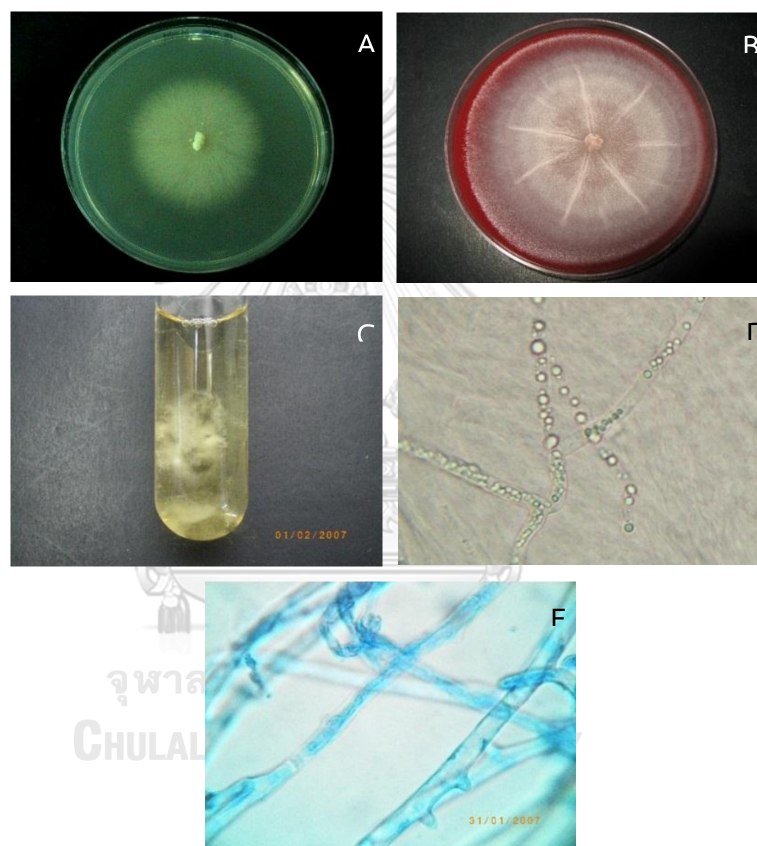

Figure 1 *Colony of P. insidiosum (A) three days old on SDA at 35°C, (B) five days old on BA at 35°C, (C) two days old on SB at 35°C, (D,E) P. insidiosum showing broad non-septate and sparsely septate hyphae in 10% KOH and LCB, respectively (400x).*

3. Life cycle

In nature, both sexual and asexual spores of *P. insidiosum* are produced depending on the environmental conditions. Interestingly, the appearance of both sexual and asexual spores can be presented on the same hyphae (4).

3.1 Sexual reproduction

The sexual spore of member in genus *Pythium* can reproduce by a structure called "gametangia". The gametangia composes of 1) antheridia (fairly small filamentous structures) and 2) oogonia (large and conspicuously swollen). In gametangia, both antheridia and oogonia are fertilized resulting in the creation of thick-walled structure named "diploid oospores". The gametes in antheridia are not produced as cells but only as nuclei containing the parental genome. In contrast to oogonia, oogonia produces large cellular structures called "oospheres" which containing a haploid nucleus in each cell. And finally, oospores need to wait for the suitable condition to grow (32).

3.2 Asexual reproduction

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Asexual biflagellate zoospores of *Pythium* spp. are produced and can be released from sporangium at the tip of each hyphae (Figure 3B). By two of flagella structure, zoospore are motile in water for hours supported by endogenous energy. The length of two flagella are difference, one is located in anterior called short flagellum: whiplash type for movement and another one is located in posterior called long flagellum: tinsel type for direction control. The life cycle of *P. insidiosum* in both environment and host was revealed by Mendoza *et al*. in 1993. In brief, zoospore, released from the sporangium, will present the chemotaxis motile to the host lesions, wounds, injured tissues, other damaged

skin parts or intestinal mucosa. Lesions caused by punctures and insect bites can also be a "port of entry" of *P. insidiosum.* It has been proposed that zoospore is not strong enough to penetrate the healthy skin. Generally, once zoospores contact plants or any accidental host tissues, zoospores will release their flagella and become immotile round shape cells called "encyst". After that, zoospore will secrete a sticky amorphous glycoprotein, supporting their tissue attachment before developing their germ tube which is stimulated by the host's body temperature together with infective structures which is prominent in pathogen form. However, if zoospores cannot find any susceptible host, they can also survive in the natural environment (33).

4. Molecular classification of *P. insidiosum*

By phylogenetic analysis based on Internal transcribed spacer (ITS), Cytochrome oxidase II (*Cox2*), Intergenic Spacer (IGS) and beta-glucanase (*exo1*) gene, *P. insidiosum* is classified into 3 clades related to geographical distribution (7, 9, 34). There are clade A_{TH}: mostly found in Latin America region, clade B_{TH}: usually found in Asia and Australia and clade C_{TH}: isolated from Thailand and USA (9).

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5. Epidemiology of human pythiosis

Pythiosis is one of the life-threatening diseases which mostly found in tropical, subtropical and temperate region (5). Since the first case of human pythiosis was reported in 1985, the continuously increasing trend of human cases has been revealed with the high incidence of both morbidity and mortality. So far Thailand has been claimed as an endemic area of this disease with the highest incidence in the world.

Not only in Thailand but the human cases were also found in other regions: Asia (ie. Malaysia, India China, Israel) Australia (ie. Australia, New Zealand) and America (ie. Brazil and USA) except Europe (5, 15, 19, 35-40).

In Thailand since the year 1985 (3), several human cases have been documented with high morbidity and mortality rate. In 2006, a retrospective study was performed in 9 tertiary care hospitals in Thailand during 18.5 years period (January 1985 to June 2003). A total of 102 cases with various pythiosis forms was diagnosed during this study period. Approximately, ranging from 1 to 13 pythiosis cases are diagnosed per year. Those cases are found all over Thailand. Almost half of cases found in the central part (46%) followed by the northeastern, northern, southern and eastern part with the percentage of 27, 16, 8 and 3, respectively. Age of patients is in the range of 20–60 years-old with mostly male. Most of them were associated with an agricultural occupation or water related career (1). This finding can be explained by the occupation based of Thailand. Due to Thailand being an agricultural country, there are many swampy areas that are natural habitats of *P. insidiosum*, resulting in high opportunity to acquire the infection (41). In term of definite diagnosis, this is another challenging. With the fact that rarely pythiosis cases are found and the physicians usually unfamiliar with this life-threatening disease, misdiagnosis possibly happens. Moreover, the clinical manifestation and pathogenesis of pythiosis are similar to other mycosis especially basidiomycosis and conidiomycosis resulting both underdiagnosis and misdiagnosis, making delay treatment (5). To overcome this obstacle, education of the physicians for more concerning of this rare infection is needed.

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6. Clinical correlation

The ability of *P. insidiosum* to grow and reproduce at the body temperature (37°C) is one of the outstanding adaptation for its virulence. Up to date, four types of pythiosis have been described. Interestingly, vascular and ocular form are the major form among human cases whereas (sub) cutaneous form is usually found in the animal (1).

6.1 Vascular pythiosis

Vascular type is the most common form among patients with hematological disorders such as thalassemia, aplastic anemia and paroxysmal nocturnal hemoglobinuria (PNH) which affect the arteries resulting in arterial occlusion or aneurysm. Regarding symptoms, several clinical signs are observed for example fever, paresthesia, itching, vesicle/bulla, skin ulcer, cellulitis, necrotizing fasciitis, the absence of arterial pulse, groin mass and abdominal mass (13, 42). In Thai human cases, more than 99% of vascular cases have hematological disorders as an underlying disease which is higher than other countries. One factor supporting this finding is "Thalassemic population". Thailand is an endemic area of Thalassemia containing around 1% of total populations. This might be another supporting factor related to the incidence of pythiosis in Thailand.

6.2 Ocular pythiosis

The pathogenesis of ocular pythiosis, mostly, shows ulceration in the cornea, resulting keratitis. Their clinical symptoms are a pain, irritation, decreased visual acuity, eyelid swelling, conjunctival injection and corneal infiltrates. Underlying disease is rarely found in ocular pythiosis patients which is differed from cutaneous/subcutaneous and vascular pythiosis (43). It is noted that the above criteria are only the guidelines for ocular pythiosis diagnosis, cultivation and PCR

with sequencing for definite genus and species identification are necessary. One publication reports patients infected with Lagenidiosis. Very similar clinical manifestations are presented at the first visit, however, after cultivation and sequencing processes, it is identified as *Lagenidium albertoi* (44).

7. Laboratory of pythiosis diagnosis

The earlier diagnosis is performed, the more successful treatment is revealed. However, due to various clinical manifestations of pythiosis among hosts, the experiences of physicians are absolutely indispensable. Up to the present, several diagnostic methods have been developed for example

7.1 Cultivation

Cultivation has been claimed as the "gold standard" method of diagnosis so far. To isolate *Pythium* spp. from clinical specimen, the sample is cut into small pieces around 2 mm in diameter and cultured on antifungal media such as SDA, BA, corn meal agar (CA) and potato dextrose agar (PDA). Keeping the specimen within the moisture condition which is suitable for the growth of *Pythium*, soaking with 200-500 µl sabouraud's dextrose broth (SB) at 35°C is recommended. In positive result, white to the colorless submerged colony will appear within 24 hours, approximately (41). However, false negative result usually happens by the improper specimen preparation (ie. cutting the specimen into too small pieces resulting organisms dead), improper specimen transportation or poor condition of cultivation such as dry condition, too high or too low temperature incubation.

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7.2 Serological method

Serology is another method has been developed for pythiosis diagnosis (45). However, with the principle of "antigen-antibody reaction", these serology based methods are limited only for systemic infection (vascular form). For example

7.2.1 Immunodiffusion test (17, 46)

7.2.2 Western blot analysis (47)

7.2.3 Enzyme-linked immunosorbent assay (48)

7.2.4 Immunochromatography assay (49)

7.2.5 Immunohistochemistry assay (50)

7.3 Molecular diagnosis-DNA based

Molecular diagnosis using DNA based is one of the high specificity method for *P. insidiosum* detection. This technique can be applied in both systemic and local infection using either isolates or clinical specimens. The negative result obtained by cultivation method can be proofed by fungal DNA detection directly from clinical specimen. Currently, several advance molecular methods have been developed as follow

7.3.1 Conventional polymerase chain reaction (PCR) (48)

7.3.2 Nested PCR (51-54)

7.3.3 Isothermal PCR (ie. thermophilic helicase DNA amplification: tHDA) (55)

7.3.4 Real-time PCR (ie. high resolution melting analysis: HRM) (5, 7)

However, by DNA based method, position of collecting of specimen is a key factor related to the obtained result. In vascular pythiosis cases, *P. insidiosum* hyphae usually form colonies and attached to the wall of artery, not freely flow in

the blood circulation. Thus the possibility of *P. insidiosum*'s DNA detection in blood sample (less invasive specimen) is very limited compared to the arterial clot or arterial tissue (invasive specimen). Similar to the ocular cases, the invasive specimen, cornea biopsy, is also required for the DNA based method.

With the limitations of each developed method mentioned above, high sensitivity method using less-invasive specimens such as blood samples need to be established.

7.4 Other biomarker for pythiosis diagnosis and monitoring

(1,3)-β-D-glucan is a polysaccharide which is a major component of the fungal cell wall. Presently the detection of serum (1,3)-β-D-glucan is applied from endotoxin assays, measuring activation of factor G through horseshoe crab substrates. By the optical density at wavelength 490 nm (OD_{490}) obtained from spectrophotometer readings, it can be converted into (1,3)-β-D-glucan concentration. Based on the commercial kit which is available in Thailand named "Fungitell assay" (Associates of Cape Cod, Inc., East Falmouth, MA, USA), the results are interpreted as negative (range < 60 pg/mL), indeterminate (60 pg/mL to 79 pg/mL), or positive (>80 pg/mL) (22, 56-59).

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Currently, Fungitell assay is widely used for fungal diagnosis. However, in the application of disease monitoring, Fungitell assay was applied in only some certain fungal infection disease. The decreasing trend of (1,3)-β-D-glucan level in serum was showed among the 203 cases with proven invasive candidiasis and the 40 cases with proven invasive aspergillosis who responded well to echinocandin and anidulafungin (22, 59). Similar to another study, 11 invasive aspergillosis (IA): five proven IA cases, three probable and three possible IA cases, patients with non-

responding to antifungal therapy did not show a decrease in (1,3)-β-D-glucan levels (60). These results suggested that (1,3)-β-D-glucan antigenemia is another non-invasive method and useful in predicting therapeutic outcomes in patients with IA. Currently, (1,3)-β-D-glucan measurement by "Fungitell assay" was approved by Food and Drug Administration (FDA) of United State of America as a tool to diagnose the deep-seated mycoses and fungimia and by European medical center as a presumptive diagnosis of invasive fungal disease. Moreover, the EORTC-MSG panel also included (1,3)-β-D-glucan test as one of the criteria for positive invasive fungal infection (21, 61, 62).

In general, the high sensitivity tests mostly provide less specificity. Fungitell® assay also provides high sensitivity, measuring up to picogram per milliliter with low specificity. Positive (1,3)-β-D-glucan in serum sample can be detected in most of fungal infection such as *Candida* spp. and *Aspergillus* spp. except in the member in order Mucorales: *Mucor* spp., *Rhizopus* spp.; in Phylum Basidiomycota: *Cryptococcus* spp. due to very small amount (1,3)-β-D-glucan in their cell walls. Positive (1,3)-β-D-glucan is found not only in most of fungi as mentioned above but also in both non-fungal infections such as *Pseudomonas* spp. or *Streptococcus spp.* infection and non-infectious disease conditions such as hemodialysis with cellulose membranes, intravenous immunoglobulin, use of cellulose filters for intravenous administration, albumin transfusion, gauze packing of serosal surfaces, intravenous amoxicillin-clavulanate and intravenous administration. Even some limitations of Fungitell assay was claimed, Infectious Disease Society of America (IDSA) guideline; 2016 still recommended the Fungitell assay as a biomarker for invasive fungal

infection in the high-risk population including patients with underlying hematologic malignancy or allogenic hematologic stem cell transplant (HSCT) (21, 22, 56-66).

In term of *P. insidiosum* infection, even (1,3)-β-D-glucan is also proved as a part of *Pythium* cell wall component (34, 67-69), Fungitell assay has not been applied for both diagnosis and disease monitoring. Based on the previous publication, it was reported that during *P. insidiosum* replication, (1,3)-β-D-glucan is released into blood circulation (68). Thus the application of Fungitell assay as one of the pythiosis diagnosis using blood sample (less invasive specimen) is possible. Even though only positive serum (1,3)-β-D-glucan cannot indicate the definitive pythiosis diagnosis, testing parallel with other *P. insidiosum* specific diagnosis method such as DNA-based or serology based method is practical. Another advent of (1,3)-β-D-glucan is that it can be measured the level and present as quantitative result. Therefore the application of (1,3)-β-D-glucan level for disease monitoring is also possible. Based on the previous report that (1,3)-β-D-glucan is released into the blood circulation during *P. insidiosum* replication (68), we hypothesized that the decreased serum (1,3)-β-D-glucan level should be presented in patients who have no symptom of disease recurrent. Here, the application of (1,3)-β-D-glucan level as pythiosis diagnosis and monitoring is one of the objectives in this study.

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8. Immune response of host in infection

In general, once the infection happen, immune response is the main system of host to control the disease and eliminate those pathogens.

8.1 Immune response to *P. insidiosum* infection

Presently, the knowledge of immune response to *P. insidiosum* both in humans and animals are not well understood. Especially in human, there is only one publication studied the immune response in one vascular cases after *P. insidiosum* infection so far (13). In this publication, they reported that positive *P. insidiosum* specific IgG antibody, high level of Th2 cytokines which is IL-4 and IL-5, increased number of eosinophil cells and high level of IgE compared to healthy population were found in patient with *P. insidiosum* infection. These are the information in human have been documented so far. The role of the complement system, phagocytic cells, dendritic cells, cytotoxic lymphoid cells or other related immune cells have not been studied yet.

More information related to immunity to *P. insidiosum* infection was reported in equine model with subcutaneous pythiosis. In 2005, Mendoza *et al* reported that in natural infection, after *P. insidiosum* zoospores attached to the injured tissue of a host, the encysted zoospores will develop a germ tube and penetrate into the host tissue. At the infected stage, *P. insidiosum* secretes exoantigen around their hyphae. Once those antigens expose to the antigen presenting cells (APCs), APCs will release IL-4 resulting in naïve T helper cells (Th0) activation and differentiation to be T helper 2 (Th2), in order. The stimulated Th2 subset will release IL-4, IL-5. These released cytokines will stimulate B cell to produce immunoglobulin E (IgE), IgG and IgM molecule. IgE and IL-5 will trigger the mast cells and eosinophils migration to the infected site and degranulation of cells causing tissue damage in the infected host (figure 2). (5, 17).

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By the publications mentioned above, we found that the Th2 response together with eosinophilic and IgE production after infection were revealed in both human and animal pythiosis. Interestingly, these Th2 response together with eosinophilic and IgE production are similar to the immunity to parasite infection instead of fungal infection. Even Th2 response was revealed in some fungal infection such as *Basidiobolus* spp. and *Conidiobolus* spp., that is the mechanism of immune evasion of them.

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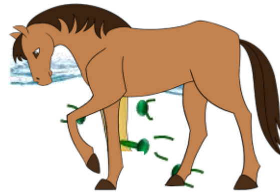

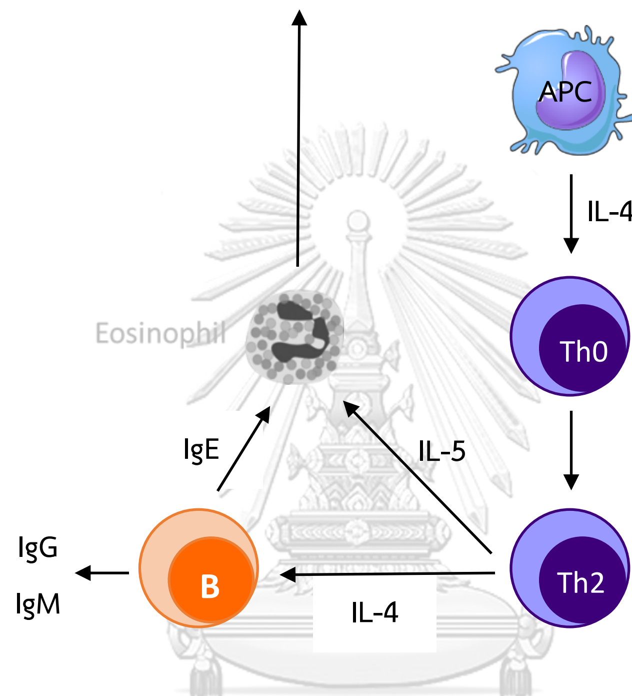

Figure 2 The immune response of *P. insidiosum* infection in equine model. Once the antigens of *P. insidosum* expose to the antigen presenting cells (APCs), IL-4 will be released to activate naïve T helper cells (Th0) to be T helper 2 (Th2), in order. Then, the IL-4 and IL-5 produced from Th2 cells will induce B-cells to release IgE, IgG and IgM, At the same time, IL-5 will activate mast cells and eosinophils migration to the infected site and degranulation of cells causing tissue damage in the infected host.

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8.2 Immune response to parasite infection

Due to the complicated cell composition of parasites, the innate immune response is not effective for them. Most of the pathogenic parasites are tolerance to phagocytosis system and able to survive in the phagocytic cells.

In parasite infection, Th2 cells is the first response after infection. By the Th2 response, the cytokines such as IL-4, IL-5 and IL-13 produced by both CD4+ and CD8+ T-cells will induce B-cells to produce the IgE and activate the fundamental immune cells in protecting against parasites such as eosinophils and basophils. The binding of IgE to the circulating basophils can induce the release of histamine and other cells related to immediate hypersensitivity reaction resulting the destruction of parasites (70, 71).

Presently, the exact cause of Th2 response after parasite infection has not been clarify. Most of the parasites present chitin (antigen drive Th2 response) as cell membrane composition during infection (70, 71) which is similar to cell wall composition of *Basidiobolus* spp. and *Conidiobolus* spp. (27, 72) whereas cell wall of fungi mainly compose of β-glucan and mannan (antigen drive Th1 response) (73).

8.3 Immune response to fungal infection

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Generally, some fungi can exist in the human body (normal flora) as commensals such as *Candida albicans* (*C. albicans*). However, once the host immune weaken, these normal flora can multiply themselves and act as a pathogen (74). Similar to several kind of fungi in environment such as *Aspergillius fumigatus* (*A. fumigatus*), *Penicillium* spp., *Cryptococcus neoformans* (*C. neoformans*) and *Histoplasma capsulatum* (*H. capsulatum*) which normally they habit as saprophytes, spore inhalation or invasion through any lesion of immunocompetent host are rarely cause the disease.

However, once the immune of host become weak such as on-going immunosuppressive drugs, long-term antibiotic treatment, and corticosteroid treatment etc., they can act as pathogens (75, 76).

8.3.1 Innate immunity (77-80)

Once the pathogen invade into the host cells, immune cells will play an important role at this stage. Most of immune cells such as monocyte, neutrophil, and dendritic cells present a set of pattern recognition receptor (PRRs) such as dectin-1, toll-like receptor *etc* which can recognize the invariant molecular structure of fungal cells called pathogen-associated molecular pattern (PAMPs) such as chitin, mannan and β-glucan *etc*. The PAMPs-PRRs interaction leads to the activation of antifungal effectors functions in phagocytosis, initiation of killing mechanisms (e.g. production of reactive oxygen species) and the development of adaptive immunity. At the same time complement system also is activated. Once the fungi invade the host cells, it will promptly activate alternative pathway resulting to C3b deposition. The deposition of C3b will serve to fungal opsonization by phagocytic cells. Moreover, via the alternative pathway, membrane attack complex (MAC) formation can be formed resulting to fungal killing activity.

After the process of fungal opsonization and PAMPs-PRRs interaction, neutrophils, macrophages, monocytes, and dendritic cells will play a role as phagocytic cells. Besides, the phagocytic activity, these immune cells also play a role as pro-inflammatory cytokine producing cells. The production of early cytokines by innate cells is a one of the

significance response to fungal infection. These mediators play a critical role in activating phagocytes and orchestrating the development of adaptive immunity. Moreover, macrophages, monocytes, and dendritic cells also can act as antigen presenting cells (APCs) to present the antigen to T-cells resulting the activation of adaptive immunity.

8.3.2 Adaptive immunity

Once the fungal antigen is presented to Th0 via APCs, Th0 will be activated to be Th1 by the supportive of IL-12 and IFN-γ which produced by the innate immune cells. Several cytokines such as IFN-γ, IL-2, TNF-β from Th1 response will promote the protective cell-mediated immune response through other T-cells activation and induce the phagocytic activity of phagocytic cells resulting to granulomatous inflammation. This reaction is very effective to kill and limit the growth of infected fungi. Moreover, IFN-γ also can inhibit Th2 response. Besides Th1 activation, Th17 was also induced through the activation of signaling pathways involving the TLR adaptor MYD88 resulting IL-17 production. In invasive fungal diseases, Th1 response which is the protective immunity plays a major role to control the invasion. Only minor response from Th2 cells are recognized as immune balancing to protect the pathogenesis caused by over response of Th1 (81, 82).

8.4 Immune evasion mechanism in *Basidiobolus* spp. and *Conidiobolus* spp.

Even though, the host provided the immune system to control the fungal infection, several fungi still can develop the mechanism to evade host immune

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system. Fungal cell wall is a dynamic structure that is continuously changing throughout the fungus cell cycle and during morphological transition. For example, (1,3)-β-D-glucans are exposed in the bud scar of *C. albicans* but are masked on hyphae which is the invasive form in host for escaping from the recognition of dectin 1 (83). The similar mechanism of immune evasion is also reported in *Basidiobolus* spp. *Conidiobolus* spp., the causative agents of similar tissue reaction in subcutaneous type of infection. Both *Basidiobolus* spp. and *Conidiobolus* spp. modify their cell wall component to present mainly chitin instead of (1,3)-β-D-glucans during infection. Chitin will activate Th2 response resulting IL-4, IL-5 and IL-10 production together with eosinophilic and IgE production which is similar to the immune response of parasite infection. Unfortunately to host, Th2 response is not able control *Basidiobolus* spp. and *Conidiobolus* spp. infection. Moreover, IL-4 and IL-10 produced from Th2 response also can inhibit the Th1 response. These mechanism leading the successful of immune evasion of *Basidiobolus* spp. and *Conidiobolus* spp. (27, 72).

