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Human Invasive Muscular Sarcocystosis Induces Th2 Cytokine Polarization and Biphasic Cytokine Changes, Based on an Investigation among Travelers Returning from Tioman Island, Malaysia

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***Sarcocystis nesbitti* is a parasite responsible for a biphasic eosinophilic febrile myositis syndrome in two recent outbreaks in Malaysia. We demonstrate Th2 cytokine polarization in infected travelers, an overall cytokine production decrease in the early phase of the disease suggestive of initial immunosuppression, and elevated levels of proinflammatory and chemotactic cytokines in the later myositic phase.**

Muscular sarcocystosis, a parasitic zoonosis that has only rarely been reported clinically in humans in Southeast Asia (1), has come to recent attention because of two concomitant outbreaks in Malaysia. One outbreak occurred in 2012 on Pangkor Island, located on the west coast of peninsular Malaysia (2, 3), and involved 89 students and teachers, mostly from Asia. The other one took place in two successive waves in 2011 and 2012 on Tioman Island, located on the east coast, and affected >100 international travelers (4–7). A third wave of infected travelers returning from Tioman Island was reported in 2014 (8), indicating an ongoing transmission from a still unknown source. Transmission on Pangkor Island has been linked to contaminated freshwater (3). Both outbreaks have been caused by *Sarcocystis nesbitti* (2, 7), a possibly snake-associated, intracellular protozoan parasite (9, 10). In the natural cycle of *Sarcocystis* sp., intramuscular encystation of the parasite occurs in the intermediate host after oral ingestion of infective oocysts which are fecally excreted by the final host. The disease has a two-phase symptomatology (3, 6, 7) consisting of an early phase characterized by fever, headache, and myalgia (phase one), followed by an asymptomatic or less symptomatic interval, and a later, again symptomatic myositic phase (phase two). Severe symptoms and prolonged and relapsing courses, even over months, have been documented (3, 6, 7, 11). Unusual for a protozoal parasite, the organism causes eosinophilia in the human accidental intermediate host. Here, we analyze lymphocyte and cytokine responses in infected German travelers returning from Tioman Island in different clinical phases of the illness.

In May and June 2014, 9 patients were seen in different travel clinics in Germany with a febrile myositis syndrome after travel to Tioman Island (2°48′47″N, 104°11′17″E). The clinical picture was identical to that of invasive muscular sarcocystosis previously reported from the island. Creatine kinase and eosinophil counts were elevated in all patients, and trichinellosis serology was negative. All patients had stayed in various villages at the northern tip of the island, as reported previously (6). According to the clinical course and the two-phase nature of invasive sarcocystosis, 3 travelers were diagnosed with phase one of the illness (2 male, 1 female; 15 to 41 years old), and 6 travelers were diagnosed with phase two of the disease (1 male, 5 female; 9 to 44 years old). In all patients, multiplex cytokine serum analyses (Bio-Rad Laboratories, Munich, Germany) were performed, and 10 sera from healthy blood donors were run in parallel. Serum cytokine level measurements from all 9 patients demonstrated no differences between phases one and two and the sera from healthy blood donors for interleukin 4 (IL-4), IL-10, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) (data not shown). However, sera of patients in phase one exhibited lower concentrations of IL-2, IL-7, IL-12, IL-15, and IL-17 than the healthy controls, with no striking difference in phase two sera (Fig. 1). Also, sera from phase one had lower concentrations of RANTES, monocyte chemoattractant protein 1 (MCP-1), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) than sera from phase two and healthy controls (Fig. 1). Sera from phase two, in contrast, showed elevations of IL-5, IL-6, IL-8, IL-9, and MIP-1α levels, compared to those in sera from phase one and healthy controls. MIP-1β levels, in contrast, were lower in phase one sera than in the healthy controls. In sera from both phases one and two, IP-10 was also elevated and, by trend, IL-1β was also (Fig. 2). Of note, two age-, sex-, and family-unrelated patients from the

