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Defective cytokine production from monocytes/macrophages of E-beta thalassemia patients in response to *Pythium insidiosum* infection

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ABSTRACT

Background: *Pythium insidiosum* has been mainly reported to cause morbidity and mortality in thalassemia patients. *P. insidiosum* zoospores can germinate to be hyphae within a few hours; therefore, it is difficult to study the initial immune response that *P. insidiosum* zoospores induce. The present study aims to compare immune responses against *P. insidiosum* zoospore infection by comparing monocytes/macrophages from thalassemia patients with those from non-thalassemia controls.

Methods: In order to keep*P. insidiosum* in the zoospore stage in vitro for inoculation, the *P. insidiosum* zoospores were preserved without germination by treatment with inorganic hypochlorite solution. CD14+ cells were isolated from peripheral blood mononuclear cells of thalassemia and non-thalassemia donors and then left to transition to macrophages. Monocytes/macrophage culture was infected with *P. insidiosum* zoospores and culture supernatants were subjected to Th1/Th2 multiplex cytokine detection.

Results: Our study of cytokine production revealed that the basal level of GM-CSF produced by thalassemia monocytes/macrophages was lower than that observed in monocytes/macrophages of non-thalassemia individuals. Higher GM-CSF and IFN-γ response was also found when cells from non-thalassemia people were stimulated with *P. insidiosum* zoospores compared to thalassemia cells. It was also found that TNF-α, GM-CSF and IFN-γ productions from monocytes/macrophages of thalassemia patients who received iron chelator treatment were significantly higher than those produced from thalassemia patients without iron chelator treatment.

Conclusion: For the first time, the present study demonstrates defective immune responses in monocytes/macrophages derived from thalassemia patients in response to*P. insidiosum* zoospore infection. The results also show an inverse correlation between iron overload and cytokine production in monocytes/macrophages of thalassemia patients. This finding could explain why thalassemia patients are susceptible to *P. insidiosum* infection.

1. Introduction

Pythium insidiosum was previously known as an aquatic fungus. However, it has recently been categorized as a fungus-like organism in the class of Oomycetes in the Kingdom Straminipila (Beakes et al., 2012). The organism is able to produce motile zoospores, which are pathogenic morphotypes that are found in aquatic environments (Mendoza et al., 1993). Zoospores contain distinctive characteristics that include tropisms for skin, hair, and plant leaves (Miller, 1983). Although *P. insidiosum* is not the only species in the *Pythium* genus that can cause diseases in human, as *P. aphanidermatum* was found in invasive wound in human (Calvano et al., 2011; Farmer et al., 2015), the first case of human pythiosis was reported in 1985 (de Cock et al., 1987; Thianprasit, 1986). It was reported that clinical presentations in human infections can occur in three forms, which are cutaneous/subcutaneous, vascular, and ocular pythiosis (Krajaejun et al., 2006; Wanachiwanawin et al., 2004). Importantly, vascular pythiosis is the most serious clinical entity with inevitable mortality once a main artery is infected. Without proper management sequential systemic pythiosis can occur resulting in death.

Interestingly, human pythiosis is usually reported in individuals with underlying hematological diseases (de Cock et al., 1987; Drouin,

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1896; Mendoza et al., 1993). The majority of these cases involve individuals with either thalassemia or paroxysmal nocturnal hemoglobinuria (Wanachiwanawin et al., 2004). In the case of thalassemia, it has been proposed that iron overload could be a predisposing factor for systemic vascular pythiosis (Wanachiwanawin et al., 1993). The role of iron level and its effect on host immune response has been studied. High serum iron was shown to interfere with the production of TNF-α, IFN-γ, as well as the ability of macrophages to destroy intracellular pathogens (de Sousa, 1989; Gordeuk et al., 1992). It was found that high iron level may be the cause of immune response defect. In the model of pythiosis, there were reports showing that iron chelator could cause damage to *P. insidiosum* hyphae (Zanette et al., 2015a). Oral iron chelator could also help anemic condition associated with iron deficiency found in *P. insidiosum*-infected animals (Zanette et al., 2013a). It also reduces the phagocytic activity of monocytes/macrophages and neutrophils (de Sousa, 1989).However, the role of iron chelator administration on host immune response against *Pythium* infection has not been evaluated. It has been hypothesized that ferritin-associated iron might be the cause of immune defects (Cunningham-Rundles et al., 2000; Vento et al., 2006), although the exact mechanism is still unclear.