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Regarding the humoral immunity which mainly involved with B lymphocytes. In fungal infection, humoral immune response plays a minor role compared to cell mediated immune response or act as a supportive mechanism. The IL-4 produced from Th2 cells will activate the B cell proliferation and differentiation to be memory B cells or plasma cells. Memory B cells are long-lived and prompt for the rapid response to the secondary antigen exposure whereas plasma cells produce antibodies called immunoglobulins specifically to the antigens. Those antibodies will activate the

immune cell such as macrophages for the pathogen clearance by the phagocytosis enhancement and the classical complement pathway activation (77).

In summary of the immunity to *P. insidiosum* infection, presently, we know very little information about them especially in human. These basic knowledge are very important to understand the pathogenesis of pythiosis and also being the fundamental information for treatment development in the future. Therefore, in this study another objective is aim to investigate the immune response in pythiosis patients both in with vascular and ocular infection.

9. Treatment of human pythiosis

As mentioned in the first part of review literature section, *P. insidiosum* is a fungus-like organism so using antifungal drugs alone is not effective for pythiosis treatment especially the antifungal drugs targeting the ergosterol. Since the year 1990, a saturated solution of potassium iodide (SSKI) and amphotericin B have been used in human pythiosis along with the aggressive surgery. It presents a good effect for cutaneous/subcutaneous form whereas almost no response is demonstrated among vascular pythiosis patients which are the major population of human cases. Moreover, the major limitation of SSKI is its toxicity (17, 41). Therefore another treatment method was investigated. Based on the hypothesis that host immune response induced by natural infection itself is not effective enough to control *P. insidiosum* infection, activate more of immune response might increase the effective of them resulting to

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P. insidiosum killing. Mendoza L *et al*. started using crude *P. insidiosum* antigen in equine model via subcutaneous injection as a therapeutic vaccine (5). And they found that Th1 response was activated instead of Th2 response as presented in natural infection together with the decreased number of *P. insidiosum* hyphae and eosinophil in the infected tissue of equine (17). This finding indicated that Th1 response can control *P. insidiosum* infection. By the study of Mendoza L *et al*., they reported that after immunotherapy, the Th1 cells subset release IFN-γ and IL-2. These cytokines can trigger the cell-mediated immunity (CMI) including cytotoxic lymphocytes (CTL) and macrophages. The CMI response can damage infected *P. insidiosum* hyphae and finally, B cells are stimulated to produce the protective IgG classes (figure 3) (5, 17).

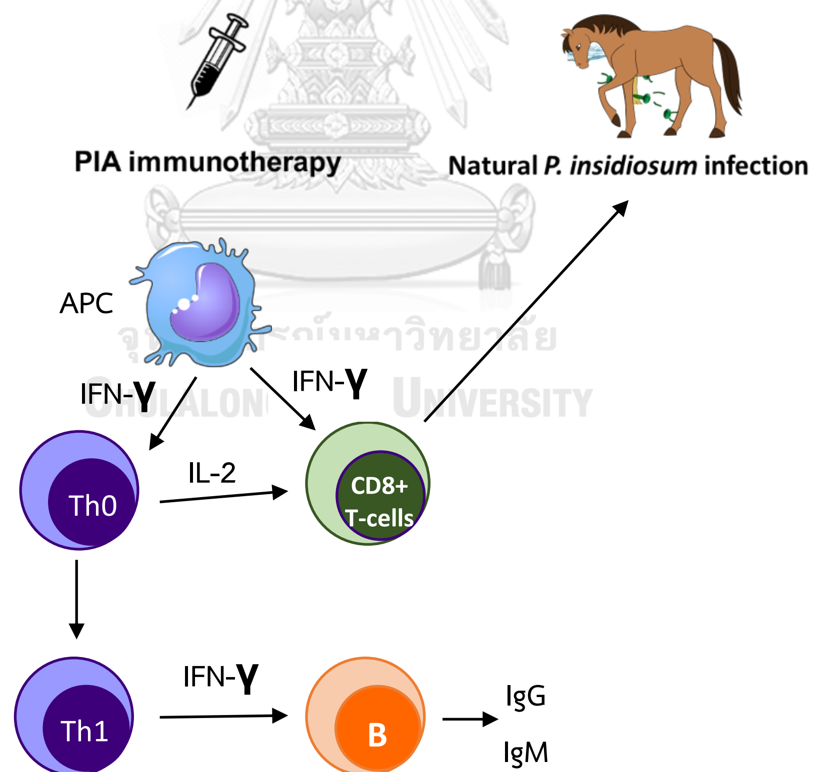

Figure 3 The immune response of equine with pythiosis after treated with PIA immunotherapy. By the activation of PIA immunotherapy, the IFN-γ and IL-2 are produced by the Th1 cells. These cytokines will trigger the cell-mediated immunity

(CMI) including cytotoxic lymphocytes (CTL) and macrophages. These response can damage the infected *P. insidiosum* hyphae and finally, B cells are stimulated to produce the protective IgG antibody.

After that this developed therapeutic vaccine has been used in animal pythiosis for years (6, 10-13, 17, 84). Until in 1998, there is a case of Thai boy with pythiosis who showed non-response to any treatment. Without any choice of treatment, the physicians decided to use the therapeutic vaccine in this case. Fortunately, he responded well with immunotherapy and survived from the disease. This is the starting point that the developed therapeutic vaccine was used in human case as an immunotherapy. Due to the developed therapeutic vaccine is the crude antigen prepared from *P. insidiosum*, it is called *P. insidiosum* antigen or PIA. Presently the standard treatment for pythiosis has not been established yet. The combination therapy of surgery, antifungal treatment and immunotherapy has been practiced based on the previous publication (6).

In vascular pythiosis, most of the physicians usually use a combination of 1) antifungal drugs, mainly by systemic itraconazole (Sporal®) and systemic terbinafine (Lamisil®), 2) organ amputation and 3) immunotherapy. However, this disease still carries very high mortality rate ranging from 36.4% to 44.4% (6, 18). Even “sterile margin amputation” is reported as a determining factor of survival in vascular cases in 2015, adequate surgery is not practical in some cases for example neck infection or in the cases with progressive disease. Therefore, early diagnosis and early surgery are one the significant condition to save the patient’s life, however, the duration time of disease onset to the first medical surgery is uncontrollable by clinical practice. Moreover, the morbidity which is dreadful among survivors of vascular cases due to the need of

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aggressive amputation to remove all infected tissues is another challenge needed to be concerned (6, 18). In case of antifungal treatment, even they were claimed as ineffective agents for pythiosis treatment, they still have been used so far. The synergistic effect is demonstrated by broth macrodilution against 17%-40% of Brazilian *P. insidiosum* isolated from animals even no synergistic reaction is reported among Thai isolates (85-93). Thai *P. insidiosum* isolates are classified into clade B_{TH} and C_{TH}, whereas those from Brazil belong to clade A_{TH} (7, 9). We hypothesized that clade is another factor of successful treatment. During that hypothesis has not been proved and no major side effect of the antifungal drug has been claimed, antifungal agents are still used as one of the combination therapy in the present.

9.1 Immunotherapy

Currently, the protocol of PIA immunotherapy in human cases is depending on the previous publication which is varied across different institutions. At King Chulalongkorn Memorial hospital, PIA schedule is composed of a single prime dose followed by subsequent 6 booster doses across a year period. The first PIA vaccine of 2 mg/mL is administered subcutaneously as soon as the definitive diagnosis is established. Subsequent booster doses are administered every 2 weeks for 1.5 months, followed by 3 doses of PIA vaccine at 3, 6 and 12 months. In some academic institute, at least 2 injections of 100 μl each of antigen, 2 mg/mL, at two weeks interval are practiced (6).

In ocular cases, similar treatment process to vascular patients has been performed. Even though no patient dies by this infection, around 50% of them become lifelong blindness due to enucleation or penetrating keratoplasty (PK). Base on the previous report, age might be one of the prognostic factors in patients

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with ocular pythiosis. Patients without underwent enucleation are significantly younger than those who underwent enucleation. In case of antifungal treatment, besides systemic itraconazole (Sporal®) and systemic terbinafine (Lamisil®), additional tropical amphotericin B (Fungizone®) have been used in ocular cases. Similar to vascular pythiosis patients, antifungal agents has been recommended so far because there is a synergistic effect based on a case report of periorbital cellulitis in a child who is completely cured by those antifungal agents. In term of PIA immunotherapy, the same schedule as vascular cases has been practiced (6).

Even though immunotherapy has been used in human for more than 30 years, the understanding of immune response in both infection and the PIA immunotherapy is very limited. In 2004, there was a study in a case of vascular pythiosis reported the switching of Th2 (IL-4 and IL-5) to Th1 (IL-2) response after one month of PIA immunotherapy along with no symptoms of disease recurrent for 7 months. This publication studied only in one time-point, 1 month, after PIA immunotherapy and only in 3 cytokines as mentioned above (13). In addition, as far as the literatures have been reviewed, no publication related to humoral immune response after PIA immunotherapy administration was found. These basic information are very essential to understand the pathogenesis of pythosis and also to improve the effective treatment of this disease. Moreover, up to the present, we still could not get the view point of immune response among the successful and unsuccessful PIA immunotherapy cases. Moreover, most of the available studies, the number of the patient sample was very small. These brought us to investigate the immune response profile for both cytokines (CMI response) and *P.*

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insidiosum specific antibody (HMI response) along a year course of PIA immunotherapy in PIA immunotherapy treated pythiosis patients during 6.5 years-period (Jan 2010 - July 2016). At the same time, we also identify the biomarkers for pythiosis diagnosis and treatment monitoring.

10. Aim of Ph.D. Thesis

From our current view of pythiosis, the aim of the Ph.D. thesis includes

- Characterize serology profile in pythiosis patients treated with PIA.
- Characterize cytokine production profile in pythiosis patients treated with PIA.
- Identify the pythiosis marker which can be applied as patients' evaluation.

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CHAPTER IV
Materials and methods

1. Study Design

The study of vascular and ocular pythiosis patients who received a combination therapy of surgery, systemic antifungal agents, and immunotherapy using PIA according research treatment protocol at King Chulalongkorn Memorial Hospital during January 2010-July 2016, totally 6.5 years period.

2. Ethical review

This study was approved by the Chulalongkorn University Institutional Review Board (IRB no. 531/58) based on the International Guidelines for Human Research Protection as Declaration of Helsinki, the Belmont Report, Council for International Organizations of Medical Sciences (CIOMS) Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

3. Patients

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Vascular and ocular pythiosis patients over the age of 18 years-old were enrolled in this study. The inclusion criteria was

3.1 Vascular cases

3.1.1 Positive at least two of these following criteria

- Successful isolation of *P. insidiosum* and zoospore production
- Positive polymerase chain reaction (PCR) and sequencing by internal transcribed spacer (ITS) gene and cytochrome oxidase II (*COX2*) regions either from the isolates or directly from the clinical specimens.

- Positive *P. insidiosum* specific IgG antibody (*Pi*-Ab) by established *in-house* ELISA

3.1.2 Treatment with the combination protocol as follow

- Surgery (amputation or debridement)
- Antifungal agents: systemic itraconazole (Sporal®), systemic terbinafine (Lamisil®)
- Immunotherapy with PIA at least 1 year period for totally 7 doses*

3.2 Ocular cases

3.2.1 Positive two of these following criteria

- Successful isolation of *P. insidiosum* and zoospore production
- Positive PCR and sequencing by internal transcribed spacer (ITS) gene and cytochrome oxidase II (*COX2*) regions either from the isolates or directly from the clinical specimens.

3.2.2 Treatment with the combination protocol as follow

- Surgery (enucleation/ penetrating keratoplasty: PK) or corneal transplantation)
- Antifungal agents: systemic itraconazole (Sporal®), systemic terbinafine (Lamisil®), tropical amphotericin B (Fungizone®)
- Immunotherapy with PIA at least 1 year period for totally 7 doses*

** By the protocol of PIA immunotherapy practiced in King Chulalongkorn Memorial hospital, totally 9 doses of PIA within 3 years is practiced. However, based on the incidence of patients monitoring after PIA treatment started, around 80% of patients lost the contact after 1 year of PIA immunotherapy which their symptoms*

disappeared. Moreover, based on our preliminary study showed that the immune responses were obviously presented by the first 3-6 months so the patients who received at least 1 year (7 doses of PIA) PIA immunotherapy according to the KCMH protocol were enrolled in this study.

4. Clinical data and clinical specimens (plasma and peripheral mononuclear cells: PBMC) collection

After receiving the definite diagnosis, the patients were started three years course of immunotherapy using PIA for totally 9 doses. The 2.0 mg/mL PIA was administered via subcutaneous route according to PIA schedule. The 1st dose (prime dose) was given to patient at the time of diagnosis (Day0) followed by 8 booster doses at 0.5 (2nd dose), 1.0 (3rd dose), 1.5 (4th dose), 3.0 (5th dose), 6.0 (6th dose), 12.0 (7th dose), 24.0 (8th dose) and 36.0 (9th dose) months after prime dose, respectively. PIA information was prepared for all patients together with the noted message that PIA was "under investigation". Both clinical data and clinical specimens were collected at the time of diagnosis and in every visit during 1 year follow-up period as follow

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4.1 Clinical data collection

The clinical data were recorded in case record (CRF) form, specifically designed for this research. The data we collected composed of

4.1.1 Vascular cases

4.1.1.1 Patient-related data

- Age
- Gender
- Occupation (agriculture related or non-agriculture related)
- History of water exposure within 3 months

- Underlying hematologic diseases
- Baseline serum ferritin level (ng/ml)
- Duration from disease onset to the 1st medical attention (month)
- Duration from diagnosis to the 1st definitive surgery (month)

4.1.1.2 Vascular pythiosis-related data

- Anatomical lesions
- Duration from disease onset to the 1st medical attention (month)
- Duration from diagnosis to the 1st definitive surgery (month)
- Type of surgery
- Types and duration of antifungal treatment
- Iron chelating therapy

4.1.1.3 Signs and symptoms for possible residual diseases (collected during 1 year follow-up period)

- Fever
- Pain
- Skin rash
- Mass at surgical sites
- Arterial insufficiency
- Syndrome (ie. claudication, paresthesia, gangrenous ulceration)
- Inflammation at surgical site (ie. redness, swelling)

4.1.2 Ocular cases

4.1.2.1 Patient-related data

- Age

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- Gender
- History of water exposure_within 3 months

4.1.2.2 Ocular pythiosis-related data

- Duration from disease onset to the 1st medical attention (month)
- Duration from diagnosis to the 1st definitive surgery (month)
- Type of surgery
- Types and duration of systemic antifungal treatment
- Types and duration of tropical antifungal treatment

4.1.2.3 Signs and symptoms for possible residual diseases (collected during 1 year follow-up period)

- Fever
- Pain

4.2 Clinical specimens' collection

To follow the level of (1,3)-β-D-glucan, *Pi*-Ab and cytokine in pythiosis patients who received PIA immunotherapy, sixteen milliliters of whole blood samples were collected using acid citrate dextrose (ACD) vacuum tube (Cat no. 364606, BD Vacutainer®, USA) prior to PIA administration both at the time of diagnosis and in every visits. In some cases who were transferred back to their home provinces during 1 year follow-up period, the PIA immunotherapies were delivered to the local hospitals where those patients follow-up. In every visit, their blood samples were collected by the local hospital-staffs and delivered back to King Chulalongkorn Memorial Hospital within 24 hours.

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4.2.1 Plasma sample preparation

The whole blood sample was centrifuged in refrigerated Hettich® Universal 320/320R centrifuge (Cat no. Z722871, Sigma-Aldrich, USA) at 3,000 round per minute (rpm) for 20 minutes at 10°C for plasma collection. The plasma was aliquot in 1.8 ml Nunc® CryoTubes (Cat no.V7884, Sigma-Aldrich, USA) and kept at -80°C systemically.

4.2.2 Peripheral blood mononuclear cell (PBMC) preparation

Human PBMC were isolated from buffy coat using Ficoll-Hypaque density gradient centrifugation. In brief, after plasma removal, the blood sample was diluted by 10% heat-inactivated fetal bovine serum: FBS (Cat no. 16000044, Gibco, USA) complete medium as 1:1 ratio v/v. The amount of 7 ml of diluted cells suspension was carefully overlaid over the same volume of Ficoll-hypaque (Cat no. 21101228, IsoPrep® Sunnyvale, Norway) in 15 ml Nunc® conical sterile polypropylene centrifuge tube (Cat no. 339650, Sigma-Aldrich, USA). Then the mixture was centrifuged at 3,000 rpm for 30 min at 10°C using a swinging bucket rotor without break of refrigerated Hettich® Universal 320/320R centrifuge (Cat no. Z722871, Sigma-Aldrich, USA). Then the upper layer was removed. The left layer represented the mononuclear cell part, contained lymphocytes, monocytes, and thrombocytes, undisturbed at the interphase. The mononuclear cell layer was carefully transferred to the new 15 ml Nunc® conical sterile polypropylene centrifuge tube (Cat no. 339650, Sigma-Aldrich, USA) and washed the cells using 5 ml of 10% FBS (Cat. no. 16000044, Gibco, USA) complete medium. After that, the washed mononuclear cells was centrifuged at 3,000 rpm for 10

min, 10°C. After centrifugation, the supernatant was completely removed and repeated the washing step twice. The viability of PBMC was determined by trypan blue staining (Trypan Blue solution Cat no. T8154, Sigma Aldrich, USA) and kept in freezing medium contains 90% FBS (Cat. no. 16000044, Gibco, USA) and 10% dimethyl sulfoxide (DMSO) (dimethyl sulfoxide solution ≥99% Cat no. M81802, Sigma-Aldrich, USA) in liquid nitrogen for long-term storage. All samples were aliquoted as $1x10^{6}$ cells/1.8 ml Nunc® CryoTubes (Cat no.V7884, Sigma-Aldrich, USA).

4.3 Control group

Age and gender-matched of both thalassemic and non-thalassemic healthy volunteers were enrolled in this study as the control group for vascular pythiosis and ocular pythiosis, respectively. All of them were collected both their basic demography (age, gender, occupation, type of thalassemia and history of water exposure) and whole blood samples.

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5. PIA immunotherapy preparation

The preparation of PIA immunotherapy was modified from the original method. In brief, 5-day-old *P. insidiosum* were cultured in Sabouraud dextrose broth (Difco™ Cat no. 238230, BD, USA) at 37°C and inactivated with 0.02% (wt/vol) Thimerosal (Thimerosal ~98% HPLC, Cat no. T2299, Sigma-Aldrich, USA) for 30 min. The antigens were precipitated from disrupted mycelial masses and culture supernatant. For long-term storage, obtained antigen were lyophilized and kept at -80°C until use (16, 94).

6. ***Pythium*** **isolation, cultivation and zoospore production**

6.1 ***P. insidiosum*** **isolation from clinical samples**

P. insidiosum isolation is one of the gold standard method to prove the infection of *P. insidiosum*. Similar to other fungal cultivation, suspected specimen (ie. vascular tissue, corneal tissue, corneal scrapes *etc*) which presented broad non-septate hyphae ranging from 4-10 micron in diameter by direct examination using 10% potassium hydroxide preparation: 10% KOH preparation (Cat no. 1310-58-3, Sigma-Aldrich, USA) was cultured on Sabouraud Dextrose Agar (Difco™ Cat no. 238230, BD, USA), Blood agar (Thermo Scientific™ Blood Agar Cat no. CM0055, France) and Corn Meal Agar (Oxoid™ Cat no. CM0103, UK) at 30°C and 35°C. The cultured plate was observed every day. In positive *P. insidiosum* isolation, the submerged colony with white to colorless, fine radiate pattern and hair-like characteristic were demonstrated by 5 days-old. The microscopic characteristic of broad non-septate hyphae of P. insidiosum can be observed using lactophenol cotton blue: LCB wet mount (Cat No. R03465, Sigma-aldrich, USA).

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6.2 ***P. insidiosum*** **cultivation**

After *P. insidiosum* was successfully isolated, it can be cultured in both fungal and bacterial medium without any antifungal and anti-bacterial agents. In this study, Sabouraud Dextrose Agar (Difco™ Cat no. 238230, BD, USA) and Blood agar (Thermo Scientific™ Blood Agar Cat no. CM0055, France) were used and incubated at 30°C and 35°C with the moist atmosphere.

6.3 Zoospore production

Zoospore production in the ions-rich condition is one of the special characteristics of *Pythium* spp. including *P. insidiosum*. In this study, *in vitro* zoospore production method was slightly modified from Mendoza *et al.* First*, P. insidiosum* was grown on corn meal agar (Oxoid™ Cat no. CM0103, UK) at 35°C for 24 h. Second, the sterile Malaysian grass leaves (*Anoxopus compressus* or Broadleaf carpet grass) was applied on the surface of that culture and incubated at 35°C for another 24 h. Third, the infected plant tissue was transferred to the induction medium and incubated at 35°C. After 2 h incubation, the zoospore could be detected by observing under the light microscope.

6.4 PCR assay

As mentioned above, positive PCR with sequencing is another criteria for definite pythiosis diagnosis. By the PCR method, the DNA sample prepared from both isolate and clinical specimen can be used as the template. Successful isolation of broad non-septate hyphae with submerging hair-like colony and/or positive zoospore production cannot be definitely identified as *P. insidiosum* infection. Those characteristics also can be observed among *Lagenidium* spp. etc. Also in the clinical specimen, broad non-septate hyphae can be observed in some fungi such as *Basidiobolus* spp., *Conidiobolus* spp., and *Rhizopus* spp. etc. Thus, PCR with sequencing is indispensable technology for definite *P. insidiosum* diagnosis.

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6.5 DNA extraction

To perform the PCR, DNA needs to be extracted from either isolates or clinical specimens and used as templates. Among the isolates, additional cell preparation step was processed. Five millimeters in diameter block of five-day *P. insidiosum* was inoculated into 50 ml Sabouraud dextrose broth (Difco^{TM} Cat no. 238230, BD, USA), shaking with 100 rpm at 35°C for 72 h. Before DNA extraction step, all cultures were killed by treated with Thimerosal solution (Cat.T2299, Sigma, USA), 0.02% (WT/V) final concentration. Here, QiAamp DNA Mini kit (Cat No. 51306, QIAGEN, USA) was used for DNA extraction in both isolated and clinical specimens. Shortly, to prepare the protoplast, the harvested mycelia were treated with 180 µl buffer ATL and proteinase K under denaturing conditions at 56°C for 1 h. After that, the 200 µl AL buffer; consists of guanidine hydrochloride which can denature protein and 200 µl ethanol (95%-100%) were added for DNA purification and precipitation, respectively. All entire lysates were filtered by the QIAamp MiniElute column and washed again using 500 µl washing buffer. Finally, DNA pellet was eluted from the membrane by sterile distilled water. NanoDrop (NanoDrop^{TM} 1000 Spectrophotometer, Thermo scientific, USA) was used for the quality and quantity of extracted DNA measurement. The absorbance at wavelength 260 nm and 280 nm were selected and the ratio of 1.8-2.0 was recommended for the high quality of DNA (95).

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6.6 PCR amplification and nucleotide sequencing

In this study, 2 proven targets were used for PCR amplification and nucleotide sequencing. There are Internal-transcribed spacer (ITS) region and Cytochrome c oxidase II (*COX2*) gene.

6.6.1 PCR Amplification: ITS region

The ITS region of *P. insidiosum* was amplified according to the method of White *et al*. The 800-900 bp amplicon was amplified using ITS-primers (synthesized by First-Base, Malaysia), the forward primer named "kit 1" and the reverse primer named "kit 4" (Table 2). Total volume of 30 μl of PCR reaction composed of 10 ng genomic DNA, 0.2 μL *i*-Taq DNA polymerase (5U/μL), 3.0 μL 10x PCR buffer, 1 μL dNTP mixture (2.5 mM each), 0.2 μL 10 mM each primer (Cat No. 25022, iNtRON Biotechnology, Korea). The positive and negative controls were run in parallel using 10 ng DNA of *P. insidiosum* strain CBS 574.85 (MTPI19) and UltraPure™ DNase/RNase-Free Distilled Water (Cat No. 10977-015, Thermo scientific, USA), respectively. The amplification reaction was carried on ThermoHybrid PCR thermocycler (Ashford, Middlesex, UK) as follow: initial denaturation step at 94°C for 3 min. and then followed by 40 cycles of the denaturation step at 94°C for 40 sec., annealing step at 55°C for 30 sec. and the extension step at 72°C for 1 min; and finally 72°C for 10 min. was used for final extension step. Gel electrophoresis was performed using 1.0 % agarose gel (Cat No. 75817, USB, USA) and 1 kb DNA Ladder (Cat No. 11300, NEB, England) as DNA marker, stained with 0.05% ethidium bromide

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(UltraPure Ethidium Bromide, Cat No. 15585011, Thermo scientific, USA), and visualized on a UV transilluminator for PCR product size determination (96).

