



A Unique Subset of $\gamma\delta$ T Cells Expands and Produces IL-10 in Patients with Naturally Acquired Immunity against *Falciparum* Malaria

Tomoyo Taniguchi^{1,2,3*}, Kaiissar Md Mannoor⁴, Daisuke Nonaka⁵, Hiromu Toma⁵, Changchun Li⁶, Miwako Narita⁷, Viengxay Vanisaveth⁸, Shigeyuki Kano⁹, Masuhiro Takahashi⁷ and Hisami Watanabe^{3,10}

¹ Department of Parasitology, Graduate School of Medicine, Gunma University, Maebashi, Japan, ² Center for Medical Education, Graduate School of Medicine, Gunma University, Maebashi, Japan, ³ Immunobiology Group, Center of Molecular Biosciences, Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Japan, ⁴ Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, United States, ⁵ Department of Parasitology and Immunopathology, Graduate School of Medicine, University of the Ryukyus, Nishihara, Japan, ⁶ Department of Health Sciences, Trans-disciplinary Research Organization for Subtropics and Island Studies, University of the Ryukyus, Nishihara, Japan, ⁷ Laboratory of Hematology and Oncology, Graduate School of Health Sciences, Niigata University, Niigata, Japan, ⁸ Center for Malariaology, Parasitology and Entomology, Vientiane, Laos, ⁹ Research Institute, National Center for Global Health and Medicine, Tokyo, Japan, ¹⁰ Infectious Diseases Research Center of Niigata University in Myanmar, Institute of Medicine and Dentistry, Niigata University, Niigata, Japan

OPEN ACCESS

Edited by:

José Roberto Mineo,
Federal University of Uberlandia, Brazil

Reviewed by:

Moriya Tsuji,
Aaron Diamond AIDS Research
Center, United States

David L. Wiest,
Fox Chase Cancer Center,
United States

*Correspondence:

Tomoyo Taniguchi
ttani@gunma-u.ac.jp

Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Microbiology

Received: 20 February 2017

Accepted: 27 June 2017

Published: 19 July 2017

Citation:

Taniguchi T, Md Mannoor K, Nonaka D, Toma H, Li C, Narita M, Vanisaveth V, Kano S, Takahashi M and Watanabe H (2017) A Unique Subset of $\gamma\delta$ T Cells Expands and Produces IL-10 in Patients with Naturally Acquired Immunity against *Falciparum* Malaria. *Front. Microbiol.* 8:1288. doi: 10.3389/fmicb.2017.01288

Although expansions in $\gamma\delta$ T cell populations are known to occur in the peripheral blood of patients infected with *Plasmodium falciparum*, the role of these cells in people with naturally acquired immunity against *P. falciparum* who live in malaria-endemic areas is poorly understood. We used a cross-sectional survey to investigate the role of peripheral blood $\gamma\delta$ T cells in people living in Lao People's Democratic Republic, a malaria-endemic area. We found that the proportion of non-V γ 9 $\gamma\delta$ T cells was higher in non-hospitalized uncomplicated falciparum malaria patients (UMPs) from this region. Notably, we found that the non-V γ 9 $\gamma\delta$ T cells in the peripheral blood of UMPs and negative controls from this region had the potential to expand and produce IL-10 and interferon- γ when cultured in the presence of IL-2 and/or crude *P. falciparum* antigens for 10 days. Furthermore, these cells were associated with plasma interleukin 10 (IL-10), which was elevated in UMPs. This is the first report demonstrating that, in UMPs living in a malaria-endemic area, a $\gamma\delta$ T cell subset, the non-V γ 9 $\gamma\delta$ T cells, expands and produces IL-10. These results contribute to understanding of the mechanisms of naturally acquired immunity against *P. falciparum*.

Keywords: $\gamma\delta$ T cells, naturally acquired immunity, *Plasmodium falciparum*, falciparum malaria, IL-10

INTRODUCTION

Malaria is caused by protozoan parasites of the genus *Plasmodium* and is widespread in tropical and subtropical regions of the world. Approximately half the world's population is at risk of malaria, and 148–304 million cases of malaria and 0.2–0.6 million associated deaths are estimated to occur each year (World Health Organization, 2016). There is still no effective vaccine for malaria (Langhorne et al., 2008; Riley and Stewart, 2013), thus posing a problem for those exposed to

Plasmodium falciparum, the species that causes the most severe form of the disease. An effective malaria vaccine must induce long-lasting protective immunity. However, it is difficult to induce sufficient immunological memory against human malarial parasites, because of their high levels of antigenic polymorphism and complex parasitic life cycle (Covell et al., 1953; Healer et al., 2004). Additionally, the life span of plasma cells in humans is short (Dorfman et al., 2005; Akpogheneta et al., 2008; Portugal et al., 2013). Nevertheless, naturally acquired immunity, which occurs after repeated infections with a parasite, does develop in people living in malaria-endemic areas; although this immunity is incomplete, it decreases clinical disease and lowers the density of parasitemia (Doolan et al., 2009). For effective vaccine development, it is important to study individuals who have developed a naturally acquired immunity against malaria.

$\gamma\delta$ T cells are T cells carrying the $\gamma\delta$ T cell receptor (TCR), and their system of antigen recognition differs from that of $\alpha\beta$ T cells. These cells have drawn much attention in relation to their involvement in host defense against infection and tumors, although they constitute a very small population of cells in the peripheral blood. $\gamma\delta$ T cells perform a variety of functions, including cytotoxic functions and initiation of acquired immunity (Bonneville et al., 2010; Vantourout and Hayday, 2013). Before $\gamma\delta$ T cells were discovered, human peripheral blood mononuclear cells (PBMCs) had been reported to proliferate in the culture supernatants and extracts of malaria-infected erythrocytes *in vitro* (Greenwood and Vick, 1975). After the concept of the $\gamma\delta$ T cell population had been established, it was confirmed that splenic $\gamma\delta$ T cell populations increase during malaria infection in both humans and mice (Minoprio et al., 1989; Bordessoule et al., 1990). There have been many conflicting reports on whether $\gamma\delta$ T cells and their subsets increase after malaria infection. Some reports claim that in patients with primary or acute falciparum malaria, $\gamma\delta$ T cells increase after antimalarial treatment and that this increase persists for 3–4 weeks after treatment (Ho et al., 1990; Roussilhon et al., 1990; Chang et al., 1992; Hviid et al., 1996, 2001; Schwartz et al., 1996; Worku et al., 1997). However, there are some reports showing that no increase occurs in $\gamma\delta$ T cells in the peripheral blood of UMPs from endemic areas (Goodier et al., 1993; Hviid et al., 1996). We have previously shown that unconventional T cells, including $\gamma\delta$ T cells, are associated with protection against malaria in murine models of the disease (Weerasinghe et al., 2001; Mannoor et al., 2002; Bakir et al., 2006; Taniguchi et al., 2007; Li et al., 2012). We have also observed both the presence and absence of an increase in $\gamma\delta$ T cells in peripheral blood samples from malaria patients in Southeast Asia (Watanabe et al., 2003). Recently, there have been reports that repeated malaria infection in malaria-endemic area is associated with a decreased percentage of $\gamma\delta$ T cells in the peripheral blood and decreased proliferation and cytokine production in response to malarial antigens (Jagannathan et al., 2014; Farrington et al., 2016).

We, therefore, hypothesized that $\gamma\delta$ T cells, which increase in primary or acute infections, do not increase in people with naturally acquired immunity to malaria. To evaluate this

hypothesis and to investigate the role of $\gamma\delta$ T cells in people with naturally acquired immunity against *P. falciparum* in more detail, we analyzed the dynamics of $\gamma\delta$ T cells in patients with falciparum malaria living in the Lao People's Democratic Republic, where malaria is endemic. We found that a $\gamma\delta$ T cell subset, the non-V γ 9 $\gamma\delta$ T cells, which increases in malaria patients living in endemic areas, may play an important role in the acquisition of natural immunity.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the National Ethics Committee for Health Research, Ministry of Health, Lao People's Democratic Republic (PDR) and the Ethics Review Board of the University of the Ryukyus, Japan. Informed consent was obtained from each participant in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later revision. Identifying information of patients of human subjects, including names, initials, addresses, or any other data that might identify patients do not be included in written descriptions in this article. Informed consent from minors was obtained from their parent before sample collection.

Study Site and Population

This cross-sectional survey was conducted at the end of each rainy season from 2005 to 2008 in villages of the Phouvong District of Attapeu Province, an area with high malaria endemicity in Lao PDR¹. The annual incidence of malaria in 2008 in this province was 14.3 cases per 1,000 people, which is the second highest incidence of malaria in the country (Jorgensen et al., 2010). Non-endemic controls (NECs) were recruited voluntarily from the population in Vientiane, the capital of Lao PDR, and Japanese healthy controls (JHCs) were recruited from volunteers in Okinawa Prefecture, Japan.

