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MAJOR ARTICLE

Innate Immunity Acts as the Major Regulator in *Talaromyces marneffei* Coinfected AIDS Patients: Cytokine Profile Surveillance During Initial 6-Month Antifungal Therapy

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Background. Talaromycosis caused by *Talaromyces marneffei* infection is a fatal systemic mycosis in immunosuppressed individuals, such as patients with AIDS. Cytokines and immunocytes play a central role against fungus infection. However, how the host immune system responds to infection and treatment has not been reported to date.

Methods. Forty-one *Talaromyces marneffei* coinfected AIDS patients were followed up, their immunocytes and cytokine profiles were obtained at different antifungal treatment stages, and data on clinical features and laboratory examinations were collected. Correlation analysis was used to identify factors associated with host immunity against *Talaromyces marneffei* infection in AIDS patients.

Results. Common diseases and conditions of these 41 patients were lymphadenopathy, hepatomegaly, and splenomegaly. $CD4^+$ T cells were extremely low in all of them. Moreover, significant increases of proinflammatory cytokines (IL-12, IL-17A, TNF-α, IFN-γ, IL-18, and IL-1β), anti-inflammatory cytokines (IL-10), and chemokines (IP-10) were observed in talaromycosis before treatment ($P < .05$), comparing to both AIDS patients and healthy controls. The cytokines IL-6, IL-8, TNF-α, IL-18, IL-17A, IL-7, IP-10, and IL-1β reached peak levels 3 days after initial antifungal therapy, and then gradually decreased. The symptoms of the patients gradually decreased. Furthermore, patients who died showed the highest levels of IL-6, TNF-α, IL-8, IL-1β, and IP-10, which were 1.4- to 164-fold higher than in surviving patients.

Conclusions. Our findings indicate that innate immune-cell-derived cytokines are critical for host defense against AIDS-associated *Talaromyces marneffei* infection; furthermore, excessive inflammatory cytokines are associated with poor outcomes.

Keywords. AIDS; antifungal therapy; cytokines; innate immunity; *Talaromyces marneffei*.

Talaromyces marneffei (*T. marneffei*), formerly designated as *Penicillium marneffei*, is a dimorphic fungus and is endemic in Southeast Asian countries, such as Thailand, Vietnam, and China [1–3]. *T. marneffei* can cause fatal systemic mycosis (talaromycosis) in immunocompromised individuals [4]. In patients infected HIV and AIDS, *talaromycosis*, after tuberculosis and *cryptococosis*, ranks third among the most fatal common opportunistic infections in Southeast Asia [4, 5], particularly in individuals with a $CD4^+$ T cells count < 50 cells/μL.

Inhalation of *T. marneffei* conidia without normally occurring clearance due to immunologic dysfunction may results in conidia dissemination throughout the body. Host immunity initiates inflammatory mechanisms to eliminate pathogens in response to *T. marneffei* invasion [6]. During the immunoinflammatory reaction, the activated immunocytes produce pro- and anti-inflammatory cytokines, which simultaneously interact and form a complex inflammatory network, the dynamic balance of which influences the progression and outcome of the disease [7]. The intracellular infection of macrophages is characteristic of *T. marneffei* pathology, and recent studies reported that the activation of macrophage-derived cytokines (ie, TNF-α and IFN-γ) is elevated and responsible for *T. marneffei* clearance [8, 9]. However, these experiments were performed in vitro by stimulating macrophages with *T. marneffei* or in animal models. In AIDS patients with severe adaptive immune deficiency, the host defense against *T. marneffei* infection remains to be elucidated. Additionally, the dynamic changes in immune parameters during both infection and treatment have not been

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reported. Furthermore, little is known about the role of inflammatory factors in vivo and the correlation between cytokines and both disease course and prognosis.

This work dynamically detected changes of inflammatory factors and immune cells at different treatment stages of AIDS co-infected with *T. marneffei*, AIDS patients without *T. marneffei* coinfection and healthy individuals were enrolled as controls. Clinical manifestations and routine laboratory examinations were analyzed and elucidated immunological changes during infection and treatment were investigated. These data provide a clinical reference for the further exploration of the relationship between immunological mechanisms and disease prognosis. This study showed that in innate immune cells, macrophage-derived cytokines and chemokines are particularly vital for the host defense against *T. marneffei* in inadequate $CD4^+$ T cells and excessive inflammatory factors may relate to poor disease prognosis and outcomes.

METHODS

Ethical Approval

Participant samples were collected according to protocols approved by the respective institutional review boards. This study was approved by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University (2018L-45). Study participants provided written informed consent in accordance with the Declaration of Helsinki.

Study Subjects

Sixty-eight AIDS patients who were coinfected with *T. marneffei* were recruited in the Yunnan Infectious Disease Hospital between May 2016 and July 2018. Patients with ongoing acute or chronic coinfections, such as viral hepatitis, tuberculosis, pneumonia, candidiasis, and cryptococcosis, were excluded and, consequently, 41 patients were recruited for this study. Among these, 14 patients were followed up before (as pretreatment) as well as 3 days, 7 days, 15 days, 30 days, 90 days, and 180 days after initial antifungal therapy. All subjects were treated and both physical examination and laboratory tests were conducted. Twelve healthy subjects were used as control to rule out possible medical conditions and a further 12 HIV-1 infected patients (HIV-1-only), who did not suffer from any other infectious diseases, were enrolled as control group.