6.6.2 PCR Amplification: *COX2* gene

The 580 bp amplicon of the partial *COX2* gene was amplified from genomic DNA using designed *COX2*-primers (synthesized by First-Base, Malaysia) named “FM58” and “FM66” (Table 2). Amplification reaction with the total volume of 30 µl, containing 10 ng genomic DNA, 0.2 µl *i*-Taq DNA polymerase (5U/µl), 3.0 µl 10x PCR buffer, 1 µl dNTP mixture (2.5 mM each), 0.2 µl of 10 mM each primer (Cat No. 25022, iNtRON Biotechnology, Korea). These reactions were performed in ThermoHybrid PCR thermocycler (Ashford, Middlesex, UK) with the cycling profile at 94°C for 2 min. for initial denature step and then follow by 40 cycles of 94°C for 20 sec., 56°C for 10 sec. and 72°C for 30 sec. Finally, the step of 72°C for 5 min was used for the final extension step. The positive (identified *P. insidiosum* strain CBS 574.85 code MTPI 19) and negative control (UltraPure™ DNase/RNase-Free Distilled Water (Cat No. 10977-015, Thermo Scientific, USA) were run in parallel. For the PCR product size, the samples were run on 1.5% agarose gel (Cat No. 75817, USB, USA) and 1 kb DNA Ladder (Cat No. 11300, NEB, England) as DNA marker, stained with 0.05% ethidium bromide (UltraPure Ethidium Bromide, Cat No. 15585011, Thermo Scientific, USA) and detected under UV light (97).

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6.6.3 Nucleotide sequencing

The amplicon was purified using QIAquick PCR purification kit (Cat No. 28106, QIAGEN, USA) for DNA sequencing process. The sequencing step was processed by 'Macrogen' (Korea) using ABI PRISM®377 DNA Sequencher by using ITS and/or *COX2*-primer and ABI BigDye kit. In brief, each of the four dideoxynucleotide chain terminators was labeled with a different fluorescent dye which each fluorescent label has a different wavelength, resulting in unequal peak heights and shapes in the electronic DNA sequence chromatogram after capillary electrophoresis process. These are detected and showed as 'peaks' of different colors which can be interpreted to determine the base sequences. Regarding reaction, each reaction was performed in 20 µl total volume contained approximately 300 ng of DNA template, 1 µl of 3.2 µM each primer, 4 µl of ready reaction premix and 2 µl of BigDye sequencing buffer. After thermocycling for 25 cycles of denaturation at 96°C for 10 s, annealing at 50 °C for 5 s, and extension at 60°C for 4 min, the extension products were purified by precipitation with ethanol acetate solution and washing with 70% ethanol. The samples were subsequently loaded on ABI PRISM®377 DNA Sequencher. In order to identify genus and species, DNA sequence of each isolate was blasted using GenBank database *(http://www.ncbi.nlm.nih.gov/genbank)*, Mycobank database *(http://www.mycobank.org)* and International Society of Human and Animal Mycology (ISHAM) ITS database *(http://its.mycologylab.org)*.

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Table 1 Primers use for tHDA, DNA Sequencing and HRM analysis

Primers	Sequences	References
ITS region		
Kit 1	5'-TCC GTA GGT GAA CCT GCG G-3'	White *et al.*, 1990 (96)
Kit 4	5'-TCC TCC GCT TAT TGA TAT GC-3'	White *et al.*, 1990 (96)
COX2 gene		
FM58	5'-CCA CAA ATT TCA CTA CAT TGA-3'	Villa *at al.*, 2006 (97)
FM66	5'-TAG GAT TTC AAG ATC CTG C-3'	Villa *et al.*, 2006 (97)

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7. *P. insidiosum* IgG antibody assay (*Pi*-Ab)

Pi-Ab is one of the immune responses generated by B-cells after *P. insidiosum* infection and/or PIA immunotherapy administration. Currently, positive *Pi*-Ab is used as one of the criteria for vascular pythiosis diagnosis with 100% sensitivity and specificity. With its very high sensitivity and specificity of *Pi*-Ab detection, we hypothesized that the pattern of *Pi*-Ab level in PIA immunotherapy-treated patients along a year course can represent the hosts' immune response against *P. insidiosum* infection and PIA immunotherapy.

7.1 *Pi*-Ab detection for pythiosis diagnosis

In the present, the established *in-house* enzyme-linked immunosorbent assay: ELISA (indirect ELISA) is the highest sensitivity and specificity method for *Pi*-Ab detection. Briefly, the 100 μl of 2 mg/ml *P. insidiosum* protein in bicarbonate buffer was coated in 96-well microtiter plates (Cat no. M0661, Polysorp®, Nunc, Rochester, NY) overnight at 4°C. The plate was washed with 1% (vol/vol) Tween 20-phosphate-buffered saline (PBS-T) and blocked with 2% Skim milk (Skim milk

powder, Cat no. LP0031, Oxoid™, UK) in PBS-T for 1 h at 37°C. After washing, the 100 ul of diluted serum in 0.5% skim milk (Skim milk powder, Cat No. LP0031, Oxoid™, UK) were added to each well, then incubated at 37°C for 1 h. After the final washing step, horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G (Cat no. P0260, Dako, Denmark) in PBS-T and O-phenylenediamine dihydrochloride: OPD (Cat no. P1526, Sigma, USA) in citric acid (Citric acid ACS reagent, ≥99.5%, Cat no. 782061, Sigma, USA) with 30% H_2O_2 (Hydrogen peroxide solution 30 % (w/w), Cat no. H1009, Sigma, USA) were used as the secondary antibody and substrate, respectively. Finally, the reaction was quenched by the addition of 1 N H_2SO_4 (Sulfuric acid 95-97%, Cat no. 109286, Merck Millipore, USA). The tested result was measured at OD_{490}. Pooled serum of healthy thalassemic volunteers (n=40) was run in parallel as a negative control (45). Due to the very high sensitivity of ELISA assay, all tests were performed in quadruplicate to obtain the high accuracy result.

7.2 *Pi*-Ab monitoring in PIA-treated pythiosis patients

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To apply the established *in-house* ELISA for *Pi*-Ab level monitoring, the concentration of 3 factors: coated antigen, serum sample and secondary antibody (2° Ab) need to be optimized. Moreover, based on the question that whether the PIA prepared from different clade influence the serum IgG levels against *P. insidiosum*, the clade effect was also proved.

***7.2.1 In-house* ELISA optimization for *Pi*-Ab monitoring**

To determine the optimal conditions of the ELISA, titration of coating antigen concentrations and serum dilution were performed. Different protein

concentrations (0.78, 1.56, 3.125, 6.25 and 12.5 ng/well) were coated on the plate to determine the appropriate protein concentration. All coated plates were tested against pooled sera for clade B_{TH} and C_{TH} (five samples each clade), but not clade A_{TH} because no human case of infection with clade A_{TH} *P. insidiosum* has been reported to date. To explore the optimal immunoreaction, pythiosis sera were also diluted in two-fold steps ranging from 1:200 to 1:12,800, whereas pooled thalassemic sera (pooled from 40 samples) were diluted 1:200 and 1:400 for controls. The reaction was performed in quadruplicate as protocol mentioned in the topic no. 9.1 (*Pi*-Ab detection for pythiosis diagnosis) by using the 100 μl of 1:2,000 of secondary antibody (horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G; Dako, Denmark). Finally, the correlations between mean OD_{490} value and the coating antigen concentrations and serum dilutions were plotted using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The optimal serum titer was defined as the titer that presented the best discrimination between the OD_{490} value of the pythiosis (positive result) and thalassemic (negative result) sera and was <1.0, which represents a high accuracy result (45).

In parallel, to determine the best dilution of secondary antibody (2° Ab), 100 μl of 2° Ab diluted in PBS-T at 1:2,000, 1:5,000, 1:10,000, 1:20,000 and 1:40,000 were used in quadruplicate with the optimal titration of coated proteins and serum dilution obtained in the previous step. The correlation between the mean OD_{490} value and 2° Ab dilution was plotted and the optimal dilution was selected according to the criteria mentioned above.

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7.2.2 Pi-Ab assay result analysis

To standardize the ELISA result at each testing batch, the OD_{490} value was presented as "ELISA value (EV)" according to the following formula:

$$EV = (OD_{sample} - OD_{background}) / (OD_{control} - OD_{background}).$$

8. (1,3)-β-D-glucan assay

(1,3)-β-D-glucan is one of the polysaccharide substances found in the main cell wall component of *P. insidiosum*. In the body, (1,3)-β-D-glucan will release in the serum during cell growth process (98). Base on the previous publications, we hypothesized that the decreased trend of (1,3)-β-D-glucan level represents the status of pathogens clearance which is related to the clinical improvement.

In this study, serum (1,3)-β-D-glucan was quantitated by using available commercial Fungitell assay kit (Cat No. FT001, Associates of Cape Cod, East Falmouth, Massachusetts, USA) according to the manufacturer's instructions in duplication. In brief, the serum sample was mixed with alkaline reagents (0.125M KOH/6M KCl) in order to convert triple-helix glucans into single-stranded glucans and to inactivate the serine protease. After the 10-min incubation at 37°C, Fungitell reagent, reconstituted in 0.1M Tris HCl, pH 7.4, was added to samples and the standard (1,3)-β-D-glucan solutions. The assay was monitored kinetically at 405 nm (OD_{405}) and 490 nm (OD_{490}) at 37°C for 40 min using Gen 5 program version 1.4.5.0. After the kinetic process and the calculation step, (1,3)-β-D-glucan concentration was presented in the unit of picogram/milliliter. Pooled serum of healthy, thalassemic volunteers (n=40) was run in parallel as a negative control. The limitation of the Fungitell assay was 7.812 pg/ml – 523.438 pg/ml; therefore, samples with (1,3)-β-D-glucan levels out of the indicated

range were reported as <7.812 pg/ml or >523.438 pg/ml. The (1,3)-β-D-glucan levels which were outside the range were processed in the statistical analysis as 7.812 pg/ml or 523.438 pg/ml for the lower limit and upper limit, respectively. Additional dilutions were not performed. Serum (1,3)-β-D-glucan level <60 pg/ml, 60-70 pg/ml, > 80 pg/ml were interpreted as negative, indeterminate, and positive respectively (22, 99).

9. Cytokine assay

Besides humoral immune response, cytokines are the main products of cell-mediated immune response (CMI). To monitor the CMI response in PIA-treated pythiosis patients, in this study, five significant cytokines as representatives of each T-cells subpopulations were assayed:

- T-helper 1: Interferon-gamma (IFN-γ)
- T-helper 2: Interleukin-4 (IL-4), Interleukin-5 (IL-5)
- T-helper 17: Interleukin-17 (IL-17)
- Regulatory T-cells: Interleukin-10 (IL-10)

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9.1 Total serum cytokine

The total cytokines were measured in the serum samples collected from the PIA-treated patients at the time of definite diagnosis and in every visit. The serum samples were kept in -80°C until use. The cytokines were measured the level by human cytokine platinum kit (eBiosciences, USA) based on the principle of direct ELISA technique. Briefly, the 96-microwell plate was coated with specific antibody against tested cytokine and incubated overnight at 4°C. Then the reaction was washed twice by soaking approximately 400 µl washing buffer per well for 10 - 15 seconds before removing all buffer. The 100 µl each of standard dilution was added

to the standard wells while the 100 μl of each serum sample was added to the test wells in duplicate. Then the 50 μl of the biotin-conjugate solution was added to all wells and incubated for 2 h at 25°C. The washing step was performed before adding the 100 μl of diluted streptavidin-HRP to all wells including the blank wells. After that, the plate was incubated at 25°C for 1 h followed by the washing step. The amount of 100 μl of TMB substrate solution was added to all wells and the plate was incubated at 25°C for 10 min in dark. Finally, the stop solution was added and detected the color development immediately by ELISA reader at 620 nm. To obtain the cytokine level, the OD_{620} result was compared with the standard curve and interpreted the concentration in the unit of pictogram/milliliter (pg/ml).

9.2 *Ex vivo* assay: PBMC Stimulation

To prove the effect of PIA immunotherapy on the immune cell activation via cytokine production, PBMC samples which were harvested from the healthy volunteers were thawed from liquid nitrogen stock and seeded 2 ml/well containing 10^6 cells/ml into the 6-microwell plate (Cat no. 140675, Polysorp®, Nunc, Rochester, NY). To challenge the PBMC, PIA solution was added to each well with the final concentration of 2 mg/ml. Purified (1,3)-β-D-glucan (Cat no. FT001, Associates of Cape Cod, East Falmouth, Massachusetts, USA) at final concentration 500 pg/ml and Phorbol myristate acetate: PMA (Cat no. P-8139, Sigma, USA)/Ionomycin (Cat no. I-0634, Sigma, USA) (final concentration at 50 ng/ml and 1 ug/ml, respectively) were used as positive control whereas RPMI1640 (Cat no. 11875-085, Thermo scientific, USA) alone was used as negative control. To determine the cytokine expression by PBMC, the reactions were incubated at 37°C for 72 h with 5% CO_2. Cell culture supernatants were collected and kept at −20°C for cytokine analysis

using Human cytokine platinum kit (eBiosciences, USA). The protocol of cytokine measurement was performed as mentioned above for total serum cytokine (topic no. 11.1) (figure 4).

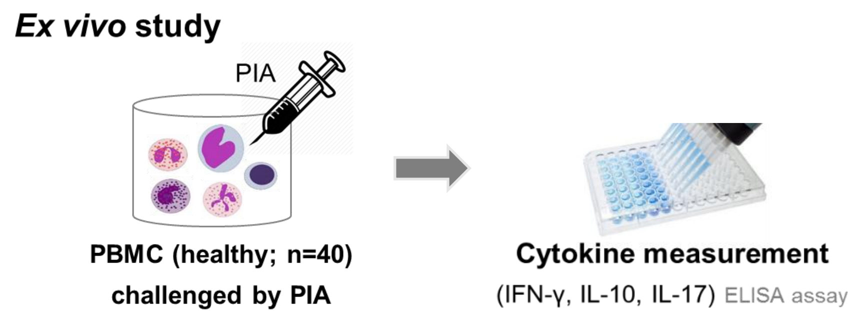

Figure 4 Schematic of *Ex-vivo* study: PBMC challenged with PIA immunotherapy

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9.3 *Ex vivo* assay: co-culture assay of $CD4^+$T-cells or $CD8^+$T-cells and monocyte-derived macrophage

To study the cell source of cytokine production, T-cells subpopulations: CD4+ T-cells or CD8+ T-cells were investigated by using *Ex-vivo* co-culture assay.

9.3.1 Purification of CD4+T-cells (T-helper cells) subpopulation and flow cytometry examination

PBMC were freshly isolated from pythiosis patients' blood sample and incubated in 20 ml incomplete media (RPMI 1640 medium (Cat no. 11875-085, Thermo scientific, USA), 1% penicillin/streptomycin (Cat no. P4333, Thermo scientific, USA), 1 mM sodium pyruvate (Sodium pyruvate ReagentPlus®, ≥99%, Cat no. P2256, Sigma-Aldrich, USA) and 10 mM HEPES (HEPES ≥99.5%, Cat no. H3375, Sigma-Aldrich, USA) supplemented with 10% FBS (Cat no. 16000044, Gibco, USA) for 1 h at 37°C and 5% CO_2 to let it familiar with the cell culture medium. Then $CD3^+CD4^+$T-cell was purified by untouched human $CD3^+CD4^+$ T

Cell Isolation Kit (Cat no. 130-096-533, MACS Miltenyi Biotec, Germany). Briefly, the 10^7 cells/ml of PBMC was stained by non-$CD4^+$ cocktail contained antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (Glycophorin A) which normally present on the $CD8^+$ T cells, monocytes, neutrophils, eosinophils, B cells, dendritic cells, NK cells, granulocytes, γ/δ T-cells, or erythroid cells for 10 minutes in the refrigerator (2–8 °C). After that, labeled cells were washed once with 1× PBS (HyClone™ Phosphate Buffered Saline Cat no. SH30256.02, GE Healthcare life Science, USA) with 5% FBS (Cat no. 16000044, Gibco, USA), and dissolved in 1 ml of PBS (HyClone™ Phosphate Buffered Saline Cat no. SH30256.02, GE Healthcare life Science, USA)–5% FBS (Cat no. 16000044, Gibco, USA) before transferred to a high-gradient magnetic separation column placed in a magnetic field. Non-target cells were magnetically labeled with the $CD4^+$ T-Cell MicroBead Cocktail. Isolation of highly pure T-cells was achieved by depletion of magnetically labeled cells. Finally, the flow-through containing unlabeled cells, representing the enriched $CD4^+$ T-cells was collected. The purity of cell was controlled by flow cytometry (FACScan; Becton Dickinson) analysis using phycoerythrin/cyanin7 (PE/Cy7) labeled anti-$CD3^+$ (Cat no. 300420, BioLegend, USA) and PE/Cy5 labeled anti-$CD4^+$ (Cat no. 300510, BioLegend, USA) monoclonal antibody (MAb). Cell viability was determined by propidium iodide: PI (Cat no. 421301, BioLegend, USA staining). At least 98% viability was accepted to avoid the auto-fluorescent signal. In parallel, isotype control of each color was used as negative control. The purities of isolated $CD3^+CD4^+$T-cells subpopulations more than 95% were accepted.

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9.3.2 Purification of $CD8^+$T-cells subpopulation and flow cytometry examination

The same protocol as $CD3^+CD4^+$T-cells purification was performed using untouched human $CD8^+$ T-Cell Isolation Kit (Cat no. 130-096-495, MACS Miltenyi Biotec, Germany). Also in the flow cytometry analysis step, only fluorescein isothiocyanate (FITC) labeled anti-$CD8^+$ (Cat no. 344704, BioLegend, USA) monoclonal antibody (MAb) was used instead of PE/Cy5 labeled anti-$CD4^+$. Other steps were processed the same.

9.3.3 Preparation of monocyte derived macrophage from PBMC sample and flow cytometry examination

Due to T-cell activation required the process of antigen presentation, in this study macrophage was derived from the monocyte harvested from PBMC of the same subject (autologous).

PBMC was freshly isolated and incubated in 20 ml incomplete media for 1 h at 37°C, 5% CO_2 to let the monocytes attach the cell culture plate (Cat no. 153066, Polysorp®, Nunc, Rochester, NY). Then the washing step using warm 1x PBS (HyClone™ Phosphate Buffered Saline Cat no. SH30256.02, GE Healthcare life Science, USA) was performed before adding the 20 ml incomplete media supplemented with 2% heat-inactivated autologous serum. The culture media was changed every 2 days for 1 week. The attached cells were observed and washed with the warm 1x PBS (HyClone™ Phosphate Buffered Saline Cat no. SH30256.02, GE Healthcare life Science, USA). Finally M1-macrophage was polarized by IFN-γ (Human IFN gamma Recombinant

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Protein, eBioscience™, Cat no. 14-8319-80, Thermo Scientific, USA) and LPS (eBioscience™ Lipopolysaccharide (LPS) Solution, Cat no. 00-4976-93, Thermo scientific, USA) in incomplete media supplemented with 2% heat-inactivated autologous serum for 24 h. Macrophage was detached from the cell culture plate (Cat no. 153066, Polysorp®, Nunc, Rochester, NY) by using cold PBS (HyClone™ Phosphate Buffered Saline Cat no. SH30256.02, GE Healthcare life Science, USA) and scrapping method. The purity of macrophage was examined by flow cytometry (FACScan; Becton Dickinson) analysis using anti-$CD3^+$ (Cat no. 300420, BioLegend, USA) marker. Briefly, cell suspension was fixed in 4% paraformaldehyde (Image-iT™ Fixative Solution, Cat no. FB002, Thermo scientific, USA) for 10 minutes before adding 1 ml of 1% FBS (Cat no. 16000044, Gibco, USA) in 1x PBS (HyClone™ Phosphate Buffered Saline Cat no. SH30256.02, GE Healthcare life Science, USA). After washing step, 0,2% TritonX (Triton™ X-100, Cat no. T8787, Sigma-Aldrich, USA) in PBS (HyClone™ Phosphate Buffered Saline Cat no. SH30256.02, GE Healthcare life Science, USA) was added and incubated for 2 min. After washing step, 50 μl of human serum was co-incubated for 20 min. Finally, 3 μl of anti-$CD68^+$ (Cat no. 137002, BioLegend, USA) was added and incubated for 30 min in dark. Cell viability was determined by propidium iodide: PI (Cat no. 421301, BioLegend, USA staining) staining. At least 98% viability was accepted to avoid the auto-fluorescent signal. In parallel, isotype control of each color was used as negative control. More than 95% purification of $CD68^+$ monocyte-derived macrophage populations was accepted.

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9.3.4 Co-cultivation of purified CD4$^+$T-cell or CD8$^+$T-cell with monocyte derived macrophage

Purified CD4$^+$T-cell or CD8$^+$T-cell (10^6 cells/ml) was co-cultured with monocyte derived macrophage and cell stimulation as 1:1 ratio in 2% heat inactivation FBS complete medium at 37°C, 5% CO_2 before stimulation by the PIA antigen (figure 5,6).

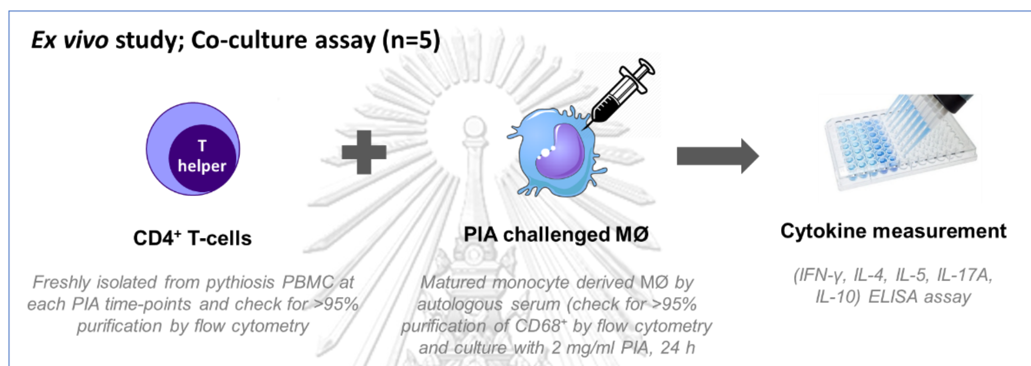

Figure 5 Schematic of *Ex-vivo* study: Co-culture assay of CD4$^+$ T-cells

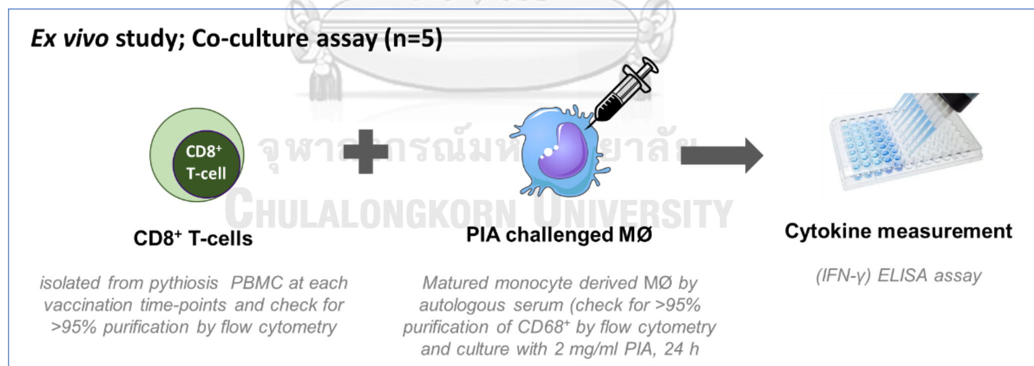

Figure 6 Schematic of *Ex-vivo* study: Co-culture assay of CD8$^+$ T-cells

9.3.5 Cell stimulation

PBMC or purified T-cells were challenged for 72 h with 2 mg/ml final concentration of PIA parallel with PMA (Cat no. P-8139, Sigma, USA) at final concentration 50 ng/ml /Ionomycin (Cat no. I-0634, Sigma, USA) at final

concentration 1 ug/ml) and 0.5 ug/ml final concentration of purified (1,3)-β-D-glucan (Cat No. FT001, Associates of Cape Cod, East Falmouth, Massachusetts, USA) as positive control whereas RPMI 1640 (Cat no. 11875-085, Thermo Scientific, USA) was used as negative control. After challenging step, cell supernatant was separated from the cells and collected for cytokine measurement.

9.3.6 Cytokine measurement

Cytokine level in the supernatant was measured by human cytokine platinum kit (eBiosciences, USA). The same protocol as mentioned above for total serum cytokine was performed.

10. Statistical analyses

All statistical analyses were conducted with SAS version 9.4 (SAS Institute, Cary, NC, USA). The t-test or the Wilcoxon rank sum test was used to compare continuous covariates between groups of patients who survived/saved globe and died/blindness during the follow-up period. The chi-square test or Fisher's exact test were used to compare categorical and binary covariates between 2 groups.

