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Functional characterization of macrophages and change of Th1/Th2 balance in patients with pythiosis after *Pythium insidiosum* antigen immunotherapy

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There has been limited research into the role of the *Pythium insidiosum* antigen (PIA) in modulating immune response in patients with pythiosis. This study investigated the balance of T helper type 2 (Th2) and T helper type 1 (Th1) responses after receiving PIA immunotherapy in patients with pythiosis. Next, the phagocytic activity and phagocytic index of IFN-γ primed PIA-treated macrophages were examined. Furthermore, the phagocytosis of infective *P. insidiosum* zoospores by macrophages was investigated. This work showed that the PIA vaccine induced Th1 response and M1 macrophages in patients with vascular pythiosis who survived and those with localized pythiosis. Phagocytic activity and phagocytic index were increased considerably in localized pythiosis patients compared to vascular pythiosis patients with hematological diseases. IFN-γ priming of PIA-treated macrophages against *P. insidiosum* zoospores enhanced the phagocytic activity and phagocytic index in vascular and localized pythiosis patients. Macrophages engulfed *P. insidiosum* zoospores, but the zoospores continued germination, resulting in macrophage death. Overall, our results suggest that PIA can modulate the immune responses, contributing to higher levels of Th1-type cytokine and potentially improving the survival of patients with vascular pythiosis. This study is the first to uncover that *P. insidiosum* zoospores can survive within macrophages.

Keywords Oomycete, Zoospore, Interferon-γ, Ocular, Vascular

Human pythiosis is a life-threatening infectious disease caused by the invasion of a pathogenic oomycete, *Pythium insidiosum*[1]. Four clinical forms of human pythiosis have been observed: vascular pythiosis, ocular (localized) pythiosis, cutaneous/subcutaneous pythiosis, and disseminated pythiosis[2]. The risk factors for pythiosis include hemoglobinopathy (thalassemia) and occupations requiring work in wet or swampy areas[3]. Over the years, *P. insidiosum* antigen (PIA) immunotherapy has shown potential benefits among patients with vascular pythiosis with residual disease or unresectable lesions[4–6]. The generalisability of successful PIA immunotherapy in animal data to humans depends on diagnosing pythiosis after the initial onset of infection. Late diagnosis results in weakened immune systems that respond poorly to immunotherapy[7]. Despite the increased success rate of PIA in treating animals with pythiosis, the efficacy of PIA immunotherapy in treating human pythiosis is limited, and further study is necessary to explore patients' immune responses to PIA immunotherapy.

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Studies in humans and animals have postulated a switch from a T helper type 2 (Th2) to a T helper type 1 (Th1) immune response as an immunotherapeutic mechanism after PIA administration in pythiosis[8–10]. *P. insidiosum*, upon infection, locks the immune system into a .

Th2-type immune response profile and triggering an eosinophilic reaction, similar to other fungi[5,7,8,11]. A previous study provided evidence of impairment in IFN-γ production and the susceptibility to *P. insidiosum* infection in patients with thalassemia, indicating a shift to Th2 dominance and weak Th1 responses[12]. A limited number of studies have assessed the effect of T helper cells (Th cells) through cytokines profile following PIA immunotherapy in humans, particularly in patients with vascular pythiosis, due to the complicated clinical status of patients. Although data from PIA immunotherapy in animals provide inferences for human responses, complexities such as underlying hematological diseases (thalassemia or paroxysmal nocturnal hemoglobinuria) have not been considered in animals, which are reported to be associated with human pythiosis[5,13,14]. The relevancy of the results of cytokine studies in animal pythiosis to humans remains insubstantial at this time[9].

Little is known about the functional ability of macrophages and M1 and M2 macrophage polarization in patients with pythiosis after receiving the PIA immunotherapy. Here, we investigated the longitudinal characterization of Th1-type cytokine (IFN-γ) and Th2-type cytokines (IL-4 and IL-5) in each month of the PIA immunotherapy schedule in patients with pythiosis for 12 months. We also examined M1 and M2 macrophages and evaluated the PIA-treated macrophage phagocytosis against *P. insidiosum* zoospores on day 0 (before 1st dose of the PIA vaccine) and day 90 after receiving the first dose of the PIA vaccine. To examine the effects of PIA-treated macrophages against *P. insidiosum* zoospores, we determined the levels of IFN-γ after 0 and 8 h on days 0 and 90 after the patients with pythiosis received the 1st PIA vaccine. We also investigated how IFN-γ priming affects the PIA-treated macrophage phagocytosis against *P. insidiosum* zoospores. Furthermore, we observed the phagocytosis behavior of phorbol 12-myristate 13-acetate (PMA)-induced THP-1 macrophages against live *P. insidiosum* zoospores using time-lapse microscopy imaging.

Results

Patient characteristics

The demographic and clinical characteristics of 28 patients with localized or vascular pythiosis patients are summarized in Table 1.

Analysis of Th1-type cytokine (IFN-γ) and Th2-type cytokines (IL-4 and IL-5)

Overall, the Th1-type cytokine (IFN-γ) levels were significantly higher than the baseline levels (month 0) in the survival group and local infection group in each month of the PIA immunotherapy schedule ($P < 0.001$). In contrast, IFN-γ levels did not increase in deceased patients after PIA immunotherapy. IFN-γ levels were significantly higher in the survival group and local infection group at 1.5 months after the initiation of PIA immunotherapy, after which the patients died in the deceased group ($P < 0.001$) (Fig. 1A).

Th2-type cytokine (IL-4) levels significantly decreased during the 1 year compared to the baseline (month 0) among the survival group ($P < 0.001$) and remained constantly lower among the local infection group. The IL-4 levels before the death of patients among the deceased group were significantly higher than the survival group and local infection group at 1.5 months after the 1st PIA vaccine ($P < 0.001$) (Fig. 1B).

The levels of Th2-type cytokine (IL-5) were significantly decreased compared to the baseline (month 0) among the survival group during the 1 year of PIA immunotherapy ($P < 0.001$). IL-5 levels were constantly lower among the local infection group. In addition, the IL-5 levels were significantly higher among the deceased group at 1.5 months after 1st dose of the PIA vaccine, after which the patients died, compared to the survival group and local infection group ($P < 0.001$) (Fig. 1C).

M1 and M2 macrophages after PIA Immunotherapy

Macrophages were gated for CD14+ cells, and in this CD14+ cell population, macrophages showing an M1 phenotype were characterized by the expression of CD86. CD14+ CD86+ cells significantly increased in survival ($P = 0.001$) and local infection ($P = 0.005$) groups on day 90 after receiving the PIA vaccine compared to the baseline on day 0. No significant difference in percentages of M1 macrophages was observed between day 0 and day 90 in the deceased group (Fig. 2A).