Blood Samples

Falciparum malaria was diagnosed at the primary schools in the villages or the village head's house. All subjects were assessed with a rapid immunochromatographic test (ICT; Paracheck Pf[®], Orchid Biomedical Laboratories, Goa, India) and a blood smear analysis. After the diagnostic tests were performed, heparinized blood samples (4 mL) were collected from the *P. falciparum*-positive subjects and some *P. falciparum*-negative volunteers (NCs) as controls. All *P. falciparum*-positive subjects with uncomplicated falciparum malaria, lacking signs of organ compromise or other severe symptoms, were classified as non-hospitalized uncomplicated malaria patients (UMPs), according to the World Health Organization guidelines (World Health Organization, 2006). Parasitemia and species identity were assessed by microscopic

¹<http://www.who.int/malaria/publications/country-profiles/2008/en/>

observation of the blood smears. The data from *P. vivax*-infected subjects and false-positive (ICT positive but microscopic observation-negative) subjects were excluded from the analysis. *P. falciparum*-positive subjects were treated with Coartem® (Novartis Pharma, Basel, Switzerland), an artemisinin-based combination therapy. After the number of white blood cells was counted, the plasma was separated from the blood by centrifugation. PBMCs were obtained by Ficoll-Paque (GE Healthcare Life Sciences, Uppsala, Sweden) gradient centrifugation. The plasma and PBMCs from *P. falciparum*-positive and -negative subjects were initially stored in liquid nitrogen (-196°C) and at -80°C until analysis.

Antibody Assays

Enzyme-linked immunosorbent assays (ELISAs) of anti-*P. falciparum* IgG antibodies (Abs) were performed as below. Each well of a 96-well plate was coated with $0.5\ \mu\text{g}$ of crude *P. falciparum* FCR-3 antigen extract (Pf Ag) in $100\ \mu\text{L}$ of $50\ \text{mM Na}_2\text{CO}_3$ buffer, a kind gift from Dr. S. Nakazawa (Department of Protozoology, Institute of Tropical Medicine, NEKKEN, and the Global COE Program, Nagasaki University, Nagasaki, Japan). The plates were incubated overnight at 4°C to allow antigen binding, after which the wells were washed four times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS/T) and then blocked for 2 h at 37°C with $150\ \mu\text{L}$ of PBS/T containing 1% bovine serum albumin (BSA; Nacalai Tesque, Kyoto, Japan) per well. Test sera ($100\ \mu\text{L}$ aliquots), each diluted 1:400 in PBS/T containing 1% BSA, were added in duplicate, and the plates were incubated for 1 h at room temperature. The wells were washed four times with PBS/T and then incubated for 30 min at room temperature with $100\ \mu\text{L}$ of horseradish-peroxidase-conjugated goat anti-human IgG (Promega, Madison, WI, United States) diluted 1:2500 in PBS/T. The wells were washed four times with PBS/T, $100\ \mu\text{L}$ of tetramethylbenzidine substrate (TMB One Solution; Promega) was added, and the plates were incubated at room temperature for up to 15 min before the reaction was stopped with $100\ \mu\text{L}$ of $0.5\ \text{M H}_2\text{SO}_4$. The optical density (OD) of the wells at $450\ \text{nm}$ (OD_{450}) was determined with a microplate reader. To generate standardized units of specific IgG reactivity, a standard curve was generated to allow the OD units to be interpolated to linearized units of antibody concentration. If a sample OD_{450} exceeded 2.0, the sample was diluted further as soon as possible to achieve an OD_{450} within the range of 0.0–2.0.

Flow Cytometric Analysis

Frozen PBMCs were thawed quickly for analysis. The surface phenotypes of the lymphocytes were determined, and the intracellular cytokine measurements were made with three- or four-color immunofluorescence tests. Fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin-conjugated monoclonal antibodies (mAbs) were used, and biotin-conjugated reagents were developed using allophycocyanin-conjugated streptavidin (Caltag Laboratories, Burlingame, CA, United States). Anti-CD3 (145-2C11), anti-V γ 9 TCR (TM- β 1), anti- $\alpha\beta$ TCR (H57-597), anti- $\gamma\delta$ TCR (GL3), anti-IL-10, and anti-interferon γ

(IFN- γ) mAbs (BD Biosciences, Mountain View, CA, United States) and anti-V δ 1 TCR (R9.12) mAb (Beckman Coulter, Marseille, France) were used. The cells were examined with a FACSCalibur flow cytometer (BD Biosciences). Dead cells were excluded with forward scatter, side scatter, and propidium iodide gating.

RT-PCR Analysis

Total RNA was extracted from the PBMCs of UMPs and NECs with the RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). To detect the TCR γ and δ chain mRNAs, the cDNA was synthesized from $2.5\ \mu\text{g}$ of RNA with random primers and Superscript VILO reverse transcriptase (Invitrogen, Carlsbad, CA, United States). The cDNA was amplified with sense primers specific for TCR V γ or V δ and antisense primers specific for TCR C γ or C δ , as previously described (Kageyama et al., 1994; Boria et al., 2008). The PCR products were visualized under UV illumination on 2% agarose gels stained with ethidium bromide.

PBMC Culture and Intracellular Cytokine Staining

Thawed PBMCs ($1\text{--}2 \times 10^6$ cells/mL) were cultured with or without interleukin 2 (IL-2, $50\ \text{U/mL}$) or IL-2 and Pf Ag ($5\ \mu\text{g/mL}$) for 10 days at 37°C under 5% CO_2 . The cultured PBMCs were restimulated with Leukocyte Activation Cocktail (BD Biosciences) containing the phorbol ester phorbol 12-myristate 13-acetate, a calcium ionophore (ionomycin), and brefeldin A for 4 h at 37°C under 5% CO_2 . The cells were then fixed, permeabilized, stained with $20\ \mu\text{L}$ of PE-conjugated rat anti-human IL-10 antibody or mouse anti-human IFN- γ antibody (BD Biosciences), and analyzed with a FACSCalibur flow cytometer (BD Biosciences).

Cytokine Detection

The cytokines in the plasma and culture supernatants were measured using a BDTM Cytometric Bead Array Flex Set and BDTM Human Soluble Protein Master Buffer kit according to the manufacturer's instructions (BD Biosciences). Aliquots ($50\ \mu\text{L}$) of the test sera, diluted 1:5 with dilution buffer, were used. IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, tumor necrosis factor α , and IFN- γ were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FCAP Array software (BD Biosciences).

Statistical Analysis

The study subjects were matched for age and sex. The Mann-Whitney *U*-test was used for two-group comparisons, the Kruskal-Wallis test was used for three-group comparisons, and paired and unpaired *t*-tests were used to analyze whole PBMC cultures. Spearman's rank correlation was used for correlational analyses. All analyses were performed with Prism version 6.0 (GraphPad Software, La Jolla, CA, United States) and JMP version 8 statistical software (SAS, Cary, NC, United States).

RESULTS

Expansion of a Unique $\gamma\delta$ T Cell Subset, the Non-V γ 9 $\gamma\delta$ T Cells, in Falciparum Malaria Patients

Despite many reports of $\gamma\delta$ T cells in malaria patients, it is unclear whether $\gamma\delta$ T cells are induced during malarial infection (Ho et al., 1990; Roussillon et al., 1990; Goerlich et al., 1991; Currier et al., 1992; Goodier et al., 1992; Zevering et al., 1992; Dick et al., 1996; Hviid et al., 1996, 2001; Rzepczyk et al., 1996; Goodier and Targett, 1997; Worku et al., 1997; Waterfall et al., 1998). To investigate this phenomenon in more detail, PBMCs and plasma obtained from UMPs and NCs living in malaria-endemic areas of Attapeu Province, Lao PDR and from NECs in Vientiane, the country's capital, were analyzed during physical examination of the subjects. The hematological characteristics of the UMPs, NCs, and NECs in this study are summarized in **Table 1** and Supplementary Figure S1. In agreement with the high incidence of malaria (Jorgensen et al., 2010), all subjects living in the Phouvong District of Attapeu Province had anti-*P. falciparum* IgG Abs in their plasma, and the subjects in the NC group had a previous history of natural malaria infection. The anti-*P. falciparum* IgG Ab titers were higher in the UMPs (median 827.9, range 86.9–8685.6) than those in the NCs (median 462.1, range 7.0–10,097.3; $p < 0.0001$). The UMPs living in the malaria-endemic area included both symptomatic (body temperature $\geq 37.5^\circ\text{C}$) and asymptomatic falciparum malaria patients (parasitemia $< 250,000/\mu\text{L}$, hematocrit $> 15\%$) (World Health Organization, 2006).

