High percentage of Gamma delta T Cells express the transcription factor, FoxP3 in Ghanaian children with or without malaria

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Abstract: Gamma delta (γδ) T cells are known as first line of defence to infectious agents and also considered as regulatory cells that form a link between innate and adaptive responses. However, there are contradictory reports on their expression of FoxP3. In this study, we examined FoxP3 expression by $\gamma\delta^+$ and $CD4^+$T cells as well as differences in expressions of FoxP3 between children with *Plasmodium falciparum* malaria and healthy donors. Peripheral blood mononuclear cells (PBMC) isolated from 29 Ghanaian children with uncomplicated malaria and age and sex-matched 14 healthy children, were stained with combinations of T-cell subset-, $CD25^-$ or FoxP3-specific monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or PE-Cy5. The antibodies were directed against CD4, CD25, TCR-γδ, Vδ1 and FoxP3. Interestingly, the results show higher proportion of TCR-$\gamma\delta^+$ cells expressing FoxP3 compared to $CD4^+$ T cells in both patients and healthy controls. Though there were no significant differences in the frequencies of TCR-$\gamma\delta^+$ $FoxP3^+$ and TCR-$\gamma\delta^+$ $V\delta1^+$ $FoxP3^+$ T cells between patients and controls, more than 40% of TCR-$\gamma\delta^+$ and 60% of $V\delta1^+$ T cells express FoxP3 in both patients and controls. In conclusion, our data demonstrate that substantial proportion of TCR-$\gamma\delta^+$ and $V\delta1^+$ T cells express FoxP3 and imply that the high frequency of $\gamma\delta^+$ or $V\delta1^+$ T cells in individuals from malaria endemic areas contributes to the pool of circulating $FoxP3^+$ cells.

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1. Introduction

Studies have shown the role of gamma delta (γδ) T cells as a first line of defence to infectious agents such as viruses and parasites (Bluestone and Matis, 1989; Born et al., 1990; Carding et al., 1990; De Paoli et al., 1990; Ho et al., 1990). γδ T cells are also known to play role in malaria protection (Taniguchi et al., 2007;Costa et al., 2011;Weidanz et al., 2010) and are now considered not only as first line of defence but also regulatory cells that form a link between innate and adaptive responses (Holtmeier and Kabelitz, 2005).

Two major subsets of human $\gamma\delta^+$T cells have been identified. One of these subsets expresses TCR variable segments Vγ9 and Vδ2 and is called $V\gamma9^+V\delta2^+$T cells and the other subset expresses Vδ1TCR V-segment and is known as $V\delta1^+$T cell. The majority subset in Caucasians is the Vγ9Vδ2 expressing cells (about 70 to 90%) whereas in malaria endemic areas $V\delta1^+$T cells constitute the dominant subset (about 30 to 50%), in both adults and children. The high levels of $V\delta1^+$T cells consequently contribute to higher levels of $\gamma\delta^+$T cells in healthy donors from malaria endemic areas (>10% of T cells) compared to Caucasians (<5% of T cells) and the levels of $V\delta1^+$ cells rise further during *P. falciparum* malaria infection (Goodier et al., 1993; Hviid et al., 1996; Hviid et al., 2001; Hviid et al., 2000). Dramatic expansion of $V\delta1^+$ cells coupled with diminished $V\gamma9^+/V\delta2^+$T cell frequency has also been observed in HIV^+ donors (Wesch et al., 1998).