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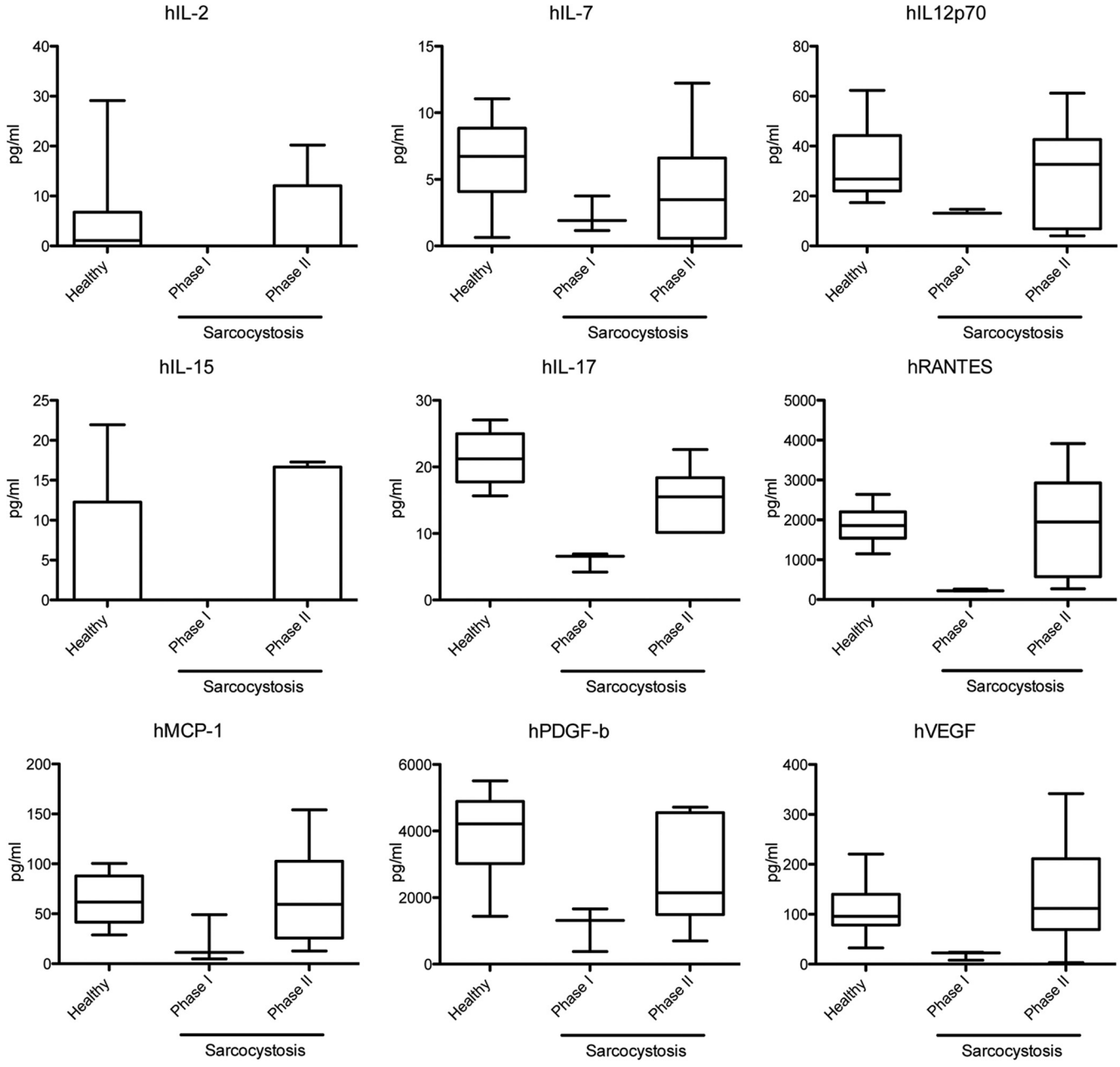

FIG 1 Cytokine levels in travelers with muscular sarcocystosis compared to healthy controls. Box and whisker plots showing median, upper, and lower quartile, minimum, and maximum values. Sera of patients in phase one (early phase) had a tendency to exhibit lower concentrations of the cytokines depicted than healthy controls and sera from phase two (late phase). IL-2 and IL-15 levels in phase one sera cluster at the bottom line. IL, interleukin; MCP-1, monocyte chemotactic protein 1; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted.