The lymphocytes in the PBMCs were phenotypically characterized by using flow cytometry. As in other acute infections, acute falciparum malaria causes a decrease in lymphocyte counts (Supplementary Table S1 and Figure S2). A decrease in lymphocyte numbers in UMPs inhabiting the malaria-endemic area and a significant increase in white blood cell numbers in NCs were observed (**Table 1** and Supplementary Figure S3); therefore, the percentage of cell populations was used for comparisons. The change in lymphocyte cell subsets

was minimal, and the percentage of $\gamma\delta$ T cells (UMPs: median 5.5%, range 1.2–13.9%; NCs: median 5.1%, range 3.5–13.4%, $p = 0.6208$) and $\alpha\beta$ T cells (UMPs: 67.9%, 50.8–79.2%; NCs: 63.4%, 47.2–75.3%, $p = 0.1917$) was not higher in the UMPs than in the NCs (**Figure 1** and Supplementary Figure S4). However, a specific $\gamma\delta$ T cell subset, the non-V γ 9 $\gamma\delta$ T cells (UMPs: 2.9%, 0.2–10.6%; NCs: 1.7%, 0.3–3.3%, $p = 0.0018$), was expanded in the UMPs (**Figure 1B**), and the proportion of non-V γ 9 $\gamma\delta$ T cells within the $\gamma\delta$ T cell subset (UMPs: 58.1%, 10.7–92.7%; NCs: 33.7%, 8.8–82.5%, $p = 0.0025$) was also higher in the UMPs (**Figure 1C**). These results differed from those of hospitalized patients with severe falciparum malaria, in whom the V γ 9 $\gamma\delta$ T cell subset increased (Supplementary Table S1 and Figures S5A,B). Although there was no change in B cell subsets between UMPs and NCs, we found a significant increase in B cells and memory B cells (IgG+CD27-CD20+) in UMPs compared with those in NECs (Supplementary Figure S6).

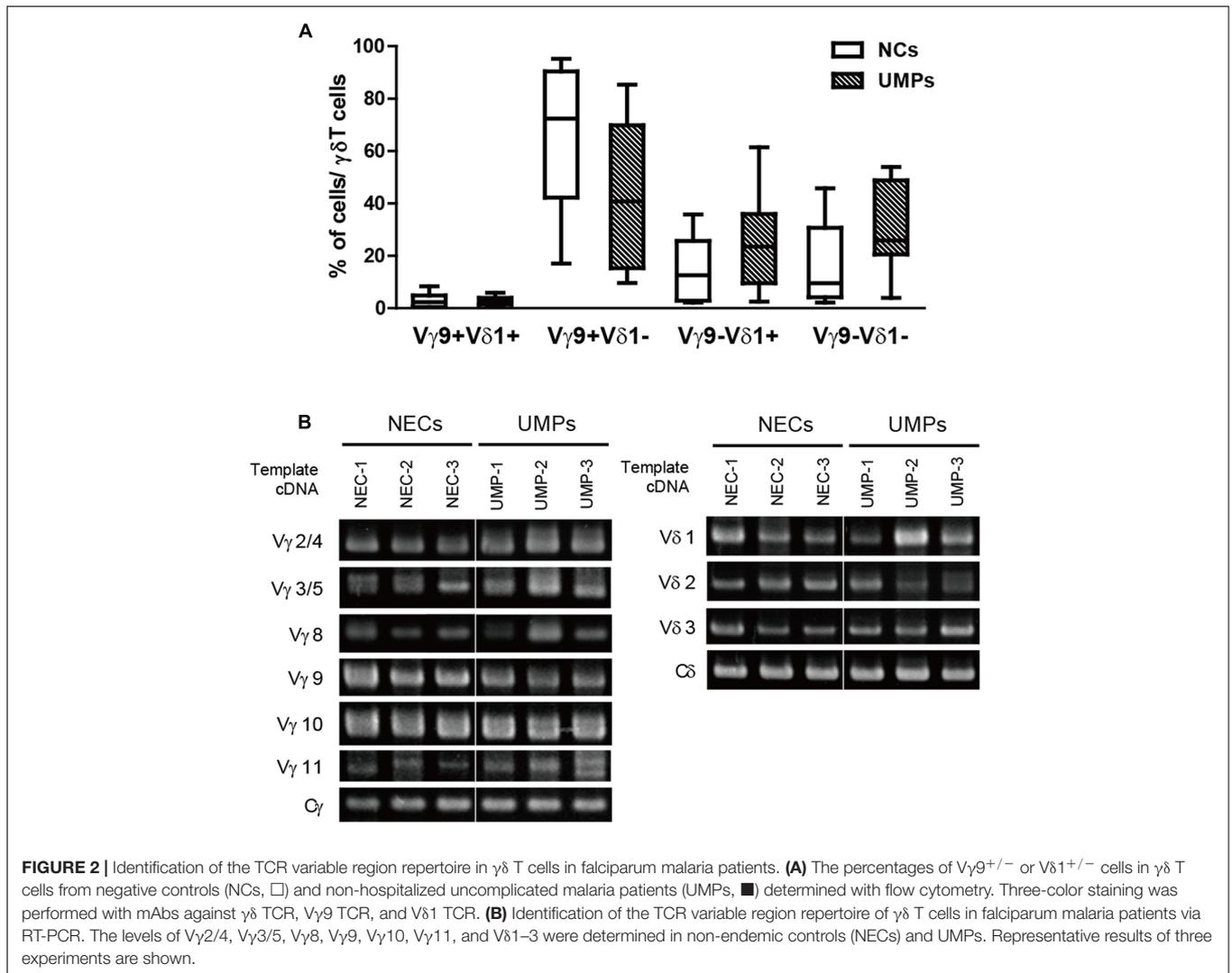
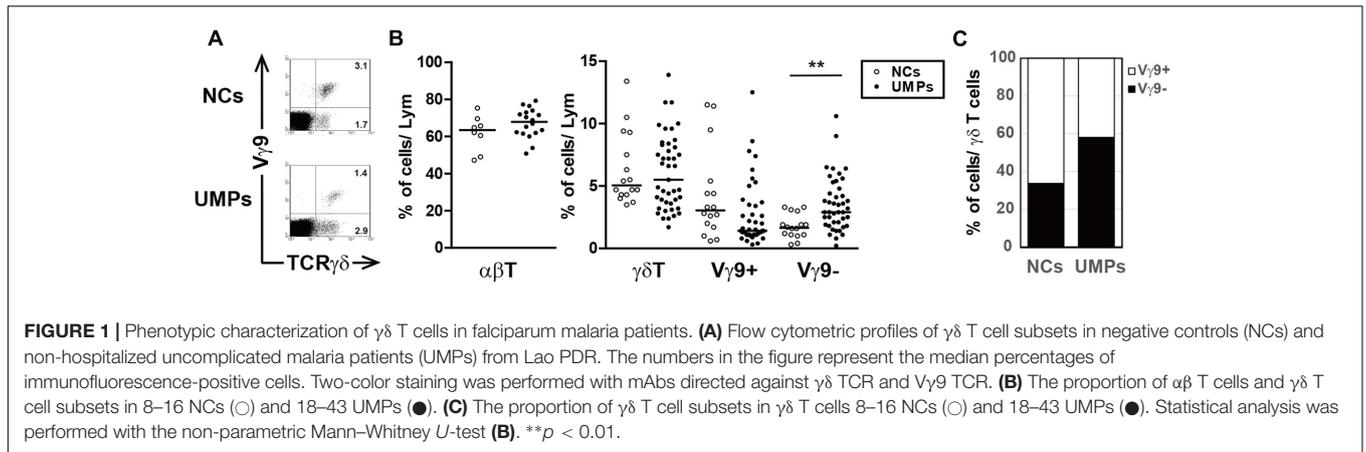
$\gamma\delta$ TCR Usage by $\gamma\delta$ T Cells in Patients with Falciparum Malaria

$\gamma\delta$ T cells with non-TCR V γ 9 chains were increased in the UMPs, and the other major subset in the peripheral blood had the V δ 1 chain; however, the predominant $\gamma\delta$ T cell subset was V γ 9V δ 2. Therefore, using flow cytometry, we examined whether these non-V γ 9 $\gamma\delta$ T cells had V δ 1 chains. The $\gamma\delta$ T cell subsets that were elevated in the UMPs living with endemic malaria were V γ 9⁻V δ 1⁺ (UMPs: 21.4%, 2.5–61.4%; NCs: 12.6%, 2.2–35.8%) and V γ 9⁻V δ 1⁻ (UMPs: 25.3%, 3.9–53.9%; NCs: 9.5%, 2.2–45.8%). In contrast, V γ 9⁺V δ 1⁻ cells were the predominant subset in the NCs, but there was no significant difference in $\gamma\delta$ TCR usage by $\gamma\delta$ T cells between NCs and UMPs, as determined by flow cytometry (**Figure 2A**). Therefore, we performed further analysis of $\gamma\delta$ TCR usage by $\gamma\delta$ T cells from UMPs compared with that in NECs by using RT-PCR (**Figure 2B**). We found that V γ and V δ were detected at the same level in the UMPs and NECs, and the V γ 9 levels tended to be lower in the UMPs than in the NECs, whereas the levels of V γ 2/V γ 4, V γ 3/V γ 5, V γ 8, V γ 11, V δ 1 and V δ 3 tended to be higher