In the CD14+ cell population, macrophages showing an M2 phenotype were characterized by the expression of CD163. As shown in Fig. 2B, percentages of CD14+ CD163+ cells were significantly lower at day 90 after

Parameters	Group 1 (survival group; $n = 12$)	Group 2 (deceased group; $n = 4$)	Group 3 (local infection group; $n = 12$)
Age[a] [years]	41.9 ± 0.5	41.1 ± 0.3	42.5 ± 0.1
Gender [n (%)]			
Male	6 (50%)	2 (50%)	6 (50%)
Hematological underlying disease [n (%)]			
β-thalassemia	3 (25%)	0 (0%)	0 (0%)
β-thalassemia/ Hemoglobin E	9 (75%)	4 (100%)	0 (0%)
Serum ferritin[a] (ng/ml)	1,965 ± 404.2 ng/ml	2,054 ± 307.6 ng/ml	109 ± 38.7 ng/ml

Table 1. Demographic and clinical characteristics of patients ($N = 28$). [a]Mean ± SD.

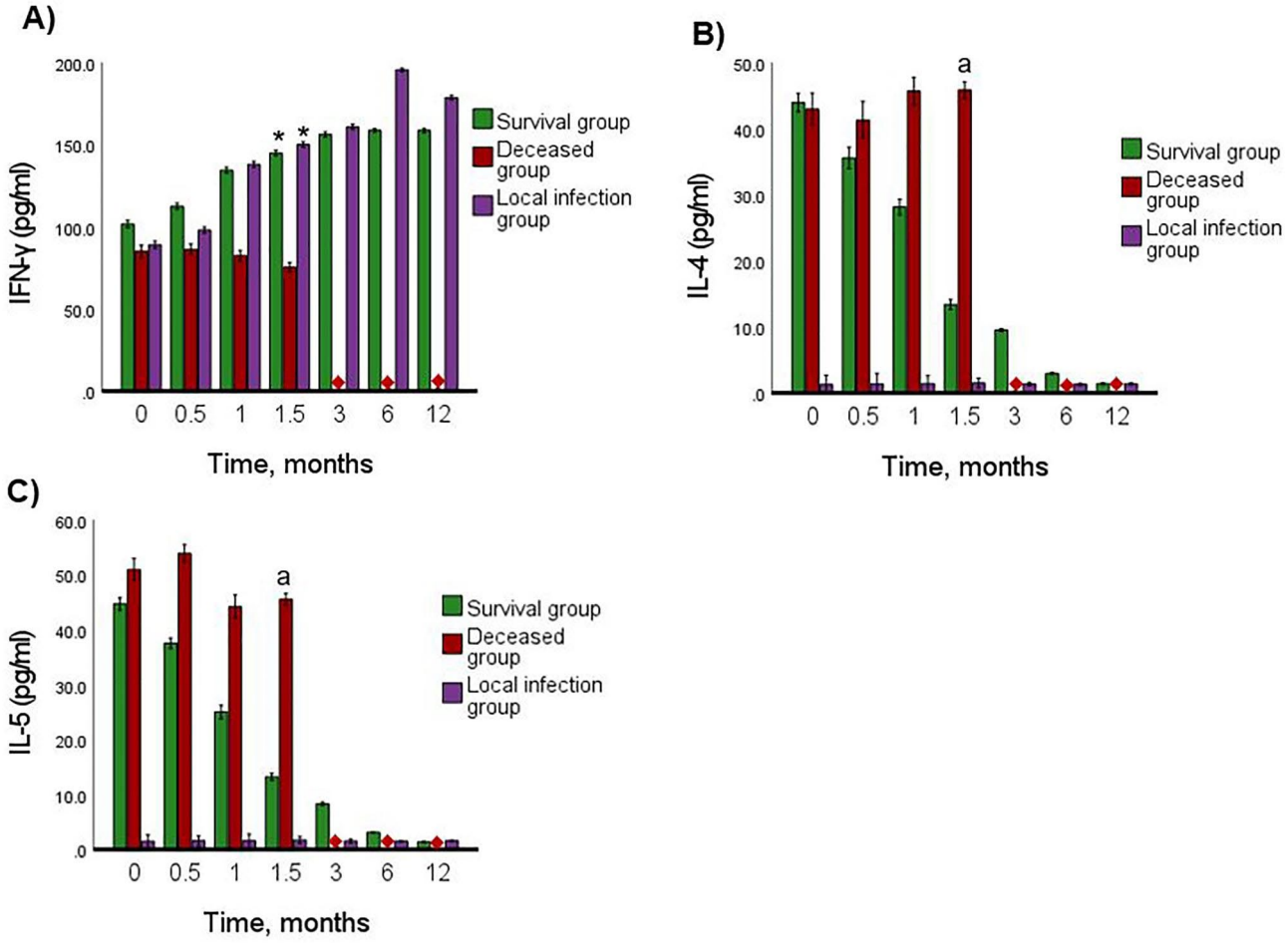

Fig. 1. Serum levels of Th1 and Th2 cytokines. Change in serum levels of Th1-related cytokine (**A**) and Th2-related cytokines (**B** and **C**) in pythiosis patients after receiving the PIA immunotherapy. Red diamonds represent the points where the patients in the deceased group died. $^{*}P < 0.001$ compared with the deceased group at 1.5 months after PIA immunotherapy; $^{a}P < 0.001$ against the survival and local infection groups at 1.5 months after PIA immunotherapy. Error bars, mean ± SEM; Statistics, repeated measures ANOVA.

receiving the 1st dose of the PIA vaccine compared to day 0 (before 1st dose of PIA vaccine) in survival ($P < 0.001$) and local infection ($P = 0.03$) groups. However, a significant increase in the percentage of M2 macrophages was observed in the deceased group at day 90 compared to day 0 after initiating PIA immunotherapy ($P < 0.001$).