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The linear mixed effect models were used to compare the differences in (1,3)-β-D-glucan levels, *Pi*-Ab levels and cytokine levels among groups of patients who survived and died at 1 year. The linear mixed effect model is a regression technique for multiple observations for each individual to allow the subset of the regression parameters to vary randomly from one individual to another, thereby accounting for sources of natural heterogeneity of the patients. In addition, this regression method can overcome unbalanced data since some patients passed away at 1.5 months;

therefore, no additional (1,3)-β-D-glucan, *Pi*-Ab, and cytokines levels from those patients for the analysis beyond the 1.5-month mark. We ran the regression models to compare (1,3)-β-D-glucan, *Pi*-Ab and cytokine levels between the 2 groups up to 3 months of the follow-up period. In this analysis, we used unstructured covariance matrix in the regression models.

CHAPTER V
Results

Base on the inclusion criteria mentioned in the method section, a total of fifty vascular pythiosis cases and thirty ocular pythiosis cases were enrolled in this research project. To simplify this section, the result will be explained group by group as follow

1. Vascular pythiosis patients

1.1 Patient characteristic

Based on the final clinical outcome after PIA treatment, proven vascular pythiosis cases recruited in this study were classified into 2 groups. There were 1) survival group: the patient who survived more than 1 year follow-up after PIA immunotherapy course started, 2) deceased group: patients who died before reaching the 1 year follow-up. Totally 50 patients, forty-five survived cases and five deceased cases were grouped. The information of patient related parameters, disease and treatment related parameter, and clinical sign/symptoms post treatment initiation were summarized in table 3. The significant difference ($p < 0.05$) between both groups were found in 4 factors: age, duration time between the disease onset to the first definitive surgery, type of surgery and the clinical sign/symptoms post treatment initiation. Patients who died before the 1 year follow-up period were significantly older than the ones who survived from the disease (mean age 49.2 vs 33.6 years respectively; $p = 0.006$). Patients in the survival group had a significant shorter time from disease onset to the first medical care

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than patients in deceased group (mean duration 1.9 vs 4.4 months respectively; $p < 0.0001$). Similar to the duration time from definitive diagnosis to definitive surgeries, the shorter waiting time was observed among the survival group compared to the deceased one (mean duration 0.6 vs 1.5 months respectively; $p < 0.0001$). The type of amputation was another factor that was found significantly difference between both groups. A higher proportion of amputation in the survival group than the deceased one was observed ($p < 0.0001$). Most patients were noted in their medical file as negative surgical margins except one patient with a carotid lesion in the deceased group, where the definitive surgery was not practical. In case of antifungal treatment, a significant shorter of treatment duration was found in the deceased group compared to the in survival group (mean duration 1.4 vs 5.9 months; $p = 0.04$); however, this was because patients in the deceased group did not live long enough to complete the therapy.

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During the follow-up period, none of the patients in the survival group complained of any symptoms of painful swelling lesions or any sign of that represented disease progression, which was contrast to patients in the deceased group. Three of the five deceased cases developed fever > 38.2 ^{o}C whereas another two cases developed low fever at 37.5 ^{o}C and 38.0 ^{o}C, respectively. In case of clinical outcomes after treatment, two cases had signs related to arterial insufficiency syndrome. One patient presented with a mass at surgical site at 1 month after debridement. Three patients complained of pain with redness and

swelling lesion at the surgical sites. One patient who presented with a lesion at his neck was diagnosed as pythiosis recurrent at 1 month after the 1st debridement. Only 1 case was examined with a computed tomography angiography (CT angiogram) for any residual disease based on the clinical symptoms. Finally, all 5 patients in deceased group died at 1.5 (n=2) and 3.0 months (n=3) after surgery.

Table 2 Characteristics of vascular pythiosis patients.

	Survival (45)	Deceased (5)	*P-value**
Patient related parameters			
Age (years)	33.6 ± 10.7	49.2 ± 17.2	0.006
Male gender	25 (56%)	4 (80%)	0.29
Occupation			0.39
- Agriculture related	39 (86.7%)	5 (100%)	
- Non-agriculture related	6 (13.3%)	-	
History of water exposure within 3 months	41 (91.1%)	5 (100%)	0.49
Underlying disease			0.87
- α-thalassemia	3 (6.7%)	-	
- β-thalassemia	3 (6.7%)	-	
- β-thalassemia Hemoglobin E disease	32 (71.1%)	4 (80%)	
- H-constant spring	2 (4.4%)	-	
- Hemoglobin H disease	5 (11.1%)	1 (20%)	
Serum ferritin (ng/ml)	1,388.40± 653	1,676 ± 402.4	0.34
Duration from onset to disease to first medical attention (months)	1.9 ± 0.7	4.4 ± 0.7	<0.0001
Duration from diagnosis to first definitive surgery (months)	0.6 ± 0.2	1.5 ± 1.2	<0.0001
Disease and treatment related parameters			
Lesions			0.05
- Brachial artery	1 (2.2%)	-	
- Radial artery	11 (24.4%)	-	
- Ulnar artery	1 (2.2%)	-	
- Femoral artery	17 (37.8%)	4 (80%)	
- Anterior tibial artery	9 (20%)	-	
- Posterior tibial artery	3 (6.7%)	-	
- Iliac artery	3 (6.7%)	-	
- External carotid artery	-	1 (20%)	
Surgery			<0.0001
- Amputation	45 (100%)	2 (40%)	
- Debridement	-	3 (60%)	

* Calculated by *the chi-square test or Fisher's exact test*

Table 2 (cont.) Characteristics of vascular pythiosis patients.

	Survived (45)	Deceased (5)	*P-value**
- Itraconazole alone	5 (11.1%)	2 (40%)	
- Itraconazole + terbinafine	34 (75.6%)	3 (60%)	
- SSKI + terbinafine	6 (13.3%)	-	
Duration of antifungal treatment (months)	5.9 ± 4.6	1.4 ± 0.9	0.04
Iron chelation drug	45 (100%)	5 (100%)	-
Clinical sign/symptoms post treatment initiation			
- Fever > 38.2°C	-	3 (60%)	0.008
- Arterial insufficiency syndrome (claudication, paresthesia, gangrenous ulceration)	-	2 (40%)	0.008
- Mass at surgical sites (arterial aneurysm)	-	1 (20%)	0.1
- New skin lesions	-	-	-
- Inflammation/infection at surgical sites	-	3 (60%)	0.008

* Calculated by *the chi-square test or Fisher's exact test*

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In summary, fifty vascular pythiosis cases were enrolled in this study. Based on the final clinical outcome, they were divided into two groups: survival and deceased group. Age, duration time from disease onset to the first medical surgery and the type of amputation were found the significant difference ($p < 0.05$) between both groups by the chi-square test or Fisher's exact test. Younger age and shorter duration time from disease onset to the first medical surgery were revealed in survival group compared to deceased group. Moreover, the higher proportion of amputation in survival group than deceased group was also revealed.

1.2 Adaptive immune response via cytokine production in vascular pythiosis patients after PIA treatment

Due to the switching of Th2 to Th1 of cytokines' profile was shown in the previous study after PIA immunotherapy treatment, the concrete evidence was revealed in here. In the first experiment of this section, the cytokines' level which represents the response of Th1 (IFN-γ), Th2 (IL-4 and IL-5), Th17 (IL-17) and Treg (IL-10) was measured in the serum of vascular pythiosis patients collected prior each PIA immunotherapy. Moreover, since all vascular cases recruited in this study have thalassemia as an underlying disease, age and gender matched thalassemic volunteers (n=40) were used as control group.

1.2.1 Total serum cytokines

In this section the cytokines' level was measured using ELISA based human cytokine measurement kit. At the time of diagnosis or at the time before PIA immunotherapy course initiation, non-significant difference ($p < 0.05$) of cytokines' level between both the survival and deceased groups was demonstrated as we hypothesized. We found only the significant difference of IL-4 and IL-5 among patients and thalassemic control group (IL-4: survived group 43.38 ± 5.36 and deceased group 58.80 + 4.05 VS control group 2.85 + 0.48; $p < 0.0239$ and IL-5: survived group 43.12 ± 4.36 and deceased group 42.89 + 2.35 VS control group 2.66 + 0.73; $p < 0.0402$). This finding is related to the previous reports that natural *P. insidiosum* infection can induce the Th2 cytokine response (figure 7). Then the cytokines's level was monitored at each PIA time-point during the PIA immunotherapy course, among the survival group. As we expected there was a significant increased trend ($p < 0.05$) of IFN-γ (Th1

cytokine), IL-10 (Treg cytokine) and IL-17 (Th17 cytokine) in parallel with a significant decreased trend ($p < 0.05$) of IL-4 and IL-5 (Th2 cytokine). We observed this conversion of cytokines during the first 3 months after PIA treatment initiation. Then there was no change in the cytokine level in all survived cases until at 12th month (figure 8A, 8B). On the other hand, in the deceased group, non-significant different ($p < 0.05$) of all cytokine levels was observed (figure 9).

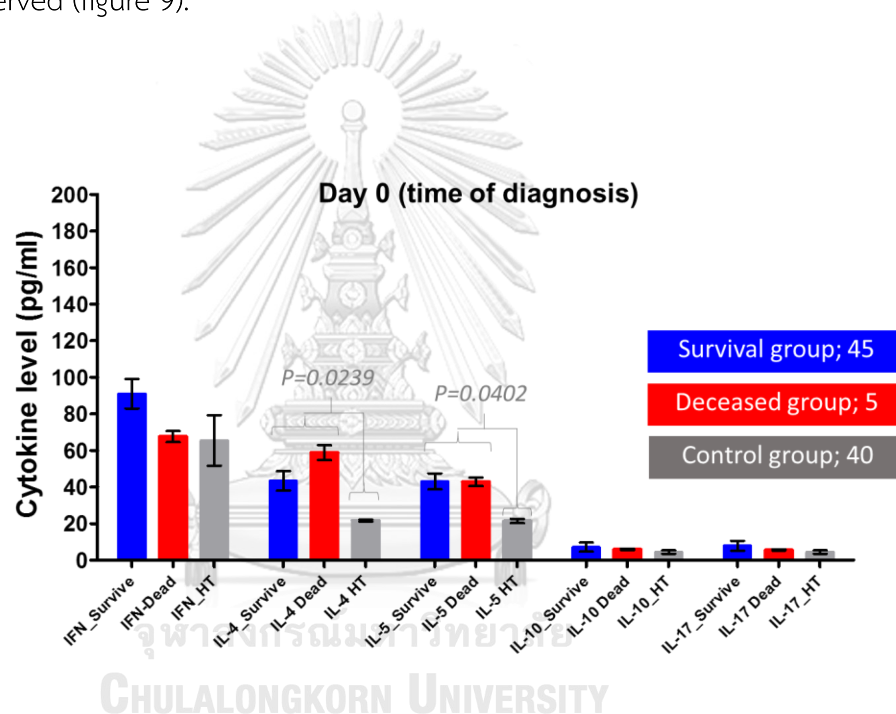

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Figure 7 The mean±SD of cytokines (IFN-γ, IL-4, IL-5, IL-10, IL-17) response of vascular pythiosis cases in both survival (n=45; blue bar) and deceased (n=5; red bar) group prior PIA immunotherapy compared with thalassemic control group (n=40; grey bar) accessed by human cytokine measurement kit based on ELISA assay.

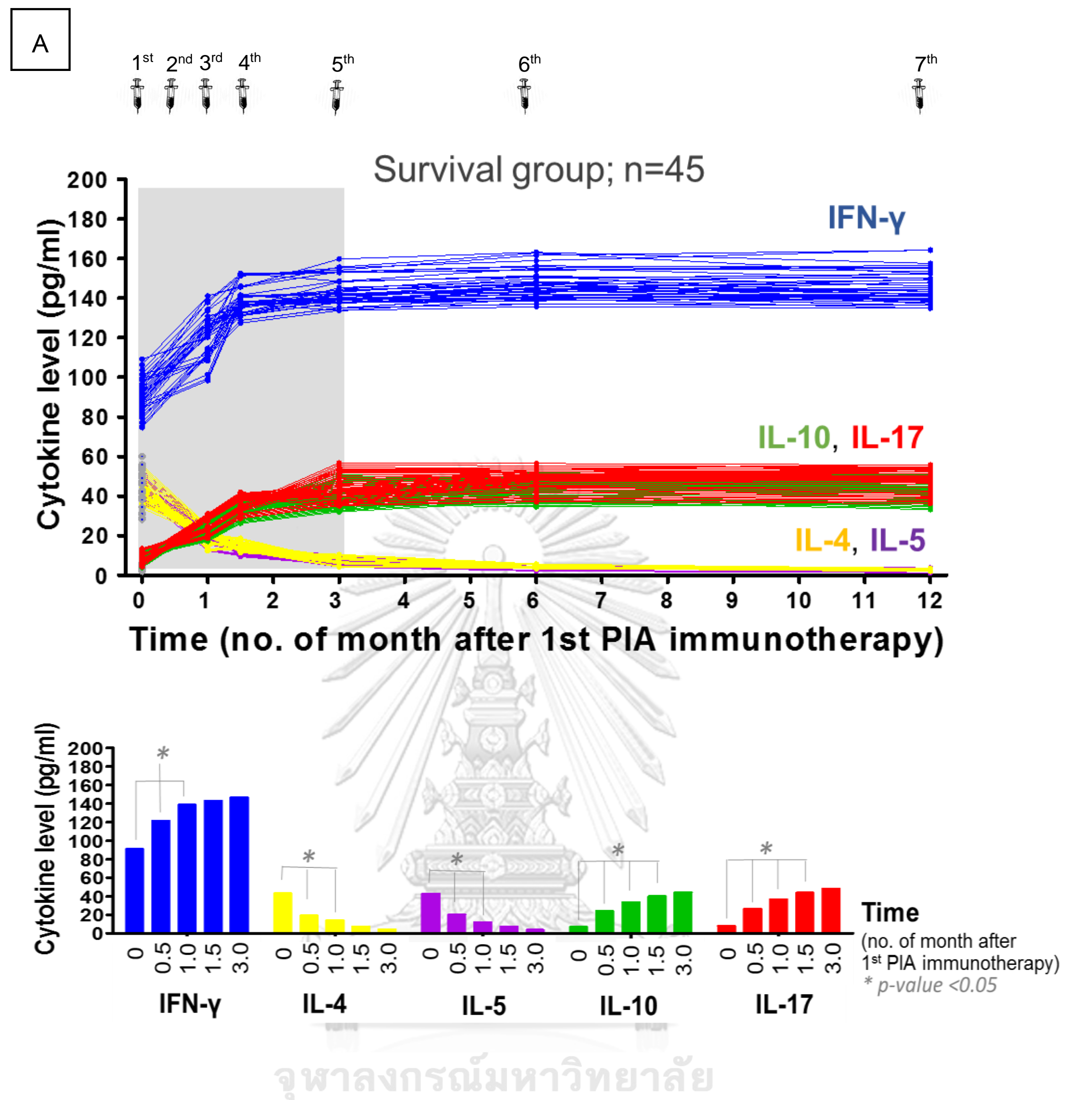

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Figure 8 The individual value (A) and mean±SD (B) of cytokines; IFN-γ (blue lines/bar), IL-4 (yellow lines/bar), IL-5 (purple lines/bar), IL-10 (green lines/bar), IL-17 (red lines/bar) response of vascular pythiosis cases in survival group (n=45) prior each PIA immunotherapy during 1 year (totally 7 doses) and first 3 month (totally 5 doses) after PIA immunotherapy, respectively compared with thalassemic control group (n=40; grey dots) accessed by human cytokine measurement kit based on ELISA assay.

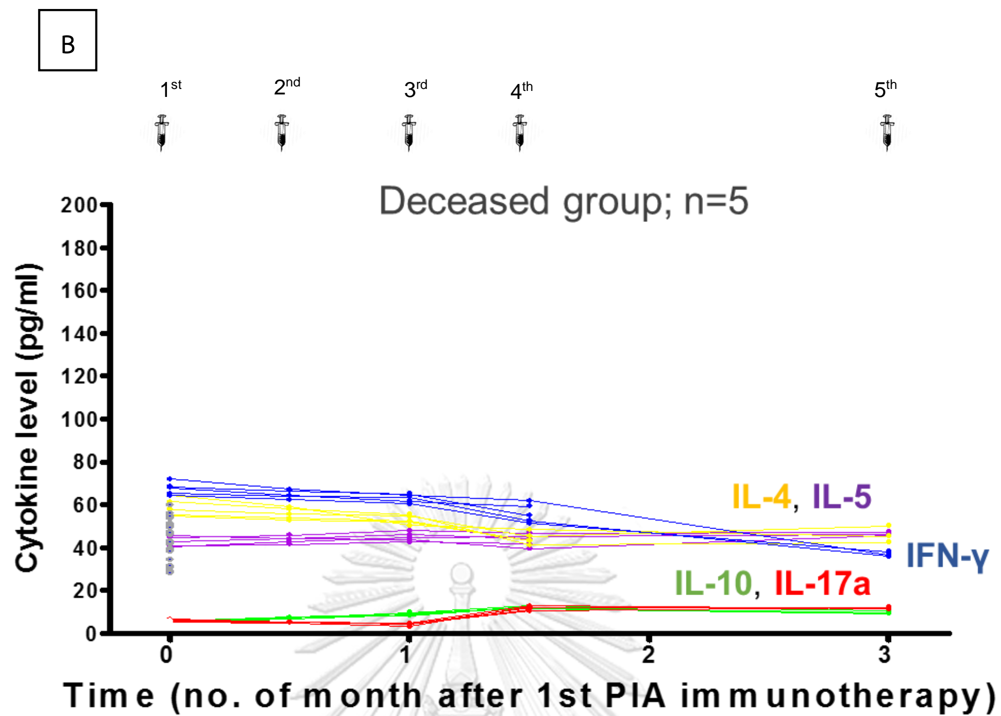

Figure 9 The individual value of cytokines; IFN-γ (blue lines), IL-4 (yellow lines), IL-5 (purple lines), IL-10 (green lines), IL-17 (red lines) response of vascular pythiosis cases in deceased group (n=5) prior each PIA immunotherapy during PIA immunotherapy started until they died compared with thalassemic control group (n=40; grey dots) accessed by human cytokine measurement kit based on ELISA assay.

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1.2.2. Cytokine response to PIA antigen

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To prove whether all tested cytokines were induced by the PIA immunotherapy, PBMCs were isolated from healthy volunteer (n=40) and challenged with 2 mg/ml PIA vaccine which was the concentration used in patients for 24 h in 5% CO_2. The cytokine, IFN-γ, IL-10 and IL-17 in cells culture supernatant were measured and compared to the positive (50 ng/ml of PMA + 1 ug/ml of Ionomycin and 0.5 ug/ml purified (1,3)-β-D-glucan) and negative (RPMI cells-culture media) controls. The mean levels of IFN-γ, IL-10 and IL-17

in the presence of PIA were significantly higher than the negative control (IFN-γ: 157 ± 9.12 and 7.67 + 1.3 respectively; $p = 0.024$, IL-10: 58.56 ± 7.43 and 5.16 ± 1.58 respectively; $p = 0.042$ and IL-17: 64.50 ± 6.05 and 6.70 ± 1.75 respectively; $p = 0.047$). These results indicated that PIA immunotherapy is capable of inducing IFN-γ, IL-10 and IL-17 secretion (figure 10A - 10C).

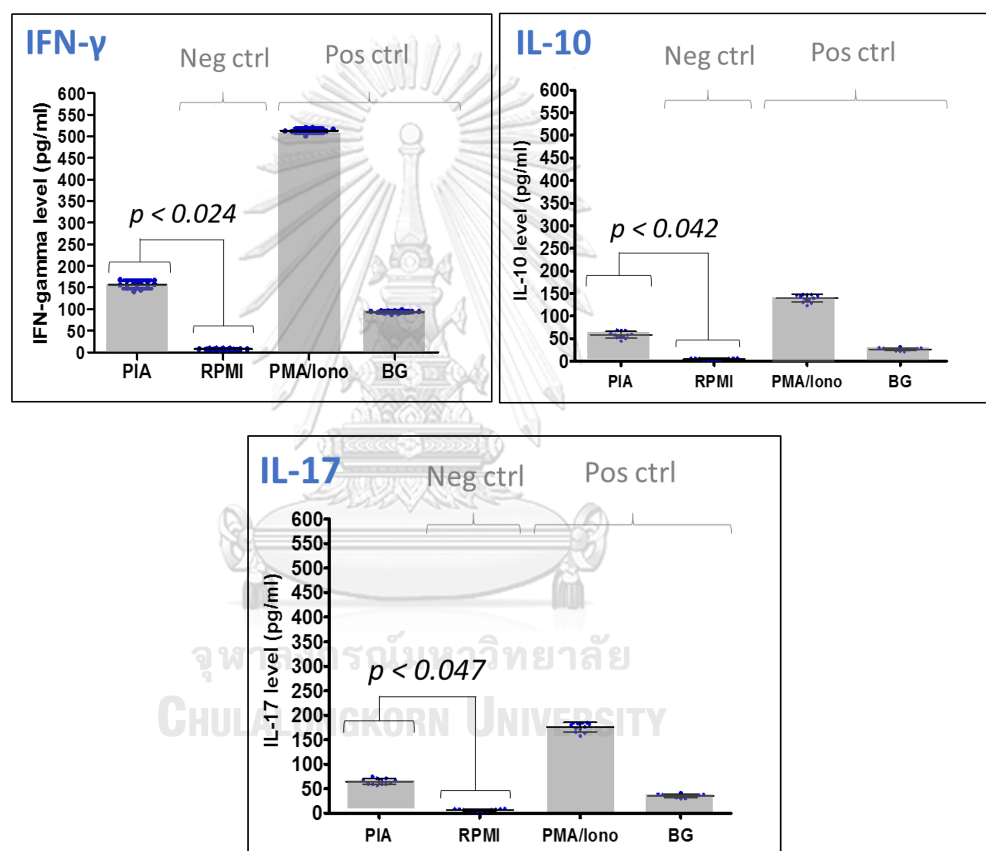

Figure 10 The mean±SD of cytokine level of IFN-γ (A), IL-10 (B) and IL-17 (C) production from healthy PBMC after challenged with PIA immunotherapy, RPMI medium (negative control), PMA/Ionomycin (positive control) and purified (1,3)-β-D-glucan (positive control).

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1.2.3 Source of cytokine response to PIA antigen

1.2.3.1 Cytokine response of $CD4^{+}$T-cells (T-helper cells) to PIA immunotherapy

To examine whether $CD4^{+}$T-cells (T-helper cells) are capable of responding to PIA immunotherapy and perhaps the major source of cytokine in the sera of pythiosis patients, $CD4^{+}$T-cells from vascular pythiosis in the survival group at each PIA immunotherapy time-point were freshly isolated. The $CD4^{+}$T-cells with >95% purification by flow cytometry analysis were co-cultured with their own matured monocyte-derived macrophage prior to further derived with 2 mg/ml of PIA immunotherapy. The cytokines, IFN-γ, IL-4, IL-5, IL-10 and IL-17 were measured by human cytokine measurement kit based on the ELISA assay. Among all tested cytokines, the level of IFN-γ shown relatively low in comparison to other cytokines and lower than expected. These results indicated that $CD4^{+}$T-cells are capable to produce IFN-γ, IL-4, IL-5, IL-10 and IL-17 cytokine in response to PIA antigen (figure 11A - 11E). However, other immune cells perhaps play a role as IFN-γ production after PIA immunotherapy activation.

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1.2.3.2 Cytokine response of $CD8^{+}$T-cells to PIA immunotherapy

In this study we were focused on $CD8^{+}$T-cells which is another immune cells that can produce IFN-γ. To examine whether the $CD8^{+}$T-cells respond to PIA antigen by IFN-γ production, $CD8^{+}$T-cells were freshly isolated from PBMCs of vascular pythiosis in the survived group at each PIA immunotherapy time-point. The $CD8^{+}$T-cells with >95% purification

by flow cytometry analysis were co-cultured with their matched monocyte-derived macrophage prior to further culture with 2 mg/ml PIA. Then IFN-γ was measured by human cytokine measurement kit. As we expected, significant higher of IFN-γ in $CD8^+$ T-cells assay than $CD8^+$ T-cells assay were revealed. These result indicated that $CD8^+$T-cells are able to produce IFN-γ in response to PIA antigen (figure 11A).