Clinical Data Collection

The clinical data of patients with AIDS and *T. marneffei* coinfection, including their routine laboratory examinations, chest computed tomography (CT) scans, chest and abdominal ultrasonography, treatment, and outcomes were collected. The demographic characteristics of all subjects were collected.

Sample Collection and Treatment

Peripheral venous blood was drawn from HIV-1-only patients, healthy control individuals, and AIDS and *T. marneffei* coinfection patients at pretreatment, as well as 3 days, 7 days, 15 days, 30 days, 90 days, and 180 days after initiation of the antifungal therapy, respectively. Every subject received an EDTA tube for the collection of peripheral blood. The blood samples were centrifugated at 3500 rpm for 10 minutes to separate plasma and blood cells. Once obtained, all plasma samples were immediately stored at -80°C for cytokine and chemokine detection.

Sample Culture and *T. marneffei* Identification

Whole blood samples, other body fluid samples, or both, were collected at different stages of antifungal therapy and were cultured at 37°C and 25°C for 7–14 days. Pathogens were identified under a microscope after medan dyeing. Brain heart infusion agar (BHI agar) was used as *T. marneffei* culture medium.

Measurement of Serum Concentrations of Cytokines

The plasma concentrantions of IL-4, IL-6, IL-7, IL-10, IL-12, IL-17A, IL-18, IL-1β, TNF-α, IFN-γ, IL-8, IP-10, and SDF-1α were measured by using the Magnetic Luminex assay with the Human Premixed Multi-Analyte kit (R&D Systems, Minneapolis, MN) according to the manufacturer's guidelines.

Statistical Analyses

Clinical data were presented as number (%), mean ($\bar{x}$), and standard deviation, or median and interquartile range, as appropriate. Comparisons of demographic data and clinical characteristics between 2 groups were performed using *t* test, Chi-square test, or Fisher's exact test, as appropriate. One-way analysis of variance was used for statistical calculations among multigroups. A P value of < 0.05 was considered statistically significant. Data analyses were performed using SPSS 22.0 software (SPSS Inc, Chicago, IL). Figures were designed by using GraphPad Prism 7 (GraphPad Software, San Diego, CA) and OriginPro software (OriginLab Corporation, Northampton, MA).

RESULTS

Demographic and Clinical Characteristics of AIDS-Associated *T. marneffei* Fungemia Patients

Forty-one *T. marneffei* strains were isolated from the blood of patients, and 8 further strains were isolated from bodily fluid samples, including bone marrow, skin lesion, sputum, and cerebrospinal fluid. The average age of the surviving group and the deceased group was 35.00 ± 9.76 years and 42.00 ± 10.29 years, respectively. No statistical difference of clinical symptoms was found between the group of patients that died and the group with noticeable improvements. In addition, no significant differences were observed for age, gender, occupation, marital status, and route of transmission between the 2 groups (Table 1).

The $CD8^+$ cell count in the deceased group (73 cells/μl, (49–105)) was significantly lower than in the surviving group (193 cells/μl, (125–346)) (P = .012). Furthermore, viral load of

Table 1. Demographic and Clinical Characteristics in *T. marneffei* Coinfected AIDS Patients