Many studies have been done on $V\gamma9^+V\delta2^+$T cells which are the majority subset of $\gamma\delta^+$T cells in donors that are not immune to malaria. Some researchers have shown that in both acute *P. falciparum* infection and *in vitro* system, the elevated subset of γδT-cells was $V\gamma9^+V\delta2^+$T cells in non-immune patients (Goodier et al., 1995;Langhorne, 1996). Costa *et al.* (Costa et al., 2011) have also demonstrated anti-parasitic activity of $V\gamma9^+V\delta2^+$T $\gamma\delta^+$ T cells against extracellular merozoites. It is therefore becoming clearer that $V\gamma9^+V\delta2^+$T $\gamma\delta^+$ T cells confer protection against at least a stage in *P. falciparum* malaria. However, the role of $V\delta1^+$T cells in protection against or pathogenesis of malaria is still not clear. Though immunoregulatory role has been attributed to them based on their activation phenotype and cytokine profile (Hviid et al., 2001; Kuhl et al.,

2009), there are contradictory reports on FoxP3 expression by $\gamma\delta^+$ T cells (Kuhl et al., 2009; Li et al., 2011). However, FoxP3 expression by $CD4^+$ T cells has been established and was found to be necessary for the development and function of naturally occurring regulatory T cells and some inducible or adaptive regulatory cells (Sakaguchi, 2005;Campbell and Ziegler, 2007). FoxP3 expressing regulatory T cells such as $CD4^+FoxP3^+$ and $CD4^+CD25^+FoxP3^+$ cells, in humans, are known to play an important role in maintaining immune homeostasis. They are found to influence the Th1/Th2 immunological balance and can negatively regulate immune responses leading to susceptibility to infection or immunopathology. (Asseman et al., 1999; Pandiyan et al., 2007; Stevenson et al., 2011; Walther et al., 2005). It has also been shown that $CD4^+CD25^-FoxP3^+$ cells secrete large amount of pro-inflammatory cytokines (Nagar et al., 2008). Foxp3 is a transcript factor known to be important for regulatory T cell development and function.

In this study, we examined expression of FoxP3 by $CD4^+$, $CD4^+CD25^+$, TCR-$\gamma\delta^+$ and $V\delta1^+$T cells as well as possible differences in expressions of FoxP3 between Ghanaian children with *P. falciparum* malaria and their healthy counterparts. We report on expression of FoxP3 by $CD4^+CD25^+$, $CD4^+CD25^-$, TCR-$\gamma\delta^+$ and $V\delta1^+$T cells and changes in its expression during malaria.

2. Material and Methods

Study subjects

Twenty-nine (29) Ghanaian children who were diagnosed of uncomplicated malaria at University of Ghana Hospital and Ghana Atomic Energy Commission (GAEC) Clinic, Kwabenya, a suburb of Accra, were included in the study (mean age: 9.0 years; range: 5-13 years). Fourteen (14) healthy children, matched for age and sex, were included as controls. Informed consent was obtained from all parents or guardians before their children were enrolled in the study. The Institutional Review Board at Noguchi Memorial Institute Ethical Committee both granted ethical approvals for the study.

Blood Sample Collection and Processing

Venous blood samples were collected into sterile 10ml heparinized vacutainer tubes. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Histopaque (Sigma-Adrich, St. Louis, MO, USA) density gradient centrifugation. The PBMC were then dispersed in a freezing mix (10% DMSO in FCS), placed in Mr Frosty (Nalgene cryo1 °C freezing container, Nalgene, Rochester, NY, USA) and frozen at -80°C overnight, and cryopreserved in liquid nitrogen. Parasitological examination for presence of parasite infected red blood cells to confirm infection with *P falciparum,* estimate parasitaemia and also to exclude asymptomatic healthy donors.

Flow cytometry

During flow cytometric analysis, the PBMCs were quickly thawed in a water bath at 37˚C, washed in RPMI1640 containing 10% heat-inactivated (FCS) supplemented with penicillin/streptomycin, and L-glutamine, adjusted to $1x10^6$/ml in a staining buffer, and stained with combinations of T-cell subset, CD25 or FoxP3-specific monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or PE-Cy5. Surface staining was done with antibodies directed against CD4 (RPA-T4; BioLegend, San Diego, CA, USA), CD25 (BC96; BioLegend), TCR-γδ (B1; BioLegend), Vδ1 (TS8.2; Thermo Scientific) and FoxP3 (PCH101, Biosciences, San Diego, CA, USA).