Phagocytic activity and phagocytic index of PIA-treated human monocyte-derived macrophages against preserved *P. Insidiosum* zoospores

We obtained macrophages from patients on days 0 before 1st dose of the PIA vaccine and 90 after the patients were vaccinated with 1st dose of PIA. The phagocytic activity and phagocytic index of PIA-treated macrophages against *P. insidiosum* zoospores on days 0 and 90 are illustrated in Fig. 3. The phagocytic activity of PIA-treated macrophages obtained from localized pythiosis patients was significantly higher than the PIA-treated macrophages obtained from survived and deceased vascular pythiosis patients after 1, 2, 3, 4 and 8 h on days 0 ($P < 0.01$) and 90 ($P < 0.01$). Phagocytic activity of the PIA-treated macrophages did not differ significantly between survived and deceased vascular pythiosis patients after 1, 2, 3, 4, and 8 h on days 0 and 90 (Fig. 3A, B). We observed a significantly higher phagocytic activity of the PIA-treated macrophages on day 90 compared to day 0 in all three groups of patients (data not shown).

Next, we found that the phagocytic index of PIA-treated macrophages obtained from localized pythiosis patients was significantly higher compared to the phagocytic index of PIA-treated macrophages obtained from survived and deceased vascular pythiosis patients after 1, 2, 3, 4, and 8 h on days 0 ($P < 0.001$) and 90 ($P < 0.01$). No significant differences could be found between the results in survived and deceased vascular pythiosis patients after 1, 2, 3, 4, and 8 h on days 0 and 90 (Fig. 3C, D). We observed a significantly higher phagocytic index of the PIA-treated macrophages on day 90 compared to day 0 in all three groups of patients (data not shown).

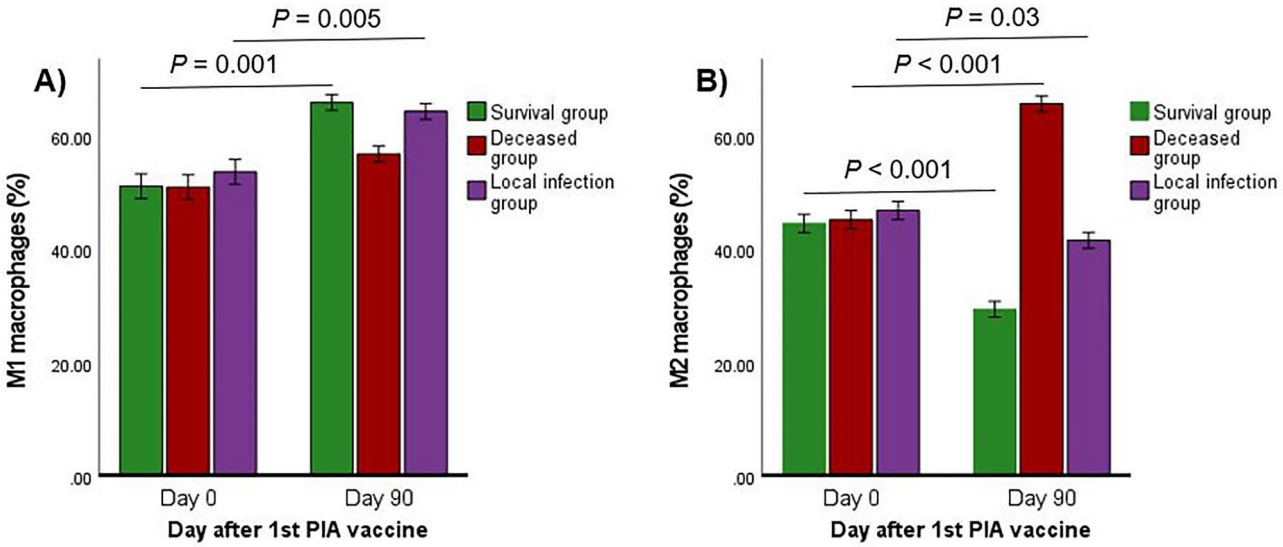

Fig. 2. M1 and M2 macrophage detection. M1 macrophages represent the percentage of CD14+ CD86+ cells over total macrophages (**A**), and M2 macrophages represent the percentage of CD14+ CD163+ cells over total macrophages (**B**) among the pythiosis patients at day 0 (before 1st dose of PIA vaccine) and day 90 after receiving the first dose of the PIA vaccine. Error bars, mean ± SEM; Statistics, repeated measures ANOVA.

Production of IFN-γ from PIA-treated human monocyte-derived macrophages against preserved *P. Insidiosum* zoospores

We measured the levels of IFN-γ to examine the effects of PIA-treated macrophages against *P. insidiosum* zoospores after 0 and 8 h on days 0 and 90 after the patients received the 1st PIA vaccine. We found that IFN-γ levels among the localized pythiosis patients were significantly higher after 8 h on days 0 and 90 compared with survived and deceased vascular pythiosis patients ($P < 0.001$) (Fig. 4A, B). However, there were no significant differences in the levels of IFN-γ between the survived and deceased vascular pythiosis patients after 8 h on days 0 and 90.

Phagocytic activity and phagocytic index of IFN-γ primed PIA-treated human monocyte-derived macrophages against preserved *P. Insidiosum* zoospores

The macrophages obtained from patients at day 0 (before the 1st PIA vaccine) and day 90 after receiving the 1st PIA vaccine were treated with PIA and primed with IFN-γ, which were co-cultured with zoospores. We assessed the phagocytic activity and phagocytic index of PIA-treated macrophages against zoospores after 1 h of IFN-γ priming. IFN-γ-treated macrophages showed a significant increase in phagocytic activity at days 0 and 90 after 1 h compared to IFN-γ-untreated macrophages in each group of patients ($P < 0.001$) (Fig. 5A, B). The phagocytic index was also significantly higher at days 0 and 90 after 1 h in IFN-γ-treated macrophages compared to IFN-γ-untreated macrophages in each group of patients ($P < 0.001$) (Fig. 5C, D). The phagocytic activity and phagocytic index of IFN-γ-treated macrophages did not differ significantly between day 0 and day 90 in survived vascular and localized pythiosis patients. However, IFN-γ-treated macrophages demonstrated a significantly higher phagocytic activity and phagocytic index on day 90 than on day 0 (data not shown).

Phagocytosis of live *P. insidiosum* zoospores by PMA-induced THP-1 macrophage

After co-culturing PMA-induced THP-1 macrophages with live *P. insidiosum* zoospores, we observed the phagocytosis behavior of macrophages using time-lapse microscopy imaging. We observed a live zoospore being eaten by a macrophage as they contact each other (Fig. 6). Following engulfment, the zoospore germinated after 2 h, resulting in lysis of the macrophage. In addition, Z-stack images obtained by confocal microscopy showed evidence of the zoospore being entirely ingested by the macrophage and not attached to the surface of the macrophage (Fig. 7).