	Total cases (n = 41)		
	Surviving group (n = 37)	Deceased group (n = 4)	*P* value
Age (years)	35.00 ± 9.76	42.00 ± 10.29	.271
Gender			.517
Male	31 (88.6%)	4 (11.4%)	
Female	6 (100%)	0 (0%)	
Nationality			.668
Han	26 (89.7%)	3 (10.3%)	
Other	11 (91.7%)	1 (8.3%)	
Marriage			.533
Married	23 (88.5%)	3 (11.5%)	
Other	14 (93.3%)	1 (6.7%)	
Occupation			.703
Farmers	12 (85.7%)	2 (14.3%)	
Unemployed	17 (94.4%)	1 (5.6%)	
Other	8 (88.9%)	1 (11.1%)	
HIV transmission route			.716
Heterosexual transmission	22 (91.7%)	2 (8.3%)	
Homosexual transmission	5 (100%)	0 (0%)	
Intravenous drug use	4 (80%)	1 (20%)	
Unknown	6 (85.7%)	1 (14.3%)	
Clinical symptoms			
Fever	33 (91.7%)	3 (8.3%)	.418
No fever	4 (80.0%)	1 (20.0%)	
Cutaneous lesions			.512
Yes	23 (92.0%)	2 (8.0%)	
No	14 (87.5%)	2 (12.5%)	
Respiratory symptoms			.598
Yes	21 (91.3%)	2 (8.7%)	
No	16 (88.9%)	2 (11.1%)	
Gastrointestinal symptoms			.598
Yes	16 (88.9%)	2 (11.1%)	
No	21 (91.3%)	2 (8.7%)	
Weight loss			.467
Yes	13 (86.7%)	2 (13.3%)	
No	24 (92.3%)	2 (7.7%)	
Other symptoms			.052
Yes	8 (72.7%)	3 (27.3%)	
No	29 (96.7%)	1 (3.3%)	
Lymphadenopathy			.533
Yes	23 (88.5%)	3 (11.5%)	
No	14 (93.3%)	1 (6.7%)	
Hepatomegaly			.245
Yes	8 (80.0%)	2 (20.0%)	
No	29 (93.5%)	2 (6.5%)	
Splenomegaly			.678
Yes	18 (90%)	2 (10.0%)	
No	19 (90.5%)	2 (9.5%)	
Abdominal or pleural cavity effusion			.623
Yes	12 (92.3%)	1 (7.7%)	
No	25 (89.3%)	3 (10.7%)	
Lung CT examination			.278
Alveolar infiltration	22 (84.6%)	4 (15.4%)	
Interstitial infiltration	8 (100%)	0 (0%)	
Normal	7 (100%)	0 (0%)	
Initial antifungal regimen			.398
Amphotericin B + Fluconazole	12 (100%)	0 (0%)	
Amphotericin B	12 (85.7%)	2 (14.3%)	
Voriconazole + Amphotericin B	13 (86.7%)	2 (13.3%)	
ART before hospitalization			.332
Yes	10 (83.3%)	2 (16.7%)	
No	27 (93.1%)	2 (6.9%)	
CD4 count (cells/μl)	15 (10–28)	7 (3–28)	.187
CD8 count (cells/μl)	193 (125–346)	73 (49–105)	.012[a]
Viral load (copies/ml)	1.0×10^6 (3.5×10^3–6.2×10^6)	3.0×10^7 (2.0×10^7–3.4×10^7)	.007[b]

Abbreviations: ART, antiretroviral therapy; CT, computed tomography.

[a] $P < .05$.

[b] $P < .01$.

the deceased group (3.0×10^7 copies/ml, (2.0×10^7–3.4×10^7)) was significantly higher than in the surviving group (1.0×10^6 copies/ml, (3.5×10^3–6.2×10^6)) ($P = .007$) (Table 1).

Dynamics of Routine Laboratory Examinations

The count and percentage of lymphocytes at pretreatment were lower than those of healthy control and HIV-infected patients, temporarily decreased during antifungal therapy, and then gradually increased and returned to normal levels within 90 days of antifungal treatment. The neutrophil percentage (NEUT%) and absolute neutrophil count at baseline were higher than in healthy control and HIV-infected patients and peaked at 3 and 7 days after initial treatment, respectively, before gradually reuturning to normal levels in response to antifungal therapy. The percentage of monocytes in *T. marneffei* coinfected AIDS patients was at a high level pretreatment; however, it deceased significantly during the 3 days after treatment, and then returned to normal levels after 15 days of treatment (Table 2). The $CD4^+$ T (14 cells/μl, (9–28)) and $CD8^+$ T (169 cells/μl, (103–331)) counts significantly decreased compared to those of HIV-infected patients (351 ± 46 and 899, (703–1522), respectively), and significantly increased in response to 30 days of treatment (130 ± 17 and 741 ± 55, respectively) (Table 2).

Hemoglobin levels significantly decreased from pretreatment to 3 days and 7 days after treatment (95.73 ± 22.25, 93.00 ± 19.35, 77.00 ± 25.01 g/L, respectively) in both HIV-1-only patients ($P < .001$) and healthy controls ($P < .001$). With ongoing treatment, the level of hemoglobin returned to normal after 90 days of treatment. Platelet levels decreased and returned to normal level with ongoing treatment. The levels of alanine aminotransferase and aspartate aminotransferase increased markedly from pretreatment (38.42 ± 33.97 IU/L, 84.86 ± 20.48 IU/L, respectively) to 7 days after treatment (47.40 ± 20.03 IU/L, 85.40 ± 27.90 IU/L, respectively), and then gradually decreased to normal levels (Table 2).

Dynamics of Pro- and Anti-Inflammatory Cytokines and Chemokines

Most proinflammatory and anti-inflammatory cytokines and chemokines of the AIDS and *T. marneffei* coinfected patients at the pretreatment stage were higher than those of healthy controls and HIV-1-only patients. Among these, TNF-α, IL-6, IL-17A, IL-1β, and IL-7 continued to increase and reached peak levels 3 days after treatment (Figure 1). Thereafter, these levels gradually decreased 7 days after treatment. IL-18 significantly increased at baseline, 3 days, and 7 days, and then sharply decreased to normal levels 15 days after treatment. However, plasma levels of IL-12 and IFN-γ had peak levels at baseline and gradually decreased after treatment, returning to nomal levels day 7. The levels of the chemokines IL-8, SDF-1α, and IP-10 increased significantly and peaked 3 days after treatment, showing a decreasing trend on day 7 (Figure 1). The level of IL-10 quickly peaked at 3 days and returned to nearly baseline level at 7 days. IL-4 showed the highest level at pretreatment and began to gradually decrease in response to treatment (Figure 1). The levels of TNF-α, IL-6, IL-8, IL-1β, and IP-10 in deceased