TABLE 1 | Hematological characteristics of patients with falciparum malaria and negative controls.

| Characteristic | UMPs ^a (n = 137) | NCs (n = 31) | NECs (n = 65) | p-Value ^b |
|--|-----------------------------|-----------------------|----------------------|----------------------|
| Age, median (range) | 10.0 (0.5–45) | 15.0 (5–31) | 12.0 (5–20) | |
| Sex (female/male) | 66/71 | 20/11 | 33/32 | |
| WBC/ μL , median (range) | 8,500 (3,500–16,400) | 10,400 (5,800–16,400) | 7,500 (3,500–13,500) | <0.0001 |
| % Neutrophil, median (range) | 42.3 (14.0–88.0) | 44.0 (13.5–72.5) | 46.0 (24.5–72.5) | 0.9103 |
| % Lymphocyte, median (range) | 38.5 (7.0–75.0) | 37.5 (21.5–85.5) | 47.0 (23.5–70.0) | 0.0037 |
| % Monocyte, median (range) | 3.0 (0.0–16.0) | 3.0 (0.0–8.5) | 1.5 (0.0–10.0) | 0.001 |
| % Eosinophil, median (range) | 9.3 (0.0–37.5) | 11.5 (0.0–28.0) | 5.0 (0.0–23.5) | 0.0002 |
| % Basophil, median (range) | 0.0 (0.0–1.5) | 0.0 (0.0–1.0) | 0.0 (0.0–1.0) | 0.0592 |
| Parasitemia/ μL , median (range) | 640 (32–180,000) | (–) | (–) | |
| Microscopic observation (<i>Pf</i>) | (+) | (–) | (–) | |
| α - <i>Pf</i> IgG Abs titer, median (range) | 827.9 (86.9–8685.6) | 462.1 (7.0–10097.3) | 0.0 (0.0–1307.1) | <0.0001 |

UMPs, uncomplicated malaria patients; NCs, negative controls; NECs, non-endemic controls; *Pf*, *Plasmodium falciparum*; Abs, antibodies. ^aDiagnosed using *Pf* rapid test and microscopic observation and excluded *Mix* (*Pf* and *P. vivax*) infection and Rapid test (+)/Microscopic observation (–). ^bCalculated from Kruskal–Wallis test.



in the UMPs. Thus, $\gamma\delta$ TCR repertoire in the non-V γ 9 $\gamma\delta$ T cells was highly polyclonal in the UMPs living in the malaria-endemic area, although the V γ 9V δ 2 T cell subset was predominant in the NCs and NECs.

IL-10 and IFN- γ Production by the Expanding Non-V γ 9 $\gamma\delta$ T Cells

The role of $\gamma\delta$ T cells in malaria infection remains unclear. However, it has been reported that these cells contribute to host defense and disease pathology during infections (Stevenson and Riley, 2004; Schofield and Grau, 2005). To investigate the function of non-V γ 9 $\gamma\delta$ T cells after *P. falciparum* infection, we cultured whole PBMCs from falciparum malaria patients in the presence of IL-2 and crude Pf Ag for 10 days. The cytokine concentrations were measured in the culture supernatants because it is difficult to culture non-V γ 9 $\gamma\delta$ T cells; these cells constitute a very small population of cells in the peripheral blood, and the specific antigen recognized by the TCR remains unknown. Surprisingly, non-V γ 9 $\gamma\delta$ T cells from NCs and UMPs in the malaria-endemic area increased 10-fold in the presence of IL-2 or IL-2 and Pf Ag after 10 days of culture (Figure 3A). Moreover, high levels of IL-10 were detected in the culture supernatants of PBMCs from the malaria-endemic groups (Figure 3B). The highest IL-10 production was observed in the supernatants of PBMCs from UMPs in the presence of IL-2 and/or Pf Ag. Interestingly, high concentrations of IFN- γ were also detected in those supernatants.

To determine whether non-V γ 9 $\gamma\delta$ T cells produce IL-10 in patients with falciparum malaria, we used intracellular staining for IL-10 and confirmed that IL-10 was indeed produced in non-V γ 9 $\gamma\delta$ T cells after whole PBMCs were cultured for 10 days. We found that non-V γ 9 $\gamma\delta$ T cells still produced IL-10, and higher levels of IL-10-producing cells were present in the PBMCs from UMPs than in those from JHCs, although many other IL-10-producing cells were present (Figures 3C,D). It has been reported that IFN- γ - or IL-17-producing V γ 9 $\gamma\delta$ T cells can be identified by using the cell surface marker CD27 (Ribot et al., 2009, 2010; Ribot and Silva-Santos, 2013). Approximately 80% of V γ 9 $\gamma\delta$ T cells in the present study were CD27-positive, but the classification of non-V γ 9 $\gamma\delta$ T cells by CD27 expression was difficult (data not shown).

We found that the non-V γ 9 $\gamma\delta$ T cells from patients living with recurrent malarial infections responded to IL-2 and/or crude Pf Ag, and these cells proliferated and produced both of IFN- γ and IL-10. Both of these are important cytokines for protecting the host against malarial infection. IFN- γ production by non-V γ 9 $\gamma\delta$ T cells was also found in the JHCs with no history of malaria, although no proliferation of non-V γ 9 $\gamma\delta$ T cells or IL-10 production was observed.

Proportion of Non-V γ 9 $\gamma\delta$ T Cells in Falciparum Malaria Patients Correlates with Plasma Levels of IL-10

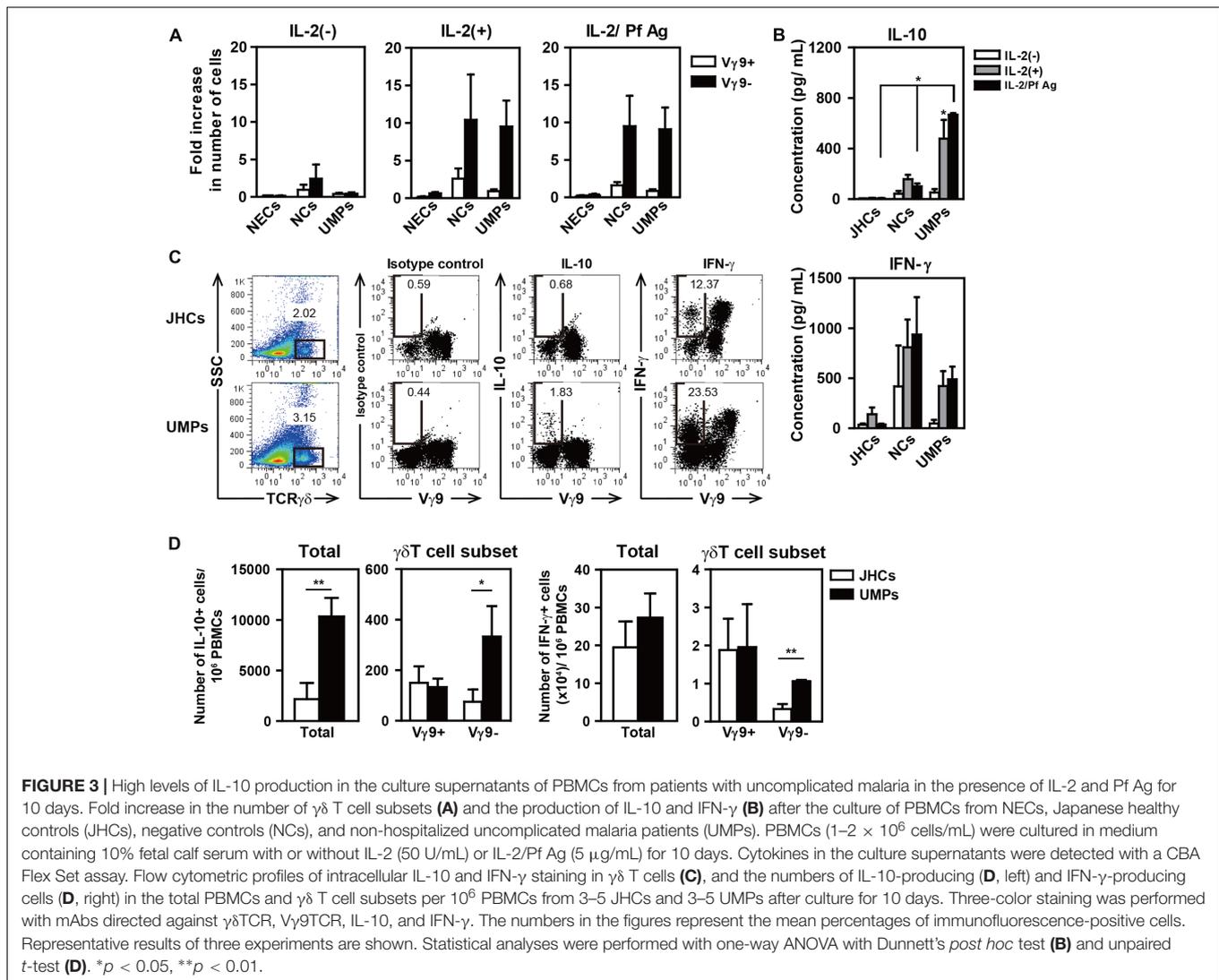
To confirm whether non-V γ 9 $\gamma\delta$ T cells correlate with levels of cytokines including IL-10 and IFN- γ in peripheral blood, we examined the plasma levels of cytokines in the infected