Discussion

PIA immunotherapy, in combination with surgery and antimicrobial drugs, has proved effective in treating human pythiosis. The anti-*P. insidiosum* response induced by PIA by activating the immune response in pythiosis patients remains unclear. The results from this study suggested a shift from a Th2 towards a Th1 cytokine profile after a 1-year course of PIA immunotherapy in patients with pythiosis. When we phenotyped macrophages in the whole blood of patients with pythiosis receiving PIA, macrophages exhibited a polarization switch towards M1 in patients with vascular pythiosis who survived and patients with localized pythiosis. In contrast, macrophages showed a polarization switch towards M2 at day 90 after receiving the 1st PIA vaccine in deceased patients with vascular pythiosis. Furthermore, assessment of phagocytic activity and phagocytic index of PIA-treated macrophages against *P. insidiosum* zoospores showed that the phagocytic activity and phagocytic index of PIA-

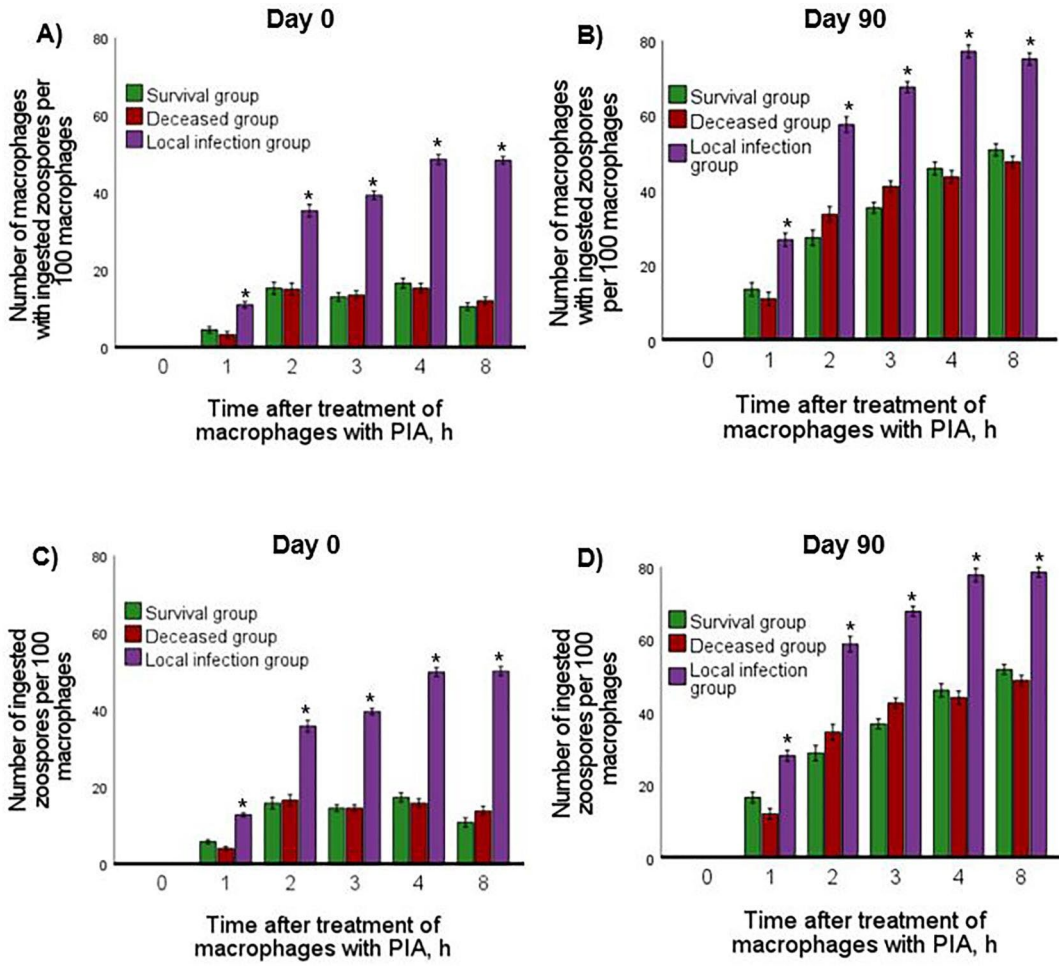

Fig. 3. Phagocytic activity and phagocytic index of PIA-treated macrophages against zoospores. The number of macrophages with ingested zoospores per 100 macrophages was counted on day 0 (**A**) and day 90 (**B**). The number of ingested zoospores was counted in 100 macrophages on days 0 (**C**) and 90 (**D**). $^{*}P < 0.05$ compared with the survival and deceased group within each time point. Error bars, mean ± SEM; Statistics, repeated measures ANOVA.

treated macrophages obtained from patients with localized pythiosis were significantly higher when compared with survived and deceased patients with vascular pythiosis. Elevated IFN-γ levels were detected in patients with localized pythiosis when the PIA-treated macrophages were co-cultured with *P. insidiosum* zoospores, and IFN-γ priming of PIA-treated macrophages exhibited increased phagocytic activity and phagocytic index compared to non-primed PIA-treated macrophages in survived and deceased patients with vascular pythiosis and patients with localized pythiosis. Moreover, confocal microscopy analysis showed that the *P. insidiosum* zoospore was engulfed by PMA-induced THP-1 macrophage. Still, the zoospore was not killed; instead, it germinated, resulting in the death of the macrophage.

The Th1 cells secrete IFN-γ, which is essential for phagocytic activity. Meanwhile, Th2 cells secrete IL-4 and IL-5, crucial for the induction and development of antibody production[15]. Based on our results, Th1 cells were generated in patients after receiving the PIA immunotherapy with increased levels of IFN-γ secretion. A trend of markedly lower levels of IFN-γ was observed among the deceased patients with vascular pythiosis compared to those with vascular pythiosis who survived. Persistently decreased IFN-γ levels among the deceased patients indicate that IFN-γ is essential for anti-*P. insidiosum* defense, however, the association between IFN-γ and death among patients with vascular pythiosis must be explored in future studies. In parallel, the time-course production of Th2 cytokines IL-4 and IL-5 after receiving the PIA vaccine was lower among vascular patients who survived than the deceased patients. This finding suggests that IFN-γ, IL-4, and IL-5 levels possess an added value in monitoring PIA therapy response in patients with vascular pythiosis. It can now be asserted that PIA immunotherapy promotes switching the Th2 response to the Th1 response in vascular pythiosis patients, which was also observed in animal data[9].