Table 2. Dynamics of Laboratory Examinations During Antifungal Therapy in *T. marneffei* Coinfected AIDS Patients

				DPT					
	Control (n = 12)	HIV (n = 12)	Pre-T (n = 14)	**3 (n = 14)**	**7 (n = 14)**	**15 (n = 14)**	**30 (n = 14)**	**90 (n = 14)**	**180 (n = 14)**
WBC ($\times 10^9$/L)	5.89 ± 0.83[a]	5.1 ± 1.08[b]	4.11 ± 2.04[c]	3.83 ± 3.39	4.30 ± 2.28[d]	3.45 ± 2.13	4.01 ± 1.6[f]	5.39 ± 2.24[g]	5.92 ± 2.17[h]
Neutrophil ($\times 10^9$/L)	3.38 ± 0.53	3.00 ± 0.78	3.18 ± 2.12	4.47 ± 3.90	4.66 ± 1.74	2.84 ± 2.61	2.28 ± 1.41	2.92 ± 1.69	3.61 ± 1.58
Neutrophil (%)	58.57 ± 6.04[a]	58.7 ± 7.06[bi]	72.54 ± 19.98	84.54 ± 12.54	83.33 ± 6.61	60.08 ± 19.37[e]	57.28 ± 15.54[fj]	53.2 ± 16.1[g]	59.86 ± 7.3[h]
Lymphocyte ($\times 10^9$/L)	2.06 ± 0.47[a]	1.89 ± 0.55[bi]	0.59 ± 0.31	0.34 ± 0.25	0.51 ± 0.23	0.77 ± 0.38[e]	0.97 ± 0.53[fj]	1.67 ± .090[g]	1.54 ± 0.49[h]
Lymphocyte (%)	34.98 ± 4.96[a]	37.69 ± 6.95[bi]	15.63 ± 9.7	11.34 ± 6.08	9.32 ± 0.82	22.07 ± 11.45[e]	24.31 ± 9.28[fj]	31.30 ± 13.46[g]	26.73 ± 6.32[h]
Monocyte ($\times 10^9$/L)	0.31 ± 0.04	0.21 ± 0.08	0.24 ± 0.15	0.16 ± 0.10	0.35 ± 0.21	0.48 ± 0.28	0.41 ± 0.14	0.39 ± 0.13	0.41 ± 0.15
Monocyte (%)	5.38 ± 1.03	3.97 ± 0.54	7.19 ± 5.50	3.66 ± 2.05	6.80 ± 3.97	13.13 ± 6.00[e]	10.97 ± 4.18[fj]	7.91 ± 3.23	7.58 ± 3.79
Hemoglobin (g/L)	156.28 ± 16.29[a]	137.36 ± 23.17[bi]	95.73 ± 22.25	93.00 ± 19.35	77.00 ± 25.01[d]	96. 43 ± 19.17	101.45 ± 15.69	137.22 ± 31.21[g]	152.62 ± 27.41[h]
Platelet ($\times 10^9$/L)	258.28 ± 67.36[a]	156.81 ± 58.71	149.42 ± 95.79	147.75 ± 55.48	104.40 ± 61.31	177.62 ± 25.30	205.55 ± 73.86[fj]	261.77 ± 107.44[g]	235.00 ± 76.71[h]
ALT (U/L)	-	46.18 ± 27.13[i]	38.42 ± 33.97	47.40 ± 20.03	42.20 ± 27.66	33.31 ± 26.72	23.85 ± 15.00[j]	21.25 ± 10.55[g]	21.25 ± 7.26[h]
AST (U/L)	-	30.63 ± 7.63	84.86 ± 20.48	85.40 ± 27.90	119.14 ± 34.61	54.91 ± 12.01	43.88 ± 11.2	33.50 ± 8.33	26.25 ± 2.90
$CD4^+$ T (cells/μl)	-	351 ± 46[i]	14 (9–28)	-	-	-	129.8 ± 17.3[j]	-	-
$CD8^+$ T (cells/μl)	-	899 (703–1522)[j]	169 (103–331)	-	-	-	740.9 ± 55.4[j]	-	-

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DPT, days posttreatment; pre-T, pretreatment; WBC, white blood cell.

[a] Statistically significant ($P < .05$) between DPT-3 group and Health control group.

[b] Statistically significant ($P < .05$) between DPT-3 group and HIV-1-only group.

[c] Statistically significant ($P < .05$) between DPT-3 group and pre-T group.

[d] Statistically significant ($P < .05$) between DPT-3 group and DPT-7 group.

[e] Statistically significant ($P < .05$) between DPT-3 group and DPT-15 group.

[f] Statistically significant ($P < .05$) between DPT-3 group and DPT-30 group.

[g] Statistically significant ($P < .05$) between DPT-3 group and DPT-90 group.

[h] Statistically significant ($P < .05$) between DPT-3 group and DPT-180 group.

[i] Statistically significant ($P < .05$) between pre-T group and HIV-1-only group.

[j] Statistically significant ($P < .05$) between pre-T group and DPT-30 group.