It was shown in vitro that exoantigens of *P. insidiosum* could induce immune response thwart Th2 dominance (Mendoza and Newton, 2005). While, Wanachiwanawin et al. showed that *P. insidiosum* immunogens induced immune modulation via a shift from the T helper 2 (Th2) response (dominated during clinical vascular/systemic pythiosis) to T helper 1 (Th1) after successful vaccination (Wanachiwanawin et al., 2004). This phenomenon was demonstrated by changes in key cytokines corresponding with the shift in Th responses, and also a reduction in anti- *P. insidiosum* IgE levels, following the introduction of *P. insidiosum* immunogens. These findings suggest that disrupted immune responses to *P. insidiosum* infection play an important role in the pathogenesis of vascular and systemic pythiosis in patients with thalassemia. Recently, Ledur et al. investigated in vitro T cell responses by co-culturing *P. insidiosum* zoospore-pulse dendritic cells and T cells derived from normal subjects and found that dead *P. insidiosum* zoospores could drive dendritic cells to induce naive T cells to produce Th1 phenotype cytokines (Ledur et al., 2018). Most of these studies explored the role of adaptive immune responses to *P. insidiosum* infection, however, the innate immune response against *Pythium* infection, i.e., *P. insidiosum* zoospore invasion, which is important in guiding the host defense against pythiosis, has not yet been explored. The present study aims to compare the initial cytokine response against *P. insidiosum* zoospores in monocytes/macrophages derived from patients with E-beta thalassemia with those in non-thalassemia controls.

2. Materials and methods

2.1. Human volunteers

In the present study, the volunteers were Thai people aged 18 to 60 who did not express general symptoms of microbial infection, such as cough, fever or headache, at the time of blood collection. The volunteers were recruited from two groups, fifteen healthy non-thalassemia people, and fifteen E-beta thalassemia patients without a history of pythiosis. The E-beta thalassemia patients, apart from a healthy general appearance without abnormal vital signs, had hemoglobin levels that were more than 6.0 g/dL. Patients, who were taking iron chelating agents and/or had a splenectomy, were not excluded. In cases where patients needed blood transfusion, whole blood was drawn the day before they were to receive transfusion. The volunteers were informed and gave documented consent before blood collection. Heparinized blood was prepared for peripheral blood mononuclear cell (PBMC) isolation. This study was approved by the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand (COA NO: Si242/2010).

2.2. Pythium insidiosum

Three clinical strains of *P. insidiosum* (SIMI1839-46, SIMI2921-45 and SIMI4763 isolated from human vascular, ocular and cutaneous tissues, respectively) were selected and used in all experiments of this study. These three strains were identified as *P. insidiosum* by zoospore induction and specific gene PCR as previous described (Chaiprasert et al., 1990; Thongsri et al., 2013). The pathogens were maintained and cultivated according to a previously described protocol for *Pythium* cultivation (Badenoch et al., 2001; Mendoza et al., 1993). Briefly, the pathogens were cultured *in vitro* and maintained on fungal media (Sabouraud agar, malt extract agar and potato agar). After approximately two weeks, *P. insidiosum* mycelia were transferred to sterile Malaysian grass in water for 48 h. Then, mycelia were induced to generate sporangium containing zoospores. Hypha and Malaysian grass in induction solution were filtrated through thick pack of sterile gauze and motile zoospores were finally obtained. As our protocol of infection required a high number of zoospores for co-cultivation and less heterogeneity of zoospore preparation was needed, hence, only clinical strain (SIMI4763) that gave the most numbers of zoospores per batch were selected for this study.