The PBMC were subsequently washed twice and acquired with appropriate isotype controls or taken through intracellular staining using FoxP3 staining buffer set (cat 00-5123, eBiosciences). Fixation and permeabilization were done according to manufacturer's instructions followed by staining with anti-human Foxp3 or isotype controls. The cells were washed after incubation and analyze with appropriate controls. A minimum of 10,000 forward- and side-scatter gated mononuclear cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Flow Cytomeric and Statistical Analyses

Flow cytometric data was analyzed using FlowJo software (Treestar, Ashland, OR, USA). Lymphocyte population was set using forward and side scatter display and gated. Isotype controls were used to establish boundaries for negative and positive regions. Statistical significance of differences in means and confidence intervals for mean differences were determined using Independent-Samples *T-T*est and confidence intervals for means were determined by One-Sample *T-Test.* They were all calculated using SPSS 16.0 software (2007). For all tests, values of $p < 0.05$ were considered significant.

3. Results

FoxP3 expression in TCR-$\gamma\delta^+$ cells

We analysed TCR-$\gamma\delta^+$ cells of children with malaria and their healthy counterparts for expression of FoxP3. The results revealed that more than 40% of TCR-$\gamma\delta^+$ and 60% of $V\delta1^+$ T cells express FoxP3 in both patients and controls. Additionally, the frequency of expression of FoxP3 by TCR- $\gamma\delta^+$ cells was more than nine times higher than its expression by $CD4^+$T cells in healthy donors [40.49 (26.59 to 54.38); 4.36 (2.14 to 6.58), respectively] and about four times in patients[48.31 (38.67 to 57.94); 12.89 (8.83 to 16.95), respectively]). However, we did not observe

significant differences in the frequencies of TCR-$\gamma\delta^+$ $FoxP3^+$ and TCR-$\gamma\delta^+$ $V\delta1^+$ $FoxP3^+$, between patients and controls (p=0.339 and 0.792 respectively). On the contrary, the frequency of CD4+FoxP3+ cells was significant higher in patients compared to controls (p=0.002) (Figure 1). Figures 2 and 3 show side and forward scatter plot, gating of cells and FoxP3 expression.

We also examined changes in the frequencies of $CD4^+CD25^+FoxP3^-$ and $CD4^+CD25^-FoxP3^+$ cell in addition to levels of $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^+$ cells in uncomplicated malaria. Whereas the frequencies of $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^+$ cells were significantly higher in patients compared to controls (p=0.002 and 0.002 respectively), there were no significant differences in the frequencies of $CD4^+CD25^+FoxP3^-$ and $CD4^+CD25^-FoxP3^+$ cells between the two categories, though higher in patients compared to controls (p=0.300 and 0.316, respectively, Table 1).

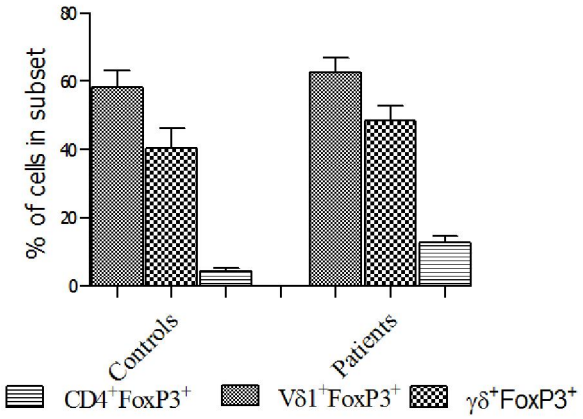

Figure 1. FoxP3 expression compared among $\gamma\delta^+$, $V\delta1^+$ and CD4+ cells, and between patients and controls