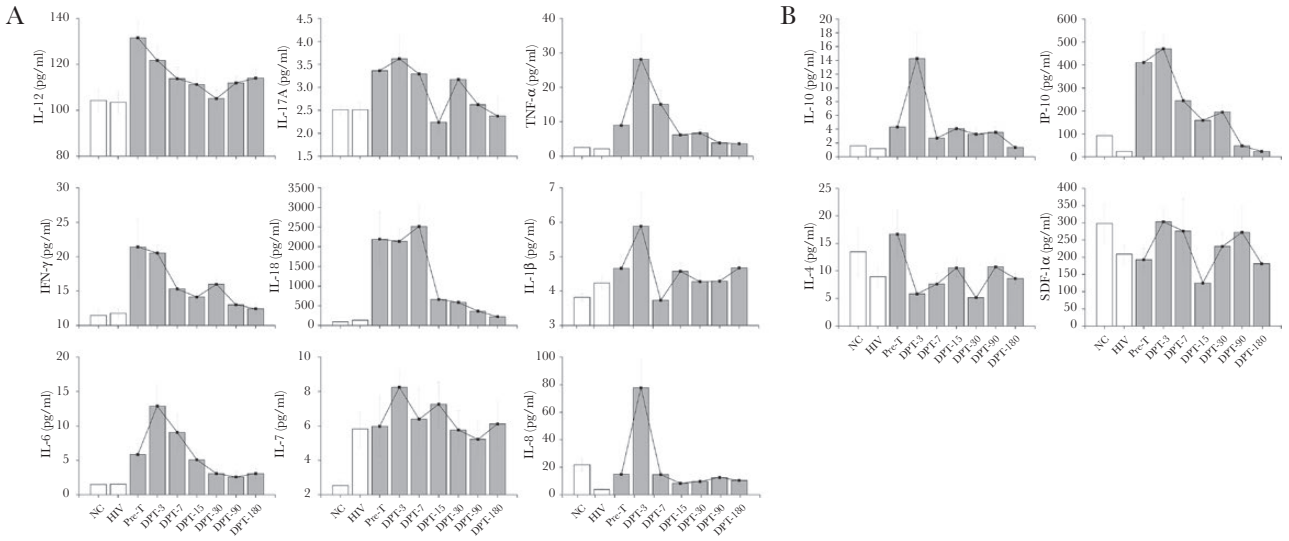

Figure 1. Cytokine profile during antifungal therapy in *T. marneffei* coinfected AIDS patients. (A) Levels of proinflammatory cytokines. TNF-α, IL-6, IL-8, IL-1β, IL-17A, IL-7, IL-18 at pretreatment were higher than those of healthy control and HIV-infected group. Besides IL-18, they continued to rise and reached the peak levels 3 days after treatment. The levels of IL-12 and IFN-γ reached peak levels at pretreatment compared to healthy controls and HIV-1-only group and then gradually decreased after treatment and returned to normal levels 7 days after treatment. (B) Levels of anti-inflammatory cytokines and chemokines. SDF-1α and IP-10 showed high levels at 3 days, and 7 days after treatment. Anti-inflammatory cytokines IL-10 quickly peaked 3 days after treatment and soon returned to almost baseline level 7 days after treatment. However, IL-4 reached peak levels at baseline and began to decrease gradually after treatment. IL indicates interleukin; IFN-γ, interferon γ; TNF-α, tumor necrosis factor-α; SDF-1α, stromal cell-derived factor-1α; IP-10, Interferon-induced protein 10.

patients were 1.4–164 fold higher than in surviving patients before treatment ($P < .05$) (Figure 2).

Correlations Between Innate Immunocytes and Inflammatory Cytokine Levels

The correlation between levels of plasma inflammatory agents levels and innate immunocytes in patients indicated positive correlations between NEUT (%) and IFN-γ (r = 0.286; $P = .03$), IL-18 (r = 0.481; $P < .001$), IL-17A (r = 0.312; $P = .017$), IP-10 (r = 0.441; $P = .002$), and IL-6 (r = 0.413; $P = .002$). IFN-γ had a significant positive correlation with IL-12 (r = 0.299; $P = .015$) (Figure 3).

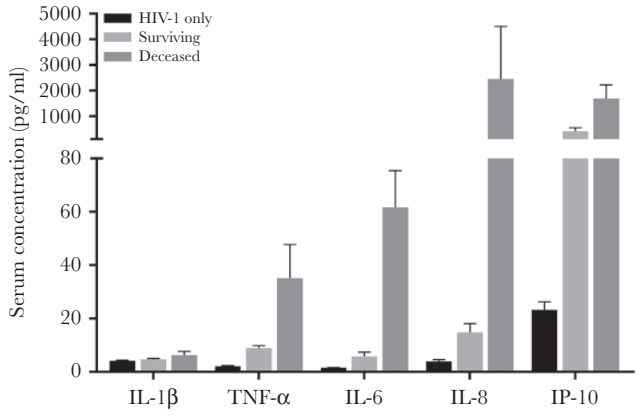

Figure 2. Plasma level of proinflammatory cytokines and chemokines in survivin and deceased patients. Pretreatment levels of TNF-α, IL-6, IL-8, IL-1β, and IP-10 in deceased patients were significantly higher than surviving patients ($P < .05$). HIV-1-only indicates the cytokine levels in patients with HIV-1 infection but without *T. marneffei* coinfection; surviving represents the cytokine levels at pretreatment stage in AIDS patients with *T. marneffei* coinfection; deceased indicates cytokine levels in the AIDS-patients with *T. marneffei* coinfection who died despite antifungal treatment.