A number studies have successfully used sodium hypochlorite at concentrations from 1 ppm to 20 ppm (1 ppm = 1 ml solute/ 1 L solvent = 0.1% v/v) to kill zoospores of other *Pythium* spp.(Baker and Matkin, 1978; Cayanan et al., 2009; Hong and Richardson, 2004). We, therefore, employed various concentrations of inorganic hypochlorite solution to treat fresh zoospores in order to produce stage-specific morphotype of *P. insidiosum* and kept them in normal saline solution at 4 °C until use.

2.3. Monocyte/macrophage preparation

For monocyte isolation, fresh heparinized blood was diluted with PBS then gently layered on the surface of a tube containing Ficoll/Hypaque® solution (GE Healthcare, Germany). The tube containing the blood and Ficoll/Hypaque® solution was centrifuged at 400 × *g* for 30 min at room temperature. After centrifugation, mononuclear cells were collected from a band at the plasma/Ficoll interface. After washing, peripheral blood mononuclear cells (PBMCs) in the pellet were enumerated and assessed for cell viability (> 95%) with 0.4% trypan blue solution. PBMCs were subjected to CD14+ magnetic bead purification following the manufacturer's instructions for CD14+ monocyte isolation (Miltenyi Biotec Asia Pacific Ltd, Singapore, Singapore).

2.4. Cytokine detection assays

In order to study cytokine responses from human monocytes/macrophages, the CD14 positive cells were suspended in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum. They were seeded to 96 well plates and incubated at 37 °C with 5% CO_2 (v/v) overnight. Subsequently, *P. insidiosum* zoospores at multiplicity of infection (MOI) of 1:5 were added to the culture. Co-cultures of 2×10^5 monocytes/macrophages and 1×10^6 *P. insidiosum* zoospores were incubated at 37°C with 5% CO_2 (v/v) for 8 h. The supernatants from the co-cultures were collected and then kept at −20°C until use. The culture supernatants were subjected to quantitate Th1/Th2 cytokine levels by Bio-Plex® Human Cytokine 9-plex kit (Bio-Rad Laboratories, USA), which it can detect IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, GM-CSF, IFN-γ and TNF-α. Each measurement of the supernatant was taken in duplicate and performed according to manufacturer's protocol. Raw data (mean fluorescent intensity) were analyzed using Bio-Plex Manager Software (Bio-Rad Laboratories) to obtain concentration values.

Table 1
Characteristic of volunteers. Demographic data of non-thalassemia and E-beta thalassemia cohorts that participated in the present study. The data included proportion of gender, age, habitat, iron chelation and anemia status (hemoglobin concentration).

Data	Thalassemia patients	Non-Thalassemia
Number	15	15
Male: Female	6:9	8:7
Male: Female (age; median)	22:32	38:34
Habitat (Bangkok : Sub-urban)	6:9	9:6
Iron chelation : Non chelation	11:4	0:15
Hemoglobin concentration (g/dL; median)	7.9	> 12

2.5. Statistical analysis

Statistical significance of results was determined using either the non-parametric, Mann-Whitney-Wilcoxon test or Wilcoxon matched-pairs signed-rank test, where appropriate. Results were determined as statistically significant when P values < 0.05 were obtained.