IFN-γ can activate macrophages with improved phagocytosis and microbe destruction[16,17]. We hypothesized that macrophages are probably the principal immune cells eliminating *P. insidiosum* through phagocytosis. Macrophages are polarized into M1 (classically activated macrophages) and M2 (alternatively activated macrophages) subtypes, which must be optimally regulated to perform the immune tasks by macrophages

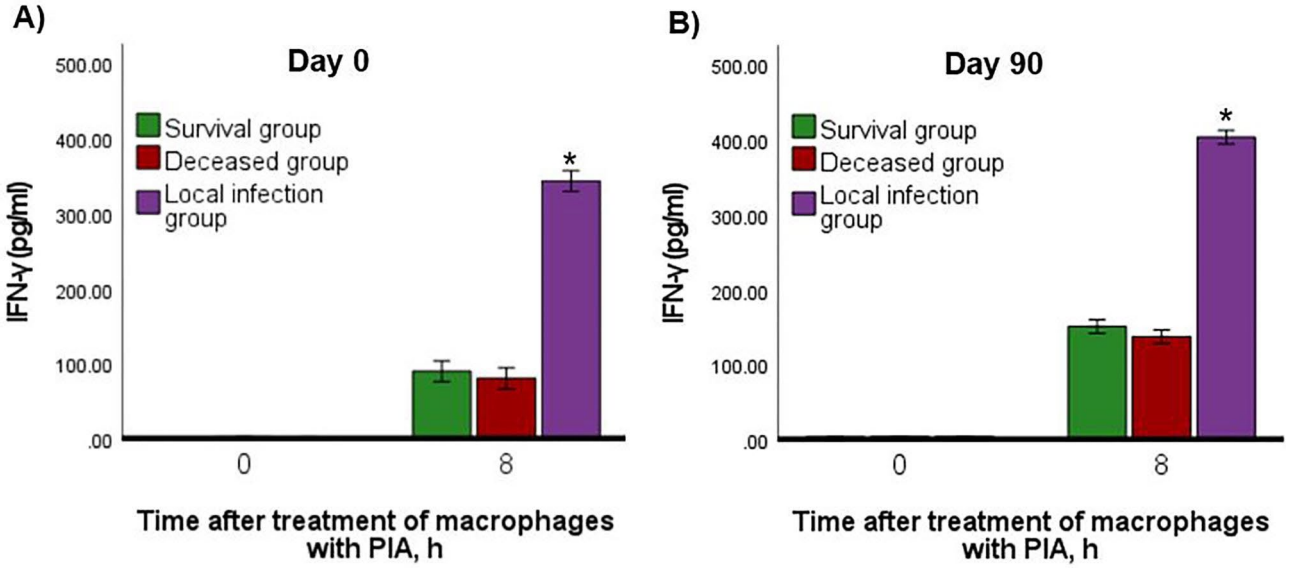

Fig. 4. Measurement of IFN-γ. Production of IFN-γ by PIA-treated human monocyte-derived macrophages against zoospores on days 0 (**A**) and 90 (**B**) after 1st PIA vaccine administration to the patients. $^{*}P < 0.001$ compared with the survival and deceased group at 8 h. Error bars, mean ± SEM; Statistics, repeated measures ANOVA.

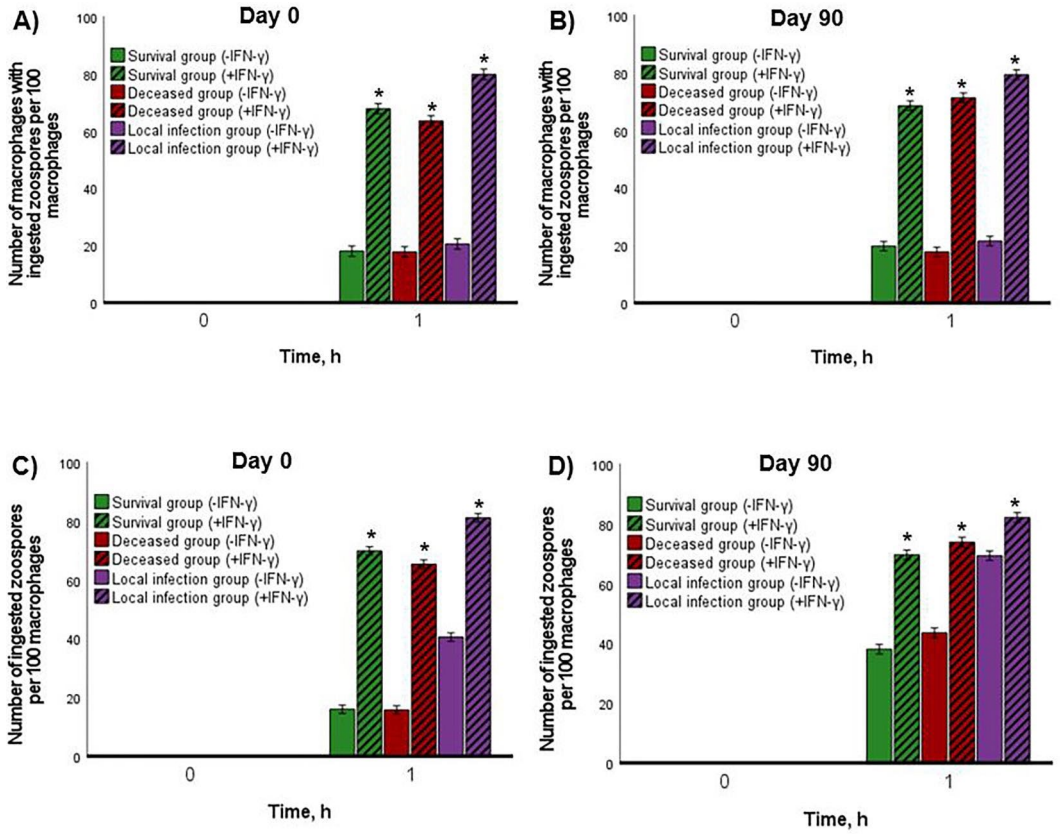

Fig. 5. Phagocytic activity and phagocytic index of IFN-γ primed PIA-treated macrophages against zoospores. The number of macrophages with ingested zoospores per 100 macrophages was counted on day 0 (**A**) and day 90 (**B**). The number of ingested zoospores was counted in 100 macrophages on days 0 (**C**) and 90 (**D**). Non-primed macrophages were used as controls. $^{*}P < 0.001$ compared to non-primed macrophages within each group at 1 h. Error bars, mean ± SEM; Statistics, repeated measures ANOVA.