DISCUSSION

For the first time, this study described the elaborate dynamics of the immune status of AIDS-associated *T. marneffei* infection both pre- and post-antifungal treatment. In innate immune cells, mainly macrophages-derived inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-12, IL-18, IL-1β) and chemokines (IL-8, IP-10) were evaluated and found to that play an important role in resistance to *T. marneffei* in patients who suffer from extreme deficiency of the adaptive immune system as a result of HIV infection. More interestingly, on the third day after initial antifungal treatment, a substantial number of inflammatory factors showed a secretion peak compared to pretreatment, while these which should have decreased in response to effective treatment. An overactive immune response may be associated with poor disease progression and disease outcomes, because inflammatory factors increased sharply in deceased patients.

Inhalation of *T. marneffei* conidia causes transient fungal pneumonia in immunodeficient patients, resulting in lung lesions and associated respiratory symptoms, such as cough and expectoration. Conidia turn into pathogenic yeast infecting alveolar macrophages, which can follow the pulmonary

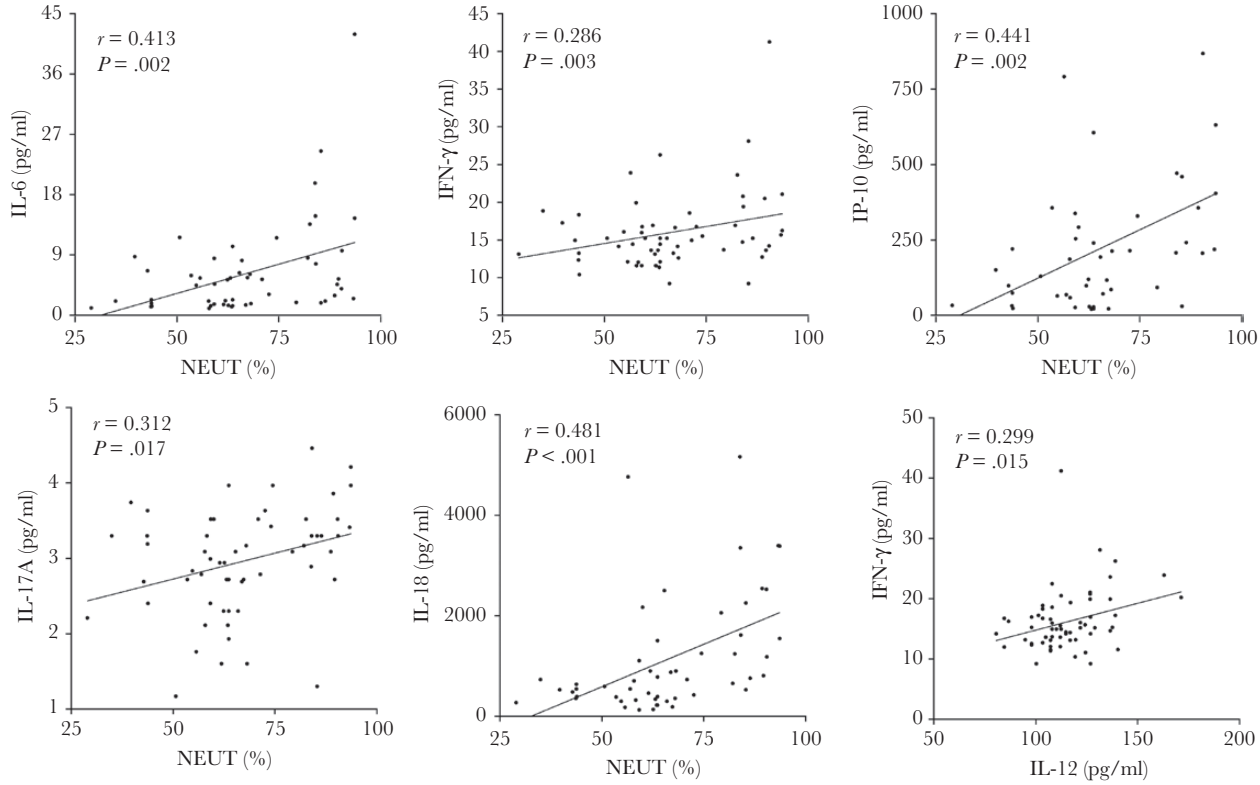

Figure 3. Correlation between levels of inflammatory cytokines and innate immunocytes. Positive correlations were found between percentage of neutrophil (%) and IFN-γ, IL-18, IL-17A, IP-10, and IL-6 ($P < .05$). IFN-γ had a significant positive correlation with IL-12 ($P = .002$).

circulation spread to the whole body causing fungaemia and exhibit sepsis like manifestations, such as fever, chills, and fatigue. Mononuclear or macrophages phagocytisis follows intracellular infection of *T. marneffei* after infection [10], as indicated by the fact that patients infected with *T. marneffei* showed hepatomegaly, splenomegaly, and lymphadenopathy in multiple organs that are rich in mononuclear-macrophages. In addition, digestive symptoms such as abdominal pain, diarrhea, poor appetite, weight loss, anemia, and other non-specific symptoms like hematuria, nausea, and a sore throat. These often lead to misdiagnosis. These reported signs and symptoms were similar to those reported previously [11, 12].