patients living in endemic areas. The plasma levels of IL-10 were significantly higher in UMPs than in NCs (UMPs: median 28.3 pg/mL, range 11.0–664.8 pg/mL; NCs: 21.9 pg/mL, 14.5–44.5 pg/mL, $p = 0.0079$; Figure 4A). None of the other cytokines (IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-12p70, tumor necrosis factor α , and IFN- γ) were elevated in UMPs living in endemic areas (Supplementary Table S2). These results were consistent with the mild symptoms of falciparum malaria in patients living in endemic areas. When we examined the relationship between plasma levels of cytokines and non-V γ 9 $\gamma\delta$ T cells proportion, we found that the plasma levels of IL-10 correlated with non-V γ 9 $\gamma\delta$ T cell proportion ($r = 0.3475$, $n = 54$, $p = 0.0100$; Figure 4B) but that of IFN- γ did not. These data suggested that non-V γ 9 $\gamma\delta$ T cells may be the source of IL-10 in UMPs living in endemic areas during infection.

DISCUSSION

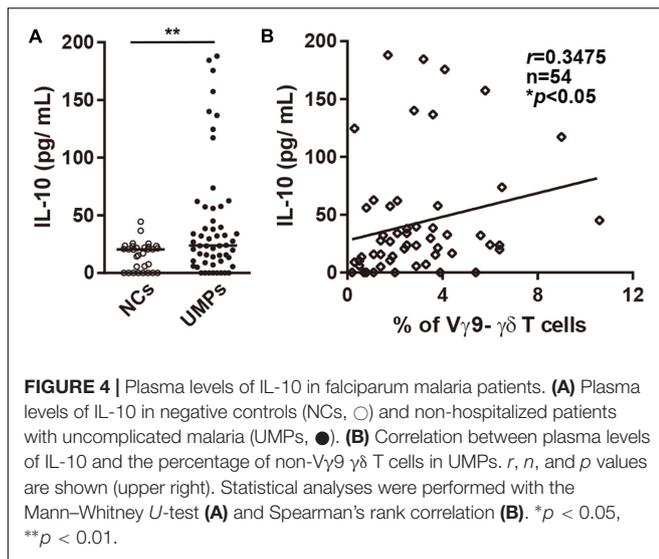
In this study, we hypothesized that $\gamma\delta$ T cells, which expand in primary or acute infections, do not do so in people with naturally acquired immunity against malaria and focused on the characteristics of these cells in people with naturally acquired immunity against *P. falciparum*. We found that non-V γ 9 $\gamma\delta$ T cells, one of the $\gamma\delta$ T cell subsets, were elevated in the peripheral blood of UMPs from a malaria-endemic area, although no increase in $\gamma\delta$ T cells was observed. In contrast, $\gamma\delta$ T cells were significantly higher in patients who were hospitalized with severe malaria than in other groups, and the major subset was V γ 9 $\gamma\delta$ T cells. These results should help to resolve the conflicting reports that (i) $\gamma\delta$ T cells in patients with primary or acute falciparum malaria expand after antimalarial treatment and this increase persists for 3–4 weeks after treatment (Ho et al., 1990; Roussillon et al., 1990; Chang et al., 1992; Hviid et al., 1996, 2001; Schwartz et al., 1996; Worku et al., 1997) and (ii) that no increase occurs in $\gamma\delta$ T cells in the peripheral blood of UMPs from endemic areas (Goodier et al., 1993; Hviid et al., 1996). We also propose that the two $\gamma\delta$ T cell subsets, the V γ 9 and non-V γ 9 $\gamma\delta$ T cells, play different roles in response to *P. falciparum* infection. V γ 9 $\gamma\delta$ T cells, which expand in malaria-naïve individuals but not in partially immune individuals following repeated infection, and these cell populations may be involved in the pathogenesis of malaria. Alternatively, non-V γ 9 $\gamma\delta$ T cells, which expand in partially immune individuals, may be involved in conferring protection against malaria and promoting the acquisition of natural immunity after reinfection.

The major $\gamma\delta$ T cell subset in the peripheral blood is V γ 9V δ 2, and the other major subset has a V δ 1 chain. From the TCR repertoire analysis, we found that the non-V γ 9 $\gamma\delta$ T cells that increased in the UMPs had polyclonal TCR repertoires, with a V γ 2, V γ 3, V γ 4, or V γ 5 chain and a V δ 1 or V δ 3 chain. This finding was consistent with results from a V δ 1 TCR analysis showing that the V δ 1 $\gamma\delta$ T cell population, which expands in falciparum malaria patients, uses the whole V γ chain repertoire and that it is highly polyclonal (Hviid et al., 2001). A polyclonal population of $\gamma\delta$ T cells may be necessary to counter the antigenic



diversity present in malarial infections (Covell et al., 1953; Healer et al., 2004). Malarial antigens for human $\gamma\delta$ T cells have been identified as soluble Pf-schizont-associated antigens, including phosphate antigens (Behr et al., 1996; Pichyangkul et al., 1997) and gametocyte-specific antigens (Ramsey et al., 2002). However, there is little information on non-V γ 9 T cell-specific antigens (Vantourout and Hayday, 2013). We used crude Pf Ag when culturing PBMCs from people living in malaria-endemic area, but the $\gamma\delta$ T cell response to crude Pf Ag in the presence of IL-2 was slightly different from that in the presence of IL-2 alone. Repeated malarial infections in endemic areas have been reported to be associated with a decreased proportion of V δ 2 $\gamma\delta$ T cells in peripheral blood and decreased proliferation and cytokine production in response to malaria antigens (Jagannathan et al., 2014; Farrington et al., 2016). This low responsiveness of $\gamma\delta$ T cells to Pf Ag may also be associated with repeated malarial infections in endemic areas. Therefore, more detailed study of the $\gamma\delta$ TCR usage and antigen recognition in non-V γ 9 $\gamma\delta$ T cells after malaria infection is required.

During malaria infection, $\gamma\delta$ T cells bridge innate and adaptive immune responses (Stevenson and Riley, 2004). These cells are stimulated by Pf antigens and proliferate and produce high levels of IFN- γ (Elloso et al., 1994; Pichyangkul et al., 1997; Troye-Blomberg et al., 1999) and other cytokines (Goodier et al., 1995). $\gamma\delta$ T cell proliferation and IFN- γ production are enhanced by IL-10, IL-12, and IL-1 β (Pichyangkul et al., 1997). A study on the function of $\gamma\delta$ T cells during malaria infection showed that these cells are significantly increased in vivax malaria patients from endemic areas with paroxysms and are positively correlated with disease severity (Perera et al., 1994). In contrast, $\gamma\delta$ T cells have been reported to play important roles in the elimination of parasites via cytotoxic activity (Roussillon et al., 1994; Costa et al., 2011), inhibition of RBC invasion by merozoites (van der Heyde et al., 1995), and inhibition of blood-stage *P. falciparum* parasite growth through direct contact (Elloso et al., 1994; van der Heyde et al., 1995; Troye-Blomberg et al., 1999). Similarly to V γ 9 $\gamma\delta$ T cells, non-V γ 9 (V δ 1) $\gamma\delta$ T cells can also produce IFN- γ when stimulated with Pf Ag (Hviid et al., 2001; D'Ombain et al., 2007)



and inhibit *P. falciparum* growth (Troye-Blomberg et al., 1999). However, the function of non-V γ 9 $\gamma\delta$ T cells during malaria infection is largely unknown.