Unlike $CD4^+CD25^+FoxP3^+$ cells and $CD4^+CD25^+$ T cells, $CD4^+CD25^+FoxP3^-$ and $CD4^+CD25^-FoxP3^+$ cell frequencies do not differ significantly between Patients and Controls

Table 1: $FoxP3^+$ and $CD4^+CD25^+$ T cell frequencies in malaria patients and healthy donors

Subset	Healthy donor(95%CI)	Malaria patients(95%CI)	Difference(95%CI)	P(t)
$CD4^+CD25^+$	2.42 (0.52 to 4.33)	9.41 (6.47 to 12.36)	6.99 (2.90 to 11.10)	0.002
$CD4^+CD25^+FoxP3^+$	1.08 (0.35 to 1.81)	4.76 (3.09 to 6.44)	3.68 (1.40 to 5.90)	0.002
$CD4^+CD25^-FoxP3^+$	9.82 (3.02 to 16.01)	15.68 (9.10 to 22.26)	5.86(-3.03 to14.75)	0.316
$CD4^+CD25^+FoxP3^-$	1.86 (0.54 to 3.18)	3.93 (2.64 to 5.23)	2.07 (0.21 to 3.90)	0.300
Figures are percentage of $CD4^+$ T cells				

4. Discussions

Foxp3 is a transcript factor known to be important for development and function of regulatory T cells. FoxP3 expression has been established in $CD4^+$ T cells and demonstrated in $CD19^+$B cells (Noh et al., 2010) and TCR-$\gamma\delta^+$ T cells. CD19+Foxp3+ B cells were found to have high spontaneous apoptotic frequency (Noh et al., 2010). FoxP3 expressing regulatory T cells, in humans, are known to play an important role in maintaining immune homeostasis but can negatively regulate immune responses leading to susceptibility to infection or immunopathology. In this study, we demonstrate FoxP3 expression in patients and healthy donors from malaria endemic region. In a recent study, Kang and his colleagues (Kang et al., 2009) have shown that peripheral blood $\gamma\delta^+$ T cells rarely express foxp3 in healthy donors as well as tumour patients. They observed an increase in its expression after stimulation, however the percentage of $\gamma\delta^+$ T cells expressing the foxp3 was small (21% maximum). We observed high proportion of both TCR- $\gamma\delta^+$ and $V\delta1^+$ T cells expressing FoxP3 in malaria patients and controls. This is the first time high percentage of TCR-$\gamma\delta^+$ $FoxP3^+$ and $V\delta1^+$ $FoxP3^+$ have been demonstrated in peripheral blood of donors, and in association with malaria in particular. The high frequency of TCR- $\gamma\delta^+$ $FoxP3^+$ cells in both patients and healthy donors is mainly due to marked expression of this marker by $V\delta1^+$ cells, which are also the majority subset of TCR- $\gamma\delta^+$ cells in donors from malaria endemic areas (Hviid et al., 2001; Hviid et al., 1996; Hviid et al., 2000).