3. Results

3.1. Demographic data of volunteers

This study recruited two cohorts consisting of non-thalassemia people (n = 15) and E-beta thalassemia patients (n = 15) as shown in Table 1. Both cohorts contained comparable proportions of males and females. Even though the median age of male thalassemia patients was lower than that in the other group, it was not a statistically significant difference. Three-fifths of thalassemia patients live in sub-urban areas, whereas three fifths of non-thalassemia people live in urban areas. Among the 15 thalassemia patients, 11 of them received iron chelation therapy either as a single agent or in combination according to the severity of iron overload, efficacy and also tolerability to the treatment. Seven patients received monotherapy, which five patients received oral deferiprone (30–100 mg/kg/day) and two patients received oral deferasirox (22–24 mg/kg/day). Two patients had combination of deferiprone (75 and 80 mg/kg/day) and deferasirox (38 and 13 mg/kg/day). Two patients were given combined therapy of deferiprone (88–96 mg/kg/day) and continuously subcutaneous infusion of deferoxamine (30–50 mg/kg/day, 3–6 days/week). Four thalassemia patients had not received any iron-chelating agent at least three years in the past and they were defined as iron chelation-untreated patients. The hemoglobin level in all thalassemia patients was higher than 7 g/dL (i.e., over inclusion criteria).

3.2. Preservation stage-specific morphotype of P. insidiosum by inorganic hypochlorite solution

The zoospore is infective stage and difficult to study because they rapidly germinate and to be hyphae. We found that treating *P. insidiosum* zoospores with 20 ppm concentrations of hypochlorite solution as other previous studies failed to prevent zoospores from forming colonies on SDA. Increasing the concentration of the inorganic hypochlorite solution to 110 ppm (11% v/v) at 4 °C for 1 h was able to kill 100% of *P. insidiosum* zoospores, as verified the lack of colony formation observed on SDA (data not shown). We also observed hypochlorite-treated zoospores under the light microscope and found that their gross morphology was unchanged as compared to fresh living zoospores (supplement Fig. 1).