T cell immunodeficient individuals are at risk for developing disseminated fungal infections. In nude or T-cell-depleted mice, mycosis is fatal. This study showed that *T. marneffei* coinfected AIDS patients had severe adaptive immunodeficiency with low levels of lymphocytes, especially $CD4^+$ T cells (Table 2). When the CD4 levels decrease below 50 cells/μl, the host enters a state of severe immunodeficiency. CD4 cell subtypes can be divided into Th1, Th2, Th17, and Treg cells. $CD4^+$ T cells are the main or sole source of Th2 anti-inflammatory cytokines (IL-4, IL-5, and IL-10), and CD4 deficiency resulted in a loss of IL-4, IL-5, and IL-10. However, residual $CD8^+$ T cells still secrete IL-2 and IFN-γ [13]. This was also found in the present study, where IL-4 increased with low amplitude due to low $CD4^+$ T levels (Figure 1 and Table 1). However, other proinflammatory factors (IFN-γ, TNF-α, IL-17A, IL-12, IL-6, and IL-10) can be secreted by Th1, Th17, and other intrinsic immunity cells. Therefore, the immune inflammatory response was much stronger in the investigated cohort compared to both HIV-1-only patients and healthy individuals, although these were at an immunosuppressed state. This indicates that substantial inflammatory factors in these patients were not only secreted by $CD4^+$ T cells. The peaks of a number of proinflammatory cytokines (IFN-γ and IL-17A) that mainly are released by T cell types were not as high as those of cytokines secreted by innate immune cells. Moreover, proinflammatory cytokines (IL-6, IL-12, IL-18, and TNF-α) and chemokines (IL-8 and IP-10) that are predominantly secreted by macrophages, increased strongly, especially in deceased patients. Thus, the phagocytes of the innate immune system, including monocytes, tissue macrophages [10], and neutrophils [14], play a prominent role in the response against *T. marneffei* infection when T cells are compromised.

As a type of multiple biological effect monocytes, macrophages are essential for mediating the first steps of an effective antifungal host defense [15]. In vitro studies have suggested that both human and mouse macrophages are central for the crucial of *T. marneffei* growth and the killing of intracellular yeast cells by secreting cytokines [16, 17]. Activated macrophages produce cytokines such as IL-6, TNF-α, IL-8, IL-18, IL-12, IL-10, and

IFN-γ. In addition, the proinflammatory cytokine TNF-α could stimulate the antifungal effector functions of macrophages and neutrophils [18].

A strongly positive correlation between IL-12 and IFN-γ levels was observed (Figure 3), and both of these levels were significantly increased compared to HIV-1-only patients and healthy controls (Figure 1). It has been reported that the IFN-γ and IL-12 pathways are essential for the initiation of adaptive responses in mucocutaneous diseases [19]. IFN-γ deficiency resulted in a higher susceptibility to *T. marneffei* infection [20] and increased *T. marneffei* proliferation [14]. Patients with anti-IFN-γ autoantibodies are more susceptible to infections *T. marneffei* [21, 22]. Because the antibody could block IFN-γ activation, inhibited STAT1 phosphorylation and IL-12 production result in a reduction of host resistance to fungal infection [23]. Mice dendritic cells also could produce IL-12 for defense against *T. marneffei* by TLR2 and dectin-1 [24]. The present study shows that the levels of IL-12 and IFN-γ increased, which may be significant for host defense against *T. marneffei* infection in vivo.

Monocyte- or macrophages-derived IL-1β and IL-18 can recruit more proinflammatory cytokines and chemokines for fungicidic efficacy, causing a cascade of inflammatory responses in the process of pyroptosis [25]. One particularly interesting observation in this study was that IL-1β and IL-18 levels increased and reached peak levels 3 days posttreatment, which were accompanied by peaks in the production of other inflammatory cytokines (TNF-α, IL-6, and IL-17A) and chemokines (IP-10 and IL-8), and they all showed the same decreasing trend in response to treatment (Figure 1). Srinoulprasert et al also demonstrated that human monocytes could recognize *T. marneffei* conidia though pattern recognition receptors and initiate the production of proinflammatory cytokines TNF-α and IL-1β [6]. Monocytes and macrophages provide the largest contribution of chemokine IP-10 [26]. High IP-10 levels were reported in HIV infection and have been associated with lower CD4 counts [27]. IL-6 is also is promptly secreted by activated macrophages. This study provides novel information about IL-6 and IP-10 in *T. marneffei* infection in AIDS patients.