In this study, we revealed that only the non-V γ 9 $\gamma\delta$ T cells from people living in malaria-endemic areas proliferated and produced IL-10 in PBMC cultures stimulated with IL-2 and/or Pf Ag for 10 days. We also showed that the plasma levels of IL-10 were elevated only in UMPs from endemic areas and that there was a slight positive correlation between the percentage of non-V γ 9 $\gamma\delta$ T cells and the plasma levels of IL-10. These non-V γ 9 $\gamma\delta$ T cells also produced IFN- γ . IFN- γ plays a key role in controlling *Plasmodium* infection in both liver and blood stages of the parasite life cycle, but it can also exacerbate the severity of malarial disease depending on its temporal and spatial production (King and Lamb, 2015). The number of IFN- γ -producing $\gamma\delta$ T cells among total producing cells is substantially higher in the V γ 9 $\gamma\delta$ T cells than in the non-V γ 9 $\gamma\delta$ T cells both in JHCs and UMPs. IFN- γ produced by non-V γ 9 $\gamma\delta$ T cells in people living in endemic areas may play an important role in the protection of hosts against malaria in the presence of fewer V γ 9 $\gamma\delta$ T cells, in agreement with findings from recent reports (Jagannathan et al., 2014; Farrington et al., 2016). IL-10 is an anti-inflammatory cytokine produced by many cell types, including T-helper type 2 (T_H2) cells, regulatory T cells, T_H17 cells, CD8⁺ T cells, B cells, dendritic cells, macrophages, mast cells, natural killer cells, eosinophils, and neutrophils (Saraiva and O'Garra, 2010). IL-10 is also an important cytokine for antibody production by B cells and their differentiation into antibody-secreting cells and plasma cells (Arpin et al., 1995; Choi, 1997; Agematsu et al., 1998; Choe and Choi, 1998; Yoon et al., 2009; Weiss et al., 2012). Interactions between $\gamma\delta$ T cells and B cells are important for class switching (Wen et al., 1996). $\gamma\delta$ T cell clones in mice produce IL-10 (Wen et al., 1998), and human V γ 9 $\gamma\delta$ T cells produce IL-10 and support antibody secretion by B cells (Caccamo et al., 2006, 2012). We suggest that the increase in non-V γ 9 $\gamma\delta$ T cells and IL-10 production by these cells during malarial reinfection

in people from endemic areas plays important roles in the acquisition of natural immunity after an increase in memory B cells and malaria-specific antibodies, although IL-10 is also produced by other cells during malaria infection (Freitas do Rosario and Langhorne, 2012; Stanisic et al., 2014). Together, these results suggest that non-V γ 9 $\gamma\delta$ T cells, similarly to other IL-10-producing cells, contribute to the acquisition of natural immunity against *P. falciparum* by stimulating the proliferation and differentiation of B cells into antibody-producing cells after IL-10 production. Surprisingly, non-V γ 9 $\gamma\delta$ T cells from NCs living in endemic areas also proliferate in the presence of IL-2 and/or Pf Ag, and they produce moderate levels of IL-10 and high levels of IFN- γ , as compared with the corresponding levels in UMPs *in vitro*. These results indicated that NCs with naturally acquired immunity against malaria-specific antibodies but no detectable parasitemia respond to *P. falciparum* infection and might have more potent naturally acquired immunity than that of UMPs. The differences in cytokine production by non-V γ 9 $\gamma\delta$ T cells between UMPs and NCs are thought to depend on the existence of *P. falciparum* parasites in the blood and the immunological status of the patients acquiring natural immunity. Further studies on non-V γ 9 $\gamma\delta$ T cells should be performed to evaluate this phenomenon.

There have been few studies on immunity against *P. falciparum* in people living in malaria-endemic areas. To develop an effective malaria vaccine, it is important to determine the factors and mechanisms that lead to the natural acquisition of immunity. Numerous *in vitro* studies have been conducted to elucidate the roles of $\gamma\delta$ T cells in malaria, especially the V γ 9 $\gamma\delta$ T cells, a major $\gamma\delta$ T cell subset, but these cells were not found to be increased in UMPs with naturally acquired immunity living in endemic areas. We emphasize the importance of studying non-V γ 9 $\gamma\delta$ T cells derived from UMPs with naturally acquired immunity rather than from patients with primary or acute falciparum malaria. Non-V γ 9 $\gamma\delta$ T cells, which increase in malaria patients living in endemic areas, may play an important role in the natural acquisition of immunity. Therefore, non-V γ 9 $\gamma\delta$ T cells are likely to be important for the development of B cell memory against falciparum malaria. Further studies on these cells should facilitate new approaches for the design of a malaria vaccine.

AUTHOR CONTRIBUTIONS

TT performed the research, analyzed the data and wrote the paper; TT, KM, DN, HT, and VV helped with sample and clinical data field collection; CL, MN, and MT provided technical advice; SK supervised field-based research; HW helped perform the research and commented on the draft manuscript.

FUNDING

This work was supported by grants from the Ministry of Education, Science and Culture, Japan; a Grant-in-Aid for JSPS

Fellows and JSPS KAKENHI JP16K20952 (TT); and a grant for International Health Cooperation Research 16-C and 19-C from the Ministry of Health, Labour and Welfare of Japan (HW).

ACKNOWLEDGMENTS

We thank the local authorities that supported this work; the villagers who participated in the study; the staff at the Center for Malariaology, Parasitology, and Entomology in Laos PDR, especially Dr. Samlane Phompida, and at the Attapeu Provincial Health Office and the Malaria Station for their contributions to this study. We are grateful to Dr. Varee Wongchotigul, Faculty of Tropical Medicine, Mahidol University, and Dr.

Fumie Kobayashi, Faculty of Medicine, Kyorin University, for information and data sharing on the hospital samples. The authors thank all our colleagues at the Center of Molecular Biosciences; the Tropical Biosphere Research Center; the University of the Ryukyus; and the Laboratory of Hematology and Oncology at the Graduate School of Health Sciences, Niigata University, for their many helpful discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.01288/full#supplementary-material>