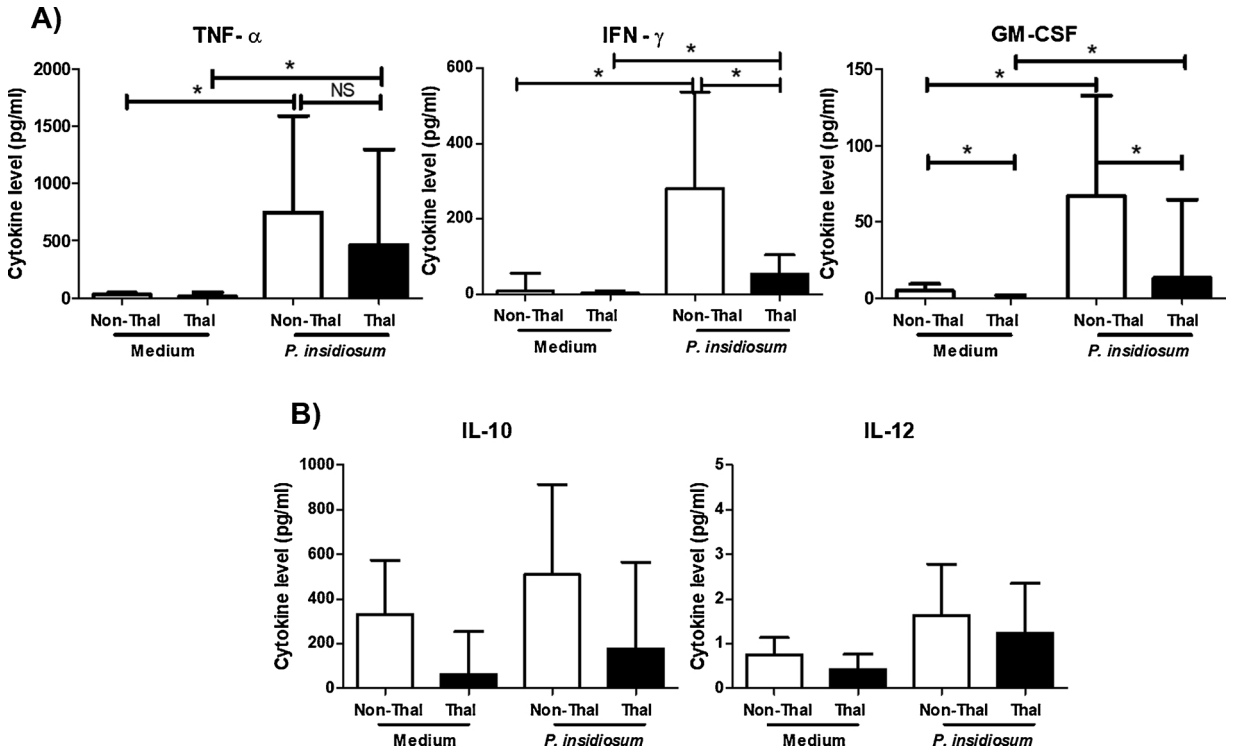

Fig. 1. A comparison of cytokine production in monocytes/macrophages of healthy individuals and thalassemia patients following *P. insidiosum* zoospore exposure. The cytokine production levels in monocytes/macrophages of healthy individuals (white bar) and E-beta thalassemia patients (black bar) following exposure to *P. insidiosum* zoospores. The level of cytokine production from monocytes/macrophages cultured in medium that did not contain zoospores was defined as the basal cytokine level. The levels of a) inflammatory cytokines (GM-CSF, TNF-α, and IFN-γ) and b) IL-10 and IL-12 production are expressed as the median ± interquartile range. * indicates statistical significance (P value < 0.05). NS stands for not significant. There is no statistical significance result in Fig. 1b.

3.3. Impaired inflammatory cytokine production against zoospores of P. insidiosum infection in PBMCs of thalassemia patients

To compare the pattern of cytokine production in human monocytes/macrophages from normal individuals and thalassemia patients in response to *P. insidiosum* zoospore exposure. Our preliminary results of cytokine production in the human monocyte cell line (THP-1) infected with *P. insidiosum* zoospores indicated that the peak period of cytokine production was at 8 h post-infection (unpublished data). Therefore, we collected supernatants to perform cytokine detection assays at 8 h post-infection. In our study, IL-2, IL-4, IL-5, and IL-13 were not detectable in cultivation of human monocytes/macrophages infected with *P. insidiosum* zoospores. However, several cytokines were produced at lower levels in monocytes/macrophages from thalassemia patients. In the absence of zoospore exposure, the basal level of GM-CSF secreted by monocytes/macrophages from patients with thalassemia was lower than that in cells from normal controls as shown in Fig. 1a.

Zoospore stimulated cytokine induction was also impaired in monocytes/macrophages from thalassemia patients. Following *P. insidiosum* zoospore exposure, significantly lower levels of GM-CSF and IFN-γ ($P < 0.05$) were secreted by monocytes/macrophages from thalassemia patients as compared to those in non-thalassemia controls (Fig. 2a). IL-10 and IL-12 were detectable and their production levels in thalassemia monocytes/macrophages were comparable to those in non-thalassemia ones ($P > 0.05$) as shown in Fig. 1b.

3.4. Iron burden in thalassemia patients

The levels of ferritin obtained on the day of blood collection were investigated to reflect iron loading in both thalassemia patients and non-thalassemia donors. The level of ferritin in sera of thalassemia patients, with and without iron chelator treatment, was approximately 10-fold higher than that in normal individuals (Fig. 3). Serum ferritin