phase two cohort exhibited the maximum levels of most of the cytokines measured. When the data of these two patients were substracted, the cytokine elevations became less pronounced than those of phase one sera (not shown). Flow cytometry lymphocyte phenotyping (Navios; Beckman Coulter, Krefeld, Germany) by following standard procedures (12) was performed in 2 patients in the late second phase of the above-described cohort (1 male, 1 female; 27 and 28 years old). Both patients showed normal relative counts of natural killer (NK) cells, total T lymphocytes, activated T lymphocytes, and $CD4^+$ and $CD8^+$ T cells (normal $CD4^+$/$CD8^+$ ratio) but low B cell counts (5.3% [109 cells/μl] and 6.2% [65 cells/μl]). Lymphocyte cytokine production and accumulation were also assessed by flow cytometry. For intracellular cytokine staining (13), stimulation with phorbol-myristate-13-acetate (PMA) and ionomycin was performed, followed by staining with monoclonal antibodies against CD3 ECD (energy coupled dye, phycoerythrin [PE] and Texas Red), CD8 PC7, CD69 ECD, IL-2 PE, IFN-γ (Beckman Coulter), IL-17A fluorescein isothiocyanate (FITC), and IL-4 PE (BioLegend, Fell, Germany). In-house age- and sex-adapted reference values from healthy blood donors were

