Parasitology International 67 (2018) 284–293

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Modulation of immune responses by *Plasmodium falciparum* infection in asymptomatic children living in the endemic region of Mbita, western Kenya

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ARTICLE INFO

Keywords:
Malaria
Asymptomatic
Immune response
T cells
Cytokine

ABSTRACT

Individuals living in malaria endemic areas become clinically immune after multiple re-infections over time and remain infected without apparent symptoms. However, it is unclear why a long period is required to gain clinical immunity to malaria, and how such immunity is maintained. Although malaria infection is reported to induce inhibition of immune responses, studies on asymptomatic individuals living in endemic regions of malaria are relatively scarce. We conducted a cross-sectional study of immune responses in asymptomatic school children aged 4–16 years living in an area where *Plasmodium falciparum* and *Schistosoma mansoni* infections are co-endemic in Kenya. Peripheral blood mononuclear cells were subjected to flow cytometric analysis and cultured to determine proliferative responses and cytokine production. The proportions of cellular subsets in children positive for *P. falciparum* infection at the level of microscopy were comparable to the negative children, except for a reduction in central memory-phenotype $CD8^+$ T cells and natural killer cells. In functional studies, the production of cytokines by peripheral blood mononuclear cells in response to *P. falciparum* crude antigens exhibited strong heterogeneity among children. In addition, production of IL-2 in response to anti-CD3 and anti-CD28 monoclonal antibodies was significantly reduced in *P. falciparum*-positive children as compared to -negative children, suggesting a state of unresponsiveness. These data suggest that the quality of T cell immune responses is heterogeneous among asymptomatic children living in the endemic region of *P. falciparum*, and that the responses are generally suppressed by active infection with *Plasmodium* parasites.

1. Introduction

It is estimated that > 40% (3.2 billion) of the world population is at risk of being infected with malaria, with the heaviest burden lying in sub-Saharan Africa [1]. Although recent control measures have reduced malaria-related morbidity and mortality in many African countries, malaria remains one of the leading causes of infection-related deaths [1,2]. Development of drug resistance among *Plasmodium* parasites to anti-malaria drugs and among vector mosquitos against insecticides as well as the lack of an effective malaria vaccine underpin the need to improve existing strategies for malaria control [2,3]. Moreover, there is long-standing evidence that individuals living in malaria-endemic areas become clinically immune after multiple re-infections over time, and remain asymptomatically infected as sterile immunity is rarely achieved by natural infection [4].

Both cellular and antibody-dependent immunity play indispensable

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https://doi.org/10.1016/j.parint.2018.01.001
Received 2 December 2017; Received in revised form 6 January 2018; Accepted 14 January 2018
Available online 31 January 2018
1383-5769/

roles for protection against blood-stage infection with *Plasmodium* parasites [5]. In addition, innate immunity such as provided by natural killer (NK) cells and macrophages has crucial function for the elimination of *Plasmodium* parasites in the host. These cellular responses are mediated by cytokines such as IFN-γ, TNF-α, and IL-12 that play key roles in the protective immunity [5–7]. However, excessive immune responses are sometimes harmful to the host and may lead to the severe symptoms of malaria. Although the mechanisms underlying severe malaria are not clearly understood, regulatory cytokines such as IL-10, IL-27, and TGF-β are considered important for the modulation of severe manifestation of this disease [8,9]. In particular, *Plasmodium*-specific $CD4^+$ T cells co-producing IFN-γ and IL-10 are predominant among children living in high endemic regions, and appear to have regulatory value for acute malaria inflammation [10,11]. Regulatory T cells that express the transcription factor Foxp3 are also induced and may modulate the immune responses as well [12]. Notably, a balance between pro- and anti-inflammatory responses may be important to attain clinical immunity.

In addition, immune responses are generally suppressed during acute infection with *Plasmodium* parasites [13]. Several mechanisms underlying such suppression have been reported, such as inhibition of T-cell production of IL-2 during *Plasmodium* infection [14], increased apoptosis of effector T cells, and down-regulated T-cell responses to unrelated antigens [15]. Moreover, $Foxp3^-$ regulatory T cells are induced and inhibit the expansion of T cells specific for unrelated antigens by producing inhibitory cytokines such as IL-27 [16,17], whereas acute malaria infection causes alterations in circulating levels of peripheral blood lymphocytes leading to a depletion of these populations, impacting the acquisition of immunity and control of infection [18–20]. These immunological findings were largely derived from studies on naturally infected patients with symptomatic malaria or animal models. However, residents in endemic regions of malaria acquire clinical immunity to malaria infection and remain asymptomatic despite maintaining low levels of infection. Studies on immunologic correlates in these individuals asymptomatic of malaria in the endemic region in Africa are relatively scarce, and there is little information available regarding the impact of infection on their immunological profiles [21–24].