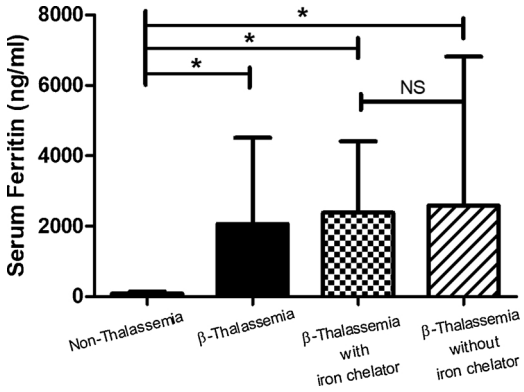

Fig. 3. Ferritin levels.
Separated clotted bloods of non-thalassemia and E-beta thalassemia donors were taken at the day of whole blood collection for PBMC isolation. The clotted blood samples were submitted to determine ferritin levels at the routine service laboratory of Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital. Serum ferritin levels in non-thalassemia volunteers (white bar) and E-beta thalassemia patients (black bar). The serum ferritin levels of E-beta thalassemia patients corresponded to iron chelation were also plotted as white bar with black spot for patients received an iron chelation and white bar with black oblique line for patients who did not receive an iron chelator, respectively. The serum ferritin levels were expressed as the median ± interquartile range. * indicates statistical significance (*P* value < 0.05). NS stands for not significant.