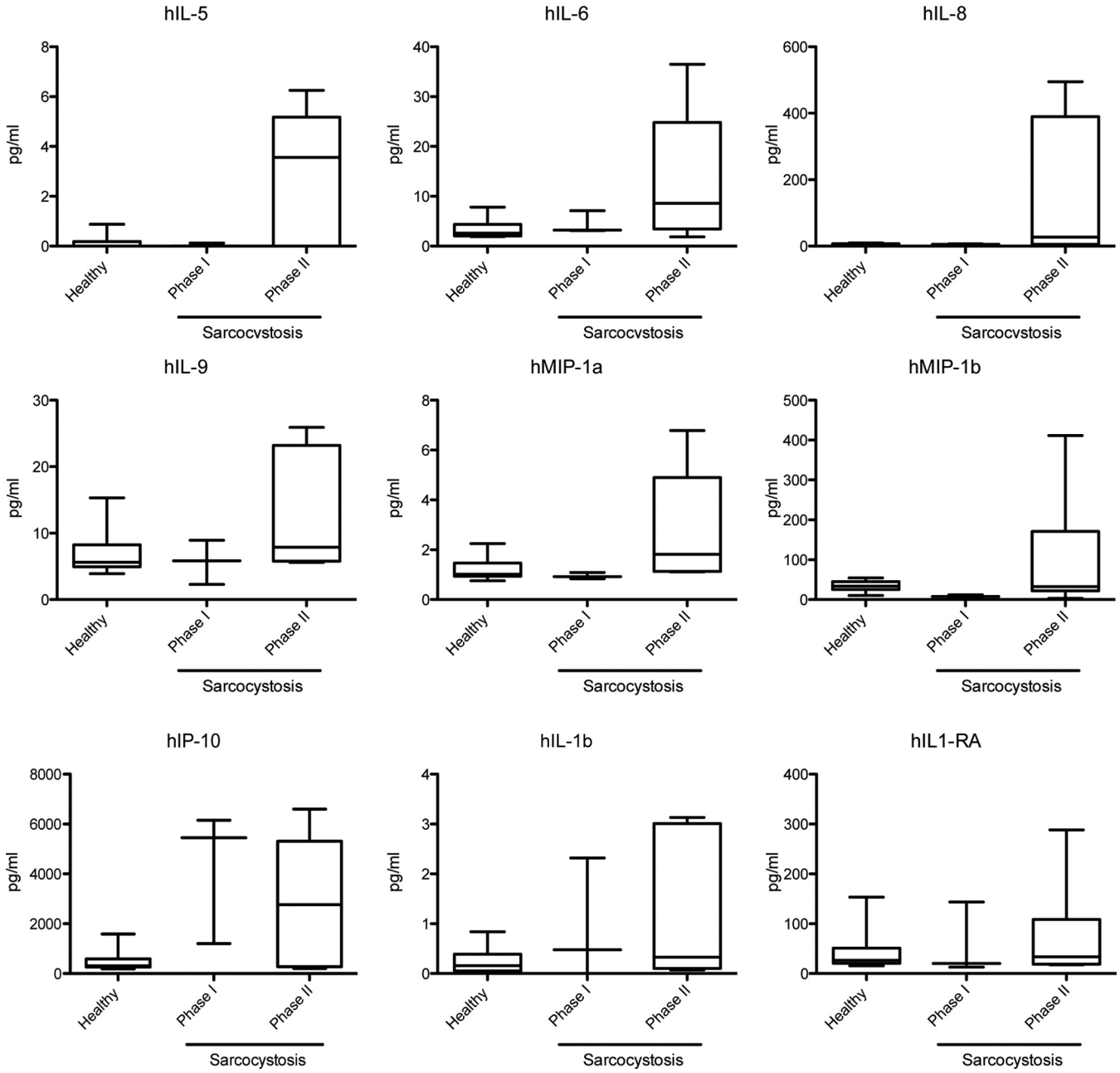

FIG 2 Cytokine levels in travelers with muscular sarcocystosis compared to healthy controls. Box and whisker plots showing median, upper, and lower quartile, minimum, and maximum values. Sera of patients in phase two (later phase) had a tendency to have higher levels of the cytokines depicted than healthy controls and, except for IP-10, sera from phase one (early phase). IL, interleukin; IP-10, interferon gamma-induced protein 10; MIP-1α/β, macrophage inflammatory protein 1 alpha/beta.

established. Both patients showed a decrease of IL-2 and IFN-γ production by $CD4^+$ lymphocytes and an age- and sex-related normal IL-4 expression. Both patients had eosinophilia (11% each [580 cells/μl]) and basophilia (1.9% [110 cells/μl] and 4.5% [240 cells/μl]) at the time of the analysis.

We here demonstrate in the two-phase disease of human invasive sarcocystosis a decrease of several serum cytokines in phase one, including the Th1 cytokines IL-2 and IL-12, paralleled by normal levels of IFN-γ. Low levels of RANTES, MCP-1, and IL-12p70 and further regulatory cytokines may point toward a decrease in T cell function and a decrease in dendritic cell activation and recruitment (14) in this initial phase of the infection. A similar T cell immunosuppression is seen in acute infections with the related parasite *Toxoplasma gondii* in a mouse model (15). The respective cytokine levels returned to values comparable with those of the healthy blood donor cohort in phase two of the disease. In contrast to phase one, in phase two of the infection several proinflammatory and chemotactic cytokines are elevated, together with IL-5, a Th2 cytokine and inductor of eosinophilia. The biphasic changes in cytokine levels observed during the two clin-

ical phases of invasive sarcocystosis likely reflect the hosts' immune response during parasite invasion and formation of schizonts in the vascular endothelium (assumed phase one of the clinical symptomatology), followed by the development of sarcocysts in muscles (phase two). Eosinophilia, a hallmark of human muscular sarcocystosis (1–8), is unusual in unicellular invasive parasitic infections and is rather seen in tissue-invasive infections involving multicellular parasites, allergy, and hematological disorders. Levels of IL-4 and IL-10, additional Th2 cytokines, were not elevated, but the observed decrease of intracellular IL-2 and IFN-γ following lymphocyte stimulation also points toward a slightly Th2-biased immune response. Characteristic biphasic immune responses have also been described in eosinophilic muscle-invasive helminth infections (trichinellosis), with a Th1 response during the initial intestinal phase and a later Th2 response during the muscle stage (16). Interestingly, B cell counts in the two examined patients were low.

This study has several limitations. First, the number of patients examined was small, thus diminishing the strength of the observation. Second, the lymphocyte analyses were performed in phase two patients only, and no data on patients in an earlier phase of sarcocystosis were obtained. Despite the fact that we show rather preliminary data, to our knowledge this is the first study investigating immune parameters in patients with sarcocystosis. Future studies will have to address lymphocyte and cytokine responses in more detail, including stimulation of immune cells with *Sarcocystis* antigen.

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