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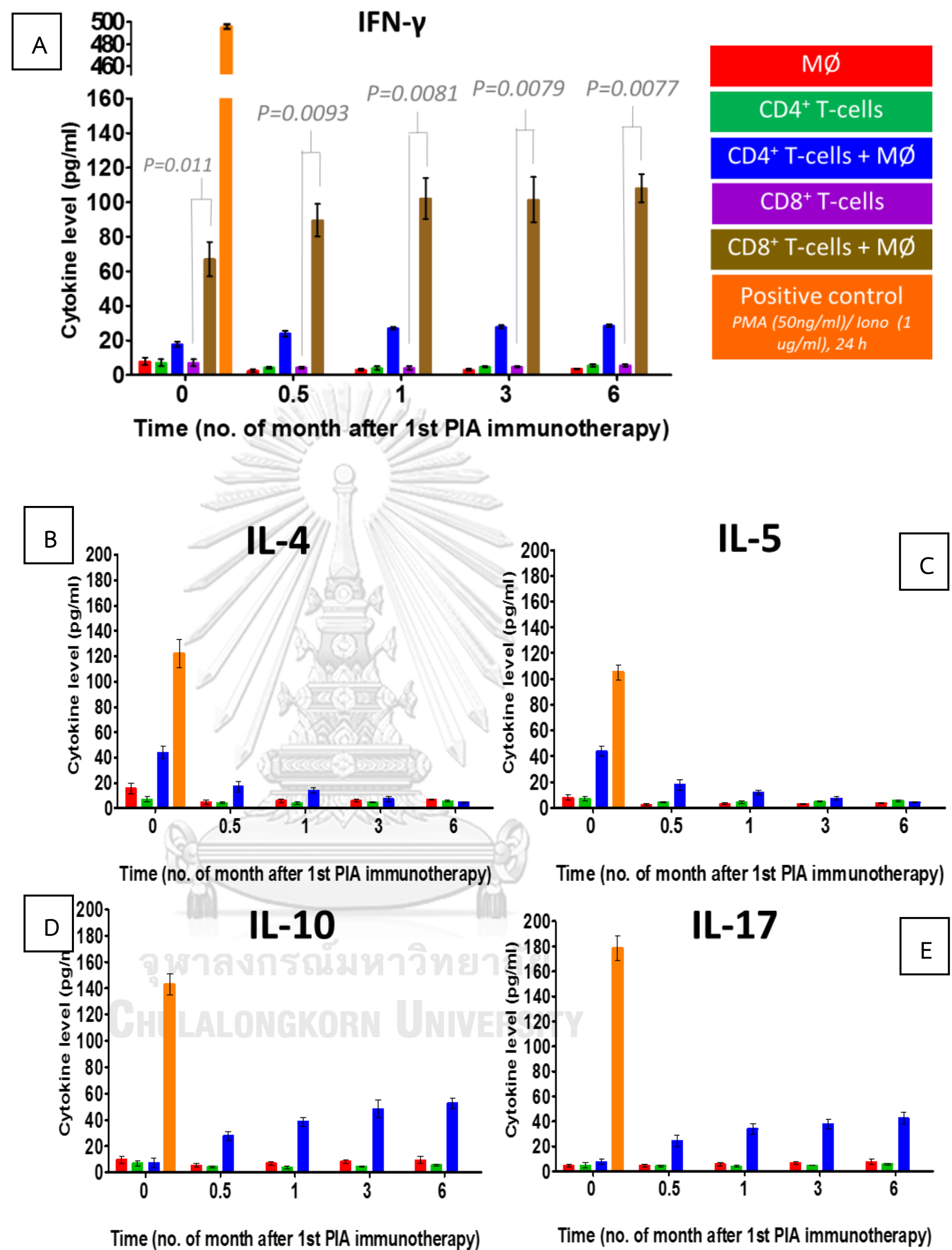

Figure 11 The mean±SD of cytokines IFN-γ (A), IL-4 (B), IL-5 (C), IL-10 (D), IL-17 (E) response of $CD4^+$ T-cells and $CD8^+$ T-cells isolated from PIA immunotherapy treated pythiosis patients who survived during 1 year follow-up along PIA immunotherapy treatment accessed by human cytokine measurement kit based on ELISA assay.

In summary, PIA immunotherapy can induce the cell mediated immune response to produce cytokines. And in PIA immunotherapy treated vascular pythiosis patients, the cytokines' profile is associated with clinical outcome. We found the "immunomodulation" of Th2 to Th1 cytokines in parallel with the increased trend of IL-10 and IL-17 in survival group at the first 1 month after PIA immunotherapy. On the other hand, high constant level of Th2 cytokines since prior PIA immunotherapy started until the patients died was shown in deceased group. In case of the source of those cytokines production, the obtained result guided that $CD4^+$ T-cells can produce all tested cytokines which are IFN-γ, IL-4, IL-5, IL-10 and IL-17 by the PIA immunotherapy activation. Moreover, we also found that $CD8^+$ T-cells is another immune cells that can produce IFN-γ by the PIA immunotherapy activation.

By the obtained result, cytokines' profile seems to be useful as biomarker for pythiosis treatment monitoring and disease prognosis. Due to in the present, CT angiogram is the only one technique used for the examination of disease status. However, high cost and the limited service only in the tertiary care or university hospital are the main obstacle. Thus biomarker that capable to evaluate the disease status or disease prognosis is indispensable. In this study we were interested in (1,3)-β-D-glucan.

1.3 Serum (1,3)-β-D-glucan level in vascular pythiosis patients

(1,3)-β-D-glucan is one of the major cell wall component of *P. insidiosum* which is released in the blood circulation during the cell replication. The studies in rabbit models revealed serum level of (1,3)-β-D-glucan was correlated with the

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severity of invasive pulmonary aspergillosis. Moreover, (1,3)-β-D-glucan level in CSF is also useful in monitoring of therapeutic response in homogenous *Candida* meningoencephalitis. Therefore, we hypothesized that (1,3)-β-D-glucan level in vascular pythiosis patients is related to the disease burden or retained fungal materials in vascular system.

Here, we monitored the level of (1,3)-β-D-glucan in serum of PIA immunotherapy treated vascular pythiosis patients prior each PIA immunotherapy time-points. Prior the PIA immunotherapy started, we expected the non-significant different ($p < 0.05$) of (1,3)-β-D-glucan level among survival and deceased group due to all vascular cases got infection by the same pathogen. However based on the practical PIA protocol used in this study, after the patients were diagnosed as pythiosis, they will start a year course of PIA immunotherapy. PIA was a crude antigen including some polysaccharide such as (1,3)-β-D-glucan. Therefore after PIA immunotherapy started, we expected to see the correlation between the serum (1,3)-β-D-glucan level and the clinical outcome. In survival group, we hypothesized that the serum (1,3)-β-D-glucan should be decreased whereas increased trend or high constant level of (1,3)-β-D-glucan should be found in deceased group.

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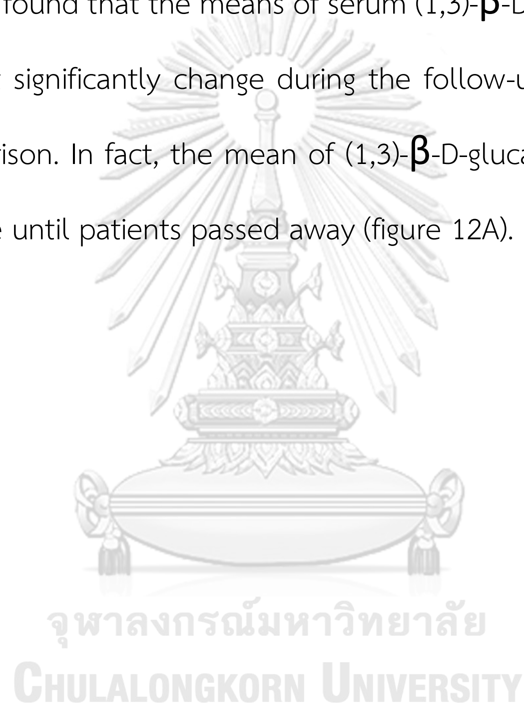
Based on the recommended cut-off value (Fungitell assay), >80 pg/ml for serum (1,3)-β-D-glucan level, all patients recruited in this study had positive result at the time of diagnosis whereas all results from matched thalassemic patients in the control group were negative (figure 11A, 11B). The means of BG level in survival group and deceased group were not statistically different (489.5 ± 39.6 pg/ml VS

514.3 ± 14.0 pg/ml respectively; $p=0.17$). However, after PIA immunotherapy started, patients in survival group had significantly $p<0.05$) lower means of serum (1,3)-β-D-glucan than deceased group (figure11A). Those significantly lower means of serum (1,3)-β-D-glucan level among survival and deceased group ($p=0.0231$, $p=0.0098$, $p=0.0074$, $p=0.0042$ at 0.5, 1.0, 1.5, 3.0 months after PIA immunotherapy started, respectively) were demonstrated until the patients died (figure 11B). Moreover, we found that the means of serum (1,3)-β-D-glucan levels in deceased group did not significantly change during the follow-up period within the same group comparison. In fact, the mean of (1,3)-β-D-glucan levels at 3 months was highly positive until patients passed away (figure 12A).



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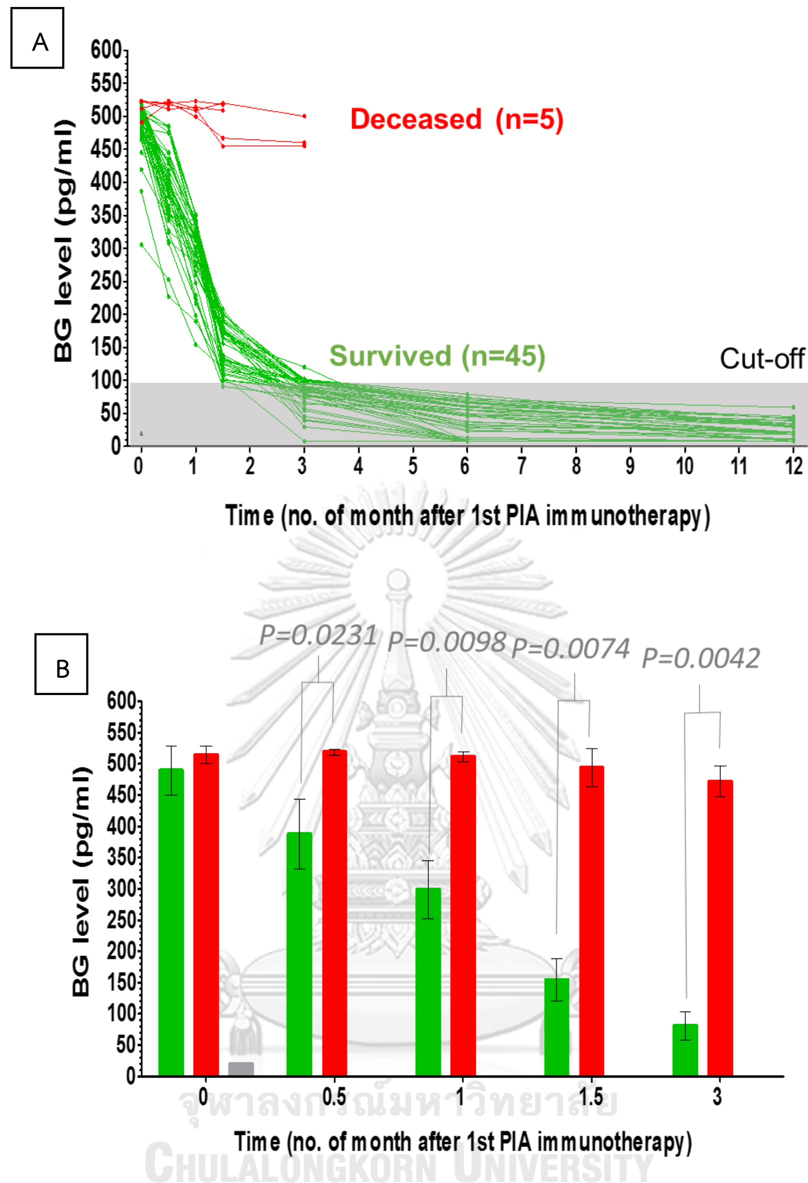

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Figure 12 The individual value (A) and the mean±SD (B) of BG levels in vascular pythiosis patients who survived (n=45; green lines/bar) and deceased (n=5; red line/bar) during 1 year follow-up along with PIA immunotherapy and thalassemic control group (n=40; gray lines/bar) accessed by Fungitell assay (Cape Cod, Falmouth, MA, USA).

By the obtained result it is obviously showed that (1,3)-β-D-glucan level is correlated with the clinical outcome of the patients. Thus it can be used as biomarker for treatment monitoring and disease prognosis.

So far we have done for the investigation of cell mediated immune response and (1,3)-β-D-glucan level. Next step we try to investigate the level of *P. insidiosum* specific antibody based on the hypothesis that the level of humoral immune response via *P. insidiosum* specific antibody production should have some correlation with the clinical outcome of vascular pythiosis patients.

1.4 Serum *P. insidiosum* specific antibody in vascular pythiosis patients

In this study we monitored the level of *P. insidiosum* specific antibody in serum among the same group as previous. We measured the *P. insidiosum* specific antibody level in both prior and after PIA immunotherapy course by *in-house* ELISA assay.

With the fact that three clades of *P. insidiosum* have been classified. By the *in-house* ELISA assay based on indirect ELISA system, plate coated protein or coated antigen is one of the factor influence the detection of specific IgG antibody levels of pythiosis. Thus, at the first step, the effect of *P. insidiosum* clade used as antigen in ELISA assay was validated.

1.4.1 The effect of *P. insidiosum* clade used as antigen in ELISA assay

To investigate the effect of *P. insidiosum* clade used as antigen in ELISA assay, the reactions were performed by the same concentration of three clades coated antigens. By this experiment, the highest EV_{490} value was demonstrated among the clade matched of antigen and antibody (EV_{490} = 19.13 ± 1.87),

followed by the reaction between clade B_{TH} and clade C_{TH} (EV_{490} = 10.63 $\pm$ 0.82). The lowest EV value was observed in the reaction between clade A_{TH} and clade B_{TH} (EV_{490} = 8.12 $\pm$ 0.31) or C_{TH} (EV_{490} = 8.09 $\pm$ 0.21). The similar results were revealed in both individual (antigen prepared from one isolate) and mix antigen (antigen prepared from isolates). Very low EV_{490} value (EV_{490} = 0.65 $\pm$ 0.24) was demonstrated among the reaction of other fungal infections (figure 13A - 13D).

Here the clade that shown the highest EV_{490} value was selected as an antigen used in this research project. Among the similar EV_{490} value of antigen from clade B_{TH} and clade C_{TH}, clade C_{TH} was selected and used as an antigen for the ELISA test by the reason of prevalence. Clade C_{TH} was the highest incidence group found in Thailand.

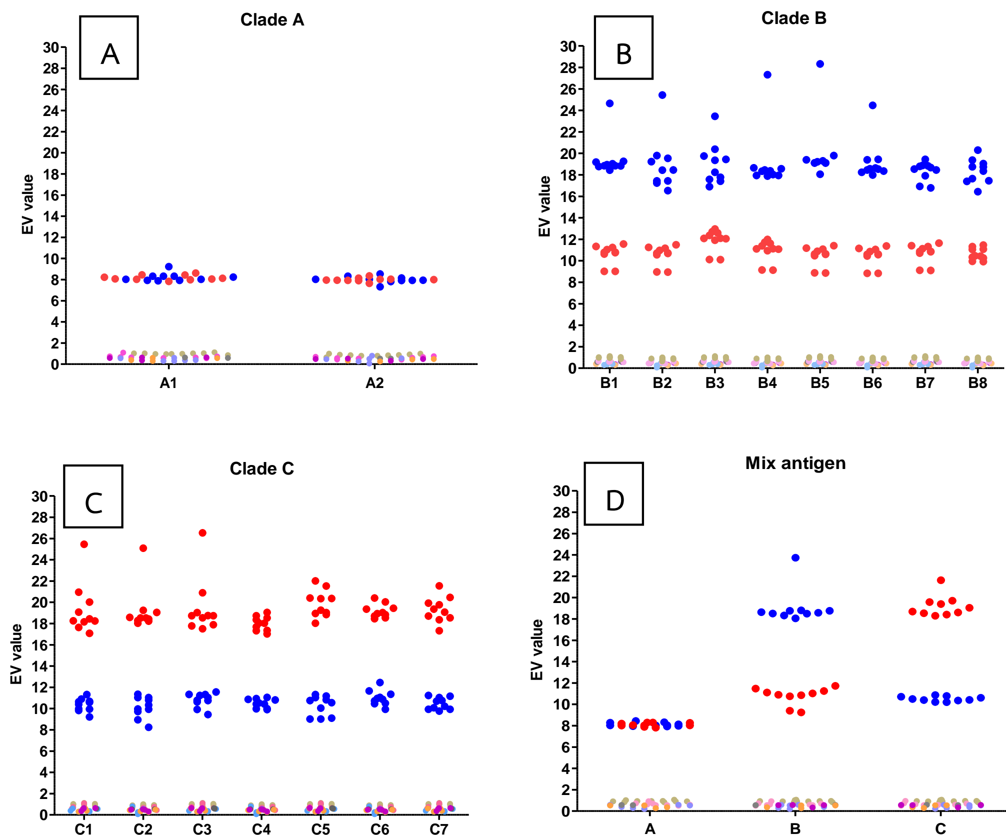

Figure 13 Comparison of EV value generated from various coated P. insidiosum antigens: individual antigen from clade A_{TH} strain 1, 2 (A1-A2), B_{TH} strain 1-8 (B1-B8), C_{TH} strain 1-7 (C1-C7) and mixed antigen of clade A_{TH}, B_{TH}, C_{TH} (D) against various types of pooled serum samples from pythiosis clade B_{TH} (•), C_{TH} (•) and other fungal infections: candidiasis (• ; n=10), cryptococcosis (• ; n=8), invasive aspergillosis (• ; n=6), penicillosis (• ; n=2), nocardiasis (• ; n=4) and histoplasmosis (• ; n=4).

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In *in-house* ELISA assay, serum dilution is another factor influences the detection of specific IgG antibody levels. Thus serum dilution optimization was examined in the next step.

1.4.2 The optimal serum dilution used in *in-house* ELISA assay

Serum dilution is another factor plays an important role in the ELISA assay. Here, the serum dilution ranging from 1:50 to 1:25,600 was tested. By this established *in-house* ELISA, the serum dilution of 1:800 presented the most significant difference of OD_{490} value among each PIA immunotherapy time-point (figure 14). Therefore, the 1:800 ratio of serum dilution was used for all tested vascular pythiosis sera recruited in this research project.

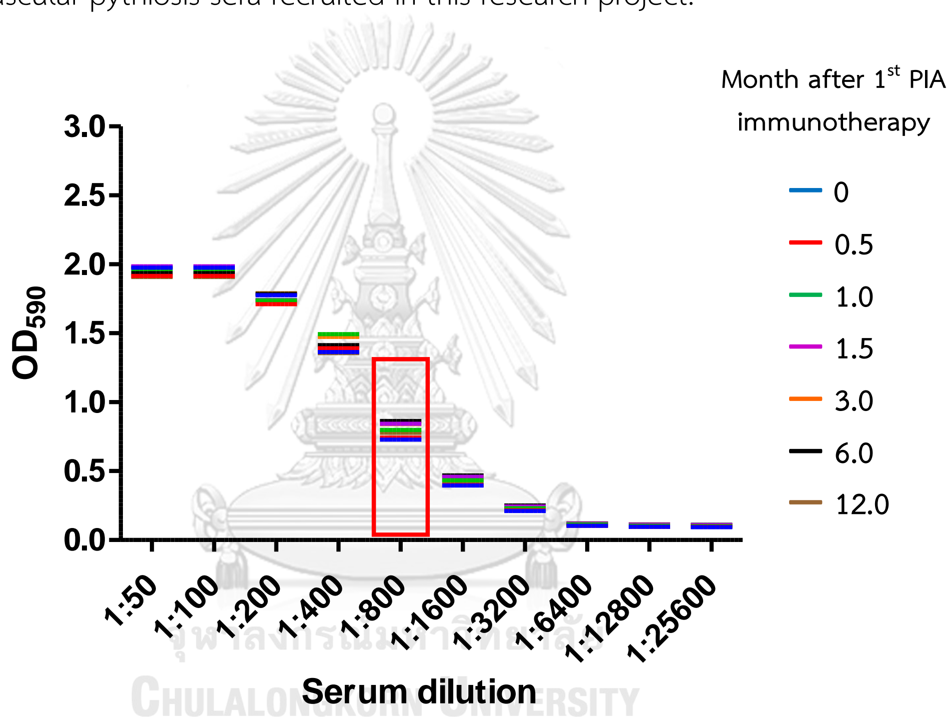

Figure 14 Comparison of OD_{490} value obtained from 7 sera collected before each PIA immunotherapy time-points by using in-house ELISA assay. Pooled serum samples were performed 2-fold serial dilution from 1:50 to 1:25,600 (x-axis). Each line demonstrated the mean of OD_{490} value (Y-axis).

After the clade of coated antigen and serum dilution were validated, the level of *P. insidiosum* specific antibody *(Pi-Ab)* was monitored in each PIA immunotherapy time-point. Based on the previous result of cytokines' profile, we hypothesized the non-significant different of *Pi-Ab* among survived and

deceased group. However, after PIA immunotherapy, we expected the correlation of *Pi-Ab* level and the clinical outcome of the patients. In survived group, we hypothesized the high constant level of *Pi-Ab* along a year course of immunotherapy whereas lower level of *Pi-Ab* compared to survival group was expected in deceased group.

1.4.3 *P. insidiosum specific antibody (Pi-Ab) level in vascular cases*

Based on the results from the linear mixed effect model, as we expected, the level of *Pi*-Ab was correlated with the clinical outcome. In survival group, the significantly higher of *Pi*-Ab than in the deceased group at the time of diagnosis (8.21 and 2.43 respectively; $p < 0.001$) was showed. At each follow-up visit, there was no significantly ($p<0.05$) change in *Pi*-Ab level among survived patients or patients in survival group can maintain their antibody levels above 6 throughout the study period. Even some patients in the deceased group showed the increased trend, all of them presented the *Pi*-Ab levels below 6 during their follow-up period (figure 15A). Moreover, we found that the *Pi*-Ab levels of survival cases showed significantly higher than patients of deceased group since the time of diagnosis until the patients in deceased group died ($p=0.0224$, $p=0.0366$, $p=0.0199$, $p=0.0173$, $p=0.0186$, $p=0.0241$ at 0, 0.5, 1.0, 1.5, 3.0, respectively) (figure 15B).

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The result obtained from this study indicated that EV of *Pi*-Ab > 6 correlates with the good treatment response and good prognosis. Thus EV of *Pi*-Ab is possible to be applied as surrogate biomarker for treatment response and disease prognosis in the future.

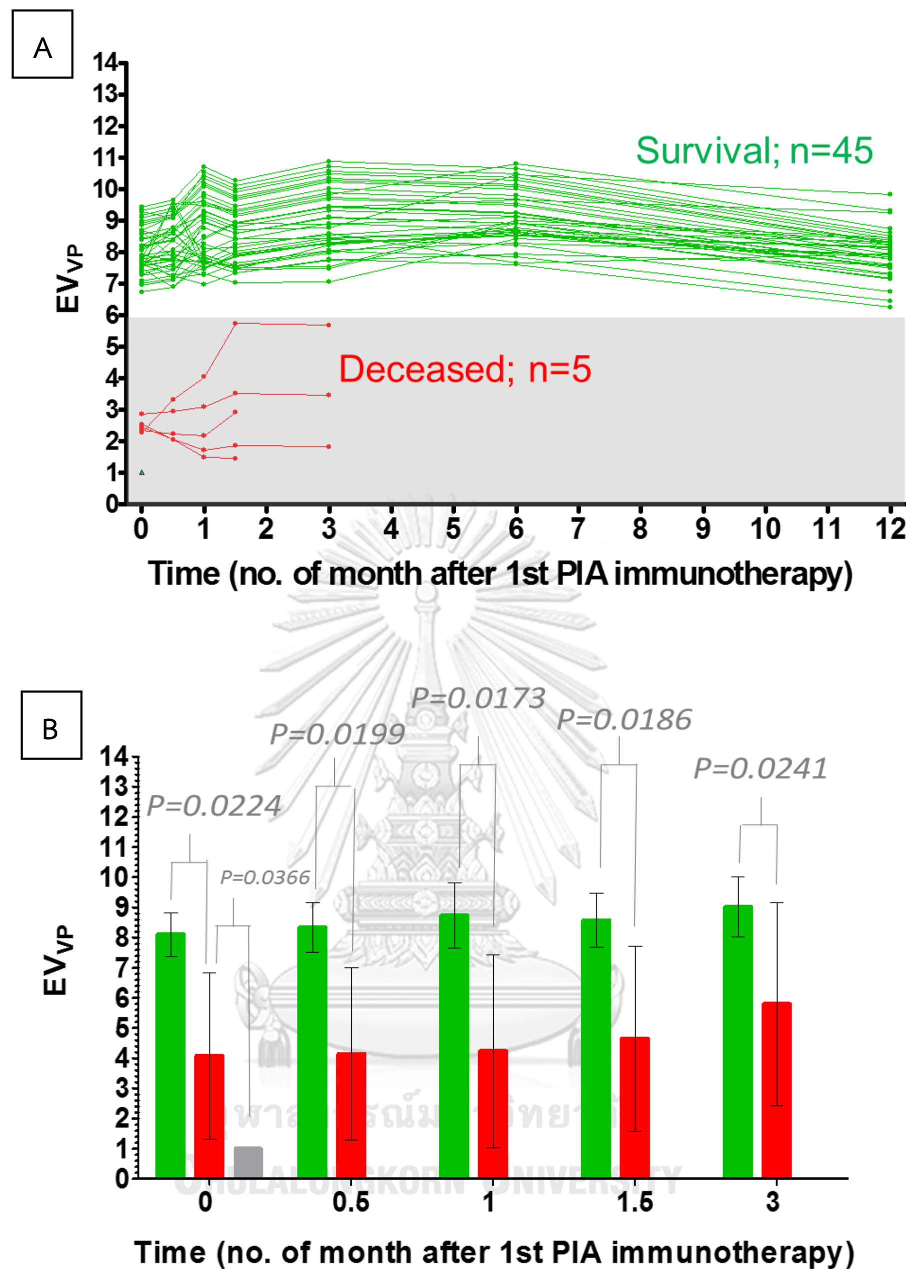

Figure 15 The individual EV value (A) and the mean±SD (B) of Pi-Ab levels in vascular pythiosis patients who survived (n=45; green lines/bar) and deceased (n=5; red line/bar) during 1 year follow-up along with PIA immunotherapy and thalassemic control group (n=40; gray lines/bar) accessed by established in-house ELISA.