In addition to macrophages, neutrophils also exhibit strong fungicidal activity in *T. marneffei* infection [14]. Various neutropenic mouse models clearly have demonstrated a significant role for neutrophils in disseminating fungal infection [28]. A relevant case report provides information on a patient with dysplastic neutrophils infected with *T.marneffei* [29]. Ellett et al reported that tissue macrophages could enhance *T. marneffei* exposure to effective neutrophil fungicidal mechanisms [14]. On the one hand, the antigens of the pathogen are capable of attracting the neutrophils to reach the inflammatory site [28]. In the present study, both the count and percentage of neutrophils in AIDS patients coinfected with *T. marneffei* increased at pretreatment and 3 days posttreatment compared to healthy controls. Therefore, it was hypothesized that it is likely that activated neutrophils were chemotactically recruited to the inflammatory site, where they activated and enhanced the function of neutrophils and promoted the fungicidic activity of the host.

On the other hand, activated neutrophils also recruit and activate other phagocytes, cytokines, and chemotaxis to kill fungal cells [28]. These results may help to understand that neutrophils showed a significant positive correlation with proinflammatory cytokines and chemokines (IL-17A, IFN-γ, IL-6, IP-10, and IL-18). IL-17A is a proinflammatory cytokine produced by Th17 cells, neutrophils, and resident macrophages, and it is a potent mediator of neutrophil recruitment, chemotaxis, and activation [30]. IL-17A could enhance host defenses against invading fungi, including *Cryptococcus neoformans*, *Aspergillus*, and *Histoplasma capsulatum* by affecting leukocyte recruitment, IFN-γ production, and antimicrobial peptides [31, 32]. It has been shown that the neutrophil relies on IL-17RA-independent antifungi activity [33].

Therefore, higher levels of innate cells and cytokines, especially monocytes and macrophage-derived cytokines (IL-8, IL-18, TNF-α, IL-12, IL-6, IP-10, and IL-1β) may play a prominent role in the response to *T. marneffei* infection in AIDS patients with substantial loss of $CD4^+$ T cells. Interestingly, the host immune inflammatory response should decline with treatment as it promotes disease improvement. However, these inflammatory factors peaked again on the third day of treatment, while the lymphocyte and monocyte counts were at their lowest level. Antifungal drugs may promote intracellular *T. marneffei* release, further causing fungaemia to promote an immune inflammatory response, and recruit further immunocytes (mainly monocytes) and inflammatory factors to the sites of infection. Systemic sepsis-like clinical manifestations in patients appear to be associated with a high inflammatory response. In surviving patients, most inflammatory factors gradually decreased after 7 days of therapy and nearly returned to normal levels after 15 days of therapy. Furthermore, the patient's clinical symptoms and signs gradually reduced about 2 weeks after treatment.

Excessive inflammatory response can lead to tissue damage and poor prognosis after infection. Immune activation and exaggerated production of various cytokines (MCP-1, TNF-α, IL-1, IL-6, CXCL-8, IL-10, IL-18, and IFN-γ) result in proinflammatory cascades or a "cytokine-storm" [34]. This has been associated with activating cell signaling pathways and overproduction of free radicals, which in turn results in oxidative stress, and might contribute to disease severity and poor clinical outcomes [35]. Both IL-4 and IL-10 moderate and balance potentially excessive immune responses during infection [36]. They have been reported to impair clearance cryptococcosis in mouse models [37]. IL-10 has been shown to inhibit the secretion of proinflammatory cytokines (IFN-γ and IL-2) and $CD4^+$ T cell proliferation [37]. In the present study, excessive immune response was found in patients who died in the hospital

and who showed the highest levels of IL-6, TNF-α, IP-10, IL-1β, and IL-8. However, anti-inflammatory cytokines IL-4 and IL-10 did not increase significantly in deceased patients due to lack of $CD4^+$ T cells. It has been reported that high levels of IL-1β and IL-6 were consistently associated with the severity of sepsis and the highest levels were associated with the worst outcomes [38, 39]. Patients who died also had a higher HIV viral load compared to patients who showed improvement; the exudation of highly inflammatory factors may be associated with high levels of HIV viral stimulation [3]. The poor outcome likely was associated with an overly strong immune response. In addition, effective antifungal treatment seems to recover overexuberant inflammatory factors.

Although the present study discovered several cryptic aspects of the immunological profile during *T. marneffei* infecton and antifugal theatment, a number of limitations remain. First, appropriate clinical samples for cytokines and chemokines observed were not obtained, and precise subcellular localization of cytokines and chemokines are not available. Second, due to low compliance of the patients and their actual treatment situation, relatively few patients could be followed up for half a year. Third, for the first time, it was observed that inflammatory factors did not decrease but increased after 3 days of antifungal treatment in AIDS patients with *T. marneffei* fungal coinfection; however, the underlying mechanisms were further explored.

In conclusion, our finding provides a deep understanding of the underlying immunological mechanisms that are responsible for host defense against fungal infection. Moreover, this information is useful to guide a more in-depth investigation on the mechanisms of intracellular parasitism pathogenesis. Additional studies are required to explore the most significant causes of changes during the peak of host inflammatory response at the third day of treatment and the specific mechanisms of innate immunity against *T. marneffei* infection.

Notes

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