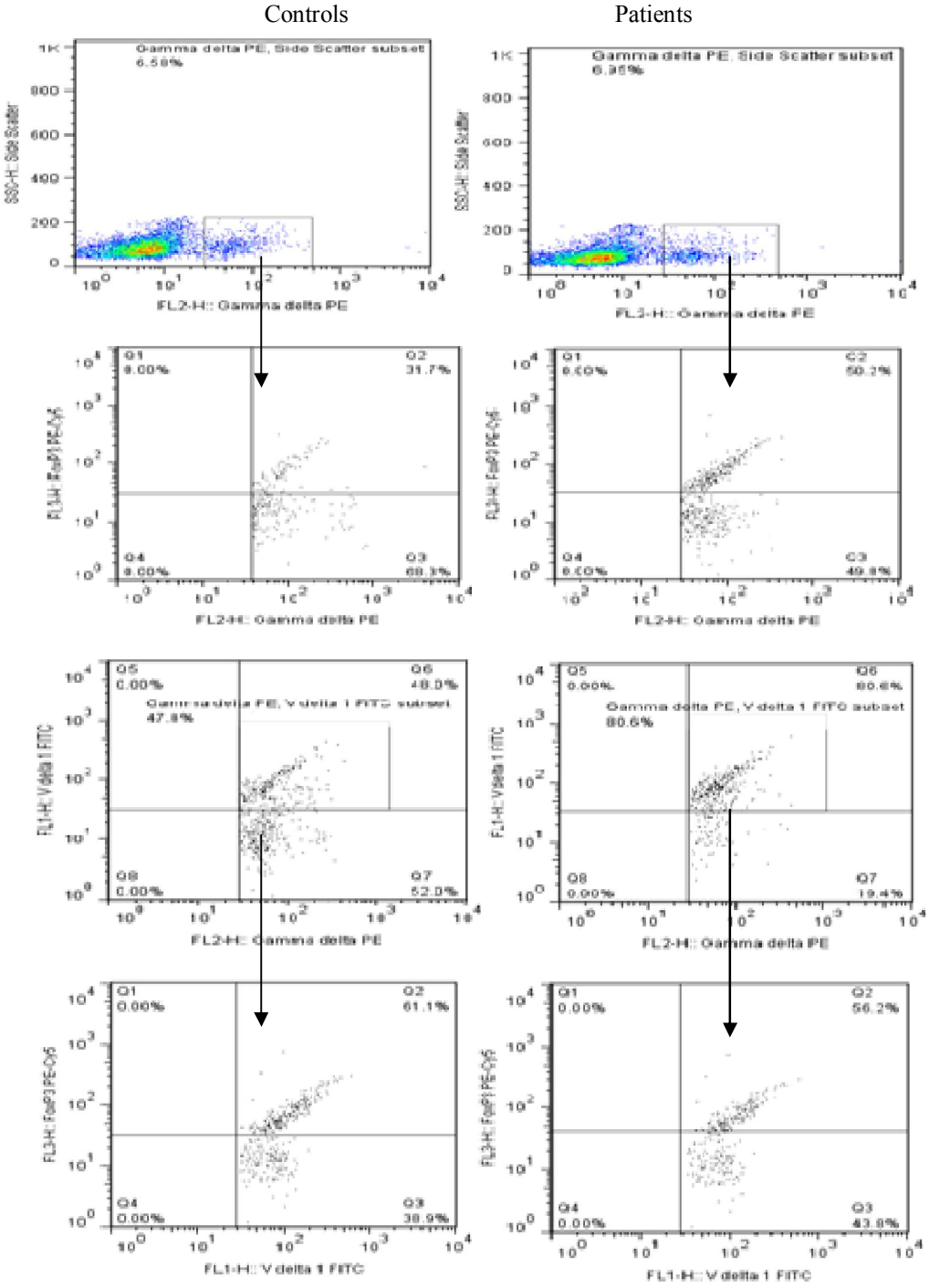

Figure 2. FoxP3 expression in TCR-$\gamma\delta^+$ V$\delta1^+$ cells

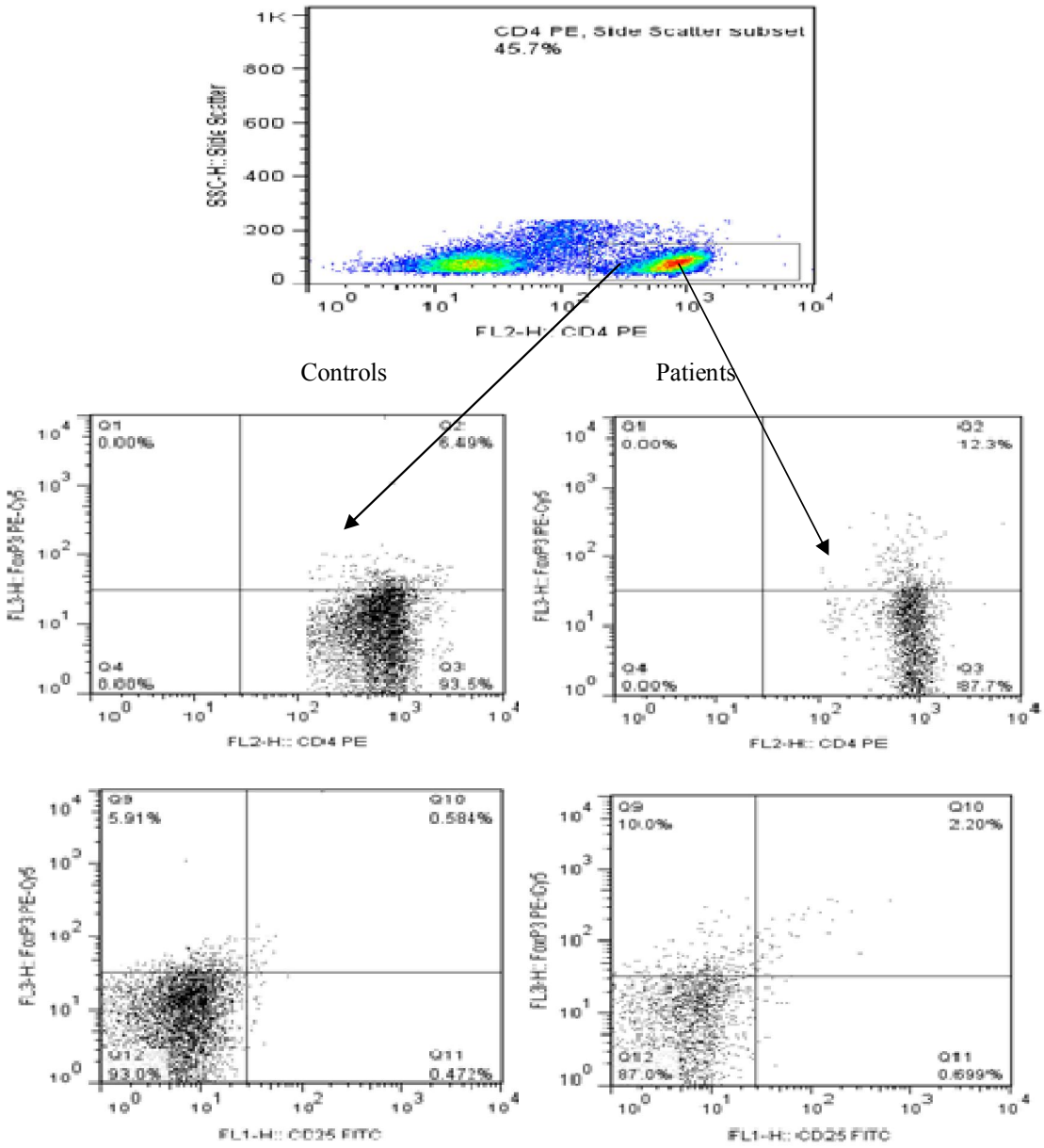

Figure 3. FoxP3 expression in $CD4^{+-}$ cells

Our data also confirmed high level of $V\delta1^+$ cells in donors and their expansion during *P. falciparum* infection (data not shown) but whereas proportions of TCR-$\gamma\delta^+$ and $V\delta1^+$ cells increased significantly in patients, no such increase has been found in the proportion of TCR-$\gamma\delta^+$ or TCR-$\gamma\delta^+$ Vδ1+ cells expressing FoxP3. However, this does not imply that these TCR-$\gamma\delta^+$ $FoxP3^+$ cells do not increase during malaria. It means that there was no significant selective increase in the frequencies of TCR-$\gamma\delta^+$ $FoxP3^+$ and $V\delta1^+$ $FoxP3^+$ over TCR-$\gamma\delta^+$ $FoxP3^-$ and $V\delta1^+$ $FoxP3^-$ respectively and as TCR-$\gamma\delta^+$ and $V\delta1^+$ cells increase remarkably during malaria, TCR-$\gamma\delta^+$ $FoxP3^+$ and TCR-$\gamma\delta^+$ Vδ1+ $FoxP3^+$ cells increase proportionately. This implies that the high frequency of $\gamma\delta^+$ or $V\delta1^+$ T cells in individuals from malaria endemic areas contributes to the pool of circulating $FoxP3^+$ cells, particularly in patients. Recent studies have shown that $\gamma\delta^+$ cells, mainly $V\delta1^+$ cells, secrete immunosuppressive cytokines or show immunoregulatory and pro-inflammatory activity similar to $CD4^+Foxp3^+$ (Nagar et al., 2008; Kuhl et al.,