Accordingly, to assess the impact of blood-stage *Plasmodium* infection on the immune responses in asymptomatic children, we conducted a cross-sectional study on the immune responses of peripheral blood mononuclear cells (PBMCs) from children in local schools of Mbita, Kenya, where malaria is holoendemic and a high prevalence of schistosomiasis has been reported [25]. We also assessed whether asymptomatic *Plasmodium* infection modifies the immune responses to *S. mansoni* antigens.

2. Materials and methods

2.1. Ethical statement

Study approval was granted by the Scientific Steering Committee and Ethical Review Committee of the Kenya Medical Research Institute, Kenya (KEMRI, SSC No. 2084) and the Ethical Review Board of the Institute of Tropical Medicine (NEKKEN), Nagasaki University, Japan (No. 140829127). Written informed consent was obtained from parents or guardians of the study participants and assent was sought orally from children.

2.2. Study location

The study was conducted in Mbita subcounty, on the Lakeside region of western Kenya, which lies in the malaria-endemic zone where there is intense malaria transmission throughout the year with some seasonal fluctuations. The estimated entomological inoculation rate of Kenya's malaria endemic zones is between 30 and 100 infectious bites per person per year [26]. Mbita is also an endemic region of schistosomiasis, with a high prevalence of *S. mansoni* infection (approximately 60%) among children attending schools within a 5-km radius from the lakeshore [25].

2.3. Study participants

School children aged 4–16 years were recruited from 5 schools in the area to participate in this cross-sectional study. The children were involved in an ongoing longitudinal study of the epidemiology of schistosomiasis in the area, in which 160 children were initially enrolled, and followed for approximately one year to investigate the immune responses to infection with *S. mansoni* (unpublished). During the cohort study, some of the children were lost to follow-up due to migration or absence on the day of sample collection etc., and total 148 children participated in this study conducted in October 2015. Blood, stool, and urine samples were collected at inclusion. A positive infection status was defined by the presence of a single *P. falciparum* parasite of either asexual or gametocyte stages in the inspection of one thick blood smear slide per individual (*P. falciparum* positive). Slides were observed by four independent, whereby subjects were included for analysis when 3 or all 4 microscopists were in agreement regarding the slide readings, and subjects were excluded when only 2 slide readings coincided. Parasite densities were expressed relative to the white blood cell count and the final number of parasites per μl of blood was calculated based on the assumption that the number of white blood cells per μl of blood is 8000 as shown in the formula; parasites/μl blood = (number of parasite counts × 8000) / (number of white blood cell counts). The results of all readings for each slide were averaged. Rapid diagnostic testing (RDT) (CareStart™ Malaria Pf (HRP2) Ag RDT, Access Bio Inc., Somerset, NJ USA, and SD Bioline Malaria Ag Pf/Pan, Standard Diagnostics Inc., Yongin, Republic of Korea) for malaria was performed to advise treatment with antimalaria drugs on site. Stool samples were analyzed in duplicate using the Kato-Katz (KK) technique for detecting eggs of *S. mansoni* [27]. Urine testing for *S. mansoni* diagnosis was evaluated using a circulating cathodic antigen cassette test (CCA, Rapid Medical, Pretoria, South Africa) [28]. Infection status was defined by a positive outcome from either KK, CCA, or both. All children positive for malaria were treated with artemether/lumefantrine per the Kenya national guidelines for the treatment of uncomplicated malaria. Children infected with schistosomes as detected by KK were treated with 40 mg/kg praziquantel and those infected with soil-transmitted helminths were treated with 400 mg albendazole according to World Health Organization guidelines.

2.4. P. falciparum culture and schizont antigen preparation

The *P. falciparum* 3D7 strain used in this study was originally reported by Dr. David Walliker (Edinburgh Univ., Edinburgh, UK) [29] and was obtained from Dr. L. H. Miller (NIAID, National Institutes of Health, Bethesda, MD, USA). Red blood cells (RBCs) were obtained from the Japanese Red Cross Blood bank. Parasites were grown as previously reported [30]. Briefly, parasites were maintained in O^+ human RBCs cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with HEPES (25 mM, Sigma-Aldrich, St. Louis, MO, USA), hypoxanthine (200 μM, Sigma-Aldrich), sodium bicarbonate (0.225%), Gentamicin (10 mg/ml, Invitrogen, Carlsbad, CA, USA), and AlbuMax I (0.5%, Invitrogen) under a gas environment of 5% O_2, 5% CO_2, and 90% N_2 at 37 °C. Parasite development was monitored by examination of Giemsa-stained thin blood smears. After two rounds of synchronizations with 5% sorbitol (Sigma-Aldrich), schizont parasites were harvested at approximately 90% parasitemia by magnetic MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany). Mature schizont-infected RBCs were lysed through five freeze-thaw cycles (− 80 °C and 37 °C) to constitute *P. falciparum* schizont extract (iRBC). Freeze-thawed preparations of