Fig. 6. Confocal microscopy time-lapse imaging of phagocytosis of live zoospore (green fluorescent) by PMA-induced THP-1 macrophage. A zoospore being phagocytosed by a macrophage is indicated by a white arrow. Germinating zoospore is indicated by a red arrow.

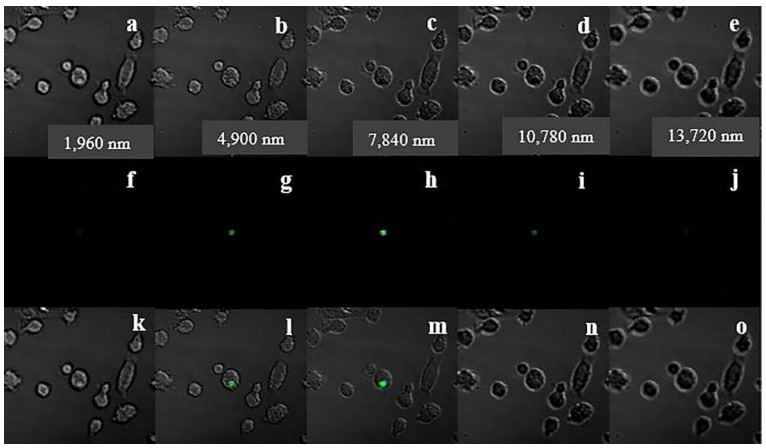

Fig. 7. Z-stack image series to analyze phagocytosis of zoospore by THP-1 macrophage. Z-stack by confocal microscope (Nikon) at 1,960 nm, 4,900 nm, 7,840 nm, 10,780 nm, and 13,720 nm in depth, respectively, representing THP-1 macrophages (**a**-**e**), FITC-zoospore (**f**-**j**), and merged figure (**k**-**o**) at 30 min incubation of PMA-induced THP-1 macrophages and live zoospore.

during infection. IFN-γ induces M1 macrophages, while IL-4 skews macrophages toward M2 macrophages[18]. M1 macrophages induce Th1 responses, whereas M2 macrophages drive Th2 responses[19]. Enhanced M1 polarization leads to increased phagocytosis of fungal pathogens[20]. In the present study, we found that the PIA vaccine stimulated the development of M1 macrophages and inhibited the production of M2 macrophages in patients with vascular pythiosis who survived and patients with localized pythiosis. This profile contrasted in the deceased patients with vascular pythiosis with no significant changes in M1 macrophages and a significant increase in M2 macrophages after PIA immunotherapy. Together with our cytokine assay findings, patterns suggest that Th1 cytokine IFN-γ is associated with macrophage phenotypes. With a significant increase in research and development investments in immunology and technological advancements in improving the detection and analysis of macrophage markers, M1 or M2 phenotype might be utilized as a biomarker in pythiosis disease monitoring after PIA immunotherapy in clinical practice.

Iron overload in thalassemia has been reported to be associated with increased susceptibility to systemic vascular pythiosis[21]. Anomalies in phagocytic functions of macrophages have been found with iron overload *in vivo*[22]. We found that phagocytosis of *P. insidiosum* zoospores by PIA-treated macrophages did not differ significantly between patients with vascular pythiosis who survived and died. The observation that phagocytosis was increased considerably in localized pythiosis patients compared to vascular pythiosis patients with hematological diseases supports the finding from previous studies that macrophage phagocytosis is impaired in patients with hematological malignancies[23–25].

Human macrophages can produce IFN-γ, a critical factor in inducing innate and acquired immune responses against infectious pathogens[26,27]. In our study, we could show that PIA-treated macrophages produced IFN-γ against *P. insidiosum* zoospores; however, macrophages from the patients with vascular pythiosis with underlying hematological diseases produced significantly lower levels of IFN-γ compared to patients with localized pythiosis with no reported hematological disorders. This observation agrees with the previous study that showed that

IFN-γ levels were lower in macrophages from thalassemia patients than in normal individuals[12]. Investigating the signaling pathways of PIA-treated macrophages against *P. insidiosum* zoospores and the production of IFN-γ in future studies is essential. Knowing the significance of IFN-γ in immune responses, we wanted to observe how IFN-γ would impact PIA-treated macrophage phagocytosis in pythiosis patients pre- and post-PIA immunotherapy. Our data demonstrate that IFN-γ contributes to improved PIA-treated macrophage phagocytosis in patients with vascular and localized pythiosis before and after receiving the PIA immunotherapy. Based on our results, IFN-γ could be administered as an adjunctive immunotherapy in pythiosis if the macrophages are proven to be critical cells in the defense against *P. insidiosum*.

Notably, we observed a *P. insidiosum* zoospore engulfed by a macrophage, but the zoospore germinated to hypha within 2 h, ultimately leading to the death of a macrophage. This is the first observation that macrophages cannot kill *P. insidiosum* zoospore, although macrophages provide effective defense in eliminating various fungal pathogens[28]. Thus, we postulate that macrophages are not the primary immune cells responsible for killing *P. insidiosum* zoospores. However, this research is a preliminary attempt, and other researchers should confirm this observation to prove the hypothesis. Recently, we reported that PIA-stimulated neutrophils can reduce the viability of *P. insidiosum* zoospores in MTT assay[29]. In another study, neutrophils killed *P. insidiosum* zoospores dependent on neutrophil extracellular traps (NETs) and phagocytosis[30]. In future studies, it is necessary to understand the mechanisms and molecules involved in impaired macrophage phagocytosis against *P. insidiosum* zoospores and the death of macrophages. Also, we recommend further studies exploring neutrophil-macrophage cooperation in achieving an increased clearance of *P. insidiosum* zoospores.

An important strength of this study is that the vascular pythiosis recruited in this study had similar underlying hematological conditions, reflecting that the results are less affected by underlying conditions between the survived and deceased patients. PIA immunotherapy in localized pythiosis had no underlying hematological diseases, which did not contribute to the outcomes of PIA immunotherapy. Despite successful PIA immunotherapy in vascular pythiosis, it has not been effective in local *P. insidiosum* eye infections[6]. Localized pythiosis in our study received an entire 1-year course of PIA immunotherapy, and the results suggest that PIA immunotherapy provides a favorable immune response in patients with localized pythiosis.

In summary, we studied the ability of PIA immunotherapy in patients with pythiosis to modulate the immune response and macrophage phagocytosis of *P. insidiosum* zoospores. Our study demonstrated that PIA immunotherapy generates Th1 response in pythiosis patients, and IFN-γ priming enhances macrophage phagocytosis. Macrophages can engulf *P. insidiosum* zoospores but cannot kill them. Overall, macrophages can orchestrate the favorable immune response in human pythiosis but are not the primary cells in the immune defense against *P. insidiosum* infection.