To further characterize the relationship between (1,3)-β-D-glucan level and cytokines or *Pi*-Ab, a correlation analysis between these parameters was performed in both survived and deceased group.

1.5 Correlation between the level of (1,3)-β-D-glucan and cytokines or *Pi*-Ab

1.5.1 Correlation between the level of (1,3)-β-D-glucan and cytokines

Based on the correlation analysis between (1,3)-β-D-glucan level and five cytokines: IFN-γ, IL-4, IL-5, IL-10 and Il-17 at each PIA immunotherapy time-points, the significant correlation ($p<0.05$) was found in survival group but not in deceased group. Among the survival group, a significant positive correlation of serum (1,3)-β-D-glucan level with IL-10 at 0.5 months (r=0.1213; $p=0.0191$) (figure 16) and IL-17 at 12 months (r=0.08713; $p=0.049$) (figure 17) after PIA immunotherapy course started was demonstrated whereas the a significant negative correlation of serum (1,3)-β-D-glucan level was found with IFN-γ at 1.5 months (r=0.1037; $p=0.0310$) (figure 18) and IL-4 at 12 months (r=0.11; $p=0.0261$) (figure 19) after PIA immunotherapy.

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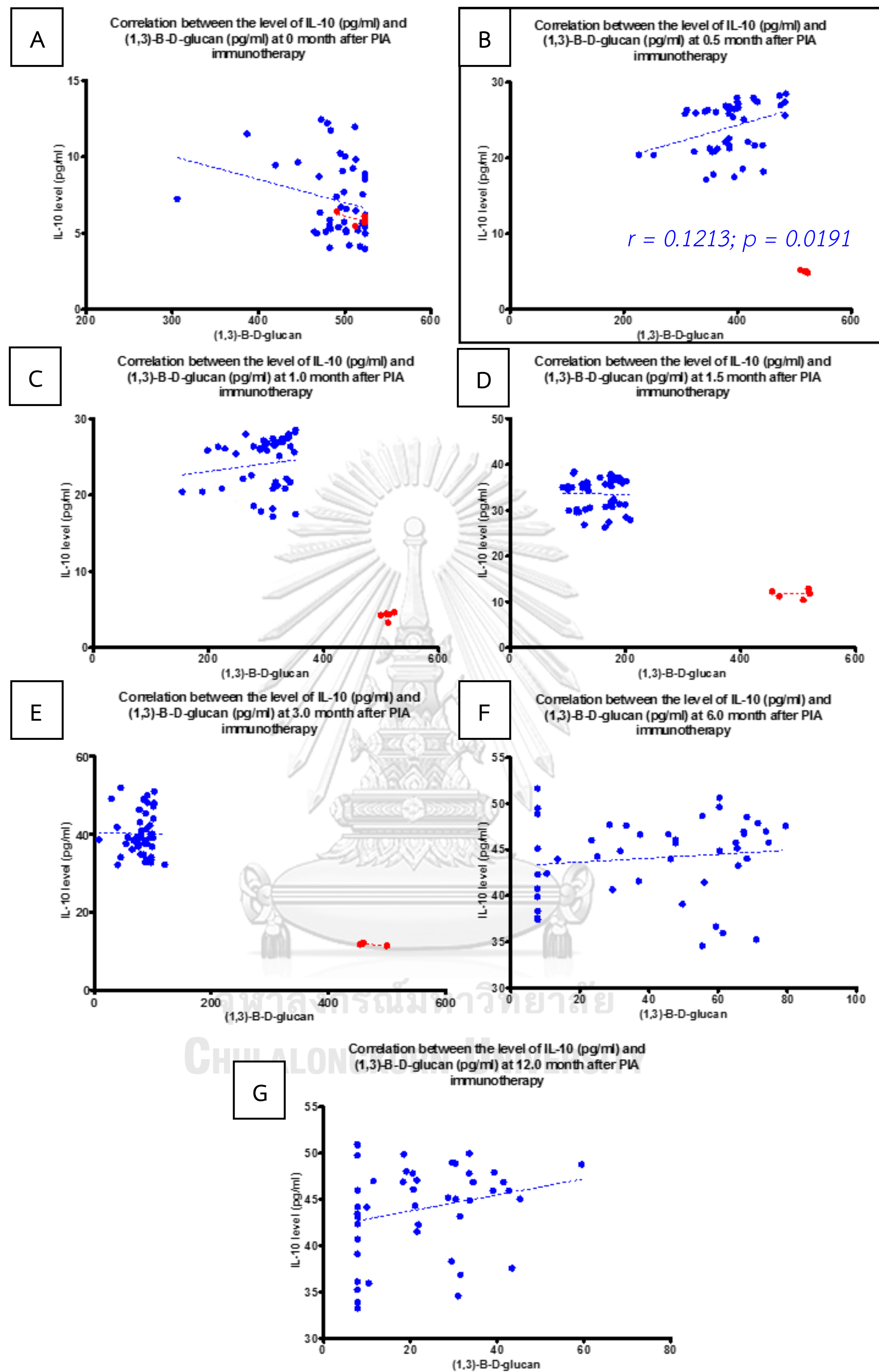

Figure 16 Correlation between the serum level of (1,3)-β-D-glucan and IL-10 at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in survival (▪ ; n=45) and deceased (▪ ; n=5) group.

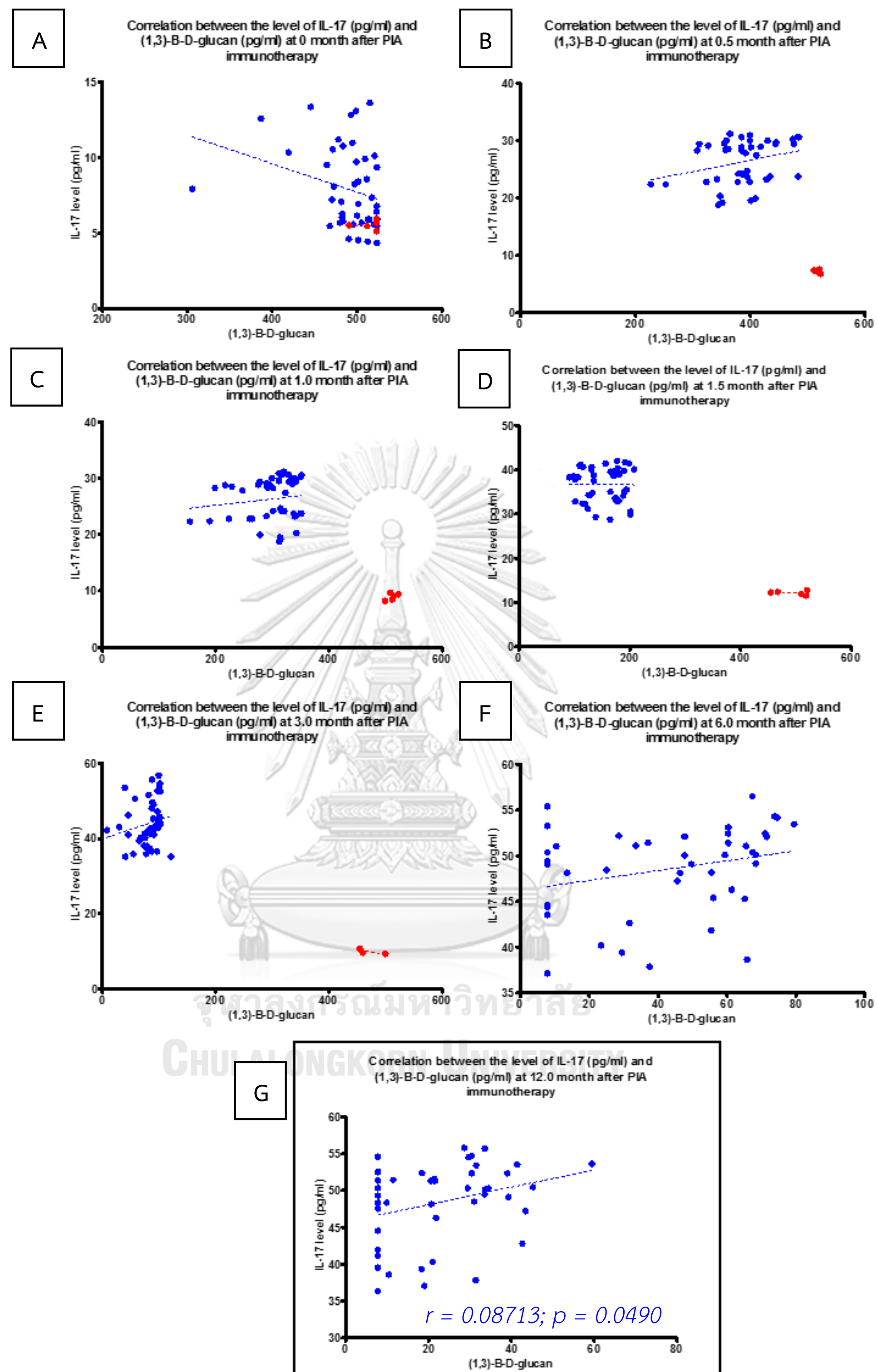

Figure 17 Correlation between the serum level of (1,3)-β-D-glucan and IL-17 at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in survival (■ ; n=45) and deceased (■ ; n=5) group.

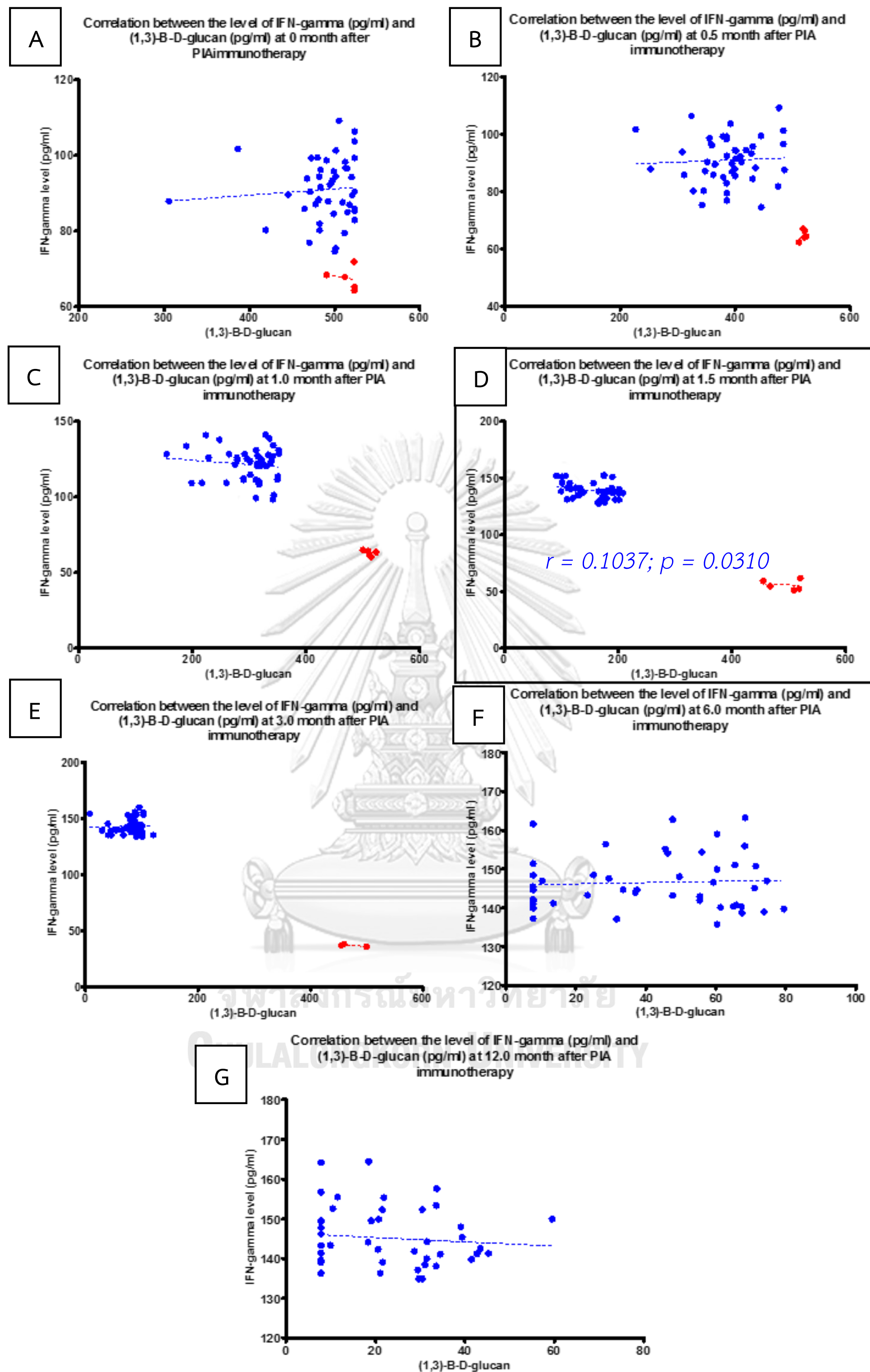

Figure 18 Correlation between the serum level of (1,3)-β-D-glucan and IFN-γ at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in survival (■ ; n=45) and deceased (■ ; n=5) group.

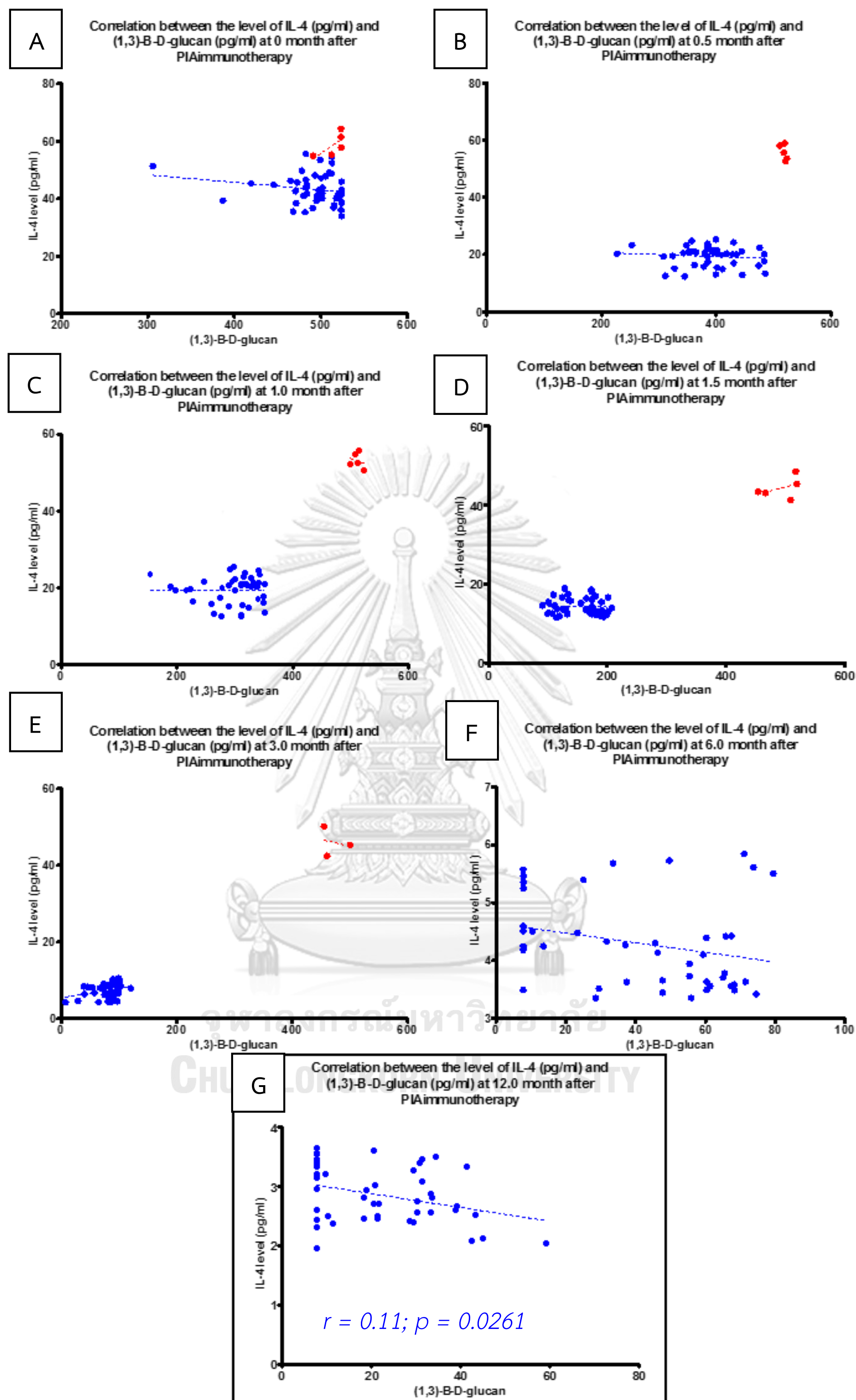

Figure 19 Correlation between the serum level of (1,3)-β-D-glucan and IL-4 at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in survival (■ ; n=45) and deceased (■ ; n=5) group.

Based on the result obtained from correlation analysis, it is quite difficult to summarize the correlation between the level of serum (1,3)-β-D-glucan and the six cytokines. It might causes by one of the limitations of the study. The upper limit of (1,3)-β-D-glucan measurement kit is 523 pg/ml. If the level of (1,3)-β-D-glucan in patients' sera is higher than that it was noted as 523 pg/ml. We did not perform the serum sample dilution to access the exactly (1,3)-β-D-glucan concentration. In case of after PIA immunotherapy started and the level of (1,3)-β-D-glucan started deceasing or become lower than the upper limit, non-correlation between (1,3)-β-D-glucan level and cytokines' level was also found. This phenomenon indicated that the trend or pattern of (1,3)-β-D-glucan and cytokines after PIA immunotherapy started is more important than the level itself.

1.5.2 Correlation between the level of (1,3)-β-D-glucan and EV of *Pi*-ab

Based on the correlation analysis between (1,3)-β-D-glucan level and EV of *Pi*-ab at each PIA immunotherapy, significant correlation ($p<0.05$) was found in survival group but not in deceased group. Among the survival group, a significant positive correlation of serum (1,3)-β-D-glucan level with EV of *Pi*-ab at 0 ($r=0.1136$; $p=0.0253$) and 0.5 months ($r=0.1566$; $p=0.0071$) (figure 20) after PIA immunotherapy course started was demonstrated.

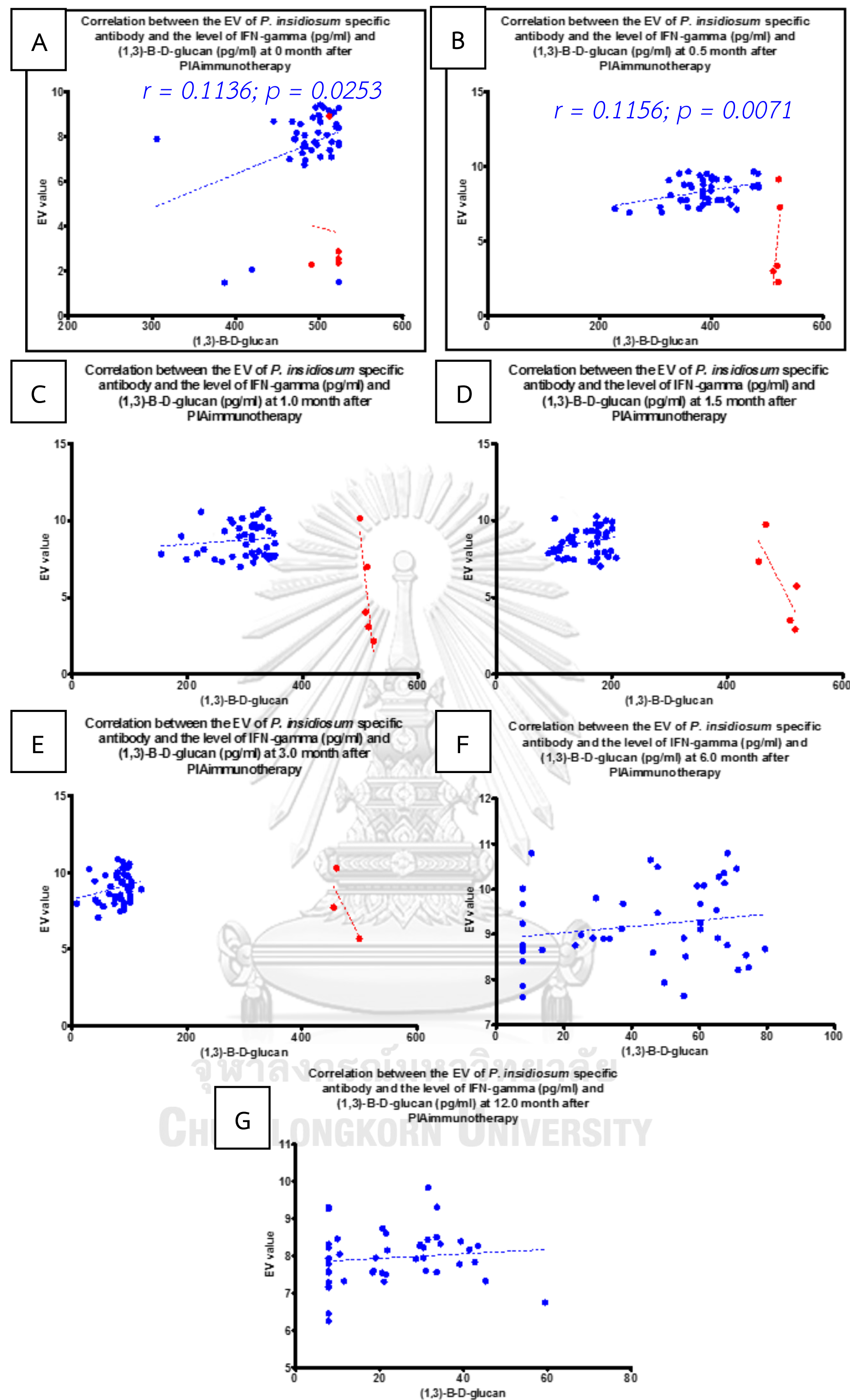

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Figure 20 Correlation between the serum level of (1,3-β-D-glucan and EV value of *Pi*-Ab at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in survival (■ ; n=45) and deceased (■ ; n=5) group.

Similar to the correlation between serum (1,3)-β-D-glucan and cytokines level, it is quite difficult to summarize the correlation between these parameters. One related cause might be the level of (1,3)-β-D-glucan which we did not access their exactly level. However, the obtained result indicated that trend or pattern of (1,3)-β-D-glucan and *Pi*-Ab after PIA immunotherapy started is more important than its level.

In summary of vascular pythiosis patients, we found that after natural *P. insidiosum* infection. The infection activated Th2 response via IL-4 and IL-5 production including *P. insidiosum* specific IgG antibody from B-cells (figure 21). However, after the patients got PIA immunotherapy, Th1 response was activated via IFN-γ production including the production of IL-10 and IL-17 from Treg and Th17. Cytotoxic T-cells is another source of IFN-γ production induced by PIA immunotherapy. Moreover, we found that PIA immunotherapy can also induce the *P. insidiosum* specific antibody production from B-cells (figure 22). All of the phenomenon appear after PIA immunotherapy are correlated with no sign of disease recurrent of pythiosis patients.