2009; Li et al., 2011). However, Vδ1$^+$ FoxP3$^+$ cells in individuals from malaria endemic areas and particularly in malaria patients may not have immunosuppressive function. Evidence for their cytokine profile, mechanism of action and expression of FoxP3 are sketchy and contradictory. In a study involving tumour patients, Vδ1$^+$ cells do not produce TGF-β or IL-10 (Peng et al., 2007), whereas in another study with healthy donors they produced such cytokines (Kuhl et al., 2009). This implies that certain conditions can alter the cytokine profile and for that matter, function or at least the mechanism of immunoregulation in these cells. The donors for the above studies were not from malaria endemic regions. It is possible that Vδ1$^+$ FoxP3$^+$ cells from inhabitants of malaria endemic areas have different cytokine profile and role. The role of Vδ1$^+$ FoxP3$^+$ cells in malaria requires further investigation.

Significantly higher levels of CD4$^+$CD25$^+$FoxP3$^+$ cells in patients compared to controls were also observed. The significant increase in the levels of CD4$^+$CD25$^+$FoxP3$^+$ during malaria corroborates what has been found in other studies (Walther et al., 2005). Though the frequency of CD4$^+$CD25$^-$FoxP3$^+$ cells was much higher than that of CD4$^+$CD25$^+$FoxP3$^+$ cells in both patients and controls, it was not significant between patients and controls and seems not to play any direct role in uncomplicated *P. falciparum* malaria. The result indicates that CD4$^+$CD25$^+$FoxP3$^+$ cells, which are activated CD4$^+$FoxP3$^+$ cells, may be more important in uncomplicated *P. falciparum* malaria than CD4$^+$CD25$^-$FoxP3$^+$cells.