REFERENCES

- Agematsu, K., Nagumo, H., Oguchi, Y., Nakazawa, T., Fukushima, K., Yasui, K., et al. (1998). Generation of plasma cells from peripheral blood memory B cells: synergistic effect of interleukin-10 and CD27/CD70 interaction. *Blood* 91, 173–180.
- Akpotheneta, O. J., Duah, N. O., Tetteh, K. K., Dunyo, S., Lanar, D. E., Pinder, M., et al. (2008). Duration of naturally acquired antibody responses to blood-stage *Plasmodium falciparum* is age dependent and antigen specific. *Infect. Immun.* 76, 1748–1755. doi: 10.1128/IAI.01333-07
- Arpin, C., Dechanet, J., Van Kooten, C., Merville, P., Grouard, G., Briere, F., et al. (1995). Generation of memory B cells and plasma cells in vitro. *Science* 268, 720–722. doi: 10.1126/science.7537388
- Bakir, H. Y., Tomiyama-Miyaji, C., Watanabe, H., Nagura, T., Kawamura, T., Sekikawa, H., et al. (2006). Reasons why DBA/2 mice are resistant to malarial infection: expansion of CD3^{int} B220⁺ gammadelta T cells with double-negative CD4⁻ CD8⁻ phenotype in the liver. *Immunology* 117, 127–135. doi: 10.1111/j.1365-2567.2005.02273.x
- Behr, C., Poupot, R., Peyrat, M. A., Poquet, Y., Constant, P., Dubois, P., et al. (1996). *Plasmodium falciparum* stimuli for human gammadelta T cells are related to phosphorylated antigens of mycobacteria. *Infect. Immun.* 64, 2892–2896.
- Bonneville, M., O'Brien, R. L., and Born, W. K. (2010). Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat. Rev. Immunol.* 10, 467–478. doi: 10.1038/nri2781
- Bordessoule, D., Gaulard, P., and Mason, D. Y. (1990). Preferential localisation of human lymphocytes bearing gamma delta T cell receptors to the red pulp of the spleen. *J. Clin. Pathol.* 43, 461–464. doi: 10.1136/jcp.43.6.461
- Boria, I., Cotella, D., Dianzani, I., Santoro, C., and Sblattero, D. (2008). Primer sets for cloning the human repertoire of T cell receptor variable regions. *BMC Immunol.* 9:50. doi: 10.1186/1471-2172-9-50
- Caccamo, N., Battistini, L., Bonneville, M., Poccia, F., Fournie, J. J., Meraviglia, S., et al. (2006). CXCR5 identifies a subset of Vgamma9Vdelta2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. *J. Immunol.* 177, 5290–5295. doi: 10.4049/jimmunol.177.8.5290
- Caccamo, N., Todaro, M., La Manna, M. P., Sireci, G., Stassi, G., and Dieli, F. (2012). IL-21 regulates the differentiation of a human gammadelta T cell subset equipped with B cell helper activity. *PLoS ONE* 7:e41940. doi: 10.1371/journal.pone.0041940
- Chang, W. L., van der Heyde, H., Maki, D. G., Malkovsky, M., and Weidanz, W. P. (1992). Subset heterogeneity among gamma delta T cells found in peripheral blood during *Plasmodium falciparum* malaria. *Immunol. Lett.* 32, 273–274. doi: 10.1016/0165-2478(92)90061-R
- Choe, J., and Choi, Y. S. (1998). IL-10 interrupts memory B cell expansion in the germinal center by inducing differentiation into plasma cells. *Eur. J. Immunol.* 28, 508–515. doi: 10.1002/(SICI)1521-4141(199802)28:02<508::AID-IMMU508>3.0.CO;2-I
- Choi, Y. S. (1997). Differentiation and apoptosis of human germinal center B-lymphocytes. *Immunol. Res.* 16, 161–174. doi: 10.1007/BF02786360
- Costa, G., Loizon, S., Guenot, M., Mocan, I., Halary, F., de Saint-Basile, G., et al. (2011). Control of *Plasmodium falciparum* erythrocytic cycle: gammadelta T cells target the red blood cell-invasive merozoites. *Blood* 118, 6952–6962. doi: 10.1182/blood-2011-08-376111
- Covell, S. G., Russell, P. F., Swellengrebel, N. H., and World Health Organization (1953). *Malaria Terminology: Report of a Drafting Committee Appointed by the World Health Organization*. Geneva: World Health Organization.
- Currier, J., Sattabongkot, J., and Good, M. F. (1992). 'Natural' T cells responsive to malaria: evidence implicating immunological cross-reactivity in the maintenance of TCR alpha beta+ malaria-specific responses from non-exposed donors. *Int. Immunol.* 4, 985–994. doi: 10.1093/intimm/4.9.985
- Dick, S., Waterfall, M., Currie, J., Maddy, A., and Riley, E. (1996). Naive human alpha beta T cells respond to membrane-associated components of malaria-infected erythrocytes by proliferation and production of interferon-gamma. *Immunology* 88, 412–420. doi: 10.1046/j.1365-2567.1996.d01-661.x
- D'Ombrain, M. C., Hansen, D. S., Simpson, K. M., and Schofield, L. (2007). Gammadelta-T cells expressing NK receptors predominate over NK cells and conventional T cells in the innate IFN-gamma response to *Plasmodium falciparum* malaria. *Eur. J. Immunol.* 37, 1864–1873. doi: 10.1002/eji.200636889
- Doolan, D. L., Dobano, C., and Baird, J. K. (2009). Acquired immunity to malaria. *Clin. Microbiol. Rev.* 22, 13–36. doi: 10.1128/CMR.00025-08
- Elloso, M. M., van der Heyde, H. C., vande Waa, J. A., Manning, D. D., and Weidanz, W. P. (1994). Inhibition of *Plasmodium falciparum* in vitro by human gamma delta T cells. *J. Immunol.* 153, 1187–1194.
- Dorfman, J. R., Bejon, P., Ndungu, F. M., Langhorne, J., Kortok, M. M., Lowe, B. S., et al. (2005). B cell memory to 3 *Plasmodium falciparum* blood-stage antigens in a malaria-endemic area. *J. Infect. Dis.* 191, 1623–1630. doi: 10.1086/429671
- Farrington, L. A., Jagannathan, P., McIntyre, T. I., Vance, H. M., Bowen, K., Boyle, M. J., et al. (2016). Frequent malaria drives progressive V δ 2 T-cell loss, dysfunction, and CD16 up-regulation during early childhood. *J. Infect. Dis.* 213, 1483–1490. doi: 10.1093/infdis/jiv600
- Freitas do Rosario, A. P., and Langhorne, J. (2012). T cell-derived IL-10 and its impact on the regulation of host responses during malaria. *Int. J. Parasitol.* 42, 549–555. doi: 10.1016/j.ijpara.2012.03.010
- Goerlich, R., Hacker, G., Pfeffer, K., Heeg, K., and Wagner, H. (1991). *Plasmodium falciparum* merozoites primarily stimulate the V gamma 9 subset of human gamma/delta T cells. *Eur. J. Immunol.* 21, 2613–2616. doi: 10.1002/eji.1830211045
- Goodier, M., Fey, P., Eichmann, K., and Langhorne, J. (1992). Human peripheral blood gamma delta T cells respond to antigens of *Plasmodium falciparum*. *Int. Immunol.* 4, 33–41. doi: 10.1093/intimm/4.1.33
- Goodier, M., Krause-Jauer, M., Sanni, A., Massougbdjji, A., Sadeler, B. C., Mitchell, G. H., et al. (1993). Gamma delta T cells in the peripheral blood of individuals from an area of holoendemic *Plasmodium falciparum* transmission. *Trans. R. Soc. Trop. Med. Hyg.* 87, 692–696. doi: 10.1016/0035-9203(93)90299-6
- Goodier, M. R., Lundqvist, C., Hammarstrom, M. L., Troye-Blomberg, M., and Langhorne, J. (1995). Cytokine profiles for human V gamma 9+ T