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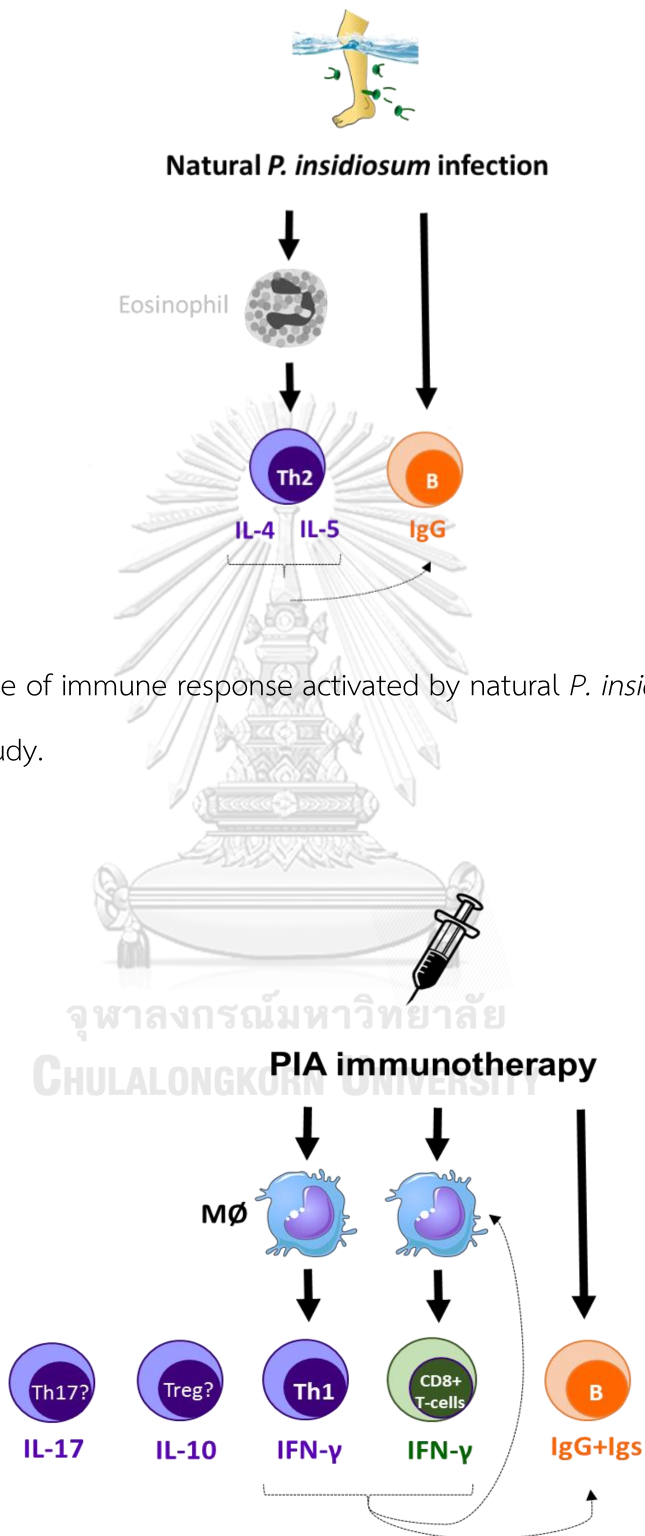

Figure 21 Scheme of immune response activated by natural *P. insidiosum* infection proved in this study.

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Figure 22 Scheme of immune response activated by PIA immunotherapy proved in this study.

2. Ocular pythiosis patients

2.1 Patient characteristic

Based on the final clinical outcome, proven ocular pythiosis cases recruited in this study was classified into 2 groups. There were the 1) saved globe group: the patient whose eye was still visual and the 2) blindness group: patients who became blind due to the pythiosis infection. A total of 30 patients, sixteen saved globe cases and fourteen blind cases were grouped. The information of patient characteristics, treatment modalities, and post treatment clinical information are summarized in table 4. We found that age of patients was significantly different between the saved globe group and the blindness group. Patients whose globe was saved were significantly younger than those who became blind (mean age 37.9 vs. 43.3 years respectively; $p = 0.042$). A shorter duration of disease onset to the first medical attention (mean age 37.9 vs. 43.3 days respectively; $p = 0.046$) and from diagnosis to the first definitive surgery (mean age 1.8 vs. 4.5 days respectively; $p < 0.0001$) were observed in the saved globe group than the blindness group. In the case of surgery type, not surprisingly, all saved globe cases underwent PK or corneal transplant whereas all cases who became blind underwent glass ball removal or evisceration which is the cause of life-long blindness. In terms of clinical signs or symptoms after treatment, no pain complaint and fever were noted in both groups.

Table 3 Characteristics of ocular pythiosis patients.

	Saved globe (16)	Blindness (14)	*P-value**
Patient related parameters			
Age (years)	37.9 ± 10.4	43.3 ± 15.6	0.042
Male gender	8 (50%)	7 (50%)	NS
History of water exposure within 3 months	4 (25%)	4 (28.6%)	NS
Duration from onset to disease to first medical attention (days)	37.9 ± 10.4	43.3 ± 15.6	0.046
Duration from diagnosis to first definitive surgery (days)	1.8 ± 0.4	4.5 ± 2.0	<0.0001
Treatment related parameters			
Surgery			<0.0001
- Glass ball removal/Evisceration	-	14 (100%)	
- PK/Corneal transplant	16 (100%)	-	
Systemic antifungal agent			NS
- Itraconazole alone	1 (6.25%)	-	
- Itraconazole + terbinafine	14 (87.5%)	14 (100%)	
- Itraconazole + ketonazole	1 (6.25%)	-	
Topical antifungl agent			
- Amphotericin B alone	-	-	
- Amphotericin B + Azole drugs	16 (100%)	14 (100%)	
Duration of sysyemic antifungal treatment (months)	6.1 ± 2.2	5.9 ± 1.4	NS
Duration of topical antifungal treatment (months)	1.9 ± 2.1	2.1 ± 1.1	NS
Clinical signs/symptoms post treatment initiation			
- Fever > 38.2°C	-	-	NS
- Pain	-	-	NS

* Calculated by *the chi-square test or Fisher's exact test*

In summary, thirty ocular cases enrolled in this study were classified into two groups: saved globe and blindness group based on the final clinical outcome. Age and duration time from disease onset to medical surgery were found the significant difference between saved globe and blindness group by the chi-square test or Fisher's exact test. Patients in saved globe group were significantly younger and

shown shorter duration time in both from disease onset to the first medical care and from definitive diagnosis to definitive surgeries than the blindness group. In case of surgery type, not surprisingly, all saved globe cases were underwent PK or corneal transplant whereas all cases who become blindness were performed the glass ball removal or evisceration which is the cause of life-long blindness. In term of clinical signs or symptoms post treatment initiation, no pain complained and fever were noted in both groups.

As mentioned above, ocular pythiosis is the localized infection at the immunoprivileged site. Thus we expected non-significant difference ($p < 0.05$) of (1,3)-β-D-glucan level, cytokines level and *Pi*-Ab between saved globe, blindness and healthy control group prior PIA immunotherapy course. However, after the patients got the PIA immunotherapy which contained 2 pg/ml of (1,3)-β-D-glucan per dose. We hypothesized that the level of serum (1,3)-β-D-glucan will increased leading to the immune response activation via cytokines and *Pi*-Ab production.

2.2 The level of (1,3)-β-D-glucan in ocular pythiosis patients

As we expected prior PIA immunotherapy, non-significant difference ($p < 0.05$) of serum (1,3)-β-D-glucan level between saved globe, blindness and healthy control group was demonstrated. Based on the recommended cut-off value, < 60 pg/ml as negative result for serum (1,3)-β-D-glucan level, all of them showed negative result at the time of diagnosis (figure 23A, 23B). Then after the PIA immunotherapy course started, the mean levels of BG in the saved globe and blindness group were also not statistically different (37.9 $\pm$ 14.0 pg/ml VS 28.7 $\pm$ 9.6 pg/ml respectively; $p = 0.12$) as showed in figure 23A, 23B.

Then at each follow-up visit during the first month, the mean level of serum (1,3)-β-D-glucan showed a significantly increase ($p < 0.05$) in all cases with the highest peak at 1 month after immunotherapy course initiation. Then the significantly decreased ($p < 0.05$) of mean of serum (1,3)-β-D-glucan levels were revealed and became negative after 3 months.

In summary, PIA immunotherapy is capable to increase the serum (1,3)-β-D-glucan level. By the practical protocol of PIA immunotherapy used in the present, the level of (1,3)-β-D-glucan showed the peak at 1 month after PIA immunotherapy followed by the increased trend and become negative (<60 pg/ml) since the 3rd month until the immunotherapy course ended. This phenomenon might be able to explain by the murine model experiment of Hong *et al* in 2004. In this study, they proved that injected (1,3)-β-D-glucan is rapidly cleared by 1-3 weeks which is shorter than the infection-related (1,3)-β-D-glucan. Thus we hypothesized that the decreased trend of (1,3)-β-D-glucan level might be the result of (1,3)-β-D-glucan degradation. Moreover, with the non-relevance of (1,3)-β-D-glucan level and the clinical outcome. The level of (1,3)-β-D-glucan is not possible to be applied as surrogate biomarker for treatment monitoring and disease prognosis.

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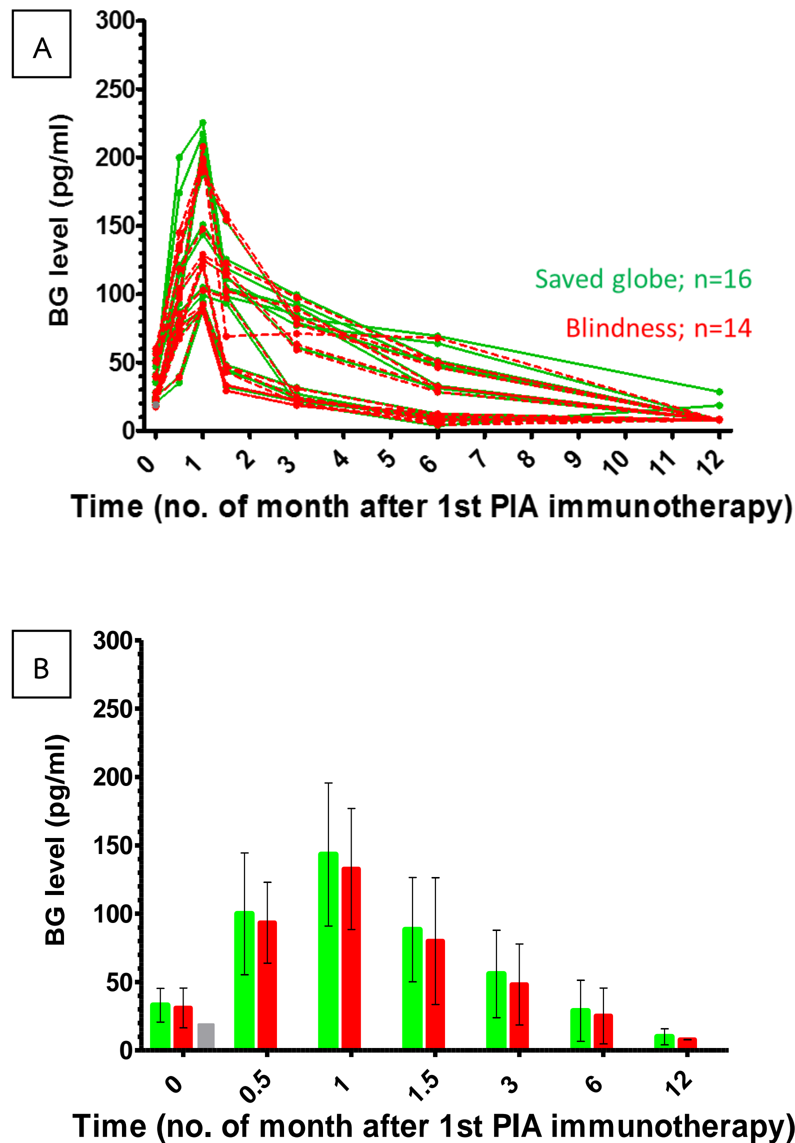

Figure 23 The individual value (A) and the mean±SD (B) of (1,3)-β-D-glucan levels in ocular pythiosis patients who the globe was saved (n=16; green lines) and blindness (n=14; red line) during 1 year follow-up along with PIA immunotherapy and healthy control group (n=40; gray dots/bar) accessed by Fungitell assay (Cape Cod, Falmouth, MA, USA).

To further investigate the capable of PIA immunotherapy to induce the cell mediated immune response via cytokines production, the cytokines level prior each PIA immunotherapy time-points was monitored.

2.3 Total serum cytokine in ocular pythiosis patients

As we expected, prior PIA immunotherapy, there was no significant difference ($p < 0.05$) of the cytokine level among the saved globe, blindness group and healthy control group for all cytokines tested in this study (figure 23). Then after PIA immunotherapy started, non-significant differences ($p < 0.05$) of all cytokines level was also presented among patients in the saved globe group and blindness group. Only one cytokine showed significantly change after PIA immunotherapy started is IFN-γ. The IFN-γ levels showed increasing during the 1.5-6.0 month period compared after PIA immunotherapy. For other cytokines tested in this study, non-significant different changing of their level was showed (figure 24).

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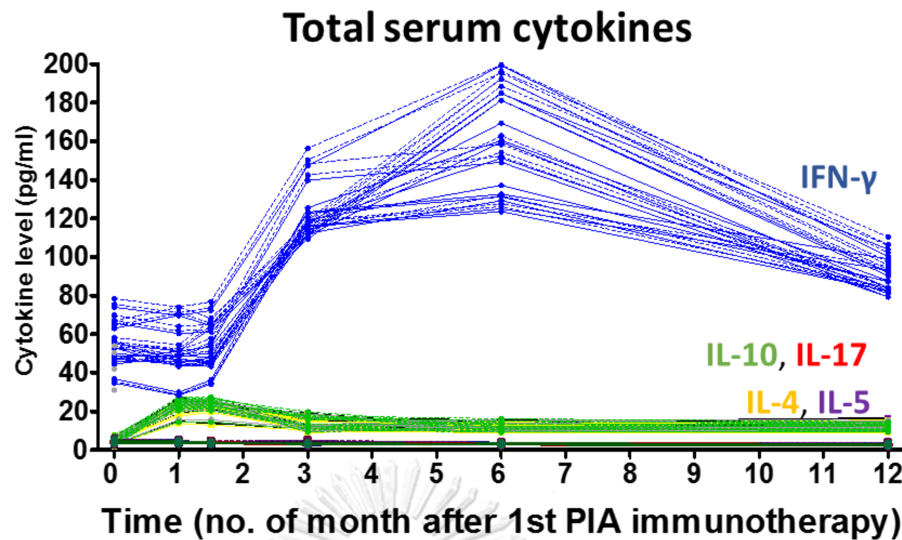

Figure 24 The individual value of cytokines; IFN-γ (blue lines), IL-4 (yellow lines), IL-5 (purple lines), IL-10 (green lines), IL-17 (red lines) response of ocular pythiosis cases both in saved globe (n=16; solid line) and blindness group (n=14; dashed line) prior each PIA immunotherapy during 1 year (totally 7 doses) compared with healthy control group (n=40; grey dots) accessed by human cytokine measurement kit based on ELISA assay.

Besides the level of cytokines, the humoral immune response after PIA immunotherapy started was also monitored in ocular pythiosis patients. The level of *Pi*-Ab was measured prior each PIA immunotherapy time-point as we did in the previous experiments.

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2.4 The EV value of *Pi*-Ab in ocular pythiosis patients

Based on the results from the linear mixed effect model, the different trend of *Pi*-Ab of ocular group from vascular cases was demonstrated. Non-significant difference of *Pi*-Ab in both at the time of diagnosis and after PIA immunotherapy started between saved glove and blindness group was revealed. At each follow-

up visit, the significant ($p < 0.05$) change in *Pi*-Ab level was found until 3 months. After that there was no significantly change in *Pi*-Ab level of all cases until the immunotherapy course ended (figure 25).

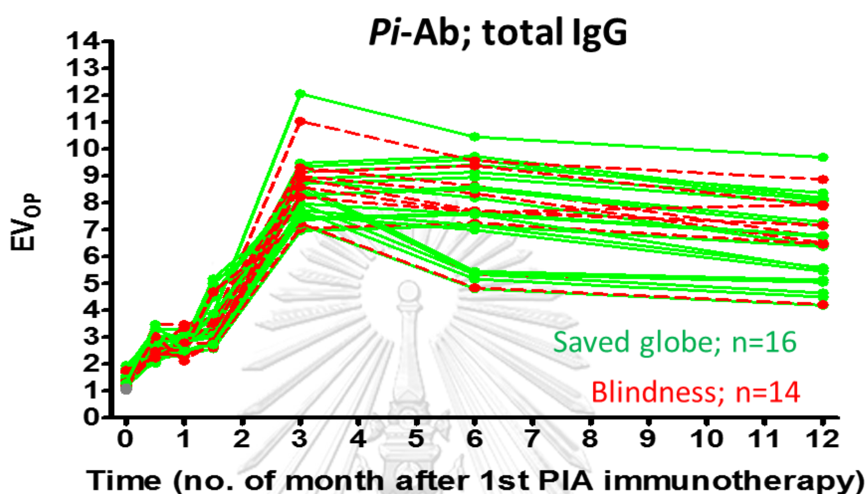

Figure 25 The individual EV value of *Pi*-Ab levels in ocular pythiosis patients who the globe was saved (n=16; green lines) and blindness group (n=14; red lines) during 1 year follow-up along with PIA immunotherapy and healthy control group (n=40; gray dots) accessed by established in-house ELISA.

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2.5 Correlation between the level of (1,3)-β-D-glucan and cytokines or *Pi*-Ab

2.5.1 Correlation between the level of (1,3)-β-D-glucan and cytokines

Based on the correlation analysis between the level of (1,3)-β-D-glucan and five cytokines: IFN-γ, IL-4, IL-5, IL-10 and Il-17 at each PIA immunotherapy time-points, the non-significant correlation ($p < 0.05$) was found in all parameters both prior and after PIA immunotherapy course (figure 26-29) except IFN-γ (figure 30). During the first 1.5 months after PIA immunotherapy

started, the negative correlation between the level of (1,3)-β-D-glucan and IFN-γ (r=0.1666; *p=0.0259,* r=0.1324; *p=0.0358,* r=0.1382; *p=0.0431 and* r=0.1866; *p=0.0172* at 0, 0.5, 1.0 and 1.5 months after PIA immunotherapy, respectively) was demonstrated. This obtained result indicated that the patients who have low level of (1,3)-β-D-glucan, the humoral immune response of them trend to produce more amount of IFN-γ.

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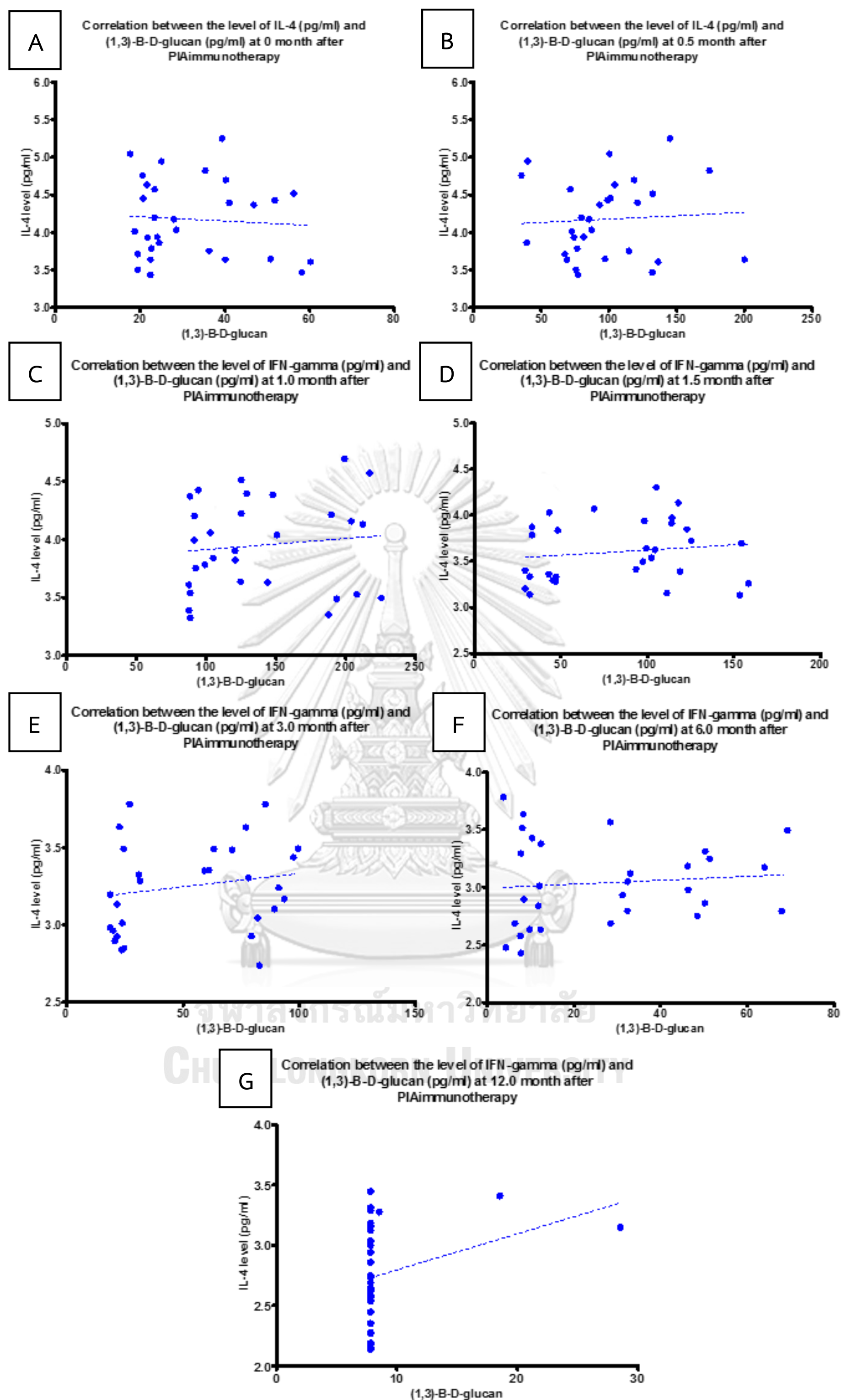

Figure 26 Correlation between the serum level of (1,3)-β-D-glucan and IL-4 at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in ocular pythiosis cases (n=30).

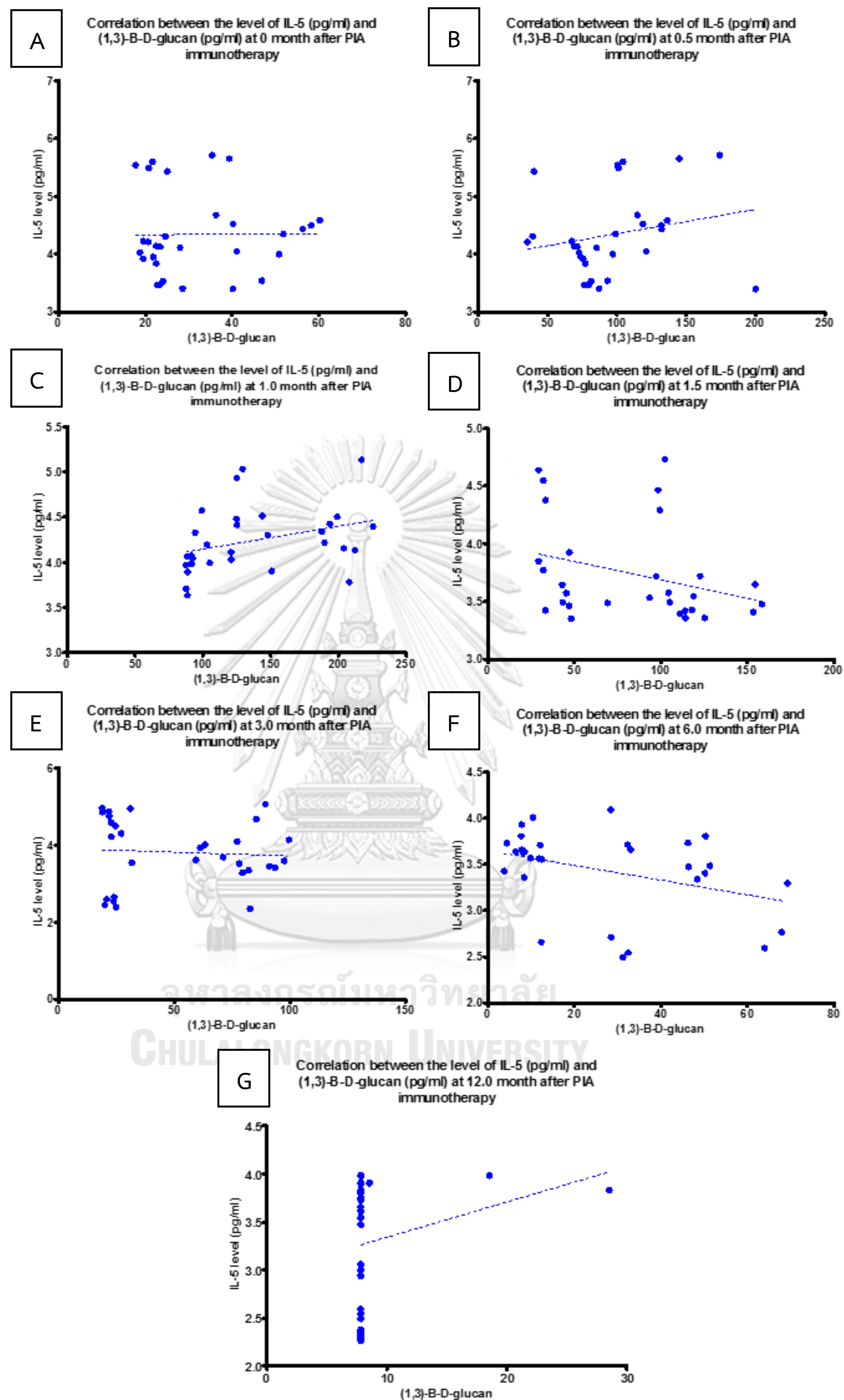

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Figure 27 Correlation between the serum level of (1,3)-β-D-glucan and IL-5 at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in ocular pythiosis cases (n=30).