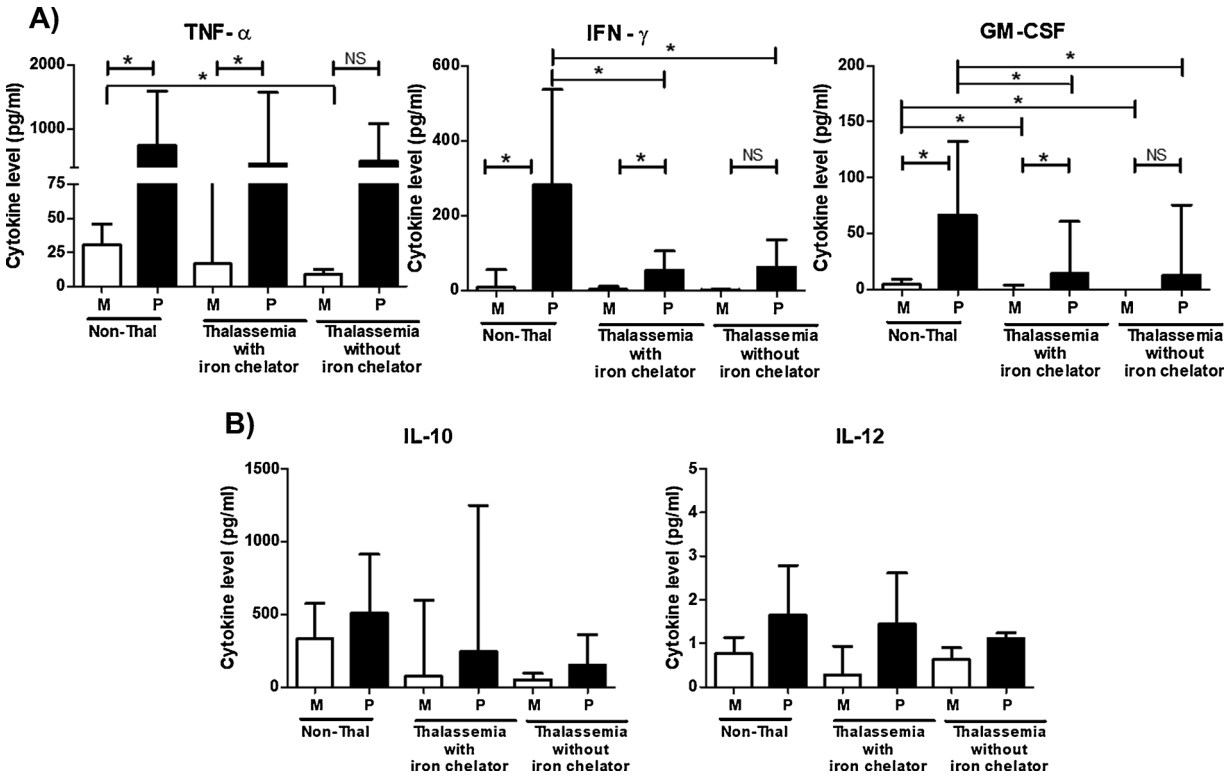

Fig. 2. Cytokine production from monocytes/macrophages of healthy individuals and thalassemia patients with and without iron chelation under *P. insidiosum* zoospore infection.
Cytokine production from monocytes/macrophages of healthy individuals and E-beta thalassemia patients (with or without iron chelation) that were grown in the absence (medium culture alone (M); white bar) or presence of *P. insidiosum* zoospores (P; black bar) is shown. The levels of cytokine production from monocytes/macrophages grown in medium with no additions were defined as basal cytokine levels. The levels of a) inflammatory cytokines (GM-CSF, TNF-α, and IFN-γ) and b) IL-10 and IL-12 production are expressed as the median ± interquartile range. * indicates statistical significance (*P* value < 0.05). NS stands for not significant. There is no statistical significance result in Fig. 2b.

levels of thalassemia patients who received iron chelator treatment were not different from those of iron chelator-treated patients (Fig. 3). Though there was no significant difference in serum ferritin level, *P. insidiosum* zoospores stimulated significantly lower productions of GM-CSF, TNF-α and IFN-γ in monocytes/macrophages from patients who did not receive iron-chelating agent compared to thalassemia patients with iron chelator administration (Fig. 2a). Basal level of TNF-α production was also lower in patients without iron-chelator treatment condition (Fig. 2a). However, iron chelator treatment had no effect on IL-10 and IL-12 productions from thalassemia monocytes/macrophages (Fig. 2b).

4. Discussion

The gender ratios in both study cohorts were comparable, so gender effects are not a concern in this study. Although the male thalassemia patients were younger than the other groups, statistical analysis revealed no significant differences (data not shown). Given that the study participants, both thalassemia and non-thalassemia subjects, lived in urban and sub-urban areas, both cohorts likely had a low chance of being exposed to *P. insidiosum* as compared to rural area. Unfortunately, the data in this study concerning non-iron chelation was represented by only four subjects. Both thalassemia and non-thalassemia cohorts were healthy without severe anemia status.

This is the first time that we were able to establish a protocol to preserve the zoospore stage of *P. insidiosum* using inorganic hypochlorite solution in refrigerated conditions. The hypochlorite solution at 110 ppm (11% v/v) is not toxic to mammalian cells as the viability of human monocytes/macrophages in hypochlorite solution was examined. There was no statistical difference between the viability of treated monocytes/macrophages and untreated ones (data not shown).

Many reports have shown that there are abnormalities in monocytes/macrophages, as well as lymphocytes in hematologic patients (Cunningham-Rundles et al., 2000; de Sousa, 1989; Gordeuk et al., 1992). Our findings revealed that the levels of proinflammatory cytokines (TNF-α, IFN-γ and GM-CSF) released from thalassemia monocytes/macrophages stimulated with or without *P. insidiosum* zoospores were significantly lower than those of non-thalassemia monocytes/macrophages. It is consistent with the finding of Gordeuk et al. (Gordeuk et al., 1992). Interestingly, the significantly lower levels of IFN-γ production in thalassemia monocytes/macrophages in response to *P. insidiosum* zoospore exposure might be a weak point for thalassemia patients due to a reduced inability to provoke Th1 responses. By contrast, the levels of IL-10 and IL-12 produced by monocytes/macrophages were similar in affected and healthy populations in the presence and absence of zoospores. In our study, the impairment of Th1 responses (TNF-α and IFN-γ production) against *P. insidiosum* zoospore exposure was consistent with results reported by Mencacci et al. (Mencacci et al., 1997). In order to revert immune responses to Th1 against *P. insidiosum* infection, previous studies of therapeutic approaches demonstrated that immunogens and zoospores of *P. insidiosum* could induce Th1 cytokine production in thalassemia patients and in vitro dendritic cells co-cultured with T cells, respectively (Ledur et al., 2018; Wanachiwanawin et al., 2004). Our findings, along with previous reports, support the hypothesis that thalassemia patients are susceptible to *P. insidiosum* infection due to the Th1 impairment. In addition, lower levels of GM-CSF at the site of infection may reduce the warning signal leading to the systemic release of neutrophils to conquer zoospore infection. Immunological defect in thalassemia patients in this study could explain why thalassemia patients may have high risk to be infected with *P. insidiosum*.