Methods

Patients

This study was approved by the Chulalongkorn University Institutional Review Board (Certificate of Approval No. 833/62) for ethical research involving human subjects in accordance with the Declaration of Helsinki. All patients provided written informed consent.

The inclusion criteria for patients were: diagnosed with localized and vascular pythiosis, received a combination therapy of surgery, antifungal agents, and a 1-year course of PIA immunotherapy according to the King Chulalongkorn Memorial Hospital research treatment protocol. Individuals were diagnosed with vascular or localized pythiosis by one of the accepted diagnostic criteria: (i) successful isolation of *P. insidiosum*, (ii) positive results for PCR-ITS/*COX2* either from the isolates or directly from the clinical specimens, (iii) and positive result for *P. insidiosum*'s specific IgG antibody (in cases of vascular pythiosis). All eligible patients were enrolled in the study at the time of vascular or localized pythiosis diagnosis, and patient characteristics were collected. Patients were classified into 3 groups: Group 1 (survival group), defined by the patients with vascular pythiosis who survived more than 1 year after first diagnosis without disease progression; Group 2 (deceased group), represented by the patients with vascular pythiosis who died during the 1-year course of PIA immunotherapy; and Group 3 (local infection group), defined by the patients with localized (ocular) pythiosis who received the 1-year course of PIA immunotherapy and did not show evidence of disease progression during the study period.

Immunotherapy of pythiosis and collection of serum samples

PIA for use in patients with pythiosis was prepared with slight modifications from the method previously described by Mendoza et al[31]. PIA was prepared from culture filtrate antigen and soluble antigen from broken hyphae of *P. insidiosum* strain. PIA immunotherapy schedule involved subcutaneous administration of one milliliter of 2 mg/ml PIA with the first dose (time zero) administered as soon as the definitive diagnosis was established, and subsequently, six booster doses administered at 0.5, 1, 1.5, 3, 6, and 12 months[32]. Blood samples were collected from each patient for one year after the primary dose of PIA and at different time points synchronized with their PIA immunotherapy schedule.

Measurement of cytokines with ELISA

T helper type 1 (Th1)-type cytokine (IFN-γ) and T helper type 2 (Th2)-type cytokines (IL-4 and IL-5) were measured by direct ELISA-human cytokine platinum kit (eBiosciences, USA) according to the manufacturer's instructions to examine the pattern of cytokines production during the 1-year course of PIA immunotherapy in pythiosis patients. 100 µl of each serum sample was added to the pre-coated anti-cytokine 96-well plate. After incubation, 50 µl of the biotin-conjugate and 100 µl of streptavidin-HRP solution were added after 2 h incubation at each step at 25 °C. Finally, 100 µl of TMB substrate solution was added, followed by a stop solution.

ELISA reader measured the optical density (OD) at 620 nm. The OD_{620} result was compared with the reference standard curve to obtain the cytokine level and the concentration expressed in the unit of pictogram/milliliter (pg/ml).

Isolation of human peripheral blood monocytes

Whole blood was collected from six enrolled patients (randomly selected two patients from three classified groups). Whole blood was collected on Day 0 (before 1st dose of PIA vaccine administration) and Day 90 after the 1st dose of PIA vaccine administration. Blood samples were immediately transferred to the laboratory to be processed to isolate peripheral blood mononuclear cells (PBMCs). Blood samples were diluted 1:2 in 1X Phosphate Buffered Saline (PBS) (HyClone™, USA) and then gently layered on the surface of a tube containing Ficoll/Hypaque® solution (GE Healthcare, Germany). Tubes were centrifuged at 400 x *g* for 30 min at room temperature. PBMCs were collected from the interface layer of plasma and Ficoll. After washing, PBMCs in the pellet were enumerated and assessed for cell viability (> 95%) with 0.4% trypan blue solution.

Analysis of macrophage phenotype

PBMCs were stained with fluorophore-anchor antibodies (FITC anti-human CD14, PE/cy7 anti-human CD86, and PE anti-human CD163) to examine the ratio of M1 and M2 macrophages. Single-cell suspension was analyzed by flow cytometry (CytoFLEX V.2; Beckman Coulter) for macrophage phenotype analysis. M1 macrophages were identified as CD14+ CD86+ and M2 macrophages were identified as CD14+ CD163+. We quantified the percent of M1 and M2 derived from each donor in total macrophages[33]. This protocol for generating M1 and M2 macrophages from PBMCs was highly reproducible across different cell batches (see Supporting Information).

Differentiation of macrophages from PBMCs

To differentiate the human monocytes into macrophages, the obtained PBMCs were incubated in 20 ml of incomplete media which was RPMI 1640 (Gibco; Life Technologies, Carlsbad, CA, USA), containing penicillin (100 U/mL), streptomycin (100 µg/mL) and 1% glutamine for 1 h at 37 °C and 5% CO_2 to let the monocytes attach to the bottom of the cell culture plate (Polysorp®, Nunc, NY). Monocytes were differentiated into M0 macrophages by using a standard stimuli protocol. After washing, 2% heat-inactivated autologous serum was added to a well containing M0 macrophages and continued incubation at 37 °C and 5% CO_2 for 7 days. Finally, M1 macrophages were polarized by 50 ng/ml IFN-γ (Thermo Scientific, USA) and 10 ng/ml LPS (Thermo Scientific, USA) in incomplete media supplemented with 2% heat-inactivated autologous serum for 24 h[34,35]. Macrophages were detached from the 6-well cell culture plate using cold PBS and gentle scrapping.

Analysis of purity and viability of the macrophages

The cell suspension was stained by anti-CD68+ (BioLegend, USA) and incubated for 30 min in the dark before running the samples on the flow cytometer (FACScan, Becton Dickinson). The cell population that resulted in more than 95% of CD68 + cells was used for the experiment. Monocyte-derived macrophages were counted with propidium iodide (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 a negative control.