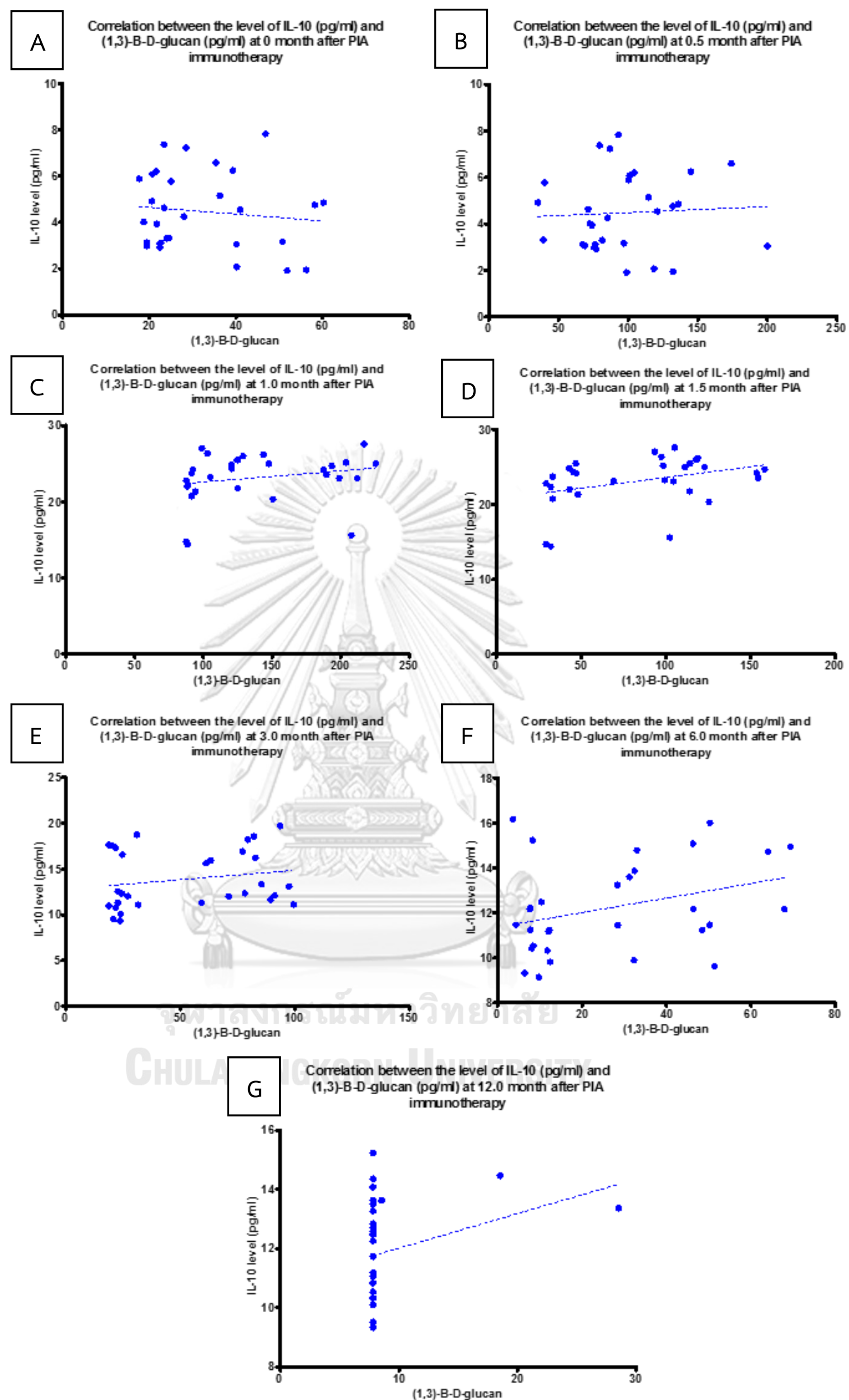

Figure 28 Correlation between the serum level of (1,3)-β-D-glucan and IL-10 at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in ocular pythiosis cases (n=30).

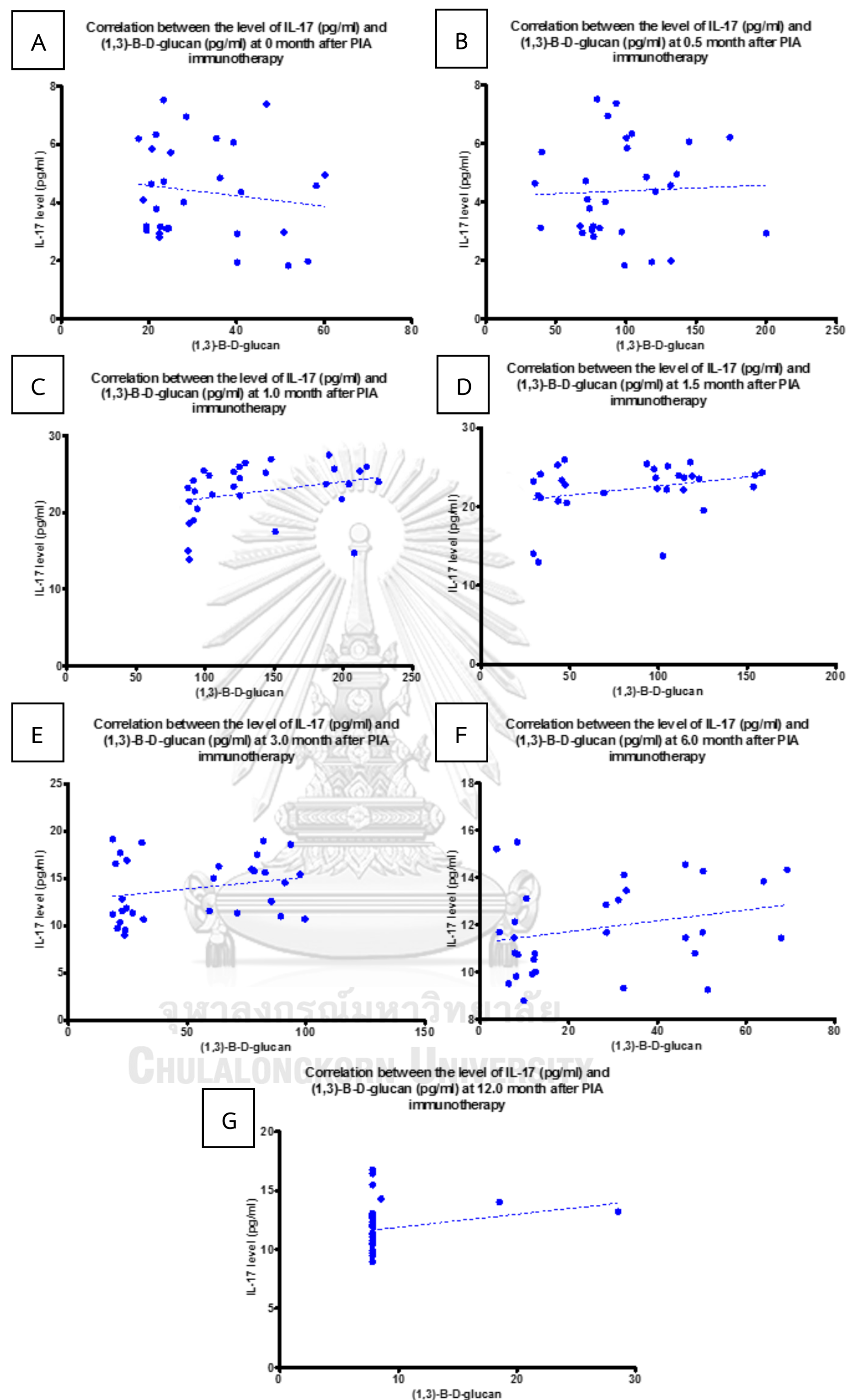

Figure 29 Correlation between the serum level of (1,3)-β-D-glucan and IL-17 at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in ocular pythiosis cases (n=30).

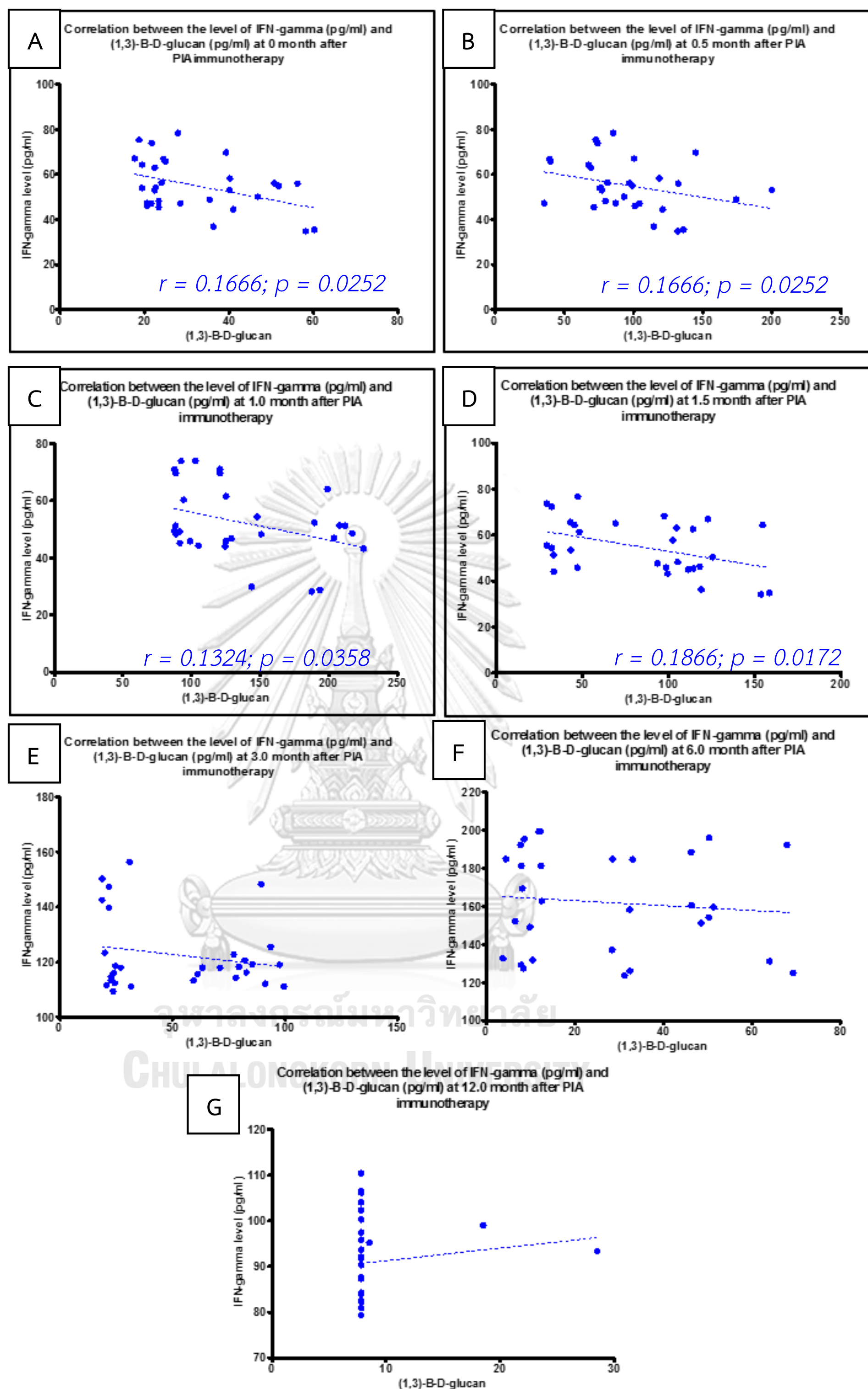

Figure 30 Correlation between the serum level of (1,3)-β-D-glucan and IFN-γ at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in ocular pythiosis cases (n=30).

2.5.2 Correlation between the level of (1,3)-β-D-glucan and EV of *Pi*-Ab

Based on the correlation analysis between (1,3)-β-D-glucan level and EV of *Pi*-Ab at each PIA immunotherapy, non-significant correlation ($p < 0.05$) was found along one year of PIA immunotherapy (figure31).

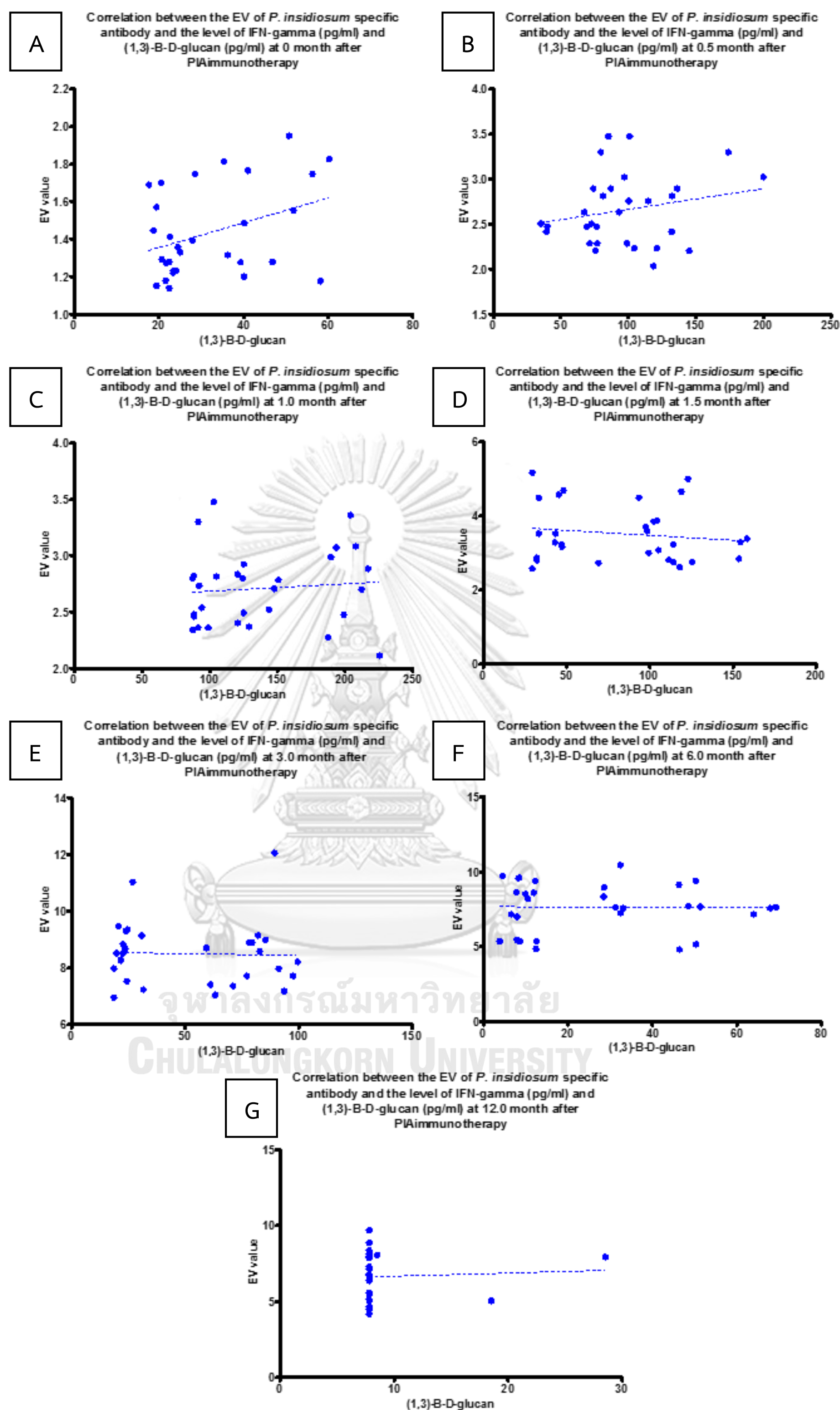

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Figure 31 Correlation between the serum level of (1,3)-β-D-glucan and EV value of *Pi*-Ab at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 3.0 (E), 6.0 (F) and 12.0 (G) after PIA immunotherapy started in ocular pythiosis cases (n=30).

In summary, we found only the negative correlation ($p < 0.05$) between the level of (1,3)-β-D-glucan and IFN-γ in PIA immunotherapy treated ocular pythiosis. This result indicated that the patients who have decreasing trend of (1,3)-β-D-glucan level trend to produce more IFN-γ. For other parameters, the non-correlation might effect by the limitation of kit to access the exactly level of (1,3)-β-D-glucan. Or it is possible that the immune response might be induced by several immunogens contained in the PIA immunotherapy, only mainly by (1,3)-β-D-glucan.

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CHAPTER VI
Discussion

PIA immunotherapy is one of the combination treatments that has been used in human since 1998 (100). Since then the increasing number of PIA-treated human cases was reported not only in Thailand where is the endemic area of pythiosis but also in neighboring countries (101). Even though PIA is widely used in human cases, the study of the immune response in PIA treated patients is very limited. This is the first study including the vascular and ocular pythiosis patients who received a combination therapy of surgery, systemic antifungal agents, and PIA immunotherapy according to research treatment protocol at King Chulalongkorn Memorial Hospital as long as 6.5 years period during January 2010 - July 2016.

By the result obtained in this study we found that after the patients were infected with *P. insidiosum*, the Th2 response of host will be induced via IL-4 and IL-5 production. However, after those patients got combination treatment including PIA immunotherapy, their immune response will be modulated. PIA immunotherapy can induce both cell mediated and humoral immune response of the host. In cell mediated immune response, PIA immunotherapy can modulated the cytokines pattern from Th2 to Th1 response. The switching of Th2 to Th1 is associated with the patients with no sign of disease recurrent. By this study $CD4^+$ T-cells was proved as one of the immune cells that is capable to produce IFN-γ, IL-4, IL-5, IL-10 and IL-17. Besides the $CD4^+$ T-cells, $CD8^+$ T-cells was also proved its capability to produce IFN-γ by the PIA immunotherapy activation. This finding is associated with the result proposed in the equine model and also in the *Leishmania* murine model with pythiosis (20). In case of

Pi-Ab, we found that the constant level of EV of *Pi*-Ab above 6 is associated with the patients who have no sign of disease recurrent. Moreover, as PIA immunotherapy itself also contains the immunogenic substances including polysaccharide substance named (1,3)-β-D-glucan, the injection of PIA also effect the level of serum (1,3)-β-D-glucan. The result of serum (1,3)-β-D-glucan level after PIA injection obtained from ocular group, clearly showed us the capability of PIA immunotherapy to increase the serum (1,3)-β-D-glucan. By this study we proved that (1,3)-β-D-glucan is capable to induce the cytokine IFN-γ, IL-10 and IL-17 production. However, the correlation between (1,3)-β-D-glucan level and other immune related parameters is quite not clear. We hypothesized that it might be effected by several factors. One is the fact that immune response might be induced by several immunogen contained in PIA immunotherapy. Another factor is the limitation in this study. In this study we measured the level of (1,3)-β-D-glucan by the Fungitell assay kit which limit the (1,3)-β-D-glucan level as 523 pg/ml. In some serum samples that the (1,3)-β-D-glucan level was higher than 523 pg/ml, we did not dilute the samples and re-measured their exact (1,3)-β-D-glucan level. We noted those cases as 523 pg/ml.

In vascular pythiosis patients, we found the correlation of (1,3)-β-D-glucan level, cytokines' pattern and *Pi*-Ab pattern with the patients outcome. These results indicated that immune response is one of the factors related to the disease recovery. In this study we found 5 deceased cases. All of them showed high constant level of (1,3)-β-D-glucan. This indicated their immune response have low ability to eliminate the pathogen. At first we hypothesized that they might be the immunocompromised host or ongoing immunosuppressive drug because they showed very low level of both

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cytokines and *Pi*-Ab after PIA immunotherapy started. However, we cannot prove that because none of them were noted as immunocompromised host or ongoing immunosuppressive drug. On the other hand, if they are immunocompetent host, another hypothesis is the strong Th2 response inhibit the Th1 cytokine production. In case of low level of *Pi*-Ab, we hypothesized that IgE which is one of the Th2 response product might inhibit the IgG production. However, those hypothesis need to be proved in the future.

In the past, type of surgery was claimed as determining factor of survival (6, 18). However, surgery is an aggressive treatment and not practical in all cases. Both disease status and the site of infection is related to the possibility of sterile amputation. Most patients who underwent sterile amputation usually survive however they become life-long disabled. More than that in this study we found 2 cases whom sterile amputation were noted but after surgery they developed the sign related to disease recurrent. One of them was proved as pythiosis recurrent by the CT angiogram and positive *Pi*-Ab whereas another case has not been proved. This situation indicated that noted as sterile amputation is not guarantee the survival. We need more biomarker to support the treatment. Presently, CT angiogram is one of the technology can be applied as disease monitoring after treatment by clot observation. However, high cost with limited service only in the tertiary care or university hospital are the main obstacle. Thus, pythiosis biomarker is indispensable. With the advantage of all parameters: (1,3)-β-D-glucan level, cytokines' pattern and *Pi*-Ab pattern examined in this study, they can be applied as treatment monitoring and disease prognosis. In 2 cases who presented the signs of disease recurrent even noted as sterile amputation, their pattern of all parameters showed different from the ones of survival group. This indicated that

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biomarker is useful method. And if the biomarker pattern showed the different pattern from survival, the patients should be considered for more consideration of treatment.

In case of ocular pythiosis group, even we cannot found the correlation between tested parameters and their clinical outcome. These are the good model to prove the efficacy of PIA immunotherapy to induce the immune response. By this study it clearly showed that PIA immunotherapy is capable to induce both cell mediated and humoral immune response. Moreover, it also indicated that PIA immunotherapy can increase the level of serum (1,3)-β-D-glucan. Even the highest peak of (1,3)-β-D-glucan showed at 1 month after PIA immunotherapy started, the decreased trend was presented in the following month even they were continuously treated by PIA. The phenomenon of (1,3)-β-D-glucan decreasing trend can be explained by the theory of "(1,3)-β-D-glucan clearance". In a murine model, the 1 mg/ml injected (1,3)-β-D-glucan is rapidly cleared by less than a few weeks when compared to infection-related (1,3)-β-D-glucan. The main reason for this situation is the slow release of hydrolytically degraded BG from conidia and hyphae that have been phagocytosed (102). In this study, we collected blood samples prior to each PIA injection time-points, so the shortest duration of blood collection after PIA injection was 2 weeks, which most of the injected (1,3)-β-D-glucan will have been degraded. This finding in pythiosis is also related to other previous publications. The studies in rabbit models reported that the serum (1,3)-β-D-glucan level was correlated with the severity of invasive pulmonary aspergillosis (57). Likewise, the studies in human with homogenous *Candida* meningoencephalitis, (1,3)-β-D-glucan levels in cerebrospinal fluid also showed the correlation with therapeutic response (59).

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The different response of cell mediated system between natural *P. insidiosun* infection and the use of PIA immunotherapy is another unclear question. PIA was also prepared from the *P. insidiosum* but Th1 response was activated rather than Th2 response as in natural infection. We suspected that chitin might be one of the causative immunogen related to cytokine production because chitin itself can induce Th2 response and can be found in *P. insidiosum*. Even though chitin is also presents in other fungi such as *C. albicans*, they can induce Th1 response after natural infection. This is due to the fact that chitin of *C. albicans* was masked by β-glucan. Moreover, some study also reported that chitin is stored in the form of chitosan, a large molecule of chitin that lack of immunogenic properties, as one of the virulence factors in *Cr. neoformans*. Similar to Mucorales group, chitosan is also in the cell wall instead of the chitin form (103, 104). So far, the real causative immunogen of Th2 induction is still under investigation.

Our study has a few limitations. We reported a relatively uncommon disease, small sample size of patients was recruited. However, so far this is the largest study that enrolled PIA-treated vascular and ocular pythiosis patients. Moreover, we cannot control the potential factors that could affect (1,3)-β-D-glucan, *Pi*-Ab and cytokines levels in some cases such as the (1,3)-β-D-glucan free protocol for blood sample collection etc because they were transferred back to their local hospitals in the rural area of Thailand after definitive surgery. Their PIA immunotherapy guided by our protocol was processed by those hospital systems and also their clinical outcomes were monitored and recorded by physicians or nurses who were not familiar with this disease.

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