- cells stimulated by *Plasmodium falciparum*. *Parasite Immunol.* 17, 413–423. doi: 10.1111/j.1365-3024.1995.tb00909.x
- Goodier, M. R., and Targett, G. A. (1997). Polyclonal T-cell responses to *Plasmodium falciparum* gametocytes in malaria nonexposed donors. *Parasite Immunol.* 19, 419–425. doi: 10.1046/j.1365-3024.1997.d01-238.x
- Greenwood, B. M., and Vick, R. M. (1975). Evidence for a malaria mitogen in human malaria. *Nature* 257, 592–594. doi: 10.1038/257592a0
- Healer, J., Murphy, V., Hodder, A. N., Masciantonio, R., Gemmill, A. W., Anders, R. F., et al. (2004). Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in *Plasmodium falciparum*. *Mol. Microbiol.* 52, 159–168. doi: 10.1111/j.1365-2958.2003.03974.x
- Ho, M., Webster, H. K., Tongtawe, P., Pattanapanyasat, K., and Weidanz, W. P. (1990). Increased gamma delta T cells in acute *Plasmodium falciparum* malaria. *Immunol. Lett.* 25, 139–141. doi: 10.1016/0165-2478(90)90105-Y
- Hviid, L., Kurtzhals, J. A., Adabayeri, V., Loizon, S., Kemp, K., Goka, B. Q., et al. (2001). Perturbation and proinflammatory type activation of V delta 1⁺ gamma delta T cells in African children with *Plasmodium falciparum* malaria. *Infect. Immun.* 69, 3190–3196. doi: 10.1128/IAI.69.5.3190-3196.2001
- Hviid, L., Kurtzhals, J. A., Dodoo, D., Rodrigues, O., Ronn, A., Commey, J. O., et al. (1996). The gamma/delta T-cell response to *Plasmodium falciparum* malaria in a population in which malaria is endemic. *Infect. Immun.* 64, 4359–4362.
- Jagannathan, P., Kim, C. C., Greenhouse, B., Nankya, F., Bowen, K., Eccles-James, I., et al. (2014). Loss and dysfunction of V δ 2⁺ $\gamma\delta$ T cells are associated with clinical tolerance to malaria. *Sci. Transl. Med.* 6:251ra117. doi: 10.1126/scitranslmed.3009793
- Jorgensen, P., Nambanya, S., Gopinath, D., Hongvanthong, B., Luangphengsok, K., Bell, D., et al. (2010). High heterogeneity in *Plasmodium falciparum* risk illustrates the need for detailed mapping to guide resource allocation: a new malaria risk map of the Lao People's Democratic Republic. *Malar. J.* 9:59. doi: 10.1186/1475-2875-9-59
- Kageyama, Y., Koide, Y., Miyamoto, S., Inoue, T., and Yoshida, T. O. (1994). The biased V gamma gene usage in the synovial fluid of patients with rheumatoid arthritis. *Eur. J. Immunol.* 24, 1122–1129. doi: 10.1002/eji.1830240517
- King, T., and Lamb, T. (2015). Interferon- γ : the Jekyll and Hyde of malaria. *PLoS Pathog.* 11:e1005118. doi: 10.1371/journal.ppat.1005118
- Langhorne, J., Ndungu, F. M., Sponaas, A. M., and Marsh, K. (2008). Immunity to malaria: more questions than answers. *Nat. Immunol.* 9, 725–732. doi: 10.1038/nri.f.205
- Li, C., Mannoor, K., Inafuku, M., Taniguchi, T., Inamine, Y., Miyazaki, T., et al. (2012). Protective function of an unconventional gammadelta T cell subset against malaria infection in apoptosis inhibitor deficient mice. *Cell. Immunol.* 279, 151–159. doi: 10.1016/j.cellimm.2012.09.012
- Mannoor, M. K., Halder, R. C., Morshed, S. R., Ariyasinghe, A., Bakir, H. Y., Kawamura, H., et al. (2002). Essential role of extrathymic T cells in protection against malaria. *J. Immunol.* 169, 301–306. doi: 10.4049/jimmunol.169.1.301
- Minoprio, P., Itohara, S., Heusser, C., Tonegawa, S., and Coutinho, A. (1989). Immunobiology of murine T. cruzi infection: the predominance of parasite-specific responses and the activation of TCRI T cells. *Immunol. Rev.* 112, 183–207. doi: 10.1111/j.1600-065X.1989.tb00558.x
- Perera, M. K., Carter, R., Goonewardene, R., and Mendis, K. N. (1994). Transient increase in circulating gamma/delta T cells during *Plasmodium vivax* malarial paroxysms. *J. Exp. Med.* 179, 311–315. doi: 10.1084/jem.179.1.311
- Pichyangkul, S., Saengkrai, P., Yongvanitchit, K., Stewart, A., and Heppner, D. G. (1997). Activation of gammadelta T cells in malaria: interaction of cytokines and a schizont-associated *Plasmodium falciparum* antigen. *J. Infect. Dis.* 176, 233–241. doi: 10.1086/514029
- Portugal, S., Pierce, S. K., and Crompton, P. D. (2013). Young lives lost as B cells falter: what we are learning about antibody responses in malaria. *J. Immunol.* 190, 3039–3046. doi: 10.4049/jimmunol.1203067
- Ramsey, J. M., Tello, A., Contreras, C. O., Ordonez, R., Chirino, N., Rojo, J., et al. (2002). *Plasmodium falciparum* and *P. vivax* gametocyte-specific exoantigens stimulate proliferation of TCR gammadelta⁺ lymphocytes. *J. Parasitol.* 88, 59–68.
- Ribot, J. C., Chaves-Ferreira, M., d'Orey, F., Wencker, M., Goncalves-Sousa, N., Decalf, J., et al. (2010). Cutting edge: adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-gamma- or IL-17-producing gammadelta T cells upon infection. *J. Immunol.* 185, 6421–6425. doi: 10.4049/jimmunol.1002283
- Ribot, J. C., deBarros, A., Pang, D. J., Neves, J. F., Peperzak, V., Roberts, S. J., et al. (2009). CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat. Immunol.* 10, 427–436. doi: 10.1038/ni.1717
- Ribot, J. C., and Silva-Santos, B. (2013). Differentiation and activation of gammadelta T lymphocytes: focus on CD27 and CD28 costimulatory receptors. *Adv. Exp. Med. Biol.* 785, 95–105. doi: 10.1007/978-1-4614-6217-0_11
- Riley, E. M., and Stewart, V. A. (2013). Immune mechanisms in malaria: new insights in vaccine development. *Nat. Med.* 19, 168–178. doi: 10.1038/nm.3083
- Roussilhon, C., Agrapart, M., Ballet, J. J., and Bensussan, A. (1990). T lymphocytes bearing the gamma delta T cell receptor in patients with acute *Plasmodium falciparum* malaria. *J. Infect. Dis.* 162, 283–285. doi: 10.1093/infdis/162.1.283-a
- Roussilhon, C., Agrapart, M., Guglielmi, P., Bensussan, A., Brasseur, P., and Ballet, J. J. (1994). Human TcR gamma delta⁺ lymphocyte response on primary exposure to *Plasmodium falciparum*. *Clin. Exp. Immunol.* 95, 91–97. doi: 10.1111/j.1365-2249.1994.tb06020.x
- Rzeczycyk, C. M., Stamatou, S., Anderson, K., Stowers, A., Cheng, Q., Saul, A., et al. (1996). Experimental human *Plasmodium falciparum* infections: longitudinal analysis of lymphocyte responses with particular reference to gamma delta T cells. *Scand. J. Immunol.* 43, 219–227. doi: 10.1046/j.1365-3083.1996.d01-24.x
- Saraiva, M., and O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10, 170–181. doi: 10.1038/nri2711
- Schofield, L., and Grau, G. E. (2005). Immunological processes in malaria pathogenesis. *Nat. Rev. Immunol.* 5, 722–735. doi: 10.1038/nri1686
- Schwartz, E., Shapiro, R., Shina, S., and Bank, I. (1996). Delayed expansion of V delta 2⁺ and V delta 1⁺ gamma delta T cells after acute *Plasmodium falciparum* and *Plasmodium vivax* malaria. *J. Allergy Clin. Immunol.* 97, 1387–1392. doi: 10.1016/S0091-6749(96)70208-7
- Stanisic, D. I., Cutts, J., Eriksson, E., Fowkes, F. J., Rosanas-Urgell, A., Siba, P., et al. (2014). $\gamma\delta$ T cells and CD14⁺ monocytes are predominant cellular sources of cytokines and chemokines associated with severe malaria. *J. Infect. Dis.* 210, 295–305. doi: 10.1093/infdis/jiu083
- Stevenson, M. M., and Riley, E. M. (2004). Innate immunity to malaria. *Nat. Rev. Immunol.* 4, 169–180. doi: 10.1038/nri1311
- Taniguchi, T., Tachikawa, S., Kanda, Y., Kawamura, T., Tomiyama-Miyaji, C., Li, C., et al. (2007). Malaria protection in beta 2-microglobulin-deficient mice lacking major histocompatibility complex class I antigens: essential role of innate immunity, including gammadelta T cells. *Immunology* 122, 514–521. doi: 10.1111/j.1365-2567.2007.02661.x
- Troye-Blomberg, M., Worku, S., Tangteerawatana, P., Jamshaid, R., Soderstrom, K., Elghazali, G., et al. (1999). Human gamma delta T cells that inhibit the in vitro growth of the asexual blood stages of the *Plasmodium falciparum* parasite express cytolytic and proinflammatory molecules. *Scand. J. Immunol.* 50, 642–650. doi: 10.1046/j.1365-3083.1999.00647.x
- van der Heyde, H. C., Elloso, M. M., vande Waa, J., Schell, K., and Weidanz, W. P. (1995). Use of hydroethidine and flow cytometry to assess the effects of leukocytes on the malarial parasite *Plasmodium falciparum*. *Clin. Diagn. Lab. Immunol.* 2, 417–425.
- Vantourout, P., and Hayday, A. (2013). Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat. Rev. Immunol.* 13, 88–100. doi: 10.1038/nri3384
- Watanabe, H., Weerasinghe, A., Miyaji, C., Sekikawa, H., Toyabe, S., Mannor, M. K., et al. (2003). Expansion of unconventional T cells with natural killer markers in malaria patients. *Parasitol. Int.* 52, 61–70. doi: 10.1016/S1383-5769(02)00085-5
- Waterfall, M., Black, A., and Riley, E. (1998). Gammadelta⁺ T cells preferentially respond to live rather than killed malaria parasites. *Infect. Immun.* 66, 2393–2398.
- Weerasinghe, A., Sekikawa, H., Watanabe, H., Mannor, K., Morshed, S. R., Halder, R. C., et al. (2001). Association of intermediate T cell receptor cells, mainly their NK1.1(-) subset, with protection from malaria. *Cell. Immunol.* 207, 28–35. doi: 10.1006/cimm.2000.1737
- Weiss, G. E., Ndungu, F. M., McKittrick, N., Li, S., Kimani, D., Crompton, P. D., et al. (2012). High efficiency human memory B cell assay and its application to studying *Plasmodium falciparum*-specific memory B cells in natural infections. *J. Immunol. Methods* 375, 68–74. doi: 10.1016/j.jim.2011.09.006
- Wen, L., Barber, D. F., Pao, W., Wong, F. S., Owen, M. J., and Hayday, A. (1998). Primary gamma delta cell clones can be defined phenotypically and functionally

- as Th1/Th2 cells and illustrate the association of CD4 with Th2 differentiation. *J. Immunol.* 160, 1965–1974.
- Wen, L., Pao, W., Wong, F. S., Peng, Q., Craft, J., Zheng, B., et al. (1996). Germinal center formation, immunoglobulin class switching, and autoantibody production driven by “non alpha/beta” T cells. *J. Exp. Med.* 183, 2271–2282. doi: 10.1084/jem.183.5.2271
- Worku, S., Bjorkman, A., Troye-Blomberg, M., Jemaneh, L., Farnert, A., and Christensson, B. (1997). Lymphocyte activation and subset redistribution in the peripheral blood in acute malaria illness: distinct gammadelta⁺ T cell patterns in *Plasmodium falciparum* and *P. vivax* infections. *Clin. Exp. Immunol.* 108, 34–41. doi: 10.1046/j.1365-2249.1997.d01-981.x
- World Health Organization (2006). *Guidelines for the Treatment of Malaria*. Geneva: World Health Organization.
- World Health Organization (2016). *World Malaria Report 2016*. Geneva: World Health Organization.
- Yoon, S. O., Zhang, X., Berner, P., and Choi, Y. S. (2009). IL-21 and IL-10 have redundant roles but differential capacities at different stages of Plasma Cell generation from human Germinal Center B cells. *J. Leukoc. Biol.* 86, 1311–1318. doi: 10.1189/jlb.0409268
- Zevering, Y., Amante, F., Smillie, A., Currier, J., Smith, G., Houghten, R. A., et al. (1992). High frequency of malaria-specific T cells in non-exposed humans. *Eur. J. Immunol.* 22, 689–696. doi: 10.1002/eji.1830220311

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Taniguchi, Md Mannoor, Nonaka, Toma, Li, Narita, Vanisaveth, Kano, Takahashi and Watanabe. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.