Studies have also shown significant rise in the levels of CD4$^+$CD25$^+$ cells in malaria (Walther et al., 2005), which has been corroborated in this study. However, in many of these studies CD4$^+$CD25$^+$ T cell frequencies included CD4$^+$CD25$^+$FoxP3$^+$ cells. When we separated the CD4$^+$CD25$^+$FoxP3$^+$ cells from CD4$^+$CD25$^+$FoxP3$^-$ cells, no significant difference was found between patients and controls. This indicates that it is the CD4$^+$CD25$^+$FoxP3$^+$ cell component of CD4$^+$CD25$^+$ cells that made the significant difference between patients and controls with regard to the frequency of CD4$^+$CD25$^+$ cells.

In conclusion, our data demonstrate for the first time that substantial proportion of TCR-γδ$^+$ and Vδ1$^+$ T cells express FoxP3 and imply that the high frequency of γδ$^+$ or Vδ1$^+$ T cells in individuals from malaria endemic areas contributes to the pool of circulating FoxP3$^+$ cells, particularly, as frequency of during malaria γδ$^+$ or Vδ1$^+$ T cells rise tremendously. The results also suggest that γδ$^+$ T cells express high levels of FoxP3 compared to CD4$^+$ T cells in both healthy and malaria children. Functional study of these cells will throw light on their role in immune responses in the children from malaria endemic areas.

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