P. Insidiosum zoospores production

We modified the method to obtain *P. insidiosum* zoospores developed by Mendoza et al[36]. to produce zoospores from PC10 isolate in our study. The hyphae of the isolate were grown on sterilized grass fragments (*Axonopus compressus*) laid down on the surface of cornmeal agar (CMA) and incubated at 37 °C for 48 h. Then, the infected grass fragments were transferred to a Petri dish containing an induction medium and incubated at 37 °C for 18–20 h. Zoospore induction occurred in media containing solution A (K_2HPO_4 87.09 g, KH_2PO_4 68.05 g, $(NH_4)_2HPO_4$ 66.04 g, distilled water 500 ml) and solution B ($MgCl_2.6\ H_2O$ 25.42 g, $CaCl_2.2\ H_2O$ 18.38 g, distilled water 250 ml)[37]. Induction media was prepared with 500 µl of solution A, 100 µl of solution B, and 1000 ml of distilled water. The zoospores of the PC10 isolate were harvested after centrifugation of the collected induction medium and were counted using a hemocytometer. Finally, harvested fresh zoospores were treated with 110 ppm (11% v/v) inorganic hypochlorite solution, not toxic to mammalian cells, at 4 °C for 1 h to preserve the gross morphology of zoospores[12].

Evaluation of phagocytic activity and phagocytic index of PIA-treated human monocyte-derived macrophages against preserved *P. Insidiosum* zoospores

A co-culture assay assessed the phagocytosis of PIA-treated human monocyte-derived macrophages in preserved zoospores. Monocyte-derived macrophages were cultured in RPMI 1640 (Gibco; Life Technologies, Carlsbad, CA, USA), containing penicillin (100 U/mL), streptomycin (100 µg/mL), 1% glutamine, and 10% heat-inactivated autologous serum in 6-well plates and incubated at 37 °C and 5% CO_2 overnight. Then, 0.4 µg/ml of PIA and the preserved *P. insidiosum* zoospores were added to the wells containing macrophages (multiplicity of infection (MOI) 1:1) and further incubated for 8 h. Results were observed at 1 h intervals during 0–4 h and 8 h after incubation, with the experiments performed in duplicate.

Cells were washed to remove uningested zoospores, and the phagocytic activity and phagocytic index were assessed at each time point. Subsequently, macrophages were detached with cold PBS and transferred to a CytoSpin chamber (Thermo Scientific) for a single-cell layer on slide preparation. Finally, cells were stained with Diff- Quick solution (Life Science Dynamic Division) and observed under a microscope for 100 macrophages. The phagocytic activity was calculated as the average number of macrophages containing zoospores/number of observed macrophages multiplied by 100. The phagocytic index was calculated as the average number of ingested zoospores per macrophage.

Evaluation of IFN-γ production by PIA-treated human monocyte-derived macrophages against preserved *P. Insidiosum* zoospores

Serum levels of IFN-γ production by PIA-treated human monocyte-derived macrophages were measured and run in parallel with the experiment of phagocytic activity mentioned above. Supernatants were collected at 4 and 8 h after incubation, the duration representing the highest cytokine levels reported previously[12], and stored at -20 °C. IFN-γ levels were estimated by direct ELISA-human cytokine platinum kit (eBiosciences, USA) as per the manufacturer's instructions and are previously described[12]. Experiments were performed in triplicate.

Evaluation of phagocytic activity and phagocytic index of IFN-γ primed PIA-treated human monocyte-derived macrophages against preserved *P. Insidiosum* zoospores

We added 1 ng/ml of recombinant human IFN-γ (Biolegend, USA) in 6-well plates containing monocyte-derived macrophages for 2 h at 37 °C and 5% CO_2 before co-culturing with preserved zoospores. As described above, we evaluated the phagocytic activity and phagocytic index of IFN-γ primed PIA-treated human monocyte-derived macrophages. The results were observed at 1 h after incubation. Non-primed macrophages were used as controls. Experiments were conducted in duplicate.

Culture of THP-1 cells and macrophage differentiation

The culture conditions and differentiation of THP-1 monocytes into macrophages were described earlier[38,39]. Human monocytic THP-1 cells (ATCC, catalog number: ATCC® TIB-202™) were cultured in RPMI 1640 (Gibco; Life Technologies, Carlsbad, CA, USA) containing 10% (v/v) heat-inactivated FBS (Gibco; Life Technologies, Carlsbad, CA, USA) and penicillin (100 U/mL)/streptomycin (100 µg/mL) in a 37 °C, 5% CO_2 incubator for 8 h. Macrophages were obtained by treating THP-1 cells for 24 h with 150 nM PMA (Sigma, USA) in 6-well culture plates with 1×10^6 cells in each well at 37 °C and 5% CO_2. Cell viability was determined by propidium iodide (BioLegend, USA) staining according to the protocol mentioned above in the analysis of purity and viability of the macrophages section.

Labeling *P. Insidiosum* zoospores with FITC

Concentrated zoospores were incubated with 0.5 mg/ml FITC (Sigma Chemical Co., St. Louis, Mo.) in 1X PBS for 10 min in the dark. Subsequently, zoospores were washed three times in cold 1X PBS or until the clear supernatant was observed[40]. An experiment was performed on ice, and cold 1X PBS was used to delay the germination of the live zoospore.

Phagocytosis of live *P. insidiosum* zoospores by PMA-induced THP-1 macrophages

The ability of PMA-induced THP-1 macrophages in phagocytosis against live *P. insidiosum* zoospores was examined using a co-culture method and monitored by time-lapse confocal microscopy. As soon as the FITC-zoospores were added to the slide containing PMA-induced THP-1 macrophages, the channel slide was mounted on a spinning disk confocal microscope (with the stage incubator set to 37 °C) for more extended period imaging. Spatial resolution was reduced via 2 × 2 binning to minimize photobleaching and phototoxicity during time-lapse 3D imaging of live cells.

Besides the clip recording using a time-lapse 3D imaging system, Z-stacks were obtained in parallel by confocal microscopy to confirm that macrophages ingested zoospores and zoospores were not attached to the surface of the macrophages. After activating the perfect focus system, Z-stacks were set up from − 1 µm to + 16 µm at 0.8 µm steps, constituting 22 Z-slices. The FITC of zoospores was detected by a green channel at a 488 nm laser.

Statistical analysis

Statistical analysis was carried out in IBM SPSS Statistics 28.0. The Shapiro–Wilk test was used to test the normality of the data. One-way repeated-measures ANOVA with the Bonferroni post hoc test was performed to compare means between and within the group. A value of $P < 0.05$ was considered statistically significant.

Data availability

The datasets and any unique materials used and analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

NP and NW conceived and supervised the study and acquired the funding. SM and NW conducted investiga-

tions. SM drafted the original manuscript. SM, NS, and NW conducted experiments. PT, RP, and NW performed a formal analysis. NS, NP, PT, RP, and NW revised the article critically for intellectual content. All authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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