Our investigation also revealed excessively high levels of ferritin in serum of thalassemia patients. These high ferritin levels reflect to iron overload and could impair human immune function, such as cytokine production and amount of lymphocyte subpopulation (de Sousa, 1989; Gordeuk et al., 1992; Schaible and Kaufmann, 2004). Our results revealed that lower levels of GM-CSF and IFN-γ production released from monocytes/macrophages of all thalassemia patients were investigated (with and without treatment of iron chelating agent) as compared to those from cells of non-thalassemia volunteer. This is consistent with previous reports indicating that high iron levels interfere with cytokine production in monocytes/macrophages in patients with pythiosis (de Sousa, 1989; Gordeuk et al., 1992). This may explain the increased susceptibility of thalassemia patients to *P. insidiosum* infection that is often observed in Thailand.

Even patients in this study received deferiprone as major iron chelator (some with deferasirox), their monocytes/macrophages under zoospore infection were able to produce higher levels of GM-CSF, TNF-α and IFN-γ as compared to the levels produced from monocytes/macrophages from patients who did not received an iron-chelating agent. In addition, even the levels of ferritin were not different between both groups of thalassemia subjects, it could be possible that the iron chelators might reduce previously higher levels of iron and improve immune function at the time of blood collection. Hence, in this study, monocytes/macrophages from iron chelation-untreated patients might be impaired to produce these cytokines as compared to cytokine levels derived from these cells of treated patients. Nevertheless, treatment of iron chelation in thalassemia patients could not be expected to reduce levels of ferritin-associated iron equivalent to that of non-thalassemia people. Our finding could imply that iron-chelating agents might provide indirect effect to improve immune responses. It was consistent with other studies revealed immunomodulatory effect and antifungal effect of the iron-chelator (deferasirox) (Zanette et al., 2013a,b; Zanette et al., 2015a, b). Although the exact mechanism by which iron overload affects immune responses is still unclear, it has been hypothesized that ferritin-associated iron might be the cause of immune defects (de Sousa, 1989; Gordeuk et al., 1992; Grady et al., 1987). Further experimental evidence supporting this role for ferritin-associated iron is required.

In summary, this study is a pioneering step to compare the innate immune responses of normal hosts with those of thalassemia patients against *P. insidiosum* infection. The lower levels of IFN-γ and GM-CSF released from thalassemia monocytes/macrophages infected with *P. insidiosum* zoospores were investigated. Interestingly, the basal levels of GM-CSF production from thalassemia monocytes/macrophages were also lower than those of non-thalassemia monocytes/macrophages. The lower levels of TNF-α, IFN-γ and GM-CSF were inversely associated with serum ferritin especially in thalassemia patients, which could infer that iron overload might cause a defect in monocyte/macrophage proinflammatory responses in thalassemia patients. Iron chelator could have immunomodulatory effect to improve cytokine production as well as immune function as previous study (Ledur et al., 2018).

Author contributions

Y.S. conceived and designed the experiments; Y.S., S.U. and T.T. performed the experiments; S.U., D.K. and Y.S. analyzed the data; W.W. recruited patients; A.C. provided clinical strains of *P. insidiosum*. Y.S., D.K., and W.W. wrote the paper.

Conflicts of interest

The authors declare no conflict of interest. The funding sponsors had no role in either the design of the study, the collection, analysis and interpretation of the data, the writing of the manuscript or the decision to publish the results.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.02.002.

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